Research Article *Pamukkale Medical Journal*

Received: 24.04.2025

Accepted: 10.07.2025

Area of Expertise: Histology and Embryology

Title: Characterization and comparison of mesenchymal stem cells derived from rat perirenal and periovarian adipose tissue.

Short title: Comparison of Rat MSCs from perirenal and periovarian adipose tissues.

Abstract

Purpose: In rats, adipose tissue with white unilocular features is found in the perigonadal region. Adipose tissue contains adipocytes, adipocyte progenitor cells, mesenchymal stem cells, fibroblasts, endothelial cells, pericytes, and immune cells. Mesenchymal stem cells are multipotent stem cells and have been isolated from many tissues, including adipose tissue and connective tissue. Our aim in this study is to isolate mesenchymal stem cells from perirenal adipose tissue and periovarian adipose tissue and compare their features with each other.

Materials and methods: Adipose tissues around the ovaries and kidneys of two 6-week-old Wistar Albino rats were used. Adipose tissue was cut into small pieces, and explant cell culture was established. Flow cytometry analysis was performed at the third passage (P3) for the characterization of isolated and produced mesenchymal stem cells. CD29, CD54, CD90, CD45, and MHC Class II were used as stem cell markers. mRNA expression changes of *CDK6*, *Cyclin-D1*, *p21*, *caspase-3*, *caspase-8*, and *caspase-9* genes involved in cell cycle regulation and apoptosis were examined in cell groups.

Results: Adherent cells that proliferated in explant cell culture were isolated and multiplied. In the flow cytometry analysis, it was observed that the cells expressed CD29, CD54, and CD90 markers, but did not express the cell surface antigens CD45 and MHC Class II. In the analyses performed with perirenal adipose tissue and periovarian adipose tissue-derived mesenchymal stem cells, no statistically significant difference was found in the expression of genes involved in the regulation of the cell cycle and apoptosis.

Conclusion: The use of adipose tissue-derived mesenchymal stem cells from different regions, which have similar molecular properties to periovarian adipose tissue-derived mesenchymal stem cells, in ovarian failures may result in better development of follicles and the acquisition of quality oocytes.

Keywords: Adipose tissue, cell culture, infertility, ovary, mesenchymal stem cell

Makale başlığı: Sıçan perirenal ve perioveryan adipoz doku kaynaklı mezenkimal kök hücrelerin karakterizasyonu ve birbirleriyle karşılaştırılması.

Kısa başlık: Sıçan perirenal ve periovaryan adipoz doku kaynaklı mezenkimal kök hücrelerin karşılaştırılması.

Öz

Amaç: Sıçanlarda, perigonodal bölgede beyaz unilokuler özellikte olan adipoz doku bulunur. Adipoz doku, adipositler yanında adiposit progenitör hücreler, mezenkimal kök hücreler, fibroblastlar, endotel hücreleri, perisitler ve immün hücreleri içerir. Mezenkimal kök hücreler multipotent özellikte olan kök hücrelerdir ve şimdiye kadar adipoz doku dahil olmak üzere bağ dokusu içeren birçok dokudan izole edilmiştirler. Bu çalışmadaki amacımız perirenal adipoz dokudan ve perioveryan adipoz dokudan mezenkimal kök hücreleri izole ederek özelliklerini birbirleriyle karşılaştırmaktır.

Gereç ve Yöntem: İki adet 6 haftalık Wistar Albino tipi sıçanın ovaryum ve böbrek çevresindeki adipoz dokuları çıkarıldı. Adipoz doku küçük parçalara ayrılarak eksplant hücre kültürü oluşturuldu. İzole edilip üretilen mezenkimal kök hücrelerin karakterizasyonu için üçüncü pasajda (P3) flow sitometri analizi yapıldı. Kök hücre belirteçleri olarak CD29, CD54, CD90, CD45 ve MHC Class II kullanıldı. Hücre döngüsünün regülasyonunda ve apoptozda rol alan genlerde *CDK6*, *Cyclin-D1*, *p21*, *caspase-3*, *caspase-8*, *caspase-9* genlerinin hücre gruplarındaki mRNA ekspresyon değişimleri incelendi.

Bulgular: Eksplant hücre kültüründe prolifere olan adherent özellikteki hücreler izole edildi ve çoğaltıldı. Yapılan flow sitometri analizinde hücrelerin CD29, CD54 ve CD90 belirteçlerini eksprese ettikleri, buna karşılık hücre yüzey antijenleri olan CD45 ve MHC Class II'yi eksprese etmedikleri gözlemlenmiştir. Perirenal adipoz doku ve perioveryan adipoz doku kökenli mezenkimal kök hücrelerle yapılan analizlerde hücre döngüsünün regülasyonunda ve apoptozda rol alan genlerin ekspresyonlarında istatistiksel olarak bir farklılık bulunamadı.

Sonuç: Perioveryan adipoz doku kaynaklı mezenkimal kök hücrelere benzer moleküler özelliklere sahip, farklı bölgelerdeki adipoz doku kökenli mezenkimal kök hücrelerin overyan yetmezliklerde kullanılması foliküllerin daha iyi gelişim göstermesine ve kaliteli oositlerin elde edilmesine neden olabilir.

Anahtar kelimeler: Adipoz doku, hücre kültürü, infertilite, ovaryum, mezenkimal kök hücre.

Introduction

MSCs are adult multipotent stem cells that can be isolated from several different tissues, including bone marrow, cartilage, adipose tissue (AT), liver, placenta, umbilical cord, and amniotic membrane. The discovery that AT is a rich source of MSCs has prompted a shift in focus towards this tissue in the study. In rats, AT is isolated from multiple sites, including subcutaneous, inguinal, perirenal, peri-epididymal, and periovarian regions.

In rats, AT surrounds the ovary, with follicles located in the cortex of the ovary, thereby creating a microenvironment for oocytes. The development of follicles is dependent on several factors, including cell-cell interactions, hormonal regulation, and other biological processes. Following puberty, some follicles commence growth and development, forming primary, secondary, and Graafian follicles [1-4]. It has been demonstrated that stromal cells, surface epithelial cells, and fibroblast-like cells within the tunica albuginea contribute to follicle development through cell-cell interactions and the secretion of paracrine factors.

Furthermore, periovarian AT plays a pivotal role in follicle development and whole-body lipid metabolism through the adipokine hormones it secretes [5]. The objective of this study was to isolate MSCs from perirenal and periovarian AT and perform a comparative analysis of their properties. Another objective was to examine the potential utility of AT-derived MSCs from other regions with molecular profiles analogous to periovarian tissue-derived MSCs in cases of ovarian failure.

Materials and methods

Our study was approved by the Pamukkale University Animal Experimentation Ethics Committee with decision number E.348411 dated 24.03.2023. In this study, MSCs were isolated from rat perirenal and periovarian AT by the explant culture method [6].

For this purpose, the ovaries and kidneys of two 6-week-old Wistar albino rats were removed, along with the surrounding AT, through a midline incision in the abdominal wall under general anesthesia. Perirenal adipose tissue is distributed bilaterally around the kidney and is encapsulated by a multilayered fibrous membrane known as renal fascia. Periovarian adipose tissue surrounds a large part of the ovary. The AT surrounding the ovary and kidney was meticulously excised under the guidance of a stereomicroscope. The periovarian and perirenal ATs were washed in phosphate-buffered saline (PBS) and placed in collection tubes containing Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA), penicillin-streptomycin (Capricorn Scientific GmbH, Germany) and fetal

bovine serum (FBS) (Capricorn Scientific GmbH, Germany). The samples were subsequently transferred to the laboratory per the requisite sterile conditions.

