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Research review paper

State of art and limitations in genetic engineering to induce stable chondrogenic phenotype

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ABSTRACT

Current protocols for chondrocyte expansion and chondrogenic differentiation of stem cells fail to reduce phenotypic loss and to mitigate hypertrophic tendency. To this end, cell genetic manipulation is gaining pace as a means of generating cells with stable chondrocyte phenotype. Herein, we provide an overview of candidate genes that either induce cartilage regeneration or inhibit cartilage degeneration. We further discuss *in vitro*, *ex vivo* and *in vivo* viral transduction and non-viral transfection strategies for targeted cells (chondrocytes, mesenchymal stem cells, induced pluripotent stem cells and synovial cells), along with the most representative results obtained in pre-clinical models and in clinical trials. We highlight current challenges and associated risks that slowdown clinical acceptance and commercialisation of gene transfer technologies.

1. Introduction

Gene therapy for the treatment of cartilage diseases was first assessed in clinical setting 20 years ago, where autologous synovial fibroblasts were transduced using a retrovirus and then intra-articularly injected in 9 patients affected by rheumatoid arthritis (RA) (Evans et al., 1996). This pioneering study assessed the feasibility rather than the efficacy of gene therapy (patients underwent metacarpophalangeal joint replacement one week after the treatment). Due to its anatomy, delivery of drugs in the joint is difficult. When drugs are systemically delivered, they enter the joint through fenestrated synovial capillaries, which restrict entry of large molecules (Simkin, 1995) and toxic side effects are often observed (Aletaha et al., 2003; Karsdal et al., 2016). When drugs are intra-articular injected, they are rapidly cleared through the lymphatic system (Wallis et al., 1987; Evans et al., 2013). Currently, the majority of the non-biological systems for the treatment of degenerative inflammatory diseases, such as osteoarthritis (OA), and autoimmune disorders, such as RA, are not able to inhibit disease progression (Karsdal et al., 2016; Bhatia et al., 2013; Zhang et al., 2016; Emery, 2006). In this context, the use of genetically engineered cells, which would constantly produce the therapeutic molecule *in situ*, seems advantageous (Evans et al., 2013; Madry and Cucchiarini, 2016), especially when gene therapy products have already received market approval for the treatment of OA (e.g. Invossa™, TissueGene C, TissueGene, Inc, USA).

Gene therapy is also under investigation for the treatment of acute cartilage defects to address limitations of naïve cell implantation approaches, such as time-limited regenerative capability (Knutsen et al., 2004; Batty et al., 2011; Vavken and Samartzis, 2010; Vasiliadis et al., 2010). Further, due to low yield of isolation, chondrocytes need to be pre-expanded *in vitro* before implantation. However, during *in vitro* expansion, chondrocytes become fibroblast-like cells that produce collagen type I instead of collagen type II (Phull et al., 2016; Rackwitz et al., 2014; Camp et al., 2014; Dehne et al., 2010). Bone marrow mesenchymal stem cells (BMSCs) (Goldberg et al., 2017; Lee and Wang, 2017) and adipose-derived stem cells (ADSCs) (Lee and Wang, 2017;

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Labusca and Mashayekhi, 2013) have also been assessed in cartilage repair and regeneration. However, when differentiated into chondrogenic lineage, they tend to differentiate into hypertrophic chondrocytes, which produce mineralised matrix and collagen type X (Chen et al., 2015; Mueller and Tuan, 2008).

Herein, we discuss candidate genes and pathways that are involved in either inducing chondrogenic differentiation or in inhibiting cartilage degeneration. We also discuss viral transduction and non-viral transfection techniques and significant advances thereof in pre-clinical and clinical setting.

2. Candidate genes

2.1. Induction of chondrogenesis

Genes used for cartilage engineering / regeneration mainly include transcription factors or growth factors physiologically involved in chondrogenic differentiation. Among the transcription factors, Sex determining region Y box (Sox) 5, 6 and 9 are the main candidates. In mesenchymal stem cells (MSCs), Sox-9 is physiologically required for condensation, for initiating chondrogenesis and for activating expression of collagen type II, aggrecan and other extracellular matrix (ECM) proteins. It possesses a high mobility group (HMG) domain and works in conjugation with Sox-5 and Sox-6, which probably facilitate recruitment of other transcription factors. Sox-5, Sox-6 and Sox-9 (known as Sox trio) are expressed during the initial stages of chondrogenesis and are turned off in hypertrophic chondrocytes (de Crombrugghe et al., 2001). In adult chondrocytes, Sox-9 represses the activity of Runx2, which is the main transcription factor responsible for hypertrophic progression (Zhou et al., 2006).

Among the growth factors commonly used for cartilage regeneration (Table 1), the main candidates are transforming growth factor β (TFG-β), growth and differentiation factors (GDFs), bone morphogenetic proteins (BMPs), insulin-like growth factor (IGF) and fibroblast growth factor (FGF). TFG-β controls MSC condensation and proliferation and initiates chondrogenesis by stimulating the expression of Sox-9. There are three TGF-β isoforms expressed in mammals that although activate the same pathways, they differ in some amino acid regions that determine affinity for the receptors (Baardsnes et al., 2009). They are expressed in different cell types and are involved in chondrogenesis (Thorp et al., 1992; Pelton et al., 1991; Kubiczkova et al., 2012). They act through the receptor TGF-β RI that recruits and phosphorylates a type II receptor, leading to activation of receptor regulated proteins Smad2 and Smad3. Once activated, these mediators form hetero-complexes with Smad4 and then activate the transcription of Sox-9, inhibiting hypertrophic progression. TGF-β members also carry out their functions through the mitogen-activated protein kinases (MAPK) cascade that results in phosphorylation and activation of various transcription factors (Mariani et al., 2014). Gene therapy with TGF-β has been widely investigated in human patients affected by OA (Ha et al., 2015; Cho et al., 2017; Lee et al., 2015a; Ha et al., 2012; Cherian et al., 2015). BMPs belong to the TGF superfamily, are involved in all phases of chondrogenesis, promote proliferation and are also essential for the endochondral ossification. They activate the Smad 1/5/8 pathway (Mariani et al., 2014; Augustyniak et al., 2015). They upregulate the expression of Sox-9 and, in cooperation with Sonic Hedgehog (Shh), they maintain the expression of Sox-9 through a positive feedback loop between Sox -9 and the homeobox containing transcription factor Bapx1 (Karamboulas et al., 2010). GDFs are members of the BMP family that regulate osteochondral differentiation (Schmierer and Hill, 2007). During chondrogenesis, GDF-5 (also known as BMP-14) promotes MSC recruitment and differentiation (Tsumaki et al., 1999), promotes mature chondrocyte proliferation, increases the size of the final skeletal elements and is also associated with the layer of cells that after joint cavitation maintain a resting cartilage phenotype (Archer et al., 2003). A pro-hypertrophic effect of GDF-5 has also been described (Coleman and Tuan, 2003; Diederichs et al., 2017).

IGF-1 is involved in early chondrogenesis. Its cellular response requires type I tyrosine kinase receptor (IGFIR) and results in the activation MAPKs (Mariani et al., 2014; Augustyniak et al., 2015). FGFs are expressed during different phases of chondrogenesis and their spatio-temporal expression is controlled by Wnt signalling and homeobox transcription factors. FGF-2 is expressed during MSC condensation and early chondrogenesis and it acts as a mitogen (Mariani et al., 2014), probably synergistically with Wnt3A (Narcisi et al., 2015). During endochondral bone development, FGF-9 and FGF-18 are produced by perichondrium and, through the STAT1 pathway and the MAPK cascade, they upregulate Sox-9 expression, supressing chondrocyte proliferation, typical of advanced chondrogenesis (Ornitz and Marie, 2015).

Ultimately, the choice of the transgene depends on the clinical need, the target cell type and the transfection system used. Although transcription factors (e.g. Sox trio) may be the main chondro-inducers (Ikeda et al., 2004), growth factors / cytokines and soluble receptors act in a paracrine manner that favours their use (Table 2).

2.2. Anti-catabolic treatment

In case of inflammatory (e.g. OA) and autoimmune (e.g. RA) diseases, gene therapy aspires to reduce cartilage degeneration (Table 3). In this context, interleukin (IL) 1 and 6 and tumour necrosis factor α (TNF-α) have attracted much attention. (Brennan and McInnes, 2008; Feldmann et al., 1996; Lubberts and van den Berg, 2003; Yoshida and Tanaka, 2014; Magyari et al., 2014; Wojdasiewicz et al., 2014; Mabey and Honsawek, 2015; Miller et al., 2014; Westacott and Sharif, 1996; Calich et al., 2010; Sokolove and Lepus, 2013). Members of the IL family mainly act through interleukin 1 receptor associated kinase (IRAK) activated pathways, such as MAPK and STAT, which lead to the activation of the nuclear factor NF-κB. ILs are mainly produced by inflammatory cells, they can be either secreted or membrane-bound and different polymorphisms are known to increase the risk of developing cartilage damage (Jotanovic et al., 2012). Soluble IL receptors are also

Table 1
Pathways activated by different growth factors and their involvement during physiological chondrogenesis.

