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# Derivation, characterization, and in vitro cell regeneration of canine white adipose tissue-derived mesenchymal stem cells obtained from a mesenteric region

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## Abstract

**Background:** The study was conducted to assess the characterization, differentiation, and in vitro cell regeneration potential of canine mesenteric white adipose tissue-derived mesenchymal stem cells (AD-MSCs). The tissue was harvested through surgical incision and digested with collagenase to obtain a stromal vascular fraction. Mesenchymal stem cells isolated from the stromal vascular fraction were characterized through flow cytometry and reverse transcription-polymerase chain reaction. Assessment of cell viability, in vitro cell regeneration, and cell senescence were carried out through MTT assay, wound healing assay, and  $\beta$ -galactosidase assay, respectively. To ascertain the trilineage differentiation potential, MSCs were stained with alizarin red for osteocytes, alcian blue for chondrocytes, and oil o red for adipocytes. In addition, differentiated cells were characterized through a reverse transcription-polymerase chain reaction.

**Results:** We observed the elongated, spindle-shaped, and fibroblast-like appearance of cells after 72 h of initial culture. Flow cytometry results showed positive expression for CD44, CD90, and negative expression for CD45 surface markers. Population doubling time was found 18–24 h for up to the fourth passage and  $30 \pm 0.5$  h for the fifth passage. A wound-healing assay was used to determine cell migration rate which was found  $136.9 \pm 4.7 \mu\text{m/h}$ . We observed long-term in vitro cell proliferation resulted in MSC senescence. Furthermore, we also found that the isolated cells were capable of differentiating into osteogenic, chondrogenic, and adipogenic lineages.

**Conclusions:** Mesenteric white adipose tissue was found to be a potential source for isolation, characterization, and differentiation of MSCs. This study might be helpful for resolving the problems regarding the paucity of information concerning the basic biology of stem cells. The large-scale use of AD-MSCs might be a remedial measure in regenerative medicine.

**Keywords:** Canine mesenteric white adipose tissue-derived mesenchymal stem cells, Stromal vascular fraction, Cell regeneration, Wound healing assay, Trilineage differentiation

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## Background

Mesenchymal stem cells (MSCs) are the promising entities in cell-based research as gripping the distinctive property of differential plasticity as well as self-renewal capability. Canine adipose tissue, a specialized type of connective tissue derived from embryonic mesenchyme, is a foremost source of MSCs owing to its abundance ( $3.5 \times 10^4 \text{ g}^{-1}$ ) and easy accessibility plus negligible donor site demolition (Mester, Opincariu, Benedek, & Benedek, 2018; Özen, Gül Sancak, Ceylan, & Özgenç, 2016). MSCs are predominantly the adult stem cells with a significant tissue repairing proficiency characterized by their inclination for cell-to-cell contact and self-proliferation (Chen, Zhou, & Pan, 2014; Tan et al., 2019). It was reported that adipose tissue-derived MSCs possess the ability to differentiate into osteocytes, chondrocytes, myocytes, and reverse to adipocytes, depending on the specific cell culture conditions (Kim, 2014). As osteocytes and chondrocytes are the essential prerequisites for bone development and cartilage formation, their prolonged expansion may be utilized in such critical wound cases where the normal recovery process fails to rejuvenate and surgery is also too hard to be done (Kriston-Pál et al., 2017). Yaneselli et al. (2018) reported that adipose tissue-derived mesenchymal stem cells preserve multi-potentiality during a higher number of passages with possessing significantly greater osteogenic potential compared to other sources of canine tissue-derived stem cells. Therefore, canine AD-MSCs may bring a new hope in the field of regenerative medicine by representing the unique differential potentials.

The functional potential of MSCs is known to decline with age, a process called senescence. Distinguishing nature of the senescence phenotype of MSCs includes growth arrest, loss of dividing capability, enlarged or flattened morphology, extracellular matrix degeneration together with increased expression of senescence-associated  $\beta$ -galactosidase, and surface marker alteration (Van Deursen, 2014). For the in vitro studies, canine adipose tissue-derived MSCs can be maintained for a longer period with stable population doubling and lower rate of senescence (Brandalise et al., 2010). MSCs are quiescent, live precursors responsible for replenishing cell deficits in healing wounds through renewing, replacement, and regeneration processes (Chagastelles & Nardi, 2011). Responding to the local developmental cues, the regeneration process mediates functional recovery of tissue injury to maintain the normal structural continuity (Facchin et al., 2018). It is a complex cellular event where early signals stimulate the production of new cells to rebuild the lost structures and subsequent signaling regulates the regrowth and patterning of newly formed ones (King & Newmark, 2012). Researchers are interested in finding direct transplantation strategies by

availing those remarkable features of MSCs which might be prospective in advanced translational biology.

A very little evidence of MSC clinical application is available in the canine model due to insufficient information regarding canine stem cell basics. In this context, we have carried out some in vitro experiments to analyze some cytological attributes of canine AD-MSCs. Therefore, the main goals of our study involve isolation, culture, and maintenance of canine white adipose tissue-derived MSCs with the differentiation and confirmatory characterization of respective cell types by evaluating major gene expression. This is a foundational step for using stem cells in orthopedic therapy.

## Methods

### Isolation and culture of canine white adipose tissue

Total 5 donor dogs were selected for collection of adipose tissue based on the history of satisfactory health status, no recent medication, proper vaccination, and owners' harmony and informed consent were written. All the experiments were conducted after receiving the approval from *Committee for the purpose of control and supervision of experiments on animals (CPCSEA)*, Government of India. A small surgical incision (~ 4 cm) was made aseptically in dog's abdomen after injecting diazepam ( $0.5 \text{ mg kg}^{-1}$ ) intravenously. About 5 g of the white adipose tissue from the mesenteric region was collected in a falcon tube containing DPBS (Sigma, USA). The collected adipose tissue sample was washed with DPBS repeatedly to remove the blood cells and then minced finely (1–2 mm in size) with a surgical scalpel inside the biosafety cabinet. The sample was digested with collagenase (Sigma, USA). Three different concentrations of collagenase were used with varying incubation periods: (a)  $100 \mu\text{g ml}^{-1}$  overnight, (b)  $500 \mu\text{g ml}^{-1}$  for 60 min, and (c)  $250 \mu\text{g ml}^{-1}$  for 60 min. The whole process of digestion was carried out at  $37^\circ\text{C}$  temperature. The digested cells were filtered through a  $70\text{-}\mu\text{m}$  cell strainer (Corning, USA) and centrifuged at  $2000 \times g$  for 5 min. Cells were grown in T25 flasks in a humidified incubator at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ . Every 72-h interval, cells were refeed with fresh DMEM-LG medium containing 20% FBS (Thermo Fisher Scientific, USA, Cat no: 16000044). Microscopic observations were done at every 24-h interval to ensure cell confluency.

