ROLE OF ESTROGEN ON THE MAINTENANCE AND HOMING CAPACITY OF BONE MARROW DERIVED RAT MESENCHYMAL STEM CELLS

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ABSTRACT

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Ph.D. in Molecular Biology and Genetics

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Mesenchymal Stem Cells (MSCs) can self renew and differentiate into different cell types like, adipocytes, osteoblasts, chondrocytes, neurons, hepatocytes and endothelial cells. Their ability to differentiate into wide variety of cell types, non-immunogenic characteristics, along with homing capacity to injured tissue and the absence of any ethical issue related to their uses, make MSCs important in regenerative medicine and tissue engineering. Their ability to migrate to the site of injury raises the opportunity of these stem cells to be considered also as *in vivo* delivery agents. However, one major obstacle in using MSCs in cell based therapies is their limited numbers. Estrogen is known to have role in growth, proliferation and apoptosis. Therefore our aim was to study the possible role and mechanism of estrogen in their maintenance and their homing capacity. We used MSCs derived from female and ovariectomized female rat bone marrow in our research. Our

results revealed that estrogen treatment increased the number of colonies and the number of cells constituting a colony. Estrogen decreased the differentiation capacity of MSCs to the adipogenic lineages as shown by reduced of Oil Red O staining Estrogen also affected MSCs' homing capacity. Estrogen treatment resulted in the migration of increased number of DiI labeled MSCs to the site of injury after partial hepatectomy (PH) compared to that of untreated MSCs. Furthermore, estrogen treatment decreased the rate of apoptosis. Our data showed that estrogen regulates apoptosis through Bcl-2 family of genes in MSCs. This regulation was at the protein level but not at the transcript level. Estrogen addition increased the expression of anti-apoptotic members of the Bcl-2 family of proteins, Bcl-2 and Bcl-$x_{L.}$ The decrease in the apoptosis was not observed when Bcl-x_L and Bcl-2 genes were knocked down. The silencing histone code H3K27me3 was also decreased in estrogen treated MSCs, suggesting an epigenetic regulation of MSCs upon estrogen treatment. Altogether our results show that estrogen increased the number of functional MSCs, decreased spontaneous apoptosis in these cells, and improved the homing capacity of rat bone marrow derived MSCs. Therefore, estrogen treatment of MSCs may offer new opportunities for the therapeutic actions of these cells.

ÖZET

ÖSTROJEN HORMONUNUN SIÇAN KEMİK İLİĞİNDEN ALINMIŞ MEZENKİMAL KÖK HÜCRELERİNİN MUHAFAZASINDA VE HASARLI DOKUYA ULAŞMASINDAKİ ROLÜ

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Mezenkimal Kök Hücreler (MKH) kendilerini yenileyebilen ve adiposit, osteosit, kondrosit, sinir hücresi, hepatosit ve endotel hücresi gibi farklı hücrelere farklılaşabilen hücrelerdir. MKHlerin yüksek farklılaşma kapasitesi, immün reaksiyon oluşturmama özelliği, hasarlı dokuya gitme özelliği ve kullanımı ile ilgili etik sorunların olmaması, bu hücrelerin rejeneratif tıp ve doku mühendisliğinde önemli bir yerde olmalarının sebebidir. MKHlerin hasarlı dokuya ulaşabilmeleri aynı zamanda bu hücrelerin *in vivo* taşıyıcı ajan olarak kullanılması olasılığını da ortaya çıkarmıştır. Fakat MKHleri terapide kullanmanın önündeki en büyük zorluk onların sayıca az olmalarıdır. Östrojen hormonu büyüme, proliferasyon ve apoptozda rol aldığı bilinmektedir. Bu yüzden amacımız, östrojenin MKHlerin hasarlı dokuya yönelme ve yerleşmesindeki ve bu hücrelerin devamlılığındaki

rolünü calışmaktı. Çalışmalarımızda dişi sıçanın kemik iliğinden alınan MKHler ve overleri çıkarılmış dişi sıçanın kemik iliğinden alınmış MKHler kullanıldı. Östrojen koloni sayısını ve kolonideki hücre sayısını arttırdı. Oil Red O boyaması ile görüldüğü gibi, östrojen aynı zamanda MKHlerin yağ hücresine farklılaşmasını azalttı. Östrojen verilen MKHler verilmeyen MKHlere gore kısmı hepatektomi yapılan sıçanların karaciğerlerine daha fazla sayıda gidip yerleştiler. Bunlara ek olarak östrojen MKHlerinin apoptozunu azalttı. Sonuçlarımız östrojenin apoptozu Bcl-2 gen ailesi üzerinden kontrol ettiğini gösterdi. Bu kontrol mRNA seviyesinde değil fakat protein seviyesindeydi. Östrojen eklenmesi Bcl-2 protein ailesinin anti-apoptotik üyelerinin protein seviyesini arttırdı. Apoptozdaki bu azalma Bcl-x_L ve Bcl-2 susturulduğu zaman görülmedi. Susturucu histon kodu olan H3K27me3 östrojen ile azalmakta, ve bu MKH gen ifadelerinin epigenetik bir yolla kontrol edildiği fikrini otaya atmaktadır. Bütün bu sonuçlar östrojenin fonksiyonel MKHlerin ve hasarlı dokuya ulaşan MKH sayısını arttırdığını, spontan olan apoptozu önlediğini göstermektedir. Bu sebeple bu hücrelerin östrojene maruz bırakılması onların tedavide kullanılmasına yeni olanaklar sunabilir.

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ABBREVIATIONS

AIF	Apoptosis Inducing Factor
AIM	Adipogenic induction media
APAF1	Pro-Apoptotic Protease-Activating Factor-1
asc	Ascorbic Acid
ASC	Adult Stem Cell
Bak	BCL2-antagonist/killer
Bax	Bcl2-associated X protein
Bcl-2	B-cell leukemia/lymphoma 2
Bcl-x_L	B cell lymphoma like X
BFB	Bromophenol Blue
BH	Bcl-2 homology
BMP	Bone Morphogenic Protein
bp	Base Pairs
BSA	Bovine Serum Albumin
CARD	Caspase Recruitment Domain
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic Acid
CFU-F	Colony-forming Units-fibroblastic
Cyt c	Cytochrome c
ddH2O	Double Distilled Water

dex	Dexamethasone
dH2O	Distilled Water
DIABLO	Direct IAP binding protein with low pI
DISC	Death Inducing Signaling Complex
DMEM	Dulbecco's Modified Eagle Medium
DMEM-LG	Low Glucose Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTP	deoxy Nucleotide Triphosphate
ER	Estrogen Receptor
ERE	Estrogen Response Elements
ERα	Estrogen Receptor Alpha
ERβ	Estrogen Receptor Beta
ESC	Embryonic Stem Cell
FADD	Fas Associated Death Domain
FBS	Fetal Bovine Serum
Fgf2	Fibroblast growth factor 2
g	Grams
GFP	Green Fluorescent Protein
GVHD	Graft-versus-host Disease
H_2O_2	Hydrogen Peroxide
H3	Histone 3

H4	Histone 4
hESC	Human Embryonic Stem Cell
HRP	Horse Raddish Peroxidase
HSC	Hematopoietic Stem Cell
IAP	Inhibitor of Apoptosis Proteins
IBMX	Isobutyl methyl xanthine
ICM	Inner Cell Mass
IF	Immunofluorescent
Indo	Indomethacine
iPS	induced Pluripotent Stem
iPSC	induced Pluripotent Stem Cells
JmjC	Jumonji-C
K	Lysine
kDa	kilo Dalton
LIF	Leukemia Inhibitory Factor
Lpl	Lipoprotein lipase
LSD1	Lysine Specific Demethylase 1
M	Molar
mA	Mili Amper
MEF	Mouse Embryonic Fibroblast
mESC	mouse Embryonic Stem Cell
MetOH	Methyl Alcohol
mg	Milli Gram

mL	milliLiter
mM	milliMolar
MPTP	Mitochondrial Permeability Transition Pore
MSCs	Mesenchymal Stem Cells
NaCl	Sodium Chloride
NCCD	Nomenclature Committee on Cell Death
NIH	National Institute of Health
NTBC	2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexanedione
o.n.	Over night
Oc	osteocalcin
OIM	Osteogenic Induction Media
Ovx	Ovariectomized
Ovx	ovariectomized
PAGE	Polyacrylamide Gel Electrophoresis
PARP-1	Poly-ADP-ribose polymerase-1
PBS	Phosphate Buffered Saline
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PH	Partial Hepatectomy
PI3K	Phosphatidylinositol 3 kinase
Pparγ	Peroxisome proliferator activated receptor gamma
PTM	Post Translational Modifictaion

PVDF	Polyvinylidenedifluoride
R	Arginine
RiPSC	RNA derived iPS Cells
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
Rpm	Revolution per minute
RT	RT
RT-PCR	Reverse-Transcriptase Polymerase Chain Reaction
Runx2	runt-related transcription factor 2
SDS	Sodium Dodecyl Sulfate
SH	Sham
shRNA	small hairpin RNA
ß-gp	beta glycerophosphate
TAE	Tris Acetate EDTA
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline with tween
TERT	Telomerase Reverse Transcriptase
TGF-ß	Transforming Growth Factor-Beta
TNF	Tumor Necrosis Factor
TRAF	TNF-associated Factor
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
V	Volt
VDAC	Voltage Dependent Anion Channel

Chapter 1

INTRODUCTION

Stem cells have attracted a lot of interest in the last three decades. Not only researchers but also the community is interested in this field due to their potential usage in regenerative therapy. Despite to the high attention and focus on these cells there is no definitive description of the stem cell concept. However, the National Institude of Health (NIH) defines stem cells as " Cells that can divide infinitely and give rise to differentiated cells *in vivo* and *in vitro*" (http://stemcells.nih.gov/). Therefore self-renewal, differentiation and *in vivo* reconstition are critical features of stem cells.

Generally stem cells can be classified according to their source of origin and their potency. During early embryonic development, inner cell mass (ICM) give rise to embryonic stem cells (ESCs). Stem cells that are isolated at later developmental stages and postnatal period collectively are called adult stem cells (ASC). Another classification is related to their potency. Totipotent stem cells give rise to the cells from the extraembryonic tissues in addition to the cells from the three germ layers, (ectoderm, endoderm and mesoderm), whereas the pluripotent stem cells give rise to the cells from the three germ layers but not to the extra embryonic cells. Multipotent stem cells differentiate into the cell types from their original germ layer and another germ layer (http://stemcells.nih.gov/). ESCs and the induced

pluripotent stem cells (iPSC) are pluripotent whereas most of the adult stem cell are multipotent, and the zygote is the only cell that is totipotent.

Use of stem cells in therapy has brought new hope in the field of regenerative medicine. The capacity of stem cells to differentiate into different types of cells after correct stimuli made them a very trustworthy agents in medicine for the treatment of several genetic, chronic and non-infectious diseases like osteogenesis imperfecta, parkinsons disease, chronic heart diseases, spinal cord injuries, liver cirrhosis, and diabetes (http://www.clinicaltrials.gov). Embryonic stem cells (ESC) are very promising agents since they can differentiate into the cells of all three germ layers. However their use is very limited due to the ethical problems since embryo is the source. To overcome this, researchers developed induced pluripotent stem (iPS) cells which are pluripotent like embryonic stem cells, but do not require the embryo therefore have no ethical problems. However, these cells are not completely characterized and it will take time before seing them in medicine. Mesenchymal stem cells (MSCs) on the other hand, are multipotent stem cells that can differentiate into many types of cells upon induction. Since they are adult stem cells there is not any ethical concern related to the usage of them. They are known for nearly 40 years and are almost fully characterized. That is why, great hope has been evolved for the usage of MSCs in therapies.

To increase the quantity of MSCs for further use, estrogen is an important factor. Estrogen can induce apoptosis in some cells and prevent apoptosis in other cells.

Treating MSCs with estrogen can have great impact in using these cells in cell based therapies.

Our aim in this study was to investigate the role of estrogen in MSC maintenance and the molecular mechanism of its effect.

In this thesis first, stem cells based on their potency will be explained. After defining the the pluripotent stem cells, ESCs and iPS cells, the multipotent stem cells will be described with an emphasis on MSCs. Estrogen and apoptosis will be explained afterwards. For the possible regulatory mechanism behind this event, epigenetic modification with an emphasis on histone post translational modification (PTM) will be defined.

1.1 PLURIPOTENT STEM CELLS

As mentioned before, pluripotent stem cells are the cells that have the capacity to differentiate into the cells of all three germ layers, not to the cells of the extraembryonic tissue (http://stemcells.nih.gov/). ESCs and iPSCs are the examples for pluripotent stem cells.

1.1.1 EMBRYONIC STEM CELL

Embryonic stem cells were first isolated by Evans and Kaufmann in 1981 from mice blastocysts [1] and by Thomson and coworkers in 1998 from human blastocysts [2]. ESCs express spesific pluripotency marker genes, which are called

the intrinsic pluripotency factors, Sox2, Nanog, and Oct4. These genes are responsible for holding them in an undifferentiated state [3-6]. They are able to form teratomas when injected into blastocysts of an immunosuppressed mouse and also contribute to the generation of chimeras [7, 8]. They form embryoid bodies *in vitro* which have cells of all the three germ layers in a disorganized manner [1]. ESCs have high telomerase activity which results in long telomeres and immortality [8]. These cells can be maintained indefinitly in undifferentiated state *in vitro* without loosing normal karyotype [8, 9]. X chromosome inactivation does not occur in undifferentiated ESCs [10]. ESCs cause immune rejection when injected into immunocompetent recipient [11]. Besides having common features of mouse ESCs (mESCs) and human ESCs (hESCs) there are also differences between them. Human ESCs are able to differentiate into trophoblast like cells while mESC can not [9]. hESCs require a feeder layer composed of mouse embryonic fibroblasts (MEF) for in vitro propagation [9]. However, mESCs do not require MEF if leukemia inhibitory factor (LIF) is added to the media [9, 12]

The signaling molecules required for ESC maintenance also differ between mESCs and hESCs. LIF and BMP are the main two extrinsic signaling pathways that are responsible for mESC self renewal. Constitutive expression of c-myc is required for self renewal. LIF activates c-myc either directly or indirectly through two different mechanism, the JAK/ STAT3 pathway [13-15] and the phosphatidylinositol 3 kinase (PI3K) dependent pathway [16]. The other pathway required for stem cell

maintanance involves the BMP signaling. Several laboratories have shown that BMP acts in a repressive manner by inhibiting the differentiation of mESC and maintaining their pluripotent state [17-19]. On the other hand, hESC self renewal is controlled via different factors like Fgf2, TGF-β, noggin, activin, and Wnt [20-24]. Greber and friends showed that hESC cultured with MEFs retained their self renewal activity when supported with Fgf2 [25]. Since there are some differences between mESCs and hESCs, the results from mESCs cannot be directly extrapolated to hESCs [9]. That is why hESC research has to be performed independent from different species. Major handicap for hESCs research is their source. During their isolation from blastocyst, the embryo is destroyed. Even though there are very strict regulations of using embryos from IVF clinics, this is a very important ethical concern around the world. This prompted the researchers to find alternative sources for pluripotent stem cells without ethical concerns. Inducing pluripotent stem cells from differentiated somatic cells through redifferentiation was a major breakthrough in stem cell research.

1.1.2 INDUCED PLURIPOTENT STEM CELL

Induced pluripotent stem cells (iPS) were first made by Yamanaka group from mouse fibroblast in 2006 [26]. They started with 24 pluripotency related genes and narrowed them down to 4 minimaly required genes, *c-Myc, Sox-2, Klf-4* and *Oct-4,* for inducing pluripotency when transfected into mouse fibroblasts. These factors are now called the Yamanaka Factors. iPS cells have similarity with ESCs in their

ability to form teratomas. However, they could not contribute to the formation of chimeras or to germ lines as efficient as ESCs. Compared to ESCs iPS cells had been shown to have incomplete promoter demethylation, and to express low levels of some pluripotency related genes [26]. Thereafter, new iPS cell lines were generated by different research groups and the features of this new lines were more similar both functionally and molecularly to ESCs [27-29]. Different laboratories developed iPSC from human, rat and monkey by using factors other than the Yamanaka Factors [30-33]. Not only fibroblasts but also B cells [34], hepatocytes [35], keratinocytes [36], and amniotic cells [37] were used as a starting cell to make iPSC. A list of the cell source of iPS cells and the factors that are used to generate them is given in table 1.1. Another improvement in iPS cell research was to use safer methods for transfections. Yamanaka group used retroviruses as delivery agents.

For this purpose different delivery methods were used; i) retroviral vectors that integrate into the genome, ii) inducible lentiviral vectors whose expression can be controlled [38, 39], iii) plasmids or nonintegrative adenovectors which do not integrate into the genome but have low transfection efficiency [40-42], iv) Cre/LoxP approach which enables to remove the vector afterwards by transient expression of Cre recombinase [43], v) PiggyBac transposons which can also be removed from the genome by expression of transposases [44, 45], vi) chemical defined approach that uses only chemical compounds for reprograming [46] , and vii) delivering the purified recombinant protein without any virus or plasmid which prevents any

integration into the host genome, but has a low transfection efficiency [47, 48]. In 2010 a group of scientist developed an efficient and safe delivery method to generate iPS cells. They used modified RNA molecules encoding the four Yamanaka Factors and Lin28 and transfected them into fetal fibroblasts, postnatal fibroblasts, and cells derived from skin biopsy from a cystic fibrosis patient. These RNA derived iPS cells (RiPSC) were generated with high efficiency and directed the iPS biology to a new path [49].