Growth factors		Involvement in chondrogenesis	Molecular pathways
IGF-1		Early chondrogenesis	MAPKs Erk 1/2 and PI3K
TGF-β		Condensation and proliferation of mesenchymal cells	Smad 2/3 or Smad 1/5/8, MAPKs P38, JNK and Erk1/2
FGF		Condensation and proliferation of mesenchymal cells Early chondrogenesis Hypertrophy	MAPKs Erk1/2, STAT1
BMP	BMP-2	All phases of chondrogenesis	Smad1/5/8 and MAPKs p38, JNK and Erk1/2
	BMP-4	Condensation and proliferation of mesenchymal cells, Early chondrogenesis	
	BMP-6	Hypertrophy	
	BMP-7	Condensation and proliferation of mesenchymal cells, Early chondrogenesis, advanced chondrogenesis	
	GDF-5	Early chondrogenesis and hypertrophy	

Table 2
Characteristics of categories of most investigated transgenes.

	Transcription factors	Growth factors/Cytokines	Soluble receptors/receptor antagonists
Biological effect	Transfected cells express the transgene, which modulates their own DNA transcription	Transfected cells produce the transgene, which is secreted and has a biological effect on surrounding cells	Transfected cells produce the transgene, which is secreted and inhibit surrounding target growth factors/cytokines
Transgenes proposed	Sox-9, Sox-5, Sox-6	TGF-β, IGF, BMPs, IFN-β, IL-10	IL-1Ra, sTNFR:Fc, sFlk1
Aim	Induce new cartilage formation	Induce new cartilage formation or reduce its degradation	Induce new cartilage formation (sFlk1) or reduce its degradation (IL-1Ra, sTNFR:Fc)
Target cells	Chondrocytes, BMSCs, ADSCs	Chondrocytes, BMSCs, ADSCs, synovial fibroblasts	Chondrocytes, BMSCs, ADSCs, synovial fibroblasts
Approach	*Ex vivo*	*Ex vivo* or *in vivo*	*Ex vivo* or *in vivo*
Disadvantages	High transfection efficiency is needed, to ensure that the transgene perform its function in all the implanted cells	Compared to transcription factors, it is more difficult to control localisation and consequent biological activity of produced growth factor/cytokine	Compared to transcription factors, it is more difficult to control localisation and consequent biological activity

known for their different biological effects. For example, soluble IL-1 receptors (sIL-1R) inhibit the actions of IL-1 (Symons et al., 1995; Smith et al., 2003), whereas soluble IL-6 receptors (sIL-6R) bound to IL-6 and stimulate a response even in cells lacking the membrane bound receptors (Rose-John et al., 2006). IL-1 receptor antagonists (IL-1Ra) is a protein, naturally produced in response to inflammation, which inhibits biological action of IL-1, by binding to its receptor. To overcome the short-life of IL-1Ra (Gouze et al., 2003a), transfection of cells with its cDNA has been proposed for the treatment of RA (Evans et al., 1996; Evans et al., 2005; Oligino et al., 1999) and OA (Zhang et al., 2015; Wang et al., 2006; Fernandes et al., 1999). Other studies generated IL receptor knockout mice (Brunger et al., 2017) or transduced cells with the tissue inhibitor of metalloproteinase 1 (TIMP-1), as TIMP-1 is induced by IL-1 (Kafienah et al., 2003).

TNF-α is a cytokine, primarily produced by monocytes and macrophages, but also by activated natural killer (NK) and lymphocyte T cells. Its expression is stimulated by NF-κB. It is expressed in trans-membrane form, which can be cleaved leading to the generation of a trimeric soluble form, which binds to dimeric receptors TNFRI and TNFR2 (Sedger and McDermott, 2014). Soluble receptors, created by fusion of TNF -α receptor with Fc of IgG1 (sTNFR:Fc), have been used to inhibit RA progression (Marotte et al., 2011; Lavagno et al., 2004; Lupia et al., 1996). Gene therapy with sTNFR:Fc has been used in antigen-induced arthritis (AIA) (Kim et al., 2002) and collagen-induced arthritis (CIA) models (Kim et al., 2003) and has been evaluated in two human clinical trials (Mease et al., 2009; Mease et al., 2010).

Cytokines and chemokines are also under investigation. For example, IL-4 and IL-10 are produced by activated immune cells (Ip et al., 2017), increase the synthesis of IL-1Ra (Jenkins et al., 1994; Fenton et al., 1992), decrease the synthesis of IL-1 and TNF-α by monocytes and macrophages, inhibit NF-κB (Ip et al., 2017; Fenton et al., 1992; Wang et al., 1995; de Waal Malefyt et al., 1991; Iannone et al., 2001; Woodward et al., 2010; Hart et al., 1989) and possess therapeutic potential in OA (Rojas-Ortega et al., 2015; Behrendt et al., 2018) and RA (van de Loo and van den Berg, 2002) animal models. IL-10 gene transfer has been shown to reduce IL-1β and IL-6 productions in a micro-mass synovial membrane culture, where certain changes associated with OA were induced by TNF-α and lipopolysaccharides (Broeren et al., 2016). It has also been shown to decrease cartilage proteoglycan (PG) depletion in RA models, induced by streptococcal cell walls (SCW) injection (Vermeij et al., 2015).

The cytokine interferon β (IFN-β) is a polypeptide belonging to the type I interferon, normally produced by fibroblasts, that possesses immune-modulatory effects. It reduces antigen presentation by antigen-presenting cells (APCs) (Chofflon, 2005), inhibits proliferation of lymphocytes T (Jungo et al., 2001; Markowitz, 2007), decreases expression of IL-1β and TNF-α (van Holten et al., 2004; van Holten et al., 2002; Smeets et al., 2000) and increases expression of IL-10, IL-4 (Rep et al., 1996) and IL-1Ra (Palmer et al., 2004). Two clinical trials, exploring gene therapy for RA and OA using IFN-β, are underway (NCT02727764 and NCT03445715, Source: clinicaltrials.gov). To restrict transgene expression in inflammatory environment and of the inflammatory cells (reducing side effects), transgene expression under the control of IL-1 enhancer / IL-6 proximal promoter (Henningsson et al., 2012; Geurts et al., 2007) or cyclooxygenase (COX) 2 promoter (Rachakonda et al., 2008) and CXC motif chemokine 10 (CXCL-10) promoter (Broeren et al., 2016) have been used in animal models.

The major limitation in successfully clinically translating gene-based therapies still remains the unsuitability of the *in vivo* models (e.g. AIA (Kim et al., 2002); SCW (Vermeij et al., 2015); CIA (Kim et al., 2003); adjuvant-induced arthritis (Sukedai et al., 2011); intra-articular injection of IL (Oligino et al., 1999; Gouze et al., 2003b) for RA, meniscectomy and ligament transection (Zhang et al., 2015; Wang et al., 2006; Fernandes et al., 1999; Guo et al., 2011; Nasi et al., 2017; Sun et al., 2016; Lee and Im, 2012); and chronic osteochondral models for OA (Frisbie et al., 2002)) to mimic the complexity of the human pathology (Miller et al., 2014; Kuyinu et al., 2016; Bevaart et al., 2010). Indeed, candidate genes that showed effective outcomes in animal models, did not show beneficial effects in human patients (e.g. TNF-α (Chevalier et al., 2015) and IL-1β (Nasi et al., 2017; Chevalier et al., 2009) inhibition in patients with OA).

Table 3
Gene therapy strategies proposed for the treatment of acute cartilage defects, osteoarthritis and rheumatoid arthritis.

	Acute cartilage defects	Osteoarthritis	Rheumatoid arthritis
Target cells	Chondrocytes, BMSCs, ADSCs, iPSCs	Synovial fibroblasts, chondrocytes, BMSCs, ADSCs, iPSCs	Mainly synovial fibroblasts
Candidate genes	Transcription factors or growth factors	Growth factors, anti-inflammatory cytokines, or soluble receptors / receptor antagonists	Growth factors, anti-inflammatory cytokines or soluble receptors / receptor antagonists
Approach	*Ex vivo* The use of scaffolds lo localise cells at defect site is beneficial	*Ex vivo* and i*n vivo*	*Ex vivo* and *in vivo*
Aim	Generate new cartilage	Reduce inflammation/cartilage degeneration and generate new cartilage tissue	Reduce inflammation/cartilage degeneration
Transgene expression	Short term expression may be sufficient	Prolonged expression is required	Prolonged expression is required

