Chapter 3

# Characteristics of Full-Term Amniotic Fluid-Derived Mesenchymal Stem Cells in Different Culture Media

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**Abstract**

Amniotic fluid contains precious therapeutic stem cells with ideal features such as they are broadly multipotent, genetically stable, and non-tumorigenic. One of the stem cells that is abundantly found in amniotic fluid is mesenchymal stem cells. Human amniotic fluid mesenchymal stem cells (hAFMSCs) had been successfully isolated from amniotic fluid obtained from second or third trimester amniocentesis. However, studies on hAFMSCs obtained during full-term delivery are still lacking. Furthermore, suitable culture media to propagate hAFMSCs for therapeutic purposes have not been fully established. Basal medium supplemented with fetal bovine serum is commonly used, and unfortunately, this condition has been associated with the risk of transmission of animal pathogens and xenogenic immune reaction. An efficient isolation and expansion method together with suitable culture conditions is essential in establishing a specific homogenous cell population, such as full-term hAFMSCs, of clinical grade. In this chapter we briefly describe the feasibility of generating hAFMSCs from full-term amniotic fluid obtained during cesarean section using serum-free medium as opposed to the conventional serum containing media. These findings would be very useful in utilizing stem cells for bench side application from a source that is accessible and devoid of ethical and safety concerns.

**Keywords:** full-term amniotic fluid, cesarean, mesenchymal stem cells, postmitotic neurons, serum-free

## 1. Introduction

The robust development in regenerative medicine, especially the use of stem cells, has opened new treatment modalities in modern medicine. Substantially supported by the scientific

evidence, stem cells promise "cures" to chronic diseases and are considered a valuable "substitute" to the conventional therapies. Thus, the dire need for "ready-to-use" or "off-the-shelf" sources of stem cells becomes very apparent because of increased demand for the stem cell therapy. Among the adult stem cells, mesenchymal stem cells (MSCs) have seized the worldwide attention of many clinicians and scientists due to their unique characteristics. The regenerative capability of MSCs with an inherent immunosuppressive ability triggers an excellent outcome in repairing tissue injuries and restoring functions of many organs in the context of inflammatory milieu. However, similar to any other cell therapies, harvesting and acquiring an adequate number of cells for the therapeutical purposes still limit the wide use of MSCs. In line with this, the current study has explored the feasibility of exploiting a human delivery waste, namely, amniotic fluid to generate and propagate fetal-derived MSCs for the potential clinical applications.

Amniotic fluid appears at about 12 days after conception in between the amnion and chorion fetal membranes. Other than nutrients such as proteins, carbohydrates, lipids, phospholipids, and urea to support the growth of the fetus, the fluid also contains heterogenous population of cells that are sloughed off from the fetal skin and the digestive, respiratory, and urogenital tracts and from the amniotic membrane [1, 2]. The total number and proportion of viable cells may vary widely between samples from different pregnancies of the same gestation period [1]. Furthermore, the cell population also found to change with gestation corresponding to the developing fetus [3]. The types of cells found in human amniotic fluid are divided into three main groups—the epithelioid E-type cells, amniotic fluid-specific AF-type cells, and fibroblastic F-type cells—which are classified according to the morphological, biochemical, and growth properties [4, 5]. E-type cells, which are round shaped and slow growing [6], are presumed to derive from the fetal skin and urine, while AF-type cells are from fetal membranes and trophoblast tissue (placenta) because these cells produce estrogen, human chorionic gonadotropin, and progesterone [1, 7]. F-type cells are considered to originate from mesenchymal tissue due to lack of any hormone production, and they do not express human leukocyte class II (HLA-DR) surface antigen [8].

Human amniotic fluid cells have been used to screen for fetal abnormalities for more than 60 years, and only recently, their therapeutic value was discovered. A number of amniotic fluid-derived cells have been identified and examined for their properties. Among all, amniotic epithelial cells have been demonstrated to express glial and neuronal stem cell markers [9]. Midterm amniotic fluid stem cells (AFSCs) have been found to express c-kit as well as mesenchymal stem cell (MSC) surface markers [10]. These AFSCs were shown to have the ability to proliferate and maintain a normal karyotype for more than 250 population doublings [10]. Furthermore, several investigators demonstrated that AFSCs from both mid- and full-term amniotic fluid positively express pluripotent markers, differentiate into derivatives of all the three primary germ layers, and form embryoid bodies under the appropriate conditions; however, they are not tumorigenic [10, 11]. The human amniotic fluid mesenchymal stem cells (hAFMSCs) on the other hand are similar to AFSCs except the c-Kit, SSEA4 and OCT4 expressions are lesser, and their in vitro expansion only could last about 30–50 doublings [12]. Apart from these, amniotic fluid was also found to contain hematopoietic progenitor cells in the first trimester [3].

