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# EMERGING GENE EXPRESSION AND GENE EXPRESSION REGULATION TECHNOLOGIES IN MEDICAL BIOTECHNOLOGY

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This report on Emerging Gene expression regulation technologies in medical biotechnology contains the conclusions of a scientific literature evaluation carried out independently by Xendo commissioned by RIVM GMO office.

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# 1 Executive Summary

This report on Emerging Gene expression regulation technologies in medical (red) biotechnology contains the conclusions of a scientific literature evaluation carried out independently by Xendo commissioned by RIVM GMO office. The report focusses on the novel molecular genetic techniques with a medical application in order to ultimately affect disease related gene expression. The major genetic engineering technology areas that have been identified are: genome and epigenome editing, gene expression regulation and gene delivery. The technologies identified are ZNF (Engineered nuclease), TALENs (Engineered nuclease), CRISPR/Cas9 (Engineered nuclease system), siRNA and miRNA, and Antisense Oligonucleotides (ASOs). Moreover, advances in both viral and nonviral delivery systems are introduced as a general driver for the described genetic engineering technologies.

Genome modification using engineered nucleases (ZFN, TALENs and CRISPR/Cas9) is of great value in research of understanding function of individual genes and as medicine of genetic disease treatment. Currently the same genome modifying complexes are developed as therapeutic agents. A critical breakthrough for this application was the discovery that creating site-specific DNA double stranded breaks (DSB) at the targeted genomic locus enhances the efficiency of homologous recombination enormously.

Engineered nucleases generally are used to introduce deletions or insertions in the genome, but in addition the complexes can be re-designed for epigenome modification and gene transcription regulation. The engineered DNA binding domains of these complexes can be fused to other functional domains such as chromatin-modifying enzymes or transcription activators/repressors. These chimeric proteins are able to modify chromatin, or regulate gene expression at transcriptional level at specific genomic loci.

A little over two decades ago small interfering RNAs (siRNAs) and microRNAs (miRNAs) were discovered as noncoding RNAs (not encoding protein) with important roles in gene regulation and with this several new RNA mediated genome regulation mechanisms were revealed. They have recently been investigated as novel classes of therapeutic agents for the treatment of a wide range of disorders including cancers and infectious diseases that involve aberrant gene expression.

Therapeutic oligonucleotides (including noncoding RNAs) that are intended to have an effect on gene expression in general need to be able to enter the targeted cells and stay biologically active to be able to reach their DNA or RNA target sequence. As nucleotides composing RNA and DNA are linked to each other by phosphodiester linkages that are easily cleaved by endo- and exonucleases, such molecules often are not suitable for the intended medical use. Many types of modifications have been described, and besides backbone modification, sugar modification (Locked Nucleic Acids, Bridged Nucleic Acids), nucleobase modification (Base Analogues), and terminal modification (coupled sugar, lipid, and peptide) have been applied to improve properties of natural oligonucleotides and make them suitable for medical purposes.

Many of the described technologies and their future development depend on efficient delivery systems. Around 70% of gene therapy clinical trials carried out so far have used modified viruses to deliver genes. Although they have substantially advanced the field of gene therapy, several limitations are associated with viral vectors, including patient safety issues and difficulty of virus production. The development of nonviral vectors is attractive because of advantages such as less safety issues and fairly simple manufacturing processes.

The most attractive aspect of the novel therapeutics based on the technologies described is their ability to target virtually any gene(s), which may not be possible with current therapeutics. While the

efficacy of these novel therapeutics has been successfully demonstrated, several technical barriers still need to be overcome for many clinical applications. The novel therapeutics allow for direct and sustained interference with disease related gene expression in most cases without the necessity to change the endogenous sequences of the genome itself. Some ethical and safety concerns of changing genome sequences are herewith circumvented and a clear paradigm shift from gene repair and replacement to gene regulation in can be observed medical biotechnology. Nevertheless some concern remains related to the transgenerational effects of medical treatments in general and specifically for treatments that strongly affect gene expression. New insights in epigenetic mechanisms reveal a new high speed evolution system independent of random DNA changes: epigenetic evolution by chromatin modifications, such as acetylation and methylation of DNA or DNA packing histone proteins, in response to environmental changes including medical treatments and even psychological experiences, which are transmitted between generations.

With the recent surge in intensive research concerning the new therapeutic mechanisms and combinations of the new tools, it can be expected that significant advances will be made for their future role in therapeutics.

# 2 Introduction

In the Netherlands the Ministry of Infrastructure and the Environment (IenM) is responsible for the regulations which aim to protect people and the environment during activities involving genetically modified organisms (GMOs). The Ministry of IenM has the task of developing policy and regulations. The Netherlands Institute for Public Health and the Environment (RIVM) and specifically the GMO Office is responsible for the processing of license applications on behalf of the Ministry. In order to prepare for future genetic engineering technologies and the required risk assessment methodology that is needed to ensure protection of people and the environment this report is presented. The report provides an overview on trending biotechnology applications that are anticipated to impact on further development within the field of red biotechnology. Parallel separate reports have been prepared to address new trends within white and green biotechnology applications.

Biotechnology is a container term for a large number of processes, products and methodologies. The Organization for Economic Cooperation and Development (OECD), defines biotechnology as “the application of science and technology to living organisms, as well as parts, products and models thereof, to alter living or non-living materials for the production of knowledge, goods and services”. Within the field of biotechnology several subgroups have been defined based on a color system (Grey, Blue, White, Green and Red). Grey biotechnology includes all those applications of biotechnology directly related to the environment. Blue biotechnology is based on the exploitation of sea resources to create products and applications of industrial interest. White biotechnology or industrial Biotechnology comprises all the biotechnology applications related to industrial processes. Green biotechnology is oriented at agricultural applications. Red biotechnology brings together biotechnology applications connected to medicine. It includes producing vaccines and antibiotics, developing new drugs, molecular diagnostics techniques, regenerative therapies and the development of genetic engineering to cure diseases. Examples of red biotechnology are cell therapy and regenerative medicine, gene therapy and medicines based on biological molecules such as therapeutic antibodies and recombinant proteins.

Discoveries of the last two decades involving genetic analysis, genome editing and gene regulation have recently resulted in novel classes of therapeutic agents for the treatment of a wide range of disorders including cancers and infections and are important drivers of the observed trend towards personalized medicine, designed to provide tailor made treatment options to individual patients based on patient specific characteristics. It was only since 1953 that the DNA helix was uncovered. In 1968 Rogers and Pfuderer demonstrated a proof-of-concept for virus mediated gene transfer. Over two decades ago the first gene therapy trials were performed and currently more than 2000 clinical trials have been approved worldwide. In 2003 the sequencing of the human genome was completed which provides new opportunities for further development of molecular medicine. Gendicine is the first gene therapy product approved for clinical use in humans. Gendicine was approved in 2003 by the Chinese State Food and Drug Administration to treat head and neck squamous cell carcinoma. In July 2012, the European Medicines Agency recommended Glybera for approval, the first recommendation for a gene therapy treatment in either Europe or the United States. With the increased understanding of molecular medicine, the field is now developing even more specific and efficient therapeutics that repair gene function, which are now producing clinical results. A paradigm shift in the conceptual strategy of genetic modification applied in the field of red biotechnology can be observed. Most applications seen so far are focusing on the introduction of new or corrected protein expression by introduction of protein encoding DNA sequences into the genome through a delivery system mostly based on a viral vector. Current pre-clinical studies indicate the future genetic therapeutics will also target gene expression regulation at the messenger-RNA level as well as at the genome transcription level or the epigenome level, by applying tools which are introduced in this

report. In addition scientists are investigating better and alternative delivery systems in order to facilitate and develop targeted and specific administration approaches of molecular medicine.

The FDA indicated personalized medicine should provide “the right patient with the right drug at the right dose at the right time”. Since 2002, FDA has been spending great efforts on building infrastructure, organizational modification, defining and clarifying regulatory pathways and policies to support the development of this field (FDA report on Personalized Medicine, 2013). Clinicians have long observed that drug responses may be determined by genetic influences as well as environmental factors. Genetic polymorphisms can account for 20-95% of variability in drug disposition and effects (Zhang and Yao, 2014). From FDA’s perspective, personalized medicine promises to enhance medical product development by improving the probability of success, and increase benefits and reduce risks for patients by improving both the safety and efficacy of medical products. The recent development in genomics largely benefits the field of designing tailored medicinal products which allow patients to be treated and monitored more precisely and effectively and in ways that better meet their individual needs (FDA report on Personalized Medicine, 2013) (Wilson and Nicholls, 2015). In the 2016 US president Obama announced a precision medicine initiative to accelerate biomedical research and deliver new treatment options to patients. Although personalized medicine may be more expensive it is believed that the healthcare system will be cheaper over all as less treatments and drugs will be prescribed that will not be efficacious. Over the past years it has been observed that pharma and biotech industries move away from the development of blockbuster drugs. As such the development seen within pharma and biotech companies seems to illustrate the trend into personalized and precision drugs development. In parallel with the development into personalized medicine researchers are working to expand their toolbox required to facilitate the development of these personalized medicine. Genetic engineering tools are important technological prerequisites that are continuously developed and contribute to the development of personalized medicine, and vice versa the need for personalized medicine is stimulating the further development of genetic engineering tools.

This report presents an inventory on new developments with respect to new molecular genetic techniques applied in red biotechnology. This report does not primarily focus on gene therapies but on the molecular genetic techniques that can be applied in red biotechnology to either affect gene expression or gene expression regulation. Nevertheless it is may be obvious that these molecular genetic techniques are fundamentals of gene therapies. Trending themes within molecular medicine can be captured by genomics based medicine, epigenetics, nanomedicine, personalized medicine and synthetic biology. All of these are impacted by the development of techniques that facilitate and improve genetic engineering. It will be genetic engineering techniques that facilitate and enable the development of these themes. We have identified four technology areas: genome and epigenome editing, gene expression regulation and gene delivery. The scope of the report is primarily on the technical developments and less on the development of the themes as a whole. Although the applications of the identified techniques are largely depending on the possible applications, these applications are not the primary focus of the report. However, some applications are mentioned as examples in order to add some perspective in relation to the techniques.

Based on literature searches using terms including “advanced genetic engineering techniques” and “trending genetic engineering techniques”, we have identified trending technologies related to these technology areas which are listed in the table below. In addition examples are provided for possible applications. The applications give some perspective to the possible application of techniques and the driving forces for their development.

| Technology area | Technology | Application |
|---|---|---|
| **Genome/epigenome editing** | CRISPR/Cas9 (Engineered nuclease) | Targeted gene mutation; Creating chromosome rearrangements; induced pluripotent stem cell disease models; Disease animal /viral disease models; Endogenous gene labeling; Targeted transgene addition; Gene therapy (modified T cell/stem cell) ; Transcription activation/inactivation; Visualization of the locus; Functional screening; saturation mutagenesis ; Genetically modified organisms; Mammalian-cell-based drug discovery; Sythetic virus/vaccine, chromatin modification |
| | TALENs (Engineered nuclease) | |
| | ZNF(Engineered nuclease) | |
| **Gene expression regulation** | siRNA and miRNA | Cell reprogramming, chromatin modification, DNA recognition, gene expression regulation |
| | Antisense Oligonucleotides (ASOs) | |
| **Gene delivery** | Viral vectors | Gene Therapy, Cell Therapies, Delivery of synthetic DNAs and microRNAs |
| | Nonviral vectors (lipids/liposomes; Polymers/polymersomes; Nanoparticles) plasmid DNA | |

## 2.1 Overview of chapters

**Chapter 3** of the current report presents an overview of genome and epigenome editing techniques. Within this theme the focus is on engineered nucleases. These engineered nucleases allow scientists to perform surgery on the level of genes, precisely changing DNA sequences at exact locations within the genome. The endonucleases discussed in this report are Zinc Finger Nucleases (ZFNs), TALENs and CRISPR/Cas9. These nucleases could make gene therapies more broadly applicable providing remedies for simple genetic disorders. Conventional gene therapies introduce new genetic material at “random” locations in the cell. The nucleases discussed in chapter 3 provide new tools for precise deletions and editing specific bits of DNA in some cases even by replacing a single base pair. This technology platform in principle would facilitate to rewrite the human genome.

**Chapter 4** presents an overview on small noncoding RNAs (ncRNAs); Micro RNAs (miRNA) and Small Interfering RNAs (siRNA). These RNAs have been discovered two decades ago and added a new dimension to our understanding of complex RNA mediated gene regulatory networks. NcRNAs are only recently investigated as novel classes of therapeutic agents. In contrast to the engineered nucleases that change the genetic code at the genome level, these RNA molecules can exert regulation of gene expression. As such molecular medicine can be applied at an additional level. These RNAs might regulate various developmental and physiological processes. It is anticipated that the use of these RNA molecules will open new opportunities when used in molecular medicine, especially for many multifactorial common diseases.

**Chapter 5** discusses modified (antisense) oligonucleotides that intend to have an effect on gene expression and therefore have to be able to enter the targeted cells and stay biologically active in order to reach their DNA or RNA target sequence. The basic concept underlying antisense technology is relatively straightforward: the use of a sequence complementary to a specific RNA or DNA sequence to influence its expression, by virtue of Watson-Crick base pair hybridization, by inducing a blockade in, or by promoting, the transfer of genetic information from DNA to protein. In Chapter 5 also variations to this basic theme will be presented.

**Chapters 3 to 5** all follow the same structure. A technical description, the conceptual mechanism of action and the introduced genetic modifications are discussed. In order to anticipate whether certain techniques can be expected on the short or long term a section on barriers and drivers is included. In the “at the horizon” section we discuss also the anticipated timescale for future development and applications within red biotechnology related to the discussed technology platforms.

For translation of red biotechnology developments into medicinal products either for human or veterinary use the importance of the delivery system of these gene modifying tools into cells is evident. Therefore a section on developments in gene delivery systems is included in **Chapter 6** as these may become an important factor to successful implementation of the genetic modification techniques. It may be evident that the delivery systems also contribute to barriers and drivers and should be translated into a horizon scan.

## 2.2 Acknowledgements

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*About HollandBIO:*
HollandBIO is the Dutch Biotech Industry Organization. HollandBio is representing Dutch companies and organizations active in medical, agro-food and industrial Biotechnology.
(www.hollandbio.nl)

*About Nefarma:*
Nefarma: the Association for innovative medicines in The Netherlands is representing Dutch branches of innovative pharmaceutical companies. The association is strongly involved with companies focused on biotechnological medicines.
(www.nefarma.nl )

## 2.3 Disclaimer & Copyright

# 3 Engineered nucleases -- genome and epigenome editing tools

## 3.1 Introduction

Genome editing is of great value in research of understanding function of individual genes and medicine of genetic disease treatment. A critical breakthrough for gene targeting approaches was the discovery that by creating a site-specific DNA double stranded break (DSB) at the targeted locus it is possible to stimulate genome editing by homologous recombination by 2-5 orders of magnitude, providing overall frequencies of 5 % or more. The basic process of genome engineering is to create DSBs at site-specific loci by nucleases and then allow the endogenous repair machinery to repair the break (Porteus, 2015). Engineered nucleases are chimeric proteins composed of DNA recognition domains and endonuclease catalytic domains. The DNA recognition domains determine the site-specificity of different engineered nucleases, while their genome editing function relies on creating DNA double stand breaks (DSBs) at targeted genomic loci. Induced DSBs stimulate endogenous cellular DNA repair processes, in which site mutations or exogenous genes can be introduced to the genome. There are 3 major types of artificial nuclease systems which are currently studied and applied in therapeutic design, namely Zinc-Finger Nuclease (ZFN), Transcription Activator-Like Effector Nuclease (TALENS), and Clustered Regulatory Interspaced Short Palindromic Repeat /associated 9 (CRISPR/Cas). These tools not only provide the opportunity of customized genome engineering, but also allow epigenome modification at specific sites or at the whole genome level. A description of the technology concept, the mechanisms of targeting and cleaving specific genomic loci by these three classes of engineered nuclease is provided. The mechanisms of endogenous DNA repair machineries-mediated genomic and epigenetic modifications will be introduced in the section on Host Effects.

### 3.1.1 Zinc-Finger Nuclease (ZFN)

Zinc-finger proteins (ZFPs) are the most abundant class of transcription factors in the human genome and the basis of designed ZFNs (Maeder and Gersbach, 2016). The modular structure of zinc finger (ZF) motifs and recognition by ZFP domains make them suitable for designing artificial DNA-binding proteins. Each ZF motif consists of two Cysteines and two Histidines which recruit zinc ions to maintain the tertiary structure, and a short 30 amino acids stretch of finger units. Each unit recognizes 3 to 4 base pairs of DNA and can be designed according to the target DNA sequences. Successful design and application of ZFNs rely on the ability to engineer ZF motifs that specifically bind defined stretches of DNA (typically 9–18 base pairs). Binding to longer DNA sequences is achieved by linking several ZF motifs in tandem to form ZFP domains (Jabalameli et al., 2015).

The DNA cleavage function of ZFNs is mostly mediated by a FokI restriction endonuclease domain which is activated by dimerization of ZFNs. Depending on the specificity of the ZFP domain, ZFNs can site specifically deliver a double stranded break (DSB) to the genome. Two ZFNs are typically designed to recognize the target sequence in a tail-to-tail configuration with each monomer binding to “half sites” (Figure 1) (Jabalameli et al., 2015).

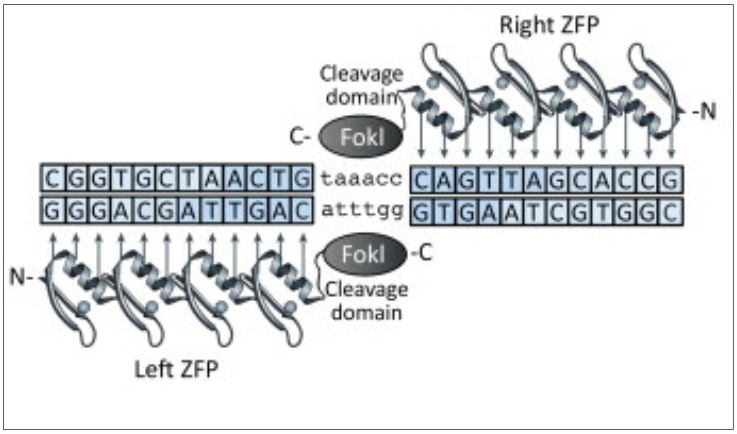



**Figure 1.** Structure of genome DNA and ZFN (Reprinted from Trends Biotechnol., 31(7), Gaj et al., ZFN, TALEN and CRISPR/Cas-baes methods for genome engineering, Page 397-405, Copyright (2013), with permission from Elsevier).

### 3.1.2 Transcription Activator-Like Effector (TALE) Nuclease (TALEN)

Following the introduction of ZFN, an alternative approach for introducing genome DNA breaks at selected sites was developed: TALEN. TALEN technology provides artificial restriction enzymes generated by fusing a TAL effector DNA binding domain to a DNA cleavage domain (mostly FokI restriction endonuclease domain). As such one can engineer restriction enzymes that cut any desired DNA sequence. The DNA-binding motifs of TAL effectors consist of a tandem repeat of typically 34 amino acids. Residues 12 and 13 of the 34-amino acid repeats, referred to as repeat variable di-residues (RVDs), define binding to a specific base. Four canonical RVDs are able to recognize and bind guanine, adenine, cytosine, and thymine, respectively. These RVDs are used to design customized TALENs which target specific DNA sequences. (Figure 2) (Hendriks et al., 2016). Similar to ZFN, dimerization of the catalytic domain is mandatory for its activity. Therefore, a pair of TALENs must be designed based on the sequences at both sites for the intended cut site (Pu et al., 2015). As ZFNs, TALENs can also be used to edit genomes by inducing DSB. Therefore, TALEN technology can be applied in host genome modification, such as creating knock-out or knock-in mutants but it is also being studied in gene correction.

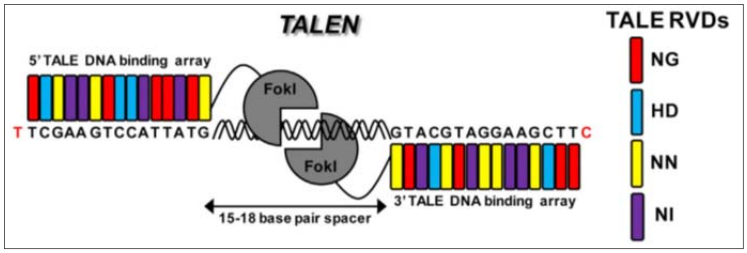



**Figure 2.** Structure of TALEN and genome DNA complex (Reprinted from Cell Stem Cell, 18, Hendriks et al., Genome Editing in Human Pluripotent Stem Cells: Approaches, Pitfalls, and Solutions, Page 53-65, Copyright (2016), with permission from Elsevier).

### 3.1.3 Clustered Regulatory Interspaced Short Palindromic Repeat (CRISPR)/associated 9 (Cas9)

The CRISPR/Cas systems are found in bacteria and archaea as the RNA-based adaptive immune system by using CRISPR RNAs (crRNAs) to guide the silencing of invading nucleic acids. In these systems, the mature crRNA that is base-paired to trans-activating crRNA (tracrRNA) forms a two-RNA

structure that directs the CRISPR-associated protein Cas9 to target DNA. Upon DNA binding, the Cas9 nuclease domains introduce DSBs by cleaving both the complementary and the non-complementary strand of target sequences (Jinek et al., 2012).

Shortly after the discovery of this mechanism, the system has been exploited as a RNA-programmable genome editing tool. Its great potential of targeting and modifying specific genome loci without complicated protein engineering makes it the most popular novel genome editing technology in recent years. Nowadays, the type II CRISPR system, which involves CRISPR-associated nuclease 9 (Cas9) derived from Streptococcus pyogenes, is widely used in genome editing after its first successful application in mammalian cells (Cong et al., 2013) (Zhang et al., 2015). Instead of using crRNA and tracrRNA, the engineered CRISPR/Cas9 system applies a chimeric single guidance RNA (sgRNA) to guide Cas9 to its target sequences (Figure 3).

