AUS DER ABTEILUNG FÜR UNFALLCHIRURGIE
LEITER: PROF. DR. MICHAEL NERLICH
DER FAKULTÄT FÜR MEDIZIN
DER UNIVERSITÄT REGENSBURG

THE ROLE OF BMP AND TGFß SIGNALLING IN THE TERMINAL DIFFERENTIATION IN THE *IN VITRO* CHONDROGENESIS OF MESENCHYMAL STEM CELLS

Inaugural – Dissertation
zur Erlangung des Doktorgrades
der Biomedizinischen Wissenschaften

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Fakultät für Medizin
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Abstract

Articular cartilage has a poor intrinsic repair capacity after injury and cartilage lesions may lead to further degeneration and osteoarthritis. Mesenchymal stem cells (MSCs) are a promising cell source for tissue engineering approaches to repair articular cartilage lesions. During *in vitro* chondrogenesis, MSCs express articular cartilage markers like collagen II and aggrecan. However, chondrogenic differentiating MSCs also express markers of hypertrophic chondrocytes such as collagen type X and alkaline phosphatise (ALP). Hypertrophy is a stage in endochondral bone development, e.g. in growth plate chondrocytes and ultimately ends in chondrocyte apoptosis and ossification. The similarity of MSC chondrogenesis and endochondral ossification raises concern for a tissue engineering application of MSCs for articular cartilage repair and suggests that similar mechanisms are involved in the regulation of hypertrophy in both biological processes. In growth plate chondrocytes, BMP signalling promotes and TGFß signalling inhibits hypertrophy. The goal of this thesis is to investigate the involvement of these pathways in the regulation of MSC hypertrophy in order to discover possible approaches for the modulation of this phenomenon.

Human MSCs were differentiated chondrogenically in pellet culture in chondrogenic medium containing TGFß and dexamathasone. In an *in vitro* hypertrophy model, the hypertrophic phenotype was enhanced by withdrawal of TGFß and dexamethasone and addition of triiodothyronine (T3). Differential gene expression analysis for ligands, receptors and modulators of BMP and TGFß signalling and confirmation of the results on protein level was carried out. The activity of the respective intracellular signalling pathways was assessed. Furthermore, functional experiments using agonists and antagonists of BMP and TGFß signalling were included.

The enhancement of the hypertrophic phenotype by the pro-hypertrophic medium conditions was clearly shown by increased cells size, collagen type X deposition and ALP activity. Differential gene expression analysis revealed up-regulation of BMP4, BMP receptor 1B and the BMP signalling associated transcription factor Runx2 upon induction of hypertrophy. Addition of BMP4 to the medium further enhanced hypertrophy, confirming the key role of BMP4 in the regulation of MSC hypertrophy in our model. In addition, it was shown that the induced hypertrophy could be blocked by the BMP antagonists noggin and dorsomorphin. On the other hand, TGFß receptor expression, the expression of the TGFß signalling associated

transcription factor Sox9 and TGFß signalling activity are reduced under pro-hypertrophic conditions.

In conclusion, the results show that increased BMP signalling and reduced TGFß signalling are both involved in the enhancement of the hypertrophic phenotype. These pathways are possible targets for the modulation of the amount of hypertrophy in MSC based cartilage tissue engineering applications.

Zusammenfassung

Artikulärer Knorpel weist eine schlechte Regenerationsfähigkeit nach Verletzungen auf und Knorpelverletzungen können zu weiteren Degenerationen und Osteoarthritis führen. Mesenchymale Stammzellen (MSCs) sind eine vielversprechende Zellquelle für die Entwicklung von Tissue Engineering Produkten zur Reparatur von lokalisierten Knorpelschäden. In den üblicherweise verwendeten *in vitro* Chondrogenese Modellen differenzieren MSCs chondrogen und exprimieren typische chondrogene Marker wie Kollagen II und Aggrecan. Chondrogen differenzierende MSCs exprimieren jedoch auch Hypertrophiemarker wie Kollagen X und Alkalische Phosphatase (ALP). Hypertrophie ist ein Stadium während der endochondralen Knochenentwicklung, insbesondere in Wachstumsfugen-chondrocyten und führt unweigerlich zur Apoptose der Chondrozyten und zur Ossifikation. Die Ähnlichkeit von MSC Chodrogenese und endochondraler Ossifikation macht den Einsatz von MSCs für die Entwicklung von Tissue Engineering Produkten zur Reparatur von Knorpelschäden bedenklich und deutet darauf hin dass ähnliche Mechanismen in der Regulation der Hypertrophie in beiden Prozessen beteiligt sind. Aus der Wachstumsfuge ist bekannt, dass verschiedene Wachstumsfaktorsysteme an der Regulation der hypertrophen Differenzierung beteiligt sind, darunter BMP und TGFß Signalling. BMP Signalling fördert die terminale Differenzierung und TGFß Signalling hemmt die Hypertrophie. Das Ziel dieser Arbeit lag darin, die Regulation der MSC Hypertrophie besser zu verstehen um Wege zu finden die Hypertrophie von MSCs zu hemmen. Insbesondere der Einfluss von BMP und TGFß Signalling auf die Hypertrophieentwicklung von MSCs wurde untersucht.

Humane MSCs wurden in einem Pellet Kultur System in chondrogenem Medium das TGFß und Dexamethazon enthält chondrogen differenziert. In einem *in vitro* Hypertrophie Modell wurde der hypertrophe Phänotyp durch den Entzug von TGFß und Dexamethazon und eine Zugabe von dem Schilddrüsenhormon T3 erhöht. Eine differentielle Genexpressionsanalyse von Liganden, Rezeptoren und Modulatoren von BMP und TGFß Signalling wurde vergleichend zwischen chondrogenen und hypertrophen Bedingungen durchgeführt. Die Aktivität der jeweiligen Signalwege wurde analysiert und funktionelle Experimente mit BMP und TGFß Agonisten und Antagonisten wurden durchgeführt.

Die Verstärkung der Hypertrophie unter pro-hypertrophen Bedingungen wurde gezeigt durch ein stark erhöhtes Zellvolumen, erhöhte Kollagen X Expression und gesteigerter ALP Aktivität. Die differentielle Genexpressionsanalyse zeigt, dass unter pro-hypertrophen Bedingungen BMP4, BMPR1B und der BMP Signalling assoziierte Transkriptionsfaktor Runx2 signifikant hochreguliert sind. Darüber hinaus führt eine Zugabe von rekombinantem BMP4 Protein zum Zellkulturmedium zu einer Verstärkung der Hypertrophie, was eine Schlüsselrolle von BMP4 in der Regulation der MSC Hypertrophie in unserem Modell untermauert. Des Weiteren konnte gezeigt werden, dass die induzierte Hypertrophie durch die BMP Antagonisten Noggin und Dorsomorphin gehemmt werden kann.

Auf der anderen Seite ist sowohl die Expression der TGFß Rezeptoren und des TGFß Signalling assoziierte Transkriptionsfaktor Sox9 als auch die Aktivität des TGFß Signalweges unter hypertrophen Bedingungen erniedrigt.

Zusammenfassend zeigen diese Ergebnisse dass verstärktes BMP Signalling und reduziertes TGFß Signalling beide zu der Erhöhung des hypertrophen Phänotyps beitragen. Diese Signalwege sind mögliche Ansatzpunkte für die Modulation der Hypertrophie in MSC basierenden Knorpelersatzprodukten.

1 Introduction

Chondrogenesis is a well-coordinated developmental differentiation program by which cartilage is formed. Mesenchymal cells condensate and differentiate into chondrocytes that synthesize and secrete a characteristic matrix into the extracellular space. Chondrogenesis is regulated by cellular interactions, growth and differentiation factors that modulate cellular signalling pathways and the transcription of specific genes. Chondrogenesis leads to transient cartilage during endochondral bone formation or to permanent articular cartilage in the joints. While endochondral ossification is well understood, the mechanisms leading to permanent articular cartilage are unclear.

1.1 Endochondral ossification

The vertebrate limb skeleton develops through a process called endochondral ossification, which involves the formation of a cartilage scaffold that is ultimately replaced by bone.

The cartilage template is formed by aggregation and condensation of loose mesenchyme. The condensing mesenchyme expresses cell adhesion and extracellular matrix (ECM) molecules such as N-cadherine, N-CAM, tenascin, versican and collagen type I. Cells located centrally differentiate to pre-chondrocytes that turn off the expression of mesenchymal and condensation markers and begin to express collagen type II and other cartilage specific markers. Cells located peripherally remain undifferentiated and form the perichondrium. Pre-chondrocytes further differentiate into fully committed chondrocytes that are characterized by an increased cell proliferation and the deposition of cartilage matrix. Chondrocytes highly express collagen type II, collagen type IX, collagen type XI and other cartilage markers like aggrecan. Initially small and round, chondrocytes become flattened and are organized into parallel, longitudinal columns. They slow down their proliferative activity and finally stop proliferating. Chondrocytes then differentiate towards pre-hypertrophic chondrocytes followed by differentiation into fully differentiated hypertrophic chondrocytes. Hypertrophic chondrocytes are characterized by an increased cell size, the expression collagen type X instead of collagen type II and expression of high levels of the enzyme alkaline phosphatase (ALP) (Lewinson, D. *et al.*, 1982; O'Keefe, R. J. *et al.*, 1994). ALP is essential for the calcification of the

matrix (Anderson, H. C., 1995) and is secreted by hypertrophic chondrocytes into the surrounding matrix. Hypertrophic chondrocytes also express vascular endothelial factor (VEGF) (Gerber, H. P. *et al.*, 1999) that promotes the invasion of blood vessels into the cartilage matrix. Finally, hypertrophic chondrocytes undergo apoptosis, blood vessel, osteoclasts and osteoprogenitor cells invade which replace the cartilage scaffold by bone (Figure 1 A) (Goldring, M. B. *et al.*, 2006) (Shimizu, H. *et al.*, 2007). Endochondral ossification takes place in the growth plate, which develops at the end of long bones and is responsible for the longitudinal growth of the bones. The growth plate is divided into different zones according to the morphology and maturation stages of the chondrocytes (Figure 1 B).

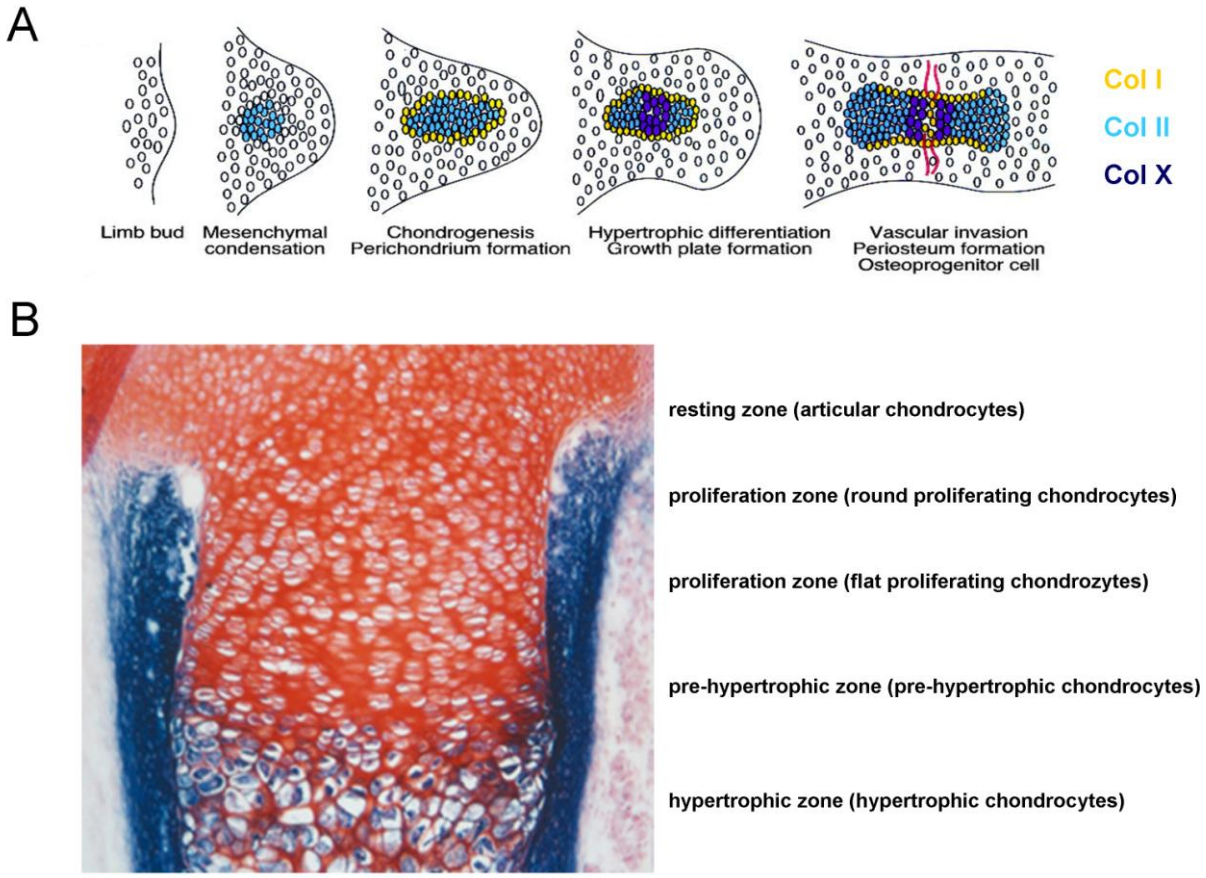

Figure 1 Endochondral bone development. **A** Schematic representation of a developing endochondral bone. Endochondral skeletal development begins with the formation of a mesenchymal condensation. Mesenchymal cells in the centre differentiate into collagen type II expressing chondrocytes (blue) that differentiate to hypertrophic chondrocytes, that are characterized by collagen type X expression (purple). Progression to the mature growth plate includes development of the perichondrium (yellow) and vascular invasion. (Minina, E. *et al.*, 2002) **B** Mouse tibial growth plate. ALP activity (blue), neutral red as counterstaining (http://www.histology-world.com /photoalbum/displayimage.php?album=77&pid=4579).

Endochondral ossification gives rise to long bones that comprise the appendicular skeleton and vertebrae. Apart from endochondral ossification there is a second developmental process that is responsible for the formation of the vertebrate skeleton, called intramembranous ossification. Intramembranous ossification gives rise to flat bones including the cranium. Both processes are initiated by condensation of mesenchyme, however in endochondral ossification a cartilagous template is formed that is subsequently transferred to bone and in intramembranous ossification mesenchymal cells differentiate directly into osteoprogenitor cells. Osteoprogenitor cells mature towards osteoblasts that deposit and mineralize the bone matrix.

Endochondral ossification also takes place during secondary fracture healing. During fracture repair, a cartilaginous template, the callus, is built which is afterwards replaced by bone (Yamagiwa, H. & Endo, N., 2009).

1.1.1 Regulation of endochondral ossification

The different steps of endochondral bone development are regulated by a number of signalling molecules including bone morphogenetic proteins (BMPs), transforming growth factor ß (TGFß), fibroblast growth factors (FGFs), parathyroid hormone-related peptide (PTHrP), Indian hedgehog (Ihh) and Wnts.

1.1.1.1 TGFß superfamily

The TGFß superfamily consists of signalling molecules including TGFß, BMPs, activins, inhibins and growth and differentiation factors (GDFs). These growth factors have been implicated in the regulation of various processes during embryonic development including cell growth and differentiation, pattern formation and tissue specification (Kingsley, D. M., 1994; Hogan, B. L., 1996).

BMP signalling pathway

BMPs form a subgroup within the TGFß superfamily. BMPs are dimeric proteins and more than 20 BMP related proteins have been characterized. In the main signalling pathway, BMPs bind to a heterodimeric receptor complex composed of type I and type II serine/threonine kinase receptors (Derynck, R. & Feng, X. H., 1997). Upon ligand binding, the type II receptor phosphorylates the type I receptor. One type II and three different type I receptors transduce BMP signals: BMP receptor 1A

(BMPR1A, ALK3), BMP receptor 1B (BMPR1B, ALK6) and ALK2. Activated type I receptor phosphorylates and thereby activates intracellular signalling molecules, the Smads. In the case of BMP signalling Smad1, 5, 8 are activated. Activated Smad1, 5, 8 build a complex with the co-Smad4 and this complex moves into the nucleus and regulates gene expression (Figure 2 A).

The BMP signalling pathway can be regulated at different levels. First, there are two inhibitory Smads, Smad6 and Smad7, that inhibit or turn off BMP type I receptor mediated phosphorylation of Smad1, 5, 8. Secondly, a considerable degree of regulation occurs on the level of ligand availability. Extracellular BMP-specific antagonists like noggin, chordin, follistatin and gremlin complex with BMPs and prevent their binding to the receptor (Zimmerman, L. B. *et al.*, 1996; Hsu, D. R. *et al.*, 1998; Iemura, S. *et al.*, 1998; Khokha, M. K. *et al.*, 2003). Furthermore co-receptors modulate BMP signalling. The pseudoreceptor BAMBI (BMP and activin membrane-bound inhibitor) is a transmembrane protein with structural similarity to type I receptors of the TGFß superfamily, but has a shorter intracellular domain which exhibits no enzymatic activity (Onichtchouk, D. *et al.*, 1999; Grotewold, L. *et al.*, 2001). BAMBI inhibits TGFß and BMP signalling by blocking the interaction between type I and type II receptors (Onichtchouk, D. *et al.*, 1999). BAMBI is tightly co-expressed with BMP4 during embryonic development and may act as a negative feedback regulator of BMP signalling (Onichtchouk, D. *et al.*, 1999; Grotewold, L. *et al.*, 2001). Finally a considerable degree of crosstalk exists between the BMP signalling pathway and other signalling pathways.

Another strategy to artificially inhibit BMP signalling is the use of small molecule inhibitors. Dorsomorphin has been shown to block BMP-induced Smad1, 5, 8 phosphorylation while having no effect on TGFß-induced Smad2, 3 activation (Yu, P. B. *et al.*, 2008). The heterocyclic core structure of dorsomorphin binds to the ATP binding site in the kinase domain of the type I receptors and inhibits their kinase domain (Yu, P. B. *et al.*, 2008; Wrighton, K. H. *et al.*, 2009) (Figure 2 B).

In addition to the Smad-dependent pathway, BMPs can also activate Smad-independent pathways including the mitogen-activated-protein kinases (MAPK) pathway. BMPs can signal by activating TGFß-activated kinase 1 (TAK1) which interacts with MEKK1 and activates p38 or by activating Ras/Erk1/2 (Derynck, R. & Feng, X. H., 1997).

TGFß signalling pathway

Similar to BMP signalling, TGFß signalling is initiated by binding of ligands to a heterodimeric serine/threonine kinase receptor complex that is composed of TGFß type I and type II receptors. The TGFß type II receptor (TGFßR2) then phosphorylates the TGFß type I receptor (TGFßR1, ALK-5) which in turn phosphorylates and thereby activates Smad proteins. In contrast to BMP signalling, TGFß signalling activates Smad2, 3. Phosphorylated Smad2, 3 forms a complex with Smad4 and accumulates in the nucleus to regulate gene transcription (Figure 2 A). Similar to BMP signalling, TGFß signalling can be modulated at different levels. Inhibitory Smad6 and Smad7 interfere with the activation of Smad2 and Smad3. Betaglycan and endoglin are TGFß co-receptors that modulate TGFß signalling. Betaglycan is a membrane-anchored proteoglycan that binds TGFß1, TGFß2 and TGFß3 with high affinity (Massague, J., 1998). Betaglycan lacks an intracellular signalling domain but can facilitate TGFß binding to the signalling receptors. Endoglin is a membrane bound glycoprotein that is able to bind TGFß1 and TGFß3 (Massague, J., 1998). Whereas betaglycan seems to increase TGFß signalling, endoglin is assumed to inhibit TGFß signalling (Letamendia, A. *et al.*, 1998; Perez-Gomez, E. *et al.*, 2007). In addition, TGFß signalling activity is controlled by the conversion of latent TGFß to active TGFß. TGFß is normally secreted in an inactive, latent form. TGFß1, TGFß2 and TGFß3 are synthesized with large amino-terminal prodomains which are required for the correct folding and dimerization of the carboxy-terminal growth factor domain (Gray, A. M. & Mason, A. J., 1990). The propeptides which are known as the latency-associated proteins (LAPs) are cleaved from the mature TGFß dimer after secretion but non-covalent association persists between the growth factor domain and prodomain. Another protein called latent TGF-ß binding protein (LTBP) binds to this complex. Activation of TGFß requires the release of TGFß from the LAP and LTBP. The process of TGFß activation is not fully understood, however, the mechanism of TGFß activation in the growth plate defined thus far includes matrix metalloproteinases or other proteases and acidic conditions (Pedrozo, H. A. *et al.*, 1999; D'Angelo, M. *et al.*, 2001).

TGFß signalling can be inhibited artificially with the TGFßR1 inhibitor SB431542. SB431542 is a potent inhibitor of TGFßR1 kinase activity and has been shown to inhibit the *in vitro* phosphorylation of Smad3 (Callahan, J. F. *et al.*, 2002) while having no influence on BMP signalling (Inman, G. J. *et al.*, 2002) (Figure 2 B).

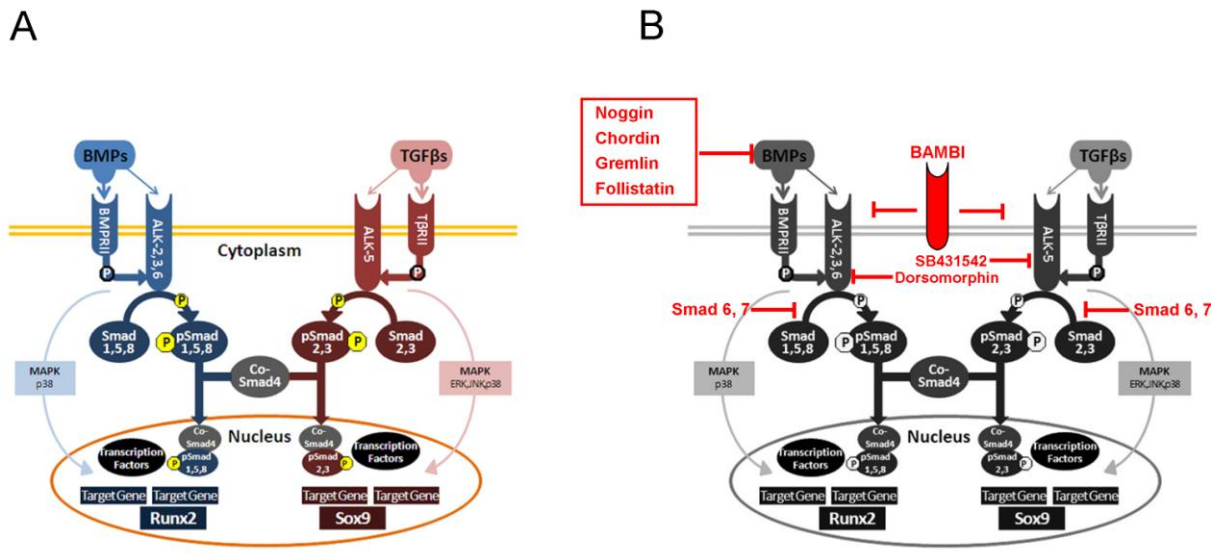

Figure 2 BMP and TGFß signalling pathways. **A** BMPs bind to their receptors and activate intracellular Smad1, 5, 8 proteins that move into the nucleus and activate target gene expression. One important downstream target of BMP signalling is Runx2. TGFßs bind to their receptors and activate intracellular Smad2, 3 proteins that move into the nucleus and activate target gene expression. One important downstream target of TGFß signalling is Sox9. **B** Modulators of BMP/TGFß signalling. Soluble BMP antagonists (Noggin, Chordin, Gremlin, Follistatin) prevent the interaction of BMPs with their receptors. Inhibitory Smad6, 7 inhibit the phosporylation of Smad1, 5, 8 and Smad2, 3 respectively. Dorsomorphin inhibits BMPR1 induced phosphorylation of Smad1, 5, 8 and SB431542 inhibits TGFßR1 induced phosphorylation of Smad2, 3. Bambi inhibits the formation of type I and II receptor complexes**.**

The role of BMP signalling in the regulation of endochondral ossification

During endochondral ossification, genes for BMPs and their receptors are expressed in distinct spatial and temporal patterns. BMP2, BMP4 and BMP7 are expressed in the perichondrium and are believed to regulate cartilage formation and development (Macias, D. *et al.*, 1997; Zou, H. *et al.*, 1997). Different studies clearly demonstrated that BMPs have multiple roles during the different stages of endochondral bone development. First, BMP signalling promotes the formation of mesenchymal condensations. Noggin expression in early chick limbs suppresses the formation of mesenchymal condensations (Pizette, S. & Niswander, L., 2000). Secondly, BMPs stimulate chondrogenic differentiation (Chen, P. *et al.*, 1991; Cancedda, R. *et al.*, 1995; Duprez, D. M. *et al.*, 1996; Pizette, S. & Niswander, L., 2000; Carlberg, A. L. *et al.*, 2001; Tsumaki, N. *et al.*, 2002). Finally, BMP signalling plays a crucial role during chondrocyte maturation. BMPs promote the terminal differentiation of growth plate chondrocytes to hypertrophic chondrocytes. *In vitro* studies showed that cultured embryonic chondrocytes are induced to undergo hypertrophy and increase the expression of hypertrophic markers including collagen

type X and ALP in the presence of BMPs (Suzuki, F., 1992; Rosen, V. *et al.*, 1994; Leboy, P. S. *et al.*, 1997; Volk, S. W. *et al.*, 1998; Grimsrud, C. D. *et al.*, 1999). In contrast, inhibition of BMP signalling prevents chondrocyte hypertrophy (Volk, S. W. *et al.*, 2000; Grimsrud, C. D. *et al.*, 2001). *In vivo* studies showed that overexpression of BMP4 in the cartilage of transgenic mice results in increased hypertrophic zone indicating increased differentiation into hypertrophic chondrocytes whereas overexpression of the BMP inhibitor noggin leads to a lack of hypertrophic chondrocytes (Tsumaki, N. *et al.*, 2002). Furthermore overexpression of noggin or chordin in the developing chick limb bud prevents chondrocyte hypertrophy and the expression of hypertrophic markers like collagen type X and ALP (Pathi, S. *et al.*, 1999; Zhang, D. *et al.*, 2002).

Other factors that induce chondrocyte hypertrophy also appear to act through BMP signalling. Thyroid hormone is one of the major systemic hormones influencing growth during childhood. Untreated hypothyroidism results in growth retardation and delayed skeletal maturation (Williams, G. R. *et al.*, 1998; Harvey, C. B. *et al.*, 2002) and hypothyroid rats display a disorganized growth plate with decreased hypertrophic zone (Stevens, D. A. *et al.*, 2000). The thyroid gland releases thyroxine (T4), which is transferred to tri-iodothyronine (T3), enters the nucleus and binds to thyroid hormone receptors (TRs) that function as ligand activated transcription factors. The receptors bind to specific DNA sequences (T3 responsive elements, TREs) and modulate transcriptional activity of thyroid hormone-responsive genes (Zhang, 2000). Thyroid hormone induces collagen type X expression and other hypertrophy associated marker in cultured mouse and chick growth plate chondrocytes (Bohme, K. *et al.*; Ballock, R. T. & Reddi, A. H.; Alini, M. *et al.*, 1996; Rabier, B. *et al.*). The effect of thyroid hormone seems to be mediated by BMP signalling. In mouse chondrocytes T4 induces hypertrophy through induction of BMP2 and this effect can be blocked by addition of the BMP antagonist noggin (Ballock, R. T. & O'Keefe, R. J., 2003). In chick chondrocytes thyroid hormone increases hypertrophy through BMP4 (Lassova, L. *et al.*, 2009). Similarly, the induction of hypertrophy by retinoic acid seems to be mediated by BMP signalling.

The role of TGFß signalling in the regulation of endochondral ossification

TGFß is expressed in condensing mesenchyme and during early stages of chondrocyte differentiation but not in hypertrophic chondrocytes (Pelton, R. W. *et al.*, 1990; Millan, F. A. *et al.*, 1991). TGFß signalling has been shown to stimulate

chondrogenesis *in vitro* and *in vivo*. *In vitro* studies showed that TGFß promotes chondrogenic differentiation in mesenchymal cells and chondroblasts (Seyedin, S. M. *et al.*, 1986; Schofield, J. N. & Wolpert, L., 1990; Leonard, C. M. *et al.*, 1991; Lorda-Diez, C. I. *et al.*). *In vivo*, it was shown that injection of TGFß1 into the periosteum induces chondrocyte differentiation and cartilage formation (Joyce, M. E. *et al.*; Chimal-Monroy, J. & Diaz de Leon, L., 1997). In addition to regulating chondrogenic differentiation, TGFß plays a significant role in the regulation of chondrocyte maturation. In contrast to BMP signalling, TGFß signalling inhibits chondrocyte terminal differentiation and hypertrophy *in vitro* and *in vivo*. *In vitro* studies showed that TGFß inhibits hypertrophy and the expression of hypertrophy associated genes like collagen type X and ALP in cultured embryonic chondrocytes (Ballock, R. T. *et al.*, 1993; Bohme, K. *et al.*, 1995; Ferguson, C. M. *et al.*, 2000). In an *in vivo* model of chick embryonic limb development, Ferguson et al showed that increased level of TGFß applied to the developing skeletal element inhibits chondrocyte hypertrophy (Ferguson, C. M. *et al.*, 2004) and loss of function models of TGFß signalling result in premature chondrocyte maturation in mice (Serra, R. *et al.*, 1997; Yang, X. *et al.*, 2001).

In summary, both BMP and TGFß signalling promote chondrogenic differentiation, but have opposing effects on later differentiation steps during skeletal development. BMP signalling promotes terminal differentiation of embryonic chondrocytes and TGFß signalling inhibits chondrocyte hypertrophy (Figure 3). Apart from BMP and TGFß signalling other signalling pathways are also involved in the regulation of endochondral ossification.

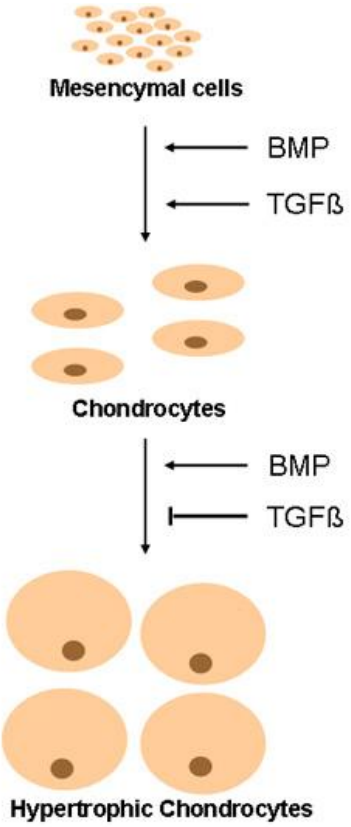

Figure 3 Regulation of endochondral ossification. BMP and TGFß signalling promote chondrogenic differentiation. BMP signalling promotes and TGFß signalling inhibits hypertrophic differentiation.

1.1.1.2 Ihh/PTHrP signalling

Ihh is part of the Ihh/PTHrP feedback loop that regulates the rate at which growth plate chondrocytes leave the proliferative zone and enter the differentiation process towards hypertrophic chondrocytes (Vortkamp, A. *et al.*). Ihh is expressed by pre-hypertrophic chondrocytes and the hedgehog receptor patched (PTCH1) is expressed in surrounding perichondrial cells. Chondrocytes that are beginning to undergo hypertrophic differentiation express Ihh which relays a signal back to the perichondrium to induce the expression of PTHrP (Vortkamp, A. *et al.*). The PTHrP receptor is primarily found in the transitional region where the switch from proliferating to hypertrophic chondrocytes takes place (Amizuka, N. *et al.*, 1994). Released PTHrP from the perichondrium binds to its receptors in the pre-hypertrophic zone and delays hypertrophic differentiation by maintaining cells in a proliferating state. *In vivo* studies showed that inhibition of PTHrP signalling in mice leads to an advanced onset of hypertrophic differentiation (Amizuka, N. *et al.*, 1994) and activation of PTHrP signalling leads to delayed hypertrophic differentiation

(Schipani, E. *et al.*, 1997). Similar to this, in mice lacking Ihh, premature hypertrophic differentiation occurs (St-Jacques, B. *et al.*, 1999).

The Ihh/PTHrP system seems to play a major role in the regulation of chondrocyte maturation during prenatal development, whereas it is believed that postnatally TGFß signalling seem to control chondrogenic maturation in adult animals. The distance between the growth plate and the articular surface is too large for paracrine Ihh/PTHrP feedback loop and recent studies have indicated that Ihh is not produced by the growth plate of postnatal animals (Iwasaki, M. *et al.*, 1997). Several lines of evidence indicate that TGFß resume the role of PTHrP as the key inhibitor of chondrocyte differentiation in adolescence. A work from Pedrozo et al demonstrated that mice deficient for the TGFß signalling associated transcription factor Smad3 have a completely normal skeleton at birth but within few weeks after birth they begin to exhibit cartilage abnormalities including premature hypertrophy (Pedrozo, H. A. *et al.*, 1998).

1.1.1.3 FGF signalling

Many FGFs and four of the FGF receptors are expressed during endochondral bone formation (Ornitz, D. M., 2005) indicating that FGF signalling is crucially involved in chondrocyte differentiation and maturation. FGF receptor 1 (FGFR1) is expressed in loose mesenchyme, while FGFR2 is expressed in condensing mesenchyme. FGFR3 is expressed by proliferating and pre-hypertrophic chondrocytes and FGFR1 is again up-regulated in pre-hypertrophic and hypertrophic chondrocytes. The function of FGFR3 is best understood. The importance of FGFR3 in skeletal development was revealed with the discovery that achondroplasia, the most common genetic form of dwarfism in humans is caused by a point mutation in the transmembrane domain of the FGFR3 (Rousseau, F. *et al.*, 1994; Shiang, R. *et al.*, 1994). The point mutation leads to an activation of the FGFR3 in the absence of the ligand. Achondroplasia is characterized by reduced growth of long bones and the growth plate of these patients shows a reduced zone of proliferating chondrocytes (Briner, J. *et al.*, 1991). Similar to this, *in vivo* studies showed that activation of FGFR3 leads to an inhibition of proliferation (Ornitz, D. M., 2005) and knockdown of FGFR3 leads to a prolonged bone growth accompanied by expansion of proliferating chondrocytes within the growth plate (Colvin, J. S. *et al.*, 1996; Deng, C. *et al.*, 1996). Thus, FGF signalling through FGFR3 inhibits proliferation. Different FGFs are capable of activating FGFR3, the most important FGF during

endochondral skeleton development seems to be FGF18 (Ornitz, D. M., 2005). Knockdown of FGF18 leads to an increase in chondrocyte proliferation that closely resembles the knockdown of FGFR3 (Ohbayashi, N. *et al.*, 2002). In addition, in FGF18 knockdown mice delayed ossification is observed indicating a role of FGF signalling in the regulation of chondrocyte maturation. This delay may reflect the lack of activation of FGFR1 in hypertrophic chondrocytes. In addition *in vitro* studies demonstrated that FGF signalling accelerates terminal differentiation of hypertrophic chondrocytes (Minina, E. *et al.*, 2002).

1.1.1.4 Wnt signalling

Canonical Wnt signalling has also been implicated in the regulation hypertrophy during endochondral ossification. The canonical Wnt/ß-catenin pathway is initiated by binding of Wnts to the Frizzled receptor and its co-receptor (low-density lipoprotein receptor-related protein 5 and 6 (LRP5/6)). In the absence of canonical Wnt signalling cytosolic ß-catenin is phosphorylated by the glycogen synthase kinase (GSK-3ß) and degraded by the ubiquitin/proteasome pathway. Upon activation of the canonical Wnt signalling pathway, the GSK-3ß is inhibited resulting in decreased ß-catenin phosphorylation and degradation. ß-catenin accumulates in the cytoplasm and moves into the nucleus to form a complex with the transcription factor Lef-1/TCF and activates transcription of Wnt target genes. The canonical Wnt signalling pathway is regulated by different extracellular and intracellular proteins. Extracellular inhibitors include secreted frizzled-related proteins (sFRP) that bind to Wnts and prevent the interaction with the receptor and dickkopfs (DKKs) that block Wnt signalling through the co-receptor LRP5/6 (Kawano, Y. & Kypta, R., 2003). Members of the canonical Wnt signalling family are highly expressed in mesenchymal cells committed to the chondrogenic lineage, are down regulated during early chondrogenic differentiation and up-regulated during hypertrophy where it promotes chondrocyte hypertrophy (Day, T. F. *et al.*, 2005; Hill, T. P. *et al.*, 2005; Tamamura, Y. *et al.*, 2005). Mice deficient for the Wnt inhibitor sFRP-1 exhibit accelerated hypertrophic chondrocyte maturation *in vivo* and *in vitro* (Gaur, T. *et al.*, 2006).

