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The Assessment of Mesenchymal Stem Cells Characteristics in Cultured Amniotic Fluid Cells

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ABSTRACT

Objectives: To evaluate the morphological characteristics, immunophenotypic features, osteogenic, adipogenic, and chondrogenic differentiation capacities of in-vitro cultured amniotic cells during the culture process based on mesenchymal stem cell (MSC).

Material/Method: This study used waste cells remaining after fetal karyotype determination with second-trimester amniotic cell culture. Cultured cells were morphologically evaluated daily. Selective subculture (SSC) was produced by the scraping method. Immunophenotypic features of primary and subculture amniotic cells were evaluated based on specific surface antigens. Specific growth mediums were used to assess their osteogenic, adipogenic, and chondrogenic differentiation abilities. Differentiation was confirmed using specific staining.

Results: Fifteen cases with 46,XY fetal cytogenetic analyses at 16^{+3}-19^{+5} gestational weeks were included in the study. Amniotic fluid cells were evaluated morphologically in three classes as Amniotic fluid specific cell-type(AF), Fibroblastoid cell-type(FB1, FB2), and Epithelioid cell-type(E). The first cell adhesions occurred in the first 24-48 hours(36%) of cell culture, while the first colonies within three days(82%). SSC was produced in AF-SSC(n=4) and FB2-SSC(n=6) directions by mechanical scraping. Primary amniotic fluid cells and AF/FB-SSCs were positive for MSC immunophenotypic markers CD29,CD73,CD166,CD44,CD49e,CD90, while negative for CD34,CD45, and HLA-DR. Cells were shown to have osteogenic (Alizarin Red-S) and chondrogenic (Alcian Blue) differentiation potentials based on histochemical staining, while adipogenic (Oil Red-O) differentiation was not obtained.

Conclusion: We believe that preliminary protocols and experiences to obtain MSCs from amniotic fluid-derived cells produced under routine prenatal diagnosis cell culture conditions can enable many promising pre-clinical/clinical studies and be the pioneer of "stem cell therapy applications" in many "incurable" diseases in the clinic.

Keywords: Amniocentesis, Cell morphology, Differentiation, Flow cytometry, Mesenchymal stem cell

Kültüre Amniyotik Sıvı Hücrelerinde Mezenkimal Kök Hücre Özelliklerinin Değerlendirilmesi

ÖZET

Amaç: Mezenkimal kök hücre (MKH) temelinde in-vitro kültüre amniyotik hücrelerin kültür işlemi sırasında morfolojik özelliklerini, immünofenotipik özelliklerini, osteojenik, adipojenik ve kondrojenik farklılaşma kapasitelerini değerlendirmek.

Materyal/Yöntem: 2. trimester amniotik hücre kültürü ile karyotip tayini yapılmış "atık" hücreler kullanıldı. Kültür hücreleri, günlük morfolojik olarak değerlendirildi. Kazıma yöntemi ile selektif subkültür (SSC) üretildi. Primer ve subkültür amniyotik hücrelerinin immünofenotipik özellikleri, spesifik yüzey antijenlerine dayalı olarak değerlendirildi. Osteojenik, adipojenik ve kondrojenik farklılaşma yeteneklerini değerlendirmek için spesifik büyüme ortamları kullanıldı. Farklılaşma, spesifik boyama kullanılarak doğrulandı.

Bulgular: 16^{+3}-19^{+5} gebelik haftasında, fetal sitogenetik analizi 46,XY olan 15 olgu çalışmaya alındı. Amniyotik sıvı hücreleri morfolojik olarak Amniyotik sıvıya özgü hücre tipi(AF), Fibroblastoid hücre tipi(FB1, FB2) ve Epitelioid hücre tipi(E) olmak üzere üç sınıfta değerlendirildi. İlk hücre adezyonları hücre kültürünün ilk 24-48 saatinde (%36), ilk koloniler ise üç gün içinde (%82) meydana geldi. SSCs, AF-SSC(n=4) ve FB2-SSC(n=6) yönlerinde mekanik kazıma ile üretildi. Primer amniyotik sıvı hücreleri ve AF/FB-SSC'ler, MKH immünofenotipik belirteçleri CD29,CD73,CD166,CD44,CD49e,CD90 için pozitifti, CD34,CD45 ve HLA-DR için negatifti. Hücrelerin histokimyasal boyamaya dayalı osteojenik (Alizarin Red-S) ve kondrojenik (Alcian Blue) farklılaşma potansiyellerine sahip olduğu gösterilirken, adipojenik (Oil Red-O) farklılaşma elde edilemedi.

Sonuç: Rutin prenatal tanı hücre kültür koşullarında üretilen amniotik sıvı kökenli hücrelerden MSC elde etmeye yönelik oluşturulan öncül protokoller ve elde edilen deneyimlerin, gelecek vaadeden pek çok preklinik/klinik araştırmaya olanak sağlayabileceği ve klinikte pekçok "çaresiz" hastalıkta, "kök hücre tedavisi uygulamaları"nın öncüsü olabileceği inancındayız.

Anahtar Kelimeler: Amniosentez, Hücre morfolojisi, Farklılaşma, Flowsitometri, Mesenkimal kök hücre

Stem cells can constantly renew themselves and differentiate into various tissues, and they can differentiate into cell types of mesodermal, ectodermal, and endodermal origin under specific conditions (1).

