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M.S. Thesis In Biology

July - 2009

SELECTIVE SILENCING OF DNA TOPOISOMERASE IIβ BY siRNA TRANSFECTION DURING NEURAL TRANSDIFFERENTIATION OF hMSCs

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APPROVAL PAGE

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

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

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July 2009

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ABSTRACT

Mesenchymal stem cells derived from human bone marrow have a potential to transdifferentiate into neural cells. In this study the differentiation was achieved by N3 cytokine combinations. During differentiation process several genes are up regulated. This increase in expression level was provided by the act of DNA Topoisomerases. Also it is known that the amount of DNA Topoisomerase IIβ enzyme increases during differentiation processes. However the effect of it during neural differentiation is not clear. In order to reveal this we decided to silence DNA topoisomerase IIβ specifically by RNA interference mechanism. To achieve this silencing we used several siRNA delivery reagents such as Lipofectamine 2000, Lipofectamine RNAiMAX, FuGENE HD and Primefect transfection reagents. Most of them delivered both GFP plasmid and siRNA into HUVEC and HEK 293 cell lines successfully. However to deliver GFP plasmid and labeled siRNA into hMSCs was not possible. Fortunately we were successful to deliver target specific siRNA (TOP 2B specific) into hMSCs with Lipofectamine RNAiMAX siRNA delivery reagent.

Keywords: Mesenchymal stem cells, neural differentiation, siRNA, transfection, topoisomerase IIβ.

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ÖZ

İnsan kemik iliğinden izole edilmiş olan mezenkimal kök hücreler nöral hücreye transfarklılaşma potansiyeline sahiptirler. Bu çalışmada nöral farklılaşma N3 sitokin karışımı ile sağlandı. Farklılaşma esnasında bazı genlerin ifadesi tekrar düzenlenip, bazılarının da ifadesi artmaktadır. DNA topoizomeraz IIβ enziminin de miktarının farklılaşma esnasında arttığı bilinmektedir. Fakat nöral farklılaşma esnasındaki rolü tam olarak bilinmemektedir. DNA topoizomeraz IIβ enziminin nöral farklılaşma esnasındaki rolünü belirleyebilmek için TOPO 2B genini RNA interferans yöntemiyle spesifik olarak susturmaya karar verildi. siRNA'yı hücreye verebilmek için Lipofectamine 2000, Lipofectamine RNAiMAX, FuGENE HD ve Primefect gibi çeşitli transfeksiyon ajanları denendi. Bu ajanların çoğu HEK 293 ve HUVEC hücre hatlarına GFP plasmidinin ve işaretli siRNA'nın girişini sağladı fakat mezenkimal kök hücrelerde başarılı bir sonuç elde edilemedi. TOPO 2B genine özgü siRNA'nın mezenkimal kök hücrelere Lipofectamine RNAiMAX transfeksiyon ajanıyla verilmesi sonucunda yeterli susturma sağlandı.

Anahtar Kelimeler: Mezenkimal kök hücre, nöral farklılaşma, siRNA, transfeksiyon, topoizomeraz IIβ.

To my parents and husband

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SYMBOL/ABBREVIATION

MSC	Mesenchymal stem cell
hMSC	Human mesenchymal stem cell
CFU-F	Colony-forming unit fibroblasts
ISCT	International Society for Cellular Therapy
CD	Cluster of differentiation
siRNA	Small interference RNA
RISC	RNA-Induced Silencing Complex
UTR	Untranslated region
HEK 293	Human Embryonic Kidney
HUVEC	Human Umbilical Vein Endothelial Cells
PTD	Protein Trunsduction Domain
PEI	Polyethylenimine
PPI	Polypropylenimine
PAMAM	Polyamidoamine
PLL	Poly-L-Lysine
PAA	Polyallylamine
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal Bovine Serum
PBS	Phosphate Buffered Saline
DMSO	Dimethyl Sulfoxide
RPM	Revolutions per minute
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal Bovine Serum

CHAPTER 1

INTRODUCTION

1.1 MESENCHYMAL STEM CELLS (MSCs)

Stem cells are precursor cells of all cells in the body. Among many types of stem cells adult stem cells are mostly used in research and clinical applications. Mesenchymal stem cells (MSCs) are also a kind of adult stem cells.

Mesenchymal stem cells are self renewing cells that are present in a number of adult and fetal tissues such as blood, placenta, amniotic fluid, heart, skeletal muscle, adipose tissue, synovial tissue and pancreas. Bone marrow is a well accepted and most studied source of MSCs. They account for 0.001-0.01% of nucleated cells of bone marrow (1, 2, 3, 4). Bone marrow derived MSCs was first described by Petrakova et al. They were derived from plastic-adherent bone marrow cells. Due to their fibroblastic morphology they were called as fibroblast colony-forming units by Friedenstein (5, 6). They are also called as colony-forming unit-fibroblasts (CFU-F), mesenchymal progenitor cells, marrow stromal cells, marrow stromal fibroblasts, stromal stem cells, and multipotent mesenchymal stromal cells in the previous studies (8, 9).

MSCs should have some essential characteristics defined by International Society for Cellular Therapy (ISCT). They should form single cell derived colonies with a fibroblastic nature. They should adhere to plastic surface in culture conditions. They should both have regeneration and differentiation potential. They should be negative for

hematopoietic markers such as CD45, CD34, and CD14 but positive for stromal markers such as CD105, CD73 and CD90 (10).

Bone marrow-derived MSCs adhere to the surface in culture and proliferate in the undifferentiated state. They can also provide hematopoiesis and differentiate into mesodermally derived tissues such as bone, cartilage, fat, muscle, connective tissue and tendon in appropriate conditions, and so that called multipotent (7).

MSCs are one of the most important stem cell types for tissue engineering and cell-based therapies due to their availability, *in vitro* expansion potential and genetic manipulation opportunity (11).

1.1.1 Isolation of hMSCs from Bone Marrow Aspirates

MSCs are present in many tissues such as adipose tissue, umbilical cord blood, amniotic fluid, chorionic villi of placenta, peripheral blood, fetal blood, liver, lung, synovial membrane, exfoliated deciduous teeth, skin tissue, muscle, trabecular bone, and pericyte. However the best source for MSCs isolation is bone marrow (64, 65). In human marrow donors, cells are often harvested from the superior iliac crest of the pelvis. In order to obtain human MSCs (hMSCs), bone marrow aspirates are fractionated by using Ficoll density gradient centrifugation (Figure 1.1). At this centrifugation a mononuclear cells enriched population is obtained. After this centrifugation there are many ways of getting a population of MSCs such as using complex set of markers and culture conditions. However, the widely used one relies on ability of MSCs to adhere to plastic surface. By simply culturing cells for several passages *in vitro*, a population of morphologically homogeneous fibroblast-like cells can be obtained. These cells are cultured in a medium such as Dulbecco's modified Eagle's medium (DMEM), with 15-20% fetal bovine serum. Cultures are maintained at 37°C in a humidified atmosphere containing 5% CO_2. Primary cultures are usually stays in culture for 10-14 days and then sub-cultured by trypsinization. The isolated cells were grown on plastic and characterized by FACS analysis for specific markers (66).

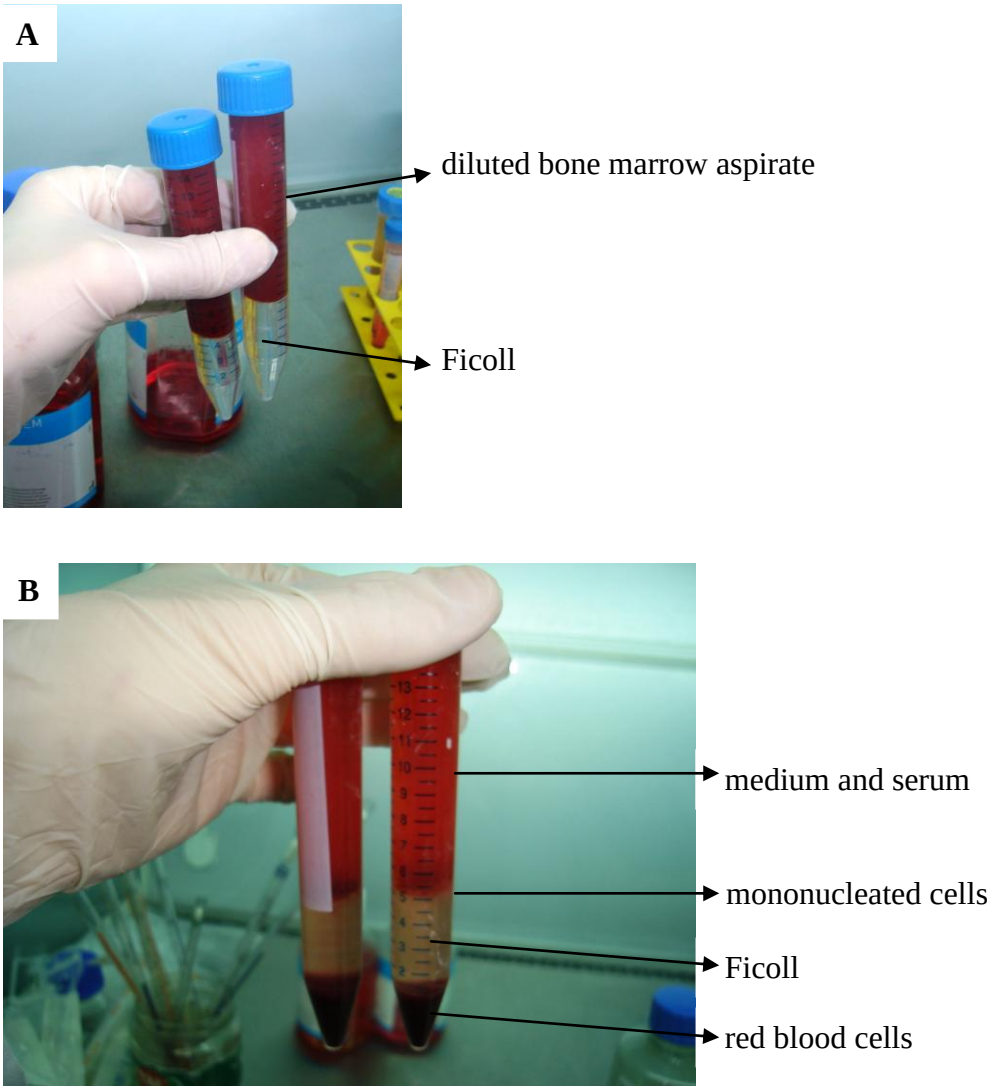


Figure 1.1 Isolation of hMSCs by Ficoll density gradient centrifugation **A.** Before centrifugation **B.** After centrifugation

1.1.2 Differentiation Potential & Neural Transdifferentiation of hMSCs

hMSCs can differentiate into many mesenchymal lineages such as osteoblasts, chondrocytes and adipocytes as well as they can be maintained in undifferentiated state *in vitro* (67). However their apparent differentiation potential is being expanded by new investigations. Recent studies indicate that hMSCs can also cross germ layers and

transdifferentiate into neural cells expressing neuronal and glial markers. Some agents such as cytokines, growth factors, neurotrophins, and are known to promote neural cell induction *in vitro* (68, 69). Many neural induction methods are used including use of neurotrophic factors and chemical inducers so far. Neurotrophic growth factors are polypeptide hormones and they are essential for the development and maintenance of the central nervous system. Neurotrophic factors also influence the proliferation, survival and differentiation of neuronal lineages (70). 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 (71). Some commonly used neurotrophic factors in neural differentiation protocols are nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and Neurothophin3 (NT-3). Potent chemical reagents used for neural induction are dimethylsulfoxide (DMSO), butylated hydroxyanisole (BHA), dibutyryl cyclic adenosine monophosphate (dbcAMP), and 3-Isobutyl-1-Methylxanthine (IBMX) (72, 73). These reagents provide rapid neuron-like morphology acquisition (74). Firstly in 2000, differentiation of rat and human marrow stromal cells into neurons by dimethylsulfoxide (DMSO) and butylated hydroxyanisole (BHA) was achieved (74). Nerve growth factor (NGF) is necessary for the development and survival of some sympathetic and sensory neurons. Brain-derived neurotrophic factor and neurothophin3 takes role in development and maintenance of neuronal populations. They also enhance the survival and differentiation of neurons (75). Cell-permeable dibutyryl cyclic adenosine monophosphate alters gene expression and affects cell growth and differentiation. It improves the survival of dopaminergic neurons in culture (76). 3-isobutyl-1-methylxanthine activates PKA by inhibiting cAMP phosphodiesterase. This leads to decreased proliferation and increased differentiation. Dimethylsulfoxid has also been shown to induce differentiation (77).

1.2 DNA TOPOISOMERASES

1.2.1 Type II DNA Topoisomerases

During cellular processes some topological problems occur in the DNA due to the helical nature of it and its length. For example, at DNA replication, recombination, chromatin remodeling, and transcription superhelical tension occurs in the adjacent double helical region. Type II topoisomerases modulate the topology of DNA and relieve supercoil stress. It provides a transient cleavage of DNA strands and allows a second double-stranded DNA segment pass through the gate. Different from other topoisomerases this transport is an ATP coupled process at type II topoisomerases. This ATP coupling is necessary for the enzyme to return its initial configuration. After the DNA transport the double-strand break is religated (Figure 1.2) (12, 14, 15, 16).

There are two subclasses of type II topoisomerases, type IIA and type IIB. Type IIA topoisomerases are the enzmes DNA gyrase, eukaryotic topoisomerase II and bacterial topoisomerase IV. Type IIB topoisomerases are distinct from type IIA and found in archea and higher plants.

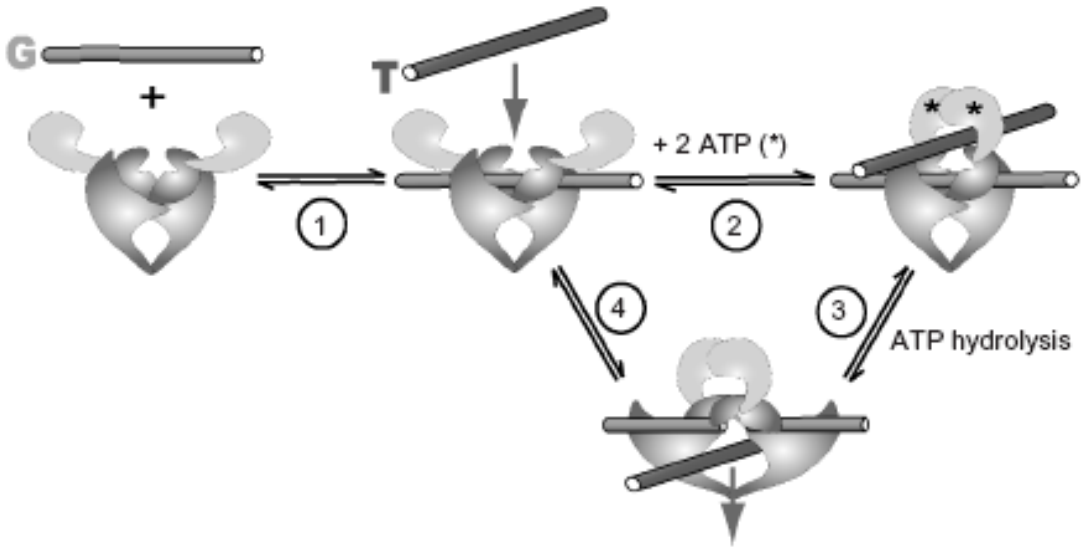


Figure 1.2 Action mechanism of DNA topoisomerase II (13)

DNA topoisomerase II enzymes have three domains; ATPase domain, DNA cleavage domain, and C-terminal domain.

In mammalian cells two isoforms of topoisomerase II have been identified; 170-kDa topo IIα and the 180-kDa topo IIβ. These isoforms are encoded by separate genes but they are similar in primary structure (72%) and they have similar catalytic properties *in vitro* (Figure 1.3).

