Thyroid Hormone-Induced Hypertrophy in Mesenchymal Stem Cell Chondrogenesis Is Mediated by Bone Morphogenetic Protein-4

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Chondrogenic differentiating mesenchymal stem cells (MSCs) express markers of hypertrophic growth plate chondrocytes. As hypertrophic cartilage undergoes ossification, this is a concern for the application of MSCs in articular cartilage tissue engineering. To identify mechanisms that elicit this phenomenon, we used an *in vitro* hypertrophy model of chondrifying MSCs for differential gene expression analysis and functional experiments with the focus on bone morphogenetic protein (BMP) signaling. Hypertrophy was induced in chondrogenic MSC pellet cultures by transforming growth factor β (TGF β) and dexamethasone withdrawal and addition of triiodothyronine. Differential gene expression analysis of BMPs and their receptors was performed. Based on these results, the *in vitro* hypertrophy model was used to investigate the effect of recombinant BMP4 and the BMP inhibitor Noggin. The enhancement of hypertrophy could be shown clearly by an increased cell size, alkaline phosphatase activity, and collagen type X deposition. Upon induction of hypertrophy, BMP4 and the BMP receptor 1B were upregulated. Addition of BMP4 further enhanced hypertrophy in the absence, but not in the presence of TGF β and dexamethasone. Thyroid hormone induced hypertrophy by upregulation of BMP4 and this induced enhancement of hypertrophy could be blocked by the BMP antagonist Noggin. BMP signaling is an important modulator of the late differentiation stages in MSC chondrogenesis and the thyroid hormone induces this pathway. As cartilage tissue engineering constructs will be exposed to this factor *in vivo*, this study provides important insight into the biology of MSC-based cartilage. Furthermore, the possibility to engineer hypertrophic cartilage may be helpful for critical bone defect repair.

Introduction

THE ENDOGENOUS REPAIR CAPACITY of articular cartilage is limited and mesenchymal stem cells (MSCs) are a promising cell source for the regeneration of mesenchymal tissue, including articular cartilage. The chondrogenic potential of MSCs has been shown in different matrix-free and matrix-based cell culture systems.^{1–8} In the commonly used pellet culture system, MSCs differentiate chondrogenically in a pellet culture system in a serum-free, strictly defined chondrogenic medium containing TGF β and dexamethasone.^{6,7} However, chondrogenic differentiating MSCs express hypertrophy markers like collagen type X and alkaline phosphatase (ALP).^{1,6,7,9–12} This indicates that MSC chondrogenesis does not stop on a developmental stage typical for articular chondrocytes, but spontaneously proceeds toward the hypertrophic stage, which is typical for growth plate chondrocytes during endochondral bone development. Hypertrophic chondrocytes are characterized by increased cell volume, the extracellular matrix calcifies, and hypertrophic chondrocytes undergo apoptosis. The tissue

is ultimately invaded by blood vessels and osteoprogenitor cells and bone are formed.^{13,14} Vascular invasion and mineralization have also been observed after ectopic transplantation of chondrogenic MSC pellet cultures *in vivo*.¹⁵ This biological behavior of chondrogenic differentiating MSCs raises concern for a tissue engineering application of MSCs in articular cartilage repair. To find strategies to inhibit this phenomenon, it is important to better understand the regulation of late differentiation stages in MSC chondrogenesis and examine the mechanisms that modulate hypertrophy.

The similarity of MSC chondrogenesis and embryonic endochondral ossification suggests that similar mechanisms play crucial roles in both biological processes.¹¹ Whereas little is known about mechanisms modulating terminal differentiation in MSC chondrogenesis, bone morphogenetic proteins (BMPs) are known to be regulators of late differentiation stages in endochondral ossification. BMPs form a subgroup of molecules within the transforming growth factor β (TGF β) superfamily. BMPs have multiple roles during embryonic skeletal development, including mesenchymal

condensation and chondrogenic differentiation of mesenchymal cells.¹⁶⁻²¹ Furthermore, 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 in the presence of BMPs.²²⁻²⁶ *In vivo* studies showed that overexpression of BMP4 in the cartilage of transgenic mice results in an increased hypertrophic zone indicating increased differentiation into hypertrophic chondrocytes, whereas overexpression of the BMP inhibitor Noggin leads to a lack of hypertrophic chondrocytes.¹⁸ Furthermore, overexpression of Noggin in the developing chick limb bud prevents chondrocyte hypertrophy and the expression of hypertrophic markers like collagen type X and ALP.²⁷