AT was minced into the smallest separable pieces with sterile tissue scissors and placed in a 100 mm petri dish containing complete medium (DMEM with 10% FBS, 50 U/ml penicillin, and 50 µg/mL streptomycin) for growth. On the second day following the procedure, the migration and proliferation of cells from AT were observed and documented. Once the cells reached 70-80% confluence on the eighth and tenth days, the cells that had adhered to the bottom of the culture dishes were removed through trypsinization with 0.25% trypsin (Hyclone, USA). Subsequently, the cells were seeded at a ratio of 1:3 on other plates and underwent repeated passages to facilitate proliferation. All of the steps mentioned above were observed using an inverted microscope. The proliferating cells were stained with trypan blue (Bio-Vision, USA) and enumerated using a Neubauer Improved chamber.

In the P3, a flow cytometric analysis was conducted to characterize the MSCs. The analysis included an examination of stem cell surface markers, including CD29, CD54, CD90, CD45, and MHC class II. To demonstrate the adipogenesis, osteogenesis, and chondrogenesis potential of isolated and characterized MSCs, StemPro Adipogenesis Differentiation Kit, StemPro Osteogenesis Differentiation Kit, and StemPro Chondrogenesis Differentiation Kit (all ready-to-use kits obtained from Gibco, Invitrogen Cell Culture, Carlsbad, CA, USA), respectively, were used according to the manufacturer's protocols. Adipogenesis is demonstrated by Oil Red O staining, osteogenesis is demonstrated by Alizarin Red S staining, and chondrogenesis is demonstrated by Alcian Blue staining (all from Sigma–Aldrich Chemie GmbH, Taufkirchen, Germany) [7].

The rat ovaries were fixed in 10% neutral buffered formaldehyde for a period of 72 hours. Following this, a routine tissue processing protocol was initiated, and the tissues were embedded in paraffin blocks (FFPE). Haematoxylin and eosin staining was performed on 5 µm sections of FFPE ovarian tissues.

Total RNA isolation, quality control of RNA samples, cDNA synthesis and RT-PCR analysis

RNA was extracted from periovarian and perirenal AT-derived MSCs at the P3. The cells were seeded in 6-well cell culture plates at a density of $3x10^5$ cells per well and incubated for 24 hours. TRIzol Reagent (Thermo Fisher Scientific, USA) (500 µl) was added to each well to lyse the cells. Then, 100 µl of chloroform was added to each tube, and the tubes were subjected to centrifugation at 15,000g for 15 minutes at 4°C. The colorless supernatant, which contained the RNA, was then collected and transferred to

separate micro tubes. To the aforementioned supernatant, 250 µL of isopropanol was added. The tubes were then subjected to centrifugation at 15,000 rpm for 10 minutes at +4°C. The supernatant was then carefully discarded, and 70% ethanol was added to the pellet, which was then subjected to centrifugation at 12.000g for 10 minutes at +4°C. Subsequently, the pellet was dissolved in 40 µL of RNase- and DNase-free water.

RNA quality was detected using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, USA). The A260/A280 and A260/A230 ratios were evaluated to assess the potential contamination of the isolated RNA by phenolic compounds, proteins, and genomic DNA. For mRNA expression analysis, cDNA synthesis was conducted. The synthesis of cDNA was conducted using the High-Capacity Kit (Cat. No. C03-01-20, ABT, Türkiye), which employs random hexamers, dNTPs, and a reverse transcriptase (RT) enzyme, under the manufacturer's instructions (see Table 1).

The expression levels of cell cycle regulatory genes and apoptosis-related genes at the mRNA level were evaluated using an RT-PCR system. SYBR Green, which can bind to double-stranded DNA, was employed as a dye. The reaction was conducted using 5X SYBR Green mix (ABT), 5 pMol forward primer (F), 5 pMol reverse primer (R), 2 µl cDNA, and sterile RNase-DNase-free water to a total volume of 20 µl, resulting in a final concentration of 1X. This mixture was prepared in sterile, instrument-compatible 96-well plates. The temperature profile of the reaction was set to 95°C for 15 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 20 seconds, and 72°C for 20 seconds. Gradual heating was performed from 60°C to 95°C with 0.5°C increments, and melting curve graphs were generated by melting curve analysis with optical measurements at each 0.5°C increment within the temperature range. The data obtained from the optical analysis of the RT-PCR were recorded as the cycle threshold (Ct), and the expression levels of the target genes were normalized to the reference gene, beta-actin (see Table 2).

Caspase-3, *-8*, and *-9*, *Cyclin D1*, *p21*, and *CDK* genes were selected for this study because they have critical roles in cell death (apoptosis) and cell cycle regulation [8-11].

Statistical analysis

The RT-PCR data will be subjected to analysis using the ΔΔCT method, with quantification performed using a computer program. Volcano plot analysis is conducted using the web-based program RT² Profiler™ PCR Array Data Analysis (Qiagen). This method entails a comparison of two expression results based on a ±3SD range. In comparing gene expression, the relative expression values of the pertinent genes in the control and dose groups are determined. The statistical evaluation of the group

comparisons is conducted using the Student's t-test analysis within the RT² Profiler™ PCR Array Data Analysis program.

Results

The method used to isolate MSCs was the explant culture technique. The tissues were then cut into small pieces and seeded on plates without the use of any enzymatic methods for this process (Figure 1). In histological examination of both perirenal adipose tissue and ovarian adipose tissue, it predominantly contained a single large lipid droplet, while sometimes multi-chambered fat cells were irregularly distributed or clustered in small groups. The nuclei of these cells were found in the periphery. Histological sections demonstrated that the ovary was surrounded by AT (Figure 2). The cells that migrated from the periovarian and perirenal ATs exhibited proliferation and confluence on days 8 and 10, respectively (Figure 3A-3D). The number of cells isolated from periovarian and perirenal AT was $1.6x10^6$ and $1.4x10^6$, respectively (Figure 4A-4B).

Subsequently, the cells were subjected to serial passaging for proliferation. Flow cytometric analysis of the cells at the P3 revealed that the cell surface markers CD45 and MHC class II were negative, while CD29, CD54, and CD90 were positively expressed (Figure 5A-5B).

Adipogenic, chondrogenic and osteogenic differentiation of stem cells were shown. Cultured cells were differentiated into adipocytes, chondrocytes and osteocytes by oil red, alcian blue and alizarin red dyes respectively (Figure 6A-6C).

RT-PCR analysis

Following the isolation of total RNA from periovarian and perirenal tissue-derived MSCs, cDNA synthesis was conducted to examine the mRNA expression changes of *Caspase-3*, *Caspase-8*, *Caspase-9*, *p21*, *CDK6*, and *Cyclin D1* genes via RT-PCR. The results demonstrated that *caspase-3* mRNA expression exhibited a 1.33-fold increase, while *caspase-9* expression demonstrated a 3.61-fold increase in perirenal tissue-derived MSCs relative to periovarian tissue-derived MSCs. In contrast, there was minimal alteration in *caspase-8* and *p21* mRNA expression. The expression of *Cyclin D1* mRNA was observed to decrease by a factor of 1.52, while *CDK6* expression decreased by a factor of 4.66 in perirenal tissue-derived MSCs. However, these changes were not statistically significant (Table 3).

A heat map illustrating the gene expression changes in periovarian and perirenal tissue-derived MSCs is presented in (Figure 7).

Discussion

AT contains a variety of cell types, including adipocyte precursors at different maturation stages, such as less differentiated stromal-vascular cells. In some ovarian regions, a fibrous sheath containing fibroblasts is situated in close proximity to the surface epithelium. The aforementioned sheath is situated between the periovarian AT and the ovarian surface epithelium. The development of follicles is influenced by interactions between multiple cell types, including ovarian stromal cells, cells in the periovarian AT, surface epithelial cells, and fibroblast-like cells in the tunica albuginea. These interactions are mediated by paracrine factors secreted by these cells [5].

