Journal of Science and Applications:
BioMedicine
Research Paper

# Isolation and Initial Characterization of Nonhuman Primate Amniotic Fluid-Derived Stem Cells

J. Koudy Williams[1]*, Silmi Mariya [2], Anthony Atala [1], Shay Soker [1]

[1]*The Wake Forest Institute for Regenerative Medicine, Wake Forest University School of medicine, Winston-Salem, NC*
[2] *The Indonesian Primate Center, The Institute Pertanian Bogor, Bogor, Indonesia*
***Corresponding Author**: J. Koudy Williams, D.V.M., Phone: 336-713-1323, Email: kwilliam@wakehealth.edu*

**Abstract:** Regenerative medicine promises to address the organ shortage by engineering replacement cells, tissues and organs using adult, progenitor, or human embryonic stem cells. However, embryonic stem cells can form teratomas and bone marrow-derived progenitor cells have limited expansion capabilities. We recently described a subpopulation of c-kit$^+$ human amniotic fluid-derived stem (hAFS) cells that are highly expansive, do not form teratomas and can yield differentiated cell derivatives corresponding to the three germ layers. To this end, and because nonhuman primates (NHP) represent a critical step towards pre-clinical testing of the AFS technology, the goal of this study was to determine if AFS cells could be isolated from amniotic samples of monkeys. Expansive cell cultures were derived from 12/28 amniotic fluid samples of cynomolgus monkey. Cells were isolated and expanded in Chang's media and c-kit$^+$ cells were isolated, further expanded and clonal populations obtained. The putative AFS cells were characterized as to their karyotype, ability to expand, identification of cell-surface and nuclear markers (qPCR) and ability to differentiate to mesodermal lineages such as bone, muscle, fat, cartilage and endothelial cells. Highly expansive multipotent cells were isolated most readily from the second trimester of pregnancy (8/15 samples, or 53%), and less so from either the first (1/3, or 33%), or third (3/10, or 30%) trimester. Cell were diploid in all cases and were consistently expanded through at least 20 passages (10 passages for the 3rd trimester cells) without losing their proliferation potential, as confirmed by cell cycle analysis (G1, G2, S phase) and doubling times. The AFS cells had low-level expression of pluripotentiality markers (Oct4, Nanog, SOX2) compared to NHP embryonic stem cells, while expressing markers similar to that of adult mesodermal-lineage cells (CD44, CD90, CD73 and CD105). nhpAFS cells express the major, but not minor, histocompatability antigen and were consistently differentiated into adipogenic, osteogenic, myogenic, chondrocytic and endothelial lineages. Because of their robust expansion capabilities and ability to differentiate into several mesodermal lineages, it is concluded that nhpAFS cells, similar to hAFS cels, have the characteristics needed to bioengineer a wide-variety of mesodermal-cell containing organs and tissues. ***Published by www.inter-use.com. Available online Dec. 10 2013 Vol. 1 Issue 1 Page 15-23.***

**Keywords:** Stem Cell, Amniotic Fluid, Nonhuman primates, nhAFS, Cell Isolation, Monkey

## 1. Introduction

A death occurs approximately every 30 seconds as a result of organ failure, primarily because the demand for replacement tissues and organs consistently exceeds their supply. This problem is likely to increase as the number of patients awaiting transplants increases by approximately 20% each year. One of the Joint Commission on the Accreditation of Healthcare Organizations recommendations to meet this impending crisis is to "support the progression of new sciences and technologies that have the potential to narrow the donation gap and decrease the risk of organ rejection". Regenerative medicine promises to address the organ shortage by engineering replacement cells, tissues and organs using adult, progenitor, or human embryonic stem cells.

However, no approach to date is capable of yielding an autologous supply of cells of the desired phenotype in sufficient numbers or purity to safely address the projected need. This shortage of autologous cells may be alleviated, in part, by using readily available amniotic fluid cells, which is known to contain multiple cell types derived from the developing fetus.

Human AFS (hAFS) cells have been isolated and expanded routinely from amniocentesis specimens obtained as backup cultures after completion of prenatal diagnostic procedures [1]. AFS cells expand extensively without the need to co-culture with "feeder cells", and typically double in about 36 hours. Clonal hAFS cell lines maintained for over 250 population doublings have retained a normal karyotype and long

telomeres. Cell cycle control appears normal and there was no evidence of malignant transformation. Importantly, hAFS cells do not form teratomas in vivo. Clonal hAFS cell lines can also yield differentiated derivatives corresponding to adipose, muscle, bone, neuronal and hepatic lineages. Although the full range of adult cell types to which AFS cells can give rise remains to be determined, we believe that AFS cells may represent a multipotent stem cell source for a variety of regenerative medicine applications.

