ROLE OF ENVIRONMENTAL FACTORS IN MESENCHYMAL STEM CELL BIOLOGY

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

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ABSTRACT

ROLE OF ENVIRONMENTAL FACTORS IN MESENCHYMAL STEM CELL BIOLOGY

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M.S. in Molecular Biology and Genetics

Supervisor: Associate. Prof. Dr. K. Can Akçalı

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Mesenchymal Stem Cells (MSCs) have the abilities of self-renewal and differentiation into fat, bone, cartilage, and muscle tissues. Besides intrinsic mechanisms that control the fate of the MSCs, extrinsic physiological factors also play role in this decision. Therefore, our aim is to explore the effects of possible environmental factors, involved in MSC maintenance by using rat MSCs as a model. We studied the effects of hypoxia and estrogen on growth regulation and cellular proliferation in MSCs. MSCs cells exhibited high colony number in hypoxic conditions and the expansion of MSCs was increased addition of the estrogen. In addition, estrogen prevents apoptosis, under hypoxic condition. The effects of estrogen on the expression levels of Notch genes (Notch1, Notch2, Notch3 and Notch4) were also investigated. In order to understand the possible mechanism of estrogen response, an experimental and *in silico* approach are used. The expression levels of Notch1 and Notch 3 were decreased treatment and the expression level of Notch 4 was increased upon estrogen treatment. In addition, bioinformatics analysis showed that, estrogen upregulates ERG family transcription factors, ELF family transcription factors, HOXL4 family transcription factors, KLF family transcription factors and transcription factor SOX3, which bind to Notch 1 transcriptional regulatory region, implying indirect effects of estrogen on Notch 1 expression. Twenty biomaterials were also investigated in order to assess whether they provide an appropriate environment for MSCs expansion. It was found that eight of the biomaterials out of twenty designated as, CA-1, CA-2, CA-3, CI-K, CI-A, CIII-1, CIII-2 and CIII-3, were appropriate candidates to expand MSCs. The combination polymers designated as HPMA/PEG provided appropriate conditions when prepared in the proportion of 1:0 (CA-1), 1:1 (CA-2) and 2:1 (CA-3). The appropriate proportion of polymers designated as HEMA/PEG/HPC was 2:1:1(CIII-1), 3:0:1 (CIII-2) and 1:1:0 (CIII-5).

Keywords: Mesenchymal Stem Cells, hypoxia, estrogen, notch receptors and biomaterials.

ÖZET

ÇEVRESEL FAKTÖRLERİN MEZENKİMAL KÖK HÜCRE BİYOLOJİSİ ÜZERİNDEKİ ROLÜ

Sinan Gültekin

Moleküler Biyoloji ve Genetik Yüksek Lisans

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Mezenkimal Kök Hücreler (MKH) hem kendilerini yenileyebilmekte hem de yağ, kemik, kıkırdak ve kas hücrelerine farklılaşabilmektedirler. MKH'ların yenilenebilme ve farklılaşabilme özellikleri, içsel mekanizmaların yanısıra, fizyolojik dış faktörler tarafından da kontrol edilir. Buradan hareketle sıçan MKH modelinde, çevre faktörlerinin, MKH devamlılığındaki olası etkilerini belirlemek amaçlanmıştır.Farklılaşma, büyüme ve çoğalma kapasitesi üzerindeki etkisi göz önüne alınarak, hipoksi ve östrojenin, MKH'ler üzerindeki roller araştırıldı. MKH'lerin koloni oluşturma özelliklerinin hipoksik ortamda artığı ve bu artışın östrojenin eklenmesiyle daha da fazlalaştığı gözlemlendi. Buna ek olarak, östrojenin hipoksik ortamda, MKH'lerin apoptoza girmelerini engellediği gösterildi. Östrojenin Notch reseptör ifadesindeki etkisi hem deneysel, hem de bioinformatik yöntemle araştırıldı. Deneysel olarak Notch1 ve Notch3 ekspresyonunun östrojenle birlikte azaldığı, ancak Notch4 ifadesinin arttığı gözlendi. Bununla birlikte, bioinformatik yöntemle östrojenin, ERG, ELF, HOXL4 transkripsiyon faktör aile gruplarının ve SOX3 transkripsiyon faktörü ifadesini arttırdığı, bu transkripsiyon faktörlerinin Notch1'in transkripsiyonu regüle eden bölgelerine bağlandığı gösterildi. MKH'lerin büyümelerine uygun ortam oluşturmak için önceden hazırlanmış 20 tane biyomateryal kullanıldı, bunlardan sadece sekiz tanesi (CA-1,CA-2,CA-3, CI-A,CI-K,CIII-I,CIII-II ve CIII-2) MKH'lerin büyümesi ve gelişmesi için iyi bir ortam oluşturduğu saptanmıştır.

Anahtar Kelimeler: Mezenkimal kök hücreler, hipoksia, östrojen, notch reseptör ailesi ve biomalzeme.

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ABBREVIATIONS

AIBN	A-A-Azoisobisbutyronitrile
ASC	Adult Stem Cells
bFGF	Basic Fibroblast Growth Factor
bHLH	Basic Helix Loop Helix
BM	Bone Marrow
bp	Base Pair
BSA	Bovine Serum Albumin
cDNA	Complementary Deoxyribonucleic Acid
$CoCl_2$	Cobalt Chloride
CSL	[Named After CBF1, Su(H) and LAG-1]
ddH_2O	Double distilled water
DEPC	Diethylpyrocarbonate
dH_2O	Distilled Water
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
EB	Embryoid Bodies
ECM	Extra Cellular Matrix
EGF	The Epidermal Growth Factor
ERE	Estrogen Response Element
ESC	Embryonic Stem Cells

FBS	Fatal Bovine Serum
FIH-1	The Protein Factor Inhibiting HIF-1α
GSC	Germ-line Stem Cells
HEMA	Hydroxyethylmethacrylate
HIF	Hypoxia-Inducible Factor
HPC	Hydroxypropylchitosan
HPMA	Hydroxypropylmethacrylate
HSCs	Hematopoietic Stem Cells
ICM	Inner Cell Mass
IDO	Indoleamine 2,3- Dioxygenase
IGF	The Insulin-Like Growth Factors
iPS	Induced Pluripotent Stem Cells
LIF	Leukemia Inhibitory Factor
M	Molar
MEF	Mouse Embryonic Fibroblast
MeOH	Methyl Alcohol
mM	milliMolar
mmHg	milli meter mercury
MOPS	3-(N-morpholino)propanesulfonic acid
MSC	Mesenchymal Stem Cells
Nicd	Notch intracellular domain
OD	Optical Density
ODDD	Oxygen Dependent Degragation Domain

PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factor
PEG	Polyethylene Glycol
PFA	Paraformaldehyde
PGC	Primordial Germ Cell
PHD	Prolyl Hydroxylase Domain
pO_2	Oxygen Partial Pressure
RNA	Ribonucleic acid
RT	Room Temperature
RT-PCR	Reverse-Transcriptase Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
TA	Transit Amplifying
TGF-b	Transforming Growth Factor-beta
VEGF	Vascular Endothelial Growth Factor
VHL	Von Hippel-Lindau
µg	Microgram
µl	Microliter

1. INTRODUCTION

In the last century, the lifespan of humans has increased due to better life conditions. Advanced technology led to progressive research results for improved clinical applications and treatments. This longer life phenomenon has brought together the concept of a better life quality, since the body functions and health conditions decline with time. The recent research areas mainly focus on different intrinsic and extrinsic factors, which might help in the treatments of diseases and disorders which are results of a longer life time; such as cancer, osteoporosis, stroke and heart attack, in addition to neurodegenerative diseases like Parkinson's disease.

Stem cells, which are an evolving research topic, have the ability for prolonged self-renewal and differentiation into mature cells of various lineages, which make them important cell sources for therapeutic applications. Their remarkable ability to replenish and differentiate *in vivo* is regulated not only by intrinsic mechanisms but also by extrinsic physiological factors. These environmental factors are important for adult stem cell maintenance. We plan to examine the effects of hypoxia and estrogen during this period.

Hypoxia is one of the important environmental factors that plays role in the stem cell behavior. The niche structures where stem cells reside are quite hypoxic, and differentiation of stem cells is inhibited in hypoxic condition, while their "stemness" feature is maintained (Poellinger and Lendahl 2008)**.** It has been shown in other cells that one of the major pathways that participate in hypoxic response is notch pathway. Notch pathway also leads to differentiation of one type of stem or progenitor cells into their specific lineages, while prevent the differentiation of other stem/progenitor cells and keep them in a self renewal state. Estrogen is another important physiological factor and plays key role in development and maintenance of normal sexual and reproductive functions. It has also effect on the proliferation and migration of different stem cells. Use of biomaterials during the maintenance of cells is drawing interest in recent years since because they mimic the extra cellular matrix (ECM) component (Tabata Y. 2009). Identification of new biomaterials has critical importance especially in stem cells since this recapitulates *in vivo* niche structures and allows manipulations *in vitro*.

To elucidate the effects of hypoxia and estrogen, mesenchymal stem cells (MSCs) were examined at hypoxic condition in the presence of estrogen. Then, we investigated the effect of estrogen on notch expression in order to understand a possible mechanism. We used

both experimental and *in silico* approaches to shed light to its mechanism. Finally, use of appropriate biomaterials for MSCs' expansion to mimic their niche structure was discussed.

1.1 Stem Cells

Stem cells are unspecialized cells which are capable of self-renewal and give rise to differentiated cells (Till and McCulloch. 1961). They are found in various sources such as the embryo, bone marrow, blood, cornea and retina of the eye, brain, skeletal muscle, dental pulp, liver, skin, lining of the gastrointestinal tract, and pancreas. Although self-renewal and differentiation to other cells are their common features; stem cells vary in their potential to differentiate, duration and pathways of self-renewal, places they are mostly found in, and division properties (Morrison *et al*. 1997).

Stem cells can be classified due to their source of origin and their potential to differentiate to different cell types. There are three main types of stem cells: Embryonic Stem Cells (ESCs), Germ Stem Cells (GSCs), and Adult Stem Cells (ASCs). In addition, a new type of stem cell- Induced Pluripotent Stem Cells (iPSs) were identified in recent years generated by reprogramming of mouse embryonic fibroblast cells by inducing four defined factors (Takashashi andYamanka. 2006).

1.1.1 Embryonic Stem Cells

ESCs were first isolated from the inner cell mass of the mouse in 1981 by Kaufman and Martin, and from humans in 1998 by Thomson *et al*. ESCs are not embryos themselves but they can form cells from all three germ-layers and undergo an unlimited number of symmetrical divisions without differentiating *in vitro* (Burdon *et al*. 2002). In order to generate cultures of mouse and human ESCs, the inner cell mass is removed from the trophectoderm and is transferred to the top of mouse embryonic fibroblast (MEFs) cells on the culture medium. These MEFs which are called feeder layer are inactivated so that they cannot divide, but they provide a sticky surface for the ESCs to attach besides releasing essential factors to the culture medium. Both mouse ESCs and human ESCs can be grown both with and without a feeder layer (Xu *et al*. 2001). A single ESC can give rise to a colony of genetically identical cells, or clones under appropriate conditions (Sell. 2003)

ESCs can stay undifferentiated for a long period of time *in vitro,* and there are several examples to prove the pluripotentiality of ESCs. When mouse ESCs derived from one blastocyst are injected to another blastocyst and is transferred to the uterus of a pseudopregnant mouse *in vivo*, chimeras form which are a mixture of tissues and organs derived both from the host and the donor blastocyst (Prelle *et al*. 1999). Also when ESCs are

injected into adult immune-deficient mice, they develop into tumors called teratomas which contain cells from all three germ layers (Martin. 1981). *In vitro* when the culture conditions are changed, ESCs form embryoid bodies (EB) which have a similar structure to teratomas. They form large structures which contain partially differentiated cells from all three germ layers in a disorganized manner (Evans and Kaufman. 1981). Both mouse and human ESCs express *oct-4* which is an important gene in the maintenance of pluripotency.

ESCs have high telomerase activity, which adds telomere repeats to the ends of chromosomes resulting in long telomeres (Armstrong *et a.*, 2000) They have stable karyotypes, and X inactivation does not occur in the undifferentiated ESCs (Xu *et al*. 2001). Unlike differentiated cells, ESCs do not require any external stimulus in order to initiate DNA replication. ESCs lack the G1 checkpoint in the cell cycle and spend most of their time in the S phase of the cell cycle synthesizing DNA. When they start to differentiate, the G1 phase of the cell cycle becomes longer with the increase in cyclin D expression and the rate of cell division slows (Burdon *et al*. 2002).

ESCs can be used in several ways in basic and clinical research. Studies on ESCs are very informative on the complex events that occur during human development. Understanding the genetic and molecular control mechanisms behind the stem cell regulation may provide an understanding on how genetic and growth abnormalities arise and suggest new strategies and methods for their therapy.

In addition to all the positive aspects of ESCs, there are several handicaps in their research and applications. Since there are many different cellular pathways regulating them, it is hard to figure out the interactions between them. ESCs cannot give rise to a full embryo but the fact that they are isolated from embryos creates problems, therefore there are ethical problems arising. In addition, when injected to immunodeficient mice, they form tumors. This indicates that due to their high differentiation potential, they can form any type of cell so they have to be controlled very strictly and all the factors affecting the differentiation of ESCs should be figured out before using them in any clinical application. Also ESCs have a potential to cause immune rejection, which is another obstacle. Until the big gaps are filled in the area of stem cell regulation and differentiation besides the ethical issues being solved, ESCs cannot be used in therapeutic applications.

1.1.2 Embryonic Germ Cells

EGCs were first identified in 1998 by John Gearheart and they were cultured from primordial germ cells (PGCs) obtained from the gonadal ridge and mesentery of the 5th to 9th week of fetal tissue. They are pluripotent cells and express *oct-4* besides other pluripotency markers similar to ESCs, and they have the capacity for long-term self-renewal. In addition they have a normal stable karyotype. The location and maintenance of germ-line stem cells are among the most studied areas and are clearly identified. Especially *Drosophila* is the best studied model in this area both in male and in female (Gilboa and Lehmann. 2004).

PGCs are diploid germ cell precursors which transiently exist in the embryo before they are committed as germ cells. In order to obtain EGCs, PGC cultures are grown in fetal bovine serum supplemented media. The PGCs are plated on a feeder layer consisting of STO fibroblasts which are non-dividing. They are cultured in a growth medium which includes the cytokine, leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF), and forskolin. At the end of three weeks, the PGCs form dense, multilayered colonies of cells resembling EGCs. Although both are pluripotent, cultures derived from embryoid bodies generated from human EGCs have less capacity for proliferation compared to ESCs. EGCs will proliferate for 40 population doublings while human ESCs can proliferate for two years through 300 population doublings or even 450 population doublings (Pedersen. 1999). Another major difference between the pluripotency of ESC and EGC is that EGC do not form teratomas when injected to immunodeficient mouse (Pedersen. 1999).

Although both are pluripotent, EGCs do not have the same proliferation and differentiation capacity as ESCs do, which makes EGCs less appealing for therapeutic applications. Also they do not form teratomas when injected to immunosuppressed mouse, and they do not participate in the formation of chimeras *in vivo;* which suggest that EGC's differentiation potential is not as wide as ESC's. In addition there is not much known about their telomerase activity either, which might be a reason why their doublings are shorter than ESCs (http://stemcells.nih.gov/).

1.1.3 Induced Pluripotent Stem Cells

iPS cells are pluripotent cells that are derived from adult stem cells using reprogramming. In 2006, a group of Japanese scientists made pluripotent stem cells by introducing of Oct4, Sox2, Klf4, and c-Myc transcription factors into murine somatic cells (Takahashi and Yamanaka 2006). The first generation iPS cells were similar to ES cells in morphology, proliferation, the expression of some ES cell marker genes, and the formation of teratomas. However, these iPS cells had a different global gene expression pattern from ES

cells and failed to produce adult chimeric mice. In 2007, germline transmission was achieved with mouse iPS cells (Yamanaka 2007), and iPS cells were generated from human fibroblasts (Yamanaka 2009). Then four groups generated iPS cells from patients with various neurodegenerative diseases—amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA) and Parkinson's disease and a variety of genetic diseases with either Mendelian or complex inheritance (Feng et al. 2009). In addition, iPS cells have been generated from both monkey and rat (Yamanaka 2009).

In addition, Thomson and colleagues were also able to reprogram human fibroblasts with a distinct set of transcription factors comprising OCT4, SOX2, NANOG, and LIN28 (OSNL), indicating that KLF4 and c-MYC could be substituted with NANOG and LIN28 (Feng et al. 2009). It was found that the orphan nuclear receptor, Esrrb, can replace Klf4 in the reprogramming of mouse embryonic fibroblasts (MEFs) when cotransduced with either OSM or OS (Feng et al. 2009). Besides transcription factors and epigenetic modifiers, micro-RNAs may play an equally important role in reprogramming, commensurate with their emerging role in the maintenance of ESCs. Only one particular microRNA, mir-302, which is expressed abundantly in human ESCs, has been implicated in reprogramming (Feng et al. 2009). In addition, several chemicals have recently been reported to either enhance reprogramming efficiencies or substitute for specific reprogramming factors. Among the reported chemicals, some are known to affect chromatin modifications, such as DNA methyltransferase inhibitor and histone deactylase inhibitors (Feng et al. 2009)

iPS cell research does not raise any ethical problems since blastocysts or embryos are not destroyed. Therefore, it has opened the possibility of autologous regenerative medicine whereby patient-specific pluripotent cells could be derived from adult somatic cells (Yamanaka 2009) However, several limitations of most existing iPSCs prohibit their usage in the clinical setting. First, virus-mediated delivery of reprogramming factors introduces unacceptable risks of permanent transgene integration into the genome. The resulting genomic alteration and possible reactivation of viral transgenes pose serious clinical concerns. Second, reprogramming factors Klf4 and c-Myc are oncogenic. Third, iPSC reprogramming is an inefficient and slow process. During the course of reprogramming, a substantial reduction in efficiency may also result from incomplete reprogramming (Feng et al. 2009).

1.1.4 Adult Stem Cells

In the pioneering experiments with ASCs in 1940-1950's, the researchers were able to repopulate the blood cells of a mouse by the infusion of bone marrow cells from a different mouse. In 1960; two different kinds of stem cell population have been identified in the bone marrow; hematopoietic stem cells (HSCs) which form all types of blood cells and bone marrow stromal cells which generate bone, cartilage, fat, and fibrous connective tissue. In the 1990's neural stem cells were discovered which are able to generate the brain's three major cell types: astrocytes, oligodendrocytes, and neurons.

ASCs are found in tissues and organs, and their primary role is to maintain homeostasis. They are important for the organ or tissue to fulfill their required task besides supplying them with the sufficient amount of stem cells for a life-time (Serakinci and Keith, 2006). ASCs are mostly found in the bone marrow, blood, cornea and retina of the eye, brain, skeletal muscle, dental pulp, liver, skin, the lining of the gastrointestinal tract, and pancreas (Figure1.1). They usually divide to generate progenitor or precursor cells, which then give rise to specialized cells with specific shapes and functions (Weissman 2000).

The number of ASCs is rare compared to other types of cells and they do not divide very often unless there is a stimulus such as tissue injuries or diseases. ASCs are scattered throughout the tissues of the mature organism and have very different roles depending on their local environment. Currently, cell surface markers and observations on stem cell differentiation patterns in test tubes and culture dishes are being used for the characterization of adult stem cells. It is also hard to expand ASCs in culture in an undifferentiated state. Many factors including growth factors, extracellular matrix contacts, cell-cell interactions, and intrinsic cell kinetics (Sherley 2002) effect their maintenance and differentiation.

Most of the ASCs are stored in specific cell compartments called niches, which are the microenvironments where ASCs are located with the same type of stem cells and differentiated cell types. These niches provide an extracellular matrix, adherens junctions, and integrins besides allowing physical interactions between cells. This enables stem cells to stay in an undifferentiated state and give rise to differentiated cell types when necessary. Niches are crucial by means of affecting the properties of stem cells but are not irreversible. They are also effective in the determination of symmetric division (Fuchs *et al*. 2004). In addition to differentiating into cells which they are programmed to, ASCs may also form the specialized cell types of other germlayers, which is known as plasticity (Horwitz 2003). If the mechanisms beneath adult stem cell plasticity can be identified and controlled, existing stem

cells from different sources might be used in the repair of diseased tissues and cells of other origins.

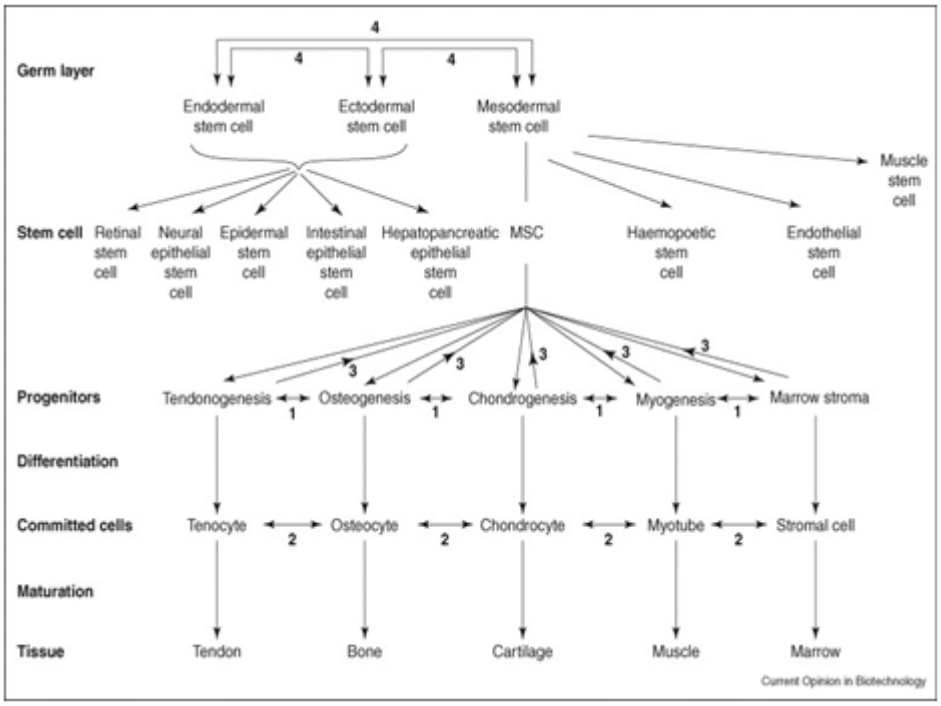


Figure 1. 1: Adult Stem Cells (Raghunath J. et.al.2005)

The most commonly studied ASCs are HSCs, MSCs, neural stem cells, epithelial stem cells, and skin stem cells. HSCs give rise to red blood cells, B lymphocytes, T lymphocytes, natural killer cells, neutrophils, basophils, eosinophils, monocytes, macrophages, and platelets. MSCs give rise to osteocytes, chondrocytes, adipocytes, and other kinds of connective tissue cells such as those in tendons. Neural stem cells in the brain give rise to three major cell types: nerve cells (neurons) and two categories of non-neuronal cells: astrocytes and oligodendrocytes. Epithelial stem cells in the lining of the digestive tract give rise to absorptive cells, goblet cells, paneth cells, and enteroendocrine cells. Skin stem cells are found in the basal layer of the epidermis and at the base of hair follicles. They give rise to keratinocytes, hair follicle, and epidermis (http://stemcells.nih.gov/).

ASCs can be used in the generation of cells and tissues in cell-based therapies**.** The organs and tissues donated are often used to replace malfunctioning or destroyed tissues, but the need for transplantable tissues and organs is a lot more compared to the available supply. ASCs offer the possibility of a renewable source for replacement of cells and tissues to treat several diseases. ASCs are potential future treatments for various diseases such as cardiac disorders, diabetes, Alzheimer disease and many others (http://stemcells.nih.gov/).

There are still many unknowns and questions to be answered such as how they stay undifferentiated in a differentiated environment, the signals regulating their differentiation and proliferation, the stimuli leading them to sites of injury, and how they can be increased in number in order to reach an adequate number to heal injuries. In addition, due to the large set of unknowns in this area, it is hard to identify, isolate, and maintain ASCs (Badge 2001). Also there are big gaps in the detection of the differentiation factors affecting their specialization, which prevent the *in vitro* tissue and organ synthesis. Mainly, the signals affecting ASC self-renewal and differentiation should be revealed in order to consider ASCs for possible treatments.

1.1.5 Mesenchymal Stem Cells

Adult MSCs were first discovered by Friedenstein et al., which were described as bone marrow-derived clonogenic, plastic-adherent cells capable of differentiating into osteoblasts, adipocytes, and chondrocytes. (Friedenstein *et. al.* 1966). After that, different populations or subsets of bone marrow stromal cells were obtained. These other cells are referred to as bone marrow stromal stem cells (BMSSC) (Gronthos *et. al.* 2003), mesenchymal stem cells/marrow stromal cells (MSC) (Caplan 1994) marrow-isolated adult multipotent inducible cells (MIAMI) (D'Ippolito *et. al.* 2004), multipotent adult progenitor cells (MAPC), and mesenchymal adult stem cells (MASCS) (Belema-Bedada *et. al.* 2008).

MSCs, in general, display significant heterogeneity. So far, there are no accepted specific surface markers for the isolation of MSCs. Instead, MSCs are defined retrospectively by a constellation of characteristics *in vitro*, including a combination of phenotypic markers and multipotent differentiation and functional properties (Liu *et. al.* 2009).

The minimal requirement for a population of cells to qualify as MSCs is to meet the three criteria, including the plastic adherence of the isolated cells in culture, the expression of CD105, CD73, and CD90 in greater than 95% of the culture, and their lack of expression of markers including CD34, CD45, CD14 or CD11b, CD79α or CD19 and HLA-DR in greater than 95% of the culture, and the differentiation of the MSCs into bone, fat and cartilage (Dominici *et. al.* 2006).

Although MSCs and MSCs-like cells are isolated from the bone marrow, they are also located in other sites including adipose tissue, periosteum, synovial membrane, synovial fluid (SF), skeletal muscle, dermis, deciduous teeth, pericytes, trabecular bone, infrapatellar fat pad, articular cartilage, umbilical cord blood, placenta, liver, spleen, and thymus (Bianco *et. al.* 2008). Among the MSCs obtained from different sources, there is not much difference by

means of yield, growth kinetics, cell senescence, multi-lineage differentiation capacity, and gene transduction capacity (Dominici et. al. 2006).

MSCs are studied in various model organisms such as human, canine, rabbit, rat, and mouse and it is shown that MSCs isolated mostly from the bone marrow differentiate to different types of cells both *in vitro* and *in vivo* (Barry and Murphy 2004). The MSC isolation from every animal is from different sites of the bone marrow using different methods. In humans it is mostly from the pelvis (Digirolamo *et al*. 1999), tibia and femur. In larger animals it is from the same site but in rodents it is usually harvested from the mid-diaphysis of the tibia and femur (Barry and Murphy 2004).

MSCs are a very small fraction of the nucleated cells in the bone marrow; only %0.01-%0.1 of the total population (Pittenger *et al*. 1999). They can be isolated and expanded in tissue culture conditions with high efficiency and they can be induced to differentiate under specific conditions. They have fibroblastic morphology and they are adherent spindle-shaped cells. Under appropriate media conditions, MSCs adhere to the culture plate leading to the formation of colonies. Usually basal mediums with serum support are used to expend MSCs in tissue culture and growth factors are added in order for MSCs to differentiate (Barry and Murphy 2004)

MSCs have a highly variable expansive potential in subculturing. Some MSC cultures can expand more than fifteen cell doublings, while others can replicate only about four cell doublings. There are several reasons for this difference such as the procedure to harvest the marrow, the low frequency of MSCs in marrow harvests, and the age or condition of the donor. Although MSCs have a high *ex vivo* expansion potential, they do not lose their normal karyotype and telomerase activity. However, in extensive subcultivation, signs of senescence and apoptosis are observed (Minguell *et al*. 2001).

MSCs are characterized by their intrinsic self-renewal capacity, which is reflected in its clonogenic property and multi-lineage differentiation potential (Fig.1.2). Although not immortal, they have the ability to expand numerous times in culture while retaining their growth and pluripotent potential. In addition to their capacity to differentiate into chondrocytes, osteoblasts, and adipocytes, MSCs may serve as hematopoiesis-supporting stromal cells (Pittenger *et. al.* 1999). MSCs can differentiate into myocytes and cardiomyocytes and even into cells of non-mesodermal origin, including hepatocytes, insulin-producing cells, and neurons (Jiang *et. al.* 2002).

