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Invited review

Targeting leukemic fusion proteins with small interfering RNAs: recent advances and therapeutic potentials[1]

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Abstract

RNA interference has become an indispensable research tool to study gene functions in a wide variety of organisms. Because of their high efficacy and specificity, RNA interference-based approaches may also translate into new therapeutic strategies to treat human diseases. In particular, oncogenes such as leukemic fusion proteins, which arise from chromosomal translocations, are promising targets for such gene silencing approaches, because they are exclusively expressed in precancerous and cancerous tissues, and because they are frequently indispensable for maintaining the malignant phenotype. This review summarizes recent developments in targeting leukemia-specific genes and discusses problems and approaches for possible clinical applications.

Introduction

The discovery of RNA interference in 1998 by Fire and coworkers provided an unanticipated new approach for studying gene functions in many different cell types and species from various kingdoms[1,2]. RNA interference (RNAi) is induced by exogenously introduced double-stranded RNAs (dsRNAs), RNA viruses, transposons and endogenous short dsRNAs, and may be an ancient defense system against viral infections and other genetic invaders[3]. The term RNA interference was first introduced in 1998, after the discovery by Andrew Fire and collaborators that injection of long dsRNA into *Caenorhabditis elegans* led to efficient and specific gene silencing[1]. However, in contrast to the situation in invertebrates, introduction of dsRNAs longer than 30 base pairs into mammalian somatic cells activates interferon responses, finally leading to a general inhibition of gene expression. Nevertheless, the identification of small interfering RNAs (siRNAs), double-stranded RNAs of 21 to 28 nucleotides in length, as intermediates of the RNAi process paved the way for applying RNAi in mammalian cells[4]. Because of their short lengths, siRNAs only rarely induce interferon responses, but cause an efficient and sequence-specific inhibition of gene expression. An increasing number of vector systems for the ectopic expression of small hairpin RNAs (shRNAs), the intracellularly expressed cousins of siRNAs, complement improved chemical synthesis of siRNAs for transient applications. All these advancements have allowed RNA interference to become a standard tool in molecular and cellular biology within 3 years. Moreover, both siRNAs and shRNAs have been successfully applied *in vivo* in animal models. Because of its high reproducibility, specificity and efficacy, in combination with rapid advances in the delivery of RNAi-inducing molecules, RNAi holds great promise for the development of new therapeutic strategies to combat diseases such as cancer[5,6].

Leukemia is a malignant disease of the hematopoietic system. Leukemic cells are characterized by impaired differentiation and increased proliferation potential, leading to an expansion of the leukemic clone and the replacement of normal hematopoiesis. In contrast to most types of solid tumors, more than 50% of all leukemia cases are associated

with distinct chromosomal changes, such as translocations or inversions. Moreover, leukemias tend to exhibit a higher genetic stability than solid tumors. For these 2 reasons, molecularly defined approaches targeting tumor-specific genes such as fusion genes may be more promising for leukemias than for solid tumors.

The leukemic fusion genes generated by chromosomal translocations are hallmarks of human leukemias. Prominent examples are the *BCR-ABL* fusion gene, which is associated with chronic myeloid leukemia and acute lymphoblastic leukemia (ALL), and *AML1/MTG8*, which is involved in acute myeloid leukemia (AML). Kinase fusion genes such as *BCR-ABL* or *TEL-PDGFβR* directly disturb signal transduction pathways and provide proliferative and survival advantages. Chimeric transcription factors such as *AML1/MTG8*, *PML-RARα* or *MLL-AF4* affect histone and DNA modifications and impair differentiation and induce cell death. If such fusion genes still played central roles in the maintenance of leukemia, they could be very promising siRNA targets for molecularly defined treatments, thereby complementing established treatment protocols or new, small molecular drug-based strategies such as inhibition of histone deacetylation or DNA methylation[7–9].

Here, we summarize a selection of such approaches to knockdown leukemic fusion proteins, and discuss the possible applications of RNAi in the development of new therapies for leukemic diseases. A overview of several fusion transcript-specific siRNAs is given in Table 1.