### Characterization of MSCs

#### Immunophenotyping

Flow cytometry analysis was done to evaluate the expression of stem cell surface marker proteins. CD44 and CD90 were selected as positive markers and CD45 as a negative marker. After a second passage, cells were trypsinized, centrifuged, and resuspended in DPBS at a concentration of about  $5 \times 10^5$  cells/tube for each test. Cells

were incubated with FITC or PE-conjugated antibodies against CD44 (11-5440-42, eBioscience, USA.), CD90 (12-5900-41, eBiosciences, USA), and CD45 (11-5450-41, eBioscience, USA) for 45 min at room temperature in the dark. After washing, cells were resuspended in fresh DPBS and ten thousand events were acquired using BD FACSCalibur flow cytometer (BD Biosciences, USA). In all experiments, negative controls (that were stained without antibodies) and isotype controls (rat IgG1 K isotype control FITC for CD 44, 11-4321-80; eBioscience, USA, rat IgG 2b K isotype control PE for CD 90, 12-4031-82; eBioscience, USA and rat IgG 2b K isotype control FITC for CD45, 11-4031-82; eBioscience, USA) were kept for references. BD CellQuest Pro software was used for flow cytometric data analysis.

#### RT-PCR

The total RNA extraction was done from well-grown cells using TRIzol reagent (Ambion, USA) following the manufacturer's guidelines. Then, 2 µg of RNA was subjected to cDNA synthesis using RevertAid reverse transcriptase (Thermo Fisher Scientific, USA) in a thermocycler (HiMedia Laboratories, India) as per the manufacturer's protocol. Twenty microliters of PCR reaction mixture was prepared with 2 µl of cDNA, 0.5 µM of forward and reverse primers, 200 µM of dNTPs, and 1.25 U of Taq polymerase. Individual PCR was performed for CD45, CD73, CD90, and CD105 to evaluate the expression of different surface markers, and GAPDH was selected as control. PCR was done also for three pluripotency markers viz., Oct4, Nanog, and Sox2. PCR profile set for each of the 30 cycles was the following: 95°C (melting temperature) for 5 min, 95°C for 30 s, 58–60°C (annealing temperature) for 30 s, 72°C (extension temperature) for 30 s followed by

72°C for 5 min. The PCR products were resolved by electrophoresis in 1.8% (w/v) agarose gel stained with ethidium bromide, and images were captured by the Chemidoc XRS system (Bio-Rad, USA) (Table 1).

#### Measurement of population doubling time

Population doubling time was measured to assess the cell growth in different passages. The time of population doublings was calculated using the formulae:  $DT = T \times \log 2 / \log (N_t / N_0)$ , where T is the incubation time in any unit,  $N_0$  is the cell number at the beginning of the incubation time,  $N_t$  is the cell number at the end of the incubation time (Greenwood et al., 2004). Doubling time (DT) was determined in cultures till passage 5.

#### Measurement of cell proliferation

Cell proliferation was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Mosmann, 1983). Cells ( $5 \times 10^3$ /well) seeded on a 96 well plate were grown for 72 h at 37°C and then supplemented with 20 µl of MTT reagent (5 mg ml<sup>-1</sup>) (HiMedia Laboratories, India). After 4 h of incubation, formazan crystals were formed. These crystals were dissolved with 100 µl of solubilization buffer (10 % SDS in 0.01 M HCl), and absorbance was measured at 590 nm with a reference wavelength of 650 nm using a microplate reader (Bio-Rad, USA).

#### Wound healing assay

From a completely confluent culture (~90%), monolayer cells were scraped with the help of a pipette tip to create a scratch of uniform width. Cell migration was assessed by capturing images using phase-contrast microscope at different times till wound closure. The migration rate of cells was calculated by measuring the distance between

**Table 1** Primers for the detection of mesenchymal stem cells markers

Primer	Forward	Reverse
CD45	GGCAAACATGTGAGGACAGA	TCCAGATCAAAAATTCACCGA
CD73	CCAGCAGTTGAAGGTCGGAT	CCATCACCAAGCCAGTTCCT
CD90	CGTGATCTATGGCACTGTGG	TATGCCCTCACACTTGACCA
CD105	AAAACAGACTACCCAGCCC	TGGGGTCTCCATGTGACAGT
NANOG	GGTGTCTACAGCAATCCAC	CAGCCAACGCTACTGAAACC
OCT4	GAGTGAGAGGCAACCTGGAG	GTGAAGTGAGGGCTCCCATA
SOX2	AGTCTCAAGCGACGAAAAA	GCAAGAAGCCTCTCCTTGAA
Osteopontin	TGAAAAGTGCAGATATGGAA	TCAGAATTTCTGGCTACC
COL1A1	GAGAGAGGTGAACAAGGTC	ACCCTTAGCACCATCATTG
Aggrecan	GGCATTGTGTTCCATTACAG	CTCATCCTGTCTCCATAGC
SOX9	AACGCCTTCATGGTGTG	GTACTIONGTAATCCGGGTGG
Leptin	TGACGACTGTTTACTGGGGC	GTTTGGGTGAGCCAGTCAGA
FABP4	TGTGCTGGATCTTTCTACTCA	GGTATGAAGTGGCAGGGCTT
GAPDH	AACATCATCCTGCTCCAC	GACCACCTGGTCTCAGTGT

the two edges of the scratch dividing by the time interval. Measurement was done at five different points for each scratch (Himal, Goyal, & Ta, 2017).

#### **Senescence cell detection**

Microscopic observation was performed to study the occurrence of senescence-specific morphological changes. For detection of typical signs of senescence,  $\beta$ -galactosidase assay was performed using the Senescence Cells Histochemical Staining Kit (Cat no: CS0030), Sigma, USA. Two types of media were used for cell growth viz., DMEM-HG+ 10% FBS and DMEM-LG+ 20% FBS for the comparative study. The growth medium of the respective cells was aspirated and cells were washed with DPBS twice. Fixation buffer was then added and cells were incubated at room temperature for 6–7 min. After fixation, cells were rinsed with DPBS thrice and incubated with 1 ml of staining solution overnight at 37°C. Blue-colored cells were observed under a microscope for the detection of cells positive for  $\beta$ -galactosidase activity.

#### **Trilineage differentiation**

Specific basal medium with respective supplement (9:1) was used for preparing three differential media (osteogenic, chondrogenic, and adipogenic) following manufacturer's protocol (Thermo Fisher Scientific, USA). Cells were grown 14 days for chondrogenesis and 21 days for osteogenesis and adipogenesis with every 48 h of media change. For the morphological study, cells were observed under a phase-contrast microscope.

In case of osteogenesis, alizarin red S (Sigma, USA) staining was carried out to detect calcified extracellular matrix deposits (Zajdel, Kałucka, Kokoszka-mikołaj, & Wilczok, 2017). Before cell staining, culture media were discarded and cells were washed with DPBS, followed by fixation with formaldehyde (4%). Then, the cells were rinsed with sterile distilled water and stained with 0.5% alizarin red S (w/v in water; pH 4.1–4.3) for 8–10 min at room temperature and observed under a phase-contrast microscope. For chondrogenesis, micromasses were stained with Alcian blue (Sigma, USA) for the confirmation of sulfated proteoglycan-rich matrix (Ogawa et al., 2004). After fixation, cells were incubated with Alcian blue (1%) staining solution for 30 min. Then, cells were washed with 0.1 N HCl followed by rinsing with sterile distilled water and observed under phase-contrast microscope. Similarly, oil red O (Sigma, USA) staining was done to examine the lipid droplet formation for the determination of adipogenesis (Neupane, Kiupel, & Yuzbasiyan-gurkan, 2008). After fixation, oil red O (5 mg ml<sup>-1</sup>) staining solution was diluted (stock: water = 2:3) and the cell was incubated in staining solution for 30 min. The cells were then washed with sterile distilled

water to remove excess stain and observed under a microscope.

