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Abstract

As a cell source, multipotent mesenchymal stromal cells or mesenchymal stem cells (MSCs) are promising candidates for chondrogenic differentiation and subsequent cartilage regeneration. From previous literature, it is known that chondrogenic differentiation of MSCs inevitably leads to hypertrophy and subsequent endochondral ossification. In this review, we examine the history of currently established protocols of chondrogenic differentiation and elaborate on the roles of individual components of chondrogenic differentiation medium. We also summarise the effects of physical, chemical and biological factors involved, and propose potential strategies to differentiate MSCs into articular chondrocytes with homogenous mature phenotypes through spatial-temporal incorporation of cell differentiation and chondrogenesis.

Keywords: Chondrogenic differentiation, chondrogenesis, mesenchymal stem cells, chondrocytes, chondrogenic differentiation factors.

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Introduction

Articular cartilage is an avascular connective tissue with limited capacity for self-regeneration. Injury and degeneration of cartilage have far-reaching impacts on personal life, healthcare expenses and reduced productivity in the work force (Ge *et al.*, 2006). Current clinical treatment strategies, such as autologous chondrocyte implantation, bone marrow stimulation and mosaicplasty, have varying success rates, but their long-term results are far from satisfactory due to the lack of structural organisation of articular cartilage, as well as inferior mechanical properties of the newly formed tissue (Hunziker, 2009). Cartilage tissue engineering has the potential to create a more durable and functional replacement for the degenerated tissue; chondrocytes and stem cells are two promising cell source candidates for this replacement tissue. Chondrocytes are potentially the ideal seed cells with mature and stable phenotype as well as lineage stability. However, they are limited by scarcity during harvest and dedifferentiation during *in vitro* proliferation (Kock *et al.*, 2012). Establishment of embryonic stem cells (ESCs) requires embryos, which leads to ethical issues for clinical applications. Induced pluripotent stem cell (iPSC) technology can provide patient-specific cells, but uncontrolled and unexpected differentiation limits their application (Blin *et al.*, 2010; Yoshida and Yamanaka, 2010). Multipotent mesenchymal stromal cells or mesenchymal stem cells (MSCs) are a broadly adopted cell source for cartilage regeneration due to ample availability from multiple tissues, high proliferation capacity and the ability to differentiate into chondrocytes (Derfoul *et al.*, 2006; Kassis *et al.*, 2006). However, concomitant premature phenotypes of the differentiated chondrocytes, undesirable hypertrophic differentiation (Studer *et al.*, 2012), lack of subtype phenotypes and subsequent immature extracellular matrix (ECM) under current chondrogenic differentiation protocols have hindered the clinical application of stem cell-differentiated chondrocytes (Huey *et al.*, 2012; Mueller and Tuan, 2008).

Weighing the advantages and disadvantages of candidate cell sources, apart from using chondrocytes, MSCs may be the most adaptive cell source for current clinical use and for cartilage tissue engineering applications. Therefore, finding ways to improve *in vitro* chondrogenic differentiation of MSCs may be the next primary focus of tissue engineering research, which is somewhat different with the well-

orchestrated embryonic chondrogenic differentiation process of MSCs. Embryonic chondrogenesis have been reviewed extensively with the focus on mesenchymal cell condensation, chondroprogenitor cells, chondrocytes as well as related mediators (Goldring *et al.*, 2006). Apart from embryonic chondrogenesis, joint specification and postnatal development have also been reviewed, though many questions remain unexplored (Onyekwelu *et al.*, 2009). MSCs have been well studied, regarding their definition, tissue origins, chemokines and receptors, as well as their *in vivo* microenvironments (Augello *et al.*, 2010); key factors like cell metabolic activity and transcript levels important for functional MSC-derived cartilage regeneration are summarised elsewhere (Chen *et al.*, 2006). Based on all these progresses, we summarise the history and challenges faced by current broadly used chondrogenic differentiation protocols with an aim to shed valuable light on the potential novel strategies to circumvent the challenges faced, which may benefit bioengineers and researchers in the field of tissue engineering and regenerative medicine.

Chondrogenic differentiation into articular chondrocytes

Chondrogenesis is initiated by cell condensation during embryonic development, before the mesenchymal stem cells differentiate into multiple lineages, such as articular, growth plate, intervertebral disc chondrocytes or endochondral ossification [some of the cells survive and transform into osteoblasts (Zhang *et al.*, 2014)]. For years, mesoderm was thought to be the origin of MSCs; however, new evidence indicates that the neuroepithelium, including the neural crest, may also be an early origin of MSCs (Morikawa *et al.*, 2009; Takashima *et al.*, 2007). MSCs from the somatopleure of the lateral plate mesoderm, neural crest cells in the neural ectoderm and sclerotome compartment of the paraxial mesoderm or somite differentiate into chondrogenic lineages and then yield the limbs, craniofacial bones and axial skeleton separately (Goldring *et al.*, 2006; Olsen *et al.*, 2000). Chondrogenesis may occur in one of two directions in the developing bone. One leads to the formation of bone *via* endochondral ossification, (at the primary and secondary centres of ossification, and in the growth plate) and the other leads to stable hyaline articular cartilage. The growth plate is composed of the resting zone, proliferative zone and hypertrophic zone (Abad *et al.*, 2002). Growth plate cartilage progresses to form the bone (Mackie *et al.*, 2008), whereas the articular cartilage does not (Pacifici *et al.*, 2005). Chondrocytes of the presumptive articular surface arise directly from a subpopulation of early chondroprogenitor cells, expressing doublecortin and growth/differentiation factor-5 (GDF-5) (Koyama *et al.*, 2008; Zhang *et al.*, 2007), but not matrilin-1 (Hyde *et al.*, 2007). Articular chondrocytes maintain the phenotype instead of resorting to hypertrophy by maintaining high expression of *ACAN* and *PRG4* (lubricin gene) and low expression of *COL2A1* with low proliferation (Lefebvre and Smits, 2005).

While articular cartilage at birth undergoes ossification and mineralisation, appositional growth from the surface forms an adult articular cartilage until puberty (Hunziker *et al.*, 2007; Onyekwelu *et al.*, 2009). These slowly dividing stem cells have bidirectional mitotic activity. In the horizontal direction, they furnish new stem cells that replenish the pool and contribute to the lateral expansion of the articular cartilage layer. In the vertical direction, the superficial zone supplies the rapidly dividing, transit-amplifying daughter-cell pool that feeds the transitional and upper radial zones and gradually forms the layered structure (Hunziker *et al.*, 2007). This growth activity of the articular surface ceases at puberty, whereas expansion of the metaphyseal growth plate continues until the time of skeletal maturity (Clark *et al.*, 1997; Hunziker *et al.*, 2007). Knowledge derived from endochondral ossification has been largely used to establish current chondrogenic differentiation protocols, while growth plate chondrocytes that are easily accessible sources are often compared to articular chondrocytes (Iwamoto *et al.*, 2013; Leijten *et al.*, 2012) (Fig. 1).

