European Cells and Materials Vol. 24 2012 (pages 118-135) DOI: 10.22203/eCM.v024a09 ISSN 1473-2262

# 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)/Sma and Mad Related Family (Smad) pathways, can regulate hypertrophy, but factors as diverse as hypoxia, co-culture, 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; co-culture.

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## 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.*, 2005; Freed *et al.*, 2006; Kuo *et al.*, 2006; Nesic *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.*, 2002; 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 (Holtzer, 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).

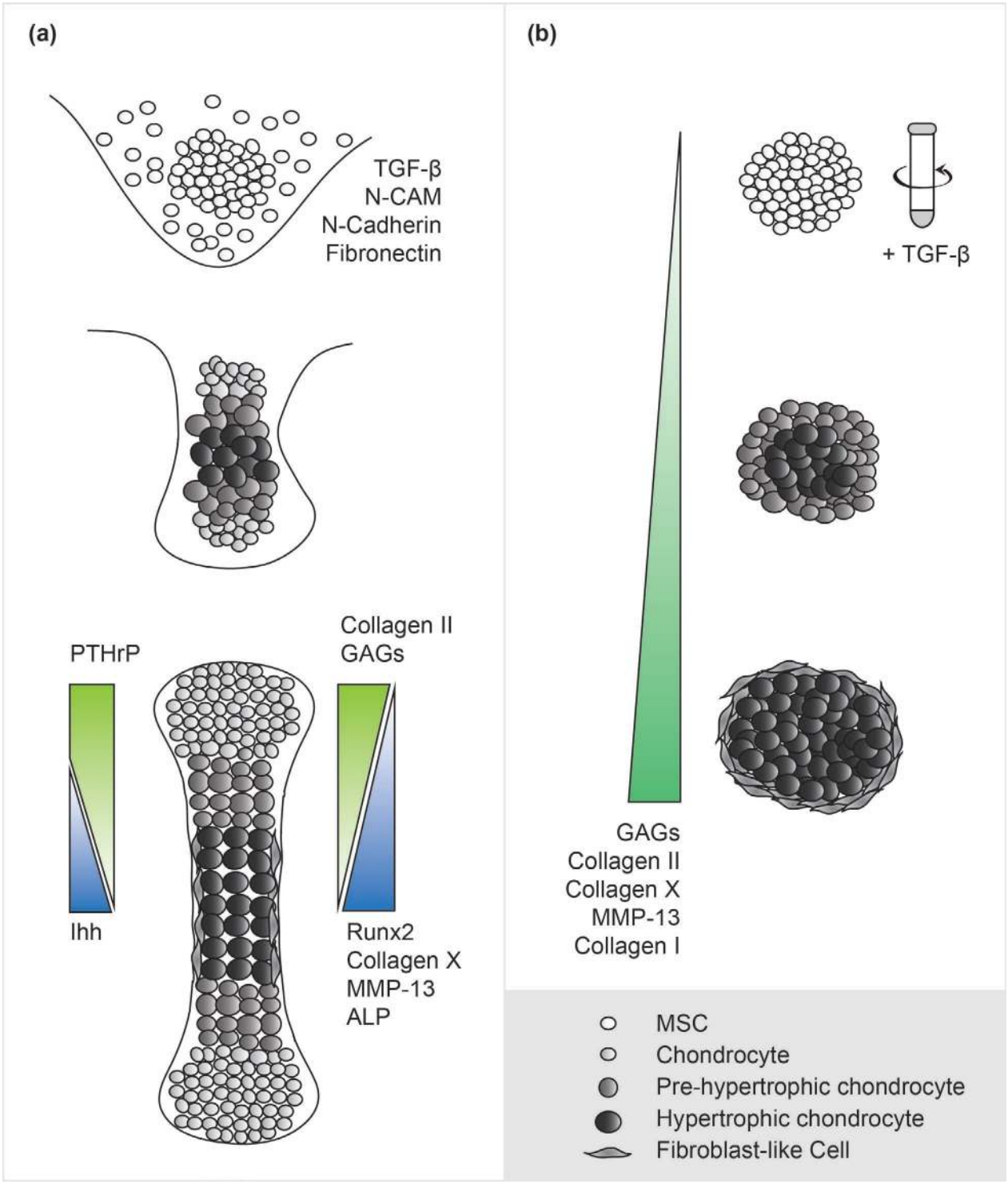
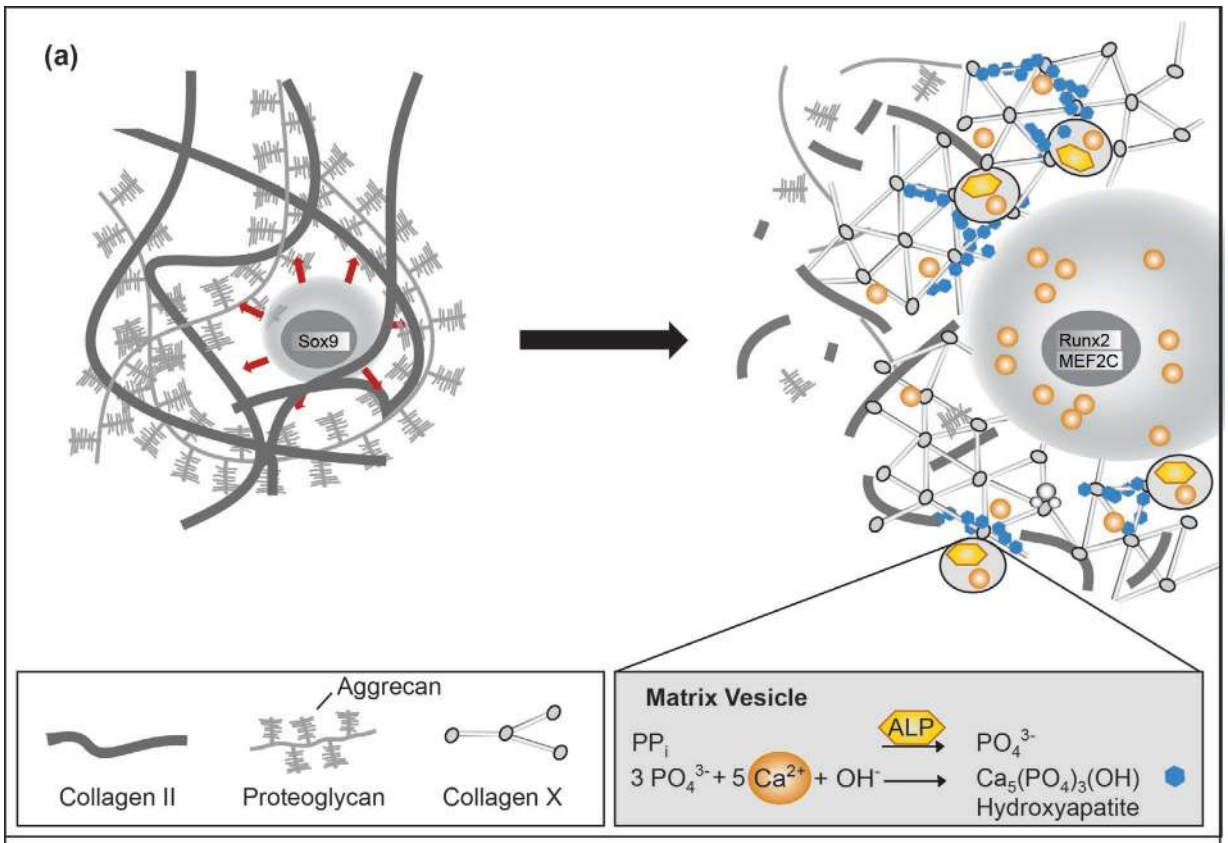



