Characterization of Mesenchymal Stromal Cells for Musculoskeletal Disorders and Regenerative Medicine

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List of Abbreviations

ALP	Alkaline phosphatase
ALPL	Alkaline phosphatase gene
BM-MSC	Bone marrow-derived mesenchymal stromal cells
COL1A1	Collagen, type I, alpha 1
hBM-MSC	Human bone marrow-derived mesenchymal stromal cells
hMSC	Human mesenchymal stromal cells
ISCT	International society for cellular therapy
nosMSC	Non-osteoporotic mesenchymal stromal cells
MSC	Mesenchymal stromal cells
OD	Optical density
oMSC	Ovine mesenchymal stromal cells
osMSC	Osteoporotic mesenchymal stromal cells
RT-PCR	Real-time polymerase chain reaction
RUNX2	Runt-related transcription factor 2
SOX9	SRY-Box transcription factor 9

1. Abstract

Recently, mesenchymal stromal cells (MSC) have come increasingly into focus as a therapeutic option for bone pathologies and regenerative medicine. While human MSCs have been extensively characterized and standardized, ovine MSCs are poorly understood. The current study reports a direct systematic comparison of human and ovine MSCs from three corresponding sources under the same conditions. All MSCs showed solid growth behavior and potent immunomodulatory capacity. In addition, common positive and negative surface markers were identified. Both human and ovine MSCs showed strong osteogenic potential.

Furthermore, the aim of this study was to isolate and characterize MSCs from the vertebral body of healthy non-osteoporotic and osteoporotic patients as the role of MSCs in osteoporosis is not fully understood. Isolated MSCs were characterized by their trilineage differentiation, surface marker expression, proliferation behavior, and immunomodulatory capacity. MSCs from both healthy and osteoporotic patients showed common morphology, proliferation behavior, expressed the typical MSC surface markers and possessed immunomodulatory capacity. Both groups demonstrated solid trilineage differentiation potential; osteogenic differentiation was further assessed by additional read outs such as optical density (OD) and free phosphate ion release.

Moreover, MSCs harvested from different tissues of the same donor have been shown to exhibit different phenotypes characterized by different cellular functionalities. In this study, we investigated the proteomic and functional properties of human bone marrow-derived MSCs (hBM-MSC) harvested either as aspirate or bone chip. Both MSC populations were profiled according to MSC markers defined by the International Society for Cellular Therapy (ISCT). hBM-MSCs derived from aspirate cultures demonstrated significantly higher osteogenic differentiation potential than MSCs grown from bone chip.

The key findings shown of this report reveal the utility of ovine MSCs in preclinical studies for MSC-based therapies. Furthermore, MSCs derived from vertebral body of osteoporotic patients were not impaired and possessed full osteogenic potential compared with MSCs from non-osteoporotic patients. Moreover, different harvesting techniques indicate the need for future standardized harvesting, processing, and phenotyping procedures to achieve better comparability in the MSC field.

2. Introduction & Aims with references

Bone is a complex and a highly dynamic tissue subject to constant turnover and remodeling, which is a well-orchestrated biological process during development and fracture healing (Einhorn et al., 2014). The performance of hard tissue regeneration depends on a balance of osteogenic cell groups, osteoinductive stimulants, and osteoconductive matrix (Giannoudis et al., 2007). However, these biological requirements seem to be limited in bone grafts and pathological bone. Mesenchymal stromal cells (MSC) are multipotent cells that possess a unique capacity for self-renewal and have therefore become a focus of interest as a possible adjuvant for tissue regeneration and therapies. Considering their osteogenic and chondrogenic potential, MSCs represent a promising cell population which offers new avenues for bone and cartilage regeneration (Fayaz et al., 2011). Recent studies have reported that there are crucial interactions between bone and immune cells. Evidence suggests that MSCs not only contribute to tissue repair but also have tremendous immunomodulatory capacity (Bernardo et al., 2013). Human (h)MSCs have been isolated from many different tissues (Pittenger et al., 1999), are well characterized and standardized according to the International Society for Cellular Therapy (ISCT) position statement (Dominici et al., 2006).

The sheep is the primary experimental large animal model for orthopedic preclinical research on bone and cartilage healing. The advantages of using sheep are their comparability to humans in terms of body weight, bone formation and anatomy (Pearce et al., 2007). Therefore, characterization of ovine (o)MSCs is necessary to investigate the effectiveness of cell therapies for bone regeneration and osseointegration of implants prior to clinical translation of human MSCs. However, the number of studies with oMSCs is still very low, oMSCs are poorly studied and not well characterized compared to hMSCs (Kolar et al., 2010). Considering the immense opportunity and promising potential of MSCs in orthopedics, oMSCs need standardization and direct comparison to hMSCs. To date, the reported oMSC characteristics do not meet the minimal criteria set by the ISCT for hMSCs (Dominici et al., 2006). To optimize the use of hMSCs and oMSCs, further efforts are needed to improve culture conditions of MSCs, identify common surface marker expression, optimize differentiation protocols, and identify gene expression markers for lineage-specific differentiation (Bottagisio et al., 2015). Only through

advances of both hMSCs and oMSCs can the translation of preclinical findings into clinical application be achieved.

Osteoporosis is a common skeletal disease characterized by low bone mass and increased fracture risk. Unfortunately, current therapies are still unsatisfactory, with osteoporosis increasingly recognized as a major public health issue (Cauley, 2013). Current treatments of osteoporosis mostly focus on preventing bone resorption and sustaining bone density, but unfortunately, also cause severe side effects (Antebi et al., 2014). Consequently, there is an urgent need for alternative innovative therapies that promote continuous bone sustainability and regeneration in patients with osteoporosis. In general, osteoporosis is the result of an imbalance between bone resorption and new bone formation (Raisz, 2014) mediated by osteoclast and osteoblast cell lineages, respectively (Teitelbaum, 2010). There is growing evidence that bone marrow-derived mesenchymal stromal cells (BMSC), the progenitors of osteoblasts, play a central role in osteoporosis (Bianco and Robey, 2015). Multipotent BMSCs contribute to bone tissue homeostasis under physiological conditions with their well-regulated osteogenic and adipogenic properties (Hu et al., 2018). However, various factors, such as menopause or aging, perturb this homeostatic equilibrium, eventually leading to an imbalanced formation of adipocytes in bone marrow and loss of bone mass (Tokuzawa et al., 2010). Recent studies have reported that the osteogenic potential of MSCs is significantly altered in osteoporotic bone (Wang et al., 2014). Specifically, MSCs from osteoporotic patients possessed a lower ability to differentiate into osteoblasts and dispayed a lower growth rate compared to MSCs from healthy patients (Rodriguez et al., 1999; Wang et al., 2006). However, most of these studies did not consider the source of MSCs. This is of particular importance as recent studies could indicate that tissue source and harvesting technique have an enormous impact on MSC performance, which is significantly underestimated in the current literature and requires further investigations (Mushahary et al., 2018). MSCs from different tissues as well as small molecules to recruit endogenous stromal cell for cell-based osteoporosis therapy have been proposed (Aghebati-Maleki et al., 2019). To date, we are only at the beginning of decoding the features of MSCs and a more profound analysis is needed to better understand how they might be used in a clinical setting (Schildberg and Donnenberg, 2018). One important MSC niche in the context of osteoporosis is the spine. It is commonly affected by osteoporosis, which is also evident

from vertebral compression fractures, which occur frequently and heal poorly in patients with osteoporosis (Tome-Bermejo et al., 2017; Chin et al., 2007). Unfortunately, comparatively few studies of the human vertebral body as a source for MSCs have been conducted due to its delicate anatomical location, which makes it difficult to access compared to other MSC niches. Consequently, the human vertebral body as a stromal cell niche is poorly studied: not only is it unclear whether functional impairment of MSCs contributes to the pathogenesis of osteoporosis, but a simple basic characterization of MSCs from vertebral bodies has not been reported.

The harvesting methods used and how the cells were treated during cell culture might have a significant impact on the phenotype and molecular cell properties of MSCs. However, researchers in the field of MSCs are just beginning to understand how these different methodological approaches affect MSC performance. Although the influence of harvesting procedure and tissue site on the osteogenic function of MSCs have been reported (Henrich et al., 2016), further systematic studies are needed to address this challenge. In this respect, classical MSC surface markers are insufficient for accurate MSC characterization. Rather, a broader analysis is crucial for accurate MSC characterization, including proteomic-like screening for surface markers, transcriptome clusters, and functional properties, such as immunomodulatory capacity and regenerative potential (Zimmerlin et al., 2013). The harvest technique of bone marrow may significantly affect heterogeneity of MSCs and thus, their regeneration capacity (Sivasubramaniyan et al, 2018). While MSCs from adipose or umbilical cord tissues (Mennan et al., 2019) are readily obtained in practice (Maria et al., 2017), bone marrow aspiration and bone reaming remain the methods most commonly described as standard.

In the current study, MSCs were derived from the same anatomical bone structure, but different harvesting techniques were used. Specifically, bone marrow aspirate was compared with bone chips from the femur. Both materials can be easily obtained during surgery procedures and are a reliable source for a clinically relevant MSC production. Direct comparison of these two harvesting techniques allowed evaluation of their effect on the cellular phenotypes and functional properties of MSCs.

The aims of the current study were, first, to compare hMSCs with oMSCs from three sources, under the same conditions and to comparatively delineate their characteristics as set by ISCT. Second, to isolate MSCs from vertebral bodies of osteoporotic and non-

osteoporotic control patients, to characterize both MSC groups and to investigate their osteogenic differentiation capability using different approaches. Third, to investigate whether different harvesting techniques from the same donor site result in the typical expression pattern of MSC markers and similar functional properties in terms of their trilineage differentiation behavior.

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3. Publications

3.1 Publication 1

International Journal of
Molecular Sciences

MDPI

Article

Characterization and Comparison of Human and Ovine Mesenchymal Stromal Cells from Three Corresponding Sources

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Abstract: Currently, there is an increasing focus on mesenchymal stromal cells (MSC) as therapeutic option in bone pathologies as well as in general regenerative medicine. Although human MSCs have been extensively characterized and standardized, ovine MSCs are poorly understood. This limitation hampers clinical progress, as sheep are an excellent large animal model for orthopedic studies. Our report describes a direct comparison of human and ovine MSCs from three corresponding sources under the same conditions. All MSCs presented solid growth behavior and potent immunomodulatory capacities. Additionally, we were able to identify common positive (CD29, CD44, CD73, CD90, CD105, CD166) and negative (CD14, CD34, CD45, HLA-DR) surface markers. Although both human and ovine MSCs showed strong osteogenic potential, direct comparison revealed a slower mineralization process in ovine MSCs. Regarding gene expression level, both human and ovine MSCs presented a comparable up-regulation of Runx2 and a trend toward down-regulation of Col1A during osteogenic differentiation. In summary, this side by side comparison defined phenotypic similarities and differences of human and ovine MSCs from three different sources, thereby contributing to a better characterization and standardization of ovine MSCs. The key findings shown in this report demonstrate the utility of ovine MSCs in preclinical studies for MSC-based therapies.

Keywords: mesenchymal stromal cells; ovine animal model; orthopedics; regenerative medicine; immunomodulation; differentiation; proliferation rate; surface markers

1. Introduction

The bone is under constant turnover and remodeling, which is a well-regulated biological process during development and fracture healing [1,2]. However, large bone defects caused by tumor, trauma, failed arthroplasty, or osteosynthesis represent an especially challenging clinical problem. The reason for this is that bone tissues cannot afford the regeneration of large bone defects and require bone graft or biomaterials to bridge the tissue gap, restore the structural support, and sustain the physiological and regenerative process. The gold standard in reconstructing large bone defects has historically been the autologous bone graft, but it is recognized that surgical stress and bone quality of the harvested tissue are significantly limiting factors of the procedure [3]. The efficiency of hard tissue regeneration depends

on a balance of osteogenic cell groups, osteoinductive stimulants, and osteoconductive matrix [4]. These biological resources, however, appear to be limited in bone grafts and in the surrounding diseased tissue. Therefore, mesenchymal stromal cell (MSC) therapies have become an area of interest as they provide a possible adjuvant for tissue regeneration. Due to their osteogenic and chondrogenic potential, they are a promising cell population which offers new ways to regenerate bone [5].

Recent studies have revealed that there are extensive interactions between bone and immune cells. New information reveals that MSCs not only contribute to tissue repair but also possess immense immunomodulatory capacity [6,7]. This immunomodulatory relevance means that MSCs are important for therapeutic modulation of disease development and degenerative processes [8,9]. Several interesting new demonstrations of the immunomodulatory capacity of MSCs showed that MSCs from different sources can influence responses and progression of various inflammatory diseases, and they have the capacity to home and integrate into impaired tissues. These immunomodulatory effects appear to be precisely coordinated with the inflammatory microenvironment [10,11].

Human (h)MSCs have been isolated from multiple different tissues [12], being well characterized and standardized according to a position statement of the International Society for Cellular Therapy (ISCT) [13,14]. However, sheep is the primary experimental large animal model for orthopedic preclinical research on bone healing, material biocompatibility, and newly developed implants. Advantages of using sheep as a primary model are their comparability to humans for multiple characteristics including body weight, bone formation, and anatomy [15–18]. Sheep are also ethically accepted and are easy to keep and handle [19]. Therefore, the characterization of MSCs from sheep is mandatory to investigate the efficacy of cell therapies for bone regeneration and implant osseointegration before clinical use of human MSCs. However, despite the convenience of utilizing sheep as a large animal model for orthopedics and the recent advantages in using MSCs, the number of studies involving ovine (o)MSCs is still very low [20].

oMSCs are poorly studied and not well characterized in comparison to hMSCs regarding their isolation, expansion, media formulation, cell surface expression, and differentiation. Due to the great opportunity and promising potential of MSCs in orthopedics, the oMSCs need standardization and direct comparison to hMSCs. Some similarities between hMSCs and oMSCs have been reported in the literature [15,20], but the reported oMSC characteristics do not meet the minimal criteria set by the ISCT for hMSCs [14]. To provide optimum use of both hMSCs and oMSCs, further efforts must be made to improve the culture conditions of MSCs, identify common surface marker expression, optimize differentiation protocols, and identify gene expression markers for lineage-specific differentiation [21]. Only through advances of both hMSCs and oMSCs can the translation of preclinical findings into clinical application come to fruition.

The current study aimed to compare hMSCs directly with oMSCs from three sources, under the same conditions, and to delineate their characteristics comparatively as set by ISCT.

2. Results

2.1. hMSC and oMSC Morphology and Proliferation Rates

Three corresponding human and ovine sources (Figure 1A) were selected for isolation of MSCs. hMSCs were harvested from donors undergoing liposuction in the abdomen (hAMSCs, adipose tissue-derived MSCs), after hip replacement (hFMSCs, femoral-derived MSCs), and during kyphoplasty procedures (hBMSCs, bone marrow-derived MSCs). oMSCs were harvested from adipose tissue (oAMSCs) in the thigh, femoral marrow fat (oFMSCs), and the tuber ischiadicum (oBMSCs). They were isolated on the basis of their ability to selectively adhere to a plastic surface. On the third day after the first culturing, the non-adherent cells were aspirated and disposed. The adherent MSCs exhibited typical mesenchymal morphology and showed flat polygonal fibroblast-like shape (Figure 1B). All MSCs from human sources (Figure 1C, left) and ovine sources (Figure 1C, right) showed a solid growth behavior. When comparing MSCs from the three human sources with each other, hFMSCs

demonstrated a trend for increased proliferation in comparison to hAMSCs and hBMSCs. Comparing MSCs from the ovine sources with each other resulted only in minor differences.

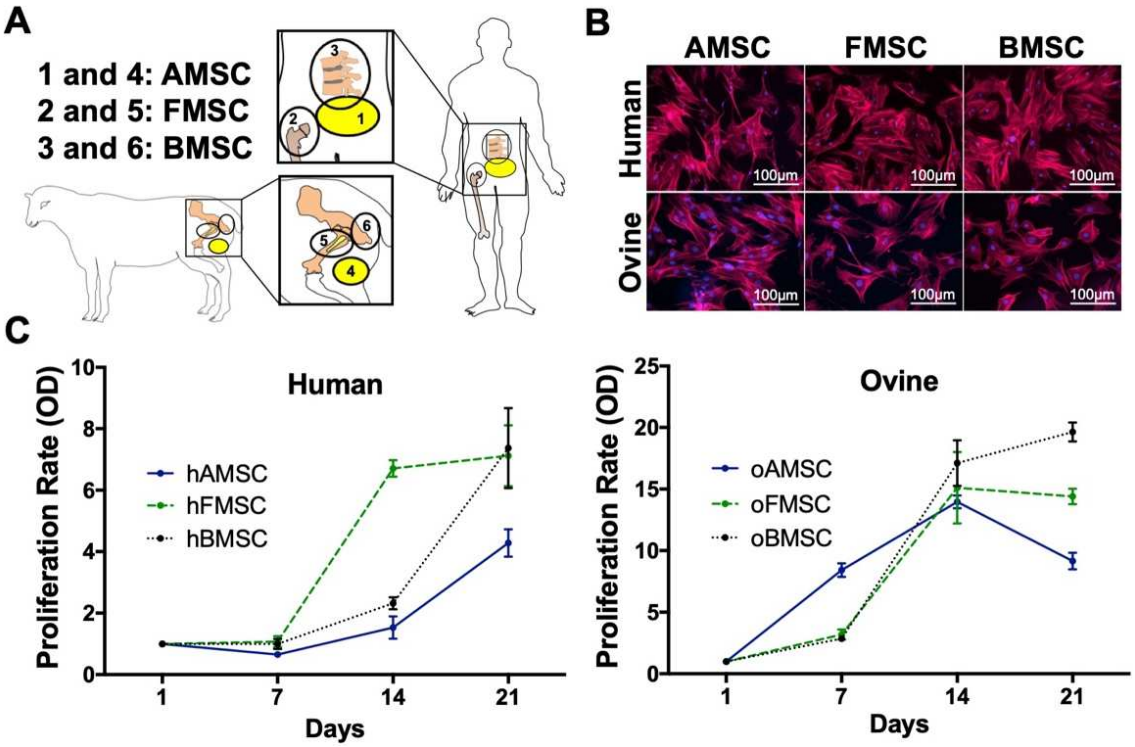

Figure 1. Graphical representation of the different human and ovine mesenchymal stromal cell (MSC) sources as well as their morphology and growth rate of corresponding MSCs. (**A**) MSC isolation from three corresponding human and ovine sources. Human (h)MSCs were harvested from donors undergoing abdomen liposuction cosmetic surgery (1), after hip replacement (2), and during kyphoplasty procedure (3). Ovine (o)MSCs were harvested from thigh adipose tissue (4), femoral marrow fat (5), and the tuber ischiadicum (6). (**B**) MSCs from human and ovine sources showed fibroblast-like morphology. Cytoskeleton-actin (red) and nucleus (blue). Representative pictures are shown. (**C**) MSC growth behavior was defined by measuring the optical density (OD) at the indicated time intervals. MSCs from human sources (left), MSCs from ovine sources (right). Data are expressed as average ± SEM of 3–5 donors per source. AMSC: adipose tissue-derived MSC, FMSC: femoral-derived MSC, BMSC: bone marrow-derived MSC.

2.2. Determination of Surface Markers

According to criteria set by the ISCT, MSCs from three human sources and their corresponding ovine sources were analyzed for surface marker expression using flow cytometry [13]. MSCs were considered to be positive for a "cluster of differentiation" (CD) surface marker if ≥95% of the MSCs expressed the marker. A negative call was given if ≤2% MSCs expressed the surface marker.

MSCs from the three human sources were positive for the following surface markers: CD29, CD44, CD73, CD90, CD105, and CD166. MSCs from the human sources were negative or low for the following surface markers: CD14, CD34, CD45, and HLA-DR (Figure 2A). Using identical surface markers for characterizing oMSCs from the three sources revealed positive staining for CD29, CD44, CD73, CD90, CD105, and CD166, and negative staining for CD14, CD34, CD45, and HLA-DR (Figure 2B). Therefore, all sources of human and ovine MSCs showed the same surface marker pattern and fulfilled the major positive and negative markers defined by ISCT.