Growth plate chondrocytes that differentiate terminally and chondrogenic differentiating MSCs that undergo hypertrophy show similar expression profiles of hypertrophy associated genes and respond similar to changes in the medium conditions. Thyroid hormones induce hypertrophy, whereas TGF β and dexamethasone inhibit hypertrophy.^{8,11,22,28,29} Similar regulatory processes may be involved in the regulation of hypertrophy in growth plate chondrocytes and chondrogenic differentiating MSCs. In mouse growth plate chondrocytes, the thyroid hormone enhances hypertrophy by induction of BMP-2.³⁰ In an *in vitro* hypertrophy model of chondrogenic differentiating human MSCs, the hypertrophic phenotype can be clearly enhanced by modulation of the culture conditions, which includes the administration of triiodothyronine (T3).^{11,12} We hypothesize that this induction of hypertrophy by the thyroid hormone is mediated by BMP. Differential expression analysis of genes involved in BMP signaling, including receptors, ligands, and transcription factors, between chondrogenic and hypertrophic MSC pellet cultures, was carried out. In functional experiments that were based on these results, the effect of the BMP agonist BMP4 and the BMP inhibitor Noggin was investigated.

Material and Methods

Isolation of MSCs

MSCs were isolated from iliac crest bone marrow aspirates of seven male patients, aged 21 to 42 years, undergoing surgery that required autologous bone grafting with approval of the local ethics committee and written consent. MSCs were isolated by Ficoll (Biochrom) gradient centrifugation followed by plastic adhesion. Cells were expanded in the Dulbecco's modified Eagle's medium (DMEM) low glucose (Invitrogen) with 10% fetal calf serum (PAN Biotech GmbH) and 1% penicillin/streptomycin (Invitrogen) at 37°C with 5% CO₂. The medium was changed twice a week and cells were trypsinized at 80% confluence and frozen for later use in liquid nitrogen. After thawing and monolayer expansion, cells were used for the experiments at passage 1.

Flow cytometry

For characterization of MSCs isolated by the method used in our laboratory, flow cytometry was done for MSCs of seven different donors (passage 1 to 7). MSCs were trypsinized and washed with PBS with 1% FCS. Surface marker staining was performed for 30 min at 4°C using directly conjugated antibodies diluted in PBS with 1% FCS. For flow

cytometry, the following antibodies were used: mouse anti CD14-FITC (10 μ g/mL; Acris Antibodies), mouse anti CD29-FITC (10 μ g/mL; Acris Antibodies), mouse anti CD34-FITC (10 μ g/mL; Acris Antibodies), mouse anti CD44-FITC (10 μ g/mL; Acris Antibodies), and mouse anti CD105 (10 μ g/mL; Acris Antibodies). The measurements were conducted with the FACSCalibur Flow Cytometer (Becton Dickinson). Dead cells were stained with propidium iodide.

Chondrogenic differentiation and enhancement of hypertrophy

MSCs were trypsinized and seeded in V-bottomed 96-well polypropylene plates at 200,000 cells per well. Pellets were formed by centrifugation at 250 g for 5 min and chondrogenically differentiated in the DMEM with high glucose (Invitrogen), 1% ITS (Sigma Aldrich), 50 μ g/mL ascorbate-2-phosphate (Sigma Aldrich), 40 μ g/mL L-proline (Sigma Aldrich), 100 nM dexamethasone (Sigma Aldrich), 1 mM sodium pyruvate (Invitrogen), and 10 ng/mL TGF β 1 (R&D Systems).

After a predifferentiation period of 14 days, medium conditions were changed to a hypertrophy enhancing medium consisting of the DMEM high glucose, 1% ITS, 50 μ g/mL ascorbate-2-phosphate, 40 μ g/mL L-proline, and 1 nM triiodothyronine (T3) (Sigma Aldrich) and the control was kept in the chondrogenic medium for the whole culture period. The medium was changed three times per week.

