







Submitted: 22/08/2024

Accepted: 02/11/2024

Published: 31/12/2024

Rabbit visceral adipose stromal cell reveals phenotype and genotype characteristics of adult mesenchymal stem cell

Ahmad Aswin¹ , Helen Susilowati¹ , Ira Sari Yudaniayanti² , Lina Susanti² , Diyantoro Diyantoro^{1,3} ,
Watchareewan Rodprasert⁴ and Suryo Kuncorojakti^{1,5*} 

¹Research Center for Vaccine Technology and Development, Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia

²Division of Veterinary Clinic, Department of Veterinary Science, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia

³Department of Health Science, Faculty of Vocational Studies, Universitas Airlangga, Surabaya, Indonesia

⁴Veterinary Stem Cell and Bioengineering Innovation Center, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand

⁵Division of Veterinary Anatomy, Department of Veterinary Science, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia

ABSTRACT

Background: As an excellent model for many animal and human diseases, rabbits are the third-most used mammal model after mice and rats. A plethora of studies on the exploration of rabbit mesenchymal stem cells still face discrepancies, especially in the standardization of phenotype and genotype characteristics to support reproducibility in both biomedical and translational research.

Aim: This study is aimed to evaluate the characterization and differentiation potential of visceral rabbit adipose-derived mesenchymal stem cells (Rab-ADMSC).

Methods: Visceral adipose tissue was obtained from three healthy male White New Zealand rabbits. Cells were further processed and cultivated aseptically. Phenotype and genotype assessments, including morphological observation, proliferation capacity, population doubling time, stemness- and senescence-related genes determination, a set panel of mesenchymal stem/stromal cell (MSC) surface markers evaluation, and multilineage differentiation, were performed in this study.

Results: Visceral Rab-ADMSC exhibited fibroblast-like shape morphology and had a plastic adherent ability, expressed stemness- (*NANOG*, *SOX2*) and senescence-related (*TP53*, *CDKN1A*) markers. Visceral Rab-ADMSC performs high expression of CD9, moderate expression of CD44 and CD49f, dimly expression of CD105, CD90, and CD73, and negative expression of CD13 and CD133 as well as CD45 as a hematopoietic stem cell marker. Despite these discrepancies, visceral Rab-ADMSC maintained its ability to differentiate into osteocytes, adipocytes, and chondrocytes.

Conclusion: To recapitulate, visceral Rab-ADMSC reveals the phenotype and genotype characteristics of adult mesenchymal stem cells. The study emphasizes how variations in tissue sources, culture conditions, and techniques can affect the reproducibility and validity of MSC obtained from different specific anatomical depots and species. Thus, the utilization of rabbit MSC as an animal model in biomedical and translational studies should be done with full caution to avoid data misinterpretation.

Keywords: Abdominal adipose tissue, *Oryctolagus cuniculus*, stem cells, regenerative medicine, process innovation.

Introduction

Rabbit is widely used as an experimental model for human and veterinary medicine because of their ease of handling and relatively economical for daily care. Their size is ideal for surgical procedures and allows for use in a large number of animals, improving the statistical significance. Rabbit is excellent for many

applications in biotechnology and translational research, because of their phenotype and genotype similarities with human (Zomer *et al.*, 2018; Tan *et al.*, 2013; Calle *et al.*, 2022). Rabbit adipose mesenchymal stem cells (Rab-ADMSC) have been reported that it has a resemblance in terms of cellular and tissue physiology to human MSC (Zomer *et al.*, 2018). Rabbit

*Corresponding Author: Suryo Kuncorojakti. Division of Veterinary Anatomy, Department of Veterinary Science, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia. Email: suryokuncorojakti@fkh.unair.ac.id



adipose mesenchymal stem cells (Rab-ADMSC) have been extensively used to support translational studies in human medicine, such as atherosclerosis, insulin resistance, osteoarthritis, and regenerative medicine (Vachkova et al., 2016; Riester et al., 2017; Tang et al., 2017; Esteves et al., 2018; Schafrum Macedo et al., 2019). Adipose tissues are an excellent alternative source for mesenchymal stem/stromal cells (MSCs) because of their less invasive procedures, less pain, and high-yield cell numbers (Guneta et al., 2016). Adipose tissue-derived MSC obtained from rabbits has been shown to adhere to plastic surfaces and undergo multilineage differentiation (Martinez-Lorenzo et al., 2009; Zomer et al., 2018; Chen et al., 2020; Almaliotis et al., 2021; Tirpakova et al., 2021; Koung Ngeun et al., 2023). Thus, it serves a high potency for the utilization of Rab-ADMSC as an MSC source in supporting biomedical and translational research (Martinez-Lorenzo et al., 2009; Lee et al., 2014; Riester et al., 2017; Zomer et al., 2018). In addition, MSCs obtained from adipose tissues show higher viability, proliferation rate, and differentiation capability compared to bone marrow-derived MSC (Torres et al., 2007; Via et al., 2012; Frese et al., 2016; Koung Ngeun et al., 2023). Rab-ADMSC can be collected from subcutaneous and visceral depots. Although it is harder to collect, visceral Rab-ADMSC showed higher osteogenic potency and high yield compared to subcutaneous depots (Peptan et al., 2006; Jurgens et al., 2008; Cawthorn et al., 2012).

Despite a great deal of research on visceral Rab-ADMSC, an inconsistent result has been found primarily due to the lack of characteristic information in cellular and molecular biology basis (Tan et al., 2013). Plethora study on rabbit MSC isolation and characterization revealed that in all studies, the rabbit MSC showed the three-lineage differentiation capability, but the study on MSC surface markers was inconsistent (Lee et al., 2014; Zomer et al., 2018). The obstacle in developing of the standardization rabbit MSC is the difficulties to obtain rabbit-specific antibody provided by the manufacturer. Thus, another approach to assess rabbit MSC surface marker was used, such as gene expression assessment in mRNA level using the RT-qPCR method (Tan et al., 2013; Lee et al., 2014; Tirpakova et al., 2021; Koung Ngeun et al., 2023). According to the International Society for Cellular Therapy (ISCT), the gold standard to evaluate the MSC surface marker should be performed by flow cytometry (Dominici et al., 2006). In addition, there are no guidelines for characterization, and the most recent guideline ISCT only addresses the human mesenchymal stem cells, which may not be valid for multiple species (Wright et al., 2021). Until now, there has been no comprehensive study evaluating the rabbit MSC on both phenotypic and genotypic bases.

This study is aimed to establish and validate the Rab-ADMSC characteristics on genotype and phenotype

bases. In addition, a set of antibody panels will be used to validate and evaluate the MSC surface marker by flow cytometry as a gold standard. Thus, the results of this study can be used to standardize visceral Rab-ADMSC to ensure reproducibility and avoid inconsistency for further downstream studies in both biomedical and translational studies.