Table 1.1: IPS cells derived from different species and cell types by different factors. O: Oct4, S: Sox2, K:Klf4, M: cMyc, L: Lin28, C: C/EBPα

SPECIES	CELL SOURCE	FACTORS	REFERENCES
mouse	fibroblast	OKSM	[26]
human	fibroblast	OKS	[30]
human	fibroblast	OSLN	[31]
rat	fibroblast	OKSM	[32]
rhesus monkey	ear skin fibroblast	OKSM	[33]
mouse	B cell	OKSM+ C	[34]
human	hepatocytes	OKSM	[35]
human	keratinocytes	OKSM/OKS	[36]
human	amniotic cells	OKSM	[37]
mouse	dermal papilla	OKM/OK	[50]
mouse	fibroblast	OKS	[30], [51]

iPS cells are promising candidates to be used in therapy since they can be isolated from the patient himself and therefore immune rejection of the transplant is not an issue. In 2007 iPS cells generated from fibroblast of a sickle cell anemia murine model was used to cure the disease. Before the fibroblast were redifferentiated, the

genetic defect in the hemoglobin gene was treated *in vitro*. The engineered iPS cells were differentiated to hematopoietic progenitor cells and these cells were given to the donor mouse. It was observed that the mature erythrocytes had no defect in hemoglobin gene [52]. Another group used iPS cells to restore liver function in FAH deficient mice model, in which they had to have the drug, 2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC), in order to prevent liver failure. After injection of the iPS cells to the blastocyst, the drug NTBC was withdrawn. It was observed that the iPS cells contributed to the formation of chimera and the mice had functional liver with FAH expressing hepatocytes [53]. Although iPS cells are promising candidates in cell therapy there is still a long way before this could happen. However, a more realistic approach of iPS cells in therapy is the use of disease specific iPS cells to mimic the disease and provide a patient spesific cell line to study the pathology in the culture. Several disease spesific iPSC have been established. A group of scientist generated a large number patient spesific iPS cell lines from patients with Adenosine deaminase deficiency-related severe combined immunodeficiency, Gaucher disease type III, Duchenne and Becker muscular dystrophy, Parkinson disease , Huntington disease , juvenile-onset, type 1 diabetes mellitus [54], spinal muscular atrophy [55], familial dysautonomia [56], and fanconi anemia [57]. Some of the iPS cell lines generated from this patients showed the disease profile in the culture and provided new opportunities to study the molecular mechanism of he disease and to search for drugs [58].

1.2 MULTIPOTENT ADULT STEM CELLS

Adult stem cells (ASC) are stem cells that are found in the adult organ or tissue of an organism. Unlike the ESCs or the iPSCs, ASCs are not pluripotent. Many of the ASCs are multipotent and a few are unipotent. ASCs can be thought of a reservoir of cells, which normaly stay in an undifferentiated state in the niche which is the environment where the ASCs reside. Like all stem cells ASCs possess two universal features; the ability to self renew and to differentiate [59], which is called stemness [60]. The maintenance of ASC pool is not clear, but the most accepted view is that ASCs divide in an assymetric fashion, giving rise to two different daughter cells with different fates. The one that is still in the niche becomes the self renewing sister for protecting the reservoir, while the other sister cell leaves the niche and differentiates into the desired cell type [61, 62]. Different ASCs have been identified. The first identified and so far the best charecterized ASCs are hematopoietic stem cells (HSCs). HSCs were first mentioned in the 60s by Till and McCulloch [63], and Siminovitch et al. [64]. These HSCs were able to reconstitude all of the blood cells [65, 66]. Two subsets of the HSCs have been classified; the Long term and short term reconstitutive cells (LT-HSC and ST-HSC, respectively) [65, 67]. In mouse in addition to these two subsets, there are also two other progenitor derived from the HSCs, Common Lyphocytes Progenitors (CLPs) and the Common Myeloid Progenitors (CMPs) [68, 69]. Different surface markers are used to separate these subtypes from each other [69].

Table 1.2: The location of different kinds of ASCs in the body and their differentiated progeny. (adopted from [70]).

CELL TYPE	TISSUE SPECIFIC LOCATION	CELLS OR TISSUE PRODUCED
Hematopoietic stem cells	bone marrow, peripheral blood	bone marrow and blood lymphohematopoietic cells
Mesenchymal stem cells	bone marrow, peripheral blood	bone, cartilage, tendon, adipose tissue, muscle, marrow stroma, neural cells
Neural stem cells	ependymal cells, subventricular zone of the central nervous system	neurons, astrocytes, oligodendrocytes
Hepatic stem cells	in or near the terminal bile ductules	oval cells that subsequently generate hepatocytes and ductular cells
Pancreatic stem cells	intraislet, nestin positive cells, oval cells, duct cells	beta cells
Skeletal-muscle stem cell/satellite cells	muscle fibers	skeletal muscle fibers
stem cells of the skin	basal layer of the epidermis, bulge zone of the hair follicles	epidermis, hair follicles
Epithelial stem cells of the lung	tracheal basal and mucus secreting cells, bronchiolar Clara cells, alveolar type II pneumocyte	mucous and ciliated cells, type I and II pneumocystis
Stem cells of the intestinal epithelium	epithelial cells located around the base of each crypt	paneths cells, brush border enterocytes, mucus secreting goblet cells, enteroendocrine cells of the villi

Other than HSCs, intestinal stem cells, neuronal stem cells, hepatic stem cells, skin stem cell, mammary stem cells, pancreatic stem cells, and mesenchymal stem cells are other well characterized ASCs. These ASCs have the capacity to differentiate into different types of cells upon tissue damage or any other means of requirement

to maintain their homeostasis [70]. A list of the ASCs and their tissue spesific localization together with the spesific cell or tissue produced from are given in table 1.2.

1.2.1 MESENCHYMAL STEM CELL

MSCs) were first isolated from rat bone marrow and described in 1966 by Friedenstein et al [71] and further characterized by Pittenger et al. [72]. MSC contribution in the bone marrow is only 0.001% to 0.01%. These small number of cells can be isolated and cultured *in vitro* and induced to differentiate into bone, fat, cartilage, muscle, cardiomyocytes, neurons, endothelial cells and blood vessel as reviewed by Barry and Murphy [73]. Cell cycle analysis revealed that most of the MSCs stay at G0/G1 phase and do not enter the cell cycle [74]. There is variation on the proliferative capacity of MSCs in culture between the research groups. This difference could be due to the isolation procedure, source of MSCs, culture conditions and donor age [75]. It has been also shown that depending on the culture condition, MSCs retain their telomerase activity and karyotype *in vitro* [72]. However culturing these cells for long time can result in spontenous apoptosis [74] and senescence [75]. MSCs are promising tool for regenerative medicine due to easy harvesting and culturing, high differentiation potential and their nonimmunogenic nature. However, their rareness is a major roadblock. Therefore new strategies should be developed to increase the number of MSCs for further use without increasing their *in vitro* culturing time. Another important feature of MSCs

which makes it easy to work, is their diverse source in the body. Several groups successfully isolated MSCs from adipose tissue, periosteum, synovium, skeletal muscle, umblical cord blood, amniotic fluid, placenta, preipheral blood, lung, dental pulp, tendon and salivary gland [73, 75-77].

1.2.1.1 CHARACTERIZATION OF MSC

For the characterization of MSCs several aspects have been used. These include their morphology, their surface markers and capacity to differentiate into specific cells.

MSCs showed fibroblastic like appearance and formed colonies in petri dish. [78, 79]. The colony numbers has ben shown to vary in different organisms and in different culture conditions [77]. Although the colonies are derived from one precursor cell, studies have shown that the colonies may also contains heterogenous cell mixtures. Several groups have defined the cells in a colony as small spindle shaped cells which proliferate at high rate, large cuboidal shape cells which proliferate at a slower rate and a much smaller cell type which has the highest self renewing rate [80-82]. This variety in colonies makes it necessary to look for other characteristics for MSCs.

MSCs isolated from bone marrow are positive for the expression of CD44, CD105, CD106, CD90, CD166, CD29, CD73, CD117, CD146, CD157, STRO-1, Sca-1, integrins α1, α5 and β5, ICAM-1,ICAM-2, LFA-3 and L-selectin and negative for

CD11b, CD14, CD31, CD33, CD34, CD133 and CD45 [72, 74, 83-86]. However no unique marker has been identified yet to characterize MSC. Several studies have shown that a combination of some of the markers listed above are sufficient and necessary to determine MSCs [77]. The source of the MSC, the isolation procedure and the culture condition can effect the surface marker profile of the MSC [77]. Therefore the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposed minimal criteria for MSC definition. According to this definition, MSCs must adhere to plastic surfaces, express CD105, CD73 and CD90 and not express CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR; and differentiate to osteoblasts, adipocytes and chondroblasts *in vitro* [87].

Besides differentiation into the mesenchymal lineage, MSCs have been also shown to differentiate to other cell types like cardiomyocytes, astrocytes, endothelial cells and epithelial cells such as retinal pigment cells, skin cells, sebaceous duct cells and epithelial cells in the kidney and to tenocytes [73, 88]. The differentiation procedure to osteoblasts, adipocytes and chondrocytes is well established [72, 73, 76, 77, 89-92]. Figure 1.1 summarizes the differentiation of MSCs to osteogenic, chondrogenic and adipogenic lineages, indicating the agents that are required to induce the differentiation and the detection methods to test the result.

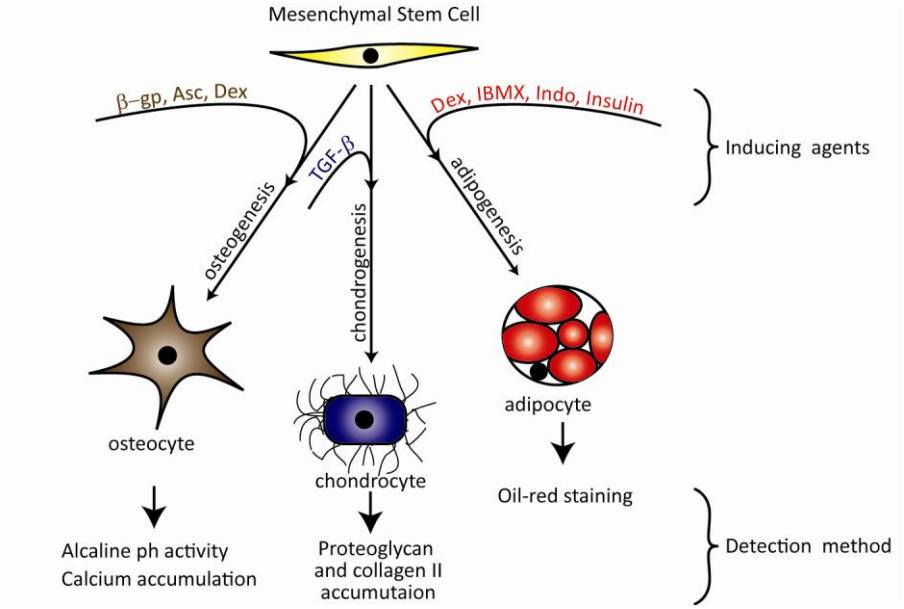

Figure 1.1: The differentiation potential of MSCs into the mesenchymal lineage. Adapted from [77]. Dex= dexamethasone, IBMX= isobuthyl methyl xanthine, Indo= indomethacine, Asc= ascorbic acid, β-gp= beta glycerophosphate.

1.2.1.2 MESENCHYMAL STEM CELL HOMING

Upon damage or aging of somatic cells, stem cells leave the niche and migrate to the site of action and differentiate into the specific cell type of demand [72, 93, 94]. The homing capacity of MSCs decreases with long term culture [73, 77]. The homing mechanism of MSCs is not known for sure however the chemokine receptors on the surface of the MSCs are thought to be responsible for this mechanism. Several groups, although with a high variety among them, have identified different chemokine receptors on MSCs [95]. The presence of different chemokine receptors on the MSCs suggests a role for trafficking the MSCs through different paths to reach various tissues in the organism. Besides the chemokine

receptors, the adhesion molecules also play a role in MSC homing. P-selectin was shown to be involved in the rolling of intravenously administered human MSCs in the blood vessels. In P-selectin deficient mice, the administered MSCs were not able to roll along the walls of the blood vessels [96]. Since the MSCs do not express the known ligands for P-selectin, it was suggested that MSCs have a novel P-selectin ligand. The same study showed that VLA-4/VCAM-1 are required for hMSCs to adhere to endothelial cells [96]. Increased number of MSCs were in the peripheral blood after vascular injury in mice compared to noninjured ones [97]. Also the mobilization of MSCs to the blood after acute burns, skeletal muscle injury and in response to chronic hypoxia has been shown [95]. Besides mobilization to the peripheral blood, MSCs also migrate from blood to the damaged tissue, which was shown by different *in vivo* studies [98, 99]. Studying the molecular mechanism of the entire process of MSC homing, may provide new knowledge to develop more effective strategies in the field of regenerative medicine. Exploring the mechanism of stem cell homing could help in reducing the needs for high number of MSCs, by developing new methods for the enhanced recruitment of ex-vivo cultured MSCs to the injured tissue [100].

It has been shown that there are two types of homing; long-distance traffic which is via the blood stream and short- distance homing which is a local movement [75]. The short distance homing of MSCs to an injured site was shown in different cases like cartilage, muscle and heart injury, bone repair, migration throughout forebrain

and cerebellum [75]. MSC when injected through the intravenous infusion was capable of migrating to a specific site of injury like in the case of bone fracture, cerebral ischemia and myocardial infarction [77].

Besides all the research being done on MSC homing there are typical problems faced by the researchers. One problem regarding the MSC research in general is the lack of a universal definition, thus the heterogeneity of MSCs used in different studies. To overcome this, a particular MSC characterization should be performed. Since there aren't any standart protocols for determining the homing efficiency and correlation, with functional effect, a direct comparison based on the outcome of the researches can not be done. A recommended action for this problem is that every paper should compare or contrast their results with previously published results. This comparison should include the number of cells that were administered, the infusion site, the time passed between MSC administration and assessment of homing, methods used to determine homing, homing efficieny and functional outcome of the experiment. Another problem that the researchers face with is the difficulty to determine the cell type that actualy has homed. The individual MSCs should be labelled in culture and tracked after administration to the organism [100]. To get a more accurate result in MSC homing research these problems should be solved and standart methods should be developed to compare different results. As the whole protocol of the experiments can effect the functional outcome every step should be explained clearly. MSCs are promising agents in stem cell therapy,

however their rareness and the high number of MSC needed for infusion makes it difficult to use them. After clearifiying the MSC homing mechanism, the MSCs could be used more efficiently in regenerative medicine. Direct targeting of the MSCs to the site of action could eliminate the demand for high number of cells to be administered.

1.2.1.3 MESENCHYMAL STEM CELLS IN REGENERATIVE MEDICINE

The discovery of stem cells opened a new era in the field of therapy. Currently there are over 3000 clinical trials involving stem cells and 174 of them are with MSCs (http://www.clinicaltrials.gov). The beneficial effects of MSCs in the treatment of different cases are via different properties of the MSCs. MSCs can support the survival of endogenous cells [101, 102], induce angiogenesis [103], inhibit immune response [104], reduce apoptosis [105] , home to the site of injury, directly differentiate to the cell of the tissue, and release some chemicals which act on the near by cells to stimulate them [106].

Two features of MSCs made them a popular candidate in stem cell therapy. One is the ability to differentiate into various types of cells as mentioned above and the other is their immunosuppressive nature. It has been shown that MSC prevent T-cell activation either by direct contact or via secreted molecules [95]. Other studies have also shown that MSCs can hinder dendritic cell and B-cell maturation [95].

The immunosuppressive nature of MSCs was used in a case study in which the patient developed Graft versus Host Disease (GvHD) after HSC transplantation. MSC treatment in this patient resulted in the healing of the gut epithelium tissue and the patient was in a good condition after 1 year of follow up [107]. MSCs have also been used in ischemic heart diseases, pancreatic regenaration, neurological disorders, hepatic cirrhosis, limb ischemia, skin regenaration, rheumatoid arthritis and bone related diseases [106]. The ability of MSC to differentiate into cardiomyocytes made them a candidate for the treatment of heart failures. After bone marrow transplantion bone marrow derived cardiomyocytes were shown to be engrafted into the heart of the patient [108]. In another study it was shown that bone marrow derived cells delivered to the infarcted heart and resulted in a dramatic increase in the heart function [109]. MSCs are also prominent candidates for the treatment of muscular dystrophy [110], and environmental induced lung disease [111], Since MSCs are able to promote remyelineation [112], and to differentiate into neurons after transplantation [113, 114], and partially recover their function [115] they can be used in neuronal injuries. Clinical trials for osteogenesis imperfecta have shown an improve in the bone formation after MSC transplantation. The bone mineral content and the growth velocity increased, whereas the bone fracture frequencies reduced [116]. Treatment of severe idiopathic aplastic anaemia with MSCs showed improvement of marrow stromal function [117].

