J Musculoskelet Neuronal Interact 2009; 9(1):53-60

Hylonome

Original Article

Apoptosis induction and reduced proliferation in human osteoblasts by rhBMP-2, -4 and -7

O.P. Gautschi[1,2], D. Cadosch[1,2], R. Zellweger[1], K.A. Joesbury[1], L. Filgueira[2]

[1]Department of Orthopaedic and Trauma Surgery, Royal Perth Hospital, Perth, Western Australia;
[2]School of Anatomy and Human Biology, University of Western Australia, Australia

Abstract

Background: The role of bone morphogenetic proteins (BMPs) in bone healing has been demonstrated in numerous *in vivo* animal models. BMP-2, -4 and -7 have also been shown to stimulate the differentiation of human and animal stem cells into osteoblasts *in vitro*. There are, however, contradictory reports of BMPs causing apoptosis and inhibition of proliferation of osteoblastic cells. Therefore, a more complete understanding of the effects of BMP-2, -4 and -7 on human osteoblasts is required. **Methods:** Cells of the immortalised human fetal osteoblastic line hFOB 1.19 were exposed to recombinant human (rh) BMP-2, -4 and -7. In addition, primary human osteoblasts were exposed to rhBMP-7. Cell proliferation was measured using a colorimetric assay. Apoptotic cells were detected using the TUNEL assay. **Results:** The hFOB cells exposed in a dose-dependent manner to rhBMP-2, -4 and -7 had significantly lower rates of proliferation than non-treated cells, ($p<0.01$ for rhBMP-2, -4 and -7). The proliferation results for rhBMP-7 were replicated using primary human osteoblasts. Additionally, rhBMP-2, -4 and -7 induced a significantly higher rate of apoptosis in the hFOB cells, with a temporal and dose-dependent pattern ($p<0.05$), irrespective of the presence of serum growth factors. **Conclusions:** Despite interest in the potential clinical application of BMPs to improve bone healing, further studies are necessary to determine their full biological function before they can be used confidently in humans.

Keywords: Osteoblasts, Apoptosis, Bone Morphogenetic Protein, Growth Factors, Molecular Pathways

Introduction

Despite improved understanding of bone biology and clinical advances in the treatment of fractures, impaired bone healing is still a major challenge[1]. Numerous treatments have been developed for patients with delayed union or non-union. Revision of internal or external skeletal fixation, with or without supplemental autologous or allogeneic bone graft, often facilitates bone healing in these patients[2]. Electrical stimulation[3,4] and ultrasound[5] have also been used as adjuvant therapies. None of these methods, however, have proven to be a panacea for the amelioration of impaired fracture healing.

In recent years, knowledge of the molecular signals that regulate the recruitment and differentiation of bone-related cells and the activity of macromolecules responsible for the bone remodeling cycle has greatly improved[6-8]. The identification and subsequent production of osteoinductive bone morphogenetic proteins (BMPs) has resulted in the augmentation and, in some cases, substitution of bone grafts. Recombinant human (rh) BMP-2 (Infuse, Medtronic Sofamor Danek, Memphis, TN, USA) and rhBMP-7 (or osteogenic protein-1, OP-1) (Stryker, Kalamazoo, MI, USA) are now commercially available and other BMP-containing materials are currently being evaluated in animal and clinical studies. However, despite significant evidence of their potential benefit in bone repair in animal and preclinical studies, there is, to date, a dearth of convincing clinical trials.

The immortalised human fetal osteoblastic cell line hFOB 1.19 consists of osteoprogenitor cells at an early stage of development[8] and provides an ideal model for the study of human osteoblast activity *in vitro*. The objective of this study was to measure proliferation and apoptosis in hFOB and primary human osteoblasts *in vitro* after exposure to rhBMP-2, rhBMP-4 and rhBMP-7.

The authors have no conflict of interest.

Corresponding author: Dr. med. Oliver P. Gautschi, Department of Neurosurgery, Cantonal Hospital of St. Gallen, Rorschacher Strasse 95, CH-9000 St. Gallen, Switzerland
E-mail: ogautschi@datacomm.ch

Accepted 12 January 2009

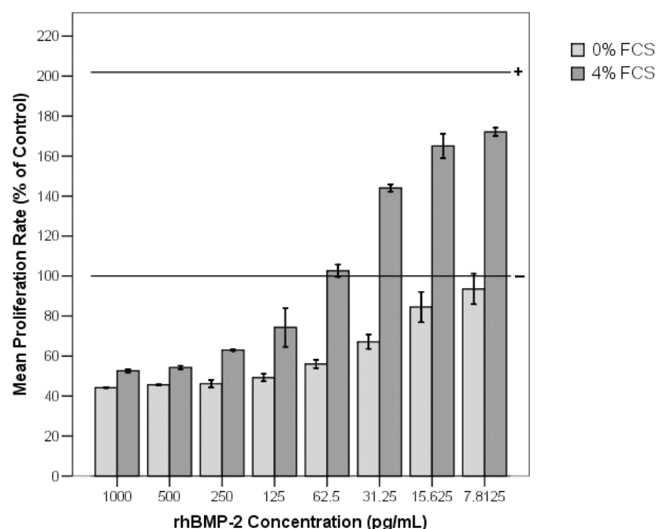

Figure 1A. Dose response of rhBMP-2 on proliferation rate of hFOB. Confluent hFOB cells (10^4 cells/well) in 96-well plates were incubated in serum-free culture medium containing varying concentrations of BMP-2 (500 pg/ml). "+ line" indicates proliferation rate of hFOB cells in the presence of 4% FCS and "– line" indicates proliferation rate of hFOB in the presence of 0% FCS. Data are represented as mean ±SD of three different experiments.