**Fig. 1**. ***In vivo* and *in vitro* chondrogenesis and hypertrophy.** (**a**) Chondrogenesis initiates in limb bud development when MSCs undergo condensation, a process facilitated by increased expression of cAMP, TGF-β, fibronectin, N-CAM, and N-cadherin. Mature chondrocytes begin secreting cartilage 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




(b)

| *Hypertrophy markers* | Function |
|---|---|
| *Runx2/MEF2C* | Transcription of collagen X, Ihh, MMP-13, VEGF |
| *MMP-13* | Degradation of collagen II and aggrecan |
| *Collagen X* | Regulation of matrix mineralization |
| *ALP* | Production of PO43- leading to mineralization |
| *Ihh* | Induction of proliferation of non-hypertrophic chondrocytes |

**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 facilities the mineralisation process through association with matrix vesicles (MV) (Shen, 2005), and alkaline phosphatase (ALP) dephosphorylates pyrophosphate ($PP_i$) to phosphate ($PO_4^{3-}$), leading to hydroxyapatite formation (Anderson, 2003).

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) and vascular endothelial growth factor (VEGF) (Kwon *et al.*, 2011), which all functionally contribute to endochondral ossification. Secreted MMP-13 degrades collagen II (Inada *et al.*, 2004) and aggrecan (Fosang *et al.*, 1996), key extracellular matrix (ECM) components of functional cartilage. Collagen X is deposited within the hypertrophic ECM and serves as a framework for subsequent calcification (Shen, 2005).

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

calcium, forms hydroxyapatite inside the MVs (Balcerzak *et al.*, 2003). Other sources of phosphate in MVs are the hydrolysis of phospholipids by PHOSPHO1 (Macrae *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 $PO_4^{3-}$ 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). Post-mitotic 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 osmolarity, 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 osmolarity 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)

Parathryroid 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 PHTrP 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 de-phosphorylation of histone deacetylase 4 (HDAC4) and the inactivation of MEF2C (Kozhemyakina *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