Short overview of the siRNA mechanism

Naturally occurring siRNAs are generated from long double-stranded RNAs by the RNase III-type enzyme Dicer. The cleavage creates 5'-phosphate and 3'-hydroxyl termini and yields 21–28 long double stranded RNAs with 2 nucleotide-long 3'-overhangs[10]. The siRNAs associate then with a multiprotein complex to form the RNA-induced silencing complex (RISC; Figure 1). RISC is activated by unwinding the siRNA duplex and discarding one of the strands. Recent data suggest that a Dicer-containing protein complex may also facilitate these 2 steps[11]. The remaining strand guides RISC to complementary RNA sequences. The RISC component AGO2 cleaves the target sequence 11 nucleotides away from the 5'-end of the siRNA, leading to the rapid degradation and, thus, inactivation of the target transcript[12,13]. Alternatively, particularly in the case of imperfect homology between target sequence and guide strand, RISC may not cleave the target transcript, but may instead inhibit its translation.

Translational interference without degradation of the transcript is also a feature of endogenously expressed micro RNAs (miRNAs). The eminent roles of miRNAs in the regulation of gene expression and the consequences for cellular processes such as differentiation or proliferation have only just recently come to the fore. In contrast to siRNAs, animal miRNAs have an imperfect homology to their binding sequence, which seems to be responsible for the inability of

Table 1. siRNAs targeting leukemic fusion transcripts.

Fusion transcript	Variant	Sequence	si/shRNA	Comments
AML1/MTG8	NA	5'-CCUCGAAAUCGUACUGAGAAG-3' 5'-UCUCAGUACGAUUUCGAGGUU-3'	siRNA	Inhibition of clonogenicity and proliferation, induction of senescence and differentiation[73,74]
BCR-ABL	b3a2	5'-GCAGAGUUCAAAAGCCCUUdTdT-3' 5'-AAGGGCUUUUGAACUCUGCdTdT-3'	siRNA	Inhibition of proliferation[30]
	b3a2	5'-GCAGAGTUUCAAAAGCCCTT-3' 5'-GGGCUUUUGAACUCUGCTT-3'	siRNA	Induction of apoptosis[29]
	b3a2	5'- CAGAGUUCAAAAGCCCUUCAG-3' 5'-CUGAAGGGCUUUUGAACUCUGCU-3'	siRNA	Inhibition of proliferation, enhanced drug sensitivity[38]
	b2a2	5'-UAAGGAAGAAGCCCUUCAGTT-3' 5'-CUGAAGGGCUUCUUCCUUATT-3'	siRNA	Induction of apoptosis, inhibition of proliferation[31]
	b3a2	5'-GCAGAGUUCAAAAGCCCUUUUCAAG AGAAAGGGCUUUUGAACUCUGCUU-3'	shRNA	Inhibition of proliferation and clonogenicity, enhanced drug sensitivity[33]
MLL-AF4	e9-e4	5'-AAGAAAAGCAGACCUACUCCA-3' 5'-UGGAGUAGGUCUGCUUUUCUUUU-3'	siRNA	Inhibition of proliferation and clonogenicity, induction of apoptosis and differentiation, prolonged survival of xenotransplanted mice[73]
	e10-e4	5'-ACUUUAAGCAGACCUACUCCA-3' 5'-UGGAGUAGGUCUGCUUAAAGUCC-3'	siRNA	

