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Targeting anti-chondrogenic factors for the stimulation of chondrogenesis: a new paradigm in cartilage repair

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ABSTRACT

Trauma and age-related cartilage disorders represent a major global cause of morbidity, resulting in chronic pain and disability in patients. A lack of effective therapies, together with a rapidly aging population, creates an impressive clinical and economic burden on healthcare systems. In this scenario, experimental therapies based on transplantation or *in situ* stimulation of skeletal Mesenchymal Stem/progenitor Cells (MSCs) have raised great interest for cartilage repair. Nevertheless, the challenge of guiding MSC differentiation and preventing cartilage hypertrophy and calcification still needs to be overcome. While research has mostly focused on the stimulation of cartilage anabolism using growth factors, several issues remain unresolved prompting the field to search for novel solutions. Recently, inhibition of anti-chondrogenic regulators has emerged as an intriguing opportunity. Anti-chondrogenic regulators include extracellular proteins as well as intracellular transcription factors and microRNAs that act as potent inhibitors of pro-chondrogenic signals. Suppression of these inhibitors can enhance MSC chondrogenesis and production of cartilage matrix. We here review the current knowledge concerning different types of anti-chondrogenic regulators. We aim to highlight novel therapeutic targets for cartilage repair and discuss suitable tools for suppressing their anti-chondrogenic functions. Further effort is needed to unveil the therapeutic perspectives of this approach and pave the way for effective treatment of cartilage injuries in patients.

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Keywords: cartilage repair, anti-chondrogenic factors, chondrogenesis, stem cells

INTRODUCTION

Regeneration of damaged cartilage represents a great challenge in orthopaedics. Due to its avascular nature and scarce cellularity, adult articular cartilage has limited potential for self-repair and poses strong barriers for therapy^{1,2}. When conservative management is inadequate, surgical interventions, e.g. bone marrow stimulation techniques and osteochondral grafting, can be considered. Unfortunately, these procedures are not successful in repairing defects with long-lasting functional hyaline cartilage. Since 1994, cell-based therapy in the form of autologous chondrocyte implantation (ACI) has provided a more advanced tool for the treatment of focal cartilage defects³. However, ACI is only indicated for a selected cohort of relatively young patients with large cartilage defects and no signs of osteoarthritis (OA)⁴. Additional limitations of the procedure are the extensive costs and time required for the *in vitro* culture of chondrocytes, with the durability of the repair tissue still being a concern. The design of novel and more effective therapies for cartilage repair remains an unmet clinical need.

Strategies using Mesenchymal Stem Cells (MSCs) have raised a growing excitement in the field⁵⁻⁸. Due to their availability and chondrogenic differentiation capacity, MSCs hold great potential to regenerate damaged cartilage⁹. The feasibility is dependent on suitable biological cues that can stimulate the process of chondrogenesis and MSCs-mediated cartilage reconstruction.

While the formation of cartilaginous tissue following microfracture surgery indicates that endogenous anabolic stimuli in the joint might be sufficient for the induction of cartilage repair, the amount and quality of the repair tissue is not optimal. So far, strategies for cartilage repair have focused almost exclusively on the stimulation of anabolism using chondro-inductive growth factors, e.g. Transforming Growth Factors- β (TGF- β s), Bone Morphogenetic Proteins (BMPs) and Fibroblast Growth Factors (FGFs). These factors can induce the differentiation of MSCs into chondrocyte-like cells and stimulate the production of cartilage matrix. The therapeutic potential of growth factor therapy has been widely investigated in experimental animals and in clinical trials^{10,11}, mainly with platelet-rich plasma (PRP), autologous-conditioned serum (ACS) and bone marrow concentrate (BMC) preparations. PRP in particular may represent a valuable option for knee OA treatment^{12,13}, but the number of randomized controlled studies remains limited and the use of standard preparations is lacking. Importantly, growth factor therapy has been questioned due to the need for high dosages, that not only leads to high production costs, but also increases the risk of side effects¹⁴. This can be caused by the exposure of joint tissues other than cartilage (synovium, tendons, ligaments, meniscus, subchondral bone) to the exogenous growth factors leading to synovial hyperplasia, joint inflammation and ectopic cartilage or bone formation, with pain