Due to the increasing application areas of stem cell researches in medicine, it has become necessary to search for stem cell sources that can be used in human applications. Fetal mesenchymal stem cell (MSC) is one of the sources determined from these studies. Amniotic cells are fetal cells that have been routinely used to diagnose genetic diseases; recent publications identify amniotic fluid-derived MSCs as potent candidate cells with particular advantages in the cell therapy approach due to their biological and genetic characteristics (2-5).

This study aimed to investigate the MSC potential of amniotic fluid cells, which were in the "waste" position after genetic analysis, and evaluate the morphological characteristics, immunophenotypic characteristics, and osteogenic, adipogenic, chondrogenic differentiation capacities of the cells during the culture process.

MATERIAL AND METHOD

Amniotic Fluid-derived Stem Cell Isolation and Cell Culture

Cell Source

Second-trimester amniotic fluid cells were used in the study. Cells were obtained from cultured cells "waste" of 15 prenatal diagnosis cases who underwent routine amniocentesis for genetic diagnosis. Fetuses with malformations detected in fetal ultrasonography and amniotic fluid cells with hemorrhagic features were excluded from the study. The local medical ethics committee (Decision no: OMU Rectorate projects, T-393, T-394) approved the study in accordance with human and animal rights and complied with the principles of the Helsinki Declaration. Informed consent was obtained before collecting samples from all patients.

Cell Culture

The 3ml cell debris obtained after the centrifugation of the amniotic fluid samples for 10 minutes at 1500rpm was suspended with Bio-AMF-1 (Biological Industries, Kibutz Beit Haemek) containing 100U/ml penicillin, 0.1mg/ml streptomycin, and 1L Glutamine (Gibco) and inoculated into three different 25 cm^2 flasks (Nunc, Rochester, MN). The flasks were named P01, P02, and P03. After seven days, a fresh medium was added to the cells adhering to the flask surface. Once the adherent cells filled the flask, the P01-flask was used for fetal karyotype determination. P02 and P03 flasks remaining in the waste cell state were used in further studies.

Cell Morphology

All flasks were examined under an inverted microscope (OLYMPUS CKX41) for morphological characteristics during the culture and subculture process.

Karyotyping

Fetal cytogenetic analysis was performed at P01-flask (n=15).

Immunophenotyping

In the P02-flask (n=15), adherent cells were removed by trypsinization with trypsin/EDTA C 10X solution (Bio. Ind., 03-053-1) once the flask was filled. Cells were washed twice with Phosphate Buffer Saline (PBS). 100µl of amniotic fluid samples were placed in the tubes with a cell concentration of $1x10^6$/ml. The tubes were vortexed by adding monoclonal antibodies in the appropriate titrations (5-10µl). They were incubated for 15 min at 4°C and in the dark. Then 1ml of washing solution (PBS + 0.1% Na azide) was added to the tubes and centrifuged at 1400rpm for 5 minutes. 0.5ml of wash solution was added to the cell precipitate again and read on the FACSCalibur (Becton Dickinson, Biosciences) flow cytometer and analyzed in the "Cell Quest Software" program [Becton Dickinson immunocytometry system, Mac OS X 10.3.6 (7R28)]. Samples prepared using autofluorescence and appropriate isotypic control were used as a negative control. Quadrants or histogram markers were adjusted relative to negative controls.

Selective Subculture (SSC)

Cell colonies adhered to the P03-flask were morphologically (OLYMPUS CKX41) monitored daily. The mechanical scraping method was used for a subculture (BD FalconTM cell scraper). Once the selected permanent colonies filled the flask, they were trypsinized with trypsin/EDTA C 10X solution (Bio. Ind., 03-053-1) and grown in two separate flasks, one for immunophenotyping and the other for differentiation studies.

Immunophenotyping in Selective Subculture (SSC)

Cells obtained from SSC-flasks (P13a) (n=10) were evaluated as immunophenotypic.

Differentiation Protocol and Histochemical Staining

Osteogenic Differentiation

In osteogenic differentiation studies, cells grown in an in-vitro culture medium were studied by a method in accordance with the literature information of Pittenger et al. (6). Trypsinized cells were treated with osteogenic medium [DMEM-LG (Euroclone Ltd.,UK) containing 10% Fetal bovine serum (FBS)(Euroclone Ltd.,UK), dexamethasone (100nM,Sigma,USA), beta glycerophosphate (10mM,Sigma,USA) and ascorbic acid (0,2mM,Sigma,USA)] after approximately 1 week of culture step. The medium was freshened every 3-4 days for 21 days. At the end of the 21st day, it was stained with "Alizarin Red-S" (6-12).

Adipogenic Differentiation

In adipogenic differentiation studies, cells grown in an in-vitro culture medium were studied by a method in accordance with the literature information of Pittenger et al. (6). Trypsinized cells have waited until they filled up the flask. Then, the medium was refreshed with adipogenic medium [DMEM-LG (Euroclone Ltd., UK) containing 10% FBS (Euroclone Ltd., UK), dexamethasone (1µM, Sigma,USA), indomethacin (60µM, Sigma,USA), 3-isobutyl-1-methylxanthine (IBMX) (500µM, Sigma,USA) and insulin (5µg/ml, Sigma, USA)] every 3-4 days and stained with "Oil Red-O" on day 21 to show the presence of adipogenic differentiation (6-9,11-13).