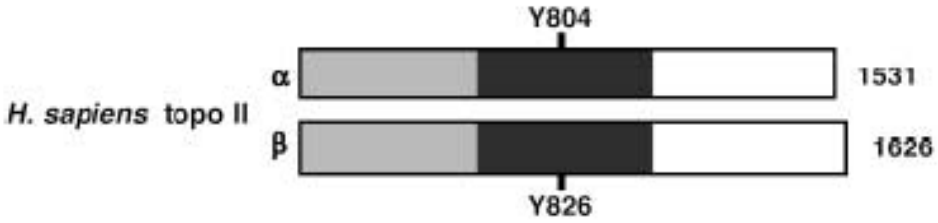


Figure 1.3 Primary structure of topo IIα and topo IIβ (17)

However they are regulated very differently. Since the timing and tissue specificity differs between them it can be assumed that they play separate roles in cellular physiology. Topo IIα isoform is mainly involved in mitotic processes and only present in proliferating tissues such as tumors. That is why there is a positive correlation between cell proliferation rate and topo IIα concentration in the cell (19, 20). 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 (21). In contrast, topo IIβ isoform is not required for normal mitotic events. It is present in all tissues including terminally differentiated tissues and its level is not changed during the cell cycle. Its presence in terminally differentiated cells indicates that it plays role in DNA metabolism especially in the transcriptional activation of some inducible genes instead of DNA replication and chromosome condensation/segregation. In some cell lines it is not expressed suggesting that it is not required for general cellular activities but important for more specific processes *in vivo*. However, its exact function is not known yet (17, 22).

1.2.1.1 DNA Topoisomerase II inhibitors

Topoisomerase inhibitors are designed to interfere with the action of topoisomerases. They have been used for cancer chemotherapy treatments. They block the ligation step of the cell cycle, generating single and double stranded breaks that harm the integrity of the genome. Introduction of these breaks subsequently lead to apoptosis and cell death. Topoisomerase inhibitors can also function as antibacterial agents.

Commonly used topo II inhibitors in order to discover the functions of topo II are 2,6- dioxopiperazines such as ICRF-159 and ICRF-187, and epipodophyllotoxins such as VP-16 and VM-26 (Figure 1.4). These inhibitors target the N-terminal ATPase domain of topo II and prevent topo II from turning over. They bind in a non-competitive manner and lock down the dimerization of the ATPase domain.

However, these inhibitors are not selective and inhibit the enzymatic activity of both topo IIα and topo IIβ (17). In order to investigate the cellular role of only topo IIβ isoform individually, topo IIβ specific inhibition should be achieved. That could be provided by RNA interference mechanism with the use of topo IIβ specific siRNAs.

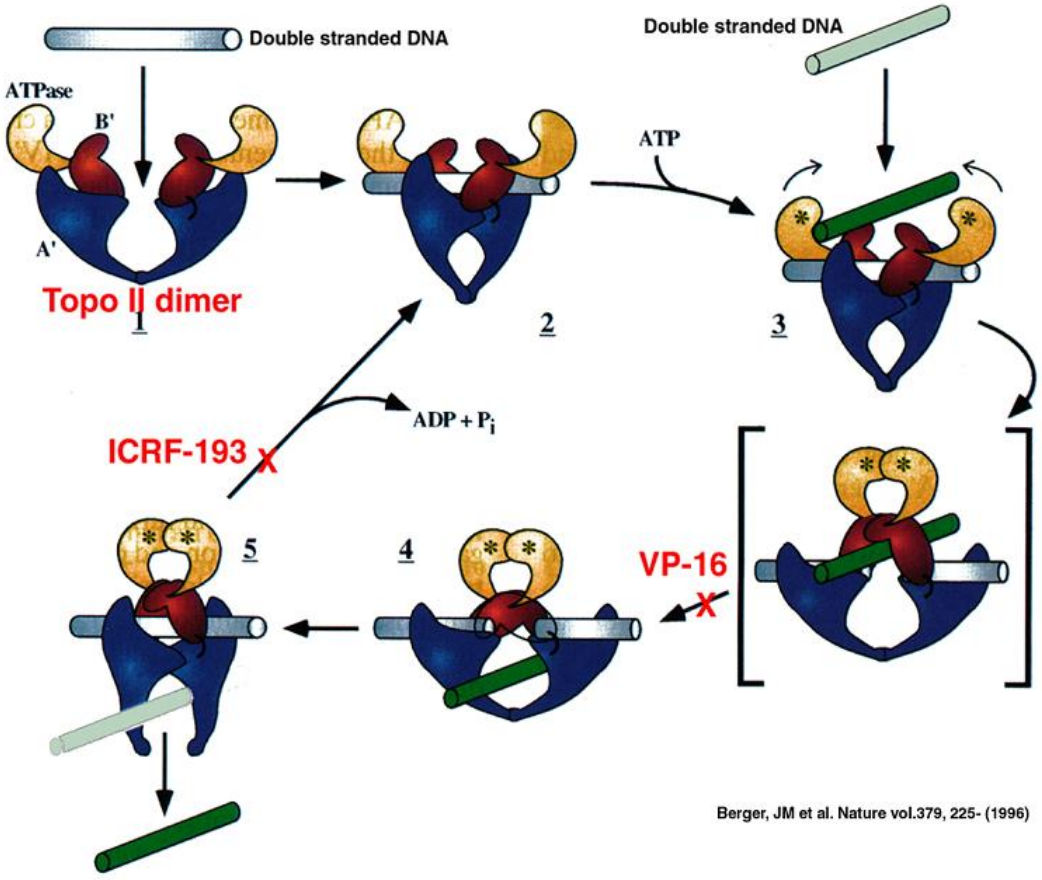


Figure 1.4 Two commonly used Toposomerase II inhibitors.

1.2.2 Role of Topoisomerase IIβ in Neural Differentiation

In some recent studies it is stated that topo IIβ plays a role in neuronal differentiation. It is found that topo IIβ is highly expressed in differentiating cerebellar neurons (18). It is suggested that topo IIβ is involved in early stages of granule cell differentiation. It makes inducible neuronal genes transcribable through alterations in higher order chromatin structure. As differentiation precedes topo IIα expression decreases. However, topo IIβ level increases through differentiation process (18) (Figure 1.5).

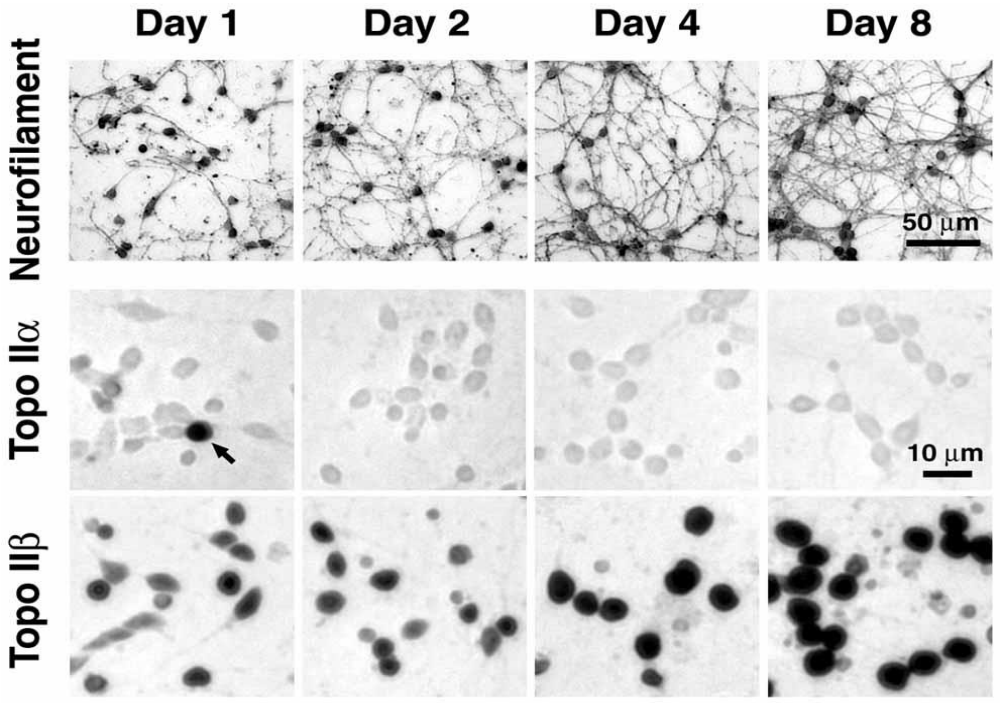


Figure 1.5 Expression of neurofilaments and topo II isoforms in the cerebellar granule cells differentiating *in vitro* (18)

It has also been previously shown that in the first two postnatal weeks the signal levels for topo IIβ mRNA increased on the cerebellar granule cells actively differentiating in the granular layer, suggesting a link between the topo IIβ expression and the cerebellar development. By use of topo II-specific inhibitors, the requirement of topo IIβ activity in the expression of neuronal genes in a primary culture of differentiating granule cells was assessed. Defects in embryonic neural development were identified in topo IIβ knockout mice (18).

At topo IIβ knockout mice during brain development a major defect in corticogenesis occured. It is demonstrated that topo IIβ has a critical role in neurite outgrowth during neuronal differentiation, possibly at the level of gene expresion (22). Studies of whole-body top2β knockout mice have demonstrated a perinatal death phenotype. It has been demonstrated that motor neuron axons fail to innervate the

diaphragm muscles and sensory neuron axons fail to enter the spinal cord, which suggests a role of topo IIβ in axon growth and/or guidance (Figure 1.6).

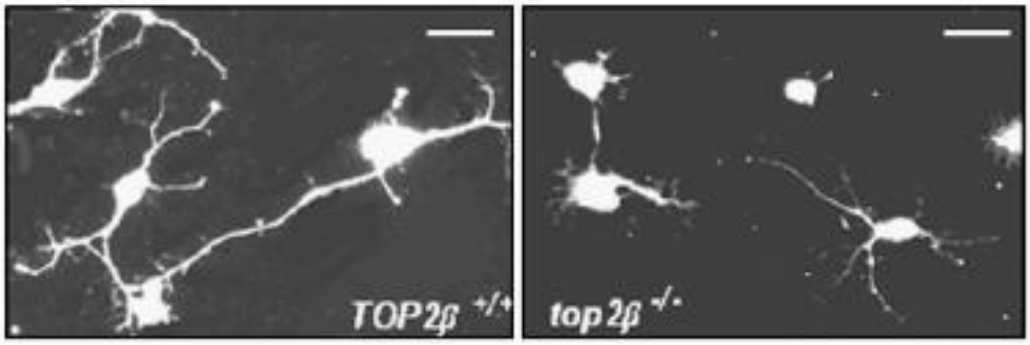


Figure 1.6 Neurons from top2β−/− mouse brains can not extend neurites in culture (22)

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 II beta dependent, transient, 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 (81).

1.3 RNA INTERFERENCE

RNA interference is a way of post transcriptional gene silencing (PTGS) in most eukaryotic cells by small interfering RNA (siRNA). They lead degradation of mRNAs in a sequence specific manner so that the homologous genes are silenced (23, 24). In this process, introduction of dsRNA into cells trigger the degradation of mRNAs with the same sequence (25, 26). RNAi was first realized in plants (petunia) and described as gene silencing or cosuppression (27). Then in 1998 Fire and Mello injected double stranded RNA in Caenorhabditis elegans and got an efficient gene silencing effect (28). Eukaryotic organisms use this mechanism to inhibit transposon mobilization and to protect their genomes against RNA molecules that can cause gene silencing. It also targets the RNA of viral genome for degradation at the times of infection (29). When

genes required for RNAi was disrupted some developmental defects occurred in *C. elegans* showing that the process is required in one of the developmental pathways (30).

Because of its high efficiency and specificity, RNAi became an important research tool for analyzing gene functions in eukaryotes via introduction of siRNAs. It is also promising for therapeutic approach as pharmaceutical products (41).

1.3.1. Mechanism of RNA Interference

siRNAs are 21-23 nucleotides long double-stranded RNAs with two nucleotides overhangs on the 3' ends. siRNAs are either generated by the enzymatic cleavage of long dsRNA by the dicer or produced synthetically (Figure 1.7).

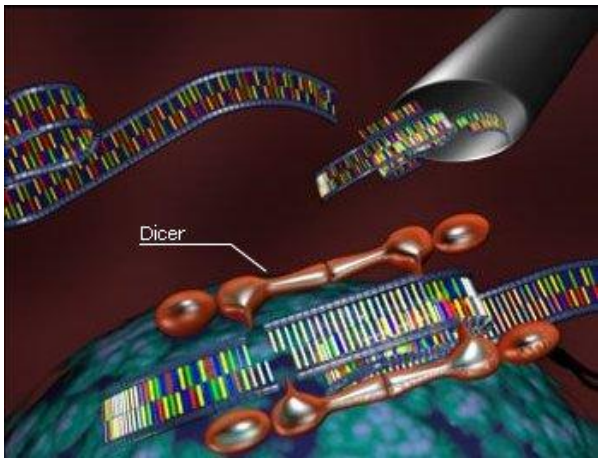


Figure 1.7 Cleavage of dsRNA by Dicer into siRNA.

RNAi is a multistep process including initiation and effector stages. The site of action for siRNA molecules is in the cytoplasm where they bind and target messenger RNA for degradation. When long dsRNAs enter the cell, they are the target for an RNAse III enzyme Dicer (31). This enzyme is a dimer and contains helicase, dsRNA binding, and PAZ (piwi, argonaute, and zwille proteins) domains (32, 33). Helicase and dsRNA binding domains are important for dsRNA unwinding and mediation of protein–RNA interactions. DICER is a dsRNA specific endonuclease that cuts it into smaller fragments and produces 21–23 nucleotide dsRNA fragments with two

nucleotide 3’ end overhangs: siRNAs. These siRNAs are then incorporated to RNA-induced Silencing Complex (RISC) which contains several proteins such as AGO2, FMRP and P100 (Figure 1.8) (34, 35). After that RISC is activated by an ATP dependent process and unwinds the double stranded siRNAs. By the guidance of siRNA it binds to the targeted mRNA containing a sequence homologous to the siRNA and an endoribonuclease cleaves the mRNA at a site located approximately in the middle of the homologous region (Figure 1.9) (32). Then it is degraded by exoribonucleases resulting in a loss of expression of the gene (36). So that, gene is specifically inactivated at a posttranscriptional level (Figure 1.10). Some of the double stranded siRNAs may be used as primers by RNA-dependent RNA polymerase in order to produce another long strand of dsRNA which can continue through the RNAi pathway. This will also enhance the efficiency of the gene silencing by dsRNA (37).