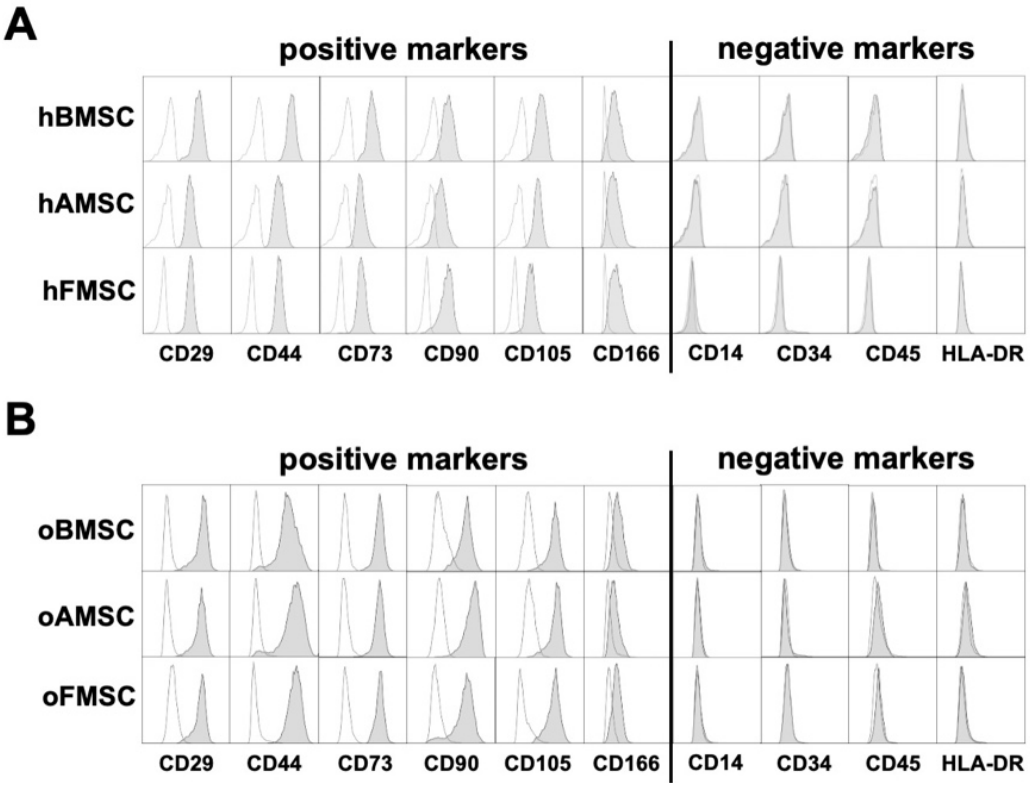

Figure 2. MSCs from human and ovine sources displayed common positive and negative surface markers. Surface marker expression analysis of (**A**) human and (**B**) ovine MSCs from the three corresponding sources was performed using flow cytometry. Representative histograms of 3-8 donors per source. AMSC: adipose tissue-derived mesenchymal stromal cells, FMSC: femoral-derived mesenchymal stromal cells, BMSC: bone marrow-derived mesenchymal stromal cells.

2.3. Immunomodulatory Capacity

To compare the ability of MSCs from human and ovine sources to exhibit immune inhibitory properties, MSCs from the three human sources and MSCs from the three corresponding ovine sources were tested. To evaluate this capacity, MSCs were measured on their inhibitory effect on lymphocyte proliferation. Carboxyfluorescein succinimidyl ester (CFSE)-labelled human and ovine lymphocytes were stimulated with phorbol myristate acetate (PMA)/ionomycin in the absence or presence of hMSCs or oMSCs, respectively. The lymphocyte proliferation was analyzed by flow cytometer using CFSE dilution after 3 days.

Proliferation of both human and ovine lymphocytes was clearly suppressed by MSCs from both human and ovine sources, respectively (Figure 3A). In detail, although all lymphocytes proliferated in the presence of PMA/ionomycin, the presence of MSCs completely inhibited lymphocyte proliferation and reduced the division index to background levels (Figure 3A). Additionally, and for verification, total lymphocyte number was determined. Absolute lymphocyte numbers confirmed MSC immunosuppression capacity, clearly indicated by inhibition of lymphocyte proliferation, as shown by cell counts in the presence of MSCs (Figure 3B).

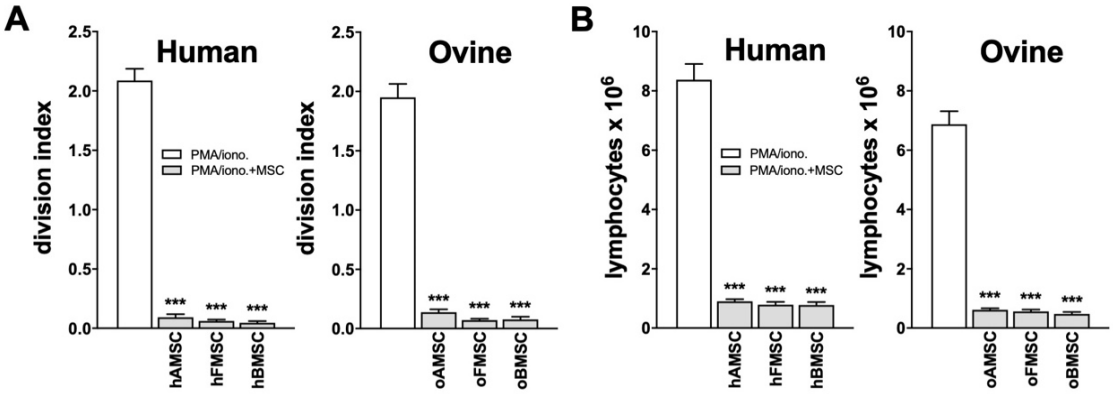

Figure 3. Immunomodulatory capacity of MSCs from human and ovine sources. Human and ovine MSCs from the three corresponding sources showed comparable immunomodulatory capacity by suppressing the proliferation of human and ovine lymphocytes, respectively. Carboxyfluorescein succinimidyl ester (CFSE)-labelled human and ovine lymphocytes were stimulated with PMA/ionomycin in the absence or presence of MSCs. (**A**) Calculations of division index and (**B**) total cell numbers of human and ovine lymphocytes are shown. Data are expressed as average ± SEM of 3-6 donors per source. *** $p < 0.001$, Mann–Whitney U test.

2.4. Differentiation towards Adipogenic and Chondrogenic Lineages

MSCs from the three human and ovine sources were investigated for their differentiation potential towards the adipogenic and chondrogenic lineages. In addition to their morphological changes, visible lipid-rich vacuoles accumulated in MSCs from all sources during adipogenic differentiation. Confirmation of adipogenic differentiation was completed via Oil Red O staining at the end of induction time (Figure 4A). For the chondrogenic differentiation, both hMSCs and oMSCs from all sources showed typical characteristics of glycosaminoglycan matrix when stained with Alcian Blue, 3 weeks after induction (Figure 4B). All controls were cultured under the same conditions, without supplementation, and did not result in adipogenic nor chondrogenic differentiation (Figure 4A,B, inserts).

To further analyze the adipogenic differentiation potential, the Oil Red O staining intensity of MSCs from human and ovine sources was evaluated by quantifying the amount of positively stained cells per image. This unbiased quantification approach confirmed a very solid adipogenic differentiation of all induced MSCs in comparison to the controls (Figure 4C left and middle). Interestingly, hMSCs from all three sources showed a significantly higher adipogenic differentiation rate compared to oMSCs from all three corresponding sources (Figure 4C, right). Further, we also quantified the chondrogenic differentiation rate of MSCs from human sources and MSCs from ovine sources by a semi-quantitative score based on Alcian Blue staining. The quantification of Alcian Blue staining indicated a clearly significant chondrogenic rate for both induced hMSCs (Figure 4D, left) and oMSCs (Figure 4D, middle) compared to their corresponding controls. MSCs derived from ovine sources showed a significantly higher chondrogenic differentiation rate compared to MSCs from human sources (Figure 4D, right).

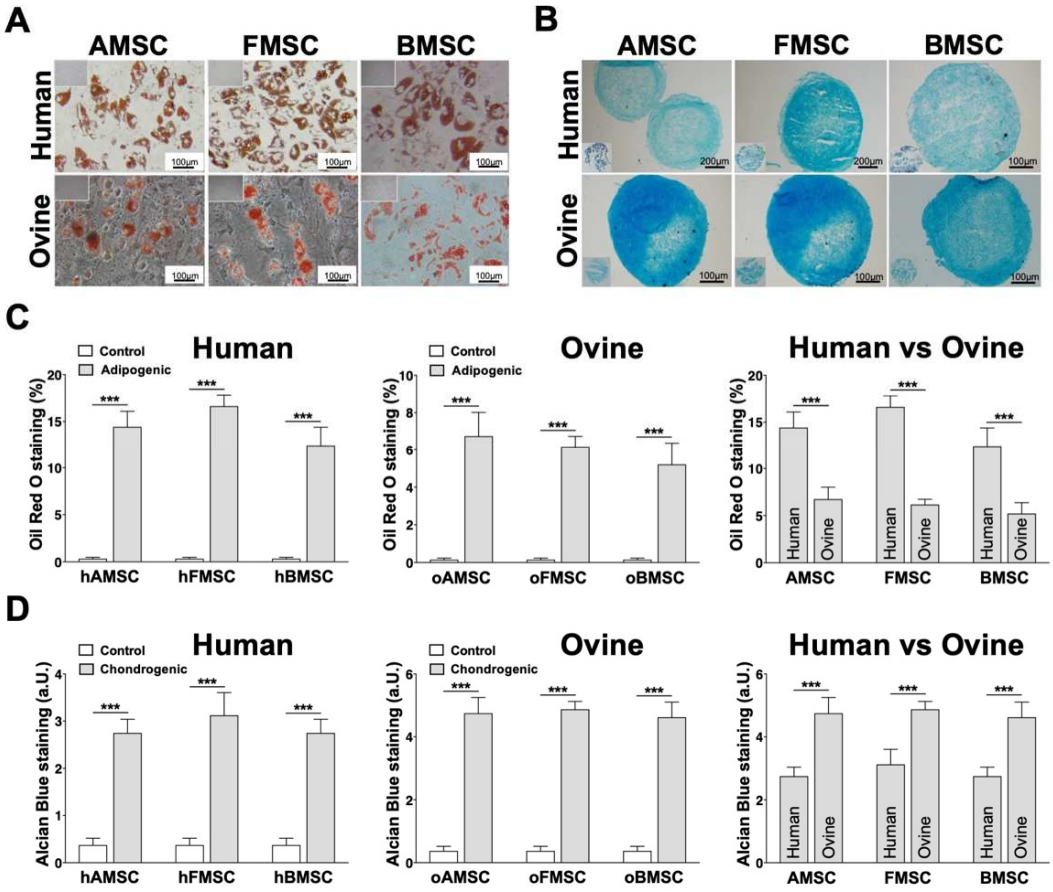

Figure 4. Adipogenic and chondrogenic differentiation of MSCs from human and ovine sources. MSCs from three human and ovine corresponding sources were induced towards the (**A**) adipogenic and (**B**) the chondrogenic lineages for 21 days. Culturing medium without any supplements was used as control. (**A**) The adipogenic differentiation was confirmed via Oil Red O and (**B**) the chondrogenic differentiation via Alcian Blue stainings. Controls are indicated in the corners. (**C**) The adipogenic differentiation rate of MSCs from human sources (left), ovine sources (middle), and human versus ovine sources (right) was evaluated by measuring the percentage of cells stained positive using the cellSens Dimension software. (**D**) The chondrogenic differentiation rate of MSCs from human sources (left), ovine sources (middle), and human versus ovine sources (right) was depicted by setting a semi-quantitative score based on the intensity of Alcian Blue staining: (1) very weakly positive, (2) weakly positive, (3) moderately positive, (4) markedly positive, and (5) strongly positive. Data are either representative pictures or expressed as average ± SEM of 3–4 donors per source. *** $p < 0.001$, Student's two-tailed unpaired *t*-test.

2.5. Assessment of Osteogenic Differentiation

For the osteogenic lineage, all MSCs were induced for 21 days and the osteogenic differentiation was confirmed via Alizarin Red S (Figure 5A, left) and alkaline phosphatase (ALP) staining (Figure 5A, right). For control cultures, identical conditions were utilized, without supplementation, and stained negative for both Alizarin Red S and ALP (Figure 5A, inserts in the top left corners).

The mineralization rate of analyzed MSCs from human sources (Figure 5B, left) and MSCs from ovine sources (Figure 5B, middle) was quantified by a semi-quantitative score based on Alizarin Red S staining, which quantifies the mineralized matrix secreted by differentiating MSCs towards osteoblasts. This staining resulted in clearly higher values for both toward osteogenic differentiation-induced hMSCs and oMSCs compared to the corresponding controls (Figure 5B, left and middle). Comparable human and ovine sources were found to have similar mineralization rates of AMSCs, FMSCs, and BMSCs (Figure 5B, right).

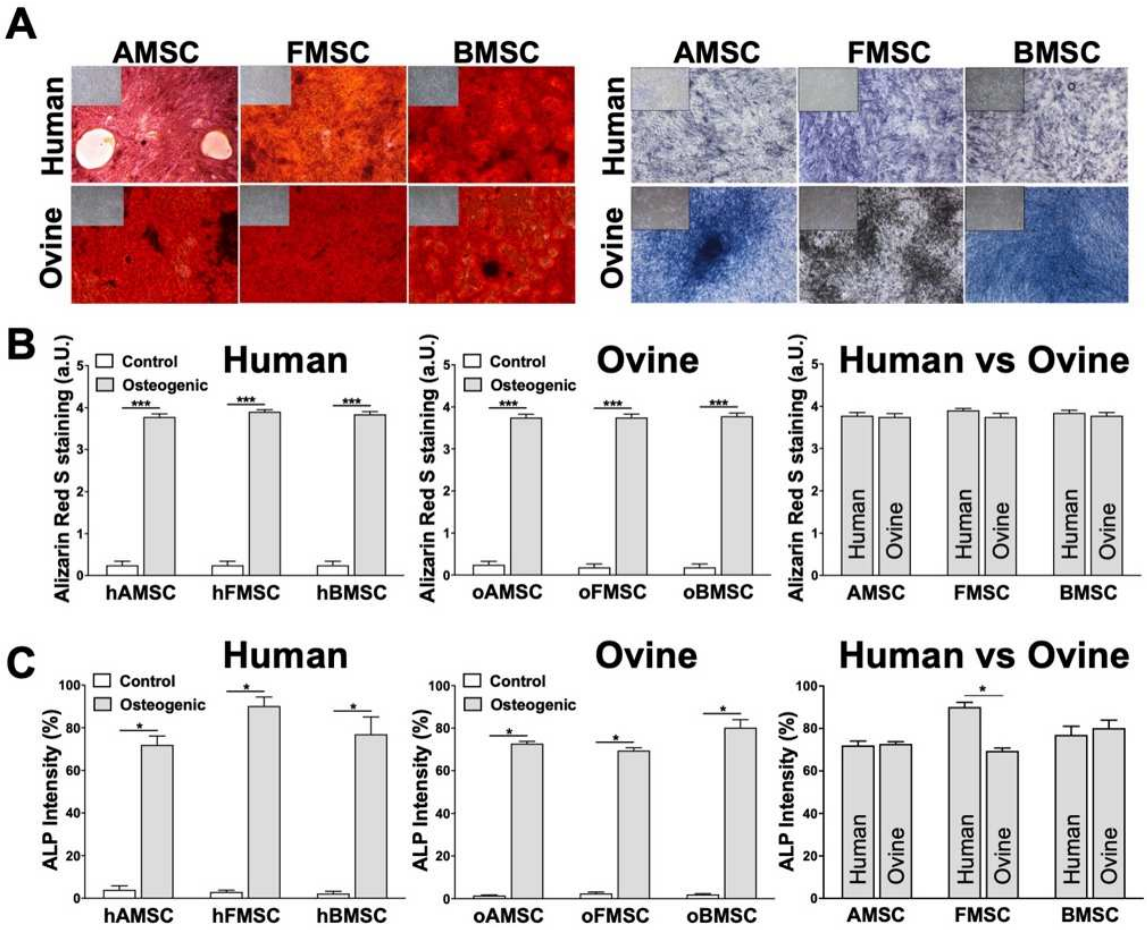

Figure 5. Strong mineralization rate and significant relative alkaline phosphatase intensity of MSCs from human and ovine sources differentiated towards the osteogenic lineage. hMSCs and oMSCs were induced towards the osteogenic lineage for 21 days. Culturing medium without any supplements was used as control. (**A**) The mineralization was confirmed via Alizarin Red S (left) and alkaline phosphatase (ALP) (right) staining. Controls are indicated in the top left corners. (**B**) The mineralization rate of MSCs from human sources (left), ovine sources (middle), and human versus ovine sources (right) was depicted by setting a semi-quantitative score based on the intensity of Alizarin Red S staining: (0) negative, (1) weakly positive, (2) moderately positive, (3) markedly positive, or (4) strongly positive. **(C)** The relative ALP staining intensity of MSCs from human sources (left), ovine sources (middle), and human versus ovine sources (right) was evaluated by measuring the percentage of cells stained positive using the cellSens Dimension software. White bars indicate control, grey bars indicate osteogenic induction. Data are either representative pictures or expressed as average ± SEM of 3–6 donors per source. * $p < 0.05$, *** $p < 0.001$, Mann–Whitney U test.

The relative ALP staining intensity of MSCs from human sources (Figure 5C, left) and MSCs from ovine sources (Figure 5C, middle) was also evaluated. ALP is an early expressed osteogenic protein marker that accumulates in the membrane and can be used to confirm osteogenic differentiation. By scoring the percentage of cells positive for ALP, we determined that the relative ALP staining intensity was comparable between all MSCs from human and ovine sources, except the human FMSC source that showed significantly increased staining (Figure 5C, right).

Moreover, the mineralization process was further assessed by optical density (OD) of monolayer cultures using a microplate reader at different time intervals during the induction period. Mineralized areas of monolayer cell cultures appear darker when measuring the OD [22], which makes this a fast approach to investigate the osteogenic differentiation. With the help of this assay, we could show that mineralized areas in osteogenic lineage-induced cells had an increased OD compared to control cultures

in both hMSCs and oMSCs. A significant shift at day 7 was seen in the osteogenic lineage-induced MSCs from both human and ovine sources. This shift continued to increase steadily compared to the corresponding controls (Figure 6A, left and middle). The overall fold change was mediated by using the ratio d17/d1 and demonstrated that the mineralization process was slightly increased in all three hMSC sources compared to the corresponding oMSC sources (Figure 6A, right), indicating a slower mineralization process for oMSCs. There were clear calcium deposits from both human and ovine sources; their mineralization was confirmed via Alizarin Red S staining, demonstrating successful osteogenic differentiation. Control MSCs showed no calcium deposits from either human or ovine sources and stained negative for Alizarin Red S.

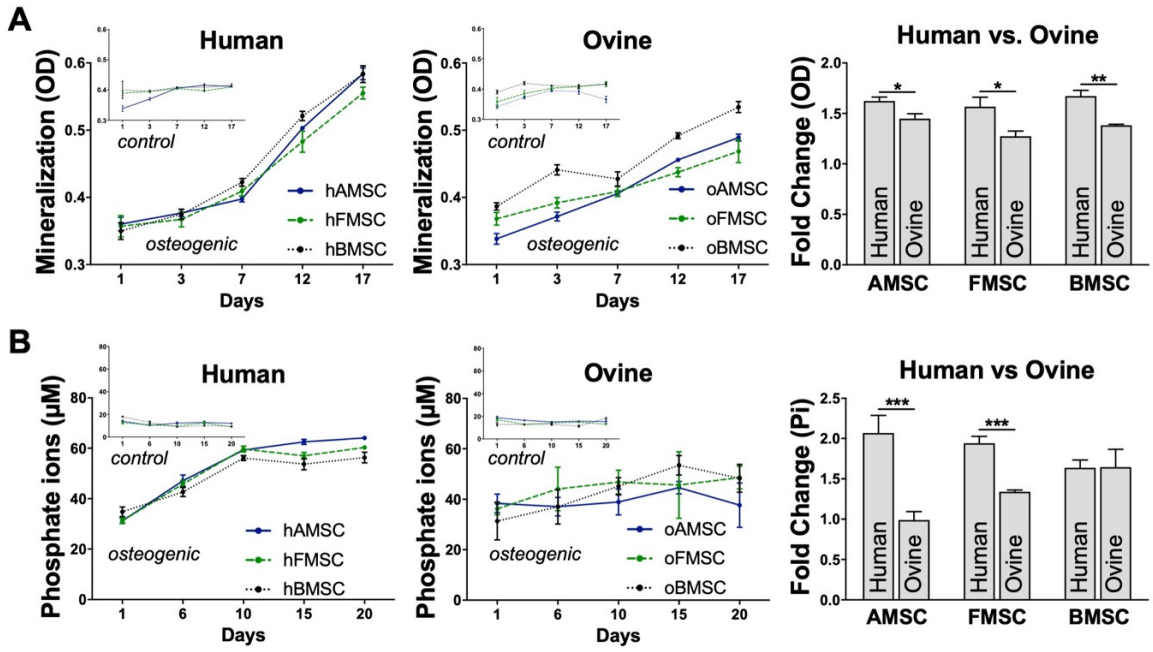

Figure 6. Mineral deposition and phosphate ion release by MSCs from human and ovine sources. MSCs from human and ovine sources were induced towards the osteogenic lineage. Culturing medium without any supplements was used as control. (**A**) The mineral deposition of MSCs from human sources (left) and MSCs from ovine sources (middle) was assessed by optical density (OD) measurement at different time intervals, as indicated. The overall mineralization fold change of MSCs from human and ovine sources was mediated using the ratio d17/d1 (right). (**B**) The osteogenic differentiation process of MSCs from human sources (left) and MSCs from ovine sources (middle) was assessed by measuring the inorganic free phosphate ions (Pi) released into the supernatant at different time intervals. The overall phosphate ion release fold change of MSCs from human sources and MSCs from ovine sources was mediated using the ratio d20/d1 (right). Data are expressed as average ± SEM of 3-6 donors per source. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Student's two-tailed unpaired *t*-test.

In addition to assessing the mineralization process, the osteogenic differentiation of MSCs from both human and ovine sources was monitored through measurement of inorganic free phosphate ions (Pi) released into the supernatant at different time intervals during the induction period. A distinct increase in Pi at all time points was noted in all osteogenic lineage-induced MSCs compared to their corresponding controls (Figure 6B, left and middle). The Pi fold change of MSCs from human sources and MSCs from ovine sources was mediated by using the ratio d20/d1 and showed that MSCs from two human sources (hAMSCs, hFMSCs) were approximately one-fold higher compared to the corresponding ovine sources (oAMSCs, oFMSCs). Interestingly, MSCs from the human BMSC source showed no significant difference compared to the ovine BMSC source (Figure 6B, right).

