Chapter 8
Controlled Release Technologies for RNAi Strategies in Regenerative Medicine

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Abstract The ultimate goal in siRNA formulation for regenerative application centers around a gradual and sustained local release of intact siRNA which is adapted to the physiological requirements of the specific tissue. Although various issues in optimizing the siRNA delivery systems in terms of safety and efficiency still need to be addressed, progress towards the potential translation of RNAi-based therapies to clinical use has been made. An increasing number of promising siRNA-based gene therapies is currently in, or advancing towards clinical trials. Looking back to a period of only 17 years since the discovery of RNAi (Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC, Nature 391(6669):806–811. doi:10.1038/35888, 1998) and the introduction of siRNAs as the underlying principle of RNAi (Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T, Nature 411(6836):494–498. doi:10.1038/35078107, 2001), it is likely that regenerative applications will be explored in clinical studies soon as well.

Keywords siRNA • Chemical modification • Cell targeting

Abbreviations

ACI	autologous chondrocyte implantation
Ago	argonaute protein
BCL2	B-cell lymphoma 2
BMSCs	bone marrow stromal cells
cbfa1	core binding factor alpha1

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CD	cyclodextrin
Chi	chitosan
Ckip-1	casein kinase 2-interacting protein-1
CPP	cell penetrating peptide
DOPE	1,2-dioleoyl-glycero-3-phosphoethanolamine
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
DOTMA	1,2-di-O-octadecenyl-3-trimethylammonium propane
ECs	endothelial cells
EEP	ethyl ethylene phosphate
EPR	enhanced permeability and retention
GNAS1	guanine nucleotide-binding protein alpha-Stimulating activity polypeptide1
HA	hyaluronic acid
hASCs	human adipose stromal cells
HIF-1	hypoxia-inducible factor1
HIV	human immunodeficiency virus
hMSCs	human mesenchymal stem cells
LbL	layer by layer
LSD1	lysine (K)-specific demethylase 1A
miRNA	micro RNA
NP	nanoparticles
PCL	poly-ε-caprolactone
PEC	polyelectrolyte complex
PEI	polyethylenimine
PEG	polyethylene glycol
PEMs	polyelectrolyte multilayers
PHD2	prolyl hydroxylase domain protein 2
PLGA	poly(lactic-co-glycolic acid)
PLA	poly(L-lactic acid)
PLK1	tumor survival genes polo-like kinase 1
PSMA	progressive spinal muscular atrophy
RANK	receptor activator of NF-κB
RNA	ribonucleic acid
RNAi	RNA interference
RSV	respiratory syncytial virus
sFIt-1	soluble fms-like tyrosine kinase-1
shRNA	short hairpin RNA
siRNA	small interfering RNA
TCP	β-tricalcium phosphate
TRIB2	tribbles homolog 2
VEGF	Vascular Endothelial Growth Factor

8.1 Introduction

In 1998, double-stranded RNA (dsRNA) molecules were described to efficiently and specifically interfere with the expression of genes in catalytic amounts (Fire et al. 1998). This revolutionary discovery earned Andrew Fire and Craig Mello the Nobel Prize in 2006. Since then, therapeutic RNA interference (RNAi) strategies have been considered as a promising approach for targeting any disease-related gene including otherwise undruggable targets.

RNAi is an endogenous process in the cells to control the gene expression through a reduced expression of undesired genes ('gene silencing'). This modulation is achieved either by cleavage of the mRNA and its subsequent degradation, or by inhibiting its translation. RNAi exerts its action through small interfering RNA (siRNA), microRNA (miRNA) or short hairpin RNA (shRNA), and can be also induced by their external delivery. Structurally, siRNA is a short double-stranded (ds) RNA of 20–25 bp in length and features a 2-nucleotide overhang at the 3′-end. Intracellularly, it is generated from longer dsRNA molecules through the action of the enzyme called Dicer. For the induction of RNAi, cells can be transfected either directly with the siRNA of choice, or with a DNA plasmid encoding for small hairpin RNA (shRNA). Upon transfection, the transcribed shRNA is then cleaved into siRNA by Dicer. Similar to siRNAs, miRNAs are small (21–25 bp), non-coding RNAs; however, in contrast to siRNA they are only partially complementary to their target mRNA molecules. Their mechanism of action comprises cleavage or degradation of mRNA. Independent of the nature of the small RNA molecule, i.e. siRNA or miRNA, their action relies on their incorporation into the endogenous RNA-induced silencing complex (RISC). The antisense strand of the siRNA duplex is incorporated as so-called guide strand into RISC and mediates, through Watson-Crick base pairing, the sequence-specific binding of RISC to its target mRNA. This brings the other RISC components such as the Argonaute (Ago) proteins into close proximity to the mRNA, leading to the subsequent mRNA cleavage by Ago. Due to its unprotected ends, the cleaved mRNA is then rapidly degraded by other nucleases. Beyond cleavage, RISC also plays an important cellular role by inhibiting endogenous mRNA translation via a related miRNA mechanism. The detailed mechanism of RNAi molecule is illustrated in Fig. 8.1 (Agrawal et al. 2003; Moore et al. 2010; Tebes and Kruk 2005).

Due to their sensitivity to nucleases, their highly negatively charged backbone and their considerable size (typically >13 kDa) (Agrawal et al. 2003), all of which contributing to poor bioavailability and cellular uptake, small RNA molecules are in principle unsuitable as therapeutic drugs. One strategy to overcome this barrier involves viral delivery vectors, which are highly efficient but may come with safety issues due to their risk of mutagenesis and immunogenicity (Pan et al. 2015; Levi et al. 2012). Alternatively, non-viral systems are explored for the formulation of DNA or small RNA molecules in nanoparticles. Nanoparticle formation may rely on, for example, ionic interaction with cationic polymers or cationic lipids, encapsulation into liposomes, or conjugation of the nucleic acid to a moiety capable of cellular entry. Since the discovery of RNAi, several clinical studies have explored