A history of the understanding of chondrogenic differentiation

Studies of the embryonic development of cartilage or cartilaginous tissues started as early as the 1960s. Ectopic implantation was a major research model during this period. The somites, which are embryonic cartilaginous progenitor cells, differentiated into chondrocytes *in vitro* when co-cultured with notochords (Lash *et al.*, 1960). Holtzer observed chondrogenesis of somites occurring in the presence of neural tubes (Holtzer, 1952; Holtzer and Detwiler, 1953). A fraction obtained from a cold perchloric acid extract of spinal cord and notochord was able to induce chondrogenesis (Lash *et al.*, 1962), which contained chondrogenic factors consisting of polypeptides, nucleotides and sugars (Holtzer, 1964; Hommes *et al.*, 1962). De-differentiation of chondrocytes was reported with morphological changes from spherical to stellate coupled with decreased synthesis of chondroitin sulphate and enhanced cell proliferation (Abbott and Holtzer, 1966). These findings led to the establishment of the pellet culture system, now broadly used in chondrogenic differentiation (Hattori and Ide, 1984).

In the 1970s and 1980s, laboratory studies evaluating the effect of cell shape, cell-cell and cell-matrix interactions on chondrogenesis were undertaken. Chondrocytes with spherical morphologies synthesised higher levels of chondroitin sulphate in comparison to those with spindle-like morphologies (Archer *et al.*, 1982). Cell-cell interactions, including both interactions amongst limb bud mesenchyme cells (Owens and Solursh, 1982) and epithelial-mesenchymal cells (Sanders, 1988), are essential for the differentiation of prechondrogenic limb mesenchyme into cartilage. Gene expression of both *COL I* and *fibronectin* increased at the onset of condensation, reached the highest point of chondrogenic differentiation and then subsided and were replaced with elevated *COL2A1* expression (Dessau *et al.*, 1980; Kulyk *et al.*,

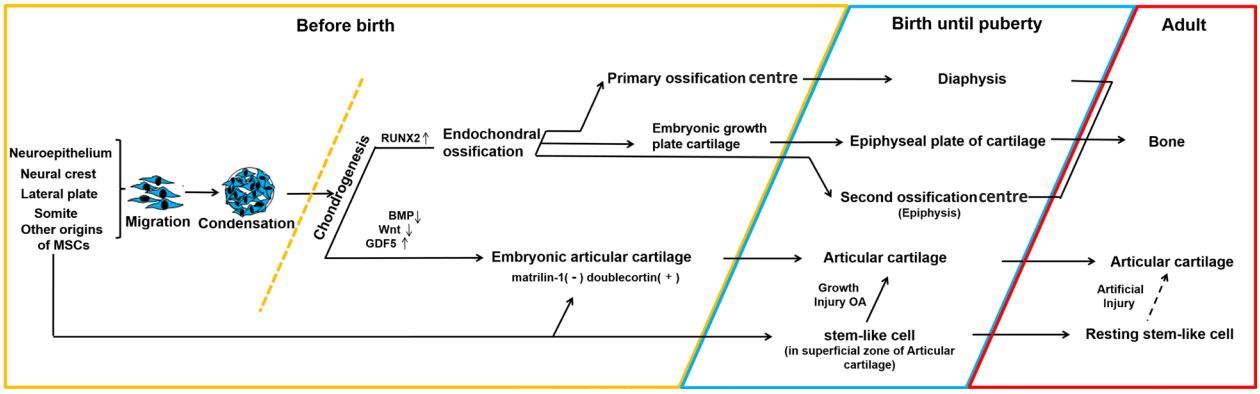

Fig. 1. A schematic representation of different stages of chondrogenesis and endochondral ossification. The development of cartilage starts with MSC condensation. After committing to chondrogenesis, cells follow one of two different directions in the developing bone. One leads to the formation of bone *via* endochondral ossification (at the primary ossification centre (POC), secondary ossification centre (SOC), and in the growth plate) (the process marked in black) and the other that leads to stable hyaline articular cartilage. All committed hyaline chondrocytes start expressing *COL2A1*; matrilin-1 expression by cells arising from the interzones that are destined to become growth plate chondrocytes distinguishes them from articular chondrocytes. Doublecortin and GDF-5 only express in the articular cartilage. Most embryonic articular cartilage present at the ends of the bone at birth is replaced by bone and new cartilage formed by stem cells in the superficial zone. Articular cartilage becomes fully mature in adulthood. With the disappearance of growth plate cartilage, the SOC connects with the POC and gradually disappears in mature mammals. Stem-like cells exist in the cell condensation stage, keep stemness in the superficial zone of AC from birth until puberty, and exist in the form of resting stem-like cell after adulthood.

1989). Furthermore, in the 1980s, transforming growth factor-β (TGF-β) was identified as a key cartilage-inducing factor (Heine *et al.*, 1987; Seyedin *et al.*, 1986).

Many components play supplementary roles in the current chondrogenic differentiation medium. For instance, ascorbate stabilises the triple helical structures of collagen through hydroxylation of proline residues in procollagen and hydroxyproline (Peterkofsky, 1972). Serum inhibits the production of cartilage matrix during *in vitro* chondrogenesis of limb bud mesodermal cells of chick embryos (Hattori and Ide, 1984), whereas serum-free medium containing insulin/transferrin/selenium (ITS) successfully keeps cells alive and active (Bottenstein and Sato, 1979).

The current broadly-used chondrogenic differentiation medium was established in the 1990s, with the presence of TGF-β in pellet cultures (Johnstone *et al.*, 1998). This chondrogenic differentiation medium includes TGF-β, ITS, high-glucose, dexamethasone (Dex), ascorbic acid, sodium pyruvate and proline, but lacks serum (Johnstone *et al.*, 1998; Vater *et al.*, 2011) (Table 1). High-glucose was found to promote MSC survival and protect cells from apoptosis by altering the metabolism of the cells in pellet cultures and increasing the synthesis of proteoglycans through respiratory repression (Crabtree effect) (Mackay *et al.*, 1998).