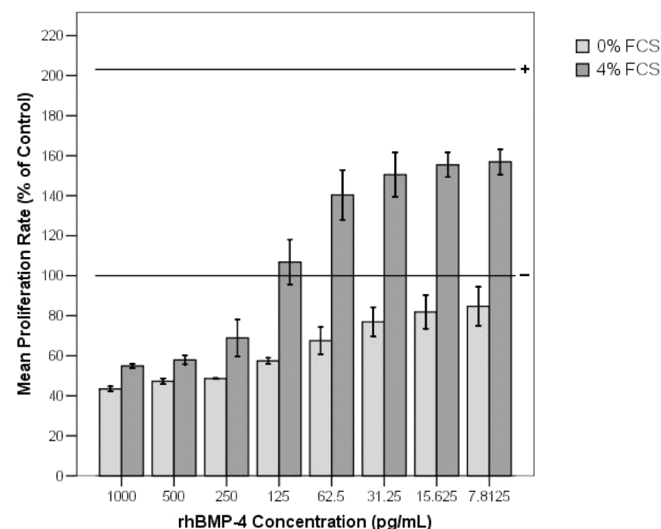

Figure 1B. Dose response of rhBMP-4 on proliferation rate of hFOB. Confluent hFOB cells (10^4 cells/well) in 96-well plates were incubated in serum-free culture medium containing varying concentrations of BMP-4 (500 pg/ml). "+ line" indicates proliferation rate of hFOB cells in the presence of 4% FCS and "– line" indicates proliferation rate of hFOB in the presence of 0% FCS. Data are represented as mean ±SD of three different experiments.

Materials and methods

Immortalised human fetal osteoblastic cells (hFOB 1.19) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Fetal calf serum (FCS) was obtained from JRH Biosciences (Lenexa, KS, USA). Dulbecco's Modified Eagle Medium (D-MEM/F-12), Phosphate Buffered Saline (PBS), Trypsin-EDTA and Antibiotics (10,000 units/ml penicillin G sodium, 10,000 μg/ml streptomycin sulfate and 25 μg/ml amphotericin B in 0.85% saline) were purchased from Gibco Invitrogen (Auckland, NZ). Purified rhBMP-2 and -4 were purchased from R&D Systems and purified rhBMP-7 from Alpha Diagnostic International (San Antonio, TX, USA). All BMPs were dissolved in PBS for the experiments according to the manufacturers instructions. The CellTiter96® AQ_{ueous} One Solution Cell Proliferation Assay was obtained from Promega Corporation (Madison, WI, USA). To detect apoptotic cells using the TUNEL assay, terminal deoxynucleotidyl transferase was purchased from Promega and dUTP-AlexaFluor555 from Molecular Probes (Eugene, OR, USA).

Cell culture

hFOB 1.19 cells were maintained in D-MEM/F-12 medium supplemented with 15% (v/v) FCS and 1% antibiotics, and cultured in a humidified atmosphere of 95% air and 5 % carbon dioxide at 34°C. The hFOB cells were incubated for a total period of 120 hours before the proliferation and TUNEL assays were performed.

Primary human osteoblast cell culture

Ethics approval was granted by the Ethics Committee of Royal Perth Hospital to obtain cancellous bone chips from patients undergoing open reduction and internal fixation of a femur or tibia fracture. After receiving written informed consent, cancellous bone chips were obtained. These bone chips were processed and primary human osteoblast cell lines were established according to our established protocol[9]. The cleaned and minced small bone fragments were cultured in 25 cm^2 tissue culture flasks (Sarstedt Inc., Newton, NC, USA) in D-MEM/F-12 medium supplemented with 15% (v/v) FCS and 1% antibiotics and cultured in a humidified atmosphere of 95% air and 5% carbon dioxide at 37°C. Only the first passage of confluent osteoblasts was used for the study.

Proliferation assay

Cell proliferation was measured using the [3-(4,5-dimethylthiazol-s-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) colorimetric assay. Results were obtained by recording the absorbance at 492nm using a 96-well plate reader (Multiskan RC, Labsystems OY, Helsinki, Finland). Proliferation was calculated as the percentage of the internal negative control (0% FCS and no