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The characteristics of amniotic fluid cells, particularly AFSCs and hAFMSCs, such as broadly multipotent, high proliferative potential, paracrine secretion activity, and non-tumorigenic in addition to devoid of ethical and safety issues will make them significant candidates in the field of regenerative medicine and drug screening.

## 2. Human full-term amniotic fluid-derived mesenchymal stem cells

The leftover amniocentesis samples of second-trimester amniotic fluid collected for the routine prenatal diagnosis are usually used for the isolation of amniotic fluid mesenchymal stem cells. At this gestational stage, it is impossible to collect a larger volume of amniotic fluid, and there are increased risks of uterine contamination and miscarriage [13]. Alternatively, stem cells could be isolated from amniotic fluid of full-term pregnancies, specifically during delivery. We attempted to generate hAFMSCs from full-term amniotic fluid obtained during cesarean section. The following sections describe their propagation using serum and serum-free media, phenotypic characterization, and in vitro differentiation potential.

### 2.1. Culture techniques

Human amniotic fluid samples were obtained under an appropriate Ethical Committee approval and after signed informed consent from 14 women prior to cesarean procedure. The mean term pregnancy duration was 38 ± 1 weeks. About 10 ml of amniotic fluid was collected by puncturing the membranes after the uterine muscle was opened for the cesarean-section delivery. Cells were isolated from the human amniotic fluid samples not more than 4 hours from the time of collection. About 10 ml of human amniotic fluid was centrifuged for 10 minutes at 1200 rpm, and 10,000 cells/cm$^2$ tumorigenic were seeded in T25 flask and grown to confluence in (1) serum-free MesenCult™-XF (Stem Cell Technologies, Canada) complete medium (SFM) according to the manufacturer protocol, (2) low-glucose DMEM with GlutaMAX™ (Gibco BRL, Invitrogen, USA) supplemented with 15% fetal bovine serum (DMEM-FBS), and (3) low-glucose DMEM with GlutaMAX™ supplemented with 15% human serum (DMEM-HS). The media were added with 1% penicillin/streptomycin (Gibco BRL, Invitrogen, USA). The cells were cultured in respective defined media at 37°C in 5% $CO_2$ incubator (RS Biotech Galaxy, Irvine, UK). The initial media change was performed at day 5 and subsequently every 3–4 days. Adherent cells achieved approximately 80% confluency around 15th day of the primary culture. The cells were harvested using 0.05% trypsin-EDTA (Gibco BRL, Invitrogen, USA) for 5 minutes at 37°C; cells were reseeded at 3000 cells/cm$^2$ and subsequently expanded up to P7. Adherent cells with fibroblast-like spindle-shaped morphology (**Figure 1**) were observed at P0 in all respective culture media. However, only SFM medium grown cells were able to expand beyond P7; others, DMEM-FBS and DMEM-HS culture grown cells, were able to attach and proliferate till P3 and P1, respectively. Therefore, the downstream experiments could not be carried out at P3 and P7 for DMEM-HS and P7 for DMEM-FBS culture conditions, respectively.