Wnts also signal through a ß-catenin independent mechanism, the non-canonical Wnt pathway. The non-canonical Wnt pathway involves the activation of the protein kinase C (PKC) leading to increased intracellular calcium concentration. A recent study demonstrated that inhibition of non-canonical Wnt signalling (via inhibition of

PKC) delays chondrocyte maturation shown by a decreased hypertrophic zone (Tu, X. *et al.*, 2007).

1.1.1.5 Transcriptional regulation during endochondral ossification

Major progress has been made over the last years in understanding the transcriptional regulation of chondrocyte differentiation during embryonic limb development. Sox9 and Runx2 seem to play a key role in the regulation of chondrogenesis. Sox9 plays a critical role in chondrogenic differentiation and inhibits terminal differentiation while Runx2 has been shown to promote chondrocyte hypertrophy (Figure 4).

Sox9

Sox9 belongs to the SRY (sex-determining region on the Y chromosome) family and contains the HMG (high mobility group) box DNA binding domain. During chondrogenesis, Sox9 is expressed in mesenchymal cells and in proliferating chondrocytes but not in hypertrophic chondrocytes (Ng, L. J. *et al.*, 1997; Zhao, Q. *et al.*, 1997). Sox9 is essential for the initiation of chondrogenesis. In chimeric mice, cells with no functional Sox9 fail to differentiate into chondrocytes and could not express chondrogenic specific markers like collagen type II and aggrecan (Bi, W. *et al.*). In addition, Sox9 is required for the maintenance of chondrogenic differentiation. Inactivation of Sox9 in the mouse embryo in collagen type II expressing cells results in the formation of pre-cartilagous condensations but pre-chondrocytes were not able to undergo further chondrogenic differentiation (Akiyama, H. *et al.*, 2002). Sox9 expression is down-regulated in hypertrophic chondrocytes indicating that Sox9 delays chondrocyte hypertrophy. Heterozygotous Sox9 mouse foetuses featured prematurely mineralized cartilages and extended hypertrophic zones in the growth plate (Bi, W. *et al.*, 1999). Furthermore, in mice that expressed Sox9 under the control of the collagen type II promoter cartilage mineralization was delayed (Akiyama, H. *et al.*, 2002). Two additional Sox family members, Sox5 and Sox6, are not expressed in early mesenchymal condensations but are co-expressed with Sox9 during chondrocyte differentiation (Lefebvre, V. *et al.*, 1998). Sox5 and Sox6 are required for sufficient expression of collagen type II and aggrecan during chondrocyte differentiation (Smits, P. *et al.*, 2001). Sox9 and Sox5/Sox6 cooperate to activate collagen type II and other cartilage specific genes (Lefebvre, V. *et al.*, 1998).

Sox9 expression seems to be regulated by TGFß (Chimal-Monroy, J. *et al.*, 2003). TGFß has been shown to promote chondrogenesis especially through Smad3 mediated TGFß signalling (Furumatsu, T. *et al.*). Smad3 stimulates transcriptional activity of Sox9 and Smad3 forms a complex with Smad2, Smad4 and Sox9 and activates expression of Collagen type II (Furumatsu, T. *et al.*, 2005; Furumatsu, T. *et al.*, 2009). In addition, Sox9 expression seems to be regulated by BMP signalling. Inactivation of both BMPR1A and BMPR1B in mice results in a lack of Sox9 expression (Yoon, B. S. *et al.*, 2005).

Sox9 has been shown to activate the expression of cartilage specific genes like collagen type II, aggrecan and collagen type XI (Lefebvre, V. *et al.*, 1997; Bridgewater, L. C. *et al.*, 1998; Sekiya, I. *et al.*, 2000).

Runx2

Runx2 is a transcription factor that belongs to the *runt*-domain gene family (Komori, T. & Kishimoto, T., 1998). It has a DNA-binding domain runt, which is homologous with the Drosophila pairrule gene runt (Kania, M. A. *et al.*, 1990). Three runt domain genes have been identified (Runx1, Runx2, Runx3). Runx2 is weakly expressed in resting and proliferating chondrocytes and its expression increases according to chondrocyte maturation with the highest expression in terminal differentiated hypertrophic chondrocytes (Inada, M. *et al.*, 1999; Kim, I. S. *et al.*, 1999; Takeda, S. *et al.*, 2001). Runx2 plays an important role in chondrocyte hypertrophy during the process of endochondral ossification. It has been shown that, *in vitro*, Runx2 stimulates chondrocyte maturation and the expression of hypertrophy markers like collagen type X and ALP (Enomoto, H. *et al.*, 2000; Ueta, C. *et al.*, 2001). *In vivo*, in Runx2 deficient mice chondrocyte differentiation is blocked before pre-hypertrophic state. The skeleton of these mice consists mainly of cartilage that is composed of collagen type II expressing chondrocytes with decreased numbers of hypertrophic collagen type X expressing chondrocytes (Komori, T. *et al.*, 1997; Inada, M. *et al.*, 1999). Ueta et al generated transgenic mice that overexpressed Runx2 or expressed a dominant negative form of Runx2 in chondrocytes under the control of the collagen type II promoter. Overexpression of Runx2 leads to accelerated chondrocyte maturation. Most skeletal elements showed massive mineralization. Interestingly, even chondrocytes that normally never undergo hypertrophy like chondrocytes that residue in articular cartilage became hypertrophic. These mice failed to form most of their joints because articular cartilage underwent the

endochondral pathway and cavitation did not take place. Transgenic expression of a dominant negative form of Runx2 blocks hypertrophic differentiation. Most skeletal elements were uncalcified cartilaginous tissues composed of immature chondrocytes expressing collagen type II but not collagen type X (Ueta, C. *et al.*, 2001). Furthermore expression of Runx2 in non-hypertrophic chondrocytes induced chondrocyte hypertrophy and endochondral ossification (Takeda, S. *et al.*, 2001). In addition to Runx2, Runx3 seems to promote chondrocyte hypertrophy (Yoshida, C. A. *et al.*, 2004).

Runx2 expression seems to be regulated by BMP signalling. BMP2, 4 and 7 have been shown to induce Runx2 expression (Tsuji, K. *et al.*, 1998; Lee, K. S. *et al.*). Other signalling molecules that have been implicated in the regulation of Runx2 expression are retinoids (Jimenez, M. J. *et al.*, 1999), FGF (Zhou, Y. X. *et al.*, 2000) and Wnts (Gaur, T. *et al.*, 2005). Furthermore, TGFß/Smad 3 and PTHrP suppress Runx2 expression (Alliston, T. *et al.*, 2001; Kang, J. S. *et al.*, 2005; Guo, J. *et al.*, 2006).

Several genes, that are specific for hypertrophic chondrocytes, have been characterized as Runx2 targets. Among them osteocalcin (Geoffroy, V. *et al.*, 2002), collagen type X (Leboy, P. *et al.*, 2001), matrix metalloproteinase (MMP)-13 (Jimenez, M. J. *et al.*, 1999) and VEGF (Zelzer, E. *et al.*, 2001). Interestingly, BMP signalling associated transcription factors Smad1, 5, 8 have been shown to interact with Runx2 at the collagen type X promoter to induce its gene expression (Leboy, P. *et al.*, 2001).

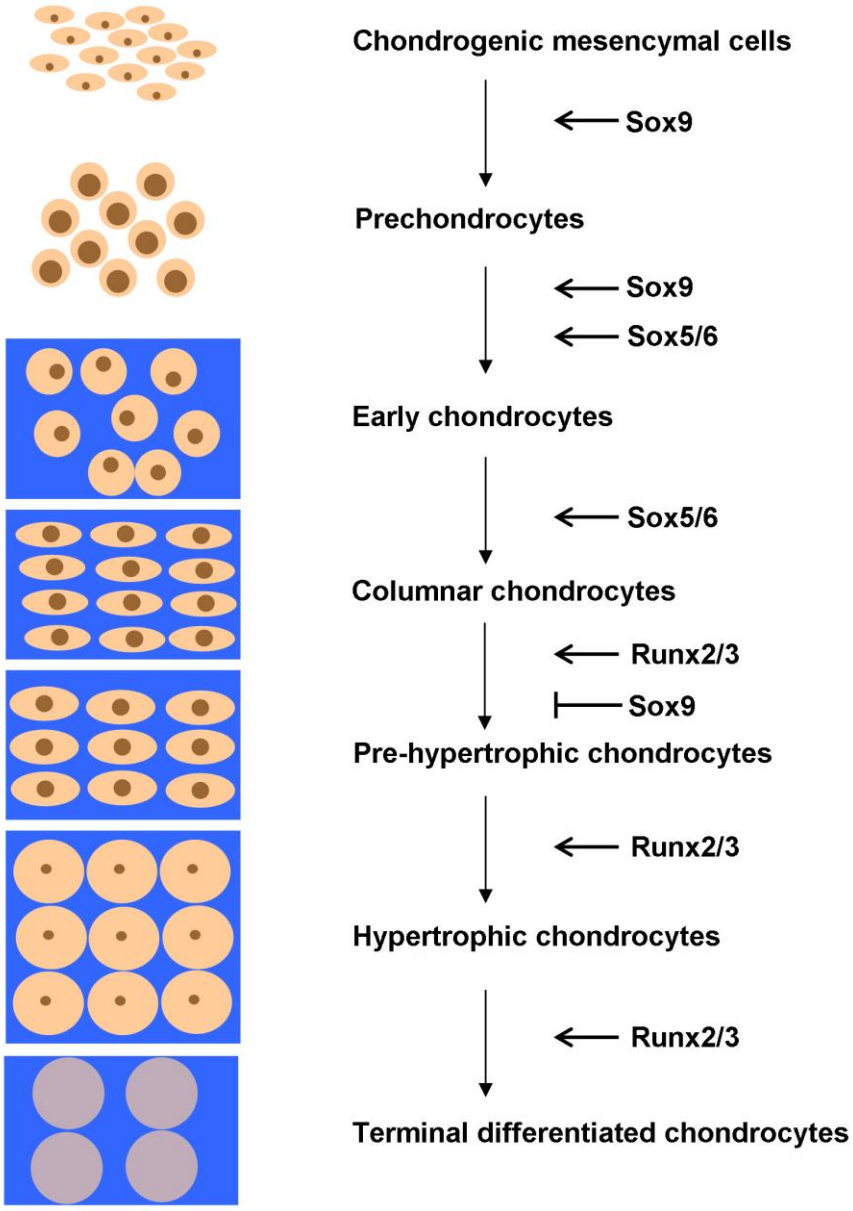

Figure 4 Transcriptional regulation of endochondral ossification. Sox9 together with Sox 5/6 promote chondrogenic differentiation and Sox9 inhibits the terminal differentiation of chondrocytes. Runx2/3 promote hypertrophic differentiation of chondrocytes.

1.2 Articular cartilage

Apart from the formation of transient cartilage during endochondral ossification, permanent articular cartilage develops in the joints, that is spared from hypertrophy. The origin of these articular chondrocytes is largely unknown. Recent evidence suggests that articular chondrocytes arise from a subpopulation of early chondrocytes (Hyde, G. *et al.*, 2007; Koyama, E. *et al.*, 2008). These cells escape the developmental program that involves the differentiation into hypertrophic chondrocytes that occurs during endochondral ossification, differentiate into articular chondroblasts and at the end of postnatal development become articular chondrocytes which maintain articular surfaces throughout life. The mechanism how articular chondroblasts escape growth plate maturation are relatively unknown. Permanent articular chondrocytes can be distinguished from transient growth plate chondrocytes by the expression of matrilin-1 and Gdf5 (Hyde, G. *et al.*, 2007; Koyama, E. *et al.*, 2008). Pacifici et al postulated that articular chondrocytes derive from Gdf5 expressing joint progenitors and growth plate chondrocytes derive from Gdf5 negative cells likely representing the bulk of the original condensing limb mesenchyme (Koyama, E. *et al.*, 2008).

After skeletal growth is completed, articular chondrocytes rarely divide and normally never undergo hypertrophy. They express cartilage specific markers like collagen type II, aggrecan and Sox9 (Davies, S. R. *et al.*, 2002). Runx2 is not expressed in articular chondrocytes (Kuboki, T. *et al.*, 2003). However in osteoarthritis (OA), a joint degenerative disease, articular chondrocytes can undergo hypertrophy and terminal differentiation and express hypertrophic markers like collagen type X and osteopontin (Girkontaite, I. *et al.*, 1996; Pullig, O. *et al.*, 2000), indicating that their differentiation state, although normally permanent, may be transient under pathologic conditions.

1.2.1 Composition of articular cartilage

The function of articular chondrocytes is to built, maintain and remodel the abundant ECM of the articular cartilage. Articular cartilage is a specialised tissue that coves the surfaces of bones in diarthrodial joints, where one bone articulates with another

and provides joints with a low-friction, gliding and wear-resistant surface that provides shock absorbance and high load bearing capability.

The articular chondrocyte is the only cell type that is present in articular cartilage. The cell density is relatively low and chondrocytes occupy less than 10% of the total volume. Each chondrocyte is surrounded by its ECM and has few cell-cell contacts. The ECM of articular cartilage consists of a collagen fibre network that is comprised primarily of collagen type II (80 % to 90 % of the collagen content) and secondary collagen type IX and collagen type XI (Poole, A. R. *et al.*, 2001). The collagen network entraps a highly hydrated gel of proteoglycans and glycoproteins. Proteoglycans consist of a core protein that is covalently linked to unbranched polysaccharide chains called glycosaminoglycans (GAGs). Aggrecan is the most abundant proteoglycan and is almost unique to cartilage. Aggrecan binds over 100 GAG chains and forms enormous aggregates by binding to linear chains of hyaluronan with the help of the link protein Crtl1. Other proteins that are present in the cartilage ECM are fibronectin, cartilage oligomeric matrix protein and the smaller proteoglycans biglycan, decorin and fibromodulin (Figure 5).

The ECM of articular cartilage is divided into different regions dependent on the proximity to the chondrocytes. These zones are the pericellular, territorial and interterritorial. The pericellular region surrounds the chondrocyte and contains proteoglycans and non-collagenous matrix components and little or no collagen fibrils. The territorial zone surrounds the pericellular region and is composed of collagen fibrils that form a fibrillar network and serves to protect chondrocytes from mechanical impact (Guilak, F. & Mow, V. C., 2000). The interterritorial zone is the largest region and contributes most to the mechanical properties of articular cartilage (Mow, V. C. & Guo, X. E., 2002). It consists of large collagen fibrils and the majority of proteoglycans.

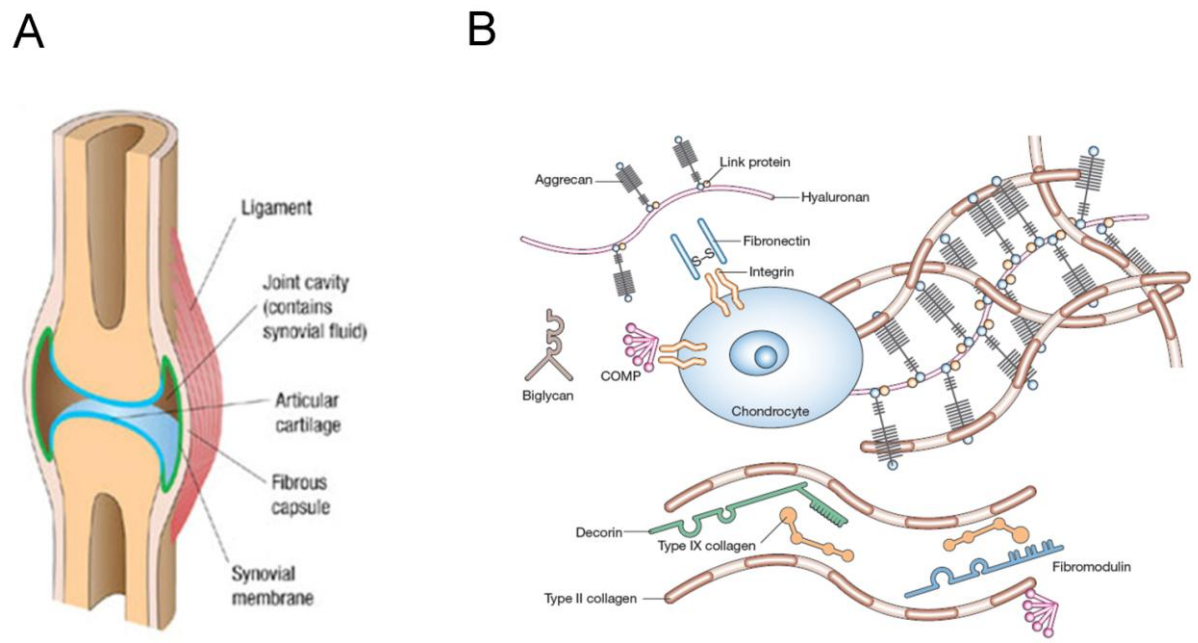

Figure 5 Articular cartilage. A Articular cartilage covers the bones in the joints (Setton, L., 2008). **B** Extracellular matrix of articular cartilage. Three classes of proteins exist in articular cartilage: collagens (mostly collagen type II); proteoglycans (primarily aggrecan); and other non-collagenous proteins (including link protein, fibronectin, cartilage oligomeric matrix protein) and the smaller proteoglycans (biglycan, decorin and fibromodulin) (Chen, F. H. *et al.*, 2006).

Furthermore articular cartilage can be divided into four distinct zones: superficial tangential, middle, deep and calcified. Within each zone the composition and organization of the ECM and the shape and arrangement of the chondrocytes varies (Figure 6).

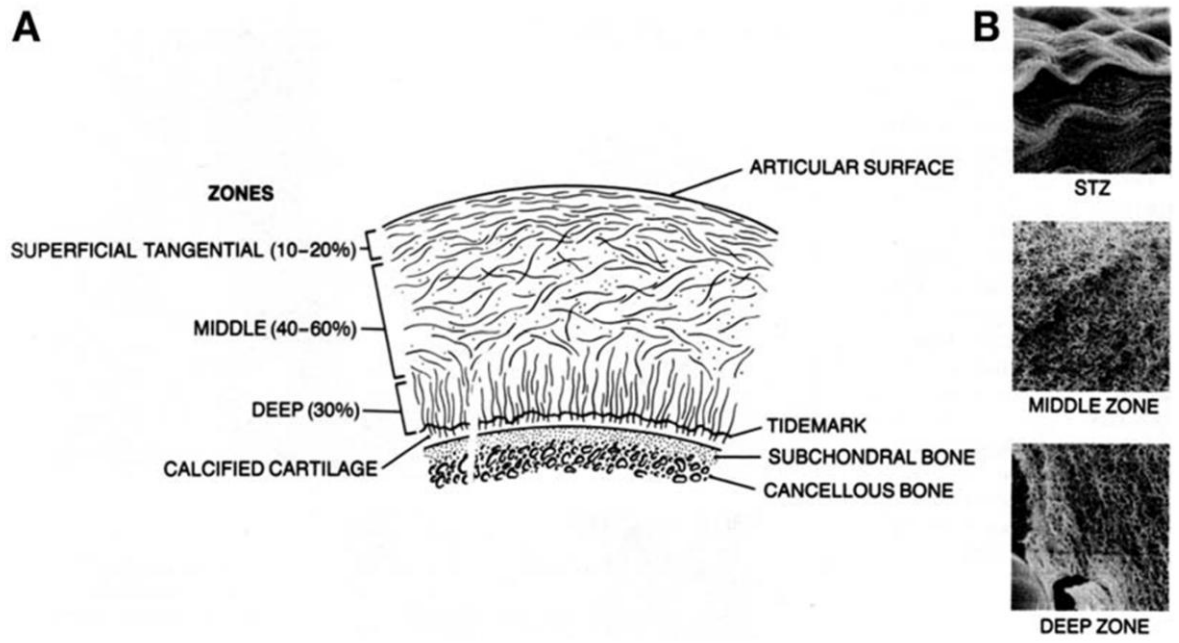

Figure 6 Schematic (A) and scanning electron micrographs (B) of the organization of articular cartilage. In the superficial tangential zone (STZ), collagen fibrils lie nearly parallel to the surface. In the middle zone, they assume a more random alignment. In the deep zone, they lie nearly perpendicular to the articular surface (Flik, 2007).

1.2.2 Repair capacity of articular cartilage

The repair capacity of articular cartilage is very limited (Buckwalter, J. A. & Mankin, H. J., 1998). Articular cartilage defects caused by trauma or disease such as osteoarthritis are not repaired properly. The low cell density within cartilage and the low proliferative activity reduces the likelihood of local chondrocytes to contribute to self-regeneration. A defect that is constricted to the cartilage layer fails to heal spontaneously (Kim, H. K. *et al.*, 1991). Resident articular chondrocytes do not migrate to the lesion and no production of reparative matrix occurs. However, if the defect penetrates the underlying layer of subchondral bone, a limited spontaneous repair occurs. Damage to the subchondral bone results in migration of mesenchymal stem and progenitor cells to the injured site and promotes cartilage repair. However, this process normally leads to the formation of less durable fibrocartilage rather than hyaline cartilage (Steadman, J. R. *et al.*, 2001).

1.2.3 Current treatment strategies for the repair of articular cartilage lesions

There are different treatment strategies for the repair of articular cartilage defects that are currently in clinical use (Figure 7).

Bone marrow stimulation

The principle of this technique is to stimulate a spontaneous repair reaction by mechanical penetration of the subchondral bone. Penetration of the subchondral bone plate disrupts the subchondral blood vessels. This leads to the formation of a fibrin clot that fills the bone defect and covers the surface of the chondral defect. Mesenchymal stem cells migrate into the clot, proliferate and form fibrocartilage.

Different modifications among this technique include subchondral drilling and microfracture. The microfracture technique was elaborated by Steadman (Steadman, J. R. *et al.*, 1999) and is now a days the most common technique. Very small micro-wholes are generated across the articular cartilage lesion at a distance 3-4 mm apart and down to a depth of 4 mm. This technique leads to the formation of a fibrocartilage repair tissue, that is inferior to normal hyaline cartilage in terms of mechanical properties (Buckwalter, J. A. & Mankin, H. J., 1998; Hunziker, E. B., 2002). However, especially in young patients good clinical results with improved joint functionality and relief from pain have been reported (Blevins, F. T. *et al.*, 1998; Sledge, S. L., 2001). This technique is limited to small sized focal cartilage lesions.

Mosaicplasty (Osteochondral grafts)

This technique was first described in 1993 and since then widely used for the treatment of chondral and osteochondral defects (Matsusue, Y. *et al.*, 1993). Cylindrical osteochondral grafts are removed from a non-affected, low-weight-bearing area and transplanted into cylindrical wholes that are prepared at the site of the cartilage defect. Good clinical results were described by different studies (Matsusue, Y. *et al.*, 1993; Jakob, R. P. *et al.*, 2002; Hangody, L. & Fules, P., 2003; Chow, J. C. *et al.*, 2004). However, removing osteochondral grafts from normal sites of a joint leads to the destruction of healthy tissue and is a major limitation for this technique. Mosaicplasty is used in small to medium sized focal defects.

Autologous chondrocyte transplantation (ACT)

The autologous chondrocyte transplantation (ACT) technique was introduced in 1987. Since then this technique has been widely performed, especially for the treatment of large chondral defects (>4 cm^2). ACT is based on the implantation of a suspension of cultured autologous chondrocytes beneath a tightly sealed periosteal flap. In the first step of the procedure a small biopsy of articular cartilage is harvested from a healthy, non-affected and minor-weight-bearing area of cartilage. The chondrocytes are released from the tissue with enzymatic digestion of the ECM and cultured *in vitro* to increase the number of cells in order to provide a sufficient number to fill a focal defect of articular cartilage. After the culture period of about 2 to 3 weeks the chondrocytes are implanted into the cartilage lesion and coved by a periosteal tissue flap. After implantation, the chondrocytes begin to produce a cartilagous matrix that gradually fills out the cartilage defect (Dell'Accio, F. *et al.*, 2003). ACT has demonstrated significant and durable benefits for the patients in terms of improved function and diminished pain with the formation of a hyaline or hyaline-like repair tissue (Brittberg, M. *et al.*, 1994; Peterson, L. *et al.*, 2000; Peterson, L. *et al.*, 2002; Peterson, L. *et al.*, 2003). However, adverse events like periosteal hypertrophy and periosteal graft delamination have been reported after classical ACT (Peterson, L. *et al.*, 2000; Marlovits, S. *et al.*, 2006; Wood, J. J. *et al.*, 2006).

Therefore modern modifications of this technique were developed to overcome these problems. In the matrix-associated autologous chondrocyte transplantation (MACT) chondrocytes are imbedded in a three-dimensional matrix and then transplanted into cartilage defects (Minas, T. & Peterson, L., 1999; Marlovits, S. *et al.*, 2006). The matrices with seeded cells are trimmed to exactly match the defect size and are implanted into the defect without the use of periosteal covers. Different biomaterials for the MACT have been tested. Among them collagen scaffolds, hyaluronan-based biodegradable polymer scaffolds (Pavesio, A. *et al.*, 2003; Marcacci, M. *et al.*, 2005) and polymers of poly-actin and poly-glactin (Erggelet, C. *et al.*, 2003).

However, there are several limitations for the use of this technique. The limited proliferative capacity of differentiated chondrocytes poses a major problem in providing adequate cell numbers for the transplantation therapy (Dozin, B. *et al.*, 2002), ex vivo monolayer expansion before transplantation leads to rapid cell

dedifferentiation and conversion into fibroblasts-like cells (Schnabel, M. *et al.*, 2002; Thirion, S. & Berenbaum, F., 2004). In addition removal of articular chondrocytes covers a risk of morbidity at the donor site.

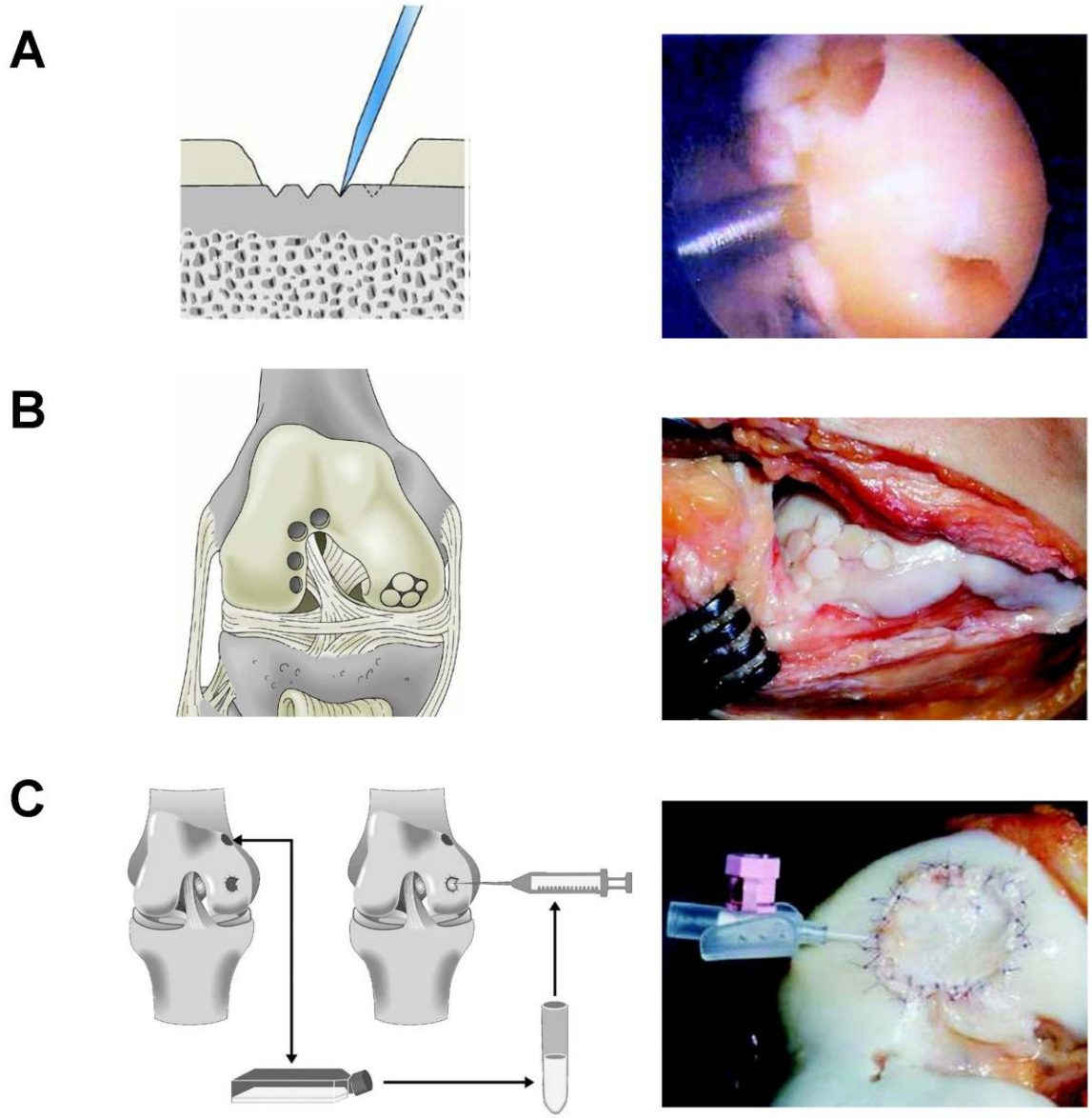

Figure 7 Cartilage repair techniques. A Microfracture (Schewe, 2008) **B** Mosaicplasty (Schewe, 2008) **C** Autologous chondrocyte transplantation (ACT) (Marlovits, S. *et al.*, 2006).

Mesenchymal stem cells for the repair of cartilage lesions

The discovery of mesenchymal stem cells (MSCs) and their potential to differentiate into cells of the chondrogenic line has opened new potential therapeutic approaches for the repair of articular cartilage lesions. The research focus in cartilage repair is

currently shifting from using autologous chondrocytes towards the utilization of MSCs. MSCs are relatively easy to isolate from different tissues in high quantities, MSCs are highly proliferative and can be extensively expanded in an undifferentiated status *in vitro*. MSCs have been transplanted with a view to inducing articular cartilage repair in various animal models with promising results (Wakitani, S. *et al.*, 1994; Kadiyala, S. *et al.*, 1997; Im, G. I. *et al.*, 2001). In humans there are only limited *in vivo* studies at present. Wakitani et al reported a clinical improvement after MSC transplantation and the defect was repaired with fibrocartilage and partly hyaline like cartilage (Wakitani, S. *et al.*, 2002). Kudora et al reported that after MSC transplantation the cartilage defect was repaired with hyaline like cartilage tissue and one year after surgery the clinical symptoms had improved significantly (Kuroda, R. *et al.*, 2007).

1.3 Mesenchymal stem cells (MSCs)

MSCs can be isolated from various tissues of the adult body including bone marrow (Pittenger, M. F. *et al.*, 1999), adipose tissue (Zuk, P. A. *et al.*, 2001), blood (Zvaifler, N. J. *et al.*, 2000) and muscle (Bosch, P. *et al.*, 2000). The most prevalent method is the isolation of MSCs from bone marrow aspirates of the iliac crest. MSCs can be cultured *in vitro* in an undifferentiated state and exhibit high proliferative activity (Prockop, D. J. *et al.*, 2001). MSCs are multipotent meaning they have the ability to differentiate into a variety of different mesenchymal tissues. Dependent on the culture conditions and on the growth factors added to the medium, MSCs are able to differentiate into bone, cartilage, tendon, muscle, fat, dermis and other connective tissues (Prockop, D. J., 1997; Pittenger, M. F. *et al.*, 1999) (Figure 8).

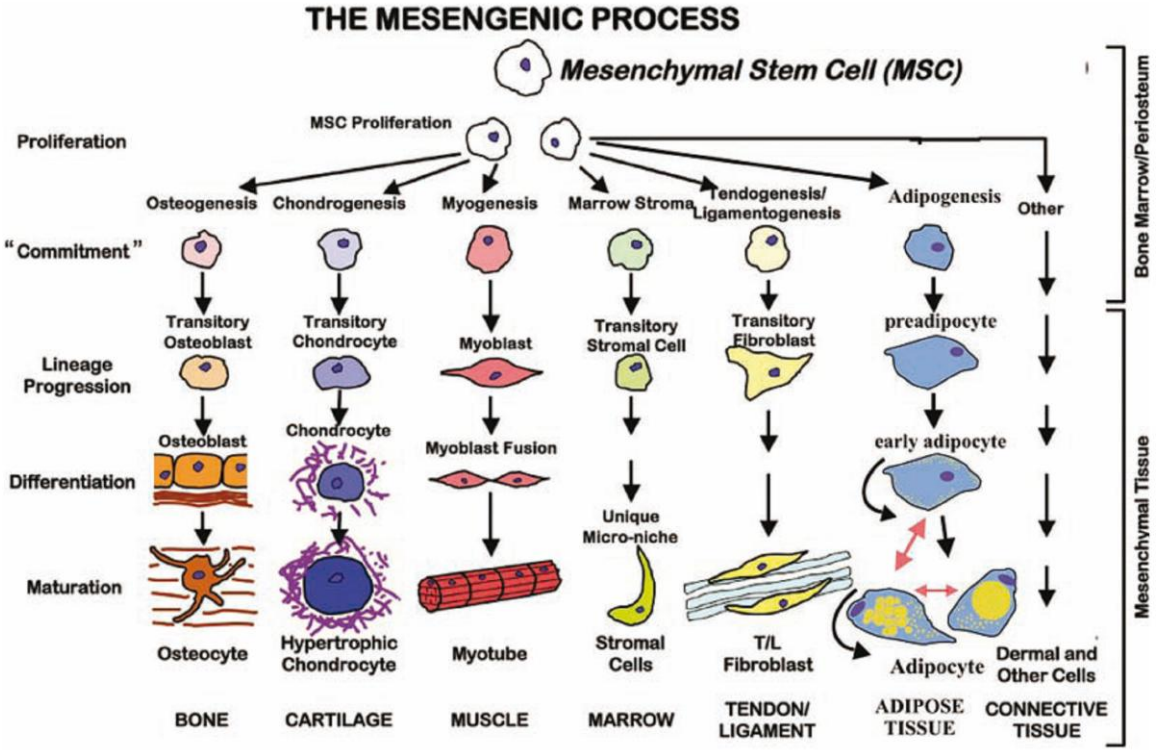

Figure 8 Mesenchymal stem cell differentiation. MSCs are able to differentiate into cell types of different tissues including bone, cartilage, muscle, marrow and adipose tissue. (Bonfield, T. L. & Caplan, A. I., 2010)

1.3.1 Chondrogenesis of MSCs

The chondrogenic potential of MSCs was first described by Owen (Ashton, B. A. *et al.*, 1980) and has been characterized in different matrix based and matrix free systems (Johnstone, B. *et al.*, 1998; Mackay, A. M. *et al.*, 1998; Yoo, J. U. *et al.*, 1998; Barry, F. *et al.*, 2001; Noth, U. *et al.*, 2002; Sekiya, I. *et al.*, 2002; Song, L. *et al.*, 2004; Ichinose, S. *et al.*, 2005). Johnstone established the classical *in vitro* chondrogenesis model for rabbit bone marrow derived MSCs (Johnstone, B. *et al.*, 1998). In this model, MSCs differentiate chondrogenically in a pellet culture system in a serum free, strictly defined chondrogenic medium containing TGFß, dexamethasone, ascorbate, pyruvate, proline and ITS. Based on this model an *in vitro* chondrogenesis model for human MSCs was developed using similar medium conditions (Yoo, J. U. *et al.*, 1998). Under these conditions, MSC pellets develop a cartilage phenotype, characterized by gene expression and synthesis of collagen

type II, collagen type XI, collagen type IX, aggrecan and other chondrogenic specific markers (Johnstone, B. *et al.*, 1998; Yoo, J. U. *et al.*, 1998; Pittenger, M. F. *et al.*, 1999; Barry, F. *et al.*, 2001; Winter, A. *et al.*, 2003).