Besides being a promising tool as regenerative agents, there are some handicaps in the therapeutic application of MSCs. Although clinical trials show promising results, long term effect of MSC transplantation is not known. Most of the knowledge of MSCs come from *in vitro* studies. However how these cells act *in vivo* is not very clear. What is the safe duration of transplanted MSCs, in other words for how long do they reside in the organism and function properly [106]? The amount of MSCs needed for regenerative medicine is high. How can we increase the number of MSCs without increasing the culture time? Short preparation times are important for urgent cell based therapies. Lastly, sufficient MSCs should reach the site of action after infusion to the organism in short time periods. These problems must be solved before using MSCs in routine therapeutic applications. The proliferative and antiapoptotic effects of estrogen makes it a promising candidate in solving the last two problems.

1.3 ESTROGEN

Estrogen is one of the steroid hormone that is produced in the ovaries from cholesterol. 17β-estradiol, estrone and estriol are found in humans and 17β-estradiol is the most abundant one among them [118]. It does not only have an effect on the reproductive system but also on many other organs and systems like, brain, bone and organs of the cardiovascular system [119, 120] in female and male. Depending on the cell type, estrogen can have a proliferative, antiapoptotic or apoptotic effect

by changing the activity of cell cycle related or cell death related genes [121-125]. After menapouse, when estrogen levels decrease, increase in osteoporosis and coronary heart diseases occur [126, 127]. This suggests a role for estrogen in MSC biology. Determining the effects of estrogen on MSCs can provide insights for development of new strategies in MSC based therapies.

Estrogen exert its effects via the estrogen receptors alpha or beta (ERα or ERβ). These receptors are members of the nuclear receptor superfamily which are ligand induceable transcription factors [128].

1.3.1 ESTROGEN RECEPTOR PATHWAY

Two types of pathways are known so far that are involved in the action of estrogen. The genomic or classical pathway and the non genomic pathway. In the classical pathway, the hormone estrogen activates estrogen receptor (ER) in the cytosol and the receptor forms homodimer. Activated ER is translocated to the nucleus and binds to the estrogen response element (ERE) located mostly at the promoter region of the target gene. Subsequently, coactivators are also recruited to the receptor–DNA complex, which results in the expression of the target gene [120, 129]. Estrogen induced genes do not always contain EREs yet they are activated by ERs.

The non ERE dependent genomic pathway involves the action of other transcription factors which bind both to the ER-ligand complex and to the target gene promoter. In this way ligand activated ER activates the transcription factor and the target gene

is expressed. It has been shown in various studies that ER enhance the activity of several genes that do not have an ERE but contains AP-1 site, CRE-like element, electrophile response element, GC-rich promoter site. Several binding partners of ER on such non-ERE sites such as Jun/Fos, Jun/ATF, ATF, SP-1, NFκB, C/EBPβ are known but there are also unknown ones [118, 129].

A third form of genomic pathway involves growth factors instead of estrogen and is called as ligand independent genomic pathway. In this model the nuclear ERs are activated via phosphorylation by protein kinases which then become active upon growth factor stimulation. The activated ERs bind to ERE sites to start the expression of the target genes.

The second type of ER signaling is the nongenomic pathway. In this pathway estrogen activates the membrane bound ER and this starts a cascade of kinase signaling pathways such as MAPK, PI3/AKT, JAK/STAT pathway [130]. The nongenomic pathway does not involve transcription or translation but translational modification for the activity, thus it results in a very rapid cell type spesific response [118]. Figure 1.2 summarizes the ER signaling pathway.

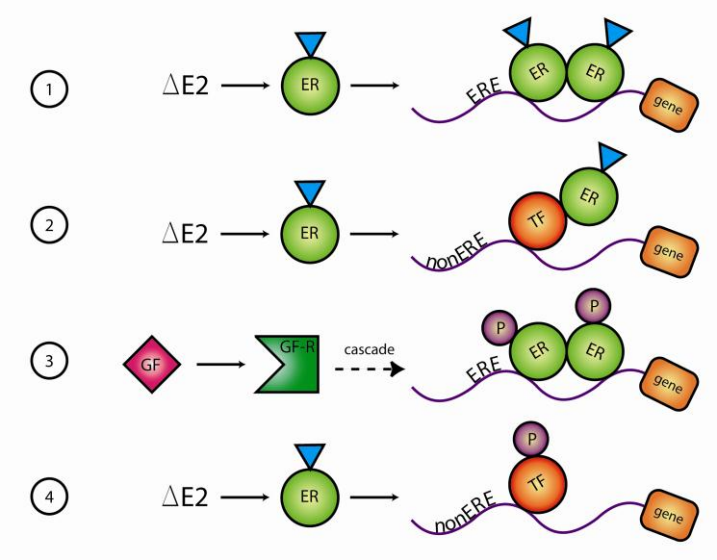

Figure 1.2: The ER signaling. 1. ligand dependent genomic pathway, 2. non-ERE dependent genomic pathway, 3. ligand independent genomic pathway and, 4. non genomic pathway

1.3.2 ESTROGEN AND MESENCHYMAL STEM CELLS

Gender specific responses to various stimuli by MSCs have been attributed to sex hormones. For example MSCs from female donor express less pro-inflammatory cytokines, TNF-α and IL-6, compared with MSCs from male donor in response to acute LPS and hypoxia [131]. Also the hypoxia induced apoptosis was different in male MSC compared to female ones. The MSCs from male donor underwent a significantly higher apoptosis compared to the female MSCs [132]. Another difference between female and male MSC was in the treatment of heart ischemia. After transplantation of MSCs from female donors, heart exhibited improved contractility compared to MSCs from male donors [133]. Estrogen also have an

effcet on the proliferation of MSC in a gender dependent fashion. MSCs from female donors shows an increase in proliferation upon estrogen treatment, while MSC from male donors do not [134]. Another relationship between estrogen and MSC is that estrogen increases the telomerase activity and hTERT expression via ERα [135]. Estrogen has also an effect on the differentiation and proliferation of MSCs. It stimulates the osteogenic differentiation [134] which can be assessed by the increase of calcium deposition and the expression of the osteogenic markers. Estrogen also increases the adipogenic differentiation evidenced by the increase in the lipid droplets [136]. However, the molecular mechanism of estrogen action on MSCs is not known. Understanding the molecular mechanism of their interaction is important in regulating MSCs and obtaining the maximum efficiency from this colloboration. Our group has previously shown that estrogen had no effect on genes related to cell cycle progression and senescence on rat bone marrow derived MSCs (Scientific and Technological Research Council of Turkey, TUBITAK, SBAG-3200(105S393)). Since estrogen also has an effect on apoptosis, apoptotic pathways are important candidates in the regulation of MSCs. After explaining apoptosis, the relation between estrogen and apoptosis will be illustrated with examples.

1.4 CELL DEATH AND APOPTOSIS

Cell death can occur through two distinct pathways called necrosis and apoptosis.

Until recently necrosis was described as cell death which is not apoptosis or autophagy. However, latest research findings have provided more clear explanation for necrosis. Defined signaling pathways and catabolic mechanisms were found to have crucial role in necrotic cell death [137, 138]. Increased intracellular Ca^{2+}, or Reactive oxygen species (ROS), decreased ATP generation [139], tumor necrosis factor- α (TNF-α) [140], poly-ADP-ribose polymerase-1 (PARP-1) [141] were shown to be important mediators of necrosis.

Apoptosis was first described by Kerr and friends upon typical morphological changes that occur in the dying cell [142]. According to the Nomenclature Committee on Cell Death (NCCD) cell death can be defined as apoptosis when it possesses all or some of the following characteristics. i) rounding-up and shrinkage of the cell, ii) retraction of pseudopods, iii) condensation of the chromatin, iv) nuclear disruption, v) preservation of the organelles, vi) apoptotic body formation while maintaining the membrane integrity and vii) phagocytosis by neighboring cells. The NCCD proposes that, programmed cell death (PCD) should not be used to exclusively define apoptosis [143] since cell death that occurs during development is programmed but not apoptotic [144, 145]. One should also not think of apoptosis merely as caspase dependent process [146]. It can also occur in a

caspase independent manner, although in some cases the outcome has a slight variation in the cytological features as compared to known apoptotic structures [146].

The molecular events that occur during necrosis and apoptosis are different as is the morphology (Table 1.3). While cells and the cytoplasmic organelles swell during necrosis, apoptotic cells shrink and the organelles undergo a minor change. Necrotic cell membrane ruptures, but the apoptotic cell membranes integrity is preserved. DNA breakage is random and caspases are not involved in necrosis but, DNA fragmentation occurs and caspases are required in apoptosis. Also necrosis leads to an inflammatory response but apoptosis does not [143, 147].

Table 1.3: Characteristics of apoptosis and necrosis

	APOPTOSIS	NECROSIS
MORPHOLOGY	cell shrinks	cell swells
MEMBRANE INTEGRITY	preserved	lost
DNA BREAKAGE	fragmentation	random
CASPASES	involved	not required
INFLAMMATORY RESPONSE	no	yes
ORGANELLES	minor modifications	swelling

1.4.1 EXTRINSIC AND INTRINSIC PATHWAY

Well defined signaling pathways have roles in the apoptotic cell death. These pathways can be divided into two: the extrinsic (death receptor) and the intrinsic (mitochondrial) pathway [148]. Figure 1.3 represents the extrinsic and intrinsic

apoptotic pathways. After explaining the pathways, and the role of mitochondria in apoptosis, special focus will be given to the Bcl-2 family members and their function in apoptosis. The relation between estrogen and apoptosis will be explained with known examples.

The extrinsic pathway is activated when death ligands, TNF-α, TRAIL and FasL bind to their cognate receptors in the plasma membrane. This binding induces receptor oligomerization and recruitment of an adaptor protein. These adaptor proteins are Fas associated death domain (FADD) and TNF associated death domain (TRADD). After the joining of pro-caspase-8 or pro-caspase-10 the complex death inducing signaling complex (DISC) is formed. The initiator caspase is activated via proteolytic cleavage. This activation triggers the effector caspases, caspase-3 or caspase-7 and apoptosis occurs [149, 150].

The intrinsic pathway occurs mainly through the mitochondria. Various stimuli can trigger the mitochondrial pathway. Hypoxia, growth factor deprivation, reactive oxygen species, DNA damage, UV/ gamma irradiation are some of the apoptotic signals activating the intrinsic pathway. Mitochondria are crucial for cell survival due to their vital role in many metabolic activities [151]. Recent studies have established important roles for mitochondria during different stages of the cell death process. They are involved in energy generation for the cell death process, calcium homeostasis, ROS generation, pro-apoptotic proteins releasing, apoptosome formation and caspase activation [151].

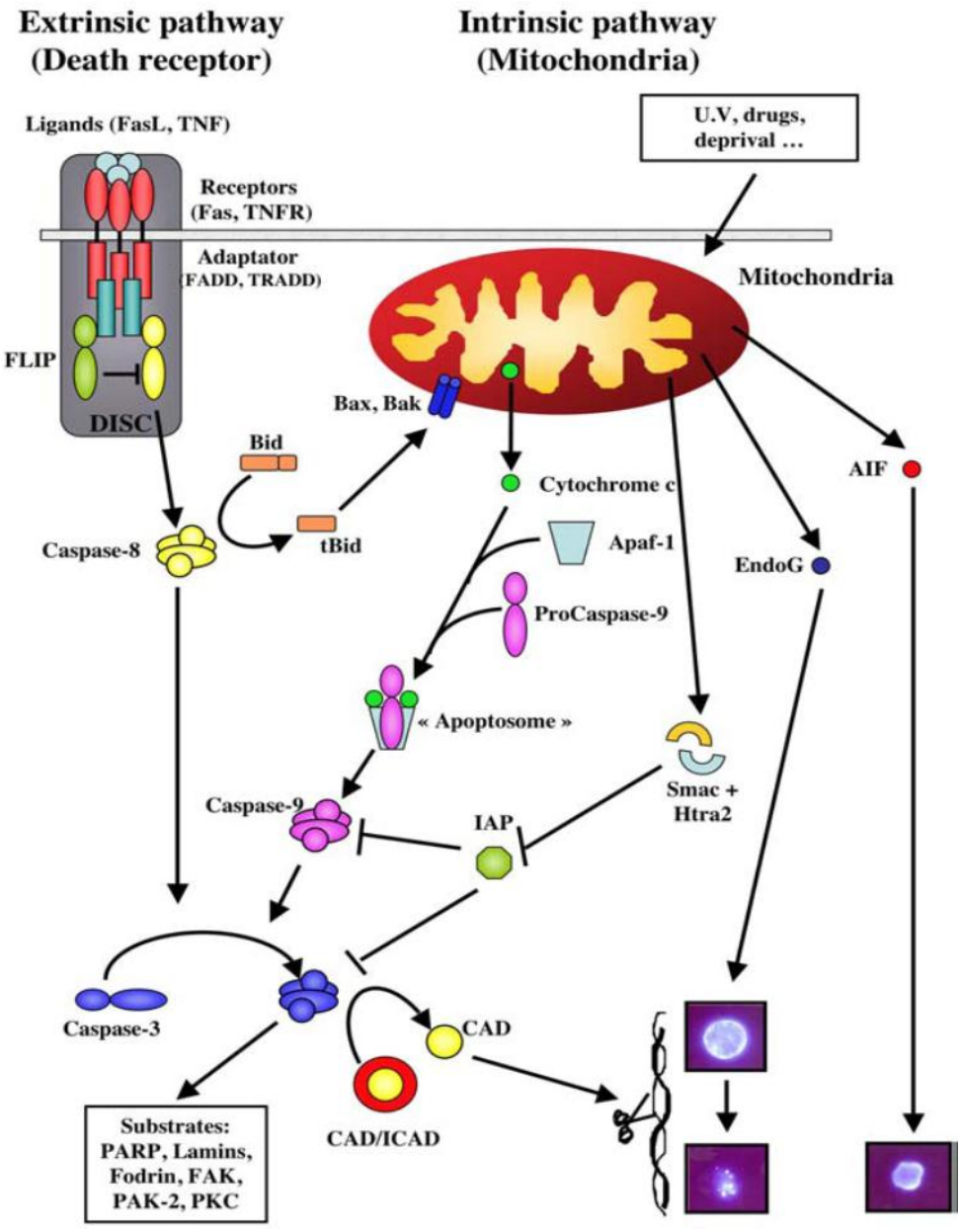

Figure 1.3: The extrinsic and intrinsic apoptotic pathways. [149]

Normally mitochondria have a transmembrane potential which creates an electrochemical gradient across the inner membrane that is essential for ATP

generation. The disruption of this transmembrane potential due to the permeabilization of the mitochondrial membranes results in cell death [151, 152]. Mitochondrial membrane permeabilization can occur through lipids *[153-155],* mitochondrial permeability transition pore (MPTP) [156, 157], or Bcl-2 family of proteins. The induction of the mitochondrial membrane permeabilization results in the release of apoptotic factors from the mitochondria to the cytosol or nucleus and initiates apoptosis.

The pro-apoptotic members, BCL2-antagonist/killer (Bak) and Bcl2-associated X protein (Bax) of the Bcl-2 family of proteins permeabilize the outer membrane of the mitochondria, which results in the release of pro-apoptotic proteins such as cytochrome c (Cyt c), apoptosis inducing factor (AIF) and endonuclease G from the mitochondria to the cytosol. While AIF and endonuclease G goes to the nucleus and induce DNA fragmentation [158, 159] in a caspase independent form [159, 160], Cyt c stays in the cytoplasm. In the cytosol, Cyt c together with apoptotic protease-activating factor (Apaf-1), dATP and pro-caspase-9 forms the apoptosome complex. After proteolytic cleavage of pro-caspase-9 in the apoptosome, it is activated and can activate the effector caspase, caspase-3 [161].

1.4.2 BCL-2 FAMILY MEMBERS

According to their function, Bcl-2 family of proteins is divided into two types, anti-apoptotic and pro-apoptotic members. The pro-apoptotic ones are further categorized as the multi-domain proteins and the BH3 only proteins due to their

structural and functional difference. More than 25 members of this family are known [162, 163]. (Figure 1.4)

All of the members share one or more conserved Bcl-2 homology (BH) domains. The four BH domains which are specific to the family members are BH1, BH2, BH3, and BH4 [164]. These domains are required for the function of the proteins [165-168]. Many of the Bcl-2 family proteins also have a carboxy terminal hydrophobic domain. The localization of the members to the mitochondria is directed through this domain [162, 169, 170].

