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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	