1.3.2 Regulation of MSC chondrogenesis

TGFß1, 2 and 3 are the classical and well established inducers of MSC chondrogenesis (Mackay, A. M. *et al.*, 1998; Barry, F. *et al.*, 2001). Although other inducers of chondrogenesis such as BMP2 have been described (Schmitt, B. *et al.*, 2003), this has not been confirmed by other investigators (Winter, A. *et al.*, 2003; Indrawattana, N. *et al.*, 2004). FGF2 and IGF promote chondroprogenitor proliferation and chondrogenic potential (Mastrogiacomo, M. *et al.*, 2001; Solchaga, L. A. *et al.*, 2010). In addition, environmental factors like mechanical stimulation and hypoxia have been reported to modulate *in vitro* chondrogenesis of MSCs (Khan, W. S. *et al.*, 2010; Potier, E. *et al.*, 2010).

1.3.3 Hypertrophy in chondrogenic differentiating MSCs

Chondrogenic induction of MSCs in pellet culture is accompanied by the up-regulation of hypertrophy markers like collagen type X and MMP-13 and the activation of ALP activity (Johnstone, B. *et al.*, 1998; Yoo, J. U. *et al.*, 1998; Barry, F. *et al.*, 2001; Winter, A. *et al.*, 2003; Mwale, F. *et al.*, 2006; Mueller, M. B. & Tuan, R. S., 2008; Mueller, M. B. *et al.*, 2010). Expression of collagen type X can be detected very early after chondrogenic induction, within 1-2 days, and before collagen type II expression is detectable. Furthermore *ex vivo* transplantation of human chondrogenic MSC pellet cultures in mice results in induction of hypertrophy, vascular invasion and extensive matrix calcification (Pelttari, K. *et al.*, 2006; Scotti, C. *et al.*, 2010). These data suggest that cultured chondrogenic differentiating MSC undergo premature hypertrophy and develop into transient endochondral cartilage instead of stable articular cartilage. This indicates that the developmental program of cultured MSCs resembles that of growth plate chondrocytes rather than that of articular chondrocytes. In fact, implanted human articular chondrocytes showed no signs of hypertrophy and terminal differentiation (Pelttari, K. *et al.*, 2006) indicating that articular chondrocytes are not prone to hypertrophic differentiation and acquire an intrinsic and stable arrest before hypertrophy.

A recent study from Mueller and Tuan further illustrates the similarity between chondrogenic differentiating MSCs and growth plate chondrocytes by demonstrated that chondrogenic differentiating MSCs undergo a differentiation program which is analogous to that observed during endochondral embryonic skeletal development (Mueller, M. B. & Tuan, R. S., 2008). Genes important for chondrogenic differentiation and hypertrophy are similarly regulated in MSCs undergoing chondrogenic differentiation and growth plate chondrocytes during endochondral ossification. Furthermore, the response of chondrogenic differentiating MSCs to changing medium conditions is very similar to that of growth plate chondrocytes. Thyroid hormone induces hypertrophy while TGFß and dexamethasone inhibit hypertrophy (Ballock, R. T. *et al.*, 1993; Leboy, P. S. *et al.*, 1997; Mackay, A. M. *et al.*, 1998; Mello, M. A. & Tuan, R. S., 2006; Mueller, M. B. & Tuan, R. S., 2008).

In order to study the regulation of MSC hypertrophy, an *in vitro* hypertrophy model for MSCs was established (Mackay, A. M. *et al.*, 1998; Mueller, M. B. & Tuan, R. S., 2008). In this model hypertrophy of MSCs can be enhanced by withdrawal of TGFß and dexamethasone and the addition of the thyroid hormone T3 (Figure 9). Under these pro-hypertrophic conditions hypertrophy associated markers like collagen type X, MMP-13 and osteocalcin are up-regulated and ALP activity is increased compared to standard chondrogenic conditions (Mueller, M. B. & Tuan, R. S., 2008).

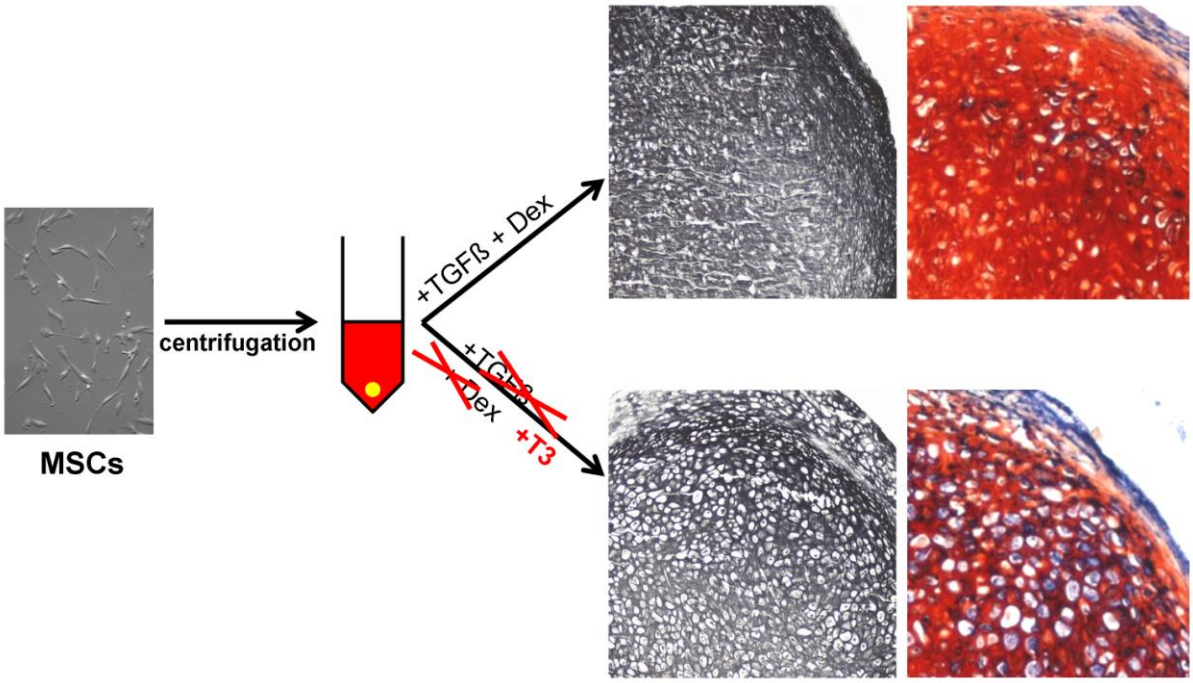

Figure 9 *In vitro* hypertrophy model. The hypertrophic phenotype of chondrogenic differentiating MSCs can be enhanced by the deprivation of TGFß and dexamethasone (Dex) and the addition of T3.

2 Aim of the study

Mesechymal stem cells (MSCs) are a promising cell source for the regeneration of articular cartilage lesions. MSCs are able to differentiate chondrogenically, however, common *in vitro* protocols for chondrogenesis of MSCs induce an inadequate, hypertrophic differentiation cascade that resembles the developmental program of growth plate chondrocytes during endochondral ossification. This biological behaviour raises concern for a tissue engineering application of MSCs in articular cartilage repair. For the use of MSCs in cartilage repair, it is imperative to improve *in vitro* chondrogenesis protocols so that permanent chondrocyte differentiation can be induced in the absence of hypertrophy. Therefore it is important to better understand the regulation of MSC hypertrophy and find ways to inhibit it. On the other hand, the tendency of chondrogenic differentiating MSCs to become hypertrophic might be a desirable trait for bone tissue engineering applications. Mechanisms that support hypertrophy of MSCs would be favourable for the use of MSCs in bone repair. The similarity of MSC chondrogenesis and embryonic endochondral ossification suggests that similar mechanisms are involved in the regulation of hypertrophy in both biological processes.

Form studies with growth plate chondrocytes it is known that BMP and TGFß signalling regulate terminal differentiation contrarily. BMP signalling promotes and TGFß signalling inhibits chondrocyte hypertrophy during endochondral ossification.

The goal of this thesis was to investigate whether BMP and TGFß signalling are involved in the regulation of MSC hypertrophy. We hypothesize, that similar to embryonic chondrocytes, BMP signalling promotes hypertrophy and TGFß signalling inhibits hypertrophy (Figure 10). In order to investigate this we used an *in vitro* hypertrophy model for chondrogenic differentiating MSCs in which the hypertrophic phenotype can be strongly enhanced by modulations in the medium conditions.

Differential expression analysis of BMP and TGFß signalling associated genes including ligands, receptors, transcription factors between chondrogenic and hypertrophic MSC cultures was carried out. BMP and TGFß signalling activity between chondrogenic and hypertrophic MSC cultures was compared. Functional experiments with modulations of BMP and TGFß activity were carried out.

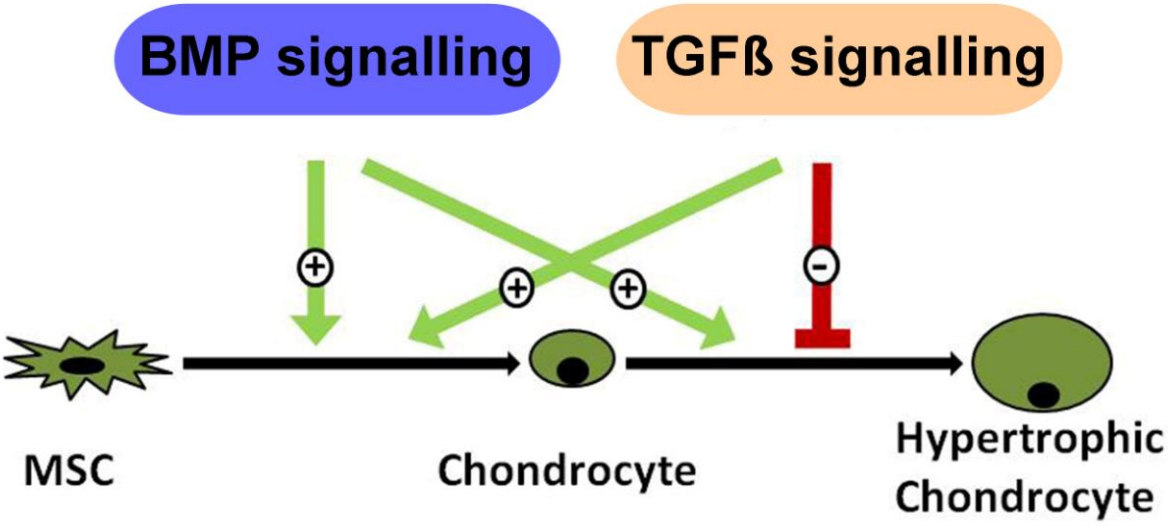

Figure 10 Hypothesis: BMP and TGFß signalling promote early chondrogenic differentiation stages of MSCs while terminal differentiation is positively regulated by BMP signalling and negatively regulated by TGFß signalling.

3 Material and Methods

3.1 Material

3.1.1 Recombinant Proteins and Inhibitors

chemical	application	concentration	company
BMP4	BMP ligand	100 ng/ml	R&D systems
dorsomorphin	Inhibitor of BMP signalling: selective inhibitor of BMP type I receptor	0,5 µM, 3 µM, 10 µM	Sigma Aldrich
noggin	Inhibitor of BMP signalling: antagonist of BMPs	10 ng/ml, 100 ng/ml	R&D systems
SB431542	Inhibitor of TGFß signalling: selective inhibitor of TGFß type I receptor	0,5 µM, 3 µM, 10 µM	Tocris
TGFß1	TGFß ligand	10 ng/ml	R&D systems

Table 1 Recombinant Proteins and Inhibitors

3.1.2 Primers

Primers were synthesized by eurofins, purified and lyophilized. Stock solutions of 100 µM (100 pmol/µl) were prepared and stored at -80°C. The following primers were used.

gene	sequence (forward)	sequence (reverse)	concen-tration
Alk2	GCCTGGAGCATTGGTAAGC	CTGCCCACAGTCCTTCAAG	150 nM
BAMBI	CGATGTTCTCTCTCCTCCCAG	AATCAGCCCTCCAGCAATGG	150 nM

BMP2	TGGATTCGTGGTGGAAG TGGC	AGGGCATTCTCCGTGGC AGTA	200 nM
BMP4	CAAACTTGCTGGAAAGG CTC	CCGCTACTGCAGGGACC TAT	200 nM
BMP7	CCCAGTGTTTACCGAGGT TTGC	TCCATCCTACTTGCTGTC CTTGTC	200 nM
BMPR1A	AAACCACTTCCAGCCCTA C	TTTGACACACACAACCTC AC	200 nM
BMPR1B	CCCTGCATTTGGGGCCG CTAT	GCCTGAAGCTGCAAAAG GCCAC	200 nM
BMPR2	CCAAGGTCTTGCTGATAC GG	CTACCATGGACCATCCTG CT	200 nM
Chordin	CTGCAGCAGCATATGAG CC	GATCAGCGCACTGTCCT GT	200 nM
Collagen type II	GGGCAATAGCAGGTTCA CGTA	TGTTTCGTGCAGCCATCC T	200 nM
Collagen type X	CCCTCTTGTTAGTGCCAA CC	AGATTCCAGTCCTTGGGT CA	200 nM
Follistatin	TCCTTGCTCAGTTCGGTC TT	TCTGCCAGTTCATGGAG GA	200 nM
Gremlin	CAGGGAAAGCTTCCAGA ACA	CCCGCTGTGGACTAGAG AAC	200 nM
HPRT	CGAGATGTGATGAAGGA GATGG	GCAGGTCAGCAAAGAATT TATAGC	150 nM
Noggin	CATGAAGCCTGGGTCGT AGT	TGGAACACCCAGACCCT ATC	200 nM
Osteocalcin	AGGAGGGCAGCGAGGTA G	GAAAGCCGATGTGGTCA GC	100 nM
Runx2	ATACCGAGTGACTTTAGG GATGC	AGTGAGGGTGGAGGGAA GAAG	200 nM
Sox9	ACACACAGCTCACTCGAC CTTG	AGGGAATTCTGGTTGGTC CTCT	200 nM
TGFß1	ACATCAACGGGTTCACTA C	CTGAAGCAATAGTTGGTG TCC	200 nM
TGFß3	ACACACAAGCAACAAACC TCAC	AACCAAACCCACACTTTC TTTACC	200 nM
TGFßR1	GAACCTGCTCTCCTGCTT G	GCCTCATCTGCTCAATCT CC	200 nM

TGFßR2	GGAAACTTGACTGCACC GTT	CTGCACATCGTCCTGTG G	200 nM

Table 2 List of Primers

3.1.3 Antibodies

Primary antibodies		
Antibody	**dilution**	**company**
BAMBI (mouse)	1:1000 (WB) 1:10 (IH)	eBioscience
Sox9 (mouse)	1:500	Millipore
Smad 1 (rabbit)	1:1000	Cell signalling
phospho-Smad 1,5 (rabbit)	1:500	Cell signalling
Smad 2 (rabbit)	1:1000	Cell signalling
phospho-Smad 2 (rabbit)	1:1000	Cell signalling
Smad 3 (rabbit)	1:1000	Cell signalling
phospho-Smad 3 (rabbit)	1:1000	Cell signalling
TGFßR1 (rabbit)	1:500	Abcam
ß-Actin (rabbit)	1:10000	Abcam
BMP4 (rabbit)	1:250	Abcam
Collagen type II (mouse)	1:100	Calbiochem
Collagen type X (mouse)	1:20	Quartett Immunodiagnostica
Secondary antibodies		
goat-anti-mouse (biotinylated)	1:100	Dianova
goat-anti-rabbit (biotinylated)	1:100	Dianova
goat-anti-mouse (HRP- conjugated)	1:1000	Pierce
goat-anti-rabbit (HRP-conjugated)	1:1000	Pierce

Table 3 List of antibodies

3.1.4 Kits

name	application	company
Alkaline Phosphatase Kit	ALP staining	Sigma Aldrich
Brilliant SYBR Green QPCR Master Mix	qPCR	Agilent Technologies
DC Protein Assay	Protein concentration	Biorad
DNA-free DNase treatment and removal	DNA digestion	Ambion by life technologies
ECL western kit	Western Blot	pierce
Re-blot Plus	Stripping of Western Blot membranes	Millipore
RNeasy Mini Kit	RNA clean up, DNA digestion	quiagen
Transcriptor First Strand cDNA Synthesis	cDNA synthesis	Roche

Table 4 List of Kits

3.1.5 Buffers and solutions

Running Buffer

50 mM MOPS

50 mM Tris base

0,1 % SDS

1 mM EDTA

Transfer Buffer

25 mM Bicine

25 mM Bis tris free base

1 mM EDTA

20 % methanol

Tris 0,2 M (ph 7,0)

24,2 g Tris base

add H_2O to 1l

Washing Buffer

780 ml Tris 0,2 M (ph 7,0)

25 g NaCl

add H_2O to 3 l

Blocking Buffer

120 ml Tris 0,2 M (ph 7,0)

2,4 g NaCl

60 ml Triton X100

1% FCS

1% goat serum

add H_2O to 250 ml

PBS

137 mM NaCl

2,7 mM KCl

10 mM Na_2HPO_4 x 2 H_2O

2 mM KH_2PO

Phosphate-Buffer (0.2 M)

29,7 g Na_2HPO_4

4,6 g NaH_2PO_4

add H_2O to 1 l

TBS

25 mM Tris/HCl (pH 7,5)

150 mM NaCl

TBST

0,1 % Tween 20 in TBS

ALP Buffer

1,5 M Tris (ph 9,0)

1 mM MgCl

1 mM ZnCl

3.1.6 Cells

Human mesenchmyal stem cells (MSCs) derived from the iliac crest of male patients aged 21 to 42 (n=10) were used for the experiments.

3.1.7 Cell culture media

Proliferation medium:

Dulbecco's modified Eagle's medium (DMEM) low glucose (Invitrogen) with 10 % fetal calf serum (PAN Biotech GmbH) and 1 % penicillin/streptomycin (Invitrogen).

Differentiation medium:

Chondrogenic medium: DMEM high glucose (Invitrogen), 1 % ITS+3 (Sigma Aldrich), 50 µg/ml ascorbate-2-phosphate (Sigma Aldrich), 40 µg/ml L-proline (Sigma Aldrich), 100 nM dexamethasone (Sigma Aldrich), 1 mM sodium pyruvate (Sigma Aldrich) and 10 ng/ml TGFß1 (R&D Systems).

Hypertrophy enhancing medium: DMEM high glucose, 1 % ITS+3, 50 µg/ml ascorbate-2-phosphate, 40 µg/ml L-proline, 1 nM triiodothyronine (T3) (Sigma Aldrich).

3.2 Methods

3.2.1 Cell culture

3.2.1.1 Isolation of MSCs

Human MSCs were isolated from iliac crest bone marrow aspirates of male patients undergoing surgery that required autologous bone grafting with approval of the ethics committee of the University of Regensburg and written consent. MSCs were isolated by Ficoll gradient centrifugation as described previously (Haynesworth et al, 1992). Therefore bone marrow was mixed 1:5 with proliferation medium (as indicated in 3.1.7). Afterwards the bone marrow medium mixture was carefully

layered onto a Ficoll (Biochrom) cushion in a 50 ml Falcon tube. The Falcon tube was then centrifuged for 35 minutes at 1680 U/min. The nucleated cell fraction, in which MSCs are located, was collected from the 1,077 g/ml density interface and mixed with fresh proliferation medium. After a further centrifugation step (10 min, 1000 U/min), the pellet was resuspended in fresh medium, the cell number was determined and the cells were plated at a density of 2 million cells per 75 cm^2 tissue culture flasks.

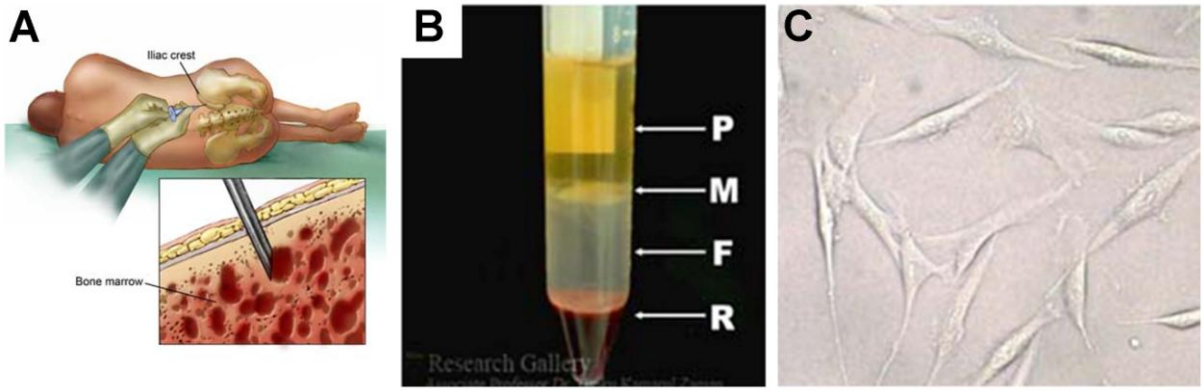

Figure 11 Isolation of MSCs. A Bone marrow aspiration from the iliac crest. (http://www.mayoclinic.com/images/image_popup/r7_bonemarrowaspiration.jpg) **B** Ficoll gradient (P=plasma, M=mononuclear cells, F=ficoll, R=erythrocytes, thrombocytes). MSCs are located in the mononuclear layer. (www.umfacts.um.edu.my). **C** Monolayer culture of MSCs

3.2.1.2 Expansion of MSCs

MSCs were cultured as monolayer in 15 ml proliferation medium in 75 cm^2 tissue culture flasks. Cells were maintained at 37 °C in a humified atmosphere containing 5 % CO_2. Medium changes were performed every three to four days and at 80 % confluence cells were trypsinized and frozen for later use in liquid nitrogen.

3.2.1.3 Chondrogenic differentiation and enhancement of hypertrophy

For chondrogenic differentiation of the MSCs, the *in vitro* chondrogenesis model established by Johnstone et al (Johnstone, B. *et al.*, 1998) was used. MSCs of passage two were used for the experiments.

MSCs were thawed, seeded in 75 cm^2 tissue flasks and expanded in proliferation medium until confluence was reached. Cells were washed with sterile PBS (PAA), 3 ml trypsin (PAN Biotech GmbH) was added and tissue flasks were softly shacked,

until the cells detached from the surface. Cells were resuspended in fresh proliferation medium to inactivate the trypsin and then centrifuged for 5 minutes at 1000 U/min. The pellet was resuspended in DMEM high glucose medium, cell number was determined and 200000 cells per well were seeded in V-bottomed 96-well polypropylene plates. Pellets were formed by centrifugation at 250 *g* for 5 minutes and chondrogenically differentiated in chondrogenic medium (as indicated in 3.1.7) for 14 days. After the chondrogenic pre-differentiation phase, pellets either stayed in chondrogenic medium for another 14 days or were transferred to hypertrophy enhancing medium (as indicated in 3.1.7).

MSC pellets were maintained at 37 °C in a humified atmosphere containing 5 % CO_2. Medium was changed three times per week. MSC pellets were isolated at different time points (as indicated in the respective section), characterized histologically, histochemically and immunohistochemically and gene expression and protein expression were analyzed.

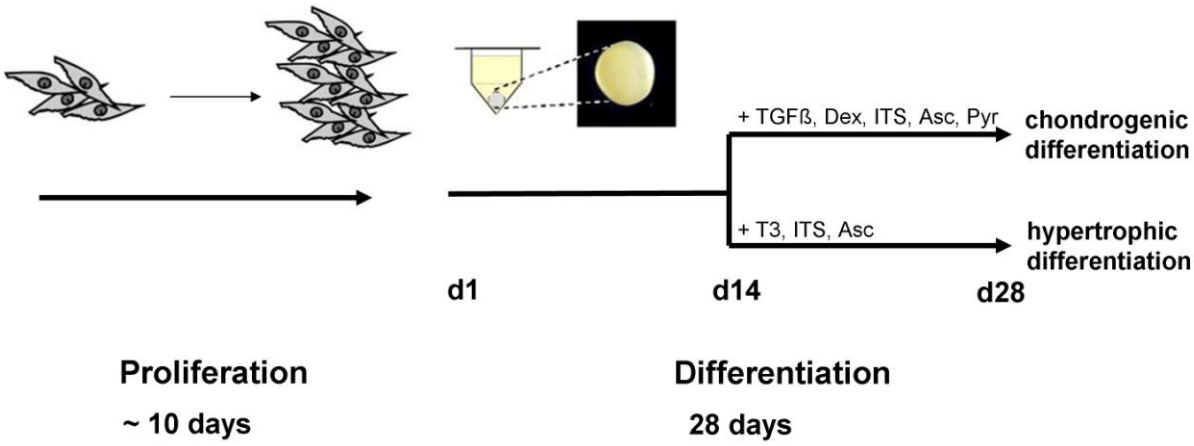

Figure 12 Paradigm. For expansion of MSCs, cells were kept in proliferation medium for about 10 days. Then cells were transferred to pellet culture system and pre-differentated in chondrogenic medium for 14 days. Afterwards pellets either stayed in chondrogenic medium or were transferred to hypertrophic medium for additional 14 days.

3.2.1.4 Modulation of hypertrophy

Modulation of hypertrophy by BMP4

MSC aggregates were formed as described and were kept in chondrogenic medium for 14 days. Then aggregates were distributed in five different groups: 1. standard chondrogenic medium; 2. chondrogenic medium with BMP4, 3. standard

hypertrophy enhancing medium including T3; 4. hypertrophy enhancing medium without T3; 5. hypertrophy enhancing medium with BMP4 instead of T3. (BMP4 was used in the concentration as described in table 1).

Aggregates for gene expression analysis were harvested on day 21 and day 28. For histological analysis aggregates were harvested on day 28 and medium supernatant was isolated on day 28 for ALP activity test.

Modulation of hypertrophy by BMP and TGFß inhibitors

MSC aggregates were formed as described and were kept in chondrogenic medium for 14 days. On day 14, aggregates were either kept in chondrogenic medium with noggin, dorsomorphin or SB431542 or were transferred to hypertrophic medium with noggin, dorsomorphin or SB431542 for additional 14 days (inhibitors were used in the concentrations as described in table 1). MSC pellets that were kept in standard chondrogenic and hypertrophic medium were taken as control.

Aggregates were harvested for gene expression analysis on day 21 and day 28 and for histological analysis on day 28. Culture medium was collected on day 28 for determination of ALP activity.

In a separate approach MSC pellets were treated with noggin or dorsomorphin over the whole culture period, from day 1 until day 28, or treated with dorsomorphin from day 1 until day 14. MSC pellets were harvested on day 28 for histological analysis.

In another experiment MSC pellets were pre-differentiated in chondrogenic medium for 14 days followed by incubation in chondrogenic medium containing BMP4 and SB431542. MSC pellets were harvested on day 14 and day 28 for histological analysis.

The noggin experiment was done in collaboration with a medical PhD student, Norman Olbrich. The results from the PCR, ALP activity and cell size measurement comprise data derived from Norman Olbrich and data from me.

3.2.2 Histology, Histochemistry and Immunohistochemistry

3.2.2.1 Fixation of MSC pellets and preparation of cryo sections

For histological, histochemical and immunohistochemical analysis, MSC pellets were harvested on day 14 and day 28 and fixed in 4 % paraformaldehyde for 1 hour. Afterwards pellets were washed in 0,1 M phosphate buffer for 1 hour followed by incubation in saccherose solutions with increasing concentrations (10 %, 20 %, 30

% saccherose solution in 0,1 M phosphate buffer), each for 1 hour. Then MSC pellets were embedded in TissueTek (Sakura) and frozen in liquid nitrogen. 10 µm thick frozen sections were prepared using the Microm HM 500 OM Cryotom (Microm, Berlin, Deutschland).

3.2.2.2 DMMB staining

DMMB (1,9-dimethylmethylenblue) stains sulphated glycosaminoglycans (GAGs) that are synthesized during chondrogenic differentiation. Cryo sections were incubated in tip water for 5 minutes followed by incubation in 0,1 % DMMB solution (Sigma Aldrich) for 5 to 10 minutes. Then sections were washed in tip water (2x 1 minute) and dehydrated (2x 100 % Propanol; 2x 100 % Xylol, 5 minutes each). Cover slips were mounted on slides with DePex-solution (Serva Electrophoresis GmbH) and afterwards investigated under the microscope.

3.2.2.3 Alkaline phosphatase (ALP) staining

ALP staining was performed using an alkaline phosphatase kit (Sigma Aldrich) according to manufacturer's instructions. Neutral red was used as counterstaining. Cover slips were mounted on slides with 70 % sorbitum solution (Caelo) and afterwards investigated under the microscope.

3.2.2.4 Immunohistochemistry

Sections were incubated in washing buffer for 5 minutes. Afterwards endogen peptidases were blocked in 3 % H_2O_2/10 % Methanol in PBS for 30 minutes. Sections were washed three times in washing buffer, followed by incubated in blocking buffer for 60 minutes at room temperature and incubation in appropriate primary antibody in blocking buffer overnight at room temperature. For collagen type II and type X staining antigen retrieval with pepsin digestion for 15 minutes at room temperature was performed prior to blocking. For collagen type X staining additional hyaluronidase digestion for 60 minutes at room temperature was performed prior to pepsin digestion. Immunolabeling was detected with a biotinylated secondary antibody, horse reddish peroxidase conjugated streptavidin (Vector Laboratories, Burlingame) and metal enhanced diaminobenzidine as substrate (Sigma Aldrich).

3.2.3 Microscopy

The following microscopes were used: Olympus BX 61, Nicon Eclipse TE2000-U.

3.2.4 Histomorphometry

Day 28 aggregates were stained with DMMB and images of three representative central sections were taken in 20 x magnification. The surface area of the cells in the sections was analyzed as correlate of the cell size using the Image J software (NIH, Bethesda, MD). Per condition three aggregates from five different patients were analyzed.

3.2.5 Determination of Alkaline Phosphatase (ALP) activity

ALP activity in the medium was measured densitometrically using the change in the absorbance at 405 nm by the conversion of p-nitrophenyl phosphate to p-nitrophenol and inorganic phosphate as described previously (Weber, M. *et al.*, 2002). Medium supernatant was harvested on day 28 and centrifuged for 5 minutes at maximum speed. 100 µl of supernatant was added to 100 µl substrate solution (4 mg/ml p-nitrophenol phosphate (Sigma Aldrich) in ALP buffer) and continuous absorbance at 405 nm was measured spectrophotometrically in a microplate reader at room temperature (Genius plate reader, Tecan). The change in A_{405} over time (dA/min) was calculated in the linear range of the reaction.

3.2.6 Gene expression analysis

3.2.6.1 RNA isolation and cDNA synthesis

For gene expression analysis RNA was isolated from MSC aggregates at day 1, day 3, day 7, day 14, day 17, day 21 and day 28. 8 to 10 pellets per condition and per time point for each donor were pooled, incubated in 1 ml TRI Reagent (Sigmal Aldrich) and homogenized using the Power Gen 1000 homogenizator (Fischer Scientific). RNA was isolated using standard protocols for Trizol isolation. Briefly, 200 µl Chloroform was added to the lysate and the solution was shacked for 15 seconds, incubated at room temperature for 15 minutes and centrifuged for 15 minutes at 12000 g at 4 °C. The aqueous phase was then transferred to a new tube and precipitated with 400 µl Isopropanol. After centrifugation (12000 g, 10 minutes, 4 °C) the obtained RNA pellet was washed with 75 % Ethanol and centrifuged (7500 g, 8 minutes, 4 °C). RNA pellet was dried for 30 minutes at room temperature and was afterwards dissolved in 20 µl RNase free water. Residual DNA was digested

using the DNA-free DNase treatment & removal kit (ambion by life technologies) according to manufacturer's instructions. RNA concentration was measured using the Picodrop analyzer (Biozym). 1 µg RNA was reverse transcribed in cDNA with the Transcriptor First Strand cDNA Synthesis kit (Roche) according to manufacturer's instructions.

3.2.6.2 Real time polymerase chain reaction (PCR)

Gene expression analysis was performed with semiquantitative real-time PCR using Brilliant SYBR Green QPCR Master Mix (Agilent Technologies) and the CFX96 Real time PCR Detection System (Biorad). The optimal concentration of the primers was tested out for each primer and is listed together with the sequences in table 2. cDNA was diluted to a concentration of 1 ng/µl and the PCR reaction was performed in a final volume of 15 µl containing 7,5 µl Brilliant SYBR Green QPCR mix, 1,5 µl primer mix, 1,5 µl DEPC water and 5 µl cDNA. The following PCR conditions were used. An initial denaturation step at 95°C for 15 minutes was followed by 40 cycles of denaturation at 95°C for 10 seconds and primer annealing and extension at 60°C for 30 seconds. Melt curve analysis was performed by slowly heating the PCR reaction from 55°C to 95°C (0,5°C per cycle) with simultaneous measurement of fluorescent intensity.
Gene expression was normalized to the housekeeping gene, hypoxanthine guanine phosphoribosyltransferase (HPRT).

3.2.7 Protein analysis

3.2.7.1 Western Blot analysis

For Western Blot analysis 5 to 8 MSC pellets per time point and per condition for each patient were pooled, washed in ice cold PBS and homogenized in 500 µl 6 M urea/2 % SDS solution containing protease inhibitor cocktail (Sigma) and phosphatase inhibitor (Sigma) using the Power Gen 1000 homogenizator (Fischer Scientific). The lysate was centrifuged for 5 minutes at 14000 rpm (4 °C) to pellet cellular debris and the supernatant was transferred to a fresh tube. The protein concentration of the supernatant was determined using the BCA Protein Assay kit (Biorad, DC Protein Assay) according to the manufacturer´s instructions.
Lysates were supplemented with 4x LDS sample buffer (Invitrogen) and 10 mM dithiothreitol (DTT) and proteins were denaturated for 5 minutes at 95 °C. For gel

electrophoresis, equal amounts of protein (10 µg) were loaded and separated on a 4-12 % bis tris gel (novex by life technologies) at 120 V. After gel electrophoresis proteins were transferred from the gel to a polyvinylidenfluoride (PVDF) membrane (millipore). Blotting was performed for 2 hours at 100 V. After transfer, membrane was blocked for 1 hour in 5 % skim milk powder in TBST. The membrane was then incubated in primary antibody in 5 % skin milk powder in TBST over night at 4 °C. The next day, the membrane was washed three times for 10 minutes in TBST and afterwards incubated in HPR-coupled secondary antibody (1:1000) in 5 % skin milk powder in TBST at room temperature for 1 hour. The membrane was washed three times in TBST for 10 minutes. Chemoluninescence was detected with the ECL western kit (pierce) and by using x-ray sensitive films (ECL Hyperfilm, Amersham). The films were developed in a photo developer (Curix 60) from AGFA.
Western Blot membranes were stripped using Re-blot Plus (Millipore) according to manufactures instructions.

3.2.8 Microarray

RNA was isolated as described. Afterwards RNA was cleaned up and residual DNA was digested using the RNeasy Mini Kit (quiagen). The samples were analysed by the Kompetenzzentrum für Fluoreszente Bioanalytik Microarray Technology (KFB) using the Affymetrix GeneChip Analysis.

3.2.9 Statistical analysis

The data from the real time-PCR analysis, ALP activity test and cell surface area analysis were expressed as mean values ± standard deviation (SD). Each experiment was carried out using cells of four to seven individual marrow preparations from different donors, as indicated in the respective experiments.
Statistical analysis was carried out by pair-wise comparison using paired, 2-tailed student´s t-test in SigmaStat software (Jandel Scientific, San Rafael, CA). A level of $p<0.05$ was considered significant.

4 Results

4.1 Characterization of MSC pellets under chondrogenic and hypertrophic conditions

Using an *in vitro* hypertrophy model for MSCs we compared MSC pellets that were kept under chondrogenic and hypertrophic conditions histologically and on gene expression level.

4.1.1 Histological analysis

The hypertrophic phenotype of chondrogenic differentiating MSCs is strongly enhanced under hypertrophic medium conditions compared to chondrogenic conditions. Histological analysis of day 28 aggregates revealed that aggregates that were kept under hypertrophy enhancing conditions clearly showed hypertrophic cell morphology with large lacunae typical for hypertrophic cartilage (Figure 13 B) whereas chondrogenic control aggregates showed a more hyaline cartilage-like morphology with little sign of cellular hypertrophy (Figure 13 A). Collagen type II staining is strong both under chondrogenic (Figure 13 C) and hypertrophic conditions (Figure 13 D). In contrast, immunostaining for the hypertrophic marker collagen type X is weak in chondrogenic aggregates (Figure 13 E) but is clearly increased in hypertrophic MSC pellets (Figure 13 F). ALP staining is strong throughout the aggregates under pro-hypertrophic conditions (Figure 13 H) whereas ALP staining is weaker and mainly located in the periphery in chondrogenic aggregates (Figure 13 G).

