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Review

Epigenetherapy, a new concept

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Abstract

Small RNAs have been shown to regulate gene transcription by interacting with the promoter region and modifying the histone code. The exact mechanism of function is still unclear but the feasibility to activate or repress endogenous gene expression with small RNA molecules has already been demonstrated *in vitro* and *in vivo*. In traditional gene therapy non-mutated or otherwise useful genes are inserted into patient's cells to treat a disease. In epigenetherapy the action of small RNAs is utilized by delivering only the small RNAs to patient's cells where they then regulate gene expression by epigenetic mechanisms. This method could be widely useful not only for basic research but also for clinical applications of small RNAs.

Keywords: epigenetics; gene therapy; miRNA; siRNA; small RNAs.

Introduction

The name 'Epigenetherapy' is derived from two disciplines, epigenetics and gene therapy. Epigenetics studies inherited changes in gene expression caused by mechanisms other than mutations in the DNA sequence, hence the name epigenetics (*epi-* in Greek standing for επι- over, above). Gene therapy means insertion of genes into individual cells to treat a disease. In epigenetherapy, endogenous epigenetic mechanisms are directed by gene delivery technologies to achieve a therapeutic effect. In this review we discuss the development of this novel field of research.

RNA interference (RNAi) is a process of silencing genes by small RNAs. Already in 1998, Fire et al. showed that double-stranded RNA (dsRNA) is effective in gene silencing (1) and since then the detailed mechanisms behind the RNAi process have been defined. dsRNAs needed for triggering this process can be from an exogenous source or transcribed from endogenous genes that produce microRNAs (miRNAs) (2). This process has also been adapted to therapeutic use and initial clinical trials are already in progress using small interfering RNAs (siRNAs) against, for example, key mediators of age-related macular degeneration which is one of the leading causes of blindness (3, 4). The RNAi approach has also been studied in cancer, viral infections and neurodegenerative diseases (5–7). It has been noted that nearly all human diseases with a gain-of-function genetic lesion are potential targets for therapeutic RNAi (8).

RNAi can occur through different mechanisms: by transcriptional or post-transcriptional gene silencing (TGS and PTGS, respectively) (9–11). PTGS and its action on mRNA degradation is still the best understood form of RNAi, in which the small RNA recognizes its target mRNA and RNA-induced silencing complex (RISC) then cleaves the mRNA at a distinct position (11, 12). TGS was first discovered in plants, where dsRNA corresponding to sequences that are not transcribed, were noticed to direct methylation of DNA and thereby also transcriptional repression (10). TGS occurs through chromatin modifications: the small RNAs incorporated into RISC recognize target sequences on the promoters and recruit chromatin modifying proteins, leading to gene silencing (10). Besides plants, it has been identified in other organisms as well. For example, Morris et al. confirmed the phenomena in human cells (13). TGS is associated with specific modifications at the epigenetic level, such as methylation of histone H3 lysine 9 (H3K9) and histone H3 lysine 27 (H3K27) in addition to the DNA methylation (14, 15). Weinberg et al. (14) have also identified the antisense strand of the siRNAs to be the one directing the histone code in human cells. Other research groups have discovered that the antisense RNAs are able to induce epigenetic changes, for example the natural antisense RNA of the tumor suppressor gene p15 triggers the heterochromatin formation (16).

In addition to gene silencing, small RNAs can induce transcriptional activation (17, 18). The dsRNAs were targeted to the non-coding regulatory regions of the promoters and they were shown to reduce H3K9 methylation, inducing gene activation via an Argonaute 2 (Ago2)-dependent manner. The role of the small RNAs in gene regulation therefore seems to be very diverse and thus might be effectively utilized for therapeutic applications.

Gene therapy

The aim of gene therapy is to bring genetic material to somatic cells in order to confer a therapeutic benefit for the

individual (19, 20) (Figure 1). One way is by introducing a functional gene into a target cell, thereby restoring protein production that is absent or deficient due to a genetic disorder. In addition, the normal functioning of a mutated gene may possibly be restored by gene correction (19). Another possible approach to curing diseases is to turn off disease-causing genes through expression of siRNAs (21). Typically, somatic cells (cells not involved in reproduction) are targeted to ensure that the genetic modification will not pass to the next generations (19, 22).

