Emerging potential of gene silencing approaches targeting anti-chondrogenic factors for cell-based cartilage repair

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Abstract

The field of cartilage repair has been exponentially growing over the past decade. Here we discuss the possibility to achieve satisfactory regeneration of articular cartilage by means of human mesenchymal stem cells (hMSCs) depleted of anti-chondrogenic factors and implanted in the site of injury. Different types of molecules including transcription factors, transcriptional co-regulators, secreted proteins and microRNAs have been recently identified as negative modulators of chondroprogenitor differentiation and chondrocyte function. We review the current knowledge about these molecules as potential targets for gene knockdown strategies using RNA interference (RNAi) tools, that allow the specific suppression of gene function. The critical issues regarding the optimization of the gene silencing approach, as well as the delivery strategies are discussed. We anticipate that further development of these techniques will lead to the generation of implantable hMSCs with enhanced potential to regenerate articular cartilage damaged by injury, disease, or aging.

Keywords

Gene silencing, RNA interference, cartilage repair, chondrogenesis, mesenchymal stem cells, anti-chondrogenic regulators

Abbreviations

AIMP1	aminoacyl tRNA synthetase complex interacting multifunctional protein 1
ANGPTL4	angiopoietin-like 4
BMP	bone morphogenetic protein
circRNA	circular RNA
ECM	extracellular matrix
EMT	epithelial-to-mesenchymal transition
ERK	extracellular signal-regulated kinase
EV	extracellular vesicle
FGF	fibroblasts growth factor

GAG	glycosaminoglycan
hMSCs	human mesenchymal stem cells
IGF	insulin-like growth factor
IHH	indian hedgehog
IKK	IkB kinase
JNK	c-Jun N-terminal kinase
KDM2A	lysine demethylase 2A
lncRNA	long non-coding RNA
MAPK	mitogen-activated protein kinase
MCC	mandibular condylar cartilage
MEK	mitogen-activated protein kinase kinase
MMP	matrix metalloproteinase
NP	nanoparticle
OA	osteoarthritis
p53R2	p53-inducible ribonucleotide reductase
PAMAM	polyamidoamine
PEI	polyethylenimine
PHD2	prolyl hydroxylase domain-containing protein 2
PLGA	poly(D,L-lactide-co-glycolide)
PLL	poly-L-lysine
PNA	peptide nucleic acid
QD	quantum dot
RISC	RNA-induced silencing complex
RNAi	RNA interference
RUNX2	Runt-related transcription factor 2
shRNA	short hairpin RNA
siRNA	short interfering RNA
SOX	SRY(sex determining region Y)-box

TF	transcription factor
TGF	transforming growth factor
TGIF1	TGF-β induced factor homeobox 1
VEGF	vascular endothelial growth factor

Introduction

Currently, trauma and age-related cartilage disorders represent a major cause of morbidity globally and result in enormous costs for health and social care systems [1]. As a consequence, there is an urgent need for interventions that can help to prevent these disorders and therapies to effectively treat them. To date, these objectives are far from being achieved and optimal cartilage reconstruction still represents an unmet clinical need [2, 3].

Cell-based therapy for cartilage repair aims at not only filling the tissue defect with a substitute, but also reconstituting the structure, physicochemical properties and functionality of the hyaline matrix, possibly promoting intimate integration with the resident tissue [4, 5]. Ideally, this is achieved by implanting a sufficient number of mature chondrocytes or undifferentiated progenitor cells with a high chondrogenic potential [6]. Recently, experimental therapies using mesenchymal stem cells (MSCs) have been receiving an increasing amount of interest, mostly due to the ease of isolation and their regenerative potential [7-9]. Unfortunately, the use of native unaltered chondrogenic cells, either chondrocytes or MSCs, hasn't fulfilled expectations, with the underlying mechanisms of tissue regeneration still poorly understood [10]. This has laid the basis for the experimental transplantation of genetically modified cells, as a revolutionary approach to exploit the full potential of the therapeutic cells. Chondrocytes and more recently MSCs have been extensively modified using a variety of techniques allowing the manipulation of critical genes that can directly or indirectly affect chondrogenesis and/or production of cartilage matrix [11]. This has been made possible by the recent advancements in cell engineering technologies, together with a progressive understanding of the molecular basis of chondrogenesis.

Chondrogenesis is the process by which cartilage is developed and occurs via mesenchymal cell condensation and chondroprogenitor cell differentiation (reviewed in [12, 13]). Several molecular pathways, mechanical stimuli and morphological cell features contribute to the activation of the signals that drive the chondrogenic process, as well as the transition through the different maturation stages. A critical role is played by (i) soluble factors, e.g. fibroblasts growth factors (FGFs), transforming growth factors (TGF-βs), insulin-like growth factors (IGFs), bone morphogenetic proteins (BMPs) and WNTs, (ii) cell adhesion molecules, e.g. N-cadherin and integrins, and (iii) intracellular signalling molecules, including mitogen-

activated protein kinase (MAPKs), c-Jun N-terminal kinases (JNKs), protein kinase A/C and protein phosphatase 2A/2B [14].

While the transcriptional control of chondrogenesis remains to be fully elucidated, it is well established that SRY (sex determining region Y)-box 9 (SOX9) is the pivotal transcription factor in developing and adult cartilage [12]. SOX9, in concert with L-SOX5 and SOX6 (collectivelly referred to as the SOX-trio), regulates cartilage formation and maintains the chondrocyte phenotype in articular cartilage by stimulating the expression of specific genes, including collagen type II, IX and XI, aggrecan and cartilage oligomeric matrix protein (COMP) [12]. SOX9 supports chondrogenesis over osteogenesis by negatively regulating Runt-related transcription factor 2 (RUNX2), the major transcription factor required for osteoblast differentiation, and WNT signaling via nuclear β-catenin phosphorylation [15]. In growth plate cartilage, inhibition of SOX proteins leads to chondrocyte maturation and hypertrophy, that is required for longitudinal bone growth. Hypertrophic chondrocytes are characterized by increased expression of parathyroid-related peptide, indian hedgehog (IHH), vascular endothelial growth factor (VEGF), collagen type X and matrix metalloproteinase 13 (MMP13) [12]. Differences between the regulation and gene expression profiles of growth plate and articular chondrocytes reflect the different functions of the two types of cartilage. This relates to the necessity to maintain cell proliferation and maturation in the growth plate, while preventing chondrocyte hypertrophy in articular cartilage [16].