The main premise of this application is that characterization of nhpAFS cells and development of nhpAFS clonal cell lines are worthwhile endeavors that would hasten the development of replacement tissues and organs for human beings. We believe this to be true in that macaque monkeys share an extremely close phylogenetic relationship with human beings and develop natural, or experimentally induced disease states that could benefit from regenerative medicine approaches to their treatment and cure. We have spent over thirty years characterizing the macaque monkey (both cynomolgus and rhesus monkeys) as an animal model for a variety of age-related chronic diseases. Proposed studies will focus on nhpAFS cells from cynomolgus monkeys which have uterine anatomy similar to that of women, a well documented menstrual and gestation period, and are of a size and weight making collection of amniotic fluid an easy and safe procedure [2]. Furthermore, cynomolgus monkeys are an established animal model of age and gender-related risk for coronary, cerebral and peripheral vascular disease, Type I and Type II diabetes, cancer, dementia, genitourinary, bone and joint diseases [2-9]. As such, this species of monkey is a well established model the chronic diseases accounting for a vast majority of morbidity, mortality and medical cost in this country.

To this end, amniotic fluid samples were collected from 28 adult female cynomolgus monkeys at various stages of pregnancy. Studies were designed to isolate and initially characterize nhpAFS cells and then compare their expression profiles to NHP bone marrow progenitor and embryonic stem cells.

## 2. Methods

### *2.1. Collection and Culture of Amniotic Fluid Samples*

We collected amniotic fluid from 28 breeding female cynomolgus monkeys (*Macaca fascicularis*) housed at the Wake Forest Primate Center. Gestational ages of the pregnant females were determined by ultrasound analysis of the gestational sac (GS), greatest length (GL), biparietal diameter (BD), occipitofrontal diameter (OFD), head area (HA), head circumference (HC), abdominal area (AA) abdominal circumference (AC), humerus length (HL) and femur length (FL). The normal values and ranges for these parameters have been established based on a study of 75 normal pregnancies in Macaca fascicularis [10] and are routinely employed by numerous other primate groups. Samples were collected from 3 monkeys in the first trimester, 15 monkeys in the second trimester and 10 monkeys in the third trimester.

Amniotic fluid was collected using 2-D ultrasound as a position guide while a 22 g. 11/2 inch needle was inserted midline into aseptically prepared ventral abdomens and sedated monkey and advanced into the amniotic sac. Approximately 2 ml of fluid was removed, diluted in 10 ml PBS and spun to a pellet at 1500 rpm for 5 minutes. The pellet was re-suspended in 1 ml of Amnio MAX-II - Complete (Gibco) and pipetted carefully to the surface of a sterile untreated glass cover slip – contained in a 35 mm culture dish – and then grown for 1 week without disturbance (Fig. 1).

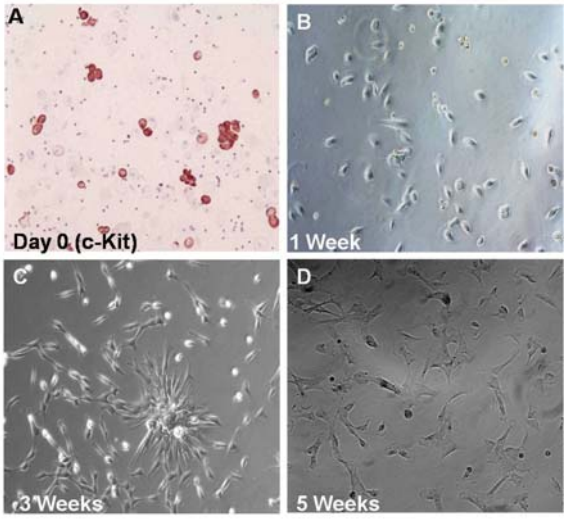


Fig. 1. Initial characterization of cell isolates. Immunocytochemical image of the amniotic fluid with c-kit$^+$ cells stained red (A), and their appearance in culture at increasing times thereafter (B-D). All images are taken at 20X.

At one week, the sample was analyzed for the presence of colony-like adherent cells (putative AFS cells) (Fig. 1). If present, the cells were trypsinized

and grown in successively larger Petri plates using α-MEM medium (Gibco, Invitrogen Corporation) containing 15% ES-fetal bovine serum, 1% glutamine and 1% Penicillin/streptomycin (Gibco), supplemented with 18% Chang B and 2% Chang C (Irvine Scientific) at 37°C with 5% $CO_2$ atmosphere. After expansion to confluence (5-7 days), a single cell suspension was prepared by gentle trypsinization.

