Gene expression profiles induced by growth factors in *in vitro* cultured osteoblasts

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**Objectives**
Effects of insulin-like growth factor 1 (IGF1), fibroblast growth factor 2 (FGF2) and bone morphogenetic protein 2 (BMP2) on the expression of genes involved in the proliferation and differentiation of osteoblasts in culture were analysed. The best sequence of growth factor addition that induces expansion of cells before their differentiation was sought.

**Methods**
Primary human osteoblasts in *in vitro* culture were treated with IGF1, BMP2 or FGF2 (10 ng/ml) for 24 hours (IGF1) or 48 hours (BMP2 and FGF2). Experiments were performed during the exponential growth phase with approximately 1e7 cells per 75 cm² flask. mRNA was reverse transcribed directly and analysed using RT-PCR Taqman assays. Expression levels of key genes involved in cell growth and differentiation (CDH11, TNFRSF11B, RUNX2, POSTN, ALP, WNT5A, LEF1, HSPA5, FOS, p21) were monitored using RT-PCR with gene-specific Taqman probes.

**Results**
Autocrine expression of BMP2 is stimulated by FGF2 and BMP2 itself. BMP2 and FGF2 act as proliferative factors as indicated by reduced expression of ALP and POSTN, whereas IGF1 exhibits a more subtle picture: the Wingless und Int-1 (Wnt) signalling pathway and the Smad pathway, but not p38 mitogen-activated protein (MAP) kinase signalling, were shown to be activated by IGF1, leading to proliferation and differentiation of the cells.

**Conclusions**
For future use of autologous bone cells in the management of bony defects, new treatment options take advantage of growth factors and differentiation factors. Thus, our results might help to guide the timely application of these factors for the expansion and subsequent differentiation of osteoblastic cells in culture.

**Strengths and limitations**
- **Strength:** Tests were performed on primary human cells without transfection and artificial over-expression of any proteins.
- **Limitation:** No studies at the protein level were performed.

**Introduction**
Tissue engineering for the treatment of a large fracture gap using *in vitro* raised cells is a promising concept. A coordinated action of various cell types is required for bone remodelling. Various growth factors are involved in the proliferation and differentiation of these cells, of which BMPs or integrins play a vital role. Growth factors bind to

**Keywords:** Osteoblasts, Tissue engineering, SMAD, Wnt, Alkaline phosphatase
specific cell surface receptors, which trigger cellular signalling pathways. The signals are transmitted to the nucleus and thereby regulate gene expression, many of which are involved in cartilage and bone formation. Through the use of in vitro models, the biology of the cells involved in bone remodelling has made important advances. Also, the favourable effect of culture expanded cells on healing fractures has been shown in animal models. In particular, sufficient numbers of cells for transplantation can be obtained by culturing precursor cells that might find applications in wound healing after appropriate expansion and differentiation. Many different culture systems have been used and a lot of biological processes need further clarification before cells from in vitro culture can be used routinely for transplantation.

Osteoblast differentiation is regulated by a number of hormones and factors that induce different signalling pathways in the cells. Major pathways that are known to modulate the number or activity of osteoblasts include the insulin-like growth factor (IGF) pathway, BMP pathway, and the Wingless und Int-1 (Wnt) pathway. The main objective of this study was to analyse the effects of BMP2, IGF1 and FGF2 (also known as bFGF) on the expression of genes involved in the proliferation (increases in the expression levels of WNT, LEF1; Table I) and differentiation (as measured by altered expression levels of CHD11, TNFRSF11B, ALP, RUNX2; Table I) of osteoblasts in culture. Thus, cellular processes involved in chromatin organisation, transcription factors, adhesion molecules and differentiation were studied by analysing expression levels using quantitative real-time PCR of appropriate genes. Analysis of these genes allowed us to also differentiate between the Smad and the p38 mitogen-activated protein (MAP) kinase pathway. In this way, we aim to elucidate the addition of growth factors that would induce expansion of cells before their differentiation.