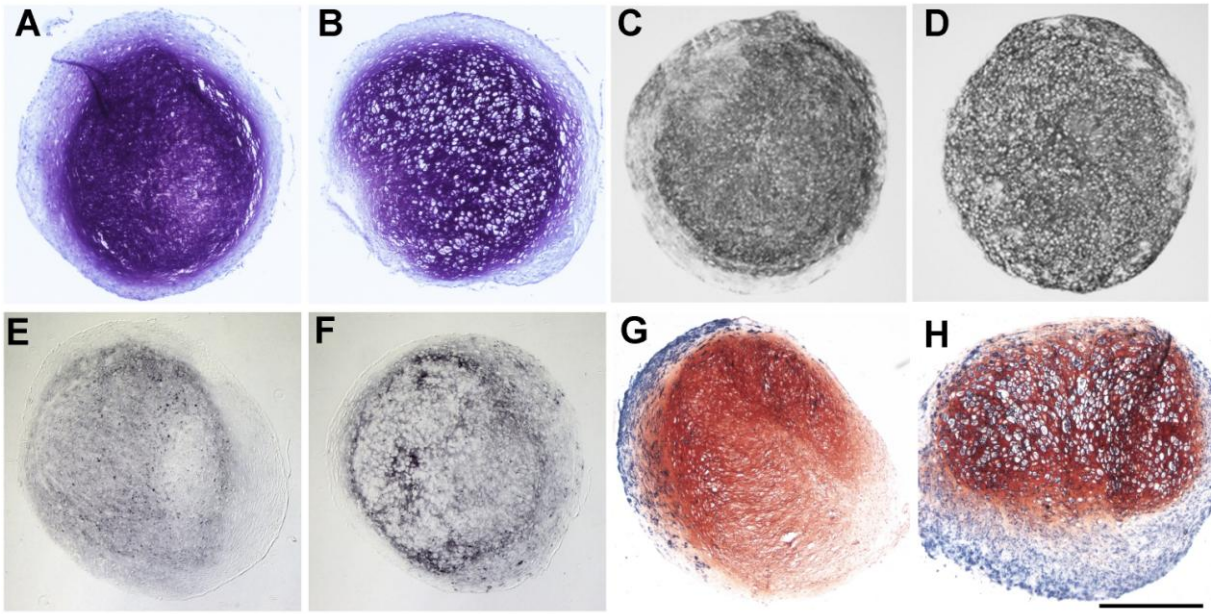

Figure 13 Histological appearance of MSC pellets on culture day 28 under chondrogenic (A, C, E, G) and hypertrophy enhancing conditions (B, D, F, H). **A, B** DMMB staining. **C, D** immunohistochemical collagen type II staining. **E, F** immunohistochemical collagen type X staining. **G, H** alkaline phosphatase (ALP) staining (blue) with neutral red as counterstaining. The hypertrophic phenotype with increased cell volume, collagen type X expression and ALP activity is strongly enhanced under hypertrophy enhancing conditions (**B, D, F, H**) compared to chondrogenic conditions (**A, C, E, G**). Scale bar = 500µm.

4.1.2 Gene expression analysis

Furthermore we analyzed the gene expression of the chondrogenic marker collagen type II and the hypertrophic markers collagen type X and osteocalcin comparatively between chondrogenic and hypertrophic MSC pellets.

Real time PCR analysis revealed that collagen type II expression increases over time both under chondrogenic and hypertrophic conditions. However no significant difference can be detected in the expression of collagen type II between chondrogenic and hypertrophic pellets (Figure 14 A). Collagen type X expression also increases over time but in contrast to the immunostaining for collagen type X, no significant difference in collagen type X expression can be detected in the PCR between chondrogenic and hypertrophic condition (Figure 14 B). MSC pellets that were kept under standard chondrogenic conditions express a high level of the hypertrophic marker collagen type X. Osteocalcin is a bone specific marker that is expressed by osteoblasts but has also been shown to be up-regulated in hypertrophic chondrocytes (Lian, J. B. *et al.*, 1993; Gerstenfeld, L. C. & Shapiro, F.

D., 1996). Gene expression analysis for osteocalcin revealed a significant enhancement in osteocalcin expression under hypertrophic conditions on day 17, day 21 and day 28 compared to chondrogenic conditions (Figure 14 C).

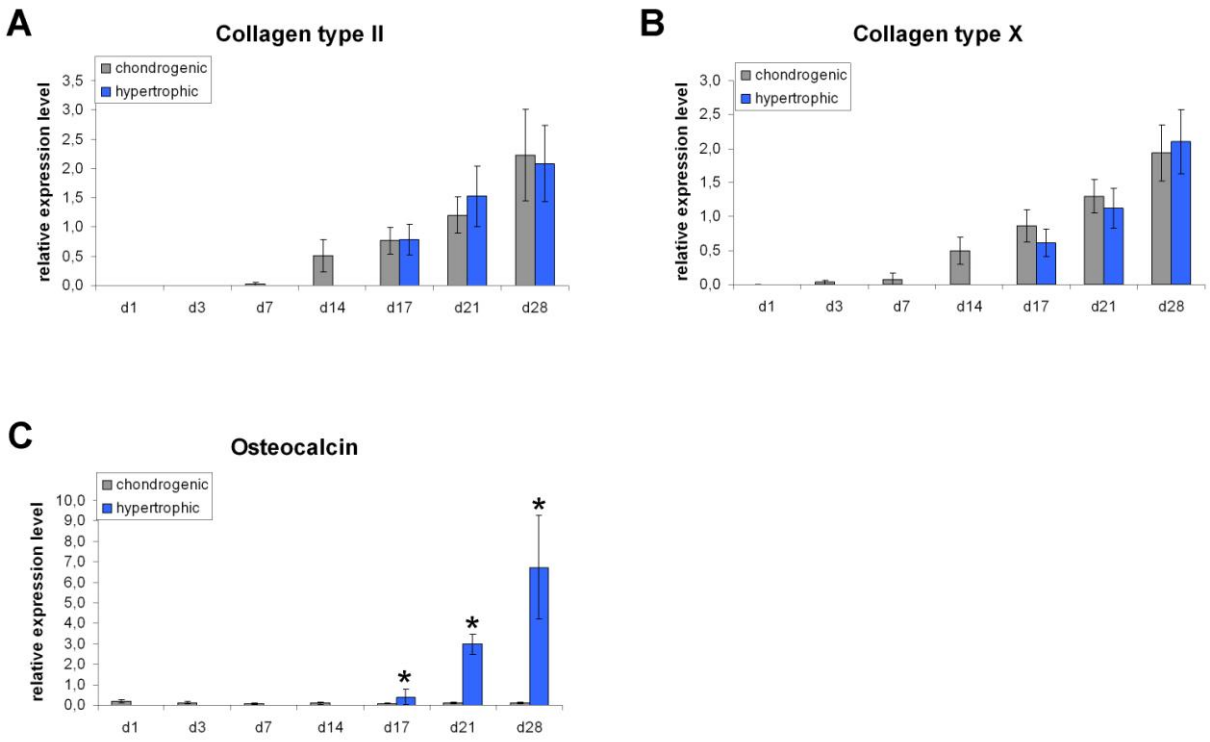

Figure 14 Gene expression analysis of collagen type II, collagen type X and osteocalcin normalized to HPRT in MSC pellet cultures under chondrogenic and hypertrophy enhancing conditions analyzed by real time PCR. Collagen type II and collagen type X expression increases over time with no significant difference between chondrogenic and hypertrophic conditions (**A, B**). Osteocalcin expression is significantly increased under hypertrophic conditions compared to chondrogenic conditions (**C**). n= 7 different donors.

These experiments clearly showed that we can increase hypertrophy of chondrogenic differentiating MSCs by switching medium conditions from chondrogenic to hypertrophic medium.

4.2 The role of BMP signalling in the induction of hypertrophy in chondrogenic differentiating MSCs

BMP signalling has been shown to promote hypertrophy in growth plate chondrocytes (1.1.1.1). In the following part we aimed to investigate whether BMP signalling is also involved in the regulation of MSC hypertrophy.

4.2.1 Regulation of BMP signalling associated genes under hypertrophy enhancing conditions

In order to elucidate a possible role of BMP signalling in the regulation of MSC terminal differentiation we compared temporal expression profiles of genes involved in BMP signalling (ligands, receptors, transcription factors) between chondrogenic and hypertrophic MSC pellets.

Real time PCR analysis showed that BMP4 is significantly up-regulated under hypertrophy enhancing conditions on day 17, day 21 and day 28 as compared to chondrogenic control conditions (Figure 15 A). Differences in BMP2 expression between chondrogenic and hypertrophic conditions were not detected (Figure 15 B). BMP7 expression was too low both in chondrogenic and hypertrophic aggregates to evaluate differences between the two conditions. The BMP receptor 1B (BMPR1B) is significantly up-regulated in the hypertrophic group on day 28 compared to the chondrogenic group (Figure 15 C). All other investigated BMP receptors (BMPR1A, Alk2, BMPR2) were not regulated as a function of hypertrophy induction (data not shown). The BMP signalling associated transcription factor Runx2 is significantly up-regulated on day 17, day 21 and day 28 under pro-hypertrophic conditions (Figure 15 D).

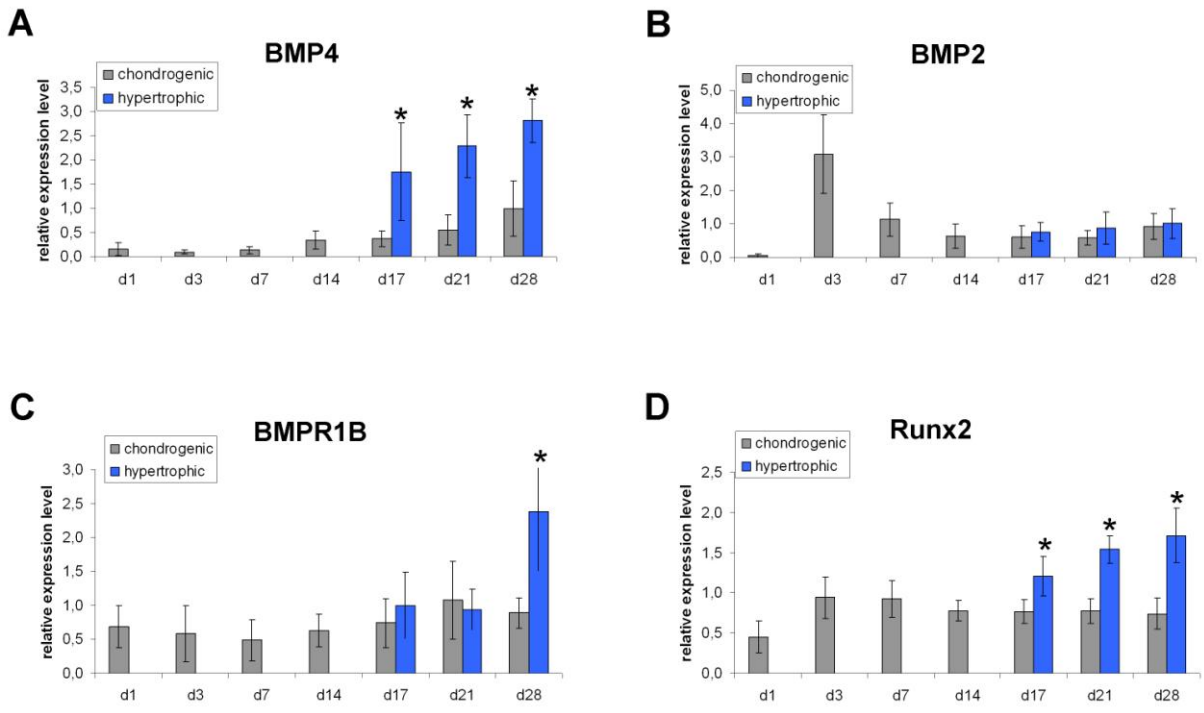

Figure 15 Gene expression analysis of BMP4, BMP2, BMPR1B and Runx2 normalized to HPRT in MSC pellet cultures under chondrogenic and hypertrophy enhancing conditions analyzed by real time PCR. BMP4 and Runx2 are significantly up-regulated on day 17, 21 and 28 under hypertrophic conditions (**A, D**). BMPR1B is up-regulated on day 28 under hypertrophic conditions (**C**) BMP2 is not regulated as a function of hypertrophy (**B**). n = 7 different donors.

On protein level, immunohistochemistry for BMP4 on day 28 showed clearly stronger staining in hypertrophic MSC pellets (Figure 16 B, D) compared to chondrogenic pellets (Figure 16 A, C). BMP4 predominantly accumulates in the membrane and cytoplasm of hypertrophic cells (Figure 16 D), whereas BMP4 staining in chondrogenic aggregates is weak in the membrane and the cytoplasm of chondrogenic cells (Figure 16 C).

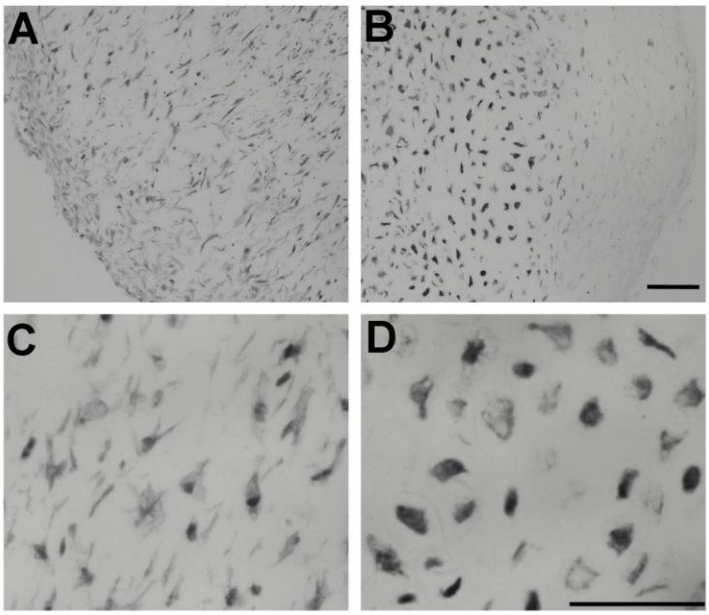

Figure 16 Immunohistochemical BMP4 staining of day 28 MSC pellets. BMP4 protein staining is increased under hypertrophy enhancing conditions (**B, D**) as compared to chondrogenic conditions (**A, C**). Scale bar = 100 µm.

4.2.2 BMP4 induces hypertrophy

The previous results showed that BMP4 expression is strongly enhanced under hypertrophic conditions. Therefore we aimed to analyse the effect of recombinant human BMP4 on chondrogenic differentiating MSCs in the *in vitro* hypertrophy model.

4.2.2.1 Histological analysis

Under standard chondrogenic conditions (chon) with continuous application of TGFß and dexamethasone, BMP4 application (chon+BMP4) did not trigger changes in the phenotype of chondrifying MSCs. Under both conditions, a similar hyaline cartilage-like morphology with little signs of hypertrophy developed (Figure 17 A, B). No difference in ALP staining could be detected between BMP4 treated and chondrogenic control aggregates (Figure 17 C, D). In addition collagen type II and collagen type X immunostaining were unchanged after BMP4 treatment (Figure 17 E, F, G, H).

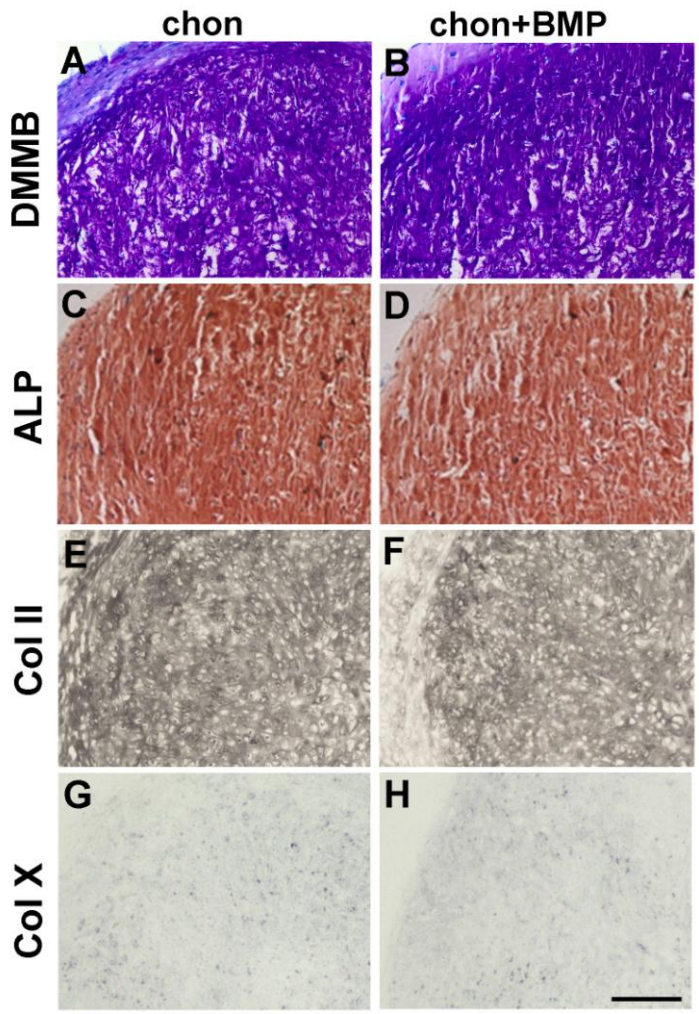

Figure 17 Histological appearance of MSC pellets on day 28 after BMP4 treatment under chondrogenic conditions. **A, C, E, G** chondrogenic control conditions. **B, D, F, H** BMP4 treated conditions. No change in DMMB (**A, B**), ALP (**C, D**), Collagen type II (**E, F**) and collagen type X (**G, H**) staining can be detected between chondrogenic control conditions and BMP4 treated conditions. Scale bar = 200 µm.

The change to standard pro-hypertrophic medium conditions with addition of T3 and withdrawal of TGFß and dexamethasone (hyp) resulted in a hypertrophic phenotype with increased cell size, ALP positivity and increased collagen type X staining (Figure 18 A, D, J). In aggregates that were incubated in medium without TGFß and dexamethasone but without addition of T3 (hyp-T3), cell size, ALP and collagen type X staining were increased compared to the regular chondrogenic control but all parameters were clearly less enhanced than in standard hypertrophic cultures that received T3 (Figure 18 B, E, K). After withdrawal of TGFß and dexamethasone and the addition of BMP4 instead of T3 (hyp-T3+BMP), the aggregates developed a very strong hypertrophic phenotype with larger cells compared to all other groups, strong

ALP and collagen type X staining (Figure 18 C, F, L). Furthermore collagen type II staining seems to be enhanced in BMP4 treated MSC pellets compared to hypertrophic medium with and without T3 (Figure 18 G, H, I).

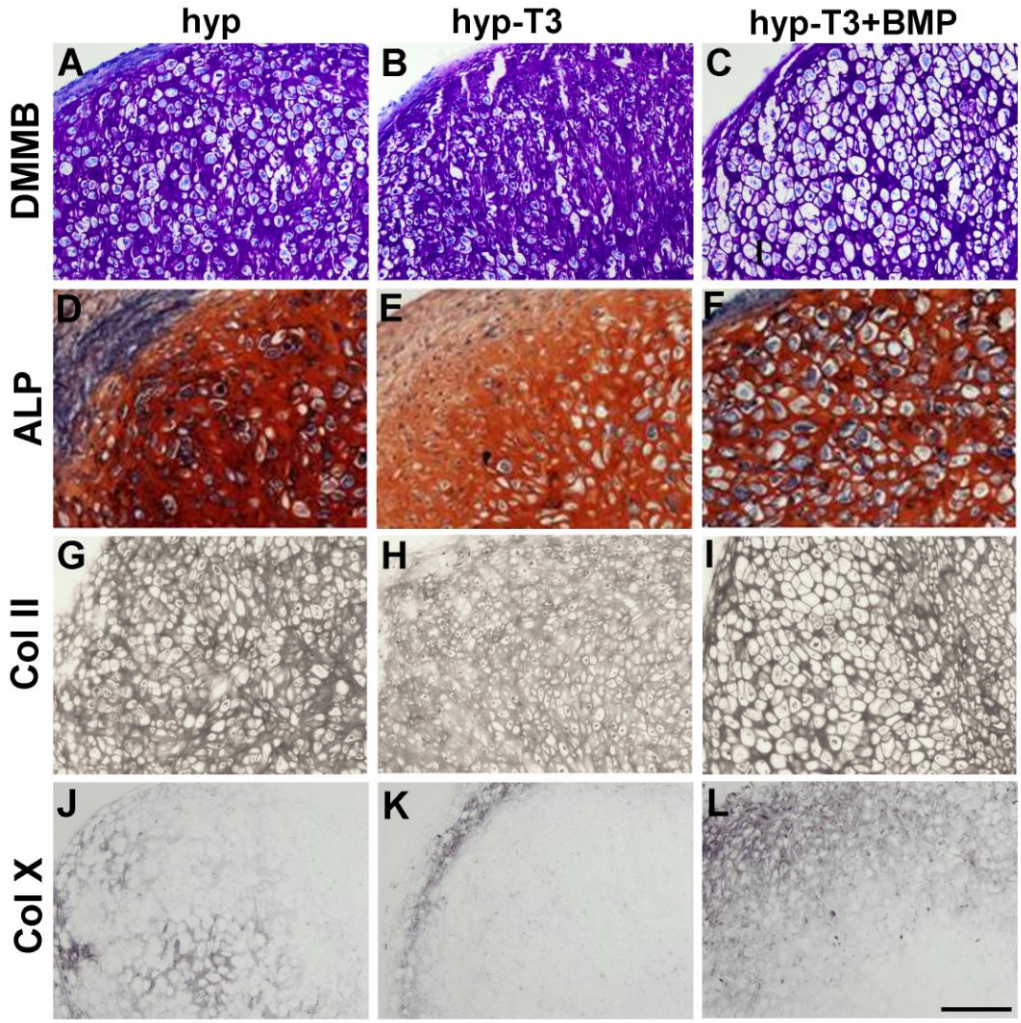

Figure 18 Histological appearance of MSC pellets on day 28 after BMP4 treatment under hypertrophic conditions. A, D, G, J hypertrophic standard medium. **B, E, H, K** hypertrophic medium without T3. **C, F, I, L** hypertrophic medium without T3 but with BMP4. Cell size (**A, B, C**), ALP staining (**D, E, F**), collagen type II (**G, H, I**) and collagen type X (**J, K, L**) staining are increased after BMP4 treatment (**C, F, I, L**) compared to hypertrophic medium with (**A, D, G, J**) and without T3 (**B, E, H, K**). Scale bar = 200 µm.

4.2.2.2 Histomorphometry

Histological analysis indicated that the cell size of MSC pellets increased after BMP4 treatment under hypertrophic conditions. In order to confirm this, we performed a histomorphometric cell size analysis of day 28 aggregates.

Under chondrogenic medium conditions histomorphometric analysis did not identify significant differences in the cell size between chondrogenic control aggregates and BMP4 treated aggregates. Histomorphometry detected significantly increased cell size under standard hypertrophic conditions compared to standard chondrogenic conditions. Furthermore, cells were significantly larger in aggregates in hypertrophic medium with T3 compared to hypertrophic medium without T3. Cells in pellets that were incubated in hypertrophic medium with BMP4 were significantly larger than cells cultured under any other condition (Figure 19 A).

Figure B shows the distribution of the cell size representative for one patient (Figure 19 B).

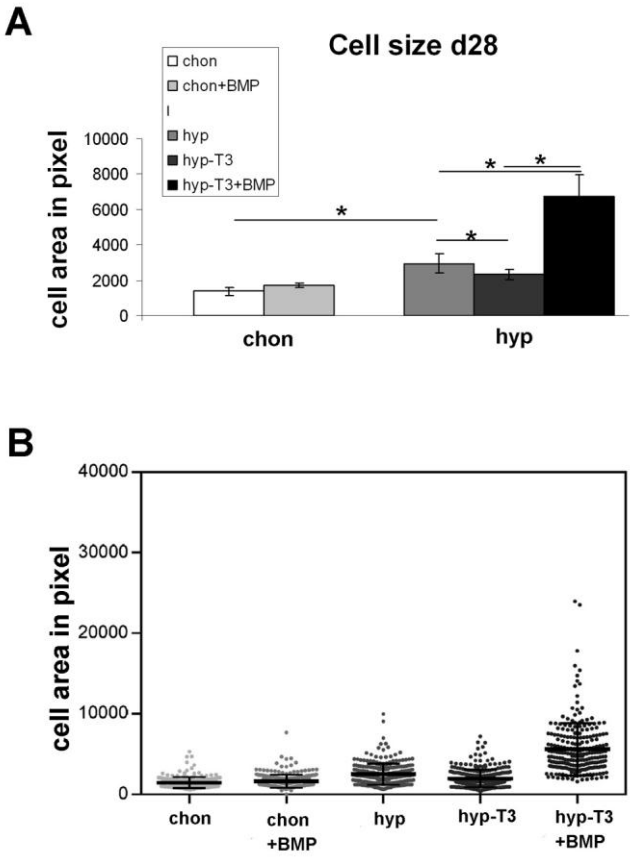

Figure 19 Histomorphometric cell size analysis of MSC pellets on day 28 after BMP4 treatment. A Under chondrogenic conditions BMP4 treatment has no influence on the cell size. Under hypertrophic conditions withdraw of T3 (hyp-T3) significantly reduces the cell size compared to hypertrophic standard conditions with T3 (hyp). Cell size of aggregates treated with BMP (hyp-T3+BMP) is significantly increased compared to hypertrophic medium with and without T3. n = 5 different donors. **B** distribution of the cell size representative for one patient.

4.2.2.3 ALP activity in the medium supernatant

Analysis of ALP activity in the medium of day 28 pellets under hypertrophic conditions revealed that ALP activity is significantly increased in pellets that were incubated in hypertrophic medium with T3 compared to hypertrophic medium without T3. ALP activity is significantly increased in the medium of pellets treated with BMP4 compared to pellets that were incubated in hypertrophic medium with and without T3 (Figure 20).

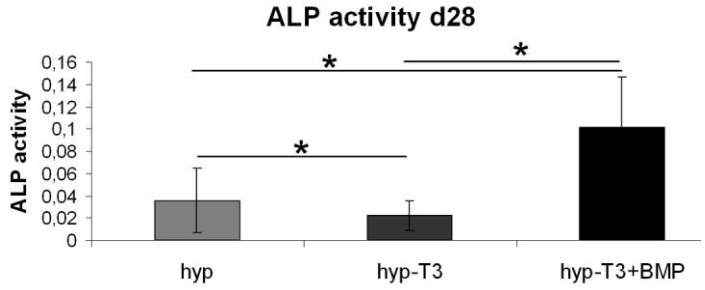

Figure 20 ALP activity in the medium of day 28 MSC pellets after BMP4 treatment under hypertrophic conditions. ALP activity in the medium with T3 (hyp) is significantly increased compared to medium without T3 (hyp-T3). BMP4 significantly increases ALP activity compared to hypertrophic medium with and without T3. n=7 different donors.

4.2.2.4 Gene expression analysis

To further characterize the effect of BMP4 on chondrogenic differentiating MSCs, gene expression of collagen type II, collagen type X and BMP4 was analysed in MSC pellets on day 21 and day 28 after BMP4 treatment.

Under chondrogenic conditions, the addition of BMP4 did not significantly affect collagen type II expression. Under pro-hypertrophic conditions, BMP4 treatment significantly increased collagen type II expression compared to hypertrophic conditions with and without T3 on day 21 and day 28 (Figure 21 A). Collagen type X expression was not affected by BMP4 treatment under chondrogenic conditions. Under hypertrophy enhancing conditions collagen type X expression was significantly increased in BMP4 treated aggregates compared to hypertrophic aggregates with and without T3 (Figure 21 B).

Histology, histomorphometry and ALP activity in the medium pointed out that in hypertrophic medium without T3 there is a less enhanced hypertrophic phenotype compared to hypertrophic medium with T3. It is important to investigate if there are

also differences in the BMP4 expression between these two conditions. Real time PCR analysis revealed that BMP4 expression is significantly increased in MSC pellets in hypertrophic medium with T3 compared to hypertrophic medium without T3 on day 21 and day 28. Furthermore the exogenous addition of BMP4 to the medium significantly suppresses BMP4 expression (Figure 21C).

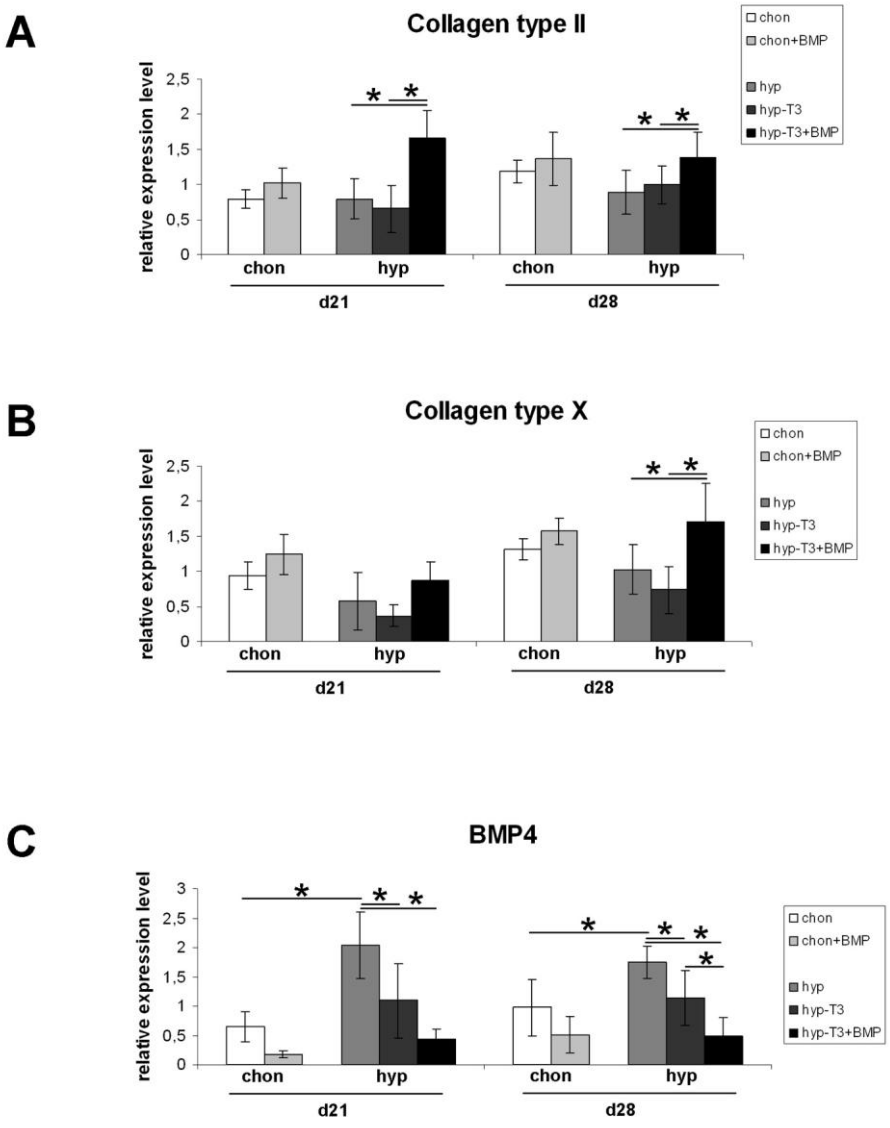

Figure 21 Gene expression analysis of collagen type II, collagen type X and BMP4 normalized to HPRT in MSC pellet cultures after BMP4 treatment under chondrogenic (chon) and hypertrophy enhancing (hyp) conditions analyzed by real time PCR. Collagen type II and collagen type X expression is significantly increased after BMP4 treatment under hypertrophic but not under chondrogenic conditions (**A, B**). BMP4 expression is significantly increased in MSC pellets incubated in hypertrophic medium with T3 (hyp) compared to hypertrophic medium without T3 (hyp-T3) on day 21 and day 28 (**C**). n = 4 different donors.

4.2.3 The BMP inhibitor noggin inhibits thyroid hormone induced hypertrophy

Previous results showed that BMP4 is able to further increase hypertrophy of chondrogenic differentiating MSCs under hypertrophic conditions. Next we aimed to investigate whether the BMP inhibitor noggin is able to block hypertrophy in our cell culture system.

4.2.3.1 Histological analysis

Under standard chondrogenic conditions histological analysis did not identify any effect of noggin on the phenotype of day 28 pellets. DMMB staining showed that noggin treated MSC pellets differentiated chondrogenically and showed hyaline cartilage like morphology (Figure 22 B, C) similar to chondrogenic control aggregates (Figure 22 A). Both, in chondrogenic control aggregates (Figure 22 D) and noggin treated chondrogenic aggregates (Figure 22 E, F) some ALP positive cells were detected throughout the aggregates, without differences between the conditions. No difference in collagen type II staining can be detected between chondrogenic control aggregates (Figure 22 G) and noggin treated aggregates (Figure 22 H, I). Collagen type X staining was weak both in chondrogenic control aggregates (Figure 22 J) and noggin treated aggregates (Figure 22 K, L) without differences between these conditions.

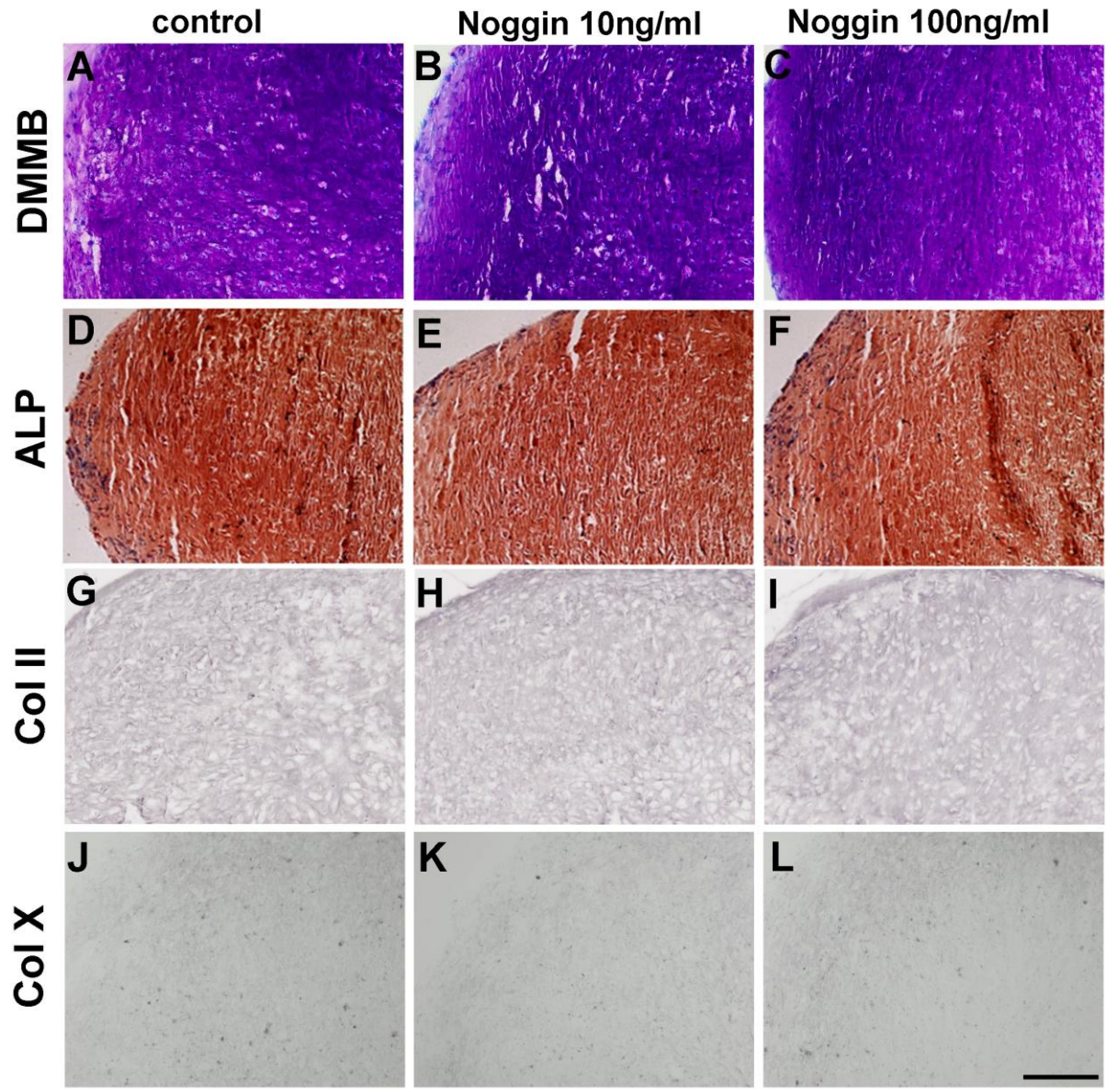

Figure 22 Histological appearance of MSC pellet cultures on day 28 after noggin treatment under chondrogenic conditions. No change in DMMB (**A, B, C**), ALP (**D, E, F**), collagen type II (**G, H, I**) and collagen type X (**J, K, L**) staining can be detected between chondrogenic control conditions (**A, D, G**) and noggin treated conditions (**B, C, D, F, H, I**). Scale bar = 200 µm.

