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Target Discovery and Validation Reviews and Protocols

Volume 2
Emerging Molecular Targets and Treatment Options

Edited by

Mouldy Sioud

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Target Discovery and Validation Reviews and Protocols

Volume 2, Emerging Molecular Targets and Treatment Options

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METHODS IN MOLECULAR BIOLOGY™

Target Discovery and Validation Reviews and Protocols

VOLUME 2

Emerging Molecular Targets and Treatment Options

Edited by

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Preface

During the last few years we have seen fundamental changes in the way scientists approach the identification and validation of new drug targets. These novel strategies for target validation are expected to maximize the likelihood of achieving target-selective inhibition with minimal in vivo side effects. For example, by the use of small interfering RNAs (siRNAs) to down regulate expression of known genes, a number of therapeutic targets have been validated both in vitro and in vivo. The technologies developed to do this have not only yielded a significant number of drug targets but have influenced our understanding of gene function, the molecular mechanisms of diseases, and the design of new therapeutic interventions. Specific gene and protein targets—on which, for example, cancer cells depend—can now be identified, along with the therapeutic agents directed against them. Several relevant examples that have been validated, and some that have reached the clinic, are featured in Volume 2, *Emerging Molecular Drug Targets and Treatment Options,* of *Target Discovery and Validation Reviews and Protocols.*

Despite knowing the molecular mechanisms of most drugs, patients vary in their responses to a medication's efficacy and side effects. Indeed, the sequence of the human genome has shown that there is extensive genetic variation among individuals that would be expected to affect the response to medication. Thus, a better understanding of the molecular mechanisms that lead to an improved treatment response should play an important role in the development of *individualized* medicine. DNA sequence alterations and the expression profiles of mRNA molecules and proteins can be used to predict drug response. These genetic and epigenetic changes may be used in turn to develop treatment algorithms adjusted for use in individual patients. Several examples of such individualized treatment, aimed at increasing drug efficacy as well as decreasing toxicity, are discussed in this edition.

In systemic autoimmune diseases, current clinical practice calls for immunosuppressive drug therapy. However, some drugs are not target-specific and some carry a high risk of side effects. New immunosuppressive strategies, such as monoclonal antibodies and receptor antagonists, are now emerging as potentially valuable discriminating agents for use in innovative combinations. Such novel opportunities for therapeutic targeting in systemic autoimmune diseases are described in Volume 2.

MicroRNAs (miRNAs) are a family of short noncoding regulatory RNA molecules expressed in a variety of different cell types. These tiny RNAs have

been shown to play important biological functions and may regulate the expression of more than 30% of human genes. Presently, evidence is emerging that particular miRNAs may play a role in human cancer pathogenesis. Thus, the identification of miRNA expression signatures in patients with cancer may help to identify subjects who are at high risk of developing cancer or those who have an early stage of cancer. In order to interfere with miRNA expression, modified antisense oligonucleotides targeting individual miRNAs have been developed and these agents have the potential to eventually progress into a new class of therapeutic agents.

Volume II, *Emerging Molecular Drug Targets and Treatment Options,* was written by leading experts in the field and presents a unique source of current information. Along with Volume I, *Emerging Strategies in Drug Targets and Biomarker Discovery,* this work will be of interest to researchers, pharmaceutical companies, clinicians, and students of biology, medicine, or pharmacy.

I would like to thank the authors for their contributions, Anne Dybwad for critical reading of the manuscripts, and all those involved in the production of the book.

Mouldy Sioud

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Contents of Volume 1:
Emerging Strategies for Targets and Biomarker Discovery

1

Druggable Signaling Proteins

Mouldy Sioud and Marianne Leirdal

Summary

In normal cells, signaling pathways are tightly regulated. However, when they are aberrantly activated, certain pathways are capable of causing diseases. In many tumors, the aberrantly activated signaling proteins include members of the epidermal growth factor receptor family, the Ras proteins, protein kinase C isoenzymes, BCR-ABL fusion protein as well as transcription factors such as signal transducers and activators of transcriptions and Myc. Accordingly, deregulation of these signaling proteins holds promise for the development of new anticancer drugs. Studies in vitro and in disease-relevant models demonstrated that blocking the activation of a key target in a constitutively activated signaling pathway could reverse disease phenotype. Moreover, constitutive activation of the target alone is sufficient to induce relevant disease phenotype. Notably, the most dramatic therapeutic advances in cancer therapy during the last decade have come from agents targeted against active thyrosine kinases. These include imatinib (anti-BCR-ABL), gefitinib (anti-EGF receptor), and herpetin (anti-ErbB-2). Here, some selected validated and drugable targets are summarized.

Key Words: Signaling pathways; Ras proteins; BCR-ABL kinase; STAT proteins; PKC; EGF receptor; MAP kinases.

1. Introduction

Cancer is a multistep process driven by progressive accumulation of genetic alterations, each of which contributes to the breakdown of mechanisms that control cell growth. During the last years both genetic and epigenetic changes associated with malignant transformation and progression in a wide variety of human cancers have been identified ***(1)***. Activation of multiple oncogenes, inactivation mutations in tumor-suppressor genes, and defects in DNA repair genes are the hallmarks of the disease. Cancer cells are often dependent on the continued activation of growth-promoting genes for maintenance of their malignant

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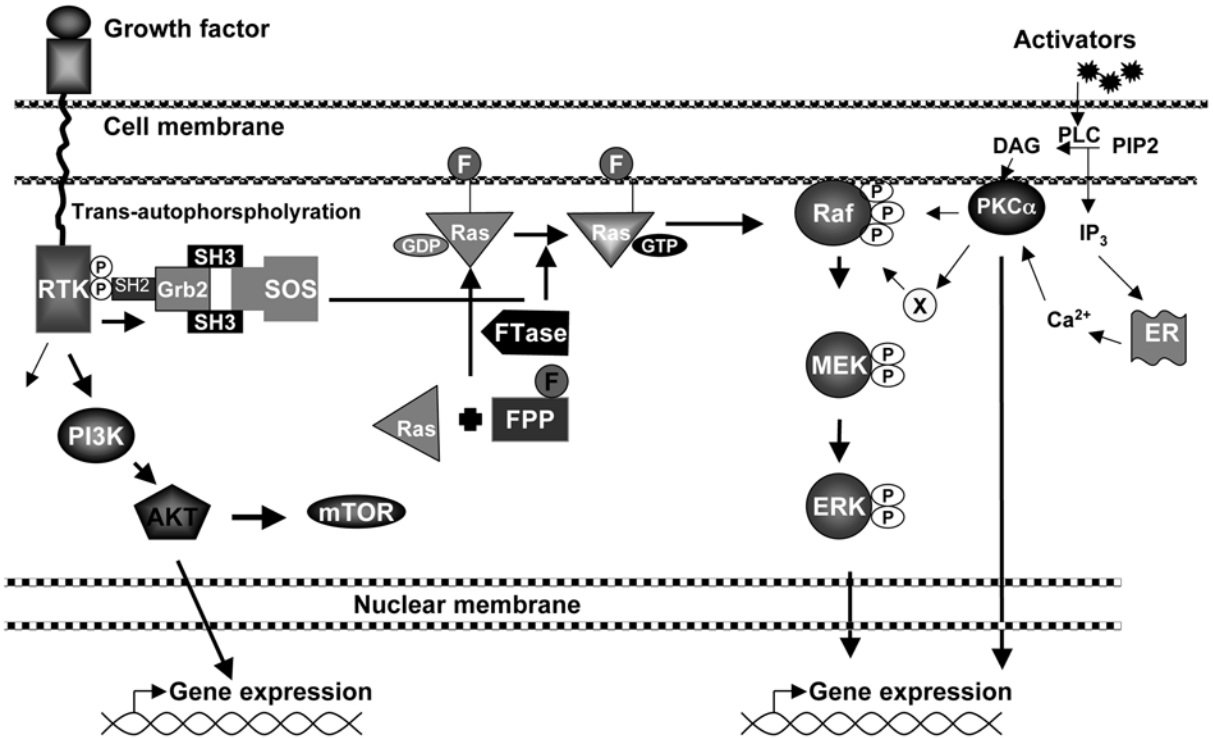
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AKT
mTOR
ERK
Nuclear membrane
Gene expression
Gene expression

phenotype *(2,3)*. Among the best-studied growth factor receptor systems has been the epidermal growth factor (EGF) receptor family and the platelet-derived growth factor receptor. These receptors contain an extracellular binding domain, a transmembrane lipophilic domain, and intracellular protein tyrosine kinase domain. Interaction of a growth factor with its receptor tyrosine kinase would activate several signaling proteins that ultimately lead to cell proliferation *(4,5)*. Thus, one way to inhibit cell proliferation is to use receptor antagonists.

The initial step of selecting an appropriate target for pharmaceutical development is of fundamental importance. The primary criterion for target selection is now established as disease relevance based on functional data in vitro and in vivo. However, before embarking on target validation it is important to investigate whether the selected candidate gene products are druggable. This would increase the rate of developing new drugs. As illustrated in **Fig. 1**, several receptor tyrosine kinases and cytokine receptors activate Ras protein, which is a major contributor to human cancer. Constitutive activation of Ras via mutations has been found in 30% of all cancer types, establishing a potential causal relation between the target (Ras) and cancer in humans *(6)*. Models organisms also confirmed the importance of Ras activation. Thus, Ras is a viable drug target.

Subsequent to activation, Ras interacts with and activates the serine/threonine protein kinase Raf-1, which in turn phosphorylates and activates mitogen-activated protein kinase (MEK). Activated MEK then phosphorylates extracellular signal-regulated protein kinase (ERK)-1 and ERK-2. Raf mutations have been identified in a range of human tumors and constitutive activation of MEK resulted in cellular transformation. Because of their important roles, both Raf and MEK kinases represent attractive targets in cancer. In addition to Ras, Raf-1 can

Fig. 1. *(Opposite page)* Schematic overview of the receptor tyrosine-kinase-RAS-ERK signaling pathway. The binding of a growth factor to its receptor tyrosine kinase (RTK) results in auto phosphorylation and activation of signaling proteins. The Src-homology 2 (SH2) domain on the adaptor molecule growth factor binding protein-2 (Grb2) binds to phosphorylated tyrosine residues on the RTK. Grb2 via its SH3 domain binds to Sos, which translocates to the plasma membrane and binds to Ras. Ras is post-translationally modified in to order to translocate into the plasma membrane. Activated Ras recruits and activates Raf to the plasma membrane. Activated Raf phosphorylates activate MEK, which further phosphorylates and activates extracellular signal regulated protein kinase (ERK). ERK translocates to the nucleus where it can regulate gene expression by phosphorylation of transcription factors. Protein kinase C (PKC) is activated by diacyl glycerol (DAG) and Ca2+. Phospholipase C (PLC) hydrolyses phosphoinositol diphosphate (PIP2) to DAG and inositol triphosphate (IP3). Cytosolic IP3 induces endoplasmatic reticulum to release Ca2+. PKCα can either directly or indirectly activate Raf kinase. Nuclear translocation of PKCα into the nucleus cans active gene expression.

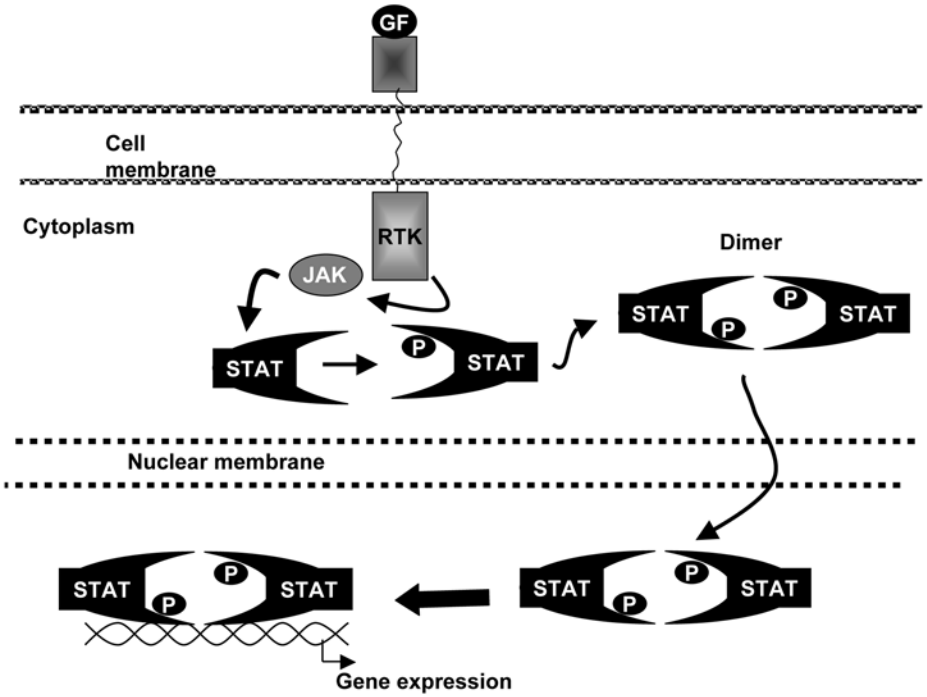

Fig. 2. Schematic representation of signal transducers and activators of transcription (STAT) signaling. STAT activation is initiated by tyrosine phosphorylation that is mediated by growth factor receptors and/or cytoplasmic protein tyrosine kinases (cPTKs), such as JAKs and Src. Phosphorylation of STAT induces dimerization, which allows STATs to translocate to the nucleus where they bind to consensus STAT-binding sequences of target genes and thereby activate gene transcription. Serine phosphorylation by protein serine kinases allows maximal transcriptional activity.

be activated by either protein kinase C (PKC)-α or the antiapoptotic protein Bcl-2. Theoretically, it could be argued that all the components of the RTK signaling pathway could represent a viable strategy to interfere with tumor growth *(7)*.

In addition to MAPK activation *(8)*, growth factor receptors also activate a family of cytoplasmic proteins known as signal transducers and activators of transcription (STAT) (**Fig. 2**). STAT proteins are also activated by cytoplasmic tyrosine kinases, particularly Janus kinase (JAK) and Src kinase families *(9)*. Because of their diverse biological functions, aberrations in STAT signaling are predicted to have a wide variety of consequences. Abnormal activity of certain STAT family members, particularly STAT-3 and STAT-5, is associated with a wide range of human malignancies, including breast and prostate cancers. And constitutive STAT-3 activation is required for oncogenic transformation by v-Src *(10)*. Importantly, a constitutively activated STAT-3 mutant alone is sufficient to

induce transformation ***(11)***, thus confirming the oncogenic function of STAT-3. Based upon these findings, STAT3 is a viable target in cancers.

Defects in Wnt signaling pathways are associated with several cancer types ***(12)*** (*see* Chapter 3). Wnt signaling acts as a positive regulator by inhibiting β-catenin degradation, which stabilizes β-catenin, and causes its accumulation and translocation to the nucleus. The mechanism by which β-catenin translocates into the nucleus is not completely clear, as it does not contain a nuclear localization signal and thus may be transported by other binding proteins. A key event in cancer is the loss of control over β-catenin levels, which can be the consequences of loss-of-function mutations in APC, originally discovered because they predispose to colorectal cancer ***(13)***. Around 80% of colorectal tumors have APC gene mutations ***(9)***. In addition to its role as a transcription factor, β-catenin is also involved in the control of cellular adhesion. It binds to the cytoplasmic domain of type I cadherins and plays an essential role in the structural organization and function of cadherins by binding to α-catenin, which in turn binds to actin cytoskeleton. Because β-catenin is the critical component of Wnt signaling, it represents an appropriate target in Wnt-causing cancers.

Several of evidences suggest that the level of PKC activity is important for certain cancer types ***(14)***. PKC is member of a family of cytoplasmic serine-threonine kinases, which are activated by several growth factors and lipid derivitives. Notably, many of the effectors molecules in inflammation are generated from phospholipids in the cell membrane. This makes the phospholipids not only building blocks in a membrane, but also very important reservoir from which cells generate intracellular and intercellular messengers. These include the oxidized fatty acids, platelet-activating factor, diacyl glycerol, and phosphatodic acid, which all are generated by a group of enzymes, the phospholipases. Phospholipase C uses phosphatidylinositol-4, 5-bis-phosphate to generate IP3 and diacyl glycerol, which lead to the activation of several PKC isoenzymes (**Fig. 1**). In addition to being involved in tumor growth, certain PKC isoenzymes were found to play an important role in the neoplastic progression of tumors and drug-resistant phenotype ***(15)***. Thus, modulation of PKC activity may improve therapies in some cancer types.

Despite some encouraging results with some therapeutic strategies, most tumors develop resistance to drug- and radiation-induced apoptosis. This resistance is mainly mediated by high expression of Bcl-2 family proteins, which can contribute to cancer cell expansion by preventing normal cell turnover initiated by physiological cell death pathways ***(16–18)***. High expression of Bcl-2 was found in a wide range of human cancers. Therefore, Bcl-2 and related proteins might be an effective therapeutic target in some cancers where altered expression profile contributes to the disease. Herein, we summarize recent work on targeting signaling pathways, a new optimism for cancer therapeutics.

2. Anticancer Drugs Targeting Receptor Tyrosine Kinase Pathways

Receptor tyrosine kinases are a family of proteins that signal cells through tyrosine phosphorylation reactions, and are the first part of a signaling pathway, which connects the signaling from membrane receptors to transcription factors that control gene expression. Hunter et al. *(7)* identified 478 typical and 40 atypical protein kinase genes in humans that correspond to approx 2% of all human genes. The family includes 385 serine/threonine kinases, 90 protein-tyrosine kinases, and 43 tyrosine-kinase-like proteins. Of the 90 protein-tyrosine kinases, 58 are receptor kinases *(7,8)*. The receptor tyrosine kinase signaling pathways play an important role in the control of fundamental cellular processes such as cell cycle, migration, survival, proliferation, and differentiation *(4)*. Examples of tyrosine kinase receptors are epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor *(4)*. Given their involvement in several cellular functions, RTK are tightly regulated *(4)*. Protein-tyrosine phosphatases have an important role in this control by dephosphorylating phospho-tyrosine residues and thereby inhibiting the activity of the receptor. In cancer, however, the receptor tyrosine kinases are often constitutively active owing to mutations, therefore leading to ligand-independent signaling *(7)*.

Among the best-studied growth factor receptor systems has been the EGF receptor family, which contains four homologous receptors: the epidermal growth factor receptor (ErbB1/EGFR/HER-1), ErbB2 (HER2/neu), ErbB3 (HER3), and ErbB4 (HER4). These receptors become activated by dimerization between two identical receptors or between different receptors of the same family *(5)*. The mechanisms that promote the formation of receptor dimers include ligand binding as well as high receptor density as a result of overexpression. The most widely studied and best-understood ErbB receptors are ErbB1 and ErbB2. Both display abnormal or enhanced expression in many types of cancer, suggesting their involvement in tumorigenesis *(19–21)*. Moreover overexpression has been shown to correlate with disease progression, survival, stage, and response to therapy. The exact mechanisms that are responsible for tumorigenic activity arising from these receptors in different types of cancer are not fully understood. EGFR seems to have a role in normal astrocyte differentiation and survival of the neural stem cell compartment. It is entirely possible that increased EGFR pathway activation may interfere with the normal differentiation process and serve to enhance malignant potential in gliomas *(22)*. Preclinical data suggest that EGFR regulates a number of important signaling pathways, including the Ras-RAF-MAPK cascade as well as the PI3K/AKT pathway, which, when upregulated via EGFR amplification/overexpression, lead to increased cellular proliferation, migration, and invasion. It is also becoming clear that upregulation of EGFR may contribute to the intrinsic radioresistance of glioma cells *(22)*.

Approximately 40% of the GBMs with EGFR amplification express a mutant form of EGFR, referred to as EGFRvIII. The EGFRvIII mutant lacks a portion of the extracellular ligand-binding domain as the result of genomic deletions of exons 2–7 in the EGFR mRNA. This results in constitutive phosphorylation, or activation, of the EGFR pathway ***(23,24)***.

The *ErbB2* gene encodes a growth factor receptor with tyrosine kinase activity that is amplified in 10–40% of breast cancer resulting in overexpression of the *ErbB2* gene. In addition to breast and ovarian cancer, overexpression has also been found in lung, gastric, and oral cancers. ErbB2 overexpression is associated with particularly aggressive disease and poor patient prognosis ***(25,26)***. A point mutation at the transmembrane domain and gene amplification are both believed to result in activation of the receptor. This is likely a result of the enhanced formation and stabilization of the receptor dimers, allowing the protein to remain in an active state. As previously mentioned, MAPK pathway has been strongly believed to participate in growth and transformation in many cell types ***(21)***. Autophosphorylation of ErbB2, leading to the activation of Ras/Raf/ERK/MAPK pathway, appears to be very important for this process of transformation ***(22)***. Another signaling molecule downstream of ErbB2 is PI3-kinase, which contains a 110-kDa catalytic and an 85-kDa regulatory subunit. Activation of this pathway is mediated by activated Ras or directly by some tyrosine kinase receptors that are under several growth factors and cytokines. Although ErbB2 protein has no p85-binding site, it may associate with PI3K, probably through heterodimerization with ErbB3. PI3-kinase is very often activated in cancer and contributes to cell cycle progression, cell survival, and metastasis. PI3-kinase activates Akt, a serine and threonine kinase, which in turn phosphorylates BCL2-antagonist of cell death (BAD) protein and inhibits apoptosis. The PI3K and AKT are considered proto-oncogenes, and the tumor-suppressor gene PTEN inhibits the pathway. Other evidence supports the interaction between the c-Src and ErbB2. Indeed, a high c-Src activity was found in ErbB2-induced mammary tumors than in the adjacent epithelium ***(21)***. The activation of c-Src correlated with its ability to form complexes with tyrosine-phosphorylated ErbB2. Also, the PI3/AKT signaling pathway is a key modulator pathway by which growth factor receptors activate a protein kinase termed mammalian target of rapamycin (mTOR). Akt indirectly activates mTOR via TSC, which in turn phosphorylates and activates several target genes (**Fig. 1**). Agents (e.g., CCI-779) targeting the rapamycin-sensitive signal transduction pathways have been developed for cancer therapy ***(27)***.

Over the last decade, several approaches were used to interfere with TKR signaling. These include the modulation of downstream signaling from the receptor by inhibiting or attenuating the activity of secondary messenger proteins, blockage of receptor expression or activity, and reducing the amount of

Table 1
Anticancer Agents Targeting Receptor Tyrosine Kinases

Drug	Type	Target	Malignancies	Development phase
Iressa® (ZD1839)	Tyrosine kinase inhibitor (quinazoline)	ErbB1 (EGFr), competitive with ATP	NSCLC, prostate, head/neck, glioma	II-III, approved for NSCLC
Tarceva® (OSI774)	Tyrosine kinase inhibitor (quinazoline)	ErbB1, competitive with ATP	NSCLC, pancreas, ovarian	II-III
Herceptin®	Monoclonal antibody	ErbB2	Breast	III/IV
Cetuximab (IMC-C225)	Monoclonal antibody	ErbB1	Colorectal, breast	II/III
Erbitux®	Monoclonal antibody	ErbB1	Colorectal, HNSCC	II/III
CI1033 (PD183805)	Tyrosine kinase inhibitor (quinizalone)	ErbB1 competitive with ATP	Squamous cell skin cancer, NSCLC, breast	I
GW2016	Tyrosine kinase inhibitor (quinazoline)	ErbB1, competitive with ATP	Lung	I

available ligands. Most progress has been made with the first two approaches (**Tables 1** and **2**). Small-molecule tyrosine kinase inhibitors represent the largest and most promising class of agents in development *(28)*. The pharmacological characteristics of Iressa® (ZD1839), a quinazoline, were first described in 1996 as a potent and selective inhibitor of the EGFR tyrosine kinase, acting as an adenosine triphosphate (ATP)-competitive inhibitor of the EGFR tyrosine kinase *(28)*. Iressa exhibited in vivo activity in a diverse human tumor xenograft models. Multiple phase I trials with Iressa have been conducted and the data showed reasonable pharmacokinetics and clinical efficacy when used as single agents in patients with advanced disease. Ireassa is now approved for NSCLC. Other selective-quinazoline-based inhibitors of the EGFR function have been developed such as CP-358, 774 which have more than 500-fold selectivity against other tyrosine kinases, such as the closely related ErbB2 kinase *(28)*. Clinical studies showed that this new generation of drugs is well tolerated at doses required for antitumor efficacy.

Table 2
Anticancer Drugs Targeting Ras

Drug	Type	Target	Malignancies	Development phase
SCH66336	Farnesyl transferase inhibitor	Farnesyl transferase	Pancreas, bladder, hematologic malignancies, gastrointestinal cancers, breast	II
R115777	Farnesyl transferase inhibitor	Farnesyl transferase	Hematologic malignancies, gastrointestinal cancers, breast	II-III
BMS214662	Farnesyl transferase inhibitor	Farnesyl transferase	Hematologic malignancies	I
ISIS2503	Antisense	Inhibiting Ha-Ras protein expression	Breast, pancreatic, NSCLC	I/II

In addition to quinazoline compounds, monoclonal antibodies (mAb), which block receptor activation, were developed. These antibodies bind to the receptors with affinity comparable to the natural ligands, compete with ligand binding, and thereby block the tyrosine kinase-receptor activity ***(5)***. Cetuximab (IMC-225) and Erbitux are two anti-EGFR (ErbB1) mAb, which are in phase II/III studies. mAb 225 induces antibody-mediated receptor dimerization (without activation of the tyrosine kinase), resulting in receptor downregulation. One of the most potent inhibitory anti-ErbB-2 antibodies was humanized for clinical applications. The resulting antibody trastuzumab (Herceptin®) exhibited a higher binding affinity for ErbB2 ***(19)***. In addition, to receptor modulations, the developed antibody also induced antibody-dependent cell-mediated cytotoxicity against ErbB2-expressing tumor cells in animal models. An early phase II with trastuzumab was conducted in 46 patients with metastatic breast carcinoma overexpressing ErbB2 resulted in one complete remission and four partial remissions, and additional 14 patients showed minimal response. A phase III clinical trial in combination with cisplatin was also conducted in patients with advanced breast cancers. An enhanced survival was obtained and the antibody is generally well tolerated ***(5)***. Laboratory data from cell cultures suggest synergism between trastuzumab and a range of chemotherapeutic agents, including docetaxel, cisplatin, and etoposide. Another

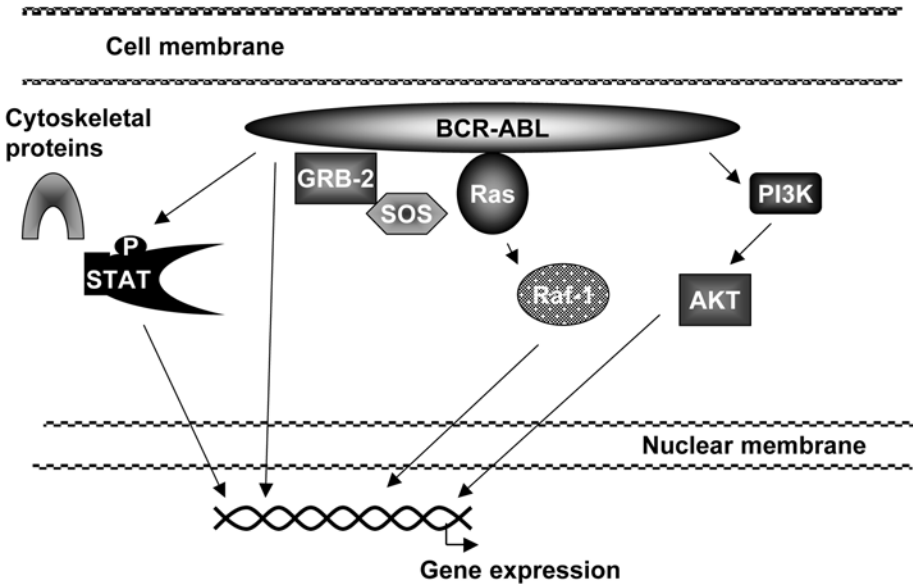

Fig. 3. Signal transduction pathways activated by BCR-ABL. The effects of BCR-ABL are mediated through its interaction with various signaling proteins leading to the activation of Ras signaling pathway, PI3K/AKT signaling pathway, and STAT proteins.

targeting approach is the use of synthetic growth factor antagonists *(23)*. One example is GFB-111, which binds to the platelet-derived growth factor receptor and was shown to inhibit glioblastoma tumor growth in mice up to 80% *(29,30)*.

As illustrated in **Fig. 1**, adaptor molecules are recruited to the active tyrosine kinase receptor. These proteins connect tyrosine kinase receptors to intracellular signaling pathways by functioning as an adapter between phosphotyrosine residues on the tyrosine kinase receptor and a proline-rich area on Sos protein *(6)*. Grb2 is an adaptor protein, which through its Src homology 2 (SH2)-domain recognizes phosphotyrosine residues on the RTK, and through its SH3-domaine recognizes Sos. Subsequently, Sos translocates to the cell membrane and binds to Ras, which are activated by exchange of GDP by GTP. Therefore, interfering with Grb2–SH2-receptor interaction or Grb2–SH3–Sos interaction could potentially inhibit the growth of malignant cells that are dependent upon activated Ras proteins. In this respect, several inhibitors have been developed *(30)*.

It is worth to note that some of the signaling proteins involved in TRK signaling also transduce the oncogenic effect of BCR-ABL kinase *(31)*. For example, by binding to BCR-ABL kinase Grb2 activates several downstream signaling pathways (**Fig. 3**). The key pathways implicated so far are those involving Ras, mitogen-activated protein kinases, PI3K/AKT as well as transcription factors such as STATs and MYC. The BCR-ABL fusion gene encodes a constitutively activated

protein tyrosine kinase that is a hallmark for chronic myeloid leukemia. Notably, BCR-ABL protein is one of the best targets in cancer because it is exclusive to the leukemic cells. During the 1990s, a number of tyrosine kinase inhibitors were purified from natural substances (e.g., herbimycin A and genistein) or chemically synthesized (e.g., tyrphostins). However, only imatinib mesylate (Glivec®, formerly known as STI 571) has resulted in excellent hematologic and cytogenetic responses in for chronic myeloid leukemia patients ***(32)***.

3. Anticancer Drugs Targeting Ras

"A number of novel therapies are currently showing promising in preclinical and clinical studies (**Table 2**). These include inhibitors targeting specific branches of TKR activated. As shown in **Fig. 1**, the main activated pathways are those involving Ras and mitogen-activated protein kinases. Ras functions as a membrane-associated biological switch that links signal from ligand-stimulated receptors to cytoplasmatic MAPK cascades. These receptors include G protein-coupled receptors, tyrosine kinase receptors (e.g., EGFR, EGF), and cytokine receptors. As previously mentioned, Ras proteins regulate cell growth and differentiation by cycling between an inactive, GDP-bound conformation and an active, GTP-bound form that stimulate downstream targets such as Raf-1. Ras is often mutated in different cancer types, and mutated Ras proteins are locked in the active GTP-bound state, thereby stimulating growth autonomously ***(33)***. When Ras is activated, effector molecules such as Raf, MEKK, and PI-3K bind to Ras and become activated. Ras must go through several posttranslational modifications to obtain full biological activity. These modifications include farnesylation by the enzyme farnesyl transferase (FTase). This posttranslational modification, which is required for Ras activity, has emerged as a major target for the development of novel anticancer agents ***(34,35)***. FTase recognizes and farnesylates the CAAX tetrapeptides with similar affinity to the full-length Ras proteins. Based upon this observation, several competitive inhibitors of FTase were designed and some of them have undergone clinical evaluation ***(34,35)***. One of the lead compounds currently in clinical development is R115777 and SCH55335 ***(36)***. R115777 was evaluated in patients with advanced solid tumors and in patients with refractory acute leukaemia, and some encouraging results were obtained. FTase inhibitors have been shown to have very little effect on the proliferation of normal cells. Thus, the antitumor activity and lack of toxicity of FTase inhibitors suggest that a farnesylated protein or proteins are critical to tumor survival and oncogenesis. In addition to FT inhibitors, GGT inhibitors were also developed.

Several new methods such as antisense, aptamers, ribozymes, and RNA interference have been developed to assign functions to a large numbers of genes. It should be noted that RNAi is now the leading technique in target validation (*see* Chapters 9–12). Despite the technical difficulties (e.g., delivery,

stability), several antisense oligomers are being subjected to phase II and phase III clinical trials *(37)*. ISIS 2503 is a 20-mer phosphorothioate oligonucleotide complementary to the translation initiation site of the human Ha-Ras mRNA. It specifically inhibits Ha-Ras expression and displayed activity against a range of different tumor types *(38)*.

4. Anticancer Drugs Targeting MAP Kinase Kinase (Raf, MEK)

The MAP kinase (MAPK) has emerged as the crucial link between membrane-bound Ras and the nucleus. This pathway involves the activation of three key kinases namely Raf, MEK (MAP kinase kinase), and ERK (MAPK), which represent novel therapeutic targets. Ras interacts with and activates the serine/threonine kinase Raf-1 in a GTP-dependent manner. Impaired Raf-1 expression has been observed in a variety of cancers including breast cancer *(39)* and small-cell lung carcinomas *(40)*. Moreover, Raf mutations have been identified in a range of human tumors *(6)*. Mutated Raf-1 is constitutively active and possesses in vitro transforming potential. Independent of mutations, Raf-1 is also activated in tumors with constitutive active TKR receptors or Ras proteins *(41)*. Once activated, Raf-1 kinase phosphorylates MEK1 and MEK2 which in turn activate ERK1 and ERK2 (ERK1/2). The potential for Raf-1 to play a broad role in tumorigenesis is evidenced by its ability to become activated by other cellular kinases such protein kinase Cα in a Ras-independent manner *(42)*. Because of its important role in cell signaling, several inhibitors for Raf activity were developed (**Table 3**). One of these inhibitors ZM336372 exhibited in vivo activity *(36)*. A search for specific pharmacological inhibitors of Raf kinase has also resulted in the identification of the bis-aryl ureas as lead active compounds *(42)*. The bis-aryl urea BAY 43-9006 has been selected as a candidate for clinical development, and has reached phase II-III studies. Similar to Ras proteins, Raf kinases were also targeted by antisense oligonucleotides and DNAzymes (*see* Chapter 7). In this respect, we recently found that depletion of Raf-1 protein with a DNAzyme induced substantial inhibition of JMML cell colony formation *(44)*. Moreover, when immunodeficient mice engrafted with JMML cells were treated continuously with the DNAzyme via a peritoneal osmotic minipump for 4 wk, a profound reduction in the JMML cell numbers in the recipient murine bone marrows was found, and their survival increased *(44)*. These results indicated that cleaving Raf-1 mRNA by nucleic acid enzymes might hold promise as new therapeutic strategy in several diseases where this kinase is activated. ISIS 5132 is a 20-mer-phosphorothioate oligonucleotide targeting a site in the 3' untranslated region showed antiproliferative and antitumor activity against various tumor cell lines in vitro and in vivo *(45)*. ISIS 5132 has been tested in phase I-II studies.

Activated Raf-1 phosphorylates and activates MEK, which in turn phosphorylates and activates ERK-1 and ERK-2. MEK therefore represents an attractive

Table 3
Anticancer Agents Targeting Raf, MEK

Drug	Type	Target	Malignancies	Development phase
ZM336372	Raf inhibitor	Raf, competitive with ATP	Neuroendocrine tumors	Preclinical
PD184352 (CI-1040)	MEK inhibitor	MEK, inhibits phosphorylation	Colon, pancreas, breast	I-II
BAY 43-9006	Raf inhibitor (bis-aryl ureas)	Raf	Colorectal, breast, hepatocellular carcinoma, NSCLC, leukemia, renal cell cancers	II-III
ISIS5132	Antisense	Raf	Prostate, colon, ovarian	I-II

target for pharmacological intervention in cancer. MEK is a crucial intracellular kinase for many mitogene-signaling pathways activated by oncogenes ***(46)***. Although MEK is not identified as an oncogene, its constitutive activation can result in cellular transformation ***(46)***. In this respect, small molecular compounds that inhibit the activity of MEK have been developed ***(46)***. PD09059 is a synthetic inhibitor, which inhibits the activation of MEK1 and in lesser extent MEK2 ***(47)***. PD184352 is a second inhibitor of MEK and is shown to inhibit tumor growth in mice with colon cancer in approx 80% of the cases ***(48)***. Clinical phase I studies of this inhibitor are started ***(46)***.

5. Anticancer Drugs Targeting the MAPK Pathways (ERK)

At least five MAPK cascades have been characterized in mammalian cells, including the extracellular-regulated kinases, ERK1 and ERK2, the c-Jun NH_2-terminal kinase, also known as stress-activated MAP kinase, and the p38 kinase pathway ***(49)***. A protein kinase cascade activates each of these groups. ERK-1 and -2 are proline-directed protein kinases that phosphorylate Ser/Thr-Pro motifs in the consensus sequence Pro-$X_{1\text{-}2}$-Ser/Thr-Pro, where X is any amino acid ***(50)***. Because of their high degree of similarity, ERK1/2 are usually considered to be functionally redundant and have been implicated in the signaling cascades induced by a broad range of receptors, in virtually all cell types. In addition to their activity in the cytoplasm, they translocate into the nucleus and regulate gene expression through phosphorylation of nuclear transcription factors ***(50)***. However, neither ERK1/2 contains nuclear localization sequences or nuclear

export signals. ERK activation has been found to be required for transformation of certain cells. In this respect, constitutive activation of ERK was found to be necessary for Ras-induced transformation of fibrosarcoma cells *(51)* and for EWS/FLI-1 transformation of NIH3T3 cells *(52)*. Activated MEK phosphorylates and activates ERK. Despite their important role in cell signaling, no selective inhibitor of ERK has been described. However, ERK- and MEK-inhibitors could be expected to have the same effect because ERK only is activated by MEK *(46)*.

6. Anticancer Drugs Targeting STAT Pathways

STAT proteins are a family of cytoplasmic proteins involved in signal transduction pathways used by many growth factors and cytokines, and are activated by phosphorylation of tyrosine and serine residues by upstream JAKs and Src kinase (**Fig. 2**). Notably, Src kinase has been shown to alter the expression or activity of several gene products. JAKs are crucial signal transducers for a variety of cytokines, growth factors, and interferons *(53–55)*. JAKs relay on the signals initiated by extracellular stimuli via corresponding receptors. Either the receptor and/or JAKs can alternatively recruit the STATs proteins via recognition of the SH2 domain near the phosphorylated sites *(56)*. The phosphorylation of the STAT tyrosine residue is essential not only for dimerization, but also for the concomitant translocation of the dimmers into the nucleus. In normal cells, the signaling by STAT proteins is under tight regulation, so that the signal delivered to the cell is transient. However, abnormal activity of certain STAT family members, particularly STAT-3 and -5, has been noted in many tumors, including hematologic, breast, head and neck, and prostate cancers *(9,57–61)*. Constitutively active STAT-3 mutant alone induces transformation, and cells transformed by this active STAT-3 mutant form tumors in vivo. Using in vivo tumor models murine B16 melanoma tumors regress on inhibition of STAT-3. Aberrant STAT-3 signaling is obligatory for growth and survival of various tumors, including multiple myelomas, breast carcinomas, and head and neck squamous cell carcinomas *(9)*. The cancer-causing activity of constitutively activated STAT-3 protein and evidence of potential clinical benefits of blocking constitutive STAT-3 made this kinase an important molecular target for the development of new anticancer treatments.

Tyrosine phosphorylation of STATs constitutes an early event in the activation of these transcription factors that is required for their dimerization and DNA binding activity. Thus, one method of direct regulation of the action of STAT proteins involves the inhibition of dimerization, which is essential for nuclear translocation and transcriptional activity *(56)*. Dimerization inhibition can be brought about by the use of artificial compounds, which have high affinity for the STAT monomer, to generate compounds that are more stable than STAT-STAT homodimers *(57)*. Recently, small phosphotyrosyl-peptides have

been used; these bind at the STAT3- SH2 domain, leading to its phosphorylation, dimerization, DNA-binding, and gene activation. Importantly, they inhibited v-Src cell transformation *(61)*. STAT coactivators play an extremely important role in guaranteeing DNA binding and the transcriptional activation carried out by the STATs. Therefore, the use of pseudoactivators with high STAT affinity might block STAT natural function *(59)*.

Following dimerization, STATs translocate into the nucleus and bind to specific regulatory sequences of responsive genes to induce gene transcription. Although the mechanisms of STAT translocation are not fully understood, small-molecule mimics of the translocation machinery might block STAT nuclear translocation *(60)*. The transcriptional activity of STATs requires that they physically interact with the promoter sequences. Knowledge of the crystal structure of STAT bound to its cognate DNA sequence should be important for rational design of artificial competitors. In this respect, phosphotyrosyl peptides inhibited STAT-3 interaction with DNA and therefore cell transformation *(61)*.

Another possible therapeutic approach is the use of antisense oligonucleotides, which block the expression of specific STAT mRNA transcripts, and the use of negative-dominant STAT proteins. Recently, we have found that STAT and Src signaling pathways are extremely important in breast cancer. Inactivation of STAT-3 with a siRNA-inhibited cell proliferation in vitro and in vivo, providing evidence for a functional role of STAT-3 in human cancers.

7. Anticancer Drugs Targeting PKCs

PKCα is a member of the PKC family of serine/threonine kinases, which are activated by many extracellular signals, and are central to many signaling pathways regulating cell growth and differentiation *(62)*. This family of serine/threonine kinases consists of at least 12 isoenzymes, which have been subdivided into three groups *(14)*. The potential role of PKC in carcinogenesis was first suggested by the observation that PKC represents the primary cellular target for tumor-promoting phorbol esters *(63,64)*. Situated at the crossroads of many signal transduction pathways, PKCs are also crucial to the link of a large diversity of signals from the cytoplasm to the nucleus.

PKCs are reversibly activated by upstream signaling elements such as growth factor receptors. Once activated, they can transmit signals to the nucleus via one or more MAPK cascades, which may incorporate Raf-1, MEKs, and ERKs. Overexpressed or hyperactive PKC is among the most distinguished characteristics of malignant brain tumors, in particular gliomas *(65)*. These findings led to the hypothesis that inhibition of PKC could represent a potential therapeutic strategy against high-grade brain malignancies. Reduction of PKC activity in cultured glioma cells using nonspecific PKC inhibitors such as staurosporine, tamoxifen, and calphostin C-inhibited glioma cell growth *(65)*.

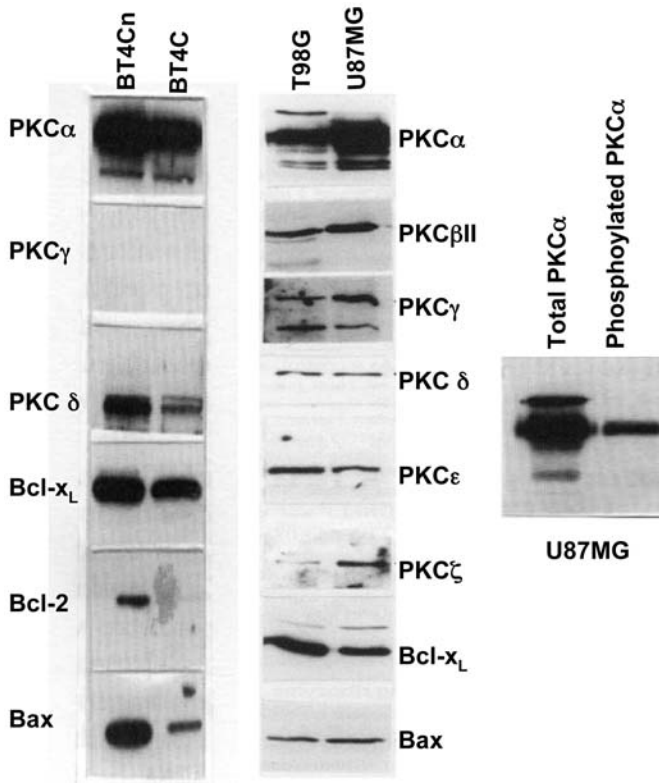

Fig. 4. Expression levels of PKC isoforms and Bcl-2-related proteins in rat (BT4C and BT4Cn) and human (T98MG and U87MG) glioma cell lines. Cytoplasmic protein extracts from each of the cell lines were analyzed by Western blotting using specific antibodies.

Although the overall PKC activity has been shown to be increased in some tumors, the precise role of each isoenzyme upon malignant cell growth is not known. To elucidate the specific role of PKC in cell survival and tumor promotion, we have used the nucleic acid enzyme strategy. Given the specificity of Watson-Crick base pairing, this strategy has the capacity to inhibit individual genes that are structurally related, such as isoenzymes. **Figure 4** shows the expression levels of several PKC isoenzyme and Bcl-2-related proteins. As shown, both human and rat glioma cells upregulated the expression of PCKα as compared to other PKC isoenzymes. In addition, a high percentage of PKCα was in vivo-activated. Specific targeting of PKCα by either a nuclease resistant ribozyme or a DNAzyme-induced apoptosis in glioma cells and inhibited tumor growth in vivo *(66–68)*. In addition, to gliomas, we have investigated the role of PKC-α in breast cancer proliferation and survival. **Figure 5** shows the

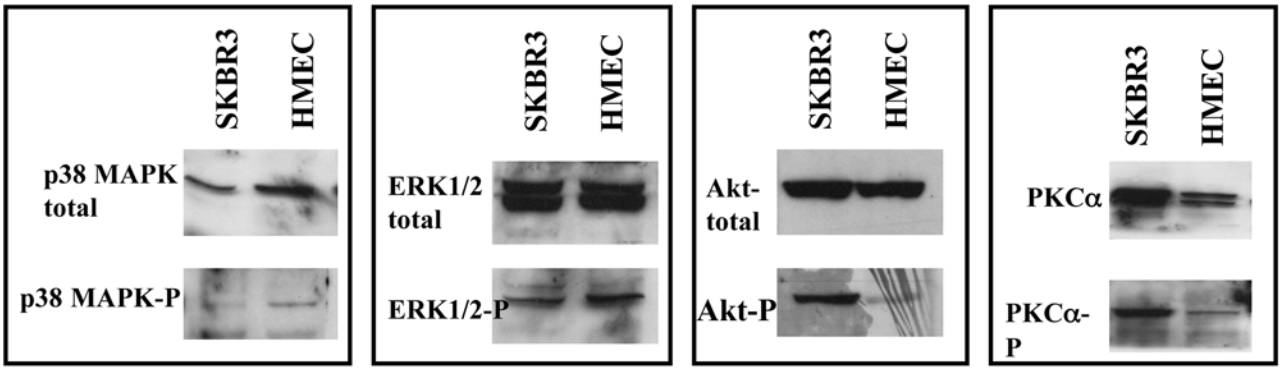

Fig. 5. Activation status of major signaling pathways in breast cancer cells. Cyoplasmic protein extracts were prepared from the breast cancer cell line SKBR3 or normal human mammary epithelial cells and then analyzed by Western blotting. Both total and phosphorylated kinases were detected using commercially available specific antibodies.

Table 4
Anticancer Agents Targeting PKC and Bcl-2

Drug	Type	Target	Malignancies	Development phase
ISIS3521	Antisense	PKCα	NSCLC, solid tumors	I-III
G3139	Antisense	Bcl-2	Melanoma, MM, CLL, NSCLC	III

activation status of the four main signaling proteins, p38, ERK, PKCα, and AKT in SKBR3 breast cancer cell line and human mammary epithelial cells. Both AKT and PKCα are constitutively activated in SKBR3 cells when compared to human mammary epithelial cells. By specifically targeting PKCα, Akt, or PI3K with either ribozymes or siRNAs, we have found that PKCα and PI3K are involved in malignant breast cancer cell survival and proliferation. Additionally, we have found that Akt can be phosphorylated by PKCα in vitro. Thus, PKCα might hold promise as a new therapeutic target in breast cancer. Recently, an antisense oligonucleotide targeting PKCα (ISIS 5132) exhibited in vitro and in vivo antitumor activity, and has reached phase I-II clinical trials (***[69]***; **Table 4**).

8. Anticancer Drugs Targeting Bcl-2 (Apoptosis)

Apoptosis is the prevalent form of programmed cell death that, when altered, contributes to a number of human diseases, including cancer. Bcl-2 and related proteins are a growing family of proteins that regulate apoptosis ***(16)***. This family includes both death antagonists such Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, and A1, and death agonists such as Bax, Bak, Bcl-x_S, Bad, Bid, Bik, Bim, Hrk, and Bok, which promote cell death ***(70)***. The ratio of death antagonists compared to agonists influences the susceptibility of cells to apoptosis ***(71)***. In general apoptosis can be divided into at least three different phases: initiation, effector, and degradation phases. During the initiation phase the cell receives apoptosis-triggering stimuli, which include ligation of certain receptors and cell stresses. The decision to die is made in the effector phase that involves the activation of the Bcl-2 proteins. The degradation phase occurs through the activation of the caspase cascade, which involves transactivation of procaspase-9 to active caspase-9, which in turn cleaves and activates downstream executioner caspases. The Fas-pathway involves the transactivation of procaspase-8 to active caspase-8.

Dysregulation of genes involved in apoptosis can contribute to cancer and resistance of cancer cells to conventional therapies. Proteins in the Bcl-2 family are crucial regulators of programmed cell death, and members that inhibit

apoptosis, such as Bcl-X_L, A1, Bcl-2, are overexpressed in many cancers and contribute to tumor initiation and progression ***(70)***. Moreover, overexpression of Bcl-2 and Bcl-X_L blocked apoptosis induced by a number of stimuli including anticancer drugs, contributing to resistance to therapy seen in patients ***(18,19)***. It is therefore essential to determine whether downregulation of these proteins would benefit cancer patients.

The three-dimensional structure of human Bcl-X_L, a close homolog of Bcl-2 has provided a structural basis for the design and discovery of Bcl-2 binding small molecules ***(72)***. Peptides containing the BH3 domain blocked protein interactions involving Bcl-2 and Bcl-X_L. Some of the developed peptide triggered apoptosis via caspase activation. Two natural products antagonized with the antiapoptotic function of Bcl-2 or Bcl-X_L. In addition, low molecular weight organic compound, HA14-1, effectively induced apoptosis in HL60 cells overexpressing Bcl-2 protein. Recently, a high-throughput nuclear magnetic resonance-based method for lead compound discovery was used to screen a chemical library to identify small molecule inhibitors. A small inhibitor ABT-737 was identified. ABT-737 induced apoptosis of cells from lymphoma and small-cell carcinoma lines, as well as primary patient-derived cells. Moreover, it exhibited in vivo antitumor activity ***(73)***. Clinical studies of this new agent are under way. This example, illustrate the importance of analyzing protein targets by three-dimensional structure for specific binding sites to evaluate their druggability for small pharmacological components. In principle, this should be done for all protein targets to minimize failures and increase specificity.

In addition to small molecules, antisense oligonucleotides targeting either Bcl-2 or Bcl-X_L induced apoptosis in cells derived from solid tumors and sensitized tumor cells to cancer therapies ***(75)***. An 18-mer phosphorothioate antisense targeting the first six codons of the Bcl-2 open reading frame (G3139) has reached phase III studie (**Table 4**). Although Bcl-X_L is similar to Bcl-2 in function, these two molecules protect cells from apoptosis nonredundantly. Antisense oligonucleotides targeting the Bcl-X_L induced apoptosis in various cancer types including NSCLC, breast cancer and prostate cancer in vitro and in vivo, and sensitize tumor cells to chemotherapy ***(74)***. Moreover, a bispecific antisense oligonucleotide targeting both Bcl-2 and Bcl-X_L sensitized breast carcinoma cells to doxorubicin, paclitaxel, and cyclophosphoamide (***76***; *see* Chapter 7).

9. Conclusion and Perspectives

As illustrated in this review, several signaling proteins have emerged as novel therapeutic targets. A new class of cancer therapies that targets this pathological constitutive activated pathway is currently under development. Notably, the most dramatic therapeutic advances in cancer during the last decade have come

from agents targeted against signaling proteins encoded by mutated genes. These include Herpetin (anti-ErbB-2), imatinib (anti-ABL-CBL), and gefitinib (anti-EGRR). Though none of these advances have resulted in cures of many patients with advanced disease, they can substantially improve and prolong lives. Several target genes are now identified (e.g., β-catenin, STAT-3, Bcl-2, PKC-α), and high-throughput analysis of drug discovery should lead to the identification of new generation of drugs.

It is worth noting that solid tumors are composed of two compartments, one consisting of neoplastic epithelial cells and other of stromal cells *(77)*. On the host side, the stromal cells together with extracellular matrix components provide the microenvironment that is important for cancer cell growth, invasion, and metastasis. In general, stroma compartment contain a variety of cell types, including immune cells, muscle and fibroblast cells, and vascular cells. These cells are modifying the phenotype of tumor cells by direct cell-to-cell contacts, via soluble factors or by modification of extracellular matrix. Thus, stromal therapy might become essential therapy in cancer prevention and intervention. By targeting either the expression of CSF-1 or its receptor *c-fms* by siRNAs, we have demonstrated that tumor growth can be inhibited in mice, through blockage of tumor-associated macrophage recruitment with concomitant reduction in local production of VEGF *(78)*.

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2

DNA Methylation and Histone Modifications in Patients With Cancer

Potential Prognostic and Therapeutic Targets

Michel Herranz and Manel Esteller

Summary

Epigenetics, a combination of DNA modifications, chromatin organization, and variations in its associated proteins, configure a new entity that regulates gene expression throughout methylation, acetylation, and chromatin remodeling. In addition to silencing as a result of mutations, loss of heterozygosity, or *classical* genetic events epigenetic modification symbolizes essential early events during carcinogenesis and tumor development. The reversion of these epigenetic processes restoring normal expression of tumor-suppressor genes has consequently become a new therapeutic target in cancer treatment. Aberrant patterns of epigenetic modifications will be, in a near future, crucial parameters in cancer diagnosis and prognosis.

Key Words: Cancer epigenetics; histone modifications; DNA methylation; prognosis; therapy.

1. Introduction

Epigenetics is concerned with the inheritance of information on the basis of differential gene expression, a process separate from genetic inheritance through gene sequence. Epigenetic modifications do not transform the DNA sequence; however, they are heritable and important in gene expression. Different components comprise the safety of the epigenome. Chromatin organization plays an important role in gene-expression regulation by modifying the tertiary structure to an open or accessible (euchromatin) status or to closed and inaccessible configuration (heterochromatin). Nuclear DNA is packaged into nucleosomes, a protein complex around which the DNA helix is wrapped. A nucleosome is a

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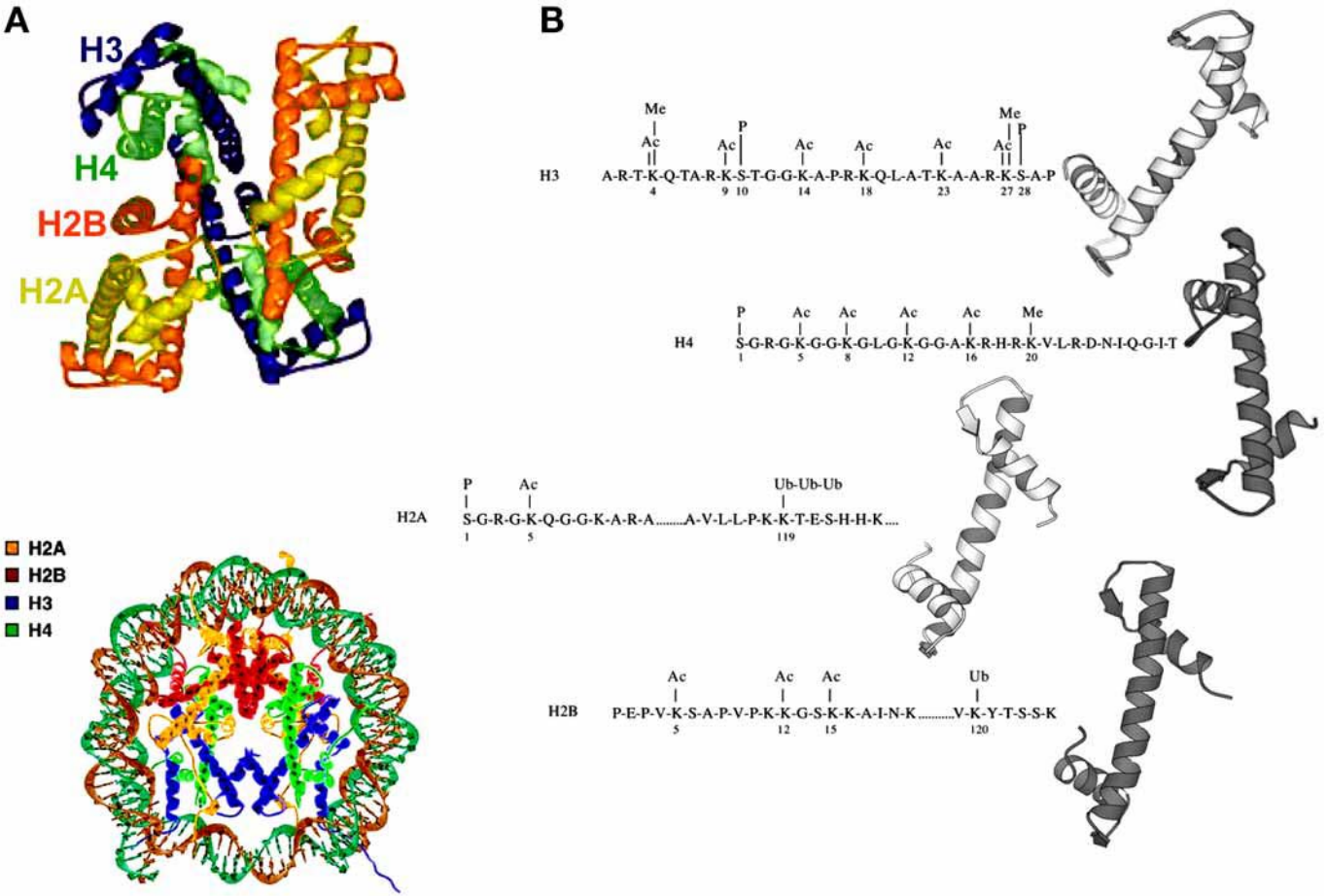

A
H3
H4
H2B
H2A
B
H3
A-R-T-K-Q-TA-R-K-S-T-G-G-K-A-P-R-K-Q-L-A-T-K-A-A-R-K-S-A-P
4
9 10
14
18
23
27 28
H4
S-G-R-G-K-G-G-K-G-L-G-K-G-G-A-K-R-H-R-K-V-L-R-D-N-I-Q-G-I-T
1
5
8
12
16
20
H2A
S-G-R-G-K-Q-G-G-K-A-R-A.....A-V-L-L-P-K-K-T-E-S-H-H-K....
1
5
119
H2B
P-E-P-V-K-S-A-P-V-P-K-K-G-S-K-K-A-I-N-K.....V-K-Y-T-S-S-K
5
12
15
120
H2A
H2B
H3
H4

histone octamer core ([H3-H4]$_2$-2[H2A-H2B]). Histone fold domain, present and described in these proteins, is completed by a basic N-terminal and a C-terminal tail ***(1)*** **(Fig. 1A)**.

Remodeling chromatin can be completed in several and unified ways: (1) covalent modification of histones, (2) intrinsic DNA modification, (3) exchange core histones with mutant or modified histone variants, and (4) disrupting DNA–nucleosome contacts. Histone modifications in the basic N-terminal tails, including acetylation, methylation, ubiquitination, phosporylation, biotinylation, and sumoylation **(Fig. 1B)** ***(2–6)***, result in changes in the cell transcriptional state. The best studied of these modifications is histone acetylation; this dynamic posttranslational modification is required for active chromatin configuration. Histone acetylation is a chemical equilibrium state. Acetylation is catalyzed by histone acetyltransferases (HAT), and deacetylation is catalyzed by histone deacetylases (HDAC) using acetyl-CoA as universal acetyl-donor **(Fig. 2)**.

In general, increased levels of histone acetylation (hyperacetylation) are found in euchromatin, a more open conformation of the nuclear chromatin, in which transcription is held in an active state; however, decreased levels of acetylation (hypoacetylation) are found in the tightly compacted chromatin (heterochromatin) associated with transcriptionally silent genomic regions. Another important histone modification in gene expression is histone methylation. Histone methyltransferases direct site-specific methylation of amino acid residues such as lysine (Lys4 and Lys9) and arginine residues. Methylation of Lys4 is important in maintenance of euchromatin structures, where genes are freely accessible and usually active; in contrast, methylation of H3Lys9 is associated with heterochromatin domains, strap, and inactive ***(7)***. Lysines can be

Fig. 1. *(Opposite page)* The histone octamer assembly in the nucleosome and modifications in histone tails. **(A)** Two molecules of each of the four core histone proteins form the histone octamer via formation of one tetramer of H3 and H4 and two dimers of H2A and H2B. Note that the nucleosome containing the two turns of DNA has the N-terminal tails of the eight-histone protein sticking out from the nucleosome like the legs of a spider. The structure of the portion of these N-terminal tails outside of the DNA is not known, and, more importantly, nor is the 30-nm chromatin fiber. Tetramerization occurs via interactions between the C-terminal halves of two histone molecules and results in a twofold axis of symmetry for the tetramer as shown. **(B)** Histone N-terminal tails are exposed from the nucleosomal interior into the aqueous surroundings. Moreover, these core histone tails have been found to be covalently modified; i.e., they can be methylated, acetylated, phosphorylated, and so on, on a single or on sets of N-terminally located serine and lysine residues. Me, methylated; Ac, acetylated; P, phosphorylated; Ub, ubiquitinated.

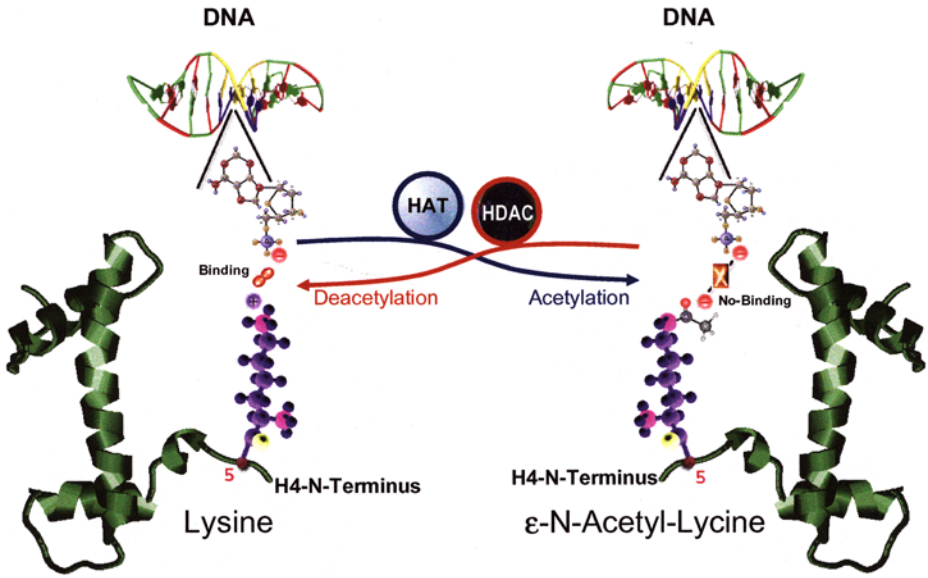


Fig. 2. Equilibrium of steady-state histone acetylation is maintained by opposing activities of histone acetyltransferases and deacetylases. Acetylation is a reversible process, in which histone acetyltransferases (HATs) transfer the acetyl moiety from acetyl coenzyme A to the ε-amino groups of internal, highly conserved lysine residues. This modification neutralizes the positively charged lysine residues of the histone N termini causing a reduction in the affinity of histone–DNA interactions, leading to increased access of transcription factors to the repressed chromatin template. Therefore, in most cases, histone acetylation enhances transcription while histone deacetylation represses transcription. Histone acetylation is catalyzed by HATs and histone deacetylation is catalyzed by histone deacetylases (denoted by HDACs).

mono-, di- or trimethylated, whereas arginines can be mono- or dimethylated, increasing the complexity of histone modifications.

DNA methylation remains the best-studied epigenetic mechanism. Methylation is needed for the normal development of cells because it facilitates static long-term gene silencing and confers genomic stability ***(8)***. Abnormal methylation, which confers growth advantages, is tightly connected to cancer development ***(9)***. Methylation of cytosines within the CpG dinucleotide (60% of human genes contain a CpG island ***[10]***) by transfer of a methyl group from the methyl donor *S*-adenosylmethionine to the carbon 5 position of cytosines (**Fig. 3**) is catalyzed by DNA methyltransferases: DNA methyltransferases 1 (DNMT1; responsible for DNA methylation maintenance during cell division, development, and cancer), DNMT3a, and DNMT3b (responsible for *de novo* methylation during early development) ***(11,12)***.

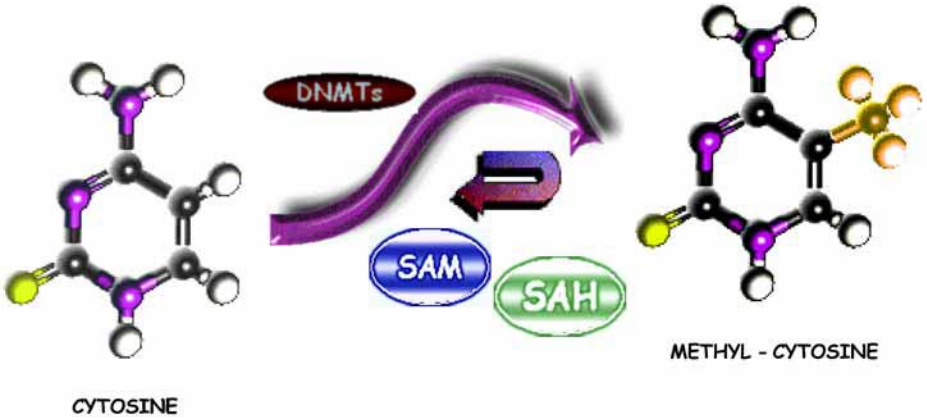


Fig. 3. Epigenetics refers to alterations in gene expression that occur without a change in DNA sequence. The chemistry behind one of these events is simple. It involves the covalent addition of a methyl group to cytosine. Of the four bases that make up DNA—adenine, thymine, cytosine, and guanine—only cytosine has the potential to be methylated in humans and most mammals. The methylation reaction involves flipping the target cytosine out of the intact double helix, so that the transfer of the methyl group from the methyl donor (*S*-adenosylmethionine) can take place. This reaction is catalyzed by the enzyme DNA methyltransferase (DNMTs). Methylation only occurs in cytosines whose 3'-carbon atom is linked by a phosphodiester bond to the 5'-carbon atom of a guanine (CpG dinucleotide). Most CpG dinucleotides are clustered in small stretches of DNA known as CpG islands.

Aberrant methylation patterns associated with cancer appear to be tumor-type specific ***(13,14)***.

It is well established that there is a good correlation between methylation state and histone modification. Genes that are methylated are usually related to deacetylated and inactive chromatin, whereas unmethylated promoters and active genes are associated with an open hypoacetylated euchromatin ***(15)***. This relationship was, at the beginning, unidirectional; DNA methylation determines histone acetylation status. The molecular event that validates this theory was the MeCP2 discovery. MeCP2 is a methylated DNA-binding protein (MBD) that recruits histone methyltransferase and histone deacetylase activity to the promoter regions of methylation-regulated genes (Jones 1998). However, now it seems to be a bidirectional control, chromatin inactivation recruits DNA methyltransferases to regulatory regions of genes ***(16,17)***.

On the basis of our current knowledge, the role of epigenetic events in cancer development, prognosis, and diagnosis is considered to be minor compared with those genetic events. However, nowadays new approaches to cancer therapy

Table 1
Epigenetic Diseases: Symptoms and Etiology[a]

Disease	Symptom	Aetiology
ATR-X syndrome	Intellectual disabilities, α-thalassaemia	Mutations in *ATRX* gene, hypomethylation of certain repeat and satellite sequences
Fragile X syndrome	Chromosome instability, intellectual disabilities	Expansion and methylation of CGG repeat in *FMR1* 5′ UTR, promoter methylation
ICF syndrome	Chromosome instability, immunodeficiency	*DNMT3b* mutations, DNA hypomethylation
Angelman's syndrome	Intellectual disabilities	Deregulation of one or more imprinted genes at 15q11–13 (maternal)
Prader–Willi syndrome	Obesity, intellectual disabilities	Deregulation of one or more imprinted genes at 15q11–13 (paternal)
BWS	Organ overgrowth	Deregulation of one or more imprinted genes at 11p15.5 (e.g. *IGF2*)
Rett syndrome	Intellectual disabilities	*MeCP2* mutations
α-Thalassaemia (one case)	Anaemia	Methylation of α2-globin CpG island, deletion of *HBA1* and *HBQ1*
Rubinstein–Taybi syndrome	Intellectual disabilities	Mutation in CREB-binding protein (histone acetylation)
Coffin–Lowry syndrome	Intellectual disabilities	Mutation in *Rsk-2* (histone phosphorylation)

[a]Neurological diseases related with deregulation of imprinted genes, mutations in MBDs (methylation-binding domain proteins), HATs (histone acetyltransferases), or DNMTs (DNA methyl-transferases).

ATR-X syndrome. α-thalassemia, mental retardation syndrome; BWS, Beckwith-Wiedemann syndrome; CREB, cAMP-response-element-binding-protien; ICF, immunodeficiency, centromeric region instability, and facial anomalies syndrome; UTR, untranslated region; DNMT, DNA methyl transferase; FMR1, Fragile X mental retardation 1; HBA1, hemoglobin alpha 1; HBQ1, hemoglobin theta 1.

based on epigenetic therapies are emerging, demethylatings agents and histone deacetylases inhibitors predominantly.

2. Epigenetic Diseases: The Neurological Achilles' Heel

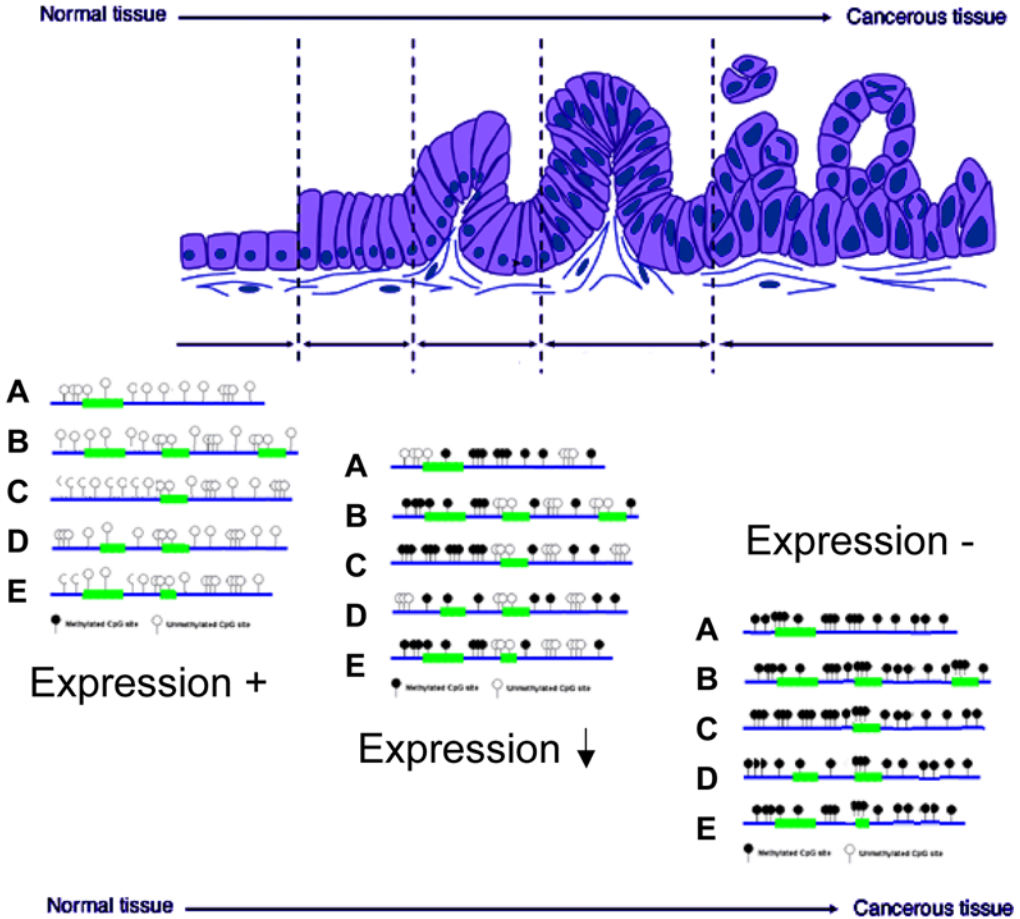
Mutations in genes that affect epigenetic profiles are inheritable or somatic acquired. Hereditable mutations in methyltransferases (DNMTs) or MBDs genes, are the phenomenon behind some human syndrome as ICF syndrome (DNMT3b mutation) or Rett syndrome (MeCP2 mutation). Curiously many of this disease results in mental retardation, chromosomal instability, and learning disabilities (**Table 1**). These new platforms of human disease could be considered as epigenetic diseases.

There are syndromes that result in deregulation of imprinted in cluster of same chromosomal location-genes as Angelman's syndrome (AS), a disorder that can be difficult to diagnose, particularly in the first few years of life. Approximately 70% of cases of AS have a deletion of 15q11-q13 in the maternally contributed chromosome. Main characteristics are developmental delay, functionally severe speech impairment, none or minimal use of words, receptive and nonverbal communication skills higher than verbal skills, movement or balance disorder (usually ataxia of gait and/or tremulous movement of limbs), behavioral uniqueness (any combination of frequent laughter/smiling), apparent happy demeanor, easily excitable personality (often with hand flapping movements), hypermotoric behavior, and short attention span.

With the same epigenetic root, but a clinically distinct disorder, is the Prader-Willi syndrome (PWS), a complex disorder, which diagnosis may be difficult to establish on clinical grounds and whose genetic basis is heterogeneous. Approximately 28% of cases of PWS are a result of maternal uniparental disomy. A disorder of chromosome 15 with a prevalence of 1:12,000–15,000 (both sexes, all races). The major characteristics of PWS are hypotonia, hypogonadism, hyperphagia, cognitive impairment, and difficult behaviors.

Finally in these examples, Beckwith-Wiedemann syndrome, and an overgrowth disorder. Wiedemann first recognized it in 1963, and in 1964 by Bruce Beckwith, a pediatric pathologist. Both doctors noted similar characteristics in their patients that were not traceable to other disorders, thereby identifying a new syndrome. The syndrome is usually sporadic, but may be inherited. These children are at risk for developing hypoglycemia and various types of tumors. The clinical picture of this syndrome can vary from mildly to greatly affected. The incidence of BWS has been reported as approx 1:15,000 births. However, exact figures of these kinds of syndromes are impossible to estimate, because so many mildly affected cases are not diagnosed.

Different collections of diseases are related to DNA methyltransferases mutations as ICF syndrome (immunodeficiency-centromeric instability-facial


Normal tissue
Cancerous tissue
A
B
C
D
E
Expression +
Expression ↓
Expression -
Normal tissue
Cancerous tissue

anomalies) is transmitted as an autosomal recessive trait. It is characterized by immune deficiency in association with unstable paracentromeric heterochromatin instability (extensively related with hypomethylated genomic regions) and facial dysmorphism. Patients are affected by recurrent respiratory infections beginning in childhood. The syndrome directly results from mutations in the gene encoding for DNA-methyltransferase 3B. This may explain the hypomethylation in the pericentromeric repeats observed in the chromosomes of patients.

3. Cancer as Epigenetic Disease

A set of human cancers are developed by *de novo* methylations in genes, mainly tumor-suppressor genes, where promoter methylation diminishes or inhibits normal cell expression and thus confers a growth advantage to the tumor cell (**Fig. 4**). Huge expectations have been raised by the large amount of genetic information relating to cancer biology that has been assembled in the past two decades. CpG island hypermethylation of tumor-suppressor genes may be a valuable tool in the essential transfer of research from the "bench" to the "bedside." The detection of hypermethylation is a "positive" signal that can be accomplished in the context of normal cells, whereas certain genetic changes such as LOH or homozygous deletions are not going to be detected in a background of normal DNA.

