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ORIGINAL ARTICLE

Isolation, culture and chondrogenic differentiation of canine adipose tissue- and bone marrow-derived mesenchymal stem cells–a comparative study

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Abstract In the dog, mesenchymal stem cells (MSCs) have been shown to reside in the bone marrow (bone marrow-derived mesenchymal stem cells: BM-MSCs) as well as in the adipose tissue (adipose tissue-derived stem cells: ADSCs). Potential application fields for these multipotent MSCs in small animal practice are joint diseases as MSCs of both sources have shown to possess chondrogenic differentiation ability. However, it is not clear whether the chondrogenic differentiation potential of cells of these two distinct tissues is truly equal. Therefore, we compared MSCs of both origins in this study in terms of their chondrogenic differentiation ability and suitability for clinical application. BM-MSCs harvested from the femoral neck and ADSCs from intra-abdominal fat tissue were examined for their morphology, population doubling time (PDT) and CD90 surface antigen expression. RT-PCR served to assess expression of pluripotency marker Oct4 and early differentiation marker genes. Chondrogenic differentiation ability was compared and validated using histochemistry, transmission electron microscopy (TEM) and quantitative RT-PCR. Both cell populations presented a highly similar morphology and marker expression in an undifferentiated stage except that freshly isolated ADSCs demonstrated a significantly faster PDT than BM-MSCs. In contrast, BM-MSCs revealed a morphological superior cartilage formation by the production of a more abundant and structured hyaline matrix and higher expression of lineage specific genes under the applied standard differentiation protocol. However, further investigations are necessary in order to find out if chondrogenic differentiation can be improved in canine ADSCs using different protocols and/or supplements.

Keywords Dog · Mesenchymal stem cells · Chondrogenic differentiation · Cartilage · Osteoarthritis

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Introduction

Mesenchymal stem cells are non-hematopoietic pluripotent progenitor cells capable to differentiate into various lineages including bone and cartilage (Barry and Murphy 2004; Arthur et al. 2009). Furthermore, MSCs secrete soluble factors which stimulate the migration, mitosis and differentiation of local stem cells, enhance angiogenesis and modulate immunoreactions which makes them interesting tools for tissue engineering and regeneration (Caplan and Dennis 2006; Le Blanc and Ringdén 2007; Kang et al. 2008). In veterinary medicine they have already found broad entrance into equine practice, most notably in the therapy of tendon injuries (Koch et al. 2009; Frisbie and Smith 2010). Besides, MSCs have been used in clinical trials in models of osteoarthritis not only in the horse but also in goats, pigs and humans (Murphy et al. 2003; Kuroda et al. 2007; Lee et al. 2007; Frisbie et al. 2009). The favourable outcome of these studies raised the idea of a MSC-based therapy for osteoarthritis in dogs where veterinarians were so far left mainly

with symptomatic treatments (Sanderson et al. 2009). First attempts were made with adipose tissue-derived nucleated cell suspensions that showed promising outcomes even though the investigators omitted the expansion and characterisation of the applied cells (Black et al. 2007; Black et al. 2008). By now, several studies confirmed the existence of MSCs in the canine adipose tissue as well as in the bone marrow and characterized them in detail (Kadiyala et al. 1997; Neupane et al. 2008; Csaki et al. 2009; Vieira et al. 2010). With the ease of harvest and the abundance of fat tissue ADSCs appear preferable to BM-MSCs. Especially in small breeds the amount of bone marrow that can be harvested is limited and the collection is more laborious and painful. However, several recent studies in humans and the horse revealed that differentiation capacity and growth kinetics of BM-MSCs and ADSCs are not equal (Lee et al. 2004; Im et al. 2005; Vidal et al. 2007; Vidal et al. 2008).

The present study should therefore unveil if similar differences exist between canine BM-MSCs and ADSCs.

Material and methods

All dog owners gave their written consent to the use of removed tissue for research purposes. All tissue samples were taken during unavoidable surgical operations or routine castrations at the local animal clinics. Donors suffering from infections, systemic diseases or with suspicion of malignoma were excluded from the study.

Isolation and cultivation of MSCs

Unless stated otherwise all cell culture reagents were purchased from PAA. Bone marrow from 9 different dogs aged from 0.5 to 9 years (mean age 3.2 years) was harvested by flushing femoral heads after routine femoral head resection due to femoral head dislocation, trauma (car accident/fall) or, in one case, hip arthroplasty. Subsequently, BM-MSCs were isolated and cultured as previously reported for the horse (Arnhold et al. 2007). Briefly, harvested cells were washed in PBS, filtered through a 70 μm falcon strainer (BD) and centrifuged at 200 g for 10 min. The resulting pellet was resuspended in alpha Minimal Essential Medium (αMEM), layered on a Ficoll gradient (LSM 1077) and centrifuged at 1200 g for 20 min. The nucleated cell fraction was washed twice in αMEM and plated at a density of 2×10^5 cells/cm^2 in αMEM containing 20% Fetal Bovine Serum (FBS Gold, fully defined) and 2% penicillin/streptomycin solution. Medium was first changed 3 days after plating in order to remove non-adherent cells.