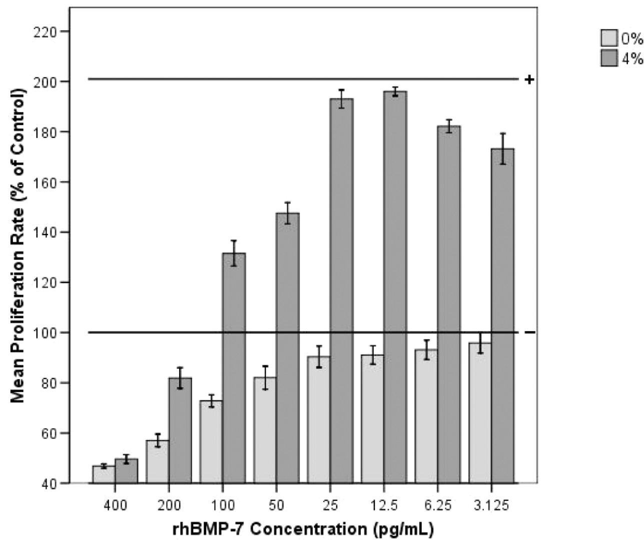

Figure 1C. Dose response of rhBMP-7 on proliferation rate of hFOB. Confluent hFOB cells (10[4] cells/well) in 96-well plates were incubated in serum-free culture medium containing varying concentrations of BMP-7 (200 pg/ml). "+ line" indicates proliferation rate of hFOB cells in the presence of 4% FCS and "– line" indicates proliferation rate of hFOB in the presence of 0% FCS. Data are represented as mean ±SD of three different experiments.

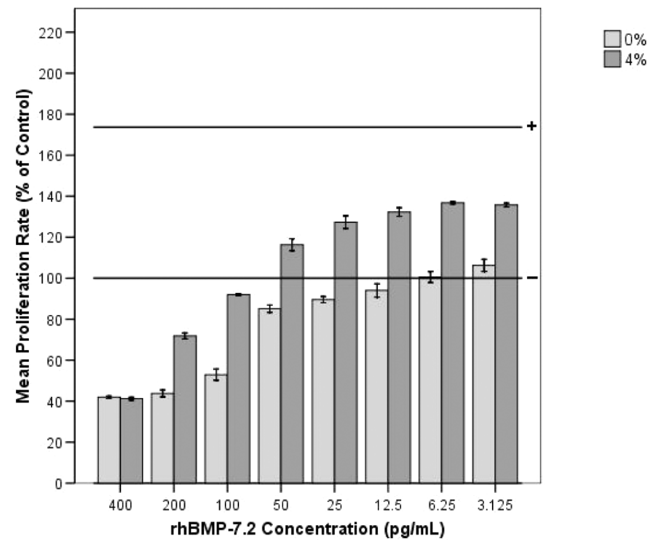

Figure 1D. Dose response of rhBMP-7 on proliferation rate of a primary human osteoblastic cell line. Confluent hFOB cells (10[4] cells/well) in 96-well plates were incubated in serum-free culture medium containing varying concentrations of BMP-7 (200 pg/ml). "+ line" indicates proliferation rate of hFOB cells in the presence of 4% FCS and "– line" indicates proliferation rate of hFOB in the presence of 0% FCS. Data are represented as mean ±SD of three different experiments.

BMP). Proliferation assays were established with the BMP-solvent PBS prior to all other experiments to exclude a confounding effect from the PBS.

TUNEL assay

Apoptotic nuclei were detected by independent investigators using the TUNEL assay, as described previously[6]. hFOB cells were cultured in serum-deprived medium or in the presence of 4% FCS and exposed to either rhBMP-2 (250pg/mL), -4 (250pg/mL) or -7 (100pg/mL). After either 6 or 24 hours' incubation, the hFOB cells were fixed with paraformaldehyde at room temperature for 5 minutes. Thereafter, cells were permeabilised with 0.1% Triton X-100 for 2 minutes and incubated for 4 hours at 37°C with the TUNEL reaction mixture that contained the terminal deoxynucleotidyl transferase (15U) and dUTP-AlexaFluor555 (25mM). Incorporated fluorescence was documented with a Nikon Eclipse TE300 inverted microscope and images were captured by a Hitachi-HV-C20M camera using Metamorph 4.5.1 software.

Statistical analysis

Data were analyzed using SPSS for Windows (Version 12.0). Average values are presented as the mean ± standard deviation (SD). The paired sample *t*-test was used to compare the TUNEL-positive cells incubated for 6 and 24 hours. Two-factor repeated-measures analysis of variance (ANOVA) was used to examine the effect of rhBMP-2, -4 and -7 and FCS (0% versus 4%) on the proliferation rate of hFOB. The Bonferroni *post hoc* test was used for multiple comparisons. Dependent variables that had a non-normal distribution were transformed by means of either the natural logarithm or square root expression. A p-value of less than 0.05 was considered statistically significant.

Results

Initially, the influence of the BMP-solvent PBS on the proliferation of hFOB was investigated. Using the same concentrations of PBS as in the rhBMP assays, there were no differences in the proliferation rates for PBS compared to the internal negative plate control (0% FCS and no BMP) ($p=0.096$) and for PBS with 4% FCS compared to the internal positive plate control (4% FCS and no BMP) ($p=0.551$).

