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Original Article

The lower in vitro chondrogenic potential of canine adipose tissue-derived mesenchymal stromal cells (MSC) compared to bone marrow-derived MSC is not improved by BMP-2 or BMP-6

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ABSTRACT

Mesenchymal stromal cells (MSC) are used for cell-based treatment for canine osteoarthritis (OA). Compared with human MSCs, detailed information on the functional characterisation of canine MSCs is limited. In particular, the chondrogenic differentiation of canine adipose tissue-derived MSCs (cAT-MSCs) is challenging. In this study, we aimed to compare cAT-MSCs with bone marrow-derived MSCs (cBM-MSCs), focusing specifically on their in vitro chondrogenic potential, with or without bone morphogenetic proteins (BMP). cBM-MSCs and cAT-MSCs were characterised using flow cytometry and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The chondrogenic differentiation potential of all cMSC preparations in the presence of TGF-β1 alone or when supplemented with 10, 100, or 250 ng/mL BMP-2 or BMP-6 was investigated using RT-qPCR, and biochemical, histochemical and immunohistological analyses.

Both cBM-MSCs and cAT-MSCs expressed the surface markers CD90, CD73, and CD29, and were negative for CD45 and CD34, although the expression of CD73 and CD271 varied with donor and tissue origin. Interestingly, expression of *ACAN* and *SOX9* was higher in cBM-MSCs than cAT-MSCs. In contrast with cBM-MSCs, cAT-MSCs could not differentiate toward the chondrogenic lineage without BMP-2/-6, and their in vitro chondrogenesis was inferior to cBM-MSCs with BMP-2/-6. Thus, cAT-MSCs have lower in vitro chondrogenic capacity than cBM-MSC under the studied culture conditions with 10, 100, or 250 ng/mL BMP-2 or BMP-6. Therefore, further characterisation is necessary to explore the potential of cAT-MSCs for cell-based OA treatments.

Introduction

Osteoarthritis (OA) is a common progressive joint disease, characterised by cartilage degradation, subchondral bone changes, and synovial inflammation. OA affects 2.5%–20% of the canine population, which increases to 80% in dogs aged >8 years (Anderson et al., 2018). Mesenchymal stromal cells (MSCs) are promising candidates for cell-based OA treatments because of their immunomodulatory properties and chondrogenic capacity (Whitworth and Banks, 2014; Im, 2018). Human MSCs isolated from the bone marrow (hBM-MSCs) possess in vitro chondrogenic potential (Johnstone et al., 1998; Pelttari et al., 2008). Compared to hBM-MSCs, human MSCs isolated from the adipose tissue (hAT-MSC) possess several advantages, including ease of isolation with minimal morbidity, relatively higher MSC concentration (Oedayrajsingh-Varma et al., 2006), higher proliferation rate (Schäffler and Büchler, 2007), and stronger immunosuppressive capabilities (Mattar and Bieback, 2015). While hAT-MSCs can differentiate toward multiple lineages in vitro and in vivo, their osteogenic and chondrogenic potential are inferior to those of hBM-MSCs (Strioga et al., 2012).

Canine MSCs (cMSCs) have been less extensively characterised than hMSCs (de Bakker et al., 2013). Particularly, the chondrogenic differentiation of cAT-MSCs is challenging; chondrogenesis in earlier studies was either unsuccessful (Russell et al., 2016), inferior to that of cBM-MSCs (Reich et al., 2012; Bearden et al., 2017), or lacked robust evidence (Robey, 2017).

Therefore, we aimed to enhance the chondrogenic capacity of cAT-MSCs to the level exhibited by cBM-MSCs by supplementing different doses of bone morphogenetic protein (BMP)-2 or BMP-6. Both BMPs and transforming growth factor (TGF)-β1 are known to synergistically affect chondrogenesis (Sekiya et al., 2005).

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Furthermore, BMP-6 may specifically induce TGFβ receptor 1 expression, which is otherwise downregulated in hAT-MSCs compared to that in hBM-MSCs, thereby recovering the effect of TGF-β1 on hAT-MSC chondrogenesis (Hennig et al., 2007). Additionally, (surface) marker expression levels in cAT-MSCs and cBM-MSCs were compared for obtaining more insights regarding differences in cell population.

Materials and methods

Animal samples

Bone marrow and adipose tissue samples were collected from healthy dogs (n = 14) after euthanasia for unrelated experiments (Appendix A: Supplementary material). The protocol was approved by the Ethics Committee on Animal Experimentation of Utrecht University (2012.III.07.068, 2013.III.08.254, 2013.III.08.054).

Isolation of cMSCs from the bone marrow and adipose tissue

cBM-MSCs and cAT-MSCs were isolated and expanded as per methods described previously (Tryfonidou et al., 2014; Malagola et al., 2016). Briefly, after isolation, the cells were plated (cBM-MSCs; 1.3–2 $\times$ 10^6 cells/cm^2, cAT-MSCs: 4 $\times$ 10^4 cells/cm^2) in MSC-expansion medium (α-MEM, Invitrogen) containing 10% foetal bovine serum (FBS; Gibco, high performance), 1% penicillin/streptomycin (P/S; GE Healthcare Life Sciences), 0.1 mM ascorbic acid 2-phosphate (Sigma), and 1 ng/mL basic fibroblast growth factor (bFGF; AbD Serotec) until confluency was reached and the cells were cryopreserved until further use. Detailed description is provided in Appendix A: Supplementary material.

