OSTEOPOROSIS IS ACCOMPANIED BY REDUCED CD274 EXPRESSION IN HUMAN BONE MARROW-DERIVED MESENCHYMAL STEM CELLS

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Abstract

Underlying pathomechanisms of osteoporosis are still not fully elucidated. Cell-based therapy approaches pose new possibilities to treat osteoporosis and its complications. The aim of this study was to quantify differences in human bone marrow-derived mesenchymal stem cells (hBMSCs) between healthy donors and those suffering from clinically manifest osteoporosis.

Cell samples of seven donors for each group were selected retrospectively from the hBMSC cell bank of the Trauma Department of Hannover Medical School. Cells were evaluated for their adipogenic, osteogenic and chondrogenic differentiation potential, for their proliferation potential and expression of surface antigens. Furthermore, a RT2 Osteoporosis Profiler PCR array, as well as quantitative real-time PCR were carried out to evaluate changes in gene expression.

Cultivated hBMSCs from osteoporotic donors showed significantly lower cell surface expression of CD274 (4.98 % ± 2.38 %) than those from the control group (26.03 % ± 13.39 %; $p = 0.007$), as assessed by flow cytometry. In osteoporotic patients, genes involved in inhibition of the anabolic WNT signalling pathway and those associated with stimulation of bone resorption were significantly upregulated. Apart from these changes, no significant differences were found for the other cell surface antigens, adipogenic, osteogenic and chondrogenic differentiation ability as well as proliferation potential.

These findings supported the theory of an influence of CD274 on the regulation of bone metabolism. CD274 might be a promising target for further investigations of the pathogenesis of osteoporosis and of cell-based therapies involving MSCs.

Keywords: Osteoporosis, mesenchymal stem cells, CD274, osteoblasts, gene expression.

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List of Abbreviations			
		DMSO	dimethyl sulphoxide
		EDTA	ethylenediaminetetraacetic acid
ALOX15	arachidonate 15-lipoxygenase	FACS	fluorescence-activated cell sorting
APC	allophycocyanin	FBS	foetal bovine serum
BMP	bone morphogenetic protein	FC	flow cytometry
CD	cluster of differentiation	FCS	foetal calf serum
CFU-F	colony forming unit-fibroblast	FGF2	basic fibroblast growth factor
CTLA-4	cytotoxic T-lymphocyte associated protein 4	FITC	fluorescein isothiocyanate
		GHRH	growth hormone releasing hormone
Ct	cycle threshold	hBMSCs	human bone marrow-derived MSCs
Cy7	cyanine dye 7	HEPES	2-[4-(2-hydroxyethyl)piperazine-1-yl]ethanesulphonic acid
DEXA	dual-energy absorptiometry		
DKK1	Dickkopf WNT signalling pathway inhibitor 1	HPRT1	hypoxanthine phosphoribosyltransferase 1
DMEM	Dulbecco's modified Eagle Medium	IBMX	3-isobutyl-1-methylxanthine

IL6	interleukin 6
IL6R	IL6 receptor
ISCT	International Society for Cellular Therapy
ITS	insulin, transferrin, sodium selenite
MRI	magnetic resonance imaging
MRONJ	medication induced necrosis of the jaw
MSCs	mesenchymal stem cells
NFATC1	nuclear factor of activated T cells 1
P1	passage 1
P3	passage 3
PBS	phosphate-buffered saline
PD-1/PDCD1	programmed cell death-1
PD-L1	PD-1 ligand
PE	phycoerythrin
PerCP	peridinin-chlorophyll-protein
PRL	prolactin
PTH	parathyroid hormone
PTHLH	parathyroid-hormone-like hormone
RANKL	receptor activator of nuclear factor kappa-B ligand
SFRP1	secreted-frizzled-related protein 1
SOST	sclerostin
TGF-β3	transforming growth factor beta 3

Introduction

Despite the increased incidence of osteoporosis in the ageing society (Burge *et al.*, 2007; Johnell and Kanis, 2005), its underlying pathomechanisms have not been fully elucidated. Immunological factors (Arron and Choi, 2000; Nagahama *et al.*, 2004) and altered mechanisms of osteogenic cell differentiation have been postulated (Rodríguez *et al.*, 1999).

As MSCs have the ability of constant self-renewal and can differentiate into several cell types *in vivo* and *in vitro*, they are of great interest for research in the fields of regenerative medicine and tissue engineering (Docheva *et al.*, 2007; Kassem *et al.*, 2004). MSCs can be found in most tissues of the human body. Besides aspirates from liposuction, they can be harvested safely by minimal invasive aspiration biopsy from bone marrow stroma (Chamberlain *et al.*, 2007). They can be identified *in vitro* by growing plastic-adherent, their differentiation potential and a set of surface markers (Bara *et al.*, 2014; Dominici *et al.*, 2006; Machado *et al.*, 2013). However, no consensus exists regarding their surface marker expression.

MSCs can differentiate into osteoblasts, adipocytes, chondrocytes and other non-mesoderm-type cells *in vitro* (Banas *et al.*, 2007; Docheva *et al.*, 2007; Kassem *et al.*, 2004). Therefore, their immense differentiation potential has raised hope for future use in standardised, cell-based therapy approaches (Ayatollahi *et al.*, 2012; Kassem *et al.*, 2004; Wang *et al.*, 2012). As MSCs are able to differentiate into osteoblasts, they have the potential to perform intramembranous bone formation, which makes their therapeutic use applicable especially in reconstructive and regenerative therapies for bone defects (Gibon *et al.*, 2017). Furthermore, MSC do not express T and B cell markers and may inhibit T cell proliferation through direct contact and cytokine secretion (Zhao *et al.*, 2004). These immune-modulatory and immune evasive properties might facilitate the use of MSCs for cell-based therapies, especially for degenerative conditions. Cell therapies for osteoporosis involving MSCs (Aghebati-Maleki *et al.*, 2019; Antebi *et al.*, 2014) have been discussed, but have so far not been established in clinical use.

