Pharmaceutical Research, Vol. 26, No. 2, February 2009 (© 2008)
DOI: 10.1007/s11095-008-9772-3

Expert Review

Antisense Makes Sense in Engineered Regenerative Medicine

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Received September 15, 2008; accepted October 28, 2008; published online November 18, 2008

Abstract. The use of antisense strategies such as ribozymes, oligodeoxynucleotides (ODNs) and small interfering RNA (siRNA) in gene therapy, in conjunction with the use of stem cells and tissue engineering, has opened up possibilities in curing degenerative diseases and injuries to non-regenerating organs and tissues. With their unique ability to down-regulate or silence gene expression, antisense oligonucleotides are uniquely suited in turning down the production of pathogenic or undesirable proteins and cytokines. Here, we review the antisense strategies and their applications in regenerative medicine with a focus on their efficacies in promoting cell viability, regulating cell functionalities as well as shaping an optimal microenvironment for therapeutic purposes.

KEY WORDS: antisense; oligodeoxynucleotides; regenerative medicine; ribozyme; RNA interference (RNAi).

INTRODUCTION

Various degenerative diseases like diabetes, cardiovascular diseases, Alzheimer's disease, Parkinson's disease, osteoarthritis, osteoporosis, rheumatoid arthritis in tandem with injuries to non-regenerating organs/tissues resulting from accidents have resulted in progressive deterioration in the quality of life of countless people. Humankind can only pin their hope on the progress of regenerative medicine, relying on the combined use of living cells, engineered materials and appropriate biological molecules (1,2). Regenerative medicine aims to repair, reconstruct or regenerate damaged tissues and organs, employing various diverse and cutting edge fields in biomedical research such as tissue engineering, stem cells and gene therapy. Tissue engineering deals with the reconstruction of degenerative tissues with three-dimensional (3D) cell-laden scaffolds, where morphogenesis is precisely induced and cell–matrix interaction is highly emphasized (3); stem cells refer to a type of progenitor cells with the capacity to differentiate into a variety of specialized cells (4); and gene therapy, which has been experimentally and clinically explored to treat severe diseases such as leukemia, cancer and AIDS, greatly contributes to regenerative medicine by introducing exogenous nucleotides into therapeutic cells, amplifying or silencing certain genes at molecular level to induce some desirable functionalities (5). Researchers in regenerative medicine adopt various approaches, established or innovative, ranging from a gene-manipulated stem cell-laden scaffold for cartilage regeneration (6) to a material-free cell therapy against neural degenerative disease (7), toward their goal of establishing a successful regeneration.

A promising strategy in regenerative medicine is the use of antisense techniques. Belonging to the field of gene therapy, antisense technology has been widely and thoroughly explored from laboratory-based investigation to clinical trials. The rationale behind antisense technology is that specific DNA or RNA can bind to a target mRNA and subsequently turn the 'undesired' gene off at post-transcriptional level. These DNA or RNA molecules are designed with a sequence complimentary to that of the target mRNA; after being introduced into the cell with suitable delivery systems, this sequence guides the 'antisense' nucleotides to the so-called 'sense' segment of the target mRNA, effectively blocking the initiation of translation or resulting in the degradation of the target mRNA (8,9).

Antisense technology, developed in 1978 (10), has been well acknowledged for its potential in inhibiting the expression of pathogenic genes involved in severe diseases like leukemia and rheumatoid arthritis (RA), the cure for which remains a substantial challenge for conventional therapies. Antisense technology has emerged to be a versatile tool that makes unique sense in engineering regenerative medicine, and its ability of suppressing certain genes has been of great assistance in promoting various kinds of tissue/organ regeneration. This review—after a brief introduction of antisense strategies—aims to discuss the application of these technologies in the various fields of regenerative medicine.

ANTISENSE STRATEGIES

Three Categories

Ribozyme

Ribozymes (from *ribo*nucleic acid en*zyme*, also called RNA enzyme or catalytic RNA) are RNA molecules with

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enzymatic activity (11), and are classified into four categories: (1) the hammerhead ribozyme, derived from the Lucerne transient streak virus in 1987 (12); (2) the hairpin ribozyme, originated from the "minus" strand of satellite RNA of tobacco ringspot virus (13); (3) the varkud satellite ribozyme, discovered in mitochondria of *Neurospora* (14); and (4) the hepatitis delta virus ribozyme, found in genomic and anti-genomic RNAs of the hepatitis delta virus (15). Type (1) and (2) ribozymes are substantially small RNAs consisting of 40–160 nucleotides and predominantly used in therapeutic practices, while types (3) and (4) require rather demanding sequence motifs to exert effective cleavage. The hairpin and hammerhead ribozymes bind to the substrate RNA *via* Watson–Crick base pairing and subsequently cleave the RNA in a non-hydrolytic reaction, degrading the target RNA. These Ribozymes can be recycled to catalyze additional multiple reactions *in vitro* (Fig. 1).

Although ribozymes are promising as therapeutic tools for various diseases—as they can down-regulate target gene expression, challenges in their design and application still remain. Most common difficulties in the use of ribozymes are low efficiency of delivery and lack of stability as well as precision of targeting. As a result, only a few ribozymes have been qualified for clinical trials so far. Various attempts are being made to overcome these shortcomings. For example, to protect against rapid degradation by ribonucleases in cells and tissues, ribozymes have been chemically modified with amino or alkyl groups to replace the 2′-OH group of the ribose (16); whereas to increase cleavage efficiency and targeting precision, researchers have developed ribozymes containing two or more minizyme units (hammerhead ribozymes without a stem-loop), like the so-called 'maxizyme' (which stands for Minimized, Active, X-shaped and Intelligent) (17) and multi-unit ribozymes (containing several units—up to nine) (18,19)

Antisense Oligonucleotides

Antisense oligonucleotides include single-stranded oligodeoxy(ribo)nucleotides (ODNs) and RNA-based antisense oligonucleotides. From bench to bedside, ODNs have been studied in laboratories since the early 1980s of the last century (20). Numerous clinical trials are ongoing and one biopharmaceutical enterprise has been launched based on ODNs. Together with RNA interference (RNAi), antisense oligonucleotides have been well acknowledged as the most operative antisense therapeutic agents. Antisense oligonucleotides are generally 13–25 nucleotides in length, single-stranded oligomers that possess a sequence complementary to a certain portion of target mRNA. After their cellular uptake, ODNs bind to specific mRNA strands *via* Watson–Crick base pairing and manage to silence gene expression through two possible mechanisms: (1) The duplex formed between the exogenous DNA and mRNA in cytoplasm can sterically block the ribosomal machinery, interfering with RNA processing and translation. (2) RNaseH dependent action resulting in cleavage of the mRNA strand (21) (Fig. 1).