Traditionally, engineering techniques for the enhancement of chondrogenesis have primarily focused on the forced expression of growth factors, mainly IGFs, FGFs, TGF-βs and BMPs, or pro-chondrogenic transcription factors, e.g. SOX5, SOX6 and SOX9. Alternatively, chondroprotective factors, cytokines and inhibitors of catabolic pathways have been proposed (a comprehensive list of these factors has been reported previously in [11]). While forcing the expression of a gene during *ex-vivo* cell culture is relatively simple, the process cannot be tightly controlled, especially in case of permanent overexpression [17]. Excessive gene overexpression may cause imbalance in gene dosage, affecting protein synthesis, folding and localization, assembly of multiprotein complexes and the machinery of gene regulation. Flooding the cell with recombinant proteins may lead to excessive consumption of cellular resources, affecting cell growth rate and metabolism [18]. While these aspects are often overlooked, they represent serious concerns in view of a wider applicability and clinical translation of overexpression-based strategies.

More recently, alternative strategies to engineer chondrocytes or MSCs for enhanced cartilage repair have emerged. These approaches are based on the silencing of anti-chondrogenic factors and aim to suppress the function of proteins negatively affecting chondrogenesis, or whose expression is detrimental for the chondrogenic potential. The main purpose of this review is to report evidence and challenges regarding the silencing of anti-chondrogenic factors as an attractive option for cell-based cartilage repair, pointing out that the optimization of such an approach is needed.

Gene silencing for chondrogenesis: potentialities and challenges

Gene silencing strategies for chondrogenesis use RNA interference (RNAi) tools, e.g. short interfering RNAs (siRNAs), short hairpin RNAs (shRNAs) and microRNA inhibitors (antimiRs), that allow the specific suppression of the function of a gene. Importantly, a number of early proof-of-principle studies in animal models and early phase clinical trials have supported the use of RNAi as therapeutic agents in different fields, without significant toxicity (reviewed in [19]). In sharp contrast with overexpression strategies, RNAi research tools are inspired by the natural phenomena of suppression of gene expression operating in various forms of life for genome integrity, defense against viruses or exogenous nucleic acids and transcriptional regulation [20]. Moreover, RNAi techniques require a limited utilization of cellular resources and do not overload the cell or the extracellular environment with transgene products [17]. Thus, it is likely that the cells are less stressed by RNAi rather than overexpression treatments.

Gene silencing represents a simple and powerful molecular tool to investigate the specific function of genes during chondrogenesis, providing critical insights into cartilage-specific regulatory mechanisms and chondro-regulators. Interestingly, the accumulating knowledge has led to the development of new therapeutic strategies based on the silencing of negative chondro-regulators. The products of these genes can be classified as anti-chondrogenic or pro-hypertrophic, as they can take part in lowering the chondrogenic potential of the cells, or in undermining the functionality of the newly formed tissue.

Research based on gene silencing for the enhancement of chondrogenesis is still in its infancy and, besides a few exceptions, has not proceeded to extensive *in vivo* testing yet. Nevertheless, increasing evidence strongly suggests that the silencing of key anti-chondrogenic regulators may be effectively employed to enhance or even induce chondrogenesis, stimulate the production of cartilage matrix, and improve or stabilize the

chondrocytic phenotype [21-24].

Without intending to be exhaustive, we provide a brief overview of critical issues related to the application of gene silencing for chondrogenesis, with a focus on MSCs, and the barriers that they pose for RNAi delivery. Here different aspects must be taken into account, as the peculiar characteristics of MSCs make them harder to transfect than conventional cell lines [25]. Transfection or transduction efficiency in MSCs is highly species, source and donor-dependent, and can be significantly affected by *in vitro* culture conditions, including composition of culture medium, cell density and proliferation rate, passage number and cell distribution [26]. This is a general principle related to transfection, but gene manipulation for chondrogenesis poses an additional obstacle which is the need to grow the cells in a 3D system, a very unfavourable condition for transfection [27]. However, 3D culture is essential to recapitulate the chondrogenic process *in vitro*, and maintain the therapeutic potential of MSCs for subsequent *in vivo* implantation.

In order to overcome the aforementioned obstacles, MSCs can be transduced with viral vectors. This rapidly induces permanent silencing of specific molecules that affect the chondrogenic process. A variety of viral vectors are available to achieve this goal, each of which has advantages and limitations [28]. The recombinant adeno-associated vectors are nowadays regarded as the most potent gene delivery vehicles, as they can efficiently and durably transduce articular chondrocytes, synoviocytes, MSCs and other relevant cell sources constituting the surrounding tissues of cartilage [11]. Unfortunately, virus-based methods for gene silencing pose many issues in regards to their clinical translation and applicability. Safety concerns have been raised in relation to their use for gene silencing *in vivo*, due to potential immune response of the host, possible mutagenesis, and lack of specificity in addition to high production costs. As a consequence, virus-free approaches of transient transfection are being widely explored.

