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RESEARCH ARTICLE

Characterizing the Amniotic Fluid-Derived Stem Cells and Optimizing the Passage Number for Targeted Applications

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A B S T R A C T

Mesenchymal stem cells are considered potent sources that can be used for tissue regeneration and treatment of various diseases and syndromes. Among the mesenchymal stem cells, amniotic fluid-derived stem cells come forward as they possess some pluripotent properties and there are no serious ethical concerns upon their derivation. Therefore, gaining a better understanding of the nature of amniotic fluid stem cells is important. Although it is known that stem cells show slightly different characteristics between passages, scientists often consider only cell numbers and the proliferation pattern of the cells when deciding the passage to use in their studies. In this study, it was aimed to characterize rat amniotic fluid-derived stem cells for their mesenchymal and pluripotent features and make inter-passage comparisons by real-time qPCR to reveal their distinctions between different passages, and eventually help decide the appropriate passage numbers to be used in future research. The outcomes of the study showed that using rat amniotic fluid-derived stem cells at P5 could be beneficial for mesodermal differentiation studies and using them in earlier passages may be more favorable for the studies requiring better ectodermal differentiation properties.

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INTRODUCTION

Stem cells are undifferentiated cells that can self-renew and differentiate into functional cell types (Reddy, 2020; Abofila *et al.*, 2021). Although totipotent and pluripotent stem cells, which are classified as embryonic stem cells, have greater cellular potencies, there are significant ethical concerns regarding their derivation (Robertson, 2001). These ethical concerns majorly arise due to the isolation process of embryonic stem cells, including the destruction of an embryo that can form an entire living organism (Robertson, 2001). Therefore, scientists focus on adult multipotent mesenchymal stem cells in stem cell research. Mesenchymal stem cells can be obtained from various sources including bone marrow, adipose tissue, umbilical cord (Hoang *et al.*, 2020), amniotic fluid (Streubel *et al.*, 1996), etc. Although bone marrow is the most preferred stem cell source in clinical studies (Kabat *et al.*, 2020), amniotic fluid is being recognized as an important stem cell source despite their comparatively later discovery, as they proved to have some pluripotent properties in addition to their mesenchymal character (Roubelakis *et al.*, 2007; Casciaro *et al.*, 2021).

As there are certain limitations on using embryonic stem cells; the amniotic fluid has become an important stem cell source as amniotic fluid-derived stem cells (AFSCs) possess higher cellular potency and they have almost no ethical concerns upon derivation (Fauza, 2004; Holden, 2007; Roubelakis *et al.*, 2007). AFSCs demonstrated to undergo mesenchymal (Pishnamazi *et al.*, 2023), endodermal (Carraro *et al.*, 2008) and ectodermal (Dikmen *et al.*, 2023) differentiations, and express important mesenchymal stem cell markers such as CD44, CD90 CD105, CD133, CD117 and SSEA-4 (Phermthai *et al.*, 2010). Also, it has been reported that AFSCs express several pluripotent markers including Nanog, SOX2, OCT 3/4, and c-Myc, which puts AFSCs in a better position in the context of cellular potency, compared to other MSC sources. Therefore, AFSCs have been suggested to be in

an intermediate form between multipotent and pluripotent stem cells in the context of cellular potency (Prusa *et al.*, 2003; Bossolasco *et al.*, 2006; Roubelakis *et al.*, 2007).

Although amniotic fluid-derived stem cells have been isolated and characterized from many different mammalian species, there is still very little research in the context of quantitative knowledge about their inter-passage differences in the expressions of important markers to choose the right passage for different research projects. In this study, we aimed to isolate AFSCs from Wistar rats and performed characterization studies to see mesenchymal and pluripotent properties of the isolated cells. In addition, the authors also intended to make inter-passage comparisons to understand the distinct characteristics of the cells in different passages to determine which passage could be favorable for separate purposes.

MATERIALS AND METHODS

All procedures were approved by the Ethical Committee of Afyon Kocatepe University (AKÜHADYEK-92-18, 11/06/2018).