In recent years, several groups have extensively mapped from most classes of human neoplasia an increasing number of gene CpG islands aberrantly hypermethylated in cancer (**Table 2**; ***[9]***).

Epigenetics can offer two components to the treatment of caner: prognostic and predictive factors. Prognostic factors will give us information about the virulence of the tumors. For example, p16INK4a hypermethylation has been linked to tumor virulence in lung and colorectal cancer patients ***(18)***. The second component is the group of factors that predict response to therapy. For example, the response to cisplatin and derivatives may be a direct function of the methylation state of the CpG island of hMLH1 ***(19)***. Nevertheless, the most compelling evidence is provided by the methylation-associated silencing of the DNA repair methyltransferase (MGMT) in gliomas and lymphomas, which indicates patients who will be sensitive to chemotherapy with carmustine (BCNU)

Fig. 4. *(Opposite page)* Epigenetic events in tumor progression, from normal epithelium (normal tissue) to dysplasia and carcinoma (cancerous tissue). Five methylation-controled-genes (A–E) represented in their promoter regions: ●, methylated CpG; ○, unmethylated CpG. Normal tissue is represented by normal gene expression and an unmethylated state in tumor-suppressor gene promoters; during progression, expression decreases and hypermethylation in promoters increases. Finally, in a cancerous tissue, promoters are extensively methylated and expression is completely inhibited.

Table 2
Examples of Genes Exhibiting Aberrant Methylation in Cancer

Evasion of apoptosis	APAF-1
	DAPK
	DLC-1
	p14ARF
	p53
	p73
	SHP1
	TMS1
	TRAIL-R1
	XAF1
Insensitivity to anti-growth signals	CyclinD2
	ERα
	LOT1
	p15INK4b
	p16INK4a
	p27KIP1
	p57KIP2
	Pax5
	PTEN
	RARα
	RASSF1A
Limitless replicative potential	pRb
	CDX1
	GATA-4 and -5
	Myf-3
	SOCS-3
Angiogenesis	THBS1
	THBS2
	VHL
Intercellular adhesion and tissue invasion	ADAM23
	E-Cadherin
	H-Cadherin
	CLCA2
	CLDN-7
	laminin-5
	Maspin
	OPCML
	TIMP3
	SLIT2
DNA repair	MLH1
	MGMT
	BRCA1

(Continued)

(20) or cyclofosfamide *(21)*. Three major clinical areas can benefit from hypermethylation-based markers: detection, tumor behavior, and treatment.

3.1. DNA Methylation

Epigenetic modifications of DNA do not alter the sequence but are hereditable and involved in gene regulation and transcription. DNA methylation is a very dynamic processes but the regulation behind this mechanism is very tight. Aberrant methylation in the CpG island-containing-promoters of genes is usually correlated with gene silencing, however in some cases abnormal methylation patterns could be related to gene activation *(22)*. Global DNA hypomethylation has been reported in several human diseases *(13,20)*. Such global hypomethylations occur mainly in repetitive elements around pericentromeric areas. In cancer, global genome hypomethylation is a common finding but, generally, is associated with specific promoter hypermethylation *(23,24)*. In normal mammalian cells, CpG islands in the regulatory regions of certain genes are not methylated, whereas CpG in the remaining genes are methylated by DNMT1. In cancer cells, global DNA hypomethylation and specific promoter hypermethylation occurs (**Fig. 4**) *(25)*.

Aberrant patterns of DNA methylation appear to be affected in several pathways: *p53* is the most frequently mutated gene in human cancers, however. *p53* can also become inactivated through methylation-mediated silencing of the tumor-suppressor gene *p14ARF (26–28)*, which normally inhibits MDM2, an oncogenic protein that induces p53 degradation. Moreover, *p73,* a *p53* homolog, has been shown to be hypermethylated in leukemia *(29)*. Hypermethylation of the cell-cycle inhibitor p16INK4a, a feature common to many tumors, enables cancer cells to escape senescence and begin to proliferate *(30–32)*. The retinoblastoma gene *(RB)* and the cell-cycle inhibitor p15INK4b can also occasionally undergo aberrant methylation *(33,34)*. DNA methylation has a major role in many repair pathways. The consequences of aberrant methylation of repair

Table 2 ***(Opposite page)***

BRCA1, breast cancer 1; DAPK, death-associated protein kinase; E-cadherin, epithelial cadherin; GSTP1, glutathione *S*-transferase p1; MLH1, mutL homolog 1; MGMT, O(6)-methylguanine-DNA methyltransferase; pRb, retinoblastoma; RASSF1a, ras-association domain family 1A; VHL, von Hippel-Lindau; APAF-1, apoptotic protease activating factor 1; DLC1, phosphodynein on microtubules; SHP1, SH2 domain-containing tyrosine phosphatase; TMS1, target of methylation-induced silencing; TRAIL, type II integral membrane protein; XAF1, XIAP-associated factor 1 (XIAP, the X-linked inhibitor of apoptosis); ER, estrogen receptor; PLAGL1/LOT1/ZAC, pleomorphic adenoma of the salivary gland gene like 1; CDX1, homeobox protein CDX-1 (caudal-type-homeobox protein 1); SOCS3, suppressor of cytokine signaling 3; THBS1, thrombospondin 1 precursor; THBS2, thrombospondin 2 precursor; ADAM23; disintegrin and metalloproteinase domain 23; CAV1, caveolin 1; CLCA2, chloride channel, calcium activated, family member 2; CLDN7, claudin 7; OPCML, opioid-binding cell adhesion molecule; TIMP3, tissue inhibitor of metalloproteinase 3; RFC, reduced folate carrier.

pathways include microsatellite instability in sporadic colorectal *(35,36)*, endometrial *(37,38)*, and gastric *(39)* tumors, owing to silencing of the DNA mismatch repair gene *hMLH1*; mutations in *K-RAS* and *p53* caused by hypermethylation of the O6-methylguanine-DNA methyltransferase promoter *(40)*; the prevention of the removal of methyl groups at the O6 position of guanine *(41,42)*; hypermethylation of the mitotic checkpoint gene CHFR *(43)*; and inactivation of *BRCA1* in breast and ovarian tumors *(44)*, which prevents the repair of DNA double-strand breaks and causing global gene-expression changes similar to those present in carriers of *BRCA1* germline mutations *(45)*. Other targets of aberrant methylation, the aberrant methylation of androgen receptors occurs in breast and uterus tumors, may render cancer cells unresponsive to treatment with steroid hormones. Some of the other genes that are affected by DNA methylation are the proapoptotic death-associated protein kinase (DAPK), a target of methylation-induced silencing gene; the von Hippel-Lindau gene in kidney tumors and hemangioblastomas; *LKB1/STK11* (a serine-threonine kinase) in hamartomatous neoplasms, the RAS-related gene, *RASSF1*; thrombospondin 1 (an antiangiogenic factor); cyclo-oxygenase 2; *TPEF*, which comprises epidermal growth-factor domains; and glutathione-*S*-transferase P1 (an electrophilic detoxifier) in tumors of the prostate, breast, and kidney (**Table 2**).

3.2. Histone Acetylation

Chromatin remodeling also plays an important role in the regulation of expression of certain genes. The basic unit of chromatin is the nucleosome, which consists of 146 bp of DNA wrapped around a histone octomer. Modification of the N-terminal group of lysine in histones by acetylation or deacetylation changes the configuration of nucleosomes. The positive charge on unacetylated lysines in the histones is attracted to the negatively charged DNA producing a compact chromatin state that is repressive for transcription. On the other hand, acetylation of the lysines by histone acetylase removes their positive charge and results in an open chromatin structure, which facilitates gene transcription. HDAC removes the acetyl groups from lysine, which reverses this process and silences gene expression (**Fig. 2**). Aberrant deacetylation of histones in nucleosomes is probably a result of a dysregulation of the specificity of HDAC and may be associated with neoplastic transformation. For example, gene translocations in some types of leukemia can generate fusion proteins that recruit HDAC and bind to promoters to silence genes involved in differentiation *(46)*.

For many years, epigenetic research focused on DNA methylation; now, a critical role in epigenetic gene control is assigned to histone modifications. Histone tails are targets for covalent posttranslational modifications, such as acetylation, methylation, and phosphorylation *(47)*. Hypoacetylation of histone-3 and –4 are usually associated with transcriptionally inactive genome regions

inside a global structure called heterochromatin. Acetylation levels and acetylation states are regulated by equilibrium of HAT and HDAC *(48,49)*.

3.3. Histone Methylation

In addition to acetylation of H3 and H4 tails, methylation in lysine residues (Lys4 and Lys9) of histone 3 has been described (**Fig. 1B**). The methylation of lysine in histones by specific histone methylases is also implicated in changes in chromatin structure and gene regulation. The methylation of lysine-4 in histone-3 is associated with an open chromatin configuration and gene expression. On the other hand, the methylation of lysine-9 in histone-3 is associated with condensed and repressive chromatin. This histone modification and the acetylation/deacetylation of histones to influence gene expression are called the histone code *(47)*. A hypermethylated promoter is surrounded by methylated lysine-9 in histone-3, whereas an unmethylated promoter is surrounded by methylated lysine-4 in histone-3 *(50)*. Treatment of tumor cells with 5-AZA reduces the level of methylated lysine-9 in histone-3 and increases the level of methylated lysine-4 in histone-3 in the promoter region of genes silenced by aberrant DNA methylation *(51)*.

As an example of cooperativity between chromatin modifications, it is interesting to note that Lys9 in histone-3 is acetylated in euchromatin (active state for gene transcription) but appears to be methylated in regions of gene-expression silencing *(52)*. Methylated K4 and K79 and acetylated K9 and K14 of H3 are associated with transcriptionally active regions. H4 methylated at K20 is present in heterochromatin regions *(53)*. Histone methylation is catalyzed by histone methyltransferases, a family of proteins with affinity for lysines and arginines. Recent studies demonstrate that peptidyl arginine deiminase 4 (PADI4) specifically deiminates arginine residues R2, R8, R17, and R26 in the H3 tail. This deimination by PADI4 prevents arginine methylation by CARM1. These results define deimination as a novel mechanism for antagonizing the transcriptional induction mediated by arginine methylation. *(54)*. Histone methylation could be involved in the replacement of histones during transcription core dislodging *(55)*. Some histone methyltransferases important in cancer are EZH2 (H3K27 histone methyltransferase), overexpressed in prostate and breast cancer *(56)*, and SMYD3 (H3K4 histone methyltransferase) overexpressed in colorectal and hepatocellular carcinomas *(57)*. Notably, there is a dynamic relationship between DNA methylation and histone modifications. Low levels of histone acetylation and H3K9 methylation recruit DNMT1 and DNA methylation to regulatory regions.

4. Epigenetic Therapy of Cancer

Tumorigenesis is known to be a multistep process in which defects in various cancer genes accumulate *(58,59)*. It is now clear that genetic alterations in human cancers will not provide a complete answer of genomic alterations

behind tumor development, progression, or metastasis. Epigenetic factors cause changes in mechanisms contributing to the malignant phenotype (**Fig. 4**). Epigenetic causes of human diseases, especially of cancer, has given impetus to the development of new therapies for reversing the processes involved. Inhibitors of DNA methylation were the first molecules to appear on the market. To date, several compounds that inhibit DNA methylation are being used in both in vitro and in vivo studies. Clinical trials have shown an incredible decrease in global methylation and specifically demethylation of tumor-suppressor-promoter-CpG-island cancer cells, recovery of the normal expression levels of these genes, and restoration of the normal phenotype. In this field, compounds such as 5-azacytidine, 5-aza-2'-deoxycytidine, zebularine, procainamide, and so on are emerging as powerful and nontoxic tools for cancer therapy.

Knowledge of CpG-island hypermethylation of tumor-suppressor genes may be an important tool in the essential transfer of research from laboratory to clinical practice. In contrast to genetic markers, in which mutations occur in various sites and can be of very different types, promoter hypermethylation occurs only within CpG islands. Furthermore, hypermethylation is a positive signal that can be observed at background levels in normal cells, whereas particular genetic changes, such as loss of heterozygosity and homozygous deletions, cannot be detected so easily. The impetus for DNA methylation studies in cancer has come from two sources. The first is the identification of well-recognized tumor-suppressor genes that undergo methylation-mediated silencing in human cancer, e.g., *BRCA1*, *hMLH1*, *p16INK4a*, *VHL* (**Table 3**) (***60,61***). The second is the emergence of a new technology to study DNA methylation, one based on bisulphite modification coupled with PCR techniques (***62***).

The study of pigenetic silencing in the last years has been on histone modifications, acetylation, methylation, and phosphorylation of histone tails, but it has already begun a shift to new transcription regulation mechanisms, those catalyzed by two groups of proteins HATs and HDACs. As a result, inhibitors of HDACs are growing as a promising therapeutic compound; inhibition of deacetylation as "word play" increases acetylation levels and maintains or remodels the chromatin to an open or gene-activation state. Some of these newly activated-genes are tumor-suppressor genes and cancer-negative-selected genes. HDAC inhibitors reduce cell growth and induce differentiation and apoptosis. Some of the classic and commonly used compounds in this field are butyric acid, valproic acid, suberoylanilide hydroxamic acid (SAHA), depsipeptide, and so on.

Links between DNA methylation and histone acetylation necessarily favor dual therapies, combining DNA methylation inhibitors with HDAC inhibitors. This synergy was profoundly studied in combinations of 5-AZA-CdR and trichostatin A (TSA) (***63–65***). Four major clinical areas can potentially benefit from hypermethylation-based markers: neoplasm detection, studies of tumor

Table 3
Hypermethylated Genes in Cancer, Role in Tumor Development and Tumor Type

SITE	GENE: role in tumor development
Brain	***MGMT:*** DNA repair and drug resistence
Breast	***APC:*** cytoskeletal reorganization
	BRCA1: DNA repair
	E-Cadherin: proliferation, invasion and metastasis
	ER: hormone response
	GSTP1: detoxification
	RASSF1A: control of cell proliferation
Colon	***hMLH1:*** DNA mistmach repair
Endometrium	***hMLH1:*** DNA mistmach repair
Esophageal	***APC:*** cytoskeletal reorganization
Gastric	***E-Cadherin:*** proliferation, invasion and metastasis
	hMLH1: DNA mistmach repair
Head and Neck	***p16:*** cyclin dependent kinase inhibitor
Kidney	***RASSF1A:*** control of cell proliferation
Leukemia	***p15:*** activation of cell proliferation
Lymphoma	***p15:*** activation of cell proliferation
Lung	***APC:*** cytoskeletal reorganization
	p16: cyclin dependent kinase inhibitor
	DAPK1:Suppression of apoptosis.
	MGMT: DNA repair and drug resistence
	RASSF1A: control of cell proliferation
Nasopharyngeal	***RASSF1A:*** control of cell proliferation
NHL	***p16:*** cyclin dependent kinase inhibitor
Oligodendroglioma	***Rb:*** DNA replication and cell division
Ovarian	***BRCA1:*** DNA repair
	hMLH1: DNA mistmach repair
	RASSF1A: control of cell proliferation
Prostate	***ER:*** hormone response
	GSTP1: detoxification
Retinoblastoma	***Rb:*** DNA replication and cell division
Renal	***GSTP1:*** detoxification
	VHL: RNA stability
Squamous cell carcinoma	***p15:*** activation of cell proliferation
Thyroid	***E-Cadherin:*** proliferation, invasion and metastasis

APC, adenomatous polyposis coli; BRCA1, breast cancer 1; CDKN2A/p16, cyclin-dependent kinase 2A; DAPK1, death-associated protein kinase 1; ER, estrogen receptor; GSTP1, glutathione *S*-transferase Pi 1; hMLH1, mut L homolog 1; MGMT, 0-6 methylguanine-DNA methyltransferase; RASSF1, ras-association domain family member; Rb, retinoblastoma; VHL, von Hippel-Lindau; GIT, gastrointestinal tract; NHL, non-Hodgkin's lymphoma.

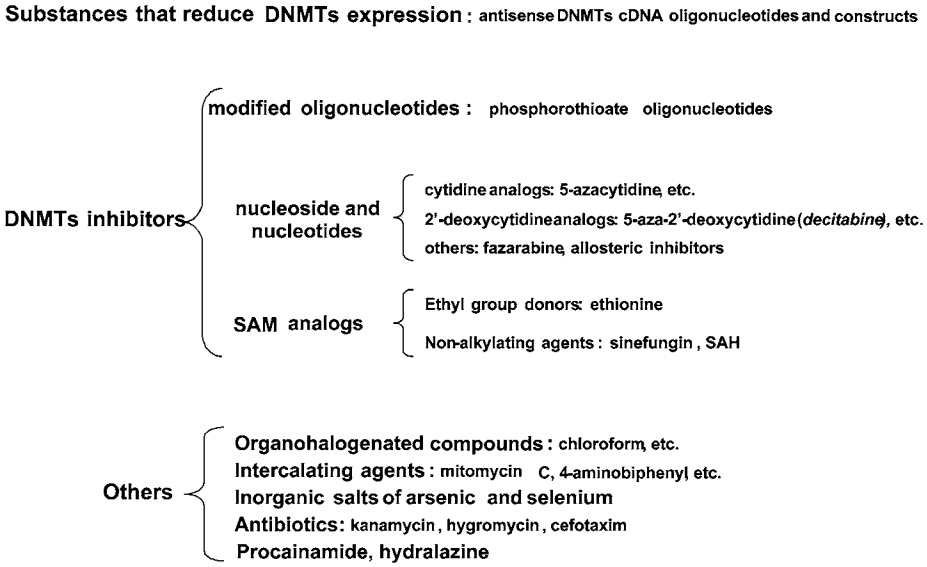


Fig. 5. DNA methylation inhibitors may be used in cancer therapy to modulate hypermethylation of genes and to reactivate antiproliferative, apoptotic, and differentiation-inducing genes in cancer cells. Although some compounds have been proposed for use as DNA methylation inhibitors, these compounds are chemically instable, have weak potency, and can generate toxic metabolites, thus preventing their use as therapeutic agents. Compounds are divided in three categories; substances that directly reduce DNMTs (DNA methyl-transferases), expression, and inhibitors of DNMT activity and others.

behavior, prediction of treatment response, and the development of therapies that target methylated tumor-suppressor genes.

4.1. DNA Methylation in Cancer Therapy

Epigenetic modifications are reversible, whereas genetic modifications are not. This feature makes epigenetic modifications a target for new human therapies. Demethylating agents are "on the crest of the wave" in pharmaceutical development of portfolio molecules (**Fig. 5**).

For several years we have been able to reactivate hypermethylated genes in vitro. One obstacle to the transfer of this technique to human primary cancers is the lack of specificity of the drugs used. Since demethylating agents such as 5-azacytidine or 5-aza-2-deoxicytidine (decitabine) (***66***) inhibit DNMTs and cause global hypomethylation, we cannot reactivate only the particular gene we are targeting. New chemical inhibitors of DNA methylation are being introduced, such as zebularine, and provide us with more hope, but the nonspecificity problem persists. If we consider that only tumor-suppressor genes are

hypermethylated, this would not be a great problem. However, we do not know if we have disrupted some essential methylation at certain sites, and global hypomethylation may be associated with even greater chromosomal instability *(67)*. Another drawback is the toxicity to normal cells, a phenomenon that was in fact observed with the initial higher doses. However, these compounds and their derivatives have been used in the clinic with some therapeutic benefit, especially in hematopoietic malignancies *(68,69)*.

Methylation-associated silencing affects many genes in all existing cellular pathways *(13,61)*. As examples of DNA methylation markers of poor prognosis, we can mention that the death-associated protein kinase, p16INK4a hypermethylation, has been linked to tumor virulence in lung and colorectal cancer patients *(61)*. Not all hypermethylation events are bad: in neuroblastoma, the CpG island hypermethylation of *HOXA9* is associated with poor survival, but the hypermethylation of *RARB2* is an excellent marker of good outcome *(70)*.

However, one of the most attractive possibilities is the establishment of clusters of CpG island hypermethylation in human tumors with prognostic value *(70)*. Studying more than 150 neuroblastomas and using an unsupervised hierarchical cluster analysis of all tumors based on methylation of 10 genes, we separated the three clinically relevant groups of tumors *(70)*. CpG island hypermethylation has been used as a tool to detect cancer cells in broncoalveolar lavage *(71)*, lymph nodes *(72)*, sputum *(73)*, urine *(74)*, semen *(75)*, ductal lavage *(76)*, and saliva *(77)*. Thus, we have shown its versatility across multiple tumor types and environments (**Table 4**).

It was possible to screen for hypermethylated promoter loci in serum DNA from lung cancer patients *(78)* , as well as from a broad spectrum of tumor types *(79,80)*, some screening even using semiquantitative and automated methodologies. The detection of DNA hypermethylation in serum or biological fluids of cancer patients (and even patients at risk of cancer) should encourage academic, governmental, and private agencies to create consortiums of different institutions (and even countries) to develop comprehensive studies to validate the use of these markers in the clinical environment. CpG island hypermethylation could be used as a predictor of response to treatment. The methylation-associated silencing of the DNA repair MGMT in human cancer provides the most compelling evidence. The MGMT protein (O6-methylguanine DNA methyltransferase) is directly responsible for repairing the addition of alkyl groups to the guanine base of the DNA *(81)*. MGMT-promoter hypermethylation predicts a good response to chemotherapy, greater overall survival, and longer time to progression in glioma patients treated with BCNU *(20)*. The potential of MGMT methylation to predict the chemoresponse of human tumors to alkylating agents is not limited to BCNU-like alkylating agents; it also extends to other drugs such as cyclophosphamide *(21)*. This has been demonstrated in diffuse large

Table 4
Detection of Cancer in Body Fluids Using DNA Methylation as Marker[a]

SPECIMEN	TUMOR TYPE	GENE	METH.(%)
Serum/plasma	Prostate	GSTP1	72
	NSCL	APC	47
		p16	73
		DAPK	73
		MGMT	73
		GSTP1	73
	Breast	p16	23
	Colorectal	p16	38
	Esophageal	APC	27
	Liver	p15	81
	Head and neck	p16	42
		DAPK	42
		MGMT	42
		GSTP1	42
Urine	Prostate	GSTP1	36
Ejaculates	Prostate	GSTP1	50
Sputum	NSCL	CDKN2A	50
Ductal lavage	Breast	Cyclin D2	85
		RAR-β	85

[a]Tumor type, gene involved, specimen of fluid, and percentage of methylation.

Meth., methylation; GSTP1, glutathione *S*-transferase Pi 1; APC, adenomatous polyposis coli; CDKN2A/p16, cyclin-dependent kinase 2A; DAPK1, death-associated protein kinase 1; MGMT, O-6 methylguanine-DNA methyltransferase.

cell lymphomas treated with cyclophosphamide, where MGMT hypermethylation was the strongest predictor of overall survival and time to progression, and was far superior to classic clinical factors such as the international prognostic index ***(21)***.

Finally, gene inactivation by promoter hypermethylation may be the key to understanding the loss of hormone response of many tumors. The inefficacy of the antisteroids estrogen–progesterone–androgen-related compounds such as tamoxifen, raloxifene, or flutemide, in certain breast, endometrial, and prostate cancer cases may be a direct consequence of the methylation-mediated silencing of their respective cellular receptors (*ER*, *PR*, and *AR* genes) ***(61,82)***.

Reactivating genes with DNA demethylating agents is an encouraging discovery with respect to avoiding toxic effects. However, it is important to note that the hypermethylation of CpG islands occurs in conjunction with the action of methyl-binding proteins, histone hypoacetylation, and histone methylation, which all contribute to formation of a closed chromatin state and transcriptional silencing *(83)*. Several clinical trials to study these and other mechanisms in patients with cancer are underway in United States and Europe. In such studies, it is essential that the clinical and molecular parameters of response are well defined. Quantitative measurement of 5-methylcytosine DNA after treatment, by use of high-performance capillary electrophoresis *(84,85)*, is an excellent surrogate marker to validate efficacy, as well as demethylation of CpG islands in tumor-suppressor genes, such as p15INK4b *(86)*.

4.2. DNA Methylation Inhibitors as Therapy

Demethylating agents such as 5-aza-cytidine or 5-aza-2-deoxycytidine inhibit DNA methyltransferases and cause global hypomethylation *(87)*. Furthermore, the demethylating effect of 5-aza-2-deoxycytidine seems to be universal, affecting all human cancer cell lines *(68)*. New inhibitors of DNA methylation are being introduced, e.g., procainamide, but the issue of nonspecificity still persists **(Fig. 5)**. Another problem is that at high doses, these agents seem to have toxic effects on normal cells. But despite their drawbacks, these compounds and their derivatives have achieved some therapeutic success in the clinic, especially in hemopoietic disorders such as myelodysplastic syndrome and acute myeloid leukemia *(68,69)*.

One of the most promising clinical scenarios for the use of demethylating drugs is acute promyelocytic leukemia(APL), which is largely caused by transcriptional disruption induced by the PML-RARa translocation. Combined treatment with inhibitors of histone deacetylases, inhibitors of DNA methylation, and differentiating factors (arsenic trioxide may have all three functions) has achieved moderate success in several patients with APL *(88)*. 5-Aza-2'-deoxycytidine alone can also induce the reexpression of silenced, but not hypermethylated, tumor-suppressor genes, such as the proapoptotic gene *APAF1* *(89)*. Although the mechanisms underlying this effect are not fully understood, this drug is known to have additional cytoxic effects, other than those resulting from demethylation, which potentiate the killing capabilities of demethylating compounds, and thus increase their effectiveness in cancer treatment.

The discovery that lower doses of 5-azacytidine associated with inhibitors of HDACs may also reactivate tumor-suppressor genes was encouraging *(63)*. Several phase I trials to test this strategy in human cancer patients are underway. 5-Aza-2'-deoxycytidine alone can even induce reexpression of certain silenced tumor-suppressor genes that do not have an apparent CpG island

hypermethylation, such as *APAF-1* (**89**). These new findings have proved very attractive to several pharmacological and biotech companies, and they are now studying how to accomplish demethylation of cancer cells using novel approaches such as antisense constructs or ribozymes against the DNMTs.

4.3. Histone Acetylation Platform for Cancer Therapy

The core histones are N-terminal tails covalently modified by acetylation, methylation, phosporylation, ubiquitination, sumoylation, and biotinylation (**3**,**90**,**91**). Modifications of specific histone residues (Lys, Arg, Ser) are essential for specific proteins interactions important in gene regulation, chromatin condensation, and remodeling and structure (**92**). Chromatin-modifying enzymes are necessary to generate an open chromatin conformation that permits transcription factors and cofactors positive accessibility to target sequences. Modification of the highly charged lysisne or arginine residues in the N-terminal histone tails is one mechanism of remodeling control (**Figs. 1** and **2**). These residues are susceptible to modifications by acetylation and/or methylation (**47**). This phenomenon appears to be controlled by a set of new emerging enzyme termed as HDACs, together with the ongoing family of HATs.

4.3.1. HDACs as Therapeutic Targets

HDACs modify chromatin by removing acetyl groups from N-terminal tail of histones and from other proteins such as p53 or tubulin (**93**,**94**). The HDAC family is divided in Zn-dependent (class I and II) and Zn-independent (class III) enzymes. Class I and II are the most extensively studies inside the HDAC family proteins. Expression profile of each member is tissue dependent and disease-dependent, critical aim in the development of new therapeutic strategies. Abnormal expression of HDACs is frequent in hematological malignancies. RAR-PML, for instance, could recruit HDACs and cause transcriptional repression and no differentiation (**95**).

4.3.2. HDACs as Therapeutic Molecules

The importance of histone modifications in cancer is illustrated by the marked antitumor activity of different inhibitors of HDAC, both in animal models and in preliminary clinical trials (**46**,**96**). The molecular mechanism of action of HDAC inhibitors is related to their activation of a subset of genes that can produce cell cycle arrest and induce differentiation or apoptosis in tumor cells (**46**,**96**).

Inhibition of HDAC includes natural and synthetic molecules. The naturally occurring antifungal antibiotic TSA was one of the first HDAC inhibitor compounds identified as having antiproliferative activity. Agents identified as HDAC inhibitors can be divided into different structural categories: hydroxamates (such as SAHA and TSA), short-chain fatty acids (such as valproic acid),

cyclic peptides (such as depsipeptide), and benzamides (MS-275). HDAC inhibitors cause differentiation, cell-cycle arrest in G1 and/or G2 and apoptosis in cultured transformed cells and tumors in animals that arise from both hematological and solid tumors. The mechanisms of genetic silencing by HDACs are associated with activation of selected genes *(97)*. Activation of these silenced genes by inhibition of HDACs contribute to repression of tumor cell growth. In practice, the results of treatment with HDAC inhibitors differ by cell type in having both activate and repressive effects *(98,99)*. However new hypotheses about the mechanism of HDAC inhibitors involvement in cancer are emerging; for example, in HepG2 cells, HDACs increased p21WAF1/CIP1 expression not through changes in chromatin structure or by enhancing promoter activity, but by mRNA stabilization *(100)*. All these findings together indicate that HDAC inhibitor treatment results in changes in chromatin structure and an increase of susceptibility to transcription factors, RNA polymerase, or topoisomerases and mRNA stabilization.

Naturally occurring and synthetic HDAC inhibitors are now of interest to pharmaceutical companies because of their great potential use against cancer and other human pathologies. These compounds can be classified according to their chemical nature and mechanism of inhibition as follows **(Fig. 6)**:

4.3.2.1. Hydroxamic Acids

This is probably the broadest set of HDAC inhibitors. Most of the chemicals in this group are very potent but reversible inhibitors of class I/II HDACs. Among these compounds we find TSA, which was one of the first HDAC inhibitors to be described *(101)* and is widely used as a reference in research in this field. However, its toxicity to patients and lack of specificity for certain HDACs has motivated the search for other substances. The design of many synthetic drugs has been inspired by TSA structure: from the simplicity of SAHA to the latest drugs including NVP-LAQ-824 *(102,103)* and PXD-101 *(104)*.

4.3.2.2. Carboxylic acids

There are few drugs in this group: butanoic *(105)*, valproic *(106,107)*, and 4-phenylbutanoic *(108)*. Despite being much less potent than the hydroxamic acids and their pleiotropic effects, these are currently among the best studied HDAC inhibitors: valproic acid and phenylbutyrate have already been approved for use in treating epilepsy and some cancers, respectively, whereas butanoic acid is undergoing clinical trials *(109,110)*.

4.3.2.3. Benzamides

MS-275 and some of its derivatives inhibit HDACs in vitro at micromolar concentrations, but the mechanism is not clearly understood. MS-275 and *N*-acetyldinaline are undergoing clinical trials *(111–113)*.

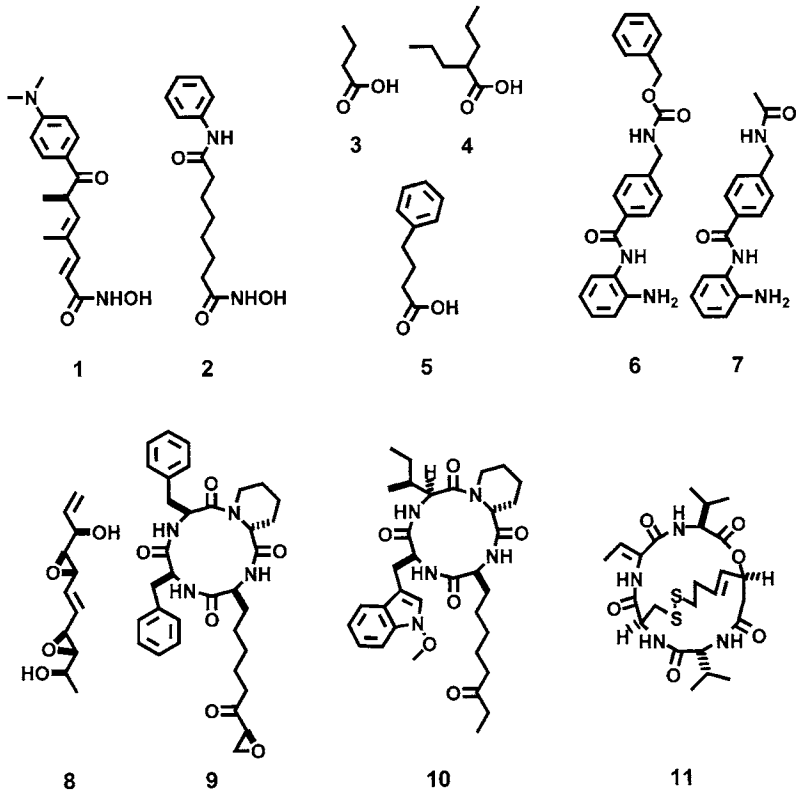


Fig. 6. Examples of chemicals included in the different HDAC inhibitor groups (*see* text). (1) TSA, (2) SAHA, (3) butanoic acid, (4) valproic acid, (5) 4-phenyobutanoic acid, (6) MS-275, (7) N-acetyldinaline, (8) depeudecin, (9) trapoxin A, (10) apicidin, and (11) depsipeptide FK228.

4.3.2.4. Epoxides

The only HDAC inhibitors in this set of compounds are a number of natural products with significant in vitro activity, such as depeudecin, trapoxin A ***(114)***.

4.3.2.5. Others

Depsipeptide FK228 (a fungal metabolite) is also undergoing clinical trials, but the mechanism by which it inhibits classical HDACs in vitro remains unknown. Apicidin A is another fungal metabolite that is able to inhibit HDACs in many organisms, from protozoa to humans, at micromolar concentrations. Apicidins B and C (also natural products) have the same structure, differing from apicidin A by a single residue. Trapoxins are also cyclic tetrapeptides that are closely related to apicidins. However, the main difference between the two

Table 5
Mammalian HATs and HDACs[a]

HDAC	HAT
Class I	**GNAT family**
HDAC1	PCAF
HDAC2	GCN5L2
HDAC3	**CREBBP family**
HDAC8	CREBBP
Class II	EP300
HDAC4	**MYST family**
HDAC5	HTATIP
HDAC6	ZNF220
HDAC7	HB01
Sirtuins	MORF
SIRT1	MYST1
SIRT2	**TAFII 250 family**
SIRT3	TAFII 250
SIRT4	**SRC family**
SIRT5	ACTR
SIRT6	SRC1
SIRT7	SRC3
	NCOA2
	Other HATs
	TCF2
	GTF3C1

[a]HATs are divided into six different families (GNAT, CREBBP, MYST, TAFII, SRC, and others) and HDAC in three classes (class I, class II, and sirtuins).

ACTR, activin receptor; CREBBP, CREB-binding protein; EP300, e1a-binding protein p300; GCN5L2, general control of amino-acid synthesis 5-like 2; GNAT, GCN5-related acetyltransferase; GTF3C1, general transcription factor 3c, polypeptide 1; HAT, histone acetyltransferase; HBO, histone acetyltransferase binding to ORC; HDAC, histone deacetylase; HTATIP, HIV tat interactive protein; HIV tat, human immunodeficiency virus type I transacting transcription factor; MORF, MOZ-related factor; MOZ, monocytic leukemia zinc finger protein; MYST, MOZ, YBF2/SAS3, SAS2, TIP60 protein family; NCOA2, nuclear receptor coactivator 2; ORC, original recognition complex; PCAF, EP300/CREBBP-associated factor; SIRT, sirtuin; SRC, steroid receptor coactivators; TAF, TATA box-associated factors; TCF2, transcription factor 2; ZNF220, zinc finger protein 220.

groups of substances is that the former bears epoxyketone functionality rather than an alkylketone functionality, which makes the compound much less stable under physiological conditions *(115–118)*.

4.3.3. HATs as Therapeutic Targets

The addition of acetyl groups from universal donor acetyl-CoA on lysine residues placed on histone tails is catalyzed by HATs (**Table 5**). Thus, histone

tails acetylation is the best-characterized of histone modifications, demonstrating a positive association between acetylation levels and gene expression profiles. In addition, acetylated histones mean actively transcribed regions of chromatin *(119)*. HATs represent an active group of proteins important in replication, apoptosis, repair, and cell cycle. This crucial function in several cellular mechanisms makes HAT damage an important step in human diseases. In cancer, hematological malignancies with chromosomal translocation express chimeric HAT proteins that gain functions *(120)*. In such solid tumors as breast, colon, and gastric cancers, mutation in HAT genes have also been reported *(119)*. Nonfunctional CBP is the main cause of the Rubinstein-Taybi syndrome *(121)* and is in the clinical etiology of neurological disorders such as Huntington disease, Alzheimer's disease, and muscular atrophy *(119,122)*.

HATs specific inhibitors or activators remain on portfolio molecules although much is known about substrates and mechanisms. To date, few molecules have been identified without clinic applicability (Lysyl.CoA, H3-CoA-20, and anacardic acid *[120]*).

5. Epigenetic Diagnosis

Such mapping of DNA methylation has highlighted the existence of a unique profile of hypermethylated CpG islands that defines each tumor type (**Table 2**) *(60,61)*. Several groups are currently attempting to define the DNA methylation signature (methylotype) of each type of human cancer. Only methylation markers that are always unmethylated in normal cells can be used for methylation profiling, but by combining three or four methylation markers, we can extract the greatest possible amount of useful information, because hypermethylation events at different loci are unrelated *(60)*.

For epigenetic markers to be clinically useful, ways of detecting hypermethylation in the CpG islands of tumor-suppressor genes that are quick, nonradioactive, and sensitive are required, such as methylation-specific PCR *(123)*. Methylation-specific primers should be developed in stringent conditions with the inclusion of positive and negative controls to avoid false-positive results. CpG island hypermethylation has been used as a tool to detect cancer cells in several types of biological fluids and biopsy samples. It was possible to screen for hypermethylated promoter loci in DNA from the serum of patients with lung cancer *(78)*. Thus, DNA hypermethylation has proved its applicability in the detection of wide range of tumor types.

The promoter hypermethylation of CpG islands in tumor-suppressor genes occurs early in tumorigenesis. But the presence of aberrant CpG island methylation alone does not necessarily indicate an invasive cancer because premalignant or precursor lesions can also carry this epigenetic marker. This finding has implications for early detection of cancer, especially in people with a high inherited

risk, because patterns of CpG island hypermethylation are the same between familial and sporadic cancers *(124)*. Aberrant DNA methylation has been found up to 3 yr before diagnosis of lung cancer in individuals, such as uranium miners and smokers, who have been exposed to large amounts of carcinogens *(73)*.

Standardization and validation of techniques for detecting changes in methylation are vital. Detection of DNA hypermethylation in the biological fluids of patients with cancer and those at risk of cancer should lead to comprehensive studies to justify the use of these markers in the clinic, through the establishment of multidisciplinary consortiums (**Table 4**).

One of the most important steps for conferring on CpG island hypermethylation a critical role in the origin and progression of a tumor is the demonstration of biological consequences of the inactivation of that particular gene. A good example is provided by the DNA repair genes *hMLH1*, *MGMT*, and *BRCA1*, in which methylation-associated inactivation may change the entire genetic environment of the cell. In the first case, there is a lack of mutations in the mismatch repair genes in sporadic tumors, and the main cause of the presence of microsatellite instability in the sporadic cases of colorectal, endometrial, and gastric cancer is the transcriptional inactivation of hMLH1 by promoter hypermethylation *(125,126)*. In the second case, the DNA repair gene O6-methylguanine DNA MGMT removes the promutagenic O6-methylguanine from the DNA. However, the DNA repair gene MGMT can be transcriptionally silenced by promoter hypermethylation in primary human tumors *(127)*. Most importantly, these MGMT-methylated tumors accumulate a considerable number of G-to-A transition mutations, some of them affecting key genes such as *K-ras* and *p53 (81)*. Finally, in the case of *BRCA1,* its hypermethylation-associated inactivation *(44)* produces the same profound disruption of expression profiles as do the *BRCA1* germline mutations *(128)*.

One of the most critical steps in giving CpG island methylation of a particular gene its true value is the fact that it should occur in the absence of gene mutations. Both events (genetic and epigenetic) abolish normal gene function and their coincidence in the same allele would be redundant. There are multiple examples but three are worth mentioning. First, the cell cycle inhibitor p16INK4a in one allele of a few colon and bladder cancer cell lines has a genetic mutation while the other is wild-type: p16INK4a hypermethylation occurs only on the wild-type allele, whereas the mutated allele is kept unmethylated *(129,130)*. A second example is that of APC, the gatekeeper of colorectal cancer, which is mutated in the vast majority of colon tumors. When APC methylation occurs in that type, it is clustered in the APC wild-type cases *(131)*. Finally, in tumors from families that harbor a germline mutation in tumor-suppressor genes, only those tumors that still retain one wild-type allele undergo CpG island hypermethylation *(124)*.

6. Epigenetic Prognosis

The most compelling evidence for predicting treatment response is provided by the methylation-associated silencing of O6-methylguanine-DNA methyltransferase. This protein is responsible for the removal of alkyl groups from guanine, which is the preferred point of DNA attack of several alkylating agents used in cancer treatment, such as carmustine, nimustine, procarbazine, streptozotocin, and temozolamide. Thus, tumors that lack function of O6-methylguanine-DNA methyltransferase owing to hypermethylation *(40)* are more sensitive to the action of alkylating agents, because there is no pathway to repair the damage these drugs cause.

In a study of patients with glioma who were treated with carmustine, we found that hypermethylation of the O6-methylguanine-DNA methyltransferase promoter was indicative of a good response to chemotherapy, greater overall survival, and longer time to progression *(20)*. The potential of O6-methylguanine-DNA methyltransferase for predicting the response of tumors to chemotherapy is not limited to carmustine-like alkylating agents, but also extends to drugs such as cyclophosphamide *(61)*. This capability has been shown for diffuse large-cell lymphomas treated with cyclophosphamide, where hypermethylation of O6-methylguanine-DNA methyltransferase was the strongest predictor of overall survival and time to progression, and was far better than classic clinical factors such as the international prognostic index *(121)*. More studies are needed to clarify this issue as the findings may have a direct effect on treatment of cancer.

Gene inactivation by promoter hypermethylation may be a crucial step in the loss of hormone responsiveness of many tumors. The lack of effectiveness of antisteroidal drugs, such as tamoxifen, raloxifene, and flutemide, in some patients with breast, endometrial, and prostate cancer may be a direct consequence of methylation-mediated silencing of their respective cellular receptors. A similar explanation can be applied to the lack of success with preventive retinoid treatment. It may be that premalignant lesions become insensitive to retinoids because of epigenetic silencing of genes that are crucial to the retinoid response, particularly the retinoic acid receptor 2 (RAR 2) *(132,133)*, and the cellular retinol-binding protein I (CRBPI) *(134)*. We have shown that supplementation of dietary retinoids prevents the aberrant methylation of RAR 2 and CRBPI in colorectal tumorigenesis *(134)*; DNA demethylating drugs can be given, if necessary, to improve treatment.

7. Clinical Applications

Most current DNA-demethylating agents (**Fig. 5**) block the action of DNMTs *(135)*. The cytidine and 2-deoxycytidine analogs of cytosine are the most extensively studied drugs. The first analog tested to determine whether

it was an inhibitor of DNA methylation was 5-azacytidine. The second analog reported was 5-aza-2-deoxycitidine (decitabine), one of the most commonly used demethylating drugs in assays with cultured cells. All of these compounds only inhibit DNMTs when incorporated into double-strand DNA ***(135)***. Zebularine (1-[beta-D-ribofuranosyl]-1,2-dihydropyrimidin-2-one) is another cytidine analog that has recently been developed ***(136,137)***. Perhaps the most interesting feature of this DNA-demethylating agent, compared with 5-azacytidine and 5-aza-2-deoxycytidine, is that it is chemically stable and of low toxicity, being the first drug in its class that can be given orally ***(136,137)***. The use of the nucleoside analogs in clinical trials has been limited by their side effects, such as thrombocytopenia and neutropenia, which are probably a result of cytotoxic effects associated with the drug's incorporation into the DNA independently of their DNA-hypomethylation value. This has encouraged the search for inhibitors of DNA methylation that are not incorporated into DNA. In this category, the drugs procainamide and procaine, approved by the FDA for the treatment of cardiac arrhythmias and as a local anesthetic, respectively, also act as nonnucleoside inhibitors of DNA methylation ***(138,139)***. The demethylating effect of 5-aza-2-deoxycytidine seems to be universal, affecting all human cancer cell lines ***(85)***. This is the conclusion that may be drawn from cancer-cell-line and mouse-tumor models, although we really do not know the molecular and cellular responses of cancer patients in their entirety.

Two phases in the clinical use of DNA-demethylating agents can be outlined. The first was during the 1970s and 1980s when high, and frequently significantly toxic, doses of 5-azacytidine and 5-aza-2-deoxycytidine were used to treat leukemia. At this time, their hypomethylating properties had not been fully recognized. The second period is marked by the acceptance of the idea that low doses of these drugs will induce cell differentiation and stop the growth of cancer cells by restoring the expression of silent tumor-suppressor genes.

Several phase I/II trials have been developed for solid tumors. However, it is in the field of hematological malignancies where DNA-demethylating agents have had their greatest success so far. Studies have found overall response rates of 40–54%, with 23–29% complete responses using 5-aza-2-deoxycytidine (decitabine) in myelodysplastic syndrome ***(68,69,140)***. For 5-azacytidine, a similar scenario can be drawn with a significant number of complete and partial remissions in myelodysplastic syndrome patients ***(141,142)***. The definitive support for an epigenetic treatment of hematological malignancies was provided in 2004, with the approval by the FDA of the use of 5-azacytidine (Vidaza) for the treatment of all myelodysplastic syndrome subtypes (http://www.fda.gov/bbs/topics/news/2004/NEW01069.html).

Table 6
Histone Deacetylase Inhibitors in Preclinical and Clinical Development[a]

Clinical Trials	HDAC Inhibitor	Family
+	Acid bishydroxamic	Hydroxamic acid
+	Acid (CBAH)	Hydroxamic acid
	Oxamflatin	Hydroxamic acid
+	Suberoylamilide	Hydroxamic acid
	SAHA	Hydroxamic acid
+	Trichostatin a (TSA)	Hydroxamic acid
+	Butyrate	Short-Chain-Fatty-Acid
+	Valproic acid	Short-Chain-Fatty-Acid
	Phenylacetate	Short-Chain-Fatty-Acid
	Apicidin	Cyclic Tetrapeptide
	Trapoxin A	Cyclic Tetrapeptide
+	Depsipeptide	Tetrapeptide
	Depudecin	Epoxide
+	MS275	Benzamide
+	LAQ824	

[a]Histone acetylation and deacetylation is modulated by the interplay between histone acetyltransferases (HATs) and histone deacetylases (HDACs), and imbalances of this process cause multisystem diseases and cancers. Biochemical structures are indicated. "+" Denotes a phase I or II clinical trials.

It is clear from in vitro and preclinical studies that HDACs have great potential as anticancer drugs, but their value will be established by the ongoing clinical trials (**Table 6**).

Multiple phase I and phase II clinical trials with many HDACs have now been completed, and others are being initiated. A phase I trial for depsipeptide in patients with postthymic lymphoma unresponsive to chemotherapeutic regimens showed partial and complete clinical responses with minimal side effects (***143***). Another phase I trial of depsipeptide in patients with refractory neoplasms yielded biologically active serum concentrations of the drug and provided a recommended dose for the phase II trials (***144***). Clinical trials of valproic acid (Mount Sinai School of Medicine), MS-275 (National Cancer Institute), and SAHA (Memorial Sloan-Kettering Cancer Center) are currently in their final stages. In the case of SAHA, a phase I trial has shown that is well

tolerated, induces histone acetylation and has antitumor activity in solid and hematological tumors *(145)*.

Clinical trials have also been undertaken to examine the combination of HDAC inhibitors with DNA-demethylating agents, such as decitabine (5-azacytidine). A treatment scheme for acute myelogenous leukemia (AML) patients entailing subcutnaeous injections of 5-azacytidine for seven consecutive days followed by 5 d of iv phenylbutyrate was well tolerated, and a reduction in bone marrow blasts and increased myeloid maturation were observed *(146)*. A similar study initiated at the Johns Hopkins University of patients with myelodysplastic syndrome and AML also indicated good tolerance, and significant hematopoietic improvements were observed in several patients.

8. Future Directions

The main goal in biomedical cancer research is to find therapies that can reverse silencing in human diseases. Epigenetic therapy places new drug discovery in a critical role. Here, it is important to remember that epigenetic diseases are developed by abnormal hypermethylation of CpG island-containing-promoters that seem to be particularly frequent in cancer cells, so DNA demethylating agents or histone deacetylating inhibitor compounds specifically targets cancer cells and certain kind of cancers. Now is just the beginning of our understanding of epigenetic causes of human diseases but is at the same time the beginning of new families of antitumoral compounds. There is much to learn about enzymes involved in the epigenetic pathways and the tight regulation of their activities, but this question will provide new opportunities for cancer therapy. The list of all possible histone modifications is not yet complete. In the future, the manipulation of the epigenetic landscape may indeed prove to be a key element of cancer therapy.

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3

Wnt Signaling as a Therapeutic Target for Cancer

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Summary

The Wnt/β-catenin signaling pathway is tightly regulated and has important functions in development, tissue homeostasis, and regeneration. Deregulation of Wnt/β-catenin signaling is frequently found in various human cancers. Eighty percent of colorectal cancers alone reveal activation of this pathway by either inactivation of the tumor-suppressor gene adenomatous polyposis coli or mutation of the proto-oncogene β-catenin. Activation of Wnt/β-catenin signaling has been found to be important for both initiation and progression of cancers of different tissues. Therefore, targeted inhibition of Wnt/β-catenin signaling is a rational and promising new approach for the therapy of cancers of various origins.

Key Words: Wnt signalling; adenomatous polyposis coli; *APC*; β-catenin; Tcf; LEF; targeted therapy; small molecules; antisense; siRNA; RNAi.

1. Introduction

The term "targeted therapy" is commonly defined as a treatment strategy focused on a specific molecular target or biological pathway involved in the genesis of a malignant process that can be detected by diagnostic testing prior to therapy *(1)*. Ideally, the target is crucial for the initiation and/or progression of the malignant process, but not for homeostasis and regeneration of normal tissues. Its inhibition only leads to regression of tumors expressing the target. Several monoclonal antibodies, small molecules, and mRNA-targeting approaches are currently under investigation. In fact, the first targeted anticancer therapies were the development of antihormonal treatments in breast, prostate, and thyroid cancer. The antiestrogen tamoxifen is directed against the estrogen receptor and is used in the treatment of breast cancer and for chemoprevention in high-risk women *(2)*.

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Trastuzumab (Herceptin®) is a humanized monoclonal antibody directed against the HER-2/neu (c-erb-B2) antigene. It is successfully used in the treatment of HER-2/neu-positive advanced breast cancer *(3)*. The chimeric monoclonal antibody cetuximab (Erbitux®) binds to the epidermal growth factor receptor (EGFR), which is frequently over expressed in several human cancers *(4)*. Cetuximab has been approved for advanced colorectal cancer in combination with the cytotoxic chemotherapeutic agent irinotecan (CPT-11). The humanized murine monoclonal antibody bevacizumab (Avastin®) targets the vascular endothelial growth factor (VEGF), which regulates vascular proliferation and survival of newly formed blood vessels *(5,6)*. It has received approval for use in metastatic colorectal cancer. Unlike trastuzumab and cetuximab, which require immunohistochemical proof of expression of the targeted receptor protein in the tumor prior to treatment, cetuximab does not require a diagnostic test. The small molecule inhibitor imatinib (Glivec®, Gleevec®) has received approval for use in chronic myelogenous leukemia (CML) and in gastrointestinal stromal tumors (GIST) *(7,8)*. It is directed against the *bcr-abl* fusion gene product in CML and the tyrosine-kinase receptor c-kit, which is oncogenically activated by mutation and overexpressed in GIST. It leads to complete hematologic and cytogenetic remissions in the early chronic phase of CML and to a strong increase in the 1-yr survival rate of patients with advanced GIST *(9,10)*. Antisense oligonucleotides are single-stranded DNA or chimeric DNA/RNA that specifically hybridize to a targeted mRNA and subsequently prevent translation of the mRNA *(11,12)*. Clinical trials are underway targeting genes deregulated in cancer, including *bcl-2* in malignant melanoma and prostate cancer, *c-myc* in prostate cancer, and protein kinase Cα in ovarian cancer *(13–16)*. These and many more targeted therapies are currently under development and clinical testing. They bear great potential for the revolution of treatment of advanced cancers.

Wnt/β-catenin signaling is an essential pathway in embryological development and tissue homeostasis. Its deregulation has been implicated in human cancers of various tissues and a still growing list of inborn diseases and developmental defects including tetra-amelia, an intersex phenotype, polycystic kidney disease, familial exsudative vitreoretinopathy, tooth agenesis, and colon cancer *(17–22)*. As deregulated Wnt/β-catenin signaling is not only involved in cancer initiation but also in cancer progression *(23–25)* its targeted disruption is a rational and promising new approach with great potential in cancer therapy.

2. Regulation of Wnt Signaling

Wnt is a fused term composed of the names of two orthologs, the *Drosophila* segment polarity gene *Wingless (Wg)* and the mouse proto-oncogene *Int-1 (26)*.

The Wnt signaling pathway, first described in *Drosophila* as *Wingless* pathway, is highly conserved among flies, frogs, and mammals. It has critical functions in embryological development, tissue morphogensis, cell-fate determination, and self-renewal of tissues ***(27)***. Physiological signaling is initiated by binding of soluble Wnt ligands to the *Frizzled* (*Fz*) family of seven-pass transmembrane receptors. This results in the activation of one of three different Wnt-regulated pathways: the canonical *Wnt/β-catenin* pathway and the two noncanonical pathways, the *Wnt/planar cell polarity* (*Wnt/PCP*) and the *Wnt/calcium* (*Wnt/Ca^{2+}*) pathway. The canonical *Wnt/β-catenin* pathway regulates the stability of the proto-oncogene β-catenin, and its activation leads to β-catenin/Tcf-dependent transcription ***(28)***. Its constitutive deregulation can result in the development of various benign and malignant tumors ***(29)***. The *Wnt/PCP* pathway acts through activation of the *c-Jun* N-terminal kinase (JNK) pathway, the *Wnt/Ca^{2+}* pathway is activated through a heterotrimeric G protein and results in an increase in intracellular calcium and activation of protein kinase C ***(30)***. Interestingly, the transcription factor c-Jun is regulated on different levels by both *Wnt/PCP* and *Wnt/β-catenin*: activation of the JNK pathway results in activation of c-Jun by phosphorylation and, in addition, c-Jun is a transcriptional target of β-catenin/Tcf ***(31)***. The JNK pathway functions in the control of cell motility, epithelial morphogenesis, and regulation of apoptosis ***(32,33)***. It has both been implicated in oncogenic transformation and tumor cell proliferation as well as in the induction of apoptosis in transformed cells ***(34)***. The function of the *Wnt/Ca^{2+}* pathway is so far unknown. In the following, this review will focus on the canonical Wnt/β-catenin signaling pathway (**Fig. 1**) and its therapeutic targeting.

2.1. Initiation of Wnt Signaling at the Cell Membrane

Wnt proteins constitute a large family of at least 19 secreted small glycoproteins, all of which contain 23–24 cysteine residues ***(35)***. They bind as ligands to the frizzled family of seven-span transmembrane receptors ***(36)***. This receptor family of at least 10 members is also characterized by a cysteine-rich domaine. However, canonical Wnt signaling is only initiated when Wnt is simultaneously complexed with both a frizzled receptor and one of the low-density lipoprotein receptor-related proteins LRP-5 or LRP-6, which act as coreceptors ***(37)***. Wnt factors and frizzled receptors can be classified in accordance with the Wnt pathway they preferentially activate. However, these preferences are not rigid as several Wnts can interact with different frizzled receptors ***(30)***. Wnt signaling can be antagonized at the membrane level by various secreted proteins which include soluble frizzled-related proteins (sFRPs) ***(38)***, Wnt inhibitory factor-1 (WIF-1) ***(39)***, and Dickkopf (Dkk) ***(40,41)***. The sFRP family consists of five members, which can squelch Wnt before binding to Fz and inhibit canonical

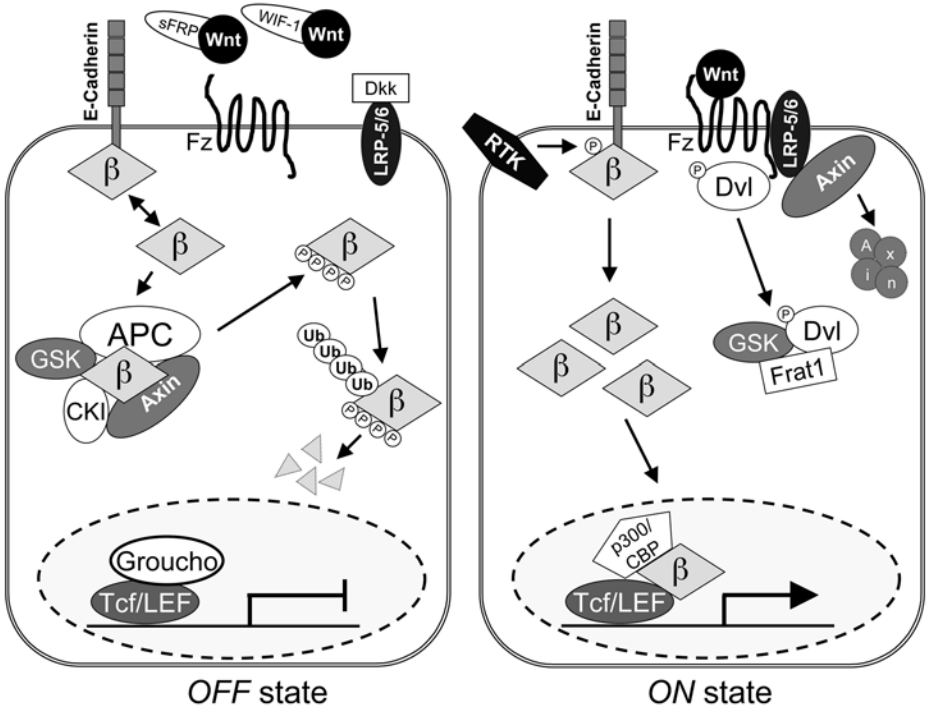

Fig. 1. The main actors of the Wnt/β-catenin signal transduction pathway in the "OFF" and the "ON" state. (Left panel) "OFF" state: β-catenin (β) is found in the cytoplasmic pool and in the E-cadherin bound pool, linking the cell membrane with the actin cytoskeleton. Soluble frizzled related proteins (SFRPs) and Wnt inhibitory factor (WIF)-1 inhibit binding of extracellular Wnt factors to frizzled receptors (Fz), Dkk (Dickkopf) blocks the LRP-5/6 coreceptors. In the absence of Wnt factors binding to Fz the free cytoplasmic pool of the proto-oncogene β-catenin is tightly regulated: the destruction complex composed of the tumor suppressor protein APC (*adenomatous polyposis coli*), the scaffold protein axin, and the kinases glykogen synthase kinase 3β (GSK) and casein kinase I α (CKI) facilitates phosphorylation (P) of 4 serine and threonine residues in the amino-terminus of β-catenin. This is the prerequisite for ubiquitination (Ub) and subsequent proteasomal degradation of β-catenin. In absence of β-catenin in the nucleus the transcriptional repressor Groucho is bound to the Tcf/LEF transcription factors and no target genes are transcribed. (Right panel) "ON" state: upon binding of Wnt ligands to Fz and the LRP-5/6 coreceptors dishevelled (Dvl) is phosphorylated. Phosphorylated Dvl inhibits GSK by forming a complex with GSK and Frat1. Axin is degraded after binding to LRP-5/6. In consequence, the destruction complex can no longer be constituted and β-catenin is stabilized. β-catenin can be liberated from its binding to E-cadherin by phosphorylation of tyrosine residues by receptor tyrosine kinases (RTK). Upon translocation of β-catenin to the nucleus it binds to Tcf/LEF factors and other proteins including p300/CBP and activates the transcription of multiple target genes.

and noncanonical Wnt signaling. Dkk proteins (Dkk-1, -2, -3) block canonical Wnt signaling by binding to LRP-5/6, which inhibits Wnt binding and induces endocytosis of LRP-5/6 *(42,43)*.

2.2. Regulation of β-Catenin in the Cytoplasm

β-catenin is synthesized continuously. The majority of cellular β-catenin is found within the cadherin bound pool at the cell membrane, linking adherens junctions with the cytoskeleton *(44)*. Phosphorylation of tyrosine residues of β-catenin by receptor tyrosine kinases such as c-RON, EGFR, and c-ErbB2 can lead to dissociation of β-catenin from adherens junctions and to its liberation into the cytoplasm *(45–47)*. The free cytoplasmic pool of β-catenin is tightly regulated. This involves three steps: (1) β-catenin phosphorylation of amino-terminal serine and threonine residues, (2) β-catenin ubiquitination, and (3) β-catenin degradation. (1) In the absence of a Wnt signal a multiprotein β-catenin destruction complex is formed in order to facilitate phosphorylation of β-catenin at four serine and threonine residues in its amino terminal destruction box. In addition to β-catenin this complex includes the scaffold protein axin, the tumor suppressor adenomatous polyposis coli (APC), glycogen synthase kinase 3β (GSK3β), casein kinase Iα (CK1α), and the protein phosphatase 2A (PP2A) *(48–52)*. This is the prerequisite for three phosphorylation steps to occur. First, the complex is stabilized by phosphorylation of axin and APC by GSK3β. Second, degradation of β-catenin is initiated by priming phosphorylation of serine residue 45 (S45) by CK 1α *(53,54)*. Third, the three remaining residues threonine T41, S37, and S33 are sequentially phosphorylated by GSK3β *(53)*. (2) Phosphorylated β-catenin is then recognized by β-TrCP, a subunit of an SCF-type E3 ubiquitin ligase complex and ubiquitinated *(55,56)*. (3) This targets β-catenin for degradation by the proteasome system *(57)*.

Degradation of β-catenin is inhibited upon activation of the Wnt signaling cascade. After formation of the Wnt/Fz/LRP-5/6 complex the proteins dishevelled (Dvl) and axin are recruited to the cell membrane. After phosphorylation by casein kinase Iε (CK Iε) *(58)* Dvl forms a complex with Frat1 and GSK3β resulting in the inhibition of GSK3β *(59)*. Of note, Dvl is thought to be the central switch at which the three Wnt signaling pathways branch off. Binding of axin to LRP-5/6 results in its degradation *(60)*. In consequence, the destruction complex is destabilized and β-catenin is no longer phosphorylated. This results in the accumulation of β-catenin in the cytoplasm and its subsequent translocation to the nucleus.

In addition to phosphorylation-dependent ubiquitination β-catenin can also be ubiquitinated by an alternative protein complex requiring the interaction of Siah1, a p53-induced gene, with the carboxy-terminus of APC, and the F-box

protein EBI *(61–63)*. However, the significance of this alternative way of β-catenin ubiquitination next to the main phosphorylation-dependent degradation pathway is unknown.

2.3. Execution of β-Catenin/Tcf Signaling in the Nucleus

In the presence of a Wnt signal, free cytoplasmic β-catenin can translocate to the nucleus. As β-catenin cannot bind to DNA itself it must complex with Tcf/LEF factors in order to activate transcription *(64,65)*. Tcf1, Tcf3, Tcf4, and LEF1 constitute the Tcf/LEF family of high mobility group (HMG) proteins. In the absence of nuclear β-catenin they are complexed with the transcriptional repressor Groucho *(66,67)*. Nuclear β-catenin competes with Groucho for Tcf/LEF binding. In the β-catenin/Tcf/LEF complex β-catenin serves as transcriptional activator whereas Tcf/LEF facilitates DNA-binding to the consensus motif (A/T)A/T)CAA(A/T)G *(68)*. The large β-catenin/Tcf/LEF transcriptional complex contains a number of additional proteins including the histone acetyltransferases p300/CBP, Brg-1, a component of the mammalian SWI/SNF and Rsc chromatin-remodelling complexes, and the TATA-binding protein Pontin52 *(69–73)*. The first β-catenin-regulated target genes identified were c-myc and cyclin D1 *(74–76)*. Other important target genes include the VEGF *(77)*, the matrix metallo-proteinases MMP7, MMP14, and MMP26 *(78–80)*, connexin 43 *(81)*, and the peroxisome proliferator-activated receptor δ (PPARδ) *(82)*. As the list of β-catenin target genes also includes several transcription factors, e.g., c-myc, c-Jun, ITF-2, and Sox9 *(31,74,83,84)* the number of directly and indirectly regulated genes is immense, for a comprehensive overview the reader is referred to the Wnt Homepage (http://www.stanford.edu/~rnusse/wntwindow.html). Together the β-catenin target genes have important functions in the regulation of many cellular processes including cell proliferation, cell cycle progression, apoptosis, differentiation, tissue invasion, and angiogenesis.

3. Wnt Signaling in Cancer

Deregulation of the Wnt signaling pathway can be found in many different human cancers. Changes in expression levels have been described for many components of the Wnt pathway. Overexpression of Wnt factors has been reported in several primary human malignancies including gastric cancer, head and neck squamous cell carcinoma, colon carcinoma, and chronic lymphocytic leukemia *(85–88)*. Several frizzled receptors have been found to be upregulated in esophageal, gastric, and colon cancers as well as in head and neck squamous cell carcinomas *(86,87,89,90)*. The Wnt coreceptor LRP-5 has recently been reported to be overexpressed in osteosarcoma *(91)*. Overexpression of dishevelled has been found in primary breast cancer, cervical squamous cell carcinoma, and mesothelioma *(92–94)*. Also Frat1, which

is supposed to be involved in the inhibition of GSK3β, has been described to be overexpressed in several primary human cancers including gastric, esophageal, pancreatic, cervical, and breast *(85,95)*. However, the functional consequences of overexpression of several of these factors remains to be demonstrated.

In addition to upregulation of expression levels of activators of Wnt signaling downregulation of expression has been demonstrated for the secreted inhibitors of Wnt signaling sFRPs and WIF-1. sFRPs have been found to be downregulated in breast, bladder, and colorectal cancers as well as in mesothelioma *(96–99)*. WIF-1 expression has been reported as being repressed in prostate, breast, lung, bladder, and colorectal cancers *(100–102)*. Axin, which serves as the scaffold in the multiprotein complex facilitating phosphorylation of β-catenin, has been found to be biallelically mutated and hereby inactivated in a subset of hepatocellular and colorectal cancers as well as in medulloblastoma *(103–107)*.

Taken together, up to 90% of colorectal cancers harbor inactivating mutations in the *APC* tumor-suppressor gene or activating mutations of the proto-oncogene β-catenin. The tumor-suppressor gene *APC* is inactivated in the hereditary colorectal cancer syndrome familial adenomatous coli (FAP) *(108,109)*. This inherited autosomal-dominant disease inevitably leads to the rise of hundreds to thousands of colorectal adenomas and if no proctocolectomy is performed, to the development of colorectal cancer. Although germline inactivating mutations of APC occur throughout the entire gene, somatic mutations are clustered in exon 15 between codons 1280 and 1500 *(110)*. This results in a frame shift or a premature stop codon and a truncated protein. Mutations close to codon 1300 are mostly associated with allelic loss of the second allele of chromosome 5q, whereas tumors harboring a mutation outside this region tend to have a second truncating mutation *(111,112)*. The APC gene product interacts with multiple proteins including β-catenin, axin, and GSK3β *(51,52,113–116)*. Three different motifs of the APC protein are responsible for the regulation of β-catenin: three 15-amino acid (aa) β-catenin binding repeats, seven 20 aa β-catenin binding and downregulation repeats, and three repeats responsible for axin binding *(113,117–120)*. Loss of one APC allele and truncation of the other results in the incapability to properly bind to axin and β-catenin and to form the multiprotein complex responsible for β-catenin phosphorylation *(119,121)*. APC mutations can be detected in the earliest premalignant lesions of the colon and they are found as frequently in early adenoma as in invasive carcinoma arguing that mutation of APC is a critical step in colorectal carcinogenesis *(122)*. Therefore, the APC tumor suppressor has been named the *gatekeeper* of the colon. Other human tumors that have been found to harbor APC mutations are melanoma, medulloblastoma, and desmoids *(114,123–127)*.

Mutation of one of the four serine or threonine residues in the destruction box or deletion of the whole box in the amino terminus prevents the phosphorylation and subsequent degradation of β-catenin. These molecular changes give rise to the cytoplasmic accumulation of β-catenin and after nuclear translocation, activation of β-catenin/Tcf transcription. β-catenin mutations are present in up to 50% of colorectal cancers with intact APC, adding up to approx 10% of all colorectal cancers harboring β-catenin mutations *(128–132)*. In addition to colorectal cancers, β-catenin mutations have also been described in other gastrointestinal neoplasias including hepatocellular carcinoma and hepatoblastoma, gastric cancer, gastrointestinal carcinoids, and some rare nonductal pancreatic tumors *(103,133–140)*. Other human cancers that have been found to contain β-catenin mutations include ovarian cancer, endometrial cancer, anaplastic thyroid carcinoma, prostate cancer, melanoma, medulloblastoma, and Wilms' tumor *(123,132,141–149)*. For a comprehensive review of β-catenin mutations including mutation frequencies in various tumors *see* Giles et al. *(150)*.

4. Wnt Signaling as a Therapeutic Target in Cancer

Countless in vitro and animal studies have demonstrated the importance of Wnt/β-catenin signaling in human carcinogenesis and presented evidence that this pathway is not only involved in cancer initiation but also in cancer progression *(24,25,150,151)*. Therefore, targeting elements of this cancer pathway is a promising approach for future cancer therapies (**Table 1**). As β-catenin is the central oncogenic switch in the Wnt/β-catenin pathway it is suitable to be the main therapeutic target.

4.1. Targeting the Initiation of Wnt Signaling at the Cell Membrane

Activation of canonical Wnt signaling involves binding of Wnt ligands to frizzled receptors and LRP-5/6 co-receptors. This can be antagonized by the endogenous inhibitors of Wnt signaling sFRPs, WIF-1, and Dkk. Overexpression of Dkk-3 has been demonstrated to inhibit growth of lung cancer cells and invasion and motility of osteosarcoma cells *(152,153)*. The expression of Dkk-1, which is a transcriptional target of p53, has been shown to be induced upon treatment of cancer cells with chemotherapeutic agents, and re-expression of Dkk-1 in cells lacking endogenous Dkk-1 sensitized these to chemotherapy *(154)*. These data suggest a potential of Dkk proteins as adjuvants for chemotherapy.

To directly target Wnt signaling an antibody against Wnt-1 has been utilized *(155)*. This Wnt-1 antibody inhibited β-catenin-mediated signaling, induced apoptosis in human breast and lung cancer cells, sarcoma and mesothelioma cells, and suppressed growth of xenografted tumors in mice. The apoptotic effect was limited to cancer cells expressing high levels of Wnt-1, whereas

Table 1
Overview of Proposed Approaches Targeting the Wnt/β-Catenin Signaling Pathway

Level of action	Target	Method	Evidence	References
Upstream of β-catenin	Wnt-1	Monoclonal antibody, RNA interference	In vitro, xenograft mouse model	***155,156***
	WIF-1	Expression	In vitro	***156***
	sFRPs	Expression	In vitro	***98***
β-catenin mRNA	β-catenin	Antisense oligos	In vitro, xenograft mouse model	***159–163***
	β-catenin	RNA interference	In vitro, xenograft mouse model	***164–167***
β-catenin protein	β-catenin	APC expression	In vitro	***168,169***
	β-catenin	Protein knock-down	In vitro, xenograft mouse model	***170–172***
	β-catenin	Imatinib mesylate	In vitro	***179***
β-catenin dependent transcription	β-catenin	Small molecules	In vitro, xenopus model, xenograft mouse model	***187,188***
	β-catenin	"Death inducing genes"	In vitro, xenograft mouse model	***180,181***
	β-catenin	Oncolytic viruses	In vitro, xenograft mouse model	***182–186***
Downstream effectors of β-catenin	VEGF	Monoclonal antibody	In vivo	***5,6***
	c-Myc	Antisense oligos	In vitro, phase I clinical study	***15***
	Cyclin D1	Cdk inhibitors (R-roscovitine, rapamycin)	In vitro, xenograft mouse model	***190,191***
	c-Jun	Dominant-negative c-Jun	In vitro	***192***
	COX-2	NSAIDs	In vitro, animal studies, regression of colorectal polyps in humans	***198–201***

colon cells lacking Wnt-1 expression were not affected by the antibody treatment. The same group also analyzed the expression of the Wnt antagonist WIF-1 in colorectal cancer cell lines *(156)*. WIF-1 expression was found in normal colon cells, but was missing or downregulated in the cancer cells. The reduced expression of WIF-1 was found to be caused by hypermethylation of the WIF-1 promoter. Restoration of WIF-1 expression, silencing of Wnt-1 by siRNA, or antagonizing Wnt-1 by the anti-Wnt-1 antibody inhibited β-catenin mediated signaling and induced apoptosis in colorectal cancer cell lines already carrying mutations in either β-catenin or APC. The expression of sFRPs has also been found to be downregulated by promoter hypermethylation in colorectal cancers *(98,157,158)*. As was the case with WIF-1, exogenous restoration of sFRP expression in colorectal cancer cell lines already carrying mutations in either β-catenin or APC resulted in suppression of β-catenin-mediated signaling and induction of apoptosis. These findings support the idea that upstream Wnt signaling may be important during the early development of colorectal cancer. In addition, blocking Wnt signaling at the cell membrane may also have a therapeutic potential in the treatment of cancers even if the cancer cells carry mutations in downstream effectors of the Wnt signaling pathway.

4.2. Targeting β-Catenin mRNA

Antisense oligonucleotides specifically hybridize to complimentary mRNAs (*see* Chapter 7). It is thought that the hybrids are recognized and destroyed by RNase H. Alternatively, the RNA hybrids could inhibit the expression of the targeted mRNA by interfering with splicing or blocking translation of the mRNA. Targeting β-catenin mRNA that is continuously transcribed should result in reduced β-catenin protein expression and interruption of Wnt/β-catenin signaling. Accordingly, treatment of APC mutant colon cancer cell lines with antisense oligonucleotides resulted in a reduction of β-catenin mRNA as well as of β-catenin-mediated transcription *(159)*. A reduction of neoplastic growth capabilities of transfected cells, including proliferation, anchorage-independent growth, and cellular invasiveness was observed. Application of the same oligonucleotide in a mouse xenograft model utilizing SW480 colon cancer cells resulted in reduced tumor growth compared to control mice treated with scrambled oligonucleotide *(160)*. Interestingly, transfection of this antisense oligonucleotide into esophageal cancer cell lines that do not commonly reveal mutations of *APC* or β-*catenin* genes *(161,162)* also led to a reduction of β-catenin mRNA levels, β-catenin-mediated transcription, and to reduced growth. Although β-catenin is not expressed in normal peripheral blood T cells it is expressed in tumor cells lines of hematopoietic origin. Transfection of Jurkat T-acute lymphoblastic leukemia cells with β-catenin antisense oligonucleotides reduced β-catenin

expression and resulted in the inhibition of cellular adhesion and sensitization to Fas-mediated apoptosis *(163)*.

More recently, RNA interference (RNAi) has been utilized to target β-catenin in colorectal cancer cell lines with deregulated Wnt signaling (*see* Chapters 9–12). Expression of a short-hairpin RNA (shRNA) directed against β-catenin from a plasmid in the colorectal cancer cell line LS174T which contains a mutated β-catenin allele resulted in a reduction of β-catenin-mediated transcription, cellular proliferation, and induction of differentiation *(164)*. Transfection of short-interfering RNAs (siRNA) into the colorectal cancer cell lines SW480 and HCT116, which contain mutant APC and β-catenin alleles, respectively, reduced β-catenin protein expression, β-catenin-mediated transcription, and inhibition of tumor cell growth both in vitro and in a xenograft model *(165)*. However, even though recent improvements in delivering siRNAs to their target cells are likely to give RNAi and its clinical application a boost *(166,167)* studies are needed which demonstrate that downregulation of endogenous β-catenin after systemic application of antisense oligonucleotides or siRNAs does not interfere severely with tissue homeostasis and regeneration of normal tissues.