It is now widely accepted that non-viral vectors are preferable for *in vivo* use since they are safe, easy to handle, cost-effective, and they have better chances for clinical translation (recently reviewed in [29]). In addition, this choice may be convenient for engineered cells to prime the regeneration process by triggering repair mechanisms as well as to stimulate an active involvement of the host tissues in the repair of the defect [30]. Transient techniques for gene silencing are receiving an increasing amount of interest, since the progress in RNAi techniques has led to the development and optimization of cell transfection in 3D microenvironment, with exogenous scaffolds being able to greatly increase efficiency and durability of gene

silencing [29]. Interestingly, RNAi transfection may be performed by growing cells within a 3D matrix in which the RNAi has been previously entrapped or cross-linked [31]. Alternatively, cells may be transfected with RNAi molecules prior seeding onto scaffolds or 3D-culture, under conditions that guarantee a prolonged suppression of the target gene [32].

Non-viral methods delivering RNAi for chondrogenesis

Since the ECM produced by the cells during chondrogenic differentiation represents a considerable obstacle for transfection, RNAi delivery is likely the major critical issue for successful gene silencing. Effective gene knockdown requires efficient uptake of the RNAi molecules by the cells, and their retention in the cytoplasmic compartment where the RNAi machinery is located. Only here the inhibitor can enter the endogenous RNAi pathway, integrating into the RNA-induced silencing complex (RISC) and leading to silencing of the target [20, 33]. The passage of the inhibitor into the cell through the cell membrane is therefore the limiting step. As a consequence, the effectiveness of the approach mainly relies on the physico-chemical strategy that is adopted to overcome this obstacle. Naked oligonucleotides including RNAi-based inhibitors have very little chance for a significant cell uptake, mostly due to the highly negative charge. To promote their delivery into the cell, the use of a proper carrier is required. The carrier can mask the anionic groups of the nucleotide backbone while interacting with cell surface moieties, thereby inducing internalization or endocytosis of the RNAi-based inhibitors [34]. Importantly, the specific characteristics and composition of the extracellular matrix should be considered for the choice of the appropriate delivery strategy, especially for the cartilage tissue [35]. Indeed, proteoglycans and fibrous proteins can prevent the diffusion of anionic molecules, or act as competitor during RNAi/carrier assembly, thereby affecting its cellular uptake.

Among the different methods for RNAi delivery into chondrocytes and MSCs (Table 1), liposomal-based systems are extremely popular, being easy to use and widely available as commercial products with different formulations, e.g. INTERFERin™, Oligofectamine™ and Lipofectamine™ reagents [36-38]. Interestingly, liposome-based systems in combination with scaffolds have been proposed as potential tool for efficient, controlled and localized RNAi delivery for tissue engineering and cartilage regeneration [39]. Among others, *Ollitrault et al.* developed a novel method applicable to primary chondrocytes or MSCs, by seeding the cells

on collagen sponges prior to transfection of siRNA complexes targeting collagen type 1 and *HTRA1*, a secreted enzyme that is proposed to regulate the availability of IGFs, to induce chondrogenesis [36]. Unfortunately, lack of colloidal stability, moderate cytotoxicity and potential immunoresponse restrict the therapeutic value of liposome-based carriers, as well as their *in vivo* application [40]. Nevertheless, research aimed at overcoming these issues is ongoing. Recently, non-phospholipid liposomes (stereosomes) with single-chain amphiphiles and high content of sterols were proposed as better alternatives to traditional cationic liposomes. This delivery system was successfully adopted to knockdown the expression of *Noggin*, a specific antagonist of BMP. Notably, *Noggin* knockdown in MSCs cultured both *in vitro* and *in vivo* promoted osteogenesis and bone repair [41].

Most of the recent developments in non-viral vectors for RNAi delivery into MSCs mainly rely on polymers, often in the form of nanoparticles (NPs). Natural polymers, particularly polysaccharides bearing amine groups (i.e. chitosan), can be used to deliver nucleic acids into MSCs. At the same time, synthetic polymers are commonly used in the effort to optimize the characteristics of these carriers. Various synthetic polymers have been proposed, thanks to their limited cytotoxicity, easy production and high transfection efficiency [34]. Among them are the cationic polymers poly-L-lysine (PLL) and linear or branched polyethylenimine (PEI), polyamidoamine (PAMAM) dendrimers, and poly(D,L-lactide-co-glycolide) (PLGA). *Jeon et al.* fabricated PLGA NPs loaded with *SOX9* plasmid DNA or protein and coated with PEI and *RUNX2* siRNA [21, 42]. The loaded NPs were efficiently internalized by hMSCs, increasing chondrogenic differentiation both *in vitro* and *in vivo*. In addition to polymers, cell penetrating/permeable peptides in the form of NPs have been explored as carriers for RNAi delivery. *Yan et al.* generated and delivered peptidic NPs complexed to *NF-kB* siRNA as a therapeutic approach to mediate chondroprotective effect by preserving cartilage homeostasis in chondrocytes from patients with osteoarthritis (OA) via *NF-kB* suppression [43].

Water soluble quantum dot (QDs)-based nanocarriers were initially developed as tools for investigating cellular events [44], and now represent an interesting option for RNAi delivery into chondrocytes and MSCs. QDs can be functionalized with different moieties (e.g. Arg-Gly-Asp peptide and PEG) to maximize cell uptake, biocompatibility, and targeting capacity. Interestingly, *Xu et al.* developed multifunctional QD-based nanocarriers to enhance chondrogenic differentiation and simultaneously suppress hypertrophy of human MSCs [45]. The resulting construct was capable of carrying the hydrophobic chondrogenic inducer

kartogenin while binding a siRNA against the pro-hypertrophic regulator *RUNX2* via interaction with the Arg-Gly-Asp peptide. *Wu et al.* developed QDs functionalized with sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate and fluorescently labeled to generate a novel traceable carrier for siRNA molecules. The system was successfully applied to transfect *SOX9* siRNA into hMSCs, also allowing non-invasive imaging of siRNA transport both *in vitro* and *in vivo* [46].