A heterogenic set of factors responsible for osteoporosis such as age, medication and genetics (Cummings and Melton, 2002) may be accountable for heterogeneous distribution of clinical manifestations. Donors' age has been described as a factor for reduced osteogenic and chondrogenic potential of MSCs in a murine model (Kretlow *et al.*, 2008). The co-incidence of age-related decrease in osteoblastic differentiation (Zhou *et al.*, 2008) suggests a possible link between BMSC differentiation potential and clinical manifestation of osteoporosis. Yet, osteoporosis itself seems not to be associated with a reduced osteogenic potential of MSCs (Haddouti *et al.*, 2020). On the other hand, MSCs derived from femoral heads of patients suffering from osteoporosis show reduced migration ability upon BMP-2, BMP-7 or FCS stimulation (Haasters *et al.*, 2014) and reduced but qualitatively adequate ossification (Prall *et al.*, 2013). Clinical findings and *in vitro* studies showed increased bone marrow adiposity and a negative association between bone marrow fat and rate of bone formation in osteoporotic patients (Kassem and Marie, 2011; Paccou *et al.*, 2015; Rosen and Bouxsein, 2006). Therefore, a shift from osteogenesis towards adipogenesis for MSCs from osteoporotic patients has been proposed (Kassem and Marie, 2011; Rosen and Bouxsein, 2006). Furthermore, a link between osteoporosis and decreased proliferation rates of MSCs harvested from iliac crest aspirates has been reported (Rodríguez *et al.*, 1999). Moreover, there is evidence that anti-resorptive substances routinely used in the therapy of osteoporosis may interact beneficially with MSCs for bone formation. *In vitro*, alendronate has a positive effect on osteoblastic differentiation of MSCs from healthy donors (Duque and Rivas, 2007). For osteoporotic patients, cell therapy may also play a role in the treatment of side effects of anti-resorptive substances. In a rat model, beneficial effects of growth factors and cytokines secreted by MSCs were found for cases of medication-related osteonecrosis of the jaw (Ogata *et al.*, 2015).

Čamernik *et al.* (2020) described differences between MSCs from osteoporotic patients and non-osteoporotic patients. In their work, after chondrogenic differentiation, the pellet diameter of bone-derived MSCs from osteoporotic donors was significantly smaller compared to controls. Also, deficiency in *CD152* (*CTLA-4*) and *CD279* (*PD-1*),

a receptor of CD274, have been linked to bone malformation in a murine model (Nagahama *et al.*, 2004). Hypermethylation of the CD279 gene locus, PDCD1, has recently been linked to the occurrence of osteoporosis in postmenopausal women (Cheishvili *et al.*, 2018).

As little is known about possible effects of altered MSCs on bone metabolism, the aim of the present study was to investigate differences between hBMSCs from osteoporotic and non-osteoporotic donors. The study especially focused on evaluating differences within their osteogenic, adipogenic and chondrogenic differentiation potential, proliferation ability as well as alternations in surface antigen and gene expression.

Materials and Methods

Inclusion and exclusion criteria and donors' consent
All tissue samples were acquired by bone marrow aspiration from volunteers undergoing elective surgical procedures at the Trauma Department of Hannover Medical School, Hannover, Germany. Donors suffering from clinically relevant osteoporosis were included in the study group. Criteria for inclusion were previous pathological fractures, vertebral wedge fractures and T scores ≤ − 2.5 in DEXA scan. Immunosuppressed patients and those suffering from malignant diseases were excluded from the study. An anonymous questionnaire about pre-existing conditions and lifestyle as well as reports about secondary diagnoses was obtained from all donors.

Written informed consent was obtained from all donors prior to inclusion in the study. The study protocol and process of sample donation complied with the Declaration of Helsinki and were approved by the institutional review board (Hannover Medical School, Votum number 2562).

MSC purification and proliferation
After intraoperative bone marrow aspiration from the iliac crest, samples were transferred into PBS (Biochrom) and subsequently separated by centrifugation for 30 min at 500 ×g using a synthetic polysaccharide-epichlorohydrin-copolymer (Biocoll®, Biochrom). The centrifuge was allowed to run down without the action of a break in order to prevent the layers from being swirled by the breaking force. Then, the mononuclear cell layer was extracted, washed with PBS and centrifuged for 5 min at 500 ×g. Next, the generated pellet was re-suspended in MSC specific growth medium containing DMEM FG 0415 (Biochrom) supplemented with 10 % (v/v) FBS (Hyclone® FBS, Thermo Fisher Scientific), 20 mmol/L HEPES, 1 % (100 U/mL/100 μg/mL) penicillin/streptomycin (Biochrom) and 2 ng/mL human recombinant FGF2 (PeproTech).

To facilitate BMSC proliferation, cells were incubated at 37 °C and 5 % CO_2, as previously described (Schäck *et al.*, 2013). In P1 of *in vitro* cultivation, cells were cryoconserved using freezing medium containing 95 % FBS and 5 % DMSO. Cells were stored at − 152 °C in a cell bank for hBMSCs at the Trauma Department of Hannover Medical School for further analyses.