A major challenge in the application of antisense ODNs was their inadequate stability against nucleases, impairing their efficacies in cells and tissues. Accordingly, chemical modifications have been applied to the synthesized segments, including the replacement of an oxygen atom in the phosphodiester backbone with a sulfur atom, which effectively increased nuclease resistance in the first-generation of ODNs (22,23). Following generations of antisense ODNs

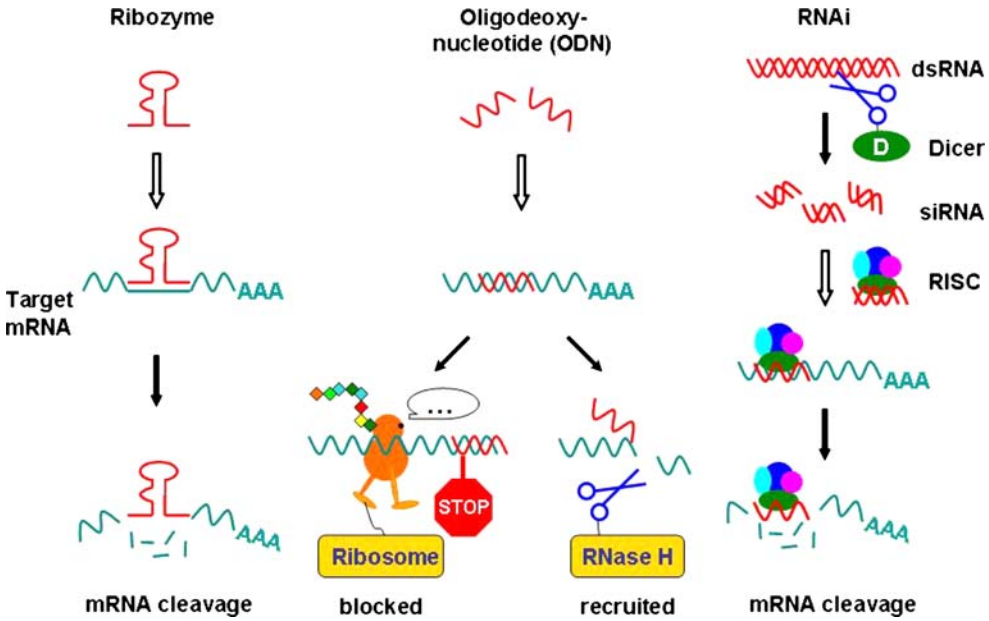

Fig. 1. Mechanisms of antisense strategies on inhibiting gene expression. Ribozyme binds specifically to the substrate RNA *via* the Watson–Crick base pairing, and subsequently attacks the RNA with a non-hydrolytic cleavage which degrades the target; ODN hybridizes with the target mRNA of a complementary sequence and blocks mRNA translation through translation arrest by blocking ribosomal machinery or RNase H-dependent cleavage; In RNAi, siRNA assembles with Argonaute proteins, Dicer and other cellular factors into the RNA-induced silencing complex (RISC) which unwinds the siRNA. Then the antisense strand guides RISC to cleave the target mRNA in a sequence-specific manner.

have resulted in products with higher affinity to the target mRNA and lower toxicity (24–26). Simultaneously, the development of more efficient non-viral carriers for ODNs delivery has drawn much attention. ODN systems for therapeutic applications have thus rapidly progressed, resulting in three cases of clinical use as can be found on ClinicalTrials.gov.

Unlike antisense ODNs that are exogenously introduced into the cytoplasm, the RNA based antisense oligonucleotides or antisense-RNA are endogenously expressed after transfection of a recombinant plasmid—encoding the antisense-RNA segment—into host cells. The endogenously expressed antisense-RNA transcripts then form a duplex with the complementary target mRNA sequence and work in a similar fashion as ODNs by blocking ribosomal translation of mRNA (27).

RNA Interference (RNAi)

In 1998, Andrew Fire *et al*. discovered that dsRNA can induce degradation of the homologous mRNA target in animals, resulting in the silencing of specific genes. This was later termed as RNAi (28). RNAi has been widely used in gene functional analysis in mammals as well as in gene therapy for various diseases. It was found that the introduction of dsRNA longer than 30 base pairs into most mammalian cells elicited an antiviral immune response (29). Therefore people started making use of small double stranded RNA fragments of 21–23 nucleotides in length, called siRNAs (small interfering RNAs) for gene silencing. (30,31). After the introduction of siRNA into the cells, the siRNA assembles with Argonaute proteins, Dicer and other cellular factors into an RNA-induced silencing complex (RISC) (32), which unwinds the dsRNAs. One of these strands, the so-called passenger strand, gets degraded, while the other strand—antisense (guide) strand, guides the RISC to cleave the target mRNA in a sequence-specific manner, leading to the degradation or translational repression of the target gene. RISC is recycled and can process several cleavage cycles (Fig. 1).

Although siRNAs can be easily introduced into the cells directly using various non-viral delivery methods, the silencing of target gene only lasts for 3–7 days, depending on the kind of target gene. Thus, plasmid vectors encoding for short hairpin RNAs (shRNAs) have been designed to increase the duration of silencing as the shRNA can be continuously expressed within the cell for a considerable period of time (33,34). shRNAs consist of 19–29 base-pair stems, i.e. sense strand and antisense strand of siRNAs with a four to nine base pair nucleotide loop at one end (35). After the plasmid encoding for shRNA enters the cell nucleus, shRNAs are expressed and then cut to form siRNAs by Dicer, whereafter they follow the same mechanism as siRNAs to induce gene silencing (Fig. 2).

siRNAs are regarded as preferred agents of gene silencing for therapeutic applications due to their low toxicity,

