Research Article

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Determination of mTOR activity depending on donor age of mesenchymal stem cells isolated from adipose tissue

Adipoz dokudan izole edilen mezenkimal kök hücrelerin donör yaşına bağlı olarak mTOR aktivitesinin belirlenmesi

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Abstract

Purpose: Aging in living organisms is an inevitable physiological consequence. Cellular senescence occurs not only in cells that have completed their differentiation, but also in stem cells. Mammalian target of Rapamycin protein complex (mTOR) has an important role in cell growth and metabolism. The mTOR, which plays an important role in cell proliferation, also regulates cellular aging and directs the bioenergetic infrastructure. The aim of the study is to determine the mTOR expression of mesenchymal stem cell (MSC) obtained from adipose tissue depending on the donor age.

Materials and methods: Six-week-old pubertal rats were named Group 1 (n=6), 10-12-week-old reproductive period rats were named Group 2 (n=6), and 20-month-old rats were named Group 3 (n=6). The isolation of MSC was performed by primary explant culture method from adipose tissue taken from groups. Characterization and differentiation experiments were performed in MSC obtained. The activity of mTOR (mTORC1 and mTORC2) in MSC was determined by qRT-PCR method. Caspase 3, 8, 9, Bax and Bcl-2 expressions were evaluated by Real-time polymerase chain reaction (qRT-PCR) method.

Results: In our study, it was determined that the highest expression of apoptotic markers was in Group 1 and the lowest expression was in Group 2. When mTOR expression was evaluated, mTORC1 was found to be highest in Group 2 and lowest in Group 1. mTORC2 expression in Group 1 was lower than in other groups. Although the expression of mTORC1 and mTORC2 in Group 3 was not as high as in Group 2, it was statistically significant (p<0.05).

Conclusion: In this study, we found that both mTORC1 and mTORC2 are differentially expressed in stem cells depending on donor age. Further studies are needed to better understand the functional consequences of this difference.

Keywords: Aging, adipose tissue, mTOR, mesenchymal stem cells.

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Öz

Amaç: Canlı organizmalarda yaşlanma kaçınılmaz bir fizyolojik sonuçtur. Hücresel yaşlanma sadece farklılaşmasını tamamlamış hücrelerde değil, kök hücrelerde de meydana gelir. Rapamisin protein kompleksinin memeli hedefi (mTOR), hücre büyümesinde ve metabolizmasında önemli bir role sahiptir. Hücre çoğalmasında önemli rol oynayan mTOR, aynı zamanda hücresel yaşlanmayı düzenler ve biyoenerjetik altyapıyı yönlendirir. Çalışmanın amacı, donör yaşına bağlı olarak yağ dokusundan elde edilen mezenkimal kök hücrelerin (MSC) mTOR ekspresyonunu belirlemektir.

Gereç ve yöntem: Altı haftalık pubertal sıçanlar Grup 1 (n=6), 10-12 haftalık reprodüktif dönem sıçanları Grup 2 (n=6) ve 20 aylık sıçanlar Grup 3 (n=6) olarak isimlendirildi. Gruplardan alınan yağ dokusundan primer eksplant kültür metodu ile MSC izolasyonu yapıldı. Elde edilen MSC'de karakterizasyon ve farklılaşma deneyleri yapıldı. Kök hücrelerdeki mTOR aktivitesi (mTORC1 ve mTORC2) qRT-PCR yöntemi ile belirlendi. Caspase 3, 8, 9, Bax ve Bcl-2 ekspresyonları Real-time polymerase chain reaction (qRT-PCR) yöntemi ile değerlendirildi.

Bulgular: Çalışmamızda apoptotik belirteçlerin en yüksek ekspresyonunun Grup 1'de, en düşük ekspresyonun Grup 2'de olduğu belirlendi. mTOR ekspresyonu değerlendirildiğinde, mTORC1 en yüksek Grup 2'de, en düşük Grup 1'de bulundu. Grup 1'deki mTORC2 ifadesi diğer gruplara göre daha düşüktü. Grup 3'teki mTORC1 ve mTORC2 ekspresyonu Grup 2'deki kadar yüksek olmasa da istatistiksel olarak anlamlıydı (p<0,05).

