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M.S. Thesis in Biology

January-2011

NEURAL DIFFERENTIATION POTENTIAL VARIABILITY OF BONE MARROW DERIVED HUMAN MESENCHYMAL STEM CELLS

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A thesis submitted to

the Graduate Institute of Sciences and Engineering

of

Fatih University

in partial fulfillment of the requirements for the degree of

Master of Science

in

Biology

January 2011

Istanbul, Turkey

APPROVAL PAGE

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

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Head of Department

This is to certify that I have read this thesis and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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January 2011

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ABSTRACT

Bone marrow derived human mesenchymal stem cells (hMSC) represent a promising cell-based therapy for a number of degenerative conditions. Many promising applications require cell expansion following harvest and involve the treatment of diseases and conditions found in an aging population. Therefore, the effect of donor age and long term passage must be understood in order to develop clinical techniques and therapeutics based on hMSCs.

Previous investigations into affects of aging on hMSC have proved contradictory due to the relative narrow age ranges of subjects assessed. This study seeks to address this controversy by increasing donor number and using a wider range of donor ages. We have established cultures of hMSCs from child (0-18 years, n = 6), adult (18-50 years, n = 6) and old (over 50 years, n = 6) donors.

In this study, we evaluated the effects of donor age on morphology, proliferation potential and differentiation ability of bone marrow derived hMSCs. In addition we examined effects of long term passage on the characteristics and trans-differentiation potential of hMSCs towards neurogenic lineage.

Differences in morphology, cell growth and differentiation potential of hMSCs obtained from donors of different age were observed. Cells from child donors maintained their fibroblast-like morphology up to higher passages and proliferated in a greater number than those from adult and old donors. Adipogenic, osteogenic and neurogenic differentiation potential decreased with age; while chondrogenic potential didn't change.

Long term serial passage affected morphology and proliferation potential of hMSCs from all ages. With increasing passage number, proliferation rate decreased and cells lost their typical morphology. In order to assess effects of long term passage on neural trans-differentiation potential, we have used RT-PCR to investigate expression levels of neural markers (β III Tubulin, NSE) and topo IIβ in populations of non-differentiated hMSCs.

hMSCs spontaneously expressed certain neural phenotype markers in culture, without specialized induction reagents. Each donor sample revealed a unique expression

pattern, demonstrating a significant variation of marker expression. Marker expression levels increased due to increasing passage number in hMSCs from adult donors, in contrast to hMSCs from child donors.

These results indicated that; even under highly standardized culture conditions, donor age and long-term passage have effects on hMSC characteristics, which should be taken into account prior to stem cell based therapies.

Keywords: human bone marrow mesenchymal stem cells, differentiation, age, neural transdifferentiation, long term passage

İNSAN KEMİK İLİĞİ KÖKENLİ MEZENKİMAL KÖK HÜCRELERİNİN NÖRAL FARKLILAŞMA POTANSİYEL DEĞİŞKENLİĞİNİN ARAŞTIRILMASI

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Yüksek Lisans Tezi – Biyoloji
Ocak 2011

Tez Danışmanı: Yrd. Doç. Dr. Sevim IŞIK

ÖZ

Hücresel tedaviler içinde kemik iliği kökenli insan mezenkimal kök hücre (iMKH) tedavisi heyecan verici ve hızla gelişen bir tedavi şeklidir. Mezenkimal kök hücrelerin, elde edildikleri dokularda çok az sayıda olmaları nedeniyle *in vitro* hücre kültür ortamında çoğaltılmalarının gerekliliği ve bu hücrelerin yaşlanan popülasyonda sıkça rastlanan hastalıkların tedavisi için kullanılmaları; klinik çalışmalar öncesi donör yaşı ve uzun süreli pasajlamanın iMKH üzerine etkisinin araştırılmasını gerektirmektedir.

Donör yaşının iMKH üzerine etkisini araştıran önceki çalışmalardan elde edilen sonuçların tutarsızlığı, seçilen donörlerin yaşlarının birbirine yakınlığından kaynaklanmaktadır. Çalışmamızda donör sayısını arttırıp, yaş aralığını geniş tutarak bu tutarsızlığı aşmayı amaçladık. iMKH kültürleri çocuk (0-18 yaş, s = 6), yetişkin (18-50 yaş, s = 6) ve yaşlı (50 yaş üzeri, s = 6) donörlerden elde edilmiştir.

Bu çalışmada, donör yaşının iMKH'nin; morfolojileri, proliferasyon potansiyelleri ve farklılaşma özellikleri üzerine olan etkisi araştırılmıştır. Aynı zamanda, *in vitro* kültür şartlarında pasajlanarak çoğlatılmalarının bu hücrelerin karakteristik özellikleri ve nöral trans-farklılaşma potansiyelleri üzerine etkisi değerlendirilmiştir.

Farklı yaş gruplarındaki tüm hastalardan izole edilen iMKH'nin; hücresel morfolojileri, çoğalmaları ve farklılaşma potansiyellerinde değişiklikler gözlenmiştir. Çocuk donörlerden izole edilen hücreler fibroblastoid-çubuk şeklindeki hücresel morfolojilerini *in vitro* koşullarda daha uzun süre korumuş; yetişkin ve yaşlı donörlerden izole edilen hücrelerle kıyaslandığında daha fazla çoğalma eğilimi göstermişlerdir. iMKH'nin adipojenik, osteojenik ve nörojenik farklılaşma kapasiteleri yaşla beraber düşmüş fakat kondrojenik farklılaşma kapasiteleri değişmemiştir.

iMKH’nin kültür ortamında pasajlanarak çoğaltılmasının bu hücrelerin; hücresel morfolojileri ve farklılaşma yeteneklerini etkilediği gözlenmiştir. Artan pasaj sayısıyla beraber hücrelerin proliferasyon oranları düşmüş ve hücreler fibroblastoid-çubuk şeklindeki morfolojilerini kaybetmişlerdir. Kültür ortamında pasajlayarak çoğaltmanın hücrelerin nöral trans-farklılaşma potansiyeli üzerine etkisini araştırmak için RT-PZR kullanılarak farklılaşmamış iMKH’nin nöral tanımlayıcılardan NSE ve β III tubulin gen ifade seviyeleri araştırılmıştır.

iMKH’nin kültür ortamında, uygun uyarıcılar olmadan, nöral dokuya özel tanımlayıcıları ifade ettikleri ve her donörün kendine özgün bir ifade modelinin olduğu gözlenmiştir. Çocuk donörlerden izole edilen MKH’nin aksine, yetişkin donörlere ait iMKH’nin, *in vitro* kültür koşullarında çoğlatılmaları sonucu; artan pasaj sayısına bağlı olarak nöral tanımlayıcı ifade düzeylerinin de arttığı sonucuna varılmıştır.

Klinik çalışmalar öncesi, yüksek derecede standardize edilmiş *in vitro* kültür koşullarında bile, donör yaşının ve hücrelerin uzun süre pasajlanmasının iMKH’nin özelliklerinde farklılıklara yol açtığı göz önünde bulundurulmalıdır.

Anahtar Kelimeler: insan kemik iliği kökenli mezenkimal kök hücreleri, farklılaşma, yaş, nöral trans-farklılaşma, uzun süreli pasaj

Dedicated to my parents Servet ZAİM and Bislim ZAİM

ACKNOWLEDGEMENT

In the first place I would like to thank to my research advisor Assist. Prof.Dr. Sevim IŞIK, for her supervision, advice, and guidance from the very early stage of this research as well as giving me extraordinary experiences through out the work. I also would like to thank to all of my instructors in Fatih University.

I express my thanks to Dr. Güven ÇETİN from İstanbul Hospital, Department of Hematology and Dr. Serap KARAMAN from Şişli Etfal Hospital, Department of Hematology for bone marrows they supplied.

I would like to thank my lab-mates, Gülsüm ARIKAN, Tuba KUNDURACI, Nihal KARAKAŞ, Aysel KARAGÖZ, Hakan ATAK and also to my colleagues for their support and friendship during master education.

My deepest gratitude goes to my family for their unflagging love and support throughout my life; this thesis is simply impossible without them.

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SYMBOL	ABBREVIATION
MSC	Mesenchymal stem cell
hMSC	Human mesenchymal stem cell
BM	Bone marrow
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal Bovine Serum
CO_2	Carbon dioxide
CFU-F	Colony Forming Unit-Fibroblast
FACS	Fluorescence activated cell sorting
PD	Population doubling
CD	Cluster of differentiation
ICAM	Intracellular cell adhesion molecule
VCAM	Vascular cell adhesion molecule
TNF	Tumor necrosis factor
IL	Interleukin
LIF	Leukemia inhibitory factor
FGF	Fibroblast growth factor
EGF	Epithelial growth factor
HGF	Hepatocyte growth factor
VLA	Very late antigen
JAM	Junctional adhesion molecules
PECAM	Platelet-endothelial cell adhesion molecule
MHC	Major histocompatibility complex
NO	Nitric oxide
DC	Dentritic cell
NK	Natural killer
PDGF	Platelet derived growth factor

BMP	Bone morphogenic protein
LFA	Lymphocyte function-associated antigen
IBMX	3-Isobutyl-1-methylxanthine
RT-PCR	Reverse transcriptase polymerase chain reaction
TGF-β	Transforming growth factor beta
IGF	Insulin-like growth factor
bFGF	Basic fibroblast growth factor
EGF	Epidermal growth factor
PDGF	Platelet-derived growth factor
LRP	Low density lipoprotein receptor related peptide
MAP-K	Mitogen activated protein kinase
PPAR γ	Peroxisome proliferation-activated receptor – γ
LPL	Lipoprotein lipase
DMSO	Dimethylsulfoxide
BDNF	Brain-derived neurotrophic factor
NeuN	Neuronal nuclear protein
GFAP	Glial fibrillary protein
BME, β-ME	β-mercaptoethanol
NSE	Neuron specific enolase
NF	Neurofilament
Topo II	Topoisomerase II
Topo IIα	Topoisomerase IIα
Topo IIβ	Topoisomerase IIβ
cDNA	Complementary DNA

CHAPTER 1

INTRODUCTION

1.1 MESENCHYMAL STEM CELLS

1.1.1 Discovery of Mesenchymal Stem Cells

The existance of nonhematopoietic stem cells in bone marrow was first suggested 130 years ago, by the observations of the german pathologist Julius Cohnheim [1]. Cohnheim, based on his observations, suggested that most of the cells contributing to wound repair are derived from the circulating system and that some of these cells had a fibroblast-like morphology.

More than a century later, Alexander Friedenstein reported evidence that bone marrow contains fibroblast-like cells that can differentiate into mesenchymal cells and these cells can be isolated from bone marrow via their inherent adherence to plastic in culture [2]. Friedenstein and his colleagues, placed whole bone marrow in plastic culture dishes and after 4 hours they discarded hematopoietic cells by removing non-adherent cells. They reported that the remaining cells were still heterogeneous in morphology, but the most tightly adherent cells were spindle shaped, which remained inactive for 2– 4 days and then began to multiply rapidly. After several passages, the adherent cells became more homogeneously fibroblastic in appearance. They also found that these cells were able to differentiate into osteocytes and chondrocytes to form bone and cartilage.

With regard to these findings, Maureen Owen suggested the presence of ‘stromal’ stem cells, with the ability to make identical copies of themselves and to

generate mature ‘stromal’ cells [4]. The stroma cells produce extracellular matrix proteins and soluble substances in the BM to provide hematopoiesis and to complete the hematopoietic stem cell niche in its totality [4].

In the 1980s further work made clear that the cells isolated by Friedenstein’s method were multipotential and could differentiate into chondrocytes, osteocytes, adipocytes, and even myoblasts, both *in vivo* and *in vitro*.

Based on Friedenstein’s studies, Caplan defined the mesengenic process of cellular differentiation from immature cells to multiple mature cell types of the mesodermal lineages (e.g. adipocytes, chondrocytes, osteocytes). In this way, to describe these cells with stem cell-like feature, Caplan introduced the term ‘mesenchymal stem cell’ (MSC) [4]. Mesenchyme originally describes the embryonic loose connective tissue that is derived from the mesoderm and that develops into hematopoietic and connective tissue [4].

MSCs were not called as mesenchymal stem cells from the beginning. They were originally defined as colony forming unit fibroblasts (CFU-Fs) by Friedenstein. They have been denoted by many different names in past literature from that time. Here are the names used for these cells until so far [9];

- Fibroblast colony-forming cells
- Colony-forming unit-fibroblasts (CFU-Fs)
- Mesenchymal progenitor cells
- Marrow stromal cells
- Marrow stromal fibroblasts
- Stromal stem cells (by Maureen Owen)
- Multipotent mesenchymal stromal cells
- Mesenchymal stem cells (first used by Caplan in 1990s)

1.1.2 Minimal Criteria to Define Human MSCs

The therapeutic potential of human mesenchymal stromal cells (hMSC) has raisen biologic and clinical interest in MSC over years. Unfortunately, researchers report studies of MSCs using different characterization, isolation and expansion methods, which causes difficulties to compare and contrast the study outcomes. Therefore, progress in the MSC field is frustrated. To eliminate this issue, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy recommends minimal criteria to define hMSCs. Briefly; the criteria to identify MSCs:

1. Plastic adherence of cells in standard culture conditions

2. Surface antigen expression:

Phenotype	
Positive (>95%/)	Negative (<2%)
CD73	CD34
CD90	CD45
CD105	CD14 or CD11b
	CD79a or CD19
	HLA-DR

3. Multilineage differentiation potential into osteocytes, adipocytes, chondrocytes *in vitro* and *in vivo* [16].

First, MSCs must adhere to tissue culture plastic when maintained in standard culture conditions. The expression of CD73, CD90 and CD105 must be greater than 95%. In addition, MSCs must lack expression (<2% positive) of hematopoietic markers such as CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II, as measured by flow cytometry. Lastly, the biological character that most uniquely identifies MSCs is their ability to differentiate into osteocytes, adipocytes and chondrocytes. Therefore, cells must be shown to differentiate into mesodermal lineages under standard *in vitro*

differentiating conditions. Trilineage mesenchymal differentiaton can be demonstrated by histochemical staining techniques.

1.1.3 Source of MSCs

The bone marrow (BM) is by far the best studied and accesible source of MSCs and almost all that is known about these cells is based on studies about BM derived MSCs. Apart from BM, MSCs have been shown to be present in a number of other foetal and adult tissues including circulating blood [12, 15], amniotic fluid [11, 15], pericytes [12, 13], trabecular bone [12]. These are only some examples in order to show diversity of MSC-containing tissues. MSC niche is not restricted to just BM. These findings show that MSCs are diversely distributed *in vivo*, and as a result may occupy a ubiquitous stem cell niche.

Table 1.1 MSCs derived from different sources

Tissue	**References**
circulating blood	[5, 12, 15]
cord blood	[5, 11, 13, 15, 27, 40, 61]
placenta	[13, 15]
amniotic fluid	[11, 15, 68]
heart	[15]
skeletal muscle	[12-15, 27, 68]
adipose tissue	[5, 12-15, 40, 61, 68]
synovial tissue	[11-14]
periosteum	[12,13]
dermis	[12,13]
dental pulp	[8, 61, 62, 68]
pericytes	[12, 13]
liver	[13, 27, 61, 68]
spleen	[5, 13]
thymus	[5, 13]

1.1.4 Isolation and Growth of MSCs

MSCs constitute only a small percentage of the total number of cells in BM. Friedenstein first described MSC as 0.01% to 0.001% of nucleated cells of BM [17]. According to recent researches, frequency of MSCs in adult BM is reported to be $1x10^5$ – $1x10^6$ nucleated cells, as estimated by using colony-forming unit fibroblast (CFU-F) assay [18]. The number of MSCs isolated from BM may vary, even when the cells are obtained from the same donor. Their number level is even lower in cord blood and peripheral blood [17]. MSCs have been isolated from several species and tissues, but the most well characterized and probably the purest preparation is from BM [19].

MSCs are generally isolated from BM aspirate harvested from the superior iliac crest of the pelvis. MSCs have also been isolated from the tibial and femoral marrow compartments in humans. In larger animals marrow is often obtained from the same site, and in rodents it is generally harvested from the mid-diaphysis of the tibia or femur [26].

There are different methods to isolate MSCs from BM ;

- Placing whole BM aspirate in plastic culture dish & removing non-adherent hematopoietic cells by discarding the medium.
- Seperating mononuclear cells form BM using density gradient centrifugation and seeding mononuclear cells. Non-adherent cells are removed by changing the medium.
- Sorting cells in BM via florescence activated cell sorter (FACS) for MSC markers.
- Sorting cells using magnetic beads.

Ficoll density gradient is generally used in order to isolate MSCs from BM aspirates. After isolation, cells are cultured in a medium such as Dulbecco's modified Eagle's medium (DMEM), with %10 fetal bovine serum (FBS) and allowed to adhere to plastic dishes for 48 hours; then, nonadherent cells are removed by discarding the medium and the remaining cells allowed to grow for 14 days. Cultures are maintained at 37^oC in a humidified atmosphere containing %5 CO_2. Confluent cells are trypsinized and allowed to expand for several passages [18].

In culture conditions, MSCs can be seen using light or contrast microscoby in their undifferentiated state, as an adherent monolayer of cells. MSC cultures are heterogeneous in their morphology even after isolation. Culture contains rapidly sel-renewing cells that have fibroblastic appearance, plus more mature cells which became larger. Self-renewing cells stay in high numbers for several passages, whereas more mature cells predominate in later passages. These cells cease proliferation at approximately the Hayflick limit of 50 population doublings [11].

Especially in long term passages, it is critical to optimize culture conditions. Before all else, fetal bovine serum is an important constant which directly effects the viability and number of MSCs. Apart from the quality of fetal bovine serum, other culture parameters, including culture medium, glucose concentration, stable glutamine, BM mononuclear cell plating density, MSC passaging frequency, and also plastic surface quality affect the final outcome [20].

Growth of MSCs *in vitro* is characterized by the occurrence of three phases, similarly to other progenitor cells :

i. **Lag phase:** An opening lag phase, which lasts for 3-4 days. Rapid cell growth occurs during which less than 50% of cells in culture complete their life span [21].
ii. **Log phase:** A rapid expansion phase. MSC doubling time is reported to be 33 hours [18].
iii. **Stationary phase:** Cells cease to divide [21].

MSCs are not limitless in their life span in culture. Depending on donor variability (such as age) and culture conditions they can be expanded *in vitro* for an average of 50 population doublings (PD) [11, 21]. The initial lag phase is characterized by a rapid cell growth during which less than 50% of cells in culture complete their life span. This rapid cell growth phase is followed by a phase of reduced growth rate during which about 50-80% of cells complete their life span. The last stage the growth arrest phase comes at which life span of more than 80% of the cells is completed [21]. Prockop et al., suggests that the shift between different stages is regulated maily by the expression of Dickkopf-1 (Dkk-1) and Wnt5a genes, which play opposite roles. The

greatest expression of Dkk-1 appears during the log phase and shortens the former stage by inhibition of Wnt5a expression, whereas Wnt5a protein level becomes maximal during the stationary phase [21].

Examination of the cell cycle profile of MSCs revealed that about 10% of these cells occurs in phases S, G2 and M of the cell cycle, while vast majority of the cells remain in the G0/G1 phase [21].

1.1.5 Immunophenotype of MSCs

MSC population is heterogenous, in terms of their proliferation and differentiation capacity and expression of cell surface antigens [21]. There are a number of reasons for this. First, by far the pioneering work of Friedenstein et al. , it has been demonstrated that all CFU-Fs were not highly proliferative and multipotential [24]. Second, numerous research groups have used a limited number of various cell surface antigens to identify *in vivo* MSCs/CFU-Fs. Taking a synthesis of these informations has led to the impression that MSCs were phenotypically and functionally heterogeneous [24].

Characterization of different cell types are usually carried out by use of cell surface antigenic profile. Expression of specific cell surface proteins is used as a sign of being a specific type of cell. These surface proteins are mainly serving as growth factor receptors, cytokines and extracellular matrices.

Although there are many studies to define MSCs in terms of specific cell surface markers, up to now, no single specific marker has been identified.

Because of the absence of a single specific marker, immuno-phenotyping of these cells is done by analysis of a combination of different markers determined to be negative or positive for these cells by different researchers. This makes identification complicated as there is a highly variable profile of markers suggested for MSCs.

Table 1.2 Cell surface markers expressed on BM derived hMSCs.

Molecule	CD (Cluster of Differentiation)	Expression on MSCs	References
CD3 complex	3	-	[26]
Integrin αL chain	11a	-	[14]
Integrin αM chain	11b	-	[14, 29]
Aminopeptidase N	13	+	[14, 30]
LPS receptor	14	-	[14, 26]
Integrin β1 chain	29	+	[14, 26]
PECAM	31	-	[26]
Hyaluronate receptor	44	+	[14, 26]
Leukocyte common antigen	45	-	[14, 26]
Integrin α chain (1, 2, 3)	49a,b,c	+	[14, 26]
ICAM-3	50	+	[26]
ICAM-1	54	+	[14, 26]
NCAM	56	+	[26]
LFA-3	58	+	[14, 26]
Integrin β3 chain	61	+	[14, 26]
E-selectin	62E	-	[26]
L-selectin	62L	+	[14, 26]
P-selection	62P	-	[26]
Transferrin receptor	71	+	[14, 26]
Ecto-5'-nucleotidase	73	+	[14, 26]
Thy-1	90	+	[14, 26]
ICAM-2	102	+	[14, 26]
Integrin β4 chain	104	+	[14, 26]
Endoglin:TGF- βR III	105	+	[14, 26]
VCAM-1	106	+/-	[14, 26]
TNF IR	120a	+	[14, 26]
TNF IIR	120b	+	[14, 26]
IL-1R (a and B)	121a,b	+	[14, 26]
IL-3Ra	123	+	[14, 26]
IL-4R	124	+	[14, 26]
IL-6R	126	+	[14, 26]
IL-7R	127	+	[14, 26]
Catherin 5	144	-	[26]

Another important issue is the stability of MSC markers in culture. Interestingly, some cell surface markers are highly expressed on freshly isolated MSCs, but their expression may disappear after a short period of cultivation [21]. Such a phenomenon

was observed in case of CD34 and CD 271 antigens [21, 24]. More likely, these markers present on MSCs *in vivo* may be induced by the BM microenvironment or be reflective of a function *in vivo* that is lost upon plastic adherence and exposure to culture media.

Although the loss of some surface markers following cultivation, MSC cultures remain multipotential, showing that these markers are unlikely to be reflective of the MSC's true 'stem cell' nature or its multipotentiality [24]. It seems that the heterogeneity in the MSC proliferative and differentiation capacities, cannot be explained on the basis of known surface antigens alone [24].

1.1.6 Self-Renewal Potential of MSCs

One of the major characteristics of stem cells is their self-renewal potential, the ability to make identical copies of themselves through mitotic division over extended time periods (even the entire lifetime of an organism). BM derived hMSCs have been shown to have a significant but highly variable self-renewal potential during *in vitro* serial reproduction [14].

Self renewal capacity is a defining property of all stem cells. It would thus be necessary to identify extracellular signalling factors, including growth factors and cytokines those not only stimulate proliferation but also retain self renewal capacity of MSC.

Some of the prominent growth factors and cytokines, which have been involved in MSC 'stemness' maintenance are leukemia inhibitory factor (LIF) [38], fibroblast growth factors (FGFs) [40], and mammalian homologues of *Drosophila* wingless (Wnts) [41, 42]. A pleiotropic cytokine, LIF, maintains the stem state of MSCs and other stem cells [38]. In addition, LIF activates and represses osteoblast and osteoclast activities. Mechanisms of LIF action in MSC self-renewal may involve paracrine crosstalk with neighboring cells, but the whole process is still unknown [33].

Several studies indicate that members of FGF-family, especially FGF-2, play an essential role to retain stem state of MSCs from a variety of species by prolonging their

viability in culture [33]. In fact, higher population doublings (i.e. >50 PDs) have been achieved as a consequence of the addition of (FGF-2), to the culture medium [14].

Wnts may also regulate MSC maintenance [42], as they do in the self-renewal of various stem cells such as hematopoietic, neural and skin stem cells [41]. Treatment of MSCs with Wnt3a is known to increase proliferation [42].

In addition, other growth factors and cytokines such as epidermal growth factor (EGF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), have a role to promote and/or maintain MSC self-renewal *in vitro* [34].

1.1.7 MSC Niche

In analyzing the biological characteristics of stem cells, it is critical to consider the influence of their tissue of origin. Depending on the source of the MSC population, differences were observed in regard to differentiation potential [10, 69, 75]. The BM microenvironment is a major site of MSC niche in the body, in which a complex cellular and noncellular interaction occurs [8]. MSCs are also isolated from a variety of foetal and adult tissues raising the question of what the common *in vivo* microenvironment of the MSC might be. Is there a common MSC niche or do MSCs function in a manner that is independent of their environment?

In 1978, Schofield first introduced the concept of a stem cell ‘niche’ [55], which includes all of the elements surrounding the stem cells when they are in their naive state, containing the non-stem cells as well as extra cellular matrix and soluble molecules found in that locale.

All of these act together to maintain the stem cells in their undifferentiated state. Specifically, endothelial cells, adipocytes, macrophages, reticular cells, fibroblasts, HSCs and their progeny are the primary cellular components of the marrow stroma [35].

It is clearly described that the niche supports MSC survival and growth, by providing the requisite factors and adhesive properties to maintain their viability, It is within this dynamic and cellular microenvironment where MSCs are presumed to exist.

1.1.8 Localization and Trafficking of MSCs

The use of MSCs for cell based therapies has been particularly hailed because of their inherent ability to home to sites of inflammation following tissue injury when injected intravenously. To understand the niche, it is important to analyze not only what keeps stem cells in their niche but also what signals them to emigrate from it.

The release of MSCs from their niche into circulation is defined as mobilization [13]. Although the knowledge about the nature of signals released from the injured tissue to mobilize MSCs is very limited, cytokines and chemokines play critical roles in regulating mobilization. It has been hypothesized that cytokines and/or chemokines that are upregulated and released into circulation under injury conditions, stimulates MSCs to down-regulate the adhesion molecules that keep them in their niche.

Homing is a process by which cells migrate to and engraft in the tissue in which they will exhibit functional and protective effects. An advantage of homing feature of MSCs is that the complications associated with site-specific injection of stem cells, is avoided, and systemic intravenous delivery with the potential for multiple dosages is possible [2].

Although the precise molecular mechanisms by which MSCs are able to migrate and home into sites of injury are not yet fully understood, the complex multistep process by which leukocytes migrate to peripheral sites of inflammation has been proposed as a paradigm.

During inflammation, the recruitment of inflammatory cells requires a coordinated sequence of multistep adhesive and signaling events, including selectin-mediated rolling fo MSCs in the blood vessels, cell activation by chemokines and cytokines, activation of integrins, integrin-mediated firm adhesion onto the endothelial cell surface lining the capilleries, transendothelial migration, and finally extravasation from the blood vessels and migration through the extracellular matrix into the target injured area [2, 13, 58, 59].

It is known that chemokines, cytokines and also integrins are important factors in trafficking and homing of MSCs and; migratory direction follows a chemokine density gradient. The increase in inflammatory chemokine concentration at the site of inflammation is a key mediator of trafficking of MSCs to the site of injury. Chemokines are released after tissue damage and MSCs express several receptors for chemokines [60]. Activation by such chemokines is also an important step during trafficking of MSCs to the site of injury.

Proposed mechanism of homing and trafficking of MSCs starts with the process of rolling and binding on the endothelium between E- and P-selectin [33], which are considered as crtitical molecules involved in the rolling of MSCs and adhesion to endothelial cells. These molecules are expressed by BM endothelial cells and on endothelium in infected tissue [33]. Rolling is followed by arrest and firm adhesion, with chemokines receptors expressed on the surface of endothelium ligating to respective chemokines and activating integrins, such as very late antigen-4 (VLA-4). VLA-4/VCAM-1 axis is responsible for mediating firm adhesion of MSCs to endothelial cells [13]. Firm adhesion is followed by transendothelial migration between endothelial cells by the action of junctional adhesion molecules (JAMs), cadherins, and platelet-endothelial cell adhesion molecule-1 (PECAM-1/CD31), mediating translocation to the extracellular matrix where they adhere to the extracellular matrix through molecules such as collagen, fibronectin via α1 integrins, hyaluronic acid, and CD44 [2].