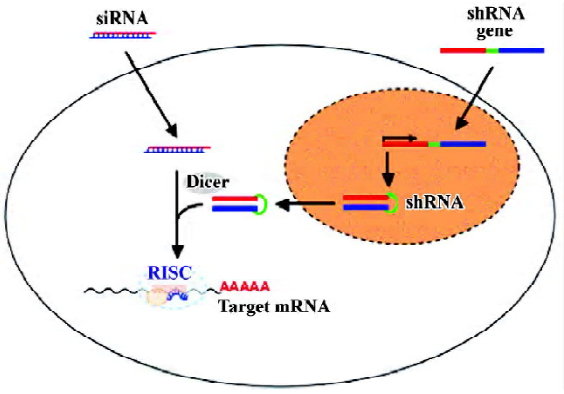

Figure 1. siRNA and shRNA delivery and processing. Delivery of preformed siRNAs is achieved by transient transfection techniques such as lipofection or electroporation. Inside the cytoplasm, a Dicer-containing complex promotes the dissociation of the double-stranded siRNA to 2 single strands and the formation of a catalytically competent RISC. In the case of shRNAs, a vector containing an shRNA expression cassette enters the nucleus via transfection or viral infection. Inside the nucleus, the transcribed shRNAs are exported into the cytoplasm. Dicer-mediated processing towards siRNAs is followed by association with the other RISC components.

miRNAs to trigger RNA cleavage[14]. Nevertheless, despite differences in the mechanism (RNA degradation versus inhibition of translation), siRNAs and miRNAs share protein components to form the corresponding nucleoprotein complexes[15].

Both siRNA-mediated RNA degradation and miRNA-mediated inhibition of translation take place in the cytoplasm[16]. However, RISC does not seem to freely diffuse through the cytoplasm, but has been shown to be part of the cytoplasmic P-bodies[17,18]. Thus, post-transcriptional gene silencing takes place in defined structures, like many other essential cellular processes.

siRNA design and delivery *ex vivo*

The efficiency and specificity of an siRNA is dependent on both siRNA and target site properties. One important point to consider is the choice of the guide strand by RISC. Strand selection is controlled by the thermodynamic stability of the siRNA termini. The strand with the lower 5'-terminal thermodynamic stability is more likely to stay with RISC. Furthermore, an adenosine residue at position 11 of the antisense strand, the complementary position of the cleavage site, can be advantageous for cleavage efficiency, as RISC prefers to cleave RNA to the 3' side of a uridine. However, these rules are not absolute, and siRNAs, which do not adhere to these rules, do not necessarily have inferior activity. Targeting the fusion site of a leukemic fusion transcript may, depending on the sequences flanking this site, not allow these rules to be strictly obeyed. Instead, a compromise sequence must be found, or, if this is not possible, "non-consensus" siRNAs can be tried. For instance, we identified active siRNAs by scanning a leukemic fusion site with several siRNAs. With this approach, we obtained active and specific siRNAs, which did not follow any of the rules mentioned here (Figure 2)[19].

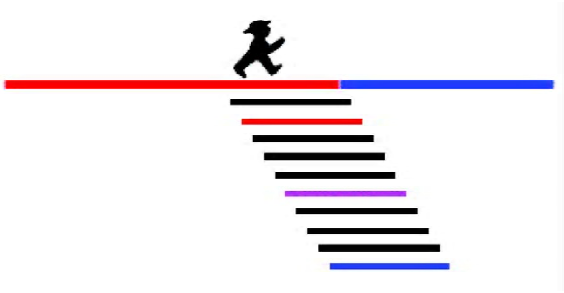

Figure 2. siRNA walk along a leukemic fusion break point site. Scanning the fusion site of a leukemic fusion transcript with overlapping siRNAs can be performed to identify active and specific antileukemic siRNAs. Active siRNAs (marked in red, violet and blue) may also affect the nonfused wild-type transcripts if the fusion site was close to the siRNA termini (blue and red), whereas the violet siRNA is likely to specifically cleave the fusion transcript. An example of such a scan has been described for the fusion site of MLL-AF4[72].