Different from ZFN and TALEN, CRISPR/Cas9 is a RNA-based targeting system. This feature gives CRISPR/Cas9 system the potential advantage to introduce multiple DSBs in the same cell via expressing distinct sgRNAs (Cox et al., 2015). Another feature of this system is that the cleavage site of double strand DNA is dependent on a short sequence which is adjacent to the target DNA sequence called the protospacer adjacent motif (PAM), which is known to play an important role in specificity of CRISPR/Cas9 system (Corrigan-Curay et al., 2015).

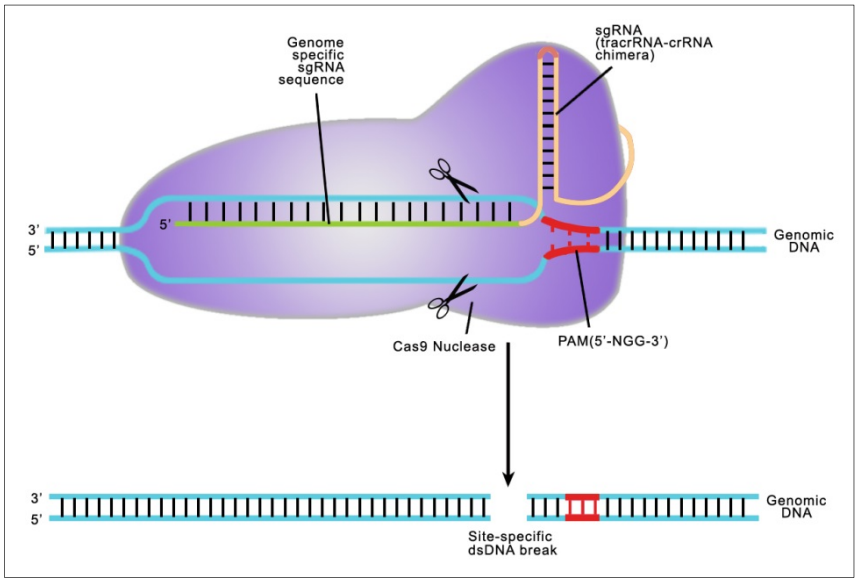



**Figure 3.** Mechanism of DNA double stranded breaks generated by CRISPR/Cas9 system (Reprinted by permission from Genecopoeia Inc., Copyright (2016): http://www.genecopoeia.com/product/crispr-cas9/).

## 3.2 Host effects

The direct modification on host genome mediated by engineered nucleases is the formation of DSBs. Genome editing including gene disruption, deletion and addition is realized by the endogenous cellular DNA repair machineries stimulated by targeted DSBs. Breaks in the DNA are typically repaired through one of two major pathways – homology-directed repair (HDR) or non-homologous end-joining (NHEJ) (Maeder and Gersbach, 2016) . These machineries are exploited to introduce specific genome modifications at the target locus.

Engineered nucleases are not only used to introduce permanent deletions or insertions in the host genome, but can be re-designed to control epigenome modification and gene transcription. The engineered DNA binding domains of these artificial endonucleases can be fused to other functional domains from chromatin-modifying enzymes or transcription activators/repressors. This type chimeric protein is able to control chromatin modification status, or regulate gene expression from the transcriptional level.

### 3.2.1 Genome modification

Once DSBs are introduced at a specific genome locus by engineered nuclease systems, one of the two endogenous DNA damage repair machineries will be applied depending on the cell state and the presence of a repair template. Unique mechanisms of these two machineries lead to different types of genome modification, respectively.

### 3.2.2 Gene disruption and deletion

In the non-homologous end-joining (NHEJ) pathway, the two ends of one DSB are directly re-ligated. Repeated DSB repair at the same loci introduces errors such as small insertions or deletions which eventually lead to the frameshift mutations. The mRNA transcripts from the mutated gene will be degraded by nonsense-mediated decay during translation, or will be translated into non-functional truncated proteins. Therefore, similar to RNAi technology, the NHEJ pathway is used in silencing or supressing target pathogenic genes (Figure 4) (Cox et al., 2015).

A combination of two DSBs could be used to delete a part of specific gene sequence between the two cleavage loci. After introducing two DSBs, the NHEJ machinery re-ligates one end of each DSB from different directions, and leads to the deletion of the sequence in between. This mechanism may achieve therapeutic effects by removing pathogenic expansions or insertions and restoring protein functions (Cox et al., 2015). Moreover, it potentially allows chromosomal deletions to be simulated in model organisms (Jabalameli et al., 2015).

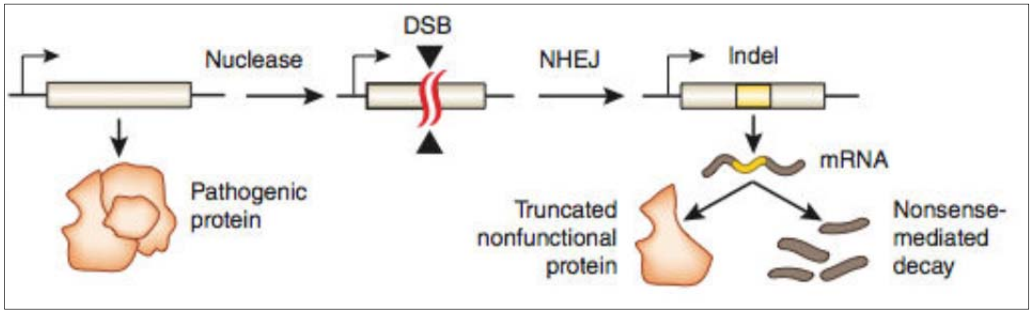


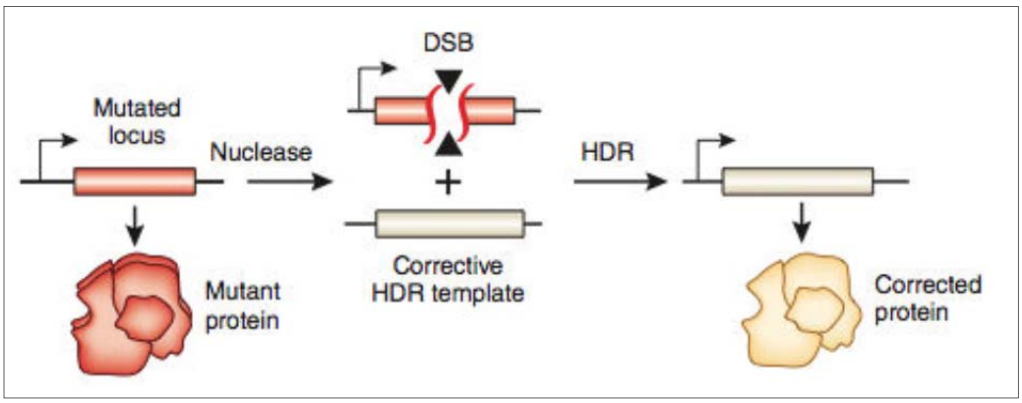

**Figure 4.** NHEJ mechanism in gene modification upon DSB introduced by engineered nucleases (Reprinted by permission from Macmillan Publishers Ltd: Nature Medicine (Cox et al., 2015), Copyright (2015)).

### 3.2.3 Gene correction and addition

In comparison to the NHEJ pathway, the homology directed repair (HDR) pathway requires a repair template and therefore provides an opportunity to introduce exogenous genes to DSB sites. As shown in Figure 5. A, upon introduction of a targeted DSB, HDR machinery may use exogenously provided single- or double-stranded DNA templates with sequence similarity to the break site to synthesize new DNA to repair the lesion. This provides the chance to incorporate desired changes in the template DNA, thereby restoring the function of a mutated gene (Cox et al., 2015).

An alternative way of applying the HDR machinery is to insert a full-length gene in replacement of the original mutated one at the native locus or a "safe harbour" locus (Figure 5 .B). Safe harbour loci could be regions of the genome whose disruption does not lead to discernible phenotypic effects and therefore provides the flexibility of choosing the target loci. Successful examples applying this approach have been seen in both mice and human cell lines. However, when a therapeutic transgene is introduced in a safe harbour locus, its expression is not under control of the natural physiological mechanism since upstream regulatory elements are missing (Cox et al., 2015).

(A)




(B)

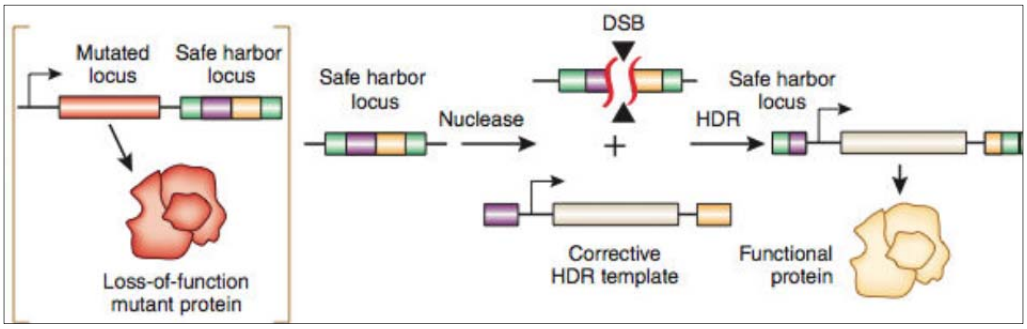



**Figure 5.** HDR mechanism in gene modification upon DSB introduced by engineered nucleases (Reprinted by permission from Macmillan Publishers Ltd: Nature Medicine (Cox et al., 2015), Copyright (2015)).

### 3.2.4 Epigenome modification (modulation of epigenetic marks)

Since chromatin epigenome modifications have direct impact on gene expression and are involved in a wide range of disease mechanisms, nowadays the epigenome research raises a growing attention in the field of disease mechanism study and therapeutics development. Precise knock-out of DNA methyltransferase by ZFN, TALEN, or CRISPR/Cas9 has been developed as the approach to study the change of genome methylation in both *in vitro* and *in vivo* models. Moreover, studies done in mouse models and human cell lines have proven that engineered nucleases can be used to introduce epigenome modifications by targeting and deleting nucleotide positions which are DNA methylation sites or histone binding sites, or which are crucial for maintaining chromatin structure (Laufer and Singh, 2015) (White and Khalili, 2016).

The other major contribution of engineered nucleases to epigenome modification is to facilitate designing of fusion proteins (de Groote et al., 2012). In order to precisely and temporarily modulate the epigenome, their DNA recognition domains (RNA binding domain in case of CRISPR/Cas9 system) are fused to chromatin-modifying enzyme domains (from DNA methyltransferases and demethylases, histone acetyltransferases and deacetylases, and histone lysine methyltransferases or

demethylases) to create synthetic proteins called EpiEffectors. Depending on different chromatin-modifying domains, EpiEffectors can successfully introduce deposition or removal of different chromatin modifications including DNA methylation (Figure 6 .A), histone modification (Figure 6.B), acetylation, or ubiquitination. Successful application of targeted EpiEffectors in animal models has been already documented (Kungulovski and Jeltsch, 2016). (See Chapter 4.4.2.1(Please see for more information on epigenetics.) 4.4.2.1)

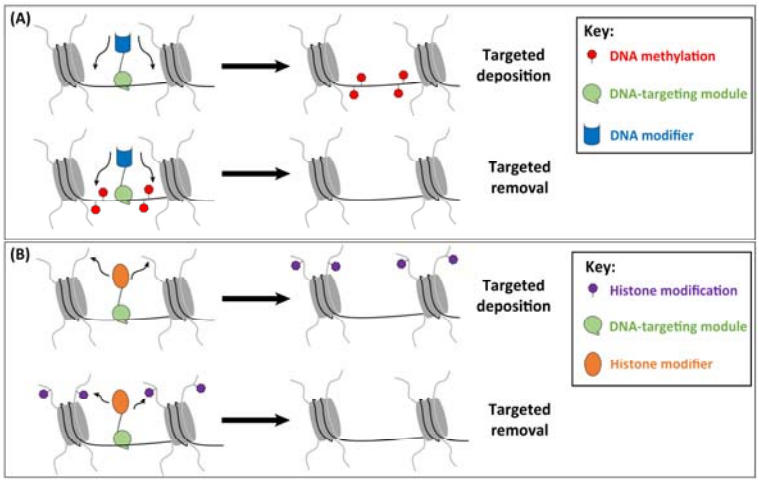



**Figure 6.** The mechanism of targeted epigenome editing. (A) DNA methylation modification. (B) Histone modification (Reprinted from Trends in Genetics, Vol.32, No.2, Kungulovski and Jeltsch, Epigenome Editing: State of the Art, Concepts, and Perspectives, Page 101-113, Copyright (2016), with permission from Elsevier).

### 3.2.5 Transcription regulation

Another category of epigenetic tools are fusion proteins that consist of DNA binding domains (RNA-protein complex in the case of CRISPR/Cas9) of nucleases and varieties of transcriptional activators and repressors which regulate target gene expression. Similar to the mechanism of EpiEffectors, in these fusion proteins the DNA binding domains (or RNA-protein complex) of nucleases interacts with target sequences and serve as genomic anchors, thereby provide localization of protein modulators to specific gene locations (Figure 7. A, B). This technology can be combined with optogenetics to enable temporally specific modulation of epigenetic states on a designed time scale, enable cell- or even projection-specific epigenetic modulation in different subtypes of cells. In more detail (Figure 7. C), the DNA binding domain of TALE is fused with the light-sensitive protein Cryptochrome 2 (Cry2), while the effector protein is fused with Calmyrin (CIB1). Upon photostimulation with a blue light source, Cry2 undergoes conformational changes and recruits its binding partner CIB1. Consequently, the transcriptional regulation of the target gene is induced (Day, 2014).

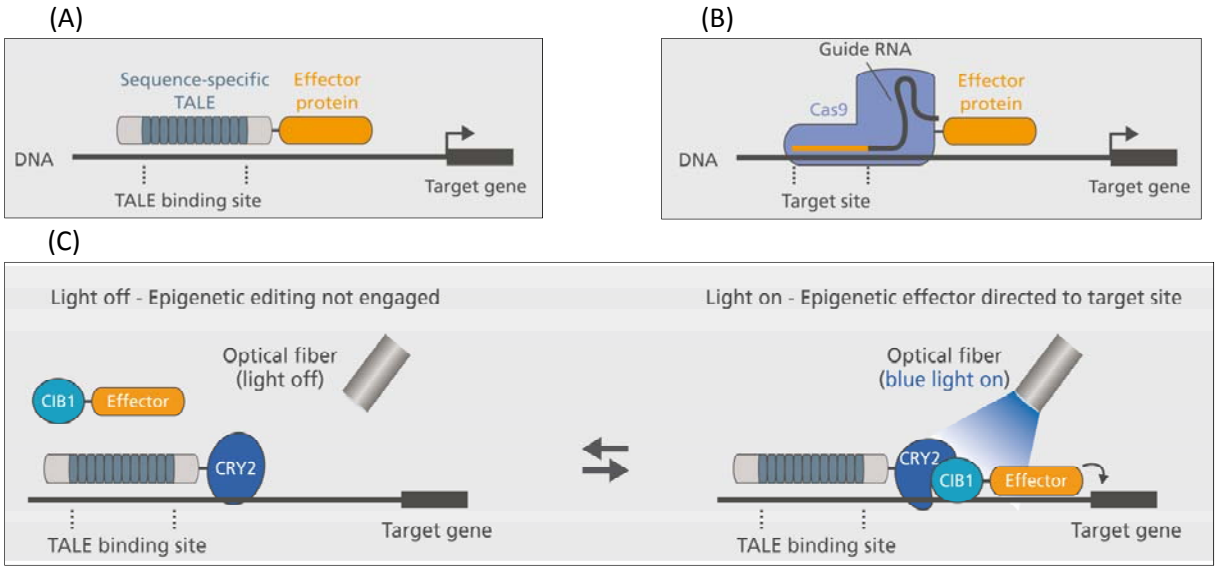



**Figure 7.** The mechanism of sequence-specific gene expression modulation with designer DNA targeting tools. (A) Fusion protein consists of the DNA binding domain of TALE and an effector protein. (B) Fusion protein consists of the Cas9-sgRNA complex and an effector protein. (C) Epigenetic regulation tool combined with optogenetic technology (Reprinted from Dialogues in Clinical Neuroscience with the permission of Institut La Conférence Hippocrate (Day, 2014), Copyright (2014)).

## 3.3 Application areas (medicinal applications)

The great potential of engineered nucleases in introducing genetic and epigenetic modifications to targeted genome loci has been widely exploited in medicinal studies and therapeutic development. In this report, the emerging trends of applying ZFN, TALEN and CRISPR/Cas9 systems in animal model creation, disease mechanism study, and treatment development are briefly introduced. Currently, besides the first clinical trials using ZFN engineered T-cells in HIV treatment (Tebas et al., 2014), most of these applications are still at the preclinical development stage. Nevertheless, the new medicinal solutions provided by these novel technologies will largely change the picture of disease mechanism study and treatment in future.

### 3.3.1 Genetically modified disease animal models

Engineered nuclease technologies are used in developing disease animal models by introducing germline modification or targeting somatic cells of adult animals. ZFN was successfully used to generate gene knock-out animals (Butler et al., 2015). In addition, it has been reported that TALEN and CRISPR/Cas9 are capable of introducing modifications to specific gene loci (from several hundred bases), and induce large genomic deletions or inversions (up to nearly 1 Mb) in animal models such as zebrafish, mouse and pig. As mentioned before, one of the significant advantages of CRISPR7/Cas9 over other engineered nuclease systems is its ability to modify multiple genes. This feature is valuable for generating animal model for multi-genic diseases (such as cancer), which is very challenging for traditional technologies. Researchers have demonstrated the success of generating mice carrying multiple genetic alterations by co-injection of Cas9 construct and sgRNAs into mice embryonic stem cells or fertilized egg. Delivery of constructs of engineered nucleases by viral vectors is also proven to be successful in generating cancer models in adult animals (Torres-Ruiz and Rodriguez-Perales, 2015) (Whitelaw et al., 2016).

### 3.3.2 Disease mechanism study

Engineered nucleases have been widely used as knockout and expression regulation tools in Loss of Function (LOF) studies. Importantly, CRISPR/Cas9 system has recently been developed into a tool for genome-scale LOF screens by several laboratories (Humphrey and Kasinski, 2015). Moreover, their potential in disease mechanism study has being exploited further. For example, in a recent study CRISPR/Cas9 system was introduced to the cell with a fusion green fluorescent protein (GFP), in order to unravel the mechanism of dynamic chromatin structure and genome organization during gene expression in living cells. This allows scientists to monitor the location of target loci in the genome (Falahi et al., 2015) (Fujita and Fujii, 2015).

### 3.3.3 Disease treatment

#### *3.3.3.1 Monogenic disorders:*

Engineered nucleases can be applied in the treatment of monogenetic disorders which are caused by single gene defects. Researchers have proven that somatic gene correction by delivering CRISPR/Cas9 agents and a homologous donor template successfully rescues the disease phenotype of tyrosinemia in mice. This suggests the potential application of this technology in human somatic cells, bypassing embryonic manipulations (Xiao-Jie et al., 2015).

Besides somatic gene correction, engineered nucleases can also be used in editing specific genes in induced pluripotent stem cells (iPSCs) derived from patients in *ex vivo* culture. Patient-derived iPSCs can be modified *in vitro*, then differentiated into desired cells for therapeutic autologous transplantation. For example, using engineered nuclease technology, modified iPSCs have been successfully generated from cells of monogenic disorder patients with loss-of functions mutations, or gene duplicates including cystic fibrosis, Duchenne muscular dystrophy, sickle cell anemia and β-thalassemia, primary immune deficiencies, and hemophilia (Xiao-Jie et al., 2015) (Prakash et al., 2016). Moreover, the success of applying CRISPR/Cas9 *in vivo* has been achieved in a mouse model of type I tryrosinemia (Savić and Schwank, 2016). In future, it may be expected that engineered nucleases will be widely applied in development of personalized therapeutics for inherited monogenic diseases.

#### *3.3.3.2 Cancers:*

It is well-established that many cancers are caused by acquisition of multiple mutations in the cellular genome. Therefore, engineered nucleases can be used in designing cancer treatments in different aspects. First of all, genome editing tools are able to precisely modify sequences in order to inactivate oncogenes (for example: ErbB, Ras, Raf, and Myc) and activate tumour suppressors (for example: pRb, p53, PTEN, BRCA1/2, and ATM). Mutations can also be introduced to genes that confer chemo-resistance (for example: MDR-1, MRP, and GST-p). Moreover, deletion of specific DNA methyltransferases allows us to silence the hyper-methylation of tumour suppressors on the epigenetic level (White and Khalili, 2016) (Vasileva et al., 2015). The recent development of epigenetic tools based on fusion proteins has demonstrated examples of precisely and temporarily modulating epigenetic marks and regulating gene expression in cancer cells (Falahi et al., 2015). Considering the fact that many viral infections are associated with carcinogenesis, inactivation or clearance of oncogenic virus such as hepatitis B/C virus, Epstein-Barr virus, human papillomavirus by using engineered nucleases provides a promising option for prevention and treatment of virus-associated cancers. Examples have already existed of CRISPR/Cas9 mediated antiviral and antiproliferation effects in virus-infected cancer cell lines (Xiao-Jie et al., 2015) (Wen et al., 2016).

Creation of genetically modified T cells is another major application of engineered nucleases in cancer therapy. To increase therapeutic responses, T cells are genetically engineered *ex vivo* with viral vectors to express various types of genes enhancing their immuno-activities towards cancer cells or facilitating their proliferation and survival. ZFN, TALENS and CRISPR/Cas9 can be applied to modify T cell receptors or knock-out genes to improves the efficacy and safety of adoptive immuno-therapy (June and Levine, 2015).

#### *3.3.3.3 Infectious disease:*

Nucleoside analogues and interferon are the only two currently available types of treatment for hepatitis B virus (HBV). However, none of them directly target the stable nuclear covalent closed circular DNA (cccDNA) and therefore only very a few treated patients achieve sustained viral response. Engineered nuclease technology provides a promising future therapy for HBV virus eradication (Lin et al., 2015). Moreover, *in vitro* studies have also shown the success of removal of the integrated proviral HIV DNA from host cells by mutating long terminal repeat (LTR) sequence of HIV-1, and a significant reduction of virus expression by using CRISPR/Cas9 (Xiao-Jie et al., 2015). CRISPR/Cas9 has been further successfully applied in *in vivo* treatment of HBV in a hydrodynamics-HBV persistence mouse model (Savić and Schwank, 2016).