For immune-selection of c-kit positive monkey AFS cells from single cell suspensions, the cells were incubated with a rabbit polyclonal antibody to CD117 (c-kit), specific for the protein's extracellular domain (amino acids 23-322) (Santa Cruz Biotechnology). The CD117-positive cells were purified by incubation with magnetic Goat Anti-Rabbit IgG MicroBeads and selection on a Mini-MACS apparatus (Miltenyi Biotec, Auburn, CA) following the protocol recommended by the manufacturer. AFS cells were sub-cultured routinely at a dilution of 1:4 to 1:8 and not permitted to expand beyond approximately 70 percent of confluence. AFS cells were cloned by limiting dilution.

*2.2. Collection of Monkey Bone Marrow Cells*

Bone marrow samples were taken from the breeding females. The sample was collected from the humerus of sedated monkeys by inserting a 3 inch 18g needle, attached to a 20 cc syringe, into the proximal joint space and directing the needle distally down the length of the shaft. Approximately 6 cc of marrow was added to 0.5 cc a heparin solution (100U/ml) and the sample immediately prepared for culture. The white blood cell layer was isolated from the remaining part of the sample by centrifugation using a Histopaque 1077 gradient. The buffy coat was washed in PBS and the mesenchymal progenitor cells immuno-isolated using anti-CD105 and/or anti-CD90 antibodies in a similar method used to isolate c-kit$^+$ cells from amniotic fluid (Miltenyi Biotec kits). The isolated cells were re-suspended in DMEM+10% FBS+1% antibiotic and plated on a 10 cm culture dish. The samples were incubated at 37 ºC with 5% $CO_2$. Stromal cell colonies appeared within 3-4 days and were expanded.

*2.3. Monkey ES Cells*

Cynomolgus embryonic stem cells were obtained from the University of Washington National Primate Research Center as a kind gift from Dr. Eric Hayes.

*2.4. Karyotyping*

The cells were incubated with colcemid (10 micrograms/ml) for 2 hours followed by washing with 0.075 M KCl, dropwise at first, and then with gentle agitation to the centrifuge tube. The cells were re-suspended in fixative at room temperature for 30 minutes. Small amount of cell suspension was streaked on a glass slide placed over dry ice and the cells allowed to dry. The chromosomes were stained with giemsa stain for 5 minutes. A band is an area of a chromosome was clearly distinct from its neighboring area and representative areas of the slide were photographed to produce high contrast photographs of the chromosome spreads. The chromosome from the photograph was arranged according to size and position of the centromere.

*2.5. Cell cycle analysis*

Cell cycle analysis of cells was performed at different passages to determine if they had normal DNA content and normal cell cycle and division. The assay was done using propidium iodide labeling.

*2.6. Cell Differentiation protocols*

Undifferentiated AFSCs from each designated source were exposed to the following protocols for their respective differentiation.

*2.6.1. Adipogenic*

Cells were seeded at a density of 3,000 cells/cm$^2$ and were cultured in DMEM low-glucose medium with 10% FBS, antibiotics (Pen/Strep, Gibco/BRL), and adipogenic supplements (1 M dexamethasone, 1 mM 3-isobutyl-1-methylxanthine, 10 g/ml insulin, 60 M indomethacin (Sigma-Aldrich).

*2.6.2. Osteogenic*

Cells were seeded at a density of 3,000 cells/cm$^2$ and were cultured in DMEM low-glucose medium with 10% FBS (FBS, Gibco/BRL), Pen/Strep and osteogenic supplements (100 nM dexamethasone, 10 mM beta-glycerophosphate (Sigma-Aldrich), 0.05 mM ascorbic acid-2-phosphate (Wako Chemicals)).

*2.6.3. Myogenic*

Cells were seeded at a density of 3,000 cells/cm$^2$ on plastic plates pre-coated with Matrigel (Collaborative Biomedical Products; incubation for 1 h at 37 °C at 1 mg/ml in DMEM) in DMEM low-glucose formulation containing 10% horse serum (Gibco/BRL), 0.5% chick embryo extract (Gibco/BRL) and Pen/Strep. Twelve hours after seeding, 3 M 5-aza-2'-deoxycytidine (5-azaC; Sigma-Aldrich) was added to the culture medium for 24 h. Incubation then continued in complete medium lacking 5-azaC, with medium changes every 3 d.

*2.6.4. Chondrocytic*

Cells were differentiated into chondrocytes using the methods described in the Nonhematopoietic (NH) stem cell media protocol using NH ChondroDiff medium (Miltenyi Biotech - Auburn, Ca).