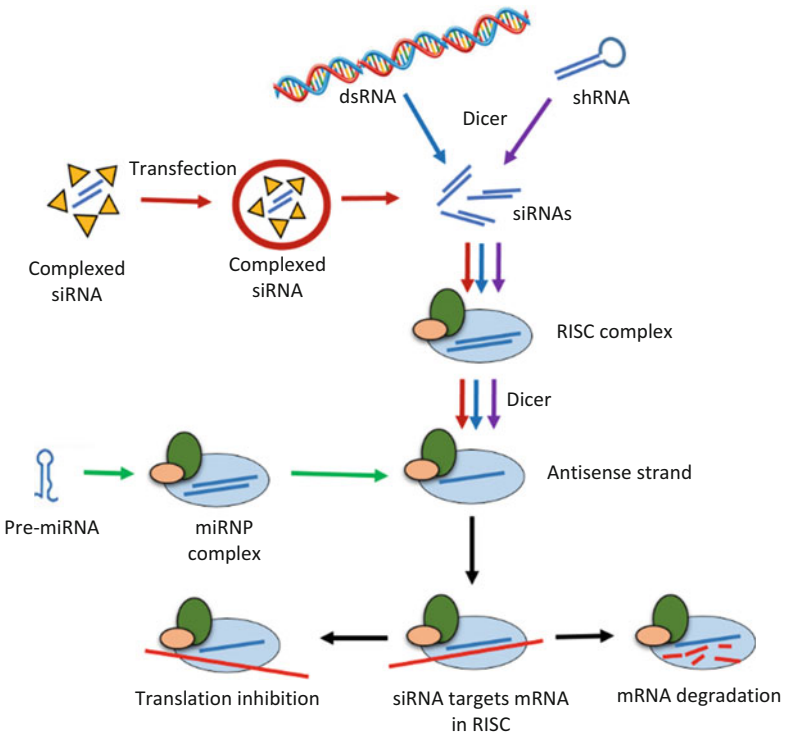

Fig. 8.1 RNAi mechanisms of shRNA, siRNA and miRNA in mammalian cells. *Purple and blue arrows*: Short hairpin RNA (shRNA) and double stranded RNA are processed by Dicer to form siRNAs in the cytoplasm. *Red arrows*: siRNA can also be delivered through transfection via endocytosis. The double stranded siRNA then complexes with argonate proteins to form the RISC complex. *Green arrows*: pre-miRNAs are processed into miRNAs which then complexes with argonaute proteins to form the miRNP complex. After elimination of sense strand from the double stranded RNAs, the antisense strand anneals to the target mRNA. *Black arrows* represent the last stages of the silencing process by siRNA, shRNA and miRNA; perfect or non-perfect match leads to mRNA degradation or inhibition of translation

its potential, with initially unsatisfactory results related to unwanted 'off-target effects' and/or issues regarding siRNA delivery (Kanasty et al. 2012). This indicates the requirement of further optimization of the delivery vectors, careful selection of the siRNAs and optimal application concepts. Nevertheless, several clinical studies are in progress (see below) (Kanasty et al. 2013).

The focus of this chapter will be on delivery systems and controlled release concepts for RNAi therapeutics and in particular on their use in regenerative medicine. Although RNAi-based therapies often target cancer due to the availability of attractive target molecules, there is an ever increasing number of approaches in other pathologies including regenerative medicine. In the latter case, these are often focussed on local delivery, that also allow for efficient delivery of siRNA to stem cells or other target cells for tissue engineering purposes. Thus, RNAi-based strategies

could for instance improve bone regeneration through targeting genes that have been recognized to negatively affect bone formation or stimulate bone resorption (Novina and Sharp 2004).

8.2 Background and Principles

Despite concerns regarding systemically administered siRNA due to adverse effects on other non-target tissues (López-Fraga et al. 2009), certain diseases, including viral infections, require administration via the blood stream. Systemic applications include intravenous and subcutaneous injection, delivery through the skin, lung (Lam et al. 2012) or nasal mucosa (Kanazawa et al. 2014). The reluctance to systemic siRNA delivery originates from the risk of systemic toxicity, inefficient targeting to the desired tissues as well as the above mentioned unfavourable physicochemical properties of siRNA (Durcan et al. 2008). Likewise, the highly negative charge of naked siRNA leads to rapid blood clearance caused by the reticuloendothelial system (RES), hampering efficient systemic application of siRNA due to short circulation times (Watts and Corey 2010). The introduction of chemical modifications is one possible approach to modulate the poor pharmacokinetic properties of siRNA, increase its stability and selectivity and avoid non-specific or off-target effects. For example, modifications at the 2′-position such as O-methyl (2′-O-Me) and fluoro (2′-F) substituents have been introduced (Corey 2007) (see below for further discussion).

Other formulation concepts to overcome the problems of low stability against nucleases and poor membrane permeability aim at neutralizing or overcompensating the negative charge by complexation with cationic polymers or lipids that improve the interaction with the negatively charged cell membranes and increase cellular uptake. Another way to address the uptake problem, is to hide the negative charge by encapsulation in neutral polymer nanoparticles, e.g. biodegradable polyesters. Apart from optimizing complex stability and transfection efficiency, the carrier nanoparticles can be chemically modified with targeting moieties, e.g. antibodies or cell penetrating peptides in order to improve biocompatibility and/or enable targeted delivery to the respective tissues or cells.

Standard systemic administration concepts achieve a comparably short protein knockdown. Many therapeutic applications, however, require more prolonged effects and thus a controlled delivery strategy. Prolonged delivery can be achieved by nanoparticle incorporation into implants that slowly release the nucleic acids over time and allow for fine-tuning of the release profiles. Biomaterials that are designed for the local delivery of RNAi therapeutics often feature a hierarchical structure. This means that the nucleic acids that are typically formulated in nanoparticles for stabilization and charge control are further embedded in a micro- and/or macroscopic matrix, such as microparticles, fibers, macroporous scaffolds or even combinations of these systems. The macroscopic devices thereby serve as controlled release systems for siRNA nanoparticles as well as a physical support, providing a large attachment area and direct contact area for invading cells ready to support tissue formation.

8.3 Formulation Strategies for siRNA and Nanoparticulate Systems

8.3.1 Naked siRNA

Direct injection of naked siRNA to the site of administration without any complexation has been shown inefficient in several cases, due to enzymatic degradation resulting in low siRNA half-life and efficiency. However, in some organs, such as eye, brain, skin and heart, the delivery of naked siRNA was reported to be effective in gene knock-down (Pan et al. 2015).

8.3.2 Chemically Conjugated siRNA

siRNA can be subject to chemical conjugation with (bio-)molecules to improve physicochemical properties and enhance cellular uptake. For example, a study published by Zhu et al. showed that siRNA conjugated with galactose-PEG at the sense strand was taken up more efficiently by hepatocytes via the asialoglycoprotein receptor. Cleavage of the disulfide bond between PEG and siRNA in the reducing intracellular environment by, e.g., glutathione caused the siRNA to be released (Zhu and Mahato 2010).