Colony-forming unit-fibroblast (CFU-F) assay

Cryopreserved cells were thawed on ice and plated in expansion medium at a density of 17 cells/cm^2 (corresponding to approximately 1000 cells/Petri dish (CellStar, 664160, Greiner Bio-One). After 10–14 days, the cells were fixed with methanol and stained with Crystal Violet (0.5% in methanol (100%), Sigma) for 15 min at room temperature (RT). After rinsing, the colonies (consisting of >50 cells) were counted.

FACS analysis

Seven cryopreserved donor-matched cAT-MSCs and cBM-MSCs (2 $\times$ 10^6 cells/donor) at passage 2 were analysed. The cells were thawed on ice, washed in Hanks' buffered saline solution (HBBS) and resuspended in stain buffer (FSB, BD Pharmingen) at a density of 0.5–1 $\times$ 10^5 cells per reaction. Subsequently, the cells were incubated on ice in the dark for at least 15 min with canine-specific phycoerythrin (PE), fluorescein isothiocyanate (FITC), or isotype control antibodies (Table 1). After washing, the cells were stained with 7-aminoactinomycin D (7AAD, 0.25 mg/test; BD Biosciences) to differentiate between dead and live cells. Data were collected using the FACS Diva software (v8.0) on a CANTO II (BD Biosciences) and analysed using the FlowJo software (v10.0).

Chondrogenic differentiation

The protocol used for chondrogenic differentiation of the cMSCs was adapted from Johnstone et al. (1998) and was first performed using TGF-β1 (10 ng/mL, R and D Systems) and BMP-6 (10 ng/mL, Peprotech; Hennig et al., 2007) in chondrogenic differentiation medium (Dulbecco's modified Eagle medium [DMEM] high glucose, Invitrogen) containing 1% P/S, 1% ITS + premix (BD), 0.04 mg/mL proline (Sigma), 0.1 mM ascorbic acid 2-phosphate, and 10^{-7} M dexamethasone (Sigma; Appendix A: Supplementary material). Pellets were collected for reverse transcription-quantitative polymerase chain reaction (RT-qPCR), glycosaminoglycan (GAG), and DNA analyses, and immunohistochemical evaluation after 35 days.

As this did not result in chondrogenic differentiation of the cAT-MSCs, follow-up experiments were performed with higher concentrations of BMP-6 and BMP-2 (10, 100, and 250 ng/mL). RT-qPCR was performed after 7 days to detect early transcriptional changes that precede the changes at the protein level. Additionally, culture period of 21 days was used based on methods reported by Mackay et al. (1998; Appendix A: Supplementary material).

GAG and DNA content

Three pellets per donor were pooled for each condition and digested overnight at 60 °C using 600 μL papain digestion solution (11.9 μL/mL papain [Sigma] and 15.7 μg/mL cysteine HCl [Sigma] in papain buffer [13 mg/mL H_2NaPO_4 .2 H_2O and 3.26 mg/mL EDTA, pH 6]). GAG content was determined using dimethyl methylene blue (DMMB, Sigma) assay as per protocols described previously (Bach et al., 2015). DNA content was measured according to manufacturer's instructions with the Qubit™ dsDNA HS assay (ThermoFisher Scientific).

Immunohistochemical evaluation

Each pellet was fixed in 4% neutral buffered formalin (Klinipath B.V.) with 0.1% eosin (Boom BV Memmel) and embedded in 2.4% alginate and paraffin. Five-micrometre sections were stained for Safranin O/Fast Green staining (0.125% Safranin O, Sigma; 0.4% Fast Green, Sigma) and collagen type II (COL-II) and I (COL-I) immunohistochemistry as per methods described previously (Bach et al., 2015) using COL-I mouse monoclonal antibody (0.07 μg/mL, Abcam, ab6308) and COL-II mouse monoclonal antibody (0.02 μg/mL, DSSHB, II-II6B3), respectively. Normal

Table 1
The antibodies used for FACS analysis.

Target	Host	Reactivity	Clone	Fluorochrome	Manufacturer	Catalog number
CD90	Rat	Dog	YKIX337.217	PE	eBioscience	12-5900-42
CD73	Rabbit	Human, Mouse, Rat, Dog, Chicken	Unknown	FITC	Bioss antibodies	bs-4834R
CD29	Mouse	Human, Cow, Dog	TS2/16	PE	BioLegend	303004
CD271	Mouse	Dog, Human, Mouse	ME20.4	PE	Invitrogen	12-9400-42
CD146	Mouse	Dog, Human, Mouse, Rabbit	P1H12	FITC	Invitrogen	11-1469-42
CD45	Rat	Dog	Unknown	PE	LSBio	LS-C127720
CD34	Mouse	Dog	IH6	PE	BD Pharmingen	559369
Rat IgGK	Rat	Isotype control	eB149/10H5	PE	eBioscience	12-4031-82
Rabbit IgGk	Rabbit	Isotype control	Unknown	PE	Antibodies online	ABIN376422
Mouse IgGk	Mouse	Isotype control	MOPC-21	PE	BioLegend	400112

IgGk, immunoglobulin kappa; PE, phycoerythrin; FITC, fluorescein isothiocyanate.

mouse isotype IgG (IgG_1 (R and D, HAF007) was used as control. Sections were stained for 5 min with Toluidine Blue O (0.04%, Sigma; Basic Blue 17, 86% dye, dissolved in 0.2 M acetate buffer).