Cell Isolation: Wistar rats were sacrificed on the 17th day of pregnancy and all the amniotic sacs were collected into sterile petri dishes under sterile conditions. The sacs were washed with 2% penicillin-streptomycin (Gibco, UK) and 0.2% amphotericin-B (Biowest, France) supplemented sterile phosphate-buffered saline (PBS). The sacs were punctured, and the amniotic fluids of all sacs from the same mother were collected into a sterile centrifuge tube. The tube was centrifuged at 250g for 10 minutes. After discarding the supernatant, the cell pellet was diluted with growth medium which consisted of 20% fetal bovine serum (FBS) (Biowest, South America), 1% penicillin-streptomycin, 0.1% amphotericin-B, 1% L-Glutamine (Gibco, UK), 20 ng/ml basic fibroblast growth factor (FGF) (R&D Systems, USA) in DMEM-LG (Sigma, USA). Cells were seeded in T25 flasks and incubated at 37°C. The culture medium was changed every 3rd day until reaching 70-80% confluency. After reaching confluence, cells were trypsinized and passaged.

Growth Curve: Growth curve studies were conducted to determine proliferation patterns of rat AFSCs (RAFSCs) in passage (P) 3 and P5. Cells were seeded at a concentration of 15×10^4 cells/well into 4-well plates. Cells were cultured for 14 days, and cell counting was performed at 2-day intervals for each sample (Kibria *et al.*, 2020a).

Multi-Lineage Differentiation: As a part of the mesenchymal stem cell characterization experiments, RAFSCs were tested for their multi-lineage differentiation capacities. For this purpose, osteogenic, chondrogenic, and adipogenic differentiation experiments were performed. Osteogenic differentiation of RAFSCs was induced using α-MEM (Lonza, Belgium) supplemented with 5% FBS, 1% penicillin-streptomycin, 1% L-glutamine, 0.1µM dexamethasone (Sigma, USA), 50µM L-ascorbic acid (Sigma, USA), and 10nM ß-glycerophosphate (Sigma, USA). Cells were cultured with this osteogenic induction medium for 21 days. The culture medium was refreshed every 3 days. Following the differentiation, cells were stained with 80mM Alizarin Red S (pH: 4,2) (Merck, Germany) to confirm the differentiation by visualizing orange-red calcium accumulations in the wells (Özden Akkaya *et al.*, 2023).

For adipogenic differentiation, cells were cultured with the adipogenic induction medium and the adipogenic maintenance medium throughout the 21-day experiment. The adipogenic induction medium consisted of 10% FBS, %1 penicillin-streptomycin, 1µM dexamethasone, 500µM IBMX (Gibco, UK), 100µM indomethacin (Sigma, USA), and 10µg/ml insulin in DMEM-LG. The adipogenic maintenance medium consisted of 10% FBS, %1 penicillin-streptomycin, and 10µg/ml insulin (Sigma, USA) in DMEM-LG. The adipogenic maintenance medium was used only in culture medium changes on the 7th and 15th days. In all the other medium changes, the induction medium was used. Following the adipogenic differentiation, cells were stained with Oil Red O to confirm successful differentiation by demonstrating red-stained oil droplets in the differentiated cells (Pittenger *et al.*, 1999).

A monolayer differentiation method was used for chondrogenic differentiation. For this, cells were cultured with DMEM-HG (Sigma, USA) supplemented with 5% FBS, 0.1mM dexamethasone, 50mM L-ascorbic acid, 1% ITS-premix (Gibco, UK), 1mM sodium pyruvate (Capricorn, Germany), 0.35mM proline (Sigma, USA), %1 non-essential amino acid (Lonza, Belgium), %1 penicillin-streptomycin and 10ng/ml TGF-ß3 (Peprotech, USA). The differentiation medium was replenished every 3 days. After the 21-day differentiation process, cells were stained with Alcian Blue (pH: 2.5) (AlfaAesar, USA) and glycose-amino-glycans were stained in blue to confirm chondrogenic differentiation (Kibria, 2018).

Immunofluorescence Staining: The P5 RAFSCs were checked for their protein expressions against pluripotent markers SOX2 and OCT3/4. Cells were fixed with 4% paraformaldehyde for 30 minutes and treated with 0.1% Triton-X (Biomatik, USA) for 10 minutes. Then, RAFSCs were blocked with 10% goat serum (Abcam, UK). Anti-SOX2 (Merck, Mab5603, 1/50 dilution), and Anti-OCT3/4 (Santa Cruz, Sc-5279, 1/50 dilution) were used as primary antibodies. A goat anti-mouse (Abcam, ab6787, Texas Red Conjugated, 1/1000 dilution) secondary antibody was used for signaling. To stain the nuclei of the cells, DAPI Fluoroshield Mounting Medium (Abcam, UK) was applied, and the slides were mounted. The slides were checked under Zeiss Axio Observer Z.1 microscope and photographed.