8.3.3 Viral Vectors

Viral vectors, such as lentiviruses, adenovirus-associated vectors and retroviruses have been developed for the delivery of siRNA-encoding DNA. Among them, adenovirus-associated vectors and lentivirus are the most commonly used viral vectors, due to their comparatively general safety, low immunogenicity and proper transduction efficiencies. Various applications of viral siRNA delivery have been investigated. For instance, by knocking down the BMP-2 inhibitor Noggin with lentiviral techniques, Levi et al. could increase the osteogenic capability of hASCs (Levi et al. 2012). However, the risk of mutagenic effects as well as immunogenic and inflammatory responses in body organs discourages to the use of viral vectors in clinical studies (Pan et al. 2015).

8.3.4 Liposomal Delivery

Liposome-based siRNA delivery vehicles are among the most common non-viral gene nanocarriers. In such liposome nanoparticles, the siRNA molecules are encapsulated in an aqueous core surrounded by a phospholipid bilayer. Liposomes for nucleic

acid delivery can contain various neutral and/or positively charged lipids. However, cationic lipids are preferable and commonly used in commercially available transfection agents such as Lipofectamine®. Cationic lipids facilitate loading of the negatively charged nucleic acids into the liposomes and expedite uptake through the lipid bilayer into the cells as well as endosomal escape. This approach was first described by Felgner et al. (1987). Lipoplex formation is achieved by simply mixing the cationic liposomes with the anionic nucleic acids, leading to self-assembly of the liposome/siRNA complexes as a result of electrostatic interaction (Buyens et al. 2012).

Commonly used cationic lipids include DOTMA and DOTAP. However, the use of cationic lipids *in vivo* is limited due to the surface charge and relatively large size of the lipoplexes. Thus, rapid clearance, immunogenicity and toxic side effects are induced, among others, by nonspecific interactions with serum proteins (Filion and Phillips 1997; Lappalainen et al. 1994). Stabilization of the liposomes is often achieved by the use of neutral helper lipids such as DOPE or cholesterol, which enhance cellular uptake by tailoring the lipoplex composition to the lipid composition of the cell membrane, facilitating the formation of endocytic vesicles (Marchini and Howie 2010).

8.3.5 Polymeric, siRNA Loaded Nanoparticles

Tightly packed polymeric nanoparticles (NPs) are formed by electrostatic interactions between polycationic polymers and negatively charged siRNA. Packing of siRNA as compared to longer nucleic acid molecules like for example plasmid DNA is considered as more challenging because siRNA molecules are comparably short and thus rather rigid, which is why they cannot be wrapped around the nanoparticle and condensed in the same way (Xu and Anchordoquy 2011). The net charge of the resulting NP depends on the ratio of positive charges (mostly from cationic amines or imines) of the polymers (nitrogen; N) and negative charges of the siRNA (phosphate; P) and is referred to as N/P ratio. In order to ensure high complexation efficacy and improve cellular uptake, the N/P ratio has to be sufficiently high, thus leading to positively charged NP. Since the adjustment of the N/P ratio is crucial as it has a notable impact on complex stability and cellular uptake efficacy, these interactions with the specific polymer should be well characterized in advance. A large number of studies exist on cationic polymers such as poly(ethylene imine) (PEI).

Among the cationic polymers, PEI is considered as a gold standard. This is based on its high transfection efficiency and favourable endosomal release characteristics for cytosolic delivery of siRNA after endocytosis, which is the prevalent uptake route of polycationic delivery vectors. The endosomolytic properties of PEI are caused by its high buffering capacity due to the high number of basic amino groups, acting as proton acceptors. This provokes more protons to be pumped into the endocytic vesicles to keep the acidic pH characteristic of endosomes. Eventually, this leads to osmotic swelling and rupture of the lipid bilayer. The phenomenon is commonly referred to as the proton sponge effect (Behr 1997). Branched PEI can be synthesized

by ring opening polymerization of aziridine, while the synthesis of linear PEI relies on the hydrolysis of poly(2-ethyl-2-oxazoline). On the negative side, the high charge density may lead to significant cytotoxicity, which depends on the molecular weight and structure of the PEI. Branched PEI is usually more toxic than linear PEI due to the higher density of amino groups, which in turn makes it more favourable for nucleic acid packing. Likewise, higher molecular weight PEIs like 25 kDa linear PEI show higher transfection efficiencies but at the same time exert higher toxicity. Thus, defined molecular weights and degrees of branching are required. Certain PEIs like the linear jetPEI or the branched PEI F25-LMW (Werth et al. 2006), meet these criteria and have been extensively used *in vitro* and *in vivo* (Höbel and Aigner 2013). Another way to circumvent this biocompatibility issue offer biodegradable PEIs, which are cleaved into smaller fragments with low toxicity and higher availability for rapid body clearance. Biodegradability is achieved using cleavable linkers based on, e.g., esters, carbamates, ketal linkages or disulfide bridges (Breunig et al. 2008). Concomitantly, transfection is supported by facilitated unpacking of nucleic acids after decomposition of the polymer (Islam et al. 2014).

An alternative approach to improve systemic biocompatibility is the grafting of polyethylene glycol (PEG) chains onto the PEI polymer, which shield the positive charge of the nanoparticle, thus reducing unspecific ionic interactions. The shielding is increased with the PEG chain length and the degree of grafting. The latter inversely correlates with transfection efficiency, whereas this unfavourable effect is less pronounced for siRNA delivery as compared to DNA (Malek et al. 2008). Enhanced biocompatibility was also observed by grafting of other natural oligomers/polymers like for example the non-ligand oligosaccharide oligomaltose (Gutsch et al. 2013; Höbel et al. 2010).

A formulation of siRNA with a PEI derivative, namely Staramine – a functionalized lipopolyamine – showed high cellular uptake with low systemic absorption and side effects after instillation. Intravenous (i.v.) administration of Staramine/siRNA nanocomplexes modified with methoxy-PEG enabled efficient siRNA delivery to the lung in mice, with reduced gene knockdown in liver, spleen, and kidney (Polach et al. 2012).

PEI-containing lipopolyplexes have been synthesized in order to combine the favourable properties of lipid delivery systems such as high transfection efficiency and comparably low cytotoxicity with the excellent siRNA complexation and endosomal release characteristics of PEI (Schäfer et al. 2010). In addition, lipopolyplexes showed higher storage stability as compared to polyplexes and retained biological activity over a prolonged time even in the presence of serum (Ewe et al. 2014).

8.3.6 Cyclodextrins

Modified cationic cyclodextrins are a possible alternative for cationic polymers in siRNA delivery, since they feature comparably high transfection efficiency paired with relatively low cytotoxicity (Islam et al. 2014). Grafts of β-CD and PEI were significantly less toxic than unmodified PEI (Forrest et al. 2005).