The functions and mechanisms of differentiation-related growth factors, such as bone morphogenetic protein (BMP), fibroblast growth factor (FGF), parathyroid hormone related protein (PTHrP) and Indian hedgehog (IHh), were studied in depth during this period (Murtaugh *et al.*, 1999; Shukunami *et al.*, 1996). Though these growth factors can benefit chondrogenesis *in vitro*, they are not routinely included in the current medium due to several limitations. BMPs have been shown to promote the expression of *SOX9* and *COL II,* however, they also inevitably result in a hypertrophic phenotype of cells (Enomoto-Iwamoto *et al.*, 1998; Nonaka *et al.*, 1999). FGFs can maintain the proliferation of cells but result in chondrodysplasia when overexpressed (Minowada *et al.*, 1999). Additionally, PTHrP can reverse chondrocytes from a hypertrophic phenotype to a prehypertrophic proliferating phenotype, and prevent terminal differentiation of chondrocytes *in vitro* (Zerega *et al.*, 1999). IHh expressed in prehypertrophic chondrocytes enhances hypertrophic differentiation. Misexpression of IHh can prevent hypertrophic differentiation of chondrocytes (Vortkamp A, 1996).

In the late 1990s, the role of integrins in mediating cell attachment to extracellular matrix proteins was highlighted. These interactions regulated morphogenesis and cell differentiation. Treatment with integrin β1 antibodies inhibited early chondrogenesis of limb bud cells from mouse embryos *in vitro* (Shakibaei, 1998). Additionally, *SOX9* was found to be a master gene which regulated downstream *COL2A1* expression directly (Healy *et al.*, 1999).

Understanding of chondrogenic differentiation has accelerated since the year 2000, attributed to the application of a variety of genetic modification techniques. Epigenetics then emerged as an important field. MicroRNAs (miRNAs) proved effective mediators of key pathways during MSC differentiation through regulating transcription factors (Hong and Reddi, 2012). DNA methylation and histone modification (including histone acetylation and deacetylation) are involved in chondrogenesis through modulating chromatin structures, which directly influence the activity of DNA in transcription, replication and

Table 1. Chondrogenic differentiation medium and effects of individual components on chondrogenesis

	Factors	Function	Signalling	Dose	Synergy	Subtypes
The well-established chondrogenic differentiation serum-free media (Johnstone *et al.*, 1998; Vater *et al.*, 2011)	**TGF-β** (J.Zuscik *et al.*, 2004; Lutz and Knaus, 2002; Park *et al.*, 2011; Schmierer and Hill, 2007)	Promotes chondrogenesis, enhances extracellular matrix production and downregulates collagen I expression	MAPK, Wnt, β-catenin, Smad	10 ng/mL	IGF-I, BMPs, PHTrP	TGF-β1, β2 and β3, have similar functions
	Insulin (Mueller *et al.*, 2013; Quarto *et al.*, 1992)	Stimulates collagen synthesis, inhibits hypertrophy	Enhances tyrosine kinase activity of the insulin receptor	1-10 μg/mL	Transferrin, selenium	
	Transferrin (Cigan, 2013; Kisiday *et al.*, 2005)	Iron-binding		1-30 μg/mL	Insulin, selenium	
	Selenium (Yan *et al.*, 2012)	Anti-oxidant and enhances proliferation		5 μg/mL	Insulin, transferrin	
	Dex (M.Florine *et al.*, 2013; Thomas M.Randua, 2013)	Supports cell viability and delays the appearance of type X collagen	GRs, Scrapie Responsive Gene 1	10^{-7} M	TGF-β	
	Glucose (Cigan, 2013; Han *et al.*, 2004; Tsai, 2013)	Major energy source, precursor for the synthesis of glycosaminoglycans	PKCα, p38MAPK, ERK	4.5 g/L		
	Ascorbic acid (Choi *et al.*, 2008; Franceschi, 1992; Peterkofsky, 1972; Stone and Meister, 1962)	Proliferation, DNA synthesis, collagen biosynthesis and reductant	Reduction of iron to its ferrous state	50 mg/mL	Proline	L-ascorbate, sodium L-ascorbate, and L-ascorbate-2-phosphate
	Pyruvate (Andrae *et al.*, 1985; Geshi *et al.*, 2000)	Energy source, against hydrogen peroxide-induced cytotoxicity		1 mM		
	Proline (Peterkofsky, 1972; Washington *et al.*, 2010)	Stabilisation of the collagen triple helix , promotes ES cell differentiation	mTOR signalling pathway	40 μg/mL	Ascorbic acid	
Selected growth factors regulators	**BMPs** (Cao and Chen, 2005; Hassel *et al.*, 2003; Hatakeyama *et al.*, 2004; Jin *et al.*, 2006; Sekiya *et al.*, 2005; Shen *et al.*, 2010)	Enhances cartilage formation	MAPK, Wnt, Smad	10-500 ng/mL	TGF-β	BMP-2, enhances proliferation and ECM, and downregulates Col I expression. BMP-4, accelerates the progression of cartilage differentiation to maturation BMP-6, enhances chondrogenesis in special subpopulation of MSCs, increases proteoglycans BMP-7, inhibits cell proliferation, induces chondrogenic differentiation BMP-9, enhances Sox9, Col2A1 and aggrecan, overcome the inhibitory effect of IL-1. GDF-6(BMP-13), inhibits hypertrophic chondrocyte specific marker, upregulate of proteoglycan
	GDF-5(BMP-14) (Coleman and Tuan, 2003; Hatakeyama *et al.*, 2004)	Enhances cell condensation, chondrogenesis, and ECM production	Gap junction intercellular communication, p38MAPK	150 ng/mL	TGF-β, BMP	
	PHTrP (Barbara Zerega, 1999; Kim *et al.*, 2008; Mau *et al.*, 2007)	Inhibits the TGF-β-induced hypertrophic differentiation	PTHrP and IHH feedback loop	10 μM	TGF-β	PHTrP1-34
	FGF (Ng *et al.*, 2008; Solchaga *et al.*, 2005; Tsutsumi *et al.*, 2001)	Regulates proliferation, and increases cartilaginous ECM production	ERK	10 ng/mL FGF-2 10^{-9} M FGF-18	TGF-β	FGF-2, increases proliferation, and proteoglycan production FGF-18, inhibits cell proliferation and induces chondrogenic differentiation

recombination (Furumatsu and Ozaki, 2010; Roth *et al.*, 2001). Recently, several groups found an important relationship between epigenetic marking and gene expression in chondrogenesis. Herlofsen *et al*. found that modifications can provide primary epigenetic control of the early differentiation of MSCs toward the chondrogenic lineage (Herlofsen *et al.*, 2013).