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Sonuç: Bu çalışmada hem mTORC1 hem de mTORC2'nin donör yaşına bağlı olarak kök hücrelerde farklı şekilde eksprese edildiğini bulduk. Bu farkın fonksiyonel sonuçlarını daha iyi anlamak için daha fazla çalışmaya ihtiyaç vardır.

Anahtar kelimeler: Yaşlanma, yağ doku, mTOR, mezenkimal kök hücreler.

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Introduction

Aging in living organisms is an inevitable physiological outcome. Cellular senescence does not only occur in differentiated cells. It also happens in stem cells. Mesenchymal stem cells (MSCs), which are easy to obtain and have fewer ethical problems, have become a promising method for treating cartilage, bone and spinal cord injuries as well as metabolic diseases. However, many of the stem cells transplanted to treat tissue damage have been found to die after transplantation. Although molecular, epigenetic and metabolic activity-related changes have been demonstrated in studies suggesting that this problem may be related to stem cell aging, stem cell aging has not yet been fully elucidated [1].

Studies show that the Mammalian Target of Rapamycin (mTOR) pathway plays an important role in cellular and organismal aging. The mTOR is a protein that acts as a sensor in anabolic and catabolic processes in our body; coordinates adequate nutrient, energy, oxygen availability and growth factors; and plays a central role in cell proliferation. There are two signaling complexes called mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Playing an important role in cell proliferation, mTOR also regulates cellular senescence and directs the bionergic infrastructure. Attenuation of mTOR signaling by rapamycin delayed senescence in many cell types [2]. The mTORC2 plays an important role in endothelial senescence, which is manifested by increased binding of mTORC2-directed mTOR to Ser2481 and Rictor, an important component of AKT (phospho-AKT) phosphorylation [3]. Rapamycin mTOR is a regulator of cell growth in mammals, depending on hormone, nutrient and oxygen levels. Activation of both complexes facilitates cell growth and survival. The mTORC1 is involved in protein and lipid synthesis and cellular growth and proliferation, early cellular senescence and autophagy. The mTORC2 is involved in the organization of the actin cytoskeleton, control of ion transport, and anti-apoptotic events through stimulation of the AKT-FOXO pathway. The mTOR complex rapamycin is effective in the treatment of cancer immunology and genetic diseases, and in the prevention of degenerative and age-related pathologies in the heart and brain [4, 5]. In particular, mTORC1 has been reported to be a regulator in events such as cellular aging, telomere attrition, genomic imbalance and mitochondrial dysfunction [5].

Studies on stem cell senescence are generally related to cell activity after advanced passage (such as passage 45). In our study, we aimed to investigate mTORC-1 and mTORC-2 signaling complexes in adipose tissues derived MSCs of puberty, adult and aged rats.

Materials and methods

With the decision of Pamukkele University Animal Experiments Ethics Committee dated 03/02/2020 and numbered PAUHADYEK-2020/11, 18 female 200-250 gr rats were used. The rats were kept at 22°C a for 12 hours in a light-dark cycle and their access to water and food was arranged ad libitum. Groups were designated 6-week-old puberty as Group-1 (n=6), 10-12-week old reproductive rats as Group-2 (n=6), and 20-month-old rats as Group-3 (n=6). Retroperitoneal adipose tissues of the rats were removed through laparotomy incision. The adipose tissues were washed in 50 ml phosphate buffered saline (PBS). Adipose tissue pieces of the groups were transferred to the Cell Culture Laboratory under sterile conditions. Adipose tissue was dissected with sterile thin tissue scissors into the smallest pieces that could be separated in a petri dish containing complete medium. Each group was placed in 5 ml of medium Dulbecco's Modified Eagle's Medium (L-DMEM), 10% Fetal Bovine Serum (FBS), 50 U/ml penicillin, 50 µg/ml streptomycin, 0.1 mM nonessential amino acids and 2 mM L-glutamine) in the culture flask prepared for initial inoculation and incubated

separately at 37°C in a humid environment with 5% CO_2. Subculture was initiated when cells reached 70% confluence. At passage 3, cells were harvested with 0.25% trypsin. Cell counts were done. Flow cytometry analysis and differentiation assays were performed.