Transient transfections of siRNA duplexes into mammalian cells can be performed with various commercially available cationic lipid formulations, or with electroporation[20,21]. The advantage of such a transient approach is the lack of cell adaptation to reduced target protein levels. Furthermore, genes essential for cell proliferation can be targeted by transient siRNA approaches. However, there is no single transfection method that can be successfully applied to all cell types under all experimental conditions. It is therefore important to optimize transfection conditions so that maximum gene silencing is achieved. The following transfection parameters have been shown to affect transfection and gene silencing efficacy: cell culture conditions, including cell density and medium composition; the type and amount of transfection agent; the quality and quantity of siRNA; and the length of time that the cells are exposed to the siRNA. Differences have been reported in the ability to transfect and silence gene expression between adherent and nonadherent cells. Post mitotic cells such as neurons and muscle cells tend to be more difficult to transfect using liposomes compared with mitotic cells such as stem cells, fibroblasts and tumor cells.

A major disadvantage of transfections using preformed siRNA lies directly in their transient nature: a long-lived protein may not be affected by such an approach. To overcome this limitation, several siRNA expression systems based on plasmid and viral vectors have been developed to achieve a sustained "knockdown" of the gene of interest[22]. In most cases, the expression cassettes contain an RNA polymerase III-dependent promoter such as the U6 or the H1 promoters. The siRNAs are expressed as hairpin structures known as short hairpin RNAs (shRNAs), which are processed by Dicer to the mature siRNAs[23].

Because the application of plasmid vectors is frequently hampered by inefficient stable transfection rates and by silencing of the shRNA genes, viral vector systems for the expression of shRNAs have been developed. In particular, lentiviral and oncoretroviral systems have been demonstrated to be effective in most cell lines and primary cell types. Because lentiviruses are able to infect non-cycling and postmitotic cells, they are now widely used to establish a stable RNAi in, for example, neuronal cells, stem cells and transgenic mouse models[24].

Targeting kinases with siRNAs

BCR-ABL The vast majority of all chronic myeloid leukemia (CML) cases and a significant fraction of ALL cases are associated with a chromosomal translocation t(9;22)(q34; q11), also known as the Philadelphia chromosome. This translocation involves the reciprocal transfer of the 3' Abelson proto-oncogene (*ABL1*) sequence of chromosome 9 to variable locations in the 3'-breakpoint cluster region (*BCR*) of chromosome 22. The resulting fusion gene *BCR-ABL* is transcribed into 2 differently spliced chimeric mRNA encoding p210 or p190 BCR-ABL proteins. Both isoforms exhibit constitutively active tyrosine kinase activity. The development of the *BCR-ABL* rearrangement in hemopoietic stem cells (HSC) is the determining event for the development of CML, leading to a cell clone with a competitive growth advantage over the normal stem cells[25].

A major breakthrough in the treatment of BCR-ABL-associated leukemia was the therapeutic application of the tyrosine kinase inhibitor imatinib mesylate (STI571, Gleevec). Administration of this drug results in marked clinical and cytogenetic remissions. However, this compound is not perfectly specific for BCR-ABL, but also inhibits the tyrosine kinase activities of ABL1, KIT and PDGFR. Moreover, and more importantly, clinical resistance due to point mutations in the ABL kinase domain of BCR-ABL leading to an increasing population of drug-resistant patients is frequently observed[26,27]. These limitations necessitate the development of alternative strategies to complement existing treatment protocols.

Different approaches have been used to deactivate the chimeric protein, such as single-stranded antisense oligonucleotides, which, however, yielded conflicting results with respect to specificity and functionality[28]. To overcome this, several groups used siRNAs to target *BCR-ABL* mRNA as an alternative approach[29–31]. Consistently, such siRNAs decreased *BCR-ABL* expression without affecting *BCR* and *ABL1* expression. BCR-ABL depletion was associated with a decreased leukemic proliferation and an increased extent of apoptosis. Despite the fact that cell lines were used in these studies, they provide a basis for a possible siRNA application not only for functional analysis but also as a therapeutic tool. The potential therapeutic value of a lentiviral gene transfer strategy was evaluated as an alternative to the exogenous delivery of chemically synthesized siRNAs[32,33]. The resultant stable expression of BCR-ABL shRNAs yielded a sustained reduction of BCR-ABL, and, consequently, inhibited proliferation, decreased cell survival and compromised colony formation of $CD34^+$ CML cells. Withey *et al* targeted *BCR-ABL* in primary chronic phase CML[34]. The siRNA treatment inhibited the expansion of granulocyte-macrophage progenitors expressing the b3a2 variant of *BCR-ABL* without having any obvious effects on cells expressing the b2a2 variant. This study underlines the role of *BCR-ABL* in driving aberrant myeloid progenitor amplification.

