Tuba KUNDURACI

M.S. Thesis In Biology

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ANALYSIS OF DNA TOPOISOMERASE IIβ DEPENDENT GENE EXPRESSION PROFILE OF NEURALLY TRANSDIFFERENTIATED HUMAN MESENCHYMAL STEM CELLS

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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.

_____
Assist. Prof. Dr. Sevim IŞIK
Head of Department

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

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September 2010

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ABSTRACT

In mammalian cells, there are two isoforms of DNA topoisomerase II (topo II). While topo IIα is essential for chromosome segregation in mitotic cells, topo IIβ is involved in more specialized cellular functions. According to recent studies, topo IIβ plays an important role in the initiation of selective gene transcription. Although it is shown that the functional topo IIβ expressed in cerebellar granule neurons differentiating in a primary culture is required for the induction of a subset of neuronal genes. When topo IIβ activity is inhibited, the transcriptional induction of differentiation-related genes is supressed.

Stem cells can transdifferentiate into different cell types including neuronal cells. The transdifferentiation process of stem cells appears to be different substantially from the terminal differentiation of granule neurons. Therefore, the functional significance of topo IIβ may also be different in these differentiation systems. There have been no reports that analyzed the roles of topo IIβ in the transdifferentiation process of stem cells.

To understand the role of topo IIβ, we used bone marrow isolated human mesenchymal stem cells (hMSCs). Since these cells are proliferating cells, they also express topo IIα which may substitute topo IIβ at the initiation stage of differentiation. Topo IIβ specific small interfering RNAs (siRNAs) in Lipofectamine RNAiMAX transfection reagent were used to knockdown topo IIβ selectively. Then the cells were induced to differentiate by N3 cytokine combination. Transdifferentiation process was monitored by immunostaining and RT-PCR with antibodies and primers against neural

tissue proteins such as nestin, beta-3 tubulin, neuron specific enolase, neurofilament and microtubule- associated protein 2. Transfection efficiency was checked by RT-PCR.

The induced or silenced genes in the presence and absence of siRNA treatments were analyzed by DNA microarray analysis. Neural differentiation efficiency of hMSCs by N3 cytokine combination was found nearly 60% according to changes in morphology. RT-PCR and immunostaining results also confirmed these results by expression of early and late neural markers in transdifferentiated hMSCs. Silencing of topo IIβ was about 85-90% that was checked by RT-PCR and transfection was topo IIβ specific since the expression level of topo IIα did not change. The axon length in siRNA tranfected neural cells was shorter than untrasfected neural cells. Upregulated or downregulated topo IIβ related genes were scanned by microarray analysis.

siRNA transfection and neural induction of hMSCs provided to understand the role of topo IIβ and find the genes related to topo IIβ in neural differentiation process.

Keywords: DNA topoisomerase IIβ, siRNA transfection, human mesenchymal stem cells, neural differentiation, microarray analysis

NÖRAL TRANS-FARKLILAŞAN İNSAN MEZENKİMAL KÖK HÜCRELERİN DNA TOPOİZOMERAZ IIβ BAĞIMLI GEN İFADE PROFİLİNİN ANALİZİ

Tuba KUNDURACI

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ÖZ

Memeli hücrelerinde iki DNA topoizomeraz II (topo II) izomeri bulunmaktadır. Topo IIα, mitotik hücrelerde homolog kromozomların birbirinden ayrılmasını sağlayan segregasyon olayında temel enzim iken, topo IIβ ise daha özelleşmiş hücresel olaylarda görev almaktadır. Yakın zamanda yapılan çalışmalara göre topo IIβ'nın gen transkripsiyonun tetiklenmesi, başlatılması esnasında önemli bir rol almaktadır. Fare primer nöron kültür hücrelerinde fonksiyonel topo IIβ'nın bazı gen setlerinin açılmasında gerekli olduğu gösterilmiştir. Topo IIβ aktivitesi durdurulduunda, nöral hücre farklılaşması ile ilgili bazı genler baskılanmaktadır.

Kök hücreler nöron hücreleri dahil değişik hücre tiplerine dönüşebilme yeteneğine sahip hücrelerdir. Fakat kök hücrelerinin başka hücrelere farklılaşması (transdifferensiyasyon) işlemi, primer nöron hücrelerin farklılaşmasından daha farklıdır. Bunun için, topo IIβ'nın kök hücrelerin transdifferensiyasyon işleminde primer hücrelerin farklılaşmasındakinden farklı davranması muhtemeldir. Literatürde, kök hücrelerin transdifferensiyasyonu esnasında topo IIβ aktivitesine bağlı olarak tetiklenen genlerle alakalı bir yayın bulunmamaktadır. Topo IIβ'nın fonksiyonunun anlaşılması için kemik iliğinden izole edilen insan mezenkimal kök hücreleri (iMKH) kullanılmıştır. Bu hücreler proliferatif hücreler oldukları için hem topo IIβ hem de farklılaşmanın ilk aşamasında topo IIβ ile yer değiştirme ihtimali olan topo IIα ifade etmektedirler. Lipofektamin RNAiMAX transfeksiyon ajanı içerisinde topo IIβ'ya özel kısa interferans RNAlar (siRNAs) ile iMKH transfekte edilmiştir.Daha sonra nöral farklılaşma işlemi nöral dokuya ait Nestin, Beta-3 tubulin, Nöron Spesifik Anolaz, Nörofilament ve Mikrotübül Alakalı Protein-2 gibi proteinlere karşı kullanılan antikor ve primerler ile immunboyama ve RT-PCR teknikleriyle takip edilmiştir. Transfeksiyon verimliliği RT-PCR ile kontrol edilmiştir. siRNA varlığında ya da yokluğunda indüklenen ya da baskılanan genler mikroarray analizi ile gerçekleştirilmiştir.

N3 sitokin kombinasyonu kullanılarak iMKH'lerde nöral farklılaşma verimliliği morfolojik değişime bakılarak %60 civarında bulunmuştur. RT-PCR ve immunboyama sonuçları da farklılaşmış iMKH'lerde erken ve geç nöral markerların ekspresyonu ile dedoğrulanmıştır. RT-PCR sonucuna göre topo IIβ susturulma oranı %85-90'dır ve siRNA ile baskılanan nöral hücrelerde akson uzamasının daha kısa kaldığı gözlenmiştir. Topo IIβ bağımlı artış ve azalış genler mikroarray ile analiz edilmiştir.

iMKH'lerin siRNA transfeksiyonu ve nöral indüklenmesi ile nöral farklılaşma süresince topo IIβ'nın rolü ve topo IIβ'ya bağımlı genler anlaşılmıştır.

Anahtar Kelimeler: DNA topoisomerz IIβ, siRNA transfeksiyonu, insan mezenkimal kök hücreleri, nöral farklılaşma, mikroarray analizi

To my parents and husband

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This thesis was like milestone of my academic career. The path to my future was defined when I started to this project. I wish that we all meet at future stations of this path.

TABLE OF CONTENTS

LIST OF TABLES

TABLE

LIST OF FIGURES

FIGURE

LIST OF SYMBOLS AND ABBREVIATIONS

SYMBOL	ABBREVIATION
MSC	Mesenchymal stem cell
hMSC	Human mesenchymal stem cell
BM	Bone marrow
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal Bovine Serum
CO_2	Carbon dioxide
FACS	Fluorescence activated cell sorting
IBMX	3-Isobutyl-1-methylxanthine
RT-PCR	Reverse transcriptase polymerase chain reaction
TGF-β	Transforming growth factor beta
IGF-1	Insulin-like growth factor 1
bFGF	Basic fibroblast growth factor
EGF	Epidermal growth factor
PDGF	Platelet-derived growth factor
VEGF	Vascular endothelial growth factor
DMSO	Dimethylsulfoxide
RA	Retinoic acid
BDNF	Brain-derived neurotrophic factor
NeuN	Neuronal nuclear protein
GFAP	Glial fibrillary protein
BME, β-ME	β-mercaptoethanol
BHA	Butylated hydroxyanisole
MAP-2	Microtubule associated protein-2
NSE	Neuron specific enolase

NF	Neurofilament
Topo II	Topoisomerase II
Topo IIα	Topoisomerase IIα
Topo IIβ	Topoisomerase IIβ
RNAi	RNA interference
siRNA	Small interference RNA
RISC	RNA-Induced Silencing Complex
mRNA	Messenger RNA
cDNA	Complementary DNA
Cy3	Cyanine 3
Cy5	Cyanine 5

CHAPTER 1

INTRODUCTION

1.1 MESENCHYMAL STEM CELLS

1.1.1 General information about MSCs

Mesenchymal Stem Cells (MSCs) are one type of adult stem cells which were firstly discovered by Friedenstein in 1966. MSCs were reported as fibroblastic colony forming unit. Thereafter, MSCs have been described differently by various research groups such as; bone marrow stromal stem cells (BMSSC), marrow stromal cells, marrow isolated adult multipotent inducible cells (MIAMI), multipotent adult progenitor cells (MAPC) and mesenchymal adult stem cells (MASCS) (1, 2, 3, 4).

Since mesenchymal stem cell displays significant heterogeneity, there are some characteristic and specific phenotype or cell surface markers to define a cell as mesenchymal stem cell. According to International Society for Cytotherapy, the minimal criteria to characterize a cell as mesenchymal stem cell are (5):

a) Cells should have adherence capacity to plastic under cell culture conditions
b) Cells should express CD105, CD 73 and CD90 with a ration greater than 95 %
c) Cells should be negative for markers including CD34. CD45, CD14 or CD11b, CD79α or CD19 and HLA-DR
d) Cells should have ability to differentiate into bone, fat and cartilage

In addition to these criteria, mesenchymal stem cells also have self renewal capacity and they should form single cell derived colonies in the culture and should have fibroblastic morphology. Mesenchymal stem cells are not immortal but they can proliferate numerously in culture conditions, still they have expansion and differentiation capacity (5).

There are some factors that they affect some expansion, differentiation and immunogenic features of mesenchymal stem cells in culture. These factors are method used in isolation, growth medium, culture surface, cell seeding density and in addition to those also growth factors and chemicals used in the culture (6). Also age of donor and stage of disease can affect yield of MSC obtained, proliferation and differentiation capacity of MSC.

Mesenchymal stem cells are mainly isolated from bone marrow but there are also other sources in various other sides such as adipose tissue, periosteum, synovial membrane, synovial fluid (SF), skeldatal muscle, dermis, deciduous teeth, pericytes, trabecular bone, infrapatellar fatpad, articular, cartilage, umbilical cord blood, placenta, liver, spleen and thymus (7).

1.1.2 Isolation of hMSCs from bone marrow aspirates and their expansion

MSC has a low frequency (0.001- 0.01% of nucleated cells) in bone marrow (8). Human bone marrow (BM) MSCs are usually aspirated from posterior iliac crest of pelvis or tibial and femoral marrow compartments. Ficoll density gradient centrifugation is a method that is used to isolate hMSCs from bone marrow aspirates. In this method, a layer which has an enriched population of mononuclear cells is formed. After this step, standard cell culture techniques are used to plate and enrich MSCs *in vitro*. So adherence to plastic in culture conditions ability of MSCs is used to distinguish MSCs from other cells in bone marrow mononuclear cells (9). Cells are plated at a density of approximately 1500 cells/cm^2 and then cultured in a medium such as Dulbecco’s modified Eagle’s medium (DMEM), containing 10-15% fetal bovine

serum (FBS) (10). Cultures are maintained at 37°C in a humidified atmosphere containing 5% CO_2. 10-14 days are enough to obtain primary cultures of MSCs and then cells are trypsinized and subcultured (11). The isolated cells were grown on plastic culture flasks and characterized by FACS analysis for specific markers (5).

1.1.3 Multi-lineage Differentiation potential of MSCs

Mesenchymal stem cells have two important properties; self renewal capacity and multi-lineage differentiation potential (Figure 1.1) (12). Mesenchymal stem cells can generate identical copies of themselves by mitotic division over their lifespan. This property of MSCs is known as self renewal. Beside self renewal capacity, MSCs have ability to differentiate into chondrocytes, osteoblasts and adipocytes. In addition to this, it was reported that MSCs also differentiate into myocytes and cardiomyocytes and even into cells of non-mesodermal origin, including hepatocytes, insulin-producing cells and neurons (13). Prockop and Pittenger et al. suggested that MSCs may also serve as hematopoiesis-supporting stromal cells (14, 15).

Growth factors and chemicals have important roles in multi-lineage differentiation of MSCs.

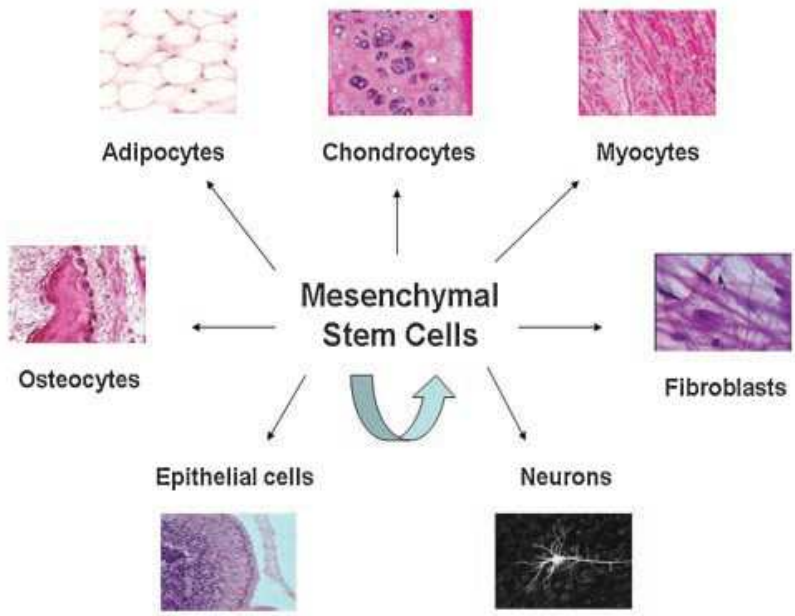


Figure 1.1 Pluripotent capacity of MSC to differentiate into mesodermal and non-mesodermal cell lineages, including osteocytes, adipocytes, chondrocytes, myocytes,

fibroblasts, epithelial cells, and neurons (solid arrows). In addition, MSCs are capable to self-renewal (block arrow) (12).

1.1.3.1 Osteogenic Differentiation

In order to obtain osteogeneic lineage differentiation of MSCs, glycerol-phosphate, ascorbic acid-2-phosphate, dexamethasone and fetal bovine serum have to be present in the medium. If cells are cultured monolayer in the presence of these molecules, there is an increase of alkaline phosphatase activity and deposition of a calcium-rich mineralized extra cellular matrix to obtain osteoblastic morphology (16).

1.1.3.2 Chondrogenic Differentiation

In order to obtain chondrogenic differentiation, MSCs have to loose their classical fibroblastic morphology in the culture conditions including; the addition of transforming growth factors, a three-dimensional culture format and finally a serum-free nutrient medium. Hence differentiation begins expression of a number of cartilage-specific extra cellular matrix components starts such as rapid biosynthesis of sulfated glycosaminoglycans that has a role in important changes of cell shape (17, 18).

1.1.3.3 Adipogenic Differentiation

MSC has to be treated with a hormonal combination including dexamethasone, isobutyl methyl xanthine (IBMX) and indomethacin (19, 20). To confirm the differentiation, oil-red staining technique and RT-PCR for detection of adipsin, the fatty acid binding protein aP2 and PPAR γ expression are performed (15).

Table1.1 Summary for Regulatory Signals for Multiple-Lineage Differentiation of MSCs (12)

	Multilineage differentiation potential	**Representative Refs.**
Biological signals		
TGF-β	Chondrogenic	Barry et al. [2001] Sekiya et al. [2005]
IGF-1	Chondrogenic	Matsuda et al. [2005]
bFGF	Chondrogenic, Osteogenic	Ito et al. [2008]
EGF	Chondrogenic	Kratchmarova et al. [2005]
PDGF	Chondrogenic, myofibroblastic	Kratchmarova et al. [2005] Nedeau et al. [2008]
VEGF	Endothelial	Liu et al. [2007]
Wnt	Chondrogenic, osteogenic, neural	Baksh and Tuan [2007]
Chemical signals		
Dexamethasone+isobutyl methylxanthine+indomethacin	Adipogenic	Pittenger et al. [1999]
5-azacytidine+amphotericin B	Myoblastic	Wakitani et al. [1995]
Nicotinamide+2-ME	Islet β-cell-like	Chen et al. [2004]
2-ME DMSO	Neural	Kohyama et al. [2001] Woodbury et al. [2000]
Retinoic acid	Neural	Mareschi et al. [2006]

1.1.3.4 Neural Transdifferentiation of hMSCs

Recent studies have shown that mesenchymal stem cells can transdifferentiate into non-mesodermal cells such as neural cells expressing neuronal and glial markers. Neural differentiation capability of MSCs was firstly shown by Sanchez-Ramos et al. (12) and Woodbury et al (20) in 2000. They illustrated in their studies that induction of

MSCs originated from rat, mouse and human with combination of chemicals or growth factors resulted in changes in morphology as neural shape and expression of neural markers. Sanchsez-Ramos used growth factors and Woodbury used chemicals in order to obtain neural differentiation. In the study of Sanchez-Ramos, cells were treated with epidermal growth factor (EGF) and retinoic acid (RA) or RA with brain-derived neurotrophic factor (BDNF) and cells expressed neural markers; nestin, neuron-specific nuclear protein (Neu-N), and GFAP. But in the study of Woodbury, cells were treated with β-mercaptoethanol (BME), dimethylsulfoxide (DMSO) and butylated hydroxyanisole (BHA) in DMEM and Kabos *et al.* demonstrated that cytokines, growth factors, neurotrophins and retionic acid are used to induce neural differentiation (21), (12). For example, when mouse marrow stromal cells were treated with epidermal growth factor (EGF) and brain derived neurotrophic factor (BDNF), neuronal markers such as NeuN and MAP2 were detected (12). In addition to this, injection of MSCs into brains of mouse or rat induces differentiation of MSCs into neurons and astrocytes. And also these cells can be used to cure Parkinson disease (22, 23, 24).

Moreover, there are some objections against transdifferentiation of neurons from MSCs. According to some authors, it was shown that only a small amount of pluripotent stem cells within the adult mesenchymal stem cells can differentiate into electro physiologically active neurons with correct neural morphology (25). But there are other studies against this opinion and it was claimed that MSCs transdifferentiate robust and very quickly into neural like cells *in vitro* (20). In many studies, it was shown that undifferentiated MSCs can also express neural markers such as NSE, NF-M, NeuN and GFAP (26). Also there is a contradiction about the time needed for MSC neuronal conversion. In some researches, it was said that several days or weeks are necessary to obtain neural-like phenotype after induction of MSCs (27). But in the other studies in which chemical methods are used a few hours are enough to obtain a fast neuronal differentiation (28).

1.2. TOPOISOMERASE IIβ AND ITS ROLE IN NEURAL DIFFERENTIATION

1.2.1. DNA Topoisomerases

There are topological problems since the structure of DNA is double helix. To resolve these kinds of topological problems, DNA topos (topoisomerases) are used in

cells. The DNA topoisomerases are essential for DNA replication, transcription, recombination, as well as for chromosome compaction and segregation (28). DNA topoisomerases form transient single or double-strand breaks in the DNA and allow DNA strands or double helices to pass through each other

There are two types of DNA topoisomerases; type I and II, on the basis of their different architectures and mechanisms (29). Type I enzymes introduce a single stranded break in DNA while type II enzymes are dimeric enzymes that can introduce a break in two strands at the same time. Different from type I topoisomerases, type II topoisomerases use ATP during changing DNA topology (30). There are two different type of enzymes; type IIA and type IIB on the basis of sequence and structure. For type IIA topoisomerases there are the enzymes DNA gyrase, eukaryotic topoisomerase II and bacterial topoisomerase IV are the members. Type IIB topoisomerases are found in archea and higher plants.

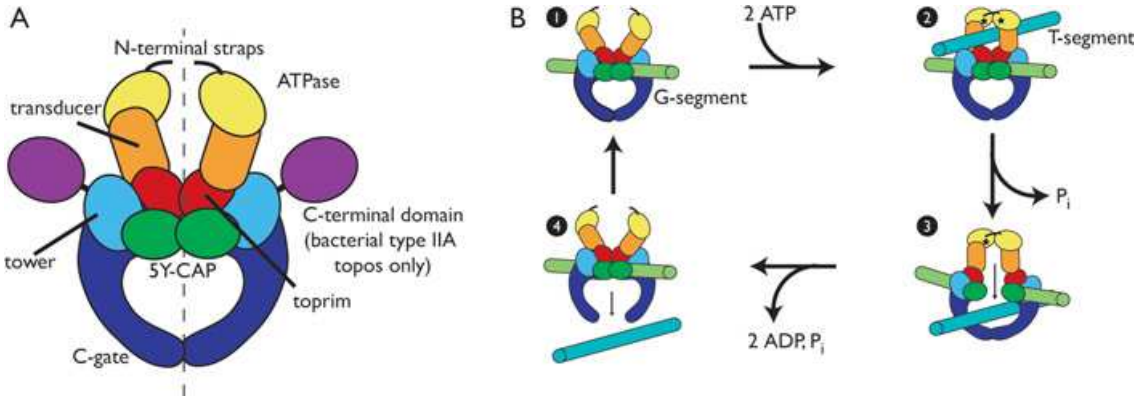


Figure 1.2 A. Proposed general quaternary structure B. Catalytic mechanism of type IIA topos (31)

In mammalian cells, two isoforms of topoisomerase II have been identified; 170-kDa topoisomerase IIα (topo IIα) and the 180-kDa topoisomerase IIβ (topo IIβ). These isoforms are encoded by separate genes but they are similar in their primary structure (72%) and they have similar catalytic properties but regulation of these enzymes is very different. These enzymes play different roles in cellular physiology because of the fact that they differ in cell cycle dependency and tissue specificity. Topo IIα plays a role in mitotic processes and only present in proliferating tissues such as

tumors. As a result in cancer research, there is an interest in topo IIα. So while cell proliferation rate increases, concentration of topo IIα also increases (32, 33).The level of topo IIα enzyme increases at G2/M phase of cell cycle and it is localized at the centromeres of metaphase chromosomes indicating that it is essential for chromosome segregation in mitotic cells (34).

However there is no need for topo IIβ isoform in mitotic events. All tissues including terminally differentiated tissues contain topo IIβ and the level of enzyme does not change during the cell cycle. Since it is found in terminally differentiated cells, it is assumed that it may play a role in DNA metabolism especially in the transcriptional activation of some inducible genes instead of DNA replication and chromosome condensation/segregation (35). It can be said that there is no need for topo IIβ in general cellular activities so it may have more specific roles but exact function of it has not been known yet (36).But there are different studies trying to explain exact function of topo IIβ. In some of these studies, topo IIβ is searched *in vivo* and in others it is studied during differentiation.

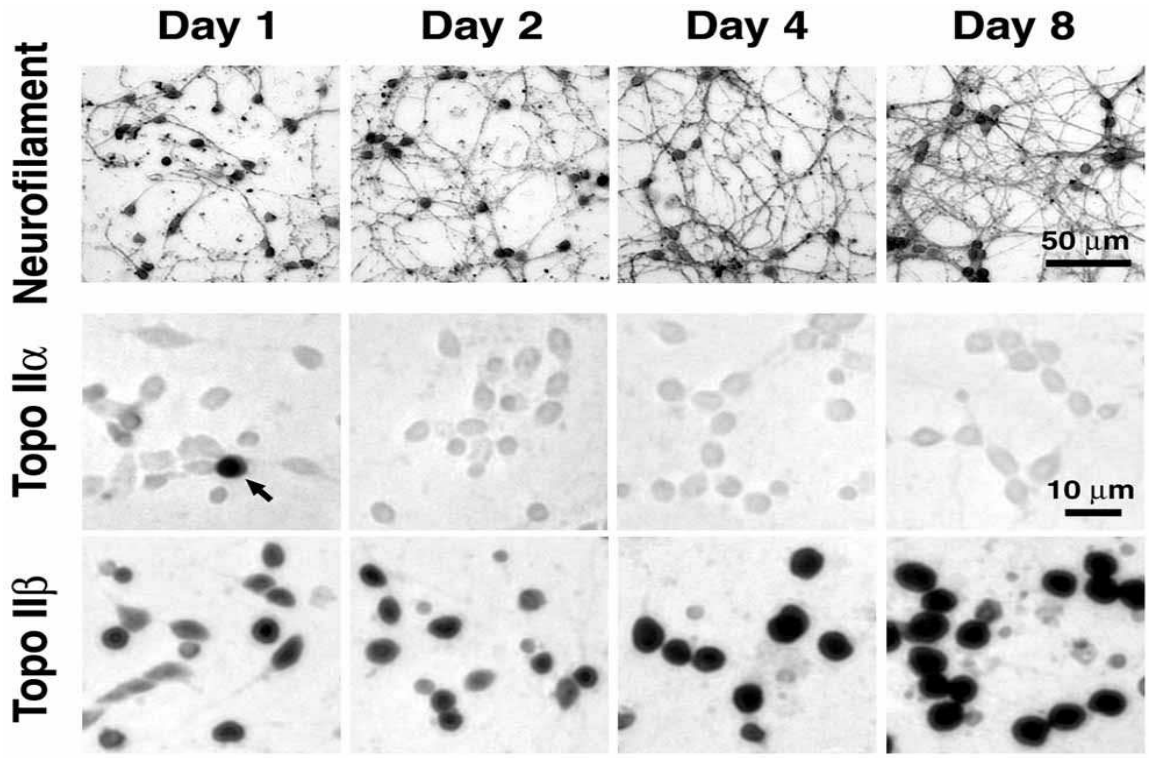


Figure 1.3 shows expression of neurofilaments and topo II isoforms in the cerebellar granule cells differentiating *in vitro* (36).