Differentiated cells were characterized through RT-PCR as described earlier. Expression of osteopontin and collagen I was assessed for detection of osteogenic differentiation. Likewise, aggrecan and Sox9 expression were examined for validating chondrocytes. Similarly, expression of FABP4 and leptin was evaluated in both differentiated and undifferentiated cells for confirmation of adipogenic differentiation. GAPDH was used as a loading control.

#### **Statistical analysis**

Statistical analysis was assessed by Student's *t* test. All *P* values were derived from a two-tailed statistical test using the SPSS software (version 22.0.0.0). A value of *P* < 0.05 was considered statistically significant.

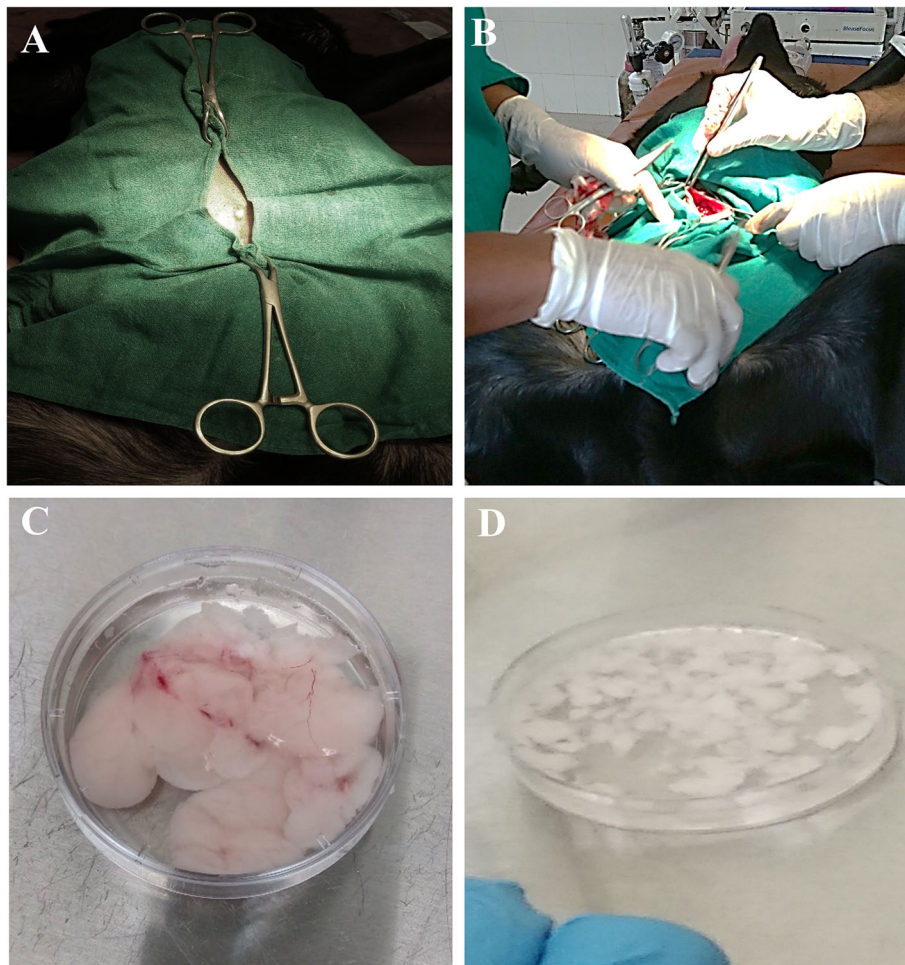
## **Results**

### **Establishment of canine white adipose tissue-derived primary culture for MSCs**

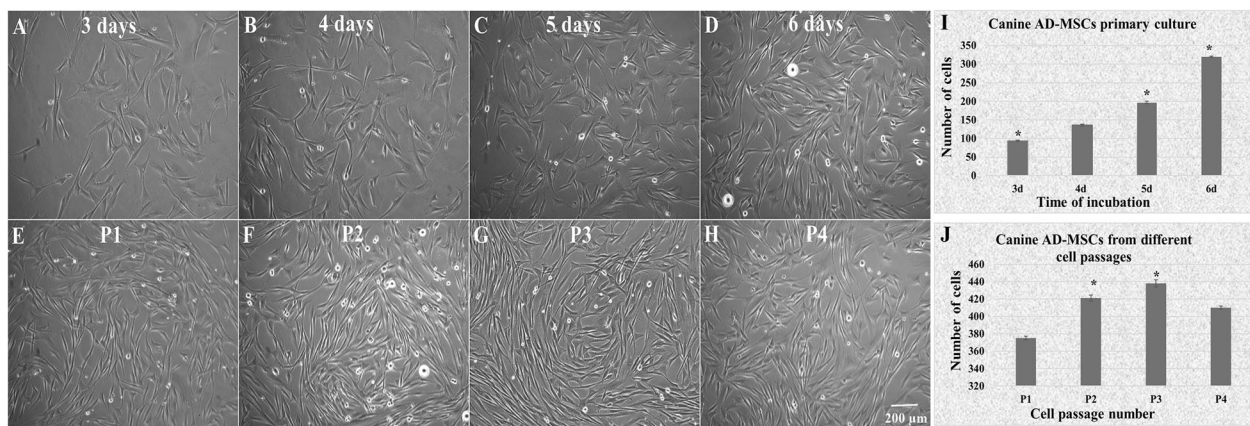
Mesenteric white adipose tissue was collected aseptically from the operating theater during surgery. The tissue was excised with sterilized scissors and immersed in DPBS. It was then transported to the stem cell laboratory of the West Bengal University of Animal and Fishery Sciences (WBUAFS) for MSCs culture (Fig. 1). The minced tissue was digested in three different ways, among which 250  $\mu$ g ml<sup>-1</sup> collagenase used for tissue digestion in a 60-min incubation showed the highest efficiency in terms of the number of attached cells followed by 500  $\mu$ g ml<sup>-1</sup> for 60 min and 100  $\mu$ g ml<sup>-1</sup> for overnight (data not shown). We found that under suitable growth conditions, cells reached approximately 30–80% confluency from 3 to 6 days of incubation (Fig. 2a–d). The bar diagram also represents a higher number of cells with the time of incubation (Fig. 2i). Cell passaging was performed to keep them viable and to grow for an extended period of time. Cells were passaged every 7-day interval till several passages. No phenotypic change in cells was observed from passage one to four (Fig. 2e–h). However, a number of cells remain almost the same after 7 days of each passage, because the cells entered the plateau phase on the seventh day (Fig. 2j).