and loss of mobility due to joint obstruction¹⁵. Several studies have confirmed that repeated injections of TGF- β , BMP2 or BMP9 and adenoviral overexpression of TGF- β in murine knee joints can cause the formation of osteophytes¹⁶⁻¹⁸. It also remains unclear whether growth factors are an optimal strategy for guiding MSC chondrogenesis. *In vitro* and *in vivo* implantation experiments have showed that MSCs that are chondrogenically differentiated with growth factors tend to acquire typical features of growth plate chondrocytes, and this can lead to formation of calcified repair tissue, rather than hyaline cartilage^{19,20}. In this regard, extensive effort is ongoing to identify factors that can suppress hypertrophic differentiation of MSC-derived chondrocytes to retain the articular cartilage phenotype. Whereas the clinical relevance of growth factor therapy could be improved by the implementation of advanced delivery and targeting strategies, the pursuit of alternative options to guide cartilage repair must continue.

With the focus remaining on the search for chondro-inductive growth factors, not much attention has been paid to anti-chondrogenic regulators that can prevent MSCs to obtain or maintain a chondrocyte-like phenotype. This might be surprising given the variable quantity and quality of cartilage tissue that is formed following microfracture surgery, suggesting the presence of blocking or inhibitory factors. Inhibition of chondrogenesis can physiologically be exerted at two levels; 1. at the extracellular level by growth factors, growth factor inhibitors and pro-inflammatory cytokines, and 2. at transcriptional/translational level by transcriptional (co-)regulators and microRNAs (miRNAs) (Table 1). Targeted inhibition of anti-chondrogenic molecules may “release the brakes”, creating more favourable conditions for MSCs to acquire a chondrocyte phenotype and produce stable cartilage.

We hereby provide a brief overview of relevant anti-chondrogenic regulators, with the aim to highlight how suppression of these signals may represent a feasible and effective way to guide chondrogenesis and cartilage repair.

Table 1. Overview of anti-chondrogenic regulators.

family	name	anti-chondrogenic role	ref.
growth factor inhibitors	NOGGIN, FOLLISTATIN, GREMLIN, CHORDIN	BMP antagonists	22,23
	TSG	Direct binding and inhibition of BMP-2 and BMP-4	22,23
growth factors	FGF-2	Inhibition of hMSCs chondrogenesis. Counteraction of the pro-chondrogenic effect of BMP-2, hedgehog, TGF- β and BMP-6	26-28
	GDF11	Inhibition of cartilage nodule formation and chondrocyte hypertrophy	30
	WNT1, WNT4, WNT7A, WNT8, WNT9A	Inhibition of chondrogenesis and stimulation of hypertrophic differentiation	33

Table 1. Overview of anti-chondrogenic regulators. (*continued*)

family	name	anti-chondrogenic role	ref.
transmembrane proteins	NOTCH	Inhibition of chondrogenesis following constitutive activation	31
pro-inflammatory cytokines	IL-1 β , TNF α , IL-6, IL-8	Inhibition of chondrogenic differentiation and stimulation of cartilage catabolism	41,42
transcription factors	TWIST1	Inhibition of chondrogenesis via competitive binding of the SOX9 DNA-binding domain	50-52
	DEC2	Inhibition of proliferation and chondrogenesis in hMSCs	54
	SLUG/SNAIL2	Inhibition of collagen II and aggrecan. SLUG silencing induced chondrogenesis of hMSCs in the absence of growth factors	55-57,104
	ZFP60	Inhibition of ATDC5 differentiation	58
	HOXA2	Chondrodysplasia after COL2A1-driven overexpression of HOXA2 <i>in vivo</i>	59
	HOXD4, HOXC8	Delayed chondrogenesis following HOXD4 and HOXC8 overexpression <i>in vivo</i>	60
	AP-2 α	Suppression of chondrocyte differentiation and ECM production	62
	YAP1/TAZ	Inhibition of chondrogenesis in hMSCs and chondrocytes. YAP1/TAZ knockdown stimulated the expression of chondrogenic markers	64-67
	NF- κ B	Inhibition of chondrogenic differentiation and stimulation of cartilage catabolism	68
miRNAs	miR-193b	Suppression of chondrocytes markers via inhibition of TGF- β 2 and TGF- β RIII	72
	miR-483	Inhibition of chondrogenesis by direct targeting of SMAD4	73
	miR-199a	Targeting of SMAD1	74
	miR-146a	Targeting of SMAD2/3	75
	miR-195	Targeting of FGF-18. Suppression of miR-195 led to enhanced chondrogenesis and protective effects on cartilage lesions	76
	miR-145	Targeting of SOX9 leading to inhibition of chondrogenesis and ECM production, and stimulation of hypertrophy	77-79
	miR-30, miR-495, miR-1247	Targeting of SOX9	80-82
	miR-146b, miR-194	Targeting of SOX5	83,84
	miR-21	Targeting of SOX2 with inhibition of proliferation and chondrogenesis, and stimulation of osteogenesis	85
	miR-499a	Targeting of LEF1	86
	miR-29a/b	Targeting of FOXO3A and COL2A1	87,88
	miR-221	Inhibition of hMSCs chondrogenesis. Implantation of miR-221-depleted hMSCs in a cartilage defect model led to enhanced cartilage repair	57,90
	miR-222	miR-222 silencing induced <i>in vivo</i> chondrogenesis in a rat fracture model	91

