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Review Article

RNA-Mediated Gene Silencing in Hematopoietic Cells

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In the past few years, the discovery of RNA-mediated gene silencing mechanisms, like RNA interference (RNAi), has revolutionized our understanding of eukaryotic gene expression. These mechanisms are activated by double-stranded RNA (dsRNA) and mediate gene silencing either by inducing the sequence-specific degradation of complementary mRNA or by inhibiting mRNA translation. RNAi now provides a powerful experimental tool to elucidate gene function in vitro and in vivo, thereby opening new exciting perspectives in the fields of molecular analysis and eventually therapy of several diseases such as infections and cancer. In hematology, numerous studies have described the successful application of RNAi to better define the role of oncogenic fusion proteins in leukemogenesis and to explore therapeutic approaches in hematological malignancies. In this review, we highlight recent advances and caveats relating to the application of this powerful new methodology to hematopoiesis.

INTRODUCTION

The discovery of RNAi

In 1998 Andrew Fire and Craig Mello discovered in a series of experiments in *Caenorhabditis elegans* that injection of sense or antisense RNAs led to negligible decreases of target RNA, whereas introduction of dsRNA resulted in effective and specific degradation of cytoplasmic mRNA. Furthermore, these silencing effects of dsRNA in *C elegans* were systemic and heritable [1]. Later on, the sequence-specific gene silencing capacity of dsRNA, now known as RNA interference (RNAi), has been linked to previously described gene silencing phenomena such as posttranscriptional gene silencing (PTGS), co-suppression in plants, and quelling in fungi [2–7]. Following this exciting and fundamental discovery, intense studies were undertaken with the purpose to dissect the molecular mechanism of RNAi. Indeed, in the past few years, many details of the biogenesis of small dsRNAs have been elucidated, and components, structure, and function of protein complexes of the RNAi machinery have been identified. RNAi has thus emerged as an evolutionarily highly conserved and fundamental mechanism for the regulation of gene expression and has rapidly been developed into a tool to analyze gene function.

RNAi molecular pathways

The detailed molecular mechanism of RNAi has been the subject of numerous recent reviews [8–16]. As this review concentrates on delivery of RNAi in the hematopoietic system and discusses its potential diagnostic and therapeutic applications in hematology, we will only briefly resume the principal phases of dsRNA-mediated gene silencing pathways as schematically represented in Figure 1.

Two types of small noncoding dsRNA can serve as effector molecules and trigger RNAi: small interfering RNA (siRNA) and micro RNA (miRNA). Long dsRNAs, introduced experimentally into the cell or generated as intermediates during viral infections [17] or as aberrant transcripts derived from inverted tandem repeats and transposons [18], are processed in the cytoplasm by a ribonuclease (RNase) III-like enzyme called Dicer [19, 20] into siRNA duplexes of 21–25 nt in length with 3′ dinucleotide overhangs, 5′-phosphates, and 3′-hydroxyl termini [21].

With the discovery of micro RNAs (miRNAs) the important physiological role of RNA-mediated gene silencing for regulating gene expression during development [22], differentiation [23], and apoptosis [9, 24], as well as its possible involvement in diseases like cancer, soon became manifest (see [8, 9] for review). miRNAs constitute a large class of endogenously expressed, highly conserved, noncoding small RNA molecules which act as negative regulators of gene expression in a variety of organisms ranging from plants to mammals [9]. Usually miRNAs are transcribed from endogenous genes by RNA polymerase II [25, 26] as long primary transcripts (pri-miRNAs) (see [27] for review) possessing 5′-caps and 3′-poly A tails [25, 28] (Figure 1). Recent studies

