New Techniques in Molecular Biology

HORMONE RESEARCH IN PÆDIATRICS

Horm Res Paediatr 2011;75:63–69
DOI: 10.1159/000322817

Received: August 26, 2010
Accepted: November 12, 2010
Published online: January 17, 2011

RNA Interference in Mammalian Cell Systems

Didier Lochmatter Primus-E. Mullis

Paediatric Endocrinology/Diabetology and Metabolism, University Children's Hospital, Inselspital, Bern, Switzerland

Key Words
MicroRNA · Noncoding RNA · RNA interference · Short interfering RNA

Abstract
In the last decade, few areas of biology have been transformed as thoroughly as RNA molecular biology. Without any doubt, one of the most significant advances has been the discovery of small (20–30 nucleotide) noncoding RNAs that regulate genes and genomes. The effects of small RNAs on gene expression and control are generally inhibitory, and the corresponding regulatory mechanisms are therefore collectively subsumed under the heading of RNA silencing and/or RNA interference. Two primary categories of these small RNAs – short interfering RNAs (siRNAs) and microRNAs (miRNAs) – act in both somatic and germline lineages of eukaryotic species to regulate endogenous genes and to defend the genome from invasive nucleic acids. Recent advances have revealed unexpected diversity in their biogenesis pathways and the regulatory mechanisms that they access. Our understanding of siRNA and miRNA-based regulation has direct implications for fundamental biology as well as disease aetiology and treatment as it is discussed in this review on 'new techniques in molecular biology'.

Introduction

The modern era of molecular biology has been highlighted by the development of methods that can alter the expression of target genes in intact cells and organisms. While applying 'transgenic' techniques, it is possible to generate organisms that express a specific gene, normal or mutated, in either a specific tissue or throughout the whole organism for the purpose of studying its effects. However, as it is a much simpler task to study the biology of a specific biochemical pathway in cultured cells system than it is to study an intact organism, it is often desirable to turn down or turn off the expression of a specific protein when studying its role. One of the places where this form of gene regulation occurs naturally is in cellular defences against the infection by specific classes of viruses. Cells have developed a mechanism that responds to the presence of double-stranded RNA (dsRNA), which is often associated with infection by these specific viruses. When the system is activated, specific enzyme complexes are able to degrade the RNA represented by the double stranded sequence, and to silence the expression of the gene product. Learning to co-opt this mechanism and to utilize it in order to study the function of specific genes has provided a powerful new tool for scientific research.

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1663–2818/11/0751–0063$38.00/0

Accessible online at:
www.karger.com/hrp

Prof. Dr. Primus-E. Mullis
Paediatric Endocrinology/Diabetology and Metabolism
University Children's Hospital, Inselspital
CH–3010 Bern (Switzerland)
Tel. +41 31 632 9552, Fax +41 31 632 9550, E-Mail primus.mullis@insel.ch