Flow cytometry analysis

Cells from Group 1 and Group 2 were sent to Pamukkele University Hospitals Tissue Typing Laboratory for mesenchymal stem cell characterization. Cells in Group 3 could not be analyzed because they could not be obtained sufficiently. The cells were characterized using CD34, CD73, CD90, mesenchymal stem cell surface markers in Flow Cytometry (Navios EX, Beckman Coulter Life Sciences, Houston, Texas).

Mesenchymal stem cell differentiation assays

Adipogenic differentiation

Cells from Group 1 and Group 2 at the third passage at 70% confluent were seeded in 12 well plates with 1×10^4 cm² cells. Since the cells in Group 3 could not be obtained sufficiently, differentiation experiments could not be performed. For 15 days, adipogenic culture was induced in media. The medium was changed twice a week with adipogenic medium (45 ml Basal Medium, 5 ml Adipocyte Supplement, 25 µl penicillin-streptomycin). At the end of 15 days, adipogenesis was demonstrated by oil red O. Cells were washed twice in PBS and fixed in 10% formalin. Cell staining was performed in filtered Oil Red O for 1 hour. At the end, the cells were washed under running water, Hemotoxylene was added to wells, left for 1 minute and rinse in distilled water. Cells of each group were examined and photographed under phase contrast microscope.

Osteogenic differentiation

At the third passage, $3.5x10^6$ live cells were added to the cells of Group 1 and Group 2 from 1 ml of cell suspension prepared after cell counting. Medium and 20µl of cell suspension were added to the culture dish and incubated. Twice a week, the medium was changed. At the end of the 21st day, the medium was removed. Cells were fixed (4% formaldehyde) and incubated for 30 minutes. The cells were stained with a solution of Alizerin Red S for 2 to 3 minutes. Cells of each group were examined and photographed under phase contrast microscope.

Chondrogenic differentiation

Viable cells ($1.6x10^7$ /ml) from 1 ml of the prepared cell suspension was added to the center of the 12-well plate. It was incubated at 37°C for 2 hours. The prepared chondrogenic differentiation medium was added and placed in the incubator. The medium was changed twice a week. At the end of the 14th day, 4% formaldehyde was added to the cells and kept for 30 minutes. The fixed cells were kept in 1% Alcian blue dissolved in 0.1 N HCl. After removal of Alcian blue and washing with 0.1N HCl, cells from each group were examined and photographed under a phase-contrast microscope.

Real-time polymerase chain reaction (qRT-PCR)

The RNA isolation by TRIzol Reagent was performed on cells obtained from Group 1, Group 2 and Group 3. Cells were resuspended in 500 µl TRIzol and Total RNA was extracted. Complementary DNA (cDNA) synthesis was performed with cDNA Synthesis Kit with Rnase Inhibitor. After cDNA synthesis, expression level differences in mRNA levels were determined by qRT-PCR using SYBR green assay. The data obtained were recorded as Cq. The primary sequences of the genes were analyzed and the housekeeper gene (beta-actin) was used as a reference gene for normalization (Table 1).

Statistical analysis

The analysis of the data was quantified by using the ΔΔCT method with a computer program. Volcano Plot in the "RT2Profiles™PCR Array Data Analysis" program was used to compare the groups. The groups were statistically evaluated by "Student's t-test" analysis. Statistical significance was determined at $p \leq 0.05$.

Table 1. Real Time PCR forward and reverse primer sequences

Gene names	Primer Sequence
Beta Actin	F: GCGAGTACAACCTTCTTGCAGCTC R: TGGCATGAGGGAGCGCGTAA
CD 34	F: AGCCATGTGCTCACACATCA R: CAAACACTCGGGCCTAACCT
CD 45	F: TTGCTCCCCATCCGATAAGAC R: AGCGTGGATGAAAAACCATCG
CD 73	F: TGCATCGATATGGCCAGTCC R: AATCCATCCCCACCGTTGAC
CD 105	F: ACTGAGTTGCACATCTGGGG R: TTCCGAAGTGGTGGTAAGCC
CD 106	F: GGTGGCTGCACAGGTTGGGG R: ACCCACAGGGCTCAGCGTCA
Bcl 2	F: ATCGCCCTGTGGATGACTGAGT R: GCCAGGAGAAATCAAACAGAGGC
Bax	F: TCAGGATGCGTCCACCAAGAAG R: TGTGTCCACGGCGGCAATCATC
mTOR	F: TGAGAGAGGAGATGGAGGAA R: TTCAGAGCGGAGAAAGCA
mTORC 1	F: TGACTTACCGAGAGCACACA R: ACATTCACAGACTCAGGCATC
mTORC 2	F: GAAGGTGCTAAAACTGAAGGTG R: CAGAACTCGGAAACAAGGAA
Caspase 3	F: GGAAGCGAATCAATGGACTCTGG R: GCATCGACATCTGTACCAGACC
Caspase 8	F: AGAAGAGGGTCATCCTGGGAGA R: TCAGGACTTCCTTCAAGGCTGC
Caspase 9	F: GTTTGAGGACCTTCGACCAGCT R: CAACGTACCAGGAGCCACTCTT