By the recommendations of the International Society for Cell Therapy (ISCT), MSCs are defined by three characteristics:

1. The cells must adhere to plastic in standard culture conditions.

2. The cells are positive for CD105, CD73, and CD90, and negative for CD45 and CD34.

3. The capacity to differentiate into bone, fat, and cartilage cells in vitro.

AT functions as a dynamic endocrine organ, playing a pivotal role in metabolic regulation. The functional differences are associated with the regional distribution of AT. MSCs derived from AT are the subject of extensive research due to their potential for regenerative and tissue repair applications [12].

Hendawy et al. [13] isolated mesenchymal stem cells from adipose tissues in the periovarian, perirenal, and mesenteric regions and examined them. They detected non-significant differences in the morphology and immunophenotypes of mesenchymal stem cells taken from these regions. There are numerous techniques for the isolation of MSCs from AT, with the enzymatic method being the most prevalent. This method entails mincing the AT and utilizing a collagenase enzyme. An alternative approach is the explant tissue culture method, which was utilized in this study. This method offers several advantages, including easier cell isolation, reduced costs, the elimination of the need for enzymes (collagenase and trypsin), and minimal alteration in cell surface antigens [6, 14-18].

In rodents, white AT is situated in the peri-gonadal region. Although humans lack this peri-ovarian fat depot, its function is analogous to that of visceral omental AT. In addition to adipocytes, AT comprises other cell types, including adipocyte progenitor cells, fibroblasts, endothelial cells, pericytes, and immune cells. As a consequence of advanced reproductive age, there is an increase in the size of the adipocytes in the periovarian AT, which results in a reduction in the number of adipocytes per unit area [19].

AT, the primary energy storage organ, is composed of two distinct types: white AT and brown AT. Each type serves a unique function. The surgical removal of unilateral periovarian AT in prepubertal mice has been observed to result in delayed antral follicle development and an increased number of atretic follicles in the ovaries. Additionally, it has been demonstrated that the removal of unilateral periovarian AT in prepubertal mice results in a reduction in the expression of adipokine hormones, growth factors, and steroidogenic enzymes within the ovaries [20].

Periovarian AT has a pivotal role in follicular development and systemic lipid metabolism. In studies on mice with bilateral periovarian AT removal, an increase in follicular atresia and a reduction in the expression of genes responsible for follicle development were observed [21].

It has been demonstrated that AT can contribute to the development of hyperandrogenism and metabolic issues in women with obesity. Wagner et al. [22] observed elevated testosterone levels in the periovarian AT of prepubertal rats relative to inguinal AT. In adult obese rats, testosterone levels in periovarian and inguinal ATs were found to be nearly equal.

Brown adipocytes are thermogenic cells that play a pivotal role in maintaining energy balance. Their function is driven by mitochondrial uncoupling protein (UCP) activity. It was previously assumed that brown adipocytes in rodents were confined to classical brown AT. It has been demonstrated that periovarian AT in rats maintained at 24°C contains cells with mitochondria that are characteristic of brown AT and lipid droplets with multiple openings, indicating that there is a potential for plasticity between brown and white ATs [23, 24].

Histological analysis of periovarian AT from obese mice revealed increased adipocyte size and a higher infiltration of immune cells. This indicates that diet-induced obesity increases the expression of inflammatory genes in both ovaries and periovarian AT, which may impair ovarian function [25].

Vitamin D plays a pivotal role in follicle development, and vitamin D3 metabolism is disrupted in polycystic ovary syndrome. In rats with letrozole-induced experimental polycystic ovary syndrome, the amount of 1,25(OH)2D3 is reduced in both ovaries and periovarian AT [26].

In an experimental menopause model created by bilateral ovariectomy and vitamin D3 restriction in mice, there was a notable increase in periovarian AT inflammation. The adipocytes in the periovarian AT of these mice exhibited hypertrophy, and the levels of proinflammatory cytokines and inflammatory markers increased systemically [27].

Pleiotrophin, a heparin-binding growth factor, is indispensable for the maintenance of insulin sensitivity, energy metabolism, and the regulation of AT lipids. In an experimental model of hepatic steatosis in pleiotrophin-deficient mice on a high-fat diet, periovarian AT transformed brown and multilocular adipocytes with elevated UCP-1 expression [28].

El Husseiny et al. [29] and colleagues isolated and characterized MSCs derived from inguinal, epididymal, perirenal, and subcutaneous AT in rats. The researchers reported that MSCs from epididymal and inguinal AT exhibited high capacities for adipogenic and osteogenic differentiation. Subcutaneous MSCs demonstrated a greater propensity for chondrogenic differentiation than other MSCs.

Another study demonstrated that estradiol and progesterone treatment did not affect plasma leptin levels in ovariectomized rats. Furthermore, the gene expressions of leptin and UCP1, 2, and 3 in subcutaneous, periovarian, mesenteric white AT, and interscapular brown AT remained unaltered. These findings suggest that steroids do not exert a substantial influence on AT metabolism regulation [30].

Hendawy et al. used periovarian, perirenal, mesenteric and omental regions to analyze adipose tissues. Then, they isolated and examined stromal vascular fraction (SVF) cells. The expression of mesenchymal stem cells and hematopoietic markers in the SVF cell subpopulation and their pluripotent gene expression profiles were evaluated. It was observed that CD34 expression in omental AT was significantly higher than in other anatomical regions. It was determined that omental AT had a high SVF cell fraction and living cells. CD90 and CD44 were highly expressed in SVF cells isolated from omental AT. It was also observed that Oct4, Sox2 and Rex-1 gene levels were higher 31].

So far, only a few studies have been conducted on the molecular characteristics of periovarian adipose tissue. In our study, no statistically significant difference was found between perirenal adipose tissue and periovarian adipose tissue in terms of cell cycle regulation and apoptotic gene expression. Additionally, it was demonstrated that mesenchymal stem cells could be efficiently obtained from both adipose tissues using the explant method. The reason for choosing the explant method was that, compared to other isolation techniques, it leads to fewer losses in cell surface antigens during mesenchymal stem cell extraction.

Studies have demonstrated that periovarian adipose tissue (AT) plays a pivotal role in the development of ovarian follicles in rats. In humans, periovarian AT is absent; yet, analogous functions are fulfilled by intraovarian AT and visceral AT in surrounding organs. An investigation into the effects of AT on ovarian function may yield new

approaches to infertility treatment. The utilization of AT-derived MSCs from disparate regions, which exhibit molecular similarities to periovarian tissue-derived MSCs, may prove beneficial in cases of ovarian failure, facilitating enhanced follicle development and improved oocyte quality.

In a previous study, we transplanted ovarian tissue containing periovarian adipose tissue in rats using a heterotopic method (unpublished data). There is a fibrous sheath along the surface epithelium of the ovary and visceral periovarian adipose tissue immediately adjacent to it. In our study, the tunica albuginea, fibrous sheath, and periovarian adipose tissue were transplanted as a whole with the ovary. Fourteen days after transplantation, it was observed that the periovarian adipose tissue had infiltrated well into the muscle tissue surrounding the ovary, and angiogenesis had occurred in both the ovarian tissue and the surrounding tissue. Hypoxia and ischemia following ovarian transplantation often lead to significant follicular losses. Based on our findings, periovarian adipose tissue plays a crucial role in preventing ovarian tissue ischemia and preserving the ovarian follicle reserve, without the need for scaffolds or bioengineering applications. These effects are caused by the proangiogenic effect of the mesenchymal cells of the periovarian adipose tissue, secreting bioactive molecules such as VEGF, EGF, bFGF, and HGF through a paracrine mechanism. In addition, adipokine hormones secreted by the adipose tissue play important roles in the development of follicles in the rat ovary.