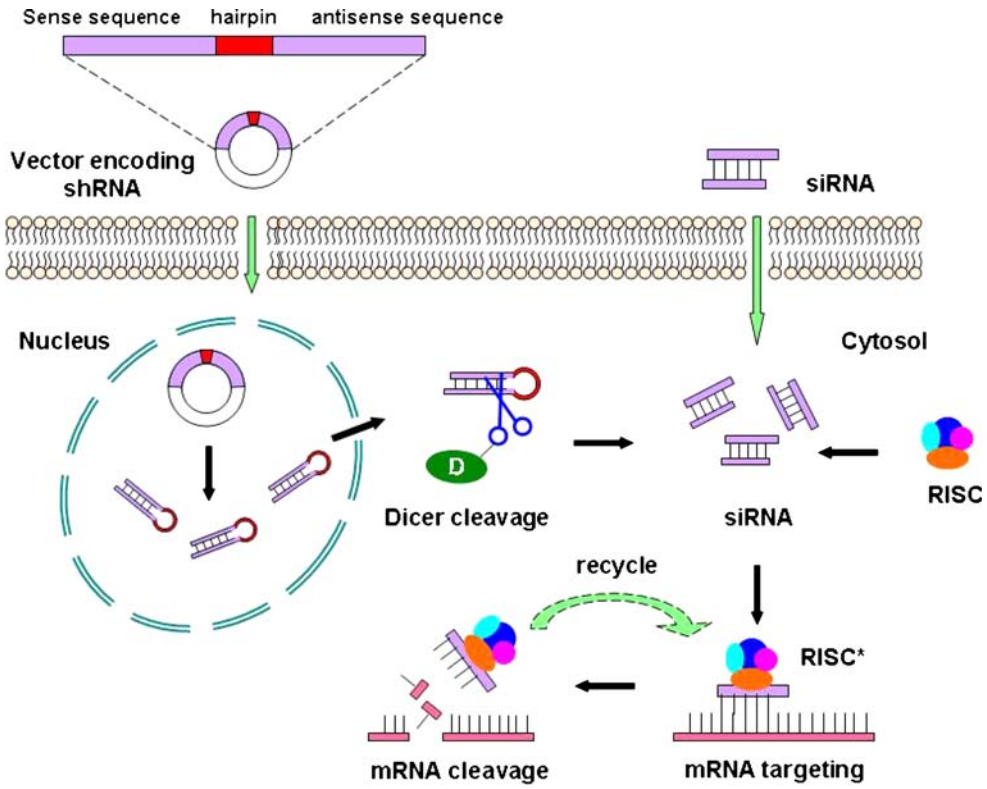

Fig. 2. Mechanism of RNAi in mammalian cells. Short hairpin RNA (shRNA) is endogenously expressed *via* expression vectors, and then migrates into cytosol where it is cut by Dicer into smaller siRNA. Alternatively, double-strand siRNA is directly introduced into cells. siRNA produced by both means are recognized and incorporated into RISC (RNA-induced silencing complex). The antisense-combined RISC* binds to the target mRNA with a complementary sequence and cleaves it. RISC* can be recycled to carry out additional multiple reactions.

high sequence specificity and ability to induce RNAi at low concentrations (36). However, there are still some issues such as "off-target effects" and cellular stability that need to be addressed (37). Chemical modification and proper sequence design may be helpful to ameliorate these issues to some extent (38).

Recently, microRNAs (miRNAs) have also attracted interests from researchers engaged in regenerative medicine. As endogenous small RNAs with essential roles in animal development, miRNAs regulate various physiological and pathological processes, and have implications in some severe diseases like colon cancer and leukemia (39,40). Although it is a bit earlier to comprehensively summarize their applications, a handful of pilot trials have already reported the potential of miRNA in treating cardiovascular disorders (41,42). It is predictable that more exciting works based on miRNA would emerge in the near future.

In this review, we focus on ODNs, ribozymes and siRNAs. All these antisense techniques have a common ground—to down-regulate the expression of a target gene—at mRNA level—so as to prevent its translation into a protein pathogenic to the cell or to study gene functionalities. In general, antisense oligonucleotides and siRNAs accomplish this by employing the cellular machinery to prevent translation or to degrade mRNA, while ribozymes form catalytic centres by folding into specific three-dimensional structures that catalyse mRNA degradation. Table I indicates the comparison of these three methods.

Delivery Systems

Both viral- and non-viral-based nucleic acid delivery systems serve well in antisense transfer for regenerative medicine. Non-viral gene delivery utilizes chemical reagents including lipids and cationic polymers, or physical means such as electroporation and microinjection (5). Primary advantages of non-viral methods include low immune responses and facileness in handling, while their relatively-low transfection efficiencies and cell toxicity remain major challenges. Though manufacturers have always aimed at developing non-viral delivery systems with both higher transfection efficiency and lower toxicity, and huge commercial success has been attained with products such as FuGene® and Lipofectamine™, people are still cautious in their use in clinical applications.

On the other hand, viral delivery systems, consisting of retrovirus (including lentivirus), adenovirus, adeno-associated virus and herpes virus, have attracted more attention in antisense delivery on the account of their higher transfection efficiency, while concerns regarding their immunogenicity remain. Each type of viral vector has its own characteristics and thus meets the requirements in different applications. For instance, retroviruses and lentiviruses can lead to the integration of exogenous genes into the host genome with the drawback of introducing insertional mutations (43), while adenoviruses do not have this capability. Therefore, retroviruses and lentiviruses can be used to attain a stable gene knockdown while adenoviruses are more suitable to attain transient gene silencing. Lentiviral and adenoviral vectors work in both dividing and non-dividing cells, and consequently can be used to infect terminally differentiated cells such as neurons, muscle and liver cells; in contrast, retroviruses only infect replicative cells and hence can be reliably used in cancer therapy *in vivo* (44). Having been used in several preclinical and clinical trials, viral vectors with specific properties provide various choices and flexibility in targeting inherited and acquired diseases with antisense strategies. However, serious concerns remain in the use of viral vectors. For example retroviruses have a tendency to integrate near oncogenes in the host genome, resulting in lethal neoplastic diseases in the patients. Such issues would need to be addressed before viral vectors can be effectively used in clinical practice. *Ex-vivo* gene transfer into *in-vitro* cultured cells may result in the elimination of toxicity and immunogenicity associated with non-viral and viral vectors to a certain extent before the use of these cells *in-vivo*. For example, when adenoviral vectors are used for transfection, the virus undergoes disassembly inside the cells, where the capsid (an immunogen) gets degraded during the period of *in-vitro* culture, while the genetic material gets transferred to the nucleus.

ANTISENSE IN REGENERATIVE MEDICINE

All therapeutic strategies in regenerative medicine rely either on autologous or allogenous cells. These cells are delivered to desired sites, in presence or absence of material vehicles and with or without gene manipulations. Demands of high cell viability, normal functionalities as well as favorable settlement in the new microenvironment make it a complicated and difficult task to establish a successful regenerative medicine therapy or strategy. The order of requirement in cell transplantation towards an established regeneration should be: (1) Viability: cells can survive; (2) Functionality: cells play their roles against degeneration; and (3) Integration: cells can communicate and get along with their neighbors (including host cells and ECM components such as glycosaminoglycans, collagen and hyaluronic acid). Here we discuss the role

Table I. Most Commonly Used Antisense Strategies in Regenerative Medicine

	Characteristics	Limitations	Delivery means
Ribozyme	Simple catalytic domain Recycled in multiple cycles	Vulnerable to ribonucleases Off-target effects	Viral and non-viral
ODN	Convenient to synthesize and modify	Unsustainable effect Large dose required Impossible to endogenously express	Non-viral only
RNAi	High sequence specificity Small dose effective Recycled use	Off-target effects High requirement for synthesis	Viral and non-viral

antisense strategies can play in facilitating the above requirements in regenerative medicine.

Antisense in Anti-apoptosis

Researchers in regenerative medicine frequently encounter cell apoptosis. In many cases, cell death itself is the cause of degeneration, and the *in situ* prevention of apoptosis could directly and effectively restore the organ's functions. In other cases where the two-dimensionally cultured cells are transplanted to three-dimensional scaffolds, their fates are significantly influenced by the complicated biophysical and biochemical signals in the new environment, resulting in a percentage of cells undergoing programmed cell death, i.e. apoptosis—triggered and regulated by a variety of signaling pathways. A large class of pro-apoptotic proteins including Bcl-2 family (45) and Caspase family (46,47) are involved in this process. As a result, the implanted constructs have low cell viability, compromising their therapeutic efficacies. Hence, researchers have been developing antisense strategies to block the expression of pro-apoptotic proteins that lead the cells to death.