Materials and Methods

Visceral Rab-ADMSC isolation and culture

Visceral Rab-ADMSC was isolated from visceral adipose tissues (mesenteric depot) of three healthy male White New Zealand rabbits (2-kg BW on average, 2 old months). Briefly, visceral tissue from rabbit was washed with PBS and then processed with mechanical and enzymatic dissociation methods by TrypLE Express (Gibco, Paisley, UK) for 1 hour under constant stirring at 37 °C. Cells were cultured with alpha minimum essential medium (α MEM) (Gibco, Paisley, UK) and maintained at 37 °C in a humidified incubator with 5% CO₂. The medium was supplemented with 10% fetal bovine serum (FBS) (Gibco, New York, NY), 1% penicillin–streptomycin (Gibco, New York, NY), and 1% Amphotericin B (Gibco, New York, NY). After cells achieved 80% confluence, the cells were subcultured. Cells in passages 3–5 were used for all experiments.

Cell proliferation assay

To evaluate the proliferation capacity, a number of 5×10^3 cells were plated in 96-well plates and incubated for 24, 48, 72, and 96 hours. The cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and after incubating with 100 μ l of MTT solution (0.5 mg/ml in PBS) for an additional 30 minutes at 37 °C, the supernatant solution was removed and 100 μ l of DMSO (Sigma Aldrich, Darmstadt, Germany) was added to dissolve the formazan crystal. The absorbance of each well was measured with a plate reader (BIOBASE, Shandong, China) at a 570-nm wavelength.

Cell population doubling time (PDT)

The cell PDT was obtained by counting the starting and final cells over time (passages 4–6) according to Roth's algorithm (Zomer et al., 2018).

Multilineage differentiation assay

For adipogenic differentiation, 5×10^3 cells were plated onto 24-well plates (NEST, Jiangsu, China) for 14 days in α MEM (Gibco, Paisley, UK) containing 10% FBS (Gibco, New York, NY), 10-mM isobutylmethylxanthine (Thermo Scientific, Fair Lawn, NJ), 100-mM indomethacin (Sigma Aldrich, MO, USA), 1-mM dexamethasone (Sigma Aldrich, Darmstadt, Germany), and 10- μ g/ml insulin (Sigma Aldrich, Darmstadt, Germany). At day 14, the intracellular lipid droplets were stained with Oil Red O (Sigma Aldrich, Darmstadt, Germany) staining, and RT-qPCR was used to assess the gene expression of *LPL* and *PPAR* adipogenic mRNA markers.

For chondrogenic differentiation, 5×10^3 cells were plated onto 24-well plates (NEST, Jiangsu, China) and treated with a chondrogenic induction medium α MEM (Gibco, Paisley, UK) 10% FBS (Gibco, New York, NY) supplemented with $1 \times$ insulin–transferrin–sodium selenite (Sigma Aldrich, Darmstadt, Germany), 40-mg/ml L-proline (Sigma Aldrich, Darmstadt, Germany), 50-mg/ml L-ascorbic acid-2-phosphate (Sigma Aldrich, Darmstadt, Germany), 1-ng/ml transforming growth factor- β 1 (Thermo Scientific, CA, USA), and 100-nM dexamethasone (Sigma Aldrich, Darmstadt, Germany) for 21 days. Afterward, the glycosaminoglycan was stained with Alcian Blue (Sigma Aldrich, Darmstadt, Germany) staining, and RT-qPCR was used to quantify the gene expression of *COL21A* and *SOX9* chondrogenic-related markers.

For osteogenic differentiation, 3×10^4 cells were plated onto 24-well plates (NEST, Jiangsu, China) and induced with osteogenic induction medium containing α MEM (Gibco, Paisley, UK) supplemented with 10% FBS (Gibco, New York, NY) supplemented with 50-mg/ml L-ascorbic acid-2-phosphate (Sigma Aldrich, Darmstadt, Germany), 10-mM β -glycerophosphate (Sigma Aldrich, Darmstadt, Germany), and 100-nM dexamethasone (Sigma Aldrich, Darmstadt, Germany) for 21 days. The extracellular matrix mineralization was stained with 0.2% Alizarin Red (Merck, Darmstadt, Germany) staining, and RT-qPCR was performed to evaluate the gene expression of *RUNX2*, *BMP2*, and *OPN* osteogenic mRNA markers.

Immunophenotyping

Visceral Rab-ADMSC at passages 4 and 5 were harvested and resuspended in PBS at a 1×10^6 cells/ml concentration. The surface markers were evaluated using Attune™ CytPix™ Flow Cytometer (Invitrogen, USA) against specific antibodies: CD105, CD44, CD49f, CD9, CD113, CD73, CD90, CD13, and CD45 (Table 1). All of the staining protocols were performed according to the manufacturer's instructions.

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using RNAsimple Total RNA Kit® (Tiangen, Beijing, China). The quantity of RNA was determined by Qubit (Thermo Scientific, USA). The cDNA was synthesized from 273-ng mRNA of each sample using FastKing gDNA Dispelling RT SuperMix® (Tiangen, Beijing, China) according to the manufacturer's instructions and was performed using MiniAmp Plus Thermal Cycler (Thermo Scientific, USA), and the cDNA can be kept at -80°C for further downstream study. Forget-Me-Not™ EvaGreen® qPCR Master Mix (Biotium, CA, USA) was utilized for a real-time quantitative PCR reaction. The RT-qPCR was performed by using QuantStudio™ 5 Real-Time PCR (Thermo Fisher, USA). The set of primers for both target and housekeeping genes are listed in Table 2. Target mRNA was normalized to *GAPDH* (housekeeping gene) using the formula $2^{-(\Delta\Delta\text{CT})}$ to determine the relative mRNA expression.

Statistical analysis

The results of this study were visualized as a bar chart and analyzed by nonparametric statistic ($n = 3$). The Mann–Whitney U test was used to compare two independent groups. A statistically significant difference was set when the p -value was <0.05 .

Ethical approval

All of the protocols involving the animal subject were performed according to the animal bioethics guidelines standard (Council, 2010) and were reviewed and approved by the Institutional Animal Care and Use Committee of the Faculty of Veterinary Medicine Universitas Airlangga with approval number 1.KEH.112.07.2024.

Results

Cell morphology and proliferation capacity

Microscopical observation reveals that the Rab-ADMSC morphology was a spindle-shaped fibroblast-like cell attached to the bottom of container (Fig. 1A). The proliferation rate curve showed an increase in the

Table 1. List of antibodies.

