Development of Human Mesenchymal Stem Cells for Treatment of Multiple Disorders

MANUEL CABALLERO, CIV

HUI XIA

JAMIE L. MYERS

GHULAM J. CHAUDRY

FINAL REPORT

August 2018

59th Medical Wing
Office of the Chief Scientist
2520 Ladd Street, BLDG. 3885
JBSA Lackland AFB, TX 78236-7517

DISTRIBUTION A. Approved for public release; distribution is unlimited.

DECLARATION OF INTEREST

The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Air Force, Department of Defense, nor the U.S. Government. This work was funded by Project Code Number AC12EM01. Authors are military service members, employees, or contractors of the US Government. This work was prepared as part of their official duties. Title 17 USC §105 provides that 'copyright protection under this title is not available for any work of the US Government.' Title 17 USC §101 defines a US Government work as a work prepared by a military service member, employee, or contractor of the US Government as part of that person's official duties and that the author's do not endorse any products in this report.

NOTICE AND SIGNATURE PAGE

Using Government drawings, specifications, or other data included in this document for any purpose other than Government procurement does not in any way obligate the U.S. Government. The fact that the Government formulated or supplied the drawings, specifications, or other data does not license the holder or any other person or corporation or convey any rights or permission to manufacture, use, or sell any patented invention that may relate to them.

Qualified requestors may obtain copies of this report from the Defense Technical Information Center (DTIC) (http://www.dtic.mil).

Development of Human Mesenchymal Stem Cells for Treatment of Multiple Disorders

JOSEPH H. LYNCH, DAF

Medical Modernization Program Analyst
Integrative/Clinical Medicine Program
59 Medical Wing-Science & Technology

CARLTON C. BRINKLEY, Ph.D., DAF

Dir, Diagnostics & Therapeutics Research
59 Medical Wing-Science & Technology

This report is published in the interest of scientific and technical information exchange, and its publication does not constitute the Government's approval or disapproval of its ideas or findings

TABLE OF CONTENTS

Development of Human Mesenchymal Stem Cells for Treatment of Multiple Disorders

Hui Xia[1], Jamie Myers[2], Manuel Y. Caballero[1], and Ghulam J. Chaudry[1]

[1]Center for Advanced Molecular Detection, Chief Scientist's Office, Science and Technology, 59th Medical Wing, US Air Force, JBSA-Lackland, San Antonio, Texas 78236

[2]Current Address: Hematology and Oncology Division, Department of Medicine, The University of Texas Health Science Center, San Antonio, Texas 78229

Keywords

Mesenchymal, stem cells, pluripotent, potency, differentiation, cytokine

1.0 ABSTRACT

Stem cells are those that can differentiate into other functionally varied cell types, such as nerve, muscle, bone, and skin cells. Stem cells represent various temporal and spatial stages in organismal development, and therefore their capacity to differentiate into other cells is variable. Embryonic stem cells are those that are isolated from a very early embryonic development, the blastocyst stage. Such cells retain the capacity to form all tissue types of an organism. Adult stem cells are those isolated from different tissues once such tissues have definitively formed, including any adult stage. Such cells have different capacities to differentiate into other cell types, but no capacity to form all tissue types that make an organism. A common type of adult stem cells is the mesenchymal stem cells (MSCs), typically isolated from bone marrow, adipose tissue, and umbilical cord blood. Due to their capacity to differentiate into other cell types, stem cells have potential therapeutic utility in replacing various terminally differentiated cells when such cells are lost due to injury, disease, or aging. However, when adult stem cells are isolated as primary populations, they do not exhibit uniform differentiation properties; some have greater differentiation capacity, some lesser, and some may even lack any such capacity. Using human mesenchymal stem cells, this project aimed to develop subpopulations and individual lines with greater differentiation capacity. Further, the work also aimed to identify protein and gene expression markers characteristic of the cells with greater potency. The MSCs for this work were purchased from a commercial source. These cells were at passage two. The results presented here show that these MSCs had the capacity to differentiate into adipocytes and osteocytes. However, upon directed induction for differentiation into osteocytes, chondrocytes, or adipocytes, not all cells differentiated, suggesting that within the induced populations the potency of cells varied considerably. We also assessed the expression of markers characteristic of MSCs, both positive and negative. The results also show that with successive passaging the MSC gradually lost their potency, altogether entirely losing it around passage 15. Another notable result was that the expression levels of positive and negative markers commonly used for MSCs were not commensurate with loss of potency; whereas the cells lost potency at late passages, the marker expression levels did not appreciably change, except CD34, a negative marker of MSCs. Analysis of a panel of cytokines in the culture media collected at each passage revealed that for most of these proteins the levels went up at first, peaking at passage 10 or 11, and then gradually declined. However, the HGF and IL-1 levels were initially high, but then gradually decreased from passage 1 to 17. In contrast, the IL-17A level was very low at first, but then gradually increased with successive passaging. The IL-2 and TNF-alpha levels did not change appreciably.

2.0 INTRODUCTION

The cells' ability to depart from a defined set of properties to differentiate into other types of cells is a fundamental principle of multicellular life-forms. Indeed for life to proceed beyond simple unicellular forms, cellular differentiation had to be an inevitable evolutionary development. Further, regulation of differentiation had to be spatial and temporal for organismal development. Thus, certain types of cells must differentiate into certain other types at various stages during development, and the developmental patterns must follow well defined paths to make normal organisms.

That differentiation is a fundamental principle of life has been a self-evident fact for long, given that complex organisms develop from a single cell, the fertilized egg or zygote. Adult tissues have very small populations of certain types of cells, generally called stem cells, that are not terminally differentiated, and that retain the capacity to develop into various other cell types [Caplan, 1991]. Such cells have come to be called stem cells. Aside from these cells and the zygote, stem cells have varying capacities to differentiate into other cells, including those terminally differentiated. Thus, there exist stem cells that can differentiate into a variety of cell types with vastly different phenotypes, and those that can differentiate into only one kind of cell, to which they retain spatial, temporal, and developmental proximity. This highly variable differentiation capacity of stem cells is termed potency [Kfoury, 2016].

Although stem cells markedly differ in their potency, they have two common basic characteristics: the ability to differentiate into other cell types, and the ability to sustain their stem cell phenotype, a property termed self-renewal and originally recognized in stromal cells [Evans, 1981; Martin, 1981; Thomson, 1995]. Given their varying potency, stem cells have great therapeutic potentials where replacement of damaged, lost, or malfunctioning cells and tissues is needed. But they are also potentially useful in modulating the immune response, e.g., in inflammation [Aurora, 2014; Bernardo, 2013]. The translational aspects of stem cell properties are clearly very important in regenerative or restorative medicine.

Clearly, the most fundamental of the stem cells are embryonic stem cells (ESCs), derived from very early stage embryos. ESCs are pluripotent; that is, they can differentiate into all cell types required to make an organism. ESCs were first isolated from mice in 1981 [Evans, 1981; Martin, 1981]. The primate ESC lines were isolated much later [Thomson, 1995], and the human ESC lines even later [Thomson, 1998]. In contrast, the adult stem cells are multipotent; that is, they can differentiate into a few different cell types, but lack the potential to differentiate into all the cell types required to make an organism. The adult stem cells include the widely studied mesenchymal stem cells, described below.

