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Mesenchymal stem cells from osteoporotic patients reveal reduced migration and invasion upon stimulation with BMP-2 or BMP-7

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ABSTRACT

Fractures to the osteoporotic bone feature a delay in callus formation and reduced enchondral ossification. Human mesenchymal stem cells (hMSC), the cellular source of fracture healing, are recruited to the fracture site by cytokines, such as BMP-2 and BMP-7. Aim of the study was to scrutinize hMSC for osteoporosis associated alterations in BMP mediated migration and invasion as well as in extracellular matrix (ECM) binding integrin expression.

HMSC were isolated from 18 healthy or osteoporotic donors. Migration was assessed using a collagen IV coated micro-slide linear gradient chamber and time-lapse microscopy. Invasion was analyzed utilizing an ECM coated transmembrane invasion assay. Quantitative real-time RT PCR was performed for the ECM binding integrins α1, α2, α3, α4, α5, α11, αv and β1.

HMSC from osteoporotic patients showed a significant increase of migration upon BMP-2 or FCS stimulation, as well as a significant increase of invasion upon BMP-2, BMP-7 or FCS stimulation. Nevertheless, the migration and invasion capacity was significantly decreased compared to healthy controls. Out of all integrins analyzed, collagen binding integrin α2 was significantly downregulated in hMSC from osteoporotic patients.

In conclusion, we here demonstrate for the first time osteoporosis associated alterations in BMP mediated hMSC recruitment. These findings may underlie the reduced healing of osteoporotic fractures. Nevertheless, the maintained migration and invasion response upon BMP stimulation illustrates the therapeutic potential of these clinically approved substances in the treatment of osteoporotic fractures. Another therapeutic target may be the downregulation of the collagen binding integrin α2 in hMSC from osteoporotic patients.

1. Introduction

Fractures to the osteoporotic bone typically feature more complex fracture patterns, reduced cancellous and cortical bone, leading to an impaired stability of the osteosynthetic fixation [1]. Furthermore, the fracture healing itself seems to be reduced [2]. Animal models revealed a delay in callus formation and enchondral ossification of the osteoporotic fracture [3,4]. Mesenchymal stem cells are the cellular sources of fracture healing. Upon stimulation with cytokines and chemokines hMSC migrate to the fracture site, where they proliferate and differentiate into osteogenic precursor cells [5,6]. Recent studies on hMSC from osteoporotic patients showed a significantly reduced osteogenic differentiation capacity [7–10]. Unfortunately, there are no studies investigating osteoporosis associated alterations of the recruitment process prior to differentiation. Recruitment of hMSC to the fracture site requires active migration towards chemokines or cytokines and invasion by proteolytic interaction with the extracellular matrix (ECM) [11]. Some of the most potent chemoattractants belong to the tumor necrosis factor (TNF) and the transforming growth factor beta (TGF-beta) superfamilies [12,13]. As a member of the TGF-beta family, bone morphogenetic protein (BMP)-2 and BMP-7 are known to induce migration in hMSC [14,15]. BMP-2 and BMP-7 are both clinically approved for the treatment of

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distinct fracture entities [16,17]. The BMP induced migration seems to be mediated via activation of different integrins, such as integrin α5 and β1 [18,19]. Preliminary data from our group recently suggested a downregulation of the collagen binding integrin α2 in hMSC from osteoporotic patients [20]. Studies in cell lines featuring downregulation of integrin α2 revealed decreased cellular adhesion and impaired migration on collagen I and IV, whereas upregulation of integrin α2 lead to an increased migratory activity [21,22].

Therefore, aim of the present study was to evaluate whether our preliminary findings of integrin α2 downregulation in hMSC from osteoporotic patients could be confirmed in a larger patient cohort. In addition, the expression of other relevant ECM binding integrins should be analyzed. Furthermore, we intended to scrutinize hMSC with regards to osteoporosis associated alterations in BMP-2 and BMP-7 mediated migration and invasion. Such findings could contribute to a deeper understanding of the impaired fracture healing in osteoporotic patients and lead to new therapeutic approaches.