Further strategies for siRNA delivery were recently introduced thanks to the increasing knowledge on membrane vesicles. Mounting evidence suggests that extracellular vesicles (EV), particularly exosomes, play a crucial role in the transfer of RNA molecules between cells as part of cell communication processes. Different encapsulation approaches have been explored for loading exosomes with DNA or siRNA molecules [47, 48]. Thus, exosomes represent interesting natural carriers potentially exploitable for the delivery of RNAi to a variety of cell types and tissues, including cartilage. Among membrane vesicles-based delivery systems, nanoghosts derived from mesenchymal stem cells are currently under investigation [49, 50]. Interestingly, nanoghosts exhibit inherent targeting capabilities, versatile loading capacity and immuno evasiveness, making them highly attractive for the development of novel gene silencing-based strategies.

Identifying candidate targets for gene silencing to promote chondrogenesis

Through the gene silencing approach numerous scenarios have been explored, including increase of chondrogenic potential of cell populations, stabilization of the differentiated phenotype, or improvement of cell survival and anabolic properties. This has led to the identification of many anti-chondrogenic factors that can be potentially targeted in primary cell populations to promote cartilage tissue repair *in vivo*. Since many factors and complex networks of interactions are responsible for the regulation of the chondrogenic potential, different classes of candidate targets need to be considered. These factors include intracellular molecules, e.g. transcriptional (co-)regulators, matrix components, extracellular signaling molecules and non-coding RNAs.

Silencing of transcription factors

Transcription factors (TFs) have long been considered as the central regulators of gene expression and, as such, among the major drivers of cell differentiation and production of ECM. While many TFs have been

shown to exert a negative role with respect to chondrogenesis and cartilage production [51], only few have been validated in primary chondroprogenitors or chondrocytes for gene silencing approaches aimed at inducing chondrogenesis (Table 2).

RUNX2 is the best known chondro-inhibitory TF during the commitment of mesenchymal progenitors, as it competes with SOX9 to induce osteogenic determination. It also promotes the terminal differentiation of chondrocytes during the later stages of chondrogenesis, contributing to cartilage hypertrophy and calcification [52]. Attempts have been made to target *RUNX2* for the enhancement of the chondrogenic potential, or for inhibiting hypertrophy and improving the stability of the newly formed cartilage. Jeon and co-workers were able to differentiate hMSCs into chondrocytes *in vitro* and *in vivo* by treating the cells with PLGA nanoparticles coated with a *RUNX2*-targeting siRNA and loaded either with *SOX9* plasmid DNA or protein [21, 42]. In other studies, *RUNX2* knockdown could significantly enhance the chondrogenic potential of human cartilage progenitor cells [53] and OA-like chondrocytes [54].

TGF-β induced factor homeobox 1 (TGIF1) is a highly conserved transcriptional regulator that participates in the transmission of nuclear signals during development and in the adult. It is a transcriptional target of TGF-β and activin signaling, and as transcriptional repressor of SOX9 was found to be downregulated during chondrogenesis [55]. *In vivo* implantation of *Tgif1*-depleted MSCs enhanced fibrocartilage production and healing of tendon-to-bone insertion in an animal model of supraspinatus tendon tear-and-repair [56, 57]. Notably, the newly-formed tissue displayed stronger expression of chondrogenic proteins and greater maximum load at failure and stiffness, performing better both histologically and functionally. SHOX2 is another member of the homeobox protein family. Interestingly, *Shox2* deletion in early chondrocytes stimulated hypertrophy, while deletion in MSCs enhanced early chondrogenesis due to increased BMP activity, without signs of hypertrophic maturation [58].

Epithelial-to-mesenchymal transition (EMT) regulators play a decisive role in chondrogenesis. These proteins are directly responsible for the determination and stability of the cell phenotype, as they regulate the expression of lineage-specific transcription factors (e.g. SOX9 and RUNX2) and adhesion molecules. Among the EMT-regulators, TWIST1 and SLUG/SNAIL2 were validated as targets for enhancing the chondrogenic potential [59-61]. We previously showed that treating hMSCs from different sources with a siRNA against *SLUG* induced chondrogenesis and production of cartilage ECM in a 3D-microenvironment.

Interestingly, even in the absence of TGF-β, *SLUG* depletion alone was sufficient to direct hMSCs towards the chondrocyte lineage [61].

Silencing of intracellular co-regulators and enzymes

While transcription factors are traditionally regarded as the major drivers of differentiation, the role of transcriptional co-regulators, cell cycle regulators and intracellular enzymes should not be underestimated. Different factors belonging to these families have been proposed as candidate targets for gene silencing to improve chondrogenic differentiation (Table 3).

Cell cycle regulators are responsible for the integration of diverse extracellular signals and their participation in coordinated proliferation and differentation of chondrocytes [62]. Thus, manipulation of these genes may greatly affect chondrogenesis. Knockdown of the transcriptional coactivator *YAP* in articular chondrocytes increased the expression of *Sox9*, collagen type II and aggrecan, with a concomitant decrease of collagen type I [63]. The cyclin-dependent kinase inhibitor *p16* is overexpressed in human OA chondrocytes, and its knockdown induced proliferation and recovered the expression of collagen type II and aggrecan [64]. Similarly, lentiviral-mediated knockdown of *p21* in murine induced pluripotent stem cells stimulated proliferation during expansion and increased matrix production, while limiting the synthesis of collagen type I and X [22]. Silencing of the cell growth regulator *Gadd45b* blocked terminal differentiation and expression of catabolic enzymes and collagen type X in 3D-pellet cultured murine chondrocytes [65]. Importantly, this study proposed *Gadd45b* as a critical regulator of the hypertrophic transition, hence a relevant target to stabilize the chondrogenic phenotype of implanted cells.