RT-qPCR analysis

The surface marker and chondrogenic gene expression profiles were investigated in expansion passage 2 of five matched, undifferentiated cAT-MSC and cBM-MSC donors (0.1×10^6 cells/donor); the bone marrow was used as the control. The chondrogenic gene expression of the differentiated cMSCs was determined in three pooled pellets per donor for each condition. The pellets were snap-frozen and crushed with a pestle (Argos technologies Inc, 9951-901). Total RNA was isolated using the RNeasy minikit (Qiagen, 74134) or microkit (Qiagen, 74004), which included an on-column DNase step. RNA was quantified using the NanoDrop ND-1000 spectophotometer (Isogen Life Science). cDNA was synthesised using the iScript™ cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions.

RT-qPCR was performed using the BioRad CFX-384 cycler and IQ SYBRGreen supermix (Bio-Rad) and surface marker- and chondrogenic lineage-specific primers (Table 2). Relative expression was estimated using the efficiency-corrected delta-delta Ct ($\Delta\Delta$Ct) method and six reference genes; *HNRPH, RPL8*, *GUSB*, *SRPR*, *YWHAZ*, and *sDHA* (Table 2).

Statistical analysis

Detailed description of the statistical analyses using R software (version 3.0.2) is provided in Appendix A: Supplementary material. In short, linear mixed models were used for normally distributed data and the Kruskal-Wallis rank sum test for data that were not distributed normally. Differences were considered significant if P values were <0.05 after multiple comparison correction. Additionally, the effect size (ES) was calculated using Cohen's d (Cohen, 1977). Effect size of >0.8 was considered to be large, and >2.0 was considered to be huge (Cohen, 1977; Sawilowsky, 2009).

Results

cBM-MSCs and cAT-MSCs were plastic-adherent and form colonies

All donors demonstrated the typical fibroblast-like phenotype (Fig. 1). Approximately 80% confluency was reached within 5 days for cAT-MSCs (range; 3–7 days) and within 8 days for cBM-MSCs (range; 7–9 days). On average, cAT-MSCs and cBM-MSCs produced

Table 2
Primers used in RT-qPCR.

Marker	Gene	Primer Sequence	Amplicon Size (bp)	Annealing temperature (°C)	NCBI accession no.
Surface marker expression	*CD90*	F: CAGCATGACCCGGGAGAAAAAG R:TGGTGGTGAAGCCGGATAAGTAGA	134	63.5	NM_001287129
	CD146	F: GGGAATGCTGAAGGAAGG R: CTTGGTGCTGAGGTTCTG	99	63	XM_022418207
	CD166	F: AAGCGTCATAAACCAAACAG R: TATAGCAGAGACATTCAAGGAG	150	61	NM_001313804
	CD73	F: CTCCAACACATTCCTTTACAC R: ACTCAACCTTCAAATAGCCT	150	61	XM_532221
	CD105	F: CATCCTTCACCACCAAGAG R: CAGATTGCAGAAGGACGG	139	60	XM_005625330
	CD44	F: CTTCTGCAGATCCGAACACA R: GAGTAGAAGCCGTTGGATGG	147	60	NM_001197022
	CD14	F: CCCGGCGCTCACCACCTTAGAC R: CCTGGAGGGCCGGGAACTTTTG	98	60	XM_843653
	CD45	F: GACCATGGGGTGCCTGAAGAT R: CACAATGGGGCCACTGAAGAAG	90	58–64	XM_005622282
	CD31	F: GTTCTGCGTGTCAAGGTG R: TGTCCTTCCCAAACTCCA	85	65	XM_022422841
	CD34	F: TCAGGGCCCCCGACATCTC R: TCTCTGCTCACCCCTCTGGAAAAA	115	66	NM_001003341
Chondrogenesis	*ACAN*	F: GGACACTCCTTGCAATTTGAG R: GTCATTCCACTCTCCCTTCTC	110	61–62	NM_001113455
	COL2A1	F: GCAGCAAGAGCAAGGAC R: TTCTGAGAGCCCTCGGT	150	60.5–65	NM_001006951
	SOX9	F: CGCTCGCAGTACGACTACAC R: GGGGTTCATGTAGGTGAAGG	105	62–63	NM_001002978
	COL1A1	F: GTGTGTACAGAACGGCCTCA R: TCGCAAATCACGTCATCG	109	61	NM_001003090
	COL10A1	F: CCAACACCAAGACACAG R: CAGGAATACCTTGCTCTC	80	61	XM_849417
	TGFβR1	F:CAGTCACCGAGACCACAGACAAAGT R:TGAAGATGGTGCACAAACAAATGG	100	54	XM_014117881
House keeping genes	*HNRPH*	F: CTCACTATGATCCACCACG R: TAGCCTCCATAACCTCCAC	151	61,2	XM_538576
	RPL8	F: CCATGAATCCTGTGGAGC R: GTAGAGGGTTTGCCGATG	64	55	XM_532360
	GUSB	F: AGACGCTTCCAAGTACCCC R: AGGTGTGGTGTAGAGGAGCAC	103	62	NM_001003191
	SRPR	F: GCTTCAGGATCTGGACTGC R: GTTCCCTTGGTAGCACTGG	81	61,2	XM_546411
	YWHAZ	F: CGAAGTTGCTGCTGGTGA R: TTGCATTTCCTTTTTGCTGA	94	58	XM_843951
	sDHA	F: GCCTTGGATCTCTTGATGGA R: TTCTTGGCTCTTATGCGATG	92	61	DQ402985