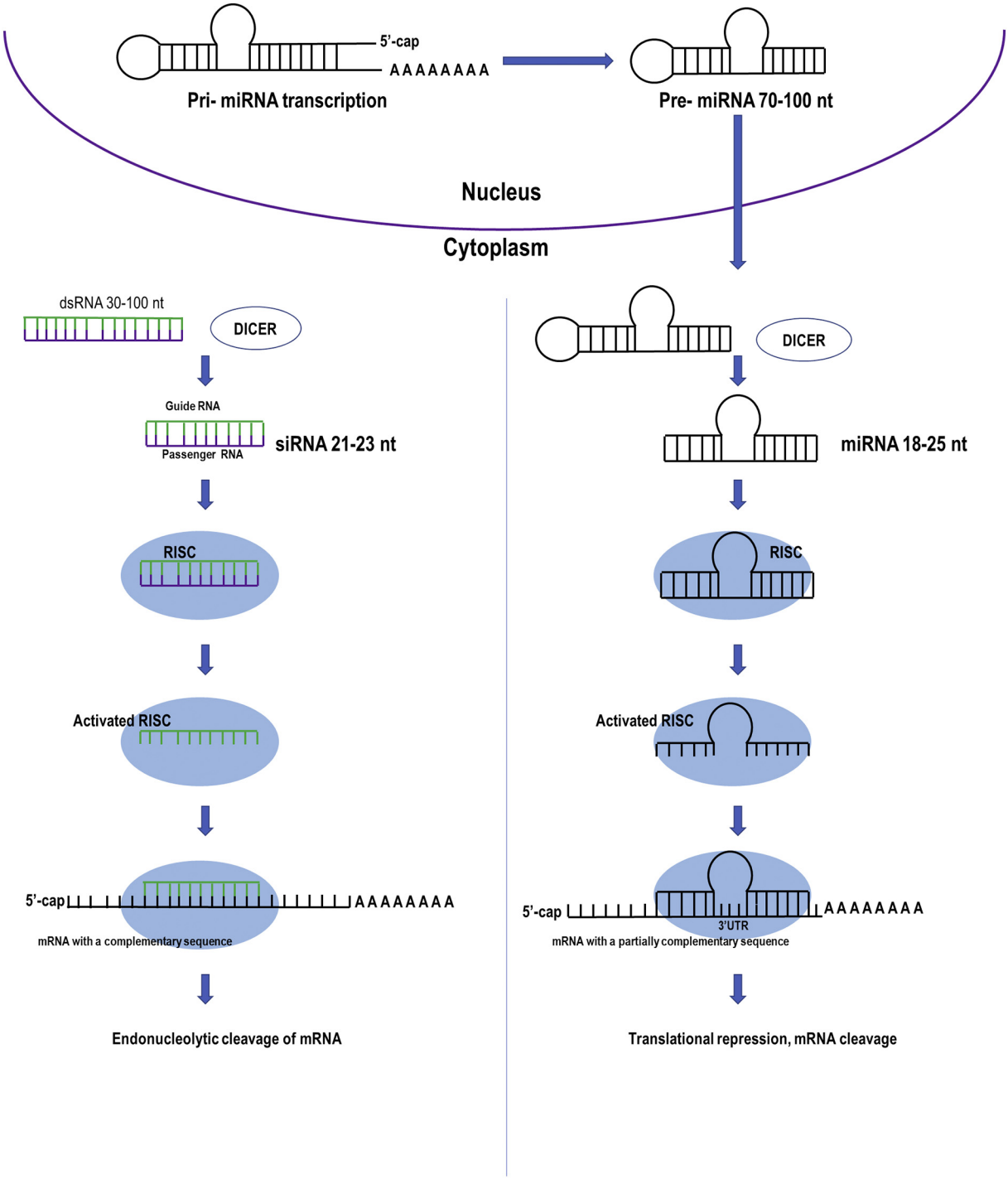

Fig. 1. siRNA and miRNA mediated RNA silencing. Foreign dsRNAs are cleaved by DICER into siRNAs, which contain a guide RNA (complementary to the target mRNA) and a passenger RNA filament. Pri-miRNAs derive from transcription of endogenous genes (and contain a polyadenylated tail and a 5′-cap) and are processed into pre-miRNA. Pre-miRNAs translocate to the cytoplasm where they are processed by DICER into mature miRNA. Mature miRNAs and siRNAs are recognised by RISC complex, which cleaves/discards the passenger RNA. After binding between the guide RNA filament and a fully complementary (in the case of siRNA) or a partially complementary (in the case of miRNA) sequence of an endogenous mRNA, the target mRNA is cleaved or translationally repressed (in the case of miRNA).

2.3. RNA interference

RNA interference (RNAi) is the process by which endogenous mRNA are silenced by double strand RNA (dsRNA), which can be either small-interfering RNAs (siRNAs) or a microRNAs (miRNAs) (Fig. 1) (Lam et al., 2015). siRNAs have been proposed to reduce synthesis of fibro-cartilaginous ECM proteins. Specifically, gene encoding the α1 chain of collagen type I, which is the main component of fibro-cartilaginous tissue has been primarily targeted in chondrocytes (Perrier-Groult et al., 2013; Ollitrault et al., 2015; Legendre et al., 2013) and MSCs (Branly et al., 2018). Silencing of mRNAs for α1 and for α2 chains of collagen type I has significantly reduced collagen type I synthesis in equine BMSCs, compared to the silencing of α1 chain alone (Branly et al., 2018). Core-binding factor alpha 1 (Cbfa), which is an HMG transcription factor involved in osteogenic differentiation, has also been targeted (Sun et al., 2012; Jeon et al., 2012). Knockdown of Cbfa and

Runx2 decreased expression of collagen type I and alkaline phosphatase, which is an osteogenic marker, without affecting expression of chondrogenic markers in murine BMSCs and it also inhibited mineralised matrix synthesis, when the cells were exposed to osteogenic induction media (Gordeladze et al., 2008). High-temperature requirement A serine peptidase 1 (HtrA1) is a serine protease which inhibits TGF-β signalling and is upregulated in OA. Knockdown of HtrA1 in de-differentiated chondrocytes increased the ratios of collagen type II / collagen type I and aggrecan / collagen type I (Ollitrault et al., 2015; Rakic et al., 2017). Overexpression of few miRNAs has also shown to either increase hyaline matrix synthesis (e.g. miR-92a-3p) (Mao et al., 2017a) or to decrease ECM catabolism mediated by MMP-13 (e.g. miR-320, miR-92a-3p and miR-127-5p) (Mao et al., 2017b; Meng et al., 2016; Park et al., 2013). siRNA targeting NF-κB, which is activated in response to IL-1β and TNF-α and regulates different inflammatory genes, has also been developed (Chen et al., 2008; Lianxu et al., 2006).

Compared to siRNAs, miRNAs are easier to design, since databases are available and most of protein-encoding genes contain conserved binding sites of miRNAs (Ha and Kim, 2014). However, each miRNAs has multiple target mRNAs, whilst siRNAs are specific for only one mRNA (Shivdasani, 2006). Either the DNA encoding for the required pri-miRNA or the pre-miRNA/mature miRNA (Fig. 1) can be delivered. The first approach (delivery of DNA encoding for pri-miRNA) belongs to the general DNA delivery systems: it requires nuclear targeting, it exploits endogenous nuclear RNA processing apparatus (Zhang et al., 2013) and can guarantee the inheritance of the miRNA. However, oversaturation of nuclear exporting apparatus (Grimm et al., 2006) can reduce knockdown efficiency and identification of silenced genes is more difficult, as one pri-miRNA can give rise to more mature miRNAs (Kim and Nam, 2006). Delivery of both siRNAs or mature /pre-miRNAs may be used for an initial, not inheritable, biological stimulation. Poor RNA stability, risk of IFN -γ stimulation (for dsRNA molecules longer than 30bp (Barik, 2005; Gantier and Williams, 2007; Elbashir et al., 2001; Bridge et al., 2003; Sledz et al., 2003)) and likelihood of inflammation via Toll like receptors (TLRs) (Fabbri et al., 2013; Heil et al., 2004; Kleinman et al., 2008) limit their clinical use.

3. Gene transfer methods

Gene transfer methods, independently on whether viral vectors are used, are broadly classified as *ex vivo* and *in vivo* and each approach exhibits distinct characteristics, advantages and disadvantages (Table 4). In the *ex vivo* approach, gene transfer takes place *in vitro* and the cells are implanted into the joint afterwards. The alternative strategy is based on *in situ* gene transfer. The *ex vivo* approach brings the advantage of delivering also cells and has been proposed for treating both cartilage defects and chronic diseases. When used for cartilage defects, cells are generally implanted using a carrier system to increase localisation in the defect (Im and Kim, 2011; Yang et al., 2011; Hidaka et al., 2003). When used for the treatment of chronic inflammatory (OA) or autoimmune (RA) diseases, transfected cells are generally intra-articularly injected (Evans et al., 1996; Evans et al., 2005; Wehling et al., 2009).

3.1. Viral vectors

Difficulties in transfecting chondrocytes and MSCs through standard methods have encouraged the use of viral vectors (Dinser et al., 2001; Parreno et al., 2016). Adenoviruses (Goodrich et al., 2006; Hemphill et al., 2014; Oberholzer et al., 2007), adeno-associated viruses (AAV) (Hemphill et al., 2014; Goodrich et al., 2009), retroviruses and lentiviruses (Brunger et al., 2014; Li et al., 2004) and baculoviruses (Ho et al., 2004a; Ho et al., 2006) have been the most widely investigated (Table 5). Disadvantages of viral vectors include the restriction in transgene size, complexity of preparation and risk of development of immune response (Lee et al., 2017).