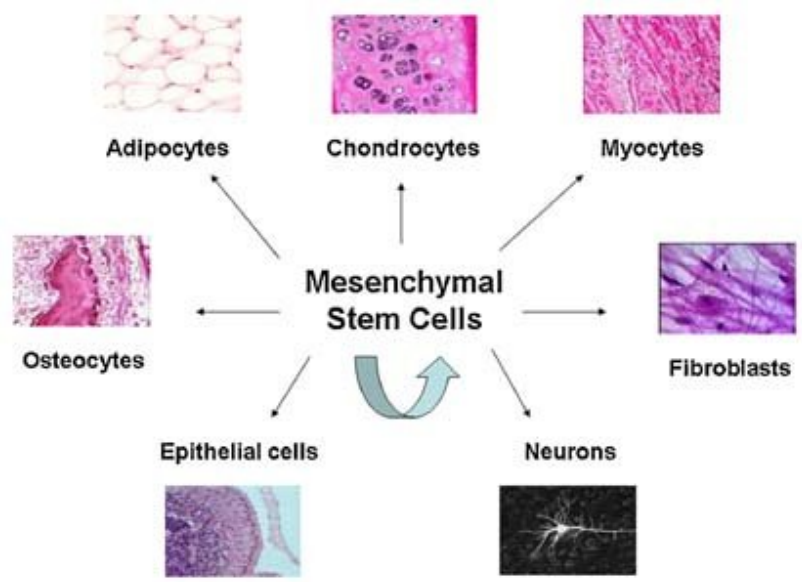


Figure 1. 2 Pluripotent capacity of MSC to differentiate into mesodermal and non-mesodermal cell lineages, including osteocytes, adipocytes, chondrocytes, myocytes, fibroblasts, epithelial cells, and neurons In addition, MSCs are capable to self-renewal. (Chen F et.al.2008)

The specific lineage commitment of MSCs is largely influenced by culture condition, especially growth factors. Growth factors that have regulatory effects on MSCs include members of the transforming growth factor-beta (TGF-b) superfamily, the insulin-like growth factors (IGF) , the fibroblast growth factors (FGF), the epidermal growth factor (EGF), the platelet-derived growth factor (PDGF), the vascular endothelial growth factor (VEGF), and the family of growth factors known as Wnt (Liu et al. 2009).

MSCs actively inhibit T-cell proliferation and due to this reason they are considered as nonimmunogenic or hypoimmunogenic which is important in host response to allogeneic MSC therapy (Uccelli et al. 2008). There are few mechanisms in immunospuression by MSCs. Contact-dependent mechanisms and soluble factors are thought to collaborate for the induction of MSC-mediated immunosupression. The interaction between MSCs and their target cells involves cell-cell contact mediated by adhesion molecules, such as PD1 and its ligands. Several soluble immunosuppressive factors have reported which either produced by MSCs or released following cross-talk with target cells, such as nitric oxide and indoleamine 2,3- dioxygenase (IDO).

Another important feature of MSCs is long-term homing and engraftment of implanted cells to various tissues even after the development of immunocompetence. The implanted MSCs can migrate to the specific site of injury by the help of factors that are released from the wound. This has been observed in cases like bone fractures, myocardial infarction, meniscus, and ischemic cerebral injury. Also at specific sites it has been observed

that the stem cells injected differentiate according to the local signals at the local site of injury (Miguell *et al*. 2001). MSCs have a high potential for regenerative medicine and tissue engineering besides being used as a gene delivery method, which makes their therapeutic value very high. Researchers are looking for different ways to use specialized cells derived from MSCs in targeting specific cancerous cells and delivering treatments that will destroy them or make them benign.

Besides these advantages of MSC in therapeutic applications, there are some unknowns like long-term effect and safety which require more toxicology studies. In addition, the efficiency is not clear. There are some evidences on homing and differentiation, but they are insufficient. Also large-scale culture, storage and distribution are important for their applications of MSCs (Miguell *et al*. 2001).

1.1.6 Stem Cell Niches

Stem cells unique ability to replenish themselves during the development, maintenance of tissue homeostasis, and repair of many tissues through self-renewal and differentiation are regulated by both intrinsic programming and input from their local environment, often referred to as the stem cell niche or microenvironment (Watt *et. al.* 2000).

Germ stem cells in the *Drosophila* ovariole and testis require physical interaction with supporting cap or hub cells, respectively, to retain stem cell identity. In the *C. elegans* gonad, the niche consists primarily of a single distal tip cell whose long cytoplasmic processes make extensive physical contact with germ stem cells (Keith *et. al.* 2007).

In mammals, spatially defined stem cell niches have also been identified in multiple tissues, including the gonad, skin, intestine, and brain. A number of molecular factors that control stem cell identity have identified. These factors typically supplied by the supporting cells of the niche include components of the BMP, Notch, Wnt, JAK-STAT, and Sonic hedgehog (Shh) signaling pathways, which provide intercellular cues that regulate stem cell identity and differentiation (Keith *et. al.* 2007).

In addition, niche structure provides mechanical stimuli and low oxygen tension. These features are shared by many types of adult stem cells. Indeed, increasing evidence demonstrates that oxygen tension is not only a metabolic substrate but also a powerful signaling molecule that regulates stem cell proliferation and differentiation (Ma *et. al.* 2009). Further information about effects of hypoxia on stem cell is discussed later.

1.2 Hypoxia

Oxygen is one of the essential elements that influence the evolution of life. Primitive life is considered to have started less than 4 billion years ago, and the first organisms that had the ability to convert and store the energy of light in the form of biochemical molecules with higher energetic potential appeared less than 3 billion years ago. The concentration of the oxygen started to increase in the atmosphere through the photosynthesis done by these organisms, during the next billion years. About 2 billion years ago, the concentration of O_2 in atmosphere reached a level that influence life. Several adaptation mechanisms have been evolved by living organisms in order to detoxify the toxic effects of oxygen in parallel with the increase of O_2 concentrations in atmosphere which continued until 500 million years ago (Ivanovic 2009).

Therefore, the eukaryotes, whose physiology based on the aerobic metabolism, are built during the evolution and became dependent on oxygen. Since inadequate oxygen availability can lead to cellular dysfunction and, since it causes cell death if its level is sufficiently profound in aerobic organisms, sophisticated systems are required to adjust the oxygen level, such as the cardiovascular system and the respiratory system (Kaelin and Ratcliffe 2008).

The blood circulation carries oxygen to tissues and a physiological tissue distribution of oxygen occurs as a result of progressive consumption of the oxygen in the blood circulation as it passes through different organs. The oxygen partial pressure (pO_2) of inspired air is around 160 mmHg at sea level (Fig. 1.3). This drops progressively first in the lungs; in part due to water vapor and diffusion, then in the blood flowing from the alveolar capillaries that carry the oxygen, at a pO_2 of around 104 mmHg, towards organs and tissues for their oxygenation. A further drop in the pO_2 is observed in the venous system. The pO_2 of a given tissue depends on the type of organ; rat spleen has a measured pO_2 of around 16 mmHg, while the thymus was measured at 10 mmHg (Brahimi-Horn and Pouyssegur 2007). This “normal” tissue pO_2 can be considered as “hypoxic” from a molecular standpoint. The low vascularization of the rat retina also makes it relatively hypoxic (2–25 mmHg) (Brahimi-Horn and Pouyssegur 2007), while tissues of the rat brain is even more hypoxic, 0.4–8.0 mmHg (Brahimi-Horn and Pouyssegur 2007). The diffusion distance of oxygen in a tissue is approximately 100–200 μm and an oxygen partial pressure of almost zero has been reported at only 100 μm from blood vessels (Brahimi-Horn and Pouyssegur 2007)

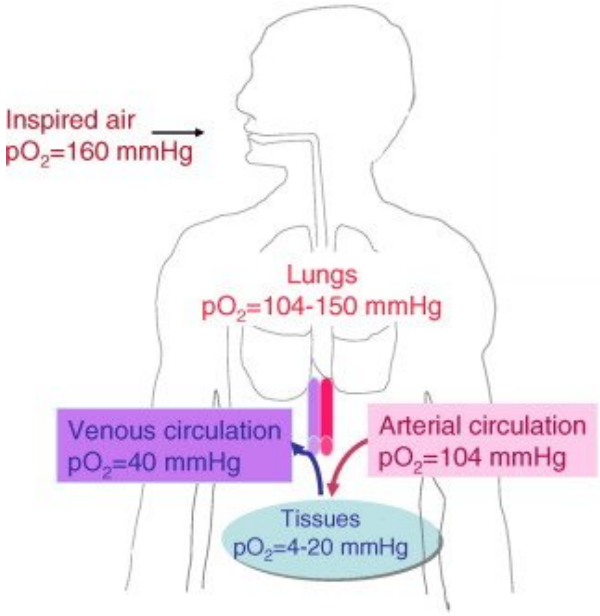


Figure 1. 3 Physiological and pathophysiological oxygen partial pressures (pO_2). The pO_2 of inspired air drops progressively as oxygen passes from the lungs into the circulation, which irrigates the tissues of major organs, and then in the venous system before re-oxygenation in the lungs. (Brahmi et al. (2009)).

In mammals an increase in altitude results in an initial physiological response that increases the rate and depth of breathing. Low levels of oxygen in tissues (hypoxia) also arise in normal development and as a consequence of a number of pathophysiological conditions where there is a diminished oxygen supply due to a defective vasculature. Such conditions include ischemic disorders (cerebral or cardiovascular), diabetes, atherosclerosis, inflammatory diseases, psoriasis, pre-eclampsia, chronic obstructive pulmonary disease and cancer (Brahimi-Horn and Pouyssegur 2007).

In these responses, at the molecular level, cells have ability to sense the oxygen availability whether it is insufficient, and thus undergo adaptive changes in gene expression that either enhance oxygen delivery or promote survival in a low oxygen (hypoxic) environment. An evolutionarily conserved pathway mediated by oxygen-dependent posttranslational hydroxylation of a transcription factor called hypoxia-inducible factor (HIF) plays a pivotal role in this process (Kaelin and Ratcliffe 2008).

There are three isoforms of hypoxia inducible factors. All three factors bind to a hypoxia response element (HRE; 5'-RCGTG-3') found in the target genes. (Semenza *et. al.*, 1991). HIF-1α belongs to the basic helix-loop-helix Per-ARNT-Sim (bHLH–PAS) protein family (Fig. 1.4) (Wang *et. al.* 1995), where it composes, in hypoxic condition, a

heterodimeric complex with HIF-1β which is also known as the aryl hydrocarbon nuclear translocator (ARNT).

HIF-2α is a closely related protein to HIF1-α and is also termed endothelial PAS protein, HIF-like factor (HLF), HIF-related factor (HRF), and member of the PAS superfamily 2 (MOP2) (Tian *et. al.* 1997). 48% amino acid sequence identity of HIF-2α is similar to HIF-1α. It also share structural and biochemical similarities with HIF-1α, such as heterodimerization with HIF-1β and binding HREs. Although HIF- 1α is ubiquitously expressed, expression of HIF-2α is tissue specific such as in the lung, endothelium, and carotid body (Ke and Costa 2006).

HIF-3α is the latest discovered isoform between hypoxia inducible factors. This transcription factor is also expressed in a different tissues and can dimerize with HIF-1β and binds to HREs. HIF-3α has splice variant, inhibitory PAS (IPAS), which is predominantly expressed in the Purkinje cells of the cerebellum and corneal epithelium. IPAS possesses no endogenous transactivation activity. Instead, it interacts with the amino-terminal region of HIF-1α and prevents its DNA binding, acting as a dominant negative regulator of HIF-1. (Ke and Costa 2006)

The bHLH and PAS motifs are required for heterodimer formation between the HIF-1α and HIF-1β subunits, and the downstream basic regions required for specific binding to the HRE DNA sequence (Crews 1998). HIF-1α has two transactivation domains, called N-terminal (N-TAD) and C-terminal (C-TAD), in the C-terminal half of the HIF-1α protein (Ruas *et. al.* 2002). The C-TAD interacts with coactivators such as CBP/p300 to activate gene transcription (Lando *et. al.* 2002). HIF-1_ also contains an oxygen-dependent degradation domain (ODDD) that mediates oxygen-regulated stability (Pugh *et. al.* 1997).

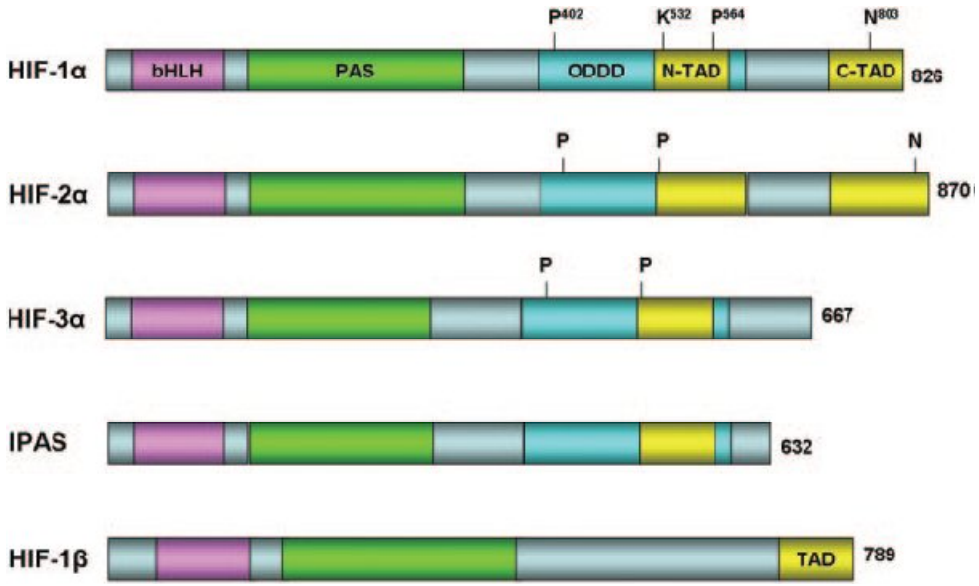


Figure 1. 4 Domain structures of human HIF-α and HIF-1β. HIF-α (HIF-1α, HIF-2α, HIF-3α IPAS) and HIF-1β belong to the bHLH and PAS protein family. HIF-α contains an ODDD that mediates oxygen-regulated stability through the hydroxylation of two proline (P) residues and the acetylation of a lysine (K). HIF-1α and HIF-2α also contain two transaction domains (C-TAD and N-TAD), whereas HIF-1β has only one TAD (KE et.al. (2006).

Despite of the fact that HIF-1 beta protein is constitutively expressed and its mRNA and protein level are constant regardless of oxygen availability, HIF-1 alpha protein is highly controlled by oxygen (Salceda and Caro 1997). In normoxia, HIFα becomes hydroxylated at one (or both) of two highly conserved prolyl residues located near the NTAD by prolyl hydroxylase domain (PHD) family (Kaelin 2005). In normoxia, hydroxylation of two proline residues and acetylation of a lysine residue in its ODDD promote interaction of HIF-1α with the von Hippel-Lindau (pVHL) ubiquitin E3 ligase complex, which is a component of a ubiquitin ligase complex (Masson *et. al.* 2001). As a result, HIFα is polyubiquitylated and subjected to proteasomal degradation by 26S proteasome when oxygen is available.

The PHD proteins belong to the Fe(II) and 2-oxoglutarate-dependent oxygenase superfamily, whose activity is dependent on oxygen. Under low oxygen conditions, HIFα accumulates then dimerizes with an HIFβ family member, translocates to the nucleus, and transcriptionally activates 100–200 genes, including genes involved in erythropoiesis, angiogenesis, autophagy, and energy metabolism (Kaelin and Ratcliffe 2008)

FIH1, like the PHD family members, is an Fe(II)- and 2-oxoglutarate-dependent dioxygenase. When oxygen is available, FIH1 hydroxylates a conserved asparaginyl residue within the HIF1α and HIF2α CTADs, and thus prevents the recruitment of the coactivators p300 and CBP (Kaelin and Ratcliffe 2008). FIH1 remains active at lower oxygen

concentrations than the PHDs and so might suppress the activity of HIFα proteins that escape destruction in moderate hypoxia (Dayan *et. al.* 2006).

Moreover, HIF-1α is acetylated by an acetyltransferase named arrest-defective-1 (ARD1) at Lysine residue located in the ODDD domain, which favors the interaction of HIF-1α with pVHL, and thus destabilizes HIF-1α (Jeong *et. al.* 2002).

Despite the central importance of hydroxylases in sensing oxygen tension and regulating HIF-1 activity, there are other mechanisms that contribute to the control of HIF-1. Phosphorylation is well known to be crucial in controlling protein activities. Direct phosphorylation of HIF-1α has been reported, and the mitogen-activated protein kinase (MAPK) pathway seems to play a role (Minet *et. al.* 2001). Phosphorylation does not affect stability or DNA binding of HIF-1α; instead, it increases the transcriptional activity of HIF-1. In addition to the post-translational modification of HIF-1α , SUMOylation of HIF-1α contributes to repressing transactivation (Brahimi-Horn *et. al.* 2005).

HIF-1 is also regulated in an oxygen-independent manner. Cytokines, growth factors, environmental stimuli, and other signaling molecules have been implicated in controlling HIF-1 under non-hypoxic condition. Although complex and cell-type dependent, some stimulate HIF-1 transactivation or synthesis by activation of the MAPK or the phosphatidylinositol 3-kinase (PI3K) signaling pathways (Ke and Costa 2006).

The PHDs and FIH1, like other 2-oxoglutarate-dependent oxygenases, employ a two-histidine-one-carboxylate facial triad to coordinate the catalytic Fe(II) center, leaving two positions for binding 2-oxoglutarate, and one for molecular oxygen. During catalysis, the splitting of molecular oxygen is coupled to the hydroxylation of HIFα and to the oxidative decarboxylation of 2-oxoglutarate to succinate and CO_2. The reaction proceeds via the formation of a highly reactive ferryl ($Fe^{IV} = O$) intermediate that oxidizes the target amino acid residue. Ascorbate is required for full catalytic activity and likely functions to reduce the catalytic iron in the event of uncoupled turnover, in which failure to oxidize the HIFα substrate leaves the iron center in an oxidized and inactive form (Schofield and Ratcliffe, 2004). Therefore, The HIF hydroxylases are also inhibited *in vivo* by iron chelators (Page *et. al.* 2007).

1.2.1 Hypoxia and Stem Cells

The effect of oxygen tension on stem cell physiology has been studied for over 30 years (Toya *et. al.*,1976). For hematopoietic stem cells (HSCs), it has been found that cultivation under low oxygen tensions maintained a significantly higher number of long-term

colony initiating cells (LTC-ICs) relative to cultures under ambient (20%, v/v) oxygen concentrations.(Cipolleschi *et. al.* 2000). Recently, it has also been observed, in several other stem and progenitor cell populations, that cultivation under hypoxic conditions resulted in enhanced proliferation and maintenance of their naïve states (Csete *et. al.* 2006). *In vivo* studies have shown that mesenchymal stem and progenitor cells home specifically to hypoxic niches (Ma *et. al.* 2009). Although the identity of cellular oxygen sensor is being debated, emerging evidence indicate that some of the effects of hypoxia on stem cell function are directly regulated by hypoxia-inducible factor (HIF) proteins (Keith *et. al.* 2007).

1.2.2 Bone Marrow Microenvironment

The oxygen distribution within the bone marrow has been studied almost exclusively in the context of hematopoietic stem cells (HSCs) and to a much lesser extent in the context of MSCs. However, bone marrow has a hierarchical structure, in which the haematopoietic compartments are bound by stromal elements (Weiss *et. al.* 1976) - mainly mesenchymal stem cells (MSCs) - and such that the two cell types form an integral part of each other's niche (Figure 1.5). It has long been proposed that hematopoietic progenitors exist at high concentration at the endosteal surface and release via the central venous sinus as they differentiate and mature (Lord *et. al.* 1990). Early direct measurement revealed that bone marrow in general is hypoxic, where some regions are as low as ~1-2% O_2 (Ma *et. al.* 2009).

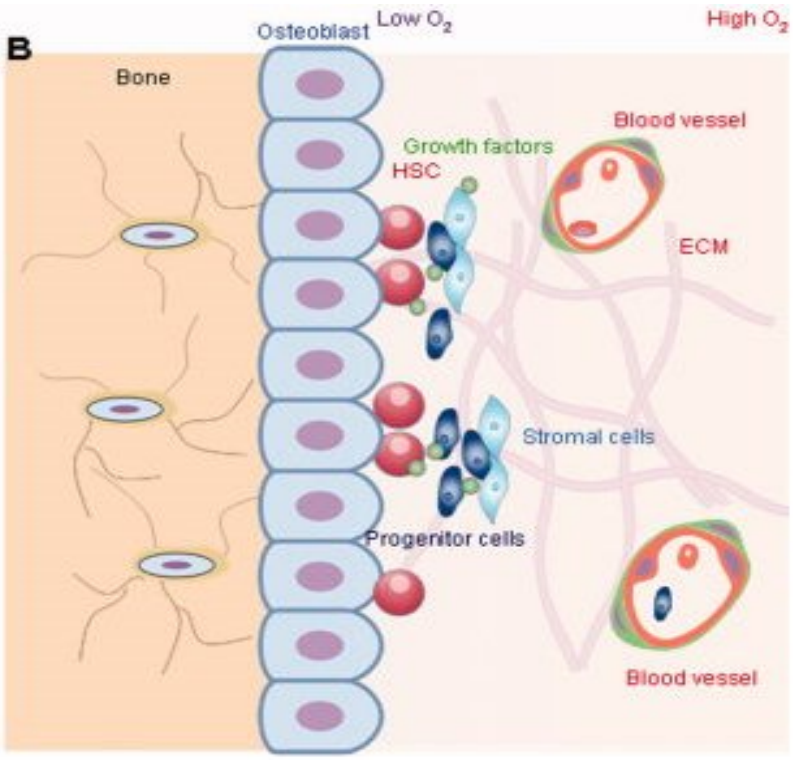


Figure 1. 5. Oxygen tension in bone marrow is well below ambient oxygen environment in the range of 3–7% O_2 with oxygen concentrations decreasing with distance away from sinuses. (Ma T. et.al. 2008)

1.2.2.1. Effects of Hypoxia on Haematopoietic and Embryonic Stem Cells

Embryonic stem (ES) cells grow more efficiently under low O_2 conditions, as opposed to in ambient air that is supplemented with 5% carbon dioxide. It was previously noted that bovine blastocysts produced under reduced O_2 tensions exhibited significantly more inner cell mass (ICM) cells than those that were maintained at higher O_2 levels (Simon M. C. and Keith B. 2008). The ICM and its ES cell counterparts are pluripotent. Roberts *et al.* demonstrated that human ES cells proliferate at a similar rate when cultured at 3–5% O_2 as they do under 21% O_2. The authors concluded that hypoxic conditions are required to maintain the full pluripotency of mammalian ES cells (Simon M. C. and Keith B. 2008). HSCs and their proliferating progenitors are naturally occupying the most hypoxic niches. Furthermore, Danet *et al.* demonstrated that culturing bone marrow HSCs at 1.5% O_2 promoted their ability to engraft and repopulate the haematopoietic organ of immunocompromised recipient mice (Simon M. C. and Keith B. 2008)

1.2.2.2. Effects of Hypoxia on Bone Marrow-Derived Mesenchymal Stem Cells

In general, MSCs exhibited greater colony-forming potential, proliferated faster, and longer, and maintained their undifferentiated characteristics better under low oxygen conditions (Ma *et. al.* 2009). It has been shown that hMSCs grown in 3D scaffolds under extended hypoxic conditions (2% O_2) increased their expression of pluripotent genes Oct-4 and Rex-1 and had elevated CFU-F ability while maintaining their ability to differentiate along osteogenic or adipogenic lineages (Grayson *et. al.* 2006). In two-dimensional (2D) culture studies, hMSCs under hypoxic conditions exhibited 30-fold greater expansion potential over a 6-week period than normoxic cells, homogenously maintained their spindle morphology, and formed multiple cell-layers with high expression of connexin-43 (Grayson *et. al.* 2007). Hypoxia affects the differentiation characteristics of MSCs in ways that may be correlated with the physiological oxygen requirements of the differentiated cells (Fink *et. al.* 2004).

1.2.3 The role of HIFs in regulating stem cells

Some of the effects of hypoxia on stem cell function are directly mediated by the HIF proteins. Targeted mutation of the ARNT subunit eliminates both HIF-1α and HIF-2α function and results in a decreased number of progenitor cells of all hematopoietic lineages in the embryonic yolk sac of $Arnt^{-/-}$ mouse embryos (Keith *et. al.* 2007).

Recently, new molecular mechanisms by which HIFs directly modify cellular differentiation and stem cell function have been defined. Lendahl, Poellinger, and colleagues reported that hypoxia blocked the differentiation of myogenic satellite cells, a myogenic cell line (C2C12), and primary neural stem cells in a Notch-dependent manner (Gustafsson *et. al.* 2005). It was shown that HIF-1α was physically recruited to a DNA-binding complex containing the Notch intracellular domain in hypoxic cells. Hypoxic induction of Notch target genes was dependent on the Notch intracellular domain and also required the functional C-terminal transactivation domain of HIF-1α, which interacts directly with p300/CBP.

The links between the HIFs, Notch, and Oct4 reveal specific molecular mechanisms whereby oxygen responses can inhibit differentiation and, possibly, promote stem cell identity. They also raise the possibility of crosstalk between hypoxia and other stem cell signaling pathways. TGF-β has been reported to induce HIF-α stabilization by inhibiting PHD2 (McMahon *et. al.* 2006). In addition, a recent paper describing physical interaction between β-catenin and HIF-1α suggests at least one mechanism by which Wnt signaling might affect HIF activity in stem cells (Kaidi *et. al.* 2007).

1.3 Estrogen

Estrogen is a hormone which is produced by the ovaries and testes. It stimulates the development of secondary sexual characteristics, induces menstruation, and regulates growth, differentiation, cell proliferation, metabolic activities, reproduction, homeostasis, cardiovascular health, bone integrity, cognition, and behavior. It targets many organs like the brain, heart, bone, breast, uterus, and prostate. This wide range of organs, which estrogen is involved in the functioning of, makes estrogen a very important hormone for therapy and therefore it is crucial to understand the imbalances of estrogen, gene networks controlled by estrogen, and regulation of its targets (Deroo and Korach 2006).

Estrogen controls the expression of a wide variety of genes through distinct genomic and nongenomic pathways. In the classical genomic pathway, estrogen signals are mediated through the estrogen receptor (ER), which functions as a transcription factor for target genes. In the nongenomic pathway, estrogen regulates the functions of factors in cells through various mechanisms, including protein phosphorylation (Norman *et. al.* 2004).

1.3.1 Estrogen Signals

1.3.1.1. Ligand-Dependent Genomic Pathways

Estrogen exerts its biological effects by binding to ER, which mainly exists in the nucleus as a member of the nuclear receptor superfamily of transcription factors (Figure 1.6). ER acts through the formation of homo- or heterodimers of ERα and ERβ. In the classical pathway, estrogen-bound ERs dimerize and function as a transcription factor which binds to a specific DNA sequence named the estrogen response element (ERE) present in the promoter or enhancer regions of target genes. ER binds to ERE through its DNA-binding domain (DBD) and recruits coactivators such as SRC-1, AIB1 and p300/CBP to form a functional ER complex. It is now known that ER target genes which have full or half ERE sites (Smith *et. al.* 2004).

In the genomic pathway, ER can also regulate transcription without binding directly to DNA. ER acts as a coactivator, and interacts with other transcription factors such as AP-1, SP-1 and NF-κB via protein–protein interactions, and it could regulate the transcription of genes that lack ERE but has a binding element for its partner's protein (Hayashi *et. al.* 2009).

1.3.1.2. Nongenomic Pathways

Estrogen also exerts its effects that are not accounted for by transcriptional mechanisms (Figure 1.6). There is accumulating evidence that estrogen receptors are also located at the plasma membrane and are responsible for nongenomic actions (Hayashi *et. al.* 2008) Membrane ER associates with many growth factor receptors, such as IGF-1R, EGFR, HER2. In the activation of IGF-1R, E2 induces the formation of a ternary complex among ERα, IGF1-R and Shc, the adaptor protein, in the plasma membrane, which induces phosphorylation of IGF-1R (Song *et. al.* 2004). The estrogen-bounded membrane ER activates several signals in a cell type-specific manner, including calcium currents, cAMP, inositol phosphate, G proteins, Src, and Shc, which leads to the activation of downstream kinases, such as mitogen-activated protein kianse (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt (Norman *et. al.* 2004).