Baba and colleagues targeted an siRNA against a downstream sequence in the *ABL* portion instead of the fusion site[35,36]. Thus, this siRNA has the potential to interfere with both *BCR-ABL* and *ABL1* expression. They demonstrated that this siRNA markedly decreased target mRNA levels, consequently leading to the inhibition of protein tyrosine kinase activity and suppression of cell proliferation. Microarray analysis revealed cross talk between siRNA-mediated suppression of the *BCR-ABL* oncogene and expression of several apoptotic/antiapoptotic and cell proliferation factors. Bartova and colleagues studied the dependence of nuclear topography and expression of the *BCR-ABL* fusion gene in the leukemic cell line K562 on the expression of nonfused *ABL1*[37]. They showed that not only *BCR-ABL* suppression but also inhibition of *ABL* expression induced downregulation of BCR-ABL and ABL1 proteins, which appeared to be sufficient for the stimulation of apoptosis.

Of the particular interest is the work of Wohlbold and collaborators, who demonstrated that combined treatment

with BCR-ABL siRNAs together with imatinib mesylate could overcome a partial imatinib mesylate resistance[38]. In this case, decreasing the quantity of BCR-ABL protein using siRNA could antagonize the 2 major mechanisms of imatinib mesylate resistance, namely overexpression of the protein and the occurrence of point mutations. Such applications of siRNAs as supplementary therapy could be of clinical significance. The existence of several *BCR-ABL* fusion transcript variants (eg b3a2 or b2a2) is a major limitation for the application of fusion site-specific siRNAs. However, Wohlbold *et al* demonstrated in a follow-up study that all common BCR-ABL transcript variants could be successfully targeted with siRNAs that are homologous to the corresponding fusion sites.

TEL-PDGFβR The chromosomal translocation t(5;12)(q33;p13) associated with chronic myelomonocytic leukemia (CMML) generates the *TEL-PDGFβR* fusion gene[39]. Similar to BCR-ABL, the TEL-PDGFbR fusion protein exhibits a constitutively active tyrosine kinase activity, which can be inhibited with imatinib mesylate or rapamycin. However, as with BCR-ABL, drug resistance due to point mutations limits the therapeutic efficacy of these kinase inhibitors.

Chen *et al* applied an oncoretroviral transduction system for the delivery of TEL-PDGFβR shRNAs[40]. The siRNAs were transcribed using an H1-promoter-based short hairpin RNA expression system in a self-inactivating retroviral vector. The TEL-PDGFβR siRNAs potently downmodulated TEL-PDGFβR, which affected signal transduction through PI3 kinase and mammalian target of rapamycin (MTOR), and, consequently, inhibited the proliferation of TEL-PDGFβR transformed cells. Furthermore, TEL-PDGFβR depletion sensitized Ba/F3 cells expressing either wild-type TEL-PDGFβR or a mutated, chemoresistant form to the small molecule inhibitors. Moreover, to evaluate the therapeutic efficacy of the siRNA *in vivo,* TEL-PDGFβR-transformed Ba/F3 cells with or without coexpression of the active siRNA were injected into the tail vein of nude mice. Whereas Ba/F3 cells stably expressing TEL-PDGFβR alone caused tumor development and death with a median latency of 24 d after injection, expression of TEL-PDGFβR siRNA resulted in significantly prolonged survival with a median latency of 41 d.

