MOLECULAR AND BIOPHYSICAL MECHANISMS REGULATING HYPERTROPHIC DIFFERENTIATION IN CHONDROCYTES AND MESENCHYMAL STEM CELLS

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Abstract

Chondrocyte hypertrophy is one of the key physiological processes involved in the longitudinal growth of long bones, yet regulation of hypertrophy is becoming increasingly relevant for clinical application of mesenchymal stem cells (MSCs) and screening for drugs to treat hypertrophic osteoarthritis. The extraordinary cell volume increase during hypertrophy is accompanied by an up-regulation of collagen X, matrix metalloproteinases (MMPs), and vascular endothelial growth factor (VEGF), all which are targets of the runt-related transcription factor 2 (Runx2). Many pathways, including parathyroid hormone-related protein (PTHrP)/Indian Hedgehog, Wingless/Int (Wnt)/β-catenin, and transforming growth factor beta (TGF-β)/Smad and Mad Related Family (Smad) pathways, can regulate hypertrophy, but factors as diverse as hypoxia, coculture, epigenetics and biomaterial composition can also potently affect Runx2 expression. Control of hypertrophic differentiation can be exploited both for cartilage repair, where a stable phenotype is desired, but also in bone regeneration, where hypertrophic cartilage could act as a template for endochondral bone formation. We hope this review will motivate the design of novel engineered microenvironments for skeletal regeneration applications.

Keywords: Hypertrophy; mesenchymal stem cells; chondrogenesis; biomaterials; epigenetics; hypoxia; coculture.

Introduction

Due to its avascular nature, cartilage tissue does not have access to the body’s healing mechanisms and has a very low capacity for regeneration. Current clinical treatment of diseased or injured cartilage does not sufficiently restore long-term function and relieve joint pain. The material properties of native cartilage, which contains large quantities of collagen II fibrils for tensile strength and glycosaminoglycans (GAGs) such as aggrecan for osmotic swelling properties that confer compressive strength, have yet to be duplicated in vitro. Cartilage engineering, which has been the subject of many reviews (Hutmacher, 2000; Beris et al., 2006; Kuo et al., 2006; Swieszkowski et al., 2007; Giannoni and Narcisi, 2009; Vinatier et al., 2009; Mauck and Burdick, 2011), presents numerous challenges. Isolation of chondrocytes from healthy joint tissue causes donor site morbidity. Further, 2D expansion of chondrocytes to achieve sufficient numbers of cells for transplantation results in loss of the chondrogenic phenotype at early passage (Benya and Shaffer, 1982). Mesenchymal stem cells (MSCs) could be a potentially suitable substitute as a cell source as they are present in large quantities in adipose tissue, bone marrow, synovium and cartilage (Williams et al., 2010) and can be expanded for a number of passages without losing their ability to undergo chondrogenic differentiation. Unfortunately, the phenotype of MSCs in cartilage repair is unstable so that differentiation continues along the endochondral ossification pathway (Sekiya et al., 2008; Pelttari et al., 2006). This review summarises the biomaterial, molecular, epigenetic and environmental conditions which regulate hypertrophy, with an aim to develop therapies to control MSCs in clinical applications or to target hypertrophic osteoarthritis.

Chondrogenesis in the growth plate

Many clues for controlling hypertrophy can be found following the process of skeletal development which begins in humans between 4 and 5 weeks after fertilisation (Holzter, 1964; Reddi, 2000). Chondroprogenitor cells of diverse embryonic origin (Quintana et al., 2008) begin to up-regulate expression of transforming growth factor beta (TGF-β), fibronectin, N-CAM, and N-cadherin which initiates cell condensation and cartilage differentiation (Hall and Miyake, 2000) (Fig. 1a). The differentiation phase of stem cells towards chondrogenesis can be divided into distinct stages including cell attachment, proliferation/differentiation and differentiation/hypertrophy (Mrugala et al., 2009).

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Fig. 1. **In vivo and in vitro chondrogenesis and hypertrophy.** (a) Chondrogenesis initiates in limb bud development when MSCs undergo condensation, a process facilitated by increased expression of cAMP, TGF-β, fibronectin, N-CAM, and N-cadherin. Mature chondrocytes begin secreting cartilage matrix components primarily consisting of collagen II and glycosaminoglycans (GAGs). Chondrocytes outside the signalling range of PTHrP secreted by resting zone chondrocytes produce Ihh, a potent inducer of cell hypertrophy. Hypertrophic chondrocytes secrete collagen X which establishes the framework for subsequent calcification and endochondral ossification. (b) During *in vitro* chondrogenesis, MSCs are centrifuged into a micromass pellet and cultured in medium containing TGF-β to induce differentiation. The critical spatiotemporal cues are not present in this method, and the majority of the MSC population continues to express both collagen II and collagen X concomitantly.