*2.7. FACS Analysis*

To determine the antigenic profile of AFS cells, staining and analysis with monoclonal antibodies were performed according to standard protocols using a FACS Calibur analyzer (BD Biosciences, San Jose, CA) and FlowJo software (Tree Star, Inc., Palo Alto, CA) or an Epics analyzer and CELLQuest software (Beckman Coulter). Assays for surface antigens were carried out with live cells. The cells were grown to approximately 50% of confluence, detached from culture dishes by mild trypsinization, and washed with phosphate buffered saline (PBS). For assay of Oct4 and Nanog, the cells were fixed for 10 minutes with 4% paraformaldehyde in PBS and then made permeable by incubation with cold ethanol for 1 minute. Monoclonal antibodies were obtained from the sources indicated: CD29, CD44, CD117, Stro-1, CD73, CD90, CD105, HLA-DR (Immunotech, Beckman Coulter, Marseille, France); HLA-ABC (BD Biosciences Pharmingen, Europe); Oct-4, and Nanog (Santa Cruz Biotechnology); CD133 (Miltenyi Biotech). Primary antibodies were diluted to the concentration recommended by the supplier (generally 1 μg/ml). Secondary antibodies will be FITC-goat anti-mouse IgG (Beckmann Coulter) or AlexaFluor488-goat anti-mouse IgG (Molecular Probes / Invitrogen), diluted 1:100.

*2.8. Immunostaining*

It was necessary to confirm cellular location of antigen staining – which cannot be done with flow cytometry. Therefore, cells from each passage will be grown in chamber slides and antigen location confirmed using immunocytochemistry. The cells were fixed with 4% paraformaldehyde permeabilized and washed with PBS. Primary antibodies against markers of embryonic and mesenchymal stem cells that were described above were used for immunostaining followed by incubation with fluorescently-labeled secondary antibodies. Specific staining was confirmed using matched IgG's. The stained cells were examined under fluorescent microscope and representative images were recorded to confirm specific staining and localization of the antigens.

*2.9. RT-PCR*

Analysis of mRNA expression by reverse transcription (RT)-PCR was carried out using standard protocols. Primer sequences, PCR conditions and DNA fragment sizes are available on request. Transcripts encoding the following proteins were assessed for the specified lineages. Oct 4, Nanog, SOX2, EpCAM, MCAM, CD44, CD105 and HLA-A.

## 3. Results

*3.1. Collection and Isolation of nhpAFS Cells*

Amniotic fluid was collected from 28 adult female monkeys ranging in age from 7-10 years. Three monkeys were in the first trimester (1-52 days), 15 monkeys were in the second trimester (53-105 days) and 10 monkeys were in the third trimester (54-155 days). Collection of the samples did not cause abortion and the offspring were delivered normally (Table 1). Successful cultures (those yielding c-kit$^+$ cells that expanded through many passages) were obtained more readily from second trimester samples (53%), than either the first (33%), or third trimesters (30%), although the number of samples was not equal among the three different trimesters (Table 1). Cells were readily expanded through 80-100 population doublings (20-25 passages) except for those isolated from third trimester animals where they routinely expanded only to around 10 passages before becoming senescent

(Table 1). Three clones were routinely derived from each sample.

The clones were diploid and maintained a 36 hour doubling time through their passages until senescence at the above referenced passage number. AFS cells maintain their normal G1/G2/S phase cell cycle through 20 passages (Fig. 2). In contrast NHP bone-marrow-derived mesenchymal progenitor cells have reduced percentage of cells in the G2 phase by passage 5, suggesting that they are not actively dividing at that point.

Table 1. Characterization and differentiation potential of cells isolated from the amniotic fluid of cynomolgus monkeys. Data presented are the percent of positive cultures obtained from the different gestation periods of the monkeys, whether clones were obtained, their karyotype and the mesehcnymal differentiation potential of the isolates.

| N=28 adult female cynomolgus monkeys | Number of C-Kit positive cultures/total number collected | Cloned/karyotype and current passage# | Differentiation |
|---|---|---|---|
| Trimester (gestation = 155 days) | | | |
| 1st (1-52 days) | 1/3 = 33% | Yes/diploid/20 | Osteo/adipo/chondro/muscle |
| 2nd (53-105 days) | 8/15 = 53% | Yes/diploid/25 | Osteo/adipo/chondro/muscle |
| 3rd (105-155 days) | 3/10 = 30% | Yes/diploid/10 | Osteo/adpio/chondro/muscle |
| Osteo - osteocyte; adipo - adipocyte; chondro - chondtrocyte; muscle - skeletal muscle | | | |