NPM-ALK Up to 75% of childhood and adolescent anaplastic large cell lymphomas (ALCL) carry the translocation t(2;5)(p23;q35). In this case, the ubiquitously expressed nucleophosmin (NPM) is fused to the cytoplasmic tail of the anaplastic lymphoma tyrosine kinase (ALK). The NPM-ALK fusion protein codes for a constitutively active tyrosine kinase, which is necessary for cell transformation. This kinase enhances factor-independent proliferation and inhibits apoptosis mostly via PI3K–Akt, Jak3/2-Stat3/5 and phospholipase Cγ signaling pathways. Ritter and collaborators designed and evaluated 3 chemically synthesized siRNAs for downregulation of the *NPM-ALK* fusion mRNA[41]. The most potent of them reduced the levels of NPM-ALK mRNA expression in SR786 ALCL cells by 50%–60%. However, repeated transfections were needed for significant reductions in the protein level, probably due to the long half-life of the NPM-ALK protein. Nevertheless, the siRNAs in this case were successfully used to dissect the signaling pathways employed by NPM-ALK. Similar to TEL-PDGFβR, stable expression of an NPM-ALK shRNA sensitized *NPM-ALK*-transformed Ba/F3 cells to rapamycin-induced cell death[40].

FLT3 Due to internal tandem duplication (ITD) within the juxtamembrane domain or due to point mutations, FMS-like tyrosine kinase 3 (FLT3) is constitutively activated in 35% of all AML cases. In ALL carrying mixed lineage leukemia gene (MLL) rearrangements, constitutive FLT3 activation is caused by overexpression of this kinase. Similar to other studies[38], Walters *et al* examined the effects of siRNA-mediated *FLT3* suppression on the sensitivity towards a small-molecule FLT3 inhibitor, MLN518[42]. SiRNA-mediated FLT3 depletion diminished the phosphorylation of several downstream molecules, thereby leading to the decreased viability of Ba/F3 and Molm-14 cells, which have internal tandem duplication of FLT3. The combination of FLT3 siRNAs together with the specific FLT3 inhibitor MLN518 led to synergistic effects on cell proliferation and apoptosis induction.

LYN As already described earlier, imatinib mesylate resistance due to BCR-ABL point mutations is a major problem in CML therapy. Therefore, new, complementary approaches to interfere with BCR-ABL function such as siRNAs are currently being intensively studied. Alternatively, proteins other than BCR-ABL, which are also crucial for maintaining the leukemic phenotype, may be promising targets. Members of the SRC kinase family such as SRC or LYN play a central role in BCR-ABL-associated leukemogenesis. For that reason, Ptasznik *et al* studied the effects of LYN depletion on the survival of imatinib mesylate-resistant CML and ALL blast crisis cells[43]. LYN siRNA reduced LYN protein in both normal hemopoietic cells and BCR-ABL-expressing blasts by 80%–95%. Within 48 h, both siRNA-treated CML and ALL blasts underwent apoptosis, whereas normal cells remained viable. Notably, ALL blasts seemed to be more affected by LYN depletion than CML blasts. This increased dependence of BCR-ABL-positive leukemic blasts on LYN

signaling provides the rationale for a complementary treatment of imatinib mesylate-resistant CML blast crisis, particularly when lymphoid in nature.

Targeting transcriptional modulators with siRNAs

AML1/MTG8 The chromosomal translocation t (8;21) (q22;q22), which is the most frequent aberration associated with 10%–15% of all cases of AML, fuses the DNA-binding domain of the transcription factor RUNX1 (also called AML1 or CBFa) to the almost complete open reading frame of RUNXT1 (also named MTG8, ETO or CBFA2T1)[44]. Whereas RUNX1 is an essential transcription factor for definitive hemopoiesis, RUNXT1 or MTG8 is part of histone deacetylase-containing complexes. Thus, the translocation converts a transcriptional modulator to a constitutive repressor of gene expression. The resulting fusion protein AML1/MTG8 (AML1-ETO) inhibits myelopoiesis and supports the clonal expansion of hemopoietic stem cells. Moreover, by directly binding to and sequestering transcription factors, such as SMAD3, C/EBPα or vitamin D receptor, AML1/MTG8 interferes with signal transduction pathways controlling differentiation and proliferation[45].