2.1 Mesenchymal Stem Cells (MSCs)

Caplan [1991] is widely credited with the original coinage of the term "mesenchymal stem cells". MSCs are multipotent; that is, they can differentiate into a number of cell types, but not all cell types required to make an organism. It has been shown, for example, that MSCs isolated from bone marrow have adipogenic, osteogenic, and chondrogenic potency, and that this property is retained even by some clonal populations derived from the parental population [Pittenger, 1999]. MSCs reside in a number of different adult tissues, e.g., adipose, bone marrow, umbilical cord, dental pulp, and synovial tissue. But bone marrow and adipose tissue have been the two common sources [Kfoury, 2015]. Because of their multiple origins and heterogeneous properties, MSCs have different differentiation potentials. For example, cells isolated from certain tissues have greater differentiation potential than cells isolated from some others [Sakaguchi, 2005]. They are able to differentiate into a number of different cell types, e.g., myocytes, osteocytes, chondrocytes, and adipocytes [Caplan, 1991; Pittenger, 1999; Zuk, 2001; Zuk, 2002]. MSCs and other stem cells derived from adult tissues have certain advantages over embryonic stem cells: work with MSCs and other adult stem cells does not invoke the ethical and legal issues that work with embryonic stem cells does, and adult stem cells are easier to harvest, notably from adipose tissues [Kfoury, 2016]. MSCs exhibit markedly low frequency of teratoma formation, as well as low signature for invoking the host immune response, which consequently leads to better host acceptance. MSCs have also proven important players in modulating the inflammation, a pathologic response that accompanies innumerable diseases. MSCs therefore have strong potential from the viewpoint of translational applications in medicine [Caplan, 2017; Grompe, 2012].

Following their discovery, a certain confusion about the precise nature of cells that different researchers considered MSCs persisted, which underscored the need to develop uniform criteria for identifying and defining these cells. Based on different findings about the origin and properties of the cells generally described as mesenchymal stem cells, the International Society for Cellular Therapy (ISCT) set three types of criteria for cells to be considered MSCs: **1)** adherence to plastic surfaces when grown under standard *in vitro* culture conditions. **2)** measured by FACS analysis, $\geq 95\%$ with expression of the surface antigens CD73, CD90 (Thy-1), and CD105; $\leq 2\%$ with expression of CD14 or CD11b, CD34, CD45, CD79a or CD19, and HLA-DR. **3)** *in vitro* multilineage differentiation capacity, e.g., into adipocytes, osteoblasts, and osteocytes [Dominici, 2006]. In its 2005 position paper, ISCT recommended that these cells be referred to as "multipotent mesenchymal stromal cells" [Horwitz, 2005]. This was unlike the previous terminology of "mesenchymal stem cells," a term coined by Caplan [Caplan, 1991]. It should be noted, however, that confusion over the usage of these terms to describe MSCs has nonetheless persisted in various aspects, e.g., their potency, adult tissue source, and the therapeutic applications. Indeed in a 2017 account of the historical confusion about the nature and properties of MSCs, Caplan disfavored his original designation, and instead reemphasized

the usage of the term “medicinal stromal cells,” a term whose use he had urged earlier [Caplan, 2010; Caplan, 2017]. But the term “medicinal stromal cells” has gained little currency. The most commonly used terms remain “mesenchymal stem cells” and mesenchymal stromal cells.” Of the two terms, the former appears to be the most widely used one, and therefore in this report we have kept the term “mesenchymal stem cells”, because of its widespread usage.

2.2 Genetic and Phenotypic Heterogeneity of Stem Cells

As with any population of cells, e.g., primary cells isolated from adult tissues, stem cells are not uniform in their genetic and phenotypic constitution [Grompe, 2012]. Such variations exist even within a given type of stem cell population, such as MSCs isolated from a particular type of tissue [Pittenger, 2004]. These variations inevitably can lead to cellular differences such that in a given population there exist subpopulations, potentially even individual cells, with markedly variable differentiation and therapeutic potential. Obviously it would be desirable to use stem cells with uniform optimal properties for therapy, unless a condition warrants treatment with a mixture of stem cell types exhibiting the needed sets of properties. Likewise, biochemical, cellular, and molecular genetic studies may also warrant work with stem cells that exhibit uniform properties. One way to achieve genetic and phenotypic homogeneity is to develop clonal cell lines. Since clonal lines arise from single cells, they represent the maximum genetic and phenotypic uniformity.

The overall goal of this work was to segregate and identify stem cell populations and develop clonal lines with greater differentiation potentials. We reasoned that such stem cells may have greater therapeutic potential as well. The work reported here utilized MSCs purchased from commercial sources.

3.0 MATERIALS AND METHODS

3.1 Mesenchymal Stem Cell Culturing

Human adipose-derived mesenchymal stem cells (Ad-MSC; ATCC® PCS-500-011™) at passage 2 were purchased from ATCC (Manassas, VA). These Ad-MSC were originally from a 52 year old Caucasian female. The mesenchymal stem cell basal medium (cat. # ATCC® PCS-500-030) and the mesenchymal stem cell growth kit (ATCC® PCS-500-040™) were also from ATCC. The growth kit contained fetal bovine serum (FBS), recombinant basic human fibroblast growth factor (rhFGF-b), recombinant acidic human fibroblast growth factor (rhFGF-a), and L-alanyl-L-glutamine. The cells were thawed and propagated in the basal medium supplemented with the growth kit constituents. At passage 2, the supplier-reported fraction of Ad-MSC was about 99% and of negative markers about 0.2%. The positive markers listed were CD29, CD44, CD73, CD90, CD105, and CD166. The negative markers were CD14, CD34 and CD45.

3.2 Adipocyte Differentiation

Ad-MSCs were seeded in 24-well plates at the density of 18,000 cells/cm^2. After 48 hours, the cells were induced and maintained in Adipocyte Differentiation Toolkit for Adipose-Derived MSCs and Preadipocytes (cat. # ATCC® PCS-500-050™) as described by the supplier. After 17 days from initial plating of cells, the cells were fixed with 4% paraformaldehyde and stained with Oil Red O (Millipore Sigma, St. Louis, MO; catalog # O0625) or HCS LipidTOX Green Neutral Lipid Stain (Invitrogen, Carlsbad, CA; catalog # H34475). DAPI was used for nuclear staining. The green fluorescent lipid droplets were visualized with Zoe Fluorescence Cell Imager (Bio-Rad, Hercules, CA).

3.3 Osteocyte Differentiation

Ad-MSC were seeded into 24-well plates at 18,000 cells/cm^2 (~ 36,000 cells/well). After 48 hours, the Ad-MSC growth medium was replaced with 1 ml of Osteocyte Differentiation Tool medium (cat. # ATCC® PCS-500-052™). After additional three to four days, 0.5 ml of the old medium was removed from each well and replaced with 1 mL of fresh, pre-warmed Osteocyte Differentiation Medium to each well, and this was repeated every 3-4 days, until the cells had been in the differentiation medium for 19 days. At the end of the 19^{th} day, the cells were washed with 1X Dulbecco's phosphate buffered saline (D-PBS; ATCC® 30-2200) and fixed in 4% paraformaldehyde. The cells were then stained with Alizarin Red S (Millipore Sigma, catalog # A5533) to gauge calcium accumulation.

3.4 Chondrocyte Differentiation

Micromass culture was used to assess the chondrogenic potency of the Ad-MSC [Zhang, 2010]. The Ad-MSC solution of 1.6 X 10^7 cells/ml was prepared, and 5 µl droplets of cell solution (80,000 cells) were seeded in a 24-well plate, placing the suspensions in the middle of each well.