Introduction of therapeutic DNA into cells is achieved using one of the several types of DNA carrying 'vehicles' called vectors. Non-viral technologies rely on the use of liposomes or cationic compounds, such as dendrimers, for delivery of transgenes (23) and are limited in their effectiveness. Viral vectors, such as adenoviruses, adeno-associated viruses, retroviruses and lentiviruses are more efficient for *in vivo* gene delivery applications. Retroviral vectors are widely used in gene therapy protocols (21% of the clinical trials; http://www.wiley.com/legacy/wileychi/genmed/clinical/). Their advantages come from the unique life cycle of retroviruses that enables the viral genome to become permanently incorporated as a part of the cellular chromatin (19, 22).

Delivery of small RNAs as a therapeutic approach

RNA interference (RNAi) represents a promising new strategy for using small RNA molecules to silence specific genes associated with disease processes. Several clinical trials are currently underway and many are in pre-clinical development. Bevasiranib, the first small interfering RNA agent developed for the treatment of neovascular age-related macular degeneration, held clinical promise for therapeutic use of RNAi [24]. Unfortunately, Opko Health decided to close the clinical program due to the lack of efficiency (http://www.opko.com). Although bevasiranib showed activity when used in conjunction with Lucentis (ranibizumab, Genentech), the trial was unlikely to meet its primary endpoint. Luckily, no systemic or local ocular safety issues were identified in the trial. The use of siRNA oligos for RNAi is easy and fast but unfortunately only mediates a transient effect. Also, non-viral delivery of RNA oligonucleotides is a major limiting factor for the use of RNAi *in vivo*.

RNAi can also be used to knockdown target gene expression with a virus vector-mediated delivery of small hairpin RNA molecules (shRNAs). These molecules are driven by