4.3. Targeting β-Catenin Protein

APC tumor-suppressor function is lost in the majority of colorectal cancers and up to 50% of colorectal cancers with wild-type APC harbor activating β-catenin mutations. In consequence, regulation of β-catenin degradation is severely impaired and β-catenin-mediated signaling is activated. Consistent with the critical function of APC in colorectal carcinogenesis, reexpression of APC in the APC-deficient colorectal cancer cell line HT29 led as a proof of principle to the reduction of β-catenin level, growth arrest, and induction of apoptosis *(168)*. Later studies demonstrated that the central third of the APC protein, which contains the β-catenin binding sites, is sufficient to downregulate β-catenin and to suppress β-catenin-mediated transcription *(169)*. Infection of colorectal cancer cells with an adenovirus expressing this mini-APC fragment resulted in growth inhibition and activation of apoptosis.

Another approach to downregulate β-catenin in cell lines focuses on the ubiquitination and degradation machinery. Phosphorylation is a prerequisite for β-catenin to be recognized by the F-Box protein β-TrCP and subsequent proteasomal degradation. Therefore, bypassing the impaired phosphorylation machinery in cancer cells with deregulated β-catenin would be an intriguing approach targeting deregulated β-catenin expression. To overcome β-catenin from escaping its destruction chimeric F-box proteins have been generated that recognize β-catenin in a phosphorylation-independent manner and promote its

proteasomal degradation. One approach engineered a chimeric protein with the β-catenin binding domain of E-cadherin fused to the F-box protein β-TrCP *(170)*. This chimeric protein recruits β-catenin protein to the SCF ubiquitin ligase complex and targets it for degradation by ubiquitination. Expression of the β-TrCP-E-cadherin chimera in DLD-1 colorectal carcinoma cells selectively reduced cytosolic but not E-cadherin-bound β-catenin, resulting in impaired growth and inhibition of colony formation in vitro, as well as loss of tumorigenicity in xenografted mice. Another chimeric protein was constructed to contain multiple copies of a minimal β-catenin-binding element of 15 amino acids derived from the APC protein fused to the F-Box motif of β-TrCP *(171)*. Like the previously mentioned approach, this chimeric protein suppressed the activity of β-catenin-mediated transcription and downregulated the β-catenin target gene *c-myc* by reduction of nuclear and cytosolic β-catenin, but also preserved the membrane-bound pool. Growth of colorectal cancer cells was substantially inhibited in vitro and in vivo. A third chimeric F-box protein, which replaces the WD40 repeat of β-TrCP with the β-catenin-binding domains of Tcf-4 and E-cadherin, has been generated *(172)*. Also this chimera has been shown to downregulate β-catenin independent of phosphorylation. The main advantage of these three approaches is that the cytosolic- and nuclear- but not the membrane-bound pools of β-catenin are reduced. Only the pools responsible for oncogenic signaling but not the membrane-bound pool of β-catenin involved in cell adhesion are targeted. This might be a major advantage when compared to approaches targeting β-catenin mRNA. Discriminating between cytosolic/nuclear and E-cadherin-bound β-catenin might minimize side effects of this therapeutic approach. However, the major drawback of these chimera targeting β-catenin degradation is the delivery of the chimeric proteins to tumors.

Receptor tyrosine kinases have been shown to be involved in the regulation of free cytoplasmic β-catenin *(173–178)*. Phosphorylation of tyrosine residue 654 of β-catenin blocks the E-cadherin/β-catenin interaction and results in an increase of β-catenin/Tcf-regulated transcription. It has been demonstrated that the tyrosine kinase inhibitor imatinib downregulates β-catenin-mediated signaling and suppresses growth of colorectal cancer cells *(179)*. Similar experiments with other tyrosine kinase inhibitors like gefitinib and erlotinib are pending.

4.4. Targeting β-Catenin/Tcf Transcription

Another interesting approach targeting active Wnt/β-catenin signaling is the use of recombinant viruses, which either carry a "death-inducing gene" under the control of a Tcf-dependent promoter or the replication of which is dependent on active β-catenin/Tcf signaling. A recombinant adenovirus containing the apoptosis inducing gene FADD (Fas-associated death domain-containing

protein) under the control of a Tcf-dependent promoter has been generated ***(180)***. Colorectal cancer cells with activated β-catenin/Tcf signaling were selectively and efficiently killed by the virus, supporting the idea that aberrantly activated β-catenin can be used to selectively target colon cancer cells. In order to maximize the tumor-killing effect in colorectal cancer cells with deregulated Wnt signaling a recombinant adenovirus was generated which carries a herpes simplex virus thymidine kinase gene (HSV-TK) under the control of a Tcf-responsive promoter ***(181)***. Treatment of nude mice xenografted with human DLD-1 colon cancer cells with the recombinant adenovirus and ganciclovir significantly suppressed the growth of the tumor cells. Control mice xenografted with a human hepatoma cell line did not respond to this treatment, demonstrating that this approach selectively targets tumor cells with aberrant activation of β-catenin.

Oncolytic viruses selectively lyse malignant cells by cytopathic effects ***(182)***. The major advantage of this strategy is the amplification of the virus at the site of the tumor. Brunori and coworkers engineered an oncolytic adenovirus that selectively replicates in cells with aberrantly high β-catenin expression ***(183)***. This adenovirus expresses the viral E1B and E2 genes from promoters controlled by b-catenin/Tcf. The Tcf-E1B and Tcf-E2 promoters were found to be active in many cell lines with activated Wnt signaling. Viruses with Tcf-dependent regulation of E2 expression replicated efficiently in SW480 colon cancer cells, but showed a significantly reduced replication in H1299 lung cancer cells and WI38 normal fibroblasts. As a proof of concept, the authors introduced a stable β-catenin mutant into normal WI38 fibroblasts, which rendered these cells permissive for virus replication. Another oncolytic virus with Tcf-binding sites integrated into the promoters of the *E1A, E1B, E2*, and *E4* genes exhibited a strong selectivity for cells with deregulated Wnt signaling ***(184)***. This adenovirus preferentially replicated in cells with activated Wnt signaling and resulted in a dramatic increased efficacy in cytopathic assays. Based on a similar idea, Toth and coworkers generated an adenovirus with a synthetic promoter containing five Tcf-consensus binding sites replacing the wild-type E4 promoter ***(185)***. The virus preferentially replicated in cells with activated Wnt signaling. In a xenograft model the virus effectively suppressed the growth of SW480 colorectal cancer cells but not of control cells without deregulated Wnt signaling. Instead of using adenoviral vectors Malerba et al. ***(186)*** modified the minute parvovirus of mice to contain Tcf-binding sites within the P4 promoter. Replication and cytopathic effects of this recombinant virus were also strongly dependent on β-catenin/Tcf activity.

So far, two screens for small molecular compounds targeting active Wnt/β-catenin signaling in cancer have been published. In search for small molecule inhibitors of Wnt signaling, Lepourcelet and coworkers performed a systematic screen of libraries of natural compounds that specifically disrupt the

β-catenin/Tcf-4 complex *(187)*. Among 7000 compounds screened, 8 showed dose-dependent inhibition of the β-catenin/Tcf-4 complex. Each of the compounds was able to inhibit β-catenin/Tcf reporter gene activity and transcription of known β-catenin/Tcf target genes. Subsequent analyses revealed that three of the compounds abrogated axis duplication in *Xenopus* embryos, which was induced by injection of β-catenin mRNA. Finally, two compounds of fungal origin were identified as specific inhibitors of the β-catenin/Tcf complex in vitro and in vivo. However, the compounds were also found to inhibit β-catenin binding to APC, which might affect β-catenin phosphorylation by inhibition of its binding to the multiprotein phosphorylation complex. Another screen of 5000 compounds for small-molecule inhibitors of β-catenin-mediated signaling identified a compound, ICG-001, which specifically inhibits the cyclic AMP response element-binding protein, a p300-related coactivator of β-catenin-mediated transcription *(188)*. Treatment of cells with ICG-001 inhibited transcription of a reporter gene and the transcription of β-catenin/Tcf target genes was downregulated. ICG-001 inhibited the proliferation of colorectal cancer cells in vitro and of tumor xenografts in nude mice. These two studies are encouraging as they demonstrate the feasibility of identifying small molecule inhibitors that target protein–protein interactions. However, more studies are needed to exclude toxic side effects and negative effects on tissue homeostasis and regeneration as a compound effective against cancer cells would be expected to also target normal cells that depend on the same pathway, i.e., intestinal epithelium.

4.5. Targeting Downstream Effectors of β-Catenin

β-catenin/Tcf regulate the expression of multiple genes some of which are known to promote tumor growth, including c-Myc, cyclin D1, c-Jun, VEGF, and COX-2. Therefore, targeting these genes in cancers with deregulated Wnt signaling might be a promising alternative to the previously mentioned concepts. The oncogene c-Myc is overexpressed in a variety of human cancers including leukemia, lymphoma, melanoma, breast cancer, and colorectal cancer *(189)*. Being a direct target gene of β-catenin, c-Myc is crucial for the development of cancer and inhibiton of c-Myc is an important step to limit tumor growth. An antisense oligonucleotide targeting c-Myc mRNA was able to significantly reduce the growth and induce apoptosis in prostate cancer cells *(15)*. When tested in a xenograft model, the antisense oligonucleotide caused a dramatic reduction of tumor load. Moreover, antisense oligonucleotides against c-Myc have been shown to limit proliferation in leukemia, lymphoma, melanoma, prostate, breast and liver cancer cells *(15)*. Several approaches have been done to target the cell cycle regulator cyclin D1. The function of cyclin D1 to phosphorylate the tumor suppressor Rb in complex

with the cyclin-dependent kinases (CDK) 4 and 6 is inhibited by the CDK inhibitor R-roscovitine (CYC202) and by rapamycin *(190,191)*.

C-Jun and Fos proteins form hetero- and homodimers to constitute the activator protein (AP)-1. To investigate the role of AP-1 in colorectal cancer, a dominant-negative mutant of c (DN)-Jun lacking the transcription activation domain was used *(192)*. Transfection of HT29 colorectal cancer cells with DN-Jun inhibited tumor cell proliferation in vitro and in vivo, suggesting that the β-catenin-regulated gene *c-Jun* might be a useful target in colorectal cancer. VEGF stimulates the vascular permeability and acts a mitogen and survival factor for endothelial cells through interaction with its cognate cell surface receptors. Treatment of HCT116 colorectal cancer cells with β-catenin antisense oligonucleotides led to a reduction of VEGF expression by more than 50% *(193)*. Therefore, targeting VEGF by small molecules or antibodies will indirectly inhibit part of β-catenin function in cancer.

Cyclooxygenase-2 is overexpressed in many tumors and its direct or indirect regulation by Wnt/β-catenin signaling has been demonstrated *(82,194–196,208)*. Its activity can be inhibited by selective cyclooxygenase (COX)-2 inhibitors and nonselective nonsteroidal antiinflammatory drugs *(197)*. Epidemiological and experimental studies have demonstrated that COX-2 inhibitors and nonselective nonsteroidal antiinflammatory drugs can inhibit the development of colorectal cancer *(198–201)*. The COX-2 inhibitor celecoxib has been approved for reduction and regression of colorectal polyps in FAP patients. However, to effectively downregulate Wnt/β-catenin signaling by inhibiting downstream effectors of the pathway more than one β-catenin/Tcf-regulated gene needs to be targeted. This kind of an approach is appealing but specific studies are pending.

5. Conclusions and Future Perspectives

Targeting deregulated Wnt/β-catenin signaling in cancer is a rationale therapeutic approach. Accordingly, many different strategies focusing on different levels of the pathway have been developed. However, none of these has so far reached clinics. It needs to be kept in mind with all new approaches targeting Wnt signaling that the Wnt pathway has vital functions in the maintenance and self-renewal of various pluripotent stem cells and progenitor cells *(202–204,209)*. Interruption of Wnt signaling in the intestinal crypt may lead to depletion of the epithelial stem-cell compartment and consequently to a loss of intestinal regeneration *(205,206)*. Moreover, not every single cancer cell with deregulated Wnt/β-catenin signaling might also be dependent on this pathway for maintenance of the transformed phenotype as has been demonstrated by direct targeting of β-catenin by knock-out of the mutant allele in the colorectal cancer cell line HCT116 *(207)*. The individual genetic background of a tumor

might be of great importance for its response to a targeted therapy even if the targeted pathway is indeed deregulated. As our understanding of the Wnt/β-catenin pathway is progressing with new activators, inhibitors, target genes, and cross-connections to other pathways being identified, more therapeutic approaches to target this pathway will appear. In conclusion, as most tumors carry mutations in several genes and reveal various deregulated pathways, the majority of tumors will most likely not be treated successfully by targeting only one single pathway. As multiple pathways in a single cell contribute to the transformed phenotype, only a treatment focusing on more than one target will ultimately be successful in the majority of cases.

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4

NG2/HMP Proteoglycan as a Cancer Therapeutic Target

Martha Chekenya and Heike Immervoll

Summary

Neuroepithelial cells of the central nervous system constitute neuroglia (astrocytes, oligodendrocytes, and microglia), ependyma, and neurons, which make up the stromal cells of the brain. The stromal tissue organization of the brain is tightly regulated, but occasionally the signals that define the normal contexts become disrupted and result in cancer. Malignant progression is then maintained by cross-talks between the tumor and its stroma, where the activated stroma nurtures the proliferative and invasive neoplastic cells, by providing neovasculature, extracelluar matrix components, and stimulatory growth factors. The NG2/HMP plays a major role in tumor–stroma activation through alterations in cellular adhesion, migration, proliferation, and vascular morphogenesis. Therapeutic strategies specifically targeting NG2/HMP may be useful in normalizing the tumor stroma and may reduce the toxic side effects when used in combination with conventional treatments.

Key Words: NG2; siRNA; immunotherapy; glioma; melanoma.

1. Introduction

1.1. The Brain Stroma as the Glioma Backdrop

The human brain is made up of numerous cell types that interact physically through cell–cell, and cell–extracellular matrix (ECM) contacts, and biochemically via soluble and insoluble signaling molecules. Neuroepithelial cells of the central nervous system (CNS) constitute neuroglia (astrocytes, oligodendrocytes, and microglia) and neurons, which are derived from the neuroectoderm. These cells produce the ECM, an important feature of the normal stroma, which provides structural scaffolding as well as contextual information to the cells. However, with the exception of the vascular basement membrane and the *glia limitans externa*, the adult CNS is poorly endowed with the classical ECM

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components, such as collagens, laminin, tenascin, vitronectin, elastin, and fibronectin *(1)*. These glycoproteins contain an Arg-Gly-Asp (RGD)-peptide adhesion domain that is recognized by members of the integrin family of *(2,3)* adhesion receptors. The brain ECM is composed of several proteoglycans, including chondroitin sulphate proteoglycans (CSPG) *(1)*. These macromolecules, with core proteins covalently linked to glycosaminoglycan chains are believed to mediate multiple cellular functions, such as the regulation of ECM structural organization, proliferation, trophic-factor binding, cellular adhesion, and motility *(4,5)*. The vascular and perivascular cells of the endothelium interact with the basement membrane, a specialized form of ECM that provides structural support to cells. The vascular system provides nutrients, oxygen, and circulating immune cells that combat pathogens and remove apoptotic cell debris. The normal brain is considered an "immune-privileged site" because of the lack of resident immune-competent cells (T cells and B cells). This stromal tissue organization of the brain is tightly regulated, but occasionally the signals that define the normal contexts become disrupted in cancer. Malignant progression is thus maintained by cross-talks between the tumor and its stroma, where the activated stroma nurtures the proliferative and invasive neoplastic cells, by providing neovasculature, ECM components, and stimulatory growth factors (**Fig. 1**).

1.2. NG2 at the Tumor–Stroma Interface

NG2 is a 300-kDa membrane spanning CSPG that was originally identified as a surface epitope on a subset of tumor cell lines derived from rat embryos after ethylnitrosourea administration *(6)*. The cell lines were characterized by the presence of Na^+ and K^+ ion channels, but an inability to generate full-fledged action potentials, features characteristic of neural precursor cells. NG2 positive neural precursors can differentiate into either neuroglia or neurons, hence the designation nerve/glial antigen 2 (NG2) *(6)*. Structurally, NG2 is composed of an extensive extracellular domain, which is further segregated into three functionally distinct subunits (designated domains 1–3) (**Fig. 2**). Domain 1 comprises two globular "dumb-bell" structures stabilized by intramolecular disulphide bonds *(2,3)*. Domain 2 contains one chondroitin sulfate chain at serine –999 *(7)*, and an α-helical site for collagen V and VI binding *(2,3)*. Domain 3 is the globular juxtamebrane site for proteolysis of NG2 that leads to its cleavage and release from the cell surface *(8)*. Although four internal repeats resembling the Ca^{2+}-binding motifs of cadherins are scattered throughout the ectodomain, no homology is found between NG2 and other proteoglycans, e.g., syndecans, aggrecans, glypicans, and so on *(9,10)*. NG2/HMP has single transmembrane domain, and a short cytoplasmic tail. The extreme C terminus of the cytoplasmic tail contains a QYWV motif responsible for PDZ-binding *(11)* and

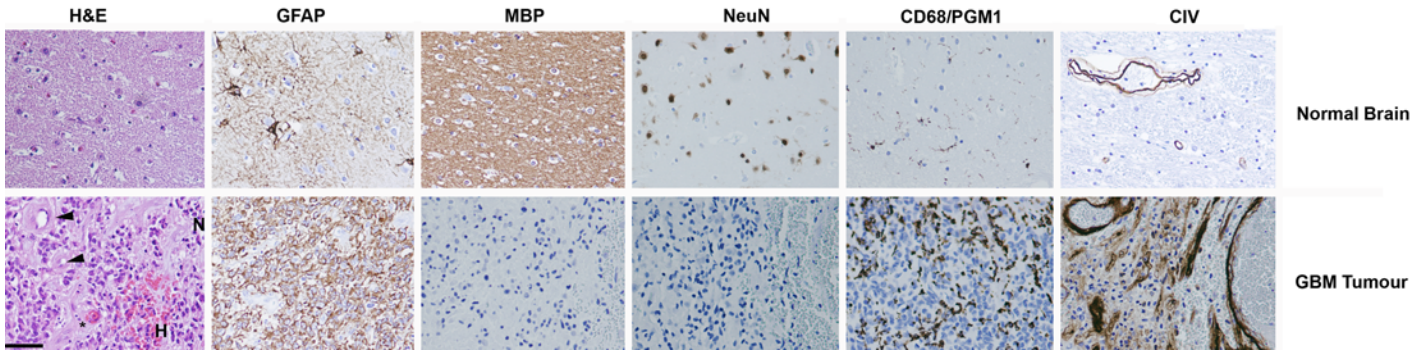

Fig. 1. The normal brain versus malignant brain tumor. The normal brain shows a highly structured and segregated architecture, indicated here by immunohistochemistry and H&E staining **(Top)**. The normal brain is composed of several cell types: astrocytes, oligodendrocytes, neurons, and microglia (visualized here with immunohistochemical staining for glial fibrilliary acidic protein [GFAP]; myelin basic protein [MBP]; NeuN; and CD68, respectively). The basement membrane is denoted here with immunostaining for collagen IV (CIV) and highlights blood vessels. These tissue components are embedded within a finely textured eosinophilic background "feltwork" that is the neurophil **(Bottom)**. The glioblastoma multiforme tissue is less well organized (H&E). There is evidence of tissue necrosis (N), hemorrhage (H), microvascular proliferations, which are abnormal blood vessels (*); numerous blood vessels (arrowheads) and mitotic figures indicative of cellular proliferation. Tumor angiogenesis produces poorly defined, dilated blood vessels (shown here with CIV staining), and glioma cells are intermingled with all the stromal elements. In this tumor, there is a complete loss of oligodendrocytes and neurons, and hyperplasia of astrocytes and microglial cells. This hypertrophic response, which results in an increase in cell size and cytoplasmic prominence, occurs quickly and is indicative of both reactive gliosis and tumor upregulation of the GFAP-intermediate filaments. This image comprises sections consecutively cut and stained using standard immunohistochemistry methods of paraffin-embedded tissue. Original magnification ×200, scale bar 100 μm.

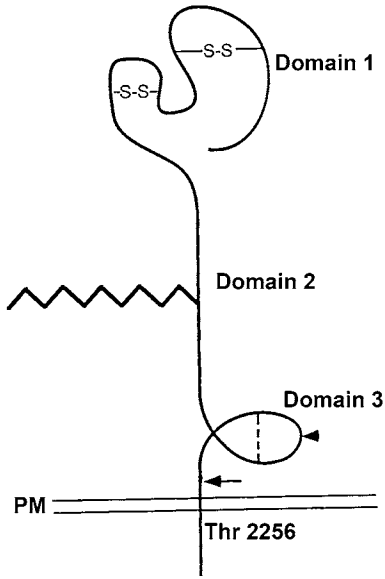

Fig. 2. The schematic structure of NG2. Domain 1 comprises the ligand binding globular head that is stabilized by disulphide bonds. Domain 2 contains type VI collagen binding sites and a single chondroitin sulfate chain (jagged line). Domain 3 is the site for proteolytic cleavage (arrowheads). The cytoplasmic tail contains PDZ-binding motifs and proline-rich regions and several sites for phosphorylation, including threonine 2256, which has been shown to be phosphorylated by PKCα, *(156)*.

may be the site for NG2 interaction with MUPP1, a multi-PDZ domain-containing cytoplasmic scaffolding protein *(12)*. The NG2 cytoplasmic domain also contains phospho-threonine residues, four of which are candidates for phoshorylation *(13)*. Recently, the threonine residue 2256 has been identified as a primary phosphorylation site for PKCα. Finally, although a classical PXXP SH3-binding motif is lacking *(14)*, the C terminal half of the cytoplasmic tail is rich in prolines, the significance of which remains to be elucidated *(6)*.

NG2 shares high-sequence homology with the high-molecular-weight melanoma-associated antigen (HMW-MAA) or human melanoma proteoglycan (HMP) *(15–18)*, where 18 residues of the NG2 amino-terminus are identical to the HMP, identifying it as the human homolog, and shall hereon be denoted NG2/HMP. In contrast, HMP contains three nucleotides in the juxtamembrane domain that are absent from the NG2 sequence *(13)*. The mouse NG2 homolog, designated AN2, has also been identified *(19,20)* indicating that it is evolutionary conserved. NG2/HMP is a well-established marker for glial progenitor cells in the CNS *(21–26)*. Although differentiation of NG2/HMP-positive progenitors into oligodendrocytes in vivo has been demonstrated, derivation of astrocytes

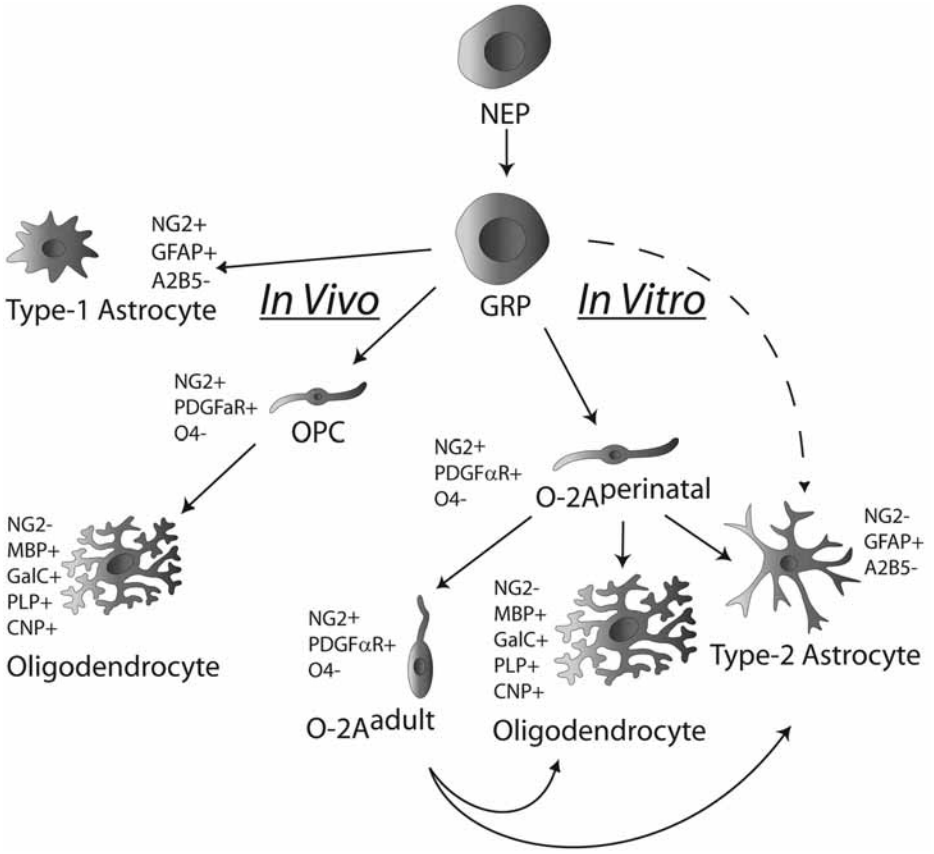

Fig. 3. Lineage of NG2-positive oligodendrocyte precursors in the CNS. Neuroepithelial stem cells (NEP) become lineage restricted to give glial-restricted precursors (GRP), which are tri-potent, giving rise to oligodendrocytes and two astrocytes: type 1 and type 2 astrocytes (O-2A cells). This lineage has only been demonstrated in vitro. The generation of oligodendrocytes in vivo has been demonstrated from oligodendrocyte precursors (OPC), which are lineage restricted from GRP. Type 1 astrocytes have also been demonstrated in vivo to be derived from GRP cells.

from the same lineage has been elusive *(21–23,25,27–30)* (**Fig. 3**). NG2/HMP expression by oligodendrocyte progenitor cells is preceded by expression of the PDGFα receptor, which is critical for progenitor development and provides another stringency marker for this population *(31–33)*. Oligodendrocyte progenitors expressing both NG2/HMP and PDGFα receptor in the rodent CNS are present as early as embryonic day 16–17 *(24)* in the ventral spinal cord. Their distribution expands with time to the first postnatal week, when they are located throughout both the gray and white matter in the CNS. Oligodendrocyte progenitor cells have a characteristic unipolar or bipolar morphology in vitro and

small cell bodies (10- to 15-μm in diameter) that give rise to multiple stellate branches in vivo *(34,35)*. The processes have a radial orientation in the gray matter, but are more longitudinal in the white matter *(35)*.

2. The Role of NG2/HMP in Tumor–Stroma Activation

2.1. Reactive Response to Injury

NG2/HMP positive cells undergo morphological changes in response to a variety of injuries *(36–41)*, characterized by (1) upregulation of NG2/HMP expression *(42)*, (2) an increased density and change in morphology to become shorter and thicker *(34,35,39,40)*, and (3) appearance of fine filopodia on their cell bodies and processes *(39)*. The onset is rapid (within hours of injury) and may persist for several weeks, indicating that these dynamic cells *(39,40)* can be involved in stromal activation.

2.1.1. Cell Adhesion and Migration

Cell adhesion and migration on ECM components are promoted by a number of membrane receptors, of which, integrins represent a major class *(43)*. Several lines of evidence indicate that NG2/HMP is also capable of modulating both cell–ECM and cell–cell adhesion. For example, NG2/HMP is localized on microspikes in melanoma cells where it participates in cellular adhesion *(44)*. Furthermore, the human 9.2.27 monoclonal antibody (mAb), which recognizes the NG2/HMP human homolog, HMW-MAA, inhibits melanoma cell migration on ECM proteins *(45)*. The extracellular domain of NG2 can be proteolytically cleaved from the cell surface both in vitro and in vivo *(8,42)* and is then deposited at the interface between the substrata and the migrating tumor *(46)*, and endothelial cells *(47)*. Although brain CSPGs can promote transient adhesion of neuronal cells, they generally inhibit stable cell adhesion and neurite outgrowth *(48)*. NG2/HMP negatively modulates adhesion of melanoma cells via cluster of differentiation 44 (CD44), hyaluronic acid (HA), fibronectin, and α4β1 integrin *(49)*. CD44 is ubiquitously expressed in several tumors *(50)*, including gliomas *(51)*, where it mediates tumor cell adhesion and migration *(52,53)*.

NG2/HMP may indirectly regulate cell–ECM interactions by serving as a co-receptor for α4β1 integrin, which it communicates with by outside–in and inside–out signaling mechanisms *(54,55)* during adhesion and migration *(55)*. NG2/HMP also interacts with the β1 integrin-subunit, where it modulates adhesion to type II and VI collagen *(54,56)*. NG2/HMP is best characterized as a cell surface receptor for type VI collagen *(2,57–59)*, which is a major component of the basement membrane in some types of vasculature *(60,61)*. The normal brain stroma is composed of very little classical ECM, but glioma cells and activated stromal astrocytes can synthesize tenascin, HA, and collagen *de novo* during invasion and migration *(62,63)*. The fact that NG2/HMP interacts with these ECM

components *(2,3,58,59)*, and it is highly expressed on some gliomas, suggests that NG2/HMP may modulate neoplastic cell adhesion to various ECM components. Indeed, we previously reported that NG2/HMP expressing glioma cells failed to migrate on laminin, vitronectin, and fibronectin substrates, and only migrated efficiently on collagen IV-coated substrates in vitro *(64)*. Others have shown that NG2/HMP-expressing glioma cells migrate most efficiently on type VI collagen *(3)*, and that this can be inhibited by NG2/HMP blocking antibodies or by mutations in NG2/HMP's collagen-binding domain. We have also compared the NG2/HMP spatial distribution in neoplastic tissue that originated from the tumor main mass with that obtained from the interface between the tumor and the infiltrated brain. The results indicated that the NG2/HMP expression was confined to the tumor main mass but was markedly reduced in the infiltrative edge *(65)*. It has been reported that NG2/HMP poses a barrier to cell migration and axonal growth on laminin substrates *(66–70)* through Gi protein receptor signaling *(70)*. Because laminin is abundantly deposited in the border zones between the tumor tissue and the brain parenchyma in vivo *(71,72)*, we speculated whether NG2/HMP expressing cells may be restricted to the tumor main mass owing to the inhibition of cell adhesion and migration on certain ECM components *(65)*.

NG2/HMP-positive oligodendrocyte progenitor cells produced in the germinative neuroepithelium during embryogenesis migrate to the optic nerve and chiasma *(73)* by guidance of the repulsive sematophorins, Netrin1 and Sema3a, which are secreted from the optic chiasma *(74)*. However, the mechanisms by which NG2/HMP induces tumor cell motility involve modification of the cytoskeletal dynamics *(75)*, because NG2/HMP localizes to radial actin spikes associated with filopodia in migrating cells. This indicates a role for NG2/HMP in cell migration through Rho-dependent mechanisms *(7,75,76)*.

2.1.2. Cell Proliferation and Tumor Growth

Fibroblast growth factor (FGF)-2 and PDGF AA are critical mitogens and chemoattractants, which alter the differentiation of cultured oligodendrocyte progenitor cells *(77,78)*, and are upregulated during pathological conditions *(79)*. Both NG2/HMP and PDGF receptors are coexpressed on oligodendrocyte progenitor cells *(24)*, and on the surface of vascular pericytes and smooth muscle cells *(23,24,80,81)*. NG2/HMP binds and activates both PDGF-AA and FGF-2, modulating their pleiotropic effects on the signaling receptors. Moreover, antibodies against NG2/HMP block the mitogenic effects of PDGF-AA on both oligodendrocyte progenitor and aortic smooth muscle cells. The PDGFα receptor is unresponsive to PDGF-AA in aortic smooth muscle cells derived from NG2/HMP knockout mice *(23,63,80,81)*, indicating that NG2/HMP is important for both the proliferative and migratory responses to the PDGF-AA/PDGFα receptor pathway.

Using Ki67 (MIB-1) immunolabeling, which recognizes proliferation associated nuclear proteins expressed during G1 to M phases of the cell cycle, we showed that NG2/HMP expressing glioblastoma cells were more proliferative than their NG2/HMP negative counterparts *(65,82)*, and similar observations have been reported for melanoma cells, where NG2/HMP expression increased their proliferation rates in vitro, tumorigenicity, and their metastatic potential in vivo *(49)*. Using magnetic resonance imaging (MRI) we showed that NG2/HMP expressing xenografts grew faster and exhibited greater spread within the brain compared to NG2/HMP negative control tumors *(82,83)*. NG2/HMP's binding and activation of the PDGF-AA/PDGFαR pathway is one putative mechanism for enhancing the growth of neoplastic cells. The *PDGFαR* gene on chromosome 4q11-p12 is amplified in some glioblastomas *(84)* and is over expressed in most astrocytomas *(85,86)* and this may lead to both autocrine and paracrine growth factor stimulation. In addition, NG2/HMP–collagen interactions may also potentiate mitogen-driven proliferative and migratory responses, because the collagen types are capable of binding PDGF. These findings might indicate that NG2/HMP expressing cancer cells generate a supportive microenvironment by producing stroma-modulating growth factors. These disrupt normal homeostasis and act in paracrine manner to induce stromal reactions such as angiogenesis *(87)*, inflammatory responses, as well as activating other surrounding stromal cells, such as microglia and macrophages, resulting in secretion of additional growth factors and proteases.

2.1.3. Vascular Morphogenesis and Tumor Angiogenesis

NG2/HMP is widely expressed during both normal and pathological angiogenesis *(65,82,83,88,89)* by the mural cell component of the neovasculature. NG2/HMP is expressed by pericytes on immature brain capillaries as early as embryonic day 10–12 (E10–12) in the rat and continues throughout the period of rapid expansion of the brain vasculature *(23,80,88,90)*. Microvascular pericytes are characterized by their coexpression of NG2/HMP and PDGF β-receptor, which is just as important to their development *(91,92)* as the PDGFα receptor is to oligodendrocyte progenitors. However, as the brain matures and CNS capillaries become quiescent, NG2/HMP expression decreases *(90)*. Outside the CNS, the earliest and most prominent expression of NG2/HMP is in the heart, on cardiomyocytes *(80,89)* and smooth-muscle cells in the dorsal aorta from E10 to 14 *(89)*. NG2/HMP expression is primarily confined to perivascular cells along arterioles and capillaries, and continuous expression is not observed along venules and never beyond the immediate postcapillary vessels *(93)*. During tumor angiogenesis and wound healing, NG2/HMP expressing mural cells respond to environmental cues by migrating to sites where vessel growth and repair are occurring *(94–96)*. The close apposition of pericytes to

endothelial cells also indicates a seminal role during angiogenesis *(97)*. NG2/HMP is proteolytically cleaved into a soluble form *(8,98)*, which is highly angiogenic, stimulating endothelial and pericytic tube formation both in vitro and in vivo *(47)*.

Furthermore, NG2/HMP binds specifically and saturably to plasminogen, which can be proteolytically cleaved to release the endogenous angiogenesis inhibitors, angiostatin consisting of kringle domains (K1–4) and miniplasminogen (K5) *(99)*. In agreement with these findings, we have shown that overexpression of NG2/HMP in human glioblastoma cells directly increased tumor angiogenesis *(83)*, by binding and sequestration of angiostatin. Furthermore, we have recently demonstrated that the tumor vasculature in NG2/HMP-positive tumors is structurally and functionally defected compared to that in NG2/HMP-negative tumors *(82)*. As a cell surface component of mural cells, NG2/HMP is adequately positioned to sequester angiostatin, which otherwise would be available to inhibit proliferation and migration of endothelial cells *(83,99)*.

3. Normalizing the Stroma Through Targeting NG2/HMP

3.1. mAb-Directed Therapy

Antibody-based immunotherapy has been greatly investigated as a therapeutic strategy against NG2/HMP-expressing melanomas. This method utilizes a molecular vehicle, mAb, to selectively deliver radionuclides or toxins to tumor cells *(100)*. Several studies have used various mAbs that recognize the NG2/HMP because the majority of melanomas extensively express it. There is limited intra- and intertumor heterogeneity, where it has been detected on >90% of melanoma cells *(49,101,102)*. Furthermore, because it is expressed on both tumor cells and the associated vasculature, it offers several advantages over therapies that are strictly tumor-directed. Therapies that target only tumor cells, are limited by both the heterogeneous expression of the NG2/HMP within the tumor, as well as by the high rate of tumor cell mutation *(103,104)*. In contrast, the host activated-stromal vascular cells are relatively homogenous and lack the problems associated with drug resistance *(103,105–107)*. Other than the tumor-associated vascular cells, and oligodendrocyte precursors in the brain, the NG2/HMP has a restricted distribution in normal tissues *(108,109)*.

The 9.2.27 mAb has been most frequently chosen as the drug-targeting device because it effectively targets and has a high affinity for melanoma cells *(108–110)*. The antibody does not cause antigen modulation in vivo and expression of the NG2/HMP antigen it recognizes is not cell cycle dependent *(111)*. Although it is not clear at this point whether NG2/HMP antigen is internalised, other mechanisms can still mediate the clinical benefits of mA3-directed therapy. These mechanisms involve both immunological and nonimmunological effector functions (**Fig. 4**).

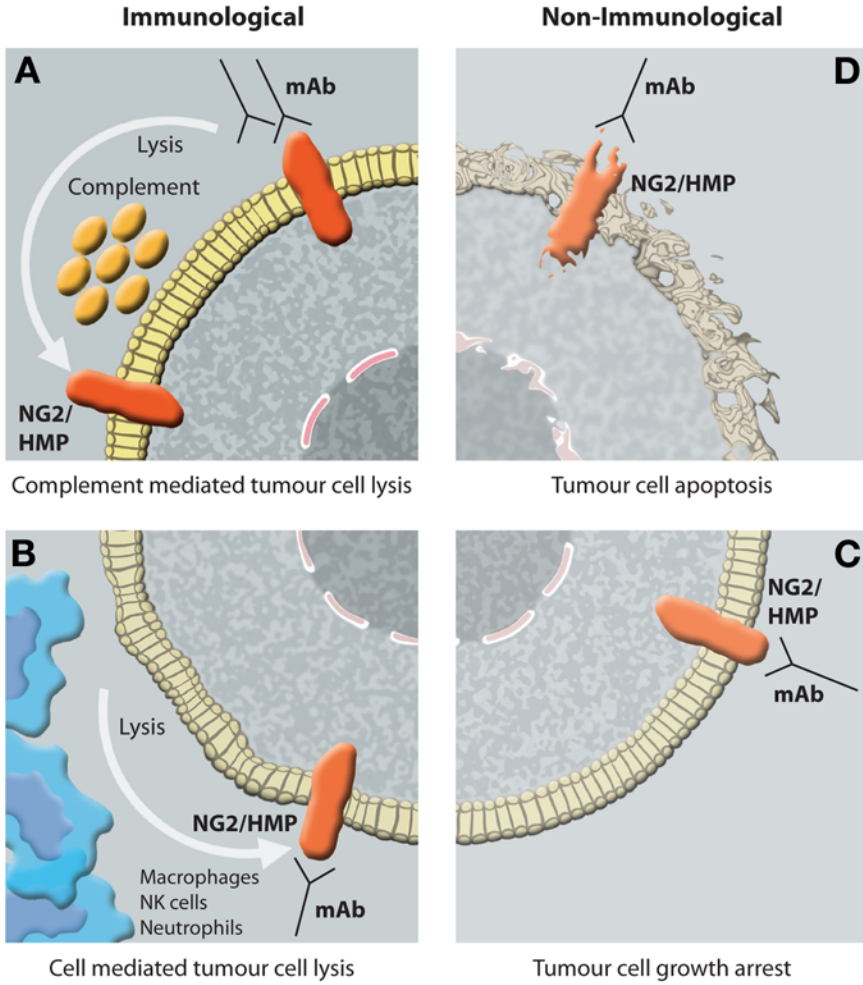

Fig. 4. Mechanisms underlying antibody-based immunotherapy. Immunological mechanisms include **(A)** complement dependent, where membrane-bound tumor-associated antigens (e.g., NG2/HMP) bound to mAb are recognized and destroyed by complement membrane attack complexes. Alternatively, **(B)** lysis of tumor cells is initiated by macrophages bearing Fcγ III receptors, natural killer cells, and neutrophils. Nonimmunological mechanisms include **(C)**, tumor cell growth arrest, and **(D)**, tumor cell apoptosis induced by mAb-specific antibody binding to the antigens that trigger signal transduction events in the tumor cells. Models are proposed from the literature (*157–159*).

The methods of preperation for mAbs and antibody fragments include hybridoma technology (*112*), antibody phage display (*113*), ribosome display (*114*), and iterative colony filter screening (*115*). The dissociation constants for

mAbs are in micromolar to picomolar range but might require further affinity maturation procedures *(116)*. Antibody formats include single-chain variable fragments, Fab fragments, miniantibodies, or immunoglobulins, which all show different pharmacokinetics and tumor-targeting properties *(117)*.

Although this treatment strategy is highly attractive, the therapeutic efficacy of antibody-directed immunotherapy has been limited by several shortcomings *(118)* including (1) the loss or downregulation of antigenic epitopes on tumor cells; (2) lack of tumor-specific antigens; (3) antibody toxicity; (4) failure of the large antibody complexes to cross the blood brain barrier *(119)*; (5) high interstitial pressure leading to poor diffusion of antibodies into all parts of the tumor *(120)*; (6) dehalogenation or removal of radioactive label from mAb; (7) enzymatic removal of mAb from tumors; and (8) lack of mAb humanization. Nonetheless, several preclinical experimental studies and clinical trials targeting the tumor- and vessel-associated NG2/HMP have been developed (*see* **Table 1**).

3.2. Targeting Peptides

In order to circumvent the problems associated with antibody-directed immunotherapeutic strategies, an alternative approach can involve using small peptides. Phage display of random peptide libraries has previously enabled isolation of peptides that successfully bind to integrin receptors *(121–124)*, growth factor receptors *(125)*, and other tumor-associated proteins *(126,127)*. NG2/HMP-binding decapeptides that home directly to the angiogenic neovasculature of melanoma xenografts in mice have been developed *(128)*. The localization and accessibility of NG2/HMP on pericytes suggested a potential use of these vessel homing sequences for targeted delivery of therapeutic agents to tumors. Several reports have suggested that pericytes play an important role in controlling endothelial cell proliferation and vessel stabilization during angiogenesis *(91,129–131)*. Thus, anticancer strategies targeting pericytes in the angiogenic vasculature may complement approaches targeting endothelial cells. Because NG2/HMP is also expressed on tumor cells *(132–134)*, the peptides could deliver therapeutic agents both to the tumor cells and their vasculature. The small peptides may prove superior to antibodies in terms of penetration into tumors.

Methods of preparation for peptides include phage display of random peptide libraries *(135,136)*, including in vivo panning *(137)* and solid phase parallel synthesis *(138)*. Typical dissociation constants are in micromolar range, although avidity can be improved by multimerisation *(139)*. The in vivo stability may differ greatly among peptides.

3.3. siRNA Targeted Therapy

RNA interference (RNAi) is a process whereby small, noncoding double stranded RNAs posttranscriptionally silence specific genes (*see* Chapters 9, 11,

Table 1
Monoclonal Antibody-Mediated Immunotherapeutic Strategies For Targeting NG2/HMP in Tumor Stroma

Monoclonal antibody	IgG class	Conjugate	Tumor type	Reference
9.2.27 mAb	γ2a	^{125} I	Human melanoma xenografts in athymic mice	*(148)*
9.2.27 mAb	γ2a	Diphtheria toxin A chain	Human melanoma xenografts in athymic mice	*(17)*
9.2.27 mAb	γ2a	Doxorubicin via cis-acotinic anhydride	Human melanoma xenografts in athymic mice	*(149,150)*
Me1–14 F(ab')$_2$	IgG2a	^{131}I-labeled	D54 MG human glioblastoma xenografts in athymic mice	*(151,152)*
Me1–14	IgG2a	^{131}I-labeled	Human glioblastoma patients	*(153)*
Me1–14 F(ab')$_2$	IgG2a	^{131}I-labeled	Phase I trial of a patient with neoplastic meningitis secondary to melanoma	*(154)*
Me1–14 F(ab')$_2$	IgG2a	(^{211}At)	Human glioblastoma and melanoma cells in vitro	*(155)*

and 12). RNAi is a highly conserved mechanism *(140)* that was first recognized as an antiviral immune response in plants to protect against random transposable elements. Double stranded RNAs are processed into short interfering RNAs (siRNA), about 22 nucleotides in length, by the RNA enzyme Dicer. These siRNAs are then incorporated into RNA-induced silencing complexes (RISC), which identify and silence complementary messenger RNAs **(Fig. 4)**.

RNAi has produced a paradigm shift in the process of drug discovery. Its strong appeal in therapeutics is the potency and specificity with which gene expression can be silenced. However, two key challenges to the use of RNAi in therapeutics are avoiding off-target effects and ensuring efficient delivery. The potential risk for side effects stems from the inherent use of host cellular machinery for directing the sequence specific silencing. The use of RNAi to target specific cellular transcripts essentially hijacks the endogenous RNAi pathway,

which can potentially be saturated. The issue of delivery has been the greatest hindrance to the successful treatment of gliomas. Because siRNAs are double-stranded molecules, delivery and cellular uptake is more of a challenge than for single stranded antisense agents, which are taken up by binding to serum proteins *(141)*. It is feasible to modify the backbone of synthetic siRNAs in order to enable resistance to serum nucleases and increase their half-life in vivo *(142)*. Nevertheless achieving intracellular delivery at therapeutically effective concentrations is a still major challenge. The alternative approach is viral-vector mediated delivery, but there are several safety concerns and systemic delivery of viral vectors is still a major hurdle.

The presence of a blood brain barrier precludes passage of macromolecules with molecular weights more than 100,000 Da, including mAbs, liposomes, and gene therapy vectors, which are generally excluded from more than 95% of the tumor tissue *(143,144)*. Once present in the tumor microvasculature the siRNA must be transported across the capillary walls, and penetrate the ECM to reach the tumor cells. Transport through the ECM involves both diffusion and convection owing to the absence of osmotic or pressure gradients in solid tumors. These are relatively slow processes, because the time required to move some distance by diffusion is proportional to the square of that distance *(145)*. During these processes the siRNA might further miss its target because of nonspecific binding to proteins or other tissue components. Moreover, because the siRNA must travel through the vasculature to reach its target, a functional and well distributed vascular network facilitating the tumor blood flow is necessary for efficient drug delivery *(146,147)*. From a clinical point of view this represents a therapeutic quagmire, because tumor blood flow also improves tumor growth. The tumor interstitium is characterized by elevated levels of interstitial fluid pressure, which reduces fluid filtration and consequently impedes the influx of therapeutic agents *(120)*. One strategy used to circumvent these barriers is to administer large enough doses of the drug to achieve therapeutic concentrations to the brain. However, this often results in significant systemic toxicity. Furthermore, because all perivascular cells along arterioles express NG2, therapeutic strategies targeting it in brain tumors must involve local delivery. Nevertheless some of the delivery issues previously discussed need to be taken into account to ensure an adequate biodistribution within the tumor bed. However, should the delivery be optimized and the highest intracellular concentration of the siRNA is achieved, gene silencing may be limited by their transient effects and restricted by the rate of cell division. Mammalian cells do not have mechanisms to amplify and propagate RNAi-like *C. elegans* and plants.

We have recently uncovered a novel role of NG2/HMP in glioma cell death resistance and confirmed it using RNAi. We demonstrated that the expression of NG2/HMP results in significant resistance to death induced by tumor necrosis factor (TNF)-α, daunorubicin, or etoposide. NG2/HMP expression in various

glioma cells was transiently inhibited by siRNAs, which resulted in their increased sensitivity to apoptotic stimuli such as TNF-α. Furthermore, stable downregulation of NG2/HMP in glioblastoma tumors in vivo led to marked reduction of tumor growth rates and volumes, identifying it as an antitumor target. The growth of these tumors in vivo was considerably retarded after TNF-α treatment indicating that the glioma sensitivity to cell death mediators can be restored by siRNA gene silencing (unpublished data).

4. Conclusion and Future Directions

Agents that target the tumor microenvironment represent an important strategy in cancer therapy. Just as the normal brain exists in dynamic equilibrium to maintain normal tissue function, likewise, the tumor adopts many mechanisms to maintain its functional disorder and to evade anticancer therapies. By expressing NG2, the tumor can interact with growth factors to modulate proliferation and angiogenesis; can interact with integrin receptors and ECM components to mediate cellular motility, as well as stimulate signal transduction pathways to avoid apoptosis. Several questions remain to be answered. Can the therapeutic effects of anti-NG2/HMP mAb-mediated immunotherapy be enhanced to overcome the hurdles to efficient delivery and penetrance? Can the administration of small molecule inhibitors of the NG2/HMP or the different signal transduction pathways mediated by the NG2/HMP be targeted? With the use of RNAi in whole animals increasing, its growing implementation in experimental therapy is anticipated. Despite considerable hurdles to overcome, it seems likely that RNAi might find a place alongside conventional approaches in brain tumor treatment, although it is unclear how long it will be before the first RNAi-based drug.

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5

Heterotrimeric G Proteins and Disease

Øyvind Melien

Summary

Heterotrimeric G proteins attached to the cell membrane convey signals from G protein-coupled receptors in response to stimulation by a number of hormones, neurotransmitters, chemokines, and pharmacological agents to intracellular signaling cascades. The heterotrimeric G proteins are also located in the cell interior, and receptor-independent mechanisms may elicit their activation. Thus, G proteins may possibly exert cellular functions other than acting as signaling transducers. There is also increasing evidence for roles in different diseases including infections, inflammation, neurological diseases, cardiovascular diseases, cancer, and endocrine disorders. This review describes characteristics of the heterotrimeric G proteins, evidence for their involvement in different diseases, and outlines some of the therapeutic options utilizing G protein targets.

Key Words: Heterotrimeric G proteins; disease; therapy.

1. Introduction

Heterotrimeric guanine nucleotide-binding proteins (G proteins) belong to a superfamily of guanosine triphosphate (GTP)ases acting as key transducing components of cellular signaling cascades *(1–8)*. Attached to the plasma membrane they transmit signals from G protein-coupled receptors (GPCR) in response to hormones, neurotransmitters, chemokines as well as a number of pharmacological agents to intracellular pathways, which include adenylyl cyclases (AC), phospholipases, and ion channels (**Fig. 1**).

The G proteins are composed of an α- (39–52 kDa), β- (35 kDa), and γ- (7–8 kDa) subunit. Altogether, 21 different Gα-subunits (from 17 genes) have been described sharing approx 20% conserved amino acids. Based on the amino acid sequence of the α-subunits, the heterotrimeric G proteins are

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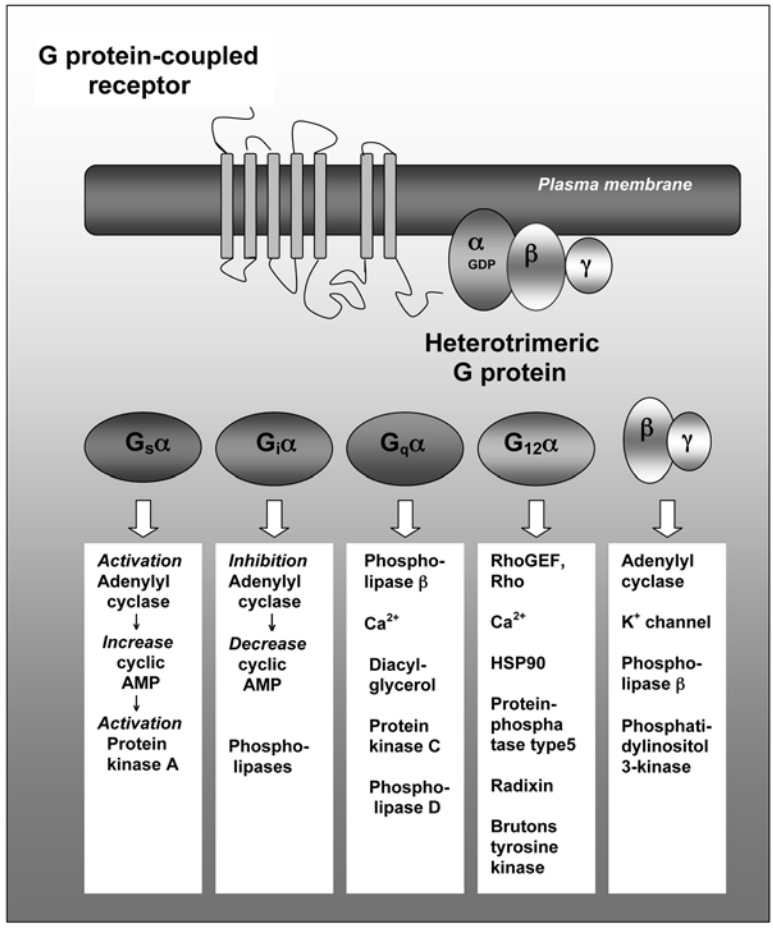

Fig. 1. G protein signaling. Heterotrimeric G proteins are activated in response to stimulation of G protein-coupled receptors by numerous hormones, neurotransmitters, chemokines, pharmacological agents, and other substances. Upon activation, the heterotrimeric G protein dissociates into its α- and βγ-subunits, which transduce signals to various effectors in the cell illustrated here for the different subunit groups.

divided into the four main families G_s, G_i, $G_{q/11}$, and $G_{12/13}$. Six β- and 12 γ-subunits have been identified. The β- and γ-subunits form tightly bound functional dimers that only dissociate after denaturation. Whereas combinations of β- and γ-subunits assembling with specific α-subunits have been defined in various cell types, there is still limited understanding with regard to the biological significance and regulation of G protein heterotrimeric composition.

The GTPase activity of the G proteins is localized within the α-subunit, which also possess a single guanine–nucleotide-binding site. In the resting, inactive state the G protein exists as a heterotrimer with the α-subunit in a GDP-bound form. The activation of G proteins is believed to involve two major events, a guanine–nucleotide-exchange step and dissociation of the α- and βγ-subunits. Upon receptor stimulation by hormones, neurotransmitters, or other agents, the activated receptor interacts with the G protein resulting in release of GDP. Subsequently, a complex is formed between receptor and the G protein in its guanine–nucleotide-deficient ("empty pocket") state before GTP is inserted ***(8)***. The binding of GTP to the α-subunit produces conformational changes resulting in decreased affinity of α for the βγ dimer allowing them to separate and signal to various effectors. The G protein deactivation involves hydrolysis of GTP bound to the α-subunit, mediated by the intrinsic GTPase activity, resulting in subunit reassociation and termination of signaling.

In addition to the intrinsic GTPase activity of heterotrimeric G proteins, the group of regulators of G protein signaling (RGS) ***(9,10)*** act as GTPase-activating proteins (GAP) accelerating the termination of the GTP bound state of Gα, resulting in a shortened timespan of Gα to exert signaling. In addition, the RGS proteins may counteract the interaction between Gα and its effector molecules. Thus, the RGS proteins, presently counting over 30 members, add to the mechanisms of a temporal fine-tuning of signal transduction mediated through heterotrimeric G proteins.

It has been claimed that the dissociation of the α- and βγ-subunits during G protein activation may imply reassociation in an accidental, unregulated manner possibly affecting the signaling outputs that are generated through successive cycles of G protein activation ***(11)***. An alternative model to activation through subunit dissociation would be conformational changes of α and βγ within the frame of a heterotrimeric structure still allowing subunit access to effector molecules. This mechanism is supported by observations indicating that G proteins retain their heterotrimeric state during signaling ***(11–14)***.

Although the activation of heterotrimeric G proteins mainly results from stimulation of GPCRs ***(15)***, receptor-independent mechanisms involving activators of G protein signaling (AGS) have also been identified ***(16,17)***. These discoveries, as well as the observations of several intracellular locations for G proteins, such as in the Golgi apparatus or the nucleus ***(18–24)*** suggest additional roles for G proteins beyond acting as signal transducers. Furthermore, the knowledge of G protein interactions with AGS, RGS, and other accessory proteins ***(17)***, which may also function as scaffolding proteins, as well as with particular cellular microdomains, like caveolae and the Golgi apparatus, suggests additional mechanisms for the achievement of signaling specificity and effects.

2. Heterotrimeric G Proteins Families

2.1. G_s

G_s proteins (44–52 kDa) were the first heterotrimeric G proteins to be discovered *(25–27)*. G_s stimulates AC directly, through the α- or βγ-subunit, to produce cyclic adenosine monophosphate (AMP), which is a central signaling molecule for a range of hormones. The increase in cyclic AMP levels activates protein kinase A, resulting in phosphorylation of several enzymes, ion channels, and transcription factors thereby regulating various metabolic and growth-related processes. G_s proteins may also regulate ion channels independent of AC activation *(18)*. In HEK293 cells, β_2-adrenoceptors switched their coupling from G_s proteins to G_i to convey signals that stimulated extracellular signal-regulated kinase (ERK)1/2 *(28)*. Notably, β-adrenoceptor regulation of ERK1/2 in COS-7 cells was conveyed through G_s proteins in a bimodal manner, i.e., the βγ-subunit-mediated activation, whereas the α_s-subunit exerted inhibition *(29)*. Moreover, in PC12 cells, G_s is involved in stimulation of ERK1/2 through protein kinase A-dependent activation of the small G protein Rap1 and B-Raf *(30)*.

Evidence for active signaling role(s) of G proteins in their heterotrimeric state is obtained for G_s *(11,31)*. Furthermore, a notable observation is the ability of EGF to activate AC in cardiac cells *(32)* involving the $G_s\alpha$-subunit. This supports data from other cells suggesting the existence of signaling interactions between receptor tyrosine kinases and heterotrimeric G proteins *(33)*.

The G_s proteins occur in several splice variants, also within a single cell type. In addition, observations in pituitary cells have provided evidence for the presence of biologically active $G_s\alpha$ variants resulting from limited proteolytic cleavage by calpain *(34)*. The $G_s\alpha$ proteins share the susceptibility to be adenosine diphosphate (ADP)-ribosylated at a highly conserved arginine residue in the N-terminal end of the α-subunit by cholera toxin (CTX), an enterotoxin from *Vibrio cholerae* *(35)*. This modification constitutively activates G_s, by inactivating intrinsic GTPase function, as well as inhibiting the interaction between $G_s\alpha$ and Gβγ, resulting in marked elevation of cyclic AMP. Notably, Gα-subunits belonging to other G protein families also contain the CTX-sensitive arginine residue, and members of the G_i family may be ADP-ribosylated by CTX under certain conditions.

G_{olf} is a member of the G_s family distributed in olfactory epithelium capable of stimulating AC and susceptible to ADP-ribosylation by CTX.

2.2. Gi

G_i proteins (40–41 kDa) include several subtypes. G_{i1}, G_{i2}, G_{i3}, G_o, and G_z inhibit AC, thereby decreasing cyclic AMP production, whereas the retinal subtypes G_{t1} and G_{t2} activate cyclic guanosine monophosphate (cGMP)-specific

phosphodiesterase ***(18)***. All members of the G_i family except G_z are sensitive to pertussis toxin (PTX) ***(36)*** produced by the bacteria *Bordetella pertussis*. Using nicotinamide adenine dinucleotide as a substrate, PTX ADP-ribosylates the α-subunit of G_i at a cystein residue four positions from the C-terminal end, thereby inactivating the G_i protein. It has been observed in various cell types that G_i proteins are more abundant than other members of the G protein family, and $G_o\alpha$ constitutes up to 1–2% of membrane protein in brain.

G_i proteins are involved in regulation of ion channels for K^+, Ca^{2+}, Na^+, or Cl^- ***(37)***. The regulation of the cardiac K^+ channel is conceivably mediated by G_i in a direct manner. Activation of PLCβ enzymes may involve G_i proteins, conceivably their βγ-subunits ***(38)***, and also phosphoinositide 3 (PI 3) kinases are reported to be activated by G_i proteins, through both α and βγ-subunits ***(37)***. The G_i-mediated activation of ERK is promoted through various signaling pathways. In some cells activation of ERK1/2 through G_i proteins involves the βγ-subunit in Ras-dependent and protein kinase C (PKC)-independent pathways ***(39)***. There is also evidence that G_i proteins mediate activation of ERK1/2 in a βγ-independent manner; i.e., suggesting a role for α_i, and as well independent of Ras ***(40)***. In CHO cells a G_i-dependent pathway to ERK1/2 was identified which bypassed Ras and Raf, but involved PI 3-kinase γ and atypical PKC-ζ ***(41)***. A complex formed of a 100-kDa tyrosine-phosphorylated protein (p100) and Grb2, but not Src kinases or Shc, was found to be critical for G_i-mediated ERK activation in Rat-1 fibroblasts and COS cells ***(42)***.

There are also observations suggesting that certain receptor tyrosine kinases activate ERK in part involving G_i-dependent mechanisms ***(43)***. A role for heterotrimeric G proteins in signaling pathways from receptor tyrosine kinases ***(33)*** has support from observations in different experimental models. In hematopoietic cells both colony-stimulating factor 1 and erythropoietin activate mechanisms that conceivably involve G_i proteins ***(44–45)***. In breast cancer cells, epidermal growth factor (EGF) activation was found to implicate G_i proteins ***(46)***. A direct interaction between the EGF receptor and G_i protein was suggested in rat hepatocytes ***(47)*** and similar interactions were reported for the insulin receptor and G_i in hepatoma cells ***(48)***. Furthermore, a recent report suggests that Gβγ-subunits are needed in EGF-induced formation of complexes between EGF receptor and G protein-coupled receptor kinase 2 (GRK2) ***(49)***. These and other data suggest that the function of heterotrimeric G proteins is not confined to signaling from seven transmembrane receptors, although the molecular basis for this interplay is rather elusive.

Observations in different cell types support role(s) of G_i proteins, especially G_{i3}, in functions of the Golgi apparatus ***(18,50–52)***. Data suggest that Gβγ-subunits may mediate several of these Golgi regulatory functions through direct pleckstrin homology domain interactions with protein kinase D, a

subtype of PKC (53). Furthermore, G_i has also been implicated in nuclear import of proteins, although the mechanism for this is unclear (19,54), and $G_{i2}\alpha$ is believed to play a role in the biogenesis of tight junctions (55).

The major roles of G_o proteins have not been clearly determined. As they are both relatively weak inhibitors of AC and highly abundant in certain tissues, other functions have been sought. It may be that G_o proteins are involved in regulation of various ion channels. Furthermore, G_o is implicated in activation of ERK (39) conceivably through a particular mechanism depending on the GTPase activating protein Rap1GAP (56). G_o is involved in pathways that activate the signal transducer and activator of transcription 3 (STAT3) (57), and it appears that the protein GAP43 exerts a regulatory control of G_o (58).

G_z is primarily distributed in brain, adrenal medulla, and platelets. Whereas the understanding of the biological role of G_z are still only partially understood, it is believed that G_z is involved in functions of the nervous system and in platelets and recently it has been reported a role for G_z in the pancreatic islet β cells (59). In addition to its inhibitory effect on AC, it is reported that βγ dimers from G_z mediate regulation of G protein-activated K^+ channels from muscarinic m2 receptors (60), and in a previous report a role for G_z in production of tumor necrosis factor-α was suggested (61). A well described effector for G_z is Rap1GAP, which is a GAP for the Ras family protein Rap1 (62). Activated $G\alpha_z$ recruit Rap1GAP from cytosol to the cell membrane, thus resulting in downregulation of Rap1-signaling (63). $G\alpha_z$ also interacts with a specific regulator of G protein signaling, RGSZ1 as well as the transcriptional cofactor Eya2 (64).

2.3. $G_{q/11}$

Members of the phospholipase(PLC)β family are main substrates for the $G_{q/11}$ proteins (40–41 kDa) (38,65). This activation is mediated through the α-subunit and results in generation of inositol (1,4,5)-trisphosphate (IP_3) from phosphatidyl-inositol 4,5-bisphosphate together with diacylglycerol, which activates PKC. Also Bruton's tyrosine kinase is reported to be substrate for G_q (66), and $G_{q/11}$ proteins are as well involved in ERK activation (39) and growth regulation (67). The G_q-mediated activation of ERK is reported to involve PKC in Ras-independent pathways (39), or may converge with pathways from βγ of G_i that involve Ras (68). G_q is activated by pasteurella multocida toxin (69) causing athrophic rhinitis in pigs.

G_{14}, G_{15}, and G_{16}, which are members of the $G_{q/11}$ family, also activate the β forms of PLC. The distribution of G_{14} is mainly restricted to spleen, kidney, lung, testis, bone marrow, and early myeloid and progenitor B cells, whereas G_{15} and G_{16} are expressed in hematopoietic cells. $G_{14}\alpha$ is reported to stimulate

c-Jun N-terminal kinases (JNK), ERK, and STAT3 *(70,71)*. G_{16} has also been found to be involved in activation of mitogen-activated protein (MAP) kinases as well as STAT3. Thus, melatonin stimulates JNK involving G_{16} *(72)*, and constitutive active $G_{16}\alpha$ is reported to stimulate STAT3 involving c-Src/Janus kinase and ERK *(73)*. Moreover, G_{16} appears to couple a wide range of different GPCRs, and has attained interest as a tool to compare agonist properties *(74)*.

2.4. $G_{12/13}$

The widely distributed G_{12} and G_{13} proteins (42–43 kDa) constitute the most recently discovered class of heterotrimeric G proteins *(65,75,76)*. Although G_{12} and G_{13} are insensitive to PTX, novel observations suggest that pasteurella multocida toxin activates these proteins *(77)*, in addition to its effect on G_q. G_{12} and G_{13} exhibit 67% amino acid identity and couple thrombin, thromboxan A_2 and lysophosphatidic acid receptors. Actin polymerization, phospholipase A_2 activity, and activation of Na^+/H^+ exchanger are reported to implicate $G_{12/13}$. On the other hand, $G_{12/13}$ proteins do not stimulate IP_3 generation *(76)*, but may elicit increases in intracellular Ca^{2+} levels *(78)*. Several of the effects mediated by $G_{12/13}$ such as the stimulation of JNK and activation of the cyclooxygenase-2 promoter involve small G proteins like Rho, Rac, or Ras *(79,80)*. $G_{13}\alpha$ activate Rho through direct stimulation of guanine nucleotide exchange factor RhoGEF *(81)* and $G_{12}\alpha$ is reported to directly stimulate Brutons tyrosine kinase and a Ras GTPase-activating protein *(82)*. In COS-7 cells, ERK was reported to be inhibited by $G_{12/13}$ *(83)*. Moreover, there is evidence that G_{12} interacts with the heat shock protein 90 and that G_{13} interacts with radixin whereas both G proteins interact with protein phosphatase type 5 *(84)*. Despite intensive research the understanding of the roles and signaling mediated by G_{12} and G_{13} proteins is still limited, in part as a result of a lack of identified effector systems *(85)*.

3. G Proteins in Disease

The heterotrimeric G proteins are involved in various diseases through mechanisms involving modifications of Gα-subunits, genetic changes and conceivably by acting as transducers of signals implicated in pathogenetic processes, which, in some cases, may involve quantitative changes in certain subgroups of G proteins. However, it is difficult to define how tight the observed alterations in G protein levels are connected to the disease mechanisms; i.e., whether they only reflect secondary changes or if they are integrated in significant pathogenetic steps. The disease groups covered here, that could have been even more extended, represent main clinical areas wherere there is evidence, although to a variable extent, for an involvement of G proteins.

3.1. Infections

Bacteria, viruses as well as parasites may utilize G protein-dependent pathways in disease mechanisms. One of the early recognized G protein diseases was cholera involving modification of the Gα-subunit. The watery diarrhea induced by *V. cholerae* is a result of the actions mediated by CTX, which ADP-ribosylates $G_s\alpha$ *(35)*. This modification strongly activates AC leading to an increase in intracellular cyclic AMP levels in intestinal cells and, finally, secretion of salt and water. In whooping cough or pertussis, caused by the bacteria *B. pertussis*, the main secreted toxin, PTX, inactivates $G_i\alpha$ *(36)*. The precise role of this particular molecular mechanism in the pathogenesis of pertussis is, however, still not fully understood. Furthermore, it is reported that nontypeable *Haemophilus influenza* invades host cells in a G_i-dependent manner *(86)*.

Disease states induced by several viruses involve GPCRs through different mechanisms *(87)*. Herpesviruses and poxviruses encode GPCRs that are homologues to chemokine receptors and may act in a constitutive manner. For example, there is evidence that the human herpesvirus 8 or Kaposi's sarcoma-associated virus encodes a chemokine GPCR, which promotes the development of Kaposi's sarcomas *(88,89)*. Also the Epstein–Barr virus, which is involved in diseases like infectious mononucleosis and Burkitt's lymphoma, encode a GPCR acting through G_i-dependent mechanisms *(90)*. On the other hand, the molecular pathways utilized by HIV involve chemokine receptors representing coreceptors for virus cell entry, primarily the CXC-chemokine receptor-4 and the chemokine-receptor-5 *(87)*. The receptors, which couple to heterotrimeric G proteins, may be affected by genetic defects, and thus it was previously reported that homozygous loss of function in such coreceptors provides resistance to HIV-1 cell entry *(91)*.

The *GNB3* C825T polymorphism, affecting the G-protein β3-subunit, is reported to be associated with increased function of immune cells in humans *(92)*. On the other hand, recent findings in patients with hepatitis C who carry the *GNB3* 825 CC genotype, showed an association to a lack of treatment effect in response to the combination of interferon-α and ribavirin *(93)*. The *GNB3* 825 CC genotype has recently also been found to be associated with sudden infant death as a result of infection *(94)*. Thus, it appears that the *GNB3* 825 CC genotype somehow is unfavorable in relation to certain infectious states.

Also parasite infections have been reported to involve heterotrimeric G proteins, as it was detected that the β_2-adrenergic receptor with its cognate $G_s\alpha$ protein regulates the entry of *Plasmodium falciparum* into erythrocytes *(95)*.

3.2. Inflammation

Several observations indicate a role for heterotrimeric G proteins in inflammatory responses. Mice deficient of $G_{i2}\alpha$ develop a lethal diffuse colitis, which is

related to ulcerative colitis ***(96)***, and the development of the colitis was preceded by activation of the immune system in the intestine ***(97)***. Recent findings have shown that long-term treatment with anti-α4-integrin antibodies aggravated the colitis in these animals, whereas short-term treatments performed in earlier studies counteracted the inflammation ***(98)***.

In other experimental models, it has been shown that lysophospholipids produced from activated platelets and acting as wound-healing factors in the endothelium involve G_i-dependendent mechanisms ***(99)***. A role for $G_{i2}\alpha$ has also recently been reported in a model of pulmonary immune complex inflammation ***(100)***. Based on studies in $G_{i2}\alpha$-deficient mice, it is suggested that the $G_{i3}\alpha$ protein may be involved in homing of T cells. Also G_{12} and G_{13} proteins are believed to be implicated in B- and T-cell functions, including homing and motility. Lsc, which is the murine homolog to the human guanine exchange factor RhoGEF, is activated by $G_{13}\alpha$ and plays an important role in actin polymerization in both B and T cells, marginal zone B-cell homeostasis and immune responses ***(101)***.

Furthermore, previous experiments in a human lymphoblastic B-cell line also indicated a role for heterotrimeric Gβγ-proteins in the assembly of terminal complement complexes (C5b-7, C5b-8, and C5b-9) in inflammatory reactions ***(102)***. On the other hand, G_s signaling in response to stimulation of the A2A adenosine receptor attenuates proinflammatoric transcription and appears to play an important role in regulating overactive immune cells ***(103)***.

3.3. Neurological Diseases

Certain data suggest that G protein pathways may play a role in neurological diseases including epilepsy and Alzheimer's disease. Various experimental models used in studies of epilepsy shed light on mechanisms involving GPCRs and their heterotrimeric G proteins. In mice it was found that the neuropeptide Y receptor, Y5R, mediated antiepileptic actions of neuropeptide Y regulating limbic seizures ***(104)***. Limbic status epilepticus in a rat model was also recently reported to be counteracted by galanin through the GPCRs, GalR1, and GalR2, whereas inhibition with PTX increased the severity of the epileptic state ***(105)***. In rats with genetic absence epilepsy from Strasbourg (GAERS), injections with PTX decreased seizures suggesting a role for G_i-dependent mechanisms ***(106)***.

In a rat-kindling model of epilepsy, where epileptogenesis is induced in response to electric stimulation, increases in mRNA levels in brain for both $G_s\alpha$ (bilaterally) and $G_{i2}\alpha$ (only on stimulated side) were detected ***(107)***. In accordance with a possible role for $G_s\alpha$, it was earlier demonstrated that intrahippocampal administration of CTX into rats resulted in epileptic seizures ***(108)***. Interestingly, recent observations have also demonstrated that γ-subunits of

G proteins may play a role in epilepsy, as mice deficient of the γ_3-subunit are more susceptible to seizures *(109)*.

In Alzheimer's disease, which involves dysfynctional cholinergic signaling observed at early stages, there is evidence of a decreased coupling of muscarinic M_1-receptors to heterotrimeric G proteins assessed in postmortem neocortex of Alzheimer patients *(110)*. This is in accordance with earlier observations suggesting an uncoupling between receptors and G proteins in Alzheimer's disease *(111)*. In familial Alzheimer's disease, a majority of the patients have a mutation in genes for presenilin. Presenilin is a membrane protein localized in the endoplasmatic reticulum as well as in other areas, and it has been reported that presenilin-1 interacts with the G_o protein and thereby possibly regulating the G protein activity *(112)*.

Also the G_q group of G proteins may play a role in the pathogenesis of neurological disease as it has been described ataxia and dysregulation of motor coordination in $G_q\alpha$-deficient mice *(113)*.

3.4. Cardiovascular Diseases

Different disease states in the cardiovascular system appear to implicate components of G protein pathways. Thus, G protein-dependent mechanisms have been reported with relation to hypertension, cardiac hypertrophy, heart failure, as well as atrial fibrillation (AF).

G proteins may influence hypertension both with regard to the control mediated by the autonomic nervous system and the peripheral vasoregulatory control exerted by opposing actions of vasodilators and vasoconstrictors acting on GPCRs. One of the mechanisms characterized in hypertensive patients is an impaired vasodilator response *(114)*. Thus, it was observed early that there was a decreased β-adrenergic response in lymphocytes of hypertensive patients *(115)*. The explanation for this might relate to a weakened interaction between the GPCR and its G protein, an interaction that is modulated, in part, by the phosphorylation state of the receptor. Phosphorylation of the GPCRs are mediated by GPCR kinase family members (GRK) as well as other kinases, and it has been shown in various hypertensive models that the GRKs are overexpressed *(114)*. These findings thus suggest a role for components exerting a regulatory control of G protein pathways in the disease mechanism. This is also the case in a recent study indicating a role for regulator of G protein signaling 2 (RGS2) in hypertension *(116)*. In this study of hypertensive patients, two single-nucleotide polymorphisms were found to be associated with the hypertensive phenotype *(116)*. These observations are of particular interest with regard to the regulatory role of RGS2 proteins toward G protein signaling. RGS2 acts selectively toward the G protein subunit $G_q\alpha$, which is a main signal transducer for vasoconstrictory agents. Thus, an intact RGS2 function with the

ability to attenuate $G_q\alpha$ activity is presumably an important mechanism to counteract the effect of vasoconstrictors and thereby regulate bloodpressure. In concert with this is the recent reported finding that RGS2 acts as an effector of the nitric oxide-cyclic GMP pathway, which itself is a principal counteractor of vasoconstriction ***(117)***.

Other G protein mechanisms may also play a role in hypertension. Thus, it has been found that expression of the $G_{i2}\alpha$ protein was significantly higher in fibroblasts from hypertensive individuals compared to controls ***(118)***, and the *GNB3* C825T polymorphism, affecting the G protein β3-subunit ***(119)***, has been found associated with hypertension. Recent findings also suggest a role for G_s proteins in hypertension as an association was found to a GNAS1 gene variant ***(120)***.

GPCRs, G proteins and their downstream effectors are involved in normal and pathological regulation of the mammalian myocardium. Cardiac hypertrophy, which is an independent risk factor for heart failure and death, may result from a number of different physiological and pathological conditions. A G protein-dependent pathway, which is believed to play an important role in the development of hypertrophy, involves enhanced $G_{\alpha q}$ signaling ***(121,122)***. Agonists such as angiotensin II, α-adrenergic agents and endothelin-1 stimulate through their membrane receptors $G_q\alpha$ signaling and have also been linked to hypertrophic responses. Furthermore, a recent report also describes an association of the *GNB3* C825T polymorphism with left-ventricular hypertrophy ***(123)***.

The underlying molecular mechanisms in heart failure are reported to involve an impaired β-adrenergic receptor function in heart that affects both receptor density as well as sensitivity ***(124)***. The amount of β-adrenergic receptors of the β1-subtype decreases, whereas the sensitivity both in β-1- and β-2-adrenergic receptors is lowered through desensitization, which in part is mediated by GRK, denoted β-adrenergic receptor kinases (βARK). There are typical changes in βARK function in heart failure involving increases in the level of βARK-subtype 1 appearing even before the disease is precipitated ***(124)***. Thus, inhibition of βARK function is a potential treatment strategy in heart failure ***(124)***. In heart failure models it has also been detected changes in the levels of G proteins including increases of $G_q\alpha$ and $G_i\alpha$ ***(122,125)***.