8.3.7 Natural Cationic Polymers

Minakuchi et al. introduced siRNA delivery using atelocollagen, which is positively charged and derived from natural collagen type I by pepsin protease-treatment (Minakuchi et al. 2004). Atelocollagen is less immunogenic as compared to collagen. Another protein-based nanoparticle system was developed by Zimmer et al., who used naturally occurring, arginine-rich protamine to tightly pack siRNA via electrostatic complex formation (Scheicher et al. 2015).

8.3.8 Micelles

Spontaneously forming micelles of fairly complex composition were introduced by Nelson et al. Micelles formed from a raft polymerized diblock-copolymer which consisted of a siRNA complexing hydrophilic block of polymerized dimethylaminoethylmethacrylate (DMAEMA) and a pH responsive, hydrophobic block of DMAEMA, butylmethacrylate and 2-propylacrylate that enables endosomal escape of siRNA from internalized micelles (Nelson et al. 2012). This concept of self-assembling polymers that can be tailored by their functional components holds great promise for siRNA applications (Jhaveri and Torchilin 2014).

8.3.9 Concepts Involving Biodegradable Polymers for Nanoparticle Formulation

Other concepts of NP formulation that involve biodegradable polymers for encapsulation of siRNA have been described for example for intradermal delivery. Jacobson et al. used supercritical carbon dioxide (SC-CO_2) to encapsulate siRNA in biodegradable PLA nanoparticles and showed a release siRNA *in vivo* over 80 days. Encapsulated siRNA showed minimal initial burst release from the surfaces of the nanoparticles (Jacobson et al. 2010). Methodically, siRNA and PLA were dissolved in a microemulsion with dichloromethane as lipophilic component and the microemulsion was sprayed into an extraction chamber with supercritical CO_2. The process allowed for precise adjustment of shape and particle size of the nanoparticles by varying extraction conditions, such as pressure of the supercritical extraction phase.

In another example, encapsulation of siRNA was achieved using PLGA by single- or double-emulsion solvent evaporation techniques. Addition of a small amount of cationic polymers such as PEI was shown to significantly improve siRNA complexation (Pantazis et al. 2012). PLGA/siRNA microspheres (with an average size of 5.45 ± 0.88 μm) have been successfully incorporated within calcium-based injectable bone cement (Wang et al. 2012) that is clinically used for fixation in osteoporosis patients.

A study by Jensen et al. demonstrated the delivery of siRNA by cationic lipid-modified PLGA nanoparticles as a dry powder formulation to the lungs after pulmo-

nary administration. The nanoparticles were prepared using a double emulsion solvent evaporation technique with DOTAP and PLGA dissolved in the organic phase. The use of DOTAP-modified PLGA facilitated gene silencing activity of the siRNA-loaded nanoparticles. In a next step, the authors demonstrated a spray drying step to successfully incorporate the nanoparticles in mannitol microparticles. The study introduces spray-drying as an optimal technique for engineering dry powder formulations of siRNA nanoparticles, enabling the local delivery of siRNA to lung tissue (Jensen et al. 2012).

8.3.10 Layer-by-Layer Techniques

A layer-by-layer (LbL) delivery method for siRNA uses electrostatic forces to fabricate multilayered films which could cover an implant material surface. The substrate is alternately dipped into polyanion and polycation solutions generating polyelectrolyte multilayers (PEM) in order to incorporate the nucleic acid and provide a controllable loading and release profile.

Song et al. used an LbL approach involving sodium hyaluronate and chitosan/siRNA nanoparticles to coat a titanium surface with a PEM film for gradual siRNA release over approximately 1 week (Song et al. 2015).

8.3.11 Cell-Penetrating Peptides

Cell-penetrating peptides (CPP), also known as protein transduction domains, are a class of peptides that are able to autonomously penetrate cellular membranes either directly or via endocytosis. They are usually short (up to 30 amino acids) and often positively charged. They can be either derived from naturally occurring protein transduction domains such as the HIV transactivator of transcription (Tat), low molecular weight protamine, or other rationally designed peptides. An example of the latter is the chimeric peptide Transportan that contains the amino terminus of the neuropeptide galanin and the wasp toxin mastoparan. They feature comparably low cytotoxicity and low risk of immunogenicity. Their positive charge enables them to form electrostatic nanoparticle complexes with nucleic acids, even though covalent approaches have been described as well (Hoyer and Neundorf 2012).

8.4 Biomaterials as Scaffolds for Sustained Release of RNAi Therapeutics

Tissue regeneration requires longer time periods, and siRNA-based approaches should thus be able to release siRNA over a relatively long period. RNAi technology is hence only applicable in regenerative medicine if the drug can exert its effect in a sustained manner. Local delivery of siRNA by simple injection of nanoparticles is

insufficient, due to the small size of nanoparticles which may cause their rapid dispersion from the target site, thus preventing continuous silencing at the target site over the desired longer time period (Krebs and Alsberg 2011). Biomaterial-based scaffolds have been widely applied to overcome such limitations. Biodegradable and biocompatible scaffolds, implanted in defect sites, are frequently used in tissue engineering approaches as cell carriers and / or to provide large attachment areas for invading cells and to promote cell proliferation and differentiation. By incorporating siRNA or siRNA-encapsulated nanoparticles into the scaffolds, a sustained release of siRNA over time is intended. The released siRNA can then locally exert its silencing effect, while the scaffold itself can aid in tissue regeneration by providing a conductive or inductive support for cellular growth and tissue formation inside the defect site. The siRNA release kinetics are governed by diffusion processes through the scaffold pores. Diffusion depends on interactions of the siRNA or the respective nanoparticles with the scaffold material by, e.g., electrostatic or hydrophilic interaction as well as on pore sizes, diffusion distances and the degradation rate of the biomaterial. Hence, the release over time can be adjusted to the physiological or therapeutic needs.

Considering the challenges in the delivery of siRNA to its specific site of action, scaffold-based siRNA delivery systems are generally designed to also meet various other criteria besides sustained and controlled release of the drug, such as high loading efficiency, structural integrity and suitable mechanical properties, favourable biophysical and morphological properties and high silencing efficiencies. To this end, the rational design of the biomaterial-based siRNA release systems is of high importance. The loading efficiency of siRNA into the carrier system naturally plays a critical role in determining silencing efficiencies. It is defined as the total amount of siRNA released over time plus the amount of remaining siRNA extracted afterwards divided by the theoretical siRNA loading. Integrity and cellular uptake of siRNA after *in situ* release also constitutes an important factor determining its activity (Krebs and Alsberg 2011).

