CELL SURFACE LOCALIZATION AND EXPRESSION OF CELL SURFACE RECEPTOR INTEGRIN ALPHA5/BETA1 DURING PROLIFERATION AND NEURAL DIFFERENTIATION OF HUMAN BONE MARROW MESENCHYMAL STEM CELLS (hMSCs)

by

Nihal KARAKAŞ

May, 2009
Istanbul, Turkey

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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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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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ABSTRACT

Integrins are transmembrane receptors that act in cellular processes such as cell proliferation, cell motility and cell differentiation related to stimulation of signal pathways and regulation of cellular events. The complex effect of integrins was detected in these cellular processes by the studies with one of the multipotent adult cell types, Mesenchymal Stem Cells (MSCs). Since they were defined as typical sources for tissue repair and regeneration, human MSCs (hMSCs) are one of the potential candidates leading to tissue engineering approaches. Neurons have less ability in tissue regeneration as they are compared to other cell types. Therefore, stem cells became crucial origins in derivation of neurons and bone marrow MSCs are one of those key stem cell populations. In this study, various induction protocols composed of dissimilar factors were applied in order to afford neural differentiation *in vitro* and neurons at distinct stages of neural maturation were arised. Beside apparent morphological modifications, neuronal existance was revealed by use of immunofluorescence staining of neuron spesific markers such as NSE, βIII Tubulin and NF, GFAP which are known as widely expressed in neurons. In addition to that, neuron induction methods were eliminated according to their efficiency due to optimize the best one among applied protocols. Since cell adhesion molecules have a main contribution to neural differentiation of hMSCs cell surface localization and expression of integrin α5β1, which is one of the fundamental moderator proteins in regulation of cellular events, were detected during proliferation and neural differentiation of hMSCs. On the other hand, extracellular ligand binding through fibronectin and cytoskeletal interaction with actin filaments were investigated within this period. Accumulated data concluded that integrin α5β1 expressions were upregulated begining from the first day as hMSCs undergone neural differentiation whereas decreasing in expression levels occurs towards the 14th day, at which *in vitro* neural differentiation of MSCs is deemed as terminated. Moreover, fibronectin binding and interaction with the actin skeleton effectively facilitate integrin α5β1 participation in neural differentiation of hMSCs. Accordingly, our outcomes denoted that integrin α5β1 has a role at molecular basis throughout the period of neural differentiation of hMSCs.

Keywords: Mesenchymal stem cells, neural differentiation, integrin α5β1

HÜCRE YÜZEY RESEPTÖRÜ İNTEGRİN ALFA5/BETA1'in İNSAN KEMİK İLİĞİ MEZENKİMAL KÖK HÜCRE (hMSC) PROLİFERASYONU VE NÖRAL FARKLILAŞMA SIRASINDA HÜCRE YÜZEYİ LOKALİZASYONU VE EKSPRESYONU

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ÖZ

İntegrinler, hücre yüzey reseptörleri olmakla birlikte hücre çoğalması, hücre hareketi, hücre farklılaşması gibi önemli hücre olaylarında, çeşitli sinyal yollarının uyarılmasına ve bu olayların yönlendirilmesine katkı sağlamaktadır. Hücre farklılaşmasında multipotent özelliğe sahip olan yetişkin hücre tiplerinden Mezenkimal Kök Hücreler (MKH) ile yapılan çalışmalarda, integrinlerin bu hücresel olaylarda kompleks etkileri saptanmıştır. İnsan MKH, dokularda meydana gelen tahribatı tamir edici ve yeni doku oluşumundaki fonksiyonlarından dolayı doku mühendisliğinde başvurulan potansiyel kaynaklardan birisi olmuştur. Farklılaşmada hedeflenen nöron hücreleri, diğer vücut hücrelerine kıyasla doku yenilenmesi düşük hücrelerdir. Bu nedenle, kök hücreleri sinir dokusu eldesinde çok önemli bir kaynaktır ve kemik iliği MKH'leri ise bu kaynağın önemli bir kısmını oluşturur. Bu çalışmada, insan MKH'den spesifik farklılaşma faktörleri kullanılarak çeşitli *in vitro* nöron oluşum protokolleri uygulanmış ve farklı olgunlaşma basamağında nöron hücreleri elde edilmiştir. Morfolojik bulguların yanında kültür ortamında elde edilen nöronlar NSE, βIII Tubulin, NF, GFAP gibi, bu hücrelerde ifade edilen markerlar immunofloresan boyama yöntemiyle işaretlenmiş ve farklılaşmanın gerçekleştiği gösterilmiştir. Nöron eldesinde kullanılan protokollerin karşılaştırılması yapılarak en etkili yöntemin optimizasyonu sağlanmıştır. İnsan MKH-nöron farklılaşması sürecinde, hücre yüzeyi adezyon moleküllerinin önemi büyüktür. Farklılaşma gibi hücresel olayların regülasyonunda önemli bir integrin çeşidi olan α5β1'in, MKH'lerin nöral farklılaşmasındaki ifade edilme düzeyleri ve hücre yüzeyindeki lokalizasyonu incelenmiş olup, bu süreçte sitoplazmik proteinlerden aktin ve ekstraselüler matriks proteinlerinden fibronektin ile ilişkisi tespit edilmiştir. Elde edilen bulgular, MKH'lerde nöral farklılaşma ile birlikte ilk günden itibaren integrin α5β1 ekspresyonunun arttığını ve *in vitro* farklılaşmanın tamamlandığı düşünülen 14. güne yaklaştıkça ekpresyon hızının azaldığını göstermiştir. İntegrin α5β1'in özgün olarak tutunduğu matriks yapılarından fibronektin ile bağlantısının farklılaşmayı tetiklediği ve hücre içi uzantısının sağlandığı aktin filemanları ile koordineli olarak farklılaşmada rol aldığı saptanmıştır. Sonuç olarak, MKH'lerin nöral farklılaşması sürecinde integrin α5β1'in moleküler düzeyde bir rol üstlendiği gösterilmiştir.

Anahtar Kelimeler: Mezenkimal Kök Hücreler, Nöral Farklılaşma, İntegrin α5β1

Dedicated to my parents and husband.

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LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOL	ABBREVIATION
MSC	Mesenchymal stem cell
hMSC	Human mesenchymal stem cell
SPC	Stromal precursor cell
RS	Recycling stem cells
CFU-F	Colony forming unit-fibroblasts
FACS	Fluorescence activated cell sorting
MACS	Magnetic activated cell sorting
ISCT	International Society for Cellular Therapy
RA	Retinoic acid
BM	Bone marrow
PD	Population doubling
FBS	Fetal Bovine Serum
bFGF	Basic fibroblast growth factor
NeuN	Neuronal nuclear protein
GAD	Glutamic acid decarboxylase
NSE	Neuron specific enolase
NF	Neurofilament
GFAP	Glial fibrillary protein
NSE	Neuron specific enolase
PBS	Phosphate buffer saline
CD	Clusture of differentiation
HCAM	Homing-associated cell adhesion molecule
VCAM	Vascular cell adhesion molecule
ALCAM	Activated leukocyte cell adhesion molecule
ICAM	Intercellular cell adhesion molecule
NCAM	Neural cell adhesion molecule

LFA-3	Lymphocyte function-associated antigen-3
IL	Interleukin
RT-PCR	Reverse transcriptase polymerase chain reaction
IBMX	3-Isobutyl-1-methylxanthine
CBB	Coomassie brilliant blue
FGFR	Fibroblast growth factor receptor
PDGFR	Platelet derived growth factor receptor
EGFR	Epidermal growth factor receptor
SCFR	Stem cell factor receptor
LIFR	Leukemia inhibitory factor receptor
GCSFR	Granulocyte colony-stimulating factor receptor
TGFβR	Transforming growth factor β receptor
Sca-1	Stem cell antigen-1
HSC	Hematopoetic stem cell
GM-SCF	Granulocyte-macrophage colony-stimulating factor
M-SCF	Macrophage colony-stimulating factor
G-SCF	Granulocyte colony-stimulating factor
HGF	Hepatocyte growth factor
SDF-1	Stromal derived factor-1
IBMX	Isobutylmethylxanthine
HLA	Human leukocyte antigen
MAP-2	Microtubule associated protein-2
BME, β-ME	β-mercaptoethanol
DMSO	Dimethylsulfoxide
BHA	Butylated hydroxyanisole
DMEM	Dulbecco’s modified essential medium
hEGF	Human epidermal growth factor
bFGF	Basic fibroblast growth factor
BDNF	Brain-derived neurotrophic factor
NGF	Nerve growth factor
cAMP	Cyclic adenosine monophosphate
dbcAMP	Dibutyryl cyclic adenosine monophosphate
DMEM-LG	Dulbecco’s modified essential medium-low glucose
RT	Room temperature

MSC-FBS	Mesenchymal stem cell qualified fetal bovine serum
NBCM	Neuroblastoma conditioned medium
NHS	Normal horse serum
PBS-Tx	0.3 % Triton X / PBS
DAPI	4',6-diamidino-2-phenylindole
BSA	Bovine serum albumin
HSA	Human serum albumin
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
APS	Amonium peroxy sulphate
HRP	Horse readish peroxidase
VLA	Very late activated antigen
Ag	Antigen
SH	src homology protein
CB	Cord blood
PB	Peripheral blood
ECM	Extra cellular matrix
DC	Differentiated cells
FAK	Focal adhesion kinase

CHAPTER 1

INTRODUCTION

1. 1 MESENCHYMAL STEM CELLS

1.1.1 Overview of MSCs

The field of stem cell biology continues to evolve with the ongoing characterization of multiple types of stem cells with their inherent potential for experimental and clinical applications. Mesenchymal stem cells (MSCs) are one of the most promising stem cell types due to their availability and relatively simple requirements for *in vitro* expansion and genetic manipulation. [1].

MSCs are roughly defined as “mesenchymal” because of their ability to differentiate into cells or as “marrow stromal cells” because they appear to arise from the complex array of supporting structures found in marrow.

Cumulative data have been documented on the multipotential differentiation of MSCs since the method to isolate and culture-expand MSCs was developed by Friedenstein and coworkers in the mid-1970’s and modified by other groups in the 1990’s [2].

MSCs have great appeal for tissue engineering and therapeutic applications because of their general multipotentiality and relative ease of isolation from numerous tissues [3]. The potential plasticity and self renewal capacity of MSCs offer a huge potential for clinical tissue regeneration [7]. The most studied and accessible source of MSCs is the bone marrow (BM), although MSCs have been isolated from a number of tissues, including the river, foetal blood, cord blood and amniotic fluid [5].

Bone marrow derived human mesenchymal stem cells (hMSCs) represent an appealing source of adult stem cells for cell therapy and tissue engineering, as they're easily obtained and expanded while maintaining their multilineage differentiation potential [56]. MSCs can be isolated from bone marrow and expanded *ex vivo* without any apparent modification in phenotype or loss of function [4].

Non-hematopoietic MSCs were firstly described as clonal, plastic adherent cells from bone marrow capable of differentiating into members of three germ layers such as osteoblasts, adipocytes, and chondrocytes. hMSCs are capable of differentiating into multiple mesenchymal lineages. They differentiate into the lineage and the cells move through stages of differentiation that involve several layers of commitment and alterations in gene expression before becoming a mature differentiated cell type.

Table 1. 1 Representative examples of terms given to mesenchymal stem cells.

DEFINITION TERMS FOR MSCs		
TERM	**CELL TYPE(S) IDENTIFIED**	**ANIMAL SOURCE/REFERENCE**
Precursors of non-hematopoietic tissue	Adherent cells of bone marrow that include fibroblast-like cells, endothelial cells, and monocytes/macrophage	Guinea pig[8] Mouse[9]
Colony forming unit-fibroblast	Colonies of fibroblastic cells, with the occasional monocyte/macrophage present	Human [10] Mouse [11, 12] Rabbit [13]
Mesenchymal stem cells	Cells defined by their selective attachment to a solid surface	Human [14]
Marrow stromal cells	Adherent cells of bone marrow that include and/or adherent fibroblast-like cells, endothelial cells and colonies monocytes/macrophage	Mouse [15, 16, 17]
Bone marrow stromal cells (BMSSCs) and/or Stromal precursors cells (SPCs)	Non-hematopoietic cells of mesenchymal origin, displaying fibroblastic morphology	Mouse [18) Human [19, 20]
Multipotent adult progenitor cells	Culture-derived bone marrow-derived progenitor cells	Humans [21] Murine [22] Rat [22]
RS-1, RS-2, mMSCs (RS: Recycling stem cell) (m: mature)	RS-1: thin, spindle-shaped cells RS-2: moderately thin, spindle shaped cells mMSCs: wider, spindle-shaped cells	Human [23], Murine [51], Rat [51]

Putative mesenchymal cell progenitors have been identified in human marrow by their ability to generate colonies of fibroblast-like cells originating from single clonogenic progenitors termed fibroblast colony-forming units (CFU-F) [4]. These progenitors have multilineage differentiation capacity and supporting the “stromal stem cell hypothesis”.

1.1.1 Key Characteristics of MSC Phenotype

In most studies, it remains to be determined whether true stem cells are present or whether the population is instead a diverse mixture of lineage-spesific progenitors. Inconsistency in published reports of the growth charactheristics and differentiation potential of MSCs underscores the need for a functional definition of these cells [6]. The majority of data regarding the phenotypic properties of MSCs is based on the analysis of *in vitro* culture expanded MSCs, whereas little is known about the precise phenotypic characteristics of the primary clonogenic stromal precursors in the bone marrow that are responsible for initiating stromal cell growth *in vivo* [5]. Sorting a common cell type within a mixture of cell populations is usually carried out by either FACS (Flourescent-Activated Cell Sorting) or MACS (Magnetic-Activated Cell Sorting) methods which require investigation of cell surface markers. These surface molecules are variously responsible for hetero- and homotypic interactions among cell types and also serve as receptors for growth factors, cytokines, or extracellular matrices.

Typical MSC phenotype have been under consideration via profilling the expression of essential surface antigens and identifying the culture properties. In line with this, International Society for Cellular Therapy (ISCT) reported three criteria to define MSCs:

1. MSCs must be adherent to plastic when maintained in culture.
2. MSCs must be positive for several Ags such as CD105, CD73 and CD90.

MSCs must be lack the expression of hematopoietic antigens like CD45, CD34 and markers for monocytes, macrophages and B cells.

3. MSCs must be able to differentiate at least into chondroblasts, osteoblasts and adipocytes.

Table 1. 2 Examples of hMSC frequency and phenotypic properties calculated from representative studies [24].

Cell fraction isolated	**Frequency**	**Major cell properties**	**Reference**
1.07 g/ml	68-10 in $5x10^6$	Adherent fibroblastic-like cells	[10]
70% Percoll (1.03 g/ml)	1 in $1x10^5$	Adherent fibroblastic-like cells CD45, CD14	[42]
70% Percoll (1.073 g/ml)	1 in $1x10^5$	Adherent fibroblastic like cells $SH2^+$, $SH3^+$, $CD29^+$, $CD44^+$, $CD71^+$, $CD90^+$, $CD106^+$, $CD120a^+$, $CD124^+$	[43]
Percoll (1.073 g/ml) 23.4-5.9 ml BM	1.4-0.7 in $1x10^{5(a)}$	Adherent fusiform fibroblastic-like cells $SH2^+$, $SH3^+$, $SH4^+$, $CD45^-$, CD14, $CD34^-$	[44]
BM aspirates	34.2-6 in $1x10^5$	Adherent colonies of fibroblastic-like cells	[45]
Ficol-Paque (1.077 g/ml)	1 in $1x10^6$	Clusters of small adherent cells $CD34^-$, CD44low, $CD45^-$, $CD117^-$, class I-HLA^-, class II	[46]

		HLA-DR $CD45^-$GlyA cells	
NGFR+ cells	1,584 in $1x10^6$	NGFR+ cells: Isolated fraction consists of small round cells that rapidly adhere to plastic NGFR+ cells express $CD34^+$ (44.1- 45.8%), $CD113^+$ (49.4-29.9%)	[47]
STRO-1+VCAM+	1 in 3 STRO-1+VCAM+ cells	Adherent fibroblastic-like cells with occasional cluster of cells 0.02% STRO-1+VCAM+ cells in BM MNC population >90% of cells stained for collagen type 1 $CD45^-$ Quiescent in vivo No detection of mature mesenchymal cell markers	[48]
Ficoll-Paque (1.077 g/ml)	1 in 13,000	$CD45^-$, $CD14^-$, $CD34^-$, $CD11b^-$, $CD90^+$, HLA-ABC^+	[49]

(a) A mean of $1.4\text{-}0.7x10^5$ MSCs are recovered at the first passage from $1x10^6$ input BM MNC.

Table 1. 3 Surface markers exspressed in human bone marrow MSCs [27-41]

SURFACE MARKERS FOR hMSCs		
Class	**ID**	**Antigen**
Cytokine and Growth Factor Receptors	IL-1R, IL-3R, IL-4R, IL-6R, IL-7R (IL-R, interleukin receptor), SCFR (stem cell factor receptor), LIFR (leukemia inhibitor factor receptor), GCSFR (gronulocyte colony stimulating factor), Interferon R, TNF-α_{1R}, TNF-α_{2R} (TNF, tumor necrosis factor), TGF-β_{1R}, TGF-β_{2R}, (TGF, transforming growth factor) FGFR (fibroblast growth factor receptor), PDGFR (platelet-derived growth factor receptor), EGFR (epidermal growth factor receptor)	CD121 CD123 CD124 CD126 CD127 CD117 CD114 CDw119 CD120a CD120b CD140a
Adhesion Molecules	ICAM-1 ICAM-2 ICAM-3 (ICAM, intercellular adhesion molecule)	CD54 CD102 CD50
	VCAM-1 (vascular cell adhesion molecule)	CD106
	ALCAM (activated leukocyte cell adhesion molecule)	CD166
	HCAM (hyaluronate receptor)	CD44
	NCAM (neural cell adhesion molecule)	CD56
	L-selectin	CD62L
	P-selectin	CD62P
	E-selectin	CD62E
	LFA-3 (leukocyte function associated antigen 3)	CD58
	Endoglin	CD105
	Hyaluronate receptor	CD44
	Fibronectin	

Extracellular Matrix Molecules	Laminin	
	Vimentin	
	Hyaluronan	
	Collagen type I, III, IV, V and VI	

Table 1. 3 Surface markers exspressed in human bone marrow MSCs [27-41]

Class	**ID**	**Antigen**
Integrins	VLA-α1 VLA-α5 (VLA, very late activated antigen) β1 integrin β3 integrin	CD49a CD49e CD29 CD61
Additional Markers	SH-2 SH-3 SH-4 (SH, src homology protein) STRO-1 Thy-1	CD105 CD73 CD73 CD90

Mesenchymal stem cells are known to express a wide range of adhesion molecules (CD44, CD29, CD90), stromal cell markers (SH-2, SH-3, SH-4) and cytokine receptors [interleukin (IL)- 1R, tumor necrosis factor (TNF)- αR]. Therefore, alongside removing contaminating hemopoietic cells by negative selection using antibodies to CD45, CD34 and CD11b, these MSC markers can be collectively used to help positively identify and isolate MSCs in culture [5].

1.1.2 Bone Marrow Derived hMSCs

MSCs have been isolated from several species and tissues, but the most well characterized and probably the purest preparation is from human bone marrow [52].
BM hMSCs are generally isolated from an aspirate of bone marrow harvested from the superior iliac crest of the pelvis in humans. Technically, it is possible to obtain 50-375 million cells per 10 ml bone marrow aspirate from adult donors. In addition to that, hMSCs represent a very minor fraction (0.0001%) of the total nucleated cell population in marrow, but can be plated and enriched using standard cell culture techniques [26]. To isolate MSCs from a bone marrow aspirate, cord blood (CB) or peripheral blood (PB), the samples are subjected to fractination on a density gradient solution such as Percoll, after which the cells are plated at densities ranging from $1x10^4$ to $0.4x10^6$

cells/cm^2 for mononuclear isolation, resuspended in appropriate culture medium containing selected batches of fetal bovine serum and allowed to adhere to plastic dishes for about 2 days [25, 50].

MSCs in culture have a fibroblastic morphology and adhere to the surface of plastic culture dishes. Mixture of nonadherent hematopoietic cells have to be removed from the culture flasks to obtain purified MSCs within a time period after isolation process. Primary cultures are usually maintained for 12-16 days, during which time the hematopoietic cell populations are depleted while MSCs adhere to the tissue culture substrate; hence, remaining cells are allowed to grow for 2-3 weeks. It's required to evaluate isolation techniques with care, and to identify new cell-spesific markers due to complexity of subpopulations of bone marrow cells.