In human heterotopic transplantation, co-transplanting ovarian cortex grafts with visceral adipose tissue after thawing may promote rapid angiogenesis, thereby preventing tissue necrosis. Additionally, the molecular similarities between mesenchymal stem cells derived from perirenal and periovarian adipose tissues suggest that they could be used interchangeably in clinical applications.

Acknowledgements: We also place on record our sense of gratitude to one and all who directly or indirectly, have lent their hand in this venture.

Funding: This study was supported by The Pamukkale University Scientific Research Projects Coordination Unit (Grant 2023HZDP005).

Authors' contributions: M.S.U., A.U., and S.T.G were involved in the design of the experiments and drafted the manuscript. M.S.U., N.C., S.T.G., E.O., and M.S were involved in data analysis. M.S.U., S.T., and G.M. reviewed and edited the manuscript. All authors read and approved the final version of the manuscript.

Conflict of interest: No conflict of interest was declared by the authors.

References

1. Ross MH, Pawlina W. Histology. Lippincott Williams & Wilkins; 2006.
2. Junqueira LCU, Carneiro J. Basic histology: text & atlas. Vol 3: McGraw-Hill New York; 2005.
3. Kierszenbaum AL, Tres L. Histology and cell biology: an introduction to pathology. (No Title). 2003.
4. Ünal MS, Tan S, Seçme M. Changes in structure during the corpus luteum's formation. Pam Med J. 2024;17(2):285-301. doi:10.31362/patd.1383988
5. Unal MS, Secme M. Does the ovarian surface epithelium differentiate into primordial follicle and primary follicle precursor structures? *Cukurova Med J.* 2022;47(3):1256-1262. doi:10.17826/cumj.1134852
6. Niyaz M, Gürpinar ÖA, Günaydin S, Onur MA. Isolation, culturing and characterization of rat adipose tissue-derived mesenchymal stem cells: a simple technique. *Turkish Journal of Biology.* 2012;36(6):658-664. doi:10.3906/biy-1109-31
7. Çil N, Yaka M, Ünal MS, et al. Adipose derived mesenchymal stem cell treatment in experimental asherman syndrome induced rats. *Mol Biol Rep*. 2020;47(6):4541-4552. doi:10.1007/s11033-020-05505-4
8. Kus G, Oztopcu-Vatan P, Uyar R, et al. Cytotoxic and apoptotic functions of licofelone on rat glioma cells. Acta Biol Hung. 2013;64(4):438–452. doi:10.1556/ABiol.64.2013.4.4
9. Sepehri B, Darbani R, Mesgari-Abbasi M, et al. The effects of short-time air pollution, SO_2, and ozone on biochemical, histo-pathological, oxidative stress, and carcinogenesis-related gene expressions in the liver of rats. Hum Exp Toxicol. 2024;43:9603271241263569. doi:10.1177/09603271241263569
10. Ashrafi M, Bathaie SZ, Abroun S. High expression of Cyclin D1 and p21 in N-nitroso-N-methylurea-induced breast cancer in Wistar albino female rats. Cell J. 2012;14(3):193–202.
11. Liu M, Li M, Liu J, et al. Elevated urinary urea by high-protein diet could be one of the inducements of bladder disorders. J Transl Med. 2016;14:53. doi:10.1186/s12967-016-0809-9
12. Baglioni S, Francalanci M, Squecco R, et al. Characterization of human adult stem-cell populations isolated from visceral and subcutaneous adipose tissue. *FASEB J*. 2009;23(10):3494-3505. doi:10.1096/fj.08-126946
13. Hendawy H, Kaneda M, Metwally E, Shimada K, Tanaka T, Tanaka R. A Comparative Study of the Effect of Anatomical Site on Multiple Differentiation of

Adipose-Derived Stem Cells in Rats. *Cells*. 2021;10(9):2469. doi:10.3390/cells10092469

14. Balducci L, Alessandri G. Isolation, Expansion, and Immortalization of Human Adipose-Derived Mesenchymal Stromal Cells from Biopsies and Liposuction Specimens. *Methods Mol Biol*. 2016;1416:259-274. doi:10.1007/978-1-4939-3584-0_15
15. Sherman LS, Condé Green A, Kotamarti VS, Lee ES, Rameshwar P. Enzyme-Free Isolation of Adipose-Derived Mesenchymal Stem Cells. *Methods Mol Biol*. 2018;1842:203-206. doi:10.1007/978-1-4939-8697-2_14
16. Li J, Li H, Tian W. Isolation of Murine Adipose-Derived Stromal/Stem Cells Using an Explant Culture Method. *Methods Mol Biol*. 2018;1773:167-171. doi:10.1007/978-1-4939-7799-4_14
17. Francis MP, Sachs PC, Elmore LW, Holt SE. Isolating adipose-derived mesenchymal stem cells from lipoaspirate blood and saline fraction. *Organogenesis*. 2010;6(1):11-14. doi:10.4161/org.6.1.10019
18. Zuk PA, Zhu M, Ashjian P, et al. Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell*. 2002;13(12):4279-4295. doi:10.1091/mbc.e02-02-0105
19. Dipali SS, Ferreira CR, Zhou LT, Pritchard MT, Duncan FE. Histologic analysis and lipid profiling reveal reproductive age-associated changes in peri-ovarian adipose tissue. *Reprod Biol Endocrinol*. 2019;17(1):46. doi:10.1186/s12958-019-0487-6
20. Yang L, Chen L, Lu X, et al. Peri-ovarian adipose tissue contributes to intraovarian control during folliculogenesis in mice. *Reproduction*. 2018;156(2):133-144. doi:10.1530/REP-18-0120
21. Zhu M, Shen Q, Li X, Kang J. Removal of peri-ovarian adipose tissue affects follicular development and lipid metabolism†. *Biol Reprod*. 2020;103(6):1199-1208. doi:10.1093/biolre/ioaa144
22. Wagner IV, Sahlin L, Savchuk I, Klöting N, Svechnikov K, Söder O. Adipose Tissue is a Potential Source of Hyperandrogenism in Obese Female Rats. *Obesity (Silver Spring)*. 2018;26(7):1161-1167. doi:10.1002/oby.22198
23. Cousin B, Cinti S, Morroni M, et al. Occurrence of brown adipocytes in rat white adipose tissue: molecular and morphological characterization. *J Cell Sci*. 1992;103(4):931-942. doi:10.1242/jcs.103.4.931
24. Zhang L, An G, Wu S, et al. Long-term intermittent cold exposure affects peri-ovarian adipose tissue and ovarian microenvironment in rats. *J Ovarian Res*. 2021;14(1):107. doi:10.1186/s13048-021-00851-8