In recent studies it has been suggested that topo IIβ may play a role in neuronal differentiation. Tsutsui,K et al. showed that topo IIβ is highly expressed in differentiating cerebellar neurons (36). And whole body top2β knockout mice have shown prenatal death. It was found that topo IIβ play a role in activation or repression of developmentally regulated genes at late stages of neuronal differentiation. Regulation the expression of developmentally regulated genes at late stages of neuronal differentiation may be performed by controlling chromatin structure (37). Yang et al found that topo IIβ plays a role in axon growth and regulation. In addition to this, brain specific top2β knockout mice had a problem in brain development (38). Nur-E-Kamal et al. reported that topo IIβ plays critical role in forming and maintaining the growth cone morphology and may affect neurite outgrowth through its regulation of the expression of certain neuronal genes (39).

Ju et al. reported that the signal dependent activation of gene transcription by nuclear receptors and other classes of DNA binding transcription factors requires DNA topo IIβ dependent, transient and site-specific dsDNA break formation. According to this study, there is a link between topo IIβ dependent dsDNA break formation and DNA repair machinery in regulated gene transcription (40).

To clarify the function of topoisomerases, topoisomerase inhibitors are designed. There are common inhibitors such as ICRF-159, ICRF-187 and ICRF-193 and epipodophyllotoxins such as VP-16 and VM-26. The way that these inhibitors use to inhibit function of enzyme is to block the N-terminal ATPase domain of topo II and prevent topo II from turning over. So they bind in a non-competitive manner and lock down the dimerization of the ATPase domain (41).

But selective inhibition of these enzymes can not be obtained because these inhibitors target both topo IIβ and topo IIα. To understand exact function of these enzymes distinctly, specific inhibition has to be obtained. That could be provided by RNA interference mechanism with the use of specific siRNAs.

1.3 RNA INTERFERENCE

RNA interference (RNAi) becomes one of the most interesting and important techniques in modern biology. By the help of RNA interference, it can be possible to

observe the effects of the loss of function of specific genes in mammalian systems. RNA interference is used in studying gene function, protein knockdown studies, phenotype analysis, function recovery, pathway analysis, *in vivo* knockdown, and drug target discovery.

RNA interference is a way of post transcriptional gene silencing (PTGS) in most eukaryotic cells by small interfering RNA (siRNA). It was firstly identified in the nematode worm *Caenorhabditis elegans and* sequence-specific gene silencing was obtained against double-stranded RNA (dsRNA) (42). After Guo and Kemphues found that as antisense RNA, also sense RNA was effective in silencing gene expression in worms. Fire and Mello used antisense RNA in order to inhibit gene expression (42, 43).

In 2001, in mammalian cells, RNAi could be obtained by direct use of siRNA without any nonspecific effects. This success in RNAi brought award of the Nobel Prize in Physiology or Medicine to Fire and Mello for their discovery in 2006 (44). In nature, RNAi is used to protect genome against invasion by mobile genetic elements such as transposons and viruses, which produce aberrant RNA or dsRNA in the host cell when they become active. It also targets the RNA of viral genome for degradation at the times of infection.

1.3.1 Principle of RNAi

The exact molecules that led to RNAi are short dsRNA oligonucleotides. These molecules are called as siRNAs and they are in 21-23 nucleotides length with two nucleotides overhangs on the 3’ ends. siRNAs can be produced by the cleavage of dsRNA with enzyme Dicer or produces synthetically. Then, RNA-induced Silencing Complex (RISC) was introduced to these siRNAs. This RISC contains several proteins such as AGO2, FMRP and P100 (45, 46). The function of RISC is to remove sense strand and pair antisense strand with a complementary region in the cognate mRNA (47, 48, 49). So this action starts cleavage and slicing at a region of bases 10 and 11, relative to the 50 end of the antisense strand. Finally many different cellular nucleases fully degregade 5′ and 3′ mRNA fragments (50, 51).

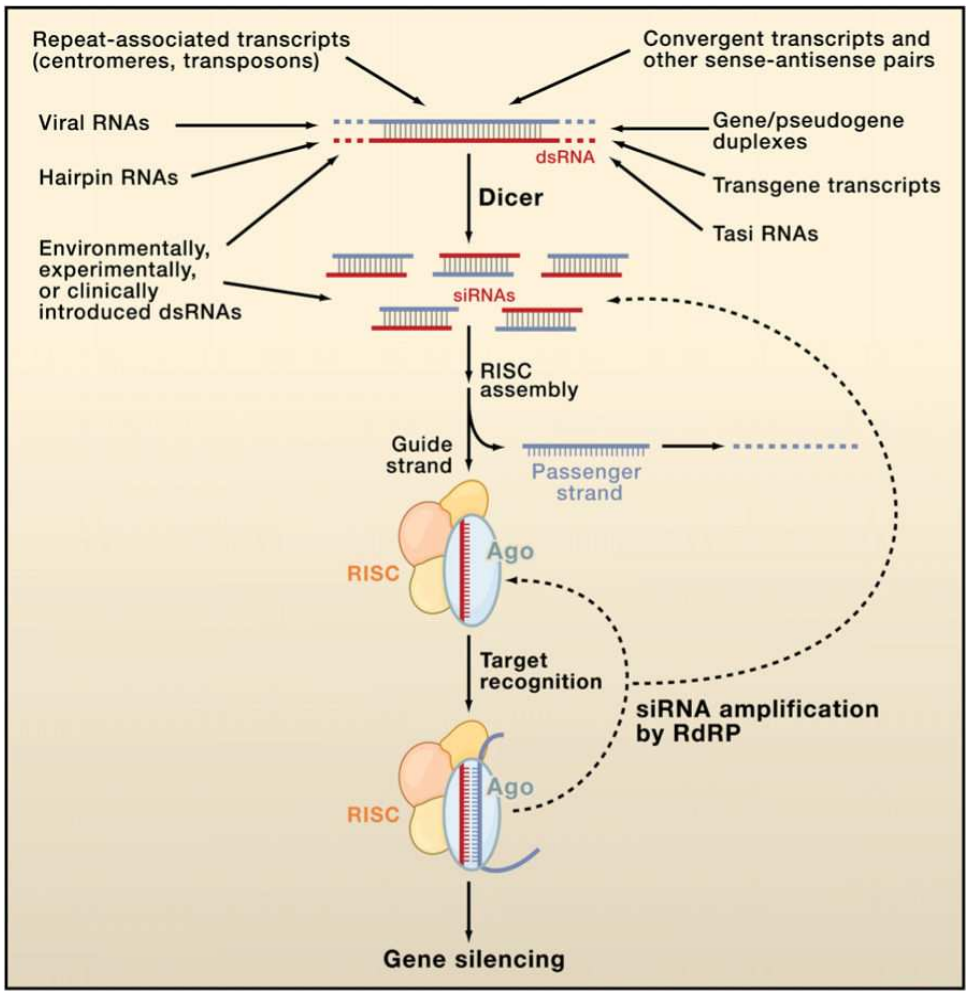


Figure 1.4 A Diversity of siRNA Sources (52)

1.3.2 RNAi delivery methods

There are some factors that affect down regulation of individual gene expression by RNAi for example transcription rate of the selected gene, the overall stability of mRNA transcript and corresponding protein, effectiveness of selected siRNA sequence design and the characteristic of the chosen cell line type (53).

Chemical and biological stability of siRNAs are very important but in addition to this, delivering of siRNAs into cells is also essential. Under normal conditions, siRNAs are negatively charged because of net negative charge on the sugar-phosphate backbone. So to deliver negatively charged siRNAs into cell, siRNAs have to make contact with a lipid blayer of the cell membrane whose head groups are also negatively charged (52).

There are three general methods to introduce siRNAs into target cells; transfection, electroporation and viral gene transfer (54, 55).

Table1.2 Summary of different siRNA delivery methods (from www.SABIOSCIENCES.com) (54)

Technique	**Delivery mode**	**Advantages**	**Disadvantages**
Transfection	Cationic liposomes Polymer nanoparticles Lipid conjugation	High efficiency and reproducibility Easy to use in most cells Many commercial offerings	Low efficiency in primary and non-dividing cells Low *in vivo* efficiency
Electroporation	Voltage Pulse	Delivery into difficult to transfect cells	High cell death Parameter optimization for different cell types
Viral Vector	Retrovirus Adeno-associated virus Lentivirus	Deliver into primary and non-dividing cells Compatible for *in vivo* delivery Stable expression	Generation and titration of virus particles Possible multigenic and immunogenic effects

1.3.3 Stem cell RNAi

Most of the primary cells and non-dividing cells are very hard to transfect. Delivering of siRNAs into stem cells, electroporation and transfection methods are used. Although, stem cells are well suited to transfect, as they start to differentiate, they become harder to transfect. To increase transfection efficiency; lentiviral vectors are used with long lasting RNAi knockdown in non-dividing and terminally differentiated cells such as hematopoietic stem cells, neurons and macrophages (56).

In MSCs, different approaches are used to deliver plasmid DNA or siRNAs into cells such as electroporation techniques and Nucleofector technology. Amaxa Nucleofector Technology offers the first non-viral transfection method for efficient gene transfer into mesenchymal stem cells with up to %60 transfection efficiency (57, 58). Austin *et al* achieved %50 transfection efficiency at MSCs by using Lipofectamine 2000.

1.4 MICROARRAY

In multi cellular organisms, whole genome of the organism is made of genes. But these genes are expressed differently according to type of tissue where they are expressed. After identification of major events in the cell by using molecular biology and DNA techniques, it is clarified that these events are regulated by factors that change expression of genes. To understand the expression profile of the genes has become major goal in the molecular biology. To clarify this, following questions have to be answered (59):

• How does gene expression level differ in various cell types and states?

• What are the functional roles of different genes and how their expression varies in response to physiological changes within the cellular environment?

• How is gene expression effected by various diseases? Which genes are responsible for specific hereditary diseases?

• What genes are affected by treatment with pharmacological agents such as drugs?

• What are the profiles of gene expression changes during a time dependent series of cellular events?

Microarrays are based on hybridization technique which is used to identify and quantitate nucleic acids in biological samples (e.g. Southern (60) and Northern blots (61), colony hybridizations, dot blots (62)). In hybridization, cDNA probes are prepared firstly according to complementary copies of mRNA. Two diffeent probes; one of them is for control group and one for experimental group were prepared and then by

analyzing diffrences between them, gene profiles can be determined. This technique is efficient but it is limited since only limted number of genes can be analyzed at once.

In the past decade, microarray technique has been developed. It was used firstly in 1995 by using 46 cDNA probes (63). This number increases to 1000 probes until 1996 (64, 65). When compared with hybridization technique, it can analyze ten to thousand of genes efficiently at one. So it is very rapid. Ultimately microarray has provided large scale analysis by using automated robotic tools (59).

Prior to microarray, the idea of ordering DNAs is used in arrays on porous membranes have been used for years (66, 67, 68). In microarray, firstly huge amount of cDNA or oligonucleotide DNA sequences were probed on a glass, nylon, or quartz membrane. In the first experiments, cDNA libraries and Bacterial Artificial Chromosomes (BAC) sets have been used to obtain probes for microarray slides (69). In later years, oligonucleotides were preferred since they are shorter in length and more specific (70). Two sets of mRNAs are labelled with two different fluorescent probes. After hybridization of labelled mRNAs and probes on chip, microarary is scanned bu using generally a laser beam to generate an image of all the spots. The intensity of fluorescent dye in spots determine the expression level of mRNA associated with the specific sequence at that spot. By using sophisticated software programs, images are analyzed. So general gene expression profile can be determined by using microarray (59).

There are three main steps in microarray (71);

1.Preparation of microarray slide

2. Preparation of fluorescently labelled probes and hybridization.

3.Scanning, image and data analysis

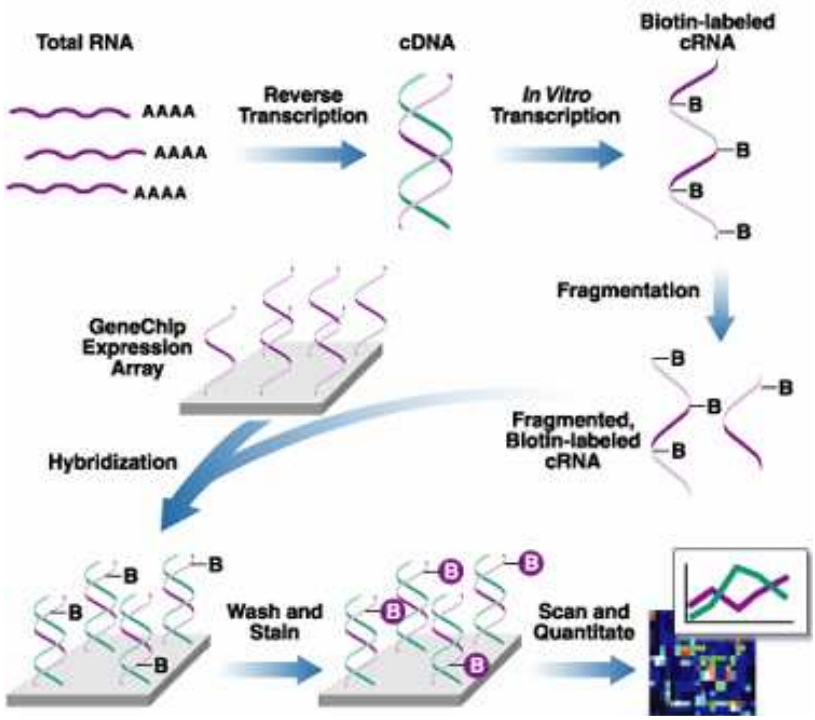


Figure 1.5 The process of fluorescently labeled RNA probe production (From Affymetrix web site)

1.4.1 Preparation of microarray slide

There are two types of microarrays; short-oligomer microarrays (oligonucleotide microarray), (e.g. Affymetrix Genechips, NimbleGen, Agilent and Febit), and long-probe microarrays, i.e. cDNA microarrays, with probes of up to a few hundreds bases in length (72). There are two important factors in this step; selection of the DNA sequences that will be arrayed on the slide and type of technique that is used to fix sequences on the slide (73).

1.4.2 Preparation of fluorescently labelled probes and hybridization

To obtain labeled probes, the first step is to extract mRNA from the sample. Then mRNA is reverse transcribed to cDNA and then cDNA is converted to cRNA and fluorescently labeled. There are direct labeling by Cy3 and Cy5 and indirect labeling methods. After that, labelled samples were applied on the microarray slides to allow the hybridization. RNAs that are complementary to the molecules on the microarray

hybridize with the strands on the microarray. Excess of labelled oligonucleotides were washed of and then the microarray slide is scanned.

1.4.3 Scanning, image and data analysis

After scanning of the slide, next step is to analyze the image by using bioinformatics methods. But before this analysis, summary of the measured data was obtained. After that spatial effects on the chip are investigated and corrected. Thereafter, analysis of the intensity value distribution of raw data was performed and if it is necessary normalization was done. After these steps, the normalized microarray data can be analyzed using statistical methods, clustering techniques and addition to these more advanced pattern recognition approaches.

A spatial distribution analysis is used to understand if there is a correlation with the position of the probes and intensities of the probes. There are several factors that cause spatial variability such as unequal amounts of RNA, the intensity-dependent dye effect and the set-up of the detection process. If adjustment is required, global background subtraction is carried out.

In experiments in which more than one microarray slides are used, there are always differences arising from non-biological variation between arrays such as amount of RNA, dye biases, sample preparation or hybridization differences, calibration of scanner, variations in slide printing etc. Data normalization is used to adjust microarray data for effects of these variabilities. So normalization between samples can help to increase the quality of data by ensuring that different samples can be comparable to each other. Different methods are used to obtain normalization such as median or mean normalization, quantile normalization (74) and variance stabilizing normalization (75). For a graphical representation of the results, boxplots or violinplots are generated.

Following to normalization, statistical approaches are used which are analysis methods which show good correlations. Correlation anlaysis and scatter plots are some of the tools to obtain statistical values.

Cluster analysis is a method to identify the samples which show similar intensity patterns and thus are similar to each other. Many different methods are used in

clustering such as hierarchical clustering (76, 77), quality threshold clustering (78), *k*-means clustering (79) and self-organizing maps (80).

For the detection of differentially regulated mRNAs, quotation of mean, median, or variance, parametric t-test, non-parametric Wilcoxon-Mann-Whitney test (81, 82), Empirical Bayes Statistics (83) and the area under the receiver operator characteristics curve (84) are used.

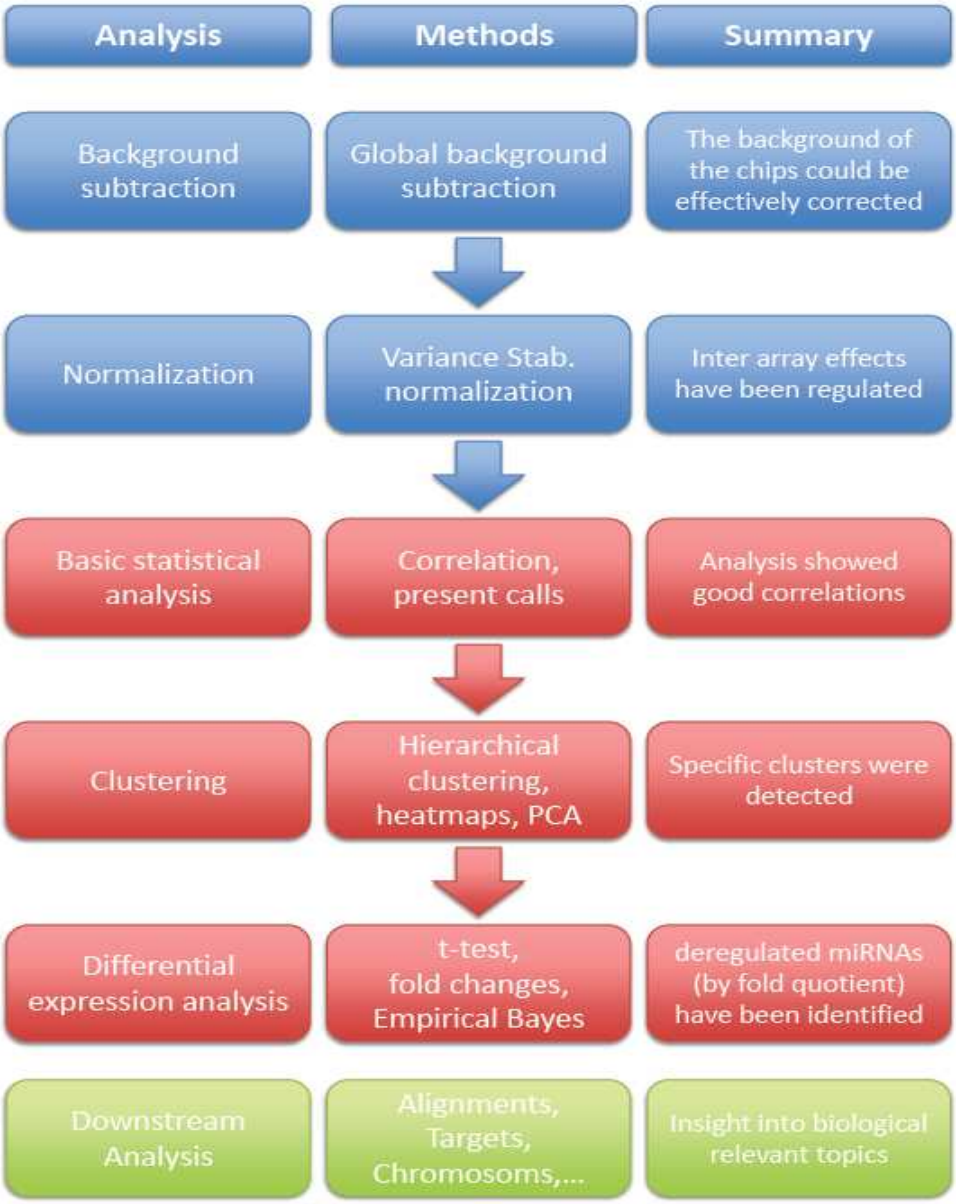


Figure 1.6 Bioinformatics workflow used in this thesis

CHAPTER 2

MATERIALS AND METHODS

2.1 CELL CULTURE

2.1.1 Isolation of hMSCs from Bone Marrow

Human bone marrow aspirates were provided by Karadeniz Technical University, Department of Hematology. Ficoll density gradient centrifugation method was used to isolate human mesenchymal stem cells from bone marrow. Firstly bone marrow aspirates were diluted with PBS (Gibco) at 1:10 ratio. Then 5 ml Ficoll (Biochrom) was put in 15 ml falcon tube and 10 ml diluted bone marrow aspirate was poured onto ficoll drop by drop carefully. It was centrifuged for 25 minutes at 800g (2500 rpm) at room temperature. After centrifugation, separately four different layers were formed; red blood cells, ficoll, mononuclear cells and serum from bottom to top. Since mononuclear cells part includes mesenchymal stem cells, serum part was discarded. 10 ml serologic pipette was used to obtain the cloudy part between serum and ficoll layers and transferred into a new 15 ml falcon tube. Dulbecco's modified Eagle's medium with low glucose (DMEM-LG, Gibco) was used to complete the cloudy part to 10 ml and then centrifuged at 350g (1500 rpm) for 10 minutes. After centrifugation, supernatant was discarded and only 0.5 ml pellet with medium was left. Cells in this part were resuspended flicking and pipetting. Falcon tube containing cells was

completed to 15 ml with DMEM in order to get rid of ficoll completely again centrifuged at 350g (1500 rpm) for 10 min. Supernatant was removed again and pellet was resuspended. Then cells were seeded into a 25 cm^2 tissue culture flask (BD Falcon) in 10 ml DMEM including 20% hMSCs-qualified Fetal Bovine Serum (MSCs-FBS, Gibco) and 100µg/ml Primocin (InvivoGen). Cells were incubated in 37^oC, 5% CO_2 incubator and the screw cap of the flask was kept loose in order to allow circulation of carbon dioxide into flask. At the end of 3 days after isolation, non-adherent hematopoietic cells were removed via medium refreshment and this was repeated until the first subculture. If enough amounts of colonies formed after 10-14 days, the first subculture was performed.

2.1.2 Seeding and Subculture of hMSCs

After seeding of cells into culture flasks, cells were attached to the surface of flasks and colonies were started to form. When cells became 80-90% confluent they were subcultured. Before the subculture, water bath was used to warm DMEM, FBS, PBS and trypsin to 37^oC. Medium in the flask was removed by a sterile pipette and then 5 ml of calcium and magnesium free Phosphate Buffered Saline (PBS, Biochrom) to remove residual medium. After removal of PBS, 4 ml of pre-warmed 0.25% Trypsin/EDTA (GIBCO) was added to the flask and kept at room temperature for 1-2 min. Then cells were observed under invert microscope. If most of the cells were detached from the surface of the flask, 1 ml of FBS added to the flask to inactivate the function of trypsin. The cells in the flask with trypsin and FBS was transferred into new 15 ml falcon tube and centrifuged at 1500 rpm for 10 min at room temperature. After centrifugation, supernatant was discarded by leaving about 0.5 ml of the cell suspension at the bottom. Pellet was finger mixed and volume was up to 10 ml with DMEM medium in order to remove the remaining any tyripsin. Centrifugation step was repeated once more and then cells were counted by hemocytometer. After counting, hMSCs were seeded at a density of 1500 cells/cm^2 with 15% hMSCs qualified FBS containing DMEM for expansion. Subculture of cells was repeated at about 5-6 days intervals and medium was refreshed once in between two passages. Mesenchymal stem cells at 3-5 passages were used for neural differentiation and transfection experiments.

2.1.3 Seeding and Subculture Human Umbilical Vein Endothelial Cells (HUVEC)

10000 cells/cm^2 HUVECs were seeded in 10% FBS (Biochrom) containing DMEM or RPMI, penicylin-streptomycin after each subculture. Cells were subcultured twice in a week.

2.1.4 Freezing and Thawing Human Umbilical Vein Endothelial Cells (HUVEC)

After subculture, cells were resuspended in FBS at density of 1-2x10^6 cells/ml. Cryovial tubes were placed on ice and 900 µl cell suspension was transferred into each tube. 100 µl DMSO (Dimethyl Sulfoxide, Applichem) was added into each tube drop by drop and after each drop the mixture was mixed by pipetting. Tubes were left at -20^oC for 1 hr and then kept at -80 ^oC overnight .The next day cryovial tubes were transferred to liquid nitrogen tank (-196 ^oC) for long term.

Frozen cells can be used later, so for this purpose cells in cryovial tubes have to be thawn. Before thawing process, 10 ml of pre-warmed DMEM to 37 ^oC was added into 15 ml falcon tubes. Then cryovial tubes were taken from the nitrogen tank and transferred to 37^oC water bath. As soon as possible, liquid content in the tube was transferred to the tube containing medium and centrifuged at 1500 rpm for 10 min. The supernatant was discarded and the pellet of the cells was resuspended in 10 ml medium and centrifugation was repeated once more in order to get rid of DMSO. All of the cells in the pellet were seeded with DMEM containing 15% FBS and the next day medium was refreshed in order to get rid of dead cells.