## 3.4 Barriers and drivers

Major barriers and drivers of genome editing technology from both technical and ethical aspects are identified from web-scanning exercise and document analysis.

### 3.4.1 Barriers

Obviously engineered nucleases have the potential to be powerful tools for gene therapy because of their ability to inactivate genes, correct mutated sequences, insert intact genes, or regulate gene expression from the epigenetic level (Corrigan-Curay et al., 2015). However, there are barriers from both technological and regulatory aspects before their wide clinical application.

#### *3.4.1.1 Technological challenges*

The specificity of genome editing tools is one of the main safety concerns for clinical application (Cox et al., 2015). The problem of off-target cleavage activity at genomic regions has been addressed for all three kinds of engineered nucleases. For example, the targeting specificity of CRISPR/Cas9 is believed to be tightly controlled by the paring between a 20-nt sgRNA sequence and the genome target sequence adjacent to a PAM. However, varieties of factors could lead to the off-target binding and cleavage. Even 3-5 base pair mismatches in the PAM-distal part of the sgRNA-guiding sequence could lead to off-target cleavage. Different sgRNA structures can also affect its target-specific binding. Moreover, researchers have suggested that the off-target effect might depend on the double-stranded breaks repairing capacity and therefore is cell-type-specific (Zhang et al., 2015).

As the off-target effect is inevitable in all currently applied engineered endonuclease systems, toxicity (cytotoxicity and genotoxicity) is a very important concern in genome/epigenome editing. Considering increasing the concentration of a given nuclease is often related to an increased toxicity, a “Good” nuclease should have a high on-target activity and only a low off-target activity at a relatively high concentration. A green fluorescent protein+ (GFP+) cell assay (commonly used in measuring cytotoxicity) suggests the concentration of selected ZFNs and TALENs is inversely related to the cell viability (Corrigan-Curay et al., 2015). Therefore, concentration optimizing is crucial to safety control when engineered endonucleases are applied in the clinic.

Another common issue for engineered nucleases is that the natural conformation of chromatin in different types of cells raises the ambiguity of targeting. For example, in differentiated cells, only the part of actively expressing genome is amenable to cleavage. The incorporation of silencing histones and condensation of chromatin prevent the inactive part from being accessible to nucleases (Jabalameli et al., 2015) (Fujita and Fujii, 2015).

Genomic modification by engineered nucleases requires the activity of endogenous NHEJ and HDR pathways. Generally speaking, the NHEJ pathway is more active than the HDR pathway. On the contrary, increasing the efficiency of HDR is to date still the primary challenge for applying genomic editing tools in cell types other than dividing cells. Therefore, further studies which enable precise gene correction in postp-mitotic cells are crucial to developing therapeutics specially for untreatable neurological diseases (Cox et al., 2015).

The efficiency of delivery systems is another important concern which needs to be addressed when translating the engineered nuclease systems to clinical treatments. For example, in the HBV treatment design, it is essential to deliver the engineered nucleases to every infected cell in order to eradicate HBV (Lin et al., 2015). Therefore, even though the treatment with engineered nucleases achieved a high efficiency in *in vitro* cell culture, there is still a lot of further research needed before bringing this technology to clinic.

By comparing different features of the three technologies (Table 1), it is shown that every system has its unique advantages. Nevertheless, there are disadvantages for each nuclease. For example, ZFN and TALEN technologies are often limited by the complexity of protein design. The large size of TALEN and Cas9 proteins limits the choice of delivery system and is considered as one big challenge of TALEN and CRISPR/Cas9 systems. All these factors deserve attention in technology application and development.

| | ZFN | TALEN | CRISPR/Cas9 |
|---|---|---|---|
| **Advantage** | Low immunogenicity (Human protein origin)<br><br>Can recognize modified DNA bases<br><br>Small size | High specificity to targets<br><br>Can recognize modified DNA bases | Does not depend on protein engineering |
| **Disadvantage** | Depends on protein engineering<br><br>Off-target activity | Depends on protein engineering;<br><br>Immunogenicity (Bacterial protein origin)<br><br>Large size | Off-target activity<br><br>Immunogenicity (Bacterial protein origin)<br><br>Large size |

**Table 1.** Comparison of advantages and disadvantages of ZFN, TALEN and CRISPR/Cas9 (Falahi et al., 2015)(Kungulovski and Jeltsch, 2016).

#### *3.4.1.2 Regulatory environment and ethical concern*

It is encouraging that engineered nuclease systems have a promising future in treating various types of diseases. However, the ethical issue of where should be the boundary of applying this type of technology in germline genome editing (genomic modification oocytes, sperm, zygotes, and embryos) has been under debate for a long time.

Researchers have shown the success of TALEN and CRISPR/Cas9 technologies in mammalian (e.g. mouse, rat, porcine, monkey) including human zygote genome modification. Fifteen countries including Belgium, Canada, Denmark, Japan, and the UK permit research that creates human embryos with a purpose of improving or providing instruction in assisted reproductive technology (ART). The indicated purpose potentially implies that germline genome editing research may be permitted after prior consultation or permission from the authorities if the gene modification is associated with improving embryo viability, implantation, or the pregnancy rate. Notably, the UK explicitly sanctions genetically modifying human embryos under the Human Fertilisation and Embryology Act if a license is obtained from the Human Fertilisation and Embryology Authority (HFEA). It is also to be mentioned that, even if researchers do not have permission to create human embryos for research purposes, they can alternatively use existing embryos derived from surplus *in vitro* fertilization (IVF) embryos, or embryos screened out by preimplantation genetic diagnosis (PGD) because of a genetic defect in the course of ART (Ishii, 2015).

Although the worldwide regulatory landscape is permissive for human embryo research applying genome editing technologies, the relevant clinical applications with a purpose of reproductive use of edited embryos or gametes is prohibited in many countries. As shown in Figure 8, 29 of 39 countries (including the Netherlands) ban human germline gene modification for reproductive purposes, while the guidelines from China, India, Ireland and Japan are less strictly enforced and are subject to amendments. In the USA, the clinical application of genetically modified human embryos is reviewed by the FDA, and is restricted according to the National Institutes of Health (NIH) Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules (2013) (Ishii, 2015).

**Figure 8.** The international regulatory landscape of human germline gene modification. Pink: ban (legislation); Light pink: ban (guidelines); Grey: restrictive; Light Grey: ambiguous (Reprinted from Trends in Molecular Medicine, Vol.21, No.8, Ishii, Germline genome-editing research and its socioethical implications, Page 473-481, Copyright (2015), with permission from Elsevier).

Considering potential socio-ethical implications, the application of human germline genome editing is in favour if the purpose is preventing definitive inheritance of a serious genetic disease. However, due to the off-target effect of engineered nuclease systems, it is difficult to precisely predict and control the risk in modified embryos. Meanwhile, the worry of potential nonmedical abuse of these technologies remains. Therefore, the future technology development and updates of regulation in different countries should be paid attention to.

### 3.4.2 Drives

As mentioned before (3.4.4.1), off-target effect is the most considered issue when evaluating the safety and efficacy of genome editing tools. To improve the specificity of these systems, synthetic biology approaches are applied in modifying both DNA recognition domain and nuclease domain of engineered nucleases. Moreover, un-biased detection systems are desired for off-targets detection.

#### *3.4.2.1 Synthetic biology*

The progress in synthetic biology is a driver for improving specificity of engineered nuclease. For example, in ZFNs, the linkers between zinc finger units and the links between the Fok1 nuclease and zinc finger units can be altered to maximize engagement of the preferred sequence. In addition, the Fok1 domains can be engineered to require heterodimer binding. Moreover, a so-called Cas9 nickase (Cas9D10A) has been engineered from wild-type Cas9. Instead of a DSB, Cas9 nickase creates a DNA nick. In order to create a DSB, co-expression of two sgRNAs in each other's vicinity with Cas9 nickase is required. The dual nickase approach has been shown to increase specificity of gene editing (Hendriks et al., 2016). Furthermore, protein engineering helps to reduce the size of nucleases. For example, the size of Cas9 protein is an obstacle for delivery. Modified constructs expressing two domains of Cas9 respectively provide a solution for this issue (White and Khalili, 2016).

Many other approaches have also being developed to maximize the specificity of engineered nucleases. Recent publications suggest that chemical modifications of sgRNAs enhance the editing efficiency of CRISPR/Cas9 system in human primary T cells and CD34+ hematopoietic stem cells and progenitor cells (Hendel et al., 2015).

#### *3.4.2.2 Detection technologies*

Development of sequencing technologies provides various methods of identifying specificity of engineered nuclease systems when different criteria (time, cost, or sensitivity) are considered. In the following table (table 2), major technologies for detecting off-target events for engineered nuclease systems are introduced. For example, several studies performed ChIP-seq to determine CRISPR/Cas9 binding specificity on a genome-wide scale and the results validate the binding at the target sites. However, the signal from off-target sites varied between different groups. The performance of the latest generation of sequencing technology (such as: GUIDE-seq and HTGTS) are largely improved compared with ChIP-seq in terms of sensitivity (O'Geen et al., 2015). The fluorescence in situ hybridization (FISH)-based methods for off-target identification, which is fast but less precise, can be also used as an alternative (Zhang et al., 2015).

| Technologies | Advantage | Disadvantage |
|---|---|---|
| T7E1 assay | Simple | Poor sensitivity, not cost-effective |
| Deep sequencing | Precise | Biased, misses potential off-target sites elsewhere in the genome |
| In silico prediction | Predicts some off-target mutation sites | Fails to predict bona-fide off-target sites |
| ChIP-seq | Unbiased detection of Cas9 binding sites genome-wide | Most off-target DNA-binding sites recognized by dCas9 are not cleaved at all by Cas9 in cells |
| GUIDE-seq | Unbiased, sensitive (0.1%), qualitative translocations, identifies breakpoint hotspots | False negatives present, limited by chromatin accessibility. |
| HTGTS | Identifies translocations | False negatives present, limited by chromatin accessibility. |
| IDLV | Programmable, sensitive (1%) | Many bona-fide off-target sites cannot |

| | | |
|---|---|---|
| | | be captured |
| Digenome-seq | Sensitive (0.1% or lower), unbiased and cost-effective | Not widely used |
| FISH | Quick | Less precise |

**Table 2.** Technologies of off-target detection. T7E1, T7 Nuclease I; ChIP-seq, Chromatin Immunoprecipitation followed by high throughput sequencing ; GUIDE-seq, Genome-wide, Unbiased Identification of DSBs Enabled by Sequencing; HTGTS, High-throughput, Genome-wide, Translocation Sequencing; IDLV, Integrase-Defective Lentiviral Vectors; FISH, Fluorescence *in situ* Hybridization (Reprinted by permission from Macmillan Publishers Ltd: Nature Molecular Therapy (Zhang et al., 2015), Copyright (2015)).

## 3.5 At the horizon

ZFN is the most studied nuclease and has low immunogenicity because of its human origin. Until today it is the only engineered nuclease technology which has been used in clinical trials. In the first clinical trial, viral vector-delivered ZFNs were applied to generate modified T cell for HIV treatment (Tebas et al., 2014). Currently follow-up safety studies and clinical trials for the same disease indication (using different ZFN protein or cell model) are still ongoing (Table 3). Moreover, future clinical trials applying ZFN technologies for other disease indications are expected (Table 3). In 2015, a TALEN based system was used in a clinical setting for treatment of a one year old girl suffering from a very aggressive leukemia. This treatment was developed by a French biopharmaceutical company Cellectis and approved by the ethics committee specifically to try the TALENs treatment on this girl.

| ClinicalTrials.gov Identifier | Clinical trial phase | Status | Disease indication | Application | Delivery method |
|---|---|---|---|---|---|
| NCT00842634 | Phase 1 | Completed | HIV Infections | Genetically modified T-cells | Adenoviral vector |
| NCT01252641 | Phase 1/2 | Completed | HIV Infections | Genetically modified T-cells | (Not mentioned) |
| NCT01044654 | Phase 1 | Completed | HIV Infections | Genetically modified T-cells | (Not mentioned) |
| NCT02500849 | Phase 1 | Recruiting | HIV Infections | Genetically modified Hematopoietic Stem/Progenitor Cells | (Not mentioned) |
| NCT02388594 | Phase 1 | Recruiting | HIV Infections | Genetically modified T-cells | (Not mentioned) |
| NCT02225665 | Phase 1/2 | Active, not recruiting | HIV Infections | Genetically modified T-cells | (Not mentioned) |
| NCT02695160 | Phase 1 | Not yet recruiting | Severe Hemophilia B | Genetically modified hepatocytes | (Not mentioned) |
| NCT02702115 | Phase 1 | Not yet recruiting | Mucopolysaccharidosis I | Gene therapy: inserting the gene encoding leukocyte and plasma iduronidase (IDUA) | Recombinant Adeno-associated Virus (rAAV)2/6 |

**Table 3.** Overview of ongoing clinical trials applying ZNF technology (source: ClinicalTrials.gov).

With the development CRISPR/Cas9 nuclease, the barrier for performing genome and epigenome modification for investigators was decreased dramatically. Therefore, CRISPR/Cas9 is often considered as the most potential genome editing tool with the unique RNA-guided targeting feature. However, the CRISPR/Cas9 system is facing the issue of a higher off-target rate compared with TALEN in *in vivo* studies and has so far only been tested in animals and non-viable human embryos. Its first clinical trial in United States may be expected in 2017 for treating a rare eye disease led by an American gene therapy company Editas.

All three systems are actively applied in research aiming in therapeutic gene-editing approaches development for monogenic diseases (Table 4). Experimental models used in these studies include somatic and stem cells from patients and humanized mice. Viral vectors are still major delivery tools applied in these studies. For multi-genic disease treatment, CRISPR/Cas9 system is the most promising candidate, considering its unique feature of allowing multi-genetic modifications.

| Disease | Technology | Experimental model | Delivery method |
|---|---|---|---|
| **Cystic fibrosis** | ZFN | Patient epithelia cells, iPSCs | Plasmid transfection |
| | TALEN | iPSCs | Plasmid electroporation |
| | CRISPR/Cas9 | Stem cell organoids, iPSCs | Plasmid transfection/ electroporation |
| **Duchenne muscular dystrophy** | ZFN | Immortalised patient myoblasts | Plasmid electroporation |
| | TALEN | Patient fibroblasts or iPSCs | Plasmid electroporation |
| | CRISPR/Cas9 | Immortalised patient Myoblasts, zygote, patient fibroblasts or iPSCs | Plasmid electroporation, Cas9 mRNA injection |
| **Sickle cell anemia** | ZFN | Patient iPSCs, healthy donor and patient CD34+ cells, | Plasmid electroporation, ZNF mRNA electroporation, mRNA transfection |
| **&** | TALEN | K562 cell line, patient iPSCs, mobilized human (adult) CD34+ HSCs | Plasmid electroporation, mRNA transfection |
| **B-thalassemia** | CRISPR/Cas9 | Patient iPSCs, immortalized human CD34+ and CD34+ HSPCs | Plasmid electroporation, Lentiviral transduction |
| **Hemophilia** | ZFN | Humanized hemophilia A/B Neonatal from adult mice | AAV-8 ZFN transduction |
| | TALEN | Patient iPSCs | Plasmid electroporation |
| | CRISPR/Cas9 | Patient iPSCs | Cas9 protein and *in vitro* transcribed |

| Disease | Technology | Experimental model | Delivery method |
|---|---|---|---|
| | | | gRNA electroporation |
| **Primary immune deficiencies** | ZFN | K562, mouse embryonic stem cells and CD34+ cells, patient iPSCs, mouse primary fibroblast, iPSCs, healthy donor CD34+ cells | Integrase-defective lentiviral vectors ZFN transduction, ZFN mRNA transfection/electroporation, Plasmid transfection/ electroporation |
| | TALEN | Jurkat cells, patient iPSCs | Plasmid electroporation |

**Table 4.** Overview of engineered nuclease systems in therapeutic development of monogenic diseases. iPSCs: induced Pluripotent Stem Cells; HSCs: Hematopoietic Stem Cells (Reprinted by permission from Macmillan Publishers Ltd: Nature Molecular Therapy, (Prakash et al., 2016) Copyright (2016)).

Extraordinary progress in engineered nuclease technologies in the last few years has shown us the possibility of precisely modifying genome and epigenome. Nonetheless, important issues including developing a tailored regulatory framework (taking both the ethical and scientific issues into account) and improving safe and effective use of these tools should arise enough attention (Porteus, 2015).

# 4 Small Noncoding (nc)RNAs; MicroRNAs (mi)RNAs, Small Interfering (si)RNAs); Technical Description

## 4.1 Introduction

Discovered a little over two decades ago, small interfering RNAs (siRNAs) and microRNAs (miRNAs) are noncoding RNAs with important roles in gene regulation. They have recently been investigated as novel classes of therapeutic agents for the treatment of a wide range of disorders including cancers and infections. Both siRNAs and miRNAs are short duplex RNA molecules that can exert regulation of gene expression. The discrimination between siRNAs and miRNAs is based on the targeted sequence; siRNA has one target while miRNA has multiple targets. Endogenous siRNAs are found in plants while mammals appear to express only miRNAs and to have no endogenous siRNAs. Polymorphisms in miRNA sequences and perturbation of miRNA expression has been correlated with cancer (Seto, 2010) (Obsteter et al., 2015) and a variety of other common diseases such as diabetes (Regulus Therapeutics Inc.) and Alzheimer's (Fan et al., 2015). Some miRNAs show tissue-specific expression and miRNA expression profiling in a variety of normal tissues and their matched disease types have revealed that diseased cells can be classified by their miRNA signatures. The striking new observation is that manipulation of miRNA levels can control disease phenotypes. Hence, the race to bring the first si/miRNA therapeutic to the market has begun.

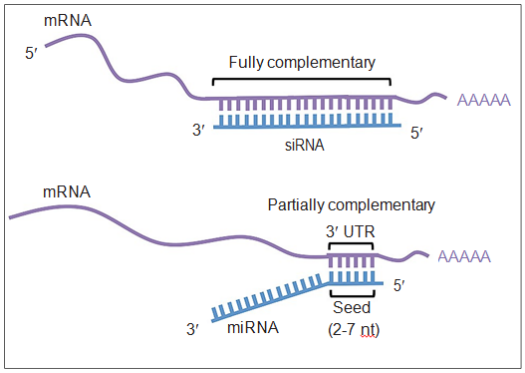



**Figure 9:** siRNAs have one target to which they are fully complementary, whereas miRNA's have multiple targets to which they are only partially complementary (Reprinted by permission from Macmillan Publishers Ltd: Mol. Ther. — Nucleic Acids (Lam et al., 2015), Copyright (2015)).

### 4.1.1 Endogenous RNA mediated regulation

Most miRNAs are transcribed by RNA polymerase II from individual miRNA genes, introns of protein-coding genes, or polycistronic clusters encoding several miRNAs in a single transcript. The relatively long precursors, primary miRNAs (pri-miRNAs), are processed by the Microprocessor Complex, containing the enzyme Drosha, into a 70-100 nucleotide hairpin precursor miRNA (pre-miRNA). After Dicer processing, the 18-24 nucleotide double-stranded molecule can be incorporated into the RNA-induced silencing complex RISC (including the Argonoute effector protein) where it is unwound into its mature, single-stranded form for binding to its target sequence(s). Argonaute-bound single stranded RNA may target mRNA to regulate translation in the cytoplasm (RNA interference; RNAi). Additionally there is an increasing awareness that already in the cell nucleus RNAi mechanisms may be involved in the regulation of splicing of primary transcripts into mRNA via recognition of splice sites and interaction of AGO1 with splicing factors. Besides the interactions with mRNA it is found

that RNAi control mechanisms also interact with nuclear non-coding RNAs such as promoter transcripts and enhancer transcripts (nascent transcripts) (Kalantari et al., 2016). These interactions can result both in up and down regulation of gene transcription (see figures 11-13 below).

### 4.1.2 Therapeutic approaches

Therapeutic approaches based on siRNA generally involve the introduction of a synthetic siRNA into the target cells to elicit RNA interference (RNAi), thereby inhibiting the expression of a specific messenger RNA (mRNA) to produce a gene silencing effect. By contrast, miRNA-based therapeutics comprise two approaches: miRNA inhibition and miRNA replacement. The former approach is Argonaute independent with synthetic single stranded RNAs acting as miRNA antagonists (also known as antagomirs or anti-miRs) to inhibit the action of the endogenous miRNAs, and is therefore classified as antisense therapy (see (Antisense) oligonucleotides based therapies). In the replacement approach, synthetic miRNAs (also known as miRNA mimics) are used to mimic the function of the endogenous miRNAs (Lam et al., 2015).

