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Ph.D. THESIS

ROLE OF DNA TOPOISOMERASE IIβ IN OSTEOGENIC DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS

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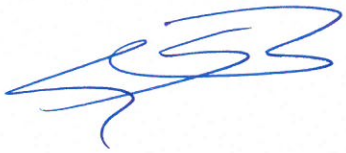

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I dedicate this PhD Thesis to My Dear Family Members and days ahead better than we left behind.

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbol	Explanation
µl	**:** Microliter
µg	**:** Microgram
h	**:** Hour
min	**:** Minute
ml	**:** Mililiter
nM	**:** Nanomolar
sec	**:** Second
#	**:** Number

Abbreviation	Explanation
ALP/L	**:** Alkaline phosphatase/liver bone kidney
ARS	**:** Alizarin red staining
BMD	**:** Bone mineral density
BMP	**:** Bone morphogenetic protein
BMSC	**:** Bone marrow derived stem cells
BSP	**:** Bone sialoprotein
cDNA	**:** Complementary DNA
CGN	**:** Cerebellar granule neuron
COL	**:** Collagen
CSC	**:** Cancer stem cell
CT	**:** Calcitonin
CTGF	**:** Connective tissue growth factor
CTSK	**:** Cathepsin K
DMEM	**:** Dulbecco’s modified Eagle’s medium
DMSO	**:** Dimethylsulfoxide
dsRNA	**:** Double stranded RNA
ECM	**:** Extracellular matrix
ESR	**:** Estrogen receptor

ESC : Embryonic stem cell
EGF : Epidermal growth factor
FBS : Fetal bovine serum
F-CFU : Fibroblast colony forming unit
FGF/R : Fibroblast growth factor/receptor
gDNA : Genomic DNA
GF : Growth factor
GFP : Green Fluorescence Protein
GI : Gastrointestinal
GVHD : Graft versus host disease
GM-CSF : Granulocyte macrophage colony stimulating factor
hMSC : Human mesenchymal stem cell
HSC : Hematopoietic stem cell
IGF : Insulin like growth factor
IL : Interleukin
iPSC : Induced pluripotent stem cell
MAPK : Mitogen activated protein kinase
M-CSF : Macrophage colony stimulating factor
mRNA : Messenger RNA
miRNA : Micro RNA
MMP : Matrix metalloproteinase
NK : Natural killer
NT : Non-treated
OBF : Osteoclast binding factor
OCN : Osteocalcin
OCIF : Osteoclast inhibitor factor
OE : Overexpression
OI : Osteogenesis imperfecta
OST : Osteogenic Differentiation
OP : Osteoporosis
OPG : Osteoprotegerin
OPN : Osteopontin
PBS : Phosphate buffered saline
pDNA : Plasmid DNA

PDGF : Platelet-derived growth factor
RCPTR : Receptor
PTH : Parathyroid hormone
RISC : RNA induced silencing complex
RNAi : RNA interference
RT-qPCR : Real time quantitative polymerase chain reaction
Runx2 : Runt related transcription factor 2
siRNA : Small interfering RNA
SC : Stem cell
TB : Toluidine blue
TF : Transcription factor
TGF-β : Transforming growth factor-beta
TNF : Tumor necrosis factor
Topo IIα/β : Topoisomerase II alpha/beta
UD : Undifferentiated
VDR : Vitamin D receptor
VEGF : Vascular endothelial growth factor
w/wo : With/Without
WT : Wild type

ÖZET

DOKTORA TEZİ

İNSAN MEZENKİMAL KÖK HÜCRELERİNİN OSTEOGENİK FARKLILAŞMASINDA DNA TOPOİZOMERAZ IIβ'NIN ROLÜ

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Memeli hücrelerinde transkripsiyonel düzenleme bir dizi enzim ve protein aktivitesi ile sağlanmaktadır. DNA topoizomeraz II (topo II) enzimleri hücrede replikasyon, kromatin organizasyonu, transkripsiyon gibi hücrenin kaderini belirleyen birçok hayatsal faaliyette aktif rol oynamaktadır. Memeli hücrelerinde α ve β izoformu bulunan topo II enzimleri, yapısal olarak benzer ancak hücre çoğalması ve farklılaşma sürecinde işlev olarak değişkenlik göstermektedir. Topo IIα, mitoz geçiren hücrelerde homolog kromozomların ayrılmasında görevli ana enzim iken, topo IIβ çoğalan ve çoğalmayan (farklılaşmış) bütün hücrelerde ifade edilmektedir. Bu durum, topo IIβ'nın kromozomların birbirlerinden ayrılması haricinde, transkripsiyon, DNA tamiri gibi özelleşmiş başka hücresel olaylarda görev yapabileceğini akla getirmektedir. Son yıllardaki araştırmalar topo IIβ'nın DNA üzerinde bazı genlerin transkripsiyon esnasında açılması ve ifadesinin düzenlenmesini sağladığını göstermektedir. Özellikle nöral farklılaşma esnasında akson uzamasındaki rolü ile ilgili yoğun çalışmalar bulunmaktadır. Ayrıca DNA tamir mekanizmasında da gerekli bir enzim olduğu bildirilmiştir.

İnsan mezenkimal kök hücreleri (iMKH) farklılaşma potansiyelleriyle araştırma ve klinik uygulama açısından rol model olarak kullanıma açık hücrelerdir. Ancak primer iMKH'lerin elde edilmesindeki zorluklar, hücre alımının gercekleştiği bireyin yaşına bağlı olarak laboratuvar kosullarına alınan hücrelerin farklılaşma potansiyelini etkileyen faktörler ve sınırlı bölünebilme özellikleri nedeniyle *in vitro* çalışmalarda primer hücrelerden ziyade iMKH hattı kullanımı avantaj sağlamaktadır. Farklılaşma süreci hücrede transkripsiyonel düzenleme ve değişim aşamalarını ifade etmektedir. Yapılan *in vivo* ve *in vitro* çalışmalar ile topo IIβ'nın bu noktada etkili bir faktör olduğu belirlenmiştir. Literatürde iMKH'lerin nöral farklılaşma sürecindeki etkisine yoğunlaşılmış ancak diğer farklılaşma türlerindeki olası ve genel fonksiyonu çalışılmamıştır. Bu nedenle, topo IIβ enziminin nöral farklılaşmaya özgün bir fonksiyonu mu olduğu, yoksa diğer hücre farklıklaşmalarında da genel bir kontrol faktörü mü olduğu bilinmemektedir. Çalışmanın cıkış noktasını oluşturan bu fikir ile topo IIβ enziminin iMKH'lerin osteogenik farklılaşma yolağındaki genlerin transkripsiyonu üzerine etkisi araştırılmıştır. Bu amaçla, yüksek verimlilikte osteogenik farklılaşma potansiyeline sahip iMKH hattında, topo IIβ'nın osteoblastik genlerin aktivasyonunda rolü olup olmadığı araştırılmıştır.

Çalışmada, iMKH hattında, ticari olarak elde edilen osteogenik farklılaşma faktörleri kullanılarak indüklenmiş osteogenik farklılaşma gerçekleştirilmistir. Farklılaşma sürecinin teyidi, özel osteogenik belirteçlerin, histolojik boyamalar (Toluidine mavisi, Alizarin kırmızısı) ve moleküler düzeyde gen ifade profilinin (BSP, OPN, OCN). RT-qPCR (Gerçek zamanlı kantitatif polimeraz zincir reaksiyonu) yöntemiyle belirlenmesiyle gerçekleştirilmiştir.

Osteoblastik hücre populasyonu eldesinin teyidi sonrası, topo IIβ geninin belirlenen zaman dilimlerinde siRNA (small interfering RNA) tekniği ile susturulması ve nukleofeksiyon teknigi ile aşırı ifadesi calışmaları yapılmıştır. Belirlenen zamanlarda osteogenik farklılaşma ile birlikte topo IIβ gen ifadesinin engellenmesi ve aşırı ifadesi ile başlatılan deneysel süreç, son aşamada topo IIβ susturulan gruplarda daha güvenilir sonuçların gözlemlenmesinden dolayı yalnızca bu gruplar ile devam etmiştir. Son aşamada belirlenen günlerde ve topo IIβ susturularak elde edilmiş deneysel koşullarda osteogenez ve osteoporoz (OP) ile alakalı genlerin kıyaslanması PCR array tekniği ile yapılmıştır.

Deneylerde kulllanılan gruplar: herhangi bir uygulama veya osteogenik indükleme yapılmamış iMKH kontrol grubu (NT-UD), osteogenik farklılaşmaya indüklenmiş iMKH grubu (Ost.), osteogenik farklılaşmaya indüklenmiş ve özgün siRNA'lar ile topo IIβ susturulmuş iMKH grubu (Ost.+siTopo IIβ) ve osteogenik farklılaşmaya indüklenmiş ve topo IIβ aşırı ifadesi yapılmış iMKH'lerden (Ost+OE) oluşmaktadır.

siRNA indüklü topo IIβ gen susturulması belirlenen tüm zaman aralıklarında, çeşitli yöntemlerle analiz edilen ilgili belirteçlerin ifadesinde ve osteogenik farklılaşmanın ilerleyişinde azalmalara sebep olmuştur. Topo IIβ aşırı ifadesi ise kullanılan transfeksiyon yönteminin iMKH'lerin yoğun strese maruz kalmaları ve ölümüne sebep olması nedeniyle hücrelerin toparlanma süreçlerini uzatmış ve osteogenik farklılaşmanın ilerleyişi planlanan şekilde gözlemlenememiştir.

Osteogenez ve OP ile ilgili gen taramalarını içeren PCR array sonuçları da topo IIβ gen susturulması gerçekleşmiş örneklerde osteoblastik farklılaşma ile ilgili genlerin negatif

yönde etkilendiğini göstermiştir. Yalnızca osteogenik farklılaşmaya yönlendirilen iMKH'lerde ise bu genlerin oldukça fazla ifade edildiği gösterilmiştir.

Sonuç olarak, topo IIβ aktivitesinin iMKH'nın osteogenik farklılaşmada gerekli olduğu ve osteblastik hücre populasyonunun elde edilmesinde önemli olduğu gösterilmiştir. Osteoblast populasyonunun devamlılığı hücre farklılaşması aracılığıyla sağlandığından iMKH'lerin ostegenik farklılaşmasında etkili olan biyolojik moleküllerin ve ayırıcı gen ifade profillerinininin belirlenmesi, hem osteopatolojik hastalıkların teşhis ve tedavisi açısından yol gösterici olacak hem de hücre tipine özel farkılaşma sürecinin moleküler mekanizmalarının aydınlatılmasına katkıda bulunacaktır. Bu yönüyle tez benzer calışmalar ve tedavi temelli yaklaşımlar için yardımcı kaynak olacaktır.

Temmuz 2017, 139 sayfa.

Anahtar kelimeler: Topo IIβ, iMKH hattı, osteogenik farklılaşma, siRNA.

SUMMARY

Ph.D. THESIS

ROLE OF DNA TOPOISOMERASE IIβ IN OSTEOGENIC DIFFERENTIATION OF HUMAN MESENCHYMAL STEM CELLS

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In mammalian cells transcriptional regulation is provided with the activity of a serial enzyme and protein members. DNA topoisomerase II (topo II) enzymes play active roles in many crucial functions including replication, chromatin organization and transcription that determine the fate of the cell. In mammalian cells topo II enzymes that have two isoforms as α and β, have similar structures but they show variable functions in proliferation and differentiation processes. While topo IIα is main enzyme, functional in chromosome segregation of mitotic cells, topo IIβ is expressed in all proliferative and non-proliferative (differentiated) cells. This suggests that topo IIβ might have functions on some other specialized cellular processes such as transcription and DNA repair except for segregation of chromosomes. Recent studies show that topo IIβ provides opening of some genes on DNA during transcription and regulation of its expression. Especially there are intensive studies related to its roles in axon growth during neural differentiation. It was also reported that it is an essential enzyme for DNA repair mechanisms.

Because of their differentiation potential, human mesenchymal stem cells (hMSCs) are open sources for using as model systems in researches and clinical applications. However due to the difficulties in obtaining of cells, factors that affect differentiation potential of cells in laboratory conditions which depends on the age of donor and limited cell division capacity give opportunity to use hMSC line *in vitro* studies rather than primer hMSCs. Differentiation process refers transcriptional regulation and changing phases in cell. Via *in vivo* and *in vitro* studies it has been determined that topo IIβ is an effective factor for this fact. In the literature studies has been focused on roles of topo IIβ in neural differentiation process but its probable and general role in other types of differentiation hasn't been studied. So, it is not clear if topo IIβ has a specialized function for neuronal cells or it has a general controlling factor involved in cell differentiation processes. By using this idea that is the starting point of study, effects of topo IIβ in transcription of genes involved in osteogenic differentiation pathway have been investigated. For this purpose it has been investigated that whether topo IIβ has effects in activation of osteoblastic genes' expression in hMSC line that has highly efficient osteogenic differentiation potential.

In the study induced osteogenic differentiation process in hMSCs was conducted by using commercially available osteogenic differentiation factors. Osteogenic differentiation process has been confirmed via determining the presence of osteogenic markers by using histological stainings (Toluidine blue (TB), Alizarin red (AR)) and gene expression profiles at molecular level (BSP, OPN, OCN) via RT-qPCR (Real time quantitative polymerase chain reaction) technique.

After confirmation of osteblastic cell population gain, studies of topo IIβ gene silencing via siRNA (small interfering RNA) method and overexpression via nucleofection technique at indicated time points were done. Experimental progression, which was started with the osteogenic differentiation in addition to the gene silencing and overexpression of topo IIβ, was only proceeded with topo IIβ silencing at last stage of experiments due to the observation of more reliable results in these groups. At last stage, in experimental conditions that were obtained with topo IIβ silencing and at indicated time points, comparison of genes related to osteogenesis and osteoporosis (OP) were done with PCR array technique.

Groups used in experiments were composed as: hMSCs as control group which didn't have any treatments or osteogenic induction (NT-UD), osteogenically induced hMSCs (Ost), osteogenic induction in topo IIβ silenced hMSCs via specific siRNAs (Ost+siTopo IIβ) and osteogenic induction in topo IIβ overexpressed hMSCs (Ost+OE).

siRNA induced silencing of topo IIβ caused decreasing effects in expression of related markers that were analyzed with several methods and progression of osteogenic differentiation at all indicated time points. In overexpression of topo IIβ due to the method that was used in transfection, hMSCs got into intense stress and caused cell death took long time of cells to be recovered and progression of osteogenic differentiation wasn't observed as planned.

PCR array results that involved gene scannings related to osteogenesis and OP also showed that genes related to osteoblastic differentiation were negatively affected in topo IIβ silenced samples. In hMSCs that were committed into osteogenic differentiation alone, it has been shown that these genes were expressed quite a lot.

As a result it has been shown that activity of topo IIβ is required in osteogenic differentiation of hMSCs and it is important for obtaining the osteoblastic cell population. Since the continuity of osteoblastic cell population is provided via cell differentiation process, determination of effective biological molecules and differential gene expression profile of hMSCs' osteogenic differentiation will be guided for both diagnosis and treatments of osteo-pathological disorders and contribute the detection of cell specific mechanisms of differentiation at molecular level. In this aspect study will be helpful and a source for similar studies and treatment based approaches.

July 2017, 139 pages.

Keywords: Topo IIβ, hMSC line, osteogenic differentiation, siRNA.

1. INTRODUCTION

1.1. DNA TOPOISOMERASES

Inside the cell, during proceedings on DNA, there might be some topological changes that effect transition and expression of genetic information. In addition to the helical coiling of the strands to form a double helix, the double stranded DNA molecule can also twist upon itself and form supercoils (coiled coils) that cause torsional tension. Naturally obtained DNA from cells are negatively supercoiled and in right hand form as a natural product of coiled structure of double helix. When DNA twist upon itself around own axis, storage of free energy in molecule is occurred and this energy is released by ATP hydrolysis during relaxation of supercoiling structures. DNA twist at the same direction of helix forms positive supercoil while twisting at opposite direction forms negative supercoil. Negatively supercoiled DNA is underwound and favors unwinding of the helix while positive supercoils are overwound. DNA topology strongly related to transcriptional regulation of genes [1, 2].

Supercoiling occurs in almost all chromosomes even it is circular or linear. DNA should be relaxed from these supercoil structures. DNA topo lead to resolve such topological entanglements by generating a transient break through which topological changes can occur. DNA topo are essential for removing excess amount of supercoils while maintaining the sufficient supercoils to favor formation of compact genome [3].

DNA topo family members grouped into two according to mechanisms on single or double strand of DNA. Topo I cut DNA in single site and found in both prokaryotes and eukaryotes. It was the first enzyme discovered in that family [4]. Topo II family has two subgroups and isoforms known as α and β. They are enzymes of eukaryotic organisms such as human, fruit fly *Drosophila melanogaster* and *Saccharomyces cerevisiae.* Topo II family members have functions in replication, chromosome condensation, segregation of chromosomes and DNA repair [5]. They can generate double strand breaks on DNA. Topo IIα is essential for mitotic processes and present in proliferating cells with higher expression rate at G2 and M phase of cycle. Expression level of topo IIβ during cell

cycle is stable and found in all types of terminally differentiated cells [6, 7].

General mechanism on DNA and main steps of DNA segment transport was indicated in Figure 1.1. Two independent double strands refered as G and T segments interact with topo II enzymes for relaxation. Ways that were followed by T segment as transported strand can be separated into 3 as; N-gate for entrance to the system, DNA gate for transition and C gate for output. First of all, the G segment as gate segment binds to enzyme and cleaved through both strands. Cleavage causes formation of opening DNA gate. Second double strand known as T segment and transported strand passes through opened DNA gate and captured by C gate. Reaction requires ATP hydrolysis to reset. At the end, G segment is resealed and both G and T segments are released from topo II enzyme.

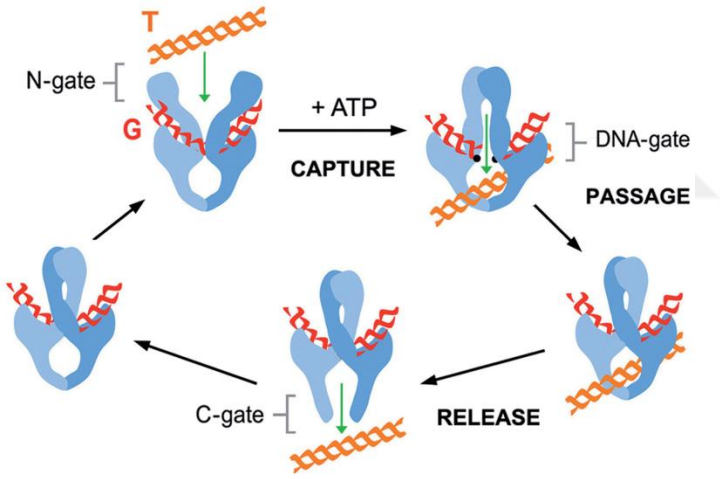


Figure 1.1: Transporting of T segment DNA strand through a double strand break in G segment by topo II enzyme [8].

1.1.1. Role of DNA Topo IIβ in Biological Processes

Expression pattern of topo II differs according to cellular processes. Cells of proliferating stage of cell cycle express topo IIα while topo IIβ expression remains stable or increase in that conditions. Unlike topo IIα with lower expression rate, stationary cells of cycle mainly express topo IIβ [9].

Supercoiling affects interaction of DNA with other molecules and required for members of genomic processes such as nucleosomes, members of transcription factors and

chromatin organizers [10]. Nucleosomes favor conformational changes in its structure to control mechanisms on gene expression. Especially topo IIβ have roles in relaxation of nucleosomes by associating with the promoter regions to form site-specific cuts on DNA strands [11, 12]. Also it was shown that activity of topo IIβ depends on its interaction with RNA-protein complex [13]. In addition to that specific binding region and sequences of topo IIβ in genome were studied [14, 15]. Similar results were also shown in a study that focused on relations with several stages of differentiation [16].

Recent studies have focused on functions of topo IIβ in post-mitotic and terminally differentiated cells especially in neural cells [17]. Results demonstrate that topo IIβ have more specific roles in DNA metabolism rather than functions in chromosome segregation or DNA repair. *In vivo* and *in vitro* studies show strong relation between expression of topo IIβ and neural development.

Effects of topo IIβ in neural differentiation is related to gene regulation by repression or activation of developmentally controlled genes at late stages of neural differentiation. In an *in vitro* study that analyze gene expression pattern of granule neurons in developing rat cerebellum showed that expression pattern of DNA topo isoforms switched from topo IIα to topo IIβ [18]. Analyzing the gene expression profiles of cerebellar granule neurons (CGNs) in primary culture showed that topo IIβ affects genes related to ion channels, receptors and signal tranductions required for neuronal functions [19].

In a study activity of topo IIβ in neural development was shown in an *in vitro* aging model of CGNs that resulted in observation of its activity in DNA repair efficiency [20]. Another study supports this finding by showing the activity of topo IIβ in base excision repair capacity of aging CGNs [21]. Studies that used topo IIβ specific knock out mice have demonstrated multiple brain defects in corticogenesis [18, 22] while whole body topo IIβ knockout cause prenatal death [23]. Neurite outgrowth and axon guidance are important processes for formation of neuronal network. Role of topo IIβ in these processes have been studied in cultured CGNs and topo IIβ knockout mice showed failures in neural development [24]. A study that used retina-specific knockout mouse models showed that topo IIβ deficient retinas have reduced cell number, delayed cell differentiation and degenerations in photoreceptor regions [25]. Relation between topo IIβ expression and DNA repair in neural cells was also studied. *In vivo* and *in vitro*

studies in topo IIβ knockdown or knockout conditions show relation in between aging of cells and activity of topo IIβ [18]. According to the literature, topo IIβ is absolutely required for neural differentiation of cells and studies focus on that area.

1.2. STEM CELLS

Degenerative diseases that cause painful and suffering conditions of patient increase in worldwide. So that prognostic and diagnostic studies of diseases are important for human health. In this respect, changes in human body and symptoms of the disease that cannot be based on any reasons should be studied and solved at molecular level. That helps understanding the mechanism of disease. Degenerative diseases cause cell and tissue loss and highlight the importance of studies for therapeutic purposes. Changing and supporting of unhealthy cells with healthy ones are helpful approaches for treatment. Stem cells offer multifunctional supports for this type of therapies especially in regenerative medicine.

Stem cells are type of cells in multicellular organisms characterized by having potential to self renew and differentiate into several types of cells in the body. So they are sources of newly formed specialized cells. Stem cells are grouped according to their spectra of differentiation and self-renewal potential. Their distribution is limited in the body but via self-renewal potential, they can proliferate and increase through mitosis to form more stem cells. Understanding the self-renewal mechanism of stem cells' lead to solve some cases related to cell division and proliferation such as cancers. It is still unclear that some types of stem cells like embryonic stem cells can divide infinitely about six months to one year without differentiating while others like adult stem cells cannot. So that, understanding stem cells in detail is both important for their usages as therapeutic purposes and model systems of molecular mechanisms [26].

1.2.1. Sources and Types of Stem Cells

Stem cells are categorized according to their main properties: rate of differentiation capacity (plasticity) and self-renewal. From the beginning of life there is a hierarchy between stem cell populations. Totipotent cell that take part at top of this hierarchical system is characterized by unrestricted differentiation potential and give rise to obtain

functional entire organism such as zygote, spore and morula. Fertilized egg is a totipotent cell, as having ability to form endoderm, mesoderm, ectoderm layers and germ cells of whole body and it also forms trophoblasts to support nutrition for embryo and keep it alive. Other groups of stem cells are embryonic stem cells (ESCs) and embryonic germ cells (EGCs) that are isolated by using inner cell part of blastocytes or obtained from early embryos' primordial germ cells. They have potential to form endoderm, mesoderm, ectoderm layers and germ cells but cannot form other embryonic tissues that is required to form whole functional organism, so they are members of pluripotent cells. As being partially differentiated cells, adult stem cells are present in adult tissues, induced to form specialized cells and tissues and termed as progenitor cells and they are multipotent (multi-lineage differentiation). Cells that are derived from these stem cells and committed into one type of specialized cells are called as precursor cells and they are unipotent (uni-lineage differentiation). In addition to these groups, genetically programmed forms of non-pluripotent adult somatic cells that are obtained by artificial expression of some genes are induced pluripotent cells (iPSCs). Also there is one group of stem cells unlike having therapeutic properties, they cause formation of tumors in a tissue, known as cancer stem cells (CSCs). It is believed that they are responsible from cancer cells to relapse after treatments by having more resistant properties against cancer drugs and therapies [27].

1.2.2. Embryonic Stem Cells

Studies that are focused on using of stem cells as model systems are important for cell-based therapies to treat some diseases. ESCs are primarily used stem cell type in 1980s. Human embryo at blastocyst stage has 50-150 cells at days 3-5 after fertilization and ESCs are obtained from that stage of blastocyst's inner cell mass. ESCs are obtained from *in vitro* fertilization clinics that are donated for researches. There are some criterias that ESCs must have. First of all, ESCs should be divide and proliferate long term without differentiating. Also there are some indicators at gene expression level. Transcription factors are defined for ESCs that are essential for genes' on and off position according to cellular processes. Most important transcription factors of ESCs are Nanog, Oct4, SOX2 and c-Myc. Surface markers of ESCs should be confirmed via several techniques. Detection of possible mutations in cells should also be checked for number of chromosomes or for damaged chromosomes. ESCs' pluripotency is

confirmed with *in vivo* experiments. Immunologically suppressed mouse models are injected with ESCs and checked for formation of different cell types. In addition to that, promotion of cells' differentiation through target cell type is not sufficient for therapeutic usages. Obtaining the functional and metabolically active cells is important. For example, insulin secreting cells, myelinated neurons, beating cardiac cells etc. These functional cells should repair damaged or injured tissue in therapeutic uses of regenerative medicine. ESCs' pluripotency serve higher rate of differentiation potential for therapeutic usages but there are many problems that makes process unsafe. ESCs' uncontrolled proliferation and differentiation potential is a big concern for therapeutic uses. ESCs may form uncontrolled and heterogeneous tumor cell population called as teratoma. These risk factors make clinical applications more complicated [27].

1.2.3. Induced Pluripotent Stem Cells

iPSCs are genetically forced pluripotent stem cells by using transfection of specific genes into the non-pluripotent cells of adult tissues. Newly formed iPSCs should have all properties of ECSs even expression of stem cell markers, specific protein production, chromatin methylation arrangements, doubling times, risk of teratoma formation and production of cells of all three germ layers. iPSCs were firstly obtained from mouse model in 2006, and from human in 2007. There are some risks for using of iPSCs for therapeutic uses but they are good sources for obtaining model systems of diseases and drug development. Unlike ESCs they are useful for prevention of immune rejection reactions and graft-versus-host disease (GVHD) since they are obtained from individuals' own tissues. Most important problem is about method of gene injection. Generally viral methods are used but this may cause expression of cancer causing genes known as oncogenes [27].

1.2.4. Cancer Stem Cells

Theory refers that CSCs are localized inside tumor tissue for relapsing of tumor development. They are found in tumor tissue in a small rate about 0.1-1 % and they can form all types of tumor cells via self-renewal and differentiation potential. They can be selected from other tumor cells by detection of specific cell surface antigens such as CD34+. CSCs have same properties of stem cells' stemness. In addition to self-renewal and multipotential of differentiation properties, CSCs are distinguished from normal

stem cells on their uncontrolled growth and proliferation rate. As seen in somatic cells of body contact inhibition of growth is important for cell growth, development, wound healing and organogenesis. Mechanism refers interaction of cells with each other that cause them to stop replicating and keep mitotic activity and cell area stable. Unfortunately this mechanism is inactivated in CSCs. Cancer chemotherapies are effective on existing tumors but CSCs are able to escape from chemotherapies and left behind for supplying new cancer cells via their self-renewal and differentiation potentials. Therapies against CSCs may be focused on increasing the differentiation rate of CSCs and prevent them to remain after chemotherapies. Researches showed that tumor tissue has heterogeneous cell population that comes from a single cell. These findings highlight the presence and theory of CSCs. If these heterogeneous cells have the same ancestor cell that is able to form multiple cell types it has common stem cell properties and called as CSCs. CSCs cannot be obtained *in vitro* lab conditions and they are isolated from tumors at last stage instead of initial stage. Also some of the researchers claimed that CSCs are products of mutated cells of developmental stage such as progenitor cells and adult stem cells and it is easy to form tumors from these mutated cell sources [28].

As mentioned before triggering of cell differentiation may be helpful for elimination of CSCs during chemotherapy. This approach is successfully used in blood cancer therapy of children's acute promyelocytic leukemia but it is hard to apply in solid tumors due to the penetration problem inside tumor tissue [29].

1.2.5. Adult Stem Cells

As understood from the name of cells, adult stem cells are spread to postnatal individual's whole body parts, tissues and organs. They are progenitors of several tissues and precursor cells of related tissue. Multi-lineage progenitor cells yield precursors that devoted to uni-lineage differentiation. Entire body parts are composed of three germ layers known as endoderm, mesoderm and ectoderm. Formation and development of body structures from these germ layers are controlled genetically at molecular level during embryogenesis. Genetic control mechanism allows cells to determine amounts of cell division, proliferation and cell size to obtain newly formed tissue and organs. For the following stages of development and during lifespan, tissues

and organs may have trauma, injury or disease and they should be repaired and committed with healthy cells. Adult stem cells are essential for these conditions. They are obtained from almost all types of mature tissues. They can be taken from different sources of endoderm, mesoderm and ectodermal origin such as; pulmonary epithelial stem cells (SCs), gastrointestinal region SCs, SCs of pancreas, prostatic gland SCs, SCs of gonads as endodermal origin, hematopoietic SCs, mesenchymal stroma SCs, mesenchymal SCs, adult progenitor cells with multipotency, non-hematopoietic SCs of bone marrow, fetal somatic SCs, cardiac SCs as mesodermal origin and neural SCs, skin SCs, as ectodermal origin. Adult SCs are excellent sources for studies that try to overcome irreparable tissue and organ loss. Due to the problems of restricted organ donation and GVHD, researches on adult SCs' multifunctional differentiation potential are valuable [30].

There are successful *in vivo* preclinical studies and clinical trials on transplantation of precursor adult SCs into the animal models and individuals such as transplanted hepatic SC precursors that form functional hepatocytes [31], transplanting of bone marrow derived hematopoietic SCs to patients of myocardial infarction that cause formation of functional cardiomyocytes and endothelial cells [32], grafting of *in vitro* grown corneal SCs that successfully caused recovering of vision [33], using of mesenchymal stem cells (MSCs) to facilitate wound healing [34] transplantation of bone marrow derived MSCs into children of osteogenesis imperfecta (OI) that cause increasing in total bone mineral content and bone mass [35].

But still it is hard to predict long-term effects of therapies. In addition, clinical responses on MSC transplanted children patient with OI who has followed up to 36 mounts after transplantation, was reported by Horwitz et al. at 2001. Observations on increasing rate of growth, enhanced presence of total bone mineral, stable proceedings of bone fractures was mentioned in addition to complications of toxicity. Data of the study should be improved because of some missing points such as lack of healthy control individuals, restricted numbers of patient and measurements, but short time benefits of process was mentioned [36].

As discussed above adult SCs serve increasing therapeutic application areas for adult to children patients. They are also supportive for regeneration of cells and tissues via

prevention of apoptosis and immunosuppression properties [37].

All of these instances are related to great contribution of adult SC plasticity on tissue and organ development. SC plasticity focuses on multipotential differentiation property of a single cell. Adult SCs have been described as maintaining and renewing within their specific tissue. In addition to that, findings propose that single cell can differentiate into several cell types by crossing borders and restrictions through its specific tissue. Term SC plasticity supposes that differentiated cells should be functional *in vitro* and *in vivo* and stable when engrafted to individual. Different types of cells or tissues originated from SCs' of a specific tissue are in wide range. For instance, bone marrow derived hematopoietic stem cells (HSCs) have ability to differentiate into blood lympho-hematopoietic cells; MSCs from the same origin can form bone, cartilage, adipose tissue, tendon, muscle and neural cells of central nervous system that differentiate into neurons, astrocytes and oligodendrocytes. MSCs can be obtained from several tissues of postnatal organism. In murine, MSCs can be isolated from femurs and tibias of mice by flushing the culture medium through femurs and tibias and transferring the obtained cell suspension into the culture conditions for expansion while isolation from human is occurred by taking of bone marrow aspirates from healthy volunteers' iliac crest and providing suitable culture conditions for expanding of hMSCs [38].

Specifically bone marrow derived stem cells (BMSCs) serve wide range of therapeutic applications for cell replacement therapy due to having heterogeneous cell population of SCs and target cell's progenitors, including HSCs and MSCs that can be obtained from individuals' own body and prevent immunological problems [39].

1.2.5.1. Hematopoietic stem cells

HSCs are bone marrow derived SC population that can differentiate into all types of blood-cell lineages including red blood cells, granulocytes/monocytes, platelets, B-cells and T-cells required for the whole life of adult mammals' hematopoiesis [40]. Hematopoiesis process, which is cooperatively managed with different organs such as liver, spleen and supplied with HSCs in bone marrow, is divided into two classes: lymphoid and myeloid lineages. B and T cells and natural killer (NK) cells come from lymphoid lineages while monocytes, erythrocytes, granulocytes and megakaryocytes come from myeloid lineages. Using HSCs as direct transplantation or *in vitro*

genetically manipulated forms in patients suffer from hematopoietic diseases is an approach of therapy. Once transplantation of healthy HSCs into patients' body via autologous or allogeneic ways is occurred, HSCs can be replaced and replenish whole hematopoietic system and supply cured cells [41].

1.2.5.2. Mesenchymal stem cells

MSCs are multipotent progenitor cells of initially bone marrow and also reside in adipose tissue and almost all types of connective tissue. MSCs are non-hematopoietic cell population of bone marrow. Non-hematopoietic cell population of bone marrow was firstly recognized by Julius Cohnheim in 1867 that suggest damaged tissue repair was achieved by cells come from bloodstream. At that time his hypothesis wasn't supported but it was the basic finding for these time's research areas about bone marrow stromal cells in different research approaches [42]. MSCs are cell populations of bone marrow and mainly obtained from bone marrow but distributed lots of tissues in the body. Potential for its action in body depends on conditions such as age [43].

Friedenstein and his colleagues have a series of studies and papers about MSCs. Early terms of studies showed formation of fibroblast colony forming units (F-CFU) [44]. Although this factor cells can differentiate into several types of body cells including osteoblasts, chondroblasts, adipocytes and neural cells in *in vitro* conditions. In addition to basic findings of Friedenstein, cell surface markers of MSCs have been identified like STRO-1, HOP-26, CD49. Also differential expression markers of hematopoietic and non-hematopoietic cell population are being negative for CD11B, CD14, CD34, CD45 and (HLA)-DR but expressing CD73, CD90 and CD105 [45].