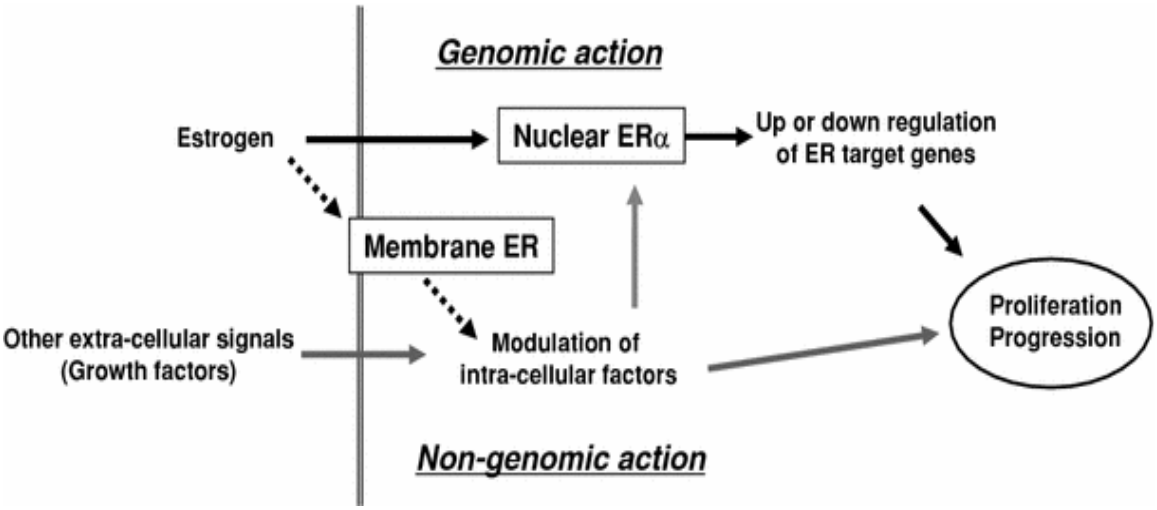


Figure 1. 6: Outline of estrogen signaling pathways. Estrogen evokes genomic and nongenomic actions via nuclear ER and membrane-associated ER. Moreover, these signals are also stimulated or modulated by crosstalk with the intracellular protein kinase-mediated phosphorylation signaling cascade (Hayashi S. et.al 2008).

1.3.2 Estrogen and Hypoxia

The relationship between hypoxia and estrogen-mediated pathways comes from breast cancer. It has been shown that, in T47 human breast cancer cells, the expression level of 31 genes out of 8000 changes in hypoxic condition increase in the presence of estrogen. These genes are involved in cell growth, differentiation, angiogenesis, protein transport, metabolism, and apoptosis (Yi. *et. al.* 2009**)**.

So far, there have been two possible mechanistic explanations for the relationship between estrogen and hypoxia. One mechanism is that HIF-1α is a coactivator for ERα itself. Other is that HIF-1α activates intermediary cofactors for ERα that further facilitate ERα transactivation (Fig. 1.7), such cofactors as SRC, CBP, ASC (Seifeddine *et. al.*, 2007). In addition, HIF-1α directly controls ERs activation and degradation, where the level of ERα decreases in hypoxia. The mechanisms of ERa downregulation during hypoxia are probably dependent on both increased proteasomal degradation of ERa and decreased transcriptional ERa-activation. The transcriptional inhibition of ERa during hypoxia seems to be mediated by the ERK pathway (Kronblad *et. al.* 2005).

However, there is no interplay between ERβ and HIF-1α in contrast to ERα. It was shown that estrogen-occupied ERα activates HIF-1α by the MAPK and PI3K pathways in the uterus (Kazi and Koos 2007). Even if mechanism is not known HIF-1α is a coactivator only for ERα, but not ERβ. HIF-1 α mediated ERα activation involves the C-terminal domain of estrogen in which C-terminal AF-2 is found, and is important for the transcription activation. Some coregulators, such as SRC-1, TIF-2, and AIB-1, interact with the AF-2 of ERα, whereas

other cofactors, such as p68 RNA helicase protein, interact with AF-1 in potentiating ERα function (Yi.*et. al.* 2009**)**

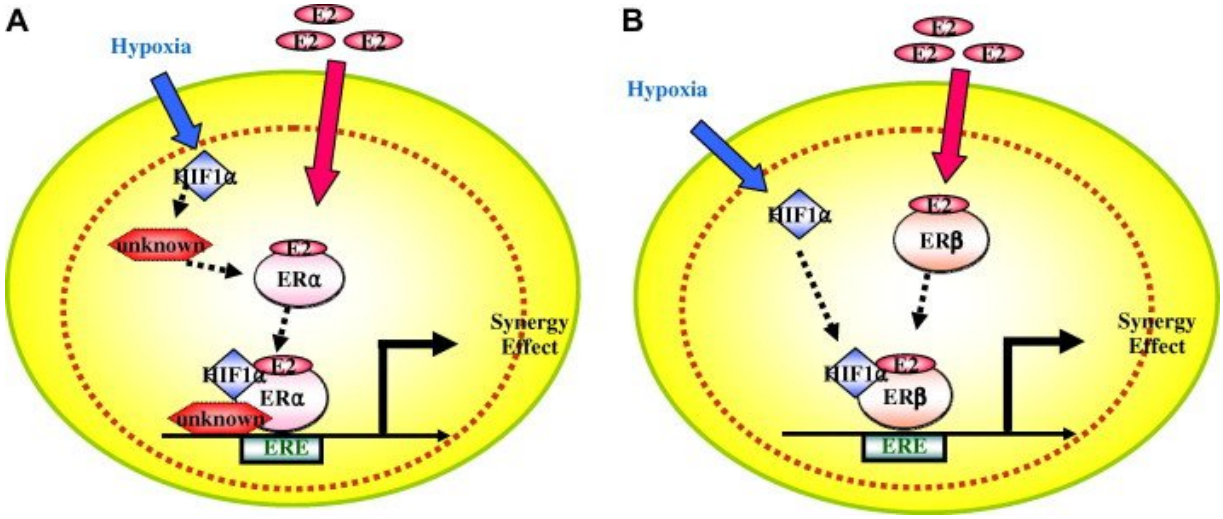


Figure 1. 7: A proposed model of the effects of E_2 and hypoxia on transactivation of ERs. Unknown factor(s) recruited by HIF1α specifically interact(s) with ERα and synergistically activate(s) transactivation of ERα (A). Such synergism is not observed with ERβ (B) (Yi et. al. 2009).

1.3.3 Estrogen and Notch

There are no direct results showing the relationship between Notch pathway and estrogen. The direct link between Notch signaling pathway and estrogen comes from breast cancer. It has been shown that the amount of Notch-1 was increased by 17β-estradiol treatment in breast cancer cell lines such as T47D or MCF-7 (Rizzo *et. al.* 2008). Rizzo *et. al*. (2009) showed that loss of estrogen signaling caused Notch re-activation because Notch-1 is found primarily on cell surface and nuclear levels N^{IC} are reduced when cells are treated with estrogen, suggesting that cross-talk between estrogen and Notch signaling leads to Notch re-activation.

1.3.4 Estrogen and Stem Cells

Gender differences, by the differential effects of sex-specific hormones, exist in a variety of cardiovascular, cardiopulmonary, neurodegenerative, endocrine and metabolic bone diseases such as osteoporosis (Figure 1.8). Recent studies reported the presence of estrogen receptor on stem cells, suggesting that estrogen may modify the function of those cells (Ray R. *et. al.* 2008). 17β-estradiol enhances the proliferation and migrations of some stem cells, such as endothelial progenitor cells (EPCs), to the injured vessels, or ischemic myocardial tissues through the process of homing and help in repair and regeneration to compensate for the lost tissue. Thus, modification of the function of stem cells through estrogenic stimulus may increase the function of stem cells (Ray R *et. al.* 2008).

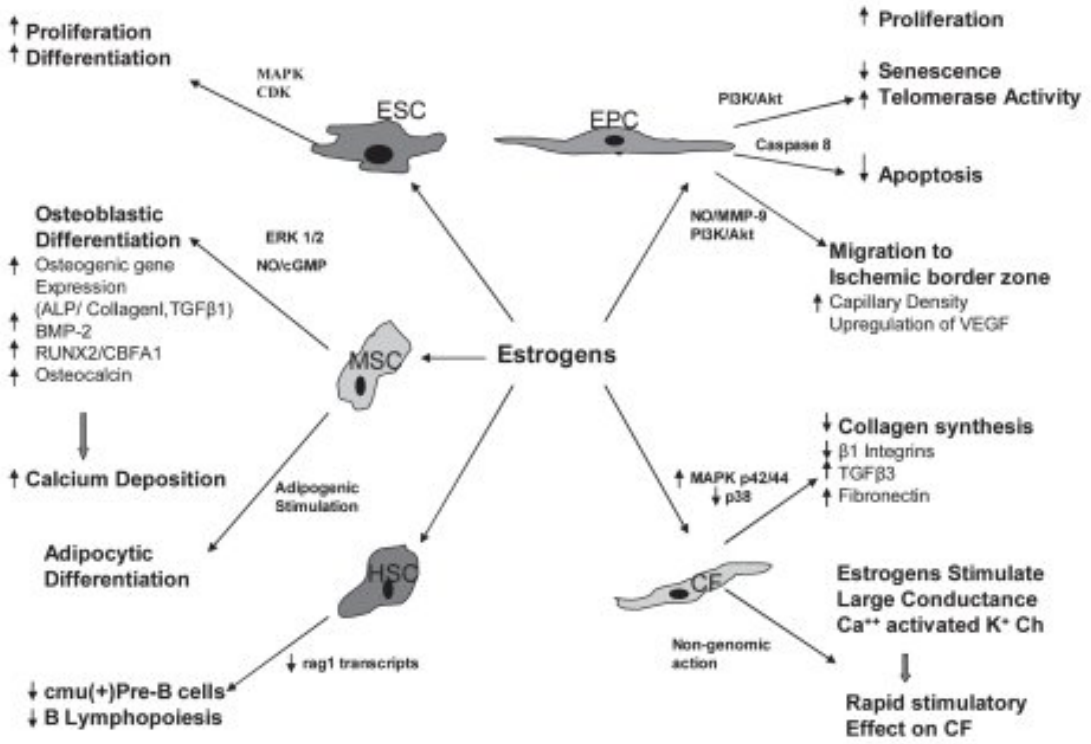


Figure 1. 8: Effects of estrogen on various stem cells and progenitor cells. ESC (embryonic stem cell); EPC (endothelial progenitor cell); MSC (mesenchymal stem cell); HSC (hematopoietic stem cell); CF (cardiac fibroblast); BMP (bone matrix protein); RUNX2/ CBFA1 (runt-related transcription factor 2/core-binding factor alpha) (Ray R. et.al.2008)

Estrogens may enhance the protective function of MSCs by increasing or decreasing cytokine and growth factor production (Crisostomo *et. al.*2008). Female MSCs express less amount of proinflammatory cytokines, TNF-α and IL-6, when compared with male MSCs in inflammatory reactions (Crisostomo *et. al.* 2006).

Furthermore, estrogen plays a role in osteogenic differentiation of MSCs through the ERα receptor, and support growth and differentiation (Wang *et. al.* 2006). When bone marrow MSCs are subjected to osteogenic differentiation medium supplemented with 17β-estradiol, the expression levels of bone morphogenetic protein (BMP) and osteocalsin increase together with increase calcium deposition (Hong *et. al.* 2006). 17β-estradiol also stimulates the expression of osteogenic genes for ALP, collagen I, and TGF-β1 in MSCs.

1.4 Notch Signaling Pathway

Notch signaling has been shown to regulate a broad range of events during embryonic and post-natal development, including proliferation, apoptosis, border formation, and cell fate decisions. In self-renewing organs in vertebrates, inhibition of differentiation, lineage specification at developmental branch points and induction of differentiation are relevant

functions of Notch signaling (Artavanis-Tsakonas *et. al.* 1999). In addition, Notch signaling plays a role in stem cell maintenance (Rajan *et. al.* 2009).

Both Notch receptor and its ligands, Delta and Serrate (known as Jagged in mammals), are transmembrane proteins with large extracellular domains that consist primarily of epidermal growth factor (EGF)-like repeats (Figure 1.10). There are four Notch receptors in mammals: (Notch1–4), two in *Caenorhabditis elegans* (LIN-12 and GLP-1*)* and one in *Drosophila melanogaster* (Notch).

Ligand binding promotes two proteolytic cleavage events in the Notch receptor (Fig. 1.9). The first cleavage is catalysed by ADAM-family metalloproteases, whereas the second is mediated by γ-secretase, an enzyme complex that contains presenilin, nicastrin, PEN2 and APH1 (Fortini 2002). The second cleavage releases the Notch intracellular domain (Nicd), which then translocates to the nucleus and cooperates with the DNA-binding protein CSL (named after CBF1, Su(H) and LAG-1) and its coactivator Mastermind (Mam) to promote transcription (Fortini 2009).

Notch signaling is important for 'lateral inhibition, a transcriptional feedback mechanism that explains how Notch signaling can drive two initially identical progenitor cells to adopt different fates (Fortini 2009).

Initially, the two interacting cells are equivalent, each expressing comparable levels of the Notch receptor and DSL ligands, and thus possessing equivalent signal-sending and signal-receiving activities. Over time, a small difference in some aspect of signaling arises between the two cells. This initial difference is amplified by a transcriptional feedback loop wherein Notch signaling activates transcription of the Enhancer of split gene family, which encodes bHLH transcription factors that in turn repress target genes (Fortini 2009).

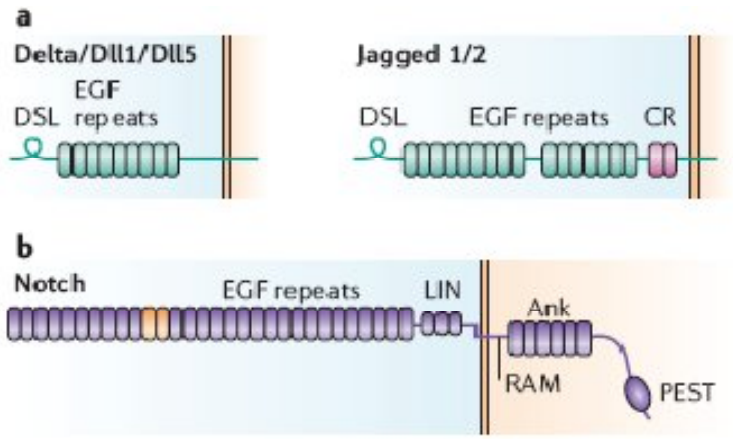


Figure 1. 9: DSL ligands and Notch receptors: (a) Notch ligands are transmembrane proteins that are characterized by an N-terminal DSL (Delta, Serrate and LAG-2) domain that is essential for interactions with the Notch receptor. The extracellular domains of the ligands contain varying numbers of epidermal growth factor (EGF)-repeats. The ligands are subdivided into two classes, Delta or Delta-like (Dll) and Serrate (Jagged in mammals), depending on the presence or absence of a cysteine rich (CR) domain. (b) Notch extracellular domains contain 29–36 EGF repeats, 3 cysteine rich LIN repeats and a region that links to the transmembrane and intracellular fragment. This linker region is important in preventing premature activation of the receptor. EGF-repeats 11 and 12 are essential for ligand binding. The intracellular portion consists of a RAM domain, six ankyrin (Ank) repeats and a C-terminal PEST domain. It also contains nuclear localization signals. Individual types of Notch receptor have additional protein–protein interaction motifs. (Bray 2006)

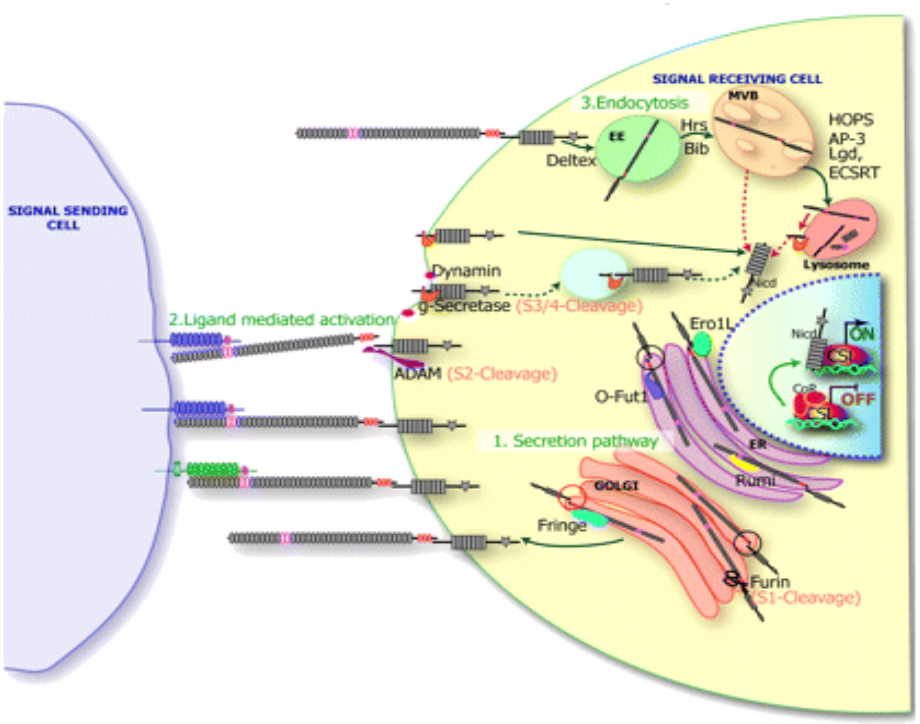


Figure 1. 10 Schematic illustration of Notch and the pathway**.** (1) Secretory pathway: Notch is translated inside ER, where it is glycosylated by an *O*-fucosyltransferase O-Fut1 and *O*-glucosyltransferase Rumi. Notch is then translocated into Golgi, where it is cleaved by Furin protease at the S1 site and further modified by the *N*-acetylglucosaminyltransferase, Fringe. (2) Ligand-mediated activation: Notch interacts with the DSL ligands, Delta and Serrate, resulting in a series of proteolytic cleavage events induced by ligand binding. The S2 cleavage is mediated by ADAM protease, whereas the S3/4 cleavage event is mediated by γ-secretase. (3) The endocytic regulation of the Notch receptor: Full-length Notch can undergo endocytosis, leading to translocation of Notch into EEs, MVB, and lysosomes (Kopan R. et.al 2009).

1.4.1 CSL proteins

CSL proteins are the essential effectors of the Notch pathway. Nicd forms a trimeric complex with CSL and the co-activator Mam, which is essential for Nicd-dependent transcription (Nam *et. al.* 2006). Another Nicd-interacting protein, SKIP (Ski-interacting protein), a transcriptional coregulator and component of spliceosomes, is also recruited to promoters at the same time (Fryer *et. al.* 2004). Mam in turn recruits the histone acetylase p300, which promotes assembly of initiation and elongation complexes (Bray 2006).

The assembly of the co-activator complex not only promotes transcription, but also results in turnover of Nicd, which is achieved by recruitment of cyclin-dependent kinase-8 (CDK8), which phosphorylates Nicd, rendering it into a substrate for the nuclear ubiquitin ligase SEL10 (Fryer *et. al.* 2004). Destruction of Nicd would result in the dissociation of Mam and other co-activator.

1.4.2 Epigenetic regulators

In the absence of Notch activity, CSL proteins remain on the DNA and recruit co-repressors. In *D. melanogaster*, the adaptor Hairless tethers the more global repressors Groucho and CtBP, which recruit histone deacetylases (Nagel *et. al.* 2005). In mammalian cells, co-repressors interact with CSL, including SMRT (also known as NcoR) and SHARP (also known as MINT/ SPEN), which in turn recruit CtBP or other global co-repressor (Oswald *et. al.*, 2005). However, number cofactors interact with CSL, including the histone acetyl transferase GCN5 and the SWI/SNF chromatin-remodelling enzyme Brahma (also known as BRM) (Bray 2006).

1.4.3 Notch Signaling Pathway and Stem Cells

A diversity of stem cell subsets require Notch signaling for the productive maintenance of tissues. In skeletal muscle, expression of the Notch ligand Delta is essential for activation of endogenous stem cells (satellite cells) after injury (Conboy *et. al.* 2003)

Likewise, Notch plays a key role in the self renewal of intestinal and hematopoietic stem cells, and is required during various stages of stem cell commitment to lineage specification. Interestingly, Notch is likely to operate in other less well-defined systems, such as in osteogenesis (Carlson and Conboy 2007)**.**

The function of endogenous Notch signaling in mammalian ESCs is just beginning to be understood in detail. mRNAs encoding Notch1–3 and DLL1 are present at high levels in hESCs. Conversely, Notch2 is expressed at much lower levels. Notch signaling is thought to be relatively inactive in undifferentiated hESCs (Rho *et. al.* 2006).

However, Notch might not be a required signaling component for ESC self-renewal, rather, Notch is likely to be involved during specific differentiation intervals of ESCs. For example, it could play a role in regulating the decisions between mesodermal and neuroectodermal cell-fates. Neural specification of ESCs is influenced by Notch signaling, as Notch1 activation promotes neural lineages and suppresses ESC differentiation into mesodermal cell fates (Noggle *et. al.* 2006).

The mammalian intestinal epithelium has one of the highest turnover rates in the body and comprises stem cells, transit amplifying (TA) cells and terminally differentiated cells. One function of Notch signaling within the small intestine is to maintain proliferative crypt progenitors in the undifferentiated state, while a second function is to influence a binary cell fate decision of TA cells that have to choose between the adsorptive and the secretory lineages such as goblet cells and enteroendocrine cells. This process seems to be regulated by the Notch target gene Hes1, which transcriptionally represses Math1. Math1 is required for the development of secretory lineages while Hes1 expression favors the development of adsorptive cells (Wilsona and Radtke 2006).

In fetal hematopoiesis, Notch1 signaling is necessary for developing stem cells within the AGM (aorta-gonad-mesonephros) region. In adult BM (bone marrow) progenitors Notch signaling inhibits differentiation of stem cells (HSCs). Downregulation of Notch1 signaling is required in BM B cell progenitors to allow normal B cell development. In the thymus Notch1 signaling is essential for T lineage specification in an early thymocyte progenitor, while at subsequent developmental stages it promotes differentiation of pro-T cells into pre-T cells of the αβ*T* lineage (Wilsona and Radtke 2006).

In the skin, Notch1 signaling induces expression of early differentiation markers such as Keratin1 and Involucrin, and partially represses the expression of Loricrin and Filagrin, two late differentiation markers. Moreover Notch1 induces expression of the cell cycle regulator $p21^{CIP1/WAF}$ by two mechanisms. First, Notch1 targets the $p21^{CIP1/WAF}$ promoter directly, and second Notch1 upregulates $p21^{CIP1/WAF}$ through the activation of calcineurin/NFAT activity mediated by the downregulation of calcipressin via the Notch target gene Hes1. Both Wnt- and Shh-mediated signaling are normally repressed in the murine epidermis by Notch1. Repression of the Wnt pathway is at least partially mediated by the downregulation of Wnt4 through a $p21^{CIP1/WAF}$:E2F-1-dependent mechanism (Wilsona and Radtke 2006).

1.4.4 Notch Pathway and Hypoxia in Stem Cells

Notch signaling and the cellular hypoxic response synergize at various levels in the pathways (Figure 1.11). A link between Notch and hypoxia was first suggested by that the Notch target gene Hes1 was upregulated by hypoxia in neuroblastoma cell lines. It has also been shown that hypoxic response requires functional Notch signaling, demonstrated for myogenic and neural stem cell differentiation and more recently for hypoxia-induced epithelial-to-mesenchymal transition in tumor cells (Poellinger and Lendahl 2008).

At the molecular level, there are a number of interaction points between the Notch and hypoxia pathways, and various potential transcriptional outcomes of this cross-talk are outlined in Figure 6. HIF-1α is recruited to a DNA-binding complex containing the Notch intracellular domain in hypoxic cells. Hypoxic induction of Notch target genes is dependent on the Notch intracellular domain and also required the functional C-terminal transactivation domain of HIF-1α, which interacts directly with p300/CBP (Keith and Simon 2007).

It is not clear how hypoxia affects the activation of Notch-responsive, but it is speculated that there is a cell-context-dependent transcriptional response because the activation of Hes and Hey genes are observed in different cell types (Poellinger and Lendahl 2008). Some genes, such as Hey 1, Hey 2, and Dll4, contain both HRE-binding and CSL-binding sites, and hypoxia and Notch may therefore have additive effects on transcription (Diez *et. al.*, 2007). An alternative way of interaction is hypoxia-induced elevation of Notch ligand expression, which has been observed for Dll4 (Sahlgren *et. al.* 2008).

A recent link between Notch and hypoxia involves the protein factor inhibiting HIF-1α (FIH-1) in which FIH-1 hydroxylates HIF-1α at asparagine residue 803 in the C-terminal transactivation domain (Poellinger and Lendahl 2008). Recent reports show that FIH-1 also hydroxylates Notch ICD, and leads to the reduced Notch signaling. Since FIH-1 binds more strongly to Notch ICD than to HIF-1α, it raises the possibility that Notch ICD sequesters FIH-1 away from HIF-1α, relieving HIF-1α from repression by FIH-1, and thus leads to an increased recruitment of HIF-1α to HRE-containing regions in hypoxia-induced genes (Zheng *et. al.* 2008).

Therefore, it might speculate that a stem cell residing in a hypoxic niche may require HIF-α proteins to fully activate Notch target genes that inhibit differentiation, thereby contributing to stem cell self renewal and multipotency (Keith and Simon 2007).

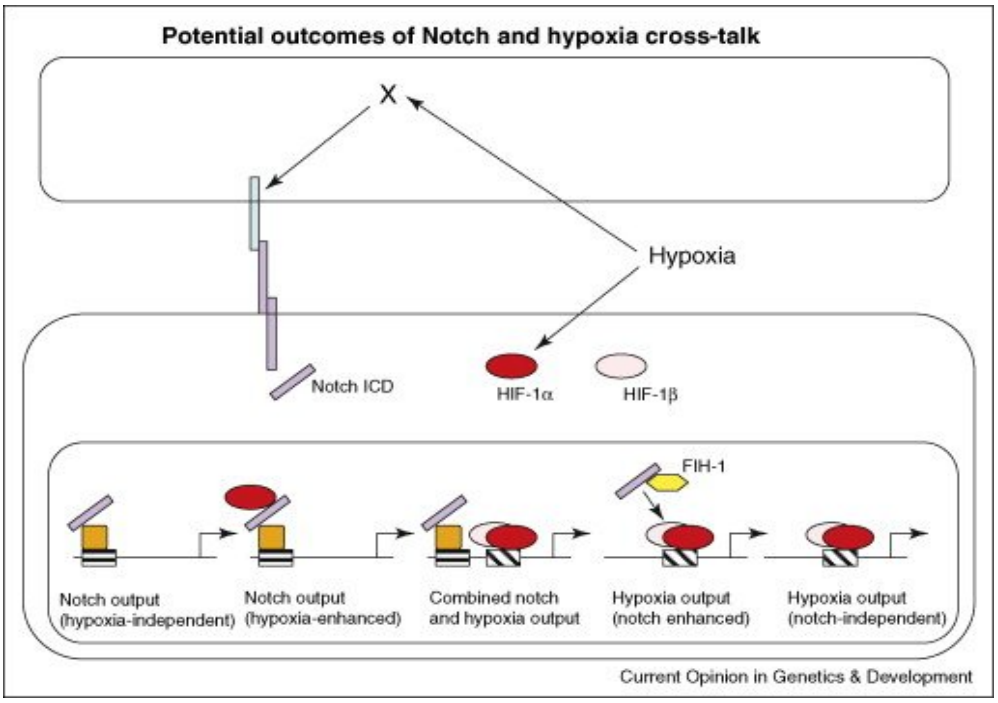


Figure 1. 11: Schematic depiction of different levels of interaction between Notch and the cellular hypoxic response and the potential transcriptional outcome of this cross-talk. Hypoxia can elevate the expression of Notch ligands in the Notch signal-transmitting (upper) cell, leading to enhanced Notch signaling in the signal-receiving (lower) cell. In the signal-receiving cell, transcription of genes, as a result of the interplay between Notch ICD and HIF-1α, can fall into at least five potential categories. From left to right: first, a Notch transcriptional output that is not affected by hypoxia. Second, HIF-1α is recruited to Notch ICD and enhances the Notch transcriptional output (via CSL). Third, HIF-1α and Notch ICD are bound to distinct binding sties and have additive effects on transcription. Fourth, Notch indirectly enhances HIF-1α recruitment to HRE sites, possibly via sequestering of FIH-1 away from HIF-1α, which may lead to enhance expression of HIF-1α-responsive genes. Fifth, a HIF-1α transcriptional output that is independent of Notch (Poellinger and Lendahl 2008)**.**

1.5 Biomaterials

Tissue engineering is the use of cells, biological factors, and biomaterials, alone or in combination, with the goal of restoring normal tissue structure and function. Basically, body tissue is composed of two components: cells and the surrounding environment. The latter includes the extracellular matrix (ECM) for cell proliferation and differentiation as the living place of cells and, biosignalling molecules as the nutrients of cells. There are some cases where tissue regeneration is achieved by the single or combinational use of the components in an appropriate way. Biomaterials play a key role in designing and creating substitutes for ECM and biosignalling molecules to enhance their biological activities. In addition to therapeutic applications, biomaterials are also useful in the progress of research and development of stem cell biology and medicine (Tabata Y. 2009).

Various synthetic and natural materials, such as polymers, ceramics and metals or their composites have been used in different manners. Among them, biodegradable biomaterials are important, which is degraded or water solubilized by any process in the body to disappear

from the site implanted. There are two ways of material disappearance. First, the main chain of the material is hydrolysed or enzymatically digested to decrease the molecular weight, and finally disappears. Second, the material is chemically cross-linked to form a hydrogel insoluble in water. When the cross-linking bond is degraded to generate water-soluble fragments, the fragments are washed out from the site implanted, resulting in the disappearance of material (Tabata Y. 2009).