### 4.1.3 RNA delivery systems

Delivery of the therapeutic RNA molecules, for instance as short hairpin RNA (shRNA (Mockenhaupt et al., 2015)), into the right cells at the right dose is an important hurdle to take to enable effective and safe treatment. In addition to chemical modification of RNA to improve *in vivo* stability or to improve functionality, a major consideration is targeting of the molecule to the proper cells within a tissue or organ, as in general small RNAs are not efficiently taken up into target cells and are degraded in circulation and cleared via kidneys or liver. When systemic or local delivery is not feasible, targeted delivery may be obtained by conjugation of a targeting moiety to the RNA molecule, or encapsulation/binding by a nanoparticle that contains a targeting component. Viral vectors are becoming less attractive as there are serious safety concerns associated with the use of viral vectors, including high immunogenicity (especially in adenoviruses) and the risk of insertional mutagenesis (especially in lentiviruses). Additionally, low packaging capacity (especially in adeno-associated virus – AAV) and high production cost have also limited their clinical applications (Place et al., 2012) (Ozpolat et al., 2010) (Lam et al., 2015). The relatively limited packaging capacity of AAV of 4.7 kb can be a disadvantage when designing vectors for gene replacement but not for RNAi-based applications, which typically employ smaller-sized expression cassettes (Borel et al., 2014). (See table 5 and Figure 10 below)

| Delivery system | Disease | miRNA/siRNA | Route of administration |
|---|---|---|---|
| **Unmodified Pei (Synthetic polyethylenimine polymer)** | | | |
| | Asthma | siRNA targeting IL-13 | Intravenous |
| | Sepsis | siRNA targeting IL-6 and TNFα | Intravenous; intraperitoneal |
| | Colon cancer | miRNA-145; miRNA-33a | Intratumoral; intraperitoneal |
| **Modified Pei** | | | |
| SA-PEI-CNT | Melanoma | siRNA targeting Braf | Topical |
| PU-PEI | Lung cancer | miRNA-145 | Intratumoral |
| | Gliobastoma | miRNA-145 | Intratumoral |
| **Dendrimers (Poly-amidoamine, dendrimers with nucleic acids)** | | | |
| PAMAM | Ovarian cancer | siRNA targeting Akt | Intratumoral |
| | Drug-resistant prostate cancer | siRNA targeting Hsp27 | Intratumoral |
| PAMAM-folic acid | Glioma | miRNA-7 | Intratumoral; intravenous |
| | Ovarian cancer | siRNA targeting CD44 | Intraperitoneal |

| Delivery system | Disease | miRNA/siRNA | Route of administration |
|---|---|---|---|
| **Natural polymers** | | | |
| Glycol chitosan | Drug-resistant breast cancer | siRNA targeting P-glycoprotein | Intravenous |
| Hyaluronic acid-chitosan | Breast cancer | miRNA-34a | Intravenous |
| Atelocollagen | Prostate cancer | siRNA targeting Bcl-xL | Intravenous |
| | Muscular dys- trophy | siRNA targeting Mst | Intramuscular |
| | Metastatic pros- tate cancer | miRNA-16 | Intravenous |
| **PLGA poly(lactic-co-glycolic acid)** | | | |
| PLGA microspheres with PEI | Sarcoma | siRNA targeting VEGF | Intratumoral |
| | Joint inflammation | siRNA targeting FcγRIII | Intra-articular |
| PLGA nanoparticles with PEI | Lung cancer | siRNA targeting STAT3 | Intraperitoneal |
| **Other nanoparticles** | | | |
| Tf-targeted nanoparticles of CDP | Subcutaneous tumor | siRNA targeting RRM2 | Intravenous |
| Mesoporous silica nanopar- ticles with pDMAEMA | Cervical cancer | siRNA targeting PLK1 | Intravenous |
| Mesoporous silica nanopar- ticles with KALA peptide- PEG-PEI | Ovarian cancer | siRNA targeting VEGF | Intravenous |
| Porous silica nanoparticles with GD2 antibody | Neuroblastoma | miRNA-34a | Intravenous |
| **Lipoplexes** | | | |
| Cationic liposomes | Melanoma with lung metastasis | siRNA targeting Mcl1 | Intrapulmonary |
| PEG-cationic liposomes | Prostate and pan- creatic cancer | siRNA targeting PKN3 | Intravenous |
| | Drug-resistant renal cancer | siRNA targeting PLK1 | Intravenous |
| RGD peptide -PEG-cationic liposomes | Melanoma with lung metastasis | siRNA targeting c-Myc, MDM2 and VEGF | Intravenous |
| Peptides-modified PEG-cationic liposomes | Glioma | siRNA targeting VEGF | Intratumoral; Intravenous |
| Cationic liposomes (Lipofectamine™) | Colon cancer | miRNA-143 | Intratumoral; Intravenous |
| Cationic liposomes DOTMA/cholesterol/TPGS | Non–small-cell lung cancer | miRNA-29b | Intravenous |
| Neutral lipid emulsion (RNALancerII) | Non–small-cell lung cancer | miRNA-34a, let-7 | Intravenous |
| **Lipid-based nanoparticles (SNALPs, SLNs and LPH nanoparticles)** | | | |
| SNALP | Ebola infection | siRNA targeting polymerase of Ebola virus | Intraperitoneal |
| SLN | Lung cancer | miRNA-34a | Intravenous |
| LPH with single chain anti- body fragment | LPH with single chain anti- body fragment | Combined miRNA-34a and siRNA targeting MDM2, c-myc and VEGF | Intravenous |
| **Lipopolymer** | | | |
| StA-PEI | Melanoma | siRNA targeting STAT3 | Intratumoral |
| DA-PEI | Colorectal cancer | siRNA targeting XIAP | Intratumoral |
| | Myocardial infarc- tion | siRNA targeting RAGE | Intra-myocardial |
| Cholesterol-PEI | Prostate cancer | siRNA targeting VEGF | Intratumoral |

**Table 5:** A selected set of examples of nonviral vectors evaluated for therapeutic siRNA and miRNA candidates in preclinical studies (for more information please see chapter 6 in this report and (Lam et al., 2015), (Ozpolat et al., 2010) and (Coelho et al., 2010))(Adapted by permission from Macmillan Publishers Ltd: Mol. Ther. — Nucleic Acids (Lam et al., 2015), Copyright (2015)).

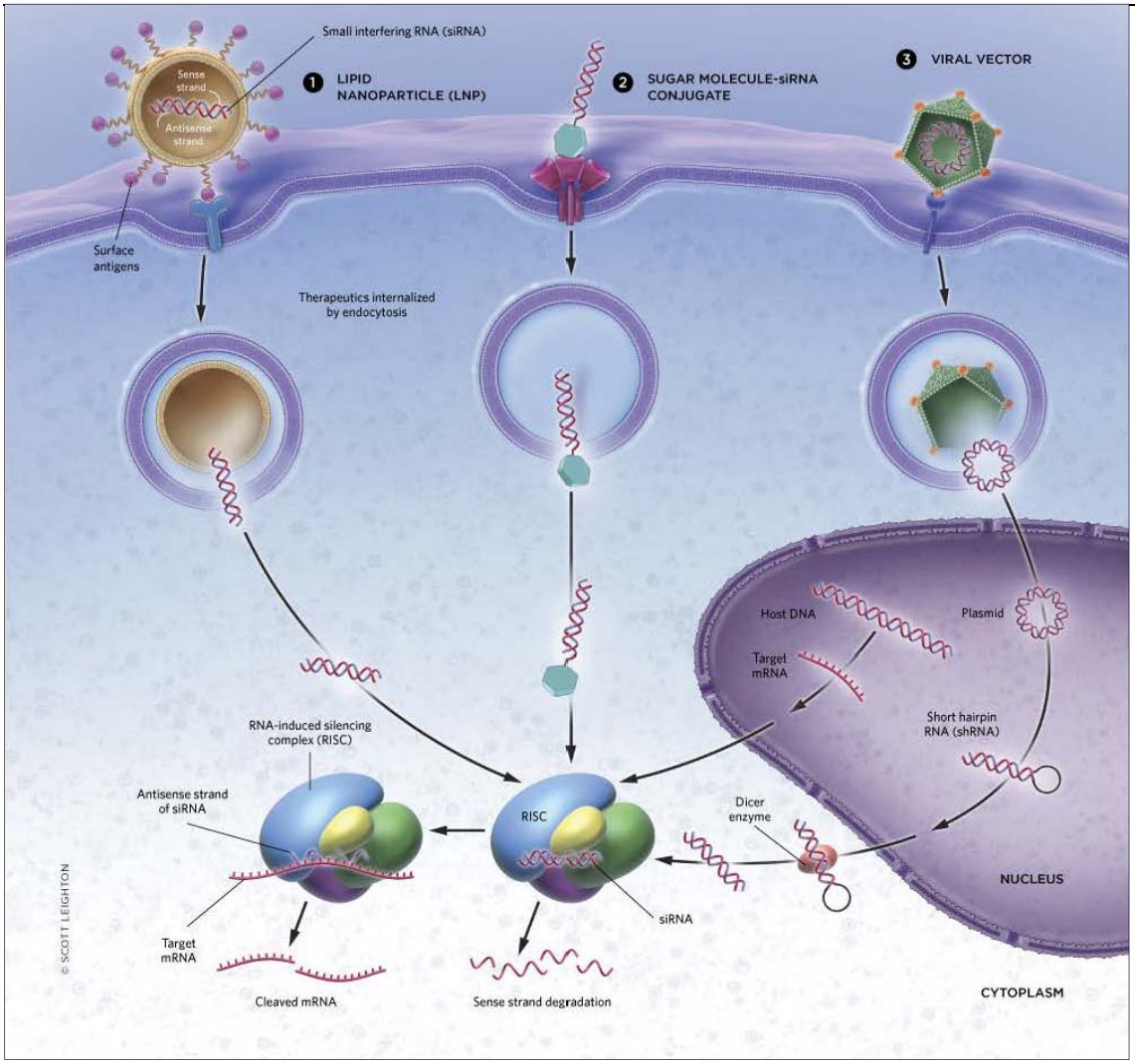



**Figure 10:** The siRNA or miRNA molecules can be encapsulated into lipid nanoparticles (LNPs), which protect against degradation in the blood stream and can be decorated with surface antigens to deliver the RNA to target cells, where the LNPs are taken up by endocytosis (1). Alternatively, some drug developers are conjugating the siRNAs with other molecules such as sugars to aid uptake by specific cells (2). Once in the cytoplasm, the siRNA's antisense strand is incorporated into an RNA-induced silencing complex (RISC), where, as most common target, messenger RNA is degraded. An alternative approach is to deliver the genes encoding the RNA sequences via a viral vector, taking advantage of the natural role of RNAi: the Dicer enzyme processes the short hairpin RNAs (shRNAs) generated after transcription of the inserted DNA into siRNAs that interact with RISC to inhibit protein translation (3) (http://www.the-scientist.com/Sept2014/RNAi_full_new.jpg)

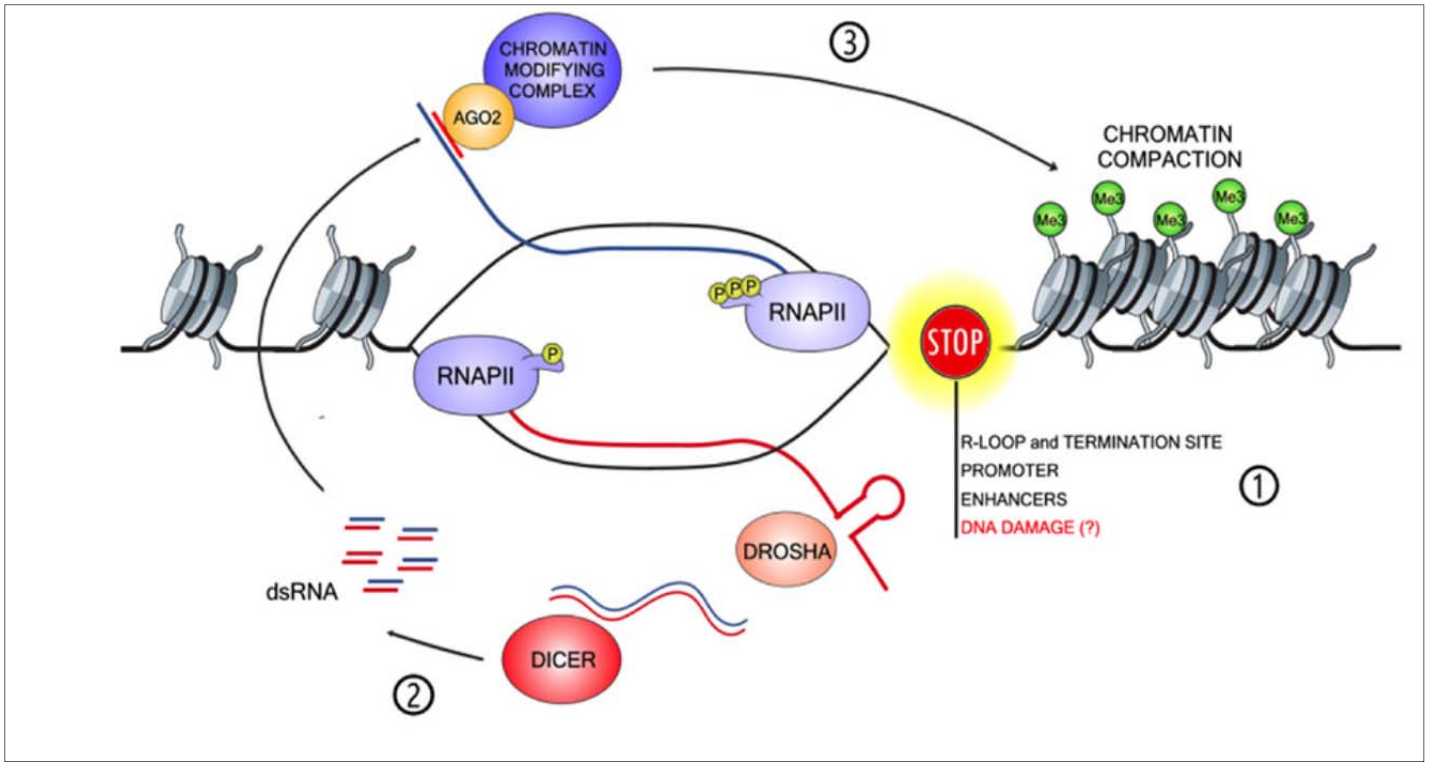



**Figure 11:** A model to explain an additional mechanism of action of small non coding RNAs (sncRNAs); chromatin modulation. For currently developed miRNA suppletion therapies this might be a mechanism of action, however , for miRNA's all the mechanisms of action are not always known as they may have many targets, see 4.1, siRNA's would need to be specifically targeted. (1) Pausing of RNAPII is induced in physiological condition at promoter and enhancer sequences and at transcription-termination sites prone to form R-loops, but could also occur in the presence of DNA lesions. (2) RNAPII pausing stimulates the loading of another RNAPII in opposite orientation on the complementary DNA template and generates antisense transcripts. DsRNA precursors are then processed by the RNAi-machinery into sncRNA. (3) Argonaute 2 (AGO2) forms a complex with such sequence-specific sncRNA and guide chromatin-modifying enzymes to the pausing site via nascent RNA:sncRNA interaction (Reprinted by permission from Frontiers Media S.A: Genetics (Francia, 2015), Copyright (2015)).

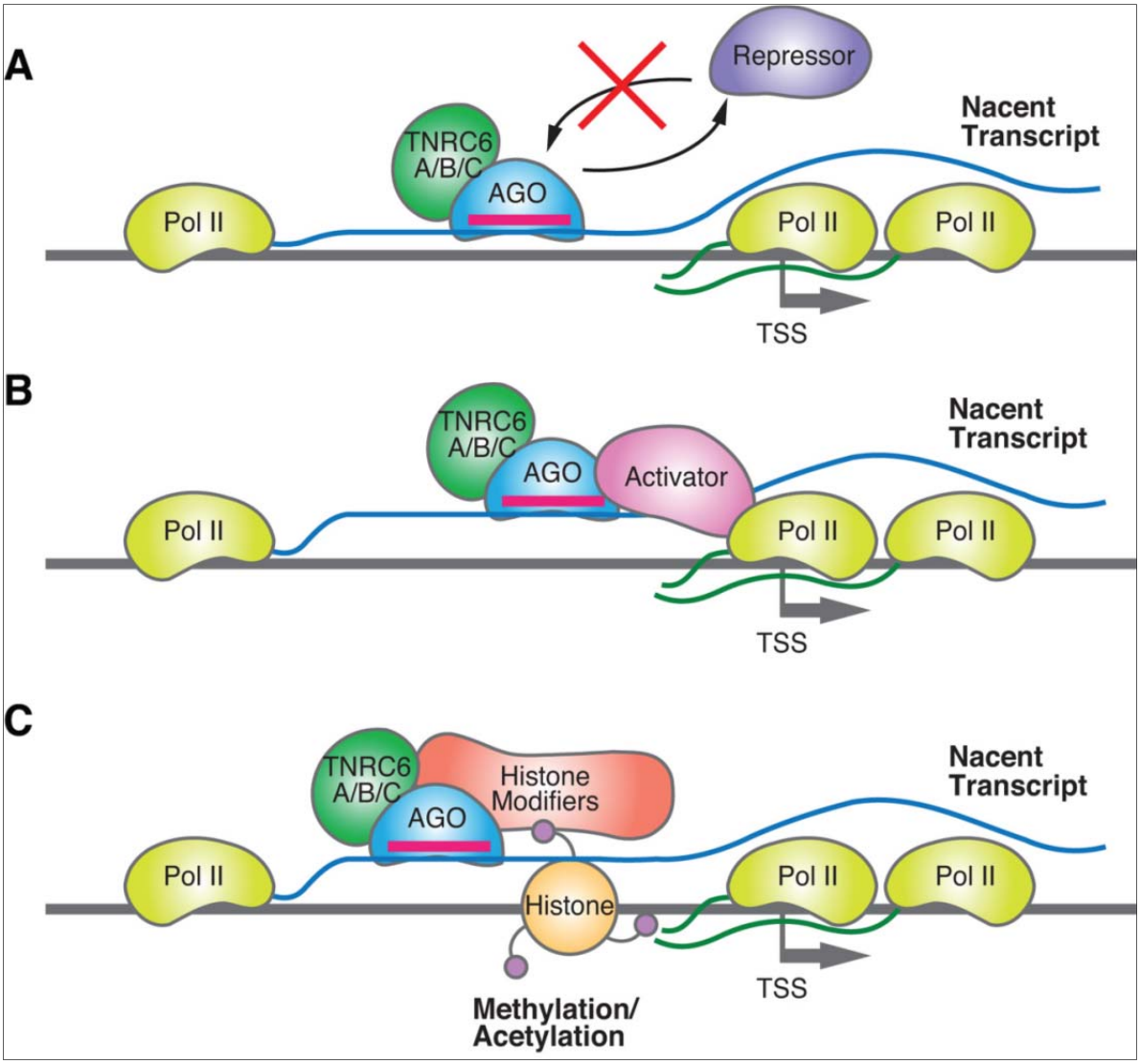



**Figure 12.** Scheme showing potential mechanisms for RNA-mediated gene activation via binding to nascent (promoter transcripts) that are based on known mechanisms for activation by protein transcription factors. For miRNA therapies this might be one of the mechanisms of action, see 4.1, siRNA's would need to be specifically targeted.
(A) Activation by blocking the binding of one or more proteins needed for repression. (B)Activation by promoting the binding of an activating factor. (C) Activation by inducing histone modifications (Reprinted by permission from Oxford University Press: Nucleic Acids Research (Kalantari et al., 2016), Copyright (2016)).

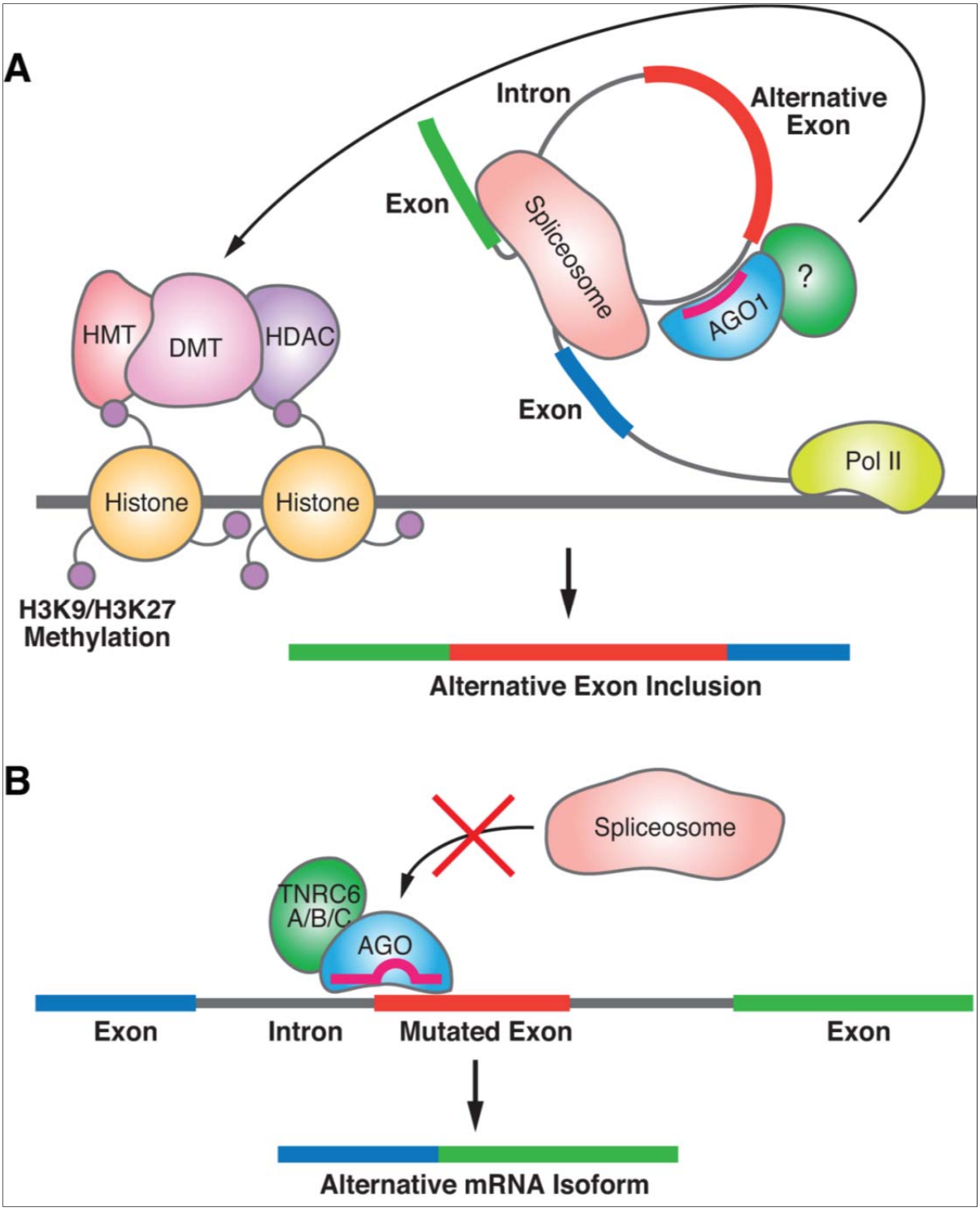



**Figure 13.** Scheme showing potential mechanism for RNA-mediated control of alternative splicing. For miRNAs this might be one of the mechanisms of action, see 4.1, siRNA's would need to be specifically targeted. (A) RNA-mediated binding of AGO1 alters histone modifications, affects the rate of transcription, and alters alternative splicing. (B) Binding of an RNA: RNAi factor complex near a splice site blocks association of the spliceosome and redirects alternative splicing (Reprinted by permission from Oxford University Press: Nucleic Acids Research (Kalantari et al., 2016), Copyright (2016)).