After 2 hours of incubation under humidified conditions, prewarmed Chondrocyte Differentiation medium (ATCC® PCS500051) was added. The cultures were then fed with fresh medium every 2-3 days. After 14 days, cells were fixed in 4% paraformaldehyde and stained with Alcian Blue (Millipore Sigma, catalog # A5268) to assess presence of the proteoglycans characteristic of chondrocytes.

3.5 Neuronal Induction

Ad-MSC were maintained in the Ad-MSC growth medium as described above, but supplemented with 1 mM β-mercaptoethanol for 24 hours. To initiate neuronal differentiation, the preinduction medium was replaced with the FBS-free neuronal differentiation medium (MSC Basal medium, 200 µM butylated hydroxyanisole, 2% DMSO). Cells were observed daily to detect any neuron-like morphologic characteristics [Woodbury, 2000]. NeuroTrace™ 500/525 Green Fluorescence Nissl Stain – Solution in DMSO (Molecular Probes, Eugene, OR, catalog # N21480) was used to stain the Nissl bodies characteristic of neuronal cells.

3.6 Assessment of Relative Ad-MSC Marker Gene Expression

Total RNA was isolated from Ad-MSC at different passages with Qiagen RNeasyPlus Mini Kit (QIAGEN Inc., Germantown, MD; catalog # 74134) as directed by the manufacturer. RNA concentration in each preparation was determined by UV spectrophotometry (Nanodrop, Thermo Fisher Scientific). cDNA was synthesized using the SuperScript IV First-Strand Synthesis System (Invitrogen, Carlsbad, CA, catalog # 18091050) as directed: 10-min incubation of reaction mixture at 50^{o}C, followed by 10-min incubation at 80^{o}C. The marker gene expression analysis was done by real-time PCR on QuantStudio 6 (Applied Biosystems, Foster City, CA). For marker analysis, TaqMan Array Human Ad-MSC plates with TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific, Waltham, MA, catalog # 4444558) were used. Each reaction mixture contained cDNA equivalent to 10 ng of total RNA. Thermocycling protocol was as follows: 50^{o}C for 2 min; 95^{o}C for 20 sec; 40 cycles of 95^{o}C for 3 sec and 60^{o}C for 30 sec. Relative gene expression levels were determined by normalizing to 18S rRNA and GAPDH transcript levels, calculated using the expression $2^{-\Delta\Delta Ct}$. The MSC positive markers analyzed were CD29, CD44, CD73, CD90, CD105 and CD166. The negative markers analyzed were CD11b, CD14, CD19, CD34, CD45, and CD79A.

3.7 Multiplex Analysis of Cytokines Secreted by Ad-MSC at Different Passages

The cytokine analysis was performed on culture media. Prior to collection of media for this analysis, the cells were incubated in serum-free Ad-MSC basal culture medium for 48 hours. The collected media were centrifuged at 3000xg to pellet any particulate matter. The supernatants were then recovered and stored at -80^{o}C. Cells at each passage were cultured in triplicate. Cytokine concentrations (pg/ml) in supernatants were determined in duplicate using the Luminex MAGPIX™ Multiplex Reader using the Cytokine 35-Plex Human Panel (Invitrogen, catalog # LHC6005M). The total protein concentration in each cell lysate (µg/ml) was determined by the

bicinchoninic acid assay (BCA) with a commercial kit (Thermo Fisher Scientific; Cat# 23227). The cytokine concentrations in each supernatant were normalized to total protein concentration and expressed as mean ± standard error of the mean (pg/µg).

4.0 RESULTS

The adipose mesenchymal stem cells (MSCs) we used for the work reported here were purchased from ATCC, and they were reported to have the properties characteristic of mesenchymal stem cells, as described in the Materials and Methods section. Nonetheless, we assessed these cells for two main properties: expression of the characteristic markers and the cells' differentiation capacity. As shown in Figure 1, the adipose tissue derived MSCs (Ad-MSCs) showed adherent growth and fibroblast-like morphology. Adherence in culture is an important requisite property for considering newly isolated primary cells mesenchymal stem cells [Dominici, 2006].

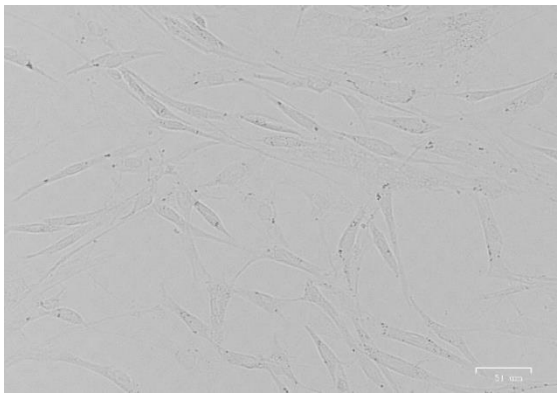

Figure 1. General fibroblast-like morphology of cultured adipose tissue derived mesenchymal stem cells (MSC)

As stated elsewhere in this report, the International Society for Cellular Therapy has listed the property of adherence to plastic surfaces under routine growth conditions as one of the defining properties for cells to be considered MSCs [ISCT position paper; Dominici, 2006].

4.1 Relative Expression Levels of MSC Markers in Ad-MSCs

To better ascertain the stem cell status of Ad-MSC, we analyzed the expression levels of the reported positive and negative markers for such stem cells. The positive markers analyzed were CD29, CD44, CD73, CD90, CD105 and CD166. The negative markers were CD11b, CD14, CD19, CD34, CD45, and CD79A. The expression levels at each passage were determined in terms of the mRNA levels, as described in Materials and Methods. The results are shown in Figure 2. CD29, CD90, and CD166 showed a general upward trend in levels, although CD166 had the highest level at P4. CD73 levels showed a slight downward trend with propagation. CD44 and CD105 levels did not change very much. The negative marker expression levels

shown as per cent of the expression levels of the positive marker CD29 at the corresponding passages. All negative markers tested showed very little expression in comparison to CD29, except CD34, which exhibited rapidly increasing levels; at P3 and P4, its levels were about 0.02%, whereas at P15 its level had gone up to 2%, a 100-fold increase.