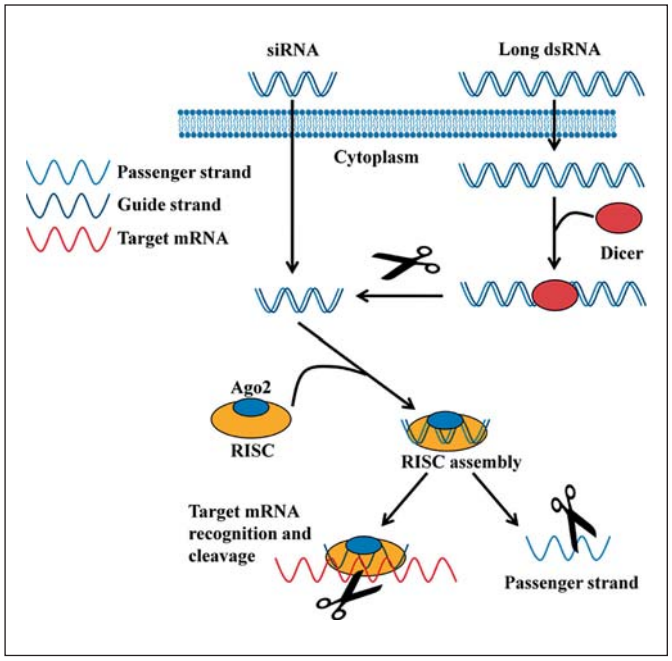

Fig. 1. RNA interference mechanism. Long double-stranded RNA is introduced into the cytoplasm, where it is cleaved into small interfering RNA (siRNA) by the enzyme Dicer. Alternatively, siRNA can be introduced directly into the cell. The siRNA is then incorporated into the RNA-induced silencing complex (RISC), resulting in the cleavage of the passenger strand of RNA by argonaute2 (Ago2). The activated RISC-siRNA complex seeks out, binds to and degrades complementary mRNA, which leads to the silencing of the target gene.

In 2006, Andrew Fire and Craig C. Mello shared the Nobel Prize in Physiology or Medicine for their work on RNA interference (RNAi) in the nematode worm *Caenorhabditis elegans (C. elegans)*, which they published in 1998 [1]. Nowadays, the use of RNAi to evoke gene silencing in mammalian cells has almost become routine laboratory practice and the capacity of RNAi to effect changes in gene expression has been proven in laboratories over and over again [2, 3]. One of the challenges of using this therapy for medical needs will be the development of methods that will allow for the introduction of interfering RNAs into various cells and tissues of the body [2]. Access to the blood stream and its cells is easy; the same cannot be said for most other cells and tissues. Delivery systems that allow this technology to be applied to many tissues will be a great advantage in its future use.

The aim of this review is to describe to a broad readership of clinical endocrinologists the basics of this excellent new methodology, which will be commonly used not only in laboratories, and therefore research, but also at the treatment and clinical levels. It summarizes the mechanisms underlying RNAi, and discusses some of the current methods used to ensure potent gene knockdown.

History of Small RNAs

In the last decade, biology has been transformed by the discovery of small noncoding RNAs [20–30 nucleotide (nt)] that can regulate the expression of genes and genomes. Everything started when 2 research groups found that a small 21-nt RNA molecule called lin-4 controlled developmental timing in *C. elegans* by posttranscriptional regulation of the gene *lin-14*. They also observed that *lin-4* did not code for a protein, but that it rather contained antisense sequences being complementary to a repeated sequence element in the 3′ untranslated region (UTR) of this very specific lin-14 mRNA. Therefore, it was hypothesized that lin-4 downregulates *lin-14* expression via an antisense RNA-RNA interaction [4, 5]. First, the discovery of this new class of RNA was taken as an oddity of the *C. elegans* genome. However, 5 years later, Fire et al. [1] reported that exogenous dsRNA silenced specifically genes through a mechanism named RNAi. Later focusing on the mechanism it became clear that RNAi relies on a cellular post-transcriptional gene regulatory mechanism that uses endogenously encoded 22-nt single-stranded RNAs to guide a ribonucleoprotein complex (the RNA-induced silencing complex, RISC) to target the mRNAs (fig. 1) [6]. Further, at least 1000 of these microRNAs (miRNAs) are found in the human genome; they are believed to have a key role in regulating vertebrate differentiation and development [6]. The miRNA biogenesis pathway includes 3 distinct RNA intermediates: the initial pri-miRNA, the pre-miRNA hairpin and the miRNA duplex (fig. 2). All can be used as entry points to allow programming of RISC with artificial miRNAs call short interfering RNAs (siRNAs). Therefore, miRNAs and siRNAs appeared to be distinguished in 2 primary ways. Firstly, miRNAs were viewed as endogenous and purposefully expressed products of an organism's own genome, whereas siRNAs were thought to be primarily exogenous in origin, derived directly from the virus, transposon, or transgene trigger. Secondly, miRNAs appeared to be processed from stem-loop precursors with incomplete double-stranded character, whereas siRNAs were found to be excised from long, fully complementary dsRNAs. It seemed clear that that the biogenesis of these small RNAs was different and by this point, the 2 catego-