Antibody	Clone	Clonality	Isotype	Conjugated fluorophore	Brand and catalog number
CD105	SN-6	Monoclonal	Mouse IgG1	PE	GeneTex (GTX75142)
CD44	N/a	Polyclonal	Rabbit IgG	FITC	Bioss (BS-0521R-FITC)
CD49f	GoH3	Monoclonal	Rat IgG2a, k	PE	Biolegend (313612)
CD9	HI9a	Monoclonal	Mouse IgG1, k	PE	Biolegend (312106)
CD133	N/a	Polyclonal	Rabbit IgG	FITC	Biorbyt (orb15325)
CD73	N/a	Polyclonal	Rabbit IgG	FITC	Bioss (BS-4834R-FITC)
CD90	N/a	Polyclonal	Rabbit IgG	FITC	Bioss (BS-10430R-FITC)
CD13	WM15	Monoclonal	Mouse IgG1	FITC	GeneTex (GTX74833)
CD45	H130	Monoclonal	Mouse IgG1, k	FITC	Biolegend (304006)

Table 2. List of primers.

Gene	Accession Number	Forward primer reverse primer	Tm (°C)	Length (bp)
NANOG	XM_002712762.1	5' – TCAGCCTTCAGCAGATGCAAGA – 3'	62.26	185
		5' – AGCAGGGTAGAAGCCTGGGTA – 3'	62.39	
SOX2	XM_008266557.3	5' – GACAGCTACGCGCACATGAA – 3'	61.35	200
		5' – CTGTAGGTGGGAGAGCCGTT – 3'	61.26	
CDKN1A	XM_051854805.1	5' – TTGGGCGTGGAGATAAGGTGG – 3'	62.13	140
		5' – GTCCGACGGCTGCGACAT – 3'	62.80	
TP53	NM_001082404.1	5' – TGACACGCTCTCTGAGGACT – 3'	62.61	70
		5' – CGAGGCTGAGATCCGACTGC – 3'	62.68	
LPL	NM_001177330.1	5' – CGACTGGGAACGTGTGTGTA – 3'	59.97	139
		5' – CCACACACAACCCTCTCTCC – 3'	59.96	
PPAR γ	XM_051851047.1	5' – CAAGTACGGCGTCCATGAGA – 3'	59.83	94
		5' – CGTCATGAAGCCTTGTCCT – 3'	60.04	
COL2A1	NM_001195671.2	5' – GGATAGACCCCAACCAAGGC – 3'	60.11	117
		5' – TCCACCAGTTCTTCTTGGGC – 3'	59.89	
SOX9	XM_051825911.1	5' – GCCCAGAAGAGCCTCAAAGT – 3'	59.96	95
		5' – TAAGAGAGGTGGGGAGGGTG – 3'	59.96	
RUNX2	XM_051855680.1	5' – CTTCAAGGTGGTAGCCCTCG – 3'	60.11	154
		5' – CCGGCCACAAATCTCAGAT – 3'	60.11	
BMP2	NM_001082650.1	5' – GGAAACGCCTCAAATCCAGC – 3'	59.83	106
		5' – TAAAAGGCGTGATACCCCGG – 3'	59.82	
OPN	XM_051840601.1	5' – AGACCCTCCCGAGTAAGTCC – 3'	60.03	111
		5' – CGGCATCGTCGGATTCAATTG – 3'	59.77	
GAPDH	NM_001082253.1	5' – AGCTGGTATCAACGGGAAG – 3'	60.04	110
		5' – GAAGACGCCAGTGGATTCCA – 3'	60.04	

cell viability within 3 days (Fig. 1B) and the PDT was also increased from 23.53 ± 4.67 and 37.27 ± 8.66 to 105.83 ± 45.54 hours in passages 4 and 5, respectively. The relative mRNA expression of stemness-related marker (*NANOG* and *SOX2*) and senescence-related genes (*CDKN1A* and *TP53*) was detected by RT-qPCR (Fig. 1D–E)

Cell surface marker expressions

Phenotypic analysis of visceral Rab-ADMSC surface markers by flow cytometry revealed that the cells expressed in high positivity of CD9 ($98.94 \pm 1.36\%$) and moderately positive of CD44 and CD49f expression $25.08 \pm 4.71\%$ and $50.54 \pm 2.96\%$, respectively. A dimly positive of CD105 ($9.18 \pm 0.45\%$), CD90 ($3.94 \pm 0.80\%$), and CD73 ($2.52 \pm 0.29\%$) surface marker was detected in Rab-ADMSC, while negative expression of CD13 ($0.85 \pm 0.13\%$) and CD 133 ($1.70 \pm 0.12\%$) was detected. The hematopoietic surface marker of CD45 was not expressed in Rab ADMSC. The MSC surface marker expression was represented as the mean (%) \pm SD, separately for each marker in Figure 2.

Multilineage differentiation

In a multilineage differentiation study of visceral Rab-ADMSC, after the induction, the cells were committed to differentiate into mature adipocytes, chondrocytes, and osteocytes. Phenotypic evaluation by histochemical staining revealed that the differentiated cells are positive in oil red O, Alcian Blue, and Alizarin Red staining (Fig. 3A–C) showing that visceral Rab-ADMSCs were differentiated into adipogenic, chondrogenic, and osteogenic lineage. The genotypic study of three lineage differentiations of visceral Rab-ADMSC was in agreement with the phenotypic results. The expression of gene-related markers for adipogenic (*LPL* and *PPAR γ*), chondrogenic (*SOX9* and *COL2A1*), and osteogenic (*OPN*, *BMP2*, and *RUNX2*) was elevated compared to undifferentiated cells (Fig. 4A–C).

Discussion

According to the previous study, Rab-ADMSC characteristics might be defined using the same set of parameters as human MSC. The criteria should include