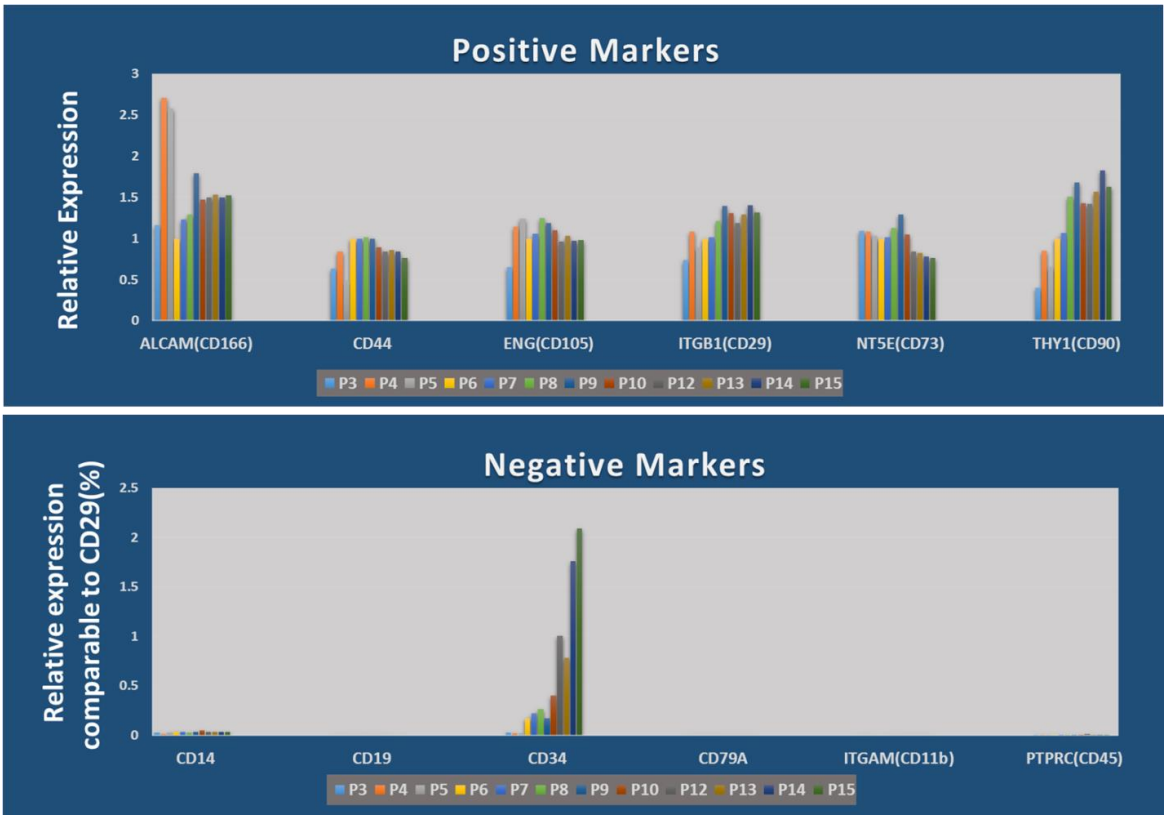

Figure 2. Analysis of positive and negative marker expression at the mRNA level in cultured adipose tissue MSCs

The cells were grown and the samples prepared for the marker analysis as described in detail in the Materials and Methods section. The negative marker expression levels were normalized to those of CD29 at the corresponding passages.

4.2 Osteocyte, Adipocyte and Chondrocyte Differentiation

One way we assessed the potency of Ad-MSCs was to see whether they would differentiate into adipocytes. To do that, Ad-MSCs were grown in the adipogenic medium purchased from the supplier of cells (ATCC). The cells were grown in this medium as directed. The results in Figure 4 show that these cells had the adipocyte differentiation potency. As evidenced by the results shown in Figure 3, Oil Red O stained the cells very well following growth in the adipogenic medium, whereas the dye did not appreciably stain the cells grown in the regular medium for MSCs.

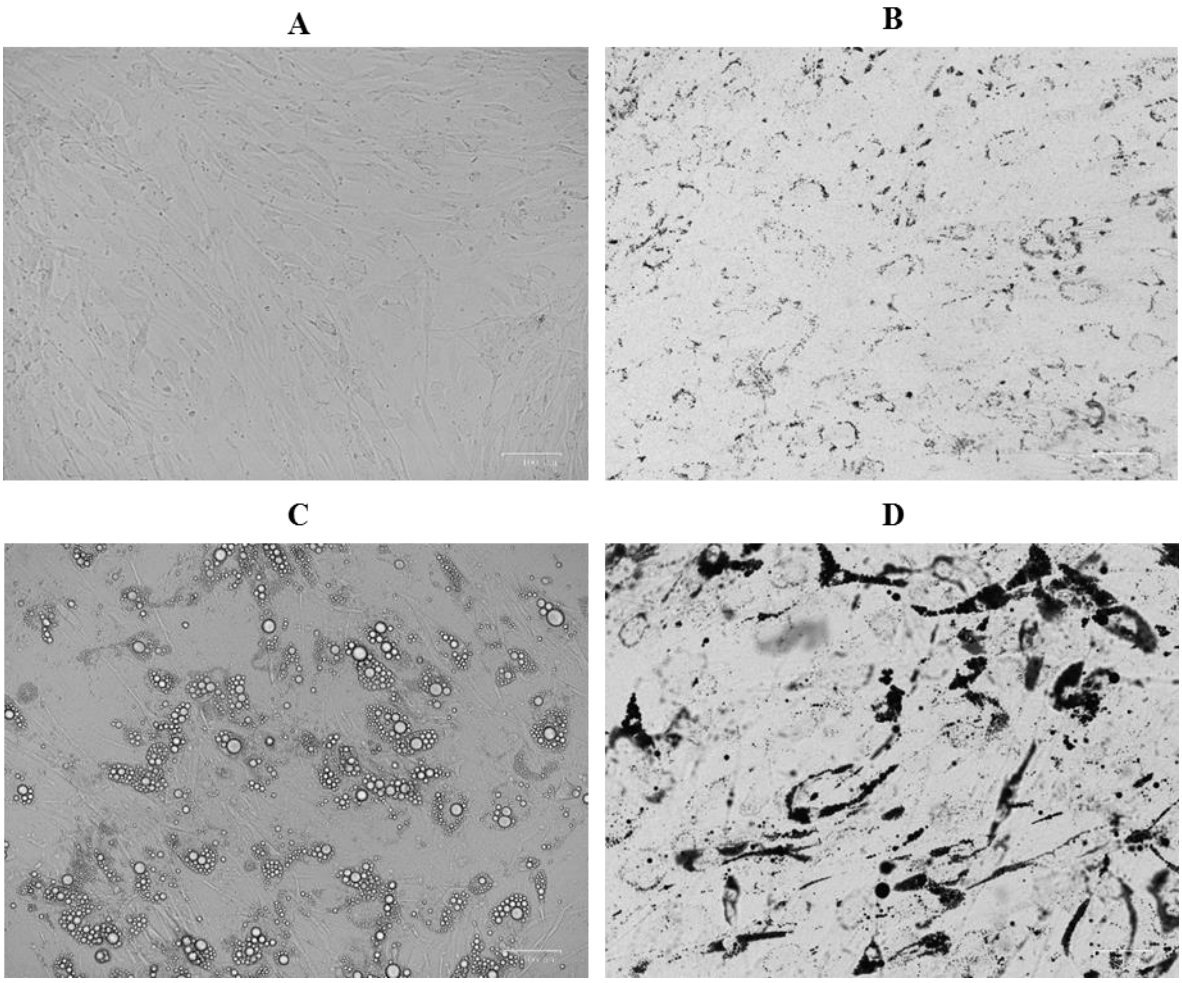

Figure 3. Differentiation of passage 5 Ad-MSC into adipocytes. A, undifferentiated MSCs without Oil Red O staining. B, undifferentiated MSCs stained with Oil Red O. C, unstained MSCs following growth in adipocyte differentiation medium. D, MSCs grown in adipocyte differentiation medium and stained with Oil Red O

Cells were in the adipocyte differentiation medium for 14 days, at which point vacuolar fat deposits became markedly noticeable. The deposits can be stained with Oil Red O, a marker for lipid-rich intracellular sites. Another way we assessed the Ad-MSCs potency was to see if they would differentiate into osteocytes. For each differentiation, the cells were grown in the appropriate induction media. The results show that Ad-MSCs had the potency to differentiate into osteocytes, as was evident from markedly greater Alizarin Red S staining of the induced cells than the uninduced ones (Figure 4).