Aggregates were harvested at day 1, 3, 7, 14, 17, 21, and 28 for gene expression analysis. Aggregates for histological analysis were harvested on day 14 and 28.

Modulation of hypertrophy by BMP4

MSC aggregates were cultured in the chondrogenic medium for 14 days. Then, aggregates were distributed in five different groups: (1) the chondrogenic medium; (2) the chondrogenic medium with BMP4 (R&D systems) (100 ng/mL); (3) the hypertrophy enhancing medium, including T3 as previously described; (4) the hypertrophy enhancing medium without T3; and (5) the hypertrophy enhancing medium with BMP4 (100 ng/mL) instead of T3.

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

Modulation of hypertrophy by Noggin

Aggregates were differentiated chondrogenically for 14 days. On day 14, aggregates were either kept in the chondrogenic medium without or with 10 ng/mL or 100 ng/mL recombinant human Noggin (R&D systems) or transferred to the standard hypertrophic medium without or with 10 ng/mL or 100 ng/mL Noggin for an additional 14 days.

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

Histology, histochemistry, and immunohistochemistry

Aggregates were harvested on day 14 and 28, fixed in 4% paraformaldehyde, and 10- μ m-thick frozen sections were prepared.

Sections were stained with dimethylmethylen blue (DMMB) (Sigma Aldrich). Histochemical ALP staining was performed with an alkaline phosphatase kit (Sigma Aldrich) with neutral red as counterstaining.

For immunohistochemistry, the following antibodies were used: mouse anti-collagen type X (1:20; Quartett Immunodiagnostika und Biotechnologie GmbH); mouse anti-collagen type II (1:100; Calbiochem); and mouse anti-BMP4 (1:200; Abcam).

After blocking of endogen peptidases (3% H₂O₂/10% methanol in PBS) for 30 min, sections were incubated in a blocking buffer (10% fetal bovine serum/10% goat serum in PBS) for 60 min at room temperature (RT) followed by incubation with an appropriate primary antibody in the blocking buffer overnight at 4°C. For collagen type II and type X staining, antigen retrieval with pepsin digestion for 15 min at RT and for collagen type X staining, additional hyaluronidase digestion for 60 min at RT were performed before blocking. Immunolabeling was detected with a biotinylated secondary antibody (1:100; Dianova), horseradish peroxidase-conjugated streptavidin (Vector Laboratories), and metal enhanced diaminobenzidine as substrate (Sigma Aldrich). For each antibody, a negative control without a primary antibody was conducted.

RNA isolation, cDNA synthesis, and gene expression analysis

Eight to ten aggregates per condition and time point for each donor were pooled, homogenized in 1 mL TRI Reagent (Sigma Aldrich) using the Power Gen 1000 homogenizator (Fischer Scientific), and RNA was isolated by the Trizol method. Reverse transcription was performed with the Transcriptor First-Strand cDNA Synthesis kit (Roche). Semiquantitative real-time polymerase chain reaction (PCR) was performed with Brilliant SYBR Green QPCR mix (Stratagene) and the Mx3000P QPCR System (Stratagene). Gene expression was normalized to hypoxanthine guanine phosphoribosyltransferase (HPRT) using the delta-Ct method. Primer sequences are shown in Table 1.

Histomorphometry

Images of three representative central sections of DMMB stained day 28 cultures were taken in 20× magnification. The surface area of the cells was analyzed as correlate of the cell size using the ImageJ software (NIH). Per condition three sections of three aggregates from five different patients were analyzed.

ALP activity

The medium was harvested on day 28 and centrifuged for 5 min at maximum speed. A 100 µL supernatant was added to a 100 µL substrate solution (4 mg/mL p-nitrophenol phosphate (Sigma Aldrich) in 1.5 M Tris, 1 mM ZnCl₂, 1 mM MgCl₂, pH 9.0) and continuous absorbance at 405 nm was measured spectrophotometrically in a microplate reader at room temperature (Genius plate reader; Tecan). The change in A₄₀₅ over time (dA/min) was calculated in the linear range of the reaction.³¹

Statistical analysis

The data from the RT-PCR analysis, ALP activity test, and cell surface area analysis are expressed as mean value ± 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 using the Tukey's test. A level of $p < 0.05$ was considered significant.