Results

Cells isolated from adipose tissue taken from the retroperitoneal region of Groups rats were observed to adhere to the plastic surface in the first 24 hours and had a fibroblast-like appearance. At the second and third passages, fibroblast-like cells were homogeneous and dense. In group 3, adhesion to the plastic surface occurred on the 8th day (Figure 1). In the following days, it was observed that the cells did not proliferate and deaths started and these deaths increased day by day. Therefore, since the cell density required for characterization and differentiation experiments could not be reached, these analyses could not be performed in this group. Only RT- PCR analyses could be performed.

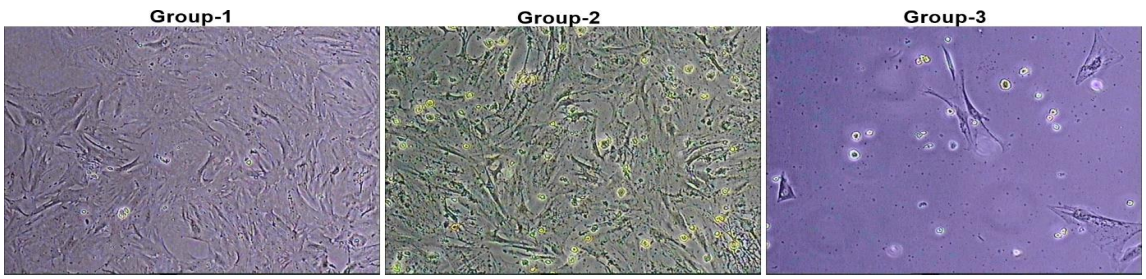

Figure 1. Mesenchymal stem cells obtained from rat adipose tissue by primary cell culture. X20, inverted microscope

Flow cytometry analysis

In flow cytometry analysis performed for stem cell characterization, CD73 and CD90, which are mesenchymal stem cell markers, were measured as 86.08% and 83.76% in Group 1. In Group 2, CD73 was 86.46% and CD90 was 90.46%. CD34, a hematopoietic stem cell marker, was negative (0%) in both groups (Figure 2). According to flow cytometry analysis, surface markers of MSC were demonstrated in Group 1 and Group 2. In Group 3, analysis could not be performed because of insufficient number of cells.