CFU-F assay
During hBMSC characterisation, a CFU-F assay was carried out at P1 to investigate cells' proliferation potential. Cells were seeded in duplicates in ascending concentrations of 125, 250 and 500 cells per well in a six-well plate. Cells were fixed with methanol (Merck) after 10 d of incubation at 37 °C and 5 % CO_2. After staining the cells using 1 % crystal violet solution (Merck), macroscopically visible BMSC colonies were counted to calculate the number of colonies per 100 cells seeded.

FC
To assess CD274 expression and to confirm identifying criteria for MSCs, cell samples were analysed by FC. In addition to the criteria defined by Dominici *et al.* (2006) (expression of CD105, CD73 and CD90 but not of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR), expression of CD29, CD44, CD166, CD11c, CD15 and CD31 was analysed, as postulated in the literature (Bara *et al.*, 2014; Dominici *et al.*, 2006; Machado *et al.*, 2013).

After controlled thawing, cells were incubated, as previously described (Schäck *et al.*, 2013), in MSC specific growth medium. P3 cells were detached using 0.05 %/0.02 % trypsin-EDTA solution (Biochrom) and washed twice using FC buffer [2 % (v/v) FBS in PBS]. All centrifugation steps were performed at 400 ×g and 4 °C for 1 min. For each sample, 1×10^5 cells were used and incubated with appropriate fluorochrome-conjugated antibodies for 60 min at 4 °C in the dark. The following monoclonal antibodies (mouse anti-human) were used: CD11b APC, CD15 FITC, CD19 PerCP, CD29 APC, CD31 FITC, CD34 PE-Cy7, CD44 FITC, CD45 APC-Cy7, CD73 APC, CD90 PerCP Cy5.5, CD105 PE, CD166 FITC. All antibodies were purchased from BioLegend (San Diego, CA, USA) apart from CD166 FITC, which was purchased from MBL (Woburn, MA, USA). CD274 expression was evaluated during FC analysis using PE anti-human CD274 (B7-H1, PD-L1) antibodies (clone 29E.2A3, Biolegend) (Table 1).

After two washing steps with FC buffer, cells were analysed using a FACS Canto (BD Biosciences) as described before (Schäck *et al.*, 2013). Briefly, for each flow cytometric analysis, 3×10^4 cells were recorded. Cell debris was excluded by using scatter parameters in BD FACSDiva Software and Flowing Software version 2.5.0. Within the analysed surface antigens, minimal standards regarding expression were expected to be met according to those commonly described in the literature (Bara *et al.*, 2014; Dominici *et al.*, 2006; Machado *et al.*, 2013).

Table 1. Antibodies used for FC Analysis. The following monoclonal antibodies (mouse anti-human) were used for FC analysis.

Antibody	Dye	Volume (µL) per 100 µL buffer	Clone	Catalogue number	Company
CD105	PE	1.25	43A3	323206	Biolegend
CD11b	APC	2.50	ICRF44	301310	Biolegend
CD15	FITC	0.63	W6D3	323004	Biolegend
CD166	FITC	0.40	3A6	K0044-4	MBL
CD19	PerCP Cy5.5	2.50	HIB19	302230	Biolegend
CD274	PE	0.63	29E.2A3	329706	Biolegend
CD29	APC	0.03	TS2/16	303018	Biolegend
CD31	FITC	1.25	WM59	303104	Biolegend
CD34	PE-Cy7	2.50	581	343516	Biolegend
CD44	FITC	0.20	BJ18	338804	Biolegend
CD45	APC-Cy7	2.00	H130	304014	Biolegend
CD73	APC	0.01	AD2	344006	Biolegend
CD90	PerCP Cy5.5	0.50	5E 07	328118	Biolegend

Gene expression

RT2 Profiler PCR Array Osteoporosis (Gene Globe ID: PAHS-170ZC-2, Qiagen) was carried out as previously described (Bundkirchen *et al.*, 2018). Summarised, RNA was isolated from hBMSCs pellets from three healthy and three osteoporotic donors using the Direct-zol RNA extraction kit (Zymo Research), according to the manufacturer's protocol. RNA quality was determined using an Epoch-Reader (BioTek Instruments). cDNA was synthesised starting from 1 µg RNA using the High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystem) according to the manufacturer's protocol.

For determination of involved signalling pathways, the RT2 Profiler PCR Array Osteoporosis was performed using the Step-OnePlus Real-Time PCR System (Applied Biosystems). For all samples, the threshold was set at 0.075 ΔRn [ΔRn = Rn (post-PCR read) − Rn (pre-PCR read), where Rn = normalised reporter]. Ct values were exported to an Excel file. Then, this table was uploaded to a data analysis web portal (Web ref. 1). Samples were assigned to control (healthy donors) and test group (osteoporotic donors). Ct values were normalised based on the reference gene HPRT1. The data analysis web portal calculates fold change/regulation using the ΔΔCt method, in which the ΔCt is calculated between a gene of interest and an average of reference genes, followed by ΔΔCt calculations [ΔCt (test group) − ΔCt (control group)]. Then, fold change is calculated using the $2^{-\Delta\Delta Ct}$ formula. A fold change of 0.5 or less was considered as a down-regulation, of 2.0 or more as an up-regulation.

Determination of gene expression was performed using TaqMan probes (Applied Biosystems) for the following genes: CD274 (also known as PD-L1; Hs00204257_m1), CD279 (also known as PD-1; Hs01550088_m1) and CD152 (also known as CTLA4; Hs00175480_m1). As a housekeeping gene, eukaryotic 18S rRNA (Hs9999901 s1) was used. Experiments were carried out with the Step-OnePlus Real-Time PCR System and the corresponding Mastermix (Applied Biosystems). The threshold was set at 0.2 ΔRn for all samples. The evaluation of the Ct values was performed using the ΔΔCt method. Statistical analysis was performed with fold change values ($2^{-\Delta\Delta Ct}$).

