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Mesenchymal stem cells from osteoporotic patients feature impaired signal transduction but sustained osteoinduction in response to BMP-2 stimulation

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ABSTRACT

Osteoporotic fractures show reduced callus formation and delayed bone healing. Cellular sources of fracture healing are mesenchymal stem cells (MSC) that differentiate into osteoblasts by stimulation with osteoinductive cytokines, such as BMP-2. We hypothesized that impaired signal transduction and reduced osteogenic differentiation capacity in response to BMP-2 may underlie the delayed fracture healing.

Therefore, MSC were isolated from femoral heads of healthy and osteoporotic patients. Grouping was carried out by bone mineral densitometry in an age-matched manner. MSC were stimulated with BMP-2. Signal transduction was assessed by western blotting of pSMAD1/5/8 and pERK1/2 as well as by quantitative RT-PCR of Runx-2, Dlx5, and Osteocalcin. Osteogenic differentiation was assessed by quantifying Alizarin Red staining.

Osteoporotic MSC featured an accurate phosphorylation pattern of SMAD1/5/8 but a significantly reduced activation of ERK1/2 by BMP-2 stimulation. Furthermore, osteoporotic MSC showed significantly reduced basal expression levels of Runx-2 and Dlx5. However, Runx-2, Dlx5, and Osteocalcin expression showed adequate up-regulation due to BMP-2 stimulation. The global osteogenic differentiation in standard osteogenic differentiation media was reduced in osteoporotic MSC. Nevertheless, osteoporotic MSC were shown to feature an adequate induction of osteogenic differentiation due to BMP-2 stimulation.

Taken together, we here demonstrate osteoporosis associated alterations in BMP-2 signaling but sustained specific osteogenic differentiation capacity in response to BMP-2. Therefore, BMP-2 may represent a promising therapeutic agent for the treatment of fractures in osteoporotic patients.

1. Introduction

The treatment of osteoporotic fractures is often hindered by reduced bone healing and higher rates of complications [1–3]. Studies in osteoporotic animal models revealed delayed callus formation and enchondral ossification, resulting in impaired biomechanical properties of the bone [4,5]. The cellular sources of fracture healing are mesenchymal stem cells (MSC). MSC migrate to the fracture site, where they proliferate and differentiate into osteoblasts upon stimulation with osteoinductive cytokines. Molecular biological alterations of MSC, such as decreased proliferative capacity [6], production of collagen I deficient matrix [7], preferableness of adipogenic differentiation [8], and impaired osteogenic differentiation have been described in osteoporotic patients [9]. With regards to the osteoinduction of MSC, key agents are bone morphogenic proteins (BMPs) [10]. Of these, BMP-2 is one of the most potent osteoinductive cytokines [11] which physiologically contributes to the early phase of fracture healing [12,13]. Beyond that, BMP-2 is already clinically approved for the treatment of distinct fracture entities [14]. The predominant role of BMP-2 in osteoinduction and bone formation led to a scientific quest regarding its involvement in the pathophysiology of osteoporosis. Osteoporotic animal models revealed inconsistent data with regard to BMP-2 expression levels. BMP-2 was found to be overexpressed in the callus of mandibles [15] and down-regulated in MSC derived from tibial and femoral bone [16]. In humans, genetic polymorphisms in BMP-2 have been identified as risk factors for the development of familial osteoporosis and osteoporotic fractures [17–19]. All these findings directly link the BMP pathways to osteoporosis. Other studies investigated the therapeutic

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potential of BMP-2 in osteoporotic animal models. The systemic administration of rhBMP-2 increased the volume of trabecular bone and stimulated bone formation in osteoporotic mice [20]. The local application of adenoviral BMP-2 at the site of injury enhanced callus formation and improved mechanical properties of the healing bone in osteoporotic sheep [21].

Nevertheless, there has not been any study analyzing osteoporosis associated alteration in the BMP-2 signal transduction and its osteoinductive potency on MSC. This issue is of elementary interest, in such that molecular biological alterations could account for the observed delay in facture healing. A sustained osteogenic differentiation capacity in response to BMP-2, on the other hand, could provide a therapeutic approach in the treatment of osteoporotic fractures.