2.2 TRANSFECTION

2.2.1 Transfection with Lipofectamine RNAiMAX Reagent

The day before transfection, cells were seeded into 6-well culture plates with complete medium at 30-50% confluence. (3x10^3 HUVEC/well, 5x10^4 hMSCs/well). Lipofectamine RNAiMAX was diluted with Opti-MEM in 1:50 ratio. 2 different validated siRNAs (TOP2B_5 and TOP2B_6 from Qiagen) were used for silencing topo IIβ. Sequences of TOP2B_5 and TOP2B_6 are TCGGGCTAGGAAAGAAGTAAA and CAGCCGAAAGACCTAAATACA respectively. Opti-MEM was used to dilute siRNAs in 1:100 ratio. Diluted siRNA and diluted reagent were mixed in one tube and

incubated for 15 min at room temperature to allow the siRNA-Lipofectamine RNAiMAX complexes to form. 500 µl of siRNA-Lipofectamine RNAiMAX complexes were added into the wells containing cells and medium, by rocking the plate back and forth. Cells were incubated at 37°C in CO_2 incubator for 24-48 hrs. After 24 hrs incubation, the medium containing complex was removed and replaced by complete medium. Since transfection was performed during neural differentiation, medium was replaced with neurobasal medium containing N3 cytokine combination. Efficiency of transfection was checked by RT-PCT.

2.3 NEURAL TRANSDIFFERENTIATION OF hMSCs

2.3.1 Neural Differentiation with N3 Cytokine Combinations

The day before induction, transfection experiments were performed. After transfection, the expansion medium was replaced with 4 ml Neurobasal Medium (GIBCO) containing 2% (v/v) B-27 Supplement (GIBCO), 0.5mg/ml dbcAMP (dibutyryl cyclic AMP, SIGMA), 0.5mM IBMX (3-isobutyl-1-methylxanthine, SIGMA), 20 ng/ml hEGF (human epidermal growth factor, SIGMA), 40 ng/ml rhFGF (recombinant human fibroblast growth factor, R&D systems), 10 ng/ml FGF-8 (fibroblast growth factor-8, PeproTech), 10 ng/ml rhBDNF (recombinant human brain-derived neurotrophic factor, R&D systems) and 2mM L-Glutamine (GIBCO). This induction medium was refreshed every 48 hrs. So during medium refreshment, the following transfection was also performed. Morphologies of the cells were observed under light microscope during 12 days and neural marker expressions were monitored at mRNA level with RT-PCR.

Table 2.1 Summary of N3 cytokine combination used in neural differentiation of hMSCs

INDUCTION MEDIUM WITH N3	**FINAL**
NEUROBASAL MEDIUM	50X
B27 SUPPLEMENT	1X
dbCAMP (100 mg/ml)	0,125mg/ml
IBMX (1M)	0,5 mM

Table 2.1(continued) Summary of N3 cytokine combination used in neural differentiation of hMSCs

hEGF (100 mg/ml)	20 ng/ml
BFGF (100 mg/ml)	40 ng/ml
FGF-8 (10 mg/ml)	10 ng/ml
BDNF (10 mg/ml)	10 ng/ml
L-GLUTAMINE (200mM)	2 mM
NGF (100 mg/ml)	40ng/ml

2.4 REVERSE TRANSCRIPTION PCR

siRNA silencing and expression of neural markers, actin, topo IIα and topo II β were checked at the mRNA level. RT-PCR is a technique used to determine the change in mRNA concentration in various siRNA treated samples and also samples of neural differentiation of hMSCs. RNA of samples was extracted by using RNeasy kit (Qiagen). Then the concentration of samples was quantified. 0.5 µg of total RNA was reverse transcribed to obtain cDNA by Quantitect reverse transcription kit (Qiagen). cDNA underwent 30 or 35 cycles of amplification (PCR core kit, Qiagen).

To prepare the genomic DNA elimination reaction, 14 µl reverse transcription master mix , 2 µl of gDNA Wipeout Buffer , and the volume of RNA corresponding to 0,5 µg were added in eppendorf tube and then volume was completed to 14 µl with RNase-DNase free water. Then the tube was incubated for 2 min at 42 °C and by following this, tube was placed on ice. The reverse-transcription master mix was obtained by adding of 1 µl RT Primer Mix, 4 µl Quantiscript RT Buffer and 1 µl Quantiscript Reverse Transcriptase d on the mixture to obtain totally 20 µl volume ice .Then tube was incubated at 42 °C for 15 min. To inactivate Quantiscript Reverse Transcriptase, tube was incubated for 3 min at 95°C.

A 25 µl reaction was prepared by mixing 1 µl of cDNA, 16.8 µl of double distilled water, 0.2 µl of Taq polymerase, 0.5 µl of 10 mM dNTP, 2 µl of 25 mM $MgCl_2$, 1 µl of forward primer, 1 µl of reverse primer and 2.5 µl of 10X Buffer. The

PCR conditions were 5 min at 94 °C for initial incubation, 30 seconds at 94 °C for denaturation, 45 seconds at 60 °C for annealing of topo IIβ primer, 1 min at 72 °C for extension and 7 min at 72 °C final incubation. The PCR was done for 30 or 35 cycles.

Agarose gel electrophoresis is used to separate PCR products according to their sizes. To visualize the products under UV light ethidium bromide or safe DNA staining solution were used. To prepare % 2 (w/v) agarose gel, 1.6 gr of agarose powder (Sigma) and 80 ml of 0.5 X TBE buffer (Fluka) were added an erlenmayer flask and boiled in microwave oven. Then the flask was cooled and 8µl/80ml ethidium bromide or 12µl/80ml safe DNA staining solution was added. Just before it solidifies comb is placed in order to generate 20 wells into 13 x 14x 0.5 horizontal agarose gel platform and then the solution was poured. 100 bp DNA Ladder (Bioron) was used as molecular size marker. To load PCR products on gel, bromophenol blue loading dye was used. Gel was run at 110 V for 45 min.

Table 2.2 List of primers used for quantative RT-PCR

Gene	**Forward(5'-3')**	**Reverse(5'-3')**	**Annealing Temperature**
topo IIα	ACCATTGCAGCCTGTA	GCTCTTCCCATATTATCC	54^{o}C
topo IIβ	ATCAAAAGCCACTC-CAGAAAAATC	AGAAGGTGGCTCAGTAGGGAAGTC	60^{o}C
Actin	CGCACCACTGGCATTGTCAT	GTGGCCATCTCCTGCTCGAA	60^{o}C
Nestin	TGGCTCAGAGGAAGAGTCTGA	TCCCCCATTTACATGCTGTGA	65^{o}C
NF-L	TCCTACTACACCAGCCATGT	TCCCCAGCACCTTCAACTTT	59^{o}C
NSE	CCCACTGATCCTTCCCGATACAT	CCGATCTGGTTGACCTTGAGCA	65^{o}C
BIII tubulin	AGTGATGAGCATGGCATCGA	AGGCAGTCGCAGTTTTCACA	60^{o}C

2.5 IMMUNOFLOURESCENT STAINING

The day before experiment, hMSCs were seeded into 24-well culture for immunofluorescence staining. At the day of staining, medium was aspirated from wells. To permeabilize cells, pre-warmed (37^{o}C) 500 µl/well TZN buffer (10 mM pH 7.5 Tris-HCl, 0.5% Nondet P40, 0.2 mM $ZnCl_2$were with) was added to each well and then incubated for 15 min by mixing on rocking shaker at very low speed (approx. 10 rpm). Then the cells were fixed with 4% Paraformaldehyde / PBS (500 µl/well) for 10 min at RT. Cells were washed with 750 µl/well PBS (Phosphate Buffered Saline, pH 7.4, SIGMA) for 3 times, 5 min at each time on rocking shaker. 500 µl/well of 10% Normal Goat Serum (Gibco) and 10% Normal Horse Serum (Biochrom) in 0.3% TritonX / PBS (PBS-Tx) were used to block cells for 30 min at RT. Then, cells were incubated with 60 µl/well specific primary antibodies for target proteins for 2 hrs at RT. Antibodies diluted in PBS-Tx with 3% NHS. Washing steps with PBS was repeated for three times. Cells were then treated with 50 µl/well Alexa Flour labeled anti mouse or anti rabbit secondary antibodies at RT for 1 hr. After this incubation, cells were washed 3 times with PBS and treated with 150 µl/well, 1/15000X DAPI (Sigma) for 10 min. Then washing with PBS was repeated for three times and finally cells were washed with dH_2O. 4 µl Prolong Gold Antifade Reagent (Invitrogen) was applied on glass coverslips and they were placed on cells in the wells. Wells were observed under fluorescent microscope (Nikon) and images were taken next day.

Antibodies against NF-H (1:100, Chemicon), MAP2 (1:50, Cell Signalling), Nestin (1:200, Santa Cruz) and NSE (1:100, Chemicon) were used at indicated dilutions. Secondary antibodies GAM-IgG-Alexa Fluor 488 (1:100) and GAR-IgG-Alexa Fluor 594 (1:100) were purchased from Invitrogen.

2.6 MICROARRAY

2.6.1 Microarray slide preparation

2.6.1.1 Biochip design

Biochips were designed by Febit group. Probes complementary to the transcripts from the organism homo sapiens were designed. The intensities of blank probes which consist only of one single "T" nucleotide were used for background corrections.

2.6.1.2 Biochip synthesis

The Geniom biochips used were synthesized with the Geniom One instrument applying Febit's standard shortmer kit for oligonucleotide synthesis. The light-activated in-situ oligonucleotide synthesis using a digital micromirror device was performed within the Geniom One instrument on an activated three-dimensional reaction carrier consisting of a glass-silicon-glass sandwich (Biochip). Using standard DNA synthesis reagents and 3'-phosphoramidites carrying a 5'-photolabile protective group, oligonucleotides were synthesized in parallel in eight individually accessible microchannels (referred to as arrays) of one biochip. Prior to synthesis, the glass surface was activated by coating with a spacer to facilitate probe-target interaction and to avoid probe-probe interference.

2.6.2 Microarray sample preparation

2.6.2.1 Quality Control

All incoming samples undergo a quality check to determine the quality and the quantity of the sample RNA. The quality control was done with the Agilent 2100 Bioanalyzer, using the RNA 6000 Nano Kit according to the manufacturer's instructions.

2.6.2.2 Sample labeling, Washing, Detection

MessageAmpTMII-Biotin Enhanced RNA Kit from Ambion was used for the labeling of RNA for mRNA expression analysis. The kit provides a transcription of RNA in cDNA, following a transcription in cRNA while enrichment of all nucleic acid molecules is included. For each array of total RNA was labeled according to the manufacturer's instructions.

Reverse Transcription to Synthesize First Strand cDNA:

1 µg of total RNA was placed into a nonstick, sterile, RNase-free tube. 1 µl of T7 Oligo (dT) Primer was added into tube. 12 µl of final volume was obtained by

adding Nuclease-free Water and vortexed briefly. Sample was incubated 10 min at 70°C in a thermal cycler and then centrifuged briefly (~5 sec) to collect them at the bottom of the tube. The mixtures were placedon ice. Reverse Transcription Master Mix was prepared by adding 2 µl 10x First Strand Buffer, 4 µl dNTP Mix, 1 µl RNase Inhibitor and 1 µl ArrayScript. Mixture was vortexed gently and centrifuged briefly and placed on ice. 8 µl of Reverse Transcription Master Mix was transferred to each RNA sample and mixtures was mixed thoroughly by pipetting up and down 2–3 times, then flicking the tube 3–4 times and centrifuged briefly to collect the reaction in the bottom of the tube. Samples were placed at 42°C in a thermal cycler and incubated for 2 hr at 42°C. After incubation, sample was centrifuged briefly (~5 sec) to collect the reaction at the bottom of the tube and placed on ice and immediately proceed to second strand cDNA synthesis.

Second Strand cDNA Synthesis:

Second Strand Master Mix in a nuclease-free tube was prepared on ice by adding 63 µl Nuclease-free Water, 10 µl 10XSecond Strand Buffer, 4 µl dNTP Mix, 2 µl DNA Polymerase, 1 µl RNase H. 80 µl of Second Strand Master Mix was transfered to each sample and mixed thoroughly by pipetting up and down 2–3 times, then flicking the tube 3–4 times and centrifuged briefly to collect the reaction in the bottom of the tube. Tubes were placed in a 16°C thermal cycler and incubated 2 hrs in a 16°C thermal cycler. After incubation tubes were placed on ice briefly or freezed immediately.

cDNA Purification:

Firstly, 24 ml 100% ethanol (ACS grade or better) was added to the bottle labelled Wash Buffer and mixed well and marked the label to indicate that the ethanol was added. Nuclease-free Water was preheated to 50–55°C for at least 10 min. 250 µl of cDNA Binding Buffer was added to each sample and mixed thoroughly by pipetting up and down 2–3 times, then flicking the tube 3–4 times and spinned down. cDNA sample\cDNA Binding Buffer was pipetted onto the center of the cDNA Filter Cartridge and centrifuged for ~1 min at 10,000 x g. Flow-through was discarded and the cDNA Filter Cartridge in the wash tubewas replaced. 500 µl Wash Buffer was applied to each cDNA Filter Cartridge and centrifuged for ~1 min at 10,000 X g. Flow-through was

discarded and the cDNA Filter Cartridge was spinned for an additional 1 min to remove trace amounts of Wash Buffer. cDNA Filter Cartridge was transfered to a cDNA Elution Tube. 12 µl of Nuclease-free Water (preheated to 50–55°C) was applied to the center of the fitler in the cDNA Filter Cartridge. Tube was left at room temperature for 2 min and then centrifuged for ~1.5 min at 10,000 x g. Tube was eluted with a second 12 µl of preheated Nuclease-free Water. The double-stranded cDNA was obtained now in the eluate (~20 µl).

In Vitro Transcription to Synthesize Biotin-labeled aRNA:

An IVT Master Mix was prepared by adding 12 µl Biotin-NTP Mix,4 µl T7 10X Reaction Buffer,4 µl Enzyme Mix at RT and mixture was vortexed gently, centrifuged briefly and placed on ice. 20 µL of IVT Master Mix was transferred to 20 µl of each sample and mixture was mixed thoroughly by pipetting up and down 2–3 times, then flicking the tube 3–4 times and centrifuged briefly to collect the reaction in the bottom of the tube. Tubes were incubated for 4–14 hr at 37°C. 60 µl Nuclease-free Water was added to stop the reaction by completing the final volume to 100 µl. Final solution was mixed gently.

aRNA Purification:

Before starting, Nuclease-free Water was preheated to 50–60°C for at least 10 min. For each sample, an aRNA Filter Cartridge was placed into an aRNA Collection tube.350 µl of aRNA Binding Buffer was added to each aRNA sample and proceed to the next step immediately. 250 µl of ACS grade 100% ethanol was added to each aRNA sample and mixed by pipetting he mixture up and down 3 times. There was no vortex and centrifuge steps. Each sample mixture from step 2 was added onto the center of the filter in the aRNA Fitler Cartridge and centrifuged for ~1 min at 10,000 X g. Flow through was discarded and the aRNA Filter Cartridge was replaced back into the aRNA Collection tube. 650 µl of Wash Buffer was added to each aRNA Filter Cartridge and centrifuged for ~1 min at 10,000 X g. Flow-through was discarded and the aRNA Filter Cartridge was spinned for an additional ~1 min to remove trace amounts of Wash Buffer. Filter Cartridge was transferfed to a fresh aRNA Collection tube. Preheated 100 µl Nuclease-free Water was added to the center of the fitler and left at RT for 2 min and

then centrifuged for ~1.5 min at 10,000 X g.The aRNA was now in the aRNA Collection Tube in ~100 µl of Nuclease-free Water. It can be stored at –80°C for up to 1 year.

Fragmentation of labeled cRNA Samples

To obtain 15 µg fragmented cRNA: cRNA was dissolved in 2.5 µl DEPC-H_2O in 0.2 ml tube and 2.5 µl of diluted fragmentation buffer 1 was aded. The mixture was incubated for 35 min at 94°C (Thermo Cycler) and placed on ice. To check cRNA fragmentation, either a denaturing gel or a Bioanalyzer (200ng/1 µl) was used. The fragments should have an average length of 200 nucleotides.

After all of these steps, Febit's proprietary standard Hybridization Buffer (20 *µl* per array) was added. For each array the RNA was suspended in Febit's proprietary mRNA Hybridization Buffer (25 *µl* per array). Hybridization was done automatically for 16 hrs at 45^{o}C using the Geniom RT Analyzer. Then the biochip underwent a stringent wash. Following the labeling procedure, the microuidic-based primer extension assay was applied. The elongation was done with Klenow Fragment and biotinylated nucleotides at 37°C for 15 min. Finally, Biochip was washed automatically. For maximum sensitivity, biotin and its detection with streptavidin-phycoerythrin (SAPE) was used by consecutive Signal Enhancement (CSE) procedure.

Consecutive Signal Enhancement (CSE)

Firstly, the required solutions were prepared:

- 12 x MES buffer: MES hydrate 64.61 g, MES sodium salt 193.3 g, deionized (DI) H2O 800 ml, adjust pH to 6.5-6.8 and fill to 1000 ml with DI H2O
- IgG stock solution: resuspend 50 mg goat IgG in 5 ml 150 mM NaCl
- Biotinylated anti-Streptavidin antibody: resuspend 50 mg in 1 ml DI H2O 2 x Stain buffer: 41.7 ml 12 x MES buffer, 92.5 ml 5 M NaCl, 2.5 ml 10% Tween-20,113.3 ml DI H2O, fitler sterilize
- Antibody solution: 3500 µl 2 x Stain buffer, 280 µl 50 mg/ml BSA, 70 µl Goat IgG stock, 42 µl biotinylated anti-Streptavidin antibody, 3108 µl DI H2O

Antibody solution was placed into port Buffer-3. In the Hybridization module, the hybridization profile “Febit Signal Amplification (CSE)” was selected. Standard procedures for Hybridizations/Detections were followed. It was essential to clean port Buffer-3 after use as the antibodies will form aggregates which can contaminate the system. Antibody solution was replaced with 50 ml 0.5x sodium hypochlorite. In the module Maintenance Hyb_CleanPort Buffer_3 was started. After that, 50 ml DI H2O was added into Port Buffer 3. Hyb_CleanPort Buffer 3 start was started again. And then HYB_WASH_80C was performed.

2.6.3. Bioinformatics analysis

The bioinformatics analysis started with a summary of the measured data Thereafter, spatial effects on the chip were investigated and corrected. Then, the intensity value distribution of raw data was analyzed and if required, normalized. For the detection of differentially regulated mRNAs, the following comparison was done:

	Comparison
1	Group 1 vs. Group 2
2	Group 1 vs. Group 3
3	Group 2 vs. Group 3

Figure 2.1 Comparison of Sample Groups (Group 1 is untreated control hMSCs, Group 2 is hMSCs those are treated with N3 cytokine combination and Group 3 is hMSCs those are transfected during neural differentiation.)

CHAPTER 3

RESULTS

3.1 SURFACE MARKERS OF BONE MARROW CELLS

Cell surface markers of hMSCs at passage three were analyzed by fluorescence activated cell sorting (FACS). Figure 3.1 gives graphical results that show percentage of cell surface markers and table 3.1 summarize the results in this figure.

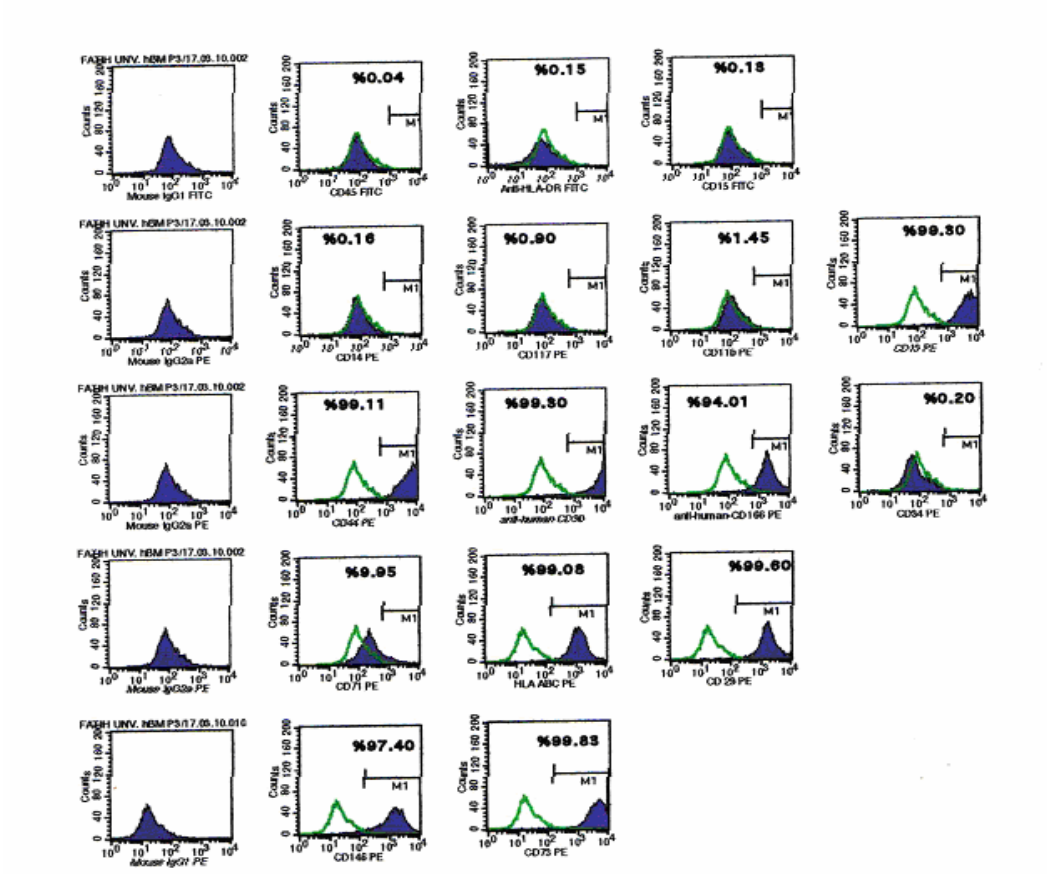


Figure 3.1 representative flow cytometry analyses of cell surface markers in hBM-MSCs at passage 3

Table 3.1 Summary for percentage of cell surface markers according to FACS results

Cell Surface Antigen	**(%) Presence in hMSCs at Passage 3**
CD 13	99.3
CD 14	0.16
CD 15	0.13
CD 29	99.6
CD 34	0.2
CD 44	99.11
CD 45	0.04
CD 71	9.95
CD 73	99.83
CD 90	99.3
CD 116	1.45
CD 117	0.90
CD 146	97.4
CD 166	94.01
HLA-DR	0.15
HLA-ABC	99.08

At passage three most of the cells (about 99%) express stromal markers (CD73 and CD90) where as expression of hematopoetic markers (CD45, CD34, and HLADR) is less than 0.2%.