2. Materials and methods

2.1. Cells and cell culture

HMSC were isolated from femoral heads of patients undergoing hip joint replacement due to a proximal femoral fracture as published previously [7,23,24]. Inclusion criteria were female gender, age >60 years and a T-Score of either >−1.0 SD for the healthy control group or <−2.5 SD for the osteoporosis group as measured by bone mineral density (DXA). Furthermore, only patients who suffered a fracture due to an adequate trauma were included in the healthy control group. In the osteoporosis group patients were included who suffered a fragility fracture due to a low impact trauma. The study was approved by the LMU ethical commission (No. 311-04) and performed according to the Declaration of Helsinki.

The hMSC characteristics were verified according to Dominici et al. [25]. 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 cytometry. Furthermore, cells were differentiable into osteoblasts, adipocytes and chondroblasts under standard in vitro differentiating conditions [7].

2.2. Migration assay

The assessment of 2-D migration was carried out in linear gradient micro slide chemotaxis chambers coated with collagen IV (IBIDI, Germany) as published earlier [11]. In brief, cells were seeded in a concentration of 1×10^6/ml along the cultivation channel and incubated for 3 h. Subsequently, culture medium was washed out and replaced by serum free medium. Microslide reservoirs were filled with serum free culture medium and the chemoattractant gradient was applied. BMP-2 and BMP-7 (R&S Systems, USA) were used in a concentration of 100 ng/ml and FCS in a concentration of 10%. Serum free culture medium served as a control. After 1 h stabilization and equilibration of the chemotaxis gradient time lapse analyses were performed over 15 h in a controlled bio-chamber (Pecon, Germany) at 5% CO_2 and 37 °C. Microscopy, cell tracking and data processing were carried out as published earlier [11]. The migration towards the chemokine gradient was quantified by calculating the forward migration index (FMI). The FMI is the ratio of covered distance along the chemokine gradient and the cell's overall path length. At least three independent experiments were performed for each donor. Aged matched samples were analyzed in parallel.

2.3. Invasion assay

3-D invasion was analyzed using a transwell invasion assay as published earlier [11]. The invasion chamber cell culture inserts (HTS FluoroBlok) were coated with 10 μg of human ECM (BD Biosciences, USA). Cells (5×10^3) were seeded in 200 μl serum free culture media into the upper compartment. The lower compartment of the invasion chamber was filled with 600 μl serum free culture media as control or one of the chemoattractant. BMP-2 and BMP-7 were used in a concentration of 30 ng/ml and FCS in a concentration of 10%. After incubation, staining, large-field microscopy and cell counting were performed as published earlier. Three independent experiments were carried out in triplicates for each donor. Aged matched samples were analyzed in parallel.

2.4. Quantitative real-time RT-PCR

Total RNA was extracted with RNeasy Mini Kit (Qiagen). For cDNA synthesis, 1 μg total RNA and AMV First-Strand cDNA Synthesis Kit (Invitrogen) were used. For Quantitative RT-PCR LightCycler Fast Start DNA Master SYBR Green kit (Roche, Munich, Germany) and primer kits for Integrins α1, α2, α3, α4, α5, α11, αv and β1 as well as for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Search-LC, Heidelberg, Germany) were used. 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 and data is given as fold changes compared to the healthy patients. All PCR results have been reproduced minimum three times.

2.5. Statistical analysis

Statistical calculations were performed using GraphPad Prism v5.02 (Graphpad Software Inc., USA). Significance was fixed at $p < 0.05$ and tested by two-tailed unpaired *t*-test or one way ANOVA with Bonferroni corrections for multiple comparisons tests.

3. Results

3.1. Patient data

A total of 18 patients were included in our study. The mean age of the healthy donors was 83 (±12 SD) years with a mean T-Score of 0.4 (±0.8 SD) at the lumbar spine and 0.7 (±0.2 SD) at the proximal femur. The mean age of the osteoporotic donors was 82 (±7 SD) years with a mean T-Score of −2.7 (±1.1 SD) and −3.0 (±0.6 SD) at the spine and the proximal femur, respectively (Table 1).