Osteogenic differentiation

To assess the osteogenic potential of each specimen, BMSCs from each donor were transferred onto six-well plates with each well containing a total of 150,000 cells. Samples were incubated either in 3 mL control medium containing DMEM FG0415 (Biochrom) supplemented with 20 mmol/L HEPES (Biochrom), 10 % FBS (Thermo Fisher) and 1 % penicillin/streptomycin (100 U/mL/100 µg/mL; Biochrom) or 3 mL induction medium for osteogenic differentiation supplemented with 10 nmol/L dexamethsone (Merck), 50 µmol/L ascorbate-2-phosphate (Merck) and 3 mmol/L Na_2HPO_4/NaH_2PO_4 at pH 7.4 (Merck) added to the control medium. Medium was changed on a weekly basis. Histological specimens were harvested at days 28 and 42, fixed for 30 min in 4 % formalin solution and consequently stained using 0.5 % alizarin red S (pH 4.5; Carl Roth).

Adipogenic differentiation

Apart from a different medium to induce adipogenic differentiation, all specimens underwent the identical procedure as described for osteogenic differentiation. The induction medium for adipogenic differentiation contained 1 µmol/L dexamethasone (Merck), 500 µmol/L IBMX (Merck), 60 µmol/L indomethacin (Merck) and 172 nmol/L insulin in addition to the aforementioned control medium. Medium was changed on a weekly basis. Histological specimens were harvested at day 28, fixed in 4 % formalin

Table 2. Basic parameters within both groups measured by FC analysis. Mean expression and standard deviation in percentage. CD274 expression was significantly decreased in patients with osteoporosis compared to control group ($p = 0.007$). No significant differences were found for all other antigens between the groups.

Antigen	Control group	Osteoporosis group
CD11b	1.17 % ± 0.53 %	1.10 % ± 0.33 %
CD11c	4.22 % ± 1.92 %	3.23 % ± 1.54 %
CD15	0.78 % ± 0.34 %	0.87 % ± 0.18 %
CD19	5.19 % ± 4.61 %	2.66 % ± 0.65 %
CD29	89.72 % ± 19.44 %	98.09 % ± 1.76 %
CD31	0.99 % ± 0.43 %	1.23 % ± 0.44 %
CD34	14.65 % ± 11.56 %	14.53 % ± 4.78 %
CD44	81.72 % ± 28.83 %	91.85 % ± 5.25 %
CD45	2.96 % ± 1.22 %	2.46 % ± 0.93 %
CD73	94.14 % ± 9.52 %	94.74 % ± 3.31%
CD90	95.28 % ± 4.54 %	97.55 % ± 0.82 %
CD105	98.77 % ± 1.49 %	99.35 % ± 0.38 %
CD166	89.30 % ± 19.42 %	97.45 % ± 1.21 %
CD274	26.03 % ± 13.39 %	4.98 % ± 2.38 %

solution for 30 min and consequently stained with oil red O (5 g/L in 60 % isopropanol; Merck).

Chondrogenic differentiation

To assess the chondrogenic potential of each specimen, 2.5×10^5 hBMSCs from each donor were pelleted by centrifugation at 200 ×*g* for 5 min. Pellets were incubated in 0.5 mL control medium containing DMEM FG0435 (Biochrom) supplemented with 20 mmol/L HEPES-buffer (Biochrom), 1 % penicillin/streptomycin (100 U/mL/100 µg/mL; Biochrom), 0.1 µmol/L dexamethasone (Merck), 10 µL/mL ITS (Sigma-Aldrich), 170 µmol/L ascorbate-2-phosphate (Sigma-Aldrich), 1 mmol/L Na-pyruvate (Biochrom) and 350 µmol/L proline (Carl Roth). After 1 d, control medium was replaced by induction medium, with 10 ng/mL TGF-β3 added to the control medium, for chondrogenic differentiation. Each medium was changed on a weekly basis. Histological specimens were harvested at day 28. They were fixed in 4 % formalin solution for 30 min and embedded in Tissue-Tek® O.C.T. (Sakura Finetek, Tokyo, Japan) while using liquid nitrogen. 5 µm-thick frozen sections were stained using 0.1 % safranin O and covered using Vitro Clud® (Langenbrinck, Freiburg, Germany). At 40× magnification, the quality of chondrogenic differentiation was assessed and the area covered by the pellet in relation to the total area was evaluated to assess the pellet growth.

Digital processing and statistics

All samples were assessed by light microscopy. Images were evaluated by automatic threshold-based segmentation with an in-house-coded, Java-based software. Results were collected in Excel for Mac 14.7.7 (Microsoft) and processed using Wizard Statistics 1.9.16 (Evan Miller, Chicago, IL, USA). Normal distribution of the data was evaluated by Kolmogorov-Smirnov test. Non-normally distributed groups were compared by their median using Mann-Whitney U test. A $p \leq 0.05$ was considered to be
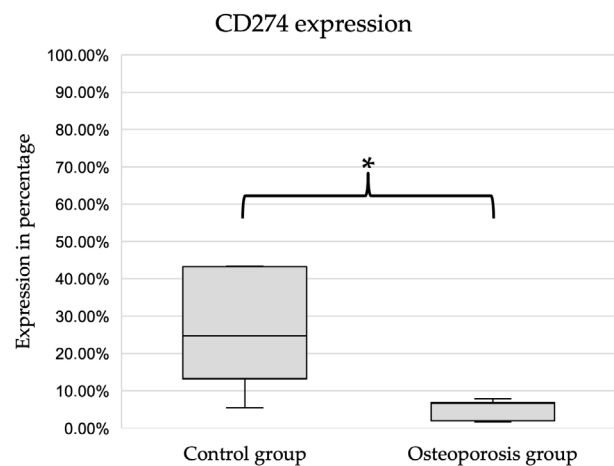

Fig. 1. Expression of CD274 was significantly lower in hBMSCs of the osteoporosis group than in control group (* $p = 0.007$).

statistically significant. Further statistical analyses were carried out using SPSS Statistics 24 (IBM).