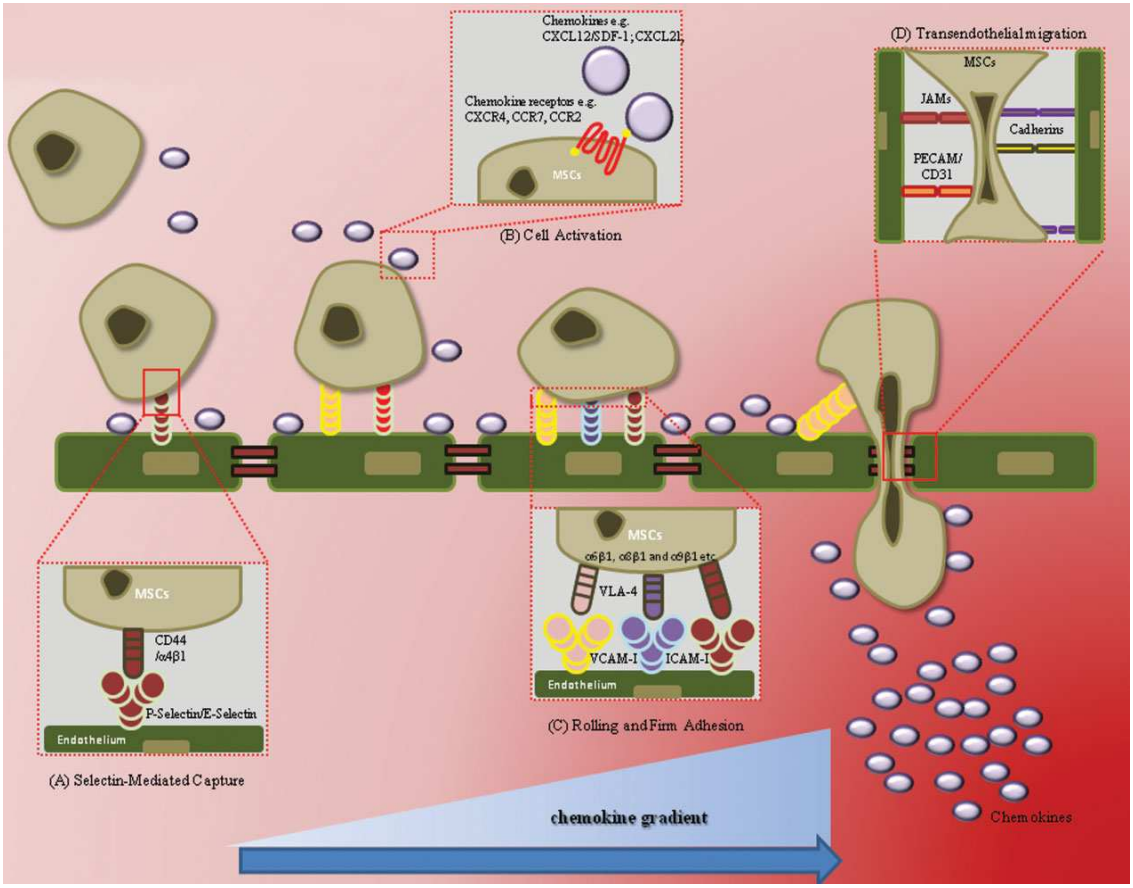

Figure 1.1 Proposed mechanisms involved in the homing and trafficking of MSCs to sites of injury after infusion [2].

1.1.9 Role of MSCs in Immunomodulation

MSCs have gained increasing interest in their use of transplantation medicine. The regenerative ability of MSCs through their potential plasticity was seen as the driving force behind interest in MSCs, however their role in modulating the immune system is now attracting greater interest.

The immunological characteristic of MSCs (generally defined as MHC I^+, MHC II^-, $CD40^-$, $CD80^-$, $CD86^-$) is regarded as nonimmunogenic and, therefore, transplantation into an allogeneic host may not require immunosuppression [1].

Numerous studies have shown that MSCs modulate the function of T cells including cell activation [6]. T cells (T lymphocytes) are a major executor of the

adaptive immune response. MSCs lack expression of MHC class II and most of the classical costimulatory molecules such as CD80, CD86, or CD40. MHC Class I may activate T cells, but with the lack of expression of T cell costimulatory molecules a secondary signal would not engage, thus T-cell activity may result in anergy (immune unresponsiveness) that may contribute to the observed immune tolerance [1, 2].

Many groups have reported that MSCs also have immunosupressive properties. Some reports have demonstrated that production of soluble factors cause suppressor activity whereas others have shown that direct cell-cell contact is required for suppression [1]. Release of soluble factors such as nitric oxide (NO) and interleukin (IL) by MSCs, has been implicated as a potential mechanism by which MSCs inhibit T-cell proliferation [39].

MSCs also appear to reduce T-cell activation through indirect mechanisms. Dentritic cells (DCs) have an important role in antigen presentation to naive T-cells immediately after maturation from monocytes. MSCs inhibite the maturation of monocytes into DCs [6, 43, 52].

NK cells are key effector molecules of innate immunity. The interaction between MSCs and natural killer (NK) cells may contribute to the immunomodulatory effects of MSCs [6]. Sotiropolou et al., referred that cell-cell contact between MSC-NK cells and release of some soluble factors by MSCs, together, cause supressed proliferation of NK cells [55].

The mechanisms by which MSCs exhibit their function on immune cells are pleiotropic and redundant, and it is clear that our understanding is far from complete.

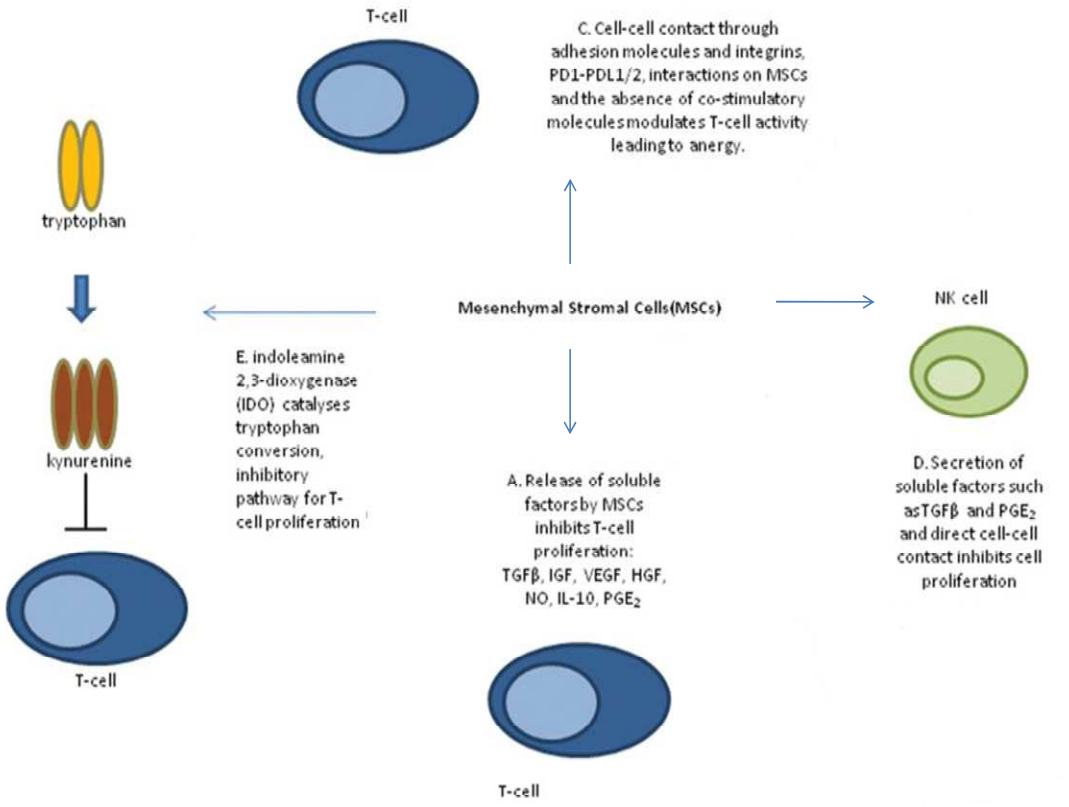

Figure 1.2 Possible effects of MSCs on immune cells [2] (modified).

1.1.10 Clinical Applications of MSC-Based Therapy

MSCs have shown great promise in cell and gene therapy applications, because of their multipotential differentiation ability and self-renewal capacity. These cells have high expansion potential and also, can be easily collected and shipped from the laboratory to the bedside and are compatible with different delivery methods. In addition, MSCs have other extraordinary characteristics: they can migrate to sites of injury and have strong immunosuppressive properties that can be used for successful autologous as well as heterologous transplantations [45].

According to animal transplantation studies, MSCs expanded *ex vivo* were able to differentiate into cells of the tissue where they reside in, repair the damaged tissue due to trauma, and partially restore its normal function [14].

Apart from these features, MSCs not only regenerate tissues of mesenchymal lineages, but also differentiate into cells derived from other embryonic layers, including neurons [48] and epithelia in skin, lung, liver, intestine, kidney, and spleen [50].

There is considerable interest in combining gene therapy with stem cell therapy, which offers the prospect of molecular engineering of stem cells. MSCs are shown to be an ideal carrier to deliver genes into the tissues of interest for gene therapy applications, because neither autologous nor allogeneic MSCs induce any immunoreactivity in the host upon systemic administration or local transplantation [47, 50].

Various studies have been examined to introduce exogenous DNA into MSCs. Viral transduction, particularly using adenovirus mediated gene transfer, can generate stable cell clones with high transfection efficiency and low cell mortality, thus making rendering it a popular option in gene therapy. However, alternative non-viral gene delivery approaches should be investigated because of the safety concerns associated with viral transduction. Traditional transfection methods, like lipofection and electroporation, have shown little success, usually resulting in less than 1% transfection efficiency and high cell death [53]; therefore, these methods are not convenient for producing adequate transfected cells for gene delivery and transplantation.

Despite their tremendous potential, one of the major disadvantage in the use of MSCs has been their limited numbers. Many clinical applications, such as regeneration of large segmental bone defects, need significant cell numbers to achieve a successful result [53]. The number of MSCs obtained from the primary tissue source is inadequate for such clinical applications. The low frequency of MSCs necessitates their *in vitro* expansion prior to clinical use. MSCs, which lack telomerase activity [54], show defined *ex vivo* proliferation capability. They reach senescence and lose their multilineage differentiation potential after 40-50 population doublings *in vitro*. Thus, it is essential and critical to develop new strategies to maintain proliferation capacity of MSCs without loosing their multipotentiality.

The web site of the United States sponsored by the National Institutes of Health (www.clinicaltrials.gov) provides information about the current clinical trials based on the use of MSCs.

Table 1.3 Current clinical studies using MSCs

Study	Condition	Cell type	Site of injection	Status	Sponsor
Mesenchymal stem cells in multiple sclerosis	Multiple sclerosis	BM-derived autologous MSC	Intravenous	Active, not yet recruiting	University of Cambridge
Mesenchymal stem cells and subclinical rejection	Organ transplantation	BM-derived allogenic MSC	Intravenous	Not yer recruiting	Leiden University Medical Center
Autologous implantation of mesenchymal stem cells for the treatment of distal tibial fractures	Tibial fracture	BM-derived autologous MSC	Local implantation	Active	Hadassah Medical Organization
Cord blood expansion on mesenchymal stem cells	Myelodysplastic syndrome; leukemia	CB-derived allogenic MSC	Intravenous	Recruiting	M.D. Anderson Cancer Center
Mesenchymal stem cell transplantation in the treatment of chronic allograft nephropathy	Kidney transplant, chronic allograft nephropathy	BM-derived MSC	Intravenous	Not yet recruiting	Fuzhou General Hospital
Autologous mesenchymal stromal cell therapy in heart failure	Congestive heart failure	BM- derived autologous MSC	Intramyocardial injection	Not yet recruiting	Rigshospitalet University Hospital
Marrow mesenchymal cell therapy for osteogenesis imparfecta	Osteogenesis imperfecta	BM- derived allogeneic MSC	Intravenous	Completed	St. Jude Children's Research Hospital

Table 1.3 shows some of the ongoing studies based on MSCs. At october 2010, there are 137 clinical trials currently exploring the application of MSCs.

Looking at ongoing clinical trails, it is too early to tell whether all therapies based on stem cells will prove to be clinically effective.

1.2 Differentiation Potential of MSCs

1.2.1 Multilineage Mesodermal Differentiation of MSCs

In their niches, stem cells remain as undifferentiated cells that do not show mature tissue-specific cell characteristics until a specific stimulus cause them to produce progenitor cells that then differentiate into specific mature cells that are needed.

The multilineage differentiation potential of MSC populations has been broadly studied *in vitro* since 1960s. Up to now, studies demonstrate that human BM derived MSCs have the ability to differentiate into osteogenic, chondrogenic and adipogenic lineages, when placed in appropriate *in vitro* or *in vivo* conditions [11]. *In vitro* differentiation into a specific lineage requires incubating cells with a proper mixture of specific differentiation factors. Basal nutrients, growth factors, cell density, spatial organization, mechanical forces and cytokines, all play a role in MSC differentiation process. To obtain efficient outcome, each factor should be optimized.

1.2.1.1 Molecular Regulation of Osteogenesis

Differentiation of MSCs into osteocytes is a highly programmed process, best illustrated *in vitro* [12]. In order to obtain osteogenic differentiation, MSCs should be incubated with a mixture containing dexamethasone, β-glycerophosphate and ascorbic acid, throughout the period of 3-4 weeks [21].

Treatment of MSCs with dexamethasone, a synthetic glucocorticoid, stimulates cell proliferation and supports osteogenic lineage differentiation. Organic phosphates also support osteogenesis. β-glycerophosphate is essential for mineralization and modulation of osteoblast activities. Other commonly used supplements are ascorbic acid

and 1,25-dihydroxyvitamin D3, which are involved in increasing alkaline phosphatase activity in osteogenic cultures and promoting the production of osteocalcin [12].

In addition, members of the bone morphogenetic protein (BMP) family of growth factors are also used for osteoinduction. Among BMP-2, 4, 6, and 7, BMP-6 is the most consistent and potent regulator of osteoblast differentiation and, of these BMPs, only BMP-6 gene expression is detected prior to hMSC osteoblast differentiation [78]. Commonly BMP-6 is used with BMP-2 to support osteogenic differentiation. BMP-2 increases bone nodule formation and the calcium amount of osteogenic cultures *in vitro*. Basic fibroblast growth factor (b-FGF) also plays a role in osteoinduction. Using BMPs and b-FGF together, increased osteogenesis were observed both *in vivo* and *in vitro* [12]. Other important factors involved in osteogenic regulation are: insulin-like growth factor (IGF), brain-derived growth factor (BDGF), FGF-2, leptin and parathyroid hormone related peptide (PTHrP). These proteins organize secretion of matrix proteins and the expression of signals necessary for bone remodeling through osteoclast activation [21].

There are a number of signaling pathways involved in MSC osteogenesis. Wnts are known as signaling proteins, which have been implicated in numerous differentiation programs, including osteogenesis. Low-density lipoprotein receptor-related peptide 5 (LRP-5) is an estanblished Wnt coreceptor. In mice, LRP-5 mediates Wnt signaling via the canonical pathway. In humans, LRP-5 has been related to osteoporosis–pseudoglioma syndrome [12]. Patients with this syndrome are prone to fracture and bone deformation because of low bone mass, and have an overall decrease in trabecular bone volume. It has been demonstrated that trabecular bone is a source of MSCs [35]. MSCs may be affected in this disease, thereby leading to alterations in bone formation and remodeling. It has also been shown that mice with disruptions of LRP-5 expression have a decreased level of osteoblast proliferation and display a phenotype similar to humans with osteoporosis–pseudoglioma syndrome [35].

Knockout and dosage compensation in Wnt-pathway-related transgenic animals provide the strongest proof that high levels of endogenous Wnts promote osteogenesis, whereas low levels inhibit osteogenesis [33].

Osteogenic differentaition might be measured through calcium accumulation and alkaline phosphatase activity. The MSCs generate aggregates or nodules and the expression of alkaline phosphatase increases; calcium accumulation can be seen over time. These bone nodules stain positively by Toluidine Blue, Alizarin Red and Von Kossa techniques [1].

1.2.1.2 Molecular Regulation of Chondrogenesis

Chondrogenic differentiation of MSCs *in vitro* mimics cartilage development *in vivo* [33]. The induction of chondrogenesis in MSCs depends on many factors, including parameters such as cell density, cell adhesion, and growth factors. For example, chondrogenic induction of MSCs require high-density pelleting and serum-free medium containing specific growth factors and supplements.

The TGF-β superfamily of proteins and their members, such as the bone morphogenetic proteins (BMPs), are well-established regulatory factors in chondrogenesis [7]. Although, TGF-β3 has recently been shown to induce rapid and proper expression of chondrogenic markers, TGF-β1 was initially used to induce chondrogenesis *in vitro*. BMPs, such as BMP-6, increases the amount of matrix proteoglycan size and weight of pellet cultures. BMP-2 and BMP-9, which can induce markers of chondrogenesis, have been used in three-dimensional MSC culture systems [12].

Wnt and Wnt-related family of signaling proteins have a role in chondrogenesis and adult cartilage homeostasis during development [12]. Wnt-4 and Wnt-14 were shown to display high expression at sites of future joint development [21].

Other signaling pathways involved in crosstalk with TGF-β include the mitogen-activated protein kinase (MAPK) pathways. [12].

In hMSCs, permanent expression of Wnt7a is chondroinhibitory, while transient upregulation of Wnt7a enhances chondrogenesis through various TGF-β–MAPK signaling pathways [33].

As recent data indicate, the signaling triggered by the FGF receptor 3 is sufficient to induce chondrogenic differentiation. TGF-β and related cytokines are able to induce signal transduction pathways specific for chondrogenesis, mostly via activation of MAPKs such as: ERK-1, p38, PKC and Jun, whereas FGF receptor acts through Smad protein signaling [48]. The activation leads to induction of specific transcription factors Sox9, Msx2 expression [7]. They were shown to activate the expression of chondrocyte-specific genes, like aggrecan and collagen II [21]. Further investigations should focus on the crosstalk between pathways, such as those of TGF-βs and Wnts [33].

After 2-3 weeks remaining in differentiation culture, chondrogenic formation, except from multilayered, matrix rich morphology, may be confirmed by histological staining for the presence of proteoglycan. Cell pellets show strong staining with Alcian Blue. Differentiated cells also produce collagen type II, which are typical of articular cartilage.

1.2.1.3 Molecular Regulation of Adipogenesis

To induce adipogenic differentiation, MSC cultures are treated with indomethacin, dexamethasone, insulin and isobutyl methyl xanthine. There is an accumulation of lipid rich vacuoles within differentiated cells, and they express peroxisome proliferation-activated receptor - γ (PPAR γ), lipoprotein lipase (LPL), and the fatty acid-binding protein aP2 [1]. A nonsteroidal anti-imflamoatory drug, Indomethacin, binds to and activates the transcription factor PPARγ, which is significant for adipogenesis [12].

The nuclear hormone receptor, PPARγ, is a critical adipogenic coordinator promoting MSC adipogenesis while repressing osteogenesis. The binding of PPARγ to long chain fatty acids and thiazolidinedione compounds, triggers the transactivation and transrepression of PPARγ [33].

Wnt signaling proteins are also involved in adipogenic differentiation pathways. During the adipogenesis, Wnt signaling, possibly through Wnt-10b expression by pre-adipocytes, is known to decrease adipogenic differentiation of MSCs *in vitro.*

It is thought that endogenous, canonical Wnt signaling maintains pre-adipocytes in an undifferentiated state by inhibiting C/EBP-α and PPAR- γ. When Wnt signaling is suppressed in pre-adipocytes, they proceed down the adipogenic lineage [12].

Lipid vacuoles in adipocytes are observed following staining with Oil Red O solution after 2-3 weeks. These adipocytes remain healthy in culture for at least 3 months [18]. The differentiation also might be confirmed controlling the expression of specific proteins such as PPAR- γ2, LPL and the fatty acid binding protein aP2 [21].

MSCs are heterogenous with respect to their multilineage differentiation potential. Although various studies show them to be multipotent, with a mesodermal differentiation potential, in clonal assays it could be observed that only one third of these MSCs are multipotent [4]. Thus, most of them have bi- or only uni-lineage differentiation ability. It is hypothesized that heterogeneous MSC population contains a minority of immature cells with tri-lineage multipotency. Remaining ones have lost their tri-lineage differentiation potential while passing through various stages of maturation, ending with an only unipotent differentiation capacity [4].

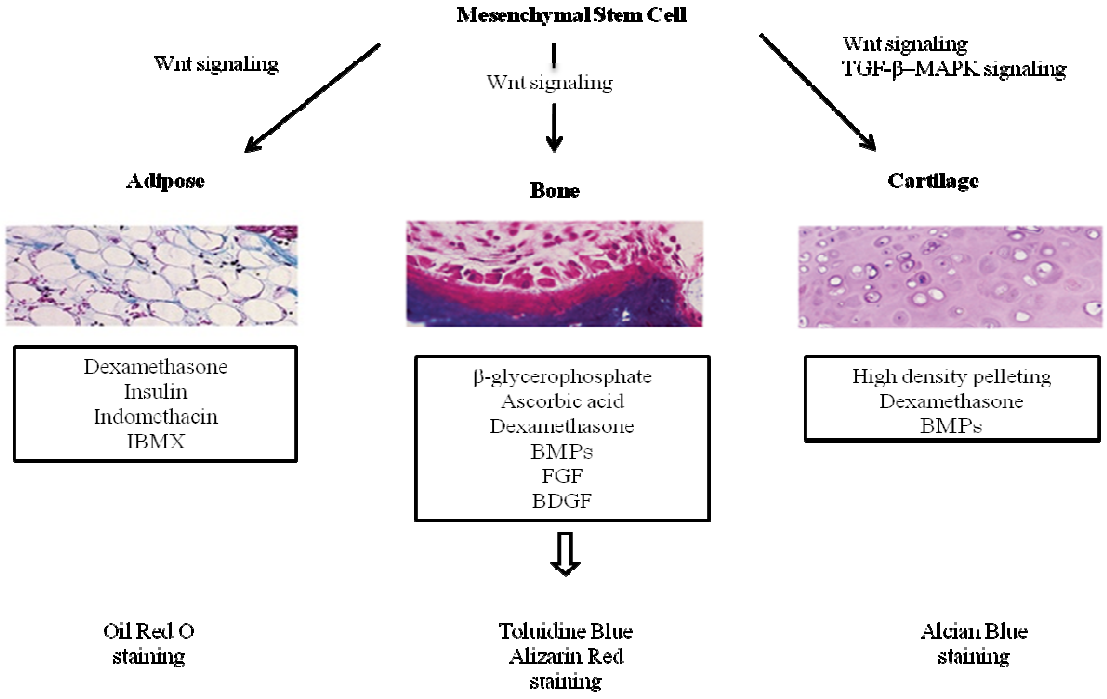

Figure 1.3 MSC differentiation into the three mesenchymal lineages.

1.2.2 Transdifferentiation of MSCs Into Neurons

In earlier studies, it was believed that adult stem cell plasticity was restricted to the tissue in which they reside and embryonic germ layer boundaries cannot be passed. Recently, according to large-scale studies on MSC biology, this dogma has been changed. It has been reported that adult stem cells can be converted into the cells of other germ layers under specific conditions which is defined as 'transdifferentiation' [83]. Studies indicate that, under appropriate *in vitro* conditions, MSCs can cross germ layers and transdifferentiate into tenocytes, myocytes and even neurons [1, 27].

Neural transdifferentiation of MSCs *in vitro* started to attract attention in 2000 with two simultaneous studies by Sanchez-Ramos et al. [87] and Woodbury et al [64]. They reported that rat, mouse and human MSCs can be converted into cells with neuronal morphology expressing neural markers after treatment with combinations of different chemicals or growth factors. These two studies were the first ones establishing the potential of MSCs to differentiate into neural cells.

Some agents such as growth factors, cytokines, neurotrophins are known to promote neural cell induction *in vitro* [87]. Many neural induction methods are used including use of chemical inducers and neurotrophic factors so far.

Potent chemical reagents used for neural induction are 3-Isobutyl-1-Methylxanthine (IBMX), dibutyryl cyclic adenosine monophosphate (dbcAMP) and dimethylsulfoxide (DMSO) [31, 36]. These reagents provide rapid neuron-like morphology acquisition [64]. Neurotrophic growth factors are essential polypeptide hormones for the development and the maintenance of the central nervous system. Some commonly used neurotrophic growth factors are brain derived neurotrophic factor (BDNF), nerve growth factor (NGF) and neurotrophin3 (NT-3). BDNF and NT-3 take role in development and maintenance of neural populations. NGF is necessary for the development and survival of some sympathetic and sensory neurons [65].

Molecular pathways that can be effective in neural differentiation of MSCs [79];

- Protein kinase A pathway; activated by an increase in cAMP levels.
- MEK-ERK signalling.
- CaMKII activity.

1.3 Aging of MSCs

1.3.1 Defining Aging

If the definition of MSC is elusive, a definition of aging is even more intimidating. In numerous cytological studies, further challanges arise in distinguishing between aging *in vivo* and long term cultivation *in vitro* that might or might not stimulate 'true' aging ('*in vitro* aging'). According to Sames and Stolzing et al., definition of aging is 'the sum of primary restrictions in regenerative mechanisms of multicellular organisms' [71].

MSCs have a limited lifespan *in vitro* as any normal, somatic cell. After a certain number of cell division, MSC enter senescence. Senescent MSCs show abnormalities typical of the Hayflick model of cellular aging. Cell size increases, proliferation rate decays, differential potential is affected, chromosomal instabilities may arise.

Aging can be conceptually distinguished from senescence, with the latter emphasising the cellular level. Campisi et al., equates senescence with replicative senescence by defining it as ''an essentially irreversible arrest of cell division'' [72].

Cellular senescence is a complex phenotype that causes alterations in reproduction mechanism and also functions of cells. Various culture conditions, protocols and cell types give rise to different kinds of senescence. Typically, senescent cells show enlarged, flattened morphology. These cells are characterized by an irreversible G1 growth arrest including upregulation of cell cycle inhibitors such as p53/p21 and p16/RB and supression of genes that drive cell cycle progression. There are remarkable differences between senescent states induced by the p53 and p16/RB pathways; there is a recent co-decision that senescence occurs by one pathway or the

other, with p53 mediating senescence due primarily to telomere dysfunction and DNA damage and the p16/RB pathway mediating senescence due primarily to oncogenes, chromatin disruption, and various stresses [74].

MSCs are both exposed to and causes of organismal aging. MSCs are exposed to aging directly, as they experience time-related stress such as oxidative stress and indirectly, as surrounding tissue becomes silent in time, thus hindering the differentiation ability of MSCs. MSCs are causes of aging at the tissue and organ level when their age-related inability to renew progenitor cells give rise to functional impairment [66].

1.3.2 Effects of Age on MSCs

1.3.2.1 MSC Number

Although MSCs are present through the entire lifetime, their total number is inversely correlated to the age [3, 12]. Age related decline in the number of MSCs in the BM of rodents, monkeys and human have been reported [86, 90, 91, 95].

The highest number of MSCs are found in newborn baby, than it is reduced to about one-half at the age of 80 [19]. As for circulating fetal MSCs, the highest number is detected in the first trimester and declines during the second trimester to about 0.0001% and further to 0.00003% of nucleated cells in cord blood [21]. In embryos, mesenchymal tissues constitute from a relatively high local density of progenitors within a very loose, extracellular matrix. From birth to teens, many of these progenitors are differentiated to specific cell types, and the relative number of MSCs dramatically decreased [19]. From this point of view, it seems reasonable to expect regeneratively repair of tissue injury of a child below the age of 5 years due to relatively high MSC numbers; in a 25 years old, the same injury might only repair with fibrous scar tissue. To take this comparison even further, 50 years old would have fewer MSCs than 20 years olds.

Accurate amount of MSCs in the body cannot be determined, due to the lack of unique probe for MSCs. Colony forming units-fibroblasts can be measured as an

estimate. Even in this situation, variations in BM aspiration techniques, growth medium and serum level prevent determining the experimental agreement on the absolute numbers of MSCs in marrow [19]. What everybody seems to agree on is the decrease of MSCs with age.

1.3.2.2 Morphology

In the initial phase of growth, MSCs of all ages have fibroblast like morphology in culture. With continued growth, cells become larger and a gradual loss of spindle type occurs. Replicative senescence led to previously mentioned typical morphological changes: cells gain irregular and flat shape, and nuclie became more circumscribed in phase contrast microscoby. The cytoplasm began to be granular with many inclusions appearing to be cell debris increased [22].

1.3.2.3 CFU Numbers and Colony Size

There is a notable tendency of CFU numbers to decrease according to aging. In addition to decreases in total CFU numbers, there is also evidence that the average colony size decreases in aged MSCs [66].