F, Forward; R, Reverse; bp, base pair; no, number.

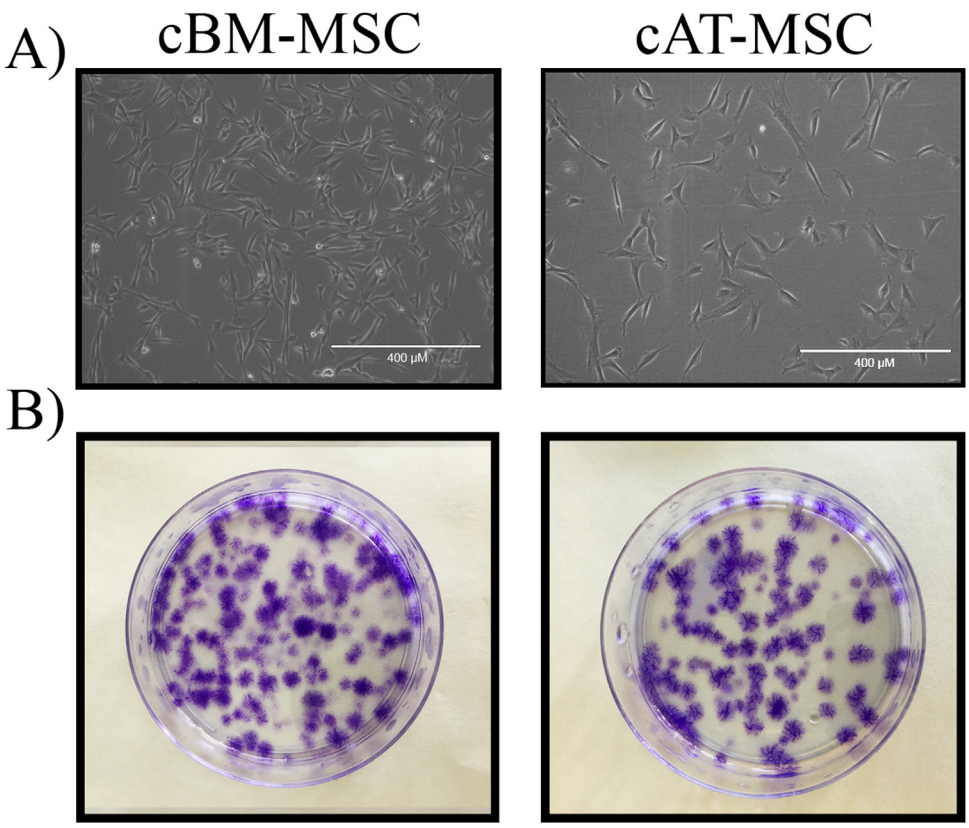

Fig. 1. MSC morphology and CFU-F assays. (a) Bright light phase contrast images of canine bone marrow (BM)- and adipose tissue (AT)-derived mesenchymal stromal cells (MSC) demonstrate a typical fibroblast-like phenotype. Scale bars, 400 µM (b) Representative images of the colony-forming-units (CFU) assay after staining with Crystal Violet; cAT-MSCs, 101 ± 32 colonies; cBM-MSCs, 111 ± 49 colonies per 1000 plated cells.

101 ± 32 and 111 ± 49 colonies per 1000 plated cells, respectively (no significant difference).

Passage 2 cAT-MSCs had low CD73, ACAN, and SOX9 expression levels

The expression profile, investigated using FACS analysis, was similar for passage 2 cBM-MSCs and cAT-MSCs, with high frequencies of $CD90^+$ and $CD29^+$ cells and negligible number of $CD271^+$, $CD146^+$, $CD34^+$, and $CD45^+$ cells (Fig. 2a). The number of $CD271^+$ cells were significantly lower in cAT-MSCs than that in cBM-MSCs ($P < 0.001$). Low but variable frequencies of $CD73^+$ cells were observed (cBM-MSC: 17.7% ± 18.7%; cAT-MSC: 14.7% ± 8.8%).