## *4.2* Host effects

### 4.2.1 Anticipated effects

When applied as therapeutics siRNAs and miRNAs are intended to (down)regulate the expression of undesired (ectopic expression) or mutated genes, or correct under- or overexpression of normal genes. The mode of action general is inhibition of translation, siRNAs and miRNAs could also interact directly or indirectly with transcription (including alternative slicing) of targeted genes. The siRNAs and miRNAs based therapies do therefore not change the genetic DNA code of the treated patient, however, epigenetic modification is very well possible and epigenetic changes that are transmitted though the germline have been described (Slatkin, 2009) (Lim and Brunet, 2013) see also 4.4.2.

### 4.2.2 Unintended effects

Although one of the distinctive features that differentiate siRNA from miRNA is that siRNA is designed to silence the expression of a specific target mRNA, siRNA may lead to the downregulation of unintended, unpredicted targets, resulting in off-target effects. Indeed, one of the major challenges of siRNA therapy is to reduce off-target effects, as these compromise the therapeutic effect and can even lead to cell death, e.g. by deregulating apoptosis (Jovanovic and Hengartner, 2006). The most common type of off-target effect of siRNA is the miRNA-like effect. This occurs when the 5’ end of the guide strand of siRNA is complementary to the 3’UTR of the mRNA. In some situations, this off-target effect occurs simply due to the poor design of the siRNA, as siRNA can tolerate several mismatches at the mRNA (imperfect complementarity) without losing gene silencing ability. Under these circumstances, siRNA behaves like a miRNA molecule: it enters the natural miRNA pathway leading to the inhibition or degradation of multiple mRNAs. In certain cases, this type of off-target effect is nearly as efficient as the on- target effect in reducing the protein levels(Lam et al., 2015).

Another type of off-target effect is not sequence-dependent, but due to the saturation of the RNAi machinery. When synthetic siRNAs (or miRNAs) are introduced into the cells, they compete with the endogenous miRNAs for common proteins such as RISC and other factors. As a result, gene regulation by endogenous miRNAs is perturbed, leading to unpredictable off-target effects (Grimm, 2011).

A third type of unwanted effect is due to the fact that siRNAs can cause immune responses mediated by receptors of the innate immune system. Immune-stimulatory sequence motifs should be avoided to reduce the siRNA immunogenicity. Alternatively, immune response could be minimized by the use of delivery agents that exclude siRNA endosomal delivery or by chemical modification of the immune-stimulatory sequences to render them unrecognizable by the relevant receptors. As the rules of siRNA immune activation are still poorly understood, all therapeutic siRNAs must be carefully tested for any possible immunostimulatory adverse effects (Lam et al., 2015).

## 4.3 Application areas

Both siRNAs and miRNAs have huge potential as therapeutic agents. They can overcome the major limitation of traditional small drug molecules, which can only target certain classes of proteins. Even for protein-based drugs including monoclonal antibodies that are highly specific, their targets are mainly limited to cell-surface receptors or circulating proteins. By contrast, siRNAs and miRNAs can regulate the expression of virtually all genes and their mRNA transcripts. Since many diseases result from the expression of undesired or mutated genes, or from under- or overexpression of certain normal genes, the discovery of siRNA and miRNA opens up a whole new therapeutic approach for the treatment of diseases by targeting genes that are involved causally in the pathological process. An incomplete summary of selected examples of therapeutic siRNAs and miRNAs focused on cancer treatments is given under 4.1. It is however important to emphasize that also for other common diseases also candidate miRNAs have been identified for treatments such as; Diabetes type2 (Natarajan et al., 2012), neurodegenerative diseases (e.g. Alzheimer's (Long et al., 2014)) and viral infections (e.g. Hepatitis-C (Li et al., 2011)). Many (non-infectious) common diseases, such as ageing related diseases, with a contributing genetic component are so called multifactorial common diseases, which implicates that the disease occurs as a consequence of the interaction between genetic predisposition and environmental factors. The genetic predisposition often involves unfavorable expression of contributing genes. This unfavorable expression can be corrected by siRNAs and miRNAs based therapies.

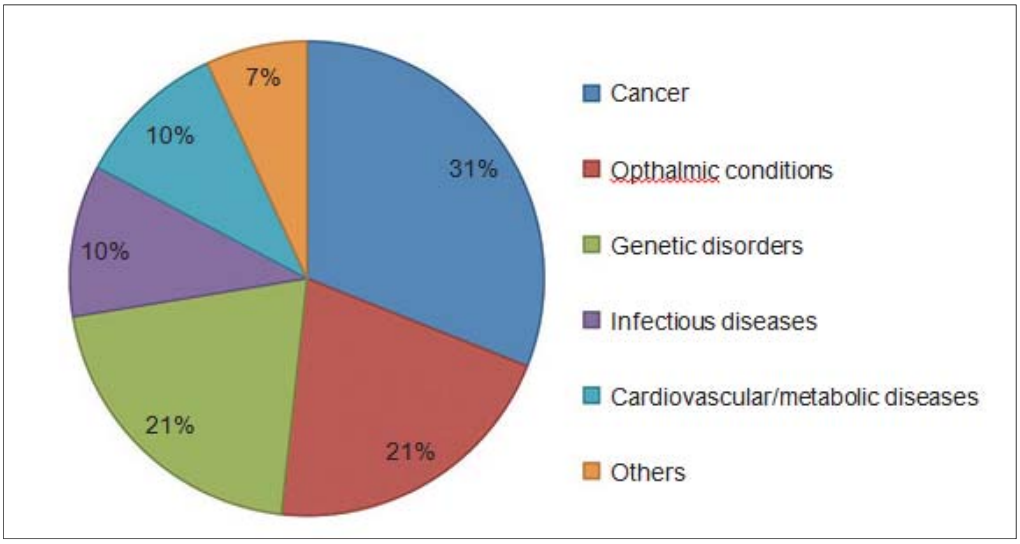



**Figure 14:** Proportions of conditions addressed by miRNA and siRNA therapeutics in clinical trials (registered with clinicaltrials.gov, last accessed 13 June 2015) is given (Reprinted by permission from Macmillan Publishers Ltd: Mol. Ther. — Nucleic Acids (Lam et al., 2015), Copyright (2015)).

## 4.4 Barriers and Drivers

### 4.4.1 Technical Barriers and Drivers

The most attractive aspect of siRNA and miRNA therapeutics is their ability to target virtually any gene(s), which may not be possible with small molecules or protein-based drugs. siRNA and miRNA therapeutics are therefore studied for the treatment of various human diseases including cancers, viral infections, ocular conditions, genetic disorders, and cardiovascular diseases. For proper therapeutic use, the RNA sequences must be carefully designed to avoid any specific or nonspecific unwanted effects and immune responses. It should also be realized that an intended RNAi effect almost never results in 100% knock down, but also that 100% may not be required for clinical effect.

While a large number of RNA molecules and targets have been identified with therapeutic potential, the duration of therapeutic effect has not been properly investigated or reported, and it may affect the dose interval and length of treatment. The stability of the RNA molecules, the rate of RNA release from the delivery system, the type of target tissues, as well as the half-life and turnover rate of the target proteins may influence the duration of the treatment effect.

RNA-based therapy therefore depends heavily on the availability of a safe, clinically relevant delivery system that can facilitate cellular uptake of the RNA into target tissues/cells and offer protection against nuclease degradation. For instance PEGylated nanoparticles incorporated with targeting ligands are frequently employed to prolong circulation time and achieve targeting to specific sites following systemic administration.

Overcoming the delivery barrier, and better understanding of the duration and possible consequences of gene up or down regulation, will be necessary to allow siRNAs and miRNAs to become practical therapeutics in the clinic in the near future. Barriers and drivers are well illustrated in the comparison below between small molecules, protein-based drugs (including monoclonal antibodies) and siRNA/miRNA-based drugs in table 6 below (Lam et al., 2015).

| Properties | Small molecules | Protein-based drugs | siRNA/miRNA-based drugs |
|---|---|---|---|
| Nature of action | Activation or inhibition of targets | Activation or inhibition of targets | Inhibition or activation of targets |
| Site of target proteins | Extracellular and Intracellular | Mainly extracellular | Virtually any sites |
| Selectivity and potency | Variable (depending on binding-site and ligand specificity, their affinity and efficacy etc.) | Highly specific and potent | Highly specific and potent |
| Lead optimization | Slow | Slow | Rapid |
| Manufacture | Easy | Difficult | Easy |
| Stability | Stable | Unstable | Unstable |
| Delivery | Easy | Difficult | Difficult |

**Table 6:** A comparison between small molecules and protein based drugs (including monoclonal antibodies) and siRNA/miRNA based drugs (Reprinted by permission from Macmillan Publishers Ltd: Mol. Ther. — Nucleic Acids (Lam et al., 2015), Copyright (2015)).

The most important general drivers behind genetic engineering therapies are without doubt the novel fast and high throughput sequencing technologies that enable elucidation of molecular disease mechanisms and fast individual diagnoses (Pareek et al., 2011). A very important general barrier for many genetic engineering therapies will be the development of a suitable delivery system for the RNA molecules or find stable and targetable alternatives for the endogenous RNA molecules (see chapters on delivery systems and modified (antisense) oligonucleotides.

### 4.4.2 Socio-ethical Barriers and Drivers

#### *4.4.2.1 Introduction into Epigenetics*

siRNA and miRNA based therapeutics provide a huge potential for new treatments of various human diseases including cancers, viral infections, ocular conditions, genetic disorders, and cardiovascular diseases. The siRNAs and miRNAs based therapies do not change the genetic DNA code of a treated patient, however, epigenetic modification is very well possible. As epigenetic changes can be transmitted though the germline (Slatkin, 2009) (Lim and Brunet, 2013) and consequentially safety aspects may affect multiple generations, epigenetic changes are an important subject of the ongoing Socio-ethical debate concerning medicinal treatments that may induce such changes.

Epigenetic changes are alterations in the chemical modification of DNA that occur in humans and other organisms. The genetic code has been compared to the hardware of a computer, whereas

epigenetic information has been compared to computer software that controls the operation of the hardware. Further, the factors that affect the epigenetic information may be analogized as parameters for operating the software (Rothstein et al., 2009). Although epigenetic effects do not change the genetic code per se, they leave “marks” on the DNA sequence, which in turn affect whether, when, and how specific segments of the genetic code are turned on or “expressed.” Some epigenetic changes involve chemical alterations to the DNA molecule itself, most commonly the addition of a methyl group to cytosine bases (the “C’s”) to form methyl-cytosine, which makes the DNA in that region less likely to be expressed. Other epigenetic changes involve chemical alterations to the proteins that bind with DNA to form chromosomes, including methylation or acetylation of histone proteins that bind with DNA and affect the higher-order structure of chromosomes and the nucleus. The acetylation of histone proteins signals an open configuration of the chromosomal region that promotes expression. A third type of epigenetic effect is RNA interference, which involves RNA molecules produced from an DNA region binding back to the same DNA at specific sites to turn off gene expression. A significant crosstalk exists between these different epigenetic pathways.

#### *4.4.2.2 Epigenetic risks*

Epigenetics link environmental and genetic influences on the traits and characteristics of an individual, and new discoveries reveal that a large range of environmental, dietary, behavioral, and medical experiences can significantly affect the future development and health of an individual and their offspring. Identical twins born with identical genotypes increasingly diverge in their epigenetic profiles as they age, with the extent of divergence increasing as the twins got older, had different lifestyles, or spent less of their lives together. Strong evidence supports the notion that predisposition to various types of diseases that do not manifest until later in life may be encoded epigenetically at early embryonic developmental stages. Animal tests suggest the effects of maternal DES (diethylstilbestrol) exposure were transmitted through the maternal germline to offspring via both genetic and epigenetic mechanisms. DES ingestion increased the risk of reproductive disorders and rare forms of cancer in DES daughters and granddaughters. It is now widely accepted that epigenetics play a key role in many cancers (Hou et al., 2012).

#### *4.4.2.3 Evaluation*

The observation that (early) life experiences including drug treatment, may alter epigenetic programming may also have implications for drug safety and approval. Epigenetic changes to critical genes could affect subsequent health in individuals and/or their offspring. Especially as epigenetic changes tend to occur at a much higher frequency than mutations in the DNA sequences. Genotoxic agents will usually only result in mutations in less than 0.01 percent of offspring, whereas epigenetic processes often affect the majority of offspring. The mechanism(s) involved in transmission of epigenetic changes to the germline remain(s) to be elucidated. However, the epigenetic state of an organism has a “lifecycle”, epigenomics must therefore consider not only the magnitude but also the timing of a certain exposure as epigenetic changes are intrinsically reversible. Further it should be emphasized that epigenetic changes also tend to be species-specific, so a carcinogenic or toxic response in a laboratory study using rodents may be less predictive. Numerous legal and ethical issues are raised by epigenetics, especially regarding individual and societal responsibilities to prevent hazardous exposures, monitor health status, and provide care. Epigenetics represents a new class of biological effects from harmful exposures and adds a multigenerational dimension to environmentally/medical treatment-caused adverse health effects. Given the trans-generational nature of many epigenetic disruptions, transgenerational studies will be needed to evaluate some epigenetic-mediated toxicity, as this has significant scientific, economic and legal implications. For example, insurance policy claims and liability may have a “long tail” if the toxic effects from agents acting via an epigenetic mechanism are not manifested until one or more generations into the

future. It should be prevented however, that the costs of transgenerational studies would interfere with the availability of new generations of medicines such as siRNA or miRNA based medicines.

### 4.4.3 Patent Situation siRNAs and miRNA's based therapies

Up to 2013 genes and gene-based diagnostic tests were patent eligible in the US. Diagnostic gene patents have been a source of debate for several reasons. Pierce et al., 2009, studied the impact of patents on the development of genome-based clinical diagnostics and found that fragmented ownership of these patents made it difficult and expensive for a single party to assemble the patent rights necessary to develop a panel of genetic tests for clinical purposes and this was considered a barrier to patient care and medical innovation. In 2013 the US Supreme Court ruled that natural human genes cannot be patented but that cDNA still is patentable (AMP v. Myriad Genetics). In the EU methods for treatment of the human or animal body by surgery or therapy and diagnostic methods practiced on the human or animal body are not patentable; this provision does not apply to products, in particular substances or compositions, for use in any of these methods. In Japan methods of surgery, therapy, and the diagnosis of human diseases cannot be patented. In 2015 the Australian high court ruled that naturally occurring genes cannot be patented.

An Australian study analyzed a large number of patents that encircled high-cost drugs. The majority of these patents relate to medicines that contain the active pharmaceutical ingredient (API) of the drug – either patents for a combination of the API with other pharmaceutical compounds, or patents for a delivery mechanism or a formulation for the API. Patents for a method of treatment (both same and different ATC class) using the API were also prevalent. Among 736 patents connected to high-cost drugs in Australia, 3 in every 4 were owned by entities other than the drug's originator. Patents most commonly held by the API originator are for delivery mechanisms or formulations for the API, and for processes for making or formulating the API. The focus of API originators on these areas of innovation is probably not surprising, given that these areas are most closely connected with the original innovation, the API. Non-originators patented heavily in three areas: delivery mechanisms or formulations for the API; methods of treatment (different ATC class); and intermediates or different forms of the API. The focus of non-originators on intermediate and alternative forms is logical: they are likely to be exploring these compounds in preparation for manufacture of the API once the original patent on it expires. The "one drug, one patent" perception is popular, but appears inaccurate.(Christie et al., 2013)

It is expected that the patent situation can be a driver as well as a barrier for most innovative therapeutic applications for relevant therapeutic approaches and production methods. A relevant overview of the patent situation for the new siRNAs and miRNAs based therapies is available (Santiago Grijalvo, 2014), but the impact analysis of the patent situation can only be done by patent experts and is therefore considered out of scope here.

## 4.5 At the horizon

siRNA and miRNA therapeutics are studied for the treatment of various human diseases including cancers, viral infections, ocular conditions, genetic disorders, and cardiovascular diseases. The first generation licensed RNAi drugs, Pegabtanib (anti VEGF, wet macular degeneration), Fomivirsen (anti cytomegalovirus) and Mipomersen (anti Apolipoprotein B, cholesterol reducing) are administered to elicit an inhibiting effect on their mRNA targets. An ongoing developments overview is listed in table 7 and 8 below and provides some insight on what treatments could be expected to enter the market in the near future.

| Name | indications | siRNA target | Phase | Delivery system |
|---|---|---|---|---|
| ALN-VSP02 | Advanced solid tumors with liver involvement | KSP and VEGF | 1, completed | Lipid nanoparticles |
| Atu027 | Advanced solid tumor | PKN3 | 1, ongoing | Liposomal particles (AtuPLEX®) |
| CALAA-01 | Solid tumor | RRM2 | 1, terminated | Polymer-based targeted nanoparticles |
| DCR-MYC (Dicer-substrate siRNA) | Solid tumor, multiple myeloma, non-Hodgkin's lymphomas | MYC oncogene | 1, ongoing | Lipid nanoparticles (EnCore) |
| | Hepatocellular carcinoma | | 1/2, ongoing | |
| siG12D LODER | Advanced pancreatic cancer | mutated KRAS oncogene | 1, completed; 2, ongoing | Biodegradable polymer-based scaffold |
| siRNA-EphA2-DOPC | Advanced cancers | EphA2 | 1, ongoing | Neutral liposomes |
| TKM-080301 (TKM-PLK1) | Primary or secondary liver cancer | PLK1 | 1, completed | Lipid nanoparticles |
| | Neuroendocrine tumors and adrenocortical carcinoma | | 1/2 ongoing | |
| **infectious Diseases** | | | | |
| ALN-RSV01 | RSV infection RSV infection in lung transplant patients | RSV nucleocapsid | 2, completed 2, completed | Naked oligonuleotide |
| ARC-520 | Chronic HBV infection | conserved regions of HBV | 1, completed; 2, ongoing | DPC (membrane lytic peptides with cholesterol conjugated siRNA |
| TKM-100201 TKM-100802 | Ebola virus infection | Ebola L polymerase, VP24 and VP35 | 1, terminated 1, ongoing | Lipid nanoparticles |
| **Ocular Conditions** | | | | |
| AGN211745 (Sirna-027) | CNV, AMD | VEGF receptor 1 | 1/2, completed; II, terminated | Naked oligonuleotide |
| Bamosiran (SYL040012) | Ocular hypertension, glaucoma | ADRB2 | 1, completed; 1/2 completed | Naked oligonuleotide |
| | Ocular hypertension, open-angle glaucoma | | 2, completed; 2, ongoing | |
| Bevasiranib (Cand5) | Wet AMD | VEGF | 2, completed | Naked oligonucleotide |
| | Diabetic macular edema VEGF | | | |
| | Wet AMD | | 3, terminated | |
| | AMD | | 3, withdrawn | |
| PF-04523655 (PF-655) | AMD | RTP801 (hypoxia-inducible factor 1 responsive gene) | 1, 2 completed | Naked oligonucleotide |
| | CNV, diabetic retinopathy, diabetic macular edema | | 2, completed | |
| | Diabetic retinopathy, diabetic complications | | 2, terminated | |
| QPI-1007 | Optic atrophy, nonarteritic anterior ischemic optic neuropathy | CASP2 | 1, completed | Naked oligonucleotide |
| SYL1001 | Ocular pain, dry eye syndrome | Capsaicin receptor TRPV1 | 1, completed; 1/2, completed | Naked oligonucleotide |

| Name | indications | siRNA target | Phase | Delivery system |
|---|---|---|---|---|
| **Cardiovascular** | | | | |
| ALN-PCS02 | Hypercholesterolemia | PCSK9 | 1, complete | Lipid nanoparticles |
| ALN-PCSsc | | | 1, ongoing | GalNAc-siRNA conjugation |
| PRO-040201 (TKM-ApoB) | Hypercholesterolemia | ApoB | 1, terminated | Lipid nanoparticles |

**Table 7**: A summary of siRNA therapeutics in clinical trials (registered with clinicaltrials.gov, last data 13 June 2015) (Adapted by permission from Macmillan Publishers Ltd: Mol. Ther. — Nucleic Acids (Lam et al., 2015), Copyright (2015)).

| Company | miRNA target | Mode of action | Delivery system | Indication | Status |
|---|---|---|---|---|---|
| Santaris Pharma | | | | | |
| | miR-122 | antimiR | Naked modified RNA | Hepatitis C virus | Clinical Phase II |
| Mirna Therapeutics | | | | | |
| | miR-34 | mimic | Liposomal nanoparticle | Unresectable primary liver cancer | Clinical Phase I |
| | let-7 | mimic | neutral lipid emulsion | Cancer | Preclinical |
| Regulus Therapeutics | | | | | |
| | miR-122 | antimiR | GalNAc-conjugated | Hepatitis C virus | Clinical Phase I |
| | miR-221 | antimiR | Unknown | Hepatocellular carcinoma | Preclinical |
| | miR-10b | antimiR | Unknown | Glioblastoma | Preclinical |
| | miR-21 | antimiR | Unknown | Hepatocellular carcinoma | Preclinical |
| | miR-21 | antimiR | Unknown | Kidney fibrosis | Preclinical |
| | miR-33 | antimiR | Unknown | Atherosclerosis | Preclinical |
| miRagen Therapeutics | | | | | |
| | miR-208 | antimiR | Unknown | Heart failure | Preclinical |
| | miR-15/195 | antimiR | Unknown | Post-myocardial infarction remodeling | Preclinical |
| | miR-145 | antimiR | Unknown | Vascular disease | Preclinical |
| | miR-451 | antimiR | Unknown | Myeloproliferative | Preclinical |
| | miR-29 | mimic | Unknown | Fibrosis | Preclinical |
| | miR-208 | antimiR | Unknown | Cardiometabolic disease | Preclinical |
| | miR-92 | antimiR | Unknown | Peripheral artery disease | Preclinical |
| TargomiRs | | | | | |
| | miRNA-16 | mimic | EDV nanocells | Malignant pleural mesothelioma; non–small-cell lung cancer | Phase I |

**Table 8** A summary of miRNA therapeutics in clinical trials (Adapted by permission from EMBO: EMBO Molecular Medicine (van Rooij and Kauppinen, 2014), Copyright (2014)).