As an example for a scaffold system, electrospun nanofiber scaffolds from a copolymer of caprolactone (CL) and 1 % ethyl ethylene phosphate (EEP), named PCLEEP, have been investigated by Rujitanaro et al. as controlled release system for siRNA. Morphologically, incorporating siRNA compexed with TKO (a commercially available siRNA transfection reagent with a cationic polymer/lipid formulation) into an electrospun PCLEEP scaffold led to bead-free fibers with narrowly distributed fiber diameters. When CL was copolymerized with EEP, siRNA release was significantly enhanced compared to release from PCL fibers. The transfection efficiency of released siRNA against GAPDH determined in NIH 3T3 cells seeded onto the scaffolds was partially retained for at least 30 days. Interestingly, siRNA entrapped alone in the scaffold was able to silence the desired gene by 21 % whereas release of the complexed siRNA showed slightly improved silencing (31 %) (Rujitanaroj et al. 2011).

In contrast to pure PCL which released only 3 % of naked siRNA (Cao et al. 2010), PCLEEP scaffolds showed about 90 % release of complexed siRNA and even more when naked siRNA was used (Rujitanaroj et al. 2011). However, the

authors determined the loading efficiency and found only about 60 % of the theoretical siRNA in the scaffolds. The release was normalized to these 60 %.

Another system involving polyesters was introduced by Nelson et al. who presented a new class of easily tunable biodegradable scaffolds for the delivery of siRNA loaded micellar NPs. This involved an *in situ* forming scaffold consisting of a biodegradable polyester urethane (PEUR) formed by an isocyanate functionalized three-armed cross-linker and biodegradable polyester macromers (PCL, PLA, PGA) (Nelson et al. 2012). In another study Nelson et al. showed that PEUR scaffolds are favourable in regeneration of excisional cutaneous wounds (Nelson et al. 2013) and allow for improved angiogenesis of regenerated tissue (Nelson et al. 2014). Unlike previous approaches, this study demonstrated the local delivery of siRNA for up to 3–5 weeks and the capability to fine tune the release kinetics from a scaffold *in vivo*. Therefore, siRNA release, scaffold degradation, and cell/tissue infiltration could be modified to optimize tissue regeneration (Nelson et al. 2014).

Chitosan scaffolds have been used by many groups for tissue engineering applications. Chitosan, as an example of a positively charged naturally derived polymer, can be easily processed to sponge-like implants via a freeze-drying procedure. These chitosan sponges provide very interesting properties for tissue engineering applications due to efficient swelling on the one hand and their porous structure, enabling cell migration into the implant, on the other hand. In a study by Jia et al., two fluorescently labelled siRNAs complexed with Lipofectamine® were loaded onto chitosan sponges in order to improve bone regeneration in a rat calvarial bone defect model. siRNA loading was performed by incubation of the chitosan sponge with the complexed siRNA and subsequent freeze drying. The *in vitro* release profile of siRNA was determined over 4 weeks, showing 20 % release within the first 8 days and a more rapid release in the following days along with the structural breakdown of the scaffold due to dissolution of the chitosan matrix. The release was determined relative to the theoretical loading and showed that the amount loaded to the scaffolds was equal to the released amount. The sponge-loaded fluorescently labelled siRNA demonstrated at least a fivefold increase in fluorescence intensity compared to the controls (sponges loaded with non-labelled, non-coding siRNA) (Jia et al. 2014).

A hydrogel delivery system composed of the biodegradable hydrogel Glycosil® (a thiol-modified analogue of heparin and thiol-modified hyaluronan cross-linked with a PEG diacrylate) and a chemically modified single-stranded RNA termed "agomirs" (miRNA enhancer) has been used to enhance endogenous miRNA-26a expression. miRNA-26a is known to modulate osteogenic differentiation of adipose tissue derived stem cells (ASC) (Luzi et al. 2008). The hydrogel served as scaffold for cell ingrowth and differentiation as well as a system for controlled and sustained release of the miRNA enhancer *in vivo*; however, data show a burst release of agomir which was released within a time span of 2 days. Still, significantly enhanced vascularization as well as bone formation in a controlled and sustained manner was observed (Li et al. 2013).

Zhang et al. introduced a new type of LbL film coatings based on polyelectrolyte multilayers containing poly(L-lysine) and negatively charged shRNA carrying cal-

cium phosphate nanoparticles. shRNAs against osteocalcin and osteopontin served as a proof of principle to show functional gene silencing in human osteoblasts. Using multilayered films they showed a more efficient silencing than for one layer only (Zhang et al. 2010).

A different concept for controlled nucleic acid release was introduced by Qureshi et al. (Qureshi et al. 2013). They used silver nanoparticles loaded with miRNA via a photocleavable linker. The nanoparticles provided stabilisation and immobilization of the miRNA as long as the nucleic acid remained immobilized. Upon UV-light exposure, the miRNA was released. Loaded silver-nanoparticles can be integrated into various biomaterials, such as PCL scaffolds and hydrogels and flashed upon implantation (Qureshi et al. 2015).

8.5 Modification of Delivery Systems for Cellular Targeting

Targeted delivery of therapeutics by linkage with targeting moieties as ligands for cell-type specific receptors offers the advantage of a higher local concentration of the drug in the desired tissue, thus requiring lower dosages and reducing off-target adverse effects. In this context, aptamers and antibodies as well as small organic molecules and peptides have been explored. These approaches have been mostly used for cancer targeting, e.g. (Zhou and Rossi 2014; Höbel et al. 2014), but there is also an application for bone regeneration (Zhang et al. 2012) that will be shortly described here.

An approach involving aptamers, i.e. oligonucleotides that bind to specific epitopes on the cell surface, has recently been developed which enable targeting of various diseases in different tissues in a cell-specific manner. This approach has been used, e.g., to target prostate-specific membrane antigen (PSMA)-expressing cells to treat prostate cancer in a mouse model after systemic delivery of siRNA directed against tumor survival genes like polo-like kinase 1 (PLK1) (Zhou and Rossi 2014).

Antibody-functionalized PEI nanoparticles for tumor cell-specific delivery of siRNA have been described as well. Cetuximab, an antibody specific for the epidermal growth factor receptor (EGFR), which is overexpressed in many human carcinomas, was covalently linked to the nanoparticles via a PEG spacer prior to siRNA complexation (Höbel et al. 2014).

Zhang et al. introduced a targeting peptide consisting of six repetitive sequences of the tripeptide aspartate-serin-serin grafted to DOTAP based liposomes for systemic intravenous siRNA application. The authors used this targeting system for delivery of siRNA against casein-kinase 2 interacting protein (Ckip) targeting to bone formation surfaces characterized by low crystalline hydroxyapatite. They compared *in vivo* targeting effectiveness of their peptide with a peptide of eight aspartates known to target highly crystalline hydroxyapatite typically found in bone resorption sites (Zhang et al. 2012).