3.2 hMSCs IN CULTURE

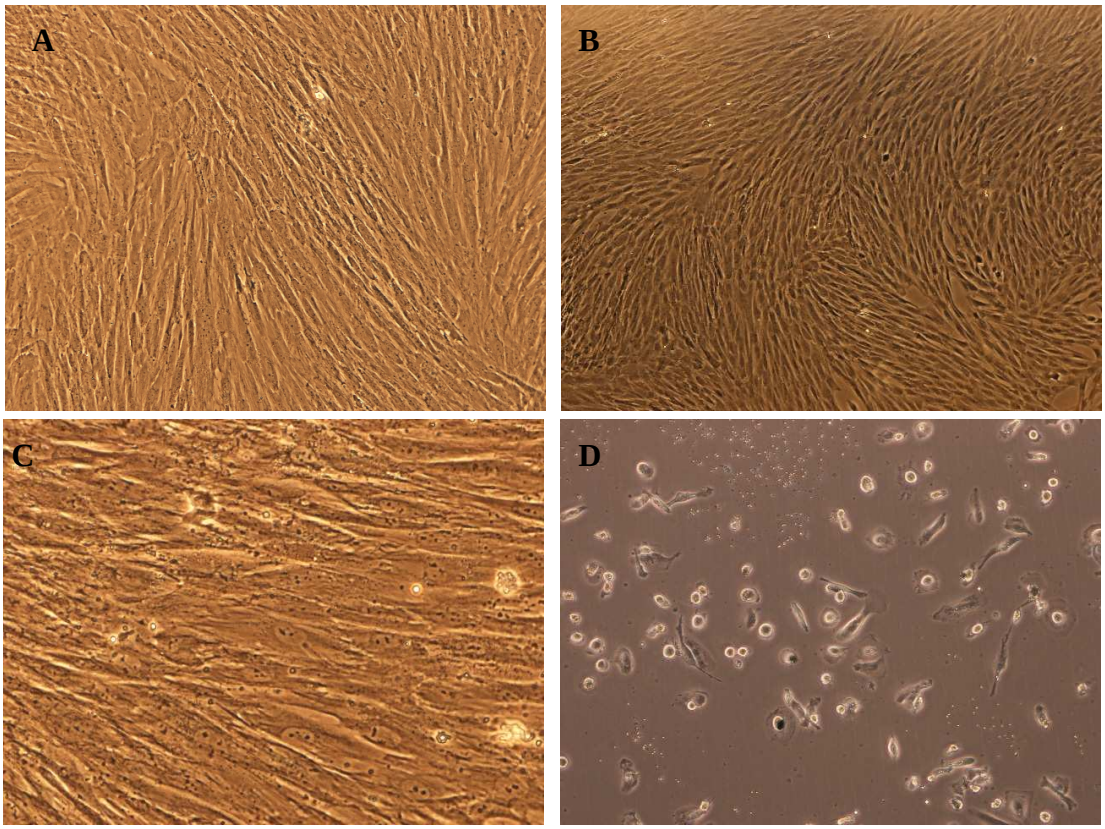
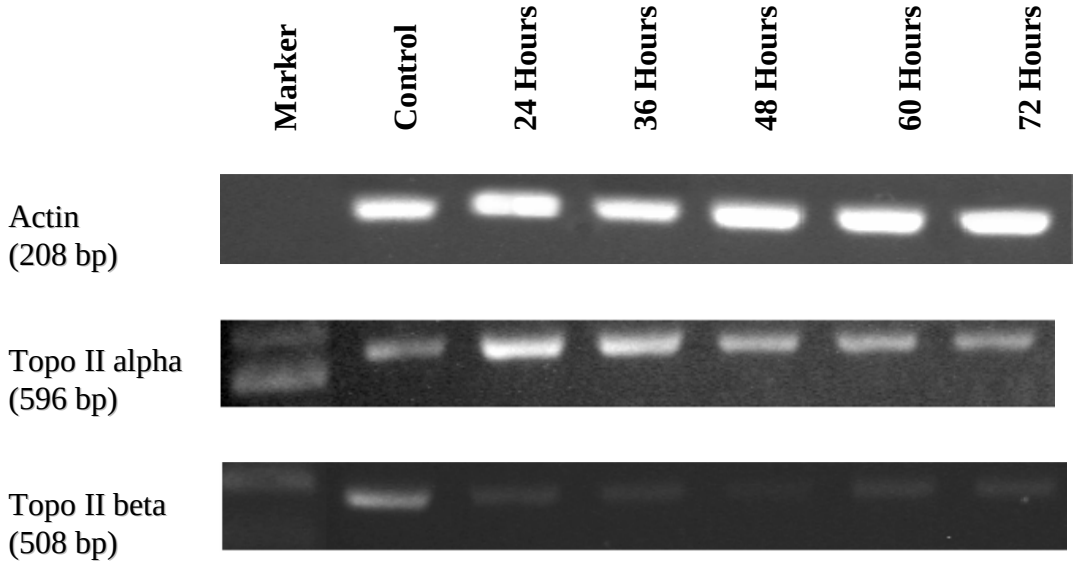


Figure 3.2 Light microscope images of primary hMSCs in culture flask surface.**A. B. C.** and **D.** hMSCs colonies with fibroblastic morphology at passage 3. **A**.4X **B**.10X **C.**20X and **D.** Primary culture including a mixture of adherent cells

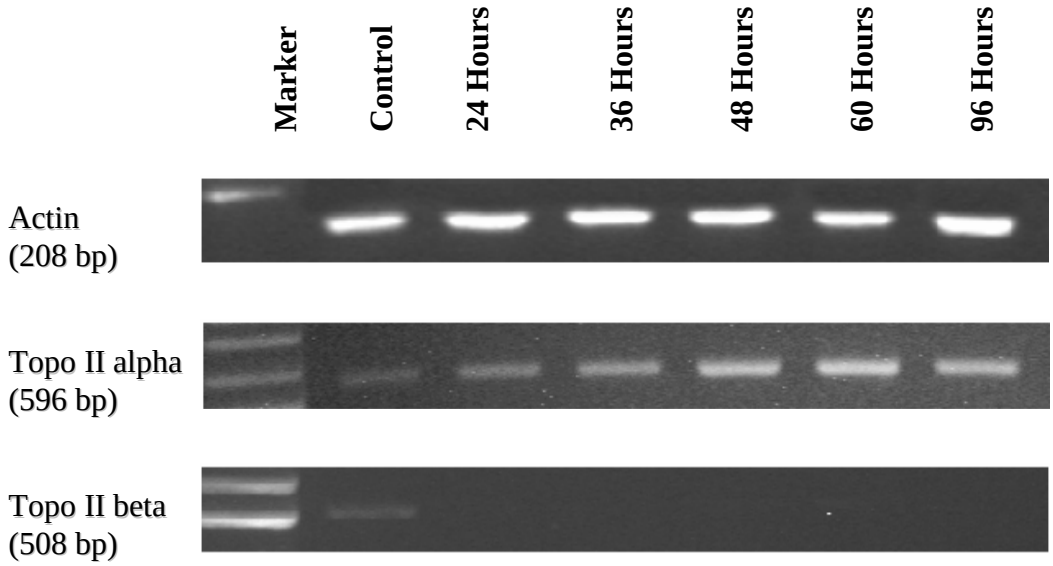
3.3 TRANSFECTION WITH LIPOFECTAMINE RNAiMAX TRANSFECTION REAGENT

3.3.1 RT-PCR results after topo IIβ specific siRNA transfection

A)



B)



C)

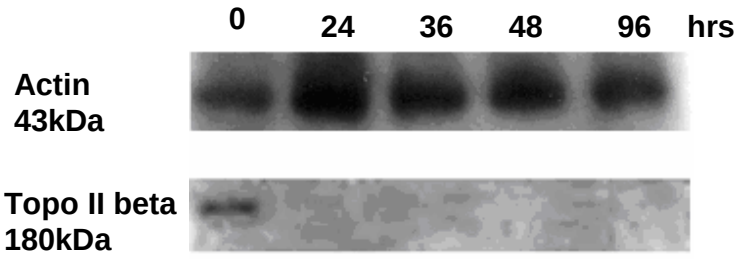


Figure 3.3 Transfection of hMSCs with TOP2β siRNA by LipofectamineRNAiMAX transfection reagent.

A. RT-PCR analysis for topo IIβ after siRNA transfection. Transfection was performed once at hour "0" (0, 24, 36, 48, 60, 72: hours after siRNA transfection). **B.** and **C.** results are for actin, topo IIα and topo IIβ according to RT-PCR and Western-Blot. Transfections were repeated two times at hours "0" and "48".

According to these results, transfection was achieved succesfully. In part A only one shoot of transfection was performed at hour "0" and topo IIβ appeared again at 60 hours. When transfections were repeated at "0" and "48" hours, about 85-90% silencing of topo IIβ was achieved. At mRNA and protein level. And also selective silencing of topo IIβ was obtained since expression level of topo IIα did not change.

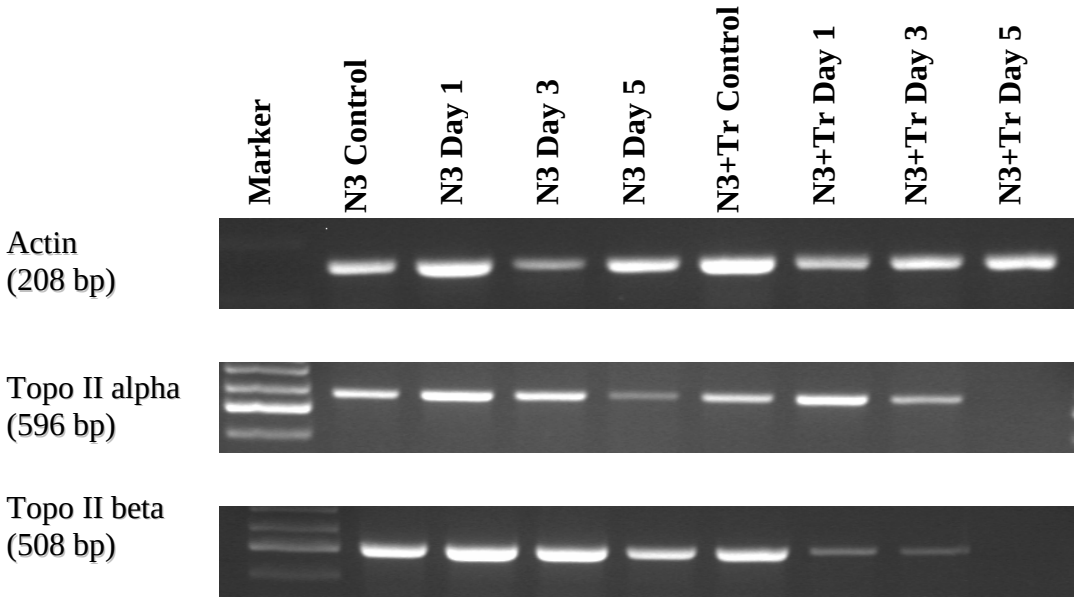


Figure 3.4 RT–PCR results for actin, topo IIα and topo IIβ. Neural differentiation was abbreviated N3 and transfection during neural differentiation was abbreviated N3+Tr.

In these results, neural differentiation was achieved since topo IIα level decreases as differentiation progresses. Transfection was obtained succesfully during neural differentiation since topo IIβ level decreases and disappears according to control. Neural differentiaition and transfection during neural differentiaition repeated for 12 days.

3.4 NEURAL DIFFERENTIATION WITH N3 CYTOKINE COMBINATIONS

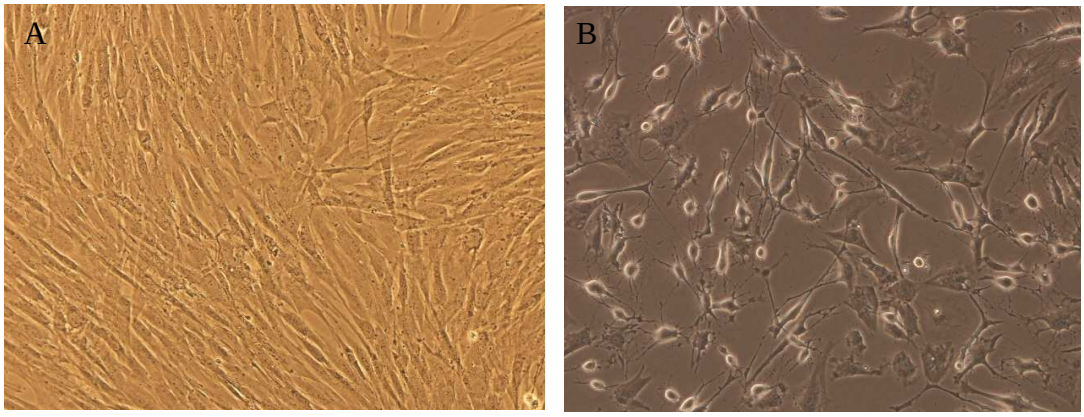


Figure 3.5 Neural differentiation of hMSCs with N3 cytokine combinations was observed under light microscope. Images are at 10X. A. Control in expansion medium B. Changes in morphology of hMSCs after neural induction 50-60% of hMSCs show neural like morphology in B.

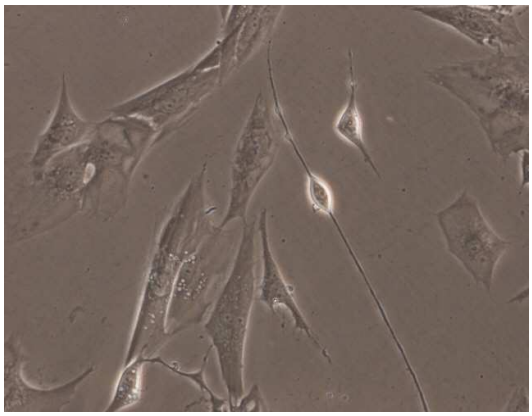


Figure 3.6 shows hMSCs treated with N3 cytokine combination at 20X

In control group, fibroblastic morphology of untreated hMSCs did not change. But hMSCs treated with N3 cytokine combination started to change morphology and look like neural cells.

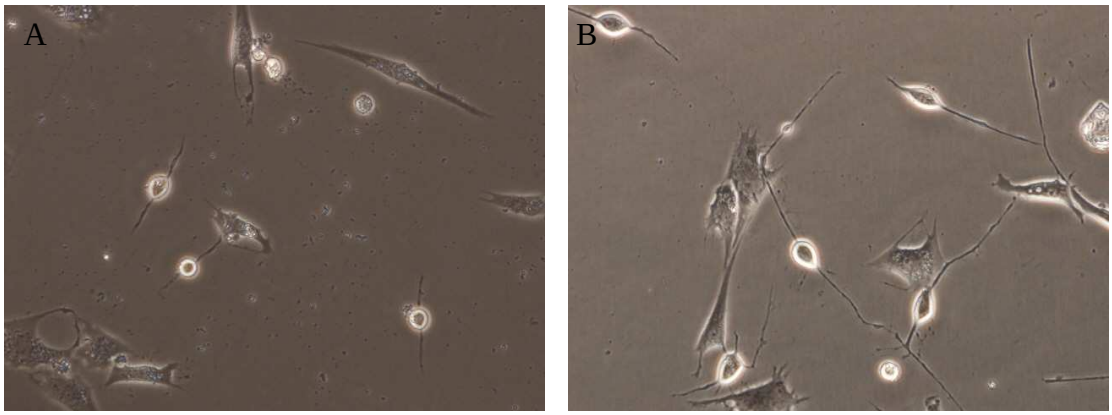


Figure 3.7 shows difference in the axon length between N3+Tr and N3 groups A. Transfected hMSCs with topo IIβ specific siRNA during neural differentiation B. Neurally differentiated hMSCs

In the N3+Tr group, length of axon decreases as shown in the above figure. However, in neurally differentiated hMSCs, cells have longer axons. So topo IIβ probably play a role in axon length. When these two groups compared, the length of axon decreases about 60%.

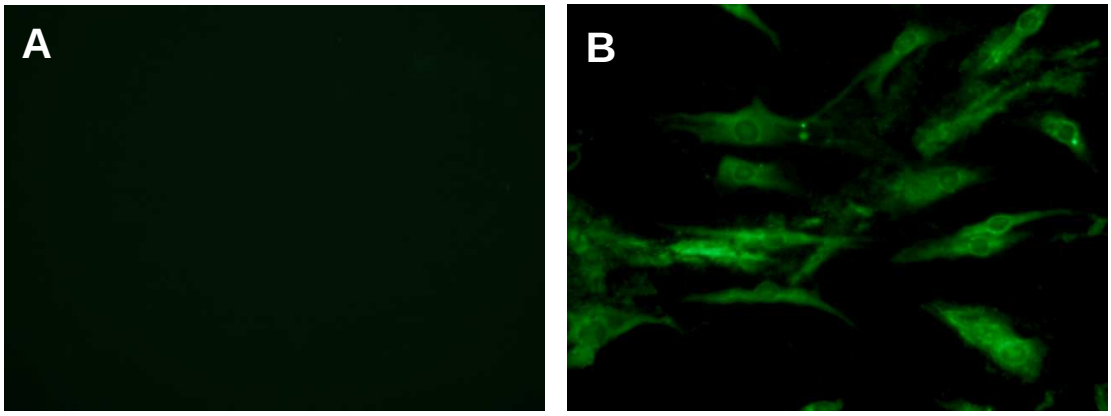


Figure 3.8 shows neural markers during neural differentiaition of hMSCs A.Control B. hMSCs expressing neural marker Nestin.

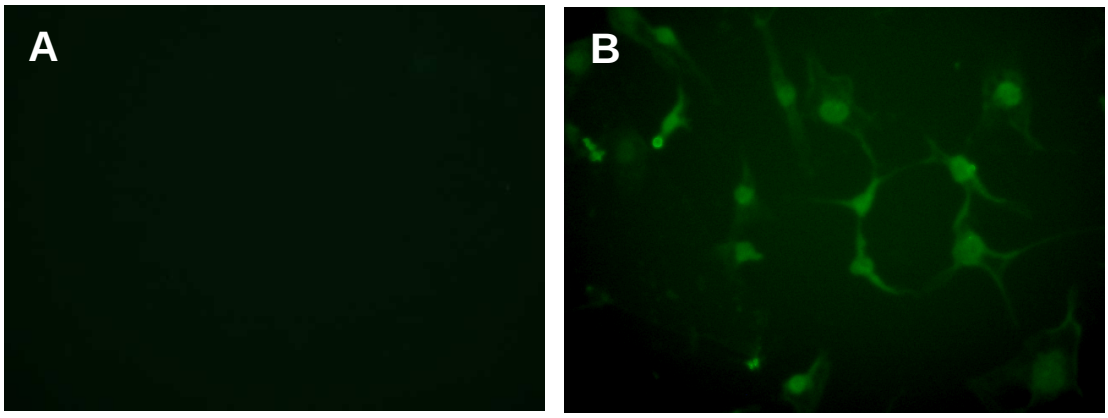


Figure 3.9 shows neural markers during neural differentiation of hMSCs A. Control B. hMSCs expressing neural marker NF.

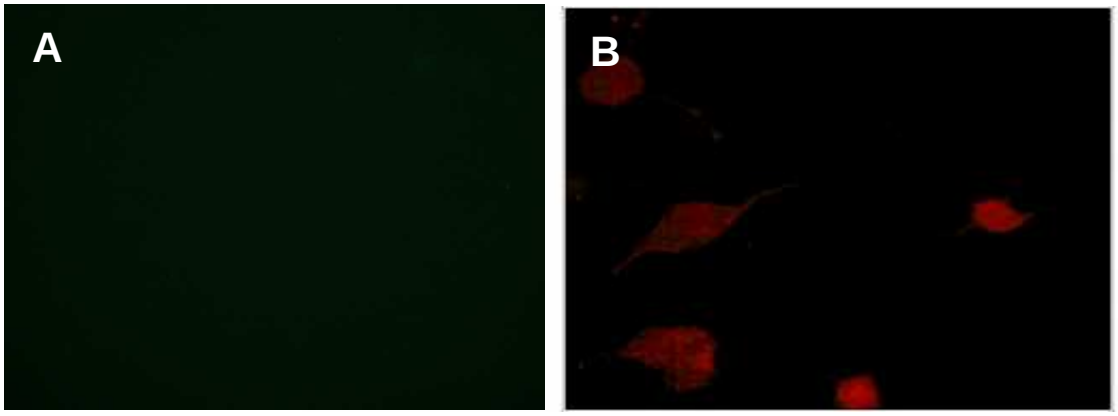


Figure 3.10 indicates expression of NSE in neurally differentiated hMSCs.

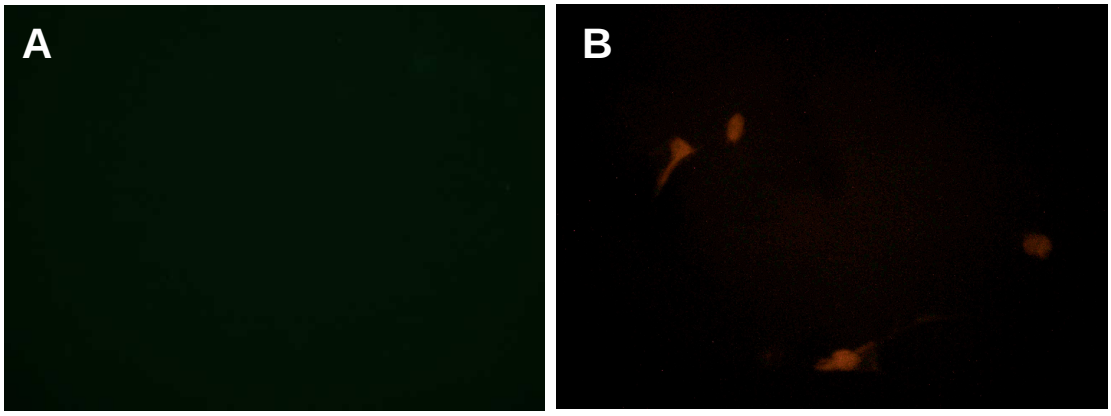


Figure 3.11 shows expression of late neural marker MAP2 in neurally differentiated hMSCs at 14^{th} day of differentiating process.

Accordig to imunofluorescent staining results, hMSCs that are treated with N3 cytokine combination started to differentiate neurally and express early neural markers Nestin and NSE, late neural markers NF and MAP2. For early neural markers, imunofluorescent staining was performed at 6th day and for late markers was performed at 12th day. Neural markers were not expressed in control groups.

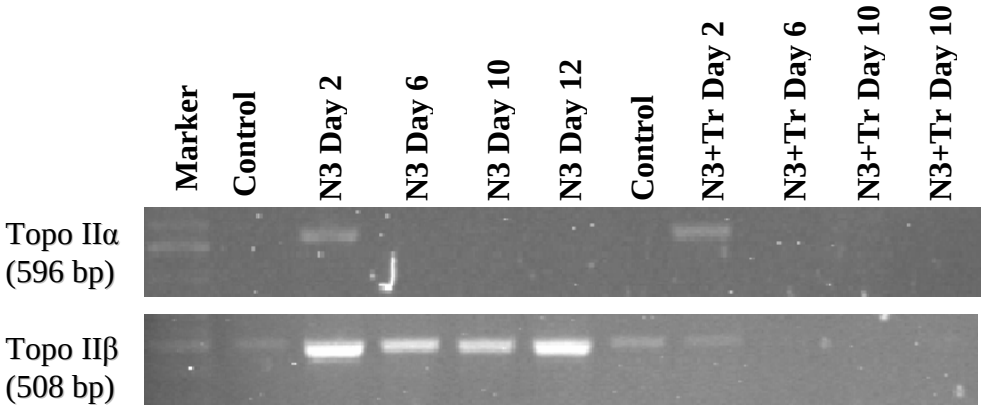
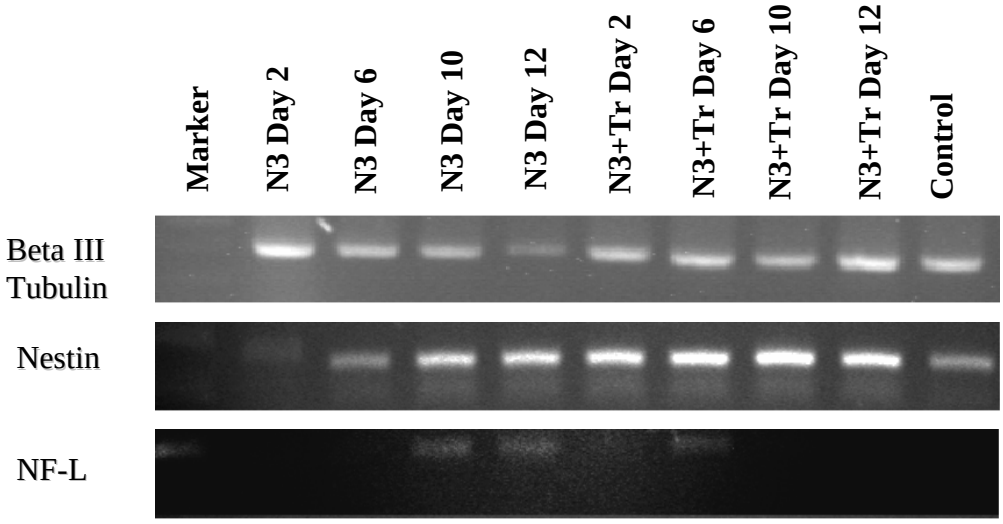


Figure 3.12 RT-PCR analysis for topo IIα (596 bp) and topo IIβ (508 bp). N3 indicates neural differentiaiton group and N3+Tr indicates transfection during neural differentiation group.



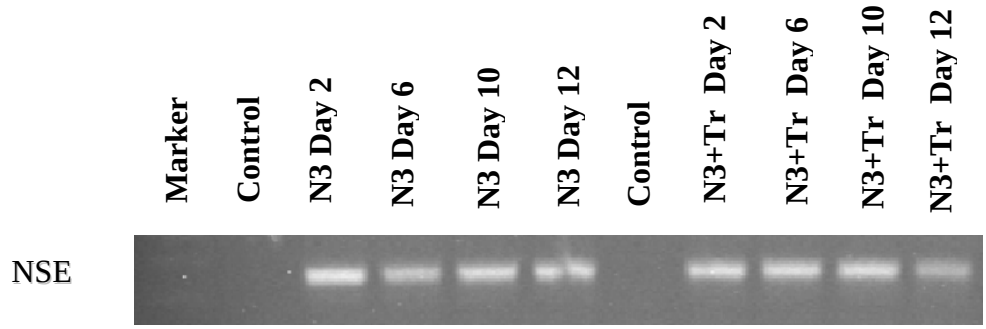


Figure 3.13 RT-PCR analysis for Beta III tubulin, Nestin, NF-L and NSE

According to these results, neural differentiation and transfection during neural differentiation were achieved succesfully. When neural differentiation and transfection during neural differentiation groups were compared, level of topo IIα increases at day 2 according to control group and decreases at other days. It was shown that, level of topo IIβ increased at N3 group and decreased at N3+Tr group so transfection was obtained succesfully. And early neural markers; Beta III tubulin, Nestin and NSE, late neural marker NF-L were expressed during neural differentiation. NF-L was not expressed at day 2 and 6 but started to express at day 10 and 12 for N3 group.

3.5 MICROARRAY RESULTS

3.5.1.Quality Control

After RNA of samples were isolated, all samples underwent a quality check to determine the quality and the quantity of the sample RNA. The quality control was done with the Agilent 2100 Bioanalyzer, using the RNA 6000 Nano Kit according to the manufacturer's instructions. Within the resulting electropherogram, high quality RNA was characterized by two distinct bands, representing 18 and 28S rRNA. In this experiment there are three different groups. The first group is control group which is consist of RNA samples of untreated hMSCs and represented by 1-1, 1-2, 1-3. The second group is RNA of hMSCs which are treated with N3 cytokine combinaton and this group contains 2-1, 2-2 and 2-3. Final group is group 3 and consist of RNAs of

hMSCs that aret treated with N3 cytokine combination and at the same time transfected with topo IIβ specific siRNA.3-1, 3-2 and 3-3 are members of this group.

Table 3.2 *OD*260/280 of the samples

Sample	$OD_{260/280}$	
1-1	2.1	✔
1-2	2.1	✔
1-3	2.1	✔
2-1	2.1	✔
2-2	2.1	✔
2-3	2.1	✔
3-1	2.1	✔
3-2	2.1	✔
3-3	2.1	✔

The quality of samples is very well and thus they passed quality check.