25. Nteeba J, Ortinau LC, Perfield JW 2nd, Keating AF. Diet-induced obesity alters immune cell infiltration and expression of inflammatory cytokine genes in mouse ovarian and peri-ovarian adipose depot tissues. *Mol Reprod Dev*. 2013;80(11):948-958. doi:10.1002/mrd.22231
26. Grzesiak M, Burzawa G, Kurowska P, et al. Altered vitamin D_3 metabolism in the ovary and periovarian adipose tissue of rats with letrozole-induced PCOS. *Histochem Cell Biol*. 2021;155(1):101-116. doi:10.1007/s00418-020-01928-z
27. Borges CC, Bringhenti I, Aguila MB, Mandarim de Lacerda CA. Vitamin D restriction enhances periovarian adipose tissue inflammation in a model of menopause. *Climacteric*. 2020;23(1):99-104. doi:10.1080/13697137.2019.1597841
28. Zuccaro A, Zapatería B, Sánchez Alonso MG, et al. Pleiotrophin Deficiency Induces Browning of Periovarian Adipose Tissue and Protects against High-Fat Diet-Induced Hepatic Steatosis. *Int J Mol Sci*. 2021;22(17):9261. doi:10.3390/ijms22179261
29. El Husseiny HM, Kaneda M, Mady EA, Yoshida T, Doghish AS, Tanaka R. Impact of Adipose Tissue Depot Harvesting Site on the Multilineage Induction Capacity of Male Rat Adipose-Derived Mesenchymal Stem Cells: An In Vitro Study. *Int J Mol Sci*. 2023;24(8):7513. doi:10.3390/ijms24087513
30. Luukkaa V, Savontaus E, Rouru J, et al. Effects of estrous cycle and steroid replacement on the expression of leptin and uncoupling proteins in adipose tissue in the rat. *Gynecol Endocrinol.* 2001;15(2):103-112. doi:10.1080/gye.15.2.103.112
31. Hendawy H, Kaneda M, Yoshida T, et al. Heterogeneity of adipose stromal vascular fraction cells from the different harvesting sites in rats. Anat Rec (Hoboken). 2022;315(4):789-802. doi:10.1002/ar.24915.

Table 1. cDNA Synthesis Mixture (20 µl reaction volume)

	Volume
Total RNA	Variable (1 ng - 2 µg/rxn)
Reaction Buffer	2 µl
20X dNTP mix (2.5 mM each)	1 µl
Random hexamer (50 µM)	2 µl
Reverse Transcriptase (200 U/µl)	1 µl
RNase Inhibitor	0,5 µl
Easyscript plus RTase (200U/µL)	1 µl
RNAase-free water	Variable
Final Volume	20 µl

The procedure for synthesizing cDNA is outlined in Table 1. Following preparation, the mixture was incubated at 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 minutes to inhibit the enzyme

Table 2. Primer sequences of the genes were used in this study

Gene Name	**Forward**	**Reverse**
Actin beta	GCGAGTACAACCTTCTTGCAGCTC	TGGCATGAGGGAGCGCGTAA
CASPASE-3	CCTTGCCTCAAACAAAGGTT	CCTGAGTGGGTTCACAATGTT
CASPASE-8	CTGGGAAGGATCGACGATTA	CATGTCCTGCATTTTGATGG
CASPASE-9	CGACATGATCGAGGATATTCAG	TGCCTCCCTCGAGTCTCA
CCND1 (Cyclin D1)	CAGACCAGCCTAACAGATTTC	TGACCCACAGCAGAAGAAG
P21	CTGGATGCTAGAGGTCTGC	AGAGTTGTCAGTGTAGATGC
CDK6	AGTGTTGGCTGCATCTTTGC	CCTGTCTGGGAAGAGCAACA

Table 3. Demonstration of gene expression changes in perirenal tissue-derived mesenchymal stem cells as fold regulation when periovarian tissue-derived mesenchymal stem cells are compared with perirenal tissue-derived mesenchymal stem cells

		Fold Regulation	***p* value**
1	**CASPASE-3**	1.33	0.600673
2	**CASPASE-8**	-1.10	0.682357
3	**CASPASE-9**	3.61	0.377600
4	**p21**	-1.05	0.734690
5	**Cyclin D1**	-1.52	0.409162
6	**CDK6**	-4.66	0.236692

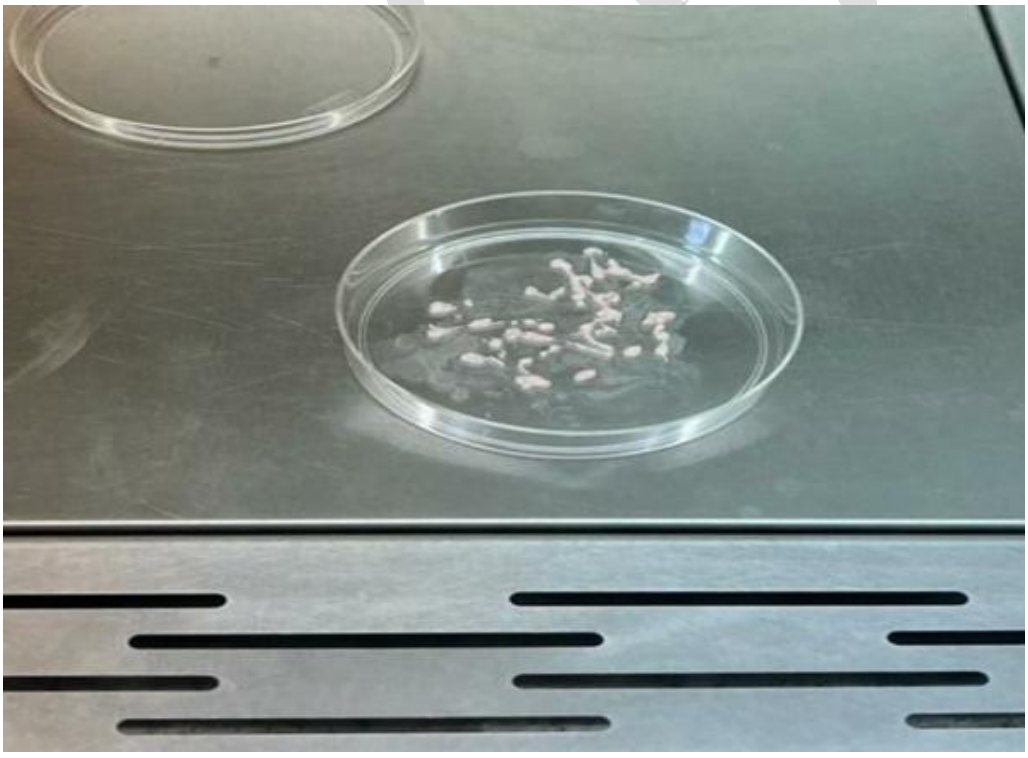


Figure 1. Explants obtained from adipose tissue were placed in a petri dish in small sizes and allowed to adhere to the culture dish