1.3.2.4 Differentiation Potential

The multilineage differentiation ability of MSCs derived from various species seems to change with age. According to literature, there is conflicting evidence with some groups reporting no change, while a majority finds age-related decrease.

It has been demonstrated that long-term culture has an impact on differentiation potential of MSCs. Recent studies have indicated that murine and human MSCs exhibit reduced differentiation potential upon prolonged *in vitro* culture [17, 22].

1.3.2.5 Proliferation Potential

One sign of '*in vitro* aging' is a decreased replication ability. A donor age associated decline is found in replicative life span of somatic cells [22], and this was

also shown for MSCs. In contrast to embryonic stem cells that show no loss of proliferative potency, MSCs can be dublicated for approximately 30-40 population doublings [77]. Proliferation/expansion potential of hMSCs is affected by *in vitro* culture [10]. Proliferation gradually decreases in the course of long-term cultivation until the cells finally stop to proliferate **[**22**].**

1.3.2.6 Marker Phenotype

The expression of some cell surface markers, which are highly expressed on freshly isolated MSCs, may disappear after a short period of cultivation. Significant age related changes in the expression levels of some markers such as CD44, CD90, CD105 and Stro-1 were found when correlated the expression of these markers with the age range of samples used in the research [70]. These results show that the expression level of surface antigens varies according to long term passage [22].

1.3.2.7 Telomere Length

Gradual shortening of the telomeres during a cell's life continues, until the presence of critically short telomeres triggers a p53/Rb senescence pathway, which results in proliferation arrest. Because of that, a normal human cell can only divide 50 to 100 times in *in vitro* conditions [10]. hMSCs do not express telomerase and therefore telomere length decreases approximately 50-200 nucleotides per cell cycle. There is evidence that telomere shortening occurs also upon aging *in vivo* [44].

1.3.2.8 Gene Expression

Long term culture induces continuous changes in global gene expression profile [22]. Genes involved in cell cycle, DNA replication, mitosis and DNA repair are significantly down-regulated in late passages [44]; strenghting the hypothesis that celular aging is driven by an organized process rather than a random accumulation of cellular defects[56]. Also, long-term culture associated gene expression changes were related to age-associated changes in MSCs from young versus elderly donors [57]. This indicates that cellular aging might be related aging of the organism.

The underlying molecular mechanisms of aging and senescence is a complex process and the sequence of its molecular events is thus far unknown; however it evidently has consequences for cellular theraphy [91, 88].

With all of these in mind, we designed a study to evaluate the effects of donor age on both differentiation and trans-differentiation capacity of BM derived hMSCs by differentiating cells into adipogenic, chondrogenic, osteogenic and neurogenic lineages of child and adult donors. In paralel, we investigated how morphology, proliferation potential and neural trans-differentiation capacity of MSCs is affected by long term serial passage. The objective of our study was to provide a controlled analysis of two variables (donor age and long term serial passage) and possible interactions between these crucial factors in developing stem cell based therapeutics for which no consensus exists with respect to their effect in MSC differentiation.

CHAPTER 2

MATERIALS AND METHODS

2.1 ISOLATION OF hMSCs FROM BONE MARROW

BM aspirates were obtained from healthy volunteer donors (age 0-80). Child BM aspirates (age 0-18) were obtained from Şişli Etfal Hospital; adult and old BM aspirates (age 18-50 and over 50 years) were obtained from SSK Samatya Hospital. MSCs were isolated from BM by ficol density gradient centrifugation. 1 ml BM sample was diluted with 9 ml Phosphate Buffered Saline (PBS, Biochrom) in a 15 ml falcon tube. 1:10 diluted BM aspirate was added on 5 ml Ficoll (Biochrom) in 2:1 ratio very slowly in a 15 ml falcon tube. Samples were centrifuged at 800g (2500 rpm) for 25 minutes (min) at room temperature (RT). Samples were seperated into different layers; the bottom, red layer contains red blood cells; above red blood cells the colorless liquid layer contains Ficoll; white, cloudy layer located on top of Ficoll contains mononuclear cells and on the top, yellow layer contains serum. Mononuclear cells that include mesenchymal stem cells were collected by rotating pipet and transferred into a new 15 ml centrifuge tube. The volume was completed to 10 ml by adding Dulbecco's modified Eagle's medium with low glucose (DMEM-LG, Gibco) and centrifuged at 350g (1500 rpm), RT, for 10 min to remove remaining Ficoll. Supernatant was discarded and pellet was resuspended in 10 ml DMEM-LG and centrifuged again at 350g (1500 rpm), RT, 10 min in order to get rid of the Ficoll completely. Supernatant was discarded and pellet was resuspended. Cells were seeded into a 25 cm^2 tissue culture flask (BD Falcon) in 10 ml DMEM-LG including 10% hMSC qualified fetal bovine serum (MSC-FBS, Gibco) and 0.1 mg/ml primocin (InvivoGen) and incubated in 37^oC, 5% CO_2 incubator After 3 days, medium was refreshed in order to remove non-adherent hematopoietic cells.

2.2. SEEDING AND EXPANSION

Adherent primary hMSCs were grown in culture and formed colonies as they were left for proliferation after isolation process. Culture medium (expansion medium; DMEM-LG, 10% MSC-FBS, 0.1 mg/ml primocin) refreshment was done twice weekly. 12-14 days later when culture reaches 80-90% confluency, primary hMSCs were subcultured and seeded into a 75 cm^2 tissue culture flask (BD Falcon). Medium was discarded from the flask and adherent cells were washed with 10 ml prewarmed PBS. Cells were trypsinized with prewarmed 4 ml of 0.25% Trypsin/EDTA (Gibco) solution for 2-3 min. Cells were observed under microscobe and after most cells round up and start to dissociate from tissue culture flask, Trypsin was inactivated by adding 1 ml FBS into culture flask (20% of total volume). Neutralized cells were transferred into a 15 ml centrifuge tube and centrifuged at 350 g (1500 rpm), at RT, for 10 min. Supernatant was discarded leaving average 0.5 ml of liquid at the bottom. Pellet was finger mixed and suspended in 10 ml DMEM-LG and second centrifuge was done at 350g (1500 rpm), RT, 10 min in order to remove remaining trypsin. Pellet was resuspended in 2 ml DMEM-LG and cells were counted on hemocytometer. Population doubling and cell viability (assesed by Trypan Blue (Sigma) dye exclusion) were recorded. Cells were seeded at a density of 1500 cells/cm^2 with DMEM-LG containing 10% MSC-FBS and incubated in 37^oC, 5% CO_2 incubator. Subculture of hMSC was repeated every 5-6 days and hMSCs could be expanded up to 15 passages with this method.

2.3 IMMUNOPHONOTYPE OF hMSCs BY FLUORESCENT ACTIVATED CELL SORTER (FACS)

Undifferentiated hMSCs in expansion cultures were examined at passage 3 for the expression of specific surface antigens commonly used to characterize hMSC populations. The monoclonal antibodies: CD45 fluorescein isothiocyanate (FITC), Anti-HLA-DR (FITC), CD15 (FITC), CD14 phycoerythrin (PE), CD117 (PE), CD 116 (PE), CD13 (PE), CD44 (PE), anti-human CD90, anti-human CD166 (PE), CD34 (PE), CD71 (PE), CD29 (PE), HLA ABC (PE) were used.

2.4 MULTILINEAGE MESODERMAL DIFFERENTIATION

2.4.1 Adipogenic Differentiation

For adipogenic differentiation, hMSCs were harvested at P3 by trypsinization and seeded into 24-well plate at a density of 5000 cells per cm^2. Cells were incubated in culture medium (DMEM-LG ,10% MSC-FBS, 0.1 mg/ml primocin) at 37^oC in a humidified atmosphere of 5% CO_2 for a minimum of 2 hrs up to 24 hrs. Expansion medium was replaced with pre-warmed Complete MesenCult Adipogenic Medium containing MesenCult MSC Basal Medium (Stemcell) and 10% Adipogenic Stimulatory Supplement (Stemcell). Complete MesenCult Adipogenic Medium was refreshed every 3 days. Morpohologies of cells were observed under light microscobe. After 21 days adipogenic cultures can be processed for oil red o staining.

2.4.1.1 Oil Red O Stain Analysis

2.4.1.1.1 Procedure

1. After 21 days under differentiating condition, media was removed from 24-well plate.
2. Wells were rinsed with 1X PBS.
3. Cells were fixed with 4% paraformaldehyde solution for 30 min at RT.
4. Samples were incubated at 60% isopropanol for 5 min at RT.
5. Wells were rinsed once with 1X PBS and once with dH_2O.
6. Cells were stained with Oil Red O (Sigma) solution for 40-50 min at 37^oC.
7. Wells were rinsed again once with dH_2O.
8. Cells were counterstained in hematoxylen solution for 2 min.
9. Wells were rinsed with dH_2O.
10. Samples were mounted with mounting medium.

2.4.1.1.2 Results:

Lipids _____ red

Nuclei _____ pale blue

2.4.1.2 Mayer's Hematoxylen

Table 2.1 Mayer's hematoxylen solution

Chemicals	**Amount**	**Function**
Aluminum potassium sulfate (alum)	50 g	mordant
Distilled water	1000 ml	solvent
Hematoxylen	1 g	dye
Sodium iodate	0.2 g	oxidizing agent
Glacial acetic acid	20 ml	pH control

To prepare hematoxylen solution, first aliminum potassium sulfate was dissolved in dH_2O. When alum was completely dissolved, hematoxylen was added. When hematoxylen was completely dissolved, sodium iodate and glacial acetic acid were added. The solution was boiled and cooled. Finally, hematoxylen solution was filtered.

2.4.2 Chondrogenic Differentiation

For chondrogenic differentiation, hMSCs were harvested at P3 by trypsinization and seeded into 24-well plate at a density of 750.000 cells per cm^2. Cells were incubated in culture medium (DMEM-LG, 10% MSC-FBS, 0.1 mg/ml primocin) at 37^oC in a humidified atmosphere of 5% CO_2 for minimum of 2 hrs in order to get cells attached to the plate wells. To stimulate chondrogenic differentiation culture medium was replaced with pre-warmed Stempro Chondrocyte Differentiation Basal Medium (Gibco) containing 10% Stempro Chondrogenesis Supplement (Gibco). The medium was changed every 3 days. After 21 days of cultivation, chondrogenic pellet can be processed for Alcian blue staining.

2.4.2.1 Alcian Blue Stain Analysis

2.4.2.1.1 Solutions and Reagents:

1. Alcian Blue solution (pH 2.5)

Alcian Blue (Sigma)_____1.0 g
Distilled water _____97.0 ml
Glacial acetic acid_____3.0 ml

Alcian Blue was dissolved in distilled water, than; acid was added and the solution was mixed well. Solution was filtered into the reagent bottle and filtered before use.

2. Alcian Blue solution (pH 1.0)

Alcian Blue (Sigma)_____1.0 g
Distilled water_____90 ml
Hydrochloric acid_____10 ml

1 g of Alcian Blue was dissolved in 90 ml of distilled water and 10 ml of 1N hydrochloric acid.

3. Alcian Blue solution (pH 0.2)

Alcian Blue (Sigma)_____1.0 g
Sulphuric acid (10%) _____100 ml

1 g Alcian Blue was dissolved in 100 ml of 10% sulphuric acid.

2.4.2.1.2 Procedure:

1. After 21 days under differentiaitng condition, media was removed from 24 well plate.
2. Wells were rinsed with 1X PBS.
3. Cells were fixed with 4% paraformaldehyde solution for 30 min at RT.
4. Wells were rinsed with 1X PBS.

5. Cells were stained with 1% Alcian Blue solution (pH 2.5) for 40-50 min at RT.
6. For lower pH solutions ie pH 1.0 or pH 0.2 samples were drained and blotted dry, to prevent removal of stain in water.
7. Cells were counterstain in hematoxylen solution for 2 min.
8. Wells were rinsed with dH_2O.
9. Samples were mounted with mounting medium.

2.4.2.1.3 Results:

At pH 2.5 most acid mucins (except some of the strongly sulphated groups) _____ blue
At pH 1.0 only weakly and strongly sulphated acid mucins _____ blue
At pH 0.2 only strongly sulphated acid mucins _____ blue

2.4.3 Osteogenic Differentiation

For osteogenic differentiation, hMSCs were harvested at P3 by trypsinization and seeded into 24-well plate at a density of 2.10^5 cells per cm^2. To stimulate osteogenic differentiation, Complete MesenCult Osteogenic Medium including MesenCult MSC Basal Medium, Osteogenic Stimulatory Supplement, β-Glycerophosphate, Dexamethasone, Ascorbic acid (all from Stemcell) was prepared. Cells were incubated in Complete MesenCult Osteogenic Medium (without β-Glycerophosphate) at 37^oC in a humidified atmosphere of 5% CO_2. After 5 days, culture was replanished with Complete MesenCult Osteogenic Medium (without β-Glycerophosphate), unless cell multilayering has been noted. Cell multilayering is the layering of cells on top of each other. Multilayering is indicative of the beginning of bone generation. Once multilayering has been observed, β-Glycerophosphate was added to Complete MesenCult Osteogenic Medium as directed. Cultures were replenished with β-Glycerophosphate-containing medium every 3 days. After 5 weeks of cultivation, chondrogenic pellet was processed for Toluidine Blue staining.

2.4.3.1 Toluidine Blue Stain Analysis

2.4.3.1.1 Procedure

1. After 5 weeks under differentiating condition, remove media from 24-well plate.
2. Wells were rinsed with 1X PBS.
3. Cells were fixed with 4% paraformaldehyde solution for 30 min at RT.
4. Wells were rinsed with 1X PBS.
5. Cells were stained with 1% Toluidine Blue (Sigma) solution (in 50% isopropanol) for 40-50 min at 37^{o}C.
6. Samples were incubated in absolute isopropanol for 1 min.
7. Cells were counterstained in hematoxylen solution for 2 min.
8. Wells were rinsed with dH_2O
9. Samples were mounted with mounting medium.

2.4.3.1.2 Results

Calcium deposits _____dark blue
Background _____blue

2.5 REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

2.5.1 RNA Isolation

RNA was extracted using RNeasy kit (Qiagen). $2.5x10^5$ cells were seeded into 10 cm^2 tissue culture dish. After 2 days, cells were disrupted by adding Buffer RLT. Buffer RLT was added the appropriate volume (see Table 2.2) and vortex to mix for 1 min.

Table 2.2 Volumes of buffer RLT for lysing pelleted cells

Number of cells	**Volume of buffer RLT (µl)**
$<5x10^6$	350
$5x10^6 – 1x10^7$	600

Ethanol (70%) was added 1 volume to the homogenized lysate, and mixed well by pipetting. The sample was transfered up to 700 µl, into an RNeasy spin column placed in a 2 ml collection tube. The lid was closed gently and centrifuged for 15s at 8000 x g. Buffer RW1 added in 700 µl to the RNeasy spin column. The lid was closed gently and centrifuged for 15s at 8000 x g. Buffer RPE was added in 500 µl to RNeasy spin column. The spin column membrane was centrifuged for 15s at 8000 x g to wash. Buffer RPE was added in 500 µl to the RNeasy spin column. The lid was closed gently and centrifuged for 2 min at 8000 x g. the RNeasy spin column was placed in a new 1.5 ml collection tube. RNase-free water was added 30 µl directly into the spin column membrane and centrifuged for 1min at 8000 x g to elute the RNA. For the RNA quantification sample tubes were set up as follows and with the components as prescribed in Table 2.3.

Quant-iT working solution was prepared by diluting the Quant-iT reagent 1:200 in Quant-iT buffer. 200 µl of working solution was required for each sample and standard. Assay tubes were prepared according to the table and read in Qubit fluorometer.

Table 2.3 RNA Quantification Kit ingredients and amounts (µl) required for assay

	Standard Assay Tubes	**User Sample Assay Tubes**
Volume of working solution to add	190 µl	180-199 µl
Volume of standard to add	10 µl	—
Volume of sample to add	—	1–20 µl
Total volume in each assay tube	200 µl	200 µl

2.5.2 cDNA Synthesis

RNA was reverse transcribed to obtain cDNA by quantitect reverse transcription kit (Qiagen). Template RNA, gDNA wipeout buffer, quantiscript reverse transcriptase, quantiscript rt buffer, rt primer mix, and rnase-free water were thawed at RT. The genomic DNA elimination reaction was prepared on ice according to Table 2.4 and mixed well and then stored on ice.

Table 2.4 Genomic DNA elimination reaction components

Component	**Volume/Reaction**	**Final Concentration**
gDNA wipeout buffer,7x	2 µl	1x
Template RNA (0.1µg)	variable	
RNase-free water	variable	
Total volume	14 µl	

Reaction mixture was incubated for 2 min at 42°C and then placed on ice. Then, the reverse-transcription master mix was prepared on ice, according to Table 2.5.

Table 2.5 Reverse-transcription reaction components

Component	**Volume/Reaction**	**Final Concentration**
Quantiscript-reverse transcriptase	1 µl	
Quantiscript-rt buffer,5x	4µl	1x
RT primer mix	1 µl	
Entire genomic DNA elimination reaction	14 µl	
Total	20 µl	

Template RNA was added (14 µl) to each tube containing reverse-transcription master mix. It was mixed and then stored on ice. Master mix was incubated for 15 min at 42°C and incubated for 3min at 95°C to inactivate quantiscript reverse transcriptase. Then it was stored at -20°C.

2.5.3 RT-PCR

PCR reactions were performed in reaction mixture of 28 µl containing the components in Table 2.6.

Table 2.6 PCR solutions and their initial and final concentrations with final volumes.

Reagent	**Initial Concentration**	**Final Concentration**	**Final Volume**
Taq buffer	10X	1X	2.5 µl
dNTP	2 mM	0.2 µM	1.5 µl
$MgCl_2$	25 mM	2 mM	2.5 µl
Primers (x2)	12,5 pmol/µl	12,5 pmol	2 µl
ddH2O	-	-	16.8 µl
Taq DNA polymerase	5 U/µl	1 U	0.2 µl
cDNA			2.5 µl
Total reaction volume			28 µl

The denaturation of the dsDNA at 94°C 30s, so that the two strands separated and the primers bound again at lower temperatures and began a new reaction. Then, the temperature decreased until it reaches the annealing temperature (see table 2.7).

Table 2.7 PCR procedure of primers

Primers	Denaturation temp. / time	Annealing temp./ time	Extension temp. / time	Number of cycle
Actin	94^{o}C 30s	60^{o}C 45s	72^{o}C 1min	30
Topo IIα	94^{o}C 30s	54^{o}C 45s	72^{o}C 1min	35
Topo IIβ	94^{o}C 30s	60^{o}C 45s	72^{o}C 1min	35
β III Tubulin	94^{o}C 30s	60^{o}C 45s	72^{o}C 1min	35
NSE	94^{o}C 30s	65^{o}C 45s	72^{o}C 1min	35

The final step of PCR amplification is cDNA extension from the primers. This was done with thermostable Taq DNA polymerase, usually at 72°C, the temperature at which the enzyme works optimally. The length of the incubation at each temperature, the temperature alterations, and the number of cycles were controlled by a programmable thermal cycler components.

2.5.3.1 Agarose Gel Electrophoresis

PCR products were resolved on 2% agarose gels. Gel is prepared adding 1.6 g of powdered agarose gel into 80 ml of 0.5 x TBE buffer (Fluka) solution and it is boiled until the agarose is completely dissolved in the buffer solution. 12 µl of safe DNA staining solutionwas added when the boiled solutions began to cool down. Solution was mixed homogenously by making hand-shaking. It was directly poured into horizontal agarose gel platform and 20 wells were placed one side of the gel. Gels were let to solidify for at least 10 min.

2.5.3.2 Loading and Visualization of the Gel

12.5 µl PCR products were loaded in each slot. 3.5 µl of a 100 bp DNA Ladder (Bioron) was mixed with 2 µl bromophenolblue. Then 5.5 µl of this mix was put into usually the first slot as a molecular size marker. The gel was run at 110 V in 0.5X TBE buffer for 45 min. Bands were detected under UV transilluminator.

2.6 NEURAL TRANSDIFFERENTIATION

For neural differentiation, hMSCs were harvested at passage 3 and at passage 15. Cells were seeded into 24-well plate at a density of 1500 cells per cm^2 prior to neural induction. Cells were incubated in culture medium (DMEM-LG, 10% MSC-FBS, 0.1 mg/ml primocin) at 37^oC in a humidified atmosphere of 5% CO_2 . On the day of induction, medium was replaced with neural induction medium.

Induction medium which is composed of several cytokines and growth factors includes 0,5 mg/ml dbcAMP (dibutyryl cyclic AMP, SIGMA), 0.5 mM IBMX (3-isobutyl-1-methylxanthine, SIGMA), 20 ng/ml hEGF (human epidermal growth factor, SIGMA), 40 ng/ml rhFGF (recombinant human fibroblast growth factor, R&D systems), 10 ng/ml FGF-8 (fibroblast growth factor-8, Pepro Tech), 10 ng/ml rhBDNF (recombinant human brainderived neurotrophic factor, R&D systems) 2 mM L-Glutamine (GIBCO) and 40 ng/ml NGF in Neurobasal medium (GIBCO) supplemented with 2% B27 Supplement (GIBCO). Induction media was refreshed per 48 hrs during 12 days. Morphologies of the cells were observed under light microscobe.

Table 2.8 N3 cytokine combination used in neural differentiation of hMSCs

Induction Medium With N3	**Final**
Neurobasal Medium	50X
B27 Supplement	1X
dbCAMP (100 mg/ml)	0.125mg/ml
IBMX (1M)	0.5 mM
hEGF (100 mg/ml)	20 ng/ml
BFGF (100 mg/ml)	40 ng/ml
FGF-8 (10 mg/ml)	10 ng/ml
BDNF (10 mg/ml)	10 ng/ml
L-Glutamine (200mM)	2 mM
NGF (100 mg/ml)	40ng/ml

2.7 IMMUNOFLOURESCENT STAINING

The day before experiment, hMSCs were seeded into 24-well culture for immunofluorescence staining. At the day of staining, medium was aspirated from wells. To permeabilize cells, pre-warmed (37^{o} C) 500 µl/well TZN buffer (10 mM pH 7.5 Tris-HCl, 0.5% Nondet P40, 0.2 mM ZnCl2 were with) was added to each well and then incubated for 15 min by mixing on rocking shaker at very low speed (approx. 10 rpm). Then the cells were fixed with 4% paraformaldehyde / PBS (500 µl/well) for 10 min at RT. Cells were washed with 750 µl/well PBS (Phosphate Buffered Saline, pH 7.4, SIGMA) for 3 times, 5 min at each time on rocking shaker. 500 µl/well of 10% Normal Goat Serum (Gibco) and 10% Normal Horse Serum (Biochrom) in 0.3% TritonX / PBS (PBS-Tx) were used to block cells for 30 min at RT. Then, cells were incubated with 60.l/well specific primary antibodies for target proteins for 2 hrs at RT. Antibodies diluted in PBS-Tx with 3% NHS. Washing steps with PBS was repeated for three times. Cells were then treated with 50 µl/well Alexa Flour labeled anti mouse or anti rabbit secondary antibodies at RT for 1 hr. After this incubation, cells were washed 3 times with PBS and treated with 150 µl/well, 1/15000X DAPI (Sigma) for 10 min. Then washing with PBS was repeated for three times and finally cells were washed with dH2O. 4 µl Prolong Gold Antifade Reagent (Invitrogen) was applied on glass coverslips and they were placed on cells in the wells. Wells were observed under fluorescent microscope (Nikon) and images were taken next day.

Antibodiy against NSE (1:100, Chemicon) and NF (1:100, Chemicon) was used at indicated dilutions. Secondary antibodies GAM-IgG-Alexa Fluor 488 (1:100) and GAR-IgGAlexa Fluor 594 (1:100) were purchased from Invitrogen.

CHAPTER 3

RESULTS

3.1 CHARACTERIZATION OF hMSCs BY FLUORESCENT ACTIVATED CELL SORTING (FACS)

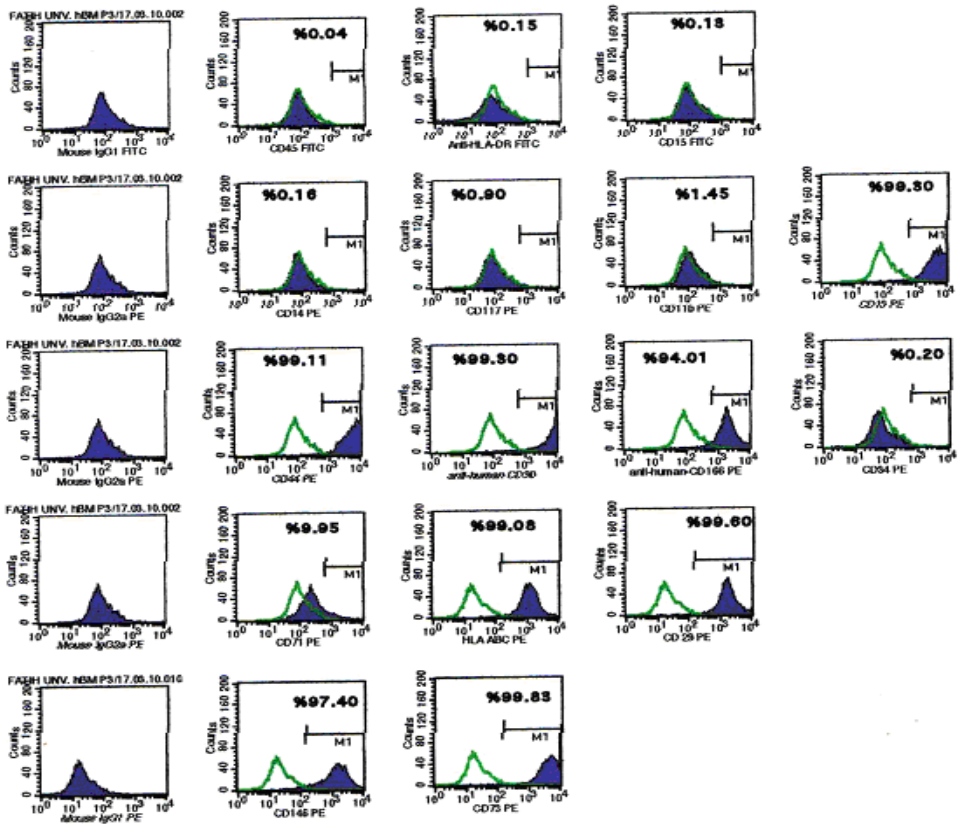

Figure 3.1 Surface marker expressions of hMSCs.

Immunophenotypic surface profile for CD45, anti-HLA-DR, CD15, CD14, CD117, CD116, CD13,CD44, anti-human CD90, anti-human CD166, CD34, CD71, HLA ABC, CD29, CD146 and CD73 of isolated hMSCs at passage 3 were analyzed by FACS.

Green histograms represent the fluorescence from negative control cells incubated with only secondary antibody; blue histograms represent the counts of cells incubated with the relevant primary antibody. The logarithm on the X-axis represents the intensity of the fluorescent signal and Y-axis represents number of cells.

hMSCs at passage 3 were positive for the markers CD13, CD44, CD90, CD166, HLA ABC, CD29, CD146, CD73 but negative for CD45, HLA-DR, CD15, CD14, CD117, CD116, CD34.

3.2 STUDY POPULATION

Cell Name	Donor Age	Donor Sex	Group
Child 1	11	female	C
Child 2	5	female	H
Child 3	8	male	I
Child 4	3	female	L
Child 5	9	male	D
Child 6	2	female	(0 - 18 years)
Adult 1	44	female	A
Adult 2	26	male	D
Adult 3	33	female	U
Adult 4	48	female	L
Adult 5	42	male	T
Adult 6	50	male	(18 - 50 years)
Old 1	57	female	
Old 2	57	female	O
Old 3	77	male	L
Old 4	70	male	D
Old 5	53	male	
Old 6	65	male	(over 50 years)

Table 3.1 Bone marrow samples used in the project.

Fifteen individuals participated in the study: five children (three females and three males) aged 0-18 years; five adults (three females and three males) aged 18-50 years; five old donors (four males and two females) aged over 50 years.

The participants were recruited from the local community and they had no history of concurrent illness or intake of medication that could affect bone metabolism. All participants signed an informed written consent.