1.2.5.3. Differentiation Potential of MSCs

Differentiation of MSCs into several cell types can be controlled and manipulated by using some inducible factors such as hormones, growth factors, cytokines and vitamins in *in vitro* conditions. They have ability to differentiate into chondrocyte, adipocyte and osteoblastic cell lineages and beyond these types of differentiation originated from mesenchymal derivatives they can transdifferentiate into cells involved in neural and myogenic cells. Both growth factors and some chemicals are effective on driving of MSCs into several lineages. For chondrogenic differentiation mainly transforming growth factor beta (TGF-β) superfamily members (TGF-β-1, TGF-β2, TGF-β3) and

BMPs have regulatory effects. Promoting of cells into chondrogenic lineage cause formation of collagen type II (COL2) and proteoglycans in culture conditions. In addition to growth factors cell signalling molecules such as mitogen activated protein kinases (MAPK), Smads that induce specific transcription factors like sox9 that is known as main molecule for chondrogenesis and cartilage formation. For promotion of MSCs into adipogenic lineage specific chemicals are required such as dexamethasone, insulin, isobuthyl methyl xanthine and indomethacine. When cells get into adipogenesis lipid rich vacuoles are observed and can be detected as histologically by oil red O staining. Supplementation with nicotinamide and beta mercaptoethanol drives MSCs into formation of islet β-cells [46]. Differentiation status of MSCs is confirmed by using some assays of histological staining, measuring of specific protein levels that belongs to target tissue and gene expressions patterns.

1.2.5.4. Osteogenic Differentiation Potential of MSCs

Obtaining of bone tissue depends on multiple biochemical factors and activities of osteoblast cells. Process has multisteps and involves molecular pathways and biochemical requirements that result morphological and functional changes of cells. These changes are related to differential expression of specific genes and formation of calcified and mineralized bone matrix by the activity of osteoblasts. Development through osteoblastic lineage from multipotent MSC results proliferation and formation of multilayering of cells. Process is followed by increasing synthesis of extracellular matrix (ECM) proteins such as collagen type I (COL1) and alkaline phosphatase (ALP) activity, then increasing calcium production for mineralized ECM is occurred [47].

All of these mentioned factors are some indicators of differentiation such as higher ALP level, expression of bone specific genes, histological staining of calcified (mineralized) bone matrix [48]. Both morphological and molecular indicators of osteogenesis can be followed by using these detection methods. Cells in osteogenic commitment become cuboidal shape from fibroblastic morphology, start to produce ECM that involves COL1, synthesize calcium and show increasing expression of ALP levels [49]. *In vitro* conditions of osteogenic differentiation requires basal medium, osteogenic supplement, dexamethasone, β-glyserophosphate and ascorbic acid treatment for 3-4 weeks [50].

Commitment of hMSCs into several lineages depends on local and hormonal factors.

Dexamethasone is essential for starting of osteogenic differentiation while ascorbic acid is required for matrix mineralization by synthesizing of COLs and other inorganic molecules such as phosphates. Genetic regulation of osteogenesis is also essential. Runt related transcription factor 2 (Runx2) is an absolutely required transcription factor, effects osteoblast specific genes' activity such as bone sialoprotein (BSP), osteopontin (OPN), osteocalcin (OCN) and ALP that are expressed at different stages of differentiation. In addition to these factors several growth factors such as insulin like growth factor (IGF-I), TGF-β [51], epidermal growth factor (EGF) and connective tissue growth factor (CTGF) [52] and hormones such as parathyroid hormone (PTH), estrogens are also related in osteogenic differentiation [53] attended to process. These factors were discussed in detail at further topic 1.5.4.

Identification of cells that are committed into osteogenic lineage can be confirmed such ways like histological staining of differentiated cells, detection of specific markers' expression level. As an indicator of calcified bone matrix, AR staining is one of the way that can be used for identification. Nodular aggregates that refer calcium deposites produced by osteoblastic cells are stained with AR [54]. Other type of staining used for detection of osteoblasts is TB [55].

1.3. BONE FORMATION

Bone tissue contains different parts and cells in it. Osteoblasts and osteocytes are supporter cells of bone while osteoclasts are bone remodeling cells. Bone matrix has COL as a non-mineral matrix and non-collagenaous proteins called osteoids contain inorganic mineral salts stored in matrix. Bone formation is a balanced process inside the cell as a function of osteoblasts and osteoclasts and called as osteogenesis or ossification. It refers formation of new bone tissue by osteoblast cells. Osteogenesis has three important sequential steps; synthesis of osteoid as an extracellular organic matrix, matrix mineralization required for bone formation, bone remodeling as a results of resorption and formation processes [56].

1.3.1. Members of Bone Tissue

1.3.1.1. Osteoblasts

A group of cell types in bone tissue and originated from bone marrow stroma derived MSCs. After propagation of MSCs through osteogenic differentiation, they are called as osteoprogenitor cells. When they differentiate into osteoblasts, they have ability to produce bone matrix and bone related proteins. Then process is continued with matrix mineralization. Some osteblasts stop production of osteoid (bone matrix) and function as osteocytes. Osteocytes are most abundant cell type in bone tissue and responsible for regulation of bone formation and resorbtion. They control both osteoblasts for formation of bone and osteoclasts for disruption of bone when required [66].

1.3.1.2. Bone matrix

Bone is composed of both organic (22%) and inorganic components (69%). Organic part has COL type proteins and non-collagenaous structural proteins, such as proteoglycans, sialoproteins, gla containing proteins and 2HS-glycoprotein. Growth factors and cytokines are important components of bone. Whole inorganic part almost made up of hydroxyapatite. Hydroxyapatite is a complex of phosphate and calcium crystal and makes bone rigid and hard. That part of the bone contains 25% of organic components, 5% of water and 70% of inorganic mineral hydroxyapatite. 90-95% of organic part contains COL1, which is produced as a function of osteoblasts. As a non-collagenaous protein, OCN production is also occurred by osteoblasts [66].

1.3.1.3. Bone minerals

Hydroxyapatite crystals that contain inorganic phosphate and calcium that are obtained from the blood plasma are highly deposited in adult bone. Vitamin D and PTH metabolism is important for regulation of bone minerals. As an end product of adult bone, mixture of proteins mainly COL, minerals mainly hydroxyapatite give bone to its mechanical integrity and function of skeleton [66].

1.3.1.4. Osteocytes

They are fully differentiated forms of osteoblasts and localized inside bone tissue for keeping its structure and minerals stable. Apoptosis of osteocytes in response to different conditions cause bone tissue being fragile. Osteocytes communicate each other via gap junctions known as cell-matrix interactions. Lack of communication or

interaction between osteocytes cause cells to undergo apoptosis. Also estrogen metabolism is important for prevention of apoptosis. Insufficient estrogen or glucocorticoid treatments are reasons of apoptosis in osteocytes [57].

1.3.2. Osteoblastogenesis and Ossification

Terms refer the process which bone tissue is formed. Ossification of bone occurs both in connective tissue and derived from a cartilage precursor. Ossification process originated fom connective tissue is known as intramembranous ossification and observed in development of flat bones of skull and cranial bones. Connective tissue such as mesenchym is essential for this process. Also it is important for repairing of bone fractures. MSCs found in mesenchyme activate the process of intramembranous ossification. MSCs are precursors of osteoblasts so called as osteoprogenitor cells. During ossification, MSCs proliferate and condense around capillary vessels network that localizes inside bone tissue. Process is related to mineralization of that vascular connective tissue. Ossification begins at certain points and known as centers of ossification. MSCs start to differentiate into osteoblasts to form osteoid. Osteoprogenitors start assembling in that center, than interaction is occurred by synthesis of COL between cells. Immature bone tissue is arranged by these osteoblasts and some of them integrate into the bone tissue to form osteocytes.

Ossification of bone from a cartilage precurcor is called as endochondral (intracartilaginous) ossification. Process occurs in long bones and actually most of the bones in the body. Compared to intramembranous ossification, process needs development and growing of a cartilage model, than primary and secondary ossification centers are built up and completed with vascularization [66].

1.3.3. Biological Factors That Affect Bone Formation

Bone mineral density (BMD) is affected by some factors such as nutritional habits and exercise. Observation of low BMD causes bone tissue to be less compact and weak. Smoking is another effective environmental factor. Illness, that cause long term resting with sedentary lifestyle and no exercise, during bone development years also have negative effects on bone density. Changes in hormones such as estrogen, testosterone and growth hormones, are also effective. As seen in explanations, bone formation can

be affected and controlled by multiple metabolic ways. Also bone is reshaped according to some physiological outcomes such as mechanical forces on bones. As a response of these biomechanical forces, bone can be reshaped by the function of osteoblasts and osteoclasts.

1.4. BONE METABOLISM

In addition to the functions of the bone as a skeleton in the body, they have important metabolic roles in regulation of minerals, fat metabolism, growth hormones, cytokine contents, acid-base composition, hormones and act as detoxifying agents in tissues and organs. Bones regulate mineral metabolism by adjusting the amount of calcium and phosphorus in blood and other organs. Mineral homeostasis is controlled by the amount of magnesium in addition to calcium and phosphorus. Amount of these minerals in blood is supplied from bone, renal and gastrointestinal (GI) tract cells. Also mineral exchange is occurred in between these supportive regions from blood. Bones contain 99% of total body calcium, 85% of phosphorus and 65% of magnesium. Some hormones cooperate to keep the amount of bone and bone metabolism stable. For this purpose, PTH, calcitonin (CT) and vitamin D function at three target regions, bone, kidneys and GI tract. Regulation of that mechanism is achieved by the function of cells in bone, called as osteoblasts and osteoclasts. Osteoblasts express some bone supportive agents' receptors, such as to PTH, parathyroid related protein (PTHrP), vitamin D metabolites, steroids, cytokines and growth factors. Also COL1, which is the main component of bone matrix or osteoid is produced by osteoblasts. Osteocytes that are derived from osteoblasts have functions in mineralized or mature bone and essential for osteoblasts in their secreted products. Osteocytes have interaction with outer part of the bone and cells via channels known as canaliculi with gap junctions. Removal of waste products and nutritional changes are occurred with these interactions. Calcium in skeleton is supplied and controlled by the function of these cells through the pathways of GI tract and kidney. There are some regulators that have important roles in skeletal development metabolism such as calciotropic hormones, PTH, CT, vitamin D [1,25(OH_2)D], PTHrP, thyroid hormones, gonadal and adrenal steroids, growth factors and cytokines.

Mineralized bone matrix is an important reservoir for growth factors and cytokines. IGFs, TGF-β and bone morphogenetic proteins (BMP) functions are regulated by bone via releasing and resorbing of them. Fatty acids are deposited in yellow bone marrow. Blood pH is stabilized via regulating the accumulation or decreasing of alkaline salts of blood. Bone tissue acts as detoxifying agent by its ability to accumulate heavy toxic metals and prevent them to join circulation. Bone also regulates endocrine functions. In kidneys, phosphate reabsorption is prevented by the function of bones. A protein known as OCN is produced by bone, which is essential for regulation of blood sugar and fat metabolism by increasing the insulin secretion via activate the production of insulin producing β cells and prevent fat storage.

1.5. BONE REMODELLING

Bone remodeling is permanent process that is characterized by continuously replacement of old bone tissue with newly formed bone. Removal of bone is called as bone resorption while bone formation is called as ossification or osteogenesis. Remodeling refers function of osteoclasts for removal meanwhile function of osteoblasts for formation and production of matrix proteins and resulting with mineralized bone tissue. These sequential processes are also essential during normal development and repair that requires reshaping or replacement of bone. This dynamic process continuously occurs in skeleton as a metabolically active organ for maintaining its integrity and homeostasis of mineral content. Bone remodeling cycle is controlled by following of the mesenchymal osteoblastic cell lineage and hematopoietic osteoclastic cell lineage that results in formation of osteoblasts and osteoclasts (Figure 1.2). Osteoblasts are responsible for increasing of bone mass and building of bone tissue via inhibitory function against activity of osteoclasts. Osteoclasts are derived from HSC precursors of monocyte macrophage lineage. They are multinucleated cells. Especially bone marrow derived mononuclear monocyte macrophage lineage specific cells lead to formation of osteoclasts.

Bone remodeling occurs throughout life, in a young skeleton removal and formation of bone tissue is equal. Till bone has its maximum mass, process is in balanced, but after the age of 50 resorption getting higher than formation. Going through menopause also

affects bone remodeling. Increasing bone resorption decreases bone mass and causes breaking down of this balanced process in term of menopause. Osteoclasts have ion channels in their cell membrane that cause releasing of protons into the extracellular space. By this way pH value is kept lower in that environment and cause releasing of bone mineral. Bone remodeling refers balancing of differences in bone resorption and formation. The importance of bone remodeling process is indicated as, keeping bone mechanical strenght stable by removing of older bone result in obtaining less damaged, healthier bone and balanced calcium and phosphorus homeostasis.

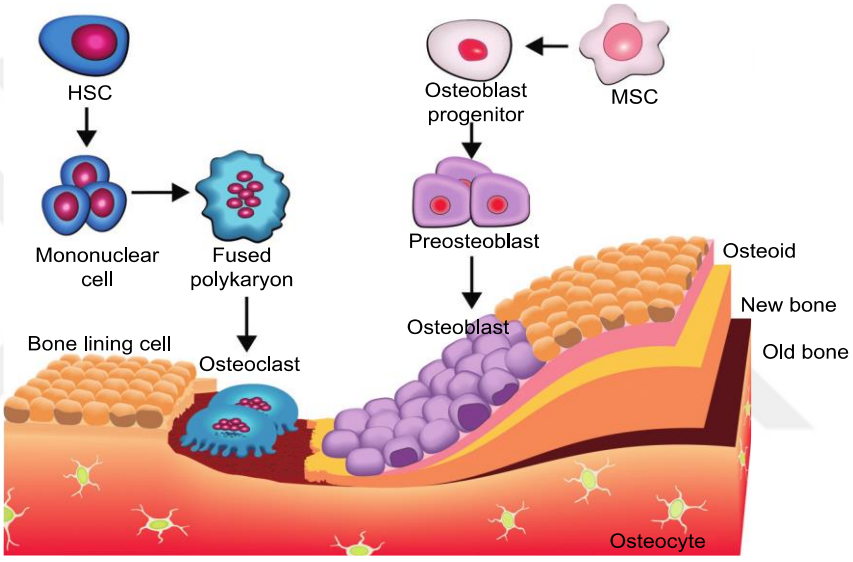


Figure 1.2: Members of bone remodeling process [58].

1.5.1. Factors of Bone Remodeling

As mentioned before osteoblasts and osteoclasts are functionalized cells of bone resorption and formation. Bone remodeling process requires cooperation of cells, cell signaling pathways and activity of transcription factors, cell surface receptors and inhibitors.

One of the cell surface receptor that have roles in that process is RANK and it is important for driving of osteoclast precursor cells to become terminally differentiated osteoclast cells. RANK is activated by its ligand RANKL that locates in tumor necrosis factor (TNF) superfamily. RANKL is one of the signaling molecule, that coordinates bone remodeling via helping interaction of osteoblasts and osteoclasts. In addition to

RANKL, macrophage colony-stimulating factor (M-CSF) that affects HSCs to differentiate into macrophages is another important factor for osteoclast formation. Osteoprotegerin (OPG) is osteoblast-releasing protein that prevents interaction of RANK and RANKL. So it is also known as osteoclast inhibiting factor (OCIF) or osteoclast binding factor (OBF). OPG also belongs to TNF receptor family. Dissociation of RANK/RANKL causes inhibiton of osteoclast proliferation and bone resorption. If the balanced interaction of these molecules for bone resorption is broken, bone resorption become uncontrolled and lead to observation of osteopathologic diseases such as osteoporosis (OP), Paget's disease, rheumatoid arthritis and bone loss caused by cancer. Bone resorption mechanism is controlled by secretion of H^+ ions by the help of osteoclasts and dissolving of bone minerals at lower pH. In addition to that, activity of enzyme cathepsin K (CTSK) causes disruption of osteoid or protein matrix. When osteoclasts undergo apoptosis, mononuclear cells complete resorption. As it appears, signaling pathways are very important for regulation of bone remodeling [59].

1.5.2. Phases of Bone Remodeling

Bone remodeling can be divided as six sequential steps named as; quiescent, activation, resorption, reversal, formation and mineralization phases. At quiescent phase bone is in resting mode and there is no information about the initiative factors of bone remodeling. Activation phase refers awaken of mononuclear monocyte macrophage osteoclast precursor cells to become differentiated and interact with osteoblastic precursors. Osteoclasts attach to the mineralized bone surface and start to disrupt bone matrix via hydrogen ions and lysosomal enzymes at low pH. At resorption phase osteoclasts start to dissolve osteoid matrix and process is completed via activity of macrophages. Also growth factors such as TGF-β, platelet-derived growth factor (PDGF), and IGF-I and II are induced for the following steps. Reversal phase makes switch process from resorption to formation. After bone resorption is completed, monocytes, osteoblast precursor cells and osteocytes released from bone matrix are accumulated in environment. Formation phase is characterized with proliferation of preosteoblasts and starting the synthesis of bone matrix and cause production of BMPs that are responsible for differentiation by newly formed bone tissue. Mineralization phase starts after accumulation of osteoid matrix and at the end of the process amount of bone resorped should be equal to amount of bone formed [66].

1.5.3. Regulatory Factors of Bone Remodeling

Many factors categorized as systemic (genetic, mechanical, vascular, nutritional, hormonal) and local have effects on bone homeostasis. Systemic factors are related to physiological and biochemical conditions of body. Genetic characters that transmit from parent to children are important identifier of bone mass. 60-80% of bone mass is determined genetically. As a result of genetic properties, differences in bone mass according to nations are observed. For example Negroes have higher bone mass than Whites and Asians. Mechanical factors refer mechanical loadings on bone tissue. Physical activity directly affects interaction of muscles with bones and bone remodeling. It is proposed that mechanical or physical activity in muscles transmit bones and sensed by osteocytes. So that less physical activity and sedentary lifestyle cause increasing in bone resorption. Vascular/neural networks are another effective factors of bone remodeling. Vascularization is important for transferring of nutrition, oxygen, minerals, ions, hormones, growth factors and interaction of cells. Also process is required for repairing of bone fractures and bone regeneration. Formation of neural network is necessary for bone physiology. The bone has autonomous nerve system and sensorial nerve fibers. So patients of neurological diseases have osteopenia, characterized by low bone density and bone fragility. Nutritional intake of calcium is necessary for mineralization. Also smoking and high consumption of alcohol, caffeine and salt are toxic and risk for bone loss. Hormonal regulation of development depends on correct activity of endocrine system. Lots of the hormones are related to normal skeletal development. Thyroid hormone cooperates with IGF-I to stimulate formation of osteoid matrix and mineralization by osteoblasts. Also activity of osteoclasts is controlled by thyroid hormone to regulate bone resoption. So that bone defects in hyper- and hypothyroidism is observed. PTH is related to homeostasis of calcium. PTH favors both bone resorption and formation via interacting with molecular pathway of osteoclastogenesis and inhibiting the apoptosis of osteoblasts and increasing the amount of growth factors. CT affects bone resorption temporarly. Number and the activity of osteoclasts are reduced via syththesis of CT, but osteoclasts can sense the adverse effects of CT and make cell membrane impermeable. $1,25(OH)_2$ Vitamin D_3 or Calcitriol regulate intestinal resorption of calcium and phosphate and affect mineralization. Androgens affects bone faith in puberty. Hormone's action is related to

acting as a receptor and a mediator. Receptors of osteoblasts and mediators of growth hormones are activated by androgens and influences bone density and mass. Androgens prevent OP disease in males. Estrogens are essential hormones for promoting of bone formation via increasing the number and activity of osteoblasts and decreasing bone resorption. Osteoblasts, osteocytes and osteoclasts have estrogen receptors. Also estrogen is effective on other factors that favor or inhibit bone formation. So that estrogen hormone metabolism is important in menopause that cause bone loss because of OP. Both disorders in amount of androgens and estrogens cause abnormalities of bone remodeling by affecting the lifespan of osteoblasts and osteoclasts. Progesterone supports bone formation via having receptors for osteoblasts. Insulin secretion is related to matrix synthesis. IGF-I synthesis increases amount of osteoid matrix production. Glucocorticoids are required for osteoblast differentiation during development but following birth (postnatal) it has negative effects on bone formation by inhibiting the synthesis of IGF-I by osteoblasts and suppression of BMP2 [60]. This effect is a result of glucocorticoid-induced OP as a pathological condition. Growth hormone increases the activity of osteoblasts for production of bone formation proteins such as ALP, OCN and COL. As mentioned before osteoblasts have receptors of hormone that cause appearing of these effects. Also stimulatory effects on synthesis of IGF I-II positively affect bone formation. As a summary Table 1.1 indicates the effects of these factors on bone metabolism. Amounts of indicated factors that were indicated as 'increasing of… and decreasing of…' cause increasing of bone formation and resorption as shown with arrows.

Table 1.1: Effects of hormones on regulation of bone metabolism.

Effects on bone metabolism	**Bone formation ↑**	**Bone resorption ↑**
Increasing of	Growth hormone Metabolites of Vit. D Androgens Insulin Lower dose PTH/PTHrp Progesterons	PTH/PTHrP Glucocorticoids Thyroid hormones High dose vit. D
Decreasing of	Glucocorticoids	Calcitonin Estrogens

Local regulation mechanism includes activities of growth factors, matrix proteins and cytokines. Bone cells activity affect secretion of these local regulation factors. Growth factors that control cellular activities include cell proliferation, differentiation and growth. In bone metabolism they have effects on bone cells' cellular processes. Inside this system they are produced either by bone cells or found in osseous tissues. Their activities are related to associated proteins and binding targets. As being examples of growth factors, IGFs, TGF-β, PDGF, PTHrGF and fibroblast growth factor (FGF) can be given. In addition to bone remodeling these factors have roles in skeletal development and repair. IGF-I and II are proteins that are similar to insulin and synthesized by liver and osteoblasts. Higher amount of those is found in osteoid matrix and stimulate proliferation of osteoblasts and synthesis of COL. They are found in linked form with IGF binding proteins and this may have positive and negative effects on bone. Secretion of IGF is also related with other growth factors and hormones such as growth hormone, estrogen and progesterone and their activity increase synthesis while glucocorticoid inhibit formation of IGF. Also osteoblast-osteoclast interaction is controlled by IGFs. During embryogenesis IGF-II functions are important.

TGF-β is a group of protein family commonly present in bone matrix as well as IGFs [61]. It controls osteoblast formation and resorption by stimulating the apoptosis of osteoclasts during bone remodeling. It supports formation of osteoid matrix via inhibiting the activity of matrix metalloproteinase proteins (MMPs).

BMPs are members of TGF-β family. They have role in converting of connective tissue into bone tissue (osteoinductive). They also activate differentiation of SCs into several cell types such as cartilage, bone and adipose tissue. During embryogenesis development of cartilage and bone tissue is affected by activity of these proteins. Osteoblastic cell differentiation is strongly stimulated by BMPs [62].

PDGF is related to synthesis of proteins that cause formation of osteoblasts. Neovascularization and COL synthesis are also supported by PDGF. FGF has stimulative effects on osteoblast formation [63]. EGF stimulates formation of cells originated from mesoderm and ectoderm [64]. Vascular endothelial growth factor (VEGF) has roles in primer fracture repair and regeneration of bone tissue [65]. Granulocyte/macrophage-colony stimulating factor (GM-CSF) has role in

osteoclastogenesis and important for pathologic conditions. M-CSF has role in early term of osteoclastogenesis and formation of multinucleated cells but doesn't affect activity of osteoclasts. TNF is related to bone loss in osteopathologic conditions and bone resorption.

Matrix proteins are stimulators of growth factors. Matrix proteins have important roles during differentiation of osteoblastic cell lineage. Proteins such as COL, ALP and OCN are synthesized during osteoblastic cell lineages are indicators of cellular phase of differentiation.

Cytokines are related to immunologic regulation in the body. They are polypeptides secreted by lymphocytic and monocytic cells. They have different roles in cellular processes and some of them are important for bone such as Interleukin 1 (IL-1), IL-6, IL-11, Prostaglandins (PG) and Leukotrienes. Totally they have similar roles by increasing number and activity of osteoblasts, stimulate bone resorption and osteoclast activity [66].

1.5.4. Gene Expression Markers of Bone Metabolism

Indicator factors as gene expression markers of bone formation and bone resorption give idea about the dynamic changes in bone tissue. Markers can be classified into bone formation and bone resorption markers.

ALP, OCN, protocollagen peptides are indicators of bone formation while biochemical measurements of several molecules such as hydroxyproline-containing peptides, hydroxylysine glycosides, acid phosphatase are indicators of bone resorption. ALP is commonly used enzyme for indicator of bone formation and localized in cell surface of osteoblasts and other cell types. It is hard to use ALP for diagnostic purposes because its secretion is not specific to bone cells and can be found in different places in the body. Both liver and bone synthesize ALP. And amount of enzyme can be different at some conditions such as intestinal release and secretion during pregnancy. Source of ALP can be detected for bone cells via presence of sialic acid residues. ALP secretion is also indicator for some osteo-pathological conditions. Detection of biochemical substances gives idea about resorption of bone tissue. Urinary measurements of hydroxyproline-containing peptides, hydroxylysine glycosides, acid phosphatase

indicates rate of bone resorption. Amounts of these biochemical differ in pathological conditions of bone resorption. As a result of post-translational hydroxylation of lysine and ploline, hydroxylysine and hydroxyproline are produced. Researches showed that hydroxyproline amount of urine is increased after menopause and decreased after treatment with estrogen and CT. Glycosylated hydroxylysine can be used for detection of bone resorption in OP disease. Also detecting the amount of calcium, growth factors, vitamin-D in proper tissues may be helpful for bone resorption metabolism that cause formation of osteo-pathological conditions.

Ostoegenic differentiation or osteoblastogenesis can be characterized at 4 phases as: osteogenic lineage commitment of hMSCs; proliferation and expansion of osteoprogenitor cells; synthesis of bone matrix proteins in ECM by osteoblasts and starting of mineralization and exiting from cell cycle; maturation of ECM and cells of bone tissue. All of these stages are controlled by the transcriptional regulation of osteoblast specific genes according to the transitions between stages [67]. First step starts and controlled with activity of related transcription factors and their co-regulators. Main transcription factor required for this step is Runx2. Also Sox9, Osterix, TGFβ/BMPs genes [68], glucocorticoids, Vitamin D3, PTH and estrogen attend to the process. In second stages growth related genes are active for supporting of cell proliferation such as histones and c-Myc. At this stage cells are in heterogenic populations with osteo- chondro- progenitors, preosteoblasts and UD hMSCs. Genes responsible for production of matrix proteins also become active such as COL1 and fibronectin. Third stage of ostegenic differentiation is expressed as assembling of osteoblasts and observation of condensed multilayering. Accumulation of matrix components that promotes interaction of cell-cell and cell-matrix to support related gene expression is observed. Also non-collagenaous proteins such as BSP, OPN, OCN are synthesized. Last stage involves maturation of matrix components by synthesizing of hydroxyapatite crystals and transition of cuboidal cell morphology to form osteocytes (Figure 1.3) [69]. Some of the pathways that have specific roles in osteogenic differentiation are Wnt-β cathenin [70, 71], Notch pathway [72], FGF signaling, RANK/RANKL/OPG signaling [73].

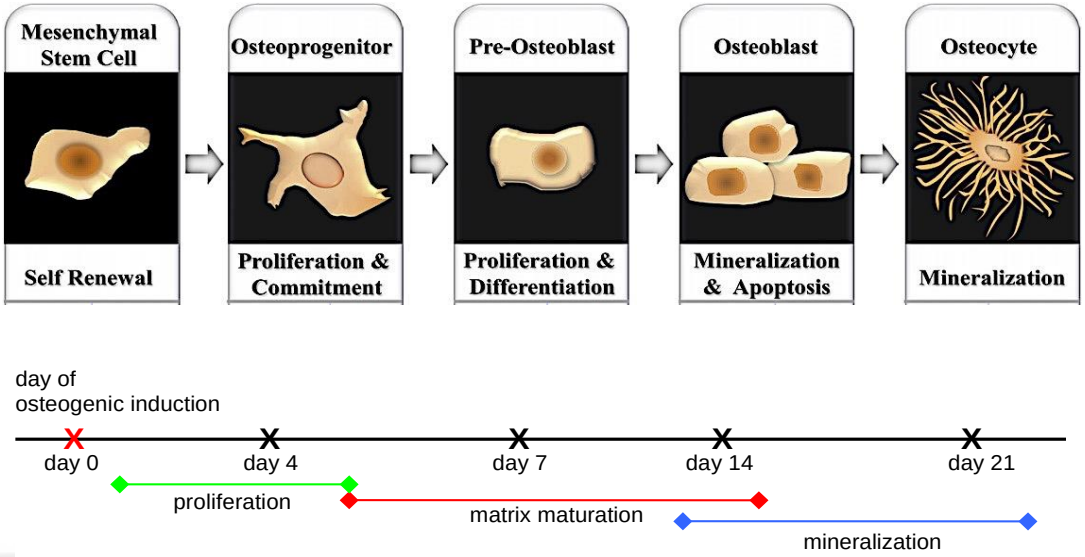


Figure 1.3: Progression of hMSCs through osteoblastic lineage and phenotypic and morphological changes day by day at different stages of differentation. (adapted from ref. 69 and 67 respectively).

1.5.5. Osteo-Pathological Conditions of Bone Metabolism

Instabilities in bone formation and resorption cause observation of pathological conditions such as OP, Paget's disease, osteopetrosis and other orthopedic disorders. According to type of pathological conditions, abnormalities may be observed in both bone mass and bone strength as a result of hormonal activity, viral infections and cellular processes. Most common disorder of bone is OP characterized by loss of bone committed with increased risk of fracture, failure in increasing of bone mass and higher amount of bone resorption. Abnormalities in osteoblast function trigger these disorders. Deformity of osteoblast function may be result of cellular senescence or irregular secretion of growth factors or hormones. Clinical diagnostics of OP depends on measuring bone mass with densitometry techniques and combining the results with biochemical measurements of indicators such as ALP, OCN in plasma and hydroxyproline and calcium in urine. These measurements give idea about the rate of bone formation and resorption. In Paget's disease osteoclast activity cause formation of irregular bone resorption and this cause strong osteoblast function and newly formed bone tissue is in abnormal architecture and woven form. Reason of these abnormalities is viral infection. Also result of incomplete mineralization of osteoid, observation of growth plates both in adults (osteomalacia) and children (rickets) is occurred [66].

1.5.5.1. Osteoporosis

Bone is dynamic tissue with the balance of bone formation and resorption. Organic and inorganic content of bone is effective on bone tissue's rigidity and density. It may be both in dense and rigid form and weak and fragile form. Most of the metabolic bone diseases have role in arising of these forms of bone tissue that cause deformities. OP is a metabolic disease characterized by deteriorated bone tissue, resulting in low bone mass and bone fragility. Worldwide, incidence rate of disease is getting higher depends on age, sex, ethnicity, weight, smoking, alcohol intake, diet, lifestyle and some other diseases. Disease is asymptomatic till patient has bone fracture. In different populations, OP related fractures in hip, wrist and spine are observed at different incidence rate. Having bone fragility in women at postmenopausal term and men over the age of 50 is generally detected. Wide scope of epidemiologic studies on OP is published in 2012 that include 50 years of period. In 2004, as reported by US Surgeon General, 10 million Americans, more than age of 50 have OP and 34 million are at risk for the disease. In Europe another report obtained in 2010 indicate that 6.6% of men and 22.1% of women aged over 50 years had OP. In a British study estimations are familiar with others with the result of 1 in 2 women and 1 in 5 men aged 50 years are assumed to have osteoporotic fractures during this period of their life [74]. Closest case studies in Turkey were evaluated in 2009 that involve detection of bone fractures in population of age 50 or higher that were obtained from different regions via different ways. BMD of 1965 men and women patients were evaluated and fracture incidence in several parts of body such as neck and hip were analyzed. Results of 2009 that were compared with a previous report were higher than those obtained in 1988/1989. And in 2009 approximately 24000 hip fractures that involve 73% of women patients were detected and it is proposed that incidence rate of bone fracture will be increased to 64000 in 2035 that doesn't matter sex or age specificity. As it seems prevalence of osteoporotic hip fractures are lower than world incidence in Turkey but remarkable increasing of cases should be noticed and evaluated according to these predictions [75].

Factors such as hormones are effective on bone metabolism disorders. Controlling of related hormone secretions might have both anabolic and catabolic effects on bone metabolism. As an important hormone PTH have roles in releasing and absorption of calcium, phosphate and 1,25 dihydroxyvitamin D3 through its actions in bone, kidney

and GI tract. Quantitating the rate of OP can be determined in three ways; radiological measurements of bone density (radiograms, computed tomography, ultrasound), laboratory tests (circulating serum calcium, phosphate, 1,25 dihydroxyvitamin D3, ALP, PTH, serum OCN, COL1 and urine calcium) and bone biopsy.

As mentioned before, combination of different conditions are responsible for the disease. For the therapy, strategies that cause rebuilding of bone is acceptable such as calcium and vitamin D supplementations, patient specific exercises, hormone and agent treatments that prevent bone resorption like estrogen, CT. In addition to these conditions genetic basis of disease is worth to study. Genetic factors are effective up to 80% on BMD as a strong predictor of OP. Polymorphisms in candidate genes such as vitamin D receptor (VDR), estrogen receptor (ESR), COL1A1 are indicators of disease [76]. Mutations in VDR gene cause 1,25-dihydroxyvitamin D-resistant rickets. Researches in this gene polymorphism are occurred in small population and suggest the importance of relations in bone loss and VDR genotype. Also it is proposed that, interaction of VDR gene polymorphisms and BMD is still unclear [77].

1.6. GENE SILENCING AND OVEREXPRESSION

1.6.1. Methods of Gene Delivery into the Cell for Gene Silencing and Overexpression

Transfection is a powerful tool for studying the functions of genes and gene products in cells [78]. In its simplest form, transfection is the general name given to the process of transferring foreign DNA fragments to the nucleus in the eukaryotic cell. DNA is delivered into the cell in plasmid form. For general purposes transfection can be divided roughly to two parts: Transient transfection or stable transfection (Figure 1.4) [79].