Real-Time qPCR: RAFSCs from P1, P3, and P5 were used in real-time qPCR analyses. Total RNA extraction was performed by using a commercial kit (TRIzol Reagent, Thermo 15596026). Total RNA amounts in samples were determined by using Qubit RNA HS Assay Kit and Qubit 2.0 Fluorometry device (In Vitrogen). Samples were treated with RNase-free DNase I to avoid DNA contamination and cDNA synthesis was made using the Maxima First Strand cDNA synthesis kit. RealQ Plus 2X Master Mix Green Rox Kit was used in real-time

qPCR analysis. The analyses were performed using Applied Biosystems ViiA 7 real-time PCR device. All samples were run in duplicates, and triplicates were performed if necessary. GAPDH was used as the housekeeping gene. Mesenchymal markers CD90, CD105, and CD44; hematopoietic markers CD34 and CD45; pluripotent markers SOX2, OCT3/4, and Nanog were analyzed in real-time qPCR studies. The fold change in expression was calculated with the $2^{\Delta\Delta ct}$ method for all used genes (Nawaz, 2020). All the used PCR primers are presented in Table 1.

Statistics: All the statistical tests were performed by using SPSS (v20) software. Samples from each group were checked for their normal distribution by the Shapiro-Wilk test. Data from real-time qPCR studies were analyzed by one-way ANOVA with Tukey's multiple comparisons test. The data of growth curve studies were analyzed by t-test. All the graphs were made using GraphPad Prism 8 software.

RESULTS

Cell Morphology: The RAFSCs exhibited a diverse morphology in the initial passage (P0) as fibroblast-like cells, epithelial-like cells, and stromal cells were visible (Fig. 1, A, E, F). Cells had attached to the surface within the first 6 hours and later, they started to aggregate (Fig.1, A). Fibroblast-like morphology had become the dominant morphology beginning with the first passage. Although fibroblast-like cells were dominant in P1, still other morphologies were present in smaller portions. However, after P3, only fibroblast-like cells were seen in the culture and the morphology remained persistent through all the passages (Fig. 1, B, C, D).

Growth Curve: The growth curve analysis showed that the cells from P3 and P5, both entered the logarithmic phase right after the second day and cells reached the peak at day 6 for both passages. Afterward, cells entered the plateau phase until the end of the study, on day 14. No changes were observed related to the proliferation patterns between P3 and P5 cells. Furthermore, there was no statistical difference between the cell numbers of both passages in any time interval ($p>0.05$) (Fig. 2).

Multi-Lineage Differentiation: To confirm their multi-lineage differentiation capabilities as a part of their mesenchymal character, isolated RAFSCs were differentiated into osteogenic, chondrogenic and adipogenic cells at P3. After 21 days of induction, differentiations were confirmed by their respective staining techniques. The orange-red stained calcium deposits were observed after alizarin red s staining to confirm osteogenic differentiation (Fig. 3, B) while the presence of oil droplets, which were stained in red after oil red o staining, confirmed the adipogenic differentiation (Fig. 3, D). Although blue-stained glycosaminoglycans were visible after the alcian blue staining, these glycosaminoglycans were very few, and the chondrogenic differentiation of RAFSCs observed to be weaker in monolayer culture conditions compared to the results of other differentiation experiments (Fig. 3, F). The negative control cells remained unstained in all differentiation studies (Fig. 3, A, C, E).