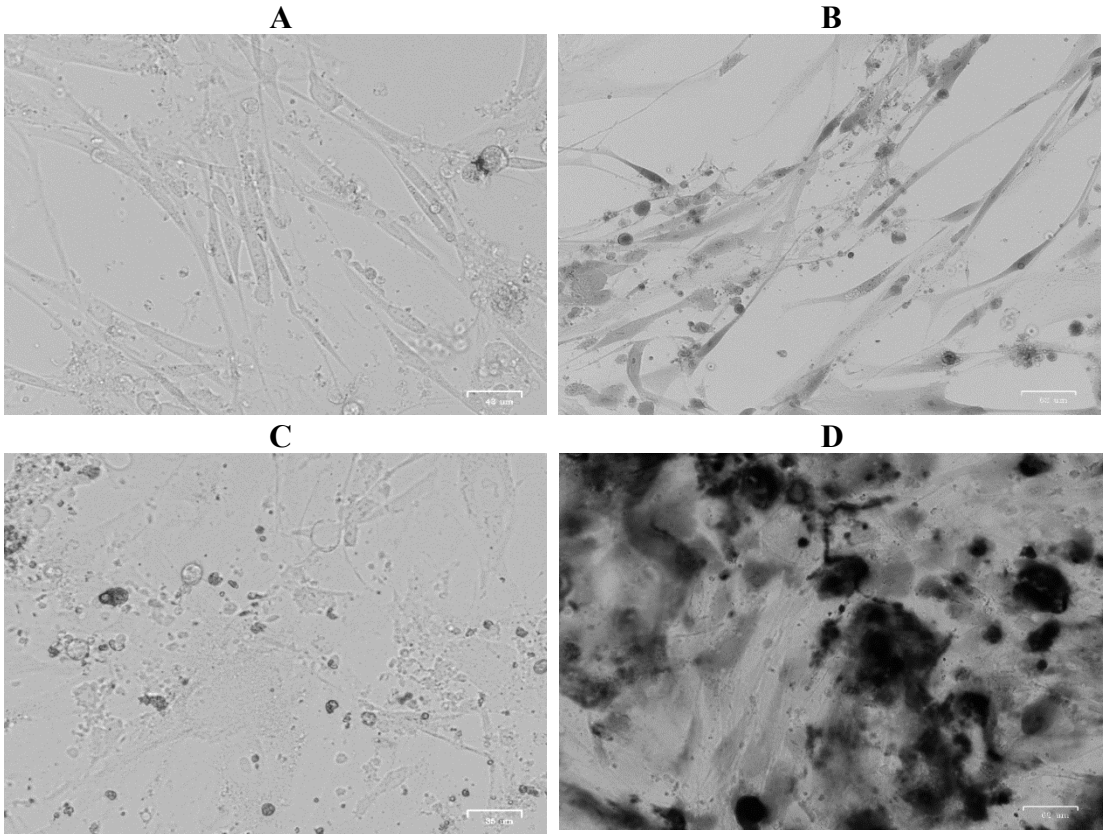

Figure 4. Differentiation of passage 5 adipose tissue MSCs into osteocytes. A and B, Cells grown in normal growth medium without (A) and with (B) Alizarin Red S staining. C and D, cells grown in osteocyte differentiation medium without (C) and with (D) Alizarin Red S staining. The dye stains the calcium deposits red, which appear as dark areas in the black and white photographs (B, D).

The third potency check that we did was to see whether the Ad-MSCs had the capacity to differentiate into chondrocytes. This was done by staining the cells with Alcian Blue to gauge the extent of aggrecans in the cells. Figure 5 shows that the cells indeed had this capacity.

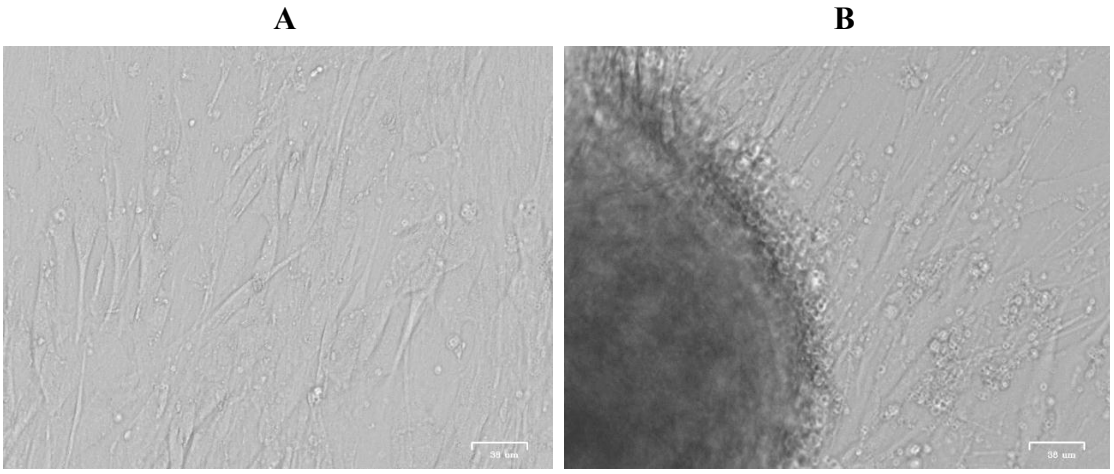

Figure 5. Differentiation of passage 5 adipose tissue MSCs into chondrocytes. A, cells stained with Alcian Blue following growth in regular MSC medium. B, cells stained with Alcian Blue after 14-day growth in chondrocyte differentiation medium. The Alcian Blue staining (blue) appears as dark because the photographs are in black and white.

4.3 Neuronal Differentiation

The neuronal differentiation potential of Ad-MSCs was tested and differentiation was gauged by employing a fluorescent Nissl body staining method using a commercial kit. The procedural details are described in the Materials and Methods section. The results show that Ad-MSCs exhibited neuron-like differentiation, as suggested by retraction or shrinkage of the cell body and intense concentrated Nissl staining (Figure 6).