2. Material and methods

2.1. Cells and cell culture

Human mesenchymal stem cells (hMSC) were isolated from femoral heads of patients with fractures to the proximal femur undergoing hip joint replacement [22]. The study was approved by the LMU ethical commission and performed according to the Declaration of Helsinki. Inclusion criteria were female gender, 60 or more years of age and a T-Score of either >−1.0 SD for the healthy control group or <−2.5 SD for the osteoporosis group as revealed by bone mineral density. Furthermore, the histories of all patients were taken regarding the mechanism of injury. We only included patients in the healthy control group who suffered the fracture due to an adequate trauma with a high impact. All patients included in the osteoporosis group suffered a fragility fracture due to a low impact trauma. In order to minimize age related alterations, samples from both groups were age matched and analyzed in parallel. A total of 18 patients were included. The mean age of the healthy donors was 83.4 years (11.9 SD) with a mean T-Score of 0.3 (0.9 SD) and −0.7 (0.2 SD) of the spine and proximal femur, respectively. The mean age of the osteoporotic donors was 84.3 years (8.4 SD) with a T-Score of −3.5 (1.3 SD) and −3.1 (0.6 SD) of the spine and proximal femur, respectively (Table 1). Cells were isolated using Ficoll gradient centrifugation and cultured in MEM Alpha GlutaMAX media (Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (Sigma, Taufkirchen, Germany) [23]. Cells were maintained at 60–80% confluency in T-75 plastic culture flasks at 37 °C and 5% CO_2. The hMSC characteristics were verified according to Dominici et al. [24]. In brief, hMSC were plastic adherent as well as proven to be positive (>95%) for the hMSC-related markers CD105, CD90, CD73 and negative (<2%) for the hematopoiesis and leucocytes related markers CD45, CD34, CD19, CD14, HLA-DR using flow cytometric analyses. Furthermore, cells were differentiable into osteoblasts, adipocytes and chondroblasts under standard in vitro differentiating conditions. Experiments were carried out using hMSC at first and second passage. Stimulation was performed using recombinant human BMP-2 (R&S Systems, Minneapolis, MN, USA) in a concentration of 100 ng/ml. In order to minimize age-related biases, grouping was carried out in an age matched manner for western blotting and quantitative RT-PCR.

Table 1
HMSC were isolated from the femoral heads of 18 patients undergoing hip joint replacement due to a fracture to the proximal femur. Grouping into Healthy and Osteoporosis was carried out according to the T-Scores of bone mineral density, patient history and mechanism of injury. Patient age is given in years. SD = standard deviation.

Healthy			**Osteoporosis**		
Age	T-Score (SD)		Age	T-Score (SD)	
	Spine	Femur		Spine	Femur
66	1.2	−0.6	69	−3.5	−3.1
69	1.3	−0.5	76	n/a	−2.9
83	0.7	−0.7	77	−1.2	−3.0
86	−1	−0.9	80	−2.9	−2.1
89	−0.8	−1	82	−2.8	−2.9
94	0.5	−0.9	84	−4	−2.4
97	0.2	−0.6	86	−4.1	−3.5
			91	−4.6	−3.5
			93	−3.8	n/a
			94	−5.6	n/a
			95	−2.2	−4.1

2.2. Western blotting

Matched pairs of hMSC were handled simultaneously, cultured in serum free Alpha MEM media for 12 h and stimulated with BMP-2 for 5, 30 and 180 min. Adherent cells were directly lysed in 1× Laemmli buffer (200 mM Tris–HCl pH 6.8, 40% glycerol, 10% SDS, 30% 2-mercaptoethanol, 0.02% bromphenolblue and 0.2 M dithiothreitol). The lysates were homogenized, denatured at 99 °C for 5 and centrifuged at 4 °C for 10 min. The total protein amount in the supernatant was determined using bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. Equal volumes of the protein lysates were loaded on 10% SDS–PAGE gels and transferred onto polyvinylidene fluoride membranes. For blocking, 5% skim milk (Merck, Darmstadt, Germany) in Tris-buffered saline buffer (0.05% Tween20) was used. The following primary anti-human antibodies were applied: phosphor-Smad1/5/8 and phosphor-ERK1/2 (both R&D Systems, Minneapolis, MN, USA). Anti-human ß-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a loading control. The antibodies were diluted in the blocking solution and incubated overnight at 4 °C. Secondary horseradish peroxidase-conjugated antibodies (Rockland, Gilbertsville, PA, USA or Santa Cruz) were applied for 1 h at room temperature. Electrochemiluminescence solution (GE Healthcare, Waukesha, WI, USA) and Lumi-detection films (Roche, Munich, Germany) were used for protein visualization. Western blot bands were digitized using a flatbed scanner (HP Scanjet, Böblingen, Germany) and analyzed using the open source imaging software ImageJ v1.43 (ImageJ, NIH, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/). All bands were quantified as integrals in 8-bit mode with no background correction as previously recommended by others [25]. The relative band density was calculated as a ratio to unstimulated controls.