Results

Characterization of human MSCs

Flow cytometry showed that MSCs were positive for the MSC markers CD29, CD44, and CD105 and negative for the hematopoietic markers CD14 and CD34.

Enhancement of hypertrophy

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 a large lacunae typical for hypertrophic cartilage (Fig. 1b), whereas chondrogenic control aggregates showed a more hyaline cartilage-like morphology with little sign of cellular hypertrophy (Fig. 1a). Collagen type II staining is strong both under chondrogenic (Fig. 1c) and hypertrophic conditions (Fig. 1d). In contrast, collagen type X immunostaining is weak in chondrogenic aggregates (Fig. 1e), but is strongly increased in hypertrophic aggregates (Fig. 1f). ALP staining is increased throughout the aggregates under

TABLE 1. PRIMER SEQUENCES FOR REAL-TIME PCR

Gene	Sequence (forward)	Sequence (reverse)
Alk2	GCCTGGAGCATTTGGTAAGC	CTGCCCACAGTCCTTCAAG
BMP2	TGGATTTCGTGGTGGAAGTGGC	AGGGCATTCTCCGTGGCAGTA
BMP4	CAAACCTGCTGGAAAGGCTC	CCGCTACTGCGAGGGACCTAT
BMP7	CCCAGTGTTTACCGAGGTTTGC	TCCATCCTACTTGCTGTCCTTGTC
BMPR1A	AAACCACTTCCAGCCCTAC	TTTGACACACACAACCTCAC
BMPR1B	CCCTGCATTTGGGGCCGCTAT	GCCTGAAGCTGCAAAAGGCCAC
BMPR2	CCAAGGTCTTGCTGATACGG	CTACCATGGACCATCCTGCT
Col2a1	GGGCAATAGCAGGTTACACGTA	TGTTTCGTGCAGCCATCCT
Col10a1	CCCTCTTGTTAGTGCCAACC	AGATTCCAGTCCTTGGGTCA
HPRT	CGAGATGTGATGAAGGAGATGG	GCAGGTCAGCAAGAATTTATAGC
Runx2	ATACCGAGTGACTTTAGGGATGC	AGTGAGGGTGGAGGGAAGAAG

PCR, polymerase chain reaction.

FIG. 1. Histological appearance of mesenchymal stem cell (MSC) aggregates on culture day 28 under chondrogenic (a, c, e, g) and hypertrophy enhancing conditions (b, d, f, h). (a, b) Dimethylmethylene blue (DMMB) staining. (c, d) Immunohistochemical collagen type II staining. (e, f) Immunohistochemical collagen type X staining. (g, h) Alkaline phosphatase (ALP) staining with neutral red 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) as compared to chondrogenic conditions (a, c, e, g). Scale bar=500 μ m. Negative control for collagen type II (i), collagen type X (j). Scale bar=200 μ m. Color images available online at www.liebertpub.com/tea

prohypertrophic conditions (Fig. 1h), whereas ALP was weaker and mainly located in the periphery in chondrogenic aggregates (Fig. 1g).

Regulation of BMP signaling associated genes under hypertrophy enhancing conditions

BMP4 is significantly upregulated under hypertrophy enhancing conditions on day 17, 21, and 28 as compared to chondrogenic control conditions (Fig. 2a). Differences in BMP2 expression between the chondrogenic and hypertrophic conditions were not found (Fig. 2b). BMP7 expression was too low both in chondrogenic and hypertrophic aggregates to evaluate differences between the two conditions. The BMP receptor 1B (BMPRI1B) is significantly upregulated in the hypertrophic group on day 28 compared to the chondrogenic group (Fig. 2c). All other investigated BMP receptors were not regulated as a function of hypertrophy induction (data not shown). The BMP signaling associated transcription factor Runx2 is significantly upregulated on day 17, 21, and 28 under prohypertrophic conditions (Fig. 2d).

On the protein level, immunohistochemistry for BMP4 on day 28 showed a clearly stronger staining in hypertrophic

(Fig. 2f, h) compared to chondrogenic aggregates (Fig. 2e, g). BMP4 predominantly accumulates in the membrane and cytoplasm of hypertrophic cells (Fig. 2h), whereas BMP4 staining in chondrogenic aggregates is weak in the membrane and the cytoplasm of chondrogenic cells (Fig. 2g).