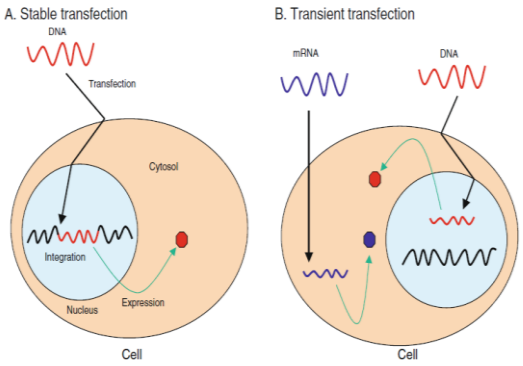


Figure 1.4: Schematic diagrams of stable and transient transfection [79].

In transient transfection, plasmid is not integrated into the genome. Plasmid amount decreases over time due to degradation of the plasmid over time or a decrease due to cell division [79]. For stable transfection, DNA must be integrated into the genome [78]. The plasmid is linearized before transfection, in order to increase the chance of correct integration of the plasmid [79]. Although, DNA can be integrated into the genome, the efficiency of transfection is very low in this method compared to transient transfection. Selection markers are used to identify clones in which stable transfection has occurred correctly. Selection markers can be aminoglycoside-phosphotransferase, hygromycin-phosphotransferase, puromycin-acetyltransferase, dihydrofolat-reductase (DHFR), and GFP-Fusion proteins [79].

Although there are many different methods for transfection, general factors that influence transfection efficiency can be summarized as follows: Cells must be in logarithmic phase of growth, cells must be grown under optimal conditions, cell density during transfection should be between 40-80% depending on cell type [74]. Another factor in the success of transfection is the high quality of DNA and the absence of protein, RNA, and chemicals in DNA [80].

DNA is unable to pass the amphipathic plasma membrane due to its polar property [81]. Hence, various tools exist in order to transfer DNA into the cell. Transfection methods can be divided into three main categories as chemical, physical and viral transfection methods.

1.6.1.1. Lipid Maintained Gene Delivery

The genetic material is transported into the cell via liposomes. Lipid mediated gene delivery's yield is very high, but it is a disadvantage that it cannot be applied to all cell types. In this technique, a mixture of cationic lipid reagent and DNA is mixed and after a while DNA-liposome complex forms. This mixture is then given to the culture to be transfected. Sometimes toxic effects or changes in the cell membrane are observed in this technique [79].

1.6.1.2. Calcium Phosphate Maintained Gene Delivery

It works the same way as lipid mediated gene delivery. The difference is Calcium Chloride is mixed with DNA [80]. Genetic material is taken up by cells via endocytosis or phagocytosis. It provides transient and stable transfection. With this technique, high-level transfection can be achieved in HEK 293 cells known as easy to transfect cells [79]. This technique, which is often used to generate recombinant vectors, is only suitable for cells growing as monolayer [81].

1.6.1.3. DEAE-Dextran Mediated Gene Delivery

DEAE-Dextran, positively charged carbohydrate, interact electrostatically with negatively charged DNA and forms a complex. This complex is internalized through endocytosis. This technique is only suitable for transient transfection. The efficiency of gene delivery can be improved by using osmotic shock agents such as glycerol or DMSO [81].

1.6.1.4. Cationic Polymers

Other chemicals such as polyethylenimine or dendrimers can be used for gene delivery too. These chemicals are completely soluble in water. They can be synthesized in different lengths and different 3-D structure [80]. For simplicity only polyethylenimine mediated gene delivery method will be discussed under this topic. Polyethylenimine (PEI) is a polycationic high molecular weight polymer. Gene transfer is achieved via introducing PEI-DNA complex into the cell. Due to the cytotoxicity of PEI necrotic cell death may occur in this method (Figure 1.5) [81].

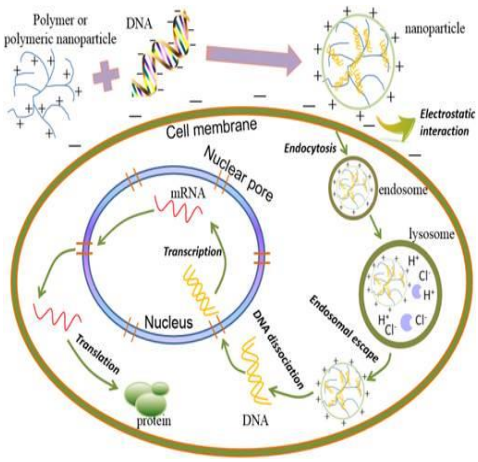


Figure 1.5: Schematic representation of polymer mediated gene delivery system [82].

1.6.1.5. Physical Transfection Methods

Different tools such as direct injection, biolistic particle delivery, electroporation, laser irradiation, sonoporation, and magnetic nanoparticles are used to deliver DNA into the cell [78].

Direct injection is a method to deliver naked DNA to the cell. This technique is hard to practice, because only one cell can be transfected at a time [80]. In electroporation pores in cell membrane are destabilized temporarily via using a pulse of electricity and genetic material is delivered to the cell [80]. Electroporation is commonly used with suspension cells [79]. Biolistic particle delivery is another method in which heavy metal coated with DNA is delivered to the cell via high-velocity [80]. Laser irradiation operates in the same way as electroporation. A focused pulse laser is used to form a transient pore in the membrane [78]. Due to osmotic difference DNA is transferred to the cell [80]. Pore can be formed in any region of the cell via laser irradiation [80]. Last but not least, sonoporation uses ultrasound for transient pore formation in the membrane [81].

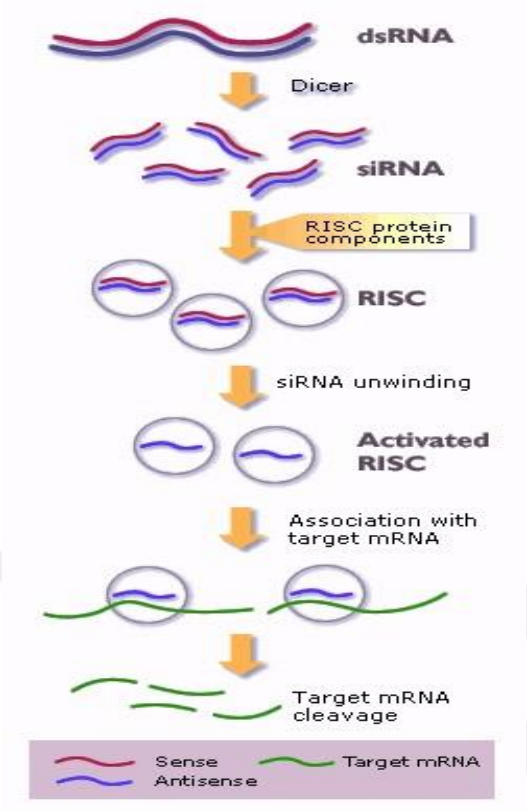
1.6.1.6. Viral Transfection Methods

The detailed elucidation of the life cycle of the virus through molecular biology has been preceded by the viral transfection. Virus mediated transfection is also called as transduction. Gene delivery efficiencies are very high in this method. Hence, it is suitable for introducing highly expressed genes. DNA viruses (adenovirus, adeno-associated virus (AAV), herpes simpex virus) and retroviruses (murine leukemia virus,

human immunodeficiency virus, and human T-cell lymphotropic virus) are some of the viruses used for transduction [80].

1.6.2. RNA Interference (RNAi) Mechanism

Gene silencing or overexpression studies give opportunity to observe the effects of the loss or gain of function of specific genes in mammalian systems. RNAi is a molecular technique, which inhibits gene expression at translation step or prevents transcription by degradation of target messenger RNA (mRNA). Its mechanism is complex. In mammalian systems, micro RNAs (miRNA) and siRNA use common action mechanisms. miRNAs locate in genome and endogeneously activated while siRNAs come from endogeneous sources [83]. For RNAi actions on target gene silencing, antisense strand of RNA that refers non-coding regions are essential. Double stranded RNAs (dsRNA) that are transported into the cell are cleavage into small interfering RNAs by ribonuclease enzyme Dicer. siRNA molecules interact with RISC (RNA induced silencing complex) and unwind into sense and antisense strands. Antisense strand of siRNA guides RISC for interaction of target mRNA. At the end of the process target mRNA cleavage is occurred and suppressed gene expression (Figure 1.6) [84]. Designing of suitable siRNA requires some optimizations related to localization of siRNA sequence in target gene and GC content. Also experimental controls are important for checking of some conditions such as cell line control that should has higher transfection efficiency, endogeneous positive control without siRNA treatment, optimal concentrations of siRNA to obtain gene knockdown and its half life and stability inside the cell, confirmation of siRNA by using tagged molecules transferred to the cell, checking of toxicity that affect cell viability, scrambled RNAs to check possible nonspecific effects. Checking of gene silencing is achieved by using moleculer techniques such as RT-qPCR and western blotting [85].



1. Entering of dsRNAs into the cell.
2. Cleavage of dsRNAs into small siRNAs by DICER enzyme.
3. Interaction of siRNAs with RISC and unwinding of strands.
4. Activation of RISC for binding to target mRNA by antisense strand of siRNA.
5. Degredation of target mRNA and gene knockdown.

Figure 1.6: Shematic representation and explanation of of RNAi mechanism inside cells [86].

1.6.3. Nucleofection

Nucleofector technology is a member of electroporation method. As a non-viral transfection method, it serves efficient, rapid and reproducible gene delivery into the mammalian cells. System gives opportunity to use cell specific commercially available products and electrical parameters. Even primary cells and hard to transfect cell types can be transfected by using nucleofector technology successfully. In other transfection methods cell division is required for transporting of gene into the cell and nucleus while nuclefection can be used for non-divided and proliferated cells of interest [87].

1.7. PCR ARRAY

PCR array system is useful and easy technique to analyze gene clusters related to a biological system or disease pathway. Technique uses SYBR green based RT-qPCR and broad range of gene scanning property of microarray that requires small amount of RNA sample. Array system is specifically designed according to interested biological event or disease by the companies and/or customize by the researcher. PCR array

technology is highly sensitive, robust and reproducible that tolerates possible problems of differences in individuals and laboratory conditions. PCR array technique is cost, time and labor intensive friendly that provides rapid information about up- and downregulation of genes related to specific pathways and doens't require intensive bioinformatical analysis. PCR array technology is alternative to costly and time and labor consuming microarray analysis that requires complicated bioinformatical analysis. Each array reaction contains 84 pathway specific genes, genomic DNA (gDNA) control to check the non-transcribed, repetitive gDNA, reverse transcription controls that demonstrate efficiency of PCR reaction that use external RNA control, positive PCR controls to check efficiency of polymerase chain reaction itself via the wells that contain artificial DNA and primers (Figure 1.7) [88].

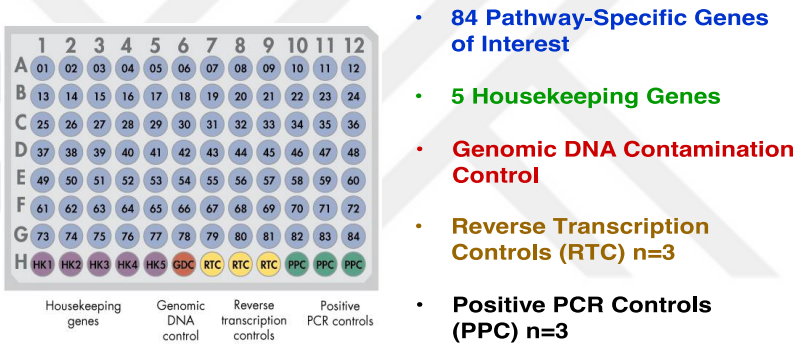


Figure 1.7: Explanation of RT^2 Profiler PCR array designed for 96 well plate format [89].

2. MATERIALS AND METHODS

2.1. CELL CULTURE

2.1.1. Thawing and Freezing of Cells

Cell culture process was started with maintaining and storage of cells. UE7T-13 line known as hMSC line was obtained from Riken Science Institute in Japan in catalog number of RBRC-RCB2161. Cells have been stored at liquid nitrogen tank for long-term preservation. Cell culture experiments were started with thawing of cells. Before starting of thawing process, 10 ml of pre-warmed culture media (Gibco, DMEM, Glutamax) at 37ºC and transferred to the 15 ml tube. Then cryovials were taken from the liquid nitrogen tank (-196ºC), transferred into the 37ºC water bath and swirled gently until melting. Liquid content of cryovial tube was transferred to the 15 ml tube, containing medium as quickly as possible and centrifuged at 2000 rpm for 5 min. Supernatant was discard, and get rid of DMSO (Dimethyl Sulfoxide, Applichem) and other waste products, by leaving behind pellet in medium. Pellet was suspended mixing by finger. Then cells were seeded into a 25 cm^2 tissue culture flask (Greiner) in 10 ml medium including 20% Fetal Bovine Serum (FBS) and 0.1% primocin as antibiotic and antifungal agent. Cells were incubated at 37^oC, 5% CO_2 incubator and the screw cap of the flask was kept loose in order to allow circulation of CO_2 into the flask and the next day medium was refreshed in order to remove dead cells and waste products. When cells become confluent they were subcultured and prepared for cell freezing of long-term preservation. Cells were resuspended in fetal bovine serum (FBS, Gibco) at density of 1-2x10^6 cells/ml. Cryovial tubes were placed on ice and 950 µl cell suspension was transferred into each tube. 50 µl DMSO was added into each tube drop by drop and after each drop cell suspension was mixed by pipetting, final concentration of DMSO was 5%. Tubes were left at -20^oC for 1h and then kept at -80^oC overnight. The next day cryovial tubes were transferred to the liquid nitrogen tank (-196^oC) for long-term preservation. Remaining cells were cultured for following experimental processes.

2.1.2. Subculturing of hMSC Line

After seeding of cells into culture flasks, cells were allowed to attached to the surface of flasks and started to proliferate and colony formation. When cells became 80-90% confluent, they were sub-cultured. DMEM (Dulbecco's Modified Eagle Medium) contains 1 g/L glucose with glutamax (Gibco), FBS, phosphate buffered saline (PBS, Lonza) and 0.25% Trypsin-EDTA (Gibco) were pre-warmed to 37^{o}C at water bath. Culture medium was removed from the flask by using a sterile pipette and then cells were washed with appropriate volume of PBS with calcium and magnesium. After removal of PBS, cells were trypsinized with appropriate volume of pre-warmed 0.25% Trypsin-EDTA at 37 ^{o}C in the incubator for 5-10 min. Then cells were observed under inverted microscope. When most of the cells were detached from the surface of the flask, appropriate volume of FBS was added to the flask to inactivate the activity of 0.25% Trypsin-EDTA. Cells in the flask with 0.25% Trypsin-EDTA and FBS were transferred into a new 15 ml falcon tube and centrifuged at 2000 rpm for 5 min at RT. After centrifugation, supernatant was discarded by leaving about 0.5 ml of cell suspension at the bottom of the tube. Pellet was finger mixed and volume was filled up to 10 ml with DMEM in order to remove 0.25% Trypsin-EDTA remained in the tube. Centrifugation step was repeated once again and then the cells were counted in Thoma hemocytometer. After counting, cells were seeded at a density of 1×10^{4} cells/cm^{2} with DMEM containing 10% FBS for expansion. Subculture of cells was repeated at about 3-4 days intervals.

2.2. MESODERMAL DIFFERENTIATION OF CELLS

2.2.1. Osteogenic Differentiation of hMSCs

For osteogenic differentiation, hMSCs were harvested by trypsinization and seeded into the plates at a density of $3.2x10^{4}$ cells/cm^{2}. To stimulate osteogenic differentiation, MesenCult Osteogenic Stimulatory Kit (StemcellTechnologies) including MesenCult Basal Medium, Osteogenic Stimulatory Supplement, β-glycerophosphate, dexamethasone, ascorbic acid was prepared as indicated in Table 2.1. Cells were incubated in Complete MesenCult Osteogenic Medium (without β-Glycerophosphate) at 37^{o}C in a humidified atmosphere of 5% CO_2 for 2 weeks by

refreshing of medium 3 times a week. Once cell multi-layering was observed, addition of β-Glycerophosphate to complete mesenCult osteogenic medium was started. Cell multi-layering is the layering of cells on top of each other and an indicator of bone tissue formation. Cultures were replenished with β-Glycerophosphate-containing osteogenic medium every 2-3 days. After 4 weeks of cultivation, cells were processed for TB staining. TB staining is an indicator of osteoblastic cells that produce calcium nodules and minerals. Staining process was utilized for demonstration of mineralized bone tissue.

Table 2.1: Contents of MesenCult Osteogenic Stimulatory Kit.

Product name	**Final concentration**
MesenCult MSC Basal Medium (Human)	Final volume
MesenCult Osteogenic Stimulatory Supplement (Human)	15% final volume
β-Glycerophosphate (1 M)	3.5 mM
Dexamethasone (10^{-4} M)	10^{-8} M
Ascorbic Acid (10 mg/ml)	50 µg/ml

2.2.2. TB Staining of Calcified Bone Matrix

Cells under osteogenic differentiation conditions were assessed via TB staining at indicated time points. Process was started with removing of media from the plate. Culture plates were rinsed with 1X PBS and started fixation with 4% paraformaldehyde solution for 30 min at RT. Wells were rinsed with 1X PBS again and cells were stained with 1% of TB (Sigma) staining solution (in 50% isopropanol) for 40-50 min at 37^{o}C. Samples were incubated in absolute isopropanol for 1 min. Wells were rinsed with dH_2O and observed under inverted microscope.

2.2.3. Alizarin Red Staining of Ossified Matrix

For determination of ossified bone matrix ARS was proceeded. hMSCs were seeded into suitable culture vessesls at a confluency of $3.2x10^4$ cells/cm^2. Cells were kept under osteogenic differentiation conditions at indicated time points. At the end of these time points process was started with fixation of cells. Culture medium was removed and cells

were washed with PBS 3 times and distilled water respectively. They were fixed with ice-cold methanol for 5 min at RT. Then washed with PBS again and stored at +4°C till staining of samples. Before staining of cells AR dye was prepared. 1% of AR was dissolved in distilled water and pH was adjusted to approximately 5 via addition of 1M sodium hydroxide and 0.1M hydrochloric acid. Cells were stained for 45-60 min at RT and washed with distilled water and observed under microscope for detection of stained calcified regions of bone matrix.

2.2.4. Determining the Expression Level of Osteogenesis Specific Genes

hMSCs that were induced into osteogenic differentiation were used for isolation of RNA by using RNeasy kit (Qiagen) at the end of 4th week.

2.2.4.1. RNA Isolation

Cells were seeded at a density of 3.2×10^4 cells/cm^2 into 35 mm in diameter tissue culture dishes. Cells were disrupted by adding of buffer RLT in appropriate volumes as indicated in Table 2.2 and vortex by mixing for 1 min.

Table 2.2: Amount of buffer RLT required for cell lysis process.

Cell Number	Amount of buffer RLT (µl)
$<5x10^6$	350
$5x10^6 – 1x10^7$	600

70% of ethanol was added 1 volume to homogenize lysate, and mixed well by pipetting. The sample up to 700 µl was transferred into an RNeasy spin column placed in a 2 ml collection tube and centrifuged for 15s at 8000 x g. Buffer RW1 was added in 700 µl to the RNeasy spin column and centrifuged for 15s at 8000 x g. 500 µl of buffer RPE was added into RNeasy spin column. The spin column membrane was centrifuged for 15s at 8000 x g for washing. 500 µl of buffer RPE was added into the RNeasy spin column and centrifuged for 2 min at 8000 x g. The RNeasy spin column was placed in a new 1.5 ml collection tube. 30 µl of RNase-free water was added into the spin column membrane directly and centrifuged for 1 min at 8000 x g to elute the RNA. For quantifying the amount of RNA sample, Nanodrop 2000 (Thermo) was used.

2.2.4.2. Synthesis of Complementary DNA (cDNA)

RNA was reverse transcribed to obtain cDNA by quantitect reverse transcription kit (Qiagen). Template RNA, gDNA wipeout buffer, quantiscript reverse transcriptase (RT), quantiscript RT buffer, RT primer mix, and RNase-free water were thawed at room temperature. The gDNA elimination reaction was prepared on ice according to Table 2.3 and mixed well then stored on ice.

Table 2.3: gDNA elimination reaction components.

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

Reaction mixture was incubated for 2 min at 42°C and then placed on ice. Then, the reverse-transcription master mix was prepared on ice, according to Table 2.4.

Table 2.4: Reverse-transcription reaction components.

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

Template RNA was added (14 µl) to each tube containing reverse-transcription master mix and stored on ice. Master mix was incubated for 15 min at 42°C and 3 min at 95°C to inactivate quantiscript reverse transcriptase and stored at -20°C. Then cDNA samples were used for RT-qPCR experiments for confirmation of the process at molecular level.

2.2.4.3. RT-qPCR Analysis

PCR amplification was carried out using gene specific primers indicated in Table 2.5 and the SYBR Premix Ex Taq (Tli RNase H Plus) (Cat. no: RR420) on Rotor Gene 6000 Real Time PCR instrument (Corbett Life Science) under given thermocycling conditions: 95˚C for 5 min, 40 repetitive of 95˚C for 10 sec as denaturation step, 60˚C for 30 sec as annealing step, 72˚C for 20 sec as an extension step. Actin gamma was used as housekeeping gene to normalize amplified products.

Table 2.5: Primer sequences of interested genes.

Primer name	**(5'-3')_Forward**	**(5'-3')_Reverse**	**Annealing temp. °C**
Actin gamma	CGCACCACTGGCATTGTCAT	GTGGCCATCTCCTGCTCGAA	59,4 °C, 60 sec
BSP	CACTGGAGCCAATGCAGAAGA	TGGTGGGGTTGTAGGTTCAAA	60 °C, 60 sec
OPN	CTCCATTGACTCGAACGACTC	CAGGTCTGCGAAACTTCTTAGAT	60 °C, 60 sec
OCN	CACTCCTCGCCCTATTGGC	CCCTCCTGCTTGGACACAAAG	60 °C, 60 sec

2.3. TRANSFECTION

2.3.1. Overexpression of Topo IIβ in hMSCs

pEGFP-N1 plasmid including topo IIβ gene was a kindly donated from Okayama University, Medical School, Prof. Tsutsui to our lab. (Fig.2.1). Before starting of transfection studies, confirmation of topo IIβ gene insert was occured.

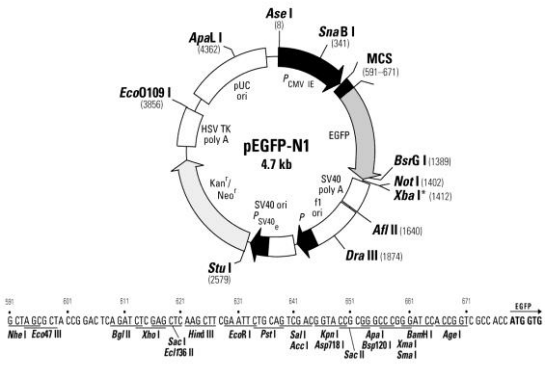


Figure 2.1: pEGFP-N1 vector, 4.7 kb in length from BD Biosciences Clontech. (GenBank #U55762).

2.3.1.1. Preperation of Calcium Shocked Competent E.coli DH5α

First of all, calcium shocked competent *E.coli* DH5α strain was obtained and transformed with Topo IIβ-EGFP plasmid to amplify the plasmid in order to make large quantities of it. Chosen *E.coli* was cultured overnight in 5 ml LB medium without antibiotics at 37°C at 200-250 rpm. 16-18h later 1 ml of culture was taken and added into 200 ml of LB medium (-ab) and incubated at 37°C at 200-250 rpm for 1.5-3h. Values at OD_{600} was measured (0.3-0.6 /ml) and culture was cool down on ice for 10 min. Cells were transferred to falcon tube and centrifuged at 3600 rpm x15 min x 4°C and supernatant was discarded. 10 ml ice cold 0.1 M $CaCl_2$ was added and pellet was resuspended carefully. Culture was centrifuged at 1200 rpm x 10 min and supernatant was discarded. 6.5 ml 0.1 M $CaCl_2$ was added again and cells were resuspended to incubate at 0°C for 1-2h. 1.2 ml of autoclaved glycerol was added (final concentration was 15%) and incubated on ice for 10 min. cells were aliquoted into pre-cool down 1.5 ml tubes at appropriated volumes (50-200 µl).

2.3.1.2. Transformation of EGFP- Topo IIβ plasmid into E.coli DH5α

Transformation process was occurred via heat shock. Competent cell vial was thawed on ice and 2 µl 0.5 M of β-mercaptoethanol was added to tube and mixed by tapping gently. 10 ng-100 ng interested plasmid DNA (pDNA) was added to tube and incubated on ice for 30 min. Following incubation on ice cells were transferred to heat block at 42°C for 30 sec and quickly put on ice for 2 min. 450 µl of prewarmed (RT) LB medium was added to vial and incubated at 37°C for 1h at 200 rpm. Tubes were placed on ice again and spread to agar plates at appropriate selected antibiotic (kanamycin).

2.3.1.2. Plasmid Isolation from Transformed Bacterial Cultures

Plasmid isolation was applied via Qiagen Midiprep #12145. Transformed cells were streaked to kanamycin resistant plates (30 µg/ml). Then a single colony was picked up by using loop and seeded to 5 ml LB broth and incubated at 37°C 200 rpm for 8h. 8h later 100 µl culture was transferred to 100 ml LB broth and incubated at 37°C 200 rpm overnight. 16h later concentration of bacterial culture was measured at OD_{600}. Bacterial cells were harvested by centrifugation at 6000 x g for 15 min at 4°C. Bacterial pellet was resuspend in 4 ml Buffer P1. The bacterial pellet was resuspended completely by vortexing (or pipetting up and down) until no cell clumps remained. 4

ml of Buffer P2 was added and mixed gently inverting the sealed tube 4–6 times, and incubated at RT (15–25°C) for 5 min. 4 ml of chilled Buffer P3 was added and mixed immediately and vigorously inverting 4–6 times, and incubated on ice for 15 min. Bacterial lysate was centrifuged at ≥20,000 x g for 30 min at 4°C. Supernatant containing pDNA was removed promptly. Supernatant was centrifuged again at ≥20,000 x g for 15 min at 4°C. Supernatant containing pDNA was removed promptly. QIAGEN-tip 100 was equilibrated by applying 4 ml Buffer QBT, and allowed the column to empty by gravity flow. Supernatant obtained from previous step was applied to the QIAGEN-tip and allowed to enter the resin by gravity flow. QIAGEN-tip was washed with 2 x 10 ml Buffer QC. Eluate was collected in a 15 ml tube and pDNA was precipitated with isopropanol then DNA precipitate was washed with 2 ml of room-temperature 70% ethanol and centrifuged at ≥15,000 x g for 10 min. Carefully supernatant was decanted without disturbing the pellet. Pellet was air dried for 5–10 min, and redissolved in a suitable volume of TE buffer, pH 8.0. Following step of the isolation of topo IIβ inserted EGFP-N1 plasmid, confirmation studies were started.

2.3.1.3. Confirmation of Topo IIβ DNA insert into EGFP-N1 plasmid

Process was achieved in two ways; colony PCR and digestion of plasmid with appropriate restriction enzymes.

After transformation procees, bacteria were seeded into LB agar plate, which contain kanamycin as a selective antibiotic. 16h later, plate was checked for growing bacteria. From the plate, 5 different colonies were picked and directly transferred to PCR mixture, analyzed with PCR experiment. After that, samples were loaded into agarose gel for obtaining the results of true insert.

For restriction digestion process, vector plasmid's restriction points were detected. Suitable restriction enzymes for cutting of the pDNA were indicated. Three types of enzyme can be used for detection of topo IIβ gene insert. XhoI, SmaI and BamHI were appropriate enzymes for this reaction. When XhoI, SmaI are used, plasmid is cut into two pieces (insert 4.8kb, lineer plasmid 4.7 kb). If XhoI, BamHI are used, plasmid cut into three pieces (insert 2.3+2.5 kb, lineer plasmid 4.7 kb.) Both of the enzyme couples were used to check presence of topo IIβ gene insert. Digested DNA samples were analysed via DNA gel electrophoresis.

2.3.2. Optimizations of EGFP-Topo IIβ plasmid concentration for Overexpression in hMSCs

To determine the possible effects of topo IIβ gene on osteogenic differentiation of hMSCs, overexpression studies were carried out. Topo IIβ inserted pEGFP-N1 plasmid (Figure 2.1) was used to transfect hMSC line by nucleofection technique (Amaxa).

In this part, some of the conditions such as plasmid concentration and cell number seeded on plates were critical. In addition to the topo IIβ gene, GFP plasmid was used as a positive control. The Amaxa Nucleofection (4D Nucleofector^{TM} system, Lonza) was used for transfecting hMSCs with topo IIβ gene. This method was essential for increasing the transfer efficiency of topo IIβ gene. hMSCs were deattached by 50 µl/cm^2 0.25% Trysin-EDTA solution then FBS was added to stop the activity of tyrpsin. Cells were counted and cell density was determined. 5 × 10^6 cells were centrifuged at approximately 600 x g for 5 minutes at RT and supernatant was completely removed. Cells were resuspended in total 100 µl solution including 82 µl nucleofector solution and 18 µl supplement. 2 µg pmaxGFP plasmid and 5, 6 µg topo IIβ plasmid were added to each 100 µl solution and transferred into 100 µl nucleocuvettes. System is adjusted to suitable programme for hMSC line, FF-104 (high efficiency) program was applied. After nucleofection, cells were resuspended in 500 µl prewarmed RPMI containing 10% FBS and incubated at 37^{o}C for 10 min and seeded into the prewarmed DMEM and 10% FBS containing well plates.

2.3.3. Optimization of Topo IIβ siRNA Transfection

In this part, topo IIβ gene silencing was achieved by using siRNA delivery. Gene silencing process was applied by using four different validated siRNAs at 48h. Four different validated siRNAs (TOP2B_5, TOP2B_6, TOP2B_7, TOP2B_8 from Qiagen) were used for silencing of topo IIβ as indicated in table 2.6. Lipofectamine RNAiMAX was used as a transfection agent. Cells were seeded to 35 mm in diameter tissue culture dishes at a density of 3.2×10^4 cells/cm^2 and meanwhile siRNA treatment was started. siRNAs were diluted with Opti-MEM at a range of 1:100 and Lipofectamine RNAiMAX was diluted with Opti-MEM in 1:50 ratio. Diluted siRNAs (20 nM, 15 nM, 12.5 nM, 10 nM, and 7.5 nM topo IIβ-specific siRNAs) and diluted

reagent were mixed and incubated for 15 min at RT to allow the formation of siRNA-Lipofectamine RNAiMAX complexes. After incubation siRNA-Lipofectamine RNAiMAX complexes were added to the wells directly. Cells were incubated at 48h, RNA was isolated and gene silencing was confirmed at molecular level via RT-qPCR. RNA was extracted by using RNeasy kit (Qiagen).

Table 2.6: Sequences of Topo IIβ siRNA produced by Qiagen.

siRNA	Target sequence_5'-3'	Sense sequence_5'-3'	Antisense sequence_5'-3'
TOP2β_5	TTCCTTTATGATG ATAATCAA	CCUUUAUGAUGAUA AUCAATT	UUGAUUAUCAUCAU AAAGGAA
TOP2β_6	GAGATAAATAAT ATTATTAAA	GAUAAAUAAUAUUA U UAAATT	UUUAAUAAUAUUAU UUAUCTC
TOP2β_7	AAGAGGAATGAT AAACGTGAA	GAGGAAUGAUAAAC GUGAATT	UUCACGUUUAUCAU UCCUCTT
TOP2β_8	CTGATTGAAGTAG TTAAGAAA	GAUUGAAGUAGUUA AGAAATT	UUUCUUAACUACUUC AAUCAG

2.3.4. Confirmation of Topo IIβ Gene Overexpression and Silencing via RT-qPCR

Both for overexpression and silencing studies, RNA samples of indicated time points transfected with several concentrations of topo IIβ plasmid and siRNA were isolated, cDNA synthesis was occurred and RT-qPCR analysis was done as previously explained in parts 2.2.4.1, 2.2.4.2 and 2.2.4.3. Following table indicates the primer sequence and conditions of experiments for Topo IIβ.

Table 2.7: Primer sequences and conditions of interested genes.

Primer name	(5'-3')_Forward	(5'-3')_Reverse	Annealing temp. (^{o}C)
Topo IIbeta	TTTTTCACCATCATTTGGTCTG	GGGCTTAGGGACTGTATCTGAA	60 ^{o}C, 60 sec.

2.3.5. Osteogenic Differentiation of Topo IIβ Induced and Silenced hMSC Line

2.3.5.1. Osteogenic differentiation of hMSC line transfected with topo IIβ-EGFP

To determine the possible effects of topo IIβ gene expression on osteogenic differentiation of hMSCs, overexpression studies were performed. Topo IIβ inserted pEGFP-N1 plasmid (Figure 2.1) was used to transfect hMSC line by using a physical method, known as nucleofection technique (Amaxa). In this part, conditions such as

plasmid concentration and cell seeding number were used as indicated before. These conditions were combined with osteogenic differentiation process.

Before starting of osteogenic differentiation, transfection of topo IIβ plasmid is proceeded via Amaxa nucleofection (4D Nucleofector™ system, Lonza). Cells are seeded into 1x75 cm^2 and 1x175 cm^2 cell culture flasks 4 days before nucleofection. hMSCs were deattached with 50 µl/cm^2 0.25% Trypsin-EDTA solution then, FBS was added to inactivate the tyrpsin. Cells were counted and number of cells required for nucleofection was determined. Because of high mortality rate high number of cells are required. For preperation of 2 x 35 mm diameter plates, $7.5x10^6$ cells were reqired. At least 4 fold increased cell number was used that $4x10^6$ cells were counted for obtaining $7.5x10^6$ topo IIβ-EGFP transfected cells. $4x10^6$ cells were centrifuged at approximately 600 x g for 5 min at RT and supernatant was completely removed. Amaxa nucleocuvette vessels were used. In each cuvette $5x10^5$ hMSCs can be transfected, so totally 8 cuvettes were used. Nucleofector machine is suitable for transfection of 2 nucleocuvette in each usage, $4x10^6$ cells were divided into $1x10^6$ cells/tube. 1.5 ml eppendorf tubes were centrifuged at 200 g for 10 minutes, supernatant was removed and cell pellet was resuspended in 100 µl solution, including 80 µl nucleofector solution and 18 µl supplement. 6 µg topo IIβ plasmid were added to each 100 µl solution and transferred into 100 µl nucleocuvettes. System was adjusted to suitable programme for hMSC line, FF-104 (high efficiency) program was applied. After nucleofection, cells were resuspended in 500 µl prewarmed RPMI containing 10% FBS and incubated at 37^oC for 10 min and seeded into the prewarmed DMEM+10% FBS containing well plates. Cell confluency wasn't sufficient for osteogenic differention process so that cells were incubated 7 days to obtain required confluency. After that differentiation process was initiated and proceeded during 14 days.