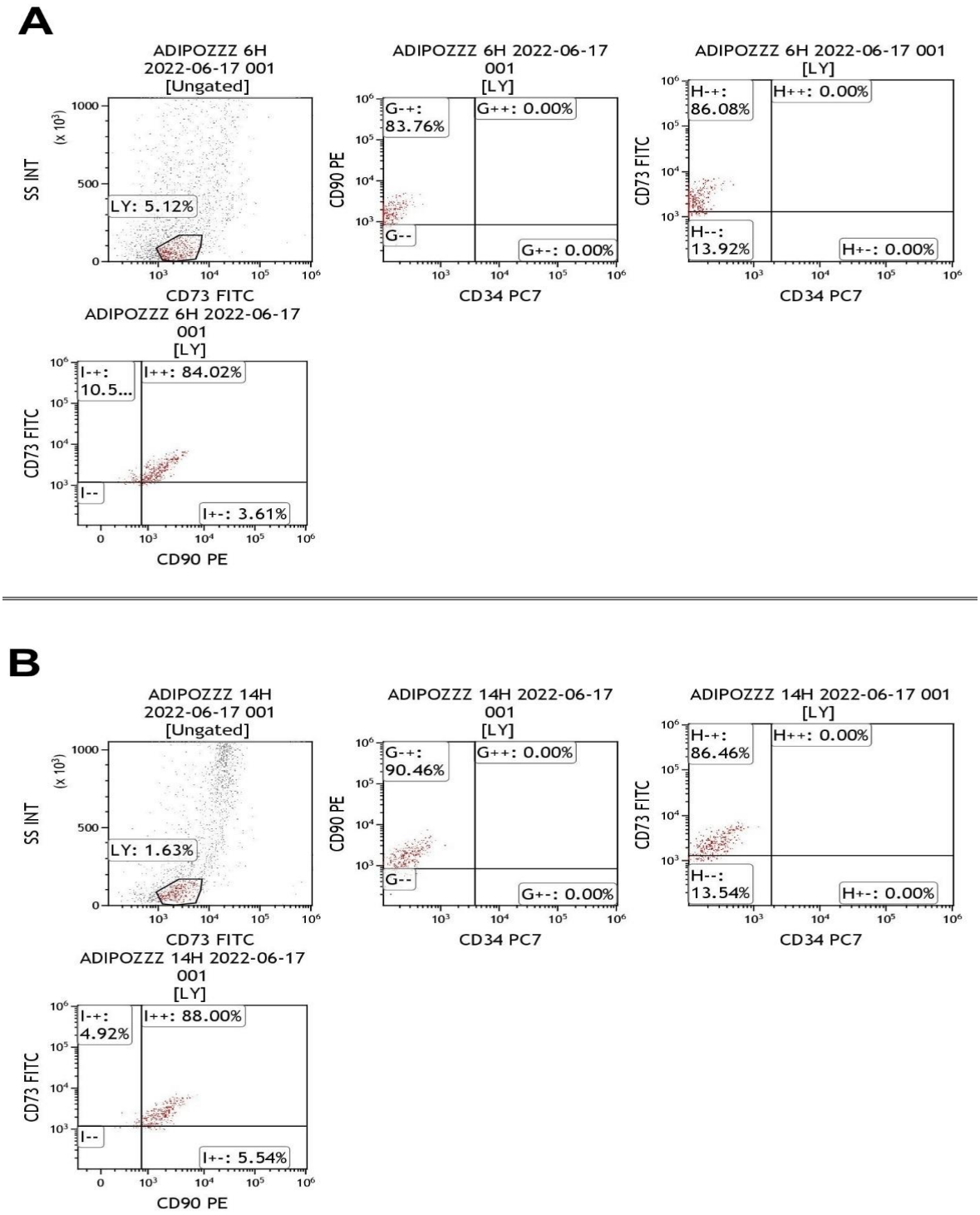

Figure 2. Expression of MSCs surface markers measured by Flow cytometry analysis in MSCs derived from adipose tissue from rat. A: In Group 1, CD73 was 86.08% CD90 83.76%. B: In Group 2, CD73 was 86.46% CD90 90.46%. CD34 was measured as negative (0%) in both groups

Differentiation experiments

As a result of differentiation experiments performed on stem cells in Group 1 and Group 2, osteogenic, adipogenic and chondrogenic differentiation was observed. Thus, mesenchymal stem cells were demonstrated by differentiation experiments (Figure 3). In Group 3, differentiation experiments could not be performed because sufficient number of cells could not be obtained.