Figure 1.8 RNA-induced Silencing Complex (RISC)

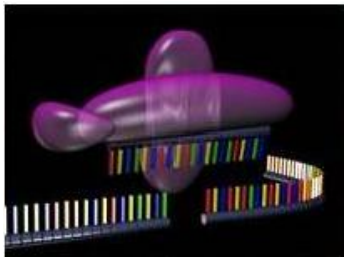


Figure 1.9 Cleavage of target mRNA by RISC

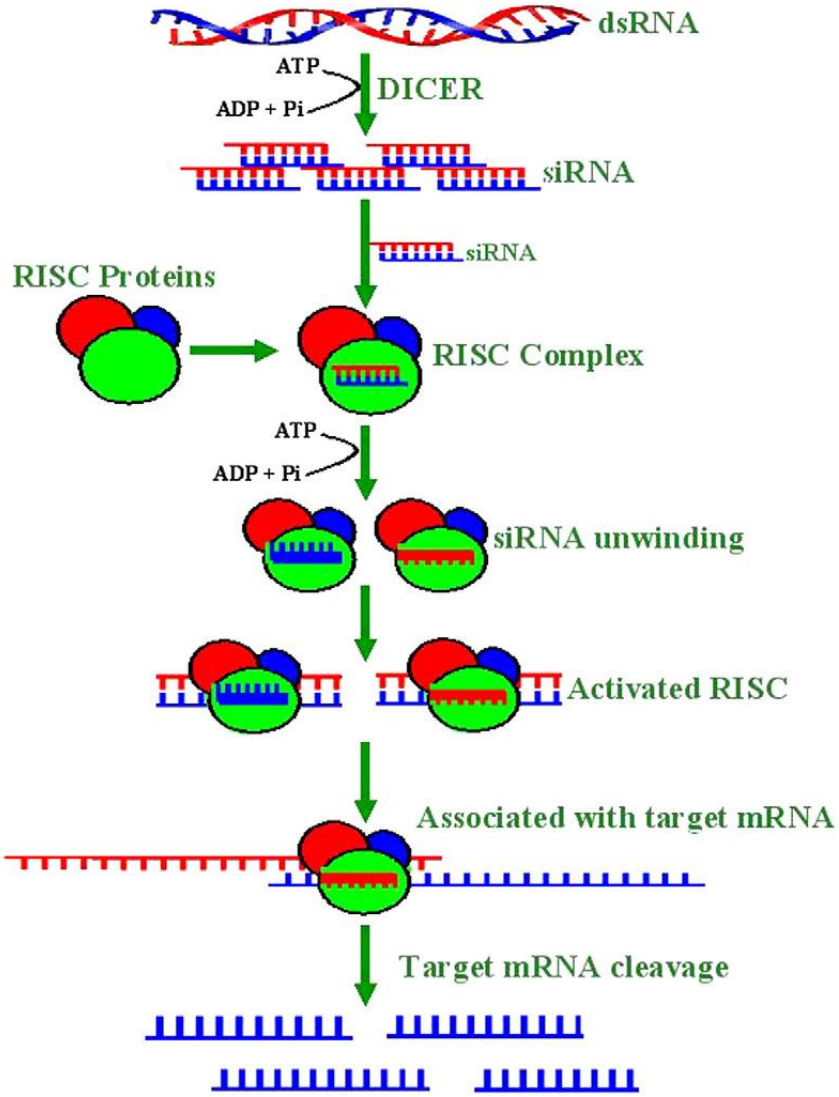


Figure 1.10 Mechanism of RNA interference (37)

It was also realized in *C. elegans* that RNAi can spread between cells. A transmembrane protein, SID-1 acts as a channel for long dsRNA, siRNA, or a currently undiscovered RNAi-related signal (38).

1.3.2. Sources of siRNA

In applications, the double stranded RNA is introduced into the cells in several ways (Figure 1.11). dsRNA can be introduced into simple organisms such as *C. elegans* and Paramecium by feeding the organism with the bacteria engineered to express

dsRNA or by soaking the bacteria in a dsRNA solution (39). In some cells dsRNA can be injected (37). Then RNAi starts by processing the dsRNA into siRNA in the presence of ATP.

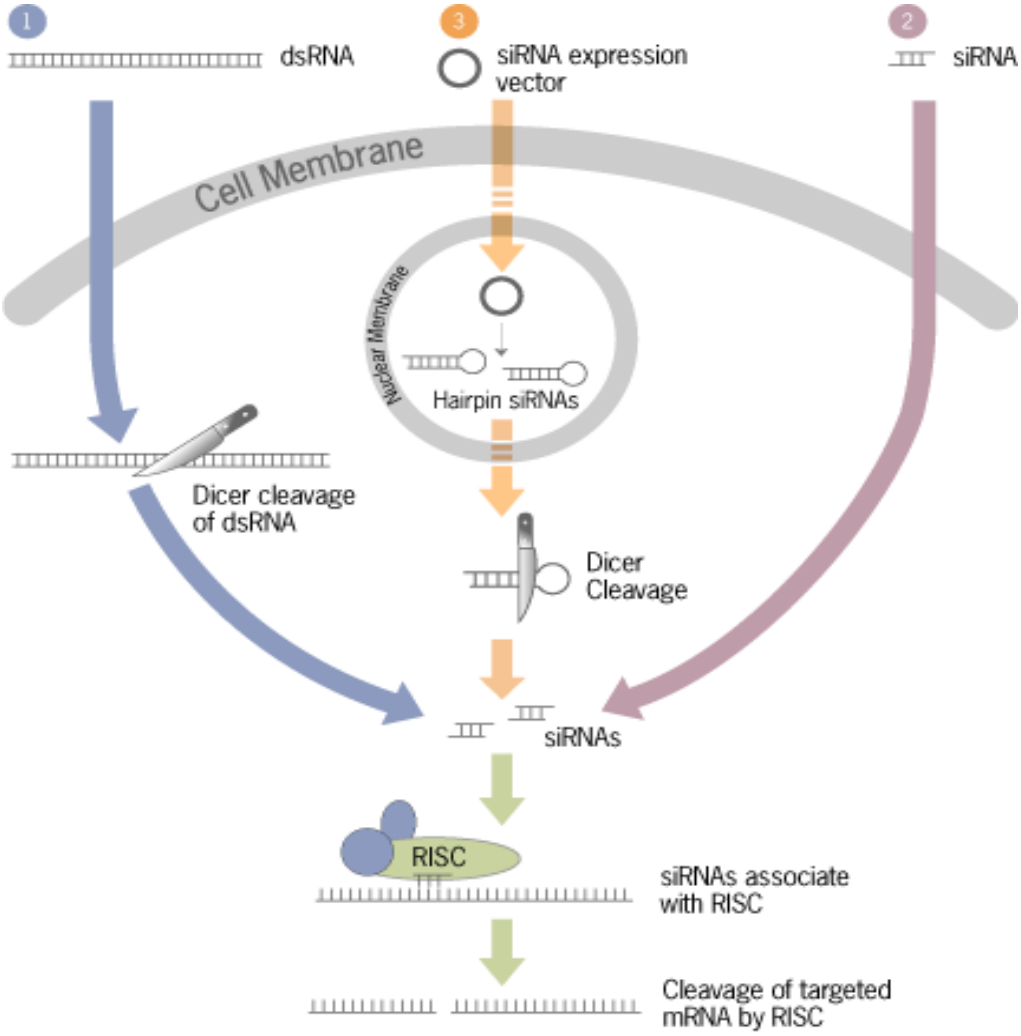


Figure 1.11 Sources of siRNA for RNAi mechanism

DNA plasmid vector producing hairpin siRNAs can be used as a source of siRNA. It is a more economical way of producing siRNA for especially multiple sequences and also stable RNAi can be achieved. However, it is laborious to generate and difficult to transfect. siRNAs can also directly delivered to the cells. Chemical synthesis of them is rapid but expensive. In general, the transfection efficiency of

siRNA is higher than for plasmid DNA, independent of the transfection method and cell line used. It is most likely this is due to the fact that plasmid DNA is larger and has to be delivered to the nucleus, whereas siRNA is small and is active in the cytoplasm. Suppression of gene expression by siRNA is generally a transient phenomenon. Gene expression usually recovers after 96 to 120 h or 3–5 cell divisions posttransfection, due to both dilution and degradation of siRNAs.

There are expanding libraries of validated, commercially available siRNAs directed toward some commonly targeted genes. If the gene of the interest has not been targeted using siRNA before, a novel siRNA must be developed.

siRNA Design

Given a siRNA sequence alone, it is not currently possible to predict with complete certainty the degree of gene suppression a particular siRNA will produce. However, a number of observations have been made which can be used to advantage, increasing the probability of producing an effective siRNA. The principal variable is the gene target site. It is usually recommended that a target site located at least 100–200 nucleotides from the AUG initiation codon be chosen (40). Targets within 50–100 nucleotides of the termination codon should also be avoided. The 5' and 3' untranslated region (UTR) should ideally also be avoided as associated regulatory proteins could theoretically compromise RNAi. Numerous on-line design tools will produce a list of suitable gene target sites. It is important to ensure that the sequence is specific to the target gene by performing a BLAST search.

Characteristics of the siRNA are equally important as the choice of target sequence. Twenty-one-nucleotide siRNAs with 3'-d(TT) or (UU) overhangs are the most effective (41) and most commonly used ones. For optimal siRNA secondary structure, the GC ratio should ideally be between 45 and 55%, and multiple identical nucleotides in series, particularly poly(C) and poly(G), should be avoided.

1.3.3 siRNA Delivery Methods

In order to obtain RNAi effect a sufficient amount of synthetic siRNA or plasmid DNA must be delivered to cells. They usually can not pass cell membranes but with the help of transfection reagents they can enter cells. Many transfection methods have been developed for DNA and siRNA delivery. Under certain conditions siRNAs may enter cells easily without any transfection reagent. dsRNA delivery could be achieved by injection or soaking the nematode *C. elegans* in a dsRNA solution. Methods developed during past few decades for DNA transfection are based on calcium phosphate-DNA coprecipitates (1973), polycations such as DEAE-dextran (1965) or polylysines (1991), electroporation (1982), cationic lipid-formed liposomes (1987), dendrimers (1996) and scraping (1995). Most commonly used ones are lipid based and amine based transfection reagents. Transfection of many cell lines with a high efficiency can be achieved by some of these methods. However, most primary cells are resistant to these methods. In order to overcome this alternative methods such as microinjection, retroviral vectors or adenovirus-mediated cell infection are used.

Transfection efficiency also depends on cell density, transfection time, the ratio of siRNA-to-transfection reagent, the cell passage number, and also antibiotic use.

1.3.3.1 Cationic Lipid Based Transfections

Lipid based transfections are carried out by using commercialized cationic lipids. These cationic liposomes are also known as lipoplexes and they are capable of delivering both siRNA and siRNA encoding plasmid through the cell membrane (42, 43). Cationic lipids affect requirements for transfection such as adhesion to cell surface, internalization, translocation of DNA into the nucleus (46, 47). These cationic lipids are used *in vitro* and *in vivo* for delivery of oligonucleotides for therapeutic and research purposes. Synthesizing lipophilic derivatives of siRNA by coupling it for example with cholesterol is another way of improving cellular uptake (44). Also surface modifications of cationic lipids with specific ligands targeting the required tissue are being applied (45).

Lipofectamine 2000, Lipofectamine RNAiMAX, FuGENE6, and FuGENE HD are some frequently used commercially available cationic lipids. They contain a blend of lipids in a non-liposomal formulation.

In cationic lipid based transfections several factors determine the transfection efficieny. The most important one is DNA or siRNA to lipid ratio. Negative charges on DNA/siRNA interact with positively charged lipids. However the net charge should be positive for efficient interaction with plasma membrane. Also serum inhibits interactions between DNA/siRNA and lipids so decrease efficiency. To prevent this serum free mediums sould be used to prepare complexes. Another factor effecting transfection efficiency is cell seeding density. Lower densities are more efficient (48).

In cell lines such as HEK293, HUVEC, and HeLa cationic lipids provide satisfying siRNA/DNA delivery. They show nearly complete transfection. However primary cells and differentiated cells have resistance to transfection due to presence of tight junctions, cilia or microvilli. Also cationic lipids are more toxic *in vivo*.

1.3.3.2 Peptide Based Transfection Reagents

Several peptide based gene delivery systems that overcome extracellular and intracellular limitations have been developed. Peptide carriers combine DNA binding and membrane destabilizing properties and promote gene transer into cultured cells and living animals. Synthetic peptides containing nuclear localization sequences (NLS) improve nuclear delivery of DNA. Peptide based transfection reagents also provide improved DNA delivery by an endosome independent pathway by the aid of protein transduction domains (PTD). An example of peptide-based gene delivery system, MPG, is a bipartate amphipathic peptide derived from the fusion protein of HIV-1 gp41 protein and the nuclear localization sequence of SV40 large T antigen (50, 60).

Wang et al. developed a peptide targeted delivery method to deliver siRNA efficiently in receptor mediated manner. This system was prepared by in situ functionalization of a pH-sensitive surfactant and self assembly with siRNA. The pH-

sensitive amphiphilicity of the surfactant at pH 5-6 is able to induce cell membrane disruption at endosomal pH and facilitate endosomal escape of siRNA after internalization (59).

1.3.3.3 Viral Delivery

Viral vectors provide high transfection efficiency and interest is growing in viral vector mediated RNAi. Even in the absence of selective agent, adenoviral and retroviral vectors produce siRNAs *in vivo* and provide stable RNAi. However, survival of the infected cells in some cell types is a few days. Also production of the recombinant virus is complex and time consuming (50, 51, 52, 53).

1.3.3.4 Electroporation-Mediated Transfection

Electroporation is application of short electric pulses and permeabilize cellular membranes transiently. It is used for transfection of bacterial, plant, and mammalian cells with siRNA or DNA. It is also a promising method to deliver siRNA into differentiated cells. However cell toxicity is high with this technique (54, 55).

1.3.3.5 Cationic Polymers

Natural and synthetic polymers are used to mediate non-viral delivery systems. They have several advantages such as safety, ease of preparation, reproducibility, ability to carry large nucleic acid constructs and stability. Some cationic polymers are polyethylenimine nanoparticles (PEI), polyamidoamine (PAMAM), polyproprylenimine (PPI), poly-L-lysine (PLL), polyallylamine (PAA), cationic dextran, and chitosan. Most successful and widely studied one is PEI. It has membrane destabilization potential, high charge density (DNA condensation capability), and ability to protect DNA from enzymatic digestion. Cationic polymers are developed to transfer nucleic acids (i.e., DNA, siRNA) into tissue specific sites (55, 57, 58).

When chitosan and plasmid DNA multilayered structures are fabricated by layer-by-layer assembly, they show significant higher transfection efficiency at MSCs (56).

For delivery of DNA into cells Au nanoparticles and silica nanoparticles have also been the choice since the surface chemistries, physicochemical and optoelectronic properties of them are well established. Nucleic acid hybridization via base pairing and Au-thiol chemistry offer a unique opportunity (79, 80).

1.3.4 siRNA Transfection of hMSCs

Many primary cells especially stem cells are very hard to transfect and achieve gene knockdown by conventional methods. Many groups have developed techniques to deliver plasmid DNA or siRNA into hMSCs at different efficiencies. Some of them managed to transfer genes into hMSCs by viral or electroporation techniques. Although cell viability was reduced (16.5%) with Nucleofector technology in hMSCs, an efficiency of 45% were achieved which is a high transient transfection efficiency compared to other non-viral methods (61, 62, 63). Austin *et al*. managed nonviral nucleic acid (DNA and siRNA) delivery in hMSCs by using Lipofectamine 2000 with 50% efficiency. This was the first demonstration of efficient nonviral, liposomal delivery of both nucleic acids (DNA and siRNA) into hMSCs. In this study Metafectene and Lipofectamine Plus were also tested and 22% and 13% efficiencies were obtained respectively. When transfection efficiency and cell viability were combined, the highest overall yield was achieved with Lipofectamine 2000. Adenoviruses are used as a valid tool for knockdown of target genes by siRNA however MSCs are poorly transfected by conventional adenoviruses.

It was investigated by Imaizumi et al. that GFP plasmid transfection efficiency of hMSCs with Lipofectamine 2000 is dependent on both passage number and incubation time of the cells with reagent and DNA complex before medium refreshment (78). It is evident that transfection efficincy decreases signifacantly after passage 3. The percentage of transfection efficiency of GFP to hMSCs was investigated with 3-, 6-, 18-

, and 24-hour incubations of cells with reagent and DNA complex and each passage from P1 to P4. The highest transfection efficiency was with either 3- or 6-h incubation and up to three Ps (Figure 1.12).

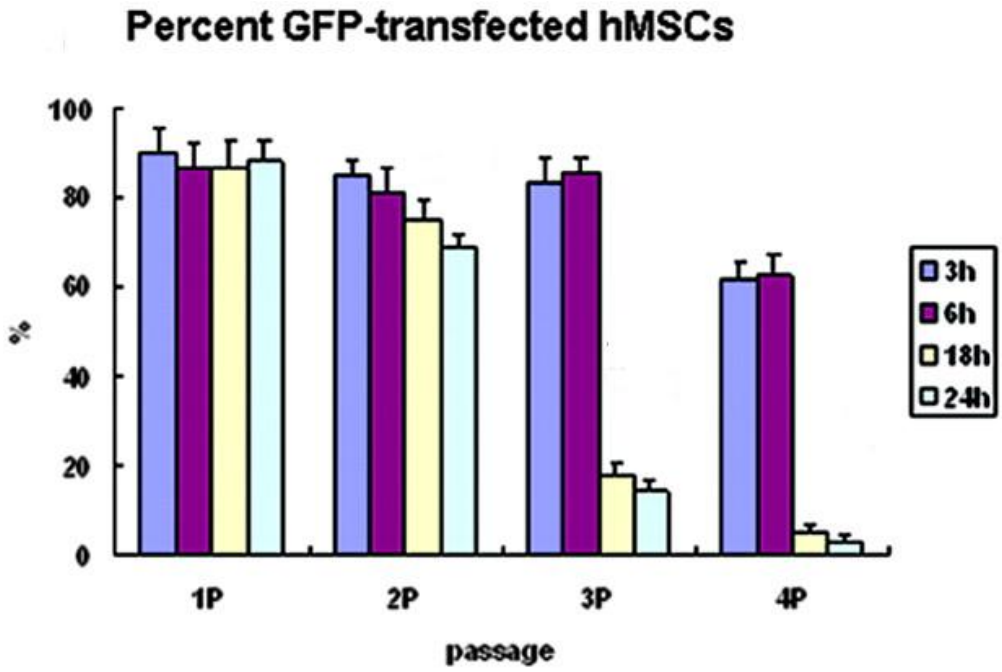


Figure 1.12 The effect of passage number and incubation time on transfection efficiency (78).