# 5 Modified (Antisense) oligonucleotides based therapies Technical Description

## 5.1 Introduction

As the mechanisms of activity may be 100% overlapping, the most relevant differences between miRNAs/siRNAs and modified (Antisense) oligonucleotides may actually be the applied chemical modifications, and in these cases modified (antisense) oligonucleotides simply provide an approach to overcome the delivery hurdles associated with natural RNA (and DNA) molecules. However, in addition modified (Antisense) oligonucleotides may also have therapeutic applications that mechanistically are not RNAi/Argonaute related.

Therapeutic oligonucleotides that intend to have an effect on gene expression in general need to be able to enter the targeted cells and stay biologically active to be able to reach their DNA or RNA target sequence. As nucleotides composing RNA and DNA are linked to each other by phosphodiester linkages that are easily cleaved by endo- and exonucleases such molecules often are not suitable for the intended medical use. Besides many other possible modifications, modification of the natural backbone is typically the basis to enhance oligonucleotide resistance against nuclease degradation. The modifications result in molecules that strictly speaking are no longer RNA or DNA molecules, but this should not affect the ability to recognize the same target and induce the same biological effect as the natural RNA or DNA counterparts. An interesting example of such modification is the so-called PNA; Peptide Nucleic acid. Synthetic peptide nucleic acid oligomers have been used in recent years in molecular biology; in diagnostic assays and antisense therapies. Due to their high binding strength PNA usually requires oligonucleotide synthesis of only 20–25 bases. PNA oligomers also show greater specificity in binding to complementary DNAs, with a PNA/DNA base mismatch being more destabilizing than a similar mismatch in a DNA/DNA duplex. This binding strength and specificity also applies to PNA/RNA duplexes. PNAs are not easily recognized by either nucleases or proteases, making them resistant to degradation by enzymes. PNAs are also stable over a wide pH range. Though an unmodified PNA cannot readily cross cell membranes to enter the cytosol, covalently coupling a cell penetrating peptide to a PNA can improve cytosolic delivery. However, many types of modifications have been described, and besides backbone modification; sugar modification (Locked Nucleic Acids, Bridged Nucleic Acids), nucleobase modification (Base Analogues), and terminal modification (coupled sugar, lipid, peptide) have been applied to improve oligonucleotides properties (Nielsen and Egholm, 1999)(van Rooij and Kauppinen, 2014)(Guo et al., 2010). The figure below gives some information concerning common modifications of oligonucleotides.

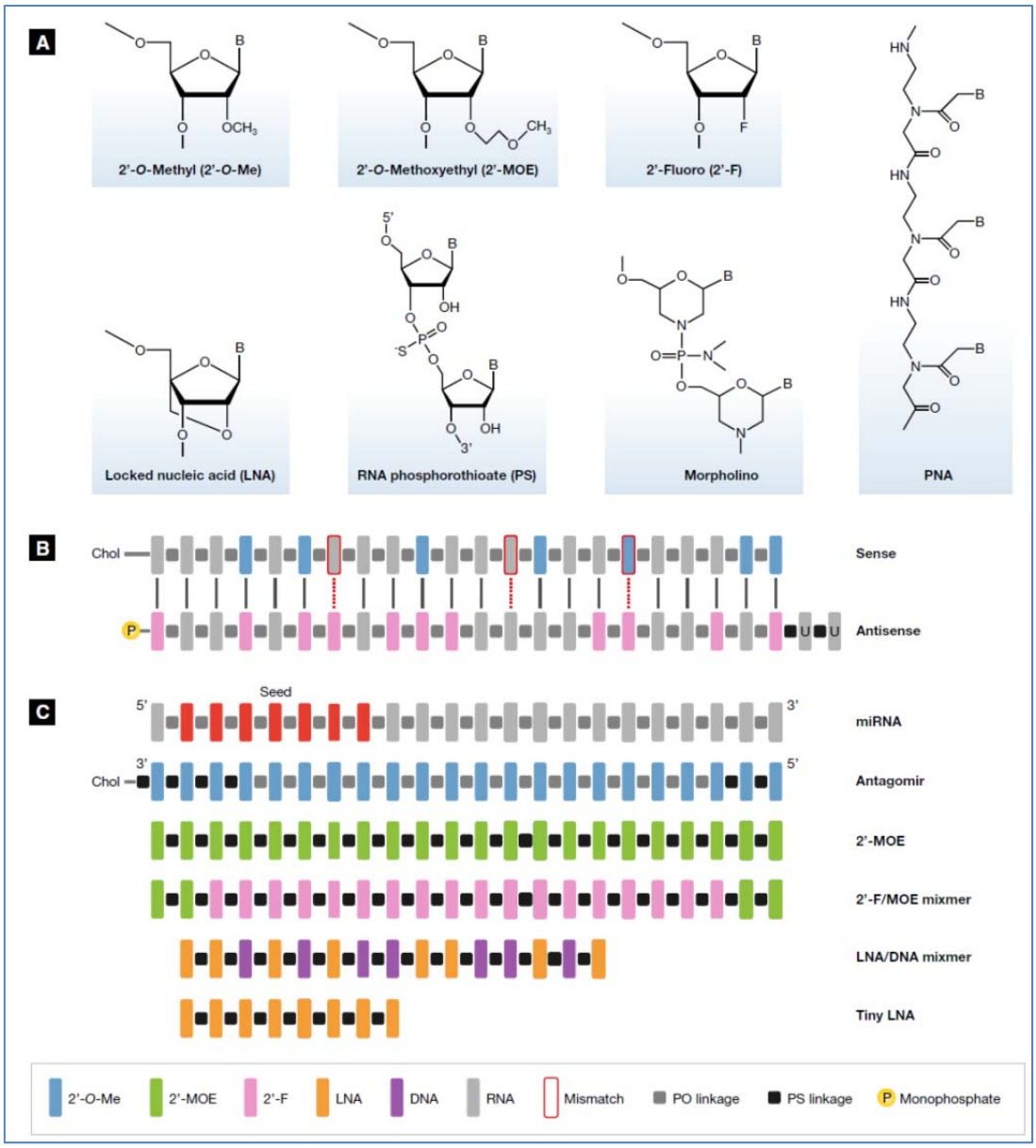



**Figure 15**. Design of chemically modified oligonuclotides.
(A) The 20-O-methyl (20-O-Me), 20-O-methoxyethyl (20-MOE) and 20-fluoro (20-F) nucleotides are modified at the 20 position of the sugar moiety, whereas locked nucleic acid (LNA) is a bicyclic RNA analogue in which the ribose is locked in a C30-endo conformation by introduction of a 20-O,40-C methylene bridge. To increase nuclease resistance and enhance the pharmacokinetic properties, most oligonucleotides harbor phosphorothioate (PS) backbone linkages, in which sulfur replaces one of the non-bridging oxygen atoms in the phosphate group. In morpholino oligomers, a six-membered morpholine ring replaces the sugar moiety. Morpholinos are uncharged and exhibit a slight increase in binding affinity to their cognate miRNAs. PNA oligomers are uncharged oligonucleotide analogues, in which the sugar–phosphate backbone has been replaced by a peptide-like backbone consisting of N-(2-aminoethyl)-glycine units. (B) An example of a synthetic double-stranded miRNA mimic. One way to therapeutically mimic a miRNA is by using synthetic RNA duplexes that harbor chemical modifications for improved stability and cellular uptake. In such constructs, the antisense (guide) strand is identical to the miRNA of interest, while the sense (passenger) strand is modified and can be linked to a molecule, such as cholesterol, for enhanced cellular uptake. The sense strand contains chemical modifications to prevent miRISC loading. Several mismatches can be introduced to prevent this strand from functioning as an antimiR,while it is further left unmodified to ensure rapid

degradation. The 20-F modification helps to protect the antisense strand against exonucleases, hence making the guide strand more stable, while it does not interfere with miRISC loading.
(C) Design of chemically modified antimiR oligonucleotides (see also 5.2.1). Antagomirs can for instance be 30 cholesterol-conjugated, 20-O-Me oligonucleotides fully complementary to the mature miRNA sequence with several PS moieties to increase their *in vivo* stability. The use of unconjugated 20-F/MOE-, 20-MOE-, PNA or LNA-modified antimiR oligonucleotides harboring a complete PS backbone represents another approach for inhibition of miRNA function *in vivo*. The high duplex melting temperature of LNA-modified oligonucleotides allows efficient miRNA inhibition using truncated, high-affinity 15–16-nucleotide LNA/DNA antimiR oligonucleotides targeting the 50 region of the mature miRNA. Furthermore, the high binding affinity of fully LNA-modified 8-mer PS oligonucleotides, designated as tiny LNAs, facilitates simultaneous inhibition of entire miRNA seed families by targeting the shared seed sequence. (Reprinted by permission from EMBO: EMBO Molecular Medicine (van Rooij and Kauppinen, 2014), Copyright (2014)).

## *5.2* Mechanisms of action of modified oligonucleotides

### 5.2.1 miRNA inhibition

The first generation antisense oligonucleotides (ASOs or AOs) drugs, Pegabtanib (anti VEGF, wet macular degeneration), Fomivirsen (anti cytomegalovirus retinitis) and Mipomersen (anti Apolipoprotein B, cholesterol reducing) are administered (as oligo's in solution by intravitreal injection, intraocular injection and injection, as described for these new drugs respectively) to elicit an RNAi effect on their mRNA targets using the mechanisms as described for miRNAs and siRNAs. Another target of an antisense oligonucleotide may be a miRNA. The inhibition of the miRNA by an antisense oligonucleotide (anti-miR) is based on the specific annealing between the miRNA and the anti-miR. A stable, high-affinity binding of the anti-miR to the miRNA will compete with the binding to the miRNA target and effectively sequester the miRNA. For example, miR-509-3p, a microRNA that was identified as being able to inhibit the expression of the Cystic Fibrosis Transmembrane Regulator (CFTR) disease-gene of Cystic Fibrosis (CF) and CFTR-Related Disorders (CFTR-RD), was shown to be inhibited using peptide nucleic acids as inhibitors. miR-509-3p is generally found to be over expressed in patients (Amato et al., 2014). PNAs that were designed to be at least complementary to the "seed region" of miR-509-3p (i.e. the first seven bases at its 5'end) were shown to be effective inhibitors. See figure 16.

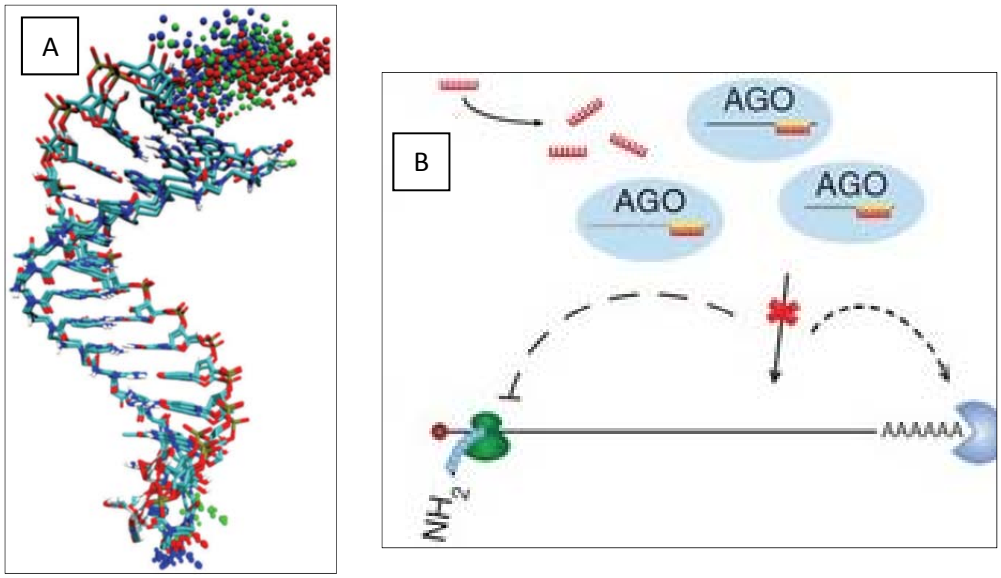



**Figure 16**: Peptide nucleic acid (PNA) hybridized with target miRNA sequence.

(A) The PNA, with a more red backbone and a FITC tail (fluorescent) for detection, forms a double stranded polymer and inhibits target finding of miRNA by blocking the seed sequence (more blue backbone) (Reprinted by permission from Hindawi Publishing Corporation: BioMed Research International (Amato et al., 2014), Copyright (2014)). (B) The blocked seed sequence incorporated in the Argonaute protein (AGO) will result in an inactive complex (Reprinted by permission from Macmillan Publishers Ltd: Nature Genetics (Obad et al., 2011), Copyright (2011)).

### 5.2.2 Exon skipping

Modified oligonucleotides may be designed to elicit other effects than RNAi. ASOs targeted at exons or specific splice factor recognition sites can induce exon skipping resulting in alternative mRNA splicing, which may contribute to decreased melanoma growth (Dewaele et al., 2016) or restoration of a coding frame of the dystrophin gene(Jirka et al., 2015) (see figures below). The mechanisms involved in these examples appear to be different from those described for Argonaute/RNA complexes as they involve DNA oligos, which are not described to form functional complexes with Argonaute proteins, although this is still under discussion (Smalheiser and Gomes, 2014).

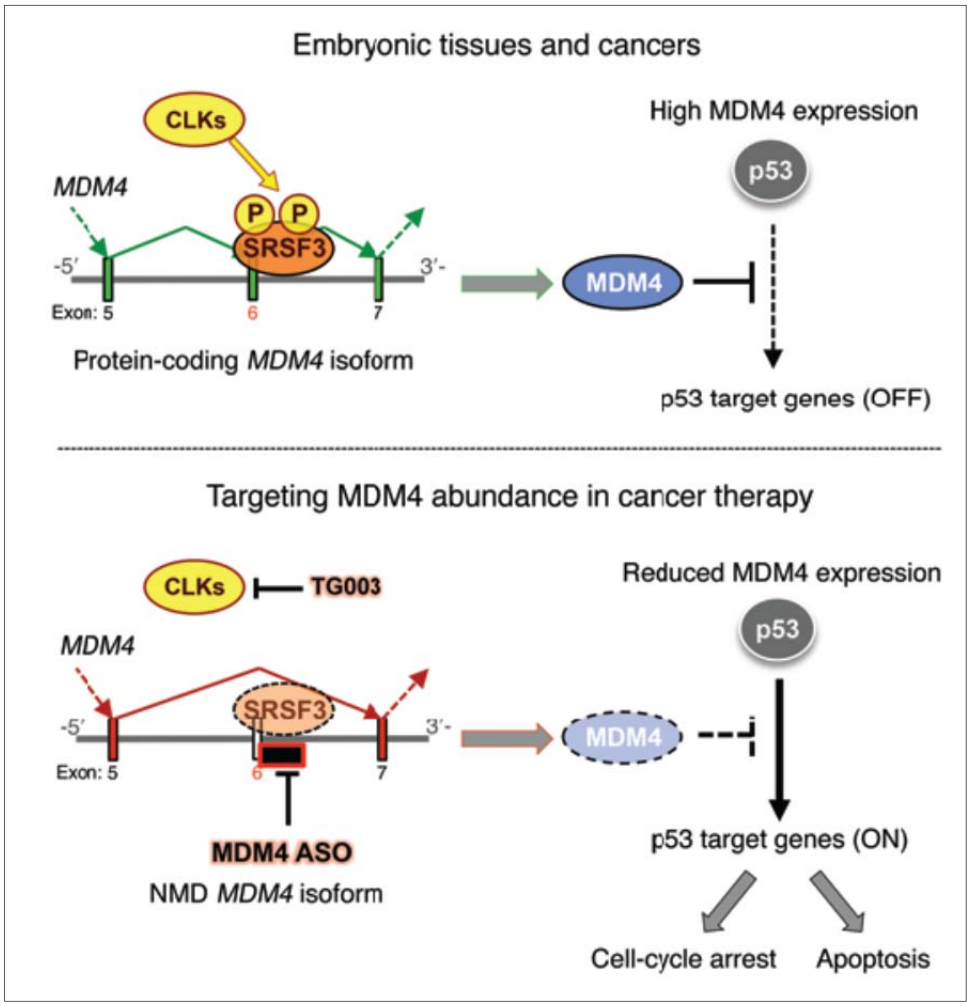



**Figure 17.** Targeting MDM4 splicing in cancer therapy.
Whereas MDM4 is unproductively spliced in most normal adult tissues, MDM4 protein is highly expressed in embryonic tissues and in cancers (such as melanoma) as a result of enhanced exon 6 inclusion. Splice factor SRSF3 is the only SRSF family member that promotes exon 6 inclusion. TG003 is a CLK (Splice factor activator) inhibitor that affects the phosphorylation of multiple SR proteins. Inducing MDM4 exon 6 skipping via ASO is a very specific, efficient, and clinically compatible

approach to inhibiting p53-dependent MDM4 oncogenic functions (Reprinted by permission from Macmillan Publishers Ltd: Journal of Clinical Investigation (Dewaele et al., 2016), Copyright (2016)).

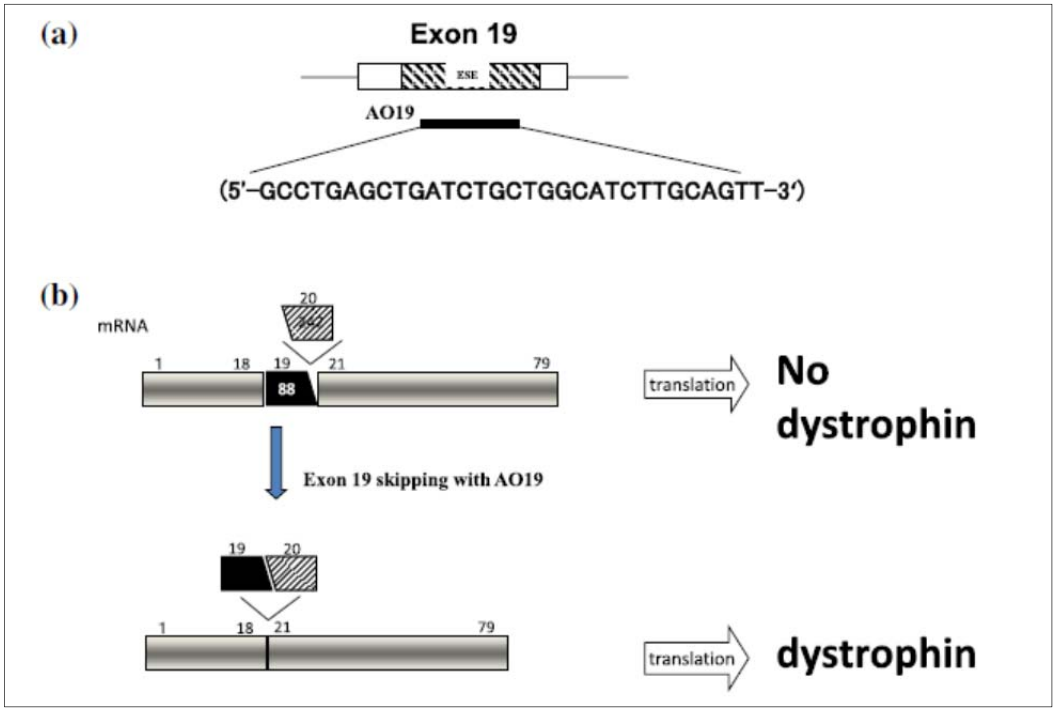



**Figure 18.** Dystrophin expression treatment with antisense oligonucleotides.
(a) AO(ASO) 19 is complementary to the exonic splicing enhancer sequence (ESE) in exon 19 and consists of a 31-nt phosphorothioate DNA oligonucleotide.
(b) Induction of dystrophin expression. When exon 20 is deleted, an out-of-frame dystrophin mRNA is produced. This creates a premature stop codon, and no dystrophin protein is produced (top). With AO19 mediated exon 19 skipping, the dystrophin mRNA became in-frame because of the removal of exons 19 and 20 (bottom). Then dystrophin protein can be produced, albeit slightly shortened. Boxes indicate exons and numbers over the box indicate exon number. Numbers inside the boxes indicate the number of nucleotides (Jirka et al., 2015; Matsuo et al., 2016) (Reprinted from Brain & Development, 38, Matsuo et al., Contributions of Japanese patients to development of antisense therapy for DMD, Page 4–9, Copyright (2016), with permission from Elsevier).

### 5.2.3 Transcription activation

In addition to the described single stranded (ss) oligo nucleic acids also activities specifically of double stranded (ds) RNA and DNA oligos have been found to be useful. Specifically for dsRNA transcriptional activation of targeted genes is described, such activity is named RNAa (RNA activated transcription), the dsRNAs are also named saRNAs (small activating RNAs). dsRNAs activate gene expression by targeting noncoding regulatory regions in gene promoters. Mechanistically, the dsRNA induced gene activation requires the Argonaute 2 (Ago2) protein and is associated with a loss of lysine-9 methylation on histone 3 at dsRNA-target sites, which results in a relatively long lasting activating effect. (Li et al., 2014) saRNAs are described to work via an onsite mechanism by binding to target genomic promoter DNA (see fig below), which is different from the RNA binding mechanism of RNAi (Meng et al., 2016). However, the described mechanism via promotor transcripts seems equally feasible (Kalantari et al., 2016). Nevertheless, Argonaute proteins are also here involved and target binding is in a seed-region-dependent manner, reminiscent of miRNA-like target recognition. These findings reveal a more diverse role for small RNA molecules in the regulation of gene expression than previously recognized and identify a potential therapeutic use for dsRNA in targeted gene activation.