3.3 GROWTH KINETICS AND MORPHOLOGY

3.3.1 Growth Curve

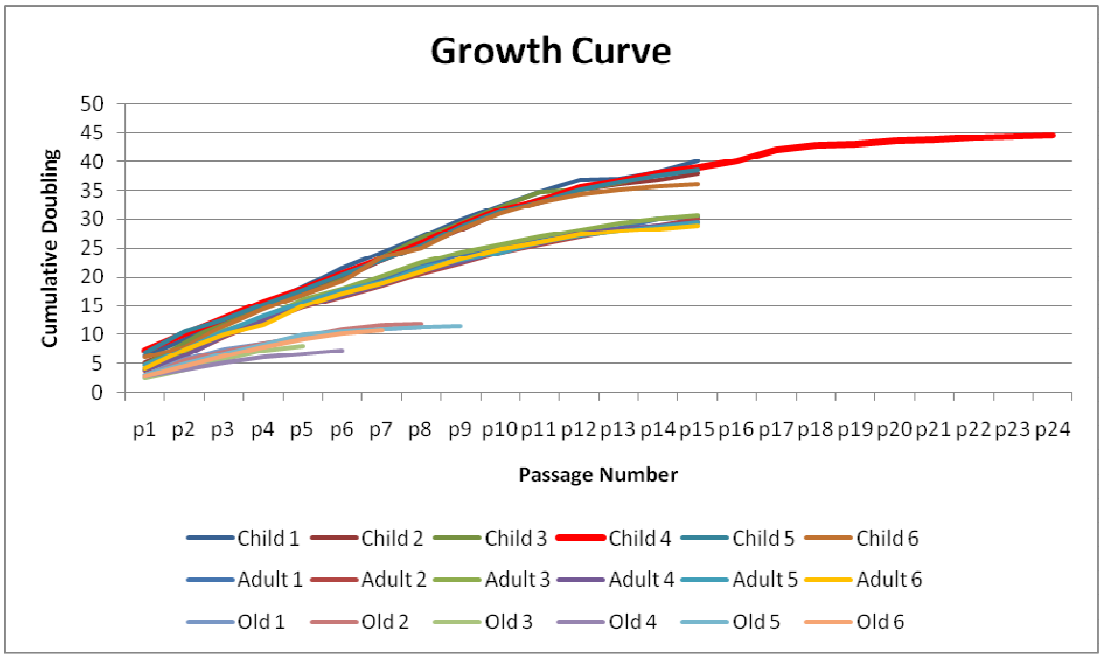

Figure 3.2.1 Long term growth curves; each obtained from an individual donor.

To examine long term growth kinetics of hMSC cultures, cumulative population doublings were measured with respect to passage number in multiple donors.

Cell growth was monitored by determining the number of PD using the formula log N/log 2, where N is the cell number of the confluent monolayer divided by the initial number of cells seeded. This procedure was repeated in every passage.

In 120 days, the average number of cumulative PD was 37 for hMSCs from child donors; 25 for hMSCs from adult donors and 10 for hMSCS obtained from old donors.

MSC from old donors exhibited a decreased maximal life span compared with cells from child and adult donors and mean PD rate was lower in old donor cells compared with young and adult donor cells. A reduction in the proliferation rate was observed in MSCs from all ages according to increasing passage number.

MSCs harvested from adult donors stopped proliferating at about P15, and hMSCs form old donors stopped proliferating at P7. However, cells from child donors continued to divide. To determine maximal life span of MSCs from child donors, we passaged cells until they did not proliferate. Our results indicated that, MSCs obtained from child donors reach their maximal life span at P24 (182 days) with the 45 cumulative population doublings.

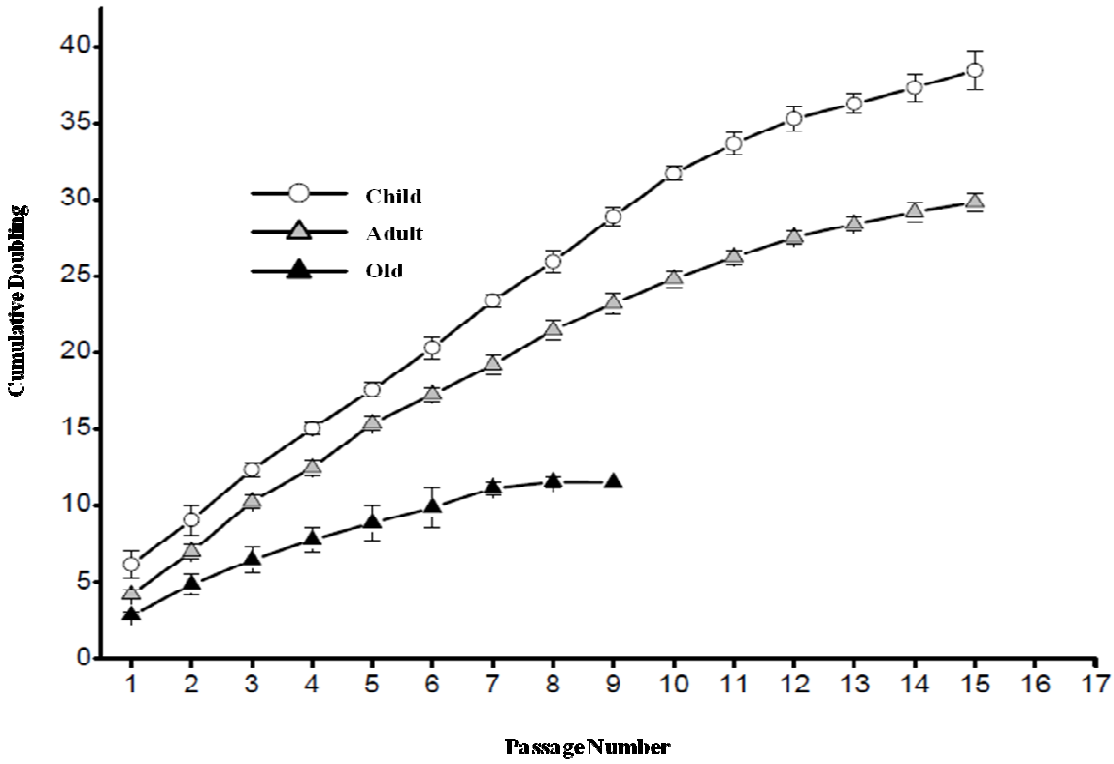

Figure 3.2.2 Long term growth curves; average cumulative population doublings of each group.

3.3.2 Long-term Culture of hMSCs

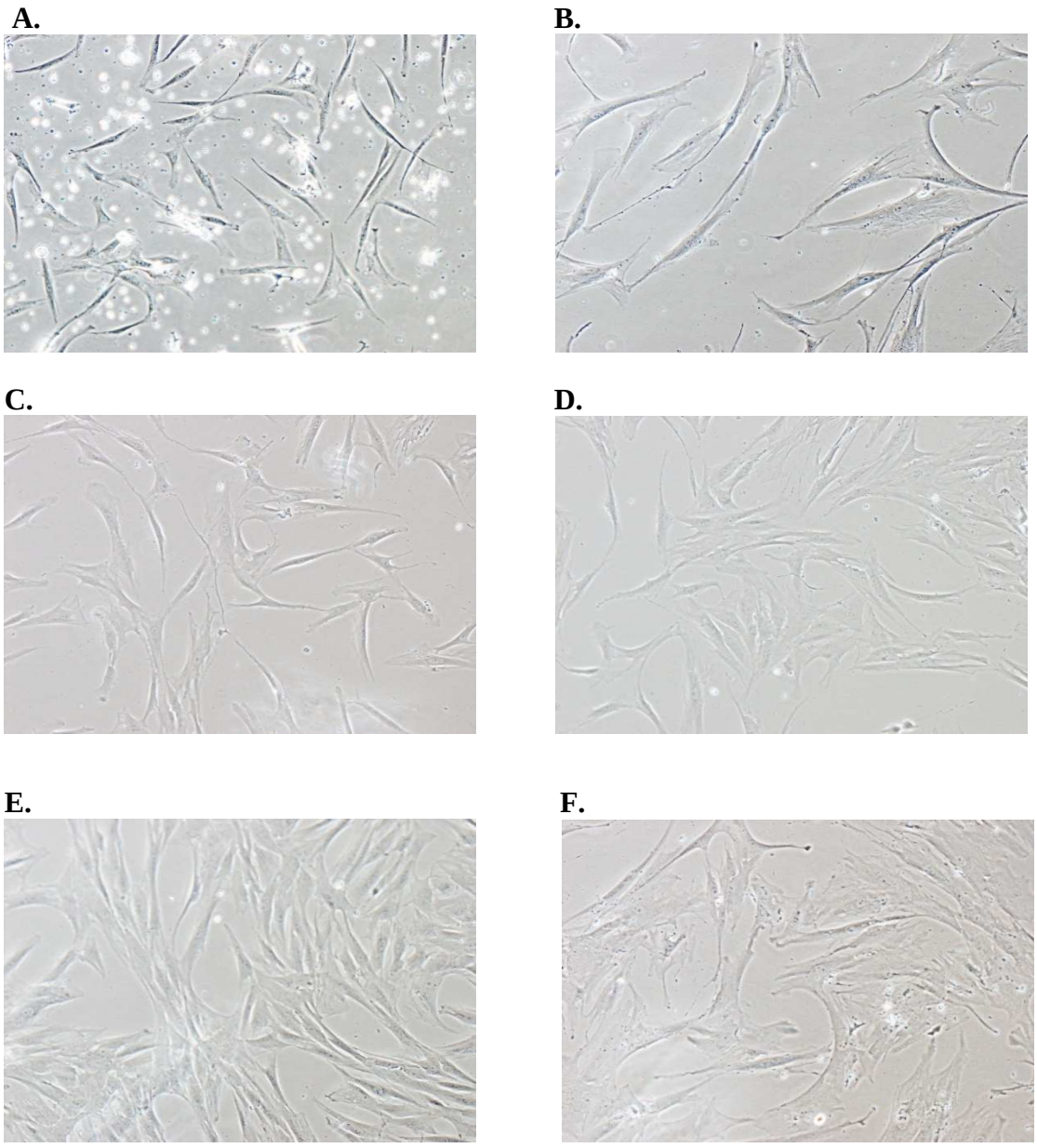

Figure 3.3 Phase-contrast images of hMSCs at different passages.
Primary cells on culture (A), at passage 3 (B), at passage 6 (C), at passage 9 (D), at passage 12 (E) and at passage 15 (F). All images are taken under 10X magnification.

Primary colonies of hMSCs (P0) show fibroblast-like appearance. With continued *in vitro* serial propagation, hMSCs gradually lost their morphology. At P15, 17 weeks after primoculture, cells were enlarged and flat and difficult to harvest by trypsinization.

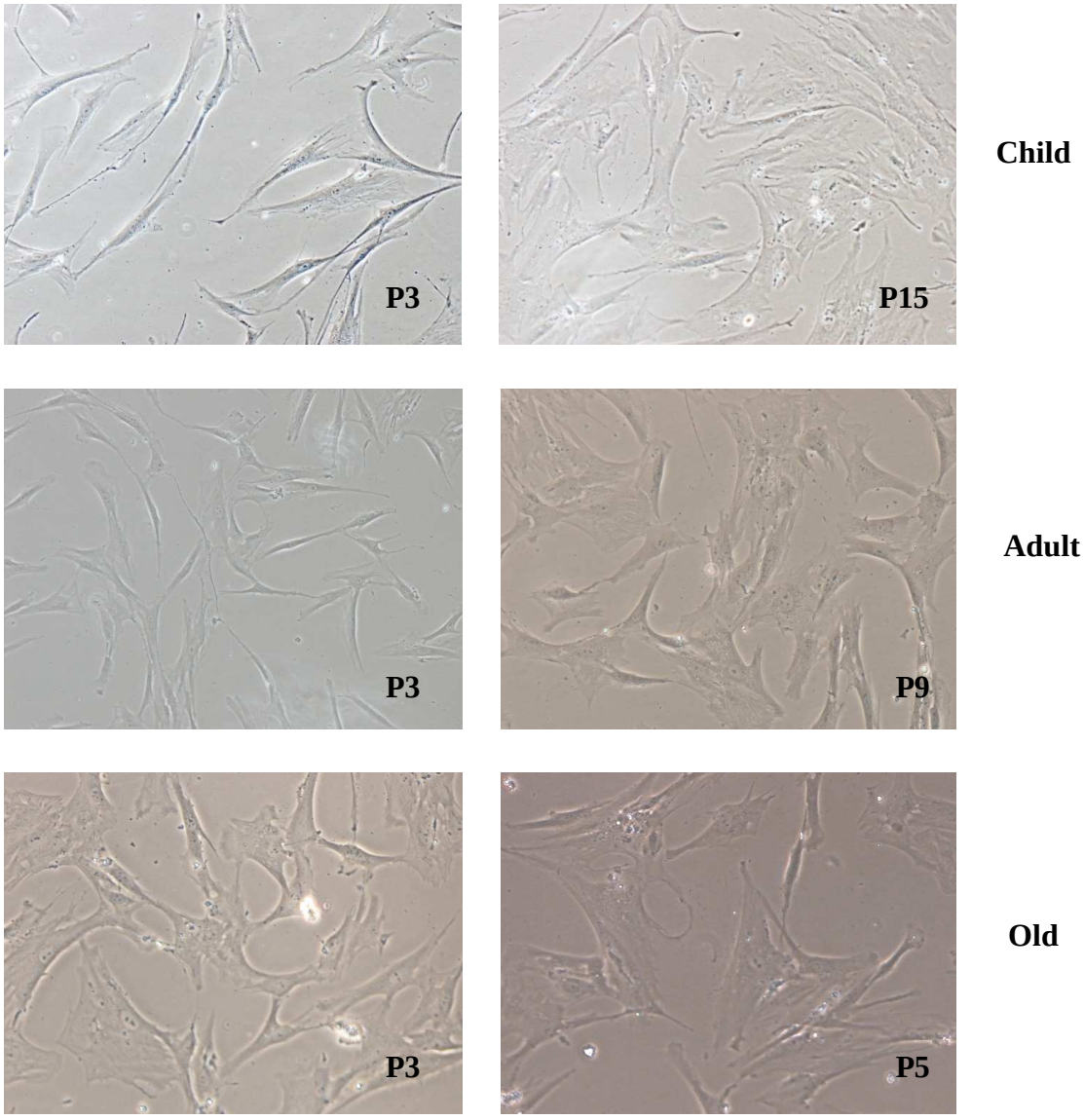

Figure 3.4 Comparison of hMSCs from child, adult and old donors in terms of morphology. All images are taken under 10X magnification.

In the initial phases of cultivation (P3), hMSCs from all ages were spindle shaped. However, during long term serial passage, hMSCs gradually lost their fibroblast like morphology. hMSCs from child donors gain irregular and flat shape when they were at P15. Cells obtained from adult donors gained the same morhology at P9. hMSCs obtained from old donors became enlarged and flat when they were at P5.

3.4 MESODERMAL DIFFERENTIATION OF hMSCs

3.4.1 Adipogenic Differentiation

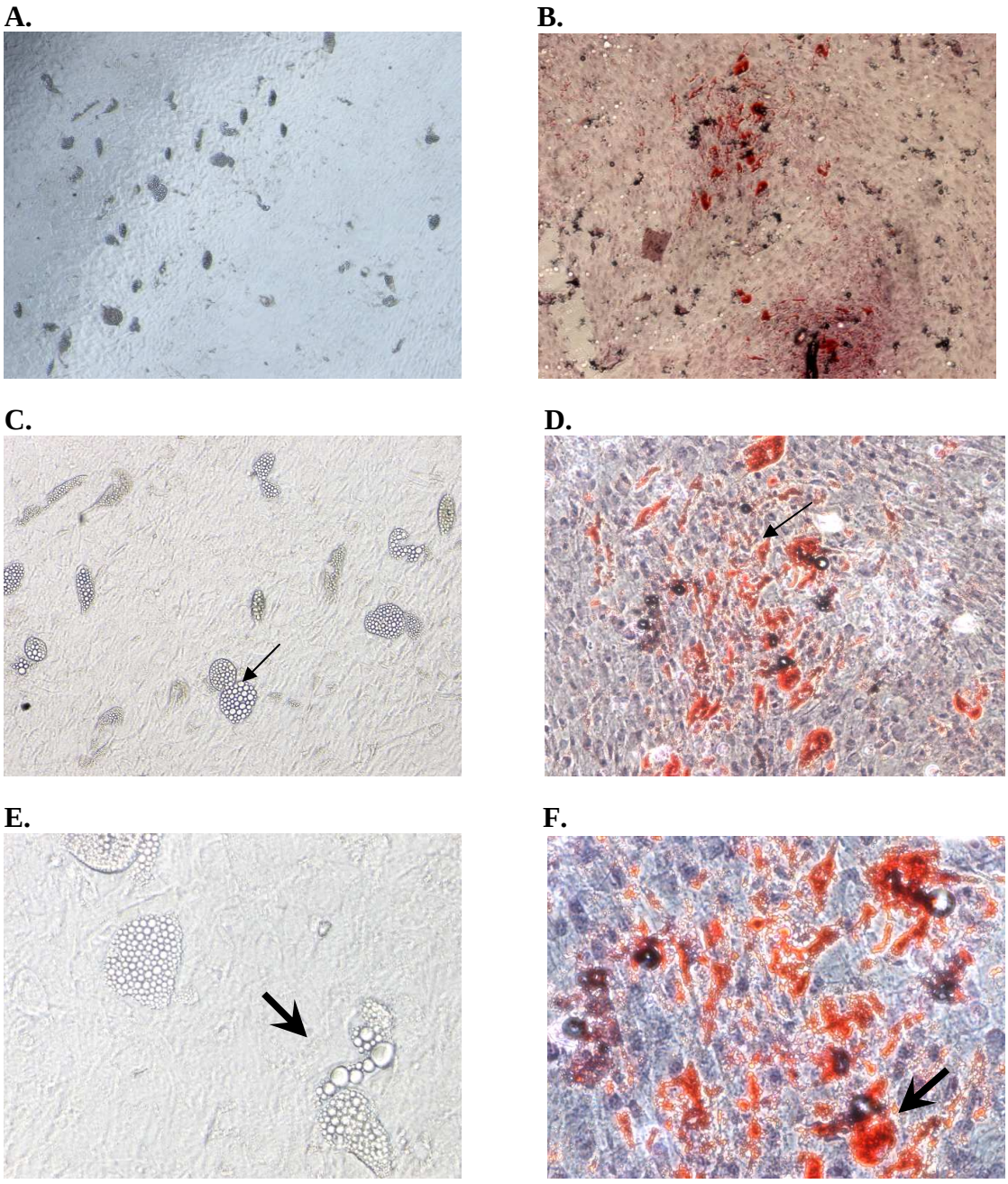

Figure 3.5 Adipogenic differentiation of hMSCs obtained from child donors (A-F).

(A-C-E) Differentiated hMSCs before staining (A 4X, C 10X, E 20X magnification).

(B-D-F) Differentiated hMSCs after staining with Oil Red O (with hematoxylen nuclear counterstain) (B 4X, D 10X, F 20X magnification).

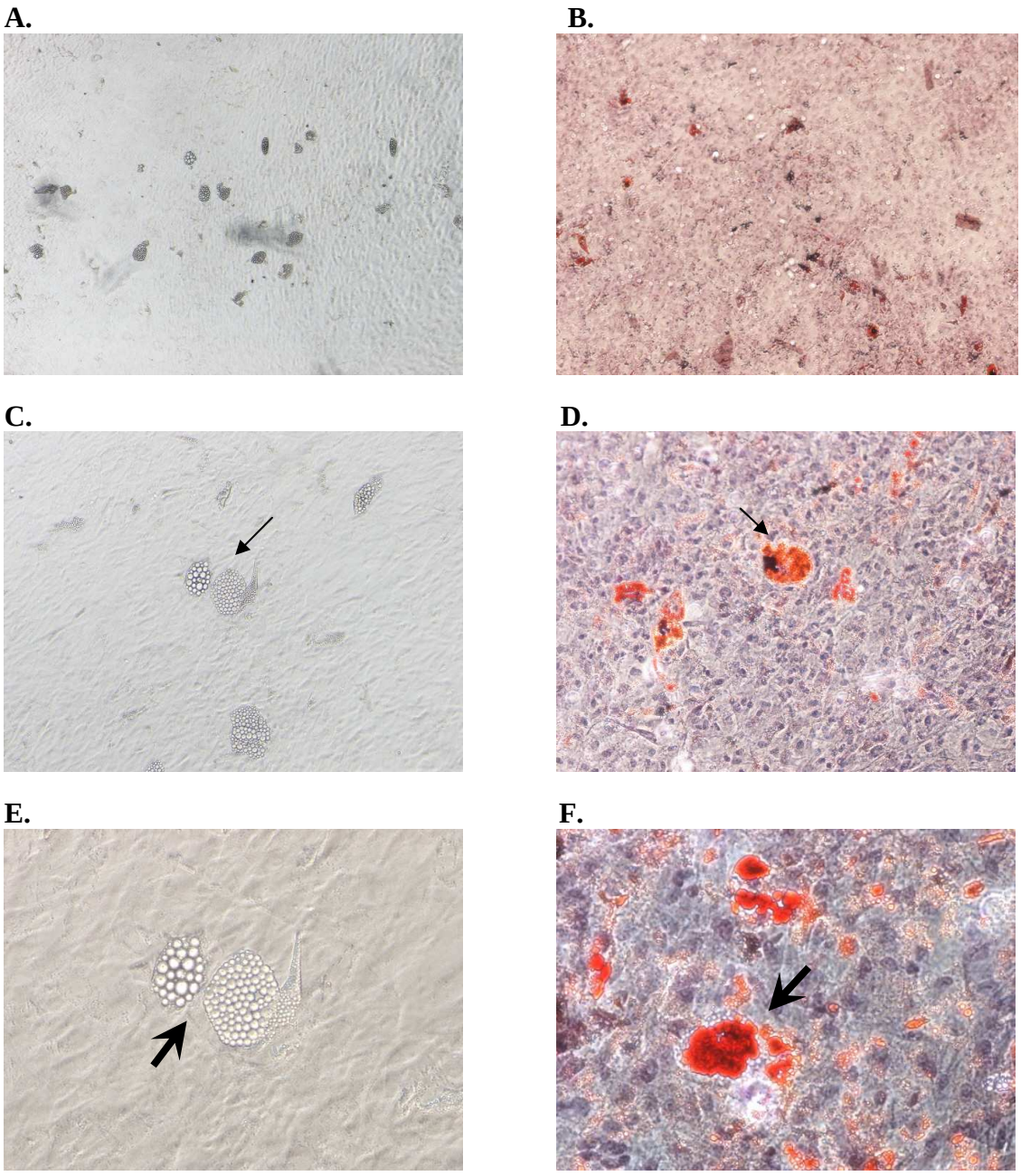

Figure 3.6 Adipogenic differentiation of hMSCs obtained from adult donors (A-F).

(A-C-E) Differentiated hMSCs before staining (A 4X, C 10X, E 20X magnification)

(B-D-F) Differentiated hMSCs after staining with Oil Red O (with hematoxylen nuclear counterstain) B 4X, D 10X, F 20X magnification).

The arrow indicates lipid vacoules.

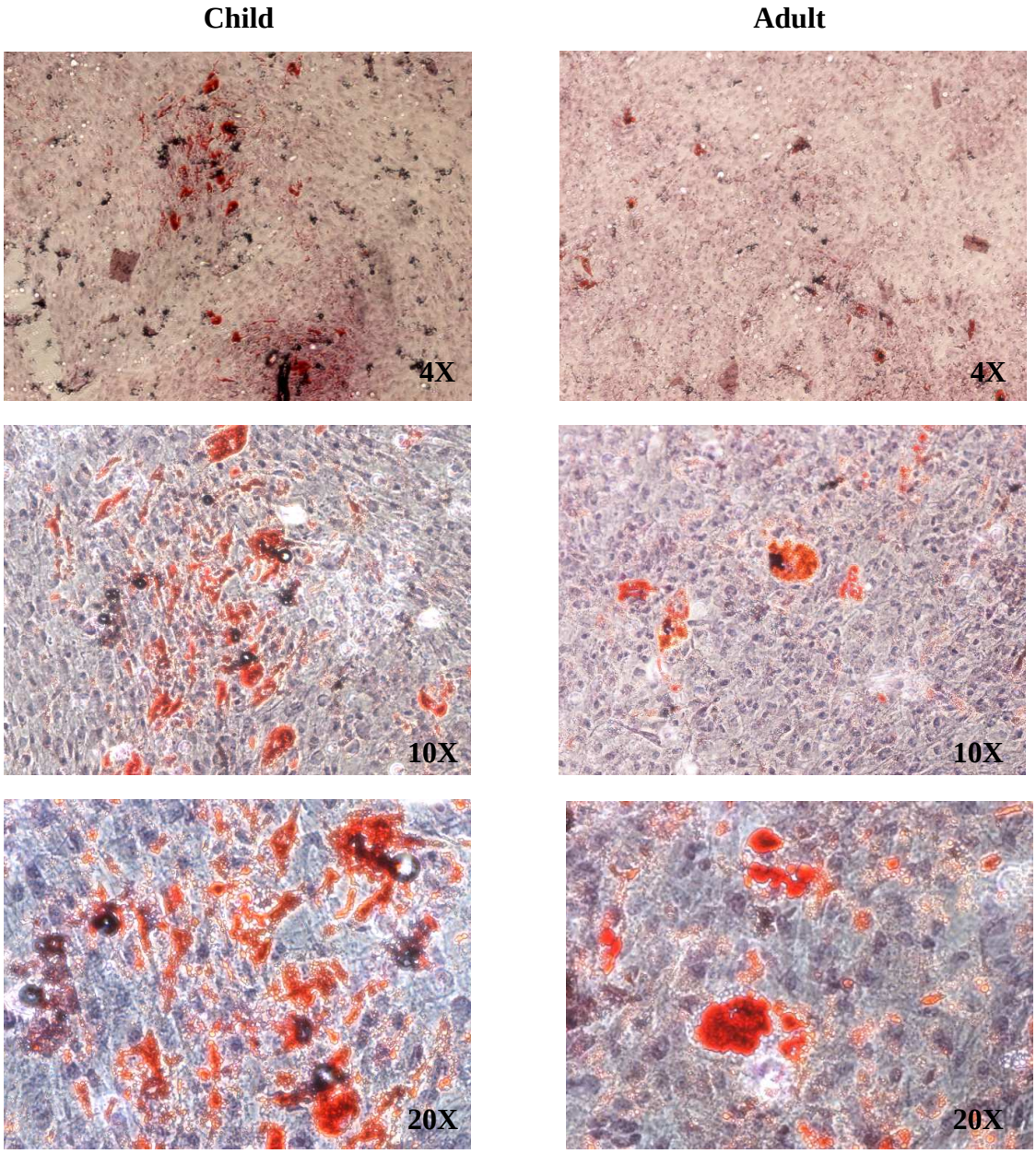

Figure 3.7 Adipogenic differentiation of hMSCs obtained from child and adult donors after staining with Oil Red O (with hematoxylen nuclear counterstain).

To assess the adipogenic differentiation potential of hMSCs, Oil Red O staining was quantified after 3 weeks in differentiation conditions. Cells containing a visibly Oil Red O stained lipid vacuoles were considered to be positively stained. Significantly lower cells stained positive with Oil Red O, which stains lipid vacuoles, in adult versus child donors.

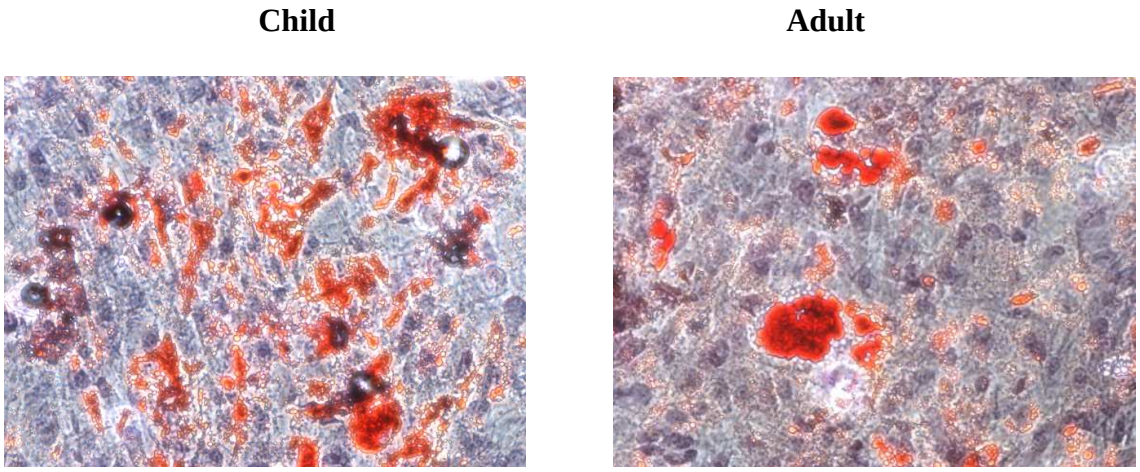

Figure 3.8 Comparison of adipogenic differentiation.