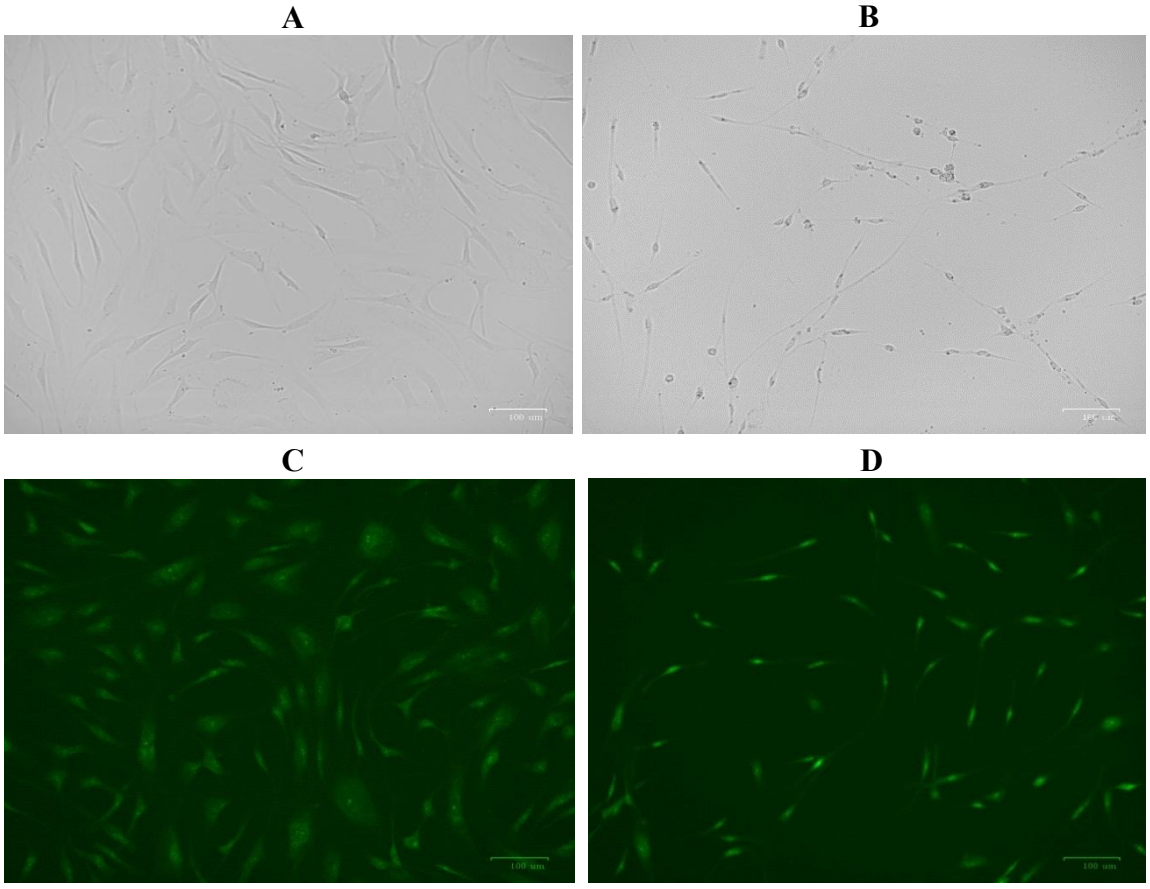

Figure 6. Differentiation of passage 5 adipose tissue MSCs into neuron-like cells

Morphological changes on differentiation of p5 Ad-MSC into neural-like cell. After 3 days induction with DMSO/BHA, Undifferentiated fibroblast-like-shaped Ad-MSCs (Fig#, a) appear neuron-like morphology. **A, C,** uninduced cells. Notice the fibroblast-like morphology. **B, D,** induced cells. Notice the retracted (shrunken) cell body and the long, slender processes (B). Also notice the intense staining within the cell body, a feature indicative of Nissl body presence (D). The Nissl bodies are considered an important characteristic of neuron-like cells. A, B, unstained. C, D, green fluorescence staining for the Nissl body presence (see the Materials and Methods section).

4.4 Effect of Continued *in vitro* Growth on Potency

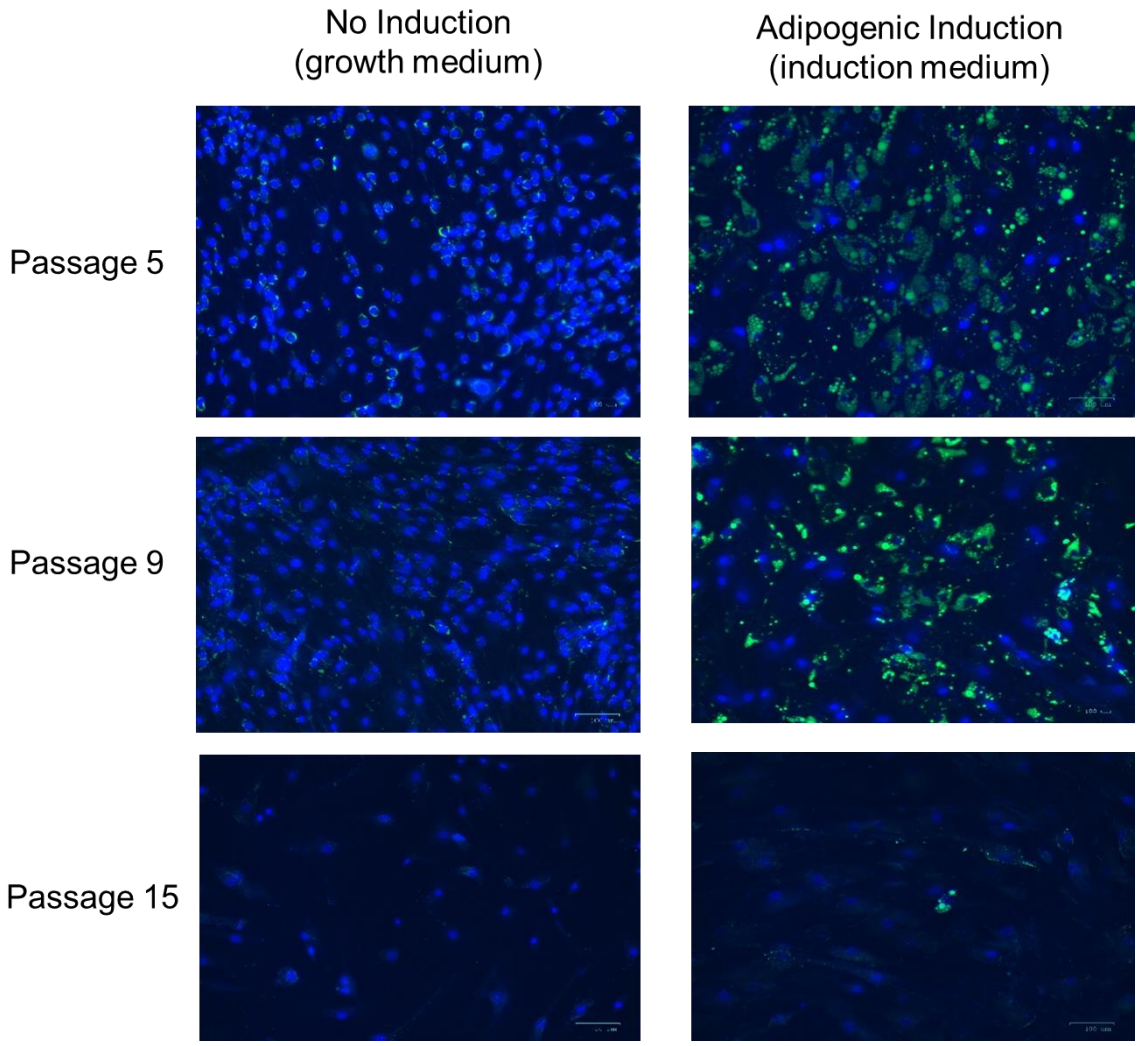

Figure 7. Adipogenic potency of MSCs at different passages

At some point during successive passaging, stem cell potency dramatically diminishes or altogether disappears. The results here show that these adipose tissue derived stem cells essentially retain their potency up to passage number 9, but lose it at passage number 15. A rapid decline in potency occurred between passages 9 and 15 (not shown).

4.5 Cytokine Expression Profile of Cultured Ad-MSC at Various Passages

The concentrations were determined by a multiplex microbeads-based immunofluorescence assay, described in the Materials and Methods section. The results here show that the levels of twenty six cytokines in the culture medium generally increased with each passage, achieving the peak levels at passage 10 or 11 (**A**). The levels of HGF and IL-1RA dramatically decrease with successive passaging, whereas the levels of IL-17A show a gradual increase (**B**). IL-2 and TNF-α appear not to exhibit much changes, except that they peak at passage 11.Then the levels gradually decreased (**C**).