BMP4 induces hypertrophy

As BMP4 expression is clearly regulated, we investigated the effect of recombinant human BMP4 on chondrogenic differentiating MSCs.

Under standard chondrogenic conditions with continuous application of TGF β and dexamethasone, 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 (Fig. 3a, b). No difference in ALP staining could be detected between BMP4-treated and chondrogenic control aggregates (Fig. 3f, g) and histomorphometry did not show significant differences in the cell size between chondrogenic aggregates with or without BMP4.

The change to prohypertrophic medium conditions with addition of T3 and withdrawal of TGF β and dexamethasone resulted in a hypertrophic phenotype with increased cell size

FIG. 2. (a–d) Gene expression analysis of BMP4, BMP2, BMPR1b, and Runx2 normalized to hypoxanthine guanine phosphoribosyltransferase (HPRT) in MSC pellet cultures under chondrogenic and hypertrophy enhancing conditions analyzed by real-time PCR. BMP4 and Runx2 are significantly upregulated on day 17, 21, and 28 under hypertrophic conditions (a, d). BMPR1b is upregulated on day 28 under hypertrophic conditions (c). BMP2 is not regulated as a function of hypertrophy (b). Values are the mean $\pm$ SD. * $p < 0.05$. $n = 7$ different donors. (e–h) Immunohistochemical BMP4 staining of day 28 MSC aggregates. BMP4 protein staining is increased under hypertrophy enhancing conditions (f, h) as compared to chondrogenic conditions (e, g). Negative control for BMP4 (i). Scale bars = 100 μ m.

and ALP positivity (Fig. 3c, h). In aggregates that were incubated in a medium without TGF β and dexamethasone, but without the addition of T3 (referred to as hyp-T3), the cell size and ALP staining were increased compared to the regular chondrogenic control, but both parameters were clearly less enhanced than in standard hypertrophic cultures that received T3 (Fig. 3d, i). After withdrawal of TGF β and dexamethasone and the addition of BMP4 instead of T3 (referred to as hyp-T3+BMP4), the aggregates developed a very strong hypertrophic phenotype with larger cells com-

pared to all other groups and strong ALP staining (Fig. 3e, j). Histomorphometry detected a significantly increased cell size under all three prohypertrophic conditions compared to standard chondrogenic conditions. Cells were significantly larger in aggregates in the hypertrophic medium with BMP4 compared to the hypertrophic medium with and without T3 (Fig. 3k). The graph in Figure 3l shows the distribution of the cell size representative for cells from one donor.

Under chondrogenic conditions, the addition of BMP4 did not significantly affect collagen type II expression. Under

FIG. 3. (a–j) Histological appearance of MSC pellet cultures on day 28 after BMP4 treatment under chondrogenic (a–b), (f–g) and hypertrophy enhancing conditions (c–e, h–j). (a–e) DMMB staining. (f–j) ALP staining with neutral red as counterstaining. Under chondrogenic conditions, there is no difference in hypertrophic extend between chondrogenic control aggregates (a, f) and BMP4-treated aggregates (b, g). In the standard hypertrophic medium with addition of T3 (c, h) a hypertrophic phenotype is induced. Under hypertrophic conditions without T3 (d, i) the hypertrophic phenotype is less enhanced compared to the standard hypertrophic medium with T3. Addition of BMP4 instead of T3 to the hypertrophic medium further increased the hypertrophic phenotype (e, j). Scale bar = 200 μ m. (k) Histomorphometric cell size analysis of MSC pellets on day 28. Under standard chondrogenic conditions, BMP4 treatment has no influence on the cell size (chon, chon + BMP). Under hypertrophic conditions, the cell size of aggregates treated with BMP (hyp-T3 + BMP) is significantly increased compared to the hypertrophic medium with and without T3 (hyp, hyp-T3). Values are the mean $\pm$ SD. * p < 0.05. n = 5 different donors. (l) Representative histomorphometric analysis for aggregates from one cell donor showing the distribution of cell size on day 28. * p < 0.05. Color images available online at www.liebertpub.com/tea

prohypertrophic conditions, BMP4 treatment (hyp-T3 + BMP4) significantly increased collagen type II expression compared to hypertrophic conditions without BMP4 (hyp and hyp-T3) on day 21 (Fig. 4a). 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 (hyp-T3 + BMP4) compared to hypertrophic aggregates without BMP4 (hyp and hyp-T3) (Fig. 3b).