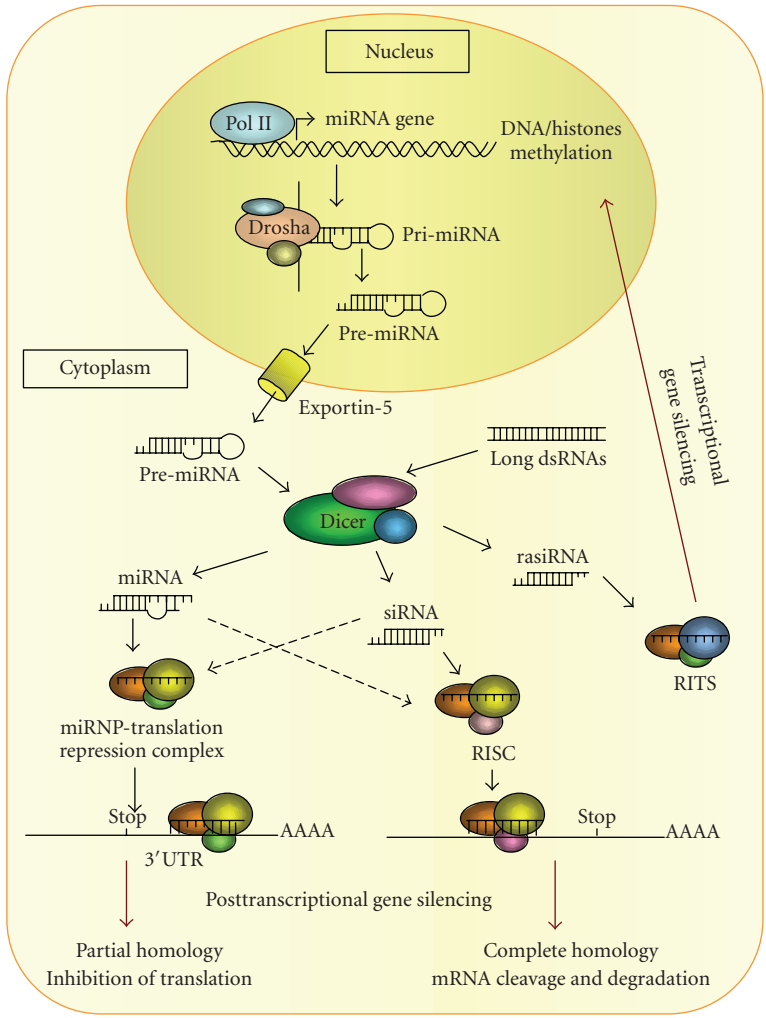

Figure 1: *Schematic representation of RNA-mediated gene silencing pathways.* miRNAs are transcribed from endogenous genes by RNA polymerase II as long primary transcripts (pri-miRNAs). In the nucleus, Drosha, an RNase III-like enzyme, releases the characteristic stem-loop structure of the ~ 70 bp precursor-miRNA from the primary transcript (pre-miRNA). The pre-miRNAs are then transported from the nucleus to the cytoplasm by exportin-5, where they are processed by Dicer to generate the mature miRNA of 21–23 nt in length. Similarily, siRNA duplexes of 21–25 nt in length are generated from long dsRNAs in the cytoplasm by Dicer as well. Only the antisense strand of the mature si/miRNAs is retained in the active ribonucleoprotein effector complexes (RISC or miRNP) and acts as a guide to target the homologous mRNA. Normally, siRNAs are fully complementary and induce the cleavage and degradation of their target mRNA transcript. In contrast, miRNAs usually bind only with partial complementarity to the 3′ untranslated region (UTR) of their cognate mRNAs and lead to translational inhibition. Depending on the cell context and the degree of complementarity between the small RNA effector and the mRNA target, both siRNA and miRNA can induce posttranscriptional gene silencing through degradation or translational inhibition. In addition, transcriptional gene repression has been described by repeat-associated siRNA (rasiRNA). They are believed to be loaded into RNAi-induced transcriptional gene silencing (RITS) complexes and may target homologous genomic loci. However, this mechanism is not yet fully understood.

suggest that miRNA expression is regulated at the level of transcription [29–31], similarly to mRNA and likely involving a similar or identical molecular machinery. The pri-miRNAs undergo a two-step processing to give rise to the mature miRNAs: in the nucleus, the characteristic stem-loop structure of the precursor-miRNA (pre-miRNA) of ~ 70 bp is released from the primary transcript by another RNase III-like enzyme called Drosha [32–36]. The pre-mRNAs are then transported from the nucleus to the cytoplasm by exportin-5 [37–40], where its 2 nt 3′-hydroxyl terminus is recognized by Dicer, which generates the mature miRNA of 21–23 nt length [41–43]. Dicer thus represents the core of the RNAi machinery where the different RNA-mediated gene silencing processes converge (Figure 1).

Only one strand of the mature si/miRNAs, distinguished by the lower thermodynamic stability at its 5′ terminus

[44, 45], is retained in the active ribonucleoprotein effector complexes and acts as a guide to target the homologous mRNA (Figure 1). Normally, siRNAs are fully complementary to their cognate mRNAs and guide the RNA-induced silencing complex (RISC) [46, 47] to the target transcripts, followed by cleavage and degradation. A member of the Argonaute (Ago) family of proteins [48, 49], which contain a small RNA-binding domain (PAZ [50]) and an RNase activity (PIWI domain [51]), constitutes the catalytic ribonuclease component of RISC responsible for cleaving the target mRNA at the center of the region complementary to the guide RNA [48, 49]. RISC is then released and the cleaved mRNA is degraded by cellular exonucleases. Except in plants where they have perfect homology to their targets and act exactly as siRNAs inducing mRNA degradation [52] upon integration into complexes usually referred to as miRNA-ribonucleoproteins (miRNPs), most miRNAs bind only with partial complementarity to the 3′ untranslated region (UTR) and lead to translational inhibition downstream of the initiation step [53]. In fact, apart from the distinction based on their biogenesis, a precise boundary between si- and miRNA functionality cannot always be established, since both have been shown to be capable to target mRNA for degradation or translational inhibition, depending on the cellular context and the degree of complementarity between the RNA effector and the mRNA target [46, 54–58].