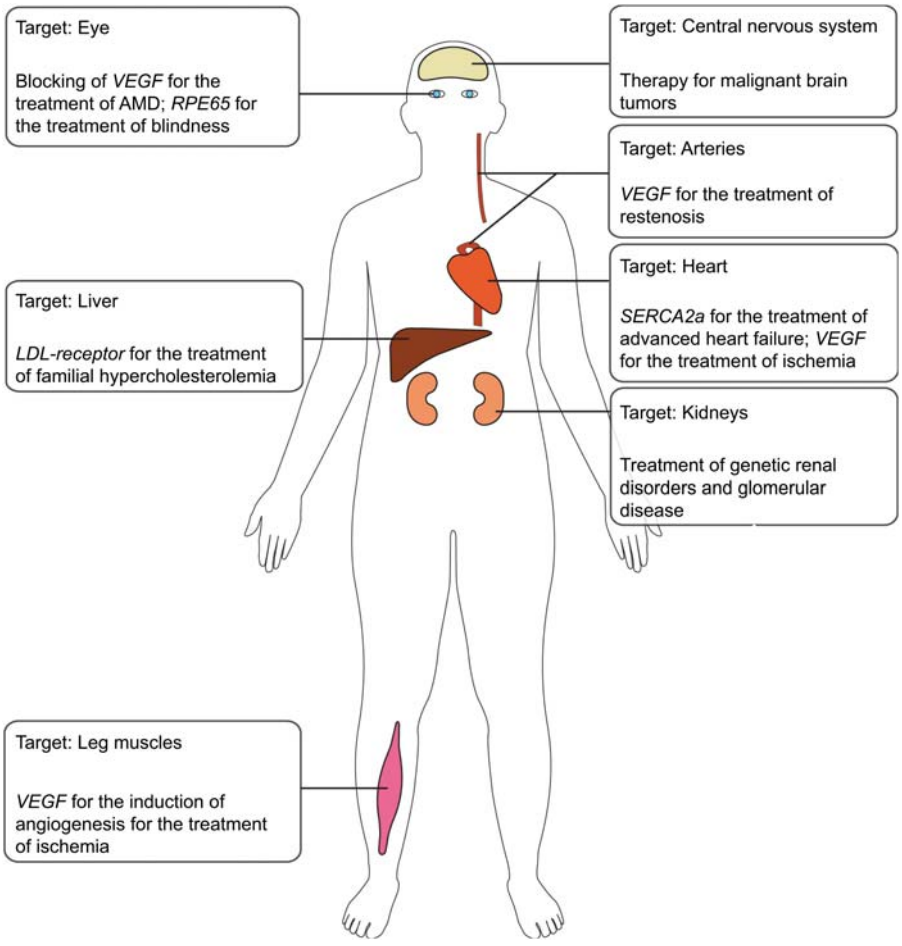

Figure 1 Examples of different gene therapy treatments for various target organs.

polymerase II or III promoters as hairpin structures. They are processed in the cytoplasm by an enzyme called Dicer into mature duplex RNAs, which then bind to RISC complex and mediate RNAi mediated knockdown of the targeted mRNA. By using a virus vector-based delivery technology, one can design regulated and/or tissue specific expression systems (24).

Epigenetic mechanisms

Epigenetics includes all heritable information that occurs beyond the DNA sequence itself (25). Epigenetic inheritance takes place through mechanisms involving modifications of chromatin and proteins associated with it: DNA methylation and covalent and non-covalent modifications of histones (26). These events can control gene expression independently of the DNA sequence and are involved in many physiological and pathological conditions (25).

Histone modifications are considered to form a histone code that is read by chromatin-associated proteins and translated into a transcriptionally active or repressed genetic state (27). The epigenetic histone modifications are predominantly located at specific positions in the amino-terminal tails of histones (28). These protease-sensitive tails protrude from the chromatin surface and comprise approximately 25% of the mass of core histones thus providing an exposed surface for interactions with other proteins (29). The modifications of histones are dynamic so that there are histone-modifying enzymes that add and remove the modifications (30). For acetylation and methylation, the histone acetyltransferases and histone methyltransferases add the acetyl and methyl groups to histone residues, respectively. Accordingly, the histone deacetylases and histone demethylases remove these modifications. Many coactivators contain the histone acetyltransferase activity (e.g., p300/CBP) whereas some global transcriptional repressors are associated with histone deacetylases (e.g., complex of NCoR and Sin3), which is strongly correlated with the fact that acetylation of histone residues mark active genes (31, 32). Histone methyltransferases seem to be the most specific of the histone modifying enzymes, like SUV39H1, which only methylates H3K9 (33). Examples of demethylating proteins are the family of Jarid1 of JmjC proteins and the nuclear amine oxidase homologue lysine-specific demethylase 1 (LSD1) (34, 35).

The levels of histone modifications seem to be quantitatively related to gene expression levels (36). Gene expression levels can be predicted by analyzing a set of histone modifications on the gene promoter. Wang et al. (37) identified a backbone of 17 histone modifications associated with increased gene expression. Karlic et al. (36) reduced the number of significant modifications into four, but the set of four modifications seems to be different with high CpG content promoters and with low CpG content promoters. Recently Ernst and Kellis introduced a computational method for annotating genome with different chromatin states including diverse epigenetic information (38). They were able to differentiate chromatin states (e.g., promoter-associated states, enhancers) using genome-wide chromatin immunoprecipitation sequencing data for 38 histone methylation and acetylation marks, histone variant H2AZ, RNA polymerase II and CTCF. H3K4me2, H3K9ac and H3K14ac marks have, for example been associated with transcription start sites (TSS) in the human genome and they may be required for transcription initiation, but their proportion is reduced downstream of the TSS implying that these marks are not needed for elongation by polymerases (39).

Small RNAs in epigenetic regulation

Small RNA molecules have been shown to regulate gene transcription by interacting with the promoter region and modifying the histone code (13, 17, 18, 40). Transcriptional gene silencing involves promoter-targeted siRNA and leads to silent state epigenetic profile containing H3K9me2 and H3K27me3 (14). This process was first recognized in plants but has since been identified in mammals (10, 13). Besides TGS, the small RNAs have been reported to induce gene activation (17, 18). This is associated with a loss of H3K9me2 at the targeted promoter sequences and seems to require a member of the RISC complex, Ago2. TGS has earlier been noticed to require Ago 1 and 2 (41). The more precise mechanisms for both gene activation and silencing are still largely unknown.