8.6 Application

Biomaterials for the controlled release of RNAi therapeutics have been widely applied in tissue engineering. These systems address for example bone, cartilage or nerve regeneration as well as angiogenesis and skin repair for wound healing. In the following, applications in bone regeneration are given.

8.6.1 Bone and Cartilage

Two targets have attracted great attention for their application in bone regeneration: the receptor activator of NF-κB (RANK), which plays a major role in differentiation of bone-resorbing osteoclasts and antagonists of bone morphogenetic proteins (BMPs), especially noggin.

8.6.1.1 Targeting RANK

The receptor activator of NF-κB (RANK) and its ligand RANKL play a role as key molecules in differentiation and activation of osteoclasts. Binding of RANKL to its receptor triggers downstream signalling which leads to the transformation of precursor cells into mature osteoclasts. Therefore, inhibition of this pathway by silencing RANK is a promising approach to suppress osteoclast maturation and prevent bone resorption (Koide et al. 2010; Wang and Grainger 2010).

PLGA microspheres of about 5 μm in diameter were used to passively target osteoclast-precursor phagocytes and to deliver RANK siRNA (Wang et al. 2012). siRNA complexed with branched PEI (NP ratio = 20) was integrated into the microsphere by a double emulsion technique. In order to prevent passive release of siRNA from the microspheres (Wang et al. 1999) and to favour uptake by phagocyting osteoclastic cells, PLGA of a molecular weight of 57 kDa was used. siRNA released after phagocytosis in the osteoclastic cells exerted its function through inhibiting osteoclast precursors to differentiate along the osteoclastic line. PLGA microsperes were successfully integrated into a calcium phosphate cement for application (Wang et al. 2012).

Ma et al. used a thermosensitive chitosan hydrogel for delivery of siRNA directed against RANK (Ma et al. 2014). The fluorescence signal from labeled siRNA in the hydrogel showed a prolonged siRNA release profile *in vitro*, followed by a sustained gene silencing effect in a murine macrophage cell line for a period of up to 9 days. *In vivo* release investigations of Cy3 labeled siRNA in a subcutaneous local delivery setup in mice showed a burst release of about 75 % within the first day while the remaining siRNA was slowly released for 14 days.

8.6.1.2 Targeting BMP-2 Antagonists

BMP-2 as an osteoinductive factor was shown by several groups to play a major role in enhancing osteogenesis *in vitro* and *in vivo* (Cohen 2002; Wozney 1992). The effectiveness of this growth factor to improve bone formation lead to the registration of a BMP-2 releasing class 3 medical device, Infuse Bone Graft®. However, BMP-2 signalling is strongly regulated, e.g. by internal antagonists such as noggin, chordin and others (Yanagita 2005). In order to overcome this regulation, BMP-2 has to be delivered in high dosages to be effective, coming along with unwanted side effects. Therefore, siRNA application to increase the endogenous availability of BMP-2 by suppressing its active antagonists has been shown to synergistically improve BMP-2 effects (Schneider et al. 2014). Levi et al. investigated the effects of noggin silencing using lentiviral transfection. They found convincing effects of small hairpin RNA (shRNA) alone and in combination with BMP-2 *in vitro* on osteogenic differentiation of hASC and *in vivo* in a critical size defect that was treated with transfected hASC loaded onto a hydroxyapatite coated PLA scaffold. In the same study, the authors demonstrated that the system was also effective when a scaffold loaded with lentiviral/shRNA particles and BMP-2 was combined with pristine hASC. In another approach involving noggin silencing, Manaka et al. used a biodegradable hydrogel consisting of a PLA-*p*-dioxanone-PEG triblock co-polymer with a central PEG block (Saito et al. 2001) as delivery system for naked noggin siRNA (Manaka et al. 2011). They evaluated the biological efficiency of 1 nmol noggin siRNA in presence of extra BMP-2 (2.5 μg) *in vivo* in a mouse model. The results indicated that the siRNA releasing polymer melt implanted in a muscle pouch of mice caused ectopic bone formation with improved size and mineral content as compared to the control containing only BMP-2. Recently, another report on noggin silencing was published that involved a new transfection system based on cationic sterosomes (Cui et al. 2015). The authors investigated a transfection system consisting of cholesterol and stearylamin and found that it was more effective to silence noggin in mouse derived ASCs than Lipofectamine® 2000. They combined their transfection system with a hydrogel consisting of crosslinked methacrylated glycol chitosan and tested the siRNA loaded hydrogel in a critical sized calvaria defect. However, bone formation was not convincingly improved, possibly in part due to a lack of BMP-2 supplementation. Takayama et al, in contrast, injected siRNA against noggin in muscle tissue of mice, applied electroporation and eventually placed a BMP-2 loaded collagen sponge on top. The *in vivo* approach was unable to increase bone formation beyond the BMP-2 group, however, the density of newly formed bone was enhanced in the group with noggin siRNA and BMP-2 (Takayama et al. 2009). A study from the Alsberg group on PEG-based biodegradable and *in situ* forming hydrogels involving noggin silencing was recently published. They showed that release of PEI-complexed siRNA against noggin was controlled by the degradation rate of their hydrogel and allowed a sustained release over a 3–6 weeks period depending on the hydrogel composition. hMSC encapsulated in the siRNA containing hydrogel were successfully transfected and showed improved osteogenic differentiation (Nguyen et al. 2014). A study published by Kowalczewski and Saul

showed the adsorption of Lipofectamine® 2000 complexed siRNA against noggin on fibrin hydrogels. Complexed siRNA was not released in buffer but taken up by MC3T3-E1 mouse fibroblasts seeded onto the gels. Transfection was shown to correlate with the amount of complexed siRNA on the surface.

Kwong et al. investigated effects of siRNA against another BMP-antagonist, chordin, in hMSC (Kwong et al. 2008) and Schneider et al. performed similar experiments on hASC. Both studies found that silencing chordin improved osteogenic differentiation of human osteogenic cells in the absence of BMP-2. Moreover, in the presence of BMP-2, synergistic effects of the combination of siRNA against chordin and BMP-2 were found for hASC (Schneider et al. 2014). No release system, however, has been shown for this application yet.

8.6.1.3 Other Targets for Improved Bone and Cartilage Formation

Casein kinase 2 interacting protein 1 (Ckip-1) is another interesting target to improve osteogeneic differentiation that works via a bone morphogenetic protein-related signalling pathway. Song et al. used a multilayered film (LbL) of hyaluronic acid and chitosan for siRNA delivery from titanium surfaces (Song et al. 2015). siRNA was complexed with chitosan first and loaded to the LbL films as nanoparticle dispersion.