The use of ribozyme in silencing Fas ligand might be among the first attempts in the use of antisense technology in preventing apoptosis. Fas is a member of the tumor necrosis factor (TNF) receptor superfamily and a key mediator of cell apoptosis—*via* a pathway similar to that of TNF receptor but slightly simpler (48). Klein *et al.* (49) made use of ribozyme to specifically silence the expression of Fas at mRNA level *in vitro*. Around 5000 copies of anti-Fas ribozyme transcripts per cell were found to be expressed in the transfected beta TC-3 cells and 80% less FAS was expressed in these transfected beta TC-3 cells compared to mock-transfected cells.

In a pioneering work (50), researchers attempted to inhibit the expression of p53 gene to modulate liver regeneration *in vivo*. p53 plays a key role in regulating cell cycle and cell fate (51). p53 expression was blocked using antisense ODN resulting in a decrease in the number of cells in G1 phase of the cell cycle and in the promotion of mitosis and PCNA (proliferating cell nuclear antigen) expression, all of which are beneficial in liver restoration. Another group (52) designed ODNs targeted to Fas ligand and transfected it into Jurkat cells and found that the transfected cells underwent apoptosis at a slower rate. More interestingly, certain ODN products (not primarily designed to be anti-apoptotic, helped in maintaining and increasing cell viability *in-vitro* and *in-vivo*) which are non-specific to apoptotic genes proved to be versatile once introduced into cells. One example is the PyNTTTTGT prototype of immunostimulatory oligonucleotides—IMT504 (53); it significantly increased the viability of adult mesenchymal stem cells (MSCs) both *in vitro* and *in vivo* and additionally helped the cells to maintain their capacities to differentiate to osteogenic and adipogenic lineages. Encouraging results from IMT504 and other ODNs in tissue repair have attracted more researchers to develop novel antisense technologies with anti-apoptotic properties.

The explosive development in RNAi techniques in recent years has led to innovative approaches in the prevention of cell apoptosis. For instance, diabetes may arise due to inadequate production of insulin from the islet β-cells. Aiming to preserve cell mass as well as restore the function of these cells, Mussmann and colleagues (54) inactivated glycogen synthase kinase-3 (GSK3) in islet β-cells by siRNA. This resulted in the prevention of cell death from high concentration of glucose and the saturated fatty acid palmitate. Various developments, like improvements in siRNA targeting (55) and the regulation of GSKs without exceeding permissible thresholds (56), followed that were aimed at avoiding adverse effects generated by long-term GSK3-inhibition, promoting β-cell proliferation and preventing tumorigenesis at the same time. Another anti-apoptotic siRNA has been reported in a very recent study (57). The researchers demonstrated that using an siRNA to silence the expression of PTP-1B gene—a regulator of cardiomyocytic apoptosis—may be effective in protecting cardiomyocytes from hypoxia-reoxygenation (H/R). A more comprehensive study by neurologists (58) tested the efficacies of three siRNAs in retinal ganglion cells (RGC), respectively targeting an early gene c-Jun, a pro-apoptotic gene Bax and an apoptosome constituent Apaf-1. Aside from confirming the important roles that these genes play in RGC apoptosis, the research showed a significant increase in cell population after the administration of these siRNA *in vivo* (Fig. 3).

Recent studies by Mahato group (59) demonstrate the application of iNOS-targeting siRNA in preventing cell death in human islets, which would clearly facilitate the practice of human islet transplantation. The transplanted islets, which show promise in treating diabetes, are prone to host immune reaction and are severely attacked by pro-inflammatory cytokines. As a result, in these exogenous cells, the expression of inducible nitric oxide synthase (iNOS) is significantly up-regulated to produce radical nitric oxide (rNO), which is an important signaling molecule initiating cell apoptosis (60). This results in a loss in cell population at an early stage, even before the implant can be considered as functional islets. In Li and Mahato's work, the role of iNOS in this process was highlighted and an siRNA was constructed and administered to silence iNOS gene in the islets, resulting in a decrease in NO production and consequently reduction in cell death. Specifically, rat β-cells pre-incubated with cytokine cocktails of IL-β (50 pg/mL), TNF-α (5 ng/mL), and IFN-γ (50 ng/mL) were transfected with siRNA-iNOS or control siRNA. As illustrated in Fig. 3, almost all the cells treated with control siRNA aggregated and turned round, apparently indicating the occurrence of apoptosis. In contrast, high viability and normal morphology were maintained in the siRNA-iNOS group (Fig. 3).

RNAi thus proves to be an effective tool in inhibiting apoptosis by silencing genes involved in this complicated process. Efforts are still on for the development of siRNA (and also other types of antisense oligonucleotide) techniques to cover a wider range of applications in regenerative medicine.

Antisense in Regulating Cell Functionalities

After the survival of transplanted cells is ensured, a successful regenerative medicine treatment requires that the cells express desired functionalities by either differentiating into a specific lineage or producing biomolecules like cytokines and extracellular matrix components. Toward this goal, expression of some genes may need to be inhibited or silenced in order to achieve optimal therapeutic effects. For instance, transcripts leading to suppression of differentiation