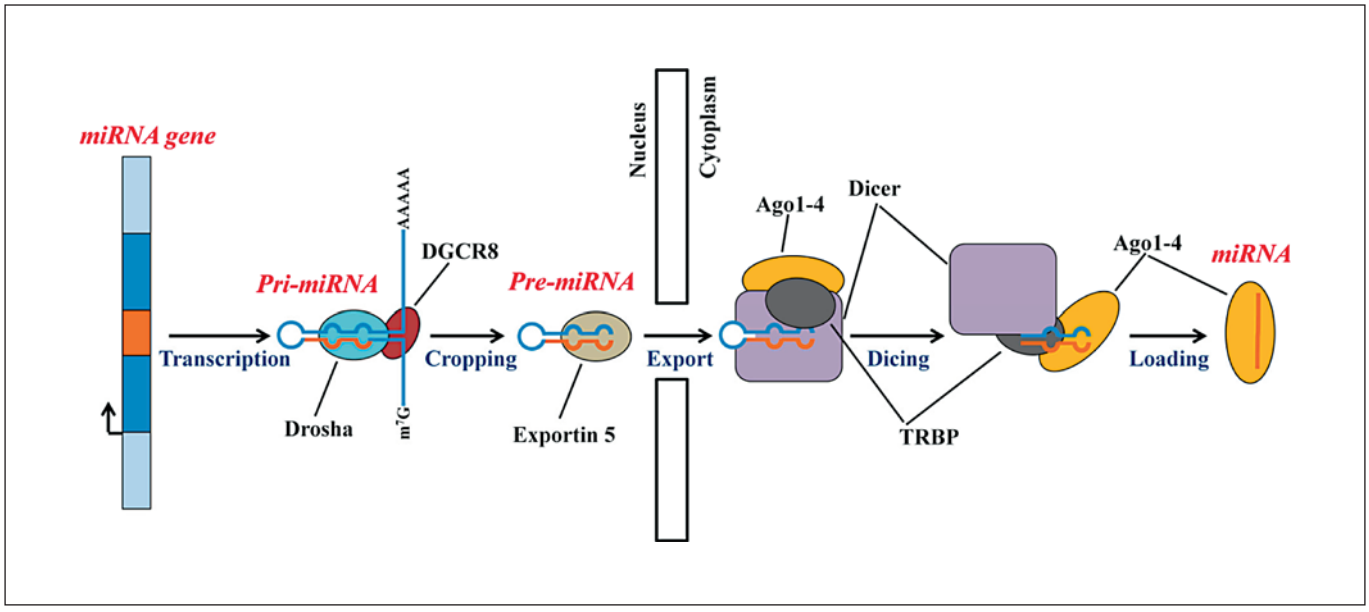

Fig. 2. Animal miRNAs biogenesis pathway. Most microRNAs (miRNAs) are processed from primary transcripts (pri-miRNAs) which have a particular hairpin structure containing imperfectly base-paired stems. They are usually encoded within specific miRNA genes or within introns of protein coding transcription units and are transcribed by RNA polymerase II. Therefore pri-miRNAs can be caped by 7-methylguanosine (m^7G) at their 5′ site as well as polyadenylated at their 3′ end (AAAAA). Following transcription, the initiation step (cropping) is mediated by the Drosha-DiGeorge syndrome critical region gene 8 (DGCR8) complex that generates ~65-nucleotide pre-miRNAs. Pre-miRNAs have a short stem plus a ~2-nucleotide 3′ overhang, which is recognized by the nuclear export factor exportin 5. Upon export from the nucleus, the cytoplasmic RNase III Dicer catalyses the second processing (dicing) step to produce miRNA duplexes. Dicer, TRBP (TAR RNA-binding protein), and argonaute (Ago)1-4 mediate the processing of pre-miRNA into a mature miRNA guide of 20–22 nucleotides which is incorporated into the RNA-induced silencing complex (RISC), which in turn induce gene silencing at a posttranscriptional level.