Biophysical, chemical and mechanical factors were found to be heavily involved in chondrogenesis (Leijten *et al.*, 2014). Small chemical molecules (Benoit *et al.*, 2008), matrix elasticity (Engler *et al.*, 2006; Park *et al.*, 2011), cell material property (cell softness) (Chowdhury *et al.*, 2010) and many more factors, are now known to guide MSCs to differentiate into specific cell lineages. A breakthrough in the regeneration of articular cartilage illustrated that an entire articular surface can be regenerated using adult stem/progenitor cells recruited from the host with the help of biomaterial-based scaffold and growth factors (Lee *et al.*, 2010).

Key elements in current chondrogenic differentiation medium

At present, MSCs cultured in pellet and micromass systems in the presence of chondrogenic differentiation medium are the two major methods for initiating chondrogenic differentiation *in vitro*. The following sections will describe

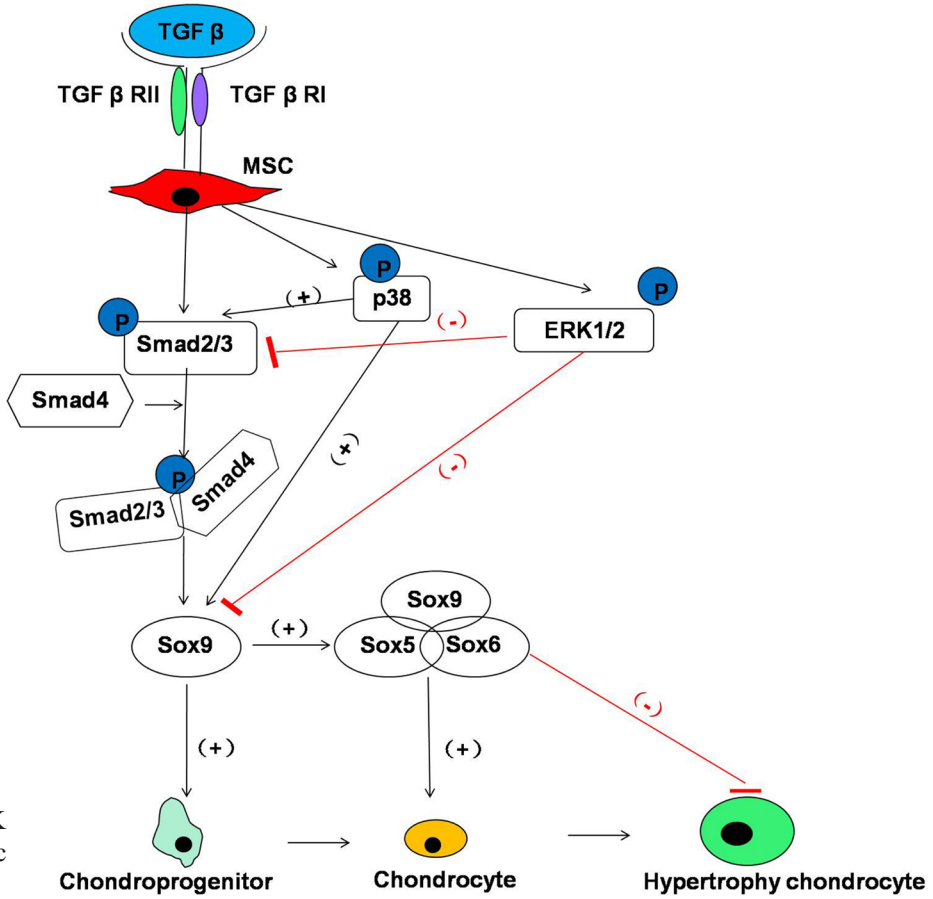

Fig. 2. TGF-β and MAPK signalling in chondrogenic differentiation of MSCs.

the in-depth role of the ingredients potentially used in chondrogenic differentiation medium.

TGF-β is the core component in most chondrogenic differentiation protocols used currently. TGF-βs exist in three homologous homodimeric forms (β1, β2 and β3) and all of them signal through the same receptors [TGF-β receptor I (TGF-β RI) and II (TGF-β RII)]. After activation of the TGF-β receptors, TGF-β induces chondrogenesis mainly through TGF-β/drosophila mothers against decapentaplegic protein (Smad) and mitogen-activated protein kinase (MAPK) signalling pathways, that cross-talk with each other (Li *et al.*, 2010; Lutz and Knaus, 2002; Schmierer and Hill, 2007) (Fig. 2). Before phosphorylation by TGF-β RII, TGF-β RI is catalytically inactive due to a wedge-shaped GS region (a highly conserved 30-amino acid region with a characteristic SGSGSG sequence) inserted into the kinase domain (Massagué, 1998). In Smad signalling, TGF-β RII phosphorylates the GS region after forming a ligand-receptor complex with TGF-β, which results in the activation of TGF-β RI (Huse *et al.*, 1999). The activated TGF-β RI phosphorylates Smad2/3, which recruits Smad4, and the complex translocates into the nucleus (Massagué and Wotton, 2000). The translocated Smad2/3 associates with Sox9 and CBP/p300 (cAMP-response element-binding protein, which has an intrinsic histone acetyltransferase activity and acts as an important co-activator for the expression of the *COL2A1*) on the *COL II* enhancer region containing the Sox9-binding site that is important for the initiation of chondrogenic differentiation (Furumatsu *et al.*, 2005). As for MAPK signalling, the major subtypes of MAPK include p38, extracellular signal-regulated kinase-1 (ERK-1) and c-jun N-terminal kinase (JNK). In addition, p38 is an enhancer of chondrogenesis, whereas ERK-1 is a repressor of chondrogenesis; JNK plays a minor role in chondrogenesis. Activation of MAPK involves indirect modulation of cell adhesion molecules, including N-cadherin and integrin $\alpha_5\beta_1$, during precartilage condensation and progression to chondrogenic differentiation (Tuli *et al.*, 2003). TGF-β induces the expression of the chondrogenic factor *SOX9* and the function of the main chondrogenic trio (*SOX5*, *SOX6* and *SOX9*) (Lefebvre *et al.*, 1998; Lefebvre and Smits, 2005) that further regulates chondrogenic differentiation and ECM formation. It is known that the genes for many ECM components, such as *ACAN*, *COL2A1* and *Matrilin-1*, are only expressed when the three Sox proteins bind to the high-mobility group (HMG)-domain sites in their enhancers (Akiyama and Lefebvre, 2011). On the contrary, TGF-β also plays an inhibitory role in chondrocyte maturation (Ballock *et al.*, 1993; Kato *et al.*, 1988; Serra *et al.*, 1997).