Gene and Protein Expressions: The availability of protein expressions of pluripotent markers OCT 3/4 and SOX2 were investigated by IF staining in P5 cells. The staining showed that all the seeded cells at P5 were positive against both SOX2 and OCT 3/4 (Fig. 4). Gene expressions of mesenchymal, hematopoietic, and pluripotent markers were investigated in real-time qPCR at P1, P3, and P5 to make inter-passage comparisons. For mesenchymal markers CD105 and CD44, there were no significant differences between P1, P3, and P5 cells ($p >0.05$) (Fig. 5, B, C). However, there was a significant upregulation for CD90 gene expression in P5 cells compared to P1 and P3 ($p <0.05$) (Fig. 5, A). For hematopoietic markers, the expression of CD34 was significantly downregulated at P3 and P5 compared to P1 ($p <0.05$) (Fig. 5, G). CD45 expression was also significantly downregulated at P3 ($p<0.01$) and P5 ($p<0.001$) compared to P1 (Fig. 5, H). SOX2 expression is found to be significantly downregulated at both P3 ($p<0.01$) and P5 ($p<0.05$) compared to P1 (Fig. 5, D). For expressions of both OCT3/4 and Nanog, no statistical differences were noted between P1 and P3 cells. Yet, significant upregulations were detected in P5 cells for both OCT 3/4 ($p<0.01$) (Fig. 5, E) and Nanog ($p<0.05$) (Fig. 5, F) gene expressions.

DISCUSSION

In this study, it was aimed to isolate RAFSCs and confirm their mesenchymal character by testing their multi-lineage differentiation capacity and their expressed markers, according to the minimum criteria set by Dominici *et al.* (2006). As the AFSCs were reported to have some pluripotent features, the expressions of pluripotent markers were also tested. After confirming their mesenchymal character and examining their pluripotent properties, RAFSCs were further investigated for their inter-passage differences to evaluate their potential for different purposes.

After isolation, a diverse morphology consisting of fibroblast-like, stromal, and epithelial-like cells were observed at P0. The fibroblast-like morphology had become the dominant morphology starting with the 1st passage. Although still other cell types were visible in P1 and P2, after the 3rd passage, cell morphology became homogenous, and all cells exhibited fibroblast-like morphology. In parallel to our results, Savickiene *et al.* (2015), demonstrated the existence of stromal, epithelial-like, and fibroblast-like cells in their study, based on human AFSCs, and Nawaz (2020) showed these three morphologies in RAFSCs.

The growth curve analysis showed no statistical difference between the cell numbers and the proliferation patterns of RAFSCs from P3 and P5. These results show parallelism of the results of You *et al.* (2009) and Minocha *et al.* (2019) as they also reported no difference in their growth curve analyses until P10.

Dominici *et al.* (2006) outlined minimum criteria for cells to be defined as mesenchymal cells. According to that, cells must be capable of undergoing multi-lineage differentiations; express markers including CD 90, CD105, and CD44; and not express hematopoietic markers such as CD34 and CD45. To confirm the multi-

Table 1: PCR primers used in real-time PCR analyses.

Gene	Forward (5'→3')	Reverse (5'→3')	Tm (°C)
SOX2	GGCGGAAAACCAAGACGCTC	TGTAGCTGCCGTTGCTCCAGC	58
OCT 3/4	CAGACAACCATCTGCCGCTTCG	CACCAGGGTCTCCGATTTGCA	58
NANOG	TGCTACTGAGATGCTCTGCAC	CCTGAGAGAACACAGTCCGCA	58
CD90	GGTCCTTACTCTAGCCAACTTCAC	AACCAGCAGGCTTATGCCACC	58
CD105	GCGTCACACTTGAATGGCAACC	GGATGAGAACGGCATCCCCA	58
CD44	GCATCCAACACCTCCCACTATGAC	CTGGTCCATCGAAGGAATTGGGTA	58
CD45	GATGTACCACCAGGGACTCACAA	TGTAGAGGACTTCCGCAGCAC	58
CD34	GCCATCTCAGAGACCACGGTC	GGTGGAGTGTTCCACTTCTGGA	58