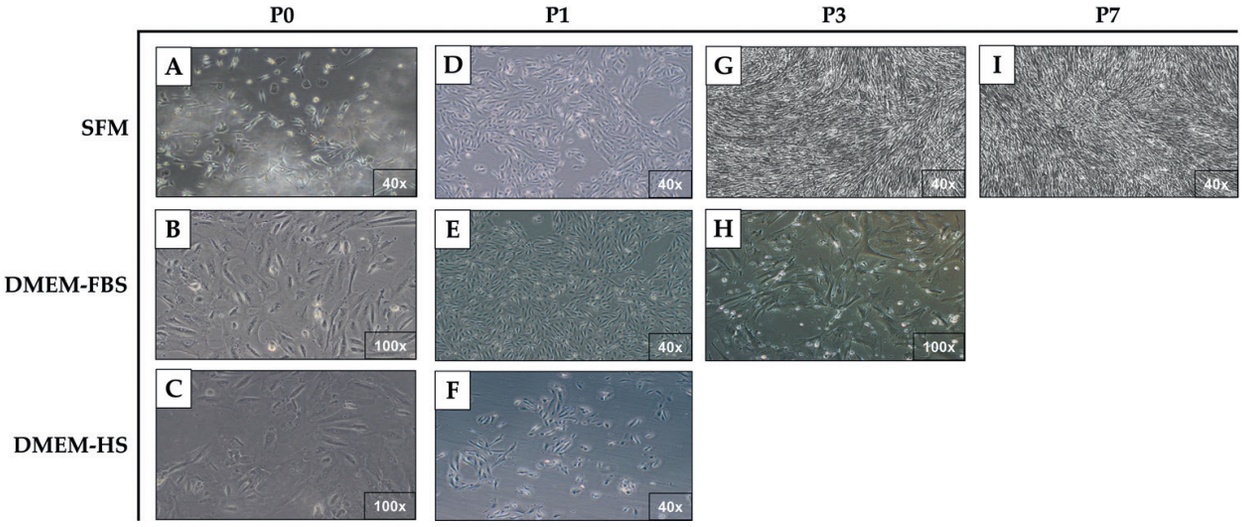


**Figure 1.** Primary culture of full-term amniotic fluid-derived adherent cells in different culture media. (A, B, and C) Adherent cells were noticed from day 7 onward and took 2 weeks, 25 days, and 38 days to confluence in P0, respectively. (D and E) More spindle-shaped fibroblast-like cells were observed in P1. (F) Cells attached but failed to reach confluency. (G, H, and I) Higher percentage of homogenous population of spindle-shaped fibroblast-like cells was observed in DMEM-FBS and SFM at P3 and P7, respectively.

Morphologically, MSCs derived from human amniotic fluid resembled MSCs from other human tissues, especially the bone marrow. Adherence to the plastic surface and assuming spindle cell morphology are the criteria that minimally define MSCs along with stipulated immunophenotyping and the mesodermal differentiation. Despite the fact that all P0 cells from the respective media acquired an MSC-like morphology, the adherent cells from SFM media showed a much smaller, denser, and defined cell population as compared to other culture systems. These features reflect the nature of the expanding cells, where cells with rapid turnover appear small and with defined spindle morphology (**Figure 1G** and **I**) compared to the slowly growing cells with a broader and polygonal shape (**Figure 1H**). The morphological observation of the SFM-expanded cells confers the high proliferative nature of the culture system (**Figure 1G** and **I**). Although, the content of SFM media is not fully disclosed, it might contain cocktail of growth factors that enhances cell proliferation, preserves the telomere length and prevents early cellular senescence.

## 2.2. Colony forming unit assay and population doubling time

The proliferations of hAFMSCs in these media were examined by colony forming unit-fibroblast (CFU-F) assay and population doubling time (PDT). The ability to form CFU-F is one of the characteristics of MSCs [14]. PDT was carried out by seeding cells at P3 and P7 in respective T75 flasks with density of 10,000 cells/cm$^2$ until cultures reach confluency. PDT was calculated using the following formula: PDT = CT/log (Nf/Ni), where CT = culture time, Nf = final number of cells, and Ni = initial number of cells at culture initiation. CFU-F assays were performed by plating 100 cells in the respective culturing media in 100 mm cell culture plate and incubated at 37°C in 5% $CO_2$. On the 14th day, the cells were washed with

Dulbecco's phosphate buffered saline (DPBS) (pH 7.4) and stained using 10 ml 0.5% crystal violet (Sigma-Aldrich, USA) in methanol for 1 hour at room temperature. Plates were washed with DPBS twice, and colonies containing 50 or more cells were counted.

The hAFMSCs doubled in 36 hours and formed more colonies when cultured in SFM, which is defined for MSCs, while it took 6 days when cultured in DMEM-FBS (**Table 1**). The slow and poor growth of these cells in DMEM-FBS and DMEM-HS most likely because of the media was not supplemented with any additional growth factors. It could be possible that the lack of optimized serum batch selection for FBS and HS that support MSC colony formation and expansion rendered the observed non-conducive proliferation. The selection of serum batch for a particular cell type, especially stem cells, is crucial since the halted cellular expansion is often noticed due to senescence [15].

| Medium | Passages | PDT (hours) | CFU-F (no. of colonies) |
|---|---|---|---|
| SFM | 3 | 35.63 ± 0.16 | 82.11 ± 2.14 |
| SFM | 7 | 37.15 ± 1.22 | 75.89 ± 2.61 |
| DMEM-FBS | 3 | 146.1 ± 0.97 | 5.88 ± 1.53 |

The proliferation of the hAF cells is slower in DMEM-FBS than in SFM. Results are of three independent experiments.

**Table 1.** Population doubling time (PDT) and colony forming unit-fibroblast (CFU-F) analyses of full-term amniotic fluid-derived mesenchymal stem cells.

### 2.3. Immunophenotyping by flow cytometry analysis

Second-trimester hAFMSCs were reported to positively express CD44, CD73, CD90, CD105, and CD166 and lack of expression of CD14, CD19, CD34, CD45, and HLA-DR [16, 17]. In order to investigate the expression of MSC surface markers on the full-term hAFMSCs grown in different culture media, fluorescence-activated cell sorting analysis was carried out. Cultured cells were harvested and resuspended in DPBS supplemented with 1% FBS at a cell density of $1.0 \times 10^6$ cells/ml. Approximately $1 \times 10^5$ cells were incubated with 3 ul of labeled mouse antihuman monoclonal antibodies at 4°C for 30 minutes in dark and then washed with 1 ml 1× DPBS supplemented with 1% FBS. The following antibodies were used: CD44, CD73, CD90, CD105, CD166, CD14, CD19, CD34, CD45, and HLA-DR (BD Pharmingen, San Diego, CA). All analyses were normalized against negative control cells incubated with isotype specific to the respective antibodies. At least 10,000 events were acquired on Guava easyCyte™ flow cytometer, and the results were analyzed using guavaSoft software (Millipore, USA). We found that more than 90% of the cells grown in SFM expressed all MSC-positive markers at P3 and P7 (**Figure 2**). However, in DMEM-FBS, only about 50% of the P3 cultured cells expressed CD105 and CD166. Lower percentage of cells expressing CD105 and CD166 was also reported by several researchers when second- and third-trimester hAFMSCs were cultured in different types of media supplemented with FBS [13, 16, 18].