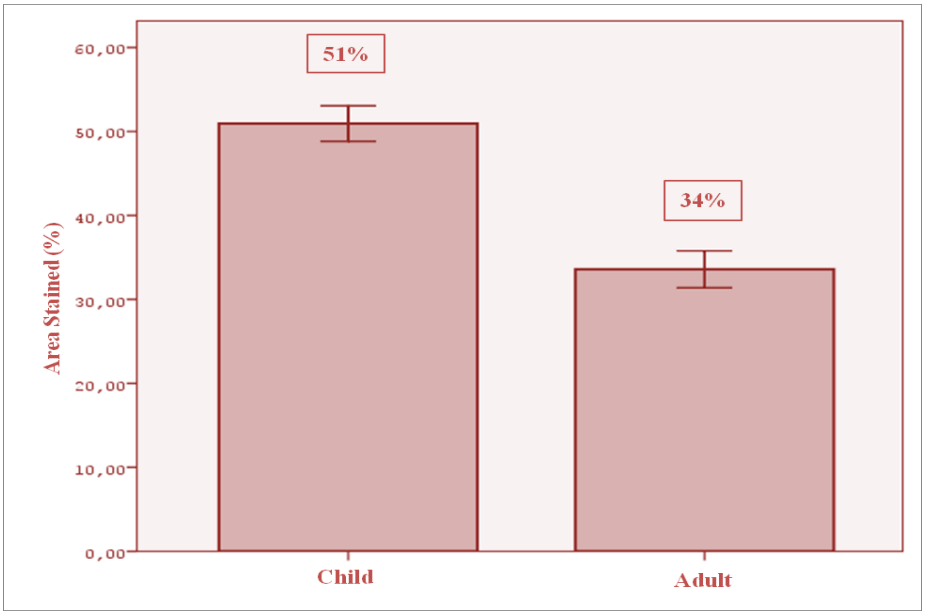

Figure 3.9 Percentage of total area that was positively stained with Oil Red O.

Quantification was performed by determining the percentage of total area that contianed Oil Red O stained lipid laden vacuoles using ImageJ (NIH, Bethesda, MD). The percentage of cells that were posively stained decreased from 51% to 34% due to increasing donor age. Donor age related decline was observed between child and adult donors.

3.4.2 Chondrogenic Differentiation

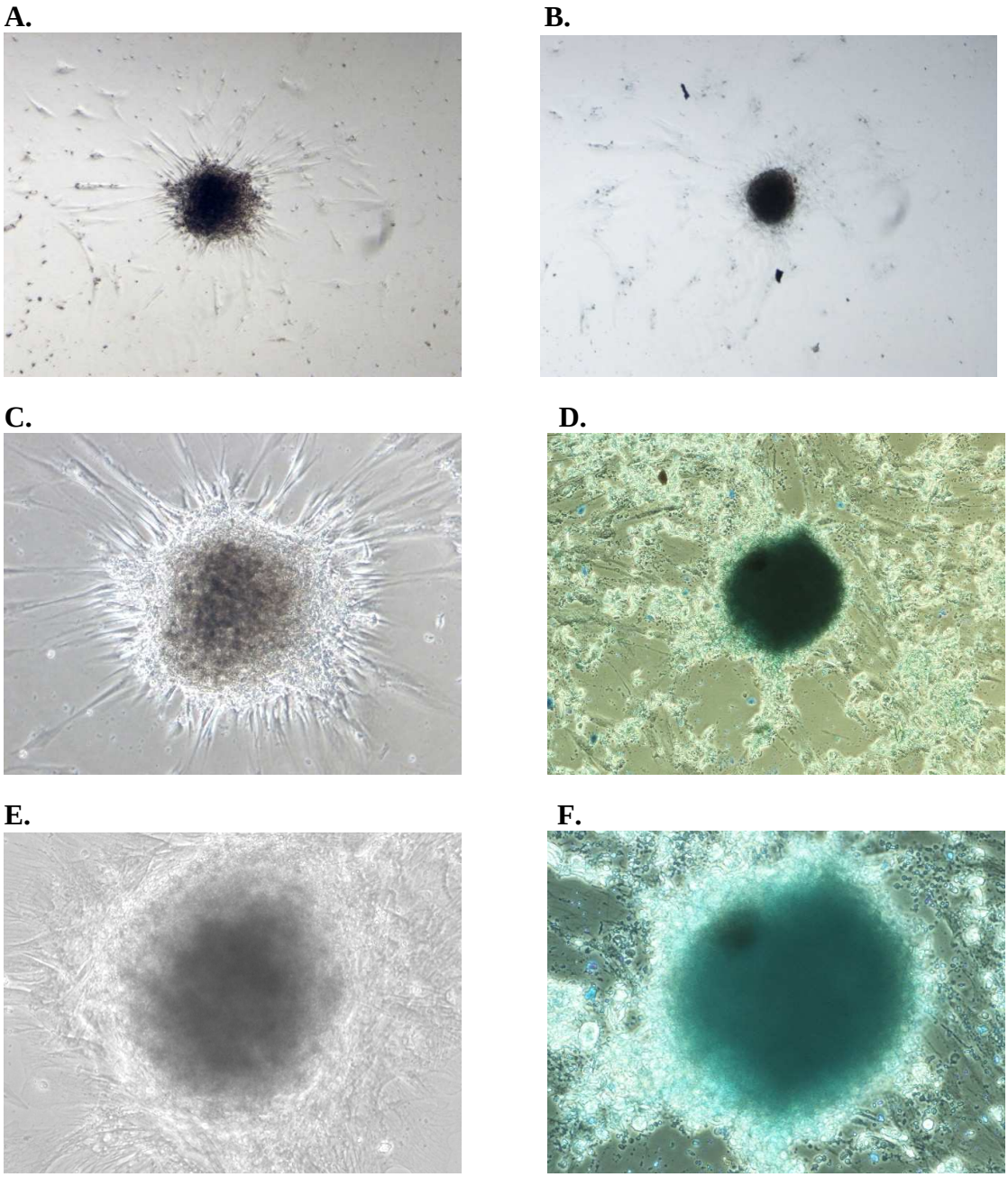

Figure 3.10 Chondrogenic differentiation of hMSCs obtained from child donors (A-F).

(A-C-E) Differentiated hMSCs before staining (A 4X, B 10X, C 20X magnification)

(B-D-F) Differentiated hMSCs after staining with Alcian blue (D 4X, E 10X, F 20X magnification).

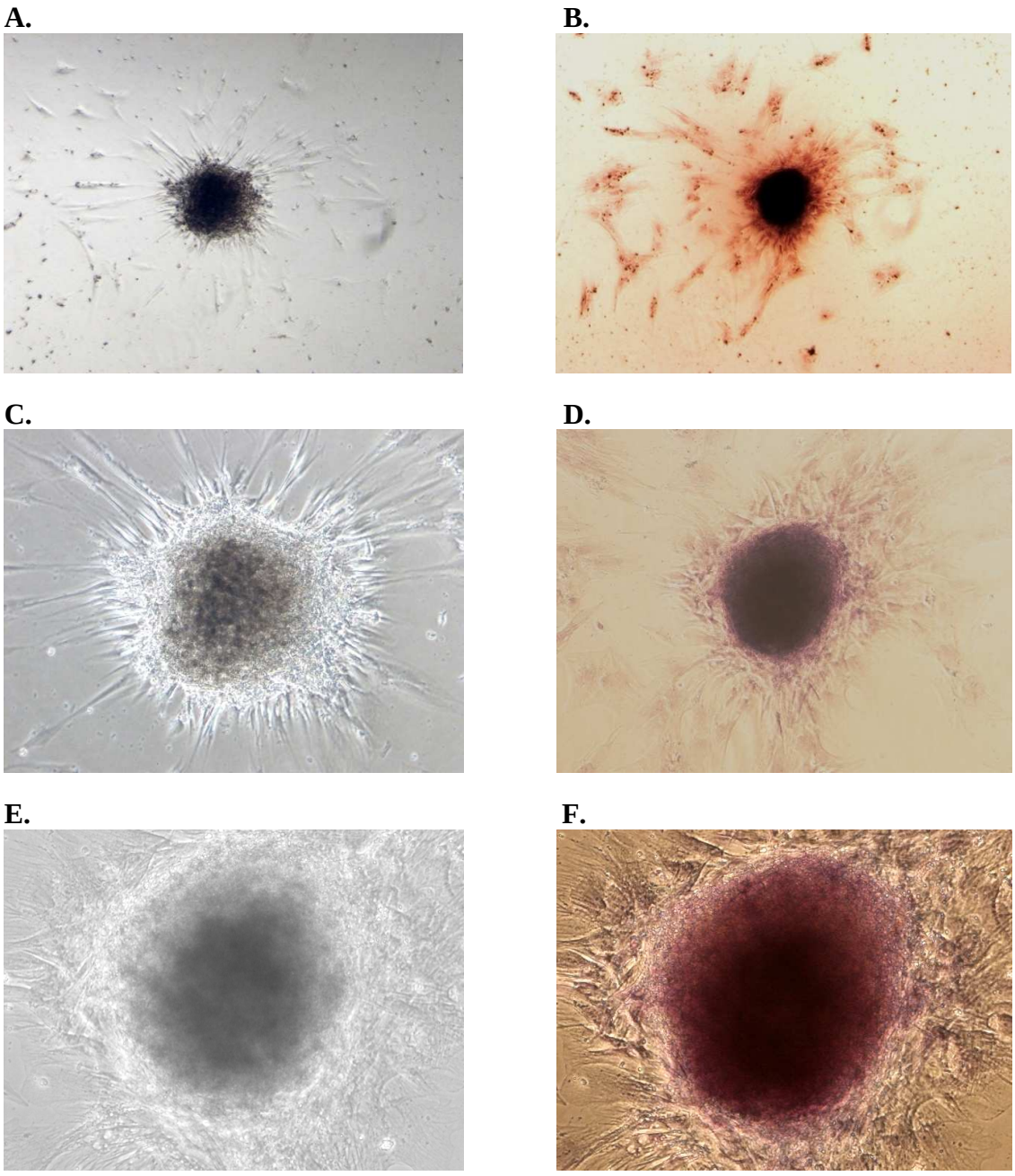

Figure 3.11 Chondrogenic differentiation of hMSCs obtained from child donors (A-F).

(A-B-C) Differentiated hMSCs before staining (A 4X, B 10X, C 20X magnification)

(D-E-F) Differentiated hMSCs after staining with hematoxylen (D 4X, E 10X, F 20X magnification).

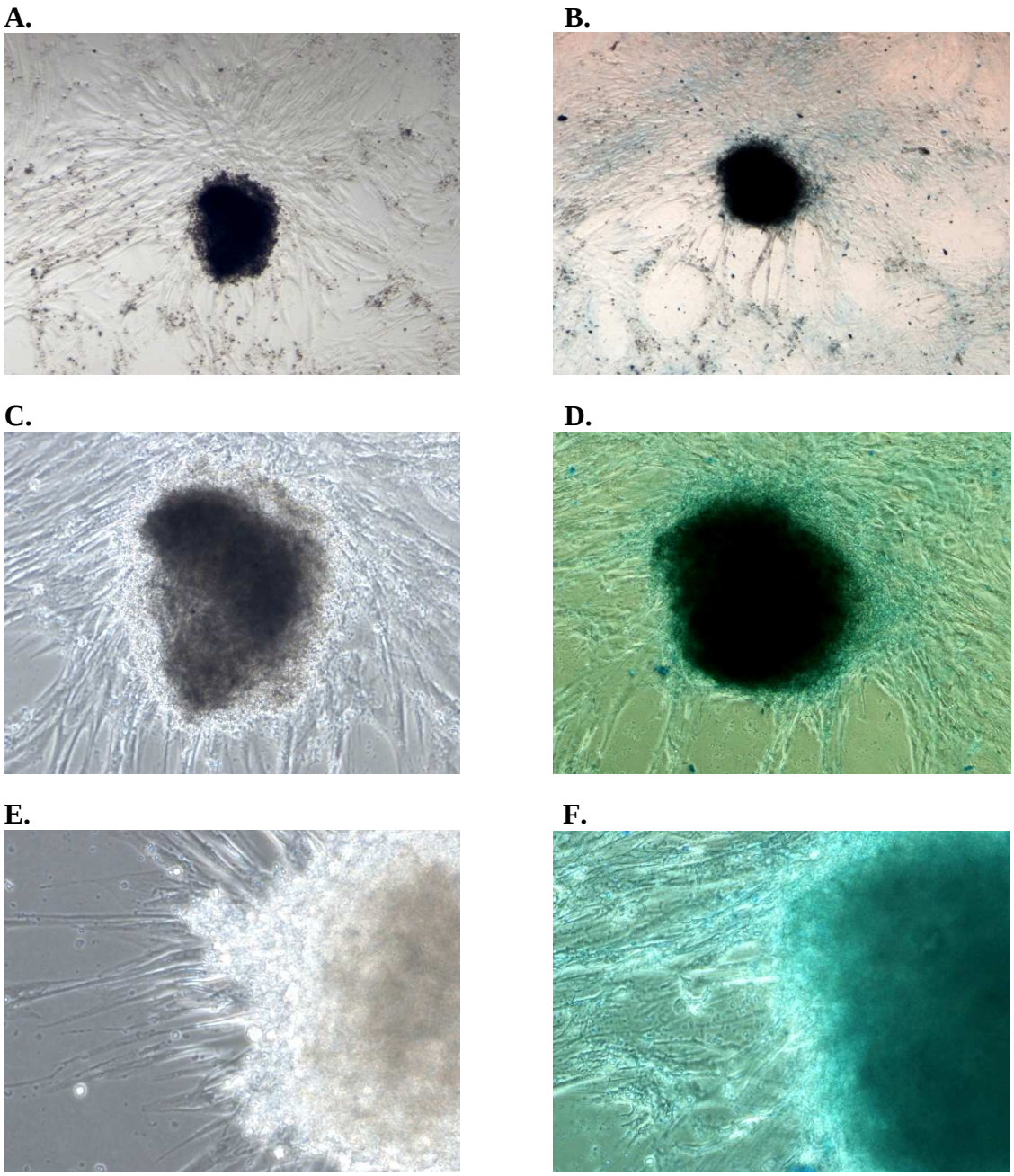

Figure 3.12 Chondrogenic differentiation of hMSCs obtained from adult donors (A-F).

(A-C-E) Differentiated hMSCs before staining(A 4X, B 10X, C 20X magnification)

(B-D-F) Differentiated hMSCs after staining with Alcian blue (D 4X, E 10X, F 20X magnification).

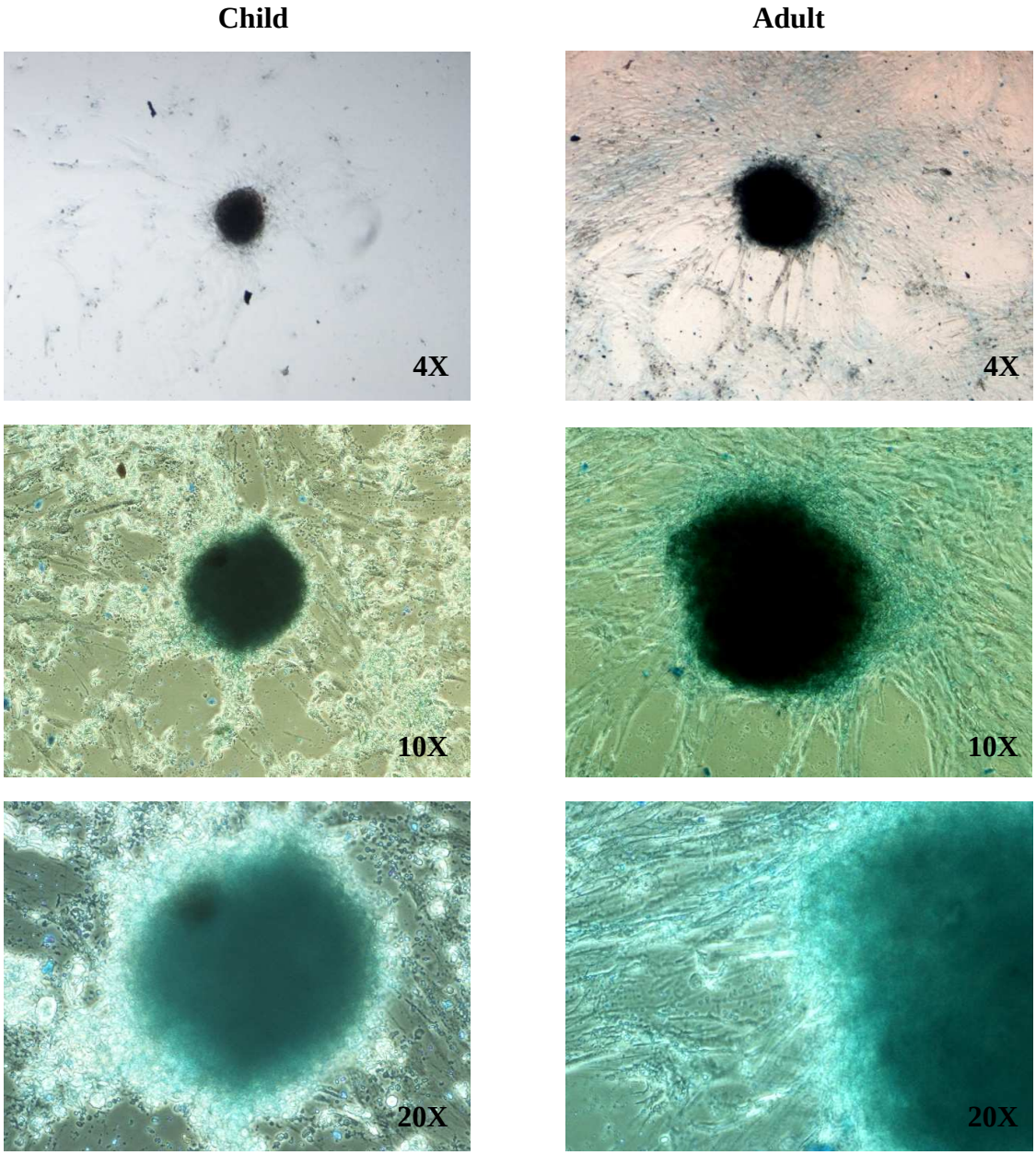

Figure 3.13 Chondrogenic differentiation of hMSCs obtained from child and adult donors, after staining with Alcian Blue.

The effect of donor age on chondrogenesis was more obscure. hMSCs obtained from both child and adult donor generated chondrogenic pellet after 5 weeks under chondrogenic differentiation culture. Chondrogenic pellets stained positively with Alcian Blue, which stains proteoglycans in chondrocytes. Due to our findings, we can say that chondrogenic differentiation potential of hMSCs was independent of donor age.

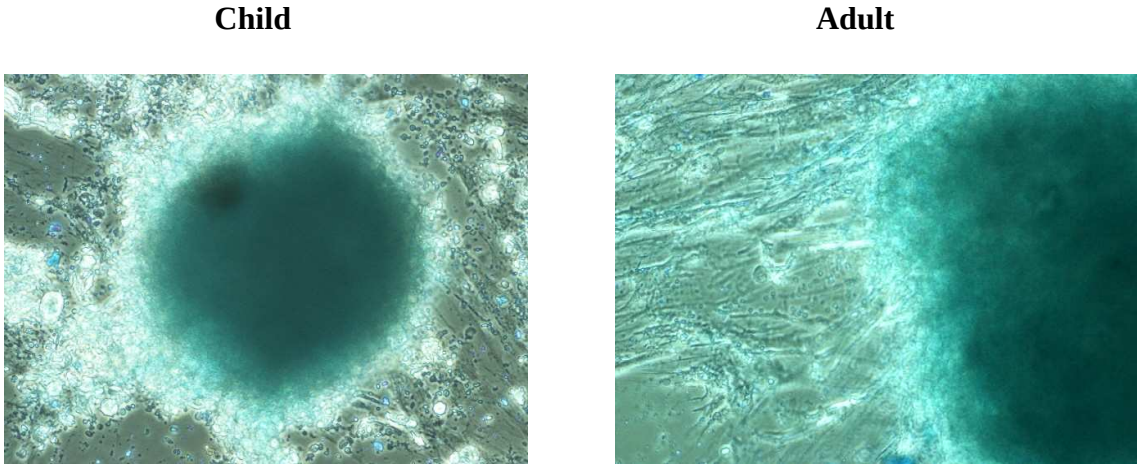

Figure 3.14 Comparison of chondrogenic differentiation.

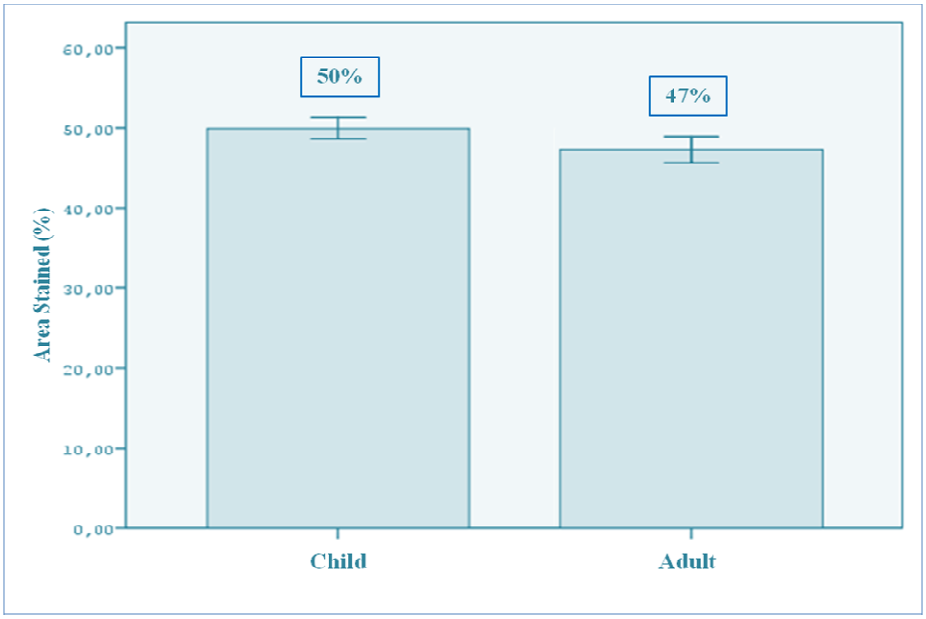

Figure 3.15 Percentage of total area that was positively stained with Alcian Blue.

Quantifying the area stained with Alcian Blue showed no statistically siginificant difference in chondrogenic differentiation of hMSCs obtained from child and adult donors.

3.4.3 Osteogenic Differentiation

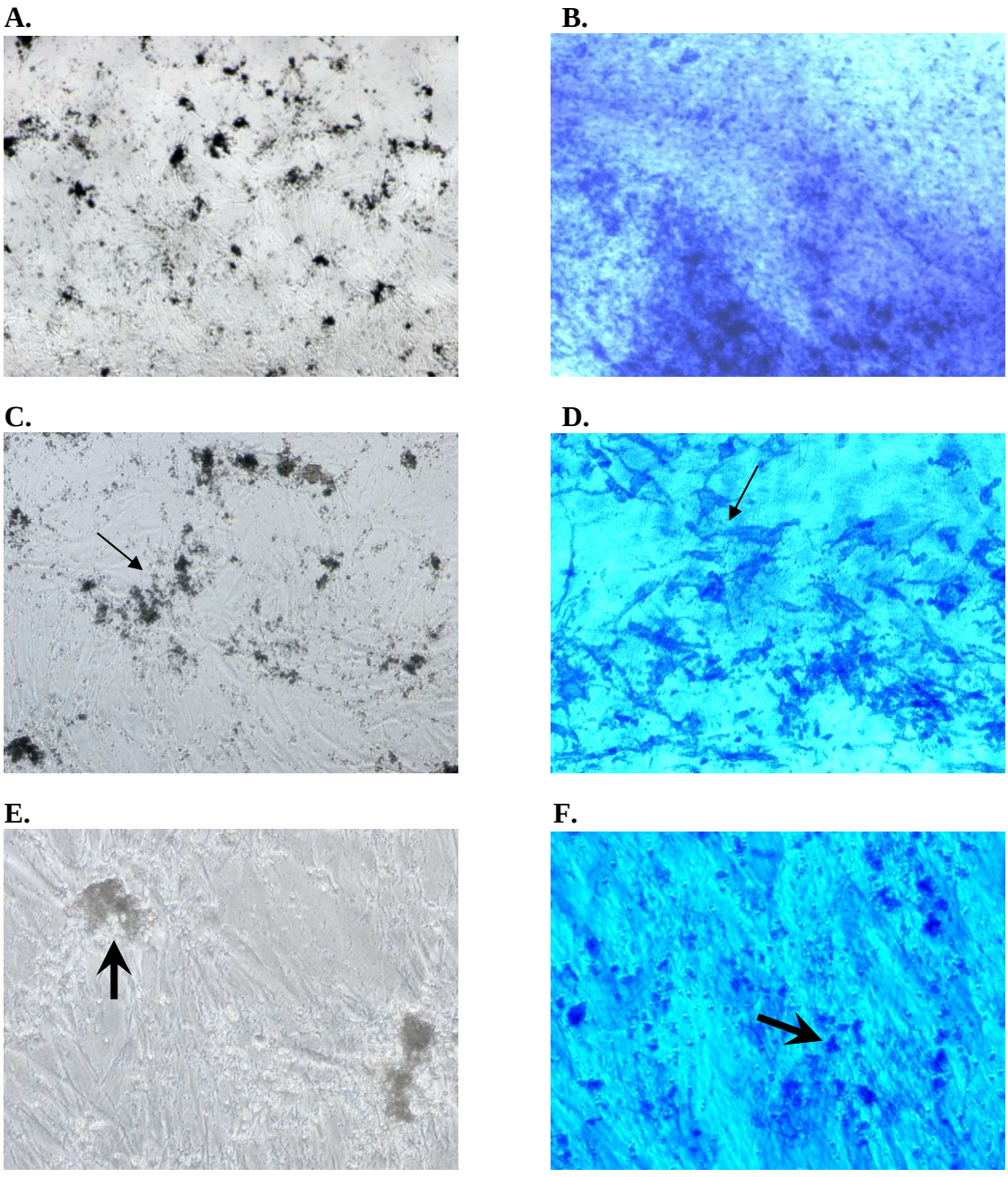

Figure 3.16 Osteogenic differentiation of hMSCs obtained from child donors (A-F).

(A-B-C) Differentiated hMSCs before staining (A 4X, B 10X, C 20X magnification).

(D-E-F) Differentiated hMSCs after staining with Toluidine Blue (D 4X, E 10X, F 20X magnification).