3.2. Migration assay

2-D migration of hMSC was assessed over a period of 24 h utilizing a collagen IV coated μ-slide linear gradient chemotaxis chamber. FCS is known to be a strong chemoattractant and served as a positive control. HMSC from healthy donors revealed a FMI of 0.39 (±0.12 SD) towards the FCS gradient, while the FMI of hMSC from osteoporotic patients was significantly lower (0.24 (±0.08 SD)). The FMI of hMSC from healthy donors towards BMP-2 and BMP-7 was 0.13 (±0.05 SD) and 0.09 (±0.08 SD), respectively. HMSC from osteoporotic patients showed significantly lower FMIs towards both chemokines with a FMI of 0.06 (±0.04 SD) and 0.01 (±0.04 SD), respectively. Even though hMSC from osteoporotic patients showed a significantly lowered FMI, the 2-D migration was significantly increased upon BMP-2 or FCS stimulation

Table 1
Patient data.

	Healthy				Osteoporosis		
	Age	T-Score (SD)			Age	T-Score (SD)	
		Spine	Femur			Spine	Femur
	66	1.2	−0.6		69	−3.5	−3.1
					77	−1.2	−3.0
	69	1.3	−0.5		72	−1.6	−2.6
	83	0.7	−0.7		82	−2.8	−2.9
					81	−1.3	−2.7
	86	−1.0	−0.9		86	−2.6	−1.7
					84	−4.0	−2.4
	87	−0.1	−1.0		87	−2.2	−3.2
					87	−3.7	−3.5
	94	0.5	−0.9		86	−4.1	−3.5
	97	0.2	−0.6		95	−2.2	−4.1
Mean	83	0.4	0.7	Mean	82	−2.7	−3.0
SD	12	0.8	0.2	SD	7	1.1	0.6

compared to the negative control (0.0 (±0.01 SD)). Upon BMP-7 stimulation HMSC from osteoporotic patients showed only very low migratory response. In hMSC from healthy patients we found a significantly increase of 2D migration upon stimulation with all three analyzed chemoattractants (Fig. 1).

3.3. Invasion assay

3-D invasion was analyzed using an ECM coated transwell invasion assay. A mean number of 749 (±93 SD) cells from healthy donors crossed the coated porous membrane upon FCS stimulation. Upon BMP-2 or BMP-7 stimulation a mean number of 316 (±81 SD) and 286 (±120 SD) was found in the lower compartment, respectively. HMSC from osteoporotic patients revealed a significantly lower invasion upon FCS stimulation, BMP-2 and BMP-7 with 397 (±94 SD), 183 (±63 SD) and 145 (±37 SD) cells crossing the coated membrane, respectively. Compared to the negative control BMP-2, BMP-7 and FCS significantly increased 3D invasion of hMSC from healthy and osteoporotic patients (Fig. 2).