ries of small RNAs had become firmly embedded in our view of the gene regulatory landscape: miRNAs, as regulators of endogenous genes, and siRNAs, as defenders of genome integrity in response to foreign or invasive nucleic acids such as viruses, transposons, and transgenes [7]. In the following years more and more evidence suggested that miRNAs play an essential role in multiple biological processes and today they are suggested to regulate about 30% of protein-coding genes [8].

siRNA and miRNA Biogenesis in Mammalian Cells

A common characteristic of single-stranded forms of both miRNAs and siRNAs is that they associate with a multiprotein effector assembly known as the RISC [9]. Dicer, an RNA III endoribonuclease first cleaves dsRNAs and, in association with TAR RNA-binding protein (TRBP) and argonaute 2 (Ago2) nucleate the formation of an active RISC to induce gene silencing [10, 11]. Although the pathways followed by these 2 types of small RNAs are relatively similar, both have their own characteristics.

siRNAs were originally believed to be mainly produced as a defence mechanism in response to foreign nucleic acid such as viral dsRNAs but more and more studies uncovered their endogenous genomic origin (endo-siRNA), and new roles related to transposons and gene regulation are emerging for this new class of small RNAs [12]. In general, RNAi can be triggered by the presence of long pieces of dsRNAs (endogenous or exogenous), which are cleaved by Dicer into the fragments with 5′ phosphates and 2-nucleotide 3′ overhangs known as siRNAs (21–23 nt) [13]. This phenomenon can be circumvented by the introduction of a synthetic siRNA into the cells therefore avoiding processing from Dicer. Once in the cytoplasm, siRNA is incorporated into the RISC [14] and its main catalytic protein Ago2, incorporate the siRNA guide strand (or antisense strand), whereas the passenger strand (or sense strand) is cleaved [15]. The activated RISC selectively seeks out

and degrades mRNA that is complementary to the antisense strand (fig. 1) [16].

In contrast, endogenous miRNAs are typically encoded within genes, introns or as separate transcription units and generated by a multiple-step process starting from a miRNA gene that will finally produce a mature miRNA. They are usually transcribed by polymerase II (POL II) and possess a specific hairpin-shaped stem-loop secondary structure that lacks perfect Watson-Crick complementarities [17] which can, like protein coding mRNAs, be caped by 7-methylguanosine at its 5′ site as well as being polyadenylated at its 3′ end [18]. The pri-miRNA hairpin enters a large microprocessor complex which is constituted of Drosha (RNase III endonuclease) and an essential cofactor, DiGeorge syndrome critical region gene 8 (DGCR8) (a protein containing 2 double-stranded RNA binding domains). DGCR8 first recognizes the distinct stem-loop structures and binds to the pri-miRNA. Drosha asymmetrically and specifically cuts both strands of the hairpin-shaped stem at the sites near the base of the stem loop and finally releases a 60- to 70-nucleotide pre-miRNA that has a 5′ phosphate and a 3′ two-nucleotide overhang. The pre-miRNA is then transported to the cytoplasm by Exportin-5 (EXP5). Like siRNAs, once in the cytoplasm, pre-miRNAs are further processed by Dicer. Finally, miRNA is unwound, one strand of the duplex is degraded whereas the other strand, a mature miRNA guide of 20–22 nucleotides, is incorporated into RISC complex (fig. 2) which in turn, induces gene silencing at a posttranscriptional level [7, 11, 17].