2.3.5.2. Osteogenic Differentiation of Topo IIβ Knockdown hMSC Line

Determination of optimum concentration of siRNA enabled starting of osteogenic differentiation in topo IIβ silenced hMSCs. Before starting of differentiation, gene knockdown was proceed at 48h as indicated previously. Cells were seeded to 35 mm diameter cell culture plates at a concentration of $3.2x10^4$ cells/cm^2. Then

differentiation process was started. Osteogenic medium was changed per 48h in addition to siRNA refreshment. 7^{th} and 14^{th} days of differentiation were selected for determination of osteogenic differentiation potential. Indicated time points were also important for gene expression detection of differentiation.

2.3.5.3. Checking the Osteogenic Differential Procees at Indicated Time Points

Induction of osteogenic differentiation at coditions of manipulated topo IIβ gene expressions via silencing or overexpression was checked with both microscopic observation of calcification and RT-qPCR of related genes. Procedures were conducted as previously explained according to type of experiments.

2.4. RT^2 PROFILER PCR ARRAY

2.4.1. Silencing of Topo IIβ and Induction of hMSCs into Osteogenic Differentiation at Days 1, 3, 5, 7

As previously described hMSC line was subcultured and seeded for gene silencing studies. After seeding of cells into culture flasks, cells attached to the surface of flasks and started to proliferate and colony formation. When cells became 80-90% confluent, they were subcultured and started to preparation of experimental groups. Meanwhile silencing of topo IIβ was started. Four different validated siRNAs (TOP2β_5, TOP2β_6, TOP2β_7, TOP2β_8 from Qiagen) were used for silencing of topo IIβ. Lipofectamine RNAiMAX was used as a siRNA transfection agent. Cells were seeded to 35 mm in diameter tissue culture dishes at a density of 3.2×10^4 cells/cm^2 for starting of siRNA treatment. siRNAs were diluted with Opti-MEM medium at a ratio of 1:100 and Lipofectamine RNAiMAX was diluted with Opti-MEM in 1:50 ratio. Diluted siRNAs (10 nM per topo IIβ-specific siRNAs) and diluted reagent were incubated at RT for 5 min then mixed and incubated for 20 min at RT to allow the formation of siRNA-Lipofectamine RNAiMAX complexes. After incubation siRNA-Lipofectamine RNAiMAX complexes were added to the plates directly. Cells were incubated at 48h to allow silencing and started to osteogenic differentiation. To stimulate osteogenic differentiation, Complete MesenCult Osteogenic Medium including MesenCult MSC Basal Medium, Osteogenic Stimulatory Supplement, β-Glycerophosphate, Dexamethasone, Ascorbic acid (all from Stemcell company) was prepared. Cells were

incubated in Complete MesenCult Osteogenic Medium at 37^{o}C in a humidified atmosphere of 5% CO_2. According to indicated time points, culture was replenished with Complete MesenCult Osteogenic Medium and required supplements. Process was continuously repeated per 48h for 7 days. Required samples at days 1, 3, 5 and 7 were prepared for the following experiments.

2.4.2. Total RNA Extraction

RNA samples of each days' topo IIβ silenced and differentiated samples were isolated by using RNeasy Plus minikit (Qiagen). Procedure was same as part 2.2.4.1 RNA isolation. Only one step was added to the previous procedure, after collection of cell homogenate. Before addition of 70% ethanol homogenized lysate was transferred to a gDNA Eliminator spin column placed in a 2 ml collection tube and centrifuged for 30 sec at ≥8000 x g (≥10,000 rpm). For quantifying the amount of RNA samples, NanoDrop 2000 spectrophotometer (Thermo scientific) was used. After quantification, RNA samples were reverse transcribed to obtain cDNA by using RT² first strand kit (Qiagen).

2.4.3. cDNA Synthesis Using the RT² First Strand Kit Components

Reagents of the RT^2 First Strand Kit (Tables 2.8, 2.9) were thawed and briefly centrifuged (10– 15 sec) to bring the contents to the bottom of the tubes. gDNA elimination mix for each RNA sample according to Table 2.8 was prepared and mixed gently by pipetting up and down, then centrifuged briefly.

Table 2.8: gDNA elimination reaction components.

Component	Volume/Reaction
Template RNA (0.5µg)	variable
Buffer GE	2µl
RNase-free water	variable
Total volume	10 µl

gDNA elimination mix was incubated for 5 min at 42°C, then placed immediately on ice for at least 1 min. Reverse transcription mix was prepared according to table 2.9.

Table 2.9: Reverse-transcription reaction components.

Component	Volume/Reaction
5x Buffer BC3	4 µl
Control P2	1 µl
R3 Reverse Transcriptase Mix	2 µl
RNase-free water	3 µl
Total volume	10 µl

10 µl reverse-transcription mix was added to each tube containing 10 µl gDNA elimination mix and mixed gently by pipetting up and down. Mixture was incubated at 42°C for exactly 15 min. Then the reaction was stopped immediately by incubating at 95°C for 5 min. At the end 91µl RNase-free water was added to each reaction tube, mixed by pipetting up and down several times. Reactions were stored at –15 to –30°C.

2.4.4. Real-Time PCR of RT^2 Profiler PCR Array

This protocol combines RT^2 profiler PCR array with RT^2 SYBR green mastermix. RT^2 SYBR green mastermix was briefly centrifuged (10–15 sec) to bring the contents to the bottom of the tube at room temperature. PCR components were prepared and mixed in a 5 ml tube, as indicated in table 2.10.

Table 2.10: PCR components' mix.

Component	Volume/Reaction
2x RT^2 SYBR Green Mastermix	1350µl
cDNA sample	102µl
RNase-free water	1248µl
Total volume	2700µl

Note: Mixture resides an excess volume of 300 µl (to allow for pipetting errors). Remaining 9 µl cDNA synthesis reaction was stored at –15 to –30°C, as it may be needed to perform quality control analysis.

PCR components mix was dispensed into the RT^2 Profiler PCR Array. Pipet tips were changed following each pipetting step to avoid cross-contamination between the wells. Carefully RT^2 Profiler PCR Array plate was removed from its sealed bag. 25µl PCR components mix was added to each well of the RT^2 Profiler PCR Array. RT^2 profiler PCR array plate was carefully and tightly sealed with optical adhesive film and

centrifuged for 1 min at 1000 *g* at room temperature (15–25°C) to remove bubbles. Plate was placed on ice while setting up the PCR cycling program.

Note: The RT^2 Profiler PCR Array containing PCR components mix may be stored at –15 to –30°C wrapped in aluminum foil for up to one week if desired.

Set-up instructions of Roche LightCycler 480 real-time PCR for Running of RT^2 Profiler PCR Arrays:

In the setup area of program software, following setup parameters were specified.

- Detection Format: SYBR Green I
- Block Type: block format 96
- Reaction Volume: 25µl for 96 well plate array format

Protocol is multistep process and set up according to required conditions as indicated below.

First program is "Heat Activation" that is essential for polymerase activity. Number of cycles left at "1" and analysis mode was set up as "None." In the program temperature targets section, values was entered for the following parameters (see Table 2.11).

Table 2.11: Heat Activation program conditions.

Target	Acq. mode	Hold (hh:mm:ss)	Rate of Ramp
95 °C	None	00:10:00	4.8 °C/s

New program was added for "PCR Cycling" conditions. Number of cycles and analysis mode were changed to "45" and "Quantification" respectively. Following conditions were identified ın Table 2.12.

Table 2.12: PCR Cycling conditions.

Target	Acq. mode	Hold (hh:mm:ss)	Rate of Ramp
95 °C	None	00:00:15	1.5 °C/s
60°C	Single	00:01:00	1.5 °C/s

Program name of the 3rd program was changed to "Melt Curve". Number of cycles and analysis mode were changed to "1" and "Melting Curves" respectively. Following

conditions were identified in Table 2.13.

Table 2.13: Melt Curve conditions.

Target	Acq. mode	Hold (hh:mm:ss)	Ramp	Acquisition/°C
60°C	None	00:00:15	4.8 °C/s	
95°C	Continuous		0.03°C/s	20

After running the protocol obtained results were analyzed by using data analysis software that is offered by Qiagen Company. Changes in target genes' expressions that were referred as critical threshould (CT) value were calculated with ΔΔCT method and normalized to B2M and HPRT1 genes. CT values>40 were detected as non-transcribed and haven't been expressed. For each group of experiment, arrays were done in single set.

2.4.4.1. Osteogenesis PCR Array

Osteoblastogenesis takes several weeks with differential expression rate of gene clusters. PCR array of osteogenesis pathway serves opportunity to analyze genes, related to development of skeletal, bone mineral metabolism, extracellular matrix, cell-adhesion molecules, growth factors and transcription factors [90]. Experimental design and localization of genes at 96 well plate was given in Table 2.14.

Table 2.14: Gene list of Qiagen Osteogenesis PCR array, PAHS-026Z [90].

Position	Unigene	Refseq	Symbol	Description	Gname
A01	Hs.470316	NM_001105	ACVR1	Activin A receptor, type I	ACTRI/ACVR1A/ ACVRLK2/ALK2/ FOP/SKR1/TSRI
A02	Hs.324746	NM_001622	AHSG	Alpha-2-HS-glycoprotein	A2HS/AHS/FETU A/HSGA
A03	Hs.75431	NM_000478	ALPL	Alkaline phosphatase, liver/bone/kidney	AP-TNAP/APTNAP/H OPS/TNAP/TNSA LP
A04	Hs.480653	NM_001154	ANXA5	Annexin A5	ANX5/ENX2/HEL -S-7/PP4/RPRGL3
A05	Hs.654541	NM_199173	BGLAP	Bone gamma-carboxyglutamate (gla) protein	BGP/OC/OCN
A06	Hs.821	NM_001711	BGN	Biglycan	DSPG1/PG-S1/PGI/SLRR1A
A07	Hs.1274	NM_006129	BMP1	Bone morphogenetic protein 1	OI13/PCOLC/PCP/ PCP2/TLD
A08	Hs.73853	NM_001200	BMP2	Bone morphogenetic protein 2	BDA2/BMP2A

Table 2.14 (continue): Gene list of Qiagen Osteogenesis PCR array, PAHS-026Z [90].

A09	Hs.387411	NM_001201	BMP3	Bone morphogenetic protein 3	BMP-3A
A10	Hs.68879	NM_130851	BMP4	Bone morphogenetic protein 4	BMP2B/BMP2B1/MCOPS6/OFC11/ZYME
A11	Hs.296648	NM_021073	BMP5	Bone morphogenetic protein 5	-
A12	Hs.285671	NM_001718	BMP6	Bone morphogenetic protein 6	VGR/VGR1
B01	Hs.473163	NM_001719	BMP7	Bone morphogenetic protein 7	OP-1
B02	Hs.524477	NM_004329	BMPR1A	Bone morphogenetic protein receptor, type IA	10q23del/ACVRLK3/ALK3/CD292/SKR5
B03	Hs.598475	NM_001203	BMPR1B	Bone morphogenetic protein receptor, type IB	ALK-6/ALK6/CDw293
B04	Hs.471119	NM_001204	BMPR2	Bone morphogenetic protein receptor, type II (serine/threonine kinase)	BMPR-II/BMPR3/BMR2/BRK-3/POVD1/PPH1/T-ALK
B05	Hs.489127	NM_001742	CALCR	CALCITONIN RECEPTOR	CRT/CT-R/CTR/CTR1
B06	Hs.120949	NM_000072	CD36	CD36 molecule (thrombospondin receptor)	BDPLT10/CHDS7/FAT/GP3B/GP4/GPIV/PASIV/SCARB3
B07	Hs.596112	NM_001797	CDH11	Cadherin 11, type 2, OB-cadherin (osteoblast)	CAD11/CDHOB/OB/OSF-4
B08	Hs.166186	NM_003741	CHRD	Chordin	-
B09	Hs.520339	NM_000493	COL10A1	Collagen, type X, alpha 1	-
B10	Hs.409662	NM_021110	COL14A1	Collagen, type XIV, alpha 1	UND
B11	Hs.409034	NM_001855	COL15A1	Collagen, type XV, alpha 1	-
B12	Hs.172928	NM_000088	COL1A1	Collagen, type I, alpha 1	EDSC/OI1/OI2/OI3/OI4
C01	Hs.489142	NM_000089	COL1A2	Collagen, type I, alpha 2	OI4
C02	Hs.408182	NM_001844	COL2A1	Collagen, type II, alpha 1	ANFH/AOM/COL11A3/SEDC/STL1
C03	Hs.443625	NM_000090	COL3A1	Collagen, type III, alpha 1	EDS4A
C04	Hs.210283	NM_000093	COL5A1	Collagen, type V, alpha 1	EDSC
C05	Hs.1584	NM_000095	COMP	Cartilage oligomeric matrix protein	EDM1/EPD1/MED/PSACH/THBS5
C06	Hs.173894	NM_000757	CSF1	Colony stimulating factor 1 (macrophage)	CSF-1/MCSF
C07	Hs.1349	NM_000758	CSF2	Colony stimulating factor 2 (granulocyte-macrophage)	GMCSF
C08	Hs.2233	NM_000759	CSF3	Colony stimulating factor 3 (granulocyte)	C17orf33/CSF3OS/GCSF
C09	Hs.632466	NM_000396	CTSK	Cathepsin K	CTS02/CTSO/CTSO1/CTSO2/PKND/PYCD
C10	Hs.99348	NM_005221	DLX5	Distal-less homeobox 5	SHFM1D
C11	Hs.419815	NM_001963	EGF	Epidermal growth factor	HOMG4/URG
C12	Hs.488293	NM_005228	EGFR	Epidermal growth factor	ERBB/ERBB1/HE

Table 2.14 (continue): Gene list of Qiagen Osteogenesis PCR array, PAHS-026Z [90].

				receptor	R1/NISBD2/PIG61 /mENA
D01	Hs.483635	NM_000800	FGF1	Fibroblast growth factor 1 (acidic)	AFGF/ECGF/ECGF-beta/ECGFA/ECGFB/FGF-1/FGF-alpha/FGFA/GLIO703/HBGF-1/HBGF1
D02	Hs.284244	NM_002006	FGF2	Fibroblast growth factor 2 (basic)	BFGF/FGF-2/FGFB/HBGF-2
D03	Hs.264887	NM_015850	FGFR1	Fibroblast growth factor receptor 1	BFGFR/CD331/CEK/FGFBR/FGFR-1/FLG/FLT-2/FLT2/HBGFR/HH2/HRTFDS/KAL2/N-SAM/OGD/bFGF-R-1
D04	Hs.533683	NM_000141	FGFR2	Fibroblast growth factor receptor 2	BBDS/BEK/BFR-1/CD332/CEK3/CFD1/ECT1/JWS/K-SAM/KGFR/TK14/TK25
D05	Hs.594454	NM_002019	FLT1	Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	FLT/FLT-1/VEGFR-1/VEGFR1
D06	Hs.203717	NM_002026	FN1	Fibronectin 1	CIG/ED-B/FINC/FN/FNZ/GFND/GFND2/LETS/MSF
D07	Hs.2171	NM_004962	GDF10	Growth differentiation factor 10	BMP-3b/BMP3B
D08	Hs.632702	NM_005269	GLI1	GLI family zinc finger 1	GLI
D09	Hs.643447	NM_000201	ICAM1	Intercellular adhesion molecule 1	BB2/CD54/P3.58
D10	Hs.160562	NM_000618	IGF1	Insulin-like growth factor 1 (somatomedin C)	IGF-I/IGFI/MGF
D11	Hs.643120	NM_000875	IGF1R	Insulin-like growth factor 1 receptor	CD221/IGFIR/IGFR/JTK13
D12	Hs.700350	NM_000612	IGF2	Insulin-like growth factor 2 (somatomedin A)	C11orf43/GRDF/IGF-II/PP9974
E01	Hs.654504	NM_002181	IHH	Indian hedgehog	BDA1/HHG2
E02	Hs.644352	NM_181501	ITGA1	Integrin, alpha 1	CD49a/VLA1
E03	Hs.482077	NM_002203	ITGA2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	BR/CD49B/GPIa/HPA-5/VLA-2/VLAA2
E04	Hs.265829	NM_002204	ITGA3	Integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)	CD49C/GAP-B3/GAPB3/ILNEB/MSK18/VCA-2/VL3A/VLA3a
E05	Hs.172631	NM_000632	ITGAM	Integrin, alpha M (complement component 3 receptor 3 subunit)	CD11B/CR3A/MAC-1/MAC1A/MO1A/SLEB6
E06	Hs.643813	NM_002211	ITGB1	Integrin, beta 1 (fibronectin receptor,	CD29/FNRB/GPIIA/MDF2/MSK12/VLA-

Table 2.14 (continue): Gene list of Qiagen Osteogenesis PCR array, PAHS-026Z [90].

				beta polypeptide, antigen CD29 includes MDF2, MSK12)	BETA/VLAB
E07	Hs.2258	NM_002425	MMP10	Matrix metallopeptidase 10 (stromelysin 2)	SL-2/STMY2
E08	Hs.513617	NM_004530	MMP2	Matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	CLG4/CLG4A/MMP-2/MMP-II/MONA/TBE-1
E09	Hs.161839	NM_002424	MMP8	Matrix metallopeptidase 8 (neutrophil collagenase)	CLG1/HNC/MMP-8/PMNL-CL
E10	Hs.297413	NM_004994	MMP9	Matrix metallopeptidase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	CLG4B/GELB/MANDP2/MMP-9
E11	Hs.618430	NM_003998	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	EBP-1/KBF1/NF-kB1/NF-kappa-B/NF-kappaB/NFKB-p105/NFKB-p50/NFkappaB/p105/p50
E12	Hs.248201	NM_005450	NOG	Noggin	SYM1/SYNS1
F01	Hs.535898	NM_002607	PDGFA	Platelet-derived growth factor alpha polypeptide	PDGF-A/PDGF1
F02	Hs.495834	NM_000444	PHEX	Phosphate regulating endopeptidase homolog, X-linked	HPDR/HPDR1/HYP/HYP1/LXHR/PEX/XLH
F03	Hs.535845	NM_004348	RUNX2	Runt-related transcription factor 2	AML3/CBF-alpha-1/CBFA1/CCD/CCD1/CLCD/OSF-2/OSF2/PEA2aA/PEBP2aA
F04	Hs.596449	NM_001235	SERPINH1	Serpin peptidase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)	AsTP3/CBP1/CBP2/HSP47/OI10/PIG14/PPROM/RA-A47/SERPINH2/gp46
F05	Hs.604588	NM_005900	SMAD1	SMAD family member 1	BSP-1/BSP1/JV4-1/JV41/MADH1/MADR1
F06	Hs.12253	NM_005901	SMAD2	SMAD family member 2	JV18/JV18-1/MADH2/MADR2/hMAD-2/hSMAD2
F07	Hs.727986	NM_005902	SMAD3	SMAD family member 3	HSPC193/HsT17436/JV15-2/LDS1C/LDS3/MADH3
F08	Hs.75862	NM_005359	SMAD4	SMAD family member 4	DPC4/JIP/MADH4/MYHRS
F09	Hs.167700	NM_005903	SMAD5	SMAD family member 5	DWFC/JV5-1/MADH5
F10	Hs.647409	NM_000346	SOX9	SRY (sex determining region Y)-box 9	CMD1/CMPD1/SRA1/SRXX2/SRXY10
F11	Hs.742807	NM_152860	SP7	Sp7 transcription factor	OI11/OI12/OSX/osterix
F12	Hs.313	NM_000582	SPP1	Secreted phosphoprotein 1	BNSP/BSPI/ETA-1/OPN

Table 2.14 (continue): Gene list of Qiagen Osteogenesis PCR array, PAHS-026Z [90].

G01	Hs.645227	NM_000660	TGFB1	Transforming growth factor, beta 1	CED/DPD1/LAP/TGFB/TGFbeta
G02	Hs.133379	NM_003238	TGFB2	Transforming growth factor, beta 2	LDS4/TGF-beta2
G03	Hs.713281	NM_003239	TGFB3	Transforming growth factor, beta 3	ARVD/ARVD1/RNHF/TGF-beta3
G04	Hs.494622	NM_004612	TGFBR1	Transforming growth factor, beta receptor 1	AAT5/ACVRLK4/ALK-5/ALK5/ESS1/LDS1/LDS1A/LDS2A/MSSE/SKR4/TGFR-1/tbetaR-I
G05	Hs.604277	NM_003242	TGFBR2	Transforming growth factor, beta receptor II (70/80kDa)	AAT3/FAA3/LDS1B/LDS2/LDS2B/MFS2/RIIC/TAAD2/TGFR-2/TGFbeta-RII
G06	Hs.241570	NM_000594	TNF	Tumor necrosis factor	DIF/TNF-alpha/TNFA/TNFSF2
G07	Hs.333791	NM_003701	TNFSF11	Tumor necrosis factor (ligand) superfamily, member 11	CD254/ODF/OPGL/OPTB2/RANKL/TRANCE/hRANKL2/sOdf
G08	Hs.66744	NM_000474	TWIST1	Twist homolog 1 (Drosophila)	ACS3/BPES2/BPES3/CRS/CRS1/CSO/SCS/TWIST/bHLHa38
G09	Hs.109225	NM_001078	VCAM1	Vascular cell adhesion molecule 1	CD106/INCAM-100
G10	Hs.524368	NM_000376	VDR	Vitamin D (1,25-dihydroxyvitamin D3) receptor	NR1I1/PPP1R163
G11	Hs.73793	NM_003376	VEGFA	Vascular endothelial growth factor A	MVCD1/VEGF/VPF
G12	Hs.732095	NM_003377	VEGFB	Vascular endothelial growth factor B	VEGFL/VRF
H01	Hs.520640	NM_001101	ACTB	Actin, beta	BRWS1/PS1TP5BP1
H02	Hs.534255	NM_004048	B2M	Beta-2-microglobulin	-
H03	Hs.592355	NM_002046	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	G3PD/GAPD/HEL-S-162eP
H04	Hs.412707	NM_000194	HPRT1	Hypoxanthine phosphoribosyltransferase 1	HGPRT/HPRT
H05	Hs.546285	NM_001002	RPLP0	Ribosomal protein, large, P0	L10E/LP0/P0/PRLP0/RPP0
H06	N/A	SA_00105	HGDC	Human Genomic DNA Contamination	HIGX1A
H07	N/A	SA_00104	RTC	Reverse Transcription Control	RTC
H08	N/A	SA_00104	RTC	Reverse Transcription Control	RTC
H09	N/A	SA_00104	RTC	Reverse Transcription Control	RTC
H10	N/A	SA_00103	PPC	Positive PCR Control	PPC
H11	N/A	SA_00103	PPC	Positive PCR Control	PPC
H12	N/A	SA_00103	PPC	Positive PCR Control	PPC

2.4.4.2. Osteoporosis PCR Array

Gene groups designed by the company related to OP are remodeling of bone and bone morphogenic protein signaling, calciotropic hormones and receptors, cytokines, growth factors and receptors, activity and differentiation osteoblast, RANK/RANKL/OPG signaling, WNT/-β Catenin signaling and several genes of osteoporosis (Table 2.15).

Table 2.15: Gene list of Qiagen Osteoporosis PCR array, PAHS-170Z [91].

Position	Unigene	Refseq	Symbol	Description	Gname
A01	Hs.1211	NM_001611	ACP5	Acid phosphatase 5, tartrate resistant	HPAP/SPENCDI/TRAP
A02	Hs.320892	NM_018417	ADCY10	Adenylate cyclase 10 (soluble)	HCA2/HEL-S-7a/SAC/SACI/Sacy/hsAC
A03	Hs.654431	NM_000697	ALOX12	Arachidonate 12-lipoxygenase	12-LOX/12S-LOX/LOG12
A04	Hs.73809	NM_001140	ALOX15	Arachidonate 15-lipoxygenase	12-LOX/15-LOX-1/15LOX-1
A05	Hs.89499	NM_000698	ALOX5	Arachidonate 5-lipoxygenase	5-LO/5-LOX/5LPG/LOG5
A06	Hs.75431	NM_000478	ALPL	Alkaline phosphatase, liver/bone/kidney	AP-TNAP/APTNAP/HOPS/TNAP/TNSALP
A07	Hs.76704	NM_000044	AR	Androgen receptor	AIS/AR8/DHTR/HUMARA/HYSP1/KD/NR3C4/SBMA/SMAX1/TFM
A08	Hs.654541	NM_199173	BGLAP	Bone gamma-carboxyglutamate (gla) protein	BGP/OC/OCN
A09	Hs.73853	NM_001200	BMP2	Bone morphogenetic protein 2	BDA2/BMP2A
A10	Hs.473163	NM_001719	BMP7	Bone morphogenetic protein 7	OP-1
A11	Hs.155097	NM_000067	CA2	Carbonic anhydrase II	CA-II/CAC/CAII/Car2/HEL-76
A12	Hs.37058	NM_001741	CALCA	Calcitonin-related polypeptide alpha	CALC1/CGRP/CGRP-I/CGRP1/CT/KC
B01	Hs.489127	NM_001742	CALCR	CALCITONIN RECEPTOR	CRT/CT-R/CTR/CTR1
B02	Hs.435615	NM_000388	CASR	Calcium-sensing receptor	CAR/EIG8/FHH/FIH/GPRC2A/HHC/HHC1/HYPOC1/NSHPT/PCAR1
B03	Hs.472860	NM_001250	CD40	CD40 molecule, TNF receptor superfamily member 5	Bp50/CDW40/TNFRSF5/p50
B04	Hs.459649	NM_001287	CLCN7	Chloride channel 7	CLC-7/CLC7/OPTA2/OPTB4/PPP1R63

Table 2.15 (continue): Gene list of Qiagen Osteoporosis PCR array, PAHS-170Z [91].

B05	Hs.73037	NM_001841	CNR2	Cannabinoid receptor 2 (macrophage)	CB-2/CB2/CX5
B06	Hs.172928	NM_000088	COL1A1	Collagen, type I, alpha 1	EDSC/OI1/OI2/OI3/OI4
B07	Hs.489142	NM_000089	COL1A2	Collagen, type I, alpha 2	OI4
B08	Hs.370408	NM_000754	COMT	Catechol-O-methyltransferase	HEL-S-98n
B09	Hs.517888	NM_006371	CRTAP	Cartilage associated protein	CASP/LEPREL3/OI7/P3H5
B10	Hs.632466	NM_000396	CTSK	Cathepsin K	CTS02/CTSO/CTSO1/CTSO2/PKND/PYCD
B11	Hs.438016	NM_000102	CYP17A1	Cytochrome P450, family 17, subfamily A, polypeptide 1	CPT7/CYP17/P450C17/S17AH
B12	Hs.260074	NM_000103	CYP19A1	Cytochrome P450, family 19, subfamily A, polypeptide 1	ARO/ARO1/CPV1/CYAR/CYP19/CYPXIX/P-450AROM
C01	Hs.414480	NM_001352	DBP	D site of albumin promoter (albumin D-box) binding protein	DABP
C02	Hs.40499	NM_012242	DKK1	Dickkopf homolog 1 (Xenopus laevis)	DKK-1/SK
C03	Hs.527295	NM_006208	ENPP1	Ectonucleotide pyrophosphatase/phosphodiesterase 1	ARHR2/COLED/M6S1/NPP1/NPPS/PC-1/PCA1/PDNP1
C04	Hs.744830	NM_000125	ESR1	Estrogen receptor 1	ER/ESR/ESRA/ESTRR/Era/NR3A1
C05	Hs.660607	NM_001437	ESR2	Estrogen receptor 2 (ER beta)	ER-BETA/ESR-BETA/ESRB/ESTRB/Erb/NR3A2
C06	Hs.110849	NM_004451	ESRRA	Estrogen-related receptor alpha	ERR1/ERRa/ERRalpha/ESRL1/NR3B1
C07	Hs.264887	NM_015850	FGFR1	Fibroblast growth factor receptor 1	BFGFR/CD331/CEK/FGFBR/FGFR-1/FLG/FLT-2/FLT2/HBGFR/HH2/HRTFDS/KAL2/N-SAM/OGD/bFGF-R-1
C08	Hs.533683	NM_000141	FGFR2	Fibroblast growth factor receptor 2	BBDS/BEK/BFR-1/CD332/CEK3/CFD1/ECT1/JWS/K-SAM/KGFR/TK14/TK25
C09	Hs.37023	NM_021081	GHRH	Growth hormone releasing hormone	GHRF/GRF/INN
C10	Hs.195040	NM_005525	HSD11B1	Hydroxysteroid (11-beta) dehydrogenase 1	11-DH/11-beta-HSD1/CORTRD2/HDL/HSD11/HSD11B/HSD11L/SDR26C1
C11	Hs.160562	NM_000618	IGF1	Insulin-like growth factor 1 (somatomedin C)	IGF-I/IGFI/MGF

Table 2.15 (continue): Gene list of Qiagen Osteoporosis PCR array, PAHS-170Z [91].

C12	Hs.438102	NM_000597	IGFBP2	Insulin-like growth factor binding protein 2, 36kDa	IBP2/IGF-BP53
D01	Hs.168132	NM_000585	IL15	Interleukin 15	IL-15
D02	Hs.654458	NM_000600	IL6	Interleukin 6 (interferon, beta 2)	BSF2/HGF/HSF/IFNB2/IL-6
D03	Hs.135087	NM_000565	IL6R	Interleukin 6 receptor	CD126/IL-6R-1/IL-6RA/IL6Q/IL6RA/IL6RQ/gp80
D04	Hs.644352	NM_181501	ITGA1	Integrin, alpha 1	CD49a/VLA1
D05	Hs.218040	NM_000212	ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	BDPLT16/BDPLT2/CD61/GP3A/GPIIIa/GT
D06	Hs.194236	NM_000230	LEP	Leptin	LEPD/OB/OBS
D07	Hs.720014	NM_022356	P3H1	Leucine proline-enriched proteoglycan (leprecan) 1	GROS1/LEPRE1/OI8
D08	Hs.162757	NM_002332	LRP1	Low density lipoprotein receptor-related protein 1	A2MR/APOER/APR/CD91/IGFBP3R/LRP/LRP1A/TGFBR5
D09	Hs.6347	NM_002335	LRP5	Low density lipoprotein receptor-related protein 5	BMND1/EVR1/EVR4/HBM/LR3/LRP-5/LRP7/OPPG/OPS/OPTA1/VBCH2
D10	Hs.584775	NM_002336	LRP6	Low density lipoprotein receptor-related protein 6	ADCAD2
D11	Hs.36	NM_000595	LTA	Lymphotoxin alpha (TNF superfamily, member 1)	LT/TNFB/TNFSF1
D12	Hs.512776	NM_000428	LTBP2	Latent transforming growth factor beta binding protein 2	C14orf141/GLC3D/LTBP3/MSPKA/MSTP031/WMS3
E01	Hs.584852	NM_006439	MAB21L2	Mab-21-like 2 (C. elegans)	MCOPS14
E02	Hs.513617	NM_004530	MMP2	Matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	CLG4/CLG4A/MMP-2/MMP-II/MONA/TBE-1
E03	Hs.41565	NM_005259	MSTN	Myostatin	GDF8/MSLHP
E04	Hs.214142	NM_005957	MTHFR	Methylenetetrahydrofolate reductase (NAD(P)H)	-
E05	Hs.534074	NM_172390	NFATC1	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1	NF-ATC/NF-ATc1.2/NFAT2/NFATc
E06	Hs.248201	NM_005450	NOG	Noggin	SYM1/SYNS1
E07	Hs.647092	NM_000603	NOS3	Nitric oxide synthase 3 (endothelial cell)	ECNOS/eNOS
E08	Hs.1832	NM_000905	NPY	Neuropeptide Y	PYY4
E09	Hs.122926	NM_000176	NR3C1	Nuclear receptor subfamily 3, group	GCCR/GCR/GCRST/GR/GRL

Table 2.15 (continue): Gene list of Qiagen Osteoporosis PCR array, PAHS-170Z [91].

				C, member 1 (glucocorticoid receptor)	
E10	Hs.729169	NM_002562	P2RX7	Purinergic receptor P2X, ligand-gated ion channel, 7	P2X7
E11	Hs.477866	NM_182943	PLOD2	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	BRKS2/LH2/TLH
E12	Hs.1905	NM_000948	PRL	Prolactin	-
F01	Hs.37045	NM_000315	PTH	Parathyroid hormone	PTH1
F02	Hs.1019	NM_000316	PTH1R	Parathyroid hormone 1 receptor	PFE/PTHR/PTHR1
F03	Hs.591159	NM_002820	PTHLH	Parathyroid hormone-like hormone	BDE2/HHM/PLP/PTHR/PTHRP
F04	Hs.535845	NM_004348	RUNX2	Runt-related transcription factor 2	AML3/CBF-alpha-1/CBFA1/CCD/CCD1/CLCD/OSF-2/OSF2/PEA2aA/PEBP2aA
F05	Hs.213424	NM_003012	SFRP1	Secreted frizzled-related protein 1	FRP/FRP-1/FRP1/FrzA/SARP2
F06	Hs.658169	NM_003014	SFRP4	Secreted frizzled-related protein 4	FRP-4/FRPHE/sFRP-4
F07	Hs.632235	NM_001040	SHBG	Sex hormone-binding globulin	ABP/SBP/TEBG
F08	Hs.349204	NM_025237	SOST	Sclerostin	CDD/SOST1/VBCH
F09	Hs.111779	NM_003118	SPARC	Secreted protein, acidic, cysteine-rich (osteonectin)	BM-40/ON
F10	Hs.313	NM_000582	SPP1	Secreted phosphoprotein 1	BNSP/BSPI/ETA-1/OPN
F11	Hs.642990	NM_007315	STAT1	Signal transducer and activator of transcription 1, 91kDa	CANDF7/IMD31A/IMD31B/IMD31C/ISGF-3/STAT91
F12	Hs.645227	NM_000660	TGFB1	Transforming growth factor, beta 1	CED/DPD1/LAP/TGFB/TGFbeta
G01	Hs.633514	NM_003255	TIMP2	TIMP metallopeptidase inhibitor 2	CSC-21K/DDC8
G02	Hs.211600	NM_006290	TNFAIP3	Tumor necrosis factor, alpha-induced protein 3	A20/OTUD7C/TNFA1P2
G03	Hs.204044	NM_003839	TNFRSF11A	Tumor necrosis factor receptor superfamily, member 11a, NFKB activator	CD265/FEO/LOH18CR1/ODFR/OFE/OPTB7/OSTS/PDB2/RANK/TRANCER
G04	Hs.81791	NM_002546	TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11b	OCIF/OPG/PDB5/TR1
G05	Hs.256278	NM_001066	TNFRSF1B	Tumor necrosis factor receptor superfamily, member	CD120b/TBPII/TNF-R-II/TNF-R75/TNFBR/TNFR

Table 2.15 (continue): Gene list of Qiagen Osteoporosis PCR array, PAHS-170Z [91].