2.3. Quantitative real time RT-PCR

Grouped hMSC were handled simultaneously and stimulated with BMP-2 for 72 h. Total RNA was extracted with RNeasy Mini Kit (Qiagen, Hilden, Germany). For cDNA synthesis, 1 mg total RNA and AMV First-Strand cDNA Synthesis Kit (Invitrogen, Karlsruhe, Germany) were used. Quantitative RT-PCR was carried out using LightCycler Fast Start DNA Master SYBR Green kit (Roche) and primer kits for Runx2, Dlx5, Osteocalcin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Search-LC, Heidelberg, Germany). The PCR was performed in a LightCycler1.5 instrument (Roche) equipped with LightCycler3.5.3 software. Crossing points for each sample were determined by the second derivative maximum method and relative quantification was performed using the comparative DDCt method according to the manufacturer's protocol. The relative gene expression was calculated as a ratio to GAPDH. Experiments were carried out in duplicates.

2.4. Osteogenic differentiation assay

HMSC were plated in twelve-well dishes and cultured to a density of 80%. Osteogenic differentiation media consisting of Dulbecco's modified Eagle's medium with high glucose (PAA Laboratories, Cölbe, Germany) supplemented with 10% fetal bovine serum, 100 nM dexamethasone, 10 mM ß-glycerophosphate, and 50 mM L-ascorbic acid 2-phosphate (Sigma) was applied for 10 days and changed every 4 days. BMP-2 was added to the osteogenic differentiation media for the initial four days. Osteogenic differentiation was evaluated by Alizarin Red (AR) staining, visualizing calcium-rich deposits produced by the cells. AR staining and quantification were performed using the Osteogenic Quantification Kit (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Micrographs were taken with an Axiovert100 microscope and an AxioCam ICc3 camera (CarlZeiss, Jena, Germany). Afterwards, AR was extracted with 10% acetic acid and neutralized with 10% NH_4OH. Optical density measurements were taken at 405 nm on a microplate reader (Microtek, Overath, Germany). The AR concentration was calculated against an AR standard curve. The experiments were carried out in triplicates.

2.5. Statistics

Results are given in means and standard deviations. Statistical evaluation was performed using the GraphPrism software (GraphPad, La Jolla, CA, USA). The Kolmogorow–Smirnow-Test was carried out testing data's Gaussian distribution. Subsequently, one-way ANOVA analysis with Bonferroni's Multiple Comparison testing or Kruskal–Wallis with Dunn's Multiple Comparison testing were applied. A p-value <0.05 was considered significant. Graphs and bars charts show mean values and standard deviation.

3. Results

3.1. Western blotting

Phosphorylation of SMAD1/5/8 increased due to the stimulation of BMP-2. In healthy hMSC, there was a 2.68 (SD 0.74), 5.26 (SD 2.64) and 2.29 (SD 1.05) fold induction after 5, 30 and 180 min, respectively. The peak induction after 30 min was statistically significant. In osteoporotic hMSC SMAD1/5/8 featured a comparable phosphorylation pattern in response to BMP-2. The basal phosphorylation was insignificantly lower by 0.8 with a standard deviation (SD) of 0.46. After 5, 30 and 180 min there were inductions by 2.48 (SD 2.65), 5.64 (SD 4.26) and 2.09 (SD 2.66), respectively. The peak induction after 30 min was again statistically significant. There were no significant differences in phosphorylation of SMAD1/5/8 between healthy and osteoporotic hMSC at any investigated time point. In healthy hMSC, stimulation with BMP-2 resulted in a significant induction of ERK1/2 phosphorylation after 5 min as shown by a fold change of 8.01 (SD 5.3). This early phosphorylation decreased after 30 and 180 min with a declining fold change of 3.57 (SD 1.38) and 1.14 (SD 0.81), respectively. In osteoporotic hMSC, there was a comparable basal phosphorylation of ERK1/2 in the unstimulated control with a ratio of 1.05 and a standard deviation of 0.97. In comparison to healthy hMSC, there was a significantly lower induction of phosphorylation by BMP-2 stimulation after 5 min. The fold changes after 5, 30 and 180 min were 1.94 (SD 1.34), 1.18 (SD 1.09) and 1.32 (SD 1.55), respectively (Fig. 1).