ADSCs were obtained from intra-abdominal or subcutaneous fat tissue from 6 different dogs aged from 1 to 11 years (mean age 6.6 years) following a routine operation or castration at one of our local animal clinics. ADSCs were isolated as described previously in the horse (Raabe et al. 2010). Briefly, fat tissue was minced, washed in PBS Buffer and digested for 30 min with an equivalent amount of collagenase I solution containing 1 mg/ml collagenase I (Biochrom) and 0.1 mg/ml BSA (BSA fraction V). The resulting cell suspension was filtered through a 70 μm falcon strainer (BD) and collagenase I activity was stopped by a double volume of PBS. After centrifugation cells were resuspended in Dulbecco's modified eagle medium, low glucose (DMEM LG) containing 10% FBS Gold and 1% penicillin/streptomycin and plated at a density of 2×10^5 cells/cm^2. After 24 h non-adherent cells were washed off.

Both, BM-MSCs and ADSCs were cultured until 80% confluence was reached, detached with accutase and first passaged. Again, cells were maintained until 80% confluence, detached and cryopreserved in a freezing medium consisting of αMEM/DMEM LG containing 30% FBS Gold and 5% DMSO (Sigma-Aldrich) for later use.

Population doubling time of freshly isolated cells

For PDT assessment non-cryopreserved cells at passage 1 were seeded into a 24-well plate (Greiner Bio-One) at a density of 2×10^3 cells/cm^2. After a 2 day recovery, the cell number of three wells was assessed daily over a period of 6 days. Cell numbers were plotted in a semi-logarithmical chart against the culture time and a regression line was adjusted. The PDT was then calculated using the formula $P_D = \log_{10}2/m$ where m is the slope of the regression line.

Wound-healing assay (WH-assay)

For the assessment of the migration potential a WH-assay was conducted (Liang et al. 2007). Cryopreserved cells of passage 1 were first cultured, detached and seeded at passage 3 with a density of 5×10^5 cells/cm^2 in a 3.5 cm cell culture dish (Greiner Bio-One). At 80–90% confluence a scratch was applied using a 1000 μl pipette tip. After washing twice with PBS the dish was placed in a live cell incubator providing 37°C, 5% CO_2 in a humidified atmosphere under a phase contrast microscope (Axio Observer and PM S1, Zeiss). Pictures were taken every 5 min over 14 h and data analyzed with the ImageJ software (NIH). The percentage of uncovered area was plotted against the time and a regression line was adjusted. The slope of the regression line was used to compare migration velocity of both cell populations.

Flow cytometry

For the detection of CD90 surface protein cells of 5 different individuals from each group were detached as described

above and incubated with a monoclonal rat-anti-dog CD90 antibody (MCA 1036G, AbD Serotec) in a 1:50 dilution. As isotype control served a rat IgG2b antibody (MCA 1125, AbD Serotec) and as secondary antibody an R-Phycoerythrin-conjugated goat anti rat IgG antibody (Dianova) in a 1:100 dilution.

A minimum of 10.000 events were acquired for each sample and were analysed with a FACSCalibur™ flow cytometer (Becton-Dickinson) using the software Cell Quest Pro (Becton-Dickinson). Data were exported and analysed with the software FCS Express 2 (De Novo-Software). The gate was set on the forward-scattered light (FSC) vs. side-scattered light (SSC) plot to define and characterize the size and granularity of each cell population. Following, gated cells were plotted in a dual-colour histogram to detect positively stained cells by a shift from the lower left to the lower right. The percentage of cells in the lower right was outlined for each origin of cells for several replicates.

Chondrogenic differentiation

For the evaluation of chondrogenic differentiation potential, $0.8–1.2\times10^5$ cells at passage 2 were centrifuged in falcon tubes (BD) at 100 g for 5 min. Cells were kept overnight at 37°C, 5% CO_2 in a humidified atmosphere. Formed cell pellets were placed in a 24-well plate with a number of 4 pellets per well and cultivated in chondrogenic differentiation medium composed of DMEM LG, 1% FBS Gold, 1% penicillin/streptomycin, 0.05% ITS ×100 (insulin, transferrin, sodium selenite I3146, Sigma-Aldrich), 50 μM ascorbic acid (A8960, Sigma-Aldrich), 100 nM dexamethasone (D4902, Sigma-Aldrich) and 10 ng/ml TGF β1 (T5050, Sigma-Aldrich) (Tondreau et al. 2004). For histological analysis two pellets of each sample were cultivated in normal culture medium as negative controls. Differentiation was conducted over 10 and 24 days, respectively.