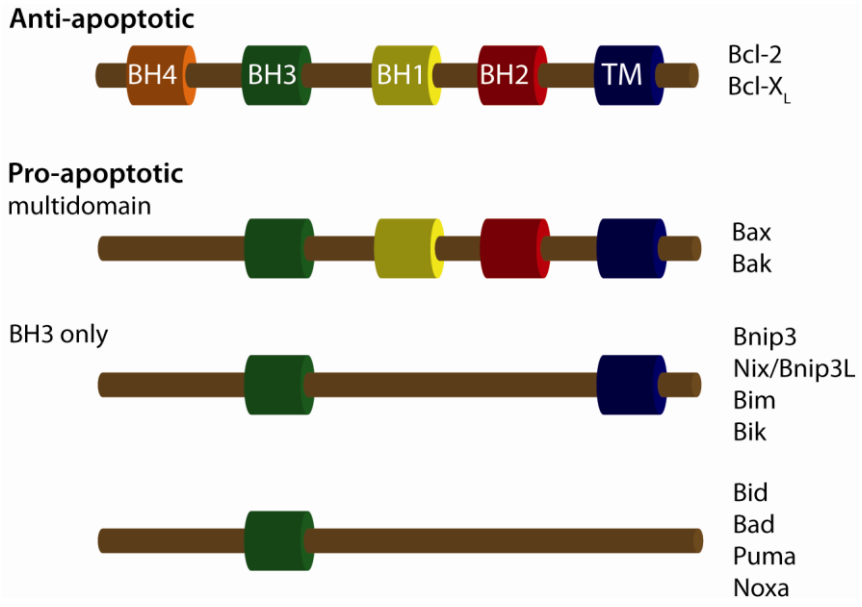

Figure 1.4: The structure of the Bcl-2 family members. BH= Bcl-2 homology domain and TM= transmembrane domain.[170]

Among anti-apoptotic members of the family, B cell lymphoma like X (Bcl-x_L) and B-cell leukemia/lymphoma 2 (Bcl-2), have all the four BH domains and are

generally integrated mostly to the mitochondrial outer membrane but also can be found in the endoplasmic reticulum membrane or the cytosol [171, 172]. Bcl-2 prevents cell death by inhibiting free radical production, suppressing caspase activation, regulating calcium sequestration, and by preventing the pro-apoptotic factors from inducing apoptosis [173]. Bcl-2 is widely expressed during embryogenesis but it decreases and becomes much more restricted postnatally in the nervous system [174]. Bcl-2 and Bcl-x_L prevent apoptosis either by segmenting caspases or by preventing the release of mitochondrial apoptogenic factors such as cytochrome c and AIF into the cytoplasm [175].

The multi domain pro-apoptotic members have the BH1-3 in common and lack the BH4 domain. The localizations of the proteins are different in that in healthy cells, Bak is in the outer mitochondrial membrane whereas Bax is mostly in the cytosol and travels to the mitochondria upon apoptotic stimuli [176-179]. The presence of either Bax or Bak, which are thought to function mainly at the mitochondria, seem to be crucial for apoptosis in many cell types since most death stimuli converge on their activation [180, 181]. Bax heterodimerizes with Bcl-2 and homodimerizes with itself. When Bax is overexpressed, apoptotic death in response to a death signal accelerates, but when Bcl-2 is overexpressed, it heterodimerizes with Bax and apoptosis is repressed [182]. Therefore the ratio of Bax and Bcl-2 is important in regulating apoptosis.

The BH3 only proteins possess only the conserved BH3 domain. The BH3 only proteins are known to function in two distinct mechanisms. Some, like Bid, can either directly activate the multidomain pro-apoptotic members while others, like Bad and NOXA, inactivate the anti-apoptotic members [183, 184].

The interaction between the anti-apoptotic, pro-apoptotic and BH3 only proteins determines apoptosis [171]. Several distinct models have been proposed to explain the relationship between the anti-apoptotic, the pro-apoptotic and the BH3 only proteins during regulating apoptosis (Figure 1.5). In the first model the pro-apoptotic members are sequestered and hold in an inactive state, by the anti-apoptotic ones when there is no need for apoptosis. Upon apoptotic stimulus the BH3 only proteins bind to the anti-apoptotic members and set free the pro-apoptotic ones. This release results in activation of the pro-apoptotic members and subsequently apoptosis. In the second proposed model again the anti-apoptotic ones prevent the pro-apoptotic ones from inducing apoptosis, however this time the pro-apoptotic members are activated by the BH3 only members via direct interaction between these two members. In the third model the anti-apoptotic members hinder the BH3 only members to activate the pro-apoptotic ones. Upon apoptotic stimuli the BH3 only proteins overcome the hurdle and activate the pro-apoptotic members [170].

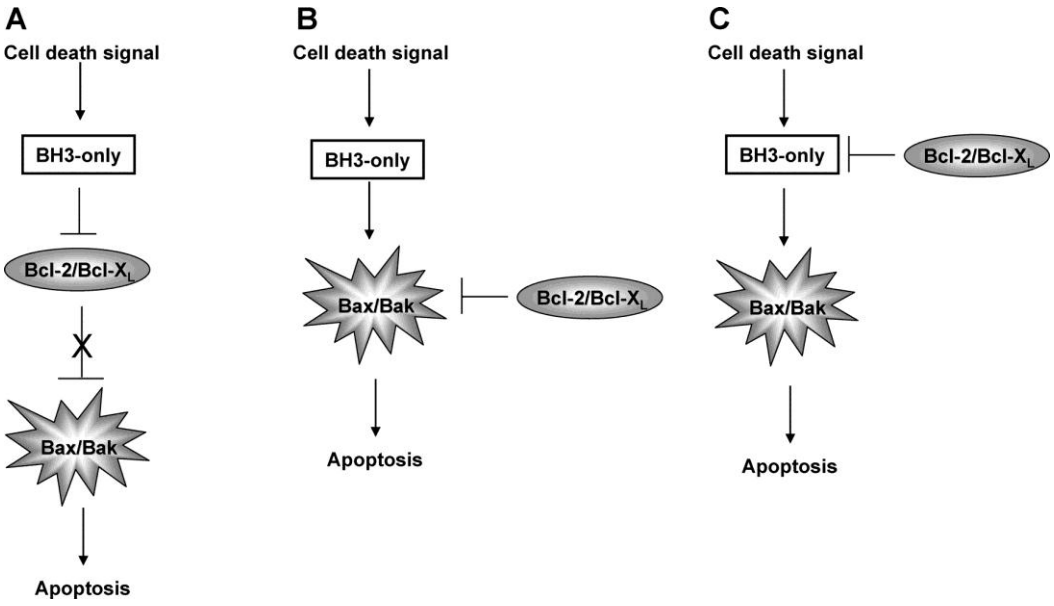

Figure 1.5: Regulation of Bcl-2 proteins. A)BH3-only proteins sequester anti-apoptotic proteins and allow the pro-apoptotic ones to initiate apoptosis. B) BH3-only proteins directly activate pro-apoptotic members. C)the anti-apoptotic proteins sequester the BH3-only proteins and thereby preventing apoptosis. [170]

1.4.2.1 SIGNALING PATHWAY

After activating the intrinsic pathway through various stimuli like growth factor deprivation or DNA damage, the signaling pathway starts. The first reaction involves the activation of the multi-domain pro-apoptotic proteins. This activation is mainly at post translational level where the inactive protein becomes activated through conformational changes. Upon apoptotic stimuli, the C-terminal and N-terminal domains of Bax undergo a conformational modification, which results in the translocation of the protein from the cytosol to the mitochondrial outer membrane where it forms oligomers [176, 185, 186]. Since Bak is already in the mitochondrial outer membrane, there is no need for its translocation. However,

studies have shown that also at the N-terminus of Bak a conformational alteration occurs after an apoptotic stimulus [187] suggesting that there are other consequences of the modification than translocating to the mitochondria. Pro-apoptotic proteins, Bak and Bax form channels on the outer mitochondrial membrane by homo- or hetero-oligomerization [151]. These channels cause the release of the pro-apoptotic activators from the mitochondria to the cytosol. There are also other models to explain the role of Bak and Bax oligomers on inducing apoptosis. One of these models is that, Bak and Bax cause a change in the lipid bilayer of the outer mitochondrial membrane. This change facilitates the release of the apoptotic factors like Cyt *c* to translocate to the cytosol [188, 189]. Other mechanism by which Cyt *c* is released includes the interaction of Bax with the mitochondrial protein voltage dependent anion channel (VDAC) [190] but the detailed function of VDAC in this process is not known [152]. Once in the cytosol, Cyt C, binds to APAF-1. As discussed above Cyt C, APAF-1 and dATP forms a complex called the apoptosome. In this complex APAF-1 undergoes a conformational change and as a result of this, its caspase recruitment domain (CARD) becomes exposed. The pro-caspase 9 is recruited to the apoptosome and proteolytic cleavage occurs to activate the caspase. The active caspase activates the effector caspases, caspase 3 or 7, which leads to apoptosis [161, 191]. Other mitochondrial proteins which are involved in this caspase dependent cell death pathway, is Smac/DIABLO and HtrA2/Omi. Both proteins bind and sequester the

inhibitor of apoptosis proteins (IAP), whereby they prevent them from inhibiting the caspases [192-195].

1.4.3 ESTROGEN, APOPTOSIS AND THE BCL-2 FAMILY OF GENES

The effect of estrogen on apoptosis depends on the cell type, the duration and amount of estrogen and the downstream target signaling pathway. It has been shown that estrogen prevents apoptosis in mouse neurons by upregulating the anti-apoptotic genes *bcl-2, bcl-x_L, bcl-W, blf-1* and by downregulating TRADD [196]. It has also been shown that estrogen had an antiapoptotic effect on rat osteocytes [197], human umbilical vein endothelial cells [198], rat hippocampal neurons by increasing the expression of Bcl-x_L [199], mice neurons by inhibiting Fas-mediated-apoptosis after ischemia [200], some breast cancer cell lines by increasing the expression of Bcl-2 [123]. On the other hand, it promoted apoptosis in osteoclasts [201, 202], thymocytes [203], neurons via ERβ [204]. It has been shown that estrogen could have opposing effect on the apoptosis of neurons. It may function as a neuroprotective agent or an inducer of apoptosis, depending on the estrogen receptor-subtype present in the cell. Thus, ERα has a neuroprotective effect, while ERβ mediates the induction of apoptosis in neuronal cells [204]. Although the mechanism of the apoptotic or antiapoptotic function of estrogen is not always through the same pathway, the Bcl-2 family of genes are one of the major candidates in this process. Several studies have shown a direct or an indirect

relation between estrogen and the expression status of the members of the Bcl-2 family of genes. It is thought that estrogen increases the expression of b*cl-x*$_L$ through a putative ERE site on *Bcl-X* gene [199]. Several ERE sites are found on the *bcl-2* gene [205]. Estrogen also prevents the upregulation of Bax in ovariectomized rats neurons [206]. All these data show that estrogen influences the expression of the Bcl-2 family of genes either via the ERE dependent classical pathway or through the non-genomic pathway.

1.5 EPIGENETICS

Different definitions for the term "epigenetics" have been made since mid 1900s. The first scientists who used the epigenetic concept were mainly developmental biologists who were aware of the fact that although the genetic sequences were same, the phenotype might not be same in every cell. Conrad Waddington is the scientist who came up with the word epigenetics and defined as "the interaction between genes and their products which brings the phenotype into being", more than 60 years ago [207]. Today it is accepted that epigenetics involves two main concepts; first, without changing the DNA sequence, modifications occuring in the chromosome or in the proteins changes the outcome, and second, these modifications are mitotically inherited [208, 209]. The mechanism beyond this is now a huge area to study. Several distinct pathways have been identified that lead to epigenetic changes. The molecular mediators of these pathways can be divided into

three; the epigenators which are the environmental signals and give the signal for that particular pathway to start, the epigenic initiators like DNA binding factors which locate the precise place on the chromatin to be modified upon the epigenator signals, and lastly, the epigenetic maintainers like the histone/DNA modifiers, histone variants which keep the modification in every generation [210].

1.5.1 TYPES OF EPIGENETIC MODIFICATIONS

Two of the best known epigenetic modifications are DNA methylation and histone protein post translational modifications (PTM). The concept of DNA methylation can be traced back to 1970s. The importance of this phenomena was understood when researchers found out that mostly the promoters of inactive genes were methylated whereas the the active gene promoters were not or ligthly methylated [211, 212]. The specific Cytosine that is methylated in the DNA sequence is a Cytosine nucleotide preceeding a Guanidine nucleotide. This dinucleotide sequence in the DNA is called CpG island and is required for the DNA methyltransferase enzyme family to add a methyl group to the cytosine. Members of this family can start a de novo methylation in the early developmental stage or/and fully methylate the hemimethylated CpG in a daughter cell. This feature of the DNA methyltransferase enzymes and the semiconservative replication nature of the DNA makes this modification sustained in the next generations. DNA methylatransferase searches for the hemimethylated CpG island in the new cell and methylates the C in the newly synthesized strand [209, 213].

Another epigenetic modification that gained high attention is histone modification. The eucaryotic genomic DNA is wrapped around nucleosome which consists of the nucleosome core particle and an octamer formed by the four core histone proteins, H2A, H2B, H3, and H4, and 146 bp of DNA [214], [215]. For other DNA binding proteins, like transcription or replication factors to reach the DNA sequence of interest, these histone proteins have to be modified to allow access at the right time to the right position of the DNA [216]. These modifications are either histone PTM namely methylation, acetylation, ubiquitylation, sumoylation and phosphorylation or the alteration of the chromatin packaging directly by moving of the histones namely remodeling. Histone PTMs are seen on the canonical histones H2A, H2B, H3 and H4 and on the variant histones H3.1, H3.3 and HTZ.1. Most of the modifications are on the amino- and carboxy-terminal domain whereas few modifications are localized to the histone globular domains (Figure 1.6) [217].

Histone modifications render the DNA in an active or inactive state. However there is not always a direct correlation between the histone mark and the on or off state. It is found that several histone modifications functioning in activating a gene may also have repressing effects or visa versa [218]. The type of modification does not always indicate the function of it. For example, although acetylation is mainly activating, the effect of methylation is context dependent meaning that the other PTMs are also important. For example 3 methylation of nineth lysine residue on histone 3 (H3K9me3) is known as a repressive mark however when it is found on

chromosomes with H3K4me3 mark than they function in activating a gene. Besides, also the residue to which the methy moiety is attached to is important in defining the expression state of a gene. For example euchromatins have mostly acetylation in their histone tails and H3K4, H3K36 and H3K79 methylation, whereas the heterochromatins are defined by H3K9, H3K27 and H4K20 methylation [218]. Several histone marks and their regulatory role are listed in table 1.4.

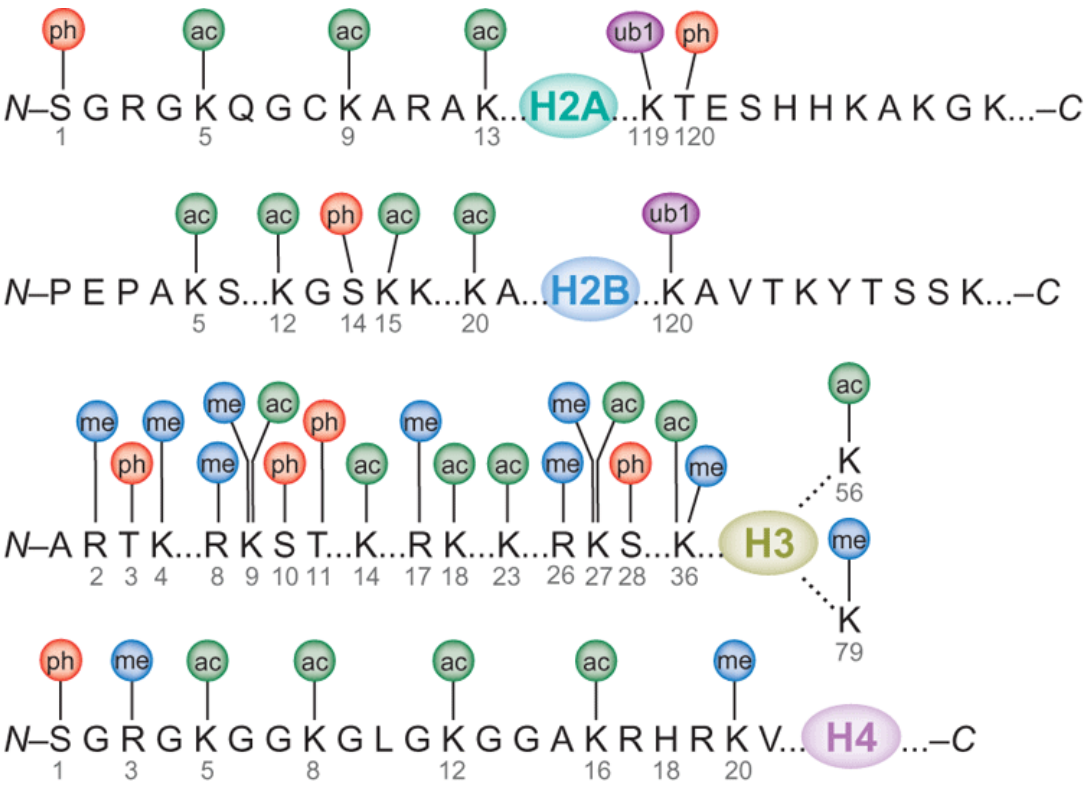

Figure 1.6: Covalent modifications of the core histone proteins. Ph; phosphorylation, ac; acetylation, ub1; ubiquitylation, me; methylation. N; amino terminal domain, C; carboxy terminal domain [217].