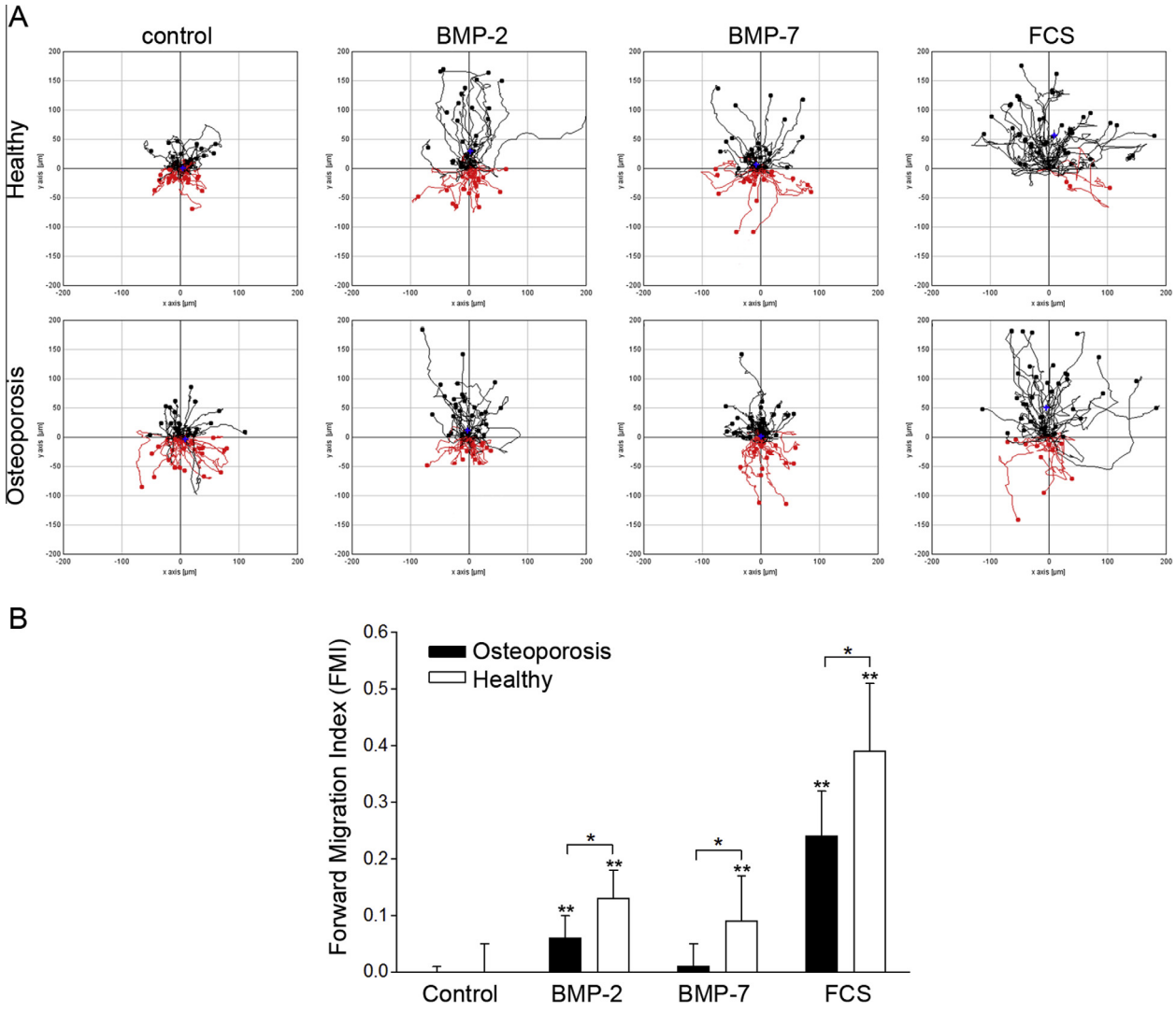

Fig. 1. 2-D migration assays on μ-slide chemotaxis chambers upon BMP-2, BMP-7 or FCS stimulation. (A) Representative scatter plots of a healthy and an osteoporotic patient. Migration towards the higher chemoattractant concentration is indicated by black cell paths, towards the lower concentration by red cell paths. (B) Grouped column graph of all donors showing mean values with standard deviation. HMSC from osteoporotic patients revealed a significantly decreased migration towards BMP-2, BMP-7 and FCS compared to cells from healthy donors. Nevertheless, hMSC from osteoporotic patients showed a significant increase of migration on BMP-2 and FCS stimulation. * = $p < 0.05$. ** = $p < 0.05$ compared to respective control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