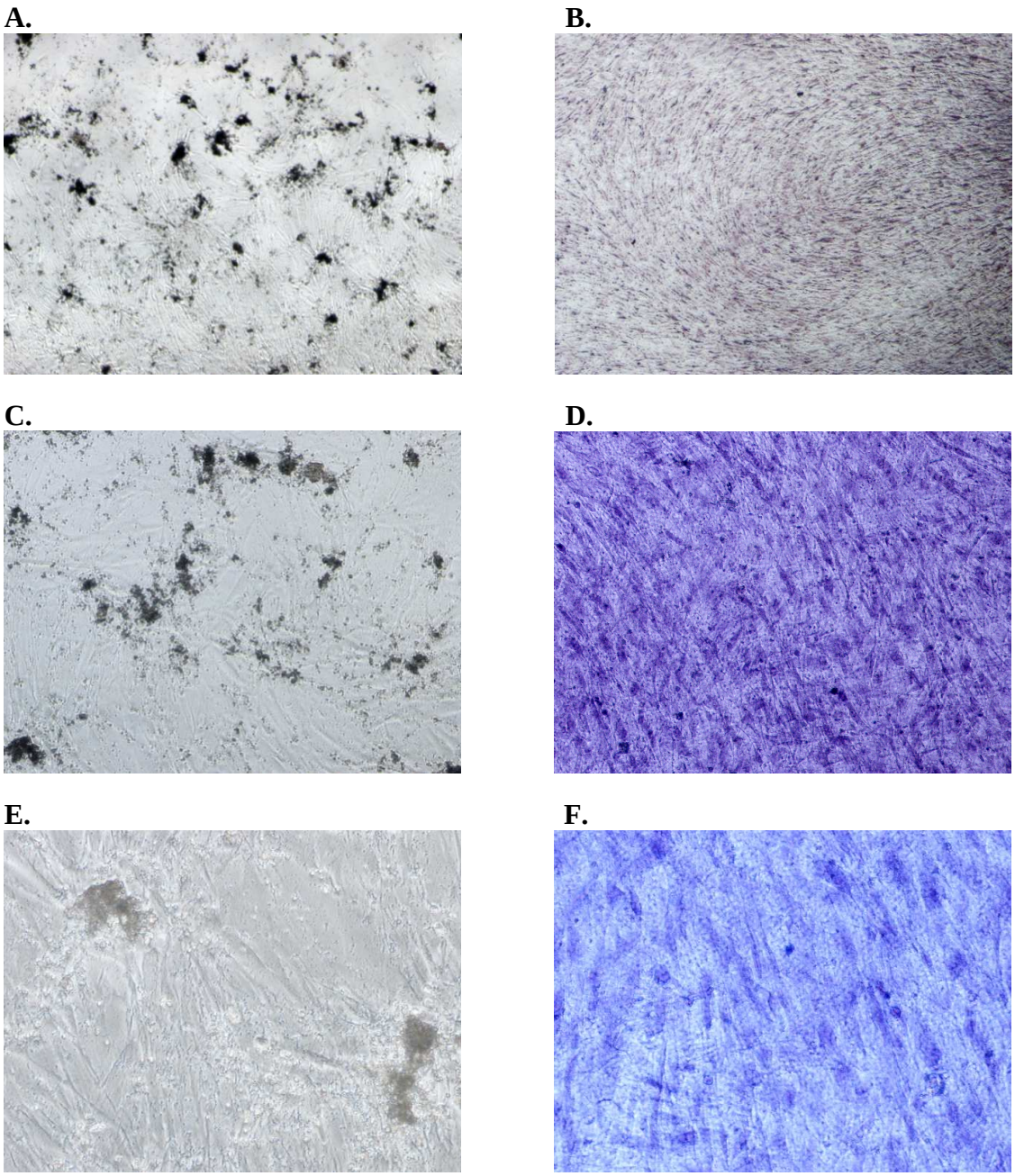

Figure 3.17 Osteogenic differentiation of hMSCs obtained from child donors (A-F).

(A-C-E) Differentiated hMSCs before staining (A 4X, B 10X, C 20X magnification)

(B-D-F) Differentiated hMSCs after staining with hematoxylen (D 4X, E 10X, F 20X magnification)

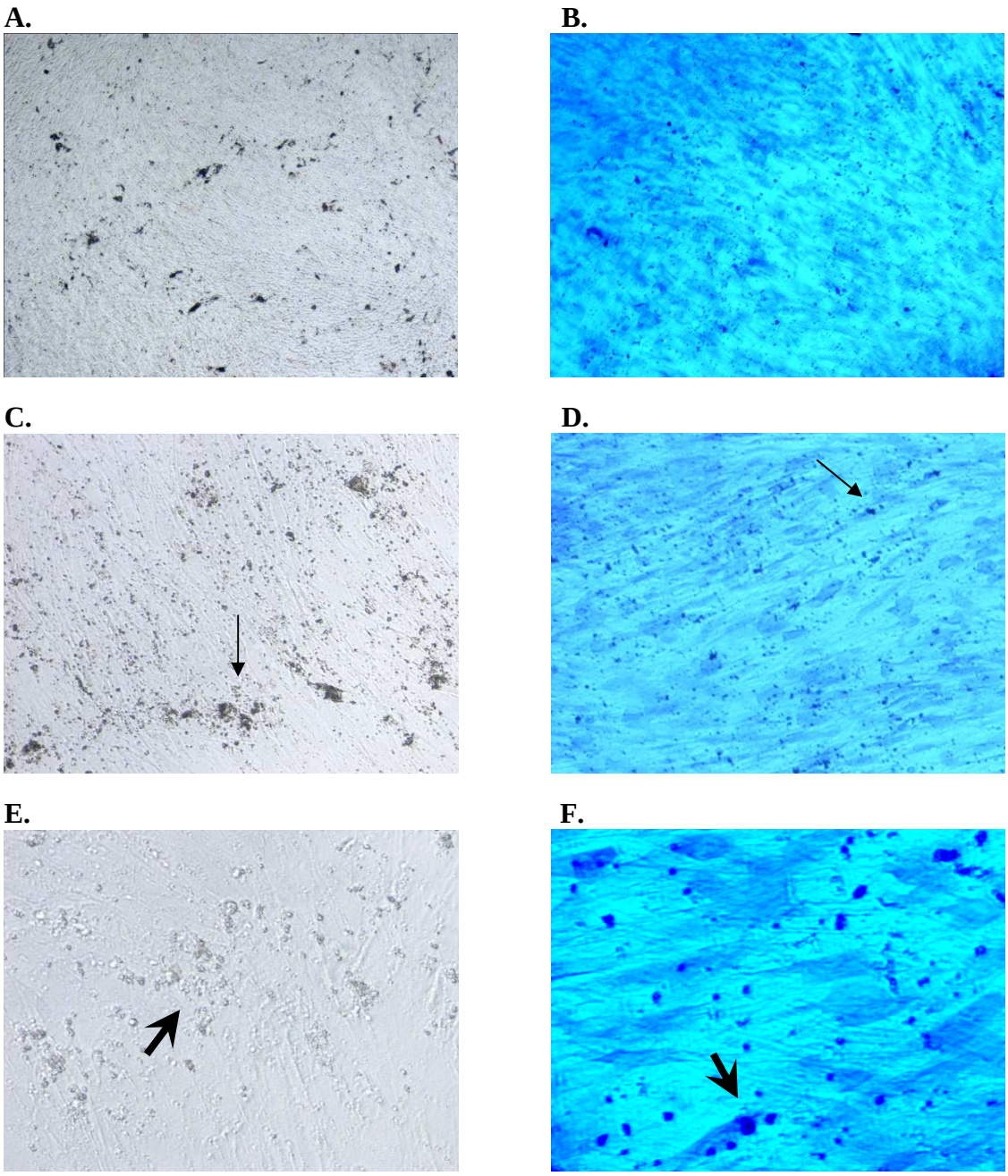

Figure 3.18 Osteogenic differentiation of hMSCs obtained from adult donors (A-F).

(A-C-E) Differentiated hMSCs before staining (A 4X, B 10X, C 20X magnification).

(B-D-F) Differentiated hMSCs after staining with Toluidine Blue (D 4X, E 10X, F 20X magnification).

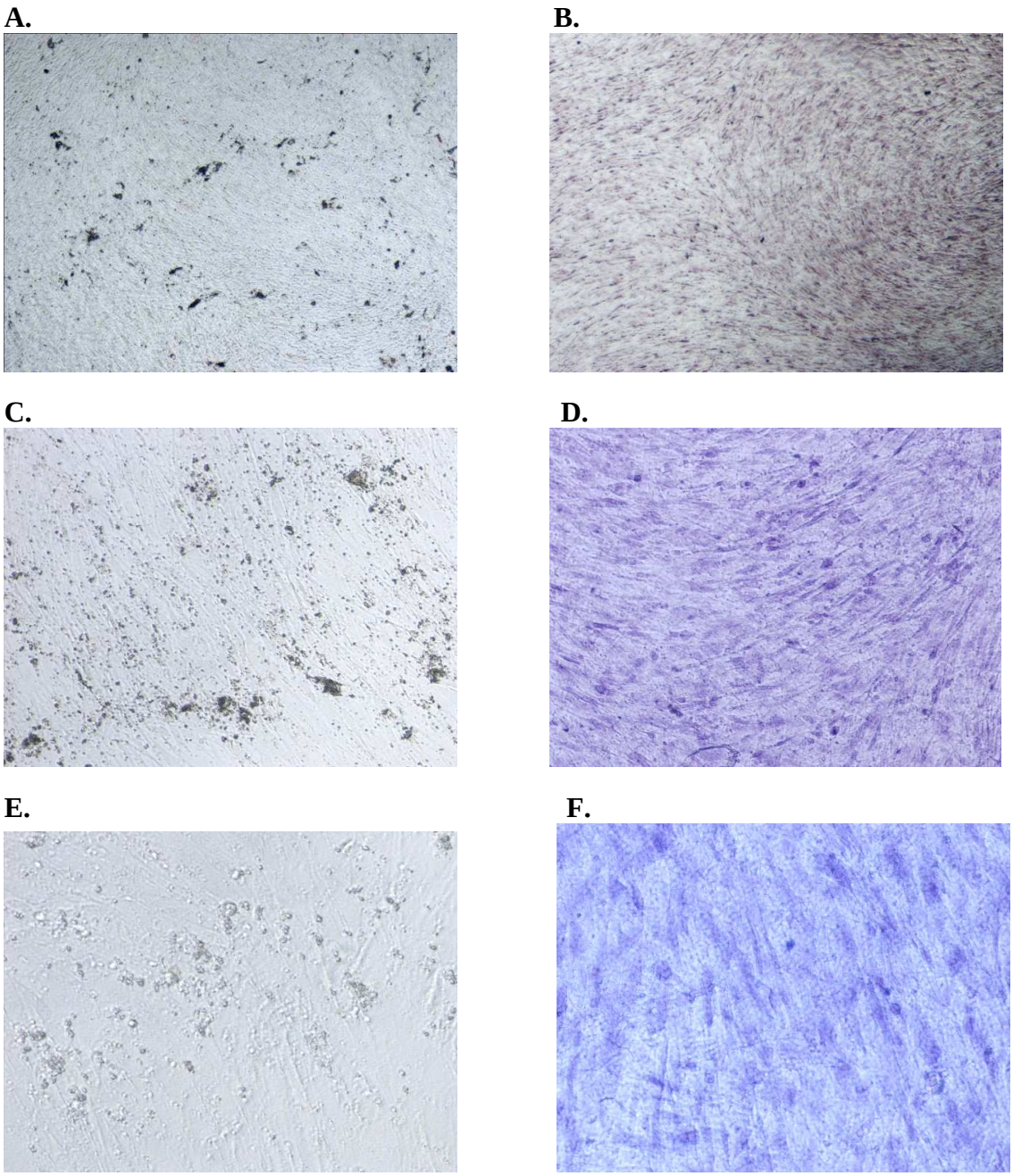

Figure 3.19 Osteogenic differentiation of hMSCs obtained from adult donors (A-F).

(A-B-C) Differentiated hMSCs before staining (A 4X, B 10X, C 20X magnification).

(D-E-F) Differentiated hMSCs after staining with hematoxylen (D 4X, E 10X, F 20X magnification).

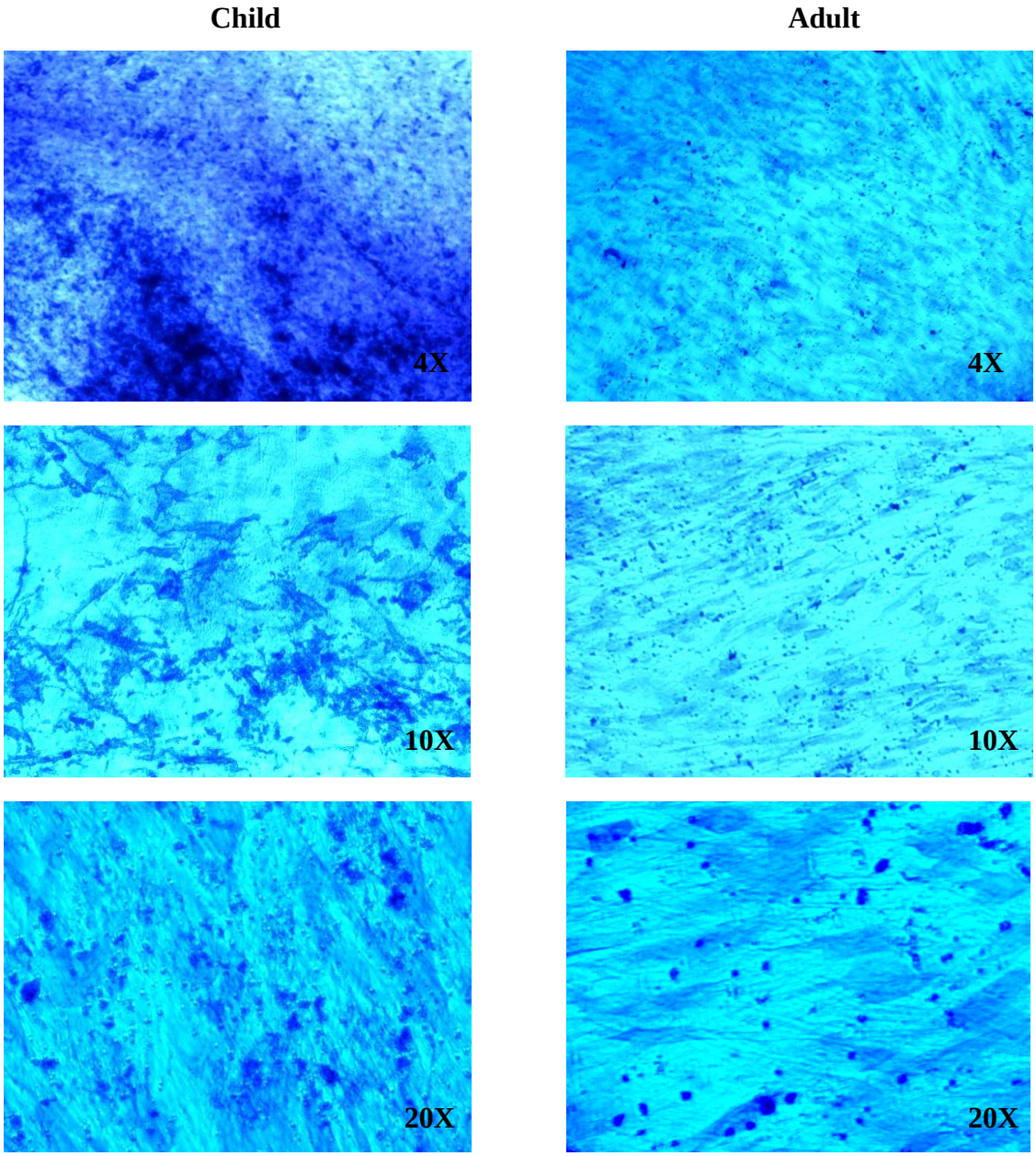

Figure 3.20 Osteogenic differentiation of hMSCs obtained from child and adult donors, after staining with Toluidine Blue .

Osteogenic differentiation potential of hMSCs were adversly affected by increased donor age in terms of calcium content, which is stained by Toluidine blue.

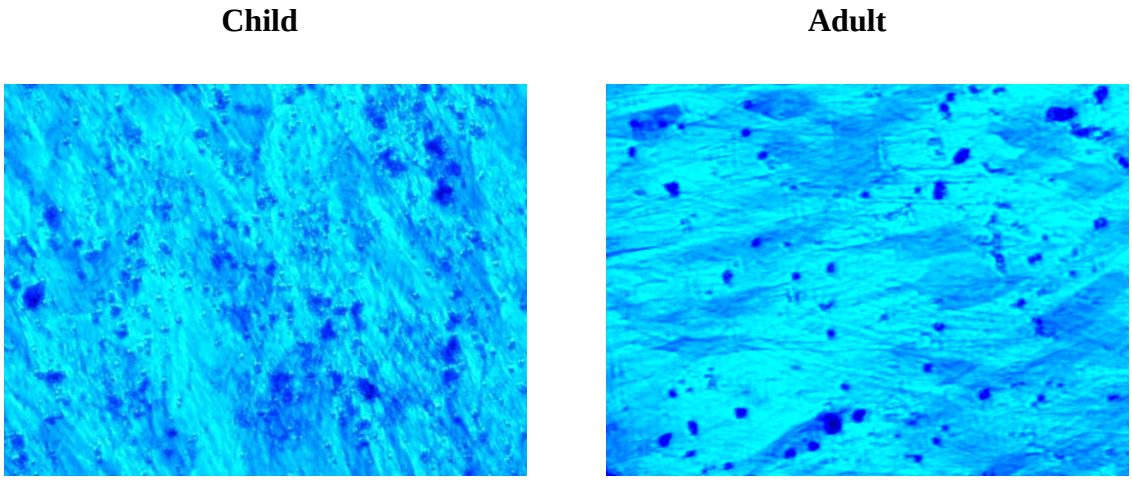

Figure 3.21 Comparison of osteogenic differentiation.

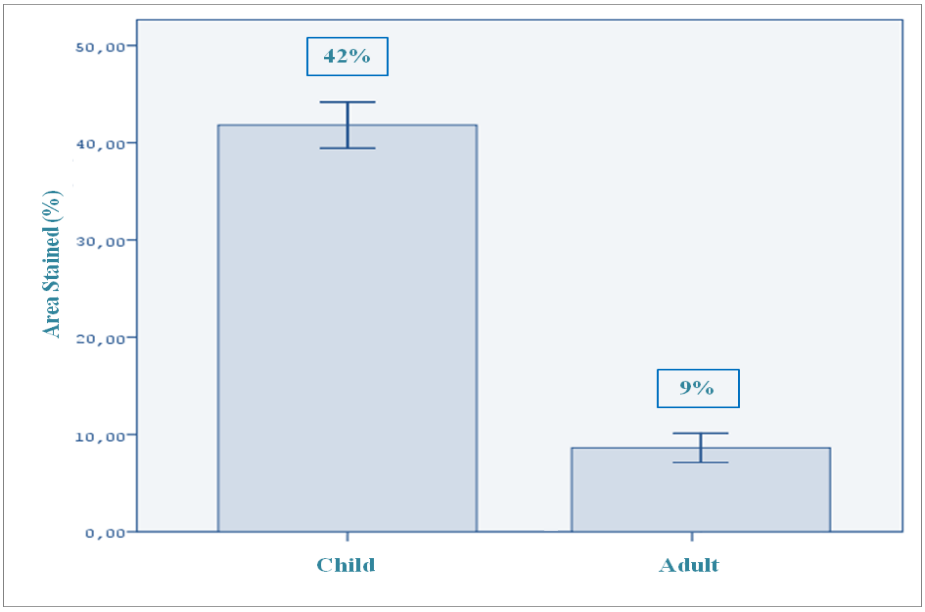

Figure 3.22 Percentage of total area that was positively stained with Toluidine Blue.

Differentiation potential of hMSCs to osteocytes dropped from 42% in child donors to 9% in adult donors. Great number of hMSCs lost their osteogenic differentiation potential due to increasing donor age. Our study also demonstrated that, donor age affected osteogenic differentiation potential of hMSCs more than it affected adipogenic potential.

3.5 NEURAL TRANSDIFFERENTIATION OF hMSCs

A. **B.**

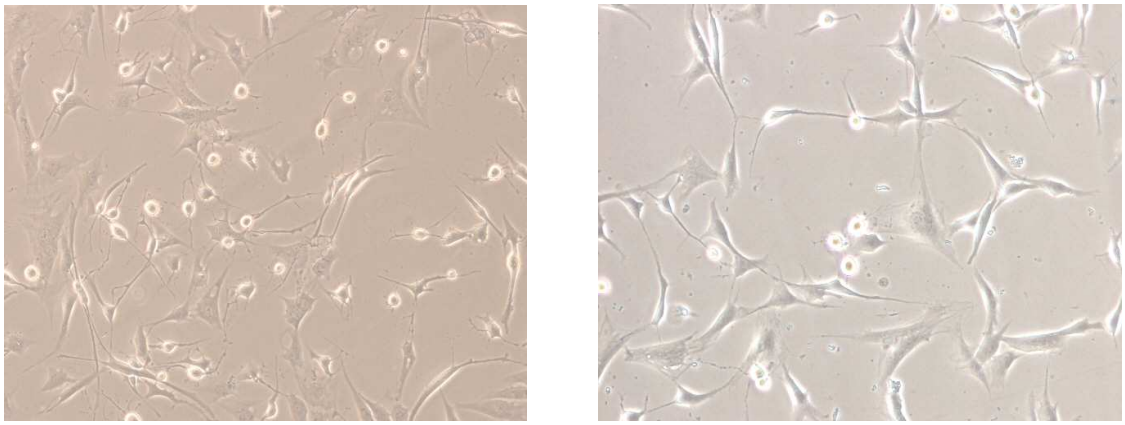

Figure 3.23 Neural transdifferentiation of hMSCs obtained from child and adult donors, with N3 cytokine combination (A-B).

(A) Differentiated hMSCs obtained from child donors.

(B) Differentiated hMSCs obtained from adult donors

Images are taken under 10X magnification.

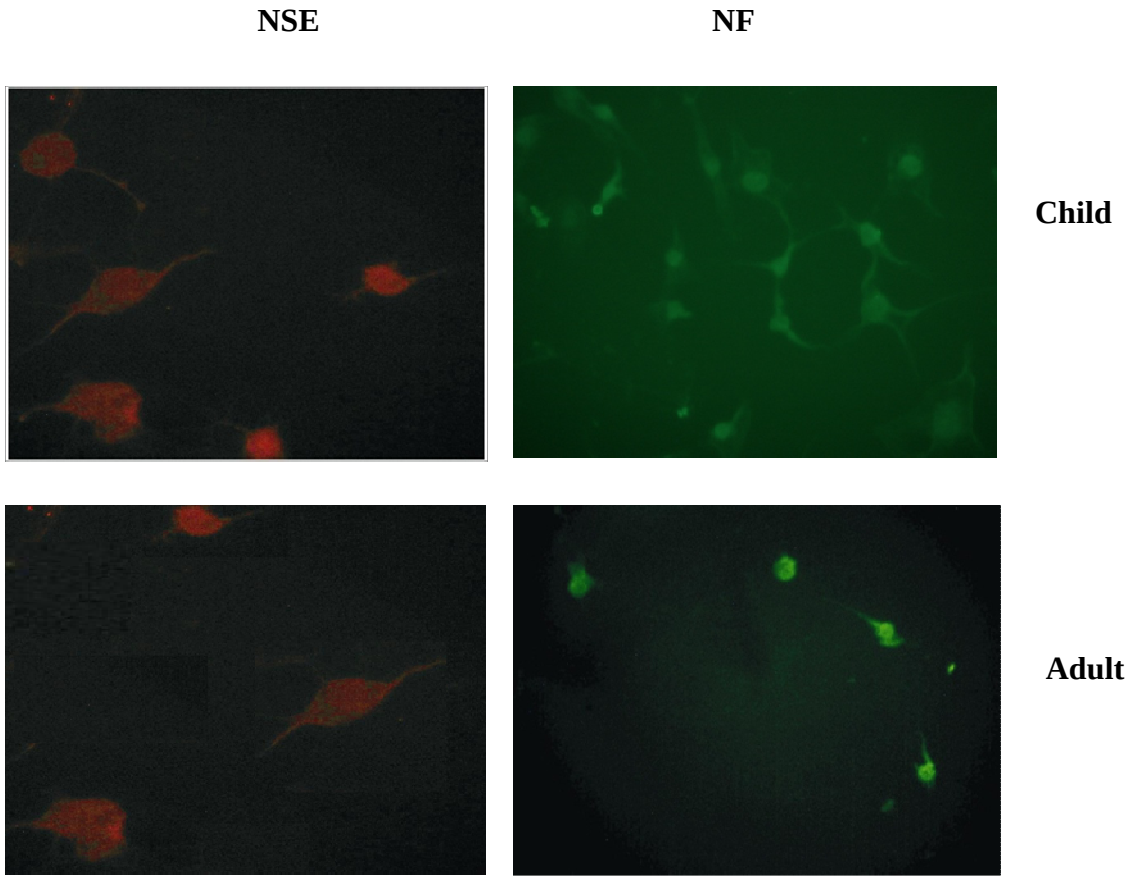

Figure 3.24 Immunostaining of neural markers (NSE and NF) during neural transdifferentiation of hMSCs obtained from child and adult donors.

MSCs harvested from both child and adult donors transdifferentiated into neural phenotype when treated with N3 cytokine combination. Immunostaining results of early neural marker NSE and late neural marker NF confirmed neural transdifferentiation of these cells. hMSCs from child donors had greater number of differentiated cells; which showed higher expression of neural markers than adult donors.

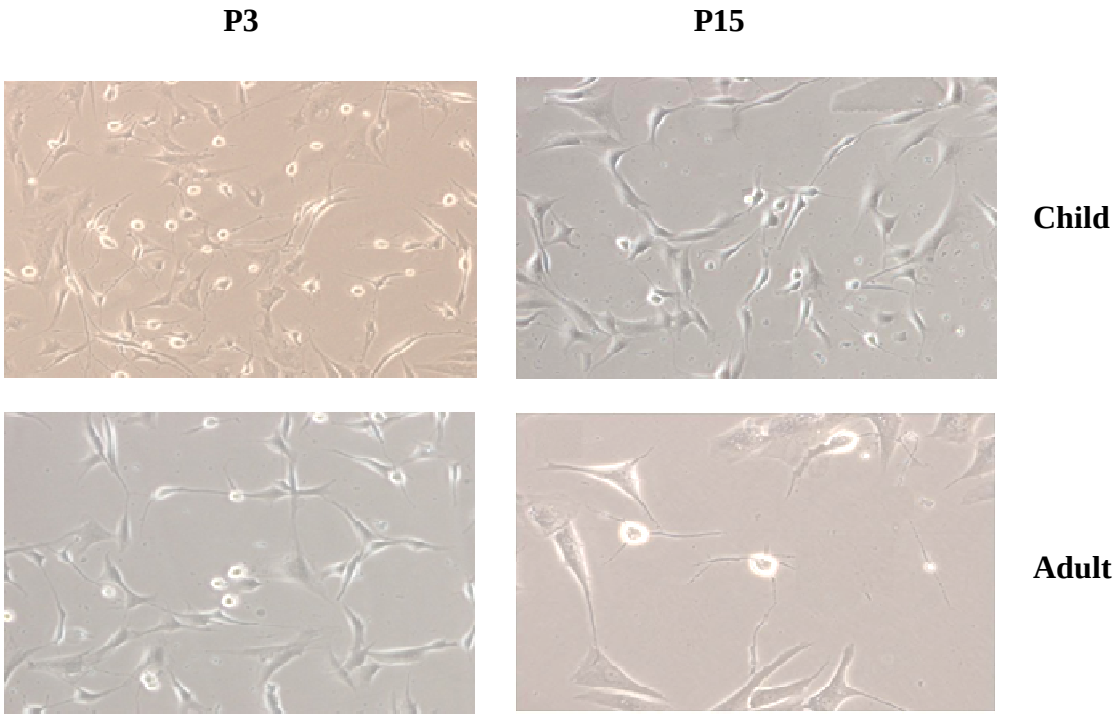

Figure 3.25 Comparison of N3 mediated neural transdifferentiation.

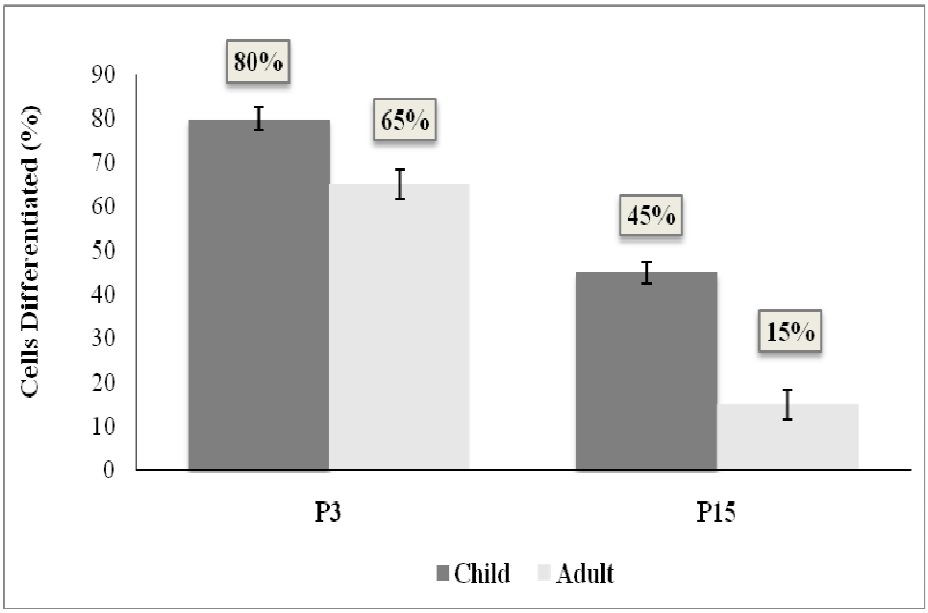

Figure 3.26 Percentage of neural transdifferentiated cells.

Neural differentiation efficiency was quantified by determining the percentage of morphologically neural differentiated cells. Neural transdifferentiation potential of hMSCs decreased with age and repeated passage nearly abrogates neural transdifferentiation in adult donors.