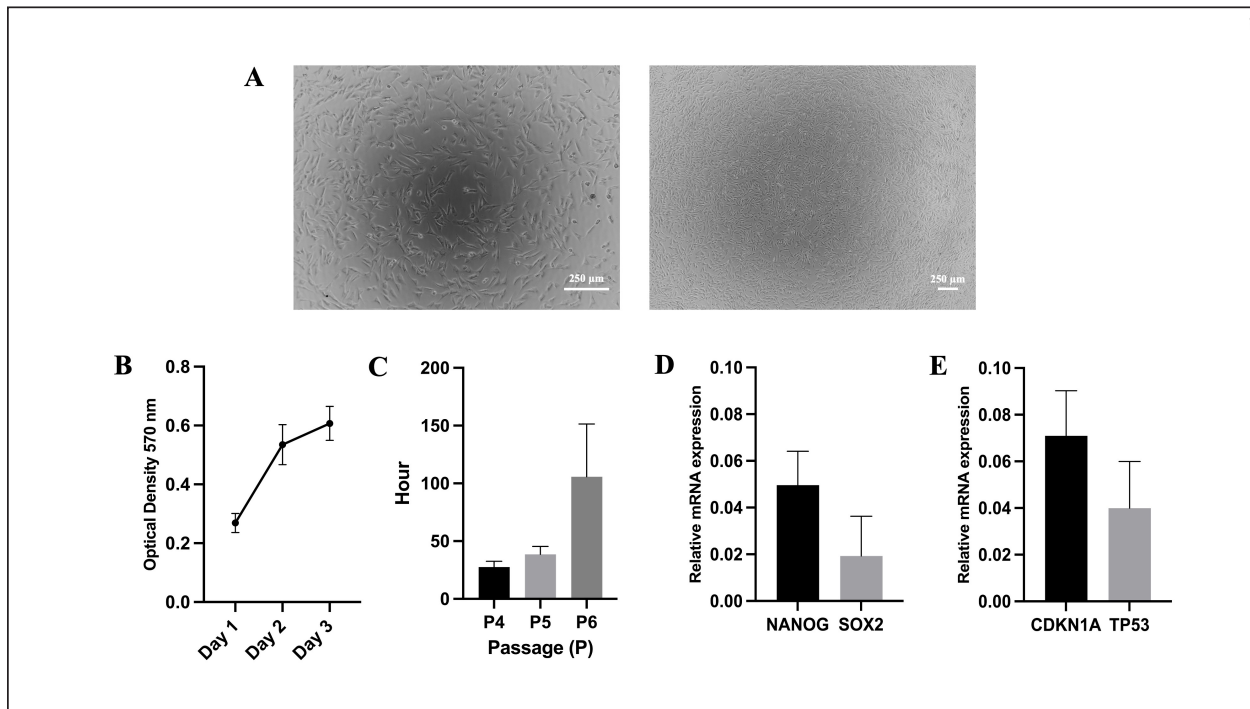


Fig. 1. Cell morphology after 24 hours of culturing in the high magnification and low magnification (100× and 40×, respectively). (A) Proliferation assay using MTT assay. (B) PDT. (C) Stemness. (D) Senescence. (E) related marker quantification by RT-qPCR.

plastic adherent behavior, morphological appearances, the expression of specific MSCs surface markers, and the ability for tri-lineage differentiation (Tan *et al.*, 2013). Visceral Rab-ADMSC showed fibroblast-like cells with two processes adhering to the bottom of culture plastic, as described in the previous study (Zomer *et al.*, 2018; Tirpakova *et al.*, 2021; Koung Ngeun *et al.*, 2023). Furthermore, the expression of stemness-related markers (*NANOG* and *SOX2*) and senescence-related markers (*CDKN1A* and *TP53*) indicates the multipotent, proliferative, and senescence characteristics of Visceral Rab-ADMSC. *OCT4* and *SOX2* are normally expressed in embryonic and adult stem cells. In the early passage of MSCs, both stemness-related markers are usually expressed at low levels, and as the number of passages increases, those markers eventually decreased. Co-expressing *OCT4* and *SOX2* in MSCs will improve their capacity for both proliferation and differentiation. Moreover, *OCT4* and *NANOG* are necessary to maintain the pluripotency of embryonic stem cells and MSC properties (Frese *et al.*, 2016). When expanded in vitro, MSCs inevitably undergo senescence and loss of stem cell characteristics, as described by low expression of *TP53* and *CDKN1A* (senescence markers) in the early passage, and tend to increase at late passage (Liu *et al.*, 2020; Gao *et al.*, 2023). *TP53* is a precursor of the P53 protein that induces the expression of *CDKN1A*, which

inhibits cell cycle progression (Matveeva *et al.*, 2024). Visceral Rab-ADMSC showed an elevated growth rate day by day, but the PDT increased, simultaneously with the increase of cell passage. The doubling times for rabbit adipose mesenchymal stem cells were previously reported to be 1.6 ± 0.2 days (Tirpakova *et al.*, 2021), 3.5 days (Koung Ngeun *et al.*, 2023), and 1.2 days (Zomer *et al.*, 2018).

This current study's findings revealed the negative expression of hematopoietic surface marker of CD45 and high expression MSC surface makers of CD9, and these results are in agreement with previous results on rabbit adipose MSC obtained from subcutaneous tissue (Koung Ngeun *et al.*, 2023). CD9 is a key player in intercellular communication and is expressed in human MSCs and exosomes produced from MSCs (Hsieh *et al.*, 2021). The first description of a high proportion of CD9 in rabbits was published by Koung Ngeun *et al.* (2023). The detailed comparison of the current study on the MSC surface markers study compared to other studies is summarized in Table 3. Contrary to previous studies that reported the high expression of CD105, CD90, and CD73 (Tan *et al.*, 2013; Zomer *et al.*, 2018; Almaliotis *et al.*, 2021), these three surface markers were dimly expressed in the current study. It might have occurred due to the discrepancy of antibody used in this study. Similar results were also shown by the previous study on the low expression of CD13, CD133,