An approach using a dual siRNA release system was published recently by Jia et al. (2014). In addition to Ckip-1 they silenced Flt-1, a soluble VEGF receptor, to increase the amount of free VEGF in tissue and eventually improve bone healing. siRNA complexed with Lipofectamine® 2000 was loaded onto chitosan scaffolds. Rat-derived MSCs showed improved osteogenic differentiation and expression of von Willebrand factor *in vitro*, whereas proliferation in Lipofectamine containing sponges was found to be reduced compared to control sponges.

8.6.1.4 Cartilage

Regarding cartilage engineering, an interesting study was published by Perrier-Groult (Perrier-Groult et al. 2013). They addressed the problem that expansion of isolated chondrocytes in 2D culture is known to dedifferentiate the primary chondrocytes and eventually causes fibrocartilage formation. In order to keep the cells in a differentiated state they used siRNA against collagen type 1. After treatment of primary mouse chondrocytes with siRNA and electroporation, they could show that BMP-2 was more effective than TGF-β1 to induce typical chondrogenic marker when expanded cells were differentiated in an agarose hydrogel 3D culture.

8.6.1.5 miRNA for Bone Applications

Besides siRNA delivery, which has been the mostly investigated type of nucleic acid used in RNAi approaches, miRNA and shRNA-mediated gene silencing have also been subject to recent research projects. Li et al. investigated an RNAi approach using an identified miRNA, which positively modulates the coupling of angiogenesis and osteogenesis. They investigated the expression of different literature-known miRNAs in hMSC, mouse MSCs and mouse preosteoblast MC3T3-E1 cells during osteogenic differentiation. In contrast to other investigated miRNAs, miRNA-26a was found to be strongly upregulated in differentiated cells. A miRNA enhancer structure was used to investigate the effectiveness of miRNA-26a *in vivo* employing an hMSC loaded biodegradable hydrogel delivery system in a critical size calvaria defect model in mice. They found intriguing effects of miRNA-26a with 100 % bone regeneration compared to only 40 % for the cell loaded hydrogel system without miRNA-26a enhancer.

Another miRNA formulation was presented by Qureshi et al. in order to improve closure of critical size mouse calvarial defects. miRNA-148b was linked to silver nanoparticles via a photolabile linker. The conjugate PC-miR-148b-SNP, a derivative of miRNA-148b tethered to silver nanoparticles (SNPs), regulated the gene expression to enhance cortical defect closure in mice. They were added to hASCs and loaded to polycaprolactone (PCL) scaffolds showing enhanced levels of defect healing compared to controls (Qureshi et al. 2015).

Deng et al. investigated the effects of anti-miRNA-31 by lentiviral transfection of rat- and canine-derived ASC (Deng et al. 2013, 2014). miRNA-31 targets the special AT-rich sequence binding protein-2 (Satb2) that is involved in transcriptional regulation and chromatin remodeling and is relevant for osteogenic commitment of ASC downstream of Runx-2. Employing an anti-miRNA-31, they showed improved osteogenic differentiation *in vitro*. Additionally, Deng et al. loaded anti-miRNA-31 transfected ASC onto macroporous β-tricalcium phosphate (TCP) scaffold and showed improved bone healing compared to the non-transfected and miRNA-31 transfected cell-loaded scaffolds in critical size orbital defect models of different species (Deng et al. 2014).

8.6.2 Other Tissues

Local siRNA delivery enables a direct interaction with target genes in the intended tissue. In the following, a number of studies on various tissues that have been targeted with siRNA strategies are described.

A large body of evidence from preclinical studies confirms the successful use of pulmonary siRNA delivery in preclinical models, however none of them could yet proceed to clinical phase (Merkel et al. 2014). A study by Jensen et al. demonstrated a successful delivery of siRNA using cationic lipid-modified PLGA nanoparticles

as a dry powder formulation to treat severe lung diseases through pulmonary administration of siRNA (Jensen et al. 2012).

An anti-TGFβ1 hydrogel pressure-sensitive adhesives transdermal patch composed of polyvinylpyrrolidon, polyvinylalcohol and polyethylene glycol terephthalate could successfully reduce the expression of targeted gene and treat hypertrophic scars (Zhao et al. 2013). This study found that siRNA against TGFβ1 reduced TGFβ1-mediated collagen I synthesis and led to a more homogenous orientation of collagen I fibers.

Mittnacht et al. used hollow resorbable block-copolymer filaments as scaffolds to promote peripheral nerve regeneration. Scaffolds were loaded with chitosan/siRNA nanoparticles for local delivery of nanotherapeutics and the enhancement of nerve regeneration. The stable nanoparticles were instantly internalized by the cells without cytotoxic effects. The targeted gene was silenced by 65–75 % and neurite outgrowth was promoted even in an inhibitory environment (Mittnacht et al. 2010). Chitosan imidazole /siRNA nanoplexes in polyelectrolyte multilayers (PEM) were introduced by Hartmann et al. and an efficacious delivery, uptake and silencing in neuronal cell culture as well as in biologically functional neuronal implants was shown (Hartmann et al. 2013).

Hossfeld et al. successfully delivered chitosan/siRNA NPs from PEM to endothelial cells in porcine artery walls. RNAi strategies were also used in coronary arteries when inflammation occurred after stent placement. Chitosan/siRNA NP, incorporated into PEM that consisted of hyaluronic acid and chitosan were constantly delivered to the porcine artery wall *ex vivo*, suggesting that stents coated with biologically active siRNAs against cytokine receptors or adhesion molecules might be efficient in restenosis prevention (Hossfeld et al. 2013). Monaghan et al. reported on *in vitro* and *in vivo* effects of collagen based scaffold loaded with miRNA in wound healing processes. They found a specific impact on ECM remodelling after injury, which was shown to synergistically improve wound healing controlled by the dose of miR-29B present in the scaffold (Monaghan et al. 2014).

8.7 Barriers to Practice…

Several siRNA based therapies have entered clinical trial phases for FDA approval. None of these trials, however, is focussed on regenerative applications yet. When quantifying the extent of RNAi-based preclinical research during the past years, a steady increase of publications in the field is noticeable as a consequence of successful basic research and the promising general concept of RNAi. A Pubmed search using the keywords “siRNA” and “tissue engineering” or “regenerative medicine” shows that the annual number of publications has steadily increased over the last 10 years and still is increasing to date (Fig. 8.2).