				1B	1B/TNFR2/TNFR80/p75/p75TNFR
G06	Hs.333791	NM_003701	TNFSF11	Tumor necrosis factor (ligand) superfamily, member 11	CD254/ODF/OPGL/OPTB2/RANKL/TRANCE/hRANKL2/sOdf
G07	Hs.160411	NM_000369	TSHR	Thyroid stimulating hormone receptor	CHNG1/LGR3/hTSHR-I
G08	Hs.66744	NM_000474	TWIST1	Twist homolog 1 (Drosophila)	ACS3/BPES2/BPES3/CRS/CRS1/CSO/SCS/TWIST/bHLHa38
G09	Hs.524368	NM_000376	VDR	Vitamin D (1,25-dihydroxyvitamin D3) receptor	NR1I1/PPP1R163
G10	Hs.73793	NM_003376	VEGFA	Vascular endothelial growth factor A	MVCD1/VEGF/VPF
G11	Hs.91985	NM_003394	WNT10B	Wingless-type MMTV integration site family, member 10B	SHFM6/WNT-12
G12	Hs.336930	NM_033131	WNT3A	Wingless-type MMTV integration site family, member 3A	-
H01	Hs.520640	NM_001101	ACTB	Actin, beta	BRWS1/PS1TP5BP1
H02	Hs.534255	NM_004048	B2M	Beta-2-microglobulin	-
H03	Hs.592355	NM_002046	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	G3PD/GAPD/HEL-S-162eP
H04	Hs.412707	NM_000194	HPRT1	Hypoxanthine phosphoribosyltransferase 1	HGPRT/HPRT
H05	Hs.546285	NM_001002	RPLP0	Ribosomal protein, large, P0	L10E/LP0/P0/PRLP0/RPP0
H06	N/A	SA_00105	HGDC	Human Genomic DNA Contamination	HIGX1A
H07	N/A	SA_00104	RTC	Reverse Transcription Control	RTC
H08	N/A	SA_00104	RTC	Reverse Transcription Control	RTC
H09	N/A	SA_00104	RTC	Reverse Transcription Control	RTC
H10	N/A	SA_00103	PPC	Positive PCR Control	PPC
H11	N/A	SA_00103	PPC	Positive PCR Control	PPC
H12	N/A	SA_00103	PPC	Positive PCR Control	PPC

3. RESULTS

Results part of the thesis can be separated into three sections as: following and conformation of osteogenic differentiation, optimizations of transfection and PCR array. Osteogenic differentiation and transfection parts were essential for obtaining required conditions of experiments and optimizations. Also optimized results were confirmed by using different techniques according to type of experiment. PCR array part involved combination of osteogenic differentiation and transfection parts. Optimized conditions were used at indicated time points for human osteogenesis and OP PCR array to scan specific gene panel.

3.1. OSTEOGENIC INDUCTION OF CELLS AND ANALYZING THE PRESENCE OF OSTEOGENIC LINEAGE MARKERS

3.1.1. Histological staining: TB

Osteogenic differentiation of hMSCs takes 4 weeks. In each week, cells were observed under microscope for detection of differentiation and its indicators. Deposition of calcium rich mineralized bone matrix synthesized by osteoprogenitors and osteoblastic cell morphology were observed. As seen in Figure 3.1 in 2^{nd} week of the process, calcium deposits or nodules were started to observe as an indicator of osteogenic differentiation. In the following week, nodule formation was observed greater than previous days. At 4^{th} week of differentiation, calcification was clearly observed as black dots or irregular black regions.

In Figure 3.2 calcified matrix of bone synthesized by cells in osteoblastic lineage were stained with TB as dark blue regions. Stainings areas of osteoblastic cells were remarkably different compared with UD hMCSs.

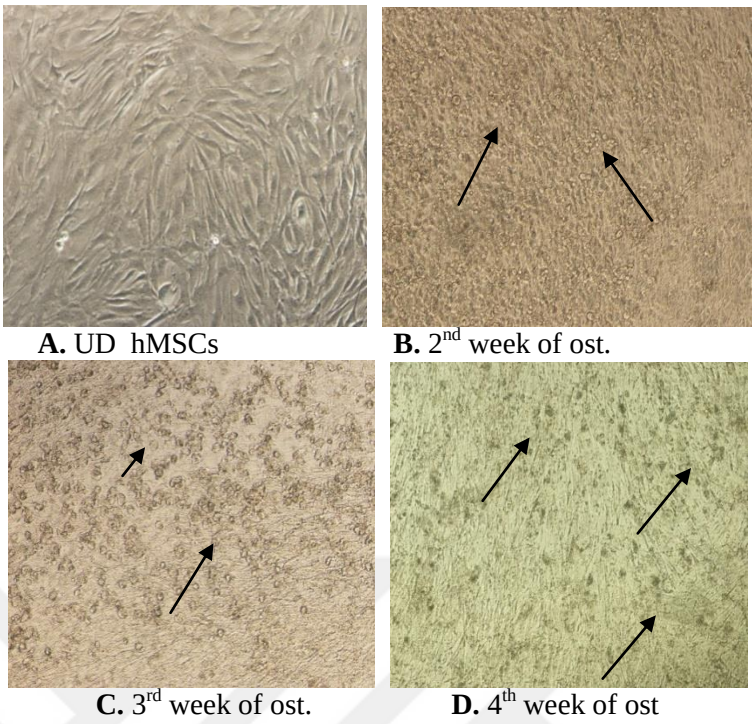


Figure 3.1: Light microscope images of osteogenic differentiation for 4 weeks before staining at 10X magnification. Calcium deposites were indicated with arrows.

.

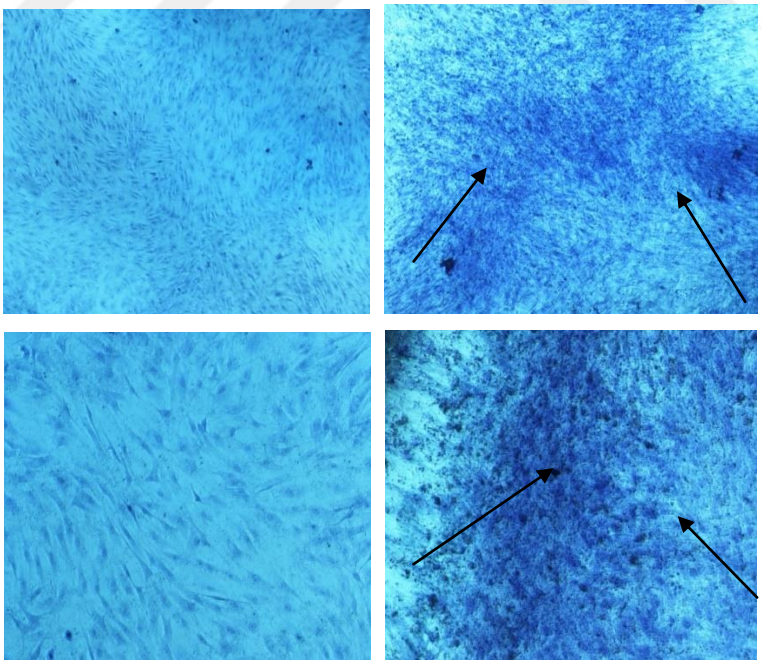


Figure 3.2: Light microscope images of osteogenic differentiation at 4 weeks after staining. A, C UD hMSCs 4x, 10x, B, D ost. 28 days, 4x, 10x respectively.

3.1.2. RT-qPCR Results of Osteogenic Differentiation

Osteogenic differentiation of hMSCs was also confirmed at gene expression level by using selected bone specific markers that belong to different stages of osteogenesis. As seen in Figure 3.3 OCN known as late marker of osteoblastogenesis showed higher expression level while BSP and OPN expressions seemed irrelevant with process. This is because BSP belongs to early terms of differentiation and its expression level was lower at 4th week of differentiation while OPN peaks twice during early stage of differentiation, thus it wasn't possible to catch right time point for following of their expressions.

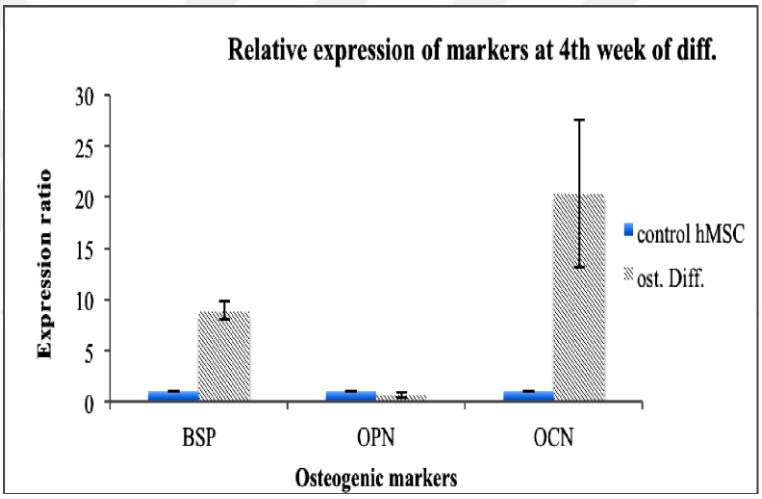


Figure 3.3: Expression levels of osteogenic markers at 4 weeks' differentiation samples.

3.2. OPTIMIZATIONS OF GENE KNOCKDOWN AND OVEREXPRESSION

3.2.1. Confirmation of Topo IIβ gene insert in pEGFP

The plasmid topo IIβ-EGFP construct was requested and obtained from Ken Tsutsui in Japan. Topo IIβ insertion into EGFP plasmid was checked before starting of transfection studies in two ways. Figure 3.4 was the result of colony PCR of selected colonies from bacterial culture. First lane was molecular marker. Others were the samples of colonies and molecular weight of topo IIβ primer used in PCR was 281 bp. As seen in Figure 3.4 in each colony topo IIβ gene inserted plasmids were successfully observed.

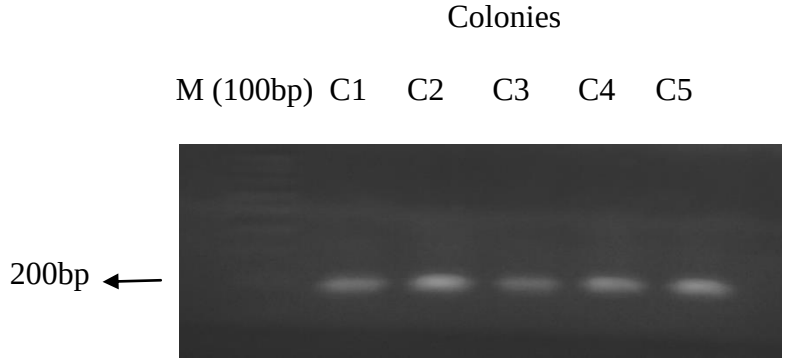


Figure 3.4: Results of colony PCR. M; 100bp DNA ladder.

Results showed that each colony contained and expressed topo IIβ, thus just one colony (#2) was selected and used for the following confirmations. Plasmid isolation was proceeded for colony #2. By using isolated plasmids, digestion of pDNA with suitable restriction enzymes was performed for application of other checking point of insertion. According to the determined restriction sites and suitable enzymes, confirmation of pDNA containing true topo IIβ insert was showed. In Figure 3.5 lane 3 refered plasmid fragments separated into 3 pieces by XhoI and BamHI restriction enzymes. In this result topo IIβ-EGFP plasmid was both separated from each other (4.8 kb topo IIβ+4.7 kb GFP) and topo IIβ insert was also separated into 2 pieces (2.3 kb+2.5kb). Obtained results were suitable with expected. As a result of this experiment, presence of topo IIβ gene insert was confirmed. Next step includes, plasmid isolation from selected colonies by using Qiagen mini prep #12145 kit to obtain large quantities of plasmids for transfection studies. Isolation was preceeded according to the protocol to obtain high plasmid yield.

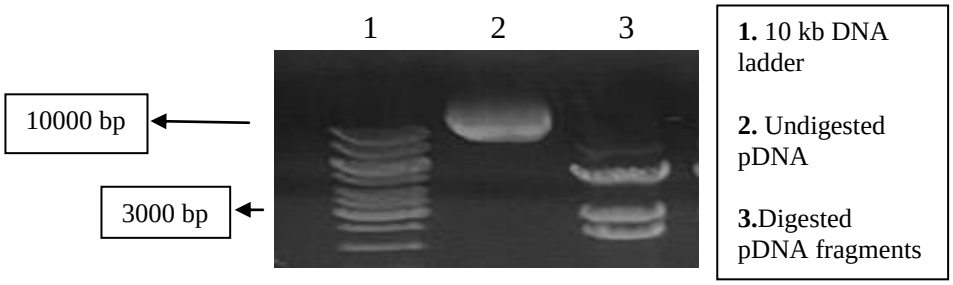


Figure 3.5: Plasmids that were restricted with XhoI, BamHI enzymes.

3.2.2. Overexpression of Topo IIβ

Transfection of topo IIβ-EGFP plasmid into hMSC was hard. Several methods were used to obtain efficient gene transfection. These methods were suitable for transfection

of GFP plasmid used as positive control but EGFP tagged topo IIβ become larger and made process difficult.

Lonza Company serves a specific electroporation device known as Nucleofector, which has specific conditions of electrical parameters and kits according to type of cells to be transfected. In Figure 3.6 hMSCs transfected with GFP was observed at 4X and 10X magnification. Both high viability and high efficiency programs were optional served by device software. Although technique was suitable for efficient transfection, it caused higher cell mortality.

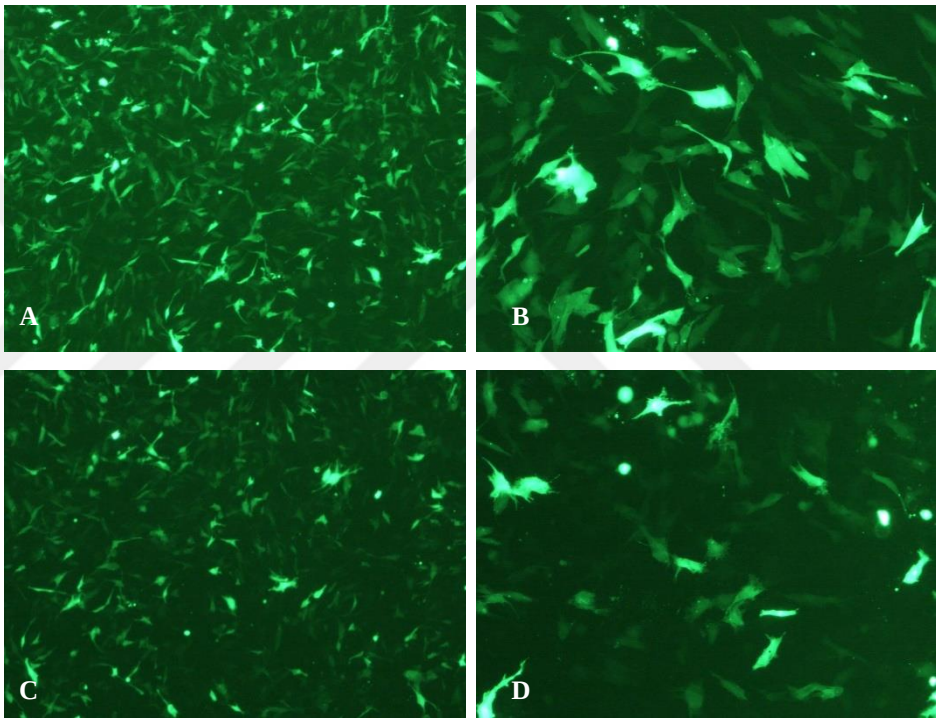


Figure 3.6: GFP transfected hMSCs 24h post nucleofection. EW-104 (high viability) program (A. 4X, B. 10X) and FF-104 (high efficiency) program (C. 4X, D. 10X) of 4D Nucleofector™ system (Lonza) were used.

After succesful transfection of GFP, studies were focused on topo IIβ-EGFP transfection into hMSC line. Optimizations in plasmid concentration were done by using 4, 5, 6, 8 µg of pDNA. In each concentration trials transfection was succesfully occurred but rate of efficiency were different. As seen in Figure 3.7, 4 µg (A), 5 µg (B), 6 µg (C), 8 µg (D) of plasmid concenrations were selected. Since topo IIβ is nuclear protein its expression was observed in nucleus, green dots refer topo IIβ expressed cell's nucleus. According to the images 4 µg plasmid concentrations caused

low transfection efficiency while 8 µg caused high cell mortality. So that these conditions were discarded. Images showed that both 5 and 6 µg of pDNA concentrations were suitable for avoiding of low transfection efficiency and high cell mortalitiy. Cells transfected with 5 and 6 µg pDNA were followed till day 7 post nucleofection. Then RNA samples were isolated for detection of of topo IIβ expression at late stage of nucleofection.

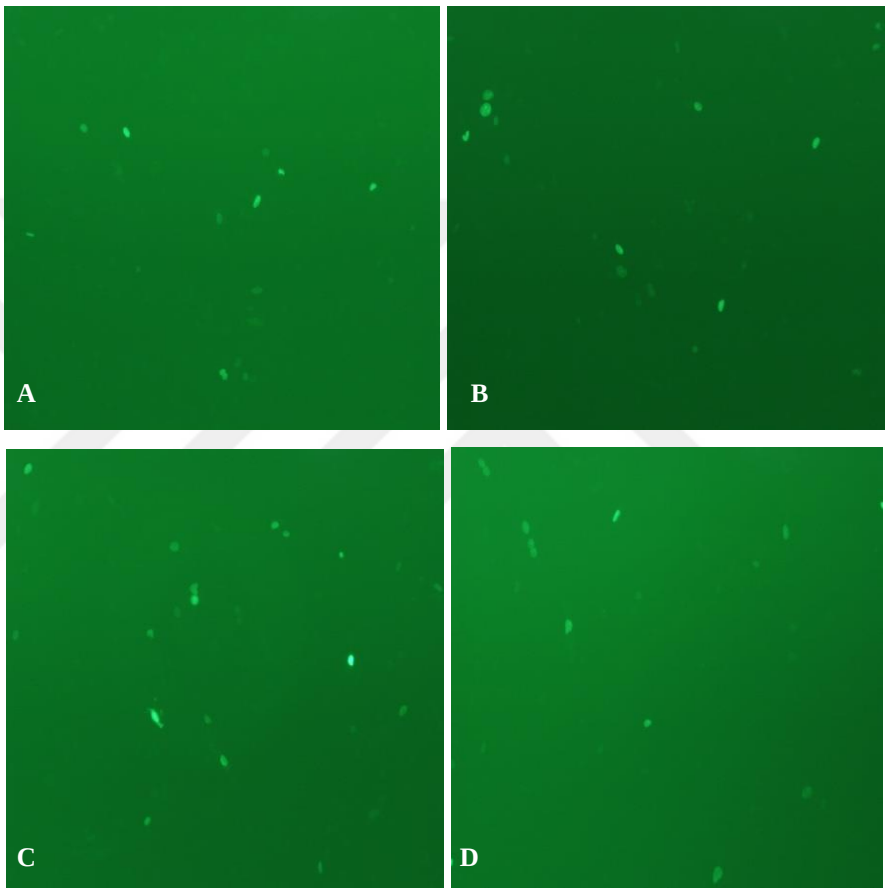


Figure 3.7: Fluorescent microscopy images of topo IIβ-EGFP transfected hMSCs with different pDNA concentrations (A. 4 µg, B. 5 µg, C. 6 µg ve D. 8 µg) 24h post nucleofection.

In order to confirm topo IIβ gene expression with RT-qPCR, total RNA of transfected hMSCs were extracted using RNeasy kit (Qiagen) at 24h and 7 days postnucleofection. The concentration of extracted RNA was quantified by using Qubit (Invitrogen). For RT-qPCR analysis, RNA was reverse transcribed to obtain cDNA by the Quantitect ReverseTranscription Kit (Qiagen) according to the manufacturers' protocol. Untransfected hMSCs were used as negative control and the expression of topo IIβ were analyzed with RT-qPCR at mRNA level.

As seen in Figure 3.8 RT-qPCR results showed that expression of topo IIβ was higher at 6 µg pDNA even at day 7. Cell proliferation cause continuous expression of topo IIβ with increasing rate.

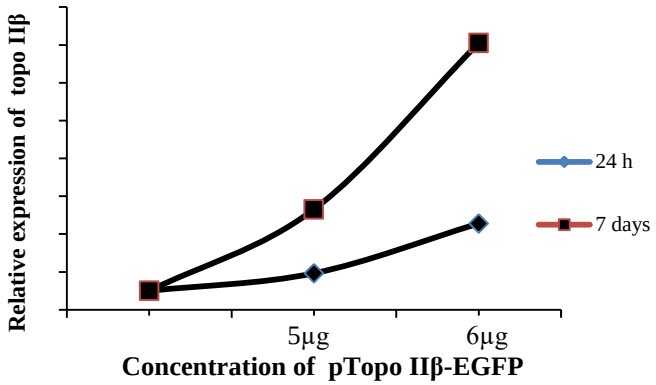


Figure 3.8: Expression level of topo IIβ in transfected hMSCs at indicated conditions.

3.2.3. Determination of the Optimum Concentration of siRNA via RT-qPCR

Determining the optimum conditions for gene silencing was also essential. Conditions should be designed according to number of cells to be transfected, current transfection reagent and required duration of siRNA refreshment for continuous silencing. For osteogenic commitment of hMSCs, high cell number was required. Also osteogenic medium should be refreshed per 48h, thus silencing conditions should be repeated per 48h. For these requirements, cells were transfected with 7.5, 10, 12.5, 15 and 20 nM of siRNA for 48h. Then RNA samples were isolated at 48h posttransfection, cDNA synthesis was performed and gene expression profile of topo IIβ was detected at mRNA level. Data was normalized with actin gamma housekeeping gene. Experiments were done in triplicate. As seen in Figure 3.9 each concentration cause significant gene silencing. Most efficient and significant gene knockdown was observed at 10 nM. For further experiments silencing was proceeded at 10 nM siRNA by refreshing per 48h according to the required time scale of experimental design.

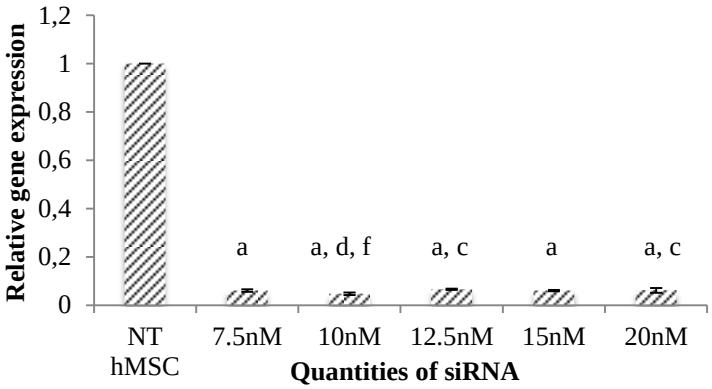


Figure 3.9: Gene expression level of topo IIβ against different concetration of siRNA treatment. *Significantly different from NT hMSC (a), 7.5 nM (b), 10 nM (c), 12.5 nM(d), 15 nM(e), 20 nM (f), (p< 0.05, one-way ANOVA with Tukey's HSD post-hoc procedure).

3.3 CONFIRMATION OF OSTEOGENIC DIFFERENTIATION AT DAYS 7/14 IN PRESENCE OR ABSENCE OF TOPO IIB

After optimization studies both transfection and osteogenic differentiation conditions were combined by induction of hMSCs into osteogenic lineage in addition to gene silencing and overexpression of topo IIβ. According to the hypothesis it was expected that effect of topo IIβ observed at early terms of differentiation. Thus time points of experiments were restricted to day 7 and 14 at the begining. First of all TB and AR staining of osteogenically induced cells were done and confirmed that osteogenesis was also proceeded at that time points (Figures 3.10, 3.11).

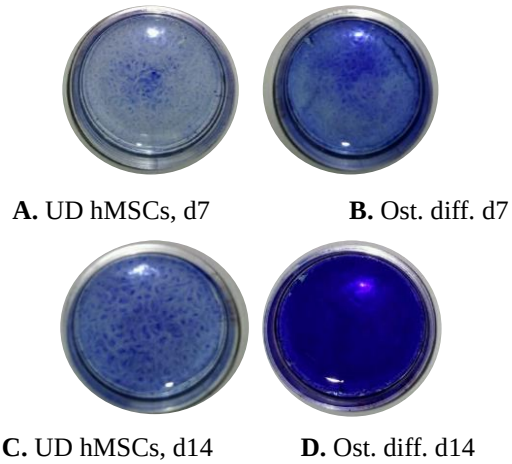


Figure 3.10: TB stained areas of osteogenically induced hMSCs at days 7 and 14.

Cells of osteogenic lineage cause synthesizing of calcified bone matrix and staining of these calcium condense areas with dark blue (Figure 3.10, B, D). As seen in Figure 3.10 comparing the images of UD hMSCs and osteogenically induced hMSCs showed remarkable differences in calcified bone matrix regions. ARS of days 7 and 14 were also evaluated. In Figure 3.11 plates of UD hMSCs and osteogenically induced hMSCs samples of days 7/14 were fixed and stained with AR.

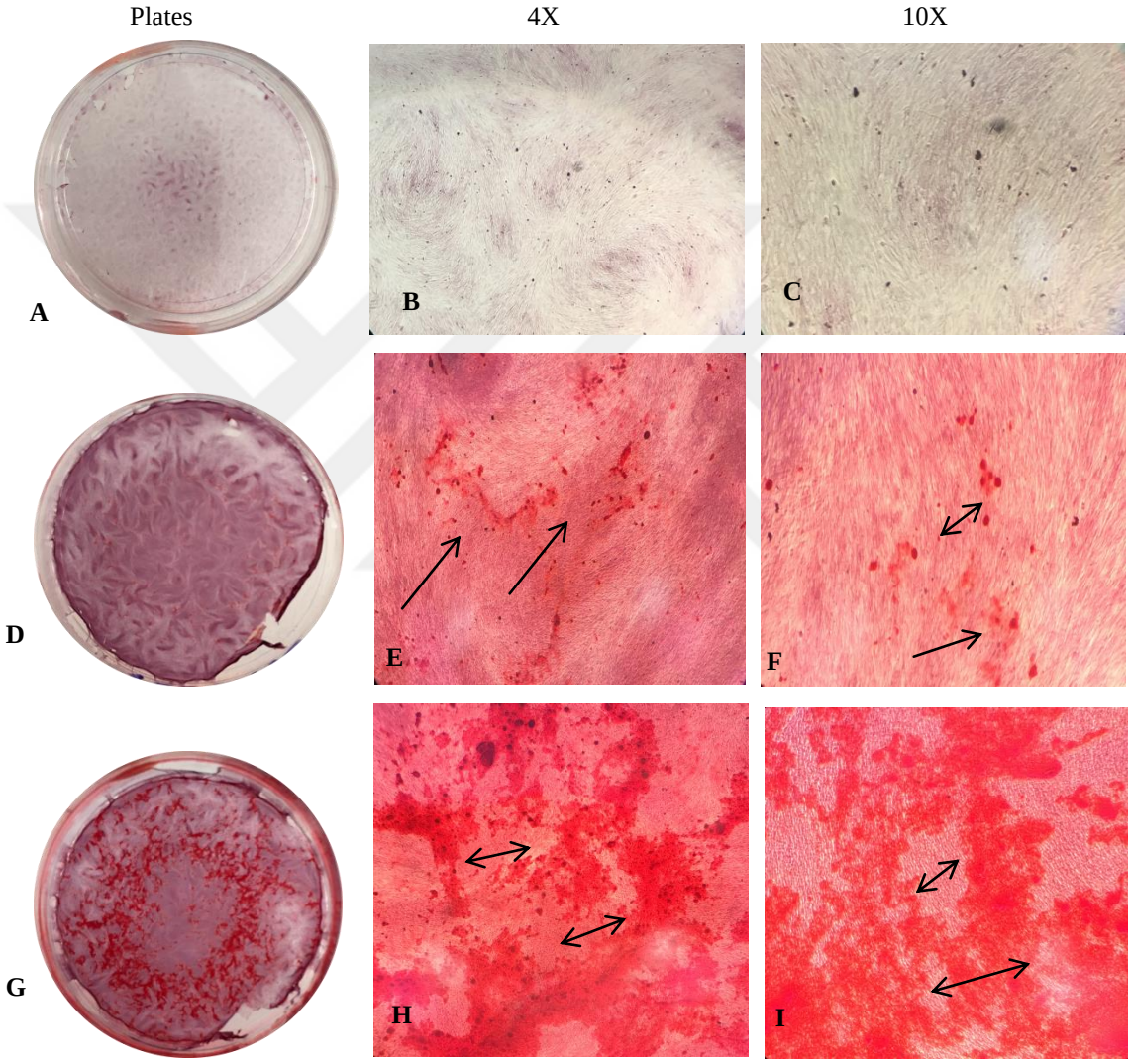


Figure 3.11: ARS of calcified bone matrix at days 7 and 14. UD hMSC (A, B, C), osteogenic induction at d7 (D, E, F), osteogenic induction at d14 (G, H, I) under microscope at 4x, 10x magnification and images of plates. Arrows show stained calcified regions.

Areas stained with red were indicators of calcified bone matrix. Differences of stained areas between NT-UD hMSCs and osteogenically induced samples were clearly observed. Even in naked eye observations (Figure 3.11, A, D, G), differences in rate of AR stained areas between differentiated versus UD samples were clearly observed.

In both TB and AR stainings amounts of calcified and stained areas were increased during progression of osteogenic differentiation. And those results were confirmation of osteoblastic cell formation and their functionalities at days 7 and 14.

3.3.1. Observation of Osteogenic Differentiation at Indicated Time Points

Experimental groups of days 7 and 14 were determined as: UD hMSCs, osteogenic induction with wild type and natural (WT) expression of topo IIβ (ost.), osteogenic differentiation with silenced topo IIβ (ost+siTopo IIβ) and osteogenic differentiation with overexpressed topo IIβ (ost.+oe).

Results that involve osteogenic commitment of hMSCs in presence or absence of topo IIβ were evaluated. Osteogenic induction was started meanwhile with topo IIβ silencing while starting of osteogenic induction took 1 week post overexpression. Due to observation of high cell mortality caused by nucleofection, obtaining the required cell confluency for osteogenic induction took 1 week. This means osteogenic induction was started 1 week later after overexpression. In Figure 3.12 images represented the increasing number of cells day by day post nucleofection. Required cell confluency was observed at day 7 and osteogenic differentiation was started after that point.

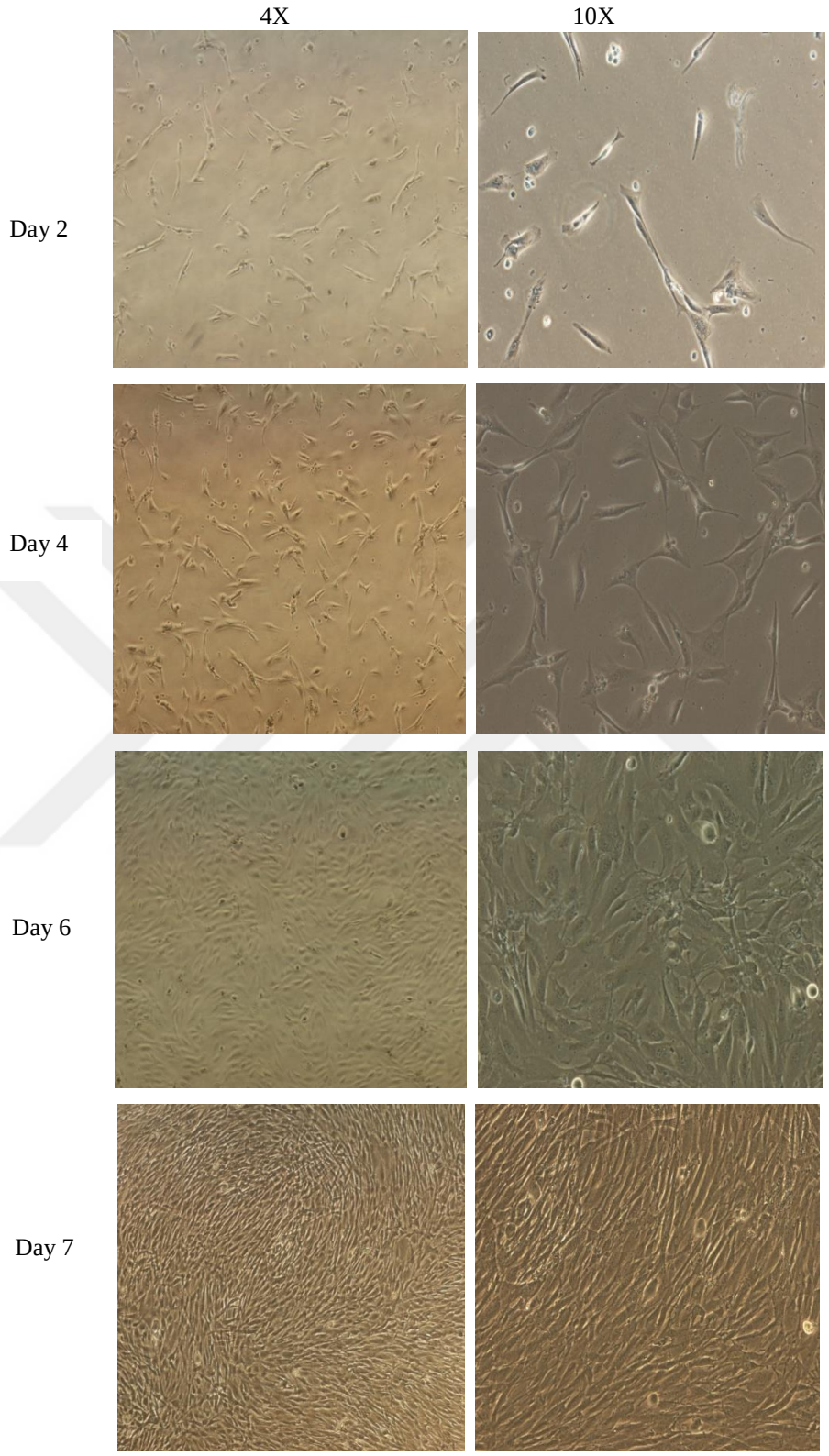


Figure 3.12: Light microscope images of topo IIβ transfected hMSCs at indicated conditions before starting of osteogenic differentiation.