RT-qPCR was performed to investigate the expression of surface markers, against which canine-specific FACS antibodies were not available. High *CD90*, *CD105*, and *CD166* expression and low *CD146* expression were observed in both cell types (Fig. 2b). *CD44* expression was low in cBM-MSCs and was undetectable in cAT-MSCs ($P = 0.26$). *CD73* expression was significantly higher in cBM-MSC than that in cAT-MSC ($P = 0.0088$). *CD34*, *CD45*, *CD14*, and *CD31* were undetectable in both cell types. In undifferentiated cBM-MSCs, the expression levels of *SOX9* ($P = 0.02$) and *ACAN* ($P = 0.055$) were higher than those in cAT-MSC (Fig. 2c). *COL2A1* and *COL1A1* were similarly expressed in both cell types.

cAT-MSCs did not undergo chondrogenesis without high concentrations of BMP-2 or BMP-6

Differentiation with TGF-β1 and 10 ng/mL BMP-6 assessed at day 35

After addition of TGF-β1, DNA content increased in both cell types ($P = 0.00015$) compared to that in the control group, without additional effect of BMP-6 (Fig. 3a). This was also reflected in the increase in pellet size after TGF-β1 addition (Fig. 3c). The increase in GAG deposition, observed only in cBM-MSCs (Fig. 3a), was confirmed by increase in *ACAN* expression ($P = 0.007$, Fig. 3b) and positivity to Safranin O and Toluidine Blue staining (Fig. 3c). Deposition of COL-II by cBM-MSCs was confirmed using immunohistochemistry and was in agreement with the increase in *COL2A1* expression (Fig. 3b and 3c). *COL1A1* expression was significantly upregulated in cBM-MSCs and cAT-MSCs upon addition of TGF-β1 alone ($P = 0.00003$) or with BMP-6 ($P = 0.00003$). Further, all cBM-MSC and cAT-MSC donors expressed COL-I. Expression of TGFβR1 was significantly lower in cAT-MSCs than that in cBM-MSCs ($P = 0.0008$), and its expression did not increase with TGF-β1 or BMP-6 treatment. *COL10A1* expression was not detected, irrespective of the condition and donor.

Differentiation with TGF-β1 and different concentrations of BMP-2 and BMP-6

RT-qPCR was performed after 7 days to assess early transcriptional changes (Fig. 4a). A BMP dose-dependent effect was observed in cBM-MSCs, with significant increase in *ACAN* expression upon addition of 100 or 250 ng/mL BMP-6 ($P = 0.014$ and 0.049, respectively), and 100 or 250 ng/mL BMP-2 ($P = 0.0017$ and 0.0059, respectively), compared to that in the control. Additionally, compared to that in the control, *COL2A1* expression significantly increased with 100 and 250 ng/mL BMP-2 ($P = 0.046$ and 0.042, respectively), and 250 ng/mL BMP-6 ($P = 0.049$). In cAT-MSCs, a moderate dose-dependent increase was observed only for *SOX9*, *ACAN*, and *COL1A1* after stimulation with BMP-2 or BMP-6. *TGFBR1* expression was significantly lower in cBM-MSCs treated with 100 or 250 ng/mL BMP-6 ($P < 0.001$) than in those treated with TGF-β1 alone. This downregulation was also significant for cAT-MSCs in the presence of 250 ng/mL of BMP-6 ($P = 0.02$). Expression of *TGFBR1* in cAT-MSCs was significantly lower than