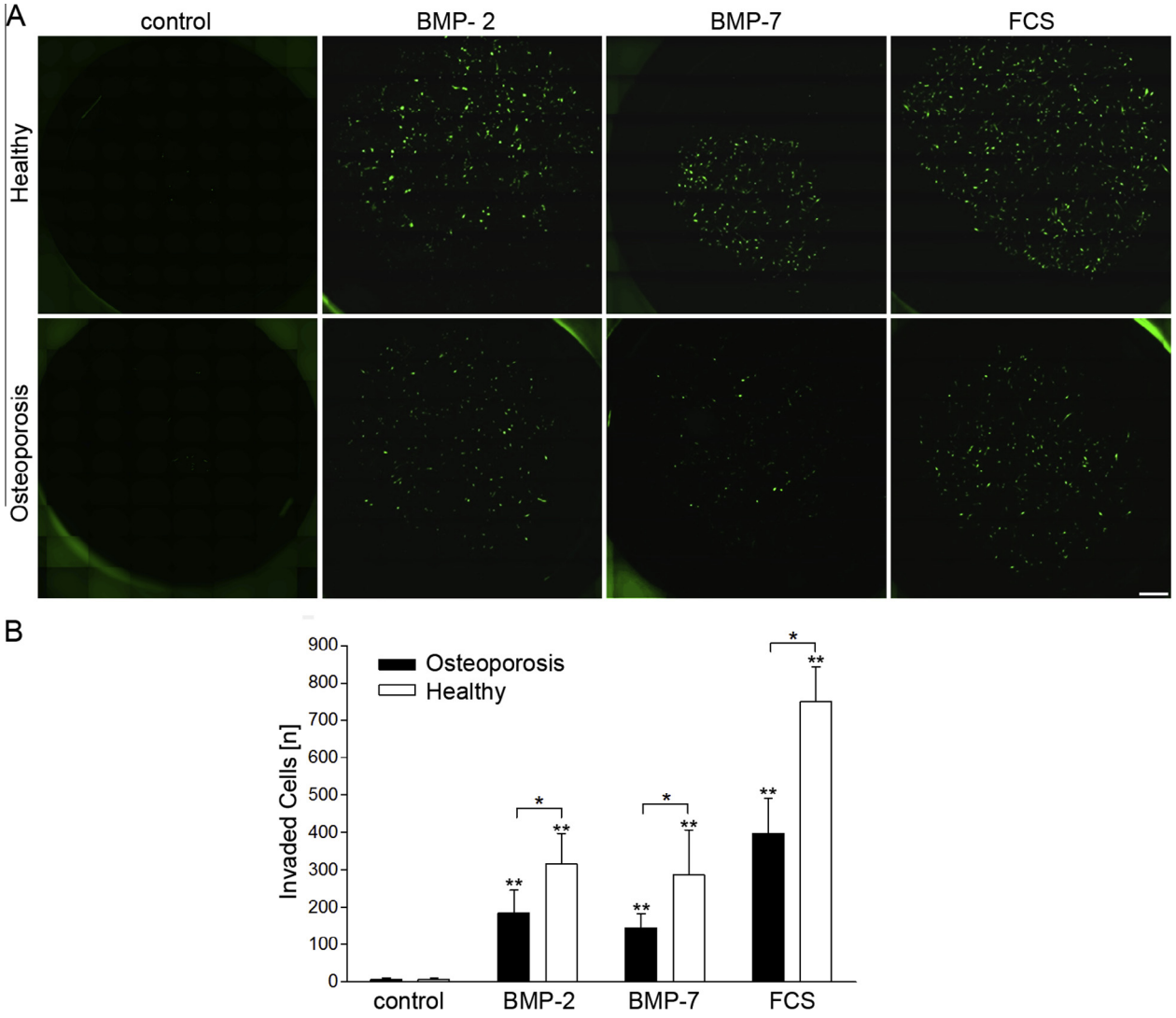

Fig. 2. 3-D invasion assay across an extracellular matrix barrier upon BMP-2, BMP-7 or FCS stimulation. (A) Representative fluorescence stainings of hMSC from a healthy and an osteoporotic donor on the lower side of the porous insert after 12 h of incubation with the chemoattractant. (B) Grouped column graph of all donors showing mean values with standard deviation. Invasion towards BMP-2, BMP-7 and FCS is significantly reduced in hMSC from osteoporotic patients compared to cells from healthy donors. Bar = 1500 μm. * = $p < 0.05$. ** = $p < 0.05$ compared to respective control.