**Materials and Methods**

Primary human osteoblasts were isolated from femoral trabecular bone tissue from the knee or hip joint (obtained from PromoCell, Heidelberg, Germany; C-12760, no specific source information is available) and suspended in an osteoblast growth medium from PromoCell (C-27001; 50 000 cells per 25 cm² flask). Media were supplemented with Supplement Mix (PromoCell, C-39615), containing all supplements necessary for the optimal growth of human osteoblasts according to the manufacturer’s protocol, Pen/Strep/Fungizone (PromoCell, C-42020), and 10% foetal bovine serum (PAA, A15-101). Cells were cultured at 37°C and 5% CO₂. Daily visual inspection of the cells during expansion indicated mostly undifferentiated cells. Cells were cryopreserved at passage 2 and passed another time prior to the experiments. Growth factors were obtained from Sigma-Aldrich (St. Louis, Missouri). Experiments were performed during the exponential growth phase, with approximately 1e7 cells per 75 cm² flask. Osteoblasts were treated with IGF1, BMP2 or FGF2 (10 ng/ml) for 24 hours (IGF1), or 48 hours (BMP2 and FGF2), based on previous studies in the literature. Control cells were kept in a medium without added growth factors. After incubation, cell plates (two plates for all experiments) were rinsed with Trizol™ (Life Technologies, Darmstadt, Germany) for immediate mRNA extraction after the culture medium was removed and mRNA was purified according to the manufacturer’s instructions. mRNA was reverse transcribed directly and the cDNA was stored at -70°C until the point of analysis. This protocol ensured the highest possible quality of mRNA, preserving the mRNA within seconds from nuclease digest. Random primers, deoxyribonucleotide triphosphates, protector RNase inhibitor and reverse transcriptase were obtained from Roche (Basel, Switzerland). RT-PCR was performed using Roche FastStart DNA Master HybProbe (Roche) and Taqman primers obtained from Life Technologies (CDH11: Hs00901475_m1; TNFRSF11B: Hs00900358_m1; RUNX2: Hs00231692_m1; POSTN: Hs01566734_m1; ALP: Hs01062534_m1; TNNTA: Hs00998537_m1; LEF1: Hs01547250_m1; HSPAS: Hs00607129_gH; FOS: Hs04194186_s1; p21: Hs00355782_m1; IGF1: Hs01547656_m1; FGF2: Hs00266445_m1; BMP2: Hs00154192_m1). RT-PCR was performed on an ABI Prims 7000 detection system (Life Technologies).

6-Carboxyfluorescein (FAM) fluorescence was used as a readout. The amplification blots were checked visually and the baseline was set manually. Every RT-PCR reaction was run in triplicate for every cDNA (Table I). Expression levels of the various genes are

**Table I. Genes in the current study**

| Gene        | Comment                                                                 |
|-------------|-------------------------------------------------------------------------|
| CDH11       | Encodes a type II classical cadherin from the cadherin superfamily. Proteins from this family mediate calcium-dependent cell–cell adhesion6|
| TNFRSF11B   | Osteoproterin (OPG), also known as osteoclastogenesis inhibitory factor (OCIF), or tumour necrosis factor receptor superfamily member 11B decoy receptor for the receptor activator of nuclear factor kappa B ligand (RANKL). OPG can reduce the production of osteoclasts by inhibiting the differentiation of osteoclast precursors7 |
| RUNX2       | Runt-related transcription factor 2 (RUNX2), also known as core-binding factor subunit alpha-1 (CBF-alpha-1). Key transcription factor associated with osteoblast differentiation9 |
| POSTN       | Matricellular protein involved in adhesion, proliferation and differentiation of osteoblasts9 |
| ALP         | Alkaline phosphatase tissue-nonspecific, important for mineralisation of bone matrix and differentiation of osteoblasts10 |
| WNT5A       | Signal protein stimulating proliferation and differentiation of osteoblasts. Blocks apoptosis in osteoblasts11 |
| LEF1        | Transcription factor signalling via the canonical WNT/β-catenin pathway. Stimulates proliferation, development, and regeneration of osteoblasts12 |
| HSPAS       | Heat shock protein induced by p38 mitogen-activated protein (MAP) kinase signalling14 |
| FOS         | Transcription factor induced by SMAD signalling15 |
| p21         | Also known as CDKN1a, is induced by SMAD signalling15 |
shown as means plus standard deviation of the triplicate qPCR measurements.