Within the class of intracellular enzymes, four protein kinases were successfully targeted for the enhancement of the chondrogenic potential, i.e. mitogen-activated protein kinase kinase 5 (MEK5), extracellular signal–regulated kinase 5 (ERK5), and the IkB kinases (IKK) α/β. siRNA-mediated knockdown of *MEK5* and *ERK5* in hMSCs enhanced the production of glycosaminoglycans (GAGs), cartilage ECM proteins and pro-chodrogenic regulators, even in the absence of TGF-β supplementation [66]. Silencing of both *IKK* kinases in human OA chondrocytes led to increased production of cartilage matrix and concomitant inhibition of collagen type X and reduced formation of calcium deposits [67]. To date, targeting of 3 non-kinase intracellular enzymes has been reported, i.e. the p53-inducible ribonucleotide reductase (p53R2), the

prolyl hydroxylase domain-containing protein 2 (PHD2), and the lysine demethylase 2A (KDM2A) [68-70]. These studies not only identified novel candidate targets for the induction of chondrogenesis, but also highlighted the feasibility of manipulating cellular oxygen sensors (PHD2) and the epigenetic machinery (KDM2A) in order to guide the chondrogenic process and cartilage synthesis.

Additional intracellular targets have been suggested, but the effectiveness to induce production of cartilage ECM by silencing these genes needs to be investigated. Among them are the RNA-binding protein TTP [71], the phospholipase Cγ1 [72], the Ras-related proteins RALA [73] and RAB3B [74], the components of the ubiquitin-proteasome pathway E6-AP and UBC9 [75], the anti-apoptotic protein BRE [76] and the actin filament-associated protein AFAP [74].

Silencing of matrix and secreted proteins

As described so far, the targeting of intracellular regulators by gene silencing aims to re-program the cell behavior in order to enhance the chondrogenic phenotype. At the same time, a careful manipulation of the extracellular milieu may offer an attractive alternative, in the effort to remodel the joint microenvironment for optimal cartilage repair. This goal may be achieved by suppression of (i) matrix components whose presence is not desirable in the newly formed tissue, (ii) pro-catabolic enzymes and (iii) anti-chondrogenic extracellular signals (Table 4).

Suboptimal cartilage repair normally leads to the production of tissue containing abundant collagen type I, in addition to collagen type II. This is a serious concern, as such newly formed fibrocartilage lacks the desired mechanical strength that is typical of hyaline cartilage and required to repair joint lesions. Encouragingly, different studies have succeded in circumventing this issue by silencing collagen type I [36, 77]. Remarkably, this strategy did not only prevent production of collagen type I, but also enhanced the synthesis of typical components of cartilage matrix (collagen type II, aggrecan, GAGs) both *in vitro* and *in vivo*. Suppression of collagen type I may therefore help in preventing fibrogenesis while improving the hyaline features of the newly synthesized ECM. Interestingly, other ECM proteins have been targeted to improve the quality of neocartilage produced by chondrocytes and hMSCs, i.e. decorin [78], lumican [79], and asporin [80].

MMPs and aggrecanases are key degradative enzymes in articular cartilage. Strong evidence suggests that

their knockdown via gene silencing can inhibit matrix degradation and help to preserve the integrity of articular cartilage. Lentiviral-mediated knockdown of aggrecanases in primary chondrocytes cultured on a chitosan-gelatin scaffold facilitated the *in vitro* formation of engineered cartilage [81]. In a second study, treatment with MMP2 siRNA increased the expression of adhesion molecules in chicken limb mesenchymal cells, overall stimulating precartilage condensation, chondrogenesis and production of GAGs [82].

Secreted proteins that function as anti-chondrogenic extracellular signals are susceptible of gene silencing-mediated manipulation. Interesting findings concern aminoacyl tRNA synthetase complex interacting multifunctional protein 1 (AIMP1), angiopoietin-like 4 (ANGPTL*4*) and VEGF. AIMP1 negatively regulates the TGF-β signaling by preventing SMAD2/3 phosphorilation, and a siRNA against *AIMP1* was effective in rescuing the chondrogenic potential of dedifferentiated and OA human chondrocytes, *in vitro* and *in vivo* [23]. Treatment of hMSCs with a specific siRNA against *ANGPTL4* prior to chondrogenesis increased the expression of collagen type II and aggrecan, while repressing the expression of MMPs [37]. Finally, suppression of *VEGF* was showed to enhance the chondrogenic potential of human chondrocytes, while providing protection from hypertrophy-inducing stimuli [83].

Other candidate extracellular targets have been proposed but extensive investigations are missing, i.e. IHH [84], NOTCH1 [85], the Wnt-related proteins DKK3 [74] and WNT5A [86], OB-cadherin [87], Fibulin-3 [88] and the orphan G-protein coupled receptor RDC1 [89].

Silencing of non-coding RNAs

Increasing evidence demonstrate that non-coding RNAs, and especially microRNAs, are crucial for the homeostasis and integrity of articular cartilage [90]. Thanks to their ability to simultaneously inhibit the expression of many genes, microRNAs exert a tight and complex control on both chondrogenic differentiation and maintenance of cartilage ECM. This makes them highly attractive targets for gene manipulation strategies (Table 5).