Under pro-hypertrophic conditions noggin treatment inhibited hypertrophy induction in a dose dependant manner. DMMB staining showed the distinct hypertrophic phenotype with increased cell size in hypertrophic control conditions (Figure 23 A). 10 ng/ml noggin clearly reduced the amount and size of hypertrophic cells (Figure 23 B) and at 100 ng/ml noggin no hypertrophic cells could be detected (Figure 23 C). The amount of ALP positive cells was decreased in noggin treated aggregates in the centre of the aggregates (Figure 23 E, F) compared to hypertrophic control aggregates (Figure 23 D). Cells in the periphery were ALP positive in both, control

and noggin treated hypertrophic conditions. Collagen type X immunostaining was strong in hypertrophic control aggregates (Figure 23 J) and decreased with increasing noggin concentration (Figure 23 K, L). Of note, high doses of noggin lead to a dedifferentiation of the cells shown by decreased metachromatic DMMB staining (Figure 23 C) and decreased collagen type II staining (Figure 23 H, I).

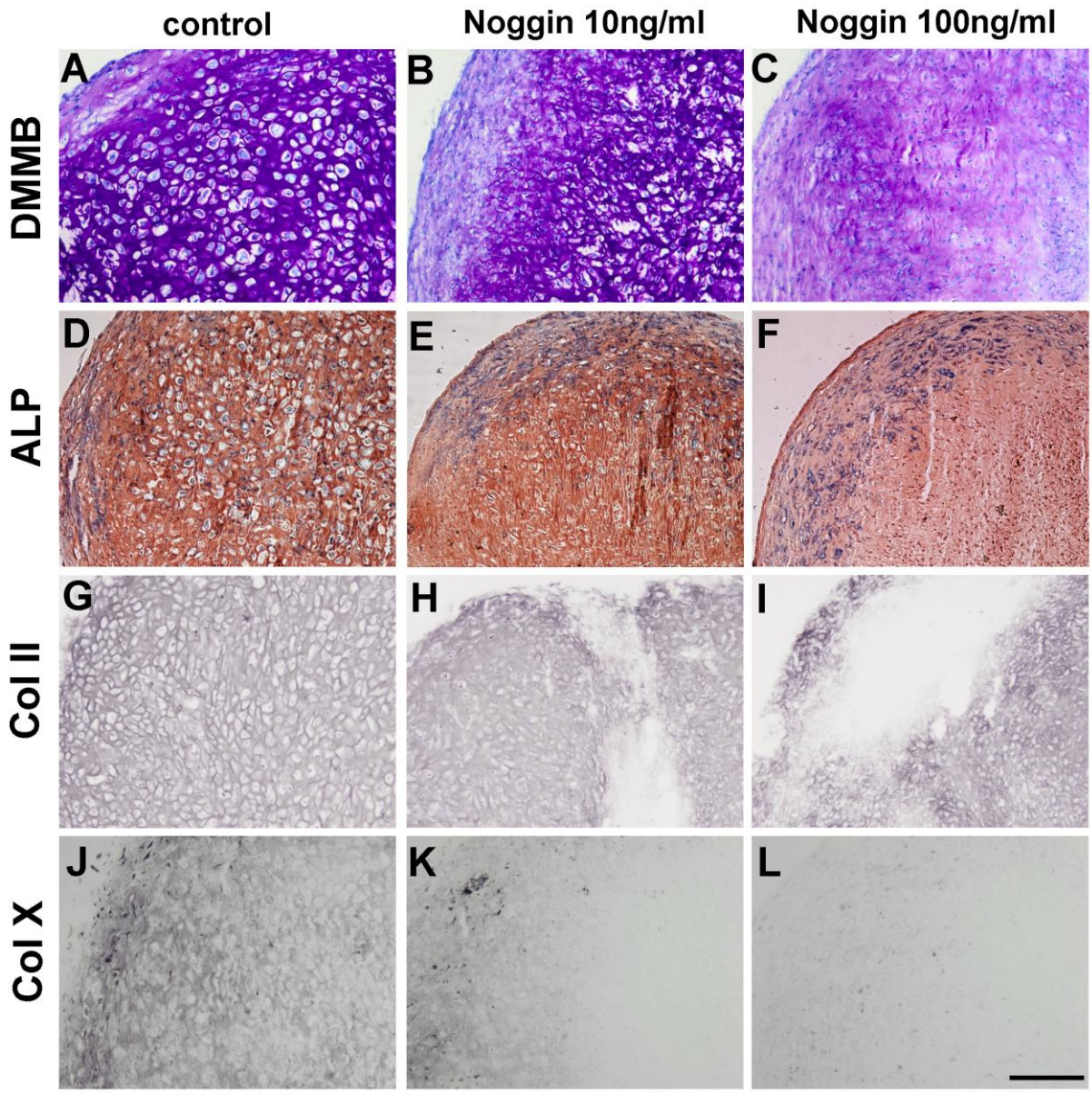

Figure 23 Histological appearance of MSC pellet cultures on day 28 after noggin treatment under hypertrophy enhancing conditions. Noggin treatment inhibits hypertrophy shown by decreased amount of hypertrophic (**A, B, C**) and ALP positive cells (**D, E, F**) and decreased collagen type X staining (**J, K, L**) in noggin treated aggregates (**B, C, E, F, K, L**) compared to hypertrophic control aggregates (**A, D, J**) . Collagen type II is decreased in noggin treated pellets (**H, I**) compared to hypertrophic control pellets (**G**). Scale bar = 200 µm.

4.2.3.2 Histomorphometry

Histological analysis indicated that the cell size of MSC pellets decreased after noggin treatment under hypertrophic conditions. In order to confirm this, we performed a histomorphometric cell size analysis of day 28 aggregates.

Under chondrogenic conditions, noggin treatment had no effect on the cell size. There is no difference in the cell size between chondrogenic control aggregates and noggin treated aggregates. Under hypertrophic conditions, noggin treatment significantly decreased the cell size (Figure 24).

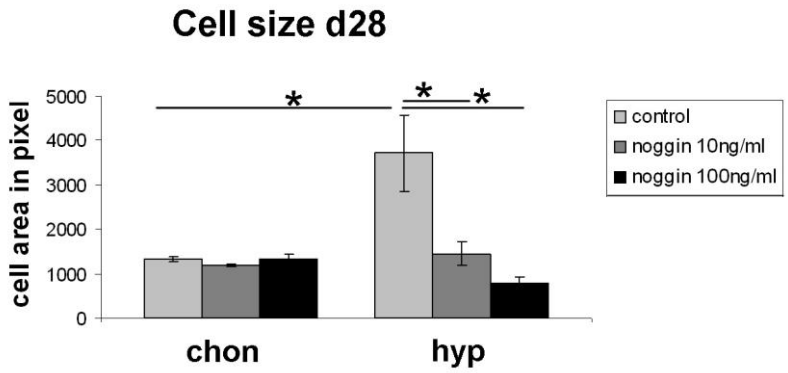

Figure 24 Histomorphometric cell size analysis of MSC pellets on day 28 after noggin treatment. Under chondrogenic conditions (chon) noggin treatment has no influence on the cell size. Under hypertrophic conditions (hyp) noggin treatment significantly reduces the cell size. n = 4 different donors.

4.2.3.3 ALP activity in the medium supernatant

Additionally, ALP activity in the medium supernatant was investigated on day 28 after noggin treatment. Noggin treatment did not significantly change ALP activity under chondrogenic conditions. Under hypertrophic conditions, ALP activity was reduced dose-dependently and significantly by noggin treatment. In addition, ALP activity is significantly increased under hypertrophic control conditions compared to chondrogenic control conditions (Figure 25).

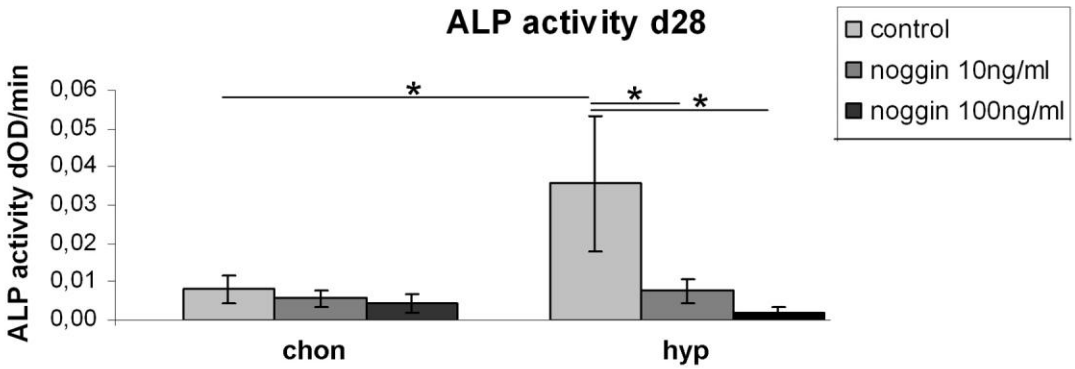

Figure 25 ALP activity in the medium of day 28 MSC pellets after noggin treatment. Under chondrogenic conditions (chon) noggin treatment has no influence on ALP activity. Under hypertrophic conditions (hyp) noggin treatment significantly reduces ALP activity. ALP activity under hypertrophic standard conditions is significantly increased compared to chondrogenic standard conditions. n=4 different donors.

4.2.3.4 Gene expression analysis

To further investigate the effect of noggin we performed gene expression analysis of collagen type II, collagen type X and BMP4 after noggin treatment.

Under chondrogenic conditions, noggin treatment did not have a significant effect on collagen type II or X expression (Figure 26 A, B). Under hypertrophic conditions collagen type II expression is significantly reduced by noggin treatment on day 28 (Figure 26 A) and collagen type X expression is significantly decreased on day 21 and day 28 in noggin treated pellets compared to hypertrophic control pellets (Figure 26 B). The ratio collagen type II / collagen type I can be taken as a chondrogenic differentiation index (Martin, I. *et al.*, 2001). Here we use the ratio collagen type X / collagen type II as a hypertrophic differentiation index. The ratio collagen type X / collagen type II on day 28 under hypertrophic control conditions is 1,8. Noggin treatment decreases this ratio. Treatment with 10 ng/ml noggin slightly decreases the ratio to 1,5 and after treatment with 100 ng/ml noggin the ratio is reduced to 0,95. This indicates that noggin has a stronger inhibitory effect on the hypertrophic differentiation than chondrogenic differentiation.

BMP4 gene expression was not influenced by noggin under chondrogenic conditions whereas under hypertrophic conditions 100 ng/ml noggin significantly increased BMP4 expression on day 21 and day 28 (Figure 26 C).

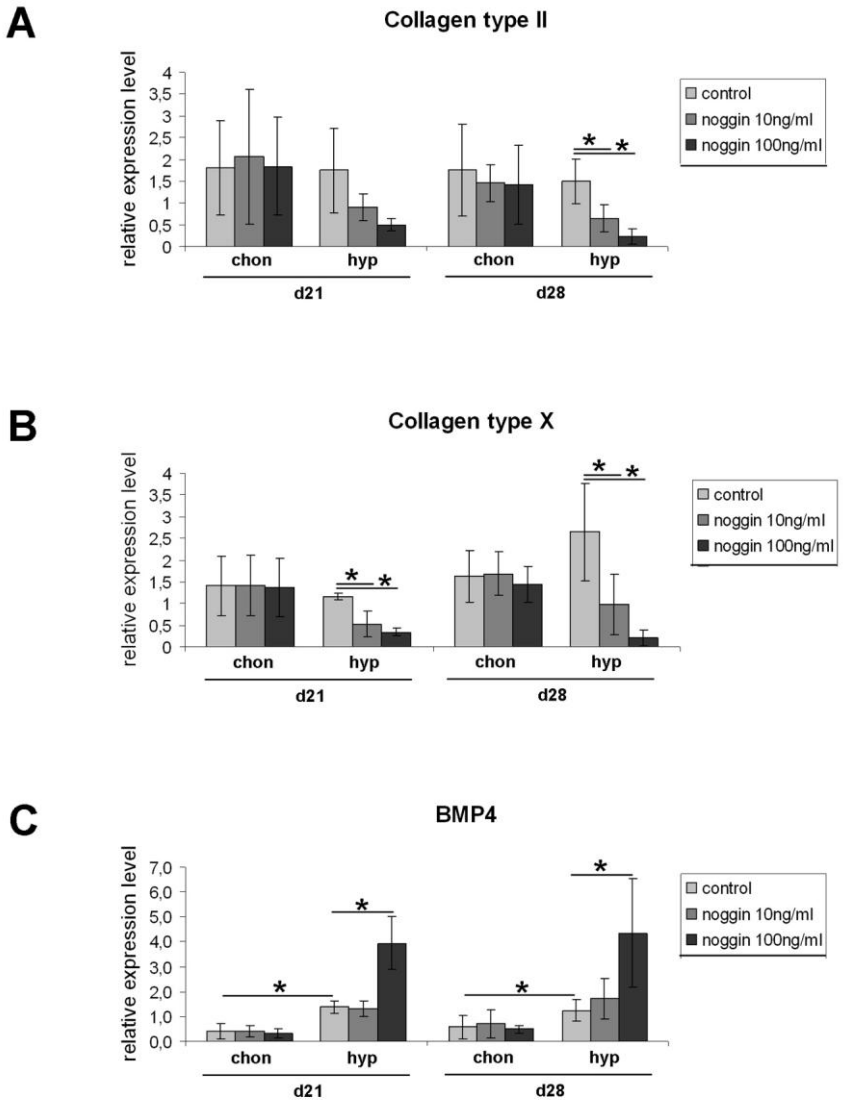

Figure 26 Gene expression analysis of collagen type II, collagen type X and BMP4 normalized to HPRT in MSC pellet cultures after noggin treatment under chondrogenic (chon) and hypertrophy enhancing (hyp) conditions analyzed by real time PCR. Collagen type II and collagen type X expression is significantly down-regulated after noggin treatment under hypertrophic conditions but not under chondrogenic conditions (**A, B**). Noggin treatment in high doses (100 ng/ml) significantly increases BMP4 expression under hypertrophic but not under chondrogenic conditions (**C**). n = 4 different donors.

4.2.4 BMP signalling activity

The principal effectors downstream of BMP receptors are the Smad transcription factors, Smad1, 5 and 8. Upon activation of the BMP signalling pathway, Smad1, 5, 8 get phosphorylated. In order to investigate Smad activation we performed Western Blot analysis using antibodies specific for the phosphorylated form of Smad1, 5.

Previous results pointed towards an increased BMP signalling activity under hypertrophic conditions. In the Western Blot analysis we did not detect a clear difference in the amount of phospho-Smad1, 5 between chondrogenic and hypertrophic MSC pellets on day 21 and day 28. On day 28 a tendency towards decreased phospho-Smad 1, 5 was visible. The total amount of Smad1 protein and ß actin was taken as loading control (Figure 27 A).

Activated Smad complexes accumulate in the nucleus. Therefore we performed immunohistochemistry for phospho-Smad1, 5, 8 in order to investigate whether we can detect differences in the localisation of phospho-Smad 1, 5, 8 between chondrogenic and hypertrophic conditions. In chondrogenic pellets we detected a diffuse staining of the cytoplasm of the cells (Figure 27 B) whereas under hypertrophic conditions we saw a strong staining of the nucleus indicating an accumulation of phospho-Smad1, 5, 8 in the nucleus of hypertrophic cells (Figure 27 C).

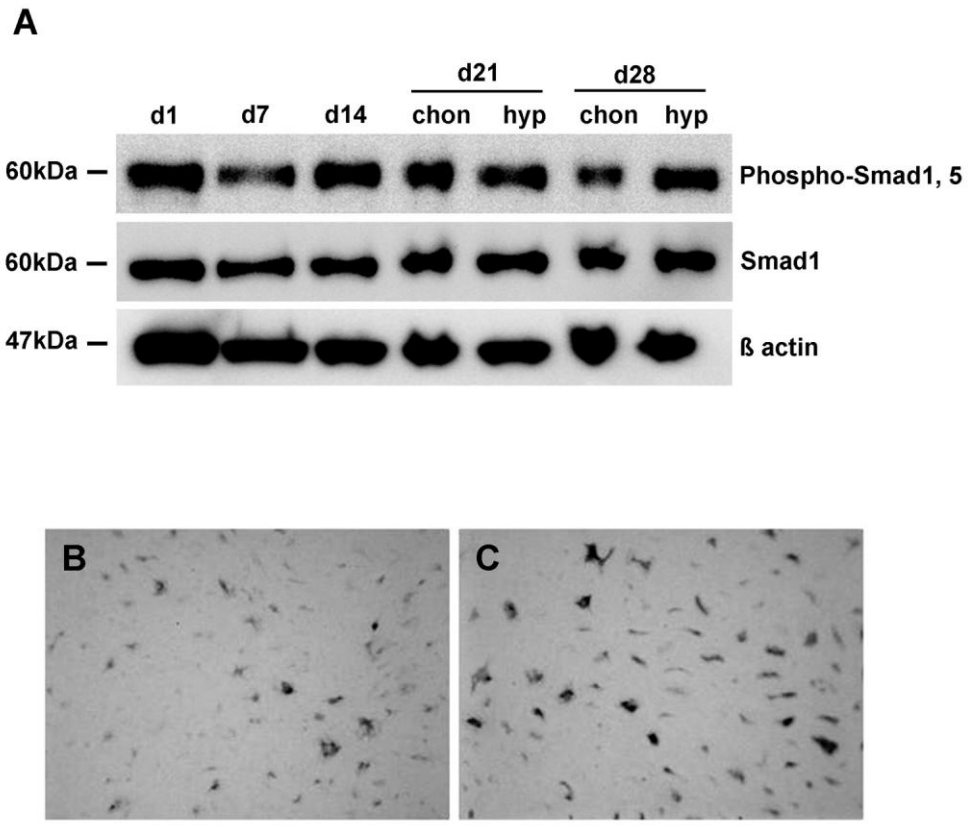

Figure 27 BMP signalling activity. A Western Blot analysis of Smad 1, 5 protein. No clear difference in the amount of phospho-Smad1, 5 can be detected between chondrogenic and hypertrophic conditions. The total amount of Smad1 and ß actin was taken as loading control. **B, C** Immunohistochemistry for phospho-Smad1, 5, 8. Increased accumulation of phospho-Smad1, 5, 8 in the nucleus under hypertrophic conditions (**C**) compared to chondrogenic conditions (**B**)**.**

4.2.5 The BMP inhibitor dorsomorphin inhibits thyroid hormone induced hypertrophy

We clearly showed that BMP signalling associated genes are up-regulated under hypertrophic conditions and that under hypertrophic conditions, BMP4 further increases hypertrophy whereas hypertrophy is blocked by the BMP inhibitor noggin. This strongly suggests an involvement of BMP signalling in the regulation of MSC hypertrophy and increased BMP signalling under hypertrophic conditions. We did not detect a clear difference in phospho-Smad1,5 between chondrogenic and hypertrophic conditions in the Western Blot analysis. It is possible that BMP4 does not act via the Smad signalling cascade but activates a non-Smad signalling pathway to induce hypertrophy in chondrogenic differentiating MSCs. It has been shown that BMPs are able to activate various members of different MAPK pathways such as p38 and ERK1/2 and the AKT/PKB pathway independent of Smad proteins (Shim, J. H. *et al.*, 2009).

In order to test this we investigated the effect of the BMP inhibitor dorsomorphin in the *in vitro* hypertrophy model. Dorsomorphin, in contrast to noggin, does not block soluble BMPs but blocks BMP induced Smad1, 5, 8 phosphorylation. If BMP4 acts via the Smad signalling cascade, dorsomorphin should have a similar effect as noggin.

Histological analysis of day 28 pellets showed that under chondrogenic conditions there is no difference in the phenotype of dorsomorphin treated pellets and chondrogenic control pellets. Dorsomorphin treated pellets differentiated chondrogenically characterized by a hyaline cartilage like morphology with little sign of hypertrophy (Figure 28 B, C, D) similar to chondrogenic control pellets (Figure 28 A). ALP staining revealed that both under chondrogenic control conditions (Figure 28 E) and dorsomorphin treated conditions (Figure 28 F, G, H) some ALP positive cells are present. In addition there is no difference in collagen type II staining between chondrogenic control pellets (Figure 28 I) and pellets that were treated with dorsomorphin (Figure 28 J, K, L). Collagen type X staining is weak in chondrogenic control pellets (Figure 28 M) and in dorsomorphin treated pellets (Figure 28 N, O, P). But no differences can be detected between the conditions.

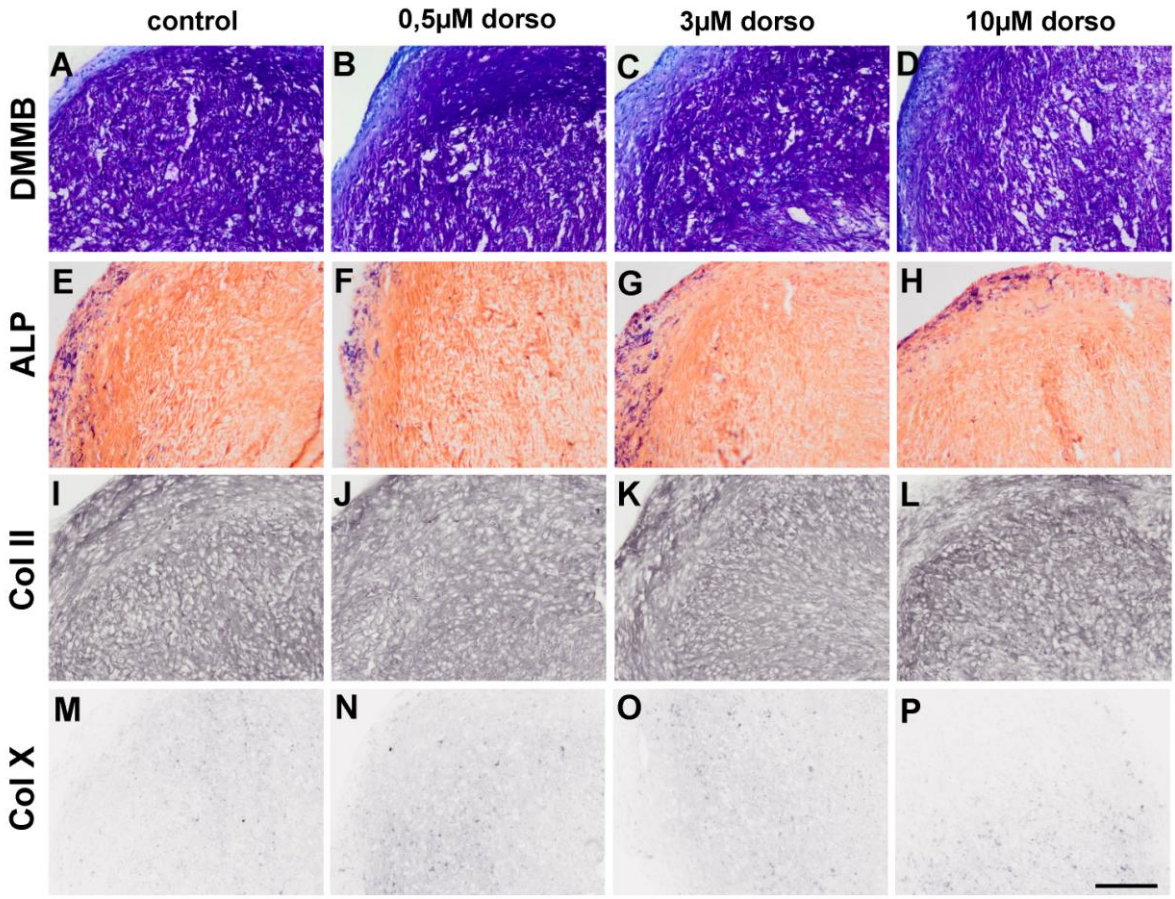

Figure 28 Histological appearance of MSC pellet cultures on day 28 after dorsomorphin treatment under chondrogenic conditions. No change in DMMB (**A-D**), ALP (**E-H**), collagen type II (**I-L**) and collagen type X (**M-P**) staining can be detected between chondrogenic control conditions (**A, E, I, M**) and dorsomorphin treated conditions (**B, C, D, F, G, H, J, K, L, N, O, P**). Scale bar = 200 µm.

Under hypertrophic conditions, dorsomorphin inhibits thyroid hormone induced hypertrophy similar to noggin. DMMB staining revealed that under hypertrophic control conditions the hypertrophic phenotype with plenty of hypertrophic cells appears (Figure 29 A). Dorsomorphin treatment inhibits hypertrophy in a dose dependent manner (Figure 29 B, C, D). 0,5 µM dorsomorphin clearly reduced the amount of hypertrophic cells (Figure 29 B). In MSC pellets that were treated with 3 µM dorsomorphin only few hypertrophic cells can be detected (Figure 29 C) and application 10 µM dorsomorphin blocked the appearance of hypertrophic cells and cells dedifferentiated (Figure 29 D). In hypertrophic control pellets lots of ALP positive cells can be detected (Figure 29 E). The number of ALP positive cells is dose dependently reduced after dorsomorphin treatment (Figure 29 F, G, H). Collagen type II staining is strong in hypertrophic control aggregates (Figure 29 I).

Treatment with 0,5 µM dorsomorphin does not alter collagen type II staining (Figure 29 J) but addition of 3 µM and 10 µM dorsomorphin clearly reduced collagen type II staining (Figure 29 K, L). Hypertrophic control pellets showed a strong collagen type X staining (Figure 29 M). Collagen type X staining decreases with increasing dorsomorphin concentration (Figure 29 N, O, P). In pellets that were treated with 10 µM dorsomorphin no collagen type X staining can be detected (Figure 29 P).

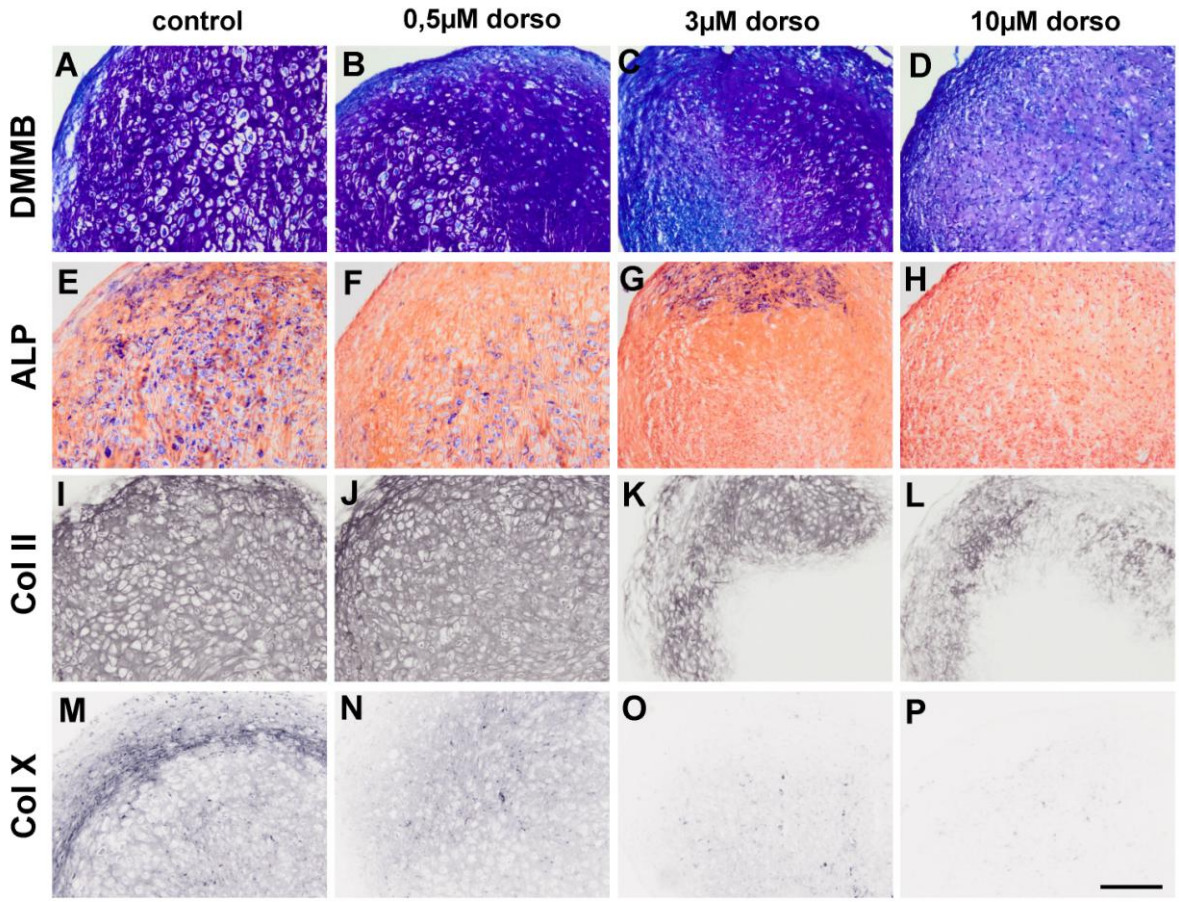

Figure 29 Histological appearance of MSC pellet cultures on day 28 after dorsomorphin treatment under hypertrophic conditions. Dorsomorphin inhibits hypertrophy shown by a decreased amount of hypertrophic (**A-D**) and ALP positive (**E-H**) cells and decreased collagen type X staining (**M-P**). Furthermore dorsomorphin reduces collagen type II staining (**I-L**). Scale bar = 200 µm.

The data obtained here clearly show that dorsomorphin, similar to noggin, blocks thyroid hormone induced hypertrophy. This confirms the assumption that BMP signalling is important for the establishment of hypertrophy in chondrogenic differentiating MSC and that BMP signalling acts, at least in part, via a Smad 1, 5, 8 dependent pathway.

In the previous experiments MSC pellets were treated with dorsomorphin from day 14 until day 28. In the following we wanted to investigate the effect of dorsomorphin treatment from day 1 until day 14. This was done in order to investigate the effect of an early inhibition of BMP signalling on the establishment of hypertrophy as real time PCR detected a strong up-regulation of BMP2 on day 3. Furthermore another part of the MSC pellets was treated with dorsomorphin over the whole culture period, from day 1 until day 28.

Histological analysis of day 28 MSC pellets revealed that under chondrogenic conditions treatment with dorsomorphin from day 1 until day 14 has no morphological effect. Dorsomorphin treated MSC pellets differentiated chondrogenically (Figure 30 B, C, D) similar to chondrogenic control pellets (Figure 30 A). Only few ALP positive cells appear in chondrogenic control pellets (Figure 30 E) and dorsomorphin treated pellets (Figure 30 F, G, H). Furthermore no difference in collagen type II staining can be detected between chondrogenic control pellets (Figure 30 I) and dorsomorphin treated pellets (Figure 30 J, K, L). Collagen type X staining is weak in chondrogenic control pellets (Figure 30 M) and in dorsomorphin treated pellets (Figure 30 N, O, P).

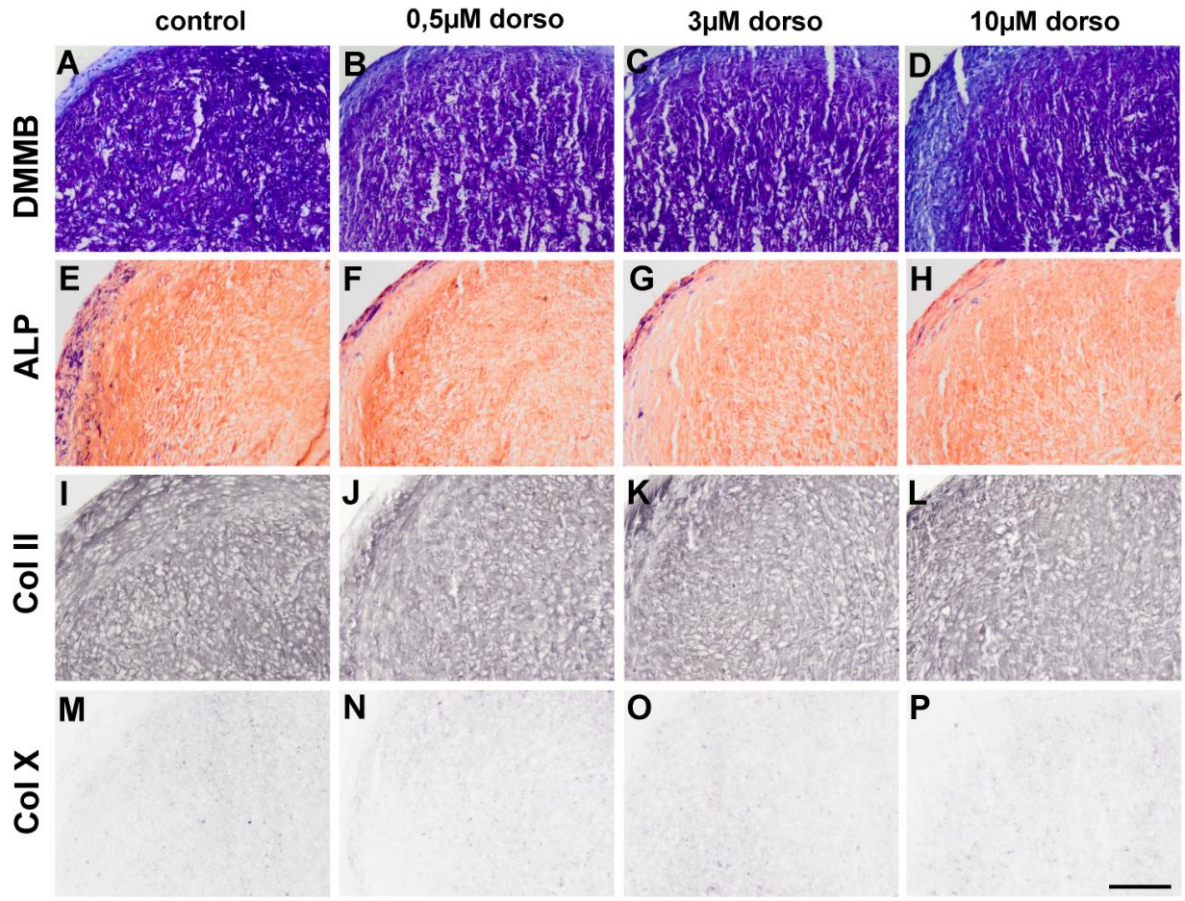

Figure 30 Histological appearance of MSC pellet cultures on day 28 after dorsomorphin treatment from day 1 until day 14 under chondrogenic conditions. No difference in DMMB (**A-D**), ALP (**E-H**), collagen type II (**I-L**) and collagen type X (**M-O**) staining can be detected between chondrogenic control pellets (**A, E, I, M**) and dorsomorphin treated pellets (**B, C, D, F, G, H, J, K, L, N, O, P**). Scale bar = 200 µm.