2.6. Osteogenic Lineage-Specific Gene Expression

Finally, MSCs from the three human and ovine sources were induced towards the osteogenic lineage for 21 days to allow for gene expression quantitation. Controls were cultured in medium without supplementation. The osteogenic differentiation was assessed using RT-PCR to investigate the relative mRNA expression of two osteogenic lineage-specific genes, Runx2 and Col1A. These were quantitated on day 1 and day 21 of the osteogenic induction.

Initially, the mRNA expression of Runx2 was slightly up-regulated on day 1 after induction in FMSCs and BMSCs from both human and ovine sources compared to controls (Figure 7A,B, top panels). However, on day 21 of induction, mRNA expression of Runx2 was up-regulated in all MSCs from both human and ovine sources compared to the corresponding controls as well as compared to day 1 of induction (Figure 7A,B, top panels). The second osteogenic lineage-specific gene, Col1A, showed no significant change on day 1, but was clearly down-regulated on day 21 in both MSCs from human and ovine sources compared to the corresponding controls (Figure 7A,B, bottom panels), suggesting a feedback down-regulation as has been described previously both at mRNA [23] and protein level [24].

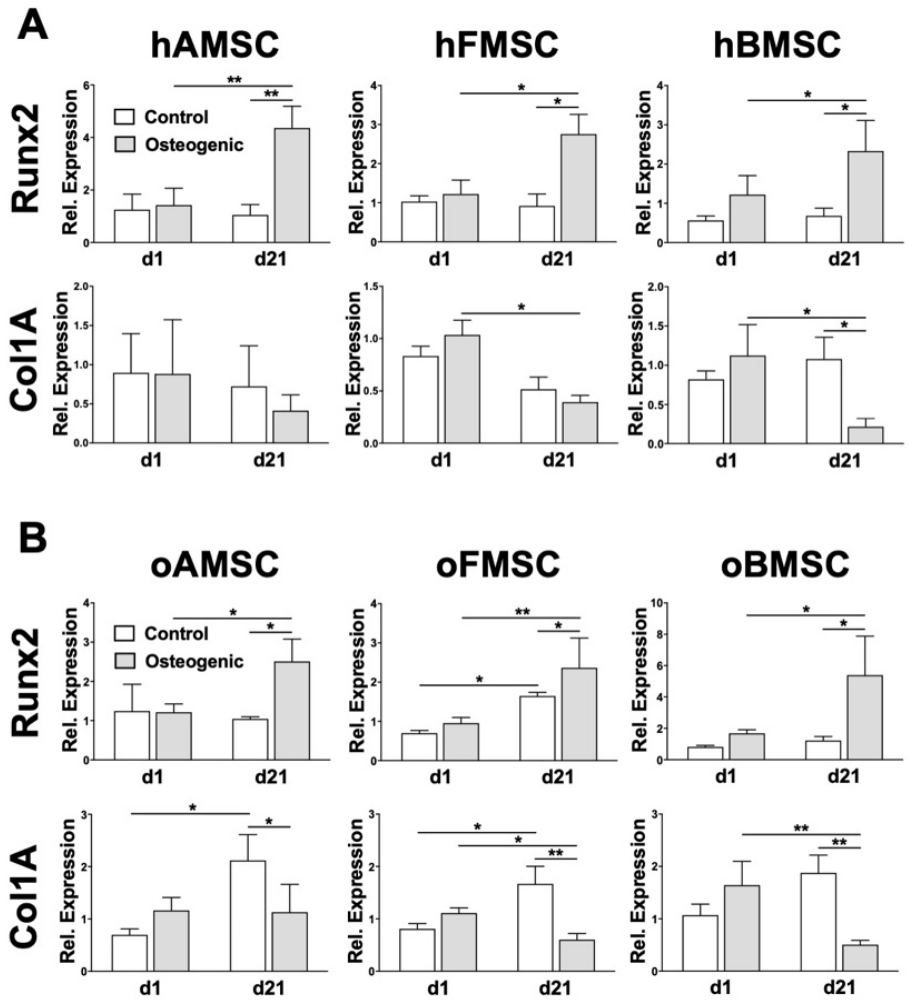

Figure 7. MSCs from (**A**) human and (**B**) ovine sources expressed common osteogenic gene marker Col1A and Runx2. MSCs from three corresponding human and ovine sources were induced towards the osteogenic lineage for 21 days. Culturing medium without any supplements was used as control. The relative expression of Col1A and Runx2 genes was investigated on day 1 and day 21. Data analysis was performed using delta-delta-Ct (ddCT) values normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the corresponding samples harvested on day 0. Data are expressed as average ± SEM of 3–5 donors per source. * $p < 0.05$, ** $p < 0.01$, two-way ANOVA.

3. Discussion

MSCs play a key role in processes important for health and disease [25]. Considering their role in multiple tissues and organs, advanced studies are dedicated to deciphering the basic biology and potential clinical applications of MSCs [26–30]. Some advantages to MSC manipulation are ease of harvest, minimal ethical concern, and that they do not tend to form tumors. They also exhibit the unique property of self-renewal and the remarkable ability to differentiate into diverse cell types including adipocytes, osteoblasts, and chondrocytes when cultured under specific growth conditions in vitro [31]. Additionally, MSCs from different sources have been demonstrated to possess a significant immunomodulatory capacity. In inflammatory diseases, MSCs uniquely respond by homing and integrating into impaired tissues [10,11,27]. These unique immunomodulatory properties establish MSCs as a cell type of primary interest for clinical advancement in many fields of research [32]. More specifically, MSCs show great potential as future therapeutic option in the pathophysiology of orthopedic injury and disease, and MSCs have been identified for their promising potential in regenerative medicine [33].

Human MSCs have been well characterized and standardized and their minimal criteria fulfillment have been outlined in a position statement of the ISCT in 2006 [13,14]. oMSCs, however, are poorly characterized, and remain un-standardized [34–37]. Recently, several interesting studies have partially characterized bone marrow-derived MSCs for bone formation in a sheep model [38]. The reported results from that study demonstrated that oMSCs have a high impact on implants and bone-engineered tissue testing in sheep; therefore, oMSCs have been further investigated regarding their growth and differentiation potential with various culture media and differentiation protocols. Interestingly, it has been reported that proliferation, surface marker expression, and differentiation of oMSCs are culture medium-dependent [39], which further underlines the need of a continued thorough characterization and comparison of human and ovine MSCs.

The first study comparing human and ovine bone marrow- and adipose tissue-derived MSCs by Kalaszczynska et al. investigated the MSC responses to various osteogenic differentiation media. The mineralization of oMSCs was not possible though, which was in stark contrast to hMSCs [40]. Later studies attempted osteogenic differentiation of human and ovine bone marrow-derived MSCs by utilizing different protocols including supplementation with bone morphogenetic protein 2 (BMP-2). Again, oMSCs responded poorly compared to hMSCs [21]. Most recently, a thorough review of oMSC isolation and characterization was published, which discussed the previously conflicting results and challenges in oMSCs. The review also delineated the important similarities between hMSCs and oMSCs [41].

In the current study, MSCs were isolated from three human and three corresponding ovine sources and expanded by applying the same protocol. All MSCs exhibited typical fibroblast morphology with spindle shape and showed robust proliferation behavior. Further confirming previous reports pertaining to MSC proliferation by other investigators [21,42], research found that proliferation of MSCs from ovine sources was 2-3-fold higher when compared to MSCs from the corresponding human sources cultured under same conditions. However, a direct comparison of the growth behavior between human and ovine MSCs is only possible with limitations, as there are still several open questions, such as whether isolated MSCs have the same developmental stage or how age affects this interspecies comparison.

In contrast to hMSCs, the cell surface expression profile of oMSCs has not previously been well characterized. A recent literature review [41] of MSC comparisons indicated that the field is still missing consensus for a common surface marker panel. Although some studies found relevant expression of CD44, CD73, CD90, CD105, CD166, and CD271 [43,44], other studies have reported the expression of CD29 and absence of CD90 in oMSCs [35,39], thereby yielding a sum of conflicting reports. In our study, CD29, CD44, CD73, CD90, CD105, and CD166 were identified as positive markers in both hMSCs and oMSCs, in addition to CD14, CD34, CD45, and HLA-DR as common negative surface

markers for both hMSCs and oMSCs. These results prove oMSCs to be even more comparable to hMSCs and contribute to a long discussion about their MSC-specific surface markers.

Immunomodulation is important for therapeutic advances, yet most of the reported immunomodulatory properties of MSCs have been investigated using human and mouse MSCs [45]. To date, only a limited number of studies has demonstrated the immunosuppressive potential of oMSCs [46]. Our direct comparison now demonstrates that MSCs from both human and ovine sources show comparable immunomodulatory capacity by suppressing lymphocyte proliferation.

In another confirmation of current literature [39,42,47], our study describes the adipogenic differentiation potential of MSCs from all three human and ovine sources as seen by the accumulation of large lipid-rich vacuoles. Interestingly, in our direct comparison, hMSCs showed significantly increased adipogenic potential in comparison to oMSCs. Previous studies have already shown that oBMSCs show no or only low adipogenic differentiation potential, even if different protocols were used [39,42]. However, to our knowledge, a direct comparison of adipogenic differentiation of human and ovine MSCs from several sources has not been reported before. In contrast to adipogenic differentiation, our comparative study demonstrated that oMSCs have a significantly higher capacity for chondrogenic differentiation compared to hMSCs. This evidence is of particular importance as it aids MSC-based strategies for cartilage repair, a subdiscipline that has increasingly been focused on the comparison of human and ovine MSCs. Such direct comparisons are needed for translating the findings in sheep cartilage repair models into the clinic for human use [41,47].

Further, our study demonstrated that both human and ovine MSCs from the three corresponding sources showed strong mineralization rates. It also indicated significant relative ALP intensity after differentiation towards the osteogenic lineage. Recently, conflicting results arose when human and ovine MSCs were compared for their mineralization capacity using β-glycerophosphate and sodium dihydrogen phosphate (NaH_2PO_4) as a source of phosphate ions [21,40]. hBMSCs have been reported to mineralize in the presence of β-glycerophosphate, but not with NaH_2PO_4, whereas hAMSCs behaved the opposite way. Interestingly, oBMSCs and oAMSCs were able to mineralize in the presence of NaH_2PO_4 but not with glycerophosphate [40]. In another study, the phosphate ion sources NaH_2PO_4 and glycerophosphate were combined with BMP-2, and osteogenic potential of hBMSCs and oBMSCs were investigated. Although oBMSCs responded poorly compared to hBMSCs, the study also revealed that matrix deposition was improved in NaH_2PO_4 and showed no mineralization in β-glycerophosphate [21]. These studies nicely foster the need for a reliable osteogenic induction for both human and ovine MSCs.

In our study, we evaluated the β-glycerophosphate-mediated osteogenic differentiation of hMSCs and oMSCs from three sources at different time points. Overall, strong mineralization rates could be seen in MSCs from both human and ovine corresponding sources using Alizarin Red S staining. This report is the first assessment of the mineralization process of MSCs from three different ovine sources in comparison to MSCs from three corresponding human sources and therefore lays the fundament for future studies utilizing the osteogenic capacity of oMSCs. As Alizarin Red S staining, however, is suboptimal to detect delicate differences in osteogenic differentiation, we employed further sophisticated assays to quantify the mineralization process. In detail, we utilized a methodology to analyze the osteogenic process by monitoring the OD of monolayer cultures of hMSCs and oMSCs, as described previously by Loebel et al. [22,48]. This technique can be used as an additional measure at early stages of mineralization during osteogenic differentiation and is particularly advantageous because there is no need for staining or biochemical assays in contrast to assays relying on Alizarin Red S. Although the Alizarin Red S staining did not result in significant differences, the OD assay indicated significantly higher mineralization rates in hMSCs from all three sources compared to the corresponding oMSCs. This could be explained by the sensitivity of the OD measurements and the fact that MSCs responded differently to the osteogenic induction medium, suggesting that oMSCs possess a reduced mineralization capacity.

Further, we analyzed the mineralization process by measuring the free phosphate ion release at various time points during the osteogenic differentiation, as they play a crucial role in bone matrix mineralization [49]. Both hMSCs and oMSCs from the corresponding sources demonstrated a distinct increase of phosphate ion release during the osteogenic lineage progression. Calculated fold changes indicated higher phosphate ion release in two human sources, AMSCs and FMSCs, when compared to their corresponding ovine sources. When comparing phosphate ion release of hBMSCs and oBMSCs, however, there was no significant difference.

Moreover, osteogenic differentiation was analyzed at the gene expression level utilizing RT-PCR at two different time points of the osteogenic differentiation process. Clearly, MSCs from both human and ovine sources demonstrated an increase of the osteogenic marker Runx2 at day 21 compared to day 1. Col1A demonstrated a slight increase at induction day 1 but showed a significant decrease at day 21. These relative mRNA expression differences are in line with the reported findings in hMSCs [23,50,51]; however, thus far there has not been a consensus for oMSCs [21,47].

To our knowledge, we reported for the first time an investigation characterizing and comparing hMSCs from three sources with oMSCs from three corresponding sources, side by side under the same conditions and using only one protocol. Here, we specifically assessed the mineralization process via OD measurement, free phosphate ion release, and osteogenic gene expression. Common positive and negative surface markers were also identified on hMSCs and oMSCs from the three sources. In summary, this direct comparison defines phenotypic similarities and differences of human and ovine MSCs from three different sources, thereby contributing to a better characterization and standardization of ovine MSCs. The key findings supplied in this report demonstrate the utility of ovine MSCs in preclinical studies for MSC-based therapies.

4. Materials and Methods

4.1. Tissue Donors and Study Design

Recruitment of human subjects was approved by the ethics committee of the University Hospital Bonn (project IDs: 122/09 and 102/19) and was conducted in accordance with the approved guidelines as well as the declaration of Helsinki. All animal experiments were approved by the official state animal care and use committee (LANUV NRW, 8.87-50.10.35.08.308). Experiments were performed in accordance with the German federal law regarding the protection of animals, institutional guidelines, and the criteria in "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health publication 8th Edition, 2011) were followed.

This study was designed to characterize and compare human and ovine MSCs from three sources under the same conditions. Due to the anatomical structure and musculoskeletal function, we defined corresponding sources (Figure 1A) for the isolation of adipose tissue-derived MSCs (hAMSCs), femoral-derived MSCs (hFMSCs), and bone marrow-derived MSCs (hBMSCs). hMSCs were harvested from donors undergoing liposuction in the abdomen (hAMSCs, $n = 4$), after hip replacement (hFMSCs, $n = 8$), and during kyphoplasty procedures (hBMSCs $n = 5$) (Figure 1A). Ovine subjects, more specifically, Merino sheep, had oMSCs harvested from adipose tissue in the thigh (oAMSCs, $n = 4$), femoral marrow fat (oFMSCs, $n = 4$), and the tuber ischiadicum (oBMSCs, $n = 7$). After successful isolation of human and ovine MSCs from the indicated sources, we investigated their morphology, proliferation rate, surface marker expression, immunomodulatory capacity, and differentiation potential towards the three lineages (adipogenic, chondrogenic, and osteogenic). More detailed experiments were performed to elucidate and compare the osteogenic differentiation process, including measurement of the mineralization process via optical density (OD), quantification of the free phosphate ion release, and RT-PCR.

4.2. MSC Isolation and Culture

hAMSCs and oAMSCs (Figure 1: 1 and 4) were isolated by mixing adipose tissues with pre-warmed (37 °C) Dulbecco's phosphate-buffered saline (DPBS; 1:1) and shaken thoroughly, followed by room temperature incubation for 30 min. The bottom fluid phase was then aspirated and DPBS was added to the upper phase (1:1). Vigorous shaking and collagenase digestion (0.15 U/mL; Sigma Aldrich, Darmstadt, Germany) followed for 60 minutes in a shaking water bath at 37 °C. Human and ovine FMSCs (Figure 1: 2 and 5) and BMSCs (Figure 1: 3 and 6) were isolated through gradient centrifugation (800× *g* for 30 min without brake) using Biocoll separating solution (Biochrom AG, Berlin, Germany). All human and ovine cells were plated in cell culture flasks (Greiner Bio-One GmbH, Frickenhausen, Germany) with Dulbecco's modified Eagle's medium (DMEM) (Gibco by Life Technologies, Darmstadt, Germany) containing 10% serum, 1% L-glutamine, and 1% penicillin-streptomycin (Biochrom AG, Berlin, Germany). Incubation took place under standard conditions at 37 °C in a humidified atmosphere with 5% CO_2.

4.3. MSC Morphology

All cells, hMSCs and oMSCs, were cultured as a monolayer and grown to optimal confluency, fixed with 4% paraformaldehyde (PFA) (5 min), followed by a washing step with DPBS. Next, MSCs were treated with Triton X-100 for 5 min for membrane permeabilization. Actin stock solution (Abcam plc, Cambridge, United Kingdom) was diluted (1:1000) and applied to MSCs for 10 min while nuclear counterstains were completed with 4′,6-diamidino-2-phenylindole (DAPI).

4.4. MSC Proliferation

The proliferation and growth characteristics of human and ovine MSCs were investigated. Cells were plated in 96-well plates as a monolayer at a density of 2×10^3 cells per well with standard culture medium for 21 days. Every third day of the growth period, medium was changed. At the indicated time points, cellular optical density (OD) was determined at 570 nm according to the manufacturer's instructions utilizing the MTT cell proliferation assay (Boster Biological Technology Co., Ltd, Pleasanton, CA, USA).

4.5. Immune Modulation

For examination of MSC immune inhibitory capacity, hMSCs and oMSCs were seeded in 24-well plates and cultured to confluence. For the isolation of peripheral blood mononuclear cells (PBMC), human and ovine peripheral blood was mixed with DPBS (1:1), then gently layered on a Biocoll separating solution (Biochrom AG, Berlin, Germany) and centrifuged at 800× *g* for 30 min without brake. Mononuclear cells were collected from the liquid interface and washed with DPBS. Without further purification, the naive freshly isolated human and ovine lymphocytes were labelled with CFSE (Molecular Probes, Leiden, Netherlands) and added to hMSCs or oMSCs. Lymphocytes were stimulated with PMA/ionomycin (Thermo Fisher Scientific, Karlsruhe, Germany). After 3 days, flow cytometry was performed to quantify lymphocyte proliferation by CFSE dilution, as described previously [52,53], and data were analyzed using FlowJo software 10 (BD Biosciences, Heidelberg, Germany).

4.6. MSC Surface Marker Expression

Flow cytometry was used to evaluate surface marker expression on MSCs. MSCs were resuspended in DPBS with 1% fetal bovine serum (FBS)/2 mM ethylenediaminetetraacetic acid (EDTA) and were stained with saturating concentrations of antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany) for 20 min. Flow cytometry data were acquired on a BD FACS Canto ll flow cytometer (BD Biosciences, Heidelberg, Germany) and analyzed using FlowJo software (BD Biosciences, Heidelberg, Germany). Human and ovine MSCs were tested for CD14, CD29, CD34, CD44, CD45, CD73, CD90, CD105, CD166,

and HLA-DR. All antibodies have been validated to work in sheep by previous papers and/or according to manufacturers' instructions.

4.7. Adipogenic Differentiation

For adipogenic lineage differentiation, hMSCs and oMSCs at a density of 1×10^4 cells/cm^2 were induced through incubation with culture medium supplemented with 1 µM dexamethasone, 1 µM insulin, and 200 µM indomethacin (Sigma Aldrich, Darmstadt, Germany) for 21 days. Culture medium lacking supplementation was used as control. At the end of the adipogenic differentiation period, cells were washed with DPBS, fixed with 4% formalin at 37 °C for 30 min, and incubated with 0.1% Oil Red O staining (Sigma Aldrich, Darmstadt, Germany) for 30 min. A collection of images was taken using light microscopy and the relative intensity of the adipogenic staining was quantified using the cellSens Dimension software (Olympus Corporation, Hamburg, Germany).

4.8. Chondrogenic Differentiation

The chondrogenic lineage differentiation of hMSCs and oMSCs was induced using high-glucose DMEM medium supplemented with 1 µg/mL insulin, 1 ng/mL transferrin, 1 ng/mL sodium selenite, 0.1 µM dexamethasone, 50 µM 2-phosphate-L-ascorbic acid trisodium salt, and 10 ng/mL transforming growth factor beta-1 (TGF-β1) (Sigma Aldrich, Darmstadt, Germany). MSCs were cultured on agarose gel to allow self-formation of 3D microspheres, as described previously [54]. On top of 60 µL solidified 2% agarose in 200 µL corresponding medium, 2.5×10^4 cells were cultured for 21 days. The 3D microspheres were fixed with 4% PFA overnight at 4 °C and cut into 15 µm cryosections (Microm 550, Thermo Scientific, Schwerte, Germany). Staining was completed with Alcian Blue (Sigma Aldrich, Darmstadt, Germany). The chondrogenic differentiation rate was analyzed by setting a semi-quantitative score based on the intensity of Alcian Blue staining: (1) very weakly positive, (2) weakly positive, (3) moderately positive, (4) markedly positive, or (5) strongly positive.

4.9. Osteogenic Differentiation

Induction towards the osteogenic lineage was performed by supplementing culture medium with 0.1 µM dexamethasone, 10 mM β-glycerophosphate disodium salt hydrate, and 50 µM 2-phosphate-L-ascorbic acid trisodium salt (Sigma Aldrich, Darmstadt, Germany) for both hMSCs and oMSCs. MSCs were seeded at a density of 10^4 cells/cm^2 and cultured for 21 days. When differentiation was complete, cells were fixed in 4% formalin and stained with 40 mM Alizarin Red S (Sigma Aldrich, Darmstadt, Germany) and ALP (Dako, Hamburg, Germany). ALP staining was performed using the 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) substrate system (Dako, Hamburg, Germany) according to the manufacturer´s instructions. A collection of images of all samples was taken by using a light microscope and the mineralization rate was depicted by setting a semi-quantitative score based on the intensity of Alizarin Red S staining: (0) negative, (1) weakly positive, (2) moderately positive, (3) markedly positive, or (4) strongly positive. The relative ALP staining intensity was analyzed by measuring the percentage of stained cells using the cellSens Dimension software (Olympus Corporation, Hamburg, Germany).