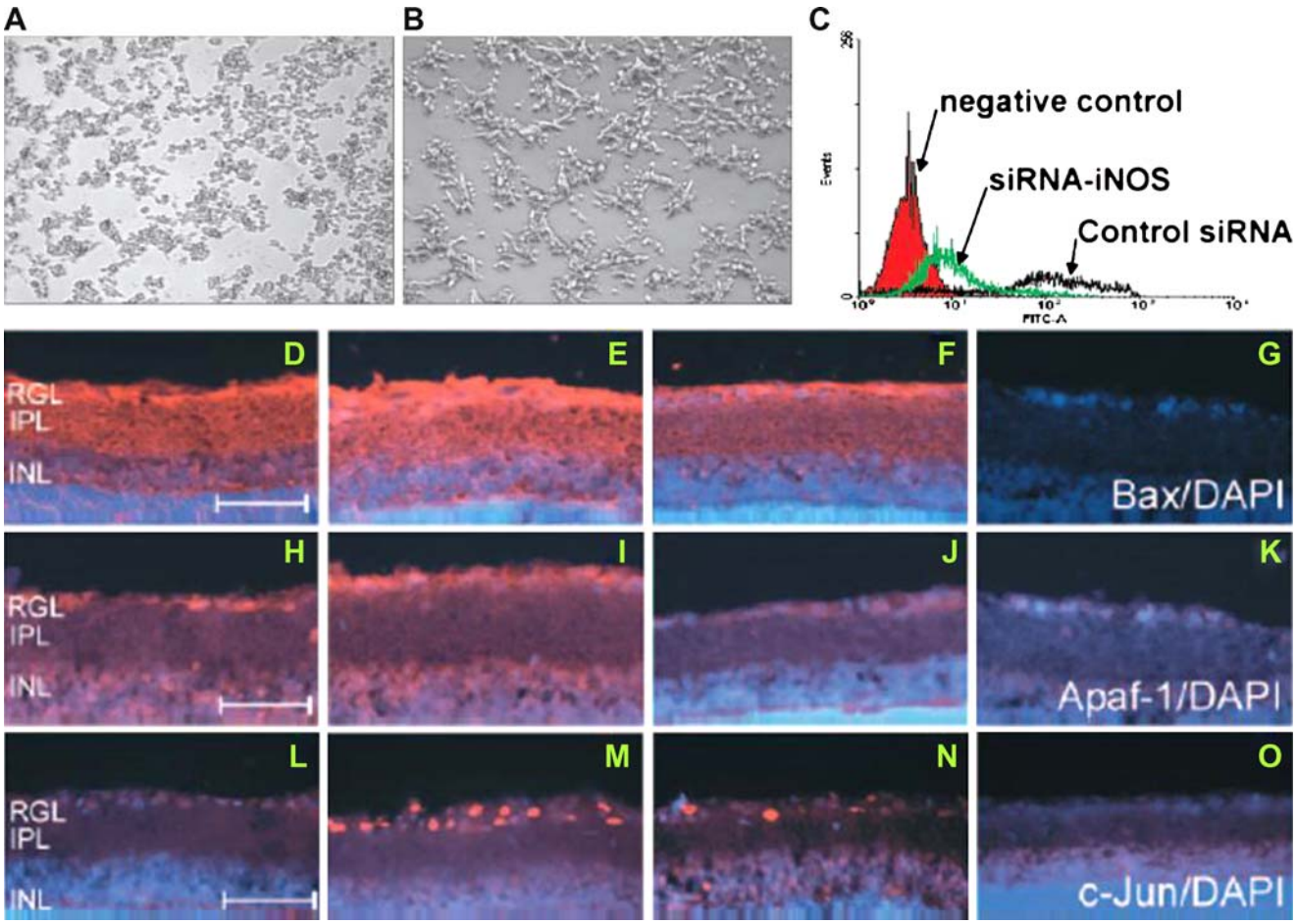

Fig. 3. Antisense strategies in preventing cell death. **A** Apoptosis could be seen in the islet β-cells in the control group, indicated by cell clumping and turning round; **B** Normal spreading morphology was observed in the siRNA-iNOS-treated groups; **C** INS-1 E cells treated with no cytokine and siRNA, or with siRNA-iNOS and cytokine, or with control siRNA and cytokine were analyzed by TUNEL assay. The values indicated the percentage of live cells. (**A**–**C** from reference 59); (**D**–**O**) Micrographs of retinal sections indicating the effect of siRNA on regulating expression of target protein related to apoptosis. **D**, **H**, **L** In the RGC layer in non-axotomized animals, expression of Bax, c-Jun and Apaf-1 was observed; **E**, **I**, **M** in axotomized animals with control siRNA, expression of these three proteins was visibly enhanced; **F**, **J**, **N** in axotomized animals with siRNA targeting Bax, Apaf-a and c-Jun respectively, expression of these three proteins was distinctly suppressed. **G**, **K**, **O** Sections without treatment with primary antibody were used as controls (**D**–**O** from reference 58).

would hamper the process of tissue repair, and some genes *per se* are pathogenic as they code for cytokines or enzymes that cause the destruction of the local microenvironment. Antisense strategies down-regulate gene expression and are thus particularly suited in fulfilling this gene-silencing requirement, providing a unique way of facilitating regeneration—especially in treating some complicated diseases. Antisense techniques have been widely investigated for their efficacies in the regeneration of various tissue/organ types, and in this section we focus on their utility in several areas including osteo-/chondro-, cardiovascular and neural regeneration.

Bone and Cartilage: (1) Rheumatoid Arthritis(RA)

Researchers in regenerative medicine are confronted by one of the greatest-ever challenges—treating bone/cartilage destruction caused by RA, which is a severe degenerative disease and a chronic inflammatory disorder. It leads to the destruction of cartilage and bone whilst bringing much suffering, pain and agony to the patients, heavily decreasing their quality of life. Although the detailed pathogeny of RA remains unclear, it is widely considered that many inflammatory cytokines play a vital role in RA (61–63). The elevation in the levels of these cytokines and some other related genes in rheumatoid arthritic synovium and related joint lesions has led researchers to attempt to silence their expression using antisense technology and consequently prevent degeneration and even promote regeneration.

Most studies focus on the tumor necrosis factor-α (TNF-α) cytokine. It has been well acknowledged that this inflammatory factor is a key participant in cartilage/bone degradation and induction of immune responses (64). Blockage of TNF-α by its antibody soundly inhibits the secretion of several inflammatory cytokines by synovial cells *in vitro* (65, 66) and improves the condition of joint lesions *in vivo* (67, 68). However, it would be more efficient and safer to block TNF-α at post-transcriptional level compared to the antibody-based therapies. Takahashi *et al.* (69) transfected RA synovial cells with a synthesized hammerhead ribozyme against TNF-α and demonstrated its presence in the intracellular space for more than 48 h. Transcription of TNF-α as well as the production of cytokines TNF-α and IL-6 was effec-

tively suppressed as long as 48 h but no significant cytotoxicity was observed. Another group (70) designed and administered a hammerhead ribozyme Rz666 against TNF-α intravenously in mice, discovering that the inhibition of TNF-α could prevent the destruction of cartilage and bone and reduce the development of established collagen-induced arthritis (CIA), thus providing evidence for the first time that a ribozyme used to inhibit the expression of TNF-α could be efficient in regulating arthritis *in vivo* without triggering immune responses. These findings suggest that antisense products such as modified ribozymes could be adopted as a promising clinical tool for the treatment of RA and other TNF-α-related diseases.

Aside from TNF-α, some other genes involved in inflammation and cartilage destruction have also been targeted and silenced with corresponding antisense strands. One example is the nuclear factor κB (NF-κB), a transcription factor widely involved in immune responses and other cellular responses (71). NF-κB can be activated by, and then turn around to regulate the expression of, cytokines such as IL-1β and TNF-α (72,73). Its potential role in joint destruction has been well documented (74). Tomita *et al.* (75) transferred an NF-κB decoy ODN with liposome intra-articularly to synovial cells of CIA rat, and confirmed its presence in the cells even 28 days post-administration. Amounts of cytokines such as IL-1 and TNF-α were found to be much lower in the synovium of arthritic joints, together with alleviation in symptoms as indicated in histological and radiographic analysis. Similarly, siRNA-based strategies (76) against NF-κB were investigated with an adenoviral delivery system and positive results were observed in the *in vivo* osteoarthritis (OA) models. In addition, genes coding for matrix-degrading enzymes such as Cathepsin L (CL) (77) and matrix metalloproteinase-1 (MMP-1) (78) have also been targeted and related ribozymes administered using retroviral vectors, leading to satisfactory outcomes including amelioration of cartilage degradation *in vitro* and *in vivo*.