We examined the efficacy and specificity of siRNAs homologous to the fusion site of *AML1/MTG8* transcripts. Such siRNAs efficiently and specifically suppressed *AML1/MTG8* without interfering with *RUNX1* expression. AML1/MTG8 depletion led to severely impaired clonogenicity, a senescence-associated G1 cell cycle arrest, and an increased responsiveness to Tumor Growth Factor-β (TGFβ)/vitamin D_3-induced myelo-monocytic differentiation in t(8;21)-positive leukemic cells[46]. The data imply a central role of AML1/MTG8 not only in the expansion of preleukemic progenitor cells, but also in the maintenance of the leukemia. Moreover, AML1/MTG8 siRNAs are not only of value for the functional analysis of this fusion gene, but may also be useful for an antileukemic therapy.

MLL-AF4 The chromosomal translocations involving human mixed lineage leukemia gene MLL located on chromosome 11 are associated with aggressive lymphoid and myeloid leukemias[47]. MLL is the human trithorax homologue and stabilizes unmethylated CpG islands. It is part of a several MDa large protein complex involved in the regulation of DNA methylation and histone acetylation and methylation. MLL can be fused to more than 40 different partner genes, yielding a very diverse collection of chimeric fusion proteins[48]. The translocation partners do not share any unifying properties. Nevertheless, 2 different classes can be distinguished. One group of partners such as AF1 or GAS7 cause oligomerization of the corresponding fusion protein[49], whereas a second group (eg AF4, AF9, ENL) might be part of a single multiprotein complex[50,51]. The molecular mechanisms of MLL-associated leukemogenesis are currently being intensively studied. Despite the diversity of the fusion proteins, characteristic features of MLL-associated leukemias are an inappropriate expression of a subset of homeotic genes, such as *MEIS1* and *HOXA9,* and the overexpression of FMS-like tyrosine kinase 3 (*FLT3*)[52].

The translocation t(4;11) is associated with a very aggressive and therapy-resistant acute lymphoblastic leukemia in infants and with therapy-related secondary leukemias[53,54]. Recently, we showed that transient inhibition of *MLL-AF4* expression with small interfering RNAs impaired the proliferation and clonogenicity of the t(4;11)-positive human leukemic cell lines SEM and RS4;11[19]. Reduction of MLL-AF4 levels induced apoptosis associated with caspase-3 activation and diminished *BCL-*X_L expression. Suppression of *MLL-AF4* was paralleled by a decreased expression of the homeotic genes *HOXA7, HOXA9,* and *MEIS1*. Moreover, MLL-AF4 depletion inhibited expression of the hemopoietic stem cell marker CD133, indicating hemopoietic differentiation. Finally, transfection of leukemic cells with MLL-AF4 siRNAs reduced leukemia-associated morbidity and mortality in xenotransplanted *SCID* mice, suggesting that MLL-AF4 depletion negatively affects leukemia-initiating cells. Our findings demonstrate that *MLL-AF4* is important for leukemic clonogenicity and engraftment of this highly aggressive leukemia.

To date, the presence of MLL rearrangements are indicative of a poor clinical outcome, partially due to the lack of specific pharmacological inhibitors[55]. Therefore, the siRNA approach might be an option for developing new strategies for the treatment of these aggressive leukemias.

Challenges and promises

The examples discussed in the previous sections strongly suggest that siRNA-mediated oncogene suppression may become a promising option in antileukemic therapy. However, there are major obstacles to overcome, such as specificity, induction of an interferon response, emergence of escape mutations, or inefficient systemic siRNA delivery *in vivo*. Only if these challenges can be successfully addressed will siRNA technology fulfill its promises in cancer therapy.