3.1.1. Ex vivo approaches

Adenoviruses and AAV transduce irrespectively of the cell cycle and allow a transient expression of the transgene, which resides in episomal form (AAV can rarely integrate). Adenoviruses can carry large DNA segments (up to 36 kb, although the most commonly used has a size limitation of 7.5kb) (Lee et al., 2017) and are the most used vectors in clinical trials (Ginn et al., 2018). More than 100 AAVs, classified in 12 serotypes, are known. Different primary receptors and co-receptors determine their tropism and through capsid engineering or isolation of naturally occurring AAVs, cell specificity can theoretically be modulated (Hauck et al., 2003), although relatively little has been done so far in this context. Serotype 2 (AAV.2), which is widely used, uses heparan sulphate PG (HSPG) as primary receptor (Lisowski et al., 2015). Different AAV serotypes have exhibited similar *in vitro* transduction capability in synovial cells and chondrocytes (apart from AAV.4 which led to significantly lower transduction) (Hemphill et al., 2014; Goodrich et al., 2009). AAV.2 and AAV.3, compared to AAV.1, AAV.4, AAV.5 and AAV.6, transduced at significantly higher levels cartilage and synovium explant, indicating that they possessed higher capability of penetrating the ECM (Hemphill et al., 2014). Recombinant chimeric vectors of the serotype 2 have been tested and the AAV.2.5, compared to AAV.2.1, AAV.2.2, AAV.2.6, AAV.2.7, AAV.2.8 and AAV.2.9, showed higher capability to transduce chondrocytes, synovial cells and BMSCs and synovial and cartilage explants (it is worth noting that after 30 days and 60 days, similar results were obtained with AV.2.2) (Mason et al., 2012). AAV2.5 showed similar transduction efficiency in cartilage monolayer, cartilage explant and synovial cell cultures, compared to traditional AAV.2 (Hemphill et al., 2014; Goodrich et al., 2013).

The genome of natural and first-generation recombinant AAVs (rAAVs) is a single-stranded DNA that requires conversion (generally mediated by host cell activities) to double-stranded DNA form to be transcribed. Self-complementary AAVs possess in their genome complementary sequences that lead to intra-molecular double-stranded DNA formation, which facilitate transgene transcription. A study that compared *in vitro* the transduction efficiency of self-complementary

Table 4
Characteristics of *in vivo* and *ex vivo* approaches.

	In vivo	*Ex vivo*
Possibility to control which cell type is transfected	No	Yes
Cell type transfected	Cells in the synovium are easily transfected. Chondrocytes are difficult to transfect. Transfection of cells in the infrapatellar fat pad (Payne et al., 2011) and in the capsule and ligaments (Gouze et al., 2007) has been reported.	Chondrocytes, synovial fibroblasts, BMSCs, ADSCs, iPSCs have been used
Transgenes	Soluble molecules (e.g. growth factors, cytokines, soluble receptors)	Soluble molecules or transcription factors
Application	OA and RA	Acute cartilage defects, OA and RA
Disadvantages	Immune response and virus spreading, when viral methods are used. *In vivo* transfection through non-viral method has not been widely investigated	More expensive, compared to the *in vivo* approach.

Table 5
Characteristics of various viruses and numbers of studies available (Source: PubMed; Date:06-04-2018).

Virus	DNA capability	Transgene expression	Infectivity	Disadvantages	Term searched	No. of studies
Adenovirus	8–36 Kb	Episomal	Dividing and non-dividing cells	Immune response	Adenovirus cartilage engineering	54
AAV	5 Kb	Episomal (it can rarely integrate)	Dividing and non-dividing cells	Immune response	Adeno associated virus cartilage engineering	12
Lentivirus	9 Kb	Integrated	Dividing and non-dividing cells	Risk of insertional mutagenesis and of replication competent viruses	Lentivirus cartilage engineering	18
Retrovirus	8 Kb	Integrated	Dividing cells	Immune response, risk of insertional mutagenesis	Retrovirus cartilage engineering	28
Herpes virus	30–40 Kb	Episomal	Dividing and non-dividing cells	Tropism for neuronal cells. No expression during latent infection	Herpes virus cartilage engineering	2
Baculovirus	300 Kb	Episomal or integrated	Dividing and non-dividing cells	Limited mammalian host range, immune response	Baculovirus cartilage engineering	13

AAV.2 (scAAV) using equine synovial cells and chondrocytes showed higher and more persistent transduction when scAAVs were used (100 times less scAAVs resulted in the same transduction efficiency of rAAVs) and transgene expression was detected for up to 42 days (Ishihara et al., 2012). Transduction efficiency using adenovirus has also been correlated to the cell expansion system used prior to infection (e.g. monolayer compared to alginate beads) (Oberholzer et al., 2007).

Contrarily to adenoviruses and AAVs, lentiviruses and retroviruses integrate in the host genome, offering the possibility of a permanent expression of the transgene. However, this may pose a risk of insertional mutagenesis. Most retroviruses infect only dividing cells, whilst lentivirus infection is independent of cell cycle. The likelihood of replication-competent virus generation has been reduced through deletion / inactivation of multiple viral genes (Zufferey et al., 1997) and regulatory sequences (e.g. self-inactivating lentiviruses (Bosticardo et al., 2009; Zufferey et al., 1998)) and by transferring viral genes from the lentiviral vectors to the packaging cell line and packaging vector(s) (e.g. second and third generations (Dull et al., 1998)). However, lentiviruses have not been largely used in clinical trials (Lee et al., 2017). It is also worth noting that the *in vitro* transduction efficiency of primary chondrocytes through lentiviruses or retroviruses has been similar (higher than 70 %) (Li et al., 2004).

Only few studies have used baculoviruses for transduction of primary chondrocytes (Ho et al., 2004b) and MSCs (Ho et al., 2006; Lo et al., 2009). Baculoviruses infect both dividing and non-dividing cells and guarantee a transient expression of the transgene, which remains in episomal form (van Oers et al., 2015). The use of herpes viruses, which can carry large DNA (up to 40 kb), infects both dividing and non-dividing cells and allows a transient expression of the transgene, has also been reported (Oligino et al., 1999).

The main advantages of using viruses include their high transduction efficiency, the possibility to infect even non-dividing cells and the permanent expression of the transgene (for viruses which integrate into the host genome). Further investigation on *ex vivo* approaches and on behaviour of transduced cells once implanted, need to be performed.

3.1.2. Intra-articular injection of viruses

Viral vectors have also been directly injected intra-articularly to transduce host cells. This approach is cheaper compared to the *ex vivo* one, but does not permit control of the target cells, thus has been generally used for delivering secreted molecules (e.g. growth factors). It has not been used for acute cartilage defects; in this case, the *ex vivo* approach would better restrict transgene expression at the defect site (Gelse et al., 2001).

When herpes viruses were intra-articularly injected, since they possess a strong tropism for neuronal cells, they transduced even neurons innervating the joint (Oligino et al., 1999). Adenoviruses, compared to AAVs, have been associated with higher inflammatory response, as was evidenced by infiltration of white blood cells in the synovial fluid, higher decrease in joint motion and increase in IL-1β in synovial fluid (Ishihara et al., 2012). Among the various serotypes of AAVs, it has been reported the natural occurrence of neutralising antibodies anti-AAV.5 over AAV.2, AAV.3 and AAV.6 in synovial fluids from horses (non-deliberately exposed to AAV antigens) (Hemphill et al., 2014). Higher expression of the transgene has been reported after intra-articular injection of AAV.5, compared to AAV.2 (Adriaansen et al., 2005) and of AAV.2.5, compared to AAV.2.2 and AAV.2.1 (Apparailly et al., 2005).

It has been hypothesized that when viruses are injected intra-articularly, synovial fibroblasts are easily reached and infected, whilst chondrocytes in deep zone of articular cartilage can be infected only if AAVs, which possess small dimensions (20 nm of diameter, compared to 100 nm of adenoviruses), are used (Evans et al., 2018). Yet again, a few studies have actually shown transfection of both articular chondrocytes and synovial cells after intra-articular injection of AAV.2 in horses and rabbits (Goodrich et al., 2013; Ulrich-Vinther et al., 2004). Another one showed that after intra-articular injection of AAV.2, AAV.5 and AAV.8, the majority of transduced cells were synovial fibroblasts, whilst articular chondrocytes were transduced only to a lesser extend (Watson et al., 2013). Another one revealed that after intra-articular injection of AAV.2 in joints of rats, all the transfected cells belonged to the synovium and infrapatellar fat pad (Payne et al., 2011).

For the treatment of chronic diseases, intra-articular virus injection must ensure a sufficiently prolonged transgene expression. Among the different factors determining the duration of the transgene expression, inflammatory response plays key role. Differences after intra-articular injection of the same lentiviruses in immunocompromised or immunocompetent rats have been described (up to 20 days in immunocompetent (Gouze et al., 2002; Gouze et al., 2007) compared to more than 6 weeks (Gouze et al., 2002) and 5 months (Gouze et al., 2007) in immunocompromised rats). Transgene expression was detected 35 days after the adenoviruses were injected in immunocompetent horses (Goodrich et al., 2006), whilst in Sprague Dawley (albino) rats, transgene expression was detectable even one year after the intra-articular injection of the AAVs (Payne et al., 2011).