Under hypertrophic conditions treatment with the BMP inhibitor dorsomorphin from day 1 until day 14 clearly reduced hypertrophy. Treatment with 0,5 µM dorsomorphin reduced the amount of hypertrophic cells (Figure 31 B) and treatment with 3 µM and 10 µM dorsomorphin totally abolished the appearance of hypertrophic cells and the cells dedifferentiated (Figure 31 C, D). ALP staining showed that in pellets that were treated with 0,5 µM dorsomorphin less hypertrophic cells developed (Figure 31 F) compared to hypertrophic control conditions (Figure 31 E). After treatment with high doses of dorsomorphin (3 µM, 10 µM) no ALP positive cells can be detected (Figure 31 G, H). There is no distinct difference in collagen type II staining between hypertrophic control pellets (Figure 31 I) and pellets that were treated with 0,5 µM dorsomorphin (Figure 31 J). However, in pellets that were treated with 3 µM and 10 µM dorsomorphin only very weak and no collagen type II staining can be detected (Figure 31 K, L). Collagen type X staining is strong in

hypertrophic control pellets (Figure 31 M). Some collagen type X staining can be detected in MSC pellets that were treated with 0,5 µM dorsomorphin (Figure 31 N). No collagen type X staining can be detected in MSC pellets that were treated with higher dorsomorphin doses (Figure 31 O, P). Of note, MSC pellets that were treated with high doses of dorsomorphin were smaller compared to hypertrophic control pellets and dedifferentiated completely.

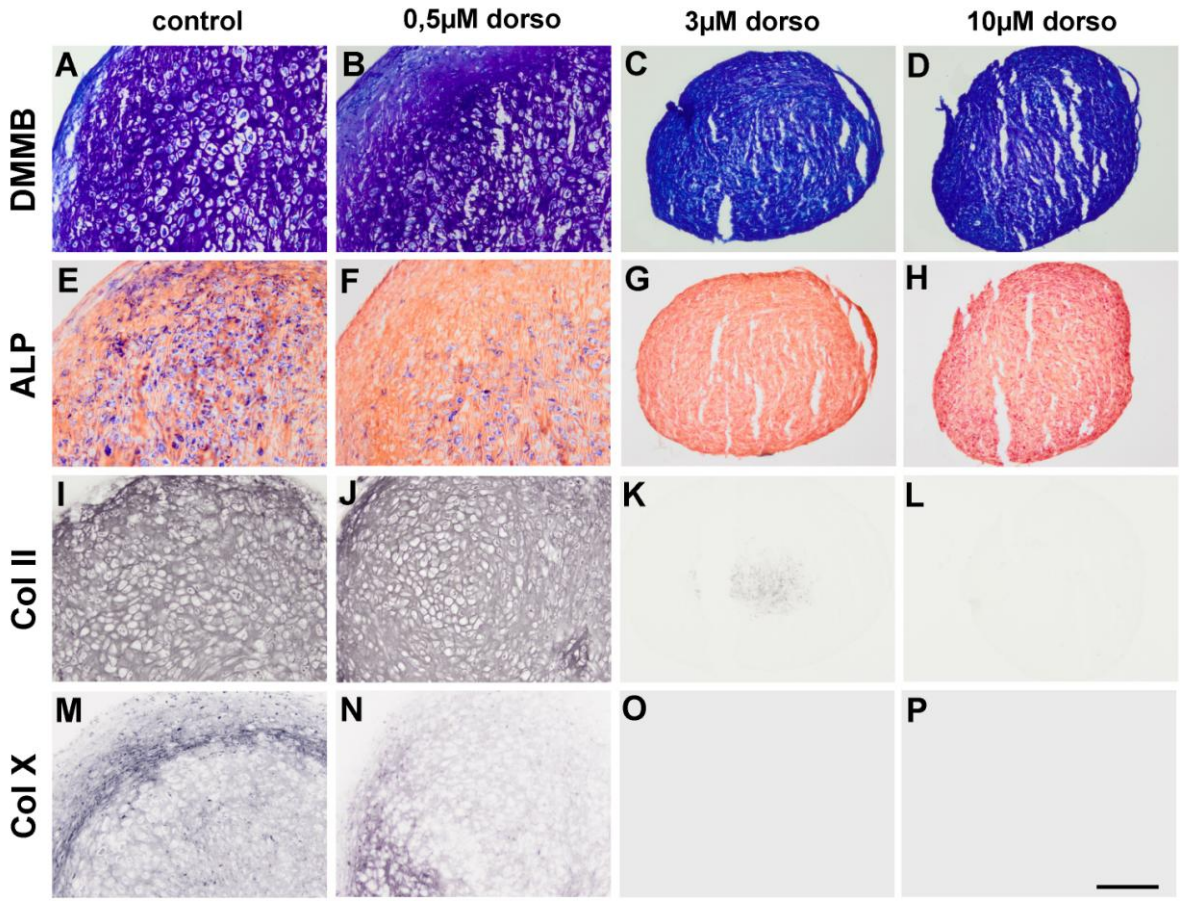

Figure 31 Histological appearance of MSC pellet cultures on day 28 after dorsomorphin treatment from day 1 until day 14 under hypertrophic conditions. Low doses of dorsomophin reduce the amount of hypertrophic (**B**), ALP positive (**F**) cells and collagen type X staining (**N**) compared to hypertrophic control conditions (**A, E, I, M**). High doses of dorsomorphin lead to a dedifferentiation of the cells and the pellets size is reduced (**C, D, G, H, K, L, O, P**). Scale bar = 200 µm.

In a further experiment the impact of dorsomorphin treatment over the whole culture period from day 1 until day 28 was investigated.

Histological analysis of day 28 MSC pellets showed that under chondrogenic conditions, dorsomorphin treated pellets differentiated chondrogenically and showed a hyaline cartilage like morphology (Figure 32 B, C, D) similar to that of

chondrogenic control pellets (Figure 32 A). In chondrogenic control conditions some ALP positive cells can be detected (Figure 32 E) but with increasing dorsomorphin concentration the amount of ALP positive cells decreases (Figure 32 F, G, H). In pellets that were treated with 10 µM dorsomorphin no ALP positive cells can be detected (Figure 32 H). Collagen type II staining is unchanged in dorsomorphin treated MSC pellets (Figure 32 J, K, L) compared to chondrogenic control pellets (Figure 32 I). Collagen type X staining is weak in chondrogenic control pellets (Figure 32 M) and MSC pellets that were treated with low doses of dorsomorphin (0,5 µM, 3 µM) (Figure 32 N, O). In MSC pellets that were treated with 10 µM dorsomorphin over the whole culture period no collagen type X staining can be detected (Figure 32 P).

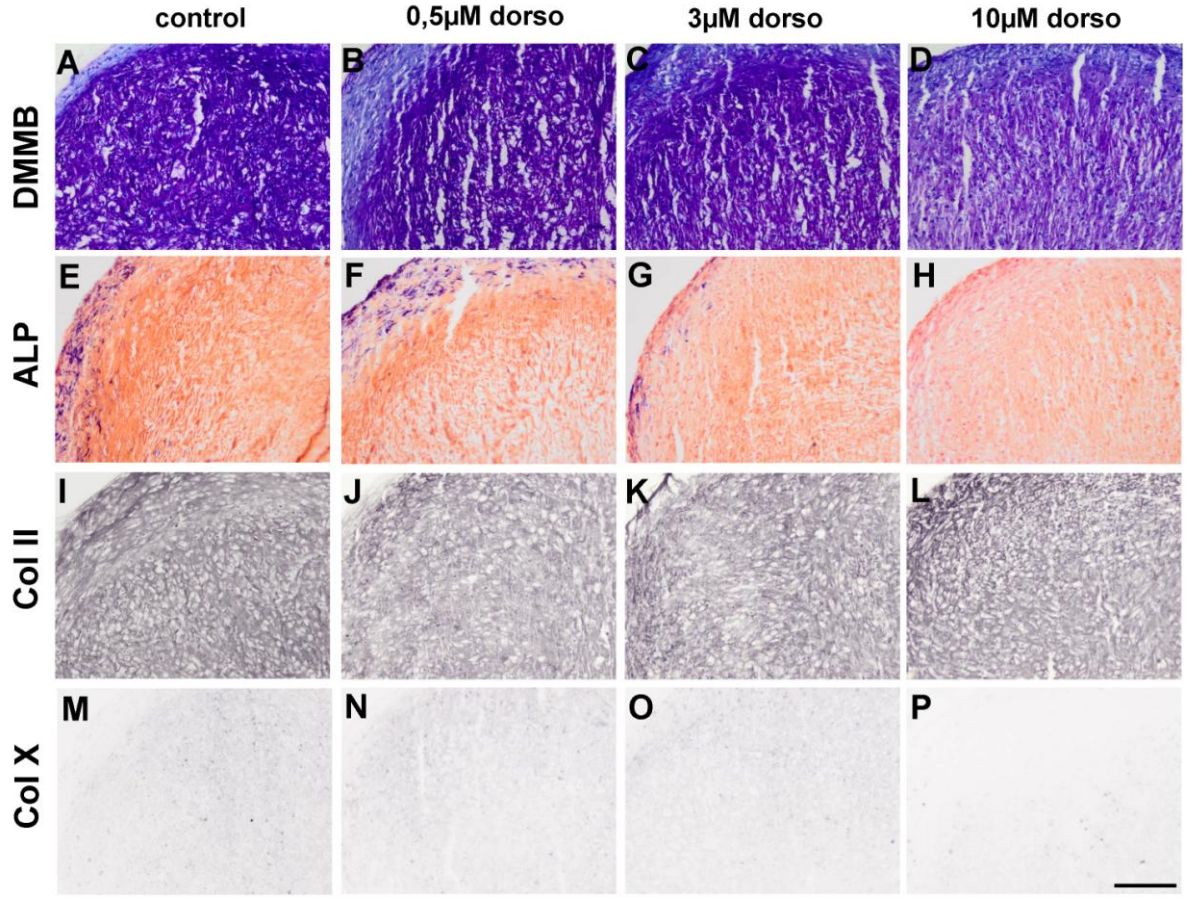

Figure 32 Histological appearance of MSC pellet cultures on day 28 after dorsomorphin treatment from day 1 until day 28 under chondrogenic conditions. No difference in DMMB, ALP, collagen type II, collagen type X staining can be detected between chondrogenic control pellets (**A, E, I, M**) and dorsomorphin treated pellets with low doses (**B, C, F, G, J, K, N, O**). High doses of dorsomorphin reduce the amount of ALP positive cells (**H**) and collagen type X staining (**P**) without affecting collagen type II staining (**L**). Scale bar = 200 µm.

Under hypertrophic conditions treatment with dorsomorphin over the whole culture period clearly reduced hypertrophy. In MSC pellets that were treated with 0,5 µM dorsomorphin some hypertrophic and ALP positive cells developed (Figure 33 B, F). The number of hypertrophic, ALP positive cells is however reduced compared to hypertrophic control conditions (Figure 33 A, F). MSC pellets that were treated with 3 µM and 10 µM dorsomorphin dedifferentiated and no hypertrophic cells and no ALP positive cells can be detected (Figure 33 C, D, G, H). There is no difference in collagen type II staining between hypertrophic control conditions (Figure 33 I) and MSC pellets that were treated with 0,5 µM dorsomorphin (Figure 33 J). However in MSC pellets that were treated with higher dorsomorphin concentrations (3 µM, 10 µM) only very weak collagen type II staining can be detected (Figure 33 K, L). In addition, collagen type X staining is clearly reduced after dorsomorphin treatment. After application of 0,5 µM dorsomorphin collagen type X staining is weak (Figure 33 N) and with 3 µM and 10 µM dorsomorphin no collagen type X staining can be detected (Figure 33 O, P). Of note, the size of MSC pellets that were treated with 3 µM and 10 µM dorsomorphin was clearly reduced compared to hypertrophic control pellets.

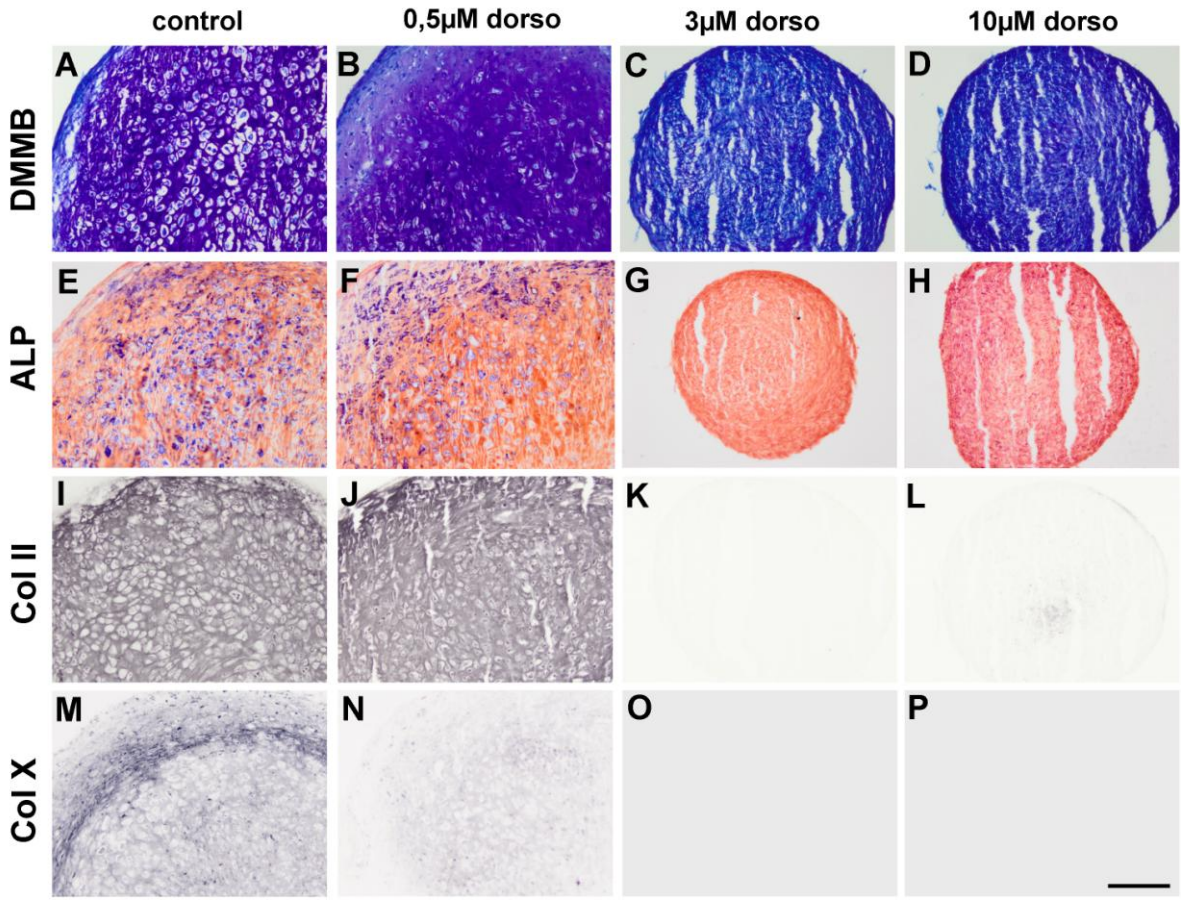

Figure 33 Histological appearance of MSC pellet cultures on day 28 after dorsomorphin treatment from day 1 until day 28 under hypertrophic conditions. Low doses of dorsomorphin reduce the amount of hypertrophic, ALP positive cells and collagen type X staining (**B, F, N**) compared to hypertrophic control conditions (**A, E, I, M**). High doses of dorsomorphin lead to a dedifferentiation of the cells and the pellets size is reduced (**C, D, G, H, K, L, O, P**). Scale bar = 200 µm.

Taken together, these results showed that under chondrogenic conditions dorsomorphin has no effect on chondrogenic differentiation and hypertrophic extend when applied from day 1 until day 14 or from day 14 to day 28. However application of dorsomorphin over the whole culture period reduces hypertrophic markers but has no influence on chondrogenic markers.

Under hypertrophic conditions administration of dorsomorphin from day 14 until day 28 lead to a dose dependent reduction in the amount of hypertrophic cells, ALP positive cells and a reduction in collagen type X. High doses of dorsomorphin lead to a dedifferentiation of the cells. Administrations of low doses dorsomorphin from d1 until day 14 or over the whole culture period reduce the amount of hypertrophic cells, whereas higher dorsomorphin concentrations lead to a dedifferentiation of the cells.

4.2.6 The role of BMP signalling modulators in MSC hypertrophy

The BMP signalling pathway is regulated and modulated by a network of different regulators. Soluble antagonists specifically bind to the growth factors and prevent their interaction with their receptors. Co-receptors modulate the transmission of the extracellular signal to the cytosol. In the following part we aimed to analyze whether there are differences in the expression of different BMP antagonists between chondrogenic and hypertrophic conditions.

4.2.6.1 Soluble BMP antagonists

The family of BMP antagonists consists of structurally unrelated proteins that differ in its specificity and affinity for different BMPs. We investigated the expression of the most prominent BMP antagonists, noggin, chordin, follistatin and gremlin, comparative between chondrogenic and hypertrophic MSC pellets. Differences in the level at which BMP antagonists are expressed may lead to differences in BMP signalling activities.

Real time PCR revealed that noggin is significantly down-regulated in hypertrophic MSC pellets on day 17. On culture day 21 there is a tendency towards reduced noggin expression in hypertrophic aggregates but on day 28 there is no difference in the expression of noggin between chondrogenic and hypertrophic conditions (Figure 34 A). The BMP antagonist chordin is significantly down-regulated in hypertrophic aggregates on day 28 (Figure 34 B). Surprisingly, the BMP antagonist gremlin is significantly up-regulated under hypertrophic condition on day 17, day 21 and day 28 (Figure 34 C). Follistatin is not regulated as a function of hypertrophy. There is no difference in the expression of follistatin between chondrogenic and hypertrophic MSC pellets on day 17, day 21 and day 28 (Figure 34 D).

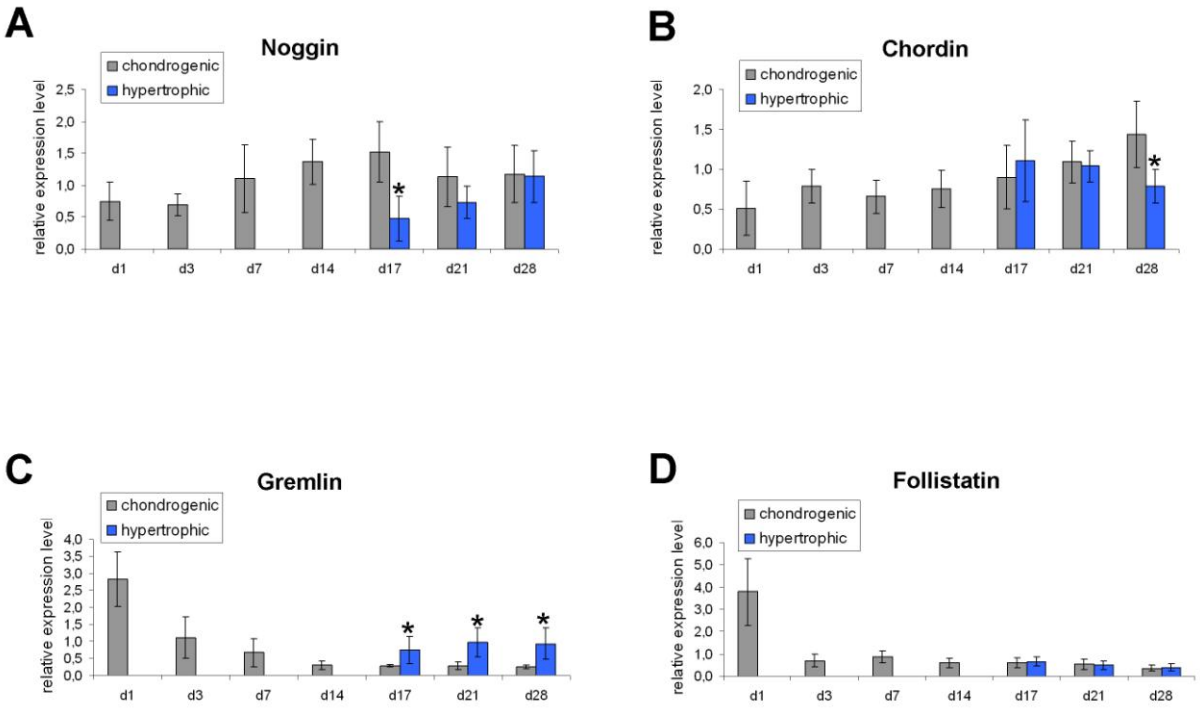

Figure 34 Gene expression analysis of noggin, chordin, gremlin and follistatin normalized to HPRT in MSC pellet cultures under chondrogenic and hypertrophy enhancing conditions analyzed by real time PCR. Noggin is significantly down-regulated under hypertrophic conditions on day 17 (**A**) and chordin is down-regulated on day 28 under hypertrophic conditions (**B**). Gremlin is up-regulated under hypertrophic conditions on day 17, 21 and 28 (**C**). Follistatin is not regulated as a function of hypertrophy (**D**). n= 7 different donors.

4.2.6.2 The co-receptor BAMBI

The pseudoreceptor BAMBI (BMP and activin membrane-bound inhibitor) is known as an inhibitor of the TGFß and BMP signalling pathway (Onichtchouk, D. *et al.*, 1999; Sekiya, T. *et al.*). BAMBI is a transmembrane protein that has structural similarity to type I receptors of the TGFß family but lacks the intracellular kinase domain (Onichtchouk, D. *et al.*, 1999; Loveland, K. L. *et al.*, 2003). BAMBI is able to bind to type I receptors and thereby inhibits the formation of type I/II receptor complexes and consequently inhibits signal transduction (Figure 35 A).

Real time PCR analysis of BAMBI revealed a pronounced increase in BAMBI expression under hypertrophic conditions. BAMBI expression is significantly up-regulated on culture day 17, day 21 and day 28 in hypertrophic MSC pellets compared to chondrogenic pellets (Figure 35 B).

To confirm this result on protein level we performed immunohistochemistry and Western Blot analysis for BAMBI. Immunohistochemistry revealed increased BAMBI

staining in hypertrophic aggregates compared to chondrogenic aggregates. Hypertrophic cells show a strong BAMBI staining in the cytoplasm and membrane whereas BAMBI staining of chondrogenic cells is only weak (Figure 35 C). Furthermore Western Blot analysis of BAMBI demonstrated that the amount of BAMBI protein is strongly enhanced under hypertrophic conditions. Under chondrogenic conditions nearly no BAMBI protein can be detected whereas on day 21 and especially on day 28 under hypertrophic conditions a strong BAMBI protein band can be detected (Figure 35 D).

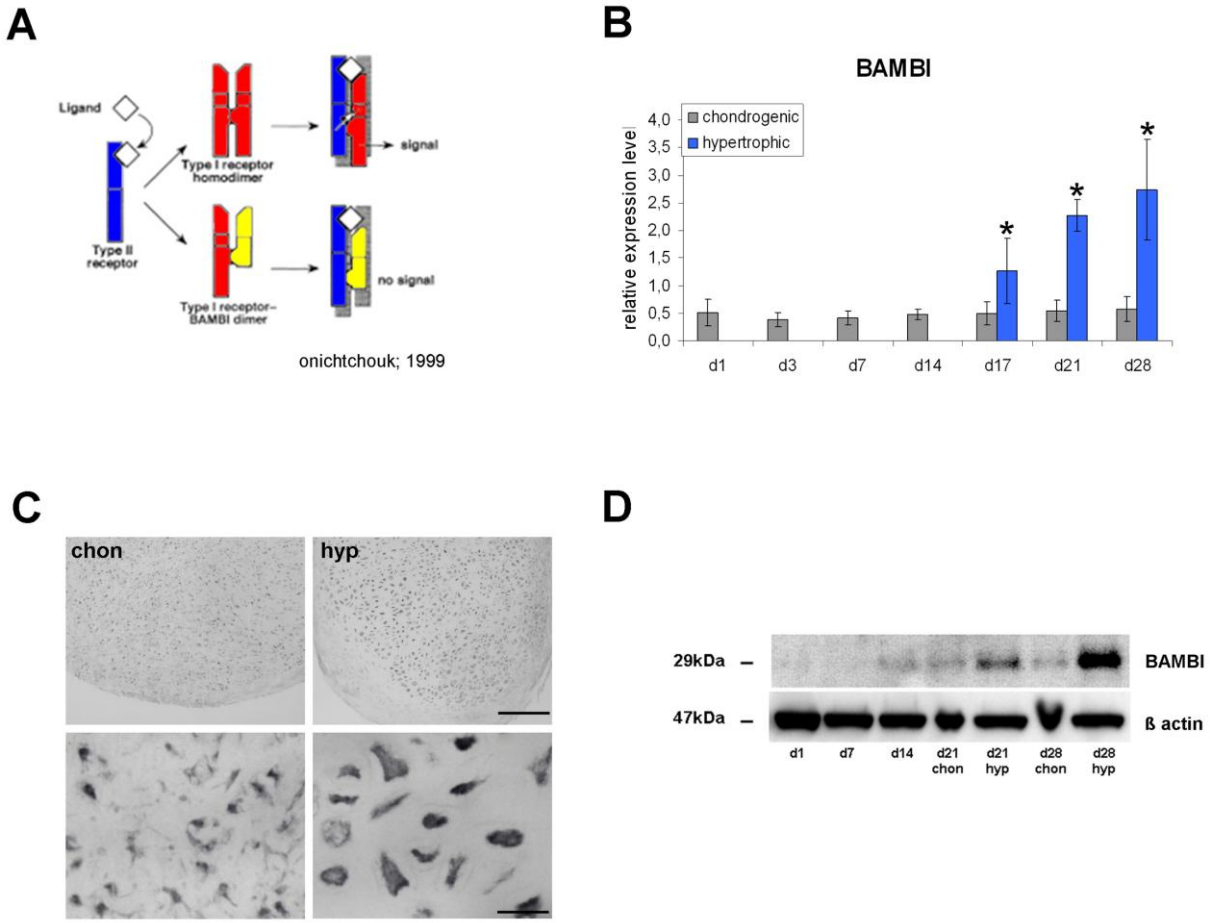

Figure 35 BAMBI expression in chondrogenic and hypertrophic MSC pellets. A Model of BMP/TGFß signalling inhibiton by Bambi. Bambi prevents the dimerization of type I and type II receptors and thereby inhibits signal transduction. **B** Gene expression analysis of BAMBI normalized to HPRT in MSC pellet cultures under chondrogenic and hypertrophy enhancing conditions analyzed by real time PCR. Bambi is significantly up-regulated under hypertrophic conditions on day 17, day 21 and day 28 under hypertrophic conditions. n=7 different donors. **C** Immunohistochemistry of Bambi. Bambi staining is increased under hypertrophic conditions compared to chondrogenic conditions. Scale bar=200 µm and 50 µm. **D** Western Blot analysis of Bambi. Increased amount of Bambi protein can be detected under hypertrophic conditions on day 21 and day 28 compared to chondrogenic conditions. ß actin was taken as loading control.

To further investigate the role of BAMBI in the induction of hypertrophy, we analysed the expression of BAMBI after BMP4 and noggin treatment. BMP4 treatment significantly increases BAMBI expression on day 28 under chondrogenic conditions. Under hypertrophic conditions BAMBI expression is significantly increased in MSC pellets that were treated with BMP4 compared to hypertrophic medium without T3 (Figure 36 A).

Treatment with the BMP inhibitor noggin does not alter BAMBI expression under chondrogenic conditions. Under hypertrophic conditions, noggin treatment significantly decreases BAMBI expression on day 21 and day 28 (Figure 36 B).

These experiments showed that the expression of the pseudoreceptor BAMBI is strongly up-regulated under hypertrophy enhancing conditions and BMP4 and noggin treatment are able to modulate BAMBI expression. The function of BAMBI is not clear and will be investigated by functional experiments with lentiviral knock-down and over-expression of BAMBI.

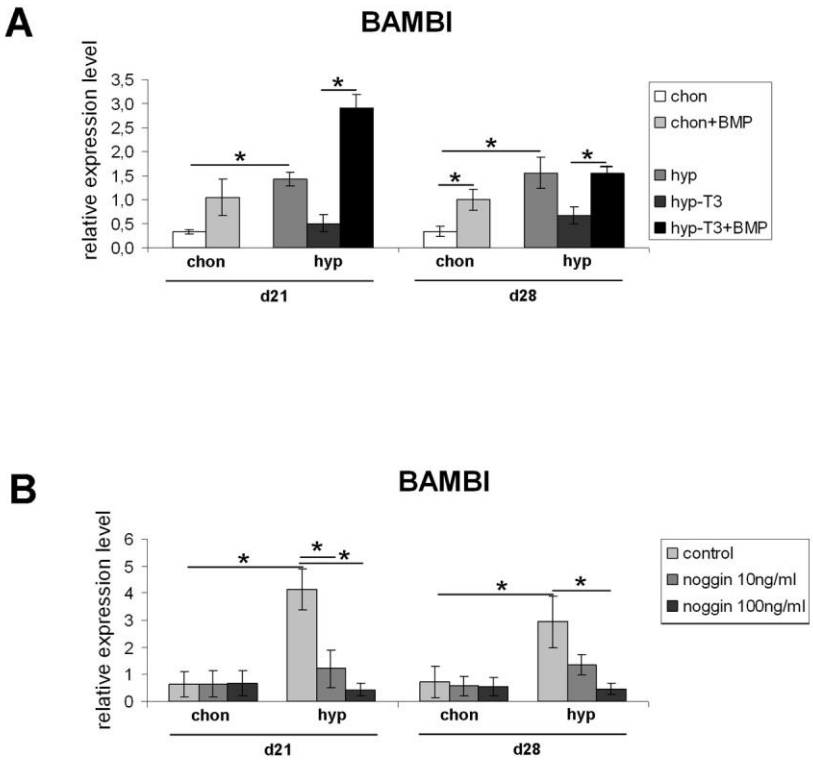

Figure 36 Gene expression analysis of BAMBI normalized to HPRT in MSC pellet cultures after BMP4 treatment (A) and noggin treatment (B) under chondrogenic (chon) and hypertrophy enhancing (hyp) conditions analyzed by real time PCR. BAMBI expression is increased under chondrogenic and hypertrophic conditions after BMP4 treatment (**A**). Noggin treatment significantly decreases BAMBI expression under hypertrophic conditions (**B**). n=4 different donors.

4.3 The role of TGFß signalling in the regulation of hypertrophy in chondrogenic differentiating MSCs

TGFß signalling has been shown to inhibit terminal differentiation of growth plate chondrocytes (Ballock, R. T. *et al.*, 1993; Ferguson, C. M. *et al.*, 2000; Ferguson, C. M. *et al.*, 2004). The role of TGFß signalling in the suppression of hypertrophy in chondrogenic differentiating MSCs is largely unknown. Therefore, in the following part, we aimed to clarify if TGFß signalling is involved in the regulation of MSC hypertrophy.

4.3.1 TGFß signalling associated genes are down-regulated under hypertrophic conditions

Comparison of genes associated with TGFß signalling between chondrogenic and hypertrophic MSC pellets using real time PCR, revealed that the TGFß receptor 1 (TGFßR1) is significantly down-regulated under hypertrophic conditions on day 17, day 21 and day 28 compared to chondrogenic conditions (Figure 37 A). In addition, the TGFß receptor 2 (TGFßR2) is significantly down-regulated on day 28 in hypertrophic MSC pellets (Figure 37 B). Sox9 is known to be an important transcription factor for chondrocyte differentiation and is a downstream target of TGFß signalling. Real time PCR analysis showed that Sox9 is significantly down-regulated on day 17 and day 21 under hypertrophy enhancing conditions. On day 28 there is a tendency towards decreased Sox9 expression under hypertrophic conditions but not significantly (Figure 37 C).

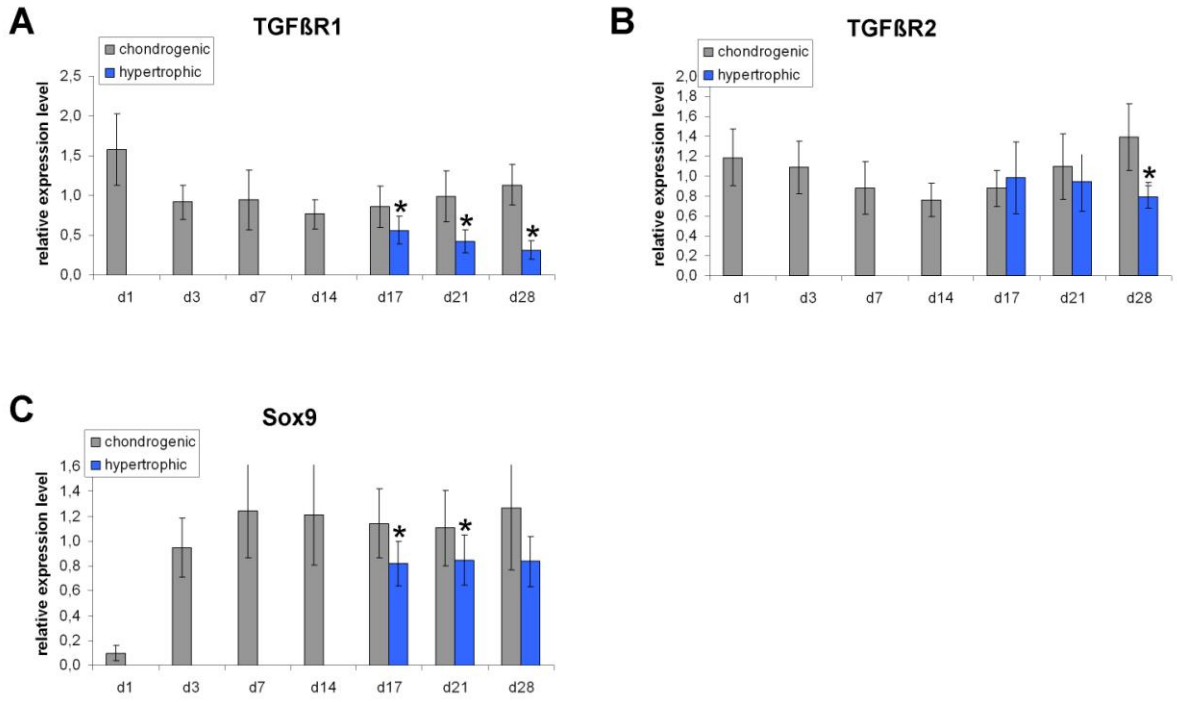

Figure 37 Gene expression analysis of TGFßR1, TGFßR2 and Sox9 normalized to HPRT in MSC pellet cultures under chondrogenic and hypertrophy enhancing conditions analyzed by real time PCR. TGFßR1 is significantly down-regulated under hypertrophic conditions on day 17, 21 and 28 (**A**). TGFßR2 is down-regulated under hypertrophic conditions on day 28 (**B**). Sox9 is down-regulated on day 17 and 21 in hypertrophic MSC pellets (**C**). n= 7 different donors.

Analysis of whole cell lysates with Western Blot revealed that, similar to the gene expression analysis, the TGFßR1 protein amount is decreased under hypertrophic conditions on day 21 and day 28 compared to chondrogenic conditions (Figure 38 A). Sox9 protein amount is reduced on day 21 in hypertrophic cell pellets but not on day 28 (Figure 38 B).

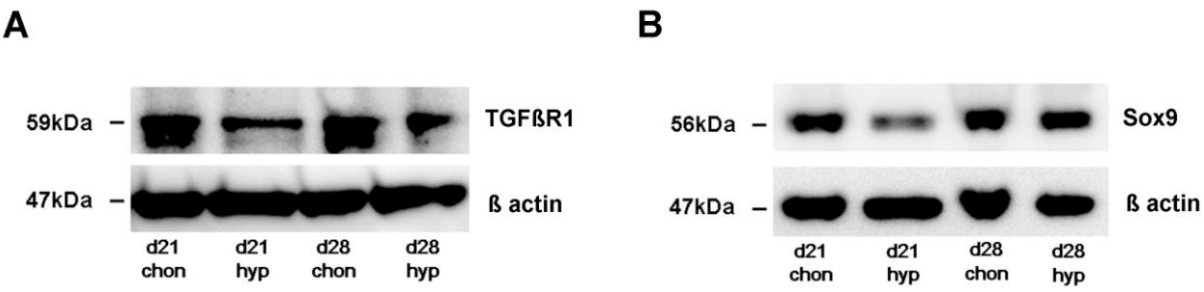

Figure 38 Western Blot analysis of TGFßR1 and Sox9. TGFßR1 protein amount is reduced on day 21 and day 28 under hypertrophic conditions (**A**). Sox9 protein level is decreased on day 21 under hypertrophic conditions (B). ß actin was taken as loading control.

4.3.2 TGFß signalling activity

Similar to BMP signalling, TGFß signalling utilizes intracellular Smad proteins to transduce the signal from the activated receptor to the nucleus. In contrast to BMP signalling that activates Smad1, 5, 8, TGFß signalling activates Smad2, 3. In order to determine whether there are differences in TGFß signalling activity between chondrogenic and hypertrophic MSC pellets Western Blot analysis for the phosphorylated forms of Smad2 and Smad3 were performed.