Chondrocyte hypertrophy is marked by a 10-fold increase in cell volume (Bush *et al.*, 2008), extracellular matrix remodelling (Fig. 2a) and expression of terminal differentiation markers including runt-related transcription factor 2 (Runx2), matrix metalloprotease 13 (MMP-13), collagen X, alkaline phosphatase (ALP) and Indian Hedgehog (Ihh) (Mueller and Tuan, 2008) (Fig. 2b). The transcription factors Runx2 and myocyte enhancer...
factor-2C (MEF2C) drive the expression of the terminal differentiation markers including MMP-13 (Shimizu et al., 2010), collagen X (Takeda et al., 2001; Stanton et al., 2004; Arnold et al., 2007), Ihh (Yoshida et al., 2004), MMP-13 (Shimizu et al., 2010) and VEGF (Kwon et al., 2011). MMP-13 degrades aggrecan and collagen II (Inada et al., 2004), Ihh increases non-hypertrophic chondrocyte proliferation (Van Donkelaar and Huiskes, 2007), collagen X facilities the mineralisation process through association with matrix vesicles (MV) (Shen, 2005), and alkaline phosphatase (ALP) dephosphorylates pyrophosphate (PPi) to phosphate (PO$_4^{3-}$), leading to hydroxyapatite formation (Anderson, 2003).

The large gradient of calcium concentration from a high extracellular to a lower intracellular concentration is most probably the major driving force of mineralisation, triggering the influx of calcium and subsequent volume increase (Bawden, 1989; Grandolfo et al., 1992). Calcification of the cartilage ECM originates at matrix vesicles (MVs) containing ALP, which are secreted from chondrocytes in response to increasing calcium concentrations (Iannotti et al., 1994) and are anchored to the collagen X matrix (Shen, 2005). In the first step of mineralisation, ALP hydrolyses pyrophosphate (PPi) to inorganic phosphate (PO$_4^{3-}$, Pi) which, in the presence of

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**Fig. 2. Hypertrophy and the main actors.** (a) Hypertrophy is marked by cell volume increase (red arrows) and ECM remodelling (b). These changes are regulated by the transcription factors Runx2/MEF2C, which regulate transcription of collagen X (Takeda et al., 2001; Stanton et al., 2004; Arnold et al., 2007), Ihh (Yoshida et al., 2004), MMP-13 (Shimizu et al., 2010) and VEGF (Kwon et al., 2011). MMP-13 degrades aggrecan and collagen II (Inada et al., 2004), Ihh increases non-hypertrophic chondrocyte proliferation (Van Donkelaar and Huiskes, 2007), collagen X facilitates the mineralisation process through association with matrix vesicles (MV) (Shen, 2005), and alkaline phosphatase (ALP) dephosphorylates pyrophosphate (PPi) to phosphate (PO$_4^{3-}$), leading to hydroxyapatite formation (Anderson, 2003).
Calcium, forms hydroxyapatite inside the MVs (Balcerzak et al., 2003). Other sources of phosphate in MVs are the hydrolysis of phospholipids by PHOSPHO1 (Macr even et al., 2010) as well as the influx through the type-III Na/Pi co-transporter. Calcium influx into matrix vesicles is regulated by annexin II, V and VI with annexin V activity directly simulated by binding to collagen II and X fibrils (Kirsch, 2003). The second step of mineralisation is initiated by the penetration of hydroxyapatite crystals into the extracellular space where their prolongation is regulated by the PO₄³⁻ production by tissue non-specific alkaline phosphatase (TNAP) as well as PC-1, a nucleoside triphosphate pyrophosphohydrolase, inhibiting mineralisation through the production of Ppi (Orimo, 2010).

The final stages of endochondral ossification, including degradation of the calcified matrix, VEGF-mediated vascular invasion of the calcified zone and deposition of osteoid on the calcified trabeculae by osteoblasts, are all under the control of MMPs (Ortega et al., 2004). Postmitotic hypertrophic chondrocytes pass through a transient autophagy stage to help meet the energy demands of cells (Srinivas et al., 2009) before undergoing apoptosis and removal from the growth plate (Shapiro et al., 2005).

**Biophysical changes during cell volume increase**

The explosive increase in the volume of the hypertrophic chondrocyte involves changes in intracellular and extracellular osmolality, ECM degradation around the cell (Mackie et al., 2011), and an increase in the amount of organelles per cell. Osmotic swelling had been shown stereologically to be responsible for most of the cell volume increase (Buckwalter et al., 1986). Swelling can be the result of either an increase in cytoplasmic concentration or a decrease in extracellular osmolality followed by aquaporin-mediated (Wang and Zhu, 2011) movement of water to re-establish iso-osmotic conditions. Ion concentration in the cell can be regulated by active transport across the cell membrane by the Na-K-Cl co-transporter (NKCC1) (Bush et al., 2010) and to a lesser degree by osmolite accumulation (Farnum et al., 2002). Although it is not completely understood if expression of terminal markers results in increased cell volume, or if the cell volume increase contributes to the expression of hypertrophic markers, it is clear that physical volume increase can affect cell function (Chao et al., 2006; Negoro et al., 2008; Clark et al., 2010) possibly through changes in the shape and size of the nucleus (Finan et al., 2009) and chromatin structure (Richter et al., 2007). Due to macromolecular crowding, even small changes in water content can have large influences on protein activity and interactions.

Of all the ECM molecules, aggrecan is the prime contributor to the osmotic pressure generated in cartilage, both due to its abundance and its high negative fixed charge. Although direct experimental evidence linking degraded ECM to increased cell volume is lacking, both the hypertrophic region of the growth plate (Hunziker et al., 1999) and the pericellular region surrounding hypertrophic osteoarthritic chondrocytes show decreases in GAG density. This weakening of the ECM could lead to higher cell volumes as the matrix is less able to counteract osmotic swelling forces.