Cell proliferation after transfection was also effected by passage number. Cells at P1 proliferated significantly while any proliferation could not be observed at P2 and P3 after transfection (Figure 1.13).

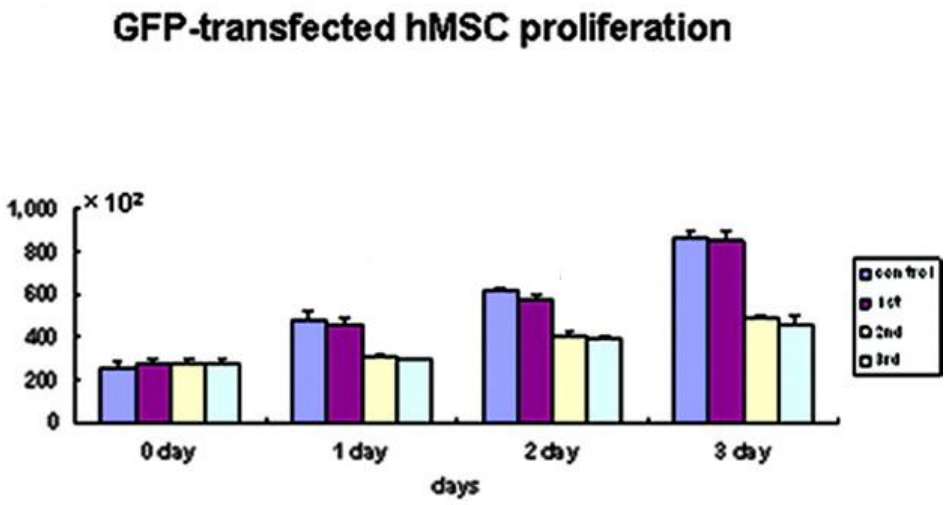


Figure 1.13 The effect of passage number on cell proliferation after transfection (78).

CHAPTER 2

MATERIALS AND METHODS

2.1 CELL CULTURE

2.1.1 Isolation of hMSCs from Bone Marrow

1:10 diluted human bone marrow aspirates were provided by Karadeniz Technical University, Department of Hematology. Human mesenchymal stem cells were isolated from bone marrow by ficoll density gradient centrifugation. 10 ml diluted bone marrow aspirate was loaded on 5 ml Ficoll (Biochrom) carefully in 15 ml falcon tube. It was

centrifuged for 25 minutes at 800g (2500 rpm) at room temperature. After centrifugation, the 4 layers are red blood cells, ficoll, mononuclear cells and serum form bottom to top. In order to get the mononuclear cells that include mesenchymal stem cells, serum part was discarded. Between serum and ficoll layers the cloudy part was removed and transferred into a new 15 ml falcon tube. The volume was completed to 10 ml with Dulbecco's modified Eagle's medium with low glucose (DMEM-LG, Gibco) and centrifuged at 350g (1500 rpm) for 10 minutes. Supernatant was discarded and cells at the pellet in 0.5 ml medium were resuspended by flicking and pipetting. In order to get rid of the ficoll completely cells were washed with medium again by centrifugation. Again the pellet was resuspended and 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 0.1 mg/ml Primocin (InvivoGen). Cells were incubated in 5% CO_2 incubator at 37^oC and the screw cap of the flask was kept loose in order to allow circulation of carbon dioxide into flask. Until the first subculture, medium was refreshed at 3 days intervals. Non-adherent cells are removed at these medium refreshments. When sufficient colonies were formed after 10-14 days, first subculture was carried out.

2.1.2 Seeding and Subculture of hMSCs

The growth of cells were observed under microscope and when cells became 80-90% confluent they were subcultured. First of all, medium, trypsin, FBS and PBS were warmed to 37 C in water bath. For subculturing the media from the culture flask was aspirated with the help of sterile pipette and cells were washed with 5 ml of Calcium and Magnesium free Phosphate Buffered Saline (PBS, Biochrom) to remove residual medium. After aspirating this PBS, 4 ml of 0.25% Trypsin/EDTA (GIBCO) pre-warmed to 37 C was added and cell surfaces were exposed to it for 1-2 minutes. Cells were observed under microscope for detachment from the bottom. When most of the cells were detached from the surface 1 ml of FBS was added to the flask to inactivate the trypsin. Content of the flask that contains detached cells was centrifuged at 1500 rpm for 10 min at room temperature. Pellet was kept and after suspending it in 10 ml

DMEM second centrifugation was done in order to get rid of trypsin completely. Pellet was resuspended in 1-5 ml DMEM and cells were counted on hemocytometer. From this cell suspension, hMSCs were seeded at a density of 1500 cells/cm^2 with 15% hMSCs qualified FBS containing DMEM for expansion. Subculture was repeated at about 5-6 days intervals. Between two passages medium was refreshed one times. Cells were used at 3-5 passages for differentiation and transfection experiments.

2.1.3 Seeding and Subculture of Human Embryonic Kidney 293 (HEK-293) Cells and Human Umbilical Vein Endothelial Cells (HUVEC)

Similar subculture procedure was applied to HUVEC and HEK 293 cells. However after subculture for expansion, 10000 cells/cm^2 were seeded in 10% FBS (Biochrom) containing DMEM.

2.1.4 Freezing and Thawing of Human Embryonic Kidney 293 (HEK-293) Cells and Human Umbilical Vein Endothelial Cells (HUVEC)

After subculture the pellet of cells was resuspended in FBS instead of DMEM. The cell density was 1-2x10^6 cells/ml. Cryovial tubes were placed on ice and 900 µl cell suspension was delivered into each tube. 100 µl DMSO (Dimethyl Sulfoxide, Applichem) was added into each tube drop by drop and after each drop the tube was mixed. Tubes were left at -20^{o}C for 1 hour and then transferred to liquid nitrogen tank (-196 ^{o}C) for storage.

In order to thaw and seed the frozen cells the cryovials are transferred to 37^{o}C water bath. 10 ml pre-warmed DMEM was delivered into falcon tubes. As soon as the contents of the vial came in liquid state they were transferred to the tube containing medium. They were mixed and centrifuged at 1500 rpm for 10 minutes. The

supernatant was discarded and the pellet of the cells was resuspended in 10 ml medium and centrifugation was repeated in order to get rid of DMSO. Then cells were seeded with 15% FBS containing DMEM and on the next day media was refreshed in order to get rid of dead cells.

2.2 TRANSFECTION

2.2.1 Transfection with Lipofectamine 2000 Reagent

Plasmid DNA or siRNA transfections of HEK-293, HUVEC and hMSCs with Lipofectamine 2000 (Invitrogen) were carried out according to manufacturer's instructions. The day before transfection cells were seeded into 96-well culture plate with complete medium at 90-95% confluence (10000 HEK-293/well, 10000 HUVEC/well, and 8000 hMSCs/well). On the day of transfection, medium was refreshed with 100 µl antibiotic free, 10% (v/v) FBS containing medium. Appropriate amount (0.2 µl or 0.4 µl or 0.6 µl) of Lipofectamine 2000 was diluted in 25 µl Opti-MEM® I Reduced Serum Medium (Gibco) without serum. The mixture was mixed gently and incubated for 5 min at room temperature. At plasmid DNA transfections, Green Fluorescent Protein encoding plasmid DNA was used. 0.5 µl plasmid DNA (0.5 µg/µl) was diluted in 25 µl Opti-MEM I by mixing gently. Diluted plasmid DNA and diluted reagent were mixed in one tube and incubated for 20 minutes at room temperature to allow the DNA-Lipofectamine 2000 complexes to form. 50 µl of DNA-Lipofectamine 2000 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 hours. After 12 hours incubation, the medium containing complex was removed and replaced by complete medium. Transfection efficiency was observed under fluorescent microscope (Carl-Zeiss). Alexa Flour 488 labelled siRNAs (Qiagen) were used for siRNA transfection. In that case 5 µl siRNA (1µM) was diluted in 25 µl Opti-MEM I. Same procedure as plasmid transfection was followed at next steps.

2.2.2 Transfection with Lipofectamine RNAiMAX Reagent

siRNA transfections of HEK 293, HUVEC and hMSCs with Lipofectamine 2000 (Invitrogen) were carried out according to manufacturer's instructions. The day before transfection cells were seeded into 96-well culture plate with complete medium at 30-50% confluence. ($3x10^3$ HEK 293/well, $3x10^3$ HUVEC/well, and $2x10^3$ hMSCs/well). On the day of transfection, medium was refreshed with 100 µl antibiotic free, 10% (v/v) FBS containing medium. Appropriate amount (0.1 µl or 0.2 µl or 0.3 µl) of Lipofectamine RNAiMAX was diluted in 10 µl Opti-MEM® I Reduced Serum Medium (Gibco). The mixture was mixed gently. 1.2 µl Alexa Flour 488 labelled siRNA (µM) was diluted in 10 µl Opti-MEM I by mixing gently. For silencing topo II IIβ 2 different validated siRNAs (TOP2B_5 and TOP2B_6 from Qiagen) were used. Sequences of TOP2B_5 and TOP2B_6 are TCGGGCTAGGAAAGAAGTAAA and CAGCCGAAAGACCTAAATACA respectively. MAPK1 spesific siRNA with a sequence of AATGCTGACTCCAAAGCTCTG was also used as control. Diluted siRNA and diluted reagent were mixed in one tube and incubated for 20 minutes at room temperature to allow the siRNA-Lipofectamine RNAiMAX complexes to form. 20 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 hours. After 12 hours incubation, the medium containing complex was removed and replaced by complete medium. Transfection efficiency of labeled siRNA was observed under fluorescent microscope (Carl-Zeiss).

2.2.3 Transfection with FuGENE HD Reagent

Plasmid DNA transfections of HEK-293 and hMSCs with FuGENE HD (Roche) were carried out according to manufacturer's instructions. The day before transfection, cells were seeded into 96-well culture plate with complete medium at 80-90% confluence. ($1x10^4$ HEK 293/well, and $8x10^3$ hMSCs/well). On the day of transfection, medium was refreshed with 100 µl antibiotic free, 10% (v/v) FBS containing medium. 1 µl plasmid DNA (0.5 µg/µl) was diluted in 25 µl Opti-MEM I by mixing gently.

Appropriate amount (0.5 µl or 1 µl or 1.5 µl) of FuGENE HD was pipetted directly into the medium containing the diluted DNA without allowing contact with the walls of plastic tubes. The mixture was mixed vigorously and incubated for 15 minutes at room temperature to allow the DNA-FuGENE HD complexes to form. 5 µl of DNA-FuGENE HD complexes were added dropwise into the wells containing cells and medium,. Cells were incubated at 37°C in CO_2 incubator for 24-48 h. After 12 hours incubation the medium containing complex was removed and replaced by complete medium. Transfection efficiency was observed under fluorescent microscope (Carl-Zeiss).

2.2.4 Transfection with PrimeFect siRNA Reagent

siRNA transfections of HUVEC and hMSCs with PrimeFect (Lonza) were carried out according to manufacturer's instructions. For silencing topo IIβ, 2 different validated siRNAs (TOP2B_5 and TOP2B_6 from Qiagen) were used. Sequences of TOP2B_5 and TOP2B_6 are TCGGGCTAGGAAAGAAGTAAA and CAGCCGAAAGACCTAAATACA respectively. MAPK1 spesific siRNA with a sequence of AATGCTGACTCCAAAGCTCTG was also used as control. The day before transfection, cells were seeded into 24-well culture plate with complete medium at 50% confluence. ($1x10^4$ HUVEC/well and $8x10^3$ hMSCs/well). On the day of transfection, medium was removed from cells and replaced with 250 µl antibiotic free, 10% (v/v) FBS containing fresh medium. 0.5 µl PrimeFect siRNA Transfection Reagent was diluted in 50 µl PrimeFect Diluent and mixed by flicking. Mixture was incubated at room temperature for 15 minutes. Then 0.75 µl from each siRNA (10 µM) was added and components were mixed by pipetting. The mixture was incubated at room temperature for 15 minutes to allow the siRNA-reagent complexes to form. To the wells containing cells and medium, 50 µl of siRNA-reagent complex was added dropwise by rocking the plate side-to-side to distribute the reagents evenly. Cells were incubated at 37°C in CO_2 incubator for 24-48 hours. After 12 hours incubation the medium containing complex was removed and replaced by complete medium. Silencing efficiency was observed by RT-PCR

2.3 NEURAL TRANSDIFFERENTIATION OF hMSCs

2.3.1 Neural Differentiation with HyClone Advancestem Neural Differentiation Kit

The day before induction 10^5 cells per 25 cm^2 culture plate (Falcon BD) was seeded in expansion medium. On the day of induction, the expansion medium was replaced with 4 ml basal HyClone Neural Differentiation Medium (Thermo Scientific) including 10% HyClone AdvanceSTEM Growth Supplement (Thermo Scientific). This induction medium was refreshed at each 48 hours. Morphologies of the cells are observed during 14 days.

2.3.2 Neural Differentiation with N3 Cytokine Combinations

The day before induction 10^5 cells per 25 cm^2 culture plate (Falcon BD) was seeded in expansion medium. On the day of induction 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), 0.5 mM IBMX (3-isobutyl-1-methylxanthine, SIGMA), 20 ng/ml hEGF (human epidermal growth factor, SIGMA), 40 ng/ml rhFGF (recombinant human fibroblast growth factor, R&D systems), 10 ng/ml FGF-8 (fibroblast growth factor-8, PeproTech), 10 ng/ml rhBDNF (recombinant human brain-derived neurotrophic factor, R&D systems) and 2mM L-Glutamine (GIBCO). This induction medium was refreshed at each 72 hours. Morphologies of the cells were observed under light microscope during 14 days and neural marker expressions were monitored at mRNA level with RT-PCR.

2.4 REVERSE TRANSCRIPTION PCR

siRNA silencing was monitored at the mRNA level. RT-PCR was used to determine the change in mRNA concentration in various siRNA treated samples. From

the samples RNA isolation was done by total RNA isolation kit (Qiagen) according to manufacturer's instructions. Then cDNAs were synthesized by Quantitect reverse transcription kit (Qiagen).These cDNAs were multiplied by PCR with qiagen PCR core kit.

Reverse transcription master mix was prepared on ice by adding 1 µl RT Primer Mix, 4 µl Quantiscript RT Buffer, and 1 µl Quantiscript Reverse Transcriptase to the 14 µl RNA mixture. The mixture was incubated 30 minutes at 42 °C first, and then incubated 3 minutes at 95°C. At this reverse-transcription reaction first strand cDNA was synthesized and this cDNA was used at PCR.