Additionally, chondrogenic differentiation was conducted in a high density monolayer over a 10 day period for easier RNA extraction. Therefore 2.5×10^4 cells/well were seeded in a 24-well plate. Five wells were defined as positive controls and cultured with chondrogenic medium whereas another five wells cultured with basal medium served as negative controls. After 10 days 4 wells of each cell population and well group were taken in Tri Reagent (Sigma-Aldrich) for RNA extraction. One well of each group was fixed with 4% paraformaldehyde and stained with alcian blue staining (data not shown).

Histomorphological evaluation

For histological examination pellets were fixed in 4% paraformaldehyde, embedded in paraffin and cut into 4 μm thin sections. Adherent cells were fixed in the tissue culture well with 4% paraformaldehyde and directly stained (data not shown). Staining was performed using alcian blue 8GX (Merck) for the detection of mucopolysaccharides and glycosaminoglycans followed by a nuclear fast red counterstain (Merck).

Transmission electron microscopy

For TEM, pellets were fixed in yellow fix solution (2% paraformaldehyde, 2% glutaraldehyde, 0.02% picric acid) (Roth), stained with 1% osmium tetroxide (Roth) and embedded in Epon (Serva). Ultrathin sections (80 nm) were counterstained with uranyl acetate and lead citrate (Reichert Ultrastainer, Leica) and examined in a Zeiss EM 109 transmission electron microscope.

RT-PCR and quantitative RT-PCR (RT-qPCR)

Total RNA was extracted from a minimum of 5×10^5 cells using Tri Reagent (Sigma-Aldrich) according to the manufacturer's protocol. Specimens were adjusted to 200 ng/μl RNA, treated with a recombinant DNAse I (Roche) and subsequently reverse transcribed using GeneAmp® Gold RNA PCR Core Kit (Applied Biosystems) according to the manufacturer's protocol. Minus RT samples for each specimen were included. PCR was conducted using 10 μl cDNA, 2 μl $MgCl_2$, 4 μl 10× PCR Gold Buffer, 32.75 μl nuclease-free water, 0.25 μl AmpliTaq Gold® (Applied Biosystems) and 1 μl of a 10 pmol forward and reverse primer mix (sequences see Table 1). All primers were purchased from Eurofins MWG Operon. Cycling conditions were as follows: 95°C for 10 min, following 39 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 1.30 min and finally 72°C for 10 min. PCR products were separated using a 2% agarose gel electrophoresis and visualized by SYBR Green (Sigma-Aldrich).

RT-qPCR for Sox9 and Collagen 2A1 (Coll2A1) was carried out with specimens from 2D-chondrogenic differentiation on a CFX96 Realtime Cycler (Bio-Rad) using IQ SybrGreen Supermix (Bio-Rad) with the following protocol: 3 min 95°C, following 40 cycles of 15 s 95°C and 1 min 60°C, with a subsequent melting curve. Data was analyzed using the CFX Manager software 1.6 (Bio-Rad) applying the ΔΔCT-method for relative gene expression relative to GAPDH as housekeeping gene.

Statistical analyses

A Welch-test was performed to calculate the significance for differences in the mean PDT. WH-assay data were compared using a pooled *t*-test on the logarithmized mean slope values. Expression of Sox9 was analyzed by bi-factorial ANOVA with repeated measurements. A paired *t*-test served to evaluate data from Coll2A1 gene expression of

Table 1 Primer sequences

Primer	Sequence (5'-3')	Accession no.	Amplicon length	Annealing temperature
GAPDH	for GCTGCCAAATATGACGACATCA rev GTAGCCCAGGATGCCTTTGAG	NM_001003142	75 bp	60°C
Oct4	for AGAGGCAACCTGGAGAACATG rev GGGCAATGTGGCTGATCTG	XM_538830	71 bp	60°C
PPARγ2	for TGGTTGACACAGAGATGCCATT rev GTGGTCATCCATTACGGACAGA	AJ972913	77 bp	60°C
Runx2	for TGTCATGGCGGGTAACGAT rev TCCGGCCCACAAATCTCA	AY738265	107 bp	60°C
Sox 9	for AGTACCCGCACCTGCACAAC rev CGCTTCTCGCTCTCGTTCAG	NM_001002978	79 bp	60°C
Coll2A1	for GGTGGAGCAGCAAGAGCAA rev GTGTTGGGAGCCAGGTTGTC	NM_001006951	103 bp	60°C

BM-MSCs after data logarithmization to achieve an approximately normal distribution. Because of relatively high inter-individual differences (variance), qPCR data may statistically only be interpreted as a tendency. All data was analyzed using BMDP Statistical software.