**Chondrogenesis in vitro**

Chondrogenesis of MSCs is typically induced in vitro by incubation of cells in defined media containing TGF-β, ascorbic acid and dexamethasone in combination with a 3D culture system such as micromass pellets (Johnstone et al., 1998; Mackay et al., 1998; Mello and Tuan, 1999; Pittenger et al., 1999), hanging drop culture (Bohrnsen et al., 2009; Elvenes et al., 2009) or cellular encapsulation in hydrogels (Li et al., 2005; Mauck et al., 2006; Varghese et al., 2008; Liu et al., 2010; Bian et al., 2011b). While up-regulation of the chondrogenic genes Sox9 and collagen II can be observed during in vitro induction of chondrogenesis, the differentiating cells also exhibit markers of hypertrophy including collagen X, MMP-13, and ALP (Sekiya et al., 2002; Pelttari et al., 2006; Mueller and Tuan, 2008) (Fig. 1b).

**Molecular Pathways of Hypertrophy**

**PTHRP signalling** (Fig. 3, yellow shading)

Parathyroid hormone-related protein (PTHRP) is emerging as one of the key anti-hypertrophy factors. The knockout of PTHrP (Vortkamp et al., 1996; Mak et al., 2008) or its receptor (Lanske et al., 1996; Guo et al., 2002; Kobayashi et al., 2002) leads to accelerated hypertrophy in vivo. PTHrP participates in a negative feedback loop with Indian Hedgehog (Ihh), a stimulatory factor of hypertrophy and chondrocyte proliferation (Lanske et al., 1996; Kobayashi et al., 2002; Kronenberg, 2006). Resting chondrocytes at the ends of long bones secrete PTHrP subsequently suppressing Ihh production in the proliferating zone. Chondrocytes outside of this paracrine signalling range, however, do produce Ihh and undergo hypertrophy (Fig. 1a). Ihh also leads to hypertrophy independently of PTHrP (Mak et al., 2008).

PTHRP functions by activating protein kinase A and C (PKA and PKC) (Li et al., 2004) and also interferes with the calcium pathway (see section “Calcium”) by dephosphorylation of calcium/calmodulin-dependent protein kinase (CaMKII) (Li et al., 2011a). Cyclic adenosine monophosphate (cAMP)-dependent PKA phosphorylates Sox9 (Huang et al., 2000) and activates protein phosphatase II (PP2A) leading to the dephosphorylation of histone deacetylase 4 (HDAC4) and the inactivation of MEF2C (Kozhenyakina et al., 2009) (see sections “Transcription factors” and “Epigenetics”). PKC inhibits the activity of the mitogen-activated protein kinase (MAPK) p38 (Zhen et al., 2001) reducing MEF2C phosphorylation (Stanton et al., 2004) and, ultimately, hypertrophic gene expression.

**In vitro studies with MSCs in pellet culture** showed that PTHrP administration leads to suppression of hypertrophy but also down-regulates collagen II (Weiss et al., 2010). Conversely, Kim et al. (Kim et al., 2008) and Kafienah et al. (Kafienah et al., 2007) observed a selective hypertrophic inhibition upon PTHrP treatment with stable or even up-regulated expression of collagen II. This discrepancy
might be linked to the existence of both PTHrP receptor 1 (PTH1R)-dependent and PTH1R-independent pathways. PTH1R is not upregulated until day 21 of pellet culture in chondrogenic media (Mueller and Tuan, 2008; Weiss et al., 2010), whereas groups using bone morphogenetic protein 2 (BMP-2)-supplemented medium observed its up-regulation at day 0 (Hoffmann et al., 2002). PTH1R knockout mice showed accelerated hypertrophy and were unaffected by treatment with PTHrP, indicating that the inhibitory action on hypertrophy is dependent on PTH1R receptor binding (Lanske et al., 2005). The choice of the PTHrP isoform has further been shown to affect the suppressive action on hypertrophy with isoform 1-34 being the most effective in promotion of chondrogenesis as well as hypertrophy inhibition (Lee and Im, 2012).

**Calcium** (Fig. 3, pink shading)

The cytoplasmic and extracellular calcium concentration increases in growth plate chondrocytes between the reserve and hypertrophic zones (Iannotti and Brighton, 1989) where its presence is required for mineralisation. Calcium further acts inside the cell as a second messenger. This cytoplasmic calcium accumulation occurs through at least two distinct pathways. Extracellular Ca$^2+$ can directly pass through ion channels in the cell membrane or alternatively it can activate G-protein coupled receptors (such as the calcium-sensing receptor CaR, which is expressed in elevated levels in hypertrophic chondrocytes (Chang et al., 1999)) that stimulate intracellular Ca$^2+$ release from the endoplasmic reticulum (ER) (Lamprecht and Lipkin, 2003; Hendy et al., 2009). The release from the ER is regulated by
phospholipase C (PLC) activation, prompting hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-triphosphate (IP_3), which triggers the opening of ER ion-channels (IP_3R) (D’Andrea et al., 2000). Which of the two accumulation pathways is primarily involved in hypertrophy remains a subject of debate (D’Andrea et al., 2000; Guilak et al., 1999; Wu et al., 2004). Increase in extracellular calcium (Bonen and Schmid, 1991) and activity of CaR (Wu et al., 2004) were linked to collagen X up-regulation. Hypertrophic induction via this pathway is further regulated by calcium/calmodulin-dependent protein kinase (CaMKII) (Bucks et al., 2008; Li et al., 2011a), which is activated by autophosphorylation upon binding of calcium to calmodulin. Activated CaMKII upregulates expression of hypertrophic genes (see “Epigenetics” section). PTHrP is also involved, inactivating CaMKII through dephosphorylation (Li et al., 2011a). Calmodulin, in addition, binds and activates calcineurin leading to the dephosphorylation of nuclear factors of activated T-cells (NFATs). NFAT4 was shown to induce chondrogenesis of MSCs (Tomita et al., 2002) whereas NFATc2 was established as a downstream target of Runx2 involved in hypertrophy of MSCs (Thirunavukkarasu et al., 2007). Physiologic toxicity and the inhibition of calcineurin by the inhibitor FK506 decreased hypertrophic marker expression in osteoarthritic chondrocytes (van der Windt et al., 2012).