Chondrogenic Differentiation

Chondrogenic differentiation studies were done by a method according to the literature information of Pittenger et al. (6). Trypsinized cells were placed in 2 separate 15-ml polypropylene centrifuge tubes ($2.5x10^5$ cells), centrifuged at 1000rpm for 5 minutes, and formed into pellets. The pellet was incubated for 24 hours at 37°C, 5% CO2. Then, it was treated with chondrogenic medium [DMEM-HG (Euroclone Ltd.,UK) containing dexamethasone (100nM,Sigma,USA), TGF-B3 (10ng/ml,Peprotech,USA), ascorbic acid (50µg/ml,Sigma,USA), ITS+Premix (50mg/ml 500 B&D Biosciences)] and the medium was refreshed every 3-4 days for 21 days. At the end of the 21st day, 5µm thick "frozen" sections were taken with a semi-automatic rotating arm microtome (RM2125RT Leica), and the preparations were stained with "Alcian Blue" and "Hematoxylin/Eosin." (8,14-16).

RESULTS

Amniotic Fluid-derived Cell Source and Karyotyping

Fifteen male (46,XY) amniocentesis cases with a gestational age of 16^{+3}-19^{+5} weeks, maternal age between 24-44 years, were included in the study.

Cell Culture and Cell Morphology

Cells seeded from amniotic fluid in 3 separate flasks under sterile conditions consisted of amniocytes of different maturity (young and old). Initial cell adhesions were observed within the first 24-48 hours of primary amniotic fluid cell culture. Adhesion was observed in 20%, 36%, and 82% of flasks at 24, 48, and 72 hours, respectively. The first colonies were observed on the 3-5th day, and on the 8th-11th days, the cell density filled 2/3 of the culture dish.

During the 2-week follow-up period, colonies were observed in 3 cell types: Amniotic fluid specific cell-type (AF), Fibroblastoid cell-type (FB1, FB2), and Epitheloid cell-type (E).

The fibroblastoid-type cells were spindle-shaped cells parallel to each other. The long and thin structure of FB1 cells was more homogeneous than FB2 cells. AF-type cells were non-tightly organized, round-shaped, polygonal cells, whereas epithelioid-type cells were tightly organized, forming small colonies, and had a rounded, sharp-edged, manlike morphology.

At the initial stage of culture, the cell population was heterogeneous. AF-type cells were observed throughout the culture process; E-type cells were significantly reduced after a short time. FB1-type cells emerged relatively late in the culture process, on average ten days (Fig.1).

Immunophenotyping

On days 15-18 of the primary cell culture (P02) (n=15), in immunophenotyping, CD90, CD73, CD166, CD49e, CD44, CD29, HLA-ABC were positive, CD34, CD45, HLA-DR were negative. "Dot-plot" and "histogram graphs" of all cases were constituted. In 10 cases, cell surface antigen with CD14 antibody was also studied and found negative (Table.1) (Fig.2).