Synthetic polymers are generally degraded by simple hydrolysis while natural polymers are mainly degraded enzymatically. The synthetic polymers can be modified with ease to change their chemical composition and molecular weight, which affect the physicochemical property of the materials. Natural polymers of proteins, polysaccharides and nucleic acids are available. Their degree of freedom for property modification is small when compared with that of synthetic polymers, but they can be chemically modified to produce various derivatives (Tabata Y. 2009).

Biomaterials have different applications areas in tissue regeneration therapy and the basic stem cell research. Biomaterials play an important role in the preparation of cell scaffolds in order to promote cell proliferation and differentiation for *in vivo* tissue regeneration. The biomaterial should be in porous structure for the infiltration of cells, the supply of oxygen and nutriens and washout of cell wastes (Lutolf and Hubbell 2005; Chan and Mooney 2008). The mechanical property of biomaterial scaffolds is also important. If the mechanical strength is low, the scaffold readily deforms in the body

Biomaterials are also used for preparation of a large number of stem cells of high quality, because, currently, several stem cells with a high potential for proliferation and differentiation are being prepared and applied to a tissue defect to induce tissue regeneration (Fernyhough et al. 2008). For example, many researchers of tissue regeneration therapy with stem cells, especially MSC, have reported their therapeutic feasibility in tissue regeneration (Docheva et al. 2007). In addition, neural stem cells and stem cells isolatable from fat tissue have been extensively investigated. Embryonic stem (ES) and inducible pluripotent stem (iPS) cells have been established and expected as cell sources for transplantation therapy (Tabata Y. 2009).

For this purpose, the efficient preparation and proliferation of cells are achieved by providing cell culture substrates as the artificial ECM. The three-dimensional substrate can be designed and prepared from biomaterials of cytocompatibility. From the viewpoint of nutrients and oxygen supplies, research and development of cell culture methods and bioreactors are required (Mironov et al. 2008).

2. AIM OF STUDY

MSCs are potential therapeutic agents which are applicable in tissue engineering, gene delivery, carry high potential for regenerative medicine and do not induce any immune reaction. They reside in the niche structures and in order to keep homeostasis, they migrate to the site of injury. Despite they are future promise for many disease treatments; there are still hurdles to tackle. The amount of MSCs is very low *in vivo,* therefore their yield should be increased. Our aim is to examine the role of environmental factors or conditions on MSC biology.

By using female rat MSCs, we aimed to investigate the following in this thesis;

i. The role of hypoxia and estrogen on MSCs' maintenance.
ii. The effects of estrogen on the expression of Notch receptor in hypoxia.
iii. Determination of appropriate biomaterials for MSCs expansion.

3. MATERIALS AND METHODS

3.1 Animals

Adult female Spraque Dawley rats were used for the experiments. The animals were kept in the animal holding facility of the Department of Molecular Biology and Genetics at Bilkent University under controlled conditions at 22^{o} C with 12 hour light and 12 hour dark cycles. They were provided with unlimited access of food and water. The experimental procedures have been approved by the animal ethical committee of Bilkent University (Bil-AEC 2005/2)

3.2 Isolation of the Bone Marrow from the Animals

After the animals were sacrificed by cervical dislocation for the isolation of the bone marrow, the gastrocnemius and biceps regions were shaved with a razor. Then the femur and tibia were removed, and the ends of bones were cut and flushed with Dulbecco's Modified Eagle Medium (DMEM) (Hyclone) which contains 10% Fetal Bovine Serum (Hyclone) and 1% penicillin/streptomycin solution (Hyclone), by using a 5ml syringe (Figure 3.1). The mixed media and bone marrow started to be centrifuged at 3000 rpm for 5 min then the media was removed and the cells were washed with 1X PBS buffer once. The cells mixed in 1X PBS were centrifuged at 2500 rpm for 5 min and then the buffer was removed and cells were resuspended in 1X PBS buffer again. The mixture was centrifuged at 2000 rpm for 5 min and then the buffer was removed. 1X PBS buffer was added to the cell pellet and cells were resuspended once more. The buffer and cells mixture were centrifuged at 1500 rpm for 5 min and then cells were resuspended in MesenCult (Stem Cell technologies) media to prepare for tissue culturing.

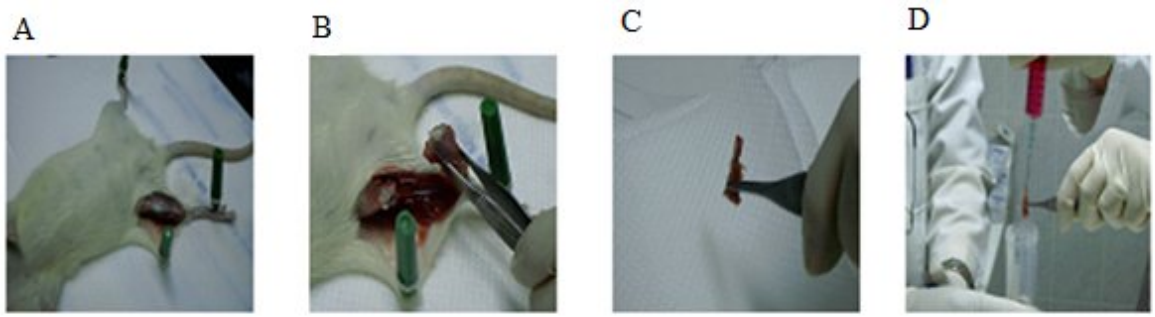


Figure 3. 1: The isolation of the bone marrow of the rats. A- The gastrocnemius and biceps regions were shaved with a razor. B- The femur and tibia were removed. C- The ends of bones were cut. D- Cells were flushed with DMEM

3.3 Cell Culture

3.3.1 Cell Number Detection with Cell Count

After the heterogenous bone marrow cells was washed and precipitated, they were suspended in 10 ml of MesenCult (Stem Cell Technologies) media and the mixture was micropipetted into a hemocytometer. The number of cells in the chamber was determined by counting under the light microscope. The cell number was calculated according to the following formula:

concentration of cells in original mixture =

$$\left(\frac{\text{number of cells counted}}{(\text{proportion of chamber counted})(\text{volume of chamber})}\right)\left(\frac{\text{volume of sample dilution}}{\text{volume of original mixture in sample}}\right)$$

(http://en.wikipedia.org/wiki/Hemocytometer)

3.3.2 MSC Culture

MSCs were cultured by MesenCult media (Stem Cell technologies) with supplement (Stem Cell Technologies), and 1% penicillin/streptomycin solution (Hyclone). Next day, the media of the tissue culture plates were changed and the majority of the non-adherent cells were removed. The media of the cells were changed in every 4-5 days, after washing with sterile 1X PBS prior to the change. The adherent cells on the 14^{th} day of the cell culture were mostly MSCs.

3.3.3 Estrogen Treatment

For the estrogen treatment of cells, 17 ß-estradiol (Sigma) was used, which will be referred to as "estrogen" in the following sections. The cells which will be treated with estrogen were cultured with MesenCult (Stem Cell Technologies) media at first, without estrogen. At every media change including the first day, estrogen was added to the media of the cells at a physiological concentration of 10^{-7} M (Zhou *et al*, 2001).

3.4 Hypoxic Condition

On the 14^{th} day of cultured rat MSCs cells were treated with 100 µM $CoCl_2$ prepared, from the stock solution, in MesenCult (Stem Cell Technologies) medium for 14 h. If necessary, estrogen was added at a concentration of 10^{-7} M after 100 µM $CoCl_2$ solution was prepared. Next day, cells were collected for RNA and protein. Immunohistochemistry was performed on cells grown on cover slip.

3.5 Biomaterials

Four different biomaterials were used in this study. Biomaterial designated as 'CI' was done by mixing hydroxyethyl methacrylate (HEMA) (Sigma, Germany) and hydroxypropylchitosan (HPC) (Sigma, Germany) monomers. Biomaterial designated as 'CII' was prepared by combination of hydroxypropylmethacrylate (HPMA) (Sigma, Germany), polyethylene glycol (PEG) (Sigma, Germany) and HPC monomers. Biomaterial designated as 'CIII' was done by mixing HEMA, PEG and HPC monomers. Biommaterial designated as 'C' was done by combination of HPMA and PEG monomers. Five different biomaterials were obtained from each group through combination of the monomers with different concentration. From CI, CI-A, CI-B, CI-D, CI-E and CI-K were produced. From CII, CII-1, CII-2, CII-3, CII-4 and CII-5 were obtained. From CIII, CIII-1, CIII-2, CIII-3, CIII-4 and CIII-5 were produced. From C, CA1, CA2, CA3, CA4 and CA5 were done. All biomaterials were produced in Gazi University.

3.5.1 Preparation of Biomaterials

The membrane poly HEMA:HPC, poly HPMA:PEG:HPC, poly HEMA:PEG: HPC and poly HPMA:PEG were prepared by photopolymerization. HEMA was distilled under reduced pressure before use. The polymerization was carried out in a glass mold (9 cm diameter). Initiator and a-a-azobisisobutyronitrile (AIBN) (Sigma, Germany) (20 mg) were dissolved in water and was mixed with monomers mixture as indicated in table1. The resulting mixture was equilibrated at 25 ºC for 15 min in the water bath. It was then purged with nitrogen for 2 min and was transferred into moulds. It was sealed and exposed to long-wave ultraviolet radiation for 1 h. After polymerization, the membrane were washed with purified water and sterilized with heat at 121 ºC and 15 min. They were stored at 4 ºC. Combination of the biomaterials was listed in table 3.1.

Table 3. 1: Different composition with varying monomer ratio

CI

HEMA/HPC	v/v
CI-A	2:1
CI-B	5:1
CI-D	11:1
CI-E	1:1
CI-K	1:0

CII

HPMA/PEG/HPC	v/v/v
CII-1	1:1:0
CII-2	1:0:1
CII-3	3:1:2
CII-4	3:2:1
CII-5	3:0:0

CIII

HEMA/PEG/HPC	v/v/v
CIII-1	2:1:1
CIII-2	3:0:1
CIII-3	3:1:0
CIII-4	1:0:1
CIII5	1:1:0

C

HPMA/PEG	v/v
CA-1	**1:0**
CA-2	**1:1**
CA-3	2:1
CA-4	3:1
CA-5	4:1

3.5.2 Growing Rat Bone Barrow Cells on Biomaterials

Before biomaterials were used in the cell culture, they were sterilized under UV light for 15 min and then were put on either 24 well plate or 12 well plate. Bone marrow isolated cells were seeded directly on biomaterials. One day after cells were seeded, MesenCult (Stem Cell Technologies) media was changed. The media was changed every 2 days and cells were washed with 1X PBS buffer. On 14th day, the plates were taken and experiments were performed.

3.6 Standard Solutions and Buffers can be found in Appendix A

3.7 Colony Forming Assay

MSCs were washed with 1X PBS, then they were air dried. MeOH (cold) was used for fixation and left for 5 minutes. Then the cells were washed again with 1X PBS buffer and giemsa staining reagent (Carlo Erba) was added and left for 5 min. The reaction of giemsa staining was stopped by the addition of tap water and at the end the colonies were stained in purple.

3.8 Determination of the Gene Expression

3.8.1 Total RNA Isolation from Rat MSCs

On the 14th day of the cell culture, total RNAs from the MSCs were isolated. The cells were first washed with 1X PBS buffer in order to eliminate the non-adherent cells in the media. Then the cells were trypsinized at 37^{o}C for 5 min. DMEM (Hyclone) which contains 10% Fetal Bovine Serum (Hyclone) and 1% penicillin/streptomycin solution (Hyclone) was added to the trypsinized cells and centrifuged at 1500 rpm for 5 min. Then the media was removed and the precipitated cells were washed with 1X PBS buffer and centrifuged again at 1500 rpm for 5 min. Next the buffer was removed and the total mRNA was isolated from the precipitate by using RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The RNA isolated was stored at -80^{o}C.

3.8.2 Concentration and Integrity of RNA

The concentration of RNA samples were measured via NanoDrop ND-1000 (Nano Drop Technologies, USA) and the quality of RNAs was determined by measuring the ratio A_{260}/ <A_{280}. The O.D.$_{260}$/ O.D.$_{280}$ ratios were calculated in order to determine if there is a phenol, protein or DNA contamination in the RNA samples. The expected values are between 1,6-2,0. In order to check the integrity of the isolated RNAs, denaturing agarose gel electrophoresis was performed.

3.8.3 Denaturing Gel Electrophpresis

Denaturing gel electrophoresis was performed to check the integrity of the isolated RNAs. 1.2% agarose gel was prepared with DEPC-treated water, including 10X MOPS, 3.6% formaldehyde and 30 ng/mL ethidium bromide solution. The electrophoresis tank contained 1X MOPS during running the gels. The samples were prepared by adding 2µl of Agarose Gel Loading Dye, 3µl RNA sample and 3µl DEPC-treated H_2O and were kept at 65 ^{o}C for 15 minutes in order to denature the RNA. The gel was run at 90V, for 30 minutes and visualized under the transilluminator (Gel-Doc Bio-Rad, USA or Vilber Lourmat, France). Chemicapture (Vilber Lourmat, France) software was used in order to take the photographs of the gels.

3.8.4 The cDNA Synthesis

The cDNAs were synthesized from the total RNA samples with DyNAmo cDNA Synthesis Kit (Finnzymes, Finland) according to the manufacturer's protocol. 2µg RNA was mixed with DEPC- ddH2O to a total volume of 14µl and 2µl of oligo(dT) primer was added to a total volume of 16 µl. They were incubated at 65 ^{0}C for 5 minutes and then chilled on ice for 3 minutes. Then 20µl of 2X RT buffer including dNTP mix and 10mM MgCl2 and 4 µl of M-MuLV RT RNase H enzyme was added to the mixture and incubated at 25 ^{0}C for 10 min, 45 ^{0}C 45 min, and 85^{0}C 5 minutes, respectively. The cDNAs were stored at -20 ^{0}

3.9 RT- PCR

3.9.1 Primer Design

We have designed primers (Table 3.2) for*, Notch1, Notch2, Notch3, Notch4,* beta-actin, CD90, CD34, CD29, CD45 and CD71. The cDNA sequences of the rat homologues of these genes are available at the Ensemble database. The Ensemble Accession numbers of the rat homologues of *Notch1* is ENSRNOT00000008312; *Notch2* is ENSRNOT00000025718, *Notch3* is ENSRNOT00000037456, *Notch4* is ENSRNOT00000000513, CD90 is ENSRNOT00000008685, CD 34 is ENSRNOT00000010148, CD 29 is ENSRNOT00000014785, CD 71 is ENSRNOT00000002407, CD 45 is ENSRNOT00000060292 and beta- actin is ENSRNOT00000042459

Table 3. 2: The sequences and the sizes of the primers

Notch 1 (200 bp)	Forward Reverse	: 5'-TCAACACACTGGGCTCTTTC -3' : 3'-CCCTCATAACCTGGCCATACA-5'
Notch 2 (220 bp)	Forward Reverse	:5'-TGATGAGCAGGACAGGAGA-3' :5'AACCGCAAGCAGATAGAGGA-3'
Notch 3 (225 bp)	Forward Reverse	:5'-AACTGCCGAAGCGACATAGA -3' :5'-GAAGCCAGGAAGACAAGCAC-3'
Notch 4 (193 bp)	Forward Reverse	:5'-ACCTTTCATCTCTGCCTCTGTC -3' :5'- CCTGGGCTTCACATTCATCTC-3'
CD 90 (394 bp)	Forward Reverse	:5'- CCAGTCATCAGCATCACTCT-3' :5'- AGCTTGTCTCTGATCACATT- 3'
CD 34 (281 bp)	Forward Reverse	: 5'- TGTCTGCTCCTTGAATCT -3' : 5'- CCTGTGGGACTCCAACT- 3'
CD 29 (190 bp)	Forward Reverse	: 5'-ACTTCAGACTTCCGCATTGG -3' : 5'- GCTGCTGACCAACAAGTTCA-3'
CD 71 (182 bp)	Forward Reverse	:5'-ATGGTTCGTACAGCAGCAGA -3' :5'- CGAGCAGAATACAGCCATTG-3'
CD 45 *(175 bp)*	Forward Reverse	: 5'- ATGTTATTGGGAGGGTGCAA-3' : 5'- AAAATGTAACGCGCTTCAGG-3'
Beta actin *(65 bp)*	Forward Reverse	: 5'-CTGGCCTCACTGTCCACCTT-3' : 5'-GGGCCGGACTCATCGTACT-3'

The RT-PCR reaction was performed by using two different Taq polymerase enzymes. The reaction ingredients used in RT- PCR reactions are shown in Table 3.3 and Table 3.4, and the conditions of notch receptor PCR reactions are shown in Figure 3.2 and the conditions of PCR reactions for CD markers are shown in Figure 3.3.

Table 3. 3 PCR mixture with Taq Polymerase

PCR Mixture	Volume
cDNA	1µl
10x Taq Buffer	2.5µl (1X)
10mM dNTP	0.5µl (0.2µM)
50mM $MgCl_2$	0.75µl (1,5mM)
Forward Primer	1µl (10 pmol)
Reverse Primer	1µl (10 pmol)
Taq DNA polymerase	0.5µl (1U)
ddH_2O	17.75 µl
Total	**25µl**

Table 3. 4: PCR mixture with Phire Hot Start DNA Polymerase

Reaction Mixture	Volume
cDNA	1 µl
5x Reaction Buffer (Finnzymes, Finnland)	4 µl (1X)
Forward Primer (Invitrogen)	1 µl (10 pmol)
Reverse Primer (Invitrogen)	1 µl (10 pmol)
10 mM dNTP (MBI Fermentas)	0,4 µl (0,2mM)
Phire Hot Start DNA Polymerase (Finnzyme, Finland)	0,4 µl (1U)
ddH_2O	12,2 µl
Total	**20 µl**

Notch1

Denaturation 5 min at 95°C

30 sec. at 94°C
30 sec. at 55°C
30 sec. at 72°C
} 32 cycles

Extension: 5 min. at 72 °C

Notch2

Denaturation 30 sec at 98°C

5 sec. at 98 °C
5 sec. at 60 °C
4 sec. at 72 °C
} 30 cycles

Extension: 1 min. at 72 °C

Notch3

Denaturation 30 sec at 98°C

5 sec. at 98 °C
5 sec. at 63 °C
4 sec. at 72 °C
} 30 cycles

Extension: 1 min. at 72 °C

Notch4

Denaturation 30 sec at 98°C

5 sec. at 98 °C
5 sec. at 63 °C
4 sec. at 72 °C
} 30 cycles

Extension: 1 min. at 72 °C

CD90

Denaturation 5 min at 95°C

30 sec. at 94 °C
30 sec. at 55 °C
30 sec. at 72 °C
} 30 cycles

Extension: 5 min. at 72 °C

CD34

Denaturation 5 min at 95°C

30 sec. at 94 °C
30 sec. at 55 °C
30 sec. at 72 °C
} 30 cycles

Extension: 5 min. at 72 °C

CD71

Denaturation 5 min at 95°C

45 sec. at 94 °C
60 sec. at 66 °C
45 sec. at 72 °C
} 35 cycles

Extension: 5 min. at 72 °C

CD29

Denaturation 5 min at 95°C

30 sec. at 94 °C
30 sec. at 60 °C
30 sec. at 72 °C
} 23 cycles

Extension: 5 min. at 72 °C

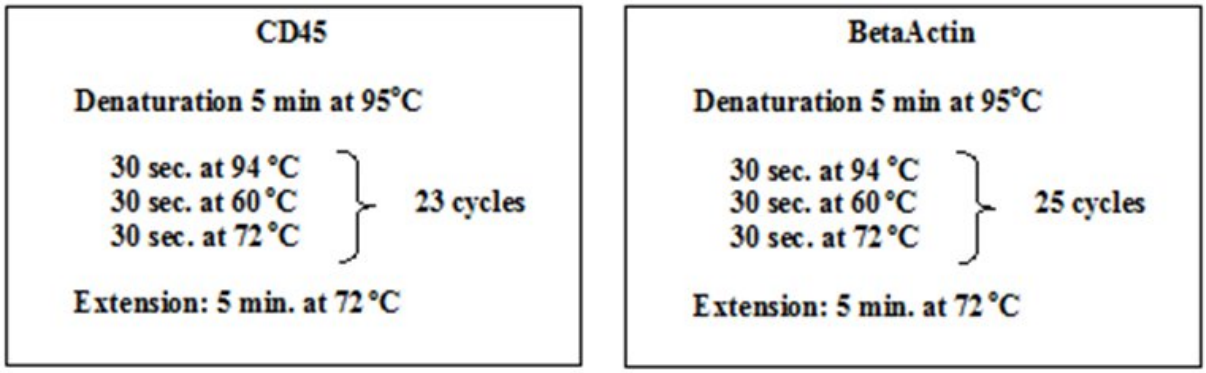


Figure 3. 2: PCR conditions and cycles

3.10 Agarose Gel Electrophoresis

2% agarose gel was prepared with 1X TAE Buffer and 1 mg/ml ethidium bromide solution. Samples were prepared by addition of 3µl Agarose Gel Loading Dye to 10µl of cDNA sample and loaded to the agarose gel. The gel was run at 120V for 30 minutes and visualized under transilluminator (Gel-Doc Bio-Rad, USA or Vilber Lourmat, France). Chemicapture (Vilber Lourmat, France) software was used to take photographs of the gels. The gene Ruler DNA Ladder Mix (Fermentas, #SM0331) was used as a marker and 5µl was loaded to every gel.

3.11 Protein Expression

3.11.1 Total Protein Isolation from the MSCs

The total proteins of the MSCs were isolated on the 14^{th} days of the cell culture. The cells were washed with 1X PBS buffer twice in order to wash out the non adherent cells. Then the cells were scraped with a scraper and put into a 15 ml falcon tube. Then the mixture was centrifuged at 1500 rpm for 5 minutes. The 1X PBS was removed and the precipitate was suspended in lysis buffer which was prepared freshly and checked against any protein contamination with Bradford reagent prior to use. The mixture was left on ice for 30 min and mixed gently every 5 -10 minutes. Then the mixture was centrifuged at 13000 rpm for 20 minutes. The supernatant which contained the proteins was taken to a separate eppendorf tube. It was first dipped into liquid nitrogen for a few seconds and then stored at -80^0C.

3.11.2 Protein Quantification

Protein quantification was made with Bradford assay. A standard curve was made by using 10 different BSA concentrations as in Table 3.5. A standard curve was formed by measuring the different BSA concentrations at a fixed wavelength of 595 nm. A formula was

derived from the graph derived by putting the O.D. measurements to the y axis and BSA amount to the x axis. Then a second set of measurements was made by taking the lysis buffer sample containing 900µl Bradford reagent+98µl ddH2O+ 2µl lysis buffer as blank. The other samples contained 900µl Bradford reagent+98µl ddH2O+ 2µl protein sample and was measured at 595 nm fixed wavelength as well. The O.D. measurements were put to the formula derived from the standard BSA concentrations, and the concentrations of the proteins were derived.

Table 3. 5 The standards used for the derivation of the standard curve for protein concentration detection

Standards	Std1	Std2	Std3	Std4	Std5	Std6	Std7	Std8	Std9	Std10
BSA(1µg/µl)	0 µl	2,5µl	5 µl	7,5µl	10µl	12,5µl	15µl	20µl	25µl	35µl
ddH2O	100µl	97.5µl	95µl	92.5µl	90µl	87.5µl	85µl	80µl	75µl	65 µl
Bradford Reagent	900µl	900µl	900µl	900µl	900µl	900µl	900µl	900µl	900µl	900µl

3.12 Western Blotting

3.12.1 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The protein samples were separated by SDS-PAGE gel with 7.5% resolution gel, and 5% stacking gel. The proteins were loaded with a concentration of 50ug. A total volume of 25µl was loaded to the gel which contained the protein, 2% cracking buffer containing 10% ß-mercaptoethanol. The samples were left at 90^0C for 5 minutes and then they were put on ice for 5 minutes. The PageRuler Prestained Protein Ladder (SM06510, MBI Fermentas, Ontario, Canada) was used as a protein molecular weight marker and 5µl was loaded for each gel. The gel was run at 80 volts in 1X cold running buffer until the proteins passed from the stacking gel to the resolution gel. Then the gel was run at 120 volts until the dyes reached the end of the glass and was stopped. The gel was removed from between the glasses. After the stacking part of the gel was removed and washed with dH_2O, it was put in the semi-dry transfer buffer.

3.12.2 Transfer of Proteins to the Membrane

The proteins were transferred onto the membrane by semi-dry transfer. 4 Whatman papers, and the resolution part of the SDS-PAGE gel which are at the sizes of 5,5 cm to 8,5cm were left in the semi-dry transfer buffer for 10 minutes. The membrane was left in MetOH for 30 seconds, in dH_2O for 2 minutes, and then in semi-dry transfer buffer for 10 minutes. Then two of the Whatman papers, the membrane, the gel, and 2 more Whatman papers were put on

to the semi-dry transfer apparat (BioRad) in this order. The bubbles between the layers were removed and the transfer was made at 0,16 Ampere for 55 minutes. After the transfer was completed, the membrane was washed 5 minutes with 1X TBS-T buffer before the proteins were immunologically detected.

3.12.3 Immunological Detection of Immobilized Proteins

After washing, the membrane was left in the blocking solution for 1 hour at RT on an orbital shaker. Primary antibody of Hydroxy-HIF-1alpha (D43B5 Cell Signaling Technology, USA) was prepared in 5% BSA dilution buffer and was left overnight at 4^{o}C. Primary antibodies of ER-a (MC-20 sc-542 Santa Cruz Biotechnologies), ER-b (H-150 sc-8974 Santa Cruz Biotechnologies) were prepared in block solution at 1/200 concentration and was left overnight at 4^{o}C. The following day the membrane was washed with 1X TBS-T three times for 5 minutes. They were incubated with the secondary antibody of Anti-Rabbit IgG, HRP (Cell Signaling Technology, USA) at a concentration of 1:2500 for 1 hour at RT on an orbital shaker (Thermolyne). The membrane was washed again three times for 5 minutes. ECL Plus Detection Reagent (Amersham Biosciences, New Jersey, USA) was added on to the membrane and left for 5 minutes. The excess detection reagent was drained off, the membrane was placed on glass, and covered with stretch film. The wrapped glass was placed in a x-ray film cassette. Films were exposed to x-ray for between 30 seconds and 1 minute, respectively. After exposure, the films were developed (Amersham).

3.12.4 Coomassie Blue Staining of the Gel and Membrane

The membrane and gel were left in the staining solution for 30 min, and then they were left in destaining solution overnight, by changing the solution on the membrane and gel each time the colour of the destaining solution turned into blue.

3.13 Immunohistochemistry Staining

The cells were grown on cover slips (Deckglaser 100 No.1 12mm) in the cell culture plates. The plates were taken from the 37^{o}C incubator and the media was removed. The cells were washed twice with 1X PBS and fixed with cold MeOH for 10 minutes. The cells were washed again with 1X PBS twice and left in 3% H_2O_2 for 15 minutes. After washing three times with 1X PBS, the cells were left in blocking solution for 30 minutes at RT. After the completion of the blocking step, the cells were incubated with Notch 1 antibody (M-20 sc-6015 Santa Cruz Biotechnologies) at a concentration of 1:200, Notch 3 antibody (M-20 sc-832 Santa Cruz Biotechnologies) at a concentration of 1:200, Notch 4 antibody (M-18 sc-32636 Santa Cruz Biotechnologies) at a concentration of 1:200. The samples were left in the

antibody solution for 16 hours at 4°C. The next day the cells were taken and washed with 1X PBS twice. The secondary antibodies of Anti-Goat-FITC (Sigma) with 1:5000 dilution for Notch1, Notch3 and Notch4 antibodies were added and incubated for 1 hour at RT in the dark. After this incubation, the cells were washed twice with 1X PBS. 100µl of Biotin-link yellow solution (Dako, Carpinteria, USA) was added and left for 10 min. The cells were washed with 1X PBS twice and 100µl Streptavidin-HRP red solution (Dako, Carpinteria, USA) was added and left for 10 min. Cells were washed with 1X PBS twice and then DAB+Chromogen (Dako, Carpinteria, USA) substrate prepared by dissolving 1 drop of DAP+Chromogen reagent in 1ml DAB + Substrate Buffer (Dako, Carpinteria, USA) solution was added and incubated until brownish color was observed. Cells then were washed with tap water three times. Hemoatoxylene solution was added for counterstain and left for 1-1.5 min. Cells were washed with tap water for three times and then the specimens were mounted using mounting medium (Dako,USA) and covered for further studies. The photos of the slides were taken with the light microscope 40X objectives (Leica TCS/SP5, JAPAN) without water immersion equipped with Lecic software.