3.4. Quantitative real-time RT PCR

In order to investigate a relationship between osteoporosis and the expression of ECM binding integrins, we performed a quantitative real-time RT PCR for the integrins α1, α2, α3, α4, α5, α11, αv and β1. Integrin expression of HMSC from healthy donors was set to one. Compared to the healthy donors we found a 1.185 (±0.232 SD) fold upregulation for integrin α1, a 1.017 (±0.266 SD) fold upregulation for integrin α3, a 0.87 (±0.076 SD) fold downregulation for integrin α5, a 1.004 (±0.236 SD) fold upregulation for integrin α11, a 0.865 (±0.197 SD) fold downregulation for integrin αv and a 0.897 (±0.138 SD) fold downregulation for integrin β1. Most strikingly, hMSC from osteoporotic patients revealed a significant 0.273 (±0.136 SD) fold downregulation of integrin α2 (Fig. 3).

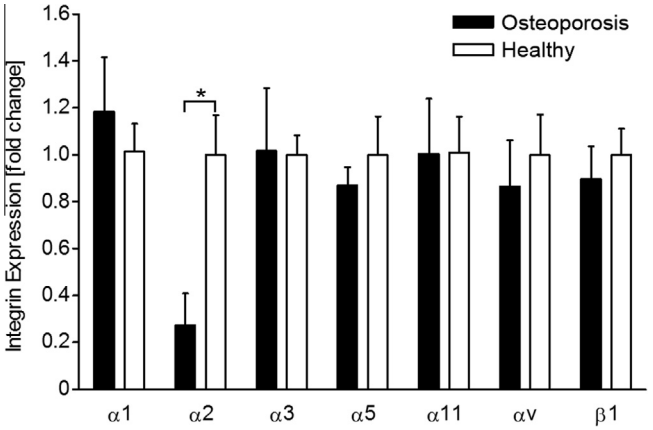

Fig. 3. Quantitative real-time RT-PCR of ECM binding integrins. Grouped column graph of all donors showing mean values with standard deviation. HMSC from osteoporotic patients revealed a significant downregulation of integrin α2 in comparison to hMSC from healthy donors. * = $p < 0.05$.

4. Discussion

Aim of present study was to verify our hypothesis that hMSC from osteoporotic patients feature a significantly reduced expression of the collagen binding integrins associated with an impaired migration and invasion towards chemoattractants, such

as BMPs. For this purpose, hMSC from 18 healthy donors or osteoporotic patients were analyzed in an age matched manner. Prior to inclusion all hMSC were characterized with regards to the minimal criteria of the International Society for Cellular Therapy defining multipotent mesenchymal stem cells [25].

In order to assess migration of hMSC we performed time-lapse analyses over a period of 24 h utilizing a two-dimensional (2-D) collagen IV coated micro slide chemotaxis chamber. This elaborate method is well established [11] allowing a precise migration analyses on a single cell level with constant linear chemoattractant gradients. We found a significantly reduced migration activity of hMSC from osteoporotic patients upon BMP-2 or BMP-7 stimulation (Fig. 1). Apart from the migration upon stimulation with distinct chemoattractants involved in fracture healing, we could detect a significantly reduced migration upon stimulation with FCS, a highly potent conglomerate of different chemoattractants. Although on a reduced level, a significant increase of migration could still be induced by BMP-2 in hMSC from osteoporotic patients. Comparing the effect of BMP-2 and BMP-7 on hMSC migration, BMP-2 revealed to be a more potent stimulus in hMSC from healthy donors and osteoporotic patients. These finding go well in line with Mishima et al. who detected similar results in human articular chondrocytes [26]. Among different BMPs analyzed, Lind et al. found BMP-2 to be the most potent chemoattractant for human osteoblasts [27].