Bone and Cartilage: (2) Other Disorders

The application of antisense techniques also extends to other skeletal disorders. In a study (79) involving osteogenesis imperfecta (OI), a genetic bone disorder caused by the mutation of type I collagen, researchers delivered a hammerhead ribozyme Col1A1Rz547 aiming to specifically cleave a mutant Col1A1 gene in the murine calvarial osteoblast line MC3T3-E1. The use of a vaccinia-based delivery system successfully led to the stabilization of ribozyme levels in the cells, thus drastically decreasing the expression of the mutant gene and the corresponding mutant protein. In another study focusing on osteopenia, a condition in which bone mineral density is abnormally lower, Gazzerro *et al.* made use of siRNA (80) to down-regulate the expression of gremlin, an antagonist to bone morphogenetic proteins (BMPs), resulting in an elevated expression of osteocalcin and Runx-2 and enhanced bone formation.

Chondrocyte dedifferentiation is one major intrinsic cause for cartilage degeneration in adults. Cathepsin B is a protease that indicates the extent of dedifferentiation of chondrocytes and is involved in cartilage destruction resulting from OA or RA. A trial by Zwicky *et al.* (81) demonstrated that by means of dsRNA and antisense DNA, silencing of Cathepsin B can be capable of preventing chondrocytes from dedifferentiation and thus protecting the cartilage from degeneration. This finding additionally provided tissue engineers and biomaterialists a unique way to prevent dedifferentiation during *in vitro* cultivation and expansion of primary chondrocytes.

The use of an implant is a commonly adopted strategy in bone/cartilage regeneration. However, implant failure such as aseptic loosening of total joint replacement can cause serious problems and can not be neglected. In most cases, a revision surgery has to be performed and the patients seriously suffer as a result. Realizing that particle-induced osteolysis is a primary cause of implant failure, Dong and colleagues (82) contrived novel therapeutic tactics intending to block osteoclastogenesis with antisense techniques. Again, the cytokine TNF-α was chosen as the target of their designed ODN product and the osteolysis induced in murine calvarial models was effectively suppressed. This is a clear demonstration that antisense-based methods, with further developments, can be used effectively in overcoming difficulties like secondary failure in clinical practice with much lower invasion and damage to patients (Fig. 4).

Cardiovascular Degeneration

Cardiovascular disease refers to diseases that affect the heart and blood vessels. It is a leading cause of death and disability in the world. Efforts have been made in vascular tissue engineering and other cardio-regenerative areas in investigating the use of antisense strategies in preventing or curing heart/vessel ailments. Natarajan *et al.* (83–85) carried out in-line studies on ischemia–reperfusion (I/R) injury using RNAi. First, they tried an siRNA against prolyl 4-hydroxylase-2 gene (PHD2) to inhibit the expression of PHD2 *in vivo*, which proved to promote the transcription of hypoxia-inducible factor-1 (HIF-1) and attenuate myocardial IR injury. Next, they found that after activating HIF-1 *via* siRNA-silenced PHD2, the amounts of certain I/R-induced cytokines and chemokines were decreased both *in vitro* and *in vivo*. Subsequently in their most recent report, by following the same methodology, the researchers detected up-regulation of a cardioprotective cytokine—adiponectin, which dramatically decreases in diabetic patients. These studies, conducted on murine microvascular endothelium and intact murine hearts of both wild type and obese/diabetic ones suggest a feasible antisense-based method to protect diabetic hearts from I/R injury.

The deployment of siRNA targeting phospholamban (PLB) in cardiomyocytes effectively silenced PLB expression in neonatal rat myocytes and increased Ca^{2+} uptake affinity, providing another potential candidate for treating heart failure (86). Nevertheless, more efforts are required as the siRNA based treatment had its shortcomings—transient gene silencing and relatively low transfection efficiency, especially *in vivo*. Compared to conventional delivery means such as lipofection, Fechner *et al.* (87) innovatively used a short hairpin RNA (shRNA) against rat PLB, delivered using an adenoviral vector, which kept producing shRNA at an average level over a 13-day period and eventually led to inhibition of PLB with higher specificity and stability.

Known as a negative regulator of cardiac contractility (88), PKCα attracted the attention of both antisense investigators and heart tissue engineers. El-Armouche and co-