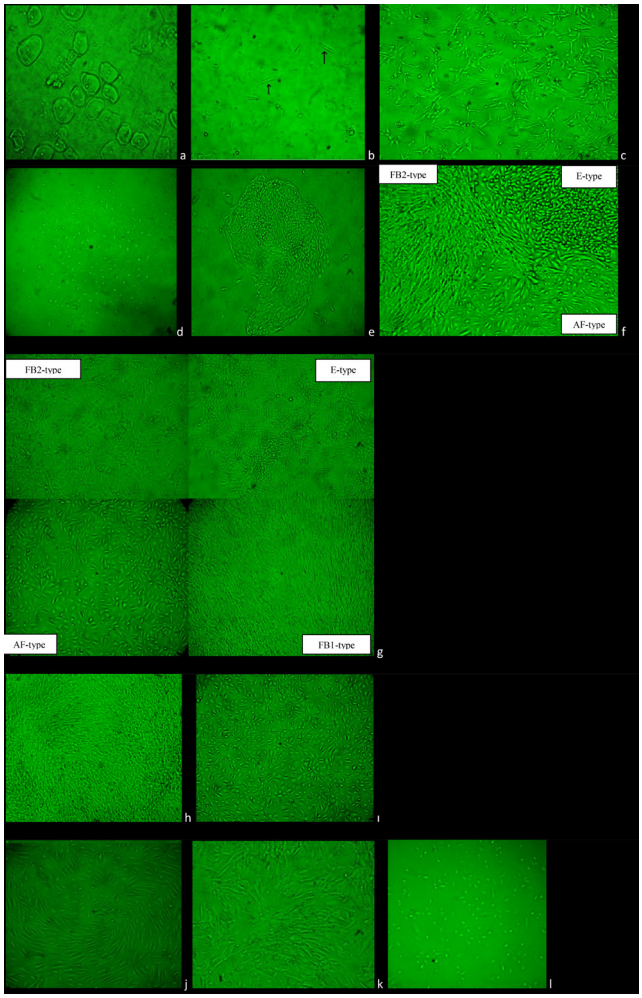

Figure 1: Amniotic fluid cell morphology; a- First-day appearance of amniotic fluid cells in culture (OLYMPUS CKX41, 10X); b- First 24-48 hours, first cell adhesions (OLYMPUS CKX41, 10X); c- FB2-type cell adhesions (first colonies) in the first 3-5 days (OLYMPUS CKX41, 4X); d- AF-type cell adhesions (first colonies) in the first 3-5 days (OLYMPUS CKX41, 4X); e- Epithelioid-type cell adhesions (first colonies) in the first 3-5 days (OLYMPUS CKX41, 4X); f- Cell types in amniotic cell culture (OLYMPUS CKX41, 10X); g- Cell types in amniotic cell culture (OLYMPUS CKX41, 10X); h- AF selective subculture image (Olympus CKX41, 10X); I- FB2 selective subculture image (Olympus CKX41, 10X); j- Post-trypsinization images of AF selective subcultures (Olympus CKX41, 10X); k- Image of FB2 selective subcultures after trypsinization (Olympus CKX41, 10X); l- AF selective subcultures degenerated (Olympus CKX41,10X)

Selective Subculture (SSC)

Adhering cells in the P03-flask were monitored daily and mechanically scraped to form SSCs (BD FalconTM cell scraper) specific to a single cell (FB2/AF) colony. E-type cells could not be included in the study because they disappeared rapidly in culture. In some cultures, mechanical scraping could not be achieved. Out of 10 cases, AF-type SSC was done in 4 and FB2-type in 6. When the culture flask was nearly full, the SSC-flask was trypsinized with trypsin/EDTA C 10X solution (Bio.Ind., 03-053-1) and grown in two separate flasks. FB2/AF-SSCs retained their morphological features after trypsinization. "Embryoid body"-like spheroid structures were observed in AF-SSCs. While FB2-SSCs ensured culture continuity after the third passage, AF-SSCs degenerated at the same time (Fig.1).

Table 1: Immunophenotyping Protocol

Tube No	Antibody (Becton Dickinson, Biosciences)
1	CD 45 (FITC) / (Bazen CD 14) (PE)
2	Ig G1κ (FITC) / Ig G1κ (PE) (isotypic control)
3	CD 34 (FITC) / CD 73 (PE)
4	HLA-DR (FITC) / CD 166 (PE)
5	/ CD 29 (PE)
6	/ CD 44 (PE)
7	/ CD 90 (PE)
8	/ CD 49e (PE)
9	HLA-ABC (FITC) /
10	Autofluorescent control (amnion cells were treated in the same way without staining with any monoclonal antibody)

"Fluorescein isothiocyanate" (FITC), "Phycoerythrin" (PE), "Cluster of Differantiation" (CD), Immunoglobulin G1-kappa (Ig G1κ)

Selective Subculture (SSC) Immunophenotyping

10 cases underwent SSC in the P13a-flask were immunophenotypically determined as CD90, CD73, CD166, CD49e, CD44, CD29, HLA-ABC positive, CD34, CD45, HLA DR negative. Cell surface antigen with CD14 antibody was studied in 3 cases and found negative. "Dot-plot" and "histogram graphs" of all cases were constituted (Table.1) (Fig.2).

Differentiation

Adipogenic and Osteogenic Differentiation

FB2-SSCs could be done in a total of 8 cases. Differentiation studies were initiated at passage-2 in 4 cases, at passage-3 in 3 cases, and passage-4 in 1 case. In FB2-SSCs, in the wells in which differentiation study with the osteogenic medium was initiated, accumulation of white material also seen macroscopically, indicating calcium accumulation, was observed and osteogenic differentiation was demonstrated with Alizarin Red-S in all cases in different passages. However, in adipogenic differentiation processes, intracytoplasmic lipid accumulation could not be demonstrated with Oil Red-O, and adipogenic differentiation was not detected. In AF-SSCs, early degeneration of cells was observed in 5 cases. One heterogeneous case was included in the study in passage-1, and while osteogenic differentiation was detected in this case, adipogenic differentiation was not observed (Table.3) (Fig.3).