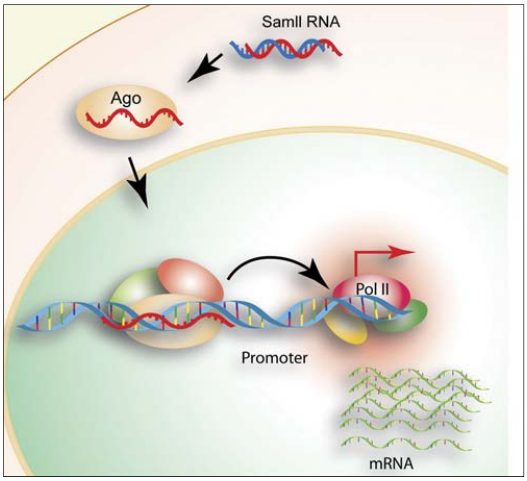



**Figure 19:** RNAa by promoter-binding of a saRNA/Argonaute complex. (Reprinted by permission granted by prof. Long-Cheng Li, MD, Laboratory of Molecular Medicine, Peking Union Medical College Hospital Chinese Academy of Medical Sciences, Beijing, China: http://www.urogene.org/index.html).

### 5.2.4 Decoy sequences

To induce alterations in gene expression to correct disease pathogenesis, transcription factors and other regulators of gene expression have become an increasingly attractive target for potential therapeutic intervention. Transcription factors are generally nuclear proteins that play a critical role in gene regulation and can exert either a positive or negative effect on gene expression. These regulatory proteins may bind specific sequences found in the promoter regions of their target genes. These binding sequences are generally 6–10 bp in length and are occasionally found in multiple iterations. Because transcription factors can recognize their relatively short binding sequences even in the absence of surrounding genomic DNA, oligodeoxynucleotides (ODNs) bearing the consensus binding sequence of a specific transcription factor have been explored as tools for manipulating gene expression in living cells. This strategy involves the intracellular delivery of such "decoy" ODNs, which are then recognized and bound by the target factor. A therapeutic target selected for such treatment was human bypass vein graft failure, a process characterized by neointimal hyperplasia and accelerated atherosclerosis, with long-term failure rates that approach 50% (Mann and Dzau, 2000). Autologous vein grafts are the most widely used for surgical revascularization in patients who suffer from occlusive disease of the coronary or lower extremity circulations. Treatment with decoys targeting the E2F transcription factor can prevent Neointimal Hyperplasia. (see figures below)

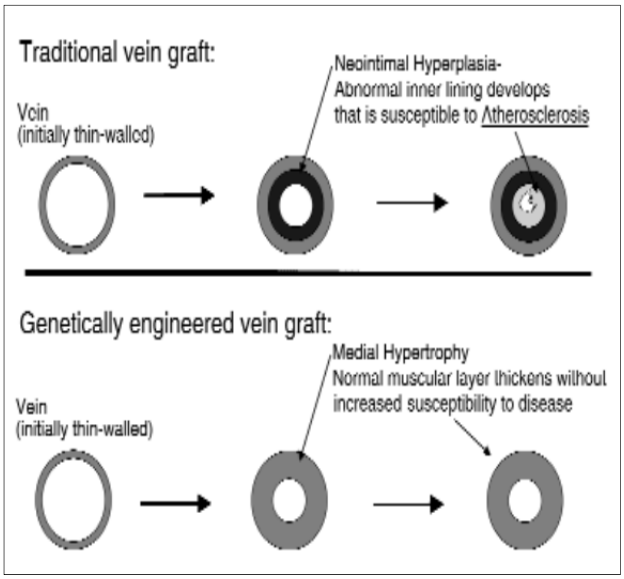



**Figure 20:** Vein grafts are initially thin-walled vessels that must undergo wall thickening to resist increased stress in the arterial circulation. The neointimal hyperplasia that produces this thickening, however, involves the proliferation of activated smooth muscle cells that create a substrate for accelerated atherosclerosis and subsequent graft occlusion. Blocking neointimal hyperplasia, as done using an E2F decoy oligonucleotide, induces an adaptive hypertrophic thickening of the medial layer of the vessel, yielding hemodynamic stability without increased susceptibility to atherosclerotic disease (Reprinted by permission from American Society for Clinical Investigation: Journal of

Clinical Investigation (Mann and Dzau, 2000), Copyright (2000)).

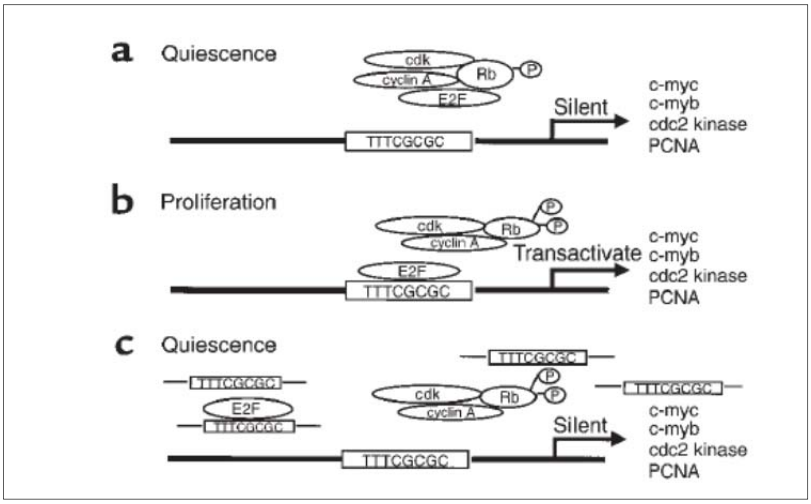



**Figure 21:** The decoy oligonucleotide approach to block the function of the transcription factor E2F. In quiescent cells (a), the factor is sequestered in a protein complex. During cell cycle progression (b), the complex is phosphorylated and free E2F is released. The factor binds to its consensus binding sequence in the promoter regions of multiple cell cycle regulatory genes. The introduction into the nucleus of decoy oligonucleotides that bear the consensus binding sequence (c) prevents interaction of the factor with its promoter targets, thus inhibiting the upregulation of cell cycle genes and blocking proliferation (Reprinted by permission from American Society for Clinical Investigation: Journal of Clinical Investigation (Mann and Dzau, 2000), Copyright (2000)).

A different type of decoy is represented by artificial miRNA-binding RNA transcripts designed to sequester and thereby inhibit specific miRNAs (see figure 22). Such miRNA decoys could provide an inexpensive alternative to proprietary oligonucleotide chemistries and delivery formulations, enabling research laboratories to examine the consequence of inhibiting each known miRNA in any particular model. Interestingly, miRNA 'decoys' or 'sponges', are found to be naturally expressed (Ebert and Sharp, 2010).Therapeutic applications for miRNA sponges are found in cases where families of miRNA's need to be inhibited, e.g. the design of a miRNA sponge for the miR-17 miRNA family as a therapeutic strategy against vulvar carcinoma (de Melo Maia et al., 2015; Stenvang et al., 2012)

Specific adaptations of the miRNA sponge concept allow longer-term inhibition of miRNAs; for the TuD RNAs (tough decoy RNAs), the binding site is perfectly complementary to the miRNA, but contains four nucleotides inserted at the site of Ago2 cleavage to prevent the TuD RNA from being inactivated. (Broderick and Zamore, 2011).

### 5.2.5 miRNA Masking

In contrast to miRNA sponges, miR-masks consist of single-stranded modified antisense oligonucleotides fully complementary to predicted miRNA binding sites in the 3'-UTR of a specific target mRNA (see Figure 22). Although unwanted effects or off-target effects can be dramatically reduced with this approach, this may be a disadvantage for cancer therapy for which the targeting of multiple pathways might be desirable. The miR-mask is able to cover up the access of the miRNA to its binding site on the target mRNA, so as to impair its inhibitory function and provide an additional system to interfere with unwanted miRNA activity (Iorio and Croce, 2012).

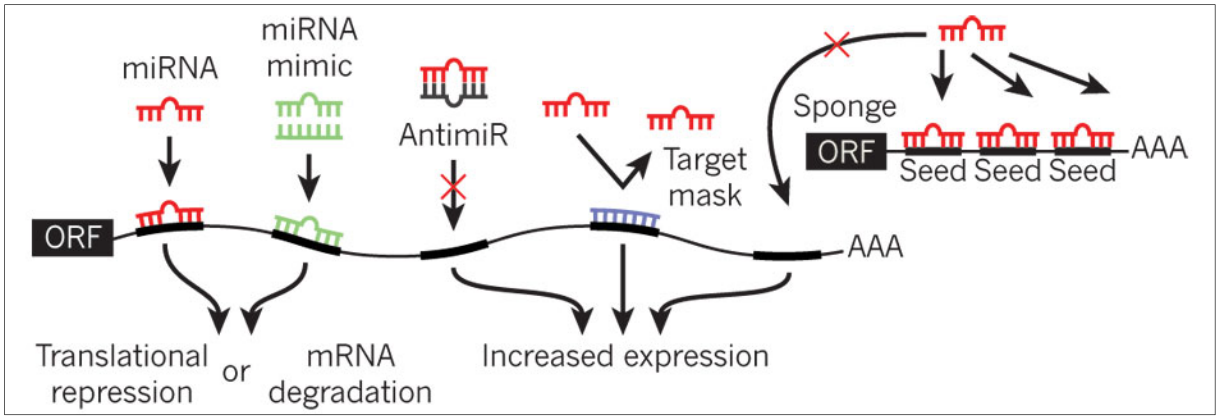



**Figure 22:**Depicted are mRNA inhibition by miRNA activity (miRNA and miRNA mimic) and three mechanisms for therapeutic interference with unwanted miRNA activity; by antimiR (anti sense oligos targeted at de miRNA seed sequence), by masking (anti sense oligos that block miRNA target sites) and by miRNA sponges/decoys (artificial RNA with tandem repeats of the miRNA target sequence) (Reprinted by permission from Macmillan Publishers Ltd: Nature (Small and Olson, 2011), Copyright (2011)) (see above and also chapter 4 for therapeutic examples).

## 5.3 Host effects

### 5.3.1 Anticipated effects

Host effects of (Antisense) oligonucleotides based therapies may often be very similar to those of siRNAs and miRNAs when intended to (down)regulate the expression of undesired (ectopic expression) or mutated genes, or correct under- or overexpression of normal genes. The mode of action can be the same and involve the same factors when it is intended to inhibit of translation or interfere directly or indirectly with transcription of targeted genes. Like for siRNAs and miRNAs in principle (Antisense) oligonucleotides based therapies do not change the genetic DNA code of the treated patient, however, epigenetic modification is very well possible and epigenetic changes that are transmitted though the germline have been described (Slatkin, 2009) (Lim and Brunet, 2013).

### 5.3.2 Unintended effects

Specifically for DNA oligo's it is theoretically possible that they occasionally would be incorporated into somatic cellular genomes. The possibility of germline changes is expected to be negligible due to the physiological barriers that exist between the blood stream and the Primordial germ cells; the blood–testis barrier and the low number and relatively inactive (non-proliferative) state of primitive ovum. Additionally in ovaries, blood in the capillaries is separated from the developing oocyte by a basal lamina, but no literature is found with a clear conclusion on possibility of germline changes. However, for DNA oligo's with stabilizing modifications such as backbone modifications incorporation into genome sequences appears biochemically impossible.
As described for siRNAs modified antisense oligonucleotides are in most cases designed to target a specific sequence, nevertheless antisense oligonucleotides may affect unintended, unpredicted targets, resulting in off-target effects. Additionally, both single stranded- and double stranded oligonucleotides can cause immune responses mediated by receptors of the innate immune system. Immune-stimulatory sequence motifs should be avoided to reduce the immunogenicity. As the rules of oligonucleotide immune activation are still poorly understood, all therapeutic oligonucleotides must be carefully tested for any possible immunostimulatory adverse effects.

## 5.4 Application areas

As (Antisense) oligonucleotides based therapies mechanistically largely overlap with siRNAs and miRNAs based therapies and have applications even in addition to these, their potential as therapeutic agents is also huge. Like siRNAs and miRNAs, (Antisense) oligonucleotides can regulate the expression of virtually all genes and their mRNA transcripts and open up a whole new therapeutic approach for the treatment of diseases by targeting genes that are involved causally in the pathological process. The development of (Antisense) oligonucleotides based therapies therefore heavily depends on pathological knowledge obtained with miRNA Research. The use of modified (Antisense) oligonucleotides instead of RNA molecules could have big advantages such as improved stability, specificity and targeting. The nucleotide modification technology developed for (Antisense) oligonucleotide based therapies can be seen as a delivery technology that can be used for siRNAs and miRNAs based therapies as well.

## 5.5 Barriers and Drivers

### 5.5.1 Technical Barriers

The high mechanistic overlap between RNA and modified oligo technologies is illustrated by the fact that only one example of a modified (antisense) oligo nucleotide based therapy is described that has definitively no relation to siRNA and miRNA therapeutics; the transcription factor decoy therapy. But even in that case a siRNA or miRNA based inhibition of a transcription factor is very well possible as an alternative approach.

siRNA therapies generally involve the introduction of a synthetic siRNA molecule into the target cells while miRNA-based therapeutics implicate two approaches: miRNA inhibition and miRNA replacement. Modified (antisense) oligonucleotides in general provide an approach to overcome the delivery hurdles associated with therapeutically used RNA molecules, as described in 5.1. full length miRNAs have to be processed to become active and any (backbone) modification of miRNA is likely to interfere with the necessary processing, however even for synthetic double-stranded miRNA mimics stabilizing approaches are available (see 5.1), and necessary chemical processes seem therefore no barriers for modified (antisense) oligo nucleotide based therapies, except for maybe the involved cost factor. Depending on the modifications applied an oligonucleotide might be able to reach the target as ‘naked’ oligo or by applying the therapeutic molecule in combination with an additional delivery system (see chapter 6). Viruses or plasmids are not feasible as delivery system for modified oligonucleotides as the chemical modifications cannot be encoded.

Due to the high mechanistical overlap the described RNA and modified oligo nucleotide technologies are driving each other, and important drivers as described for siRNA and miRNA therapeutics are important for modified (antisense) oligo nucleotides as well. Except for the molecular stability and delivery issues that are specific for natural siRNA and miRNA molecules most drivers and barriers listed under 4.4 in table 6 are therefore considered applicable for Modified (antisense) oligo nucleotide based therapeutics as well.

The most important general drivers behind genetic engineering therapies, and therefore also for modified (antisense) oligo nucleotides, are without doubt the novel fast and high throughput sequencing and detection technologies that enable elucidation of molecular disease mechanisms and fast individual diagnoses.

### 5.5.2 Socio-ethical Barriers and Drivers

Due to the high mechanistical overlap with miRNA and siRNA based therapeutics the socio-ethical drivers and barriers listed under 4.4.2 are considered applicable for Modified (antisense) oligo nucleotide based therapeutics as well, please refer therefore to this section for further information.

### 5.5.3 Patent Situation

Another factor important for most innovative therapeutic applications is the patent situation for relevant therapeutic approaches and production methods. The general patent situation for genetic engineering therapies as described under 4.4.3 is considered relevant for modified oligonucleotide technologies as well. A relevant review of the patent situation for modified oligo nucleotide technologies is available (Santiago Grijalvo, 2014), but the impact analysis of the patent situation can only be done by patent experts and is therefore considered out of scope here.

## 5.6 At the horizon

Modified (antisense) oligo nucleotide based therapeutics are studied for the treatment of various human diseases including cancers, viral infections, ocular conditions, genetic disorders, and cardiovascular diseases. The first generation licensed RNAi drugs, Pegabtanib (anti VEGF, wet macular degeneration), Fomivirsen (anti cytomegalovirus) and Mipomersen (anti Apolipoprotein B, cholesterol reducing) are actually modified (antisense) oligo nucleotides administered to elicit an inhibiting effect on their mRNA targets. In addition to therapeutic RNAi applications the development of modified (antisense) oligo nucleotides with other therapeutic activities, e.g. exon skipping, is expected on a somewhat longer term. An ongoing developments overview is listed in the table below and provides some insight on what treatments could be expected to enter the market in the near future.

| Therapies | Topic | Delivery system | Application method | Phase |
|---|---|---|---|---|
| EGFR Antisense | Carcinoma, Squamous Cell | Naked oligonucleotide | intratumoral EGFR antisense DNA | Phase 1<br>Phase 2 |
| IGF-1R Antisense | Malignant Glioma | 10 diffusion chambers | implantation in the rectus | Phase 1 |
| Transthyretin Antisense | Amyloidosis | Naked oligonucleotide | Subcutaneous injection | Phase 2 |
| TGF-β2 antisense | Glaucoma | Naked oligonucleotide | Intravitreal injection | Phase 1 |
| STAT3 Antisense | Ovarian Cancer | Naked oligonucleotide | Systemic delivery | Phase 2 |
| L-Grb-2 Antisense | Leukemia | liposomal | injection | Phase 1 |
| QR-010 antisense | Cystic Fibrosis | isoosmolar solution | intranasal administration | Phase 1<br>Phase 2 |
| Hsp27 antisense | Lung Cancer | Naked oligonucleotide | Intravenous injection | Phase 2 |
| GATA-3 antisense | Colitis, Ulcerative | Naked oligonucleotide | Intrarectally Applied | Phase 1<br>Phase 2 |
| Exon skipping ologonucleotide | Duchenne Muscular Dystrophy | Naked oligonucleotide | Subcutaneous injection | Phase 1<br>Phase 2 |

**Table 9** Summary of oligonucleotides based ongoing trials registered with clinicaltrials.gov, last accessed in March 2016.

# 6 Delivery Systems

## 6.1 Introduction

Many of the described technologies and their future development will depend on efficient delivery systems. In the field of gene delivery several developments should be taken into consideration to anticipate on the future perspective. There are different viral and nonviral vectors for gene delivery, but all gene therapy applications depend on the fact that the genetic material needs to be delivered across the cell membrane and ultimately to the cell nucleus. Therapeutic genome editing can be achieved by either *ex vivo* or *in vivo* modification. *Ex vivo* mode allows the target cell population to be manipulated with a wide range of delivery platforms such as, electroporation, cationic lipids, cell penetrating peptides, carbon nanowires and viral vectors. *In vivo* genome editing involves direct delivery of genetic material to diseased cells in the body. To date, clinical *in vivo* editing has largely been achieved through the use of viral vectors in limited types of organs such as the liver, muscle and eye. Major barriers for *in vivo* viral delivery systems include the immune response that may be raised in response to the large amounts of virus necessary for treatment, and difficulties in controlling distribution of viruses and dosage of genetic materials (Cox et al., 2015).

## 6.2 Viral vectors

In fact, around 70% of gene therapy clinical trials carried out so far have used modified viruses amongst which are retroviruses, lentiviruses, adenoviruses and adeno-associated viruses (AAVs) to deliver genes (Yin et al., 2014). Although they have substantially advanced the field of gene therapy, several limitations are associated with viral vectors, including carcinogenesis, immunogenicity, tropism, limited DNA packaging capacity and difficulty of vector production (Yin et al., 2014). Advantages and disadvantages of these major four types of viral vectors are summarized in Table 12. In general, lentiviral vectors are widely used in *ex vivo* modification. For *in vivo* applications, the most promising delivery system are AVV vectors, which have demonstrated high delivery efficacy for a variety of tissue types and recently been approved for clinical use, but are limited by their cargo capacity (Cox et al., 2015). An overview of vectors applied in gene therapy (Figure 23) shows a trend of slightly reduced popularity of viral vectors: from 68 % in 2008 to 66% in 2015. Considering majority (78 %) of undergoing clinical trials are still in early stage (phase I and phase I/II) (http://www.abedia.com/wiley/phases.php), it is likely that within the next 5-10 years viral vectors will still be the most applied delivery systems. Nevertheless, it is interesting that this overview (Figure 23) also reveals the increase in other vectors applied in clinical trials during the last 8 years.

| | Retroviral vectors | Lentiviral vectors | HSV vectors | Adenoviral vectors | AAV vectors |
|---|---|---|---|---|---|
| **Advantage** | Effective over long periods; efficient transfection. | Can mediate gene transfer to both dividing and non-dividing cells; reduced oncogenic potential | Large packaging capacity; effective on many cell types, safe for immuno-compromised patients. | Mediate gene transfer to both dividing and non-dividing cells; can be manufactured at high titers. | Can mediate gene transfer to both dividing and non-dividing cells; can exist stably in an episomal state with a low rate of genomic integration; exhibit no pathogenicity or cytotoxicity; very mild immunogenicity; can be manufactured at high titers |
| **Disadvantage** | Small packaging capacity; cannot be used for gene transfer to non-dividing cells; safety concerns regarding proviral integration | Small packaging capacity | Difficult to produce in large quantities | Small packaging capacity; strong innate immune response and continuous secondary immune response | Small packaging capacity; drive constitutive expression |
| **Application** | *ex vivo* gene therapy | *ex vivo* gene therapy | *in vivo* gene therapy | *in vivo* gene therapy | *in vivo* gene therapy |
| **Advances** | | Delivery of ZFN and CRISRP/Cas9 systems in human cells | | Delivery of TALEN transgenes *in vitro* | Delivery of ZFN, CRISRP/Cas9 system in mouse models and human cells |

**Table 10** . Comparison of currently commonly used viral vectors in gene therapy. AAV: Adeno-Associated Virus; HSV: Herpes Simplex Virus (Choudhury et al., 2016) (Jo et al., 2015) (Edwards 2014) (LaFountaine et al., 2015).