**Results**

For the quantitative analysis of mRNA expression levels, the amount of expression of target genes was compared with the expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (comparative CT method - ΔCT). Different effects of the various growth factors on the expression of target genes were found:

- IGF1 leads to increased expression of all genes analysed and, most especially, it shows a strong induction of ALP, whereas the other growth factors act to repress ALP.

- Both BMP2 and FGF2 increase the expression levels of WNT5A and lymphoid enhancer-binding factor 1 (LEF1) as IGF1 does, and this upregulation is accompanied by a higher expression of CDH11, and RUNX2. TNFRSF11B expression is not increased markedly by FGF2, but is upregulated highly in cells treated with IGF1 and BMP2 (Fig. 1). FGF2 reduces the expression of POSTN, a regulator of osteoblast differentiation. For BMP2 this reduction is not significant (Fig. 1). Both growth factors decrease the expression of ALP, indicating that differentiation (as measured by expression of ALP) is suppressed by FGF2 and BMP2, but not by IGF1.

- None of the growth factors seem to signal via the p38 MAP kinase pathway as HSPA5 expression is not markedly altered. However, genes involved in Wnt signalling (WNT5A, LEF1) are changed markedly by all three growth factors. The FOS gene, which is regulated by the Smad pathway, is repressed by IGF1 and FGF2. Surprisingly, expression of p21 remains totally constant under all the conditions that we applied, even though this gene has also been described as being regulated via the Smad pathway (Fig. 2).

Interestingly, the BMP2 gene is expressed strongly in the osteoblasts. This expression is further induced by FGF2 (Fig. 3).
cells express the master regulatory transcription factor RUNX2. Major pathways that are known to modulate the number or activity of osteoblasts include the IGF, BMP, and Wnt pathways. BMPs are members of the transforming growth factor-β (TGF-β) superfamily that play important roles in morphogenetic development. IGF1 signalling is known to increase osteogenesis significantly and may be used for tissue engineering purposes. The combination of PDGF and IGF1 may be more beneficial than either alone.

Thus, it was of interest to stimulate osteoblasts in \textit{in vitro} cultures by various growth factors and highlight their effects on the cells by assessing the expression of key genes. Genes involved in proliferation, maintenance and differentiation of the cells were monitored before and after addition of three growth factors (Table I).

Clearly, IGF1, FGF2, and BMP2 trigger separate responses in the cells; IGF1 leads to increased expression of all genes analysed, however, the other two growth factors showed a much subtler and more differentiated response. Both BMP2 and FGF2 increase the expression levels of WNT5A and LEF1, developmental genes that regulate cell fate and patterning during embryogenesis. This upregulation is accompanied by higher expression of CDH11 and RUNX2. RUNX2 and CDH11 are associated with osteoblast differentiation and both genes have specific functions in bone development and maintenance. RUNX2, especially, is a major transcription factor of osteoprogenitor cells. Consequently, its increased expression substantiates the proliferative response of the cells, and it can be assumed that the cell culture harbours both osteoblasts and osteoprogenitor cells. TNFRSF11B – which is critical for adequate bone metabolism via its surface-bound protein product that activates osteoclasts – is not changed by FGF2 but is higher with BMP2. Both growth factors decrease the expression of POSTN – which codes for Periostin, a regulator of osteoblast differentiation and bone formation, and ALP, indicating that differentiation is suppressed by FGF2 and BMP2 but not by IGF1. This may seem paradoxical because it would imply that IGF1 induces proliferation and differentiation simultaneously, but it again indicates that proliferation or differentiation occur in different cell fractions.