Histology and histomorphometry indicated that in the hypertrophic medium without T3, there is a less enhanced hypertrophic phenotype compared to the hypertrophic medium with T3. BMP4 expression is significantly increased in aggregates in the hypertrophic medium with T3 compared to the hypertrophic medium without T3 on day 21 and 28. The exogenous addition of BMP4 to the medium significantly suppressed BMP4 expression (Fig. 3c).

The BMP inhibitor Noggin inhibits thyroid hormone-induced hypertrophy

Histological analysis did not show any effect of Noggin on the phenotype of day 28 pellets under standard chondrogenic conditions. DMMB staining showed that Noggin-

treated MSC pellets differentiated chondrogenically and showed hyaline cartilage-like morphology (Fig. 5b, c) similar to chondrogenic control aggregates (Fig. 5a). Both in chondrogenic control aggregates (Fig. 5g) and Noggin-treated chondrogenic aggregates (Fig. 5h, i), some ALP-positive cells were detected throughout the aggregates. Collagen type X staining was weak both in chondrogenic control aggregates (Fig. 5m) and Noggin-treated aggregates (Fig. 5n, o) without differences between these conditions.

Under prohypertrophic conditions, Noggin treatment inhibited hypertrophy induction in a dose-dependent manner. DMMB staining showed a clearly hypertrophic phenotype with increased cell size in hypertrophic control conditions (Fig. 5d). About 10 ng/mL of Noggin clearly reduced the amount and size of hypertrophic cells (Fig. 5e) and at 100 ng/mL Noggin, no hypertrophic cells could be detected (Fig. 5f). The amount of ALP-positive cells was decreased in Noggin-treated aggregates in the center of the aggregates (Fig. 5k, l) compared to hypertrophic control aggregates (Fig. 5j). 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 (Fig. 5p) and decreased with increasing Noggin concentrations (Fig. 5q, r).

FIG. 4. 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 the hypertrophic medium with T3 compared to the hypertrophic medium without T3 on day 21 and 28 (c). Values are the mean $\pm$ SD. * $p < 0.05$. $n = 4$ different donors.

Histomorphometry detected a significantly decreased cell size after Noggin treatment under hypertrophic conditions. Under chondrogenic conditions, Noggin treatment had no effect on the cell size (Fig. 5s).

The ALP activity in the medium is significantly increased under hypertrophic control conditions compared to chondrogenic control conditions on day 28. Noggin treatment did not significantly change the ALP activity under chondrogenic conditions. The ALP activity under hypertrophic conditions was reduced dose dependently and significantly by Noggin treatment (Fig. 5t).

Under chondrogenic conditions, Noggin treatment did not have a significant effect on collagen type II or X expression (Fig. 6a, b). Under hypertrophic conditions, collagen type II expression is significantly reduced by Noggin treatment on day 21 and 28 (Fig. 6a) and collagen type X expression is significantly decreased on day 21 and 28 compared to hypertrophic control aggregates (Fig. 6b). BMP4 gene expression was not influenced by Noggin under chondrogenic conditions, whereas under hypertrophic conditions, 100 ng/mL Noggin significantly increased BMP4 expression on days 21 and 28 (Fig. 6c).

Discussion

Changing the medium conditions from the chondrogenic to hypertrophy enhancing medium, including withdrawal of TGF β and dexamethasone and addition of T3, clearly enhanced the hypertrophic phenotype of chondrogenic differentiating MSCs as shown by the increased cell size, a stronger collagen type X staining, and a higher ALP activity in hypertrophic aggregates. These hypertrophy markers are not only

expressed under prohypertrophic conditions, but also under standard chondrogenic conditions but to a lower degree.