Thereafter, the influence of varying concentrations of rhBMPs on the proliferation of hFOB was investigated in the presence or absence of FCS (4%=positive control, 0% = negative control). Incubation of rhBMP-2, -4 and -7 resulted in significantly lower rates of proliferation as compared to non-treated cells, in a dilution-related and dose-dependent manner, which was independent of the presence or absence of FCS (Figures 1A, 1B and 1C). For rhBMP-2, dilution was shown to have a significant effect on rates of proliferation ($p<0.01$) for the sample as a whole, and independently for

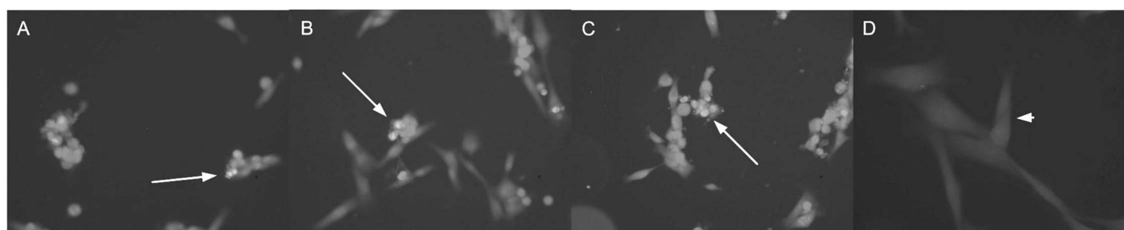

Figure 2A-D. TUNEL stain of hFOB incubated with rhBMP-2 (250 pg/ml) (1), BMP-4 (250 pg/ml) (B), BMP-7 (100 pg/ml) (1) and a control (D) with culture medium for 6 hours. Arrows are pointing to apoptotic hFOB nuclei (A-C) and the arrowhead is showing a normal hFOB cell (D).

the 0% and 4% FCS samples ($p<0.01$) (Figure 1A). Concentration was also significant in regard to the proliferation rate of hFOB treated with rhBMP-4 ($p<0.01$), and for both 0% and 4% FCS samples ($p<0.01$) (Figure 1B). Likewise, concentration had a significant effect on proliferation rates for hFOB treated with rhBMP-7 ($p<0.01$), and independently for 0% and 4% FCS samples ($p<0.01$) (Figure 1C).

To confirm the results obtained with the commercially available human osteoblastic cell line hFOB 1.19, the proliferation assay was repeated using a primary (non-immortalised) human osteoblastic cell line incubated with rhBMP-7. Incubation of rhBMP-7 resulted in significantly lower rates of proliferation as compared to non-treated cells, in a dilution-related and dose-dependent manner, which was independent of the presence or absence of FCS. For rhBMP-7, dilution was shown to have a significant effect on rates of proliferation ($p<0.01$) for the sample as a whole, and independently for the 0% and 4% FCS samples ($p<0.01$) (Figure 1D). Although the proliferation rates of rhBMP-7 with 0% FCS incubated with primary human osteoblasts had a trend towards the negative control, the proliferation rates with 4% FCS did not converge towards the positive control.

The influence of FCS and rhBMPs on cell proliferation was examined by incubating hFOB cells with each rhBMP, in the presence or absence of 4% FCS, for 24 hours. Proliferation rates for 4% FCS-treated samples were significantly higher than FCS-deprived samples for rhBMP-2 ($p<0.01$), rhBMP-4 ($p<0.01$) and rhBMP-7 ($p<0.01$). In respect of rhBMP-7, the rate of proliferation tended to increase with decreasing rhBMP-7 concentrations for samples with and without FCS. Of note, concentrations of 400 and 200pg/ml with 4% FCS had a proliferation rate below the value of the negative control.

The effect of rhBMP-2, -4 and -7 on hFOB cell apoptosis was determined by using the TUNEL assay (Figure 2A-C, including the control Figure 2D). Mean TUNEL-positive cell (%) values for rhBMP-2, -4 and -7 at 6 and 24 hours incubation are presented in Table 1. As shown, significant mean differences are evident for all rhBMPs, but not the FCS-only control.

	TUNEL-Positive Cells (1)		
	Mean at 6 Hours	Mean at 24 Hours	*P* value*
Control	8.2	9.3	0.146
rhBMP-2	39.4**	43.8**	0.005
rhBMP-4	31.8**	36.9**	0.009
rhBMP-7	24.3**	34.7**	0.002

* Paired sample *t* test; ** $p<0.005$ compared to the Control

Table 1. Percentage of TUNEL-positive hFOB 1.19 cells after six and 24 hours' incubation with rhBMP-2, -4 and -7.

Discussion

Bone healing depends on three factors: osteoregenerative cells (mesenchymal stem cells, committed osteoblastic cells), osteoinductive proteins (growth factors) and the local environment (provided by a carrier, matrix and native tissues)[7,10]. Tissue engineering with bioactive factors, such as BMPs, has the ability to cause a paradigm shift away from the utilisation of bone-grafting procedures currently employed in orthopedic surgery. BMPs belong to the transforming growth factors beta (TGFβ) superfamily, and play an important role in embryonic development, including brain[11] and bone formation[12,13]. At least 20 types of BMPs have been identified in humans. BMP signal transduction is induced via interaction with the heterodimeric complex of two transmembrane serine/threonine kinase receptors[14,15]. The activated receptor kinases phosphorylate the transcription factors Smads 1, 5, and/or 8. The phosphorylated Smads then form a heterodimeric complex with Smad 4 in the nucleus and activate the expression of target genes in concert with other coactivators[16-18]. The BMPs set themselves apart from other bioactive factors by virtue of this action at the cellular level, which results in mesenchymal and osteoblastic cell differentiation into mature bone-forming cells. Uniquely, the application of a single BMP can induce the complex cellular response leading to bone formation. However, it is likely that multiple

BMPs in combination with other growth factors in a specific temporal sequence are necessary for the complete process of new-bone formation *in vivo*.