The *GNB3* C825T polymorphism has recently been linked also to the risk for AF ***(126)***. In a study of 292 patients with AF compared to an equal control group the prevalence of GNB3 TT genotype was 5.8% in the group of AF-patients and 12.0% in the controls. Thus, homozygous carriers of the T allele had a 54% lower risk for AF. However, this risk reduction did not apply to the heterozygous T-allele carriers. The explanation for the observed risk reduction in the homozygous T-allele carriers is not clear. Findings of increased activity

in atrial inward rectifier potassium currents in homozygous T-allele carriers might be of relevance *(127)*. The *GNB3* C825T polymorphism presumably results in an increased signal transduction possibly mediated through G_i proteins *(119)*, which in some way may be favorable with regard to the risk of AF. Of note are novel findings using a gene therapeutic strategy by overexpression of the $G_{i2}\alpha$ protein in porcine atrioventricular node resulting in improved heart rate control in a model of persistent AF *(128)*.

3.5. Cancer

Heterotrimeric G proteins are involved in cell growth and proliferation through their integration with intracellular cascades and networks *(129–131)*. However, the more direct evidence for their transforming potentials are derived primarily from studies of mutant forms of their G protein α-subunits.

Several data support a role of G_i proteins in regulation of gene expression *(132)* and cell growth *(133–135)*, and in hepatocellular carcinoma increased levels of G_i proteins have been detected *(136)*. The mutated $G_{i2}\alpha$ protein *gip2* has been found in ovarian sex chord tumors and adrenal cortical tumors and transforms Rat1a fibroblasts *(137–140)*. The constitutive active Q205L $G_o\alpha$ protein is also reported to transform NIH3T3 cells, murine fibroblasts, through activation of STAT3 *(141)*.

$G_{q/11}$ proteins are involved in ERK activation *(39)* as well as growth regulation *(67)*, but mutant forms of these proteins have so far not been identified in any tumors *(131)*. Expression of the GTPase-deficient $G_q\alpha$ mutant, $G_{\alpha q}$Q209L was observed to cause either cell death *(142)* or transformation *(143)*. In addition, certain data suggest that $G_{q/11}$ proteins may somehow be involved in development of hepatocellular carcinoma *(144)*.

$G_{12/13}$ proteins are involved in growth regulation and exhibit marked transforming properties in fibroblasts *(145–147)*, but no mutations have been detected so far in human cancers. However, overexpression of the native $G_{12/13}$ proteins has been detected in prostate, breast, and colon adenocarcinoma cells *(131)*, but the precise role of these changes are not clear. Recent studies in NIH3T3 murine fibroblasts stimulating protease-activated receptor-1 with thrombin, suggest a more concerted action by different G proteins in the processes of cellular transformation *(148)*. Although $G_i\alpha$ was found to be involved in ERK activation, also $G_q\alpha$ and/or $G_{13}\alpha$ were needed to achieve cellular transformation.

GTPase-deficient mutants of $G_s\alpha$, initially discovered in various endocrine tumors, activate the cyclic AMP/protein kinase A pathways constitutively *(140)*. Although expression of these mutated G proteins do not result in transformation of Swiss 3T3 cells *(149)*, and suppressed Ras-induced transformation of NIH 3T3 cells *(150)*, findings in prostate cancer cells support a role for

$G_s\alpha$-induced pathways in tumor progression and metastatic capability ***(151)***. Furthermore, recent data indicate that a polymorphism in the $G_s\alpha$ protein, GNAS1 T393C is a marker for survival in colorectal cancer stages I and II ***(152)***. The GNB3 C825T polymorphism has also recently been reported to influence disease progression in bladder cancer ***(153)***.

3.6. Endocrine Disorders

Defects in G protein-signaling have been detected in certain endocrine disorders caused by mutations in the $G_s\alpha$ gene, *GNAS1*, and are thoroughly reviewed ***(154,155)***. To date, no mutations resulting in monogenic diseases in humans have been detected in the genes for the β- or γ-subunits. In principle, the mutations in the $G_s\alpha$ gene are either gain-of-function mutations resulting in increased signal transduction owing to a constitutive G protein activity thereby mimicking states of endocrine hyperfunction, or loss-of-function mutations causing hormonal resistance and endocrine hypofunction. An example is the McCune-Albright syndrome involving a somatic gain-of-function mutation in the $G_s\alpha$ gene causing polyostotic fibrous dysplasia, café-au-lait skin hyperpigmentation, and autonome hyperfunction of endocrine organs. This syndrome is not believed to be inherited, although possible exceptions may have been discovered, and presents a variable clinical picture presumably dependent on the time in embryogenesis for the occurrence of the mutation.

Albright's hereditary osteodystrophy is caused by a heterozygous loss-of-function mutation in the $G_s\alpha$ gene resulting in a clinical picture with presentation of short stature, short fingers and toes, obesity, mild mental retardation, as well as other components. However, clinical presentations may vary. Furthermore, it has become evident that the phenomena of genetic imprinting, affects $G_s\alpha$. Thus, patients who receive $G_s\alpha$ mutations maternally develop hormonal resistance (pseudohypoparathyroidism type Ia) in addition to the Albright's hereditary osteodystrophy, because the maternal defect allele tends to be expressed in various organs under hormonal control. On the other hand, the paternally derived defect $G_s\alpha$ alleles are silenced owing to genetic imprinting, and thus the phenotype will be limited to Albright's hereditary osteodystrophy.

Several observations suggest that heterotrimeric G proteins can be linked to actions and regulation of insulin. G_i proteins have been implicated in mediating insulin signaling as well as in the development of insulin resistance, and also G_q might play a role for insulin action. ***(48,118,156)***. Furthermore, recent observations support a physiological role of the G_z protein in the pancreatic β-cell function ***(59)***. Somatostatin, prostaglandin E_1, and other agents have the ability to reduce the insulin secretion from β cells in response to glucose. The recent findings demonstrated that $G_z\alpha$ is necessary for the prostaglandin E_1-induced inhibition of the glucose-stimulated insulin secretion in these cells.

The data also show that the components of the specific $G_z\alpha$ axis, i.e., $G_z\alpha$, Rap1GAP, and Rap1, are functionally active in the β cells. These observations thus implicate G proteins in the regulatory control of pancreatic islet β-cell function.

A recently discovered polymorphism affecting a β-subunit of G proteins, the so called *GNB3* C825T polymorphism is reported to be associated with diabetes type 2 and obesity *(119,157)*. This polymorphism, affecting the $G\beta_3$ gene, results in a truncated splice variant of the $G\beta_3$ protein, denoted $G\beta_{3s}$, lacking 41 amino acids compared to the native protein. The expression of the $G\beta_{3s}$-splice variant is reported to enhance G protein activation, preferentially through G_i proteins.

4. Concluding Remarks and Potentials for Therapeutic Options

Heterotrimeric G proteins transduce signals in response to a number of hormones and neurotransmitters. Furthermore, about 50% of all drugs used in therapy act through GPCRs, thus emphasizing the importance of GPCR/G protein signaling pathways in medical treatment. In addition to the role of G proteins in signaling mechanisms, the identification of their localization also intracellularly as well as the discovery of novel receptor-independent mechanisms for their activation, suggest that G proteins may have other important cellular functions than acting as signal transducers. In light of this, the growing body of evidence that connects heterotrimeric G proteins to various diseases, is not surprising. Added to the initial discoveries that toxin-mediated modifications of G proteins may cause infectious disease as well as the delineation of $G_s\alpha$ mutations as the cause of certain endocrine disorders, is an emerging understanding of a more complex role for G proteins in disease mechanisms. Thus, it is conceivable that G proteins may contribute as transducers or components within more integrated signaling networks in diseases of multigenic/multifactorial origin. For example, the recently discovered *GNB3* C825T polymorphism has been associated with various diseases such as hypertension, diabetes type 2, AF, disease progression in bladder cancer, and response to a certain treatment of hepatitis C. Presently, it is not known how this particular single-nucleotide polymorphism may affect disease development in these different clinical settings. However, it is possible that the *GNB3* C825T polymorphism provides a mechanism, possibly enhanced G_i protein signaling, which somehow might facilitate or act in a permissive manner related to different pathogenetic processes. In any case, this example of a potential G protein-dependent pathogenetic mechanism challenges the approach of understanding more complex disease states.

The possibility that heterotrimeric G proteins may become candidates for medical treatment will depend on several conditions, first, the identification

of significant and clinically relevant targets as well as the development of efficient and selective molecular strategies. Of the numerous possible G protein targets, both the Gβγ dimer and the G_i protein have been explored in experimental models with relevance for cardiovascular diseases. A molecular strategy for targeting the Gβγ dimer utilizes an inhibitor of the enzyme β-adrenergic receptor kinase, βARK1, denoted βARKct, which is a peptide consisting of the last 194 amino acids of βARK1 ***(158)***. This sequence also contains a specific Gβγ-binding domain, and the βARK1ct can thus be used to counteract Gβγ-mediated signaling, which, in turn, also blocks the Gβγ-dependent translocation of βARK1 to the membrane. This translocation is necessary for the activation of βARK1. These inhibitory actions of βARK1ct have been studied in various models. Thus, inhibition of the Gβγ-subunit in arterial vascular smooth muscle through adenovirus-mediated gene transfer of βARKct was shown to dramatically reduce intima hyperplasia and thereby may reveal a novel strategy to treat restenosis after percutaneous transluminal coronary angioplasty ***(158)***. Furthermore, as increased expression and function of the βARK1 is believed to be implicated in heart failure by mediating downregulation of β-adrenergic receptors, the use of βARKct may represent a potential treatment strategy ***(124)***. Recent studies with gene transfer of βARKct in a rabbit model for cardiac dysfunction showed increased right-ventricular afterload owing to pulmonary artery banding (inducing early right ventricular hypertrophy and dilatation), also resulted in improvement of ventricular performance ***(159)***.

Other models have explored direct manipulation of the levels of G proteins. Notably, in studies of persistent AF the overexpression of G_i-protein resulted in improved heart rate control ***(128)***. The further characterization of possible strategies to target G proteins in various disease-related systems may also take advantage of tools such as ribozymes and RNA interference ***(160)***.

The increasing knowledge of the mechanisms in G protein signaling including the discovery of a number of accessory G protein partners, may uncover novel targets for drug treatment. In this regard, both AGS as well as regulators of G protein signaling (RGS) might be candidates. The RGS2 protein, which appears to be implicated in hypertension ***(116)***, mediating actions of nitric oxide, is one possible target.

Finally, an important issue concering the potential of targeting intracellular signaling components in medical therapy, such as G proteins, is the risk level for unwanted effects. As outlined in the first part of this review, G proteins are key elements in numerous signaling pathways, and may exert other important cellular functions. Therefore, the possibility of provoking serious side effects, by targeting key signaling molecules, must obviously be carefully evaluated. This circumstance is an essential condition, which needs to be addressed in

terms of demands for a high degree of cell and target selectivity, if a further development of G protein therapeutic strategies shall succeed.

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6

High-Mobility Group Box-1 Isoforms as Potential Therapeutic Targets in Sepsis

William Parrish and Luis Ulloa

Summary

High-mobility group box-1 (HMGB1) protein was originally described as a nuclear DNA-binding protein that functions as a structural cofactor critical for proper transcriptional regulation and gene expression. Recent studies indicate that damaged, necrotic cells liberate HMGB1 into the extracellular milieu where it functions as a proinflammatory cytokine. Indeed, HMGB1 represents a novel family of inflammatory cytokines composed of intracellular proteins that can be recognized by the innate immune system as a signal of tissue damage. Posttranslational modifications of HMGB1 determine its interactions with other proteins and modulate its biological activity. However, very little is known about how these posttranslational modifications of HMGB1 affect its extracellular inflammatory activity and pathological potential. These studies can provide more efficient therapeutic strategies directed against specific HMGB1 isoforms. Therapeutic strategies against these specific HMGB1 isoforms can serve as models for more efficient therapeutic strategies against rheumatoid arthritis or sepsis. This article reviews the recent studies on HMGB1 regulation and their impact on the inflammatory activity and pathological contribution of HMGB1 to infectious and inflammatory disorders.

Key Words: HMGB1; proinflammatory cytokines; sepsis; posttranslational modifications.

1. Introduction

High-mobility group box-1 (HMGB1) was originally identified as a nuclear DNA-binding protein that participates in the assembly of transcriptional complexes in somatic cells *(1,2)*. Recent studies indicate that damaged necrotic cells or activated leukocytes can liberate HMGB1 into the extracellular milieu where it functions as a proinflammatory cytokine *(1–6)*. Thus, HMGB1 represents an intracellular protein that when present in the extracellular milieu is recognized by the innate immune system as a signal of tissue damage. In this context, extracellular

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HMGB1 appears to be a sufficient mediator of systemic inflammation because administration of exogenous HMGB1 causes a constellation of symptoms similar to that found in clinical trauma or severe sepsis *(4–7)*. HMGB1 is a powerful chemotactic and activating signal for inflammation-mediating immune cells such as neutrophils, monocytes, and macrophages *(8–10)*. HMGB1 also acts as a chemotactic and mitogenic signal for smooth muscle cells *(11)*, and specific types of tissue stem cells such as mesoangioblasts, which can differentiate into most mesoderm-derived cell types, including endothelium and muscle *(12,13)*. Collectively, these results suggest that HMGB1 represents a comprehensive cytokine that is able to orchestrate the regulation of both inflammation and tissue regeneration to promote wound healing.

There are two basic mechanisms for cells to liberate HMGB1 into the extracellular milieu (**Fig. 1**). The first mechanism is a "passive release" of HMGB1 from damaged or necrotic cells. In this context, the extracellular HMGB1 released during necrosis acts as an immune-stimulatory signal that indicates the extent of tissue injury *(7)*. The second mechanism is by "active secretion" of HMGB1 from immune cells. During an immunological challenge, extracellular HMGB1 secreted by immune cells acts as a conventional proinflammatory cytokine *(14,15)*. Both mechanisms result in significant levels of extracellular HMGB1 that can trigger a systemic inflammatory response to ischemia, trauma, burn, infection, or sepsis. Active secretion of HMGB1 requires the execution of a cellular program in activated cells of the innate immune system, especially macrophages and monocytes *(15)*. Although the molecular details of HMGB1 secretion remain unknown, an obligate event in this process is the accumulation of HMGB1 in the cytosol *(14,15)*. Normally, HMGB1 protein is translocated from the cytosol into the nucleus where it binds to DNA and regulates transcription. Nuclear translocation of HMGB1 is controlled by at least two nuclear localization signals (NLS): NLS1 is composed of amino acids 28–44, and NLS2, amino acids 180–185 *(14)*. However, during an immunological challenge, macrophages are activated and HMGB1 becomes acetylated on groups of lysine residues within the NLS (**Fig. 2**). Extensive acetylation of these domains is thought to inhibit nuclear HMGB1 translocation, and, therefore, hyperacetylated forms of HMGB1 accumulate in the cytosol where they are packaged through an unknown mechanism into specialized secretory lysosomes *(14,15)*. The fusion of these secretory lysosomes with the plasma membrane liberates HMGB1 into the extracellular environment. Consequently, immune cells secrete hyperacetylated isoforms of HMGB1, which are molecularly different from the predominantly hypoacetylated forms released from necrotic cells (**Fig. 1**).

Although the functional consequences of the mode of HMGB1 release are currently unknown, acetylation modulates HMGB1 interactions with other

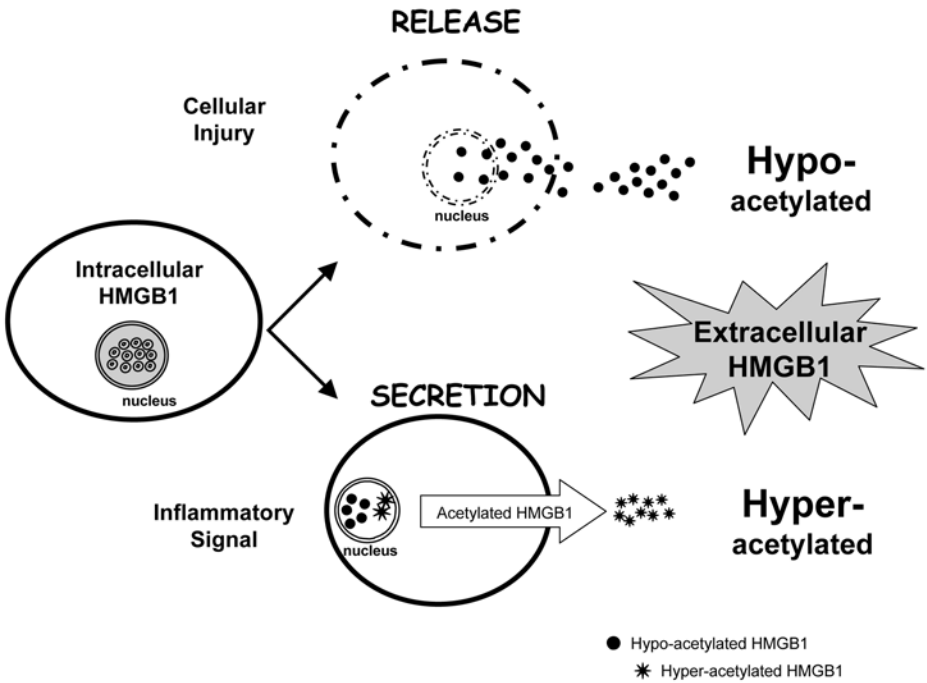
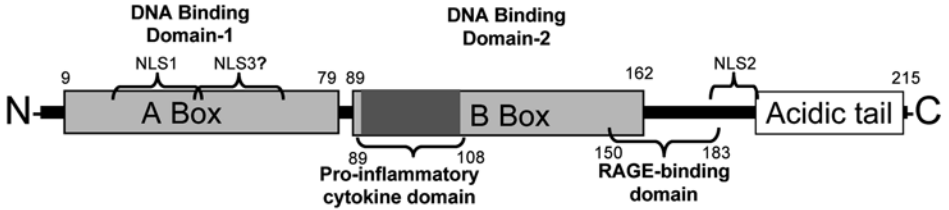
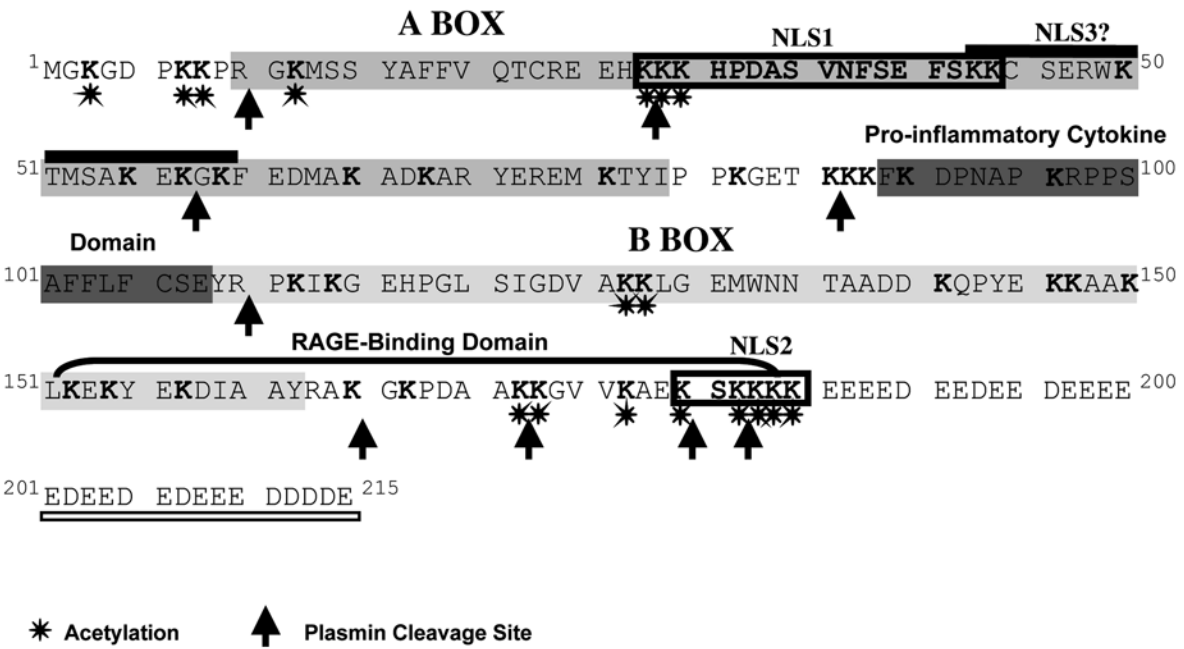


Fig. 1. "Passive release" of hypoacetylated high-mobility group box-1 (HMGB1) isoforms from necrotic cells vs "active secretion" of hyperacetylated HMGB1 isoforms from activated immune cells. Somatic cells contain large amounts of hypoacetylated nuclear HMGB1 that is passively released into the environment following cell membrane perturbation during necrotic death. Alternatively, macrophages, monocytes, and dendritic cells have a specialized regulated secretory mechanism that leads to the active secretion of HMGB1 in response to proinflammatory stimuli. Activated immune cells hyperacetylate nuclear HMGB1 on several lysine residues, which presumably blocks the function of nuclear localization signals and promotes the cytoplasmic accumulation of these HMGB1 isoforms. These hyperacetylated HMGB1 isoforms are then sequestered into specialized secretory lysosomes, which will fuse with the plasma membrane to release hyperacetylated HMGB1 into the extracellular milieu. Note that actively secreted hyperacetylated HMGB1 isoforms are molecularly different from passively released hypoacetylated isoforms. Posttranslational modifications such as acetylation are important for modulating HMGB1 interaction with other proteins and might determine its ability to bind to and activate cell surface receptors, determining its pathological potential.

nuclear proteins *(16–18)*. Likewise, acetylation can impact HMGB1 interactions with specific cell surface receptors and modulate its cytokine and inflammatory activity. The extent to which acetylation or other posttranslational modifications determine extracellular HMGB1-inflammatory activity and


DNA Binding Domain-1
NLS1
NLS3?
DNA Binding Domain-2
NLS2
9
79
89
162
215
N
A Box
B Box
Acidic tail
C
89
108
Pro-inflammatory cytokine domain
150
183
RAGE-binding domain


A BOX
NLS1
NLS3?
1 MGKGD PKKPR GKMSS YAFFV QTCRE EHKKK HPDAS VNFSE FSKKC SERWK 50
Pro-inflammatory Cytokine
51 TMSAK EKGKF EDMAK ADKAR YEREM KTYIP PKGET KKKFK DPNAP KRPPS 100
Domain
B BOX
101 AFFLF CSEYR PKIKG EHPGL SIGDV AKKLG EMWNN TAADD KQPYE KKAAK 150
RAGE-Binding Domain
NLS2
151 LKEKY EKDIA AYRAK GKPDA AKKGV VKAEK SKKKK EEEED EEDEE DEEEE 200
201 EDEED EDEEE DDDDE 215
Acetylation
Plasmin Cleavage Site

pathological contribution to infectious and immunoinflammatory disorders is presently unknown. Currently, there is a great deal of interest in the characterization of HMGB1 isoforms and their relative contributions to infectious and inflammatory disorders *(4,6)*. This renewed interest in the inflammatory activity of HMGB1 has fueled the need for precise methods to purify contaminant-free HMGB1 that retains biological activity. Different methods for the purification and characterization of functionally active HMGB1 are under current development.

2. HMGB1 Purification

There are several recent reports describing refined methods for the purification of biologically active HMGB1 from either prokaryotic (recombinant) or eukaryotic (endogenous or transfected) sources *(19,20)*. These methods represent a major advance in the ability to purify significant amount of HMGB1 and study its cytokine activity and pathological potential under diverse conditions. As the methods employed in these studies are very detailed in the respective publications, the purpose here is to discuss differences in the procedures that affect the levels of contaminants, and the biological activity of the purified HMGB1 protein.

HMGB1 purification from prokaryotic sources. HMGB1 is a proinflammatory cytokine that activates the innate immune system. A major potential problem with expression and purification of HMGB1 from microbial sources is that contamination with bacterial cellular debris, including endotoxin (LPS), can also activate the innate immune system and evoke very similar responses as HMGB1. Complicating purification is the fact that HMGB1 contains an inordinately large number of charged residues (43 lysines and 9 arginines in the N-terminal

Fig. 2. *(Opposite page)* Structural characteristics of high-mobility group box-1 (HMGB1). Primary amino acid sequence and structural organization scheme of human HMGB1 (PubMed accession no. NP_002119). The acetylation state of bovine HMGB1 lysine residues have been previously studied *(14,58)*. (Note that HMGB1 is extremely well conserved in evolution, and human and bovine HMGB1 differ only at amino acid 206, which is aspartic acid in human and glutamic acid in the cow *[58]*). Lysine residues that were found acetylated are indicated with asterisk. Sequences corresponding to the two DNA-binding A- and B-box are highlighted in light gray. Sequences comprising the minimal proinflammatory cytokine-inducing domain of the B-box are highlighted in dark gray. The two characterized HMGB1 nuclear localization signal (NLS) sequences (amino acids 28–44, and amino acids 180–185) are boxed *(14)*, and sequences that comprise a third potential NLS predicted by PROSITE (amino acids 43–59) are underlined. The RAGE-binding domain (amino acids 150–183) is indicated with a bracket *(41)*. Sites that match preferred plasmin digestion sequences *(42)* are denoted by black arrowheads.

DNA-binding domains, and 36 glutamic acids and 20 aspartic acids in the C-terminal acidic tail) that confer strong dipolar, charged properties to the protein (**Fig. 2**). This dipolar feature promotes the binding and copurification of contaminating bacterial components, including bacterial CpG DNA and LPS *(19)*. These contaminants induce immune responses similar to that of recombinant HMGB1, and can potentially obscure specific cytokine effects in different immune cells, including macrophages and neutrophils *(21,22)*. Therefore, a critical step in the purification of recombinant HMGB1 is to avoid bacterial contaminants. The different methods described for HMGB1 purification lead to differences in the final concentration of contaminant endotoxin that can range from 6 pg/mL to >50 ng/mL. The highest reported levels of purity can be best attributed to polymyxin B chromatography followed by a further fractionation using triton X-114 phase separation *(19)*. Recombinant HMGB1 purified in this manner elicits a tumor necrosis factor (TNF) response from primary human blood cells similar to that of protein purified by other methods, indicating that multiple procedures result in recombinant HMGB1 that retains a similar level of biological activity *(19,20)*. Future studies are warranted to determine whether differences in the HMGB1 obtained by these methods might affect other immune responses induced in different cell types.

HMGB1 purification from eukaryotic sources. An important limitation of studies using recombinant protein generated in bacteria is that eukaryotic HMGB1 is extensively modified posttranslationally by mechanisms that are lacking in prokaryote. Therefore, modifications that could potentially modulate the cytokine activity of HMGB1 would be missing from bacterially purified protein. For these reasons, it is important to compare the effects of recombinant and eukaryote-derived HMGB1. Eukaryote-derived HMGB1 has been purified from cultures of Chinese hamster ovary (CHO) *(19,20)*. The methods described in these reports include the generation of stably transfected CHO cells expressing rat HMGB1 *(19)* and the isolation of a CHO cell line that naturally secreted large quantities of endogenous hamster HMGB1 *(20)*. These methods differ primarily in the efficacy of purification. Transfected rat HMGB1 was N-terminally tagged with three tandem copies of the FLAG epitope to facilitate purification from the medium, resulting in highly pure (>90%) HMGB1 at about 50 µg/L of conditioned medium with few chromatography steps *(19)*. Hamster HMGB1, naturally secreted in large quantities, was purified from the culture medium using a series of chromatography steps that resulted in a roughly 90% pure preparation at about 500 µg/L of culture supernatant *(20)*. Similar to the bacterially expressed HMGB1, both eukaryotic preparations result in a similar cytokine activity, and both recombinant and native HMGB1 induce TNF production in human blood, murine RAW264.7 and monocytic THP1 cells in a dose-dependent manner. Both recombinant and native HMGB1

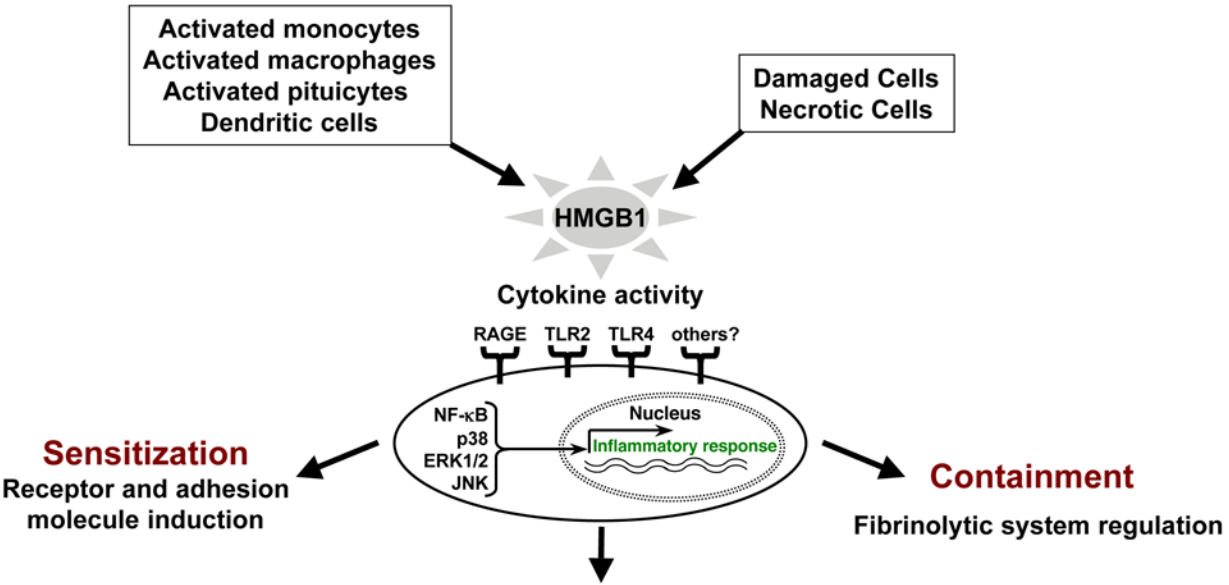
also induce similar proliferation of NIH/3T3 fibroblasts. However, bacterial expressed recombinant HMGB1 elicits stronger responses from cultured cells than equivalent doses of HMGB1 purified from eukaryotic sources *(19,20)*. These results suggest that eukaryotic posttranslational modifications are not required for HMGB1 to function as a proinflammatory cytokine on primary human blood cells.

Since recombinant unmodified HMGB1 is more robust at evoking inflammatory responses than secreted HMGB1 purified from culture supernatants, modifications such as acetylation may actually restrict the ability of HMGB1 to activate TNF or other immune responses. These observations might reflect differences in the physiological mode by which HMGB1 is liberated into the extracellular milieu. For example, nuclear hypoacetylated HMGB1 leaked into the environment by damaged or necrotic cells could signify local tissue damage requiring a strong and immediate immune response to protect against further injury or dissemination of infection. On the other hand, hyperacetylated HMGB1, secreted by activated immune cells even hours after the initial immune insult *(23)*, could serve the more limited purpose of prolonging the initial inflammatory response to promote wound healing and the resolution of tissue damage. A major limitation of these studies is that the test of analysis in vitro, are normally performed at very low concentrations of inactivated fetal serum, which may not mimic the stability of HMGB1 in serum. Future studies will determine whether these results can be translated in vivo, and whether specific responses in different cell types are affected by posttranslational modifications of HMGB1.

3. Cellular Responses to Extracellular HMGB1

The cellular responses to extracellular HMGB1 vary considerably depending upon the cell type. As such, cell-type specific responses to HMGB1 stimulation, and their relative contributions to inflammatory responses are addressed specifically (**Fig. 3**).

Immune cells: HMGB1 is a proinflammatory cytokine that acts as a very potent activator of macrophages and monocytes. HMGB1 stimulates the migration and phagocytosis of these cells *(24,25)*, and activates the production and secretion of a battery of proinflammatory cytokines including TNF, interleukin (IL)-1, IL-6, IL-8, macrophage inflammatory protein-1, and HMGB1 *(8,23,24)*. Thus, HMGB1 can stimulate a self-perpetuating, positive-feedback autocrine loop in macrophages that amplifies and sustains inflammatory cascades. Similar to that described for macrophages, HMGB1 also acts as a chemotactic factor to drive the recruitment of neutrophils to sites of inflammation *(24)*. HMGB1-activated neutrophils produce and secrete proinflammatory cytokines such as TNF, IL-1, and IL-8 *(9)*. HMGB1 also stimulates dendritic cell maturation



Endothelium:	ICAM, VCAM, RAGE, tPA, PAI-1, TNF, IL8
Macrophages:	TNF, IL1, IL6, IL8, MIP1, HMGB1
Neutrophils:	TNF, IL1, IL8, chemo-attraction, migration
Dendritic cells:	TNF, IL1, IL6, IL8, IL12, enhanced immune synapses
Epithelium:	iNOS, increased permeability
Smooth muscle:	actin re-organization, chemo-attraction, migration
Neurons:	TNF, IL1, pyrogens, neurotrophic factor

and subsequent secretion of TNF, IL-1, IL-6, IL-8, and IL-12, which functions to further enhance immune reactions in other immune cell types ***(5,26,27)***.

Endothelium: in response to HMGB1, microvascular endothelial cells upregulate the expression of cellular adhesion molecules like intracellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1, which promote the attachment of different immune cells to the endothelial vessel wall. HMGB1 also disrupts endothelial barrier functions and facilitates the extravasation of immune cells into tissues, but also increases the risk of vascular leakage and edema, which can contribute to tissue damage ***(13)***. HMGB1 also stimulates endothelial cells to produce a variety of proinflammatory cytokines including TNF and IL-8, and causes the secretion of tissue-type plasminogen activator as well as plasminogen activator inhibitor, which influence hemostasis ***(28)***. Therefore, stimulation of the endothelium by HMGB1 provokes inflammatory responses that are important during injury or infection.

Smooth muscle: HMGB1 acts as a strong chemotactic signal that promotes the reorganization of the actin cytoskeleton, leading to smooth muscle cell migration ***(11)***, which is critical to the process of wound healing. The chemotactic induction of smooth muscle cells in response to HMGB1 is also thought to play a role in the pathogenesis of certain vascular diseases such as atherosclerosis and restenosis ***(29)***.

Other cell types: HMGB1 can also alter the permeability of cultured enterocytes through the stimulation of a nitric oxide-dependent pathway and impair the intestinal barrier function in mice ***(30)***. HMGB1 is also a key neurotrophic factor that plays an important role in directing neurite outgrowth during brain development ***(31,32)***. As a ligand for RAGE, HMGB1 has also been implicated in tumor cell metastasis ***(33)***. HMGB1 abundance at the invasive front of solid tumors correlates with matrix metalloprotease activation and the depth of tumor invasion and lymph node metastasis ***(34)***.

Overall, HMGB1 is nearly ubiquitous, and participates in a large number of molecular processes in a variety of cell types. This feature allows HMGB1 to

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Fig. 3. *(Opposite page)* Cytokine activity of high-mobility group box-1 (HMGB1). HMGB1 can be secreted by mononuclear leukocytes as a delayed response to activation by inflammatory signals such as endotoxin, tumor necrosis factor, or interleukin-1, as serum levels of HMGB1 are not elevated until 16–20 h postimmunological challenge in animals, and peaks about 24 h after the onset of sepsis ***(23)***. Once released, HMGB1 functions as a convential proinflammatory cytokine, exerting its effects through cell surface receptors like advanced glycation end-products, Toll-like receptors-2, and Toll-like receptors-4, which activate a variety of cellular responses that vary depending upon the cell type. Typical responses from cell types that have key roles in the normal and pathophysiological outcomes of HMGB1 signaling are indicated.

orchestrate a plethora of cellular signaling events in diverse cell types to coordinate specific biological process (**Fig. 3**). During infection, trauma, or shock, stimulated macrophages can secrete large amounts of HMGB1 into the extracellular milieu *(35)*. Extracellular HMGB1 promotes macrophage and dendritic cell maturation, and induces the production of other proinflammatory cytokines, which further enhance immune reactions *(5,26)*. HMGB1 can also activate endothelial cells, inducing the expression of cellular adhesion molecules and tissue-type plasminogen activator *(2)*. The expression of these molecules promotes the adhesion of immune cells to the endothelial vessel wall and permits their extravasation into tissues. Although this process is critical for the proper resolution of infection or injury, excessive secretion of HMGB1 may result in the disruption of endothelial barrier functions, leading to vascular leakage similar to that observed in sepsis *(13)*. Likewise, HMGB1 can also alter the permeability of cultured enterocytes and impair the intestinal barrier function in mice *(30)*. Thus, similar to other proinflammatory cytokines, HMGB1 can elicit a beneficial defensive immune response against injury or infection. However, excessive release of HMGB1 can become more dangerous than the original stimuli and thus contribute to the pathogenesis of different inflammatory disorders *(1,2)*.

4. Receptors and Cytokine Activity of HMGB1

HMGB1 is considered a cytokine because it is secreted by activated immune cells, transduces signals through cell surface receptors, and induces conventional inflammatory responses in immune and endothelial cells *(1,2)*. Extracellular HMGB1 binds to the cellular receptor for advanced glycation end-products (RAGE) in a concentration-dependent manner *(36)*. RAGE is a transmembrane protein that belongs to the immunoglobulin super-family, and acts as a receptor for diverse ligands including RAGE, amyloid peptide, members of the S100 family of inflammatory-mediating peptides, and is currently the best characterized cellular receptor for HMGB1 *(37–39)*. RAGE is expressed on the surface of a variety of cell types including endothelium, vascular smooth muscle, neurons, macrophages, and monocytes *(38)*. Stimulation of RAGE results in the activation of multiple intracellular signaling pathways including the small Rho-like GTPases Rac and CDC42, mitogen-activated protein kinases (p38, c-Jun N-terminal kinase, and ERK1/2) and the nuclear factor (NF)-κB pathway *(37)*. It is noteworthy that both RAGE and HMGB1 are NF-κB-responsive genes, and their expression can be dramatically enhanced upon RAGE stimulation in various cell types *(38)*. This self-enhancing property of RAGE signaling could function to further sensitize cells to RAGE ligands such as HMGB1 or S100 proinflammatory peptides, and has important implications for the progression of inflammatory responses. One specific example of this mechanism is the

requirement of HMGB1-RAGE signaling for the maturation of plasmacytoid dendritic cells (PDCs) and their contribution to T-cell activation *(27)*. Activated PDCs accumulate HMGB1 in the cytosol in response to bacterial CpG DNA-mediated Toll-like receptor (TLR)-9 signaling, and the subsequent secretion of HMGB1 by these PDCs is crucial to direct their maturation in an autocrine fashion *(27)*. The pools of HMGB1 secreted from activated dendritic cells appears to be essential for the clonal expansion, survival, and functional polarization of naive T cells *(5,26)*, which then drive adaptive immune responses. These observations may in part explain why certain therapies that target HMGB1 can short-circuit the progression of experimental sepsis *(4,6,23,40)*.

Recent studies indicate that specific stimulation of RAGE alone cannot account for the full array of cellular responses induced by HMGB1 *(39)*. First, neutralizing antibodies directed against RAGE only partially inhibit HMGB1-mediated cytokine activity *(10,28,29)*. Second, although the RAGE-binding domain has been mapped to the C-terminus (amino acids 150–183) of HMGB1 *(41)* **(Fig. 2)**, structure/function studies of HMGB1 show that the first 20 amino acids of the DNA-binding B-box (corresponding to residues 89–108) are sufficient for stimulating macrophage TNF secretion *(10)*. Also consistent with alternative cellular HMGB1 receptors is the observation that the RAGE-binding domain of HMGB1 contains several preferred plasmin recognition sequences **(Fig. 2)** whose availability for digestion is likely to be controlled by acetylation *(42)*. Therefore, acetylation might play multiple roles in controlling HMGB1–RAGE interactions, not only by potentially modulating receptor affinity through targeting the RAGE-binding domain of HMGB1, but also by potentially protecting it from proteolytic inactivation. Taken together, these observations suggest that the cytokine-inducing activity of HMGB1 might be partially independent of RAGE. Recent studies suggest that in addition to RAGE, TLR-2 and TLR-4 also mediate the cytokine activity of HMGB1. The ability of necrotic cells to maximally stimulate NF-κB activity in macrophages is at least in part dependent on a TLR-mediated signaling pathway *(43)*, and both TLR-2 and TLR-4 have been implicated in the activation of macrophages by HMGB1 *(21)*. The specific contributions of RAGE, TLR-2, and TLR-4 to the cytokine activity of HMGB1 remain unclear in part because these receptors share common downstream effector molecules (especially NF-κB, c-Jun N-terminal kinase, and p38 mitogen-activated protein kinases) that stimulate similar intracellular signaling events *(2,40)*. This sharing of signaling components makes genetic studies using dominant-negative constructs and heterologous expression systems difficult to interpret, and has lead to contradictory conclusions in the literature regarding the roles of RAGE, TLR-2, and TLR-4 in executing the cytokine activity of HMGB1 *(2,21,39)*. The TLR receptors also form homo- and hetero-oligomers that function in signaling, and the oligomeric state of TLRs

can dramatically increase the complexity of receptor isoforms *(22,36,44)*. Each oligomeric state might have alternative substrate specificities and different capacities for the recruitment and activation of downstream signaling adaptors in a cell type-specific manner. This phenomenon has been already described for G protein-coupled receptors; the biological significance of G protein-coupled receptors oligomerization has only recently coming to light *(45,46)*, and could potentially explain some apparent discrepancies regarding HMGB1 signal transduction.

HMGB1 has the capacity to act through nonreceptor-mediated mechanisms to facilitate delivery of other molecules across cellular membranes *(47,48)*. Because HMGB1 can effectively transport plasmid DNA into cells for transfection, it might also facilitate the delivery of other biologically active molecules into cells, especially those derived from necrotic cells or invading pathogens. In this respect, HMGB1 has similar properties to proteins like lactoferrin and the HIV-1 Tat protein, which contain protein transduction domains and promote cargo delivery across cell and organelle membranes *(49)*. These protein transduction domains are typically rich in basic amino acids (lysine, arginine, and histidine), and convey the ability to transport other proteins, DNA, and RNA into cells *(50)*. This property of HMGB1 adds another potential dimension to its signaling capabilities, as it could potentially act as a carrier to present unmethylated CpG DNA to TLR-9, an intracellular TLR expressed in dendritic cells and B cells that responds to bacterial or viral DNA *(51)*. This mechanism could be analogous to TLR-4, which requires additional cofactors such as LPS-binding protein, CD14, and MD-2 to respond to LPS. The potential for HMGB1 as an alternative carrier for the presentation of LPS to TLR-4 could represent an interesting mechanism to explain why HMGB1 and LPS can elicit similar responses from macrophages in culture *(21)*. Detailed studies are needed to define the relative contribution of RAGE, TLR-2, and TLR-4 to HMGB1 cytokine activity and to its roles in the pathological progression of infectious and inflammatory disorders *(4,40,52)*.

5. Future Directions

HMGB1 was recently described as a therapeutic target for different infectious and inflammatory disorders *(1,2,4,52)*. However, little is known about the contribution of posttranslational modifications to the extracellular regulation of HMGB1 cytokine activity, or its pathological effects in infectious and inflammatory disorders. Indeed, HMGB1 is a member of a novel family of inflammatory cytokines composed of intracellular proteins that when present in the extracellular milieu, are recognized by the innate immune system as a signal of tissue damage. Posttranslational modifications of HMGB1 modulate its intracellular biological activity by controlling its interactions with other proteins

(14,18). It is now proposed that posttranslational modifications of HMGB1 modulate its cytokine and inflammatory activity by determining its interactions with cell surface receptors.

HMGB1 purified from calf thymus exists in several distinct isoforms including at least 10 acetylated species *(14)*, which may also bear ADP ribose, glycosylation, phosphorylation, and methylation moieties *(1,2)*, each of which may contribute differentially to determine the biological activity of HMGB1. The acetylated isoforms of HMGB1 were described for protein purified from the thymus of healthy animals *(14)*, and thus it is uncertain whether they reflect the acetylation state of HMGB1 secreted during pathological or inflammatory conditions. Thus, it is probable that lysine residues found unmodified in this study could represent important sites to control the inflammatory activity of HMGB1. For instance, several studies indicate that under normal conditions HMGB1 moves and accumulates into the nucleus. During immune challenges, acetylation of lysine residues within NLS (NLS1 and NLS2) is thought to prevent HMGB1 translocation into the nucleus **(Fig. 2)**. However, lysine residues in NLS1 (lysines 28, 29, 30), and in NLS2 (lysines 180, 182, 183, 184, and 185) were acetylated in healthy animals, and a mutant HMGB1 protein compromised for the function of both NLS sequences by substitution of six of these lysines (either to alanine or to glutamine) still showed nuclear localization *(14)*. These results suggest a putative third NLS (NLS3) within HMGB1. Accordingly, a PROSITE scan of HMGB1 reveals a putative NLS3 corresponding to the amino acids 43–59 **(Fig. 2)**. Because none of the lysine residues within this putative NLS were found acetylated, this motif could represent a potential target for preventing HMGB1 nuclear translocation during immune stimulation. Hyperacetylation of HMGB1 on lysines within NLS2 (lysines 180, 182, 183, 184, and 185), which are located inside the RAGE-binding domain **(Fig. 2)**, is thought to promote secretion during immune challenges *(14)*. Therefore, hyperacetylated HMGB1 (in the RAGE-binding domain) might have lower affinity for RAGE and induce a weaker immune response, perhaps mimicking the weaker responses induced by HMGB1 purified from eukaryotic sources in vitro *(19,20)*. However, hypoacetylated nuclear HMGB1 that leaks into the extracellular milieu during injury or necrosis might have higher affinity for RAGE, and induce a stronger immune response, similar to that described for bacterial expressed recombinant HMGB1 *(19,20)*.

Posttranslational modifications can also determine HMGB1 interaction with specific proteases and modulate its extracellular stability. Although there is currently no data regarding the stability of HMGB1 in the extracellular environment, one possibility is that acetylation can provide greater stability to HMGB1 in the extracellular environment. Acetylation in lysine residues during immune active secretion of HMGB1 can block lysine residues that constitute sensitive

protease digestion sites for tissue and serum proteases, such as a plasmin. In support of this hypothesis, HMGB1 interacts with tissue-type plasminogen activator and stimulates the conversion of the zymogen plasminogen to plasmin, a highly active lysine-specific endoprotease *(53)*. Because HMGB1 is itself a lysine-rich protein that serves as a plasmin substrate, hypoacetylated extracellular HMGB1 would be more susceptible to degradation, restricting it to a local environment *(42)*. This same mechanism would potentially allow hyperacetylated HMGB1 secreted from macrophages and other immune cells to disseminate into the serum and contribute to systemic inflammation, which appears to be the case in severe sepsis.

In addition to acetylation, HMGB1 has also been found to be methylated, glycosylated, ADP-ribosylated, and phosphorylated *(2)*, all of which can contribute to the regulation its biological functions. Indeed, HMGB1 can be polyADP-ribosylated, which is a common phenotypic change in proteins present in cells with damaged DNA. HMGB1 is also a substrate for protein kinase C (PKC) phosphorylation *(54)*, and the activity of several PKC isoforms is stimulated upon leukocyte activation *(55–57)*. Although the PKC phosphorylation sites on HMGB1 remain uncharacterized, it is noteworthy that there are PKC consensus phosphorylation sites in each of the NLS sequences (NLS1, NLS2, and the putative NLS3), in the proinflammatory cytokine domain of the B-box, and in the RAGE-binding domain (**Fig. 2**). These potential phosphorylation sites may represent additional mechanisms for regulating HMGB1 cytokine and inflammatory activity. Because of recent methodological advances in the ability to generate large quantities of relatively contaminant-free HMGB1 preparations *(19,20)*, in vivo experimental approaches can now be targeted to determine the relative contribution of these posttranslational modifications of HMGB1 to its proinflammatory cytokine activity and pathological contribution to infectious or inflammatory disorders *(1,2,4,52)*.

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7

Antisense Oligonucleotides

Target Validation and Development of Systemically Delivered Therapeutic Nanoparticles

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Summary

Antisense oligonucleotides (ASO) against specific molecular targets (e.g., Bcl-2 and Raf-1) are important reagents in cancer biology and therapy. Phosphorothioate modification of the ASO backbone has resulted in an increased stability of ASO in vivo without compromising, in general, their target selectivity. Although the power of antisense technology remains unsurpassed, dose-limiting side effects of modified ASO and inadequate penetration into the tumor tissue have necessitated further improvements in ASO chemistry and delivery systems. Oligonucleotide delivery systems may increase stability of the unmodified or minimally modified ASO in plasma, enhance uptake of ASO by tumor tissue, and offer an improved therapy response. Here, we provide an overview of ASO design and in vivo delivery systems, and focus on preclinical validation of a liposomal nanoparticle containing minimally modified *raf* antisense oligodeoxynucleotide (LErafAON). Intact rafAON (15-mer) is present in plasma and in normal and tumor tissues of athymic mice systemically treated with LErafAON. Raf-1 expression is decreased in normal and tumor tissues of LErafAON-treated mice. Therapeutic benefit of a combination of LErafAON and radiation or an anticancer drug exceeds radiation or drug alone against human prostate, breast, and pancreatic tumors grown in athymic mice. Further improvements in ASO chemistry and nanoparticles are promising avenues in antisense therapy of cancer.

Key Words: Raf-1, antisense oligonucleotides; liposomes; radiation; chemotherapeutic drugs; preclinical validation; cancer therapy.

1. Introduction

Sequence-specific depletion of molecular targets via antisense strategies, including antisense oligonucleotides (ASO) and short-interfering RNA, has enormous potential in cancer biology and therapy (*1*,*2*; *see* Chapters 9 and 12).

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During the past two decades, considerable progress has been made in the design and application of synthetic ASO *(3–8)*. The applications of ASO in target discovery and validation and in functional genomics have been well established. Further improvements in ASO chemistry and development of safe and effective tumor-targeted ASO nanoparticles are likely to close the gap between ASO drug discovery and clinical practice. This chapter provides an overview of current state-of-the-art ASO technology, and focuses on our efforts to develop and validate a therapeutic nanoparticle containing minimally modified c-*raf* ASO.

2. ASO Design and Validation

Ideally, an antisense therapeutic is expected to fulfill all of the following criteria: (1) nuclease resistance; (2) lack of cytosine–guanine motifs; (3) acceptable pharmacokinetics and bioavailability; (4) nontoxicity in animal models; (5) transport through cell membrane; (6) maximal inhibition of the target protein preferentially in tumor tissues; (7) preclinical efficacy; (8) cost-effective scale-up and production; (9) clinical tolerability; and (10) therapeutic benefit over existing treatment modalities. Various modifications of ASO chemistry and in vitro and in vivo applications of ASO targeted against specific molecules in cancer cells are summarized in **Table 1**. The reader is referred to recent reviews for further details of ASO chemistries and their advantages and disadvantages *(4,7)*. In general, phosphorothioate and chimeric modifications, which include partial or complete modification of the backbone and/or ribose moiety (2'-OH group), have resulted in significant improvements in pharmacokinetics, bioavailability, and target inhibition. As a prototype example, Raf-1 protein expression is inhibited in several human cancer cells exposed, albeit in the presence of lipofectin, to a phosphorothioate ASO targeted to the 3'-untranslated region of c-*raf*-1 mRNA sequence (ISIS 5132) (**Fig. 1**). Similar effects have been seen using a phosphorothioate ASO targeted to the same region of c-*raf*-1 mRNA and it also has a 2' methoxyethyl group on the sugar moiety (ISIS 13650) (**Fig. 1**). ISIS 5132, ISIS 13650, and a mismatch phosphorothioate oligo ISIS 10353 were kindly provided by Dr. Brett Monia (ISIS Pharmaceuticals) *(9,10)*. Currents efforts are directed at further improvements in chemistry so that the clinical side effects are minimized and access to the target site and penetration/retention of ASO into tumor cells are maximized. In preclinical studies, as single agents, most ASO exhibit tumor growth arrest properties, consistent with the oncogenic/prosurvival phenotype of their target molecules. The combinatorial antisense approach, whereby disease-specific signaling pathway(s) can be simultaneously disrupted by approaching redundant and multiple targets, and has the potential to enhance therapeutic efficacy of radiation or anticancer drugs against refractory cancers.

Table 1
Antisense Oligonucleotides in Cancer Biology and Therapy[a]

Molecular target	ASO design and application (I.D., size, chemistry, in vitro, in vivo)[b]	Ref.
Bcl-2	G3139, 18-mer, PS, in vitro, and in vivo	35
Bcl-xL	ISIS 16009, 20-mer, PS, and 5 nt at the 5'- and 3'-ends with 2'-MOE, in vitro	36
Bcl-x pre-mRNA	ISIS 22783, 20-mer, PS with 2'-MOE, in vitro	37
Bcl-2/Bcl-XL	4625, 20-mer, PS, and 5 nt at the 5'- and 3'-ends with 2'-MOE, in vitro	38
	5005, 20-mer, PS, and LNA modification of 5 nt at the 5'- and 3'-ends, in vitro	39
Bcr-Abl	NLS-asPNA, 13-mer, basic peptide (VKRKKKP)-linked PNA, in vitro	40
Clusterin	OGX-011, 21-mer, PS, chimeric 2' MOE/2'-deoxynucleotide, in vitro, in vivo	41
CRE-transcription factor	CRE-decoy, 24-mer CRE palindrome, PS, in vitro, in vivo	42
CSF-1	CSF-1 ODN-196, 15-mer, PS, in vivo	43
HER2/ErbB2/Neu	AS HER-2 ODN, PS, 15-mer, in vitro	44
	AP7-2, 15-mer, PS, ASO containing dithiodipyridine group at 3'-end and conjugated to a peptide (LTVSPWYC), in vitro	45
Mcl-1	ISIS 20408, 20-mer, PS, chimeric 2'-MOE/2'-deoxynucleotide, in vitro, in vivo	46
MDM2	AS, 20-mer, PS, MBO, in vitro, in vivo	47
	T5-12-Acr, Acr-PNA, in vitro	48
c-Myc	INX-6295, 16-mer, PS, in vitro, in vivo	49
Translocated c-Myc	PNAE μwt, 18-mer, NLS peptide (PKKKRKV)-linked PNA, in vitro	50
PKA (RIα)	GEM 231, 18-mer, PS, MBO, in vitro, in vivo	51
PKC-α	ISIS 3521, 20-mer, PS, in vitro, in vivo	52
PML/PML-RAR-α	PNA no 1 15-mer, adamantyl(*Ada*)-linked PNA, in vitro	53
B-Raf	BRAF-AS, 18-mer, PS, in vitro	54
c-Raf	As-raf/rafAON, 15-mer, one base at 5'- and 3'-ends has PS-linkage, in vitro	30,55
	ISIS 5132, 20-mer, PS, in vitro, and in vivo	9
	ISIS 13650, 20-mer, PS with 2'-MOE, in vitro	10

(Continued)

Table 1 ***(Continued)***

Molecular target	ASO design and application (I.D., size, chemistry, in vitro, in vivo)[b]	Ref.
Ribonucleotide reductase (R2)	GTI-2040, 20-mer, PS, in vitro, in vivo	*56*
Telomerase	ISIS 24691, 13-mer, PS, 2'-MOE RNA, in vitro	*57*
XIAP	GEM640/AEG 35156, 19-mer, MBO, in vitro	*58*

[a]Selected ASO and their applications are listed. ASO tested using specialized in vivo delivery systems are listed in **Table 2**.

[b]In vitro, cultured cells; in vivo, animal models; PO, phosphodiester; PS, phosphorothioate; PNA, peptide nucleic acid; Acr-PNA, 9-aminoacridine conjugated PNA; LNA, locked nucleic acid; 2' MOE, 2'-*O*-(2-methoxy)ethyl; MBO: modified oligodeoxynucleotide or oligoribonucleotide segment (2'-*O*-methylribonucleoside PS/2'-*O*-methylribonucleoside PO/deoxynucleoside methylphosphonate *(59)*.

3. In Vivo Delivery of Antisense Oligonucleotides

The criteria of a clinically and commercially compatible ASO delivery system include nanoparticle size (<1 μ in diameter), biodegradability, high encapsulation/entrapment efficiency, ASO stability and favorable pharmacokinetics, tumor-specific drug delivery, easy formulation, scalability, and safety profiles in animals and humans. Such an optimal in vivo delivery system offers merits that may complement not only the chemically modified ASO but also natural ASO. First, a suitable vehicle may protect the unmodified/minimally modified ASO from nuclease degradation and alleviate chemistry-related side effects. Second, it may preferentially carry ASO to its desired destination, decreasing the dose required and normal tissue toxicity. Third, it may facilitate ASO transport through the cell membrane and enhance its retention in cytoplasm or transport to the nucleus, effectively blocking translation and/or transcription of the target molecule. Limited studies of ASO in vivo delivery systems have been reported (**Table 2**). Nontargeted and tumor-targeted cationic liposomes have been shown to deliver minimally modified ASO and modified ASO, respectively (**Figs. 2** and **3**; **Table 2**). In addition, several laboratories have reported the use of stabilized antisense lipid particles. Further investigations are necessary to develop validated composition(s) of safe and efficacious ASO nanoparticles. Efforts in our laboratory have been focused on development of liposome-entrapped ASO nanoparticles. One of these formulations, liposomal nanoparticle containing minimally modified *raf* antisense oligodeoxynucleotide (LErafAON), is discussed in the following section.

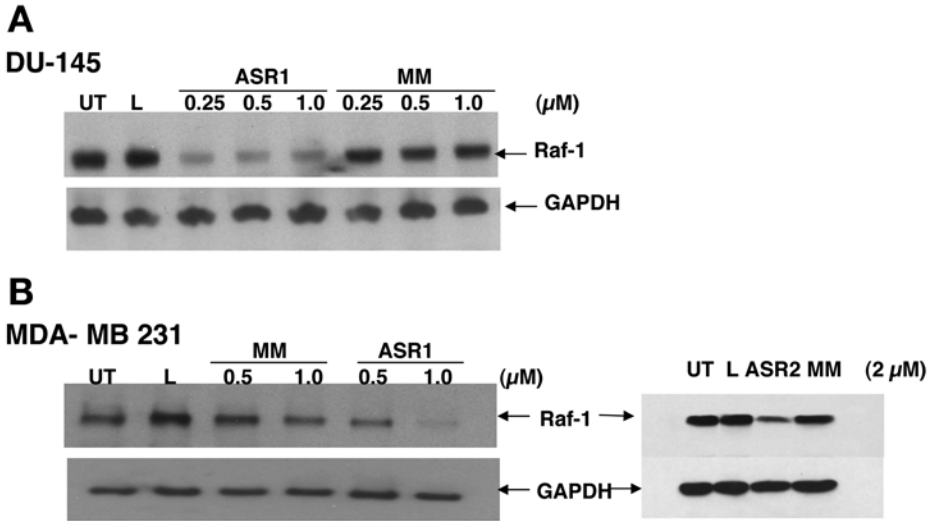


Fig. 1. Inhibition of Raf-1 protein expression in human prostate (DU-145) and breast cancer cells (MDA-MB 231). Tumor cells were treated with indicated concentration of phosphorothioate *c-raf* ASO (ASR1, ISIS 13650; ASR2, ISIS 5132) or a mismatch ASO (MM, ISIS 10353). Cells were grown to 80% confluency in improved minimum essential medium containing 10% fetal bovine serum (FBS) and 2 m*M* L-glutamine. For ASR1 treatment, on day 1 cells were treated with indicated concentration of ASO or MM for 6 h in the presence of lipofectin (15 μg/mL) in medium containing 1% FBS. Cells were then washed twice with 10% FBS containing medium and maintained overnight in 1% FBS containing medium in the presence of indicated concentration of antisense oligonucleotides (ASO) or MM. On day 2, cells were treated as on day 1. On day 3, Raf-1 expression was detected in cell lysates by Western blotting using monoclonal anti-Raf-1 antibody. The blots were reprobed with polyclonal anti-GAPDH antibody. For ASR2 treatment, cells were washed twice with serum-free medium and then treated with indicated concentration of ASO or MM for 6 h in serum-free medium containing 20 μg/mL lipofectin. Cells were washed twice with 10% FBS containing medium and incubated overnight in 10% FBS containing medium in the presence of indicated concentration of ASO or MM. On day 2, cells were treated as on day 1, followed by cell lysis and Western blotting on day 3. UT, untreated; L, lipofectin control.

4. Liposome-Entrapped raf Antisense Oligonucleotide: A Therapeutic Nanoparticle

4.1. Raf-1 Is a Validated Target in Cancer Therapy

Raf-1 (c-Raf), B-Raf, and A-Raf are members of a family of serine-threonine kinases known to regulate extracellular signal-regulated kinase -mediated mitogenic signal transduction pathways (*11*). Numerous reports from our laboratory and others have shown that Raf-1 plays a key role in cell proliferation, survival,

Table 2
In Vivo Delivery Systems of Antisense Oligonucleotides[a]

In vivo delivery system	Molecular target	Formulation I.D. and ASO parameters in vivo[b]	Ref.
Cationic liposomes (first generation)	Raf-1	LE-ATG-AS, one base at 5'- and 3'-ends has PS-linkage, 15-mer, IV/IT, PK, TIB, TI	*34*
		LE-5132, PS, 20-mer, IV, PK, TIB, TI, EF	*60*
Cationic liposomes (second generation)	Raf-1	LErafAON, one base at 5'- and 3'-ends has PS-linkage, 15-mer, IV, EntEf, S, TX, PK, TIB, TUB, TI, EF	*26–28*
Cationic liposomes (easy to use)	Raf-1	LErafAON-ETU, one base at 5'- and 3'-ends has PS-linkage, 15-mer, IV, PK, TIB, E	*32,61*
Folate-liposomes	HER2	AS HER-2 ODN, PS, 15-mer, S, IV, TT, TI, EF	*62*
Cationic and immunoliposomes-(scFv against TfR)	HER2	scL-AS HER-2 ODN, PS, 15-mer, IV, TT, TI, EF	*63*, Personal communication
Coated-cationic immunoliposomes (GD2-targeted)	c-Myc	aGD_2-CCL-myc-as, PS, 16-mer, IV, TT, PK, TI, EF	*64*
	c-Myb	Targeted liposome-CpG-myb-as, PS, 24-mer, IV, EF	*65*
PIHCA nanoparticles	MutatedHa-Ras	AS-VAL, unmodified 12-mer, IT, TI, EF	*66*
PACA nanocapsules	EWS-FLi-1	NC AS, PS, 25-mer, IT, EF	*67*
SALP	c-Myc	SALP INX-6295, PS, 16-mer, IV, PK, TUB, TI, EF	*68*

[a]In vivo refers to preclinical studies in animal models. Selected in vivo ASO delivery systems in cancer biology or therapy are listed.

[b]EntEf, ASO entrappment efficiency; S, ASO stability; IV intravenous, IT intratumoral; TT, tumor-targeted; PK, plasma pharmacokinetics; TIB, normal tissue biodistribution; TUB, tumor tissue biodistribution; TX, toxicology; TI, target inhibition; EF, in vivo antitumor efficacy; PIHCA, polyisohexylcyanoacrylate; PACA, polyisobutylcyanoacrylate; TfR, transferrin receptor; GD2, disialoganglioside; SALP, stabilized antisense lipid particles.

damage-induced signal transduction, and metabolism *(12–17)*. Growing evidence also suggests an extracellular signal-regulated kinase-independent role of Raf-1 in cell survival *(5,18,19)*. Using sense and antisense c-*raf*-1 cDNA

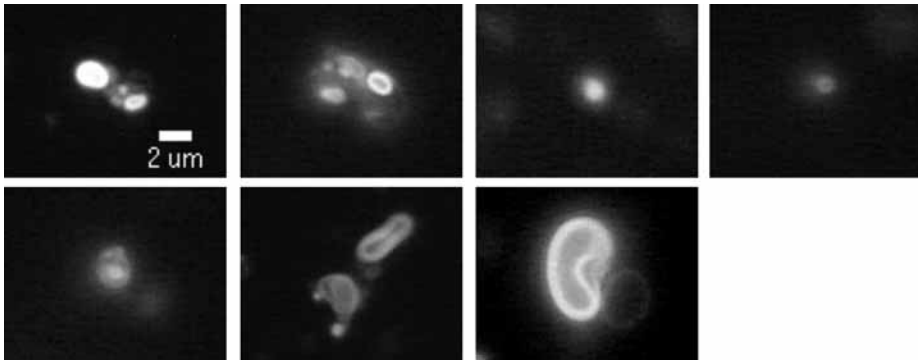


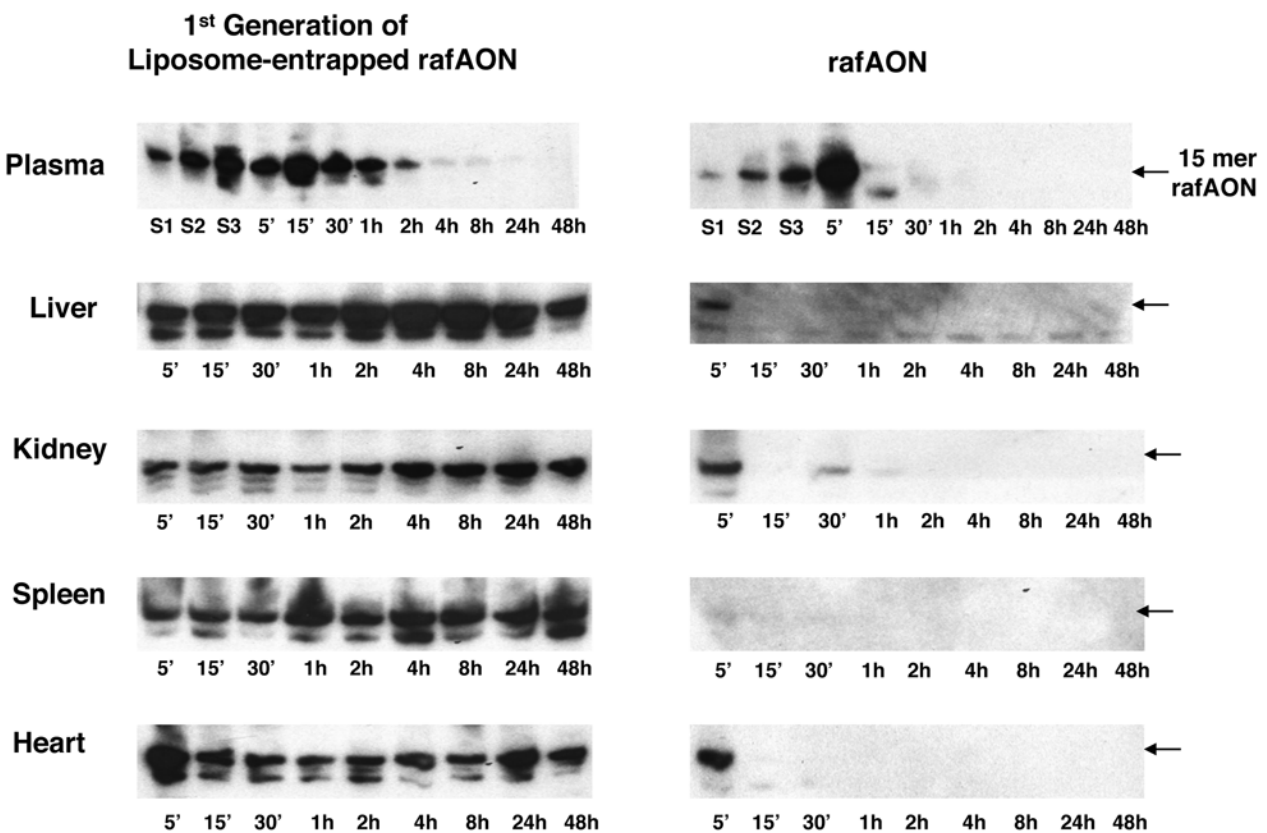
Fig. 2. Fluorescence microscopy showing first generation of liposome-entrapped *raf* antisense oligodeoxynucleotide (LE-ATG-AS *raf* ODN). 5'-Fluorescein-labeled minimally modified rafAON (ATG-AS *raf* ODN) was entrapped in liposomes as described earlier ***(34)***.

molecules and Raf short-interfering RNA, expression of Raf-1 has been associated with resistance of human tumors to radiation and chemotherapeutic drugs ***(2,20–25)***. A correlation between ASO-specific inhibition of Raf-1 in vivo, tumor growth arrest, and enhanced sensitivity to radiation or chemotherapeutic drugs has also been demonstrated, further establishing Raf-1 as a validated target in cancer drug discovery ***(5,9)***. We summarize next our recently reported preclinical and clinical studies of liposome-entrapped *raf* antisense oligonucleotide (LErafAON) as a novel liposomal antisense therapeutic ***(26–29)***.

4.2. Preclinical Development and Validation of Liposome-Entrapped raf Antisense Oligonucleotide

A schematic diagram of LErafAON formulation is shown in **Fig. 4A**. The rafAON is a 15-mer ASO with phosphorothioate linkage limited to one base at 5'- and 3'-ends ***(30)***. The rafAON sequence is targeted against the translation initiation region of c-*raf*-1 mRNA. The liposome formulation consists of a mixture of a cationic lipid (dimethyldioctadecyl ammonium bromide), egg phosphatidylchloline, and cholesterol in a molar ratio of 1:3.2:1.6. The rafAON to lipid ratio is 1:15 (w/w). The particle size was found to be approx 500 nm and entrapment efficiency was >85%. The LErafAON formulation was stable at room temperature for at least 1 wk as shown by the presence of 15-mer rafAON (**Fig. 4B**).

In CD2F1 mice, systemic administration (intravenous via tail vein [iv]) of LErafAON produced no morbidity/mortality (35 mg/kg/dose, iv, ×12). Dose-related elevations in liver enzymes (alanine aminotransferase [ALT] and aspartate aminotransferase [AST]) and histopathological changes in liver were noted


1st Generation of
Liposome-entrapped rafAON
rafAON
Plasma
Liver
Kidney
Spleen
Heart
15 mer
rafAON
S1 S2 S3 5' 15' 30' 1h 2h 4h 8h 24h 48h
5' 15' 30' 1h 2h 4h 8h 24h 48h

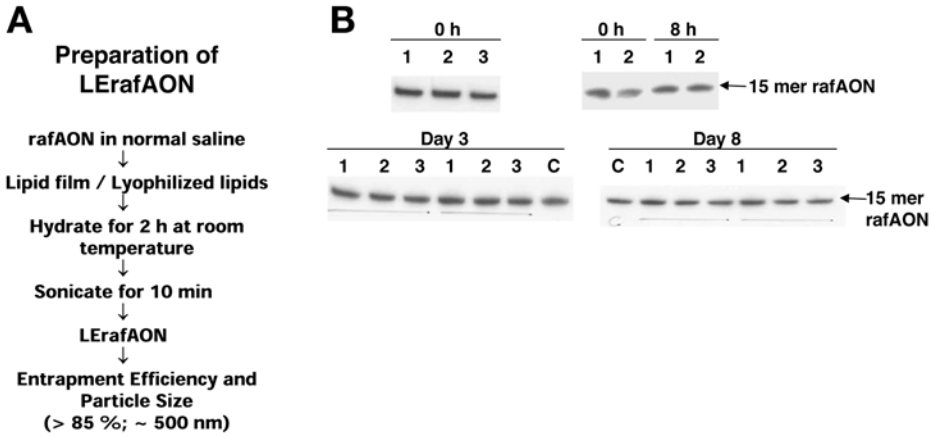


Fig. 4. Formulation **(A)** and stability **(B)** of a second generation of liposome-entrapped 15-mer *raf* antisense oligodeoxynucleotide (rafAON) with PS linkage of one base at 5'- and 3'-ends (LErafAON). The assays of LErafAON preparation, entrapment efficiency, particle size, and stability at room temperature have been reported in details earlier *(26)*. The rafAON to lipid ratio was 1:15 (w/w). C, control freshly prepared LErafAON.

in LErafAON and blank liposome groups. No morbidity/mortality and changes in clinical chemistry or histopathology were observed in NZW rabbits using two dose groups (3.75 mg/kg/dose, iv, ×8; 6.5 mg/kg/dose, iv, ×6) or in cynomolgous monkeys (3.75 or 6.25 mg/kg/dose, iv, ×9). Transient decrease in total hemolytic complement activity (CH50, ~62–74%) and increases in C3a (3-fold) and Bb levels (~5- to 12-fold) were observed in LErafAON and blank liposome groups of monkeys.

A 30-mg/kg iv dose of LErafAON in human prostate tumor (PC-3)-bearing Balb/c athymic mice gave a terminal plasma half-life of 27 h, and intact rafAON could be detected in plasma, and in normal and tumor tissues for up to at least 48 h **(Fig. 5)**. In monkeys, the terminal plasma half-life of 30.36 ± 23.87 h was

Fig. 3. *(Opposite page)* A comparison of the plasma pharmacokinetics and tissue distribution profiles of first generation of liposome-entrapped (LE-ATG-AS *raf* ODN) and unentrapped 15-mer rafAON (ATG-AS *raf* ODN) with PS linkage of one base at 5'- and 3'-ends. The dried lipids (DDAB:PC:CHOL in a molar ratio of 1:3.2:1.6) and rafAON (1.0 mg/mL) were hydrated overnight at 4°C in phosphate buffered saline, followed by vigorous vortexing and sonication for 5 min. The rafAON to lipid ratio was 1:30 (w/w). Balb/c nu/nu mice received 30 mg/kg, iv of LE-ATG-AS *raf* ODN or ATG-AS *raf* ODN. Blood and tissue samples were collected at indicated times post-injection, and antisense oligonucleotides concentrations in plasma and tissue samples were quantified by electrophoresis and autoradiography. (Modified from **ref.** *34*).