Results

Morphology and population doubling time of freshly isolated cells

Both, BM-MSCs and ADSCs showed a fibroblastic and spindle-shaped cell morphology at early passages in adherent cell culture (Fig. 1a and c). Flow cytometry analysis revealed a high similarity in granularity and cell size for the detached cells of both populations (Fig. 1b and d).

A significantly faster (p=0.02) population doubling at passage 1 could be detected in ADSCs with a mean value of 29.1 h compared to BM-MSCs with a mean value of 46.4 h (Fig. 2a).

Wound-healing assay (WH-assay)

No significant differences (p=0.12) in the migration abilities were observed between both cell populations (Fig. 2b). Differences in the velocity of wound closure were rather dependent on the patient than on the cell origin and showed a similar variance in both groups.

Expression of mesenchymal stem cell and early differentiation markers

CD90 surface protein was detected in 96.1±0.6% of BM-MSCs (n=3) and in 94.8±2.9% of ADSCs (n=4) by flow cytometry (Fig. 1e and f). The expression of the pluripotency marker Oct4 on mRNA level could be proven for 4 different specimens of each cell population (Fig. 2c).

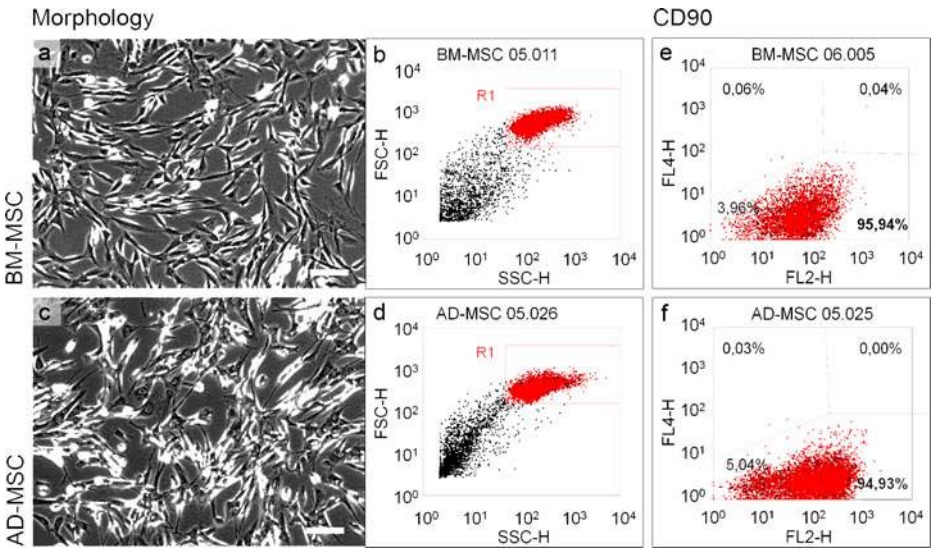

Fig. 1 Morphology and CD90 expression. **a**, **c** Phase contrast microscopy of BM-MSCs and ADSCs showed a highly similar spindle-shaped, fibroblastic cell morphology (bar = 100 μm). **b**, **d** Flow cytometry additionally confirmed the resemblance of both cell types in terms of size, as measured by the forward scattered light (FSC), and granularity, measured by the sideward scattered light (SSC). **e**, **f** Fluorescent labeling with a monoclonal anti-dog CD90 antibody verified over 94% CD90-positive cells in both populations