An interesting synergistic relationship exists between the inhibitory effect of PTHrP and the stimulatory effect of calcium on hypertrophy. A Ca^{2+} sensitive sequence on the PTHrP promoter was identified, which highlights its calcium-dependent transcription (Zuscik et al., 2002). Administration of PTHrP or the overexpression of its receptor abolished the effect of elevated calcium to increase terminal differentiation markers (Rodriguez et al., 2005). Additionally, a feedback loop between PTH/PTHrP signalling and Ca^{2+} level has been established. PTH/PTHrP was shown to inhibit the activity of p38 mitogen-activated protein kinase (MAPK) (Zhen et al., 2001), which leads to an increase in B-cell lymphoma 2 (Bcl-2) activity, a known regulator of apoptosis, which was further shown to inhibit IP_3R channel opening (Amling et al., 1997; He et al., 1997; Bassik et al., 2004). Despite the evidence of calcium depletion as an effective hypertrophy inhibitor in vivo, to our knowledge, there is no study applying a similar strategy to MSC chondrogenesis in vitro.

**TGF-β** (Fig. 3, grey shading)

Transforming growth factor-β (TGF-β) is a potent inducer of chondrogenesis in vitro (Barry et al., 2001; Weiss et al., 2010). Signalling pathways of the TGF-β superfamily have been reviewed (van der Kraan et al., 2009) including its downstream phosphorylation of Smad and Mad Related Family (Smads), which exhibit both stimulatory and inhibitory effects on chondrocyte hypertrophy. The Smad1/5/8 route, through which BMPs signal and control FGF pathways (Yoon et al., 2006; Maruyama et al., 2010), induces hypertrophy by epigenetic changes (see section “Epigenetics”) and blocking phosphorylation of Smad1/5/8 inhibited expression of 3 terminal differentiation markers (Hellingman et al., 2011). The Smad2/3 pathway is activated by TGF-β directly leading to inhibition of hypertrophy and induction of chondrogenesis due to stabilisation of the Sox9 transcription complex by Smad2/3 (Yang et al., 2001; Furumatsu et al., 2005). TGF-β activation of Smad3 also leads to Runx2 inhibition through epigenetic regulation (see “Epigenetics”) (Kang et al., 2005). Though TGF-β is clearly crucial in the regulation of chondrocyte hypertrophy, its addition to induction media during pellet culture of MSCs is not sufficient to suppress the onset of hypertrophy (Sekiya et al., 2002; Pelttari et al., 2006; Mueller and Tuan, 2008). TGF-β1-supplemented expansion media has further shown to redirect chondrocytes towards hypertrophy (Narcisi et al., 2012).

**Wnt** (Fig. 3, red shading)

Activation of β-catenin by canonical Wingless/Int (Wnt) signalling is another pathway involved in regulation of chondrocyte hypertrophy. Wnt signalling controls the fate of β-catenin via the Frizzled receptor (Angers and Moon, 2009). In the absence of Wnt, β-catenin is bound by a degradation complex consisting of adenomatous polyposis coli (APD), axin, glycosyn synthase kinase 3 (GSK3), and casein kinase 1α (CK-1) which phosphorylate β-catenin initiating its ubiquitination and proteosomal degradation. Wnt activation of the Frizzled receptor interferes with the degradation complex and β-catenin can translocate to the nucleus where it binds to lymphoid enhancer factor (LEF) and T cell factor (TCF) proteins. The LEF/TCF/β-catenin complex promotes Runx2-expression inducing hypertrophy (Dong et al., 2006). Sox9 inhibits this signalling through phosphorylation of β-catenin, marking it for degradation (Topol et al., 2009). The inhibition of canonical Wnt led to an increase in collagen II and aggrecan expression but did not affect collagen X expression in MSC pellet culture (Im et al., 2011).

The non-canonical Wnts (such as Wnt5a) exhibit dual functions during chondrogenesis of MSCs. At early stages, Wnt5a induces chondrogenesis and hypertrophy through intracellular calcium release via G-protein coupled receptor (GPCR) activation. Later, though, it can act as an inhibitor of hypertrophy by activating the phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB or Akt)-dependent pathway, which in-turn activates NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells), an inhibitor of Runx2 (Bradley and Drissi, 2010).