3.14 Immunofluorescent Staining

The cells were grown on cover slips (Deckglaser 100 No.1 12mm) in the cell culture plates. The plates were taken from the 37°C incubator and the media was removed. The cells were washed twice with 1X PBS and fixed with MeOH for 10 minutes. Then the cells were washed again with 1X PBS twice and left in 3% H_2O_2 for 15 minutes. After washing three with 1X PBS, the cells were left in blocking solution for 30 minutes at RT. After the completion of the blocking step, the cells were incubated with CD90 antibody (Chemicon-MAB1406) at a concentration of 1:500 and CD34 antibody at a concentration of 1:500 (sc-7324, Santa Cruz Biotechnologies). The samples were left in the antibody solution for 1 hour at RT. The cells were taken and washed with 1X PBS twice. The secondary antibodies of Anti-Mouse-FITC (Sigma-F2012-1ML) with 1:100 dilution for CD34 and CD 90 antibody in block solution were added and incubated for 1 hour at RT in the dark. After the secondary incubation, the cells were washed twice with 1X PBS. After the washing, the specimens were mounted using UltraCruz (Santa Cruz) mounting medium with DAPI. CD90, CD34 and DAPI staining were investigated under the fluorescent microscope (Leica TCS/SP5, JAPAN) equipped with Lecic software for image analysis at an excitation wavelength 490nm for FITC and 359nm for DAPI. The images were taken by 40X objectives without water immersion.

3.15 In Situ Cell Death Detection (TUNEL ASSAY)

DNA fragmentation was detected in situ by TdT- (terminal deoxynucleotidyl transferase) mediated fluorescein-dUTP labeling kit (Roche Diagnostics, Mannheim, Germany). 14th day of cells which was adherent to the cover slips (Deckglaser 100 No.1 12mm) washed with 1X PBS twice and then some groups of cells were treated with MesenCult medium, some groups of cells treated with MesenCult medium with $CoCl_2$, some groups of cells were treated MesenCult medium with estrogen and, some group of cells were treated with MesenCult medium containing $CoCl_2$ and estrogen, for 14h. Next day, cells were washed with 1X PBS and were fixed in freshly prepared 4% paraformaldehyde in PBS for 1 hour at room temperature. Then the cells were washed with 1X PBS for 3 times and incubated with a freshly prepared permeabilisation solution (0.1 % Triton X-100, 0.1 % sodium citrate) for two minutes on ice. Then the cells were washed again with 1X PBS twice, and 50 µl of TUNEL reaction mixture was added onto each sample and incubated for one hour at 37^{0}C in the dark in a humidified chamber. The samples were washed with 1XPBS twice and cover slips then were closed by UltraCruz (Santa Cruz) mounting medium with DAPI. Slides were then analyzed by fluorescent microscopy (Leica TCS/SP5, JAPAN) equipped with Lecic software for image analysis at an excitation wavelength 490nm and 359nm for DAPI. Positive cells were counted by 40X objectives. As negative control, we incubated the slides in the absence of terminal deoxynucleotidyl transferase. For positive controls, the samples were first treated for 10 minutes with DNaseI (Fermentas- EN0521) at a concentration of 0,4u/µl at RT to induce DNA strand breaks prior to labeling procedures, and then incubated with 50 µl of TUNEL reaction mixture. The samples were kept at dark at 4^{0}C.

3.16 BrdU incorporation assay

14th day of rat bone marrow cells grown on 75cm flask (Greiner Bio-One, Germany) were trypsinized at 37^{o}C for 2 min after washing with 1X PBS twice. DMEM (Hyclone) which contains 10% Fetal Bovine Serum (Hyclone) and 1% penicillin/streptomycin solution (Hyclone) was added to the trypsinized cells and centrifuged at 1500 rpm for 5 min. Then the media was removed and was washed with cold 1X PBS and centrifuged again at 1500 rpm for 5 min. Cells were resuspended in MesenCult medium with or without estrogen and 50000 cells were seeded on 18mm cover slip (Dechglasser). After 50% confluency was obtained, some groups of cells were treated with MesenCult medium, some groups of cells treated with MesenCult medium containg $CoCl_2$, some group of cells treated with MesenCult medium containg estrogen, and some groups of cells were treated with MesenCult medium containing

$CoCl_2$ and estrogen, for 14h. Next day, cells were treated with BrdU prepared by mixing 10mg/ml BrdU in 10mL MesenCult medium, in MesenCult with estrogen, in MesenCult with $CoCl_2$ and in MesenCult with estrogen and $CoCl_2$. Cells were incubated at 37^{o}C for 1h. After incubation of BrdU, cells were washed with 1X PBS twice then fixed with 70% cold ethanol for 10 min. Cells were washed with 1X PBS twice again and then cells were incubated with 2N HCl for 20 min at RT. Cells were washed with 1X PBS for three times. Block solution was added and left for 15 min. After the removal of the block solution, mouse anti-BrdU primary antibody (MO744,Dako, USA) prepared in block solution was added at a concentration of 1:500 and incubated for 1h at RT. The cells were washed with block solution three times and as a secondary antibody, Anti-Mouse-FITC (Sigma-F2012-1ML) with 1:100 dilution was added and incubated for another 1h at RT in the dark. Cells were washed with the block solution three times and cover slips then were closed on slides by UltraCruz (Santa Cruz) mounting medium with DAPI. Slides were then analyzed by fluorescent microscopy (Leica TCS/SP5, JAPAN) equipped with Lecic software for image analysis at an excitation wavelength 490nm for FITC and 359nm for DAPI. Positive cells were counted.

3.17 *In Silico* Analysis

3.17.1 Finding Transcription Factors

10000bp upstream sequence of Notch1 from the first exon was found by using BioMart. Transcription factors that bind this region was found by using MatInspector database, which is a software tool that utilizes a large library of matrix descriptions for transcription factor binding sites to locate matches in DNA sequences.(Cartharius et al., 2005). The sequence of 10000bp upstream region of Notch1 was entered as FASTA format to the MatInspector. Transcription factor binding sites weight matrices then was chosen. Transcription factors binding to the sequence was selected whose core similarity between 0.9-1, and whose matrix similarity is greater than 0.8. The maximum core similarity of 1.0 is only reached when the highest conserved bases of a matrix match exactly in the sequence. Matrix similarity takes into account all bases over the whole matrix length. A perfect match to the matrix gets a score of 1.00 (each sequence position corresponds to the highest conserved nucleotide at that position in the matrix), a "good" match to the matrix usually has a similarity of >0.80 (Cartharius et al., 2005).

3.17.2 Finding Estrogen induced Transcription Factors

Publicly available datasets were collected from Gene Expression Omnibus (GEO) (Table 3.6). 12 microarrays were used for this study. The datasets were normalized with RMA using BRB-tool which uses Bioconductor R packages when raw .cel files were available, if unavailable, the series matrix files were used and global median normalization was performed over the normalized data for each GSM, separately. After normalization step, one-way ANOVA ($p<0.001$) was applied to the normalized data. Estrogen upregulated genes were selected by drawing scatter plot in BRB-tool, according to the experimental setup of each analyzed microarray

Table 3. 6: Publicly available gene expression data from estrogen studies on cell lines

GSE NO	Institution	Author	Cell Type	Platform	Data Source	Number of Arrays	Data Process	Pubmed ID
GSE1153	University of Illinois	Stossi F. et.al., 2004	U2OS	HG_U95A	Series Matrix	20	MAS 5.0	15033914
GSE2292	Mayo Clinic	Monreo DG. et.al., 2005	U2OS	HG-Focus	Cel File	45	RMA	15802376
GSE9757	University of Rochester	Nott SL. et.al., 2009	MDA-MB-231	HG-U133_Plus_2	Cel File	12	Affy. signal method	19321454
GSE9758	University of Rochester	Nott SL. et.al., 2009	MDA-MB-231	HG-U133_Plus_2	Cel File	12	Affy. signal method	19321454
GSE9759	University of Rochester	Nott SL. et.al., 2009	MDA-MB-231	HG-U133_Plus_2	Cel File	18	Affy. signal method	19321454
GSE11352	Genome Institute of Singapore	Lin CY et.al., 2oo7	MCF-7	HG-U133 Plus2	Cel File	18	RMA	17542648
GSE11506	Northwestern University	Lin Z. et.al., 2007	MCF-7	HG-U133 Plus2	Cel File	9	RMA	17510434
GSE2251	Unilever	Moggs JG. et.al., 2005	MDA-MB-231	HG-U133A	Series Matrix	12	MAS 5.0	15821115
GSE4025	University of Illinois	Frasor J. et.al., 2006	MCF-7	HG-U133A	Series Matrix	17	RMA	16849584
GSE4648	Mass General Hospital Cancer Center	Coser KG. et.al., 2003	MCF/BUS	HG-U133A	Series Matrix	34	MAS.5.0	14610279
GSE4006	University of Illinois	Chang EC. et.al., 2006	MCF-7	HG-U133A	Series Matrix	12	GCRMA	16809442
GSE11324	Baylor College of Medicine	Carroll JS. et.al, 2006	MCF-7	HG-U133 Plus2	Cel File	12	RMA	17013392

Summary of the mentioned microarray studies is explained below:

Gse1153: U2OS human osteosarcoma cells were stably transfected with human ERα or ERß The U2OS-ER cells were grown in MEM with phenol red supplemented with 15% fetal bovine serum, 100 U penicillin/ml, 100 µg streptomycin/ml, and 400 µg G418/ml. Before use in experiments, cells were grown in MEM without phenol red and supplemented with 5% charcoal-dextran-treated fetal bovine serum for at least 4 d before the start of E2 treatment. Cells were treated with 10 nM E2 for 4, 8, 24, or 48 h. Control cell samples were also treated with 0.1% ethanol control vehicle for 48 h (Stossi F. et.al., 2004).

Gse2292: The U2OS-ERα, U2OS-ER, and U2OS-ERα/ß cells pretreated with 100 ng/ml Dox for 24 h in CS-FBS media. The cells were then treated with 100 ng/ml Dox plus ethanol control, 10 nM E2, or 10 nM 4HT for 24 h (Monroe DG. et.al., 2005)

Gse9757: Cells were infected with the recombinant adenovirus bearing no cDNA or cDNA for ERα for 48h. Infected cells were then treated with 1 nM Estradiol 17beta for 6h (Nott SL. et.al., 2009).

Gse9758: Cells were infected with the recombinant adenovirus bearing no cDNA or cDNA for ERα 203/204/211E (3411E) for 48h. Infected cells were then treated with 1 nM Estradiol 17beta for 6h (Nott SL. et.al., 2009) .

Gse9759: Cells were infected with the recombinant adenovirus bearing no cDNA or a cDNA for ERβ, or cDNA for ERβ mutant defective in binding to ERE (ERbDBD) for 48 hours. Infected cells were then treated with 1 nM Estradiol 17beta for 6h (Nott SL. et.al., 2009).

GSE11352: MCF-7 cells were grown in D-MEM/F-12 supplemented with 10% FBS. Cells were washed with PBS and incubated in phenol red-free D-MEM/F-12 medium supplemented with 0.5% charcoal-dextran stripped FBS for 24 h in preparation for 17β-estradiol (E2) treatment. MCF-7 cells were treated with 10 nM E2 for 12, 24, and 48 h (Lin CY. et.al., 2007)

Gse11506: MCF7 cells were treated with vehicle or E2 at a concentration of 10E-9 mol/L for 3 and 6 h (Lin Z.et.al., 2007)

GSE 2251: ER-negative MDA-MB-231 breast cancer cells were infected with recombinant adenovirus encoding full-length human ERα (Ad-ERα) or control vector (Ad-LacZ) for 24 h, and treated with 0·01% ethanol (vehicle control) or 10-8 M 17beta-estradiol (E2) for 48h (Moggs JG. et.al., 2005)

GSE 4025: MCF-7 cells expressing endogenous ERalpha were infected with adenovirus carrying either estrogen receptor beta (AdERb) or no insert (Ad) at multiplicity of infection (moi) of 10. Cells were infected with adenovirus for a period of 48hr before treatment with ligand (vehicle control, 10nM 17beta-estradiol, or 10nM hydroxytamoxifen) for a additional period of 24hr before harvest (Frasor J. et.al., 2006)

GSE4668: MCF7/BUS cells were maintained in DMEM supplemented with 5%FBS.Before the treatment, cells were washed three times with phenol red-free DMEM supplemented with 5% charcoal-dextran stripped FBS and then grown in the presence of varying concentrations of E2 (0 pM, 10 pM, 30 pM, 60 pM, and 100 pM) for 48 hours. In another experiment, MCF7/BUS cells were subjected to hormone starvation for 0, 1 or 2 days (Coser KR. et.al., 2003)

Gse4006: MCF-7 cells expressing endogenous ERalpha were infected with adenovirus carrying either estrogen receptor beta (AdERb) or no insert (Ad) at multiplicity of infection (moi) of 5 or 50. Cells were infected with adenovirus for a period of 48hr before treatment with ligand (vehicle control or 10nM 17beta-estradiol) for a additional period of 24hr before harvest (Chang et al, 2006)

GSE11324: MCF7 cells were grown in a hormone-deprived media for 24 hours before the treatment. Then, cells were stimulated with 100 nM estrogen for 0, 3, 6 or 12 hours (Carroll, Meyer et al. 2006).

3.17.3 Finding Common Genes

The intersect of the genes found by MatInspector (section 3.17.1) and estrogen upregulated genes (section 3.17.2) was found by WebGestalt tools, stands for WEB-based GEne SeT AnaLysis Toolkit, incorporated information from different public resources. WebGestalt is designed for functional genomic, proteomic and large scale genetic studies from which high-throughput data are continuously produced (Zhang B. et. al., 2005). Transcription factors list coming from MatInspector was uploaded into Webgestalt. The gene lists coming from each reanalyzed microarray were uploaded into the database as well.

3.18 Statistical Analysis

The statistical difference between two groups was analyzed by the two-tailed, Student's *t* test using Microsoft Office Excel. Significant difference between two groups was declared if $p < 0.05$.

4. RESULTS

4.1 Characterization of Rat MSCs

First isolated bone marrow cells were examined for the expression of mesenchymal stem cell markers (CD29, CD71 and CD90) and hematopoietic cell markers (CD34, CD45). By using RT-PCR, we showed that these cells express CD29, CD71 and CD90 but not CD34 and CD45 (Figure 4.1) Immunoflourescence staining of these cells revealed the presence of CD90 and absence of CD34 at the protein level (Figure 4.2).

Our results showed that isolated bone marrow cells are MSCs, suggesting that isolated cells are not mixed with HSC or other cell types. Therefore, we obtained a population of MSCs after 14 days.

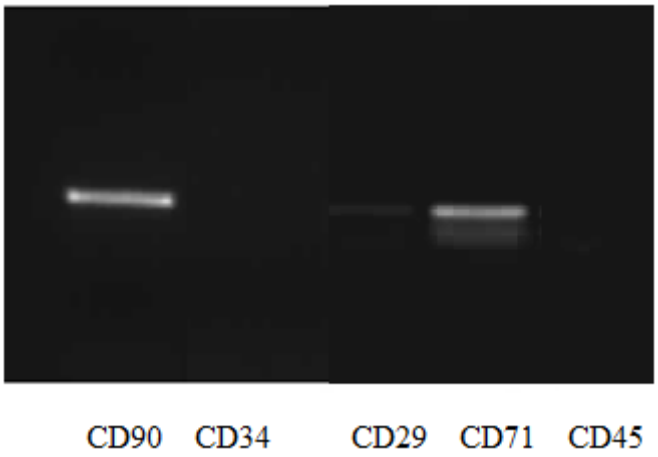
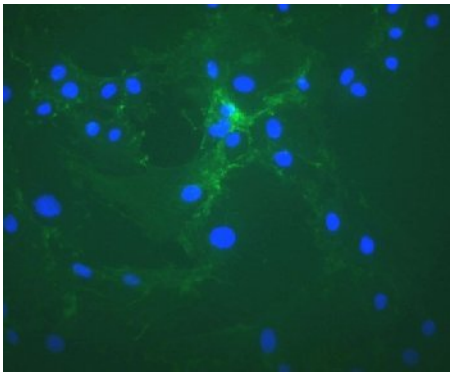
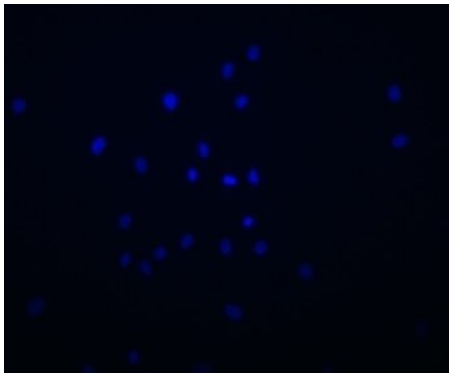


Figure 4. 1: The expression of CD markers in rat bone marrow isolated cells after 14 days.

A



B



C

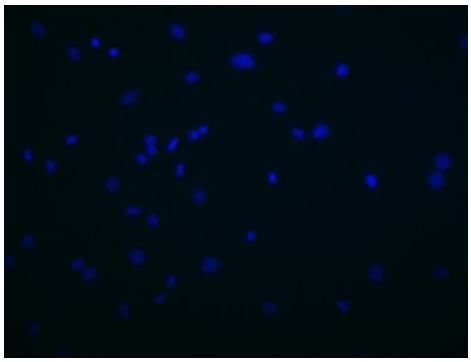


Figure 4. 2: The expression of CD90 (A) and CD34 (B) in bone marrow isolated cells after 14 days. (C) is negative control. Green fluorescence signal was from FITC-secondary antibody, while blue fluorescence signal was from nuclear DNA counterstained with DAPI. The magnification was 40X.

4.2 Role of Hypoxia and Estrogen in MSCs Biology

We wanted to test whether two physiological stimulus play a role in MSCs biology by acting together. In order to do this, hypoxic conditions were mimicked by treating cells by $CoCl_2$. Estrogen was at the dose of 10^{-7} M given together with 100μM $CoCl_2$ in order to compare the effects of estrogen on MSCs in hypoxic condition.

4.3 Characterization of MSCs after $CoCl_2$ treatment

In order to establish a hypoxic condition, female rat bone marrow isolated cells were treated with 100 μM $CoCl_2$, as indicated in materials and methods, after 14 days. In order to investigate the effect of estrogen in hypoxic condition 10^{-7} M estrogen was also mixed with $CoCl_2$ and cells were treated with this mixture.

In order to detect whether bone marrow MSCs cells displayed the feature of MSCs after $CoCl_2$ treatment, the expression level of MSCs markers were controlled by RT-PCR (Figure 4.3).

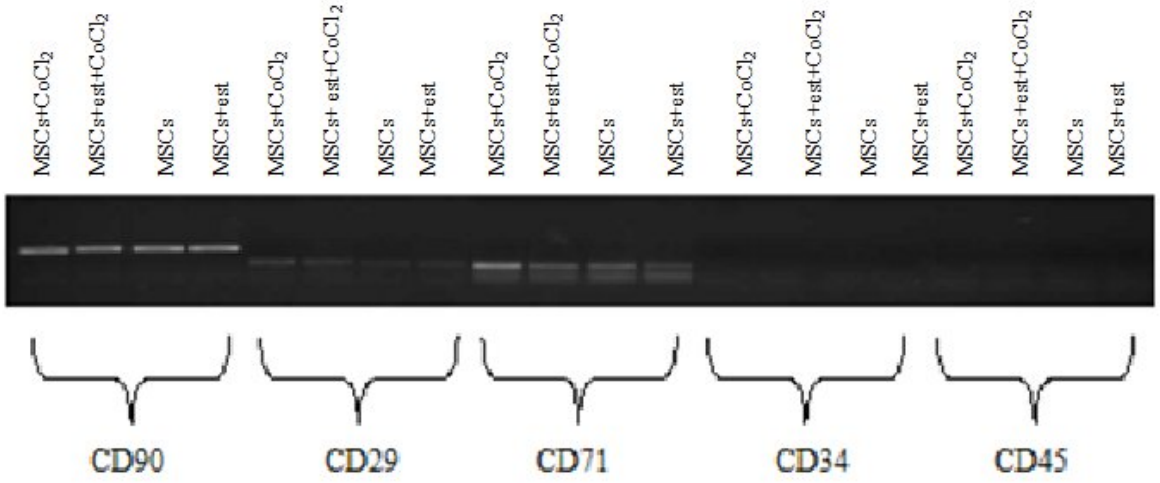


Figure 4. 3: Expression levels of CD90, CD29, CD71, CD34, CD45. MSCs+$CoCl_2$ represents mesenchymal stem cell treated with $CoCl_2$, MSCs+est+$CoCl_2$ represents mesenchymal stem cell treated with estrogen and $CoCl_2$, MSCs represents mesencymal stem cell without treatment, MSCs +est represents mesencymal stem cells treated with estrogen

As shown in Figure 4.3, the cells expressed mesenchymal stem cell markers but not hematopoetic markers after $CoCl_2$ treatment, suggesting that $CoCl_2$ treatment did not alter the characteristics of these cells.

4.4 Expression of HIF-1α upon $CoCl_2$ treatment.

Cellular response to hypoxia is manifested by the activation of the hypoxia-inducible factor-1 (HIF-1) which consists of the O_2-regulated HIF-1α subunit and the O_2-independent HIF-1β subunit. Under normoxia, HIF-1α protein becomes hydroxylated at proline-402 and proline-564 in its O_2-dependent degradation (ODD) domain and is targeted by the von Hippel-Lindau protein for proteasome-mediated degradation. As pO_2 decreases to hypoxic levels, HIF-1α is no longer hydroxylated and thus becomes stabilized. Upon nuclear translocation, HIF-1α dimerizes with the O_2-independent HIF-1α to initiate gene transcription (Lin Q. et.al.2006). Therefore, we investigated the expression level of Hif-1α upon $CoCl_2$ treatment. Since our antibody recognizes hydroxylated Hif-1α, we expected to see the accumulation of hydroxylated Hif-1α in normoxia, but not in the hypoxic environment which was provided by the treatment with $CoCl_2$. As shown in Figure 4.4, hydroxylated Hif -1α was present in normoxic but not in hypoxic condition suggesting that we induced hypoxia with $CoCl_2$.

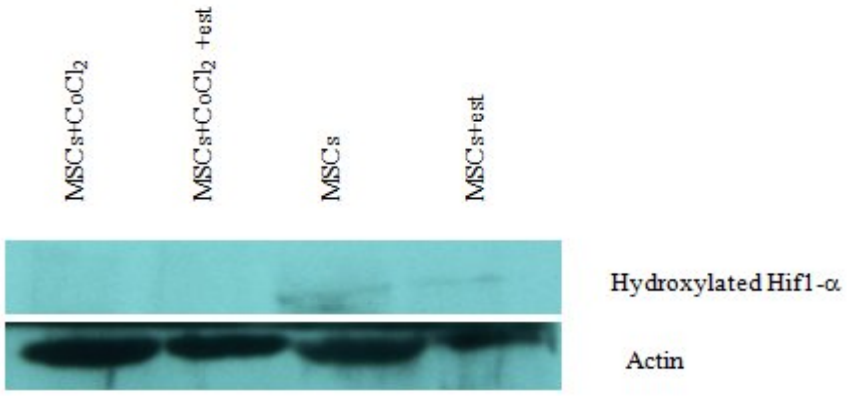


Figure 4. 4: Expression level of hydroxylated Hif-1α in samples treated with or without $CoCl_2$. Actin was used as a loading control. MSCs+$CoCl_2$ represents mesenchymal stem cell treated with $CoCl_2$, MSCs+est+$CoCl_2$ represents mesenchymal stem cell treated with estrogen and $CoCl_2$, MSCs represents mesencymal stem cell without treatment, MSCs +est represents mesencymal stem cells treated with estrogen.

4.5 Expression of ERα and ERβ in Mesenchymal Stem Cells.

Since estrogen exerts its effects through its receptor, it is important to determine whether MSCs express ERα and/or ERβ . Our western blot data showed that MSCs cell are positive for both estrogen receptor (Figure 4.5).

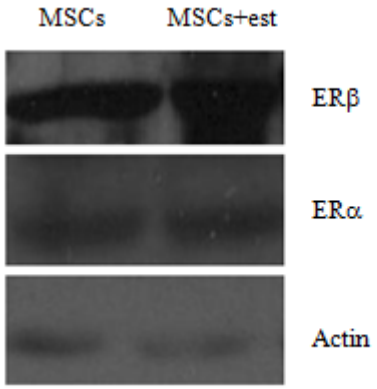


Figure 4. 5: The expression level of ERβ and ERα. Actin is used as a loading control. MSCs represents mesencymal stem cell without treatment, MSCs +est represents mesencymal stem cells treated with estrogen.

4.6 Colony Forming Assay

Colony forming is one of the distinguishing characteristics of MSCs and reflects their functionality. We examined colony formation capability of MSCs in hypoxia and determined the role of estrogen treatment in hypoxic condition. Colony forming assay was performed in the cells treated only with $CoCl_2$, treated both with $CoCl_2$ and estrogen, treated only with estrogen and with no treatment. At least two samples from each sample were taken and colonies were counted. Then the average number of colonies among each group was

calculated and the results were compared between samples (Figure 4.6). Average number of colonies counted on cells treated with $CoCl_2$ was 16.25, average number of colonies counted on cells treated with estrogen and $CoCl_2$ was 25, average number of colonies counted on cells with no treatment was 13.25 and average number of colonies counted on cells treated with estrogen was 19.

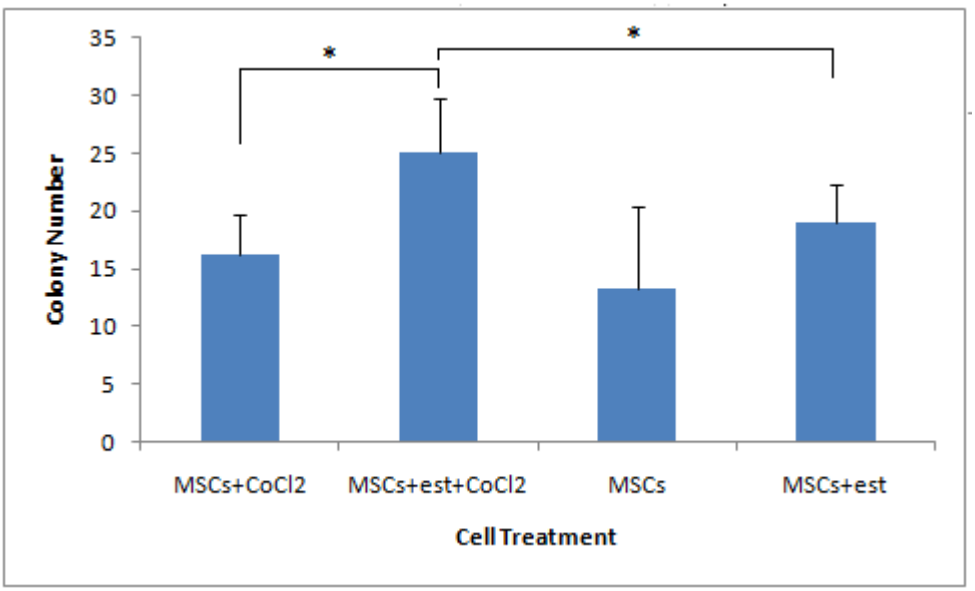


Figure 4. 6 The number of colonies after treatment with $CoCl_2$, with $CoCl_2$ and estrogen, with no treatment and with only estrogen treatment. *P < 0.05. MSCs+$CoCl_2$ represents mesenchymal stem cell treated with $CoCl_2$, MSCs+est+$CoCl_2$ represents mesenchymal stem cell treated with estrogen and $CoCl_2$, MSCs represents mesencymal stem cell without treatment, MSCs +est represents mesencymal stem cells treated with estrogen.

Our results showed that (Figure 4.6), number of colonies increased in cells treated with $CoCl_2$ when compared to the normal samples (Fe). Addition of estrogen to the samples treated with $CoCl_2$ leads further increase in the colony number. Increase in colony number was also observed between samples both treated with estrogen. In addition, increase in colony number is higher in cells treated with estrogen and $CoCl_2$.

4.7 TUNEL Assay

At least two samples from each group were counted for both their total number of cells and apoptotic cells. Then the percentage of apoptotic cells to total cells was calculated and their averages were taken. The percentage of apoptosis in cells treated with $CoCl_2$ was 4.25, the percentage of apoptosis in cells treated with estrogen and $CoCl_2$ was 3.42, the percentage of apoptosis in cells with no treatment was 3.12 and the percentage of apoptosis in cells treated with estrogen was 1.18. While the cells were being counted, only the colonies were considered in order to be sure that the cells counted were MSCs. When cells are subjected to

hypoxia, they undergo apoptosis and it is known that estrogen prevent apoptosis in some cell types. In order to see whether treatment of $CoCl_2$ leads increase in apoptosis in MSCs and whether estrogen decrease apoptosis in MSCs, we performed In Situ Cell death detection as described in materials and methods. According to the Figure 4.7, the number of the apoptotic cell increased upon $CoCl_2$ treatment when compared to the non-treated samples. However, the rate of apoptosis was decreased when the sample treated with $CoCl_2$ and with estrogen together. The percentage of apoptosis was also high in cells treated with $CoCl_2$ and estrogen when compared with sample treated with estrogen alone. In addition, percentage of apoptosis was also decreased in cells treated only with estrogen when compared with the non-treated samples.