1.1.3.1 Self Renewal Potential of MSCs

One of the typical characteristics of stem cells is their self-renewal potential, the ability to generate identical copies of themselves through mitotic division over extended time periods -even the entire lifetime of an organism. Self-renewal mechanism refers to undifferentiated stem state. Genomic arrays have been used to identify molecular signatures that maintain the stem cell state, including that of MSCs [3]. As a population, bone marrow derived MSCs have been demonstrated to have a significant but highly variable self-renewal potential during in vitro serial propagation.

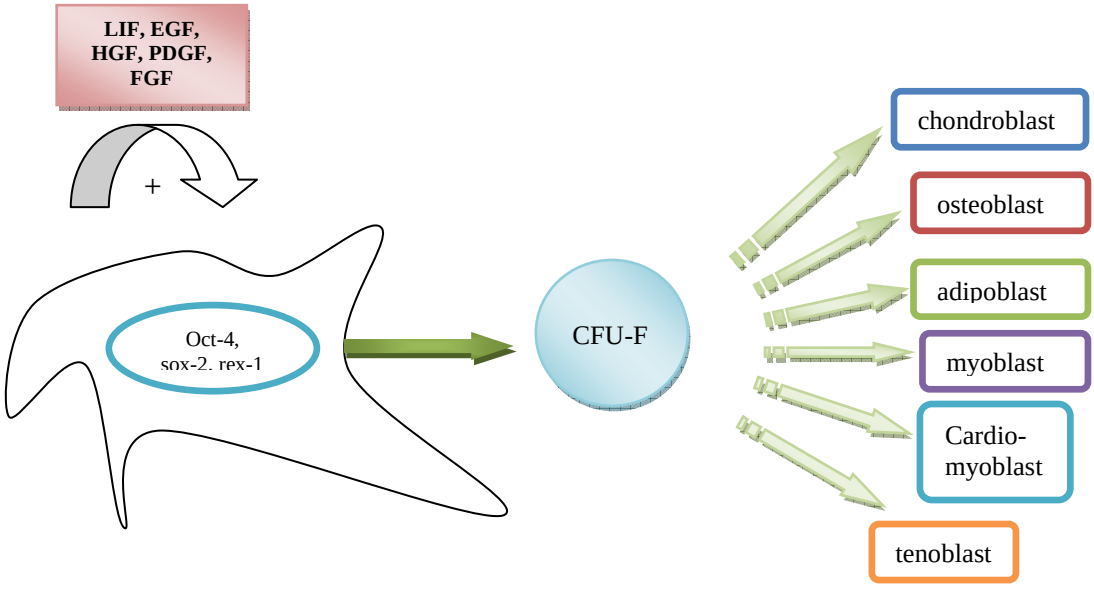


Figure 1. 1 Self renewal and cytodifferentiation scheme of MSCs.

Extracellular signalling factors, inluding growth factors and cytokines (LIF, leukemia inhibitory factor; EGF, epidermal growth factor; HGF, hepatocyte growth factor; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor) have a role to promote and/or maintain MSC self-renewal *in vitro*. Gene markers characteristic of MSC self-renewal include oct-4, sox-2 and rex-1 that are expressed as being embroyonic stem cell gene markers [3].

1.1.3.2 In Vitro Expansion of MSCs

Life span of MSCs is not infinite and limitless in culture . In other words, MSCs can be succesfully expanded in vitro for an average of 30 population doublings (PD) without any chromosomal aberrations related to donor variability and culture conditions [61] It is reasonable they are limited in life as they are found to contain no telomerase activity [60]. Essentially, MSCs *in vivo* have telomerase activity but they lose this ability during *ex vivo* expansion [62].

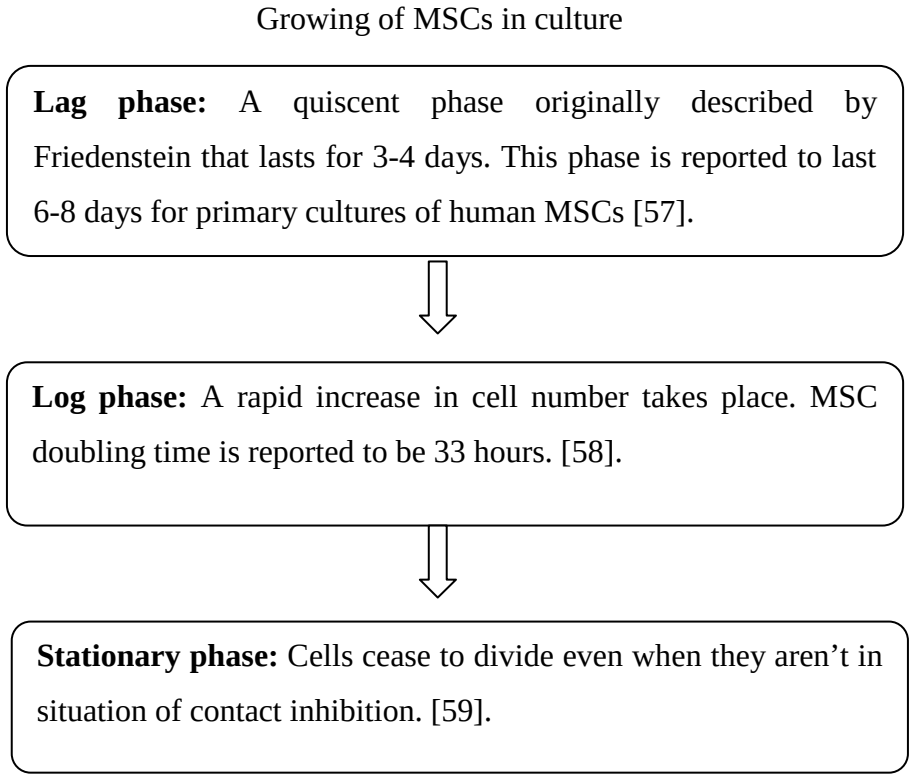


Figure 1. 2 Reported consecutively growing phases of MSCs *in vitro*.

MSC life span can be seperated into three phases in terms of *in vitro* aging. [60]

	Phase	% of cells completed life span in culture
1.	Rapid Cell Growth	< 50%
2.	Reduced-Growth Rate	50% - 80 %
3.	Growth Arrest	> 50%

1.1.3 MSC Niche and Regulation of Differentiation

In analyzing the differentiation of stem cells, it is critical to consider the influence of their tissue of origin presuming that the requisite microenvironment for stem cell survival and maintanance, will be modified depending upon that basis. It has been matter for reflection what defines and constitutes the mesenchymal stem cell microenvironment, considering whether there is a MSC niche that is common to all of MSC derivating tissues or MSCs function autonomously, in a manner that is independent of their environment.

Since Schofield first inroduced the concept of a stem cell ‘niche’ in 1978 [75] , the idea has gained wide support, particularly in recent years. In brief, the niche encompasses all of the elements immediately surrounding the stem cells when they are in their naive state, including the non-stem cells that might be in direct contact with them as well as exta cellular matrix (ECM) and soluable molecules found in that locale. All of these act together to maintain the stem cells in their undifferentiated state. It’s then assumed that certain cues must find their way into the niche to signal to the stem cells that their differentiation potential is needed for the regeneration or repopulation of a tissue [3].

It’s clearly described that distinct niches exist within the bone marrow that support hematopoietic stem cell (HSC) survival and growth, by providing the requisite factors and adhesive properties to maintain their viability, while facilitating an appropriate balanced output of mature progeny for the lifetime of an organism. The stroma, and stromal cells, together, provide a physical support for maturing precursors of blood cells, and serve as a repository of a broad range of cell derived cues and signals driving the commitment, differentiation and maturation of hematopoietic cells [24]. In line with

this, it's relatively crucial to define MSC niche according to its presumptive own residence amidst hematopoietic stem cells or same microenvironment with hematopoietic cells.

This field of stem cell studies also requires a lineage-spesific investigation for each essential factor (soluable agents, extra- and intra- cellular signaling pathways corresponding to a diverse array of adhesion molecules) that regulate stem cell microenvironment, in order to create, for instance, proper conditions for in vitro cell expansion.

Effects of several hormones, vitamins, growth factors and cytokines on MSC proliferation and differentiation have been tested applying in vitro differentiation assay, studying mainly osteogenic, chondrogenic and adipogenic differentiation [7]. However, there is evidence that ECM alone can regulate MSC differentiation, with potential applications for tissue engineering. Molecular information on ECM-MSC interactions, most probably involving integrins, which have already been implicated in niche biology in other systems, is clearly needed [3, 66, 67].

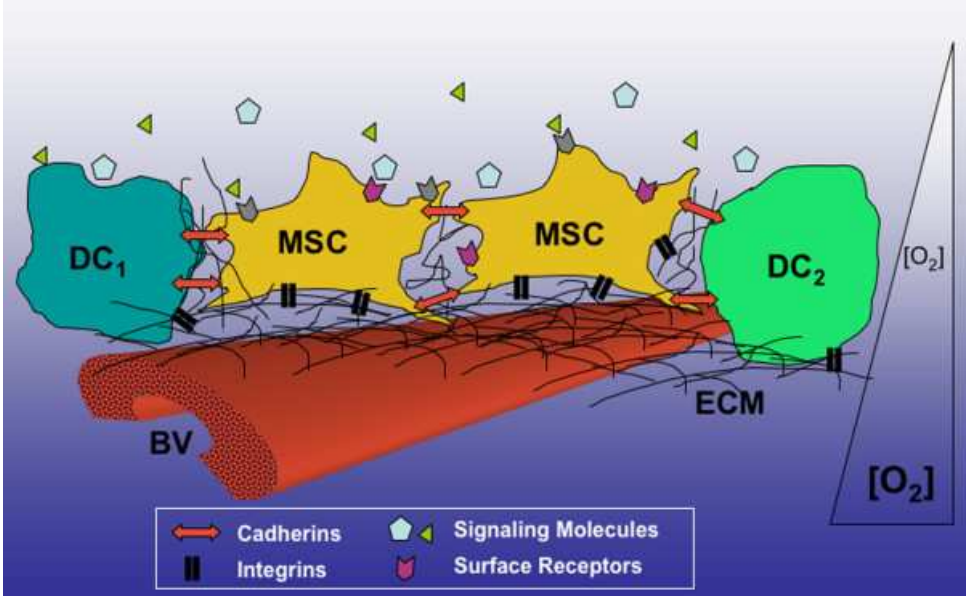


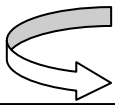
Figure 1. 3 Mesenchymal stem cell niche

MSCs are shown in their putative perivascular niche (BV, blood vessel), interacting with (1) various other differentiated cells (DC1, DC2, etc.) by means of cell-

adhesion molecules, such as cadherins, (2) ECM deposited by the niche cells mediated by integrin receptors, and (3) signaling molecules, which may include autocrine, paracrine, and endocrine factors. Another variable is O_2 tension, with hypoxia associated with MSCs in the bone marrow niche [3].

Fist Step: The progression of MSCs in precursor cells

MSCs undergo transcriptional modification, yielding precursor cells without appearent change in phenotype and self-renewal capacity. Until stimulated, the majority of MSCs cultured in vitro remain quiescent and growth arrested in G0/G1, smilar to MSCs located in adult bone marrow. Upon stimulation, multipotent, uncommitted MSCs divides asymetrically, giving rise to two daughter cells, one is the exact copy of the mother cell and it maintains multilineage potential, and the other daughter cell becoming a precursor cell, with a more restricted developmental program. In this model, the precursor cell continues to divide symetrically, generating more tripotent and bipotent precursor cells. These newly-formed cells are morphologically smilar to the multipotent MSCs, but differ in their gene transcription repertoire, and therefore, still reside in the stem cell compartment [24].



Second Step: Existance from the stem cell compartment

The transition from the stem cell compartment to the commitment compartment occurs when precursor cells continue to divide symetrically to generate unipotent progenitor cells, simultaneous with the acqusition of lineage spesific properties, rendering them fully committed mature cells with distinguishable phenotypes [24].

Figure 1. 4 Model for regulation of differentiation

Even though it is far too early to conclude which growth factors are essential, it's highly presumed that such growth factors not only promote proliferation but also retain self-renewal capacity of MSCs and maintain their multilineage potential.

MSCs are able to acquire charactheristics of cells derived from embriyonic mesoderm, such as osteoblasts, chondrocytes, adipocytes, tendon cells, as well as cells possessing ectodermal and neuronal properties [24]. For regulation of adult stem cell differentiation, a model was described related to previously published genetic and genomic information. This model incorporates two continues distinct compartments (Figure 1.5).

1.1.4 In Vitro Studies with MSCs

The fundamental principle behind stem cell therapy is that as undifferentiated cells are delivered to the injured host and migrate to the site of injury, under the influence local signals they differentiate into the appropriate phenotype [26]. A broad range of evidence indicates that these specialized cells then contribute to the repair of the injured tissue, but the data concerning the spesific signals which give rise to differentiation *in situ* have been under investigation. In line with this, underlying mechanisms leading to MSC differentiation down spesific cellular pathways has been estimated by the discovery of *in vitro* culture conditions.

Stromal long-term culture was originally developed in the murine system and subsequently adapted for human bone marrow. Functional stroma involving macrophages, adipcyes, endothelial cells and fibroblasts, which have the ability to support hemopoiesis, are characteristically produced in culture conditions [68, 69].

Human bone marrow derived MSCs can be maintained in an undifferentiated state *in vitro*, but have the ability to generate functional stroma, to support hematopoiesis, or to differentiate along osteogenic, chondrogenic and adipogenic lineages, when exposed to appropriate *in vitro* or *in vivo* environments [5].

1.1.5.1 Osteogenic Differentiation

The induction of MSCs into osteogenesis is a highly programmed process, best illustrated *in vitro*. Treatment with the synthetic glucocorticoid dexamethasome stimulates MSC proliferation and supports osteogeneic lineage differentiation [73, 74] Osteogenic differentiation is induced by the presence of β-glycerophospate, ascorbic acid-2-phosphate, dexamethasone and FBS. Under these culture conditions, cells upregulate alkaline phosphatase, osteocalcin and osteopontin activity, and also calcium deposition within the ECM [5].

1.1.5.2 Chondrogenic Differentiation

Chondrogenic differentiation is typically carried out when MSCs are cultured certain conditions as follows [26] :

- A three dimensional culture format,
- A serum free nutrient medium,
- The addition of a member of the transforming growth factor-β superfamily.

TGF-β appears to induce chondrogenesis via protein kinases including extracellular signal-regulated kinase 1, p38, protein kinase A, protein kinase C, and Jun kinase. The TGF-β mediated kinase activation also includes expression of adhesion molecule N-cadherin related to Wnt expression. [70, 71, 54]

1.1.5.3 Adipogenic Differentiation

In vitro adipogenic induction requires spesific medium supplementations, including dexamethasone and 3-isobutyl-1-methylxanthine. Molecular regulation of adipogenesis is controlled by several transcription factors including peroxisome proliferator-activated receptor gamma (PPAR-γ) which is crucial for adipogeneic process [72] and signaling.

1.1.5.4 Neural Differentiation

Novel studies with neural differentiation of MSCs *in vitro* arised from two simultaneous studies by Sanchez-Ramos et al. and Woodbury et al . in 2000. They reported that rat, mouse and human MSCs can be differentiated 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. Woodbury treated MSCs with β-mercaptoethanol (BME), dimethylsulfoxide (DMSO) and butylated hydroxyanisole (BHA) in DMEM and observed that about 80% of cells changed morphology. Treated MSCs were found to be positive for nestin, an immature neural marker, after 5h while they were negative after 6 days. Few cells were stained positive for neural markers TrkA, Tau and neuron-specific enolase (NSE) after treatment while none of them were positive for GFAP. Sanchez-Ramos treated cells with epidermal growth factor (EGF) and retinoic acid (RA) or RA with brain-derived neurotrophic factor (BDNF) and observed expression of neuron-specific nuclear protein (Neu-N), nestin and GFAP.

Neural induction of hMSCs was followed with distinct inducers like retinoic acid (a derivative of vitamin A), cytokines, growth factors (EGF, bFGF), neurotrophins (NGF, BDNF, NT-3, NT-4/5, NT-6), antioxidants (BME, DMSO, BHA, SF), demethylating agents (5-azacytidine), compounds that can upregulate intracellular cAMP levels (such as forskolin), noggin (a protein that acts antagonistically to BMPs, promotes neural differentiation and supress glial differentiation). In line with this, there are copious arguments about hMSCs originated neural-like cells such as how real those generated neurons are, whether they are functional or not, how long they can survive, whether they are transient or not and etc. Those challenges are resulted in a competetive arena of *in vitro* neural differentiation studies for the purpose of obtaining closest neuronal cells beside the need of much more animal studies.

1.2 INTEGRINS

1.2.1 Integrin Family

Integrins are a large family of transmembrane receptors which stimulate interior signaling pathways caused by cell to cell and cell to extracellular matrix (ECM) attachments. Thus, they mobilize a diverse array of cellular processes including cell adhesion, cell motility, cell proliferation and differentiation through inside-out and outside-in signals.

Since the discovery of the integrin receptor family around 15 years ago (Hynes, 1987), they have become the best understood cell adhesion receptors. Integrins and their

ligands play key roles in development, immune responses, leukocyte traffic, hemostasis, and cancer and are at the heart of many human diseases such as genetic, autoimmune and etc. They are the target of effective therapeutic drugs against thrombosis and inflammation, and integrins are receptors for many viruses and bacteria [78]. There are several reports that deregulated integrin function contributes to the pathogenesis of many diseases including cancer. Not only is integrin engagement established to promote proliferation and apoptotic resistance of cancer cells but it can also contribute to invasiveness and cell migration during metastasis. Indeed, reagents that target integrins are now known to be clinically effective as anticancer agents [80].

As for their structural composition, integrins result in a heterodimeric conformation, and an individual integrin molecule consists of two non-covalently bound main subunits, alpha (α) and beta (β).

Each subunit is a type I transmembrane glycoprotein that has a relatively large extracellular domain and short cytoplasmic tail. Mammals contain 18 α and 8 β subunits that combine to produce at least 24 different heterodimers, each of which can bind to a spesific repertoire of cell-surface-, ECM or soluable protein-ligands [77]. Many members of the integrin family, including α5β1, α8β1, αIIbβ3, αVβ3, αVβ5, αVβ6 and αVβ8, recognize an Arg-Gly-Asp (RGD) motif within their ligands. Peptides containing this RGD region can effectively block these integrin-ligand interactions. However, it is the residues outside the RGD motif that provide specificity as well as high affinity for each integrin-ligand pair [79]. Besides, at the inner face of the cell membrane, there are a lot of associated proteins, which can interact with the integrin transmembrane or cytoplasmic domains and their number is growing constantly. As they are bound to their ligands, they move laterally in the plain of the membrane to form specialized clusters called “focal adhesion sites”. These ECM attachment organelles and signalling centers assure substrate adhesion as well as targeted location actin filaments and signalling components and hence they are essential for establishing cell polarity, directed cell migration, and maintaining cell growth and survival [77].

There is also a high level of redundancy with respect to ligand spesificity between the different integrin species, and even within the same integrin subclass. These properties endow integrins with the potential to elicit a large number of different cellular responses, depending on the type of integrin receptor expressed, the developmental stage of the cell, and according to the composition of the surrounding ECM [81].

Table 1.4 Integrin receptors and their protein ligands [77].

Subunits		Ligands
β1	α1	Collagens, laminins
	α2	Collagens, laminins
	α3	Collagens, laminins, fibronectin, entactin
	α4	Fibronectin, VCAM-1
	α5	Fibronectin
	α6	Laminins
	α7	Laminins
	α8	Vitronectin, fibronectin, tenascin
	α9	Vitronectin, fibronectin, tenascin
	α10	Collagens
	α11	Collagens
	αV	Fibronectin, vitronectin
β2	αL	ICAM-1, ICAM-2, ICAM-3
	αM	iC3b, fibrinogen, ICAM-1, coagulation factor X
	αX	Fibrinogen, iC3b
	αD	ICAM-3
β3	αII β	Fibrinogen, fibronectin, von Willebrand factor, vitronectin, thrombospondin, tenascin
	αV	Fibrinogen, fibronectin, von Willebrand factor, vitronectin, thrombospondin, osteopontin, collagens
β4	α6	Laminins
β5	αV	Vitronectin, fibronectin
β6	αV	Fibonectin, tenascin
β7	α4	Fibronectin, VCAM-1, MAdCAM-1
	αE	E-cadherin
β8	αV	Vitronectin

As for study of integrin trafficking, in the late 1980s and early 1990s, as a result of the biochemical recycling assays pioneered by Mark Bretscher, it became clear that certain integrin heterodimers were continually internalized from the plama membrane into endosomal compartments and then recycled back to the cell surface, thus completing an endo-exocytic cycle. Therefore, these findings highlighted that integrin recycling is a highly efficient process, i.e. most internalized integrin is returned to the plasma membrane, rather than being targeted for degradation.