Fig. 8A

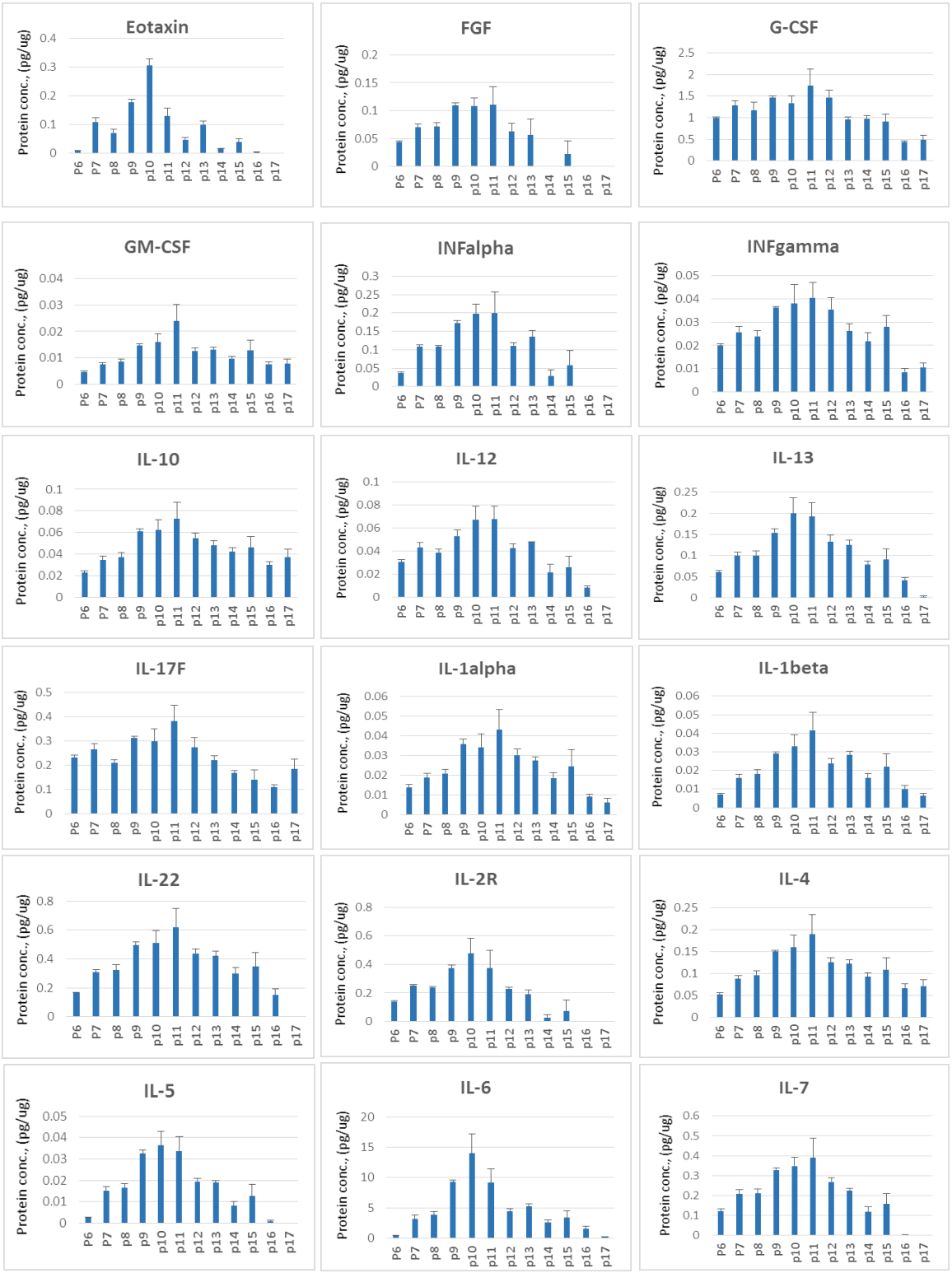
Eotaxin
FGF
G-CSF
GM-CSF
INFalpha
INFgamma
IL-10
IL-12
IL-13
IL-17F
IL-1alpha
IL-1beta
IL-22
IL-2R
IL-4
IL-5
IL-6
IL-7
Protein conc., (pg/ug)
P6 P7 p8 p9 p10 p11 p12 p13 p14 p15 p16 p17

Fig. 8A (continued)