ITS premix is a substitute for foetal bovine serum that maintains cellular activity in biosynthesis and cell division. Serum has some disadvantages including poor characterisation and inconsistency in batch-to-batch composition and quality (Barnes and Sato, 1980; Cigan, 2013). Insulin is a primary factor involved in the onset and progression of chondrogenesis (Quarto *et al.*, 1992) and can facilitate chondrogenesis in a dose-dependent manner (1-10 μg/mL) with added TGF-β (Mueller *et al.*,

2013a). Furthermore, insulin can facilitate glucose uptake (Kono *et al.*, 1982) and enhance DNA synthesis and proteoglycan production in cartilage cultures (Maor *et al.*, 1993; Rosen, 1987). Transferrin promotes cell proliferation and differentiation through detoxifying oxygen radicals and peroxides (Ned *et al.*, 2003; Schäfer *et al.*, 2007). Selenite, a co-factor for glutathione peroxidase and other proteins, enhances proliferation of chondrocytes through acceleration of G1-phase cell cycle progression and the induction of cyclin D1 (Wei *et al.*, 1986). This effect is mediated by the enhancement of intracellular adenosine triphosphate (ATP) content (Yan *et al.*, 2012).

Dex, a synthetic glucocorticoid, promotes chondrogenic differentiation through enhancing related gene expression (Florine *et al.*, 2013; Jakobsen *et al.*, 2014; Randua *et al.*, 2013). The chondrogenic effects of glucocorticoids are predominantly mediated *via* the glucocorticoid receptors (GRs). GRα constitutes the main active GR form while GRβ constitutes a negative isoform of GRα (Oakley *et al.*, 1999). Dex promotes chondrogenic differentiation by down-regulating GRβ levels and maintaining GRα levels (Derfoul *et al.*, 2006). Dex enhances the gene expression of *COL XI* under chondrogenic conditions (collagen XI acts as both the template for collagen II fibrillogenesis and a regulator for maintaining fibril diameters in cartilage) (Chen *et al.*, 2005; Derfoul *et al.*, 2006). Previous studies have shown that Dex enhanced the TGF-β induced expression of *ACAN*, *COL2A1* and cartilage oligomeric matrix protein (*COMP*) and supported cell viability, as well as delayed the expression of *COL X* when used in conjunction with TGF-β (Derfoul *et al.*, 2006; Quarto *et al.*, 1992). The tissue origin of MSCs determines the effect of Dex on chondrogenic differentiation. Dex enhanced TGF-β1-induced chondrogenesis in bone marrow-derived MSCs (BMSCs) but had no significant impact on the TGF-β1- or BMP2-induced response in synovial derived MSCs (Kurth *et al.*, 2007; Park *et al.*, 2005; Shintani and Hunziker, 2011; Shintani *et al.*, 2007). This may be due to the different cell survival rates and microenvironment which could be tissue-source dependent (Buxton *et al.*, 2011).

Glucose, a major energy source and precursor of ECM for most mammalian cells, promotes chondrogenic differentiation and inhibits apoptosis at high levels (4.5 g/L or 25 mM) (Mackay *et al.*, 1998; Shikhman *et al.*, 2001; Tsai *et al.*, 2013). Glucose participates in multiple metabolic cycles of MSCs during chondrogenic differentiation, including oxygen consumption, glucose consumption and lactate production. High levels of glucose in the medium enhance cell differentiation by changing the metabolic patterns and modulating subsequent cell signalling pathways. The synthesis of proteoglycans increases through progressively inhibiting the respiration of chondrocytes cultured *in vitro,* as the glucose concentrations (1-10 mM) increase (Derfoul *et al.*, 2006; Otte, 1991). High doses of glucose enhance the differentiation of stem cells into chondrogenic lineages through the down-regulation of ERK and protein kinase C α (PKCα) activities, up-regulation of p38, as well as through modulating the expression of adhesion molecules such as fibronectin, integrin β1 and N-cadherin (Han *et al.*, 2004).

Both ascorbic acid and proline are essential for the production of collagen in cartilage. Ascorbic acid enhances cell proliferation, synthesis and extracellular deposition of collagenous matrix, in particular collagen I and II (Choi *et al.*, 2008; Franceschi, 1992; Potdar and D'Souza, 2010; Stone and Meister, 1962). Ascorbic acid stabilises the collagen triple helical structure through reduction of iron to its ferrous state, which is important for the hydroxylation of proline residues in procollagen and hydroxyproline and the maturation of protocollagen to collagen (Padh, 1991; Peterkofsky, 1991). Proline has been shown to regulate ESC differentiation into early primitive ectoderm-like (EPL) cells, through mammalian targeting of the rapamycin (mTOR) signalling pathway (Washington *et al.*, 2010).

Sodium pyruvate is essential for enhancing the energy metabolism in the Krebs cycle (Geshi *et al.*, 2000). Mammalian cells secrete pyruvate and α-ketoacids in culture to protect them against hydrogen peroxide-induced cytotoxicity (Andrae *et al.*, 1985; O'Donnell-Tormey *et al.*, 1987). Pyruvate up-regulates the expression of genes involved in free radical scavenging which, in turn, enhances the antioxidative machinery and is essential for maintaining mitochondrial activity. Super-physiological concentrations of pyruvate (50 mM, 50 folds of physiological concentrations) enhance mitochondrial mass and functionality (Wilson *et al.*, 2007).

Phenol red, a commonly used pH indicator in tissue culture medium can inhibit the chondrogenic differentiation of MSCs (Lysdahl *et al.*, 2013). During chondrogenic differentiation, phenol red in Dulbecco's Modified Eagle Medium (DMEM) has been shown to decrease *SOX9*, *COL2A1* and *ACAN* on days 14 and 21 and proteoglycan synthesis on days 21 and 28 in culture (Lysdahl *et al.*, 2013). This phenomenon may have occurred due to phenol red's structural resemblance to certain nonsteroidal oestrogens and weak oestrogen agonist activity (Berthois *et al.*, 1986).