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 minutes at 94 °C for initial incubation, 30 seconds at 94 °C for denaturation, 45 seconds at 60 °C for annealing of topo IIβ primer, 1minute at 72 °C for extension and 7 minutes at 72 °C final incubation. The PCR was done for 30 cycles. Thermocycler Techne was used for amplification reactions. The primers used were TOP2B forward, 5'-AGCATCTGGCTCTGAAAATGA-3', TOP2B reverse, 5'-TGGCTCAGTAGGGAAGT-CTGA-3' yielding a 139 bp PCR product, TOP2B forward, 5'-ATCAAAAGCCACTC-CAGAAAAATC-3', reverse, 5'-AGAAGGT GGCTCAGTAGGGAAGTC-3' yielding a 508 bp PCR product, MAPK1 forward, 5'-CCCAAATGCTGACTCCAAAGC-3', reverse, 5'-GCTCGTCACTCGGGTCGTAAT-3', and BETA actin?? Forward 5'-CGCA-CCACTGGCATTGTCAT-3', reverse, 5'-GTGGCCATCTCCTGCTCGAA-3'. Primers for neural markers also were NSE forward, 5'-CCCACTGATCCTTCCCGATACAT-3', reverse, 5'-CCGATCTGGTTGACCTTGAGCA-3'; Nestin forward, 5'-TGGCTCA-GAGGAAGAGTCTGA-3', reverse, 5'-TCCCCCATTTACATGCTGTGA-3', Beta-tubulin III forward, 5'-AGTGATGAGCATGGCATCGA-3', reverse, 5'-AGGCAGTC-GCAGTTTTCACA-3'.

Amplification products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. % 2 (w/v) agarose gel was prepared by boiling agarose (Sigma) in 0.5 X TBE buffer (Fluka) solution. Then it was cooled and 5µl/80ml Ethidium Bromide was added. Just before it solidifies, it was poured into 13 x 14x 0.5 horizontal agarose gel platform and then comb is placed in order to generate 20 wells. 100 bp DNA Ladder (Bioron) was used as molecular size marker. Gel was run at 110 V for 45 min.

2.5 IMMUNOFLOURESCENT STAINING

hMSCs were seeded into 24-well culture plate for immunofluorescence staining. At the day of staining medium was aspirated from wells and cells were permeabilized with warmed (37°C) 500 µl/well TZN buffer (10 mM pH 7.5 Tris-HCl, 0.5% Nondet P40, 0.2 mM $ZnCl_2$) for 15 min and fixed with 500 µl/well 4% Paraformaldehyde / PBS for 10 min at RT. Wells were washed with 750 µl/well PBS (Phosphate Buffered Saline, pH 7.4, SIGMA) 3 times, 5 minutes at each time on rocking shaker. Cells were blocked with 500 µl/well 10% Normal Goat Serum (Gibco) and 10% Normal Horse Serum (Biochrom) in 0.3% TritonX / PBS (PBS-Tx) for 30 minutes at RT. After that, cells were treated with 60 µl/well specific primary antibodies for target proteins for 2 h at room temperature. Antibodies were diluted in PBS-Tx with 3% NHS. Following 3 washing steps with PBS, cells were then treated with 50 µl/well Alexa Fluor labeled antimouse or antirabbit secondary antibodies at room temperature for 1 hr. Cells were washed 3 times with PBS and treated with 150 µl/well, 1/15000X DAPI (Sigma) for 10 minutes and after final washings with PBS and 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 (Carl-Zeiss) 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.

CHAPTER 3

RESULTS

3.1 SURFACE MARKERS OF BONE MARROW CELLS

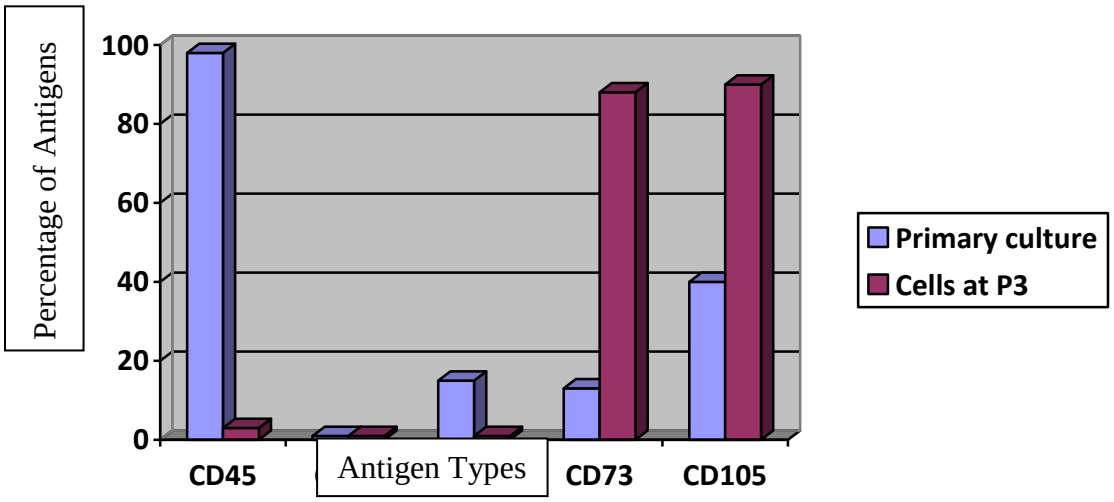


Figure 3.1 FACS (Fluorescent activated cell sorter) analysis of bone marrow derived cells.

At passage three most of the cells (about 90%) express stromal markers (CD73 and CD90) where as expression of hematopoetic markers (CD45, CD34, and HLADR) is less than 5%.

3.2 hMSCs IN CULTURE

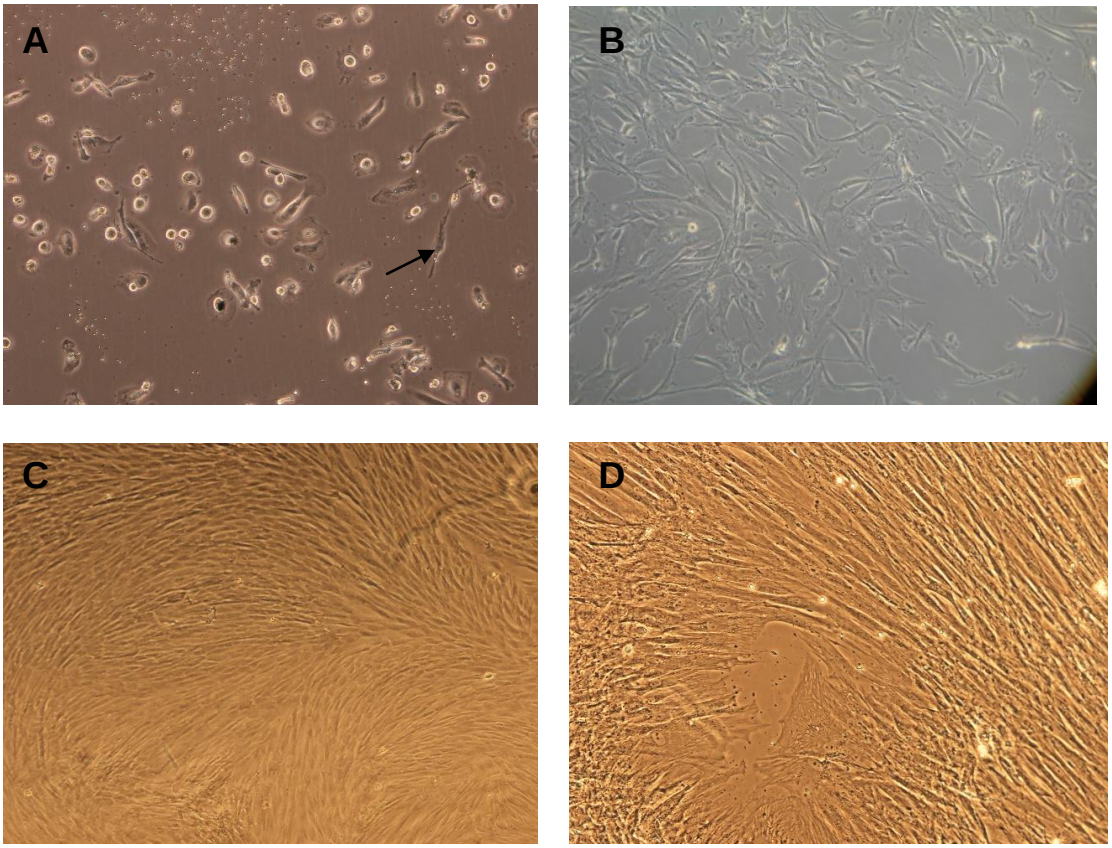


Figure 3.2 Light microscope images of primary hMSCs in culture flask surface.

A. Primary culture including a mixture of adherent cells, **B.** Proliferating primary culture **C.** and **D.** hMSCs colonies with fibroblastic morphology at primary culture.

All images are taken under 10X magnification.

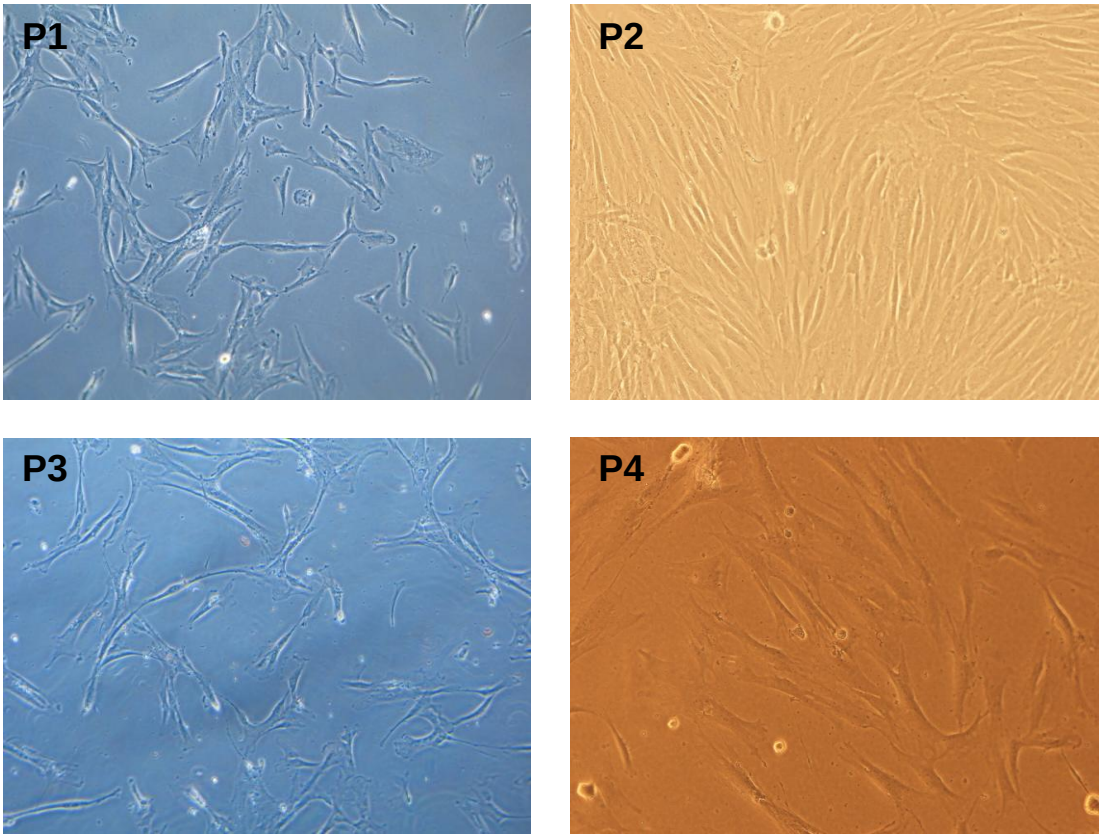


Figure 3.3 Light microscope images of hMSCs at different passages (P1-P4: refer to the passage numbers of hMSCs). hMSCs maintain their fibroblastic morphology during their subculture.

Images are at 10X magnification.

3.3 GFP PLASMID TRANSFECTION

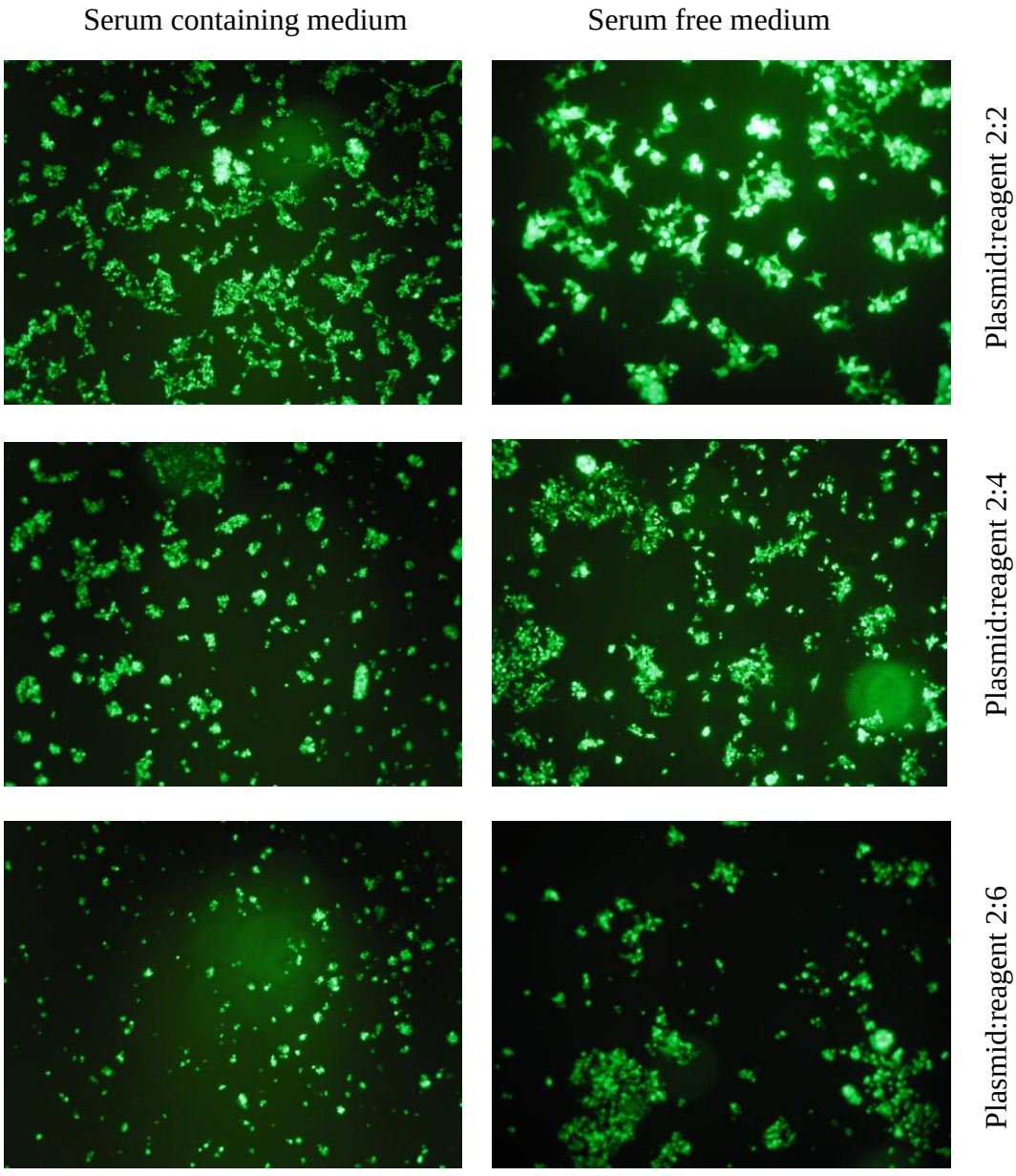


Figure 3.4 GFP plasmid transfection of HEK 293 cells with Lipofectamine 2000 transfection reagent. Transfections are carried out at different reagent:plasmid ratios and serum containing and serum free conditions. About 90% transfection effifciency was achieved. More cytotoxic effect was observed at serum free transfections.

Cytotoxicity increases as the ratio of the reagent increases. Photographs were taken at 4X, 48 hours after transfection.