EXTRACELLULAR ANTI-CHONDROGENIC REGULATORS

Growth factors and related regulators

Growth factors play a pivotal role in the regulation of chondrogenesis. During embryogenesis and adult life, many growth factors regulate tissue formation, maintenance and repair, depending on their spatiotemporal patterns^{10,21}. In parallel, other factors are needed to control these pathways, to create gradients and boundaries that prevent excessive activation and interrupt the signalling when necessary^{21,22}. These effectors are inhibitory molecules that suppress the activation of growth factor-dependent pathways via different mechanisms.

Several extracellular inhibitors can block the activity of pro-chondrogenic growth factors²². NOGGIN, FOLLISTATIN, GREMLIN and CHORDIN act as BMP antagonists, diffusing through extracellular matrices and preventing the interaction of BMPs with their receptors^{22,23}. Under physiological conditions, the expression of BMP inhibitors is directly stimulated by BMPs themselves, highlighting a self-control of their activity through negative feedback mechanisms²³. Interestingly, NOGGIN was also shown to bind GDF5 and GDF6, crucial regulators of MSC condensation and cartilage formation^{22,23}. Twisted gastrulation (TSG) can both promote and inhibit BMP signals, by suppressing the activity of chordin or directly binding BMP-2 and BMP-4, respectively^{22,23}.

Although growth factors are normally associated with the stimulation of chondrogenesis, even these proteins can, under certain conditions, exert anti-chondrogenic roles. FGF-2 is known for stimulating the expansion and chondrogenic priming of MSCs^{24,25}, but various studies have reported anti-chondrogenic actions during cell differentiation depending on the context and time of exposure. FGF-2 could counteract the synergistic pro-chondrogenic effect of BMP-2 and hedgehog proteins in RMD-1 pre-chondrogenic cells²⁶. Additionally, FGF-2 was shown to inhibit chondrogenesis and matrix production in adipose-derived²⁷ and bone marrow-derived hMSCs^{28,29}.

GDF11 was shown to exert a negative effect on chondrogenesis both *in vitro* and *in vivo*³⁰. In chick limb-derived MSC micromass cultures, GDF11 caused strong inhibition of chondrocyte differentiation and cartilage nodule formation³⁰. NOTCH proteins are a family of transmembrane proteins whose extracellular domain contains several epidermal growth factor (EGF) sequences. Constitutive activation of NOTCH1 strongly repressed the expression of chondrocyte markers and cartilage production³¹. Chondrogenesis could thus be enhanced by inhibition of GDF11 or NOTCH1 at specific moments during the process of chondrogenesis.

WNT signalling has a complex and major role in the regulation of cartilage development by controlling the specification of skeletal progenitor cells and

differentiation of chondrocytes³². Several WNT proteins including WNT1, WNT4, WNT7A, WNT8 and WNT9A were shown to inhibit chondrogenic differentiation of progenitor cells, thus representing potential targets for the stimulation of cartilage repair³³. Inhibition of endogenous WNT production during MSC chondrogenesis was shown to prevent calcification and supported cartilage stability³⁴. During OA, a reduction of natural WNT inhibitors (e.g. DKK1 and FRZB) is suggested to be responsible for cartilage degeneration, thus further indicating that WNT inhibition may be beneficial for improving cartilage repair^{35,36}.

Pro-inflammatory factors

Environmental factors in the joint greatly influence the processes of chondrogenesis. When cartilage is damaged, high levels of extracellular mediators of inflammation including pro-inflammatory cytokines and chemokines are produced by joint tissues and released in the synovial fluid³⁷. While low levels of these factors are required as initial stimulus for tissue repair, their increased or chronic production can impair chondrogenesis and stimulate the degeneration of newly-formed cartilage³⁸⁻⁴⁰. Among pro-inflammatory mediators, IL-1 β , TNF α , members of the IL-6 family and IL-8 are well recognized as potent anti-chondrogenic factors⁴¹. These cytokines induce the transcription factor NF- κ B, which can inhibit the expression of SOX9 and TGF- β receptor type II, and block SMADs phosphorylation³⁸. Thus, a chronic inflammatory milieu in the joint poses a serious obstacle for cartilage repair⁴².