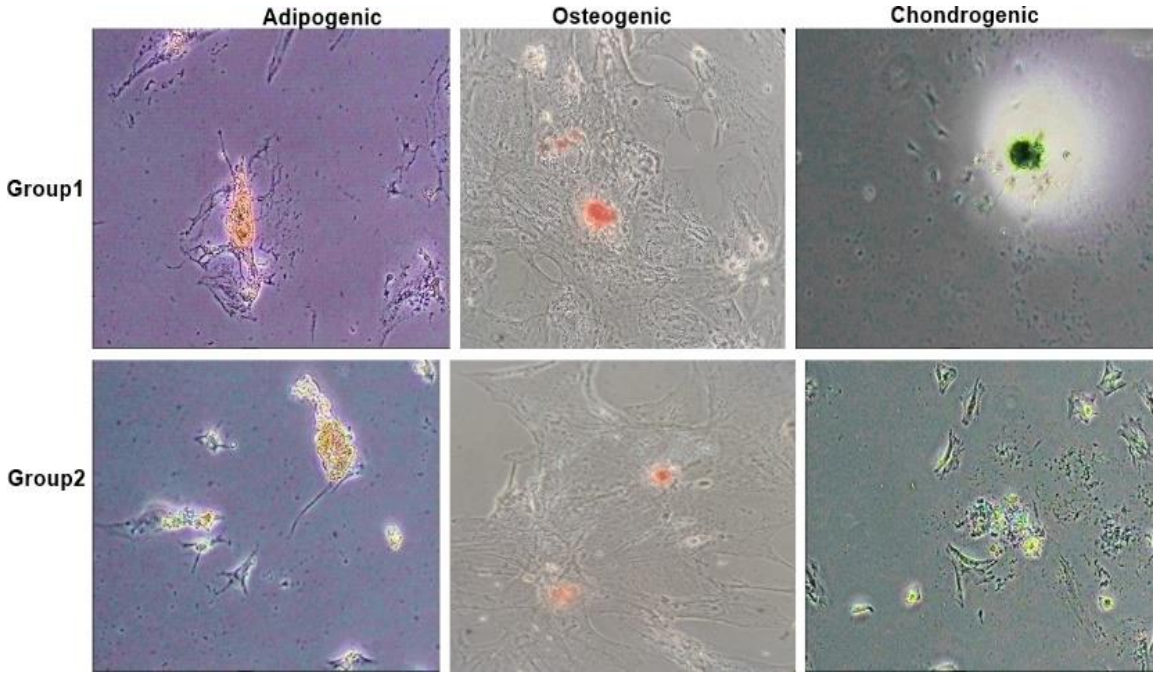

Figure 3. Differentiation of mesenchymal stem cells into adipogenic, osteogenic and chondrogenic tissue in Group 1 and Group 2. X20, Inverted microscope

qRT-PCR analysis results

As a result of qRT-PCR analysis performed on Group 3, in which flow cytometry and differentiation experiments could not be performed, the expression of CD45 was significantly lower and CD 10^6 expression was significantly higher, proving that Group 3 was also mesenchymal stem cells. Fibroblast-like morphologic images also supported this (Table 2). According to qRT-PCR analysis results, the highest expression of mTORC1 and mTORC2 was found in Group 2 and the lowest expression was found in Group 1. The expression of Group 3 was lower than in Group 2 and higher than in Group 1 and was statistically significant ($p<0.05$). Among the pro-apoptotic markers, Bax, caspase 8 and caspase 3 expression was higher in Group 1, while the lowest caspase 8 and Bax values were observed in Group 2. Caspase 9 was close to each other in Group 2 and Group 3 and higher than Group 1. Among the anti-apoptotic markers, Bcl-2 was lowest in Group 1 and highest in Group 2 (Table 2).

Table 2. The mRNA expression changes of genes in Mesenchymal Stem Cell related with groups. Data were obtained by qRT-PCR assay via ΔΔCt method in RT2 Profiles PCR array data analysis online program

Gene names	Group 1		Group 2		Group 3	
	Fold Regulation	*p* value	Fold Regulation	*p* value	Fold Regulation	*p* value
Beta Actin	1.00		1.00		1.00	
CD 34	-1.09	0.70	3.52	0.09	3.22	0.045*
CD 45	-23.59	0.34	-4.37	0.91	-103.01	0.002*
CD 73	-48.06	0.26	118.33	0.26	2.46	0.30
CD 105	1.57	0.10	1.02	0.84	1.59	0.058
CD 106	1.90	0.79	4.03	0.17	7.66	0.008*
Bcl 2	-2.87	0.37	4.39	0.37	1.53	0.95
Bax	8.75	0.34	-13.83	0.37	-1.58	0.37
mTOR	-3.72	0.37	1.29	0.54	-2.89	0.35
mTORC 1	-9.43	0.37	27.10	0.37	2.87	0.03*
mTORC 2	-1.61	0.41	3.39	0.37	2.11	0.02*
Caspase 3	1.70	0.10	-1.50	0.45	1.13	0.81
Caspase 8	4.72	0.17	-8.82	0.26	-2.09	0.34
Caspase 9	1.01	0.39	3.70	0.38	3.72	0.70