In addition to posttranscriptional gene silencing, RNAi has also been demonstrated to induce transcriptional repression through RNA-directed DNA methylation in plants [59], and recent studies have reported similar effects even in human cells [60, 61], inducing heterochromatin formation (see [62, 63] for review).

RNAi IN MAMMALIAN CELLS: DESIGN, DELIVERY, AND TARGET VALIDATION

After its discovery, RNAi was rapidly employed as a powerful tool for large-scale reverse genetic screens in *C elegans*, where it can be easily induced by direct injection of dsRNAs, by feeding worms with bacteria engineered to express siRNAs, or simply by soaking the animals in medium containing siRNAs. Furthermore, in nematodes, RNAi acts systemically and is a long-lasting heritable event due to the presence of RNA-dependent RNA polymerases (RdRPs) which allow the amplification of the trigger and the perpetuation of the silencing process [64–66].

In mammals, however, initial efforts to use RNAi for specific gene silencing encountered more difficulties, especially due to the induction of nonspecific inhibition of gene expression resulting from the activation of the interferon (IFN) response pathway by dsRNAs longer than 30 bp [67]. The discovery from Elbashir et al [68] and Caplen et al [69] that RNA duplexes of 21 nt in length, mimicking the Dicer cleavage products, were able to mediate efficient and specific RNAi upon transfection into mammalian cells without eliciting the INF response, finally enabled loss-of-function studies of specific target genes in mammalian systems as well.

Designing RNAi effector molecules

To design efficient siRNAs several parameters should be considered. Although duplex RNAs of 21 nt in length were shown to be the most effective RNAi triggers, recent studies suggest that 27- to 29-mers may be more active than 21-mers [70–72]. This could be due to the fact that these dsRNAs are processed by Dicer and thus may be incorporated directly and more efficiently into RISC [73]. Reynolds et al [74] conducted systematic analyses to evaluate physicochemical characteristics associated with highly functional siRNAs and set up an algorithm with several criteria which significantly improved selection of potent siRNAs. To comply with the rules promoting asymmetric incorporation into RISC, the base pair at the 5′ end of the siRNA antisense (guide) strand should have a lower thermodynamic stability compared with the 3′-end [44, 45]. Accordingly, the presence of three or more A/U nucleotides at the 3′-terminus of the sense strand was defined as a criterion for siRNA functionality [74]. Low G-C content (30%–52%), lack of internal inverted repeats which can form secondary structures, and specific nucleotide preferences at positions 3 (A), 10 (U), 13 (absence of G), and 19 (A, absence of G or C) on the sense strand also increased the probability of selecting a potent siRNA in this study [74]. Concerning the specificity of the sequence match necessary to achieve efficient gene silencing, different groups have reported varying degrees of mismatch tolerance for siRNA-mediated silencing [75–81]. Mismatched small RNAs may still be competent and can function like miRNAs which may bind to mRNAs with multiple target sites in the 3′-UTR [82]. miRNA binding is not limited to the 3′-UTR but can include the coding sequence of an mRNA as well [83]. These results demonstrate the possibility of off-target effects by siRNAs used for experimental or therapeutic purposes. Therefore, for prevention of cross-reactive silencing, a BLAST search of potential target sequences should be performed in order to exclude candidate siRNAs with some degree of homology with other genes [74, 84]. For the efficacy of gene silencing, the overall stability of the antisense RNA/RISC-mRNA complex is probably more important than the absolute number of mismatches. Accordingly, base pairing at the center of the duplex is critical, while one or two mismatches located at the 3′- or 5′-end of the siRNA may be well tolerated [85]. Since regions which are not involved in intramolecular folding have been demonstrated to be optimal targets [86], computational approaches have been reported to analyze the secondary structure and the local folding of the target mRNA. However, all predictions based on physicochemical characteristics, sequence homologies, or secondary structure can not guarantee the generation of an efficient siRNA, and functional testing is still always required. Similarly, different siRNAs targeting the same gene may have different silencing efficacies not always predictable by the parameters discussed above. Therefore, more than one target sequence should be tested empirically to identify the optimal small RNA for efficient and specific silencing of a given target mRNA.

RNAi delivery in mammalian cells

RNAi in mammalian cells can be triggered by direct introduction through injection, electroporation, lipid-mediated transfection, nanoparticles, or antibody bound enzymatically generated or chemically synthesized siRNAs, among others. Alternatively, siRNAs or small hairpin RNAs (shRNAs) can be delivered by vector-based intracellular expression.

Synthetic siRNA-mediated RNAi

siRNAs can be synthesized chemically [75, 87, 88], generated enzymatically through in vitro transcription by T7 phage polymerase [89, 90], or through endonuclease digestion by recombinant Dicer of in vitro transcribed long dsRNA [91, 92]. In mammalian cells, direct delivery of siRNAs can only induce transient silencing due to their limited half-life and to their dilution during cell division.