**Integrin signalling** (Fig. 3, green shading)

The supporting matrix of chondrocytes is under constant remodelling to which cells respond by translating stimuli into intracellular signalling, a process mediated by integrin signalling (Millward-Sadler and Salter, 2004). Upon the exposure of a cell to ECM proteins, integrins cluster together and small Rho GTPases (RhoA, Rac1, Cdc42) are activated regulating polymerisation and assembly of actin filaments which lead to the formation of focal adhesions and stress fibres (Amano et al., 1997).

The influence of RhoA/Rock on chondrogenesis and hypertrophy is somewhat controversial. Overexpression of RhoA/Rock was shown to suppress ALP and mineralisation in chondrocytes, whereas their counterparts, Rac1/Cdc42
induced hypertrophy (Wang et al., 2004; Wang and Beier, 2005). Rock is also involved in the phosphorylation of Sox9 (Haudenschild et al., 2010). In contrast, down-regulation of RhoA/Rock was shown to drive chondrogenic differentiation of mesenchymal stem cells by both inducing a round shape morphology (Loeser et al., 1995; Connelly et al., 2008; Lu et al., 2008) and by increasing Sox9 activity (Woods and Beier, 2006). Rac1/Cdc42 are activated by integrin-β1, which also induces GTP-bound transglutaminase 2 (TG2) recruitment to the cell surface and phosphorylation of p38 MAPK, which in turn activates MEF2C (Tanaka et al., 2007; Johnson et al., 2008). The inhibition of p38 MAPK effectively delayed hypertrophy in chondrocytes (Stanton et al., 2004). Blocking intergrin-β1, a collagen II and X binding integrin (Loeser et al., 1995; Loeser et al., 2000; Luckman et al., 2003), was shown to be a successful strategy to suppress collagen X expression in chondrocytes and to decrease cell volume (Hirsch et al., 1997; Johnson et al., 2008).

**Epigenetics**

Histone (de)acetylation, DNA methylation and microRNAs are epigenetic events strongly involved in chondro-specific differentiation (Barter et al., 2012; Furumatsu and Ozaki, 2010) (Fig. 4). When DNA is present in the nucleus in a highly packed form wrapped around histones, access of transcription factors is restricted and transcription is consequently downregulated. Histone deacetylases (HDACs) keep the chromatin in this form, whereas histone acetyl transferases (HATs) decrease the association of the histone with the DNA strand allowing the access of transcription factors. The release of HDACs from the histone complex is performed through kinase-induced phosphorylation, which creates chaperone docking sites (such as “14-3-3”) docking sites that, once bound, transport HDACs out of the nucleus. Phosphatases dephosphorylate HDACs to activate and relocate them into the nucleus. Another epigenetic control is the introduction of methylation sites on cytosine-guanine dinucleotides (CpG) by DNA methyltransferases (Chen and Riggs, 2011). CpG islands (areas with high concentration of CpGs) are mostly found in the promoter region of genes where methylation causes gene silencing. MicroRNAs are post-transcriptional regulators of messenger RNAs (mRNAs) that, by binding to their complementary sequence, repress translation or lead to the degradation of mRNAs (Wienholds and Plasterk, 2005).

**Histone deacetylases**

HDAC4 is a potent regulator of chondrocyte hypertrophy. Adenoviral expression of HDAC4 in synovial-derived MSCs increased chondrogenesis as well as suppressed hypertrophy (Pei et al., 2009) by inhibiting Runx2 (Vega et al., 2004) and MEF2C (Wang et al., 1999). HDAC4 nuclear localisation is enhanced by TGF-β and PTHrP, whereas increased calcium concentration and PTH lead to its phosphorylation and nuclear export. The transport of HDAC4 into the nucleus is initiated by TGF-β through the activation of Smad3-complexes (Kang et al., 2005; Pei et al., 2009) and by PTHrP in a protein phosphatase II (PP2A)-dependent way (Kozhemyakina et al., 2009). PTH signals through PKA/cAMP to induce hypertrophy by escorting HDAC4 out of the nucleus (Shimizu et al., 2010). Besides PTH, an increased intracellular calcium concentration also leads to the phosphorylation of HDAC4 induced by the calcium-activated CaMKII (Backs et al., 2006). In addition to HDAC4, HDAC5 (Backs et al., 2008) and HDAC7 (Jensen et al., 2009) suppress MEF2C and Runx2 respectively.

**DNA methylation**

The role of gene silencing through DNA methylation during chondrogenesis and hypertrophy is still in its
infancy in comparison to our knowledge of histone (de)acetylation. COL10A1, the gene encoding collagen Xα1, was found to be methylated in articular chondrocytes, whereas in mesenchymal stem cells this gene silencing was not identified (Zimmermann et al., 2008). This discrepancy may explain why hypertrophy is less likely in chondrocyte pellet cultures compared to MSC pellets. On the other hand, Runx2 and Sox9 were shown to keep their methylation status during in vitro chondrogenesis of synovium-derived MSCs (Ezura et al., 2009). During osteoarthritis, the methylation of matrix metalloproteases present in healthy cartilage is lost. This de-methylation leads to the expression of MMP-3, -9, -13 and ADAMTS-4 (A Disintegrin And Metalloproteinase with Thrombospondin Motifs 4) (Roach et al., 2005; Cheung et al., 2009). The application of the unspecific de-methylating agent 5-aza-deoxycytidine on chondrogenic differentiation has yielded mixed results, where both increased osteogenesis (El-Serafi et al., 2011) and chondrogenesis (Caporali, 2011) have been found.