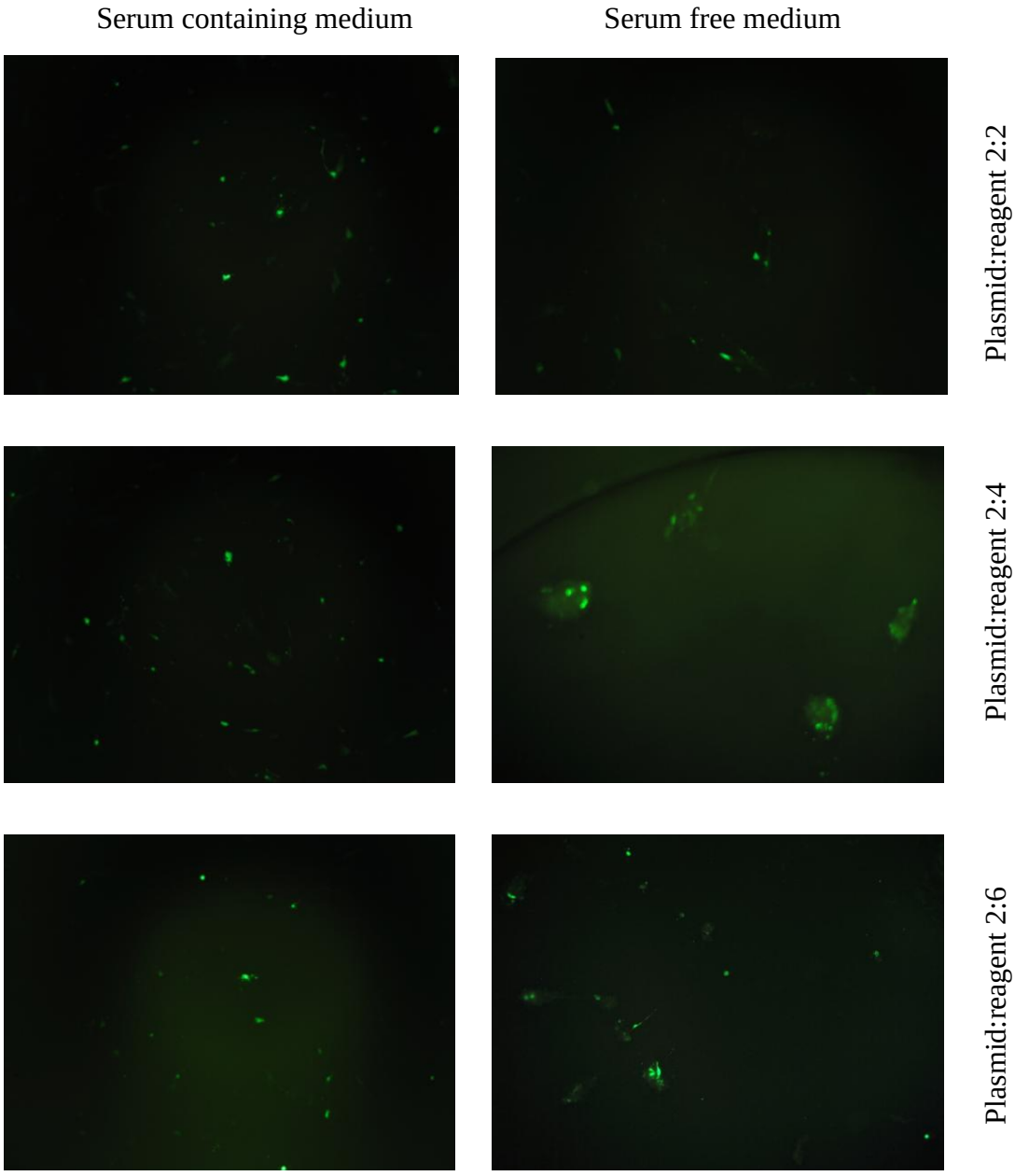


Figure 3.5 GFP plasmid transfection of hMSCs with Lipofectamine 2000 transfection reagent. Transfections are carried out at different reagent:plasmid ratios and serum containing and serum free conditions.

About 10% transfection effifciency was achieved. More cytotoxic effect was observed at serum free transfections. Cytotoxicity increases as the ratio of the reagent increases. Photographs were taken at 4X, 48 hours after transfection.

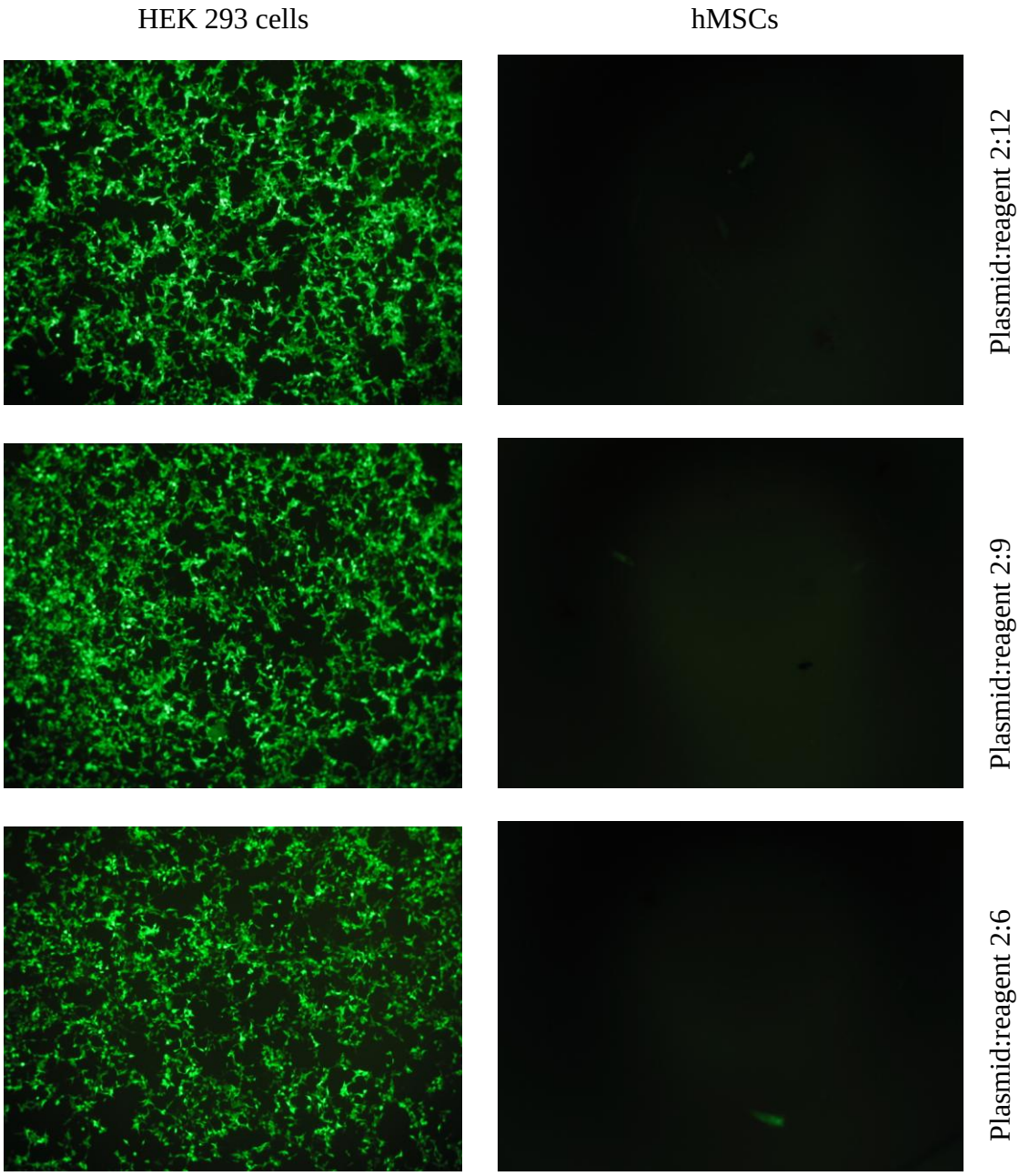


Figure 3.6 GFP plasmid transfection of HEK 293 and hMSCs with FuGENE transfection reagent. Transfections are carried out at different reagent:plasmid ratios.

Transfection efficiency at HEK 293 cells is almost 100%, while it is at most 5% at hMSCs. All transfections are carried out in serum containing medium. Photographs were taken at 4X, 48 hours after transfection.

3.4 siRNA TRANSFECTION

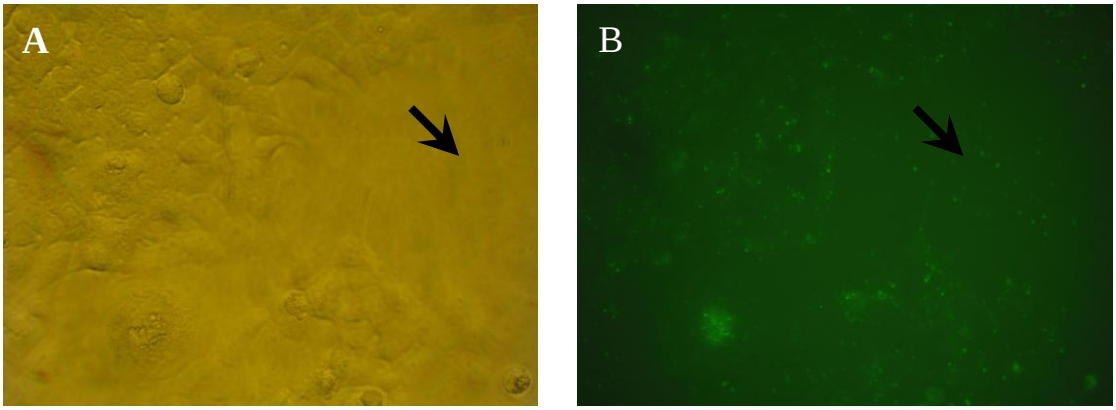
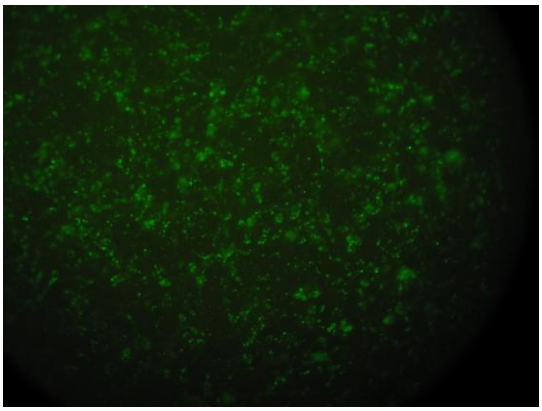


Figure 3.7 Alexa Flour488 labelled siRNA transfection of HEK 293 and hMSCs with Lipofectamine RNAiMAX transfection reagent.

A. HEK 293 cells under light microscope (20X); **B.** Transfected HEK 293 cells under fluorescence microscope (20X). Although transfection efficiency is almost 100%, there is nonspecific illumination at cell lacking places. Also any transfection was not observed at hMSCs.

HUVEC hMSCs

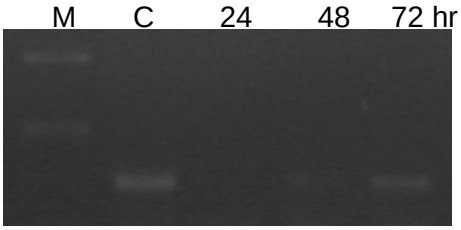


No transfection

Figure 3.8 Alexa Flour488 labelled siRNA transfection of HUVEC and hMSCs with Lipofectamine 2000 transfection reagent. Transfected HUVEC under fluorescence microscope (4X).

Transfection efficiency is almost 100% at HUVEC. However any transfection was not observed at hMSCs.

A



B

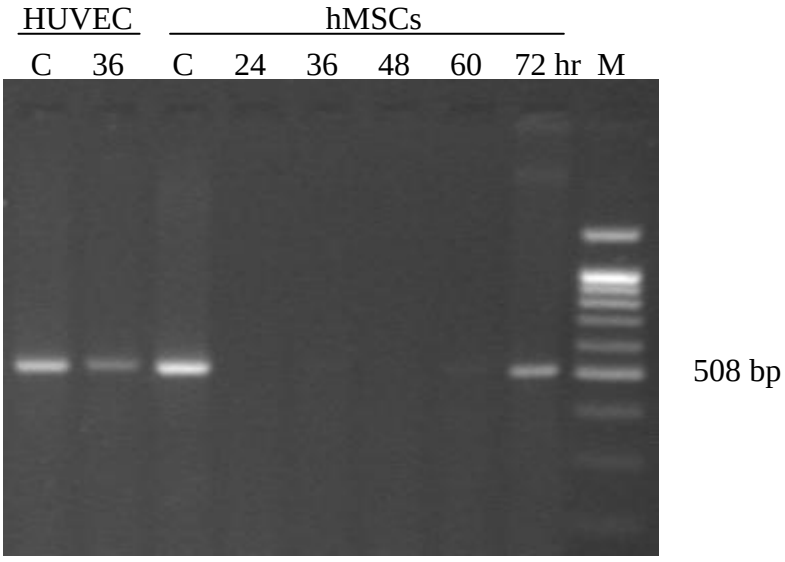
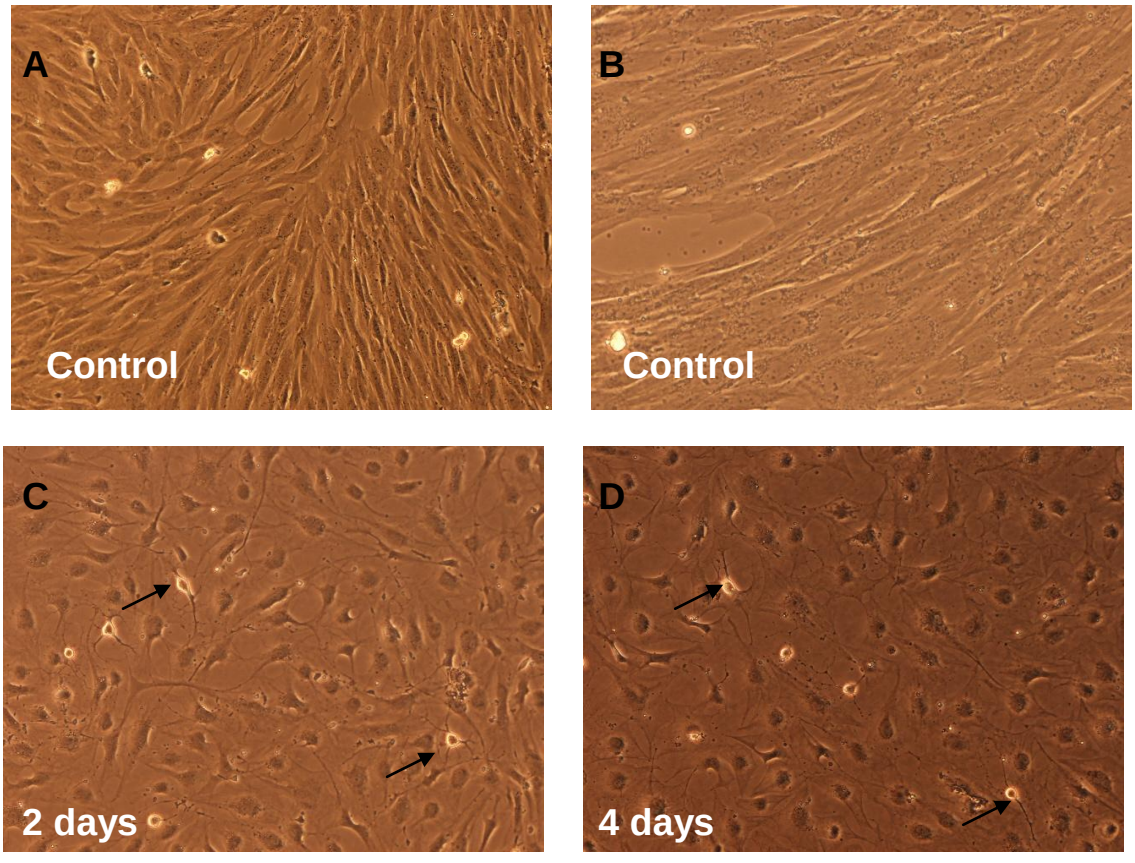


Figure 3.9. Transfection of hMSCs with TOP2β siRNA by LipofectamineRNAiMAX Transfection Reagent.