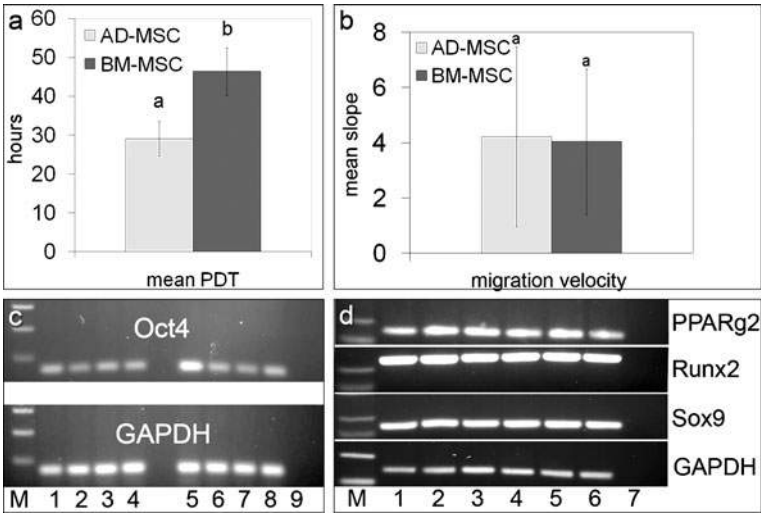

Fig. 2 Population doubling, migration velocity and marker gene expression. **a** Population doubling time at passage 1 occurred with an average of 29.1 h significantly faster in ADSCs than in BM-MSCs with a mean of 46.4 h (p=0.02). **b** A WH-Assay revealed no significant differences in the migration velocities of both cell types (p=0.12). Different letters resemble significant differences ($p \leq 0.05$). **c** Expression of stem cell marker Oct4 was detected in 4 different specimens of each group. GAPDH served as housekeeping gene (1–4 BM-MSCs, 5–8 ADSCs, 9 No template control (NTC)). **d** Early transcription factors of the adipogenic (PPARγ2), the osteogenic (Runx2) and the chondrogenic pathway (Sox9) were regularly expressed in 3 different specimens of each group, respectively. Again GAPDH served as housekeeping gene (1–3 BM-MSCs, 4–6 ADSCs, 7 NTC)

BM-MSCs as well as ADSCs regularly expressed early differentiation markers of the main mesenchymal differentiation pathways, namely PPARγ2 for adipogenesis, Sox9 for chondrogenesis and Runx2 for osteogenesis (Fig. 2d).

Morphological, ultrastructural and molecularbiological evaluation of in vitro chondrogenesis

Pellet cultures after 10 and 24 days of chondrogenic differentiation revealed a deposition of a glycosaminoglycan (GAG)-rich matrix as detected by alcian blue staining in both cell types (Fig. 3a–d and g–j). BM-MSCs showed a chondrocyte-like appearance with cartilage-typical lacunae as early as day 10 of culture and cells were progressively pushed apart by high amounts of self-produced matrix (Fig. 3a and b). After 24 days of differentiation time, the GAG-rich matrix in BM-MSCs pellets appeared well-organized and numerous chondrone-like areas could be observed (Fig. 3c and d). In contrast, ADSCs stayed spindle shaped and densely packed producing only sparse matrix between cells (Fig. 3g and h). After 24 days the centre of the ADSCs pellets was increasingly subjected to necrosis and numerous lipid vacuoles could be observed (Fig. 3i and j).

Under TEM examination BM-MSCs pellets displayed multiple star-shaped, highly active cells with long cytoplasmic processes (CP) surrounded by a well-organized matrix of fine fibers and fibrils (Fig. 3e and f). On the contrary, in ADSCs the matrix seemed unarranged and loosely packed. Numerous lipid vacuoles could be found in- and outside the cells which mostly lacked active cell organelles such as an endoplasmatic reticulum and showed signs of necrosis (Fig. 3k and l).

RT-qPCR of high density cultures after 10 days of differentiation showed a 2- to 6.25-fold upregulation of Sox9 gene expression in BM-MSCs whereas in ADSCs Sox9 expression was downregulated between 7.3- and 14-fold compared to the negative controls. There was a clear significance for the difference between groups (p=0.041) and between positive and negative controls (p=0.025). Moreover, a high significance could be shown for the correlation between group affiliation and differentiation meaning the response to differentiation medium was highly dependent on cell origin (p=0.0064) (Fig. 4a). Collagen 2A1 (Coll2A1), the most abundant collagen in hyaline cartilage, was upregulated between 14.4- and 700-fold in BM-MSCs (p=0.057) while it stayed unregulated or even undetectable in ADSCs (Fig. 4b).

Discussion

Mesenchymal stem cells with their broad regenerative potential, their paracrine and immunomodulatory effects have already found entrance into veterinary practice in the field of equine medicine (Koch et al. 2009; Frisbie and Smith 2010). Today, MSCs are routinely used by many practitioners in the field of tendon or ligament disease as well as in damaged joints (Smith 2008; Frisbie et al. 2009; Godwin et al. 2011). In equine orthopaedics, ADSCs are by now likewise applied as bone marrow derived MSCs (Nixon et al. 2008; Smith 2008; Frisbie et al. 2009). Compared with this, the