Behaviour of target cells and viral integration capacity also plays an important role in determining the length of transgene expression. Six months after AAV injection in horses, chondrocytes, which *in vivo* do not divide, were still expressing the transgene, whilst synovial fibroblasts, which possess a high *in vivo* turnover rate, were no longer expressing the transgene (Goodrich et al., 2013). However, intra-articular injection in nude rats showed a similar expression profile when the transgene was delivered through adenoviruses (which remain in an episomal form) and lentiviruses (which integrate into the host genome). In that study, articular chondrocytes were not reached by the viruses and did not express the transgene. Synovial fibroblasts expressing the transgene were lost from day 5 to 168 (independently on whether adenoviruses or lentiviruses were used). At both day 5 and 168, non-

dividing cells belonging to connective tissues (mainly capsule and ligaments) were expressing the transgene. The authors concluded that synovial cells lost their phenotype due to their short life span and that since cells expressing the transgene were non-dividing, integration capability of the virus is not the main parameter to consider (Gouze et al., 2007).

A drawback of the intra-articular injection has been the difficulty in controlling localisation of the viruses and the development of immune response (Goodrich et al., 2006; Gelse et al., 2001). A study injected adenoviruses carrying the BMP -2 or IGF -1 genes into mice knees and not only detected viral DNA in liver, lung and spleen and anti-adenoviruses antibodies in the blood, but also showed formation of ectopic cartilage in the peri-articular mesenchyme (with consequently osteophyte formation) after 3 weeks. On the contrary, when synovial fibroblasts were transduced with the same viruses and then intraarticularly injected, viral DNA was rarely detected in lung and spleen, no antibodies were produced and new cartilage was formed only close to injection points (Gelse et al., 2001). However, the limited virus spreading reported in this study may be attributed to the difficulty of intra-articular injection in mice.

To better control timing of transgene expression, inducible gene expression system (Payne et al., 2011; Moutos et al., 2016; Ma et al., 2016) have been investigated. In a study, for example, articular cartilage defects were created in a rabbit models, the articular cartilage surface was then irradiated with a flash of ultra-violet (UV) light (UV irradiation induce the host DNA polymerase responsible of the synthesis of AAV second DNA strand), the wounds were sutured the wound and AAVs were intra-articularly injected. The UV irradiation increased the transduction efficiency of articular chondrocytes adjacent to the defect, without affecting that of synovium. This approach may permit to reduce the number of injected AAVs and increase effective viral concentration in selected regions (Ulrich-Vinther et al., 2004). To better control virus localisation, it has been proposed to mix viral vectors with bone marrow aspirate and implant the resulting clots in cartilage defects (Ivkovic et al., 2010; Pascher et al., 2004).

Difficulties associated with targeting the appropriate cells, controlling virus spreading and the risk of developing an inflammatory response, which can lead to adverse events and in reducing the length of transgene expression, are probably the main limitations of these approaches. A few systems (e.g. non-constitutive promoters, UV light irradiation, bone marrow clot) have been proposed to reduce risks associated with intra-articular virus injections, but more investigations in these directions are needed. For the treatment of chronic diseases, intra-articular virus injection must ensure a sufficiently prolonged transgene expression, which actually has been difficult to be achieved.

3.2. Non-viral vectors

Compared to viral vectors, non-viral vectors do not have restrictions in transgene size and are generally more cost effective and easier to produce. They also possess a lower risk of immune response stimulation, although inflammation, due to the high frequency of unmethylated cytosine-phosphate-guanine (CpG) dinucleotide motifs, has been reported after *in vivo* delivery of naked DNA or cationic-lipid DNA (Yew and Cheng, 2004). Indeed, non-viral vectors are generally expanded in bacterial cells, which lack the DNA methylation apparatus, thus their CpGs are unmethylated. Immune system recognises unmethylated CpGs, through TLRs, as part of defence against microorganism infection (Schwartz et al., 1997; Krieg, 1999; Krieg et al., 1995). Non-viral vectors generally induce transient expression and their transfection efficiency is lower (less than 40-50 % of transfection efficiency (Saraf and Mikos, 2006; Elsler et al., 2012) compared to 80-90 % of viral vectors (Hemphill et al., 2014; Goodrich et al., 2009; Li et al., 2004), they generally do not work well in non-dividing cells, since DNA enters the nucleus when the nuclear envelope is dissolved during mitosis (Dean et al., 2005) and have not been much used in pre-clinical models and clinical trials (Ginn et al., 2018). Non-viral methods can be used either for *ex vivo* or in *in vivo* approaches.

3.2.1. Ex vivo approaches

Electroporation methods, which use electrical field to deliver DNA, have been used extensively for chondrocytes and MSCs (Sukedai et al., 2011; Im and Kim, 2011; Katayama et al., 2003; Helledie et al., 2008; Welter et al., 2004; Kim et al., 2008a), but their cytotoxicity (Kim et al., 2008b) raises concerns. Among electroporation methods, Nucleofection™ (developed by Amaxa, US, and commercialised by Lonza, Switzerland), which creates pores in the nuclear membrane facilitating transfection of non-dividing cells, has been shown to reach up to 70 % of transfection efficiency in chondrocytes (Parreno et al., 2016) and MSCs (Kim and Im, 2011). Microporation (NanoEnTek, South Korea), which uses a pipette tip as an electroporation space to reduce generated heat and cell detrimental effect, has been the most efficient technique in transfecting ADSCs, compared to standard electroporation, cationic polymer and a calcium phosphate methods (Abdul Halim et al., 2014), reaching transfection efficiency of 70 % (using a mock plasmid, with no insert) (Madry et al., 2013; Wang et al., 2009).

A non-liposomal lipid formulation (FuGene6™, commercialised by Roche, Switzerland and Promega, USA) reached 35 % transfection efficiency in chondrocytes (Madry et al., 2005) and appeared to be the most efficient method in transfecting chondrocytes, compared to $CaCl_2$ and a liposomal system (Lipofectin™, Thermo Fisher Scientific, USA) (Stove et al., 2002). A protocol to transfect chondrocytes in suspension (reduces ECM proteins interference) reached 80 % transfection efficiency; it worth mentioning that cells were transfected with siRNA which has the advantage that it functions cytoplasmically and therefore does not need to penetrate the nuclear membrane (Makki et al., 2017). Using a poly-L-lysine lipid system, with the cells in the detergent to permeabilise the membrane, a 71 % transfection efficiency of chondrocytes was obtained (Goomer et al., 2000).

Gene-activated matrices (GAM), which are scaffolds containing DNA in the form of lypoplexis and polyplexis, have also been investigated for the treatment of cartilage defects, bringing the advantage to increase cell localisation in the defect site after implantation. GAMs are generally three-dimensional scaffolds bound through covalent and biodegradable or weak interactions with the DNA complexes, ensuring a constant release (Raisin et al., 2016). Two-dimensional films allowing substrate-mediated delivery of naked plasmids have been also described and transfection efficiency has been related with the spreading propensity of the cells on the film (the authors also associated transfection efficiency with activation of the β-integrin pathway) (Huang et al., 2015). GAMs in the form of three-dimensional scaffolds are more frequently used than two-dimensional films, as a three-dimensional environment is necessary for cells to maintain / acquire a chondrogenic phenotype. GAMs ensure higher and longer transfection efficiency, compared to two-step methods, during which cells are first transfected and then seeded onto the scaffolds. One should consider that when cells are first transduced and then seeded on scaffolds, they replicate to populate the scaffolds. The more they replicate, the more they lose the transgene, when transient transfection methods are used. If instead they are seeded on GAMs, even when transient methods are used, the risk of loss of transgene expression due to cell division is lower. It is worth noting that in transient transfection, a quick decline in the number of transfected rabbit chondrocytes has been reported (Baragi et al., 1997; Shui et al., 2013).

cDNA release profile changes as a function of the architecture and composition (Rey-Rico et al., 2016) of the GAM system (e.g. natural (Diao et al., 2009) or synthetic (Needham et al., 2014; Im et al., 2011; Li et al., 2013)) for the transfection of transcription factors (Needham et al., 2014; Im et al., 2011) or growth factors (Diao et al., 2009; Li et al., 2013). When poly(lactic-co-glycolic acid) (PLGA) scaffolds were impregnated with polyplexes encoding Sox-5, Sox-6 and Sox-9 and seeded with ADSCs, the highest transgene expression was detected after

14 days, with 70 % of cells expressing the transgene and 90 % of the loaded polyplexes were released over 8 weeks period (Im et al., 2011). PLGA GAMs seeded with BMSCs allowed expression of the transgene (TGF-β1) for up to 3 weeks after implantation in rabbit cartilage defects (Li et al., 2013), whilst another study that used chitosan/gelatin GAMs seeded with MSCs and assessed the efficacy of the system in rabbit defects reported increased transgene expression (TGF-β1) for up to 2 weeks (Diao et al., 2009). High transfection efficiencies achieved using new non-viral transfection technologies and their relatively easy preparation encourage their use; however, difficulty in inducing plasmid integration and selecting stable transgene expressing clones (Maier and Schafer, 1999; Smith, 2001; Brinster et al., 1985; Bire and Rouleux-Bonnin, 2013) limit their applicability. In general, the choice of the most suited non-viral system depends on the cell type and clinical need (e.g. use of GAMs may be more advantageous in case of cartilage defects, to localise cells at the defect site).