MicroRNA
MicroRNAs (miRs) bind to 3’ untranslated regions of mRNA where they repress the translational step from mRNA to protein. Table 1 lists miRs implicated in chondrogenesis and hypertrophy and their targets.

Other publications have established whole sets of Runx2-regulating miRs (Zhang et al., 2011) as well as miRs involved in the decision between osteogenic and chondrogenic lineage (Suomi et al., 2008). Recently, the first mechanico-inductive miR was discovered. In tension experiments, miR-365 was upregulated in chicken chondrocytes. MiR-365 was identified to target HDAC4 and to induce the expression of Ihh and collagen X (Guan et al., 2009). The application of the unspecific de-methylating agent 5-aza-deoxycytidine on chondrogenic differentiation has yielded mixed results, where both increased osteogenesis (El-Serafi et al., 2011) and chondrogenesis (Caporali, 2011) have been found.

MSC/Chondrocyte Co-Culture

It has long been observed that the chondrogenic phenotype in differentiated limb bud mesenchymal cells is more stable when exposed to paracrine factors produced by mature chondrocytes (Solursh and Reiter, 1975). Co-culture of chondrocytes and adult stem cells (Fischer et al., 2010; Cooke et al., 2011; Acharya et al., 2012) in micromass pellet culture showed decreases in collagen X and MMP-13 expression compared to pellets containing stem cells alone. The combination of MSCs and chondrocytes in hyaluronic acid (HA) hydrogels at various ratios demonstrated decreases in collagen X expression with a MSC/chondrocyte ratio of 4:1 (Bian et al., 2011a). This effect was lost when the two cell types were seeded in separate gels within the same well, indicating that some physical interaction may be necessary to realise suppression of hypertrophy. Conversely, micromass pellets with limited heterotypic cell-cell interactions by spatially arranging the two cell types by first pelleting MSCs and encapsulating them by adding an outer layer of chondrocytes, resulted in reduction of collagen X, Runx2, and MMP-13 expression (Cooke et al., 2011).

Many details as to the specifics of the heterotypic cell interactions remain unclear. However, PTHrP-secretion of chondrocytes was identified as responsible for the repression of collagen X in co-culture (Fischer et al., 2010). Studies have also shown collagen II matrix deposition in pellet co-culture originates exclusively from chondrocytes and not MSCs (Mo et al., 2009). These results point to mutual benefits for phenotype maintenance in both cell types – the presence of MSCs in co-cultures stimulates chondrocyte production of cartilage matrix, and chondrocytes seem to prevent or slow hypertrophy of differentiating MSCs. It is further possible that secreted factors from the MSCs or chondrocytes could be involved in epigenetic regulation of the other cell type by inducing or inhibiting the activity of DNA methyltransferases (Benton et al., 2009), HDACs or HATS.

### Table 1: MicroRNAs implied in hypertrophy of chondrocytes with their target.

<table>
<thead>
<tr>
<th>miR #</th>
<th>Target</th>
<th>Species</th>
<th>Effect on hypertrophy</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>26b</td>
<td>COL10A1, IGF-1 etc.</td>
<td>human MSC</td>
<td>inhibitory</td>
<td>Han et al., 2010</td>
</tr>
<tr>
<td>140</td>
<td>ADAMTS-5</td>
<td>mice</td>
<td>inhibitory</td>
<td>Miyaki et al., 2010</td>
</tr>
<tr>
<td>140</td>
<td>HDAC4</td>
<td>mice</td>
<td>stimulatory</td>
<td>Tuddenham et al., 2006</td>
</tr>
<tr>
<td>140-5p</td>
<td>Smad2/3</td>
<td>human C</td>
<td>stimulatory</td>
<td>Swingler et al., 2011</td>
</tr>
<tr>
<td>145</td>
<td>Sox9</td>
<td>murine MSC</td>
<td>stimulatory</td>
<td>Yang et al., 2011</td>
</tr>
<tr>
<td>152</td>
<td>COL2, -4, -6A1, BMP3</td>
<td>human MSC</td>
<td>stimulatory</td>
<td>Han et al., 2010</td>
</tr>
<tr>
<td>199*</td>
<td>Smad1</td>
<td>murine MSC</td>
<td>stimulatory</td>
<td>Lin et al., 2009</td>
</tr>
<tr>
<td>204</td>
<td>Runx2</td>
<td>human MSC</td>
<td>inhibitory</td>
<td>Huang et al., 2010</td>
</tr>
<tr>
<td>365</td>
<td>HDAC4</td>
<td>chicken C</td>
<td>inhibitory</td>
<td>Guan et al., 2011</td>
</tr>
<tr>
<td>453-3p</td>
<td>Smad2/3</td>
<td>human C</td>
<td>stimulatory</td>
<td>Swingler et al., 2011</td>
</tr>
<tr>
<td>675</td>
<td>COL2A1 repressor</td>
<td>human C</td>
<td>inhibitory</td>
<td>Dudek et al., 2010</td>
</tr>
</tbody>
</table>

Classification into miRs that inhibit or stimulate hypertrophy. Abbreviations: mesenchymal stem cell (MSC), chondrocyte (C).
in a number of different scaffold and surface types including synthetic and natural polymer combinations (Awad et al., 2004; Betre et al., 2006; Park et al., 2011) as well as scaffolds from de-cellularised tissues (Choi et al., 2010). Surface functionalisation may further provide an important tool in arresting the chondrogenic phenotype prior to terminal differentiation of MSCs (Mwale et al., 2006; Woodfield et al., 2006).