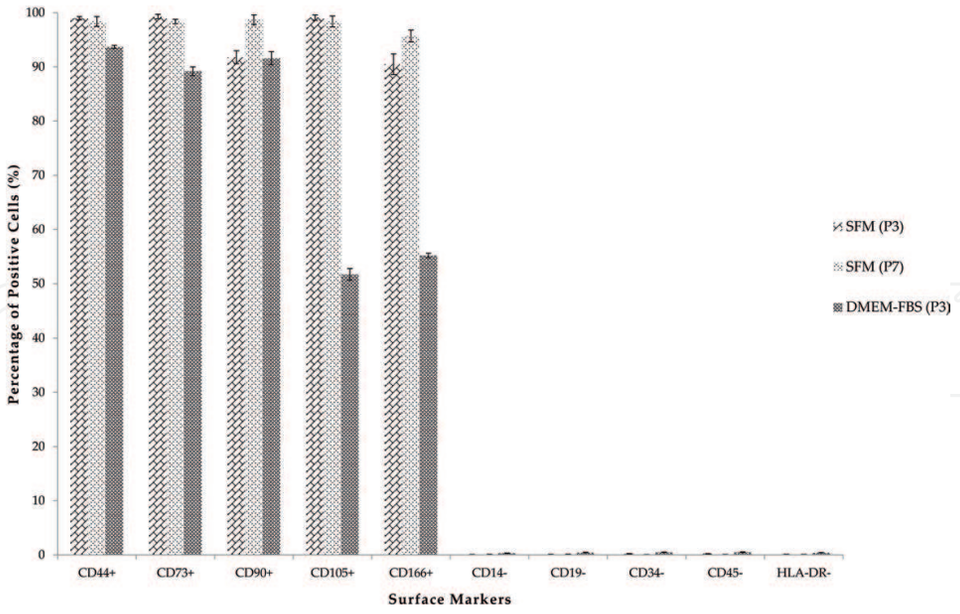


**Figure 2.** Human full-term amniotic fluid-derived adherent cells grown in SFM and DMEM-FBS showed typical mesenchymal stem cell molecular marker expression. Flow cytometry results showed that the adherent cells were absolutely positive for CD44, CD73, CD90, CD105, and CD166 while negative for CD14, CD19, CD34, CD45, and HLA-DR. Results are of three independent experiments.

## 2.4. Differentiation into mesodermal lineage

To study the multilineage capacity, hAFMSCs were subjected to differentiate under the conditions that promote osteogenesis, adipogenesis, and chondrogenesis (**Figure 3**). The