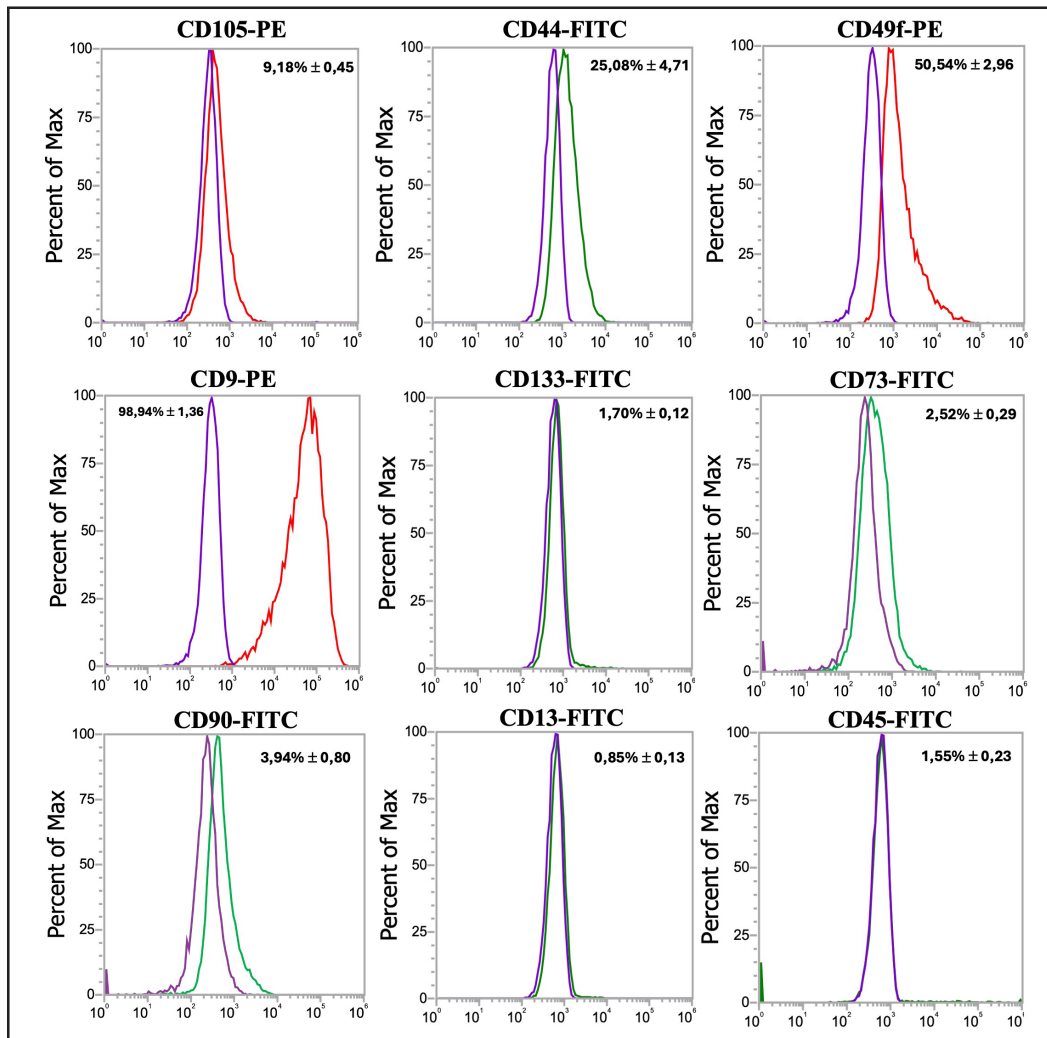


Fig. 2. Representative histograms showing the expression of CD45 as a hematopoietic surface marker and CD9, CD44, CD49f, CD13, CD45, CD73, CD90, CD105, and CD133 MSC surface markers. The purple histogram represents isotype control, the green histogram represents FITC-conjugated antibodies, and the red histogram represents PE-conjugated antibodies.

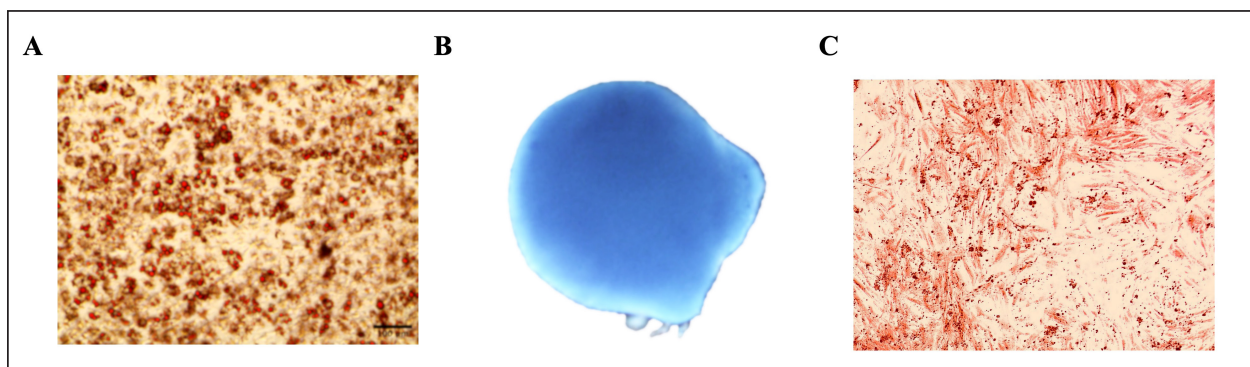


Fig. 3. Multilineage differentiation staining. Adipogenic staining with oil red O revealed red-stained lipid droplets. (A) Chondrogenic staining with Alcian Blue revealed a uniform blue color indicating glycosaminoglycans and cartilage-specific extracellular matrix, (B) whereas osteogenic staining with Alizarin Red revealed a calcium deposit (C).

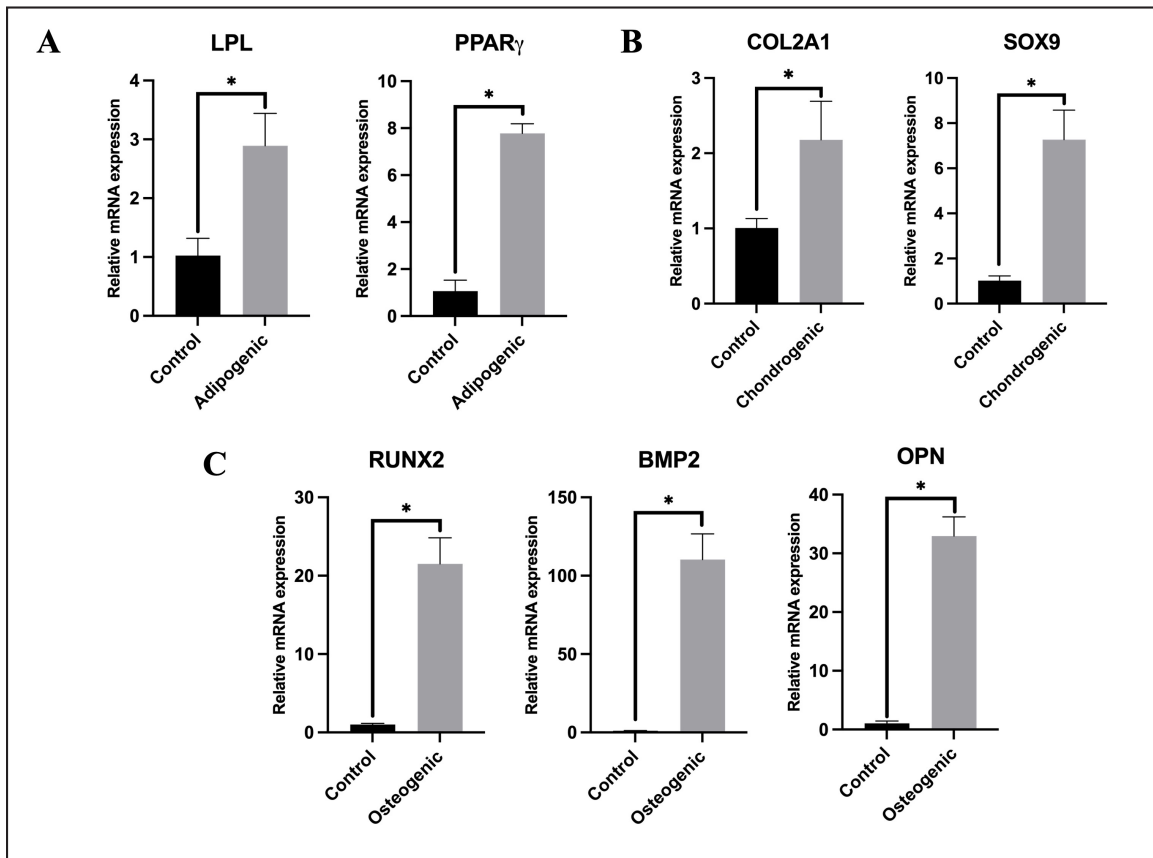


Fig. 4. Gene expression of multilineage differentiation. (A) Adipogenic-related genes (*LPL* and *PPAR γ*). (B) Chondrogenic-related genes (*COL2A1* and *SOX9*). (C) Osteogenic-related genes (*RUNX2*, *BMP2*, and *OPN*).