We recently designed some shRNAs targeted on vascular endothelial growth factor A (VEGF-A) promoter and delivered them to mouse endothelial cell lines by lentiviral transduction (18). We identified a shRNA significantly inducing VEGF-A expression whereas another one downregulated it. The downregulating shRNA caused demethylation of H3K4me2 and deacetylation of H3K9ac at the promoter and TSS but had no effect on H3K9me2 level. It also enriched nucleosome positioning both at the promoter and TSS. The upregulating shRNA increased H3K4me2 at TSS but not at the targeted promoter and had no effect on nucleosome positioning. The epigenetic changes were initially observed 14 hours after transduction and had further increased by seven days. The effect is cell- and tissue-specific since the up- and downregulation and epigenetic changes were only noticed in some cell lines and not in others. We also wanted to evaluate the effects of shRNAs *in vivo*. Thus, we transduced mouse ischemic hindlimbs with the lentiviral vectors encoding the up- and downregulating shRNAs. The observed effect and epigenetic profile in muscle tissue was similar to that found in cell culture. Ultrasound analysis also showed that upregulating shRNA significantly increased vascularity and improved blood flow in the hindlimb, demonstrating that promoter targeted shRNAs can have therapeutic effects *in vivo* (18).

It was recently shown that a long intergenic non-coding RNA (lincRNA) HOTAIR acts as a molecular scaffold by binding a histone methylase (polycomb repressive complex2) and a demethylase (LSD1/CoREST/REST complex) with distinct domains and thus directing a specific combination of histone methylations to the target gene chromatin (42). The

report suggests a possibility that other lincRNAs could also guide distinct histone modification patterns to specific genes and therefore affect the epigenetic state of the chromatin during development and disease progression.

Small RNAs in physiological and pathological conditions

Small RNAs have been studied in many cancer cell types. Small activating RNA (saRNA) targeted on p21 promoter inhibited cell proliferation and induced G1-phase arrest and apoptosis by upregulating the p21 expression in bladder cancer cells (43). Targeting the promoters of E-cadherin, p21 and VEGF in human cells resulted in induction of the indicated gene in the studies by Li et al. and the gene activation involved epigenetic changes in the promoter (17). The group also transfected African green monkey and chimpanzee cells with the same promoter targeted small RNAs (44). Since the promoter sequence is highly conserved between humans and primates, the activation of the genes were also induced in these cell lines. They also tested saRNAs targeting promoters of genes p53, PAR4, WT1, RB1, p27, NKX3-1, VDR, IL2 and pS2 in primate cell lines but succeeded in the induction of only p53, PAR4, WT1 and NKX3-1. Huang et al. also successfully designed saRNAs for mouse and rat, targeting the promoters of Cyclin B1 and chemokine receptor CXCR4, respectively (44). Cyclin B1 is known to promote mitosis entry and the saRNAs did increase H3S10 phosphorylation, correlating with chromosome condensation in mitosis.

Kloc et al. showed that endogenous siRNAs are involved in histone modifications needed for chromosome replication during the S phase in fission yeast (45). The phosphorylation of H3S10 during mitosis leads to the dissociation of heterochromatin protein 1 (HP-1), since H3S10ph prevents binding of the HP-1 to H3K9me2 via the 'phospho-methyl switch'. This allows heterochromatic transcripts to accumulate in the S phase of the cell cycle. The transcripts are further processed into siRNAs and they in turn promote the methylation of H3K9 and formation of heterochromatin so that the gene silencing pattern is inherited after cell division.