Modulation of inflammation via targeted inhibition of pro-inflammatory signals could offer great therapeutic benefits, by reducing cartilage degeneration and creating a favourable environment for repair. *Kawaguchi et al.* showed that the repair of osteochondral defects in rabbit could be improved by injection of the TNF-inhibitor etanercept⁴⁰. However, modulation of inflammation represents a considerable challenge since non-selective inhibition of inflammation via non-steroidal anti-inflammatory drugs (NSAIDs) was found to inhibit chondrogenesis and cartilage production⁴³. Moreover, transient activation of NF- κ B with low expression of pro-inflammatory mediators (e.g. cyclooxygenase-2, inducible nitric oxide synthase, IL-6 and TNF α) was shown to be required during the early stages of chondrogenesis⁴⁴. Pro-inflammatory mediators are thus not only associated with cartilage degeneration, but their effect on chondrogenic cells depends on the differentiation status of the cells and largely on the magnitude, timing and duration of the stimulus. This remains a main challenge for the application of inflammation modulators for cartilage repair.

INTRACELLULAR ANTI-CHONDROGENIC REGULATORS

Transcription factors

Chondrogenesis is precisely regulated by several transcription factors including the master regulators SOX9 and RUNX2/3, which act as crucial regulators of MSC commitment and cartilage development. Inhibition of anti-chondrogenic transcription factors may represent a feasible strategy to stimulate cartilage repair by intervening on gene expression level. The therapeutic blockage of “negative” transcription factors is a concept which is gaining increasing interest and that has already been transposed to a pre-clinical level e.g. in cancer therapy, with the use of BRD4 and HOXA9 inhibitors⁴⁵.

TWIST1 is a member of the helix-loop-helix family of transcription factors and plays a major role in development, mesoderm specification and differentiation and joint homeostasis^{46,47}. TWIST1 was initially reported as negative regulator of myogenesis and osteogenesis^{48,49}, and was later characterized as a key mediator of the canonical WNT signalling in repressing chondrogenesis in ATDC5 cells⁵⁰. Interestingly, TWIST1 overexpression in growth plate-derived chondrocytes and depletion in ATDC5 cells showed inhibition and enhancement of chondrogenesis, respectively^{50,51}. TWIST1 regulates the early stages of chondrogenesis through a competitive binding to SOX9 DNA-binding domain, causing reduced expression of SOX9 downstream chondrocyte-specific genes⁵². However, *in vivo* work based on TWIST1 overexpression in COL2A1-expressing cells revealed a protective effect on cartilage degeneration in OA, likely due to a functional role in the maintenance of chondro-progenitor cells⁵³. These seemingly contrasting effects of TWIST1 highlight how the therapeutic targeting of anti-chondrogenic factors should take into account the temporal, spatial and cell type-specific effects in different pathophysiological conditions. Differentiated embryo chondrocyte 2 (DEC2) is another member of the helix-loop-helix family of transcription factors that was described as negative regulator of proliferation and chondrogenesis in bone marrow hMSCs⁵⁴. Overexpression of DEC2 in bone marrow hMSC pellet cultures inhibited cell proliferation and GAG accumulation⁵⁴.

SLUG/SNAIL2 is a crucial regulator of MSC fate belonging to the Snail family of zinc-finger transcription factors. SLUG overexpression during chondrogenesis of ATDC5 strongly inhibited collagen II and aggrecan expression⁵⁵. Interestingly, SLUG silencing in bone marrow or umbilical cord-derived hMSCs had a strong pro-chondrogenic effect, and stimulated the expression of chondrogenic markers such as SOX9 and collagen II, even in the absence of growth factors^{56,57}. This does not only confirm a pivotal anti-chondrogenic role of SLUG during MSC differentiation, but also provides a proof-of-concept for the application of gene silencing as

an alternative to growth factors for directing MSC chondrogenesis. Another zinc-finger transcription factor, ZFP60, was characterized as inhibitor of chondrogenesis by transient overexpression in ATDC5 cells⁵⁸.