Chondroitin sulphate (CS) and other nutraceuticals have long been postulated as being beneficial to joint health. MSC chondrogenesis in synthetic PEG hydrogels containing CS moieties (Fig. 5a) showed reduced collagen X mRNA and almost no Runx2 expression (Fig. 5b) compared to pure PEG gels (Varghese et al., 2008). However, another group explored a similar PEG/CS composite gel versus pure PEG gels but saw little difference in collagen X expression (Nguyen et al., 2010). These conflicting results indicate that the role of chondroitin sulphate in MSC differentiation is complex. The conformation and concentration of chondroitin sulphate within the hydrogel may influence its chondrogenic properties, particularly since sulphates have an affinity for growth factors such as TGF-β (Re’em et al., 2011). Inclusion of the over-sulphated isoform of the marine polysaccharide GY785 DRS in chondrogenic induction media during pellet culture also results in reduced collagen X expression after 4 weeks in culture (Merceron et al., 2012). The relationship between sulphate groups and the growth factors involved in chondrogenesis has emerged as an intriguing one that should continue to be explored in future cartilage-engineering concepts.

Finally, de-cellularised tissues have been investigated as alternatives to synthetic scaffolds for regenerative medicine therapies (Hoshiba et al., 2010; Crapo et al., 2011; Song and Ott, 2011). Compared to polyglycolic acid (PGA) controls, MSCs seeded in de-cellularised porcine micromass pellet scaffolds were much slower to produce collagen X and did not attract vascularisation when implanted subcutaneously in nude mice (Choi et al., 2010).

Surface functionalisation is another important parameter dictating stem cell fate (Fig. 5c). Simply limiting the adhesion area was enough to switch MSC differentiation from the smooth muscle cell pathway to the chondrogenic pathway during cultivation in the presence of TGF-β (Gao et al., 2010). In another study, increasing substrate charge density was shown to be beneficial for collagen II and aggrecan production in chondroprogenitor cells cultured in insulin-free media (Kwon et al., 2010).

Glass surfaces modified with carboxyl (-COOH) functional groups produced higher quantities of collagen II and lower amounts of Runx2 than controls or other functionalisation chemistries (Curran et al., 2005). Furthermore, it was shown that N-doped plasma-polymerised ethylene surfaces containing >30% nitrogen almost completely suppressed collagen X expression in MSCs cultured in growth media (Mwale et al., 2006). Generally, surface chemistries that promote a rounded cell morphology appear to be chondrogenic.

Oxygen Tension

Tissue engineering approaches have mostly involved expansion and differentiation of MSCs under normoxic conditions (20% pO₂); however, the in vivo MSC niche is hypoxic (2-8% pO₂) (Mohyeldin et al., 2010). Expansion
under hypoxia was reported to enhance preservation of stemness properties (D’Ippolito et al., 2006; Grayson et al., 2006) and subsequent differentiation potential of bone marrow-derived MSCs in vitro (Lennon et al., 2001; Zscharnack et al., 2009). Expansion of adipose-derived human MSCs under hypoxic conditions suppressed the hypertrophic phenotype assessed by down-regulation of collagen X and MMP-13 (Ronziere et al., 2010; Sheehy et al., 2012). Hypoxic repression of hypertrophy is a combined effect of regulation through HDAC4 and Nkx3.2 activation as well as Smad6 inhibition, all resulting in the suppression of Runx2 and hence collagen X (Hirao et al., 2006; Kawato et al., 2011). HIF-2α is shown to be a chondrogenic factor supporting collagen II and aggrecan expression of human articular chondrocytes under hypoxic conditions (Murphy and Sambanis, 2001; Murphy and Polak, 2004; Lafont et al., 2007). On the other hand, HIF-2α also induces chondrocyte hypertrophy with collagen X, MMP-13 and VEGF as its transcriptional targets (Saito et al., 2010). Runx2 is up-regulated with HIF-2α leading to further expression of these genes (Tamiya et al., 2008). HIF-2α induction of this catabolic pathway is triggered by stresses such as inflammation making HIF-2α a putative target for osteoarthritis (Saito et al., 2010; Yang et al., 2010).