Different microRNAs exert an anti-chondrogenic role by direct repression of SOX9. Among them are *miR-30a* [38], *miR-145* [91, 92], *miR-199a* [93], *miR-495* [94] and *miR-1247* [95]. Silencing of these microRNAs in human articular chondrocytes or hMSCs led to relieved repression of SOX9, whose increased levels stimulate the synthesis of cartilage ECM components. A second member of the SOX-trio, SOX5, was

identified as the direct target of *miR-194*. Suppression of *miR-194* in hMSCs enhanced chondrogenesis and production of cartilage ECM [96].

miR-34a, *miR-142* and *miR-375* were found to be downregulated during the chondrogenesis of limb bud mesenchymal cells [97-100]. *miR-34a* silencing induced the expression of collagen type II and GAGs production, while inhibition of *miR-142* or *miR-375* by peptide nucleic acid (PNA)-based inhibitors promoted proliferation, migration and pre-cartilage condensations of mesenchymal cells *in vitro*. Recently, the BMP receptor type 2, a crucial regulator of endochondral bone formation, was identified as the direct target of *miR-99a*. Knockdown of *miR-99a* led to promotion of early chondrogenesis of rat MSCs, and increased production of cartilage ECM [101]. *Umeda et al.* identified *miR-200a* as a major regulator of the formation of cartilage during mandibular condylar cartilage (MCC) development. Transfection of antimiR-200a in MCC cells or organ culture positively influenced chondrogenesis, inducing the expression of *Sox9* and collagen type II [24].

We previously characterized *miR-221* as a novel regulator of chondrogenesis. Silencing *miR-221* in hMSCs proved effective and sufficient to induce differentiation into chondrocytes, without requiring supplementation with growth factors [32]. Notably, seeding of *miR-221*-depleted hMSCs in cartilage defects led to enhanced cartilage repair *in vivo*, providing a proof of concept for the implantation of miRNA-depleted hMSCs for improved cartilage repair. Interestingly, *Yoshizuka et al.* showed that silencing of the paralogue of *miR-221*, *miR-222*, promoted chondrogenesis and osteogenesis of hMSCs, as well as angiogenesis and bone healing in a rat fracture model [102].

Additional microRNAs have been shown to inhibit the chondrogenesis of hMSCs, i.e. *miR-29a* [103], *miR-138* [104], *miR-181b* [105] and *miR-499a* [106]. However, the feasibility of targeting these microRNAs for the guidance of chondrogenesis still needs to be assessed.

Finally, other types of non-coding RNAs have been shown to regulate chondrogenesis, thereby providing additional candidate targets for gene silencing. Circular RNAs (circRNAs) and long non-coding RNAs (lncRNAs) are large classes of non-coding RNAs, not completely characterized, and whose role in cartilage homeostasis and disease is still obscure [107-109]. *circRNA-CER* and *lncRNA-CIR* were recently found to be overexpressed in OA cartilage. Interestingly, silencing of *circRNA-CER* or *lncRNA-CIR* in OA chondrocytes led to enhanced expression of pro-chondrogenic genes and suppression of catabolic enzymes (MMPs and

aggrecanases) [108, 109].

Concluding remarks and open questions

The idea of producing hMSCs depleted of anti-chondrogenic factors ("silenced hMSCs") represents an intriguing challenge from different perspectives. On the one hand, "silenced hMSCs" represent an ideal system to elucidate and validate the function of a gene or a microRNA in the context of chondrogenesis and cartilage repair. On the other, they may provide a novel therapeutic tool for enhanced cartilage repair (Fig.1). In this review, we present the current knowledge related to anti-chondrogenic genes that can be targeted to enhance the therapeutic potential of primary chondrogenic cells. While a growing body of evidence thus supports the great potential of RNAi-based approaches in this field, diverse issues remain to be addressed, in the effort to generate functional and durable articular cartilage, and to address patient-specific needs.

Cartilage regeneration is not regulated by simple mechanisms supported by one factor, but rather by the interplay of multiple biological factors and downstream signaling cascades [110]. To date, these aspects are not fully understood. Thus, researchers are wondering how to generate the best "silenced hMSCs" for efficient cartilage repair. Which is the anti-chondrogenic factor that once silenced can guarantee the best effect on cartilage formation? While this certainly depends on the biological context and the size of the damage to be repaired, only further experiments with more complex models of cartilage defect will give an adequate response. In this regard, it is imperative to point out that extensive *in vivo* studies are still lacking, and further efforts are needed to unveil the true potential of gene silencing approaches for cartilage repair.

First of all, it is essential to understand how the "silenced hMSCs" are able to influence the microenvironment once implanted, and how the local pathophysiological conditions may affect the performance of the "silenced hMSCs". It is well established that subchondral bone [111], synovium [112] and articular fat tissue [113] may greatly affect the process of chondrogenesis and cartilage repair. Histological and molecular analysis detecting the tissue response and changes that occur following implantation could help in optimizing the "silenced hMSCs" for the repair of a specific type of damage. This aspect deserves particular attention and directly relates to the achievement of a "dynamic state of communication" between endogenous and implanted cells (Fig. 1), and ultimately to the formation of well organized neocartilage with proper biomechanical and functional properties [114]. In this context, a major

contribution will derive from preclinical studies implanting “silenced hMSCs” into critical size chondral or osteochondral defects in large animal models, such as the equine model, that better mimic the human physiology.

Another important aspect is the combination of the “silenced hMSCs” with exogenous scaffolds. This is particularly relevant, since delivering a sufficient amount of RNAi molecules to cells is a notoriously difficult task. An important branch of biomaterials science is aimed at studying the optimal combination of the cells with convenient chemically or physically-modified scaffolds. This can sustain the gene silencing process, while providing a favourable microenvironment for the “silenced hMSCs” at the defect site. In addition, the presence of a scaffold can be important for the newly formed cartilage to achieve the proper biomechanical properties. At the same time, it should be considered that side effects due to the presence of exogenous materials, in terms of short- and long-term foreign body reaction, may eventually arise.

Finally, it is important to mention that accumulated evidence is pushing research in the direction of endogenous cartilage repair. It is well established that bone marrow and synovium-derived MSCs have the ability to migrate to the site of cartilage damage, and initiate the repair of partial or full-thickness cartilage defects [115, 116]. Based on this concept, endogenous repair strategies aim to direct the migration of autologous MSCs toward the site of damage, and to induce *in situ* chondrogenic differentiation for local tissue repair. In such a context, the targeting of autologous cells with RNAi molecules against anti-chondrogenic factors, previously validated by using the approaches described in this review, would represent an intriguing option to accelerate and direct the process of endogenous repair.