Members of the BMP family are currently being evaluated for their potential clinical use in bone healing because of their known stimulatory effects on bone formation in cell cultures and animal models[19-21]. Most pre-clinical trials have focused on the effects of BMP-2, -4 and -7 on improving fracture healing. The results of these studies have been variable and several factors, such as the use of different treatment regimes, BMP dosing schedules and carriers, make them difficult to compare.

In two recent randomized clinical trials involving humans, one study investigated the addition of BMP-2 for the treatment of open tibia fractures[22], and the other used either BMP-7 or autologous bone graft for the treatment of pseudoarthroses[23]. Neither study provided conclusive evidence that administration of BMPs is superior to reaming or autologous bone graft alone. Most patients in published BMP clinical trials were treated with an intramedullary tibia nail using a reamed technique[20,24]. The process of reaming *per se* provides internal bone grafting. Additionally, it stimulates new periosteal bone formation, which makes differentiating the independent effect of the endosteal bone graft or the exogenous BMP in the healing process difficult without inclusion of non-reamed controls. A number of human clinical trials of rhBMP-7 and rhBMP-2 using a collagen carrier have been conducted[22,23,25-27]. Methodological limitations of these studies include the potentially beneficial nature of the carriers used in the delivery of BMPs at the site of intervention[28,29]. This makes it difficult to determine whether the effect of treatment is due to the BMPs *per se* or that of the carrier or both. Furthermore, quantification of bone consolidation using X-rays is difficult, especially when there is no true loss of bone substance. The timing of application is also important in terms of clinical outcome. For instance BMP-2 appears to be most active at 1-3 days after fracture whereas BMP-7 appears to be most effective at day 2-5, although this is based on animal models[30,31].

This study aimed to investigate the effect of the most commonly studied BMPs (2, 4 and 7) on human osteoblastic cells *in vitro* to provide an insight into the cellular events that are manifested in clinical trials. rhBMP-2, -4 and -7 have been shown to be osteogenic *in vitro* and *in vivo* using mainly rodent cellular and animal models[32,33]. In that respect, BMPs are considered to play a major role in embryologic bone development and to act on early stages of cell differentiation within the osteogenic lineage[34]. Furthermore, in rodents, BMPs induce heterotopic bone formation and enhance bone fracture healing[33,35,36]. There is increasing evidence that rodents and humans react differently towards BMPs, especially when focusing on bone forming cells[37]. Therefore, a human *in vitro* model using the fetal osteoblastic cell line hFOB1.19 was chosen in this study. Yen ML et al. demonstrated that the preosteoblastic hFOB1.19 cell line are still at an early stage of differentiation and capable to become other cell types of other stromal cell lineages when cultured under corresponding conditions[37]. In addition, we used primary human osteoblasts to confirm the results for BMP-7. BMP-2 and BMP-4 were not tested with the primary human osteoblasts as similar experiments have already been published, indicating that those BMPs induce apoptosis in human and mouse osteoblasts[39-41]. This study used BMP concentrations up to 1 ng/ml although much higher concentrations are recommended for clinical trials and have been used in animal studies[33,42-44]. Previous preliminary experiments had indicated a linear relationship between BMP concentrations and the cellular response *in vitro*, even at higher BMP concentrations. In addition, the few studies documenting BMP blood plasma and tissue concentrations in humans stated that they are in the range of 10 to 1000 pg/ml[45,46]. Furthermore, other published studies investigating BMPs' activities using human cells indicate that physiological activity may well be at concentrations below 1 ng/ml[47,48]. However, our results show that BMPs have a significant effect on human osteoblast cells *in vitro* in the range of 10 to 1000 pg/ml.

In the first instance, this study reveals that treatment of the human osteoblasts with BMPs results in decreased cell numbers in comparison with untreated cells. For that purpose the MTS test was preferred in our study as this assay measures and compares numbers of viable cells. The BrdU incorporation assay, which is also available in our laboratory, was disregarded for this study because it may give a false positive impression of proliferation, as cells often undergo DNA synthesis and pass through the S-phase of the cell cycle before undergoing apoptosis[49].

Once we realized that the BMPs decrease cell numbers, it was interesting to discover that human osteoblasts undergo apoptosis in a concentration-dependent manner. In that respect, apoptosis was measured with the TUNEL method as this indicates the final and irreversible stage of the apoptotic process at the single cell level. Testing caspase activation, often used as an indication of apoptosis, was also considered but then disregarded as caspases also play an important role in the differentiation process of various cell types, including osteoblasts, and caspase measurements may thus give false positive results[50]. However, most important was the observation that induction of apoptosis by BMPs was independent of the presence of serum growth factors and the cells were not rescued by the addition of FCS. This indicates that a specific independent signaling pathway is involved in the induction of apoptosis by BMPs[39,41]. Most interestingly, the BMP-2 apoptotic signal in human osteoblasts seems to be Smad-independent, but uses a protein kinase C-dependent signaling pathway[40]. Future studies have to further elucidate the apoptotic BMP-signaling pathway in human osteoblasts.