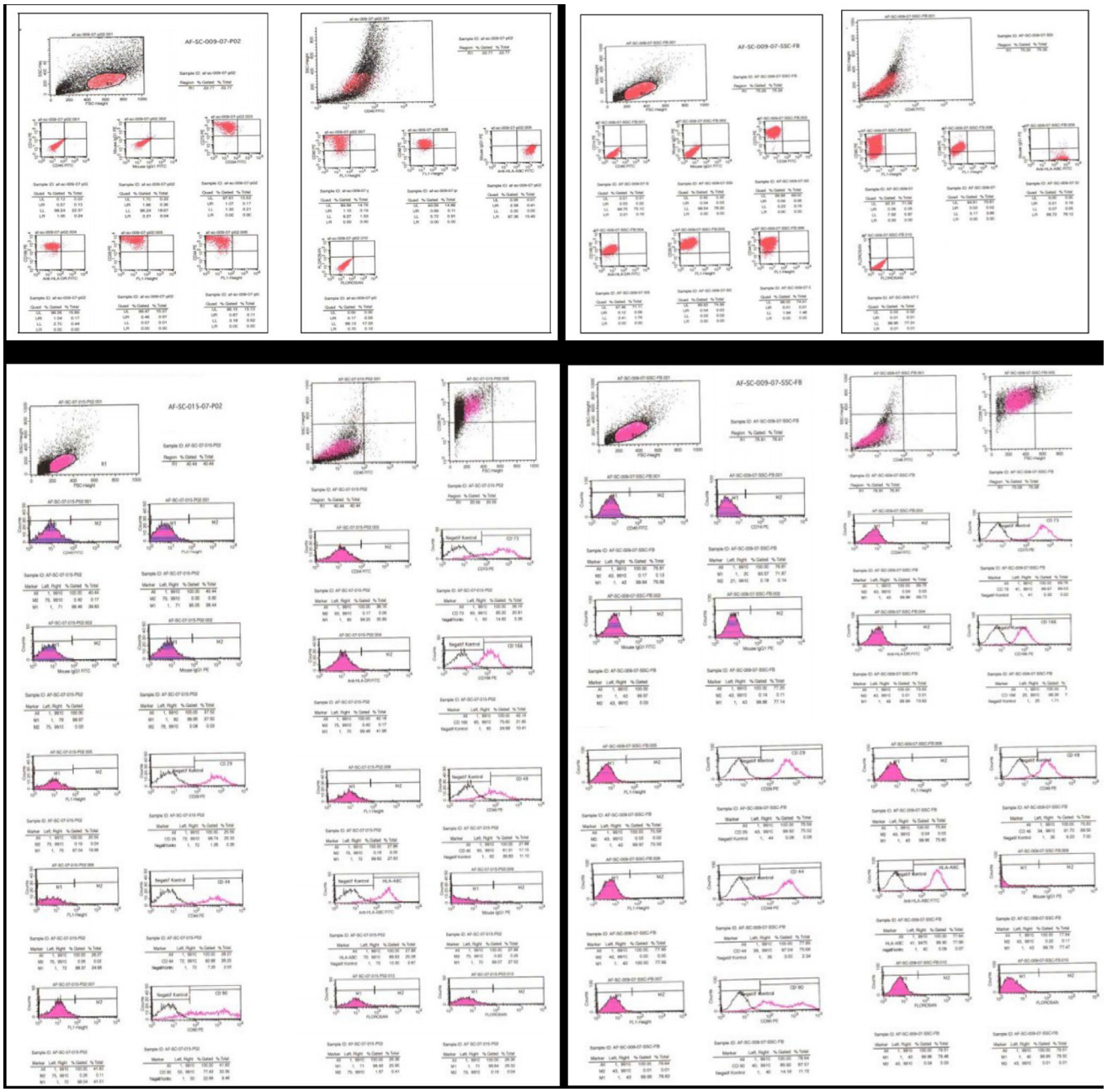

Figure 2: Example Dot-Plot and Histogram graph for primary cell culture and SSC

Chondrogenic Differentiation

Cartilage-like differentiation was detected with Alcian Blue and Hematoxylin/Eosin, in a total of 11 cases, 8 in FB2-SSCs, 2 in AF-SSCs, and 1 in heterogeneous character (Table.3) (Fig.3).

Statistical Analysis

Statistical analysis was performed using IBM SPSS version 22.0, and all data were expressed as descriptive statistics and percentages. $P<0.05$ was considered statistically significant. Normality analyzes of the surface marker data of the immunophenotyped primary cultured cells (Po) and AF/FB2-SSCs were first performed. Since the data did not show a normal distribution, three groups (PO/AF/FB) were compared using the non-parametric statistical Kruskal-Wallis method. $P<0.05$ was accepted as the level of significance.

Table 2: Primary Cell Culture and SSC "Dot-plot" Immunophenotyping Results

Primary cell culture "Dot-plot" Immunophenotyping results							
Case code	CD73	CD90	CD166	CD49e	CD44	CD29	HLA-ABC
1	96.85	94.06	99.52	99.01	99.73	100.00	99.82
2	99.60	93.57	99.62	98.29	99.58	100.00	99.79
3	99.55	84.95	99.64	99.21	99.80	100.00	100.00
4	96.30	84.18	99.18	98.62	100.00	100.00	99.85
5	98.51	89.34	99.76	98.20	99.95	100.00	99.87
6	97.63	87.43	99.77	99.61	100.00	100.00	100.00
7	89.77	82.44	93.13	87.74	97.69	99.30	97.84
8	100.00	-	98.15	98.07	99.87	99.94	99.95
9	98.68	90.73	97.30	94.28	96.82	99.93	99.94
10	99.16	83.00	98.89	97.21	99.76	99.91	98.89
11	98.43	96.97	88.08	96.11	98.66	100.00	99.08
12	85.93	37.09	90.65	75.93	99.21	98.26	97.47
13	84.97	80.56	46.66	63.41	96.76	98.45	80.95
14	93.36	78.86	90.53	62.73	98.75	99.68	92.76
15	99.38	90.04	94.78	89.73	98.53	99.94	98.14
Mean	95.87	83.80	93.04	90.54	99.01	99.69	97.62
SSC "Dot-Plot" Immunophenotyping results							
Case code	CD73	CD90	CD166	CD49e	CD44	CD29	HLA-ABC
001 AF	96.64	29.80	98.14	96.64	99.44	99.73	98.06
006 AF	98.30	26.05	99.20	98.92	99.98	100.00	99.67
008 FB2	99.84	98.89	98.48	99.53	98.84	100.00	98.20
009 FB2	99.77	92.37	97.58	94.84	98.06	99.97	99.93
010 AF	99.52	33.51	99.59	96.60	99.86	99.95	99.93
011 FB2	99.70	90.28	95.91	99.07	95.58	99.96	98.42
012 FB2	98.92	89.41	95.20	84.54	99.24	99.81	87.38
013 AF	99.59	76.20	99.17	94.51	99.90	99.68	98.84
014 FB2	99.81	75.62	98.11	91.74	99.53	99.99	99.25
015 FB2	95.25	87.53	96.70	91.97	98.44	99.82	97.35
Mean	98.73	69.96	97.80	94.83	98.88	99.89	97.70