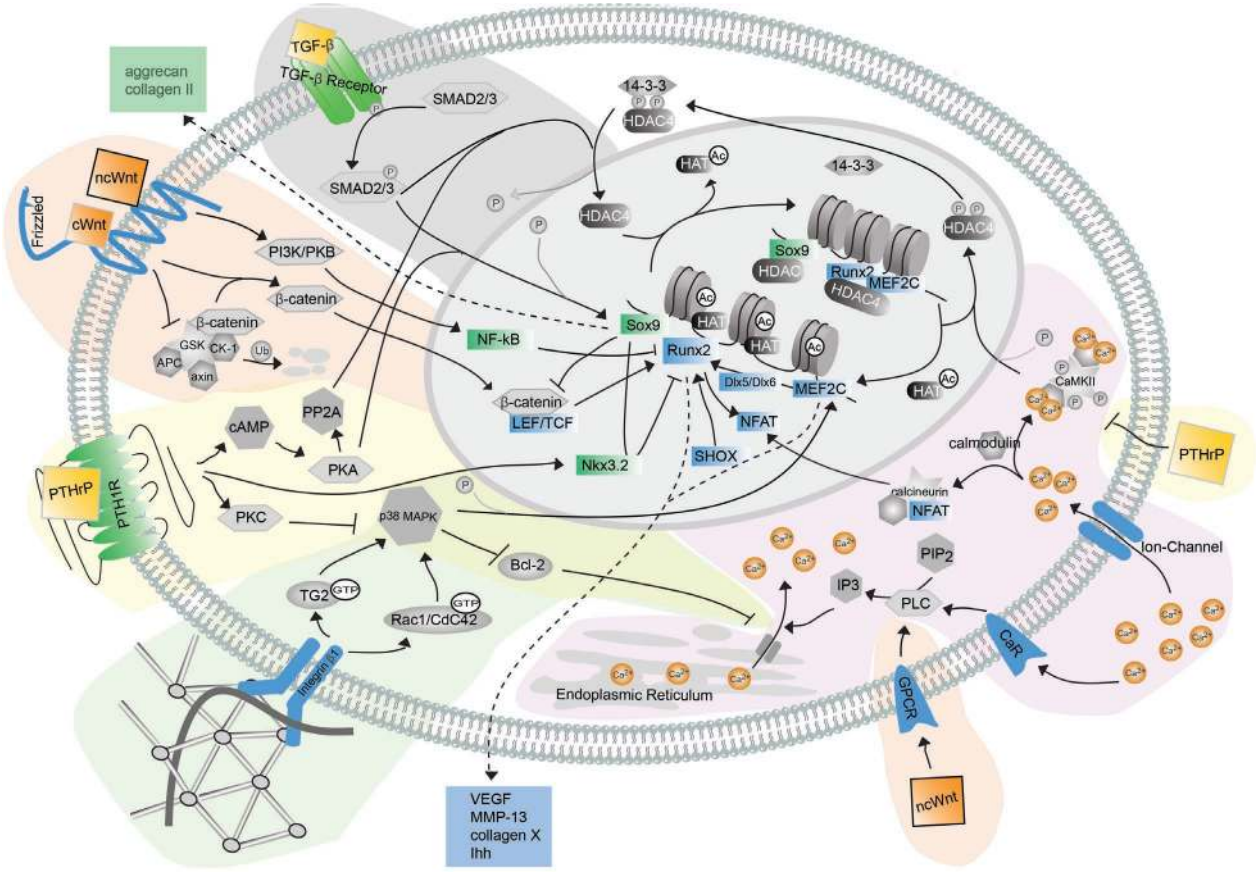



**Fig. 3**. **Molecular pathways of chondrocyte hypertrophy.** Signalling pathways initiated by TGF-β (grey shading), PTHrP/PTH (yellow shading), Wnt (orange shading), integrin-β1 interaction with collagen X (Luckman *et al.*, 2003) (green shading) and calcium ions (pink shading). The main transcription factors (rectangular boxes) regulating hypertrophy are Sox9, which is responsible for the expression of collagen II and aggrecan, and Runx2 which regulates transcription of collagen X, MMP-13, VEGF and Ihh genes. Sox9 was shown to inhibit Runx2 (Dy *et al.*, 2012) via Nkx3.2 (Yamashita *et al.*, 2009; Zhou *et al.*, 2006). MEF2C is proposed to be the main regulator of Runx2 (Arnold *et al.*, 2007), which acts through Dlx5/Dlx6 (Verzi *et al.*, 2007). SHOX/Shox2 is yet another upstream activator of Runx2. Nkx3.2 is induced by PTHrP (Provot *et al.*, 2006) and acts synergistically with Sox9 (Yamashita *et al.*, 2009) to inhibit Runx2 (Lengner *et al.*, 2005). Ihh triggers the protein degradation of Nkx3.2 (Choi *et al.*, 2012). Histone deacetylases (HDACs) participate in epigenetic regulation preventing transcription by stabilising DNA packing around histones (grey cylinders), whereas histone acetylases (HATs) provide access to the DNA strands. HDACs are released via phosphorylation by kinases that provide a phosphate (P) which can then be bound by chaperones (14-3-3) and exported out of the nucleus.

Green colour indicates a pathway involved in the suppression and blue in the promotion of hypertrophy. Dashed lines represent the expression of marker genes. "P" depicts phosphorylation/dephosphorylation and "Ub" ubiquitination.

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.*, 1996). 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) (Backs *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 tonicity 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 Sma 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, glycogen 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-κB (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 chondrogeneis and hypertrophy is somewhat controversial. Overexpression of RhoA/Rock was shown to suppress ALP and mineralisation in chondrocytes, whereas their counterparts, Rac1/CdC42