For this study, cells were used that have already committed to the osteoblast lineage. Other studies looking into osteoblast biology *in vitro* have used bone marrow or muscle-derived pluripotent stromal stem cells that are at an early stage of differentiation. In both cases, treatment of those

stem cells with BMPs, and especially with BMP-7, induces differentiation of the stromal stem cells into osteoblasts[51-56]. Therefore, it can be postulated that in a clinical setting, topical application of BMPs may induce apoptosis in cells committed towards osteoblasts, but new undifferentiated stromal stem cells may be recruited from the adjacent muscles and bone marrow and subsequently stimulated to become new osteoblasts. However, future studies have to further elucidate the mechanisms of BMPs' action on bone healing.

In the context of clinical applications of BMPs for the treatment of delayed or deficient bone fracture healing, one has to consider published histological studies indicating that BMPs may play a significant role in bone fracture healing in humans[57]. In that respect, detection of expression of BMPs by fibroblasts in most cases of non-union may indicate that BMPs could even be responsible for the pathology of fracture healing deficiencies[58], similar to the biology of cranial sutures, where BMPs and apoptosis of osteoblasts seem to play an essential role in sustaining or closing the cranial sutures[59,60]. However, apoptosis of cells, including bone forming cells, is an important process for the balance between induction of new bone formation and preventing extraneous bone formation. BMPs may play a key role in that process and controlled therapeutic application of certain BMPs may help with rescuing bone healing deficiencies by bringing the balance back to physiological conditions.

Conclusion

The findings of this study using primary human osteoblasts and the human osteoblast cell line hFOB1.19 suggest that rhBMP-2, -4 and -7 have anti-proliferative and pro-apoptotic effects *in vitro*. This contrasts with the stimulatory effect of BMPs on osteoblast differentiation, reported repeatedly in the literature. More studies are needed to better understand the complex biological functions of BMPs in the human bone in health and disease, aiming to develop new therapeutic strategies and applying these very promising factors.

Acknowledgements

We thank Tricia Knox for her excellent assistance in the laboratory of the Royal Perth Hospital. Dr. Gautschi was financially supported by a research grant from the Novartis Foundation, formerly Ciba-Geigy Jubilee Foundation, and the Freiwillige Akademische Gesellschaft Basel, Switzerland. This study was funded by the SUVA Stiftung, Switzerland.

References

1. Sykaras N, Opperman LA. Bone morphogenetic proteins (BMPs): how do they function and what can they offer the clinician? J Oral Sci 2003;45:57-73.
2. Wiss DA, Stetson WB. Tibial nonunion: treatment alternatives. J Am Acad Orthop Surg 1996;4:249-57.
3. Bassett CA, Pilla AA, Pawluk RJ. A non-operative salvage of surgically-resistant pseudarthroses and non-unions by pulsing electromagnetic fields. A preliminary report. Clin Orthop Relat Res 1977;124:128-43.
4. Brighton CT, Friedenberg ZB, Zemsky LM, Pollis PR. Direct-current stimulation of non-union and congenital pseudarthrosis. Exploration of its clinical application. J Bone Joint Surg Am 1975;57:368-77.
5. Heckman JD, Ryaby JP, McCabe J, Frey JJ, Kilcoyne RF. Acceleration of tibial fracture-healing by non-invasive, low-intensity pulsed ultrasound. J Bone Joint Surg Am 1994;76:26-34.
6. Groscurth P, Filgueira L. Killing mechanisms of cytotoxic T lymphocytes. News Physiol Sci 1998; 13:17-21.
7. Ripamonti U. Soluble osteogenic molecular signals and the induction of bone formation. Biomaterials 2006; 27:807-22.
8. Subramaniam M, Jalal SM, Rickard DJ, Harris SA, Bolander ME, Spelsberg TC. Further characterization of human fetal osteoblastic hFOB 1.19 and hFOB/ER alpha cells: bone formation *in vivo* and karyotype analysis using multicolor fluorescent *in situ* hybridization. J Cell Biochem 2002;87:9-15.
9. Filgueira L, Meagher J, Al-Mushaiqri M, Skirving AP, Zellweger R. Fulvestrant, an estrogen receptor antagonist, blocks proliferation and differentiation of cultured primary human osteoblasts. Bone 2005;36(Suppl.): S463-4.
10. Seeherman H, Wozney JM. Delivery of bone morphogenetic proteins for orthopedic tissue regeneration. Cytokine Growth Factor Rev 2005;16:329-45.
11. Adachi T, Takanaga H, Kunimoto M, Asou H. Influence of LIF and BMP-2 on differentiation and development of glial cells in primary cultures of embryonic rat cerebral hemisphere. J Neurosci Res 2005; 79:608-15.
12. Hogan BL. Bone morphogenetic proteins: multifunctional regulators of vertebrate development. Genes Dev 1996;10:1580-94.
13. Zou H, Choe KM, Lu Y, Massague J, Niswander L. BMP signaling and vertebrate limb development. Cold Spring Harb Symp Quant Biol 1997;62:269-72.
14. Massague J, Weis-Garcia F. Serine/threonine kinase receptors: mediators of transforming growth factor beta family signals. Cancer Surv 1996;27:41-64.
15. Yamashita H, Ten Dijke P, Heldin CH, Miyazono K. Bone morphogenetic protein receptors. Bone 1996; 19:569-74.
16. Heldin CH, Miyazono K, ten Dijke P. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. Nature 1997;390:465-71.
17. Massague J. TGF-beta signal transduction. Annu Rev Biochem 1998;67:753-91.
18. Wrana JL. Regulation of Smad activity. Cell 2000; 100:189-92.
19. Urist MR. Bone morphogenetic protein: the molecularization of skeletal system development. J Bone Miner