BMPs are morphogens in the TGF-β superfamily and regulate chondrogenesis and skeletogenesis during normal embryonic development (Hogan, 1996). They are involved in various stages of chondrogenic differentiation, from initiation of chondrogenic differentiation, to regulation of chondrocyte maturation and terminal differentiation (Pizette and Niswander, 2000). BMP2, BMP4, BMP6 and BMP7 are the most commonly used BMPs for chondrogenic differentiation (Kramer *et al.*, 2000; Sekiya *et al.*, 2005; Shen *et al.*, 2010). BMPs signal through Smad or non-Smad signalling pathways. The Smad1/5/8 route is an important Smad-dependent pathway that induces hypertrophy and bone formation (Cao and Chen, 2005). The alternative Smad-independent pathway in BMP regulates chondrogenesis, mediated by activating several MAPKs including ERKs and p38 kinases (Hassel *et al.*, 2003). p38 enhances chondrogenesis by up-regulating *SOX9* expression directly or by inhibiting the Wnt7a/beta catenin pathway indirectly (Jin *et al.*, 2006). MSCs from different species and tissue sources vary in their responsiveness to BMPs, partly due to their distinct cell receptor repertoires (Hennig *et al.*, 2007; Osyczka *et al.*, 2004).

GDF5, also known as BMP14 in humans, is a member of the TGF-β superfamily and plays an important

role in limb bud mesenchymal cell condensation and chondrogenesis during embryogenesis (Coleman and Tuan, 2003; Storm *et al.*, 1994). GDF5 stimulates the recruitment and differentiation of chondrogenic cells at early stages and increases chondroprogenitor cell condensation and cartilaginous nodules without altering the overall pattern of differentiation. It causes a more sustained elevated expression level of *SOX9* than *BMP4* (Hatakeyama *et al.*, 2004). GDF5 increases mesenchymal cell condensation independent of cell density or N-cadherin-mediated adhesion and signalling; however, it is dependent on gap junction-mediated cellular communication (Coleman and Tuan, 2003; Sun *et al.*, 2012). GDF5 can promote chondrogenesis *via* signalling cross-talk, when used together with TGF-β3 and BMP2 *in vitro* (Murphy *et al.*, 2015). GDF5 has also been shown to increase the expression of *COL2A1* and hypertrophic marker, alkaline phosphatase (*ALP*) by enhancing the phosphorylation of Smad1/5/8 (Coleman *et al.*, 2013), which is activated by BMPs. GDF5's effect on the enhancement of *ACAN* and the inhibition of *MMP13* expression occurs by up-regulating the canonical Wnt inhibitors DKK1 (Enochson *et al.*, 2014).

PTHrP inhibits TGF-β-induced hypertrophic differentiation by up-regulating the chondrogenic markers *COL2A1* and *SOX9* and down-regulating the hypertrophic markers *COL10A1* and *RUNX2* during *in vitro* chondrogenesis of MSCs (Kim *et al.*, 2008; Weiss *et al.*, 2010). PTHrP exerts its effects *in vivo via* the PTHrP and IHh feedback loop by binding to its receptor (PTHR1) and activating a range of signalling molecules, *e.g.* protein kinase A (PKA), PKC and inositol 1, 4, 5-tris-phosphate (IP3). Some signalling molecules act as negative regulators for IHh. Hence, PTHrP inhibits the terminal differentiation of chondrocytes driven by IHh (Kim *et al.*, 2008; Mau *et al.*, 2007; Rabie *et al.*, 2003). The chondrogenic effects of PTHrP vary amongst different PTHrP isoforms. PTHrP 1-34, added in chondrogenic medium containing TGF-β3, significantly increases chondrogenic differentiation with less hypertrophic differentiation, while other isoforms (PTHrP 1-86, 7-34 and 107-139) show inconsistent effects (Lee and Im, 2012). When culturing MSC pellets under hypertrophy-enhancing conditions [hypertrophy-enhancing medium consisting of DMEM, 1 % ITS, 50 μg/mL ascorbate-2-phosphate, 40 μg/mL L-proline and 1 nM triiodothyronine (T3)], adding PTHrP (1-40) does not diminish the induced enhancement of hypertrophy (Mueller *et al.*, 2013b).

The FGF family, especially FGF [basic FGF (bFGF); also known as FGF2], can maintain MSCs in an immature state during *in vitro* expansion and enhance MSC proliferation and differentiation potential (Mastrogiacomo *et al.*, 2001; Solchaga *et al.*, 2005; Tsutsumi *et al.*, 2001). Literature suggests that aggregates of human MSCs that were expanded in culture in the presence of bFGF lacked the collagen I-positive and collagen II-negative outer layer characteristics of aggregates (Ng *et al.*, 2008; Solchaga *et al.*, 2005). However, in pellet cultures, the prolonged exposure of MSCs to bFGF caused the reduction of glycosaminoglycan (GAG) production (Hellingman *et al.*, 2010). Therefore, bFGFs can be added into the medium to culture MSCs, but in differentiation medium, it may not be necessary.

Physical, chemical and biological factors

In addition to biochemical molecules used in current chondrogenic differentiation medium, many other factors are involved in chondrogenic differentiation. These factors include physical (oxygen tension, mechanical, topography, *etc*.), chemical (small molecules, chemical properties, *etc*.) and biological factors (gene modification, bioactivity, epigenetics, *etc*.). Apart from biochemical factors (growth factors), biophysical factors (co-culture) and biophysicochemical factors (biomaterials) are equally important (Fig. 3). The influence of biophysical factors on chondrogenesis *in vivo* and *in vitro* have been reviewed (Huang *et al.*, 2010a; Studer *et al.*, 2012; Tate *et al.*, 2008). In this section, we focus on the use of biophysical and biochemical cues for chondrogenesis and recent advances in the field of tissue engineering.

Hypoxia can facilitate chondrogenic differentiation of MSCs, in addition to maintaining an undifferentiated state and inducing apoptosis. The microenvironment or the niche of MSCs in the bone marrow has low oxygen tension (< 32 mm Hg, 4.2 % oxygen) (Oze *et al.*, 2012; Spencer *et al.*, 2014). Therefore, chondrogenic differentiation is enhanced when MSCs are cultured in hypoxia (5 % oxygen) in comparison to normoxia (20 % oxygen) (Lee *et al.*, 2013). Hypoxia has a positive influence on chondrogenic differentiation of MSCs, mainly through phosphorylation of AKT (known as protein kinase B) and p38 MAPK, thereby down-regulating hypoxia-inducible factor (HIF-1) (Kanichai *et al.*, 2008).