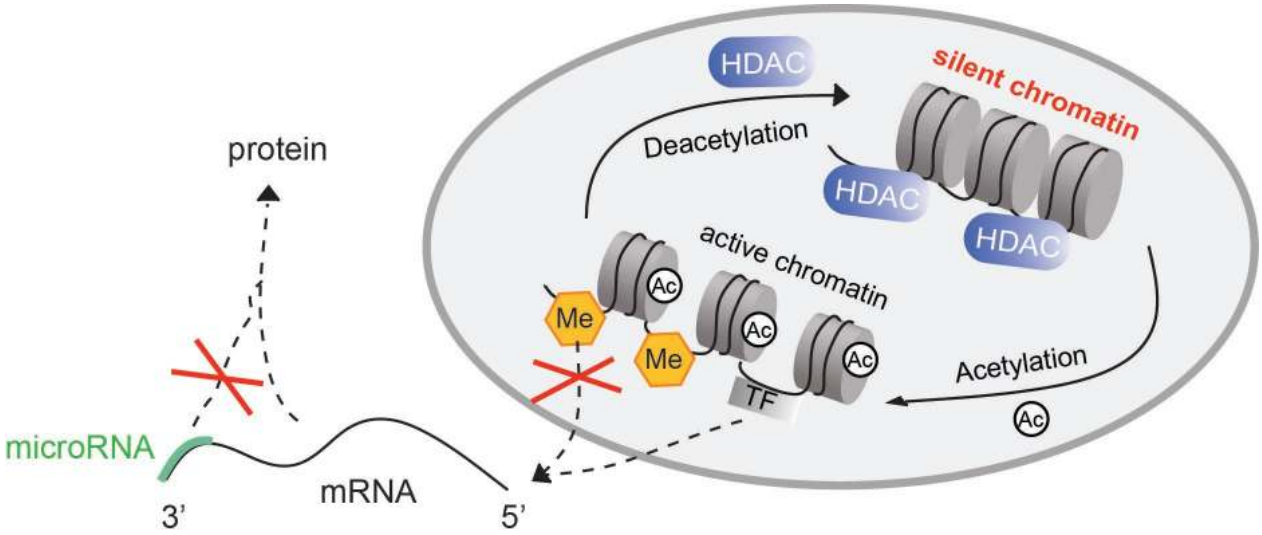



**Fig. 4**. The epigenetic regulation of hypertrophy involves histone deacetylases (HDACs), which act inside the nucleus to compact the chromatin into a "silent" structure. The acetylation (Ac) of histones opens up the chromatin for transcription of DNA by transcription factors (TF). Methylation (Me) of the DNA inhibits its transcription to messenger RNA (mRNA), whereas the post-transcriptional regulation of microRNAs inhibits the translation of mRNA by binding to its 3' untranslated region.

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 deactylases (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 mechano-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.*, 2011). The mechanosensitivity of certain MiRs and HDACs (Li *et al.*, 2011b) suggests an important role for epigenetics in mechanotransduction.

## 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.

## Biomaterials

Chondrogenesis of MSCs has been shown to be induced and maintained by simply tailoring cell-matrix interactions

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

| *miR #* | Target | Species | Effect on hypertrophy | Reference |
|---|---|---|---|---|
| *26b* | COL10A1, IGF-1 etc. | human MSC | inhibitory | (Han *et al.*, 2010) |
| *140* | ADAMTS-5 | mice | inhibitory | (Miyaki *et al.*, 2010) |
| *140* | HDAC4 | mice | stimulatory | (Tuddenham *et al.*, 2006) |
| *140-5p* | Smad2/3 | human C | stimulatory | (Swingler *et al.*, 2011) |
| *145* | Sox9 | murine MSC | stimulatory | (Yang *et al.*, 2011) |
| *152* | COL2, -4, -6A1, BMP3 | human MSC | stimulatory | (Han *et al.*, 2010) |
| *199** | Smad1 | murine MSC | stimulatory | (Lin *et al.*, 2009) |
| *204* | Runx2 | human MSC | inhibitory | (Huang *et al.*, 2010) |
| *365* | HDAC4 | chicken C | inhibitory | (Guan *et al.*, 2011) |
| *455-3p* | Smad2/3 | human C | stimulatory | (Swingler *et al.*, 2011) |
| *675* | COL2A1 repressor | human C | inhibitory | (Dudek *et al.*, 2010) |

Classification into miRs that inhibit or stimulate hypertrophy. Abbreviations: mesenchymal stem cell (MSC), chondrocyte (C).