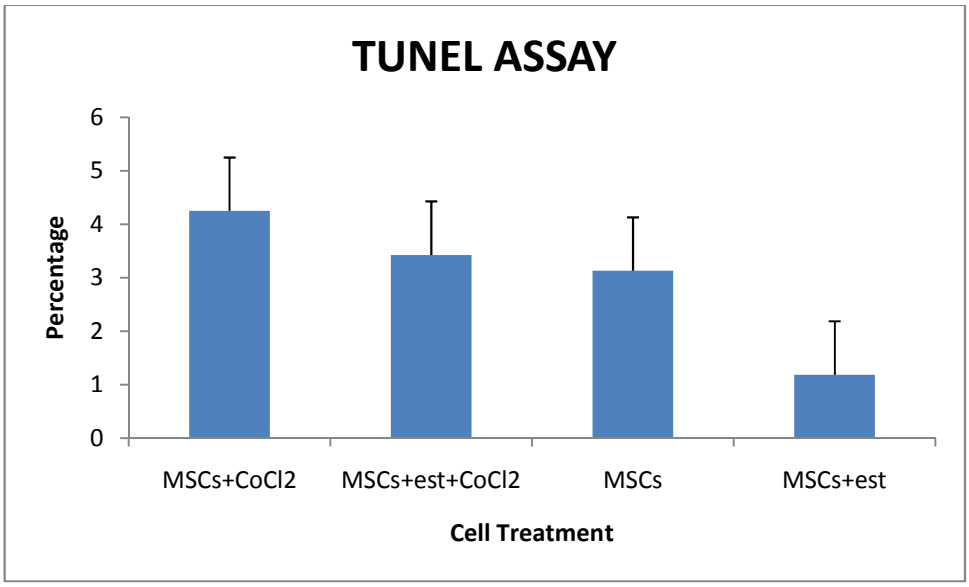


Figure 4. 7: The apoptotic cell ratios of cells after treatment with $CoCl_2$, with $CoCl_2$ and estrogen, with no treatment and with only estrogen treatment. The number of TUNEL positive cells was divided to the total number of cells. MSCs+$CoCl_2$ represents mesenchymal stem cell treated with $CoCl_2$, MSCs+est+$CoCl_2$ represents mesenchymal stem cell treated with estrogen and $CoCl_2$, MSCs represents mesencymal stem cell without treatment, MSCs +est represents mesencymal stem cells treated with estrogen.

4.8 BrdU Incorporation Assay

At least two samples from each group were counted for both their total number of cells and BrdU positive cells. Then the percentage of BrdU positive cells to total cells was calculated and their averages were taken. The percentage of proliferation in cells treated with $CoCl_2$ was 18.2, the percentage of proliferation in cells treated with estrogen and $CoCl_2$ was 20.4, the percentage of proliferation in cells with no treatment was 22.6 and the percentage of proliferation in cells treated with estrogen was 26.3. While the cells were being counted, only the colonies were considered in order to be sure that the cells counted were MSCs. While the cells were being counted, only the colonies were considered in order to be sure that the cells

counted were MSCs Since hypoxia is an important factor for the stem cell self-renewal, we investigated how proliferation of MSCs was changed upon $CoCl_2$ treatment and estrogen treatment with BrdU incorporation assay (Figure 4.8).

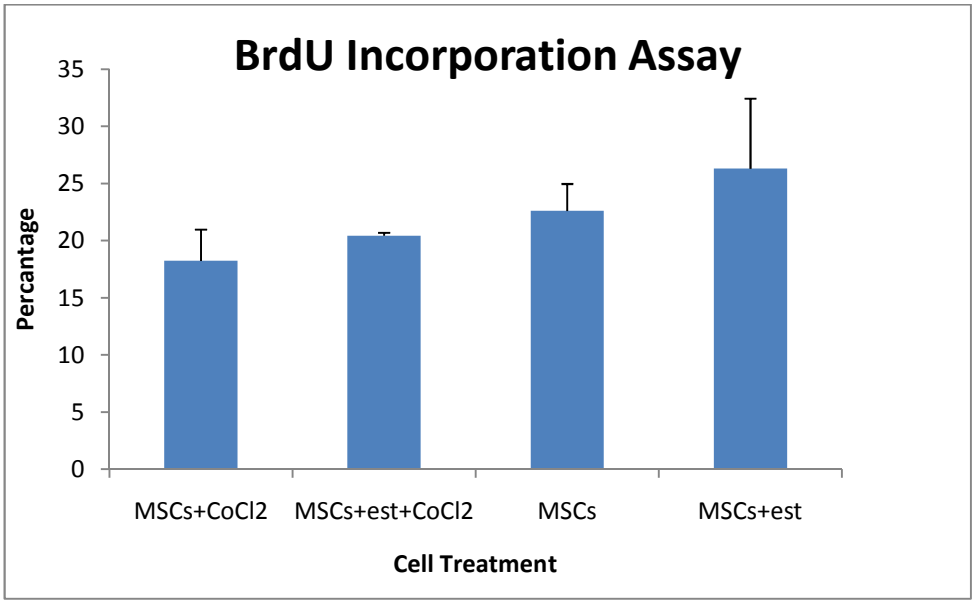


Figure 4. 8: The proliferation rate of MSC after treatment with $CoCl_2$, with $CoCl_2$ and estrogen, with no treatment and with only estrogen treatment. Cells were incubated BrdU for 1h. The number of BrdU positive cells was divided to the total number of cells. MSCs+$CoCl_2$ represents mesenchymal stem cell treated with $CoCl_2$, MSCs+est+$CoCl_2$ represents mesenchymal stem cell treated with estrogen and $CoCl_2$, MSCs represents mesencymal stem cell without treatment, MSCs +est represents mesencymal stem cells treated with estrogen.

Parallel to our TUNEL data, our BrdU incorporation assay result revealed that proliferation of MSC was decreased in cells treated with $CoCl_2$ when compared with non-treated samples. However, proliferation of MSC increased when estrogen was added to the cells treated with $CoCl_2$. The proliferation rate increased upon estrogen treatment in non-treated samples.

4.9 Expression of Notch Receptors in hypoxic condition and upon estrogen treatment in MSCs.

We investigated the expression levels of notch receptors when cells treated with $CoCl_2$. Moreover, we also examined whether estrogen treatment affects the expression level of notch receptors in normoxic and hypoxic conditions. We used RT- PCR to determine the expression of notch receptors at transcript and used immunohistochemisty for protein level, from 14^{th} day cells after treatment with $CoCl_2$, with $CoCl_2$ and estrogen, with no treatment and with only estrogen treatment.

4.9.1 Expression of Notch Receptors

In order to check how expression levels of Notch receptors were changed in hypoxic condition with or without estrogen, RT-PCR (Figure 4.9) and immunohistochemistry (Figure 4.10, 4.11. and 4.12) was performed on cells 14th day after treatment with $CoCl_2$, with $CoCl_2$ and estrogen, with no treatment and with only estrogen treatment.

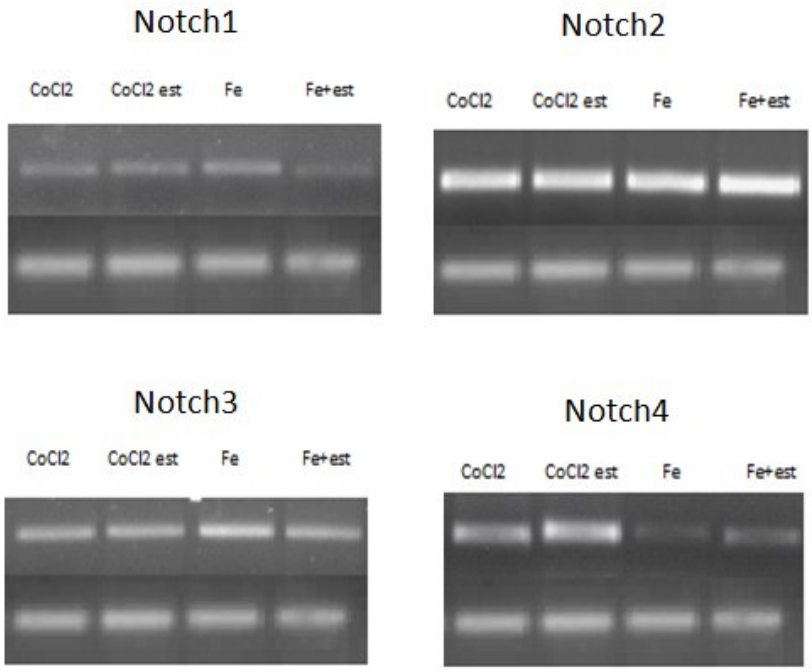


Figure 4. 9: The expression profile of Notch receptors. The first lines represent Notch expression. Second line represents loading control done with beta-actin.

Our results showed that (Figure 4.9.), the expression of Notch 1 did not change with $CoCl_2$ treatment with compared to untreated cells. Moreover addition of estrogen at the hypoxic environment did not change the expression. However notch 1 receptor expression was decreased when MSCs treated only with estrogen. The expression of notch 2 receptor did not show any change amongst the experimental groups. Notch 3 receptor expression decreased in hypoxic conditions compared to normoxia. Its expression remained unchanged upon estrogen treatment at the hypoxic environment. However, Notch 3 level decreased when cells were treated with estrogen in normoxic condition. The expression level of Notch 4 was increased in the hypoxia compared to normoxia and its expression was further increased when estrogen was added to the hypoxic cells. On the other hand, expression level of Notch 4 was increased when normoxic cells were treated with estrogen.

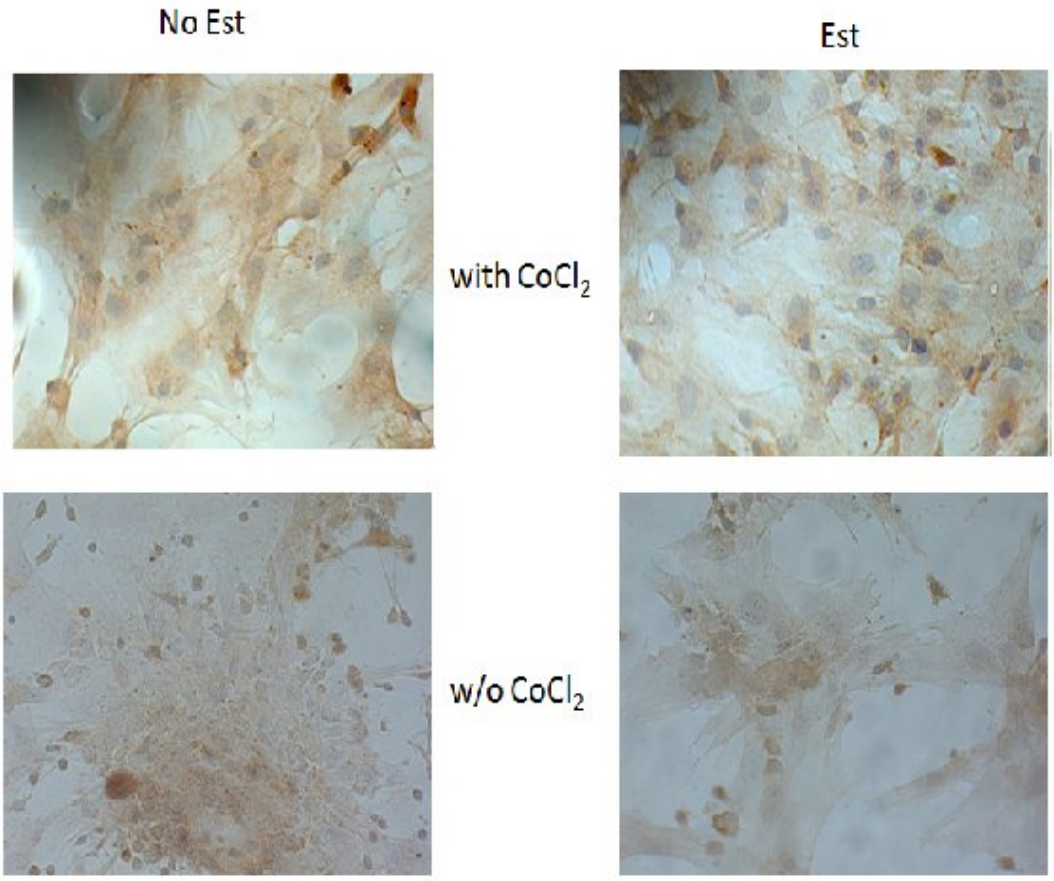


Figure 4. 10 : The expression of Notch 1 in cells after treatment with $CoCl_2$, with $CoCl_2$ and estrogen, with no treatment and with only estrogen treatment. 40X

At the protein level, the expression level of Notch 1 was denser in cells treated with $CoCl_2$ when compared to cells treated without $CoCl_2$ (Figure 4.10). However, unfortunately we were not able to measure the levels of notch 1 protein by western blot. Therefore we are not able to quantitate its levels.

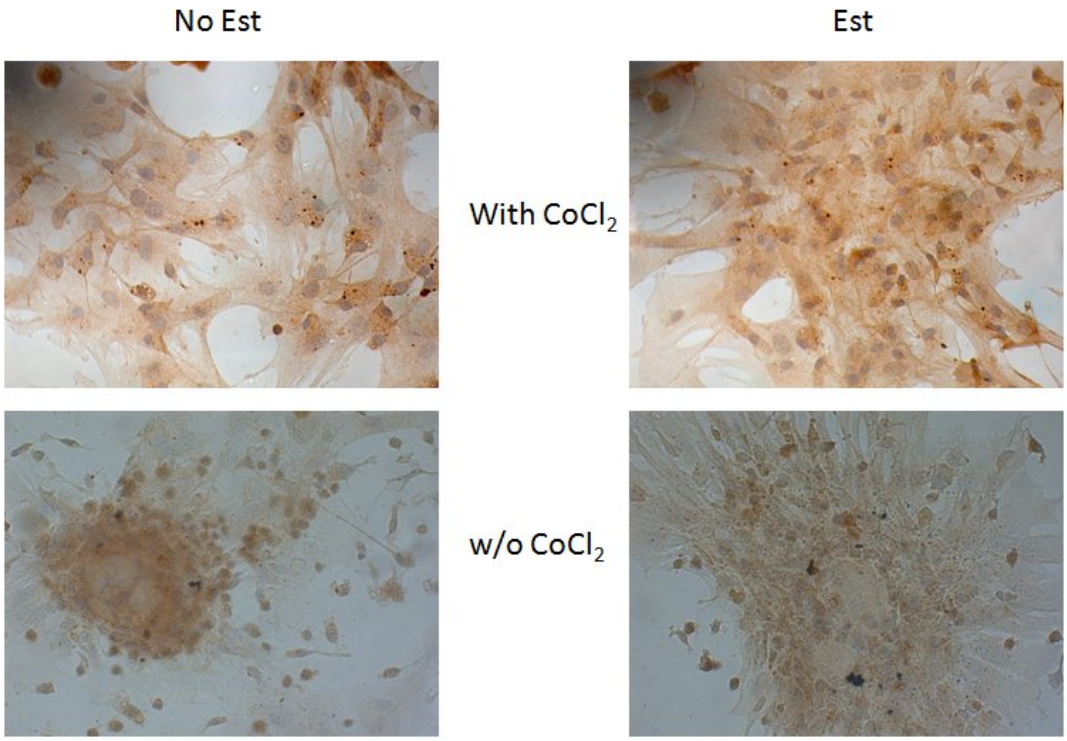


Figure 4. 11: The expression of Notch 3 in cells after treatment with $CoCl_2$, with $CoCl_2$ and estrogen, with no treatment and with only estrogen treatment. 40X

When we examined notch 3 by immunohistochemistry, we found that in the hypoxic conditions MSCs were stained denser than the normoxic condition (Figure4.11). Again we were not able to measure the levels of notch 3 protein by western blot. Therefore we are not able to quantitate its levels.

Finally, notch 4 staining was denser in MSCs in hypoxic conditions than normoxia (Figure 4.12). Similar to other notch receptors we were not able to measure the levels of notch 1 protein by western blot. Therefore we are not able to quantitate its levels

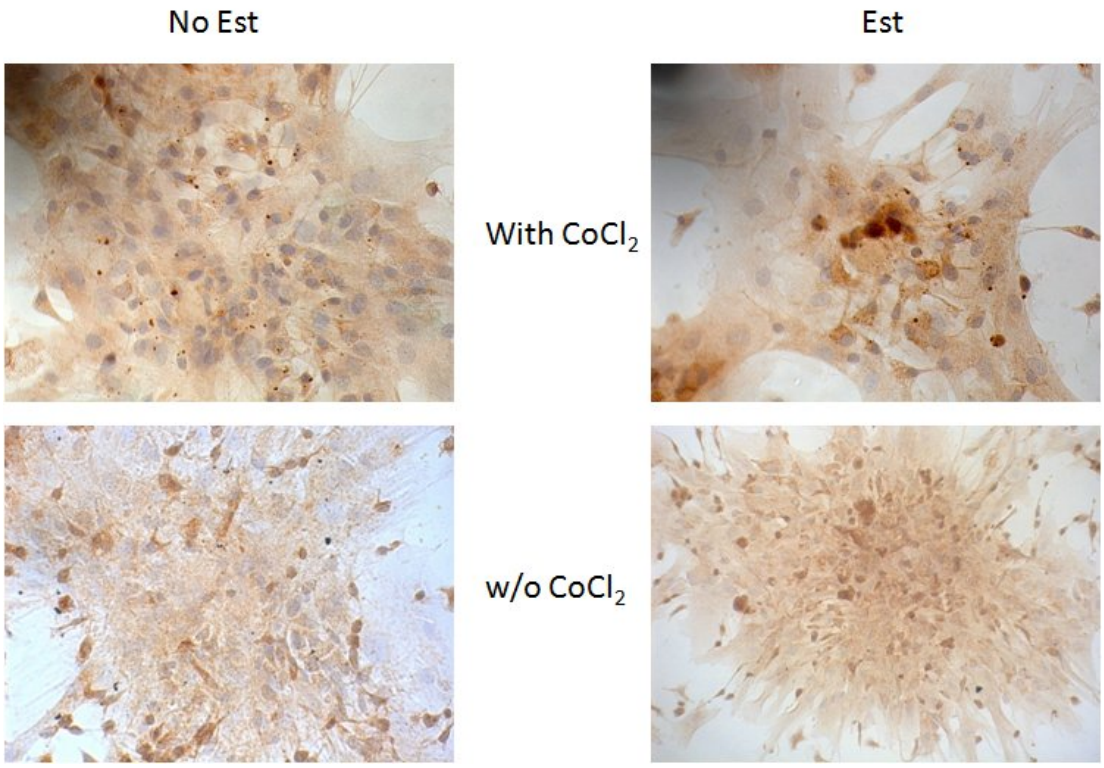


Figure 4. 12: The expression of Notch 4 in cells after treatment with $CoCl_2$, with $CoCl_2$ and estrogen, with no treatment and with only estrogen treatment. 40X

4.10 Bone Marrow Isolated Cells Grown on Biomaterials

MSCs were growth on 20 different biomaterials, which were produced combinations of the different polymer (Table 4.1). In order to determine whether MSCs were able to attach the biomaterials after 14 days, we investigated their morphology their colony forming activities. Our data showed that we were able to observe MSC growth on the biomaterials designated as "CIII-1", "CIII-2", and "CIII-5" (Figure 4.13). In addition, when the bio materials were stained with Giemsa to reveal their CFU-F activity, we found that they were capable of forming colonies suggesting that biomaterials designated "CIII-1", "CIII-2", and "CIII-5" are compatible for MSCs (Figure 4.14). Even if, cells grown on CIII-3 displayed MSC morphology, they were not able to form colonies. Similar to CIII-3, no MSC-like cells were observed on CIII-4 (table 4.2).

Table 4. 1: Different composition with varying monomer ratio

CI

HEMA/HPC	v/v
CI-A	2:1
CI-B	5:1
CI-D	11:1
CI-E	1:1
CI-K	1:0

CII

HPMA/PEG/HPC	v/v/v
CII-1	1:1:0
CII-2	1:0:1
CII-3	3:1:2
CII-4	3:2:1
CII-5	3:0:0

CIII

HEMA/PEG/HPC	v/v/v
CIII-1	2:1:1
CIII-2	3:0:1
CIII-3	3:1:0
CIII-4	1:0:1
CIII5	1:1:0

C

HPMA/PEG	v/v
CA-1	**1:0**
CA-2	**1:1**
CA-3	2:1
CA-4	3:1
CA-5	4:1

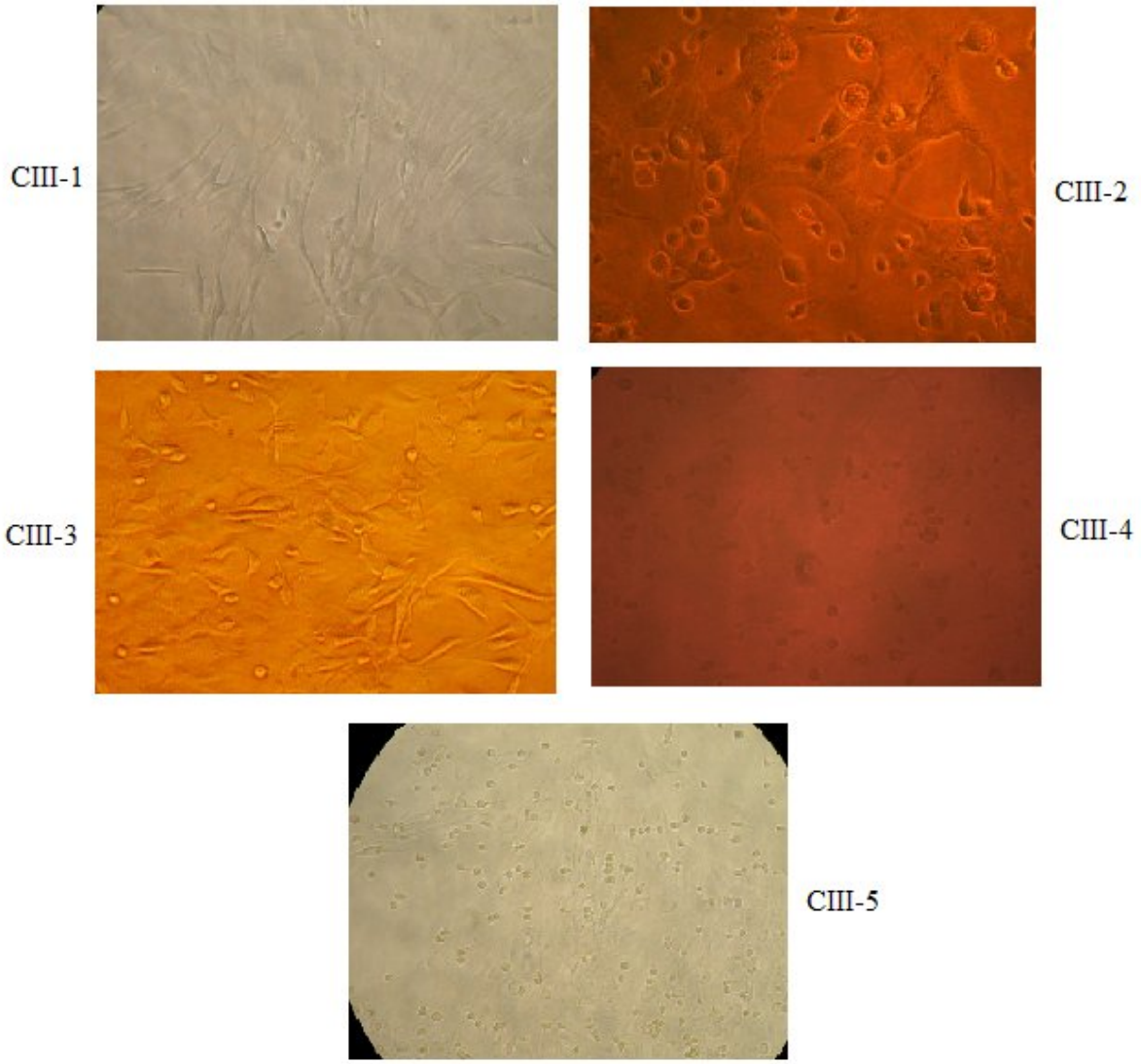


Figure 4. 13: On the 14th day pictures of rat female bone marrow isolated cells grown on biomaterials "CIII". 20X objective was used in all pictures but magnification varied between them to obtain better resolution for each biomaterial.

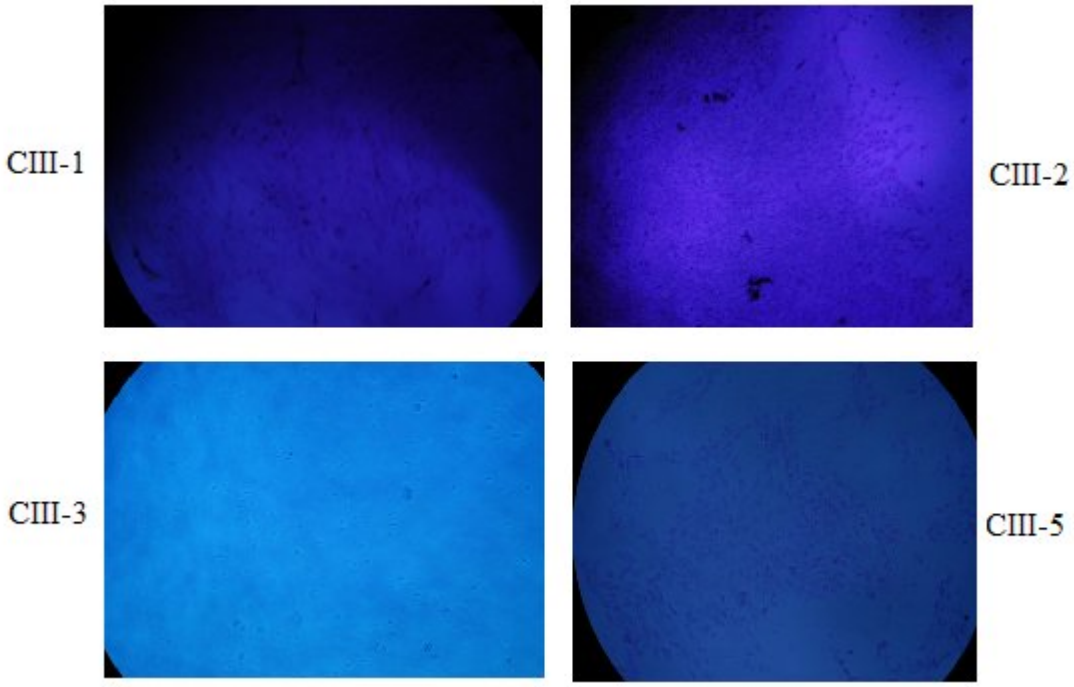


Figure 4. 14: Colonies formed on biomaterials designated as CIII after 14 days culture. 20X objective was used in all pictures but magnification varied between them to obtain better resolution for each biomaterial.

Amongst "CI" series of biomaterials "CI-K" provided the best condition to support the MSCs compared to other "CI" biomaterials (Figure 4.15). Cells that were grown on "CI-K" form colonies (Figure 4.16). MSCs were also able to grow and form colonies when grown in biomaterial "CI-A" but not in CI-B and CI-D (Figure 4.15). Cells grown on CI-E were not visualized due to thickness and lack of transparency of the biomaterial. (Table 4.2).