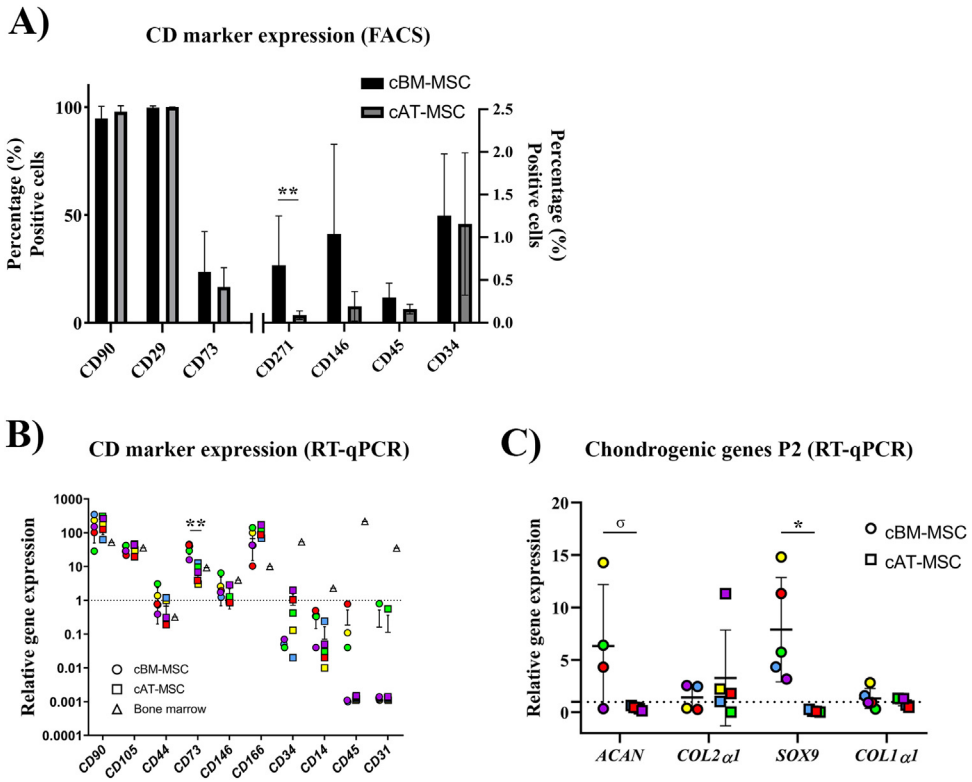

Fig. 2. Surface and chondrogenic marker expression in canine bone marrow (BM)- and adipose tissue (AT)-derived mesenchymal stromal cells (MSC) in passage 2. (a) Mean ± standard deviation (SD) of the percentage of cells positive for CD markers in cBM-MSCs (black bars) and cAT-MSCs (grey bars), assessed using FACS analysis. (b) Mean ± SD of the normalised relative expression of CD markers in the bone marrow (triangle), cBM-MSCs (circles), and cAT-MSCs (squares) compared to the mean gene expression (dotted line). (c) Mean ± SD of the normalised relative expression of *ACAN*, *COL2A1*, *SOX9*, and *COL1A1* of cBM-MSCs (circles) and cAT-MSCs (squares) compared to the mean gene expression (dotted line). Individual donors are shown in different colours. *, Significant difference between cell types within a gene or marker (* $P < 0.05$; ** $P < 0.01$). σ, Difference between the two cell types with $P < 0.1$ and an effect size > 0.8.

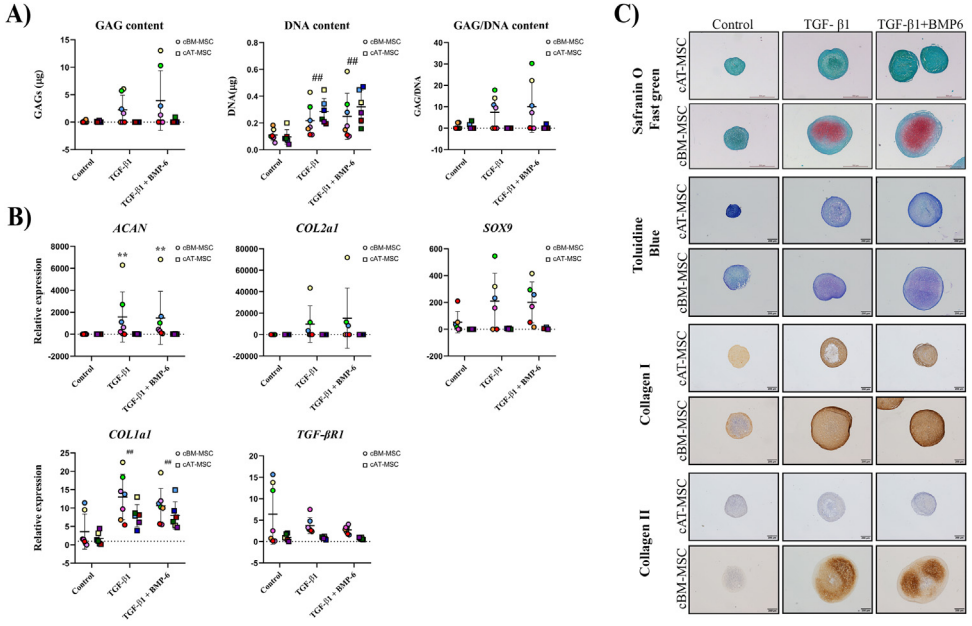

Fig. 3. Biochemical, histological, and RT-qPCR analyses of canine bone marrow (cBM, circles)- and canine adipose tissue (cAT, squares)-derived mesenchymal stromal cells (MSC), differentiated towards the chondrogenic lineage for 35 days in pellet culture with or without 10 ng/mL TGF-β1 and 10 ng/mL BMP-6. (a) Biochemical analysis of the glycosaminoglycan (GAG) content, DNA content, and GAG content corrected for DNA content (mean ± standard deviation (SD)). (b) Mean ± SD of the normalised relative expression of *ACAN*, *COL2A1*, *SOX9*, *COL1A1*, and *TGFBR1* compared to the mean expression of the control samples of both cell types (dotted line). Individual donors are shown in different colours. ##, Significant difference in both cell types in this condition vs. the control group ($P < 0.01$); **, significant difference in the cBM-MSC group compared to that in the cBM-MSC control group and the corresponding cAT-MSC group; $P < 0.01$. (c) Histological analysis of deposited glycosaminoglycans (GAG), using Safranin O/Fast Green and Toluidine Blue, and immunohistochemical analysis of collagen type I and collagen type II. Scale bars, 200 µm.