3.6 RT-PCR RESULTS

3.6.1 RT-PCR results obtained from child donors.

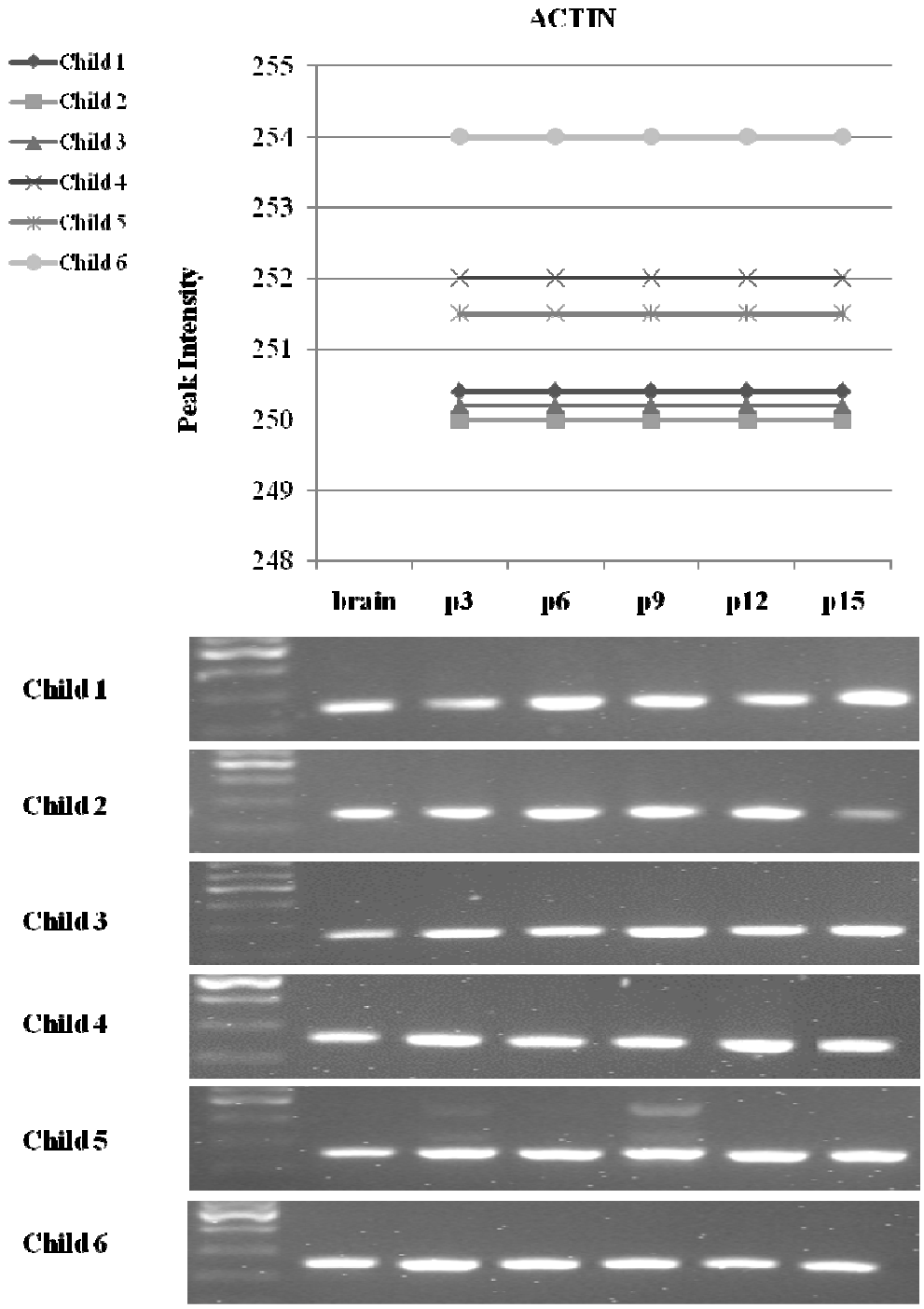

Figure 3.27 Actin (208 bp) expressions of hMSCs, obtained from child donors, at different passages.

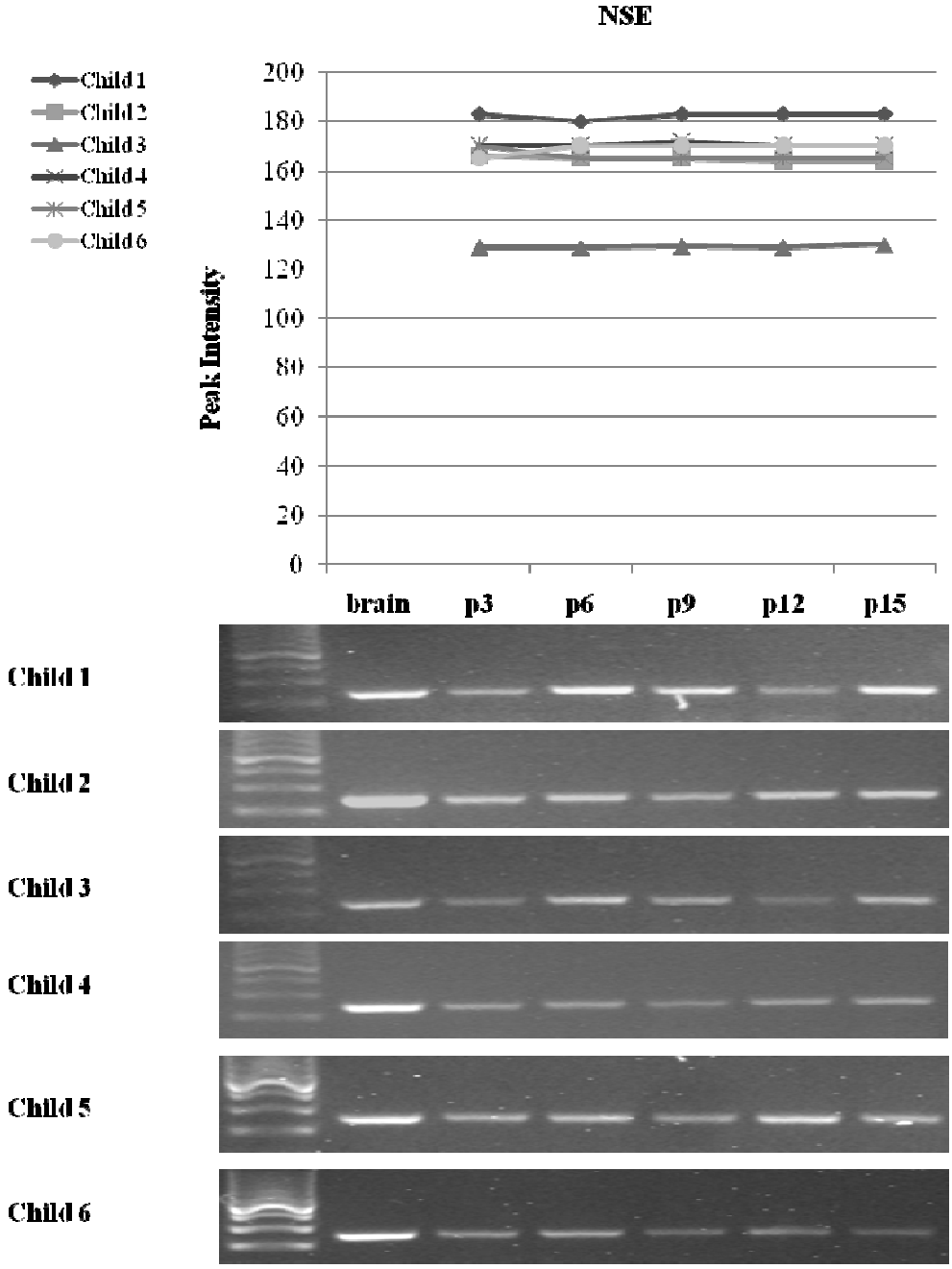

Figure 3.28 NSE (254 bp) expressions of hMSCs, obtained from child donors, at different passages.

RT-PCR results of NSE expression of undifferentiated hMSCs indicated that, there was donor-related heterogeneity in the expression level of NSE marker. Each donor revealed a unique expression pattern.

Except inter-donor variability, there was no passage related increase in the expression of this marker.

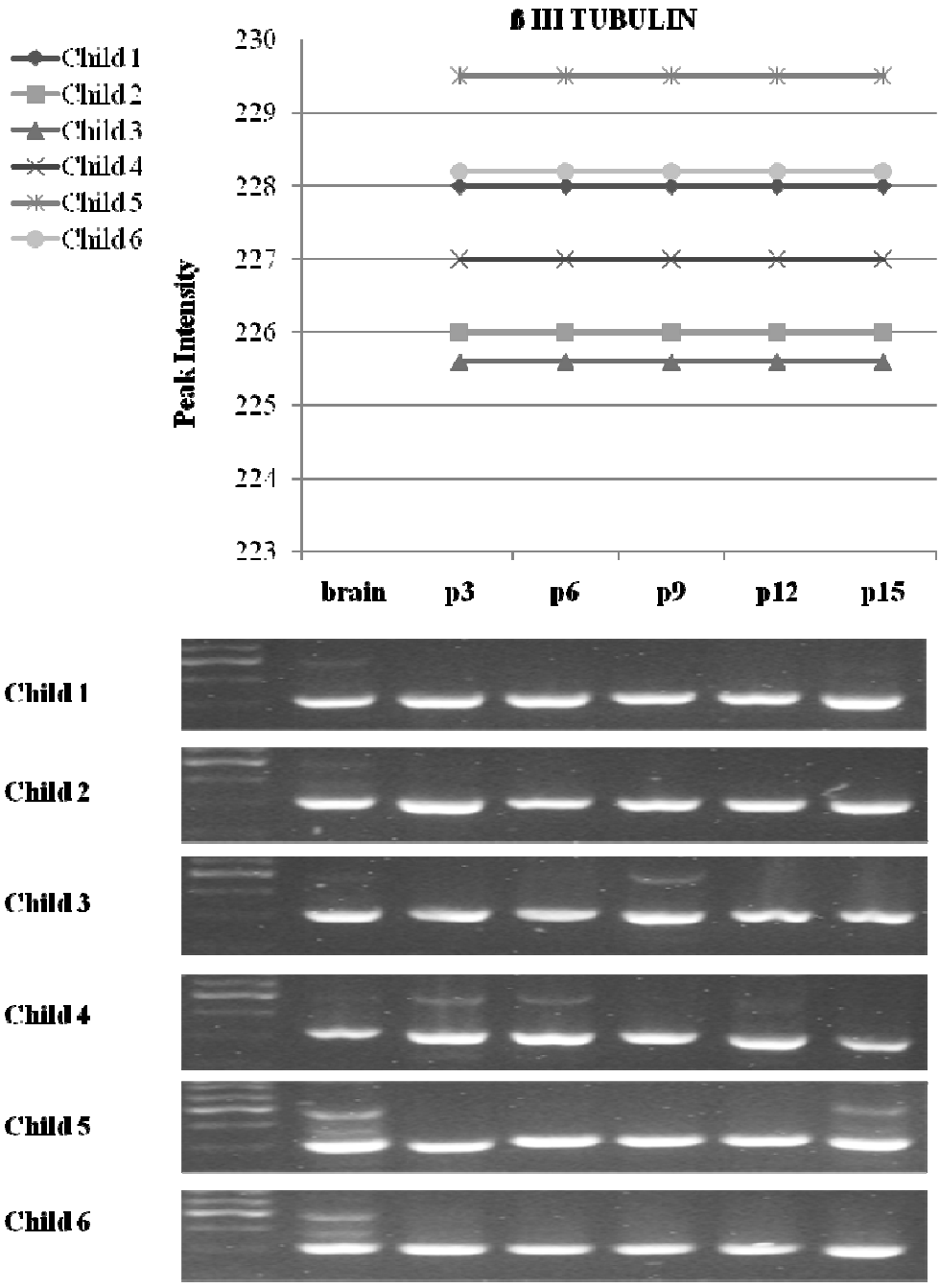

Figure 3.29 β III Tubulin (317 bp) expressions of hMSCs, obtained from child donors, at different passages.

Expression level of β III Tubulin changed according to donor, but expression of this marker didn't increase due to increasing passage number.

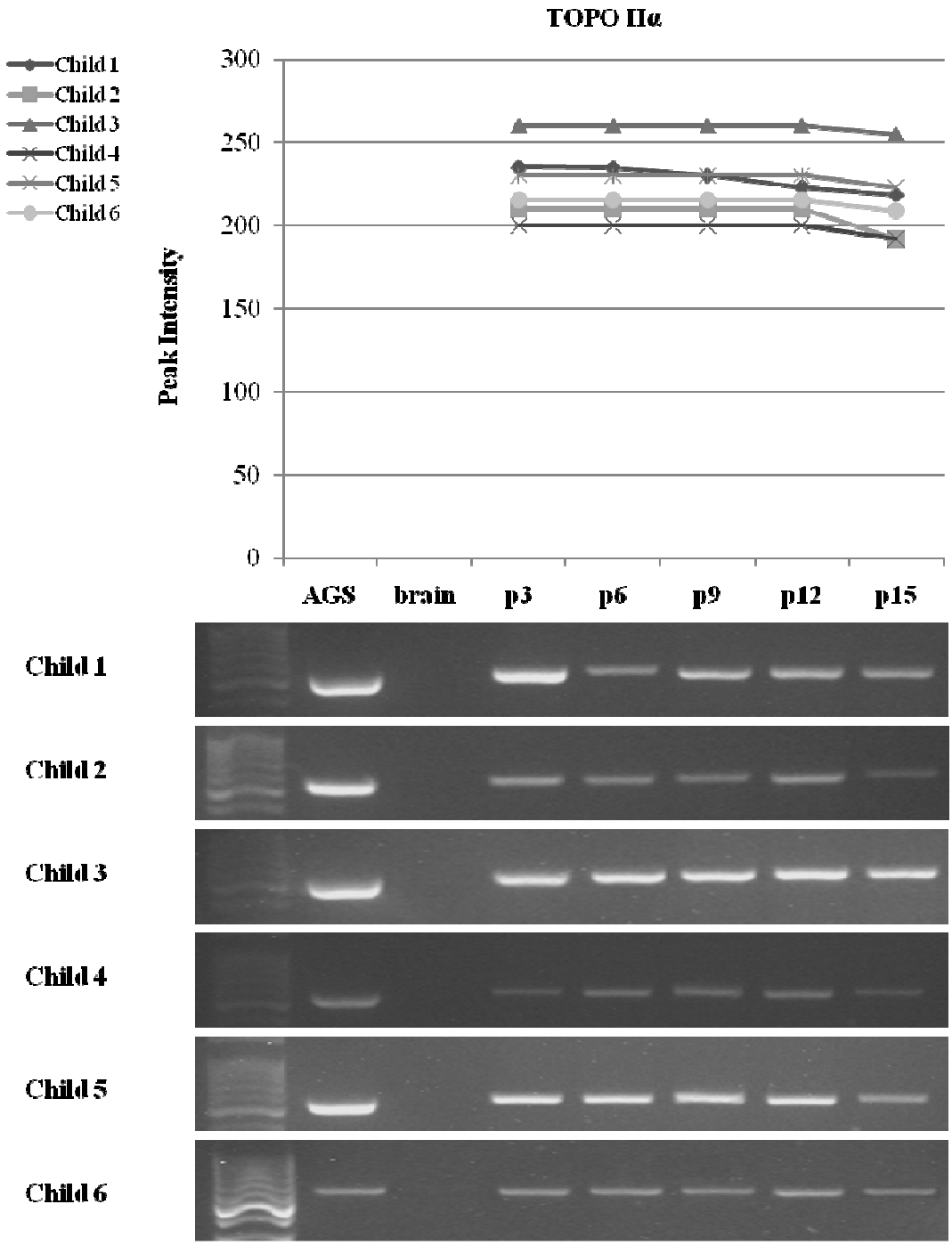

Figure 3.30 Topo IIα (596 bp) expressions of hMSCs, obtained from child donors, at different passages. AGS was used as (+) control, Brain was used as (-) control.

RT-PCR results of topo IIα, which is found only in dividing cells, showed that proliferation potential of MSCs from child donors decrease after P12.

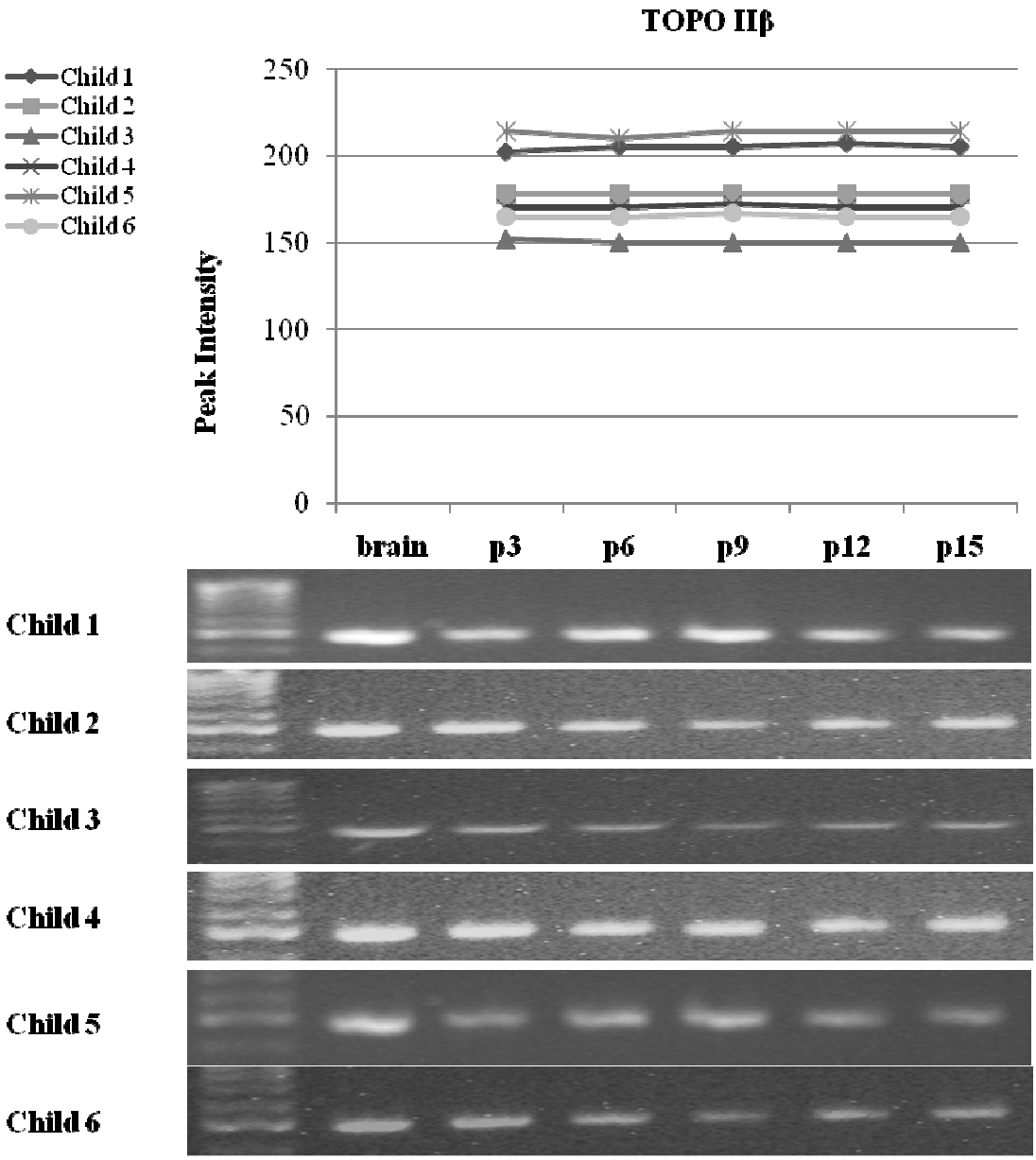

Figure 3.31 Topo IIβ (508 bp) expressions of hMSCs, obtained from child donors, at different passages.

Except high degree of donor heterogeneity, there was no significant difference between passage numbers in topo IIβ expression of hMSCs.

3.6.2 RT-PCR results obtained from adult donors.

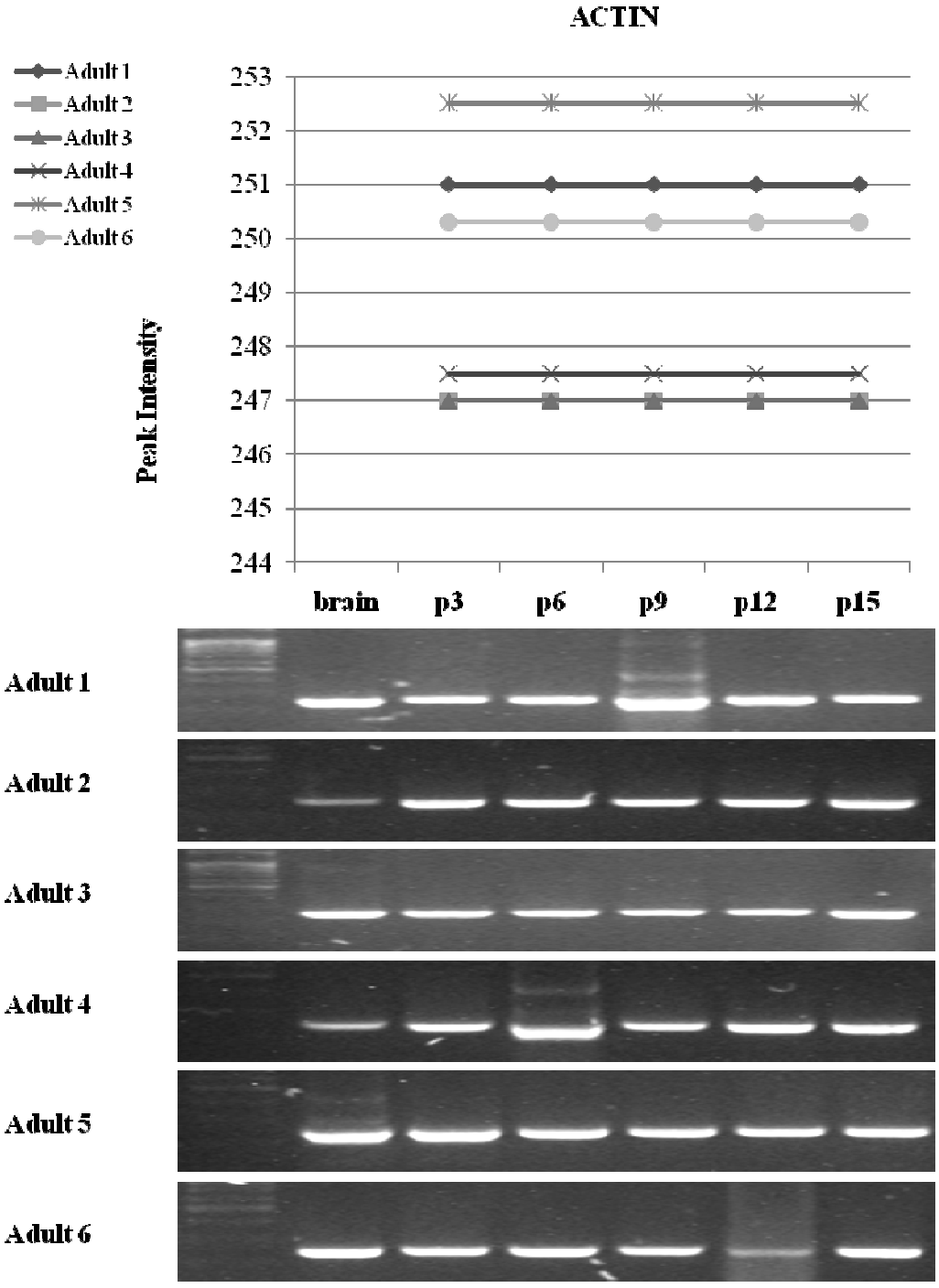

Figure 3.32 Actin (208 bp) expressions of hMSCs, obtained from adult donors, at different passages.

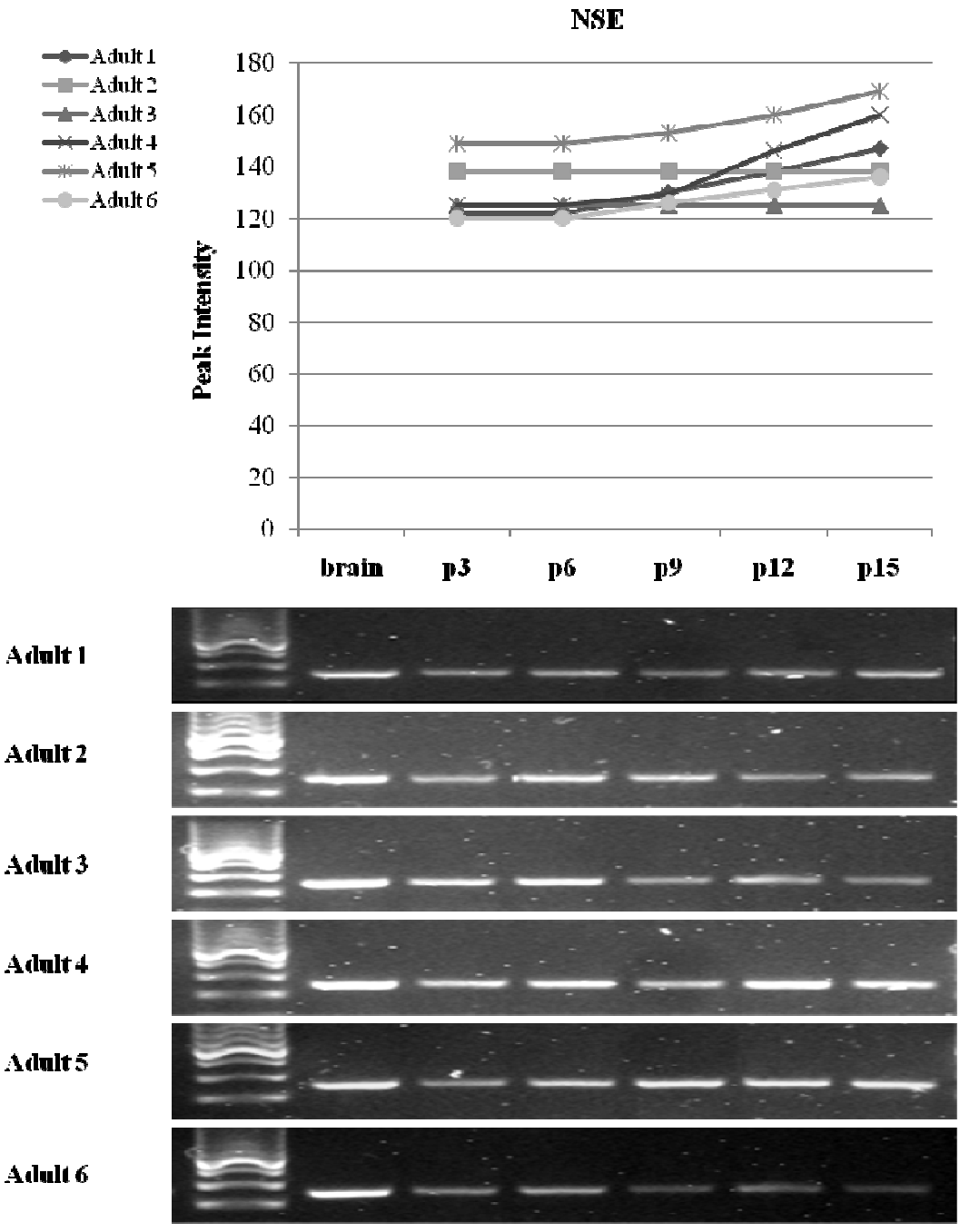

Figure 3.33 NSE (254 bp) expressions of hMSCs, obtained from adult donors, at different passages.

Donor related heterogeneity was observed between adult donors in the expression level of NSE. However; MSCs from all donors revealed increasing expression pattern due to increasing passage number.