In previous work, the inherent heterogeneity of bone cell cultures has been addressed in detail. Interestingly, distinct patterns of gene expression were associated with the major signalling pathways (Wnt and p38 MAP kinase). Our results imply that FGF2 is best suited to expanding osteoblastic cells in culture, because this growth factor only stimulates the expression of WNT5 and LEF, whereas markers of differentiation (ALP and POSTN) are strongly down-regulated. IGF1 on the other side does stimulate proliferation, but most probably certain fractions of the cells also respond with heightened expressions of ALP and POSTN.

The mode of action of BMP2 stimulation on osteoblasts has been a matter of debate. BMP2 signals via two types of receptors, namely BMP receptor type I and type II (BR-I and BR-II). These receptors are expressed on the surface of the cells as homomeric as well as heteromeric complexes. A total of two possible responses of the cells to BMP2 are under discussion: 1) binding of BMP2 to preformed receptor complexes activates the Smad pathway, whereas 2) BMP2-induced recruitment of receptors activates a different pathway, independent of Smad, resulting in the induction of alkaline phosphatase activity and possibly apoptosis via p38 MAP kinase. We have investigated a cell’s two possible responses to BMP2 stimulation by measuring the expression levels of genes implicated in the Smad pathway. Our results show that the strong downregulation of ALP by BMP2 is accompanied by unaltered expression of genes regulated by p38 MAP kinase. This suggests that the cells show preformed receptor complexities on their surface for the binding of BMP2 (= response 1). The ensuing signalling via the Smad pathway is thus accompanied by the downregulation of alkaline phosphatase.

Generally, the addition of BMP2 to osteoblastic cells is reported to stimulate differentiation of the cells by increased levels of ALP and COL I. Interestingly, our study shows a strong downregulation of ALP by BMP2, whereas expression of RUNX2 and CDH11 is markedly increased. It can be speculated that this specific growth factor response is due to our cell system, because most cells used in previous studies are mouse osteoblasts from calvaria. As mentioned above, BMP2-induced downregulation of ALP is mediated by the Smad pathway.

Clearly, there was mainly stimulation of proliferation of the cells in FGF2, proliferation and some differentiation in BMP2 and strong stimulation of both proliferation and differentiation in IGF1. Thus, our results might help to guide the timely application of these factors for the expansion and subsequent differentiation of osteoblastic cells in culture.

References

1. Schmidt-Rohlfing B, Trioupia C, Menzel CL, Pape HC. Tissue engineering of bone tissue. Principles and clinical applications. Unfallchirurg 2009;112:795-794;quiz795. [Article in German].
2. Stern AR, Stern MM, Van Dyke ME, et al. Isolation and culture of primary osteocytes from the long bones of skeletally mature and aged mice. Biotechniques 2012;52:361-373.
3. Kawakami Y, Li M, Alev C, et al. Local transplantation of ex vivo expanded bone marrow-derived CD34-positive cells accelerates fracture healing. Cell Transplant 2012;21:2689-2709.
4. Czekanska EM, Stoddart MJ, Richards RG, Hayes JS. In search of an osteoblast cell model for in vitro research. Eur Cell Mater 2012;21:1-17.
5. Aubin JE. Advances in the osteoblast lineage. Biochem Cell Biol 1998;76:889-910.
6. Cheng SL, Lecanda F, Davidson MK, et al. Human osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell 1997;89:308-319.
7. Simonet WS, Lacey DL, Dunstan CR, et al. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Tissue Eng A 2013;19:254-263.
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9. Merle B, Garnero P. The multiple facets of periostin in bone metabolism. Osteoporos Int 2012;23:1198–1212.

10. Deng FY, Tan LJ, Shen H, et al. SNP rs6265 regulates protein phosphorylation and osteoblast differentiation and influences BMD in humans. J Bone Miner Res 2013;28:2498–2507.

11. Almeida M, Han L, Bellido T, Manolagas SC, Kousteni S. Wnt proteins prevent apoptosis of both uncommitted osteoblast progenitors and differentiated osteoblasts by beta-catenin-dependent and -independent signaling cascades involving Src/ERK and phosphatidylinositol 3-kinase/AKT. J Biol Chem 2005;280:41342–41351.