Table 1.4: The regulatory roles of several histone marks. (Adopted from [218])

PTM	RESIDUE	TRANSCRIPTIONAL STATUS
Acetylated lysine (K,ac)	H3 (9, 14, 18, 56),	Activation
	H4 (5, 8, 13, 16),	Activation
	H2A, H2B	Activation
Methylated arginine (R,me)	H3 (17, 23),	Activation
	H4 (3)	Activation
Methylated lysine (K,me)	H3 (4, 36, 79)	Activation
	H3 (9, 27),	Repression
	H4 (20)	Repression

The activating effect of acetylation comes from the rendering the chromatin in a more open state which facilitates the binding of coactivators. Indeed some coactivators like, CBP-p300, SRN-1 have an intrinsic histone acetylase activity [217].

Histone methylation occurs via histone methylatransferases. In contrast to histone acetylases these enzymes are more spesific to their substrates. Methylation can occur on arginine (R) and lysine (K) residues [217]. For a long time, it was thought that histone methylation was an irreversible modification, since only histone methylases were known but no demethylases. Only recently lysine demethylases have been found [219]. These enzymes remove the methyl moiety from the N terminal tail of the histones through complex biochemical reactions [219]. Lysine Specific Demethylase 1 (LSD1) is the first demethylase that was discovered. It demethylates mono-dimethylated histone 3 at lysine 4 (H3K4) through amine

oxidation reaction [220]. After LSD1 other demethylases have been identified. The Jumonji-C (JmjC) domain proteins catalyze the removal of methyl moieties from various methylated histone tails [219]. The list of demethylases with their substrates is given in table 1.5.

Table 1.5: Demethylases and their substrates. (Adopted from [217]).

ENZYME	SPECIFICITY
LSD1	H3K4me2, me1, H3K9me2
D.melanogaster Lid	H3K4me3
UTX, JMJD3	H3K27me3, me2
JHDM1	H3K36me2, me1
JHDM2	H3K9me2, me1
JHDM3 (JMJD2)	H3K9 and K36 me2, me1
JARID1A (RBP2), JARID1B (PLU-1), JARID1C (SMCX)	HEK4me3, me2

1.5.2 HISTONE MODIFICATIONS, STEM CELL, APOPTOSIS, AND ESTROGEN

The pluripotency and differentiation of stem cells are controlled by various epigenetic modifications. In the undifferentiated state, the chromatin of the ESCs are mostly in euchromatin state and upon differentiation they gain heterochromatic structure [221]. The epigenetic modification on the stem cell chromosomes are stable enough to be inherited in one lineage, and also reversible to be changed during differentiation. The first feature is important for the self renewal and the second feature is important for the potency of the stem cells [222]. The histone proteins that bind to the chromatin of undifferentiated ESCs are more loosely attached compared to histone proteins binding to differentiated cells [223]. The promoters of lineage spesific genes are mostly occupied by repressive H3K27me3,

and activating H3K4me, histone mark, which is called bivalent chromatin marks. These genes are silenced, however can be activated upon correct stimuli [224]. If the gene is spesifically expressed by the committed lineage, then the histone mark on the promoter of that gene will be an activating mark, (H3K4me) otherwise it will be a repressive mark, (H3K27me3) [225]. This phenomena could also explain how the normaly inactive genes during the undifferentiated state are turned on quickly upon differentiation [226].

The relationship between apoptosis and some types of histone PTMs have been identified. Phosphorylation, dephosphorylation, acetylation, methylation and deubiqutylation of some histone variants are related to apoptosis [227]. Some of these modifications are phosphorylation of H1, H2A, acetylation of H4, methylation of H3K27, phosphorylation of H2BS14 [227].

The effect of estrogen on histone PTMs have been shown mostly in breast cancers, but other researches have also linked estrogen to histone PTM. In Mcf-7 cell line the demethylation of H3K9me3 and the methylation of H3K4 occurs as a result of ERα signaling pathway. The enzyme complex that is responsible for that is JMJD2B/MLL2. JMJD2B is a target of ERα signaling pathway and also required for the expression of estrogen responsive genes [228].

Another histone modification enzyme that is involved in ERα pathway is Sirt1 which is an histone deacetylase. In the absence of this enzyme the ligand activated

ERα pathway is impaired [229]. In an early study it was shown that estrogen increased the global acetylation in rat uterus [230]. It was also responsible for an increase in acetylation of spesific histones in endometrial stromal cells [231].

Two different studies showed the linkage between enhancer of zeste homolog 2 (EZH2), the methyltransferase for H3K27me3 mark and estrogen. First Hwang and friends showed that when overexpressed, EZH2 repressed the expression of estrogen regulated genes in the presence of a protein called the repressor of estrogen receptor activity (REA) [232]. Later Bredfeldt and friends showed that phosphorylation of EZH2 was followed by the decrease in H3K27me3 upon estrogen and xenoestrogen addition both in Mcf-7 cells and uterine tissue. The ceasing of the activity of EZH2 resulted in increased expression of estrogen regulated genes that were substrates for EZH2 [233]. In both of the studies there were a direct connection between estrogen and H3K27me3 mark.

Chapter 2

AIM OF THE STUDY

MSCs have high therapeutic value due to their immuno suppressive characteristics, capacity to differentiate into various cell types, ability to home to the site of injury, ease of handling and no ethical concerns. However, a major handicap in using MSCs in medicine is their limited numbers. Therefore the number of MSCs should be increased effectively. Estrogen is a promising candidate in the regulation of MSC maintenance due to its role in proliferation and apoptosis. It is known that apoptosis is an important regulatory mechanism in all organisms and is a conserved system which emphasizes its importance throughout evolution. The Bcl-2 family of proteins have critical members that are controlled by estrogen in other systems. They are regulated either directly or indirectly via estrogen. Therefore the members of the Bcl-2 family of proteins are strong candidates in the regulation of MSC. In this thesis we aimed to tudy the effect of estrogen on MSC maintenance and the molecular mechanism of this effect.

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Chapter 3

MATERIALS AND METHOD

3.1 ANIMALS

Normal female and ovariectomized Spraque Dawley rats were used in the experiments. They were kept in the animal facility laboratory of the Molecular Biology and Genetics Department at Bilkent University with unlimited access of food and water and under a controlled environment with 12 hour light/dark cycles and at 22^0C. The experimental procedures have been approved by Bilkent University Local Ethical Committee (BILHADYEK).

3.1.1 OVARIECTOMIZATION

The ovariectomization procedure was performed under full anesthesia with Ketamine (Park Davis, Michigan, USA) at a concentration of 30mg/kg. Two incisions were made on both sides of the vertebrate, ovaries at the tip of each uteri horn were tied with silk and then removed. The animals were sutured and kept under supervision until they fully recovered from the anesthesia. Ovariectomized animals were used approximately after 2 months after the operation to ensure the total deprivation of estrogen from their bodies.

3.1.2 PARTIAL HEPATECTOMY (PH)

Liver resections consisting of 70% of the liver mass were surgically performed after injecting Ketamine (Park Davis) subcutaneously at a dose of 30mg/kg. Sham (SH) group of animals had undergone the same preoperative anesthesia procedure and all the surgical operations same as PH, but the liver lobes were not resected. Operations were performed between 8:00 AM and 12:00 PM to minimize diurnal effects. After completion of the procedure, the animals were placed under a lamp to prevent the hypotermy and then put into cages (one animal per cage) with continuous supply of food and water.

3.2 HARVESTING AND CULTURING OF THE BONE MARROW DERIVED MESENCHYMAL STEM CELLS

The bone marrow cells were isolated from the tibia and femur of the rats. Animals were sacrificed with cervical dislocation. Tibia and femur were removed and cleaned from the adherent tissue. Both ends of the bones were cut open. The bone marrow was flushed with DMEM (HyClone, Logan, USA) containing 10% Fetal Bovine Serum (HyClone) and 1% penicillin/streptomycin antibiotic solution (HyClone), by using a 5 ml syringe. The suspension was centrifuged at 1500 rpm for 5 minutes and the supernatant was removed. To wash the cells the pellet was resuspended in 10 mL of 1XPBS and centrifuged again at 1500 rpm for 5 minutes. Supernatant was removed and the washing step with the same conditions was

performed two more times. The bone marrow cells were resuspendet in MesenCult media (Stem Cell technologies, Vancouver, Canada) with supplement (Stem Cell Technologies, Vancouver, Canada), and 1% penicillin/streptomycin antibiotic solution (HyClone). The cells were counted with hemacytometer and equal number of cells were plated onto the culture flasks. The calculation was done according to the formula below

Total number of cells= number of cells in mm^3 X 10^4 X dilution factor

The media was changed at the first day and then every 4-5 days until 14 days of culture. 10^{-7} M estrogen (17 ß-estradiol, Sigma, Germany) was added to the culture plates from the first day of culture and until the last day to obtain the estrogen treated counterparts. The tamoxifen treated samples were prepared by adding 10^{-6}M tamoxifen (Sigma) to the culture plates from the first day of culture until the last day. For every experiment at least three sets of animals was used. summary of the animal models is shown in figure 3.1.

3.3 ADIPOGENIC AND OSTEOGENIC DIFFERENTIATION

MSCs were trypsinized and plated onto appropriate culture dishes or flasks at day 14 of their culture and incubated with Mesencult media until they reached 85-95% confluence. After that, the media was replaced with adipogenic induction media (AIM) containing 1µM dexamethasone (Sigma), 100µM indomethacine (Sigma),

0.5mM IBMX (Sigma), 10µg/mL insulin (Sigma, Germany), 10% FBS (HyClone), 1% penicillin/streptomycin (HyClone) in DMEM-LG (HyClone) or with osteogenic induction media (OIM) containing 0.1µM dexamethasone, 0.2mM ascorbic acid ɣ-irradiated (Sigma), 10mM glycerol phosphate disodium salt hydrate (Sigma), 10% FBS and 1% penicillin/streptomycin in DMEM-LG. The media was changed three times a week. AIM and OIM were prepared freshly before every medium change.

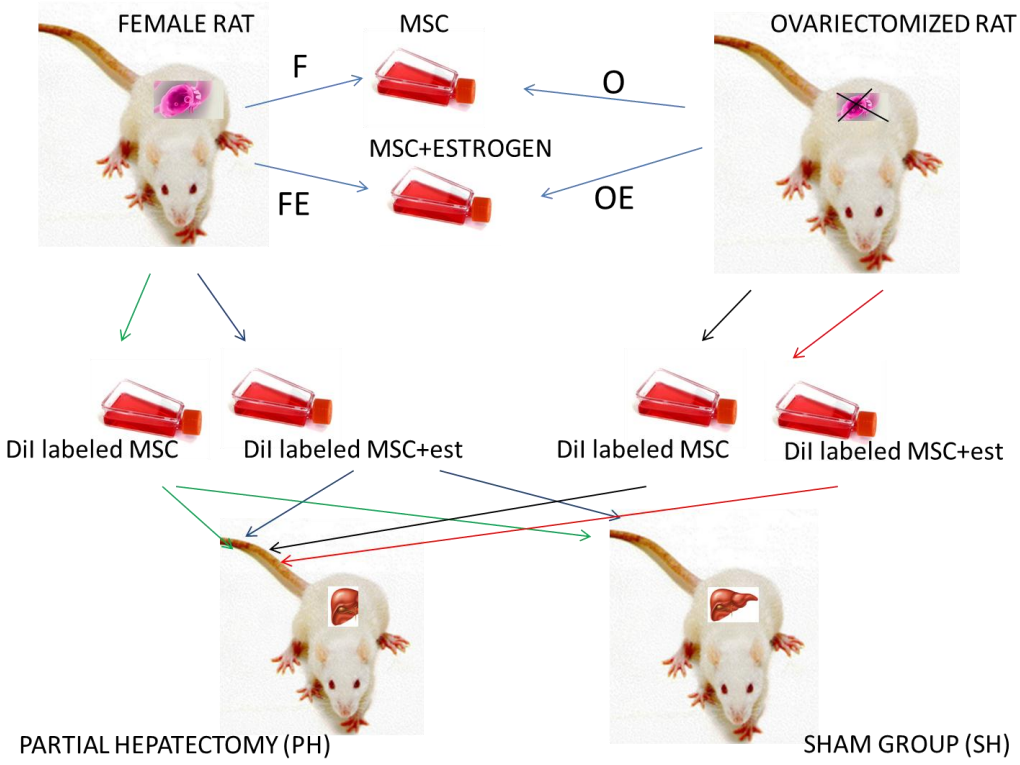


Figure 3.1: Animal models and MSCs groups used in the thesis.

3.4 OIL RED O STAINING

The medium was aspirated and cells were washed with 1XPBS. Afterwards they were incubated in 10% formaldehyde for 10 minutes at room temperature (RT). Then they were washed first with 1XPBS and then with water. Cells were incubated with Oil red O (Sigma) solution for 50 minutes at RT. Subsequently Oil red O solution was removed and the cells were washed with tap water. Photos were taken under light microscope.

3.5 ALIZARIN RED S STAINING

The medium was removed and cells were washed with 1XPBS. Next they were fixed with 70% ethanol at RT for 1 hour. Next the cells were washed with ddH2O and stained with 40mM Alizarin Red S (Sigma) (pH:4.1) for 30 minutes at RT. Afterwards the cells were washed with tap water. Images were taken under light microscope.

3.6 DiI LABELING

A stock solution of 2 mg/mL CM-DiI (C7001 Molecular Probes) was prepared in dimethyl sulfoxide (DMSO) and stored at -20°C. The stock solution was diluted in 1X PBS to achieve a final concentration of 5 μg/mL. The MSCs to be labeled were washed with 1X PBS and afterwards the 5 μg/mL DiI working solution was added

directly into flasks. The flasks were incubated for 5 minutes at 37°C and for 15 minutes at 4°C. After washing twice with 1X PBS, MesenCult (StamCell Technologies) was added to the flasks.

In the fifth day of CM-DiI labeling, the cells were taken from flasks by trypsinizing. The cell concentration was adjusted to $1X10^5$cells/ml in 1X PBS for all injections and chilled on ice. $7.5X10^5$ labeled cells were injected to the, 3 day PH and SH rats under anesthesia through the tail vein. After 3 days the animals were sacrificed and their livers were taken and frozen in liquid nitrogen. 5µm liver sections were taken and visualized under fluorescence microscope after mounting with DAPI (Santa Cruz, CA, USA Biotechnology).

3.7 COLONY FORMING UNIT (CFU) ASSAY

MSCs were washed with 1X PBS and let to air dry. Subsequently the cells were left in ice cold methanol for 5 minutes for fixation and let to air dry again. Afterwards giemsa (Carlo Erba, Italy) was added to the samples for 5 minutes. The reaction was stopped by the addition of tap water. The purple colonies were counted under light microscope. To count the cells in a colony MSCs were fixed with ice cold methanol for 10 minutes and than mounted with UltraCruz (Santa Cruz) mounting medium with DAPI. The cells in the colonies were counted under fluorescence microscope. 359nm wavelength was used to detect the DAPI staining.

3.8 TUNEL ASSAY

TUNEL assay is based on the detection of fragmented internucleosomal DNA. The fragmented DNA causes free 3'-hydroxytermini where the enzyme terminal deoxynucleotidyl transferase (TdT) adds labeled dUTP, hence the name, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). TUNEL assay was performed on the MSCs adhered to the cover slip with In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's manual. The cells were washed with 1XPBS and fixed with 4% freshly prepared paraformaldehyde in 1XPBS. The incubation was done in dark for 1 hour at RT (RT). After washing the cells with 1XPBS, they were incubated for 2 minutes at $+4^0$C with cold permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) which was also freshly prepared. Next the cells were washed again with 1XPBS and incubated with TUNEL reaction mixture for 1 hour at 37^0C in an humidified environment. At the end the samples were washed with 1XPBS and mounted with the UltraCruz (Santa Cruz) mounting medium with DAPI. Samples were analysed under fluorescent microscope. The excitation wavelength of FITC was 490nm and the excitation wavelength for DAPI filter was 359nm.