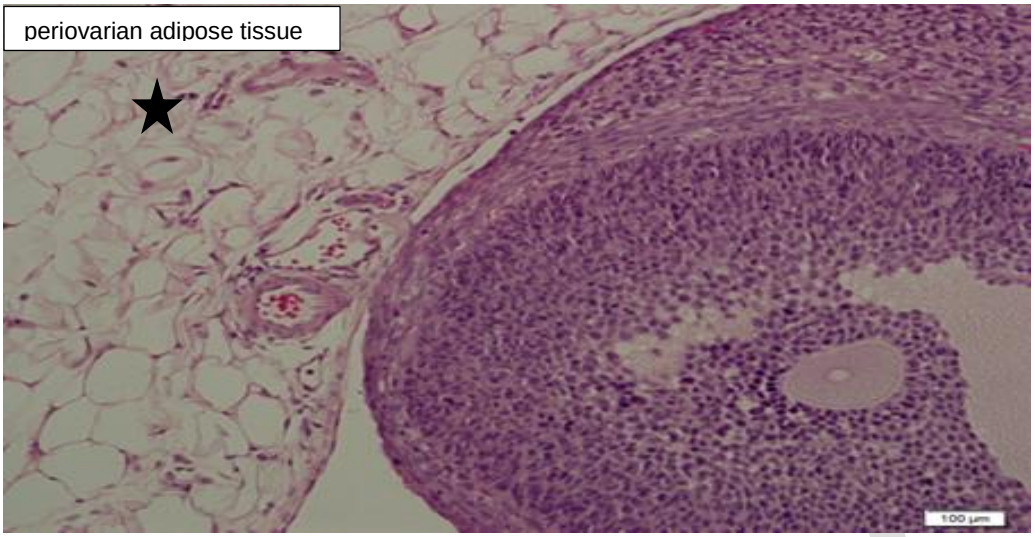


Figure 2. Histological appearance of periovarian adipose tissue

*: Periovarian adipose tissue

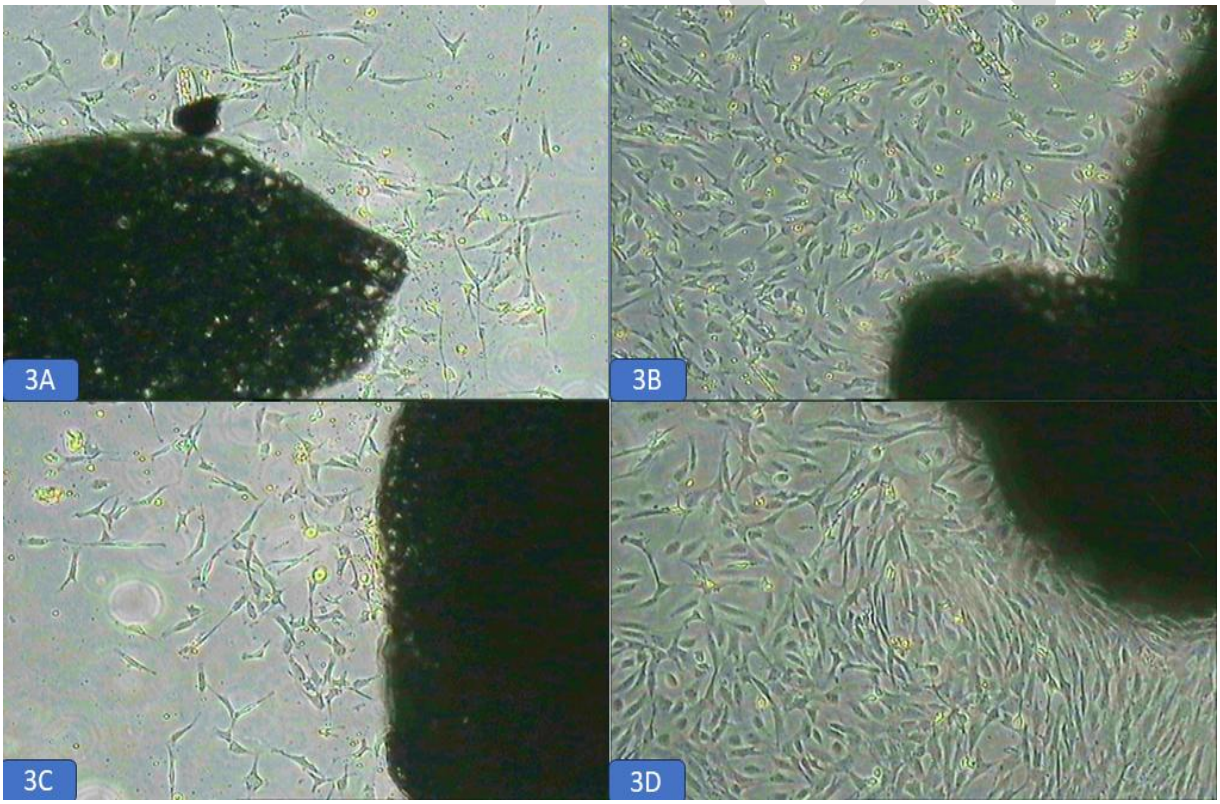


Figure 3. A- In initial cultures, the first cells appeared migrating out from the periovarian adipose tissue fragments after about a week, (x4) B- Cells reached to an adequate number throughout the culture during the second week, (x10) C- In initial cultures, the first cells appeared migrating out from the perirenal adipose tissue fragments after about a week, (x4) D- Cells reached to an adequate number throughout the culture during the second week (x10)

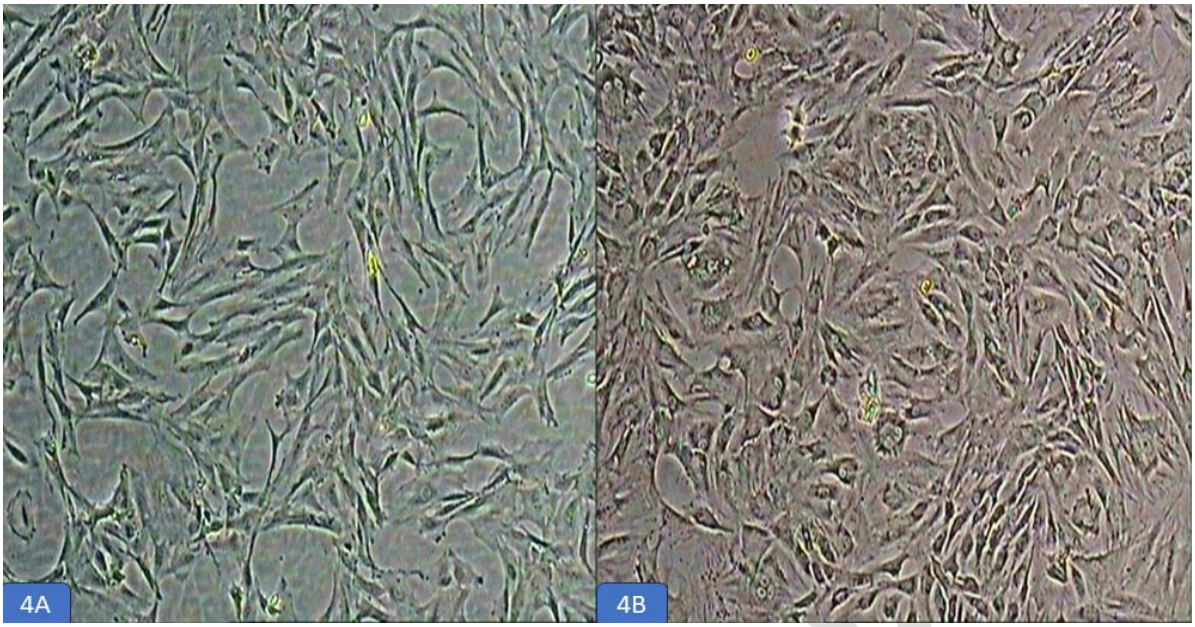
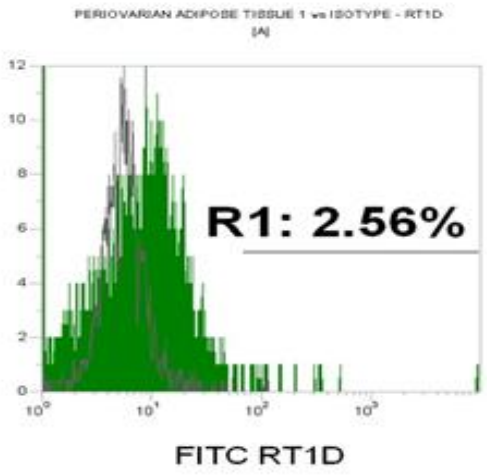
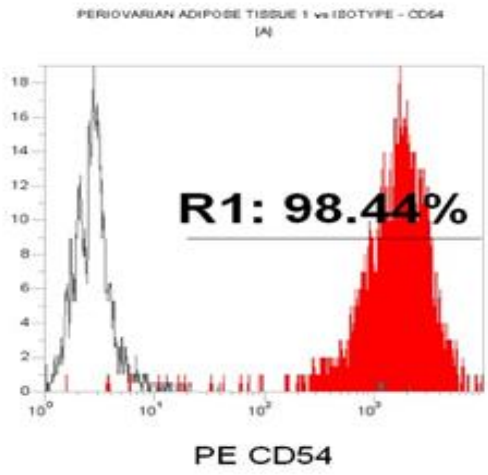