Animal miRNA Biogenesis and Gene Regulation

miRNA can trigger post-transcriptional gene regulation by base pairing of the guide strand with a target mRNA and subsequent mediation of translational repression or mRNA degradation [19]. Both siRNAs and miRNAs perfectly complementary to their target mRNA usually promote endonucleolytic cleavage. However, if mismatches and bulges are present during base pairing with mRNA, translational repression followed by deadenylation, decapping, and exonucleolytic cleavage (miRNA mediated mRNA decay) generally occurs [7, 20]. These activities account for most miRNAs but other types of regulation, such as translational activation [21] and heterochromatin formation [22], have also been described.

With few exceptions, animal miRNA-binding sites lie in the 3′ UTR sequence and are usually present in multiple copies. A key feature of target mRNA recognition was determined by experimental and bioinformatic analyses, and involves Watson-Crick base pairing of miRNA nucleotides 2–8, representing a so-called seed region. In addition to this, it was observed that GU pairs or mismatches in the seed region greatly affect repression, but are usually present in the central region of the miRNA-mRNA duplex. Finally, reasonable complementarity to the miRNA 3′ half is required to stabilize the duplex interaction [19]. Animal mRNAs can be targeted simultaneously by more than one miRNA species at multiple sites in the 3′ UTR, leading to a degree of translational repression thought to be associated with the degree of miRNA-binding. In addition, the seed region and mRNA recognition with mismatches, enables the base pairing of a single miRNA with several targets of similar sequences [23]. In contrast, most plant miRNAs bind with near-perfect complementarity to sites within the coding sequence of their targets and trigger endonucleolytic mRNA cleavage by an RNAi-like mechanism. In rare instances, a similar mechanism is used by vertebrates [19]. However, when synthetic siRNAs are specifically designed to engage targets with imperfect complementarity they become virtually indistinguishable from miRNAs in their silencing effects, and mediate translational repression and exonucleolytic degradation in a manner similar to miRNA silencing (fig. 3) [24]. All these structural and functional aspects of endogenous miRNA can potentially lead to off-target mRNA silencing [25, 26] and therefore experimental testing is required when designing synthetic siRNAs or miRNAs.

Exogenous siRNA/miRNA Delivery, and Safety Concerns

There are several ways to artificially generate RNA molecules to silence gene expression. Historically, the first method used, mainly in worms and plants, involved the introduction of long dsRNAs complementary to a specific target sequence. However, long dsRNAs (>30 nt) trigger an interferon response and the shutdown of cellular protein expression in mammalian cells [27]. This can be circumvented by the introduction of chemically synthesized siRNAs directly into the cytoplasm bypassing the 'dicing' step. RNAi may also be mediated by short hairpin RNA (shRNA) cloned in a plasmid or a viral vector [28]. The core of these constructs is constituted of a sequence of 21–29 nt, a short loop region, and the reverse complement of 21–29 nt region usually driven by a poly-