Following figures showed images of cells at experimental conditions during progression of osteogenic differentiation. These images were essential for following of osteogenic cell morphology and calcified bone matrix. Dark black dots or regions of images reflected calcified bone matrix (Figures 3.13/14/15/16). Topo IIβ-EGFP transfected cells were glowing under fluorescence as an indicator of gene transfection.

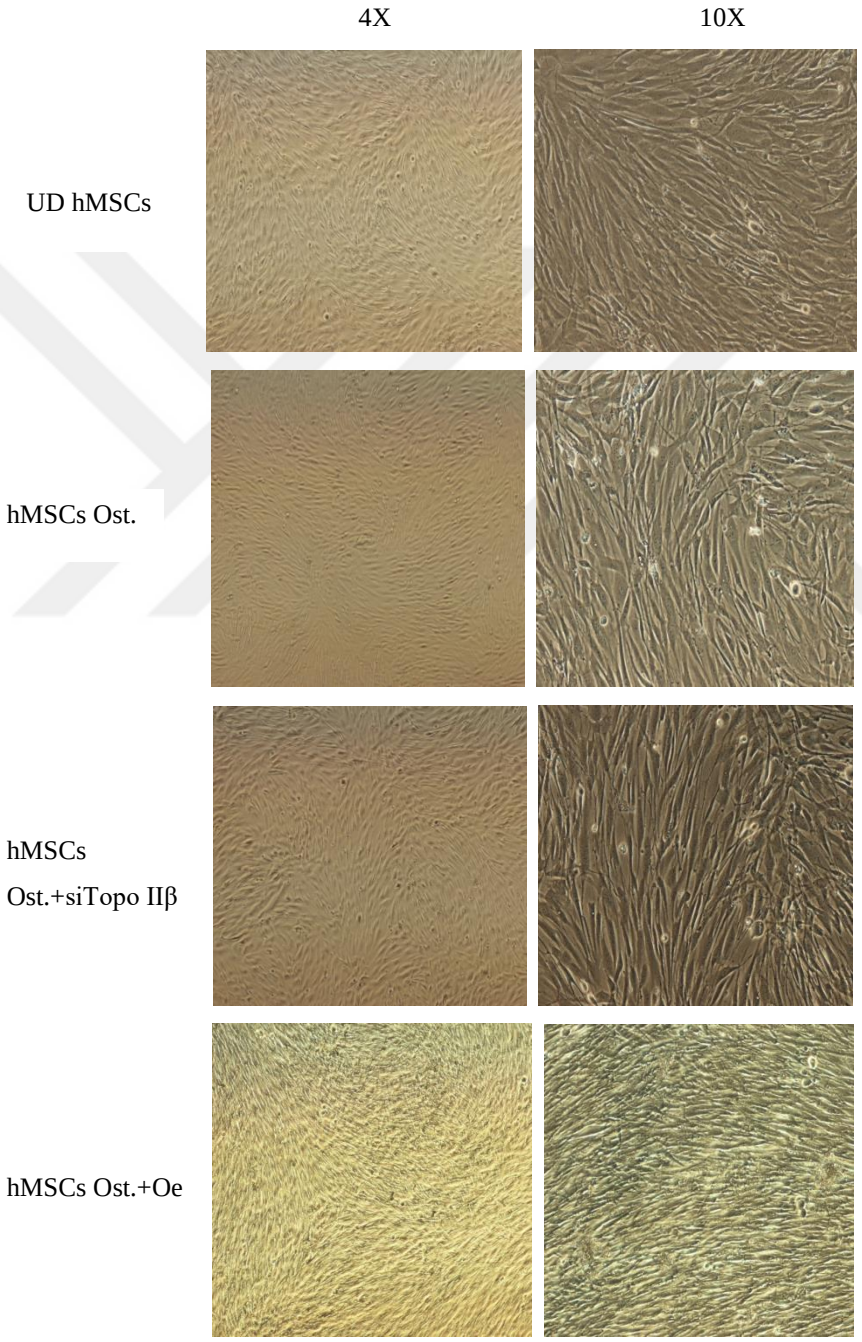


Figure 3.13: Light microscope images of osteogenically induced hMSCs at 48h.

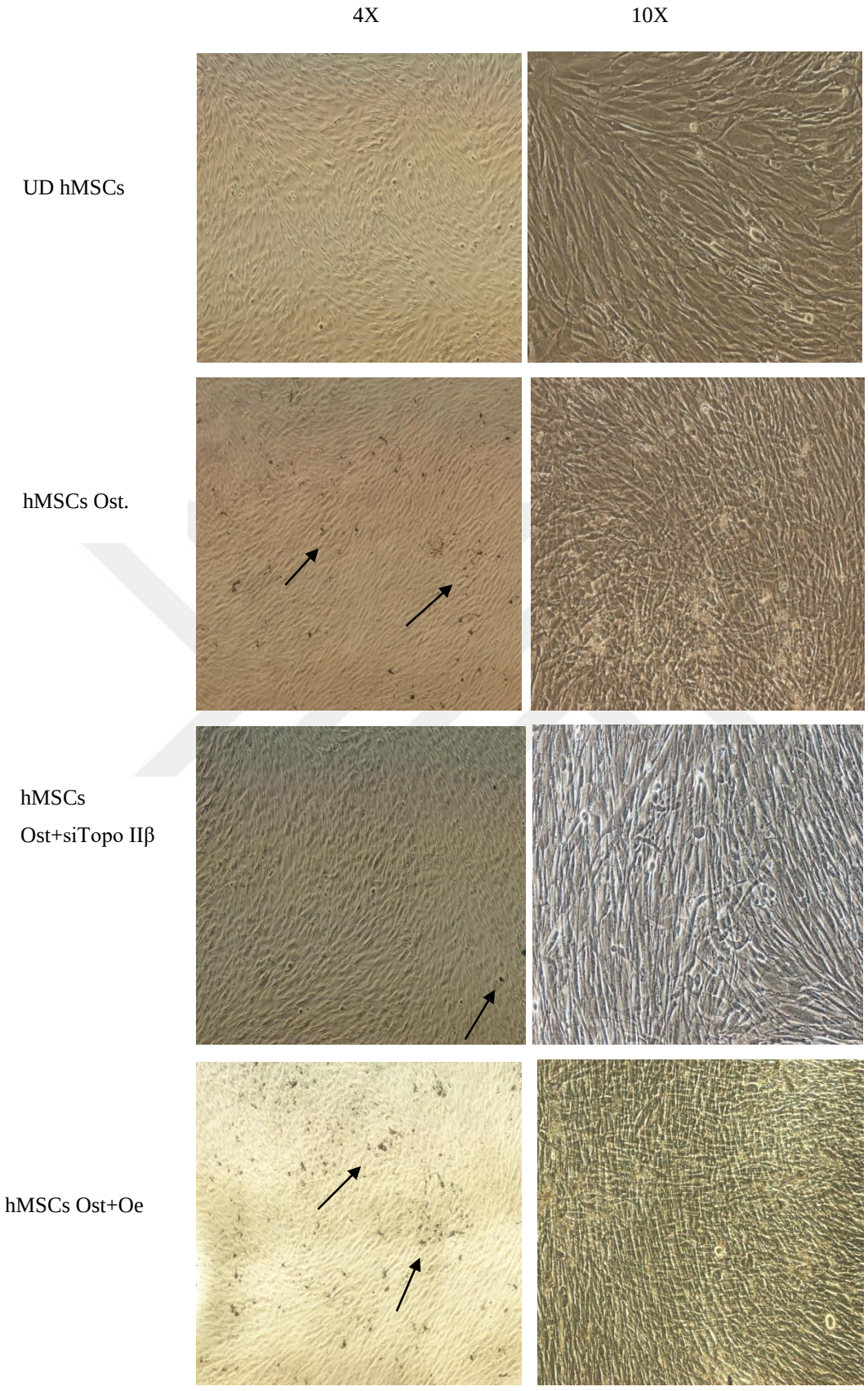


Figure 3.14: Images of experimental groups at 96h post osteogenic induction.

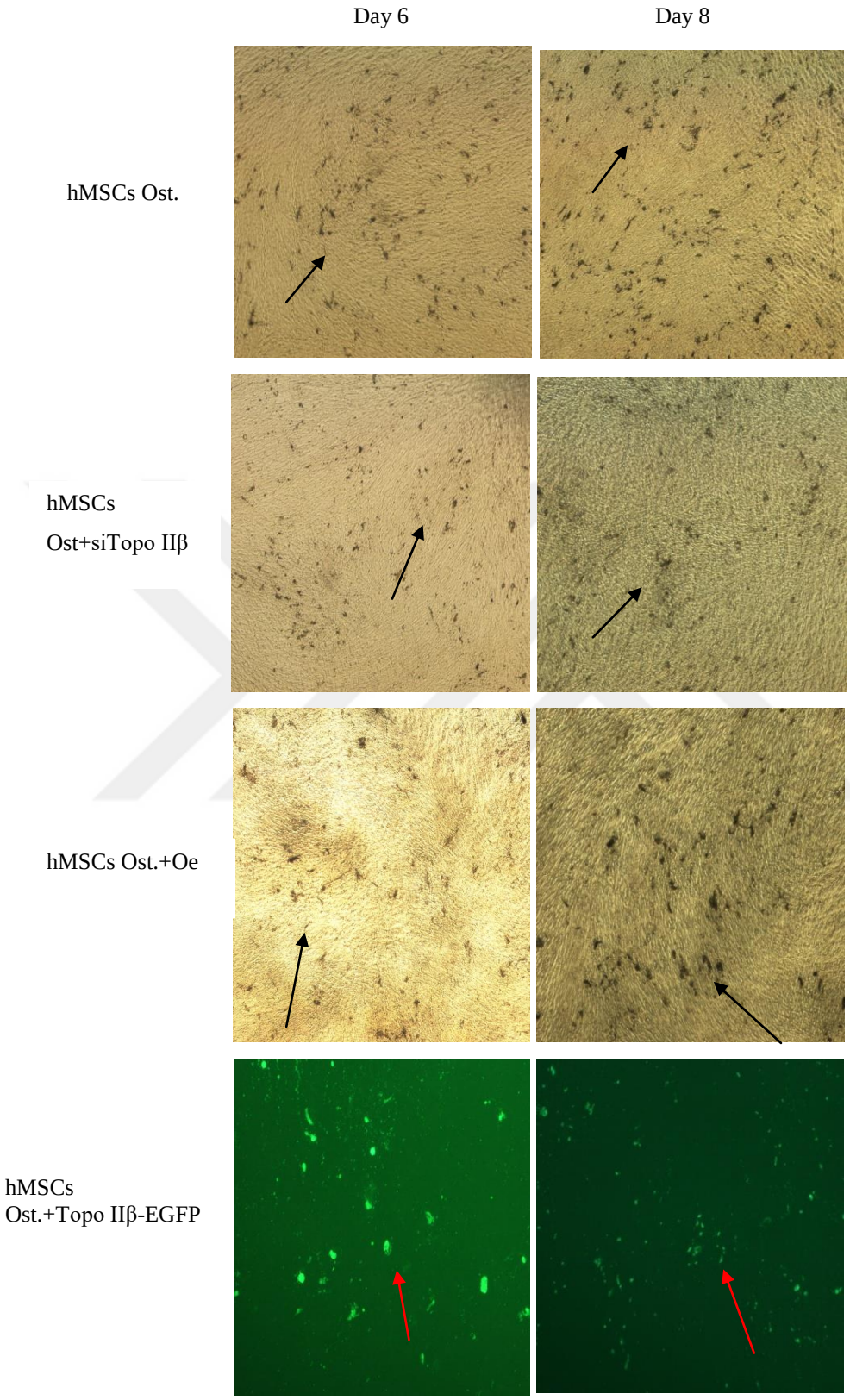


Figure 3.15: Images of experimental groups at 4X mag. and indicated conditions.

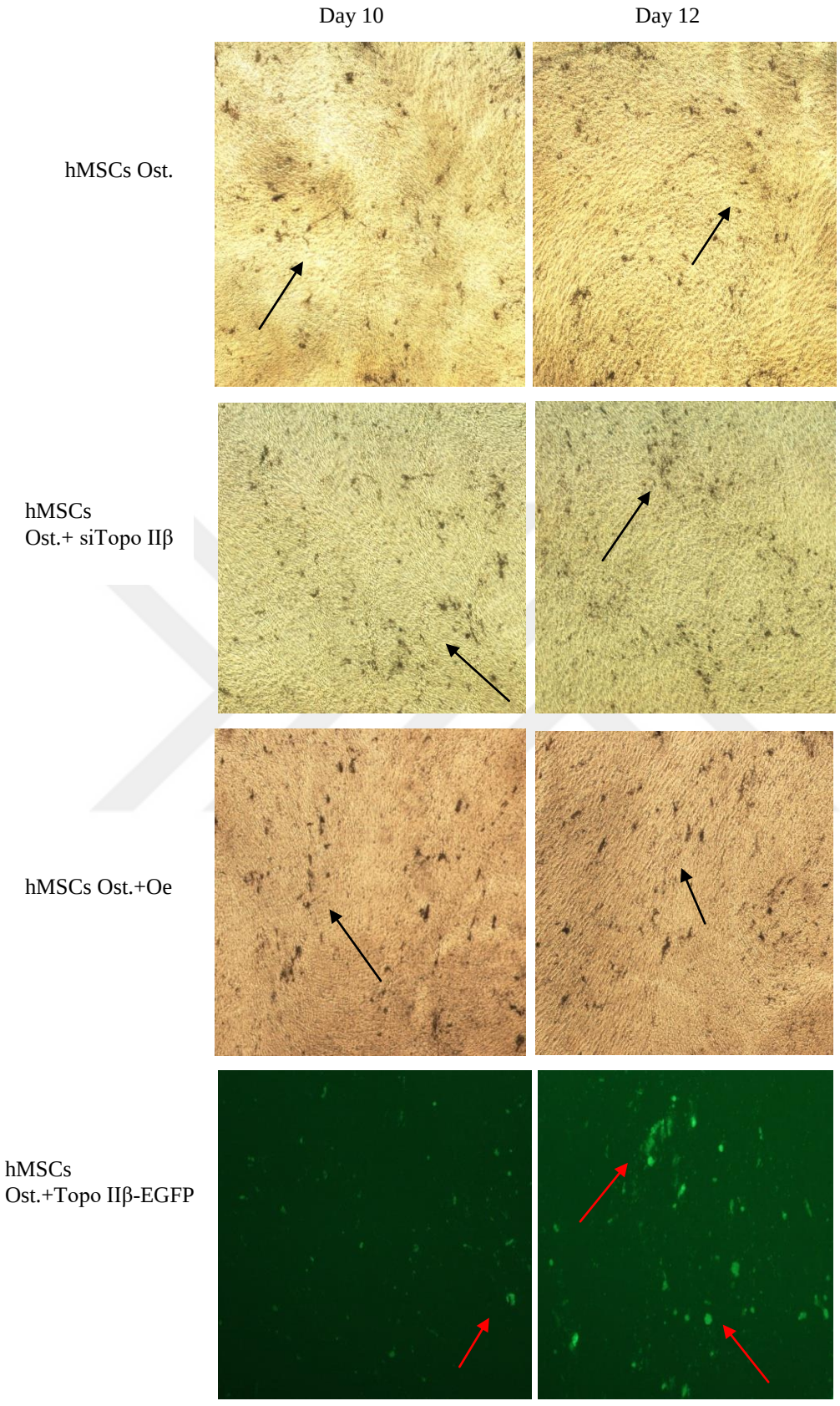


Figure 3.16: Images of experimental groups at 4X mag. and indicated conditions.

According to the hypothesis if topo IIβ has effects in osteogenic differentiation of hMSCs indicators of osteoblastogenesis shouldn't be observed in topo IIβ silenced samples. As seen in figures even in topo IIβ silenced samples, production of calcified bone matrix was observed but amount of them was lower than others. In topo IIβ overexpressed samples rate of calcification was higher compared with osteogenically induced hMSCs with WT and silenced topo IIβ. Since microscopic observations didn't prove any differences analyzing the amount of calcified areas was done by ImageJ program (Figure 3.17). At least 3 areas were scanned for each experimental group and indicated as % of calcified area without compared or normalized with any groups. Due to detection of calcified areas in NT-UD hMSCs didn't possible, only experimental groups' results were given and statistically analyzed. As seen in Figure 3.17 % of calcified areas were lower in topo IIβ silenced samples. Especially at day 7 decreasing was significant with all other samples. Also in topo IIβ overexpressed samples increasing calcified areas ere observed without any significance compared with others and significance was observed between samples of overexpression.

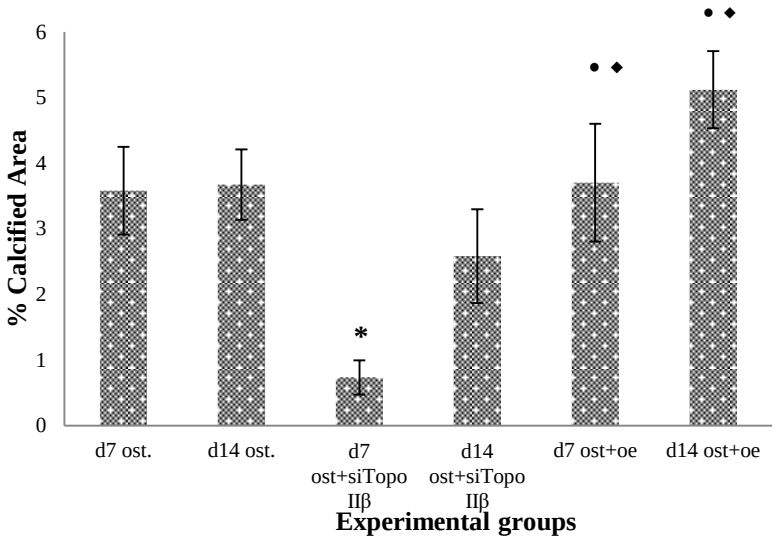


Figure 3.17: % of mineralized areas at days 7/14 and indicated conditions analyzed with ImageJ programme. • Significantly different from day7 ost, * significantly different from all samples, ◆ significantly different between groups ($p < 0.05$, one-way ANOVA with Tukey's HSD post-hoc procedure).

3.3.2. RT-qPCR Results of Day 7 and 14 Differentiated Samples

Indicated experimental conditions related to topo IIβ expression was checked at molecular level by using RT-qPCR. As indicated in Figure 3.18 both silencing and

overexpression of topo IIβ was achieved. This was the confirmation of topo IIβ targeting conditions of experimental groups. Results were statistically analyzed.

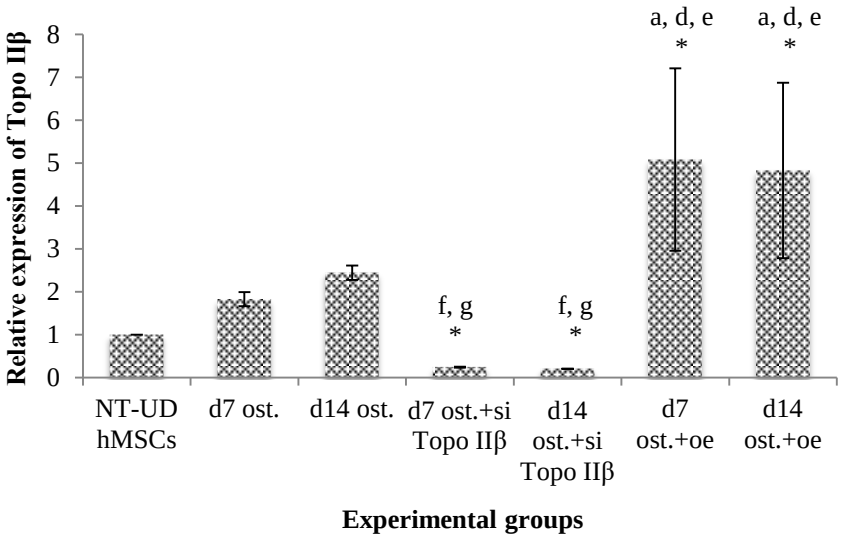
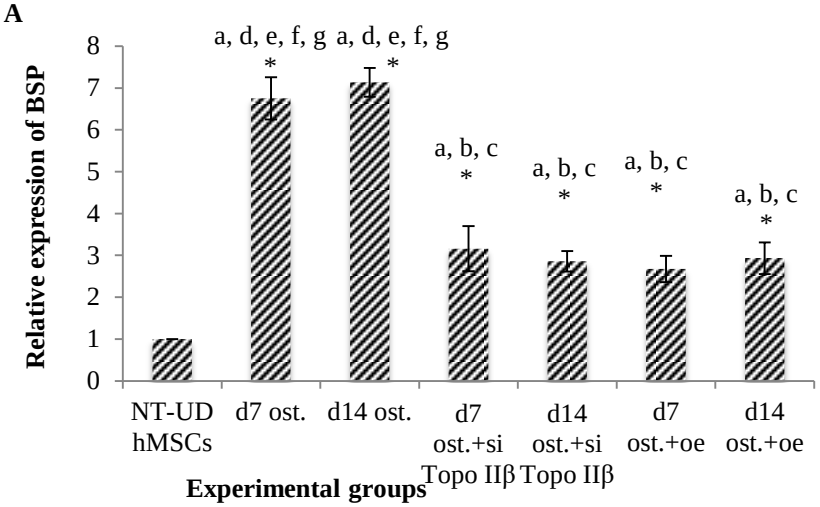


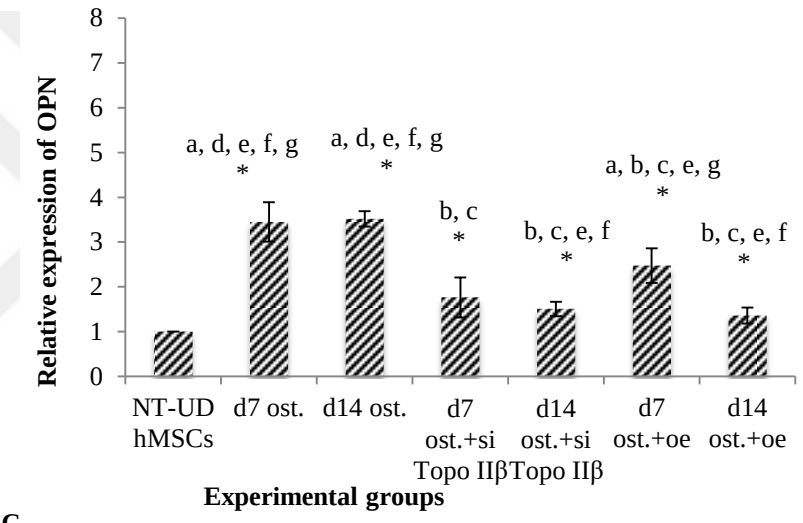
Figure 3.18: Gene expression levels of topo IIβ at indicated experimental groups. *significantly different from UD hMSC (a), D7 ost. (b), d14 ost. (c), d7 ost.+si Topo IIβ (d), d14 ost.+si Topo IIβ (e), d7 ost.+oe (f), d14 ost.+oe (g), ($p < 0.05$, one-way ANOVA with Tukey's HSD post-hoc procedure).

Expression levels of specific bone markers indicated for different stages of osteogenic differentiation were checked via RT-qPCR. Results were statistically analyzed. As seen in Figure 3.19 for all osteogenic markers, expression levels were dramatically and significantly decreased in topo IIβ silenced samples compared with osteogenically induced WT topo IIβ samples. For topo IIβ overexpressed samples it was expected that opposite effect should be observed compared with topo IIβ silenced samples. But almost same decreasing effect in gene expression was observed. As an absolutely required gene for osteogenic differentiation Runx2 expression was surprisingly peaked at topo IIβ overexpressed sample of day 7. This may be the result of recovery process post nucleofection. This is because nucleofection cause transfected cells to be under stress conditions and recovering of cells may be time dependent. And it may be hard for osteogenically induced hMSCs to enter osteogenic lineage. Also it wasn't possible to analyze transfection efficiency in that samples. As a result we have decided that silencing conditions were more suitable and reliable for further experiments. Also restriction in duration of osteogenic induction was indicated as days 1, 3, 5 and 7.

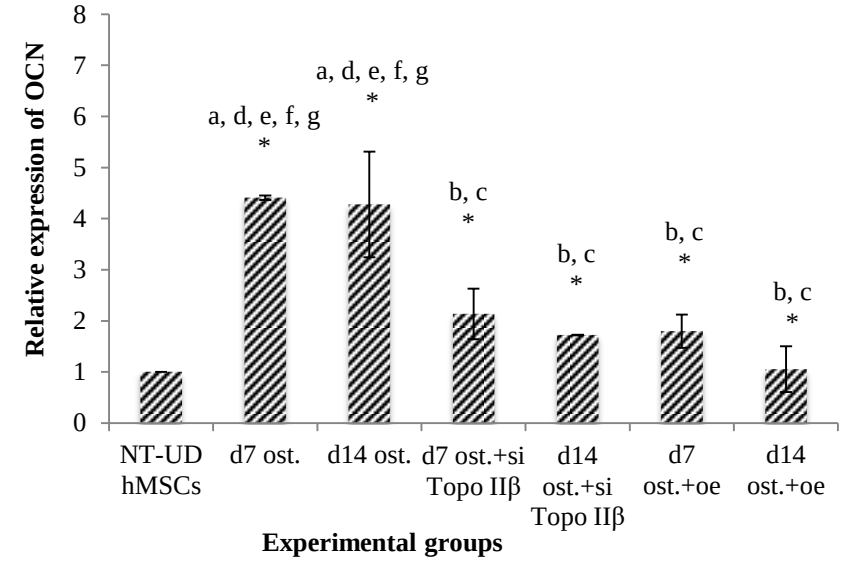
A


Relative expression of BSP
a, d, e, f, g
a, d, e, f, g
a, b, c
a, b, c
a, b, c
a, b, c
NT-UD hMSCs
d7 ost.
d14 ost.
d7 ost.+si Topo IIβ
d14 ost.+si Topo IIβ
d7 ost.+oe
d14 ost.+oe
Experimental groups

B


Relative expression of OPN
a, d, e, f, g
a, d, e, f, g
b, c
b, c, e, f
a, b, c, e, g
b, c, e, f
NT-UD hMSCs
d7 ost.
d14 ost.
d7 ost.+si Topo IIβ
d14 ost.+si Topo IIβ
d7 ost.+oe
d14 ost.+oe
Experimental groups

C


Relative expression of OCN
a, d, e, f, g
a, d, e, f, g
b, c
b, c
b, c
b, c
NT-UD hMSCs
d7 ost.
d14 ost.
d7 ost.+si Topo IIβ
d14 ost.+si Topo IIβ
d7 ost.+oe
d14 ost.+oe
Experimental groups

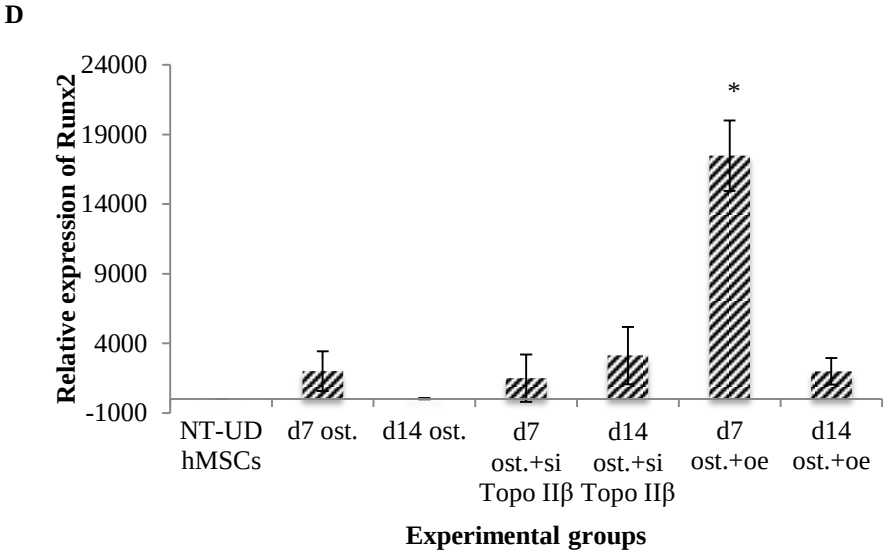


Figure 3.19: Gene expression levels of osteogenic markers A. BSP, B. OPN, C.OCN, D. Runx2. *significantly different from UD hMSC control (a), ost d7 (b), ost d14 (c), si+ost d7 (d), si+ost d14 (e), oe+ost d7 (f), oe+ost d14 (g), Gene expression level of Runx2 * was significantly different from all experimental groups ($p < 0.05$, one-way ANOVA with Tukey's HSD post-hoc procedure).

3.4. RT^2 PROFILER PCR ARRAY

3.4.1. Checking of Topoisomerase IIβ Gene Silencing at Days 1, 3, 5 And 7 by Using RT-qPCR

Before starting of PCR array experiments, silencing of topo IIβ at indicated time points was checked via RT-qPCR. As seen in Figure 3.20 silencing was succesfully achieved both at days 1, 3, 5 and 7. Statistical analysis showed that significant difference from NT-UD hMSC was observed in topo IIβ silenced samples while no significant difference was observed in between osteogenically induced hMSCs and NT-UD hMSCs. Also significant difference was detected in between osteogenically induced w/wo siTopo IIβ groups of each time points. Results were statistically analyzed and represented as indicated in Figure 3.20.

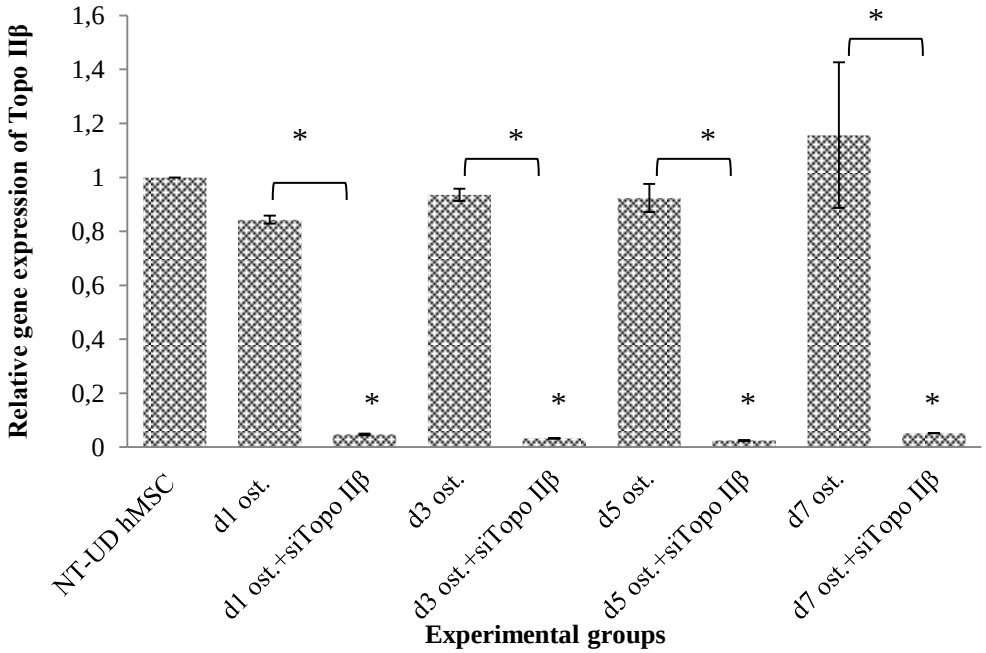


Figure 3.20: Topo IIβ gene expression in osteogenically induced and siRNA treated samples at days 1, 3, 5, and 7 (n=3). * Significantly different from; NT-UD hMSC and each time points' topo IIβ silenced samples were also significant according to its NT form. ($p<$ 0.05, one-way ANOVA with Tukey's HSD post-hoc procedure).

3.4.2. ARS of Experimental Groups at Days 1, 3, 5 and 7

ARS of cells in osteoblastic lineage were evaluated at days 1, 3, 5 and 7. Both cells that had naturally expressed and silenced forms of topo IIβ were committed into osteogenic differentiation at indicated time points. As seen in images of Figures 3.21/3.22/3.23, silencing of topo IIβ inhibited commitment of hMSCs into ostegenic differentiation and observation of ossified bone matrix synthesized by osteoblasts. As seen in Figures 3.21/3.22/3.23 naked eye and microscobic observations showed increasing amount of calcification from day 1 to 7 during progression of osteogenesis while almost no stainings were observed at topo IIβ silenced samples of each day. These findings were also supportive for regulatory effects of topo IIβ in osteogenic differentiation.

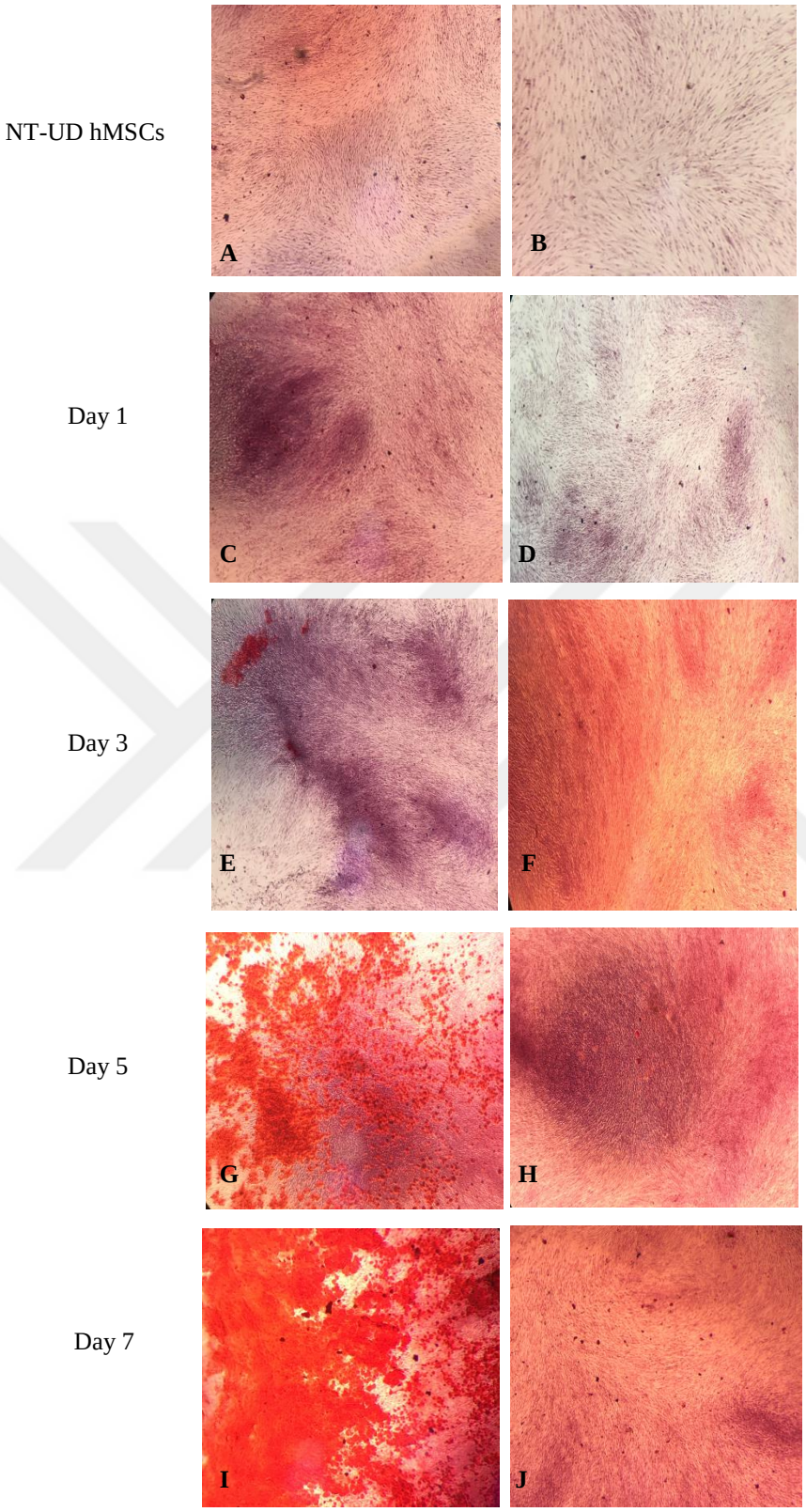


Figure 3.21: Images of calcified bone matrix stained with AR at days 1, 3, 5 and 7 in presence (WT topo IIβ, C, E, G, I) and absence (siTopo IIβ, D, F, H, J) of topo IIβ in osteogenic commitment, UD-NT hMSCs (A, B) (4x) (n=3).