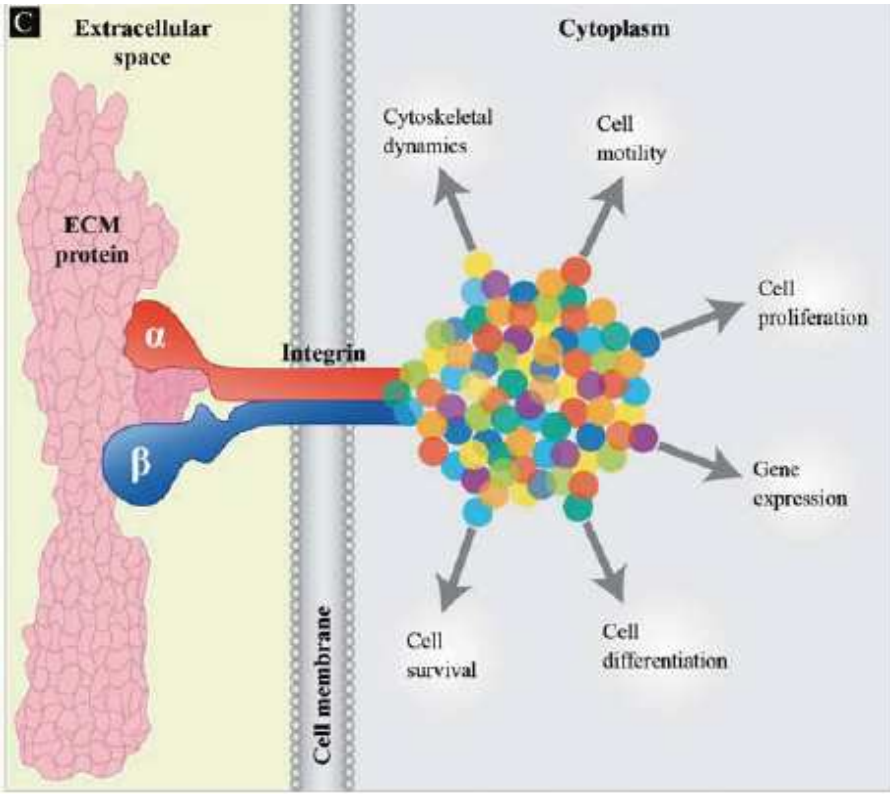


Figure 1.5 Schematic Model of Integrin Contacts with Inside and Outside of The Cell [77].

1.2.2 Integrin Interaction with the Framework and Its Role in Cellular Processses

In addition to their roles in adhesion to ECM ligands or counterreceptors on neighbouring cells, integrins serve as transmembrane mechanical links from those extracellular contacts to the cytoskeleton interior of the cell. For all integrin types except α6β4, the linkage is to the actin based microfilament system, which integrins also regulate and modulate [78].

Integrin interactions with the focal adhesion sites through their cytoplasmic tails, coordinate various signalling complexes leading to diverse cellular behaviours. For example, differences in the focal adhesion and actin cytoskeleton properties determine the variety of cell morphology. The actin cytoskeleton provides a structural framework around which cell shape and polarity are defined. Its dynamic properties provide the driving force for cells to move and divide.

In case of cell motility, it has been suggested that the strength of focal adhesions influences cell motility. Cell migration is diminished in cells exhibiting strong adhesion, as characterized by abundant actin stress fibers and numerous focal contacts, therefore preventing the cells from releasing its cytoskeleton-ECM linkages. Intermediate state of adhesion facilitates cell migration whereas weak adhesion does not generate the contractile force necessary for directed cell movement. Integrin signalling promotes cell migration by inducing changes in the cytoskeletal organization and by induced cellular contractility.

The initiation of integrin mediated cell adhesion has also an impact on proliferation. Integrins can regulate, in a cooperative manner some members of the Cyclin family and thereby progression through the cell cycle.

Establishment of specific integrin-ECM stimuli can lead to the augmentation of gene expression related to differentiation [77].

1.2.3 Human Mesenchymal Stem Cells in contact with the Integrin System

Human MSCs Express a large number of different cell surface proteins, including various integrins, growth factor receptors (bFGFR, PDGFR, EGFR, TGFβIR/IIR), chemokine receptors (some interleukins, CC and CXC receptors) and cell adhesion molecules (VCAM-1, ICAM-1/', ALCAM-1, L-selectin, CD105, CD44). Moreover, hMSCs produce a vast array of matrix molecules including fibronectin, collogens, laminin and proteoglycans [77].

Plenty are the articles, describing integrin subunits detected on hMSCs and their effects in many cellular events have been under investigation for last few years. The flourescence activated cell sorting (FACS) method has been mostly used for the identification of integrin subunits presented on the cell surface of hMSCs. Expression of itegrin subunits like α1, α2, α3, α5, α6, αV, β1, β3, β4 among others have been independently reported. Nevertheless, contradictory results exist and it is still unclear if

all subunits indeed expressed and how their importance differs during cellular events including both survival and differentiation processes [77]. There are few studies rely on RNA and protein based approaches. In line with this, very few reports not only provide evidence of integrin expressions but also that of integrin engagement in the biology of hMSCs.

As a first significant finding, in 2001, Gronthos and his colleagues examined the mechanisms mediating the growth of hMSCs on different ECM components and they found that hMSCs demonstrate a higher colony-forming efficiency when seeded onto collogen type IV, fibronectin, vitronectin and laminin coated surfaces in comparison with collogen type I and III. This may be caused by their functional origin in the BM, stage of commitment and unique integrin expression. Moreover, this study revealed that β1 integrin seems to be important for *in vitro* differentiation of hMSCs into osteoblasts. Furthermore, a plenty of data has being recorded by the aid of subsequent studies about integrin involvement in osteogenic, chondrogenic and adipogenic differentiation programme of hMSCs *in vitro.* It's a promising area of research which of the integrin molecules are expressed and utilized in hMSCs during the fate of a cell. These results, which emphasize underlying mechanism of hMSC differentiation in terms of integrin utilization, may lead to arrange new ways for therapeutic and tissue engineering approaches.

1.2.4 Integrin α5β1 : Fibronectin Common Receptor VLA-5

Integrin α5β1 is a type of transmembrane receptor which binds to fibronectin (Fn) through its extracellular domain and its cytoplasmic tail interacts with actin filaments in the cell. Integrin α5β1 is the only unambiguously proangiogenic integrin; genetic ablation experiments and pharmacological results are consistent and strongly support its importance in neovascularization processes and it has a known effect in cancer issues as it is expected to move into the forefront of research for new effective anti-cancer drugs.

Among other members of the integrin family receptors**,** α5β1 is a fundamental fibronectin specific integrin that can be found in different adhesion structures, and has been implicated in the control of differentiation of various cell types, such as precursor cell osteogenic differentiation.

Integrin α5β1 is also called as “fibronectin common receptor” due to its specific binding property on this core ECM protein as its unique physiological ligand while other integrin molecules are able to interact with distinct ECM components such as laminin, vitronectin and etc. Fibronectin is a widely found member of extracellular network in many tissues, it regulates a variety of cell activities predominantly through direct interactions with cell surface integrin receptors. Functionally, α5β1 interaction requires both the traditional integrin-binding sequence (RGD) as well as the synergy sequence (PHSRN) whereas most other RGD dependent integrins do not require PHSRN [76]. Besides, this receptor-ligand pair (integrin α5β1- Fn) is functionally very important because it mediates fibronectin fibril formation and governs ECM assembly, which is vital to cell function *in vivo*. The interaction between α5β1 and Fn is fundamental for vertebrate development, since lack of α5β1 or Fn results in early embryonic lethality [79].

Since Fn was termed as one of the significant component within the framework of hMSCs, as being common fibronectin receptor, integrin α5β1 has been a fundamental candidate to investigate for hMSC-integrin engagement. Recently, M. Martino and his friends were demonstrated that α5β1 has an important role in the control of MSC osteogenic differentiation [76].

1.2.5 Integrin Mediated Signal Processing

Integrin activation and stimulation of interior signals in the cell occur mainly as follows:

- Ligand binding through ECM components
- Aggregation of integrins and FAK activation by autophosphorylation
- Binding of Src kinase family members to FAK at autophosphorylated sites & phosphorylation of FAK at other tyrosine residues
- Binding of downstream signal molecules including Grb2-Sos complex to those phosphotyrosines
- Activation of Ras & PLC-γ
- Stimulation of cell signaling pathways (ERK)
- Initiation of cellular processes

CHAPTER 2

MATERIALS & METHODS

2.1 ISOLATION OF hMSCs FROM BONE MARROW

Human bone marrow aspirates were supplied from Karadeniz Technical University, Faculty of Medicine, Hematology Department. 10 ml bone marrow sample was diluted with 10 ml DMEM-low glucose (DMEM-LG, Gibco) culture medium and poured into 50 ml falcon tube.

Mixture was shaked gently and homogenized by pipetting. Differential centrifugation method was used for isolation of hMSCs from bone marrow sample; 10 ml of diluted bone marrow sample was poured onto the layer of 5 ml Ficoll (Biochrom) in the ratio of 2:1 by gently pipetting in a 15 ml centrifuge tube. All tubes were centrifuged at 800 g (2500 rpm) at room temperature (RT) for 25 minutes.

Afterwards, samples were seperated into 4 different layers; the bottom, red layer contains red blood cells; above this layer the colorless liquid contains Ficoll; white, cloudy layer located on top of Ficoll contains mononuclear cells in bone marrow which is a composition of various hematopoietic cells including stem cells and on the top, yellow layer contains sera. After discarding sera, mononuclear cell layer was removed from the interface by rotating pipette over the cloudy layer, and transferred into a new 15 ml centrifuge tube. As volume was up to 10 ml with DMEM-LG, it was centrifuged at 350 g at RT for 10 minutes to remove any remaining Ficoll. Supernatant was then discarded , pellet was resuspended in 10 ml DMEM-LG and centrifuged again at 350g at RT for 10 minutes. This time, supernatant was discarded and pellet was resuspended in 10 ml DMEM-LG containing, 20% Mesenchymal stem cell qualified fetal bovine serum (MSC-FBS, Gibco) and 1% penicillin-streptomycin (Gibco). Cell suspension was inserted into a 25 cm^2 tissue culture flask (BD Falcon) and incubated in 37^oC, 5% CO_2 incubator. At the end of 3 days after isolation, non-adherent cells were removed via medium refreshment.

2.1 CULTURE OF hMSCs

2.2.1 Seeding and Expansion

Adherent primary hMSCs were grown in culture and formed colonies as they were left for proliferation after isolation process. Expansion medium; DMEM-LG ,15% MSC-FBS, 1% penicillin-streptomycin) was refreshed per 72 hours after first medium replacement. Nearly 13-14 days later, primary cells were subcultured and seeded into a 75 cm^2 culture flask (BD, Falcon)

Subculture of hMSCs:

1. Medium was discarded from the flask
2. Adherent cells were washed with prewarmed 10 ml PBS (phosphate buffer saline) (Biochrom)
3. Cells were then trypsinized with prewarmed 8 ml of 0.25% Trypsin/EDTA solution (GIBCO) for 1-2 minutes.
4. As the cells start to detach from the culture surface, tyripsin was neutralized with 2 ml of MSC-FBS.
5. Cell suspension was transferred into a 15 ml centrifuge tube and centrifuged at 350 g (1500 rpm) speed for 15 minutes at room temperature (RT).
6. Supernatant was discarded by leaving ~0.5 ml of the cell suspension at the bottom (which was only required for the first passage after isolation)
7. Pellet was finger mixed and volume was up to 10 ml with DMEM medium in order to remove the remaining any tyripsin.
8. Centrifuge was repeated once more. Finally cells were counted by using a hemacytometer (counting chamber) and applied to viability test with Tyrpan Blue (Sigma)
9. 1500 cells/ cm^2 were then seeded into tissue culture flask with DMEM + 15% MSC-FBS and incubated in 37^oC, 5% CO_2 incubator.

Subculture of hMSCs was performed per 6-7 days and expansion was maintained up to the 6^{th} passage at the most.

2.2.2 Cryopreservation of hMSCs

Freezing was applied to preserve hMSCs for future use. Harvested cells were centrifuged at 350g for 10 minutes at RT twice and finally obtained pellet was resuspended in FBS. After cell counting, 900 µl of previously diluted cell suspension (including at least 10^6 cells /ml was added in a cryovial and 100 µl (at the ratio of 10%) of DMSO (Applichem) was added drop by drop very slowly. DMSO was mixed with cells as it was dropped each time. In addition to that, transferring procedure was performed with cryotubes placed on ice. Subsequently, cryovials were transferred to -20 ^oC for 1 hour (at least 30 minutes) and kept at -80^oC overnight. In the end, they were stored in -196 ^oC liquid nitrogen tank for long term preservation.

2. 2 SURFACE COATING ASSAY

2.3.1 Fibronectin Coating

Prior to cell seeding for neural induction of hMSCs, 24 well plates (immunostaining), 6 well plates (morphological followings) and 10cm petri dishes (immunoblotting) were coated with 2 µg/cm^2 fibronectin matrix protein (CHEMICON). Coated wells were then incubated at 37°C for 1 hour. After that, unstuck part was removed and washed with prewarmed PBS so as to prepare for cell seeding.

2.3.2 Matri-Gel Coating

Thin-gel method was followed in order to coat the tissue culture wells with matri-gel surface material.

At the preliminary stage, it's recommended to prepare coating material before starting the assay. For this purpose, matrigel (BD Biosciences) was thawed one day before use and all culture plastics that will have been used for this coating assay were precooled at 4°C . Besides, matrigel at -20°C was embedded into an ice box and stored overnight at 4°C due to liquify the solid phase.

Secondly, culture wells were overlaid with previously liquified 50 μL/cm^2 matrigel mixture by using precooled plastics and keeping culture plates on ice. Plates were then incubated at 37°C for 30 minutes to start the seeding process.

2. 3 NEURAL INDUCTION PROTOCOLS FOR hMSCs

2.4.1 Retinoic Acid Treatment

Retinoic acid treatment was followed by two different induction protocols and prior to treatment, 10 mM stock RA was diluted to 30 μM final concentration by serial dilution steps using DMSO and basic culture medium DMEM-LG. Care should be taken during RA dilution because of its rapidly crystallization property.

2.4.1.1 Under Serum-Induced Conditions

Before cell seeding for immunostaining of neuronal induced hMSCs, 12 mm glass coverslips were put in each 2 cm^2 well of 24 well plates. After that, cells in the absence or presence of surface coating material (fibronectin/matri-gel) were prepared as previously described (2 μg/cm^2 , 4μg/well fibronectin; 50 μL/cm^2, 100 μL/well matri-gel). Cell harvest at passage three were then seeded into each well including 2500 cells/ cm^2 (5000 cells/well) within induction media composed of DMEM-LG, 10% MSC-FBS and 1% Penicillin-Streptomycin. Afterwards, cells were incubated in 37^{o}C, 5% CO_2 incubator for 48 hours. At the end of two days, the cells attached and spred on culture wells.

As for neural induction, expansion medium was discarded and replaced with neural induction media containing 10 μM RA in DMEM-LG, 10% FBS (Biochrom), 1% Penicillin-Streptomycin. Medium refreshment was executed per 72 hours after induction and neural differentiation was observed along fourteen days period.

In case of immunoblotting and neural phenotype analysis experiments, cells were cultured on coated surfaces without putting any glass coverslips. On the other hand, non-coating process also wasn't required any coverslip insertion. Besides, following cell preperation steps were the same as neural induction for immunostaining.

2.4.1.2 Serum-Free Media Supplemented Protocol

Basically, the difference between two protocol related to RA treatment were firstly seeding concentration of cells and secondly reducing in serum percentage within neural induction media. Expansion was maintained by use of 1000 cells/well rather than seeding 5000 cells/well and neural induction was followed with the media including 10 μM RA in DMEM-LG, 0.5% FBS (Biochrom), 1% Penicillin-Streptomycin. After that, cells were incubated in 37^{o}C, 5% CO_2 incubator for forteen days and medium with the same contents was changed per 72 hours.

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2.4.2 Use of Multiple Chemical Agents by Altering the Induction Media

Table 2. 1 Neural Induction (NI) combinations by use of various chemical agents including some antioxidants.

Well #	**Well ID**	**Preinduction**	**Induction**
1	Control	no treatment	no treatment
2	NI_1	1mM β-ME (Merck), 20% FBS, DMEM-LG	10 μM RA, 10% FBS, 50% NBCM, DMEM-LG
3	NI_2	-	10 μM RA, 10% FBS, 50% NBCM, DMEM-LG
4	NI_3	1mM β-ME, 20% FBS, DMEM-LG	10 μM RA, 2% DMSO, 10mM KCl, 10% FBS, 50% NBCM, DMEM-LG
5	NI_4	-	10 μM RA, 2% DMSO, 10mM KCl, 10% FBS, 50% NBCM, DMEM-LG
6	NI_5	-	10 μM RA, 10% FBS, DMEM-LG

hMSC harvest at passage 4 was seeded into 6-well plates (BD Falcon) at the proportion of 2000 cells/cm^2 and incubated in DMEM-LG, 10% MSC-FBS with 1% Penicillin-Streptomycin for two days. Expansion medium was replaced with five

different neural induction media. First well was left as a control keeping expansion and it was maintained to culture in expansion medium during sixteen days of differentiation.

Neural induction media was refreshed per three days and differentiation was observed during sixteen days. Neuroblastoma Conditioned Medium (NBCM) was collected by discarding medium (DMEM-LG, 10% FBS and 1% Penicillin-Streptomycin) of cultured neuroblastoma cells which have been releasing various cytokines and growth factors that may lead to facilitate and speed the duration up.

2.4.3 Exposing of Enriched Cytokine Combinations within Neurobasal Media (N3)

hMSCs at passage three were seeded on culture dishes prior to neural induction. Cell frequency was optimized to 3500 cells/ cm^2 for immunostaining, 8000 cells/ cm^2 for immunoblotting and 3000 cells/cm^2 for RT-PCR. Culture was maintained via using DMEM containing 10% MSC-FBS and cells were incubated in 37^oC, 5% CO_2 incubator for two days in terms of attachment to the polystyrene growth surface.

Neural induction media which is composed of several cytokines and growth factors was prepared with 1 mM dbcAMP (dibutyryl cyclic AMP), 0.5 mM IBMX (3-isobutyl-1-methylxanthine), 20 ng/ml hEGF (human epidermal growth factor), 40 ng/ml bFGF (basic fibroblast growth factor), 10 ng/ml FGF-8 (fibroblast growth factor-8), 10 ng/ml human BDNF (human brain-derived neurotrophic factor) in Neurobasal medium + B27 supplement in the absence of serum [82]. Cells were then treated with neural induction media by medium refreshment per 72 hours during fourteen days. In line with this, samples were collected per two days for western blotting and RT-PCR according to preset time points (1 day, 2 day, 3 day, 4 day, 6 day, 9 day, 14 day) while following any morphological change through cells related to neural differentiation.

2. 4 SCREENING OF PROTEIN EXPRESSION LEVELS DURING PROLIFERATION AND NEURAL DIFFERENTIATION OF hMSCs

2.5.1 Immunofluorescence Staining

Medium in wells was aspirated off and cells were permeablized with prewarmed TZN buffer (10 mM pH 7.5 Tris-HCl, 0.5% Nondet P40, 0.2 mM $ZnCl_2$) at RT for 15 minutes by mixing on rocking shaker at very low speed. Besides, cells that were left for immunostaining of membrane bounded proteins such as integrins, weren't treated with TZN buffer because of its detergent content for permeabilization. After removal of buffer, cells were fixed with 4% paraformaldehyde in PBS (Sigma) and incubated at RT for 10 minutes. Accordingly, fixed specimens were washed three times with PBS for 5 minutes on rocking shaker at high speed. After washing with PBS, cells were blocked with the solution containing 10% normal goat serum (NGS, Gibco), 10% normal horse serum (NHS, Biochrom) and 0.3% Triton X in PBS (PBS-Tx) due to prevent non-spesific binding. Blocking solution, which is a combination of 10% NGS and 10% NHS in PBS, was modified for membrane proteins considering needlessly use of Triton X as a detergent. After discarding blocking solution, cells were then treated with primary antibodies at given concentrations (Table 2.2), both diluted in PBS-Tx containing 3% normal human serum (NHS, Biochrom) that bind specifically to target proteins and incubated at 30^{o}C for two hours. Normal human serum was applied onto the negative control wells instead of primary antibodies and after antibody exposure both wells were washed with PBS for three times. Wells were incubated at 30^{o}C for one hour with fluorescence-conjugated (Alexa Fluor 488 and Alexa Fluor 594, Invitrogen) secondary goat antibodies to mouse or rabbit IgG which were prepared in PBS containing 0.5% BSA by 100 times dilution of stock solution resulted in 20 μg/ml final concentration and then washed with PBS for three times. For nuclear staining, cells were treated with 1/15000X DAPI solution and incubated at RT for 10 minutes. Henceforth, procedure was carried by sealing the plate with aluminum foil due to avoid any light transition. Then, three washing were done beside further washing with distilled water (dH_2O). Finally, glass coverslips were drawn off and they were mounted onto the microscope slides covered with Prolong Antifade Kit (Molecular Probes) or Prolong Gold Antifade Reagent (Invitrogen) as the cells interacted with the sticky medium. During fourteen days of neural differentiation, cell images for each spesific time points, were taken under fluorescent microscope (Carl Zeiss).