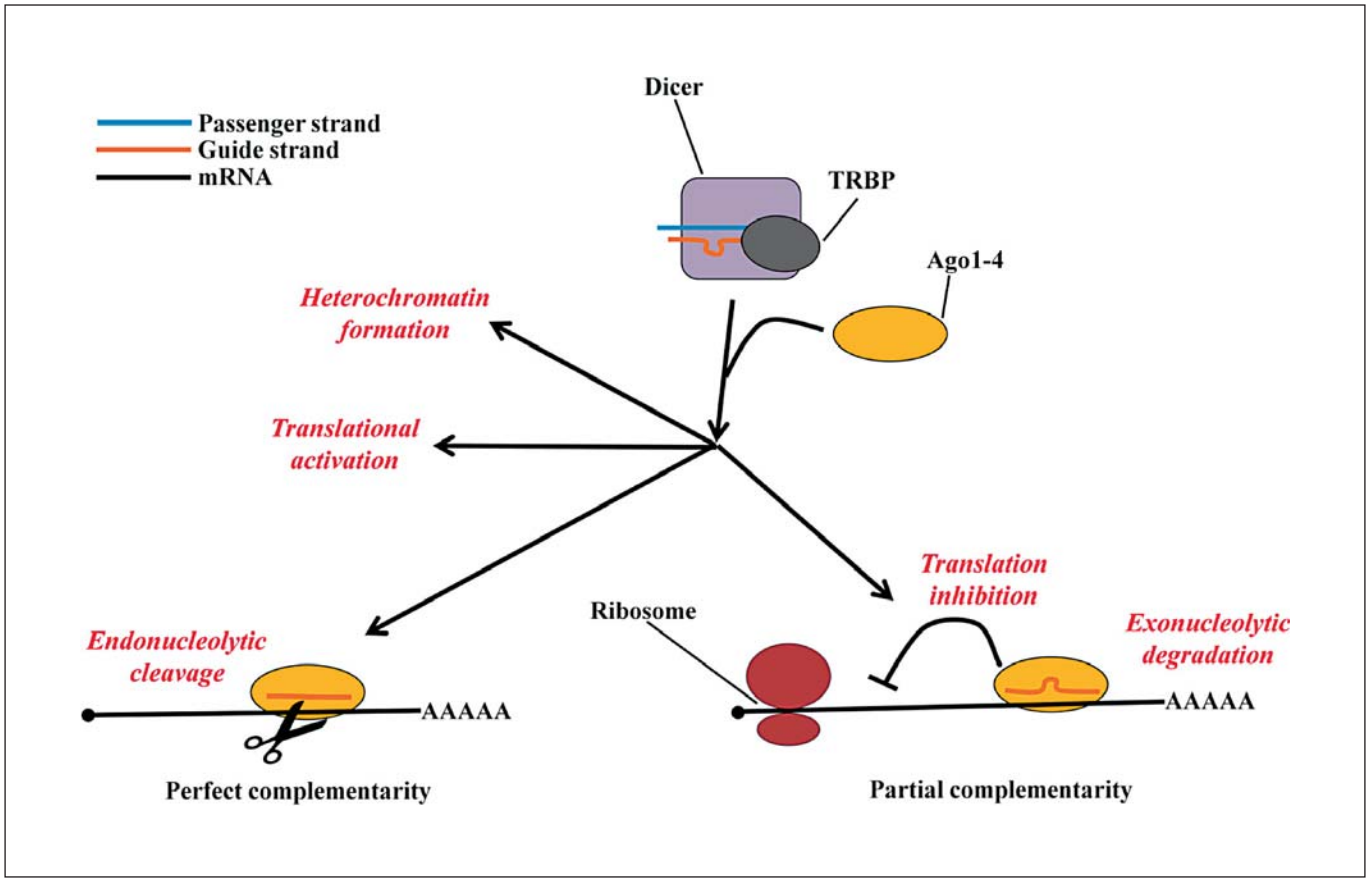

Fig. 3. siRNA and miRNAs mode of action. Upon export from the nucleus, Dicer catalyses the dicing step to produce miRNA duplexes. Dicer, TAR RNA-binding protein (TRBP) and argonaute (Ago)1-4 process a mature miRNA guide and mediate its incorporation into the RNA-induced silencing complex (RISC). In animals, miRNAs usually recognize their complementary mRNA imperfectly and block their expression by translation inhibition and further exonucleolytic degradation. miRNAs can follow the same pathway as observed for siRNAs of perfect complementarity with their target mRNA, leading to Ago2-catalyzed mRNA cleavage (endonucleolytic cleavage) and further degradation of the remaining fragments. Other types of regulation have been recently discovered, such as heterochromatin formation or activation of translation, but these are not yet well characterized.

merase III (POL III) promoter such as U6 or H1. When transcribed in vivo, this short transcript folds back on itself to form a hairpin structure which is transported in the cytoplasm and processed by Dicer to finally produce duplex RNA similar to siRNA. As the understanding of miRNA biogenesis advanced, a new generation of constructs expressing RNA hairpin more closely resembling endogenous miRNAs appeared. Zeng et al. [24] first developed a vector based on human miR-30, and showed that potent RNAi could be triggered by substituting the stem sequences of the miRNA precursor with unrelated base-paired sequences targeting a gene of interest. This initial work has led to the development of a new generation of miRNA-adapted shRNA (shRNAmir) vectors [29] that enter the endogenous miRNA pathway starting with the transcription of an initial pri-mRNA.