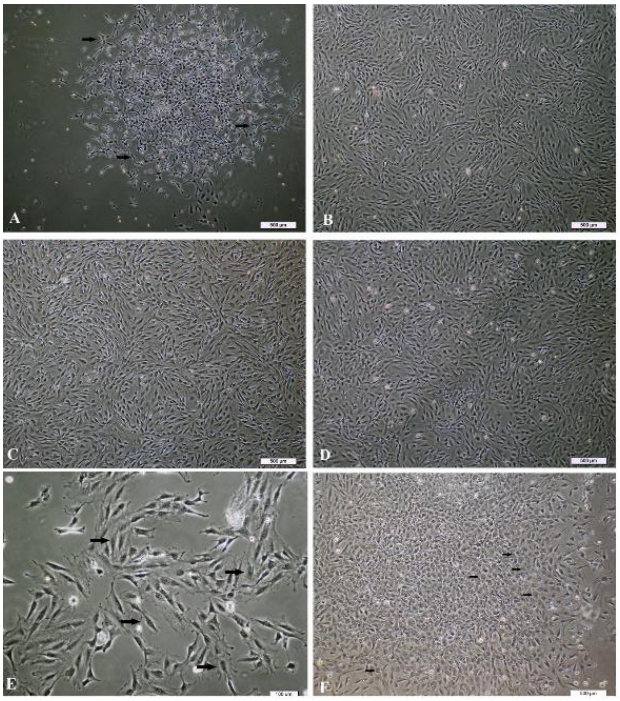

Fig. 1: Cells exhibited diverse morphology at P0 and fibroblast-like morphology had become the dominant morphology after the first passage. A: Fibroblast-like cells at P0, B: Fibroblast-like cells at P1, C: Fibroblast-like cells at P3, D: Fibroblast-like cells at P5, E: Stromal cells at P0, F: Epithelial-like cells at P0. Bar= 500µm (A, B, C, D), 100µm (E).

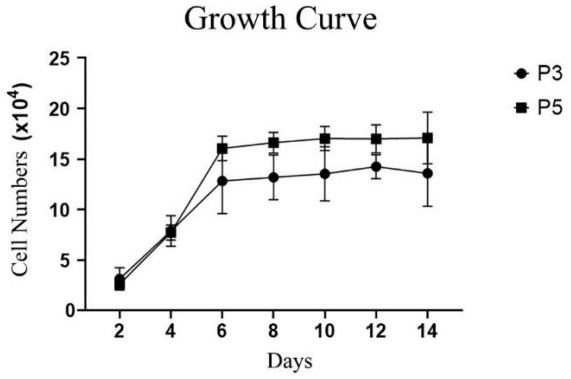

Fig. 2: Growth curve of P3 and P5 cells. The growth curve analysis showed no difference between P3 and P5 cells for both proliferation patterns and cell numbers.

lineage differentiation capacity of isolated RAFSCs; osteogenic, adipogenic, and chondrogenic differentiations were performed. In addition to that, expressions of mesenchymal markers CD90, CD44, and CD105; and hematopoietic markers CD34 and CD45 were investigated in P1, P3, and P5 cells by real-time qPCR. The differentiation studies were concluded on the 21st day and each differentiation was verified with their respective staining. Previously, AFSCs from various mammalian

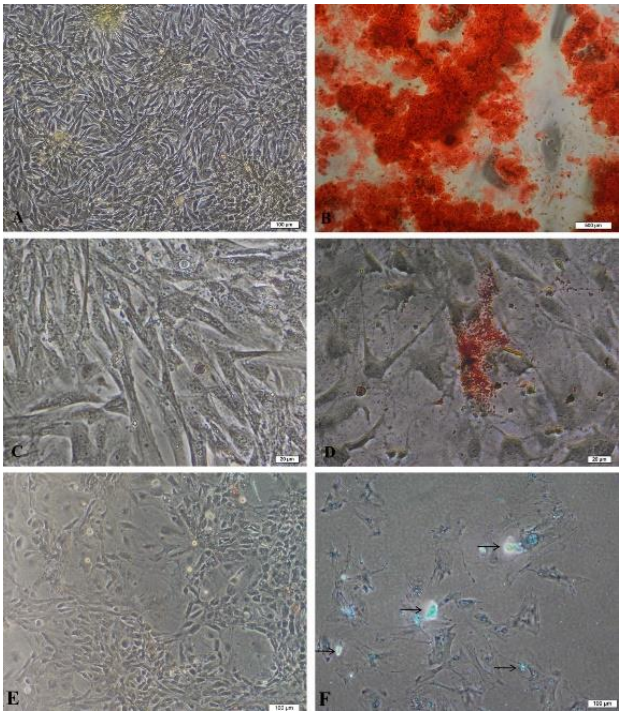

Fig. 3: Multi-lineage differentiation of RAFSCs. A, C, E: Negative control cells remained unstained, B: Osteogenic differentiation confirmed by demonstrating red-orange calcium deposits after alizarin red s staining, D: Adipogenic differentiation confirmed by demonstrating red oil droplets after oil red o staining, F: Bright blue stained glycosaminoglycans were observed after alcian blue staining to confirm chondrogenic differentiation. Bars= 500 µm (B), 100 µm (A, E, F), 20 µm (C, D).