3.9 TOTAL RNA ISOLATION FROM CULTURED MSC

The cells were washed twice with 1XPBS and trypsinized (HyClone). The cell suspension was transferred into a falcon tube and centrifuged at 1500rpm for 5

minutes. The total RNA from the cell pellet was isolated by using the NucleoSpin^{R} RNA II Kit (Macherey-Nagel, Germany) according to the manufacturer's protocol. The cell pellet was lysed with 350µl RA1 containing 3.5µl β-Mercaptoethanol and 350µl 70% DEPC-treated ethanol was added to the lysate. The lysate was transferred into the column supplied by the manufacturer and centrifuged at 13000 rpm for 1 minute. The flow through was discarded and 350µl MDB solution was added on the column containing the lysate and centrifuged again at 13000 rpm for 1 minute. 95µl of the DNase reaction mixture was added slowly onto the samples in the column and incubated at RT for 20 minutes. Next the column was removed and placed in another collection tube and washed with 200µl RA2 and centrifuged at 13000 rpm for 1 minute. After that the flow through was discarded and the sample was washed with 600µl RA3 and centrifuged at 13000 for 1 minute. After removing the flow through the sample was washed for the last time with 250µl RA3 and centrifuged at 13000 rpm for 2 minutes. The RNA was eluted with RNase free water and the concentration was determined with NanoDrop ND1000 spectrophotometer (Nanodrop technology, USA).

3.10 cDNA SYNTHESIS

2µg of RNA was used for each cDNA synthesis reaction. The synthesis was performed with the DyNAmo cDNA Synthesis Kit (Finnzymes, Finland) according to the manufacturer's protocol. 2µg RNA was mixed with DEPC treated ddH_2O to a

total volume of 12µl and 2µl of Oligo (dT) primer was added to the mixture. The samples were incubated at 65^0C for 5 minutes and then chilled on ice for 3 minutes. 20µl of 2X RT buffer including dNTP mix and $MgCl_2$, and 4µl of M-MuLV RT RNase H^+ enzyme was added to the samples and then they were incubated at 25^0C for 10 minutes, 45^0C for 45 minutes and 85^0C for 5 minutes. The cDNAs were kept at -20^0C.

3.11 PRIMER DESIGN

Primers were designed by using Primer exe and Primer3 programs. The amplicon size of the PCR products of each primer and the specificity of each primer were controlled with publically available NCBI and ENSEMBLE databases.

3.12 SEMI QUANTITATIVE PCR

PCR for the anti-apoptotic genes, *bcl2, bcl-x_L,* the pro-apoptotic genes *bak, bax*, the positive and negative markers of MSCs, CD90, CD71, CD29, CD45, CD34, the estrogen responsive gene, *mmp12*, and for the housekeeping genes g*apdh*, and *β-Actin* was performed by using DyNAzyme II (Finnzymes). The primers and RT-PCR conditions are indicated in table 3.1 and table 3.2 respectively.

The RT-PCR mixture contained 1X Taq Buffer (Finnzymes), 1U Taq DNA polymerase (Finnzymes), 1.5 mM $MgCl_2$ (Finnzymes), 1 µL 10 pM forward and 1

µL 10 pM reverse primers, 0.2 µM dNTP (Finnzymes),and 1 µL cDNA in a total of volume 25 µL that was completed with ddH_2O.

3.13 AGAROSE GEL ELECTROPHORESIS

1X TAE was used to prepare 1.5 % agarose gel. 1mg/mL ethidium bromide was added to the gel prior to polymerization of the gel. Agarose gel loading dye was added to the samples at a final concentration of 1X and the samples were loaded onto the gel. The gel was run at 80 Volt for 25-40 min and visualized with transluminator (Vilber Lourmat, France). ChemiCap (Vilber Lourmat) software was used to take the photos and Bio1D (Vilber Lourmat) software was used to calculate the band intensities. Gene ruler DNA ladder mix (Fermentas, Canada) and 50bp DNA Ladder (New England, Biolabs, MA, USA) was used as markers.

3.14 TOTAL PROTEIN ISOLATION AND QUANTIFICATION

MSCs were scraped with a scraper and centrifuged at 1500 rpm for 5 minutes. The pellet was dissolved in lysis buffer and put on ice for 30 minutes with finger tipping every 5 minutes. Afterwards the samples were centrifuged at 13000 rpm for 20 minutes at 4^0C. The supernatant was taken and kept at -80^0C. The protein concentration was detected with the Bradford assay [234].

Table 3.1: Primer used in the study

gapdh	Forward Reverse	5'-CCTCCTCATTGACCTCAACTAC -3' 5'-CATGGTGGTGAAGACGCCAG-3'	210 bp
bak	Forward Reverse	5'-CCGGAATTCCAGGACACAGAGGA-3' 5'-CCAAGCTTGCCCAACAGAACCAC-3'	536 bp
bax	Forward Reverse	5'-AATCATGGACGGGTCC-3' 5'- GCCCATCTTCTTCCAG-3'	578 bp
bcl-2	Forward Reverse	5'-CCTGGCATCTTCTCCTTC-3' 5'-TGCTGACCTCACTTGTGG-3'	584 bp
bcl-x_L	Forward Reverse	5'-TCAATGGCAACCCTTCCTGG-3' 5'-ATCCGACTCACCAATACCTG-3'	346 bp
beta actin	Forward Reverse	5'-CTGGCCTCACTGTCCACCTT- 3' 5'-GGGCCGGACTCATCGTACT- 3'	65 bp
CD 90	Forward Reverse	5'- CCAGTCATCAGCATCACTCT- 3' 5'- AGCTTGTCTCTGATCACATT- 3'	374 bp
CD 34	Forward Reverse	5'- TGTCTGCTCCTTGAATCT -3' 5'- CCTGTGGGACTCCAACT- 3'	281 bp
CD 71	Forward Reverse	5'- ATGGTTCGTACAGCAGCAGA- 3' 5'- CGAGCAGAATACAGCCATTG- 3'	182 bp
CD 29	Forward Reverse	5'-ACTTCAGACTTCCGCATTGG -3' 5'- GCTGCTGACCAACAAGTTCA- 3'	190 bp
CD 45	Forward Reverse	5'- ATGTTATTGGGAGGGTGCAA-3' 5'- AAAATGTAACGCGCTTCAGG-3'	175 bp
mmp 12	Forward Reverse	5'- GGCAACTGGACACCTCAACT -3' 5'- GTCCAGGTTTCTGCCTCATC -3'	382 bp
lpl	Forward Reverse	5'-ACTGCCACTTCAACCACAGC -3' 5'-AATACTTCGACCAGGCGACC-3'	467 bp
pparγ	Forward Reverse	5'-GGACCTCTCTGTGATGGATGA-3' 5'-GGACGCAGGCTCTACTTTGA-3'	198 bp
runX	Forward Reverse	5'-CTACGAAATGCCTCTGCTGT-3' 5'-TGTCTGTGCCTTCTTGGTTC-3'	194 bp
oc	Forward Reverse	5'-ATGAGGACCCTCTCTCTGCTC-3' 5'-GTGGTGCCATAGATGCGCTTG-3'	293 bp

Table 3.2: RT-PCR conditions for each primer

Genes	Initial denaturation	Denaturation	Annealing	Extension	Cycle	Final extension
gapdh	95°C, 5 min	94°C 45 sec	55°C 40 sec	72°C 40 sec	23	72°C, 5 min
bak	95°C, 5 min	94°C 30 sec	55°C 30 sec	72°C 30 sec	27	72°C, 5 min
bax	95°C, 5 min	94°C 30 sec	60°C 30 sec	72°C 30 sec	28	72°C, 5 min
bcl-2	95°C, 5 min	94°C 40 sec	63°C 35 sec	72°C 40 sec	36	72°C, 5 min
bcl-x_L	95°C, 5 min	94°C 30 sec	52°C 30 sec	72°C 30 sec	31	72°C, 5 min
beta actin	95°C, 5 min	94°C 40 sec	60°C 35 sec	72°C 40 sec	25	72°C, 5 min
CD 90	95°C, 5 min	94°C 30 sec	55°C 30 sec	72°C 30 sec	30	72°C, 5 min
CD 34	95°C, 5 min	94°C 30 sec	55°C 30 sec	72°C 30 sec	30	72°C, 5 min
CD 71	95°C, 5 min	94°C 40 sec	66°C 60 sec	72°C 40 sec	35	72°C, 5 min
CD 29	95°C, 5 min	94°C 30 sec	60°C 30 sec	72°C 30 sec	29	72°C, 5 min
CD 45	95°C, 5 min	94°C 30 sec	60°C 30 sec	72°C 30 sec	23	72°C, 5 min
mmp 12	95°C, 10 min	94°C 40 sec	48°C 60 sec	72°C 40 sec	28	72°C, 5 min
lpl	95°C, 10 min	94°C 30 sec	61°C 45 sec	72°C 30 sec	30	72°C, 5 min
pparγ	95°C, 10 min	94°C 30 sec	61°C 45 sec	72°C 30 sec	30	72°C, 5 min
oc	95°C, 10 min	94°C 30 sec	61°C 45 sec	72°C 30 sec	30	72°C, 5 min
runX	95°C, 10 min	94°C 30 sec	61°C 45 sec	72°C 30 sec	30	72°C, 5 min

3.15 HISTONE PROTEIN EXTRACTION FROM CELL CULTURE (ACID EXTRACTION)

Cells were scraped and centrifuged at 1200 rpm for 10 minutes and the supernatant was removed. The pellet was re-suspended in 1X PBS and centrifuged as before. The cell pellet was dissolved in 400 µL TEB buffer (1XPBS/0.5%Triton X-

100/0.02%NaN3/1X protease inhibitor) by pipetting up and down and incubated on ice for 10 minutes. After that the samples were centrifuged at 2000 rpm for 10 minutes at $+4^0$C. The supernatant was removed and half of the TEB buffer was added to the samples. The samples were centrifuged again at 2000 rpm for 10 minutes at $+4^0$C. The pellet was re-suspended in 150 µL 0.2N HCl and incubated at $+4^0$C with constant rotating for over night (o.n.) Afterwards the samples were centrifuged at 2000 rpm for 10 minutes at $+4^0$C. The supernatant was collected and the protein concentration was determined with the Bradford assay.

3.16 WESTERN BLOT

3.16.1 SDS- POLYACRYLAMIDE GEL ELECTROPHORESIS

The protein samples, were separated by SDS-PAGE gel with 10% resolution gel, and 5% stacking gel while the histone proteins were separated with 17% resolution gel. 30µg protein sample for the former group and 5-10µg samples for the histone proteins were mixed in, 1% cracking buffer with 10% β-mercapthoethanol and boiled at 90^0C for 10 minutes. The samples and the prestained protein marker (Fermentas) were loaded on the gel and run at 70V until leaving the stacking gel. Then the voltage was increased first to 90V and after 30 minutes to 110V while the samples were in the resolution gel. The running was terminated when the samples had run enough by comparing the separation of the bands of the marker.

3.16.2 TRANSFER OF PROTEINS TO THE MEMBRANE

3.16.2.1 SEMI DRY TRANSFER

Proteins in the range of 20-75 kDa were transferred by semi dry transfer method. The PVDF transfer membrane (0,45µm, Thermo Scientific, MA, USA) was put first in methanol for 20 seconds for its activation and then in ddH_2O for 2 minutes to remove the excess methanol and lastly in semidry transfer buffer. The gel was taken and washed with ddH_2O and the resolution part was cut and left in the semidry transfer buffer. 4 whatman papers were cut according to the size of the gel and left also in the semidry transfer buffer. They were placed onto the semidry transfer apparatus (Bio-Rad, CA, USA) in a specific order. At the bottom two whatman papers were placed, on top of them the membrane, and gel and lastly two more Whatman papers. Transfer was performed on the semi-dry transfer apparatus with a current of 3,5 mA/cm^2 membrane for 25 minutes. The gel was stained with comassie brilliant blue and destained with destaining solution for o.n. to detect the transfer of the proteins from the gel to the membrane.

3.16.2.2 WET TRANSFER

The histone proteins which are smaller than 20kDa were transferred with the wet transfer method. The PVDF transfer membrane (0,45µm, Thermo Scientific) was put first in methanol for 20 seconds for its activation and then in ddH_2O for 2 minutes. The gel was taken and washed with ddH_2O. Then the gel, membrane, 4

whatman papers and 2 sponges were soaked into the wet transfer buffer for small proteins. The proteins from the gel were transferred to the membrane with the BioRad gel tank (Bio-Rad). Transfer was done at 40V and lasted for 2.5 hours at $+4^0$C. Afterwards the gel was stained with comassie dye for 15 minutes and destained with destaining solution for o.n.

3.16.3 IMMUNOLOGICAL DETECTION OF THE PROTEINS ON THE MEMBRANE

Membrane was washed for 5 minutes with 1X 0.3% tween 20-TBS (0.3%TBS-T). Afterwards it was incubated with the blocking solution as indicated in table 3.3 for different antibodies for 2 hours. The primary antibodies were diluted in block solution at various concentrations as indicated in table 3.3. The membrane was left in the primary antibodies for o.n. at 4^0C with constant shaking. Next, the membrane was washed 3 times for 10 minutes with 1X 0.3%TBS-T and incubated with the secondary antibody of Anti-Rabbit-HRP (Cell signaling, MA, USA) at a concentration of 1:2000 for 1 hour at RT on an orbital shaker (RotaMix, Thermolyne, IA, USA). Subsequently the membrane was washed for 4 times for 10 minutes with 1X 0.3% TBS-T. Detection was done with Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific). The reagent was added to the membrane and incubated for 4 minutes the excess reagent was drained off and then radiography was detected.

Table 3.3: Antibodies used in western blotting

antibody	Block solution	dilution
Bcl-2 (Santa Cruz, CA, USA)	4% milk powder in 0.3% TBS-T	1:500
Bcl-x_L (Santa Cruz, CA, USA)	4% milk powder in 0.3% TBS-T	1:500
Bak (Santa Cruz, CA, USA)	4% milk powder in 0.3% TBS-T	1:500
Bax (Santa Cruz, CA, USA)	4% milk powder in 0.3% TBS-T	1:500
ERα (Santa Cruz, CA, USA)	4% milk powder in 0.3% TBS-T	1:200
ERβ (Santa Cruz, CA, USA)	4% milk powder in 0.3% TBS-T	1:200
Actin (Santa Cruz, CA, USA)	4% milk powder in 0.3% TBS-T	1:1000
Anti-Histone H3 (Upstate)	5% BSA in 0.1% TBS-T	1:1000
Anti-Histone H4 (Upstate)	5% BSA in 0.1% TBS-T	1:1000
Anti-trimethyl-Histone H3 (Lys27) (Upstate)	5% BSA in 0.1% TBS-T	1:750

3.17 IMMUNOFLUORESCENCE STAINING FOR NON-HISTONE PROTEINS

For immunofluorescence staining the cells were grown on cover slips (Deckglaser 100 No.1 12mm) in the cell culture plates. At the 14^{th} day of culture cells were washed twice with 1X PBS and fixed with Methanol for 7 minutes. Then they were washed again twice with 1X PBS and left in 3% H_2O_2 for 15 minutes. Next the cells

were washed again twice with 1XPBS, and left in blocking solution for 30 minutes at RT. Without any further washing, the blocking solution was removed and the cells were incubated with the primary antibodies, diluted in the block solution, for o.n at +4 ^{0}C with the concentrations stated in table 3.4. The next day, the cells were taken and washed twice with 1X PBS and incubated with FITC labeled secondary antibodies diluted in the block solution, for 1 hour at RT at the concentrations written in table 3.4. Lastly the cells were washed again with 1XPBS and mounted with UltraCruz (Santa Cruz) mounting medium with DAPI. The samples were investigated under fluorescent microscope with the wavelength 490nm and 359nm for FITC and DAPI respectively.

3.18 IMMUNOCYTOCHEMISTRY FOR HISTONE PROTEINS

Samples were fixed with freshly prepared 4% paraformaldehyde for 30 minutes and washed once with ddH_2O and twice with 1XPBS. The permeabilization was done with 1XPBS/0.5% saponin/0.3% tritonX-100 three times for 5 minutes each. After the permeabilization the samples were blocked with 1XPBS containing 10% FBS and 0.3% tritonX-100 for one hour at 37^{0}C. Before the addition of the primary antibody the samples were washed with 1XPBS. The primary antibodies were diluted in 1XPBS containing 2% FBS and 0.3% tritonX-100 as stated in table 3.4 and left for o.n. incubation at +4^{0}C. The next day the samples were washed first with 1XPBS and than three times with 1XPBS containing 2% FBS and 0.3%

tritonX-100 for 5 minutes each. Then the cells were incubated with universal biotinylated link (DakoCytomation, Denmark) for 10 minutes at RT and washed with 1XPBS afterwards. Next the samples were incubated with streptavidinHRP (DakoCytomation) for 10 minutes at RT and washed afterwards first with 1XPBS and subsequently with 1XPBS containing 2% FBS and 0.3% tritonX-100. For the brown color development DAB Chromogen (DakoCytomation) was used. This solution was applied to the samples and waited for 2-4 minutes for color development. The reaction was stopped with ddH_2O. The samples were counterstained with eosin for 1 minute and mounted with mounting medium (DakoCytomation).