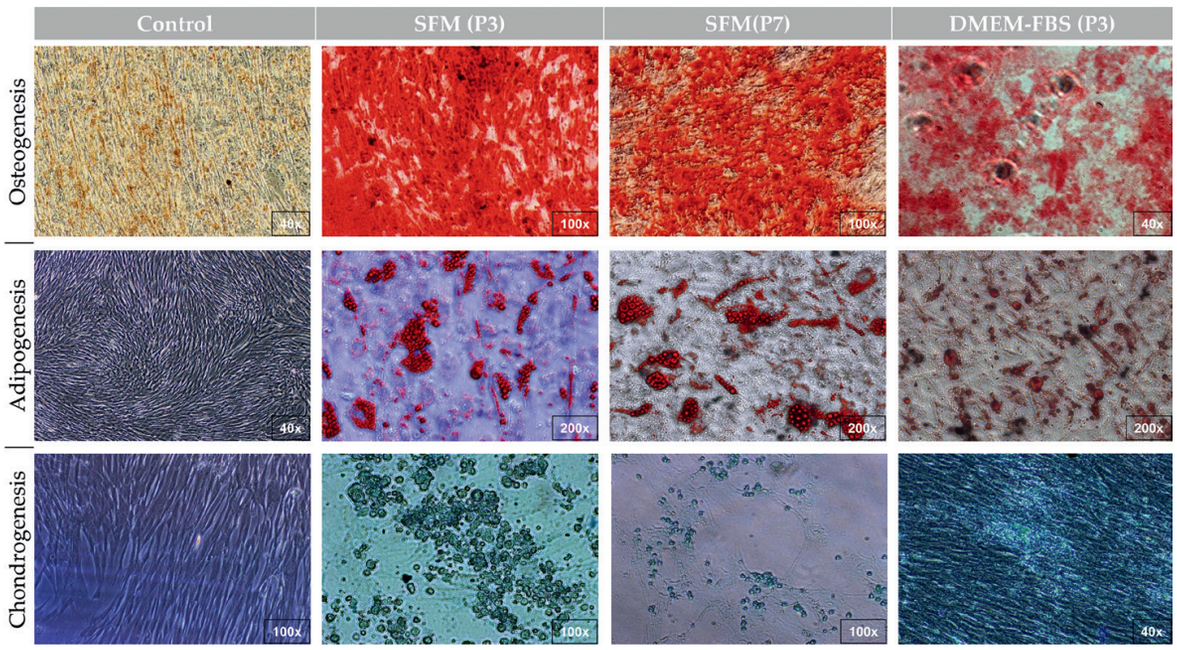


**Figure 3.** Differentiation potential of SFM and DMEM-FBS cultured human full-term amniotic fluid mesenchymal stem cells into mesodermal lineages. Adherent cells were grown to confluency and subjected to the relevant induction media as per manufacturer's protocol. Osteogenic, adipogenic, and chondrogenic differentiations were evidenced by stained calcium deposits, lipid droplets, and proteoglycan aggregates, respectively.

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differentiations were performed using StemPro Adipogenesis, osteogenesis, and chondrogenesis differentiation kits (Gibco, Invitrogen, USA) according to manufacturer's protocol. Differentiation of MSCs into osteoblasts was demonstrated by staining the calcium deposition with Alizarin red. The adipogenic phenotype was determined by staining the cell monolayers with Oil Red O. Multiple intracellular lipid-filled droplets were observed which is consistent with the phenotype of mature adipocytes. Chondrogenic differentiation was detected by staining extracellular proteoglycan aggrecan produced by the differentiated cells with alcian blue stain. Similar to the morphological and proliferation analyses, the mesodermal differentiation of hAFMSCs grown in serum and serum-free media also varied based on the passage numbers and culture media. In agreement with others, the early passage (P3) of SFM-grown hAFMSCs differentiated at the greater extent as compared to the P7 hAFMSCs. Although the degree of differentiation was not quantitatively captured, the distribution and density of the relevant biochemical stainings have indicated the early passaged cells induced into maturation with high magnitudes. When P3 cells from SFM and DMEM-FBS were compared, the degree of differentiation was lesser in the FBS-supplemented medium.

### 2.5. Spontaneous differentiation into postmitotic neurons

We also observed that the adherent cells derived from full-term amniotic fluid of few samples were growing in atypical manner compared to some of the propagated hAFMSCs when cultured in SFM medium. Morphology of spindle-shaped fibroblast-like cells with neurite-like branching was noticed during expansion at P2 (**Figure 4**) as seen during standard in vitro neural differentiation process.

Preliminary tests were conducted to verify whether these cells had undergone neuronal differentiation. The cells were confirmed positive for class III β-tubulin expression, a specific neuronal marker that can be detected during early neuronal differentiation and neurite outgrowth [19]; however, they were negative for microtubule-associated protein 2 (MAP-2), a marker for mature neurons. Immunocytochemistry (**Figure 5**) and flow cytometry (**Figure 6**) analyses revealed that more than 90% of the cell population expressed the class III β-tubulin protein as well as MSC markers (CD105, CD73, CD44, and GD2), however negative for MAP-2. These results indicate that the cells exhibited postmitotic neuronal cell identity while maintaining the MSC properties.

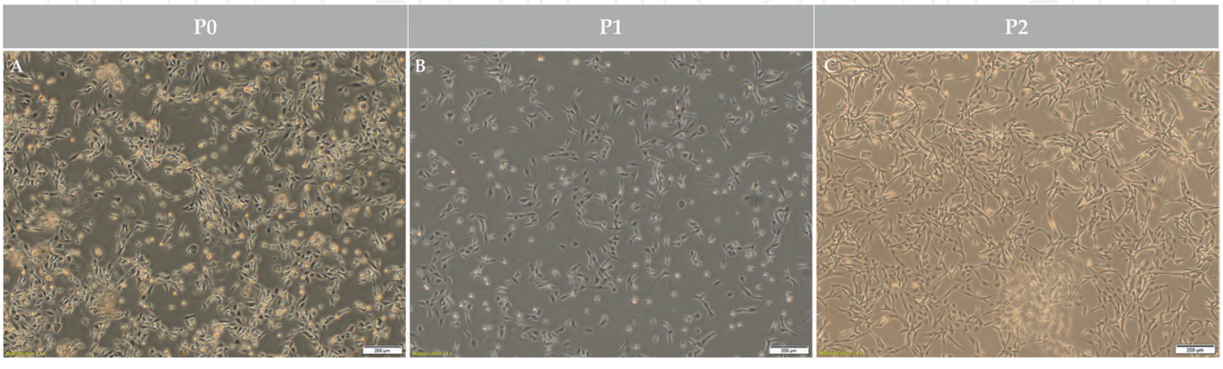


**Figure 4.** Neurite-like branching in primary culture of human full-term amniotic fluid-derived cells grown in SFM. (A) Heterogenous population of amniotic fluid cells was observed in P0. (B) Spindle-shaped fibroblast-like cells seen in P1. (C) Spindle-shaped fibroblast-like cells with neurite-like branching were noticed in P2.