Homeobox (HOX) proteins are transcription factors expressed throughout life, which play a crucial role during embryonic development. *HOX* genes are expressed during cell condensation in skeletogenesis but are switched off when chondrogenic differentiation is initiated. Notably, COL2A1-driven expression of Homeobox protein Hox-A2 (HOXA2) and overexpression of HOXD4 and HOXC8 in their own expression domains led to chondrodysplasia and delayed chondrogenesis *in vivo*^{59,60}.

AP-2 α belongs to the family of highly homologous genes AP-2 and its involvement in chondrogenesis and skeletogenesis was first demonstrated in knockout mice by severe skeletal and craniofacial defects⁶¹. Retroviral overexpression of AP-2 α in ATDC5 confirmed its anti-chondrogenic role, with suppression of cartilage nodule formation, proteoglycan production and expression of chondrocyte markers after TGF- β or insulin stimulation⁶².

Yes-associated protein (YAP1) and its paralogue TAZ are transcriptional cofactors that act as central effectors of the Hippo pathway, regulating MSC commitment⁶³. The role of YAP/TAZ as negative regulators of chondrogenesis was extensively described in chondrocytes and MSCs⁶⁴⁻⁶⁷. YAP knockdown in rat chondrocytes grown on stiff matrices led to maintenance of the chondrocyte phenotype^{65,66}. Accordingly, YAP overexpression in C3H10T1/2 cells determined decreased chondrogenic differentiation⁶⁷, while increased expression of chondrogenic markers was observed after YAP/TAZ knockdown in rat MSCs⁶⁴.

NF- κ B is the main transcriptional player in inflammation and an attractive intracellular target to counteract inflammation and thus favour cartilage repair. NF- κ B activation in cartilage and synovium enhances the production of degradative enzymes, catabolic cytokines, and pro-inflammatory signals which all contribute to cartilage damage⁶⁸. Because it is induced by a number of pro-inflammatory mediators, blocking NF- κ B may provide more comprehensive protection than targeting individual cytokines for regenerating cartilage⁶⁹. Nevertheless, since nuclear translocation of NF- κ B is a necessary step during early chondrogenesis, the timing of intervention is of critical relevance for therapeutic strategies inhibiting the NF- κ B signalling⁴⁴.

microRNAs

Post-transcriptional mechanisms play a major role in the regulation of chondrogenesis and cartilage production. miRNAs are short non-coding RNAs that fine-tune gene expression by base-pairing with complementary mRNA targets to elicit transcriptional repression. This level of control is very potent since a single miRNA can target hundreds of mRNAs.

A recent miRNA expression profile study showed that 169 miRNAs were modulated during hMSC chondrogenesis⁷⁰. Notably, 93 of these miRNAs were significantly downregulated, with the expression of 62 miRNAs being completely lost in the transition from MSC to pre-chondrocyte stage. Similar evidence was provided by other studies⁷¹, suggesting that several microRNAs might exert anti-chondrogenic functions and that suppression of these regulators may be required for chondrogenesis to take place. Recently, increasing effort has been put into the characterization of chondro-inhibitory miRNAs, leading not only to a better understanding of the molecular basis of chondrogenesis, but also to the identification of novel targets to stimulate cartilage repair. Anti-chondrogenic miRNAs control various processes involved in cartilage homeostasis, including condensation and differentiation of mesenchymal progenitors, maintenance of the chondrocyte phenotype and production of ECM components. At a molecular level, this is explained by the ability of these miRNAs to fine-tune the expression of chondro-regulatory growth factors and transcription factors, as well as cartilage matrix proteins. In the following section we provide relevant examples (Table 1).

Several microRNAs were shown to directly target TGF- β growth factors and receptors, as well as effectors of the TGF- β pathway e.g. SMAD proteins. *miR-193b* targets both TGF- β 2 and TGF- β RIII and inhibits the phosphorylation of SMAD3, leading to suppression of the early chondrogenic markers SOX9, collagen II and COMP⁷². Overexpression of *miR-483* in hMSCs reduced the expression of chondrogenic markers and GAGs production and this effect was shown to be achieved via direct targeting of SMAD4⁷³. *miR-199a** and *miR-146a* exert anti-chondrogenic roles by targeting SMAD1 and SMAD2/3, respectively^{74,75}. Pro-chondrogenic growth factors other than TGF- β were also shown to be regulated by miRNAs. *miR-195* was found to be highly expressed in the joint fluid of aged animals and patients with chronic cartilage lesions⁷⁶. Interestingly, *miR-195* exerted an anti-chondrogenic function by targeting FGF-18, and its suppression promoted chondrogenesis and had a protective effect on cartilage lesions *in vivo*.