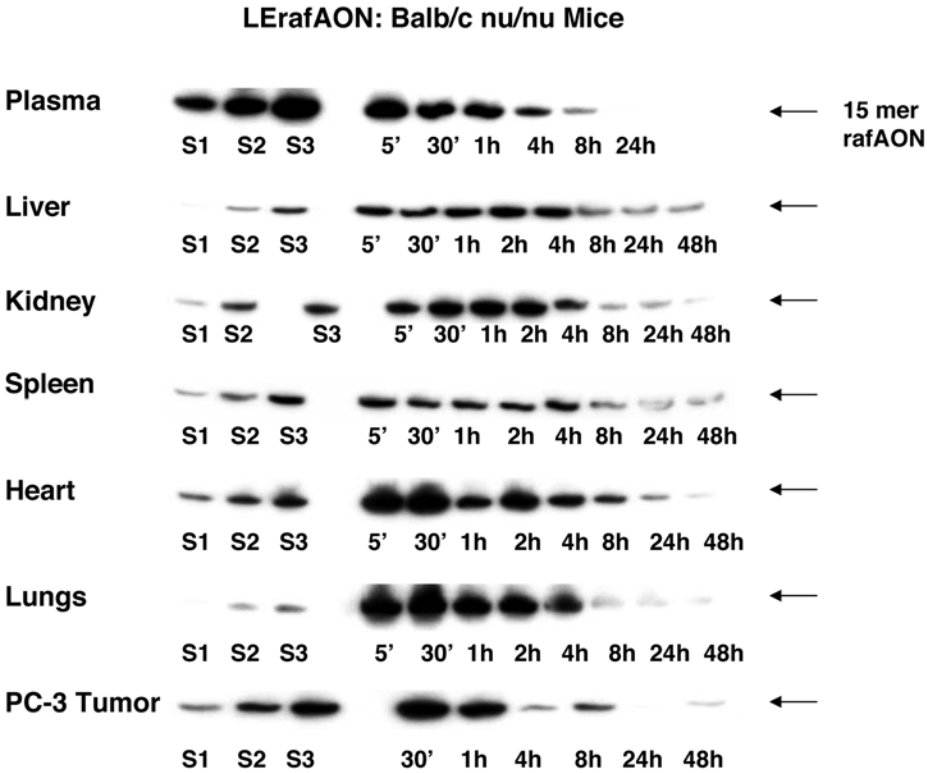


Fig. 5. Plasma pharmacokinetics and tissue distribution profiles of liposomal nanoparticle containing minimally modified *raf* antisense oligodeoxynucleotide (LErafAON). Balb/c nu/nu mice received 30 mg/kg, iv of LErafAON. Blood and tissue samples were collected at indicated times postinjection. Various samples may be differently diluted before electrophoresis to improve resolution of the bands following autoradiography. Different concentrations of the standard rafAON (S1, S2, S3) were used in different tissues. Antisense oligodeoxynucleotide concentrations in plasma and tissue samples were quantified as described earlier ***(26)***.

observed at an iv dose of 6.25 mg/kg (**Fig. 6**) LErafAON treatment (25 mg/kg/dose, iv, ×10) caused inhibition of Raf-1 protein expression in normal and tumor tissues of PC-3 bearing athymic mice (>50%, vs controls) (**Fig. 7**). A combination of LErafAON and ionizing radiation (IR) treatment of PC-3-tumor bearing athymic mice led to tumor growth arrest, whereas a combination of LErafAON and IR treatments resulted in tumor regression ***(26)***. Enhanced tumor growth inhibition in response to LErafAON and IR was also observed in athymic mice bearing human pancreatic tumor (Aspc-1) or hormone-independent breast tumor (MDA-MB 435) (**Fig. 8**). Previously, we have demonstrated enhanced antitumor effects of a combination of LErafAON and

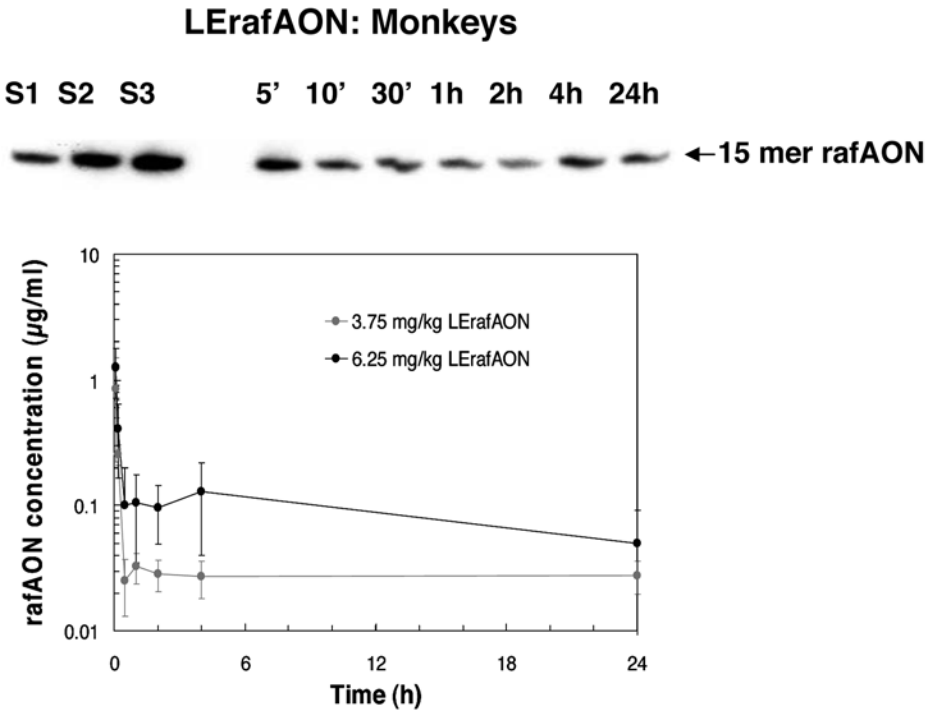


Fig. 6. The plasma concentration-time profile of liposomal nanoparticle containing minimally modified *raf* antisense oligodeoxynucleotide (LErafAON) in cynomolgus monkeys. Monkeys received a slow bolus injection of LErafAON via saphenous vein. Heparinized blood samples were collected at indicated time points postinjection and rafAON concentration was determined as described earlier *(26)*. Top, representative autoradiograph showing intact rafAON in plasma at various time points after administration of 6.25 mg/kg, iv, LErafAON in a male monkey. S1, S2, and S3, different concentrations of the standard rafAON. Bottom, plasma concentration–time curve of LErafAON. Each point represents mean ± SD (3.75 mg/kg LErafAON, $n = 3$; 6.25 mg/kg LErafAON, $n = 6$).

chemotherapeutic drugs against PC-3, pancreatic (Aspc-1 and Colo-357), lung (A549), and breast tumor xenografts grown in athymic mice (MDA-MB231) *(27,28)*. Similar observations were made in a different hormone-independent breast tumor model (MDA-MB 435) (**Fig. 9**). These data have formed a basis of the clinical phase I studies of LErafAON for cancer treatment.

4.3. Clinical Studies

LErafAON is a recent addition to the slowly growing number of ASO in the clinical trials (**Table 3**). It is also the first liposomal ASO drug tested in humans *(29,31)*.

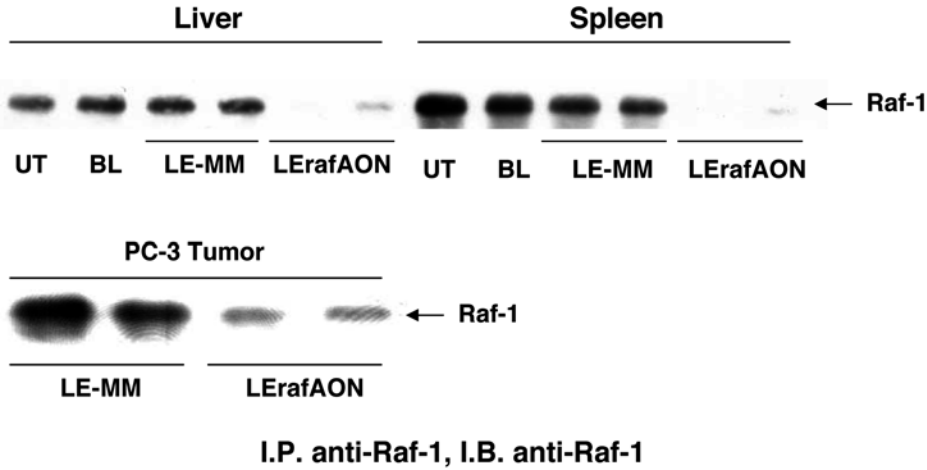


Fig. 7. LErafAON treatment inhibits Raf-1 expression in normal and tumor tissues of BALB/c nu/nu mice. Mice bearing human prostate tumor xenografts (PC-3) (>50 mm^3) received liposomal nanoparticle containing minimally modified *raf* antisense oligodeoxynucleotide (LErafAON) (25.0 mg/kg/dose, iv, ×10), a liposome-entrapped mismatch oligonucleotide, (25.0 mg/kg/dose, iv, ×10) (LE-MM), blank liposomes (iv, at the same dosing and schedule) (BL), or were left untreated (UT). Normal and tumor tissues were excised within 6–12 h after the last dose and Raf-1 expression was determined in tissue homogenates by immunoprecipitation (I.P.) followed by immunoblotting (I.B.) using anti-Raf-1 antibody as described earlier ***(26)***.

Prestudy validated methods were established to determine pharmacokinetics of LErafAON, and biomarker expression (c-*raf*-1 mRNA and Raf-1 protein) in clinical specimens. Bioanalytical method validation experiments were performed to quantify rafAON in human plasma using a gel electrophoresis method. Concentration standards were prepared by adding known amounts of rafAON to blank human plasma, followed by extraction of rafAON using the phenol chloroform extraction method. The validation experiments determined the precision and accuracy of the limits of quantification of rafAON in experiments, and the effects of dilution, freeze–thaw cycles, and storage at –80°C and 4°C. Other parameters included the integrity of rafAON during sample processing, specificity of rafAON sequence, and interference of lipids in the assays. The acceptance criteria were <25% coefficient of variation for precision and 100 ± 25% analytical recovery (AR). A maximum dilution of 500-fold from a 5-µg/mL rafAON specimen was acceptable in clinical studies. The rafAON samples could be stored at –80ºC for up to 14 d. Three cycles of freeze–thaw did not impair the quality of the rafAON specimens at tested concentrations. The integrity of 15-mer rafAON was determined in two independent experiments

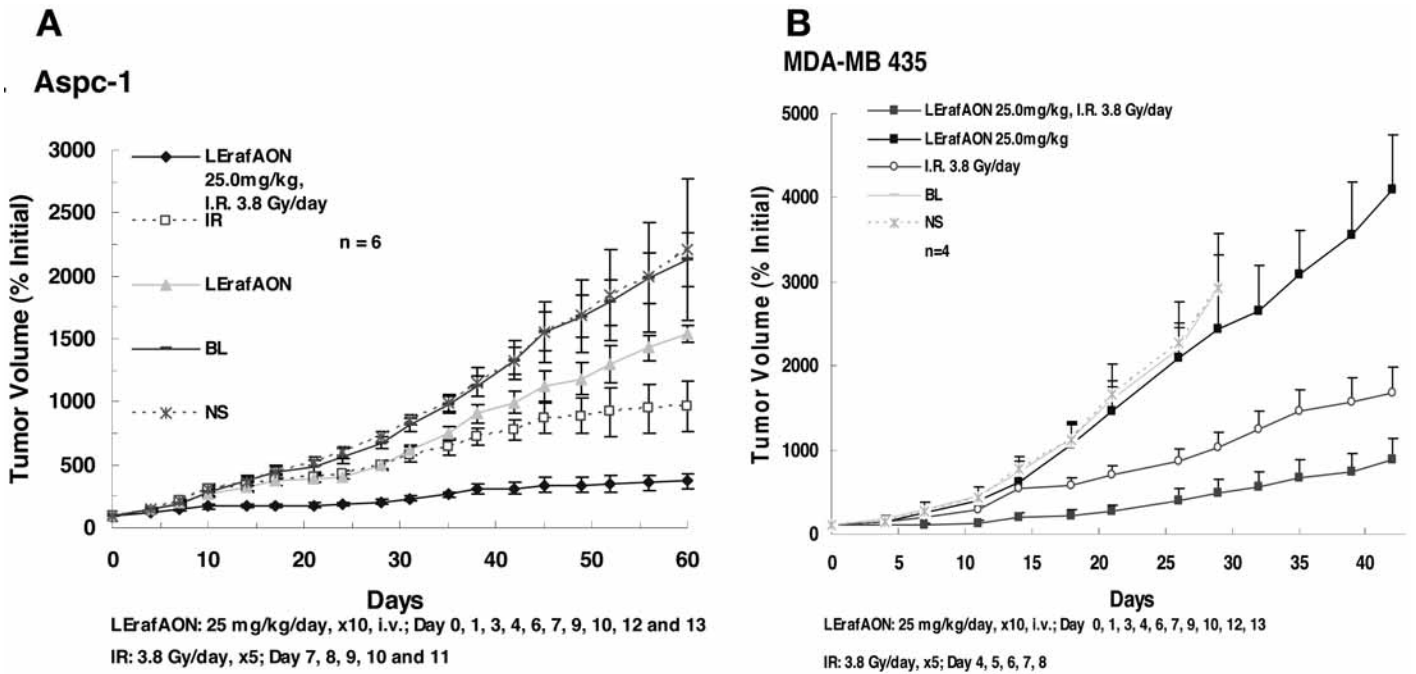


Fig. 8. Enhanced antitumor activity of a combination of liposomal nanoparticle containing minimally modified *raf* antisense oligodeoxynucleotide (LErafAON) and ionizing radiation (IR) against human pancreatic **(A)** and breast cancer models **(B)**. Mice bearing human pancreatic tumor xenografts (Aspc-1) or hormone-independent breast tumors (MDA-MB 435) (>50 mm^3) received indicated doses of LErafAON and/or IR as described earlier *(26,28)*. Control groups received blank liposomes (BL), iv, at the same dosing and schedule as LErafAON or normal saline (NS). Day 0 is the first day of treatment. For pancreatic tumor model, tumor growth was monitored for 47 d after the last LErafAON treatment (day 13) and study was terminated on day 60. For breast tumor model, tumor growth was monitored for 29 d after the last LErafAON treatment (day 13) and study was terminated on day 42. The values shown are mean ± S.E.

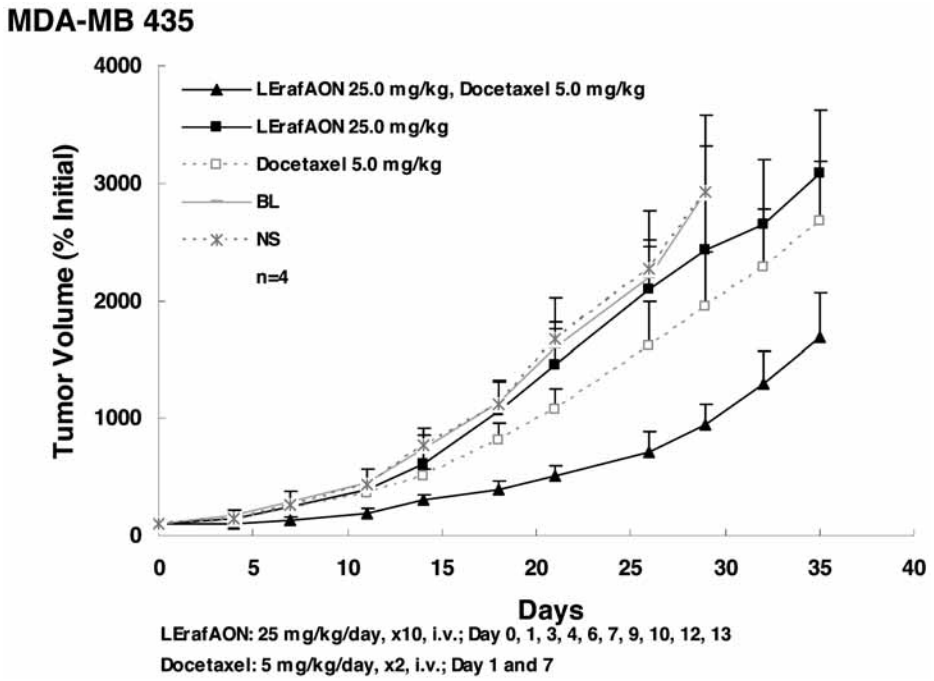


Fig. 9. Enhanced antitumor activity of a combination of liposomal nanoparticle containing minimally modified *raf* antisense oligodeoxynucleotide (LErafAON) and docetaxel (Taxotere) against human breast cancer model (MDA-MB 435). Mice bearing human hormone-independent breast tumors (MDA-MB 435) (>50 mm^3) received indicated doses of LErafAON and/or docetaxel as described earlier ***(28)***. Control groups received blank liposomes (BL), iv, at the same dosing and schedule as LErafAON or normal saline (NS). Day 0 is the first day of treatment. Tumor growth was monitored for at least 22 d after the last LErafAON treatment (day 13). The values shown are mean ± S.E.

by comparing the size of the 15-mer rafAON with a size marker representing a mixture of 15-mer, 14-mer, and 13-mer rafAON. The sample processing procedure was acceptable with accuracy of the 15-mer rafAON controls in the 84.0– 90.0% AR range. Overall, lipids do not interfere in the quantification of rafAON in human plasma.

Experiments were also performed to establish certified methods for expression of biomarkers, c-*raf*-1 mRNA and Raf-1 protein in human peripheral blood lymphocytes. Relative levels of c-*raf*-1 mRNA and Raf-1 protein in human lymphocytes were quantified using a RT-PCR method for RNA and a Western blotting method for protein. The acceptance criteria were <25% coefficient of variation for precision and 100 ± 25% AR. For the mRNA validation experiments, leukocytes were isolated from heparinized human blood obtained from

Table 3
Summary of Antisense Oligonucleotides in Clinical Oncology Trials

Molecular target	Drug	Company	Size, ASO chemistry[a]	Cancer type	Clinical status (ref.)
Bcl-2	G3139	Genta	18-mer, PS	Solid tumors, MM, AML, HRPC, melanoma	Phase I–II *69–74*
PKA-R1α	GEM 231	Hybridon	18-mer, MBO	Refractory solid tumors	Phase I–II *75–77*
PKC-α	ISIS 3521	ISIS	20-mer, PS	Solid tumors, non-Hodgkin's lymphoma	Phase I–II *78–80*
Raf-1	ISIS 5132	ISIS	20-mer, PS	Solid tumors	Phase I-II *81,82*
LErafAON	NeoPharm		15-mer, liposome-entrapped, minimally modified	Solid tumors	Phase I *29,31*
LErafAON-ETU	NeoPharm		15-mer, liposome-entrapped, minimally modified	Advanced cancer	Phase I *33*
RNR (R2)	GTI-2040	Lorus	20-mer, PS	Solid tumors	Phase I *83*

[a]PS, phosphorothioate; MBO, mixed-backbone; minimally modified, one base at 5'- and 3'-ends has PS-linkage; MM, multiple myeloma; AML, acute myeloid leukemia; HRPC, hormone-refractory prostate cancer.

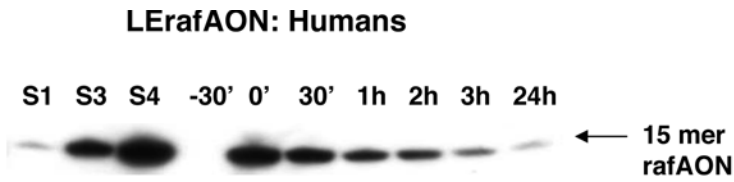


Fig. 10. Plasma pharmacokinetics of liposomal nanoparticle containing minimally modified *raf* antisense oligodeoxynucleotide (LErafAON) in clinical specimens. Representative autoradiograph showing intact circulating rafAON at various time points in a patient treated with LErafAON (Week1:2 mg/kg/wk dose level) as described earlier *(29)*. Sample aliquots were diluted as follows: 0', 4X; and 24 h, 0.5X; or left undiluted before electrophoresis. S1, S3, and S4 represent 0.01, 0.1, and 0.5 µg/mL of rafAON standard, respectively. –30', predose sample.

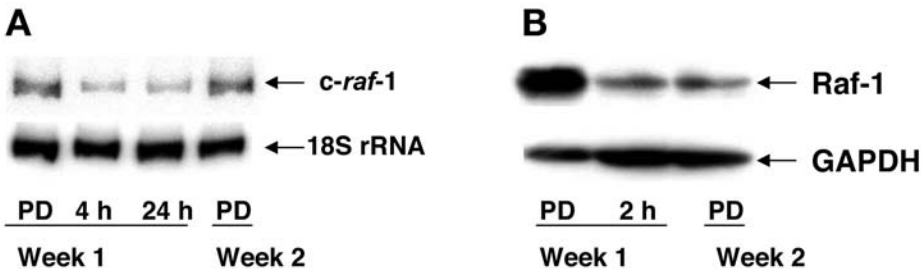


Fig. 11. Expression of c-*raf*-1 in clinical specimens. **(A)** Representative autoradiographs showing inhibition of c-*raf*-1 mRNA relative to the predose (PD) at 4 and 24 h in peripheral blood mononuclear cells of a patient treated with LErafAON (Week1:6 mg/kg/wk dose level) as described before *(29)*. The c-*raf*-1 mRNA expression was determined by quantitative RT-PCR using *raf*-1 and 18S specific primers. **(B)** Representative immunoblot showing inhibition of Raf-1 expression relative to the predose (PD) at 2 h in a different patient in the same cohort as **(A)**. Raf-1 protein expression was analyzed in peripheral blood mononuclear cells by Western blotting using anti-Raf-1 antibody followed by reprobing of the same blot with anti-GAPDH antibody.

healthy donors. Based on the data obtained, lowest and highest amounts of total RNA to be used in clinical studies were 40 and 160 ng, respectively. Total RNA samples were unacceptable for expression analysis following three cycles of freeze–thaw and/or storage at –80°C for 3 wk prior to analysis.

The Raf-1 protein validation experiments were performed using whole cell lysates from donor human lymphocytes. Based on the data obtained, lowest and highest amounts of total protein to be used in clinical studies were 100 and 300 µg, respectively. Lymphocytes subjected to three cycles of freeze–thaw were unacceptable for Raf-1 expression determinations in clinical studies. However,

lymphocytes stored frozen at –80°C for approx 2 mo prior to assay were found to be acceptable for Raf-1 expression in clinical studies.

In a phase I clinical study of LErafAON in patients with advanced solid tumors, dose-independent hypersensitivity reactions and dose-dependent thrombocytopenia were observed *(29)*. In this report, pharmacokinetic studies of LErafAON showed persistence of circulating rafAON for up to 24 h **(Fig. 10)**. Inhibition of *c-raf*-1 expression was seen in peripheral blood mononuclear cells in some of the evaluable clinical specimens **(Fig. 11)**. An improved formulation of LErafAON (LErafAON-ETU) is being tested in a clinical study *(32,33)*.

Acknowledgments

The authors thank colleagues and collaborators for their contributions to studies discussed in this review. The number of citations included does not reflect all of the literature in this field. The research work was supported by grants from the National Institutes of Health and NeoPharm, Inc.

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8

Nucleic Acid-Based Aptamers as Promising Therapeutics in Neoplastic Diseases

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Summary

Isolated through combinatorial libraries by an iterative in vitro selection process, small single-stranded nucleic acid compounds, named aptamers, have been developed as high-affinity ligands for a variety of targets, ranging from small chemical compounds to large proteins. In the last years, an increasing number of aptamers has been generated that represent potential antagonists of the disease-associated target proteins. These molecules have been shown to discriminate between even closely related targets, thus representing a valid alternative to antibodies or other biomimetic receptors for the development of biosensors and other bioanalytical methods. Moreover, they can be easily stabilized by chemical modifications for in vivo applications and numerous examples have shown that stabilized aptamers against extracellular targets such as growth factors, receptors, hormones, or coagulation factors are very effective inhibitors of the corresponding protein function, thus resulting as useful reagents for target validation in a variety of diseases, including cancer. Indeed, many signaling proteins involved in diverse functions such as cell growth and differentiation can act as oncogenes and cause cellular transformation, thus making these high affinity ligands promising tools for cancer diagnosis or therapy.

Key Words: Aptamer; RNA ligand; SELEX; therapy; cancer.

1. Introduction

Cancers are generally considered to be a result of the accumulation of multiple genetic alterations that affect the activity and/or expression of proteins involved in the cellular signaling pathways, confering proliferative and invasive characteristics of growth on tumor cells (***1***, *see* Chapters 1 and 2).

The completion of the sequence of the human genome has provided us with a partial list of known and putative human genes, the total number of which is estimated between 30,000 and 45,000 ***(2,3)***. Based on this knowledge, the

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availability of such novel genomic and proteomic techniques as the high-throughput screens and microarrays analysis has led in the last few years to the accumulation of huge amounts of information about genes that are potential targets in cancers. These include proteins already known to govern different functions in tumor progression, such as cell cycle, proliferation, survival, invasion, or angiogenesis. Indeed, in malignant tumors the expression and function of key cellular proteins, including the loss of tumor-suppressor genes or activation oncogenes, is altered through multiple mechanisms such as enhanced or ectopic expression, deletions, single-point mutations, and generation of chimeric proteins *(1)*. These proteins may thus provide primary targets in the rational approach to target cancer cells.

Despite the intrinsic genetic complexity of the neoplastic phenotype, in several cases highly selective molecular ligands have been developed as target-specific therapeutics to stop or delay the growth of cancers *(4)*, thus encouraging the ongoing search for oncogenes as selective targets for diagnosis or therapy. Indeed, the nature and the role played by the oncogene products largely vary across cancer types, and some tumors show a surprisingly tight dependence on the continued activity of a specific oncogene, even in the presence of additional tumorigenic lesions. This is best illustrated by targeting the Bcr/Abl oncoprotein, generated by the fusion of c-Bcr and the tyrosine kinase c-Abl, that is responsible for a wide range of human leukemias *(4,5)*. In chronic myeloid leukemia, the tyrosine kinase inhibitor, imatinib mesylate (known as Gleevec®), can cause complete regression of advanced tumors by specifically inhibiting the tyrosine kinase activity of the overexpressed Bcr-Abl oncoprotein *(6,7)*. Other paradigmatic examples of these target proteins are cell surface receptors with an intrinsic intracellular tyrosine kinase activity as for example the epidermal growth factor receptor that is frequently over-expressed in non-small cell lung carcinoma, bladder, cervical, ovarian, kidney, and pancreatic carcinoma *(4,5)*. Similarly, overexpression and/or gene amplification of the HER-2/neu receptor is found in various types of cancers, including breast (where it occurs in 30% of early stage cases), ovarian, gastric, lung, bladder, and kidney carcinomas *(4,5)*. Somatic rearrangements of the *Ret* gene are frequently associated to the papillary histiotype of the thyroid carcinoma, and germ-line mutations of the Ret receptor cause inheritance of multiple endocrine neoplasia type 2A and 2B and familial medullary thyroid carcinoma *(8)*. Single point mutations, which are frequently found in dominant oncogenes, often affect members of the Ras family. Activating mutations in Ras proteins (accounting for almost 30% of all human cancers) result in constitutive signaling, thereby stimulating cell proliferation and inhibiting apoptosis *(9)*. In Burkitt's lymphoma, a malignancy of immune B cells, the cancer is characterized by huge overexpression of Myc because the *Myc* gene is translocated next to the

regulatory sequences for the immunoglobulin gene. As this latter gene is often switched on in B cells, the *Myc* gene itself is switched on ***(10)***.

Hence, finding specific ligands capable to detect and inhibit the expression of all these proteins is a strategic objective for the diagnosis and therapy of cancer.

Different types of molecules have been shown to be of potential utility for cancer diagnosis and therapy, including small chemical compounds, peptides, antibodies, small-interfering RNAs, and the short nucleic acid-based ligands, named aptamers. Because of their high selectivity, monoclonal antibodies have been developed against tumor-specific antigens as highly promising molecules that have proven their worth as therapeutics. Aptamers are a new class of ligand molecules that, like antibodies, bind to their targets by complementary shape interactions. Single-stranded RNAs or DNAs-based aptamers function by folding into unique globular three-dimensional shapes that dictates high-affinity binding to a variety of targets, each structure being unique and determined by the sequence of the nucleic acid. Aptamers revealed useful for many of the applications for which antibodies are already employed because of their own definite characteristics: (1) capacity to discriminate between oncogenic and nononcogenic forms of the proteins involved in signaling pathways; (2) capacity to quantify the level of expression of the oncogenic forms; (3) are usable both for in vitro and in vivo purposes; and (4) may act as inhibitors to block the activity of the target oncogene product. Furthermore, aptamers are poor or not immunogenic when administered in animal models or in humans for therapeutic applications.

Protocols have been also developed that allow the targeting of intracellular proteins with inhibitory aptamers (named intramers) that are delivered into intracellular compartments either by direct transfection or through the use of expression systems for the aptamer sequences ***(11–13)***. Once expressed inside the cell, aptamers retain their function and can alter the phenotype of a cell by modulating the biological function of the targeted protein.

In this review, we will summarize the current status on the possible applications of aptamers as tools to understand the biological function of proteins and as novel agents in therapy and diagnosis.

2. Systematic Evolution of Ligands by Exponential Enrichment Technology to Generate Nucleic Acid Aptamers

Aptamers are single-stranded nucleic acids routinely isolated from combinatorial oligonucleotide libraries using in vitro selection methods, referred as systematic evolution of ligands by exponential enrichment (SELEX) ***(14,15)***.

By starting with a synthetic oligonucleotide library, containing up to 10^{15} different sequences (thus, virtually, 10^{15} different specific shapes), those oligonucleotides that bind with high affinity and specificity to the target

molecule of interest are enriched in the library and, after several rounds, selected. The selection scheme is based on the property of single-stranded nucleic acids to fold up into unique three dimensional shapes, thus providing a limited number of specific contact points for the target molecule. The SELEX method includes the following steps (**Fig. 1**): (1) incubating the library with the target molecule under conditions favourable for binding; (2) partitioning from other sequences those molecules that, under the conditions employed, adopt conformations that permit better binding to a specific target; (3) dissociating the nucleic acid–protein complexes; and (4) amplifying of the nucleic acids pool to generate a library of reduced complexity enriched in sequences that bind to the target. This library will be then used as starting pool for the next round of selection. After reiterating these steps for a variable number of cycles, the resulting oligonucleotides are subjected to DNA sequencing. The sequences corresponding to the initially variable region of the library are screened for conserved sequences and structural elements indicative of potential binding sites and subsequently tested for their ability to bind specifically to the target molecule.

3. Target Validation

A crucial step to validate the most promising molecular targets for drug development is to determine their biological relevance for a given disease. The possible strategies that are usually used to understand the function of a specific gene in a cell are: (1) either based on techniques that impair the expression of the candidate target gene, or alternatively (2) rely on the use of products that act by specifically interfering or inhibiting the function, but not the expression, of the final product. In both cases, the resulting phenotype turns out as a powerful source of information on the function of the target protein.

The generation of null mutants by homologous recombination of a given gene in a single cell or in an entire organism has been extensively used to create models of several human diseases, including cancer. Using this technique (known as gene knockout), in which the gene of interest is irreversibly disrupted and the synthesis of the encoded products abolished, allowed to make an incredible and rapid progress in our understanding of the function of several oncogenes and tumor suppressor genes. As an alternative strategy for highly specific gene silencing the use of RNA interference has proven to be a precious approach that permits loss-of-function phenotypic screens in mammalian somatic cells or in whole animals (*see* Chapters 9, 10, and 11). Indeed, it is now feasible to design RNAi constructs against virtually any transcript in the genome. Furthermore, in contrast to the knockout approach, the RNA interference-based RNA strategies achieve loss-of-function phenotypes without the loss of genomic information of the targeted gene (recently reviewed in **ref.** ***16***).

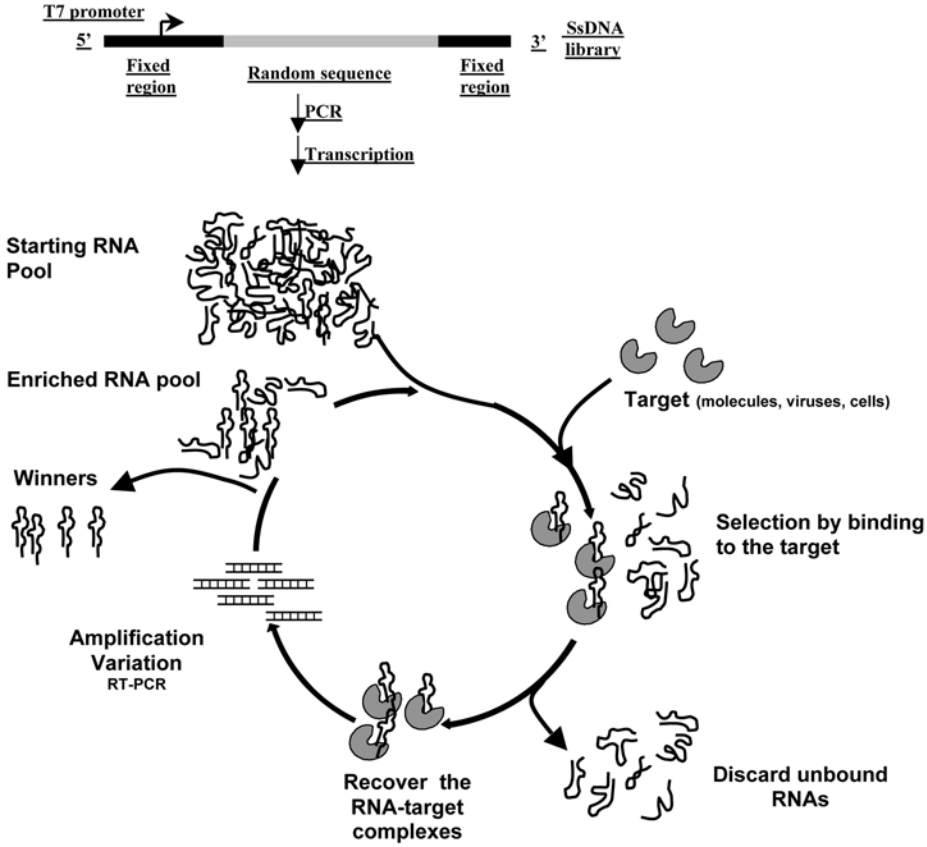

Fig. 1. Schematic representation of the systematic evolution of ligands by exponential enrichment (SELEX) process. The single-stranded (ss) DNA library is amplified by PCR to generate the double-stranded DNA pool that will be transcribed by T7 RNA polymerase. The pool of RNA molecules with different conformations will be used for the selection process (*see* **Subheading X** for details).

This leaves the possibility to restore the exact expression of the endogenous gene once the RNAi vector is silenced or removed.

However, a major disadvantage of the gene silencing approaches to obtain functional data on a given protein is that, in most cases, proteins and enzymes involved in crucial functions, such as cell growth and differentiation, act in concert with various protein partners thus forming large stable complexes. Therefore, depleting a single key protein from the cell will change, or even disrupt, one or more of these multiprotein complexes recruited by the target protein. As a consequence, the resulting phenotype will be produced by the simultaneous impairment of several protein functions and the understanding

frequently ambiguous. Furthermore, silencing a gene gives no information about which part or domain of the protein is important for its function. Therefore, to overcome these problems, additional approaches have been favored that enable to interfere with a given protein function without interfering with its expression. Indeed, addressing the process of target validation using inhibitors of protein function that directly target the protein in a drug-like manner has the advantage to interfere with a protein activity with low destabilization of the proteomic status of the cell. As excellent alternatives to monoclonal antibodies, peptides and small-molecule inhibitors, RNA-based aptamers have recently proven to be efficient inhibitors of a wide variety of protein implicated in cancer. Indeed, aptamers can recognize within the same protein different domains, or posttranslational modifications, which allows functional analysis of proteins at the molecular level. Furthermore, the SELEX procedure permits to generate aptamers that display high selectivity for the target thus allowing to easily discriminate between even very close molecules. For instance, RNA aptamers with high selectivity have been generated that bind with nanomolar affinities the protein kinase C, a potential target in cancer medicine, and are capable of discriminating between the β II from the highly related β I isoenzymes *(17)*. DNA aptamers have been obtained that recognize both the native and the denatured state of ERK-2, a member of the family of mitogen-activated protein kinases, which are central transducers of extracellular signals *(18)*. RNA ligands with high affinity for the Ras-binding domain of Raf-1 have been isolated and shown to inhibit either Ras binding to Raf-1 and Ras-induced Raf-1 activation, but they did not affect the interaction of Ras with B-Raf, a Raf-1 related protein *(19)*. Furthermore, highly specific aptamer has been generated against platelet-derived growth factor that suppress platelet-derived growth factor B-chain but not the epidermal- or fibroblast-growth-factor-2-induced proliferation *(20)*.

Among the proteins that have been reported as targets of biomedical interest for development of aptamers as therapeutics, particularly relevant is Tenascin-C (TN-C). It is a large, extracellular matrix glycoprotein that is overexpressed during tissue remodeling such as wound healing, atherosclerosis, psoriasis, and tumor growth *(21)*. To isolate oligonucleotide that can target anticancer drug delivery, selection yielded an aptamer that bound to TN-C with a Kd of 5 n*M* *(22)*. The TN-C aptamer is currently being developed for tumor-imaging applications *(23)*.

Furthermore, two aptamers have been selected against the prostate-specific membrane antigen (PSMA) overexpressed by the prostate cancer cells *(24)*. The aptamers inhibited the target with a Ki of 2.1 and 11.9 n*M*. One of the aptamers has been truncated and fluorescently end-labeled to evaluate its ability to bind PSMA-expressing cancer cells. The aptamer bound to LNCaP cells but not

PC-3, showing its specificity for PSMA and its potential in therapeutic development for this target.

Other proteins for aptamer targeting are: human epidermal growth factor receptor-3 (ErbB3/HER3; ***[25]***) and the IFN-γ-inducible CXCL10 chemokine ***(26)***. In the latter case, Marro et al. ***(26)*** identified a series of nuclease-resistant RNA aptamers with high binding affinity for human and/or mouse CXCL10. CXCL10 is a chemokine involved in a variety of inflammatory diseases. Because some of the aptamers are highly selective for CXCL10, they represent a powerful tool to further elucidate the complex cross-talk between the CXCL10/ CXCR3 receptor and other chemokine/receptor system.

As reported in **Table 1**, several aptamers are actually in clinical trials ***(27)*** and the Food and Drug Administration has recently approved one aptamer developed by Eyetech (Macugen™) that inhibits the human vascular endothelial growth factor 165 (VEGF165), for the treatment of age-related macular degeneration ***(28)***. In addition to preventing ocular neovascularization, a logical potential therapy for aptamers to VEGF is in cancer. The aptamer isolated and optimized by Ruckman et al. ***(28)*** was tested in a mouse model of Wilm's tumor or nephroblastoma ***(29)***. Renal histopathology revealed an 84% reduction in tumor weight in the aptamer-treated kidneys compared to the controls. Furthermore, lung metastases were seen in 20% of the aptamer-treated mice compared to 60% of control animals. The aptamer was also tested in a murine model of neuroblastoma, where it resulted in 53% reduction in tumor growth compared to control ***(30)***.

Even though a large number of aptamers have been selected for preferential targeting of extracellular proteins or protein epitopes the use of living cells as complex target has been recently described to develop a differential whole-cell SELEX protocol to target cell-surface-bound proteins in their natural physiological environment ***(31)***. The selection procedure was performed by using as target the RET^{C634Y} mutant expressed on PC12 cells. A library of 2'-F RNAs was incubated with parental PC12 cells to remove aptamers that bind nonspecifically to the cell surface. To select for aptamers that specifically bound the mutant receptor, the supernatant was incubated with PC12–RET^{C634Y} cells. Unbound sequences were washed off, the whole process reiterated 16 times and the bound winning sequences cloned. The resulting aptamers did not bind to a recombinant EC C634Y RET fragment highlighting the strength of the whole-cell approach. Among the selected aptamers, the best inhibitor (D4) binds specifically to the Ret receptor tyrosine kinase and blocks its downstream signaling effects on cell differentiation and transformation ***(31)***. The results suggest that the differential whole-cell SELEX approach will be useful in the isolation of other lead therapeutic compounds and diagnostic cell-surface markers.

Table 1
Therapeutic Aptamers in Cancer Treatment

	Aptamer activity		Therapeutic application
Aptamer	in vitro	in vivo	
Macugen®	Inhibition of VEGF165	Inhibition of the VEGF-induced vascular permeability	Approved by FDA for treatment of age-related macular degeneration
PDGF-B aptamer	Inhibition of PDGF binding to PDGFR	Reduction of tumor interstitial fluid pressure	Administration in tumor-bearing rats
ProMune™	Agonist for Toll-like receptor 9 (TLR 9)	Activate the immune system through TLR 9 against cancer	Phase 2-melanoma; Phase 1-renal cell carcinoma; non-Hodgkin's lymphoma; cutaneous T-cell lymphoma, nonsmall-cell lung cancer
Agro 100	Binding to nucleolin	Antiproliferative activity in a broad array of tumor cell types; enhancement of chemotherapeutic agents effects	Phase 1; Phase 2 launched in 2005 for advanced solid malignancies
HYB2055	Agonist for TLR 9	Antitumor activity in nude mouse xenografts with colon, breast, lung cancer, and glioma cell lines	Phase 2 for advanced solid malignancies
VaxImmune™ adjuvant	Agonist for TLR 9	Elicits a powerful immune response against infectious disease and cancers	Phase 2 for several different cancer indications

Aptamers that have high affinity and specificity for tissues have also been produced *(32)*, demonstrating that complex targets, including tumor tissue, are compatible with the SELEX process. "Tissue SELEX" methodology could be favourable when the precise molecular target is unknown but the target is, for example, a specific type of cells. Using human red blood cell membranes as model system, DNA aptamers binding to multiple targets have been isolated simultaneously *(32)*. A fluorescence-based SELEX-procedure was applied against transformed endothelial cells as a complex target to detect microvessels

of rat experimental glioma, a fatal brain tumor, which is highly vascularized. A secondary selection scheme, named deconvolution-SELEX, was carried out to facilitate the isolation of ligands for components of interest within the targeted mixture *(33)*.

4. In Vivo Applications of the Aptamers

To be of practical use, in vivo aptamers must possess defined molecular properties, for instance, adequate stability in the biological situation in which it will be employed, or sufficient systemic clearance in the case of aptamers used as imaging reagents.

One of the major limitations of the use of aptamers, especially RNA-based aptamers, in cell culture and animal models, is their rapid degradation by nucleases. To date, several chemical modifications have been employed to overcome this issue and a variety of approaches have been developed to improve aptamers stability. Initially, attention focused on "post-SELEX modifications", i.e., the substitution of nucleotides with the corresponding 2'-fluoro, 2'-amino or 2'-O-alkyl variants *(34,35)*. However, owing to the fact that folding rules for single stranded oligonucleotides regions change when these modifications are introduced, the binding properties of an aptamer selected in the presence of standard nucleotides might be completely different when the same sequence is synthesized with nucleotides containing a different 2'-substituent *(33,35,36)*. To circumvent this limitation selections can be performed directly in the presence of 2'-modified nucleotides, as long as the modified nucleotides are accepted by T7 RNA polymerase for the in vitro reaction steps of the selection *(28,36)*. In addition, restricted nucleotides, locked nucleic acid, have been characterized to improve further the stability against nucleases *(37)*.

An interesting application of the SELEX process is based on the selection of RNA aptamers binding to the mirror-image of an intended target molecule (e.g., an unnatural D-aminoacid peptide), followed by the chemical synthesis of the mirror-image of the selected sequence. As a consequence of molecular symmetry, the mirror-image aptamer (made from L-ribose) binds to the natural target molecule. Because of the substitution of the natural D-ribose with L-ribose, the mirror image aptamer (referred as a Spiegelmer) is totally stable. For example, Spiegelmers that bind to gonadotropin-releasing hormone I, a decapeptide associated with several malignant diseases, have been isolated and characterized *(38)*.

Despite the increasing number of aptamers isolated of potential medical importance their use in therapy is still lagging behind because of the lack of an efficient and safe delivery system to target specific cells with adequate amounts of aptamer. For therapy applications, aptamers have to cross the collagen microfibrillar network of the extracellular matrix, and reach the target tissue or cells and, most importantly, also penetrate the cell membrane. Coupling aptamers

to inert large molecules, as cholesterol or polyethylene glycol, have been used to keep them in circulation anchored to liposome bilayers *(39)*.

The application for in vivo imaging is especially promising owing to the very wide range of possibilities available to introduce changes in their structure that will enhance the bioavailability and tune the pharmacokinetics properties. Indeed, apart those previously mentioned, there are very few drawbacks for the use of aptamers in vivo, also considering the absence of immunogenicity, a very useful property for reagents that need to be administered repeatedly to the same individual for therapy or diagnostic when studying disease progression.

5. Cancer Signature Measurement

Developing methods that allow clinicians and researchers to translate signature discoveries to routine clinical use by looking simultaneously at a large number of biomarkers has now become a major challenge in cancer diagnostics. Indeed, because they are readily accessible without any need of invasive intervention measuring molecules expressed in serum or plasma is highly preferable. However, many potential cancer biomarkers in biological fluids are present at low concentrations, presumably in the low nanomolar range. Therefore, the capability to measure multiple protein markers simultaneously depends on methods having low limits of detection with elevated signals, but also coupled to very low noise, thus capable to distinguish specific protein signaling in the presence of a huge excess of unrelated proteins.

To this aim Petach et al. at SomaLogic, Inc. *(40,41)* have developed an aptamer-based array technology for analysis of multiplexed proteins. PhotoSelex technology *(42)* permits to derive a new class of capture aptamer molecules, named photoaptamers. These modified aptamers (either DNA or RNA) at specific locations include, in place of Thymdine residues, the photoreactive 5-bromodeoxyuridine (BrdU) that can form a specific covalent cross-link with the target proteins. Indeed, short pulses of ultraviolet light at 308 nm induce a chemical cross-link between the BrdU residue and the electron-rich amino acid on the target protein that is in a specific location in proximity and in the correct juxtaposition of the BrdU. Because this cross-linking event is dependent on the correct juxtaposition of the BrdU and the target amino acid, it conveys specificity to the photoaptamer–protein complex. This gives rise to multiplicative specificity by a photochemical cross-link that follows the initial affinity binding event.

To measure simultaneously large numbers of proteins, even thousands, in biological fluids multiple capture photoaptamers can be deposited and covalently linked to the appropriate chip surface. Therefore, because photoaptamers covalently bind to their targets before staining, the photoaptamer arrays can be vigorously washed to remove background proteins, thus providing the needed

potential for elevated signal-to-noise ratios. Proteins captured on the array are then measured by staining either with universal protein stain or with specific antibodies.

The aptamer array technology combined with bioinformatics could allow to discover disease-specific biomarkers and protein signatures and to verify drug compounds efficacy. Using an aptamer array, the measurement of the concentrations of a large number of proteins in a complex biological mixture, such as a clinical sample, could be obtained *(43)*. A protein profile is likely to change in the presence of disease, and different profiles may be associated with varying responses to therapeutics or other clinically relevant parameters. In this context, DNA and RNA aptamers have been used, in a study carried out at the company Archemix, as biorecognition element in optical sensors for multiplex analysis of proteins related to cancer *(44)*. Four fluorescently labeled aptamers (RNA-based aptamers against basic fibroblast growth factor, inosine monophosphate dehydrogenase, and VEGF, and an antithrombin DNA-based aptamer) were immobilized onto a glass surface within a flow cell and fluorescence polarization anisotropy was used for solid-phase measurements of target protein binding. It has been demonstrated specific detection and quantification of inosine monophosphate dehydrogenase II, VEGF, and basic fibroblast growth factor in the context of human serum as well as in cellular extracts.

To date, some photoaptamer-based chips are commercially available as highly sensitive and specific capture agents to discover disease-specific biomarkers and protein signatures (Somalogic Inc.). Furthermore, a continuous and crescent consideration is given to the challenges involved in producing multiplex aptamer chips composed of aptamers taken from disparate literature sources, and to the development of standardized methods for characterizing the performance of capture reagents used in biosensors.

6. Concluding Remarks

The encouraging results obtained with aptamers combined with their intrinsic properties make them promising candidates for diagnostic and even therapeutic applications. Several aptamers are actually in clinical trials and the Food and Drug Administration has recently approved one aptamer (Macugen) for the treatment of age-related macular degeneration *(45)*. Furthermore, the ability of aptamers to discriminate between two closely related targets even sharing common structural domains make these specific and stable ligands attractive as imaging reagents for noninvasive diagnostic procedures.

It is noteworthy that in addition to the use of aptamers in cancer medicine, in the past few years aptamers have been identified as powerful antagonists of proteins, which are associated with a number of other diseases thus emphasizing the versatility of these molecules *(45)*.

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9

Guidelines for the Selection of Effective Short-Interfering RNA Sequences for Functional Genomics

Kumiko Ui-Tei, Yuki Naito, and Kaoru Saigo

Summary

To avoid long-double-stranded-RNA-dependent interferon response, short-interfering RNAs (siRNAs) are widely used for RNA interference (RNAi) in mammalian cells. siRNA-based RNAi, however, may not be readily available for the large-scale gene silencing essential for systematic functional genomics, because only a limited fraction of siRNAs is capable of inducing effective mammalian RNAi. siRNAs correctly designed for the knockdown of a particular gene may also destroy the functions of unrelated genes. Here, we describe algorithms by which these serious setbacks can be eliminated in mammalian functional genomics using RNAi and a Web-based online software system for computing highly functional siRNA sequences with maximal target-specificity in mammalian RNAi.

Key Words: Mammalian RNAi; functional siRNA; off-target effects; target assay; siDirect.

1. Introduction

RNA interference (RNAi) is the process of nucleotide-sequence-specific posttranscriptional gene silencing *(1–4)*. In this process, long double-stranded RNA (dsRNA), either introduced into or produced within cells, is converted to many short interfering RNAs (siRNAs) 21- to 23-nt long through cytoplasmic-dicer-dependent RNase activity *(5,6)*. siRNA is a 19- to 21-bp duplex associated with 2-nt 3'-overhangs at both ends. Twenty-one-base pair-long siRNA synthesized in vitro and incorporated into cells still possesses the capability to induce RNAi *(7)*. The introduction of long dsRNA into mammalian cells frequently induces fatal interferon response, though siRNA may not do so *(8)*, indicating that siRNA is more promising as a reagent for mammalian RNAi *(9)*

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than long dsRNA *(10–14)*. siRNA generated in vivo or synthesized in vitro and introduced into cells via transfection is considered to form an RNA-induced silencing complex (RISC) with PIWI protein (Argonaute 2) and possibly other relevant proteins *(15–19)*. Active RISC as a silencing effector complex includes siRNA antisense strands (AS) but not sense strands (SS), and thus double-stranded siRNA is considered to undergo unwinding *(20)* during active RISC formation. Target mRNA is recognized by cognate siRNA AS serving as a guide strand via Watson–Crick hydrogen bonding. RISC cleaves target mRNA at a site corresponding to the center of the guide strand of siRNA in mammalian and *Drosophila* cells *(7,9,20,21)*.

siRNA-based RNAi, however, may not always be available for the large-scale gene silencing essential for mammalian systematic functional genomics, because only a limited fraction of siRNAs is capable of inducing effective RNAi in mammalian cells *(22–24)*. Thus, the first major problem when considering the use of siRNA as a mammalian RNAi reagent is the absence of a definitive way to identify functional siRNAs among hundreds or thousands of possible candidates, all homologous in sequence to a given target mRNA.

To approach this problem, examination was made of arbitrarily chosen siRNAs targeting for the firefly luciferase gene (*luc*) for their ability to induce RNAi in mammalian cells *(23)*, using human (HeLa, HEK293 and colo205), Chinese hamster ovary (CHO-K1), and mouse ES (E14TG2a) cells. As shown in **Fig. 1**, hardly any or no difference in RNAi-inducing activity was found in these cell types, suggesting that siRNA-based RNAi in mammalian cells is subject to the same rules for siRNA sequence preference. As schematically shown in **Fig. 2A** (algorithm I), all data obtained indicated four immediately apparent features of the siRNA sequence to possibly serve to discriminate highly functional siRNAs from those nonfunctional. First, the 5'-AS end of highly functional siRNAs may always be A or U, with the counterpart of nonfunctional siRNA being C or G. Second, the 5'-SS ends of highly functional siRNAs are preferably G or C, with the counterpart of nonfunctional siRNAs being A or U. Third, in the case of highly functional siRNAs, the 5'-terminal AS are A/U-rich whereas the corresponding region of nonfunctional siRNAs are G/C-rich. Fourth, highly functional siRNAs lack a long G/C stretch in the 5'-terminal two-thirds of SS. Most siRNAs associated with mixed features appeared to belong to a siRNA class with intermediate RNAi activity. From these findings, we classified siRNAs as follows: class I consisting of siRNAs possessing (1) A/U at the 5'-AS end, (2) G/C at the 5'-SS end, (3) more than four A/U nucleotides in a 7-nt 5'-terminal AS end, and (4) lacking a long G/C stretch in the 5'-terminal two-thirds of SS (*see* **Note 1**). Class III siRNAs were defined as siRNAs with opposite features except for condition 4. All other siRNAs belong to class II. Class I was subdivided into classes Ia and Ib. Class I siRNAs with

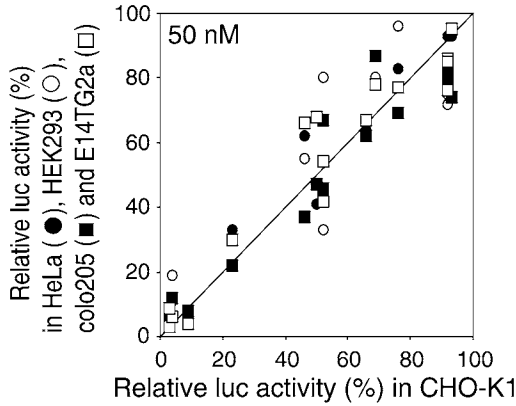

Fig. 1. Comparison of short-interfering RNAs (siRNAs)-dependent RNA interference (RNAi) activity induced in five mammalian cells. Cells were transfected with 1 of 16 arbitrarily chosen siRNAs at 50 n*M* and RNAi activity was measured using the luciferase assay 1 d after transfection. The horizontal axis shows relative *luc* activity in Chinese hamster cells, CHO-K1, whereas the vertical axis indicates *luc* activity in three human cells, HeLa, HEK293, colo205, and mouse ES cells, E14TG2a.

five to seven A/U residues in 5'-terminal one-third of AS were assumed to belong to class Ia and the remainder to class Ib. Initial experiments indicated class Ia siRNAs are highly functional, whereas class III siRNAs are inactive. Subsequent experiments showed that class Ib siRNAs possess RNAi-inducing activity almost identical to that of class Ia (Naito, Ui-Tei, and Saigo unpublished data; *see also* **Fig. 3**).

To demonstrate the validity of the previously described algorithm (algorithm I in **Fig. 2A**), 34 class Ia, 5 class Ib, and 13 class III siRNAs targeting for 2 exogeneous and 2 endogenous genes were arbitrarily chosen and assessed for their ability to give rise to RNAi in mammalian cells (***[23]***; K.U.-T., et al., unpublished data). As anticipated, all class Ia and class Ib siRNAs brought about highly effective RNAi, whereas little or no effective RNAi could be detected by transfection of class III siRNAs. The importance of base preference at the 5'-ends of both AS and SS and A/U-richness in 5'-AS-terminal one-third has been demonstrated by site-directed mutagenesis of both target and siRNA sequences (Ui-Tei et al. unpublished data). **Figure 3** shows the relationship of RNAi activity to A/U content in the 5'-terminal one-third of AS. It should be noted that, in siRNAs with A/U and G/C at the 5'-ends of AS and SS, respectively, RNAi-inducing activity dramatically decreases when the A/U content is equal to or less than 43% (3/7). It would follow then that both functional and nonfunctional siRNAs for mammalian RNAi can be quite effectively designed provided that the present algorithm, algorithm I, is used. **Figure 3** also shows that a small fraction of class II

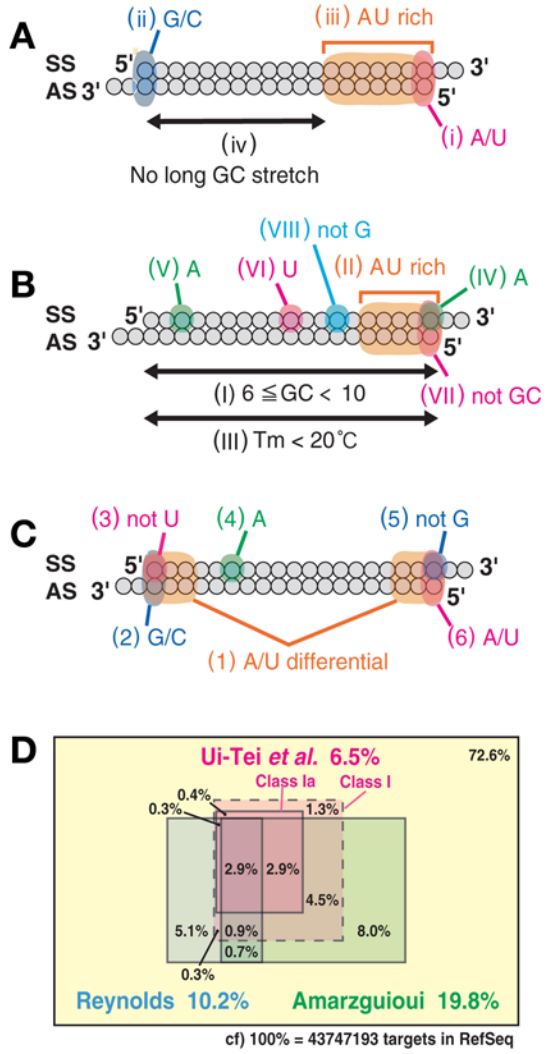

Fig. 2. Three algorithms for short-interfering RNAs (siRNA) design for functional RNA interference (RNAi) in mammalian cells. **(A)** Algorithm I. This algorithm was published by Ui-Tei et al. *(23)*. According to this algorithm, highly functional siRNAs simultaneously satisfy the following four conditions: (i) A/U at the 5' antisense strand (AS) end, (ii) G/C at the 5' sense strand (SS) end and (iii) more than four A/U nucleotides in the 5'-terminal one-third of AS, and (iv) lacking a long G/C stretch in the 5-terminal two-thirds of SS. **(B)** Algorithm II, siRNA design algorithm proposed by Reynolds et al. *(24)*. This includes eight requirements: (I) low G/C contents (30–52%), (II) three or more A/U at the SS 3'-terminus 5 bp, (III) low internal stability lacking stable inverted repeats, (IV–VIII) base preferences at SS positions 3, 10, 13, and 19. **(C)** Algorithm III, an algorithm proposed by Amarzguioui and Prydz *(25)*. This requires that A/U content in the

siRNAs is still associated with high RNAi activity, indicating that a small fraction of functional siRNAs may not be predicted by algolithm I.

Figure 2B,**C** shows other algorithms that have been proposed for selection of functional siRNA sequences for mammalian RNAi. Reynolds et al. ***(24)*** performed systematic analysis on 180 siRNAs targeting the mRNA of two genes and suggest the following characteristics to be associated with siRNA functionality: low G/C content, bias toward low internal stability at the SS 3'-terminus and lack of inverted repeats. Their algorithm (algorithm II) also maintains that the SS preferably uses A, U, and A residues at SS positions 3, 10, and 19, respectively, and that G may not be present at position 13 (**Fig. 2B**). But it is significant that only 1 of 23 highly functional class I siRNAs shown in **Fig. 3** satisfies all SS base preferences. Sixteen functional class I siRNAs exhibited none of the base preferences at both position 3 and 10.

Amarzguioui and Prydz ***(25)*** carried out statistical analysis on 46 siRNAs and identified some features of functional siRNAs (algorithm III). As in our study, A/U at the 5'-AS terminus and its SS partner and G/C at the 5'-SS terminus and its AS partner in functional siRNAs were noted to be important, opposite combination of terminal bases was maintained to cause lack of functionality. Their algorithm includes asymmetry in siRNA duplex end stability. That is, the A/U content differential for the three terminal nucleotides at both ends of the duplex is assumed essential for determining siRNA functionality. Amarzguioui and Prydz also found A to be preferably used at position 6 of functional siRNAs (**Fig. 2C**). Only 5 of 23 highly functional class I siRNAs shown in **Fig. 3** exhibited A residue at SS position 6.

Algorithms I–III may specify different siRNA sequences that are functional. Thus, using about 4.4×10^7 potential targets derived from RefSeq human sequences, determination was made of percent of siRNAs predicted to be functional by algorithm I (class Ia) that can be repredicted as functional by algorithms II or III or vice versa (Naito et al., unpublished). In **Fig. 2D**, 73% of the total possible siRNA sequences (3.2×10^7 sequences) are scored as nonfunctional ones. Class Ia was found to represent 6.5% of the total, whereas algorithms II and III, respectively, predicted 10.2 and 19.8% as functional siRNAs. About 90% of siRNAs predicted to be functional by algorithm I could be repredicted to be functional by either algorithm II or III or both. More than 60% of

Fig. 2. *(Continued)* 5'-AS end should be higher than that in the 5'-SS end. Base preferences are also required at position labeled with (2–6). **(D)** Difference in functional siRNA prediction. 97,475,268 siRNA sequences were collected from human sequences deposited in RefSeq and classified using three algorithms. About 6.5 and 13.5% of siRNAs, respectively, were assigned to class Ia and class I siRNAs by algorithm I, whereas about 10 and 20% were predicted to be functional by algorithms II and III.

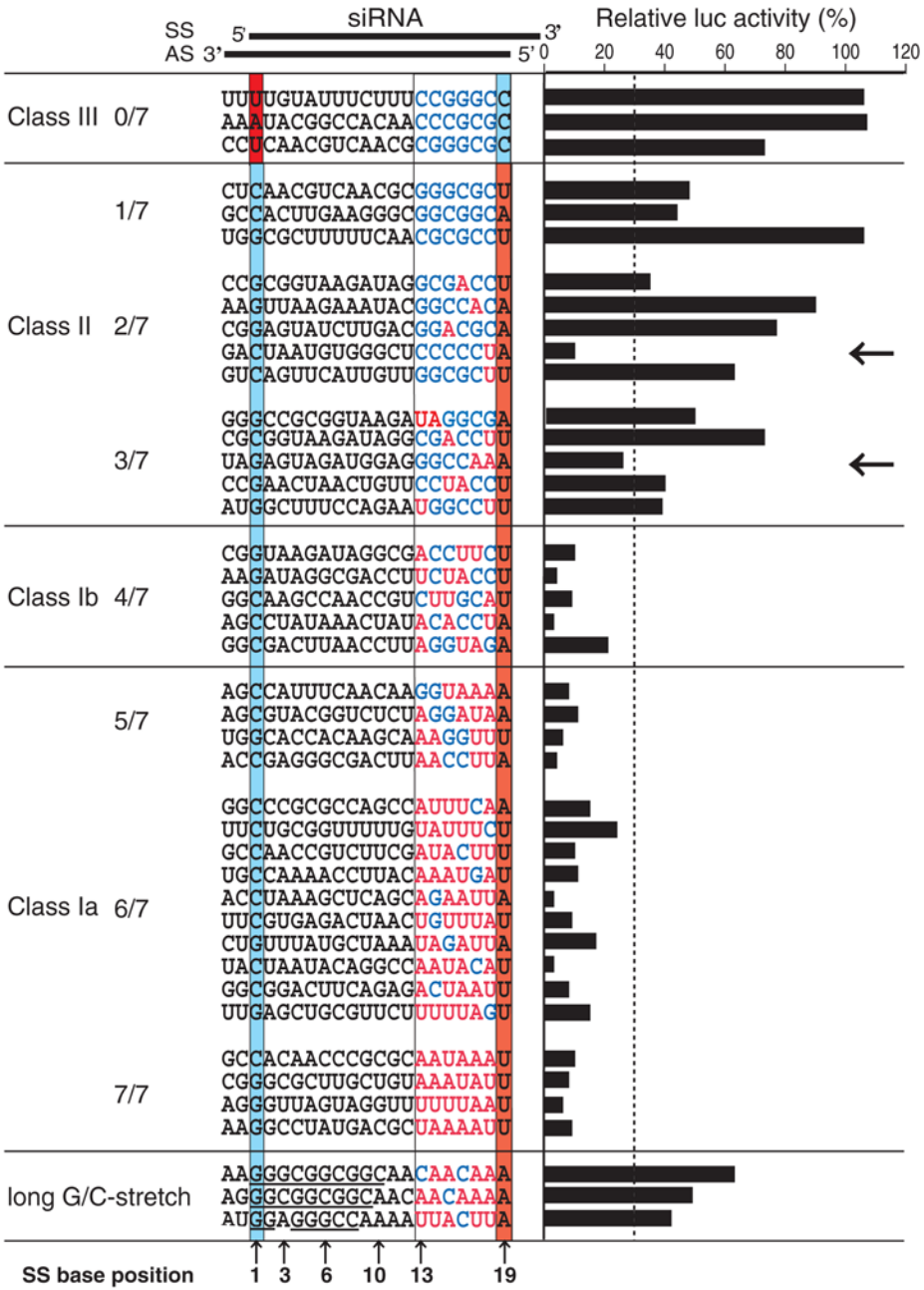

Fig. 3. Relationship between RNA interference (RNAi) activity and A/U content in the 5'-terminal one-third of AS. Thirty-nine short-interfering RNAs (siRNA) sequences, which target *luc* and possess A/U and G/C, respectively, at 5'- antisense strand (AS) and

siRNAs simultaneously predicted to be functional by algorithms II and III could be repredicted to be functional by algorithm I. This value increases to 85% in the case of class I. More than 50 and 40% of siRNAs predicted to be functional by algorithms II and III, respectively, could not be supported by other algorithms. Algorithm I is thus shown capable of predicting more reliably the functionality of siRNAs than algorithm II or III.

Our computational analysis also showed that class Ia can provide at least one functional siRNA to 99.6% of human mRNA sequences registered in RefSeq **(Fig. 4)**. Algorithm I is thus sufficient to search functional siRNAs for genome-wide gene silencing. As described previously ***(23)***, class Ia siRNAs are comprised of heterogeneous members with more than 10 times the capacity to induce RNAi. Thus, it may reasonably be considered that only siRNAs predicted simultaneously to be functional by three algorithms should be used for functional genomics. This class of siRNAs represents more than 44% of class Ia **(Fig. 2D)** and most of genes are capable of generating more than 10 class Ia siRNAs as shown in **Fig. 4**. This approach should accordingly be applicable in most cases. Note that human *src* and *vimentin* sequences, respectively, generate 27 and 64 class Ia siRNAs **(Fig. 4)**.

Mammalian Argonaute proteins (eIF2Cs) are each composed of an N-terminal proline-arginine-proline (PRP) motif and Piwi Argonaute Zwelle (PAZ) and PIWI domains ***(26)***. Crystal structural analysis of the Argonaute protein from *Pyrococcus farious* indicated the PIWI domain to have essentially the same three-dimensional structure as ribonuclease H, with a conserved active site Asp-Asp-Glu motif and that Argonaute may serve as "Slicer," the enzyme that cleaves mRNA ***(27)***. Both ends of siRNA appear to be separately recognized by two domains of the Argonaute protein. Ma et al. ***(28)*** reported the crystal structure of the complex of the PAZ domain from human Argonaute eIF2C1 and a 9-mer siRNA-like duplex and indicated that, in a sequence-independent manner, the PAZ domain is anchored to the 2-nucleotide 3' overhang of the siRNA-like duplex. The PIWI domain from *Archaeoglobus fulgidus* in complex with a siRNA-like duplex has been shown to contain a highly conserved metal-binding site that anchors the 5'-nt of the guide RNA in a sequence-independent manner ***(29)***. The first base pair of the duplex is unwound and the base at the 5'-unpaired AS end stacks on the aromatic ring of invariant Tyr 123 so that interactions between the 5'-AS end and the PIWI domain may possibly be nonsequence specific. The remaining base-paired nucleotides assume an A-form helix.

Fig. 3. *(Continued)* 5'-sense strand (SS) ends, were chosen and their RNAi inducing activity were compared at 5 n*M*. All class Ia and class Ib siRNA were found to be highly functional. siRNA functionality significantly dropped upon A/U content reduction from four to three. Arrows indicate two exceptional type II siRNAs associated with high RNAi activity.

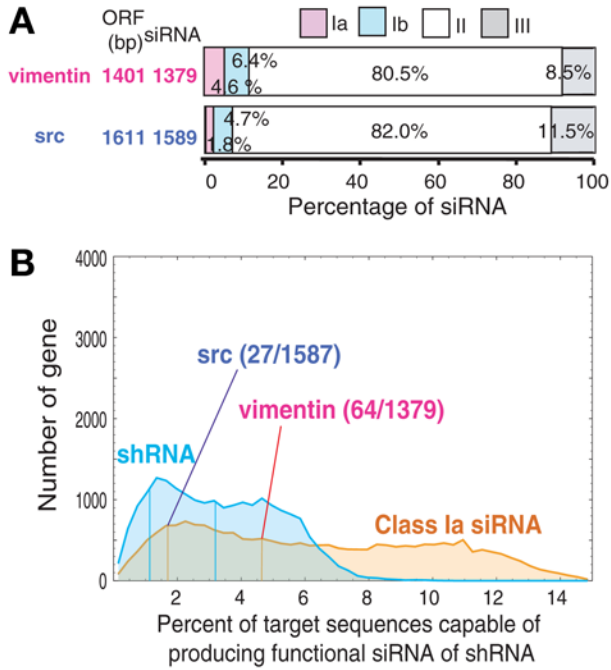

Fig. 4. Fraction of targets capable of producing functional short-interfering RNAs (siRNA) or short-hairpin RNAs (shRNA). **(A)** From vimentin and src ORF sequences, respectively, 1379 and 1589 siRNA sequences can be generated. Algorithm I predicted that 64 (4.6%) and 152 (11%) of 1379 vimentin sequences belong to class Ia and class I, respectively. Only 27 (1.8%) class Ia siRNAs can be designed from the *src* ORF sequence. (**B**) Genome-wide distribution of fraction of targets capable of producing functional siRNA or shRNA. All human sequences registered in RefSeq were examined and it was found that 99.6% of "human genes" possess at least one target sequence capable of producing class Ia sequence.

As previously described, algorithms I and III predict that functional siRNAs possess A/U and G/C at the 5'-AS and SS ends, respectively. Algorithm II maintains that the 5'-end of AS should be A. Because the GC pair is thermodynamically much more stable than the AU pair, difference in stability in terminal base pair of the siRNA duplex may determine terminal sequence preference in highly effective and ineffective siRNAs most probably by stimulating the asymmetric binding of the PIWI and PAZ domains to siRNA ends. We assume that the presence of an A/U pair at the siRNA end including the 5'-AS end is much more preferable for PIWI binding than that of the G/C pair.

The guide stand of siRNA is always designed so as to be complementary in sequence to that of its target. However, siRNA designed for a particular mRNA may also inactivate certain targets of other mRNA most probably through incomplete

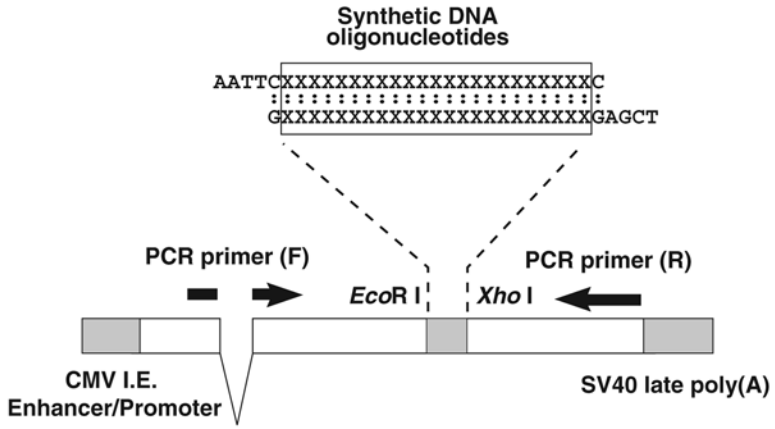

Fig. 5. Structure near the oligonucleotide insertion site of pTREC, a vector for target cleavage assay. For typical target assay, 29-bp-long single-stranded complementary oligonucleotides are annealed and the resultant double-stranded DNA oligonucleotide is inserted into *Eco*RI and *Xho*I sites of the vector. Cells are cotransfected with pTREC construct and suitable siRNA and possible change in mRNA content is examined using real time PCR.

Watson–Crick base paring. These silencing effects are collectively called off-target silencing effects. The second major problem in siRNA designing thus becomes how to eliminate possible off-target silencing effects. Molecular mechanisms of off-target effects have yet to be clarified. In some cases, even a single mismatch in the center of an siRNA appears to eliminate target mRNA silencing (*[30,31]*; our unpublished observation), whereas siRNAs with 11 contiguous matches can give rise to off-target effects *(32)*. To experimentally determine how incomplete base pairing between AS and nontargeted mRNA sequences affects silencing as a result of mRNA breakdown, a new plasmid vector, pTREC, was made, as shown in **Fig. 5**. Our unpublished observations (Naito et al.) indicate that, if the target sequence possesses three or more internal mismatches to the AS sequence, off-target effects on RNA stability should be virtually negligible.

Based on these observations on off-target silencing effects and algorithm I for selection of highly functional siRNAs for mammalian cells, a web-based online software system, siDirect *(33)* was constructed and by which it is possible to easily and very rapidly identify functional siRNAs exhibiting the least off-target effect for any human and mouse genes.

2. Materials

2.1. Small-Interfering RNAs

Sense and antisense RNA oligonucleotides, whose sequences have been selected by siDirect, are chemically synthesized, mixed in a 1:1 fashion in 10 m*M* NaCl and 20 m*M* Tris-HCl, pH 7.5, and annealed by incubating at 95°C for 15 min,

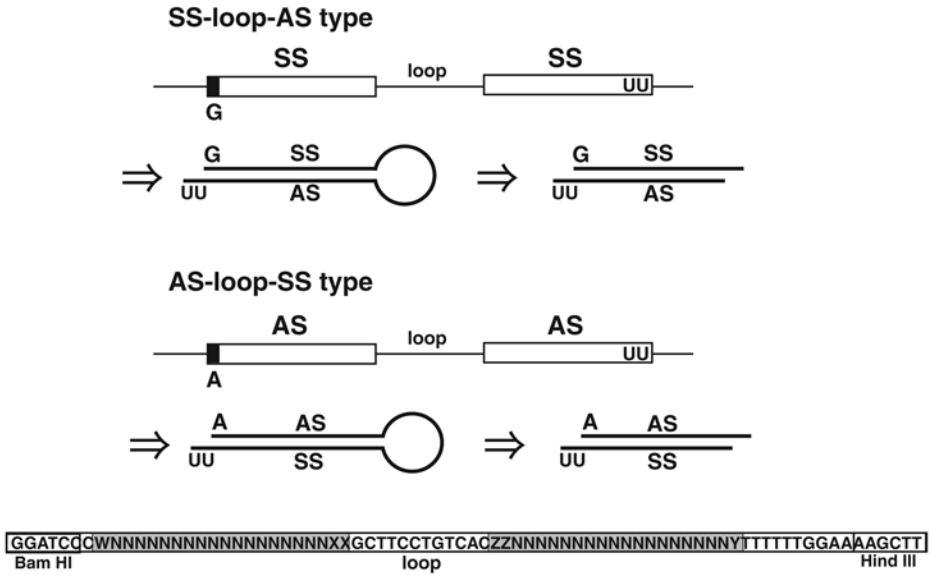

Fig. 6. Two types of DNA oligonucleotides encoding short-hairpin RNAs (shRNA). Functional short-interfering RNAs (siRNA) may be generated from two different types of shRNA. In the sense strand (SS)-loop-antisense strand (AS) type (**A**), DNA near the *Hind*III site codes for AS, whereas in the AS-loop-SS type (**B**), DNA near the *Bam*HI site encodes AS. The loop sequence is presumed to be eliminated by cytoplasmic dicer activity.

at 37°C for 30 min and at 25°C for 30 min. Annealed products may be examined using 3% agarose gel electrophoresis in TBE buffer, which can separate 21-bp-long double-stranded siRNA from 21-bp-long single stranded RNA (*see* **Note 2**).

2.2. DNA Plasmid

pSilencer 3.0-H1 (Ambion) is a plasmid vector for DNA-based RNAi *(34)*. Double-stranded oligonucleotides to be inserted vary depending on shRNA types (*see* **Fig. 6**). pTREC is a derivative of pCI-neo with cytomegalovirus immediate-early enhancer/promoter and SV40 late polyadenylation signal and can be used to express various target sequences within cells (Naito, Ui-Tei, and Saigo manuscript in preparation).

3. Methods

3.1. Identification of Functional Small-Interfering RNA and Short-Hairpin RNA Sequences for Mammalian RNA Interference

3.1.1. Design for Small-Interfering RNA

Highly functional class Ia siRNAs with maximal target-specificity for mammalian RNAi can be designed by using the web-based online software system,

siDirect (http://design.RNAi.jp/) ***(33)***, which is driven by algorithm I ***(23)*** and an accelerated off-target search algorithm developed by Yamada and Morishita ***(35)***. In siDirect, functional siRNAs for human and mouse RNAi (*see* **Note 3**) are easily designed under default conditions.

1. First, enter the accession number (GenBank or RefSeq accession number) of any human or mouse gene to be knocked down in the open box on the first page of siDirect (*see* the arrow in **Fig. 7A**).
2. Click "retrieve sequence." The nucleotide sequence of the gene corresponding to the accession number entered is automatically downloaded from the GenBank database, and full sequence appears in the box, which is labeled with double arrows in **Fig. 7A**.
3. For a off-target search for all human mRNA sequences, "Homo sapiens" should be selected in the "Off-target search" (*see* **Note 4**). For a off-target search for mouse genes, select "Mus musculus."
4. Click "design siRNA." Then, as shown in **Fig. 7B**, selected functional siRNA sequences appear on the next page with the sense DNA sequence of the target gene. The size and location of target sequences which exhibit the least off-target effect to both sense and AS are colored in blue (*see* **Fig. 7B**), whereas those with the least off-target effect only to the SS are colored in light blue (*see* **Note 5**). The sequence of siRNA selected (class Ia; *see* **Note 6**) and its off-target candidates are shown on the next page of siDirect (**Fig. 7C**) upon clicking the number in the box of the siRNA.