Table 3.4: Antibodies used in immunostaining

antibody	dilution
Bcl-2 (Santa Cruz, CA, USA)	1:500
Bcl-x_L (Santa Cruz, CA, USA)	1:500
Bak (Santa Cruz, CA, USA)	1:500
Bax (Santa Cruz, CA, USA)	1:500
Anti-trimethyl-Histone H3(Lys9) (Upstate)	1:1000
Anti-trimethyl-Histone H4(Lys20) (Upstate)	1:500
Anti-trimethyl-Histone H3(Lys4) (Upstate)	1:1000
Anti-trimethyl-Histone H3(Lys27) (Upstate)	1:1000
Anti-trimethyl-Histone H3(Lys36) (Upstate)	1:500
Anti-dimethyl-Histone H3(Arg2) (Upstate)	1:500
Anti-dimethyl-Histone H4(Arg3) (Upstate)	1:250
Anti-monomethyl-Histone H3(Lys27) (Upstate)	1:1500
FITC-anti rabbit (Sigma, Germany)	1:200

3.19 shRNA TRANSFECTION

3.19.1 shRNA PLASMID PREPARATION

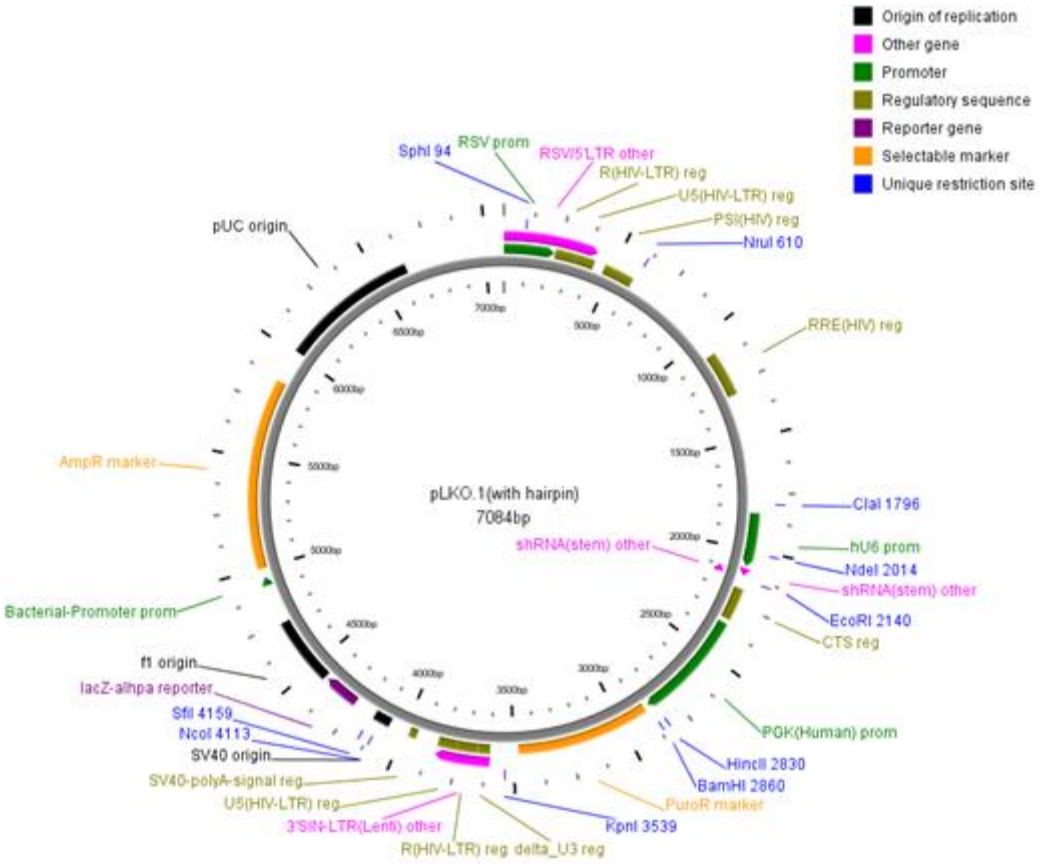


Figure 3.2: vector pLKO.1 map.

The shRNAs were in pLKO.1 vector and purchased from Openbiosystems (AL, USA). The vector map is shown in figure 3.2. Agar plates containing ampicillin were prepared. *E.coli* in glycerol stock with shRNA (shRNA Bcl-2; Openbiosystems RMM3981-9573452 and RMM3981-9573456 shRNA Bcl-x_L;

Openbiosystems RMM3981-9573460) were inoculated onto the agar plates and incubated at 37^0C for o.n. One E.coli colony was picked and transferred into 5ml ampicillin containing LB and incubated at 37^0C for 8 hours by constantly shaking at 227 rpm. 100µL from the culture was taken and transferred to 100ml ampicillin containing LB and incubated at 37^0C for 16 hours at 227 rpm. Next day plasmid isolation was done with Plasmid Midi Kit (QIAGEN, CA, USA) according to manufacturers guideline. Prior to the isolation, QIAGEN-tip 100 was equilibrated by applying 4ml QBT buffer. The column was allowed to empty by gravity. The bacterial cells were harvested by centrifugation at 6000 x g for 20 minutes at $+4^0$C. The pellet was resuspended in 4ml RNase A containing P1 buffer. Then 4ml of P2 buffer was added to the mixture and mixed thoroughly by vigorously inverting the tube several times and incubated at RT for 5 minutes. Next 4ml of chilled P3 buffer was added to the mixture and mixed immediately and vigorously. Then it was incubated on ice for 15 minutes. The samples were centrifuged at 20000 x g for 30 minutes at $+4^0$C. The supernatant containing the plasmid was removed promptly and centrifuged again at 20000 x g for 15 minutes at $+4^0$C. Then the supernatant was removed immediately and applied to QIAGEN-tip 100 and allowed to enter the resin by gravity flow. The QIAGEN-tip was washed twice with 10ml QC buffer and the DNA was eluted with 5ml QF buffer. To precipitate the DNA 3.5ml RT isopropanol was added to the eluted DNA, mixed and centrifuged immediately at 15000 x g for 30 minutes at $+4^0$C. The supernatant was removed carefully. The DNA pellet was washed with 2ml RT 70% ethanol and centrifuged at 15000 x g for

10 minutes. Then the supernatant was removed and the pellet was air dried. The DNA was resuspended in 200-400µl TE buffer, pH 8.0.

3.19.2 TRANSFECTION

MSCs were trypsinized and plated in antibiotic free Mesencult and supplement in 6 well plates for RNA isolation and in 24 well plates for TUNEL assay at day 13 of their culture. They were incubated for o.n at 37^0C with 5% CO_2. The next day 1µg shRNA (two shRNAs for *bcl-2*; Openbiosystems RMM3981-9573452 and RMM3981-9573456, 1 shRNA for *bcl-*x_L; Openbiosystems RMM3981-9573460) was added to 100µL serum free antibiotic free DMEM-LG (HyClone). Two shRNAs, 1µg of each, were used for knock down of *bcl-2* and 1 shRNA, 1µg, was used for knock down of *bcl-*$x_{L.}$ In another tube 4µL and 3µL transfection reagent (thermo scientific, dharmafect 1) was diluted with 196µL and 197µL serum free antibiotic free DMEM-LG for b*cl-2* and b*cl-*x_L respectively. After 5 minutes incubation the contents of shRNA and Transfection reagent containing tubes were mixed and incubated at RT for 20 minutes. 1200 µL antibiotic free complete DMEM-LG was added to the mixture. The cells were washed with 1X PBS and 1500µL transfection mixture was added to 6 well, 375µL to 24 well cultures. DMEM-LG and Transfection reagent only was given to the cells as two types of control. Next day the medium was changed with complete mesencult. 4 days after transfection RNA isolation and TUNEL assay was done.

3.20 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 $p<0.05$.

3.21 SOLUTIONS AND BUFFERS

Solutions and buffers used in this study are given in Appendix A.

Chapter 4

RESULTS

In this thesis MSCs were isolated from two different animal groups; normal female and ovariectomized female (ovx). Furthermore to test its effect 10^{-7}M estrogen was added to the isolated MSCs. Therefore our experimental groups were as fallows; MSCs from normal female rats (F), estrogen treated MSCs from normal female rat (FE), estrogen and tamoxifen treated MSCs from normal female rat (FET), tamoxifen treated MSCs from normal female rat (FT), MSCs from ovx female rat (O), and estrogen treated MSCs from ovx female rat (OE). estrogen and tamoxifen treated MSCs from ovx female rat (OET) and tamoxifen treated MSCs from ovx female rat (OT) The experiments were done after culturing MSCs for 14 days in tissue culture with Mesencult media. Cells were used at P0 passage.

4.1 CD MARKER EXPRESSION

To characterize MSCs several markers were used. MSCs isolated from normal female rats and from ovx female rats as well as the estrogen treated samples were positive for MSC markers, CD29, CD90 and CD71 and negative for hematopoietic markers, CD34 and CD45 (Figure 4.1). Estrogen treatment did not cause a change of the CD marker expression, indicating that estrogen treatment did not interfere

with the MSC characteristics (Figure 4.1). *Beta actin* was used as loading control. RT-PCR made with RNA template instead of cDNA template was done in order to examine genomic DNA contamination which would interfere with the results. Negative result here showed that there is no genomic DNA contamination.

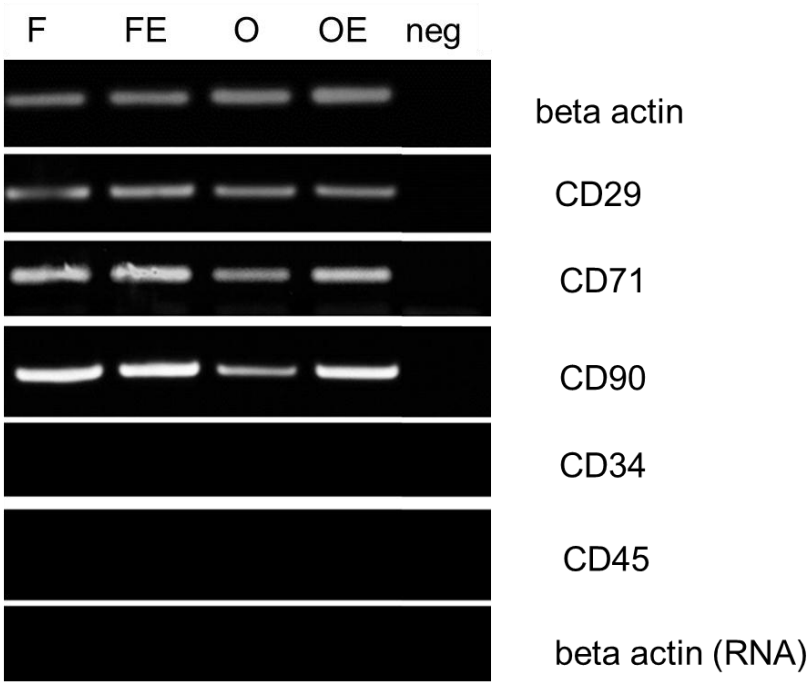


Figure 4.1: Characterization of bone marrow derived MSCs. The expression of the MSC markers CD29, CD90 and CD71 and the hematopoietic markers CD34 and CD45 by RT-PCR. *Beta actin* was used as loading control. RNA samples were used to search for genomic DNA contamination. neg= sample without cDNA.

4.2 ER PROTEIN EXPRESSION AND ESTROGEN RESPONSIVENESS OF MSCs

Estrogen exerts its effect mainly via the estrogen receptors. Since we focused on the role of estrogen on MSCs, the presence of ERα and/or ERβ is important in the

signaling of estrogen. To investigate the presence of ERs in MSCs, ER protein expression was examined by western blotting. As shown in figure 4.2 MSCs isolated from normal female and ovx female animals expressed ERα and ERβ in the presence and absence of estrogen.

To test whether estrogen addition was functional we checked the estrogen responsiveness of the MSCs. In order to test this, mRNA expression of the estrogen responsive gene *mmp12* was analyzed. The mRNA level of m*mp12* increased after treatment the MSCs with estrogen in both normal female and ovx groups as seen by RT- PCR (Figure 4.3). To test whether *mmp12* expression was mediated via ER we treated the MSCs with 10^{-6}M tamoxifen, which is an ER antagonist. Tamoxifen treatment decreased the *mmp12* expression in MSCs isolated from normal female and from ovx female rat as seen in figure 4.3.

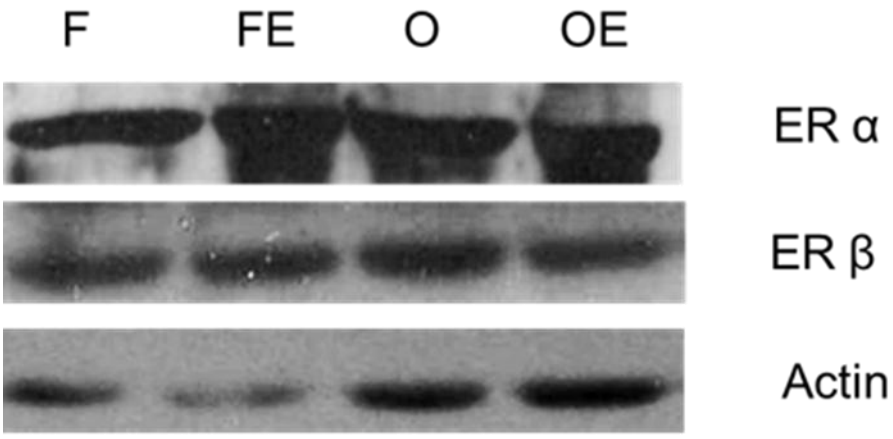


Figure 4.2: Protein expression of ER α and β in MSCs determined by western blot. Actin was used as loading control.

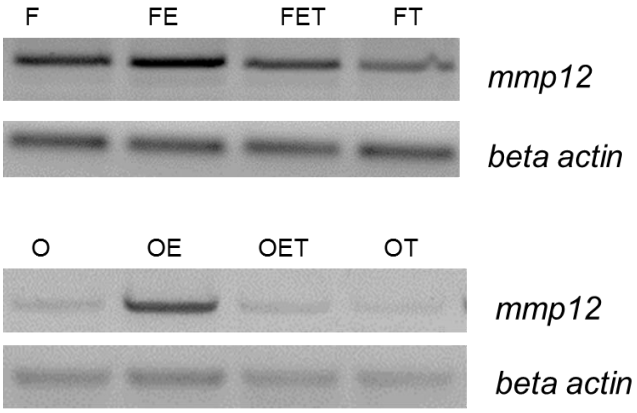


Figure 4.3: mRNA expression of estrogen responsive gene *mmp12* determined by RT-PCR. *Beta actin* was used as loading control.

4.3 CFU-F ASSAY

We also investigated the effect of estrogen on their colony forming feature. MSCs isolated from normal female and ovx female showed spindle like morphology and formed colonies when plated onto plastic surfaces in the presence and absence of estrogen (Figure 4.4A-D). The colonies were counted under light microscope with 10X objective. After staining the colonies with DAPI the cells in the colonies were counted under fluorescence microscope. Estrogen treatment increased the number of colonies both for MSCs isolated from normal female and from ovx female (Figure 4.4E). Moreover, the number of cells in the colonies also increased when MSCs were treated with estrogen (Figure 4.4F)

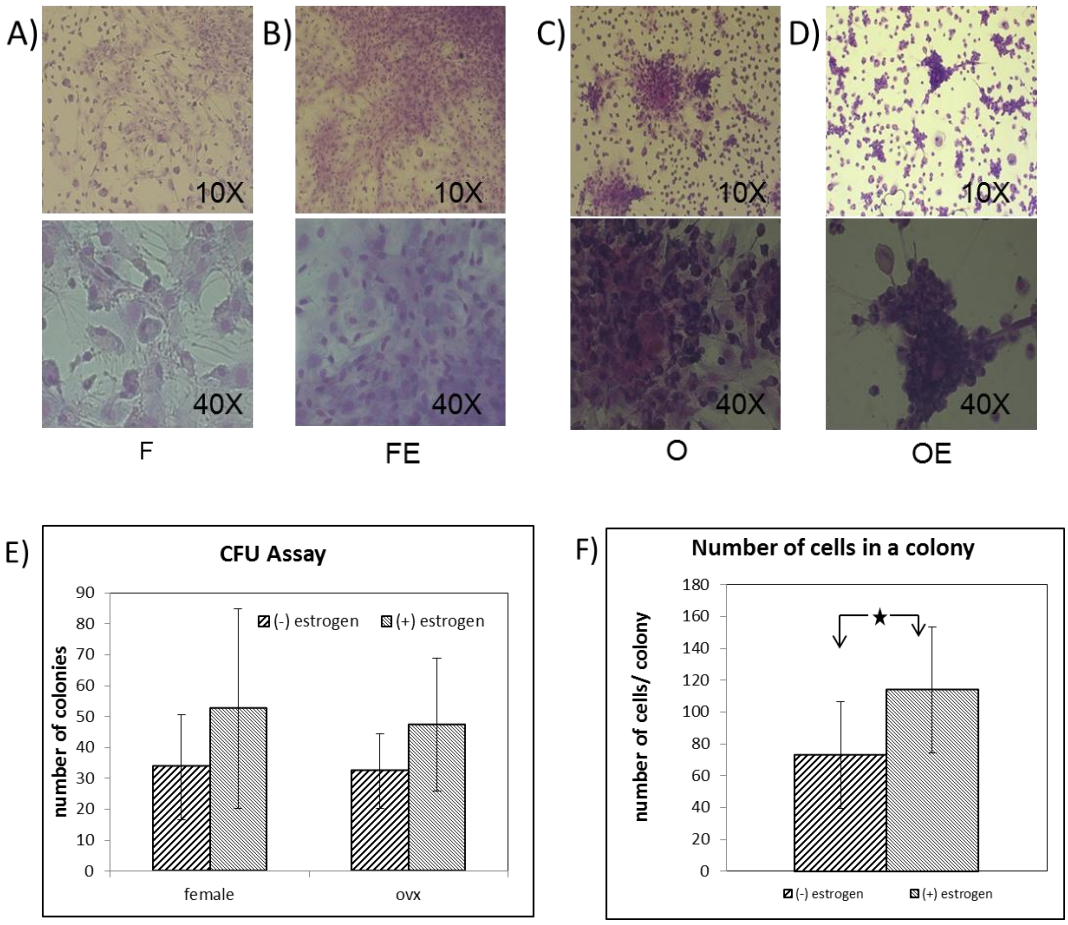


Figure 4.4: CFU activities of MSCs. Light microscope images of colonies formed by MSCs isolated from A-B) normal female, and C-D) ovx female rats. and cultured in the A-C) absence and B-D) presence of estrogen. E) Number of colonies formed by MSCs. n=3 F) Number of cells in a colony. n=7 * indicates $p<0.05$. mean ±standart deviations.