Vector-based RNAi

Transient downregulation of gene expression may not be sufficient for many applications, for example, for studies of proteins with long half-lives in rapidly dividing cells. In order to produce long-lasting RNAi in mammalian cells, plasmids and viral expression vectors have been developed to drive continuous intracellular expression of siRNA or shRNAs under the control of highly active RNA polymerase III promoters such as U6 or H1 [93, 94].

As represented in Figure 2, the two strands of an siRNA can be transcribed from distinct expression units, either cloned in tandem or in two separate vectors [95, 96], or can result from bidirectional transcription of a single 19- to 29-mer DNA fragment under the control of two opposite promoters [97, 98]. The intracellular expression and hybridization of the two strands gives rise to functionally active siRNA duplexes. However, the most commonly used approach involves the intracellular expression of shRNAs. They are transcribed as single-stranded RNAs from an expression cassette inserted immediately downstream of the pol III promoter, which contains, in the following order, a 19 nt sequence homologous to the target mRNA, a spacer 6 to 9 nucleotides in length, the antisense sequence, and the RNA pol III terminator signal composed of a stretch of about 6 thymidines. After transcription, the resulting stem-loop RNA structure, like miRNA precursors, is cleaved by Dicer to yield a functionally active siRNA [93, 99, 100] (Figure 2). A second generation of RNAi-delivering vectors is based on RNA pol II promoters driving transcription of shRNAs incorporated into a miRNA chimeric transcript, comprising flanking sequences optimized for Drosha/Dicer processing [100, 101].

A major advantage of vector-dependent RNAi is the usage of selectable markers to generate stable transfectants or of reporter genes such as green fluorescent protein (GFP) or red fluorescent protein (RFP) to identify and eventually isolate the si/shRNA expressing cells in a quantitative manner. When using shRNA-expressing vectors, however, it is absolutely necessary to confirm the sequence of the shRNA expression cassette, because genetic recombination and/or introduction of point mutations are very frequent and can occur in almost every step of the cloning strategy.

Finally, when the target gene to be silenced is essential for cell survival and/or proliferation and the constitutive knockdown of its expression is even lethal, employing inducible or tissue-specific RNAi could be especially important [100]. In the last years, various methods for inducible expression of shRNAs have been described. Tetracycline-inducible [94, 102, 103] and ecdysone-inducible [104] expression systems have been reported which mediate induced and reversible downregulation of gene expression. However, no standard technique for inducible RNAi has been established so far.

Many suspension and primary cells are difficult to transfect efficiently. However, viral transduction strategies allow stable induction of RNAi in these cells. Particularly, the capacity of lentiviruses to integrate into the genome of noncycling cells, such as stem cells or terminally differentiated cells, renders lentiviral vectors much more efficient than retroviral vectors in inducing RNAi in these cells [105, 106].

Finally, transgenic technology has also been adapted for in vivo delivered RNAi in mice. Transgenic animals have been reported which produce siRNAs constitutively [107] or conditionally, in a stage- or tissue-specific manner [108], to repress selected target genes. Tiscornia et al [106] efficiently used a lentiviral vector system to express siRNA in preimplantation mouse embryos.

In vivo systemic delivery of siRNAs

Chemical modifications are required to potentiate siRNA nuclease and thermodynamic stability in vivo without compromising their efficacy. Recently, several groups reported different approaches for systemic in vivo delivery of siRNAs. Soutschek et al [109] described intravenous injection in mice of chemically modified naked siRNAs coupled to a cholesterol group chemically linked to the terminal hydroxyl group of the sense strand to promote entry into the cells. In vivo delivery of chemically modified siRNAs encapsulated into liposome particles has been recently reported by Morrisey et al [110], and Song et al [111] described an antibody-based delivery system which could offer a possibility for systemic, cell-type-specific siRNA delivery.

Specificity of RNAi-induced gene silencing

Prior to functional analysis, monitoring the level of target mRNA expression is necessary for siRNA target validation. Quantitative RT-PCR is a fast and reliable method to measure target transcript levels in specific versus control siRNA-treated cells, to ascertain that any phenotypic changes are really due to specific gene knockdown and not to nonspecific effects of the RNAi strategy. This issue can be further addressed by verifying that the same phenotype is induced by siRNAs homologous to different regions of the target transcript. The correlation between the extent of gene silencing and dose of the delivered si/shRNA can offer some evidence of specificity. Finally, the availability of a rescue experiment