3.2.2. *In vivo gene delivery*

A few attempts of *in vivo* non-viral transfection have been described (Sun et al., 2016; Needham et al., 2014; Grossin et al., 2003; Pi et al., 2011), aiming at reaching the deep layer of cartilage and reducing risks associated with viral vectors. *In vivo* electroporation (intra-articular injection of naked plasmid, followed by electric pulse delivery) has also been proposed (Sun et al., 2016; Grossin et al., 2003), despite the cytotoxicity of the electric pulse (Lefesvre et al., 2002) and the immunogenicity of the injected naked DNA (Krieg, 1999; Krieg et al., 1995; Sawamura et al., 2005; Braun et al., 2000; McMahon et al., 1998; Suzuki et al., 1999). Electroporation of rat joints did not lead to pathological changes and resulted in a non-uniform distribution of transfected cells in the superficial, middle and deep zone of the patellar cartilage. After 2 months, fluorescent cells were present only in the deep zone of the patellar cartilage. However, authors did not show whether other tissues apart from the patellar cartilage (e.g. articular cartilage, synovium) had been transfected (Grossin et al., 2003). *In vivo* electroporation of rat cartilage has been shown to result in transgene expression for up to 4 weeks, at level similar to ones detected when the transgene was delivered through AAV.2 intra-articular injection. However, no information about which cell type had been transduced was provided (Sun et al., 2016). *In vivo* electroporation of the gastrocnemius (Kim et al., 2003) and the tibialis anterior (Sukedai et al., 2011) muscle with sTNFR:Fc and IL-1Ra have been performed in mice and rat models of arthritis. sTNFR:Fc and IL-1Ra were effectively detected in synovial fluid, despite higher amount were detected in the serum and in muscle. Electroporated muscle showed increased inflammation that strongly decreased after 20 days (Kim et al., 2003).

Specific *in vivo* targeting of chondrocytes and not cells in the synovium has been attempted through phage display of a chondrocyte-affinity peptide (CAP) that exhibited high affinity for human chondrocytes and low affinity for synovial fibroblasts. The CAP was conjugated with polyethylenimine (PEI) / DNA polyplexes and injected in rabbit knees. Although CAP peptides increased the ratio of transfected chondrocytes / synovial fibroblasts, high level of transgene expression in the synovium was reported (Pi et al., 2011).

An acellular bilayer hydrogel with polyplexes for the encoding of Sox trio and Runx2 for the simultaneous regeneration of articular cartilage and subchondral bone, respectively, in rat osteochondral defects was also developed, however *in vivo* cDNA release and cell infiltration and transfection were not described and no significant differences in terms of cartilage regeneration were observed, compared to hydrogels non-loaded with cDNA (Needham et al., 2014).

Currently, only one phase I clinical trial (NCT03477487, Source: clinicaltrials.gov) is evaluating the safety of intra-articular injection of XT-150, which is a human IL-10 variant transgene carried by a non-viral vector, for the treatment of OA. It is evidenced that although non-viral transfection methods have lower immune response than viral methods, their low transfection efficacy prohibits wide acceptance and use.

4. Cell types

4.1. *Genetically engineered chondrocytes*

Chondrocytes, being the native cell population, are the main candidate for gene therapy for cartilage. They have been generally transfected with transcription factors and growth factors involved in physiological chondrogenesis (only few studies transfected them with IL-1Ra (Baragi et al., 1995; Palmer et al., 2002)). The *ex vivo* engineering approach is preferred due to the localisation of chondrocytes as they cannot be easily infected through intra-articular injection of viral vectors (Payne et al., 2011; Gouze et al., 2002),. *In vivo* chondrocytes are resting cells (Muir, 1995) and therefore the possibility of the transgene to be lost during *in vivo* cell division is low, even when transient transfection systems are used However, in case of inflammation, chondrocyte proliferation can also be observed resulting in cell clusters *in vivo* (Sandell and Aigner, 2001).

Among the transcription factors, members of the Sox family have been used extensively in chondrocyte cultures with positive data to-date. For example, overexpression of Sox-9 in human OA chondrocytes resulted in increased collagen type II synthesis and did not affect the levels of collagen type I that remained lower than the ones of the non-transfected cells (Li et al., 2004). In another study, human healthy and OA chondrocytes were retrovirally transduced with Sox-9 and cultured in a pellet system. Transfection with Sox-9 increased levels of collagen type II and glycosaminoglycan (GAG) expression, although it did not decrease levels of expression of collagen type I (Tew et al., 2005). Simultaneous transfection with Sox trio has been proposed as an alternative to the transfection of Sox-9 alone (Ikeda et al., 2004). Dedifferentiated chondrocytes transfected with Sox trio (using PEI-PLGA nanoparticles) increased their expression of aggrecan, collagen type II and cartilage oligomeric matrix protein (COMP) and, when encapsulated in fibrin gels and injected in nude mice, were able to form a hyaline-like tissue that lacked collagen type I for 3 weeks (Yang et al., 2011).

Different studies have also transfected chondrocytes with growth factors involved in chondrogenesis, which can act through autocrine and paracrine effects. Among them, TGF-β1 has been the most widely investigated. Transduction of chondrocytes with TGF-β1 increased *in vitro* collagen type II and PG synthesis (Shuler et al., 2000; Arai et al., 1997) at level similar to those of cells expanded in the presence of exogenous TGF-β1 (Shuler et al., 2000). Contrary to the intradermal injection of TGF-β1 protein (Lee et al., 2005) and of naïve chondrocytes (Song et al., 2005), intradermal injection of stable overexpressing TGF-β1 chondrocytes resulted in ectopic cartilage formation in mice (Lee et al., 2005; Song et al., 2005). A study which implanted TGF-β1 overexpressing chondrocytes in murine, rabbit and goat chondral defects showed that significantly higher amount of hyaline-like matrix was produced by transduced cells compared to controls, in all animal models. In rabbits, the speed of new cartilage regeneration was proportional to the number of implanted cells (it is worth noting that in goat models, after one year, no significant differences were observed compared to control) (Noh et al., 2010).

Apart from TGF-β1, chondrocytes adenovirally transduced with BMP-7 incorporated in a matrix of autologous fibrin have been used for the treatment of articular cartilage defects in horses. After 4, 7, 14 and 28 days the number of inflammatory cells in the synovial fluid was comparable to ones observed when naïve cells were implanted. Four weeks after surgery, an increased tissue volume and accelerated formation of PGs and collagen type II rich matrix was observed in the BMP -7 treated defects. However, after 8 months, the levels of collagen type II and PGs and the mechanical characteristics of the treated defects, compared to the controls, were similar, due to the fact that only a few cells expressing the transgene persisted in the defect (Hidaka et al., 2003). Similarly, another study which implanted chondrocytes adenovirally transduced with IGF-1 in equine chondral defects reported the

formation of more hyaline-like tissue repair after 4 and 9 weeks, but no significative differences were observed after 8 months (Goodrich et al., 2007).

Encouraging pre-clinical results have been obtained with TGF-β1 overexpressing chondrocytes, which led to the generation of Invossa™, TissueGene C (TissueGene, Inc, USA), which is a gene therapy product composed of allogeneic human chondrocytes retrovirally transduced with TGF-β1 and non-transfected chondrocytes, at 3:1 ratio. As a precautionary measure, irradiation of transduced chondrocytes is performed to render them replication-incompetent. TissueGene C has already passed phase I (Ha et al., 2012) and phase II (Ha et al., 2015; Cho et al., 2017; Cherian et al., 2015) clinical trials. In a phase III clinical trial involving 156 patients, it reduced pain and improved sports activities one year after a single administration (Cho et al., 2016) and has recently received marketing approval for the treatment for degenerative OA.

Identification of the most suitable candidate gene for chondrocyte engineering is still under investigation. Further assessment of the behaviour of *ex vivo* transfected chondrocytes in cartilage defects of large animals is needed to identify, for example, for how long they express the transgene. The scarce availability of autologous chondrocytes coupled with their low bioactivity and the need for additional surgery (for *ex vivo* approaches) discourage their use. However, the positive experience with Invossa™, TissueGene C (TissueGene, Inc, USA) suggests that allogenic approaches may be used successful. Long term data would verify, or not, this in the years to come.