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Figure legends

Fig. 1 Schematic representation of the production and transplantation of "silenced hMSCs" for improved cartilage repair. hMSCs can be collected from different sources including adult niches (e.g. bone marrow and adipose tissue) or perinatal tissues (e.g. placenta, amnios, Wharton's jelly of umbilical cord). Following transfection with RNAi molecules, "silenced hMSCs" with enhanced chondrogenic and therapeutic potential are generated. The cells are cultured *in vitro* for a certain amount of time to obtain an implantable construct, possibly by combination with a scaffold. Different parameters (cell number, oxygen concentration, use of a bioreactor) may be modified to optimize culture conditions and to mimic as much as possible the physiological microenvironment. Following implantation into the site of injury, neoformation of cartilage can be achieved, and tissue functionality can be restored as a result of diverse events potentially supported by the "silenced hMSCs".

Delivery method		Carrier	Target	Human cells	Ref.
Lipid-based delivery systems	↑ Easy to prepare ↑ Cost-effective ↓ Cytotoxycity ↓ Low efficiency ↓ Immunoresponse	INTERFERin™	Collagen I, HTRA1	Chondrocytes	[36]
		Oligofectamine™	ANGPTL4	MSCs	[37]
		Lipofectamine 2000™	MMP13	Chondrocytes	[117]
			miR-30a	Chondrocytes	[38]
			circRNA-CER	Chondrocytes	[108]
			SLUG	MSCs	[61]
			ADAMTS5	Chondrocytes	[118]
		Lipofectamine RNAiMax™	NR1D1, BMAL1	Chondrocytes	[119]
			miR-222	MSCs	[102]
			Raptor	MSCs	[120]
			miR-221	MSCs	[32, 60]
		Lipofectamine LTX™	miR-495	MSCs	[94]
		XtremeGENE™	AIMP1	Chondrocytes	[23]
Electroporation	↑ Very effective ↑ Reproducible ↓ Cell damage	Amaxa Nucleofector™ Technology	RUNX2	Chondrogenic progenitor cells	[53]
			MEK5, ERK5	Multipotent progenitor cells	[66]
Nanoparticles (NP)	↑ Very effective ↑ Limited toxicity ↓ High costs	LNCs, Span 80™	REST	MSCs	[121]
		PLGA	RUNX2	MSCs	[21, 42]
		p5RHH peptide	NF-kB	Chondrocytes	[43]
		Chitosan	MMP3/13	Chondrocytes	[122]
		MNP/PEI	miR-335	MSCs	[123]
Quantum dot (QD)	↑ Very effective ↑ Traceability ↓ Citotoxycity	QD-SMCC	SOX9	MSCs	[46]
		RGD-β-CD-QD	RUNX2	MSCs	[45]
Extracellular vesicles (EV)	↑ Non-immunogenic ↑ Stability ↓ Expensive	HEK293T (EV)	GFP	HUVEC, MSCs	[47]

Table 1. Relevant non-viral systems for the delivery of RNAi into human cells for chondrogenesis.

Target	Main function	Biological effect of gene silencing	Ref.
RUNX2	Osteoblast differentiation	Chondrogenesis of hMSCs with reduced expression of collagen I and osteogenic markers *in vitro* and after injection in mice.	[21, 42]
		Enhanced expression of SOX9 and increased synthesis of cartilage matrix (collagen II, aggrecan) in CPCs. Reduced expression of collagen I and catabolic enzymes (MMP13, ADAMTS5).	[53]
		Enhanced expression of SOX9 and collagen II and loss of collagen I in chondrocytes derived from DDR-1 deficient mice (OA model).	[54]
TGIF1	Embryonic development	Enhanced chondrogenesis of rat tendon and bone marrow-derived MSCs *in vitro*. Improved fibrocartilage production and healing of bone-to-tendon insertion after *in vivo* implantation.	[55-57]
SHOX2	Embryonic development	Chondrogenesis of mouse MSCs in the absence of chondro-stimulation without transition to the hypertrophic stage.	[58]
TWIST1	EMT transition	Increased formation of chondrogenic nodules and expression of chondrogenic markers during micromass culture of murine limb bud mesenchymal cells.	[59]
SLUG	EMT transition	Chondrogenesis of human bone marrow or Wharton's jelly-derived hMSCs end enhanced production of cartilage ECM onto HYAFF-11 scaffold.	[60, 61]
p53	Cell cycle	Increased proliferation and expression of SOX9, collagen II and aggrecan in progenitor cells isolated from human articular cartilage.	[124]

Table 2. Validated targets for gene silencing strategies aimed at enhancing chondrogenesis – transcription factors.