*$p<0.05$ statistically significant

Discussion

In the adult, tissue-specific stem cells are responsible for the maintenance of tissue homeostasis and the generation of progenitor cells for regeneration after tissue damage. To maintain the stem cell pool while providing the progenitor cells needed to differentiate into tissue-specific cells, stem cells must undergo a process of self-renewal. Many different factors finely regulate the balance between stem cell maintenance and tissue regeneration. These include growth factors, the extracellular environment, cell-cell signalling, inflammatory mediators and cellular metabolism. However, stem cells are exposed to harmful effects in the case of disease or during the ageing process [6].

A general decline in stem cell function and specific changes in stem cell phenotype related to local tissue niches during ageing have been reported by Bengal et al. [7]. The stem cell cycle in skeletal muscle, parts of the nervous system and hair follicles of epithelial tissues is slowed during ageing and there is a general decrease in the stem cell pool in these tissues [8, 9]. In our study, we found that cell proliferation was very low in stem cells from adipose tissue obtained from Group 3, consisting of old rats, and that proliferation was unable to progress to higher passages. Morrison et al. [9] showed that stem cells in certain tissues, e.g. haematopoietic stem cell (HSC), may retain or expand their population during ageing [9]. However, other studies have shown that although HSC numbers increase with age, cell function decreases and cell longevity differs [10, 11]. In the senescence of intestinal stem cells, an increase in the number of cells and a decrease in the function of the cells have also been observed [12]. In order to understand stem cell responses to ageing and their relevance to the stem cell niche, studies suggest that further molecular investigations are required [13].

Mesenchymal stem cells have been developed and used in preclinical and clinical applications due to their important contribution to organ formation and immune modulation. In particular, a powerful and promising approach to achieve immunomodulation and tissue regeneration has been recognised through MSC transplantation. However, the mechanisms by which MSCs exert their therapeutic effects have not been elucidated. Increasing evidence suggests that engrafted MSCs are short-lived in the recipient and subsequently apoptose in the host circulation or in the engrafted tissue [14]. In our study, we found high expression of caspase-3, caspase-8, caspase-9 and Bax in

Group 1. In Group 2, caspase-3, caspase-8 and Bax were low, whereas Bcl-2, an anti-apoptotic marker, was very high. In Group 3, caspase 3 was close to Group 1, but caspase 8, 9 and Bax were lower than Group 1 and higher than Group 2. Caspase 3 elevation in Group 3 may be age-related. However, this could not explain why caspase 3 was elevated in Group 1. It has been reported in previous studies that estrogen receptors are expressed in various stem cells, including embryonic stem cells, MSCs and cardiac stem cells [15]. It has been reported that estrogen is a regulator of embryonic stem cell differentiation and an inhibitor of cell apoptosis in mesenchymal stem cells [16]. Since the rats in Group 3 were in the premenopausal period, the rats in Group 1 were in the puberty period and estrogen levels were lower than in the reproductive period. This may result in higher apoptosis in stem cells in Group 1 and Group 3 than in Group 2. In differentiation, a role for mTORC1 was identified and rapamycin was shown to inhibit adipocyte formation [17]. Other studies have demonstrated mTOR activity is required for the function of embryonic stem cells in mammalian development and mTOR activity is important for the self-renewal and differentiation of stem cells [18, 19]. Mesenchymal stem cells are multipotent that differentiate into various cell types such as adipocyte, chondrocyte and osteoblast in response to environmental cues [20]. It has been shown that rapamycin inhibits adipogenic differentiation in human preadipocytes [21]. Several other studies have highlighted the role of mTORC1 in adipogenesis. There is strong evidence that insulin-induced mTORC1 activation enhanced adipogenesis [22]. In another study, mTORC1 and mTORC2 was investigated in cell differentiation using Raptor and Rictor knocked out MSCs. In these studies, mTORC1 in adipocyte differentiation was strengthened by the reduced capacity of Raptor-deficient MSCs to form adipocytes [23]. There are also reports that Raptor-deficient MSCs have the capacity to form a mineralizing matrix similar to bone. Rictor-deficient MSCs showed an opposite differentiation program to Raptor knockout MSCs, with increased adipocyte and decreased mineralised matrix [24]. All of the above results suggest that mTORC1 activity enhances adipogenic differentiation and suppresses osteoblastic differentiation, whereas mTORC2 suppresses adipogenic differentiation and enhances osteoblastic differentiation. However, another study suggests that mTORC1 is required for osteoblast differentiation [25]. The mTORC1 is known to regulate glutamine anaplerosis, the breakdown of glutamine to tricarboxylic acid cycle intermediates, by blocking sirtuin 4 (Sirt4) transcription [26]. Glutaminase expression, which is also involved in glutamine anaplerosis, declines with age and is associated with decreased osteogenesis [27]. It has been reported that mTORC1 promotes osteogenic differentiation by restoring glutamine anaplerosis [19]. According to our qRT-PCR results, MSCs obtained from rats in Group 2 and 3 expressed higher levels of mTORC1 and mTORC2 than those in Group 1. The mTORC1 has been shown to stimulate the differentiation of adipocytes. Therefore, a higher level of adipocyte differentiation in the stem cells of Group 2 was considered to be an expected situation. The mTORC2 acts by stimulating the differentiation of osteoblasts. It would be expected that there would be less mTORC2 in Group 3. However, the fact that there was less mTORC2 in group 1 compared to both Group 2 and Group 3 was unexpected for Group 1, where we thought there would be a high level of osteoblast activation.