species such as humans (Pipino *et al.*, 2015), equines (Kibria *et al.*, 2020b), bovines (Rossi *et al.*, 2014), and rats (Nawaz, 2020) were proven to have the capacity to undergo these differentiations. Although our results of alizarin red s and oil red o staining procedures presented good results as we saw high amounts of calcium deposits and oil droplets; the alcian blue-stained glycosaminoglycans were far less following the chondrogenic differentiation experiment. The chondrogenic differentiation process heavily depends on cell-to-cell and cell-to-matrix interactions. Therefore, monolayer culture is not an optimum system for achieving strong chondrogenic differentiation. Three-dimensional (3D) culture systems are suggested for chondrogenic differentiation purposes as the provided 3D environment mimics the extracellular matrix and allows more interactions for the cells (Zuliani *et al.*, 2021). The RAFSCs had previously demonstrated to have good chondrogenic differentiation potentials in 3D culture (Nawaz, 2020). Yet, in the present study, we only performed chondrogenic differentiation to test the multilineage differentiation capability of the RAFSCs to

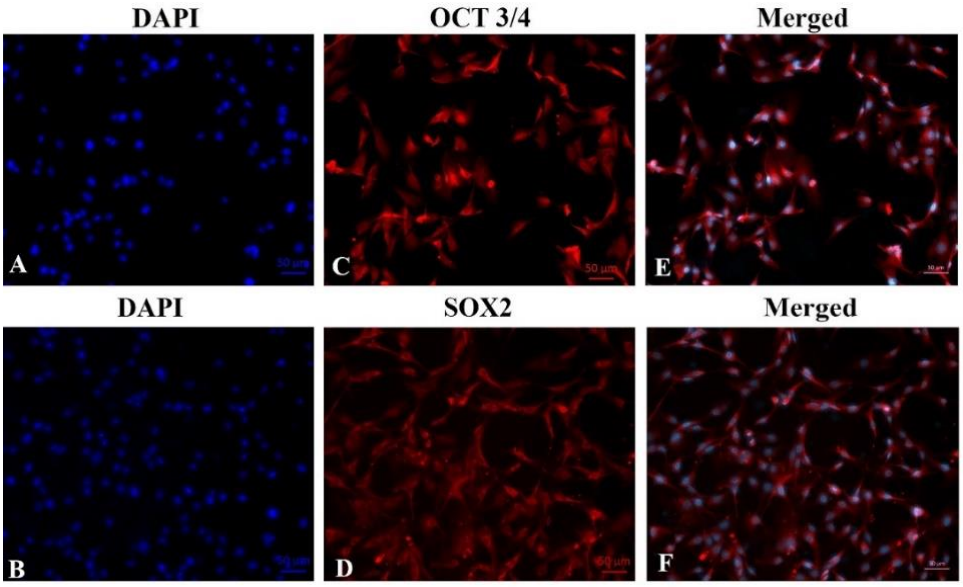

Fig. 4: The protein expressions of pluripotent markers OCT 3/4 and SOX2 were demonstrated with IF staining. A, B: Nuclei stained in blue with DAPI. C: OCT 3/4 expressions in P5 RAFSCs. D: SOX2 expressions in P5 RAFSCs. E, F: Merged images. Bar = 50µm.