Mechanical stimulation can directly influence the fate of undifferentiated stem cells (Estes *et al.*, 2004) *via* cellular and nuclear deformation and indirect biophysical factors, including osmotic and hydrostatic pressure and fluid flow (O'Conor *et al.*, 2013). Matrix elasticity directs the differentiation of stem cells; soft matrix (~ 1 kPa) has been shown to promote MSC differentiation into a chondrogenic lineage (Engler *et al.*, 2006; Park *et al.*, 2011), while activation and internalisation of integrin are actively involved in the regulation of stem cell differentiation (Du *et al.*, 2011). Cell-material properties, such as cell stiffness, dictate cell spreading and alter differentiation in response to physical stimuli (Chowdhury *et al.*, 2010). Surface topography can trigger changes in MSC morphology, gene expression and cytoskeletal structure (Huang *et al.*, 2010b). Compared to non-patterned surfaces, nano-pillar and nano-hole topographies enhanced MSC chondrogenesis and facilitated hyaline cartilage formation, whereas nano-grill topography delayed chondrogenesis and induced the formation of fibro/superficial zone cartilage (Wu *et al.*, 2014).

Small chemical molecules are also known to guide MSC differentiation (Benoit *et al.*, 2008) by binding to specific receptors that promote chondrogenic differentiation (Cho *et al.*, 2012; Johnson *et al.*, 2012; Kafienah *et al.*, 2007; Schugar *et al.*, 2008). As an illustrative example, kartogenin (KGN) has been shown to encourage

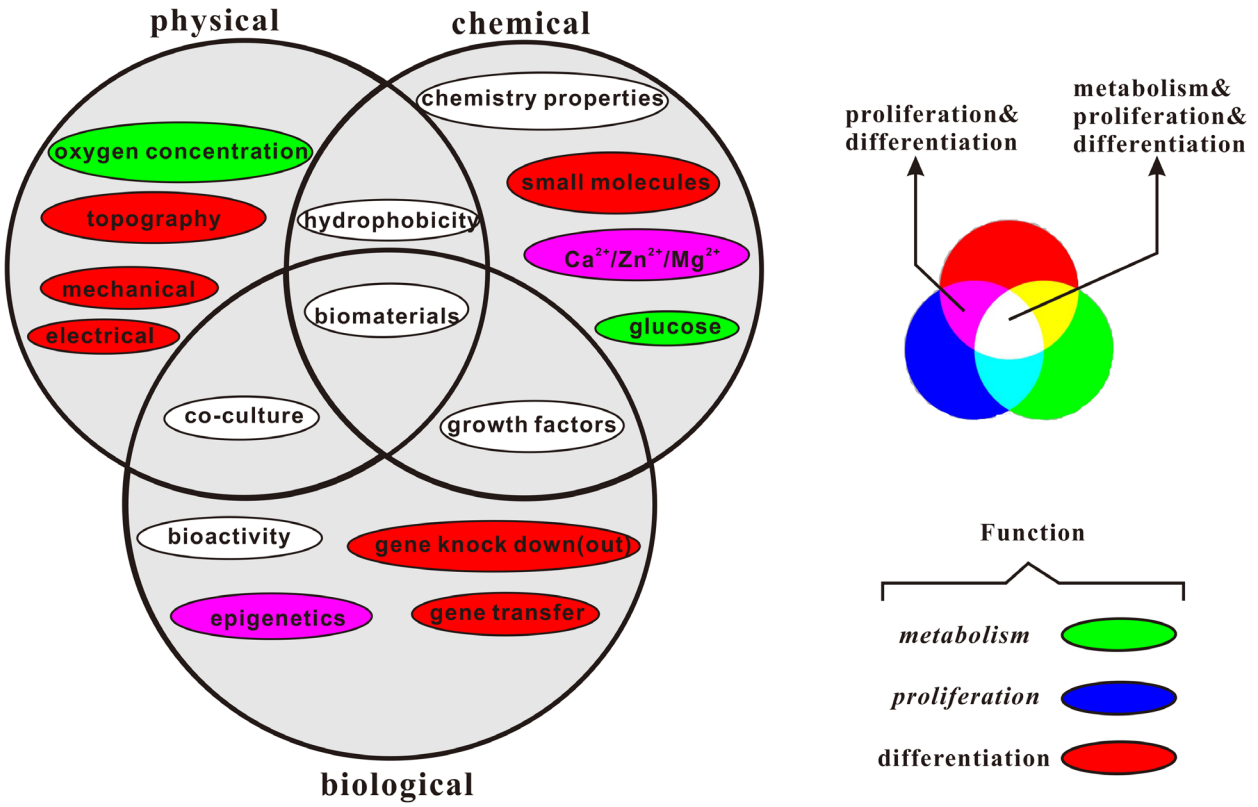

Fig. 3. Factors in the process of *in vitro* chondrogenic differentiation in MSCs.

chondrogenic differentiation of human MSCs, by significantly enhancing the expression of *COL2A1*, *SOX9* and *ACAN*. However, KGN had no significant effect on the gene products associated with chondrocyte calcification, such as osteocalcin (*OCN)*, *ALP* and *COL10A1*, in human MSCs or chondrocytes (Johnson *et al.*, 2012). KGN functions by binding to filamin A, an actin-binding protein that cross-links actin filaments. Thereby, by regulating the cytoskeletal network organisation and dynamics (Gorlin *et al.*, 1990) and disrupting its interaction with core binding factor β (CBFβ), it modulates the activity of the *RUNX* family of transcription factors (Johnson *et al.*, 2012).

Small molecule inhibitors can regulate chondrogenesis by blocking key receptors or signalling pathways. As a nuclear receptor, retinoic acid receptor β (RARβ) is down-regulated significantly during chondrogenesis. The synthetic inhibitor (LE135) enhances chondrogenesis while inhibiting hypertrophic differentiation (Kafienah *et al.*, 2007). Another small molecule inhibitor, dorsomorphin, can block Smad1/5/8 signalling, enhance chondrogenesis, elevate the production of collagen II and prevent mineralisation (Hellingman *et al.*, 2011; Retting *et al.*, 2009). Inhibition of inflammatory factors can boost the chondrogenic differentiation capacity of MSCs obtained from osteoarthritis patients through inhibiting inflammatory stimuli-induced damage, hypertrophy and apoptosis (Murphy *et al.*, 2002; Rainbow *et al.*, 2013).

Using a gene transfer/silencing strategy on MSCs can facilitate change in the complex regulatory networks of stem cell biology and regulate chondrogenic differentiation processes (Feng *et al.*, 2008; Paik *et al.*, 2012). For example, knockdown of HIF-1α by HIF-1α small interfering RNA (siRNA) leads to an increase in chondrogenesis (Kanichai *et al.*, 2008).