A. Reverse transcriptase PCR with TOP2B primer that yields 139 bp PCR product **B.** Reverse transcriptase PCR with TOP2B primer yielding 508 bp PCR product (C: Control; 24, 36, 48, 60, 72: hours after siRNA transfection; M: DNA size marker). .

Silencing is achieved for both primers after 24-48 hours. However slightly different results were obtained with each primer at further hours; Topo IIβ band appears at 48 hr with the first primer (139 bp) where it appears at 60 hr with other primer (508 bp).

3.5 NEURAL DIFFERENTIATION WITH HYCLONE KIT



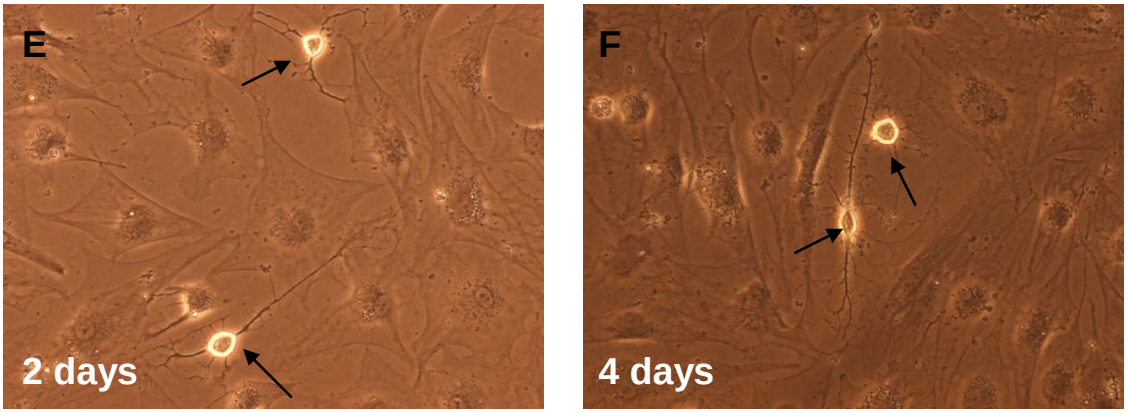
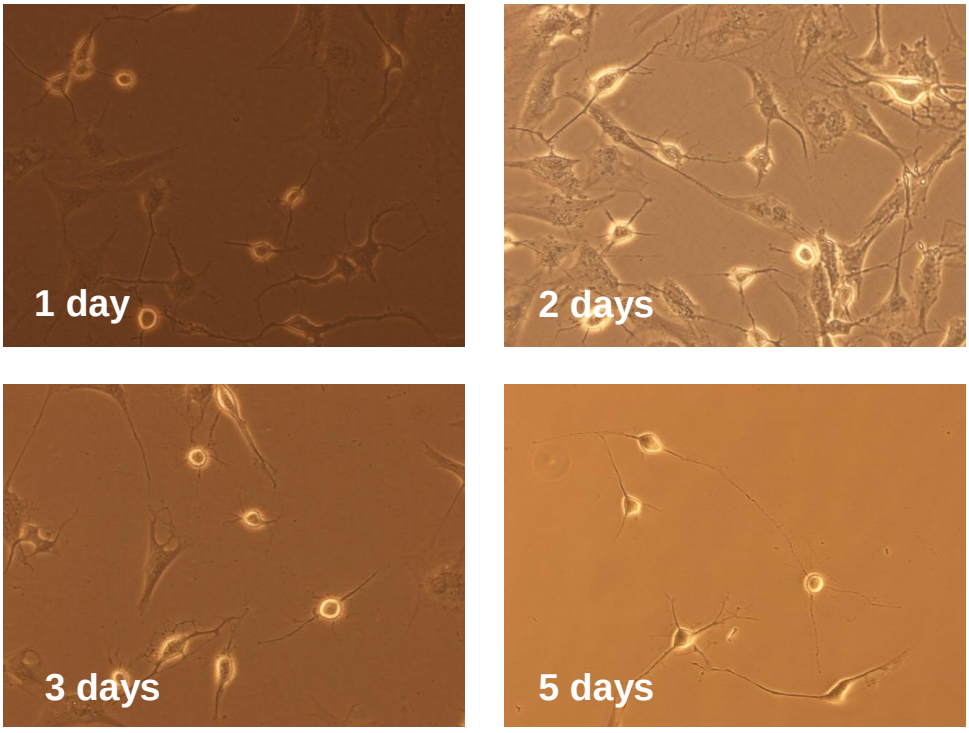


Figure 3.10 **N**eural differentiation of hMSCs with HyClone Advancestem neural differentiation kit. **A.** Control in expansion medium (10X) **B.** Control in expansion medium (20X) **C.** 2 days after induction (10X); **D.** 4 days after induction (10X) **E.** 2 days after induction (20X) **F.** 4 days after induction (20X). About 15% of hMSCs gained neuron-like morphology.

3.6 NEURAL DIFFERENTIATION WITH N3 CYTOKINE COMBINATIONS



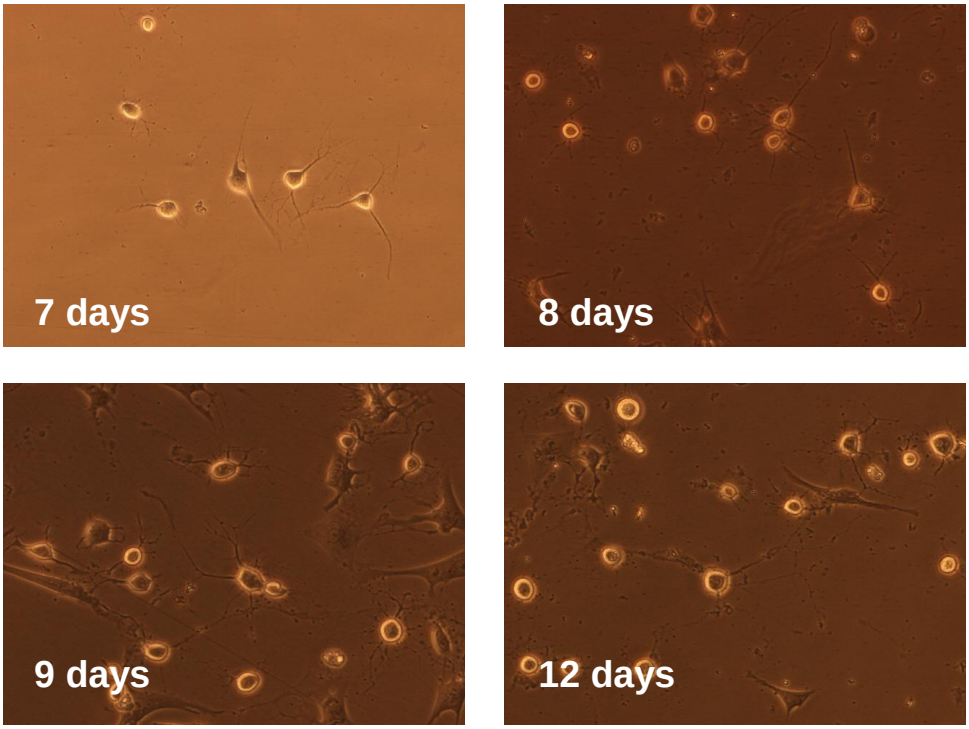
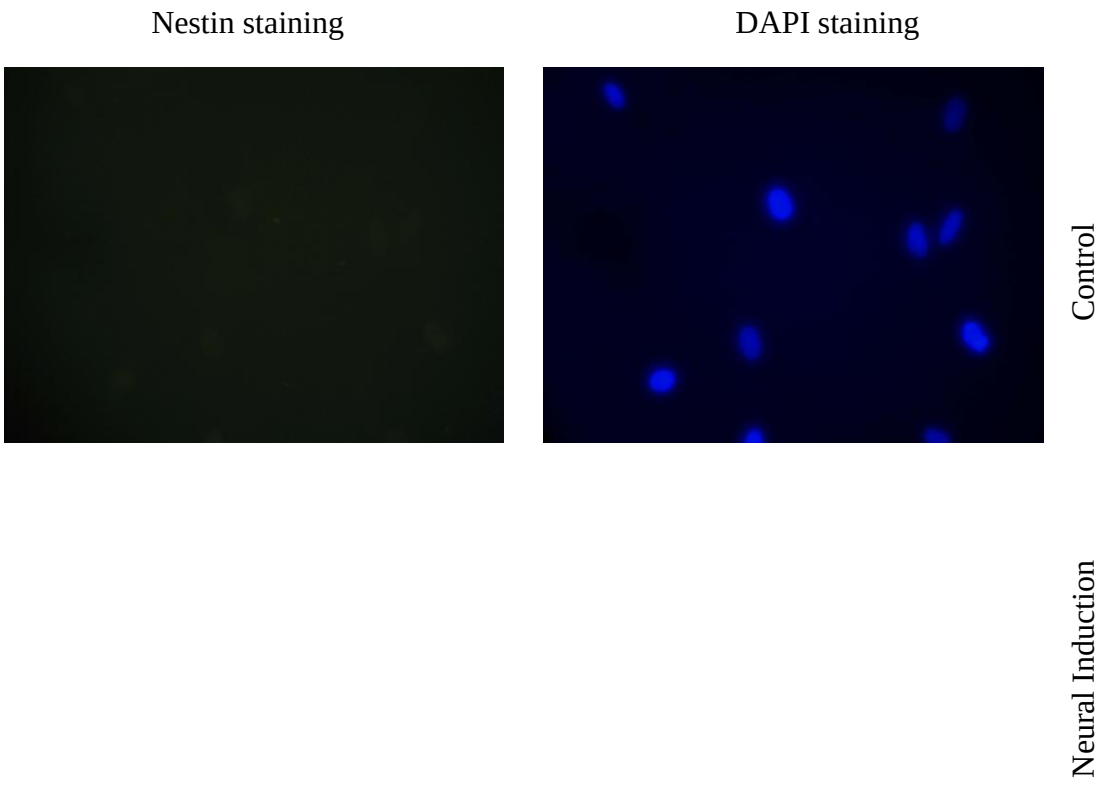


Figure 3.11 Neural differentiation of hMSCs with N3 cytokine combinations was observed at different days under light microscope. Images are at 20X.



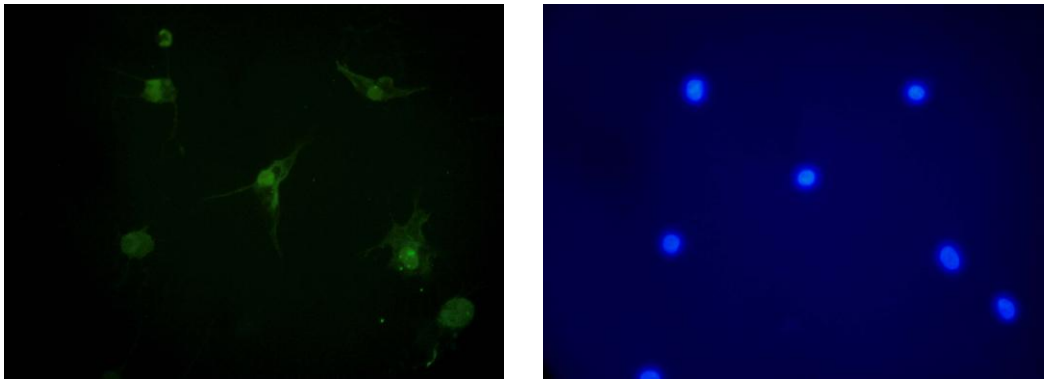


Figure 3.12 Immunofluorescent staining of hMSCs for Nestin expression 4 days after neural induction with N3.

A significant increase in Nestin expression level is observed in differentiated cells. All images are taken at 40X.

NSE staining

DAPI staining

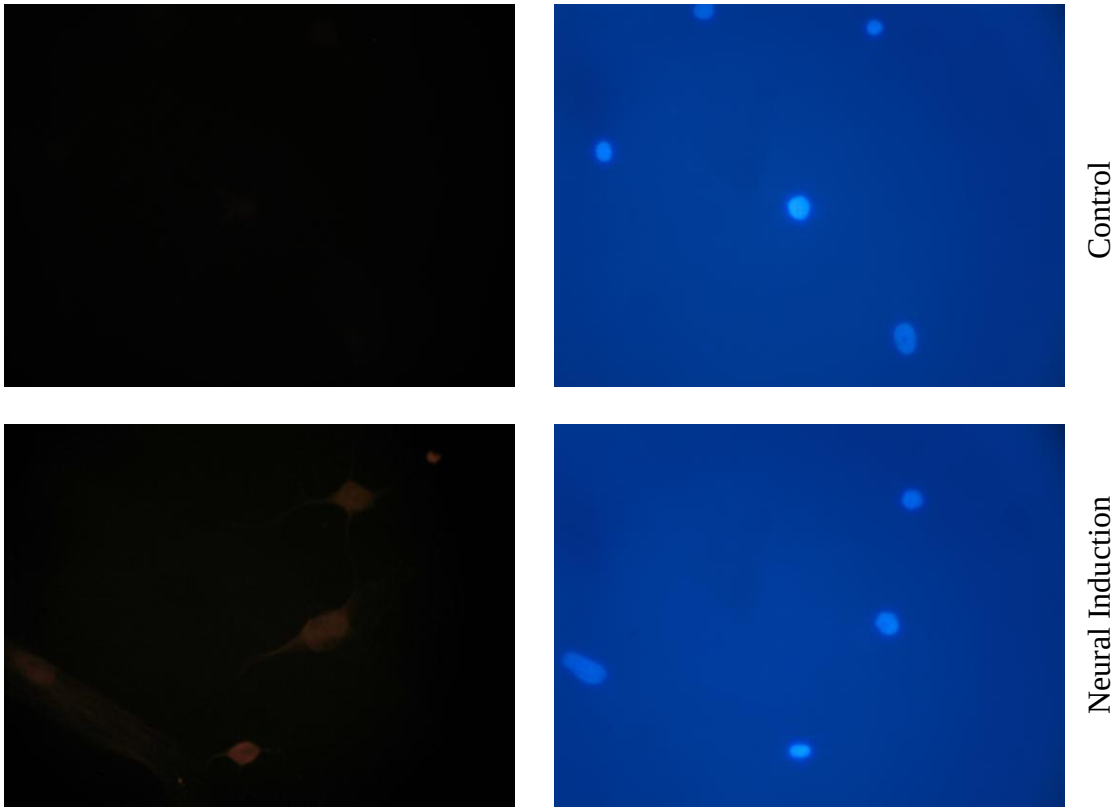


Figure 3.13 Immunofluorescent staining of hMSCs for NSE expression 4 days after neural induction with N3.

A significant increase in NSE expression level is observed in differentiated cells. All images are taken at 40X.