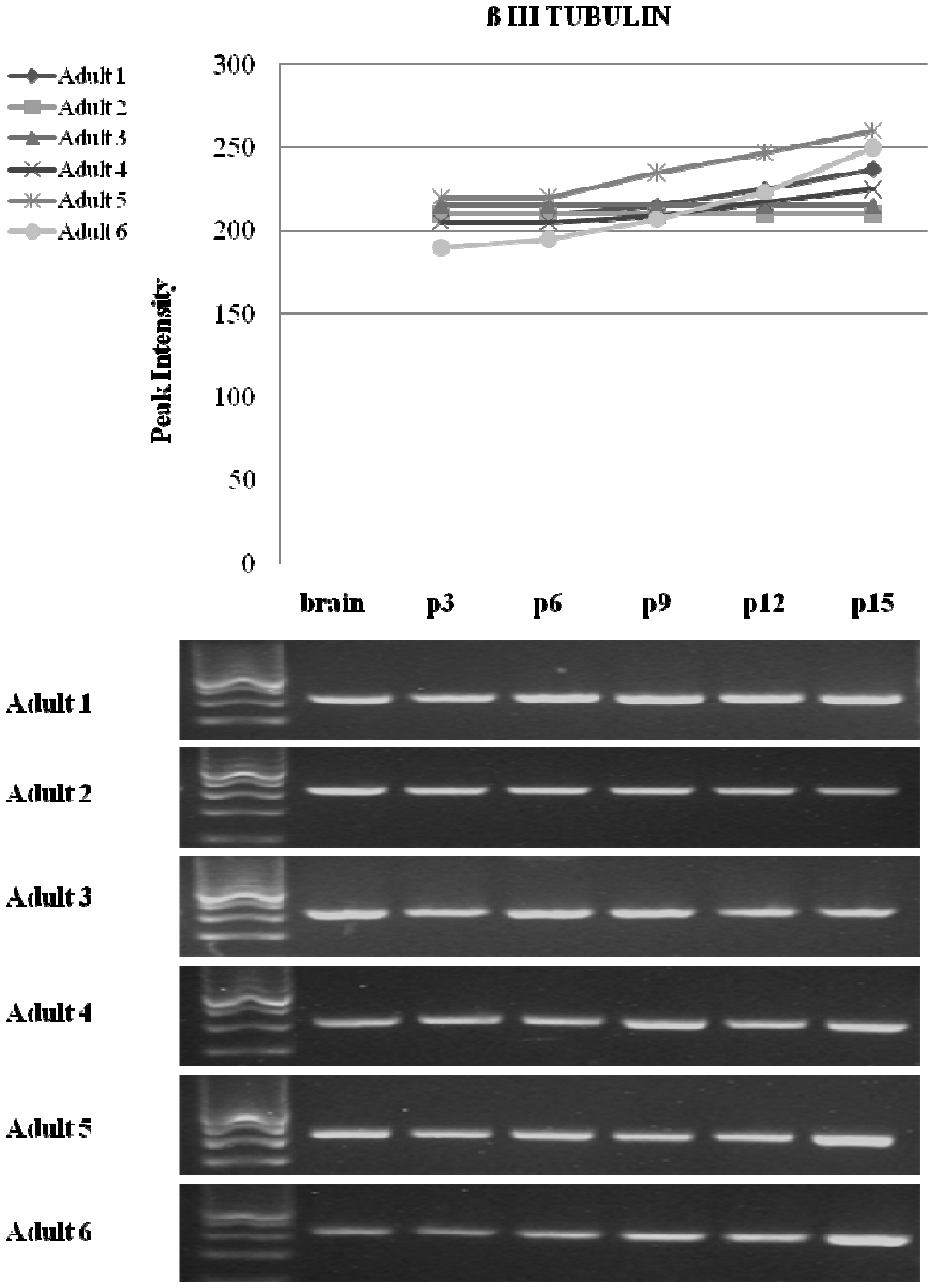

Figure 3.34 β III Tubulin (317 bp) expressions of hMSCs, obtained from adult donors, at different passages.

β III tubulin expression level increased gradully with serial passage.

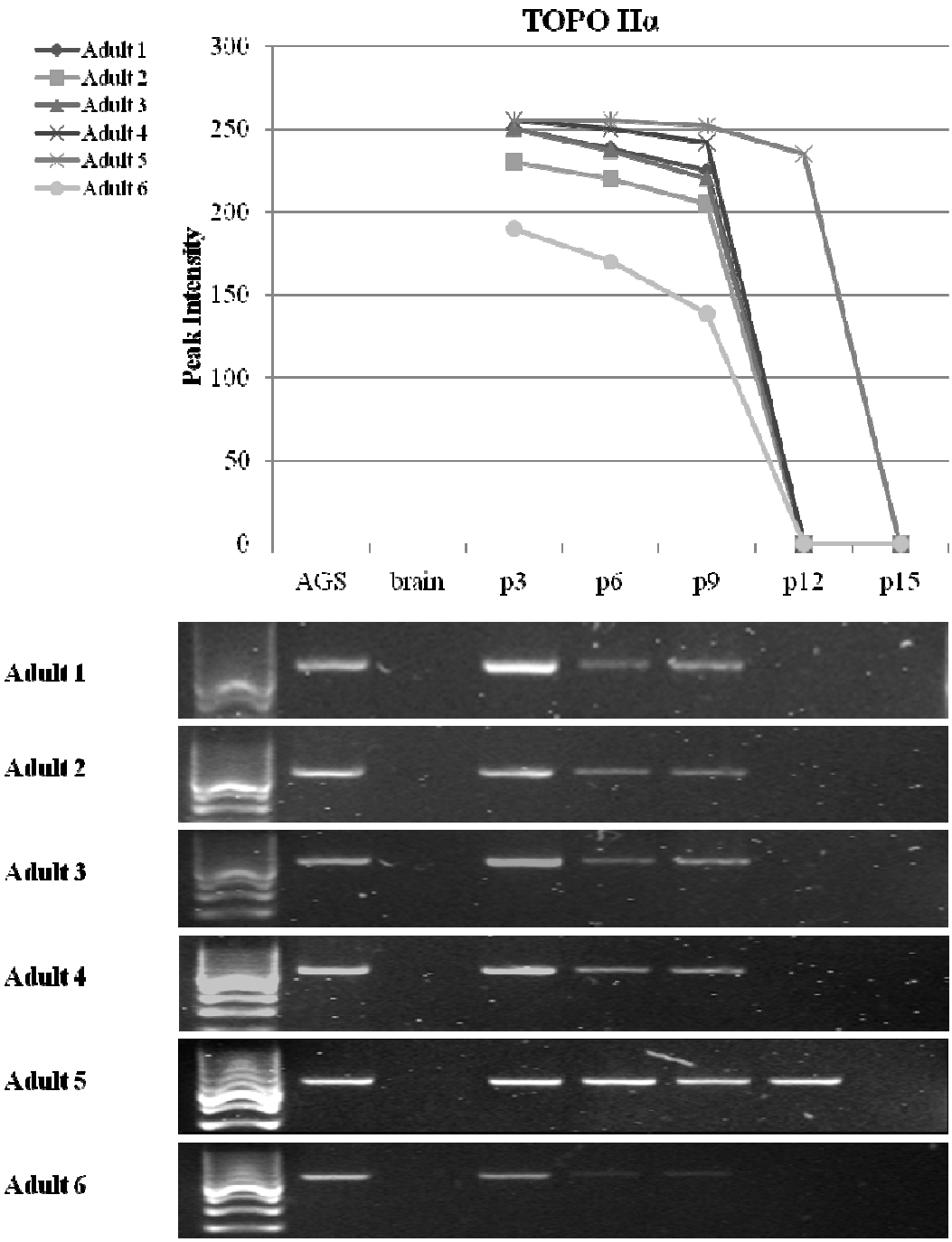

Figure 3.35 Topo IIα (596 bp) expressions of hMSCs, obtained from adult donors, at different passages. AGS was used as (+) control, Brain was used as (-) control.

RT-PCR results of topo IIα, which is found only in dividing cells, showed that proliferation potential of MSCs from adult donors decreased due to long term passage. Our result indicated that, cells stopped proliferating after P9; which corresponded with growth curve and observed morphological changes.

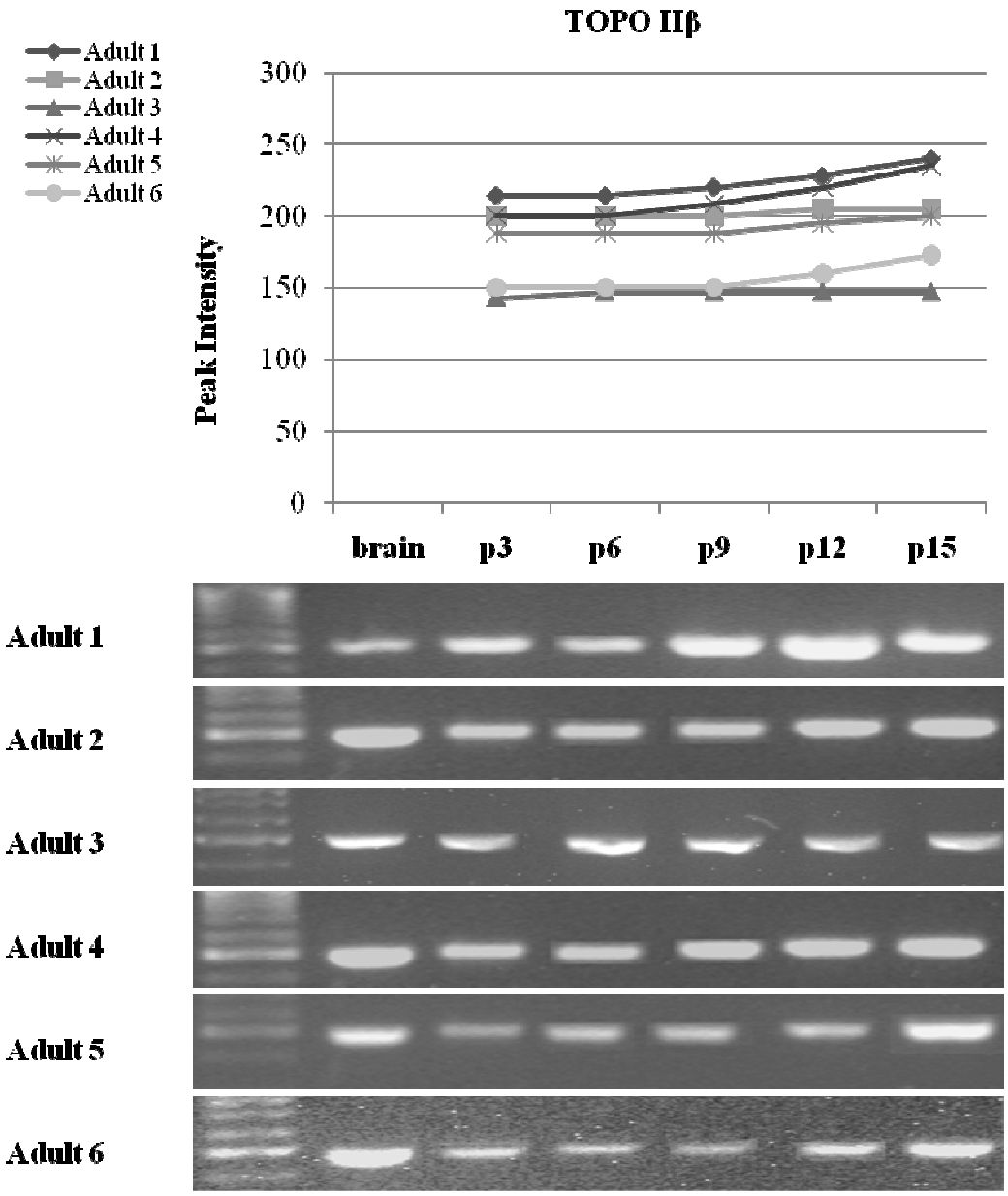

Figure 3.36 Topo IIβ (508 bp) expressions of hMSCs, obtained from adult donors, at different passages.

In addition to neural markers β III tubulin and NSE, increasing expression of topoIIβ was also observed due to *in vitro* serial propagation.

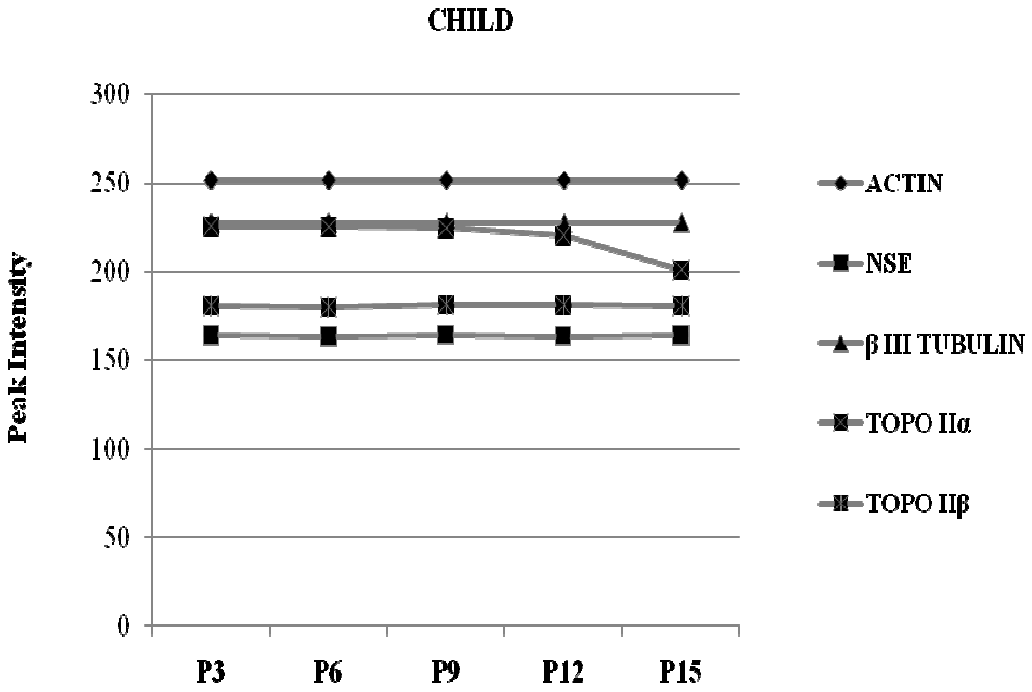

Figure 3.37 Average actin, NSE, β III tubulin, topo IIα and topo IIβ marker expressions of hMSCS from child donors.

There was no significant difference between passage numbers in neural marker (NSE and β III tubulin) and topo IIβ expression patterns of hMSCs from child donors. Increasing marker expression level didn't observed with increasing passage number.

Topo IIα level was decreased after P12, corresponding with the growth curve and observed morphological changes.

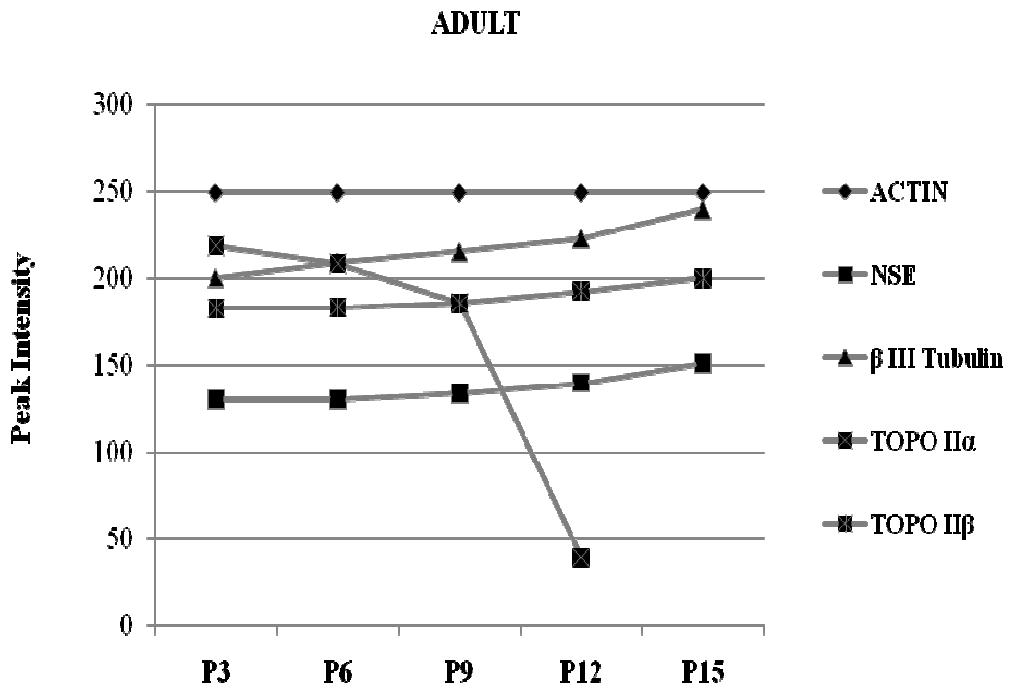

Figure 3.38 Average actin, NSE, β III tubulin, topo IIα and topo IIβ marker expressions of hMSCS from adult donors.

Neural marker (NSE and β III tubulin) expression level increased due to increasing passage number, in paralel with topo IIβ level, in hMSCs from adult donors. Depending on donor, expression levels started to increase at P9.

Topo IIα expression level decreased due to long term passage and the expression was lost after P9 confirming the lost of proliferation potential.

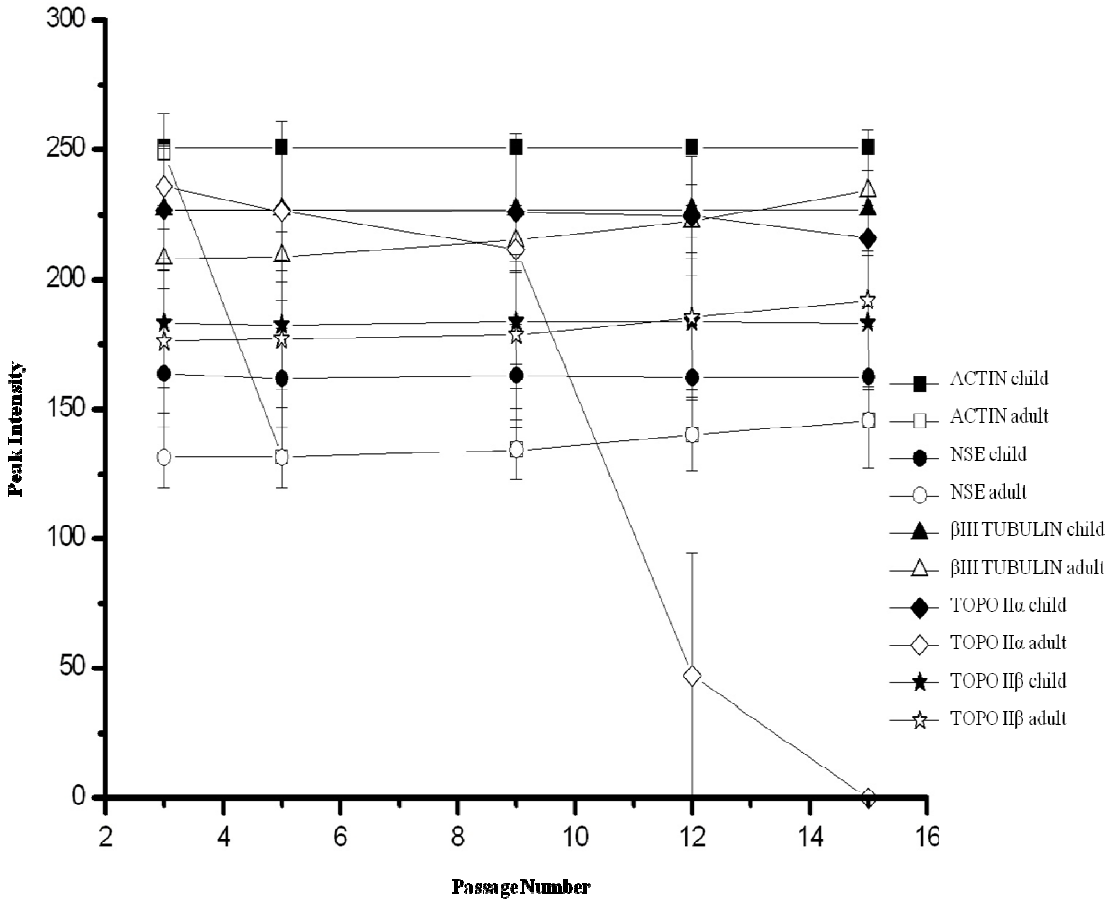

Figure 3.39 Average marker expression levels of hMSCS from child and adult donors.

Figure 3.39 shows average peak intensity levels of five different markers of hMSCs obtained from both child and adult donors at the same graphic.

CHAPTER 4

DISCUSSION & CONCLUSION

Many promising applications of tissue engineering require cell expansion and involve the treatment of diseases and conditions found in an aging population [81]. Therefore, the effect of donor age and *ex vivo* handling must be understood in order to develop clinical techniques based on MSCs.

In the present study, we investigated the effects of donor age on proliferation potential, morphology and differentiation ability of hMSCs towards adipogenic, chondrogenic, osteogenic and neurogenic lineages. There is currently little consensus and in many cases conflicting reports regarding effects of donor age on MSCs. A study have previously reported no age related differences in differentiation using human BMSCs [85]; however, many studies demonstrating no change in differentiation have found changes in proliferation, attachment or self-renewal in mouse [81], rat [88], and human [89] BMSCs. It was shown that tissue regenerative capacity, decline with age and this decline has been attributed to the reduction of number and differentiation capacities of MSC [92-94].

During the initial phases of growth, we observed hMSCs of all ages had spindle type morphology, in agreement with Stolzing et al. [70]. During *in vitro* serial propagation, cells gradually lost their morphology. Cells obtained from adult and old donors lost their fibroblast like morphology at earlier passages than child donors. Incerasing donor age accelerated changes in hMSC morphology.

We also found clear differences in growth pattern of hMSC obtained from child, adult and old donors with a decrease in the proliferation rate of hMSC with donor age. hMSCs obtained from child donors could be expanded for approximately 37

population doublings (PD) in about 120 days (17 weeks) *in vitro*; however maximal PD decreased to 30 population doublings for cells obtained from adult donors. hMSCs obtained from old donors exhibit a significant decrease in their proliferative potential with 10 PD. Our findings demonstrated a negative correlation between donor age and the proliferative potential of cells. Cells obtained from adult and old donors exhibited decreased proliferation potential compared with cells obtained from child donors.

Several clinical and histomorphometric studies have demonstrated that aging is associated with decreased bone mass and that decreased bone formation is an important pathogenic factor **[**23]. The majority of reports describe a loss of MSC osteogenic potential with donor age regardless of species [51]. Peng et al. found that expression of osteogenesis-related genes peaked very early following induction in MSCs [28].

In contrast, age related changes *in vitro* chondrogenic differentiation have not been well investigated. Im et al., found that cartilage shows an age-related decline in its repair capacity [37]. One report of Murphy et al., has shown a reduction in chondrogenesis in MSC from osteartritis patients [46], but failed to demonstrate a direct age-related decline in the chondrogenic potential in MSC from normal donors.

In this study, our results are in agreement with previous work which found donor age affected osteogenic differentiation of hMSCs. The amount of calcium accumulation in differentiated cells was lower in adult donor. BM derived hMSCs obtained from adult donor exhibited decreased potential for osteogenic differentiation than hMSCs from child donor. Great number of hMSCs lost their osteogenic differentiation potential with age.

We have found an age related decrease in osteoblastic but not chondrogenic differentiation potential. Rather than an adverse effect of increased donor age, no significant decrease was observed; supporting the hypothesis that chondrogenic potential of hMSCs is independent of age [85]. hMSCs from both child and adult donors generated chondrogenic pellet and stained positively by Alcian Blue. Similar to Hermann et al., we could conclude that condrogenesis was not age dependent [25].

Studies have found an age related decrease in osteoblastic but not adipogenic differentiation in BMSCs from rats [49] and humans [51, 70]. Several studies addressed

the hypothesis that age related decreases in bone regeneration were due to BMSC aging, resulting in a decreased osteogenic potential with a concurrent increase in adipogenic potential [80, 84].

According to our results, no absolute increase in adipogenic potential was observed with increasing age. In contrast, we have found lower percentage of cells stained positive with Oil Red O in adult donors comparing to child donors. The disparity probably arises from the choice of age groups, group size and isolation and cultivation conditions.

The potential of adult MSCs to transdifferentiate into neural cell types [83] has aroused great interest in research. Such a capacity opens extensive possibilities for autologous therapeutic treatments in a variety of neurological disorders. However, because of the low frequency of MSCs in BM [17, 44], it is necessary to expand MSCs extensively *in vitro* to acquire sufficient cells for use in research and clinical trials. Another important parameter that must be considered, particularly is the effect of cell passage on adult MSCs. Several reports on the extensive subcultivation of BM-derived hMSCs have described changes in morphology, proliferation, and differentiation capacity [63].

In this study, we focused on examining effects of long term serial passage on morphology, proliferation potential and especially transdifferentiation ability of BM derived hMSCs into neuronal phenotype. In our study, primary colonies of hMSCs at P0 contained small and fibroblast-like cells. With increasing passage number, cells gradually lost their morhology. hMSCs from child donors gained irregular and flat shape when they were at P15. hMSCs obtained from adult donors gained the same morphology at P9. Cells obtained from old donors were enlarged when they were at P5.

Our results have demonstreated that, high passage cultures included fewer dividing cells. Similar to Wagner et al., a reduction in the proliferation rate was observed in MSCs from all ages according to increasing passage number [22], which corresponded with the observed morphological changes. RT-PCR results of Topo IIα, which is only present in dividing cells, confirmed that; proliferation potential of hMSCs

harevsted from child donors decreased after P12; while proliferation potential of hMSCs from adult donors lost after P9.

To assess whether culture to high passage number alters the neural transdifferentiation potential of hMSCs, cells from P3 and P15 were exposed to neural differentiation and compared for differences in morphology and expression of neural markers. P15 hMSC culture had a reduced propensity toward acquiring a neuronal-like morphology. Also, P15 culture contained larger cells and greater amounts of cellular debris than P3 culture. Here, we have analyzed that *in vitro* trans-differentiation potential is affected by long term passage. In addition to morphological results, we also checked early and late neural markers by immunostaining methods. Cells were positive for early neural marker NSE and late neural marker NF.

MSCs have the ability to express immature and/or mature protein from other tissues without any induction [67, 73]. It has been demonstrated that MSCs express neural genes and proteins not only following exposure to neural differentiation conditions, but also before differentiation [27, 64, 67, 76, 87]. Montzka et. al., has presented inter-donor variability of expression of neural related markers in hMSCs [76]; however, small number of donors were randomly selected and therefore results obtained from this study were inadequate.

In the present investigation, to evaluate how neural transdifferentiation ability of hMSCs from child and adult donors is affected by *in vitro* serial propagation, we used RT-PCR. We investigated differences in the expression level of neural markers (β III tubulin, NSE) and topo IIβ during 17 weeks in culture. Topo IIβ is required for neural transdifferentiation and also has a regulatory role in transcriptional activation of some inducible genes. Each donor sample revealed a unique expression pattern, demonstrating a significant variation of marker expression. Even though the same criteria for isolation were followed, there was considerable donor-related heterogeneity in the expression pattern of the hMSC populations. This observation likely reflects the high degree of donor variability.

Except inter donor variability, there was no significant difference between passage numbers in neural marker and topo IIβ expression patterns of hMSCs obtained

from child donors. Increasing marker expression level didn't observed with increasing passage number. It is possible that hMSCs from child donors may have greater trans-differentiation ability than hMSCs from adult donors. Cells may not loose their transdifferentiation capability until P15. However, neural marker and topo IIβ expression levels were seem to increase according to higher passage number in hMSCs obtained from adult donors. Depending on donor, expression levels started to increase at P9. Similar to Bonab et al., we can conclude that hMSCs from adult donors loose their stem cells characteristics [17] not from the moment *in vitro* culture begins, but during *in vitro* serial propagation continues.

We obtained similar results with Khoo et al., that long-term subculture of hMSCs did not result in spontaneous neural differentiation [63], in contrast to a recent study on rat MSCs [82].

In conclusion, we have shown that donor age and long term passage are both critical factors which affected morphology, proliferation potential and differentiation ability of hMSCs. With increasing age and passage number, proliferation rate decreased and cells lost their fibroblast like morphology. Osteogenic, adipogenic and neurogenic differentiation potential decreased due to age; but chondrogenic potential was maintained. Undifferentiated hMSCs expressed neural markers β III Tubulin and NSE, with a high donor related heterogeneity. Expression level of neural markers increased due to increasing passage number, in paralel with topo IIβ, in hMSCs obtained from adult donors. Topo IIα expression level was lost after P9 confirming the lost of proliferation potential. However, there was no significant difference between passage numbers in the expression level of neural markers and topo IIβ, of hMSCs from child donors. Also, topo IIα level was decreased after P12. In the light of these results, we can conclude that increasing age have inverse effects on both differentiation and transdifferentiation potential of hMSCs. Cells from child donors have greater transdifferentiation ability than adult donors. However; hMSCs from all ages lost their stem cell characteristics upon long term passage.

Based on the results of this study and other previous studies, it appears that many parameters, such as donor age and long term passage, should be considered when choosing an ideal or appropriate cell source for a specific application.

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