4.2. Genetically engineered bone marrow stem cells

Different studies have genetically engineered BMSCs for *ex vivo* applications. The aim was either to reduce their tendency to undergo hypertrophic progression or to induce overexpression of transcription factors and growth factors to facilitate *in vivo* new cartilage formation (only a study has transfected them with IL-1Ra (He et al., 2015)). Sox-9 is a potential target for genetically engineered BMSCs, considering that induces chondrogenic lineage commitment and keeps low the expression of genes responsible of hypertrophic progression (Dy et al., 2012). One study compared the effects of adenoviral transduction of human BMSCs with Sox-9 alone or in combination with Sox-5 and Sox-6. Sox trio, as opposed to Sox-9 alone, induced higher levels of expression of collagen type II, aggrecan and chondromodulin-I and lower levels of collagen type X, collagen type I, Runx2 and osteopontin (Ikeda et al., 2004). Transduction of human BMSCs with an adenoviral vector carrying Sox-9 gene, in combination with complex multi-axial load, induced chondrogenic phenotype, with Sox-9 inducing the expression of Sox-5 and Sox-6 (Kupcsik et al., 2010). Another study showed that nucleofection of human BMSCs with Sox trio resulted in increased expression of collagen type II and Sox-9 and decreased collagen type I expression (Kim and Im, 2011). When human BMSCs were transfected with Sox trio using PEI-PLGA nanoparticles, the transfection efficiency was lower than 15 % and as such, the transfected cells were selected through FACS, encapsulated in fibrin gels and subcutaneously injected in nude mice. Within 3 weeks, they were able to form hyaline-like tissue rich in collagen type II and depleted of collagen type I (Yang et al., 2011). Another proposed approach to mitigate hypertrophic tendency of BMSCs involved the retroviral transduction with sFlk1, a decoy receptor of VEGF able to sequester endogenous VEGF. After subcutaneous injection in mice, transduced cells (with no pre-induction) spontaneously synthesised collagen II and GAGs (Marsano et al., 2016).

Transfection of BMSCs with growth factors (e.g. BMP-6 (Kayabasi et al., 2013), chondromodulin-I (Xing et al., 2015), BMP-2 and IGF-1 (Gelse et al., 2003), BMP-2 and BMP-4 (Steinert et al., 2009) and TGF-β1 (Palmer et al., 2005)) has been shown to upregulate the expression of collagen type II and other cartilage proteins. One study compared the adenoviral-mediated transduction of BMP-2 and BMP-4 in human BMSCs and found that cells overexpressing BMP-2 showed higher tendency *in vitro* to progress towards hypertrophic phenotype, as demonstrated by expression of alkaline phosphatase, annexin 5 and collagen type X (Steinert et al., 2009). Another study compared the chondrogenic differentiation of rabbit BMSCs transfected with TGF-β1, IGF-1 or BMP-2. Cells overexpressing TGF-β1 increased expression of collagen type II, at levels similar to those of cells treated with exogenous TGF-β1. Cells transduced with BMP-2 overexpressing adenoviruses augmented their collagen type II synthesis and showed the lowest level of expression of collagen type I, while cells transduced with IGF-1 did not show chondrogenic differentiation, in terms of collagen type II synthesis (Palmer et al., 2005).

The effect of genetic modification of BMSCs has been assessed in small and large animal models. A study injected bone marrow coagulates mixed with adenoviruses encoding BMP-2 or Indian hedgehog (IHH) in rabbit chondral defects. Compared to control (GFP-carrying adenoviruses), superior cartilage formation was observed when IHH was delivered. On the contrary, in 2 out of 6 animals treated with BMP-2 carrying viruses, intralesional bone formation was reported (Sieker et al., 2015). Implantation of BMSCs overexpressing TGF-β1 in polylactide (PLA) scaffolds, resulted in superior cartilaginous tissue repair in rabbit osteochondral defects, compared to implantation of naïve cells or of acellular scaffolds (Guo et al., 2006). Similarly, implantation of PLGA-based GAM encoding for TGF-β1 in rabbit osteochondral defect led to higher synthesis of collagen type II and aggrecan but also increased collagen type I and collagen type X content (compared to healthy articular cartilage). Transgene expression was detected for up to 3 weeks after implantation (Li et al., 2013). In ovine cartilage defects, implantation of bone marrow clots containing adenoviruses carrying the TGF-β1 gene led to higher collagen type II content in the repair tissue (Ivkovic et al., 2010).

Despite some encouraging preliminary *in vitro* and *in vivo* data, more pre-clinical studies are required to bring these technologies to clinical practice.

4.3. Genetically engineered adipose derived stem cells

Genetically modified ADSCs have also been investigated for *ex vivo* applications. One study compared the effects of transfection of ovine ADSCs with IGF-1, TGF-β1, FGF-2 and Sox-9 alone or in combination. Aggregates of ADSCs adenovirally co-transduced with IGF-1 and FGF-2 showed the highest expression of PGs and collagen type II, with limited expression of collagen type I and collagen type X (Garza-Veloz et al., 2013). Another study assessed the chondrogenic potential of human ADSCs transfected with Sox trio through nucleofection (cultured in the presence of endogenous TGF-β and BMP-7). While non-transfected cells increased the expression of both chondrogenic and hypertrophic markers, transfected cells increased the synthesis of GAGs and collagen type II, albeit at levels similar of those of the non-transfected cells, but they did not increase the expression of collagen type I and collagen type X. Additionally, when injected subcutaneously into nude mice, three weeks post implantation, they produced high amounts of PGs and exhibited reduced mineralisation (assessed via Alizarin red staining), in comparison to the non-transfected cells (Im and Kim, 2011). A study implanted ADSCs stably or transiently overexpressing TGF-β3 and BMP-6 in rabbit chondral defects and after 12 weeks showed that, whilst transiently transduced cells underwent osteogenesis / hypertrophy, stably transduced systems led to superior cartilaginous tissue repair (Lu et al., 2014).

In another study, rat ADSCs transfected with Sox trio using a PLGA scaffold impregnated with polyplexes and implanted in rabbit osteochondral defects for 8 weeks, resulted in higher production of PGs and collagen type II and lower expression of collagen type I and collagen type X, compared to the non-transfected cells cultured in the presence of TGF -β2 and BMP -7. The *in vitro* transfection efficiency was about 50 % and the number of transfected cells remained constant for 21 days of culture; the *in vivo* expression of the transgenes was not

reported (Im et al., 2011). Another study transduced ADCSs using retroviruses carrying Sox trio genes. Cells showed higher chondrogenic capacity and less hypertrophic tendency compared to cells transduced with empty plasmids, either *in vitro* or in rat osteochondral defects. The cells were expressing the transgene for 21 days in pellet culture and 8 weeks after implantation using a fibrin carrier. Further, the implanted in surgically-induced OA (anterior cruciate ligament transection) rat model, after 8 weeks, the cell / carrier system reduced PG loss and induced higher, but not significant, histological scores, compared to naive cells (Lee and Im, 2012).

Due to high proliferation rate and isolation efficiency of ADSCs, the use of engineered ADSCs would probably be favourable compared to other engineered cells. However, similarly to BMSCs, further *in vivo* data are needed.

4.4. Other MSCs and induced pluripotent stem cells

Engineered MSCs from perichondrium (Goomer et al., 2001), periosteum (Kobayashi et al., 2002; Mason et al., 1998) and muscle-derived stem cells (MDSCs) (Matsumoto et al., 2009; Kuroda et al., 2006) have also been generated. Rat MSCs from perichondrium have been adenovirally transduced with IGF-1 or BMP-2, mixed with fibrin and implanted in rat cartilage defects. After 8 weeks, the defects were filled with hyaline-like matrix, whilst when naïve cells were implanted fibrocartilaginous tissue was formed (Gelse et al., 2003). Periosteal cells transduced with AAVs carrying the Lac Z gene, expressed the transgene even after 12 weeks *in vitro*, but for only 2 weeks after injection in rabbit knees (Kobayashi et al., 2002). MDSCs retrovirally transduced with BMP -4 showed higher *in vitro* and *in vivo* chondrogenic capability, compared to non-transfected cells, and in athymic rat cartilage defects, transgene expression was detected for up to 12 weeks post implantation (Kuroda et al., 2006). Compared to BMSCs and ADSCs, scarce availability and difficulty in their isolation limit the use of perichondrium MSCs.

Induced pluripotent stem cells (iPSCs) possess a high degree of plasticity, which makes them suitable candidates for cartilage tissue engineering. iPSCs are somatic cells, typically adult skin fibroblasts (a study has reported generation of iPSCs from adult chondrocytes (Wei et al., 2012)), transduced with transcription factors (OCT3/4, Sox -2, KLF4 and c-MYC), that can be reprogrammed to an embryonic stem cell-like state and then differentiated into chondrogenic lineage (Wei et al., 2012; Diekman et al., 2012; Ko et al., 2014; Craft et al., 2015). Direct iPSC injections (Lee et al., 2015b) or iPSCs / scaffold systems (Liu et al., 2014) have been successfully used for repairing cartilage defects in mice (Lee et al., 2015b) and rabbits (Liu et al., 2014). Through CRISP/Cas9 gene editing, IL-1R1 gene was deleted in murine iPSCs and the cells were then differentiated into chondrocytes; in *in vitro* setting, they were resistant to IL-1α treatment (Brunger et al., 2017). However, the efficacy of this approach needs to be demonstrated *in vivo,* since other pathways may be involved in cartilage degeneration.

iPSCs can overcome limitations (e.g. availability) associated with chondrocytes and MSC. Furthermore, iPSCs possess a high expansion capacity (Diekman et al., 2012). However, the variation of the populations produced needs to be investigated in depth to accurately describe and characterise the generated cells. Before moving to human trials, one risk to consider is the difficulty to obtain uniform differentiation of iPSCs, as this can affect the efficacy of the therapy and increase the risk of tumorigenesis (Diekman et al., 2012). Furthermore, a detailed comparison between multipotent and pluripotent cells needs to be performed to ensure that pluripotent cells, under appropriate *in vitro* conditions or within the tissue *in vivo* microenvironment, effectively possess a lower hypertrophic differentiation tendency.