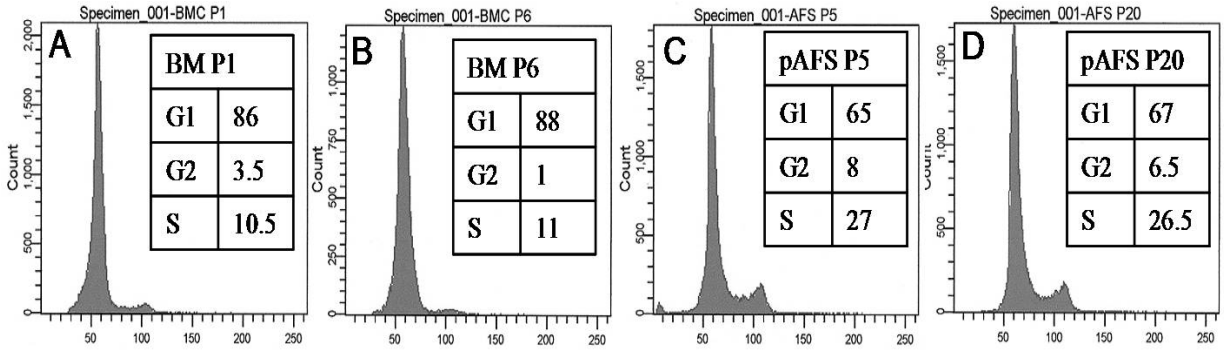


Fig. 2. Karyotyping results of isolates from different monkeys. AFS cells maintained their normal G1/G2/S phase cell cycle through 20 passages, whereas the NHP bone-marrow-derived mesenchymal progenitor cells have reduced percentage of cells in the G2 phase by passage 5.

### *3.2. Fluorescent Antibody Analysis of Cells*

While c-kit antibody was expressed in cells directly stained from the amniotic fluid (Fig. 1), once cultured, the nhpAFS cells did not express c-kit. nhpAFS cell clones consistently expressed surface markers for CD73, CD105, CD44, CD90, Stro-1 and HLA ABC (Fig. 3). However, these cells did not express Oct4, or Nanog (Fig. 3). This expression pattern did not change throughout passaging. This expression pattern was almost identical to that of NHP bone marrow-derived mesenchymal progenitor cells (data not shown).

### *3.3. Differentiation of nhpAFS Cells*

Since expression patterns were similar among the AFS samples and clones, only one sample from each monkey was differentiated into the meseodermal lineage cells. Cells readily differentiated into osteogenic, adipogenic, chontrocytic and myogenic lineage cells (Fig. 4). In this initial characterization of nhpAFS cells, differentiation into ectodermal and endodermal lineages was not attempted. Therefore, we would classify these cells, to date, as highly expansive multipotent cells.

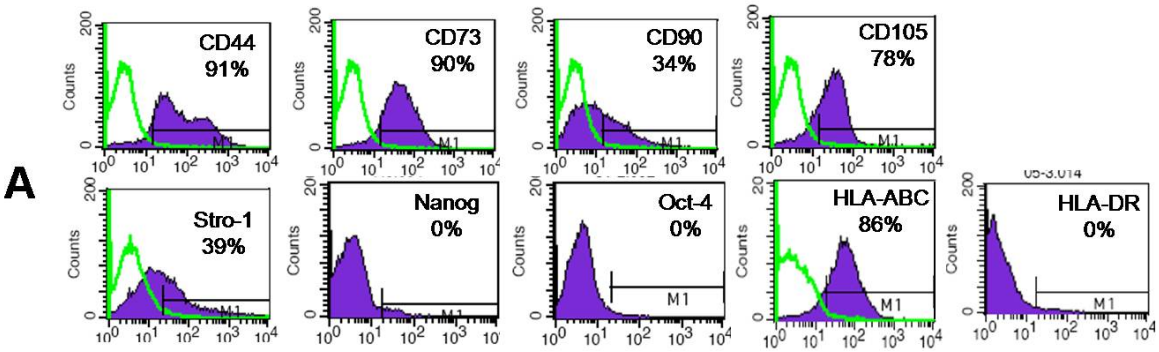


Fig. 3. Cell marker characterization. A – FACS analysis of cell cultures showing that nhpAFS cells have a cell expression phenotype similar to that of bone marrow mesenchymal cells (CD44, CC73, CD 90, CD105, Stro-1) and strongly express HLA-ABC.