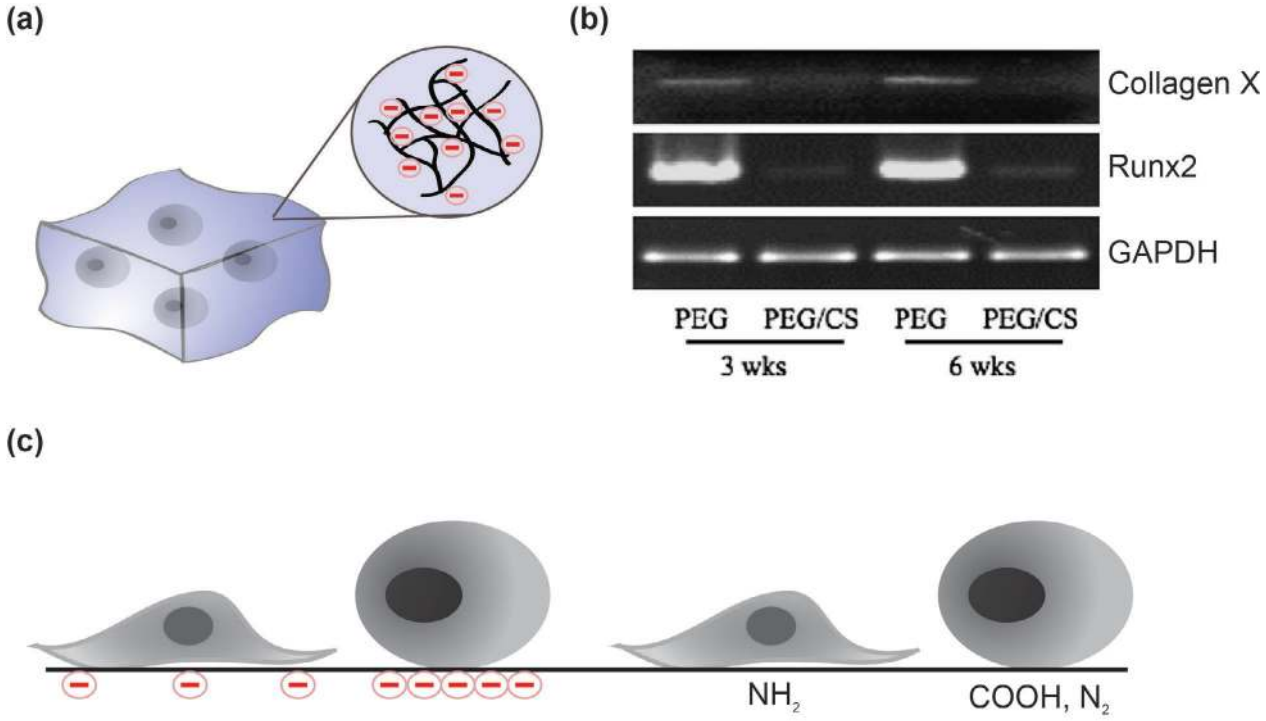



**Fig. 5**. **Control of phenotype by cell-material interactions.** (**a**) Cells encapsulated in hydrogels containing negatively charged chondroitin sulphate moieties exhibit (**b**) a significant decrease in their expression of hypertrophic markers Runx2 and collagen X. (**c**) Substrate modifications such as increasing charge density or surface functionalisation with carboxylate or $N_2$-doped plasma promote the rounded phenotype associated with mature chondrocytes and also inhibit collagen X production. (**b**) is reprinted from (Varghese *et al.*, 2008) with permission.

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 % $pO_2$); however, the *in vivo* MSC niche is hypoxic (2-8 % $pO_2$) (Mohyeldin *et al.*, 2010). Expansion