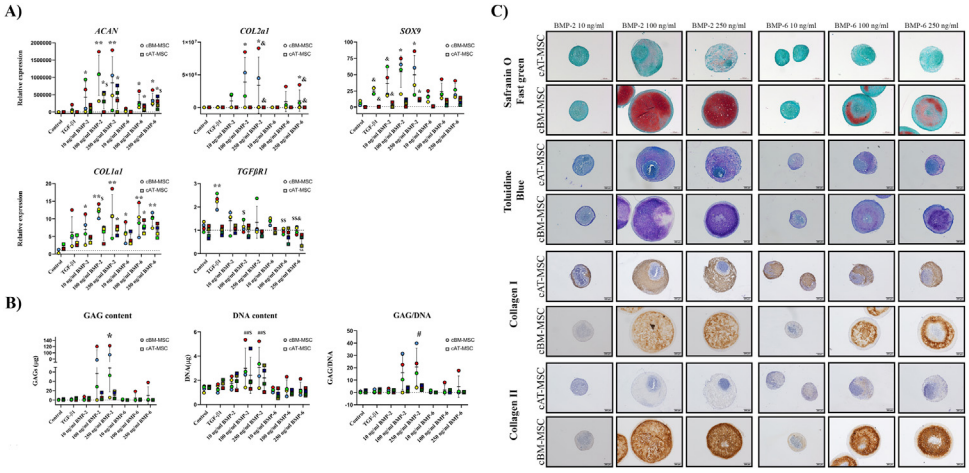

Fig. 4. Biochemical, histological, and RT-qPCR analyses of the chondrogenic differentiation with or without different doses of bone morphogenetic protein (BMP)-2 or BMP-6 of canine bone marrow (cBM, circles)- and canine adipose tissue (cAT, squares)-derived mesenchymal stromal cells (MSC). (a) Mean ± standard deviation (SD) of the normalised relative expression of *ACAN*, *COL2A1*, *SOX9*, *COL1A1*, and *TGFBR1* of cBM-MSCs and cAT-MSCs differentiated towards the chondrogenic lineage for 7 days in pellet culture with or without TGF-β1 and 10, 100, or 250 ng/mL BMP-2 or BMP-6. Gene expression has been presented as the expression compared to the mean gene expression of the control samples of both cell types (dotted line). Individual donors are shown in different colours. #, significant difference between all cell types in this condition and the control group (#, $P < 0.05$; ##, $P < 0.01$); *, significant difference vs. the control group, but only in this cell type (*, $P < 0.05$; **, $P < 0.01$); $, significant difference vs. the group treated with only TGF-β1 for cBM-MSCs (*COL1a1*, *TGFβR1*), cAT-MSCs (*ACAN*), or independent of cell type (DNA content; $P < 0.05$); &, significant difference between cell types within the same condition ($P < 0.05$). (b) Biochemical analysis of the glycosaminoglycan (GAG) content, DNA content, and GAG content corrected for DNA content (mean ± SD) of cBM-MSC and cAT-MSC, differentiated toward the chondrogenic lineage for 21 days in pellet culture with or without TGF-β1 and 10, 100, or 250 ng/mL BMP-2 or BMP-6. (c) Histological analysis of glycosaminoglycans (GAG), using Safranin O/Fast Green and Toluidine Blue, and immunohistochemical analysis of collagen I and collagen II.

that in cBM-MSCs in the presence of 250 ng/mL BMP-6 ($P = 0.049$). *COL10A1* expression was not detected under any condition or with any donor.

After 21 days, GAG content and GAG/DNA increased significantly only after addition of 250 ng/mL BMP-2 to cBM-MSCs ($P < 0.01$) and cAT-MSCs ($P = 0.07$; Fig. 4b). BMP-2 (100 ng/mL) induced chondrogenesis only in a subset of the cBM-MSC and cAT-MSC donors. GAG production and deposition was not observed after the addition of TGF-β1 or 10 ng/mL BMP-2/-6. Upon supplementation with 100 or 250 ng/mL BMP-6, chondrogenesis was limited to only one cBM-MSC donor. Irrespective of the growth factor supplemented, cBM-MSCs deposited more GAGs than cAT-MSCs. DNA content significantly increased upon addition of 100 and 250 ng/mL BMP-2 ($P < 0.01$), demonstrated by the increase in pellet size (Fig. 4c). Positive Safranin O, Toluidine Blue, and COL-II staining was observed in cBM-MSC pellets from donors that demonstrated GAG deposition at the biochemical level (Fig. 4c), while COL-II was absent in all cAT-MSCs (Fig. 4c). COL-I was deposited in pellets of all donors after growth factor stimulation (Fig. 4c).

Discussion

AT-MSCs are commonly used in dogs for treating OA (Hoffman and Dow, 2016; Gugjoo et al., 2019) owing to their immunemodulatory and regenerative abilities. However, their chondrogenic potential remains unclear. Here, we further characterised and compared the surface marker expression and chondrogenic potential of cAT-MSCs with those of cBM-MSCs, a reference standard.