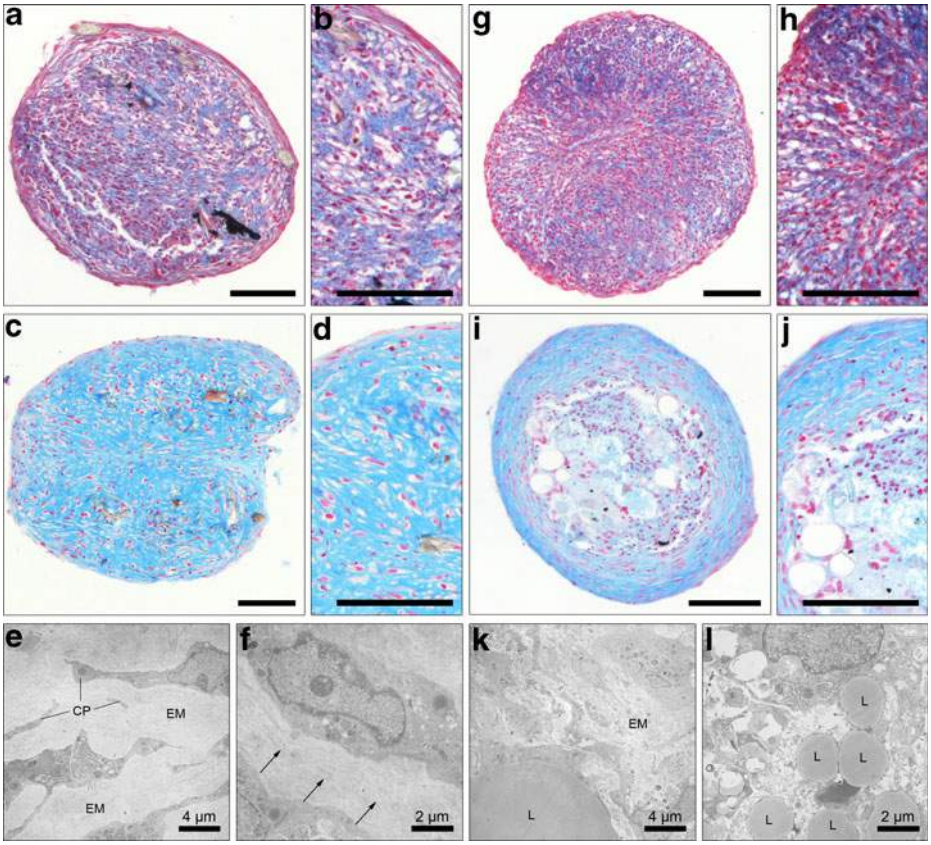

Fig. 3 Chondrogenic differentiation. **a**–**f** Chondrogenic differentiation of BM-MSCs. **a**, **b** After 10 day chondrogenic pellet culture BM-MSCs already showed a transformation from the fibroblastic to a chondrocyte-like appearance and started producing an alcian blue positive, GAG-rich matrix. **c**, **d** After 24 day of culture induced cells are dispersed by a plenty of cartilage-typical matrix and numerous chondrone-like areas could be found. Bar = 100 µm. **e**, **f** TEM displayed highly active, polymorphous cells with long cellular processes (CP) and a well-organized extracellular matrix of oriented fine fibers and fibrils (arrows). **g**–**l** Chondrogenic differentiation of ADSCs. **g**, **h** At day 10 of chondrogenic differentiation ADSCs were still spindle-shaped and densely packed with only sparse GAG-rich matrix. **i**, **j** At day 24 the centre of the pellet became increasingly subjected to necrosis and numerous lipid vacuoles emerge. Bar = 100 µm. **k**, **l** TEM of ADSCs showed only a few unoriented fibers in a loosely packed extracellular matrix (EM). Lipid vacuoles (L) could be observed in- and outside the cells and multiple residual bodies as signs of necrosis were present. EM = extracellular matrix, CP = cellular processes, L = Lipid vacuoles

clinical application of MSCs in small animal medicine is still at its beginning. So far conducted clinical studies were mainly focused on ADSCs but particularly lacked an enrichment and a subculture of the stem cells before treatment and skipped a characterisation of the differentiation potential and the cells themselves (Black et al. 2007; Black et al. 2008). The administered cell fraction in these two mentioned studies probably consisted of a mixture of cells which also contained ADSCs.