### **Study of surface marker expression to characterize MSCs**

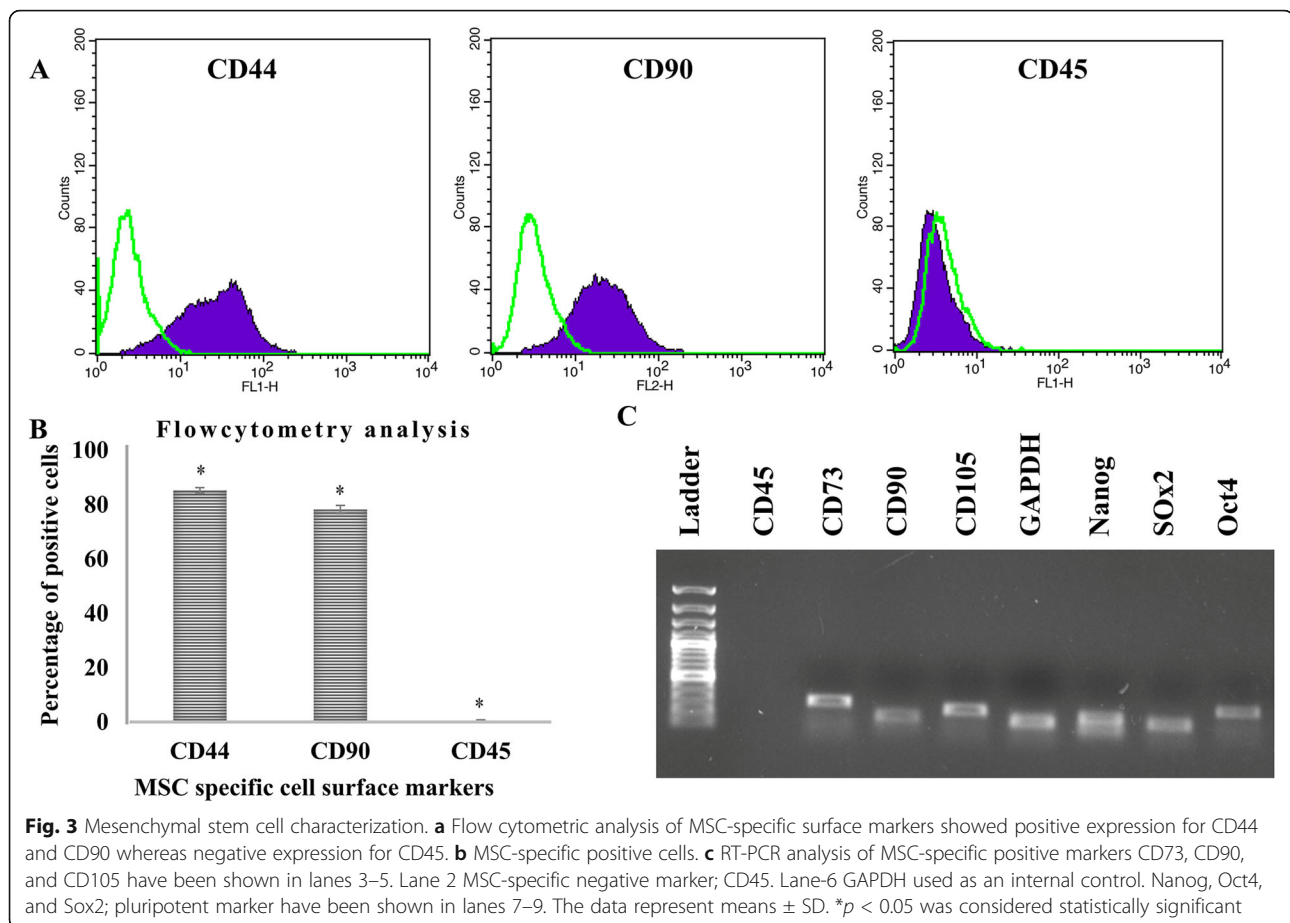
Flow cytometric analysis has given significant information for MSC characterization. Higher expression of two positive cell surface markers, CD44 and CD90, was observed in the culture of undifferentiated cells, whereas CD45, a negative marker for MSCs, had negligible expression (Fig. 3a). From the quantitative data, we found 85.3% positive expression for CD44 and 78.3% positive expression for CD90 (Fig. 3b). RT-PCR analysis revealed that the markers CD73 and CD105



**Fig. 1** Collection and processing of the canine adipose tissue. **a** Preparation of animal for tissue collection. **b** Incision in the abdomen for harvesting the adipose tissue under sterile condition. **c** Placement of the collected adipose tissue in sterile Petri plate in PBS. **d** Mincing of the adipose tissue for MSC primary culture



**Fig. 2** Canine adipose tissue-derived primary culture. **a–d** Cells from passage zero were captured at days 3, 4, 5, and 6. **e–h** 70–80% confluent monolayer cells from passage one to passage four. **e** Number of cells with the time of incubation. **j** Cells from different passages at the seventh day. The data represent means  $\pm$  SD. \* $p < 0.05$  was considered statistically significant



were expressed well than that of CD90, though it also has shown significant expression (Fig. 3c). Two pluripotency markers named Nanog and Sox2 expressed remarkably in the respective cells while the expression of another marker Oct4 is slightly lower in amount (Fig. 3c). These surface marker expression analyses through flow cytometric and RT-PCR determined mesenchymal stem cell characterization.

#### Calculation of population doubling time

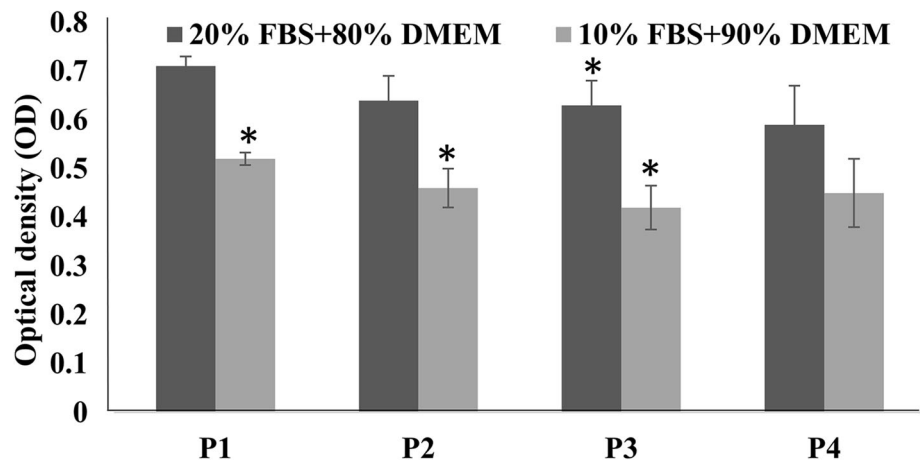
Monitoring and analyzing the quantity of cells are crucial for reliable optimization of growth rate. The proliferation of MSCs has entered the logarithmic growth phase from the first to the sixth day. According to the growth population, doubling time was determined using the formula  $DT = T \times \log 2 / \log (N_t / N_0)$ , till the fifth passage of MSCs. Up to the fourth passage, MSCs required about 18–24 h for a two-fold increment from the initial cell count. After passage four, it has taken  $30 \pm 0.5$  h for the doubling of cell population indicating the lowering of proliferation rate with the increasing passage number. This gives a significant parameter of cell growth required for further important in vitro experiments.

#### Cell proliferation through MTT assay

Breakdown of MTT reagent corresponds to the higher rate of cell proliferation. This chemical modulation is carried out by the mitochondrial succinate dehydrogenase enzyme of metabolically active cells. In this experiment, cell proliferation was measured in terms of optical density recorded in a microplate reader. Gradual passing of well-grown primary cells corresponded with serial lowering of OD values, and it was inferred that cell proliferation reduced with an increase in the number of passages. The proliferation potential is higher in cells grown in DMEM-LG+ 20% FBS than that of cells grown in DMEM-LG+ 10% FBS suggesting a higher requirement of FBS in culture media as it contains a significant amount of growth factors essential for the cellular development (Fig. 4).