Biomaterials can facilitate chondrogenesis in multiple ways, including mechanical support, surface topography and stiffness, chemical modification and bioactivity (Bian *et al.*, 2013; Lutolf *et al.*, 2009; Wise *et al.*, 2009; Zhang *et al.*, 2014). Co-cultures of chondrocytes and MSCs enhance chondrogenesis due to the trophic role of MSCs (MSCs secrete a variety of cytokines and growth factors that have both paracrine and autocrine activities), which stimulate chondrocyte proliferation and matrix production. However, MSCs in co-culture conditions do not actively undergo chondrogenic differentiation themselves (Caplan and Dennis, 2006; Meretoja *et al.*, 2012; Wu *et al.*, 2012).

Future Perspectives

Heterogeneous differentiation of differentiated MSCs is one of the main challenges faced in *in vitro* chondrogenic induction and is seldom discussed. Multiple factors are involved in these processes, such as variance in cell-cell and cell-matrix adhesions, autocrine or paracrine activities, different subpopulations of MSCs used, individual cells encapsulated in biomaterials that induces different cellular microenvironments (such as exchange of nutrient and waste, oxygen gradients), *etc*.. High-throughput technologies, such as microfluidics or microfabrication, may be able to set up large scale production of homogeneous microtissues or microenvironments to circumvent this issue

(Jakobsen *et al.*, 2014). Assembling pellets containing varying sub-phenotypes of differentiated chondrocytes layer-by-layer has previously been shown to mimic the structural properties of articular cartilage (Chen *et al.*, 2006). Defining subpopulations of MSCs may provide a good foundation; some progress in this field has been made (Houlihan *et al.*, 2012). As cells only differentiate when they exit the cell cycle, fine-tuned cell cycles at the initial stages of differentiation can influence cells to differentiate into homogeneous phenotypes (Li *et al.*, 2014).

Senescence and apoptosis inherently occur during both embryonic development and *in vitro* chondrogenic differentiation, so regulation of these two processes could potentially regulate chondrogenic differentiation. Senescence and apoptosis synergistically prevent the over-growth of chondrocytes and shapes the articular cartilage during embryonic development (Ito and Kida, 2000; Loeser, 2009). Senescence of bone marrow MSCs is associated with low proliferation activity and decline of stemness (Squillaro *et al.*, 2015), which may lead to inferior chondrogenic differentiation efficiency (Vacanti *et al.*, 2005). Efforts in hampering cell senescence during chondrogenic differentiation with growth factors, anti-inflammatory drugs, antioxidants, nutrients as well as appropriate microenvironments such as hypoxia and stem cell matrices effectively improve the efficiency and quality of tissue-engineered cartilage *in vitro* and *in vivo* (Li and Pei, 2012; Rainbow *et al.*, 2013). It is known that the majority of cells in pellet cultures undergo apoptosis (Wang *et al.*, 2010). Apoptosis may occur due to low nutrient supply to cells in the centre of the pellet, hypoxia and subsequent cell fate. Some preconditioning steps, such as use of medium with high glucose, and hypoxia preconditioning, may be able to protect MSCs from undergoing the process of apoptosis (Mackay *et al.*, 1998; Wang *et al.*, 2008).

In addition, biophysical signals are indispensable for successful chondrogenic differentiation. Mechanical properties influence phenotypes of differentiated chondrocytes and affect respective progenies (Yang *et al.*, 2014). Oxygen tension or hypoxia can facilitate differentiation. MSCs can differentiate into hyaline cartilage chondrocytes instead of hypertrophic chondrocytes which is more likely to happen under current chondrogenic differentiation protocols (Leijten *et al.*, 2014). Premature and unstable phenotypes of differentiated chondrocytes pose challenges for potential clinical use and lead to inferior quality of regenerated cartilage (Pelttari *et al.*, 2006). Mimicking embryonic articular cartilage formation instead of embryonic chondrogenesis can assist bioengineers in overcoming this challenge, which means that temporal regulation of multiple factors may be required. For example, decreased Wnt, hedgehog and BMP signalling and specification of GDF-5 expression are of great importance for the homeostasis of articular chondrocytes (Bobacz *et al.*, 2008; Leijten *et al.*, 2012). Previous studies have shown that over expression of GDF-5 led to the unsuccessful differentiation of articular chondrocytes (Feng *et al.*, 2008). However, the role of GDF-5 in embryonic arthrogenesis and postnatal development is not entirely clear. From our viewpoint, hypertrophy will not pose challenges to chondrogenic differentiation if optimised protocols can further differentiate stem cells into articular chondrocytes and maintain their classic phenotypes.

Temporally fine-tuning chondrogenic differentiation medium can profoundly enhance differentiation efficiency. Chondrogenic differentiation medium is involved in administering TGF-β and other factors throughout the entire culture period (Johnstone *et al.*, 1998). Previously, multiple factors induced step-wise mesoderm differentiation and subsequent chondrogenesis through mimicking embryonic chondrogenesis (Oldershaw *et al.*, 2010). The sequential addition of growth factors may be able to improve chondrogenesis based on gene expression of cell surface receptors and effectiveness of growth factors; withdrawal of the growth factors after nine days improves chondrogenesis (Handorf and Li, 2014). Apart from the addition of growth factors, inhibition of specific cell signalling pathways may also enhance chondrogenesis, such as the Smad1/5/8 signalling inhibitor dorsomorphin (Hellingman *et al.*, 2011; Retting *et al.*, 2009) or Wnt inhibitors [Gremlin 1, frizzled-related protein (FRP) or dickkopf (Dkk-1)] (Leijten *et al.*, 2012).

Use of mathematical models can assist researchers in regulating chondrogenic differentiation. A "seesaw model" describes a balance between mesendodermal and ectodermal specification of pluripotency (Shu and Deng, 2013) and the cross-talk between genetics and epigenetics (Leijten *et al.*, 2014). The lineage specifiers of mesendodermal and ectodermal are considered as pluripotency rivals. However, these lineage specifiers have shown to facilitate reprogramming of differentiated cells to the pluripotent state when a balance is achieved.

Summary

MSCs are a promising source of precursor cells, which are widely used in cartilage tissue engineering. By reviewing the process of cartilage development and the constituents of existing medium formulations for promoting chondrogenic differentiation, we have identified potential factors to generate a more stable and reliable hyaline cartilage *in vitro*. Cellular metabolic status, especially glucose metabolism and inhibition of inflammatory factors, need to be considered when trying to inhibit cell senescence and apoptosis. The combination and concentration of growth factors, the timing of the application of biochemical components and proper biophysical stimuli during cell culture needs further optimisation to achieve desirable differentiation outcomes.

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