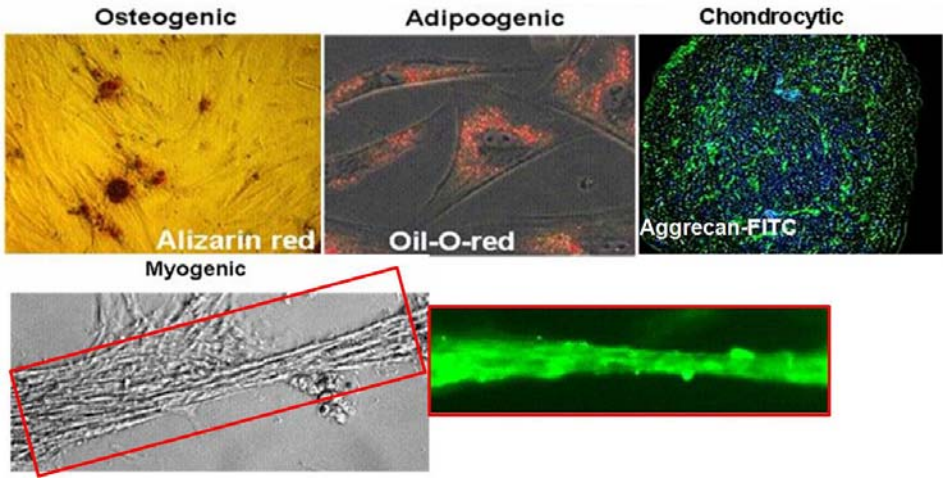


Fig. 4. Differentiation potential of nhpAFS cells. Of the isolates tested, all expressed makers of adipocytes (oil-red-O) osteocytes (Alizarin Red), chondrocytes (Aggrecan), and myocytes (formed muscle bundles and expressed desmin – green stain). Alizarin red and oil-red-o stain images photographed at 40X; aggregan and myogenic lineage images at 10X.

### *3.4. Quantitative PCR Expression of Undifferentiated and Differentiated nhpAFS Cells*

Quantitative PCR analysis was done on one clone (passage 10) of each sample. However, 3 clones from one animal were compared and showed little difference among the clones. Additionally, results were consistent among the animal samples and did not change appreciably with passage number. Results are displayed for a representative clone from one of the monkeys (Table 2). Expression of mRNA for target molecules was compared among nhp embryonic stem cells (ES cells), nhp bone marrow-derived mesenchymal progenitor cells (BMPCs), undifferentiated nhpAFS cells and AFS cells differentiated to chondrocytic, myogenic, adipogenic and osteogenic lineages (Table 2). As expected, the expression of "stemness" markers - Oct4, Nanog and SOX2 - were higher in nhpES cells than other cell types.

| | nhpES | nhpAFS | nhpBMSC |
|---|---|---|---|
| Oct4 | 18.5 | 34.5 | 35.3 |
| Nanog | 19.7 | 31.5 | 30.0 |
| SOX2 | 20.0 | 33.5 | 39.5 |
| HLA-A | 28.0 | 19.3 | 22.2 |

Table 2. Quantitative PCR results. Data presented are the number of cycles needed to obtain positive PCR expression for nonhuman primate (nhp) embryonic stem cells (ES), amniotic fluid stem cells (AFS), or bone marrow obtained stem cells (BMSC).

## 4. Discussion

The goal of this study was to isolate and characterize amniotic fluid-derived stem cells from nonhuman primates. The primary findings of this study were that: 1) C-kit positive cells can be readily isolated from the amniotic fluid of female cynomolgus monkeys during all three trimesters of pregnancy, but are more consistently obtained during the second trimester; 2) these cells have great expansion capabilities (80-100 population doublings); 3) the amniotic fluid cells readily differentiate into several mesenchymal cell lineage derivatives; and 4) the amniotic cells express cell surface antigens consistent with those of bone marrow-derived mesenchymal progenitor cells, but do not express "stemness" markers such as Oct 4, Nanog, or SOX2, as seen in embryonic stem cells from the same species.

### *4.1. Role of nonhuman primates in biomedical research*

The major rationale of the present studies was to establish AFS cell lines from nonhuman primates. Nonhuman primates are critical for translational medicine because in the spectrum of research from cells to humans. They serve to test hypotheses derived from human clinical or epidemiological experience and extend observations from non-primate model systems to species more closely resembling people. The biomedical utility of nonhuman primate results in part from their close genetic relationship to humans. The rhesus monkey genome, for example, demonstrates 93% sequence homology with that of human beings. This phylogenetic relationship underlies the many phenotypic similarities to human beings (behavior, anatomy, physiology, reproduction) and helps account for the expression in monkeys of naturally occurring or inducible variants of diseases with significant public health impact - cardiovascular disease, cancer, diabetes, osteoarthritis and osteoporosis [2-9]. Therefore, isolation and characterization of a cell source used from an animal model commonly used to receive United States Food and Drug Administration approval for clinical studies is important to translation into patients.