The use of shRNA vectors for RNAi was hampered when in 2006, Grimm et al. [30] evaluated the long-term high level expression of several shRNAs species delivered by adeno-associated vector in the liver of mice and showed that many animals presented a dose-dependent liver injury which was, in some cases, associated with morbidity. They concluded that toxicity was largely due to oversaturation of the endogenous small RNAs pathway and that this could be overcome by optimizing shRNA dose and sequence. Castanotto et al. [31] confirmed this concept when they performed studies on siRNA and found that siRNAs and shRNAs compete with the en-

dogenous miRNAs for transport and incorporation into RISC. Pri-miRNA transcripts expressed from shRNAmir vectors are first cropped by Drosha. In contrast to shRNAs that are solely processed by Dicer, or synthesised siRNA circumventing this step, shRNAmirs enter the miRNA pathway at an earlier stage that may slow their final incorporation into RISC, and make them possibly less prone to cause endogenous perturbation. The difficulty to transfect certain cell types and the need of permanent gene knockdown led to the development of retroviral vectors (RVs) and lentiviral vectors (LVs) enabling RNAi.

To summarize, gene silencing has proven its efficacy in research and has become a major method in cellular biology. Our understanding of the mechanism regulating RNAi is still incomplete; however, controlling gene expression represents an attractive way to heal human diseases. Many issues regarding unspecific gene silencing via synthetic molecules or their mode of delivery have to be solved, nevertheless the future for RNAi in research and for human therapy seems to be quite bright.

Potential for Therapeutic Applications

Short oligonucleotides including siRNA and microRNA used to degrade mRNA transcripts, and therefore suppress protein translation, and antisense oligonucleotides to manipulate splicing are of high potential for therapeutic applications. Using this methodology we were able to show that the harmful 17.5-kDa GH- variant causing the autosomal dominant GH-deficiency (IGHD II) can be blocked and, therefore, the disorder rescued [2]. Overall, as recently summarized in a review, RNAi has become the method of choice for researchers wishing to target specific genes for silencing and has provided immense potential as therapeutic tools. Without any doubt, RNAi technique will become an important and potent weapon for fighting against various diseases. However, RNAi technique has benefits and limitations in its potential clinical applications [32].

Acknowledgement

This study was financially supported by a grant from the Swiss National Foundation (SNF 320000-121998 to P.-E.M.)