Survivin is an anti-apoptotic protein that is upregulated in many human tumors and it has been studied as a therapeutic target in cancers (46). Ma et al. developed a short methylated oligonucleotide complementary to the survivin gene promoter (47). The molecule was named SurKex and treating a human non-small cell lung cancer cell line with it induced histone hypermethylation of H3K9 and H3K27 and DNA methylation in the complementary region of the survivin promoter. In a mouse model of human lung cancer, SurKex reduced tumor volume and weight in a dose-dependent manner. Another possible therapeutic target in cancer is the epithelial cell adhesion molecule E-cadherin, downregulation of which is important in tumor progression (48). Small RNAs targeted on E-cadherin promoter have been shown to upregulate E-cadherin expression in human bladder cancer cells and the upregulation also reduced migration and invasion *in vitro* (49). In breast carcinoma cells, saRNAs upregulating E-cadherin induced cell apoptosis, inhibited proliferation and reduced cancer migration and inhibited tumor growth in an animal model (50). Also, saRNAs complementary to p21 promoter upregulated its expression in T24 human bladder cancer cells and dose- and time-dependently inhibited cell proliferation and viability (43). This was related to G1-phase arrest and apoptotic cell death.

In the studies by Napoli et al., siRNA complementary to c-myc gene promoter inhibited c-myc expression (51). However, this did not involve epigenetic changes on the promoter but blocked the assembly of the transcription pre-initiation complex and was depended on Ago2 recruitment and a non-coding RNA overlapping the TSS. Silencing c-myc with siRNA treatment induced growth arrest in prostate cancer cells over-expressing c-myc but had only a minimal effect on normal cells expressing low c-myc. With small RNAs, Hawkins et al. targeted the promoter of human ubiquitin c gene, which is involved in skeletal muscle atrophy (52). Targeting lasting at least three days resulted in long-term silencing of ubiquitin c gene and was associated with increased levels of H3K9me2 and H3K27me3 and several days later increased DNA methylation. However, some other studies have found that DNA methylation is not related to the gene silencing by promoter-targeted dsRNAs. For example small RNAs targeted on CDH1 promoter in human cancer cells silenced the gene but this did not involve any changes in DNA methylation (53). They were also able to repeat the experiment successfully in a genetically modified cell line, in which the DNA methylation machinery was absent.

Mechanistic aspects of small RNA action in epigenetics

The exact mechanism of action of small RNAs in epigenetic gene regulation is largely unknown. Targeting of the VEGF-A promoter with lentiviral shRNAs resulted in Ago2 recruitment to the promoter and TSS both in small RNA mediated transcriptional repression and activation (18). The down-regulating shRNA also recruited a H3K4me2 and H3K9me2 specific histone demethylase LSD1 at the promoter and TSS. Based on these results we suggest a hypothetic model for shRNA-mediated repression and activation of VEGF-A (Figure 2). The shRNA is bound to RISC in cytoplasm and goes through processing during which also the histone modifying enzymes are attached to the complex. The complex is then transported to the nucleus where it recognizes its target sequence on the gene promoter and modulates target gene activity via histone acetylation and methylation.

One suggested mechanism of action for promoter targeted small RNA mediated gene activation and silencing is that the antisense strand of the small RNA binds to a complementary non-coding promoter-associated antisense RNA (54–56). Small RNAs targeted on the progesterone receptor gene promoter could either activate or silence the gene (55). In this study it was shown that there is an antisense transcript overlapping the progesterone receptor promoter and it was necessary for the activation of expression. The small RNAs also