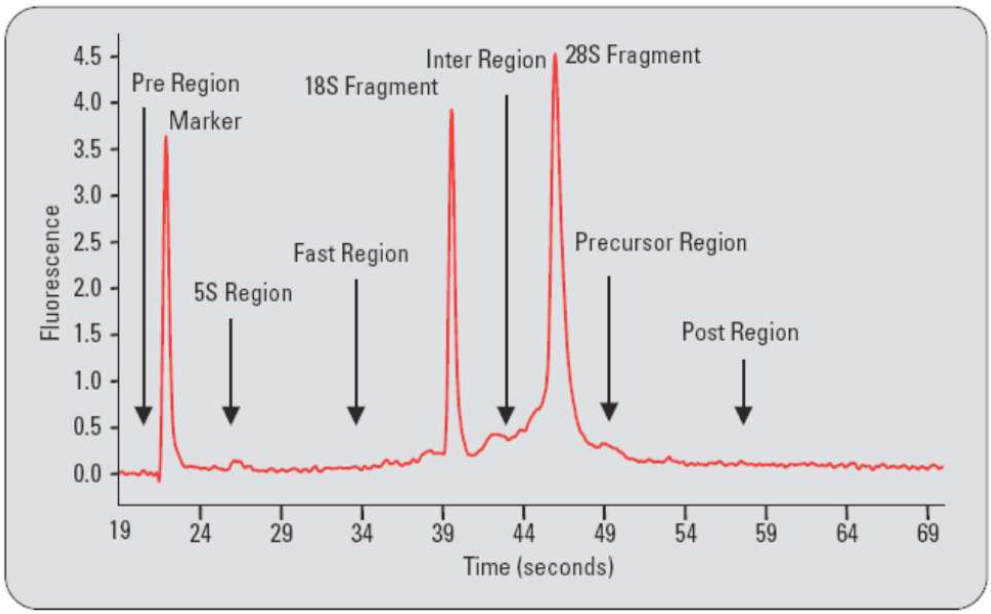


Figure 3.14 Electropherogram shows high quality RNA. High quality RNA is characterized by two distinct bands, representing the 18 and 28S rRNA.

3.5.2 Bioinformatics analysis

3.5.2.1 Properties of raw data

Properties of the raw data were presented in Table 3.3. As the tables show, we have only about 0.04% flagged values.

Table 3.3 Summary on raw data

	1-1	1-2	1-3	2-1	2-2	2-3	3-1	3-2	3-3
mean intensity	3187	3713	2970	2359	2986	5315	3469	6085	4521
sd intensity	6631	7766	5873	4883	5491	9143	6568	9945	8469
mean bg int.	640	664	736	808	1224	592	464	616	1248
sd bg. int.	247	231	247	375	523	294	106	198	228
present calls	9965	9286	7183	8885	9262	12351	10832	12796	11647
present calls %	43	40	31	39	40	54	47	56	51
flagged	0	0	90	102	108	0	0	0	0

3.5.2.2 Spatial distribution of raw data

To evaluate the distribution of signal on the biochips, we computed a spatial distribution plot. Such plots show the reactivity of each transcript of the 8 arrays at the current position of each transcript. As figure 3.15 clearly shows, a slight intensity gradient of the raw data can be observed. To adjust for this gradient, a global background subtraction was carried out i.e., for each array the global background was computed and subtracted from the measured intensity ratios. As Figure 3.16 outlines, the applied background subtraction minimized thespatial effects. We carried out all further analyses using the local background corrected data.

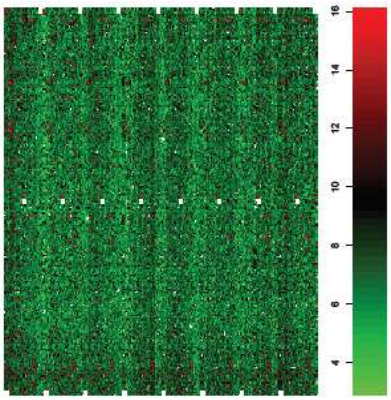


Figure 3.15 Spatial distribution of measured raw data

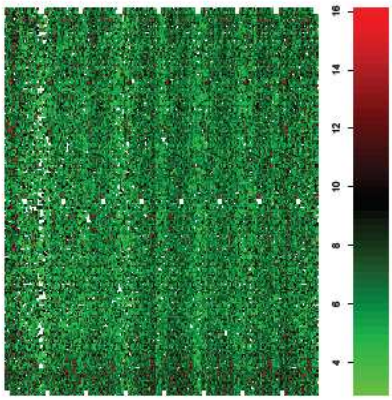


Figure 3.16 Spatial distribution of local background subtracted data

3.5.2.3 Normalization of background corrected raw data

Normalization was used to understand variations between the hybridized arrays. The mean-versus-standard deviation plot in Figure 3.19, the boxplot (Figure 3.17) and violin-plot (Figure 3.18) of the raw data provide evidence that the normalization of these data wasrequired. After normalization, the variance did not show any dependancy on the meanvalue anymore (Figure 3.22). Likewise, Figures 3.20 and 3.21 clearly presented the positive effect of the normalization on the data distribution.

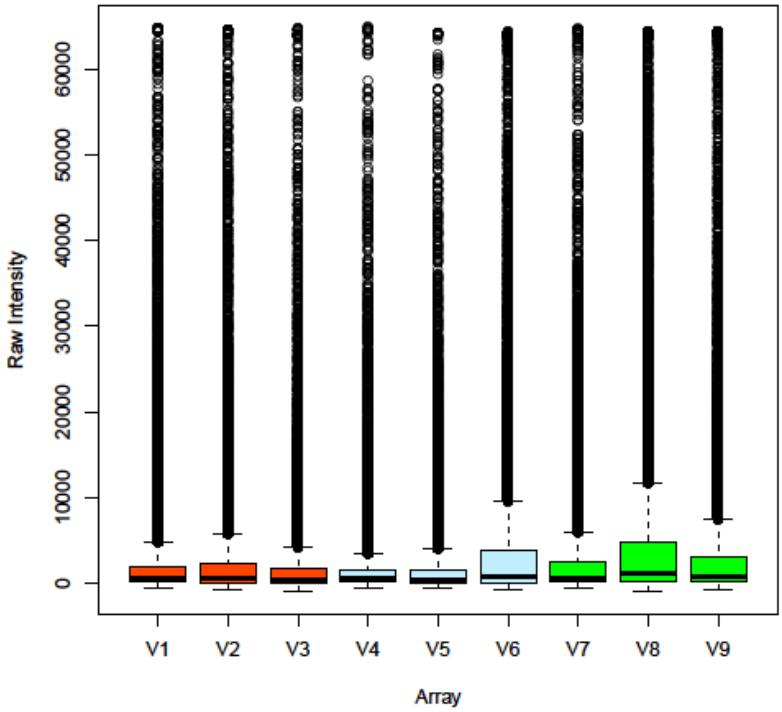


Figure 3.17 Boxplot of raw data

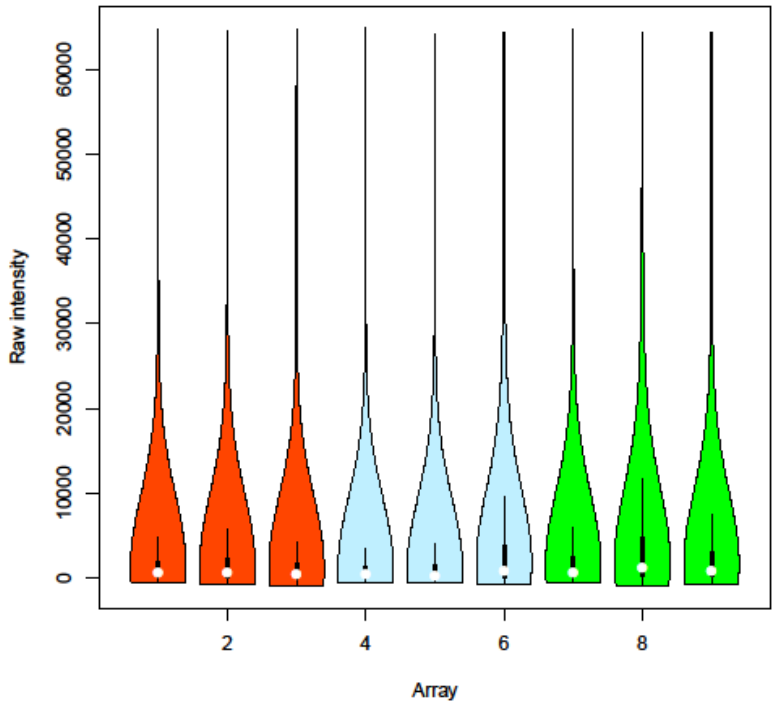


Figure 3.18 Violinplot of raw data

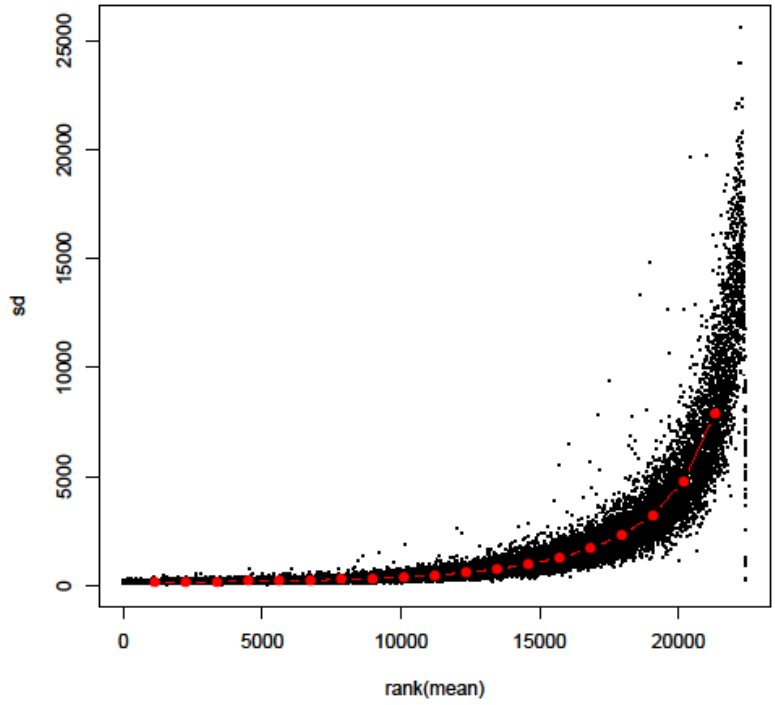


Figure 3.19 Mean-SD plot of raw data

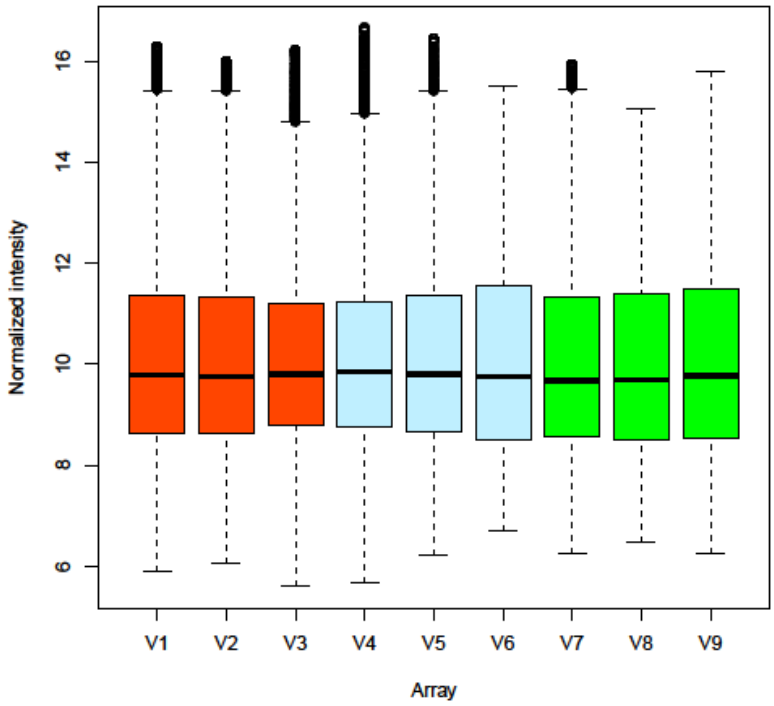


Figure 3.20 Boxplot of vsn normalized data

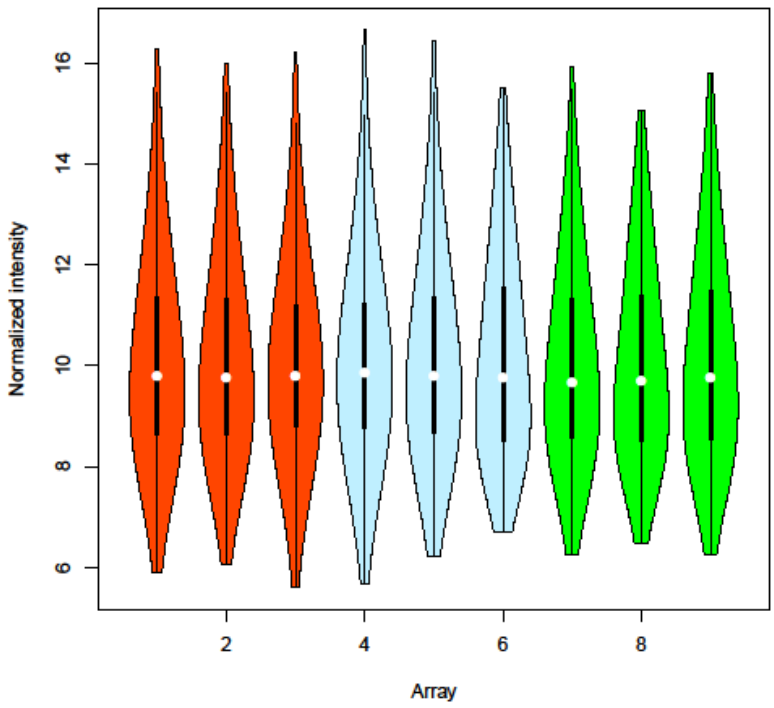


Figure 3.21 Violinplot of vsn normalized data

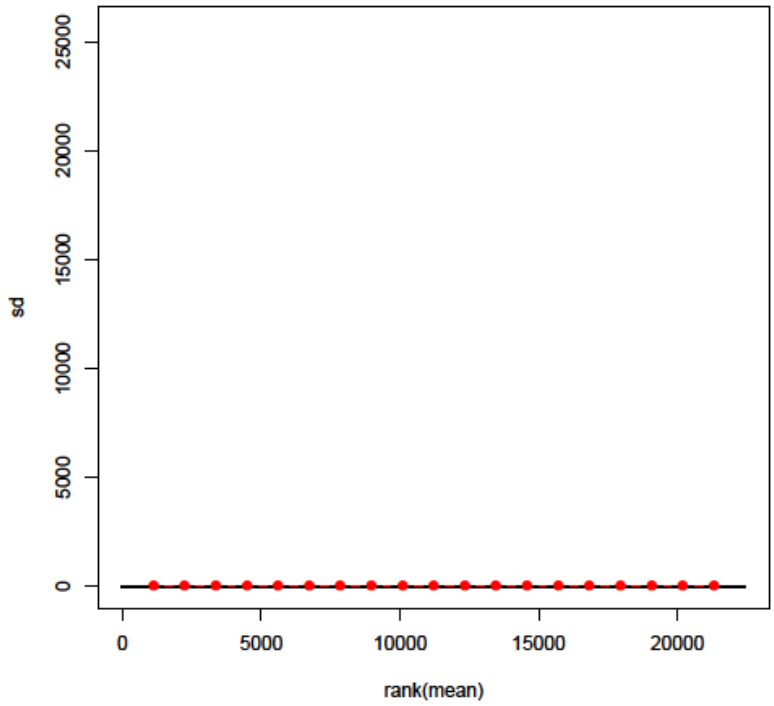


Figure 3.22 Mean-SD plot of vsn normalized data

3.5.2.4 Correlation Analysis

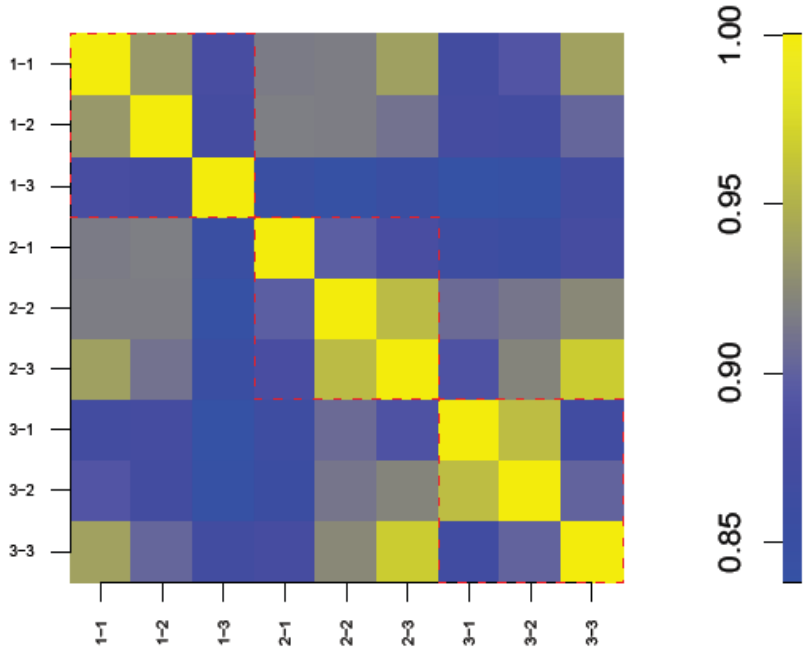


Figure 3.23 Correlation matrix. In this correlation matrix, the correlation of all probes is shown. Biological replicates are enclosed by red dashed boxes.

According to correlation results, in the same group 1-1 and 1-2, 2-2 and 2-3, 3-1 and 3-2 show high correlation. When different groups are compared, 1-1 and 2-3,1-1 and 3-3, 2-2 and 3-3 show high correlation.

3.5.2.5 Scatter Plots

To compare intensity values of two arrays, scatter plots have a role. In general, X axis show the intensity of each probe in one array and Y axis show the second. In this graphs, there is a black line that denotes the angle bisector. If all results are enough good, all points of technical replicates would lie on this line. Points that are far away from this line correspond to deregulated mRNAs. Also there is another red line which shows a regression line computed by a least squares fit. If red line fits well to the black line, it means that results are good. Also in results there is blue shaded area, corresponding to a density estimation of measured datapoints.

In the first third plots, there are examples which have the highest correlation in the groups Group1 and Group 2 (0.956 in Figure 3.24, 0.934 in Figure 3.25, 0.898 in Figure 3.26). The next two plots show the examples with the lowest correlation between these groups (0.839 in Figure 3.27, 0.841 in Figure 3.28).