Table 2. 2 Primary antibodies used in immunostaining.

Antibody	Company	Host	Reactivity	Applied concentration
Anti-Integrin α5β1, (monoclonal)	Chemicon	Mouse	Human	20 μg/ml
Anti-Actin, (polyclonal)	Santa Cruz	Rabbit	Human	10 μg/ml
Anti-Neurofilament (NF-H), (polyclonal)	Chemicon	Rabbit	Human	10 μg/ml
Anti-NSE, (monoclonal)	Chemicon	Mouse	Human	10 μg/ml
Anti-NeuN, (monoclonal)	Chemicon	Mouse	Human	50 μg/ml
Anti-GFAP, (polyclonal)	Cell Signalling	Rabbit	Human	10 μg/ml
Anti-MAP2, (polyclonal)	Cell Signalling	Rabbit	Human	1/50X
Anti-β tubulin III, (monoclonal)	Promega	Mouse	Human	10 μg/ml
Anti-Synaptophysin, (monoclonal)	Chemicon	Mouse	Human	20 μg/ml
Anti-Olig2, (polyclonal)	Chemicon	Rabbit	Human	1/200X
Anti-MAP2, (polyclonal)	Cell Signalling	Rabbit	Human	1/50X
Anti-GAD67, (monoclonal)	Chemicon	Mouse	Human	5 μg/ml

2.5.2 In Situ Hybridization for Surface Antigens

For in situ hybridization, $1X10^5$ cells were seeded on 24 well plates. After attachment by incubation for 24 hours, medium was discarded. Primary antibody which was sterilized by filtration to maintain the culture, was diluted in DMEM-LG. It's then added into the wells via leaving one of them as a control. Plates were incubated at 37^oC, 5% CO_2 incubator for 30 minutes. After removal of antibody solution inside the wells, they were washed with prewarmed fresh medium for three times. Secondary antibodies were prepared as previously described and put in the antibody treated wells. Plates were again incubated at 37^oC, 5% CO_2 incubator for 30 minutes. Residue was discarded and wells were washed with prewarmed PBS with Ca^{++} and Mg^{++}(Biochrom) to prevent the detachment of cells out from the surface layer. Cells in wells containing PBS with Ca^{++} and Mg^{++} were then visualized by flourescent microscope and then placed into 5% CO_2 incubator at 37^oC.

At this point, cells were still alive and they were kept for maintenance of culture due to monitor the progress in growth for following two or three days. Alternatively, the cells were fixed by treatment with 4% PFA solution for 20 minutes and then stored under 50:50 solution of PBS:glycerol.

2.5.3 Immunoblotting Analysis of Proteins

2.5.3.1 Cell Harvest

hMSCs seeded into 75 cm^2 petri dishes with the frequency of $1X10^6$ final outcome in cell number, were washed with PBS as the medium was discarded. Cells were scraped from 75 cm^2 petri dishes by use of lysis buffer containing 50 mM Tris-HCl (pH 6.8), 2 mM EDTA (pH 8.0), 1% SDS (MP Bio), 1% β-ME, 8% glycerol and 2% protease inhibitor cocktail (Roche). Then, lysate was transferred into microcentrifuge tubes placed on ice. Alternatively, cell lysate were frozen at -80^oC and thawed at 37^oC water bath for several times. Samples were boiled for 2 minutes and stored at -20^oC for future use in immunoblotting.

2.5.3.2 Protein Assay: Bradford Method

Total protein amount in each sample was determined by use of Bio-Rad Protein Assay Kit based on Bradford Method. Cell lysates were diluted with double distilled water (ddH_2O) to 1/100X. Protein standards (60 μg/ml, 40 μg/ml, 30 μg/ml, 20 μg/ml, 15 μg/ml, 10 μg/ml, 7.5 μg/ml, 5 μg/ml) were prepared by dilution of bovine serum albumin (BSA) in 1/100X lysis buffer (sample buffer) and as a blank, 1/100X sample buffer was used. In line with this, 160 μl of samples, standards and blank, were transferred into a 96 well-plate. Afterwards, 40 μl of 1/4X BioRad Reagent was added in order to stain for spectrophotometric measurament and solution in each well was mixed by pipetting. Besides, triplets were arranged for each sample due to correlation. Absorbance was measured at 595 nm by ELISA reader (BioTek). Standard curve was drawn and statistical data was collected. Hence, protein concentration was calculated for each sample by the aid of formula that was given with the curve.

2.5.3.3 Sodium Dodecyl Sulphate Polyacrylamid Gel Electrophoresis (SDS-PAGE)

First of all, seperating gel was loaded into the space between two glasses of the vertical gel electrophoresis apparatus and it was filled with Isopropanol (99.7%, Lachema). Gel was let to solidify for 30 minutes at RT. Polymerisation degree of the seperating gel was set according to the molecular weight of target protein (Table 2.3). After that, isopropanol was discarded and gel was washed with dH_2O for 5-6 times to remove the isopropanol residues. Then, stacking gel prepared (Table 2.4) was poured onto the solidified seperating gel and 12 well comb was inserted into the stacking gel . The gel was dried at RT for 30 minutes and gel apparatus was placed in electrophoresis tank including 1X running buffer (10X running buffer was prepared by dissolving 7.575 g Tris Base, Merck, 36 g Glycine, Applichem and 2.5 g SDS in dH_2O so that the total volume would be 250 ml). Samples were previously equalized to a proper final concentration at around 2 mg/ml and after 20 μl of each protein sample was stained with BPB (bromophenol blue), as the comb was removed they were loaded into the wells of the gel in a proper order. In addition to that, appropriate molecular markers (protein ladder, Fermentas), either unstained or prestained were used to demonstrate the size range of proteins. Subsequently the space on top of the wells was filled with 1X running

buffer due to transmit the electricity then the gel was run at 150 volt, 30 mA for 90 minutes by using a power supply.

Table 2. 3 Proportions according to the polymerization degree of seperating gel

SEPERATING GEL (6 ml)	**5%**	**6%**	**7.5%**	**8.5%**	**10%**
dH_2O	2.1 ml	1.950 ml	1.725 ml	1.564 ml	1.35 ml
1M Tris-HCl pH 8.8	2.25 ml	2.25 ml	2.25 ml	2.25 ml	2.25 ml
40% Acrylamide	0.75 ml	0.9 ml	1.125 ml	1.28 ml	1.5 ml
2% APS	0.3 ml	0.3 ml	0.3 ml	0.3 ml	0.3 ml
1% SDS	0.6 ml	0.6 ml	0.6 ml	0.6 ml	0.6 ml
TEMED	6 µl	6 µl	6 µl	6 µl	6 µl

Table 2. 4 Ingredients of stacking gel

STACKING GEL (5 ml)	
dH_2O	2.1 ml
1M Tris-HCl pH 6.8	2.25 ml
40% Acrylamide	0.75 ml
2% APS	0.3 ml
1% SDS	0.6 ml
TEMED	6 µl

After running was completed, gel apparatus was removed away from the electrophoresis tank and stacking gel was discarded whereas seperating gel was gently laid into dH_2O. Then the seperating gel was either blotted or stained with Coomassie Brilliant Blue (CBB, Applichem) staining solution (0.1% CBB in 1% Acetone-40% Methanol) for about 40 minutes and destained overnight with destaining solution composed of 1% Acetone and 40% Methanol in dH_2O.

2.5.3.4 Protein Transfer

Protein samples located into the gel were transferred to the PVDF membrane by using dry blot instrument (iBlot, Invitrogen). After blot, unstained molecular marker was cut and stained with CBB, destained with destaining solution, washed with dH_2O several times and let to dry . Since prestained marker was already stained, it was left for being dehydrated. Remaining membrane which carried the protein samples was embedded in blocking solution containing 5% Skim Milk (Applichem), 0.1% Tween 20 (Applichem) in New TBS Buffer (0.01 M Tris-HCl pH 7.5, 0.15 M NaCl, Applichem) and stored at 4^oC overnight.

2.5.3.5 Antibody Treatment

Membrane was blocked in blocking solution for 2 h at RT, washed with New TBS and TTBS (20 mM Tris-HCl pH 7.5, 0.5 M NaCl , 0.05% Tween 20) buffers. Then, membrane was incubated with specific primary antibodies against target proteins for 1 hour at 30^oC. Antibodies were diluted in antibody buffer composed of 1% gelatin (Applichem) in TTBS to a proper concentration as recommended (Table 2.5)

Table 2. 5 Primary antibodies used in western blot.

Antibody	Company	Host	Reactivity	Applied concentration
Anti-Integrin α5β1, (monoclonal)	Chemicon	Mouse	Human	0, 25 μg/ml
Anti-Integrin α5, (polyclonal)	Chemicon	Rabbit	Human	1/5000X
Anti-Integrin β1 , (polyclonal)	Chemicon	Rabbit	Human	1/500X
Anti-Actin, (polyclonal)	Santa Cruz	Rabbit	Human	1 μg/ml

After labelling with primary antibody, membrane was washed with TTBS and treated with Horse Radish Peroxidase (HRP)-labeled goat-anti mouse or goat anti rabbit secondary antibody (GAM-HRP, GAR-HRP, Chemicon) for 1 hour at RT. Secondary

antibodies were diluted with antibody buffer to 1/5000X for GAM-HRP and 1/25000X for GAR-HRP as recommended.

2.5.3.6 Detection of Band Patterns

After secondary antibody treatment, membrane was washed with TTBS and TBS (20 mM Tris-HCl pH 7.5, 0.5 M NaCl) buffers for several times and treated with chemiluminescent HRP substrate (Vector Lab) for 5 minutes. After incubation, substrate was removed by washing with 1M Tris-HCl pH 9.5 and highly pure water for 3-4 times. Under dark, membrane was exposed to an autoradiography film (KODAK) from 15 seconds up to 1 minute. Then, the film was put into the washing solutions both for 1 minute in following order :

1. Devoloping solution (KODAK)
2. Fixative solution (KODAK)
3. Tap water

In the end, it was washed with tap water and let to dry. Bands on the film was named according to loading order and scanned for data analysis.

2.6 RNA ANALYSIS BY REVERSE TRANSCRIPTASE PCR

2.6.1 Sample Distruption & Homogenization

For RNA isolation from cell culture dishes, AllPrep Isolation Kit (QIAGEN) which permits consecutively isolation of total DNA, RNA and protein component of the same sample. First of all, cell-culture medium was completely aspirated from 35mm culture dish. For direct lysis of cells grown in a monolayer, 350 µl of Buffer RLT was added to the cell-culture dish (35mm). The lysate was then collected with a rubber policeman and lysate was pipetted into a microcentrifuge tube. It was vortexed to mix until no cell clumps are visible before proceeding to next step. The homogenized lysate was transferred to an AllPrep DNA spin column placed in a 2 ml collection tube and centrifuged for 30 s at >8000 x g (>10,000 rpm). The AllPrep DNA spin column was placed in a new 2 ml collection tube, and stored at room temperature (15–25°C) or at 4°C for later DNA purification.

2.6.2 RNA Isolation

To the flow-through obtained from sample preperation step, 250 µl 96–100% ethanol was added (if 350 µl Buffer RLT was used). Then it was mixed by pipetting. 700 µl of the sample including any precipitate that may have formed was transferred to an RNeasy spin column placed in a 2 ml collection tube and centrifuged for 15 seconds at >8000 x g (>10,000 rpm). The flow-through was transferred to a 2 ml tube for protein purification in steps. 700 µl Buffer RW1 was added to the RNeasy spin column and centrifuged for 15 seconds at >8000 x g (>10,000 rpm) to wash the spin column membrane. The flow-through was then discarded. 500 µl Buffer RPE was added to the RNeasy spin column and centrifuged for 15 seconds at >8000 x g (>10,000 rpm) to wash the spin column membrane. The flow-through was then discarded and 500 µl Buffer RPE was added to the RNeasy spin column and centrifuged for 2 minutes at >8000 x g (>10,000 rpm) to wash the spin column membrane. The RNeasy spin column was placed in a new 1.5 ml collection tube and 30 µl RNase-free water was added directly to the spin column membrane. After that, it was centrifuged for 1 min at >8000 x g (>10,000 rpm) to elute the RNA.

2.6.3 cDNA Synthesis

Template RNA was thawed on ice. Then, primer solution was thawed and 10x Buffer RT, dNTP Mix, and RNase-free water were at room temperature (15–25°C). It was stored on ice immediately after thawing. Each solution was mixed by vortexing, and then centrifuged briefly to collect residual liquid from the sides of the tubes. RNase inhibitor was diluted to a final concentration of 10 units/µl in ice-cold 1x Buffer RT (diluted an aliquot of 10x Buffer RT accordingly using theRNase-free water supplied) and mixed carefully by vortexing for no more than 5 seconds, then centrifuged briefly to collect residual liquid from the sides of the tube. A fresh master mix was prepared on ice and mixed thoroughly and carefully by vortexing for no more than 5 seconds. After that it was centrifuged briefly. The master mix contains all components required for first-strand synthesis except the template RNA. If setting up more than one reverse-transcription reaction, appropriate volume of master mix was distributed into individual reaction tubes. Then they were kept on ice.Template RNA was added to the individual

tubes containing the master mix and then mixed thoroughly and carefully by vortexing for no more than 5 seconds. Finally, they were centrifuged briefly to collect residual liquid from the walls of the tubes. After that, they were incubated for 60 min at 37°C. An aliquot of the finished reverse-transcription reaction was added to the PCR mix. Reverse-transcription reactions were stored on ice and proceeded directly with PCR,* or for long-term preservation, they were stored at –20°C.

2.6.4 PCR

Master mix solution was prepared according to Table 2.6 and following RT-PCR primers (Invitrogen) were used.

Alpha 5 (584bp) :

Forward: 5'-AGCCTGTGGA GTACAAGTCC-3'

Reverse : 5'-AAGTAGGAGGCC ATCTGTTC-3'

Beta 1 (756bp) :

Forward: 5'-AGCAGGGCCA AATTGTGGGT-3'

Reverse : 5'-CCACCAAGTTTCCCATCTCC-3'

Content	**Volume**
10 X Buffer	2.5 µl
25 mM $MgCl_2$	2 µl
10 mM dNTP	0,5 µl
F.Primer	1µl
R.Primer	1µl
Taq Polimerase	0.2 µl
cDNA	1 µl
ddH_2O	16,8 µl

Table 2.6 Master mix solution used for RT-PCR

The thermal cycler (Techne, Cambridge, UK) was used for RT-PCR assay. The products were amplified under the following conditions: 5 min at 94°C for initial denaturation followed by 30 cycles of 30 sec at 94°C, 45 sec at 61°C, 1 min at 72°C, and 1 min at 94°C with a final round of 10 min at 72°C.

2.6.5 Agarose Gel Electrophoresis & Detection

2% agarose gel was used to detect PCR products.1 g of agarose (Sigma, St. Louis, USA) was mixed with 50 ml of 0.5X Tris-borate EDTA (TBE) buffer. Then it was heated until boiling. The gel was cooled to 40°C and 3.5 µl ethidium bromide was added. The gel was then poured and a comb was placed in the gel. As for loading, 10 µl PCR product was mixed with 2 µl bromophenol blue as a tracking dye.10 µl PCR product was then put in each slot. 1 µl of a 100 bp DNA Ladder (Fermentas) was mixed with 1 µl deionized water and 1 µl bromophenol blue. Then 5 µl of this mix was put into the side slot as a molecular marker. The gel was run at 95 V in 0.5X TBE buffer for 50 min. The gel was placed in Gel Doc 2000 (Biorad, Milan, Italy) apparatus and the bands were detected under UV transilluminator.

CHAPTER 3

RESULTS

3. 1 Identification of hMSCs from Bone Marrow by FACS Analysis

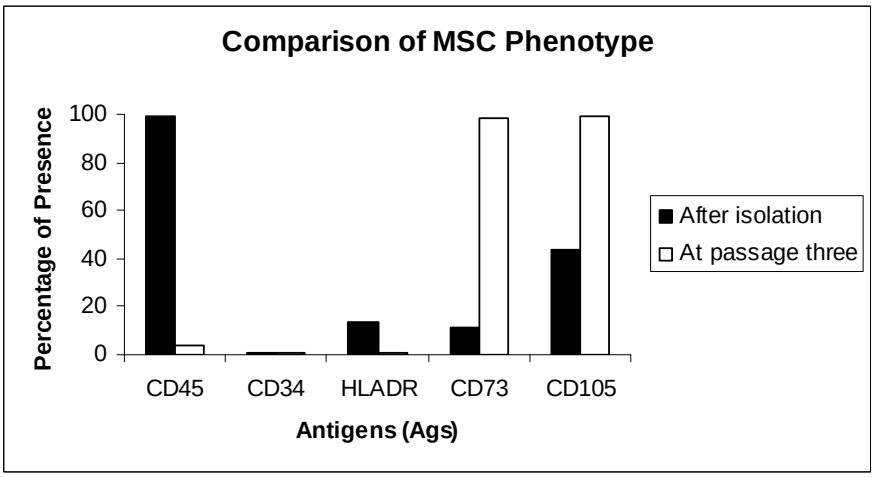


Figure 3. 1 hMSC4 immunophenotype determined by FACS.

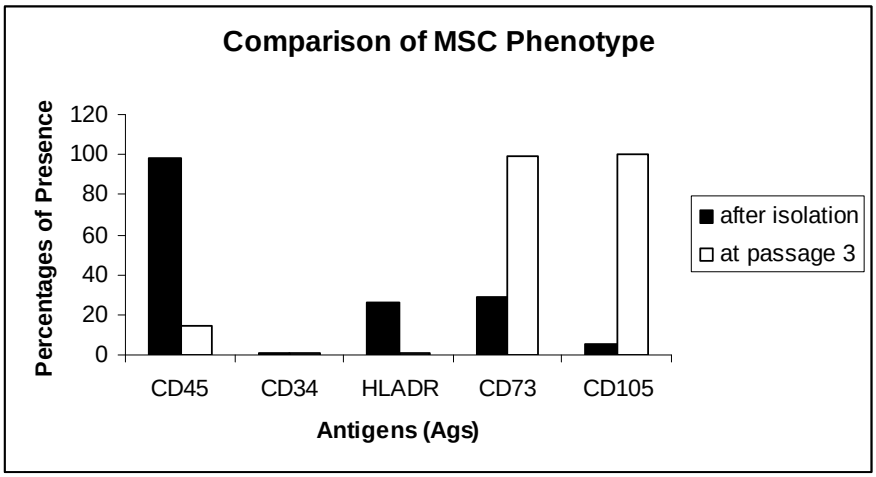


Figure 3. 2 hMSC8 immunophenotype determined by FACS.

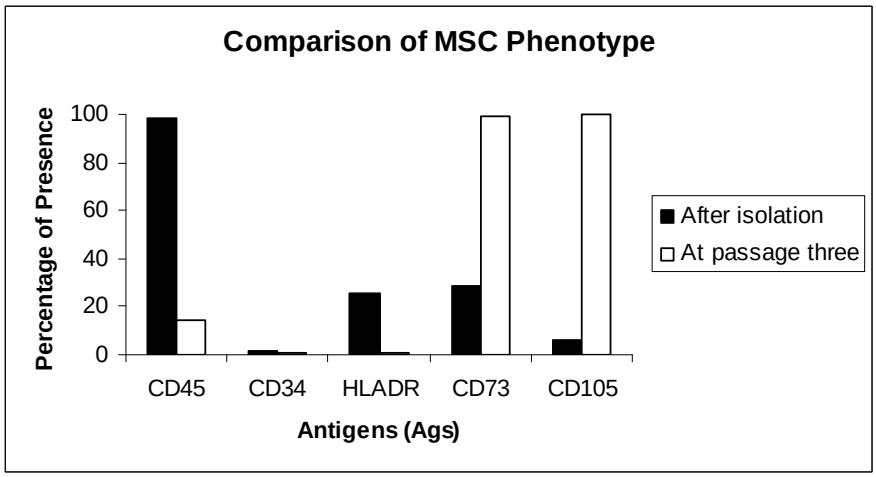


Figure 3. 1 hMSC9 immunophenotype determined by FACS.