References

1 Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC: Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 1998;391:806–811.
2 Lochmatter D, Strom M, Eble A, Petkovic V, Fluck CE, Bidlingmaier M, Robinson IC, Mullis PE: Isolated GH deficiency type II: knockdown of the harmful Delta3GH recovers wt-GH secretion in rat tumor pituitary cells. Endocrinology 2010;151:4400–4409.
3 Nlend RN, Meyer K, Schumperli D: Repair of pre-mRNA splicing: prospects for a therapy for spinal muscular atrophy. RNA Biol 2010;7:430–440.
4 Lee RC, Feinbaum RL, Ambros V: The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 1993;75:843–854.
5 Wightman B, Ha I, Ruvkun G: Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*. Cell 1993;75:855–862.
6 Cullen BR: RNAi the natural way. Nat Genet 2005;37:1163–1165.
7 Carthew RW, Sontheimer EJ: Origins and mechanisms of miRNAs and siRNAs. Cell 2009;136:642–655.
8 Lewis BP, Burge CB, Bartel DP: Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 2005;120:15–20.
9 Hammond SM, Bernstein E, Beach D, Hannon GJ: An RNA-directed nuclease mediates post-transcriptional gene silencing in drosophila cells. Nature 2000;404:293–296.
10 Meister G, Tuschl T: Mechanisms of gene silencing by double-stranded RNA. Nature 2004;431:343–349.
11 Kim VN, Han J, Siomi MC: Biogenesis of small RNAs in animals. Nat Rev Mol Cell Biol 2009;10:126–139.
12 Golden DE, Gerbasi VR, Sontheimer EJ: An inside job for siRNAs. Mol Cell 2008;31:309–312.
13 Bernstein E, Caudy AA, Hammond SM, Hannon GJ: Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 2001;409:363–366.
14 Rand TA, Ginalski K, Grishin NV, Wang X: Biochemical identification of argonaute 2 as the sole protein required for RNA-induced silencing complex activity. Proc Natl Acad Sci USA 2004;101:14385–14389.
15 Matranga C, Tomari Y, Shin C, Bartel DP, Zamore PD: Passenger-strand cleavage facilitates assembly of siRNA into ago2-containing RNAi enzyme complexes. Cell 2005;123:607–620.
16 Ameres SL, Martinez J, Schroeder R: Molecular basis for target RNA recognition and cleavage by human RISC. Cell 2007;130:101–112.
17 Zhang B, Wang Q, Pan X: MicroRNAs and their regulatory roles in animals and plants. J Cell Physiol 2007;210:279–289.
18 Cai X, Hagedorn CH, Cullen BR: Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. RNA 2004;10:1957–1966.
19 Filipowicz W, Bhattacharyya SN, Sonenberg N: Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet 2008;9:102–114.
20 Brodersen P, Voinnet O: Revisiting the principles of microRNA target recognition and mode of action. Nat Rev Mol Cell Biol 2009;10:141–148.
21 Vasudevan S, Tong Y, Steitz JA: Switching from repression to activation: microRNAs can up-regulate translation. Science 2007;318:1931–1934.

22 Kim DH, Saetrom P, Snove O Jr, Rossi JJ: MicroRNA-directed transcriptional gene silencing in mammalian cells. Proc Natl Acad Sci USA 2008;105:16230–16235.
23 Doench JG, Sharp PA: Specificity of microRNA target selection in translational repression. Genes Dev 2004;18:504–511.
24 Zeng Y, Wagner EJ, Cullen BR: Both natural and designed microRNAs can inhibit the expression of cognate mRNAs when expressed in human cells. Mol Cell 2002;9:1327–1333.
25 Fedorov Y, Anderson EM, Birmingham A, Reynolds A, Karpilow J, Robinson K, Leake D, Marshall WS, Khvorova A: Off-target effects by siRNA can induce toxic phenotype. RNA 2006;12:1188–1196.
26 Lin X, Ruan X, Anderson MG, McDowell JA, Kroeger PE, Fesik SW, Shen Y: Sirna-mediated off-target gene silencing triggered by a 7-nt complementation. Nucleic Acids Res 2005;33:4527–4535.
27 Meurs E, Chong K, Galabru J, Thomas NS, Kerr IM, Williams BR, Hovanessian AG: Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. Cell 1990;62:379–390.
28 Whitehead KA, Langer R, Anderson DG: Knocking down barriers: advances in siRNA delivery. Nat Rev Drug Discov 2009;8:129–138.
29 Fewell GD, Schmitt K: Vector-based RNAi approaches for stable, inducible and genome-wide screens. Drug Discov Today 2006;11:975–982.
30 Grimm D, Streetz KL, Jopling CL, Storm TA, Pandey K, Davis CR, Marion P, Salazar F, Kay MA: Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. Nature 2006;441:537–541.
31 Castanotto D, Sakurai K, Lingeman R, Li H, Shively L, Aagaard L, Soifer H, Gatignol A, Riggs A, Rossi JJ: Combinatorial delivery of small interfering RNAs reduces RNAi efficacy by selective incorporation into RISC. Nucleic Acids Res 2007;35:5154–5164.
32 Chen SH, Zhaori G: Potential clinical applications of siRNA technique: benefits and limitations. Eur J Clin Invest 2010, in press.