Various miRNAs inhibit the expression of pro-chondrogenic transcriptional regulators, and mainly those belonging to the SOX gene family. Being the main transcriptional player in chondrogenesis, it is not surprising that the translation of SOX9 is tightly regulated by microRNAs. *miR-145* is the best characterized SOX9-targeting miRNA. Increased *miR-145* levels cause strong reduction in the expression of cartilage ECM genes and pro-chondrogenic miRNAs (e.g. *miR-140*), as well as stimulation of hypertrophy^{77,78}. Interestingly, *miR-145* expression was negatively correlated with the chondrogenic potential of mesenchymal progenitors derived from iPS cells⁷⁹. Similar to *miR-145*, *miR-30*, *miR-495* and *miR-1247* were more recently characterized as anti-chondrogenic SOX9-targeting miRNAs⁸⁰⁻⁸². Notably, silencing of these miRNAs was proposed as an effective strategy to enhance

chondrogenesis. *miR-146b* and *miR-194* were shown to counteract chondrogenesis by targeting a second member of the SOX-trio, *SOX5*^{83,84}, while *miR-21* directly inhibits the pluripotency marker *SOX2*⁸⁵. Notably, *miR-21* was found to inhibit the clonogenic and proliferative potential of hMSCs, inducing cell cycle arrest and promoting osteogenesis over chondrogenesis. Finally, other microRNAs were shown to exert anti-chondrogenic functions by targeting additional pro-chondrogenic transcription factors, including *LEF-1 (miR-449a)*⁸⁶ and *FOXO3A (miR-29a)*⁸⁷.

Yan et al. showed that microRNAs can intervene directly on the production and secretion of cartilage ECM proteins. *miR-29a* and *miR-29b*, whose expression is directly inhibited by *SOX9*, bind to the 3'-UTR of the collagen II mRNA to inhibit its translation, thus suppressing the production of cartilage matrix⁸⁸.

Overall, these studies highlight how miRNAs can control the fate of chondroprogenitors, as well as the acquisition and maintenance of the mature chondrocyte phenotype⁸⁹. The first *in vivo* study recently demonstrated the relevance of anti-chondrogenic microRNAs as therapeutic targets for cartilage repair. *miR-221* was identified as a novel anti-chondrogenic miRNA and silencing of *miR-221* in hMSCs induced chondrogenesis *in vitro*, without requiring growth factor supplementation^{57,90}. Implantation of *miR-221* depleted hMSCs in a cartilage defect model enhanced cartilage repair *in vivo*⁹⁰. Interestingly, *Yoshizuka et al.* later showed that the paralogue of *miR-221*, *miR-222*, also exerted anti-chondrogenic effects, as its silencing promoted *in vivo* chondrogenesis of hMSCs in a rat fracture model⁹¹.

TARGETED MODULATION OF ANTI-CHONDROGENIC REGULATORS

The recent advances in molecular therapy and biotechnology have led to the development of powerful tools to inhibit the expression or function of specific extracellular and intracellular regulators (Table 2). At the extracellular level, blocking antibodies can be employed to block anti-chondrogenic growth factors and cytokines, as well as growth factor inhibitors. This strategy is already at a clinical stage for a variety of diseases, including chemotherapy for cancer and rheumatoid arthritis^{92,93}. In the case of cytokines, modified soluble receptors are also available, e.g. etanercept for the treatment of arthritis⁹². Interestingly, the possibility of using these inhibitors to target anti-chondrogenic extracellular factors and direct cartilage repair is relatively unexplored.

At the intracellular level, the RNA interference (RNAi) approach is widely applied to block the synthesis and function of regulatory proteins and miRNAs⁹⁴. This can be achieved using short interfering RNAs (siRNAs) and microRNA inhibitors, respectively. siRNAs are a class of double stranded RNA molecules that are 20-25 nucleotides in length. Once delivered into the cell, siRNAs enter the RNAi pathway

Table 2. Available tools for suppressing the expression and/or function of anti-chondrogenic regulators.