3.1.2. Design for Short-Hairpin RNA for DNA-Based RNA Interference

As described previously ***(23)***, our algorithm (algorithm I) is successfully applicable to functional shRNA design for DNA-based mammalian RNAi. However, additional sequence conditions are required for shRNA design, because RNA polymerase III is usually used for RNA synthesis in DNA-based mammalian RNAi. For shRNA design, following steps (**steps 1** and **2**) should be inserted between aforementioned steps for siRNA design, **Subheading 3.1.1.**, **steps 1–3** and **4**.

1. Check the small box of "Custom rule," and then enter "NNGNNNNNNNNNNNNNNNNNN" in the right long rectangle, which accommodate a 23-nt-long target sequence corresponding to both 21-bp-long AS and SS, if shRNA to be designed belongs to the 5'-SS-loop-AS type (**Fig. 6A**). For design of shRNAs belonging to the 5'-AS-loop-SS type (**Fig. 6B**) insert "NNNNNNNNNNNNNNNNNNNTNN" (*see* **Note 7**). Note that the 5'-end of SS corresponds to the third nucleotide of the 23-bp-long sequence.
2. Check the small box labeled with As or Ts and then select numeral 4 in the right box (*see* **Note 8**).

3.2. RNA Interference in Mammalian Cells

RNAi experiments using mammalian cultured cells should be carried out essentially as described previously (***[23]***; *see* **Note 9**)

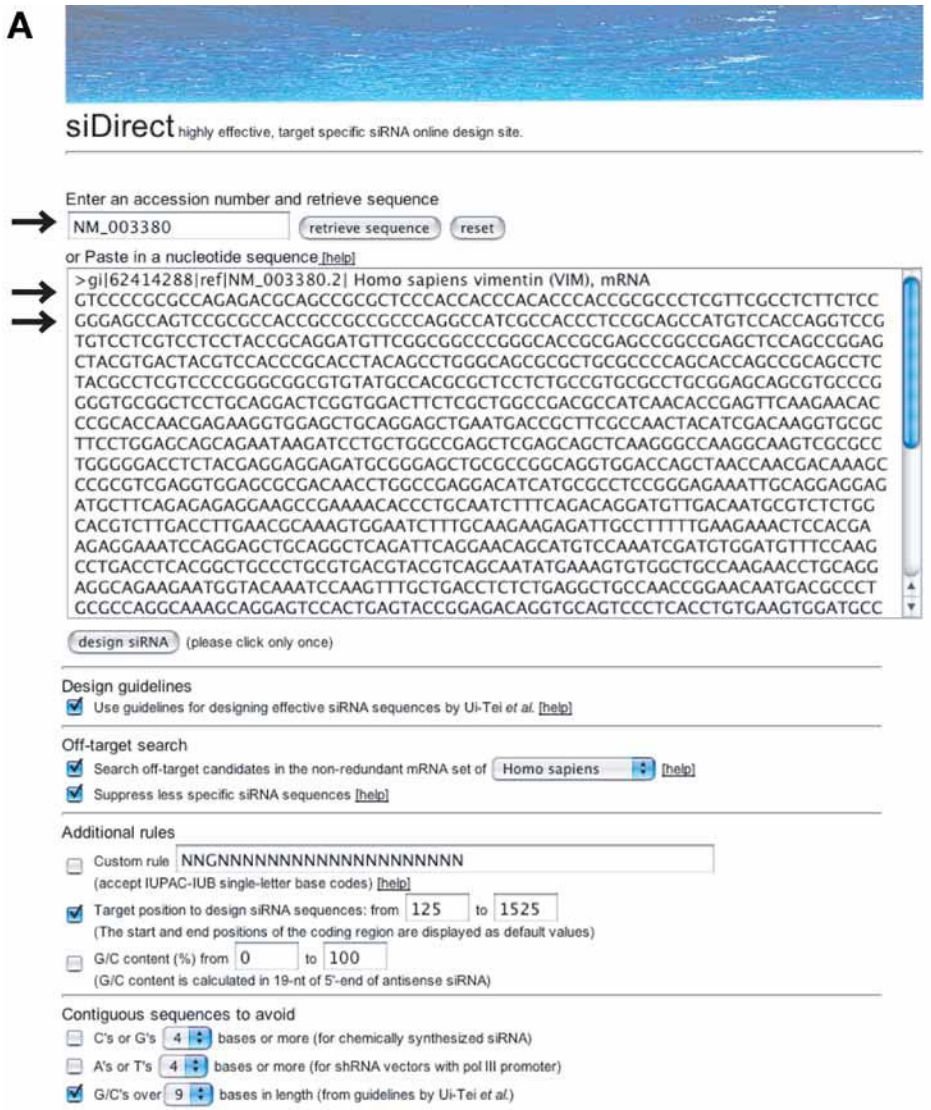

Fig. 7. The first (**A**), second (**B**) and third (**C**) pages of siDirect. *See* **Subheading 3.1.1.** for details.

3.3. Experimental Evaluation of Off-Target Effects

Although siDirect is capable of predicting highly functional siRNA sequences along with the most dangerous off-target candidate sequences against any given functional siRNA, it is important to observe how much mRNA derived from the target gene or putative off-target genes is really destroyed within cells upon

siRNA transfection (*see* **Note 10**). pTREC is a newly developed target-activity assay vector capable of accommodating any siRNA target sequence between *Eco*RI and *Xho*I sites (Naito, Ui-Tei, and Saigo in preparation). In mammalian cells transfected with pTREC, the inserted target sequence is transcribed most probably without translation.

1. Insert a desired target sequence into the *Eco*RI/*Xho*I site of pTREC (**Fig. 5**).
2. Introduce the resultant pTREC construct (0.5 μg) into cells through transfection with or without cognate siRNA and examine RNAi effects 24 h after transfection using real time PCR.
3. Total RNA is purified from transfected cells using RNeasy 96 (Qiagen) and DNase treatment (*see* **Note 11**). cDNA corresponding to extracted mRNA is synthesized using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen).
4. Real time PCR is carried out using ABI PRISM 7000 (Applied Biosystems) with SYBR Green PCR master mix (Applied Biosystems). The quantity of unbroken target or off-target mRNA is normalized with neomycin resistant mRNA simultaneously transcribed from the same construct. Universal PCR primers for any target RNA derived from the transfected construct are 5'AGGCACTGGG CAGGTGTC and 5'TGCTCGAAGCATTAACCCTCACTA, and those for neomycin gene expression are 5'ATCAGGATGATCTGGACGAAG and 5'CTCT CAGCAATATCACGGGT.

4. Notes

1. Previously, class I and class Ia siRNAs were defined as siRNAs simultaneously satisfying three conditions (*see* **Subheading 1.**). Here, for simplicity, this definition is somewhat modified by considering the fourth condition concerning to the absence of a long G/C stretch in the 5'-terminal two-thirds of SS. The permissible length of G/C stretch may vary depending on the concentration of siRNA used for transfection. In the case of RNAi with 50 n*M* siRNA, this value is 10 ***(23)***, whereas it may reduce to 7–8 when 5 n*M* siRNA is used (*see* **Fig. 3**).
2. Under the condition used here, almost all single-stranded siRNA appears transformed to double-stranded siRNA, possibly suggesting that the gel electrophoresis step may be eliminated.
3. siDirect predicts class Ia sequences corresponding to both open reading frame (ORF) and untranslated regions of the target gene, whereas requirements for functional siRNA sequences were established only based on experiments with siRNAs homologous in sequence to the ORF of the target genes. Thus, class Ia siRNAs homologous in sequence to the target-gene ORF might serve as more effective RNAi reagents.
4. At present, only “Homo sapiens” and “Mus musculus” are selections. In the planned new version, “Rattus norvegicus” and “Gallus gallus” and “Canis familiaris” will be added.
5. Theoretically, siRNA transfection may produce two types of RISCs, SS-RISC containing SS as a guide strand, and AS-RISC with AS guide within cells. In mammalian cells transfected with highly functional siRNAs, AS-RISC is considered to be

predominant. In addition, highly functional siRNAs are asymmetric in sequence preference. Thus, off-target effects owing to putative SS RISCs may not be prominent.

6. Algorithm for selection for class I siRNAs will be included in a planned new version of siDirect.
7. RNA polymerase III requires purine nucleotides for RNA start and accordingly, the first base of shRNA should be A or G. 5'-end of shRNA should be A if shRNA begins with AS, whereas it should be G if shRNA begins with SS (**Fig. 6**). Both configurations can be functional, but the latter may induce somewhat more effective RNAi (Ui-Tei unpublished data).
8. RNA polymerase III-dependent RNA polymerization is terminated in a region with continuous U residues, indicating shRNA possessing four or more nucleotide-long A stretches or U stretches is prematually terminated.
9. For RNAi assay of endogenous gene or RNAi activity observation in cells with a low-transfection efficiency, transfected cells are recommended to be preselected through cotransfection with a selective-marker-containing plasmid DNA such as pCAGIpuroEGFP possessing puromycin resistant and enhanced green fluorescent protein (EGFP) genes. pCAGIpuroEGFP plasmid DNA (0.2–0.4 μg) is simultaneously introduced into mammalian cells with siRNA via transfection. Twenty-four hours after transfection, puromycin (2 μg/mL) is added to the medium and puromycin-susceptible cells (untransfected cells) are removed 24 h after puromycin treatment. Cells are harvested 3 d after transfection for RNAi assay.
10. RNAi reaction might be prevented by secondary structures of and/or protein binding to the target mRNA.
11. DNase treatment is essential for eliminating a small amount of DNA, which cannot be eliminated by RNeasy 96.

Acknowledgments

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10

Suppression of Apoptosis in the Liver by Systemic and Local Delivery of Small-Interfering RNAs

Lars Zender and Stefan Kubicka

Summary

RNA interference (RNAi) is a sequence-specific gene-silencing mechanism triggered by double-stranded RNA. RNAi was shown to allow transient or stable knockdown of gene expression in a broad range of species and has been used successfully for functional genomic screens in mammalian cells and *Caenorhabditis elegans*. Standard therapeutic use of RNAi in clinical settings in humans has been hampered by the lack of effective methods to deliver the small-interfering RNAs (siRNAs) or short-hairpin RNA expression vectors into the diseased organs. In mice, systemic delivery of siRNAs by hydrodynamic intravascular injection leads to highly efficient uptake of siRNAs into the liver. Several groups demonstrated therapeutic use of RNAi in mouse models of acute liver failure or hepatitis B virus replication. This chapter will focus on the technical background of hydrodynamic and portal vein delivery techniques in mice and will give practical guidance for using these techniques for siRNA delivery into the liver.

Key Words: siRNA; RNA interference; hydrodynamic intravascular injection; apoptosis; acute liver failure.

1. Introduction

RNA interference (RNAi) has been shown to be a powerful tool to study gene function in a broad range of different organisms. It overcomes the limitations of classical genetic methods to knockdown gene expression in mammalian cells. Most recently, RNAi has been exploited as a high-throughput genomics tool in mammals ***(1–3)***. Furthermore, RNAi holds great promise as a therapeutic tool. Applications of RNAi directed against targets in diseases, for example cancer, infectious diseases, or dominant genetic diseases, are conceivable. Although the therapeutic use in humans is

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still some time from becoming routine in the clinic, use of experimental delivery methods in mice currently allows the study of RNAi in preclinical therapeutic settings *(4–7)*.

Because numerous comprehensive reviews about RNAi are available in the literature, this chapter will not give a detailed background on the discovery of RNAi but will focus on practical aspects of using RNAi in mice.

1.1. Efficient Delivery: the Holy Grail of Gene Therapeutic Approaches

The holy grail of all in vivo gene therapeutic approaches is to deliver effective amounts of the therapeutic gene into the desired target cells. Currently, viral vectors are most widely used for delivery of therapeutic genes in vivo. However, vector-associated side effects limit their value as a tool for clinical use.

Systemic application of plasmid DNA or antisense oligonucleotides (ASO), however, leads to very low uptake in most tissues. In part, this is owing to the fact that nucleic acids are rapidly degraded after systemic intravascular application in vivo. With regard to future therapeutic applications in humans, it is promising that under certain conditions short-interfering RNA (siRNA) duplexes seem to be more stable than chemically modified ASO *(8)*. Several approaches have been described to achieve more efficient uptake of naked DNA or ASO into tissues in vivo. Among these are direct injection into the target organ, as shown for muscle and liver *(9,10)* and electroporation *(11,12)*.

1.2. Hydrodynamic Intravascular Injection

The large volume hydrodynamic delivery technique was a major advance in the in vivo delivery of nucleic acids into mice and rats. In principle, it means the injection of a high volume into the vascular system within a short period of time. The first report that this delivery method can be even more effective than direct injection into the target organ is from Budker et al. *(13)*, who injected high volumes of either lacZ or luciferase reporter plasmids into the portal vein of mice. Using this approach approx 1% of all hepatocytes throughout the whole liver showed uptake of the plasmid. Remarkably, reporter activity was undetectable in endothelial and bile duct cells.

Because surgery on mice and intraportal injection is not feasible in every laboratory, it was an important discovery that injection of a high volume of plasmid DNA into the tail vein also leads to a very efficient uptake of DNA into the liver *(14)*. In 2002, McCaffrey et al. showed that the hydrodynamic tail-vein delivery technique could be used to effectively deliver siRNA into hepatocytes *(15)*. Subsequent studies showed that the siRNA uptake into the liver after hydrodynamic tail-vein delivery is much more effective than the

uptake of plasmid DNA using this technique; uptake of siRNA in up to 70–90% of all hepatocytes was achieved *(16,17)*.

The physiological mechanisms of the hydrodynamic intravascular injection technique are still not fully elucidated. Intravascular delivery of a large bolus of fluid is thought to result in an accumulation of the liquid in the caval vein. As the heart is swamped with the high amount of liquid, a temporary right heart failure with subsequent backflow into the liver is the consequence. In the liver, the fluid is thought to be extravasated through the pores (fenestrate) in the liver endothelium (sinusoids). It is well known that liver endothelium has a much bigger average pore size than endothelium of other organs. The observation that siRNA uptake into the liver after hydrodynamic tail-vein injection is much higher than the uptake of plasmid DNA is in line with the idea that extravasation through the endothelial pores is a prerequisite for uptake into hepatocytes. After passing the endothelium, the siRNA molecules or plasmid DNA are in direct contact with the hepatocytes. Whether the molecules are finally internalized by receptor mediated *(18)* or receptor independent processes like membrane rupture *(19)* remains unclear.

1.3. Therapeutical Use of siRNA to Prevent and Treat Acute Liver Failure in Mice

As the first demonstrations of a therapeutic application of siRNA in vivo were done in mouse models of acute liver failure (ALF), the next paragraphs will give a brief introduction into this disease, followed by a discussion of the therapeutic use of siRNA to suppress apoptosis in this model.

ALF is defined as a dramatic clinical syndrome in which a previously normal liver fails within days or weeks. Three subgroups of ALF can be distinguished, hyperacute, acute, and subacute liver failure. Despite the frequent occurrence of cerebral edema and renal failure in patients with hyperacute liver failure, prognosis without transplantation is relatively good. Survival rates in patients with acute and subacute liver failure, however, are at best 15% *(20,21)*. The etiology of ALF shows marked worldwide variation: in underdeveloped countries viral causes predominate, whereas drug-induced hepatotoxicity and seronegative hepatitis predominate in most countries of the Western world *(22)*. To this day, the management of these varying clinical scenarios is essentially supportive. It aims to identify and remove the insult that led to destruction of the liver, whereas preventing associated complications, such as acute renal and respiratory failure, bleeding diatheses, severe sepsis, cerebral edema, and encephalopathy. Overall mortality in patients with severe ALF remains high, ranging from 40 to 80%. Although liver support devices or hepatocyte transplantation may in time have a place in treatment, currently liver transplantation remains the only therapeutic option that has

been shown to significantly improve the outcome of patients with ALF. Because of limitations of donor organs and the requirement of lifelong and life-limiting immunosuppression, liver transplantation should be only performed in patients who are unlikely to recover from ALF. In patients who recover from ALF with medical support, the liver almost always returns to normal, both structurally and functionally. Consequently, prevention of destruction of liver cells in the time-course of ALF and support of liver regeneration are the most important goals in management of ALF by molecular therapies in the future.

Several molecular mechanisms can initiate liver cell injury and can further aggravate ongoing damage processes *(23)*. Mitochondria are the prominent targets for hepatotoxicity of many drugs, leading to impairment of energy metabolism and intracellular oxidative stress. Once hepatocellular function is impaired, accumulation of hydrophobic bile acids causes additional cytotoxicity. Although drug-induced hepatotoxicity appears to be mediated by both apoptosis and necrosis, viral infection predominantly induces cell death of hepatocytes by apoptosis. In contrast to necrosis, apoptosis is a highly conserved physiological process important in normal development and tissue homeostasis of multicellular organisms. Apoptosis occurs by two pathways: a death receptor pathway and a mitochondrial pathway. Signals released from the cytoplasm and/or from the cell membrane activate a well-characterized cascade of caspases (cysteine aspartase), which execute apoptotic cell death *(24–27)*. Receptor-mediated apoptosis, as triggered by the tumor necrosis factor-R, Fas-, or TNF-related apoptosis-inducing ligand (TRAIL)-receptor 1, has been reported to be involved in the pathogenesis of different liver diseases like viral hepatitis, ALF, autoimmune hepatitis, ischemia-reperfusion injury, nonalcoholic steatohepatitis, and toxic liver damage like Wilson's disease or bile acid-induced hepatotoxicity *(28–33)*. Therefore, the apoptotic pathway provides attractive targets for molecular therapy to prevent further liver damage and provides a condition for successful liver regeneration in ALF.

Recently, Song et al. *(16)* and our group *(34)* reported the therapeutic use of siRNA in mouse models of ALF. Song et al. used siRNA duplexes targeting the Fas (CD95) receptor. Three consecutive applications of Fas-siRNA led to an uptake of siRNA in more than 80% of all hepatocytes resulting in an 8- to 10-fold downregulation of Fas mRNA expression in the liver. To note, comparable downregulation of Fas had been shown before using ASO *(35)*, however, this required treating the mice with an approx 14-fold higher amount of anti-Fas ASO (6 mg/kg body weight for 12 consecutive days). In accordance with the results from Zhang et al. *(35)*, inhibition of Fas expression by siRNA protected the hepatocytes against treatment with the Fas-activating antibody Jo-2

and resulted in significantly increased survival. Remarkably, Fas-siRNA also conferred protection against ConA-mediated acute liver damage, whereas Fas-antisense did not.

As it is well established that in addition to FasL, tumor necrosis factor α and TRAIL are also involved in the pathogenesis of viral hepatitis *(36,37)*, we reasoned that an essential early downstream mediator of all death receptors would be the most suitable target to achieve the best therapeutic effects in preclinical animal models of viral hepatitis and ALF. To test this, we directed siRNA against caspase-8, which is a key downstream effector in receptor-mediated apoptosis. A single dose of 0.45–0.6 nmol/g body weight of caspase-8-siRNA resulted in very effective inhibition of caspase-8 expression in the liver, thus leading to protection against Jo-2-mediated liver damage or liver damage induced by an adenovirus overexpressing Fas ligand (Ad-FasL). With regard to potential clinical applications, it is noteworthy that caspase-8-siRNA not only prevented acute liver damage but was also highly effective when delivered into an ongoing ALF. Furthermore, it is of particular interest that in our study the therapeutic efficiency of caspase-8-siRNA was shown in acute viral hepatitis that was triggered by wild-type adenovirus, which better resembles the multiple molecular events in human acute hepatitis.

2. Materials

2.1. Preparation for Hydrodynamic Tail-Vein Injection in Mice

1. Restraining device for mice.
2. Paper towels, warm water, or alternatively heating lamp.
3. 3-mL Syringe with screw thread, 21- to 27-gauge needle.
4. 0.9% Saline or Ringer's solution.
5. siRNA duplexes (0.5–1.0 nmol/g body weight).

2.2. Preparation for Portal Vein Injection in Mice

1. Surgery pad for mice.
2. Antiseptic solution.
3. 24-Gauge iv catheter.
4. Gauze.
5. Surgical instruments (scissors, tweezers, and retractors).
6. Ketamine/xylazine, alternatively gas-anesthsia (e.g., isoflurane may be used).
7. Suture material, 4–0.
8. Optional Fibrine/Thrombine adhesive solution (*see* **Subheading 3.**).
9. 0.9% Saline or Ringer's solution.
10. Lipiodol.
11. Microvascular clamps (optional).

12. Adapter tube and infusion pump (optional).
13. siRNA duplex (0.5–1.0 nmol/g body weight).

3. Methods

3.1. Hydrodynamic/High Volume Tail-Vein Injection in Mice (see Note 1)

In principle, any mouse strain can be used for this technique. BALB/c or other strains with white fur are preferred as visualization and puncture of the tail vein, especially with bigger needles, is more difficult in black mice.

We prefer to perform standard hydrodynamic tail-vein injection without anesthesia, as the combination of the high-volume injection together with anesthesia can lead to complications in some mice. If anesthesia is used, a gentle gas-anesthesia (e.g., isoflurane) should be preferred.

First, the injection solution is prepared. In our hands, best results are obtained with desalted, lyophilized siRNA duplexes, as siRNA duplexes lyophilized in annealing buffer can result in higher morbidity of the mice. Most of the "siRNA-companies" provide siRNA duplexes in a desalted "ready-to-go" option. The desired amount of siRNA duplex is dissolved in 0.9% saline or Ringer's solution. Effective siRNAs should work in a dose of 0.5–1.0 nmol per gram body weight. The total injection volume can be calculated by dividing the mouse body weight by 10 (*see* **Note 2**).

The mouse is restrained using a suitable restraining device (numerous Plexiglas versions are available from different manufacturers) and a tail vein is dilated by application of warm water. Alternatively, some researchers dilate the tail vessels by placing the whole mouse under a heating lamp for 10 min. A 3-mL syringe with a screw thread is connected to a needle. Needles from 21- to 27-gauges are suitable. Using syringes without a screw thread can lead to disconnection during hydrodynamic injection. A tail vein is punctured approximately midway between the tail tip and middle of the tail. If the first puncture is not successful, a more proximal puncture site can be tried. After injecting a small test volume to ensure that the needle is safely placed in the vein, the whole volume is applied within 4–10 s. Longer injection times will lead to less effective uptake of siRNA into the liver. Some mice will show heavy breathing and reduced activity after injection. These symptoms should not last longer than 30 min.

3.2. Intraportal Delivery of siRNA in Mice

The hydrodynamic tail-vein injection technique leads to high and reproducible siRNA uptake into the liver. It offers the possibility to investigate the effects of siRNA-mediated gene knockdown in the "target organ" liver in

different physiological and pathophysiological settings (*see* **Note 3**). However, a criticism against hydrodynamic tail-vein injection is that it is an experimental procedure that is not feasible in the clinical situation in humans. Therefore, it is desirable to have an experimental setting in the mouse model that can resemble the clinical situation in humans.

In humans the portal vein can be reached without open surgery by puncturing the jugular vein and placing a catheter from the inferior caval vein through the liver. Thus, the direct portal vein injection in mice can serve as a model for this procedure.

Animals are anesthetized with ketamine/Xxlazine or, if preferred, with gas-anesthesia (e.g., isoflurane). The abdominal wall is cleaned with antiseptic solution. A midline ventral incision is made and the abdominal cavity is kept open with retractors. The bowels are wrapped in saline soaked gauze and placed sideward of the operation field to obtain a good view on the portal vein. The desired amount of siRNA (0.5–1.0 nmol) is dissolved in saline or Ringer's solution containing 10% lipiodol as an embolizing agent. The use of an embolizing agent leads to a temporary stasis of the siRNA solution in the liver and, therefore, enhances the siRNA uptake into the liver. Alternatively, some researchers place microvascular clamps on the suprahepatic inferior caval vein during the injection procedure. This likewise prevents a fast flow-through of the siRNA solution through the liver. The portal vein is punctured with a 24-gauge iv catheter. After the plastic catheter is placed in a safe position the needle is removed. To avoid accidental movement of the catheter during injection, the portal vein is looped with a 4–0 ligature to tighten the catheter. A total volume of up to 5% of mouse body weight can be injected within 10 s. Inexperienced researchers may find it easier to use an adapter tube to connect the catheter to an infusion-pump instead of injecting free hand. Before removing the needle, an adhesive solution of Fibrine/Thrombine is spread on the puncture site to prevent bleeding. The abdominal cavity is closed with two separate layers of 4–0 suture material (*see* **Note 4**). **Fig. 1** shows the inhibition of endogenous expression of *LacZ* gene by hydrodynamic-derived siRNAs.

4. Notes

1. Researchers who are inexperienced with hydrodynamic tail-vein injection in mice may want to test their technical proficiency by using transgenic mice carrying a reporter gene like *lacZ* or green fluorescent protein (GFP). Using this approach with established siRNA duplexes against lacZ or GFP, the efficiency of siRNA delivery into the liver can be quantified (*see also* **Fig. 1**). Alternatively, Cy-5-labeled siRNAs can be injected. An easy and precise way to quantify the percentage of liver cells with siRNA uptake is to prepare a single-cell suspension of liver cells using standard collagenase perfusion ***(38)*** and subsequently determine the Cy-5-positive fraction of hepatocytes by flow cytometry. In this context it is

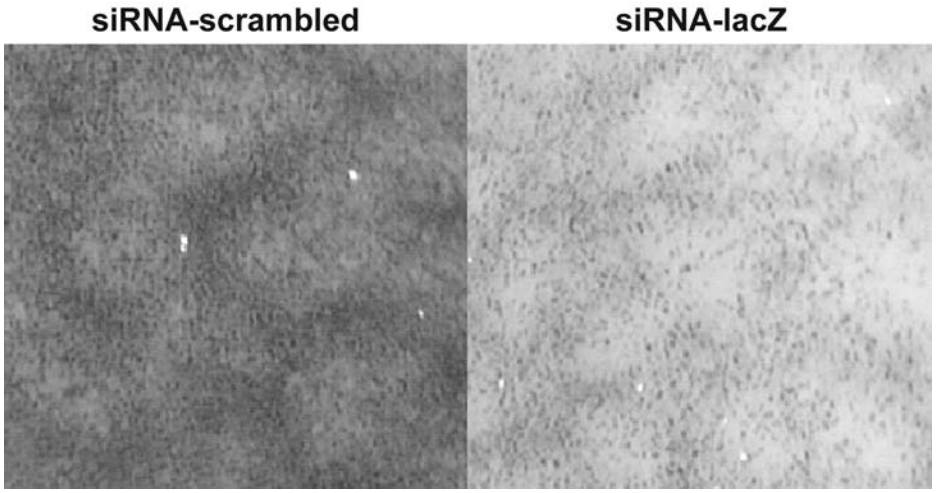

Fig. 1. Hydrodynamic tail-vein injection leads to efficient delivery of short-interfering RNAs (siRNA)-lacZ into the liver of *lacZ*-transgenic mice. Shown are photographs of the liver surface of *lacZ*-transgenic mice (C57BL/6J-TgN [MtnlacZ]) after hydrodynamic tail-vein injection of either siRNA-scrambled or siRNA-*lacZ*. *LacZ* staining was performed on whole liver (*see* **Note 5**).

important to mention that some siRNA duplexes lose their functionality when they are Cy-5-labeled.
2. To rule out off-target effects of siRNA, two independent siRNAs against every target should be used.
3. Although siRNA duplexes are effectively delivered to a target organ, the efficiency of knockdown of a specific gene must be quantified by assaying mRNA and protein expression.
4. Researchers who want to use the hydrodynamic tail-vein injection technique to deliver plasmid DNA to the liver are advised to work with supercoiled plasmid DNA that can be obtained easily by cesium chloride DNA preparations.
5. For experiments that need sustained knockdown of the target gene in the mouse liver, stability of the respective siRNA in the liver must be tested individually. Depending on the siRNA duplex, the duration of in vivo knockdown can be variable ranging from 2 d to more than 1 wk.

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11

Target Validation Using RNA Interference in Solid Tumors

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Summary

Reverse genetics is one strategy that is currently used to establish a link between a target gene and a disease phenotype. In this process, the function of a gene is inhibited and the consequence of its loss on a desired biological function, such as tumor growth and metastasis, is monitored. RNA interference (RNAi) has been found to be the most effective method to specifically inhibit gene expression. Notably, interactions between cancer cells, stromal cells, and the extracellular matrix (ECM) are crucial to angiogenesis and tumorigenesis. Tumor cells and the surrounding stroma are the principle source of growth factors and cytokines, which induce remodeling of the ECM mediated by metalloproteases (MMPs) secreted by macrophages. The production of macrophages is regulated by colony-stimulating factor (CSF)-1, which is overexpressed in several tumors. When short-interfering RNAs (siRNAs) targeting either the CSF-1 or its receptors were delivered into colon and breast cancer xenografts in mice, tumor growth was inhibited. Associated with this suppression, we observed decreased tumor vascularity, reduced expression of angiogenic factors and MMPs, and decreased macrophage recruitment to the tumors. The suppression of CSF-1 by RNA interference is therefore a powerful tool to block gene function and influence tumor–stroma interactions in solid tumor development.

Key Words: Reverse genetics; breast cancer; RNAi; siRNA; CSF-1.

1. Introduction

1.1. The Microenvironment of Cancer Cells

The development of cancer is a complex, multistage process during which a normal cell undergoes genetic changes that result in phenotypic alterations and acquisition of the ability to invade and colonize distant sites *(1,2)*. Solid tumors are composed of both malignant and normal cells. Targeting the complex

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interaction between genetically unstable neoplastic cells, the surrounding extracellular matrix (ECM), and stromal cells such as fibroblasts and inflammatory cells is a key obstacle to the cure of human cancers *(1,2)*. Changes in the tumor microenvironment can lead to ECM modification, infiltration of inflammatory cells, and alteration in the activity of matrix metalloproteases (MMPs), which are essential regulatory factors in tumor growth and invasion *(3)*. In addition, mediators released from both the stroma and tumor cells can lead to the induction of angiogenesis by shifting the balance between factors that promote and inhibit angiogenesis *(4–7)*, allowing the growth of tumors to macroscopic levels *(8–11)*.

1.2. Macrophages, Angiogenesis, and the Extracellular Matrix

Macrophages are common components of the tumor stroma *(11)* that modify the ECM and influence new capillary growth by several different mechanisms *(12,13)*. Macrophages can produce growth factors, cytokines, proteolytic enzymes, and matrix molecules that act directly to stimulate vascularization by stimulating endothelial cell proliferation, migration, and differentiation in vitro and angiogenesis in vivo *(11,12)*. Macrophages can also modify the ECM either through the direct production of ECM components or the production of proteases that alter ECM structure and composition *(14,15)*. The composition of the ECM dramatically influences endothelial cell shape and morphology and profoundly influences capillary growth *(16)*. Importantly, recruitment of macrophages to tumors can significantly increase metastatic progression *(17)*. Macrophages can also secrete cytokines that stimulate other cells to synthesize or degrade ECM molecules. Remodeling of the ECM is crucial to both angiogenesis and tumorigenesis and primarily involves the MMP family of proteolytic enzymes. MMPs degrade the ECM including the basement membrane and in conjunction with soluble growth factors, foster the migration and proliferation of endothelial cells. This process promotes angiogenesis and also allows tumors to spread locally and distantly *(3)*. Strict regulation of MMP expression is critical for maintenance of proper ECM homeostasis, however, in malignancies high levels of MMPs are often synthesized not only by cancer cells but also by adjacent and intervening stromal cells *(18)*.

2. CSF-1 Biology

2.1. CSF-1 and Macrophages

The production of macrophages is regulated by colony-stimulating factor (CSF)-1 also called macrophage-CSF (M-CSF) *(19)*. CSF-1 is produced by a variety of cell types such as fibroblasts or macrophages and prevents the death of monocytes and promotes their differentiation into macrophages *(20,21)*. CSF-1 also induces or augments the production of a variety of cytokines by macrophages such as tumor necrosis factor-α *(22)*. Macrophages most likely enhance tumor

progression through paracrine circuits involving the production of CSF-1 by tumor cells *(11)* or other host-derived stromal cells and by ECM-modulating functions mediated by MMPs *(12)* to accelerate angiogenesis in vivo *(23)*. Consistent with a proangiogenic effect, recent work suggests that CSF-1 also stimulates monocytes to secrete biologically active vascular endothelial growth factor (VEGF) *(24)*. VEGF is a key factor in tumor angiogenesis and is upregulated in numerous malignant tumors. The biological effects of VEGF are mediated by VEGF-receptor 1 (VEGF-R1, Flt-1) and VEGF-R2 (KDR/Flk-1).

2.2. CSF-1 Signaling Pathways

CSF-1 is a disulfide-linked homodimeric growth factor, which binds the integral membrane receptor tyrosine kinase (CSF-1R) product of the c-*fms* proto-oncogene *(25)*. Similar to other tyrosine kinase receptors, ligand binding stabilizes CSF-1R dimerization to activate the receptor through autophosphorylation *in trans*, thereby initiating a series of membrane-proximal tyrosine phosphorylation cascades leading to rapid stimulation of cytoskeletal remodeling, gene transcription, and protein translation *(26)*. Many of the downstream tyrosine-phosphorylated proteins, such as the p85 regulatory subunit of phosphatidyl-inositol 3-kinase, Cbl, and Gab3, have been shown to be important in regulating macrophage survival, differentiation, and motility *(27)*.

2.3. CSF-1 and Solid Tumors

CSF-1 is widely overexpressed in tumors of the reproductive system. In breast cancer, CSF-1 expression has been shown to correlate with high grade and poor prognosis associated with dense leukocytic infiltration *(28,29)*. High levels of CSF-1R mRNA have been observed in ovarian and endometrial cancers and elevated levels correlated with high histological grade and advanced clinical presentation *(30)*. Over half of invasive ovarian adenocarcinomas and endometrial cancers coexpress CSF-1 and CSF-1R *(31)*. Constitutive production of CSF-1 has been reported in normal ovarian epithelial cultures at levels comparable with ovarian cancer cell lines *(32)*. However, the coexpression of CSF-1 and CSF-1R may establish an autocrine loop that plays a role in metastatic progression. Serum levels of CSF-1 are markedly elevated in patients with endometrial cancer associated with active or recurrent disease *(33)*. Increased serum CSF-1 levels also characterize most clinical cases of epithelial ovarian cancers *(34)* and CSF-1 is considered a tumor marker for ovarian germ cell tumors *(35)*.

2.4. CSF-1 and Tumor Cell Invasion

Osteopetrotic CSF-1(op) (*op/op)* mice that have a CSF-1 gene defect and a profound macrophage deficiency *(36)* have been used as a model to examine tumor growth. These mice show an impaired tumor development (Lewis lung

carcinoma) when compared to normal littermates, which is reversed by CSF-1 treatment *(37)*. Crossing CSF-1(op) mice with a transgenic mouse susceptible to mammary cancer prevented macrophage accumulation in mammary tumors. In the macrophage-deficient mice, the incidence and initial rates of growth of primary tumors were not different from those seen in normal mice, but the rate of tumor progression was slowed and metastatic ability was almost completely abrogated when compared with mice that contained normal numbers of macrophages. Overexpression of CSF-1 in wild-type mice also accelerated tumor progression and increased rates of metastasis *(17)*. Another study has shown that CSF-1 promotes tissue invasion by enhancing ECM-degrading proteinase MMP-2 production by lung cancer cells *(38)*. In some instances, malignant cells coexpress CSF-1 and CSF-1R, raising the possibility of autocrine growth control by CSF-1 in the development of these malignancies *(39)*.

3. Target Validation of CSF-1 in Cancer

3.1. CSF-1 Antisense Treatment Suppresses Growth of Human Embryonic and Colon Carcinoma Xenografts in Mice

3.1.1. Human Embryonic Cancer Cells Upregulate Host CSF-1 Production

Human embryonic cancer cells (CRL-2073) show no detectable mRNA or protein for human CSF-1 or CSF-1R in vitro. When these cells are xenografted into the testis of SCID mice, however, mouse tissue CSF-1 gene and protein expression increases significantly compared to untreated mice. Associated with increasing CSF-1 tissue expression is an enhanced infiltration of macrophages within and surrounding the tumor. These findings indicate that human embryonic cancer cells stimulate increased host tissue expression of CSF-1. Correlated with these results, increased mouse CSF-1R expression is seen in tumor lysates *(40)*.

3.1.2. CSF-1 Oligodeoxyribonucleotide Treatment Suppresses CSF-1 Expression and the Growth of Embryonic Tumor Xenografts

Severe combined immunodeficient (SCID) mice bearing established human embryonic tumors were treated systemically with CSF-1 antisense oligodeoxyribonucleotides (ODNs), scrambled ODN or Ringer's solution. Antisense ODN treatment was well tolerated. Local inflammatory reactions were not observed and no significant changes in the complete blood count (CBC) of treated mice were seen. Treatment with CSF-1 antisense ODN significantly downregulated tissue CSF-1 mRNA and protein levels and suppressed the growth of embryonic tumors to dormant levels. Marked differences were found in the testicular weight between SCID mice with embryonic tumors treated with CSF-1 ODN for 2 wk

(89 ± 32 mg tumor weight) and those treated with Ringer's solution (285 ± 31 mg) or scrambled ODN (278 ± 27 mg) ***(40)***.

3.1.3. CSF-1 Antisense ODN Decreases Angiogenic Activity and MMP-2 Expression in Embryonic Tumor Xenografts

In human embryonic cancer cell xenografts, both intravital video microscopy and histomorphometry of embryonic tumors showed a significantly increased density of vascular sprouts in controls compared to untreated mice that returned to baseline levels after CSF-1 antisense ODN treatment in mouse testis. Similarly, VEGF-A, KDR/flk-1 and Ang-1 mRNA levels were significantly reduced in CSF-1 antisense ODN-treated mice. Protein expression of MMP-2, a key molecule in mediating tumor metastasis and angiogenesis, increased significantly in controls compared to untreated animals. Treatment with CSF-1 antisense ODN but not scrambled ODN, significantly downregulated MMP-2 protein expression in testicular tissue. Positive MMP-2 antigen staining was primarily observed in the intertubular interstitium, the capsule of testicular tubules and less frequently in the walls of vessels in untreated and ODN-treated mice, whereas in control mice, MMP-2 expression was primarily intratubular ***(40)***.

3.1.4. CSF-1 Antisense Treatment Suppresses the Growth of a Human Colon Carcinoma Xenografts and Increases Survival in Mice

The promising results with CSF-1 antisense treatment in the mouse model of human embryonic tumorogenicity encouraged us to test CSF-1 antisense treatment in other human tumor models. We chose colon carcinoma because of its poor prognosis, short median survival, and high incidence, and utilized SW-620 human colon carcinoma cells that lack expression of CSF-1 or CSF-1R. Using an established flank model in nude mice, we showed that host CSF-1 tissue mRNA and protein levels increase with tumor progression. After 2 wk of CSF-1 antisense ODN treatment at 5 mg/kg/d, CSF-1 mRNA and protein expression was significantly downregulated compared to controls. Tumor growth was markedly retarded in mice following CSF-1 blockade and tumor weights were significantly decreased compared to controls. Similar to mice bearing embryonic tumors, CSF-1 treatment was well tolerated in nude mice bearing colon carcinoma and the CBC was not significantly influenced by the treatment. MMP-2 protein expression in tumor lysates markedly increased with tumor progression and declined significantly following CSF-1 inhibition. CSF-1 ODN, but not scrambled ODN treatment, resulted in downregulation of mRNA levels of Ang-1 and the VEGF-A receptors Flt-1 and KDR/flk-1 associated with decreased angiogenesis. Long-term (6 mo) survival was observed in 8 of 14 mice following CSF-1 blockade, whereas all mice were dead after 65 d in the control groups. At sacrifice 6 mo after therapy, no metastases were detected. At

65 d (at which time the last animal in the control groups died), 85.7% of CSF-1 antisense-treated mice were still alive.

3.2. CSF-1 in Breast Cancer

The mechanism by which mammary epithelial cells undergo genetic changes that result in acquisition of the ability to invade and colonize distant sites is complex *(2,41,42)*. Normal and malignant mammary epithelium and the surrounding stromal cells produce and respond to various growth factors. Among the stromal cells, macrophages play a unique role because they are recruited into mammary gland carcinomas *(43,44)*. The fact that in the absence of such tumor-associated macrophages, metastatic progression of mammary gland tumors is profoundly reduced *(17)* as well as the fact that CSF-1 blockade suppresses tumor growth, MMP production, and macrophage recruitment in embryonic tumors and colon cancer *(40)* support the paradigm that CSF-1 enhances progression of malignancies through effects on the recruitment and control of macrophages that regulate tumor cell growth, angiogenesis, and the ECM. The recent discovery of highly specific, small-interfering (siRNA) molecules as promising candidate therapeutics to specifically and potently modify gene expression led us to hypothesize that blocking CSF-1 using this approach would efficiently suppress breast cancer development.

3.2.1. MCF-7 Cells Upregulate Mouse CSF-1 Production But Lose Their Ability to Express Human CSF-1 After Xenografting to Mice

Expression analysis showed that human MCF-7 mammary carcinoma cells express both mRNA and protein for human CSF-1 and CSF-1R in vitro. When MCF-7 cells were xenografted to immunodeficient nude mice, cancer cell expression of CSF-1 was lost but host (mouse) cells were stimulated to overexpress CSF-1.

3.2.2. CSF-1 siRNA Against CSF-1 and c-fms Downregulate Target Proteins and Suppress Mammary Tumor Growth

CSF-1 and CSF-1R siRNAs suppress target gene expression in a sequence and dose dependent manner in vitro. Mice bearing human MCF-7 mammary carcinoma xenografts were treated with five intratumoral injections of CSF-1 siRNA, CSF1-R siRNA, scrambled control siRNA, or Ringer's solution (control). siRNA treatment was well tolerated and no significant changes in the CBC of treated mice were observed. siRNA treatment against CSF-1 and CSF-1R suppressed mammary tumor growth by 45 and 40%, respectively, and selectively downregulated target protein expression in tumor lysates.

3.2.3. CSF-1 and CSF-1R Blockade Downregulate Mouse MMP-2 and MMP-12 Expression and Decrease Angiogenic Activity in MCF-7 Mammary Tumor Xenografts

After human MCF-7 cell xenografting in mice, macrophage invasion in the tumor xenografts was observed. In association with this, host (mouse) MMP-2 and MMP-12 (a macrophage-specific protease involved in ECM remodeling) were strongly expressed during tumor progression in control animals. Treatment with CSF-1 siRNA or CSF-1R siRNA reduced macrophage recruitment to the tumor and intratumoral levels of both MMP-2 and MMP-12.

Histomorphometrical analysis of mammary tumors showed an increased density of proliferating endothelial cells with tumor progression that was decreased after CSF-1 and CSF-1R siRNA blockade. In addition, VEGF-A mRNA levels increased with tumor progression and were reduced in CSF-1 and CSF-1R siRNA-treated mice. CSF-1 and CSF-1R blockade, however, did not significantly affect tissue mRNA expression of the VEGF-A receptors Flt-1 and KDR. These data indicated that blocking CSF-1 or CSF-1R is associated with decreased VEGF-A expression and reduced angiogenic activity in mammary tumor xenografts.

3.2.4. CSF-1 Blockade Increases Survival in Mice With Mammary Tumor Xenografts

The median survival of animals in the control group was 62 d, which was significantly increased in mice after treatment with CSF-1 siRNA (103 d) and slightly (but not significantly) increased after treatment with CSF-1R siRNA (76 d).

4. Conclusions

Using antisense oligonulcleotides and siRNAs, we have demonstrated that CSF-1 plays a crucial role in tumor growth and angiogenesis. Indeed, inhibition of upregulated host CSF-1 in human embryonic, colon, and breast cancer xenografts in mice suppressed tumor growth, leading to inhibition of tumor vascularity, angiogenic factors, and MMPs expression. Additionally, recruitment of host macrophages to tumors was significantly reduced ***(40,42)***. These results combined with the recently recognized role of macrophages as VEGF-secreting cells ***(24)***, suggest that certain cancer cells upregulate host CSF-1 by mechanisms that have yet to be identified leading to macrophage modification of the ECM and facilitating angiogenesis and tumor development. Moreover, some cancer cells produce CSF-1 and directly influence macrophages ***(45)***. Thus, interaction between cancer cells and the surrounding tumor microenvironment leads to upregulation of CSF-1, which in turn leads to macrophage recruitment. The tumor microenvironment educates these tumor-associated macrophages to

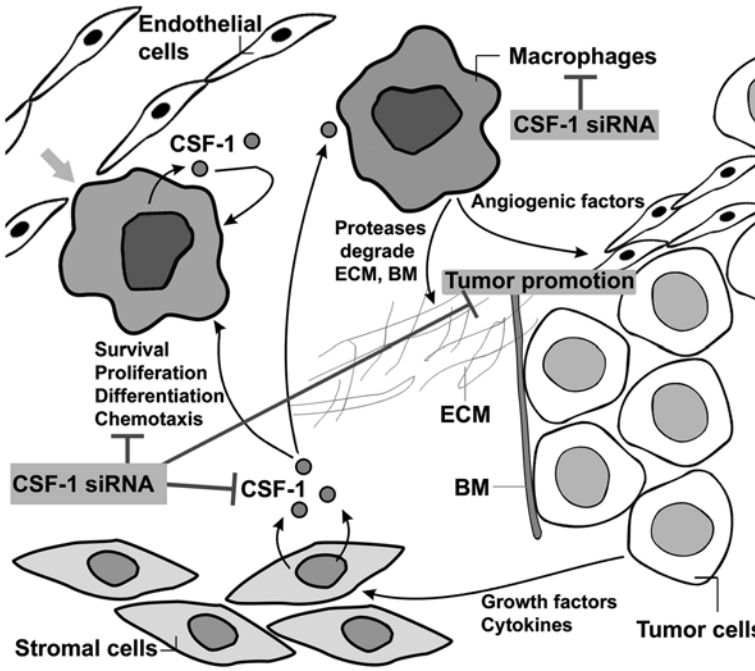

Fig. 1. Targeting colony-stimulating factor (CSF)-1 in tumor progression. Tumor cells may produce CSF-1 or secrete growth factors that upregulate CSF-1 production by stromal cells, thereby recruiting macrophages to the tumor. Tumor-associated macrophages also produce CSF-1 and stimulate angiogenesis by secreting factors such as vascular endothelial growth factor (VEGF). Macrophages also promote tumor invasion by producing proteases such as metalloproteases (MMP)-2 and MMP-9 that break down the basement membrane (BM), remodel the extracellular matrix (ECM) and promote angiogenesis. This creates a microenvironment that promotes tumor progression and invasion. CSF-1 blockade inhibits tumor progression by influencing the macrophages that regulate angiogenesis and the ECM illustrated by decreased macrophage recruitment to the tumors associated with diminished VEGF-A and MMP expression and reduced angiogenic activity. siRNA, small-interfering RNA.

perform supportive roles that promote tumor progression and metastasis *(46)*. Together, these studies demonstrate that CSF-1 and CSF-1R can be used as therapeutic targets in the treatment of solid tumors **(Fig. 1)**.

The fact that five injections of siRNA at much lower doses than antisense ODNs required for a comparable effect can block the function of the target genes effectively favors the use of these nucleic acid-based constructs for large-scale human studies *(47)* whereby a more sustained therapeutic modality may be required to increase therapeutic efficacy. Moreover, although reduction of CSF-1 by phosphorothioate antisense ODN in human tumor xenografts has

the potential to suppress tumor growth *(40,42)*, absolute sequence specificity is not attainable using oligonucleotide with phosphorothioate linkages *(48)*, which again favors the use of siRNAs that induce sequence-specific gene silencing *(49)*. RNA interference is therefore a powerful tool to block gene function and influence solid tumor development. The continuing development of stable siRNA constructs optimized to target solid tumors by systemic application therefore hold great promise for the future in cancer therapy (*see* Chapters 9 and 10).

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12

Validation of Telomerase and Survivin as Anticancer Therapeutic Targets Using Ribozymes and Small-Interfering RNAs

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Summary

In recent years expanding knowledge about basic biology and a detailed understanding of the molecular pathways involved in tumor cell growth and progression have allowed the identification of numerous genes as potential therapeutic targets. Studies in which the expression of these genes was manipulated by antisense strategies have provided clues as to how we can intervene to specifically kill tumor cells or sensitize them to conventional chemical and physical antitumor therapies. Such tumor specificity can only be obtained by exploiting a basic difference between normal and malignant cells. In this context, targeting cytoprotective factors such as telomerase and survivin is particularly attractive because of their almost selective expression in tumor cells and their proven association with disease progression. This chapter summarizes the results obtained with ribozymes and small-interfering RNAs in the functional validation of these two targets in cell cultures and animal tumor models.

Key Words: Human cancer; ribozyme; survivin; siRNA; telomerase.

1. Introduction

1.1. Telomerase

During the past decade we have seen fundamental changes in the approaches used by researchers to identify and validate therapeutic targets *(1–3)*. Human telomeres are specialized DNA-protein structures that cap the ends of linear chromosomes and are essential for the maintenance of genome integrity. Specifically, telomeres prevent the ends of linear chromosomes from being recognized as double-strand breaks, protect chromosomes from end-to-end fusion and degradation, and contribute to the organization of chromosomes during cell

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division *(4)*. Telomeres consist of double-stranded DNA tandem repeats $(T_2AG_3)_n$ that terminate with a 3'-single-strand overhang, which folds back onto duplex telomeric DNA, generating a T-loop structure *(4)*. In addition to the repeated sequences, several DNA-binding proteins regulate telomere structure *(5)*. Specifically, the telomeric repeat-binding factor 1 (TRF1), which interacts with double-stranded DNA, plays an important role in the negative regulation of telomere length; the related protein TRF2 is essential for the protection of telomere integrity by maintaining the T-loop *(6)* and preventing the T-loop insertion site from being recognized as an intermediate for the homologous recombination *(7)*. In addition, TRF2 plays a pivotal role in preventing nonhomologous end joining as demonstrated by the occurrence of telomeric fusion in cells expressing a TRF2 dominant-negative mutant *(8)*. Several other proteins bind to telomeric DNA via TRF1 and TRF2: the polyadenosine diphosphate ribosylase tankyrase and the TRF1-interacting nuclear protein 2 (Tin2) contribute to regulate TRF1 functions, whereas Rap1 and the Mre11 complex, which are involved in the control of telomere length and in the cellular response to DNA-damaging agents, interact with TRF2 *(6,7)*. The human telomere-binding protein 1 (hPOT1), which binds single-stranded DNA, has been recently identified *(9)*. The exact role of this protein in telomere dynamics is far from being completely understood *(10)*. A possible role as a negative regulator of telomerase for hPOT1 via its interaction with the TRF1 complex has been proposed. Conversely, other authors suggest that the protein acts as a telomerase-dependent positive regulator of telomere length because its forced expression in telomerase-positive human cell line lengthened telomeres, whereas hPOT1 was unable to lengthen telomeres in a telomerase-negative cell line *(11)*.

Telomeres act as a mitotic clock by which cells count divisions *(12)*. During each round of cellular replication, telomeres undergo sequence loss as a consequence of the incomplete DNA lagging-strand replication (the "end replication problem") *(13)*, which results in critically short telomeres that lead to replicative senescence and ultimately cell death. To compensate for telomere attrition, different mechanisms have evolved to maintain telomere homeostasis, and they seem to play a pivotal role for the development of human malignancies.

Telomere maintenance is mainly performed in human cells by telomerase *(14)*. The main core of telomerase consists of two subunits: a catalytic subunit, the human telomerase reverse transcriptase (hTERT) *(15)*, and the human telomerase RNA component (hTR) *(16)*. The *hTR* gene is located on chromosome 3 and encodes for the telomerase RNA component, which consists of a 451-nt-long RNA. hTR bears a sequence located at its 5'-end, which is exploited as a template for the addition of telomeric repeats at the 3'-terminus of the linear chromosomes during the enzyme's catalytic cycle. The human telomerase RNA component is consistently expressed in almost all human tissues and, for this

reason, does not represent a limiting factor for telomerase activity but is essential for the enzyme's catalytic activity through its association with the catalytic subunit ***(14)***.

The catalytic subunit of human telomerase hTERT is a 127-kDa protein comprising a specific telomerase domain (T-motif) and shares structural and functional properties with reverse transcriptases. It is encoded by a 37-kb-long gene located on chromosome 5 and composed of 16 exons and 15 introns. The catalytic component of telomerase is typically expressed in telomerase-positive tumor tissues and in those normal cells that transiently acquire telomerase activity. Such evidence has suggested that hTERT is the limiting factor for the restoration of telomerase activity and its expression is strictly regulated at multiple levels ***(14)***. In fact, the *hTERT* gene undergoes transcriptional regulation that is mediated by different transcription factors. The hTERT pre-mRNA is posttranscriptionally modified by alternative splicing, a process which generates different hTERT transcripts with opposite functions. Specifically, the α-variant (which lacks conserved residues from the catalytic core of the protein) acts as a dominant negative ***(17)***. In addition, the activity of the hTERT protein is regulated by different posttranslational mechanisms such as the assembly of telomerase in a large complex holoenzyme mediated by Hsp90 and p23 ***(14)***. The hTERT protein undergoes cellular relocalization from the cytoplasm to the nucleus (a process presumably mediated by the 14-3-3 protein) and can be sequestered in a form of enzymatically inactive complex into the nucleolus through its interaction with PinX1 ***(18)***. The activity of hTERT is also regulated through phosphorylation/dephosphorylation of specific amino acid residues, catalyzed by protein kinases (e.g., PKC, Akt, and c-Abl tyrosine kinase) and protein phosphatase 2A ***(14)***.

The reactivation of telomerase is involved in the attainment of immortality in cancer cells and therefore may contribute to tumorigenesis and neoplastic progression ***(19)***. Several lines of evidence indicate that telomerase is present in 85–90% of human cancers ***(20)*** but is generally absent in somatic cells, with a few exceptions (i.e., germ line cells, embryonic stem cells, activated lymphocytes, endometrial tissue during the menstrual cycle, and cells from the basal layer of the epidermis). Because telomerase reactivation has been identified as one of the six hallmarks of cancer ***(21)*** due to its ability to provide cancer cells with an unlimited proliferative potential and owing to its specific expression in cancer tissues, approaches aimed to inhibit the enzyme's catalytic activity in tumor cells could represent promising and innovative anticancer therapies ***(22)***. Furthermore, recent evidence has suggested that telomerase reactivation contributes to tumorigenesis by means of mechanisms other than the enzyme's catalytic activity. Specifically, it has been proposed that the hTERT serves as a physical "cap" for the telomere that can shift from a capped to an uncapped

state *(23)*. The appropriate response to uncapped telomeres is action by telomerase, which protects the telomere from signaling into cell-cycle arrest or apoptosis pathways *(23)*. Additional novel functions of telomerase, which are distinct from its telomere-maintenance activity and might have potentially important consequences in tumor cells, are related to the ability of hTERT to cross-link telomere and enhance genomic stability and DNA repair *(24)*. Moreover, it has been suggested that hTERT is involved in the maintenance of cell survival and proliferation via enzymatic activity-independent intermolecular interactions involving p53 and poly(ADP-ribose) polymerase *(25)*. The evolving understanding of telomerase composition and functions and of its interaction with telomeres is expected to contribute to improve the knowledge of the tumorigenisis process and has prompted the formulation of distinct rationales for the development of enzyme inhibitors *(22)*. Telomerase inhibitors include antisense oligonucleotides that target hTR or hTERT mRNA *(22)*, traditional reverse transcriptase inhibitors, and agents able to promote and/or stabilize high-order DNA tetraplex (G-quartet) formation in telomeres *(26)*.

1.2. Survivin

Survivin is a member of the inhibitor of apoptosis (IAP) gene family, which is positioned at the interface between mitotic progression and apoptosis inibition *(27)*.

The human *survivin* gene spans 14.7 kb on the telomeric position of chromosome 17 and is localized to band q25 *(28)*. It comprises three introns and four exons, a TATA-less proximal promoter, and approx 200-nt GC-rich regions upstream of exon 1 *(29)*. The gene encodes a 16.5-kD protein of 142 amino acids. Structurally, it is composed of a single baculovirus IAP repeat domain and an extended COOH-terminal α-helical coiled-coil domain *(30)*. Moreover, it does not contain a RING-finger domain, found in other IAPs. Splicing variants of survivin have been identified. Survivin-2B is generated by insertion of an alternative exon, survivin-ΔEx3 arises from the removal of the exon 3, and survivin-3B results from the introduction of a novel exon 3B *(31,32)*. Very recently, an additional splice variant, survivin 2α, has been identified. Structurally, the transcript consists of two exons: exon 1 and exon 2, as well as a 3' 197-bp region of intron 2. Acquisition of a new in-frame stop codon within intron 2 results in an open reading frame of 225 nt, predicting a truncated 74-amino acid protein *(33)*. Little is known about the differential functions of survivin alternative splice forms. However, preliminary data would suggest that heterodimerization of survivin with survivin-ΔEx3 is essential for the inhibition of mitochondrial-dependent apoptosis *(34)*. Moreover, it has been demonstrated in exogenous expression assays that survivin 2α attenuates the antiapoptotic activity of survivin *(33)*.

Survivin is regulated in a highly cell cycle-dependent manner, with a marked increase in the G_2/M phase ***(35)***. During this phase, survivin associates with and is phosphorylated by $p34^{cdc2}$/cyclin B1 kinase ***(36)***. It has been demonstrated that survivin exists in two immunohistochemically distinct pools, with a nuclear pool localized to kinetochores of metaphase chromosomes and to the central spindle midzone at anaphase, and a cytosolic pool associated with interphase microtubules, centrosomes, spindle poles, and mitotic spindle microtubules at metaphase and anaphase ***(37)***. However, the microtubule-associated pool appears to be quantitatively predominant and functionally relevant. These findings, together with the phenotype of knockout mice (which is characterized by a catastrophic defect of microtubule assembly, with absence of mitotic spindle, formation of multinucleated cells and 100% embryonic lethality ***[38]***), are consistent with a critical role of survivin in mitosis to preserve the mitotic apparatus and to allow normal mitotic progression. In fact, it has been demonstrated that survivin downregulation causes pleiotropic cell-division defects ***(39,40)***. Moreover, Giodini et al. ***(41)*** showed that forced expression of survivin in HeLa epithelial carcinoma cells profoundly influenced microtubule dynamics and also caused stabilization of microtubules against nocodazole-induced depolymerization, thus indicating that survivin may facilitate evasion from checkpoint mechanisms of growth arrest and, consequently, promote resistance to drugs targeting the mitotic spindle. Additional evidence indicates that survivin also participates in the regulation of chromosome segregation ***(42)***, and that the protein cooperates together with the chromosomal passenger proteins INCENP and Aurora-B to perform its mitotic duties ***(43)***. The existence of a mitochondrial pool of survivin, which is able to orchestrate a novel pathway of apoptosis inhibition in tumor cells, has been recently reported ***(44)***. Specifically, it was found that, in response to cell death stimulation, mitochondrial survivin is rapidly discharged in the cytosol, where it prevents caspase activation and inhibits apoptosis.

It has been demonstrated that Hsp90, a molecular chaperone that is the central regulator of cellular stress response, associates with survivin. Such a physical interaction, which involves the Hsp90 ATPase domain and the survivin baculovirus IAP repeat domain, is required for survivin stability and function. In fact, targeted antibody-mediated disruption of the survivin-Hsp90 complex in cancer cells resulted in proteasomal degradation of survivin ***(45)***.

In regard to the precise role of survivin in cell death, at present it is still controversial whether this protein inhibits caspases through direct binding, as other IAPs do, or indirectly, requiring intermediate proteins. In this context, a possible direct interaction of survivin with caspase-9 has been reported by O'Connor et al. ***(46)*** whereas more recently Song et al. ***(47)*** suggested an alternative model for indirect inhibition of caspases by survivin, which requires Smac/Diablo as intermediate protein. This mitochondrial factor, that is released

into the cytosol in response to different apoptotic stimuli, was found to bind to some IAPs (including XIAP, $cIAP_1$, $cIAP_2$, and livin), thus preventing them from inhibiting caspases *(48)*. The ability of survivin to physically interact with Smac/Diablo and, as a consequence, sequester it would allow other IAPs to block caspases without being antagonized.

Survivin is strongly expressed in embryonic and fetal organs but not in differentiated normal tissues with the exception of thymus, basal colonic epithelium *(49)* endothelial cells, and neural stem cells during angiogenesis *(50)*. Several reports have demonstrated survivin expression in the majority of human tumor types including lung, breast, colon, gastric, oesophageal, pancreatic, liver, bladder, uterine, and ovarian cancers, large-cell non-Hodgkin's lymphomas, leukemias, neuroblastoma, brain tumors, pheochromocytoma, soft-tissue sarcomas, melanomas, and other skin cancers *(49)*. Moreover, the expression of survivin has been also detected in a variety of preneoplastic and/or benign lesions including polyps of the colon, breast adenomas, Bowen's disease, and hypertrophic actinic keratosis *(49)*, suggesting that reexpression of survivin may occur early during malignant transformation or following disturbance in the balance between cell proliferation and death. The upregulation of survivin at the transcriptional level in human tumors has been confirmed in genome-wide searches, which indicated survivin as the fourth top "transcriptome" in cancers of various histology *(51)*. At least for some tumor types, molecular abnormalities have been described that may account for the increased expression of survivin in cancer compared to normal tissue. Specifically, in neuroblastoma a gain of 17q25 containing the survivin locus represents a frequent genetic abnormality *(52)*. Moreover, in most ovarian cancers survivin exon 1, which is silenced by methylation in normal ovarian epithelium, becomes unmethylated and, consequently, transcriptionally active *(53)*. Survivin overexpression in tumors has been recently linked to the loss of wild-type p53 *(54)*. Specifically, it was seen that accumulation of wild-type p53 in human ovarian cancer cells induced survivin transcriptional repression, which did not require direct sequence-specific DNA binding of p53 to the survivin promoter. Modifications of chromatin structure within the promoter could be the molecular explanation for silencing of the survivin gene by wild-type p53.

In the majority of tumors investigated for survivin expression (including breast, lung, colorectal, gastric, liver, bladder, and kidney cancers, neuroblastoma, gliomas, soft tissue sarcomas, leukemias, and lymphomas), high levels of the protein were predictive of tumor progression, either in terms of disease-free survival or overall survival, thus providing prognostically relevant informations *(49)*. In several neoplasms, the association with tumor progression has been also corroborated in the context of comprehensive analysis of gene-expression profiling by DNA microarray or PCR-based assays.

Considering that apoptosis is the primary mode of cell death induced by several classes of anticancer agents and ionizing radiation, a possible general role of survivin in determining the chemo- and radio-sensitivity profiles of tumor cells has been hypothesised. Moreover, because survivin is associated with microtubules and with the mitotic spindle it is likely that this protein can specifically contribute to the response of cells to microtubule-interacting agents. Li et al. ***(55)*** first demonstrated that transfection of wild-type survivin efficiently protected murine NIH3T3 fibroblasts from apoptosis induced by the microtubule-stabilizing agent taxol. In agreement with this observation, Giodini et al. ***(41)*** reported that infection of HeLa cells with an adenoviral vector expressing survivin suppressed apoptosis induced by taxol. Based on this finding, our laboratory performed a parallel investigation on cell lines and clinical specimens from ovarian carcinomas to determine whether survivin is involved in regulating cell sensitivity to taxanes. The OAW42 and IGROV-1 human ovarian cancer cell lines were transfected with the human survivin cDNA. Stable transfection with survivin cDNA was able to protect these cells from the cytotoxic effects induced by taxol and taxotere ***(56)***. In the clinical setting, when we analyzed the response of advanced ovarian cancer patients to a taxol/platinum-based regimen as a function of survivin expression, we found a significantly higher clinical or pathologic response rate in cases with absent/low protein expression than in those expressing high levels of survivin ***(56)***.

Regarding the possible role of survivin in determining the radiation response of human tumor cells, Asanuma et al. ***(57)*** reported that survivin acts as a constitutive radio-resistance factor in pancreatic cancer cells. Specifically, in a panel of established cell lines they found an inverse relationship between survivin mRNA expression and in vitro sensitivity to X-irradiation. Moreover, these authors also demonstrated that survivin mRNA expression was increased by sublethal doses of X-irradiation, which would suggest that the protein also acts as an inducible radio-resistance factor.

Very recently, Zhang et al. ***(58)*** showed that survivin mediates resistance to antiandrogen therapy with flutamide in prostate cancer cells. Specifically, these authors suggested that upregulation of survivin via insulin-like growth factor-1/AKT signaling during androgen blockade may be one of the mechanisms by which prostate cancer cells develop resistance to antiandrogens.

Overall, the results obtained in the different studies indicate survivin to be a cellular factor potentially involved in the chemo- and radio-resistant phenotypes of human tumors cells and suggest that approaches designed to inhibit survivin expression may lead to human tumor sensitization to chemical and physical agents. In recent years, considerable efforts have been made by researchers to develop strategies for modulating apoptosis in cancer and other human diseases ***(59,60)***. In this context, approaches to counteract survivin in tumor cells have been proposed with the dual aim to inhibit tumor growth

through an increase of spontaneous apoptosis, and to enhance tumor cell response to apoptosis-inducing agents *(61)*. Different kinds of survivin molecular inhibitors, including antisense oligonucleotides, dominant negative mutants, and cyclin-dependent kinase inhibitors have been used.

2. Hammerhead Ribozymes

Ribozymes are small RNA molecules that possess specific endonucleolytic activity and catalyze the hydrolysis of phosphodiester bonds, thereby leading to cleavage of RNA targets *(62)*. Naturally occurring ribozymes mediate sequence-specific RNA processing through Watson-Crick base pairing. Several catalytic domains derived from natural ribozymes have been identified, the most common of which are the hammerhead and hairpin structures *(63)*. Owing to their inherent simplicity and relatively small size, hammerhead ribozymes have received much attention in view of their potential therapeutic usefulness and ability to be incorporated into a variety of flanking sequence motifs without changing site-specific cleavage capacities. In fact, the hammerhead ribozyme consists of a 40-nt-long, highly conserved catalytic core, which cleaves substrate RNA at 5'-NHH-3' consensus sequence, where N is any nucleotide and H is any nucleotide but guanidine *(64)*. The catalytic potential of such a ribozyme can be exploited to cleave any NHH consensus sequence in a given RNA substrate by the addition of flanking arms bearing nucleotide sequences, which are complementary to the specific RNA target. Moreover, after the cleavage reaction, the substrate becomes more accessible to ribonucleases, which leads to its degradation. In addition to the catalytic activity, ribozymes possess binding capacity to the target RNA ("antisense effect") and can induce the RNase-mediated degradation of the target as a consequence of double stranded RNA formation. Because one of the major limitations to the therapeutic use of hammerhead ribozymes is the problem of their intracellular delivery different strategies have been developed. There are two main ways to deliver a ribozyme within cells. The exogenous delivery, which exploited presynthesized ribozymes that are introduced directly into the cells with the aid of conventional transfection agents, and the endogenous delivery by which the intracellular transcription of a ribozyme coding sequence is accomplished through transfection/infection of ribozyme-expressing vectors (e.g., plasmid or viruses). Using both approaches, several studies focused on experimental human tumor models have shown the possibility to obtain efficient ribozyme-mediated downregulation of cancer-associated genes *(64)*.

2.1. Ribozymes Targeting Telomerase

Telomerase is an exploitable target for strategies based on the use of antisense oligonucleotides. In fact, the template region of hTR, which naturally

binds to the 3' single-strand overhang of the telomere end to add new telomeric repeats, is inherently accessible to incoming nucleic acids and represents a suitable target site for these approaches. A number of studies on experimental human tumor models have shown the possibility to obtain efficient inhibition of telomerase through the use of hammerhead ribozymes targeting hTR. The first developed hammerhead ribozyme was engineered to target a consensus sequence located at the end of the telomerase template ***(65)***. When added to cell extracts from two hepatocellular carcinoma cell lines (HepG2 and Huh-7), the ribozyme induced dose-dependent inhibition of telomerase activity. The potential use of chemically stabilized hammerhead ribozymes to inhibit telomerase activity by cleaving the hTR component was also pursued. Specifically, hammerhead ribozymes containing 2'-*O*-methyl ribonucleotides for enhanced biologic stability and designed to be complementary to the RNA component of human telomerase exhibited dose-dependent inhibition of telomerase activity in human glioma cell lysates and induced the cleavage of the full-length hTR in intact U87-MG cells ***(66)***.

The catalytic sequence described by Kanazawa et al. ***(64)*** was exploited in our laboratory to downregulate telomerase activity in intact human tumor cells. Specifically, the JR8 human melanoma cells were transfected with the ribozyme sequence inserted into a mammalian expression vector ***(67)***. Ribozyme transfectants successfully expressing the ribozyme and characterized by reduced telomerase activity and a decreased level of telomerase RNA expression compared with mock transfectants were selected. Ribozyme-expressing clones grew more slowly than parental cells and also expressed an altered morphology with a dendritic appearance in monolayer cultures. A small but significant fraction of the cell population also expressed an apoptotic phenotype. However, no telomere shortening was observed in these clones even after a prolonged period (50 d) of growth in culture ***(67)***.

In a further study, three hammerhead ribozymes targeting GUC sequences from the 5'-end of telomerase RNA were described ***(68)***. In a cell-free system, all the ribozymes efficiently cleaved the RNA substrate. However, when the ribozymes were introduced into intact endometrial carcinoma Ishikawa cells, only the ribozyme targeting the template region was able to attenuate telomerase activity. The ribozyme sequence was then inserted into an expression vector subsequently used to transfect the endometrial carcinoma cell line AN3CA. Ribozyme-expressing clones obtained after in vitro selection showed reduced telomerase activity and telomerase RNA expression. A marked reduction of telomere length was observed in some of these clones. However, even after 30 passages in vitro, these cells still maintained their ability to proliferate. To search for more potent ribozymes targeting telomerase, the same group recently reported the use of a divalent ribozyme (referred to as 36- to 59-divalent

ribozyme) designed to cleave simultaneously the GUC triplet (which represents the most exploited target site in the template region of hTR) and the closest target sequence GUA, located 23 nt downstream from the GUC in the template region of hTR *(69)*. Data obtained by in vitro cleavage assay showed that the 36- to 59-divalent ribozyme cleaved telomerase RNA more efficiently than the related monovalent ribozymes (36- and 59-ribozyme). However, when the divalent ribozyme was introduced into Ishikawa endometrial carcinoma cells, its inhibitory effect on telomerase activity was less than that of the 36-ribozyme, whereas the 59-ribozyme did not show a significant activity on telomerase.

Recently, hammerhead ribozymes were designed against seven NHH sequences located in open loops of the hTR secondary structure and introduced through an expression vector into human breast tumor MCF-7 cells. Results showed that stable transfectants of ribozyme R1 targeting the template region of hTR induced the degradation of target and attenuated telomerase activity in breast cancer cells. Moreover, the ribozyme R1 transfectant displayed a significant telomere shortening and a lower proliferation rate than parental cells. Clones with reduced proliferation capacity showed enlarged senescence-like shapes and the occurrence of apoptotic cells was observed *(70)*.

Because the expression of hTERT is almost completely confined to tumor cells and its presence represents the rate-limiting step for telomerase activity, the antisense-mediated attenuation of hTERT mRNA expression would represent an excellent means to regulate the enzyme's activity in cancer cells *(14)*. However, hTERT is a more challenging target than hTR for antisense-based strategies. In fact, its mRNA possesses a complex secondary structure that makes it difficult to accurately predict which target site will be most accessible for hybridization. As a consequence, there are still few studies based on the use of antisense-mediated approaches to achieve telomerase inhibition through hTERT downregulation.

However, the possibility to downregulate telomerase activity by the use of hammerhead ribozymes that target the mRNA of hTERT has been exploited. Specifically, seven presynthesized ribozymes, directed against 5'-NHH-3' consensus sequences within the hTERT mRNA, were delivered into endometrial carcinoma cells by cationic lipids and demonstrated to significantly inhibit telomerase activity in intact cells *(71)*. However, a stable transfection of endometrial carcinoma cells carried out by cloning the ribozyme sequences into expression vectors confirmed the ability of only one ribozyme to suppress telomerase activity. In another study, Ludwig et al. *(72)* developed a hammerhead ribozyme directed against the consensus sequence within the T-motif of the hTERT mRNA that was able to attenuate telomerase activity in stable transfected clones of the immortal, telomerase-positive human breast epithelial cell line HBL-100 and the breast cancer cell line MCF-7. After a significant lag phase,

in ribozyme-expressing clones the decline of the enzyme's catalytic activity resulted in telomere shortening, inhibition of cell proliferation, and induction of apoptosis. In addition, such clones demonstrated an increased susceptibility to topoisomerase II inhibitors such as doxorubicin, etoposide, and mitoxantrone. Successively, the same ribozyme sequence was transduced through an adenoviral vector into four ovarian cancer cell lines with widely different telomere lengths *(73)*. The authors observed massive cell loss in mass cultures from all cell lines tested 3 d after transduction.

2.2. Ribozymes Targeting Survivin

We first demonstrated the possibility to efficiently inhibit survivin expression through the use of ribozymes. Specifically, we designed two hammerhead ribozymes targeting the 3'-end of the CUA_{110} (RZ7) and the GUC_{294} (RZ1) triplets in the survivin mRNA and transfected them into the JR8 human melanoma cell line over expressing survivin. Stably transfected clones proven to endogenously express the active ribozyme RZ1 or RZ7 were characterized by a markedly lower survivin protein level than JR8 parental cells, whereas a negligible reduction of survivin expression was observed in cells expressing a mutant ribozyme (which was produced by introducing a mutation in the catalytic core of the active ribozyme RZ1). These cells demonstrated an increased caspase-9-dependent apoptotic response to cisplatin treatment *(74)*. JR8 cells expressing RZ1 also showed a significantly increased sensitivity to the topoisomerase-I inhibitor topotecan (as detected by clonogenic cell survival) as a consequence of an enhanced rate of drug-induced caspase-9-dependent apoptosis. Moreover, an increased antitumor activity of oral topotecan was observed in ribozyme-expressing JR8 cells grown as xenograft tumors in athymic nude mice *(75)*. JR8 cells endogenously expressing the active RZ7 ribozyme also showed significantly increased sensitivity to γ-irradiation *(76)*. More recently, we constructed a Moloney-based retroviral vector expressing the RZ7 ribozyme, encoded as a chimeric RNA within adenoviral VA1 RNA. Polyclonal cell populations, obtained by infection with the retroviral vector, of two androgen-independent human prostate cancer cell lines (DU145 and PC-3) were characterized by a significant reduction of survivin expression; the cells became polyploid, underwent caspase-9-dependent apoptosis, and showed an altered pattern of gene expression, as detected by oligonucleotide array analysis. Survivin inhibition also increased the susceptibility of these cells to cisplatin-induced apoptosis and prevented tumor formation when cells were xenografted into athymic nude mice *(77)*.

Choi et al. *(78)* recently showed that two hammerhead ribozymes, able to cleave the human survivin mRNA at nucleotide position +279 and +28 and cloned into a replication-deficient adenoviral vector, were able to increase the apoptotic response to etoposide in transduced MCF-7 breast cancer cells.

3. RNA Interference

RNA interference (RNAi) is a natural mechanism of sequence-specific, post-transcriptional gene silencing. RNAi may play an important role in protecting the genome against instability caused by transposons and repetitive sequences, and it represents an evolutionary conserved antiviral defense pathway in animals and plants *(79)*. Moreover, RNAi has emerged as a powerful mechanism for sequence-specific modulation of gene expression and seems to provide a higher potency than conventional antisense strategies, presumably because it relies on natural site-directed cleavage machinery *(79)*.