Two oxygen-sensitive transcription factors, hypoxia-inducible factor 1 alpha and 2 alpha (HIF-1α and HIF-2α), have essential roles in the chondrogenic effect of hypoxia. HIF-1α was shown to improve chondrogenesis of MSCs via inducing expression of collagen II and aggrecan through activation of the Sox9 promoter (Robins et al., 2005; Kanichai et al., 2008). HIF-2α also induces chondrocyte hypertrophy with collagen X, MMP-13 and VEGF as its transcriptional targets (Saito et al., 2010). Runx2 is up-regulated with HIF-2α leading to expression of these genes (Saito et al., 2010). Runx2 is up-regulated with HIF-2α leading to further expression of these genes (Tamiya et al., 2008). HIF-2α induction of this catabolic pathway is triggered by stresses such as inflammation making HIF-2α a putative target for osteoarthritis (Saito et al., 2010; Yang et al., 2010).
as down-regulation of catabolic factors such as MMP-13. This chondrogenic and anti-catabolic effect of hypoxia was reported to disappear with the addition of an HIF-1α inhibitor (Ströbel et al., 2010). The role of HIF-2α in chondrogenesis is currently debated (Murphy, 2010). Human articular chondrocytes demonstrated increased collagen II and aggregan levels during redifferentiation under hypoxic conditions (Murphy and Sambanis, 2001; Murphy and Polak, 2004) where HIF-2α/Sox9 interactions were indicated to have a key role in the expression of these chondrogenic markers (Lafont et al., 2007) (Fig. 6c). In contrast, HIF-2α was shown to be crucial for endochondral ossification processes such as hypertrophy, matrix degradation and vascularisation via direct transactivation of collagen X, MMP-13 and VEGF (Saito et al., 2010) (Fig. 6d). Similarly, expression of the HIF-2α-encoding gene Epas1 was reported to increase during differentiation of ATDC5 cells paralleling the increase in collagen X, MMP-13 and VEGF (Saito et al., 2010) (Fig. 6d). Linking oxygen tension and epigenetic regulation of hypertrophy, HIF-2α signalling was Interestingly shown to be promoted by Sirtuin 1, a redox-sensing deacetylase, under hypoxic conditions (Dioum et al., 2009). The significance of HIF-2α for cartilage tissue being obvious, whether it is a factor supporting chondrogenic phenotype or a catabolic regulator leading to hypertrophy remains an open question.

**Outlook**

Chondrocyte hypertrophy is controlled by a complex network of signalling molecules and biophysical factors that together present a wide range of possible targets for microenvironmental engineering (Table 2). The overexpression of HDAC4, stabilisation of HIFs for normoxic conditions, miRNA targeting, reducing extracellular calcium and incorporation of negatively charged carbohydrates into smart materials represent as yet unexplored avenues for stabilising the phenotype of MSCs which could be effectively used in skeletal regenerative medicine. These, in conjunction with the activation of anti-differentiation factors, perhaps produced by differentiated chondrocytes in the same repair site, could take us a few steps closer to the goal of stably controlling stem cell fate.

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**References**


Backs J, Song K, Bezprozvannaya S, Chang S, Olson EN (2006) CaM kinase II selectively signals to histone

| Table 2: Target molecules for hypertrophy regulation. |
|---|---|
| **Hypertrophy Stimulators** | **Hypertrophy Inhibitors** |
| **Transcription factors** | Runx2 | Sox9 |
| | MEF2C | Nkx3.2 |
| **Molecular signalling** | Calcium, CaMKII, CaR | PTHrP |
| | Smad 1/5/8 | Smad 2/3 |
| | canonical Wnt | |
| | Integrin-β1 | |
| **Epigenetics** | Collagen X demethylation | HDAC4 |
| **Biomaterials** | | Negatively-charged, carbohydrate-rich |
| **Oxygen tension** | Low pO₂ | Secreted PTHrP |
| **Co-culture** | | |

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**References**


Backs J, Song K, Bezprozvannaya S, Chang S, Olson EN (2006) CaM kinase II selectively signals to histone


Caporali E (2011) Impact of the DNA methyltransferase inhibitor, 5-azacytidine, on chondrocytic phenotype. In: Vet Clinical Medicine, University of Illinois, Urbana-Champaign.


Curren J, Chen R, Hunt J (2005) Controlling the phenotype and function of mesenchymal stem cells in...
vitro by adhesion to silane-modified clean glass surfaces. Biomaterials 26: 7057-7067.


Lee J-M, Im G-I (2012) PTHrP isoforms have differing effect on chondrogenic differentiation and hypertrophy of mesenchymal stem cells. Biochem Biophys Res Commun 421: 819-824.


Sheehy EJ, Buckley CT, Kelly DJ (2012) Oxygen tension regulates the osteogenic, chondrogenic and endochondral phenotype of bone marrow derived mesenchymal stem cells. Biochem Biophys Res Commun 417: 305-310.


is regulated by HIF, mTOR and AMP kinase. Cells Tissues Organs 189: 88-92.


Discussion with Reviewers

Reviewer II: Is the expansion phase of MSCs relevant to determine the cell fate (hypertrophy)?

Authors: As is the case for chondrocyte expansion, the environmental conditions of MSC expansion are critical. One example is oxygen tension. In the study of Xu et al., expansion of adipose-derived adult stromal cells under low oxygen conditions has been reported to enhance the subsequent chondrogenic differentiation under ambient oxygen conditions. Chondrogenic markers such as collagen II and aggrecan were shown to increase whereas the level of hypertrophic markers MMP-13 and Runx2 decreased in response to expansion under hypoxia compared to normoxia. Moreover, osteogenic potential of the stem cells was shown to be suppressed by decreased levels of osteopontin and its receptor CD44 (Xu et al., 2007, additional reference). Ronziere et al. similarly showed that markers of hypertrophy such as collagen X and MMP-13 were suppressed due to expansion of adipose-derived human MSCs under hypoxic conditions. Thus, the oxygen levels that stem cells are exposed to during their expansion phase seem to play a significant role in their further differentiation potential (Ronziere et al., 2010, additional reference).

Additional References