The mTOR plays an important role in cell proliferation and differentiation and also regulates cellular senescence. Research into the relationship between mTOR and lifespan has shown that mTOR inhibition extends lifespan, but the mechanism by which this happens remains unknown. Some studies have suggested that the inhibition of mTORC1 does not delay ageing itself, but rather delays the onset of age-related diseases [28]. However, many researchers believe that the effects of mTOR inhibitors on longevity are directly due to a reduction in senescence [29].

Inhibition of mTORC1 has been reported to play a role in maintaining adult stem cell function in various tissues [30]. It has been reported that treatment of aged mice with rapamycin can indirectly increase the function of stem cells in the intestine by reducing mTORC1 signalling in the Paneth cell niche [31]. Similarly, caloric restriction has been reported to extend lifespan. During caloric restriction, mTORC1 is induced to allow intestinal stem cell proliferation [32].

The mTORC1 has many various functions in enhancing or limiting inflammatory or immune responses in the immune systems [33, 34]. Importantly, inhibition of mTORC1 is also used as an immunosuppressive therapy to limit the activation of T cells and to prevent graft rejection following organ transplantation. Inhibition of mTORC1, in turn, enhances the memory responses of CD8+ T cells that are critical for viral defence [35]. However, not much has been known about the role and activity of mTORC1 in immune cells during ageing. One study reported that increased mTORC1 activity in HSCs from aged mice was associated with an age-related decline in HSC function, which may contribute to anaemia, poor vaccination or increased tumour formation [36].

In our study, mTORC1 expression was highest in Group 2 and lowest in Group 1. Given the association of mTORC1 with longevity, the lowest expression in Group 1 could be explained. However, it was interesting that Group 2 was higher than 3. Indeed, mTORC1 is involved in many cellular events including protein synthesis, transcription, translation, DNA synthesis and autophagy. The high levels in this group could be due to the high number of these events or it could be because the end products and the pathways that are affected by mTORC1 and mTORC2 in the cellular events are very different and numerous.

In conclusion, our results show that mTOR is differentially expressed in MSCs in an age-dependent manner. To better understand the functional consequences of this difference, further studies are required. We believe that elucidating the molecular mechanisms in stem cells will be important for stem cells, which are widely used in both preclinical and clinical applications.

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

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Contributions of the authors to the article

G.A.M. and H.S. constructed the main idea and hypothesis of the study. H.S., E.O. and N.C. developed the theory and arranged/edited the material and method section. E.O. and G.A.M. evaluated the data of RT-PCR in the results section. E.O., G.A.M. and N.Ç. contributed to the histological evaluation of the results. Flow cytometry analysis was performed by E.M. The article written by H.S., N.C. and G.A.M. reviewed the article and made the necessary corrections and approved it. In addition, all authors discussed the entire study and approved the final version.