siRNA specificity and possible off-target effects should be given particular consideration. Like all other antisense molecules, siRNAs tolerate mismatches to a certain extent, possibly compromising their sequence specificity[56]. Even if mismatches prevent RISC-mediated cleavage of unintended

target transcripts, protein expression may still be affected[57]. Furthermore, siRNAs or shRNAs may compete with endogenously expressed miRNAs for RISC components, thereby affecting miRNA-regulated gene expression and cellular processes such as differentiation[58,59]. Finally, both chemically synthesized siRNAs as well as intracellularly expressed shRNAs may induce a limited interferon response both in cell culture and *in vivo*[60]. This induction proceeds via Toll-like receptors and/or protein kinase R, but the parameters responsible are just currently becoming clear[61-63]. One possible method of controlling siRNA- triggered interferon response is to examine the induction of classical interferon response genes such as STAT1 and OAS1. Furthermore, ectopic expression of an siRNA-insensitive variant of the target protein would be a suitable control for the possible side-effects of siRNAs. However, such rescue experiments are sometimes difficult to perform. For instance, ectopic expression of AML1/MTG8 or MLL-AF4 inhibits cell proliferation and induces cell death in many different cell types. An alternative might be the sequence-specific inhibition of different variants of the fusion gene. For instance, we targeted 2 different MLL-AF4 variants with specific siRNAs[19]. Each siRNA only inhibited the proliferation of that cell line expressing the perfectly complementary variant, but had no effect on the other cell line. Because both siRNAs are functional in their corresponding cell lines, such an experimental setting is equivalent to a rescue experiment.

Another point of concern is the ability of siRNAs to remain functional in the context of possible escape mutations. However, we and others have demonstrated that different fusion gene variants can be successfully targeted with siRNAs[19,38]. Thus, it may be possible to counteract an escape mutation by simply adapting the siRNA sequence.

The most challenging problem for the therapeutic application of siRNAs is the efficient delivery of siRNA into leukemic tissues, including leukemic stem cells. To date, several techniques have been examined to obtain systemic siRNA delivery in mouse models[64]. However, approaches such as high-pressure, high-volume intravenous injection of synthetic siRNAs, the so-called hydrodynamic delivery, are of limited clinical use because of the severe side effects[65].

Recently, the pharmacokinetics and efficacies of chemically modified siRNAs containing a 5'-cholesterol moiety on the sense strand in combination with limited phosphorothioate and 2'-methoxy modification were examined in a mouse model. Intravenous injection resulted in the silencing of ApoB mRNA in liver and jejunum, decreased plasma levels of ApoB protein, and reduced total cholesterol[66]. Alternatively, unmodified siRNAs have been successfully delivered by using vehicles such as polyethylenimine, atelocollagen, cationic lipids or neutral liposomes[67–71]. In all these cases, intravenous injection of the corresponding vehicle-siRNA mixture inhibited gene expression and tumor growth in mouse models.

The major goal for RNAi approaches for cancer therapy is the selective elimination of tumor cells without damaging normal cells. For that, if targeting a non-mutated gene, siRNAs should be selectively delivered to the transformed cells. Alternatively, siRNAs may target genes that are exclusively expressed in cancer cells, and that are crucially involved their growth or survival (Figure 3). The latter option is preferable when a fusion gene is involved in tumorigenesis. The examples discussed in this review demonstrate the suitability and effectiveness of this approach both in cell culture and, after siRNA delivery *ex vivo*, also in murine model systems. Given the rapid progress being made in the field of therapeutic RNA interference, antileukemic siRNAs may be not too far away from complementing existing therapeutic protocols to treat leukemia.

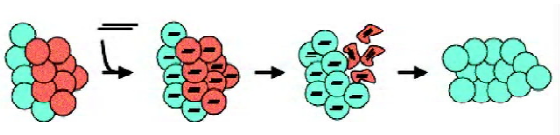

Figure 3. Possible application of antileukemic siRNAs. Antileukemic siRNAs targeting a leukemic fusion transcript may be delivered to both normal hemopoietic stem cells and progenitors (blue) and to leukemic cells (orange). Because only leukemic cells express fusion genes, and due to the siRNA targeting specificity, only leukemic cells should be affected by, for instance, inhibiting proliferation and triggering apoptosis, thus leading to a reduction of the leukemic burden and a recovery of normal hemopoiesis.

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