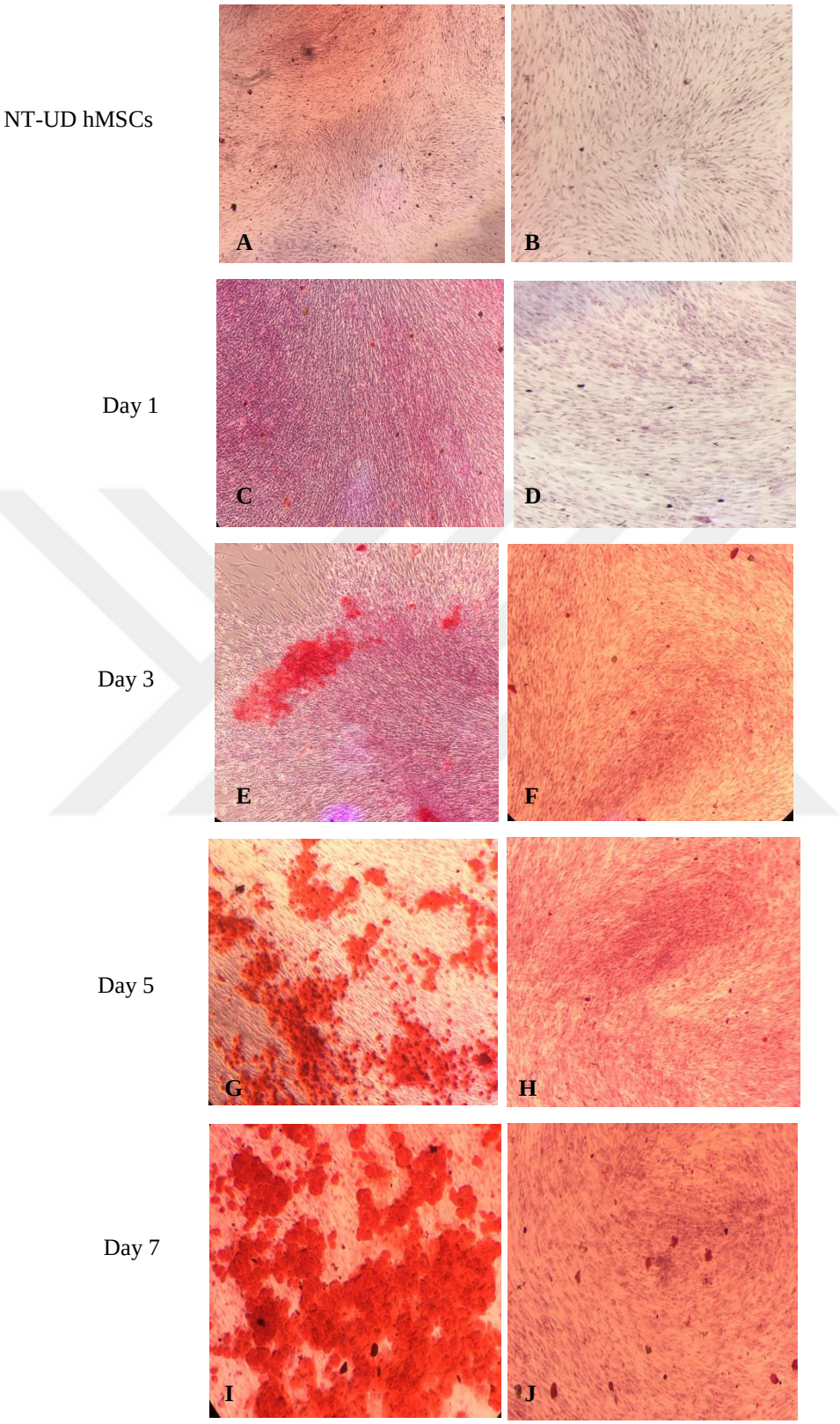


Figure 3.22: Images of calcified bone matrix stained with AR at days 1, 3, 5 and 7 in presence (WT topo IIβ, C, E, G, I) and absence (siTopo IIβ, D, F, H, J) of topo IIβ in osteogenic commitment UD-NT hMSCs (A, B) (10x) (n=3).

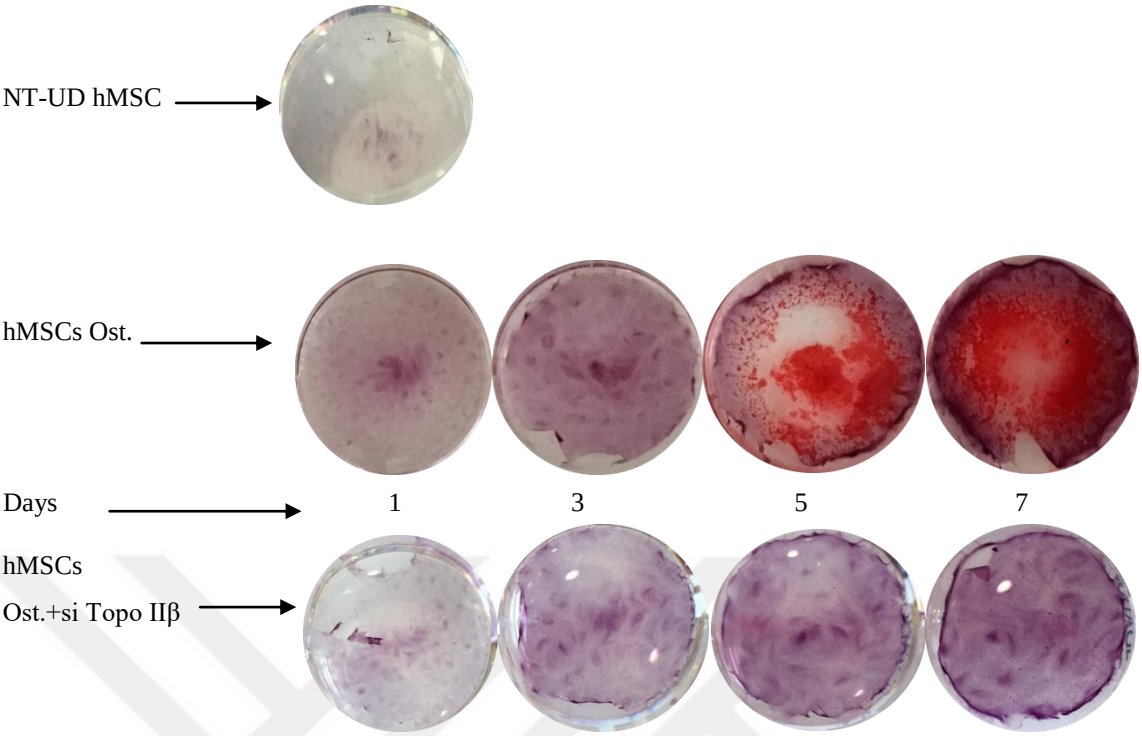


Figure 3.23: Plates of calcified bone matrix stained with AR at indicated experimental conditions. Dark red regions refer increasing amount of ossification (n=3).

3.4.3. RT^2 PCR Array of Osteogenesis and Osteoporosis

PCR array systems give opportunity to scan specific gene clusters related to selected cellular process or pathway. For our hypothesis, osteogenesis and OP arrays were used, that contain 84 different pathway specific genes, housekeeping genes for normalization, and other experimental controls. Before giving all results of PCR array, some of the gene families were picked from whole results related to specific cellular functions and they were grouped together according to their involvement in a particular function. ALPL, CTSK, Runx2 and SPP1 for osteogenic differentiation and skeletal development; COL1A1, COL1A2 as collagens and extracellular matrix (ECM) proteins; VDR for bone mineral metabolism related to calcium ion binding and homeostasis; FGFR1, TGFB1, TWIST1 and VEGFA that regulate cellular processes as growth factors, receptors, transcription factors were selected from whole array data.

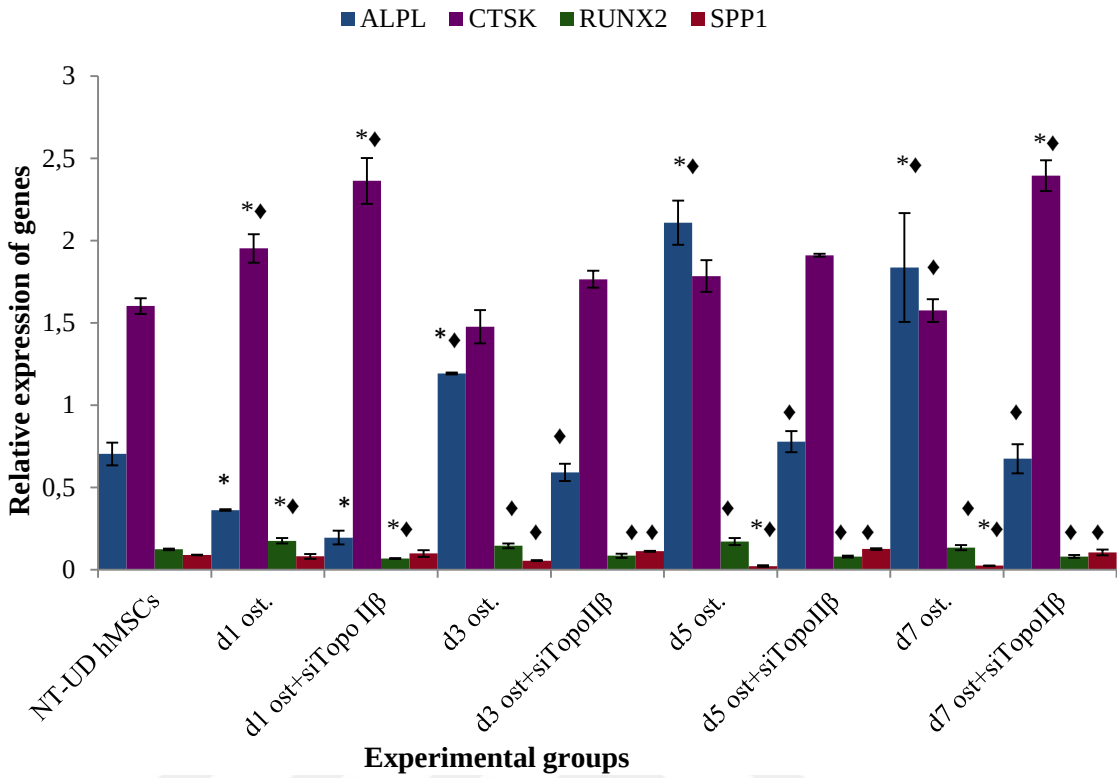


Figure 3.24: Differential expression rate of interested genes related to skeletal development, osteoblast differentiation and ossification. * significantly different from UD hMSCs, ♦ Significant difference between each time points' experimental groups w/wo siTopo IIβ. ($p < 0.05$, one-way ANOVA with Tukey's HSD post-hoc procedure. (n=2).

As seen in Figure 3.24 as positive regulators of skeletal development ALPL and Runx2 expression showed parallel expression pattern with increasing rate between ongoing days of differentiation in osteogenically induced hMSCs with WT topo IIβ expression alone and decreasing rate in osteogenically induced topo IIβ silenced samples. And as negative regulators of osteogenic differentiation and skeletal development CTSK expression showed opposite profile with ALPL and Runx2. Results were statistically analyzed and significance of groups were indicated in Figure 3.24.

In genes of ECM components same expression pattern was observed. Collagens are the essential ECM proteins of bone matrix and as seen in Figure 3.25 expression levels of COL1A1 and COL1A2 were decreased in topo IIβ silenced samples compared with osteogenic hMSCs with WT topo IIβ expression. Results were statistically analyzed.

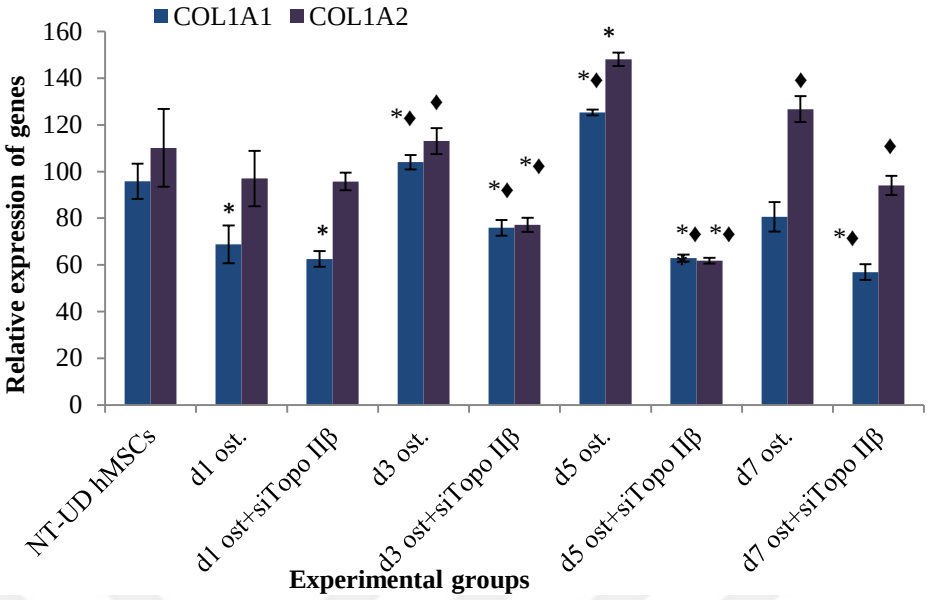


Figure 3.25: Differential expression rate of interested genes belongs to ECM required for osteoblast differentiation. *significantly different from NT-UD hMSCs. ♦significant difference between each time points' experimental groups w/wo siTopo IIβ. ($p < 0.05$, one-way ANOVA with Tukey's HSD post-hoc procedure (n=2).

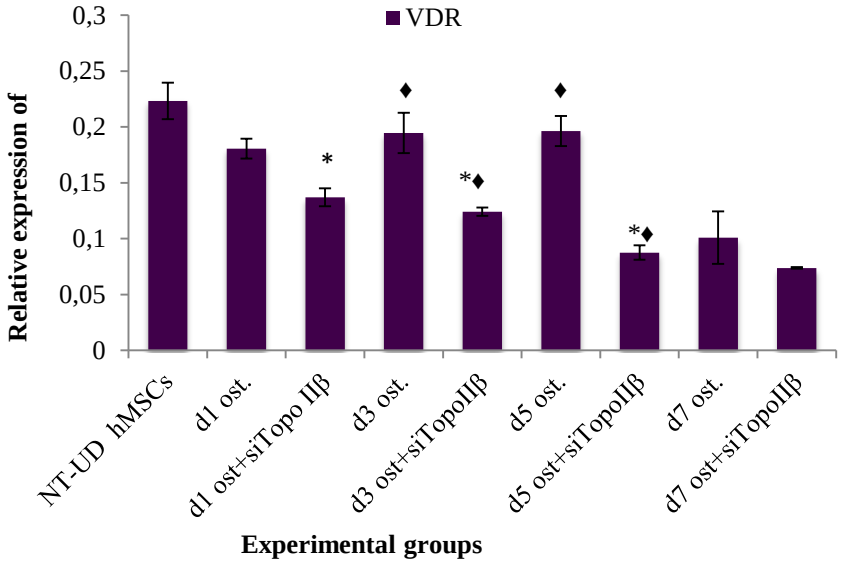


Figure 3.26: Relative expression level of VDR gene. *significantly different from NT-UD hMSCs, ♦significant difference between each time points' experimental groups w/wo siTopo IIβ. ($p < 0.05$, one-way ANOVA with Tukey's HSD post-hoc procedure) (n=2).

VDR gene that involves in calcium ion binding and homeostasis, showed compatible expression level with previous results by decreasing expression level in topo IIβ silenced samples of each day. Also time dependent effect of topo IIβ silencing was observed at day 5 in each sample (Figure 3.26).

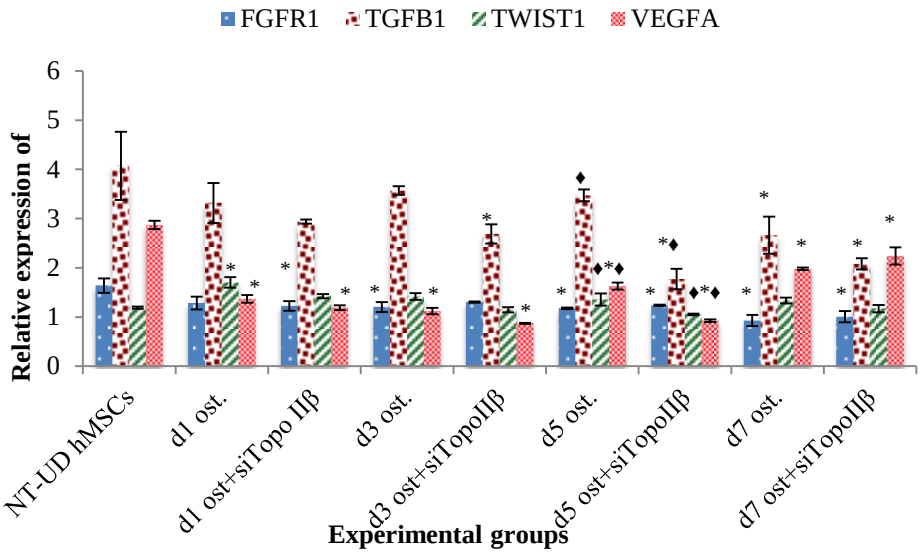


Figure 3.27: Differential expression rate of interested genes belongs to regulators (n=2)

Genes that were analyzed in that part showed compatible expression profiles with each other and were supportive for our hypothesis. In addition to these related genes a group of gene family belong to receptors, growth factors and transcription factors were analyzed. Figure 3.27 shows differential expression rates of related genes. Supportive results for hypothesis were observed for TGFβ-1 and VEGFA. They are positive regulators of osteoblastic differentiation and expression level of these genes were dramatically decreased in topo IIβ silenced forms of osteogenic differentiation. Results were statistically analyzed.

PCR array results were expressed as fold regulation rates with two-fold up- or down-regulated expression level. In following tables (3.1, 3.2) fold changes lower then 0,5 referred downregulated genes and indicated with '-' value in blue color while higher from 2 refered up-regulation of genes and indicated as '+' value in red color. Scatter plots of each comparison showed up- and downregulated genes represented with dots. Red dots refer upregulated genes in interested experimental group compared with several groups while green dots refer downregulation of genes. Also black dots refer unchanged gene expressions.

In following tables up and downregulation of genes belong to experimental group were indicated as green color. It means genes were up- or downregulated in that group labelled with green color in the table versus its compared group.

Table 3.1: Up- and downregulated genes of osteogenesis PCR array between compared groups.

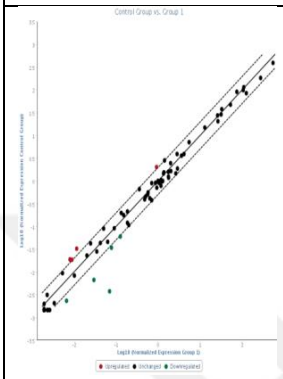
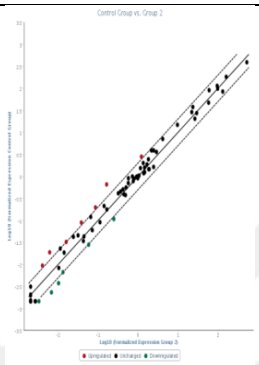
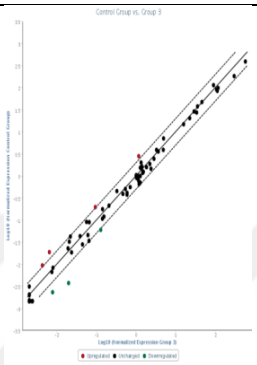
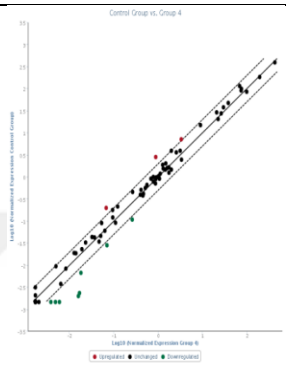
NT-UD hMSC/day1 ost.	**NT-UD hMSC/day1 ost.+si Topo IIβ**	**NT-UD hMSC/day3 ost.**	**NT-UD hMSC/day3 ost. ost./si Topo IIβ**
			
COL15A1 2,0705	ALPL 3,9724	BMP6 2,1735	MMP2 2,1435
PHEX 2,7895	BMP6 2,2974	SMAD3 2,0994	SMAD3 2,9485
SOX9 2,3457	PHEX 2,0139	SOX9 2,8879	VEGFA 3,2043
VCAM1 2,1886	SMAD3 2,2815	VEGFA 2,4116	BMP3 -3,2944
BMPR1B -2,2974	SOX9 3,0314	COMP -2,1435	CD36 -2,7132
CD36 -4,4691	TGFB3 2,3295	ITGAM -3,387	COL2A1 -4,0558
COMP -2,114	VEGFA 2,2974	NOG -5,3147	CSF2 -8
ITGAM -2,8679	CD36 -2,0139		FLT1 -2,4794
NOG -18,7654	COL2A1 -2,2974		ITGAM -7,21
	FLT1 -2,0849		MMP9 -2,6027
	ITGAM -2,969		TGFB2 -2,4116
	NOG -2,7702		
	TGFB2 -2,3295		

Table 3.1 (continue): Up- and downregulated genes of osteogenesis PCR array between compared groups.

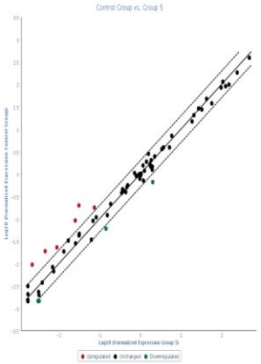
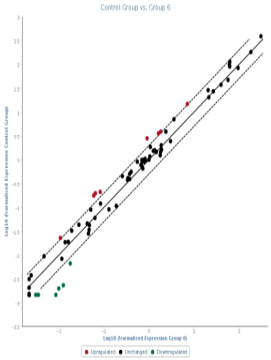
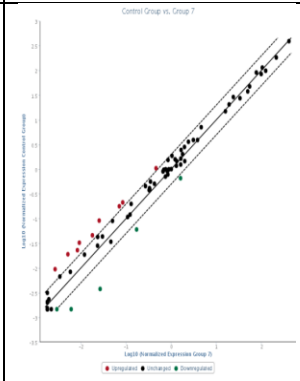
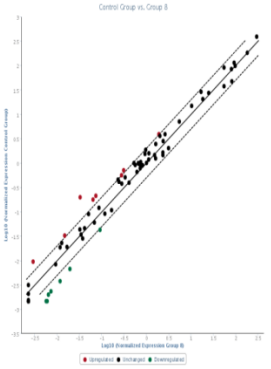
NT-UD hMSC/day5 ost.		**NT-UD hMSC/day5 ost..+si Topo IIβ**		**NT-UD hMSC/day7 ost.**		**NT-UD hMSC/day7 ost./si Topo IIβ**	
							
BMP4	2,3784	BGN	2,0849	BMP4	2,4967	BMP4	2,7895
BMP6	4,2575	BMP4	2,969	BMP6	3,5064	BMP6	3,2716
EGF	2,5669	CDH11	2,1287	EGF	2,7321	CDH11	2,0562
SMAD3	6,3643	EGF	2,114	EGFR	2,2658	NFKB1	2,0279
SOX9	4,084	SMAD3	3,0525	ITGA2	2,5491	PDGFA	2,2974
SPP1	3,605	TGFB1	2,2038	PHEX	3,5064	PHEX	2,2038
ALPL	-3,0738	VDR	2,5491	SOX9	3,6553	SMAD3	6,0629
COL10A1	-2,2501	VEGFA	3,0951	SPP1	3,5801	VDR	2,8879
COMP	-2,395	CD36	-2,6945	VDR	2,514	CD36	-2,8879
MMP9	-2,0994	COL10A1	-2,114	ALPL	-2,4453	COL14A1	-2,1435
		COL2A1	-2,4116	COL10A1	-4,1699	CSF2	-3,2043
		CSF2	-5,0281	COMP	-2,8481	CSF3	-3,8637
		ITGAM	-5,4264	CSF3	-2,0139	ITGAM	-3,1167
		MMP9	-5,8971	NOG	-7,1107	MMP9	-4,084
						NOG	-3,0951

Table 3.1 (continue): Up- and downregulated genes of osteogenesis PCR array between compared groups.

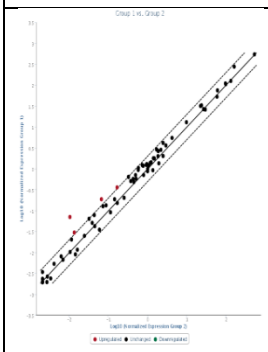
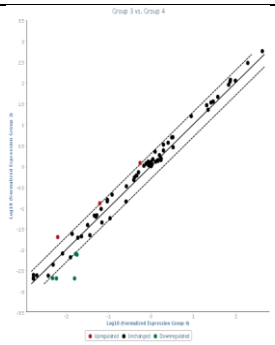
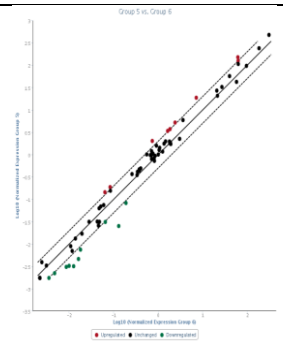
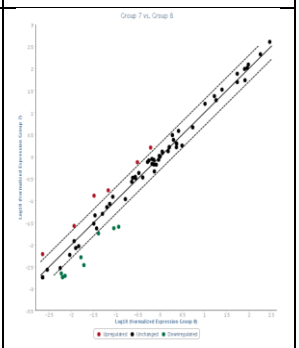
day1 ost./ ost.+si Topo IIβ	day3 ost./ost.+siTopo IIβ	day5 ost./ost. +si Topo IIβ	day7 ost./ ost.+si Topo IIβ
			
ALPL 2,1735 CD36 2,2191 NOG 6,774 RUNX2 2,8284	ALPL 2,1435 COMP 2,0562 NOG 3,1383 BMP3 -2,3295 CD36 -2,395 COL2A1 -2,9079 CSF2 -7,7812 ITGAM -2,1287	ALPL 2,7511 BGN 2,6208 CDH11 2,0279 COL1A1 2,0139 COL1A2 2,4623 COL5A1 2,1886 COMP 2,2658 TGFB1 2,0849 VDR 2,2501 BMP6 -2,1287 CD36 -2,4116 COL2A1 -2,0279 CSF2 -3,0738 ITGAM -3,9724 MMP9 -2,8089 SMAD3 -2,0849 SOX9 -3,5064 SPP1 -5,1337 TGFB2 -2,2815	ALPL 2,6208 COL10A1 2,6945 COMP 2,514 NOG 2,2974 PDGFA 2,4116 SMAD3 3,9724 CD36 -5,6178 COL14A1 -3,8637 CSF2 -3,5064 ITGA2 -2,2815 ITGAM -3,7064 MMP9 -2,6945 SOX9 -3,2043 SPP1 -4,5948

Table 3.2: Up- and downregulated genes of OP PCR array between compared groups.

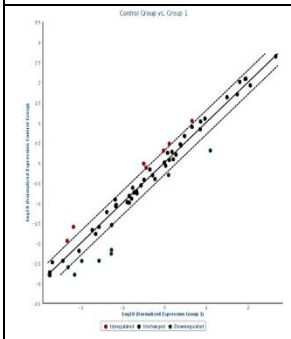
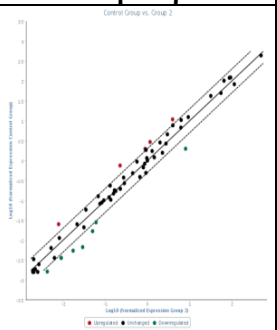
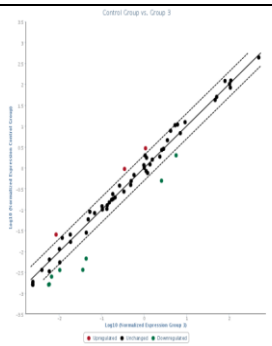
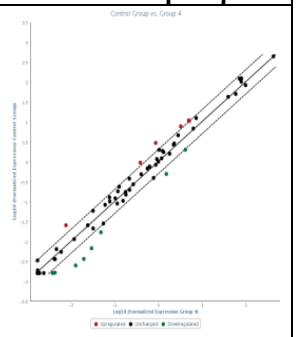
NT-UD hMSC/day1 ost.		**NT-UD hMSC/day1 ost.+si Topo IIβ**		**NT-UD hMSC/day3 ost.**		**NT-UD hMSC/day3 ost. ost./si Topo IIβ**	
							
ALPL	2,0562	ALPL	3,3404	SFRP4	2,9897	MMP2	2,3784
ENPP1	2,0705	ENPP1	2,2346	TNFRSF1 1B	2,7321	PLOD2	2,2038
P2RX7	2,395	LTBP2	2	VEGFA	2,7321	SFRP4	3,2944
PLOD2	2,3784	PLOD2	2,7511	ALOX5	-2,6574	STAT1	2,0562
SFRP4	3,7581	SFRP4	3,1821	CYP19A1	-9,9177	TNFRSF1 1B	2,4453
TNFRSF1 1B	2,9282	VEGFA	2,5315	DKK1	-2,8284	VEGFA	3,387
VEGFA	2,2346	ACP5	-2,9897	HSD11B1	-6,4086	ACP5	-2,8679
ALOX5	-2	CYP19A 1	-2,5315	IGFBP2	-5,2054	ALOX5	-5,1337
CYP19A1	-7,5685	DKK1	-4,0278	LEP	-3,5308	CYP19A1	-5,4264
DKK1	-6,3203	HSD11B 1	-4,3772	PTH1R	-2,8879	DKK1	-2,0849
HSD11B1	-8,1117	IL6R	-2,1735	SHBG	-3,5801	HSD11B1	-4,4076
IGFBP2	-2,5669	NOG	-3,2043			IGFBP2	-3,0951
NOG	-9,8492	PTH1R	-2,5491			LEP	-2,3784
PTH1R	-2,969	SHBG	-2,6208			SHBG	-2,639
SHBG	-4,3772						

Table 3.2 (continue): Up- and downregulated genes of OP PCR array between compared groups.