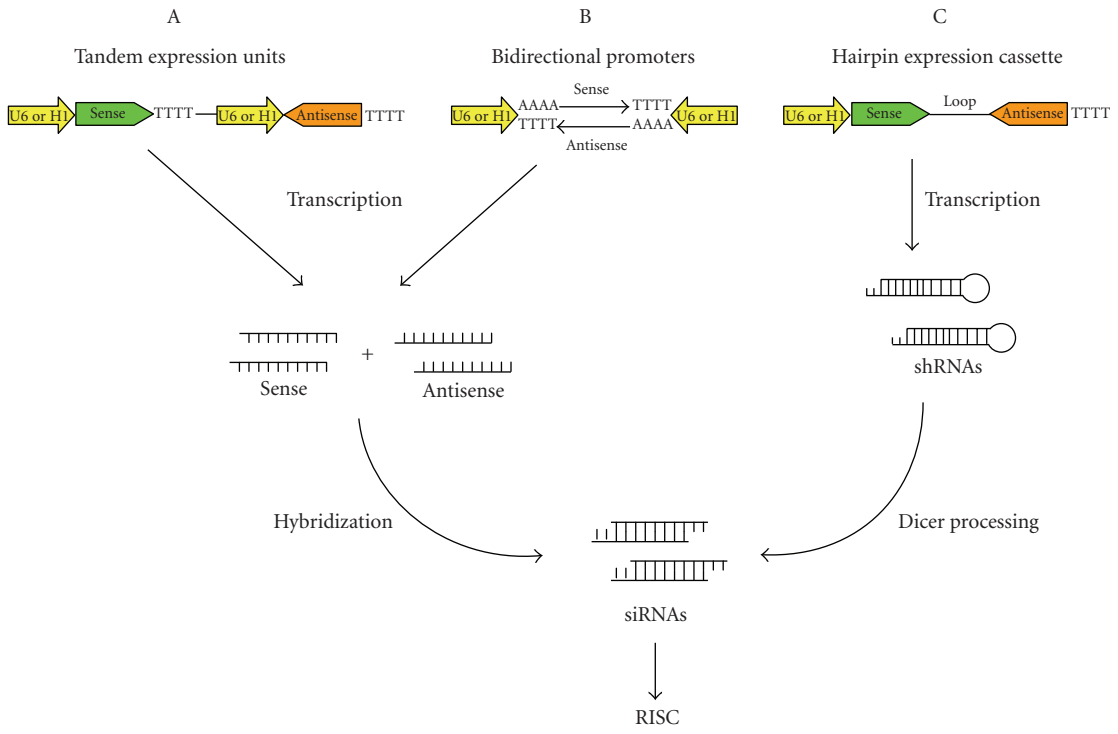

Figure 2: *Schematic representation of vector-based RNAi*. The two strands of an siRNA can be transcribed from two tandem expression units where the expression of the sense and the antisense strands is driven separately by their own respective promoter (A), or from a single DNA fragment under the control of two opposite promoters (B). After intracellular expression, hybridization of the two strands gives rise to functional siRNA duplexes. shRNAs are transcribed as single-stranded RNAs from a hairpin expression cassette cloned immediately downstream of an RNA pol III promoter containing the sense strand homologue to the target of interest followed by a 4 to 9 nt spacer, the antisense strand, and the terminator signal. After transcription the resultant stem-loop structure is cleaved by Dicer and yields a functional siRNA (C).

able to revert the loss-of-function phenotype can ensure RNAi specificity.

RNAi IN THE HEMATOPOIETIC SYSTEM

siRNAs and hematopoietic cells

Apart from its transient nature in mammals, the use of RNAi in primary hematopoietic cells is limited by the difficulty to deliver siRNA through conventional transfection methods. In contrast, lentiviruses have been shown to efficiently transduce human hematopoietic stem and NOD/SCID repopulating cells (HSCs and SRCs) as well as more committed colony forming progenitors [112–114] and can offer a useful means for effective and stable delivery of RNAi triggers in the hematopoietic system. Lentiviral transduction was successfully employed by our group to induce RNAi against the common β chain of the receptors for granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-3, and IL-5 in human $CD34^+$ SRCs and colony forming cells [115]. Similarly, Schomber et al [116] reported efficient and stable silencing of the p53 gene in human cord blood-derived $CD34^+$ cells through lentivirus-mediated RNAi.

Application of siRNAs to target leukemia-associated oncogenes

Chromosomal translocations leading to the expression of chimeric oncoproteins are frequently involved in malignant transformation in leukemias and lymphomas. In the past few years, RNAi technology has been used to specifically silence the expression of translocation products (Table 1). For example, the bcr-abl oncogene resulting from the $t(9;22)$ translocation characterizes chronic myelogenous leukemia (CML) and some variants of acute lymphoblastic leukemia (ALL). It codes for a constitutively active cytoplasmatic tyrosine kinase which is both necessary and sufficient for leukemic transformation in several models [129–131]. Different research groups [117–120] have demonstrated the feasibility of specific interference with the bcr-abl expression without affecting the expression of wild-type c-abl or c-bcr using breakpoint-specific siRNAs delivered by electroporation in bcr-abl positive hematopoietic cell lines and primary $CD34^+$ cells from CML patients. Upon siRNA treatment, inhibition of survival and proliferation, increased sensitivity to the abl-specific tyrosine kinase inhibitor imatinib mesylate (STI571) [123, 132], and increased apoptosis were observed [119, 120].