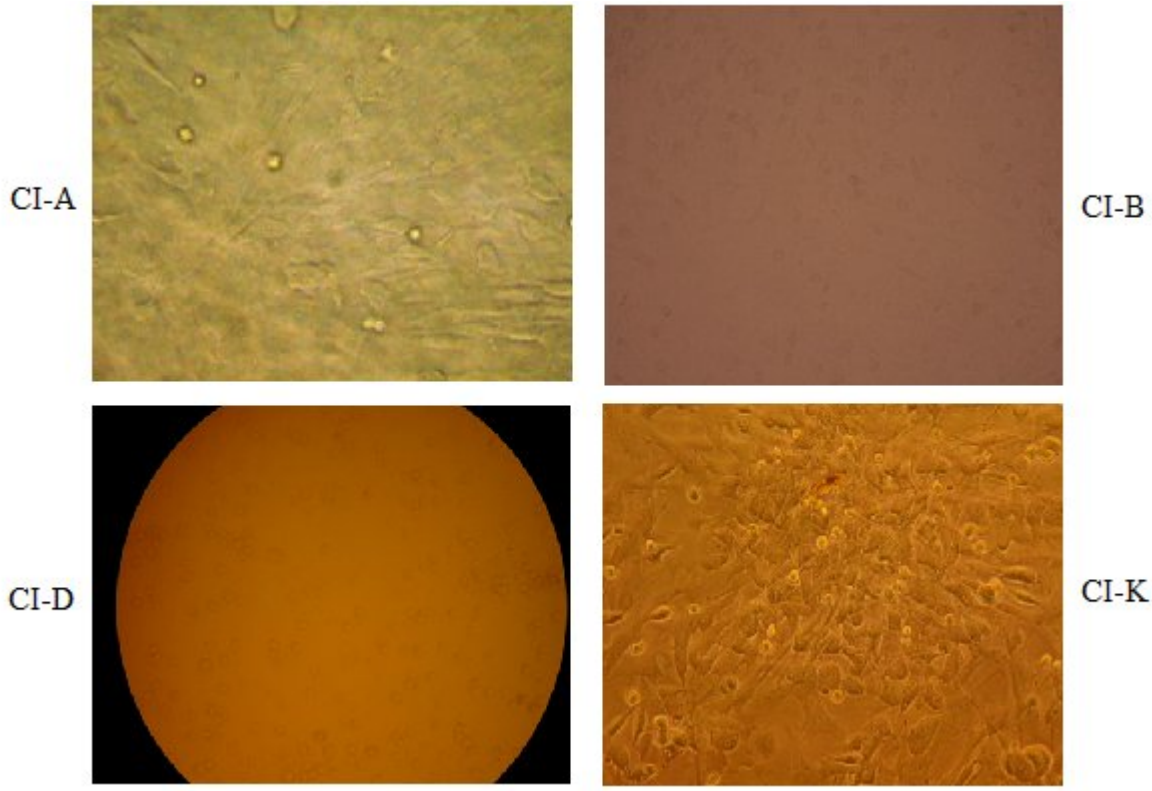


Figure 4. 15: On the 14th day pictures of rat female bone marrow isolated cells grown on biomaterials "CI". 20X objective was used in all pictures but magnification varied between them to obtain better resolution for each biomaterial

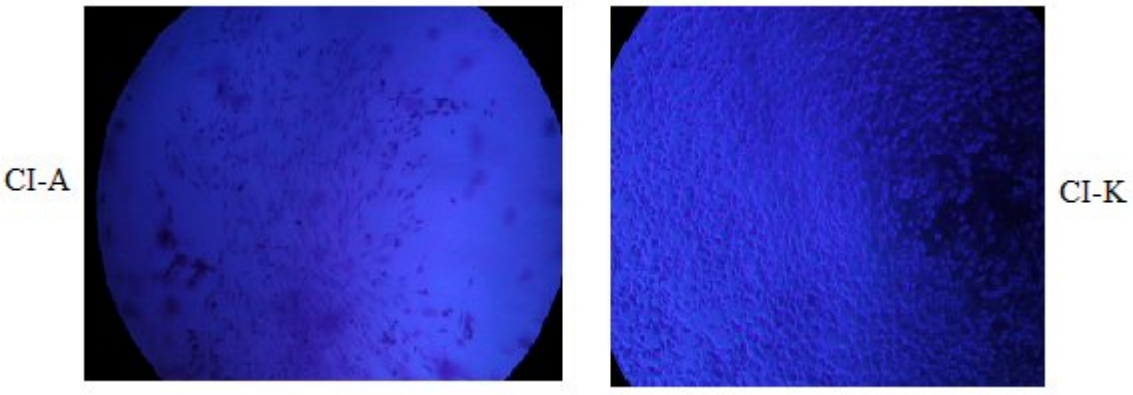


Figure 4. 16: Colonies formed on CI-A and CI-K biomaterials after 14 days culture. 20X objective was used in all pictures but magnification varied between them to obtain better resolution for each biomaterial.

Our data showed that we were able to observe MSC growing on the biomaterials designated as “CA-1” and “CA-2” (Figure 4.17). In addition, when the bio materials were stained with Giemsa, it was observed that cells grown on CA-1and CA-2 were capable of forming colonies on these biomaterials (Figure 4.18). Although we could not discriminate whether cells growing on CA-3 were MSCs (Figure 4.17), we could observe that cells growing on CA-3 could form colonies (4.18). On the other hand, no MSCs cells were observed on CA-5, and cells grown on CA-4 were not observed because cells could not visualized under microscope both before and after colony forming assay (Table 4.2).

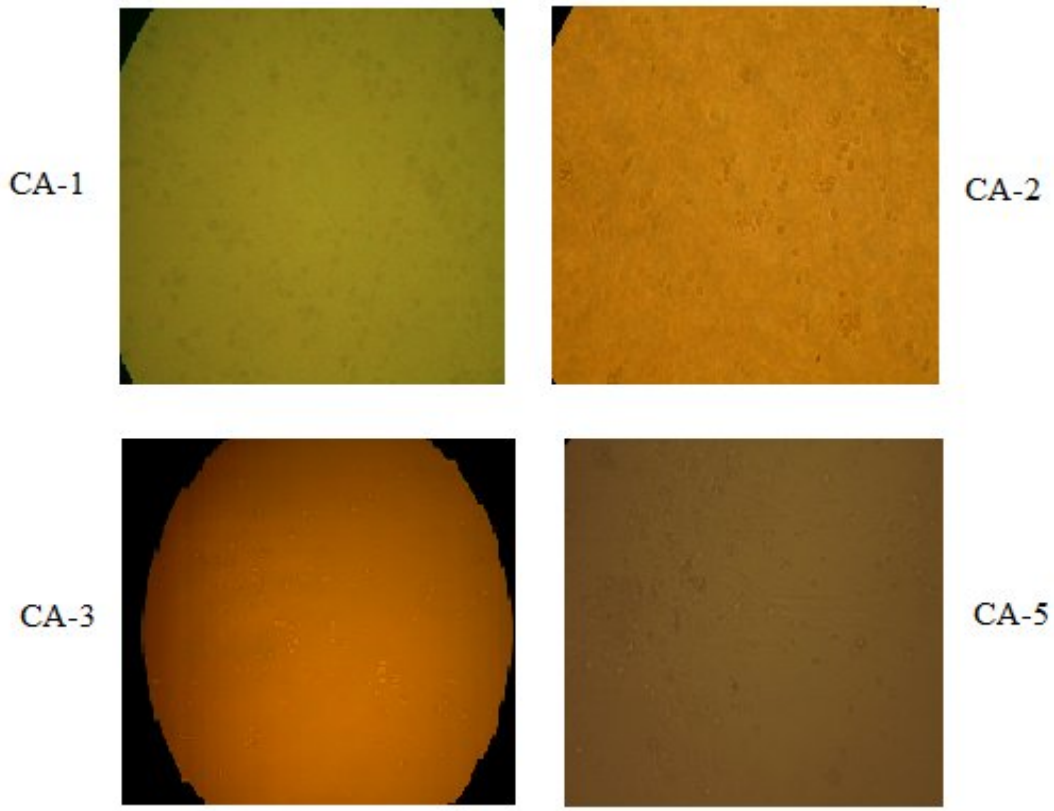


Figure 4. 17: On the 14th day pictures of rat female bone marrow isolated cells grown on biomaterials designated as “CA”. 20X

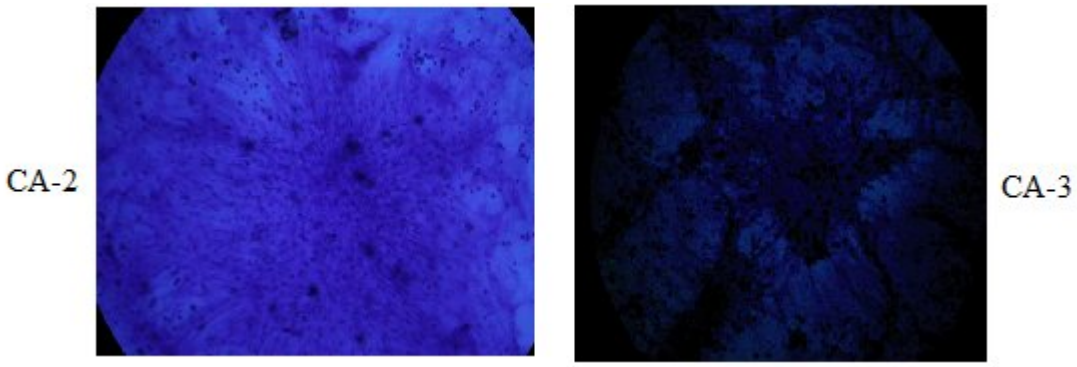


Figure 4. 18: Colonies formed on CA-2 and CA-3 biomaterials after 14 days culture. 20X objective was used in all pictures but magnification varied between them to obtain better resolution for each biomaterial.

Table 4. 2: Summary of observed cell morphology and observed colony formation on each Biomaterials. (+/-) represents both MSCs and other cells.

Bio Materials	**Presence of MSCs**	**CFU**
CA-1	+	+
CA-2	+	+
CA-3	+/-	+
CA-4	-	-
CA-5	-	-
CI-A	+	+
CI-B	+/-	-
CI-D	-	-
CI-E	-	-
CI-K	+	+
All CIIs	**- (*)**	**- (*)**
CIII-1	+	+
CIII-2	+	+
CIII-3	+	_
CIII-4	-	-
CII-5	+	+

(*) represents failure in the visualization of CII due to thickness and lack of transparency

4.11 Bioinformatics Analysis

Our aim was to understand whether estrogen regulates the expression of Notch receptors, however we were unable to perform western blot for quantification of Notch receptor protein levels, we took an indirect approach to overcome this problem. First we examined the promoter region of the notch 1 receptor gene for all the transcription factor binding sites. Then we extracted available microarray data sets to analyze estrogen-induced genes. The genes (transcription factors) that were shown to be upregulated upon estrogen

treatment were then searched for the presence of binding sites in 10000 bp upstream notch receptor 1 gene promoter. We investigated Notch 1 because it was known that its expression was increased upon estrogen treatment in MCF-7 cell lines (Soares R. et.al.2004).

4.11.1 Transcription Factors on Notch 1 10000bp upstream region

10000bp upstream sequence of Notch1 from the first exon was found by using BioMart. Transcription factors that bind this region was found (Table 5.1) by using MatInspector database, which is a software tool that utilizes a large library of matrix descriptions for transcription factor binding sites to locate matches in DNA sequences.(Cartharius et al. 2005). The sequence of 10000bp upstream region of Notch1 was entered as FASTA format to the MatInspector. Transcription factors binding to the sequence, whose core similarity was between 0.9-1 and whose matrix similarity is greater than 0.8 was selected. The maximum core similarity of 1.0 is only reached when the highest conserved bases of a matrix match exactly in the sequence. Matrix similarity takes into account all bases over the whole matrix length. A perfect match to the matrix gets a score of 1.00 (each sequence position corresponds to the highest conserved nucleotide at that position in the matrix), a "good" match to the matrix usually has a similarity of >0.80 (Cartharius et al. 2005).

Table 4. 3: 574 Transcription Factors binding to 10000bp upstream region of Notch 1 from the first exon.

IRF1	IRF2	IRF3	IRF4	IRF5	IRF6	IRF7	IRF8	ISGG	RNF31
EGR1	EGR2	EGR3	EGR4	WT1	ZBTBA	ZBTBB	EHF	ELF1	ELF2
ELF4	ELF5	ELK1	ELK3	ELK4	ERF	ERG	ETS1	ETS2	ETV1
ETV3	ETV4	ETV5	ETV6	ETV7	FEV	FLI1	FLI2	GABA	GABPAP
MGC29891	SPDEF	SPI1	SPIB	SPIC	BCL11A	BCL11B	EVI1	MDS1	PRDM16
GTF2I	GTF2IRD1	BACH1	BACH2	MAF	MAFA	MAFB	MAFF	MAFG	NFE2
NFE2L1	NFE2L2	NFE2L3	NRL	PAX3	PAX7	KLF4	EOMES	MGA	T
TBX1	TBX10	TBX15	TBX18	TBX19	TBX2	TBX20	TBX21	TBX22	TBX23P
TBX4	TBX5	TBX6	ATF1	ATF2	ATF3	ATF4	ATF5	ATF6	ATF7
CREB3	CREB3L3	CREB3L4	CREB5	CREBL1	NFIL3	EBF	EBF2	EBF3	FOXN1
TP53	TP73	TP73L	KCNIP1	KCNIP2	KCNIP3	KCNIP4	TFCP2	TFCP2L1	UBP1
NR1H3	NR1I2	NR1I3	RARA	RARB	RARG	RXRB	RXRA	RXRG	THRA
VDR	BNC1	SMAD1	SMAD2	SMAD3	SMAD4	SMAD5	SMAD9	YY1	NR5A1
CUTL1	CUTL2	GFI1	GFI1B	ONECUT1	ONECUT2	ONECUT3	CIC	HBP1	HMG4L
HMGA2	SOX1	SOX10	SOX11	SOX12	SOX13	SOX14	SOX15	SOX17	SOX18
SOX21	SOX3	SOX30	SOX5	SOX5P	SOX6	SOX7	SOX8	SOX9	SRY
STAT1	STAT2	STAT3	STAT4	STAT5A	STAT5B	STAT6	ILF2	ILF3	NFAT5
NFATC2	NFATC3	NFATC4	RBPSUH	IKZF4	ZNFN1A1	ZNFN1A2	ZNFN1A3	E2F1	E2F2
E2F4	E2F5	E2F6	E2F7	E2F8	TFDP1	TFDP2	TFDP3	INSM1	RREB1
ESR2	ESRRA	ESRRB	ESRRG	NR1D1	NR1D2	RORA	RORB	RORC	HNF4A
NR2C1	NR2C2	NR2E3	NR2F1	NR2F2	NR2F6	ZNF83	E4F1	DEAF1	HAND1
LYL1	NHLH1	NHLH2	SCXA	SCXB	TAL1	TAL2	TCF12	TCF15	TCF3
ZFHX1B	KLF6	ZNF148	ZNF202	ZNF219	ZNF281	KLF10	KLF11	KLF13	KLF16
KLF9	SP1	SP2	SP3	SP4	SP5	SP6	SP7	SP8	HIVEP1
HIVEP3	NFKB1	NFKB2	REL	RELA	RELB	CHES1	FKHL18	FOXA1	FOXA2
FOXB1	FOXB2	FOXC1	FOXC2	FOXD1	FOXD2	FOXD3	FOXD4	FOXD4L1	FOXD4L4
FOXE3	FOXF1	FOXF2	FOXG1	FOXI1	FOXJ1	FOXJ2	FOXJ3	FOXK1	FOXK2
FOXL2	FOXM1	FOXO1A	FOXO3A	FOXP1	FOXP2	FOXP3	FOXQ1	KLF4	ATF6
MAX	MGA	MLX	MLXIPL	MNT	MXI1	MYC	MYCL1	MYCL2	MYCN
TCF4	USF1	BAPX1	C10orf121	HMX1	HMX2	HMX3	NKX1-1	NKX2-2	NKX2-3
NKX2-5	NKX2-6	NKX2-8	NKX3-1	TITF1	AIRE	KLF1	KLF12	KLF15	KLF2
KLF7	KLF8	CBFA2T2	CBFA2T3	CBFB	RUNX1	RUNX1T1	RUNX2	RUNX3	NR4A1
NR4A3	CTCF	CTCFL	PAX5	PPARA	PPARAL	PPARD	PPARG	RXRA	RXRB
TFAP4	JARID1B	BCL6	BCL6B	HOXA10	HOXA3	HOXA4	HOXA6	HOXA7	HOXA9
HOXA2	HOXA5	HOXB1	HOXB3	HOXB5	HOXB6	HOXB7	HOXB8	HOXB9	HOXC10
HOXC5	HOXC6	HOXC8	HOXC9	HOXD1	HOXD10	HOXD3	HOXD4	HOXD8	HOXD9
PBX2	PBX3	SRF	TBP	HNF4A	HNF4G	NR2C1	NR2C2	NR2E3	NR2F1
NR2F6	ZNF83	POU3F2	POU3F3	POU3F4	POU4F1	POU4F2	POU4F3	POU6F1	MYT1
ST18	SMARCA3	BCL11A	BCL11B	EVI1	MDS1	PRDM16	ZNF521	CEBPA	CEBPB
CEBPE	CEBPG	CEBPZ	LHX1	LHX3	LHX4	LMX1B	NANOG	OTX1	OTX2
PHOX2B	PITX1	PITX2	PITX3	RAX	RAX2	TLX1	TLX2	TLX3	NKX6-1
TCF1	TCF2	DBP	HLF	TEF	VBP1	TEAD1	TEAD2	TEAD3	TEAD4
GFI1B	EGR1	EGR2	EGR3	EGR4	WT1	ZBTB7A	ZBTB7B	MAZ	ZNF278
RBPSUH	RBPSUHL	SPZ1	BHLHB2	BHLHB3	HELT	HES1	HES2	HES3	HES4
HES6	HES7	HEY1	HEY2	ITGB3BP	TAF1	TAF2	TAF6	TAF9	TAF9B
GTF2I	GTF2IRD1	ATOH1	NEUROD1	NEUROD2	NEUROD4	NEUROD6	NEUROG1	NEUROG2	NEUROG3
TCF12	TCF3	ZNF238	MEIS1	MEIS2	MEIS3	PKNOX1	PKNOX2	TGIF	TGIF2
TGIF2LY	GLI1	GLI2	GLI3	GLIS1	GLIS2	GLIS3	ZIC1	ZIC2	ZIC3
ZIC5	ARNT	ARNT2	ARNTL	ARNTL2	CLOCK	EPAS1	HIF1A	HIF3A	NPAS1
KCNIP1	KCNIP2	KCNIP3	KCNIP4	PLAG1	SMARCA3	PLAGL2	HSF1	HSF2	HSF4
HSFY2	ALX3	ALX4	CART1	ESX1	PURA	LEF1	SOX4	TCF7	TCF7L1
NR4A1	NR4A2	NR4A3	TFAP2A	TFAP2B	TFAP2C	TFAP2D	TFAP2E	NFIA	NFIB
NFIX	GTF2B								

4.11.2 Estrogen Induced Genes

Publicly available datasets were collected from Gene Expression Omnibus (GEO). 12 microarrays were used for this study. The datasets were normalized with RMA using BRB-tool which uses Bioconductor R packages when raw .cel files were available, if unavailable, the series matrix files were used and global median normalization was performed over the normalized data for each GSM, separately. After normalization step, one-way ANOVA ($p<0.001$) was applied to the normalized data. Estrogen upregulated genes were selected by

drawing scatter plot in BRB-tool, according to the experimental setup of each analyzed microarray. The details of these experiments are summarized below;

GSE1153

U2OS human osteosarcoma cells were stably transfected with human ERα or ERß, where cells were treated with 10 nM E2 for , 8, 24, or 48 h. Control cell samples were also treated with 0.1% ethanol control vehicle for 48 h (Stossi F. et.al. 2004)l. The series matrix files were used and global median normalization was performed over the normalized data. 50000 genes were found after normalization step. One-way ANOVA ($p<0.001$) was applied to the normalized data and 5200 genes were selected from these 50000 genes. Then, by drawing scatter plots in BRB-tool, at least 2 fold increased estrogen upregulated genes were found in samples treated with estrogen for 8h,24hand 48h by comparing with the control samples treated with ethanol (Table 4.4).

Table 4. 4: Number of estrogen up-regulated genes in GSE 1153 in different experimental setups.

Conditions	Number of estrogen up regulated genes
No estrogen vs. Era tr./w. estrogen for 8h	23
No estrogen vs. Era tr./w. estrogen for 24h	86
No estrogen vs. Era tr./w. estrogen for 48h	86
No estrogen vs. ERb tr./w. estrogen for 8h	23
No estrogen vs. ERb tr./w. estrogen for 24h	41
No estrogeb vs. ERb tr./w. estrogen for 48h	47

GSE2292

The U2OS-ERα, U2OS-ERβ, and U2OS-ERα/ß cells were treated with estrogen for 24h. GSE2292 datasets were normalized with RMA using BrB-tool which uses Bioconductor R packages because raw .cel files were available. 50000 genes were found after normalization step. One-way ANOVA ($p<0.001$) was applied to the normalized data and 5200 genes were selected from these 50000 genes. Then, by drawing scatter plot in BRB-tool, at least 2 fold increased estrogen upregulated genes were found in samples treated with estrogen by comparing with the control samples treated with ethanol (Table 4.5.).

Table 4. 5: Number of estrogen up-regulated genes in GSE 1153 in different experimental setups

Conditions	Number of estrogen up regulated genes
Era tr./w/o. estrogen vs. Era tr./w. estrogen	146
Erb tr./w/o. estrogen vs. ERb tr./w. estrogen	61
ERab tr./w/o.estrogen vs. ERab tr./w estrogen	125

GSE9757, GSE9758 and GSE9759

In Gse9757**,** cells were infected with the recombinant adenovirus bearing no cDNA or cDNA for ERα for 48h. Infected cells were then treated with 1 nM Estradiol 17beta for 6h (Nott SL. et.al. 2009). In Gse9758**,** cells were infected with the recombinant adenovirus bearing no cDNA or cDNA for ERα 203/204/211E (3411E) for 48h. Infected cells were then treated with 1 nM Estradiol 17beta for 6h (Nott SL. et.al. 2009). In Gse9759, cells were infected with the recombinant adenovirus bearing no cDNA or a cDNA for ERβ, or cDNA for ERβ mutant defective in binding to ERE (ERbDBD). for 48 hours. Infected cells were then treated with 1 nM Estradiol 17beta for 6h (Nott SL. et.al. 2009). The datasets were normalized with RMA using BrB-tool which uses Bioconductor R packages because raw .cel files were available. 50000 genes were found after normalization step. One-way ANOVA ($p<0.001$) was applied to the normalized data and 5200 genes were selected from these 50000 genes. Then, by drawing scatter plot in BRB-tool, at least 2 fold increased estrogen upregulated genes were found in samples treated with estrogen by comparing with the control samples (Table 4.6).

Table 4. 6: Number of estrogen up-regulated genes in GSE9757, GSE9758 and GSE9759 in different experimental setups

Conditions	Number of estrogen up regulated genes
No estrogen vs. Era tr./w. estrogen (GSE9757)	111
No estrogen vs. Era(def) tr./w. estrogen (GSE9758)	17
No estrogen vs. ERb tr./w. estrogen (GSE9759)	7
No estrogen vs. ERb(def) tr./w. estrogen(GSE9759)	21

GSE11352

MCF-7 cells were treated with 10 nM E2 for 12, 24, and 48 h (Lin CY. et.al. 2007) GSE11352 datasets were normalized with RMA using BrB-tool which uses Bioconductor R packages because raw .cel files were available. 50000 genes were found after normalization step. One-way ANOVA ($p<0.001$) was applied to the normalized data and 5200 genes were selected from these 50000 genes. Then, by drawing scatter plot in BRB-tool, at least 2 fold increased estrogen upregulated genes were found in samples treated with estrogen for 12h, 24h, and 48h by comparing with the control samples treated with ethanol (Table 4.7.).

Table 4. 7: Number of estrogen up-regulated genes in GSE 11352 in different experimental setups.

Conditions	Number of estrogen up regulated genes
No estrogen vs. estrogen for 12h	99
No estrogen vs. estrogen for 24h	138
No estrogen vs. estrogen for 48h	174

GSE11506

MCF7 cells were treated with vehicle or E2 at a concentration of 10E-9 mol/L for 3 and 6 h (Lin Z.et.al. 2007) GSE11506 datasets were normalized with RMA using BrB-tool which uses Bioconductor R packages because raw .cel files were available. 50000 genes were found after normalization step. One-way ANOVA ($p<0.001$) was applied to the normalized data and 5200 genes were selected from these 50000 genes. Then, by drawing scatter plot in BRB-tool, at least 2 fold increased estrogen upregulated genes were found in samples treated with estrogen for 3h and 6h by comparing with the control samples (Table 4.8.).

Table 4. 8: Number of estrogen up-regulated genes in GSE 11506 in different experimental setups.

Conditions	Number of estrogen up regulated genes
No estrogen vs. estrogen for 3h	108
No estrogen vs. estrogen for 6h	41

GSE2251

ER-negative MDA-MB-231 breast cancer cells were infected with recombinant adenovirus encoding full-length human ERα (Ad-ERα) or control vector (Ad-LacZ) for 24 h, and treated with 0·01% ethanol (vehicle control) or 10-8 M 17beta-estradiol (E2) for 48h (Moggs JG. et.al. 2005) The series matrix files were used and global median normalization was performed over the normalized data. 50000 genes were found after normalization step. One-way ANOVA ($p<0.001$) was applied to the normalized data and 5200 genes were selected from these 50000 genes. Then, by drawing scatter plots in BRB-tool, at least 2 fold increased estrogen upregulated genes were found in samples treated with estrogen by comparing with the control samples (Table 4.9.).

Table 4. 9 Number of estrogen up-regulated genes in GSE 2251 in different experimental setups

Conditions	Number of estrogen up regulated genes
ER neg. tr./w/o. estrogen vs. ER neg.tr./w. estrogen	39
ER neg. tr./w/o. estrogen vs. Era tr./w. estrogen	374

GSE4025

MCF-7 cells expressing endogenous ERalpha were infected with adenovirus carrying either estrogen receptor beta (AdERb) or no insert (Ad) at multiplicity of infection (moi) of 10. Cells were treated with vehicle control or treated with 10nM estrogen for 24h (Frasor J. et.al. 2006) The series matrix files were used and global median normalization was performed over the normalized data. 50000 genes were found after normalization step. One-way ANOVA ($p<0.001$) was applied to the normalized data and 5200 genes were selected from these 50000 genes. Then, by drawing scatter plots in BRB-tool, at least 2 fold increased estrogen upregulated genes were found in samples treated with estrogen by comparing with the control samples (Table 4.10.).

Table 4. 10: Number of estrogen up-regulated genes in GSE4025 in different experimental setups

Conditions	Number of estrogen up regulated genes
Era tr./w/o. estrogen vs. Era tr./w estrogen	553

GSE4668

MCF7/BUS cells were maintained in the presence of varying concentrations of E2 (0 pM, 10 pM, 30 pM, 60 pM, and 100 pM) for 48 hours. (Coser KR. et.al. 2003). The series matrix files were used and global median normalization was performed over the normalized data. 50000 genes were found after normalization step. One-way ANOVA ($p<0.001$) was applied to the normalized data and 5200 genes were selected from these 50000 genes. Then, by drawing scatter plots in BRB-tool, at least 2 fold increased estrogen upregulated genes were found in samples treated with estrogen by comparing with the control samples treated with ethanol (Table 4.11.).

Table 4. 11 Number of estrogen up-regulated genes in GSE4668 in different experimental setups.

Conditions	Number of estrogen up regulated genes
0 pM estrogen vs. 10 pM estrogen	108
0 pM estrogen vs. 13 pM estrogen	318
0 pM estrogen vs. 16 pM estrogen	471
0 pM estrogen vs. 100 pM estrogen	510

GSE4006

MCF-7 cells expressing endogenous ERalpha were infected with adenovirus carrying either estrogen receptor beta (AdERb) or no insert (Ad) at multiplicity of infection (moi) of 5 or 50. Cells were treated with vehicle control or treated with 10nM 17beta-estradiol for 24hr before harvest (Chang EC. et.al. 2006) The series matrix files were used and global median normalization was performed over the normalized data. 50000 genes were found after normalization step. One-way ANOVA ($p<0.001$) was applied to the normalized data and 5200 genes were selected from these 50000 genes. Then, by drawing scatter plots in BRB-tool, at least 2 fold increased estrogen upregulated genes were found in samples treated with estrogen by comparing with the control samples treated (Table 4.12.).

Table 4. 12: Number of estrogen up-regulated genes in GSE4006 in different experimental setups.

Conditions	Number of estrogen up regulated genes
Era tr./w/o. estrogen vs. Era tr./w. estrogen	272
Erab tr./w/o. estrogen vs. Erab tr./w. estrogen (5moi)	309
Erab tr./w/o. estrogen vs. Erab tr./w. estrogen (50moi)	441

GSE11324

MCF7 cells were grown in a hormone-deprived media for 24 hours before the treatment. Then, cells were stimulated with 100 nM estrogen for 0, 3, 6 or 12 hours (Carroll, Meyer et al. 2006) GSE11324 datasets were normalized with RMA using BrB-tool which uses Bioconductor R packages because raw .cel files were available. 50000 genes were found after normalization step. One-way ANOVA ($p<0.001$) was applied to the normalized data and 5200 genes were selected from these 50000 genes. Then, by drawing scatter plot in BRB-tool, at least 2 fold increased estrogen upregulated genes were found in samples treated with estrogen for 3h, 6h, and 12h by comparing with the control sample (Table 4.13.).

Table 4. 13: Number of estrogen up-regulated genes in GSE11324 in different experimental setups.

Conditions	Number of estrogen up regulated genes
Estrogen at 0h vs. estrogen at 3h	187
Estrogen at 0h vs. estrogen at 6h	393
Estrogen at 0h vs. estrogen at 12h	1060

4.11.3 Common Genes between TFs found by MatInspector and Estrogen Up-Regulated Genes.

The intersects between the gene lists of transcription factors and gene lists of estrogen upregulated genes obtained from each reanalyzed microarray datasets according to their experimental setup, were found by using WebGestalt tools. (stands for WEB-based GEne SeT AnaLysis Toolkit), which incorporated information from different public resources. WebGestalt is designed for functional genomics, proteomics and large scale genetic studies from which high-throughput data are continuously produced (Zhang B. et.al. 2005). Transcription factors list coming from MatInspector was uploaded into Webgestalt. Then, common genes were found between two different gene lists. Intersects between transcription factors and estrogen up-regulated genes were represented in the tables.