Results

Cells from seven donors suffering from symptomatic osteoporosis were compared to seven samples from healthy patients (control group). Groups were matched by age and gender. Donors suffering from cancer and autoimmune disorders were excluded from the study. Immunotherapy, chemotherapy and regular intake of steroidal drugs were further criteria for exclusion. Previous intake of anti-resorptive substances was not taken into account. By gender, distribution within the groups was equivalent to 5 female : 2 male donors. Average ages were 80.00 years (± 7.24 years) and 80.14 years (± 3.52 years), for the osteoporosis and control group, respectively.

Table 3. Results of the RT2 PCR array showing 23 upregulated and 1 downregulated gene.

Symbol	Gene name	Fold change	Function
IL6R	Interleukin 6 receptor	0.05	Receptor of IL6
TNFAIP3	TNF alpha induced protein 3	2.01	Inhibits osteoclastogenesis (Yan et al., 2020)
PTH	Parathyroid hormone	2.13	Regulates serum calcium concentrations through its effects on bone (Wein and Kronenberg, 2018)
ITGA1	Integrin subunit alpha 1	2.24	Subunit of integrin receptors. Polymorphisms and haplotypes ITGA1 are associated with bone mineral density and fracture risk in postmenopausal woman (Lee et al., 2007)
NOG	Noggin	2.25	Extracellular BMP antagonist, decreases osteoblastogenesis (Moffett et al., 2009)
TNFRSF11B/OPG	Tumour necrosis factor receptor superfamily, member 11b (osteoprotegerin)	2.28	Antagonist of RANKL (Tella and Gallagher, 2014)
SFRP1	Secreted-frizzled-related protein 1	2.30	Inhibitor of the anabolic Wnt signalling pathway (Wang et al., 2014)
SFRP4	Secreted-frizzled-related protein 4	2.32	Inhibitor of the anabolic Wnt signalling pathway (Wang et al., 2014)
NFATC1	Nuclear factor of activated T cells 1	2.36	Key transcription factor, contributes to osteoclast differentiation and bone resorption (Li et al., 2019)
DKK1	Dickkopf Wnt signalling pathway inhibitor 1	2.41	Inhibitor of the anabolic Wnt signalling pathway (Ueland et al., 2019)
IGF1	Insulin-like growth factor 1	2.64	Critical growth factor to maintain bone mass (Crane and Cao, 2014)
IGFBP2	Insulin-like growth factor binding protein 2	2.92	The role of IGFBP-2 in bone homeostasis is somewhat conflicted with evidence to support agonistic and antagonistic actions depending on the model (DeMambro et al., 2015)
IL6	Interleukin 6	3.09	Mediates the upregulation of osteoclastogenesis (Fajar and Azharuddin, 2017)
ALOX15	Arachidonate 15-lipoxygenase	3.12	Negative regulator of peak bone mineral density (Klein et al., 2004)
ADCY10	Adenylate cyclase 10	3.24	Association with osteoporosis-related phenotypes (Xu et al., 2010)
LTA	Lymphotoxin alpha	3.26	LTα, formerly known as TNFβ, is the closest homolog to TNFα (Franki et al., 2005)
CYP17A1	Cytochrome P450 family 17 subfamily A member 1	3.44	Key factor in the synthesis of oestrogen (Rodriguez-Sanz et al., 2015)
PRL	Prolacatin	4.79	In the presence of high PRL levels, bone resorption is increased and bone formation is suppressed (di Filippo et al., 2020)
GHRH	Growth hormone releasing hormone	5.12	Stimulates osteoclastic bone resorption through actions on osteoclast differentiation (Cecconi et al., 2006)
SOST	Sclerostin	5.87	Inhibitor of the anabolic Wnt signalling pathway (Ueland et al., 2019)
ACP5	Acid phosphatase 5, tartrate resistant	6.21	Involved in skeletal development, collagen synthesis and degradation, bone mineralisation, cytokine production (Hayman, 2008)
PTHLH	Parathyroid hormone like hormone	6.42	Regulates serum calcium concentration through its effects on bone (Wein and Kronenberg, 2018)
BMP7	Bone morphogenetic protein 7	7.35	BMP7 gene polymorphisms are associated with inverse relationships between vascular calcification and BMD (Freedman et al., 2009)
WNT3A	Wnt family member 3A	15.05	Members of Wnt signalling pathway, which regulates bone formation (Glass II et al., 2005)

CFU-F assay

CFU assays were carried out for both groups at P1. Cells from osteoporotic donors formed 3.74 (± 3.13) colonies per 100 cells seeded, cells from the control group 4.40 (± 4.86). The result was not statistically significant ($p = 1.000$).

Expression of MSC-specific surface antigens

Surface antigen expression of CD11b, CD11c, CD15, CD19, CD29, CD31, CD34, CD44, CD45, CD73, CD90, CD105, CD166 and CD274 was measured at P3 by FC (Table 2). Cells displayed a typical pattern of MSC-specific surface antigens. No significant differences were observed between the surface antigens in the osteoporosis and control group, except for CD274 (PD-L1). MSCs from osteoporotic donors had significantly less CD274 surface expression (4.98 % ± 2.38 %) than those from the control group (26.03 % ± 13.39 %; Fig. 1). Differences between both groups were statistically significant ($p = 0.007$).