3.2. Quantitative real time RT-PCR

Quantitative real time RT-PCR revealed a significantly reduced basal expression of Runx2 in osteoporotic hMSC with a ratio of 0.36 (SD 0.23) compared to healthy control. When stimulated with BMP-2, healthy hMSC significantly increased Runx2 expression by a fold change of 1.97 (SD 0.58). Osteoporotic hMSC also featured an increase in Runx2 expression by 1.97 due to BMP-2 stimulation, but the induction level of Runx2 was significantly lower with a ratio of 0.72 (SD 0.45) compared to healthy controls. The basal expression of Dlx5 was also revealed to be significantly lower in osteoporotic hMSC with a ratio of 0.21 (SD 0.32). In response to BMP-2 stimulation, healthy hMSC demonstrated a significant increase of Dlx5 expression by a fold change of 2.43 (SD 0.82). The expression of Dlx5 also increased in osteoporotic hMSC in response to BMP-2 by a fold change of 2.71. When compared to healthy controls, the induction level of Dlx5 was significantly lower in osteoporotic hMSC by a ratio of 0.59 (SD 0.67). The basal Osteocalcin expression was shown to be reduced in osteoporotic hMSC with a ratio of 0.54 (SD 0.22). When stimulated with BMP-2, the Osteocalcin expression increased by 2.06 (SD 0.33) in healthy hMSC and by 1.6 in osteoporotic hMSC. Stimulated osteoporotic hMSC revealed a relative fold change of 0.87 (SD 0.56) compared to healthy controls (Fig. 2).

3.3. Osteogenic differentiation assay

Extracellular calcium deposition was quantified after 10 days. Healthy and osteoporotic hMSC cultures revealed comparable concentrations of Alizarin Red in alpha MEM controls with a mean of 5.5 (SD 3.2) and 5.8 μM (SD 2.9), respectively. Using standard osteogenic differentiation media, the Alizarin Red concentration significantly increased to a mean of 53 μM (SD 24.7) in healthy cultures and 25.2 μM (SD 9.1) in osteoporotic cultures. Adding 100 μg of BMP-2 to the osteogenic differentiation media revealed a further significant increase of the Alizarin Red concentration by 2.31 (SD 0.86) in healthy cultures and an increase by 2.05 (SD 1.44) in osteoporotic cultures. For the evaluation of the additional BMP-2 dependent osteoinductive effect the Alizarin Red concentrations in response to basic osteogenic differentiation media were set as one.

4. Discussion

Aim of the study was to scrutinize hMSC with regard to osteoporosis associated alterations in signal transduction and osteogenic differentiation capacity in response to BMP-2 stimulation. To this end, hMSC of 18 healthy or osteoporotic donors were included and analyzed in an age matched manner. All samples were characterized prior to inclusion and fulfilled the hMSC criteria according to Dominici et al. [24]. Age matched samples were analyzed in parallel.

Activation patterns of two key proteins in BMP signaling and osteogenic differentiation were analyzed. The phosphorylation of SMAD 1/5/8 in response to BMP-2 was similar in osteoporotic and healthy hMSC with highest levels after 30 min. This activation pattern has repeatedly been observed by others in healthy mesenchymal progenitor cells and osteoblasts [26–28]. Nevertheless, the sustained and accurate SMAD 1/5/8 phosphorylation in osteoporotic hMSC is shown here for the first time. On the other hand, osteoporosis associated alteration regarding the phosphorylation of ERK1/2 in hMSC and osteoblasts have previously been reported. Rodriguez et al. observed a reduced phosphorylation of ERK1/2 when stimulated with osteogenic differentiation media (ODM) [9], while Perrini et al. revealed an almost abrogated increase of