#### Evaluation of in vitro cell migration through wound healing assay

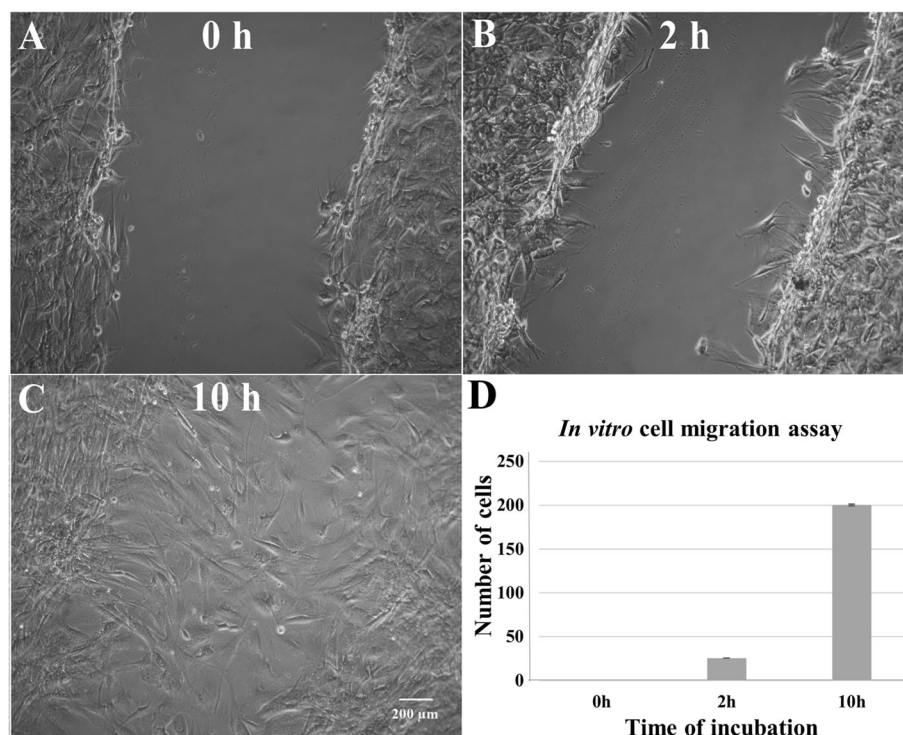
This experiment was done to evaluate in vitro cell migration. Cell migration is a fundamental process of the tissue development and driving factor for several morphogenetic events. Efficient migration of the



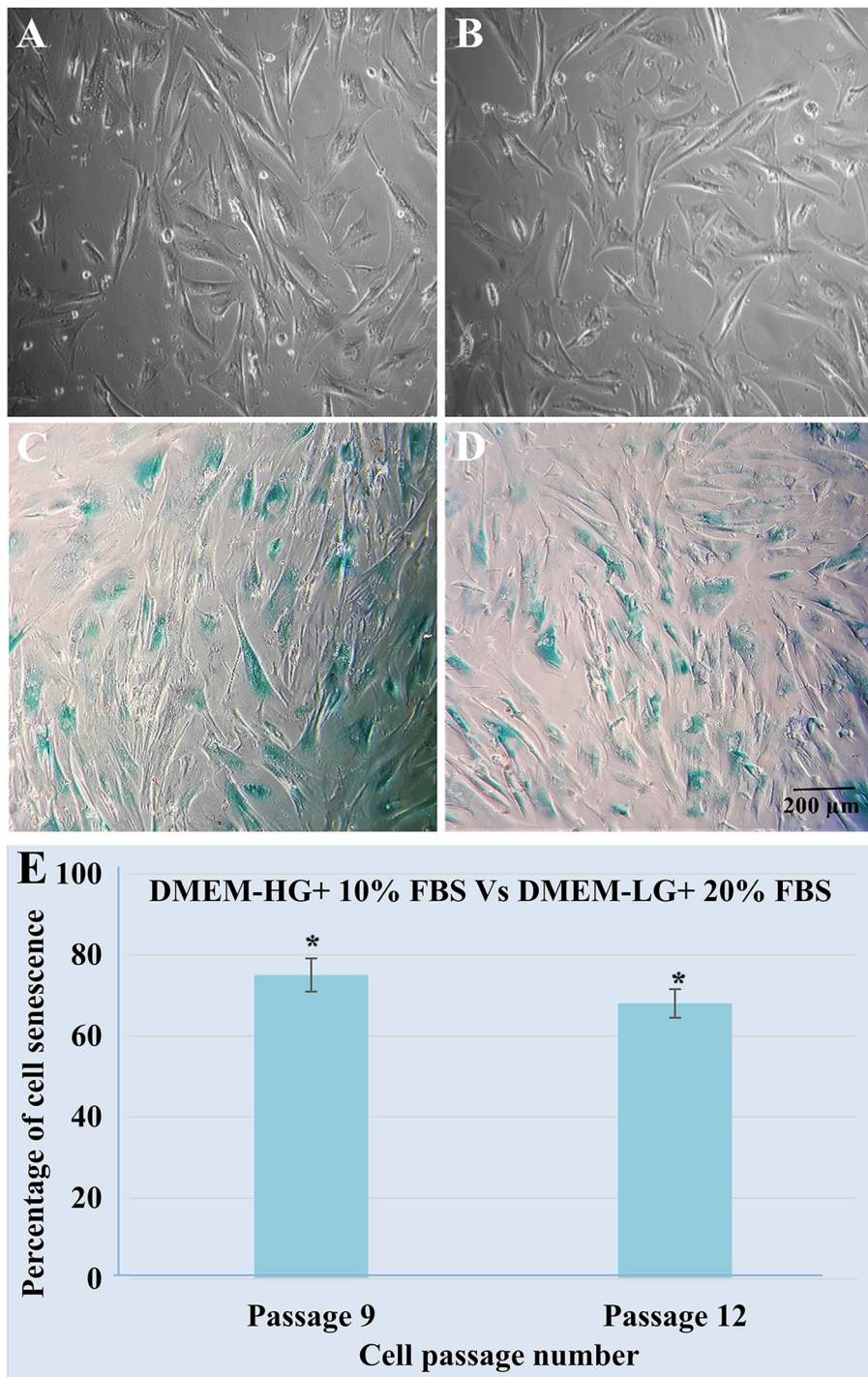
**Fig. 4** Cell proliferation through MTT assay. The proliferative capacity of canine MSCs (passages P1–P4) under two different medium compositions (20% FBS+80% DMDM-LG and 10% FBS+90% DMEM-LG) were evaluated after 72 h of incubation, and it was found that proliferation potential is higher in cells grown in 20% FBS+DMEM-LG and also observed lowering OD values with increasing number of passages. The data represent means  $\pm$  SD. \* $p < 0.05$  was considered statistically significant

respective cells to the site of the wound is an important parameter for a successful regeneration process. Gradual filling of gaps created through scratching was represented in Fig. 5. The rate of cell migration calculated from the wound healing assay was  $136.9 \pm 4.7$

$\mu\text{m}/\text{h}$ . Bar diagram data represents the number of migrated cells with incubation period (Fig. 5d). This provides significant data to fulfill the goals of our present study regarding tissue damage restoration by means of the self-renewing ability of MSCs.