CD105, CD90, and CD73 MSC surface markers (Martinez-Lorenzo *et al.*, 2009; Lee *et al.*, 2014). The moderate expression of CD49f and CD44 was still lower compared to the previous study (Martinez-Lorenzo *et al.*, 2009; Lee *et al.*, 2014; Tirpakova *et al.*, 2021). CD49f marker (integrin $\alpha 6$) is associated with cell pluripotency (Tirpakova *et al.*, 2021) and has high expression only at early passage (Martinez-Lorenzo *et al.*, 2009). In this study, the expression of CD49f is moderate, which might be caused by the higher passage compared to a previous study (Martinez-Lorenzo *et al.*, 2009). Nonetheless, it is important to note that the discrepancy in cell surface expression of several markers might be affected by substances released by accessory cells during the earlier stages. In addition, the variation of culture and isolation method, species, and tissue origin may play an important role in the discrepancy of the expression of rabbit MSC surface marker (Martinez-Lorenzo *et al.*, 2009). The differentiation capability into osteocytes, adipocytes, and chondrocytes is an important requirement for the characteristics of MSCs. According to the prior study, visceral Rab-ADMSCs have tri-lineage differentiation properties, shown by the

phenotypic characteristic of the positive histo-chemical specific staining and the elevation of expression-associated genes (Zomer *et al.*, 2018; Tirpakova *et al.*, 2021; Koung Ngeun *et al.*, 2023). Visceral Rab-ADMSC exhibited the upregulation of *LPL* and *PPAR γ* expression as well as red-stained lipid droplets inside the cells after 14-day adipogenic induction. *PPAR γ* plays a crucial role in the development of lipid droplets into adipocytes by downregulating *Perilipin* and *CIDEA*. These two factors modulate the activity of lipolytic enzymes and remove triacylglycerol from cells also tuning the size of lipid droplet (Vachkova *et al.*, 2016). The upregulation expression of *PPAR γ* and *C/EBP α* led to the upregulation expression of adipogenesis-regulated factors such as *LPL* (Kim *et al.*, 2021). The expression of *LPL* mRNA as an essential factor of adipogenic differentiation was detected at the initial stage of induction and will be stable in the late induction, resulting in mature adipocytes (Hu *et al.*, 2018). Chondrogenic gene-related marker expressions *COL2A1* and *SOX9* are upregulated and stained positively with Alcian Blue, which was performed in this study. During chondrogenic induction, at the early stage of the induction, the expression of *Col1I* and

Table 3. Specific MSC surface marker expression.

Reference	Current study	Lee et al. (2014)	Tirpakova et al. (2021)	Martinez-Lorenzo et al. (2009)	Zomer et al. (2018)	Tan et al. (2013)	Koung Ngeun et al. (2023)	Almalotit et al. (2021)	Chen et al. (2020)
Tissue origin	Visceral adipose	Bone marrow	Subcutaneous adipose	Knee adipose/ subcutaneous adipose	Adipose	Bone marrow	Subcutaneous adipose	Inguinal adipose	Inguinal adipose
Passage	4-5	3	-	1	2	2-3	4	3-4	3
CD9	98.94 ± 1.36	-	-	-	-	-	92	-	-
CD13	0.85 ± 0.13	0.75 ± 0.57	-	65.3 ± 24.4	-	-	-	-	-
CD44	25.08 ± 4.71	96.97 ± 0.58	97.15 ± 1.45	38.3 ± 19.2	-	81.6	50	98.5	95
CD45	1.55 ± 0.23	0.85 ± 0.35	1.13 ± 0.40	-	4.5	18	2.4	-	0.31
CD49f	50.54 ± 2.96	0.50 ± 0.15	98.92 ± 0.86	84.2 ± 4.05	-	-	-	-	-
CD73	2.52 ± 0.29	0.52 ± 0.21	60.50 ± 7.37	1.6 ± 0.98	54	96.4	-	99.8	-
CD90	3.94 ± 0.80	1.17 ± 0.41	95.97 ± 3.17	40.1 ± 15.4	48	96.9	-	97	-
CD105	9.18 ± 0.45	1.64 ± 0.64	4.60 ± 0.86	20.5 ± 11.4	99.9	-	-	99.3	-
CD133	1.70 ± 0.12	1.24 ± 0.41	-	13.5 ± 7.44	-	-	-	-	-

Fibronectin was upregulated and reached the highest level during the chondrogenic differentiation (middle stage). The expression of *Col1* and *Fibronectin* was subsequently decreased and replaced by higher *COL2A1* expression at the late stage (ECM-related genes) (Tang et al., 2015; Robert et al., 2020). *SOX9* is essential for the chondrogenic lineage development of MSCs. This activity is triggered when phosphorylated *SOX9* directly binds to gene targets, including *Collagen type II, IX, and XI, Aggrecan, and Link Protein*, thereby causing their transcriptional activity. *SOX9* can be found in both chondroprogenitors and mature chondrocytes, and it will directly upregulate the gene expression, specifically in cartilaginous matrix deposition (Sahu et al., 2020; Yang et al., 2022). Osteogenic differentiation potency was assessed by the upregulation of *RUNX2, BMP2, and OPN*, as well as calcium deposition was stained after 21-day induction. Hedgehog, Wnt/ β -catenin, BMP signaling, hormones, growth factors, and epigenetic regulators were all elevated during the early stages of osteogenesis. The osteogenic key factor of *RUNX2* was upregulated during the induction. *RUNX2* is a precursor of osteogenic-related marker, essential for the differentiation of osteoblasts and for controlling the expression of other osteogenic genes, such as *Col1, OPN, and ALP* (Mohamed-Ahmed et al., 2018; Robert et al., 2020). Furthermore, *BMP2* promotes the osteogenic development of MSCs by upregulating mitochondrial activity and *PGC-1 α* levels (Li et al., 2022; Koung Ngeun et al., 2023).