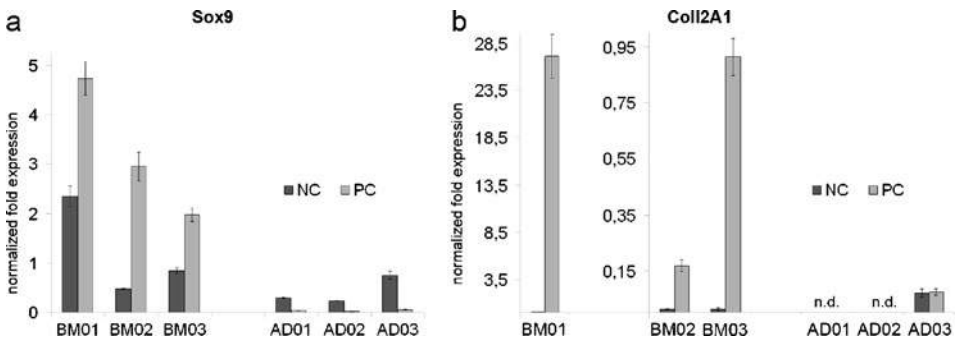

Fig. 4 Realtime RT-PCR after 10 day chondrogenic differentiation. **a** Relative mRNA expression of Sox9 for 3 different specimens of BM-MSCs (BM) and ADSCs (AD) following 10 day high density culture with (PC) or without (NC) chondrogenic induction media. **b** Relative mRNA expression of collagen 2A1 for the same specimens after 10 day cultivation. PC = positive control, NC = negative control, n.d. = not detected

Nevertheless, the outcome of these studies on canine osteoarthritis was promising as the treated dogs showed an improvement in lameness and pain. This fact made the detailed characterisation of these cells even more interesting.

Such characterisations of canine MSCs were conducted by Csaki et al. (2007) for the BM-MSCs and by Neupane et al. (2008) and Vieira et al. (2010) for ADSCs. Consistent to our study, these groups found spindle-shaped cells with high proliferative potential and multilineage differentiation abilities (Csaki et al. 2007; Vieira et al. 2010). However, so far no comparative study existed on canine MSCs of these different sources and additionally, different isolation and differentiation protocols made the outcome of the independent studies difficult to compare. Profound differences that were found in BM-MSCs and ADSCs of other mammals emphasized the importance of such a study in the dog (Lee et al. 2004; Izadpanah et al. 2006; Vidal et al. 2008).

In the presented study we confirmed the high morphological similarity of canine MSCs of the two different sources found by the mentioned authors and additionally substantiated this fact by the more objective investigation via flow cytometry. We are the first to report that canine BM-MSCs express the commonly used pluripotency marker Oct4 that was likewise found in ADSCs by Neupane et al. (2008) (Pan et al. 2002). CD90, a widely accepted surface marker on MSCs in humans, was expressed by over 90% of the cells of both origins at the same passages (Dominici et al. 2006). These findings are in accordance with those of Csaki et al. (2007) and Vieira et al. (2010) who also found the majority of isolated BM-MSCs and ADSCs CD90-positive already after a few passages. Moreover, we proved that both MSCs constitutively expressed early transcription factors of the classical three mesenchymal lineages: bone, fat and cartilage (Chawla et al. 1994; Lefebvre and de Crombrugghe 1998; Marie 2008). Such a constitutive expression of genes of early differentiation was similarly seen by other authors in different species and interpreted as another indicator for their high plasticity and multipotent differentiation ability (Zuk et al. 2002; Kamishina et al. 2006; Liu et al. 2007).

Beside the verification of basic stem cell features, we focused on properties relevant for a therapeutic application in small animal practice in our comparison. At first glance, ADSCs appear to be preferable because of their abundance and easy accessibility. Moreover, DMEM supplemented with 10% FBS Gold is sufficient for an adequate fast growth. In contrast, preliminary studies on BM-MSCs showed that it was necessary to culture them in amino acid-enriched αMEM medium and with higher serum concentration to achieve a leastwise comparable growth and to keep culturing intervals reasonably similar to ADSCs. This is a fact we already experienced culturing human and equine BM-MSC (Arnhold et al. 2006a, b; Arnhold et al. 2007). However, even under these improved culture conditions we were not able to achieve an equally fast growth in BM-MSCs. ADSCs doubled 1.6 times faster than BM-MSCs, even under lower serum concentrations. Considering these features, ADSCs could reduce cultivation costs by lower serum consumption and a shorter interval between cell harvest and clinical application. Similar differences in population doubling have been found in adipose- and bone marrow-derived MSCs of the horse and the human (Lee et al. 2004; Colleoni et al. 2009; Raabe et al. 2011).

If applied MSCs should moreover contribute to the repair of damaged tissue, their migration towards the site of injury is crucial for the progress in healing and recovery. Liang et al. (2007) therefore designed an in vitro assay that mimics the migration into a wound area and provides the possibility to compare different cell populations. In this assay BM-MSCs and ADSCs both revealed a fast migration into the artificial wound area and so seem equally suitable for the clinical application.