4.5. Fibroblasts and synovial cells

A fibroblast cell line, which has been used in rabbit models (Lee et al., 2001; Madry et al., 2010) and primary synovial fibroblasts, which have already reached human experimentation (Evans et al., 2005; Wehling et al., 2009), have been transfected with genes encoding soluble factors (e.g. IL-1Ra (Oligino et al., 1999; Watson et al., 2013; Kay et al., 2009), IFN-β *(Aalbers* et al., *2015)*, sTNFR:Fc (Khoury et al., 2007), TGF-β *(Blaney Davidson* et al., *2006)*). In animal models, synovial fibroblasts have been mainly transduced through intra-articular injection of viral vectors (Oligino et al., 1999; Adriaansen et al., 2005; Apparailly et al., 2005; Watson et al., 2013; Kay et al., 2009; Aalbers et al., 2015). Due to their *in vivo* high proliferation rate, stable transfection systems are preferred (Gouze et al., 2007). When fibroblasts overexpressing IL-1β to trigger inflammation and lentiviruses carrying the IL-1Ra were injected in rat joints, the expression of the transgene (after 5 days) in the synovium increased in relation to number of cells overexpressing IL-1β injected (Gouze et al., 2003b), suggesting that the number of synovial fibroblasts expressing the transgene was increased in response to inflammation. However, another study reported that, due to their *in vivo* short life span, the number of synovial cells expressing the transgene strongly decreased from 5 to 168 days after lentiviral injection (Gouze et al., 2007). Intra-articular injection of herpes viruses carrying IL-1Ra showed reduced inflammation (as judged by the number of inflammatory cells infiltrating and the amount of IL-1β in the synovial fluid) after 24 h in an IL-mediated experimental RA rabbit model (Oligino et al., 1999). However, to evaluate the efficacy of this approach, longer timepoints are needed, to establish the duration of the transgene expression *in vivo*. Further, in human patients, other inflammatory pathways are involved in the degeneration of cartilage, which should be considered.

Intra-articular delivery of TGF-β protein *(Allen* et al., *1990)* or gene (Bakker et al., 2001), which are strong inducers of cartilage regeneration, also resulted in synovial hyperplasia and fibrosis. To overcome this side effect, intra-articular injection of adenoviruses carrying TGF-β and Smad7, which is an inhibitor of Smad2/3 pathway, has been proposed. This approach allowed the transduced synovial cells to secrete TGF-β, but it rendered them unable to respond to it, due to Smad7 overexpression. In IL-1 induced RA and in genetically modified OA (STR/ort) mice models, this approach increased PG content in the articular cartilage, limited the increase in synovial thickness and limited collagen type I synovial content, which was observed when only TGF-β carrying adenoviruses were injected (Blaney Davidson et al., 2006).

Different clinical trials have evaluated *in vivo* and *ex vivo* approaches for synovial cells transfection. In the first clinical trial, administration of plasmid DNA encoding herpes virus thymidine kinase gene (HSV-TK), followed by systemic ganciclovir administration (OBA number 9802-237) was assessed. This study aimed at inducing synovectomy, as expression of HSV-TK conferred synovial cell sensitivity to ganciclovir. However, after only one patient had been enrolled, safety concerns raised due to the death of a patient in an unrelated gene therapy clinical trial (Raper et al., 2003) and the study was terminated. Another phase I/II clinical trial (NCT00126724, Source: clinicaltrials.gov) with 172 patients with arthritis evaluated the intra-articular injection of AAVs carrying the sTNFR:Fc gene. Twelve weeks after implantation, clinical improvement, albeit not statistically significant, was observed in treated patients compared to placebo. Although sTNFR:Fc protein was not detected in the peripheral blood, viral DNA was detected at low levels 4 and 8 weeks after the injection. Neutralising antibodies in the synovial fluid increased in a dose-dependent manner, following AAVs injection (Mease et al., 2009; Mease et al., 2010). It is worth noting that one subject underwent septic arthritis (Mease et al., 2010) and another one died due to histoplasmosis, which was not considered related with the treatment (Frank et al., 2009). Two phase I clinical trials (NCT03445715 and NCT02727764, Source: clinicaltrials.gov) are currently recruiting RA and OA patients to evaluate safety and efficacy of intraarticular injection of AAV.2.5 carrying the IFN-β gene under the control of the promoter NF-κB (ART-I02).

Ex vivo approaches have also reached clinical trials. In two studies,

Table 6
Clinical trials in gene therapy for cartilage diseases.

Name	Procedure	Disease	Clinical trial identification No.	Clinical trial phase	Status
ART-I02	Intra-articular injection of AAV.5 encoding IFN-β under control of NF-κB promoter	OA and RA	NCT02727764	Phase I	Recruiting
		RA	NCT03445715	Phase I	Recruiting
XT-150	Intra-articular injection of non-viral vector encoding IL-10	OA	NCT03477487	Phase I	Active, non recruiting
TissueGene C	Injection of allogenic chondrocytes retrovirally transduced with TGF-β1 and non-transfected cells	OA	NCT00599248 (Ha et al., 2015; Cho et al., 2017; Lee et al., 2015a; Cherian et al., 2015)	Phase I Phase II	Completed Completed
			NCT03383471	Phase III	Active, non recruiting
			NCT03412864	Phase IV (post-marketing surveillance)	Recruiting
HSV -TK	Injection of a plasmid encoding for the herpes simplex virus thymidine kinase (HSV-TK), followed by administration of ganciclovir	RA	OBA number 9802-237	Phase I	Terminated, only 1 patient has been treated
tgAAC94	Intra-articular injection of AAV.2 carrying the sTNFR:Fc	RA	NCT00617032	Phase I	Completed
			NCT00126724	Phase I/II	Completed
IL -1Ra	Intra-articular injection of autologous synovial fibroblasts retrovirally transduced with IL-1Ra	RA	(Evans et al., 1996; Evans et al., 2005; Wehling et al., 2009)	Phase I	Completed

autologous synovial fibroblasts were retrovirally transduced with IL-1Ra and injected into the joint of nine (Evans et al., 2005) and two patients (Wehling et al., 2009) with RA who had to undergo meta-carpophalangeal joint replacement. *In vivo* transgene expression was demonstrated after a month, reduced expression of matrix metallo-proteinase-3 and IL-1β was detected (Wehling et al., 2009).

No long-term clinical studies are available, questioning long-term efficacy. Identification of the most suitable candidate gene is still under investigation and comparison between *in vivo* and *ex vivo* approaches is needed.

5. Critique and conclusions

Gene therapy has been encouraged due to the limitations of cell-based and pharmacological approaches to treat acute cartilage defects and pathophysiologies. Gene therapy, although it has been approved clinically in Korea for the treatment of OA, is still a new approach for cartilage regeneration, with many technical issues and limitations to be addressed, as evidenced by the limited number of clinical studies available (Table 6). The choice of the delivered transgene / candidate gene, suitable transfection or transduction system and target cells are strictly linked to each other and they also depend on the clinical characteristics. Transcription factors (e.g. Sox) and growth factors (e.g. TGF-β and BMPs) involved in physiological chondrogenesis have been studies extensively either alone or in combination. With respect to the transfection method of choice, GAMs are probably the most promising non-viral methods for the treatment of cartilage defects, bringing also the advantage to provide a three-dimensional support for *in vitro* cell culture / differentiation and for *in vivo* implantation. On the other hand, although viral vectors lead to higher transfection efficiency, their higher safety concerns and high costs limit their applicability. The *ex vivo* approach faces the challenge to guarantee a sufficiently prolonged transgene expression and although integrative transfection systems may increase it, they carry the risk of insertional mutagenesis. Intra-articular injection of viral vectors, although effective and more economical than the *ex vivo* method, it does not offer control over which cell type is transduced. Further, intra-articular injection of viruses is associated with safety risks, which may not overwhelm the benefits (Raper et al., 2003; Frank et al., 2009).

Although significant advancements have been achieved *in vitro* and in preclinical setting, only a few clinical trials have been performed. It appears that gene engineering for the treatment of acute cartilage defects and pathophysiologies is still far from an off-the-shelf treatment. Development of safe, physiologically-informed and with higher affinity and longer *in vivo* efficacy transfection or transduction systems will enable progression of gene therapies from a research concept to clinical and commercial reality in the years to come.

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