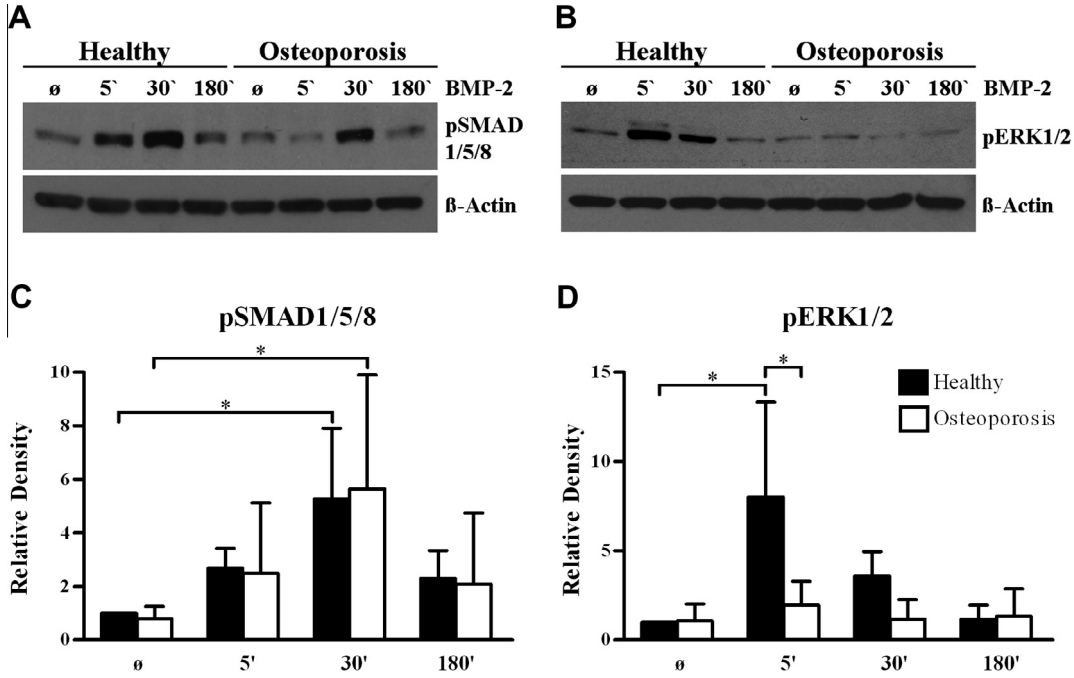

Fig. 1. Western blot of phosphorylated SMAD1/5/8 and ERK1/2. HMSC were kept in serum free media and stimulated with BMP-2 in a concentration of 100 ng/ml for 5, 30 and 120 min. Representative gel pictures are shown in (A) and (B). Semi quantitative analyses of band density were carried out and results are given as ratios to healthy controls (C) and (D).

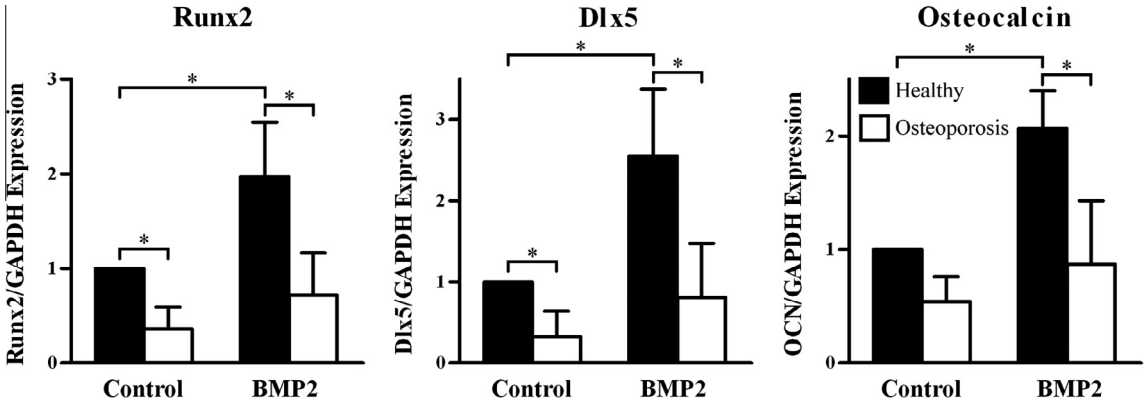

Fig. 2. Relative osteogenic marker gene expression. Quantitative RT-PCR of Runx2, Dlx5 and Osteocalcin were carried out after 72 h of cultivation in standard growth media (control) or standard growth media supplemented with BMP-2 in a concentration of 100 ng/ml. Results are given as ratios to healthy controls.