tool (inhibitor)	anti-chondrogenic target	description	stage	ref.
blocking antibodies	growth factors (including receptors and extracellular inhibitors), cytokines	blocking antibodies bind to target protein, sequestering it and/or preventing its biological activity	clinical	92,93
soluble receptors	cytokines	soluble receptors function as decoys, preventing activation of the cytokine-mediated signalling	clinical	92
siRNAs (shRNAs)	all protein-coding genes	dsRNA molecules, recognition of the target mRNAs leads to its degradation and inhibition of translation. In the case of shRNA, the siRNA is encoded by a vector, delivered into the nucleus and processed by the RNAi machinery	clinical trials	105-107
antimiRNAs	miRNAs	ssRNA molecules, inhibition of the target miRNAs is exerted mainly through steric blocking	clinical trials	90,108
miRNA sponges	miRNAs	long ssRNA molecules harbouring multiple binding sites for the target miRNAs	preclinical	100,101
small molecule inhibitors of miRNAs (SMIRs)	miRNAs	small-molecule drugs targeting and modulating the activity of specific miRNAs	preclinical	102
CRISPR/CAS9	all genes	engineered bacterial system allowing the removal/modification of genomic DNA sequences	preclinical, entering clinical trials	103,109

leading to interference with the expression of mRNAs bearing complementary sequences, usually via mRNA degradation. Theoretically, by using strong inhibitory siRNA sequences it is possible to knock-down any known gene, and a careful design can minimize dosage and toxicity. With the aim to prevent immunogenicity and off-target responses, modifications such as 2'-O-methyl functionalization of the siRNA antisense strand can be introduced⁹⁵. Other siRNA modifications including phosphorothioates and locked nucleic acids (LNA) can greatly increase the potency, specificity and transfectability of the inhibitors⁹⁶. siRNA therapy has been developed in combination with organic or inorganic delivery strategies, or with the use of viral vectors for stable knockdown approaches. siRNAs can be either delivered directly into the cytoplasm, or encoded by a vector as short hairpin (sh)RNAs, that require delivery into the nucleus and processing by the RNAi machinery of the cells to generate the mature siRNA⁹⁷. A more extensive description and examples of the recent viral and non-viral technologies for siRNA/shRNA delivery can be found elsewhere⁹⁸.

The idea of blocking anti-chondrogenic microRNAs to promote cartilage repair is becoming increasingly appealing. *In vivo* proof-of-concept studies have recently confirmed that this approach may indeed represent a powerful tool for the treatment of cartilage injuries^{90,91}. For miRNA inhibition, three types of inhibitors are available. AntimiRs (antagomiRs) represent the most common choice and are short oligonucleotides that sequester target miRNAs in highly stable complexes, thus inhibiting their activity⁹⁰. Also in the case of antimiRs, LNA chemistry has led to the development of highly potent and specific inhibitors, that are currently being investigated in clinical trials⁹⁹. miRNA sponges are longer single-stranded RNAs containing complementary binding sites to the target miRNAs (usually against the seed region), and competing with the mRNAs for interaction with the miRNAs^{100,101}. While antimiRs are usually employed to suppress a single miRNA, sponges offer opportunities for multi-targeting, since the seed sequence is normally shared within a miRNA family. The third class of molecules includes small molecules inhibitors of miRNAs (SMIRs), e.g. diazobenzene, benzothiazoles and neomycin¹⁰². Although this latter choice is far less popular, SMIRs might offer some advantages in relation to the ease of delivery and stability in body fluids.

Finally, the CRISPR/Cas9 technology has recently emerged as a revolutionary opportunity to achieve gene knockout¹⁰³. This system consists of a nuclease (Cas9) that can cut genomic DNA, and a guide RNA that recruits Cas9 to the target site. By engineering the guide RNA, Cas9 can be directed toward the desired gene target. Importantly, the application of CRISPR/Cas9 for gene silencing has already broadened our capability to study gene function in chondrogenesis. Nevertheless, it remains to be determined whether this will also serve as concrete therapeutic tool for cartilage repair.

FUTURE DIRECTIONS

A rapidly growing number of studies has started to shed light on the therapeutic potential of targeting anti-chondrogenic regulators for cartilage repair. This is made possible by the availability of highly effective and specific biotechnological tools (Table 2) that allow us to target virtually any desired anti-chondrogenic factor, regardless of the type of molecule. These tools should now be exploited to gain further insights into the molecular basis of the inhibition of cartilage repair, as well as to develop novel anti-chondrogenic factors-based therapies.