Western Blot analysis revealed a clear decrease in the amount of phospho-Smad2 and phospho-Smad3 in hypertrophic MSC pellets compared to chondrogenic pellets. During the chondrogenic pre-differentiation phase and on day 21 and day 28 under chondrogenic conditions a strong phospho-Smad2 and phospho-Smad3 protein band can be detected. In contrast under hypertrophic conditions nearly no phospho-Smad 2 and phospho-Smad3 can be detected on day 21 and day 28. The total amount of Smad2 and Smad3 protein and ß actin were taken as loading control (Figure 39). This result indicates that TGFß signalling is less active under hypertrophic conditions compared to chondrogenic conditions.

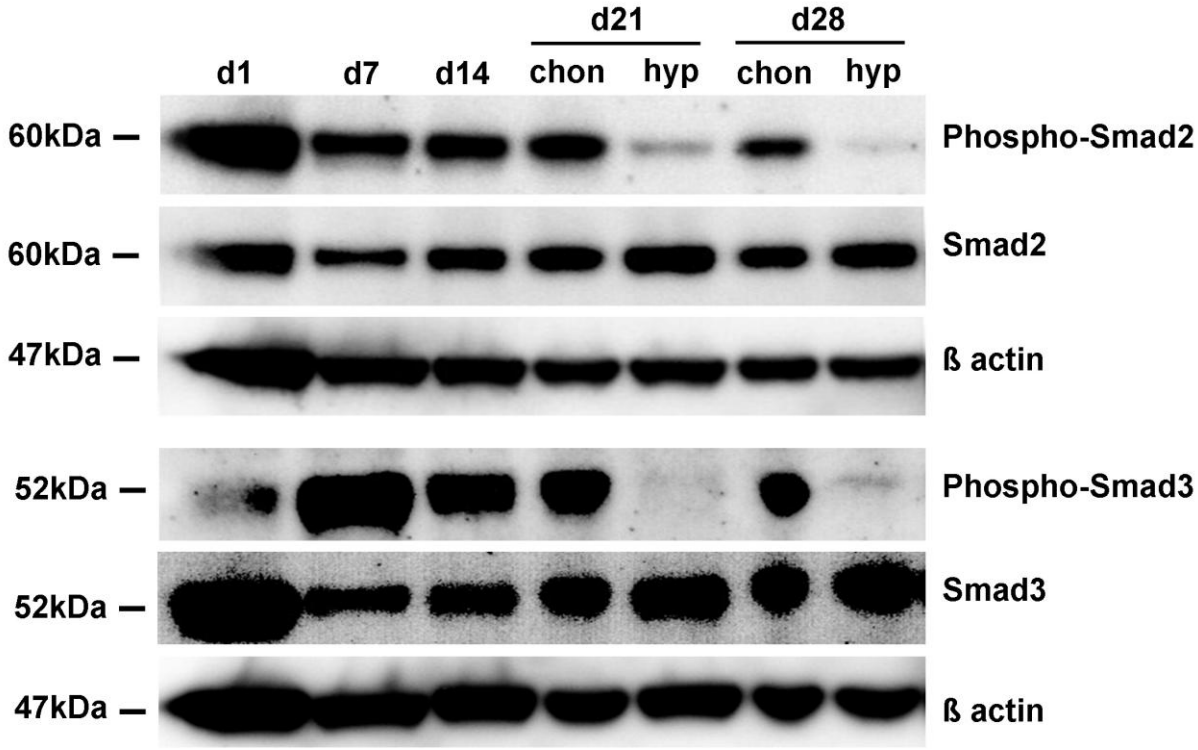

Figure 39 TGFß signalling activity. Western Blot analysis of phospho-Smad2 and phospho-Smad3 under chondrogenic and hypertrophic conditions. The amount of phospho-Smad2 and phospho-Smad3 is reduced under hypertrophic conditions on day 21 and day 28 compared to chondrogenic conditions. The total amount of Smad2/Smad3 and ß actin were taken as loading control.

4.3.3 The TGFß inhibitor (SB431542) does not increase hypertrophy

The experimental data obtained from above showed that TGFß receptors are down-regulated under hypertrophic conditions. Furthermore Western Blot analysis of the phosphorylated forms of Smad2 and Smad3 revealed a decreased TGFß signalling activity under hypertrophic conditions. We hypothesize that the reduced TGFß signalling activity under hypertrophic conditions contributes to the enhancement of hypertrophy. To confirm this hypothesis, we investigated the effect of the TGFß antagonist SB431542 on hypertrophic establishment, in the *in vitro* hypertrophy model. SB431542 inhibits TGFßR1 kinase activity and thereby inhibit the phosphorylation of Smad3 (Callahan, J. F. *et al.*, 2002).

Under chondrogenic conditions treatment with SB431542 from day 14 until day 28 does not increase hypertrophy. DMMB staining revealed no histological difference between MSC pellets that were treated with low doses of SB431542 (Figure 40 B, C) and chondrogenic control aggregates (Figure 40 A). In all conditions a hyaline cartilage like morphology developed with little sign of hypertrophy. High doses of SB431542 lead to a dedifferentiation of the pellets (Figure 40 D). Similarly, there is no difference in ALP staining between chondrogenic control aggregates (Figure 40 E) and SB431542 treated aggregates (Figure 40 F, G, H). In addition, no difference in Collagen type II staining can be detected between chondrogenic control pellets (Figure 40 I) and MSC pellets that were treated with low doses of SB431542 (Figure 40 J, K). However, Collagen type II staining is strongly reduced after treatment with 10 µM SB431542 (Figure 40 L). Collagen type X staining is weak in chondrogenic control aggregates (Figure 40 M) and in aggregates treated with low doses of SB431542 (Figure 40 N, O). In MSC pellets that were treated with 10 µM SB431542 no collagen type X staining can be detected (Figure 40 P).

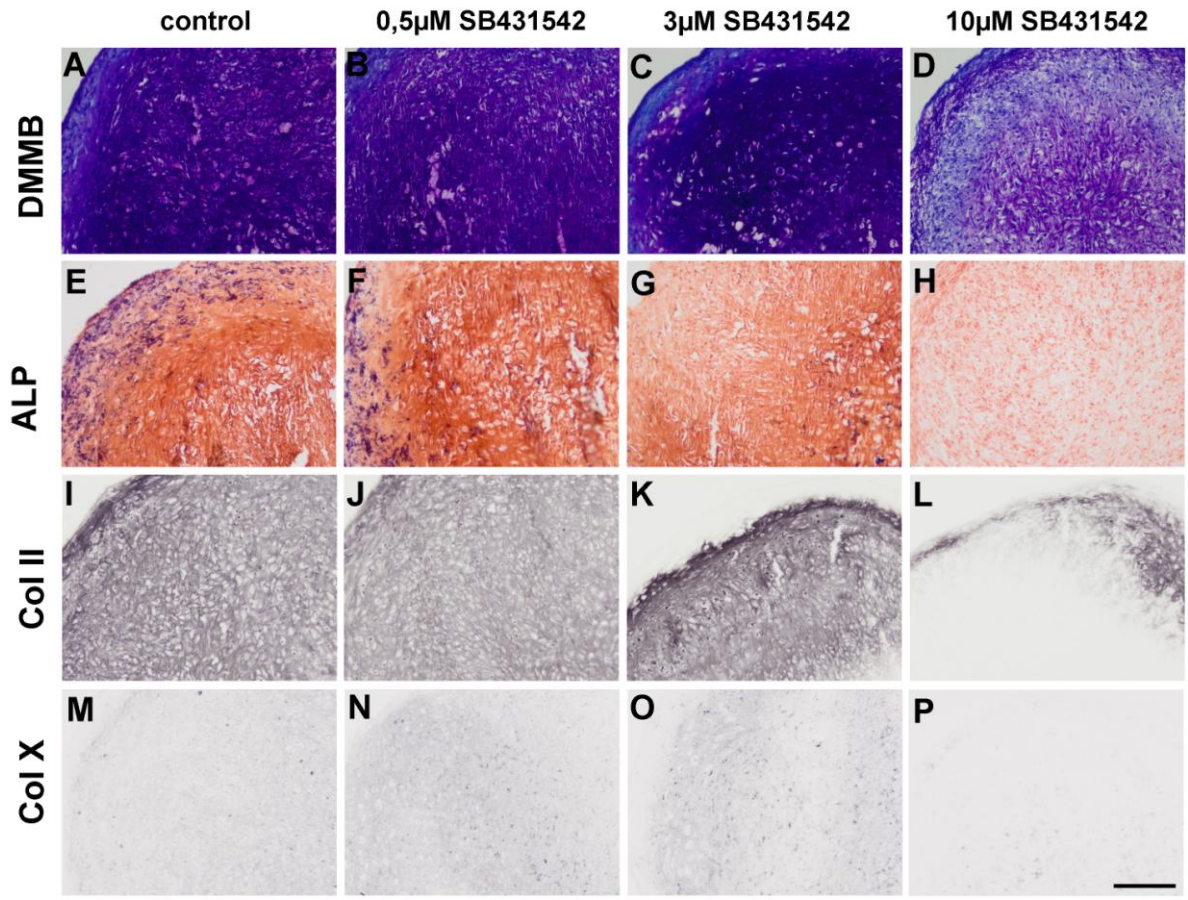

Figure 40 Histological appearance of MSC pellet cultures on day 28 after SB431542 treatment under chondrogenic conditions. No change in DMMB, ALP, collagen type II and collagen type X staining can be detected between chondrogenic control pellets (**A, E, I, M**) and pellets treated with low doses of SB431542 (**B, C, F, G, J, K, N, O**). High doses of SB431542 lead to a dedifferentiation of the cells shown by decreased DMMB staining (**D**) and collagen type II staining (**L**). Scale bar = 200 µm.

The previous results showed that inhibition of TGFß signalling with SB431542 does not enhance hypertrophy under chondrogenic conditions. Furthermore we demonstrated that BMP4 increases hypertrophy under hypertrophic conditions but not under chondrogenic conditions. Next we wanted to investigate whether we can increase hypertrophy under chondrogenic conditions by inhibiting TGFß signalling with SB431542 and simultaneous addition of BMP4. SB431542 and BMP4 were added to the medium from day 14 until day 28.

As already seen in 4.2.2.1, addition of BMP4 to chondrogenic medium has no apparent effect on the amount of hypertrophic cells (Figure 41 A). Upon addition of the TGFß inhibitor SB431542 the amount of hypertrophic cells increases with increasing SB431542 concentration (Figure 41 B, C, D). Furthermore, there are only few ALP positive cells in MSC pellets that were only treated with BMP4 (Figure 41

E). The amount of ALP positive cells increases in MSC pellets that were treated with BMP4 and the TGFß inhibitor (Figure 41 F, G, H) with the highest amount of ALP positive cells in pellets that were treated with 10 µM SB431542 (Figure 41 H). Collagen type II staining is strong in chondrogenic MSC pellets that were treated with BMP4 (Figure 41 I) and collagen type II staining is unchanged in MSC pellets that were incubated in chondrogenic medium containing BMP4 and the TGFß inhibitor (Figure 41 J, K, L). Of note, MSC pellets that were treated with the highest concentration of the TGFß inhibitor SB431542 together with BMP4 do not dedifferentiate and show a strong collagen type II staining, indicating that BMP4 preserves chondrogenic differentiation after SB431542 treatment (Figure 41 L). Collagen type X staining is low in chondrogenic MSC pellets that were treated with BMP4 (Figure 41 M). Addition of SB431542 together with BMP4 increases Collagen type X staining (Figure 41 N, O, P).

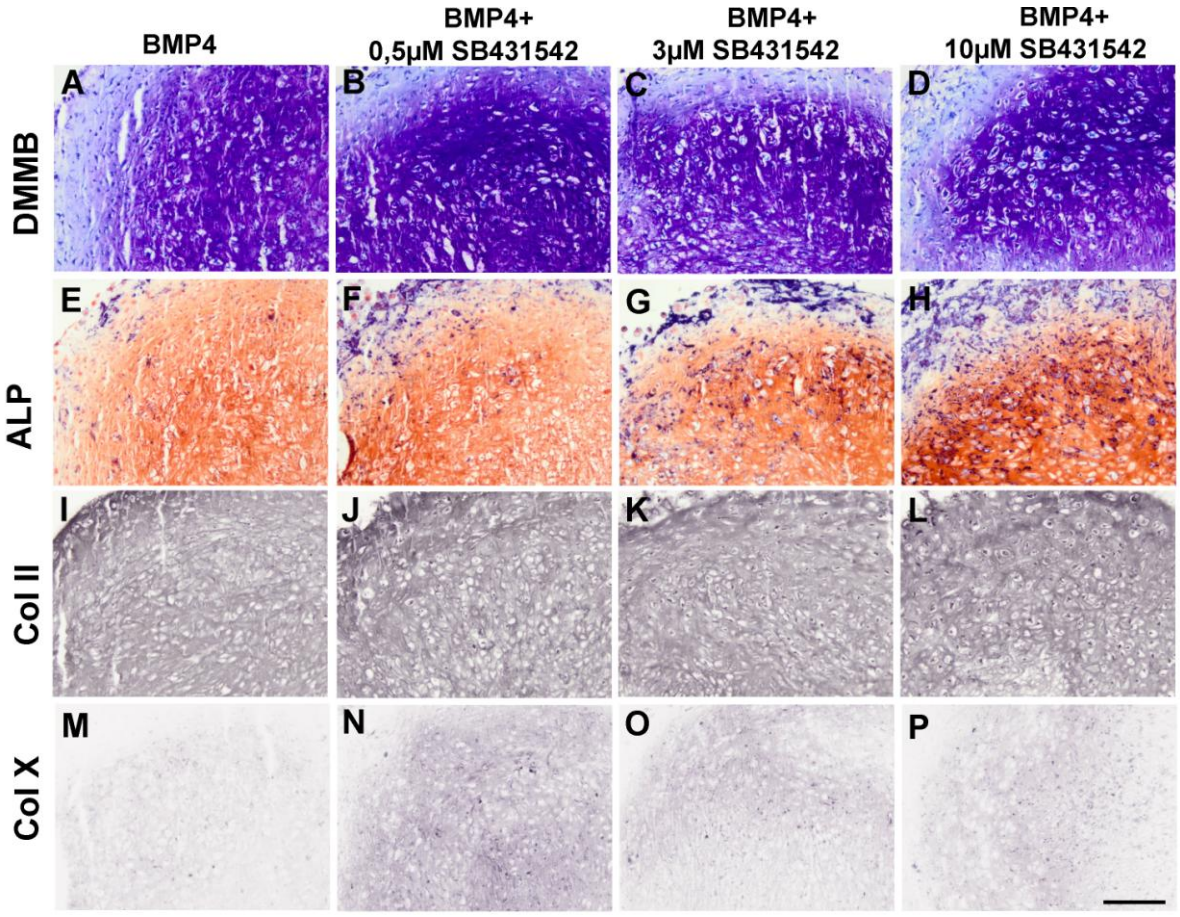

Figure 41 Histological appearance of MSC pellet cultures on day 28 after SB431542 and BMP4 treatment under chondrogenic conditions. Concomitant application of SB431542 and BMP4 increases hypertrophy in a dose dependent manner shown by and increased amount of hypertrophic (**B-D**) and ALP positive (**F-H**) cells and increased collagen type X staining (**N-P**) after SB431542 and BMP4 treatment, compared to pure BMP4 treatment (**A, E, M**). No difference in collagen type II staining can be detected between BMP4 treated control pellets (**I**) and pellets treated with SB431542 and BMP4 (**J-L**). Scale bar = 200 µm.

Under hypertrophic conditions, the TGFß inhibitor SB431542 does not additionally enhance hypertrophy. DMMB staining revealed that MSC pellets that were treated with SB431542 from day 14 until day 28 show a similar histological phenotype as hypertrophic control pellets. No difference in the amount of hypertrophic cells can be detected between hypertrophic control pellets (Figure 42 A) and pellets that were treated with the TGFß inhibitor (Figure 42 B, C, D). In contrast to chondrogenic conditions, high doses of SB431542 do not lead to a dedifferentiation of the cells (Figure 42 D). Furthermore there is no difference in the amount of ALP positive cells between hypertrophic control aggregates (Figure 42 E) and SB431542 treated aggregates (Figure 42 F, G, H). No difference in collagen type II staining can be detected between hypertrophic control pellets (Figure 42 I) and pellets that were treated with SB431542 (Figure 42 J, K, L). Of note, MSC pellets that were treated with 10 µM SB431542 show a strong collagen type II staining (Figure 42 L). Collagen type X staining is strong under hypertrophic control conditions (Figure 42 M). Treatment with 0,5 µM and 3 µM SB431542 does not alter collagen X staining (Figure 42 N, O). However, MSC pellets that were treated with 10 µM of SB431542 show a slightly reduced collagen type X staining (Figure 42 P).

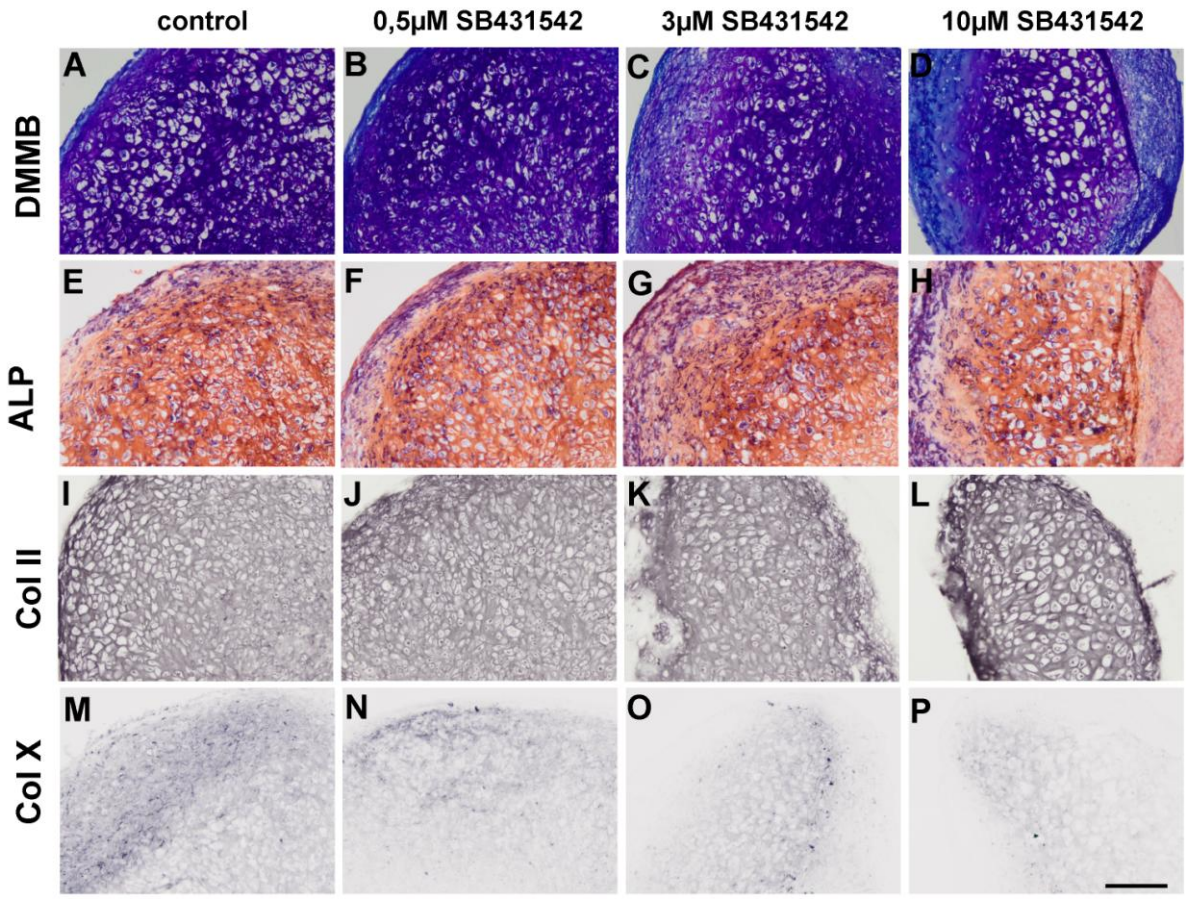

Figure 42 Histological appearance of MSC pellet cultures on day 28 after SB431542 treatment under hypertrophic conditions. No difference in DMMB, ALP, collagen type II and collagen type X staining can be detected between hypertrophic control pellets (**A, E, I, M**) and SB431542 treated pellets (**B-D, F-H, J-L, N-P**). Scale bar = 200 µm.

In summary these results showed that the TGFß inhibitor SB431542 increases hypertrophy under chondrogenic conditions when applied together with BMP4. Under hypertrophic conditions, the TGFß inhibitor does not further enhance hypertrophy.

4.4 Involvement of other signalling pathways in the regulation of hypertrophy

This study clearly showed an involvement of BMP and TGFß signalling in the regulation of MSC hypertrophy. Biological processes are regulated by different signalling pathways that work together to generate a specific outcome.
In the following we aimed to analyze the influence of other signalling pathways in the regulation of MSC hypertrophy. This was done in a preliminary experiment with whole genome microarray analysis using samples of one donor. In the evaluations we focused on the differential analysis of the expression of genes involved in signalling pathways that regulate chondrocyte differentiation, FGF, Indian hedgehog/PTHrP and Wnt signalling.

4.4.1 FGF signalling

Gene expression analysis showed that many FGF signalling associated genes are regulated in the *in vitro* hypertrophy model indicating a role of this signalling pathway in the regulation of MSC hypertrophy. For instance, FGF receptors (FGFR1, FGFR2 and FGFR3) and FGF14 and FGF7 are up-regulated under hypertrophic conditions. FGF1 is down-regulated under hypertrophic conditions (Table 5).

FGF signalling pathway				
Gene	d17	d21	d28	Gene function
BGLAP	-	+3,1	+3,9	Bone gamma-carboxyglutamate protein
CDH2	-	+2,8	+4,4	Cadherin 2
FGF1	-2,6	-3,3	-8,0	fibroblast growth factor 1
FGF14	-	+2,5	+3,9	fibroblast growth factor 14
FGF7	-	-	+2,4	fibroblast growth factor 7
FGFR1	-	+2,3	+2,4	fibroblast growth factor receptor 1
FGFR2	+2,4	+4,1	+5,8	fibroblast growth factor receptor 2

FGFR3	-	-	+2,2	fibroblast growth factor receptor 3
FOS	-3,0	-3,6	-5,4	FBJ murine osteosarcoma viral oncogene homologe transcription factor
GAB1	-	+2,2	+3,4	GRB2-associated binding protein 1
HGF	+4,8	+2,9	+4,0	hepatocyte growth factor
SPP1	-	-2,4	-2,2	Secreted phosphoprotein 1
+ fold up-regulation under hypertrophic conditions, - fold down-regulation under hypertrophic conditions				

Table 5 Regulation of FGF signalling associated genes in the *in vitro* hypertrophy model.

4.4.2 Indian hedgehog signalling

Hedgehog signalling associated genes are regulated as a function of hypertrophy. Indian hedgehog (IHH) and the IHH receptor Patched 1 (PTCH1) are up-regulated under hypertrophic conditions. Modulators of Indian hedgehog signalling like dispatched homolog 1 (DISP1) and hedgehog interacting protein (HHIP) are also up-regulated in hypertrophic MSC pellets (Table 6).

Indian Hedgehog Signalling				
Gene	d17	d21	d28	Gene function
DISP1	-	+2,4	+3,4	Dispatched homolog 1, required for hedgehog signalling
HHIP	-	+2,0	+3,0	Hedgehog interacting protein
IHH	-	+2,2	+3,0	Indian hedgehog
PTCH1	-	-	+2,3	Patched 1, receptor for sonic hedgehog
+ fold up-regulation under hypertrophic conditions, - fold down-regulation under hypertrophic conditions				

Table 6 Regulation of hedgehog signalling associated genes in the *in vitro* hypertrophy model.

4.4.3 Wnt signalling

Microarray analysis detected differences in the expression of Wnt signalling associated genes between chondrogenic and hypertrophic conditions. The Wnt receptor FZD5 (Frizzled homolog 5) and the co-receptor LRP5 (Low density lipoprotein receptor-related protein 5) are up-regulated under hypertrophic conditions. Furthermore the Wnt signalling inhibitor DKK1 (dickkopf homolog 1) is strongly down-regulated under hypertrophic conditions (Table 7).

Wnt Signalling				
Gene	d17	d21	d28	Gene function
ANKRD6	-3,4	-3,7	-3,4	Ankyrin repeat domain 6
DAB2	+2,2	+2,4	+2,1	Disabled homolog 2, mitogen-responsive phophoprotein
DKK1	-14,7	-5,9	-	Dickkopf homolog 1, inhibitor of Wnt signalling
FZD5	-	+2,2	+2,2	Frizzled homolog 5, receptor for Wnt signalling
LRP5	-	-	+2,1	Low density lipoprotein receptor-related protein 5, co receptor for Frizzled
+ fold up-regulation under hypertrophic conditions, - fold down-regulation under hypertrophic conditions				

Table 7 Regulation of Wnt signalling associated genes in the in vitro hypertrophy model.

These data point towards increased canonical Wnt signalling under hypertrophic conditions. In the absence of canonical Wnt signalling cytosolic ß-catenin is phosphorylated by the GSK-3ß and becomes ubiquitynated and directed for proteasom-assisted degradation. Upon activation of the Wnt signalling pathway, the GSK-3ß is inhibited resulting in decreased ß-catenin phosphorylation and degradation. Western Blot analysis of phospho-ß-catenin showed decreased phosphorylation of ß-catenin under hypertrophic conditions, indicating increased Wnt signalling activity in hypertrophic MSC pellets (Figure.43).

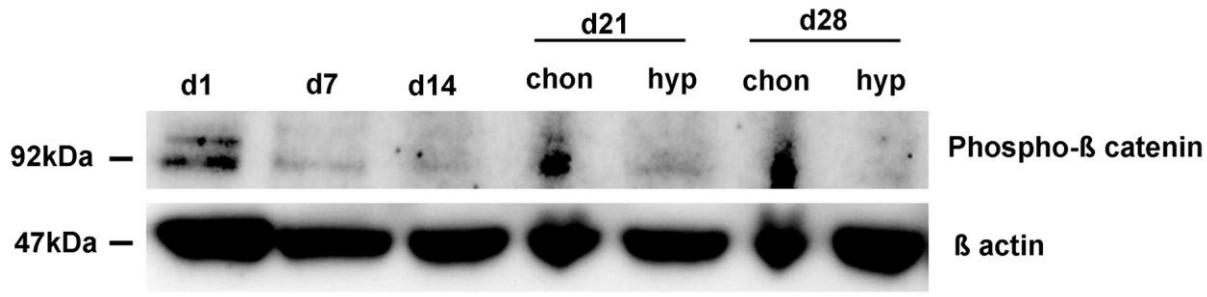

Figure 43 Western Blot analysis of phospho-ß-catenin. The amount of phospho-ß-catenin is reduced under hypertrophic conditions on day 21 and 28 compared to chondrogenic conditions. ß actin was taken as loading control.

5 Discussion

5.1 Induction of hypertrophy

Human MSCs are able to differentiate chondrogenically, however some hypertrophic markers are expressed. This hypertrophic phenotype of chondrogenic differentiating MSCs can be experimentally enhanced by changing medium conditions from chondrogenic to hypertrophy enhancing medium as described previously (Mueller, M. B. & Tuan, R. S., 2008; Mueller, M. B. *et al.*, 2010). This change in medium conditions includes withdrawal of TGFß and dexamethasone and the addition of the thyroid hormone T3. Using this *in vitro* hypertrophy model for MSCs we could significantly increase the hypertrophic phenotype of chondrogenic differentiating MSCs. The enhancement of hypertrophy was clearly shown by an increased cell size, stronger collagen type X staining, higher ALP activity and increased expression osteocalcin in hypertrophic MSC pellets. Hypertrophy markers are not exclusively expressed under pro-hypertrophic conditions but also under standard chondrogenic conditions but to a lower degree. There are some ALP positive cells in the centre of chondrogenic MSC pellets but ALP activity is mainly restricted to the periphery of the pellet. It was described recently that fibroblast like cells surround MSC pellets (Yoo, J. U. *et al.*, 1998). Based on this, we suppose that the ALP positive ring consists of fibroblast like cells rather than hypertrophic cells. On protein level we detected a clear difference in collagen type X between chondrogenic and hypertrophic MSC pellets but on gene expression level no significant differences between hypertrophic and chondrogenic conditions can be detected. This phenomenon has been described by other investigators (Barry, F. *et al.*, 2001; Sekiya, I. *et al.*, 2002; Mwale, F. *et al.*, 2006). The reason therefore is unclear. Post-transcriptional modulations may account for that phenomenon. In addition, after hypertrophic induction, areas of cellular hypertrophy as well as dedifferentiated areas were detected. The dedifferentiated areas may attenuate the collagen type X signal in the PCR under hypertrophic conditions.

5.2 Influence of BMP signalling on MSC hypertrophy

A huge amount of studies demonstrated that BMPs play a significant role during the process of endochondral ossification in skeletal development (Yoon, B. S. & Lyons, K. M., 2004). It has been shown that BMPs promote mesenchymal condensation, chondrogenic differentiation and maturation towards the hypertrophic chondrocyte. In order to examine whether the BMP signalling pathway may also be involved in the regulation of MSC hypertrophy we performed a differential gene expression analysis of components of BMP signalling including ligands, receptors and transcription factors, comparative between chondrogenic and hypertrophic MSC aggregates. We detected a clear up-regulation of BMP4 under hypertrophy enhancing conditions whereas BMP2 was not regulated and BMP7 mRNA could not be detected in an amount sufficient for proper quantification. Among BMP ligands we focused on these three growth factors as they are expressed by growth plate chondrocytes undergoing endochondral ossification (Wozney, J. M. & Rosen, V., 1998) and are all able to induce hypertrophy of cultured embryonic chondrocytes (Suzuki, F., 1992; Rosen, V. *et al.*, 1994; Leboy, P. S. *et al.*, 1997; Volk, S. W. *et al.*, 1998). BMP4 was also regulated on protein level and, in contrast to BMP2 and BMP7, BMP4 seems to play a role in the enhancement of hypertrophic differentiation in chondrogenic differentiating MSCs in the *in vitro* hypertrophy model. Consistent with these findings are studies on embryonic chondrocytes that showed that BMP2, 4 and 7 are all capable of inducing hypertrophy in chick embryonic chondrocytes but BMP4 is more effective than other BMPs (Volk, S. W. *et al.*, 1998). Furthermore *in vivo* studies showed that overexpression of BMP4 in the cartilage of transgenic mice resulted in an increased hypertrophic zone indicating increased differentiation into hypertrophic chondrocytes (Tsumaki, N. *et al.*, 2002). In mouse growth plate chondrocytes, thyroid hormone enhances hypertrophy by induction of BMP2 (Ballock, R. T. & O'Keefe, R. J., 2003). In addition a recent study showed that the thyroid hormone T3 stimulates collagen type X expression in cultured chick chondrocytes through stimulation of BMP4 expression (Lassova, L. *et al.*, 2009). These studies indicate that thyroid hormone effects on chondrocyte maturation are mediated, at least in part, by stimulation of BMP signalling. Similar to this these results indicate that in human MSCs thyroid hormone induces hypertrophy through induction of BMP4.

Analysis of BMP receptor expression revealed that the BMPR1B is significantly up-regulated in hypertrophic aggregates. In contrast, other BMP receptors (BMPR1A, Alk2, BMPR2) were not regulated upon induction of hypertrophy in our experiments. Studies with constitutive active (CA) and dominant negative (DN) BMP receptors showed that CA BMPR1B increased ALP activity and collagen type X expression in embryonic chick sternum chondrocytes. The effect of BMPR1A was less pronounced. DN BMPR1B in sternum chondrocytes blocked BMP induced hypertrophy more efficiently than DN BMPR1A. This indicates that the major type I BMP receptor involved in chondrocyte hypertrophy is BMPR1B (Enomoto-Iwamoto, M. *et al.*, 1998; Volk, S. W. *et al.*, 2000; Grimsrud, C. D. *et al.*, 2001). This assumption is consistent with our observation of increased BMPR1B expression in hypertrophic MSC aggregates. Our results suggest that BMPR1B could be the main receptor for transduction of pro-hypertrophic BMP signals in chondrifying MSCs. However, this needs to be confirmed by functional experiments.

Prior studies showed that BMP2, 4 and 7 induce the expression of the transcription factor Runx2 (Tsuji, K. *et al.*, 1998; Lee, K. S. *et al.*; Nishimura, R. *et al.*, 2002). Runx2 is expressed in chondrocytes at low level at the beginning of cartilage development and is strongly up-regulated in hypertrophic chondrocytes (Inada, M. *et al.*, 1999; Kim, I. S. *et al.*, 1999). Runx2 seems to be an essential transcription factor for chondrocyte terminal differentiation (Enomoto, H. *et al.*, 2000; Enomoto-Iwamoto, M. *et al.*, 2001; Takeda, S. *et al.*, 2001; Ueta, C. *et al.*, 2001). In Runx2 deficient mice chondrocyte maturation is decreased (Inada, M. *et al.*, 1999; Ueta, C. *et al.*, 2001) and over-expression of Runx2 in growth plate chondrocytes leads to increased chondrocyte hypertrophy (Takeda, S. *et al.*, 2001; Ueta, C. *et al.*, 2001). Based on these data we hypothesized that increased BMP4 expression in hypertrophic MSC pellets activates Runx2 expression and Runx2 in turn activates the expression of genes involved in the establishment of hypertrophy. It has been shown that different hypertrophy associated markers like osteocalcin (Geoffroy, V. *et al.*, 2002), collagen type X (Leboy, P. *et al.*, 2001), matrix metalloproteinase (MMP)-13 (Jimenez, M. J. *et al.*, 1999) and VEGF (Zelzer, E. *et al.*, 2001) are activated by Runx2. We were able to demonstrate that Runx2 is significantly up-regulated in hypertrophic aggregates compared to chondrogenic aggregates. Similar to embryonic development, during chondrocyte pre-differentiation Runx2 is expressed to a low degree and the expression strongly increases under

hypertrophic conditions with the highest Runx2 expression on day 28. This suggests that Runx2 is an essential transcription factor for the terminal differentiation of MSCs. However, the observed increase in Runx2 expression seems not to be controlled exclusively by BMP signalling as real time PCR analysis revealed that treatment of MSCs with BMP4 does not significantly increase Runx2 expression and inhibition of BMP signalling with the BMP inhibitor noggin does not significantly decrease Runx2 expression (data not shown). This indicates that Runx2 expression may be regulated by different signalling pathways. Other signalling molecules have been shown to regulate Runx2 expression, among them retinoids (Jimenez, M. J. *et al.*, 1999), PTHrP (Guo, J. *et al.*, 2006) and FGF (Zhou, Y. X. *et al.*, 2000). Wnt signalling has also been shown to induce Runx2 expression (Gaur, T. *et al.*, 2005). Microarray data showed a pronounced down-regulation of the Wnt inhibitor DKK1 under hypertrophic conditions and Western Blot analysis for phospho-ß-catenin demonstrated decreased amount of phospho-ß-catenin in hypertrophic MSC pellets. This points towards increased canonical Wnt signalling under hypertrophic conditions and may contribute to increased Runx2 expression in hypertrophic MSC pellets. In addition TGFß and dexamethasone decrease Runx2 expression (Alliston, T. *et al.*, 2001; Kang, J. S. *et al.*, 2005; Li, X. *et al.*, 2005). Under hypertrophic conditions in the *in vitro* hypertrophy model, TGFß and dexamethasone are excluded from the medium and this may lead to a reduced suppression of Runx2 expression through TGFß and dexamethasone and therefore increased Runx2 expression.