Res 1997;12:343-6.
20. Westerhuis RJ, van Bezooijen RL, Kloen P. Use of bone morphogenetic proteins in traumatology. Injury 2005;36:1405-12.
21. Wozney JM. The bone morphogenetic protein family and osteogenesis. Mol Reprod Dev 1992;32:160-7.
22. Govender S, Csimma C, Genant HK, Valentin-Opran A, Amit Y, Arbel R. Recombinant human bone morphogenetic protein-2 for treatment of open tibial fractures: a prospective, controlled, randomized study of four hundred and fifty patients. J Bone Joint Surg Am 2002;84-A:2123-34.
23. Friedlaender GE, Perry CR, Cole JD, Cook SD, Cierny G, Muschler GF, Zych GA, Calhoun JH, Laforte AJ, Yin S. Osteogenic protein-1 (bone morphogenetic protein-7) in the treatment of tibial nonunions. J Bone Joint Surg Am 2001; 83-A(Suppl.1):S151-8.
24. De Biase P, Capanna R. Clinical applications of BMPs. Injury 2005;36(Suppl.3):S43-6.
25. Boden SD, Kang J, Sandhu H, Heller JG. Use of recombinant human bone morphogenetic protein-2 to achieve posterolateral lumbar spine fusion in humans: a prospective, randomized clinical pilot trial: 2002 Volvo Award in Clinical Studies. Spine 2002;27:2662-73.
26. Kang Q, Sun MH, Cheng H, Peng Y, Montag AG, Deyrup AT, Jiang W, Luu HH, Luo J, Szatkowski JP, Vanichakarn P, Park JY, Li Y, Haydon RC, He TC. Characterization of the distinct orthotopic bone-forming activity of 14 BMPs using recombinant adenovirus-mediated gene delivery. Gene Ther 2004;11:1312-20.
27. Yeh LC, Adamo ML, Olson MS, Lee JC. Osteogenic protein-1 and insulin-like growth factor I synergistically stimulate rat osteoblastic cell differentiation and proliferation. Endocrinology 1997;138:4181-90.
28. Hulbert SF, Young FA, Mathews RS, Klawitter JJ, Talbert CD, Stelling FH. Potential of ceramic materials as permanently implantable skeletal prostheses. J Biomed Mater Res 1970;4:433-56.
29. Kuboki Y, Takita H, Kobayashi D, Tsuruga E, Inoue M, Murata M, Nagai N, Dohi Y, Ohgushi H. BMP-induced osteogenesis on the surface of hydroxyapatite with geometrically feasible and nonfeasible structures: topology of osteogenesis. J Biomed Mater Res 1998;39:190-9.
30. Cho TJ, Gerstenfeld LC, Einhorn TA. Differential temporal expression of members of the transforming growth factor beta superfamily during murine fracture healing. J Bone Miner Res 2002;17:513-20.
31. Wang EA. Bone morphogenetic proteins (BMPs): therapeutic potential in healing bony defects. Trends Biotechnol 1993;11:379-83.
32. Canalis E, Economides AN, Gazzerro E. Bone morphogenetic proteins, their antagonists, and the skeleton. Endocr Rev 2003;24:218-35.
33. Murnaghan M, McILmurray L, Mushipe MT, Li G. Time for treating bone fracture using rhBMP-2: a randomized placebo controlled mouse fracture trial. J Orthop Res 2005;23:625-31.
34. Li X, Cao X. BMP signaling and skeletogenesis. Ann NY Acad Sci 2006;1068:26-40.
35. McCullough KA, Waits CA, Garimella R, Tague SE, Sipe JB, Anderson HC. Immunohistochemical localization of bone morphogentic proteins (BMPs) 2, 4, 6, and 7 during induced heterotopic bone formation. J Orthop Res 2007;25:465-72.
36. Rosen V. BMP and BMP inhibitors in bone. Ann NY Acad Sci 2006;1068:19-25.
37. Osyczka AM, Diefenderfer DL, Bhargave G, Leboy PS. Different effects of BMP-2 on marrow stromal cells from human and rat bone. Cells Tissues Organs 2004; 176:109-19.
38. Yen ML, Chien CC, Chin IM, Huang HI, Chen YC, Hu HI, Yen BL. Multilineage differentiation and characterization of the human foetal osteoblastic 1.19 cell line: a possible *in vitro* model of human mesenchymal progenitors. Stem Cells 2007; 25:125-31.
39. Chen S, Guttridge DC, Tang E, Shi S, Guan KL, Wang CY. Suppression of tumor necrosis factor-mediated apoptosis by nuclear factor kB-independent bone morphogenetic protein/Smad signaling. J Biol Chem 2001; 276:39259-63.
40. Hay E, Lemonnier J, Fromigue O, Guenou H, Marie PJ. Bone morphogenetic protein receptor IB signaling mediates apoptosis independently of differentiation in osteoblastic cells. J Biol Chem 2004;279:1650-8.
41. Hay E, Lemonnier J, Fromigue O, Marie PJ. Bone morphogenetic protein-2 promotes osteoblast apoptosis through a Smad-independent, protein kinase C-dependent signaling pathway. J Biol Chem 2001; 276:29028-36.
42. Bilic R, Simic P, Jelic M, Stern-Padovan R, Dodiq D, Van Meerdervoort HP, Martinovic S, Ivankovic D, Pecina M, Vukicevic S. Osteogenic protein-1 (BMP-7) accelerates healing of scaphoid non-union with proximal pole sclerosis. Int Orthop 2006;30:128-34.
43. Dimitriou R, Dahabreh Z, Katsoulis E, Matthew SJ, Branfoot T, Fiannoudis PV. Application of recombinant BMP-7 on persistent upper and lower limb nonunions. Injury 2005;36S:S51-9.
44. Jones AL, Bucholz RW, Bosse MJ, Mirza SK, Lyon TR, Webb LX, Pollak AN, Golden JD, Valentin-Opran A; BMP-2 Evaluation in Surgery for Tibial Trauma-Allgraft (BESTT-ALL) Study Group. Recombinant human BMP-2 and allograft compared with autogenous bone graft for reconstruction of diaphyseal tibial fractures with cortical defects. A randomized, controlled trial. J Bone Joint Surg Am 2006; 88:1431-41.
45. Sugimoto H, Yang C, LeBleu VS, Soubasakos MA, Giraldo M, Zeisberg M, Kalluri R. BMP-7 fucntions as a nover hormone to facilitate liver regeneration. FASEB J 2007;21:256-64.
46. Wildemann B, Kadow-Romacker A, Pruss A, Haas NP, Schmidmaier G. Quantification of growth factors in