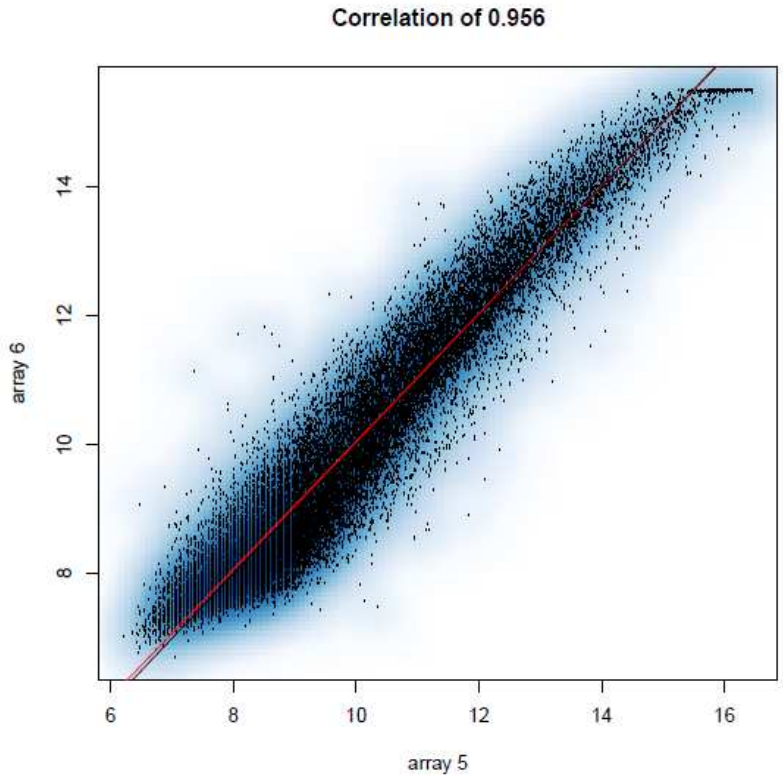


Figure 3.24 Scatterplot of sample 2-2 and sample 2-3

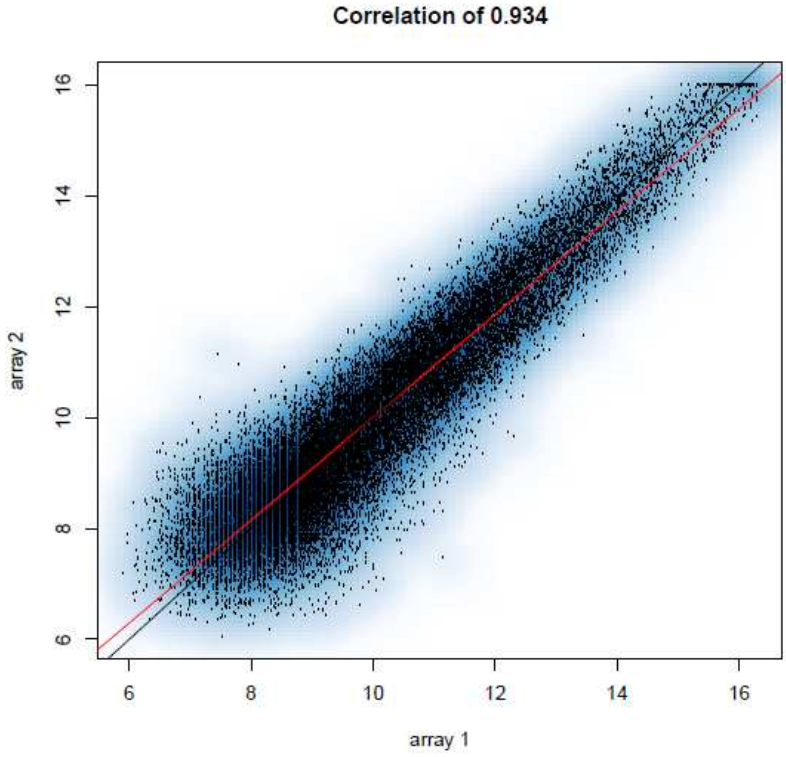


Figure 3.25 Scatterplot of sample 1-1 and sample 1-2

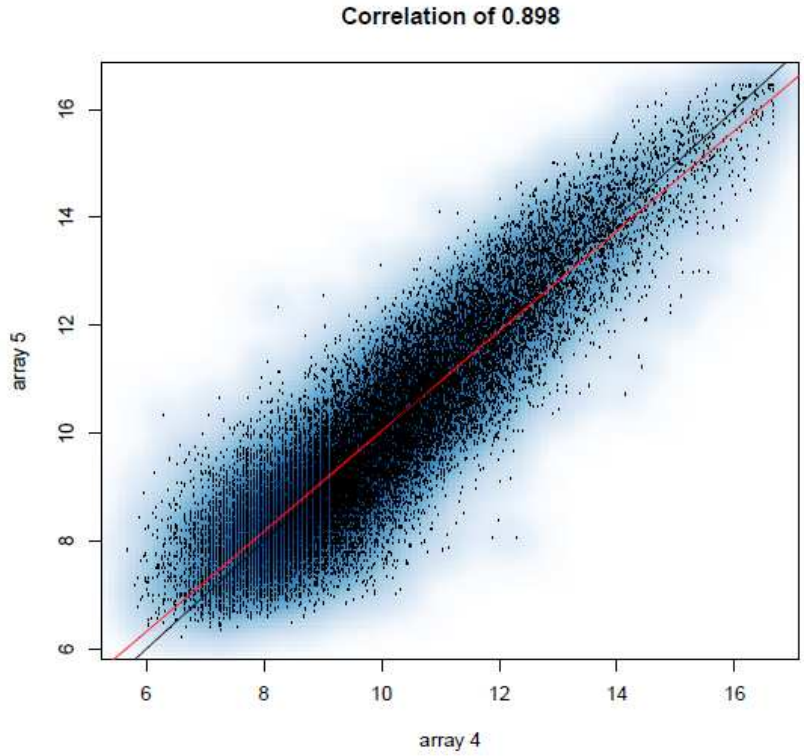


Figure 3.26 Scatterplot of sample 2-1 and sample 2-2

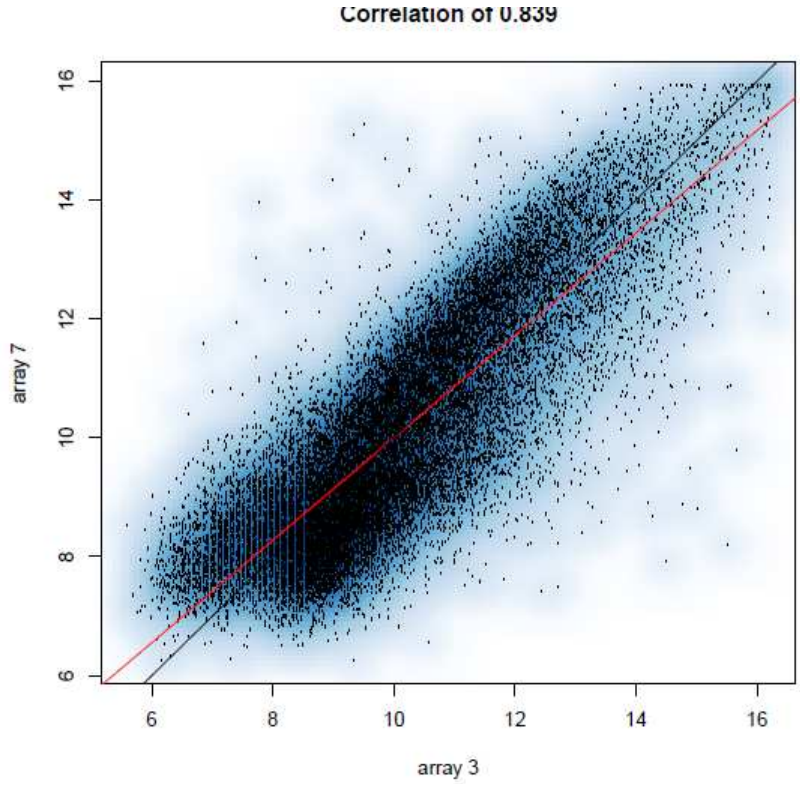


Figure 3.27 Scatterplot of sample 1-3 and sample 3-1

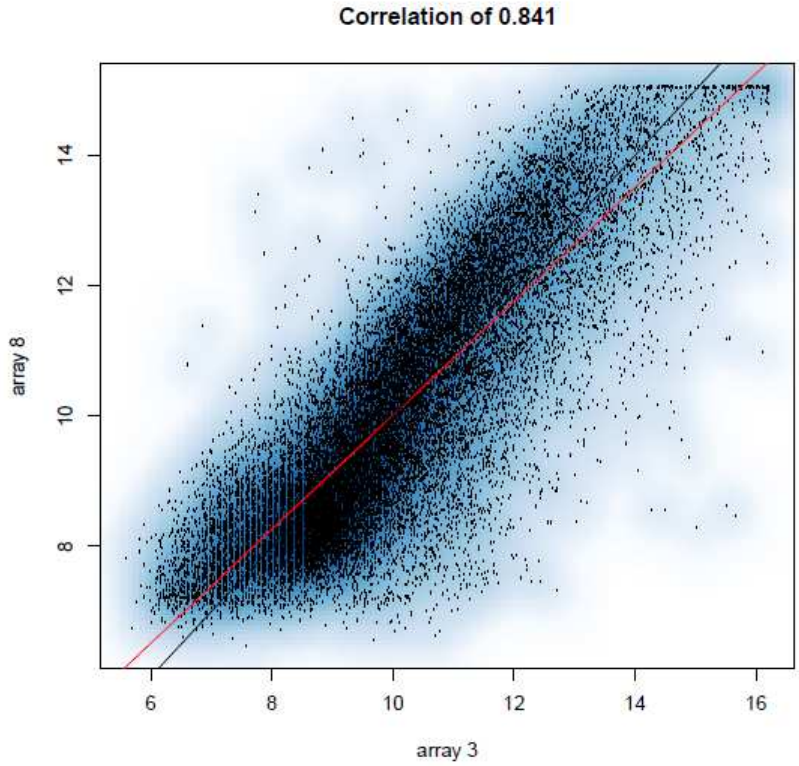


Figure 3.28 Scatterplot of sample 1-3 and sample 3-2

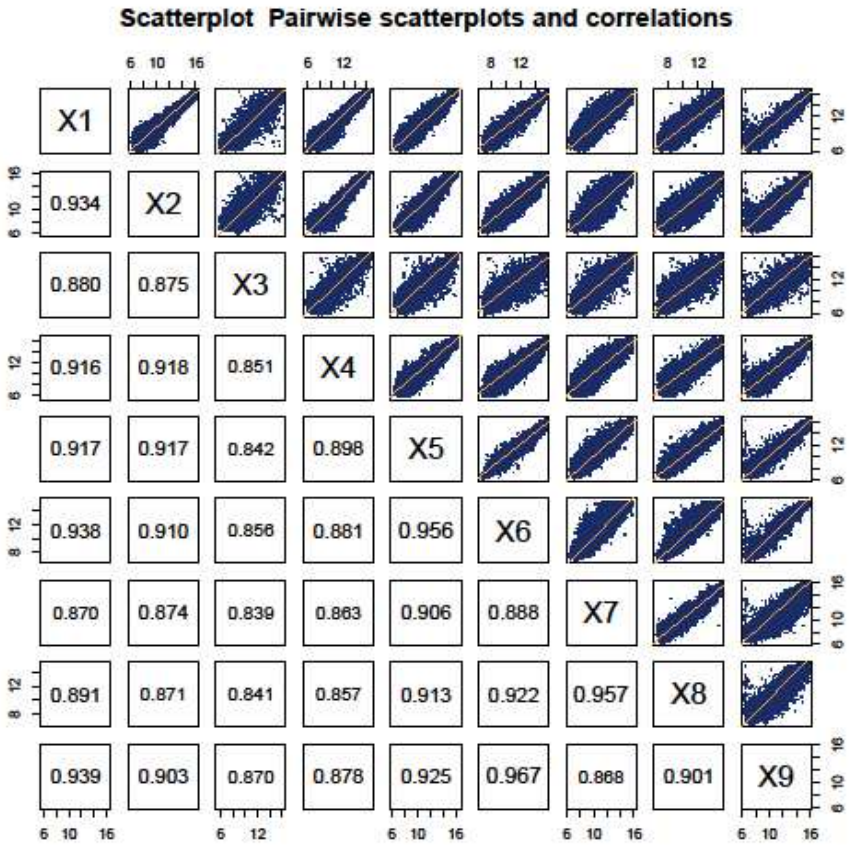


Figure 3.29 shows scatter plots and correlation results of all samples. In this figure

X1:1-1, X2:1-2, X3:1-3, X4:2-1, X5:2-2, X6:2-3, X7:3-1, X8:3-2, X9:3-3 are represented.

3.5.2.6 Cluster analysis

Samples that are similar to each other and have similar intensity values are anlayzed by cluster anlaysis. To detect possible clusters in rows (transcripts) and columns (samples) of the normalized expression matrix a hierarchical clustering was used. For this analysis, 40 mRNAs with highest overall variability were anlayzed As the dendrogram on top of the expression matrix demonstrates, the samples 3-1, 3-2 and 1-1, 1-2 and 2-1, 2-2, 2-3 cluster together (Figure 3.30).

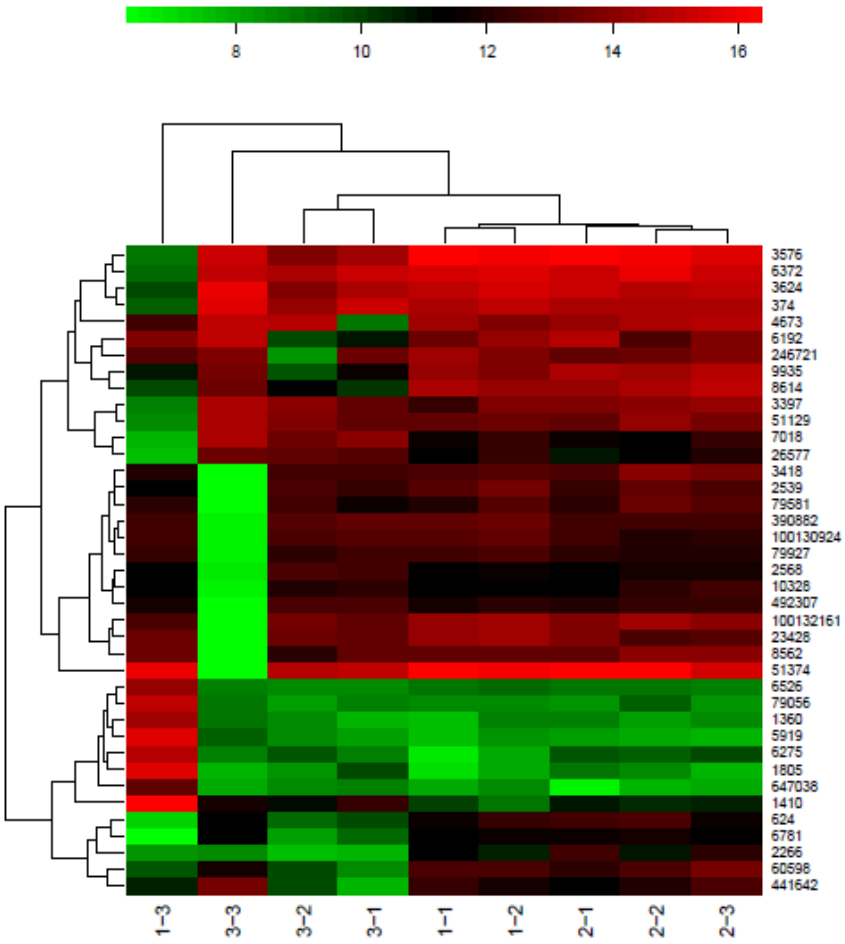


Figure 3.30 Hierarchical cluster heatmap

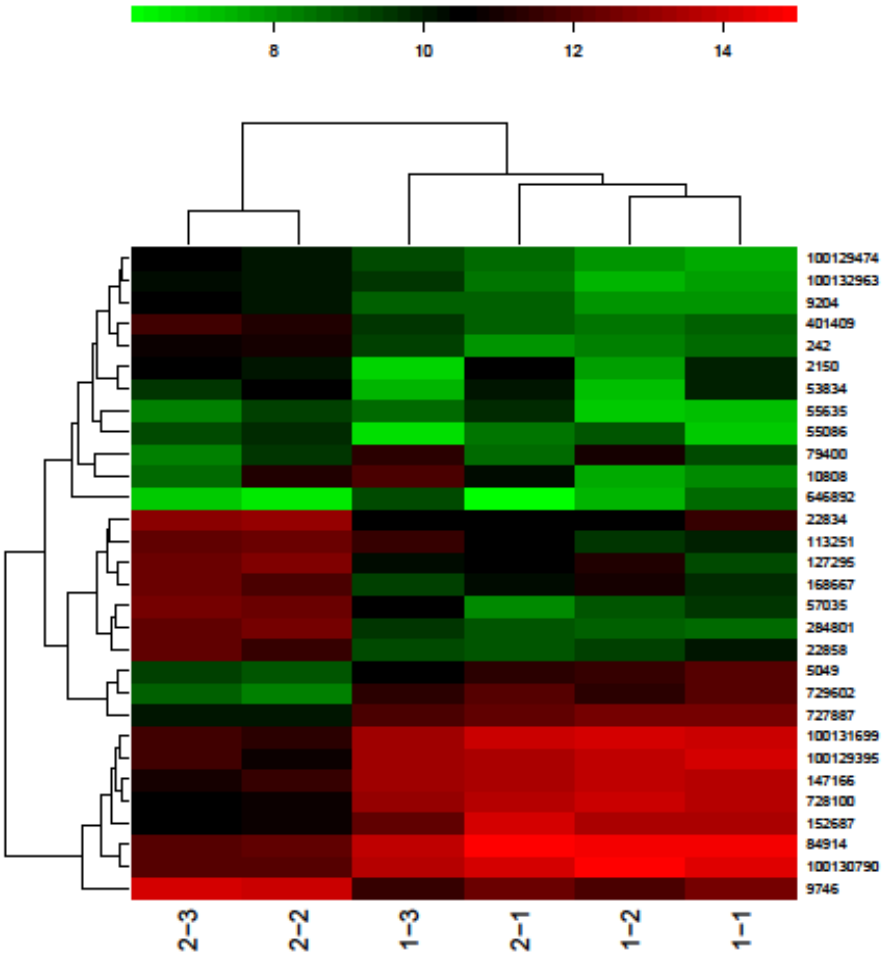


Figure 3.31 Hierarchical cluster heatmap of Group1-Group2 when most variable 30 mRNAs were compared. 2-3 and 2-2, 1-2 and 1-1 were clustered together.

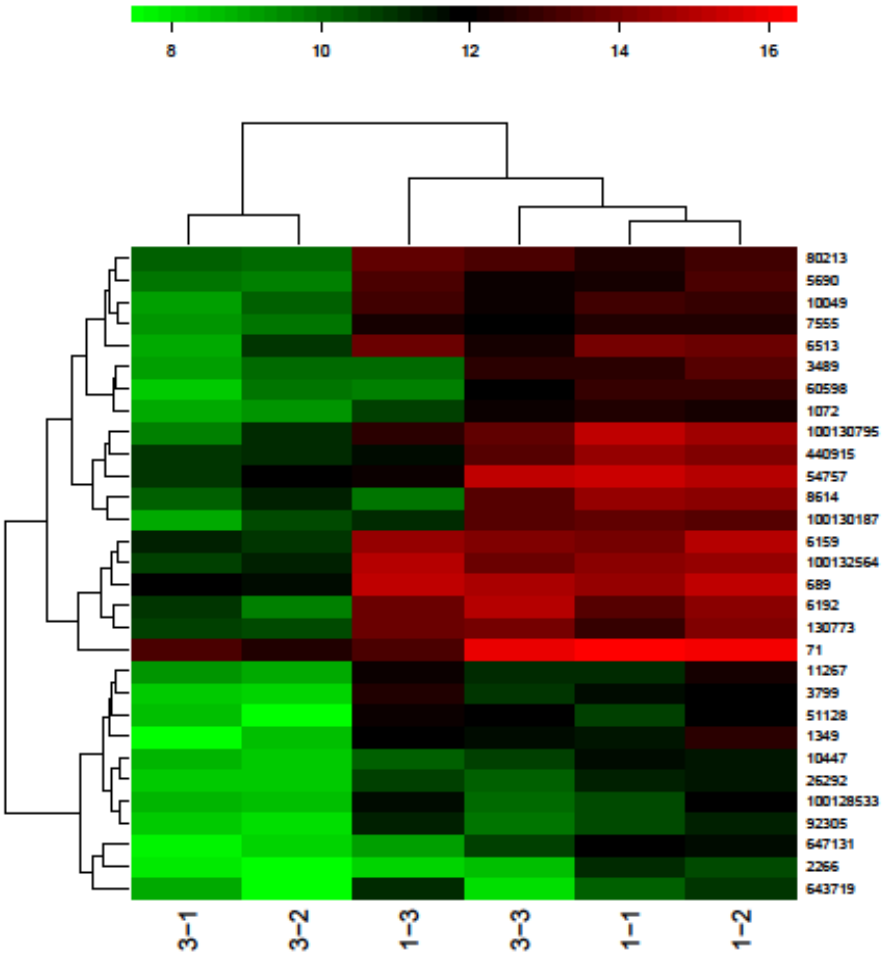


Figure 3.32 Hierarchical cluster heatmap of Group1-Group3 when most variable 30 mRNAs were compared. 3-1 and 3-2, 1-1 and 1-2 were clustered together.

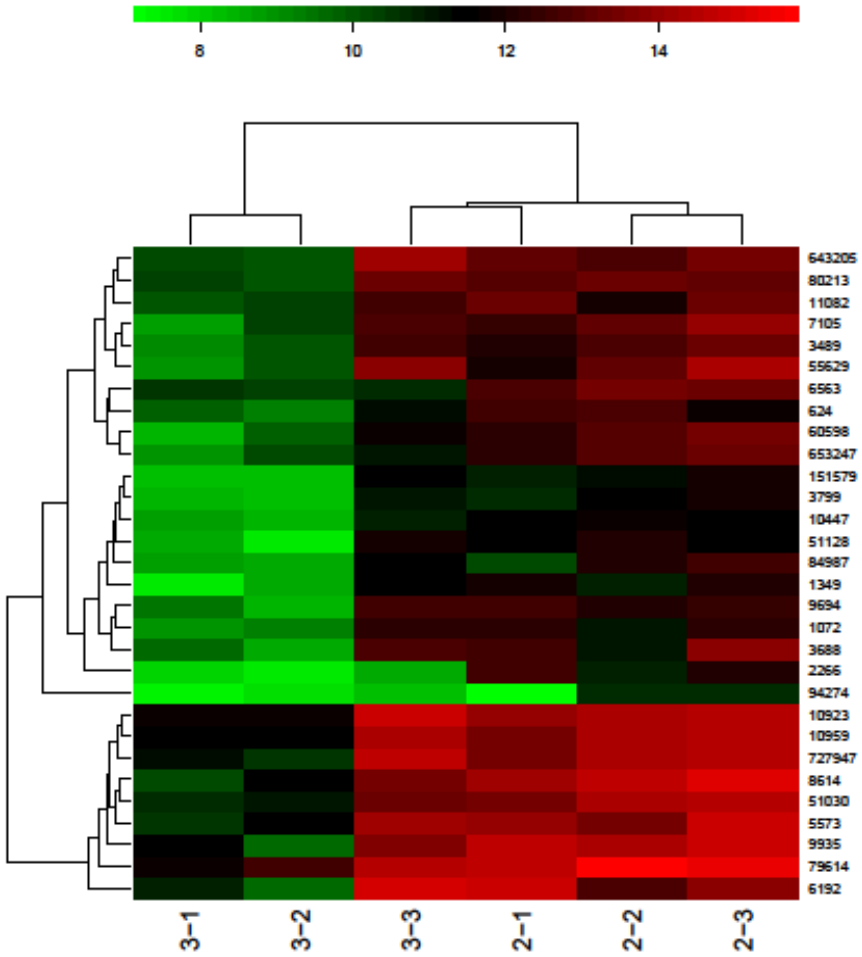


Figure 3.33 Hierarchical cluster heatmap of Group2-Group3 when most variable 30 mRNAs were compared. 3-1 and 3-2, 2-2 and 2-3 were clustered together.

3.5.2.7 Venn Diagrams

Venn diagrams graphically represent the overlap of different sets. Figure 3.33 shows Venn diagrams for samples 1 and 2. Here it can be seen that both arrays show a large overlap of 8297 mRNA features while sample 1 showed another 1668 and sample 2 989 features. In Figure 3.34, it shows the Venn diagram for samples 5 and 6. Here, it can be seen that both arrays show a similar overlap of 9165 mRNA features only and while sample 5 showed another 97 features, sample 6 showed an additional 3186 features. In Figure 3.35 shows the Venn diagram for samples 7 and 8. Both arrays show a similar overlap of 10685 mRNA features only and while sample 7 showed another 147 features, sample 8 showed an additional 2111 features.

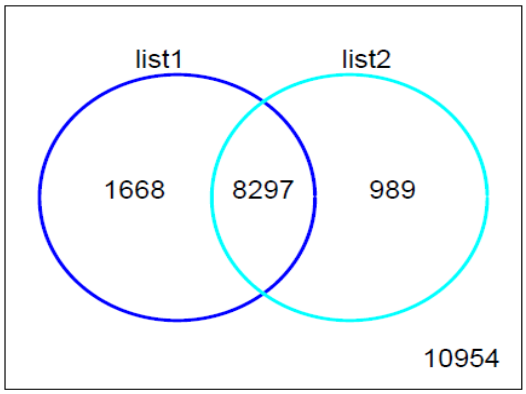


Figure 3.34 Venn diagram sample 1 and sample 2 (1-1 and 1-2)

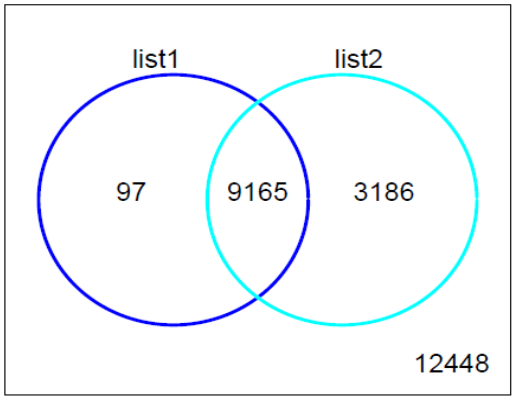


Figure 3.35 Venn diagram sample 5 and sample 6 (2-2 and 2-3)

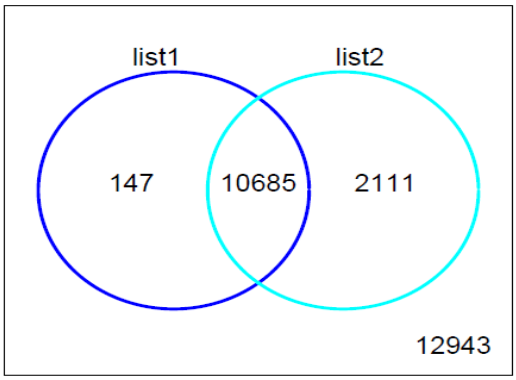


Figure 3.36 Venn diagram sample 7 and sample 8 (3-1 and 3-2)

3.5.2.8 Variance-related Boxplots

mRNAs that show a low variance in biological groups while the variance between groups should be rather high are interesting part of the microarray experiment. Thereby, transcripts that have low variance in the two biological groups (Group 1 and Group 2 versus all other samples) but an overall high variance were detected. Four examples of representative barplots were shown in Figure 3.37 (284801), Figure 3.38 (3799) and Figure 3.39 (2266).

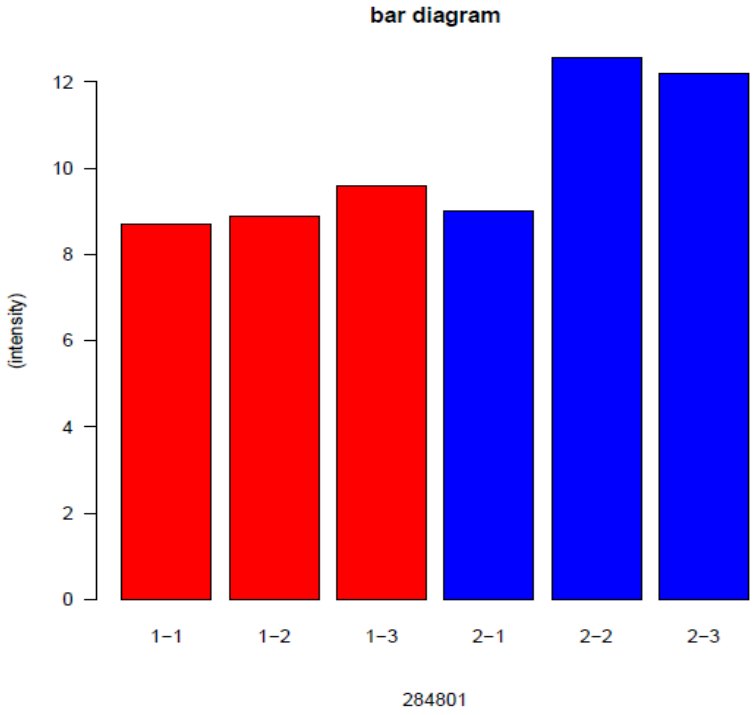


Figure 3.37 Barplot 284801 (Group 1 vs. Group 2)

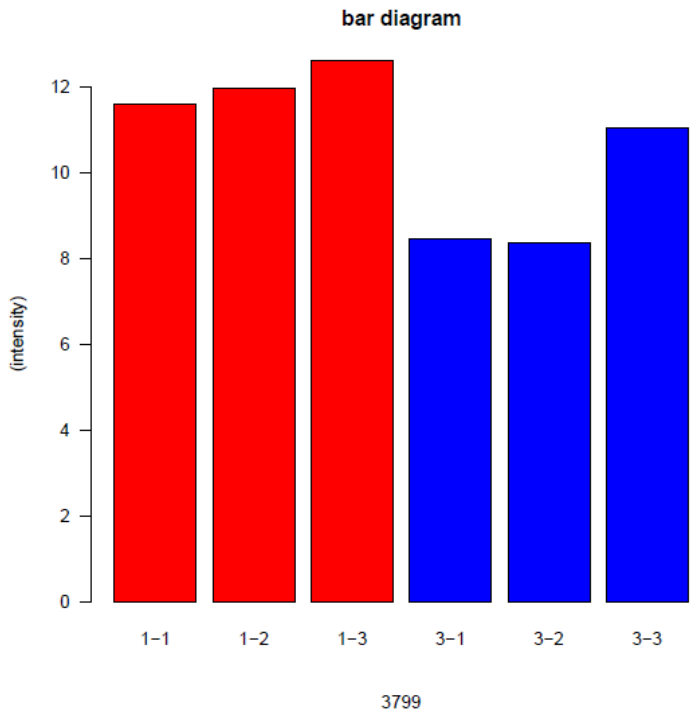


Figure 3.38 Barplot 3799 (Group 1 vs. Group 3)

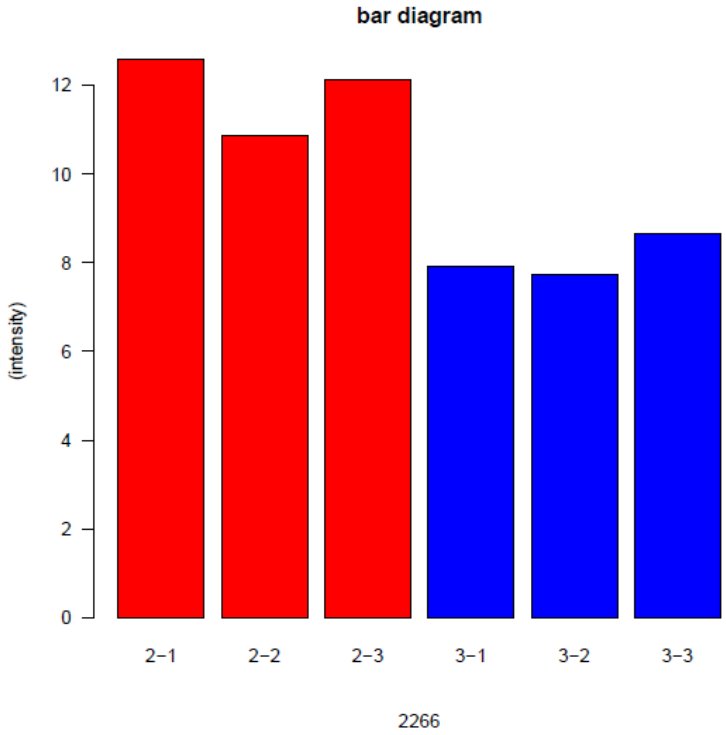


Figure 3.39 Barplot 2266 (Group 2 vs. Group 3)

3.5.2.9 Detection of differentially expressed mRNAs

To detect differentially expressed mRNAs, different approaches are used such as quotation of mean, median, or variance, parametric t-test, non-parametric Wilcoxon-Mann-Whitney test, Empirical Bayes Statistics and the area under the receiver operator characteristics curve. To understand if mRNAs are downregulated or upregulated, fold change value which is shown by" logqmedian" was used. The results below are the highest 60 fold change value for each comparison. 30 of them are positive value which represent upregulated mRNAs and the other 30 are negative values that show downregulated mRNAs. Comparisons between groups were done as Group1 vs Group 2, Group1 vs Group 3, Group2 vs Group 3.