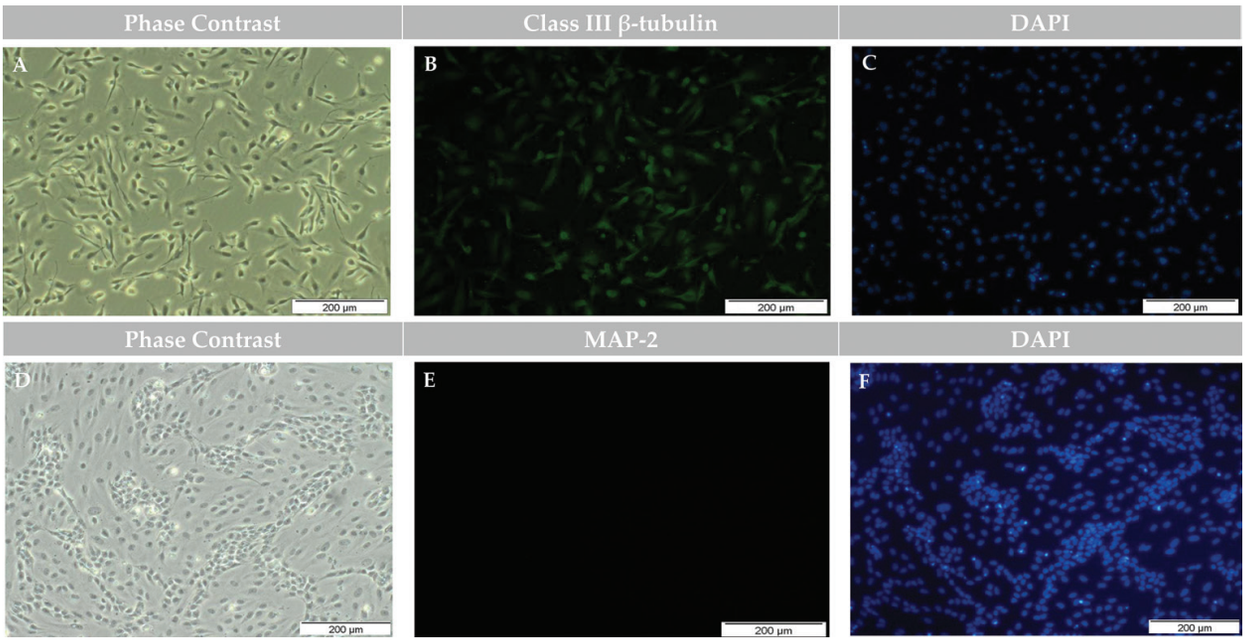


**Figure 5.** Expression of neuronal protein markers by immunocytochemistry. Passage 2 cells were stained with class III β-tubulin and MAP-2 to confirm the spontaneous neuronal differentiation. (A and D) Stained cells under phase contrast view. (C and F) Cells were counter stained with DAPI for nucleus staining. (B) Cells showing expression of class III β-tubulin indicating these cells were postmitotic neurons. (F) Cells did not express MAP-2 indicating the cells were not matured neurons.