Conclusion

In conclusion, the research offers a comprehensive understanding of the characteristic and differentiation potency of visceral Rab-ADMSC, which has a fibroblast-like shape morphology and plastic adherent. Visceral Rab-ADMSC has the proliferative capability as well as expression of senescence- and stemness-related markers such as *NANOG, SOX2, CDKN1A, and TP53*. The evaluation of mesenchymal stem cell surface markers reveals that the visceral Rab-ADMSC performs high expression of CD9, moderate expression of CD44 and CD49f, low expression of CD105, CD90, and CD73, and negative expression of CD13 and CD133 as well as CD45 as a hematopoietic stem cell marker. The visceral Rab-ADMSC fulfills the phenotype and genotype characterization multilineage differentiation into adipogenic, chondrogenic, and osteogenic lineage. Thus, this study emphasizes the necessity for the establishment of standard protocols to address the discrepancy of phenotype and genotype characterization of rabbit MSC to support reproducibility in biomedical and translational research.

Acknowledgment

The authors would like to thank the Research Center for Vaccine Technology and Development (RCVTD),

Institute of Tropical Diseases, Universitas Airlangga, for providing the facilities for this study. This study was supported by grants from the Directorate of Research, Technology and Community Service, Ministry of Education, Culture, Research and Technology Republic of Indonesia through Regular—Fundamental Research Grant (Penelitian Fundamental – Reguler) 2024 with grant number 1712/B/UN3.LPPM/PT.01.03/2024.

Conflict of interest

The authors declare that they have no conflict of interest.

Funding

This study was supported by grants from the Directorate of Research, Technology and Community Service, Ministry of Education, Culture, Research and Technology Republic of Indonesia through Regular—Fundamental Research Grant (DRTPM, Penelitian Fundamental – Reguler) 2024 with grant number 1712/B/UN3.LPPM/PT.01.03/2024.

Authors' contribution

AA: performed the experiment, analyzed data, and wrote the manuscript. SK and WR: designed, conceptualized, and supervised the study, and wrote the manuscript. ISY and LS: performed adipose tissue isolation and conceptualized the study. HS and DD: performed visceral Rab-ADMSC processing, maintained cell culture work, and analyzed the data. All authors have read and approved the final manuscript.

Data availability

All data are provided in the manuscript.

References

- Almaliotis, D., Thomas, A., Komnenou, A., Gounari, E., Almpandou, S., Siempis, T., Papaioannou, N., Koliakos, G., Papakonstantinou, E., Sotiropoulos, K. and Karampatakis, V. 2021. Evaluation of clinical and histological outcomes of adipose-derived mesenchymal stem cells in a rabbit corneal alkali burn model. *Stem Cells Int.* 2021, 6610023.
- Calle, A., Zamora-Ceballos, M., Barcena, J., Blanco, E. and Ramirez, M.A. 2022. Comparison of Biological Features of Wild European Rabbit Mesenchymal Stem Cells Derived from Different Tissues. *Int. J. Mol. Sci.* 23(12), 6420.
- Cawthorn, W.P., Scheller, E.L. and MacDougald, O.A. 2012. Adipose tissue stem cells meet preadipocyte commitment: going back to the future. *J. Lipid. Res.* 53(2), 227–246.
- Chen, G., Zhang, W., Zhang, K., Wang, S., Gao, Y., Gu, J., He, L., Li, W., Zhang, C., Zhang, W., Li, M., Hao, Q. and Zhang, Y. 2020. Hypoxia-induced mesenchymal stem cells exhibit stronger tenogenic differentiation capacities and promote patellar tendon repair in rabbits. *Stem Cells Int.* 2020, 8822609.
- Council, N.R. 2010. Guide for the care and use of laboratory animals Eighth Edition (Eighth ed.). Washington, DC: The National Academies Press.
- Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D. and Horwitz, E. 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8(4), 315–317.
- Esteves, P.J., Abrantes, J., Baldauf, H.M., BenMohamed, L., Chen, Y., Christensen, N., Gonzalez-Gallego, J., Giacani, L., Hu, J., Kaplan, G., Keppler, O.T., Knight, K.L., Kong, X. P., Lanning, D.K., Le Pendu, J., de Matos, A.L., Liu, J., Liu, S., Lopes, A.M., Mage, R. 2018. The wide utility of rabbits as models of human diseases. *Exp. Mol. Med.* 50(5), 1–10.
- Frese, L., Dijkman, P.E. and Hoerstrup, S.P. (2016). Adipose tissue-derived stem cells in regenerative medicine. *Transfus. Med. Hemother.* 43(4), 268–274.
- Gao, Y., Chi, Y., Chen, Y., Wang, W., Li, H., Zheng, W., Zhu, P., An, J., Duan, Y., Sun, T., Liu, X., Xue, F., Liu, W., Fu, R., Han, Z., Zhang, Y., Yang, R., Cheng, T., Wei, J. and Zhang, X. 2023. Multi-omics analysis of human mesenchymal stem cells shows cell aging that alters immunomodulatory activity through the downregulation of PD-L1. *Nat. Commun.* 14(1), 4373.
- Guneta, V., Tan, N.S., Chan, S.K., Tanavde, V., Lim, T.C., Wong, T.C. and Choong, C. 2016. Comparative study of adipose-derived stem cells and bone marrow-derived stem cells in similar microenvironmental conditions. *Exp. Cell Res.* 348(2), 155–164.
- Hsieh, C.C., Hsu, S.C., Yao, M. and Huang, D.M. 2021. CD9 upregulation-decreased CCL21 secretion in mesenchymal stem cells reduces cancer cell migration. *Int. J. Mol. Sci.* 22(4), 1738.
- Hu, X., Tang, J., Hu, X., Bao, P., Pan, J., Chen, Z. and Xian, J. 2018. MiR-27b impairs adipocyte differentiation of human adipose tissue-derived mesenchymal stem cells by targeting LPL. *Cell Physiol. Biochem.* 47(2), 545–555.
- Jurgens, W.J., Oedayrajsingh-Varma, M.J., Helder, M.N., Zandiehoulabi, B., Schouten, T.E., Kuik, D.J., Ritt, M.J. and van Milligen, F.J. 2008. Effect of tissue-harvesting site on yield of stem cells derived from adipose tissue: implications for cell-based therapies. *Cell Tissue Res.* 332(3), 415–426.
- Kim, J.Y., Park, E.J., Kim, S.M. and Lee, H.J. 2021. Optimization of adipogenic differentiation conditions for canine adipose-derived stem cells. *J. Vet. Sci.* 22(4), e53.
- Koung Ngeun, S., Shimizu, M. and Kaneda, M. 2023. Characterization of rabbit mesenchymal stem/stromal cells after cryopreservation. *Biology (Basel)*, 12(10), 1312.
- Lee, T.C., Lee, T.H., Huang, Y.H., Chang, N.K., Lin, Y.J., Chien, P.W., Yang, W.H. and Lin, M.H. 2014.