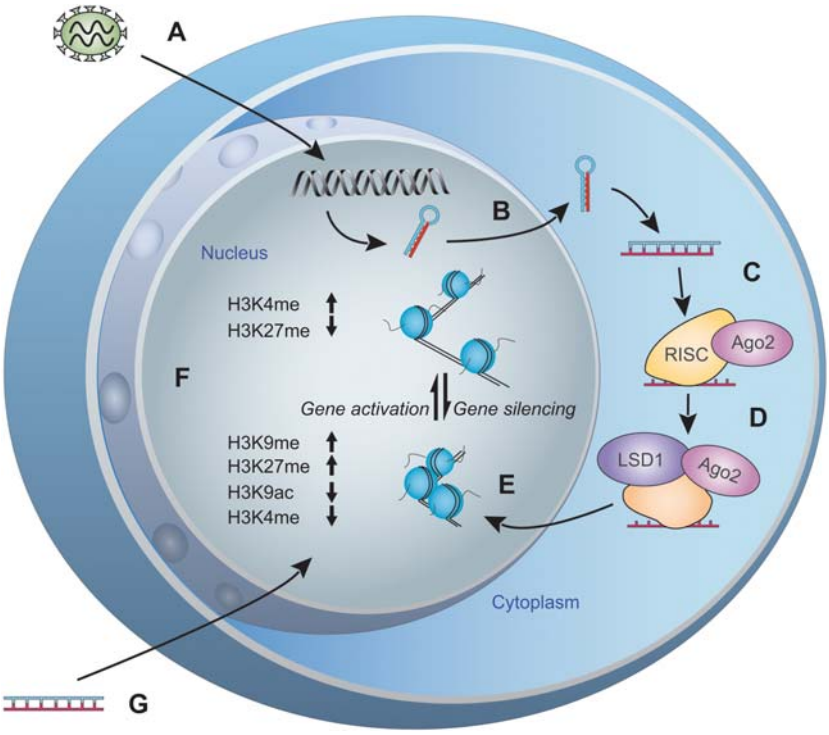

Figure 2 A model representing possible mechanisms of small RNA-mediated gene regulation.
(A) Lentiviral vectors encoding the small hairpin RNAs (shRNAs) integrate into the target cell genome. (B) Pri-shRNAs are expressed and transported to the cytoplasm. (C) Pri-shRNAs are processed into shRNA duplexes by Dicer and bound to RNA-induced silencing complex (RISC). (D) The complex goes through processing in which the histone modifying transcription factors are attached to it. (E) The complex is transported back into the nucleus where it recognizes its target sequence in the genome. (F) The complex modulates target gene activity by regulating transcription via histone acetylation and methylation. (G) The target gene activity can also be altered with other small RNAs [e.g., small interfering RNA (siRNA) transfection]. The model is based on our studies of VEGF-A regulation by shRNAs described in reference (18).

directly bound to the antisense transcripts recruiting also Ago and HP1 proteins in both transcriptional gene silencing and activation. Morris et al. suggested a more detailed model for this concept in their study of p21 (56). The p21 gene is bidirectionally transcribed and at the regular state both sense and antisense transcripts are expressed in comparable levels. In the case of gene activation, the small RNA designed for p21 bound to the antisense RNA transcript instead of direct promoter targeting, suppressing the antisense expression and thus releasing the p21 sense transcription and resulting in decreased level of H3K27me3 and gene activation. Conversely, when the level of sense transcript is decreased, the antisense RNA increases recruitment of Ago1 protein and the level of H3K27me3 resulting in p21 silencing.

In a recent study Place et al. modified double-stranded small RNA molecules that they had earlier showed to activate E-cadherin and p21 gene expression (17, 57). They were able to increase the activation by including a mismatch on the antisense strand of saRNA opposite to the most 5′ nucleotide. However, a similar mismatch on the sense strand inhibited the activation. Incorporation of 2′-O-methyl modification within the antisense strand did not interfere with the observed gene activation whereas the same modification within the sense strand inhibited the activation. Modification of the 2′-backbone could stabilize saRNAs. Off-target effects of E-cadherin saRNA were reduced by blocking the 5′-terminus of the sense strand with biotin and including the mismatched base in the antisense strand. However, in mutation studies by Li et al. for E-cadherin and p21 saRNAs, the modification of 5′-end of the antisense strand clearly inhibited the observed gene induction whereas the mutation of 3′-end hardly affected the results (17). They also observed that the length of 21 nucleotides is the most potent for saRNAs since both a shortened and extended versions of their saRNAs against E-cadherin promoter lost their ability for gene activation. However, it is clear that modification of saRNAs may improve their quality and usefulness for therapeutic use.

Expert opinion

Safety concerns have been one limiting factor in bringing gene therapy treatments to clinical use. These approaches typically consist of delivering potent transgenes, such as VEGF-A, in the form of cDNA that is driven by strong promoters. In the epigenetherapy method only small hairpin RNA is delivered to the target cells to alter the expression of an endogenous gene. This may offer several safety advantages. However, to obtain an adequate level of efficiency,

Table 1 Genes known to be regulated by small RNAs by an epigenetic mechanism and their possible therapeutic targets.