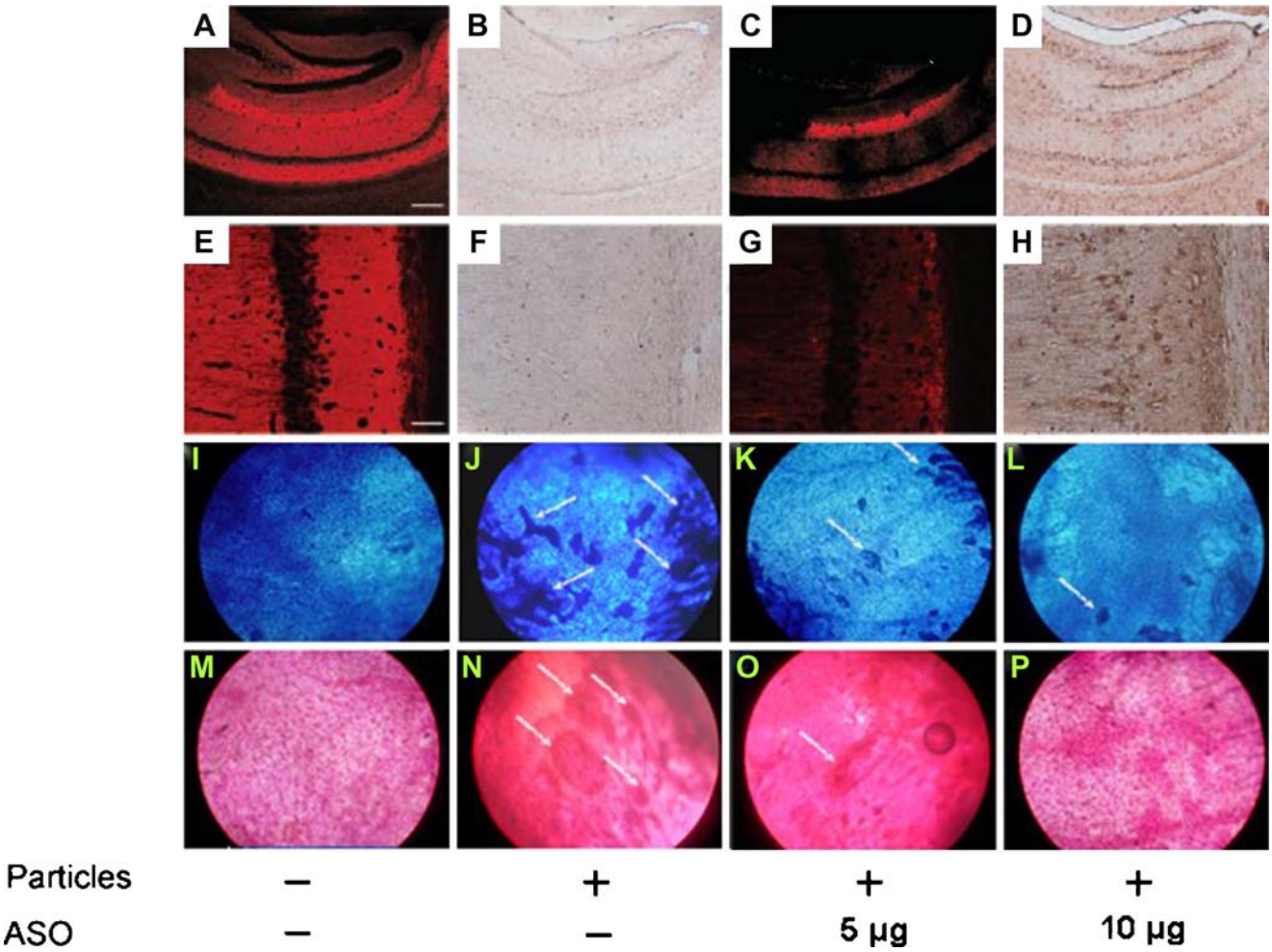

Fig. 4. Antisense strategies in enhancing tissue regeneration. **A**–**H** Silencing of PrP^{C} in chimeric mice with transgenic lentiviral shRNA. **A**, **E** Immunohistochemistry of PrP^{C} expression in hippocampal sections of a control mouse without treatment with shRNA, indicating strong expression of PrP^{C}. **C**, **G** Immunohistochemical analysis of PrP^{C} in a chinmeric mouse treated with lentiviral shRNA, showing a clear inhibition of PrP^{C} expression as compared with control. **B**, **F**, **D**, **H** Expression of EGFP in hippocampal sections of a control mouse or a chimeric mouse. **B**, **F** No obvious EGFP staining was observed in the control; **D**, **H** Visible EGFP was seen in the chimeric hippocampus, suggesting the presence of lentivirus. (**A**–**H** from reference 92) **I**–**P** Effect of antisense oligonucleotide (ASO) on Co–Cr–Mo particle-Induced osteolysis. Toluidine blue staining indicates the lacuna with bone resorption. **I**–**L** ASO proved effective in preventing osteolysis in a dose-dependent manner. **M**–**P** Histochemical staining with TRAP demonstrated that ASO treatment was effective in inhibiting the TRAP activity, i.e. suppressing the content and the activity of osteoclasts. (**I**–**P** from reference 82).

workers (89) evaluated the PKCα silencing efficacies of a certain shRNA in neonatal rat cardiac myocytes (NRCMs) and engineered heart tissues (EHTs), revealing that shRNA delivery mediated by adenoviral vector could efficiently inhibit the expression of PKCα in NRCMs and enhance contractility in EHTs. This study provides a new insight in the research and development of artificial heart organs and other cardiovascular devices, that combining antisense techniques to regenerative tactics would be of great help in practical treatment of heart/vessel failures caused by complicated physiological and pathological factors.

Neural Degeneration and Other Diseases

Multiple factors can hinder or have a negative effect on the regeneration of central nervous system (CNS), which naturally inspired researchers to silence these factors using antisense strategies. One group (90) has designed a hammerhead ribozyme against human alpha-synuclein, an important factor involved in neurodegenerative diseases such as Parkinson's disease. Delivered with adeno-associated virus (AAV) vector, the ribozyme had an obvious effect in rescuing TH-positive nigra neurons that get damaged in the MPP+ model of Parkinson's disease. Another group (91) synthesized siRNAs against three axon growth factors including Rho-A, and demonstrated that neurite outgrowth improved on silencing these genes—most notably on silencing Rho-A. Pfeifer and co-workers (92) explored the application of antisense technologies in prion disease—another fatal neurodegenerative disorder which can be characterized by PrP^{Sc} accumulation. Their efforts in using lentiviral-shRNA to inhibit PrP^{Sc} accumulation in scrapie-infected neuronal cells, together with further *in vivo* attempts, suggest the feasibility of using RNAi techniques in treating prion disease (Fig. 4).

siRNAs have also been used in the treatment of myogenesis (93) where normal myogenesis was restored in

cellular models by means of siRNA-mediated suppression of myogenic inhibitory basic helix–loop–helix factor B3 (BHLHB3), which is highly expressed in Inclusion-Body Myositis (IBM), an inflammatory muscle disease. Moreover, an ongoing trial employed ODNs to effectively suppress the expression of co-stimulatory molecules in autologous dendritic cells and block T cells in the pancreatic lymph nodes, showing that the clinical onset of Type I diabetes were prevented and even reversed. Clinical trials based on this study are being synchronously conducted (94,95). siRNA and ODNs have also been used in various other diseases/areas like liver fibrosis (96), renal regeneration (97).

Antisense in Shaping Optimal Microenvironments

With the assistance of antisense techniques, cellular survival and functions can be regulated to promote efficient regeneration. Once the cells settle down and a stable cell population is established, a suitable environment is required for their optimal performance and would eventually influence the curative effects. Besides providing appropriate extracellular matrices (ECM) to support cell growth and communication, the microenvironment should also accommodate the transplanted cells well with native residents while allowing at most mild-to-moderate immunoreactions therein. Furthermore, this niche is autonomically maintained by the cells and the creation of neo-ECM should be well controlled during the process of regeneration, as the overproduction of some ECM components will hamper the therapeutic progress. In this section, we will focus on the potential use of antisense strategies in improving cell–cell and cell–matrix/tissue interaction as a solution in treating unwanted or unexpected events taking place during tissue repair, organ transplantation and other clinical trials of regenerative medicine.

Amongst the earliest cases of the use of regenerative medicine is skin regeneration from wound and burns. During the healing process, the deposit of scar tissue is a natural step; however, its abnormal formation generated by overproduction of collagen and subsequent fibrous tissue may cause complicated functional problems and should be restrained. Transforming-growth factor-β (TGF-β) is known to be involved in repair and regeneration of tissues, but a constant TGF-β activity leads to excessive fibrosis and ultimately scarring. Accordingly, researchers designed antisense products targeting the signaling pathway of TGF-β, aiming to reduce scar formation. Cordeiro *et al.* (98) developed chemically modified ODNs targeting TGF-β and demonstrated that these antisense strands could prevent ocular scarring mediated by TGF-β *in vitro* and *in vivo*, providing a way to overcome scarring after glaucoma surgery. Alternatively, Smads, a family of intracellular regulatory proteins that modulate the activity of TGF-β ligands (99), have been chosen as target of RNAi. Investigators have reported the silencing of Smad2 and Smad3 of this family with specific siRNA (100,101), resulting in a significant reduction of pro-collagen expression and ECM deposition, as well as an attenuation of fibrosis. Although more clinical trials are in demand, this technique is potentially of great commercial value considering the huge market in scar-free wound repair and skin regeneration.