When Group 1 and Group 2 were compared it was found that SIX homeobox 3 gene responsible for brain development increases and zinc finger protein 541 responsible for cell differentiaiton increaes. But fibroblast growth factor receptor-like 1 that has role in regulation of cell growth decreases. These results confirmed the previous results.

According to results in Group 1 vs Group 2, cytochrome c oxidase subunit VIIb gene that has a role in cytochrome c activity increases and snail homolog 1 gene responsible for positive regulation of epithelial to mesenchymal transition decreases.The graph below shows the explanation of terms used in following tables.

Variable	Explanation
Qmed	Median fold change
ttest rawp	Raw pvalue of test
ttest adjp	Adjusted pvalue of test
limma rawp	Raw pvalue using emprical bayes

Table 3.4 Differentially regulated mRNAs Group 1 vs Group 2

mRNA	med g1	med g2	Qmed	logqmed	ttest rawp	ttest adjp	limma rawp	Limma adjp
728100	13.661	10.885	16.058	2.776	0.160	0.855	0.007	0.720
152687	13.358	10.726	13.900	2.632	0.990	0.997	0.123	0.747
84914	14.640	12.227	11.165	2.413	0.529	0.861	0.051	0.720
729602	11.330	8.923	11.106	2.407	0.452	0.855	0.019	0.720
79400	11.029	8.636	10.948	2.393	0.180	0.855	0.008	0.720
727887	12.466	10.090	10.759	2.376	0.142	0.855	0.013	0.720
646892	8.803	6.577	9.261	2.226	0.153	0.855	0.001	0.720
100129395	13.851	11.633	9.190	2.218	0.076	0.855	0.004	0.720
100131699	13.919	11.743	8.810	2.176	0.220	0.855	0.020	0.720
100130790	14.253	12.091	8.693	2.162	0.172	0.855	0.015	0.720
5049	11.584	9.433	8.592	2.151	0.190	0.855	0.017	0.720
147166	13.545	11.436	8.236	2.108	0.135	0.855	0.011	0.720
5334	10.743	8.690	7.789	2.053	0.164	0.855	0.009	0.720
145773	10.244	8.193	7.772	2.050	0.155	0.855	0.003	0.720
11117	10.137	8.150	7.296	1.987	0.178	0.855	0.011	0.720
6496	10.259	8.282	7.222	1.977	0.017	0.855	0.001	0.720
63906	12.114	10.139	7.212	1.976	0.142	0.855	0.017	0.720
57093	9.360	7.390	7.171	1.970	0.253	0.855	0.110	0.743
84215	11.249	9.306	6.984	1.944	0.136	0.855	0.005	0.720
6513	13.766	11.843	6.841	1.923	0.008	0.855	0.002	0.720
731789	8.693	6.783	6.753	1.910	0.163	0.855	0.013	0.720
646813	9.053	7.151	6.705	1.903	0.179	0.855	0.025	0.720
441687	9.396	7.512	6.578	1.884	0.156	0.855	0.019	0.720
54474	8.989	7.112	6.536	1.877	0.286	0.855	0.020	0.720
57546	10.853	8.977	6.522	1.875	0.126	0.855	0.002	0.720
22955	11.993	10.127	6.465	1.866	0.208	0.855	0.047	0.720
643719	10.990	9.136	6.381	1.853	0.065	0.855	0.000	0.720
8450	10.285	8.449	6.273	1.836	0.051	0.855	0.004	0.720
8412	11.018	9.186	6.243	1.831	0.255	0.855	0.008	0.720
100130768	12.487	10.658	6.226	1.829	0.373	0.855	0.048	0.720
284801	8.897	12.189	0.037	-3.292	0.199	0.855	0.005	0.720
57035	9.580	12.345	0.063	-2.765	0.218	0.855	0.113	0.744
2150	7.790	10.466	0.069	-2.676	0.043	0.855	0.002	0.720
53834	7.425	10.067	0.071	-2.641	0.103	0.855	0.005	0.720
22834	10.532	12.901	0.094	-2.369	0.205	0.855	0.033	0.720
113251	9.866	12.214	0.096	-2.347	0.201	0.855	0.030	0.720
401409	8.881	11.186	0.100	-2.305	0.213	0.855	0.013	0.720
127295	10.205	12.437	0.107	-2.232	0.182	0.855	0.008	0.720
100132963	7.887	10.068	0.113	-2.182	0.220	0.855	0.034	0.720
22858	9.376	11.531	0.116	-2.155	0.250	0.855	0.056	0.720
10808	8.233	10.365	0.119	-2.133	0.788	0.936	0.338	0.826
100129474	7.966	10.097	0.119	-2.130	0.193	0.855	0.012	0.720
9204	8.063	10.193	0.119	-2.130	0.133	0.855	0.004	0.720
55635	7.344	9.460	0.120	-2.116	0.135	0.855	0.011	0.720
168667	9.822	11.936	0.121	-2.114	0.203	0.855	0.015	0.720
9746	11.808	13.907	0.123	-2.099	0.164	0.855	0.007	0.720
55086	7.237	9.307	0.126	-2.069	0.189	0.855	0.014	0.720
242	8.748	10.808	0.127	-2.060	0.232	0.855	0.109	0.743
154386	6.591	8.649	0.128	-2.058	0.256	0.855	0.007	0.720
130	9.764	11.791	0.132	-2.027	0.104	0.855	0.004	0.720
148766	7.779	9.801	0.132	-2.023	0.076	0.855	0.001	0.720
4793	9.653	11.674	0.133	-2.020	0.270	0.855	0.091	0.737
10350	8.966	10.984	0.133	-2.018	0.042	0.855	0.002	0.720
221458	8.606	10.598	0.136	-1.992	0.048	0.855	0.001	0.720
1952	9.226	11.216	0.137	-1.990	0.171	0.855	0.010	0.720
344191	7.970	9.927	0.141	-1.956	0.217	0.855	0.001	0.720
6399	8.665	10.618	0.142	-1.954	0.060	0.855	0.001	0.720
9757	8.745	10.688	0.143	-1.943	0.199	0.855	0.026	0.720
58478	10.440	12.381	0.144	-1.941	0.204	0.855	0.046	0.720

Table 3.5 shows Gene IDs, Gene description and Gene ontology of Group 1 vs Group 2

mRNA	Gene description	Gene Ontology	logqmed
728100	hypothetical LOC728100		2.776
152687	zinc finger protein 595	DNA binding,metal ion binding,zinc ion binding,regulation of transcription DNA-dependent	2.632
84914	zinc finger protein 587	DNA binding,metal ion binding,zinc ion binding,regulation of transcription DNA-dependent	2.413
729602	nuclear pore complex interacting protein pseudogene		2.407
79400	NADPH oxidase, EF-hand calcium binding domain	FAD binding,NADP or NADPH binding,calcium ion binding,electron carrier activity,heme binding,hydrogen ion channel activity,ion channel activity,oxidoreductase activity,superoxide-generating NADPH oxidase activity,cell proliferation,cytokine secretion,cytokinesis, electron transport chain, induction of apoptosis,ion transport, oxidation reduction, regulation of fusion of sperm to egg plasma membrane,regulation of proton transport,superoxide anion generation	2.393
727887	hypothetical LOC727887		2.376
646892	SH2 domain containing 7	protein binding	2.226
100129395	NS5ATP13TP1		2.218
100131699	hypothetical LOC100131699		2.176
100130790	hypothetical LOC100130790		2.162
5049	platelet-activating factor acetylhydrolase 1b, catalytic subunit 2 (30kDa)	1-alkyl-2-acetylglycerophosphocholine esterase activity, hydrolase activity, acting on ester bonds,protein binding,lipid catabolic process,lipid metabolic process,spermatogenesis	2.151
147166	tripartite motif-containing 16-like		2.108
5334	phospholipase C-like 1	calcium ion binding, phosphoinositide phospholipase C activity,phospholipase C activity,signal transducer activity,behavior,intracellular signaling pathway,lipid metabolic process	2.053
145773	family with sequence similarity 81, member A		2.050
11117	elastin microfibril interfacer 1	extracellular matrix constituent conferring elasticity,identical protein binding,protein binding,cell adhesion,extracellular matrix organization,positive regulation of cell-substrate adhesion	1.987
6496	SIX homeobox 3	RNA polymerase II transcription factor activity, enhancer binding,receptor binding,sequence-specific DNA binding,transcription repressor activity,brain development,camera-type eye development,diencephalon development,forebrain anterior/posterior pattern formation,lens induction in camera-type eye,multicellular organismal development,negative regulation of Wnt receptor signaling pathway,negative regulation of transcription, DNA-dependent,positive regulation of transcription from RNA polymerase II promoter, protein import into nucleus,telencephalon development,visual perception	1.977

Table 3.5 (continued) shows Gene IDs, Gene description and Gene ontology of Group 1 vs Group 2

63906	G patch domain containing 3	nucleic acid binding	1.976
57093	tripartite motif-containing 49	metal ion binding,protein binding, zinc ion binding	1.970
84215	zinc finger protein 541	metal ion binding,protein binding, zinc ion binding,cell differentiation, multicellular organismal development, regulation of transcription,spermatogenesis	1.944
6513	solute carrier family 2 (facilitated glucose transporter), member 1	D-glucose transmembrane transporter activity,dehydroascorbic acid transporter activity,glucose transmembrane transporter activity,kinase binding,protein binding,substrate-specific transmembrane transporter activity,xenobiotic transporter activity,carbohydrate transport,glucose transport,response to osmotic stress, transmembrane transport	1.923
731789	hypothetical LOC731789		1.910
646813	DEAH (Asp-Glu-Ala-His) box polypeptide 9 pseudogene		1.903
441687	testis expressed 21, pseudogene		1.884
54474	keratin 20	protein binding,structural constituent of cytoskeleton,apoptosis,cellular response to stress,intermediate filament organization,regulation of protein secretion	1.877
57546	pyruvate dehyrogenase phosphatase catalytic subunit 2	[pyruvate dehydrogenase (lipoamide)] phosphatase activity,hydrolase activity,magnesium-dependent protein serine/threonine phosphatase activity,metal ion binding,protein amino acid dephosphorylation,regulation of acetyl-CoA biosynthetic process from pyruvate	1.875
22955	sex comb on midleg homolog 1 (Drosophila)	DNA binding,protein binding,transcription factor activity, transcription repressor activity,anatomical structure morphogenesis,gene silencing,multicellular organismal development ,negative regulation of transcription	1.866
643719	hypothetical LOC643719		1.853
8450	cullin 4B	Protein binding,ubiquitin protein ligase binding,DNA repair,cell cycle, ubiquitin-dependent protein catabolic process	1.836
8412	breast cancer anti-estrogen resistance 3	guanyl-nucleotide exchange factor activity,protein binding,response to drug,signal transduction,small GTPase mediated signal transduction	1.831
100130768	hypothetical LOC100130768		1.829
284801	hypothetical protein LOC284801		-3.292
57035	chromosome 1 open reading frame 63		-2.765
2150	coagulation factor II (thrombin) receptor-like 1	G-protein coupled receptor activity,receptor activity,receptor binding,thrombin receptor activity,G-protein coupled receptor protein signaling pathway,elevation of cytosolic calcium ion concentration,positive regulation of leukocyte chemotaxis,positive regulation of positive chemotaxis,regulation of blood coagulation	-2.676

Table 3.5 (continued) shows Gene IDs, Gene description and Gene ontology of Group 1 vs Group 2

53834	fibroblast growth factor receptor-like 1	fibroblast growth factor binding,fibroblast growth factor receptor activity,heparin binding,receptor activity,diaphragm development,heart valve morphogenesis,negative regulation of cell proliferation,regulation of cell growth, skeletal system development,ventricular septum morphogenesis	-2.641
22834	zinc finger protein 652	DNA binding,metal ion binding,protein binding,zinc ion binding,regulation of transcription	-2.369
113251	La ribonucleoprotein domain family, member 4	RNA binding	-2.347
401409	RAB19, member RAS oncogene family	GTP binding,nucleotide binding,protein transport,small GTPase mediated signal transduction	-2.305
127295	ribosomal protein L36 pseudogene 5		-2.232
100132963	hypothetical protein LOC100132963		-2.182
22858	intestinal cell (MAK-like) kinase	ATP binding,cyclin-dependent protein kinase activity, magnesium ion binding, nucleotide binding,protein serine/threonine kinase activity,transferase activity,intracellular protein kinase cascade,multicellular organismal developmen, protein amino acid phosphorylation,signal transduction	-2.155
10808	heat shock 105kDa/110kDa protein 1	ATP binding,nucleotide binding,response to unfolded protein	-2.133
100129474	similar to histone cluster 1,H2aa		-2.130
9204	zinc finger, MYM-type 6	DNA binding,metal ion binding, zinc ion binding,multicellular organismal development	-2.130
55635	DEP domain containing 1	GTPase activator activity,signal transduction	-2.116
168667	BMP binding endothelial regulator	protein binding,blood vessel endothelial cell proliferation involved in sprouting angiogenesis,endothelial cell activation,negative regulation of BMP signaling pathway,positive regulation of ERK1 and ERK2 cascade, regulation of endothelial cell migration, regulation of pathway-restricted SMAD protein phosphorylation,ureteric bud development	-2.114
9746	calsyntenin 3	calcium ion binding,cell adhesion,homophilic cell adhesion	-2.099
55086	chromosome X open reading frame 57		-2.069
242	rachidonate 12-lipoxygenase, 12R type	arachidonate 12-lipoxygenase activity,iron ion binding , lipoxygenase activity,metal ion binding,oxidoreductase activity	-2.060
154386	chromosome 6 open reading frame 195		-2.058

Table 3.5 (continued) shows Gene IDs, Gene description and Gene ontology of Group 1 vs Group 2

130	alcohol dehydrogenase 6 (class V)	alcohol dehydrogenase (NAD) activity,binding,electron carrier activity,metal ion binding,oxidoreductase activity,zinc ion binding,ethanol oxidation,oxidation reduction,response to ethano	-2.027
148766	similar to hCG2045075		-2.023
4793	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor	protein binding,signal transducer activity,transcription coactivator activity,cytoplasmic sequestering of NF-kappaB,signal transduction,transcription	-2.020
10350	ATP-binding cassette, sub-family A (ABC1), member 9	ATP binding,ATPase activity,nucleotide binding,transport	-2.018
221458	kinesin family member 6	ATP binding,microtubule motor activity,nucleotide binding,protein binding,microtubule-based movement	-1.992
1952	cadherin, EGF LAG seven-pass G-type receptor 2	G-protein coupled receptor activity,calcium ion binding, protein binding,G-protein coupled receptor protein signaling pathway,Wnt receptor signaling pathway,cell adhesion, dendrite morphogenesis,homophilic cell adhesion,multicellular organismal development,neural plate anterior/posterior regionalization,neuropeptide signaling pathway,regulation of cell-cell adhesion,regulation of gene-specific transcription,spermatogenesis	-1.990
344191	even-skipped homeobox 2	DNA binding,sequence-specific DNA binding,transcription factor activity,,limb morphogenesis, multicellular organismal development ,regulation of transcription, DNA-dependent	-1.956
6399	trafficking protein particle complex 2	protein binding,transcription factor binding,ER to Golgi vesicle-mediated transport,regulation of transcription, DNA-dependent IDA PubMed skeletal system development,transcription,vesicle-mediated transport	-1.954
9757	myeloid/lymphoid or mixed-lineage leukemia 4	histone methyltransferase activity (H3-K4 specific),metal ion binding,methyltransferase activity, protein binding,transcription factor activity,transferase activity, zinc ion binding,chromatin modification, chromatin-mediated maintenance of transcription,regulation of transcription, DNA-dependent	-1.943
58478	enolase-phosphatase 1	acireductone synthase activity,hydrolase activity,metal ion binding, phosphoglycolate phosphatase activity,L-methionine salvage from methylthioadenosine,cellular amino acid biosynthetic process,methionine biosynthetic process	-1.941

Table 3.6 Differentially regulated mRNAs Group 1 vs Group 3

mRNA	med g1	med g2	Qmed	logqmed	ttest rawp	Ttest adjp	limma rawp	limma adjp
3799	11.956	8.469	32.664	3.486	0.090	0.707	0.001	0.260
100130795	14.652	11.201	31.547	3.451	0.191	0.707	0.010	0.267
647131	11.682	8.408	26.422	3.274	0.209	0.707	0.035	0.333
5690	13.167	9.945	25.077	3.222	0.042	0.707	0.003	0.260
51128	11.808	8.655	23.407	3.153	0.332	0.741	0.031	0.320
6159	14.526	11.387	23.080	3.139	0.091	0.707	0.007	0.260
689	15.172	12.089	21.823	3.083	0.111	0.707	0.012	0.275
1349	11.814	8.759	21.219	3.055	0.157	0.707	0.007	0.260
100132564	14.365	11.363	20.128	3.002	0.065	0.707	0.002	0.260
130773	13.774	10.777	20.030	2.997	0.331	0.740	0.034	0.327
54757	15.038	12.070	19.442	2.967	0.441	0.778	0.167	0.530
60598	12.814	9.847	19.436	2.967	0.225	0.713	0.081	0.419
6192	13.840	10.885	19.204	2.955	0.977	0.994	0.075	0.410
11267	12.263	9.348	18.452	2.915	0.091	0.707	0.004	0.260
1072	12.321	9.407	18.436	2.914	0.311	0.733	0.051	0.364
8614	14.257	11.349	18.313	2.908	0.261	0.719	0.302	0.656
100130187	13.444	10.548	18.097	2.896	0.405	0.765	0.092	0.434
440915	14.028	11.180	17.250	2.848	0.250	0.715	0.071	0.403
6513	13.766	10.956	16.605	2.810	0.001	0.578	0.001	0.260
643719	10.990	8.182	16.565	2.807	0.058	0.707	0.000	0.260
10049	13.022	10.245	16.075	2.777	0.019	0.707	0.002	0.260
100128533	11.581	8.826	15.717	2.755	0.094	0.707	0.001	0.260
10447	11.572	8.826	15.579	2.746	0.130	0.707	0.013	0.275
3489	12.793	10.059	15.405	2.735	0.342	0.745	0.151	0.508
71	15.998	13.279	15.163	2.719	0.345	0.747	0.165	0.526
7555	12.573	9.872	14.906	2.702	0.032	0.707	0.004	0.260
2266	10.617	7.921	14.807	2.695	0.204	0.707	0.010	0.267
92305	11.304	8.610	14.796	2.694	0.043	0.707	0.001	0.260
26292	11.301	8.610	14.749	2.691	0.041	0.707	0.001	0.260
80213	12.996	10.336	14.302	2.660	0.279	0.724	0.028	0.314
284260	7.768	10.264	0.082	-2.496	0.223	0.712	0.009	0.266
100131563	12.165	14.649	0.083	-2.484	0.170	0.707	0.010	0.267
7018	11.552	13.972	0.089	-2.420	0.114	0.707	0.004	0.260
57228	8.665	11.039	0.093	-2.375	0.043	0.707	0.006	0.260
4597	10.206	12.554	0.096	-2.348	0.019	0.707	0.000	0.260
5903	8.829	11.151	0.098	-2.322	0.074	0.707	0.001	0.260
4222	9.560	11.879	0.098	-2.319	0.055	0.707	0.001	0.260
127295	10.205	12.510	0.100	-2.305	0.195	0.707	0.038	0.338
53834	7.425	9.724	0.100	-2.299	0.362	0.754	0.086	0.425
286205	7.849	10.086	0.107	-2.237	0.164	0.707	0.019	0.289
10131	8.166	10.331	0.115	-2.165	0.035	0.707	0.003	0.260
1260	10.146	12.299	0.116	-2.153	0.273	0.723	0.004	0.260
53826	9.434	11.572	0.118	-2.138	0.083	0.707	0.000	0.260
100129523	11.519	13.652	0.118	-2.134	0.148	0.707	0.006	0.260
728320	7.611	9.729	0.120	-2.118	0.097	0.707	0.016	0.280
644090	10.531	12.642	0.121	-2.111	0.182	0.707	0.015	0.278
22834	10.532	12.641	0.121	-2.108	0.061	0.707	0.002	0.260
221458	8.606	10.708	0.122	-2.102	0.015	0.691	0.001	0.260
6615	8.989	11.089	0.123	-2.100	0.134	0.707	0.001	0.260
6990	9.286	11.374	0.124	-2.089	0.073	0.707	0.004	0.260
341	6.934	9.022	0.124	-2.087	0.057	0.707	0.000	0.260
386675	9.019	11.096	0.125	-2.076	0.099	0.707	0.002	0.260
100132249	10.121	12.192	0.126	-2.070	0.043	0.707	0.001	0.260
11183	9.715	11.775	0.127	-2.060	0.119	0.707	0.002	0.260
643827	12.012	14.046	0.131	-2.034	0.169	0.707	0.013	0.275
729966	10.860	12.889	0.131	-2.030	0.034	0.707	0.000	0.260
633	12.809	14.832	0.132	-2.023	0.920	0.973	0.216	0.581
2299	7.634	9.652	0.133	-2.019	0.037	0.707	0.003	0.260
390037	7.970	9.980	0.134	-2.009	0.145	0.707	0.023	0.299

Table 3.7 shows Gene IDs. Gene description and Gene ontology of Group 1 vs Group 3

mRNA	Gene symbol	Gene description	Gene Ontology	logqmed
3799	KIF5B	Kinesin family member 5B	ATP binding. microtubule binding.microtubule motor activity.nucleotide binding.protein binding	3.486
100130795	LOC100130795	hypothetical protein LOC100130795		3.451
647131	LOC647131	hypothetical LOC647131		3.274
5690	PSMB2	proteasome (prosome. macropain) subunit. beta type. 2	peptidase activity.threonine-type endopeptidase activity	3.222
51128	SAR1B	SAR1 homolog B (S. cerevisiae)	GTP binding.metal ion binding.nucleotide binding	3.153
6159	RPL29	ribosomal protein L29	RNA binding .heparin binding .protein binding .structural constituent of ribosome	3.139
689	BTF3	Basic transcription factor 3	RNA polymerase II transcription factor activity.protein binding	3.083
1349	COX7B	cytochrome c oxidase subunit VIIb	cytochrome-c oxidase activity	3.055
100132564	LOC100132564	hypothetical protein LOC100132564		3.002
130773	RPL23AP37	ribosomal protein L23a pseudogene 37		2.997
54757	FAM20A	family with sequence similarity 20. member A	extracellular region	2.967
60598	KCNK15	potassium channel. subfamily K. member 15	potassium channel activity.voltage-gated ion channel activity	2.967
6192	RPS4Y1	ribosomal protein S4. Y-linked 1	RNA binding.rRNA binding.structural constituent of ribosome	2.955
11267	SNF8	SNF8. ESCRT-II complex subunit. homolog (S. cerevisiae)	RNA polymerase II transcription factor activity.transcription factor binding	2.915
1072	CFL1	cofilin 1 (non-muscle)	actin binding.protein binding	2.914
1072	CFL1	cofilin 1 (non-muscle)	actin binding.protein binding.Rho protein signal transduction.actin cytoskeleton organization.actin filament organization.anti-apoptosis.cellular component movement.cytokinesis.establishment of cell polarity.neural crest cell migration.neural fold formation.positive regulation of actin filament depolymerization. protein amino acid phosphorylation response to amino acid stimulus. response to virus	2.914
8614	STC2	stanniocalcin 2	hormone activity.cell surface receptor linked signaling pathway.cell-cell signaling.response to nutrient	2.908

Table 3.7 (continued) shows Gene IDs. Gene description and Gene ontology of Group 1 vs Group 3

100130187	LOC100130187	hypothetical protein LOC100130187		2.896
440915	POTEKP	POTE ankyrin domain family. member K. pseudogene	ATP binding.nucleotide binding.protein binding	2.848
6513	SLC2A1	solute carrier family 2 (facilitated glucose transporter). member 1	D-glucose transmembrane transporter activity.dehydroascorbic acid transporter activity.glucose transmembrane transporter activity.kinase binding.protein binding.substrate-specific transmembrane transporter activity.xenobiotic transporter activity.carbohydrate transport. glucose transport.response to osmotic stress.transmembrane transport	2.810
643719	LOC643719	hypothetical LOC643719		2.807
10049	DNAJB6	DnaJ (Hsp40) homolog. subfamily B. member 6	ATPase activator activity.DNA binding.chaperone binding. heat shock protein binding .protein binding.transcription repressor activity.chorio-allantoic fusion. intermediate filament organization.negative regulation of caspase activity. negative regulation of transcription. DNA-dependent.protein folding.response to unfolded protein	2.777
100128533	LOC100128533	similar to hCG2013595		2.755
10447	FAM3C	family with sequence similarity 3. member C	cytokine activity.biological process.multicellular organismal development	2.746
3489	IGFBP6	Insulin-like growth factor binding protein 6	insulin-like growth factor I binding.insulin-like growth factor II binding. regulation of cell growth. signal transduction.negative regulation of cell proliferation	2.735
71	ACTG1	actin. gamma 1	ATP binding.identical protein binding.nucleotide binding.protein binding.structural constituent of cytoskeleton.cellular component movement. response to calcium ion	2.719
7555	CNBP	CCHC-type zinc finger. Nucleic acid binding protein	metal ion binding.protein binding.transcription factor activity. zinc ion binding.cholesterol biosynthetic process.regulation of transcription. DNA-dependent	2.702
2266	FGG	fibrinogen gamma chain	eukaryotic cell surface binding.protein binding. bridging.receptor binding.platelet activation.protein polymerization.response to calcium ion.signal transduction	2.695
92305	TMEM129	Transmembrane protein 129	molecular function.biological process	2.694
26292	MYCBP	c-myc binding protein	protein binding.transcription coactivator activity.egulation of transcription.spermatogenesis	2.691
80213	TM2D3	M2 domain containing 3		2.660
284260	LOC284260	hypothetical LOC284260		-2.496