### *4.2. Isolation and Expansion of Nonhuman Primate AFS Cells*

Several nonhuman primate stem cell and progenitor cells populations have been described previously [11]. It is well-known that amniotic fluid represents a very heterogeneous population that includes cells derived from the fetal membranes as well as from the fetus itself. Amniotic fluid-derived cells have been classified on the basis of their morphological, biochemical and growth characteristics and include epithelioid-like cells, amniotic fluid-specific cells and fibroblast-like cells [12]. Both epitheliod and fibroblast-like cells could be grown from the monkey amniotic fluid samples. However, they appeared in only around 30% of the samples and did not proliferate beyond the first couple of passages. This finding is consistent with the literature [1, 12-16].

In addition, the amniotic fluid contains several different cell types, some of which have been shown to be multipotent and differentiate into cell types representing the three main germ layers of embryonic development [13, 14]. Of these cells types, there is a population of cells that are c-kit positive [1]. C-kit plays an important role in gametogenesis, melanogenesis and hematopoiesis [15]. Studies by De Coppi et al [1] report that cells from the amniotic fluid of human beings, isolated using magnetic cell sorting of c-kit positive cells, express several markers of pluripotenitality (Oct4, Nanog, SSEA4). Here, we reported that cells isolated from the amniotic fluid of adult female nonhuman primates and sorted for the c-kit antibody, are equally expansive, while maintaining their ability population doubling times up to 20-25 passages. These cells maintained a cell cycle consistent with rapidly diving cells through these passages. This is in contrast to the bone marrow-derived mesenchymal progenitor cells, which lose this dividing capability by 5 passages and approximately 10-15 population doublings. Similar to other cells found in amniotic fluid, c-kit positive cells from the nonhuman primates were diploid. C-kit cells from monkeys were obtained from all three trimesters, but more readily form the second trimester. There were not many samples collected from the first trimester because of the difficulty collecting the sample without harming the fetus. However, first trimester cells were equally expansive as second trimester cells. Third trimester cells were readily obtained. However, they were more like bone marrow cells in their limited ability to expand through many passages. The c-kit isolated cells were routinely expanded through 20 passages and 80-100 population doublings without significant reduction in doubling time (36 hours) or loss of their cell cycle proliferation (G1, G2, S phase) profile. This is in contrast with the bone marrow

mesenchymal progenitor cells which had significant reduction in the percent of cells entering the G2 phase.

*4.3. Multipotency of Nonhuman Primate AFS Cells*

Results of the present study indicate that the nonhuman primate cells express markers consistent with bone marrow MSCs (CD44, CD73, CD105, CD90, HLA-ABC). The expression of stemness markers OCT4 and Nanog was lower than that of monkey ES cells and not different than that of bone marrow MSCs. Additionally, the nonhuman primate cells could be induced to express markers of mesenchymal lineage cells (adipocytes, chondrocytes, osteocytes, myocytes). Expression of MSC-like markers by monkey AFS cells is consistent with human AFS cells isolated by De Coppi et al 2007 [1]. However, both De Coppi et al and other investigators [13-16] report that AFS cells readily express OCT-4. Whether low expression of OCT4 and Nanog represent true differences in the cell populations in the amniotic fluid of monkeys vs. humans, is unclear. Alternatively, culture and expansion protocols used for the monkey samples may have selected for a more differentiated form of the AFS cells and should be more appropriately referred to as amniotic fluid derived mesenchymal stem cells. While the monkey AFS cells appear to be multipotent, the degree to which they are pluripotent and can differentiate into cells representing all three germ layers – as reported previously [1, 13-16] – has not been determined.

*4.4. Monkey AFS cells as a tool for cell-based therapies*

Because of the expansion potential and their ability to differentiate into several mesenchymal lineage cell types, monkey AFS cells could be used to test the potential clinical utility of several cell-based and tissue-based therapies. Advancement of critical care has dramatically lowered the fatality rate of accidental or combat related injuries. Many of these injuries result in significant loss of bone, cartilage and muscle. Massive numbers of cells are needed to regenerate this tissue. AFS cells may represent a readily available, high expansive, non-tumorigenic cell source capable of fulfilling this need. However, the immunogenicity of these cells remains in question. Studies with MSC indicate that they can be transplanted successfully to unrelated recipients without the requirement to match MHC loci. This appears to result from immunomodulatory activity of the MSC, which secrete a wide variety of anti-inflammatory cytokines and chemokines. As a result the MSC are able to block mixed lymphocyte reactions (MLR) and other immune reactions, and can prove therapeutic against conditions such as graft-versus-host disease [17]. Undifferentiated AFS cells are positive for Class I major histocompatibility (MHC) antigens (HLA-ABC), but are negative for MHC Class II (HLA-DR). Further testing will be needed to determine if AFS cells have immunomodulatory properties similar to that of MSCs.