4.10. Optical Density and Free Phosphate Measurements

MSCs were induced towards the osteogenic lineage at a density of 10^4 cells/cm^2 in 96-well plates. Culture medium free of supplementation was used as control, and the medium was replaced every second or third day. The mineralization process was assessed by measuring the optical density (OD) adapted from Loebel et al. [22,48]. Briefly, the OD absorbance (450 nm) was used to evaluate the osteogenic differentiation of MSC monolayer cultures at the indicated time intervals (TECAN, Männedorf, Switzerland). Following the OD measurement, cells were washed with DPBS, and fresh medium was added to continue the differentiation process until the next measurement. The acquired OD values were corrected by the measured values of the corresponding control and

osteogenic differentiation medium. Inorganic phosphate ion (Pi) release was determined in cell culture supernatant at the indicated time points by using the Malachite Green Phosphate Assay Kit (Sigma Aldrich, Darmstadt, Germany). The amounts of released free phosphate was corrected by the measured values of the corresponding control and osteogenic differentiation medium.

4.11. Real-Time Polymerase Chain Reaction (RT-PCR)

After osteogenic lineage induction, described above, total RNA was extracted using TRIzol Reagent (Ambion, Life Technologies, Darmstadt, Germany) at indicated time points. Briefly, cells were washed with PBS and lysed in TRIzol following chloroform/isopropanol (ratio 24:1) treatment according to the manufacturer's instructions (PanReac AppliChem, Darmstadt, Germany).

After centrifugation, the upper phase with RNA was collected and precipitated by adding isopropanol. Washes with ethanol (80%) followed the precipitation. The Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany) was utilized for complementary DNA (cDNA) synthesis. RT-PCR was performed using a LightCycler 480 II and SYBR Green I Master according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). RT-PCR primer sequences are outlined in Table 1. Data analysis was performed using delta-delta-Ct (ddCT) values obtained by normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the corresponding samples harvested on day 0.

Table 1. Real-time polymerase chain reaction (RT-PCR). Primers used for the relative osteogenic gene expression of Col1A and Runx2 in hMSCs and oMSCs.

Gene	Human	Ovine
GAPDH	fwd: 5′CTCTGCTCCTCCTGTTCGAC3′ rev: 5′ACCAAATCCGTTGACTCCGA3′	fwd: 5′TCACCATCTTCCAGGAGCGA3′ rev: 5′GGTGCAGAGATGATGACCCT3′
Col1A	fwd: 5′TGCTCGTGGAAATGATGGTG3′ rev: 5′CCTCGCTTTCCTTCCTCTCC3′	fwd: 5′CATGACCGAGACGTGTGGAA3′ rev: 5′CATTCGTCCGTGGGGACTTT3′
Runx2	fwd: 5′GCGCATTCCTCATCCCAGTA3′ rev: 5′GGCTCAGGTAGGAGGGGTAA3′	fwd: 5′ CCGCCGGACTCGAACTG3′ rev: 5′GAGAGGCGCAGGTCTTGATG3′

4.12. Statistics

Data were collected in Microsoft Excel (Microsoft Corporation, Richmond, USA), and statistical analysis was carried out using GraphPad Prism 7 (GraphPad, La Jolla, CA, USA). The Shapiro–Wilk test was used to test for normal distribution. For data with Gaussian distribution, two-tailed, unpaired Student's *t*-test or two-way ANOVA were used. For non-Gaussian distributed data, Mann–Whitney U testing was used. Significance levels are marked as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ALP	Alkaline phosphatase
AMSC	Adipose tissue-derived mesenchymal stromal cells
BMP-2	Bone morphogenetic protein 2
BMSC	Bone marrow-derived mesenchymal stromal cells
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CFSE	Carboxyfluorescein succinimidyl ester
Col1A	Collagen, type I, alpha 1
DAPI	4′,6-Diamidino-2-phenylindole
ddCT	Delta-delta-Ct
DMEM	Dulbecco's modified Eagle's medium
DPBS	Dulbecco's phosphate-buffered saline
EDTA	Ethylenediaminetetraacetic acid
FMSC	Femoral-derived mesenchymal stromal cells
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
hAMSC	Human adipose tissue-derived mesenchymal stromal cells
hBMSC	Human bone marrow-derived mesenchymal stromal cells
hFMSC	Human femoral-derived mesenchymal stromal cells
HLA-DR	Human leukocyte antigen - DR isotype
hMSC	Human mesenchymal stromal cells
ISCT	International Society for Cellular Therapy
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stromal cells
oAMSC	Ovine adipose tissue-derived mesenchymal stromal cells
oBMSC	Ovine bone marrow-derived mesenchymal stromal cells
OD	Optical density
oFMSC	Ovine femoral-derived mesenchymal stromal cells
oMSC	Ovine mesenchymal stromal cells
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
Pi	Phosphate ions
PMA	Phorbol myristate acetate
RNA	Ribonucleic acid
RT-PCR	Real-time polymerase chain reaction
Runx2	Runt-related transcription factor 2

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3.2 Publication 2

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Article

Vertebral Bone Marrow-Derived Mesenchymal Stromal Cells from Osteoporotic and Healthy Patients Possess Similar Differentiation Properties In Vitro

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Abstract: Osteoporosis is a disease characterized by low bone mass and an increased risk of fractures. Although several cellular players leading to osteoporosis have been identified, the role of mesenchymal stromal cells (MSC) is still not fully elaborated. The aim of this study was, therefore, to isolate and characterize MSCs from vertebral body of healthy non-osteoporotic and osteoporotic patients, with a particular focus on their osteogenic differentiation potential. Isolated MSCs were characterized by their osteogenic, adipogenic, and chondrogenic differentiation, as well as surface marker expression, proliferation behavior, and immunomodulatory capacity. The mineralization process was confirmed using Alizarin Red S and alkaline phosphatase (ALP) stains and further evaluated by determining ALP activity, mineral deposition, and free phosphate ion release. MSCs from both healthy and osteoporotic patients showed common fibroblast-like morphology and similar proliferation behavior. They expressed the typical MSC surface markers and possessed immunomodulatory capacity. Both groups demonstrated solid trilineage differentiation potential; osteogenic differentiation was further confirmed by increased ALP activity, deposition of inorganic crystals, phosphate ion release, and expression of osteoblast marker genes. Overall, MSCs from osteoporotic and non-osteoporotic patients showed neither a difference in general MSC features nor in the detailed analysis regarding osteogenic differentiation. These data suggest that vertebral body MSCs from osteoporotic patients were not impaired; rather, they possessed full osteogenic potential compared to MSCs from non-osteoporotic patients.

Keywords: mesenchymal stromal cells; osteoporosis; immunomodulation; differentiation; proliferation rate; surface markers

1. Introduction

Osteoporosis is a common skeletal disease that is defined by a systemic deterioration of bone mass and increased fragility. Unfortunately, current therapies are still not satisfactory, with osteoporosis increasingly recognized as a major public health issue [1]. Present treatments of osteoporosis are mostly focused on preventing bone resorption and sustaining bone density, but unfortunately, also cause serious side effects [2]. There is, therefore, an urgent need for alternative innovative therapies that promote continuous bone sustainability and regeneration in patients with osteoporosis.

In general, osteoporosis is the consequence of the dysregulation between bone resorption and new bone formation [3,4], which is mediated by osteoblast and osteoclast cell lineages [5,6]. Growing evidence has indicated that bone marrow-derived mesenchymal stromal cells (BMSC), the progenitors of osteoblasts, play a crucial role in osteoporosis [7]. These cells are multipotent, and under physiological conditions, their precisely adjusted osteogenic and adipogenic properties contribute to bone tissue homeostasis [8]. However, several factors, such as menopause or aging, perturb this homeostatic equilibrium, eventually leading to a disbalanced production of bone marrow adipocytes and bone mass loss [9].

Recent publications have shown that the osteogenic potential of mesenchymal stromal cells (MSC) is significantly altered in osteoporosis [10]. Specifically, MSCs from osteoporotic patients possessed a lower ability to differentiate into osteoblasts, as well as displaying a lower growth rate compared to cells from healthy patients [11,12]. Most of these studies, however, did not consider the source of MSCs. This is of particular importance as recent studies could show that tissue source and harvesting technique have a great impact on MSC performance, which is clearly underestimated in the present literature and requires further investigation [13–16].

Currently, there are many reports considering MSC-based therapy for osteoporosis as a novel approach to overcome the limitations of the present treatments [2,17–19]. Different tissue sources for stromal cell-based therapy for osteoporosis, including bone marrow, adipose tissue, perinatal-derived MSCs, as well as small molecules for endogenous stromal cell recruitment, have been suggested [2,18–21]. Some of these led to a number of preclinical studies testing MSC transplantation in small animal models for osteoporosis; however, these studies were met with divergent outcomes [17,19]. An explanation for these inconsistent results may be the already mentioned lack of standardized protocols for MSC isolation, expansion, and characterization, as well as the use of different tissue sources and species. These parameters significantly influence MSC phenotype and functionality [13–16,22]. The present literature shows that we are still at the beginning of decoding MSC features because of their heterogeneity and that a more detailed analysis of their complex biology is needed to understand better how they can be used in a clinical setting [23].

One important MSC niche in the context of osteoporosis is the spine. It is commonly affected by osteoporosis, which is also evident from vertebral compression fractures, which are a frequent occurrence in osteoporotic patients and heal poorly [24,25]. Although several animal models of osteoporosis are available, experimental animals, such as ovariectomized rats or sheep, develop osteoporosis that is not fully consistent with the pathogenesis of human osteoporosis [26]. Unfortunately, only comparatively few examinations of the human vertebral body as a source for MSCs have hitherto been carried out because of its anatomically delicate position, which makes it a great deal less accessible and attractive than other MSC niches. Consequently, the human vertebral body as a stem cell niche is poorly studied: It is not only unclear whether dysfunction of MSCs contributes to the pathogenesis of osteoporosis; even a simple fundamental characterization of MSCs from vertebral bodies is nonexistent.

Therefore, the current study aimed to isolate MSCs from vertebral bodies of osteoporotic and non-osteoporotic control patients, to characterize both MSC groups, and to investigate their osteogenic differentiation activity using different approaches.

2. Results

2.1. Morphology and Proliferation Rate of BMSCs from Osteoporotic and Non-Osteoporotic Control Donors

Using bright-field microscopy, BMSCs from both groups showed typical bipolar spindle-shaped and fibroblast-like morphology at passage 1 (Figure 1A). They also showed a similar cell morphology after actin labeling at passage 3 (Figure 1B). We further addressed the question of whether MSCs with osteoporotic and non-osteoporotic background exert different proliferation behavior. To this end, an MTT assay was used to assess cell metabolic activity as an indirect measurement of cell proliferation

by reflecting the number of viable cells. Of note, both groups showed continuous cell growth, and we did not detect any significant difference between the cellular densities of osteoporotic MSCs (oMSCs) and non-osteoporotic healthy MSCs (hMSCs) at any given time point (Figure 1C).

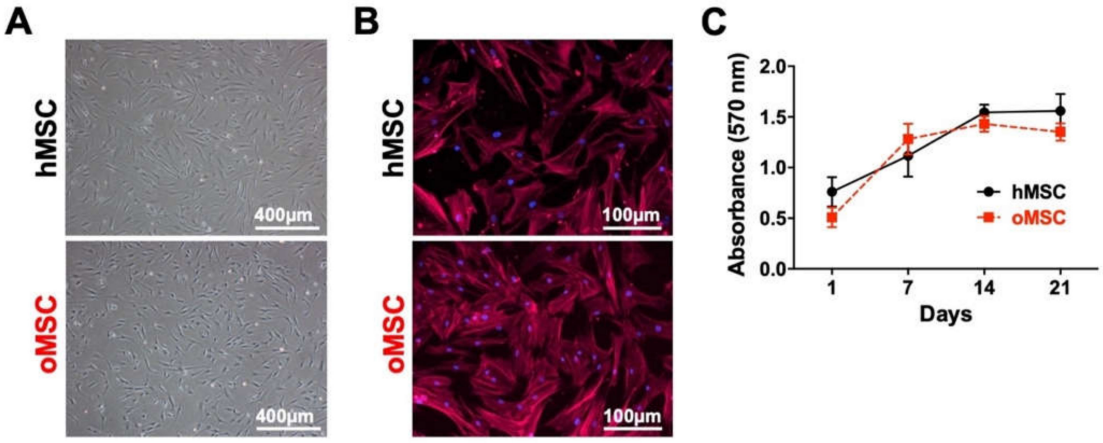

Figure 1. Mesenchymal bone marrow-derived stromal cells (BMSC) from vertebral bodies' bone marrow of osteoporotic donors (oMSC) and healthy control donors (hMSC) showed comparable growth behavior and morphology. (**A**) BMSCs from both groups showed typical fibroblastic morphology and comparable size at passage 1. (**B**) BMSCs at passage 3 were cultured as a monolayer and stained for cytoskeleton-actin (red) and nuclei (blue). (**C**) Growth behavior of BMSCs from both groups was assessed using MTT assay through absorbance measurement (570 nm) at indicated time points. Data are expressed as average ± SD of 5 donors per group.

2.2. Phenotypic Analysis and Immunomodulatory Capacity

A basic surface marker characterization was performed using flow cytometry to further analyze MSCs from osteoporotic and non-osteoporotic healthy controls. All MSCs were analyzed for the surface markers CD11b, CD19, CD45, CD73, CD90, and CD105 (Figure 2A).

BMSCs from both groups positively expressed the common surface markers CD73, CD90, and CD105, and were found to be negative for the CD11b, CD19, and CD45 (Figure 2A), which is in line with the criteria defined by the International Society for Cellular Therapy (ISCT) [27]. Furthermore, we did not find any significant difference between hMSCs and oMSCs in relation to their expression of common MSC surface markers.

BMSCs were analyzed for their immunomodulatory properties in accordance with the ISCT criteria. To this end, hMSCs and oMSCs were tested for their capacity to inhibit the proliferation of $CD8^+$ T cells. Specifically, human $CD8^+$ T cells were labeled with Carboxyfluorescein succinimidyl ester (CFSE), then stimulated with αCD3/28-coated beads in the presence or absence of MSCs from both groups, and T cell proliferation was flow cytometrically visualized by CFSE dilution after 3 days. In the presence of αCD3/28, T cells strongly proliferated, as could be seen by a CFSE proliferation profile with several peaks (Figure 2B). However, in the presence of both hMSCs and oMSCs, the proliferation of αCD3/28-activated $CD8^+$ T cells was completely abolished. We did not detect any significant difference between the immunomodulatory capacity of hMSCs and oMSCs.

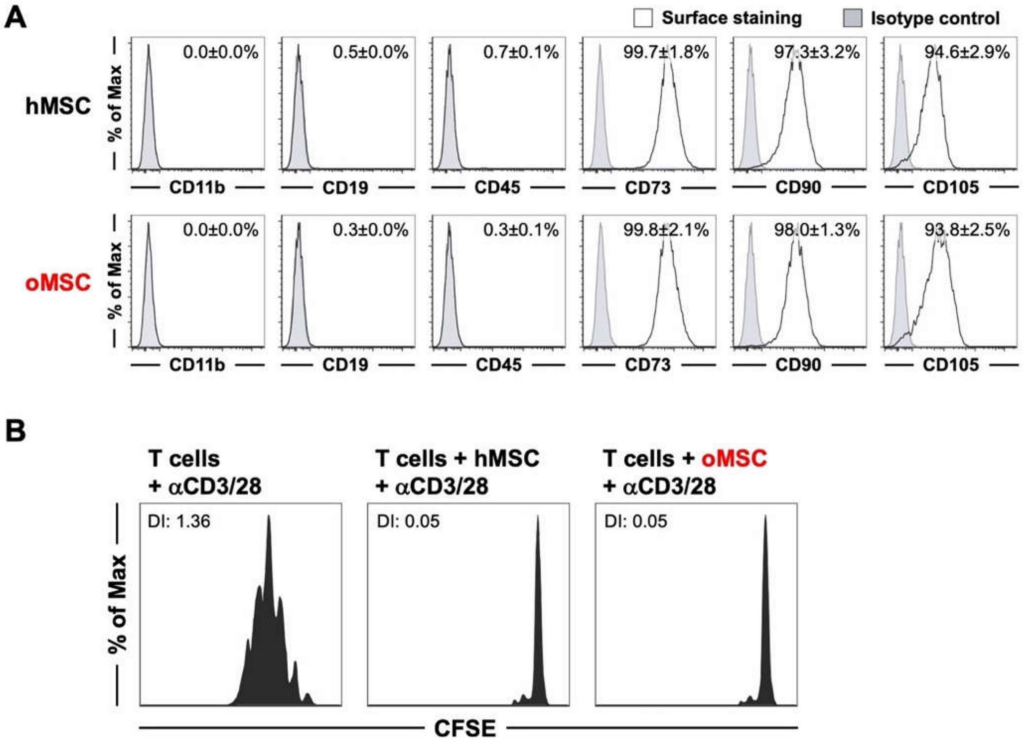

Figure 2. Mesenchymal stromal cells (MSC) of healthy control donors (hMSC) and osteoporotic donors (oMSC) exhibited comparable surface marker expression and immune-modulatory capacity. (**A**) Flow cytometric surface marker expression analysis of MSCs from osteoporotic and non-osteoporotic donors at passage 3–4. The percentage of positive cells is indicated in the top right corners. (**B**) hMSCs and oMSCs were tested for their immunomodulatory capacity by suppressing the proliferation of $CD8^+$ T cells. Carboxyfluorescein succinimidyl ester (CFSE)-labeled human $CD8^+$ T cells were stimulated with αCD3/28-coated beads in the absence or presence of hMSCs or oMSCs, and proliferation profiles were flow cytometrically analyzed. Division index (DI) as a measure of cell proliferation is depicted in the top left corners. Data are expressed as average ± SD of five donors per group.

2.3. Osteogenic, Adipogenic, and Chondrogenic Differentiation

Next, we characterized the osteogenic and chondrogenic differentiation potential of MSCs from osteoporotic and healthy control donors. First, we induced both BMSC groups towards the osteoblast lineage, and the osteogenic differentiation was confirmed via Alizarin Red S staining (Figure 3A, left).

MSCs from both osteoporotic and non-osteoporotic donors showed strong mineralization indicating their solid osteogenic capacity. BMSC control cultures from the corresponding groups were cultured under the same conditions without any osteogenic supplement and were stained negative for Alizarin Red S (Figure 3A, left, inserts in the top left corners). A direct comparison of the mineralization of hMSCs and oMSCs revealed no difference in their mineralogenic potential, suggesting that BMSCs from osteoporotic patients were not impaired. The osteogenic differentiation was further quantified by evaluating the mineralization rate by setting a semi-quantitative score based on the intensity of Alizarin Red S staining, which confirmed the successful and comparable osteogenic potential of both groups (Figure 3A, right). The mineralogenic effect was additionally assessed during the linear phase of extracellular matrix (ECM) mineralization, at day 7 and day 14, to avoid any possible overlooking of delicate differences between hMSCs and oMSCs; however, no differences in ECM mineralization was observed (Figure S1).

In the next step, we investigated the adipogenic differentiation potential of hMSCs and oMSCs. During the adipogenic differentiation process, BMSCs from both groups accumulated significant amounts of lipid-rich vacuoles that were confirmed via Oil Red O staining, indicating the successful

differentiation towards the adipocyte lineage (Figure 3B, left). Both groups generated a great number of lipid-storing cells, and we did not find any significant difference between hMSCs and oMSCs. This result was also confirmed by a quantitative evaluation of the percentage of Oil Red O positive cells (Figure 3B, right).