**Fig. 5** In vitro cell migration through wound healing assay. Representative images of cell migration at 0, 2, and 10 h after scratch are shown in panel a–c. The average migration rate during 10 h in response to the scratch was calculated from three independent experiments. **d** The number of migrated cells with incubation time. The data represent means  $\pm$  SD. \* $p < 0.05$  was considered statistically significant



**Fig. 6** Replicative senescence of canine MSCs. **a** Cells grown in DMEM-HG+ 10% FBS reached senescence at passage 9. **b** Same phenomenon was observed at passage 12 when cells were grown in DMEM-LG+ 20% FBS. Senescence cells were characterized by enlarged or flattened morphology and reduced proliferation capacity. Similarly, **c d** cell senescence in both culture medium as detected by senescence associated  $\beta$ -galactosidase activity. **e** Percentage of senescence cells at 9th and 12th passage. The data represent means  $\pm$  SD. \* $p < 0.05$  was considered statistically significant

#### Long-term in vitro proliferative leads to cell senescence

In studying the senescence of MSCs, principally two types of media compositions (DMEM-HG+ 10% FBS

and DMEM-LG+ 20% FBS) were used for several passages. Here, senescence was observed at the 9th passage when MSCs were grown in DMEM-HG+ 10% FBS,



whereas a similar phenomenon occurred at the 12th passage when cells grown in DMEM-LG+ 20% FBS (Fig. 6a, b). Similarly, senescence was also assessed with a histochemical staining kit. A Bluish-green coloration of cells after 24 h of staining was observed, and this represents cellular  $\beta$ -galactosidase activity, a typical sign of senescence (Fig. 6c, d). We also found that the percentage of senescence cells was higher at the 9th passage (medium DMEM-HG+ 10% FBS) as compared to the 12th passage (medium DMEM-LG+ 20% FBS) (Fig. 6e). This result also highlighted the greater requirement of FBS along with a small amount of glucose for the delay of senescence

### In vitro induction of trilineage differentiation

#### Osteogenesis

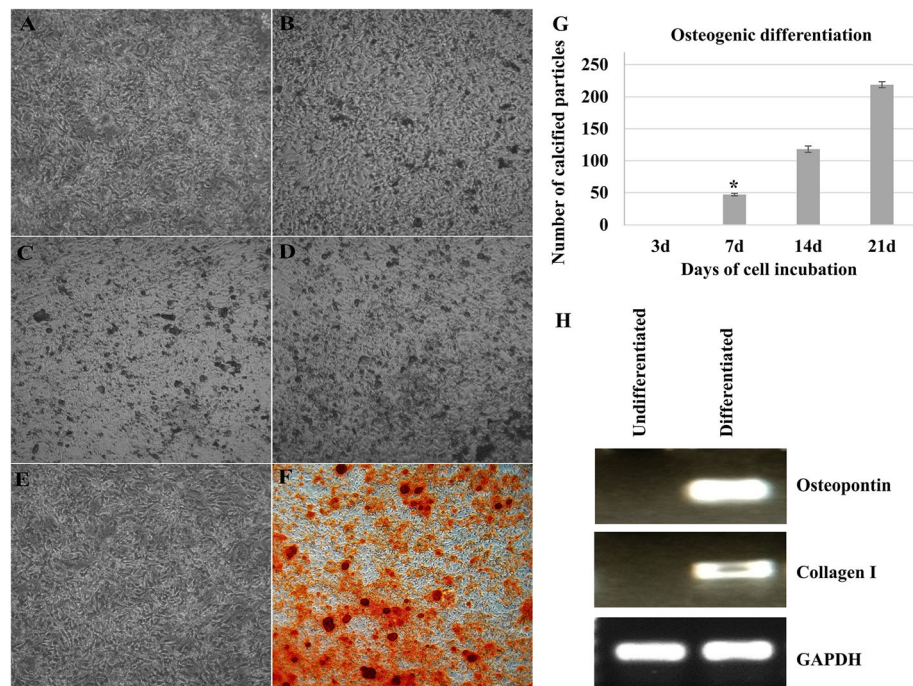
Regular observation on the cell culture of osteogenic induction has given remarkable information about the gradual changes in respective cells. Distinguishable morphological changes are presented here based on 3, 7, 14, and 21 d of observation. Very small polygonal-shaped, crystalline structures were found in the experimental set during microscopic observation (Fig. 7a–d) and those were not seen in normal undifferentiated cells (Fig. 7e). The number of calcified crystalline structures increases

with time. However, after 3 days of incubation, there were no such structures and they started appearing from day 7 (Fig. 7g). Deposited crystals of calcium compounds in the extracellular matrix were detected through the alizarin red S staining which caused the reddish-brown coloration of those calcified bodies (Fig. 7f).

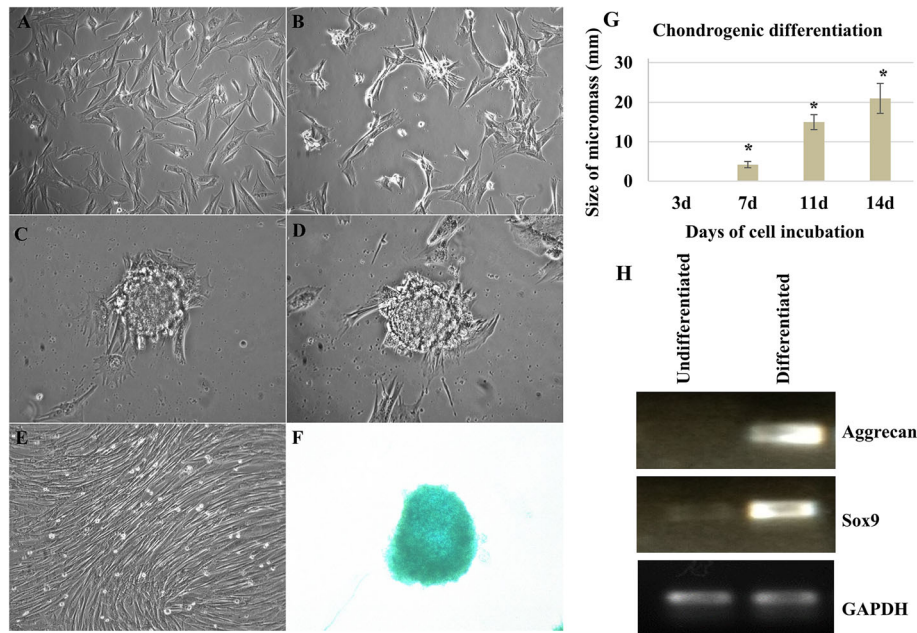
RT-PCR data have given an authentic proof of collagen I gene expression in the cells of a 21-day-old culture. Another non-collagenous extracellular matrix protein of the bone referred to as osteopontin was expressed also in response to specific media composition for osteogenesis. Those two genes were not expressed prominently in undifferentiated cells as observed in the RT-PCR study (Fig. 7h). This has given a significant indication of successful osteogenic differentiation.