## 5. Conclusion

Highly expansive, multipotent cells can be isolated from the amniotic fluid of cynomolgus monkeys. These cells express many of the cell markers of previously described amniotic fluid-derived stem cells, but appear to have lower expression of stemness markers. They can be differentiated into cells representing several mesenchymal-lineages and may be useful for pre-clinical testing of both cell and tissue-based therapies requiring regeneration of tissue.

## References

[1] De Coppi P, Bartsch G, Siddiqui MM, Xu T, Santos CC, Perin L, Mostoslavsky G, Serre AC, Snyder EY, Yoo JJ, Furth ME, Soker S, Atala A. Isolation of amniotic stem cell lines with potential for therapy. Nat Biotechnol 2007, 25(1): 100-106.

[2] Kaplan JR, Manuck SB. Status, stress, and atherosclerosis: The role of environment and individual behavior. Ann N Y Acad Sci, 1999, 896:145-61.

[3] Wood CE, Chen Z, Cline JM, Miller BE, Burk RD. Characterization and experimental transmission of an oncogenic papillomavirus in female macaques. J Virol, 2007, 81:6339-45.

[4] Xu W, Hofmann-Lehmann R, McClure HM, Ruprecht RM. Passive immunization with human neutralizing monoclonal antibodies: Correlates of protective immunity against HIV. Vaccine, 2002, 20:1956-60.

[5] Wagner JD, Kavanagh K, Ward GM, Auerbach BJ, Harwood JH, Kaplan JR. Old world nonhuman primate models of type 2 diabetes mellitus. ILAR J, 2006, 47:259-71.

[6] Kenyon NS, Chatzipetrou M, Masetti M, Ranuncoli A, Oliveira M, Wagner JL, Kirk AD, Harlan DM, Burkly LC, Ricordi C. Long-term survival and function of intrahepatic islet allografts in rhesus monkeys treated with humanized anti-CD154. Proc Natl Acad Sci U S A, 1999, 96(14): 8132-8137.

[7] Kenyon NS, Fernandez LA, Lehmann R, Masetti M, Ranuncoli A, Chatzipetrou M, Iaria G, Han D, Wagner JL, Ruiz P, Berho M, Inverardi L, Alejandro R, Mintz DH, Kirk AD, Harlan DM, Burkly LC, Ricordi C. Long-term survival and function of intrahepatic islet allografts in baboons treated with humanized anti-CD154. Diabetes, 1999, 48(7): 1473-1481.

[8] Ham KD, Loeser RF, Lindgren BR, Carlson CS. Effects of long-term estrogen replacement therapy on osteoarthritis severity in cynomolgus monkeys. Arthritis Rheum, 2002, 46:1956-64.
[9] Ham KD, Carlson CS. Effects of estrogen replacement therapy on bone turnover in subchondral bone and epiphyseal metaphyseal cancellous bone of ovariectomized cynomolgus monkeys. J Bone Miner Res, 2004, 19:823-9.
[10] Fuchs A, Buse E, Weinbauer GF. Embryo fetal development studies in nonhuman primates. Methods Mol Biol, 2013, 947:169-83.
[11] Hoffman LM, Carpenter MK. Characterization and culture of human embryonic stem cells. Nat. Biotechnol, 2005, 23, 699-708.
[12] Murphy SV, Atala A. Amniotic fluid and placental membranes: unexpected sources of highly multipotent cells. Semin Reprod Med, 2013, 31(1):62-8.
[13] Cananzi M, Atala A, De Coppi P. Stem cells derived from amniotic fluid: new potentials in regenerative medicine. Reprod Biomed Online, 2009, 18 Suppl 1:17-27.
[14] Parolini O, Soncini M, Evangelista M, Schmidt D. Amniotic membrane and amniotic fluid-derived cells: potential tools for regenerative medicine? Regen Med, 2009, 4(2):275-91.
[15] Chabot B, Stephenson DA, Chapman VM, Besmer P, Bernstein A. The proto-oncogene c-kit encoding a transmembrane tyrosine kinase receptor maps to the mouse W locus. Nature, 1988, 335, 88–89.
[16] Rennie K, Gruslin A, Hengstschläger M, Pei D, Cai J, Nikaido T, Bani-Yaghoub M. Applications of amniotic membrane and fluid in stem cell biology and regenerative medicine.Stem Cells Int, 2012, 2012:721538.
[17] Le Blanc K, Ringden O. Immunomodulation by mesenchymal stem cells and clinical experience. J Intern Med 2007, 262: 509-525.