A donor-matched comparison of MSC-related surface markers demonstrated that the expression patterns of cBM-MSCs and cAT-MSCs were similar at passage 2. Differential expression was observed for *CD73* and *CD271*, both poorly expressed in cAT-MSCs. CD73 positivity in hAT-MSCs has been associated with high chondrogenic capacity and low osteogenic capacity (Rada et al., 2011). CD271 is considered a highly selective surface marker for hBM-MSCs; $CD271^+$ cells are considered to possess higher chondrogenic potential (Lv et al., 2014; Somoza et al., 2014; Lu et al., 2020). The overall $CD271^+$ frequency was lower in both cell types than the frequencies reported in humans (Lv et al., 2014). The cAT-MSC population contains a relatively lower fraction of cells with chondrogenic potential, which possibly contributed to the poor chondrogenesis observed in this study. However, CD marker expression varies between reports and species (Uder et al., 2018); more importantly, marker profiles change during passaging (Bara et al., 2014), thereby rendering their predictive capacity of chondrogenesis debatable. For example, CD34 expression is highly affected by cell culture, which, while found to be high in cAT-MSCs immediately after isolation, disappears after culture (Lin et al., 2012). Furthermore, although CD34 expression has been reported in cAT-MSCs (Russell et al., 2016; Kriston-Pál et al., 2017), our study and studies reported by others (Ivanovska et al., 2017) have not been able to detect it. Interpretation of the CD profiles was further complicated by the previous finding that FACS-sorted subpopulations of MSCs had similar CD marker expression profiles after culturing, while retaining their functional differences (Sivasubramaniyan et al., 2018). In this complicated landscape, surface marker characterisation in the veterinary field is further challenged by the lack of species-specific antibodies. Although RT-qPCR analysis of CD markers can be used, discrepancies between mRNA and protein expression should be considered. For example, the canine MSCs were reportedly positive for CD44, while the gene expression level of this CD marker was low. Altogether, this implies that the applicability of the markers proposed by the International Society for Cellular Therapy (ISCT; Dominici et al., 2006) in dogs remains to be determined. Although CD marker expression was largely similar, higher expression of ACAN and SOX9 in passage 2 undifferentiated cBM-MSCs was detected. We speculated that these differences in gene expression were observed because cBM-MSCs were more primed toward chondrogenesis than cAT-MSCs, which was influenced by the tissue origin (Rasi Ghaemi et al., 2013). However, further investigation regarding the chondrogenic potential of specific subpopulations within cBM-MSCs or cAT-MSCs is warranted.

Ideally, upon chondrogenic differentiation, cMSCs should primarily produce COL-II rather than COL-I. Our results

demonstrated that cBM-MSCs produced both collagens, with COL-II being primarily produced under effective chondrogenic conditions, which was in agreement with the observations reported by other studies (Hodgkiss-Geere et al., 2012), while cAT-MSCs only deposited COL-I under the same culture conditions. Contrarily, COL-II deposition in cAT-MSC pellets has been demonstrated by others (Neupane et al., 2008; Vieira et al., 2010). However, a few studies that directly compared cBM- and cAT-MSCs reported that cAT-MSCs expressed less *COL2A1* (Reich et al., 2012) and exhibited limited COL-II and Toluidine Blue staining (Bearden et al., 2017) without BMP supplementation. COL-I deposition by both cMSCs is not remarkable, as TGF-β1 is known to exert fibrotic effects (Cutroneo, 2007). In the absence of sufficient chondrogenic stimuli, cMSCs produce a more fibrous instead of hyaline-based cartilaginous matrix, explaining the increase in pellet size in all TGF-β1-stimulated pellets. Furthermore, within the course of chondrogenic differentiation, hMSCs follow the endochondral ossification pathway, thereby expressing markers of hypertrophy (COL-X, ALP, and MMP13; Pelttari et al., 2008). In this study, COL-X was undetectable at the gene and protein levels. Whether cMSCs eventually also undergo hypertrophic differentiation when cultured for extended periods of time or whether additional stimuli are necessary remains to be determined.

The chondrogenic potential of human AT-MSCs may depend on growth factors other than TGF-β1 (Hennig et al., 2007). However, cAT-MSCs did not undergo chondrogenesis with the addition of 10 ng/mL BMP-6, nor was there COL-II deposition with higher doses of BMP-2/-6. Additionally, TGFβR1, which was reported to restore the chondrogenic potential of hAT-MSCs, was significantly downregulated in cAT-MSCs supplemented with high doses of BMP. This downregulation is probably the result of negative feedback loops initiated by the high BMP concentration that act at the receptor level (Yan et al., 2018). The discrepancy in outcome between this study and other studies might be due to culture protocol and/or species differences (Martínez-Lorenzo et al., 2009). In contrast to sheep or human chondrocytes, canine chondrocytes lose their ability to re-differentiate under chondrogenic induction after few passages (Giannoni et al., 2005). Alternative growth factors might therefore be required to restore the chondrogenic potential of cAT-MSCs (Boeuf and Richter, 2010). It is also possible that environmental factors arising during culture, such as mechanical stimulation, oxygen tension, and nutritional supplementation, may explain why the results of this study differ from those of other studies (Hennig et al., 2007; Neupane et al., 2008; Vieira et al., 2010), and their effect on cAT-MSCs should be further investigated.

Conclusions

This study demonstrated that the chondrogenic capacity of cAT-MSCs under standard culture conditions was inferior to that of cBM-MSCs, as demonstrated by the deposition of GAGs and COL-II, and that chondrogenic capacity could not be restored with different doses of BMP-2 or BMP-6. Further investigation of the stimuli necessary for the chondrogenic differentiation of cAT-MSCs is warranted before considering cAT-MSCs for cell-based treatment strategies for OA.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.tvjl.2020.105605.

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