Table 3: Summary of Tri-lineage Differentiation

Case Code	Differentiation Code	SSC	Passage	Adipogenic Differentiation	Osteogenic Differentiation
011	OS-1	FB2	P2	Not observed	Positive
011	OS-1	FB2	P4	Not observed	Positive
012	OS-2	FB2	P2	Not observed	Positive
016	OS-3	Heterogenous	P1	Not observed	Positive
008	OS-4	FB2	P3	Not observed	Positive
009	OS-5	FB2	P2	Not observed	Positive
009	OS-5	FB2	P3	Not observed	Positive
010	OS–6	AF	P2	no cell proliferation, inefficacious work	
010	OS–6	AF	P4	no cell proliferation, inefficacious work	
013	OS-7	AF	P4	no cell proliferation, inefficacious work	
013	OS-7	FB2	P2	Not observed	Positive
006	OS-8	AF	P3	no cell proliferation, inefficacious work	
014	OS-9	AF	P2	no cell proliferation, inefficacious work	
014	OS-9	FB2	P3	Not observed	Positive
Case Code	**Differentiation Code**	**SSC**		**Chondrogenic differentiation**	
011	OS-1,1	FB2		Positive	
011	OS-1,2	FB2		Positive	
012	OS-2	FB2		Positive	
016	OS-3	Heterogenous		Positive	
008	OS-4	FB2		Positive	
009	OS-5	FB2		Positive	
010	OS-6	AF		Positive	
013	OS-7	AF		Positive	
015	OS-8	FB2		Positive	
014	OS-9	FB2		Positive	
010	OS-10	FB2		Positive	