By differential gene expression analysis of components of BMP signaling, we detected a clear upregulation 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 ossification³² and are all able to induce hypertrophy of cultured embryonic chondrocytes.^{22,24–26} BMP4 was also regulated on the 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 our *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.²⁵ Furthermore, *in vivo* studies showed that overexpression of BMP4 in the cartilage zone resulted in an increased hypertrophic zone indicating increased differentiation into hypertrophic chondrocytes.¹⁸

Analysis of BMP receptor expression revealed that the BMPR1B expression is significantly upregulated in hypertrophic aggregates. In contrast, other BMP receptors (BMPR1A, Alk2, and 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 the ALP activity and collagen type X expression in embryonic chick sternum chondrocytes. The effect of BMPR1A was less

FIG. 5. (a–r) Histological appearance of MSC pellet cultures on day 28 after Noggin treatment under chondrogenic (a–c, g–i, m–o) and hypertrophy enhancing conditions (d–f, j–l, p–r). (a–f) DMMB staining. (g–l) ALP staining with neutral red as counterstaining. (m–r) Immunohistochemical collagen type X staining. Under chondrogenic conditions, Noggin treatment has no influence on chondrogenic differentiation and hypertrophic extent (a–c, g–i, m–o). Under hypertrophic conditions, Noggin treatment inhibits hypertrophy shown by a decreased amount of hypertrophic and ALP-positive cells and decreased collagen type X staining in Noggin-treated aggregates (d–f, j–l, p–r). Scale bar = 200 μ m. (s) Histomorphometric cell size analysis of MSC pellets on day 28. Under standard chondrogenic conditions, Noggin treatment has no influence on the cell size. Under hypertrophic conditions, the cell size of aggregates treated with Noggin is significantly decreased compared to hypertrophic control conditions. Values are the mean $\pm$ SD. * p < 0.05. n = 3 different donors. (t) The ALP activity in the medium of day 28 MSC aggregates after Noggin treatment. Under chondrogenic conditions (chon), Noggin treatment has no influence on the ALP activity. Under hypertrophic conditions (hyp), Noggin treatment significantly reduces the ALP activity. Values are the mean $\pm$ SD. * p < 0.05. n = 4 different donors. Color images available online at www.liebertpub.com/tea

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.^{33–35} 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 prohypertrophic BMP signals in chondrifying MSCs. However, this needs to be confirmed by functional experiments.

Runx2, a transcription factor that is known to be expressed upon BMP stimulation,³⁶ is upregulated in hypertrophic aggregates compared to chondrogenic aggregates. Runx2 is expressed in hypertrophic chondrocytes and has been shown to promote terminal differentiation of chondrocytes.^{37–39} Increased Runx2 expression in hypertrophic aggregates may then trigger the expression of genes involved in the establishment of hypertrophy.

As BMP4 is strongly upregulated under prohypertrophic conditions, 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 TGF β and dexamethasone and the addition of the thyroid hormone.^{8,11} The thyroid hormone has been shown to induce hypertrophy in embryonic mesenchymal cells, growth plate chondrocytes, and MSCs.^{8,11,22,40} We showed that in the hypertrophic medium after withdrawal of TGF β and dexamethasone, but without addition of T3, both the cell size and ALP activity remained relatively low. Addition of T3 to this medium, which results in our standard prohypertrophic medium, clearly enhanced hypertrophy shown by an increased cell size and amount of hypertrophic cells and increased ALP activity. The addition of BMP4 instead of T3 to the medium significantly increased the cell size and collagen type X expression compared to the hypertrophic medium with and without T3. BMP4 has a very strong prohypertrophic 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 a higher BMP4 concentration when BMP4 is added to the medium at the concentration used in this experiment. On the other hand,

FIG. 6. 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 downregulated 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). Values are the mean $\pm$ SD. * $p < 0.05$. $n = 4$ different donors.