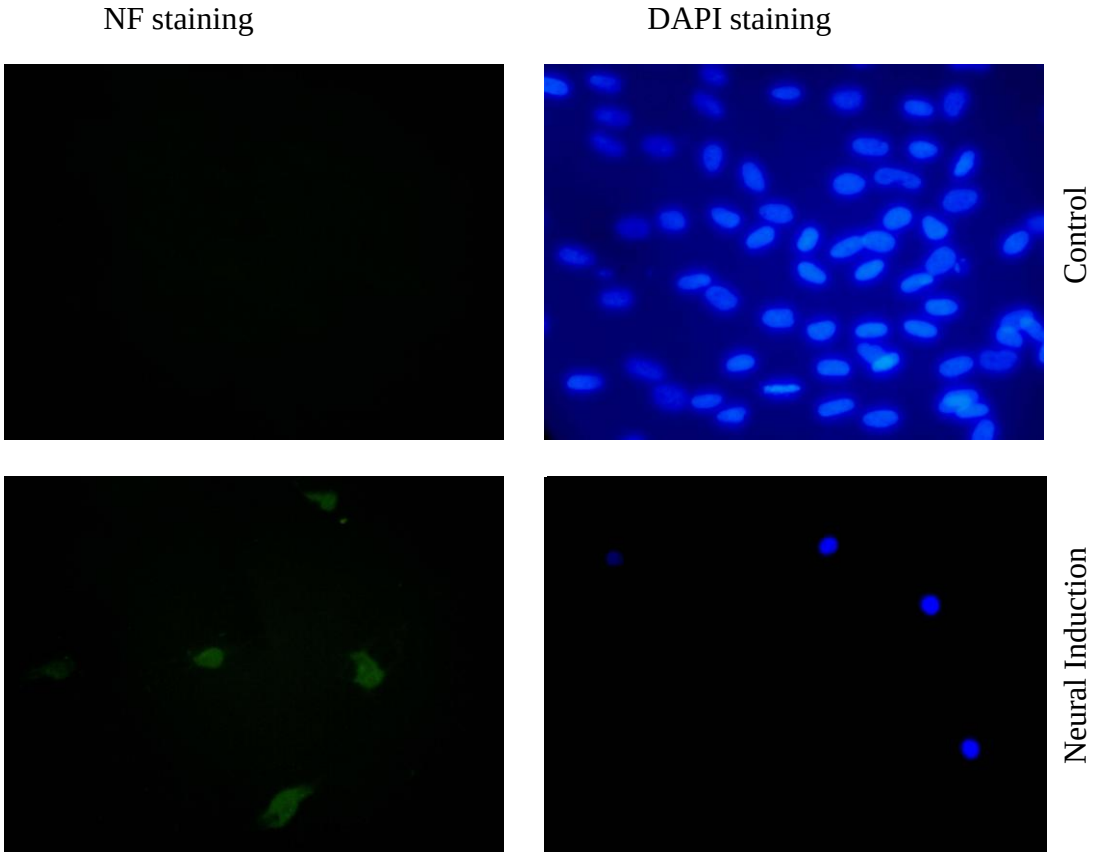


Figure 3.14 Immunofluorescent staining of hMSCs for NF expression 14 days after neural induction with N3.

A significant increase in NF expression level is observed in differentiated cells. All images are taken at 40X.

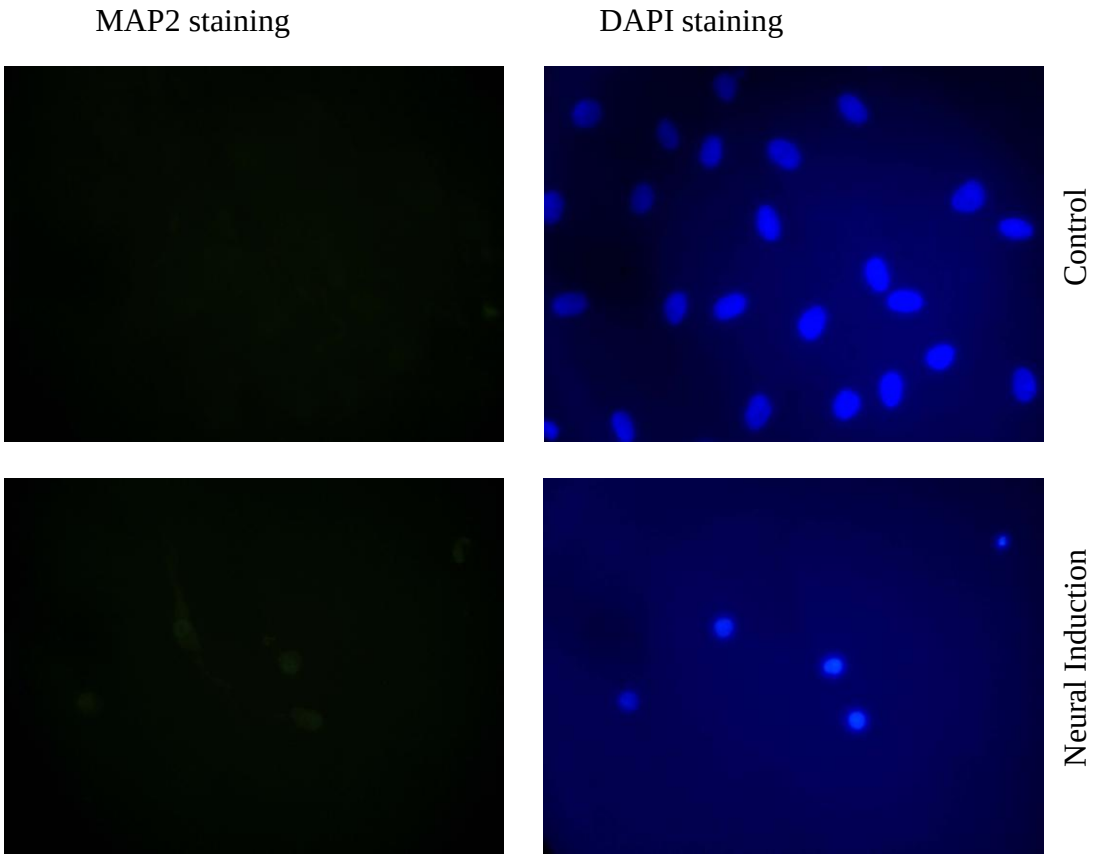


Figure 3.15 Immunofluorescent staining of hMSCs for MAP2 expression 14 days after neural induction with N3.

A significant increase in MAP2 expression level is observed in differentiated cells. All images are taken at 40X.

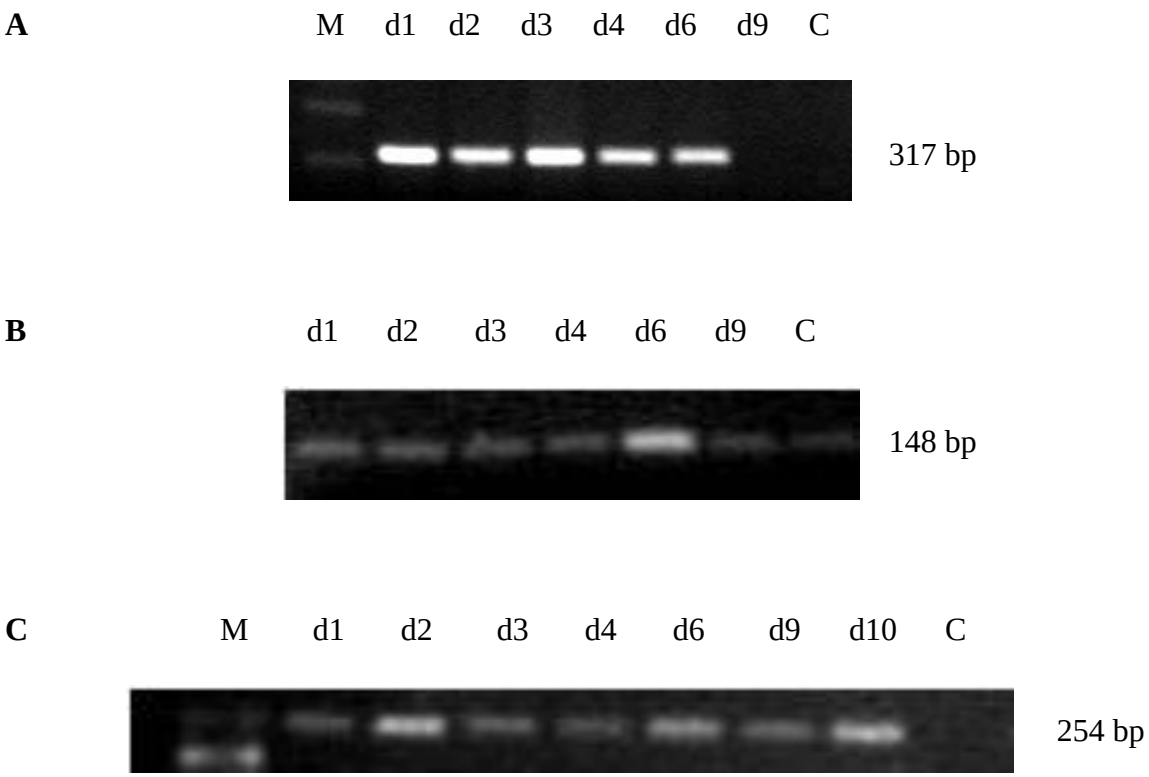


Figure 3.16 Reverse transcriptase PCR results of early markers during neural differentiation with N3.

A. Beta-III Tubulin 317 bp **B.** Nestin 148 bp, **C.** NSE 254 bp In samples at different days of induction early neural markers are expressed at higher level with respect to control samples.

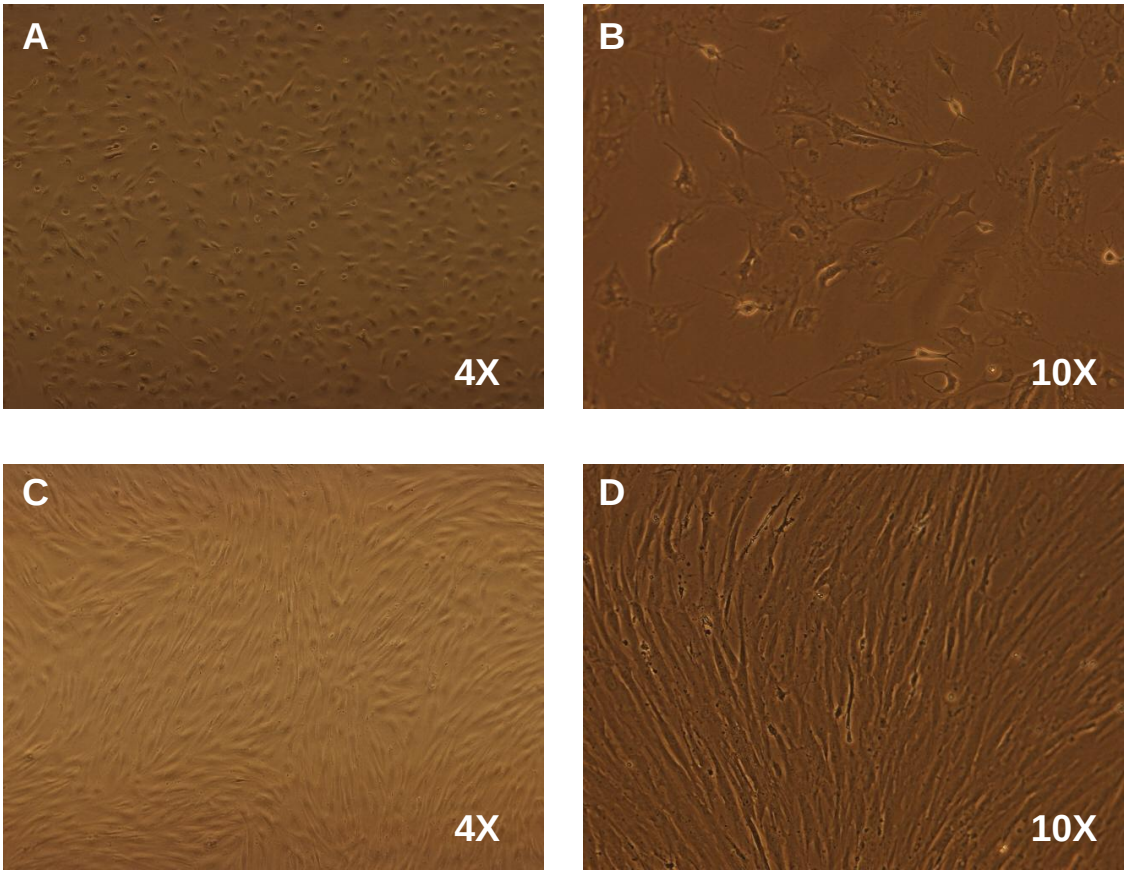


Figure 3.17 Removal of induction medium after 8 days results in loss of neuronal morphology and re-proliferation is observed.

A. Differentiated cells before removal of induction medium , **B.** Differentiated cells before removal of induction medium, **C.** Induced cells before removal of induction medium, **D.** Induced cells after removal of induction medium.

CHAPTER 4

DISCUSSION & CONCLUSION

Mesenchymal stem cells can be isolated from bone marrow aspirates by ficoll density gradient centrifugation. After this centrifugation when mononuclear cell mixture is seeded in culture flask, mesenchymal stem cells adhere to the surface. At medium refreshments non-adherent cells diminish. When adhered cells are observed under microscopy, a non-homogenous culture is recognized; round, flat cells and some short, thick cells predominate at this culture. If there are mesenchymal stem cells in the culture , spindle-like, fibroblastic cells form colonies. During subculture the first detaching cells from the surface are mesenchymal stem cells at trypsin treatment. This property provides to obtain more homogenous hMSCs culture after each subculture. At third passage mesenchymal stem cells are pure enough to be used at experiments according to FACS results. They are mostly healthy until passage 14-15 and can be used at differentiation and transfection. After passage 14, their growth rate decreases and they start to lose their fibroblastic morphology in our culture conditions; they get aged and become large, flat cells.

MSCs have neural transdifferentiation ability *in vitro*. During this process many genes are up regulated while some are down regulated. It is known that topo IIβ plays a, crucial role at transcription (81). It was also discovered that level of topo IIβ enzyme increases during neural differentiation (18). However it’s exact function at neural

differentiation of hMSCs is not obvious yet. Also there are not any studies which clarify the function of topo IIβ in the presence of topo IIα. All studies till now have chosen the cell types such as primary neurons which express only topo IIβ. There might be a possibility that topo IIβ or topo IIα may substitute each other in cellular functions. That is why in this study we deal with hMSCs expressing both topo IIα and topo IIβ enzymes and intend to reveal topo IIβ function. For this purpose we attempted to silence topo IIβ specifically since commercial topoisomerase II inhibitor drugs inhibit both isoforms of topoisomerase II (topo IIα and topo IIβ).

As many primary cells and stem cells, mesenchymal stem cells are hard to transfect with respect to cell lines. In this study four reagents were used in order to obtain desired silencing at hMSCs. With Lipofectamine 2000 and FuGENE HD transfection reagents almost 100% efficiency was observed at GFP plasmid transfection of control cell lines, HUVEC and HEK 293. However at mesenchymal stem cells, efficiency did not exceed 10%. Also carrying out transfections at serum-free medium did not cause any increase at efficiency even it increased cytotoxic effect of the reagents. These results led us to try delivering siRNA oligos since they have smaller sizes and they do not need to reach nucleus to induce RNA interference. Alexa Flour 488 labelled control siRNAs were delivered with Lipofectamine RNAiMAX and Lipofectamine 2000 to HEK 293 and HUVEC cells with about 100% efficiency. Unfortunately, this method was also unsuccessful for hMSCs. The possibility that the label at the end of oligo might be preventing efficient delivery of the siRNA into the cell made us to use non-labeled siRNAs. Topo IIβ specific siRNAs were delivered with Lipofectamine RNAiMAX to hMSCs. A significant silencing (about 95%) was observed at mRNA level of topo IIβ enzyme during 48 hrs of siRNA transfection.

Mesenchymal stem cells are pluripotent and they have capacity to differentiate into many lineages. However transdifferentiation is hard to achieve *in vitro*. When HyClone differentiation kit was used for this purpose, only 15% neuron-like morphology was observed at induced hMSCs. As an alternative differentiation method, N3 cytokine combinations including several chemicals and growth factors were used

and differentiation was obtained at higher efficiency. About 80% of hMSCs differentiated into neron-like cells. mRNA level of beta-III tubulin which is an early marker was monitored by reverse transcriptase PCR during differentiation process. It was expressed significantly at induced cells during first 6 days of the process. At day 9 it disappears indicating that it completed its work. Nestin and NSE are also expressed during differentiation. Presence of these markers is confirmed by both immunoflourescent staining and reverse transcription PCR results. Late markers, NF and MAP2 were also expressed during differentiation according to immunoflourescent results. However at this differentiation protocol when induction medium was drawn after 8 days, cells lost their neuron-like morphology and started to proliferate which indicates that the differentiation was not stable at that day. Day 8 might be an early time for terminating the differentiation and may require longer neural process *in vitro* conditions.

In this study we achieved to transfect hMSCs and also trans-differentiate hMSCs into neural-like cells with cytokine combinations. Neural-like morphology of the cells was confirmed by the neural protein expressions in immunofluorescent staining and RT-PCR methods. These results will lead us to reveal the function of topo IIβ by silencing it during neural transdifferentiation of hMSCs in our further studies.

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