The failure of regeneration in central nervous system (CNS) after injury is another area where antisense techniques can be used. One important reason of this failure is that a glial scar develops after an injury to CNS tissue and it forms a biochemical/physical barrier to axon advancement (102). Astrocytes deposit chondroitin sulfate proteoglycans (CSPGs) which take part in the intervention of axon regeneration by their glycosaminoglycan (GAG) side chains. It is hypothesized that the use of siRNA targeting chondroitin polymerizing factor (ChPF), an important enzyme in the CSPG biosynthetic pathway, could be effective in alleviating the inhibition of axon regeneration (103). Experimental data showed that the expression of ChPF could be decreased in both the Neu7 astrocyte cell line and primary rat astrocytes, resulting in a decrease in the production of CSPG-GAG chains in the conditioned media (CM). Additionally, the CM from Neu7 cells treated with ChPF siRNA led to an increase in axonal outgrowth from cerebellar granule neurons as compared to neurons cultured in CM from control cells.

Therapeutic cells employed in regenerative medicine have two main origins: autologous or allogeneic. Allogeneic cells are a desirable source thanks to their higher availability, but a major challenge in their use is the possibility of acute or chronic rejection, which results from the recognition of foreign leukocyte antigens (HLA) from the implants/grafts (104). To evade the risk of immunogenicity and thus improve histocompatibility, antisense techniques have been adopted to suppress HLA or HLA subgroups for promoting immunologic tolerance. This strategy is supposedly superior to the conventional HLA-antibody administration, which results in chronic immunosuppression leading to severe symptoms of morbidity and mortality. Individual groups have achieved stable silencing of HLA with lentiviral-based systems carrying specific shRNA. Their investigations have demonstrated that shRNA targeting β2-microglobulin (β2m) or HLA heavy-chain can be effective in inhibiting HLA class I expression in Hela, B-lymphocyte cell lines and peripheral blood monocytes (105,106), while shRNA specific to pan-Class I HLA helped in reducing surface expression of HLA in grafted cells, thereby preventing antibody-mediated cell lysis and CD8+ T-cell response (107). It should be feasible and promising to

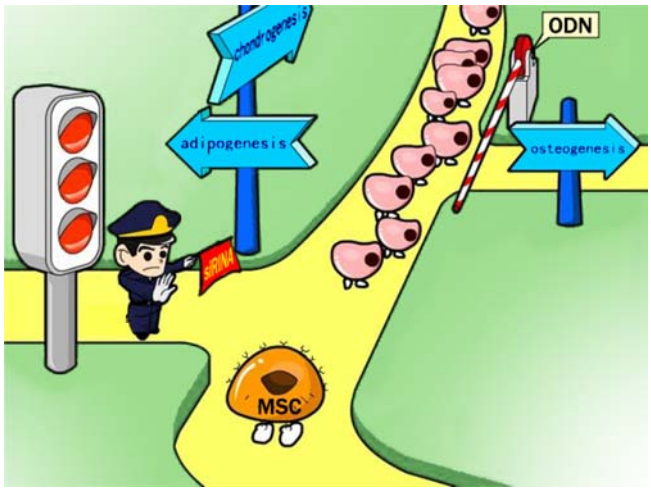

Fig. 5. The potential of antisense in regulating cell differentiation. Antisense strategies could be used to silence specific genes, directing cell differentiation towards lineage of interest.

avoid immune rejection *via* antisense strategies by regulating HLA expression in transplanted cells.

Ischemia/reperfusion injuries, caused during organ transplantation negatively affect the long-term survival of grafts. The local recruitment of inflammatory cells by cytokines and cellular surface adhesion molecules is a primary cause (108), and specifically, intercellular adhesion molecule-1 (ICAM-1) has been identified as a key element in stimulating neutrophils, T cells and macrophages and triggering subsequent injuries. According to this evidence, the donor animals were treated with ODN specific to ICAM-1 before their kidneys were transplanted into recipient rats (109). The results showed that the rats in ODN-treated group survived longer than those in control groups, and apparent changes in pathological reports were found in kidney specimens without ODN treatment, including interstitial fibrosis, focal glomerular sclerosis and other signs of macrophage/lymphocyte infiltration. Similarly, transfection with ODN against ICAM-1 had a positive effect on prevention of acute cardiac allograft rejection (110), as another proof in feasibility of using antisense approaches for immunosuppression in regenerative trials.

PERSPECTIVE: ANTISENSE LIGHTING THE CELL ROUTE?

Stem cells, including embryonic stem cells (ESC), mesenchymal stem cells (MSC) and other adult stem cells, have become more promising than ever in regenerative medicine. Their remarkable capability of differentiating into various cell types ranging from cartilage to blood to nerve cells has made them ubiquitous in tissue engineering and cell therapy. In the NIH report entitled "regenerative medicine", stem cells were in the titles of all the six chapters (five published in 2006 and one updated in 2007), which comprehensively introduced the use of stem cells in different regenerative fields. Conventional ways of inducing stem cells into specific lineages include: (1) application of conditioned medium, which has been well established; (2) application of particular biological signals such as cytokines and growth factors; (3) set-up of co-culture systems containing stem cells and tissue-derived differentiated cells (111,112). Then there are gene delivery-assisted approaches, utilizing transfection and continuous, sustainable and endogenous expression of certain cytokines or transcriptional factors to enhance differentiation.

Here we hypothesize that the application of antisense technologies could be an alternative tool to direct cell differentiation towards lineage of interest, by silencing specific genes which could block the desired route of differentiation or guide the cells to multiple lineages. Unlike elevating gene expression to promote cell development, gene silencing has its unique advantages. For example, the expression of genes in the presence of strong inhibitors can rarely meet the expectation even after successful transfection, but the suppression of such inhibitors would improve the progression towards differentiation. Some key regulators (e.g., Runx2) that play a part in osteochondral differentiation can lead the progenitor cells to differentiate into chondrocytes first and later cause the chondrocytes to go through osteogenesis. Osteogenesis is an undesirable outcome for cartilage tissue engineers. Therefore, a siRNA product could be designed to target an osteogenic inducer or a typical osteogenic marker. It is within the authors' vision that these types of antisense technologies can be individually applied or used in combination with other means of gene therapy at different stages, signaling the cells to make favorable decisions at different crossroads on their complicated journey of commitment (Fig. 5).

Recent reports by orthopedists and microbiologists provide evidence for the theoretical feasibility of our proposal. Lietman and colleagues (113) managed to inhibit the expression of Gsα with ODN and obtained an enhanced expression of both Runx2/Cbfa1 and collagen type I (α2), indicating the differentiation of MSCs towards osteoblast-like phenotype. Similarly, three other groups have regulated cardiac, adipocytic and neuronal differentiation by the means of RNAi techniques (114–116). These efforts in basic cell biology have substantial implications for the future use of antisense strategies in directing cell lineage for engineering purposes.

ACKNOWLEDGEMENTS

This study is supported by Grant ARC 10/06, Ministry of Education (MoE), Singapore. The authors are grateful to Mr. He Junjing for the drawings in Fig. 5.

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