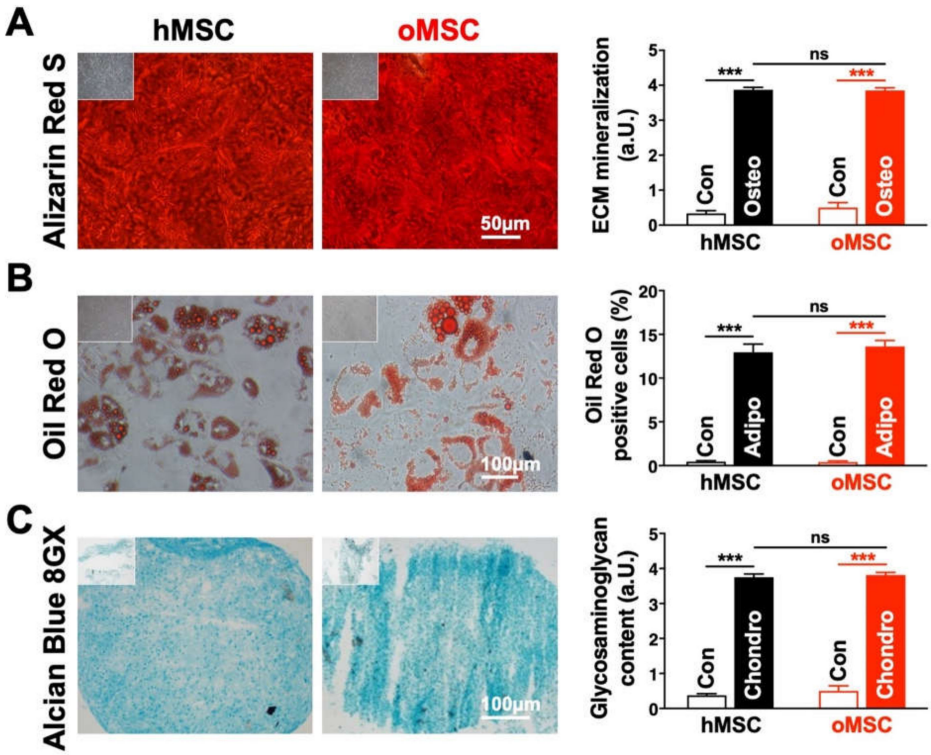

Figure 3. Comparable osteogenic, adipogenic, and chondrogenic differentiation potential. Mesenchymal stromal cells (MSC) of healthy control donors (hMSC) and osteoporotic donors (oMSC) at passage 3–4 were induced towards (**A**) osteoblast (Osteo), (**B**) adipocyte (Adipo), and (**C**) chondrocyte (Chondro) lineages. MSCs in culture medium without any osteogenic, adipogenic, or chondrogenic induction supplement were used as controls (inserts in the top left corners). Differentiation success was confirmed via (**A**) Alizarin Red S, (**B**) Oil Red O, and (**C**) Alcian Blue 8GX stains. (**A**,**C,** right) The extracellular matrix (ECM) mineralization and glycosaminoglycan content were evaluated using a semi-quantitative score based on the staining intensity and area (see Materials and Methods, 4.7 and 4.8). (**B**, right) The Oil Red O staining was determined by measuring the percentage of cells stained positive for Oil Red O using the cellSens Dimension software (see Section 4.6). The same magnification was used for all analyses. Con: control, a.U.: arbitrary unit, ns: not significant. Data are expressed as average ± SD of three donors (adipogenic and chondrogenic differentiation) and five to eight donors (osteogenic differentiation) per group. *** $p < 0.001$, Student's *t*-test.

As the last step, we also differentiated MSCs towards the chondrocyte lineage. At the end of the chondrogenic induction period, BMSCs from both groups showed typical characteristics of glycosaminoglycan matrix that were confirmed via Alcian Blue staining. This staining demonstrated the capability of both MSC groups to differentiate towards the chondrocyte lineage (Figure 3C, left) and further uncovered that both groups differentiated to the same extent. The chondrogenic differentiation rate was further assessed by a semi-quantitative scoring, which verified the similar differentiation potential (Figure 3C, right). In summary, hMSCs and oMSCs could be shown to possess a solid multilineage differentiation potential, and at the end stage of the differentiation procedure, no differences in their differentiation potential could be observed.

2.4. Alkaline Phosphatase Intensity and Activity during Osteogenic Differentiation Process

To further analyze the osteogenic differentiation potential of both groups, hMSCs and oMSCs were induced towards the osteoblast lineage and stained for alkaline phosphatase (ALP) at different time points during the differentiation process (Figure 4A, Figure S2). In comparison to the corresponding controls, the ALP staining indicated a steady increase in the ALP intensity in both MSC groups.

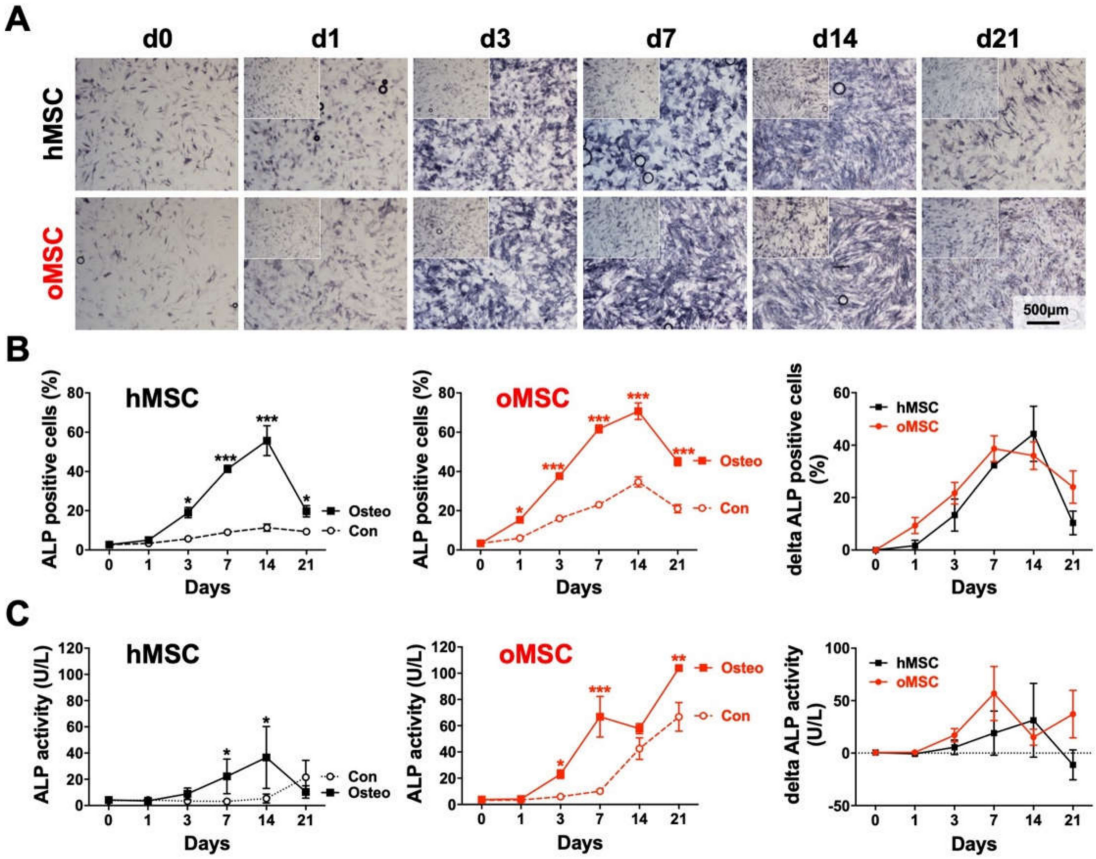

Figure 4. Alkaline Phosphatase (ALP) intensity and activity during the osteogenic differentiation process. (**A**) Mesenchymal stromal cells (MSC) of healthy control donors (hMSC) and osteoporotic donors (oMSC) at passage 3 were induced towards the osteoblast lineage for 21 days, and ALP staining was performed at indicated time points. A culture medium without any osteogenic induction supplement was used as control (inserts in the top left corners). The same magnification was used for all analyses. (**B**) The relative ALP staining intensity of both BMSCs was evaluated by measuring the percentage of cells stained positive using the cellSens Dimension software, and the delta of ALP positive cells was determined by subtracting the non-induced controls from the induced MSCs. (**C**) ALP activity of hMSCs and oMSCs was determined with the help of 4-Methylumbelliferyl phosphate disodium salt substrate using a fluorometric assay at indicated time points. The delta ALP activity was determined by subtracting non-induced from induced MSCs. Data are expressed as average ± SD of three to eight donors per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA.

The ALP staining of MSCs from both groups was further evaluated by measuring the percentage of cells stained positive for ALP. Interestingly, both hMSCs and oMSCs showed a comparable steady increase in the percentage of cells stained positive for ALP, reaching their peak at day 14 (Figure 4B). oMSCs seemingly represented a greater proportion of ALP positive cells; however, the control group of oMSCs also presented more ALP positivity, suggesting that MSCs from osteoporotic patients might exhibit higher ALP activity. When considering the real osteogenic potential, which is reflected by the difference between induced and non-induced MSCs, both hMSCs and oMSCs did not show any

significant difference (Figure 4B, right). Interestingly, the percentage of cells positive for ALP decreased from day 14 to day 21 in both groups (Figure 4B).

In parallel to the ALP staining, we also assessed the ALP activity using the same experimental setting (Figure 4C). At day 3 of induction, BMSCs from both groups already showed an increased ALP activity compared to their corresponding controls. When induced towards the osteogenic differentiation, oMSCs showed a stronger increase in ALP activity than hMSCs at all time points. However, when normalized to the control samples, no significant differences could be seen between both groups. The ALP activity peak was reached at day 7 for oMSCs and at day 14 for hMSCs, but differences were not significant.

2.5. Assessment of Osteogenic Differentiation

The mineralization process was further assessed through mineral deposition and phosphate ion release, as described previously [22]. hMSCs and oMSCs were induced to differentiate towards the osteoblast lineage. Cell culture medium without any osteogenic induction supplement was used as control. Mineralization of BMSCs was evaluated by optical density (OD) measurements of MSC monolayer cultures at different time points during the osteogenic differentiation period, thereby quantifying the deposition of inorganic crystals (Figure 5A).

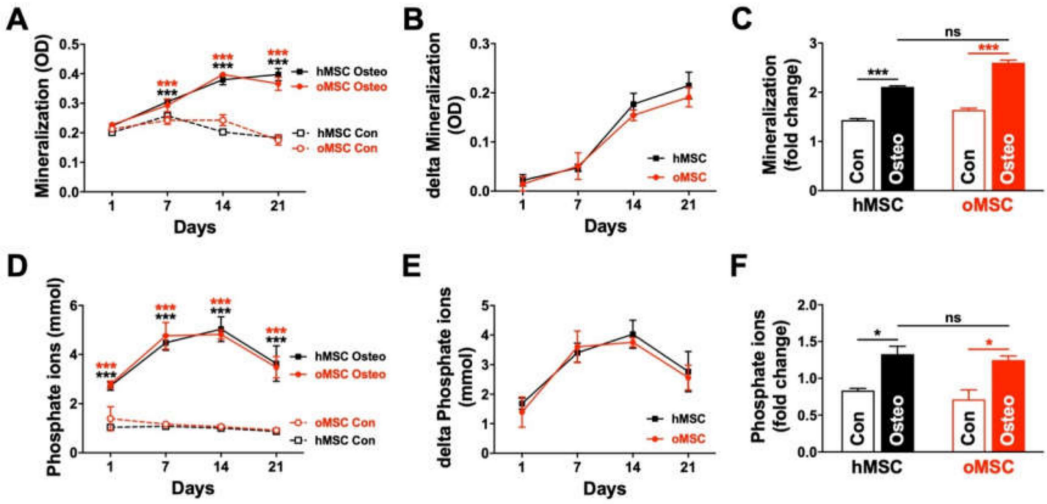

Figure 5. Comparable mineralization and phosphate ion release during the osteogenic differentiation process. Mesenchymal stromal cells (MSC) from osteoporotic (oMSC) and healthy non-osteoporotic donors (hMSC) at passage 3–4 were induced towards the osteoblast lineage for 21 days. Culture medium without any osteogenic induction supplement was used as control. (**A**) The mineralization process of both groups was assessed by optical density (OD) measurement at the indicated time points, and (**B**) the delta mineralization rate was determined by subtracting the non-induced controls from the induced MSCs. (**C**) The overall mineralization fold change was calculated using the ratio day 21/day 1. (**D**) The osteogenic differentiation process of MSCs from both groups was assessed by measuring the inorganic free phosphate ion release into the cell culture supernatant at the indicated time points, and (**E**) the delta phosphate ions release was determined by subtracting the values of non-induced controls from the induced MSCs. (**F**) The fold change of the total phosphate ion release from both MSC groups was determined using the ratio day 21/day 1. ns: not significant. Data are expressed as average ± SD of three to five donors per group. * $p < 0.05$, *** $p < 0.001$, one-way ANOVA.

Both induced MSC groups showed a continuous increase in crystal deposition (OD) over time compared to the corresponding non-induced controls. Induced MSCs from osteoporotic and healthy patients indicated a similar tendency at all time points. A minor decrease in OD values in the controls was observed between day 7 to day 21 in both hMSCs and oMSCs (Figure 5A). To better visualize the absolute increase in the mineralization, delta values between induced and non-induced samples

were calculated, which confirmed the continuous mineralization increase (Figure 5B). This was further confirmed by calculating the fold change of the OD shift over time (Figure 5C).

In addition to the OD measurements, the osteogenic differentiation process was also monitored through the determination of inorganic free phosphate ion release into the supernatant at different time points. BMSCs from both groups, osteoporotic and non-osteoporotic, demonstrated a comparable phosphate ion release at all time points during the osteogenic differentiation (Figure 5D+E). In general, phosphate ion release peaked at day 14. The phosphate ion level decreased until day 21 but was still elevated in comparison to the osteogenic initiation (day 1), which was further confirmed by the overall fold change of the phosphate ion release (Figure 5F).

2.6. Osteoblast Marker Gene Expression

Finally, the osteogenic differentiation of BMSCs from both groups was assessed using RT-PCR by investigating the relative mRNA expression of *ALPL*, *COL1A1*, *RUNX2*, and *SOX9* at different time points.

The early osteoblast marker *ALPL* showed a continuous increase from day 1 to day 7 in both groups. From day 7 to day 21, hMSCs showed decreased *ALPL* expression, whereas oMSCs slightly, but not significantly, increased gene expression from day 7 to day 21 (Figure 6). The osteoblast lineage-specific gene, *COL1A1*, showed comparable expression during the whole osteogenic differentiation process and decreased from day 7 to day 21 in both groups (Figure 6). *RUNX2* was slightly upregulated at the end of the osteogenic differentiation process in both groups (Figure 6). *SOX9*, which is a negative osteogenic marker [28–31], was downregulated for most of the differentiation period. oMSCs showed a slight but not significant increase in *SOX9* expression at day 21 (Figure 6). In summary, hMSCs and oMSCs presented a similar gene expression dynamic, and no significant differences could be detected between both groups.

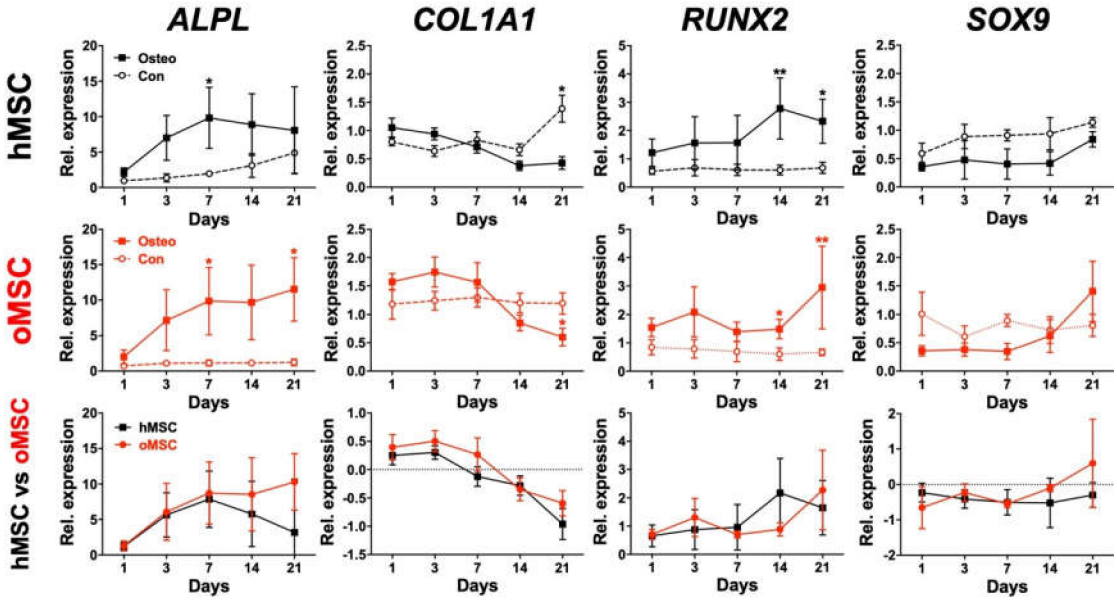

Figure 6. Expression dynamics of common osteogenic gene markers. Mesenchymal stromal cells (MSC) from osteoporotic (oMSC) and non-osteoporotic healthy control donors (hMSC) at passage 3–5 were induced towards the osteoblast lineage for 21 days. Culturing medium without any osteogenic induction supplement was used as control. The relative mRNA expression of *ALPL*, *COL1A1*, *RUNX2*, and *SOX9* was investigated at the indicated time points during the osteogenic differentiation. Data analysis was performed using ddCT values normalized to *GAPDH*. Data are expressed as average ± SD of three to four donors per group. For a direct comparison of the hMSC vs. oMSC groups (bottom panels), non-induced samples were subtracted from induced MSCs to determine the delta expression level. * $p < 0.05$, ** $p < 0.01$, Mann–Whitney U.

3. Discussion

The vertebral body as a stem cell niche is, at most, sparsely described, and it is unclear whether dysfunction of MSCs contributes to the pathogenesis of osteoporosis. Therefore, the current study aimed to isolate and characterize MSCs from the lumbar spine vertebral body of non-osteoporotic and osteoporotic patients. MSCs from both groups fulfilled the minimal MSC criteria in line with the ISCT guidelines [27]. They demonstrated fibroblast-like morphology, similar proliferation tendencies, typical MSC surface markers, immunomodulatory capacity, and comparable trilineage potential. Interestingly, none of the parameters used to investigate vertebral body-derived MSCs from osteoporotic and healthy patients demonstrated significant differences between these two groups. This is in contrast to recently reported studies that showed an increased formation of adipocytes and a reduced production of osteoblastic cells [32]. In a later study, it has been shown that muscle-derived MSCs are less deficient than femur head-derived MSCs from osteoporotic patients compared to controls, indicating that the MSC niche must be taken into consideration [33].

Most importantly, in our study, a close investigation of the osteogenic differentiation potential indicated that MSCs from osteoporotic patients were not impaired when compared to MSCs from non-osteoporotic patients. The obtained results confirmed that both hMSCs and oMSCs exhibited a potent capacity to differentiate towards the osteoblastic lineage in vitro, reflected by a steadily intensifying ALP staining from day 1 to day 14. We also noticed a decrease in ALP staining from day 14 to day 21. This effect, however, is not specific for vertebral MSCs. It has been reported before for other MSCs, but no reasonable explanation for this decline was given [34]. Indeed, ALP is an early marker of osteoblastic differentiation, whereas ECM mineralization is associated with late osteoblastic differentiation and transition towards osteocytes [35,36]. Interestingly, the relative mRNA of *ALPL* has been reported to be decreased at day 21 and day 28 in MSCs under osteogenic differentiation [37]. We also noticed a stagnation of ALP expression, which may explain the decrease in ALP staining at day 21 in our current study in both hMSCs and oMSCs. Further, the dynamic transition from osteoblasts to osteocytes should also be taken into consideration. It has been reported that primary osteoblasts from mice under osteogenic differentiation expressed osteocyte markers and showed decreased *ALPL* expression [38]. Therefore, the decrease in ALP staining at day 21 could also be due to the transition of osteoblasts to osteocytes, but this remains to be clarified.

Furthermore, we noticed a slight increase in ALP activity in osteogenically induced oMSCs and their corresponding control compared to hMSCs (Figure 4C). However, this effect did not reflect ECM mineralization determined via OD measurement, where no difference was observed (Figure 5B). It has been reported in a comparative analysis using different cells, including BMSCs and a variety of osteogenic and mineralizing media conditions, that ALP activity is not proportional to mineralization levels [39]. It has been shown that ALP activity increases in confluent monolayer MSCs during the first three weeks of differentiation [39], and in some cases, MSC cultures can produce high levels of ALP in vitro which do not fully correlate with the extent of mineralization [40].

Alizarin Red S has been traditionally used as the golden standard to evaluate and quantify ECM mineralization in vitro [41]. Nevertheless, this method presents a number of disadvantages, including culture disruption for fixation, preventing further measurements [42]. In our current study, therefore, we made use of alternative refined assays to quantitatively follow up the mineralization process continuously and accurately. To this end, we employed a methodology to analyze the mineralization process by monitoring crystal deposits by measuring the OD of monolayer cultures of hMSCs and oMSCs during the osteogenic differentiation, as reported previously [37]. In previous studies, the OD was found to correlate with Alizarin Red S quantification, which was further supported by phosphate ion release in our current study [37].

The measured OD of the deposited crystals did not indicate any significant difference between hMSCs and oMSCs; however, this does not exclude potential differences in crystal composition. A qualitative analysis of the deposited crystals to determine their composition and crystal types should be investigated further in the future. It has been shown that cultured BMSCs on collagen I/III gel

led to hydroxyapatite/calcium crystal deposition, as well as ECM proteins, in a similar manner to functional osteocytes and osteoblasts [43]. An accurate analysis to compare the chemical composition and structural properties of the deposited crystals in hMSCs and oMSCs would be of great interest as it would give deeper insights regarding the process of osteogenesis mediated by MSCs from healthy versus osteoporotic donors.

Further, we assessed the osteogenic differentiation of hMSCs and oMSCs by monitoring the gene expression of early osteogenic markers, such as *ALPL*, *COL1A1,* and *RUNX2,* utilizing RT-PCR at different time points. It has been previously reported that the relative mRNA expression of *ALPL* was elevated at day 7 with its peak at day 14, followed by a decrease at day 21 [37,44]. Our data indicated a steady increase in *ALPL* from day 1, with its peak at around day 7 for both hMSCs and oMSCs. The observed slight decrease in the relative mRNA expression of *ALPL* in hMSCs and unchanged mRNA levels in oMSCs at day 14 may explain the slight decrease in ALP staining at day 21. Additionally, the determined relative mRNA expression of *COL1A1* showed a comparable increase already at day 1 to day 7, and then a shift was observed showing a gradual downregulation towards day 21 in both hMSCs and oMSCs compared to their corresponding controls. Similar findings have been reported previously by assessing *COL1A1* gene expression during osteogenic differentiation of MSCs from healthy patients [34,37,45]. The transcription factor *RUNX2* plays a major role in osteoblast differentiation and bone formation and was shown to be expressed at a relatively similar level during in vitro differentiation of primary human osteoblasts [46–49]. In accordance with the previously reported findings, the expression of *RUNX2* was found to be increased and comparable at all time points in both hMSCs and oMSCs compared to their corresponding controls.