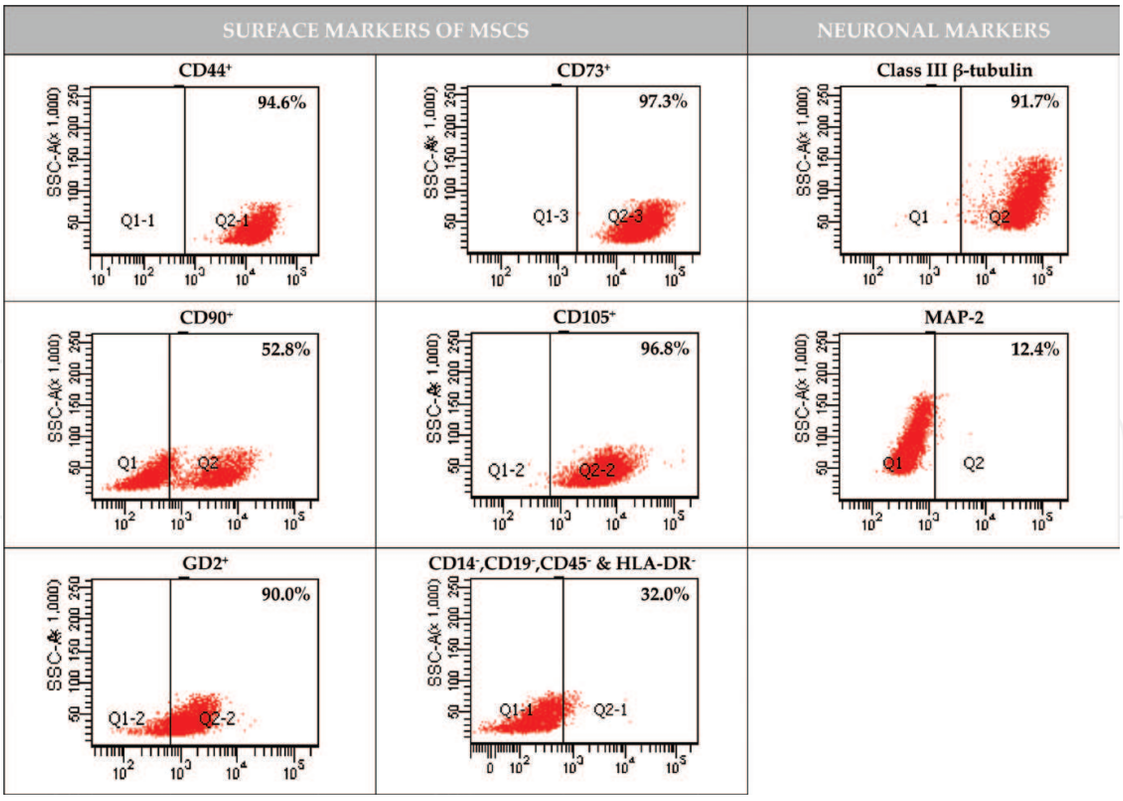


**Figure 6.** Expression of MSC surface and postmitotic neuron markers by flow cytometry of hAFMSCs grown in SFM. The adherent cells in P2 were positive for CD44, CD73, CD90, CD105, and GD2 while negative for CD14, CD19, CD45, and HLA-DR. About 91% cells exhibited positive class III β-tubulin expression; however, they were negative for MAP-2, suggesting the cells were postmitotic neurons and not matured neurons.

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## 3. Discussion

The present study evaluated the human amniotic fluid-derived MSCs in terms of morphology, expression of cell surface markers, and mesodermal differentiations in various culture conditions. The major limitation that blocks the progress of clinical trials relies on the ability of the cells to propagate in sufficient numbers for transplantation. In many cases, researchers can generate MSCs from various sources but failed to expand these cells beyond certain passages where it jeopardizes the subsequent exploration and transplantation studies. One should bear in mind that a successful use of MSCs from a respective source is only feasible when there is a culture system that allows expansion of particular cell type without compromising its molecular properties. In this study, a commercially available SFM medium was tested along with other conventional media each supplemented with FBS or HS to escalate the expansion of hAFMSCs.

The standard practice for expansion of MSCs is mainly the use of FBS as it is the basic source of growth factors and low-molecular-weight bioactive compounds [20]. FBS is simple to use and supports undifferentiated MSC expansion; however, the drawbacks of using FBS-containing medium in therapeutic application are lack of experimental reproducibility and may cause immunogenic reactions in patients [21]. In order to replace FBS, human serum has been used; nonetheless similar to FBS, there are issues such as variability between lots due to genetic diversity and lack of assurance that it will not transmit new and emerging infectious disease viruses [22]. Recently, for therapeutic purposes stem cells are being cultured in xeno-free or animal component-free media formulations to circumvent transmission of xenogenic proteins and pathogens and to improve the outcome of cell transplantation studies. Xeno-free media may contain material derived from the human plasma, while animal component-free media do not contain serum and other materials derived from animal or human sources.

In our work, commercial xeno-free, serum-free culture medium was used in conjunction with serum-free attachment substrate to support cell adhesion. The cells were also cultured in FBS and HS-containing media to evaluate the capacity of expansion and differentiation of hAFMSCs. The growth rate and morphology observations of our study were similar with other MSC studies carried out using serum-free and serum-supplemented media [21, 23, 24]. We found that the proliferation rate of the full-term amniotic fluid cells was higher in SFM, where the doubling time remained short and relatively consistent till seven passages compared to serum-containing media. Besides, we also noticed that the cells grown in SFM exhibited more elongated, spindle-shaped morphology and grow in distinct bundles of cells when continually expanded. In contrast, cells cultured in serum media displayed more flattened, fibroblast-like morphology and even monolayer of cells. Human MSCs undergo replicative senescence with decreasing proliferation and changes in cell morphology, which were observed in early passage of cells cultured in both DMEM-FBS and DMEM-HS. This could be due to undefined factors in the serum; alternatively the rapid proliferation and longer life span of hAFMSCs in SFM are possibly contributed by the higher concentrations of growth factors present in this medium. In addition to robust proliferation rate, SFM also enhanced the clonogenic potential of the full-term hAFMSCs which is one of the properties of bona fide MSCs.