With joint disorders as the probably most reasonable field for a clinical application in small animal medicine, the comparison of chondrogenic differentiation was our main focus (Chen and Tuan 2008; Csaki et al. 2008; Nöth et al. 2008). Whereas the positive outcomes of directly injected MSCs might also be due to local mediators secreted by MSCs and conceivably only transient (Caplan and Dennis 2006; Lee et al. 2011), MSCs have shown to significantly contribute to the regeneration tissue when loaded on suitable biomaterials (Kuroda et al. 2007; Jung et al. 2009). The use of those MSC-seeded biomaterials could accelerate the wound closure and improve the healing process, particularly when used with possibly pre-differentiated MSCs. Chondrogenic differentiation can be induced in MSCs in a high density monolayer (Lin et al. 2005; Liu et al. 2007) or a pellet culture (Pittenger et al. 1999) by serum deprivation, ITS-supplementation, dexamethasone and TGFβ isoforms in DMEM. In this study, both cultivation forms were conducted to compare cells of both origins in terms of their chondrogenic morphology and their cartilage-specific gene expression. The morphological differences seen in the pellet cultures reflected the findings of other authors from systematic comparisons in humans (Im et al. 2005; Liu et al. 2007) and the horse (Vidal et al. 2008). Here, chondrogenic differentiation of ADSCs equally failed when conducted under the same conditions as for BM-MSCs, similarly leading to a fibroblastic cell morphology and inferior matrix production. TEM examination in our study moreover revealed the unstructured nature of extracellular matrix in canine ADSCs. In contrast, BM-MSCs chondrogenesis displayed a well organized matrix with highly active cells that reminded of mature articular cartilage (Weiss et al. 1968).

Downregulation of Sox9, the master transcription factor for chondrogenesis, additionally indicated that chondrogenesis in ADSCs was somehow misdirected under the applied conditions. As Sox9 directly regulates Coll2A1 gene expression this finding concomitantly explains the absent or

low expression of Coll2A1 in ADSCs which is also consistent with findings in human MSCs (Bell et al. 1997; Huang et al. 2005). Recently, Hennig et al. (2007) found the disability for chondrogenic differentiation in human ADSCs to be correlated with a primary lack of expression of TGFβ-receptor 1 (TGFβ-R1). Using a combination of BMP-6 and TGF-β3 they were able to induce TGFβ-R1 in ADSCs which resulted in an upregulation of Coll2A1 expression and proteoglycan production. In another study on human ADSCs using alginate bead cultures, BMP-6 alone was able to restore the chondrogenic differentiation ability in ADSCs when dexamethasone was omitted (Estes et al. 2006). Dexamethasone is known to enhance Sox9 expression in chondroblasts in a dose-dependent manner and is therefore routinely used in chondrogenic differentiation of BM-MSCs (Sekiya et al. 2001). However, Awad et al. (2003) proved it to be rather inhibitory in human ADSCs unveiling another possible explanation for the impaired chondrogenesis of canine ADSCs in our study. The fact that Neupane et al. (2008) were able to induce Coll2A1 expression in canine ADSCs using a modified differentiation medium devoid of dexamethasone strengthens this assumption.

Although we tried to minimize serum effects by using a fully defined FBS and conducted chondrogenic differentiation under absolutely comparable conditions, we were not able to eliminate the possibility that the initial culturing of BM-MSCs under higher serum concentrations might have influenced their gene expression. Therefore, a serum-free culturing method would be useful which was so far not feasible in canine MSCs. However, the fact that Liu et al. (2007), who cultured human MSCs under the very same conditions as in our study, only detected minor differences in the gene expression of ADSCs and BM-MSCs indicates that this serum-based influence might not be that crucial. Considering the different tissue environments the cells derive from, they might be even more influenced by local, tissue-specific mediators and cytokines. Further microarray studies could help to specify such differences.

As we worked with clinical specimens we also cannot exclude individual differences as seen in other species (Phinney et al. 1999). Especially the individual differences seen in qPCR might be donor-dependent. But as the tendencies in gene expression as well as the results from the basic stem cell features were homogeneous within each group and in concert with those found in other mammals, MSCs of each origin obviously share common properties that are independent from the donor. A fact substantiated by a patient-matched study in the human that led to similar results (Huang et al. 2005).

Taken together, our study revealed both, similarities and differences between canine BM-MSCs and ADSCs. Further examinations should follow to distinguish the nature and cause of these differences before considering canine MSCs for a clinical application. ADSCs with their abundance and easy accessibility are certainly preferable for an approach in small animal practice and it therefore might be worth to figure out how their differentiation potential can be restored.

Conflict of interest None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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