FACS outcomes verify that human mesenchymal stem cells are selectively positive for the mesenchymal surface antigens at passage three while they have low expression levels of the common MSC antigenic profile after isolation.

a. **b.**

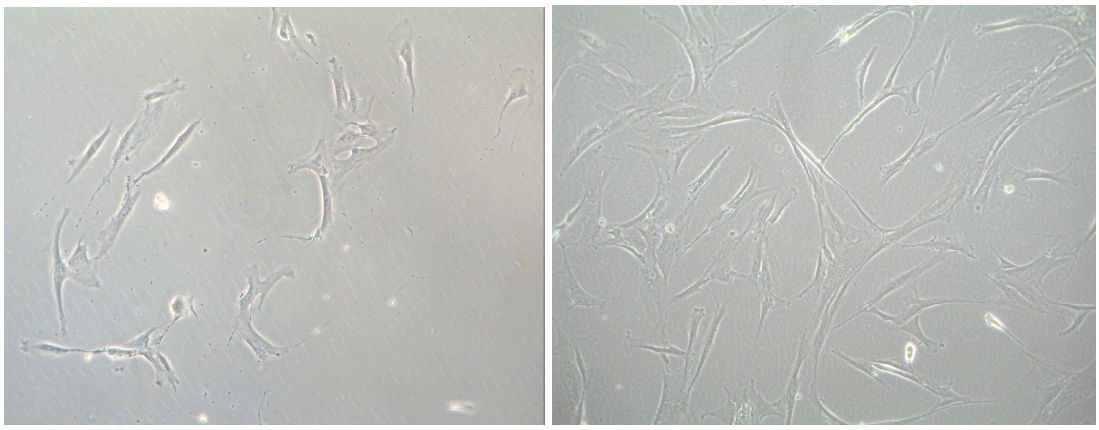


Figure 3. 2 Phase-contrast images of hMSCs after isolation, primary cells on culture. (a) and at passage three (b) both are under 10X magnification .

3. 1 Total Protein Amount Produced in hMSCs

As the cells were induced into neural-like differentiation, total protein amount in hMSCs were decreased at similar ratio according to time points. Conversely, with later proccess of differentiation, protein amounts rise again both in RA based induction and

Table 3. 1 Protein concentrations of RA mediated neural induction related to statistical outcomes caculated from standard curve analysis.

	CONTROL	RA
24 hours	1,132mg/ml	0,151 mg/ml
48 hours	0,754 mg/ml	0,604 mg/ml
day 4	1,21 mg/ml	1,43 mg/ml

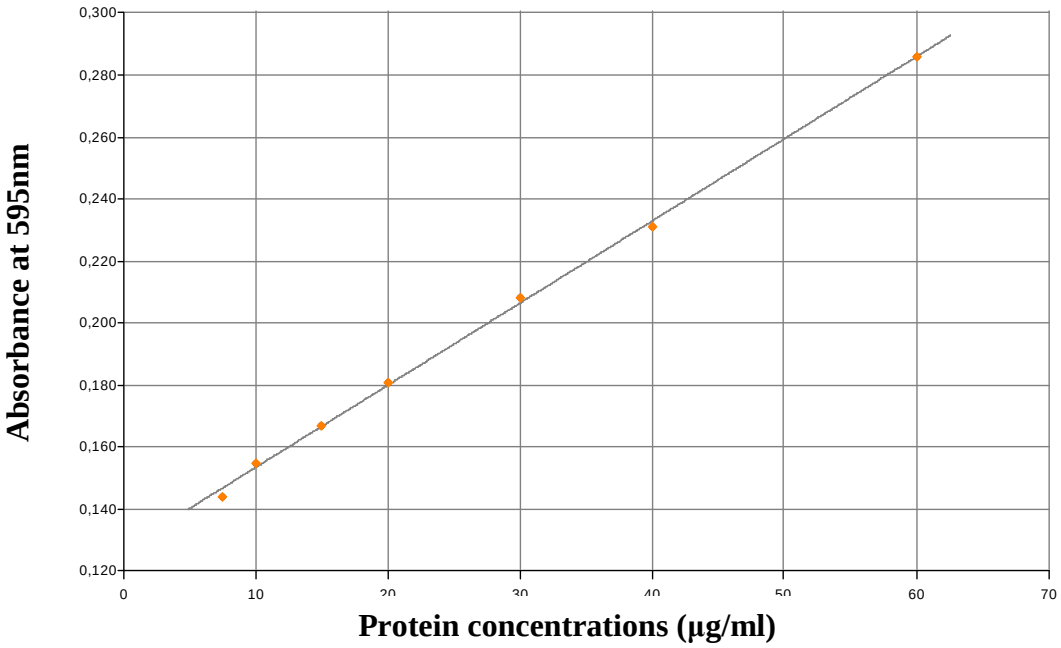


Figure 3. 3 Standard Curve for hMSCs Induced by Retinoic Acid.

Table 3. 2 Protein concentrations of N3 mediated neural induction related to statistical outcomes caculated from standard curve analysis.

	CONTROL	N3
day 2	7,6 mg/ml	
day 2	-	5,6 mg/ml
day 4	-	3,27 mg/ml
day 6	-	1,63 mg/mL
day 8	5,25 mg/ml	-
day 8	-	1,96 mg/ml
day 10	-	4,26 mg/ml
day 12	-	5,12 mg/ml
day 14	5,77 mg/ml	-

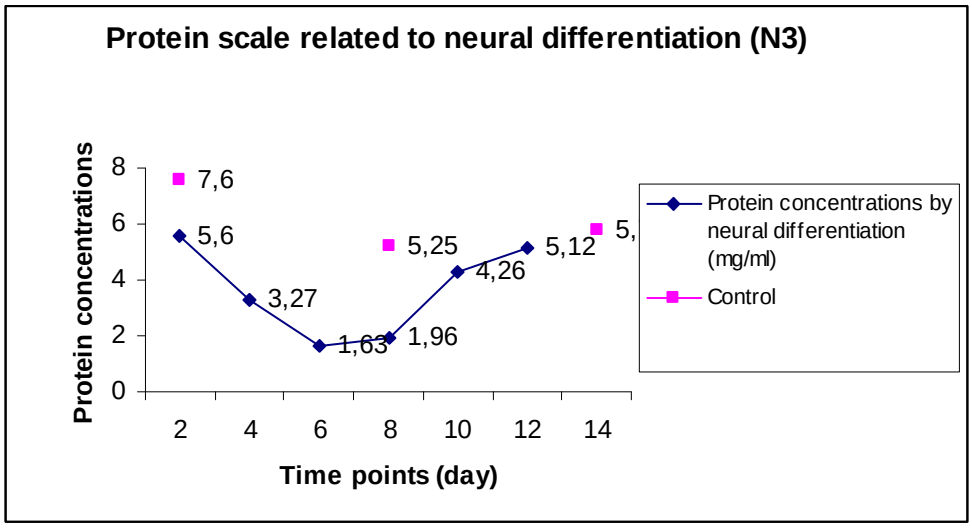


Figure 3. 4 Distribution of protein concentrations of N3 mediated neural induction.

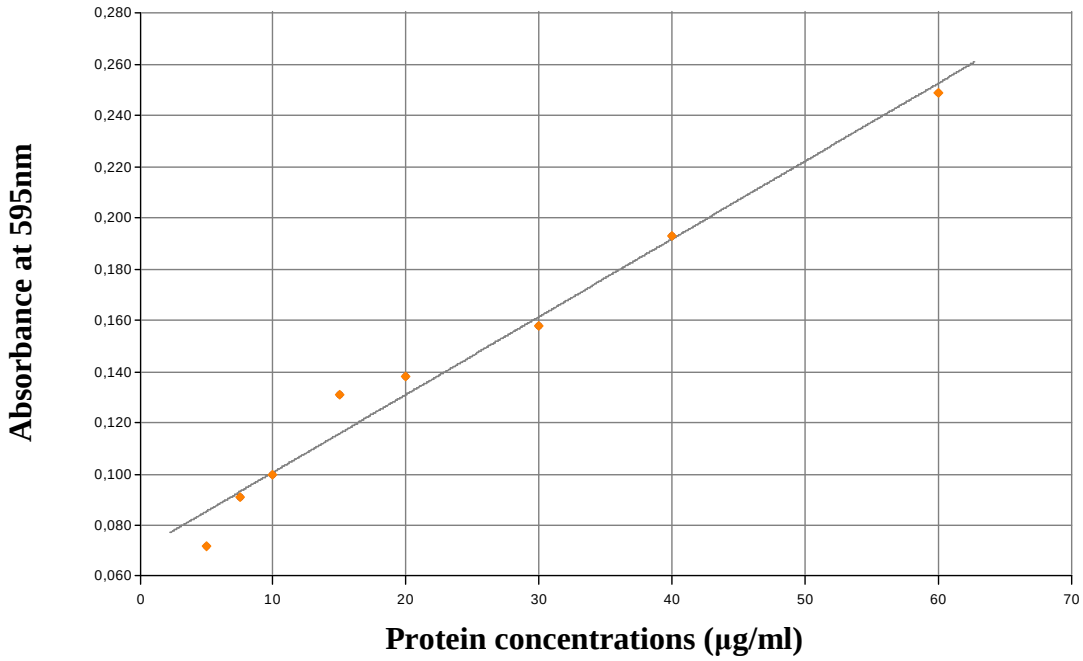


Figure 3. 7 Standard Curve for hMSCs Induced by Enriched Cytokine Combinations.

3. 2 Morphological Modifications Caused by Neural Induction with RA

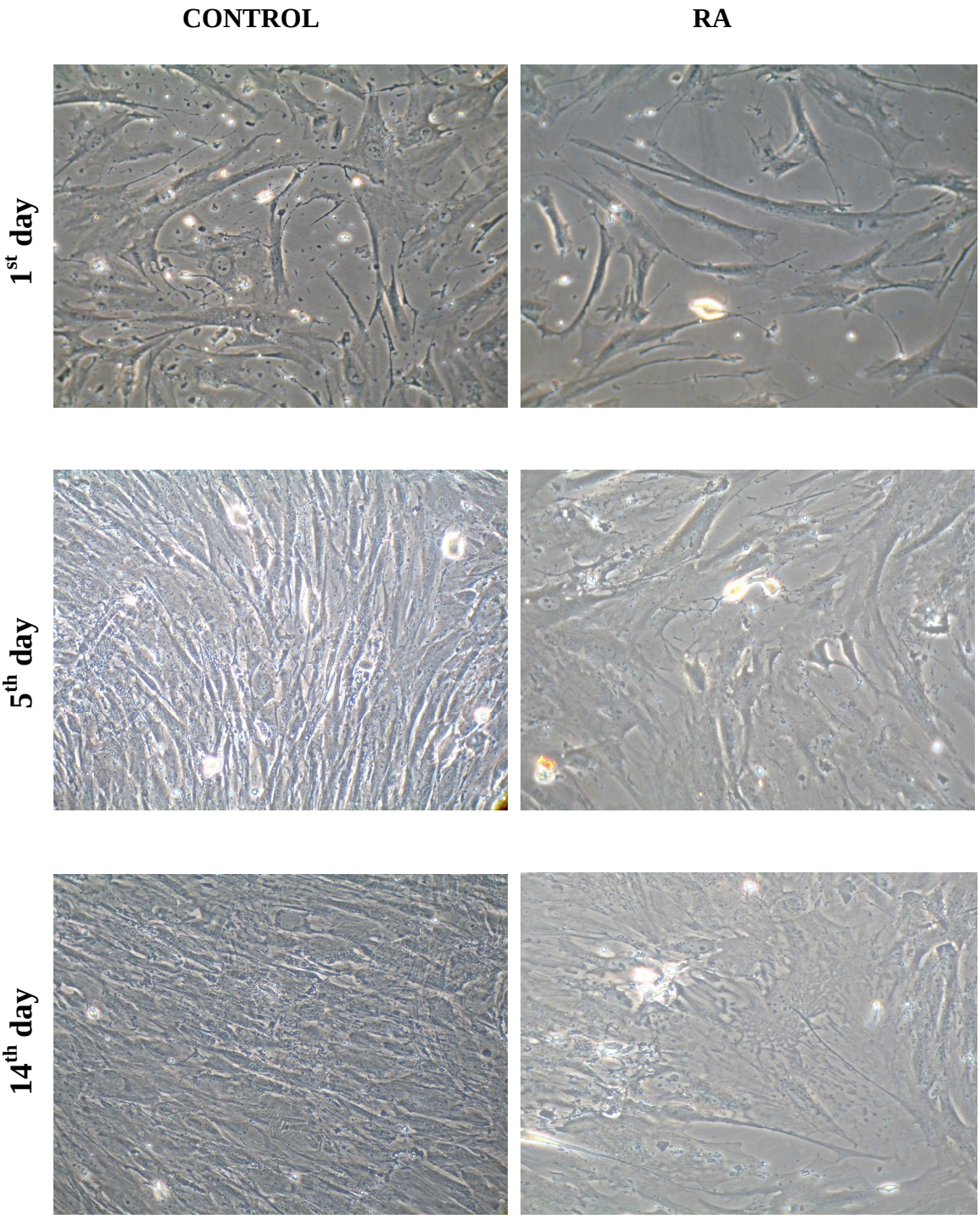


Figure 3. 8 Morphology of hMSCs treated with RA for neural induction during fourteen days. Images are taken under 20X magnification.

hMSCs in patches morphologically tend to turn into neural phenotype but not at a frequent ratio.

3. 3 Protein Variation and Complexity of Neuronal Induced hMSCs via Cytokines

Neuronal induced hMSCs have an altered protein expression pattern on SDS-PAGE. Although bands were dim because of transferring most of the proteins via blotting, it's visible to see some of proteins were gradually expressed whereas some of them were failed by neural differentiation as it's compared to control group. On the other hand, some bands appear by neural induced hMSCs while they're absent on control lanes.

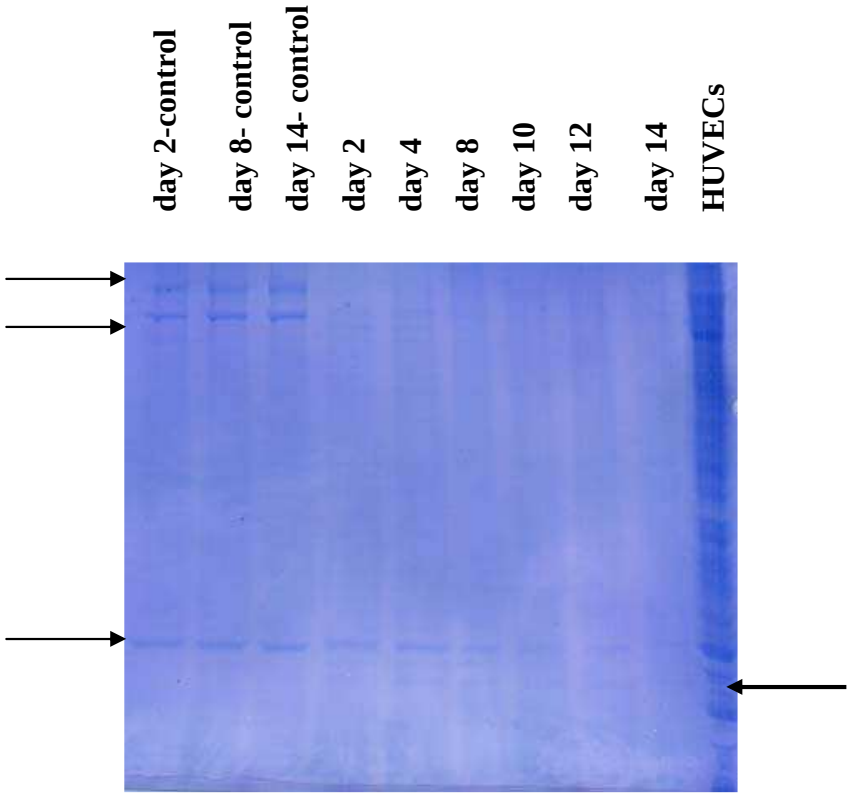


Figure 3. 9 7,5% SDS polyacrylamide gel after blot. (40 µg proteins were loaded for hMSCs and 25 µg for HUVECs.

3. 4 Existance of Early and Late Neural Markers

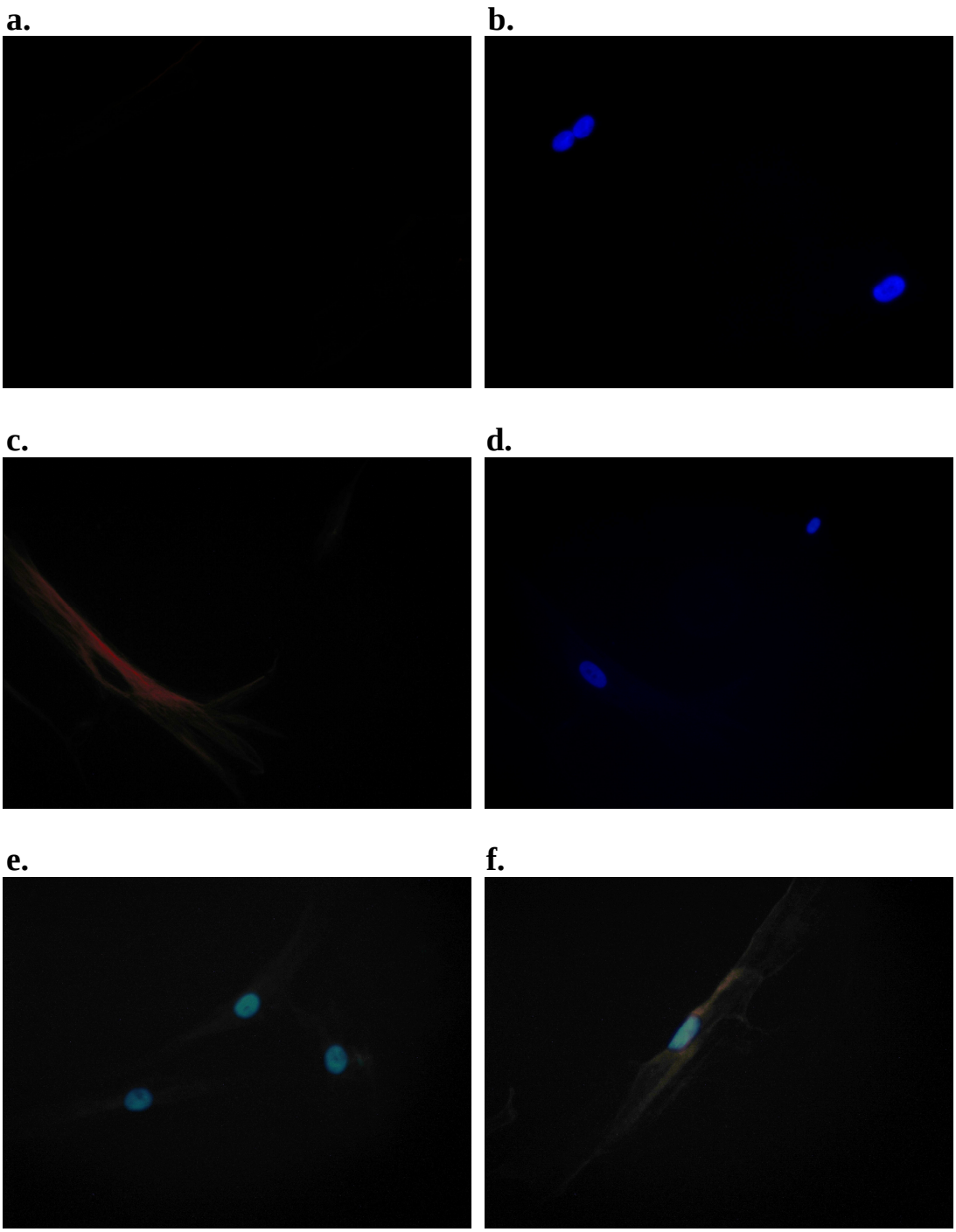


Figure 3. 10 Immunofluorescence detection of early neural markers after 48 hours neural induction of hMSCs with RA and at day 4 with N3.