The transcription factor *SOX9* is known to play a key role in chondrogenesis and endochondral bone formation [50,51] and has been shown to be a major regulator in direct osteogenesis by directly interacting with *RUNX2* [29,51]. It has been reported that *SOX9* mRNA expression was higher in the control medium compared to MSCs under osteogenic differentiation on days 2, 7, and 14, but not at day 21 [29]. Our current data indicated a clear downregulation of *SOX9* mRNA expression already at day 1 until day 14 in both hMSCs and oMSCs compared to their corresponding undifferentiated controls. Loebel et al. showed the impact of *SOX9* downregulation in mineralization of human MSCs in vitro, demonstrating that *SOX9* plays a major role in regulating direct osteogenesis. Moreover, the *RUNX2*/*SOX9* ratio has been proposed as an early indicator for osteoblastic differentiation of human MSCs in vitro [29]. Further studies are required to assess the expression of *SOX9* and its relation with *RUNX2* in the future. The comparable expression of *ALPL*, *COL1A1*, *RUNX2,* and *SOX9* in both hMSCs and oMSCs additionally supports the similarity of their osteogenic differentiation potential in vitro. Taken together, the current study presents similar differentiation properties of vertebral bone marrow-derived mesenchymal stromal cells from osteoporotic and healthy patients in vitro using different approaches.

This finding is in contrast to studies from other niches, which were performed in animal models for osteoporosis and osteoporotic patients that showed a reduced MSC proliferation rate in osteoporotic patients and, most importantly, an impaired osteogenic differentiation potential [10–12,52]. Taken together, these findings neatly show how diverse MSCs from different niches are and how important it is to investigate tissue source-specific differences. Recent studies have already demonstrated that MSCs derived from vertebrae can be maintained in vitro for a greater number of steps [53]. They further showed that MSCs from vertebral bodies were able to differentiate even more efficiently into all mesenchymal lineages under osteogenic, adipogenic, and chondrogenic conditions. Another study demonstrated that vertebral body MSCs possessed a comparable phenotype and proliferative capacity but higher chondrogenic and osteogenic properties than MSCs from the iliac crest [54]. Basically, these studies demonstrate the superiority of vertebral MSCs in terms of their osteogenic differentiation behavior. One could argue that vertebral MSCs indicate above-average osteogenic differentiation behavior under homeostatic conditions, which is highly plausible considering their anatomic location. Interestingly, in our study, we found that vertebral body MSCs from osteoporotic

patients have similar proliferation and differentiation capability in comparison to MSCs from healthy control donors, which is in contrast to previous reports from other MSCs niches, such as femur head, iliac crest, and muscle [11,32,33].

Obviously, a remaining question is why vertebral MSCs are so different in comparison to MSCs from other niches. One explanation could lie in the local microenvironment of the vertebral body bone marrow, which could shape the fate of local MSCs. This local influence could be the cellular composition that interferes with MSCs, or it could be a simple molecular trigger. A recent publication identified the histone methyltransferase enhancer of Zeste homology 2 (EZH2), which regulates the lineage commitment of MSCs and, therefore, contributes to the pathology of osteoporosis [55]. Although more and more molecular mechanisms have been identified, we are still at the beginning of understanding the fate determination of abnormal versus normal BMSCs. However, focusing on MSCs cannot be the only solution to treat osteoporosis, as our in vitro data suggest that vertebral body osteoporosis may not primarily be due to abnormal osteogenic properties of local MSCs. Certainly, ex vivo or in vivo data will be needed to formally prove this hypothesis in a more physiological context, as in vitro expanded MSCs potentially possess a different phenotype.

In summary, this study characterized MSCs from the lumbar spine vertebral body of non-osteoporotic and osteoporotic patients and found that vertebral body MSCs from osteoporotic patients were not impaired, but they rather possessed full osteogenic potential compared to MSCs from non-osteoporotic patients. These results highlight the highly important influence of the tissue source and its local microenvironment for the MSC phenotype.

4. Materials and Methods

4.1. Tissue Donors and Isolation of Bone Marrow-Derived MSCs

Recruitment of human subjects for collecting bone marrow aspirate was approved by the local ethics committee (University Hospital Bonn, project ID: 102/10, approval date: 20 July 2010) and was conducted in accordance with the approved guidelines as well as the declaration of Helsinki. BMSCs were harvested from vertebral body aspirates of the lumbar spine of osteoporotic (oMSC) and non-osteoporotic healthy control donors (hMSC), which were undergoing spondylodesis and kyphoplasty procedures, respectively. All osteoporotic patients were diagnosed with grade II osteoporosis (n = 12) and had an average age of 69 years (8 females, 4 males). Healthy patients (n = 5) had an average age of 62 years (2 females, 3 males). MSCs were isolated through gradient centrifugation using Biocoll separating solution (Biochrom AG, Berlin, Germany) and their ability to adhere to tissue culture plastic, as described previously [22]. Cells were cultured and expanded in polystyrene cell culture flasks (Greiner Bio-One GmbH, Frickenhausen, Germany) using Dulbecco's Modified Eagle Medium (DMEM) (Gibco by Life Technologies, Darmstadt, Germany) containing 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin–streptomycin (Biochrom AG, Berlin, Germany) under standard conditions (37 °C, 95% humidity, atmospheric O_2 and 5% CO_2). After isolation, BMSCs were expanded via subculturing for two passages and then stored at −150 °C until further use. All experiments reported in this study were performed using BMSCs from passage 3 to passage 5.

4.2. Morphologic Analysis

For morphological analysis, MSCs from osteoporotic and healthy control donors were grown to approximately 80% confluency and then fixed with 4% paraformaldehyde (PFA, pH 7) in PBS (ThermoFisher Scientific, Karlsruhe, Germany) for 5 min. After the washing step, BMSCs were permeabilized with 0.25% Triton X-100 (Sigma–Aldrich, Darmstadt, Germany) for 5 min, and an anti-actin antibody (10 µg/mL) (Abcam plc, Cambridge, UK) was applied for 10 min as well as 4',6-Diamidino-2-phenylindole (DAPI) nucleus counterstain.

4.3. MTT Assay

The growth properties of hMSCs and oMSCs were indirectly measured by determining their metabolic activity using an MTT assay. To this end, cells were cultured at a density of 2×10^3 cells/well in a 96-well plate as monolayer culture under standard conditions for 21 days. The culture medium was changed each third day, and the measurements were carried out at the indicated time points according to the manufacturer's protocol using the MTT assay kit (Boster Biological Technology Co., Ltd., Pleasanton, CA, USA).

4.4. Flow Cytometric Analysis

Analysis of the phenotypic surface marker expression of BMSCs from osteoporotic and healthy donors was performed by flow cytometry using a BD FACS Canto II cell analyzer and FlowJo software (BD Biosciences, Heidelberg, Germany). Briefly, MSCs were resuspended in PBS with 1% FBS/2 mM ethylenediaminetetraacetic acid (EDTA) and then incubated with saturating concentrations of antibodies (ThermoFisher Scientific, Karlsruhe, Germany) for 20 min. MSCs were tested for CD11b, CD19, CD45, CD73, CD90, and CD105. Unstained cells and isotype antibodies were used as controls.

4.5. Immunomodulatory Capacity

Peripheral blood mononuclear cells (PBMC) were isolated out of human whole blood ($n = 5$) using a Ficoll gradient, and the resulting freshly isolated naive lymphocytes were enriched for $CD8^+$ T cells using human CD8 MicroBeads (Miltenyi, Bergisch-Gladbach, Germany). Naive $CD8^+$ T cells were labeled with Carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes, Leiden, Netherlands) and then washed with PBS with 1% FBS to remove extracellular CFSE. Four times ten to the fourth hMSCs or oMSCs per 24-well were cultured for 48 h to reach confluency, and then 1×10^6 $CD8^+$ T cells and αCD3/38-coated beads (ThermoFisher Scientific, Karlsruhe, Germany) were added. The proliferation of the $CD8^+$ T cells was flow cytometrically assessed by analyzing the CFSE dilution after 3 days, as described previously [56,57].

4.6. Adipogenic Differentiation

MSCs with a cell density of 1×10^4 cells/cm^2 from osteoporotic and healthy donors were differentiated towards the adipocyte lineage by adding 1 µM dexamethasone, 1 µM insulin, and 200 µM indomethacin (Sigma–Aldrich, Darmstadt, Germany) to the cell culture medium, as described previously [13,22]. MSCs cultured in an unsupplemented medium were used as undifferentiated cell controls. After 21 days, cells were washed with Dulbecco's phosphate-buffered saline (DPBS), fixed in 4% formalin (pH 7) (Carl Roth GmbH, Karlsruhe, Germany) at 37 °C for 30 min and then stained with 0.1% Oil Red O staining (Sigma–Aldrich, Darmstadt, Germany) for 30 min. The staining solution was removed, samples were kept in PBS, and pictures of several high-power fields were taken with a light microscope within 30 min. The adipogenic differentiation rate was evaluated by analyzing the captured images and quantifying the percentage of cells stained positive for Oil Red O using the cellSens Dimension software (Olympus Corporation, Hamburg, Germany), as described previously [22].

4.7. Chondrogenic Differentiation

Differentiation of BMSCs towards the chondrocyte lineage was performed as described previously [13,22]. In detail, three dimensional (3D) pellets consisting of 2.5×10^5 cells were resuspended in a culture medium and centrifuged at 500× *g* in a 15 mL conical tube. Pellets were cultivated in chondrogenic medium with loosened cap under standard conditions (37 °C, 95% humidity, atmospheric O_2, and 5% CO_2) for 21 days using high-glucose DMEM medium supplemented with 1 µg/mL insulin, 1 ng/mL transferrin, and 1 ng/mL sodium selenite, 0.1 µM dexamethasone, 50 µM 2-phosphate-L-ascorbic acid trisodium salt, and 10 ng/mL transforming growth factor beta-1 (Sigma–Aldrich, Darmstadt, Germany). MSCs cultured in an unsupplemented medium were used as undifferentiated cell controls.

3D pellets were fixed with 4% PFA (pH 7), cut into 12 µm cryosections, and stained with Alcian Blue dye (1% *w/v* Alcian blue 8GX, in 3% acetic acid solution, containing 0.1 M $CaCl_2$, pH 1) (Sigma–Aldrich, Darmstadt, Germany). After staining, a selection of images was taken of all cryosections from the pellet cultures, and the glycosaminoglycan content was analyzed using a semi-quantitative score based on the intensity of Alcian Blue staining, as reported before [22,58]. Undifferentiated cells served as control. The Alcian Blue staining-based scoring scale was as following: (0) negative, (1) weakly positive, (2) moderately positive, (3) markedly positive, or (4) strongly positive.

4.8. Osteogenic Differentiation

BMSCs from osteoporotic and healthy donors were seeded at a density of 1×10^4 cells/cm^2 and induced towards the osteoblast lineage by using a culture medium supplemented with 0.1 µM dexamethasone, 10 mM β-glycerophosphate disodium salt hydrate, and 50 µM 2-phosphate-L-ascorbic acid trisodium salt (Sigma–Aldrich, Darmstadt, Germany). A culture medium without any osteogenic induction supplement was used as control. After 7, 14, and 21 days, differentiated cells were fixed with 4% formalin (in PBS, pH 7) (Carl Roth GmbH, Karlsruhe, Germany) and stained with 40 mM Alizarin Red S (pH 4.2) (Sigma–Aldrich, Darmstadt, Germany). The ECM mineralization was determined using a semi-quantitative score based on the intensity of Alizarin Red S staining of images taken from different high-power fields, as described before [22,34]. Scoring scale: (0) negative, (1) weakly positive, (2) moderately positive, (3) markedly positive, (4) strongly positive.

4.9. Alkaline Phosphatase Measurement, Optical Density Measurement, and Free Phosphate Assay

MSCs from both groups were induced towards the osteoblast lineage using a cell density of 10^4 cells/cm^2 in 96-well plates. A culture medium without any osteogenic induction supplement was used as control. The differentiation process was investigated through different approaches.

At different time points during the osteogenic induction, BMSCs were stained with ALP (Dako, Hamburg, Germany), and the relative ALP staining intensity was evaluated by analyzing the percentage of cells stained positive for ALP using the cellSens Dimension software (Olympus Corporation, Hamburg, Germany).

ALP activity was determined through 4-Methylumbelliferyl phosphate disodium salt substrate using a fluorometric assay kit (BioVision Inc., Milpitas, CA, USA). The resulting absorbance was measured at 360 nm using a microplate reader (TECAN, Magellan, Switzerland) according to the manufacturer's instructions.

The mineralization process was further assessed by optical density (OD) measurements at 450 nm (TECAN, Magellan, Switzerland) adapted from Loebel et al. [37]. The OD absorbance was used to assess the mineralization process during the osteogenic differentiation at different time points of the same monolayer cultures. The collected OD values were corrected by subtracting the measured values of the corresponding culture medium and osteogenic induction medium without cells. After each OD measurement, supernatants were collected, and fresh corresponding medium was added to cultures during the differentiation period.

Inorganic phosphate ion release was measured in cell culture supernatants, including media without cells, at the indicated time points using the Malachite Green Phosphate Assay Kit according to the manufacturer's instructions (Sigma–Aldrich, Darmstadt, Germany) [59].

4.10. Real-Time Polymerase Chain Reaction

To analyze the gene expression of common osteoblast markers, hMSCs and oMSCs were induced towards the osteoblast lineage, and real-time polymerase chain reaction (RT-PCR) was performed, as described previously [22]. Briefly, TRIzol reagent (Ambion, Life Technologies, Darmstadt, Germany) and chloroform:isoamyl alcohol (24:1) (PanReac AppliChem, Darmstadt, Germany) were used for mRNA extraction. Then, 1 µg mRNA was reverse transcribed using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany), and RT-PCR was conducted using

LightCycler 480 SYBR Green I Master according to the manufacturer's instructions (Roche Diagnostics GmbH). Amplifications ran at 95 °C for denaturation, 60 °C for primer annealing, and 72 °C for primer extension 10 s each for 45 cycles. Primer sequences are listed in Table 1. Data analysis was performed using the ddCT method [60] determined by normalization to *GAPDH* [44].

Table 1. Real-Time Polymerase Chain Reaction (RT-PCR). Accession numbers, size of the products, and primer sequences used for determining the relative gene expression of *ALPL*, *COL1A1*, *RUNX2*, and *SOX9* in mesenchymal stromal cells (MSCs) during osteogenic differentiation.

Gene	Primer Sequence	Product Length	Accession Number
GAPDH	fwd: 5′CTCTGCTCCTCCTGTTCGAC3′ rev: 5′ACCAAATCCGTTGACTCCGA3′	109 bp	NM_002046.5
ALPL	fwd: 5′TTTATAAGGCGGCGGGGGTG3′ rev: 5′AGCCCAGAGATGCAATCGAC3′	198 bp	NM_000478.5
COL1A1	fwd: 5′TGCTCGTGGAAATGATGGTG3′ rev: 5′CCTCGCTTTCCTTCCTCTCC3′	449 bp	NM_000088.3
RUNX2	fwd: 5′GCGCATTCCTCATCCCAGTA3′ rev: 5′GGCTCAGGTAGGAGGGGTAA3′	176 bp	NM_001024630.3
SOX9	fwd:5′AGGAAGTCGGTGAAGAACGG3′ rev: 5′AAGTCGATAGGGGGCTGTCT3′	275 bp	NM_000346.3

4.11. Statistics

Data are expressed as average ± SD of 3–8 biological replicates (donors per group) as indicated. Statistical analysis was carried out using GraphPad Prism 7 (GraphPad, La Jolla, CA, USA). The Shapiro–Wilk test was used to test for normal distribution. For data with Gaussian distribution, a two-tailed Student's *t*-test or one-way analysis of variance (ANOVA) were used. For non-Gaussian distributed data, the Mann–Whitney U testing was used. Significance levels are marked as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/21/21/8309/s1. Figure S1: Mineralization of extracellular matrix (ECM) during the osteogenic differentiation process of mesenchymal stromal cells of healthy (hMSC) and osteoporotic (oMSC) donors visualized via Alizarin Red S staining at day 7, 14, and 21; Figure S2: Control samples for alkaline phosphatase (ALP) staining during the osteogenic differentiation process of mesenchymal stromal cells of healthy (hMSC) and osteoporotic (oMSC) donors at day 0, 1, 3, 7, 14, and 21.

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Abbreviations

ALP	Alkaline phosphatase
ALPL	Alkaline phosphatase gene
BMSC	Bone marrow-derived mesenchymal stromal cells
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CFSE	Carboxyfluorescein succinimidyl ester

COL1A1	Collagen, type I, alpha 1
DAPI	4′,6-Diamidino-2-phenylindole
ddCT	Delta-delta-Ct
DMEM	Dulbecco's modified Eagle's medium
DPBS	Dulbecco's phosphate-buffered saline
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
hMSC	Healthy mesenchymal stromal cells
ISCT	International Society for Cellular Therapy
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stromal cells
OD	Optical density
oMSC	Osteoporotic mesenchymal stromal cells
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
RT-PCR	Real-time polymerase chain reaction
RUNX2	Runt-related transcription factor 2
SOX9	SRY-Box Transcription Factor 9

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Supplementary Materials

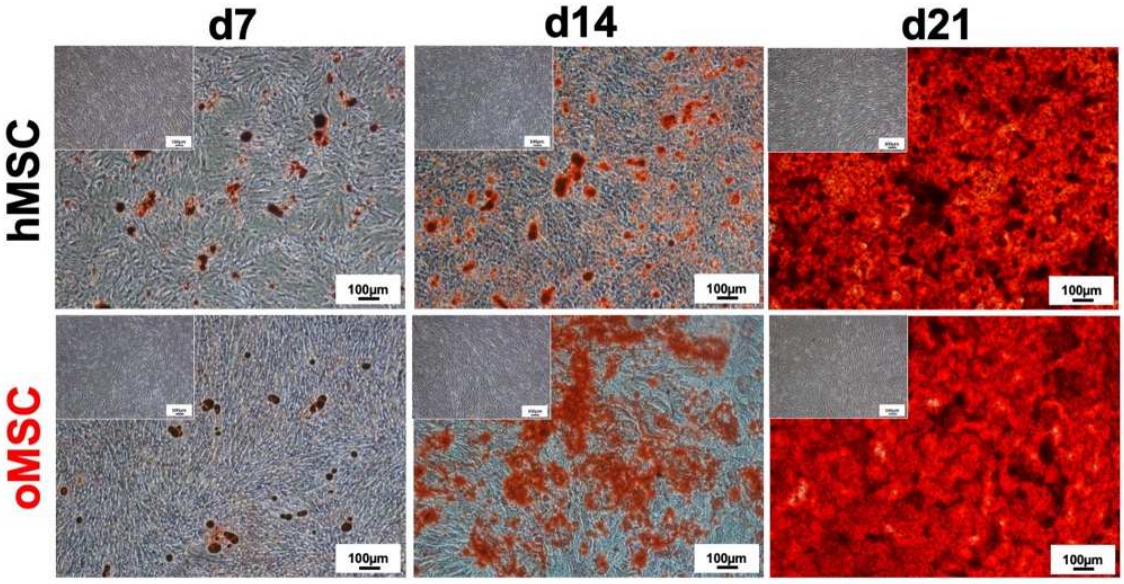

Figure S1. Mineralization of extracellular matrix (ECM) during the osteogenic differentiation process of mesenchymal stromal cells of healthy (hMSC) and osteoporotic (oMSC) donors (p3). Alizarin Red S staining was used to visualize ECM mineralization at indicated time points (d7, d14, d21). MSCs in culture medium without any osteogenic supplement were used as controls (inserts in top left corners).

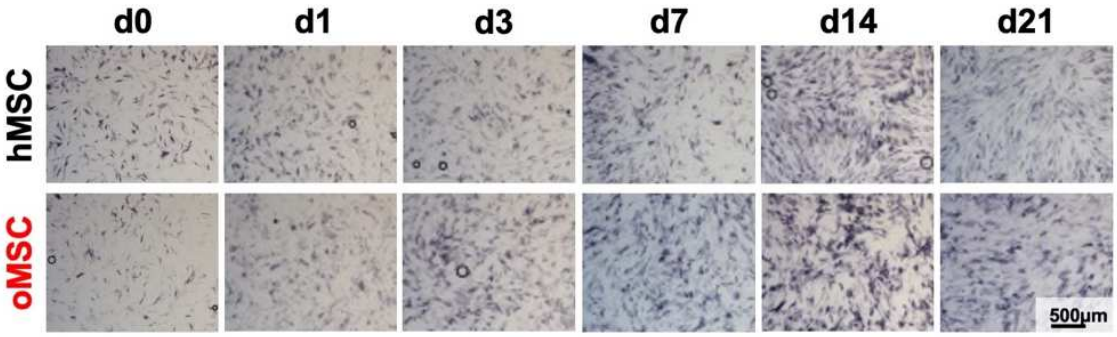

Figure S2. Control samples for alkaline phosphatase (ALP) staining. Mesenchymal stromal cells of healthy (hMSC) and osteoporotic (oMSC) donors (p3) were cultured in medium without any osteogenic induction supplement for 21 days and ALP staining was performed at indicated time points. Same magnification was used for all analyses.