HMSC invasion was analyzed using a three-dimensional (3-D) transmembrane assays through an ECM barrier. Apart from migratory activity, invasion requires additional cellular functions, such as upregulation of other ECM binding receptors and synthesis of proteolytic enzymes [11,28]. Similar to the findings in 2-D migration, the invasion analysis revealed a significantly reduced invasion capacity of hMSC from osteoporotic patients upon BMP-2, BMP-7 or FCS stimulation (Fig. 2). Nevertheless, all chemoattractants analyzed still lead to a significantly increased hMSC invasion compared to the control group. The osteoporosis associated impairment of hMSC migration and invasion upon BMP stimulation has not been described in literature before. These findings may display an osteoporosis associated limitation of hMSC recruitment to the fracture site and may underlie the reduced fracture healing described in osteoporotic animal models [3,4]. So far, studies on osteoporosis associated alterations of hMSC to some extend disregarded the aspect of cellular recruitment and mainly focused on osteogenic differentiation processes. HMSC from osteoporotic patients have been shown to feature a decreased proliferative capacity [8], the production of collagen I deficient matrix [9], a preferableness of adipogenic differentiation [29] and an impaired osteogenic differentiation [10].

Despite the impairment of migration and invasion, we could demonstrate a maintained cellular response upon BMP-2 or BMP-7 stimulation in hMSC from osteoporotic patients. Similarly, Pountos et al. observed an enhancement of proliferation and osteogenic differentiation due to BMP-2 and BMP-7 in hMSC derived from osteoporotic bone [30]. With regard to the BMP signal transduction, our group recently showed a reduced phosphorylation of ERK1/2, but an accurate activation of SMAD1/5/8 [7].

The reduced migration and invasion activity may be due to a differential expression of ECM binding integrins. Preliminary data from our group suggested an osteoporosis associated downregulation of collagen binding integrin α2 [20]. In the present study we scrutinized hMSC from a larger cohort of healthy donors and osteoporotic patients with regards to expression of integrin α1, α2, α3, α4, α5, α11, αv and β1. Quantitative real-time RT-PCR could not detect a significantly alternated expression of integrins α1, α3, α4, α5, α11, αv and β1. As a major finding and in line with our previous data, we could approve a significant downregulation of integrin α2 in hMSC derived from osteoporotic patients (Fig. 3). Studies utilizing different cell lines uncovered that migration on collagen IV and laminin is largely mediated by integrin α2 and that blocking of integrin α2 receptor significantly inhibits migration [22,31]. Apart from its important role in migration, integrin α2 is involved in osteogenic differentiation of hMSC. Hu et al. showed that integrin α2 activates ERK pathways by phosphorylation, thus promoting the expression of osteogenic transcription factors, such as Runx2. The authors could further demonstrate a dysregulation of the α2/ERK/Runx2 pathway in hMSC derived from osteoporotic patients [32]. This goes well in line with recently published data from our group showing an impaired phosphorylation of ERK upon BMP-2 stimulation. On the other hand, we further discovered an accurate activation of the SMAD1/5/8 pathway upon BMP-2 stimulation in hMSC from osteoporotic patients [7]. Both pathways, ERK and SMAD, are known to be responsible for BMP signal transduction [33,34]. Interestingly, Maegdefrau et al. revealed that blocking of the SMAD pathway lead to a significantly decreased migration and invasion upon BMP stimulation [35]. Taking these findings into account, the impairment of migration and invasion found in hMSC from osteoporotic patients may be due to a downregulation of integrin α2 and reduced phosphorylation of ERK. Nevertheless, migration and invasion can still be induced upon BMP stimulation in hMSC from osteoporotic patients, presumably via the maintained SMAD pathway.

In conclusion, we here demonstrate for the first time a significantly reduced migration and invasion upon BMP-2 or BMP-7 stimulation in hMSC derived from osteoporotic patients. These findings may underlie the reduced healing of osteoporotic fractures. Nevertheless, the maintained migration and invasion response upon BMP stimulation illustrates the therapeutic potential of these clinically approved substances in the treatment of osteoporotic fractures. Another therapeutic target may be the downregulation of the collagen binding integrin α2 or its mediated signaling in hMSC derived from osteoporotic patients.

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