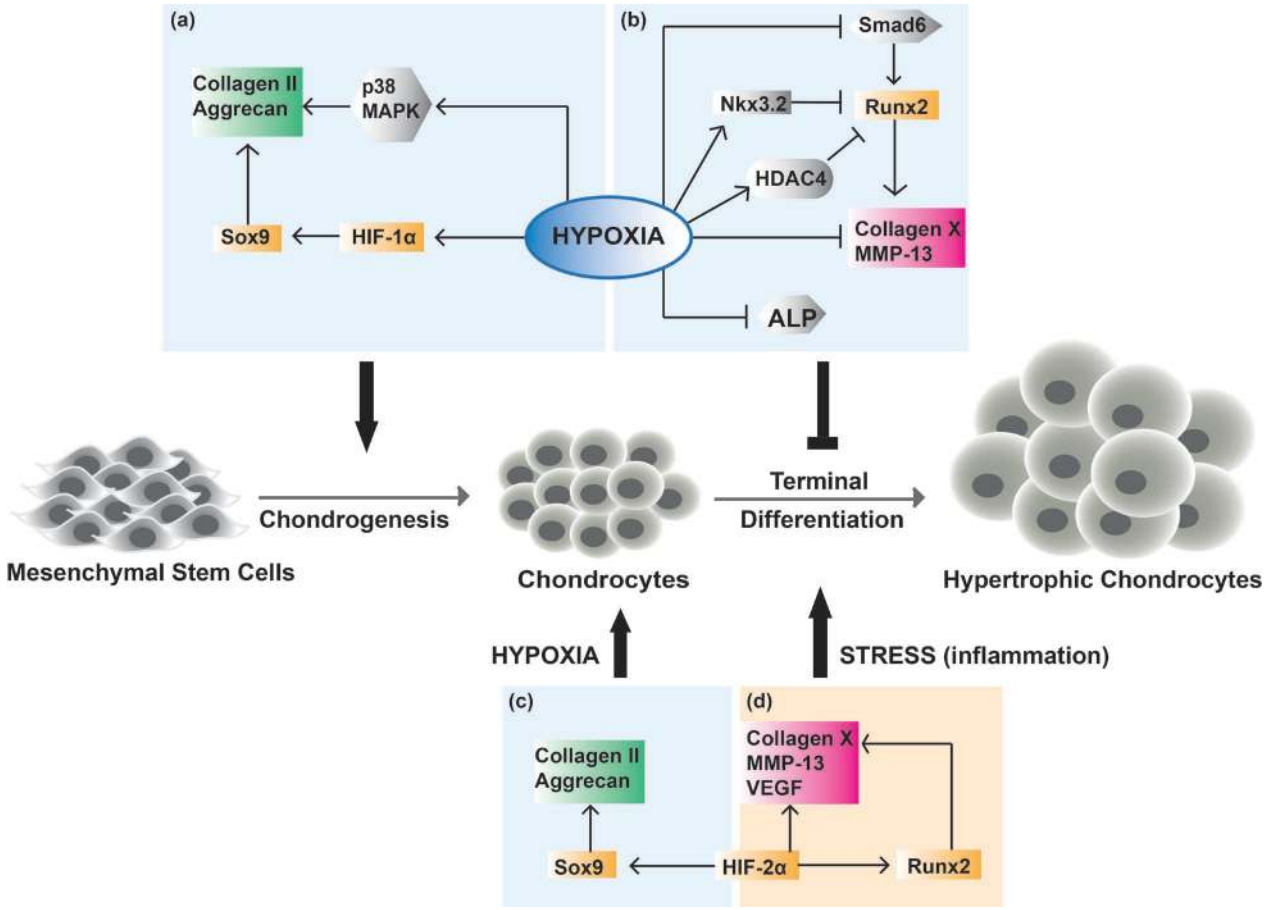



**Fig. 6**. **Hypoxia regulates chondrogenic differentiation and hypertrophy.** (**a**) Hypoxia enhances chondrogenesis of MSCs by up-regulating collagen II and aggrecan via activation of p38 MAPK pathway (Hirao *et al.*, 2006) and HIF-1α-triggered Sox9 activity (Robins *et al.*, 2005; Kanichai *et al.*, 2008). (**b**) Subsequent chondrocyte hypertrophy is suppressed by hypoxia as shown by down-regulation of collagen X, MMP-13 and ALP (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). (**c**) 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). (**d**) 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).

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). Suppression of MMP-13 and Runx2 were also observed for adipose-derived adult stromal cells due to hypoxic expansion conditions (Xu *et al.*, 2007). Similarly, hypoxia enhanced chondro-specific differentiation of the MSC line C3H10T1/2 with collagen II and aggrecan up-regulation via p38 MAPK pathway (Fig. 6a) and suppressed hypertrophy by down-regulating collagen X through Runx2 inhibition (Hirao *et al.*, 2006) (Fig. 6b). Despite induction of Runx2 by p38 MAPK pathway, hypoxic maintenance of chondrogenic phenotype was stated to be a combined effect of HDAC4 induction, Smad6 pathway repression (Hirao *et al.*, 2006) and Nkx3.2 up-regulation (Kawato *et al.*, 2011) leading to overall down-regulation of Runx2 (Fig. 6b). Moreover, hypoxic differentiation of bone marrow-derived MSCs in both pellets and hydrogels was revealed to suppress markers of hypertrophy such as collagen X and alkaline phosphatase (Sheehy *et al.*, 2012) (Fig. 6b).

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 through interaction with the Sox9 promoter (Robins *et al.*, 2005; Kanichai *et al.*, 2008) (Fig. 6a). Pellet culture of human articular chondrocytes under low oxygen tension yielded enhanced levels of collagen II, aggrecan and GAG as well

**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 $pO_2$ |
| *Co-culture* | | Secreted PTHrP |

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 aggrecan 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). Moreover, HIF-2α was shown to enhance Runx2 promoter activity bringing HIF-2α further in the context of hypertrophy (Tamiya *et al.*, 2008) (Fig. 6d). Linking oxygen tension and epigenetic regulation of hypertrophy, HIF-2α signaling 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.

## Acknowledgements

This study was supported by the Swiss National Science Foundation (CR23I2-130678/1) to MZW and KMW and the European Union Seventh Framework Programme (FP7/2007-2013) under grant agreement n° NMP4-SL-2009-229292 (Find&Bind). We acknowledge Markus Rottmar for carefully reviewing the manuscript.

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## 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).

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