Table 1: RNAi targeting hematopoietic fusion genes.

Oncogene target	Disease implication	Reference
BCR-ABL	Chronic myeloid leukemia	[117]
		[118]
		[119]
		[120]
		[121]
		[122]
AML1-MTG8	Acute myeloid leukemia	[123]
TEL-PDGFβR	Chronic myelomonocytic leukemia	[124]
MLL-AF4	Acute lymphatic leukemia	[125]
NPM1-ALK	Anaplastic large-cell lymphoma	[126]
		[127]
FLT3-ITD	Acute myeloid leukemia	[128]

In a recent study, our group used lentiviral gene transfer of shRNAs to trigger stable RNAi targeting the bcr-abl oncogene [133]. Stable, but not transient, RNAi was demonstrated to induce depletion of bcr-abl positive cells from suspension cultures. This depletion, as well as the degree of bcr-abl gene silencing, correlates with the multiplicity of lentiviral infection (MOI), the number of lentiviral integration into the host cell genome, and the expression level of the RFP reporter gene: cells with lower RFP expression and fewer lentiviral integrations could survive and were selected in suspension cultures but still showed reduced bcr-abl expression, aberrant proliferation kinetics, and enhanced sensitivity to STI571 as compared to controls. Furthermore, in contrast to transient RNAi [118], stable RNAi-induced silencing of bcr-abl inhibited the colony forming capacity of primary $CD34^+$ cells from CML patients.

Heidenreich et al [123] used siRNAs to specifically repress the AML1/MTG8 fusion product resulting from the $t(8;21)$ translocation [134] found in about 10% to 15% of all cases of de novo AML. Electroporation of siRNAs specific for the fusion site of the AML1/MTG8 into the Kasumi-1 $t(8;21)$-positive cell line specifically suppressed the expression of the fusion product, without impairing the expression of the two respective wild-type genes. Despite the transient character of the RNAi strategy employed, the authors described some functional effects due to the suppression of the fusion protein such as increased myeloid differentiation and reduced clonogenic potential upon TGFβ/vitamin D treatment.

Stable retroviral delivery of shRNAs was used by Chen et al [124] to target the fusion sequence of the TEL-PDGFβR fusion product derived from the $t(5;12)$ translocation, a recurrent cytogenetic aberration associated with chronic myelomonocytic leukemia (CMML) [135]. Stable RNAi-mediated inhibition of TEL-PDGFβR significantly reduced the proliferation of TEL-PDGFβR-transformed Ba/F3 cells, but did not restore IL-3 dependence, concordant with a marked decrease, but not abrogation of TEL-PDGFβR expression and selection of TEL-PDGFβR expressing cells. The authors also reported a significantly prolonged disease latency and survival of nude mice or Balb/C mice injected with TEL-PDGFβR-transormed Ba/F3 cells coexpressing siRNA as compared with injection of TEL-PDGFβR-transformed cells not expressing siRNA. However, as observed in cell culture, the expression of siRNA alone was not sufficient to completely abrogate TEL-PDGFβR-induced transformation in these murine models. A synergistic effect between siRNAs and small molecule inhibitors of tyrosine kinase activity, such as imatinib, was also demonstrated in TEL-PDGFβR-transformed Ba/F3 cells.

The mixed-lineage leukemia (MLL) gene is involved in numerous translocations in a variety of leukemias [136]. Most frequently, the MLL gene is fused to the AF4 gene as a consequence of the $t(4;11)$ translocation [137–139], found in acute lymphoblastic leukemia (ALL) with poor prognosis in infants [140]. Thomas et al [125] applied siRNAs to silence MLL-AF4 and demonstrated decreased proliferation and clonogenicity of $t(4;11)$-positive leukemic cells as well as induction of apoptosis through caspase-3 activation and repression of the BCL-X_L anti-apoptotic gene. They also observed that MLL-AF4 depletion resulted in a reduced expression of the homeotic genes HoxA9, MEIS1, and HoxA7, known to be upregulated by MLL fusion proteins, and of the CD133 marker for hematopoietic stem cell and early progenitors, which may suggest a reactivation of hematopoietic differentiation. Finally, using a NOD/SCID mouse xenotransplantation model, the authors showed that siRNA-mediated repression of MLL-AF4 compromised leukemic engraftment and the development of leukemia in vivo.