The targeted suppression of anti-chondrogenic factors represents a versatile approach that can be applied to either stimulate *in situ* chondrogenesis of joint-resident stem cells (endogenous repair) or deliver therapeutic stem cell populations with a higher chondrogenic potential (cell therapy). In this review we present evidence derived from various stem cell sources that are likely characterized by a

different epigenetic and differentiation status. It is important to realize that this may significantly influence the sensitivity and response of the cells to treatments such as the inhibition of anti-chondrogenic factors, and may partly explain the context-dependent differences observed following exposure to specific stimuli e.g. growth factors. While further insights into stem cell biology and epigenetics must be pursued, the development of approaches targeting anti-chondrogenic factors directed towards specific MSC populations is desirable.

In defining “anti-chondrogenic factors”, our overview included pro-inflammatory mediators as well as factors that induce hypertrophic differentiation in mature chondrocytes. Joint inflammation can strongly hinder the efficacy of therapeutic approaches for cartilage repair and the still limited progress in addressing this issue partly explains why many of the existing methods for cartilage repair have failed to provide successful long-term clinical outcomes. Importantly, anti-inflammatory strategies should carefully take into account the complex role of inflammation in cartilage repair, and possibly target specific pro-inflammatory factors in a temporally/locally regulated manner. In parallel, the challenge of preventing cartilage hypertrophy and supporting the maintenance of an articular phenotype by chondrocytes needs to be tackled. We believe that targeted suppression of pro-inflammatory and pro-hypertrophic regulators by using the approaches described in our work will help to achieve these goals.

CONCLUSIONS

There is an urgent need for more effective biological therapies for cartilage repair. Despite the enthusiasm raised by the use of growth factor preparations, variable outcomes as well as side effects have been reported, and currently hinder the process of clinical translation. Importantly, insufficient production of cartilage and/or instability and degeneration of the newly-formed tissue is commonly observed, suggesting that anti-chondrogenic factors in the joint microenvironment counteract cartilage repair. In this review, we aimed to emphasize how the targeting of anti-chondrogenic regulators may provide a novel opportunity for the field of cartilage repair. Anti-chondrogenic signals exert the physiological function of limiting chondrogenesis and cartilage production to prevent excessive or unconfined cartilage formation, and include extracellular and intracellular regulators that can act via different mechanisms (summarized in Figure 1). In a situation where the normal homeostasis of cartilage is disturbed, i.e. in the case of joint trauma or arthritic disease, anti-chondrogenic regulators create a sub-optimal microenvironment for tissue repair. Mounting evidence indicates that targeted suppression of crucial anti-chondrogenic factors may remove these blockage, providing a feasible strategy to achieve better cartilage repair. We hope that our work will push

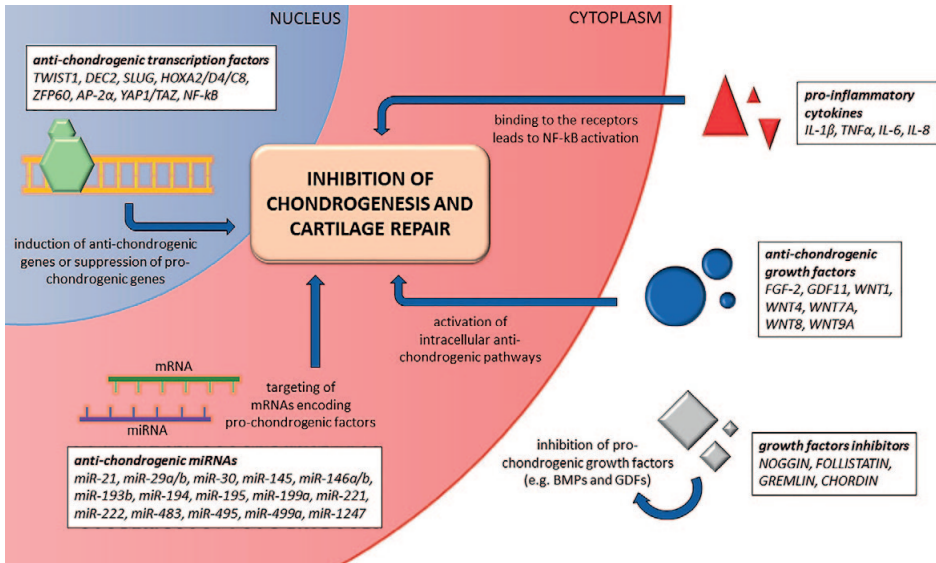


Figure 1. Anti-chondrogenic regulators in cartilage repair. Schematic representation of the main types of extracellular and intracellular anti-chondrogenic regulators and their general mechanism of action.

research in the field, that could soon lead to relevant applications for the treatment of cartilage damage in patients.

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