Images were taken under 40X magnification. a) β3 Tubulin control, b) β3 Tubulin control-DAPI, c) β3 Tubulin RA, d) β3 Tubulin RA-DAPI, e) NSE control overlapped with DAPI staining, f) β3 Tubulin RA overlap.

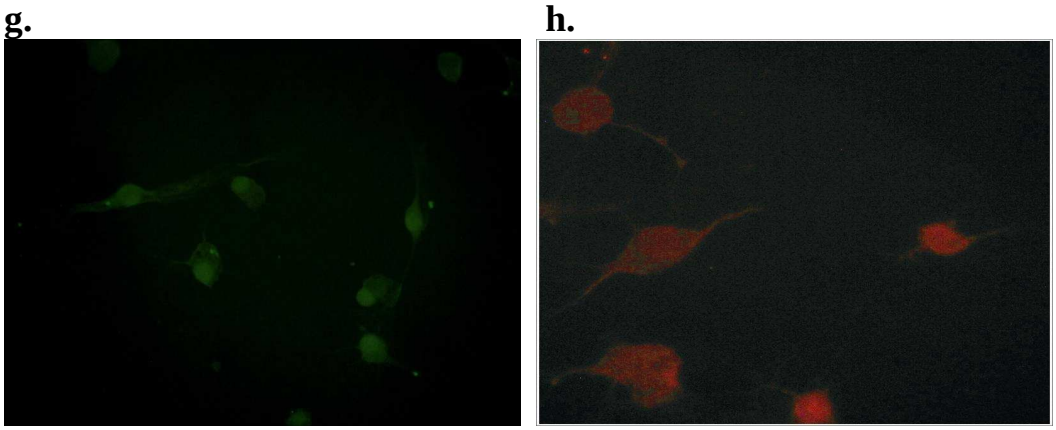


Figure 3. 10 Immunofluorescence detection of early neural markers after 48 hours neural induction of hMSCs with RA and at day 4 with N3.

g) Nestin, N3, under 40X magnification, h) NSE, N3, under 60X magnification.

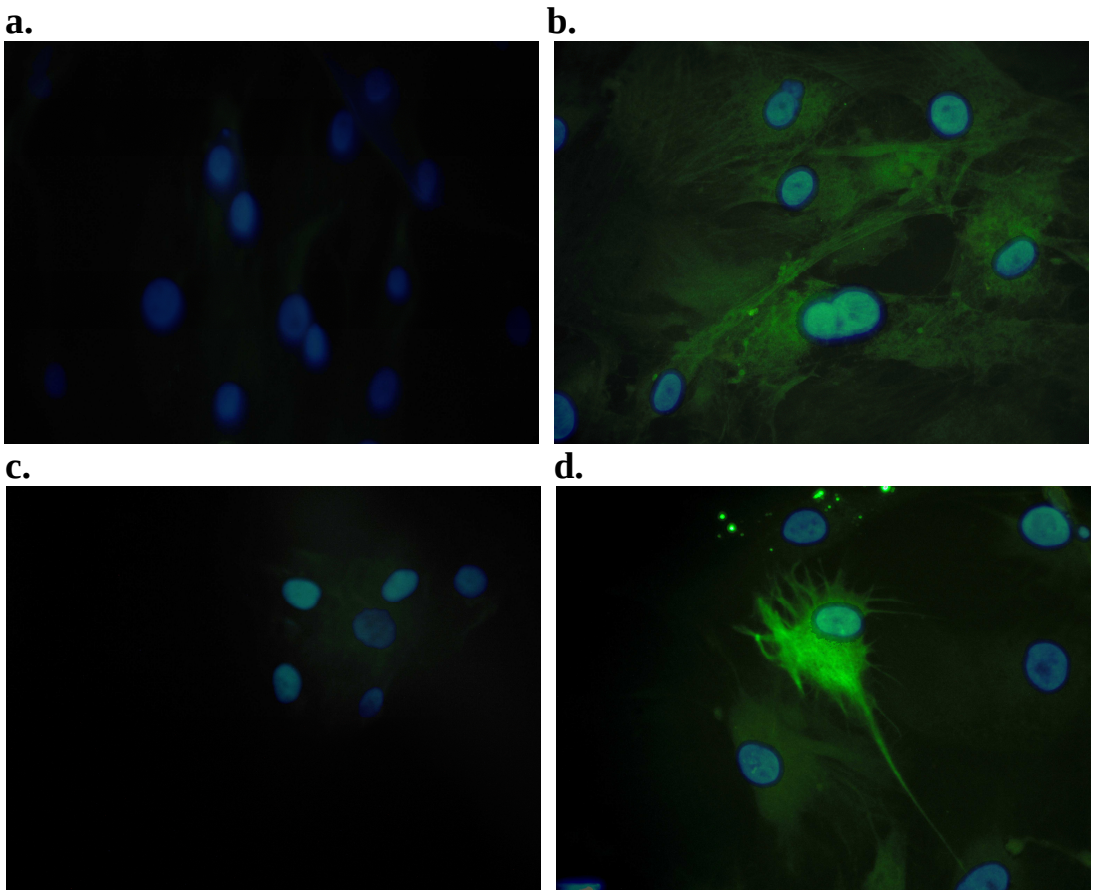


Figure 3. 11 Immunofluorescence staining of mature neuronal markers after either RA or N3 based neural induction of hMSCs. Images were taken under 40X magnification.

a) NF control, at the 14th day b) NF treated with RA, at the 14th day c) NF after RA treatment for 24 hours, d) NF after RA treatment for 14 days

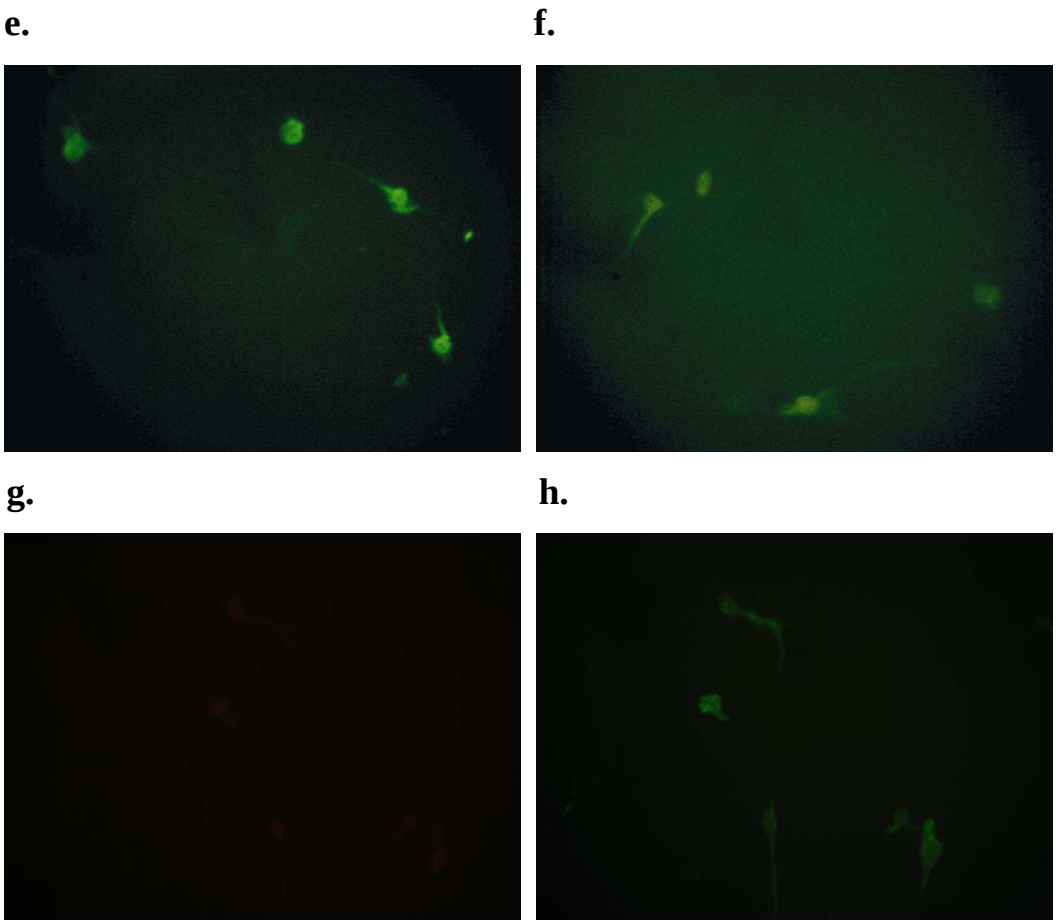


Figure 3. 11 Immunofluorescence staining of mature neuronal markers after either RA or N3 based neural induction of hMSCs .

Images were taken under 40X magnification. e) NF N3 at the end 14th day, f) MAP2 N3 at the end 14th day, g) GAD 67 N3 at the 14th day, h) GFAP N3 at the14th day.

Early neural markers exist during initial stages of neural differentiation while mature markers are detected by further phases.

3. 5 Cell Phenotype Caused by Cytokine Mediated Neural Differentiation

Enriched cytokine combinations lead to obtain the most efficient neural differentiation ability among other protocols.

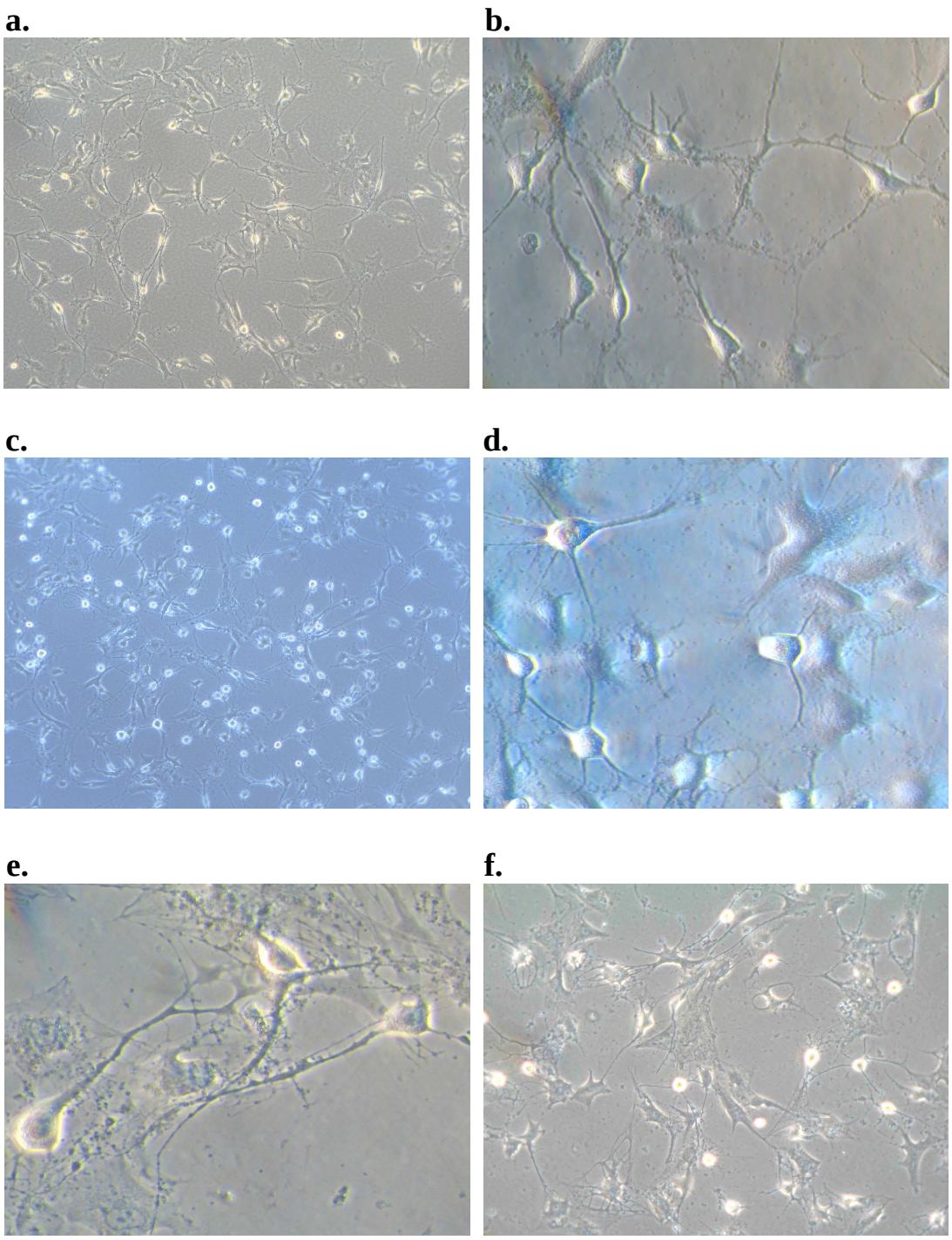


Figure 3. 12 Comparison of N3 mediated neural differentiation in terms of cell morphologies at distinct time points.

a) hMSCs at the day 5 after neural induction under 10X b) hMSCs at the day 5 after neural induction under 40X c) hMSCs at the day 6 after neural induction under 10X d) hMSCs at the day 6 after neural induction under 40X e) hMSCs at the day 9 after neural induction under 40X, f) hMSCs at the day 11 after neural induction under 20X.

3. 6 Effect of Serum Free Microenvironment in Neural Differentiation

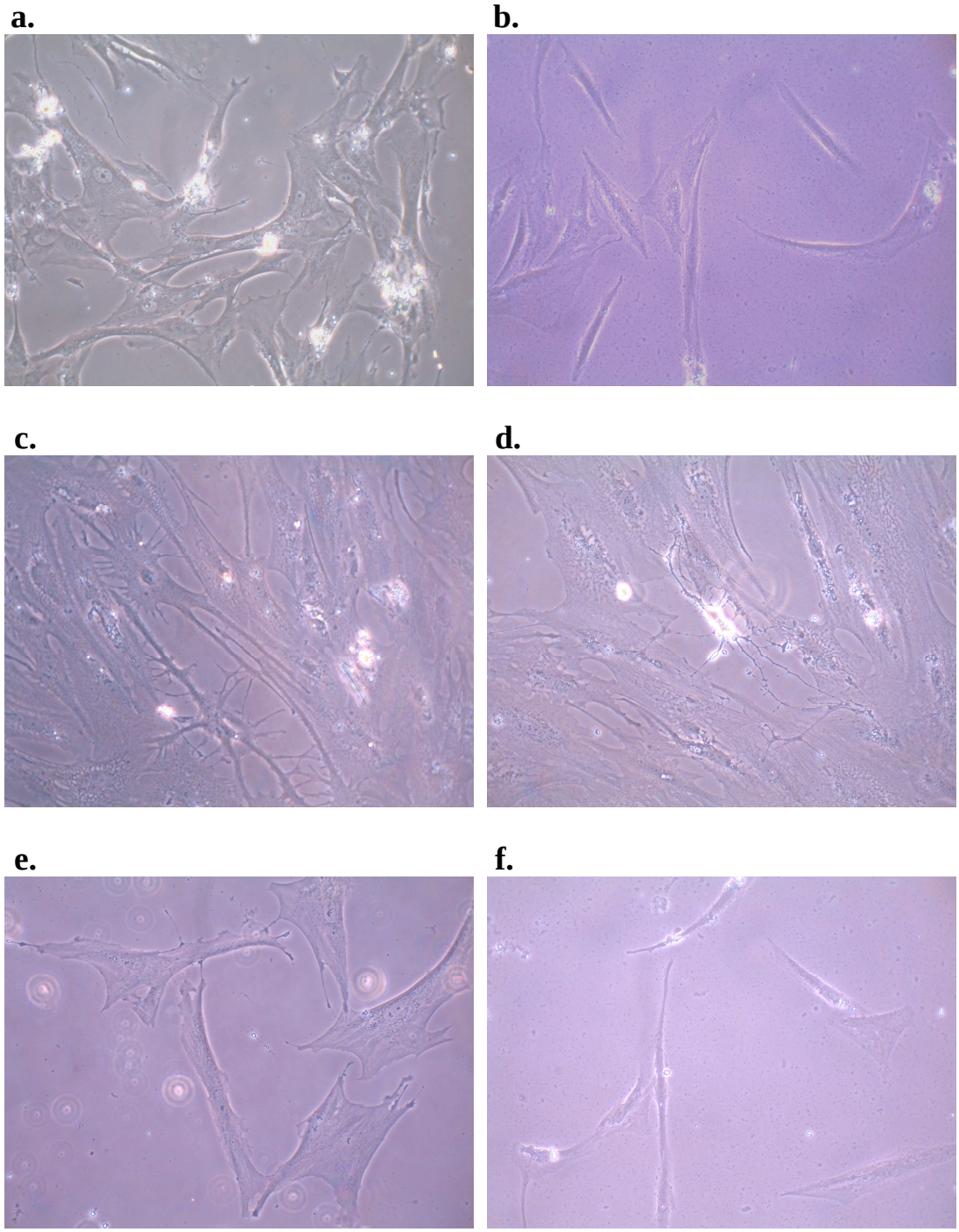


Figure 3. 13 The effect of serum free media use in neural differentiation of hMSCs.

All images were taken under 20X magnification. a) day 4-control, b) day 4-serum free RA treatment, c) day 18-serum free RA, d) day 24- serum free RA, e) day 7- RA, f) day 6- serum free RA under 40X.

Serum free conditions increase neural differentiation potential of hMSCs in vitro.

3. 8 Integrin α5β1 Expression of hMSCs in comparison with HUVECs

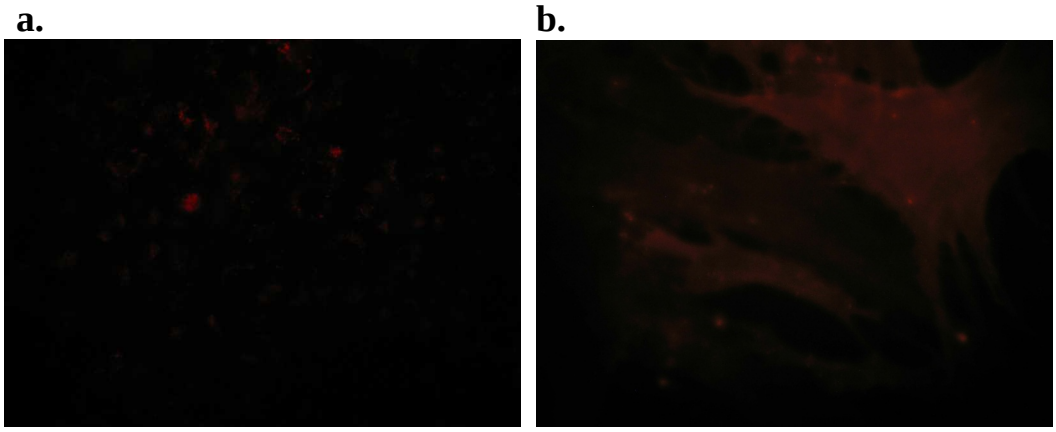


Figure 3. 14 Immunofluorescence detection of integrin α5β1 in both cancer cell lines (a) and hMSCs (b) under 40X magnification.