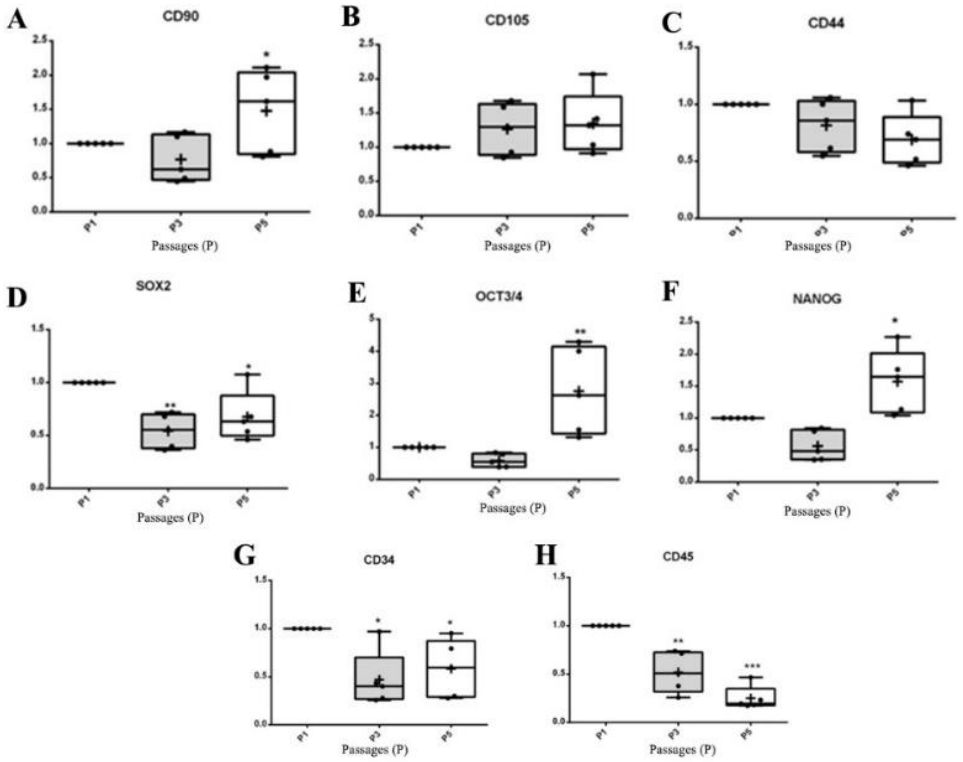

Fig. 5: The gene expressions of mesenchymal, pluripotent, and hematopoietic markers in different passages. The expression of mesenchymal markers CD105 (B) and CD44 (C) showed no significant changes between passages, while a significant increase was observed in the expression of CD90 (A) at P5. The expressions pluripotent marker SOX2 was observed to be significantly downregulated both in P3 and P5 compared to P1 (D) while other pluripotent markers, OCT ¾ (E) and NANOG (F), showed significantly increased expressions at P5. The hematopoietic markers CD34 (G) and CD45 (H) showed significantly reduced expression in P3 and P5 compared to P1 (*p<0.05, **p<0.01, ***p<0.001).

confirm their mesenchymal character. So, we did not perform a 3D culture for differentiating RAFSCs into chondrocytes. However, a future study underlying the differentiation capacities of RAFSCs towards different lineages should be made to gain a better understanding.

The real-time qPCR studies revealed that the mesenchymal markers CD90, CD105, and CD44 were all positively expressed by both P1, P3, and P5 RAFSCs. Although CD105 and CD44 showed stable expressions through the increasing passages, we saw a significant upregulation in CD90 gene expression for P5 cells. Roubelakis *et al.* (2007), Phermthai *et al.* (2010), and Pipino *et al.* (2015) described positive expressions of these markers in human AFSCs as well. Although these reports did not contain inter-passage quantitative comparisons, Corradetti *et al.* (2014) showed that CD44 is being expressed in similar levels throughout different passages by presenting PCR band images. One of the few quantitative inter-passage comparisons for RAFSCs had been performed by Nawaz (2020) in rats. However, in the study of Nawaz (2020), no statistical difference was reported for CD90 gene expressions, and a significant upregulation was described for CD105 gene expressions in P3 and P5 cells compared to P1, different than our results. The underlying reason behind these differences might be due to the different gender compositions of the fetuses in which the amniotic fluids were pooled. While collecting the amniotic fluid from rats, all the sacs from the same mother are being punctured without noting the gender of the fetus and the fluids are being pooled into one tube for each mother. Therefore, each sample had a pool of amniotic fluids collected from fetuses with

different gender distribution. Poggi *et al.* (2004) demonstrated that the gender of the fetus significantly affects the concentrations of different cytokines in amniotic fluid. Katsara *et al.* (2011) parallelly reported that the donor's gender affects the colony-forming abilities and differentiation capacities of bone marrow-derived stem cells. Therefore, the difference between the study of Nawaz (2020) and our study might be due to the difference in gender distributions of the fetuses despite using the same stem cell source. However, further investigations should be made to back this hypothesis.