First data on the efficiency of siRNAs in non-human primates were already published in 2006 (Zimmermann et al. 2006). In a proof-of-concept study on RNAi therapeutics in humans, lipid nanoparticle-formulated siRNAs directed against two

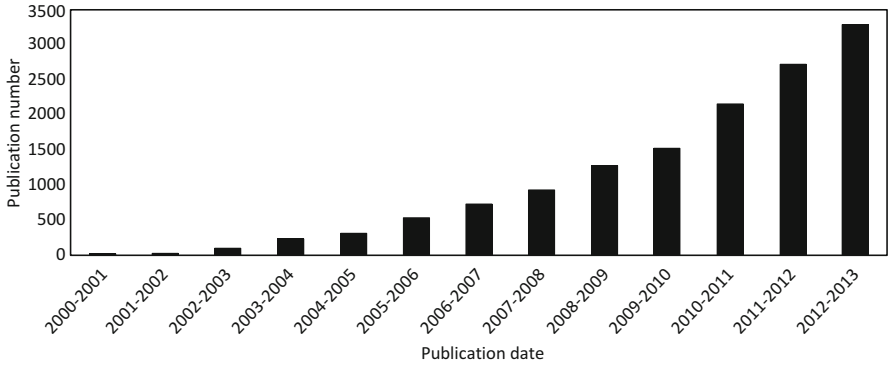

Fig. 8.2 Annual RNAi-related publications since year 2000

oncogenes in liver metastases were used (Tabernero et al. 2013). Even though siRNAs can theoretically silence any gene in the body, clinical studies still face major hurdles that have not been satisfactorily overcome. The problem of poor cellular delivery of naked siRNA due to its size, hydrophilic and anionic nature is addressed by rationally designed delivery systems. However, in many cases they still lack the efficiency of viral delivery systems and many of these vectors cannot be used in clinical studies due to safety concerns. Off-target effects of siRNA, rapid blood clearance by the RES system, immunogenicity and often cytotoxicity due to the unspecific interaction of the highly positively charged delivery systems with serum proteins remain issues that need to be tackled (Kanasty et al. 2012). Still, several drugs are currently in, or have already completed, early clinical phases (phase I or II; see e.g. (Kanasty et al. 2012; Draz et al. 2014) for review and Table 8.1 for some examples).

8.8 …and How to Overcome them

8.8.1 Approaches to Stabilize siRNA by Chemical Modifications

As described above, the problem of physiological stability due to rapid enzymatic degradation of naked siRNA leading to short half-lives in serum has been addressed by packing the nucleic acids into nanocarriers (Yamamoto et al. 2011; Dirin and Winkler 2013). On the other hand, chemical modifications of siRNAs also increase its inherent stability.

Natural oligonucleotides contain a phosphodiester (PO) backbone which is prone to degradation by nucleases *in vivo*. As defined by Dirin et al. (2013), the first generation of chemical modification to tackle poor physicochemical properties

Table 8.1 A choice of clinical trials on RNAi drugs, their targets and vehicles

Disease	Target	Vehicle	Drug name	Company	Status
Advanced solid tumours	Ephrin type-A receptor 2	Neutral liposomes	siRNA-EphA2	Anderson Cancer Center	Phase I recruiting
Metastatic solid tumors	E3 ubiquitin ligase Cbl-b	siRNA-transfected peripheral blood mononuclear cells *ex vivo* by electroporation	APN401	Comprehensive Cancer Center of Wake Forest University, Apeiron	Phase I recruiting
Choroid neovasculari-zation, age-related macular degeneration	VEGF 165 receptor	Solution of chemically modified siRNA for intravitreal injection	AGN 211745	Allergan	Phase II terminated
Advanced solid tumors	PKN3	Cationic liposomes	Atu027	Silence Therapeutics	Phase I completed
Adenocarcinoma of the pancreas	KRAS oncogene mutations	LODER (Local Drug EluteR) is a miniature biodegradable polymeric matrix	siG12D LODER (Local Drug EluteR)	Silenseed Ltd	Phase I completed
Optic atrophy	caspase 2 protein	Chemically modified siRNA	QPI-1007	Quark Pharmaceuticals	Phase I completed
Delayed graft function in patients receiving renal transplants.	pro-apoptotic p53	naked siRNA	I5NP	Quark Pharmaceuticals	Phase I completed
Treatment of relapsed or refractory cancers	M2 subunit of ribonucleotide reductase (RRM2)	Cyclodextrin nanoparticle decorated with human transferrin protein and polyethylene glycol for stability	CALAA-01	Calando Pharmaceuticals	Phase I terminated

https://clinicaltrials.gov/ and Burnett et al. (2011)

exchanged the PO group of oligodeoxynucleotide (ODN) molecules by phosphorothioate oligodeoxynucleotides (PS-ODNs). PS-ODNs show increased nuclease resistance, making a parenteral administration feasible. Furthermore, this modification improved pharmacokinetic properties and led to higher tissue distribution, decreased urinary excretion and longer residence time in various tissues as a result of higher plasma protein binding (Yu et al. 2001, 2009). However, the clinical application of PS-antisense ONs has been limited so far, due to poor pharmacodynamic properties, off-target effects and safety issues (Winkler et al. 2010; Stessl et al. 2012; Gekeler et al. 2006; Summerton 2007; Kling 2010; Pisano et al. 2008).

The second generation of chemical modifications focused on the ribose unit of the nucleic acids. The aims of these modifications include enhancement of nuclease resistance, improvement in efficacy, increased binding affinity and enhanced protein binding (Manoharan 1999). Gapmers which contain internal segments of PS-ODNs bound to 2′-OMe or 2′-O-methoxyethyl (2′-OMOE) were identified as improved structure over PS-ODNs (Prakash et al. 2008).

The third generation includes major structural changes represented by various ribose ring and phosphate modifications (Yamamoto et al. 2011). Such modifications include locked nucleic acids (LNA) and phosphorodiamidate morpholino oligomers (PMOs or morpholinos) (Summerton 1999). LNAs contain a methylene bridge between the 2′-O and 4′-C of the ribose unit, which leads to higher hybridization affinity and enzymatic stability (Fluiter et al. 2009). PMOs are constituted of an uncharged backbone, while the ribose moiety is substituted by a morpholino ring (Summerton 1999).

8.8.2 Opportunities of Local Delivery

In order to circumvent the mentioned obstacles for RNAi in regenerative applications, a combination of strategies for local delivery appears to be promising. This includes (1) the use of nanocarriers for the formulation of siRNA or other small RNA molecules, mediating protection against nucleases, efficient cellular uptake and intracellular delivery, (2) the incorporation of nanoparticle-formulated RNA into tissue-adopted implants, either macroporous scaffolds or hydrogels, that guide migration of regenerating cells into the material and (3) the targeted controlled release from the implants towards these cells in order to foster their regenerative function. In addition to chemical modifications and customization of the employed nanocarriers and implants, (4) chemical RNA modification appear to be beneficial.

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