3. 9 Integrin α5β1 Involvement During Proliferation and Neural Differentiation

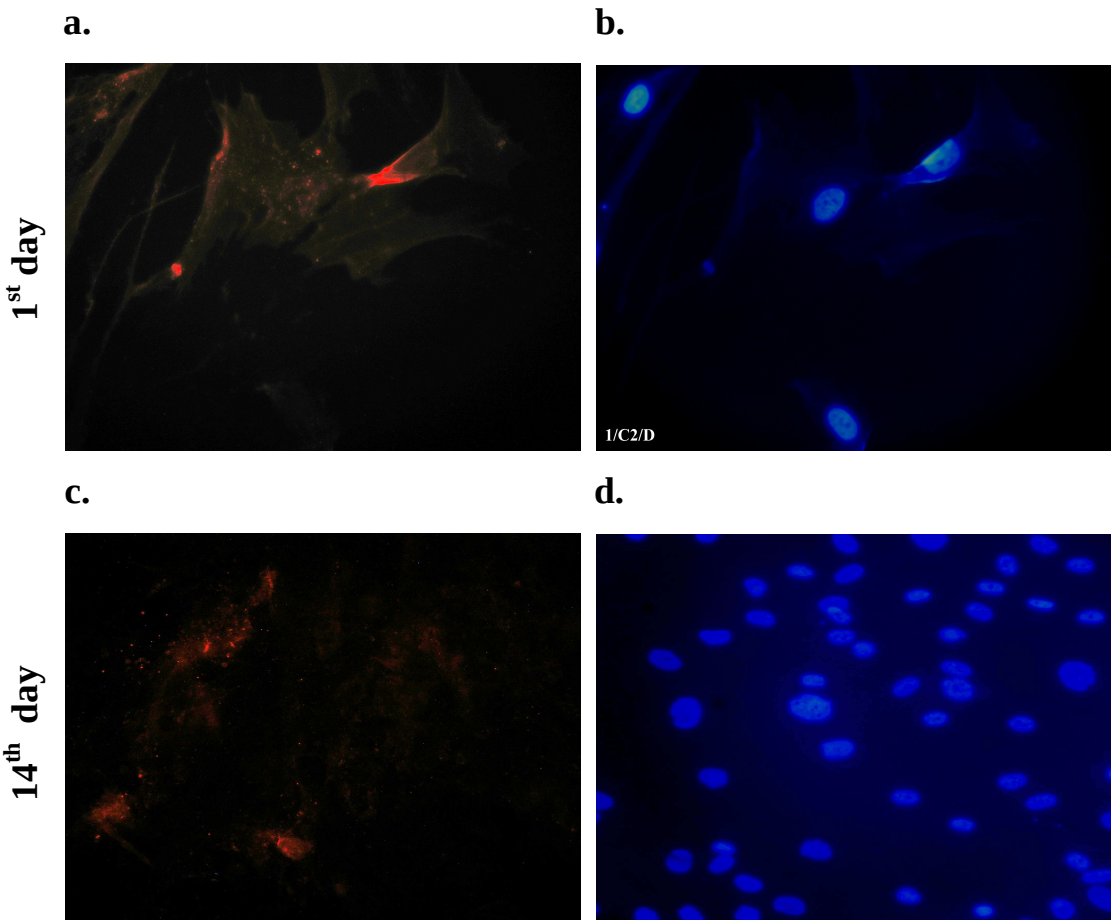


Figure 3. 15 Immunofluorescence detection of integrin α5β1 in hMSCs.

Detection at the 1st day (a) and 14th day of proliferation on culture (c) ; DAPI staining was depicted (b) for the 1st day and (d) for the 14th day; magnification was under 40X.

α5β1 presence in hMSCs increase with proliferation as it can be seen from the figure above.

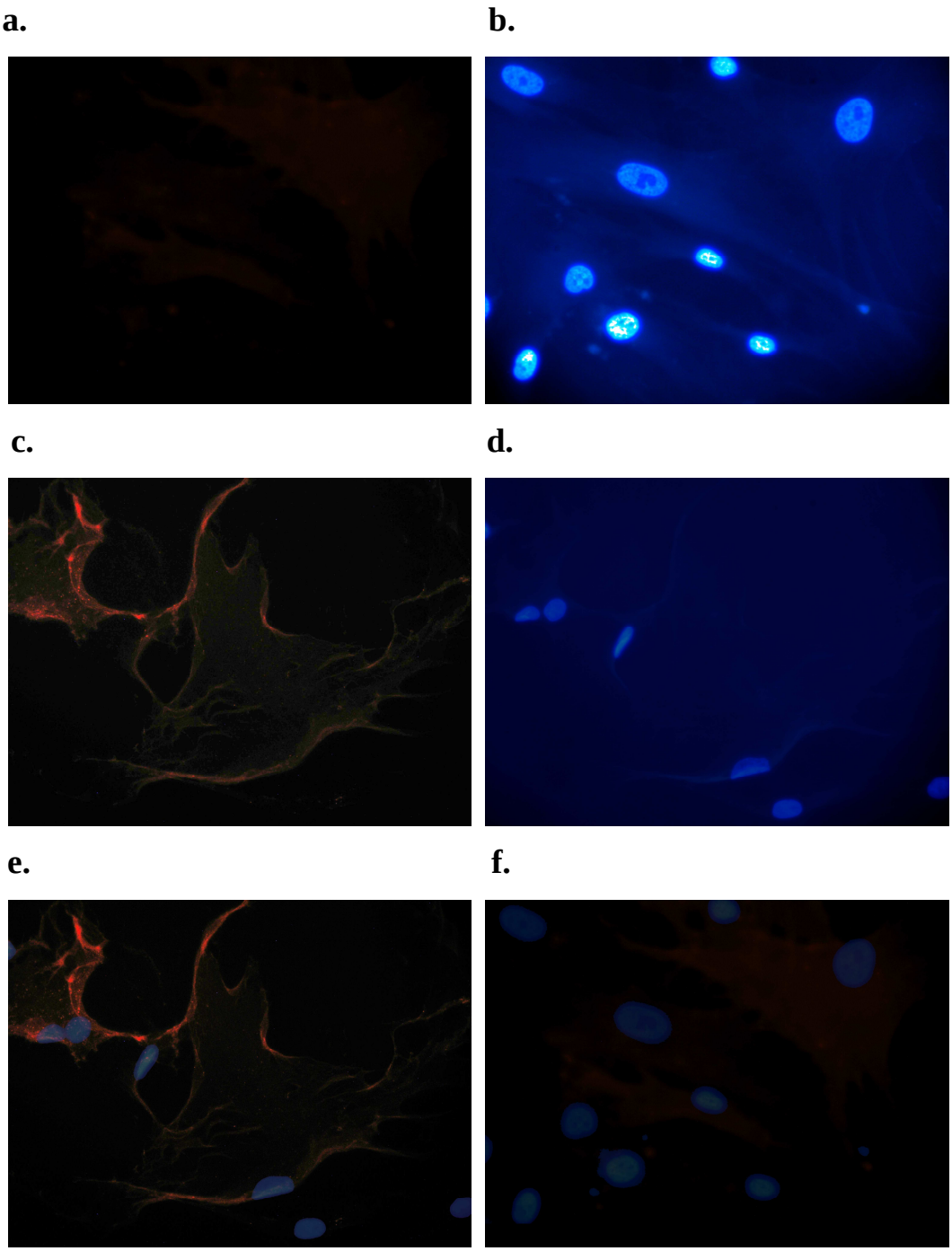


Figure 3. 16 Integrin α5β1 expression pattern of neuronal induced hMSCs at 24 hours. a) control, b) control-DAPI, c) RA, d) RA-DAPI, e) RA overlapped with DAPI staining, f) control overlapped with DAPI staining. Images were taken under 40X magnification.

Integrin α5β1 is highly expressed by the initial steps of neural differentiation as it is compared to control group.

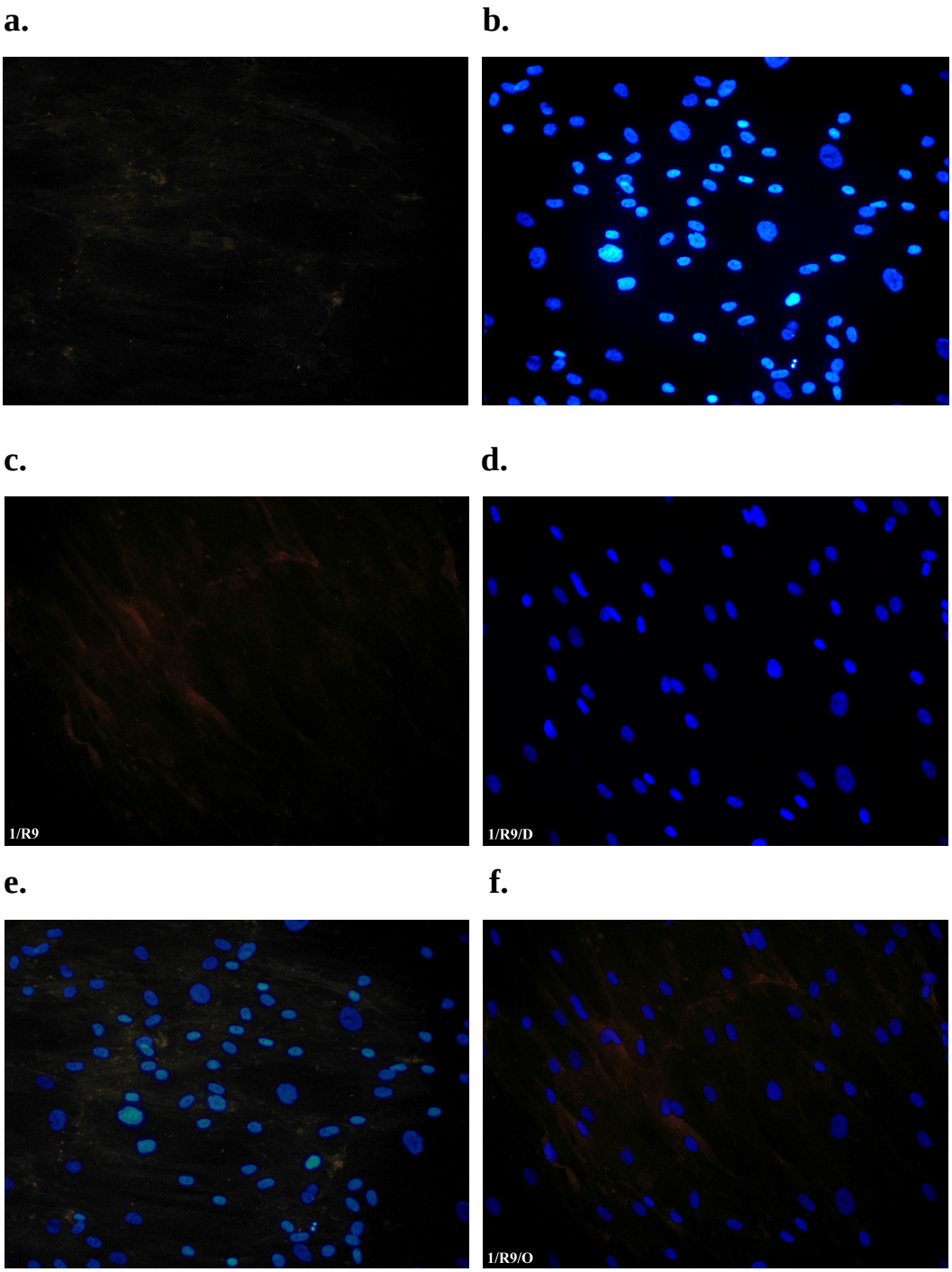


Figure 3. 17 Integrin α5β1 expression pattern of neuronal induced hMSCs at 14 days.

a) control, b) control-DAPI, c) RA, d) RA-DAPI, e) control overlapped with DAPI staining, f) RA overlapped with DAPI staining. Images were taken under 20X magnification.

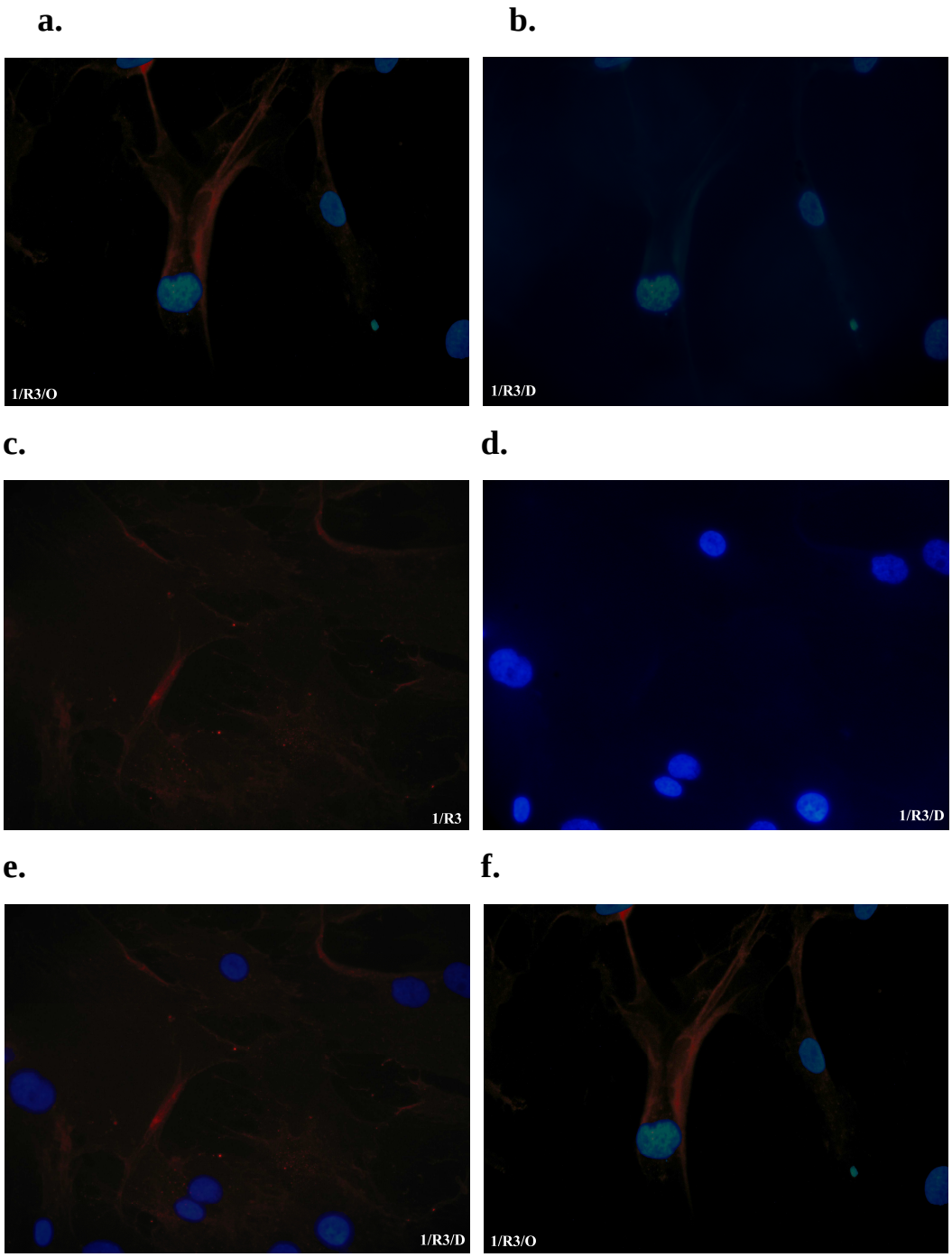


Figure 3. 18 Comparison of integrin expression and localization on neuronal induced hMSCs by RA at 1st day and 14th day.

a) 24 hours, b) 24 hours DAPI, c) 14 days, d) 14 days DAPI, e) 14 days overlap, f) 24 hours overlap. Images were taken under 40X magnification.

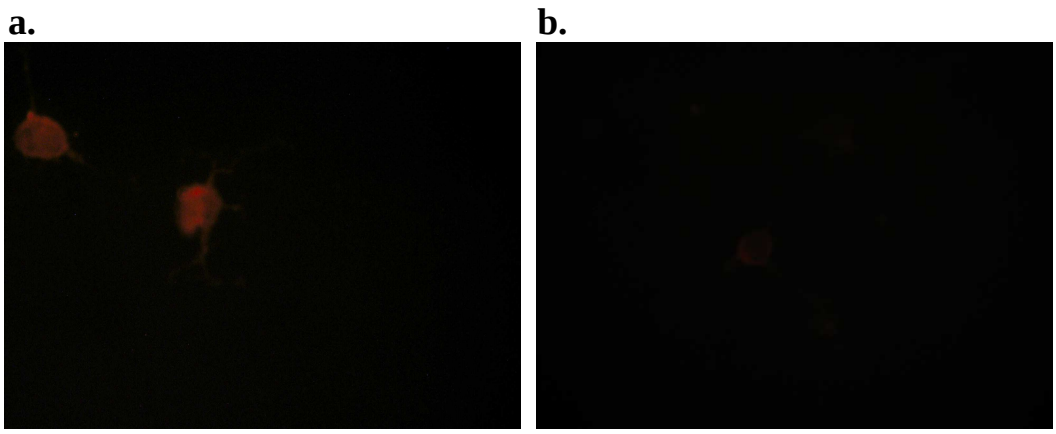


Figure 3. 19 Immunostaining of integrin α5β1 on neuronal induced hMSCs via N3 media at day 4 (a) and day14 (b) under 40X magnification.

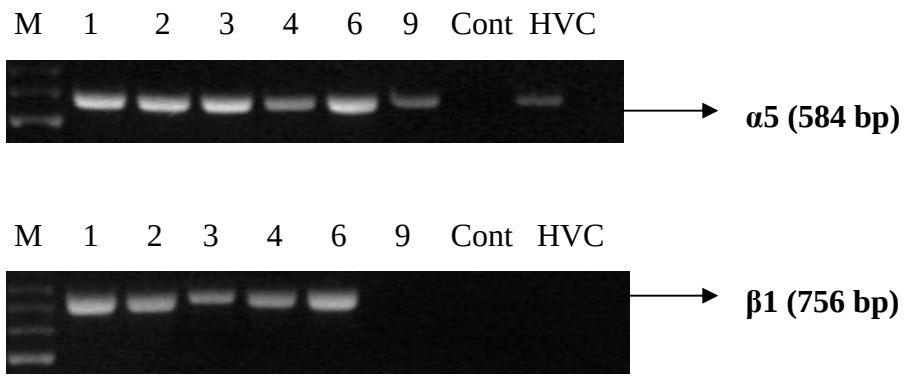


Figure 3.20 Integrin α5β1 RT-PCR results of N3 mediated neural differentiation of hMSCs at distinct time points.

Integrin α5β1 localization and expression are higher in induced hMSCs with a visible difference of decreased pattern at the late points. According to investigations at RNA level, β1 is not detected after day 6 while α5 is still found in late process, indicating that the α5 subunit belongs to another integrin receptor rather than β1 family. Additionally, control and HUVEC cells are not positive for β1 whereas α5 is seen HUVECs.

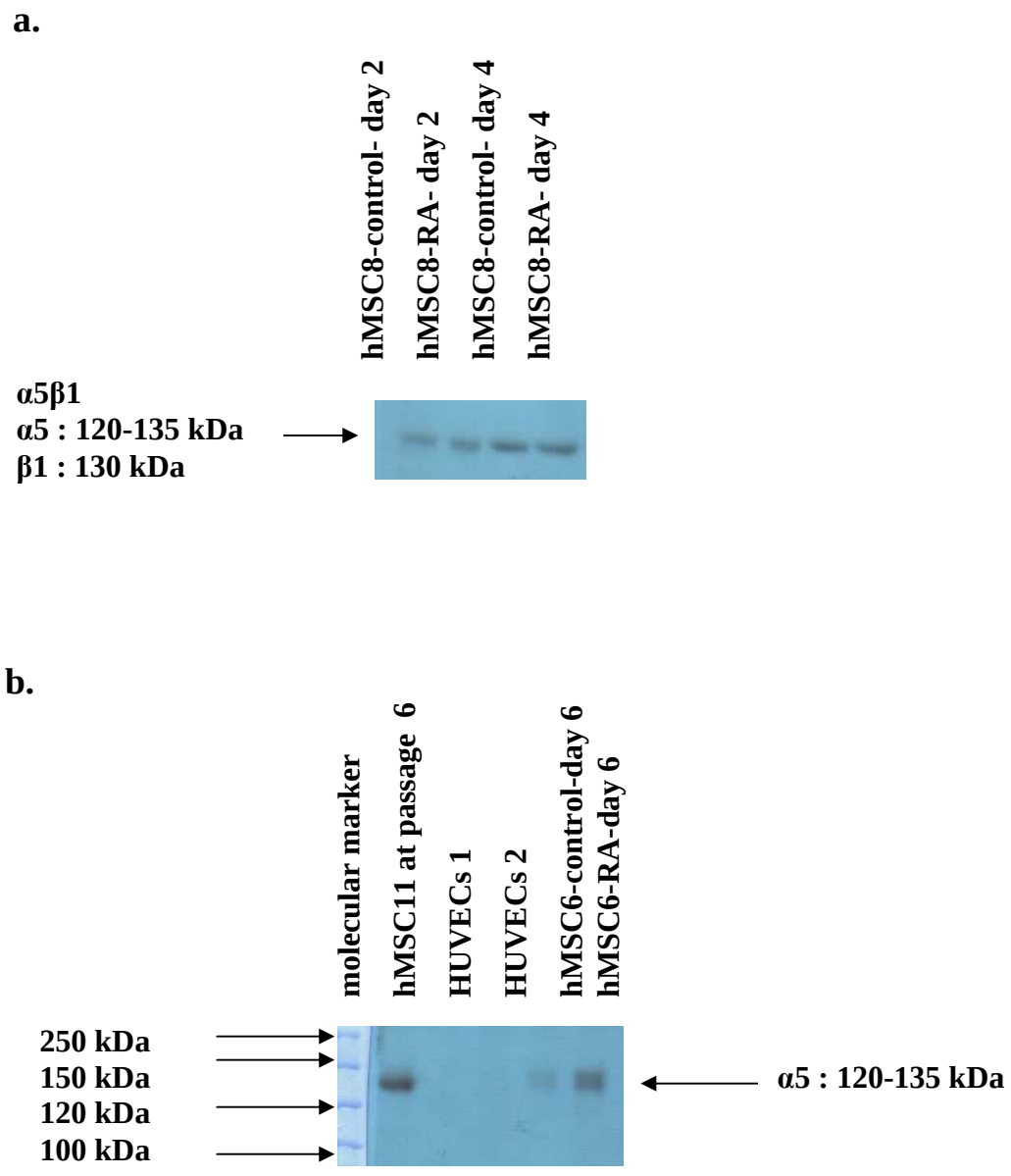
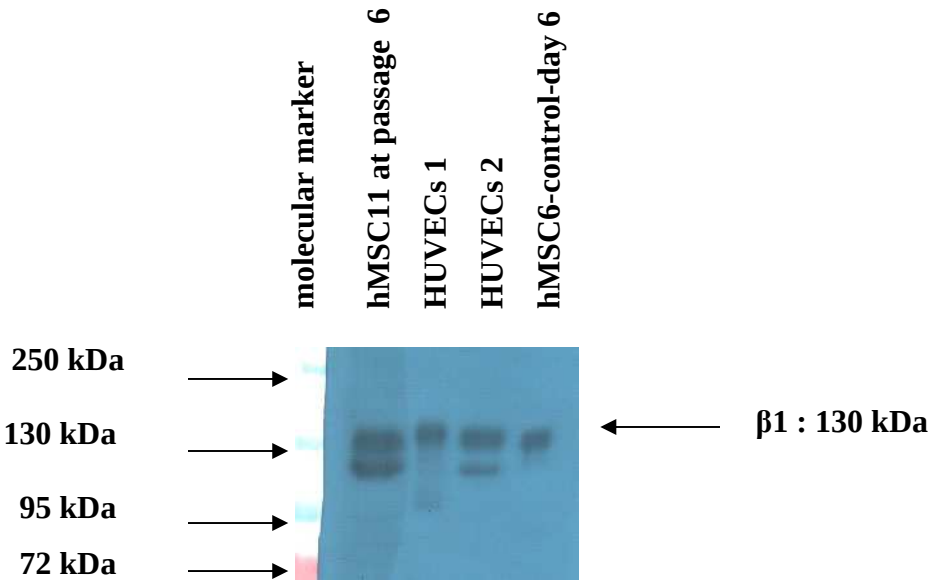


Figure 3. 21 Western blot outcomes of integrin α5β1.

a) α5β1 protein levels at day 2 and day4 of RA treatment, b) existance of α5 subunit in hMSCs according to different passage numbers.

c.



d.