ERK1/2 phosphorylation in response to IGF-1 stimulation [29]. We here observed an osteoporosis associated significantly lower increase of ERK1/2 phosphorylation in response to BMP-2 stimulation (Fig. 1).

In general, SMAD1/5/8 and ERK1/2 are able to activate Runx2 expression [30,31]. Runx2 is a transcription factor crucial for osteogenic differentiation, which can directly stimulate transcription of osteoblast related genes such as Osteocalcin. Runx2 is itself regulated by Dlx5 [30,32]. Quantitative RT-PCR revealed a significantly decreased basal expression of Runx2 and Dlx5 in osteoporotic hMSC. Nevertheless, by BMP-2 stimulation the expression levels of both genes were up-regulated by comparable fold changes in healthy and osteoporotic hMSC. The role of Runx2 in osteoporosis has been analyzed in a limited number of recently published studies. Different Runx2 polymorphisms seem to be associated with either decreased [33,34] or increased lumbal spine and proximal femur bone mineral density [35]. Valenti et al. analyzed the expression levels of Runx2 in circulating MSC-like cells and revealed significantly lower basal expression levels in MSC-like cells derived from osteoporotic patients [36,37]. Even though MSC-like cells do not fulfill the minimal hMSC criteria according to Dominici et al. [24] and probably represent a different cell entity, the findings of Valenti et al. support our data. Besides the decreased expression levels of Runx2 and Dlx5, Osteocalcin expression was also shown to be reduced in unstimulated osteoporotic hMSC. According to the up-regulation of Runx2 and Dlx5, Osteocalcin expression also increased in osteoporotic hMSC when stimulated with BMP-2 (Fig. 2). To this end, hMSC of osteoporotic patients seem to feature an impaired phosphorylation of ERK1/2 in response to BMP-2 and decreased basal expression levels of Runx2, Dlx5 and Osteocalcin, whereas the activation of SMAD1/5/8 as well as the up-regulation of Runx2, Dlx5 and Osteocalcin in response to BMP-2 seem to function accurately. Although the BMP-2 signal transduction in osteoporotic hMSC is partly impaired, the up-regulation of the osteoblast related Osteocalcin may indicate a sustained BMP-2 specific osteogenic differentiation capacity.

Functional validations of this finding were carried out by performing quantitative osteogenic differentiation assays. When cultivated in standard ODM, hMSC of osteoporotic patients showed a significant induction of calcium deposition. Nevertheless, this global osteoinductive response was significantly less pronounced compared to healthy controls, which goes well in line with observation by Rodriguez et al. [6]. Interestingly, hMSC from healthy and osteoporotic patients responded with further increase of osteogenic differentiation by a comparable fold change when additionally stimulated with BMP-2 (Fig. 3). Lacking healthy controls, Pountos et al. recently observed a likewise positive effect of BMP-2 on osteoinduction in osteoporotic hMSC [38].

Taken together, hMSC of osteoporotic patients feature limited global osteogenic differentiation capacity in response to ODM but a sufficient specific osteogenic differentiation capacity when stimulated with BMP-2. The accurate phosphorylation of SMAD1/5/8 and consecutively the up-regulation of Runx2, Dlx5 as well as Osteocalcin in response to BMP-2 may contribute to the sustained specific osteogenic differentiation capacity. On the other hand, impaired ERK1/2 activation as well as reduced basal expression of Runx2, Dlx5 and Osteocalcin are osteoporosis associated molecular alterations in hMSC and may underlie the limited global osteoinductive response. Additional investigations are required to further elucidate these molecular alterations and their impact on hMSC function as well as on the disease itself.

A limitation of the study is that the standard deviations of some experiments are high. This is rather caused by interindividual differences between the donors than by interexperimental variance. Furthermore, only seven healthy donors could be included. This is due to the fact that only a small percentage of aged patients suffer a fracture to the proximal femur and feature healthy bone mineral density. Never the less, with regard to osteoporosis associated alterations in hMSC we here present the study with the highest number of healthy controls.

Our findings demonstrate that hMSC feature sustained specific osteogenic differentiation capacity in response to BMP-2. This makes BMP-2 a promising cytokine for the treatment of osteoporotic fractures. Even though these findings require in vivo validation, BMP-2 coated implants or application of BMP-2 to the site of fracture are conceivable therapeutic approaches.