Gene regulation (RT2 PCR osteoporosis array)

Abbreviation, gene name and gene function of the upregulated and downregulated genes of the RT2 Profiler PCR Array Osteoporosis are listed in Table 3. The analysis resulted in 23 upregulated and one downregulated gene in the RNA of hBMSCs from osteoporotic patients compared to healthy ones. The most expressed gene was *WNT3A* (fold change: 15.05). Interestingly, also many inhibitors of the anabolic Wnt signalling pathway as *DKK1* (fold change: 2.41), *SFRP1* (fold change: 2.30) and *SFRP4* (fold change: 2.32) as well as *SOST* (fold change:

5.12) were significantly upregulated in samples from osteoporotic patients compared to healthy controls. Furthermore, markers known to stimulate bone resorption as *ALOX15* (fold change: 3.12), *GHRH* (fold change: 5.12), *IL6* (fold change: 3.09), *NFATC1* (fold change: 2.36), *PRL* (fold change: 4.79), *PTH* (fold change: 2.13) and *PTHLH* (fold change: 6.42) were significantly increased in osteoporotic patients. The only downregulated gene was *IL6R*, with a fold change of 0.05.

Gene expression

mRNA expression of *CD274*, *CD279* and *CD152* was examined for five randomly chosen specimens per group. While *CD279* was not expressed in hBMSCs, the mRNA expression of *CD152* showed no significant difference ($p > 0.05$) in cells from osteoporotic patients (fold change: 0.833 ± 0.623) compared to controls (fold change: 1.053 ± 0.651). In contrast, *CD274* mRNA expression was significantly ($p = 0.016$) lower in hBMSCs from osteoporotic patients (fold change: 0.648 ± 0.209) compared to controls (fold change: 1.443 ± 0.745).

Adipogenesis

Adipogenic differentiation was induced by treatment with insulin and dexamethasone. All specimens were capable of adipogenic differentiation. At day 28, within the osteoporosis group, 35.02 % (± 22.71 %) of the investigated area was covered with lipid droplets (Fig. 2**a**), while 38.10 % (± 11.31 %) of the area had undergone adipogenic differentiation within the control group. Differences were not
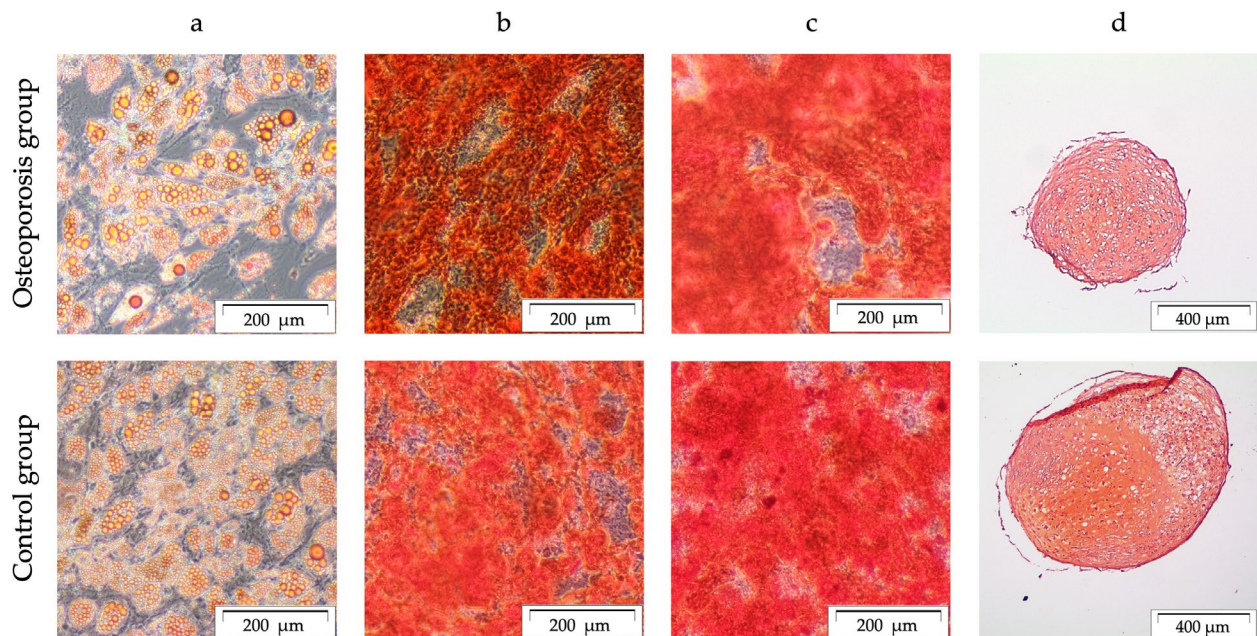

Fig. 2. **Representative images displaying the multipotent differentiation potential of hBMSCs from one donor of the osteoporosis group and one donor of the control group.** All images display samples after incubation with the respective induction medium. (**a**) Adipogenic differentiation at day 28, stained with oil red O. Images taken at 100× magnification. (**b**) Osteogenic differentiation at day 28: stained with alizarin red. Images taken at 100× magnification. (**c**) Osteogenic differentiation at day 42: stained with alizarin red. Images taken at 100× magnification. (**d**) Chondrogenic differentiation at day 28: stained with safranin O. Images taken at 40× magnification. Scale bars as indicated on the image.

statistically significant ($p > 0.05$). The non-induced samples showed no signs of major spontaneous cell differentiation.