- Comparison of surface markers between human and rabbit mesenchymal stem cells. *PLoS One* 9(11), e111390.
- Li, Y., Fu, G., Gong, Y., Li, B., Li, W., Liu, D. and Yang, X. 2022. BMP-2 promotes osteogenic differentiation of mesenchymal stem cells by enhancing mitochondrial activity. *J. Musculoskelet. Neuronal. Interact.* 22(1), 123–131.
- Liu, J., Ding, Y., Liu, Z. and Liang, X. 2020. Senescence in mesenchymal stem cells: functional alterations, molecular mechanisms, and rejuvenation strategies. *Front. Cell Dev. Biol.* 8, 258.
- Martinez-Lorenzo, M.J., Royo-Canas, M., Alegre-Aguaron, E., Desportes, P., Castiella, T., Garcia-Alvarez, F. and Larrad, L. 2009. Phenotype and chondrogenic differentiation of mesenchymal cells from adipose tissue of different species. *J. Orthop. Res.* 27(11), 1499–1507.
- Matveeva, D., Kashirina, D., Ezdakova, M., Larina, I., Buravkova, L. and Ratushnyy, A. 2024. Senescence-associated alterations in matrix of mesenchymal stem cells. *Int. J. Mol. Sci.* 25(10), 5332.
- Mohamed-Ahmed, S., Fristad, I., Lie, S. A., Suliman, S., Mustafa, K., Vindenes, H. and Idris, S. B. 2018. Adipose-derived and bone marrow mesenchymal stem cells: a donor-matched comparison. *Stem Cell Res. Ther.* 9(1), 168.
- Peptan, I.A., Hong, L. and Mao, J.J. 2006. Comparison of osteogenic potentials of visceral and subcutaneous adipose-derived cells of rabbits. *Plast. Reconstr. Surg.* 117(5), 1462–1470.
- Riester, S.M., Denbeigh, J.M., Lin, Y., Jones, D.L., de Mooij, T., Lewallen, E.A., Nie, H., Paradise, C.R., Radcliff, D.J., Dudakovic, A., Camilleri, E.T., Larson, D.R., Qu, W., Krych, A. J., Frick, M.A., Im, H.J., Dietz, A.B., Smith, J. and van Wijnen, A.J. 2017. Safety studies for use of adipose tissue-derived mesenchymal stromal/stem cells in a rabbit model for osteoarthritis to support a phase I clinical trial. *Stem Cells Transl. Med.* 6(3), 910–922.
- Robert, A.W., Marcon, B.H., Dallagiovanna, B. and Shigunov, P. 2020. Adipogenesis, osteogenesis, and chondrogenesis of human mesenchymal stem/stromal cells: a comparative transcriptome approach. *Front. Cell Dev. Biol.* 8, 561.
- Sahu, N., Budhiraja, G. and Subramanian, A. 2020. Preconditioning of mesenchymal stromal cells with low-intensity ultrasound: influence on chondrogenesis and directed SOX9 signaling pathways. *Stem Cell Res. Ther.* 11(1), 6.
- Schafrum Macedo, A., Cezaretti Feitosa, C., Yoiti Kitamura Kawamoto, F., Vinicius Tertuliano Marinho, P., Dos Santos Dal-Bo, I., Fiuza Monteiro, B., Prado, L., Bregadioli, T., Antonio Covino Diamante, G. and Ricardo Auada Ferrigno, C. 2019. Animal modeling in bone research-Should we follow the White Rabbit? *Animal Model. Exp. Med.* 2(3), 162–168.
- Tan, S.L., Ahmad, T.S., Selvaratnam, L. and Kamarul, T. 2013. Isolation, characterization and the multi-lineage differentiation potential of rabbit bone marrow-derived mesenchymal stem cells. *J. Anat.* 222(4), 437–450.
- Tang, X., Fan, L., Pei, M., Zeng, L. and Ge, Z. 2015. Evolving concepts of chondrogenic differentiation: history, state-of-the-art and future perspectives. *Eur. Cell Mater.* 30, 12–27.
- Tang, Y., Pan, Z.Y., Zou, Y., He, Y., Yang, P.Y., Tang, Q.Q. and Yin, F. 2017. A comparative assessment of adipose-derived stem cells from subcutaneous and visceral fat as a potential cell source for knee osteoarthritis treatment. *J. Cell Mol. Med.* 21(9), 2153–2162.
- Tirpakova, M., Vasicek, J., Svoradova, A., Balazi, A., Tomka, M., Bauer, M., Makarevich, A. and Chrenek, P. 2021. Phenotypical characterization and neurogenic differentiation of rabbit adipose tissue-derived mesenchymal stem cells. *Genes (Basel)*, 12(3), 431.
- Torres, F.C., Rodrigues, C.J., Stocchero, I.N. and Ferreira, M.C. 2007. Stem cells from the fat tissue of rabbits: an easy-to-find experimental source. *Aesthetic. Plast. Surg.* 31(5), 574–578.
- Vachkova, E., Bosnakovski, D., Yonkova, P., Grigorova, N., Ivanova, Z., Todorov, P., Penchev, G., Milanova, A., Simeonova, G., Stanilova, S. and Georgiev, I. P. 2016. Adipogenic potential of stem cells derived from rabbit subcutaneous and visceral adipose tissue in vitro. *In Vitro Cell Dev. Biol. Anim.* 52(8), 829–837.
- Via, A.G., Frizziero, A. and Oliva, F. 2012. Biological properties of mesenchymal stem cells from different sources. *Muscles Ligaments Tendons J.* 2(3), 154–162.
- Wright, A., Arthaud-Day, M.L. and Weiss, M.L. 2021. Therapeutic use of mesenchymal stromal cells: the need for inclusive characterization guidelines to accommodate all tissue sources and species. *Front. Cell Dev. Biol.* 9, 632717.
- Yang, X., Tian, S., Fan, L., Niu, R., Yan, M., Chen, S., Zheng, M. and Zhang, S. 2022. Integrated regulation of chondrogenic differentiation in mesenchymal stem cells and differentiation of cancer cells. *Cancer Cell Int.* 22(1), 169.
- Zomer, H.D., Roballo, K.C., Lessa, T.B., Bressan, F.F., Goncalves, N.N., Meirelles, F.V., Trentin, A.G. and Ambrosio, C.E. 2018. Distinct features of rabbit and human adipose-derived mesenchymal stem cells: implications for biotechnology and translational research. *Stem Cells Cloning* 11, 43–54.