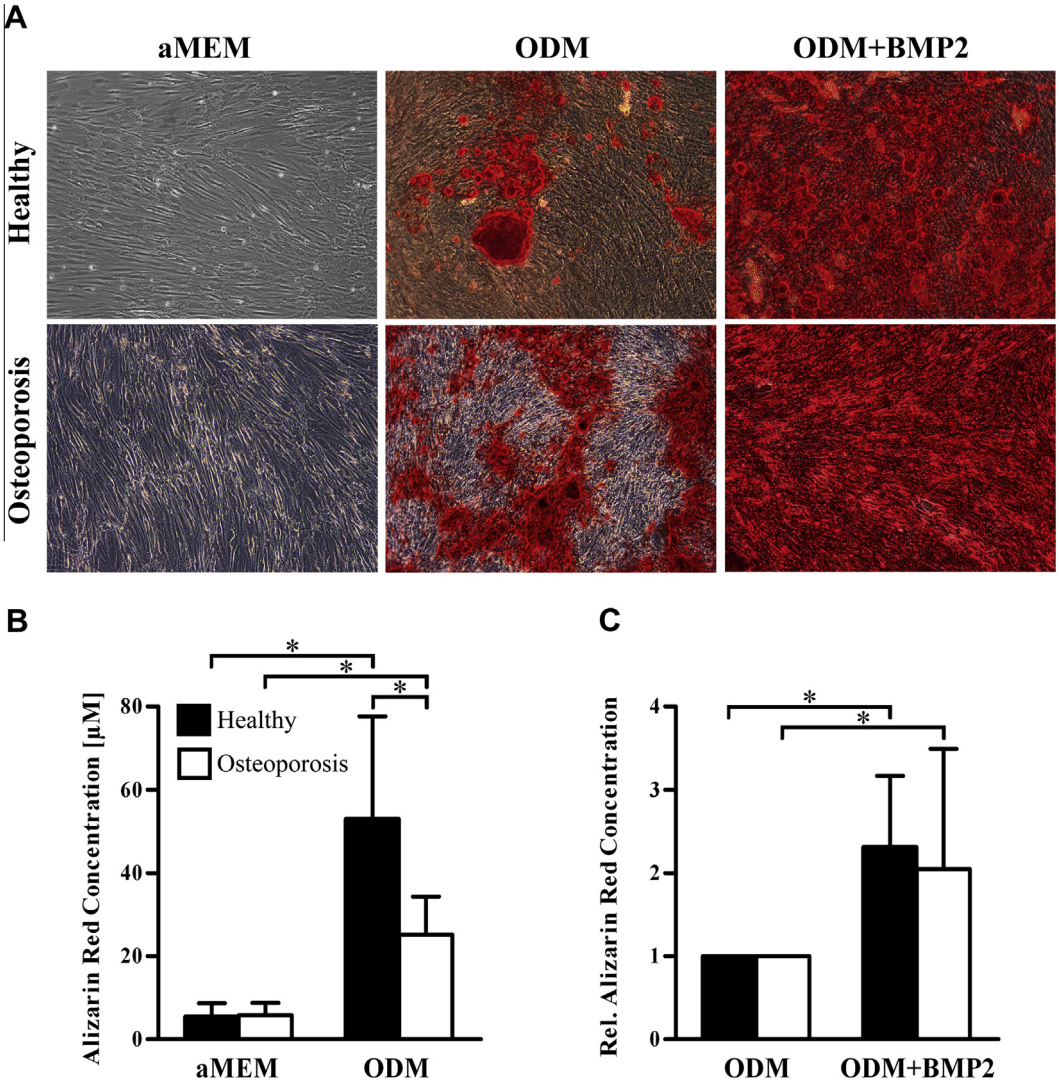

Fig. 3. Calcium deposition. HMSC were cultured in standard growth media (aMEM) or osteogenic differentiation media (ODM) with and without BMP2 in a concentration of 100 ng/ml. Osteogenic differentiation was evaluated by Alizarin Red staining (A) and quantified by photometry. Absolute concentrations of Alizarin Red measured in hMSC cultures with aMEM or ODM display the global osteoinductive capacity (B). The additional specific osteoinductive effect of BMP2 was calculated as a ratio to the global osteoinductive capacity (C).

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