#### Chondrogenesis

During the serial observational period, the micromass formation in cultured cells was clearly seen after 11 days from chondrogenic induction. After 14 days, micromasses of larger size were observed in respective induced cells while no micromass formation was observed in the control set of undifferentiated cells (Fig. 8a–e). The size of micromasses was also represented with a bar diagram (Fig. 8g). Those micromasses were turned into



**Fig. 7** Osteogenic differentiation of canine adipose tissue-derived MSCs. **a–d** Small black crystalline structures were observed at 3, 7, 14, and 21 days after osteogenic differentiation. **f** Deposited crystals were stained with alizarin red S stain which showed reddish-brown coloration. Negative control shown in panel **e** where no visible stain was observed. **g** The number of calcified crystalline structures with incubation time. **h** The characterization of osteocytes through RT-PCR. Expressions of osteopontin and collagen I, marker genes for osteocytes were compared with undifferentiated cells. We found positive expression for both the marker genes in differentiated cells whereas no expression was observed in undifferentiated cells. GAPDH was used as an internal control. The data represent means  $\pm$  SD. \* $p < 0.05$  was considered statistically significant



**Fig. 8** Chondrogenic differentiation of canine adipose tissue-derived MSCs. **a–d** Morphological changes observed at 3, 7, 11, and 14 days after chondrogenic differentiation. **f** Alcian blue staining for aggrecan bodies during chondrogenic differentiation showed bluish-green coloration. **e** Negative control where no visible stain was observed. **g** Size of micromasses represented with bar diagram. **h** RT-PCR analysis for aggrecan and Sox9, marker genes for chondrocytes. Successful expression of both the marker genes was observed in chondrogenic differentiated cells. Sox9 expression was also observed in undifferentiated cells, although expression was quite low compared to chondrocyte-differentiated cells. GAPDH was used as loading control. The data represent means  $\pm$  SD. \* $p < 0.05$  was considered statistically significant

bluish-green after staining with Alcian blue (Fig. 8f) and that indicated the presence of sulphated proteoglycans such as aggrecan in the matrix of the cell.

Sox9 involved in regulating chondrogenesis and chondrocyte proliferation was expressed significantly as observed in the RT-PCR analysis. Similarly, higher expression of aggrecan was also observed in cells grown for chondrogenesis in comparison with the control ones (Fig. 8h). These recordable data confirmed the successful chondrogenesis in this in vitro experimental study.

#### Adipogenesis

Adipogenic differentiation is characterized by the presence of lipid droplets embedded in the cytoplasm of flat, large, and wide cells. The existence of a few glossy, spheroid lipid droplets was observed after 7 days of a cellular induction for adipocyte development. On the contrary, no such morphological changes were visible in case of undifferentiated cells. It is remarkable that a higher number of conspicuous larger-sized semi-liquid fat globules were seen after 14 and 21 days of adipogenic induction (Fig. 9a–e, g). Deep brown coloration of those lipid bodies observed through oil red O staining confirmed the occurrence of adipogenesis (Fig. 9f).

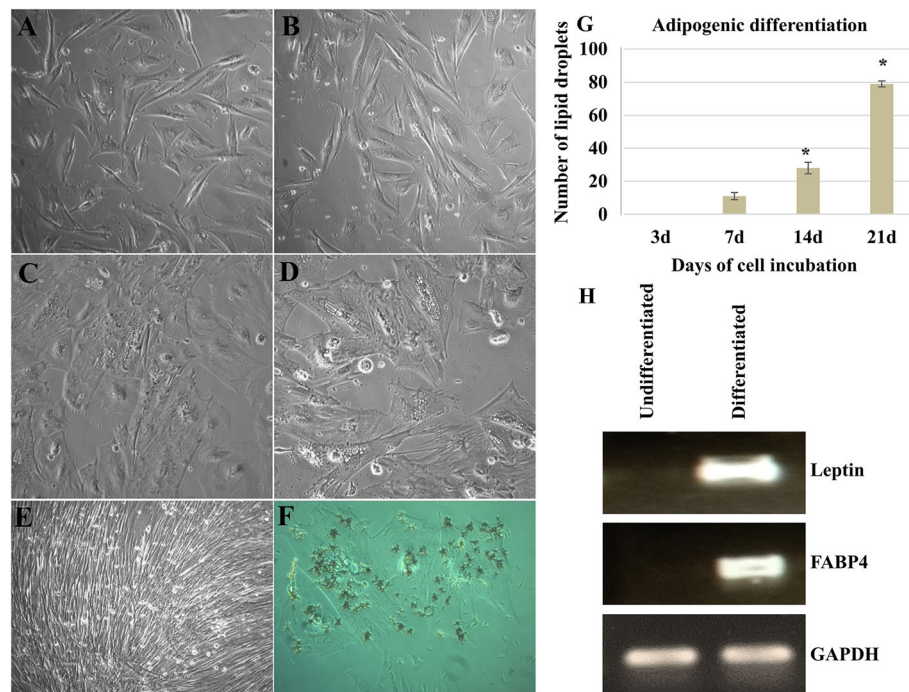
RT-PCR analysis revealed that leptin and FABP4, the two classic markers, expressed positively in the cells growing for adipogenic differentiation. Those adipogenic

progenitors were not expressed considerably in undifferentiated cells (Fig. 9h). It is noteworthy here for drawing inference for effective adipogenic differentiation of cells.

#### Discussion

According to the International Society for Cellular Therapy, the major criteria for defining (MSCs) are stated as (a) plastic adherence; (b) expression of surface markers like CD44, CD73, CD90, and CD105 (positive markers); (c) absence of some surface markers such as CD34 and CD45 (negative markers); (d) multipotent differentiation potential of chondrocytes; osteocytes; and adipocytes under different standard conditions in vitro (Dominici et al., 2006).

In this study, it may be stated that the development of stem cell proper has been done in vitro as the cells have fulfilled the criteria mentioned above. The adipose tissue collected from the veterinary clinic was transported to the laboratory for stem cell culture (Fig. 1). The elongated, spindle-shaped, fibroblast-like appearance of cells was observed after 72 h of initial culture (Fig. 2). These are the specialized phenotypic features of mesenchymal stem cells (Haasters et al., 2009). Expression of cell surface markers like CD44, CD73, CD90, and CD105 observed through RT-PCR results has confirmed the typical MSC generation from canine white adipose tissue. None of the two selected negative markers namely



**Fig. 9** Adipogenic differentiation of canine adipose tissue-derived MSCs. **a–d** Morphological changes were observed after 7, 14, 17, and 21 days of adipogenic differentiation. Formation of lipid droplet was observed in differentiated cells during this period. **f** Lipid droplets were stained with oil O red stain and **e** used as a negative control. **g** Number of lipid droplets with incubation period. **h** RT-PCR analysis for leptin and FABP4, marker genes for adipocytes. We observed much higher expression of leptin in differentiated cells, whereas FABP4 expression was observed only in adipogenic differentiated cells. GAPDH was used as loading control. The data represent means  $\pm$  SD. \* $p < 0.05$  was considered statistically significant

CD34 and CD45 were expressed and complemented the corroboration of the genesis of MSCs (Fig. 3c). In addition, higher expression of CD44 and CD90 obtained from flow cytometric analysis was also the indication of common characteristics of MSCs in this in vitro experimental study (Fig. 3a, b). Those outcomes have given supportive evidences for the agreement with the previous reports (Mercati et al., 2009; Yaneselli et al., 2018).

Expression of three pluripotency markers such as Oct4, Nanog, and Sox2 in our present study (Fig. 3c) has ensured the ability of the respective stem cells to undergo self-renewal, and those may give rise to all types of cells of a living body depending on the types of induction. Stemness of those adipose tissue-derived cells can be suggested through the detection of the above-mentioned pluripotency-associated transcription factors (Neupane et al., 2008). Normally, the gene expression level of those pluripotent markers make a correlation with population doubling of cells as cell aging starts with a longer time requirement for the doubling of cell numbers together with gradual reduction of pluripotency (Piccinato, Sertie, Torres, Ferretti, & Antonioli, 2015).