Table 3.7 (continued) shows Gene IDs. Gene description and Gene ontology of Group 1 vs Group 3

100131563	LOC100131563	hypothetical protein LOC100131563		-2.484
7018	TF	Transferring	ferric iron binding.metal ion binding.protein binding.ubiquitin protein ligase binding	-2.420
57228	SMAGP	small cell adhesion glycoprotein	cytoplasmic vesicle.cytoplasmic vesicle membrane.integral to membrane.plasma membrane	-2.375
4597	MVD	mevalonate (diphospho) decarboxylase	ATP binding.Hsp70 protein binding.diphosphomevalonate decarboxylase activity.diphosphomevalonate decarboxylase activity.kinase activity.lyase activity.nucleotide binding.protein homodimerization activity	-2.348
5903	RANBP2	RAN binding protein 2	Ran GTPase binding.isomerase activity.metal ion binding.peptidyl-prolyl cis-trans isomerase activity.protein binding.zinc ion binding	-2.322
4222	MEOX1	mesenchyme homeobox 1	chromatin binding.sequence-specific DNA binding.transcription factor activity	-2.319
127295	RPL36P5	ribosomal protein L36 pseudogene 5		-2.305
53834	FGFRL1	fibroblast growth factor receptor-like 1	fibroblast growth factor binding.fibroblast growth factor receptor activity.heparin binding.receptor activity	-2.299
286205	SCAI	suppressor of cancer cell invasion	protein binding.transcription corepressor activity	-2.237
10131	TRAP1	TNF receptor-associated protein 1	ATP binding.nucleotide binding.tumor necrosis factor receptor binding.unfolded protein binding	-2.165
1260	CNGA2	Cyclic nucleotide gated channel alpha 2	cAMP binding.intracellular cAMP activated cation channel activity.ion channel activity.nucleotide binding	-2.153
53826	FXYD6	FXYD domain containing ion transport regulator 6	ion channel activity.molecular_function	-2.138
100129523	LOC100129523	hypothetical protein LOC100129523		-2.134
728320	LOC728320	hypothetical LOC728320		-2.118
644090	LOC644090	hypothetical LOC644090		-2.111
22834	ZNF652	zinc finger protein 652	DNA binding.metal ion binding.protein binding. zinc ion binding.regulation of transcription	-2.108
221458	KIF6	Kinesin family member 6	ATP binding.microtubule motor activity.nucleotide binding.protein binding.microtubule-based movement	-2.102
6615	SNAI1	snail homolog 1 (Drosophila)	metal ion binding.protein binding.sequence-specific DNA binding. transcription repressor activity.zinc ion binding.epithelial to mesenchymal transition. hair follicle morphogenesis.mesoderm formation. multicellular organismal development.negative regulation of cell differentiation involved in embryonicplacenta development.negativ e regulation of transcription from RNA polymerase II promoter.positive regulation of epithelial to mesenchymal transition.trophoblast giant cell differentiation	-2.100

Table 3.7 (continued) shows Gene IDs. Gene description and Gene ontology of Group 1 vs Group 3

6990	DYNLT3	dynein. light chain. Tctex-type 3	motor activity.protein binding.cell cycle. cell division.mitosis.regulation of mitotic cell cycle.transport	-2.089
341	APOC1	Apolipoprotein C-I	fatty acid binding.lipase inhibitor activity.phosphatidylcholinebinding.phosphatidylcholine-sterol O-acyltransferase activator activity.phospholipase inhibitor activity	-2.087
386675	KRTAP10-7	Keratin associated protein 10-7	motor activity.protein binding	-2.076
100132249	LOC100132249	hypothetical LOC100132249		-2.070
11183	MAP4K5	mitogen-activated protein kinase kinase kinase kinase 5	ATP binding.nucleotide binding.protein binding.protein kinase activity.protein serine/threonine kinase activity.small GTPase regulator activity. transferase activity.activation of JUN kinase activity. intracellular protein kinase cascade.protein amino acid phosphorylation.response to stress	-2.060
643827	LOC643827	contactin associated protein-like 3 pseudogene		-2.034
729966	LOC729966	similar to hCG2001482		-2.030
633	BGN	Biglycan	extracellular matrix binding.extracellular matrix structural constituent. glycosaminoglycan binding.protein binding	-2.023
2299	FOXI1	forkhead box I1	DNA bending activity.promoter binding.sequence-specific DNA binding.transcription activator activity.transcription factor activity.embryo development inner ear morphogenesis.positive regulation of gene-specific transcription from RNA polymerase II promoter.positive regulation of transcription from RNA polymerase II promoter	-2.019
390037	OR52I1	olfactory receptor. family 52. subfamily I. member 1	olfactory receptor activity.receptor activity.response to stimulus.sensory perception of smell	-2.009

Table 3.8 Differentially regulated mRNAs Group 2 vs Group 3

mRNA	med g1	med g2	qmed	Logqmed	ttest rawp	ttest adjp	limma rawp	limma adjp
2266	12.119	7.921	66.494	4.197	0.129	0.877	0.000	0.089
8614	14.686	11.349	28.130	3.337	0.062	0.877	0.001	0.739
84987	12.123	8.887	25.425	3.236	0.221	0.877	0.018	0.739
51030	14.339	11.125	24.890	3.214	0.057	0.877	0.002	0.739
1349	11.867	8.759	22.367	3.108	0.175	0.877	0.014	0.739
55629	13.168	10.064	22.272	3.103	0.498	0.883	0.051	0.739
60598	12.910	9.847	21.383	3.063	0.126	0.877	0.001	0.739
727947	14.339	11.311	20.668	3.029	0.559	0.890	0.040	0.739
9935	14.599	11.572	20.638	3.027	0.010	0.877	0.002	0.739
3799	11.419	8.469	19.096	2.949	0.138	0.877	0.007	0.739
6192	13.815	10.885	18.724	2.930	0.800	0.955	0.084	0.739
643205	13.174	10.249	18.629	2.925	0.930	0.985	0.075	0.739
51128	11.572	8.655	18.500	2.918	0.303	0.877	0.018	0.739
11082	13.207	10.302	18.257	2.905	0.161	0.877	0.017	0.739
94274	10.649	7.766	17.868	2.883	0.200	0.877	0.049	0.739
151579	11.233	8.396	17.063	2.837	0.284	0.877	0.020	0.739
9694	12.396	9.568	16.925	2.829	0.288	0.877	0.019	0.739
653247	12.963	10.155	16.574	2.808	0.077	0.877	0.000	0.739
1072	12.173	9.407	15.903	2.767	0.351	0.877	0.037	0.739
3688	12.523	9.773	15.630	2.749	0.386	0.877	0.047	0.739
7105	13.052	10.307	15.564	2.745	0.204	0.877	0.008	0.739
3489	12.788	10.059	15.324	2.729	0.158	0.877	0.012	0.739
80213	13.056	10.336	15.189	2.721	0.246	0.877	0.017	0.739
10447	11.542	8.826	15.119	2.716	0.016	0.877	0.002	0.739
10959	14.230	11.521	15.015	2.709	0.324	0.877	0.033	0.739
79614	15.303	12.609	14.786	2.694	0.088	0.877	0.003	0.739
6563	13.290	10.605	14.661	2.685	0.046	0.877	0.000	0.139
624	12.547	9.890	14.259	2.657	0.079	0.877	0.001	0.739
10923	14.377	11.738	13.999	2.639	0.486	0.882	0.042	0.739
5573	13.948	11.319	13.859	2.629	0.283	0.877	0.020	0.739
7018	11.701	13.972	0.103	-2.271	0.121	0.877	0.000	0.739
768	9.259	11.362	0.122	-2.103	0.060	0.877	0.001	0.739
415117	10.356	12.303	0.143	-1.946	0.079	0.877	0.010	0.739
8905	11.425	13.310	0.152	-1.885	0.206	0.877	0.000	0.739
339500	8.550	10.428	0.153	-1.878	0.105	0.877	0.014	0.739
132320	7.321	9.178	0.156	-1.857	0.023	0.877	0.003	0.739
81607	7.390	9.243	0.157	-1.853	0.228	0.877	0.104	0.739
54578	8.779	10.618	0.159	-1.839	0.163	0.877	0.016	0.739
390162	9.466	11.302	0.159	-1.836	0.226	0.877	0.082	0.739
928	12.460	14.285	0.161	-1.825	0.052	0.877	0.002	0.739
633	13.014	14.832	0.162	-1.819	0.119	0.877	0.002	0.739
284009	8.977	10.783	0.164	-1.806	0.076	0.877	0.007	0.739
51305	8.155	9.945	0.167	-1.790	0.069	0.877	0.004	0.739
10981	9.610	11.383	0.170	-1.773	0.098	0.877	0.006	0.739
57761	10.795	12.550	0.173	-1.755	0.059	0.877	0.001	0.739
7087	8.413	10.148	0.176	-1.735	0.046	0.877	0.001	0.739
100131563	12.933	14.649	0.180	-1.716	0.206	0.877	0.057	0.739
83901	9.817	11.529	0.180	-1.713	0.225	0.877	0.092	0.739
83714	9.520	11.230	0.181	-1.711	0.168	0.877	0.020	0.739
100130276	11.589	13.294	0.182	-1.705	0.225	0.877	0.106	0.739
140701	8.744	10.425	0.186	-1.682	0.113	0.877	0.008	0.739
652827	10.500	12.158	0.191	-1.657	0.060	0.877	0.010	0.739
64426	10.922	12.570	0.193	-1.648	0.234	0.877	0.134	0.739
57459	9.732	11.376	0.193	-1.645	0.267	0.877	0.184	0.749
26577	11.503	13.141	0.194	-1.637	0.008	0.877	0.001	0.739
100131060	8.520	10.148	0.196	-1.628	0.333	0.877	0.323	0.805
85414	11.124	12.749	0.197	-1.626	0.146	0.877	0.012	0.739
653505	9.399	11.021	0.197	-1.622	0.065	0.877	0.004	0.739
10335	9.520	11.140	0.198	-1.621	0.088	0.877	0.014	0.739
219428	10.771	12.384	0.199	-1.613	0.316	0.877	0.234	0.764

Table 3.9 shows Gene IDs. Gene description and Gene ontology of Group 2 vs Group 3

mRNA	Gene symbol	Gene description	Gene Ontology	logqmed
2266	FGG	fibrinogen gamma chain	eukaryotic cell surface binding.protein binding. bridging.receptor binding	4.197
8614	STC2	stanniocalcin 2	hormone activity.cell surface receptor linked signaling pathway.cell-cell signaling.response to nutrient	3.337
84987	C12orf62	chromosome 12 open reading frame 62	integral to membrane	3.236
51030	FAM18B1	family with sequence similarity 18. member B1	integral to membrane	3.214
1349	COX7B	cytochrome c oxidase subunit VIIb	cytochrome-c oxidase activity	3.108
55629	PNRC2	proline-rich nuclear receptor coactivator 2	protein binding.deadenylation-independent decapping of nuclear-transcribed mRNA.nuclear-transcribed mRNA catabolic process. nonsense-mediated decay.regulation of transcription	3.103
60598	KCNK15	potassium channel. subfamily K. member 15	potassium channel activity .voltage-gated ion channel activity.ion transport	3.063
727947	LOC727947	ubiquinol-cytochrome c reductase binding protein pseudogene		3.029
9935	MAFB	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian)	protein binding.sequence-specific DNA binding.transcription factor activity.transcription factor binding.brain segmentation.inner ear morphogenesis.negative regulation of erythrocyte differentiation.positive regulation of transcription from RNA polymerase II promoter. respiratory gaseous exchange.rhombomere 5 development.rhombomere 6 development segment specification sensory organ development	3.027
3799	KIF5B	kinesin family member 5B	ATP binding. microtubule binding.microtubule motor activity.nucleotide binding.protein binding	2.949
6192	RPS4Y1	ribosomal protein S4. Y-linked 1	RNA binding.rRNA binding.structural constituent of ribosome	2.930
643205	RPL36P8	ribosomal protein L36 pseudogene 8		2.925
51128	SAR1B	SAR1 homolog B (S. cerevisiae)	GTP binding.metal ion binding.nucleotide binding	2.918
11082	ESM1	endothelial cell-specific molecule 1	growth factor activity.insulin-like growth factor binding.biological process regulation of cell growth	2.905
94274	PPP1R14A	protein phosphatase 1. regulatory (inhibitor) subunit 14A	phosphoprotein phosphatase inhibitor activity.protein binding.regulation of phosphorylation	2.883
151579	BZW1L1	basic leucine zipper and W2 domains 1 like 1		2.837
9694	TTC35	Etratricopeptide repeat domain 35	binding.	2.829
653247	PRB2	proline-rich protein BstNI subfamily 2	molecular function.biological process	2.808
1072	CFL1	cofilin 1 (non-muscle)	actin binding.protein binding	2.767

Table 3.9 (continued) shows Gene IDs. Gene description and Gene ontology of Group 2 vs Group 3

3688	ITGB1	integrin. beta 1 (fibronectin receptor. beta polypeptide. antigen CD29 includes MDF2. MSK12)	identical protein binding.protein binding.protein heterodimerization activity. receptor activity.B celldifferentiation.cell adhesion.cell migration. cell-cell adhesion mediated by integrin.cell-matrix adhesion.cellular defense response.homophilic cell adhesion.integrin-mediated signaling pathway. interspecies interaction between organisms.leukocyte cell-cell adhesion	2.749
7105	TSPAN6	tetraspanin 6	signal transducer activity.positive regulation of I-kappaB kinase/NF-kappaB cascade	2.745
3489	IGFBP6	insulin-like growth factor binding protein 6	insulin-like growth factor I binding.insulin-like growth factor II binding. regulation of cell growth. signal transduction.negative regulation of cell proliferation	2.729
80213	TM2D3	M2 domain containing 3		2.721
10447	FAM3C	family with sequence similarity 3. member C	cytokine activity.biological process.multicellular organismal development	2.716
10959	TMED2	transmembrane emp24 domain trafficking protein 2	protein binding.protein transport.vesicle-mediated transport	2.709
624	BDKRB2	bradykinin receptor B2	G-protein coupled receptor activity.bradykinin receptor activity. phosphoinositide phospholipase C activity.protease binding.protein binding. protein heterodimerization activity.receptor activity .type 1 angiotensin receptor binding.G-protein coupled receptor protein signaling pathway.arachidonic acid secretion. blood circulation.cell surface receptor linked signaling pathway.elevation of cytosolic calcium ion concentration. inflammatory response.negative regulation of apoptosis.negative regulation of blood pressure.negative regulation of cell proliferation. negative regulation of peptidyl-serine phosphorylation.regulation of vascular permeability.regulation of vasoconstriction.response to drug . response to salt stress .smooth muscle contraction.transmembrane receptor protein tyrosine kinase signaling pathway	2.657
79614	C5orf23	chromosome 5 open reading frame 23		2.694
6563	SLC14A1	solute carrier family 14 (urea transporter). member 1	ubiquitin-ubiquitin ligase activity.urea transmembrane transporter activity.water transmembrane transporter activity.transport.urea transport	2.685
10923	SUB1	SUB1 homolog (S. cerevisiae)	DNA binding.protein binding.single-stranded DNA bindingtranscription coactivator activity.regulation of transcription from RNA polymerase II promoter	2.639
5573	PRKAR1A	protein kinase. cAMP-dependent. regulatory. type I. alpha (tissue specific extinguisher 1)	cAMP binding.cAMP-dependent protein kinase regulator activity.nucleotide binding.protein binding.activation ofprotein kinase A activity.hormone-mediated signaling pathway.intracellular signaling pathway.mesoderm formation.regulation of protein amino acid phosphorylation.regulation of transcription from RNA polymerase II promoter.signal transduction	2.629
7018	TF	Transferring	ferric iron binding.metal ion binding. protein binding .ubiquitin protein ligase binding.SMAD protein signal transduction.cellular iron ion homeostasis.ion transport.iron ion transport	-2.271
57035	C1orf63	chromosome 1 open reading frame 63		-2.765

Table 3.9 (continued) shows Gene IDs. Gene description and Gene ontology of Group 2 vs Group 3

2150	F2RL1	coagulation factor II (thrombin) receptor-like 1	G-protein coupled receptor activity.receptor activity.receptor binding.thrombin receptor activity.G-protein coupled receptor protein signaling pathway.elevation of cytosolic calcium ion concentration.positive regulation of leukocyte chemotaxis.positive regulation of positive chemotaxis.regulation of blood coagulation	-2.676
53834	FGFRL1	fibroblast growth factor receptor-like 1	fibroblast growth factor binding.fibroblast growth factor receptor activity.heparin binding.receptor activity.diaphragm development.heart valve morphogenesis.negative regulation of cell proliferation.regulation of cell growth. skeletal system development.ventricular septum morphogenesis	-2.641
22834	ZNF652	zinc finger protein 652	DNA binding.metal ion binding.protein binding.zinc ion binding.regulation of transcription	-2.369
113251	LARP4	La ribonucleoprotein domain family. member 4	RNA binding	-2.347
401409	RAB19	RAB19. member RAS oncogene family	GTP binding.nucleotide binding.protein transport.small GTPase mediated signal transduction	-2.305
127295	RPL36P5	ribosomal protein L36 pseudogene 5		-2.232
100132963	LOC100132963	hypothetical protein LOC100132963		-2.182
22858	ICK	intestinal cell (MAK-like) kinase	ATP binding.cyclin-dependent protein kinase activity. magnesium ion binding. nucleotide binding.protein serine/threonine kinase activity.transferase activity.intracellular protein kinase cascade.multicellular organismal developmen. protein amino acid phosphorylation.signal transduction	-2.155
10808	HSPH1	heat shock 105kDa/110kDa protein 1	ATP binding.nucleotide binding.response to unfolded protein	-2.133
100129474	LOC100129474	similar to histone cluster 1.H2aa		-2.130
9204	ZMYM6	zinc finger. MYM-type 6	DNA binding.metal ion binding. zinc ion binding.multicellular organismal development	-2.130
55635	DEPDC1	DEP domain containing 1	GTPase activator activity.signal transduction	-2.116
168667	BMPER	BMP binding endothelial regulator	protein binding.blood vessel endothelial cell proliferation involved in sprouting angiogenesis.endothelial cell activation.negative regulation of BMP signaling pathway.positive regulation of ERK1 and ERK2 cascade. regulation of endothelial cell migration. regulation of pathway-restricted SMAD protein phosphorylation.ureteric bud development	-2.114
7087	ICAM5	intercellular adhesion molecule 5. telencephalin	protein binding.cell-cell adhesion.phagocytosis	-1.735
100131563	LOC100131563	hypothetical protein LOC100131563		-1.716
83901	KRTAP9-8	keratin associated protein 9-8		-1.713
83714	NRIP2	nuclear receptor interacting protein 2	aspartic-type endopeptidase activity.protein binding.transcription regulator activity.negative regulation of transcription from RNA polymerase II promoter.proteolysis	-1.711
100130276	LOC100131563	hypothetical protein LOC100131563		-1.705
140701	C20orf135	chromosome 20 open reading frame 135		-1.682

Table 3.9 (continued) shows Gene IDs. Gene description and Gene ontology of Group 2 vs Group 3

652827	LOC652827	similar to CD99-cos7		-1.657
64426	SUDS3	suppressor of defective silencing 3 homolog (S. cerevisiae)	histone deacetylase binding.identical protein binding.protein binding.chromatin modification. negative regulation of transcription	-1.648
57459	GATAD2B	GATA zinc finger domain containing 2B	sequence-specific DNA binding. transcription factor activity.metal ion binding.zinc ion binding.protein binding.regulation of transcription DNA-dependent	-1.645
26577	PCOLCE2	procollagen C-endopeptidase enhancer 2	heparin binding.protein binding	-1.637
100131060	LOC100131060	hypothetical LOC100131060		-1.628
85414	SLC45A3	solute carrier family 45. member 3	transmembrane transport.	-1.626
653505	PPIAL4B	peptidylprolyl isomerase A (cyclophilin A)-like 4B		-1.622
10335	MRVI1	murine retrovirus integration site 1 homolog		-1.621
219428	OR4C16	olfactory receptor. family 4. subfamily C. member 16	olfactory receptor activity.receptor activity.response to stimulus. sensory perception of smell	-1.613

CHAPTER 4

DISCUSSION & CONCLUSION

Topo II is an enzyme that form transient single or double-strand breaks in the DNA and allow DNA strands or double helices to pass through each other. This enzymatic activity has an important role in segregation of daughter chromosomes and thus for cell proliferation (36). It was found that topo IIα has a role in mitotic processes and only present in proliferating tissues in the study of A.J. Schoeffler et al. (31). Tsutsui, K. et al. demonstrated that the isoform topo IIβ is found in terminally differentiated cells and there is no need this enzyme in general cellular activity. It was found that topo IIβ is highly expressed in differentiating cerebellar neurons (36). So far, topo IIβ has been researched alone or by using inhibitors. Sakaguchi A. et al. used inhibitors to understand the function of topoisomerases (35). Lyu, Y. showed that whole body topo IIβ knockout mice have shown prenatal death. In addition to this it was found that topo IIβ plays a role in activation or repression of developmentally regulated genes at late stages of neuronal differentiation (37). Yang et al found that topo IIβ plays a role in axon growth and regulation (38). And in the study of Nur-E-Kamal et al., it was showed that topo IIβ plays critical role in forming and maintaining the growth cone morphology and may affect neurite outgrowth through its regulation of the expression of certain neuronal genes (37).

Stem cells can transdifferentiate into different cell types including neuronal cells. The transdiffrentiation process of stem cells appears to be different substantially from the terminal differentiation of granule neurons. Therefore, the functional significance of topo IIβ may also be different in these differentiation systems. There have been no reports that analyzed the roles of topo IIβ in the transdifferentiation process of stem cells. To clarify this problem, we used the human mesenchymal stem cells from bone marrow (hMSC). Since these cells are proliferating cells, they also express topo IIα

which may substitute topo IIβ at the initiation stage of differentiation.

In our study we also focused on topo IIβ and find the role of this enzyme in neural differentiation of hMSCs. We observed that level of topo IIβ increases during differentiation while topo IIα decreases. This study is the first one that research both topo IIα and topo IIβ in hMSCs. To knockdown topo IIβ selectively, we transfected the hMSC with small interfering RNAs (siRNAs) targeting topo IIβ in Lipofectamine RNAiMAX transfection reagent. Our study is the first study that hMSCs were transfected by topo IIβ specific siRNAs. In our study topo IIβ specific silencing was obtained since level of topo IIα was not affected by siRNAs during transfection. We obtained about 85-90% transfection efficiency by RNAiMAX transfection reagent.

We obtained similar results with Nur-E Kamal et al. We found that transfected hMSCs had shorter axons according to induced hMSCs by N3 cytokine combination. The reason for this might be that topo IIβ may have a role in maturation, so neural differentiation process has not been completed in the transfected hMSCs.

Mesenchymal stem cells have been defined as pluiropotent adult stem cells. There are many studies that show MSCs have self renewal capacity and multi-lineage differentiation potential. Jiang Y et al. showed MSCs can differentiate into chondrocytes, osteoblasts and adipocytes (13). In addition to this, it was reported that MSCs also differentiate into myocytes and cardiomyocytes and even into cells of non-mesodermal origin, including hepatocytes, insulin-producing cells and neurons. Sanchez-Ramos et al. (12) and Woodbury et al. (20) firstly found that MSCs have neural differentiation capacity. Sanchsez-Ramos used growth factors and Woodbury used chemicals in order to obtain neural differentiation of MSCs originated from rat, mouse and human. About 80% of cells changed morphology but differentiated cells reverted back after a while in the study of Woodbury.

In this study, we have obtained neural differentiation of bone marrow isolated hMSCs using N3 cytokine combination. The results of this study confirm similar studies that used animals *in vivo*. Neural differentiation efficiency in our study is about 50-60%. According to chemical methods, we obtained more neural like cells that change morphology and they did not revert back. In addition to morphological results, we also checked early and late neural markers by RT-PCR and immunostaining methods.

The induced or silenced genes in the presence and absence of siRNA treatments were analyzed by DNA microarray analysis. In this respect, our study is the first one that role of topo IIβ was analyzed during induction by N3 cytokine combination of hMSCs by microarray technique. We found up regulated or down regulated genes related to topo IIβ.

The applications of microarray technique for the analysis of neural differentiation of stem cells are well documented. Bertani N. et al. had a similar study like ours. In this study hMSCs were induced by chemical methods to obtain neural differentiation and gene expression profile was checked by microarray. It was found that the adopted neural induction protocol was no more effective in redirecting human mesenchymal stem cells toward a neural phenotype than toward an endodermal hepatic pathway (85). Lyu Y.L. et al. used microarray technique to understand the role of topo IIβ in mouse. It was found that topo IIβ has a role in activation/repression of developmentally regulated genes at late stages of neuronal differentiation (37).

In conclusion, we have shown that hMSCs can transdifferentiate into neural cells by using cytokine combination. Early and late neural markers were expressed in differentiated hMSCs. Also hard to transfect hMSCs were transfected with RNAiMAX transfection reagent successfully and topo IIβ specific silencing was obtained. We found that topo IIβ have a role in neural differentiation since level of this enzyme increases during differentiation process. Also microarray methods was used to find topo IIβ related genes and mostly up regulated and down regulated genes were analyzed by comparing transfected hMSCs and neurally differentiated hMSCs.

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