Previous results demonstrated that BMP4 is strongly up-regulated under hypertrophic conditions and points towards a significant role of BMP4 in the induction of hypertrophy. In order to investigate a functional role of this up-regulation we investigated the effect of recombinant human BMP4 on chondrogenic differentiating MSCs. Hypertrophy in the *in vitro* hypertrophy model of chondrogenic differentiating MSCs is induced by withdrawal of TGFß and dexamethasone and the addition of thyroid hormone (Mackay, A. M. *et al.*, 1998; Mueller, M. B. & Tuan, R. S., 2008). Different studies showed that thyroid hormone is able to induce hypertrophy in embryonic mesenchymal cells, growth plate chondrocytes and MSCs (Leboy, P. S. *et al.*, 1997; Mackay, A. M. *et al.*, 1998; Okubo, Y. & Reddi, A. H., 2003; Mueller, M. B. & Tuan, R. S., 2008). Here we demonstrated that in hypertrophic medium after withdrawal of TGFß and dexamethasone but without

addition of T3, both the cell size and ALP activity remained relatively low, but are increased compared to standard chondrogenic medium. Addition of T3 to this medium, which results in our standard pro-hypertrophic medium, clearly enhanced hypertrophy shown by an increased cell size, an increased amount of hypertrophic cells and increased ALP activity. The addition of BMP4 instead of T3 to the medium significantly increased cell size, ALP activity and collagen type X expression compared to hypertrophic medium with and without T3. BMP4 has a very strong pro-hypertrophic effect and seems to be more effective in inducing hypertrophy of chondrogenic differentiating MSCs than T3. This more efficient enhancement of hypertrophy could be due to higher BMP4 concentration when BMP4 is added to the medium. On the other hand, thyroid hormone could induce regulators that ameliorate the effect of up-regulated BMP4-expression. In order to confirm that the pro-hypertrophic effect of T3 is mediated by BMP4 we compared BMP4 expression between aggregates that were incubated in hypertrophic medium with and without T3 and detected significantly higher BMP4 expression in cultures that were incubated in medium with T3. This suggests that the thyroid hormone-induced enhancement of hypertrophy in this *in vitro* model is mediated by BMP4. For growth plate chondrocytes in mice it was shown that thyroid hormone induces terminal differentiation through induction of BMP2 (Ballock, R. T. *et al.*, 2000) and in chick growth plate chondrocytes thyroid hormone induces hypertrophy through increased BMP4 expression (Lassova, L. *et al.*, 2009). We could not see regulation of BMP2 expression by thyroid hormone in our model but similar to chick chondrocytes we detected regulation of BMP4. This indicates that there are species specific differences in the responsiveness to thyroid hormone. In chick chondrocytes and human MSCs thyroid hormone induces the expression of BMP4 whereas in mouse growth plate chondrocytes BMP2 expression is induced.

The pro-hypertrophic effect of BMP4 was only seen under hypertrophic conditions in which TGFß and dexamethasone were withdrawn. Under chondrogenic conditions with continuous application of TGFß and dexamethasone, BMP4 did not elicit the enhancement of the hypertrophic phenotype in chondrogenic MSC pellets. It had no effect on cell size, ALP activity and collagen type X gene expression. We suppose that this is due to TGFß and dexamethasone which are included in the chondrogenic medium. TGFß and dexamethasone are known to have anti-hypertrophic effects in growth plate and chicken sternum chondrocytes (Ballock, R.

T. *et al.*, 1993; Leboy, P. S. *et al.*, 1997; Ferguson, C. M. *et al.*, 2000) and we suppose that these anti-hypertrophic effects override the pro-hypertrophic effect of BMP4 under standard chondrogenic conditions. This phenomenon has been observed by other studies. Hanada et al showed that rat MSCs that were treated with BMP2 became hypertrophic and that effect was abolished when cells were incubated with BMP2 and TGFß (Hanada, K. *et al.*, 2001). Furthermore Sekiya et al showed that human MSCs that were treated with BMP4 in the presence of TGFß did not develop a hypertrophic phenotype (Sekiya, I. *et al.*, 2005). Other studies investigated the effect of different BMPs on MSC hypertrophy with controversially results. Schmitt et al showed that BMP2 is not able to induce hypertrophy in human MSCs. MSCs that were treated with BMP2 or a combination of BMP2 and TGFß differentiated chondrogenically with no signs of hypertrophy (Schmitt, B. *et al.*, 2003). In contrast rat periosteum-derived progenitor cells treated with BMP2 developed hypertrophic chondrocytes (Hanada, K. *et al.*, 2001). This indicates that there are species specific variations in the responsiveness to different BMPs. In human MSCs, Steinert et al showed that both BMP2 and BMP4 gene transfer into human MSCs enhance hypertrophy of MSCs (Steinert, A. F. *et al.*, 2009). This result is in line with our observation that exogenous applied BMP4 enhances hypertrophy.

As BMP4 clearly enhanced hypertrophic differentiation of MSCs in the absence of anti-hypertrophic TGFß signalling we assumed that inhibition of BMP signalling would block hypertrophy in MSCs. In order to investigate this we blocked BMP signalling in chondrogenic differentiating MSCs with the BMP inhibitor noggin. Noggin binds different BMPs with high affinity to BMP4, 2 and 7 and thereby prevents their interaction with the BMP receptor (Zimmerman, L. B. *et al.*, 1996). We could show that noggin inhibits the T3 induced hypertrophy in MSCs. Noggin treatment reduced the amount and size of hypertrophic cells, ALP activity and collagen type X gene expression as well as protein deposition in the extracellular matrix under pro-hypertrophic conditions. These result obtained here are consistent with studies on embryonic chondrogenesis. *In vivo* studies showed that over-expression of the BMP antagonist noggin in the developing chick limb bud prevents chondrocyte hypertrophy and the expression of hypertrophic markers like collagen type X and ALP (Pathi, S. *et al.*, 1999). Furthermore over-expression of Noggin in transgenic mice leads to a lack of hypertrophic chondrocytes (Tsumaki, N. *et al.*, 2002). *In vitro* it was shown that noggin prevents T3 stimulation of collagen type X

expression in chick chondrocytes (Lassova, L. *et al.*, 2009). Besides the suppression of hypertrophy markers, we detected lower expression of collagen type II and at 100 ng/ml noggin less metachromatic staining of the extracellular matrix. We think that this is due to dedifferentiation of the cells after withdrawal of the chondro-inductive growth factor TGFß and blocking of BMP signalling.

Under standard chondrogenic conditions with continuous application of TGFß and dexamethasone, hypertrophy markers are also expressed but to a lower degree than in hypertrophic cultures. This phenomenon has been described in various MSC cell culture systems and it was shown that after ex vivo transplantation of MSC pellets spontaneous hypertrophy, matrix calcification and vascular invasion occurs (Pelttari, K. *et al.*, 2006; Bian, L. *et al.*, 2011). To examine whether we can block the spontaneous induction of hypertrophy under standard chondrogenic conditions by inhibition of BMP signalling, we incubated MSC pellets under chondrogenic conditions in the presence of noggin. However, hypertrophy markers such as collagen type X expression and ALP activity were not suppressed by noggin. In addition there was no effect on collagen type II expression. This suggests that under standard chondrogenic conditions other factors than BMP signalling are involved in the development and maintenance of this phenomenon.

Our results showed that BMP signalling associated genes are up-regulated under hypertrophic conditions indicating increased BMP signalling activity under hypertrophic conditions, especially through strongly increased BMP4 expression. Although the effect of BMP signalling on chondrocyte maturation has been extensively studied, the mechanisms of BMP effects are not fully understood. It is possible that BMP4 also acts via Smad independent pathways. It has been shown that BMPs can activate certain MAPK pathways such as TAK1/p38 (Shim, J. H. *et al.*, 2009) in a Smad independent manner. In order to further investigate the signalling mechanism of BMP4 we analysed the effect of dorsomorphin, a small molecule inhibitor that inhibits BMP induced Smad1, 5, 8 phosphorylation by binding to the kinase domain of the type I receptor and inhibiting its kinase activity (Yu, P. B. *et al.*, 2008; Wrighton, K. H. *et al.*, 2009). Similar to noggin, dorsomorphin inhibits T3 induced hypertrophy in a dose dependent manner. The amount of hypertrophic, ALP positive cells and collagen type X expression is decreased after dorsomorphin treatment. Upon treatment with high dorsomorphin doses, collagen type II staining is also decreased and cells dedifferentiate. Under chondrogenic conditions,

dorsomorphin had no influence on hypertrophic extend and chondrogenic differentiation. No difference in collagen type X and collagen type II staining could be detected between chondrogenic control aggregates and dorsomorphin treated aggregates. These results are in line with the data observed after noggin treatment. Both BMP inhibitors, noggin that blocks the interaction of the ligand with the receptor and dorsomorphin that inhibits the activation of Smad1, 5, 8 through the activated BMP receptor type I, are able to inhibit T3 induced hypertrophy. This result demonstrates that BMP4 signals via Smad-dependent pathway and would suggest increased phosphorylation of BMP signalling associated Smad1, 5, 8 under hypertrophic conditions. In the Western Blot analysis we did not detect a clear difference in the level phosphorylated Smad1, 5, 8 between chondrogenic and hypertrophic conditions, whereas in the immunohistochemistry a clear accumulation of phospho-Smad1, 5, 8 in the nucleus of hypertrophic cells was detectable. A possible explanation for this phenomenon is that for Western Blot analysis whole MSC pellets were taken. Histological analysis suggests that in hypertrophic pellets less phosopho-Smad1, 5, 8 positive cells are present. We suppose that these are the hypertrophic cells that are surrounded by dedifferentiated cells or apoptotic cells that do not express phospho-Smad1, 5, 8. The staining of these hypertrophic Smad1, 5, 8 positive cells is very strong and seems to accumulate in the nucleus, where phopho-Smad1, 5, 8 acts as transcription factor to regulate gene expression. In contrast, in chondrogenic pellets more cells express phosopho-Smad1, 5, 8 but to a lower degree. We think that these are the chondrogenic cells that show low BMP signalling activity due to intrinsic BMP signalling. Recent studies furthermore showed that TGFß can also induce Smad1, 5 phosphorylation in different cell types including mesenchymal cells independent of BMP signalling (Goumans, M. J. *et al.*, 2002; Goumans, M. J. *et al.*, 2003; Daly, A. C. *et al.*, 2008; Liu, I. M. *et al.*, 2009; Wrighton, K. H. *et al.*, 2009). This may also explain the high phospho-Smad1, 5, 8 signal under chondrogenic conditions in the Western Blot analysis.

Real time PCR revealed increased BMP2 expression at the beginning of chondrogenic differentiation on culture day 3 and Western Blot analysis for Smad1, 5, 8 revealed activated BMP signalling throughout the chondrogenic pre-differentiation phase. Based on these findings we were interested whether BMP signalling during early phases of chondrogenic differentiation is important for priming cells for later hypertrophic differentiation. Maybe the time point of BMP inhibition

plays a role in the induction of hypertrophy in MSCs. In the previous experiments noggin and dorsomorphin treatment started on day 14, after the chondrogenic pre-differentiation phase. In the following MSC pellets were treated with dorsomorphin during the chondrogenic pre-differentiation phase and then transferred to hypertrophic or chondrogenic medium, or were treated with dorsomorphin over the whole culture period.

Inhibition of BMP signalling during the chondrogenic pre-differentiation phase from day 1 until day 14, followed by incubation in hypertrophic medium severely disturbs hypertrophic differentiation. Low doses of dorsomorphin reduce the amount of hypertrophic, ALP positive cells and collagen type X staining. This indicates that inhibition of BMP signalling during early phases of chondrogenic differentiation reduces hypertrophy. Higher doses of dorsomorphin lead to the dedifferentiation of the cells with no collagen type II expression. We suppose that inhibition of BMP signalling during the chondrogenic pre-differentiation phase disturbs chondrogenic differentiation and makes the cells more susceptible for the deprivation of TGFß on day 14. In general, we hypothesize that if the cells reach a certain level of chondrogenic differentiation during the chondrogenic pre-differentiation phase, they become hypertrophic after deprivation of TGFß. But if the cells do not reach this differentiation level, they dedifferentiate after deprivation of TGFß.

Under chondrogenic conditions, inhibition of BMP signalling during early stages of chondrogenic differentiation does not inhibit the expression of hypertrophy markers as there is no difference in collagen type X deposition between control and dorsomorphin treated aggregates. Interestingly, inhibition of BMP signalling with high doses of dorsomorphin over the whole culture period reduces the expression of hypertrophic markers like collagen type X and ALP. However, both inhibition of BMP signalling during the pre-differentiation phase and over the whole culture period does no influence chondrogenic differentiation and does not lead to a dedifferentiation of the cells. The fact that under chondrogenic conditions, inhibition of BMP signalling over the whole culture period inhibits the expression of the hypertrophy markers collagen type X and ALP but does not reduce the chondrogenic marker collagen type II is a promising approach for the use of MSCs in articular cartilage repair. However our results are only based on immunohistochemical analysis and additional, more sensitive assays, for example

real time PCR analysis of chondrogenic and hypertrophic markers after dorsomorphin treatment are needed to confirm this observation.

The common observation that under hypertrophic conditions after inhibition of BMP signalling with high doses of noggin or dorsomorphin cells dedifferentiated is presumably due to the combination of withdrawal of TGFß and inhibition of BMP signalling. Under chondrogenic conditions the continuous application of TGFß promotes chondrogenic differentiation and prevents the cells from dedifferentiating after inhibition of BMP signalling. TGFß signalling seems to be sufficient for chondrogenic differentiation of MSCs and BMP signalling is not needed as further pro-chondrogenic stimulus. Studies on MSC chondrogenesis pointed towards a pro-chondrogenic role of BMPs. A recent work from Sekiya demonstrated that BMP2, BMP4 and BMP6 combined with TGFß3 induced chondrogenic phenotype in cultured bone marrow derived human MSC pellets (Sekiya, I. *et al.*, 2005). In contrast, in this model of chondrogenic differentiating MSCs BMP signalling is not required for chondrogenic differentiation as long as TGFß is in the medium. Furthermore our results showed that the addition of BMP4 to chondrogenic medium does not increase chondrogenic markers like collagen type II. This observation is accordant with results from Barry et al demonstrating that TGFß3 alone is sufficient for chondrogenic differentiation in human MSCs derived from bone marrow (Barry, F. *et al.*, 2001) whereas synovium derived MSCs needed BMP2 and TGFß for optimal chondrogenic differentiation (Shirasawa, S. *et al.*, 2006).

In summary, we could show that hypertrophy in MSCs is enhanced by thyroid hormone via induction of BMP4 and BMP4 is a potent regulator in late differentiation stages in chondrifying MSCs. This thyroid hormone-induced enhancement of hypertrophy by BMP4 can be inhibited with the BMP antagonists noggin and dorsomorphin. Both BMP inhibitors had no effect under standard chondrogenic conditions when applied after the chondrogenic pre-differentiation phase, however inhibition of BMP signalling with dorsomorphin over the whole culture period reduced hypertrophic marker like collagen type X but not chondrogenic marker like collagen type II. The goal in cartilage tissue engineering is to generate phenotypically stable articular cartilage. Therefore it is important to develop effective methods to maintain the articular phenotype without hypertrophy. Attempts to avoid hypertrophic differentiation during MSC chondrogenesis based on the use of molecules that display an inhibitory effect during growth plate development have

been developed. Delayed addition of PTHrP or FGF during *in vitro* chondrogenesis of MSCs have been shown to inhibit the expression of collagen type X but also that of collagen type II (Mwale, F. *et al.*, 2010; Weiss, S. *et al.*, 2010; Mueller, M. B. *et al.*, 2013). Here we demonstrated that under chondrogenic conditions, prolonged incubation of MSC pellets with the BMP inhibitor dorsomorphin inhibits the expression of collagen type X but does not reduce collagen type II expression. The use of dorsomorphin or other BMP inhibitors may be a promising approach to inhibit hypertrophy in chondrogenic differentiating MSCs, however further analysis of gene expression of hypertrophic and chondrogenic markers after prolonged dorsomorphin treatment are needed to confirm preliminary histological analysis.

The BMP signalling pathway is regulated by different BMP antagonists. Soluble BMP antagonists bind BMPs and prevent their interaction with the receptor. During development, the functions of BMPs are spatially and temporally regulated by different specific antagonists. We hypothesized that BMP antagonists could play a role in the regulation of MSC hypertrophy by antagonizing BMP signalling. During skeletal development noggin is expressed in condensing cartilage and in immature chondrocytes (Brunet, L. J. *et al.*, 1998). This indicates that noggin antagonizes BMP signalling in immature chondrocytes and prevents them from hypertrophic differentiation. In addition, two human genetic disorders, symphalangism and multiple synostoses syndrome are caused by a mutation in the noggin gene and are characterized by bony fusions of the joints (Gong, Y. *et al.*, 1999). In the *in vitro* hypertrophy model, we found that noggin is significantly down-regulated during early phases of MSC hypertrophy indicating a role of noggin in the induction of MSC hypertrophy. Chordin, another BMP antagonist, is highly expressed in the proliferation zone and very low in the hypertrophic zone of the growth plate during limb development. This indicates a role of chordin in the regulation of BMP signalling activity during chondrocyte maturation (Zhang, D. *et al.*, 2002). Furthermore, ectopic expression of chordin in the developing limb results in a delayed chondrocyte hypertrophy (Zhang, D. *et al.*, 2002). In the *in vitro* hypertrophy model we detected a significant down-regulation of chordin on day 28 in hypertrophic pellets. It is possible that noggin and chordin cooperate to regulated BMP signalling activity during hypertrophic differentiation. During early phases of hypertrophic differentiation, noggin is down-regulated and during later phases of hypertrophic differentiation chordin is down-regulated leading to increased BMP

signalling. Surprisingly, the BMP antagonist gremlin is expressed in an opposing manner. Gremlin is up-regulated under hypertrophic conditions in the *in vitro* hypertrophy model. The function of this up-regulation is unclear. Gremlin has been shown to be highly expressed in non-dividing and terminally differentiated cells and induces apoptosis (Merino, R. *et al.*, 1999). It is possible that gremlin induces apoptosis of hypertrophic cells. Gremlin has also been shown to be pro-angiogenetic (Stabile, H. *et al.*, 2007). During endochondral ossification hypertrophic chondrocytes release angiogenetic factors like VEGF to attract blood vessels. Maybe gremlin has a similar function. Early phases of OA are characterized by hypertrophy of articular chondrocytes. Interestingly, during that phase BMP4 and gremlin are up-regulated (Nakase, T. *et al.*, 2003; Tardif, G. *et al.*, 2004; Tardif, G. *et al.*, 2009). BMP4 induces gremlin expression in OA chondrocytes (Tardif, G. *et al.*, 2004). In normal articular cartilage BMP4 is only expressed to a low degree. This indicates a critical role of BMP4 and gremlin in the induction of differentiation of normally stable articular chondrocytes towards hypertrophic chondrocytes. Similar to this we detected an increased expression of BMP4 and gremlin under hypertrophic conditions. More examinations are needed to unravel the role of gremlin in MSC hypertrophy.

BAMBI is a transmembrane protein with structural similarity to TGFß and BMP receptor type I, but lacks the intracellular kinase domain. BAMBI is able to inhibit both TGFß and BMP signalling. BAMBI has been shown to be co-expressed with BMP4 during xenopus and mouse embryogenesis (Onichtchouk, D. *et al.*, 1999; Grotewold, L. *et al.*, 2001) and BMP4 induces BAMBI expression. We detected a significant up-regulation of BAMBI under hypertrophic conditions. The function of this strong up-regulation is unclear. Preferential inhibition of TGFß signalling could diminish the anti-hypertrophic effect of TGFß which would result in further enhancement of the hypertrophic phenotype. On the other hand, predominant interaction with BMP receptors could ameliorate the BMP induced terminal differentiation in our pro-hypertrophic culture conditions. BAMBI may act as a negative feedback loop to inhibit BMP signalling in response to increased BMP4 expression. The clear up-regulation of BAMBI in our experiment suggests that BAMBI is a modulator in terminal differentiation in MSC chondrogenesis. To clarify the role of BAMBI in MSC chondrogenesis, functional experiments with knock-down and overexpression of BAMBI started.

5.3 Influence of TGFß signalling on MSC hypertrophy

Endochondral ossification is a highly regulated process that involves the interaction of different signalling pathways. Besides BMP signalling, TGFß signalling plays a crucial role during chondrocyte proliferation, differentiation and maturation and TGFß is abundantly expressed in the growth plate (Matsunaga, S. *et al.*, 1999). TGFß signalling has been shown to stimulate chondrogenesis of mesenchymal cells and embryonic chondrocytes *in vitro* and *in vivo* (Seyedin, S. M. *et al.*, 1986; Joyce, M. E. *et al.*; Schofield, J. N. & Wolpert, L., 1990; Leonard, C. M. *et al.*, 1991; Chimal-Monroy, J. & Diaz de Leon, L., 1997; Lorda-Diez, C. I. *et al.*). Similar to this, in the cell culture system we use here MSC chondrogenesis is induced by TGFß1. Prior work also demonstrated that lowering the TGFß1 concentration decreases chondrogenesis in MSC pellets (Johnstone, B. *et al.*, 1998). Besides the importance of TGFß during chondrocyte differentiation, TGFß has been shown to prevent chondrocyte maturation during skeletal development by keeping cells in a pre-hypertrophic state. *In vitro* studies with different chondrocyte cell culture models demonstrated that TGFß treatment inhibits maturation of chondrocytes into hypertrophic cells (Gelb, D. E. *et al.*, 1990; Ballock, R. T. *et al.*, 1993; Ferguson, C. M. *et al.*). In addition Ferguson et al. showed that during *in vivo* skeletogenesis increased TGFß signalling inhibits chondrocyte maturation and expression of hypertrophy associated genes like collagen type X (Ferguson, C. M. *et al.*, 2004). We believe that TGFß also inhibits MSC hypertrophy in our cell culture system. Chondrogenic differentiating MSCs are induced to increase their hypertrophic phenotype by deprivation of TGFß and the addition of T3. The withdrawal of TGFß is a prerequisite for the enhancement of hypertrophy. The continuous application of TGFß under chondrogenic standard conditions seems to slow down MSC maturation. This was confirmed by our study. We were able to demonstrate that deprivation of TGFß and dexamethasone from the medium increases hypertrophy compared to medium with TGFß and dexamethasone. This is a first hint that TGFß seems to inhibit MSC hypertrophy. In order to further investigate the role of TGFß signalling on MSC hypertrophy we compared the expression of TGFß associated genes between chondrogenic and hypertrophic conditions in the *in vitro* hypertrophy model. Besides the reduced exogenous application of TGFß under hypertrophic conditions we wanted to investigate whether intrinsic TGFß signalling is down-regulated under hypertrophic conditions. Real time PCR revealed that TGFß

receptors are down-regulated under hypertrophic conditions. The TGFßR1 is significantly down-regulated on day 17, day 21 and day 28 in hypertrophic MSC pellets. This result was confirmed on protein level using Western Blot analysis where we detected reduced TGFßR1 protein in hypertrophic MSC pellets. In addition, the TGFßR2 is down-regulated on day 28 in hypertrophic pellets. These findings are in accordance with the notion that transgenic mice that express a defective TGFßR2, have been shown to have increased hypertrophic differentiation and premature collagen type X expression in the growth plate and develop early osteoarthritis characterized by the replacement of articular cartilage by hypertrophic cartilage and bone (Serra, R. *et al.*, 1997). Based on these results we hypothesized that under hypertrophic conditions TGFß signalling is reduced through 1) deprivation of TGFß in the medium and 2) reduced TGFß receptor expression. Indeed Western Blot analysis for TGFß signalling associated Smad2 and 3 revealed decreased amounts of phospho-Smad2 and phospho-Smad3 under hypertrophic conditions. We suppose that this reduced TGFß signalling under hypertrophic conditions contributes to the enhancement of the hypertrophic phenotype. TGFß signalling has been shown to induce the expression of the transcription factor Sox9 (Chimal-Monroy, J. *et al.*, 2003; Furumatsu, T. *et al.*, 2009). During skeletal development in the growth plate, Sox9 is expressed in all chondrocyte progenitors and chondrocytes but its expression is completely turned off in hypertrophic chondrocytes (Ng, L. J. *et al.*, 1997; Zhao, Q. *et al.*, 1997). Sox9 is a key transcription factor in chondrocyte differentiation (Lefebvre, V. *et al.*, 1998) and negatively regulates maturation of chondrocytes to the hypertrophic stage. Down-regulation of Sox9 is required for chondrocyte hypertrophy. Based on this we supposed that decreased TGFß signalling under hypertrophic conditions leads to decreased Sox9 expression that then leads to hypertrophic differentiation. Gene expression of Sox9 in the *in vitro* hypertrophy model showed that Sox9 is down-regulated in hypertrophic MSC pellets on day 17 and day 21, however on day 28 there is a tendency towards reduced Sox9 expression under hypertrophic conditions, but not significantly. Protein analysis for Sox9 using Western Blot provided similar results. Sox9 protein is decreased on day 21 under hypertrophic conditions but not on day 28. The reason for this phenomenon is unclear. Loss of function models of TGFß showed that decreased TGFß signalling increases chondrocyte hypertrophy *in vivo* (Serra, R. *et al.*, 1997; Yang, X. *et al.*, 2001). To

determine whether the reduction of TGFß signalling in our cell culture system increases hypertrophy in chondrogenic differentiating MSCs we used the TGFß inhibitor SB431542, a potent inhibitor of TGFßR1 kinase activity (Callahan, J. F. *et al.*, 2002). Under chondrogenic conditions, where normally TGFß signalling activity is very high, inhibition of TGFß signalling does not increase hypertrophy. However, high doses of the TGFß inhibitor lead to a dedifferentiation of the pellets. This was shown by decreased metachromic DMMB staining and decreased collagen type II staining. This demonstrates that the pure reduction of TGFß signalling leads to a dedifferentiation of the cells rather than to an increased hypertrophy.

Our results demonstrated that BMP4 has strong pro-hypertrophic properties and is able to increases hypertrophy under hypertrophic conditions but failed to increase hypertrophy under chondrogenic conditions in the presence of TGFß. We could show that a combined treatment of MSC pellets under chondrogenic conditions with the TGFß inhibitor and the pro-hypertrophic BMP4 is able to increase hypertrophy. This was demonstrated by an increased amount of hypertrophic and ALP positive cells and increased collagen type X staining. Furthermore dedifferentiation of the pellets with high doses of the TGFß inhibitor is prevented with BMP4 treatment. In conclusion a simultaneous reduction in TGFß signalling activity together with activation of BMP signalling is needed to push the cells towards a hypertrophic cell fate. The pure reduction of TGFß signalling is not sufficient to increase hypertrophy. Similar to this we already showed that under standard chondrogenic conditions with high TGFß signalling activity, the addition of BMP4 is not sufficient to induce hypertrophy. Both TGFß and BMP signalling have to be modulated, TGFß signalling has to be decreased and BMP signalling has to be increased, to change the phenotype of the cells.

In addition to the clearly demonstrated involvement of TGFß and BMP signalling in this model, our data suggest that dexamethasone may contribute to the regulation of hypertrophy in our model. Our results showed that withdrawal of TGFß and dexamethasone (hyp-T3) increases the hypertrophic phenotype compared to standard chondrogenic conditions but to a much lower extent than after additional application of T3 (Figure 17, 18). In the experiments with the TGFß inhibitor SB431542 (Figure 40), inhibition of TGFß mediated Smad2 and Smad3 activation without withdrawal of dexamethasone does not induce this mild hypertrophic phenotype. Steroids like dexamethasone have been shown to inhibit hypertrophy in

growth plate chondrocytes (Leboy, P. S. *et al.*, 1997). One possible explanation for this difference in the response of cells to application of the TGFß inhibitor compared to the withdrawal of TGFß and dexamethasone is that dexamethasone can have an inhibitory effect on hypertrophy in chondrogenic MSC cultures. On the other hand, the activation of other signalling pathways than Smad2 and Smad3 activation by TGFß may contribute to that. Similar to the experiments with the TGFß inhibitor, the combination of TGFß and dexamethasone withdrawal and the addition of BMP4 leads to a more pronounced hypertrophic phenotype than the addition of BMP4 with blocking of TGFß mediated Smad2 and Smad3 activation in the presence of dexamethasone (Figure 18, 41). This difference may be explained again by an inhibitory effect of dexamethasone on hypertrophy. The effect of dexamethasone on MSC hypertrophy could be investigated by additional functional experiments.

Under hypertrophic conditions TGFß signalling is already relatively low due to deprivation of TGFß from the medium and eventually intrinsic TGFß signalling is decreased through a reduction in TGFß receptor expression. Inhibition of the residual TGFß signalling activity did not further increase hypertrophy. The pellets that were treated with the TGFß inhibitor differentiated to a hypertrophic phenotype similar to that of standard hypertrophic conditions. This result is not surprising as we demonstrated that TGFß signalling activity is already very weak under hypertrophic conditions and inhibition of this minor intrinsic TGFß signalling activity has no further effect on the hypertrophic outcome. Of note, high doses of the TGFß inhibitor do not lead to a dedifferentiation of the cells, presumably the activated BMP signalling under hypertrophic conditions preserves the cells form dedifferentiating.

Here we could show that under hypertrophic conditions TGFß signalling is decreased through down-regulation of TGFß receptors and the deprivation of TGFß from the medium. This contributes to the increased hypertrophic phenotype under hypertrophic conditions. Additional inhibition of intrinsic TGFß signalling does not further increase hypertrophy based on histology. Inhibition of TGFß signalling under chondrogenic conditions does not increase hypertrophy however inhibition of TGFß signalling together with addition of BMP4 increases hypertrophy. Similar to this, *In vivo* studies showed that inhibition of TGFß signalling with loss of function models of TGFß increased chondrocyte hypertrophy (Serra, R. *et al.*, 1997; Yang, X. *et al.*, 2001). In these *in vivo* studies growth factors like BMPs are expressed and available

in the growth plate and might, together with the reduced TGFß signalling, promote chondrocyte hypertrophy.

In summary the results obtained in this study bring some light into the regulation of hypertrophy in MSC chondrogenesis. Our results provide evidence that MSC hypertrophy is dependent on a balance between TGFß and BMP signalling. The current experimental data support the following working model: in the absence of both, TGFß and BMP signalling MSCs dedifferentiate characterized by decreased collagen type II expression. Reduced TGFß signalling and active BMP signalling leads to hypertrophic differentiation of the cells. Active TGFß signalling and active BMP signalling as well as active TGFß signalling and reduced BMP signalling lead to chondrogenic differentiation of the cells.

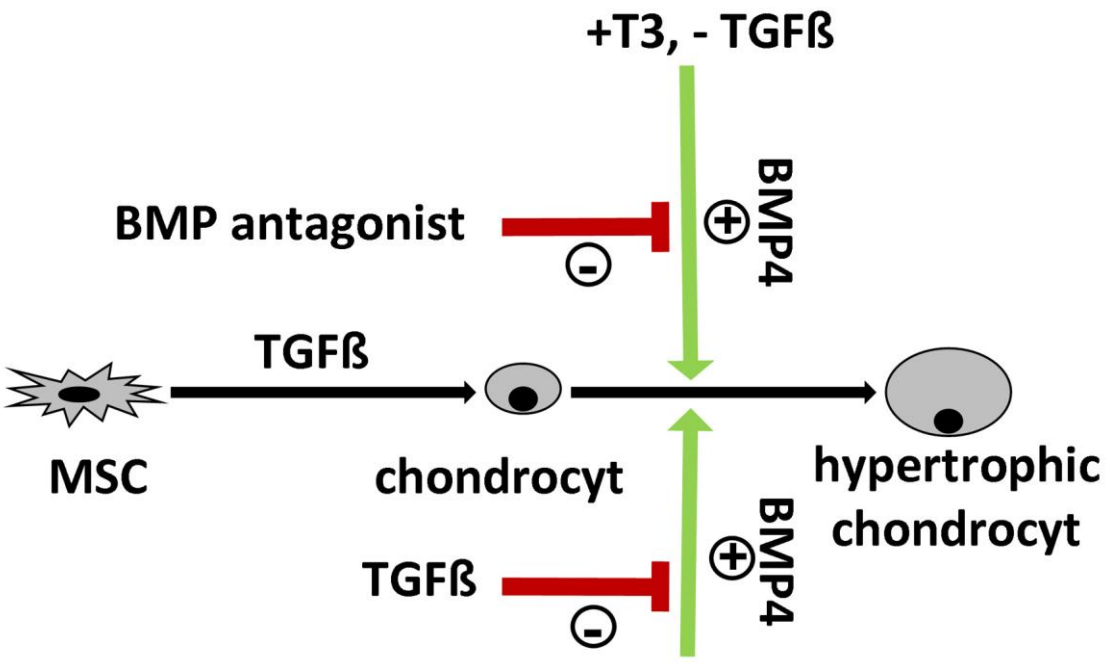

Figure 44. Model for the influence of BMP and TGFß signalling in the regulation of MSC hypertrophy.

These data provide valuable information for the use of MSCs in articular cartilage repair. Both conditions are artificial *in vitro* conditions and the ultimate goal in cartilage tissue engineering will be the generation of *in vivo* phenotypically stable articular cartilage. As tissue engineering constructs will be exposed to factors like thyroid hormone, BMPs and TGFß these results have *in vivo* relevance and add

important information to the knowledge of the biology of especially the late differentiation stages in MSC chondrogenesis. These results may help to find ways to improve the performance of MSC-based cartilage constructs *in vivo*. Finally, advantage of this for cartilage tissue engineering unfavourable enhancement of hypertrophy by thyroid hormone or BMP4 can be taken for the repair of critical size bone defects. Endochondral ossification via hypertrophic cartilage takes place in both bone development and secondary fracture healing (Kronenberg, H. M., 2003) and hypertrophic engineered cartilage has been suggested as a template for bone defect repair (Scotti, C. *et al.*, 2010; Farrell, E. *et al.*, 2011).

5.4 Involvement of other signalling pathways in MSC hypertrophy

Multiple signalling pathways are involved in the regulation of growth plate chondrogenesis and hypertrophy. Besides BMP and TGFß signalling, other signalling pathways have been implicated in the regulation of embryonic chondrogenesis including FGF signalling, Hedgehog signalling and Wnt signalling. We investigated the gene expression of signalling molecules involved in these pathways comparative between chondrogenic and hypertrophic conditions using microarray analysis. Preliminary results from one patient showed that genes involved in these pathways are differentially expressed in the *in vitro* hypertrophy model. FGF ligands and FGF receptors are differentially expressed between chondrogenic and hypertrophic conditions. Indian hedgehog (IHH) and the IHH receptor Patched 1 (PTCH1) are up-regulated under hypertrophic conditions. Wnt receptor and co-receptor expression is increased and the expression of the Wnt inhibitor DKK1 is decreased under hypertrophic conditions. These preliminary data provide evidence that these signalling pathways may be involved in the regulation of MSC chondrogenesis and hypertrophy. However the results from this analysis were derived from cells of only one donor and need to be confirmed by further experiments.

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7 List of Abbreviations

ACT	Autologous chondrocyte transplantation
ALP	Alkaline phosphatase
Asc	Ascorbat
BAMBI	BMP and activin membrane-bound inhibitor
BMBI	BMP and activin membrane-bound inhibitor
BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
Cbfa1	Core binding factor alpha 1
chon	Chondrogenic
Col	Collagen
Dex	Dexamethasone
DKKs	Dickkopfs
DMEM	Dulbecco´s modified eagle´s medium
DMMB	Dimethylmethylene blue
DN	Dominant negativ
DNA	Desoxyribonucleic acid
Dorso	Dorsomorphin
ECM	Extracellular matrix
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FGFR	FGF receptor
GAG	Glucosaminoglycan
GDF	Growth and differentiation factor

GSK	Glycogen synthase kinase
HGF	Hepatocyte growth factor
HMG	High mobility group
hyp	Hypertrophic
IH	Immunohistochemistry
Ihh	Indian hedgehog
ITS	Insulin-transferrin-sodium selenite
l	Litre
LAPs	Latency-associated proteins
LRP5/6	Low-density lipoprotein receptor-related protein 5 and 6
MACT	Matrix-associated autologous chondrocyte transplantation
MAPK	Mitogen activated protein kinase
ml	Millillitre
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
OA	Osteoarthritis
PBS	Phosphate buffered saline
PKC	Protein kinase C
PTHrP	Parathyroid hormone-regulated peptide
PVDF	Polyvinylidenfluoride
Pyr	Pyruvate
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid

Runx	Runt-related transcription factor
sFRP	Secreted frizzled-related proteins
Sox	Sry-related high mobility group box
SRY	Sex-determining region on the Y chromosome
T3	Tri-iodothyronine
T4	Thyroxine
TGF	Transforming growth factor
TGFßR	Transforming growth factor receptor
TR	Thyroid hormone receptor
TRE	T3 responsive element
VEGF	Vascular endothelial factor
WB	Western Blot

8 List of Figures

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11 Selbstständigkeitserklärung

Ich, Karl Alexandra geboren am 14.9.1982 in München erkläre hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe.

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