In mammalian cells RNAi can be triggered by several double-stranded RNAs (dsRNA) or dsRNA domain-containing molecules. Endogenously expressed dsRNA domains are converted into the nucleus by specific ribonucleases in the form of precursors that are successively processed in the cytoplasm to give rise to microRNAs (miRNAs). The miRNAs are believed to bind to sites that have partial sequence complementarity in the 3' untranslated region of their target mRNA, causing repression of translation and inhibition of protein synthesis *(80,81)*. By contrast, exogenously introduced dsRNA (e.g., viral genome) are processed in the cytoplasm by the endoribonuclease dicer into small duplex RNAs, the 21- to 23-nt-long terminal effectors molecule, known as small-interfering RNAs (siRNA), characterized by a 2- to 3-nt overhang at the 3'-terminus *(80,81)*. The resulting siRNAs are then assembled to form an RNA/protein complex, referred to as the RNA-induced silencing complex (RISC). The double-stranded siRNA is then unwound, leaving the antisense strand to guide RISC to its homologous target mRNA for endonucleolytic cleavage. The target mRNA is cleaved in the center of the duplex region arising from the annealing of the antisense strand of siRNA and the target mRNA, a process that ultimately results in the target degradation. To date, different effectors molecules have been identified as inductors of gene expression silencing in mammalian cells through the activation of the RNAi pathway. Specifically, short-hairpin RNAs (shRNAs) are transcribed from plasmid- or viral-based vectors as a pre-siRNA in which the sense and antisense strands are linked by a short spacer. The pre-siRNA is then predicted to form a 19-nt-long stem-loop structure, the terminal effectors shRNA. shRNAs are usually coded downstream of an RNA polymerase III promoter (e.g., U6 small nucleolar RNA or human RNase H1 promoters) although the use of inducible or tissue-specific RNA polymerase II promoters has been described *(80,81)*. By contrast, presynthesized siRNA are RNA duplexes formed by two complementary single strands. In this case, a siRNA may be obtained by the annealing of two in vitro transcribed 21-nt-long single strands leaving two nucleotides unpaired at the 3' terminus *(80,81)* or as preformed duplexes, usually provided by specialized companies. Presynthesized siRNAs are delivered to cells by conventional transfection approaches *(80,81)*. To date, the siRNA technology has been validated

in several mammalian experimental models but its therapeutic usefulness for human diseases is still under intensive investigation. In fact, although the effect of siRNAs on gene expression has been demonstrated to be rapid and persistent (days/weeks), the main limitation to be overcome before such technology will be exploited in the clinical practice is represented by the selectivity for the target RNA, delivery and stability inside cells, similarly to ribozyme- and antisense-based approaches.

3.1. Telomerase Silencing Through RNAi

Kosciolek et al. ***(82)*** first described the exploitation of siRNAs as telomerase inhibitors. Two types of RNA molecules targeted to the hTR and hTERT components of human telomerase were developed: chemically synthesized siRNAs and a long dsRNA expressed in the target cells as a hairpin construct. The ability of chemically synthesized siRNAs to inhibit telomerase activity was assessed in a panel of human cancer cell lines. Results showed that the siRNA targeting the hTR component was more effective in inducing inhibition of the enzyme's catalytic activity than that designed to target the catalytic component hTERT. The antitelomerase effect was concentration-dependent and relied on the transfection schedule. Furthermore, transfectant clones expressing the siRNA construct directed against the hTR subunit were characterized by a marked inhibition of the enzyme's catalytic activity, the downregulation of the hTR RNA expression levels and a reduction of telomeric DNA content. Unfortunately**,** the authors did not attempt to analyze the effects of specific telomerase inhibition on tumor cell proliferative potential.

It has been recently reported that a shRNA, expressed from a lentiviral vector and targeting the sequence encompassing the 11-nt template region of hTR, quickly inhibited the growth of p53-wild type, p53-null HCT116 colon cancer cells and LOX melanoma cells, independently of p53 status or telomere length, and without bulk telomere shortening. By contrast, no effect was detected in the immortalized, telomerase-negative VA13 cell line. Moreover, hTR downregulation did not cause telomere uncapping in these experimental models, but induced a modulation of the global gene expression profile, including suppression of specific genes implicated in angiogenesis and metastasis. This finding could be indicative of a novel response pathway distinct from the expression profile changes previously reported by the same authors and induced by telomere-uncapping mutant-template telomerase RNAs ***(83)***.

3.2. Survivin Silencing Through RNAi

Carvalho et al. ***(43)*** first used RNAi to specifically repress survivin in HeLa cells. These authors showed that survivin was no longer detectable in cultures a few days after transfection with specific siRNA and that survivin-depleted

cells were delayed in mitosis and accumulated in prometaphase with misaligned chromosomes. In this model, loss of survivin activated the mitotic checkpoint, which was mediated by induction of p53 and was associated with the increase of its downstream target, $p21^{Waf1}$. Survivin ablation also caused loss of mitochondrial membrane potential, enhanced caspase-9 proteolytic activation and spontaneous apoptosis *(43)*. More recently, survivin downregulation, accomplished through the use of siRNAs, was seen to reduce clonogenic potential and increase the percentage of multinucleated cells in a panel of human sarcoma cell lines independently of p53 gene status *(84)*. Moreover, survivin knockdown caused radio-sensitization, which was paralleled by an increased activity of caspase-3 and caspase-7, in wild-type-p53 but not in mutant-p53 sarcoma cells *(85)*. An enhanced apoptotic response to APO2L/TRAIL treatment was also observed in melanoma and renal carcinoma cell lines transfected with survivin-specific siRNAs *(86)*. Finally, Coma et al. *(87)* recently demonstrated that transfection of endothelial cells with survivin specific siRNAs induced a marked increase in the apoptotic rate, a dose-dependent inhibition of their migration on vitronectin and a decrease in capillary formation.

To prevent nonphysiological responses associated with persistent suppression of a gene that is essential for cell survival and cell cycle progression such as survivin, systems allowing an inducible regulation of RNAi have been developed. Coumoul et al. *(88)* recently demonstrated that inducible suppression of survivin was efficiently achieved in ES cells by regulating RNAi using a *Cre-Lox*P approach, as indicated by the decrease level of gene expression and reduced proliferative potential.

4. Comparison of Technologies

The new wave of interest in the antisense field arises from the discovery that dsRNAs can induce a potent targeted degradation of complementary RNA sequences, a process referred to RNAi, and that the effectors components of the RNAi pathway (siRNAs or shRNAs) can be chemically synthesized or expressed from plasmid/viral vectors, similarly to ribozymes *(79)*. There is a widely diffused opinion that RNAi provides a powerful tool for targeted inhibition of gene expression, with respect to conventional antisense strategies (i.e., ribozymes), presumably because it relies on a natural process. Despite the unique assumed potential of RNAi, limitations in the use of such an approach, such as the possibility that some mammalian cells may not be susceptible to RNAi, have been described. However, it is possible to identify many factors that most likely influence the biological efficacy of ribozymes and siRNAs *(89)*. The use of synthetic or expressed ribozymes/siRNAs to induce gene knockdown in mammalian cells requires the consideration of several common issues. To provide effectors molecules with both selectivity and specificity for the target sequence, nontarget

sequence homologies of ribozyme or both strands of a siRNA must be avoided. Moreover, thermodynamic parameters that can influence the catalytic cycle of a ribozyme and the efficiency with which siRNA is unwound and assembled into RISC complex need to be carefully determined. In addition, sequence constraints allowing the expression of ribozymes and shRNAs, from plasmid or viral vectors, that could influence the efficiency with which they are folded into an effective molecule should be taken into account ***(90)***. Finally, the presence of a suitable target site in a given RNA represents a major determinant for the biological activity of both molecules. A significant obstruction of gene knockdown mediated by ribozymes and siRNA arises from structural features of the substrate RNA. In fact, a direct correlation between the extent of gene downregulation and the local free energy in the RNA target regions has been described ***(91)***. To this purpose, the selection of accessible target sites can be made by a systematic testing of a large number of oligomers (i.e., RNase H mapping) or, alternatively, by means of specific bioinformatics tools based on thermodynamic algorithms ***(79)***. As a consequence, a systemic structural analysis of local RNA target regions can significantly improve the design of biologically active molecules ***(92)***.

Cellular uptake and colocalization to the specific target site within cells represent another main hurdle that has to be overcome for an efficient therapeutic inhibition of gene expression and the exploitation of ribozymes and siRNAs in clinical trials. To date, there are still no means to improve colocalization to the target site and to increase the efficacy of siRNAs in the presence of hardly accessible target RNA. As a consequence, the siRNA delivery is usually achieved by employing conventional methods such as cationic-lipid formulations, electroporation, or conjugation to peptides ***(80,81,90)***. In contrast, a number of specific strategies have been demonstrated to be effective in inducing a sequence-directed colocalization of ribozymes and to improve their efficacy at the target site ***(93)***.

Data reported in the literature have demonstrated that RNAi is a process that occurs in the cytoplasm. In contrast, ribozymes can act not only against cytoplasmic mRNAs but can also be exploited to target sequences localized in the nucleus (i.e., introns). This characteristic has been applied for the RNA repair, a process based on a transsplicing version of group I ribozymes, which demonstrated to be effective in inducing the repair of mutant transcripts such as sickle β-globin, p53, and mutant RNAs associated to myotonic muscular dystrophy ***(89,94)***.

The introduction of chemical modifications aimed to improve the half-life of chemically synthesized ribozymes and siRNAs makes these molecules useful for short-term experiments. In particular, modifications made at the 2' position of specific ribose residues or the introduction of inverted thymidine at the 3'-terminus can stabilize ribozymes without affecting their biological activities.

Recently, the use of phosphorothioate backbone for siRNA has shown to increase their toxicity and to reduce the silencing activity. As a consequence, alternative backbone and nucleotide modifications have been pursued, such as the introduction of boranophosphate or the incorporation of few locked nucleic acid modifications. Alternatively, the stability and delivery of siRNAs have also been improved by complexing them with polyethyleneimmine or cholesterol. Other chemical modifications, aimed to improve the stability of siRNA and to reduce the ability to induce non specific effects without affecting their biological functions, have been developed by different companies, such as the Stealth siRNAs (Invitrogen, San Giuliano Milanese, Italy) or siSATBLE siRNAs, recently developed by researchers from Dharmacon Inc. (Lafayette, CO) *(22)*.

Ribozymes as well as siRNAs can lead to nonsequence-specific effects (off-target effects) that are strongly dependent on the concentration of oligomers. However, it should be stressed that the double-stranded siRNAs may result in two single-stranded oligomers, which yield more pronounced off-target effects than those obtained with an equal molar amount of ribozymes *(89)*. Furthermore, it has been reported that ribozymes are much more sensitive to polymorphisms at the cleavage site level *(89)*. This phenomenon can contribute to reduce the possibility of ribozyme-mediated off-target effects with respect to siRNAs. Although in a large number of studies reported thus far siRNAs have been shown to be effective in a broad range of experimental models, they can be potent inducers of stress-response pathways. Such a phenomenon that could depend on the different cellular environments would compromise the efficacy of the siRNA-mediated gene silencing. In fact, it has been demonstrated that antiluciferase siRNAs were highly effective at inhibiting luciferase activity when transfected into HeLa cells, but the presence of siRNA in PC-3 prostate cancer cells led to strong nonspecific effects, as shown by the significant down-regulation of luciferase expression in the presence of a scramble siRNA *(95)*. Moreover, transfection of siRNAs can result in a global upregulation of interferon-stimulated genes as well as the activation of genes independent of an interferon response, such as the interferon-regulatory factor 3 *(96,97)*. In this regard, mammalian cells display a number of nonsequence-specific responses triggered by dsRNAs, such as those involved in viral host defense *(98)*. The effectors of such a pathway are mainly represented by the dsRNA-dependent protein-kinase PKR and 2'-5' oligoadenilate synthetase, whose activation is dependent on the size and concentration of dsRNA. Although siRNAs were initially thought to be too short to induce dsRNA-initiated response, it is becoming evident that non-specific effects are consistent for a wide range of chemically synthesized siRNA *(96)*. Moreover, it is possible that dsRNA-response pathways are not only activated by siRNA, but may also mediate the specific gene-silencing effects of RNAi, that ultimately result in nonspecific degradation of cellular

RNAs and general repression of protein synthesis *(96,98)*. Overall, siRNAs seem to induce complex signaling responses in target cells beyond the selective silencing of specific genes *(96)*. The relative efficacy and specificity of a given siRNA as well as the use of stringent controls need to be carefully established for each individual experimental model.

In conclusion, the lack of studies aimed to comparatively evaluate the efficacy of ribozymes and siRNAs in inhibiting the expression of the same genes on the same experimental systems makes it difficult to predict which is the better approach to be exploited for therapeutic purposes. It should also be stressed that hybrid RNAs, carrying ribozyme and siRNA sequences, could provide a much more powerful tool to achieve gene expression knockout with respect to ribozyme and siRNAs alone, as recently proposed *(22)*.

5. Concluding Remarks

As reported in this review, ribozyme- and siRNA-based approaches have been demonstrated to efficiently inhibit telomerase activity by targeting the RNA component hTR or the reverse transcriptase catalytic subunit hTERT. It is now well established that hTR represents a suitable target to achieve the inhibition of telomerase activity because of its natural accessibility to binding by different antisense oligonucleotides. Despite recent evidences reported by Li et al. *(83)*, a remarkable number of studies have emphasized that targeting hTR results in cancer cell growth arrest and reduced viability only after several population doublings as a consequence of interference with the telomere lengthening activity of the enzyme. Such evidence is in keeping with the classical mechanism by which telomerase inhibition would induce a delayed cell growth arrest and apoptosis as a result of critically shortened telomeres *(22)*. Such a cellular response has been proved to be largely dependent on the initial telomere length in a given tumor cell population. As a consequence, single-agent therapies based on inhibitors targeted to hTR would need long-term treatment to induce effective impairment of cancer cell growth with relatively long telomeres, thereby allowing a significant progression of the neoplastic disease *(99)*.

Conversely, recent studies have shown that treatment with telomerase inhibitors targeting hTERT was able to induce programmed cell death within a few days in different telomerase-positive tumor cell models *(22)*. Such results cannot be explained by the classical model, which predicts that long-term exposure of tumor cells to telomerase inhibitors should induce telomere shortening after a certain number of rounds of cells division and growth arrest. In fact, it is unlikely that cell death was related to telomere attrition because the cells would not have undergone enough divisions to significantly shorten their telomeres. Interference with telomerase activity might therefore affect aspects of the control of cell proliferation and apoptosis other than telomere length. Such results

would suggest that abrogation of telomerase activity may affect cell proliferation also through pathways that are not dependent on telomere erosion. In fact, it has been reported that antitelomerase approaches based on the modulation of hTERT expression could lead to an early and pronounced biological response. Such evidence gains support from recent data demonstrating that the downregulation of hTERT expression levels quickly induced programmed cell death in human breast cancer cells and that such an apoptotic response could be counteracted by the expression of an hTERT mutant lacking telomerase activity *(25)*. This finding has conferred to hTERT a putative prosurvival and antiapoptotic function, which could be independent of the specific enzymatic activity of telomerase. Such a cytoprotective function of hTERT has been confirmed by us in a recent study demonstrating that oligonucleotide-mediated inhibition of hTERT, but not of hTR, induced rapid cell growth decline and programmed cell death in the absence of telomere shortening in human prostate cancer cells *(100)*.

It should be also taken into account that prolonged treatments with telomerase inhibitors could lead to the occurrence of specific mechanisms of resistance, such as the emergence of the alternative lengthening of telomeres (ALT) phenotype *(101)*. In fact, it has been reported that some eukaryotic organisms make use of telomerase-independent pathways to maintain their telomeres and that the ALT mechanisms may often occur when telomerase is repressed. The ALT phenotype is present in a small fraction (about 10%) of tumor tissues mainly of mesenchymal origin that are telomerase negative *(101,102)*. Moreover, it has been recently demonstrated that ALT- and telomerase-based pathways may coexist in the same tumor cells *(102)*. Such evidence would suggest that even in cells that stabilize their telomeres through telomerase, a marked and prolonged inhibition of the enzyme's catalytic activity could be responsible for the selection of mutant clones that are resistant to antitelomerase agents via reactivation of the ALT pathway and emphasize the notion that all tumor cells require a solution to the "end replication problem."

Even though the efficacy of telomerase inhibitors needs to be further validated in vivo tumor models before entering clinical practice, the availability of effective antisense-based telomerase inhibitors will give further insight about the role(s) of telomerase (beyond the classical mechanism of telomere lengthening) in the tumorigenesis process. It will also provide a better rationale for developing new anticancer therapies based on the use of antitelomerase inhibitors, also in the context of combined treatments.

Through the contribution of several groups the survivin pathway has emerged as a complex and essential cellular infrastructure controlling spindle microtubule function, chromosome segregation and also the initiation of mitochondrial-dependent apoptosis. Results obtained by different experimental studies aimed at targeting survivin by means of different molecular approaches clearly

demonstrated that downregulation of this protein results in anticancer activity potentially suitable for clinical testing.

As far as the actual cellular targets of survivin antagonists are concerned, it appears that inhibition of survivin results in apoptosis of the proliferating tumor cell compartment. However, there is evidence that survivin may provide a broader cytoprotective role for the tumor microenvironment as a whole. This hypothesis is supported by the observation that survivin becomes strongly expressed in endothelial cells during the proliferating as well as the remodelling phases of angiogenesis and mediates the apoptosis resistance of these cells ***(103)***.

The evidence that survivin plays a crucial role also in tumor angiogenesis would suggest that molecular targeting of survivin may provide a dual advantage for anticancer strategies in vivo as a consequence of the ability to increase the overall tumor response to treatment not only through direct interference with the apoptotic pathways in cancer cells but also by favoring the apoptotic involution of newly formed tumor vasculature ***(104)***. Clinical testing of survivin antisense oligonucleotides is currently underway.

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Collagen-Induced Arthritis in Mice

A Major Role for Tumor Necrosis Factor-α

Richard O. Williams

Summary

Collagen-induced arthritis is the most widely used animal model for the evaluation of novel therapeutic strategies for rheumatoid arthritis. The disease is induced by immunization of genetically susceptible strains of mice or rats with type II collagen in adjuvant. Susceptibility to collagen-induced arthritis is associated with major histocompatibility complex (MHC) class II genes, although non-MHC genes also play a role. Both B- and T-lymphocytes are important in the pathogenesis of collagen-induced arthritis, with the peak of the T-cell response occurring around the time of disease onset. Histopathological assessment of the joints of animals with collagen-induced arthritis reveal a proliferative synovitis with infiltration of polymorphonuclear and mononuclear cells, the formation of an erosive pannus, cartilage degradation, and fibrosis. As in human rheumatoid arthritis, a number of both pro- and anti-inflammatory cytokines are expressed in the joints of mice with collagen-induced arthritis, including tumor necrosis factor-α (TNFα) and interleukin (IL)-1β, IL-6, IL-1Ra, IL-10, and transforming growth factor β. The use transgenic and knockout strains of mice, as well as biological inhibitors, have revealed important pathological roles for multiple cytokines. Of these, TNFα emerged as a valid therapeutic target for rheumatoid arthritis and this led to the setting up of clinical trials of anti-TNFα antibody therapy. Three anti-TNFα biologics (infliximab, etanercept, and adalimumab) are now approved for use and TNFα blockade therefore represents an important advance in our ability to treat rheumatoid arthritis.

Key Words: Rheumatoid arthritis; TNFα; autoimmunity; experimental animal models; collagen-induced arthritis; type II collagen.

1. Introduction

1.1. Rheumatoid Arthritis

Rheumatoid arthritis (RA) is an inflammatory disease of unknown etiology with a world-wide prevalence of approx 1%. The disease is often progressive

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and results in pain, stiffness, and swelling of joints. In late stages, deformity and ankylosis develop. RA is one of the most common causes of disability in the Western world and has a female to male ratio of around 3 to 1. The age of onset of RA is typically between 25 and 50, although it can strike at any age. The chief pathological features of the disease include the formation of an inflammatory erosive synovitis that ultimately leads to destruction of cartilage, bone, and soft tissues resulting in loss of joint function. Although joints are the main target of the disease process in RA, the disease is usually classified as a nonorgan specific autoimmune disease, because of the occurence of extraarticular features, such as subcutaneous nodules, vasculitis, and pulmonary fibrosis, especially in the more severe cases.

There has been considerable progress recently in characterizing the mediators that contribute to the pathogenesis of RA and a number of studies have pointed to a pivotal role for tumor necrosis factor (TNF)α. Indeed, the successful introduction of infliximab, a chimeric anti-TNFα monoclonal antibody (mAb) *(1–3)*, etanercept, a soluble p75 TNF receptor-Fc fusion protein *(4,5)*, and adalimumab, a monoclonal human antibody produced by phage display *(6–8)* into the clinic confirms the importance of TNFα in RA. However, the underlying cause of RA is still unknown and there remains an urgent need for more effective and durable remedies with low toxicity profiles. It is for this reason that animal models of arthritis are being studied.

1.2. Collagen-Induced Arthritis

Animal models of arthritis have been used in many different kinds of experiments, including the evaluation of novel therapies, the identification of proinflammatory cytokines, the identification of genes associated with disease susceptibility, and in the identification of markers of disease progression *(9)*. Collagen-induced arthritis (CIA) is probably the most widely used model for studies of therapeutic intervention although data arising from such studies should be interpreted with caution because much depends on the timing of treatment (i.e., before or after onset of arthritis). For example, a number of T-cell-targeted therapies (e.g., anti-CD4, anti-interleukin [IL]-12, CTLA4-Ig) have been shown to be effective when given at the time of immunization but ineffective when given in established disease *(10–13)*.

CIA exhibits many pathological similarities to RA *(14)*, including similar patterns of synovitis, pannus formation, erosion of cartilage and bone, fibrosis, and loss of joint function *(15)*. In addition, susceptibility to both human RA and murine CIA is associated with genes encoding major histocompatibility complex (MHC) class II molecules, implying the involvement of $CD4^+$ T cells in the pathogenesis of both forms of arthritis. Thus, susceptibility to CIA is restricted to mouse strains bearing MHC types $I\text{-}A^q$ and $I\text{-}A^r$, the

mouse homologs of human leukocyte antigen (HLA)-DQ, whereas in human RA certain subtypes of HLA-DR4 and -DR1 are associated with disease susceptibility. Another similarity is that humoral responses are thought to play a significant role in the pathogenesis of both CIA and RA *(14)* although there is a lack of convincing data pointing to a role for type II collagen-specific autoantibodies in the majority of RA patients as elevated levels of anticollagen antibody are detected only in 10–15% of patients *(16)*. Another extremely important feature of CIA that makes it a valid model for RA is the expression of proinflammatory cytokines, including TNFα, and IL-1β, in the joints of mice with arthritis *(17)*.

CIA induced by immunization of DBA/1 mice with chicken, rat, or bovine type II collagen usually leads to a relatively acute and self-remitting form of arthritis in which arachidonic acid metabolites, such as prostaglandin E_2, play an important pathological role in the development of arthritis. This was shown in a study in which cytosolic phospholipase A2α (cPLA2α) knockout mice were backcrossed onto the arthritis susceptible DBA/1 background. cPLA2α is responsible for releasing arachidonic acid from cell membranes, which is the first step in the production of prostaglandins and leukotrienes. The development of arthritis was profoundly inhibited in cPLA2α knockout mice compared with wild-type littermates, despite the fact that levels of type II collagen-specific antibodies were comparable in the two groups *(18)*. However, immunization of DBA/1 mice with mouse collagen results in a more chronic form of arthritis *(19–21)*, which is resistant to nonsteroidal anti-inflammatory drugs, such as indomethacin *(21)*. However, the drawback to the use of autologous type II collagen is that it is less arthritogenic than heterologous collagen and therefore produces a less reproducible form of arthritis. The relative lack of arthritogenicity of mouse collagen has been attributed to the low affinity of specific epitope of murine collagen (CII256-270) for I-A^q, which results in a low level of CII-specific T-cell activation *(22)*.

1.3. The Role of Proinflammatory Cytokines in Arthritis

1.3.1. Evidence From Transgenic Strains of Mice

Mice expressing a human TNFα transgene in which the 3' AU-rich region has been replaced by the 3' untranslated region of the human β-globin gene, resulting in increased mRNA stability and increased TNFα expression (**Fig. 1**), were shown to develop arthritis spontaneously *(23)*. The arthritis could be prevented by continuous administration of neutralizing antihuman TNFα mAb.

Histopathological studies of the joints of hTNFα transgenic mice revealed that the arthritis was highly erosive in nature, with subchondral bone being

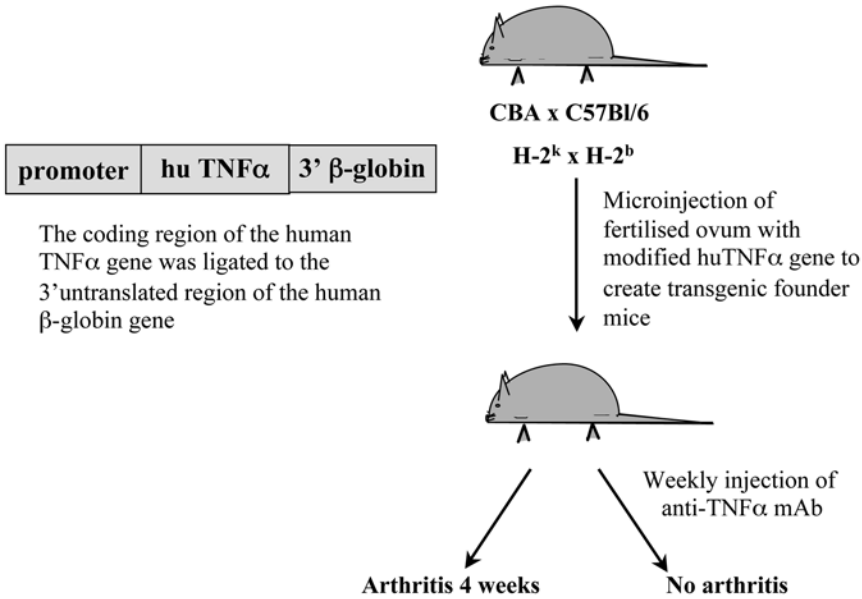

Fig. 1. Generation of trangenic mice expressing a modified human tumor necrosis factor (TNF)α gene. The replacement of the 3'-AU-rich region of the TNFα gene by the 3'-region of the β-globin gene stabilizes mRNA, resulting in over-expression of TNFα protein *(23)*.

particularly affected (**Fig. 2**). hTNFα was found to be overexpressed in various tissues, including lung, spleen, and the joint and it is not obvious why the joint should be affected pathologically whereas other tissues were apparently normal. Indeed, a second strain of TNFα over-expressing mice was generated in which the AU-rich region of the TNFα transgene was deleted by targeted disruption and these mice developed not only arthritis but also inflammatory bowel disease *(24)*. It is noteworthy that TNFα over-producing mice can be back-crossed to $RAG^{-/-}$ mice without altering the arthritis phenotype, indicating that TNFα is mostly "downstream" of the T- or B-cell response. Furthermore, we have been unable to detect autoantibodies to cartilage-derived proteins (type II collagen, type IX collagen, type XI collagen, or aggrecan) in hTNFα transgenic mice on a DBA/1 background (unpublished data).

It is of interest that treatment of hTNFα transgenic mice with an antagonistic anti-IL-1R antibody prevented the development of spontaneous arthritis *(25)*. This is compatible with studies in human RA synovial cell cultures where blockade of TNFα was found to reduce IL-1 production *(26)*, indicating that IL-1 production is downstream of TNFα.

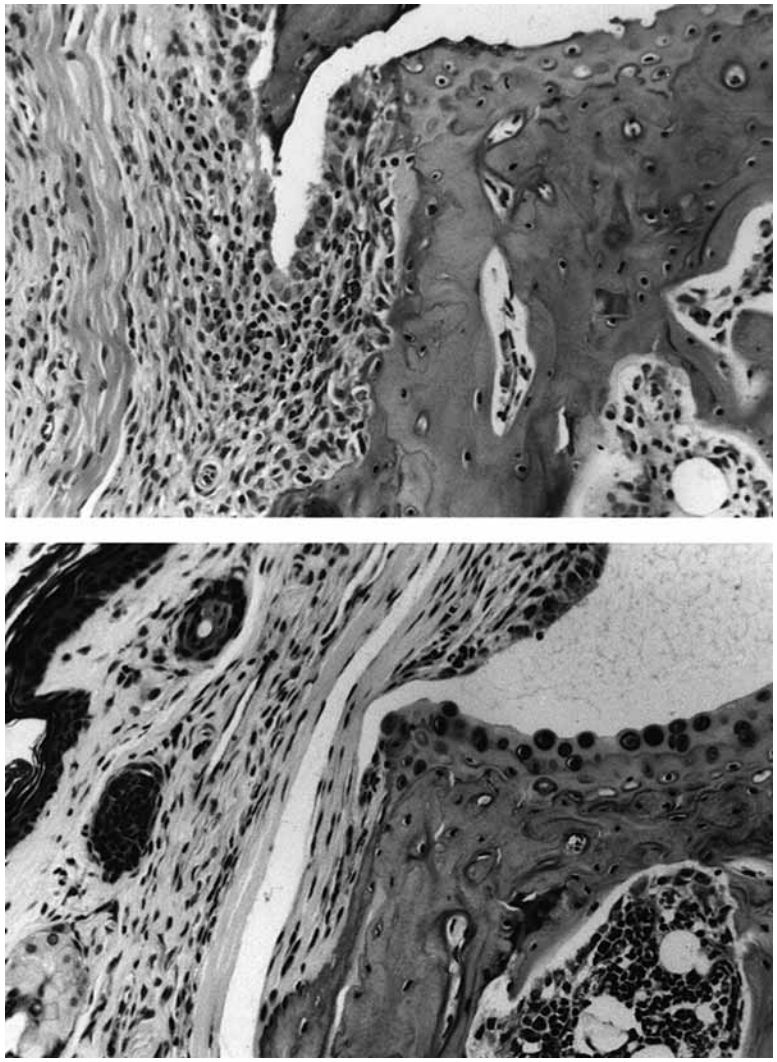

Fig. 2. Joint damage in human tumor necrosis factor (hTNF)α-transgenic mice. **(Top)** Erosive changes in the cartilage-bone-pannus region of a proximal interphalangeal joint from a hTNFα-transgenic mouse with arthritis. Note the focal erosion of subchondral bone. **(Bottom)** Normal joint from a nontransgenic littermate. Hematoxylin and eosin.

In addition to TNFα, it is also recognized that IL-1 is a major pathological mediator in arthritis and this is confirmed by the observation that IL-1α-transgenic mice develop severe polyarthritis spontaneously at around 4 wk of age *(27)*. Synovitis was observed at 2 wk of age and after 6 wk synovial lining layer

hyperplasia and the formation of pannus were seen. The cartilage was also shown to exhibit degradative changes and this confirms previous findings regarding the importance of IL-1 in cartilage breakdown *(28–30)*.

1.3.2. Evidence From Knockout Mice

The generation of gene-targeted strains of mice has provided important insights into the roles played by different cytokines in arthritis. For example, the role of IL-12 was investigated by studying the progression of CIA in IL-12$^{-/-}$ mice on a DBA/1 genetic background and the incidence and severity of disease were both found to be significantly reduced, although disease was not completely abolished *(31)*. Collagen-specific IgG2a antibodies were reduced and IFNγ production by collagen-stimulated splenocytes was inhibited, consistent with a diminished Th1 response. These results demonstrate an important role for IL-12 in the development of Th1 responses and in the pathogenesis of CIA *(31)*. This was subsequently confirmed in wild-type mice given neutralizing anti-IL-12 antibodies twice weekly from the time of collagen immunization. It was found that anti-IL-12 blockade dramatically reduced the severity of arthritis, both clinically and histopathologically *(13)*.

In a similar study, the development of CIA was followed in IL-18$^{-/-}$ mice on a DBA/1 background. IL-18$^{-/-}$ mice developed markedly reduced incidence and severity of arthritis compared with wild-type mice and this was accompanied by reduced serum anticollagen IgG2a levels and reduced proinflammatory cytokine production by spleen and lymph node cells in vitro *(32)*. Treatment of IL-18$^{-/-}$ mice with rIL-18 restored the development of arthritis to that of wild-type mice. These findings demonstrate a role for IL18 in CIA and this was subsequently confirmed in further studies in which anti-IL-18 IgG or rIL-18-binding protein were found to reduce the severity of established CIA *(33)*.

Targeted deletion of the *IL-6* gene has given conflicting results. IL-6$^{-/-}$ mice, backcrossed onto the DBA/1 genetic background for five generations were completely protected from CIA and there was a reduction in the anticollagen IgG response and an absence of inflammatory cells and tissue destruction in the joints *(34)*. In contrast, in another study in which IL-6$^{-/-}$ mice were backcrossed onto the DBA/1 genetic background for eight generations, overt arthritis developed in all of the IL-6 knockout mice, although there was a delay in disease onset and a reduction in severity *(35)*. This latter study would suggest that IL-6 is important, though not essential for the development of CIA and this is supported by therapeutic studies showing that blockade of the IL-6 receptor reduces the severity of collagen-induced arthritis in mice and cynomolgus monkeys *(36,37)*.

IL-17 is a T-cell-derived cytokine that is expressed at elevated levels in synovial tissues of RA patients and postulated to play a pathogenic role in arthritis. This was subsequently confirmed by the finding that CIA was

markedly suppressed in IL-17$^{-/-}$ mice *(38)* and that blockade of IL-17 using neutralizing antibodies was effective in reducing the severity of disease *(39,40)*.

An important pathological feature of CIA (and to a lesser extent, RA) is the preponderance of granulocytes in the joints of arthritic mice therefore it is not surprising that G-CSF-deficient mice were protected from arthritis *(41)*. The reduced arthritis severity was associated with inhibited mobilization of granulocytic cells from the bone marrow and reduced cellular infiltration in the joints. More surprising, however, was the observation that G-CSF blockade in established CIA in wild-type DBA/1 mice reduced disease severity *(41)*. These findings clearly point to a role for G-CSF in driving arthritis in the CIA model and should therefore be regarded as a potential therapeutic target for human disease.

Although the majority of gene knockout studies in CIA have focused on genes encoding proinflammatory cytokines, at least two groups have analyzed the progression of CIA in mice lacking the inflammatory cytokine, IL-10. Cuzzocrea et al. reported enhanced clinical and histological development of CIA in IL-10$^{-/-}$ mice *(42)*. In another study, IL-10$^{-/-}$ mice displayed increased incidence and severity of CIA compared to IL-10$^{+/-}$ littermates. Surprisingly, however, IL-10$^{-/-}$ were less susceptible to arthritis induced by passive transfer of anticollagen antibodies. It was concluded that endogenous IL-10 has a protective role in CIA, but exacerbates antibody-mediated joint inflammation *(43)*. However, the net effects of administration of exogenous IL-10 appears to be anti-inflammatory because daily injections of recombinant IL-10 were found to inhibit the progression of established CIA in wild-type DBA/1 mice *(44)*.

CIA is regarded as a strongly Th1-biased disease with high levels of IFNγ production in draining lymph node cells that peak at around the time of onset of disease *(45)*. Hence, one of the least anticipated findings to come out of studies of gene knockout mice was the increased severity, accelerated onset, and increased cumulative incidence of CIA in IFNγ receptor knockout mice, compared to wild-type littermates *(46,47)*. These findings are also supported by our own studies, which demonstrated enhanced progression of CIA in DBA/1 mice treated with IFNγ neutralizing mAb *(48)* and those of Boissier et al. which showed increased disease severity following blockade of IFNγ in late CIA *(49)*. Clearly, the pro- and anti-inflammatory properties of IFNγ are complex and a more comprehensive analysis will be required to elucidate the role of this cytokine in arthritis.

1.3.3. TNFα is a Valid Therapeutic Target for Rheumatoid Arthritis

There has been considerable progress in recent years in our elucidation of the pathological processes in RA and the roles played by the many cytokines, chemokines, and growth factors have come to be appreciated. Two cytokines in

particular, TNFα and IL-1, have been shown to be major inducers of both inflammation and tissue destruction. Subsequently, it was shown that blockade of TNFα in cultured synovial cells from RA patients prevented the expression of IL-1 and other proinflammatory cytokines *(26,50–52)*, suggesting a cytokine cascade in which TNFα was responsible for driving the production of multiple mediators of inflammation.

In the light of these findings a number of studies have analyzed the effect of TNFα blockade in CIA. For example, a number of studies showed that treatment of mice with monoclonal or polyclonal anti-TNFα antibodies, or soluble TNF receptors, reduced the severity of arthritis when administered before the onset of clinical arthritis *(53–55)*. Subsequently, we assessed the effect of anti-TNFα treatment in mice with established CIA *(54)*. DBA/1 mice were immunized with type II collagen in CFA. The mice were inspected daily and each mouse that exhibited clinical signs of arthritis was randomly assigned to one of three treatment groups. The mice were then given twice-weekly injections of TN3-19.12 (anti-TNFα mAb), L2 (isotype control) or PBS over a period of 14 d. The half-life of TN3-19.12 in mice had been previously estimated to be around 7 d *(56)*. There was found to be a dose-dependent reduction in the severity of arthritis following treatment with anti-TNFα mAb (**Fig. 3**). At the end of the treatment period, arthritic paws were processed for histology and it was confirmed that anti-TNFα treatment had reduced the histological severity of arthritis and protected joints from erosive changes (**Fig. 4**).

Soluble TNF receptors play an important physiological role in regulating the activity of TNFα, and two studies showed that the administration of recombinant soluble TNFRs is effective in established CIA. In the first study, a p75 TNFR–Fc fusion protein was found to reduce the severity of arthritis whether given before or after the onset of the disease *(57)*. We then showed that a p55 TNFR–Ig fusion protein was effective in reducing both the clinical and histological severity of established CIA *(58)*.

It was concluded from these studies was that TNFα is involved in the pathogenesis of CIA and this provided part of the rationale for the testing of anti-TNFα mAb therapy in human RA.

1.3.4. Effect of TNFα Blockade in Other Models of Arthritis

Although CIA is probably the most widely utilized animal model of arthritis, many other models exist, all of which mimic human RA to a greater or lesser extent *(9)*. Issekutz et al. *(59)* used neutralizing antibodies to assess the respective roles of TNFα, IL-1α, and IL-1β in adjuvant arthritis in rats. Treatment with anti-IL-1α and anti-IL-1β on day 5 of arthritis did not significantly affect infiltration of polymorphonuclear leukocytes (PML) or T-cell infiltration into the joint. In contrast, anti-TNFα treatment reduced clinical scores,

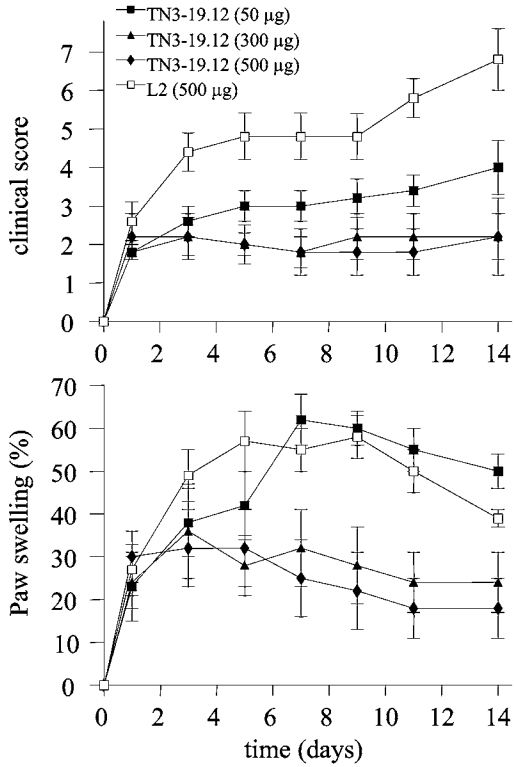

Fig. 3. Effect of antitumor necrosis factor monoclonal antibody (mAb) (TN3-19.12) on clinical progression of established collagen-induced arthritis. Arrows indicate time of injection. L2 is an isotype control mAb. There were 10 mice per group. **(Top)** Clinical score (0 = normal, 1 = slight swelling and/or erythema, 2 = pronounced edematous swelling, 3 = ankylosis). Each limb is graded, giving a maximum score of 12 per mouse. **(Bottom)** paw swelling (expressed as the percentage increment in paw-width relative to the paw-width before the onset of arthritis). (Adapted from **ref.** *54*.)

inhibited infiltration of PML by 40–50% and T lymphocytes by 30–50%. It was concluded from this study that leucocyte infiltration in adjuvant arthritis is a strongly TNFα-dependent disease with IL-1 playing a relatively minor role. This clearly differs from CIA, in which IL-1 plays a major role in both inflammatory and destructive processes *(60,61)*.

Neutralization of TNFα in antigen-induced arthritis in rabbits was found to inhibit inflammatory changes in the joint during the acute phase of the disease but had only a minor effect on proteoglycan loss from cartlilage in the long-term *(62)*. Similarly, anti-TNFα treatment failed to prevent changes in cartilage proteoglycan synthesis or proteoglycan loss in antigen-induced arthritis, zymosan-induced arthritis, immune complex-mediated arthritis, or streptococcal cell wall-induced

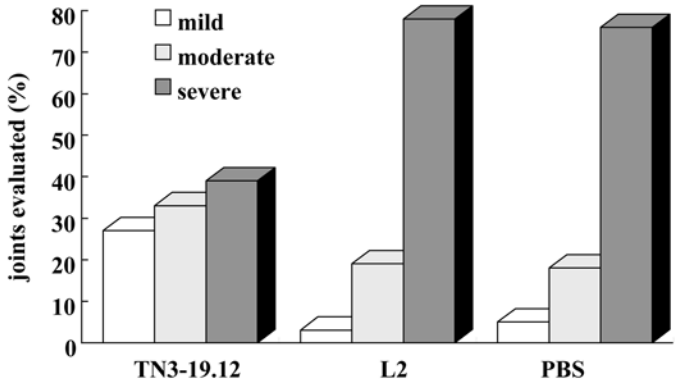

Fig. 4. Histopathological assessment of joints of arthritic DBA/1 mice treated with antitumor necrosis factor (TN3-19.12). L2 is an isotype control monoclonal antibody . The scoring system was as follows. Mild, minimal synovitis, erosions limited to discrete foci, cartilage surface intact. Moderate, synovitis, and erosions present but normal joint architecture intact. Severe, extensive erosions, joint architecture disrupted. Approximately 60 joints were examined per treatment group. (Adapted from **ref.** *54*.)

arthritis in mice *(63,64)*. It can concluded from these findings that arthritis models differ in terms of their response to TNFα blockade. Given that human RA is a relatively heterogenous disease, it seems likely that individual patients will also show different levels of response to anti-TNFα therapy.

1.3.5. What is the Effect of IL-1 Blockade?

Although this review has focused primarily on the therapeutic effect of TNFα blockade in CIA, it is clear that neutralization of anti-IL-1 also has a pronounced therapeutic effect *(60,61,65–67)*. In contrast, continuous infusion with IL-1 receptor antagonist (IL-1Ra) was found to be relatively ineffective in adjuvant arthritis *(66)* and neutralization of IL-1 in streptococcal cell wall-induced arthritis failed to affect the clinical severity of disease, although a reduction in cartilage proteoglycan depletion was observed microscopically *(64)*. In human RA, clinical trials with IL-1Ra have shown only modest reductions in disease activity *(68)* although in view of the poor pharmacokinetics of IL-1Ra, this may be owing to incomplete neutralization of IL-1 *(66)*. In the light of these conflicting findings, we evaluated the effects of anti-TNFα, anti-IL-1β, and anti-IL-1α/β therapy in CIA. Anti-TNFα, anti-IL-1β and anti-IL-1R (which blocks the activity of both IL-1α and IL-β) were all found to be effective in reducing the clinical and histological severity of arthritis, with anti-IL-1R and anti-IL-1β showing somewhat greater efficacy than anti-TNFα *(69)*. Anti-IL-1β was equally as effective as anti-IL-1R, suggesting that IL-1β plays a more important pathological role than IL-1α in CIA. An additive effect was observed between

anti-TNFα and anti-IL-1R in the prevention of joint erosion and in reduction of the levels of the acute phase protein, serum amyloid P *(69)*. On the basis of these findings it is concluded that, in addition to TNFα, IL-1 is a valid therapeutic for RA.

2. Materials

2.1. Purification of Type II Collagen

1. Powdered cartilage (*see* **Note 1**).
2. 4 *M* Guanidine-HCl, 0.05 *M* Tris-HCl, pH 7.5.
3. 0.5 and 0.1 *M* Acetic acid.
4. Sodium chloride (powder).
5. 70% (v/v) Formic acid.
6. Pepsin from porcine gastric mucosa (3X crystalized; Sigma-Aldrich).
7. 0.02 *M* Na_2HPO_4, pH 9.4.

2.2. Immunization of Mice

1. Male DBA/1 mice, 8–12 wk old (Harlan-Olac).
2. Type II collagen.
3. 0.1 *M* Acetic acid.
4. *Mycobacterium tuberculosis* H37 RA (Difco).
5. Incomplete Freund's adjuvant (IFA; Difco).
6. 0.2 mg/mL Fentanyl and 10 mg/mL fluanisol (Hypnorm®).

2.3. Measurement of Anticollagen IgG

1. Type II collagen.
2. 0.05 *M* Tris-HCl, 0.2 *M* NaCl, pH 7.4.
3. Nunc-immuno microtiter plates (Nalge-Nunc).
4. 0.05% (v/v) Tween-20 in phosphate buffered saline (PBS).
5. 2% (w/v) Bovine serum albumin (BSA) in PBS.
6. Test sera.
7. Standard serum sample.
8. HRP-conjugated anti-IgG, IgG1, and IgG2a (BD Biosciences).
9. TMB microwell peroxidase substrate system (Kirkegaard and Perry).
10. 4.5 *N* H_2SO_4.

2.4. Analysis of T-Cell Responses

1. Type II collagen immunized DBA/1 mice.
2. 70-µm Cell strainers (Falcon).
3. Hanks' balanced salt solution (HBSS).
4. Complete medium: RPM1 1640 containing 10% (v/v) heat-inactivated fetal calf serum or 1% (v/v) mouse serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 2×10^{-5} M 2-mercaptoethanol, and 20 m*M* L-glutamine.
5. 0.05% (v/v) Tween-20 in PBS.

6. 2% (w/v) BSA in PBS.
7. Recombinant IL-5, IL-10, and IFNγ (Peprotech).
8. The following capture/biotinylated detect antibody pairs. IL-5, TRFK5/TRFK4; IL-10, JES5-16E3/JES5-2A5; IFNγ, R4-6A2/XMG1.2 (Immunokontact).
9. Streptavidin-HRP (BD Biosciences).
10. TMB microwell peroxidase substrate system (Kirkegaard and Perry).
11. 4.5 *N* H_2SO_4.
12. [^{3}H]thymidine (Amersham).

3. Methods

3.1. Purification of Type II Collagen

The method of purification of type II collagen from cartilage is based on the studies of Miller ***(70)*** and Herbage et al. ***(71)***.

1. Powder cartilage in a liquid nitrogen freezer mill (Spex, Metuchen, NJ). If unavailable, the cartilage may be ground to a fine powder using a pestle and mortar placed in a bath of dry ice and liquid nitrogen.
2. To remove proteoglycans, suspend powdered cartilage in 5 vol of 4 *M* guanidine-HCl, 0.05 *M* Tris-HCl, pH 7.5, for 24 h at 4ºC. Centrifuge at 14,000*g* for 1 h at 4ºC.
3. Discard supernatant and wash cartilage pellet with 0.5 *M* acetic acid to remove guanidine-HCl. Centrifuge at 14,000*g* for 1 h at 4ºC.
4. To solubilize collagens, resuspend cartilage pellet in 20 vol of 0.5 *M* acetic acid. Adjust pH of the suspension to 2.8 using 70% formic acid. Add 1 g of pepsin for every 20 g of cartilage (wet weight). Leave stirring for 48 h at 4ºC.
5. Centrifuge at 14,000*g* for 1 h at 4°C and discard pellet. To precipitate type II collagen from the supernatant, add NaCl (powder) gradually with stirring to give a final concentration of 0.89 *M*. Leave to equilibriate overnight at 4ºC then centrifuge at 14,000*g* for 1 h at 4ºC.
6. Dissolve pellet in 0.1 *M* acetic acid. Then inactivate residual pepsin by dialyzing against 0.02 *M* Na_2HPO4, pH 9.4. The collagen will form a precipitate.
7. Centrifuge at 14,000*g* for 1 h at 4ºC, then redissolve pellet in 0.1 *M* acetic acid.
8. Dialyze against 0.1 *M* acetic acid and freeze-dry. Store at 4ºC in a dessicator.
9. Purity of the collagen can be assessed on a-5% SDS-polyacrilamide gel. In addition, the presence of contaminating proteoglycans (which may not be detected by gel electrophoresis) can be assessed according to the method of Ratcliffe ***(72)***. In brief, 40 µL of sample is added to 250 µL of 1,9-dimethylmethylene blue in formate buffer, pH 3.5, in a 96-well microtiter plate. The absorbance is then read immediately at 600 nm using an ELISA plate reader. A standard curve is constructed by titrating a known concentration of chondroitin sulfate.

3.2. Induction and Assessment of Arthritis

1. Dissolve type II collagen at 4 mg/mL in 0.1 *M* acetic acid overnight at 4ºC, with vigorous stirring. Collagen dissolved in this way may be stored at –20ºC (*see* **Note 2**).

2. To produce CFA, grind *M. tuberculosis* with a pestle and mortar to produce a fine powder, then suspend in IFA (approx 3 mg *M. tuberculosis*/mL of IFA). This should be carried out in a fume hood and with a face mask to prevent inhalation of *M. tuberculosis* powder.
3. Emulsify dissolved type II collagen with an equal volume of CFA on ice, using a syringe (preferably glass) or an Ultra-Turrax (IKA) in short bursts to prevent heating. The emulsion should be thick enough not to drip out of the vessel when inverted.
4. Sedate mice by intraperitoneal injection of 100 µL of 10% (v/v) Hypnorm, diluted in distilled water.
5. Shave rumps of mice using electric clippers to facilitate the injection.
6. Inject emulsion intradermally (as far as possible) at two or more sites at the base of the tail using a glass syringe or a latex-free syringe and a 27-gauge needle. The needle becomes blunt easily and should be changed frequently. Each mouse should receive 0.1 mL of emulsion in total. The emulsion should be shallow enough to be visible under the skin (*see* **Note 3**).
7. Some workers boost the mice with a second intraperitoneal injection of 100 µg type II collagen in 100 µL of 0.1 *M* acetic acid, 21 d after primary immunization. However, we have not found this to be necessary.
8. Monitor mice for arthritis every day from day 14 after immunization. The peak time of arthritis onset is around day 30.
9. To compare the clinical severity of arthritis a scoring system may be used where 0 = normal, 1 = slight swelling and/or erythema, 2 = pronounced swelling, 3 = ankylosis. Each limb is graded in this way, giving a maximum score of 12 per mouse. In addition, paw swelling can be monitored using calipers (Poco 2T, Kroeplin).
10. To compare histological severity, paws are removed at post mortem, fixed in buffered formalin (10% v/v), then decalcified in EDTA in buffered formalin (5.5% w/v). The tissues are then embedded in paraffin, sectioned, and stained with hematoxylin and eosin. The severity of arthritis may be graded as mild, moderate, or severe based on the following criteria: mild = minimal synovitis, cartilage loss, and bone erosions limited to discrete foci; moderate = synovitis and erosions present but normal joint architecture intact; severe = synovitis, extensive erosions, joint architecture disrupted. Alternatively, the proportion of joints with erosions (defined as demarcated defects in cartilage or bone filled with inflammatory tissue) can be determined.

3.3. Measurement of Anticollagen IgG

Serum levels of anticollagen IgG provide a marker of the magnitude of the humoral anticollagen response whereas levels of IgG1 and IgG2a serve as extremely valuable in vivo markers of Th2 and Th1 responses, respectively.

1. Make up stock solution of type II collagen in 0.05 *M* Tris-HCl, 0.2 *M* NaCl, pH 7.4, at 1 mg/mL. Aliquot and store at –20ºC (*see* **Note 2**).
2. Coat ELISA plate with type II collagen at 2–5 µg/mL in 0.05 *M* Tris-HCl, 0.2 *M* NaCl, pH 7.4, overnight at 4ºC.
3. Block for 1 h at room temperature with 2% BSA.

4. Incubate test sera (diluted in PBS/Tween-20) for 2 h at room temperature. Levels of anticollagen IgG may vary enormously between mice and it is important to serially dilute samples to ensure that comparisons are made based on the linear portion of the titration curve. A suggested starting dilution is 1/100, with seven three- to fivefold dilution steps. Include a standard serum sample on each plate. Pooled serum from collagen-immunized mice or affinity purified anticollagen IgG can be used as a standard.
5. Wash 6X with PBS/Tween-20, then detect bound IgG with HRP-conjugated anti-mouse IgG, IgG1 or IgG2a.
6. Develop with TMB substrate. Stop reaction with 4.5 *N* H_2SO_4 and read at 450 nm.

3.4. Analysis of Cell Responses

1. Proliferative responses may be measured by incorporation of [^{3}H]thymidine in response to stimulation of lymph node cells with type II collagen. Alternatively, cytokines in culture supernatants can be measured by ELISA.
2. Make up stock solution of type II collagen in 0.05 *M* Tris-HCl, 0.2 *M* NaCl, pH 7.4, at 1 mg/mL. Collagen in solution can be kept at 4ºC for up to 3 mo (for stimulation of T cells only; *see* **Note 2**).
3. Remove draining (inguinal) lymph nodes from collagen-immunized mice.
4. Push through cell strainer using syringe plunger, then wash three times in HBSS.
5. Resuspend at 5×10^6 cells/mL in complete medium and culture for 72 h (37ºC; 5% CO_2) in the presence or absence of type II collagen (50 µg/mL).
6. Remove supernatant for measurement of IL-5, IL-10, and IFNγ (*see* **Note 5**). Coat ELISA plates with relevant capture mAb (overnight at 4ºC), block with 2% BSA (1 h at room temperature), then add supernatants (overnight at 4ºC). Generate standard curve using appropriate recombinant cytokine at a range of 10,000 to 14 pg/mL for IL-5 and IL-10 and 100,000–137 pg/mL for IFNγ. Wash six times with PBS/Tween-20, then incubate with biotinylated detection mAb. Wash six times with PBS/Tween-20, then add HRP-conjugated streptavidin (1 h at room temperature), and develop with TMB substrate. Stop the reaction with 4.5 *N* H_2SO_4 and read at 450 nm.
7. To determine the rate of T-cell proliferation, pulse cells with [^{3}H]thymidine and culture for a further 16 h. Harvest cells and assess for incorporation of radioactivity.

4. Notes

1. For immunization of mice, type II collagen from bovine, porcine, or chick cartilage is normally used. Alternatively, mouse collagen (derived from mouse sternums) may be used, which results in a more chronic relapsing form of arthritis, which is reported to be more similar to human RA than conventional CIA induced with heterologous collagen *(21)*. The yield and solubility of the type II collagen is greater when derived from young animals owing to the reduced level of cross-linking. The sternum, nasal septum, or articular cartilage may be used. A good source is femoral head cartilage from a young calf. Peel off the cartilage from the surface of the bone using a scalpel wearing chain mail gloves and eye protection for safety. The cartilage is white. Avoid the underlying bone, which is pink. In the case

of sternum or nasal septum the cartilage should be chopped into small pieces. Type II collagen for immunization is also commercially available.

2. Once in solution, it is important for type II collagen to be maintained at low temperature to prevent denaturation. This is important for successful immunization and for measurement of anticollagen antibody levels whereas T-cell activity is less sensitive to the conformational state of the collagen molecule.
3. Two factors of importance in determining incidence of arthritis are the concentrations of *M. tuberculosis* in the adjuvant and collagen in the acetic acid. To establish the system, high concentrations can be used, e.g., 3 mg mycobacterium per milliliter of IFA and collagen at 4 mg/mL of acetic acid. This invariably induces high incidence but the arthritis is severe and acute in nature, therefore the amounts can subsequently be reduced once the system is known to be working.

   The most likely reasons for a failure to induce a high incidence of arthritis include the following: (1) the collagen preparation is of low purity or in a denatured form. (2) There is concurrent infection in the mouse colony, the mice are immature (less than 8 wk of age), or females rather than males are used. (3) The concentration of type II collagen or *M. tuberculosis* in the emulsion used for immunization is insufficient and the emulsion is not thick enough or is injected too deeply.
4. Following immunization, mice will develop arthritis of varying degrees of severity and novel treatment regimes may produce unexpected adverse effects. Hence, mice should be monitored on a daily basis for signs of ill-health or distress. Clearly defined humane endpoints should be strictly enforced. For example, any mouse showing severe and sustained paw swelling should be humanely killed. Any mouse which has lost 20% or more of its body weight should be humanely killed. Any mouse with severe lameness should be humanely killed. Any mouse with dyspnoea, ruffled fur, weakness, dehydration, or a hunched appearance should be humanely killed. Any mouse showing blistering at the injection site should be humanely killed. In addition, the duration of experiments involving arthritic animals should be minimized, compatible with the aims of the study.
5. In general, IL-5, IL-10, and IFNγ can be measured in culture supernatants of collagen-stimulated T cells taken from mice with active disease, whereas IL-2 and IL-4 are difficult to detect, probably because of consumption of these cytokines by proliferating T cells. An alternative is therefore to study cytokine expression by intracellular staining and fluorescence-activated cell sorting (FACS) analysis. Another important factor is that Th2 cells are usually found in low abundance in DBA/1 mice immunized with CFA, although immunomodulatory treatments may influence the Th1:Th2 ratio.

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14

Novel Opportunities for Therapeutic Targeting in Systemic Autoimmune Diseases

Meryem Ouarzane and Moncef Zouali

Summary

Systemic autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis, continue to cause significant morbidity in affected persons. In the past few years, significant progress was made in understanding their pathogenesis and the underlying molecular mechanisms. As a result, a number of new exciting therapeutic options have become available, and novel therapeutic targets have emerged, including B-cell depletion therapies, B cell-activating factor of tumor necrosis factor family (BAFF) antagonists, and FcγRIIB receptor antagonists. Also promising is the current interest centered on the development of inhibition of signal transduction pathways, such as pharmacological inhibitors that act at various levels of signal transduction pathways.

Key Words: Autoimmune diseases; B lymphocytes; TNFα; complement components; signaling; systemic lupus erythematosus; rheumatoid arthritis.

1. Introduction

The ability of the immune system to distinguish self from nonself is central to its capacity to protect against pathogens and, at the same time, maintains nonresponsiveness to its own components. This property is established at discrete checkpoints, both during early development and adulthood. To date, several early developmental checkpoint mechanisms have been identified. These include clonal deletion *(1–3)* of autoreactive lymphocytes during early development of the immune system, clonal anergy *(4)*, which converts autoreactive cells to a state that precludes them from becoming activated, and editing *(5–7)*, a mechanism for modifying self-reactive B cells that renders them nonautoreactive. Although these developmental checkpoints purge the immune repertoire from autoreactive cells, the processes of central

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tolerance remain incomplete, allowing self-reactive cells to escape into the periphery *(8,9)*.

Failure of one of these self-tolerance mechanisms results in pathogenic autoimmunity, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). SLE is a chronic systemic autoimmune disease that results in inflammation of, and damage to a range of organ systems. Approximately one-third of lupus cases have a mild form of the disease characterized by elevated titers of antinuclear antibodies, arthritis, and skin and/or mucosal membrane involvement *(10)*. However, the majority of patients will suffer additional and more severe clinical manifestations, such as renal inflammation (nephritis), and central nervous system vasculitis (cerebritis). The disease aggregates in families, suggesting an important role for genetic predisposition, and woman are about 10 times more likely to develop SLE than men. Autoantibodies directed against nuclear antigens (e.g., histones, single-stranded DNA, double-stranded DNA, Ro, La) are found in virtually all cases. RA is a chronic, systemic autoimmune disease that targets synovial joints, and is often accompanied by an array of extra-articular manifestations. The immunopathogenesis of RA is multifactorial (*see* Chapter 13). Evidence suggests that the interaction between an unknown exogenous or endogenous antigen via antigen-presenting cells and $CD4^+$ T-helper cells is involved in the induction of the immune response in RA. Subsequent recruitment and activation of monocytes and macrophages occurs with secretion of proinflammatory cytokines, in particular tumor necrosis factor-α (TNFα) and interleukin (IL)-1 into the synovial cavity. Release of these cytokines mediates tissue destruction by activation of chondrocytes and fibroblasts, which release collagenases and metalloproteinases, resulting in cartilage loss and bone erosion. B-lymphocyte dysregulation with production of rheumatoid factor and other antibodies, formation of immune complexes and release of destructive mediators also contributes to this process *(11)*.

Drugs do exist for the majority of autoimmune illnesses, but the challenge is to design new drugs that prevent autoimmune attacks without seriously affecting the body's ability to defend itself against infection. Understanding the development of autoimmunity is therefore crucial toward improving the management of autoimmune diseases. As will be discussed below, the rapid expansion of knowledge on autoimmunity is fuelling the development of a novel approach known as targeted immunotherapy. Focusing on SLE and RA, we summarize therapeutic targets that offer promising avenues for future development in systemic autoimmune diseases.

2. The Key Role for B Lymphocytes in Autoimmune Diseases

For some time, autoimmune diseases have been considered to be mediated essentially by T cells. Of late, emerging data suggest that B cells play an important role in these diseases than previously thought. While it has long been known that levels of autoantibodies such those directed to the thyroid stimulating

hormone receptor that correlate with disease severity and progression in Graves disease, and those directed to double-stranded DNA that are often elevated in lupus disease, it is becoming appreciated that B cells play more than one role in these diseases *(12)*. Not only are they the precursors of antibody-secreting plasma cells, but they also act as remarkably effective antigen-presenting cells, suggesting that they may play a potential role in autoimmunity via abnormal autoantigen presentation. B cells can also secrete cytokines, such as TNFα, that exacerbate the autoimmune and inflammatory responses. In addition, autoreactive B cells express ligands that bind costimulatory receptors on T cells. Along with presentation of self-derived peptides on cell surface major histocompatibility complex class II molecules, this can drive activation of autoreactive T cells. Therefore, targeting B cells may prove to be an effective avenue for the development of novel therapies for systemic autoimmune diseases.

2.1. B-Cell Depletion Therapy

The important role of B cells in SLE and RA etiologies is supported by the encouraging results of clinical trials aiming to eliminate B cells in affected patients. B-cell development is characterized by a series of changes of surface phenotypic markers. One of them CD20 is expressed at intermediate stages and is lost during terminal differentiation to immunoglobulin-producing plasma cells. A chimeric monoclonal antibody (MAb) against human CD20, called Rituximab, is now used in clinical trials of several autoimmune diseases. Its mode of action remains under investigation, but the available evidence suggests that this antibody acts via complement-mediated and antibody-dependent, cell mediated-toxicity, and induction of apoptosis *(13)*.

In RA, Rituximab is effective, and clinical benefits are observed 6 mo after only two infusions *(14)*. The treatment is accompanied with a major B-cell depletion and a large drop of rheumatoid factor titers, whereas total immunoglobulin levels show little changes *(15,16)*. In SLE too, the beneficial effect of Rituximab therapy has been demonstrated by a study that provides sufficient evidence of excellent tolerability and high efficacy *(17)*. Rituximab not only reduced B-cell numbers and IgG levels, but also downregulated CD40 and CD80 expression on B cells, suggesting a possible disturbance of T-cell activation through these costimulatory molecules *(18)*. Reduction of both the quantity and the functions of B cells suggest that Rituximab could improve the disease course in patients with SLE.

2.2. BAFF Antagonists

BAFF, also known as BLys, is a member of the TNF family that acts as an essential survival factor for B cells *(19,20)* and appears to play a central role in the development of some autoimmune diseases and B-cell malignancies. It has

a potent effect on B cells, but also has direct effects on other cell types. This specificity is achieved at the molecular level by binding to three different receptors (TACI, BCMA, and the BAFF receptor *[19–22]*).

Two major avenues of evidence link BAFF to autoimmunity, namely studies in animal models and measurement of BAFF levels in patients with systemic autoimmune diseases. BAFF levels are elevated in autoimmune mice, notably the (NZB × NZW) F_1 and MLR-*lpr* strains *(21)*. In patients with RA, SLE and other systemic autoimmune diseases, BAFF levels are also elevated *(23)*. During the course of SLE, BAFF levels fluctuate *(24)*, and BAFF has been proposed to be a biomarker for SLE disease activity. This information is suggestive of a link between BAFF and development of autoimmunity, and implicates BAFF in the etiology and progression of autoimmune diseases. As such, antagonism to BAFF may provide a novel therapeutic approach to the treatment of autoimmune diseases. Two types of antagonists are currently in development. First, a fully human MAb against BAFF (LymphoStat-B) has entered clinical trials. Second soluble forms of the BAFF receptor, in which the extracellular domain of a BAFF receptor is fused with the Fc domain of an immunoglobulin molecule, are in various stages of development.

2.3. The Inhibitory Coreceptor FcγRIIB: A Potential Target

The balance between stimulatory and inhibitory signals regulates the activation and expansion of lymphoid cells. Inhibitory signaling, in particular, is a critical feature of peripheral tolerance, providing a means for establishing thresholds for stimulation and for active deletion of autoreactive cells from the peripheral repertoire.

Previous work has demonstrated that the expression of the inhibitory Fc receptor FcγRIIB is required for the maintenance of self-tolerance *(25)*. C57BL/6 mice deficient in this receptor develop spontaneous lupus-like autoimmunity. Several other stains of mice that develop spontaneous autoimmune disease, such as NZB, NOD, BXSB, and MLR, have also been shown to express reduced levels of FcγRIIB on activated or germinal center B cells. This reduced expression results from a polymorphism in the promoter of the corresponding gene. These results suggest that the levels of FcγRIIB expressed on some B cells may regulate their ability to maintain tolerance, and that relatively small changes in the expression of this inhibitory receptor may permit the survival and expansion of autoreactive cells *(26)*. Thus, changes in the surface expression of this receptor appear to be critical for determining disease progression, and these changes provide a rational basis for a therapeutic approach based on manipulating the expression of this receptor to restore self-tolerance in autoimmune diseases *(27)*.

3. The Role of the Complement in Autoimmune Diseases

In addition to its important roles in the innate immune system to foreign antigens, the complement system is increasingly recognized to be causally involved in tissue injury during ischemic, inflammatory and autoimmune diseases *(28,29)*. Studies of human diseases have provided clinical evidence for activation of the alternative pathway. Indeed, the presence of activated C3 or C4 fragments and other activated components of the alternative pathway in target organs has been described in several clinical settings *(29)*. The involvement of the complement in RA has been suggested by the observation that the complement activity of the joint fluid from patients is significantly lower than that of control subjects. In addition, significant increases in soluble complement activation fragments in the joint fluid, as well as enhanced local production of complement proteins in synovial tissue, are found *(30)*.

In SLE, the first solid evidence that complement inhibitors could ameliorate target organ damage came from the finding that an inhibitory anti-C5 MAb could block the development of glomerulonephritis in the (NZB × NZW) F_1 mouse model of lupus. With regard to the alternative pathway, MRL-*lpr* mice are also protected from renal disease, as are mice in which activation of the classical and alternative pathways of C3 is partially blocked *(28)*. Thus, it is anticipated that complement inhibitors will belong to the array of therapies available for disease management.

4. Interfering With Dendritic Cell Functions

Dendritic cells contribute to central tolerance in the thymus by presenting antigens to T cells and deleting the T cells that exhibit strong autoreactivity. However, dendritic cells also play a pivotal role in peripheral tolerance. In the absence of infection or inflammation, dendritic cells remain immature and capture antigens via a number of mechanisms, such as phagocytosis and macropinocytosis *(31)*. These immature dendritic cells contribute to deleting autoreactive lymphocytes and to expanding the population of regulatory T cells, thereby ensuring peripheral tolerance. Dendritic cells mature when they come into contact with antigen via surface receptors called Toll-like receptors (TLR) and via receptors belonging to the TNF family (TNFα and CD40 ligand). Dendritic cell activation leads to T-cell activation, in particular via expression of costimulatory molecules (B7, CD40), chemokines receptors (CCR-7), and cytokines (IL-12) *(31)*.

A major role for dendritic cells has been established in lupus. Dendritic cell dysfunction may account for the loss of peripheral tolerance that characterizes lupus. Peripheral blood $CD14^+$ monocytes from lupus patients, but not from normal controls, may act as mature dendritic cells that activate potentially autoreactive T cells. Maturation of these $CD14^+$ cells is induced by elevated

circulating levels of interferon-α (INFα) produced by a population of dendritic cells that infiltrate lupus lesions. These crucial pieces of evidence suggest that INFα and dendritic cells may represent therapeutic targets in patients with lupus *(32,33)*.

5. Disruption of the Interferon-Activating Pathway

An important clue that interferon (INF) might contribute to lupus comes from a series of studies in patients who received INFα as a therapeutic agent for viral hepatitis or carcinoid tumors. Nearly a quarter of INFα-treated subjects (22%) developed a positive antinuclear antibodies blood test *(34)*, and one in five (19%) developed overt autoimmunity, including a small number who developed SLE *(35)*.

In one possible scenario, INF production promotes differentiation of SLE monocytes into activated, antigen-presenting dendritic cells, which migrate to lymph nodes and tissues, and activate autoreactive $CD4^+$ T cells *(36)*. T cells in turn stimulate self-reactive B cells to produce autoantibodies, particularly those with specificities for nucleic acids and associated proteins. These autoantibodies bind endogenous nucleic acids and chromatin derived from apoptotic material to form chromatin-containing immune complexes that stimulate further INF production by plasmacytoid dendritic cells (pDCs), and B-cell proliferation and differentiation by cross-linking of both the B-cell receptor and TLR-9. Aside from TLRs, several proteins expressed on the pDC surface might also contribute to IFN production. Monoclonal antibodies against blood dendritic cell antigen 2 (BDCA-2), a c-type lectin expressed on pDCs, prevent the production of INF by both normal and SLE pDCs in response to SLE serum and viral inducers. However, ligation of CD40 on pDCs synergizes with TLR-9 signaling to induce INF. Interestingly, activated SLE T cells express increased levels of CD40 ligand (CD40L), and levels of soluble CD40L in lupus blood correlate with disease activity. Taken together, this proposed series of events suggests new targets for therapeutic intervention aimed at disrupting the IFN activation pathway in SLE *(37)*.

6. Antitumor-Necrosis-α Therapies in RA

TNFα is an inflammatory cytokine that plays a pivotal role in the pathogenic mechanisms of RA *(11,38)*. It binds to two widely expressed receptors, type 1 (p55) and type 2 (p75), and soluble receptors also influence the activity of the cytokine. The importance of this cytokine in RA is supported by its overexpression in RA synovium, by data from in vitro synovial cell cultures with the use of anti-TNFα antibody, and by animal studies, which demonstrated development of disease in mice transgenic for TNFα and amelioration of the symptoms after treatment with anti-TNFα agents. Currently, there are three agents available which inhibit the action of TNFα. First; Infliximab is a

chimeric anti-TNFα antibody that binds soluble and membrane-bound TNFα, thereby impairing binding to its receptors. In addition, it also mediates killing of cells expressing TNFα *(39)*. Second, Adalimumab is a recombinant human MAb that binds TNFα, thereby precluding binding to its receptor. This antibody also lyses cells expressing the cytokine on their surface *(40)*. Third, Etanercept is a soluble TNFα receptor fusion protein composed of two dimers, each with a ligand-binding portion of the type 2 receptor linked to the Fc portion of human IgG1. The protein binds to both TNFα and TNFβ, preventing each from interacting with its respective receptor *(41)*.

7. RANK, RANKL, and Osteoprotegerin: New Target for RA Treatment

It has been known for some time that RA is characterized by destruction of joint cartilage and bone erosion. However, it is only recently that a major role in bone erosion has been attributed to the receptor activator of nuclear factor κB ligand (RANKL), released by activated lymphocytes and osteoblasts *(42)*. In vitro, binding of RANKL to the cognate RANK, expressed on the surface of osteoclasts, results in osteoclastrogenesis by differentiation of monocyte/macrophage progenitors to osteoclasts and activation of mature osteoclasts *(43)*.

Osteoprotegerin (OPG) produced by activated osteoblasts, is a soluble decoy receptor for RANKL and competes with RANK for RANKL binding *(44)*. Consequently, OPG is an effective inhibitor of osteoclast maturation and activation in vitro and in vivo *(45)*. These observations suggest that inhibition of the downstream RANKL effectors via OPG or other drugs should prevent bone destruction and cartilage damage in patients with RA. Therefore, modulation of these systems provides an opportunity to inhibit bone loss and deformity in chronic arthritis.

8. Anti-CD40 Ligand Therapy

Production of pathogenic antibodies in SLE requires T-cell help; along with ligation of the B-cell surface immunoglobulin receptor by antigen. It is likely that macrophages, dendritic cells, and endothelial cells are also activated by interaction with T-cells and contribute to lupus pathology. CD40 ligand (CD40L, CD154), a member of TNF family of cell surface molecules, mediates these contact-dependent signals delivered by $CD4^+$ T helper cells to $CD40^+$ target cells *(46)*.

Several tissue injuries and immune-mediated pathologies were found to involve CD40-CD40L signaling. Disruption of this pathway in animal models led to the improvement of graft survival. CD40–CD154 interactions were also shown to play a significant role in the progression of autoimmunity, and the production of auto-antibodies in SLE *(47)*. High-level expression of CD154 *(48)* has been detected in T cells from patient with SLE, RA, and other autoimmune disease,

indicating that such cells could account for the high-level expression of immune accessory molecules on B cells of patients with active disease. An increased serum level of soluble CD154 was also reported in SLE and RA in correlation with the relevant autoantibodies and with disease activity. Anti-CD154 antibody therapy prevents autoantibody production and renal immune complex deposition in lupus nephritis, indicating that disruption of this pathway could be a beneficial treatment in SLE *(49)*. Clinical trials are under way *(50)*.

9. Signal Transduction Pathways: New Targets for Treating RA and SLE

The last few years have witnessed a radical change in the treatment of immune system-mediated joint diseases, with the advent of biotherapeutic agents directed at targets identified by basic research into the mechanism of inflammation *(51)*. These agents block the proinflammatory effects of cytokines such as TNFα and IL-1β. They fall into two categories: antibodies that bind specifically to a cytokine or cytokine receptor, and soluble receptors that capture a cytokine before it binds to the cell membrane receptor. Another strategy for preventing cell activation seeks to inhibit the intracellular transduction of signals produced when ligands bind to their membrane receptors.

When the receptors that are coupled to the signal transduction pathways are stimulated, they cause activation of transcription factors that control the production of cytokines, proteases, growth factors, and many other compounds involved in the inflammatory process. Signal transduction pathways closely involved in inflammation include the mitogen-activated protein kinase pathway, the phosphatidylinositol-3 protein kinase pathway, and Janus kinase-signal transducer and activator of transcription, and nuclear factor κB. Other signal transduction pathways are key to inflammation, such as those involving immunoreceptors (integrins, selectins), receptors coupled to G proteins (chemokine receptors), and steroid nucleocytoplasmic receptors.

10. Other Promising Potential Therapeutic Targets

Several inhibitory mechanisms can act at various levels of signal transduction pathways. At least three additional main strategies hold potential for inhibiting signal transduction pathways. One strategy consists in inhibiting an enzyme that activates a signal transduction pathway by administering a pharmacological inhibitor. First, Imatinib Mesylate (Gleevec®) is a tyrosine kinase inhibitor that is highly effective in several haematological malignancies. Second, the role of p38 mitogen-activated protein kinase in the various stages of inflammation has prompted the production of several imidazole compounds capable of inhibiting p38 (SB200765A, RWJ 67657, L-167307, VX-745, and RPR200765A). These pharmacological inhibitors are cytokine-suppressive anti-inflammatory drugs responsible for in vitro and in vivo inhibition of

Table 1
Some Biological Therapies in Clinical Rials for SLE and RA

Compound	Disease	Activity
B-cell directed therapies		
Rituximab	SLE and RA	Chimeric anti-CD20 MAb
Epratuzumab	SLE	Humanized anti-CD22 MAb
Lymphostat-B®	SLE and RA	Monoclonal anti-BAFF
Abetimus sodium (LJP 394)	SLE	Synthetic toleragen molecule specific for anti-DNA antibodies
TNF inhibitors		
Infliximab	RA	Chimeric anti-TNFα antibody
Adalimumab	RA	Recombinat human monoclonal anti-TNFα antibody
Etanercept	RA	Soluble TNFα receptor fusion protein
Pegsunercept	RA	PEGlated soluble TNF receptor type I
ISIS-104838	RA	TNFα antisense inhibitor
Signaling inhibitors		
Scio-469	RA	p38 MAP kinase inhibitor
Temsirolimus (CCI-779)	RA	Cell cycle inhibitor
Interleukin-based therapies		
Anakinra	SLE	Recombinant IL-1 receptor antagonist
Atlizumab	RA	Humanized anti-IL-6 receptor MAb
Cell-adhesion molecule inhibitors		
Natalizumab	RA	Humanized anti-α4 integrin MAb
Costimulation inhibitors		
CTLA4-Ig (BMS-188667)	RA/SLE	CD28/B7 pathway inhibitor
RG2077	SLE/RA	CD28/B7 interaction inhibitor

RA, rheumatoid arthritis; SLE, systemic lupus erythematosis; IL, interleukin.

lipopolysaccharide induced TNFα expression. Third, pharmacological inhibitors of C-Jun N-terminal kinase, extracellular signal-regulated protein kinase, and phosphatidylinositol-3 protein kinase have shown in vitro and in vivo efficacy in inhibiting the production of proinflammatory compounds. The specific C-Jun N-terminal kinase inhibitor SP600125 not only diminishes the production of TNFα, INFγ, and IL-6, but also decreases joint destruction in the adjuvant model of RA.