The $t(2;5)$ chromosome translocation fuses the ALK (anaplastic lymphoma kinase) gene on chromosome 2 to the nucleophosmin (NPM1) gene on chromosome 5 and is associated with anaplastic large-cell lymphomas (ALCLs) [141]. In a recent study, Piva et al [127] reported that silencing of NPM1-ALK induced by shRNAs directed against the 3′ sequences encoding the cytoplasmatic domain of ALK-R,

which is retained in all oncogenic fusion proteins involving ALK, leads to abrogation of NPM1-ALK-mediated transformation of MEF cells and inhibition of cell growth in several human NPM1-ALK-positive cell lines. Moreover, an increased number of apoptotic cells together with caspase activation and downregulation of the anti-apoptotic protein survivin were detected in ALCL cells 4 days after lentivirus-mediated RNAi. Similar results were seen in vivo: shRNA-expressing ALCL cells injected into NOD/SCID mice revealed a reduction in tumor formation as compared to control cells. Furthermore, in a second series of experiments, injection of lentiviruses driving shRNA expression directly into ALCL tumor masses showed growth inhibition of neoplastic cells, and histologic sections of the tumors demonstrated the presence of large necrotic regions and, in areas with retained viability, many apoptotic cells.

The receptor FMS-like tyrosine kinase 3 (FLT3) is the single most frequently mutated gene in AML. It is constitutively activated by internal tandem duplications (ITDs) within the juxtamembrane domain or by point mutations within the catalytic kinase domain in approximately 30% of AML patients [142, 143] and appears to confer an unfavourable prognosis. RNAi-mediated silencing of FLT3 was reported by Walters et al [128]. The authors used an siRNA pool to effectively downregulate the expression of FLT3 in FLT3-ITD-positive human leukemia cells and showed diminished phosphorylation of downstream signalling molecules, comprising STAT5, MAPK, and Akt, inhibition of cell proliferation, and induction of apoptosis. In addition, upon siRNA treatment in these cells, they found increased sensitivity to treatment with the FLT3 inhibitor MLN518, further demonstrating the potential benefit of such combined therapeutic approaches.

miRNAs in the hematopoietic system

miRNAs associated with hematopoietic differentiation

Fine modulation of gene expression is essential for the correct realization of differentiation programs. Consistent with this, several groups recently demonstrated the implication of miRNAs in controlling hematopoietic differentiation.

Chen et al [23] described three miRNAs, miR-181, miR-223, and miR-142s, which are differentially expressed in the murine hematopoietic system, and showed that miR-181 plays a specific role in B-cell differentiation. They found that miR-181 is normally expressed at low levels in murine hematopoietic progenitors and becomes upregulated during B-cell differentiation. Overexpression of miR-181 in hematopoietic progenitors gives rise to a greater fraction of B-lymphoid cells than in wild-type progenitors, in vitro as well as in vivo.

In a recent publication, Felli et al [144] described miR-221 and miR-222 as inhibitors of normal erythropoiesis and indicated the kit receptor mRNA as a major target of these two miRNAs. Using microarray chip and Northern blot-analysis, they showed that miR-221 and miR-222 are downregulated in erythropoietic cultures of cord blood $CD34^+$ progenitors. In addition, they observed an impairment of human $CD34^+$ cell engraftment in NOD/SCID mice as well as an inhibition of cell growth in the c-kit+ TF-1 erythroleukemic cell line upon overexpression of miR-221 and miR-222.

Recent studies conducted by Fazi et al [29] revealed the implication of miR-223 in human myeloid differentiation: miR-223 expression increases during retinoic acid- (RA-) induced granulocytic differentiation of the NB4 promyelocytic cell line as well as of blasts from patients with acute promyelocytic leukemia (APL) undergoing RA treatment. The authors depicted a finely regulated network involving miR-223 and the transcription factors C/EBPα, well known for its implication in granulocytic differentiation [145–147], and NFI-A [148, 149]. C/EBPα and NFI-A, which can induce or repress miR-223 expression, respectively, are in competition for an overlapping binding site on the miR-223 promoter. In undifferentiated cells, NFI-A maintains miR-223 and consequently its translation inhibitory effect at low levels. Upon RA treatment, C/EBPα displaces NFI-A from the miR-223 promoter, thus activating its expression. Interestingly, NFI-A is a target of miR-223 which, through a positive feedback, represses NFI-A translation, reduces the competition with C/EBPα, and maintains sustained levels of its own expression.