Osteogenesis

As observable signs of osteogenic differentiation become visible later than signs of adipogenic differentiation, measurements were carried out at day 28 and 42. The non-induced samples showed no signs of major spontaneous cell differentiation. At day 28, the area covered by stained calcium deposits was 85.39 % (± 10.74 %) in the osteoporosis group and 83.40 % (± 15.40 %) in the control group (Fig. 2**b**). By day 42, 84.09 % (± 5.71 %) of the area in the osteoporosis group and 94.09 % (± 5.71 %) of the area in the control group (Fig. 2**c**) were covered by histological signs of osteogenic differentiation. Differences between groups were not statistically significant ($p > 0.05$).

Chondrogenesis

Chondrogenic differentiation was induced by treatment with TGF-β3. All induced samples in the osteoporosis or control group were capable of chondrogenic differentiation. The non-induced samples showed no signs of major spontaneous cell differentiation. By day 28, the relative area covered was 17.28 % (± 8.56 %) within the osteoporosis group *versus* 27.04 % (± 15.76 %) in control group (Fig. 2**d**). Differences were not statistically significant ($p > 0.05$).

Discussion

Osteoporosis is a disease of increasing economic importance (Kling *et al.*, 2014). Even though a variety of screening methods are available, osteoporosis is still considered to be under-diagnosed and under-treated (Golob and Laya, 2015). A variety of therapy approaches exists, some with severe side-effects (Kling *et al.*, 2014). Drugs with direct long-term effect on bone metabolism such as bisphosphonates and monoclonal antibodies (*i.e.* Denosumab) are frequently administered. Nevertheless, they come with severe long-term side effects, *e.g.* MRONJ (Fliefel *et al.*, 2015; Kling *et al.*, 2014; Lemound *et al.*, 2017; Pichardo *et al.*, 2013; Ruggiero *et al.*, 2014). Cell-based therapies might pose an alternative to current standard therapy (Antebi *et al.*, 2014; Kassem *et al.*, 2004; Mikami *et al.*, 2014). As they may be able to provide a causal therapy approach, they could be especially useful for young patients to avoid long-term drug administration. To develop these specific therapy approaches for osteoporosis, complete and profound understanding of underlying pathomechanisms is essential. Therefore, the aim of the present study was to identify differences in the stem cell characteristics of osteoporosis patients compared to healthy subjects.

Some studies in the field of bone metabolism (Rodríguez *et al.*, 1999) propose decreased osteogenic differentiation potential for BMSCs from osteoporotic patients. This is in disagreement with more recent, methodically more robust studies (Haddouti *et al.*, 2020) describing that MSCs from osteoporotic and healthy patients possess similar differentiation properties. In the present study, osteogenesis did not seem to be impaired on a cellular level *in vitro*. In line with this observation, Prall *et al.* (2013) have suggested that MSCs from osteoporotic donors might have impaired signal transduction but sustained osteoinduction upon stimulation. Therefore, an osteoporosis array was performed to examine differences between osteoporotic and healthy patients on the gene expression level. Results showed, on the one hand, a significant increase in genes associated with osteoclast activation and, on the other hand, an inhibition of the anabolic Wnt signalling pathway. These results were in line with the findings of other working groups who showed that inhibitors of the Wnt signalling, such as DKK1 or SOST, play an important role in the development of osteoporosis (Baron and Gori, 2018; Baron and Kneissel, 2013). In addition, in osteoporotic patients, the balance between bone formation by osteoblasts and bone degradation by osteoclasts is shifted in favour of osteoclasts, wherefore many therapeutic strategies have been developed that aim at inhibiting excessive bone resorption (Rachner *et al.*, 2011). Furthermore, clinical findings in MRI studies, proposing a connection between increased amounts of bone marrow fat and decreased bone density (Paccou *et al.*, 2015), suggest possible differences in adipogenic and osteogenic differentiation potential. At a molecular level, different concentrations of pro-adipogenic and pro-inflammatory regulatory factors are reported for osteoporotic bone marrow (Pino *et al.*, 2012). In the present study, no significant differences in adipogenic, osteogenic and chondrogenic differentiation potential of hBMSCs could be found *in vitro*, substantiating the assumption of a strong modulatory effect of the surrounding environment within the bone marrow *in vivo*.

The surface antigen expression on MSCs is still a subject of current research and several amendments have been made to the criteria postulated by Dominici *et al.* (2006). The meaning of the expression of some antigens such as HLA-DR (Bocelli-Tyndall *et al.*, 2010; Dominici *et al.*, 2006; Larghero *et al.*, 2008; Tarte *et al.*, 2010) and CD34 (Dominici *et al.*, 2006; Lin *et al.*, 2012) remains unclear. Regarding HLA-DR expression, it was shown that an exposure of hBMSCs to FGF2 is suspected to lead to its expression *in vitro* (Bocelli-Tyndall *et al.*, 2015). As cells in the present study were exposed to FGF2 prior to FC analysis, HLA-DR expression was, as postulated by Tarte *et al.* (2010), excluded from the minimal criteria for MSCs (Dominici *et al.*, 2006). Regarding CD34, it is highly controversial whether its non-expression should be considered as a minimal criterion, as postulated by Dominici *et al.* (2006). In the present study, more than 5 % of the cells expressed CD34. As cells were

propagated in culture, CD34 non-expression was not considered as an appropriate minimal criterion for MSCs, in agreement with Lin *et al.* (2012). The other surface antigens that should be measured according to the ISCT criteria were, as expected, positive (CD105, CD73 and CD90) or negative (CD45, CD11b, CD19) in both patients' groups without significant differences. In addition to the criteria defined by Dominici *et al.* (2006), further surface antigens were tested by FC: CD29, CD44, CD166, CD11c, CD15 and CD31, as postulated in the literature (Bara *et al.*, 2014; Dominici *et al.*, 2006; Machado *et al.*, 2013). These criteria were sufficiently met by the cells, thus supporting they represented sufficiently pure MSC populations.