3.3 Publication 3

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Article

Molecular and Functional Phenotypes of Human Bone Marrow-Derived Mesenchymal Stromal Cells Depend on Harvesting Techniques

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Abstract: Mesenchymal stromal cells (MSC) harvested in different tissues from the same donor exhibit different phenotypes. Each phenotype is not only characterized by a certain pattern of cell surface markers, but also different cellular functionalities. Only recently were different harvesting and processing techniques found to contribute to this phenomenon as well. This study was therefore set up to investigate proteomic and functional properties of human bone marrow-derived MSCs (hBM-MSC). These were taken from the same tissue and donor site but harvested either as aspirate or bone chip cultures. Both MSC populations were profiled for MSC markers defined by the International Society for Cellular Therapy (ISCT), MSC markers currently under discussion and markers of particular interest. While classic ISCT MSC markers did not show any significant difference between aspirate and outgrowth hBM-MSCs, our additional characterization panel revealed distinct patterns of differentially expressed markers. Furthermore, hBM-MSCs from aspirate cultures demonstrated a significantly higher osteogenic differentiation potential than outgrowth MSCs, which could be confirmed using a transcriptional approach. Our comparison of MSC phenotypes obtained by different harvesting techniques suggests the need of future standardized harvesting, processing and phenotyping procedures in order to gain better comparability in the MSC field.

Keywords: mesenchymal stromal cells; phenotype; characterization; differentiation; harvesting technique; osteoimmunology

1. Introduction

Bone marrow (BM)-derived mesenchymal stromal cells (MSC) are multipotent cells that possess a unique capacity for self-renewal. Although autologous MSCs retain the ability to differentiate into cartilaginous, osseous and adipose tissue, the most prevalent clinical applications have been anti-inflammatory therapy and promotion of wound healing [1,2]. As research keeps focusing on MSCs as a potential source for clinical therapies (e.g., tissue engineering), comparability of studies relies on exact characterization of MSCs used for cultivation and further processing.

In the past, research has shown that there are differences in molecular cell characteristics when applying diverse harvesting techniques or collecting MSCs from different donor sites. These findings clearly showed that MSCs are difficult to compare and that most likely a complex orchestra of factors, starting with donor site, including the harvesting methodology used and ending with the way how

cells were treated during cell culture, might have a dramatic impact on MSC phenotype. However, the MSC field is just beginning to understand how these methodological differences affect MSC biology. Even though a small selection of gene expression or proteome datasets was published in the last few years [3,4], there is still a great need for more systematic studies to tackle this problem. In this context, the MSC community also needed to agree that the classical MSC surface markers, such as CD73, CD90 and CD105, are insufficient for MSC characterization. Rather, the analysis of a broad, proteomic-like screening for surface markers, transcriptome clusters as well as description of functional properties, such as immunomodulatory capacity, regenerative potential, etc., is crucial for definitive characterization [5].

Today, the methods to harvest and purify MSCs are still very heterogeneous, differing between labs and researchers. This is highly critical and despite the potential cell biological consequences of such heterogeneity, this aspect does not get enough attention. There are several methods of harvesting MSCs: while adipose or umbilical cord-derived MSCs [6] are easy to obtain in practice [7], bone marrow aspiration and bone reaming remain the methods most often described as standard. While other authors have started to compare cells from different bones (femur versus iliac bones) with different anatomical and embryological properties, in this study, we derived MSCs from the same anatomical bone structure but used different harvesting techniques. Specifically, we compared bone marrow aspirate with bone chips from the femur. Both materials can be obtained very well during orthopedic and trauma surgery procedures and are therefore a reliable source for the production of a clinically relevant MSC product. Although in most cases it is easier to obtain bone marrow aspirates, significant amounts of bone fragments or bone chips are generated in some surgical procedures. Therefore, this direct comparison allowed us to evaluate whether these two very simple harvesting techniques have an impact on MSCs' cellular phenotypes when brought into culture and how this would potentially affect clinical outcome.

Thus, the aim of this study was to investigate whether both different harvesting techniques from the same donor site result in the typical expression pattern of MSC markers and similar functional properties regarding osteo-, chondro- and adipo-genic differentiation behavior.

2. Results

2.1. Morphology and Proliferation Behavior of MSCs from Aspirate or Outgrowth Cultures

BM-MSCs were obtained from the femoral bone during hip arthroplasty and harvested from outgrowth or aspirate cultures. Morphologically, there were no differences between outgrown and aspirated cells before and after passaging when analyzed by bright-field microscopy at P0 (Figure 1A,B) and P1 (Figure 1C,D). Also, no significant difference in optical density as pertains to cell growth was observed at any time points (Figure 1E), indicating that neither MSCs from aspirate nor MSCs from outgrowth cultures had any growth advantage.

To further analyze both MSC populations in more detail, cells were characterized using a variety of surface markers via flow cytometry. Interestingly, there was no difference in general MSC markers as defined by the International Society for Cellular Therapy (ISCT) [8]. There was no difference between MSCs from outgrowth and aspirate cultures in basic MSC marker expression of CD90, CD73, CD105, CD13, CD29 and CD44 (Figure 2A). Surface markers that were designated by definition as negative markers in MSCs, such as CD45, CD14, CD20, TCRα/β, HLA-DQ, CD11b and CD34, also showed no significant differences (Figure 2B). Further, to give a holistic impression of the MSC surface marker expression, the "% of stained cells" was analyzed to present the percentage of positive cells in the whole population and thereby indicate the relative number of cells that express a particular marker. In addition, we also analyzed the MFI (mean fluorescence intensity), which determines the relative amount of antigen that is present on the cell surface. Both parameters analyze the MSC population from a different perspective: a high MFI means that this cell population shows a high expression of the analyzed surface antigen. However, a high "% of stained cells" means that a lot of cells express this

marker, but the overall expression could be low. That is why both parameters are very useful to give a thorough impression of distribution (how many cells express this marker) and expression level (how much is expressed) of a certain marker.

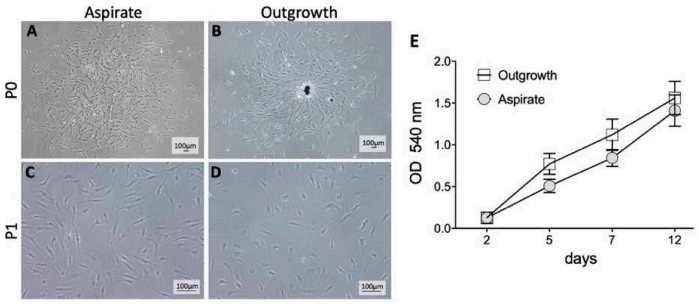

Figure 1. Morphology and proliferation behavior of mesenchymal stromal cells (MSC) from aspirate or outgrowth cultures. (**A**,**B**) The typical morphology of unpassaged MSCs is depicted. Adhered aspirate cells formed cell clones in contrast to an outgrowth culture with spare bone fragments as a source of cell growth. (**C**,**D**) All passaged cells appeared to be plastic adhered and spindle shaped. No relevant differences were observed. (**E**) Proliferation rate was measured by using an MTT Assay. Shown data were evaluated by optical density (OD) measurements. All isolated cells were viable and able to proliferate. No significant differences between both niches were detected.

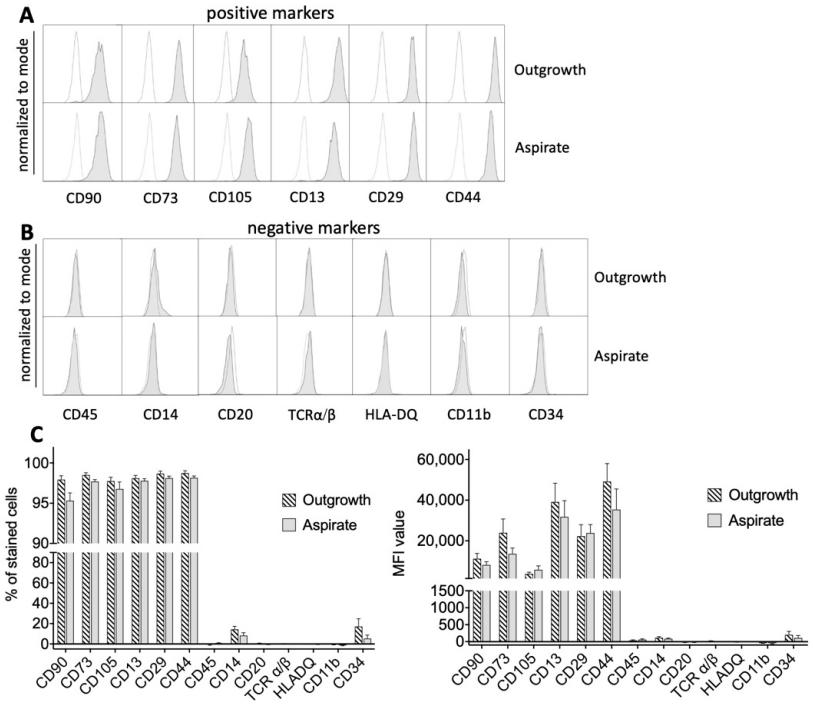

Figure 2. Expression of MSC surface markers defined by the International Society for Cellular Therapy (ISCT). (**A**) Both cells from aspirate and outgrowth cultures expressed ISCT MSC markers such as CD90, CD73, CD105, CD13, CD29 and CD44 without significant differences in expression levels (gray histograms). White histograms represent controls. (**B**) There was no significant difference for negatively expressed MSC markers defined by ISCT. (**C**) In correspondence with the previous findings, there were no significant differences between both groups regarding the percentage of stained cells for each marker or the corresponding mean fluorescence intensity (MFI) value, respectively. Only CD14 and CD34 showed a somewhat elevated expression signal.

Interestingly, there were no differences in the percentage of stained cells and the mean fluorescence intensity (MFI) between MSCs from both groups (Figure 2C). For the positively expressed markers, more than 95% of the cells expressed the antigen of interest and exhibited a high MFI value, while for negatively expressed markers, no relevant signals were detected. Supplementary Figure S1 summarizes all analyzed surface markers as a heat map.

2.2. Controversially Discussed MSC Markers and Markers of Interest

In addition, several other potentially novel MSC markers were tested for differential expression profiles in both groups [9]. These markers are currently under discussion and are not yet ratified by the broad scientific community. MSCs from both groups showed no expression of CD271 and SSEA4 and only a weak signal for CD10, MSCA, CD56 and CD200 (Figure 3A,B).

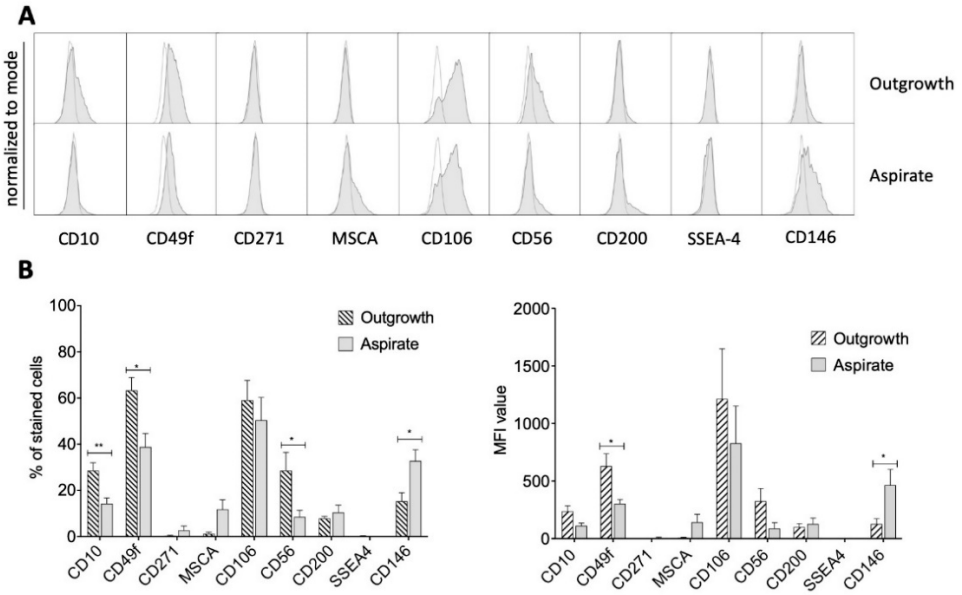

Figure 3. Expression of controversially discussed MSC markers. (**A**) Representative histograms (gray) indicated a distinct signal for CD49f and CD106. A weak signal was detected for CD10, MSCA, CD56, CD200 and both groups (aspirate and outgrowth) showed a lack of CD271 and SSEA4. White histograms represent controls. (**B**) Percentage of stained cells and their corresponding MFI confirmed the histograms. Furthermore, a statistically significant difference for the percentage of stained cells was detected for CD10, CD49f, CD56 and CD146, which was confirmed by significantly different MFI values for CD49f and CD146. * $p < 0.05$, ** $p < 0.01$.

Notably, both cell populations indicated distinct expression levels for CD49f and CD106 in histograms and there was a significant difference in the percentage of stained cells regarding CD10, CD49f, CD56 and CD146. While MSCs from outgrowth cultures expressed higher levels of CD10, CD49f and CD56, MSCs from aspirate cultures were associated with higher expression of CD146. Investigating the MFI level, significant differences were found for CD49f and CD146 (Figure 3B). While the former was significantly more expressed in outgrowth cells, CD146 showed an almost three-times increased expression in aspirate cells.

In addition to the described surface marker panel, we had previously identified further markers in preliminary surface marker screenings, which are not common MSC markers but of potential interest regarding their biology. Therefore, these markers where further analyzed in this study in order to detect differences between aspirate and outgrowth cells [10–17]. Also using these surface markers, there were significant differences between the outgrowth and aspirate group (Figure 4). While CD39, LAP, CD239, CD318 and CD36 showed low significance levels, differential expression levels of CD141

and CD54 were medium but highly significant for CD222, as was shown by the percentage of stained cells and the MFI (Figure 4).

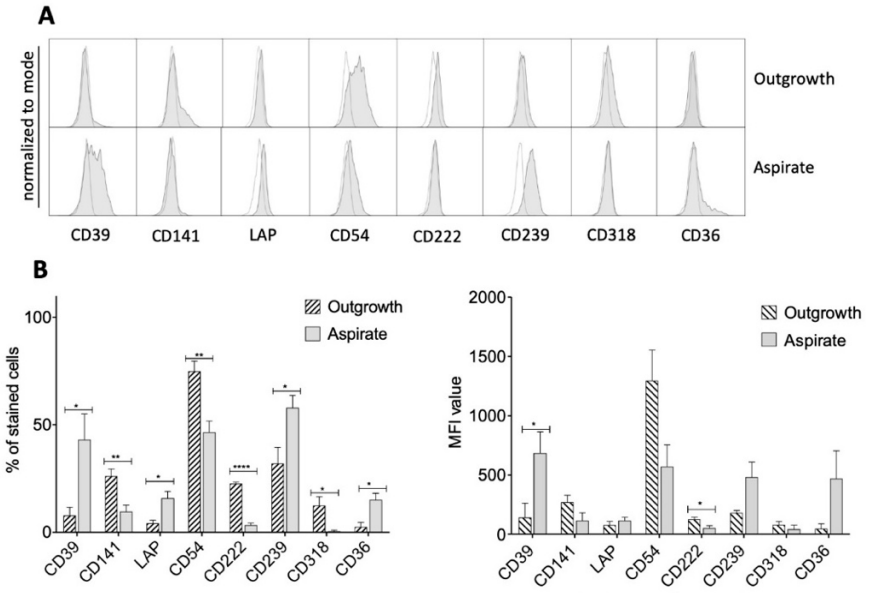

Figure 4. MSC markers of potential interest. (**A**) Representative histograms of further detected differences between outgrowth and aspirate cells (gray histograms). White histograms represent controls. (**B**) CD39, LAP, CD239, CD318 and CD36 showed low significance levels in percentages of cells stained for the given markers. For CD141 and CD54, this difference was medium, and for CD222, highly significant. MFI values indicate significant differences for CD39 and CD222 expression as well. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

2.3. Multilineage Differentiation Capacities of Outgrowth and Aspirate MSCs

Comparable chondro- and adipo-genic differentiation characteristics were found in histological analysis (Figure 5C–F). However, their level of differentiation was relatively low, which might be due to the utilized isolation procedures or the specific microenvironment of the harvested bone, which potentially tweak MSCs to slightly favor the osteogenic differentiation.

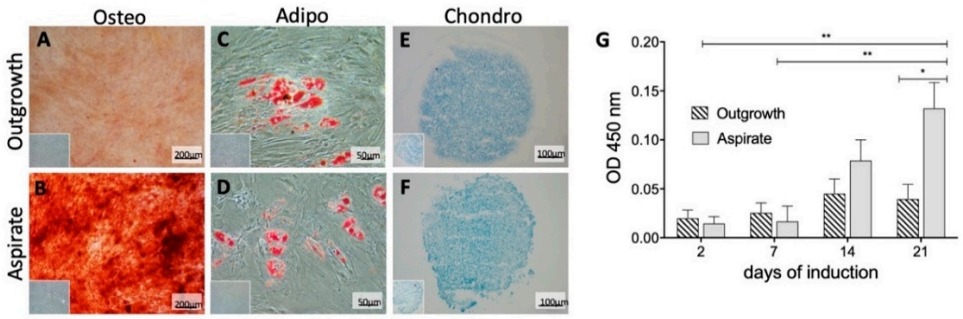

Figure 5. Differentiation capacity of MSCs from outgrowth and aspirate cultures. (**A**,**B**) Alizarin Red S, a staining for mineral deposits, indicates an osteogenic differentiation. Aspirate cells showed an increased signal compared to outgrowth cells. (**C**,**D**) Oil Red O is an indicator for lipids and visualizes adipocytes in red. Both niches were able to differentiate without significant difference. (**E**,**F**) Cell pellets with cartilaginous differentiation that were cut into 12 µm cryosections. Samples were subsequently stained with Alcian Blue to detect acid mucoids. Controls are indicated in the bottom left corners and in Supplementary Figure S2. (**G**) For quantification of the increased osseous differentiation potential of the aspirate cultures, the OD was measured at 450 nm at the indicated time points of osteogenic induction.

Increased OD correlated with enlarged mineral deposits as an indicator of osteogenic differentiation. The bar charts show delta results of unstimulated cells subtracted from induced cells. After 21 days of osteogenic induction, aspirate MSCs exhibited a significantly higher OD than outgrowth MSCs. * $p < 0.05$, ** $p < 0.01$.

Although, MSCs from both aspirate and outgrowth cultures were harvested from the same donor material, a significantly different osteogenic differentiation potential was observed after 21 days of osteogenic induction (Figure 5A,B).

In aspirate cultures, a significantly higher amount of mineral deposits was detected in alizarin red staining and, correspondingly, a significantly higher optical density (OD) at 450 nm was measured compared to outgrowth MSC cultures. At day 21, aspirate MSCs showed an OD that was almost three times as high as the outgrowth group (Figure 5G), therefore confirming its superior osteogenic potential.

To further quantify the osteogenic differentiation, the alkaline phosphatase (ALP) activity was determined in both MSC populations by using histological staining as well as a fluorometric assay. Visualizing the ALP enzyme in an MSC monolayer culture showed a stronger ALP staining in MSCs from aspirate cultures compared to outgrowth cells (Figure 6A). Using a quantitative approach to measure ALP expression confirmed these findings by detecting significantly elevated ALP concentrations in MSCs from aspirate cultures in comparison to the corresponding controls (Figure 6B). This significant difference was detected first at day five of induction and increased multifold after seven days of osteogenic differentiation (Figure 6B). This trend was even stronger at day 12; however, by then, MSCs from outgrowth cultures also showed a significant increase. Of note, the outgrowth MSCs presented only half of the ALP activity in comparison to aspirate MSCs.

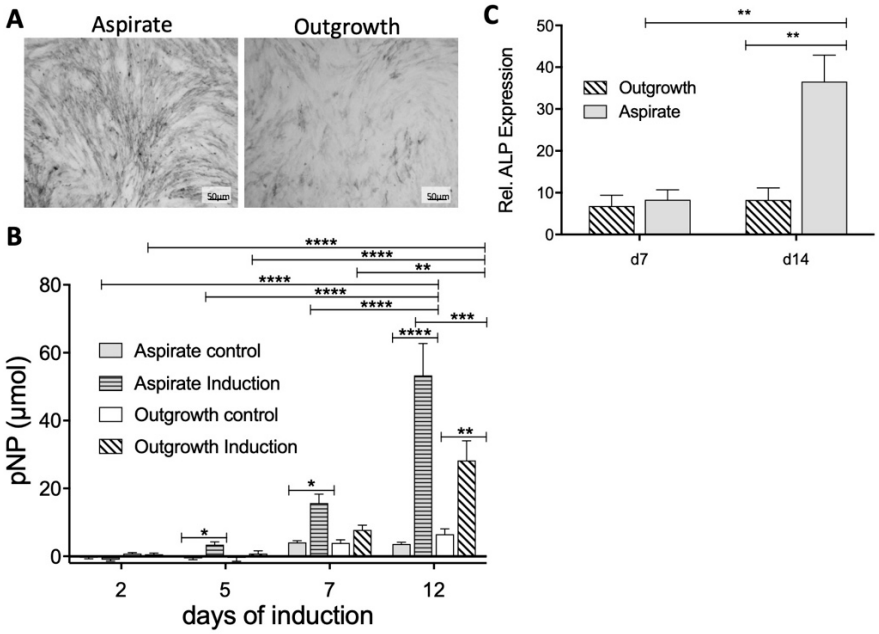

Figure 6. Comparison of alkaline phosphatase (ALP) expression by aspirate and outgrowth-derived MSCs. (**A**) ALP staining: The NBT/BCIP solution exhibited a black precipitate indicating the presence of ALP enzyme. MSCs from aspirate cultures showed a stronger ALP signal compared to outgrowth-derived MSCs. (**B**) Results of the ALP assay were evaluated by OD measurements. Starting at day 5, induced MSCs from aspirate showed higher ALP activity in comparison to the outgrowth-derived MSC group. (**C**) Relative gene expression level of ALP was determined using reverse transcriptase polymerase chain reaction (RT-PCR) for MSCs from both groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

To even further validate the functional differences between MSCs from aspirate versus outgrowth cultures and to confirm the superiority of aspirate MSCs regarding their osteogenic potential, we performed RT-PCR analyses to quantify ALP mRNA expression (Figure 6C). Indeed, MSCs from aspirate culture showed significantly increased levels of ALP mRNA after 14 days of culture, which nicely confirmed our findings that MSCs from aspirate cultures not only show a distinct surface molecule repertoire but also an enhanced osteogenic differentiation potential.