allogenic bone grafts extracted with three different methods. Cell Tissue Bank 2007;8:107-14.
47. Eichner A, Brock J, Heldin CH, Souchelnytskyi S. Bone morphogenetic protein-7 (OP1) and transforming growth factor-b1 modulate 1,25(OH)2-Vitamin D_3-induced differentiation of human osteoblasts. Exp Cell Res 2002;275:132-42.
48. Fiedler J, Roderer G, Gunther KP, Brenner RE. BMP-2, BMP-4, and PDGF-bb stimulate chemotactic migration of primary human mesenchymal progenitor cells. J Cell Biochem 2002;87:305-12.
49. Fernhead HO, Chwalinski M, Snowden RT, Ormerod MG, Cohen GM. Dexamethasone and etoposide induce apoptosis in rat thymocytes from different phases of the cell cycle. Biochem Pharmacol 1994;48:1073-9.
50. Mogi M, Togari A. Activation of caspases is required for osteoblastic differentiation. J Biol Chem 2003; 278:47477-82.
51. Chaudhary LR, Hofmeister AM, Hruska KA. Differential growth factor control of bone formation through osteoprogenitor differentiation. Bone 2004; 34:402-11.
52. Gersbach CA, Guldber RE, Garcia J. *In vitro* and *in vivo* osteoblastic differentiation of BMP2- and Runx2-engineered skeletal myoblasts. J Cell Biochem 2007; 100:1324-36.
53. Gutierrez J, Osses N, Brandan E. Changes in secreted and cell associated proteoglycan synthesis during conversion of myoblasts to osteoblasts in response to bone morphogenetic protein-2: role of decorin in cell response to BMP-2. J Cell Physiol 2006;206:58-67.
54. Komaki M, Asakura A, Rudnicki MA, Sodek J, Cheifetz S. MyoD enhances BMP7-induced osteogenic differentiation of myogenic cell cultures. J Cell Science 2003;117:1457-68.
55. Peng H, Usas A, Gearhart B, Olshanski A, Shen HC, Huard J. Converse relationship between *in vitro* osteogenic differentiation and *in vivo* bone healing elicited by different populations of muscle-derived cells genetically engineered to express BMP4. J Bone Miner Res 2004;19:630-41.
56. Wright V, Peng H, Usas A, Young B, Gearhart B, Cummins J, Huard J. BMP4-expressing muscle-derived stem cells differentiate into osteogenic lineage and improve bone healing in immunocompetent mice. Mol Ther 2002;6:169-78.
57. Kloen P, DiPaola M, Borens O, Richmond J, Perino G, Helfet DL, Goumans MJ. BMP signaling components are expressed in human callus. Bone 2003; 33:362-71.
58. Kloen P, Doty SB, Gordon E, Rubel IF, Goumans MJ, Helfet DL. Expression and activation of the BMP-signaling components in human fracture nonunions. J Bone Joint Surg Am 2002;84:1909-18.
59. Fromigue O, Modrowski D, Marie PJ. Apoptosis in membranous bone formation: role of fibroblast growth factor and bone morphogenetic protein signaling. Crit Rev Eukaryot Gene Expr 2005;15:75-92.
60. Rice D, Kim H, Thesleff I. Apoptosis in murine calvarial bone and suture development. Eur J Oral Sci 1999; 107:265-75.