the thyroid hormone could induce regulators that ameliorate the effect of upregulated BMP4 expression. To confirm that the prohypertrophic effect of T3 is mediated by BMP4 expression, we compared BMP4 expression between aggregates that were incubated in the hypertrophic medium with and without T3 and detected a significantly higher BMP4 expression in cultures that were incubated in a 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 the thyroid hormone induces terminal differentiation through induction of BMP2.⁴¹ We could not see regulation of BMP2 expression by the thyroid hormone in our model, which may be explained by the different cell types and species. This prohypertrophic effect of BMP4, however, was only seen under hypertrophic conditions in which TGF β and dexamethasone were withdrawn. Under chondrogenic conditions with continuous application of TGF β and dexamethasone, BMP4 did not elicit the enhancement of the hypertrophic phenotype in chondrogenic MSC pellets. This is probably due to antihypertrophic effects of TGF β and dexamethasone that are known from growth plate and chicken sternum chondrocytes^{22,29,42} and we suppose that these antihypertrophic effects override the prohypertrophic effects of BMP4 under standard chondrogenic conditions. This phenomenon has been observed by other studies. Hanada *et al.* showed that rat MSCs, which were treated with BMP2, became hypertrophic and that the effect was abolished when cells were incubated with BMP2 and TGF β .⁴³ Furthermore, Sekiya *et al.* showed that human MSCs, which were treated with BMP4 in the presence of TGF β , did not develop a hypertrophic phenotype.⁴⁴ Other studies investigated the effect of different BMPs on MSC hypertrophy with controversial results. Schmitt *et al.* showed that BMP2 is not able to induce

hypertrophy in human MSCs. MSCs, which were treated with BMP2 or a combination of BMP2 and TGF β , differentiated chondrogenically with no signs of hypertrophy.⁴⁵ In contrast, rat periosteum-derived progenitor cells treated with BMP2 developed hypertrophic chondrocytes.⁴³ This indicates that there are species-specific and cell source-dependent differences in the response to different BMPs. In human MSCs, Steinert *et al.* showed that both BMP2 and BMP4 gene transfer into human MSCs enhanced hypertrophy of MSCs.⁴⁶

To further confirm our hypothesis that BMP signaling promotes hypertrophic differentiation of MSCs, we blocked BMP signaling 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.⁴⁷ We could show that Noggin inhibits the T3-induced hypertrophy in our *in vitro* model. 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 prohypertrophic conditions. These results obtained here are consistent with studies on embryonic chondrogenesis. *In vivo* studies showed that overexpression of the BMP antagonist Noggin in the developing chick limb bud prevents chondrocyte hypertrophy and expression of hypertrophic markers like collagen type X and ALP.²⁷ Furthermore, overexpression of Noggin in transgenic mice leads to a lack of hypertrophic chondrocytes.¹⁸ Besides the suppression of hypertrophy markers, we detected a 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 chondroinductive growth factor TGF β and blocking of BMP signaling.

Under standard chondrogenic conditions with continuous application of TGF β and dexamethasone, hypertrophy

markers are also expressed, but to a lower degree than in hypertrophic cultures. In this group, the hypertrophy markers such as collagen type X expression and ALP activity were not suppressed by Noggin and there was no effect on collagen type II expression as well. This suggests that under standard chondrogenic conditions, factors other than BMP signaling may be involved in the development and maintenance of this phenomenon.

The discrepancy in collagen type X gene expression between control samples in the BMP4 and Noggin experiment (Figs. 4 and 6) is due to the different sets of patients used for the experiments. The experiments were done independently with different donors and the collagen type X expression varies between different patients.

In summary, we could show that hypertrophy is enhanced by the thyroid hormone through induction of BMP4 and that BMP4 is a potent regulator in the late differentiation stages in chondrifying MSCs. This thyroid hormone-induced enhancement of hypertrophy by BMP4 can be inhibited by the BMP antagonist Noggin. Noggin had no effect under standard chondrogenic conditions, but as 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, and constructs will be exposed to factors like thyroid hormones *in vivo*, these results add important information to the knowledge of the biology of especially the late differentiation stages in MSC chondrogenesis and may help to find ways to improve the performance of MSC-based cartilage constructs *in vivo*. Whereas this *in vitro* induced enhancement of the hypertrophic phenotype of chondrogenic differentiating MSCs by thyroid hormone or BMP4 is a concern for cartilage tissue engineering applications, these culture conditions can be useful for tissue engineering approaches for critical size bone defects. Endochondral ossification via hypertrophic cartilage takes place in both bone development and secondary fracture healing⁴⁸ and hypertrophic engineered cartilage has been suggested as a template for bone defect repair.^{49,50}

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Disclosure Statement

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