Target	Main function	Biological effect of gene silencing	Ref.
YAP	Proliferation and apoptosis	Increased expression of SOX9, collagen II and aggrecan, and decreased collagen I in rat articular chondrocytes.	[63]
p16	Cell cycle	Enhanced proliferation of OA chondrocytes and recovered expression of collagen II and aggrecan without increase of collagen I. Enhanced response to TGF-β1 and protection from inflammatory stimuli (IL-1α).	[64]
p21	Cell cycle	Enhanced proliferation during expansion and collagen II and GAGs production during pellet culture of murine iPS. Maintenance of the chondrogenic potential of extensively passaged cells. Suppression of collagen I and X.	[22]
GADD45β	Cell cycle	Suppressed terminal differentiation and expression of MMP13 in murine rib growth plate chondrocytes.	[65]
ERK5, MEK5	Proliferation and differentiation	Enhanced expression of SOX5, SOX6, SOX9, collagen II, aggrecan and GAGs production in hMSCs. Enhancement of the chondrogenic potential even in the absence of TGF-β.	[66]
IKKα/β	Inflammation	Increased cartilage production (collagen II, GAGs) and suppression of terminal differentiation in OA chondrocytes. Inhibition of the IL-1β mediated increase of MMP13.	[67]
p53R2	DNA repair	Increased production of collagen II, aggrecan and GAGs in OA chondrocytes after tensile strain.	[68]
PHD2	Response to hypoxia	Upregulation of SOX9 and increased production of the cartilage ECM components collagen II, IX, XI and aggrecan under normoxic or hypoxic conditions.	[69]
KDM2A	Chromatin remodeling	Enhanced chondrogenesis of apical papilla-derived hMSCs.	[70]
Raptor	Proliferation	Enhanced chondrogenesis of amniotic fluid-derived hMSCs with increased AKT activation, upregulation of HIF-2α and increased SOX9 and collagen II abundance.	[120]

Table 3. Validated targets for gene silencing strategies aimed at enhancing chondrogenesis – intracellular co-regulators and enzymes.

Target	Main function	Biological effect of gene silencing	Ref.
Collagen I	ECM structural component	Enhanced chondrogenic potential of human de-differentiated chondrocytes cultered in collagen sponges. Enhanced production of collagen II and aggrecan after subcutaneous implantation in mice.	[36]
		Increased synthesis of collagen II, aggrecan, COMP and GAGs in pig synovial MSCs.	[77]
HTRA1	Regulation of the availability of IGFs	Enhanced chondrogenic potential of human de-differentiated chondrocytes cultered in collagen sponges. Enhanced production of collagen II and aggrecan after subcutaneous implantation in mice.	[36]
Decorin	ECM structural component	Increased expression of SOX9, biglican and aggrecan in hMSCs.	[78]
Lumican	ECM structural component	Augmented production of collagen II and increased fibril diameter in bovine chondrocytes.	[79]
Asporin	ECM structural component	Increased expression of collagen II, aggrecan and TGF-β1 in human chondrocytes.	[80]
Aggrecanase-1/2	Catabolism of cartilage ECM	Enhanced proliferation, abundance of GAGs and total collagen, and expression of collagen II and aggrecan in rat chondrocytes cultured on a chitosan-gelatin scaffold.	[81]
MMP2	Catabolism of cartilage ECM	Increased expression of fibronectin, integrin α5 and β1 in chicken limb bud mesenchymal cells. Enhanced precartilage condensation and GAGs production.	[82]
VEGF	Vasculogenesis and angiogenesis	Enhanced production of collagen II, aggrecan and chondromodulin 1 during pellet culture of chondrocytes. Suppression of RUNX2, MMP13 and ALP. Protection from pro-hypertrophic stimuli (TNFα).	[83]
AIMP1	Angiogenesis and inflammation	Enhanced production of collagen II, aggrecan and GAGs in dedifferentiated and OA chondrocytes *in vitro*. Enhanced cartilage tissue formation *in vivo*.	[23]
ANGPTL4	Lipid metabolism	Increased expression of collagen II and aggrecan, and suppression of MMP1, 3 and 13 in hMSCs.	[37]

Table 4. Validated targets for gene silencing strategies aimed at enhancing chondrogenesis – matrix and secreted proteins.

Target	Biological effect of gene silencing	Ref.
miR-30a	**Increased expression of SOX9, collagen II and GAGs production by human chondrocytes. Protection from inflammatory stimuli (IL-1β).**	**[38]**
miR-145	**Increased expression of SOX9 in human chondrocytes.**	**[91]**
miR-199a	**Increased expression of SOX9, collagen II and aggrecan in human chondrocytes and MSCs.**	**[93, 125]**
miR-495	**Enhanced the TGF-β3-mediated chondrogenesis of hMSCs.**	**[94]**
miR-1247	**Increased expression of SOX9 and collagen II in human chondrocytes.**	**[95]**
miR-194	**Enhanced chondrogenesis of adipose-tissue derived hMSCs. Increased expression of collagen II, IX, XI, aggrecan and COMP.**	**[96]**
miR-34a	**Increased synthesis of cartilage ECM (collagen II, GAGs) by limb bud mesenchymal cells. Protection of human chondrocytes from inflammatory stimuli (IL-1β).**	**[98, 100]**
miR-142	**Increased cell viability, proliferation, migration and pre-cartilage condensation of mesenchymal cells.**	**[99]**
miR-375	**Increased proliferation, migration and pre-cartilage condensation of mesenchymal cells.**	**[97]**
miR-99a	**Promotion of early chondrogenesis of rat MSCs with increased production of cartilage ECM (collagen II, aggrecan, GAGs).**	**[101]**
miR-193b	**Increased expression of SOX9, collagen II and aggrecan in human chondrocytes.**	**[93]**
miR-200a	**Enhanced chondrogenesis in MCC cells and organ cultures, with increased levels of SOX9 and collagen II.**	**[24]**
miR-221	***In vitro* chondrogenesis of hMSCs in the absence of growth factors and without progression to hypertrophy. Enhanced hMSCs-mediated *in vivo* cartilage repair.**	**[32, 60]**
miR-222	**Enhanced chondrogenesis and osteogenesis of hMSCs *in vitro*. Improved angiogenesis and bone union and healing *in vivo*.**	**[102]**
circRNA-CER	**Increased expression of collagen II and aggregan in human OA chondrocytes. Suppression of MMP13.**	**[108]**
lncRNA-CIR	**Increased expression of collagen I, II, aggrecan and GAGs production in human OA chondrocytes. Suppression of MMP13 and ADAMTS5.**	**[109]**

Table 5. Validated targets for gene silencing strategies aimed at enhancing chondrogenesis – microRNAs.