It was reported that the beneficial effects of MSC transplantation for the restoration of cell deficit caused by tissue injury are mainly based on their rate of survival and capacity of proliferation (Hu, Zhang, Gu, & Guan,

2012). The self-proliferative capability of MSCs is the key factor for their sustainable growth and maintenance regarding therapeutic uses as they eventually undergo replicative senescence after a longer period of normal culture. In our present study, cultured cells underwent senescence at passage twelve in case of cells grown in medium containing DMEM-LG+ 20% FBS content whereas signs of senescence were detected at passage nine in the cells grown in the medium with DMEM-HG+ 10% FBS (Fig. 6). This has shown similarity with the report of Yang, Shen, Reece, Chen, and Yanga (2016) where cells maintained their pluripotency and ability of differentiation in low-glucose medium, but both of those were considerably impaired by the high-glucose content of the growing medium. A higher amount of FBS in a culture medium may also be the effective factor for higher cell proliferation as FBS contains a significant amount of growth factors essential for the cellular development, and its protein components play a vital role in modulating the metabolic activity of the cells (Fig. 4) (Deorosan, Byron, and Nauman (2011)). Here, the combinatorial effect of low-glucose and high-FBS content may be the possible reason for the enhanced rate of cell proliferation as well.

Cell proliferation makes a direct relationship with wound healing which determines the rate of cell

migration at the site of injury. It is a complex restorative process requiring a functional coordination among cells, growth factors, and extracellular matrix proteins (Himal et al., 2017). Through the self-renewing capability, MSCs mediate this repair mechanism by involving other cells and the secretion of growth factor plus matrix proteins (Yoo, Maxson, Danilkovitch-Miagkova, LeRoux, & Lopez, 2012). Statement of a clinical update also indicated that the secretion of cytokines and growth factors by MSCs eventually leads to epithelization through this process of regeneration (Mester et al., 2018). Our present result of the experiment (Fig. 5) conforms with previous reports where this unique restoration capacity of MSCs was mentioned (Mester et al., 2018; Yoo et al., 2012). It is a mini representative of studying the regenerative ability of MSCs before in vivo application using a suitable animal model.

Structurally, bone is a highly complex, specialized type of a connective tissue whose organic matrix is composed mainly of collagen I fibers (approx. 95%) and numerous non-collagenous proteins (about 5%). The matrix is strengthened by the deposition of calcified crystals embedded in cells, i.e., osteocytes which participate in the organization and maintenance of the bones (Barrère, van Blitterswijk, & de Groot, 2006). In our present study, calcium deposition has provided a proof for osteogenic differentiation along with collagen 1 expression (Fig. 7a–g) which is normally found in the extracellular matrix of bones as the obtained result corresponded to the analytical studies of Matsuoka et al. (1999) and Buchwald, Kozielski, and Szybowicz (2012). Another osteocyte-specific marker, osteopontin, was also expressed in the respective set of our study (Fig. 7h). This result has supported the view of Morinobu et al. (2003).

Chondrocytes consist of collagen and proteoglycan, produce, and maintain the matrix of the cartilage tissue. Aggrecan mediates chondrocyte-chondrocyte and chondrocyte-matrix interaction by using hyaluronan inside the cartilage tissue (Kiani, Chen, Wu, Yee, & Yang, 2002). Our RT-PCR data have shown significant expression of aggrecan during chondrogenic differentiation (Fig. 8). However, Mwale, Stachura, Roughley, and Antoniou (2006) disagreed that aggrecan is a good marker for chondrogenesis from human bone marrow-derived MSCs. This type of disparity may appear from the difference in tissue source along with the species variation. On the contrary, Xu et al. (2008) found a peak level of aggrecan expression during chondrogenic differentiation of human MSCs. Neupane et al. (2008) reported the higher expression of aggrecan during chondrogenesis from canine ADSCs. Our experimental result conforms with these reports. Sox9, a crucial element for cartilage development, was expressed significantly in cells grown in a chondrogenic differentiation medium. Therefore,

sox9 has presented itself as an important component in successful chondrogenic induction from mesenchymal stem cells. In this regard, our in vitro study (Fig. 8) has shown similarity with the report of Leung et al. (2011).

Leptin is an adipose tissue-derived protein that participates in a wide range of physiological functions like reproduction, thyroid function, bone density, the immune biology (Wrann & Rosen, 2012). The primary site of leptin production is the adipocyte, although it is expressed in some other tissues in a lower amount (Harris, 2014). Higher expression of leptin mRNAs indicated the remarkable adipogenic differentiation from undifferentiated MSCs in this study (Fig. 9). On account of being a marker of the population of adipocyte progenitor, FABP4 expression in cells has become one of the typical features of adipogenic differentiation (Shan, Liu, & Kuang, 2013). Neupane et al. (2008) and Vieira et al. (2010) also have demonstrated the expression of leptin and FABP4 during adipogenic differentiation observing in their own experimental studies. We have got similarity with those previous reports in our study. Reversible cellular differentiation ability was observed in this study through this adipogenic differentiation from pluripotent MSCs derived from canine white adipose tissue.

## Conclusion

Clinical investigation has approved the remarkable regenerative potentials of stem cells despite limited success in the understanding of their in vivo function (Prentice, 2019). The information obtained from this study should be given importance for rigorous analysis of the basic biology of the canine mesenchymal stem cells regarding their clinical application. In addition, preservation of cultured osteocytes and chondrocytes with proper maintenance might be utilized in bone fracture restoration through a regenerative therapeutic approach. Application of those cells to the specific target through a suitable delivery system in combination with available drug regimen may give a better treatment option for some incurable orthopedic injuries in the very near future.

## Abbreviations

SVF: Stromal vascular fraction; AD-MSCs: Adipose tissue-derived mesenchymal stem cells; DPBS: Dulbecco's phosphate-buffered saline; DMEM-HG/LG: Dulbecco's modified Eagle's medium-high glucose/low glucose; FITC: Fluorescein isothiocyanate; PE: Phycoerythrin; RT-PCR: Reverse transcription-polymerase chain reaction; MTT: [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; WBUAFS: West Bengal University of Animal and Fishery Sciences

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**Authors' contributions**

AM analyzed and interpreted all the data, conducted all lab work, and had a major contribution. AKJ prepared manuscript and assisted in cell culture. DB conducted cell culture and maintenance work, and SKG guided throughout the study and conducted surgical work. The authors read and approved the final manuscript.

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**Availability of data and materials**

All the data and materials used to reach the conclusion of this study are available from the corresponding authors on reasonable request.

**Declarations****Ethics approval and consent to participate**

The authors have obtained ethical approval vide reference number F.No. 25/15/2018-CPCSEA, dated 14/09/2018 from the concerning authority (*Committee for the purpose of control and supervision of experiments on animals*, Government of India) to isolate, expand, and apply AD-MSCs in canine osteoarthritic patients. Total 5 donor dogs were selected for collection of adipose tissue based on the history of satisfactory health status, no recent medication, proper vaccination, owners' harmony, and informed consent were written. All the experiments were conducted after receiving the approval from CPCSEA, Government of India.

**Consent for publication**

Not applicable

**Competing interests**

The authors declare that they have no competing interests.

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