12. Lambertini E, Franceschetti T, Torreggiani E, et al. SLUG: a new target of lymphoid enhancer factor-1 in human osteoblasts. BMC Mol Biol 2010;11:13.

13. Luo S, Lee AS. Requirement of the p38 mitogen-activated protein kinase signalling pathway for the induction of the 78 kDa glucose-regulated protein/immunoglobulin heavy-chain binding protein by azetidine stress: activating transcription factor 6 as a target for stress-induced phosphorylation. Biochem J 2002;366(Pt3):787–795.

14. Zhang Y, Feng XH, Derynck R. Stimulation by insulin-like growth factor-I of creatine kinase activity in skeletal-derived cells and tissues of male and female rats. J Biol Chem 1994;269:25190–25194.

15. Won KY, Kim YW, Park YK. Development of an expression profile of osteoblast lineage at defined stages of differentiation. J Biol Chem 2005;280:24618–24628.

16. Sömjen D, Kaye AM. Pathology of the TNF receptor family that activates c-Jun N-terminal kinase in T cells. J Biol Chem 1997;272:2423–2427.

17. Krattinger N, Applegate LA, Biver E, Pioletti DP, Caverzasio J. Expression of Smad and its signalling cascade in osteo-

differentiation. J Bone Miner Metab 2011;29:46–58.

18. Bard DR, Dickens MJ, Smith AU, Zarek JM. Isolation of living cells from mature mammalian bone. Nature 1972;236:314–315.

19. Hogun BL. Bone morphogenetic proteins: multifunctional regulators of vertebrate development. Genes Dev 1996;10:1580–1594.

20. Tanaka H, Wakisaka A, Ogasa H, Kawai S, Liang CT. TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. J Biol Chem 1997;272:25190–25194.

21. Saitoh T, Katoh M. Molecular cloning and characterization of human WNT5B on chromosome 12p13.3 region. Int J Oncol 2001;19:347–351.

22. Wong BR, Rho J, Arron J, et al. TRANCE is a novel ligand of the tumor necrosis factor receptor family that activates c-Jun N-terminal kinase in T cells. J Biol Chem 1997;272:25190–25194.

23. Anderson DM, Maraskovsky E, Billingsley WL, et al. A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. Nature 1997;390:175–179.

24. Xiao SM, Gao Y, Cheung CL, et al. Association of CDX1 binding site of periostin gene with bone mineral density and vertebral fracture risk. Osteoporos Int 2012;23:1877–1887.

25. Szulc P, Garnero P, Marchand F, Duboeuf F, Delmas PD. Biochemical markers of bone formation reflect endosteal bone loss in elderly men—MINOS study. Bone 2005;36:13–21.

26. Kalajicz I, Staal A, Yang WP, et al. Expression profile of osteoblast lineage at defined stages of differentiation. J Biol Chem 2005;280:24618–24628.

27. Nohe A, Hassel S, Ehrlich M, et al. The mode of bone morphogenetic protein (BMP) receptor oligomerization determines different BMP-2 signaling pathways. J Biol Chem 2002;277:5330–5338.

28. Kimura N, Matsuura S, Shibuya H, Nakashima K, Taga T. BMP2-induced apoptosis is mediated by activation of the TAK1-p38 kinase pathway that is negatively regulated by Smad6. J Biol Chem 2005;275:17847–17852.

29. Raucchi A, Bellosta P, Grassi R, Basilico C, Mansukhani A. Osteoblast proliferation or differentiation is regulated by relative strengths of opposing signaling pathways. J Cell Physiol 2008;215:442–451.

30. Park JK, Jang H, Hwang S, et al. ER stress-inducible ATF3 suppresses BMP2-induced ALP expression and activation in MC3T3-E1 cells. Biochem Biophys Res Commun 2014;443:333–338.

31. Li P, Bai Y, Yin G, et al. Synergistic and sequential effects of BMP-2, bFGF and VEGF on osteogenic differentiation of rat osteoblasts. J Bone Miner Metab 2013 (Epub).

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ICMJE Conflict of Interest:

None declared

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