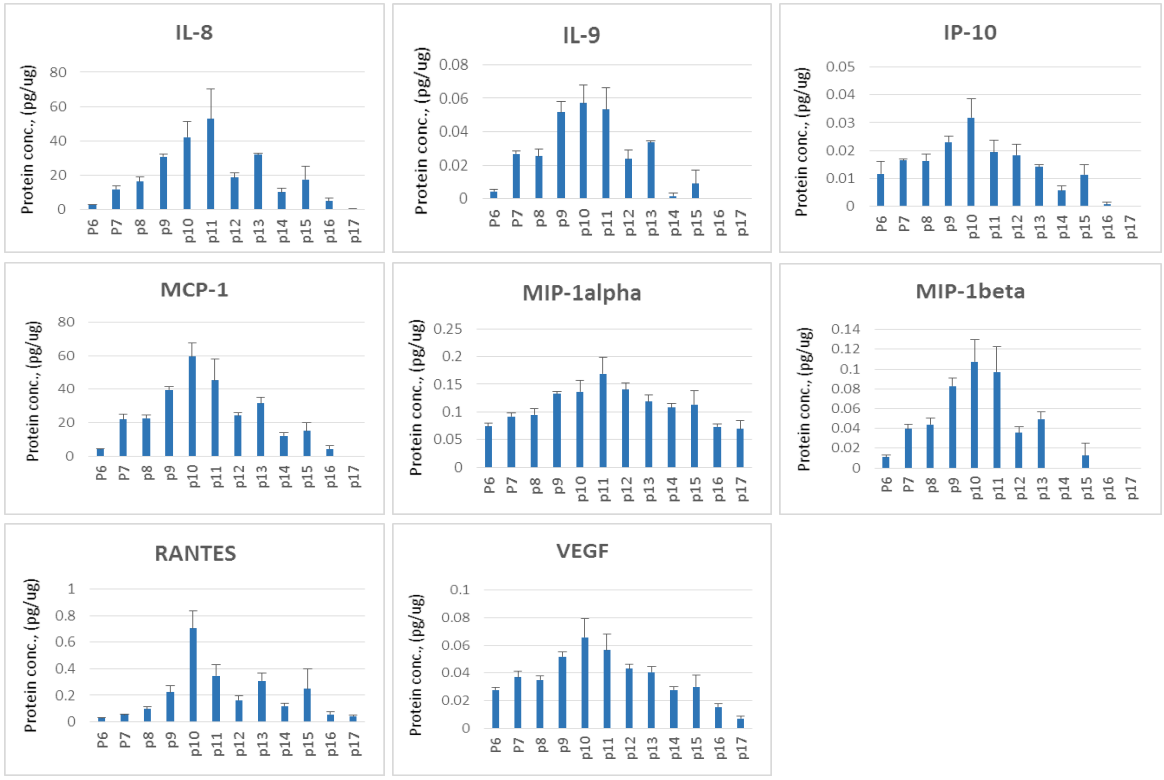

Fig. 8B

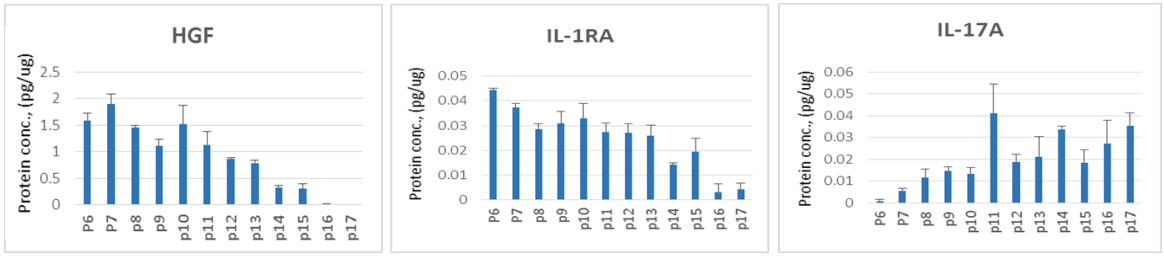

Fig. 8C

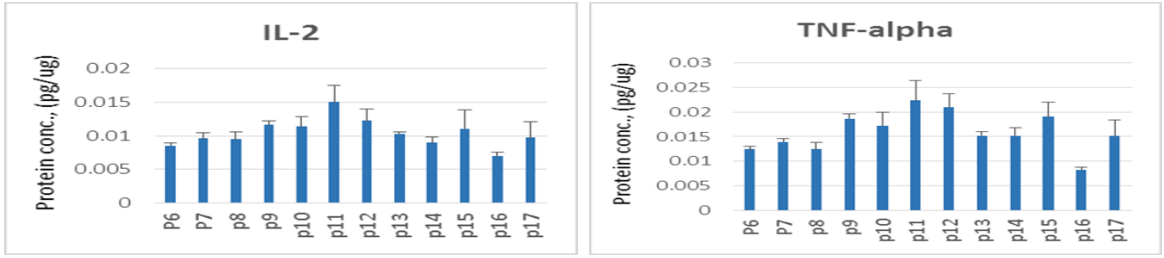

Figure 8. Quantitative analysis of cytokines secreted by Ad-MSC at various passages of growth. A, 26 cytokines whose levels increase at first, peaking at passage 10 or 11, and then decrease. B, two proteins whose levels generally decrease (HGF and IL-1RA) and one whose levels show a gradual upward trend (IL-17A). C, two cytokines whose levels do not change very much (IL-2 and TNF-α)

5.0 SUMMARY

1. Adipose tissue and bone marrow derived MSC were purchased from commercial sources.

2. The cells were propagated as directed in the recommended media. The differentiation induction media were also commercial, and the cells were grown in them as recommended.

3. The cells exhibited potency to differentiate into adipocytes, chondrocytes, and osteocytes (Figures 3-6).

4. Expression analysis of the reported markers for MSC showed that the cells expressed the markers. They showed very low levels of negative marker expression, except the negative marker CD34 (Figure 2).

5. We assessed the potency of Ad-MSC at different passages, and found that the cells' potency gradually diminished after passage 9 or 10, essentially disappearing at passage 15 (Figure 7).

6. Unlike the gradual loss of potency with propagation, the cells did not lose expression of the positive markers (Figure 2).

7. MSCs exhibit immunomodulatory activities. We therefore analyzed the secreted levels of many cytokines, including pro-inflammatory and anti-inflammatory cytokines. We used a 37-panel cytokine analysis commercial kit. The analysis was done on Ad-MSC at passages 3-17.

8. The cytokine analysis revealed that generally the levels of most cytokines initially increased, peaking around passage 9 or 10, and then gradually decreased (Figure 8A).

9. The levels of HGF (hepatocyte growth factor) and IL-1RA (interleukin-1 receptor antagonist) gradually decreased from passage 3 to 17, whereas the level of IL-17A showed a generally increasing trend from passage 3-17 (Figure 8B).

10. The levels of IL-2 and TNF-α appeared not to change appreciably from passage 3-17 (Figure 8C).

11. A parallel study conducted with the cells that we produced was with mice. The work was done at the University of Texas Health Science Center, San Antonio, Texas. One aim of that study was to gauge the cells' potential to affect the mouse cytokine levels following injections. That work will be submitted as a separate final report, and therefore we forgo presenting those results here.

12. The overall goal of this work was to segregate and characterize subpopulations of mesenchymal stem cells (MSC) with greater potency. An aim was also to develop clonal derivatives to identify and characterize those with better stem cell markers and characteristics. Both goals required a FACS machine, a large-scale gene expression analysis machine, and a fluorescence microscope. All three instruments suffered from extended purchasing delays, arriving essentially when the project funding date had ended. Unfortunately, therefore, the overall goals could not be achieved by the project closure date. However, our plan is to seek further funding for the project to achieve these objectives, and to also add the additional goal of testing the newly identified stem cells for their tissue regenerative potential in animal models.

6.0 REFERENCES

1. Aurora AB and Olson EN. Immune modulation of stem cells and regeneration. Cell Stem Cell, 15 (1): 14-25. 2014.

2. Bernardo ME and Fibbe WE. Mesenchymal stromal cells: sensors and switchers of inflammation. Cell Stem Cell, 13 (4): 392-402.

3. Caplan AI. Mesenchymal Stem Cells. J. Orth. Res., 9: 941-950. 1991.

4. Caplan AI. What's in a name? Tissue Eng. Part A., 16: 2415–2417. 2010.

5. Caplan AI. Mesenchymal Stem Cells: Time to Change the Name! Stem Cells Transl. Med., 6: 1445-1451. 2017.

6. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop DJ, and Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy, 8 (4): 315-317. 2006.

7. Evans MJ and Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature, 292 (5819): 154-6. 1981.

8. Grompe M. Tissue stem cells: new tools and functional diversity. Cell Stem Cell, 10 (6): 685-689. 2012.

9. Horwitz EM, Le Blanc K, Dominici M, Mueller I, Slaper-Cortenbach I, Marini FC, Deans RJ, Krause DS, and Keating A. Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. Cytotherapy, 7 (5): 393–395. 2005.

10. Kfoury Y and Scadden DT. Mesenchymal cell contributions to the stem cell niche. Cell Stem Cell, 16 (3): 239-253. 2015.

11. Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc. Natl. Acad. Sci. USA, 78 (12): 7634-7638. 1981.

12. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, and Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science, 284 (5411): 143-147. 1999.

13. Pittenger MF and Martin BJ. Mesenchymal stem cells and their potential as cardiac therapeutics. Circ. Res., 95 (1): 9-20. 2004.

14. Sakaguchi Y, Sekiya I, Yagishita K, and Muneta T. Comparison of Human Stem Cells Derived From Various Mesenchymal Tissues: Superiority of Synovium as a Cell Source. Arthritis & Rheumatism, 52 (8): 2521–2529. 2005.

15. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, and Jones JM. Embryonic Stem Cell Lines Derived from Human Blastocysts. Science, 282 (5391): 1145-1147. 1998.

16. Thomson JA, Kalishman J, Golos TG, Durning M, Harris CP, Becker RA, and Hearn JP. Isolation of a primate embryonic stem cell line. Proc. Natl. Acad. Sci. USA, 92 (17): 7844-7848. 1995.

17. Woodbury D, Schwarz E, Prockop D, and Black I. Adult Rat and Human Bone Marrow Stromal Cells Differentiate Into Neurons. Journal of Neuroscience Research, 61: 364–370. 2000.

18. Zhang L, Su P, Xu C, Yang J, Yu W, and Huang D. Chondrogenic differentiation of human mesenchymal stem cells: a comparison between micromass and pellet culture systems. Biotechnol. Lett., 32 (9): 1339-1346. 2010.

19. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, and Hedrick MH. Human adipose tissue is a source of multipotent stem cells. Mol. Biol. Cell, 13 (12): 4279–4295. 2002.

20. Zuk PA, Zhu M, Mizuno H, Huang JI, Futrell WJ, Katz AJ, Benhaim P, Lorenz HP, and Hedrick MH. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng., 7 (2): 211–228. 2001.