Besides degenerative factors, B and T cells influence osteoclasts *via* RANKL pathway and other immunological pathologies involving CD279 deficiency and CD40 expression have been described (Arron and Choi, 2000; Li *et al.*, 2007; Nagahama *et al.*, 2004; Pietschmann *et al.*, 2016). CD274 expression rates have been described as highly heterogenic among different hBMSC samples and to correlate negatively with donors' age (Siegel *et al.*, 2013). To the authors' knowledge, gender differences have so far not been reported. Nagahama *et al.* (2004) described a link between deficiency of CD279 and bone malformation without effect on the rate of osteogenesis *in vitro*. They showed the occurrence of an osteoporotic phenotype for CD279 deficient mice, suggesting an underlying mechanism mainly based on the modulation of the activation of osteoclast-progenitors.

As CD274 (PD-L1) is the ligand of CD279, all samples in the study were assessed for their CD274 expression by FC and real-time PCR. An increase in CD274 concentration was expected in osteoporotic patients, as a deficiency in the receptor CD279 leads to osteopetrosis. However, in contrast to expectations, a 5-fold reduction in CD274 concentration at protein and a 50 % decrease at gene level were found in osteoporotic patients. Additionally, Cheishvili *et al.* (2018) found the hypermethylation of PDCD1, the gene locus of CD279, to be associated with the occurrence of osteoporosis. As the results of the present study showed an association between CD274 deficiency and osteoporosis, they can be considered consistent with those of Cheishvili *et al.* (2018). Furthermore, IL6R blockade prevents the upregulation of CD274 (Eriksson *et al.*, 2019) and IL6 signal pathway is necessary for CD274 stability (Chan *et al.*, 2019), as well as the maximum expression of CD274 is only possible in the presence of IL6 (Jin *et al.*, 2013). As significantly downregulated IL6R values and increased IL6 concentrations were detected in the osteoporosis gene array, CD274 could be an interesting candidate in the field of bone and osteoporosis research. The underlying mechanisms and the signalling pathways involved in this setting are still unknown and are the subject of further research.

Limitations

Some common drugs used in prophylactic treatment of patients at risk of or suffering from osteoporosis, such as bisphosphonates, have a long half-life (Lemound *et al.*, 2017; Pichardo *et al.*, 2013). Prior administrations, even decades, may therefore lead to effects of these substances onto the patient's bone metabolism. As this is frequently not remembered by the patient, prior intake of anti-resorptive substances was not considered for the present study.

It cannot be completely excluded that some drugs or interventions might influence CD274 regulation. Even though it is mainly known for chemotherapeutics, whose administration was a criterion for exclusion, a bias regarding this point cannot be completely ruled out.

The results presented were based on *in vitro*-expanded hBMSCs. This was necessary due to the regulations of the institutional review board, as cells were obtained from a biobank containing samples from human donors undergoing standard surgical procedures. Yet, *in vitro* expansion may diminish the quality of cells, especially in aged individuals. Thus, further experiments should be conducted to verify the findings of the study *in vivo*.

Conclusions

CD274 expression on hBMSCs *in vitro* was found to be significantly reduced in patients suffering from osteoporosis. Interactions between CD274 (PD-L1), its receptor CD279 on T cells and bone metabolism have been described (Nagahama *et al.*, 2004). The presented findings supported the theory of strong immunological components in the pathogenesis of osteoporosis (Arron and Choi, 2000). They may be a central starting point for further investigations regarding the pathogenesis of osteoporosis and into cell-based therapies involving MSCs.

The data used to support the findings of this study are available upon request to the the corresponding author upon request.

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Web Reference

1. http://www.qiagen.com/geneglobe [20.04.21]

Discussion with Reviewers

Glen Niebur: Can the authors propose a pathway by which decreased CD274 expression might affect

immune cells and consequently bone formation or resorption? Did the results point toward a potential pathway of increased osteoclastogenesis and bone loss in the endosteal niche? Is this consistent with the results of Nagahama *et al.* (2004)?

Authors: Unfortunately, it is not possible to answer these questions satisfactorily. After observing that CD274 was significantly downregulated in hBMSCs from osteoporotic patients, it was initially thought that this was in line with the findings of Nagahama *et al.* (2004). However, on a closer inspection, it turned out that a deficiency in CD274 and CD276 led to an osteoporotic phenotype. To clarify this factual situation, more precise molecular biological investigations are required, which are planned in future.

Reviewer: There is much talk of using MSCs to treat osteoporosis. How do you envisage this happening, given that MSCs do not engraft into bone very well and, in contrast to earlier expectations, do not allograft well. Do you think autologous cells engineered to express more CD274 might be useful?

Authors: The present study showed for the first time a lower expression of CD274 in hBMSCs from osteoporotic patients and this both at the mRNA and protein level. The exact connection between this observation and the clinical picture of osteoporosis has to be analysed in more detail in further studies. However, this is a first indication on which further research should be carried out. Even though MSCs do not engraft into bone very well, stem cell therapy can still be successful, *e.g.* due to secreted factors that in turn activate surrounding cells and intervene in bone remodelling.

Editor's note: The Scientific Editor responsible for this paper was Chris Evans.