3. Discussion

In current scientific debate, heterogeneity of MSCs is well acknowledged. One important aspect is whether MSCs from different tissues and MSCs exerting different phenotypes can be designated using the same term "MSCs" or if the general definition of MSCs needs to be revised [18,19].

Until now, major focus has been put onto different tissue sources. Yet, donor characteristics, harvesting methods and processing methods represent further crucial factors affecting differentiation potential of MSCs [20,21]. The influence of the latter, however, became the object of investigation only recently. In fact, all of the above-mentioned factors might impact MSC's capacity to directed multilineage differentiation [22].

To date, most studies describing clinical and histological healing after MSC application did not characterize MSC phenotypes via a combined proteomic/flow cytometric and functional approach [23–25]. Flow cytometric cell surface proteomics represents a powerful tool to describe cell surface epitopes and allows the correlation of specific markers with functional features of the analyzed cells.

As shown in this study, isolation techniques have a major influence on MSC differentiation capacities and may be an important factor for success of translational studies; e.g., in the context of musculoskeletal tissue engineering, osteogenic differentiation potential is a crucial characteristic. MSCs that differentiate in osseous tissue may thus be the preferred cell source. It is, therefore, of high interest to define the optimal isolation methodology to generate the desired MSC phenotype. Obviously, there are several approaches to investigate the underlying MSC phenotype; however, the most common and meaningful are the characterization of surface marker expression as well as MSC differentiation potential.

The current study primarily aimed to analyze differences between MSCs generated from aspirate or outgrowth cultures. In this study, BM-MSCs that were aspirated demonstrated a better osseous differentiation capacity than BM-MSCs that were generated by outgrowth cultures. This showed that bone marrow aspiration is an important translationally relevant harvesting technique, which is further supported by the fact that this technique can more easily generate a decent amount of biomaterial and subsequently more MSCs in comparison to harvesting bone chips, which is clinically more limited in most cases.

Moreover, we found that MSCs isolated out of aspirate or outgrowth cultures showed both similarities and differences in terms of their surface marker expression. While some of these markers have been known to play a role in bone and MSC biology, for others, this association has not been so clear so far. For instance, it was shown that CD146 expression defines a subpopulation of human MSCs capable of bone formation and it was suggested to be suitable for clinical protocols of bone tissue regeneration. $CD146^+$ MSCs were also shown to pursue trans-endothelial migration and homing to injured bone sites [26]. Migratory capacity of $CD146^+$ MSCs is based on the exhibition of an enriched vascular smooth muscle cell phenotype and a smaller size and cytoskeletal morphology compared to $CD146^-$ MSCs [27,28]. Furthermore, Kevorkova et al. identified the reduced expression of CD36 as a key factor contributing to reduced deposition of osseous matrix, which is in line with the phenotype of our aspirate group [29].

It is now tempting to mechanistically tie the different surface marker expression with the functional readout of the osteogenic differentiation, but at this point, this comparison is only an association and does not prove a link between surface marker and cellular function. However, from our point of view,

the similarities and differences between these two isolation methods are of interest to the community and a discussion about potential association between surface markers and osteogenic differentiation could stimulate further studies to precisely analyze potential connections between surface marker expression and MSC function.

In this study, we investigated bone marrow-derived MSCs from the same tissue and donor site but harvested either as aspirate or bone chip cultures. This direct comparison is a novel aspect that has not been investigated so far. The study is a valuable contribution to the field, as it demonstrates the distinct impact of harvesting and processing methods on MSC quality and, thus, the importance of standardized procedures for the use of MSCs in clinical therapies. This is analogous to a study by Donnenberg and colleagues, who claimed a standard protocol for harvesting and subsequent processing in order to gain more comparability between different studies. In fact, it was suggested to investigate whether $CD44^+$ cell sorting prior to cell culture would result in more homogeneous populations as expression of this marker was strongly correlated to expression of MSC markers as defined by ISCT [20,30].

Heterogeneity of MSCs may explain a broad spectrum of success rates in clinical studies as certain subpopulations of MSCs may be more suitable for certain biological applications and superior performance in translational settings than others [31]. This is because MSCs' biological activity comprises immunomodulatory, anti-inflammatory and pro-regenerative capacities. Thus, in order to use and investigate MSCs in more detail, a more specific phenotyping of MSCs will be necessary for future studies.

In summary, for further studies investigating MSC-mediated bone regeneration, bone marrow-derived MSCs isolated by aspiration represent the source of choice because of their superior clinical relevance. This study additionally shows that a consensual standard protocol (including donor site, donor characteristics such as age, comorbidities, body mass index (BMI) and isolation as well as processing technique) urgently needs to be developed for the isolation and application of MSCs in order to achieve a better reproducibility and comparability of the results reported by different studies.

4. Materials and Methods

4.1. Tissue Donors and Isolation of Bone Marrow MSCs

Recruitment of subjects to obtain human bone marrow samples was approved by the local ethics committee (University Hospital Bonn, project ID: 122/09, approval date: 12 October 2009) and was conducted in accordance with the approved guidelines as well as the declaration of Helsinki. All included patients (n = 5) in this study were undergoing total hip arthroplasty due to primary coxarthrosis and showed no signs of congenital bone diseases, acquired diseases of the hematopoietic bone marrow, tumors or infections.

Bone marrow-derived MSCs were harvested during the procedure of hip replacement. When sawing the femoral bone, cells were either harvested by bone marrow aspiration or bone fragments were collected for outgrowth cultures. Bone chips that had to be removed for surgical reasons to perform total hip arthroplasty were washed with phosphate-buffered saline (PBS) to remove remaining blood. As fragments were not contaminated with connective tissue during surgery, no further cleaning steps were necessary.

Aspirated cells were isolated by scratching and flushing the spongious part of the femoral head or thin bone slices with PBS. The cell suspension was transferred onto a 70 µm filter and a Ficoll gradient was used in cases where a disproportionate number of erythrocytes was observed. Thus, the blood/PBS suspension was transferred on top of a 20 mL Ficoll and centrifuged for 30 min, 800 g, without break. The interphase was isolated, washed and placed into a cell culture flask.

Osseous fragments and aspirated cells were cultured under standard conditions at 37 °C/5% CO_2 in Dulbecco's modified eagle's medium (DMEM) low glucose, containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% L-glutamin. According to our standard protocol, medium was

changed twice a week. Within 1–2 weeks of incubation, a distinct outgrowth from bone fragments or cell clones from the aspirate cells were detected. To individualize cell aggregates, cells were trypsinized for 5 min with 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA). Dense cells were passaged and frozen at p1 with freezing medium containing 10% dimethyl sulfoxide (DMSO), 40% FBS and 50% DMEM until further experiments were performed. After thawing, MSCs were further expanded for two more passages (p3) and then used for all downstream assays. For flow cytometric analysis, MSCs were trypsinized, washed with PBS and filtered to generate a single cell suspension. For all other assays, MSCs were cultured as monolayer.

4.2. Phenotypic Analysis of MSCs

Phenotypic surface marker expression analysis of human MSCs was performed using flow cytometry as described previously [32]. Briefly, cells were resuspended in PBS with 1% FBS/2 mM EDTA and were stained with saturating concentrations (1:25 dilution) of antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany) for 20 min. Doublets and dead cells were excluded from the analysis. Unstained cells and isotype antibodies were used as controls. Flow cytometry data were acquired on a MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany) and analyzed using FlowJo v10 (BD Biosciences, Heidelberg, Germany). The following antibodies (Miltenyi Biotec, Bergisch Gladbach, Germany) and clones were used: CD90 (DG3), CD73 (AD2), CD105 (43A4E1), CD13 (REA263), CD29 (TS2/16), CD44 (DB105), CD45 (REA119), CD14 (TÜK4), CD20 (LT20), TCRα/β (BW242/412), HLA-DQ (REA303), CD11b (M1/70.15.11.5), CD34 (AC136), CD10 (97C5), CD49f (GoH3), CD271 (ME20.4-1.H4), MSCA (W8B2), CD106 (REA269), CD56 (AF12-7H3), CD200 (OX-104), SSEA-4 (REA101), CD146 (541-10B2), CD39 (MZ18-23C8), CD141 (AD5-14H12), LAP (CH6-17E5.1), CD54 (REA266), CD222 (REA187), CD239 (REA276), CD318 (REA194), CD36 (AC106).

4.3. Real-Time Polymerase Chain Reaction

Total RNA was extracted from both outgrowth and aspirate MSCs using TRIzol Reagent (Ambion, Life technologies, Darmstadt, Germany) at indicated time points. Cells were washed with PBS, lysed in TRIzol and chloroform/isopropanol (ratio 24:1) (PanReac AppliChem, Darmstadt, Germany) was added. After centrifugation, the upper phase containing RNA was collected and precipitated by adding isopropanol and washed twice in ethanol (80%). RNA (1 µg) was reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany) and RT-PCR was performed using LightCycler 480 SYBR Green I Master according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, German). Data analysis was performed using the delta-delta-Ct (ddCT) method by normalization to 18S rRNA and the corresponding control samples without differentiation. Previously published primers were used to analyze ALP expression [33].

4.4. Analysis of MSC Differentiation Potential

MSCs were differentiated into the osteo-, adipo- and chondro-genic lineages, as described previously [32]. For osteogenic differentiation, MSCs were induced through high-glucose DMEM medium supplemented with 0.1 µM dexamethasone, 10 mM β-glycerophosphate disodium salt hydrate, and 50 µM 2-phosphate-L-ascorbic acid trisodium salt (Sigma Aldrich, Darmstadt, Germany). Induction towards the adipogenic lineage differentiation was performed by supplementing culture medium with 1 µM dexamethasone, 1 µM insulin, and 200 µM indomethacin (Sigma Aldrich, Darmstadt, Germany). The chondrogenic differentiation was performed as cell pellet culture using high-glucose DMEM medium supplemented with 1 µg/mL insulin, 1 ng/mL transferrin, 1 ng/mL sodium selenite, 0.1 µM dexamethasone, 50 µM 2-phosphate-L-ascorbic acid trisodium salt and 10 ng/mL transforming growth factor beta-1 (TGF-β1) (Sigma Aldrich, Darmstadt, Germany). All differentiation assays were performed for 21 days and culture medium lacking supplementation was used as control. All differentiated samples were fixed with 4% paraformaldehyde (PFA) before further treatment. Chondrogenic cell pellets were cut into 12 µm cryosections (Microm 550, Thermo Scientific, Schwerte, Germany).

For histological analysis, cells were stained with Alizarin Red S (Sigma Aldrich, Darmstadt, Germany) for evaluation of osteogenic differentiation or Oil Red O staining (Sigma Aldrich, Darmstadt, Germany) in order to determine adipose differentiation, as described previously [32]. For determination of chondrogenic cell differentiation, MSCs were stained with Alcian Blue (Sigma Aldrich, Darmstadt, Germany), as described previously [32].

For further quantification of osseous cell differentiation, MSC samples at a density of 1×10^4 cells/cm^2 were treated with 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) substrate system (Dako, Hamburg, Germany) according to the manufacturer's instructions to quantify the presence of the ALP enzyme. Further, alkaline phosphatase (ALP) activity was determined with the help of 4-Methylumbelliferyl phosphate disodium salt (MUP) substrate using a fluorometric assay kit according to the manufacturer's instructions (BioVision, Inc., CA, USA). The absorbance was measured at 360 nm using a microplate reader (TECAN, Magellan, Switzerland).

4.5. Statistical Analysis

Statistical tests were performed with Prism 7 (GraphPad, La Jolla, CA, USA) using a two-tailed, unpaired Student's *t*-test with a 95% confidence interval or two-way analysis of variance (ANOVA) assuming Gaussian distribution. Significance levels are marked as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

Supplementary Materials: Supplementary Materials can be found at http://www.mdpi.com/1422-0067/21/12/4382/s1.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ALP	Alkaline phosphatase
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BM	Bone marrow
CD	Cluster of differentiation
cDNA	Complementary desoxy ribonucleic acid
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
hBM-MSC	Human bone marrow-derived MSCs
ISCT	International Society for Cellular Therapy
LAP	Latency-associated peptide
MFI	Mean fluorescence intensity
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stromal cells
MSCA	Mesenchymal stem cell antigen
MUP	4-Methylumbelliferyl phosphate disodium salt
NBT	Nitro blue tetrazolium
OD	Optical density

PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction

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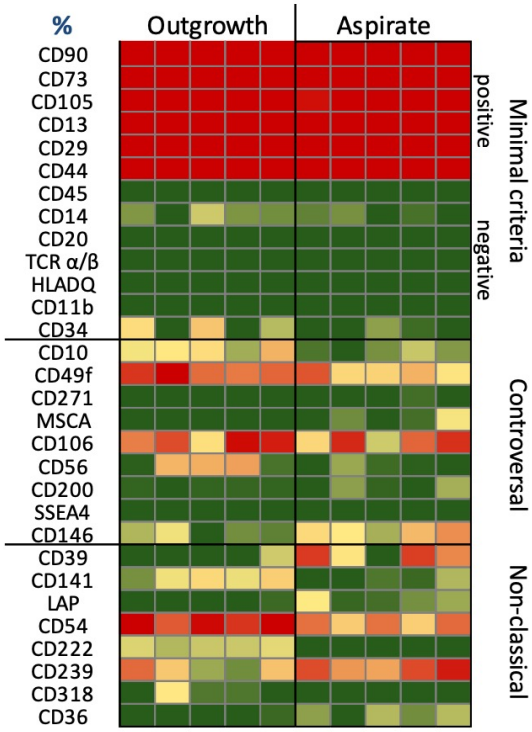
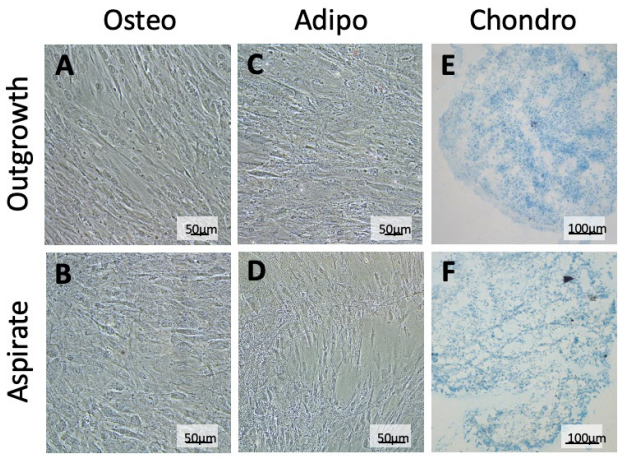
Supplementary figures



Supplementary figure 1. Heat map of expression level of all discussed markers. Depicted are the ISCT minimal criteria (positive and negative), controversially discussed markers, as well as non-classical MSC markers discussed in this study. Red color indicates high expression, green color low expression.



Supplementary figure 2. Controls for MSC lineage differentiation from outgrowth and aspirate cultures. MSCs were differentiated into the osteo-, adipo- and chondrogenic lineages. All differentiation assays were performed for 21 days and culture medium lacking supplementation was used as control. Resulting samples were fixed with 4% PFA before further treatment. Chondrogenic cell pellets were cut into 12 µm cryosections. **(A/B)** For evaluation of osteogenic differentiation, cells were stained with Alizarin Red S. **(C/D)** Adipose differentiation was determined using Oil Red O staining. **(E/F)** Alcian Blue staining was used for analyzing the chondrogenic cell differentiation.

4. Discussion with references

Mesenchymal stromal cells (MSC) are one of the most studied stem cell populations and play a major role in processes important for health and disease (Schildberg and Donnenberg, 2018). In addition to their reparative functions, MSCs have potent immunomodulatory capacities and respond in unique ways by homing and integrating into defective tissues (Ma et al., 2014). In addition, MSCs have been recognized to have great potential as a future therapeutic option in the pathophysiology of orthopedic injuries and diseases, and represent a promising potential in regenerative medicine (Berebichez-Fridman et al., 2017). Human (h)MSCs are well characterized, standardized and meet the minimal criteria outlined in a position statement of the ISCT (Dominici et al., 2006). However, ovine (o)MSCs, are poorly characterized and to date not standardized. In the current study, MSCs were isolated from three human and three corresponding ovine sources and characterized based on the same established protocols. All MSCs showed robust proliferation behavior confirming previous reports (Bottagisio et al., 2015). Our study identified common positive and negative surface markers between hMSCs and oMSCs, proving that oMSCs are comparable to hMSCs. Considering the promising therapeutic potential, the immunomodulatory properties of MSCs have been mainly investigated in humans and mice (Gao et al., 2016) and too rarely in sheep (Mrugala et al., 2008). We now show that MSCs from both human and ovine sources have comparable immunomodulatory capacity. In contrast to adipogenic differentiation, our comparative study demonstrated that oMSCs have a significantly higher capacity for chondrogenic differentiation compared with hMSCs. This evidence is important for MSC-based cartilage repair strategies needed to translate findings from sheep models into the clinic (Music et al., 2018). Further, our study demonstrated that both human and ovine MSCs showed strong mineralization rates by using specific staining, monitoring the optical density (OD) of monolayer cultures (Loebel et al., 2017), measuring the free phosphate ion release and determining the common osteogenic gene marker *COL1A1* and *RUNX2*. This report is the first investigation of the mineralization process of hMSCs compared with oMSCs from three corresponding sources, laying the fundament for future studies utilizing the osteogenic capacity of oMSCs.

The vertebral body as a stem cell niche is sparsely described, and it is unclear whether impairment of MSCs contributes to the pathogenesis of osteoporosis. Therefore, the aim

of the current study was to isolate and characterize MSCs from the lumbar spine vertebral body of non-osteoporotic and osteoporotic patients. MSCs from both groups fulfilled the minimal MSC criteria according to the ISCT guidelines (Dominici et al., 2006). They showed similar proliferation tendencies, typical MSC surface markers, immunomodulatory capacity, and comparable trilineage potentials. This is in contrast to recently reported studies indicating an alteration in the reciprocal balance between adipogenic and osteogenic differentiation pathways toward increased adipocytes formation at the expense of osteoblast differentiation, suggesting disturbance of MSC activity (Pino et al., 2012). Most importantly, in our study, close investigation of the osteogenic differentiation potential indicated that MSCs from osteoporotic patients (osMSC) were unaffected compared with MSCs from non-osteoporotic patients (nosMSC). The results obtained confirmed that both nosMSCs and osMSCs exhibited a strong ability to differentiate toward the osteoblastic lineage in vitro, as reflected by steadily increasing intensity of ALP staining, as previously reported for other MSCs (Chen et al., 2015), and increased ALP activity, as previously shown (Hoemann et al., 2009). In addition, the mineralization process was continuously followed up by monitoring crystal depositions by measuring the OD of monolayer cultures of nosMSCs and osMSCs during the osteogenic differentiation, as previously reported (Loebel et al., 2017) and by phosphate ion release in the supernatants. The measured OD of deposited crystals showed no significant difference between nosMSCs and osMSCs; however, this does not exclude possible differences in crystal composition. Furthermore, the expression of *ALPL*, *COL1A1,* and *RUNX2* was comparable in both nosMSCs and osMSCs, similar to results previously reported when assessing MSCs from healthy patients (Loebel et al., 2017). The transcription factor *SOX9* is known to play a key role in chondrogenesis and endochondral bone formation. It has been shown to be a major regulator in direct osteogenesis by directly interacting with *RUNX2*. The *RUNX2/SOX9* ratio has been proposed as an early indicator for osteoblastic differentiation of human MSCs in vitro (Loebel et al., 2015).

To date, the investigation of MSCs has focused primarily on different tissue sources. Nevertheless, donor sites, harvesting techniques, and processing methods influence differentiation potential of MSCs (Mushahary et al., 2018). In the current study, bone marrow-derived MSCs (BM-MSC) that were aspirated were shown to have better osteogenic differentiation capacity than BM-MSCs generated by outgrowth cultures. As

shown in this study, harvesting techniques have a major impact on the differentiation ability of MSCs and may be an important factor for their successful translation into the clinic. Flow cytometric cell surface proteomics provides a powerful tool to describe cell surface epitopes and allows the correlation of specific markers with functional features of the analyzed cells. We found that MSCs isolated out of aspirate or outgrowth cultures showed both similarities and differences in terms of their surface marker expression. Further studies are needed to decipher any association between surface marker expression and MSC function.

In conclusion, direct comparison of human and ovine MSCs from three different sources defines phenotypic similarities and differences, contributing to better characterization and standardization of ovine MSCs. Furthermore, MSCs from the lumbar spine vertebral body of osteoporotic patients were not impaired compared to vertebral body MSCs from non-osteoporotic patients, but possessed full osteogenic potential. Moreover, bone marrow-derived MSCs isolated by aspiration are the source of choice for MSC-mediated bone regeneration because of their superior clinical relevance.

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6. Publications

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Membership

Stem Cell Network NRW, Bonner Forum Biomedizin (Scientific Network of Bonn University)