miRNA alterations in hematological malignancies

The expression of about one-third of human mRNAs appears to be regulated by miRNAs, each of which, according to computational analysis, is predicted to regulate a broad spectrum of different mRNAs [150], revealing a very complex regulatory network. As cancers essentially derive from alteration of gene expression and/or gene function, it is not surprising that several recent publications supported the direct involvement of miRNAs in tumorigenesis. Approximately 50 percent of the known human miRNA genes are located at fragile sites and cancer-associated regions of the genome [151]. Dysregulation of various human miRNAs has been associated with leukemias and lymphomas: the precursor of miR-155 was found to be overexpressed in the majority of childhood Burkitt lymphoma [152]; the miR-15a/miR-16 cluster at locus 13q14 is frequently deleted or downregulated in patients with B-cell chronic lymphocytic leukemia (CLL), mantle cell lymphoma, and multiple myeloma [153]. As miR-15/16 was demonstrated to induce apoptosis by targeting the apoptosis inhibitor protein BCL2 in CLL cells [154], downregulation of miR-15a and miR-16-1 can contribute to malignant transformation through BCL2 upregulation and inhibition of apoptosis. The miR-17-92 polycistron, located at 13q31, is amplified in human B-cell lymphomas [155]. He et al [156] found that enforced expression of the miR-17-92 cluster can augment the oncogenic potential of *c-myc* in a mouse B-cell lymphoma model, offering the first evidence of a miRNA to act as an oncogene. O'Donnell et al [30] further confirmed the relationship between this miR cluster and cancer. Using a lymphoma cell line with inducible *c-myc* expression, they demonstrated that the miR-17 cluster is specifically and directly upregulated by *c-myc*, but at least miR-17-5p and

miR-20a downregulate E2F1-protein expression, a target of *c-myc* which promotes cell cycle progression. These findings reveal a feedback mechanism through which c-Myc activates E2F1 transcription and simultaneously induces inhibition of its translation.

Recent microarray-based studies have provided evidence that specific alterations in human miRNA expression profiles are associated with specific types of cancers. Lu et al [157] established a sensitive method to analyze the expression profiles of 217 miRNAs in a panel of 334 samples representing diverse human normal tissues and corresponding tumors. They observed a general downregulation of miRNAs in tumors compared with normal tissues and demonstrated that miRNA expression profiles correlate with the developmental origins of specific cancers. Furthermore, even within a single developmental lineage, distinct patterns of miRNA expression seem to reflect the mechanism of transformation. Indeed, clustering of miRNA profiles of bone marrow samples from patients with acute lymphoblastic leukemia (ALL) showed a nonrandom distribution into three major groups in correlation with previously characterized molecular alterations and phenotypic classifications (BCR-ABL-positive and TEL-AML1-positive samples, T-cell acute lymphoblastic leukemias, and mixed lineage leukemias). Their results suggest that miRNA expression profiles could be more accurate for the classification and diagnosis of human cancers than mRNA microarrays.

Using miRNA profiling, Calin et al [158] found that different patterns of miRNA expression distinguish CLL cells from normal CD5+ B cells. In a recent study based on genome-wide expression profiling of a large number of samples from CLL patients [159], the same group showed that a miRNA signature is associated with the presence of other known prognostic factors (levels of ZAP-70 expression and the mutational status of the immunoglobulin heavy-chain (IgV_H) gene) and with disease progression in CLL: a molecular signature composed of 13 miRNAs differentiated CLL patients with high levels of ZAP-70 expression and unmutated IgV_H from patients with low ZAP-70 expression and mutated IgV_H. Nine of these miRNAs were significantly overexpressed in the first group of patients, associated with a poor prognosis. Furthermore, 9 miRNAs of the prognostic signature were able to discriminate between patients with a short interval from diagnosis to therapy and patients with a longer interval: in the first group, 8 of the 9 miRNAs were upregulated, suggesting their involvement in disease progression. The authors also showed some functionally relevant mutations in miRNA genes in CLL. Some of these mutations are located in the flanking sequences of the pre-miRNA, compromising the correct processing and expression of the mature miRNA.

CONCLUSIONS AND FUTURE PERSPECTIVES

Despite many remaining technical problems, current advances in strategies to extend genome-wide screens with siRNA or shRNA libraries to mammalian cells [160–167] as well as specific gene silencing approaches may finally facilitate the identification of essential genes involved in human diseases and may identify new potential therapeutic targets. On the other hand, disease- and stage-specific systematic analysis of miRNA gene-expression profiles may help to establish new diagnostic and prognostic markers.

Employing RNAi in a therapeutic setting may still encounter numerous obstacles: the issue of efficient delivery in a clinical setting, as well as problems deriving from toxicity, and possible off-target effects.

While viral delivery systems are certainly of great utility for experimental models, further studies are necessary before their possible therapeutic application may become possible in the future.

If all these obstacles can be overcome, cancer-specific oncogenes, such as the fusion genes produced by chromosomal translocations involved in several types of leukemia, could be suitable candidates for tumor cell-specific targeting in RNAi-mediated therapeutic approaches. Beside those, gene expression by infectious organisms may be targeted by RNAi. Indeed, early clinical trials are under way or being started targeting vascular epidermal growth factor (VEGF) receptor in age-related macular degeneration or genes expressed by respiratory syncytial virus (RSV). Finally, as suggested by recent reports demonstrating the cooperative effects of RNAi and selective molecular inhibitors, such as imatinib for Bcr-Abl [119, 120] and TEL-PDGFβR [124], and the kinase inhibitor MLN518 for FLT3 [128], combination of targeted therapies including small molecules and RNAi could be taken into consideration, especially when drug resistance becomes a problem.

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