Figure 4. Primary cell culture images of mesenchymal stem cells obtained from rat adipose tissue

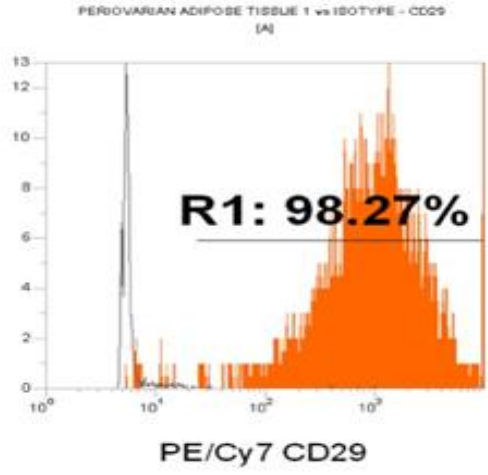
At 85-90% confluence the adipose-derived mesenchymal stem cells were harvested by explant method and the cells were subcultured (x10). A- periovarian adipose tissue, B- perirenal adipose tissue



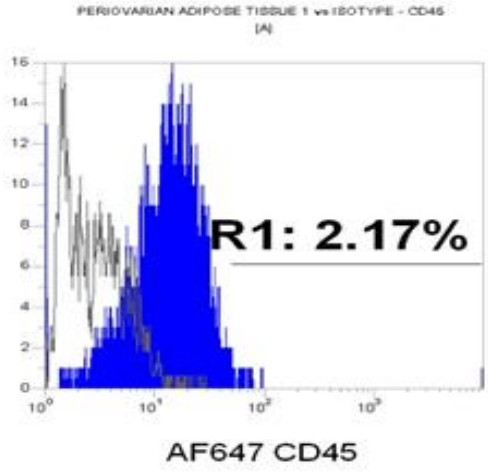
FITC RT1D



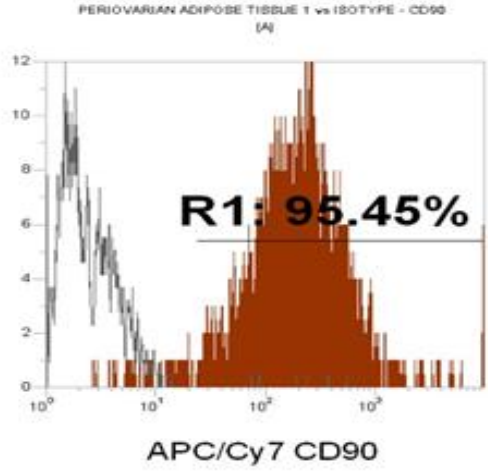
PE CD54



PE/Cy7 CD29

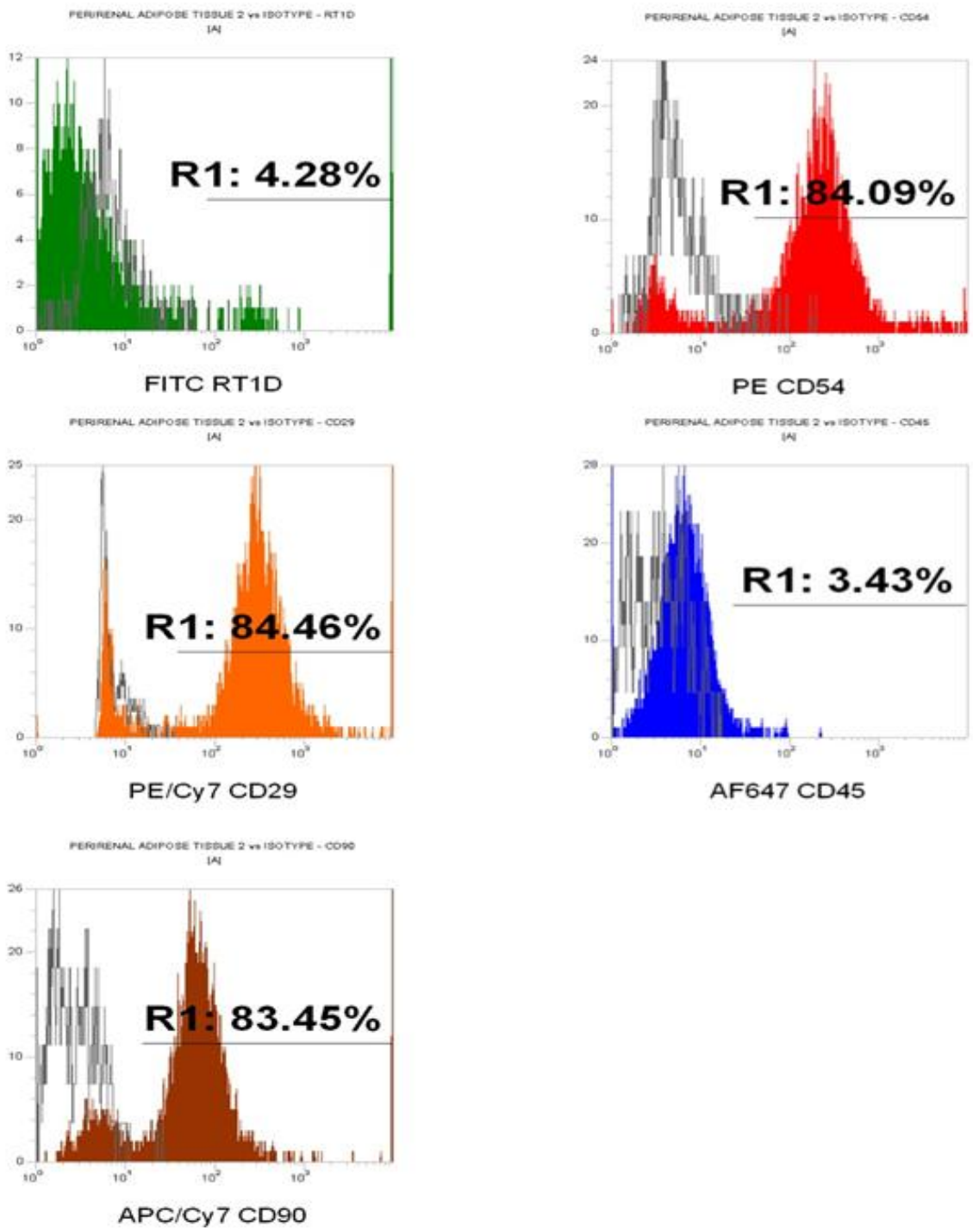


AF647 CD45



APC/Cy7 CD90

A



B

Figure 5. Flow cytometry of the isolated cells

Isolated cells were positive for the tested available cell surface markers such as CD29, CD90, and CD54, and negative for CD45, and MHC Class II revealing the immunophenotypic MSC characteristics. A- periovarian adipose tissue**,** B- perirenal adipose tissue