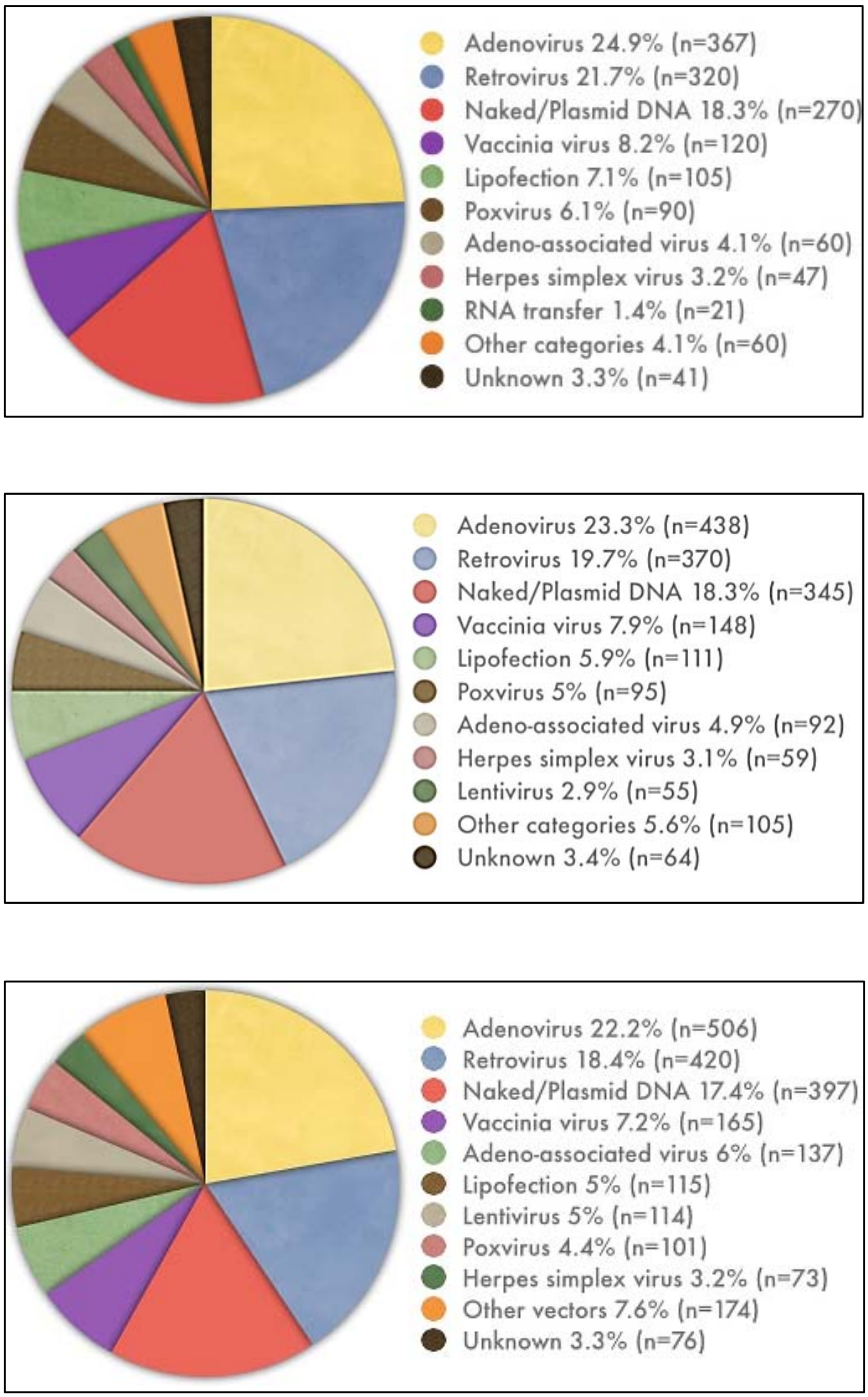



**Figure 23**. Trends in delivery vectors applied in gene therapy clinical trials worldwide. (A) Overview in 2008; (B) Overview in 2012; (C) Overview in 2015 (Adapted by permission from John Wiley & Sons, Inc, Copyright (2016): www.wiley.co.uk/genmed/clinical).

## 6.3 Nonviral vectors

Although viral vectors are current major delivery technologies considering their higher efficiency compared to nonviral vectors, the development of nonviral- vectors is attractive because of advantages such as lower immunogenicity and toxicity increased genetic loading capacities and fairly simple manufacturing processes (Valsalakumari et al., 2013). Especially for delivery of therapeutic RNA molecules, nonviral vectors are considered more attractive (described in Chapter 4 in this report).

Nonviral approaches were developed to facilitate transfer of exogenous genes into target cells without the complication of immunogenicity or insertion mutation commonly seen in viral vectors. These methods differ widely in their transfection efficiency and toxicity. In the past few years, the work continued in developing new nonviral methods, particularly in the area of chemical vectors. Many nonviral systems have been developed for delivery of genetic material, including the injection of naked DNA alone or in combination with physical methods such as gene gun, electroporation, hydrodynamic delivery, sonoporation and magnetofection. These techniques are generally less applicable to systemic gene delivery in humans than in small animals such as mice. Therefore, a range of synthetic delivery vectors has also been developed, including lipids and liposomes, polymers (linear and branched polymers, dendrimers and polysaccharides), polymersomes and inorganic nanoparticles (Yin et al., 2014). As a whole, the transfection efficiency reported so far for the nonviral approaches is still below that of the highly efficient viral vectors. Further improvements to increase the efficiency and reduce the toxicity of nonviral vectors are needed before their clinical implication can be met. These improvements will rely on a better understanding of the limiting steps that nonviral vector must overcome. Developing new vectors that are more target-specific will also be necessary. The strategies that merge nonviral and viral vectors might be helpful to achieve more, efficient, long-lasting, and nontoxic gene delivery systems (Al-Dosari and Gao, 2009; Ramamoorth and Narvekar, 2015) .

### 6.3.1 Cationic lipids and liposomes

Lipid-based vectors are most widely used nonviral delivery systems. Cationic lipids with three structural components (a cationic head group, a hydrophobic tail and a linking group between these domains) are capable of delivering DNA to various mammalian cell lines. Commonly used cationic lipids include DOTMA, DOSPA, DOTAP, DMRIE and DC-cholesterol. Neutral lipids, such as the fusogenic phospholipid DOPE or the membrane component cholesterol, are introduced to the formulations as “helper lipids” to enhance transfection activity and vector stability. Limitations of cationic lipids include low efficacy due to poor stability and rapid clearance, as well as the generation of inflammatory or anti-inflammatory responses (Yin et al., 2014).

In general, cationic lipids have the advantages of being inexpensive to produce and can be engineered to have targeted specificity. However, their transfection efficiency needs to be further improved, and the significant toxicities such as formation of aggregates in blood and the tendency to induce inflammatory response have to be solved for *in vivo* application. As of March 2009, lipoplexes have been used in clinical trials. There have been successful examples using cationic liposomes to delivery ZFN plasmids *in vitro*. However, no success of *in vivo* delivery of ZFN plasmids by nonviral vectors has been reported (LaFountaine et al., 2015).

### 6.3.2 Cationic polymers and polymersomes

Cationic polymers have also been used extensively for gene transfer. Upon mixing with DNA, these polymers form nanosized complexes, often called polyplexes. Among cationic polymers, PEI is considered one of the most effective polymer-based transfection agents. Upon systemic administration, these polyplexes of small particle size tend to aggregate to form larger complexes and accumulate in major tissues including lung and liver. Recently, more polymers with improved

biocompatibility and biodegradability have been reported demonstrating equal or superior performance comparing to nondegradable PEIs. Among these are aminoesters or oligoamines polymerized through disulfide linkages or polyamino acid derivatives with proton absorption capacities. Besides PEI and more recent polyamines of varied structures, synthetic or natural polypeptides and their derivatives have been explored as gene delivery vehicles. These include poly (l-lysine) (PLL), polyornithine, polyarginine, histones, and protamines that have excellent ability to condense DNA.
Polyplexes have been investigated in clinical trials. Other polymers such as dendromers, chitosans, synthetic amino derivatives of dextran, and cationic acrylic polymers have been shown to possess significant levels of gene transfer activity (Yin et al., 2014). TALEN plasmids complexed with cationic polymers were found to be therapeutically efficacious when delivered *in vivo* (LaFountaine et al., 2015).

### 6.3.3 Inorganic Nanoparticles

Inorganic nanoparticles are usually prepared from metals (e.g., iron, gold, silver), inorganic salts, or ceramics (e.g., phosphate or carbonate salts of calcium, magnesium, or silicon). The metal ion-based salts produce complexes with typical size range of 10–100 nm in diameter. The surface of these nanoparticles can be coated to facilitate DNA binding or targeted gene delivery. The small particle size offers several advantages including that they usually bypass most of the physiological and cellular barriers and produce higher gene expression. They can also be transported through the cellular membranes via specific membrane receptor or nucleolin which delivers nanoparticles directly to the nucleus skipping the endosomal–lysosomal degradation. Nanoparticles have the ability to efficiently transfect postmitotic cells *in vivo* and *in vitro*. Additionally, they tend to show no or low toxicity and are inert to immune responses (Al-Dosari and Gao, 2009). Magnetic nanoparticles (supermagnetic iron oxide mostly magnetite), fullerenes (soluble carbon molecules), carbon nanotubes (cylindrical fullerenes), quantum dots (semi conduction nanomaterial) and supramolecular systems all claimed some promising result in *in vitro* and animal models. Still studies require on long-term safety, surface functionalization effect of type, size, and shape on transfection efficiency to accelerate their clinical application (Ramamoorth and Narvekar, 2015).

## 6.4 At the horizon

Although most of currently undergoing clinical trials utilize viral vectors and the development of viral vectors has substantially advanced gene-delivery technology, their inherent shortcomings (limited DNA packaging capacity, complex production processes, broad tropism, cytotoxicity, immunogenicity, and tumorigenicity leave nonviral vectors a great challenge and potential to address many of these issues. Recent advances in material science, nucleic acid chemistry, and nanobiotechnology largely facilitate the development of nonviral vectors (Zhang et al., 2012). Many nonviral vector systems have successfully entered clinical trial stage (Table 11) (Yin et al., 2014). More specifically, cationic lipids and polymers are mainly used in DNA delivery while nanoparticles are more commonly seen in miRNA delivery.

| | Vector | Delivered genetic material | Clinical trial (number of cases) |
|---|---|---|---|
| **Lipids** | DOTAP–cholesterol | DNA | 2 |
| | GAP-DMORIE–DPyPE | DNA | 1 |
| | GL67A–DOPE–DMPE–PEG | DNA | 1 |
| | PEI | DNA | 5 |
| | PEG–PEI–cholesterol | DNA | 4 |
| | PEI–mannose–dextrose | DNA | 1 |
| | Poloxamer CRL1005–benzalkonium chloride | DNA | 3 |
| **Naked RNA** | Naked siRNA | siRNA | 16 |
| **Nano particles** | Lipid-based nanoparticles | siRNA | 9 |
| | CDP-based nanoparticle | siRNA | 1 |
| **Polymers** | Dynamic Poly-conjugate | siRNA | 2 |
| | siRNA–GalNAc conjugate | siRNA | 2 |
| | LODERpolymer | siRNA | 1 |

**Table 11**. Nonviral vectors in undergoing US clinical trials PEG: polyethylene glycol; PEI: polyethylenimine; CDP: cyclodextrin polymer; GalNAc: N-acetylgalactosamine (Yin et al., 2014).

# 7 Overall Discussion and conclusion

In this report an inventory of new developments with respect to new molecular genetic techniques applied in red biotechnology is presented. This report does not primarily focus on gene therapy applications but on the molecular genetic techniques that can be applied in red biotechnology in order to ultimately affect disease related gene expression. Trending themes within molecular medicine can be captured by genomics based medicine, epigenetics, nanomedicine, personalized medicine and synthetic biology and it will be genetic engineering techniques that facilitate and enable the development of these themes. The technology areas that have been identified are: genome and epigenome editing, gene expression regulation and gene delivery. The technologies identified are CRISPR/Cas9 (Engineered nuclease), TALENs (Engineered nuclease), ZNF (Engineered nuclease), siRNA and miRNA, Antisense Oligonucleotides (ASOs), (modified) nano particles and (modified) viruses.

The progress in development of new molecular genetic techniques applied in red biotechnology has been impacted by adverse event reports with innovational molecular medicine especially in pioneering phases. In 1999, the first person was publicly identified as having died in a clinical trial of gene therapy. This patient was injected with an adenoviral vector carrying a corrected ornithine transcabamylase gene and died due to a massive immune response. In 2000 trials were stopped with a gene therapy in SCID patients when it was discovered that two of ten patients in one trial had developed leukemia resulting from the insertion of the gene-carrying retrovirus near an oncogene. In 2007, four of the ten patients had developed leukemias. These cases were severe setbacks for the new gene therapy field. However, progress did not stop and the improvements have resulted recently (2016) in the product Strimvelis, which is indicated for the treatment of patients with severe combined immunodeficiency due to adenosine deaminase deficiency (ADA-SCID). The therapy involves removing stem cells from a patient's bone marrow followed by the transduction of these stem cells using a retroviral vector encoding the human ADA DNA. The defective ADA gene in these patients is consequently restored. This is remarkable as the in 2001-2007 reported leukemia's were adverse events of a related vector system aimed to treat X-linked SCID. Improvements in the vector system preventing oncogenic genome integration are key to the current success.

Fairly recent discoveries have resulted in novel precise approaches to edit the mammalian genome and additionally revealed new RNA related mechanisms to regulated genome expression, which can be used for a novel class of molecular medicines that potentially cover a very wide area of common multifactorial diseases in humans. The novel therapeutic targets allow for direct and sustained interference with disease related gene expression without changing the endogenous sequences of the genome itself. Some ethical and safety concerns of changing genome sequences are herewith circumvented and a clear paradigm shift from gene repair and replacement to gene regulation can be observed.

Nevertheless some concern remains related to the transgenerational effects of medical treatments in general and specifically for treatments that strongly affect gene expression. New insights in epigenetic mechanisms revealed a new high speed evolution system independent of random DNA changes: epigenetic evolution by chromatin modifications, such as acetylation and methylation, in response to environmental changes including medical treatments and even psychological experiences, which are transmitted between generations. Via epigenetic evolution medical treatments can affect offspring in unexpected ways as has been found for diethylstilbestrol treatment that causes cancer and reproductive problems in at least two generations daughters. For novel treatments involving siRNA or miRNA it is known that they can cause epigenetic change and may be even intended to do so, for instance when the treatments is against cancer. For many cancers epigenetic dysregulation is found to play an important role during oncogenesis. In addition to this

new general concern related to transgenerational effects several more specific barriers for new molecular genetic therapies have been identified, e.g. the rapid degradation of RNA and DNA and the immunogenicity of such molecules. An overview of barriers, drivers and horizons for emerging gene expression and gene expression regulation technologies in medical biotechnology is provided in Table 12.

Two main groups of technologies are discussed in this report: 1) The endonucleases, Zinc Finger Nucleases (ZFNs), TALENs and CRISPR/Cas9 that provide new tools for precise deletions and editing specific bits of DNA/RNA and 2) technologies related to small noncoding RNAs (ncRNAs), Micro RNAs (miRNA), Small Interfering RNAs (siRNA) and modified (antisense) oligonucleotides that intend to have an effect on gene expression. Both groups converge in the search for delivery systems that protect the nucleotides, target their activity to the right cell population and additionally facilitate cell entry. Interestingly the two groups also converge in their targeted cellular mechanisms as small oligonucleotides are being used to repair or neutralize gene defects and engineered endonucleases are used to target miRNAs or promote gene transcription. This converging is not that surprising as the basic principle is the same for these tools; targeting of enzyme activity to a specific sequence. The finding that chromatin activity depends on non-coding DNA as well as non-coding RNA has freed the way for these novel therapeutic approaches that are suitable for human use as well as veterinary use. However due to sequence differences between species exchangeability of these novel therapeutics is likely to be limited. It should be noted that the techniques described and discussed in the report can also be used in several other application fields, such as veterinary medicine, pesticides and diagnostic biosensors.

The most attractive aspect of the novel therapeutics described is their ability to target virtually any gene(s), which may not be possible with classical small molecules or protein-based drugs or even ‘classic’ genetic engineering techniques. While the therapeutic efficacy of these novel therapeutics has been successfully demonstrated *in vivo*, several technical barriers still need to be overcome in order for many clinical applications to be successful. The experience from antisense and gene therapy has contributed to the rapid progress of siRNAs and miRNAs into clinical studies. In particular, the technologies of chemical modification and delivery of nucleic acids developed previously can also be applied to both siRNAs and miRNAs. While the former possess a high specificity by targeting one single gene, the latter can target multiple related genes, often in the same cellular pathway or process, to generate pronounced therapeutic effect. Currently, the development of siRNAs is advancing ahead of miRNAs, with a larger number of candidates that have already entered clinical trials, possibly due to the more complex roles of miRNAs that require more research before progressing into therapeutic development. With the recent surge in intensive research concerning the therapeutic mechanisms and combinations of the new tools, it can be expected that significant advance will be made for their future role in therapeutics.

**Table 12**. Overview Barriers, Drivers and Horizons for Emerging Gene expression and Gene expression regulation technologies in medical biotechnology.

| Technique | Applications | Barriers | Drivers | Horizon Current | Horizon (1-5 years) | Horizon (6-10 years) |
|---|---|---|---|---|---|---|
| **Generally applicable to the red biotech field** | • Biomedical research and Molecular medicine in general | • Adverse event reporting with innovational molecular medicine especially in pioneering phases (e.g. the death of Jesse Gelsinger in 1999, the first person publicly identified as having died in a clinical trial for gene therapy, In 2000, a gene therapy "success" resulted in SCID patients with a functional immune system. These trials were stopped when it was discovered that two of ten patients in one trial had developed leukemia resulting from the insertion of the gene-carrying retrovirus near an oncogene. In 2007, four of the ten patients have developed leukemias.)<br>• Regulatory challenge and ethical concerns | • Sequencing the human genome<br>• Genomics development<br>• Synthetic Biology development<br>• Nano medicine development<br>• Market approval of innovational molecular medicine. Examples are approval of several gene and cell therapies.<br>• Governmental stimulation of personalized medicine (e.g. FDA and US government stimulating precision medicine initiative to accelerate biomedical research)<br>• Big Pharma and Biotech moving away from blockbuster development.<br>• Development of new advanced delivery systems<br>• Increased knowledge on relationship genetics and molecular disease mechanisms<br>• Development of suitable (animal) model systems<br>• Development stem cell technologies like IPCs<br>• Development genetically modified T cells | | | |
| **ZFN**<br><br>**ZFN** | • Generating genetically modified disease animal models<br>• Disease mechanism study<br>• Disease treatment (monogenic | Technological challenges<br>• Specificity (off-target effects due to the unspecific binding between target genome locus and protein binding domain of engineered nucleases;<br>• difficulty in editing non-dividing cells; | • Synthetic biology (increase the target specificity and decrease the size of engineered nucleases<br>• Detection technologies (for off-target identification in engineered nuclease-modified genome)<br>• Concentration optimization improving safety for example by | Currently most applications in laboratory and pre-clinical phases.<br><br>Use in IPCs<br><br>Development<br><br>First clinical trials are | More upcoming clinical trials<br><br>Marketing authorization applications | First generic or biosimilar products in development |

| Technique | Applications | Barriers | Drivers | Horizon Current | Horizon (1-5 years) | Horizon (6-10 years) |
|---|---|---|---|---|---|---|
| | disorders, cancers, infectious diseases) | Technology<br>• various efficiency in cell types with different chromatin confirmation;<br>• delivery efficiency by different vectors; delivery barrier due to the size of nucleases)<br><br>Toxicity<br>• Cytotoxic and genotoxic effects also due to off target effects<br>• High Dosage requirements may enhance toxicity<br><br>Delivery<br>• Efficiency of delivery systems may not be sufficient<br>• Large size of proteins especially for TALENS and CRISPR/Cas limits choice of delivery systems<br><br>Regulatory and Ethical<br>• Germline genome editing | reduced toxicity<br>• Development of systems for off target detection<br>• Protein engineering reducing the protein size for TALENS and CRISPR in order to increase delivery system options<br>• Chemical modification of sgRNA enhancing editing efficiency of CRISPR/Cas in human primary T cells and Hematopoietic stem cells<br>• Development of sequencing technologies identifying specificity | performed. | | |
| **TALEN** | | | | First clinical application has been reported but not in a clinical trial setting<br><br>Animal disease model development including those for multi genic diseases<br><br>Different delivery systems in development<br><br>Use in IPCs | Clinical trials | First marketing authorization applications |
| **CRISPR/Cas9** | | | | Animal disease model development including those for multi genic diseases<br><br>Different delivery systems in development<br><br>Development tools in loss of function (LOF) studies and genome scale LOF screens<br><br>Somatic gene correction studies in animal models<br><br>Use in IPCs<br><br>Development virus expression reduction strategies | First clinical trials | First marketing authorization applications |

| Technique | Applications | Barriers | Drivers | Horizon Current | Horizon (1-5 years) | Horizon (6-10 years) |
|---|---|---|---|---|---|---|
| **miRNA** | Disease related gene expression modification<br>Anti-viral drugs<br>Cellular reprogramming | Drug Delivery; how to get the intact product into the right cells at the right dose.<br>• Generally small RNAs are not efficiently taken up into target cells<br>• RNAs are rapidly degraded in circulation<br>• Rapid clearance via kidney or liver<br>• Low packaging capacity of current delivery systems<br>• Need for stable and targetable alternatives for delivery systems<br><br>Adverse events<br>• Off target effects<br>• Immunogenicity<br>• Incorporation into somatic cellular genomes (Antisense oligo's)<br>• Possible incorporation in genome sequences (very low probability) (Antisense oligo's)<br><br>Transgenerational effects; how to assess the risk related to hereditary epigenetic changes.<br><br>Duration of the therapeutic effect is unknown a limitation of the treatment effect may impact on treatment options and strategies<br><br>Epigenetic marks and alteration of epigenetic programming<br><br>Patents (can be drivers as well) | Disease screening technology (novel sequencing technology, detection technologies)<br><br>Synthetic biology (delivery systems, targeted nano sized particles)<br>• Encapsulation / binding by nanoparticles containing a targeting component<br>• Development of engineered delivery systems with increased packaging capacity<br><br>May provide treatment options for indications that are currently not possible to treat<br><br>Ability to target virtually any gene<br><br>Increased pathological knowledge obtained with miRNA research<br><br>Antisense oligo's have improved stability, specificity and targeting compared to other RNA molecules<br><br>(antisense) oligo's have better delivery options | | First clinical trials | First marketing authorization applications |
| **siRNA** | | | | Clinical studies ongoing mostly early phase I and II | First few products licensed<br><br>More upcoming marketing authorization applications | First generic or biosimilar products in development |
| **Modified Oligonucleotides** | | | | Clinical studies ongoing mostly early phase I and II | First few products licensed<br><br>More upcoming marketing authorization applications | First generic or biosimilar products in development |

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