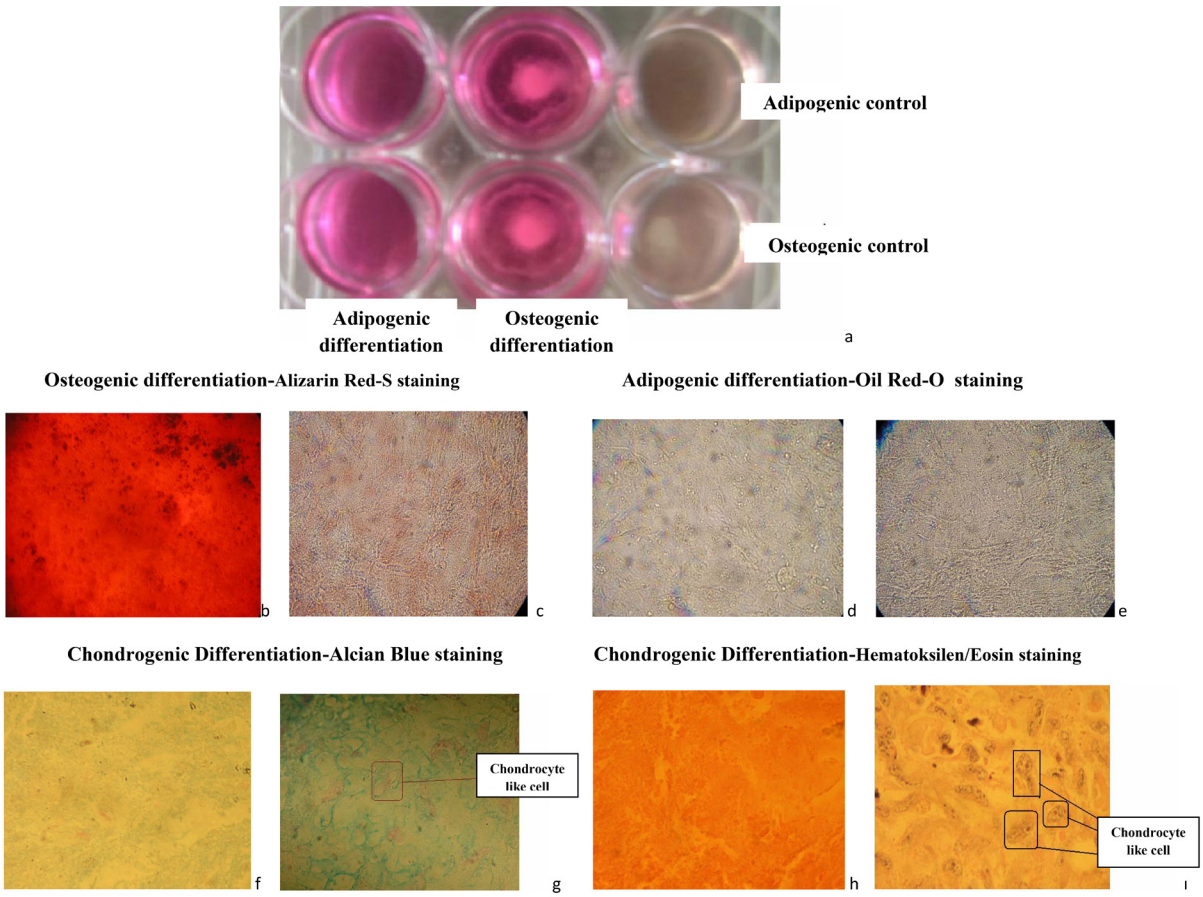

Figure 3: Tri-lineage differentiation studies; a- Macroscopic observation of osteogenic differentiation; b- Osteogenic differentiation -Alizarin Red-S staining case (Positive) (Olympus CKX41, 40X); c- Osteogenic differentiation- Alizarin Red-S staining control (Negative) (Olympus CKX41, 40X) ; d/e- Adipogenic differentiation-Oil Red-O staining case (Negative), control (Negative) (Olympus CKX41,40X); f- Chondrogenic Differentiation-Alcian Blue staining control (Negative) (OlympusBX51,100X); g- Chondrogenic Differentiation-Alcian Blue staining case (Positive) (OlympusBX51, 100X);h- Chondrogenic Differentiation-Hematoxylin/Eosin staining control (Negative) (OlympusBX51,100X); ı- Chondrogenic Differentiation-Hematoxylin/Eosin staining case (Positive) (OlympusBX51, 100X)

DISCUSSION

This study aimed to obtain MSCs from amniotic fluid-derived cultured cells routinely processed in laboratory conditions. In order to prevent possible maternal cell contamination and show the fetal origin of cultured MSCs, hemorrhagic amniotic fluid samples were not included in study, and only 46,XY cultures were used, as in some studies in the literature (2,3).

Morphological evaluation of amniotic fluid-derived cells under culture conditions is available in several studies (17-20). In the literature, some studies classify amniotic fluid cells into two as adherent and non-adherent cells and adherent and colony-forming cells into three as E-Type, AF-Type, F-Type under routine culture conditions (21). It is stated that AF-Type and E-Type cells were observed in the early stages of the culture, E-Type cells significantly decreased during the culture process, and F-Type cells appeared in the later culture stages. AF-Type cells are thought to be originated from fetal membrane and trophoblasts, F-Type cells from fibrous connective tissue and dermal fibroblasts, E-Type cells from fetal skin and urinary system (13,18,21).

Our study monitored the cell morphology from the first seeding of the cell culture, and images were evaluated on the 1st, 3rd, fifth days, first week, second week, and after trypsinization. Adherent cells were evaluated in three classes as AF-type, Fibroblastoid cell type (FB1, FB2), and E-type. FB1-type and FB2-type were determined as spindle-shaped cells parallel to each other. FB1-type cells had a long and thin structure and were much more homogeneous than FB2-type cells. AF-type cells were noted as non-tightly organized, round-shaped, and polygonal cells. E-type cells were determined to be very tightly organized, form small colonies, and be rounded and sharp-edged cells forming islets.

In our cultures, amnion cells adhered to the culture plates in the first three days (82%). The first colonies formed on the 3-5th day, and the cell density filled 2/3 of the culture plate on the 8th-11th days. At the initial stage of culture, a heterogeneous cell population, FB2, AF, and E-type, was demonstrated. While AF-type cells persisted throughout the culture process, E-type cells showed a significant decrease after a short time. FB1-type cells appeared relatively late in the culture process, with an average of 10 days.

In the literature, it is stated that normal fetus-derived amnion cells adhered to the culture plates on the 3th-4th day, whereas in the presence of fetal malformation, the cells growth rate was faster, and they started to adhere to the culture plates in less than 24 hours and had different cell morphologies (17,22). Pregnancies with fetal malformations detected in ultrasonography were not included in this study.

The "mechanical scraping" method is a practical method that can provide specific clone selection in cell cultures. Our study produced selective subcultures in parallel with the morphological evaluation. Initially, subculture studies of all three cell types were carried out. However, subculture studies could only be performed with AF-type and FB2-type cells since FB1-type cells appeared relatively late in the culture process and were contaminated with other cells, and E-type cells were reduced and disappeared. The selected cell type was allowed to proliferate in the subculture, while other cells were removed by mechanical scraping, repeated daily. Similar studies in the literature are relatively few (4,5). In some studies, a two-step culture method has been developed to obtain MSCs (13,20). In other studies, cells expressing membrane receptor c-kit (CD117) were obtained by positive selection (2,12,20).

We observed that the main morphological structure of the specific cell type was preserved after trypsinization in selective subcultures. After trypsinization, broad-based and non-dividing cells were observed in AF-type cells, which failed to proliferate, especially after the third passage. F-type cells, on the other hand, proliferated rapidly in repetitive passages.

We showed that amniotic cells grown in our routine amniotic cell culture conditions highly expressed MSCs surface antigen in primary culture cells and in their selective subcultures. This result was consistent with previous MSC immunophenotype results (2-4,7-9,11,13,23).