Immunophenotyping is one of the main criteria for characterizing MSCs. International Society for Cellular Therapy (ISCT) proposed that multipotent human MSCs must express CD105, CD73, and CD90 and lack of expression of CD45, CD34, CD14 or CD11b, CD79$\alpha$, CD19, and HLA-DR surface antigen [25]. Other positive markers currently included to define MSCs are CD166, CD44, CD29, and CD9 [26]. In our study, all the MSC-positive markers were highly expressed in full-term hAFMSCs expanded in SFM. The hAFMSCs cultured in DMEM-FBS moderately expressed CD105 and CD166. A similar expression pattern of CD105 and CD166 was also observed by other researchers during generation of MSCs derived from second- and third-trimester amniotic fluid using FBS-supplemented media [13, 16, 18]. CD105 plays a role in chondrogenic differentiation [27], while CD166 is involved in neurite extension [28]. Probably due to lower expression of CD105, hAFMSCs grown in DMEM-FBS differentiated poorly into chondrocytes. hAFMSCs isolated in SFM were found to have higher trilineage mesoderm differentiation capacity. Martinez et al. [29] studied on neural ganglioside GD2 surface antigen on bone marrow MSCs after several researchers reported on neural antigen expression on MSCs. They found that GD2 was consistently expressed at a high level on all freshly isolated or ex vivo expanded bone marrow MSCs but was not expressed in all other cells within the marrow. Likewise, Xu et al. [30] found that umbilical cord-derived MSCs were the only cells within umbilical cord that expressed this marker. These findings suggested that GD2 can be a unique marker for MSCs. It is also noteworthy that GD2 is one of the major gangliosides of the postmitotic neurons [31] and was found to increase during neurite outgrowth [32]. When spindle-shaped cells with neurite-like outgrowth were noted in most of the SFM cultures, we investigated the MSC surface antigen expressions and included GD2 marker to confirm that the cells were indeed MSCs and have neurogenic potential. Class III $\beta$-tubulin and MAP-2 expression were also analyzed to examine whether the cells were postmitotic neurons or matured neurons. As suspected, the cells had differentiated spontaneously into postmitotic neurons. Interestingly, this morphology was not seen in DMEM-FBS cultures, probably due to lower expression of CD166 on these cells. Chase et al. [24] had reported that bone marrow MSCs grown in serum-free medium significantly enhanced the expression of the intermediate filament nestin when compared to cells expanded in serum-containing medium. MSCs at the earliest developmental stage were found to harbor stronger neurodifferentiation capacity than postnatal MSCs, acquiring characteristics of postmitotic neurons [33]. It was suggested that MSCs exhibit both stem cell and precursor functions allowing neuronal differentiation through both mitotic stem cells and nonmitotic precursor pathways [34]. Many studies demonstrated that the morphological and molecular modifications of MSCs were probably due to stress response, rather than to a real differentiation into neuronal cells; however, some recent studies had demonstrated that MSC-derived cells not only showed morphological features of neurons, but that they also demonstrate functional properties of neurons [35]. There is evidence that MSCs from different sources may not have the same biological and genetic properties; probably for these reasons and neurotrophic cytokines that might be present in the microenvironment, the full-term hAFMSCs spontaneously differentiated into postmitotic neurons in our hands. These postmitotic neurons might turn into mature neurons upon further expansion. These findings suggest the possibility that the full-term

amniotic fluid-derived mesenchymal stem cells expanded in serum-free medium may serve as a source for stem cell-based regenerative medicine to a variety of therapeutic scenarios including treating the neurological disorders.

## 4. Conclusion

Our findings clearly demonstrated the feasibility of generating MSCs from full-term human amniotic fluid. SFM media were found to be most efficient in isolation and expansion of full-term hAFMSCs. It is very interesting to note that the characteristics and behavior of the established hAFMSCs change under the influence of different culture media. Keeping these observations in mind, further work needs to be done to understand differentiation potential of full-term hAFMSCs before they can be applied in bedside settings.

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## Abbreviations

| | |
|---|---|
| CFU-F | Colony forming unit-fibroblast |
| DMEM-FBS | Low-glucose DMEM with GlutaMAX™ supplemented with 15% fetal bovine serum |
| DMEM-HS | Low-glucose DMEM with GlutaMax™ supplemented with 15% human serum |
| DPBS | Dulbeccos's phosphate buffered saline |
| hAFMSC | Human amniotic fluid mesenchymal stem cell |
| HLA-DR | Human leukocyte antigen class II |
| MAP-2 | Microtubule-associated protein 2 |
| MSC | Mesenchymal stem cell |
| PDT | Population doubling time |
| SFM | Serum-free MesenCult™-XF complete medium |

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