Gene	Therapeutic target	References
VEGF-A	Cancer, cardiovascular disease, age related macular degeneration, ischemia	(17, 18)
c-myc	Cancer	(51)
p21	Cancer	(17, 43, 56)
E-cadherin	Cancer	(17, 49, 50)
p53	Cancer, ischemia	(44)
PAR4	Thrombosis	(44)
WT1	Cancer	(44)
NKX3-1	Cancer	(44)
Cyclin B1	Cancer	(44)
CXCR4	Cancer	(44)
Survivin	Cancer	(47)
Ubiquitin c	Skeletal muscle atrophy	(52)
CDH1	Cancer	(53)
Progesterone receptor	Cancer	(55)

viral vectors have to be used for the delivery of RNA hairpins. Especially with integrating vectors, such as lentiviruses, the risk of insertional mutagenesis is still a concern. This has occurred in clinical trials for X-linked severe combined immunodeficiency (X-SCID) patients, in which hematopoietic stem cells were transduced using a retrovirus, and this led to the development of T cell leukemia in some patients (58). To overcome this problem, site-specific self-inactivating integrating lentiviral vectors need to be developed (59).

Another advantage of epigenetherapy approach is that when the activity of the endogenous promoter is altered, the expression of all different splice variants of the targeted gene is present. For example human VEGF-A gene has seven different splice variants: VEGF-121, VEGF-121b, VEGF-145, VEGF-165, VEGF-165b, VEGF-189, and VEGF-206. In traditional gene therapy approach only one isoform is usually delivered. Therefore, epigenetherapy may offer a more natural treatment approach, for example to induce angiogenesis in the ischemic heart.

Outlook

J.M. Keynes has a famous quote: 'Prediction is very difficult, especially about the future'. Foreseeing the progress of research in life sciences is indeed difficult because there are still unknown mechanisms and types of gene regulation that are yet to be identified. A perfect example is the discovery of small RNAs (siRNAs, miRNAs, etc.) just little more than a decade ago and the incredible impact it has had on the whole biotech field. The Nobel Prize awarded to Fire and Mello in 2006 for the discovery of siRNAs (1) was a very significant recognition of the importance of the small RNAs, but still the fundamental importance of RNA molecules for cells and tissues may not be fully appreciated.

After the discovery of the role of small RNAs in the regulation of gene expression at the chromatin level a new 'tool' for human gene therapy – epigenetherapy – has been introduced. However, to make this method of gene regulation as efficient and safe as possible, it will be necessary to mimic naturally occurring mechanisms. There is preliminary evidence that endogenous small RNAs (miRNAs) regulate chromatin by epigenetic mechanisms (60). However, this field of research is still in its infancy. New powerful sequencing methods, such as Helicos, Solexa and SOLiD may provide a novel way to find new RNA species involved in chromatin regulation and provide new targets for clinical use.

Before initiating clinical trials one has to perform rigorous animal experiments, preferably with several species. A specific problem in this is the fact that mammalian promoters are not identical and it might be hard to draw conclusions between different species. Through carefully planned animal experiments those issues are likely to be solved. Hence, we expect that it is possible to bring this method of gene regulation into clinical testing within a few years.

Our initial discovery concerned regulating VEGF-A expression. There is already evidence (as discussed above) for similar phenomena in other therapeutically relevant genes and other promising targets will be evaluated in the future. Due to the nature of chromatin regulation and to the fact that one third of the gene promoters contain small RNAs close to the TSS (61), the number of potential genes for this application is very large. However, VEGF-A itself is a very good target for the treatment of various human diseases (see Figure 1 and Table 1).

Highlights

- Small RNAs regulate expression of genes by epigenetic mechanisms.
- They can either up- or downregulate expression of genes.
- Promoter areas and transcription start sites contain variable populations of small RNAs.
- Using gene transfer technology one can deliver siRNA or shRNA molecules targeted to the promoter areas and modulate target gene activity.
- The precise mechanism of action of these promoter targeted small RNAs is not yet fully understood.

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