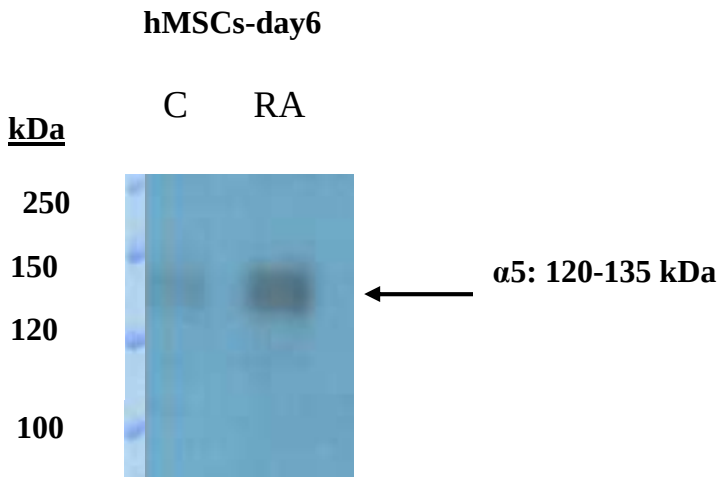


Figure 3. 21 Western blot outcomes of integrin α5β1.

c) existance of β1 subunit in hMSCs according to different passage numbers, d) α5 protein levels at day 6 of neural differentiation.

1.10 Fibronectin Coating Effect on Integrin α5β1 Expression Levels during Neural Differentiation

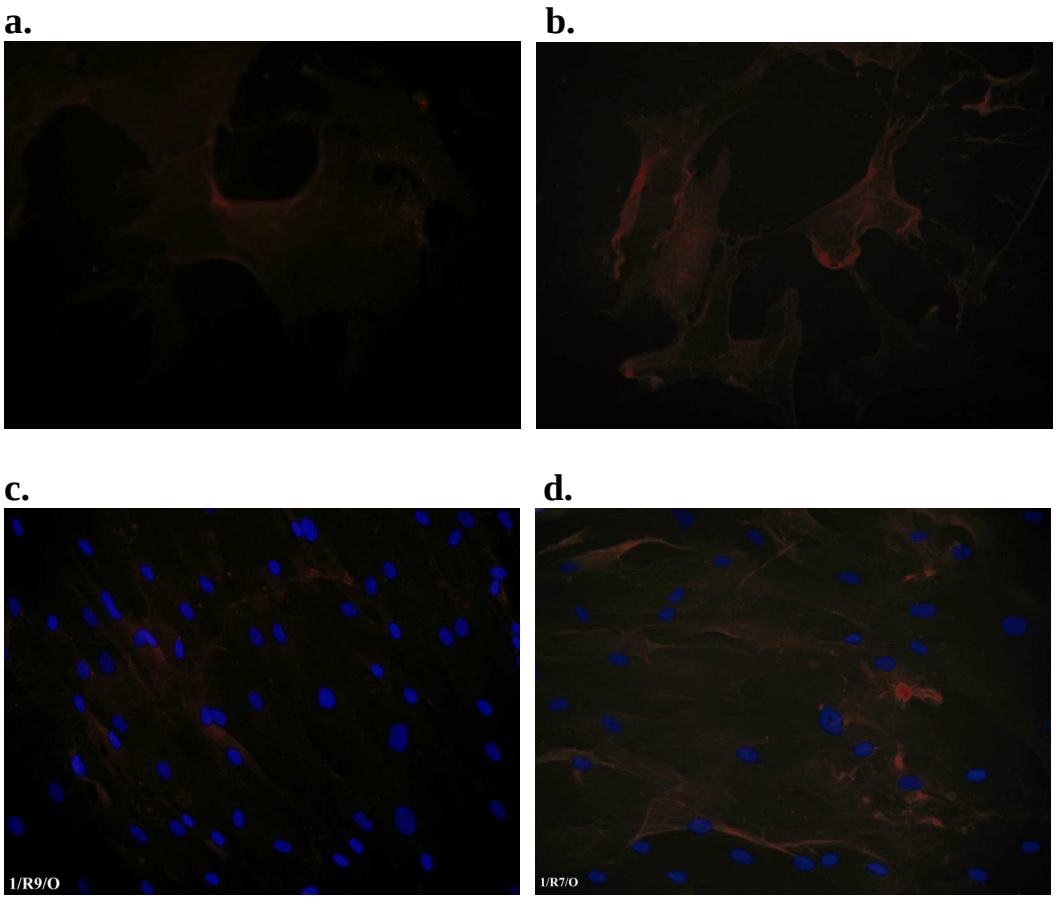


Figure 3. 22 Immunostaining of integrin α5β1 during neural differentiation of hMSCs on fibronectin coated surfaces.

a) day 1 of neural induction by RA, Fn(-), under 40X, b) day 1 of neural induction by RA, Fn(+), under 40X, c) day 14 of neural induction by RA, Fn(-), under 20X, d) day 14 of neural induction by RA, Fn(+), under 20X.

As it is visible to consider about, fibronectin coating enhances integrin α5β1 mediated neural differentiation of hMSCs during both stages of the process.

3. 11 Participation of Actin Filaments in Neural Differentiation

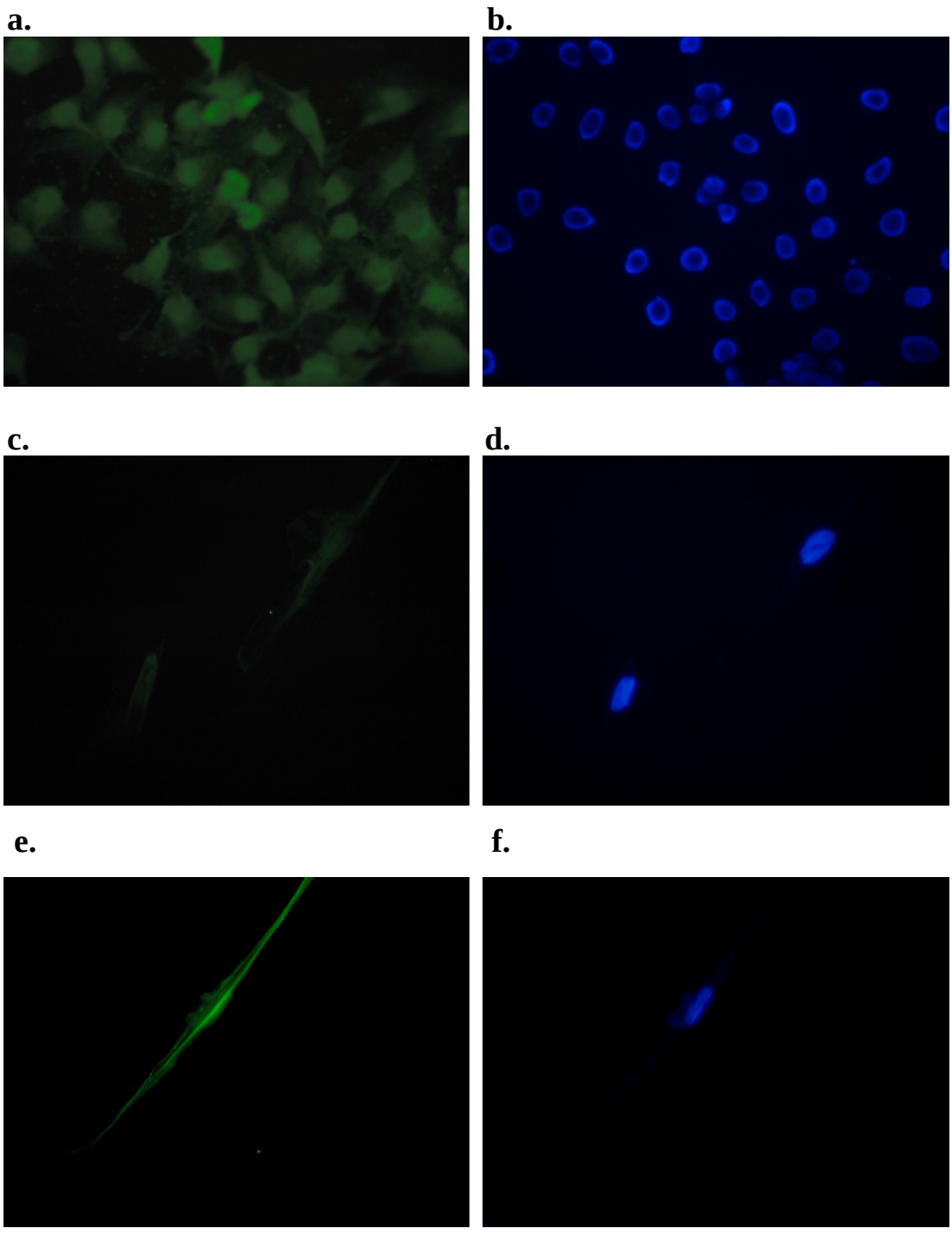


Figure 3. 23 Actin expression pattern depicted via immunostaining of hMSCs and cancer cell lines (HUVECs).

a) HUVECs-actin, b) HUVECs-actin-DAPI, c) hMSCs-actin-control, d) hMSCs-actin-DAPI, e) hMSCs-actin-RA, f) hMSCs-actin-DAPI. Images were taken under 40X magnification.

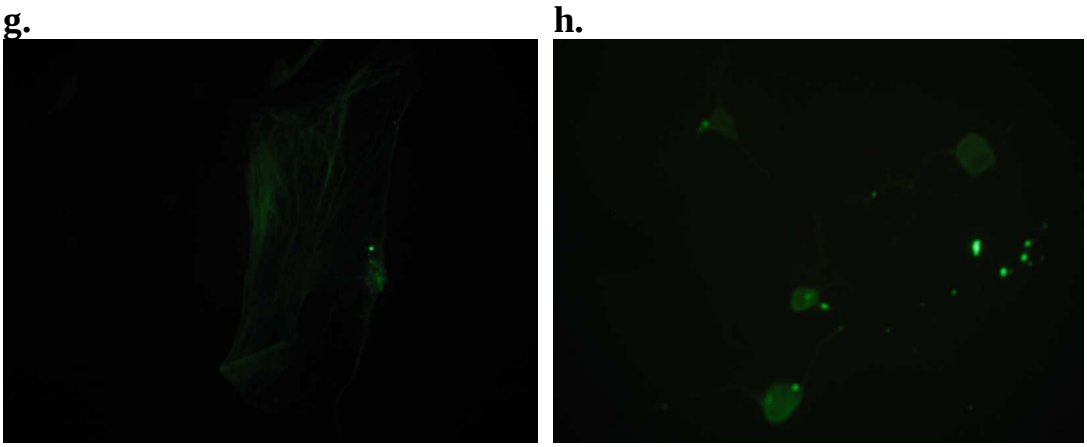


Figure 3. 23 Actin expression pattern depicted via immunostaining of hMSCs and cancer cell lines (HUVECs).

g) hMSCs-actin-RA, h) hMSCs-actin-N3. Images were taken under 40X magnification.

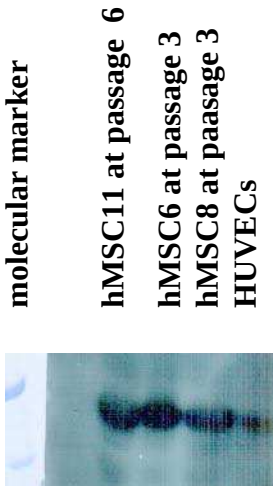


Figure 3. 24 Immunoblotting detection of actin in both hMSCs and HUVECs.

CHAPTER 4

DISCUSSIONS & CONCLUSIONS

Proper antigenic profile needs to be monitored prior to experimental initiative for the purpose of introducing the cell identity within a population. BM-hMSCs were isolated and expanded up to passage three due to examine purity of the population by the aid of FACS analysis. According to the outcomes, %98-99 of isolated cells were positive for common leukocyte antigen (CD45) while at passage 3 the ratio decreased around 8% which reveals the purity of hMSCs and elimination of hematopoietic lineages by depletion in $CD34^+$ cell population. Besides, 100% of hMSCs at passage three were almost $CD73^+$ (a marker of leukocyte differentiation) and $CD105^+$ (endoglin, TGF-β receptor) whereas the percentage was around 10 after isolation. Hence, essential markers for identification of MSCs were profiled and by passage three most of the hematopoietic cells rather than MSCs were depleted. Furthermore, structural modifications were followed during long term subculture of hMSCs and senescence was detected as fibrillar elongations and spreading on cell shape by passage five that are far away from common fibroblastic shuttled MSC morphology. Accumulated data and those results depict that hMSCs partially loose their functional abilities as the cells proliferate long-term on culture which then causes deficiencies in differentiation capacity. It was reported as it's suitable to continue for culture even until passage fifteen, in spite of that hMSCs were induced for neural differentiation not only earlier than passage three but also at passage four at the latest due to keep MSC yield and quality.

Although various induction combinations were tested as a model for *in vitro* neurogenesis of hMSCs, retinoic acid (RA) was the most applied one among the others. Our data demonstrated that RA gives rise to neurons without an apparent

neuronal phenotype. In addition to that, as for neural marker expressions, RA treated hMSCs were expressed both early (βIII Tubulin, NSE) and late (NF) neural markers along distinct stages of neural differentiation. In line with this, we predict that existance of neural markers may vary related to induction protocol and duration. In case of serum reduced differentiation media, remarkably cells have undergone morphological rearrangements and elongated cell shape in terms of neuron-like outgrowth as it's compared to serum induced microenvironment. As a result, this alternative induction procedure reveals the fundamental role of serum free environment in neural differentiation of hMSCs *in vitro*. Moreover, cells had morphological features typical of neurons, such as spherical shape and extending processes when exposed to enriched cytokine combinations. Neurite formation was detected in patches which are often packed with microtubule bundles -the growth of which is stimulated by nerve growth factor (NGF) as well as Tau proteins such as microtubule associated protein (MAP2). Fibroblast growth factor receptor (FGF-R), following tyrosine kinase phosphorylation has also a role in induction of neurite growth. N3 mediated neural differentiation yielded much more neuronal cells than RA induced cells. Neuronal induced hMSCs presented neuron-like cell morphology with progressive in neurite outgrowth. In addition to that, both early (Nestin, NSE) and late (NF, MAP2, GFAP) neural marker expressions were detected in N3 based neuronal induced hMSCs as they supported the change in hMSC cell morphology. In other words, eventhough the change was rapid, bone marrow hMSCs were differentiated into neurons via stabilizing their neural properties on culture. Highly improved integrin α5β1 expression levels were detected in neuronal induced hMSC samples when it's compared to control group.

Thereby, it's faithfully obvious that involvement of a broad range of cytokines and supporting chemical agents provided the most effective induction protocol among tried ones for *in vitro* neurogenesis. In both cases, it was clear to detect that differentiation speed was down as the cells proliferate on culture.

Integrin family receptors, deemed as the moderators of cellular responses to cell proliferation, motility and differentiation, are one of the most promising topics in discovery of the underlying mechanisms leading to stem cell fate. Our data concluded that, integrin α5β1 expression levels increase by even initial stages of neural differentiation. At the day 14, increasing in integrin α5β1 expression levels run low

whereas it was climbing up before the termination of both differentiation period. In other words, integrin α5β1 is highly important in neural differentiation of human bone marrow mesenchymal stem cells as the cells mobilize the integrin α5β1 activity which then leads to cell adhesion and motility on extracellular matrix proteins in conjunction with the stimulation of signal pathways required for in vitro neurogenesis .

Extracellular matrix components such as fibronectin, collagens, vitronectin and laminin also play a role in integrin-ligand binding as a prolongation outside of the cell. This binding promotes the signals due to transmit to the focal adhesion sites located in the cytoskeleton by phosphorylation of tyrosine kinases. In case of integrin α5β1, which is also termed as fibronectin common receptor, connection unit of α5β1 on ECM is fibronectin. In line with this, fibronectin coated surfaces improved the differentiation efficiency when it is compared to uncoated coated layers. Matri-gel coated surfaces did not give permission to immunostaining of target molecules because of its 3D solid phase. Fibroblastic cells including MSCs attach to the ECM via focal adhesion sites which is a composition of actin filaments. In addition to that, cell motility which is required for differentiation, most probably depends on aggregation of actin filaments at the movement sites. By this way, rest of the cell part migrates through the cell body via contraction. Hence, actin has also a role in transmembrane integrin activity as a cytoplasmic binding region for both cell motility and differentiation. In conclusion, we investigated actin distrubution and accumulation within the cell in terms of cellular movement during neural differentiation of hMSCs. As we estimated, actin has higher expressions by neural induction whereas control group has lower levels.

RNA studies suprisingly revealed that untreated cells have nearly no RNA translating integrin subunits neither α5 nor β1. This needs a detailed investigation if there is not any error with the samples. Besides, HUVECs are deemed to have low α5β1 utilization according to detection outcomes with both RNA and protein studies. If we turn our look to distinct time points of neural differentiation, after day 6, nearly none of the β1 subunits are fabricated in hMSCs whereas encoding for α5 subunit is maintained. This situation may be caused by need for different integrin family members by differential accumulations of integrin subunits due to combine proper dimers for desired integrin molecules, which is a result of cellular response during differentiation.

On the other hand, as the cells were expanded long-term on culture, they initially had high integrin α5β1 expression until they frequently become confluent. This rapid proliferation also cause the differentiation process to slow down related to improper microenvironment for cell to ECM attachments which then leads to deceleration in cell motility and differentiation . Both efffects have to be considered while comparing the protein expression patterns during neural differentiation of hMSCs. This study reveals the importance of integrin α5β1 in establishment of hMSC niche during in vitro neural differentiation which then pave the way for proper microenvironment so as to activate the cellular processes required for this mechanism. Accordingly, investigation the involvement of other integrin molecules is required for a general understanding of integrin dependent neural differentiation mechanism of hMSCs. Finally, this finding facilitates to build up a more realistic *in vitro* neurogenesis environment for MSC therapy and tissue engineering studies.

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