As stated in previous studies, fibroblastoid morphology similar to bone marrow-derived MSCs was detected in FB2-SSC flasks (11,15,23). It has been suggested that MSCs are FB-type amnion cells because of the rapid proliferation of FB-type cells in cultures, their more stable passage continuity, their structures being fibroblastoid-like MSCs originating from the bone marrow, and their immunophenotypic and differentiation characteristics (13).

In AF-SSC flasks, "embryoid body"-like colonies similar to spheroid structures were observed (24). In the literature, it has been reported that these cells express high levels of embryonic stem cell markers such as 0ct-4, Nanog, and SSEA-4 (24). Since selective clones could not be obtained from spheroid colonies in AF-SSC bottles in our study, Oct-4 and similar embryonic stem cell markers could not be studied, but MSC immunophenotypic markers were shown to be expressed. We thought that the MSC marker expression in AF-SSC flasks might be due to a small number of MSCs with strong colonization properties and forming spheroid structures.

In this study, cartilage-like differentiation was detected in a total of 11 cases. Eight of the cases had FB2-SSC, 2 had AF-SSC, and 1 had heterogeneous character. The chondrogenic study protocol was based on literature studies (6,25). In the literature, it has been stated that for chondrogenic differentiation, in-vitro MSCs must first be condensed, followed by cartilage-like differentiation (25). In our study, three-dimensional pellet formation was first provided to ensure in-vitro chondrogenic differentiation. After using the differentiation medium, sections were taken from the pellet, and cartilage differentiation was demonstrated in the preparations with "Alcian Blue" and "Hematoxylin/Eosin" staining (8,15).

Our osteogenic and adipogenic differentiation studies were initiated in the second passage in 4 of 8 FB2-SSC cases, the third passage in 3, and the fourth passage in one. One case with heterogeneous cell character was studied at passage-1. In 5 cases in which AF-SSC was initiated, differentiation studies could not be performed due to early degeneration of cells. In our study, differentiation was detected in all culture wells in which differentiation study was started with osteogenic medium, and it was shown with "Alizarin Red-S" staining (2,6-9,11,12). In the literature, studies indicate that osteogenic differentiation is achieved when the fibroblastoid-type is the predominant cell type in amniotic fluid cell cultures, and this differentiation is demonstrated by the expression of osteocalcin (11).

Cells stimulated with adipogenic medium simultaneously with osteogenic differentiation were stained with Oil Red-O to show the presence of differentiation. However, intracytoplasmic lipid accumulation, indicative of adipogenic differentiation, was not observed in any cases. There are studies in the literature showing the adipogenic differentiation capacity of MSCs obtained from amniotic fluid (2,7,9-13,15,20). Bossolasco et al. (11) used indomethacin approximately three times and insulin two times more in their study; they also used 0.5µM hydrocortisone, 60mM indomethacin, 100ng/ml insulin. On the other hand, Kim et al. (15) used approximately three times higher doses of indomethacin and 100 times lower doses of insulin and medium containing epidermal growth factor and FBS. De Coppi et al. (2), Delo et al. (12) used 3-isobutyl-1-methylxanthine (IBMX) and insulin two times more in their studies and obtained amniotic fluid-derived cells by c-kit positive cell selection. The stem cell rate obtained in our study may also have been insufficient for adipogenic differentiation. In the literature, there are studies using 20% FBS and 4ng/ml fibroblast growth factor (10,13,20) and 10% FBS and 20µg/ml endothelial growth factor (7,9) in MSC studies. These growth factors were not used in our study. Our study's inability to show adipogenic differentiation may be due to inadequate use of the protocol applied and chemicals and growth factors applied at different doses.

Limitations

Our study has some strengths and limitations. Although the number of patients included in the study is relatively small, it is similar to the related studies in the literature. This study was performed under "routine" laboratory conditions and achieved its goals based on mesenchymal stem cells, excluding adipogenic differentiation. Adipogenic differentiation capacity could not study with different protocols because of a one-time study. Osteogenic and chondrogenic differentiation achieved and mesenchymal stem cell markers demonstrated in both primary and selective subcultures.

CONCLUSION

This study demonstrated the "Mesenchymal Stem Cell" character of amniotic fluid-derived cells produced under "routine" laboratory culture conditions on an immunophenotypic basis and determined their osteogenic and chondrogenic differentiation potentials. Morphological evaluation data suggested that amniotic fluid MSC originated from a unique cell group (FB2-type cells) within the heterogeneous cell population of amniotic fluid.

The data obtained from similar studies in the literature and our study showed that amniotic fluid-derived MSCs could differentiate effectively in-vitro. The sufficient differentiation seen in 2 different cell lines (osteogenic and chondrogenic) in our study suggested that the fetal cell culture conditions in "routine" laboratory studies provide an advantage for amniotic cell-derived MSC isolation and a significant convenience for subsequent similar studies.

DECLARATIONS

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Conflicts of Interest/Competing Interests
None.

Ethics Committee Approval
Our study was approved by the Local Ethics Committee of Ondokuz Mayis University Faculty of Medicine (protocol ID: T-393/394).

Availability of Data
Available upon request.

Authors' Contributions
Ozlem SEZER conducted this study and wrote the article.

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