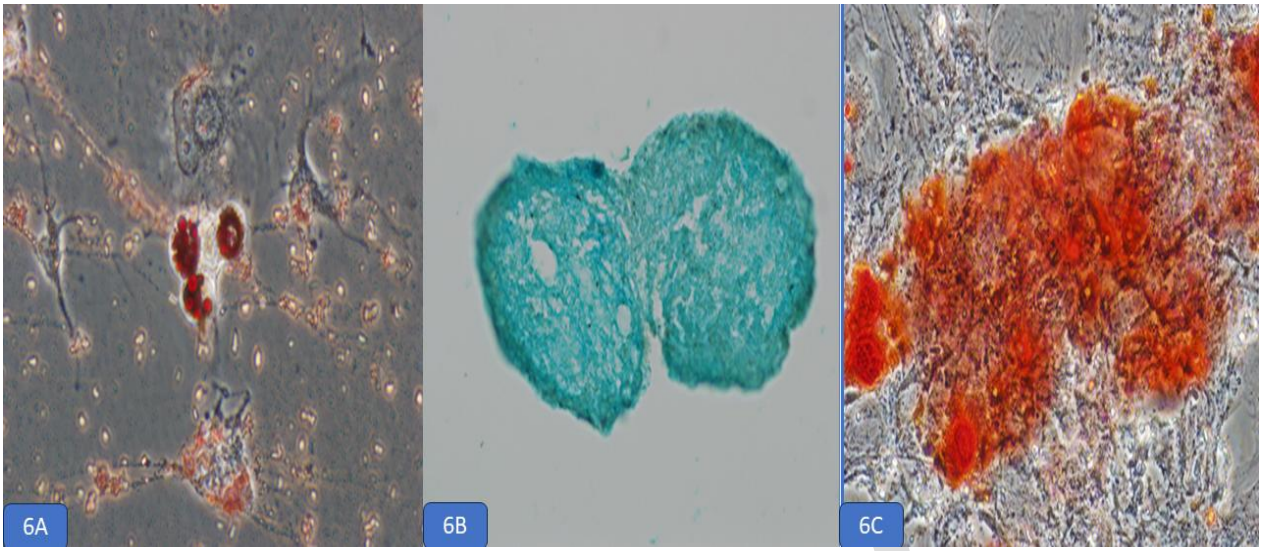


Figure 6. Demonstration of differentiation potential of periovarian adipose tissue-derived MSCs into adipocytes (A)(x4), chondrocytes (B)(x10), osteocytes (C)(x10)

Subconfluent monolayer cultures of fourth passage cells maintained in adipogenesis stimulating (AS) media for 14 days contained lipid droplets staining positive with Oil Red O, Pellet cultures of fourth passage cells maintained in chondrogenesis stimulating (CS) media for 21 days stained positive with Alcian Blue, and Subconfluent monolayer cultures of fourth passage cells maintained in osteogenesis stimulating (OS) media for 21 days stained positive with Alizarin Red S, are all shown respectedly in this figure

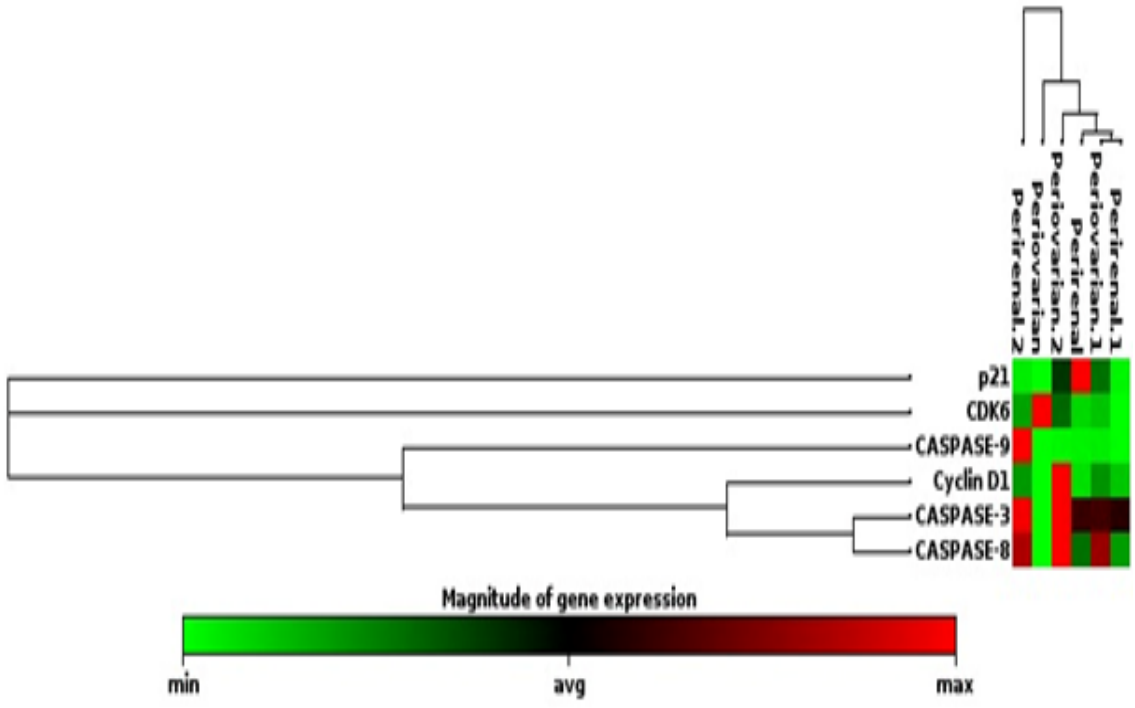


Figure 7. Clustergram analysis of gene expression changes in periovarian and perirenal tissue-derived mesenchymal stem cells

Unal MS, Uysal A, Gozlukaya TS, Onder E, Secme M, Cil N, Tabatabaei S, Abban Mete G. Characterization and comparison of mesenchymal stem cells derived from rat perirenal and periovarian adipose tissue. Pam Med J 2025;18:…-…

Ünal MS, Uysal A, Gözlükaya TS, Önder E, Seçme M, Çil N, Tabatabaei S, Abban Mete G. Sıçan perirenal ve perioveryan adipoz doku kaynaklı mezenkimal kök hücrelerin karakterizasyonu ve birbirleriyle karşılaştırılması. Pam Tıp Derg 2025;18:…-…

Murat Serkant Ünal, Asst. Prof. Department of Histology and Embryology, Faculty of Medicine, Pamukkale University, Denizli, Türkiye, e-mail: msunal@pau.edu.tr (https://orcid.org/0000-0003-1992-7909)

Aliye Uysal, Research Asst. Department of Histology and Embryology, Faculty of Medicine, Pamukkale University, Denizli, Türkiye, e-mail: aliyeu@pau.edu.tr (https://orcid.org/0000-0002-1763-2085)

Tuğçe Selda Gözlükaya, Research Asst. Department of Histology and Embryology, Faculty of Medicine, Pamukkale University, Denizli, Türkiye, e-mail: tsgozlukaya@pau.edu.tr (https://orcid.org/0000-0003-0644-2582)

Elif Önder, Asst. Prof. Department of Histology and Embryology, Faculty of Medicine, Pamukkale University, Denizli, Türkiye, e-mail: eonder@pau.edu.tr (https://orcid.org/0000-0002-7187-1669)

Mücahit Seçme, Assoc. Prof. Department of Medical Biology, Faculty of Medicine, Ordu University, Ordu, Türkiye, e-mail: mucahitsecme@odu.edu.tr (https://orcid.org/0000-0002-2084-760X)

Nazlı Çil, Assoc. Prof. Department of Histology and Embryology, Faculty of Medicine, Pamukkale University, Denizli, Türkiye, e-mail: ncil@pau.edu.tr (https://orcid.org/0000-0002-2164-8688)

Seyedmahdi Tabatabaei, MsC.Department of Histology and Embryology, Faculty of Medicine, Pamukkale University, Denizli, Türkiye, e-mail: mahdi.tabatabaei96@gmail.com (https://orcid.org/0000-0002-0616-1038) (Corresponding Author)

Gülçin Abban Mete, Prof. Department of Histology and Embryology, Faculty of Medicine, Pamukkale University, Denizli, Türkiye, e-mail: gabban@pau.edu.tr (https://orcid.org/0000-0001-6794-3685)