A second strategy involves increasing the expression of a naturally occurring signal transduction pathway inhibitor. This can be achieved either via direct administration of the recombinant protein or via gene therapy with a viral vector. A third strategy makes use of molecular biology techniques, such as antisense oligonucleotides or interfering RNAs, to directly block the synthesis of transcription factors or signaling molecules.

11. Conclusion

The last few years have been very exciting in understanding the pathogenesis of SLE and RA, with potential subsequent translation into effective treatment options. Some of the therapies reviewed here are relatively safe, more effective than placebo and slow disease progression. Research into newer pharmacological treatments and immunotherapies includes several compounds in development (**Table 1**) for patients with SLE or RA.

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15

Considerations for Target Validation and Industrial Approaches

Carlos R. Plata-Salamán and Sergey E. Ilyin

Summary

Target validation in health and disease integrates the modulation of a certain molecular target with an expected biological/biochemical/physiological or pathophysiological response or effect. The current state-of-the-art in target validation requires the interface of multiple complementary approaches and technologies to define the mechanistic connectivity between a molecular target and underlying micro- and macrobiotic processes. Target validation also represents the basis for "drug target validation" with focus on therapeutic applications. The concepts of "target validation" and "drug-based therapeutic intervention" continue to coevolve as new classes of therapeutic agents and delivery systems emerge and enable us to target or modulate previously inaccessible molecular entities.

Key Words: Allegro™; bioinformatics; biomarkers; functional informatics; gene expression; high-throughput screening; HTS; reverse transcription-PCR; RT-PCR; small-interfering RNA; siRNA; TaqMan; target validation.

1. Introduction

Target validation is a pivotal but sometimes exceedingly confusing subject in the biopharmaceutical definition compendium. In its simplest form, target validation relates to answering a question on whether modulation of a certain molecular target—such as an enzyme or receptor—would lead to an expected biological response or effect. This validation may involve confirmation in normal homeostatic regulation, involvement in a pathophysiological process or disease condition, or therapeutic applications of a molecule inducing a selective and specific modulation (e.g., inhibition or stimulation) (drug target validation). Part of the complexity and confusion of target validation arises from the multidimensional

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nature of the research directions and required data interpretation within various contexts. For example, studies performed in vitro by using functional genomic tools may not fully recapitulate the real situation in vivo, whereas the target can be modified with knockout or transgenic strategies to produce phenotypes of interest. Also, species-specific molecular, biochemical, and physiological mechanistic differences may be a potential source for the lack of translation to human clinical conditions. A concept in target validation that needs attention is the assumption that modulation of a certain molecular pathway may be fairly specific, whereas actually, in the in vivo situation, there are pleiotropic, divergent, and redundant processes and interactions that operate because of the cross-talk and connectivity of the signaling pathways. This cross-talk may result in additional biological effects (e.g., additivity, synergism, or antagonism) or in undesired side effects. Often, side effects and undesired toxicities may not be apparent in early and short-duration exploratory medicine or clinical studies.

Overall, target validation is a continuous process and the result of integrative approaches rather than a single step or a series of isolated events. One key consideration for target validation is the mechanistic connectivity between a molecular target and a biological process, which can be modulated and can be related to a pathophysiological pathway, where an association of a target with human genetics and functional genomics often exists. Technology platforms to identify and validate targets include multiple classes of tools with complementary principles, e.g., small molecules, peptides and antibodies, antisense, small-interfering RNA (siRNA), aptamers ***(1)***, ribozymes, gene knockouts ***(2,3)***, dominant negative mutants, and delivery systems, including vectors and carriers. However, there is no template for the application of various technologies to validate a particular target. The approach needs to be integrative with focus on incorporating scientific intuition with experience, e.g., into a flow scheme of testing while maintaining a flexible and continuous strategy of data-driven adjustments.

2. Automated High-Throughput Target Validation by Using siRNAs

Among multiple approaches, target validation often involves modulation of gene expression. Analysis of changes in gene expression could be achieved using various functional genomics tools, such as antisense, siRNA, and various viral vectors (***4***; Hahn; *see* Chapters 7, 9, 10, and 12). siRNA offers a compelling choice for in vitro target validation because of high potency, reliability, specificity, and a well defined mechanism of action at the mRNA level ***(5,6)***. Changes in mRNA levels are relatively easy to follow with reverse transcription (RT)-PCR and microarray assays. From a technological standpoint, automation offers a compelling solution to increase throughput and improve testing consistency of siRNA applications. Automated transfections of siRNA

Fig. 1. The Allegro™ High Throughput Screening system is amenable and adaptable to high-throughput siRNA strategies to assay for target validation. The Allegro system consists of a host PC coupled to robotic stations for the purpose of sequentially processing a large number of samples residing in 96- or 384-well microplates. The system accepts these samples as its input, processes them through precise combinations with the user-supplied reagents, and provides information and processed samples as its output. (image is a courtesy of Zymark Corporation). (From **ref.** *7* with permission.)

molecules have been reported previously *(7)*. In these experiments, fairly complex procedures were split into a series of relatively straightforward steps, each performed by an automated workstation. This approach is briefly illustrated by the following series of descriptions. **Figure 1** shows the automated high-throughput screening (HTS) platform that was used. For reliable application, the assays had been validated and reformulated in an automation-friendly format. **Figure 2** illustrates how a fairly complex experiment can be split in a series of relatively straightforward steps, each carried by an individual robotic workstation. Incorporation of reference controls allows for a quality control of each step in the process. A first step of high-throughput target validation may start with the delivery of a gene-modulating agent into the cells. This step can be monitored and verified by incorporating fluorescent tags into siRNA molecules. **Figure 3** illustrates this point. The successful transfection of siRNA molecules is not by itself a guarantee of gene-specific inhibition. The specific activity of siRNA needs to be validated using gene-specific RT-PCR assays to confirm downregulation of mRNA for the gene of interest. **Figure 4** demonstrates an example of RT-PCR analysis of siRNA-transfected

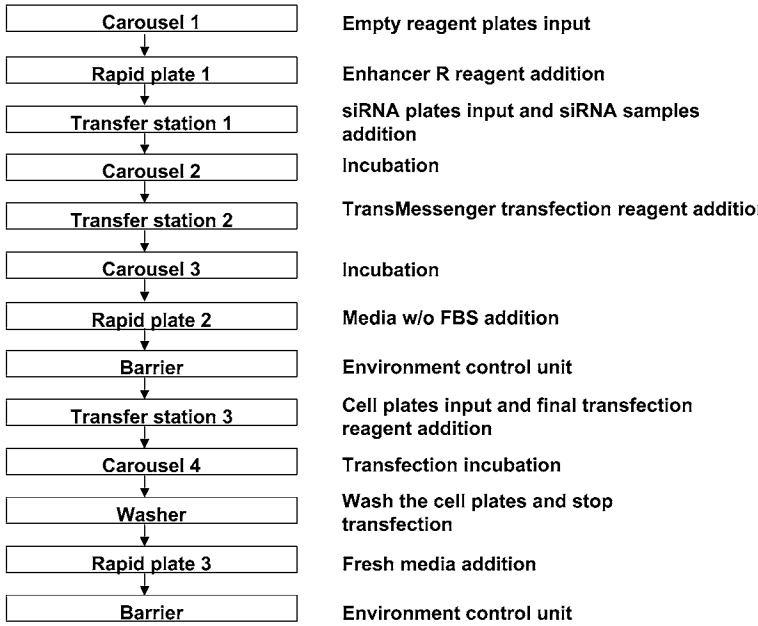

Fig. 2. The Allegro system (Zymark Corporation) modules and their operational specifics. The four modules enclosed by barrier units are environmentally controlled to maintain a temperature of 37°C. (From **ref.** *7* with permission.)

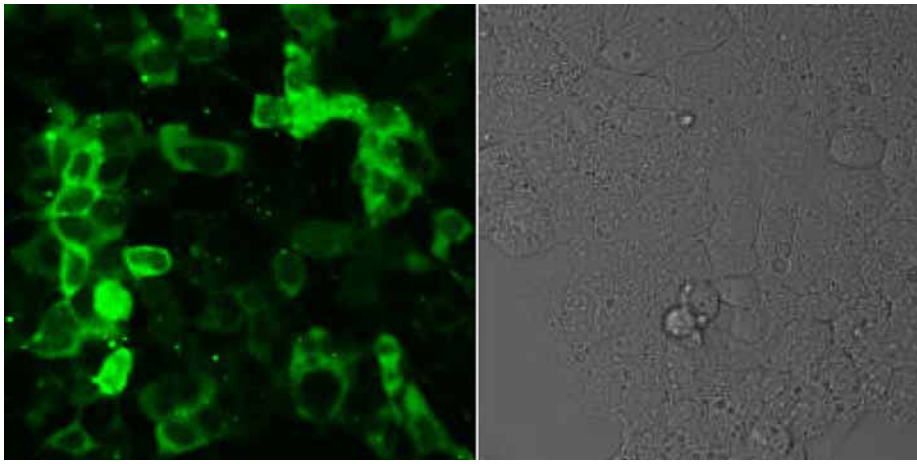

Fig. 3. Confocal laser scanning image of human embryonic kidney (HEK)293 cells transfected with siRNA. Incorporation of fluorescence at the 3' end on the sense strand allows for the monitoring of siRNA delivery. (From **ref.** *7* with permission.)

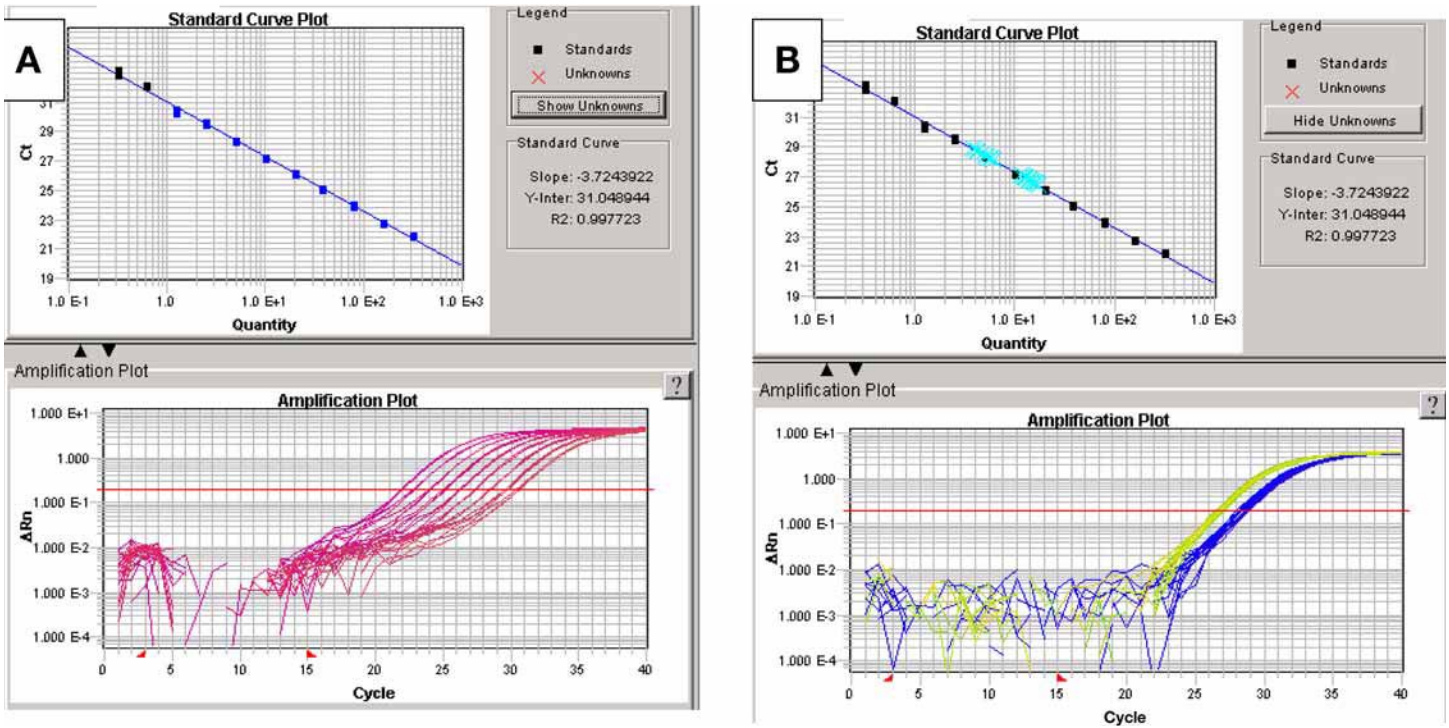

Fig. 4. TaqMan amplification plots showing amplification of reference samples and two groups of experimental samples. **(A)** Reference samples ranging from 625 ng of reverse transcriptase (RT) material to 61 pg of RT material were analyzed in triplicates. Threshold cycle (C_t) values were determined, and a standard curve plot was constructed using the SDS 2.0 Software package (Applied Biosystems, Foster City, CA). A linear relationship between amount of input material and C_t values was observed. **(B)** Standard curve plot was analyzed using SDS 2.0 to determine the amount of target transcripts in samples treated with control or target-specific siRNA. (From **ref.** *7* with permission.)

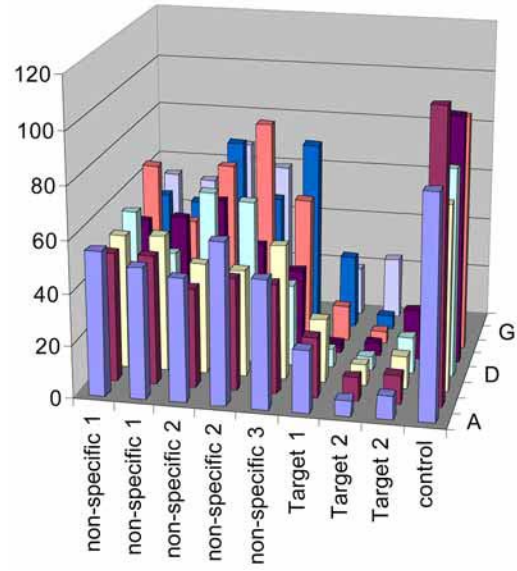

Fig. 5. Automated siRNA transfections were conducted in the 96-well format on the Allegro System. Target expression analysis was conducted on ABI Prism® 7900 HT (Applied Biosystems; http://home.appliedbiosystems.com). In this example, siRNA specifically and significantly downregulated appropriate transcripts, as detected by TaqMan quantitative RT-PCR. By designing functional assays to specific biological questions, siRNA can be used to validate targets in drug discovery. (From **ref.** *7* with permission.)

cells. RT-PCR analysis is also important to distinguish between target-specific and off-target effects of siRNA. If a gene of interest regulates a biological process, a positive relationship between the ability to downregulate the expression of a target gene and impact a biological process is expected. Thus, it is advisable to have several different siRNAs for each gene of interest (**Figs. 5** and **6**). RT-PCR assays used to be fairly time-consuming and involved hard-to-automate procedures *(8)*, but novel modifications such as those described by Maley et al. *(9)* take advantage of RNA capture plates, which simplify the assays. Modulation of gene expression is often linked to studies in in vitro and in vivo models that can be diverse and involve acute and chronic strategies where biological and behavioral observations can be associated with cellular and molecular outcomes.

It is important to note, however, that alternative splicing of a large number of genes and resulting protein isoforms with distinct posttranslational modifications increases significantly the number of potential molecular targets (individual proteins). Thus, focused inactivation of a single gene may have impact on multiple

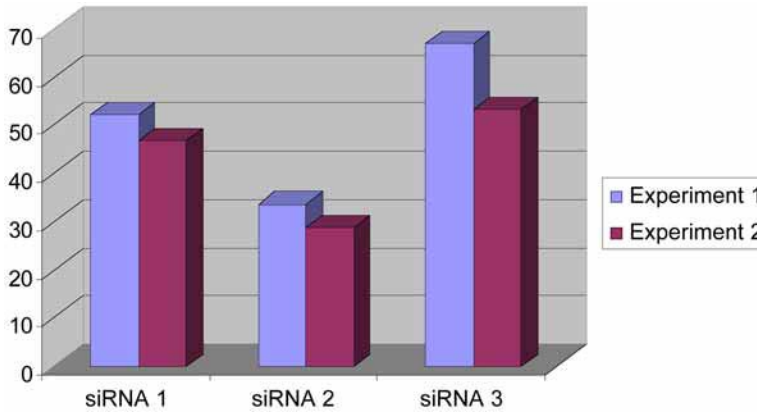

Fig. 6. Representative results in a growth inhibition assay for three different gene-specific siRNAs run in triplicate in two different experiments. Each bar represents the growth of siRNA-transfected cells as percentage of control (control or scramble siRNA-transfected cells, 100%). Growth inhibition was measured in vitro by using a sulforhodamine B assay. Cells were transfected with siRNA and allowed to grow for 72 h. (From **ref.** *7* with permission.)

proteins and functions, and caution is required when interpreting the results of a single gene modulation as evidence to assess the potential relevance of a protein as a drug target. Reduction or loss-of-function approaches to assess a protein's target validation include inhibitory peptides, aptamers, and monoclonal antibodies. Overall, the integration of data—including genetic profiling, relationship of mRNA with target protein levels as well as protein–protein interactions and derived analyses, e.g., genomics, proteomics/pharmacoproteomics, and bioinformatics—is pivotal within the context of critical "path" studies for target validation.

Automated high-throughput target validation by using existing HTS equipment offers significant advantages in terms of speed and consistency ***(10)***, but costs can be significant. For example, PCR confirmation, performed in triplicate, may cost US$0.8–$3.00, depending on the format of the assay for each of the transfected wells. Transfection and siRNA reagents further contribute to the cost, making large-scale, whole-genome experiments an expensive undertaking. An approach to cost reduction and improved efficiency is the transitioning of functional genomic studies to a microarray format. In this format, thousands or even tens of thousands of siRNAs or other gene expression-modulating reagents are printed on the slides in essentially the same way as DNA chip microarrays are fabricated. Several such systems have been successfully tested and described in the contemporary literature ***(11–15)***. For example, Carpenter

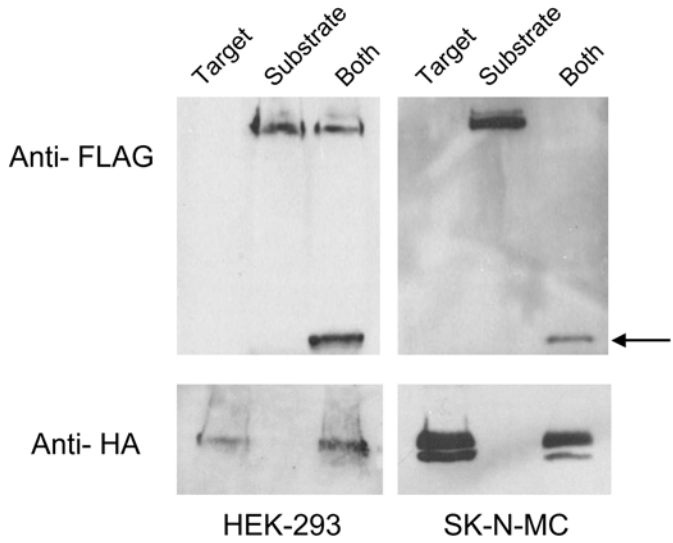

Fig. 7. Western blot analysis reveals a target-specific cleavage product. The target was detected by Western blot by using a hemagglutinin antibody conjugated to horseradish peroxidase (HRP); the substrate was detected using either a FLAG antibody–HRP conjugate (Sigma) or an HRP-conjugated antibody to the substrate. Cells (HEK293 or SK-N-MC) were infected with recombinant adenoviruses expressing either the target-green fluorescent protein (GFP)-HAHIS protein alone, or substrate-GFP-FLAGHIS protein alone, or coinfected with both of these adenoviruses. Western blots of crude cell lysates by using the anti-FLAG M2 monoclonal antibody (mAb) were used to visualize the pattern of substrate processed into C-terminal fragments (arrow). Representative Western blots are shown with the particular adenovirus, used to infect the two cell types, as indicated. The anti-HA mAb was used to detect the specific expression of the target-GFP-HAHIS recombinant protein. (From **ref.** *4* with permission.)

and Sabatini *(13)*, described an imaging-based readout performed on an siRNA array. However, even though these advances represent a promising trend, the approaches are continuously evolving and improving to solve current limitations (e.g., current physical boundaries between spots that do not allow extraction of spot-specific material for downstream applications). Translation from in vitro to in vivo testing and validation is undoubtedly a serious challenge. Although there is no single technology to answer all of the questions that may arise during this translation, certain strategies may at least help to prioritize the most promising targets. One opportunity is direct delivery of a pharmacological tool to a suspected site of action in vitro and in vivo. For example, in vivo studies using implanted minipumps helped to elucidate regulatory systems involved in the control of feeding and energy balance regulation under normal and

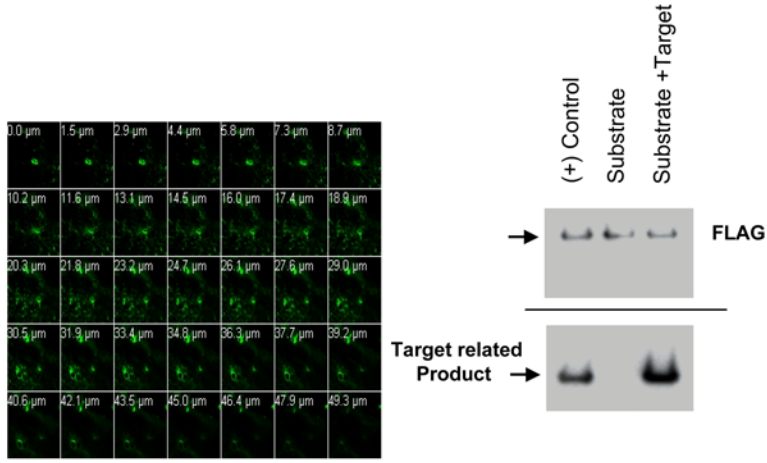

Fig. 8. GFP fluorescence in the mouse brain 48 h after intracerebral adenovirus infusion is visualized using laser scanning confocal microscopy. Western blot analysis reveals a target-specific cleavage product in samples prepared from brains cotransduced with both substrate and target protease-expressing adenoviruses. FLAG detection is used as a positive control. The positive control corresponds to the eluted fraction of the substrate and cleavage product purified using Anti-FLAG M2 agarose affinity gel target protease and substrate-expressing adenoviruses after transduction of HEK293 cells. (From **ref.** ***4*** with permission.)

pathophysiological conditions. These minipumps delivered precise doses of cytokines and peptides to target sites of action ***(16–19)***. Similarly, viral-based expression systems could be used for target validation by modulating expression of targeted genes and for monitoring biological consequences of this perturbation (**Figs. 7** and **8**; ***[4]***). In this example, expression of a target protease resulted in the accumulation of disease-specific biomarkers in several cell lines tested, and these findings were recapitulated in vivo by using intracranial injections of appropriate adenoviral constructs. Thus, this adenovirus-mediated gene transfer strategy resulted in an efficient and rapid target functionality validation, both in vitro and in vivo, and facilitated the transition between target validation and assay development for HTS.

Various examples of strategies and approaches for target validation have been briefly presented here. There are many others as well as permutations. Importantly, for human applicability, the definitive target validation occurs in normal biochemical and physiological homeostasis and in clinical medicine and clinical therapeutics.

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16

Regulatory RNAs

Future Perspectives in Diagnosis, Prognosis, and Individualized Therapy

Marjorie P. Perron, Vincent Boissonneault, Lise-Andrée Gobeil, Dominique L. Ouellet, and Patrick Provost

Summary

With potentially up to 1000 microRNAs (miRNAs) present in the human genome, altogether regulating the expression of thousands of genes, one can anticipate that miRNAs will play a significant role in health and disease. Deregulated protein expression induced by a dysfunctional miRNA-based regulatory system is thus expected to lead to the development of serious, if not lethal, genetic diseases. A relationship among miRNAs, Dicer, and cancer has recently been suggested. Further investigations will help establish specific causal links between dysfunctional miRNAs and diseases. miRNAs of foreign origin, e.g., viruses, may also be used as specific markers of viral infections. In these cases, miRNA expression profiles could represent a powerful diagnostic tool. Regulatory RNAs may also have therapeutic applications, by which disease-causing genes or viral miRNAs could be neutralized, or functional miRNAs be restored. Will bedside miRNA expression profiling eventually assist physicians in providing patients with accurate diagnosis, personalized therapy, and treatment outcome?

Key Words: microRNAs; miRNAs; small-interfering RNAs; siRNAs; RNA interference; RNAi; Dicer; individualized therapy; gene expression.

1. microRNAs as Novel Regulators of Gene Expression

microRNAs (miRNA)-guided RNA silencing is a recently discovered gene regulatory process by which endogenous miRNAs mediate translational repression of specific mRNAs through imperfect complementarity; whereas, RNA interference (RNAi) is referred to as the process initiated by exogenous small-interfering RNAs (siRNAs) that are designed to induce cleavage and degradation

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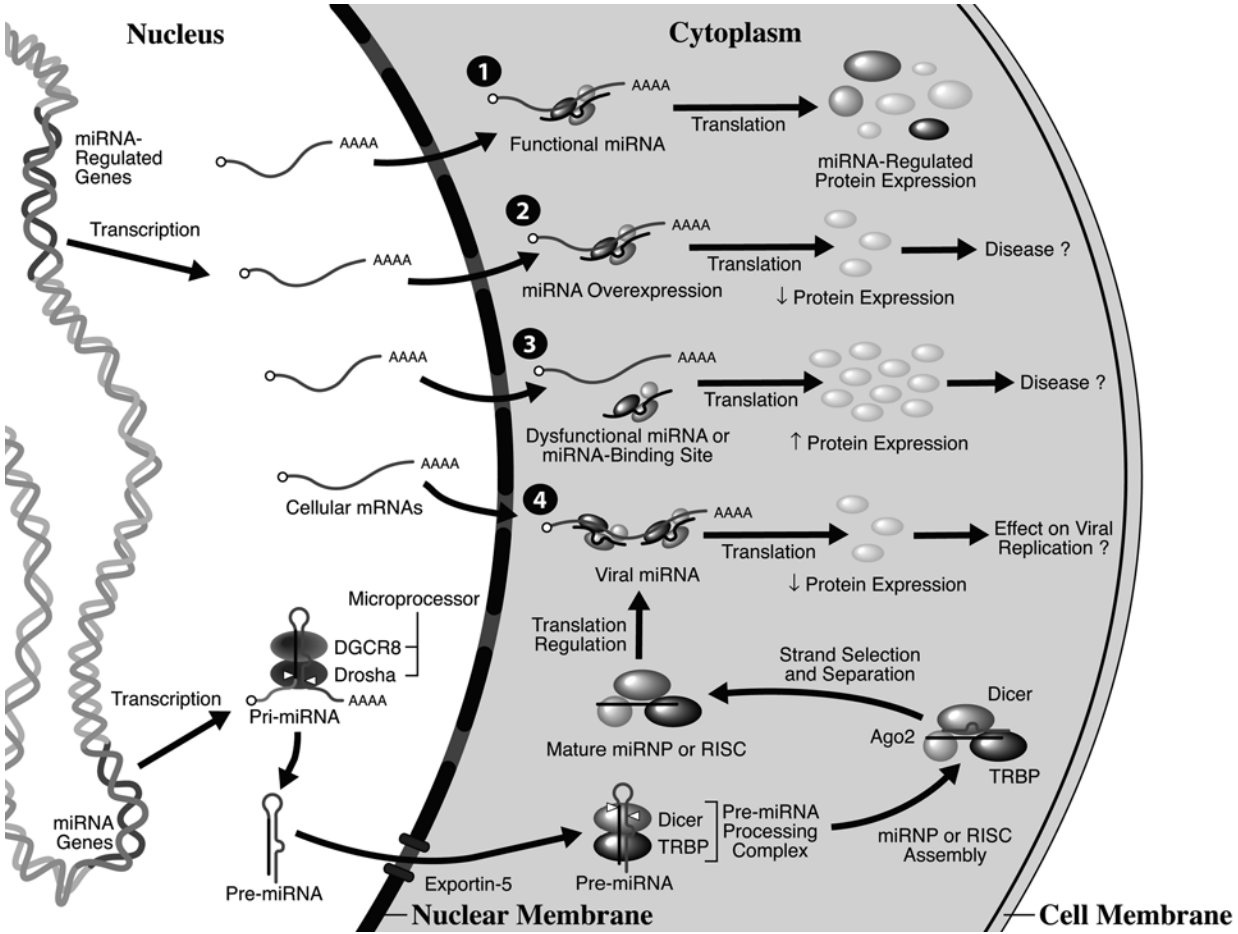
Nucleus
Cytoplasm
miRNA-Regulated Genes
Transcription
AAAA
Functional miRNA
Translation
miRNA-Regulated Protein Expression
miRNA Overexpression
↓ Protein Expression
Disease ?
Dysfunctional miRNA or miRNA-Binding Site
↑ Protein Expression
Cellular mRNAs
Viral miRNA
Effect on Viral Replication ?
Microprocessor
DGCR8
Drosha
Pri-miRNA
Translation Regulation
Strand Selection and Separation
Dicer
Ago2
TRBP
Mature miRNP or RISC
miRNA Genes
Pre-miRNA
Exportin-5
Pre-miRNA Processing Complex
miRNP or RISC Assembly
Nuclear Membrane
Cell Membrane

of specific mRNAs (*see* Chapter 9). siRNAs are thus a powerful tool to knock-down genes potently and specifically ***(1)***. Usually synthetized chemically, siRNAs are designed to be perfectly complementary to a targeted mRNA and are formed by two complementary strands of approx 21–23 nt with 2-nt 3' overhangs ***(1)***. siRNAs are phosphorylated at the 5' termini and hydroxylated at their 3'-ends ***(2)***, mimicking the endogenous miRNA:miRNA* duplexes resulting from Dicer processing of endogenous miRNA precursors (pre-miRNAs).

Several chemical modifications can be made to siRNAs to enhance some of their characteristics, such as their stability, cellular uptake, or intracellular distribution. Modifications affecting the second carbon of the pentose sugar have been widely characterized, such as 2'-*O*-methylation (2'OMe) and deoxynucleotides ***(3)***. siRNAs can also be obtained by the cleavage by Dicer of short-hairpin RNAs (shRNAs), which are transcribed from a vector or a PCR product containing this particular sequence. shRNAs are structurally homologous to the endogenous miRNAs precursors, with the difference that their stem is usually perfectly complementary. It is important to note that, when introduced into mammalian cells, siRNAs do not seem to induce an interferon response ***(1)***, opening up the possibilities for therapeutic applications.

miRNA genes can be found as clusters forming their own transcriptional units ***(4,5)***. For miRNAs that coincide with protein-encoding genes, the majority are found within introns ***(6)***. These observations strongly suggest that some miRNAs are coordinately expressed in parallel with their host proteins. Primary transcripts (pri-miRNAs) possess the signature of RNA polymerase (pol) II transcribed RNAs, characterized by a 5' methylguanosine cap and a 3' polyA tail ***(7,8)***. As shown in **Fig. 1**, the first processing step is initiated by the nuclear ribonuclease (RNase) III Drosha and produces an approx 60–70-nt stem loop named pre-miRNAs ***(4,9)***. The Drosha cleavage products harbor a classical 2-nt

Fig. 1. *(Opposite page)* Importance of microRNA (miRNA)-regulated gene expression in humans. **(1)** For more than 30% of the genes, miRNAs control the expression of their encoded proteins through recognition of their corresponding mRNAs. **(2)** miRNA overexpression may accentuate the degree of translation inhibition mediated by miRNAs. **(3)** Whereas loss of miRNA control, induced by a dysfunctional miRNA or miRNA-binding site, may lead to the overexpression of a specific protein and provoke the development of a disease. **(4)** miRNAs or small-interfering RNA can also originate from viruses and modulate host gene expression, which may result in facilitation of viral replication. Dysfunction of a protein involved in miRNA biosynthesis or function may affect miRNA expression globally and have more serious, if not lethal, consequences. TRBP, transactivating response RNA-binding protein; DGCR8, DiGeorge syndrome critical region 8.

3' overhang and a 5' phosphate that characterize cleavage of dsRNA substrates by members of the RNase III family *(10–12)*. This step is carried out in collaboration with the DiGeorge syndrome critical region 8 protein; this heterodimeric complex is known as the microprocessor *(13–16)*. The pre-miRNA is then exported from the nucleus to the cytoplasm by the Ran-GTP dependent transporter Exportin (Exp)-5 *(17–20)*. A second RNase III enzyme located in the cytoplasm, Dicer *(21–24)*, together with transactivating response RNA-binding protein (TRBP) *(25,26)*, catalyzes the second processing step by cleaving the pre-miRNA at the base of the loop to generate an imperfect miRNA:miRNA* duplex of approx 21–23 nt, which will be incorporated into an effector ribonucleoprotein (RNP) complex *(27)*, such as the RNA-induced silencing complex (RISC) *(28)*. Dicer and TRBP have recently been shown to be part of a functional RISC, thereby coupling the initiation and effector steps of RNAi *(29)*.

A difference between siRNAs and miRNAs resides in base pairing. Although siRNAs show a perfect complementarity between the nucleotides of both strands, excluding the 2-nt 3' overhangs, miRNAs show a variable number of mismatches that may confer a certain degree of instability *(30,31)*. Based on the thermodynamic stability of the duplex, a strand selection process will determine the identity of the strand to be incorporated into a functional miRNA-containing RNP (miRNP) complex *(29,32–34)*. The miRNA strand with the less stable 5'-end pairing is loaded into the miRNP, whereas the miRNA*, also called the passenger strand, is usually destroyed *(33)*.

First identified in *Drosophila* S2 cells *(28)*, the RISC is the best-characterized RNP complex in RNA silencing. It has been reported to contain Argonaute 2 (Ago2) *(35)*, the staphylococcal nuclease Tudor *(36)*, the vasa intronic gene protein *(37)*, and the fragile X mental retardation protein *(37,38)*. Although the function of these proteins within the RISC remains unclear, Ago2 has been shown to play a central role in RISC activity *(39–41)*. The PAZ domain of Ago2, a domain also present in Dicer, recognizes and binds the 3'-end of single-stranded RNAs *(42–44)*, whereas the PIWI domain binds the 5'-end *(45)*. PIWI, which shows structural similarity to RNase H *(46)*, is responsible for the mRNA cleavage activity in the RISC *(47)*. Human Ago2 binding to a mature miRNA can lead to the cleavage or translational repression of the mRNA target *(48–50)*.

2. Potential Regulatory Roles for miRNAs

As of May, 2006, 462 different miRNAs have been identified in human, which corresponds to approx 2% of the genome (http://microrna.sanger.ac.uk/) *(51)*. Little is known about their biological functions, but they may regulate more than 30% of the genes *(52,53)*. miRNAs bind to a mRNA by an imperfect

nucleotide base pairing. Generally, the critical miRNA:mRNA pairing region, referred to as the miRNA seed, is usually located in the 3' nontranslated region (NTR) of specific mRNAs and comprised of miRNA positions 2–8 in the 5' to 3' orientation. Perfect pairing of this miRNA region is a key parameter used by computational approaches that aim at predicting potential mRNA targets of miRNAs. Pairing of the miRNA 3' region appears to be less important, but may compensate a weaker binding of the 5' region ***(54)***. In humans, mRNA regulation by miRNAs is believed to consist mainly in translational repression, although a recent study reported that miRNAs downregulate a greater number of transcripts than previously appreciated ***(55)***. miRNA regulation of mRNAs is complex. For example, a given miRNA can regulate several different mRNAs. Conversely, a specific mRNA can be regulated by more than one coexpressed miRNA. This may allow for a finely tuned expression of a gene product whose levels are critical for normal cell function.

Experimental demonstration of a relevant miRNA:mRNA target interaction is arduous to achieve; only a few combinations have been experimentally validated thus far. A study of the let-7:lin-41 interaction in *Caenorhabditis elegans* by Vella et al. ***(54)*** has improved the understanding of the requirements for miRNA binding. The miRNA let-7 possesses six putative binding sites in the 3' NTR region of the mRNA lin-41. At least two sites are necessary for downregulation of lin-41 expression. The intervening 27-nt sequence also appears to be important for miRNA regulation ***(54)***, suggesting that miRNA–mRNA interactions do not rely solely to the regions of complementarity. A better comprehension of the factors involved in mRNA recognition by miRNAs will help conceive better predictive methods that are useful to limit the number of potential mRNA targets to be investigated.

Prior to initiating studies aimed at characterizing a miRNA–mRNA interaction of interest, computational approaches remain the method of choice to identify mRNAs possibly subjected to miRNA regulation, or to identify miRNAs possibly regulating a mRNA. Several different algorithms have been created and each of them uses different parameters for the sequence requirement of a miRNA–target interaction. They are designed to search for the miRNA seed and to determine the free energy of the interaction. They can also look for phylogenetic conservation and for more than one miRNA binding site in a given 3' NTR. Some bioinformatic predictive tools are available on internet: Miranda (http://www.microrna.org) ***(56)***, TargetScan (http://genes.mit.edu/targetscan/) ***(57)***, and Diana MicroT (http://www.diana.pcbi.upenn.edu/) ***(58)***. A new database web site, Argonaute (http://www.ma.uni-heidelberg.de/apps/zmf/argonaute/interface) ***(59)***, provides to scientists relevant information on different predictive algorithms, miRNA identification, and RNAi pathway components.

3. miRNAs and Their Potential Involvement in Diseases

miRNAs have been shown to regulate an increasing number of cellular processes. They can regulate development, cell proliferation, apoptosis, and other important physiological processes, as reviewed by Ouellet et al. *(60)*. Given their recognized importance in gene regulation, a link between miRNAs and several major diseases is expected. Defects in miRNA-mediated regulation of mRNA translation may lead to overexpression of specific proteins, which accumulation may cause diseases (*see* **Fig. 1**). This may be the case for mutated miRNA or miRNA-binding site on the regulated mRNA that can lead to a loss of mRNA translational control.

Clinical situations of genomic instability have brought support for a role of miRNAs in oncogenesis, as human miRNA genes have been found in fragile sites involved in cancer *(61)*. In the highly malignant human brain tumor glioblastoma, a strong overexpression of miR-21 has been observed *(62)*. This miRNA has been found to suppress apoptosis in this tumor, thereby contributing to the tumorigenesis process *(62)*. In chronic lymphocytic leukemias, the genomic region containing miR-15a and miR16-1 is deleted or downregulated *(63)*. The absence of these regulatory miRNAs allows for the overexpression of the antiapoptotic Bcl2 protein, which helps evade apoptosis *(64)*. miR-143 and miR-145 are downregulated in various human cancer cell lines, particularly those established from colorectal tumors *(65)*. Potential targets of these miRNAs have been previously implicated in oncogenesis *(65)*.

miR-155, whose precursor (pre-miR-155) was initially found to be highly expressed in pediatric Burkitt lymphoma (BL) *(66)*, was recently shown to be absent in primary cases of BL *(67)*. Although the exact link between miR-155 and BL is unclear, changes in miR-155 levels may clearly influence expression of its target genes, which remain to be identified.

The miR-17-92 cluster is often overexpressed in tumor samples from B-cell lymphomas *(68)* and human lung cancer cell lines when compared to normal cell lines *(69)*. These studies revealed that the miR-17-92 cluster can act as a potential human oncogene. The targets for this miRNA cluster, as predicted by using TargetScan *(57)*, include tumor-suppressor genes, suggesting that miR-17-92 overexpression can downregulate expression of these suppressor genes, and favor tumorigenesis *(69)*.

Members of the let-7 miRNA gene family are deleted in different forms of cancers *(61)*, supporting their implication in oncogenesis. A reduction of let-7 expression has been observed in human lung tumor samples or cancer cell lines *(70)*. In this study, patients showing a reduced let-7 expression had the worst prognosis after a potentially curative resection. In *C. elegans*, let-7 regulates let-60, the orthologue of the human *RAS* oncogene. Bioinformatic analyses revealed that the three human *RAS* genes contain multiple let-7 binding sites,

suggesting that let-7 may also regulate RAS expression in human. This is supported by an association between let-7 downregulation and an increased expression of RAS protein *(71)*, further implicating the loss of let-7-regulated RAS expression during the development of lung cancer.

Altered expression of a protein component of the miRNA-guided RNA silencing pathway may have a global impact on the expression of genes regulated by miRNAs. Indeed, a decreased expression of the RNase III Dicer was observed in nonsmall cell lung cancer (NSCLC) obtained from patients. This reduction is also associated with shorter postoperative survival *(72)*.

The emerging causal link between miRNAs and diseases suggest that health may lie on a delicate balance between the expression of miRNAs and that of the genes they are regulating. A shift in this balance may lead to a pathogenic downregulation or overexpression of the mRNA-encoded protein. Diseased organs or tissues may exhibit a unique set of miRNA expression profile, which could be used in improving diagnosis of diseases (*see* **Fig. 2**), such as cancer *(73,74)*.

In addition to cancer, cellular miRNAs can be implicated in host–virus interactions. miR-32 has been shown to restrict accumulation of the retrovirus primate foamy virus type 1 in human cells *(75)*. This virus encodes Tas, a protein inhibiting RNA silencing in mammalian cells, probably to attenuate the suppressive effects of miR-32.

As for miR-122, which is highly and specifically expressed in the liver, its sequestration caused a marked loss of autonomous replicating hepatitis C viral RNAs *(76)*. This miRNA was found to facilitate replication of hepatitis C viral by targeting the 5' NTR of its genome *(76)*.

Bioinformatical analyses aimed at identifying HIV-1 genes regulated by human miRNA targets yielded five potential targets: miR-29a and miR-29b may target the *nef* gene, miR-149, the *vpr* gene, miR-378, the *vpu* gene and miR-324-5p, the *vif* gene. Expression of these miRNAs was verified by microarray profiling of human T cells, hosts of HIV-1 infection *(77)*.

4. miRNAs as Potential Therapeutic Targets

The possible pathogenic consequences of a miRNA deregulation, associated with either miRNA downregulation or overexpression, makes them potential therapeutic targets. For example, in cases of BL cancer, miR-155 is highly overexpressed *(66,67)*. In this case, its neutralization by an antisense strategy using 2'OMe oligoribonucleotides perfectly complementary to the miR-155 sequence could be envisioned *(78)*.

It is known that viruses like Simian virus 40 *(79)*, Epstein–Barr virus *(80)*, and Kaposi's sarcoma-associated herpesvirus *(81)* encode viral miRNAs. These small RNAs may directly influence specific host and/or viral gene expression, possibly affecting antiviral host defenses or replication of the virus. Thus,

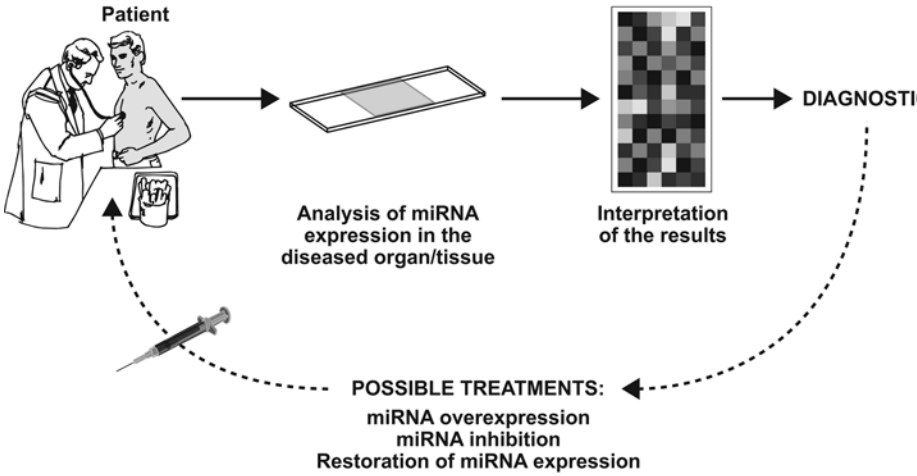

Fig. 2. Schematic illustration of the envisioned use of microRNA expression monitoring in personalizing diagnosis and medical treatment.

antisense 2'OMe oligoribonucleotides may also find applications as antiviral therapeutic agents.

In contrast, diseases, such as cancer, may also arise from a downregulated miRNA expression, as discussed for that of let-7 in the previous section. In that case, the objective would be the restoration of miRNA expression in the affected organ or tissue. Among the currently available approaches for such an intervention are the administration of miRNA duplexes, shRNA encoding cassettes, or even a viral vector encoding an shRNA.

Small-regulatory RNAs may also be used as therapeutic agents in treating non-miRNA mediated pathologies caused by overexpression of a specific gene. Here, approaches based on the administration of gene-specific siRNAs may be indicated.

Several obstacles that need to be surmounted before the launch of an RNAi-based therapy to fight genetic diseases are currently being addressed. For instance, the cost of an shRNA or siRNA therapy would be relatively high considering its relative unstability, the amount required to achieve therapeutic levels in a 75-kg human being and the anticipated duration and frequency of the treatment. As for the use of viral vectors for stable expression of therapeutic RNAs in vivo, it may face uncertainties related to gene therapy, such as immunological issues and long-term undesirable effects.

In vivo delivery of therapeutic RNAs represents one of the key hurdles to the use of RNAi as a therapeutic approach. Several aspects of delivery, such as targeting of specific tissues, cellular uptake, and genomic integration, in addition to the issues of specificity, need to be either optimized or developed. Among the

delivery methods being developed are cationic carriers, electroporation, and lentiviral-based approaches, as reviewed by Lu et al. ***(82)***. From the intense research activities on the fundamental and therapeutic aspects of RNAi in the academia as well as in biotechnology firms, we can anticipate major and significant advances in the field of therapeutic RNAs in a near future.

5. Personalized Medicine Based on Monitoring miRNA Expression

Several studies have shown that some diseases, such as cancer, are associated with a change in miRNA expression levels ***(63,70)***. But whether these changes are the cause or the effect of cancer remains to be determined. As for viral infections, miRNAs expressed from viruses like Epstein–Barr virus ***(80)*** and Kaposi's sarcoma-associated herpesvirus ***(81)*** may influence viral and host gene expression. These observations suggest that monitoring of miRNA expression levels in an affected organ or tissue may be used for diagnostic of a disease or an infection.

However, a clear link between an altered miRNA expression and a given disease first needs to be established. Comparative analyses require a "normal" range of miRNA expression in healthy organs or tissues to be defined. A number of methods that allow detection and quantification of the small miRNAs have emerged. A method to be useful for diagnostic purposes would need to be fast, reliable, reproducible, very sensitive (to detect miRNAs of lower abundance), and very specific, in order to discriminate between paralogous miRNAs that sometimes differ only from one nucleotide.

Northern blotting is a standard procedure to detect miRNAs. However, this method may not be sensitive enough, as some miRNAs are undetectable by Northern blot. In addition, it requires relatively large amounts of RNA, several separation and hybridization steps, and isotopic detection, which are far from ideal for clinical diagnostic. It is possible to improve the efficiency of Northern blot analyses by using locked nucleic acid (LNA)-modified oligonucleotide probes ***(83)***, which improve sensitivity by 10-fold via enhanced hybridization properties, and by using digoxigenin-labeled RNA probes to allow rapid (minutes to hours) and nonisotopic detection of miRNAs ***(84)***. LNA-modified oligonucleotides have also been used successfully to probe the presence of miRNAs in animal embryos *in situ* ***(85)***, an approach that could be applicable to cancer diagnosis.

In 2004, Hartig et al. ***(86)*** published a method using signal-amplifying ribozymes to detect miRNAs. This method is sequence-specific and highly sensitive, with a detection limit of 50 fmol miRNA in the reaction mixture. The probes that were used had been designed for detection of nucleic acids in vivo. However, their stability must be improved to avoid ribonuclease-mediated degradation.

Another method to detect and quantify miRNAs is the primer-extension PCR assay ***(87)***. In this assay, the first primer is used to convert an RNA template into

cDNA in order to introduce a universal PCR binding site and to extend the cDNA to facilitate subsequent monitoring by quantitative PCR. The reverse primer is LNA-modified to increase hybridization affinity and improve amplification. This method is inexpensive, sensitive (in the femtomolar range) and allows discrimination between miRNA family members. Recently, a method to quantify miRNA gene expression with a single molecule, called the Direct miRNA assay, has been published *(88)*. It uses two LNA-DNA oligonucleotide probes hybridized to the miRNA of interest. Because these oligos are spectrally distinguishable, every single tagged molecule can be directly counted on a detection instrument. This assay is fast, sensitive, and specific.

All the methods previously described cannot be used to study the overall expression profile of miRNAs. For that purpose, a modified Invader assay can be used *(89)*. This method is based on the hybridization of a miRNA to two desoxyoligonucleotides (probe and invasive oligonucleodide) that generates a structure that is specifically cleaved by a 5' nuclease (Cleavase). The released invasive oligonucleotide serves in a second cleavage reaction, which involves a fluorescent-labeled oligonucleotide substrate linked to a dye quencher. Thus, the level of miRNA can be measured by a simple fluorometric assay. This assay can be performed in unfractionated detergent lysates, is fast (2–3 h incubation time) and allows sensitive and specific high-throughput screening analyses.

Finally, the sensitive and semiquantitative microarrays are also suitable for high-throughput detection of miRNAs. Several studies using this technique have been reported during the last few years, with some of them adapting the assay to make it more specific and sensitive *(90–92)*.

As illustrated in **Fig. 2**, we can imagine that, in a near future, monitoring of miRNA expression will be used as a routine test for diagnosis and treatment of diseases. The first step would be the sampling of the affected organ or tissue, either by biopsy or withdrawal of body fluids such as plasma, saliva, or semen. The next steps would be RNA extraction, if necessary, followed by monitoring and analysis of the results by comparison with a normalized miRNA expression profile, as deduced from data obtained from a bank of normal healthy tissues or fluids. Interpretation of the results would be useful to the physician for establishing a diagnosis and offering various therapeutic avenues to the patient. If the disease is caused by a virus, the treatment would possibly aim at neutralizing the function of viral miRNAs. Whereas if the disease is caused by an abnormal miRNA or gene expression, the treatment would consist either at restoring miRNA expression or targeting the mRNA of the disease-causing gene for degradation.

From all the evidences gathered thus far, we have all the reasons to be enthusiastic about the eventual use of regulatory RNAs in diagnosis and personalized therapy.

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17

Treatment Options and Individualized Medicine

Mouldy Sioud and Øyvind Melien

Summary

Although several drug targets are identified, current strategies in therapy do not take into account that patients vary in their response to drugs, both with respect to efficacy and toxic side effects. Whereas both clinical and histopathologic predictors of prognosis are established in some diseases, a better understanding of the molecular mechanisms that determine treatment response should play an important role in the development of individualized medicine. Treatment optimization will rely on the ability to adjust treatment algorithms for use in the individual patient based on the identification and validation of the factors that critically determine treatment outcomes, including diagnosis, disease phase and characteristics, organ functions, age, and gender. Although the analysis of a single genetic marker (e.g., CYP polymorphisms) may yield significant information that predicts drug response, the prediction obtained from the analysis of several genetic and epigenetic markers is potentially more powerful in selecting patients for effective therapy, whereas sparing those who would not respond or would suffer undesirable side effects. In this chapter, several relevant examples are presented.

Key Words: Individualized medicine; genomics; proteomics; gene profiling; genetic variations; polymorphisms; breast cancer; lymphoma; leukemia.

1. Introduction

Although several factors may influence individual drug responses, including age, gender, disease state, and organ function, there is increasing evidence that a large portion of variability in drug response is genetically determined *(1,2)*. In addition to DNA polymorphisms that represent common variation in a DNA sequence leading to either reduced or increased activities of the encoded protein *(3)*, gene expression profiling has enhanced our understanding of disease mechanism, classification of tumor stages, and prediction of treatment outcome *(4)*.

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Recent studies obtained with microarrays in several malignancies indicate that it is possible to predict with high precision diagnosis, prognosis, and response to therapy *(4)*.

It should be noted that despite the development of modern molecular tools for studying individual diseases in great detail, several pathologies remain poorly understood and the identification of the genetic markers or expression signatures that affect response to therapy will also provide clinical guidelines for prognosis and treatment. Also, these studies may also reveal novel molecular targets for therapeutic intervention. Relevant genetic differences between patients may include mutations altering the function of a protein involved directly (the real target of the drug) or indirectly in drug activity and metabolism, thus rendering the patients either more or less susceptible to treatment by the drug in question *(5)*. Genetic difference may also have an effect on the expression levels of nontarget genes and proteins in cells, which may be relevant to the mechanisms underlying unwanted toxic effects precipitated by drugs *(6)*.

2. Influence of Genetic and Other Factors on Drug Effects in Cancers

Cancer progression is characterized by the accumulation of multiple genetic mutations, chromosomal instabilities, and/or epigenetic changes that cooperate to drive malignancy *(7)*. Also, changes in gene expression are further affected by the microenvironment. Indeed, it is now well established that the microenvironment of the tumor–host interface plays a proactive role during malignant disease progression, including the transition from carcinoma *in situ* to invasive cancer, tumor cell dissemination, and metastasis *(8)*. In addition to the heterogeneity of stroma cells, tumor cells themselves are heterogeneous with respect to gene expression and genetic alterations. For example, breast cancer and lymphoma comprise several pathological subtypes, indicating the presence of numerous combinations of mutated and/or aberrant regulatory proteins that are sufficient to sustain cell proliferation *(8,9)*. Likewise, each individual patient's tumor may have a unique signature of molecular defects. It is also possible that each metastasis, originating from the same primary tumors, may have a unique expression profile. Thus, in addition to the genetic make up of the patient genome, the cellular composition of each tumor is a crucial determinant of both the biological and clinical feature of an individual's disease.

Regarding cancer therapy, there are several known genetic alterations or factors/markers that are expected to affect drug response. These include, drug transporter proteins, drug metabolizing enzymes that activate, inactivate, or detoxify drugs, and drug targets. The identification and understanding of these markers has the potential to allow clinicians to select appropriate therapy, and, therefore, avoid adverse drug reactions and therapeutic failures.

However, as with all biological systems, the complexity arises out of the existence of several genetic variations that may affect drug pharmacokinetics and pharmacodynamics.

Notably, the completion of the human genome sequence has led to a detailed exploration of the DNA sequence (the genome), genes that are transcribed into mRNAs (the transcriptome), and the translated proteins (the proteome). The role of the genome in drug effects is based on the premise that genetic variation plays a key role in cancer risk and disease outcome ***(1,2)***. Polymorphisms represent common variation in a DNA sequence that may lead to reduced activity of encoded gene, but in some cases, also to increased activities ***(3)***. Unlike somatic mutations, they are stable and heritable. Polymorphisms include single-nucleotide polymorphisms (SNPs), micro-, and minisatellites.

Because of their important roles in drug pharmacokinetics and pharmacodynamics, genetic polymorphisms in phase I (oxidative) and phase II (conjugative) enzymes are likely to represent some of the most common inheritable risk factors associated with common disease phenotypes, such as adverse drug reactions. Cytochrome P450 monoxigenases (CYPs) are phase I enzymes that catalyze the oxidation of endogenous and exogenous compounds, and are responsible for the metabolism of greater than 90% of clinically prescribed drugs ***(10)***. CYPs can either detoxify anticancer drugs (e.g., epipodophyllotoxins, paclitaxel, *Vinca* alkaloids, and tamoxifen), or activate inactive prodrugs (e.g., cyclophosphamide). There are presently 57 genes in the human genome ***(10)***, of which the CYP1A, CYP2B, CYP2C, and CYP3A subfamilies are involved in the metabolism of anticancer drugs. In addition to the roles of these enzymes in metabolizing drugs, there is also evidence suggesting that polymorphisms in the CYP genes may be associated with some human cancers ***(11,12)***.

In addition to CYP genes, the situation is further complicated when one considers the impact of phase II enzyme on drug effects. These enzymes are involved in conjugating drug derivatives or the original parent drug for renal or biliary elimination. For example, glutathione-*S*-transferase (GST) conjugate glutathione to electrophilic molecules and oxidative metabolites ***(13)***. GST genes are highly polymorphic and several SNPs in GST genes that affect response to cancer drugs have been identified. For example, breast carcinoma patient with the 105 VV in *GSTP1* gene exhibited an improved progression free survival. Similarly, 105 VV homozygotes colorectal cancer patients who received 5'-fluorouracil (5'-FU)/platinum chemotherapy showed a survival advantage when compared with heterozygotes and isoleucine homozygotes ***(14)***.

A second important group of phase I enzymes is the UDP-glucuronosyl transferases (UGT), which catalyze the glucuronidation of several lipophilic

drugs *(15)*. Irinotecan, a semisynthetic derivative of the cytotoxic alkaloid camptothecin, is approved worldwide for the treatment of metastatic colorectal cancer. Although it increases survival, it also causes severe diarrhea and neutropenia in 20–35% of patients treated. Notably, irinotecan is converted by liver esterases to an active metabolite, SN-38, a potent DNA topoisomerase I inhibitor that is primary inactivated UDT1A1 into SN-38 G, which is then excreted into bile and urine. However, reduced activity of UDT1A1 places patients at high risk for severe diarrhea and leucopenia induced by SN-38. Null mutations UDT1A1 lead to Criggler–Najjar syndrome, whereas less complete defects are associated with Gilbert's syndrome. The most common functional polymorphism is a A(TA)6TAA repeat in the promoter region of *1A1* variant. The most common form carries six TA repeats, whereas *UGT1A1*28* carries seven repeats that are associated with lower UGT activity.

In addition to genetic variations in drug metabolizing phase I and II enzymes, several other polymorphisms that are involved in drug response have been described *(16)*. 5'-FU adjuvant chemotherapy is now considered the standard treatment for stage III colorectal cancer. 5'-FU, an analog of uracil, is converted intracellularly into three main active metabolites, which inactivate the thymidylate synthase (TS). The effects of 5'-FU are counteracted by certain genetic variations in the tandem repeat sequences of the promoter enhancer region (*TSER*) of the thymidylate synthase gene, which is the case in the presence of the three repeat form *TSER*3* resulting in higher expression levels of TS *(17)*. Other enzymes such as dihydropyrimidine dehydrogenase (DPD) are also important in drug activity. Deficiency in DPD activity, however, leads to severe fatal toxicity following 5'-FU therapy *(18)*. Therefore, it is important to identify patients carrying defects or polymorphisms in the genes TS and DPD prior to starting therapy.

An important genetic variation, which has been related to drug effect is the CCND1 870A > G polymorphism in the gene for cyclin D1. The presence of this polymorphism has been reported to affect the outcome of childhood acute lymphopblastic leukaemia possibly by influencing the sensitivity to methotrexate treatment *(19)*. Furthermore, certain mutations in the epidermal growth factor receptor have been demonstrated to correlate with the response to tyrosine kinase inhibition exerted by gefitinib in a subgroup of patients with nonsmall-cell lung cancer *(20)*.

Of note, the most important downstream mechanism of cytostatic drug activity is the induction of apoptosis. Therefore, its deregulation may lead to drug resistance and survival of cancer cells despite that target proteins in cancer cells have been successfully targeted by anticancer drugs. Today, there are several active anticancer drugs and large number of combination therapies that are effective in both the adjuvant and metastatic treatments. However, no

chemotherapy regimen is universally effective for all patients with the same tumor type. Although there are some available guidelines for selecting patients who are candidates for adjuvant chemotherapy, it is currently impossible to identify which of these regimens will work for a given patient before initiating therapy. Therefore, the question arises as to which particular drug and which combination of drugs is most suited for an individual tumor. In the case of breast cancer, steroid receptors and Her-2 are the tumor-based markers that are now accepted in clinical practice, having an established role in predicting hormone sensitivity or in Herceptin treatment, respectively ***(21)***. The serine protease uPA and its endogenous inhibitor uPA are used in selecting node-negative patients who may not need to receive adjuvant chemotherapy ***(22)***, thus avoiding toxic side effects of this treatment. uPA is implicated in cancer growth, invasion, and metastasis ***(23)***.

Although clinical therapy failure of tumors is multifaceted, the acquisition of multidrug resistance in cancer cells is often associated with increased expression of ATP-binding cassette transporters, which protect cancer cells through the efflux of anti-cancer drugs ***(24)***. About 50 genes encoding ATP-binding transporters, such as *ABCB1* (*MDR1*), *ABCC2* (*MRP2*), and *ABCC3* (*MRP3*), exist within the human genome, and several polymorphisms in *MDR1* gene have been identified ***(25)***. Therefore, it is possible that specific haplotypes of the *MDR1* gene might determine the efficacy and toxicity of anticancer drugs.

Considering the multitude of molecular defects in tumor cells, it may be naive to expect that a single marker could provide a highly accurate response predictor. Therefore, instead of measuring individual markers, it is desirable to combine several markers, including gene expression profiles. With a microarray, the expression of tens of thousands of genes in a tumor biopsy can be determined simultaneously. Recent studies demonstrated that microarray could predict outcomes in cancer patients (***4***; *see* Chapters of 4 and 5, Volume 1).

2.1. Gene Profiling as Part of Drug Therapy

Today, the selection of treatment is not based on either DNA variations or the molecular characteristics of the tumor, but instead consideration of the patient's prior treatment history, disease state, general activity of the drugs and regimens in question, and their expected benefits for the patient as well as the familiarity of the physician with particular drugs. Because of these uncertainties, the individualization of chemotherapy selection based on molecular characteristics of the tumor is highly desirable. Recent technologies, such as microarrays and proteomics, now afford scientist and clinicians the ability to analyze gene expression for the complete coding sequences as well as to study genome-wide DNA sequence variation ***(26)***. By providing complete genetic and genomic information, these molecular techniques, in particular microarrays will help

clinicians to predict drugs that may increase effects and decrease side effects. A particular good example of this concept is in the diagnostic entity of diffuse large B-cell lymphoma *(27)*, which has a very heterogeneous outcome pattern. Also, gene microarray studies have been able to subclassify several distinct gene expression patterns that correlate with distinct patient outcome patterns, including breast and hematologic malignancies *(27–29)*. One of the aims of these studies is to develop signatures to distinguish tumors with a favorable prognosis from those with a poor prognosis. In this respect, several subtypes of acute myeloid leukemia that may require different treatment have been identified. In most, if not all, studies, the distinct groups identified via gene expression profiling are not apparent by traditional histopathological categorization or genetic analysis of the tumors. This molecular information should have relevance for diagnostic subclassification of tumors and more importantly provide valuable information for therapeutic targeting. Clearly, medical, legal, and economic issues all play a role in the implementation of prospective gene profiling at the bedside.

3. The Influence of Genetic Variations on Drug Effects in Other Diseases

The increasing focus on the role of genetic variation in drug targets and their downstream signaling pathways will extend the pharmacogenetic knowledge platform from the field of drug metabolizing enzymes into the clinically important target area. Thus, a number of genetic polymorphisms have been detected in drug targets and signaling elements, some of which have attained interest with regard to pharmacological treatment responses.

The group of G protein-coupled receptors (GPCR), of which there are approx 1000 different members, represent the molecular targets for at least 50% of current drugs and, thus, draw particular attention with regard to the significance of their genetic variation. Also, the heterotrimeric G proteins that are directly activated in response to GPCR stimulation contain SNPs, which appears to affect drug responses as well as some intracellular elements.

In the fields of cardiovascular diseases there are several examples of genetic variants affecting drug responses including polymorphisms in the genes for GPCRs such as β_1-adrenoceptors and angiotensin II receptors, G protein subunits and the angiotensin-converting enzyme (ACE). The use of β-adrenoceptor blockers is standard therapy in heart failure established on the basis of large clinical trials. There is, however, a variation in the response to this treatment, which appears, at least in part, to be related to polymorphisms in the β_1-adrenoceptor. The impact of genetic polymorphisms at codon 49 (Ser or Gly) and 389 (Gly or Arg) have been explored and recently it was reported that heart failure patients with the genotype Arg389Arg and Gly49 exhibited a more favorable response to

β-adrenoceptor blockers than other patients when assessed as improvement in left ventricular ejection fraction ***(30)***. Also, a polymorphism in the β_2-adrenoceptor has been reported to affect drug response in heart failure patients ***(31)***. Thus, patients homozygous or heterozygous for the Glu^{27} polymorphism responded better to karvedilol, a combined nonselective β-adrenoceptor blocker and α_1-adrenoceptor blocker.

Inhibitors of ACE also constitute a fundamental therapeutic strategy in cardiovascular disease; i.e., in heart failure, hypertension, as well as in diabetic and nondiabetic nephropathy. However, the response to these inhibitors varies and the role of the so-called insertion/deletion (I/D) polymorphism of the *ACE* gene ***(32)***, denoting the presence (I) or absence (D) of a 287-bp element in intron 16 on chromosome 17, has been evaluated with relation to pharmacogenetics. Whereas the presence of the D allele has been linked to higher ACE activity ***(33)*** as well as increased mortality in heart failure ***(34)***, it has been found that the ACE DD genotype appears to favor a beneficial effect not only of ACE inhibitors, but also in response to β-adrenoceptor blockers ***(35)***.

Several polymorphisms in the genes for angiotensin II receptors have been described, and the SNP A1166C in the angiotensin II type 1 receptor has been associated with essential hypertension, myocardial infarction, and preeclampsia as well as other conditions. In addition, a role for the A1166C polymorphism with regard to pharmacological responses is suggested by studies of angiotensin II receptor 1 blockade ***(36)***. Based on experiments infusing the active metabolite of the angiotensin II receptor 1 blocker losartan, EXP3174, in hypertensive patients, it was found that under conditions of a high-salt diet the systolic blood pressure was reduced by 6.6%; i.e., 12 mmHg, in the AA patients, whereas in the CC patients the reduction was only 1.3%, i.e., 2 mmHg.

Also a polymorphism at the level of heterotrimeric G proteins is reported to affect drug responses. The C825T polymorphism of the $G\beta_3$-subunit, which originally was found to be associated with hypertension, appears to enhance signaling mediated through the inhibitory G_i protein ***(36)***. It was found that clonidine, a selective agonist acting on central α_2-adrenoceptors, and used as an antihypertensive drug resulted in a more pronounced reduction in systolic blood pressure in healthy individuals carrying the 825T allele ***(37)***. This could be interpreted as based on a more efficient signaling elicited by clonidine through the α_2-adrenoceptors in the 825T allele individuals. Also the response to nitroglycerin assessed as venodilatation was reported to be more pronounced in young, healthy individuals carrying the 825T allele of the $G\beta_3$-subunit protein, thus suggesting that heterotrimeric G proteins are implicated in the pharmacodynamic mechanisms of this drug ***(38)***.

Genetic polymorphisms on the receptors of blood platelets have been detected, and it is possible that some of these may influence drug responses.

Polymorphisms in the heterodimer glycoprotein (GP) IIb-IIIa ($\alpha_{IIb}\beta_3$), which is an integrin and fibrinogen receptor acting as the ultimate regulatory step preceding platelet aggregation and thrombus formation, has attracted particular interest as a target for antagonists like abciximab, tirofiban, and eptifibatide. The $P1^A$ polymorphism affecting the glycoprotein IIb-IIIa receptor has been explored both as disease risk factor as well as a modifier of drug response not only for the antagonists of the GP IIb-IIIa receptor, but also for the effects of aspirin ***(39)***. The clinical and therapeutic significance of the $P1^A$ polymorphism remains to be more precisely defined.

Therapy with 3-hydroxy-3-methylglutaryl-coenzyme A reductase (statins) decrease total and low-density lipoprotein cholesterol and has proven to be effective for cardiovascular risk reduction. However, considerable interindividual variation exits in response to therapy. Several SNPs across many genes known to affect cholesterol synthesis, absorption, and transport and statin metabolism were identified. Some gene variants seem to predict whether patient will benefit from treatment with statins ***(40)***.

As mentioned earlier, cytochrome p450 is a major drug-metabolizing enzyme that catalyzes the oxidative metabolism of several clinically used drugs, including some anticoagulant and antihypertensive drugs. An adenine to cytosine (A>C) transversion (*CYP29*3*) produce a protein variant with reduced activity for metabolizing warfarin, an anticoagulant. Individuals who are homozygous for *CYP2C9**3 are more likely to experience bleeding events as compared to those with the wild-type genotype. Thus, screening for CYP2C9 variant may therefore allow clinicians to develop dosing protocols and surveillance techniques for reducing the risk of adverse drug effects in patients receiving warfarin or acenocoumarol for anticoagulant therapy ***(41)***. Recent discoveries have also identified variations in the gene for vitamin K epoxide reductase complex 1 (VKORC1) to affect warfarin responses ***(42)***, and a treatment algorithm including patient age and height in addition to CYP2C9 genotype and VKORC1 genotype has been shown to assess around 55% of the variability in warfarin dose level ***(43)***.

In the field of lung diseases the impact of pharmacogenetics has attracted considerable interest especially related to asthma therapy. The pharmacological strategies toward asthma are directed against bronchoconstriction as well as inflammation and genetic variations in key target molecules for drug action have been identified. Thus, SNPs in the genes for the β_2-adrenoceptor and adenylyl cyclase as well as changes in the promoter for the 5-lipoxygenase gene have been related to altered drug response. Patients homozygous for the Arg-16 genotype affecting the amino acid residue 16 in the β_2-adrenoceptor were reported to be more susceptible to asthma exacerbations and these patients improved when discontinuing the β_2-adrenoceptor agonist treatment and

changed to ipratropium bromide ***(44–46)***. The fact that several SNPs, at least 13, are present in the β_2-adrenoceptor gene attracted the attention to evaluate the role of haplotypes. The 13 SNPs was found to be organized into 12 haplotypes out of 8192 possible combinations ***(47)*** and comparisons of haplotype structures to SNPs showed a more prominent association between bronchodilation and haplotype than between bronchodilation and SNPs ***(47)***. This may agree with the notion that evaluations of haplotype associations to drug responses can be more clinically relevant than assessments of the role of SNPs.

The β_2-adrenoceptor mediates its signaling through activation of adenylyl cyclase, and it was recently reported that the polymorphism Met772 in the adenylyl cyclase subtype 9 increased signaling exerted by the β_2-adrenoceptor under certain conditions ***(48)***. In line with this it was found that the β-adrenergic induced bronchodilation was increased in human asthma in the presence of the Met772 polymorphism when corticosteroids was given concomitantly.

Leukotrienes are implicated in asthma and inhibitors of these mediators are established as therapeutic agents. 5-lipoxygenase (ALOX5) is one of the enzymes producing leukotrienes resulting in bronchoconstriction, and drugs that counteract ALOX5 pathways may improve asthma. However, this is not the case with all asthma patients, and it was hypothesized that a lack of response to inhibition of the ALOX5 pathway would reflect that leukotrienes could not be involved in the underlying pathogenetic mechanisms of asthma in these patients ***(49)***. The promoter region for the ALOX5 gene has been shown to contain binding sites for transcription factors and genetic variations were detected in this area. In addition, it was reported that 6% of asthma patients did not have the wild-type ALOX5 core promoter locus and that patients belonging to this group did not respond to ALOX5 inhibition. These findings thereby illustrate how knowledge of individual genetic characteristics may have an impact on therapeutic strategies.

Genetic polymorphisms may also play a role for the effect of drug therapy in infectious diseases. In the case of infections with HIV-1 it has been found that the G protein-coupled chemokine receptors CCR5 and CXCR4 act as major coreceptors for virus cell entry. Furthermore, particular genetic polymorphisms detected in coreceptor genes are reported to cause resistance to HIV-1 infection and a delay of progression to AIDS ***(50)***. Notably, a recent report also suggests that the presence of particular genetic polymorphisms in HIV patients, CCR2-64I or CXCL123'A, are beneficial for the response to highly active antiretroviral therapy ***(51)***.

Heterotrimeric G proteins might somehow be involved in the susceptibility to the combination treatment of hepatitis C infection using interferon-α and ribavirin. The C825T polymorphism of the $G\beta_3$-subunit protein is reported to affect the response to this treatment in hepatitis C patients ***(52)***. It was found

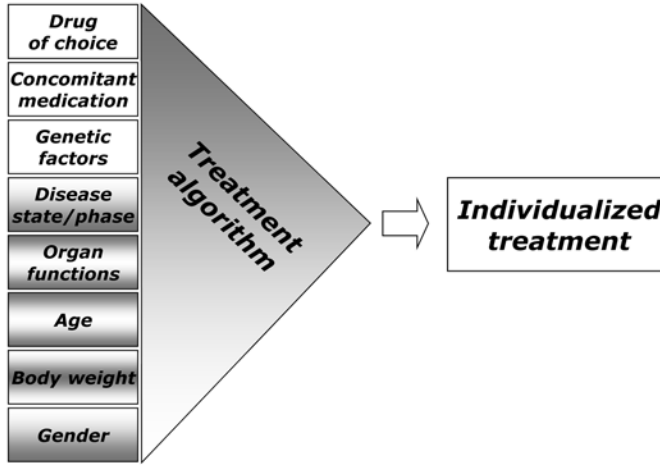

Fig. 1. Treatment algorithms in pharmacotherapy will depend on the development of models that can integrate relevant determinants with influence on drug response in combination with adjustments to the individual patient.

that patients with the GNB3 825 CC genotype did not respond the interferon-α/ribavirin treatment, albeit the explanation for this is unclear. However, it has previously been reported that the GNB3 825 CC genotype is associated with impaired immune function *(53)*. This observation may agree with recent findings that sudden infant death as a result of infection also appears to be associated with the GNB3 825 CC genotype *(54)*.

4. Toward Algorithms in Individualized Medicine

The efforts to establish a modern platform for individualized therapy is a demanding challenge to medicine. The evaluation of a rational approach to identify and handle a complex set of variable factors, which may influence drug treatment and responses, is critical. These factors include genetic variability of the drug metabolizing enzymes, drug transporters, drug targets and their signaling pathways, and as well drug–drug interactions and food–drug interactions in addition to disease state and phase, organ function/dysfunction, age, body weight, and gender. A future direction in pharmacotherapy might be to develop treatment algorithms **(Fig. 1)** for single drugs or drug regimens taking into account the relevant subset of critical determinants with adjustments to the single patient and further linked to practical implementable procedures. Algorithms aiming to integrate the premises of advanced pharmacological therapy can support a higher precision in individual treatment both with respect to optimize drug effects and to reduce the risks for unwanted toxic reactions.

The forces that can promote the previously described scenario are both the demands expressed by the patients themselves and the health authorities and in addition the results of ongoing scientific progresses, which continuously illustrate and update theses options. At the same time, it is essential to be aware of the gradual change in target molecule area for medical treatment and the impact these changes should have for establishing an individualized therapy. As a result of the last decades' intensive research and characterization of intracellular signaling pathways, there is an increasing number of novel drugs directed against molecular targets inside the cells. Several of these targets are key elements in cellular pathways in accordance with their potential of more pronounced therapeutic effects. However, because these key targets may be involved in other pathways that are also critical for different cell functions, there is a simultaneous risk for as pronounced deleterious effects. The success of using drugs acting on central intracellular molecules may thus depend on even more sophisticated and precise strategies to select the appropriate patients (based on diagnostic and disease phase criterias) and as well the ability to establish modes of specific cell delivery to ensure that the drugs predominantly reach the relevant targets. In other words, the progresses in signaling research, which contributes with novel and potentially powerful target options, may thereby further extend the challenge concerning a broad and long-term project for developing individualized therapy in medicine.

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