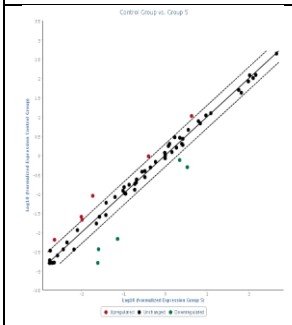
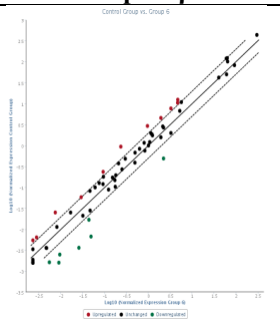
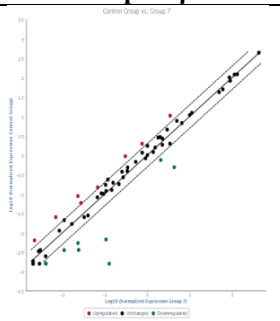
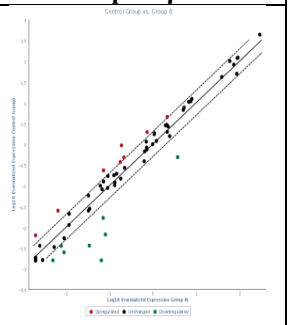
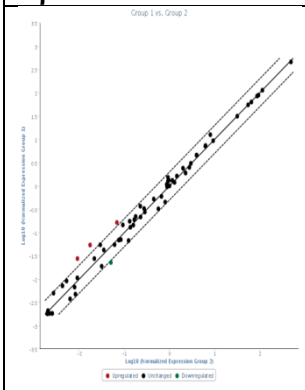
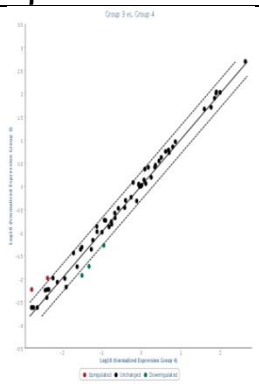
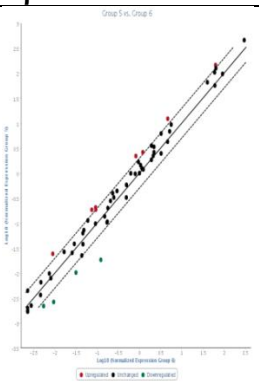
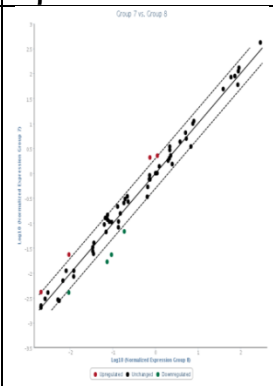
NT-UD hMSC/day5 ost.		**NT-UD hMSC/day5 ost..+si Topo IIβ**		**NT-UD hMSC/day7 ost. +si Topo IIβ**		**NT-UD hMSC/day7 ost./si Topo IIβ**	
							
PTHLH	2,0562	BGLAP	2,0139	BGLAP	2,1287	ENPP1	2,6574
SFRP4	2,5669	MMP2	2,362	ENPP1	2,6208	LRP6	2,2191
SPP1	4,7899	NOG	2,3784	IL6	2,1886	MAB21L2	2,0705
STAT1	2,4623	SFRP4	3,387	SFRP4	3,605	SFRP4	3,7842
TNFRSF11A	2,8481	STAT1	2,2658	SPP1	3,8106	TGFB1	2,114
TNFRSF11B	2,362	TGFB1	2,3784	STAT1	3,0525	TNFRSF11A	3,1383
ALPL	-2,9282	TIMP2	2,6574	TNFRSF11A	2,8879	TNFRSF11B	4,8906
CYP19A1	-7,0128	TNFRSF11A	2,2974	TNFRSF11B	3,0951	VDR	3,1602
HSD11B1	-10,7779	TNFRSF11B	4,0278	VDR	2	ACP5	-4,3169
IGFBP2	-6,8685	VDR	2,5491	ALPL	-2,7511	ALOX5	-3,6808
LEP	-15,2422	VEGFA	3,0951	CYP17A1	-2,5847	CD40	-3,1821
		GAPDH	2	CYP19A1	-6,6807	CYP19A1	-9,6465
		ACP5	-2,5847	HSD11B1	-15,8895	HSD11B1	-12,2101
		ALOX5	-3,7842	IGFBP2	-8,7543	IGFBP2	-7,6741
		CYP19A1	-7,1107	LEP	-79,3413	LEP	-41,3553
		HSD11B1	-7,2602	NOG	-4,2871	PTH1R	-2,2191
		IGFBP2	-4,5002	PTH1R	-3,0951		
		LEP	-5,6178	SHBG	-2,4794		
		SHBG	-3,3404				

Table 3.2 (continue): Up- and downregulated genes of OP PCR array between compared

day1 ost./ost.+si Topo IIβ	day3 ost./ost.+si Topo IIβ	day5 ost./ost.+si Topo IIβ	day7 ost./ost.+si Topo IIβ
			
CYP19A1 2,9897 NOG 3,0738 RUNX2 2,395 ACP5 -2,2191	ESR1 3,387 PTH1R 2,3784 ACP5 -2,6208 PTHLH -2,7321 SPP1 -2,1735	ALPL 2,6759 COL1A2 2,3295 LEP 2,7132 LTBP2 2,1886 NFATC1 2 RUNX2 2,4794 TIMP2 2,639 VDR 2,2346 ALOX5 -3,5554 PTHLH -3,0951 SHBG -2,4794 SPP1 -6,6346	ALPL 2,8089 CYP17A1 2,0279 LTBP2 2,042 NOG 2,5315 ACP5 -4,3469 ALOX5 -2,2658 IL6 -2,7132 SPP1 -3,9724

To summarize the whole results, numbers of up- and downregulated genes were given in table 3.3. and results were clarified. Conditions were grouped into 3 as; NT-UD hMSCs as Group I, hMSCs ost. as Group II, hMSCs ost.+si Topo IIβ as Group III.

Table 3.3: Up- and downregulated genes in compared groups of osteogenesis array.

Compared Groups	Number of Upregulated genes	Number of Downregulated genes
D1/I-II	4	5
D1/I-III	7	6
D1/II-III	4	-
D3/I-II	3	3
D3/I-III	6	8
D3/II-III	3	5
D5/I-II	6	4
D5/I-III	8	6
D5/II-III	9	10
D7/I-II	9	5
D7/I-III	8	7
D7/II-III	6	8

Table 3.4: Number of up- and downregulated genes in compared groups of OP array.

Compared Groups	Number of Upregulated genes	Number of Downregulated genes
D1/I-II	7	8
D1/I-III	6	8
D1/II-III	3	1
D3/I-II	3	8
D3/I-III	6	8
D3/II-III	2	3
D5/I-II	6	5
D5/I-III	12	7
D5/II-III	8	4
D7/I-II	8	9
D7/I-III	8	8
D7/II-III	4	4

According to the comparison analysis of whole gene profiles, up and down regulated genes were listed in tables 3.1, 3.2, 3.3 and 3.4 for osteogenesis and OP PCR array. Comparisons were occured in between NT-UD hMSCs and topo IIβ WT and topo IIβ silenced groups of each day. As seen in tables dynamisms in gene expression pattern were observed in groups compared with topo IIβ silenced samples of each day. Since topo IIβ has effects on DNA metabolism, silencing of it affects changes in expression pattern. In general expression profile of each array, effects on gene expression changes at day 5 was remarkable compared with topo IIβ silenced groups with both NT-UD hMSCs and groups that have WT forms of topo IIβ. Thus we have focused on analysis of genes at day 5.

3.4.4. Analyzing the Fold Regulation of Osteogenesis Specific Genes At Day 5

Regulation of genes grouped according to their functions during osteogenic differentiation and comparisons of groups were shown in Tables 3.5/3.6. Topo IIβ silenced samples were compared with both NT-UD hMSCs and osteogenically induced hMSCs with WT topo IIβ sample. Tables showed up- (+) and down-regulated (-) genes in compared groups.

Table 3.5: Up- or downregulated of genes in group I compared with group II and their functions in osteogenesis.

Name of the gene	Fold regulation	Function
BMP4	2,3784	Skeletal development
BMP6	4,2575	Skeletal development
EGF	2,5669	Growth factors
SMAD3	6,3643	Skeletal development
SOX9	4,084	Skeletal development
SPP1	3,605	Skeletal development
ALPL	-3,0738	Skeletal development
COL10A1	-2,2501	ECM
COMP	-2,395	ECM

Table 3.6: Up- or downregulated of genes in group I compared with group III and their functions in osteogenesis.

Name of the gene	Fold regulation	Function
BGN	2,0849	ECM
BMP4	2,969	Skeletal development
CDH11	2,1287	Cell adhesion
EGF	2,114	GF, TF, RCPTR
SMAD3	3,0525	Skeletal development
TGFB1	2,2038	GF, TF, RCPTR
VDR	2,5491	Calc. ion binding met.
VEGFA	3,0951	GF, TF, RCPTR
CD36	-2,6945	Cell adhesion
COL10A1	-2,114	ECM
COL2A1	-2,4116	ECM
CSF2	-5,0281	GF, TF, RCPTR
ITGAM	-5,4264	Cell adhesion
MMP9	-5,8971	ECM

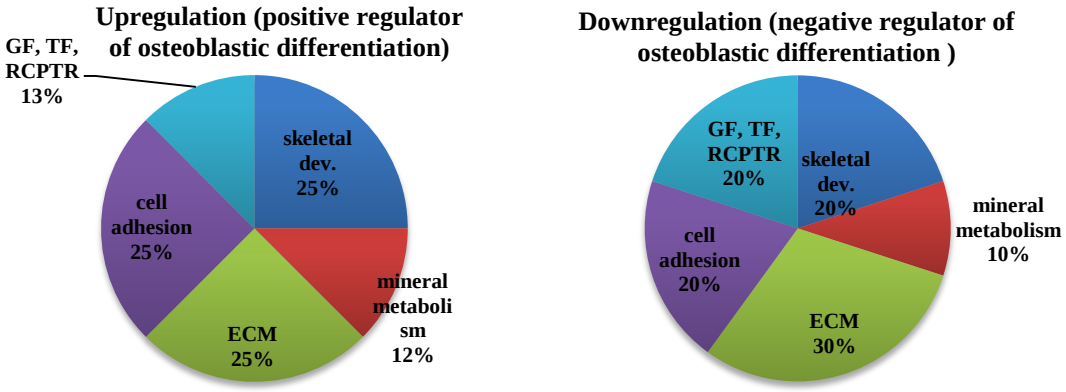


Figure 3.28: Pie chart showing the distribution and up- and downregulation of osteogenesis pathway genes in group II versus group III at day 5.

Pie chart showed percentage of gene clusters that were up- and downregulated in Group II versus Group III at day 5. Genes related to skeletal development were upregulated in Group II at a rate of 25% while downregulation was observed at 20%. Genes of bone mineral metabolism were upregulated at 12% and downregulated in 10% in Group II compared with Group III.

3.4.5. Analyzing the Fold Regulation of Osteoporosis Specific Genes At Day5

Regulation and comparison of genes related to stages of OP is shown in Tables 3.7, 3.8.

Table 3.7: Up- or downregulated of genes in group I compared with group II and their functions in OP pathway.

Name of the gene	Fold regulation	Function
PTHLH	2,0562	Calcicotropic hormones
SFRP4	2,5669	WNT-β catenin
SPP1	4,7899	Skeletal development
STAT1	2,4623	Bone remodeling
TNFRSF11A	2,8481	RANK/RANKL/OPG Signaling, Cytokine
TNFRSF11B	2,362	RANK/RANKL/OPG Signaling, Cytokine
ALPL	-2,9282	Skeletal development
CYP19A1	-7,0128	Calcicotropic hormones
HSD11B1	-10,7779	Bone remodeling
IGFBP2	-6,8685	Cytokines, GF, TF, RCPTR
LEP	-15,2422	Cytokines, GF, TF, RCPTR

Table 3.8: Up- or downregulated of genes in group I compared with group III and their functions in OP pathway.

Name of the gene	Fold regulation	Function
BGLAP	2,0139	Skeletal development
MMP2	2,362	ECM
NOG	2,3784	Bone remodeling
SFRP4	3,387	WNT-β catenin
STAT1	2,2658	Bone remodeling
TGFB1	2,3784	GF, TF, RCPTR
TIMP2	2,6574	Bone remodeling
TNFRSF11A	2,2974	RANK/RANKL/OPG Signaling, Cytokine
TNFRSF11B	4,0278	RANK/RANKL/OPG Signaling, Cytokine
VDR	2,5491	Calcicotropic hormones
VEGFA	3,0951	GF, TF, RCPTR
ACP5	-2,5847	Others
ALOX5	-3,7842	Bone remodeling
CYP19A1	-7,1107	Calcicotropic hormones
HSD11B1	-7,2602	Bone remodeling
IGFBP2	-4,5002	Cytokines, GF, TF, RCPTR
LEP	-5,6178	Cytokines, GF, TF, RCPTR
SHBG	-3,3404	Calcicotropic hormones

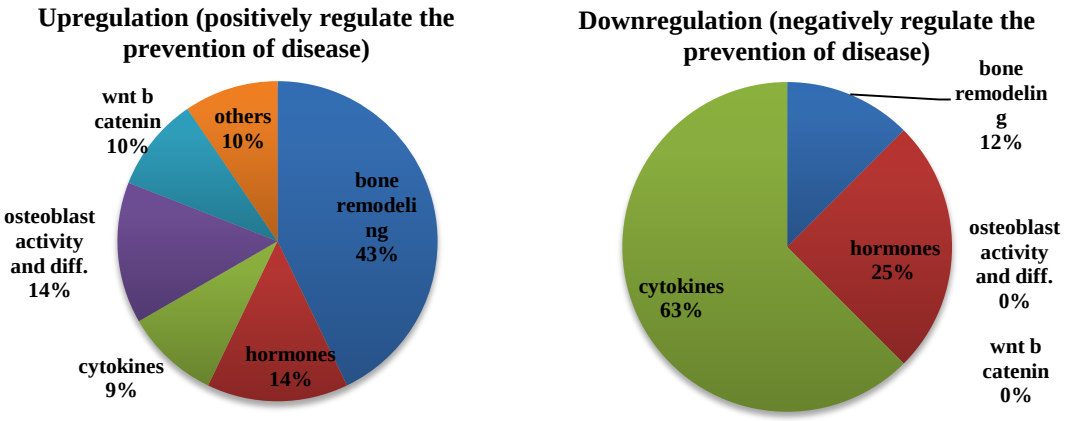


Figure 3.29: Pie chart showing the distribution and up- and downregulation of OP pathway genes in group II versus group III at day 5.

Pie chart showed percentage of gene clusters that were up- and downregulated in Group II versus Group III. Most remarkable upregulation of OP array genes in Group II compared with Group III was observed in genes of bone remodeling. In OP cases losing of bone tissue bone is a result of disorders in bone remodeling process. And both tables and pie charts indicated that silencing of topo IIβ cause downregulation of bone remodeling genes. Also genes belong to osteoblast differentiation and osteoblastic activity were upregulated in Group II versus Group III. As an important pathway for OP Wnt-β cathenin pathway genes were upregulated in Group II compared wiyh Group III again.

4. DISCUSSION

Adult MSCs were discovered as non-hematopoietic cell population of bone marrow that supply cell sources of several tissues and organs throughout life. They are spindle shaped, adherent cells and progenitors of tissues such as bone, cartilage and adipose tissue. Most important properties of that cell population are self-renewal and large range of differentiation potential. SC differentiation is controlled by several factors. Both physical and chemical conditions are effective in addition to control mechanisms at molecular level. Biological factors such as age and metabolism also regulate fate decision of MSC [43].

This study is interested in molecular mechanism that can be effective on hMSC differentiation at gene expression level. As an enzyme family, DNA topo enzymes have several functions on gene expression, replication, transcription and chromosomal segregation [92]. Their main role in DNA topology gives DNA topo leading to resolve topological entanglements by interacting with promoter regions and nucleosomes and generating a transient break through which topological changes can occur. By this way gene expression profile can be changed or controlled by DNA topo family members. So this function gives an idea that DNA topo may have effects on SC differentiation, because SC fate depends on active genes, belongs to target cell's expression profile [93].

There are two types of DNA topo enzymes in mammalian cells. In higher eukaryotes type II has 2 subgroups known as DNA topo IIα and topo IIβ. Topo IIα isoform mainly involved in mitotic prosesses and only present in proliferating cells such as tumors. Topo IIβ isoform isn't required for normal mitotic events it is present in all terminally differentiated cells. In literature almost all of the studies have been concentrated to effects of topo IIβ in neuronal differentiation potential. In this study we aimed to investigate the possible role of topo IIβ in osteogenic differentiation of hMSC line. Studies were started with optimizations and confirmations of osteogenic differentiation by using histological staining and detection of bone specific genes' expressions. First

we showed that hMSC line had capable of differentiation into osteogenic cells by using histological staining and molecular techniques for lineage specific markers at the end of 28 days (4 weeks). General progression of osteoblastic differentiation takes 28 days so that starting duration for hMSCs' differentiation was 28 days and calcified bone matrix synthesized by osteoblastic cells were stained with TB. At molecular level mRNA expressions of bone specific markers that belong to different stages of differentiation were determined as BSP, OPN and OCN and applied for RT-qPCR. Since these markers belong to several stages of osteoblastic differentiation, expression profiles varied at that time point. BSP is an early marker of differentiation, according to the literature it is transiently expressed in cells that were still in proliferative stage. Then expression is sequentially down and upregulated during differentiation. Our results showed that its expression was lower compared with OCN as a late marker of process. In the literature it is mentioned that OPN peaked twice at day 4 and between days 14-21 during proceedings of osteogenic differentiation. So that 4 weeks' osteogenic lineage cells weren't suitable for its detection. Its expression was in lower range. Results were similar with BSP expression. This is because most probably we haven't caught the correct time points for detection of their expression time of best fit during differentiation. At this time point determination of OCN expression was best suited for following of osteogenic differentiation. Its expression was peaked at the end of 28 days compared with other markers. As a result, indicators of osteogenic differentiation of hMSCs were confirmed both with histological staining and at molecular level and findings were suitable with literature knowledge and proved that hMSCs have capable of efficient osteogenic differentiation. [94].

According to the hypothesis, topo IIβ was both silenced and overexpressed before commitment of hMSCs into osteogenic differentiation. In silencing studies siRNA were used. siRNAs are small pieces of dsRNAs, usually 20-25 base pairs in lenght, with 2 nucleotides in 3' overhangs at each end that can be used to "interfere" with the translation of proteins by binding to and promoting the degradation of mRNA at specific sequences. This enables prevention of sequence dependent protein production. The process is called as RNAi, and may also be referred to as siRNA silencing or siRNA knockdown. In our study silencing was succeed by using 4 different sequences of siRNAs specific to topo IIβ. Company gives opportunity to use 4 different validated

siRNA sequences against topo IIβ gene. By using RT-qPCR technique, optimization studies were done at 48h and process was repeated per 48h for indication of required time points of gene silencing before starting of differentiation. Optimum siRNA concentration was indicated as 10 nM concentrations for every single siRNA sequence. There were missing points of thesis involved in some requirements of siRNA experiments, which were suggested for checking and confirmation of correct relation in between obtained results and hypothesis. In our study groups were designed for commitment of hMSCs into osteogenic lineage along with topo IIβ silencing. And these conditions should be applied to all groups of experiments to analyze and make results scientifically more acceptable. So that in addition to osteogenically induced hMSCs, siTopo IIβ treatment should be applied to each groups of experiment. Missing groups of these experimental requirements were UD hMSCs+siTopo IIβ that was required for proving whether the observed effect was related to osteogenic conditions or not. And scrambled RNA (negative control siRNA) treated groups known as random siRNA sequences rearranged based on target sequence should be used in each groups of experiment at each time points to show observed effects were topo IIβ specific.

For overexpression studies, nucleofection as a type of electroporation technique was used. Before starting of differentiation, obtaining the optimum cell number was required. As a disadvantage of technique high cell mortality was observed, so that 7 days were required for starting of osteogenic differentiation post overexpression. 7 days were sufficient for hMSCs' proliferation and confluency but as a result of using nucleofection technique hMSCs got into stress conditions and being recovered took more than 7 days. In overexpression studies determination of optimum pDNA concentration that wasn't toxic to cells and supplied efficient gene expression was essential. As a fluorescent indicator of gene transfection topo IIβ gene was tagged to EGFP plasmid. hMSCs' transfection was confirmed via detection of fluoresecently labeled nucleus, that express topo IIβ-EGFP. pDNA concentration was determined as 6 μg for transfection of $5x10^5$ cells.

After that gene knockdown and overexpression experiments were combined with osteogenic differentiation. At the begining, days of sample preparation during differentiation were determined as 7 and 14, because according to the hypothesis

possible effects of topo IIβ should be observed at early terms of differentiation. We didn't have information about gene expression dynamism of osteogenic lineage suited to our hypothesis so that starting of these broad ranges of time points was suitable. Firstly osteogenic lineage progression was checked at days 7 and 14 with the experiments that were used previously for determination of osteogenesis and they were applied in these conditions too. Calcium deposites were detected with ARS in addition to TB both in days 7 and 14. Time dependent differences in amounts of stained and calcified areas in samples were clearly observed both in AR and TB stainings. Detection of osteoblastic cell morphology both in topo IIβ silenced and overexpressed hMSCs at days 7 and 14 were also checked via observation of calcified regions of bone matrix without staining under inverted microscope. Results were supportive for the hypothesis but observation of osteoblastic cells morphologically alone wasn't sufficient to mention the certain effects of topo IIβ in osteogenic differentiation. Results were analyzed with ImageJ program that measured calcified areas in images and gave rate of those areas as percentage values. ImageJ analysis indicated that silencing of topo IIβ decreased calcification of bone matrix while overexpression caused inceasing amounts in these areas compared with ost. samples with WT topo IIβ . Results were statistically analyzed and sample of day 7 in ost.+siTopo IIβ conditions showed significant decreasing compared with others.

So that differences were checked at gene expression level in these experimental groups. As an indicator of osteogenesis BSP, OPN, OCN and Runx2 gene primers were used. For BSP, OPN and OCN genes, expression levels were dramatically decreased in hMSCs ost.+siTopo IIβ samples both in days 7 and 14 compared with hMSCs ost. groups. But expression profiles of related genes didn't show much more differences between ost.+siTopo IIβ and ost.+oeTopo IIβ groups at days 7 and 14. Only Runx2 cause observation of highly different result in overexpressed sample of day 7. Experimental progression, which was started with the osteogenic differentiation in addition to the gene silencing and overexpression of topo IIβ, was only proceeded with topo IIβ silencing at remaining parts of thesis due to observation of more reliable results in topo IIβ silenced groups. As a result of these observations, short-term RNA samples of defined experimental groups were decided to use in further RT^2 PCR array experiments of osteogenesis and OP. Early terms of differentiation might be important

for activity of topo IIβ in progression of osteblastic differentiation. So that RNA samples of days 1, 3, 5, 7 were prepared for ost. and ost.+siTopo IIβ samples. First of all silencing of topo IIβ at days 1, 3, 5 and 7 were checked via RT-qPCR, statistically analyzed and successful gene knockdown was confirmed. Then calcification of each days' experimental groups were checked via ARS. Results were compatible with the hypothesis. Silencing of topo IIβ caused inhibition of osteoblastic cell commitment and formation of matrix calcification or ossification during following days of osteoblastic progression.

As a part of developmental stages bone tissue is originated from mesoderm layer. Newborn bone tissue is composed of cartilage and mesodermal tissue, gradually continue with ossification to form mineralized strength bones. These stages are controlled at gene expression level. During lifespan homeostasis and calcium metabolism is controlled by bone remodeling process. Bone is metabolically active tissue with the balance of bone production and bone disruption. This balance is related to functions of osteoblasts and osteoclasts. Dysfunctions in osteoblast and osteoclasts result in disease and injuries that cause loss of bone tissue such as OP. Analyzing the expression and regulation mechanism of osteoblastogenesis and OP can be helpful for developing of cell-based therapies.

This study investigated the gene expression profiles of hMSCs conditioned with osteogenic mixture in naturally presence and silenced conditions of topo IIβ for 7 days to analyze whether osteogenic commitment was influenced by topo IIβ. Differential expression of 84 osteogenesis and OP related genes were scanned in hMSCs in indicated conditions at days 1, 3, 5 and 7 by using RT^2 PCR array technology. During progression of thesis several experimental conditions and groups were used and at the last part of thesis three different experimental conditions were used as: NT-UD hMSCs (Group I), hMSCs in osteogenic conditions alone that express topo IIβ naturally (Group II) and hMSCs in osteogenic conditions +siTopo IIβ (Group III).

Before analyzing the whole array results, some group of genes related to skeletal development and osteoblast differentiation (ALPL, CTSK, Runx2, SPP1); bone calcium metabolism (VDR); ECM genes of bone tissue (COL1A1, COL1A2); and genes that act as regulators of osteoblastogenesis (FGFR1, TWIST1, TGF-β1, VEGFA) were selected

for analysis. For each gene, experimental groups were done in replicate and analyzed statistically. Genes that are related to osteoblastic differentiation and skeletal development showed expected expression profile. Results were statistically analyzed by comparing with NT-UD hMSCs (Group I) and ost. (Group II) versus ost+siTopo IIβ (Group III) of each day. Expression levels of ALPL gene in Group II samples were increased during following days of osteogenesis. Especially its expression was peaked at day 5 and significant compared with Group I. When each days of Group II and Group III were compared its expression was dramatically and significantly (at days 3, 5 and 7) decreased in samples of Group III as expected.

Runx2 as an important gene for osteoblastic differentiation is an indicator of early stages of osteoblastic differentiation. Effects of topo IIβ silencing, in its expression was similar to expression results of ALPL gene. Decreasing through days of differentiation in Group III compared with Group II and results were significant in comparison of each day. Significant differences with Group I was only observed at day 1's samples of group II and III. As a result increasing expression levels of both ALPL and Runx2 genes involved in positive regulators of osteoblastogenesis were observed in differentiating hMSCs while silencing of topo IIβ caused decreasing of these genes' expression levels. Results were compatible with the hyphothesis as expected.

Also negative regulator genes of osteogenesis were checked. CTSK gene is a product of a proteinase family involved in bone remodeling and resorption and expressed in osteoclasts. Expression level of CTSK gene was increased in Group III versus Group II in each day. Significant differences between Group II and Group III were observed both at day 1 and 7. SPP1 gene product is involved in the attachment of osteoclasts to the mineralized bone matrix and has functions in degradation of ECM. Its expression was decreased in all samples of Group II compared with Group I without any significance at days 1 and 3 while effects were significant at days 5 and 7. Significant decreasing in Group II compared with Group III was observed at days 3, 5 and 7. As a result silencing of topo IIβ caused increasing of CTSK and SPP1 gene expressions in differentiating hMSCs compared with WT topo IIβ samples. Results were compatible with the hyphothesis as expected.

All results of these genes that have different roles in osteogenesis were meaningful for the hypothesis.

COL family members that are important components of bone tissue ECM have significant expression levels between groups. Amount of synthesized COL proteins as a product of related genes' expressions was increased during days of osteoblastic progression and peaked at day 5 in samples of Group II. As expected opposite results were observed in Group III. Similar to positive regulator genes' expression levels both COL1A1 and COL1A2 gene expressions were decreased in response to silencing of topo IIβ. In COL1A1 all results were significant compared with Group I. Group II versus Group III were significant at days 3, 5 and 7 in both COL1A1 and COL1A2.

Findings were compatible with a study that analyzed expression profiles of osteoblast related genes via RT-qPCR, in hMSCs, committed to osteogenic differentiation for 28 days. RNA samples were collected at days 7, 14, 21, 28 and expression patterns of OCN, OPN, Runx2, ALP and COL1 genes were detected. OCN expression significantly started to increase at day 21 after induction and continued on day 28, while increasing at day 7 and 14 was observed without any significance. OPN and Runx2 were significantly expressed after day 14 and continued on day 21 and 28. No difference was observed between these genes' expression on day 7. Expression pattern of COLl and ALP was similar, peaked at day 21 by increasing level and started to decrease on day 28. OCN is synthesized by mature osteoblasts and related to mineralization so its expression refers the late stages of differentiation. OPN synthesis shows different stages of differentiation and synthesized by osteoblasts. Runx2 is an early marker of differentiation and required for directing the cells through osteoblastic lineage. As a transcription factor, it interacts with the osteoblast lineage specific genes. Although being an early marker of osteogenic differentiation, its expression was still observed at day 28 in that study [95]. In an *in vivo* study, effects of Runx2 also known as core binding factor α1 (Cbfa1) overexpression in osteoblasts were analyzed. Cortical bone contains immature bone with lower number of osteocytes resulted in less dense bone tissue. Mainly Runx2 is responsible for driving of precursor cells to osteoblastic lineage and effective on bone formation and remodeling by regulating the specific genes' expression. To understand the exact function of Runx2 in late stages of bone formation,

mice bone tissue was checked for some parameters at 3th and 6th week after birth in that study. Cortical bone of Runx2 overexpressed transgenic mice was covered by osteoclasts that are originated from hemopoietic monocytic cells of the bone site, cause removing of mineralized matrix and formation of osteopenia with multiple fractures. Results showed that Runx2 inhibits osteoblast differentiation at late stages of osteoblastogenesis and essential for early stages of differentiation [96].

VDR is required for calcium ion binding metabolism and homeostasis. As supported with previous results, topo IIβ silencing was negative effector for activity of this gene. Genes that involve in growth factor, receptor and transcription factor families were also analyzed in same samples. FGFR1 gene is negative regulator of osteoblast differentiation and its expression was increased in topo IIβ silenced samples in osteogenic conditions. Results were significant compared with Group I except Group II at day 1 and Group III at day 3. TGFβ-1 regulates osteogenic differentiation by both inducing formation and resorption of bone. In our experiment expression level was significant from Group I in Group III samples at days 3, 5 and 7 and in Group II at day 7 with increasing levels. For TWIST1 gene significant expression change was observed at day 5 between Group II and Group III. VEGFA expressions were parallel to these results. Taken together genes that were analyzed in that part showed compatible expression profiles with each other and they were supportive for our results. Although they have several roles inside the cell by interacting with lots of molecules, it isn't clear whether they act specifically in osteogenesis.

PCR array results were expressed as fold regulation rates between compared groups with two-fold up- and down-regulation. Each group was compared with both NT-UD hMSCs and between w/wo siTopo IIβ samples of days in osteogenic conditions. Fold changes lower than 0,5 was referred as downregulated and indicated with '-' value, while higher from 2 referred up-regulation and indicated as '+' value of fold regulations.

In osteogenesis PCR array, osteogenic medium conditioned hMSCs that naturally express topo IIβ indicated as Group II was compared with topo IIβ silenced and osteogenically induced hMSCs named as Group III samples of each day. According to the results genes that are involved in progression of osteoblastogenesis such as: skeletal

development (ALPL); ossification and osteoblast differentiation (NOG, Runx2); calcium ion binding metabolism and homeostasis (VDR); cell-ECM adhesion (CD36, COMP); cell-cell adhesion (CDH11); collagens as important ECM proteins of bone matrix (COL1A1, COL1A2, COL5A1, COL10A1) and a growth factor (PDGFA) were upregulated in Group II while downregulated in Group III. Also upregulation of some genes that associate with more than one specific event of the osteogenic differentiation progression was observed. TGF-β1 and SMAD3 genes that interact with each other have several roles in osteoblastogenesis such as in ossification, osteoblastic differentiation, bone mineralization, calcium ion binding metabolism, cell-cell and cell-ECM adhesion and they were upregulated in Group II compared with Group III. Downregulation of genes that are negative regulators of osteoblastogenesis was observed at Group II compared with Group III. BMP3/6 gene products are suppressor of osteoblastic differentiation and negatively regulate bone density by affecting TGF-β receptor accessibility and downregulated in Group II. During differentiation of hMSCs, osteogenesis/chondrogenesis transition is important and as a major regulator of chondrogenesis and functions in cartilage condensation Sox9 was downregulated in Group II [97]. As a chondrocyte specific enhancer element, COL2A1 was also downregulated in that sample again [98]. SPP1 gene that has role in bone remodeling and attachment of osteoclasts to the mineralized bone matrix for degradation of bone tissue by mediating osteoclast recruitment and bone resorption was downregulated in Group II again compared with Group III [99]. All of these results were supportive for our hypothesis.

OP PCR array results were also analyzed with the same way. Genes that are involved in a particular event in osteoporotic pathway were grouped according to the up and dowregulation of genes at Group II versus Group III. Genes of calciotropic hormones and receptors such as CYP19A1, PTH1R and ESR1 that positively regulate osteoblastogenesis while having decreasing effect on osteoclastogenesis that affect bone remodeling process; Runx2 and NOG genes related to osteoblast differentiation and ossification; VDR gene that acts in calcium ion binding and homeostasis; ALPL gene as one of the major skeletal development gene; COL1A2 as a main ECM protein of bone tissue were all upregulated in Group II versus Group III. Also leptin gene that is secreted by white adipose tissue and controls body weight via negative feedback

mechanism enhances osteoblastic differentiation of bone marrow derived progenitors and inhibits late adipocyte differentiation [100]. Expression profile of this gene was also increased at Group II compared with Group III again. Downregulation of genes at Group II were observed in negative regulators of bone remodeling and osteclastogenesis. SPP1, ACP5, PTHLH, ALOX5, SHBG, IL6 genes' expressions showed decreasing effects in Group II.

As a specific and remarkable time point both osteogenesis and OP PCR array profile results showed differential and significant expression rates at day 5 between Group II versus Group III. Most remarkable result was observed in OP array that showed higher expression rate of bone remodeling genes at Group II. Also genes related to osteoblastic differentiation and Wnt signaling were upregulated in that samples.

Findings were compatible with a study that analyzed differential expression levels of genes related to osteoblastogenesis, osteoclastogenesis, cell proliferation and DNA repair in hMSCs of elderly patients (79-94) suffer from OP via microarray. In addition to OP cases elderly healthy people's (79-89) hMSCs were also analyzed for comparisons to check maintanence of hMSCs in OP cases. In addition to these groups hMSCs in senescence were used for checking whether hMSC-OP get into senescence. In hMSC-OP genes related to osteoblastogenesis such as IBSP, VEGFA, VEGFB, IGF2, PTH1R were upregulated. Genes that are enhancers of osteogenic differentiation and matrix minealization (Runx2, ALPL, COL1A1) were downregulated in hMSC-senescent and hMSCs-old. Sclerostin (SOST) gene that cause inhibition of Wnt pathway were also upregulated in hMSC-OP compared with hMSC-old. Genes that are susceptible for OP (LRP5, SPP1, COL1A1, SOST) were analyzed and either reduced or increased expression levels were found in hMSC-OP samples. MAB21L2 gene that is an antagonist of BMP signaling was highly expressed in hMSC-OP samples and might be a new candidate gene for OP. In addition to enhanced expression of osteoclastogenesis related genes (CSF1) in osteoporotic stem cells OP induced increasing in PTH1R gene that inactivates RANKL expression that leads to osteoclast precursor commitment. This study targeted the new therapeutic approaches as inhibition of inhibitor genes that support design of this thesis [101].

Taken together, it has been shown that topo IIβ expression is important for early terms of osteogenic differentiation commitment. This study is unique for literature as investigate the possible role of topo IIβ in different type of differentiation process in hMSCs aside from neural differentiation for the first time. For the further studies relation in between other types of mesodermal or transdifferentiation of hMSCs should be checked to mention and prove the general role of topo IIβ in differentiation of stem cells.

5. CONCLUSION AND RECOMMENDATIONS

In the literature studies about function of topo IIβ is focused on neural differentiation of stem cells. In this study it has been shown that topo IIβ have roles in commitment of hMSCs into osteogenic lineage and proceedings of osteo-pathological conditions. There aren't much more study about the specific action of topo IIβ in stem cell differentiation process except neural differentiation and development. Since topo IIβ have roles in DNA metabolism, it should have more general activities in regulation of genes and differentiation processes. Because stem cell fate is directly proportional with gene regulation that are up or down-regulated during process. This idea was the starting point of our study.

This study serves useful and successful gene delivery methods to hMSCs for silencing and overexpression of interested genes. For clinical applications, combining of gene manipulation studies with conditions that are required for efficient, easy and reproducible methods of differentiation process will be helpful for gene therapy based treatments. Also genomics based studies that involved last part of the study is helpful to clarify the molecular mechanism of interested pathway or disease.

By using PCR array, checking the regulation of genes related to osteogenesis as being a specific cellular event and osteoporosis as being interested disease pathway was big opportunity to scan broad range of gene scale. From analysis of PCR arrays, in osteoporosis pathway especially genes related to bone remodeling were strongly and positively affected by topo IIβ. This might be useful for the studies related to this disease condition's therapy. In addition to this, genes of disease specific pathways, such as Wnt/ β-cathenin was positively regulated. According to our observations these effects were time dependent at day 5 that refers the commitment and expansion of hMSCs into osteogenic lineage. This finding is supportive for study's hypothesis. Study will be helpful for further studies interested in treatment of patients' suffer from osteo-pathological diseases.

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