The mesenchymal stem cells are reported to be CD34 (+) at the start of the culture. Yet, these CD34 (+) cells are being eliminated under culture conditions and cells become CD34 (-) in time (Lin *et al.*, 2012). In this study, significant downregulations were observed in hematopoietic markers CD34 and CD45 in P3 and P5 compared to P1. These subsequential declines with increased passage numbers are in parallel to the study of Nawaz (2020) which RAFSCs were examined. Phermthai *et al.* (2010) also reported that human AFSCs had not expressed CD34 and CD45 in their flow cytometry analyses.

AFSCs had been described to have some pluripotent properties as they reported to express some pluripotency-related genes such as SOX2, OCT 3/4, Nanog, and c-Myc (Bossolasco *et al.*, 2006; Phermthai *et al.*, 2010). In this study, we demonstrated that RAFSCs express SOX2 and OCT 3/4 proteins in P5 by immunofluorescence staining and made inter-passage comparisons for SOX2, Nanog, and OCT 3/4 gene expressions by real-time qPCR. OCT 3/4 is an important marker for the self-renewal of stem cells (Zaehres *et al.*, 2005) and the decrease in its expression results in the loss of pluripotent state and de-differentiation of embryonic stem cells (Thomson *et al.*, 2011a) In addition to being a pluripotent cell marker, SOX2 is also a known enhancer of ectodermal differentiation and being used as a neural marker in differentiation studies, and it was reported that SOX2 inhibits mesodermal differentiation capacity of the cells (Johansson and Simonsson, 2010; Thomson *et al.*, 2011b). Furthermore, Thomson *et al.* (2011a) reported that the mesodermal differentiation capacity of cells is enhanced when SOX2 expression is downregulated. The same study also indicates that upregulation of OCT 3/4 expression resulted in increased mesodermal capacity of cells. Nanog, on the other hand, is known to inhibit hematopoiesis during embryonal development and is reported to have a negative correlation with hematopoiesis (Sainz de Aja *et al.*, 2019). This negative correlation between hematopoiesis and Nanog was also visible in this study as we witnessed decreasing hematopoietic marker expressions in later passages which we also observed up-regulation in the expressions of Nanog.

This study reveals that RAFSCs are stem cells of mesenchymal nature as they can undergo multi-lineage differentiation and have the ability to rapidly proliferate. Furthermore, RAFCs demonstrated to have some pluripotent properties as they express pluripotent markers SOX2, NANOG, and OCT 3/4 to an extent. The real-time qPCR studies showed that cells from P5 could be more suitable to be utilized in studies for differentiation or regeneration of mesodermal cells, like osteogenic and chondrogenic differentiation, due to the favorable expressions of genes, as SOX2 is decreased and OCT ¾ was upregulated. Yet, for research that requires better ectodermal differentiation properties, such as neural tissue regeneration studies, the RAFSCs from earlier passages might be more advantageous to use since it was demonstrated that OCT 3/4 is being increased in later passages. The drastic decrease in hematopoietic markers with the increased passage numbers also could be taken into consideration while designing research. Additionally, there is no noteworthy difference observed in the context of cell proliferation and morphology between P3 and P5 cells to consider while choosing the suitable passage to work with.

This study relies on the assessment of mesenchymal, pluripotent, and hematopoietic expression markers along with proliferation patterns of RAFSCs in P1, P3, and P5. Therefore, our results should be tested in a wider passage range, and the differences in differentiation capacities of RAFSCs of different passages to different lineages should be assessed in future experiments.

Conclusions: After assessing the literature knowledge and the results of the study, it may be suggested that using RAFSCs at P5 may favor mesodermal differentiation. Using RAFSCs at P5 also may be beneficial as Nanog, a known inhibitor of hematopoiesis capacity, is also being upregulated along with further downregulations of hematopoietic markers CD34 and CD45. Moreover, using RAFSCs in earlier passages may be more advantageous for ectodermal differentiation studies. Yet, these hypotheses should be investigated by detailed differentiation studies and the properties of RAFSCs should be examined for their other properties as well. However, future studies, including comparative studies of multi-lineage cell differentiation in different passages, are necessary to back these statements.

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Authors contributions: TD and KA conceived and designed the study. TD executed all cell culture-related experiments. ME executed real-time qPCR analysis. TD and ME analyzed the data. ME and KA critically revised the manuscript and provided supervision. All authors contributed to the writing of the manuscript and approved the final version.

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