Table 4. 14: Common genes between reanalyzed GSE1153 datasets and MatInspector

Conditions GSE1153	TF	Intersections
No estrogen vs. Era tr./w. estrogen for 8h	TF	PITX2
No estrogen vs. Era tr./w. estrogen for 24h	TF	CREB3L4, PITX2
No estrogen vs. Era tr./w. estrogen for 48h	TF	GLI3, PITX2
No estrogen vs. ERb tr./w. estrogen for 8h	TF	ELK4, GLI3,SMAD3
No estrogen vs. ERb tr./w. estrogen for 24h	TF	SMAD3
No estrogeb vs. ERb tr./w. estrogen for 48h	TF	ELK4,SMAD3

Table 4. 15: Common genes between reanalyzed GSE2292 datasets and MatInspector

Conditions	TF	Intersections
Era tr./w/o. estrogen vs. Era tr./w. estrogen	TF	ZNF283,MAFF
Erb tr./w/o. estrogen vs. ERb tr./w. estrogen	TF	HOXC10,NFE2L1
ERab tr./w/o.estrogen vs. ERab tr./w estrogen	TF	NFIL3

Table 4. 16: Common genes between reanalyzed GSE9757,GSE9758 and GSE9859 datasets and MatInspector.

Conditions	TF	Intersections
No estrogen vs. Era tr./w. estrogen (GSE9757)	TF	ERG1,ELF3, NR2F6,KLF9
No estrogen vs. Era(def) tr./w. estrogen (GSE9758)	TF	Empty
No estrogen vs. ERb tr./w. estrogen (GSE9759)	TF	ELF3,ESR2
No estrogen vs. ERb(def) tr./w. estrogen(GSE9759)	TF	ESR2

Table 4. 17 Common genes between reanalyzed GSE11352 datasets and MatInspector

Conditions	TF	Intersections
No estrogen vs. estrogen for 12h	TF	ERG3,FOXL1,HEY2,NR5A2,SOX3
No estrogen vs. estrogen for 24h	TF	ERG3,FOXL1,HEY2,NR5A2,SOX3
No estrogen vs. estrogen for 48h	TF	ERG3,FOXL1,HEY2,MAFF,NR5AS,SOX3

Table 4. 18: Common genes between reanalyzed GSE11506 datasets and MatInspector

Conditions	TF	Intersections
No estrogen vs. estrogen for 3h	TF	ERG3,ELF1,FOXL1,RARA,TGIF2, FOXN1
No estrogen vs. estrogen for 6h	TF	ERG3

Table 4. 19 Common genes between reanalyzed GSE2251 datasets and MatInspector

Conditions	TF	Intersections
ER neg. tr./w/o. estrogen vs. ER neg.tr./w. estrogen	TF	Empty
ER neg. tr./w/o. estrogen vs. Era tr./w. estrogen	TF	KLF12,ELF3,FOXO1A,HOXD1,PITX1,THRB, KLF10,NRID1,MAFB

Table 4. 20: Common genes between reanalyzed GSE4025 datasets and MatInspector

Conditions	TF	Intersections
Era tr./w/o. estrogen vs. Era tr./w estrogen	TF	LEBPB,LEBPG,ELF1,FOXM1,ITGB3BP HMGA1,HOXL4,HOXL6,FOXK2,ILF3, ATF3,TBX2

Table 4. 21: Common genes between reanalyzed GSE4668 datasets and MatInspector

Conditions	TF	Intersections
0 pM estrogen vs. 10 pM estrogen	TF	MAFG,BHLHB3,RUNX1
0 pM estrogen vs. 13 pM estrogen	TF	MAFG,BHLHB3,RUNX1
0 pM estrogen vs. 16 pM estrogen	TF	MAFG,BHLHB3,RUNX1
0 pM estrogen vs. 100 pM estrogen	TF	MAFG,BHLHB3,RUNX1

Table 4. 22: Common genes between reanalyzed GSE4006 datasets and MatInspector

Conditions	TF	Intersections
Era tr./w/o. estrogen vs. Era tr./w. estrogen	TF	EGR3,ELF1,ITGB3PB,MAFF,HOXL4,HOXL5,HOXL6,HOXL10, Sox3,KLF10,KLF4
Erab tr./w/o. estrogen vs. Erab tr./w. estrogen (5moi)	TF	EGR1,EGR3ELF1,FOXM1,FOXO1A, MAFF, HOXL4, HOXL5, HOXL6,HOXL10,NFIL3,Sox3,STAT4,TBX2,KLF10,KLF4
Erab tr./w/o. estrogen vs. Erab tr./w. estrogen (50moi)	TF	NR2F6,ETS2,HEY1,BCL6, in addition, same factors in the second raw

Table 4. 23: Common genes between reanalyzed GSE11324 datasets and MatInspector

Conditions	TF	Intersections
Estrogen at 0h vs. estrogen at 3h	TF	ELF1,HEY2,HIF1A,RUNX2
Estrogen at 0h vs. estrogen at 6h	TF	HEY2,HIF1A,SMARCA3
Estrogen at 0h vs. estrogen at 12h	TF	ATF2,E2F7,HEY2,HIF1A,ATF1,KLF3,SMARCA3 Sox3,SP3,TAF2,TAF9,TEAD1,E2F8,CLOCK

5. DISCUSSION

Stem cells are the new hope for the century since they have the potential to differentiate to several types of cells and tissues. They are future candidates for many therapeutic applications and can be the cure for many diseases such as neurodegenerative diseases, diabetes, and cancer (Fuchs and Segre, 2000). MSCs can differentiate to osteoblasts, chondrocytes, adipocytes, myocytes, cartilage, tendon, muscle, and fat tissue (Baksh *et al*, 2004; Caplan and Bruder, 2001). One of the major issues is that they form a very small population of nucleated cells in the bone marrow and their number has to be increased before they can be used for therapeutic purposes. There have been many advances to overcome this problem by mimicking their niche structure and by giving required factors, and hypoxic condition and estrogen are important players in these advances.

Mesenchymal stem cells are heterogeneous population of cells and are characterized after isolation from the bone marrow. MSCs that are cultured *in vitro* lack specific and unique marker. There is a general consensus that MSCs do not express the hematopoietic markers CD34 and CD45, whereas they do express variable levels of CD90, CD71 and CD29 (Uncelli A. et.al. 2008). In this respect, it is important to determine whether isolated bone morrow cells are MSCs cells indeed because it is possible to isolate rat MSCs with other cell types. Therefore, we also characterized the expression of specific markers and, our results showed that our cells expressed CD90, CD71 and CD29 but not CD34 and CD45, indicating that our 14^{th} day cells are MSCs. However, we cannot rule out the presence of small population of other cells because it is hard to obtain pure MSCs population (Uncelli A. et. al. 2008).

Hypoxia is one of the important environmental factors that plays role in the development, in disease formation and stem cell biology. It has been showed that hypoxic stimuli are important for the stem cell behavior (Spradling A. et. al 2001). It is believed that most of the niche structure where stem cells reside are quite hypoxic, which might be important to prevent stem cells from oxygen species, which then harm the DNA and cause formation of cancer stem cell (Parmar K. et. al. 2007). In addition, hypoxia can regulate cellular differentiation. Under hypoxic condition, the differentiation of embryonal stem cells is inhibited (Kile M. et al. 2005). Hypoxia also keeps their "stemness" feature, although its role is not fully understood (Lin Q. et. al. 2006). Bone morrow isolated MSCs cannot differentiate into adipocytes and chondrocytes when they are in hypoxic condition. The bone marrow microenvironment provides the niche structure for MSCs together with HSC and has lower oxygen concentration (Ezashi T. et. al. 2005).

Estrogen is another important physiological factor which regulates many pathways vital for the homeostasis of the body and its loss or decrease results in serious health problems such as osteoporosis, arteriosclerosis, increase in coronary heart diseases and neurodegenerative disease (Zhou *et al*, 2001). It also plays roles in development and maintenance of normal sexual and reproductive functions. It has been showed that estrogen is important for stem cell biology (Deroo and Korach, 2006). It is known that estrogen can enhance the proliferation and migration of some stem cells. Role of estrogen on MSCs biology is also known, in which estrogen gives protective role to MSCs in female by decreasing pro-inflammatory signals (Ray R et. al. 2008). Besides, it is also known that estrogen role in MSCs differentiation into adipogenesis and osteogenesis, while increase the proliferation rate of MSCs (Zhou *et al*, 2001).

Therefore, we would like to know whether two physiological stimuli play roles in MSCs biology by acting together. So far, there has been no study showing the relationship between estrogen and hypoxia in MSCs biology.

In order to reach our aim, we first generated hypoxic condition by using $CoCl_2$, which is an iron chelator and inhibits the ferrus state of PHD proteins that are the regulator of HIF1-alpha transcription factor. Since PHDs are no longer functional, Hif-1alpha escapes the posttranslational regulation and goes to nucleus and activates its target genes (Kaelin et. al. 2008). After hypoxic condition was mimicked, we performed colony forming assay, TUNEL assay and BrdU incorporation assay. According to the colony forming assay, the number of colonies increased upon $CoCl_2$ treatment when compared with normal samples, which was in accordance with result from Grayson et. al.,2007. The number of colonies was further increased by addition of estrogen in cells treated with hypoxic condition, suggesting that hypoxia led increase in the colony number and the number was further increased upon estrogen. Therefore, we can conclude that hypoxic response and estrogen pathway act together in MSC expansion. In addition, increase in colony number was also observed in cells treated with estrogen alone when compared with the normal cells, which was also shown by Zhou et al, 2001.

Although, we expected to see that the proliferation of MSCs in hypoxic condition was also correlated with increase in colony number as in other studies, such as Grayson et. al. 2006, we could not observe such correlation. The reason for this might be that $CoCl_2$ also affected the regulation of proteins playing role in cell proliferation. Since $CoCl_2$ is a heavy

metal, it may cause the repression of the activator of cell-cycle protein, which results in low rate in proliferation. In fact, most of the low oxygen tension studies were performed by using appropriate gas mixture where oxygen level was between 1-2%. However, since estrogen act as a growth factor in many cell types (Heldring N. et. al. 2007), it is expected to see that estrogen increases the proliferation rate. Indeed, the proliferation was increased again when estrogen was added to the cells treated with $CoCl_2$ and this was correlated with what was observed in colony forming assay. In addition, addition of estrogen alone to the normal MSCs led increase in the proliferation rate, which is in accordance with Zhou et al, 2001. As a result we can conclude that estrogen increase the proliferation of MSCs in both hypoxic and normoxic condition.

According to the TUNEL assay, the percentage of apoptosis was increased in hypoxic condition when compared to the normoxic cells, and the percentage was decreased when samples were treated with the estrogen, indicating that estrogen protects MSCs in hypoxic condition from undergoing apoptosis. In addition, Jung J. et. al. 2008 showed that estrodiol protects cells against $CoCl_2$ apoptosis, which was correlated with our result. There are conflicting results regarding to the effect of hypoxia on stem cells. Some studies stated that apoptosis was reduced in different stem cell populations cultured in lower oxygen conditions because of fewer reactive oxygen species are generated in culture in low vs. high oxygen conditions (Grayson W. et. al. 2007; Studer. L. et. al.2000). On the other hand, it has also been shown that hypoxia could cause apoptosis due to deprivation of growth factors (Weiquan Z. et. al. 2007 & Gui C. et. al. 2007). Our results correlated with the latter one at the hypoxic condition, where the percentage of apoptosis was reduced when these samples were treated with estrogen. Another reason why we found high apoptosis in samples treated with $CoCl_2$ was that we used $CoCl_2$, which might cause cellular toxicity and lead increase in apoptosis (Hongying R. et. al. 2006).

As a result, we may conclude that hypoxia and estrogen play synergistic role in expansion of MSCs. Our results showed that MSCs cells exhibited high colony number in hypoxic conditions and the expansion of MSCs was increased addition of the estrogen. Despite the fact that percentage of proliferation was not accordance with in colony formation rate, because of the likelihood effect of $CoCl_2$ on cell cycle proteins, increase in the proliferation upon estrogen treatment was observed in cells treated with or without $CoCl_2$, suggesting that estrogen increases the cell numbers. The increase in the percentage of apoptosis was expected because hypoxia also induces apoptosis and $CoCl_2$ might lead to

cellular toxicity. Addition of estrogen, however, decreases the pecentage in cells treated with or without estrogen, which suggests that estrogen plays a protective role for MSCs in hypoxic condition by preventing apoptosis.

Notch signaling is conserved across metazoan species, operates in most tissues and is used at various stages of decision-making in many cell lineages. Notch pathway is also important for stem cell maintenance because it leads to differentiation of one type of stem or progenitor cells into their specific lineages, while prevent the differentiation of others, and keep them in a self renewal state. Notch signaling was also important factors for niche structures and thus, important for the determination of fate of the stem cells reside in niche structure (Simon & Keith , 2008).

In the canonical Notch pathway, the single-pass transmembrane cell surface receptors (Notch 1–4 in mammals) undergo two sequential proteolytic cleavages upon binding of its ligands (Jagged-1 and Jagged-2 and Delta-like-1, Delta-like-3 and Delta-like-4 in mammals) presented on a neighboring cell surface. As a result, the Notch intracellular domain (NICD) is released from the plasma membrane and translocates to the nucleus, where it interacts with a transcription factor of the CSL family (RBP-Jk or CBF-1 in mammals) to activate transcription of target genes (Hilton M. et. al. 2008).

There is a relationship between notch signaling and hypoxia. Some hypoxic effects on progenitor cells correlate with the effects of Notch signalling in these cells. Gustaffson *et al.* have shown that hypoxia directly influences Notch activity. Hypoxia (1% O2), through the accumulation of HIF-1α, blocks the myogenic differentiation of C2C12 myoblast cells and the neuronal differentiation of P19 embryonic carcinoma cells. The authors propose a model in which HIF-1α interacts with Notch–CSLtranscriptional complexes at Notch-responsive promoters in hypoxic cells to control the differentiation status of myogenic and neuronal precursors (Simon C. & Keith B 2008). However, there is no direct relationship between notch signaling and the estrogen. All data regarding with estrogen and notch comes from the breast cancer, in which the expression of notch receptor and presence of estrogen are in reverse relation.

Therefore, we tried to investigate how expression levels of notch receptors were changed when cells treated with $CoCl_2$ because there is no direct information whether expression of notch receptors are increased upon hypoxic condition. We also tried to

understand whether estrogen affects the expression level of notch receptors in normoxia and hypoxic condition.

In order to reach our purpose, we investigated the expression level of Notch receptors in mRNA and protein level. According to RT-PCR results, expression level of Notch-1 did not changed in $CoCl_2$ treatment. The only change that we observed was that its expression level appeared to be decreased in normal samples treated with estrogen. Therefore, we can say that estrogen might decrease the expression level of Notch-1. Unfortunately, we cannot confirm this data in the protein level because there were not any western blot data because antibodies could not work for unknown reason. Besides, immunohiscochemistry result of Notch 1 indicated that expression level of Notch 1 increased upon $CoCl_2$ treatment, which is not correlated with the PCR data. In addition immunohistochemistry does not enough to show how estrogen affects the Notch-1 expression. As a result, even if we can say that estrogen might decrease the expression of Notch 1, these data is not enough to draw such a conclusion.

The information about the expression of Notch 2 came from the PCR result and its expression did not changed upon $CoCl_2$ treatment and estrogen treatment. In addition, there was no protein data about Notch 2 because there was no primary antibody for Notch 2. Therefore, we cannot say much about how hypoxia and estrogen affects the expression level of Notch 2.

The expression level of Notch 3 did not changed upon $CoCl_2$ treatment. Its expression appeared to be decreased when normal samples were treated with estrogen as in Notch 1. The protein expression seemed increase in cells treated with $CoCl_2$, but we could not say about the effects of estrogen because no western blot data could obtain because of the problem in work of primary antibody. The change in the expression level of Notch 3 was not correlated between mRNA and protein level. Therefore, it is hard to assume whether hypoxia and estrogen influence the notch expression in MSCs.

Notch 4 expression, however, exhibited more changes upon $CoCl_2$ treatment and estrogen treatment when compared with other notch receptors according to the PCR result. The expression level of Notch 4 was increased in hypoxic condition, which was accordance with the immunofluorescence, where its expression level was increased upon $CoCl_2$treatment. In addition, addition of estrogen to the samples led further increase in Notch 4 expression in cells treated with or without $CoCl_2$. Unfortunately, this was not confirmed by western blot because the Notch 4 primary antibody could not work. As a result, we can suggest that

hypoxia and estrogen have role in controlling the expression of Notch 4 expression, but further confirmation should be certainly done because the data are not enough to draw such a conclusion.

Consequently, we can conclude that the level of Notch 4 changed upon $CoCl_2$ treatment and show similar pattern with the protein level, and estrogen also increase the expression of Notch 4. We can also conclude that expression level of Notch 1, Notch 2 and Notch 3 did not change with $CoCl_2$ treatment. Its level was decreased in normal samples when treated with estrogen. Unfortunately, there is no appropriate protein data to support the assumption. In addition, there was disparity between mRNA and protein expression in Notch 1 and Notch 3, suggesting a possible posttranscriptional modification.

We also investigated the effects of estrogen on notch receptor expression by *in silico* analysis. In this time, we were not interested whether estrogen exerts its effect via ERα and ERβ receptor, instead we tried to detect if the expression level of notch receptor changed by other transcription factors whose expression are induced by estrogen. In order to reach our aim, transcription factors that bind to regulatory region of Notch 1 receptor were examined. We investigated Notch 1 because it was known that its expression was increased upon estrogen treatment in MCF-7 cells lines (Soares R. et.al.2004). Since most of the promoter region resides in 5' UTR region of the genes, we found 10000bp upstream sequence of Notch 1 from the first exon by using BioMart. The sequence then was entered to the MatInspector data base, which was developed for finding to transcription factor binding to given sequence (Cartharius et al., 2005). Transcription factors were selected according to their core and matrix similarity values which were between 0.9 and 1 as indicated by Cartharius et al., 2005. Twelve microarray datasets were reanalyzed by BrB tools. From the analysis, estrogen up-regulated genes were selected from each reanalyzed microarrays according to the experimental setups which was described in the result. Differentially expressed genes were selected after one-way ANOVA was performed. In order to combine the data obtained from MatInspector and obtained from reanalyzed microarray datasets, common genes were found by using Webgestalt tools. As a result of these common genes, we concluded that these transcription factors were upregulated upon estrogen treatment and they had the ability to bind to the 5' UTR of region Notch 1, suggesting that these transcription factors may be responsible for the Notch 1 expression upon estrogen treatment. These should be checked by wet-lab experiments for further studies. The intersection of these common genes was also investigated, and we found that ERG family transcription factors, ELF family transcription

factors, HOXL4 family transcription factors, KLF family transcription factors and transcription factors SOX3 were common, indicating that further characterization of these factor may be important to understand the relationship between estrogen and notch signaling. Besides, KLF family transcription factors and Sox3 might be more important because it has been known that Sox-2 and KLF-4 were essential player in stem cell biology in which they required for stem cell pluripotency (Keith B. et. al. 2007). Therefore, it is interesting to investigate these families in our system. We believed our results were significant because we reanalyzed 12 microarrays datasets performed with different cell lines, with different concentration of estrogen and with different incubation times. . We also believe that transcription factors found by MatInspector did not contain false positive results because we selected the factors according to their core and matrix similarity values.

Despite the fact that physiological stimulus are important for MSCs maintenance, extra cellular components of the niche structure are also important for MSC behavior. Tissue engineering has become one of the leading areas, in which an *in vivo* local environment that enables cells to promote their proliferation and differentiation is created by making use of biomaterials and technologies (Tabata Y. 2009). Biomaterials play a key role in designing and creating substitutes for ECM, which is important for cell proliferation and differentiation because it provides the living place of cells and biosignalling molecules as the nutrients of cells. In addition to therapeutic applications, biomaterials are also useful in the progress of research and development of stem cell biology and medicine (Tabata Y. 2009).

In these regards, we tried to expand female rat bone marrow MSCs on twenty different biomaterials, which were produced combinations of different polymer described in the materials and method section. Our purpose was to distinguish which biomaterials were suitable for MSCs attachment and maintenance, but not for the other cell types coming from the bone marrow. In order to determine this, we investigated whether MSCs were able to attach the biomaterials after 14 days of culture by checking the morphology of the cells and by the colony forming assay.

Our results showed that MSCs were able to form colonies on some of the biomaterials. We observed MSCs on biomaterials designated as CIII-1, CIII-2 CIII-3, CIII-5, CI-K, CI-A, CA-1 and CA-2. Among them only on biomaterial CIII-3, MSCs did not from colonies shown by CFU-F. Biomaterials CA-1, CA-2, CA-3, CI-K,CI-A, CIII-1, CIII2 and CIII-5 supports the attachment and growth of the MSCs-like cells as indicated in colony

formation and cell morphology. We can also conclude that cells grown on CI-K, CIII-1, CIII-2 and CIII-5 are more appropriate materials to use in expanding MSCs because no other cells attached to them.

In addition, we can also conclude that the combination of polymers designated as HEMA/HPC is appropriate in the proportion of 1:0 (CI-K) and 2:1(CI-A). The combination polymers designated as HPMA/PEG provided appropriate conditions when prepared in the proportion of 1:0 (CA-1), 1:1 (CA-2) and 2:1 (CA-3). The appropriate proportion of polymers designated as HEMA/PEG/HPC was 2:1:1(CIII-1), 3:0:1 (CIII-2) and 1:1:0 (CIII-5). The combination of polymers designated as HPMA, PEG and HPC did not provide suitable condition for MSCs. Moreover, different derivatives of HEMA and HPMA might be used in order to produce more suitable biomaterials because it was shown that modified HEMA or modified HPMA provided a good condition for MSCs isolated from the rat bone marrow, which then was used for the treatment of spinal cord injury (Sykova E. et. al 2006). Modification of PEG polymer might also improve the attachment of MSCs on biomaterials designated as C, and as CII (Lin C. et.al. 2008). However, although colony forming is one of the important features of MSCs cells and might give better insight whether cells are MSCs, specific markers should also be controlled for better characterization. It should be also noted that some biomaterials might toxic to the cells or they not allow to diffusion of nutrients and waste product of cells to the medium (Sridharan et. al. 2009) because of the combination of different polymer, which might be the reason of problem in observation of cells grown in some biomaterials. In addition, visualization became a big problem when investigating biomaterials, which may be result from the chemical composition of the polymers.

In general, we conclude that hypoxia and estrogen are required environmental factors for MSCs biology and estrogen might play a protective role in MSCs by inhibiting apoptosis in hypoxic condition. In addition, according to the bio informatics analysis, ERG family transcription factors, ELF family transcription factors, HOXL4 family transcription factors, KLF family transcription factors and SOX3 were upregulated upon estrogen and then they might bind to the Notch 1 transcriptional regulatory region. Lastly, biomaterials, designated as CA-1, CA-2, CA-3, CI-K, CI-A, CIII-1, CIII-2 and CIII-3, were appropriate candidates to expand MSCs cells and further studies.

6. FUTURE PERSPECTIVES

Since we hypothesizes that hypoxia and estrogen are essentials factors for MSCs expansion and since we speculate that estrogen prevent apoptosis in MSCs in hypoxic conditions, some further studies can be performed . The cell cycle stages of MSCs can be investigated in hypoxic condition with or without estrogen treatment in order get more information about how estrogen and hypoxia affect the proliferation of MSCs. In addition, the expression level of cell cycle protein can also be investigated. The role of estrogen in both pro-apoptotic and anti apoptotic genes can be investigated in hypoxic condition. The expression level of these genes can be checked in MSCs in hypoxic conditions with or without estrogen treatment. Since MSCs are multipotent stem cells, the effects of hypoxia and estrogen can be determined in MSCs differentiation. However, in this time it is better to use the appropriated gas mixture because differentiation assay takes long times, and addition of excess $CoCl_2$ may cause toxicity to cells. By analyzing the how differentiation of MSCs to adipogenesis and osteogenesis changes in hypoxia with or without estrogen gives important understanding about MSCs expansion upon physiological stimuli.

The effect of estrogen on notch expression can also be investigated. Common transcription factors found by *in silico* analysis can be controlled one by one. In order to confirm the results, Chip assay can be performed whether these genes bind to the Notch regulatory regions. Reporter gene assay can also be performed to confirm this. The expression level of these factors can be checked upon estrogen treatment to confirm the bioinformatics data. In addition, the effect of estrogen on ADAM-10 metalloproteinase expression can also be investigated because ADAM family proteins play a role in notch receptor cleave, and thus activation. Therefore, this might reveal another mechanism in the regulation of Notch receptor by estrogen.

The features of biomaterials used in our study can be improved. The composition of the polymer can be changed, and other molecules can be used to get better bio materials such as ECM components. Besides, effects of estrogen can be investigated when cells grown on the biomaterials. Since estrogen is one of the growth factors, its presence might increase the expansion of MSCs when grown on them. Since visualization poses a problem in taking the pictures, other chemicals can be added in the process of polymerization. Furthermore, MSC-like cells grown on them should be characterized by looking the expression of specific markers of MSCs.

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8. APPENDICES

Appendix A

Standard Solutions and Buffers

DEPC-Treated ddH_2O

1ml DEPC

1lt ddH_2O

Stirred in a hood for 1 hour, autoclaved in order to inactivate the DEPC

10x PBS

80 g NaCl

2 g KCl

8,01g $Na_2HPO_4.2H_2O$

2g KH_2PO_4

1 liter ddH_2O pH: 7,2

Working solution (1XPBS) was prepared by diluting 10XPBS by 10 times

50X TAE Buffer

2M Tris Base (242 g)

57,1 ml Glacial Acetic Acid

50mM EDTA

Add to 1 lt by ddH_2O

Working solution (1XTAE) prepared by diluting 50XTAE by 50 times

10 X Agarose Gel Loading Dye

0,009g BFB

0,009g XC

2,8mL ddH2O

1,2ml 0,5M EDTA.

Total volume brought to 15ml by adding glycerol, dilute 1:10 in sample prior to loading to electrophoresis gel

Cold 70% Ethanol

70 ml Ethanol

30 ml ddH_2O

Dissolved and keep at 4 C

4% PFA

4g PFA

100ml 1XPBS

Dissolved by heating, solution prepared freshly

10%SDS

100g SDS

1lt ddH_2O

1M Tris

60.55g Tris

300ml ddH2O

21ml 37% HCl

pH adjusted to 8.0 with HCl, total volume brought to 500ml by adding ddH_2O.

0.5M EDTA

93.05g EDTA

300ml ddH2O

pH adjusted to 8.0 with NaOH, total volume brought to 500ml by adding ddH_2O.

30% Acrylamide Mix

145g Acrylamide

5g bis-acrylamide

500ml ddH_2O

Filtered, stored in dark at 4^0C.

SDS-PAGE Running Buffer (5X stock solution)

15g Tris

73,2g Glycine

5g SDS

1lt ddH2O

1X working solution prepared by diluting the 5X SDS-PAGE running buffer 5 times, stored at 4^0C

Bradford Reagent

100 mg Coomassie Brilliant Blue G-250

100 ml 85%phosphoric acid

50 ml 95%EtOH

850 ml ddH_2O

Filtered through Whatman no:1

Lysis Buffer

2M NaCl

1M Tris pH:8.2

0.9% Igepal CA-630 (Sigma, Germany)

10x Protease Inhibitor Cocktail (Roche, Germany)

778.5µl ddH_2O

10X TBS

12.19 g Tris

87,76 g NaCl

pH adjusted to 8, total volume brought to 1lt by adding ddH_2O

1X TBS-T 0.3%

50 ml 10X TBS

450 ml ddH_2O

1,5 ml Tween-20

Total volume brought to 500ml by adding ddH_2O

Blocking Solution for Western Blot (5%)

2,5 g milk powder

50mL 1X TBS-T

5% BSA Solution

0.5g BSA

10mL 1X TBS

20 µl tween-20

Blocking Solution for Immunohistochemistry and Immunoflourescence Staining

4 µl 2% BSA

4 µl 1X PBS

20 µl tween-20

Block Solution for BrdU incorporation assay

1.5g BSA

50mL 1X PBS

50 µl tween-20

Cracking Buffer (2X Protein Loading Buffer)

50mM Tris HCl pH: 6,8

2mM EDTA pH: 6,8

1% SDS

20% Glycerol

0,02% BFB

Add 1% ß-mercaptoethanol prior to use

Coomassie Blue Staining Solution:

0,25 g coomassie brilliant blue

45 ml Methanol

45 ml ddH_2O

10 ml glacial acetic acid

5X Running Buffer

15 g Tris base

72 g Glycine

5 g SDS

1 liter ddH_2O

Diluted to 1X prior to use

Semi-dry Transfer Buffer

2,5 g glycine

5,8 g Tris base

0,37 g SDS

200 ml methanol

800 ml ddH_2O

Destaining Solution

100 ml methanol

35 ml acetic acid

365 ml ddH_2O

1 µM $CoCl_2$ stock solution

0.02g $CoCl_2$

10ml ddH_2O

2N HCl solution

16.38µl ddH_2O

4.3µl HCl at 37 ^oC