4.4 DIFFERENTIATION OF MSCs

To test the effect of estrogen on the differentiation capacity of the MSC samples, adipogenic and osteogenic differentiation was induced with defined stimuli. All of the samples were able to differentiate into adipogenic and osteogenic lineages.

Differentiation capacity was screened by Oil Red O staining and Alizarin Red S staining for adipogenic and osteogenic lineage respectively. We observed that the differentiation potency of MSCs into adipogenic lineage but not to osteogenic lineage could be affected by estrogen. After adipogenic induction the samples showed high lipid vacuoles as shown by the Oil Red O staining. Estrogen treatment reduced the lipid vacuole formation in MSCs isolated from normal female rats (Figure 4.5A). Alizarin detects the calcium deposits and is used for detection of mineralization and thereby assessing osteogenic differentiation. When MSCs were cultured with OIM, we detected positive alizarin staining (Figure 4.5B). The negative controls were cultured in the absence of the differentiation inductive agents. Negative samples stained neither with Oil Red O nor with Alizarin Red S.

To further evaluate the effect of estrogen on the adipogenic and osteogenic differentiation of MSCs, adipogenic and osteogenic specific marker gene expressions were analyzed. Lipoprotein lipase (*lpl*), and peroxisome proliferator activated receptor gamma (*pparγ*) were chosen as adipogenic markers and runt-related transcription factor 2 (*runx2*) and osteocalcin (*oc*) were chosen as osteogenic markers. Estrogen addition did not prevent the differentiation capacity of the MSCs however *lpl* and *pparγ* expression dropped drastically after estrogen treatment in MSCs isolated from normal female rats (Figure 4.5C), which is in correlation with the Oil Red O staining. The osteogenic marker *oc* slightly increased after estrogen

treatment in female and ovariectomized MSCs. *Runx2* expression did not change in estrogen treated MSCs.

4.5 HOMING OF MSCs

The migration of MSCs to the site of injury is called homing. To investigate the homing capacity of MSCs, liver damage was generated by performing partial hepatectomy (PH). Sham operated rats (SH) were used as control group.

4.5.1 LABELING OF MSCs WITH DiI AND DETERMINING THE OPTIMUM TIMING FOR THE ADMINISTRATION INTO THE RATS

The MSCs were labeled with DiI for 5 days in culture. After 5 days they showed a high DiI staining under the fluorescence microscope (Figure 4.6A and C). In our experimental design, PH was performed and the rats were let to recover from the surgery for 1 day (1 PH), 3 days (3 PH) and 5 days (5 PH). To find the optimum timing for the administration of MSCs to the rats after PH we injected the DiI labeled MSCs into 1 PH, 3 PH and 5PH rats through the tail vein. Our results showed that no DiI labeled MSCs migrated to the liver of 1 PH animals and estrogen addition did not change this result (Figure 4.7). We observed the highest amount of DiI labeled MSCs in the liver of 3 PH rats. In the absence of estrogen no DiI labeled MSCs were observed in the livers of 5 PH group. On the other hand,

few labeled MSCs were detected in the same group when these cells were treated with estrogen (Figure 4.7).

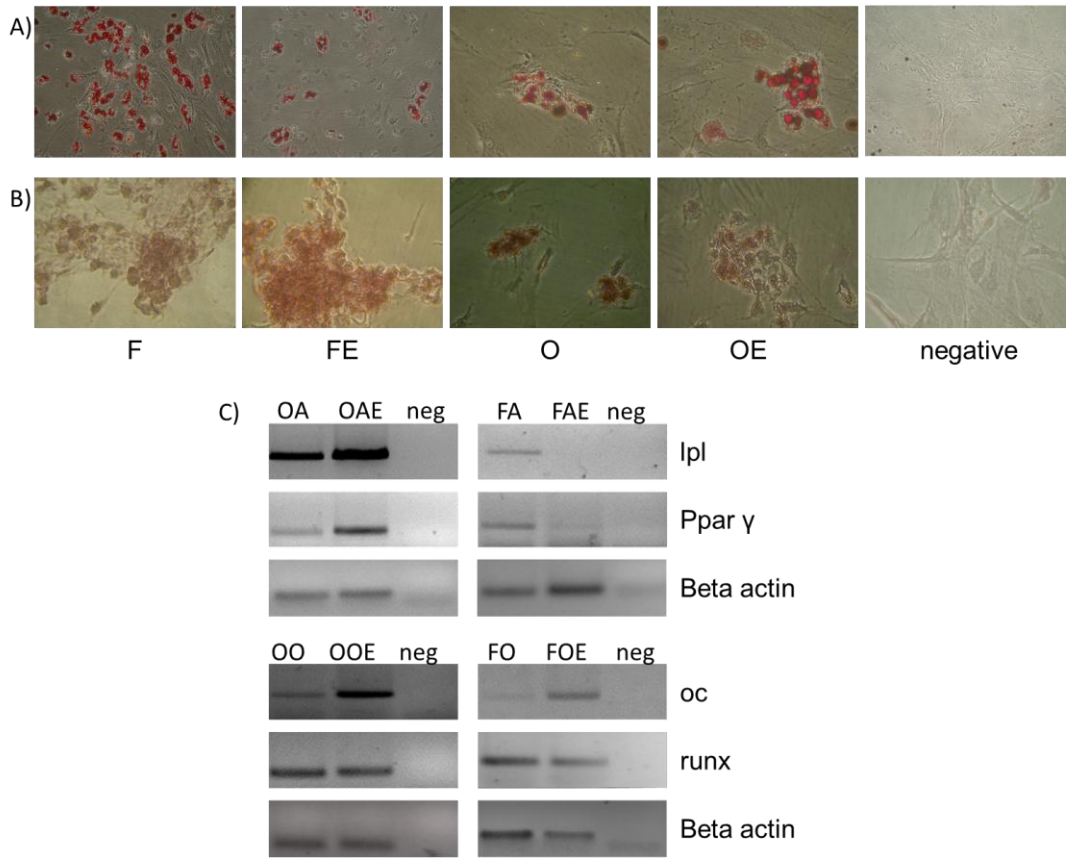


Figure 4.5: Differentiation capacity of MSCs isolated from normal female and ovx female in the absence and presence of estrogen. MSCs were stained with A) Oil Red O to detect the adipogenic differentiation and with B) Alizarin Red S to detect the osteogenic differentiation. C) mRNA expression of adipogenic (*lpl*, and *pparγ*) and osteogenic (*oc* and *runx2*) marker genes after the differentiation of MSCs. neg=without cDNA. *Beta actin* is used as loading control. (magnification 20X)

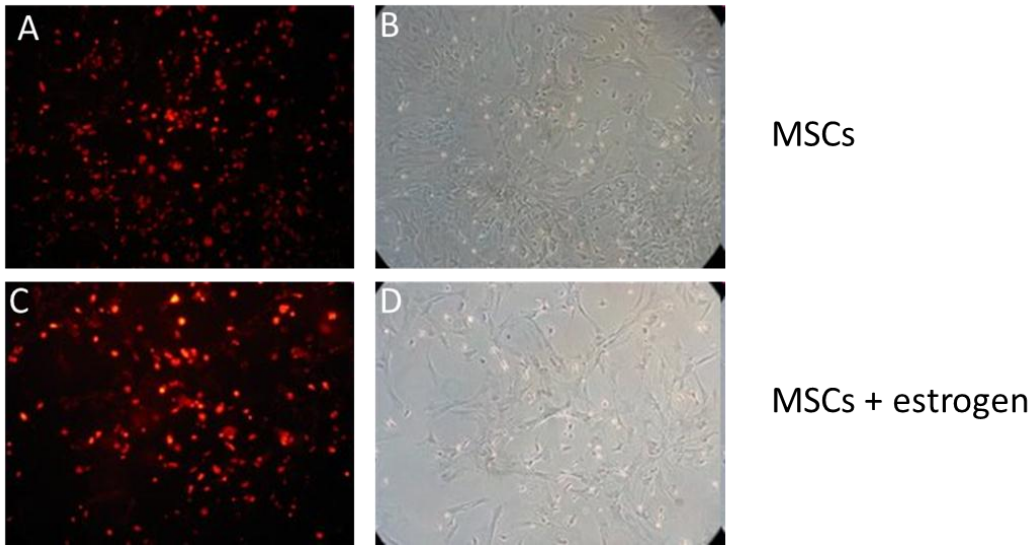


Figure 4.6: Image of in vitro DiI labeled MSCs. A,B) MSCs cultured in the absence of estrogen and C,D) MSCs cultured in the presence of estrogen. A,C) fluorescence image of DiI labeled MSCs and B,D) the same cells under light microscope

4.5.2 EFFECT OF ESTOGEN ON MSC HOMING

Labeled MSCs were injected to the 3 day PH rats and SH rats through the tail vein of the rat to test the effect of estrogen on the homing capacity of MSCs. The rats were sacrificed 3 days after the injection and the liver sections were analyzed. DiI labeled MSCs were observed under the fluorescence microscope. In addition to the transplanted MSCs, the native MSCs also migrated to the liver as observed by the expression of the MSC marker, CD90 staining (Figure 4.8E).

Our results have shown that estrogen treatment increased the homing capacity of MSCs to the injured liver (Figure 4.9E vs 4.9F). MSCs from ovx female rat did not home to the injured liver of normal female or ovx female rats and estrogen

treatment did not improve this result (Figure 4.9A-D). We did not observe any DiI labeled MSCs isolated from female rat that migrated to the liver in SH animals (Figure 4.9G and H).

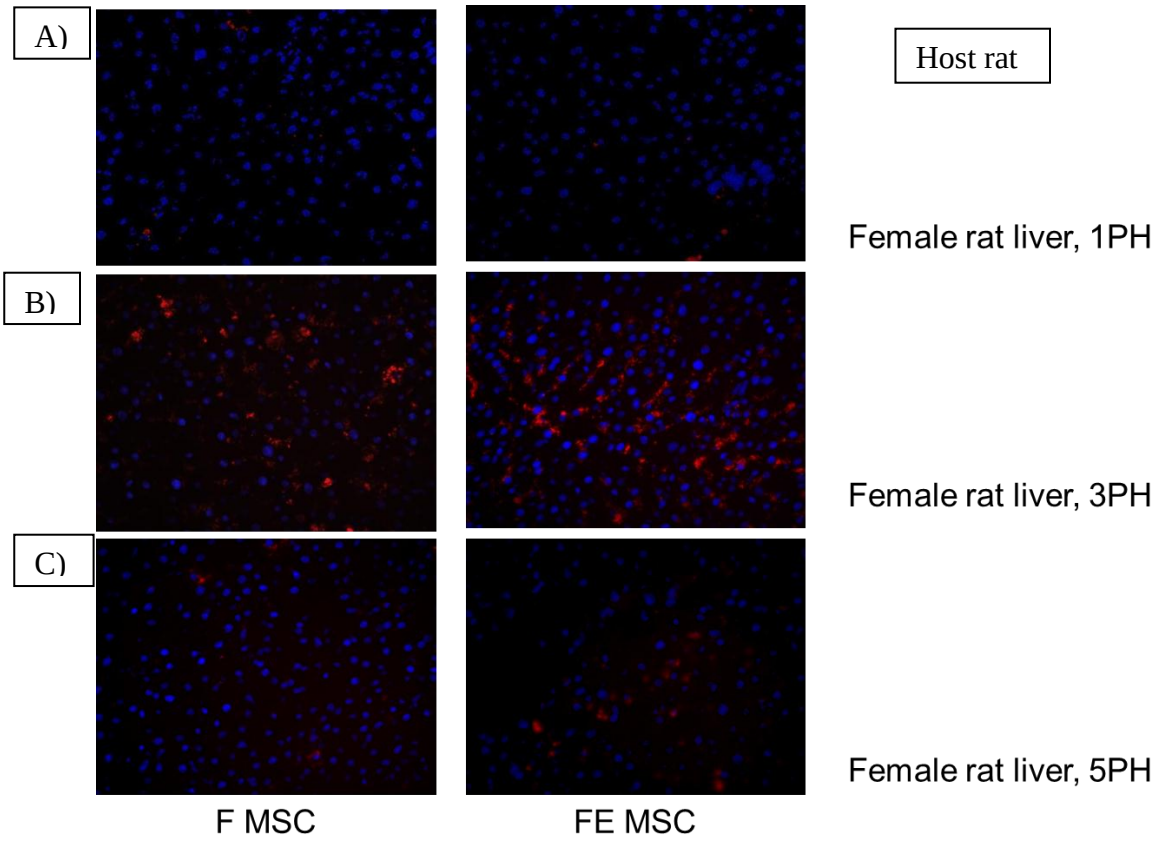


Figure 4.7: Effect of recovery time after PH on the homing capacity of DiI labeled MSCs in the liver. Liver sections from A) 1 days post PH, B) 3 day post PH and C) 5 day post PH rats after MSC injection.

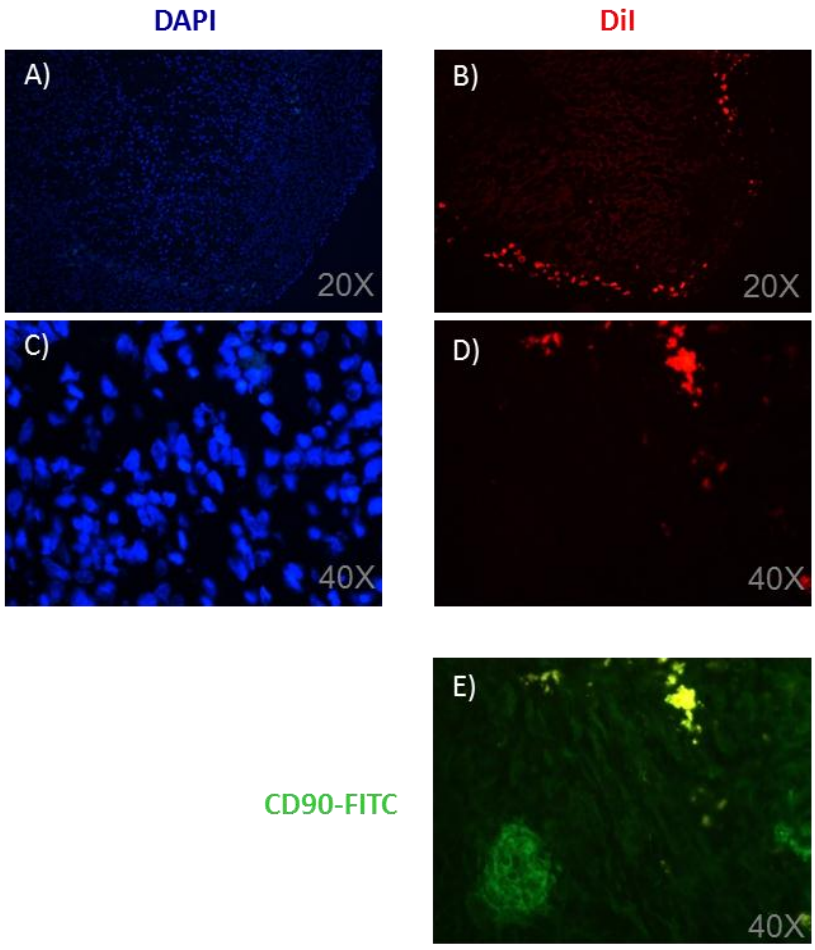


Figure 4.8: Localization of MSCs to the liver after PH. A, C) DAPI staining of the liver section. B,D) The migration of the labeled MSCs was detected by DiI staining, and E) the presence of native MSCs and the administered MSCs was detected with CD90 staining. Green staining indicates the CD90 staining. Yellow staining indicates the merging of DiI staining with the CD90 staining. C,D and E are the larger images of A and B.

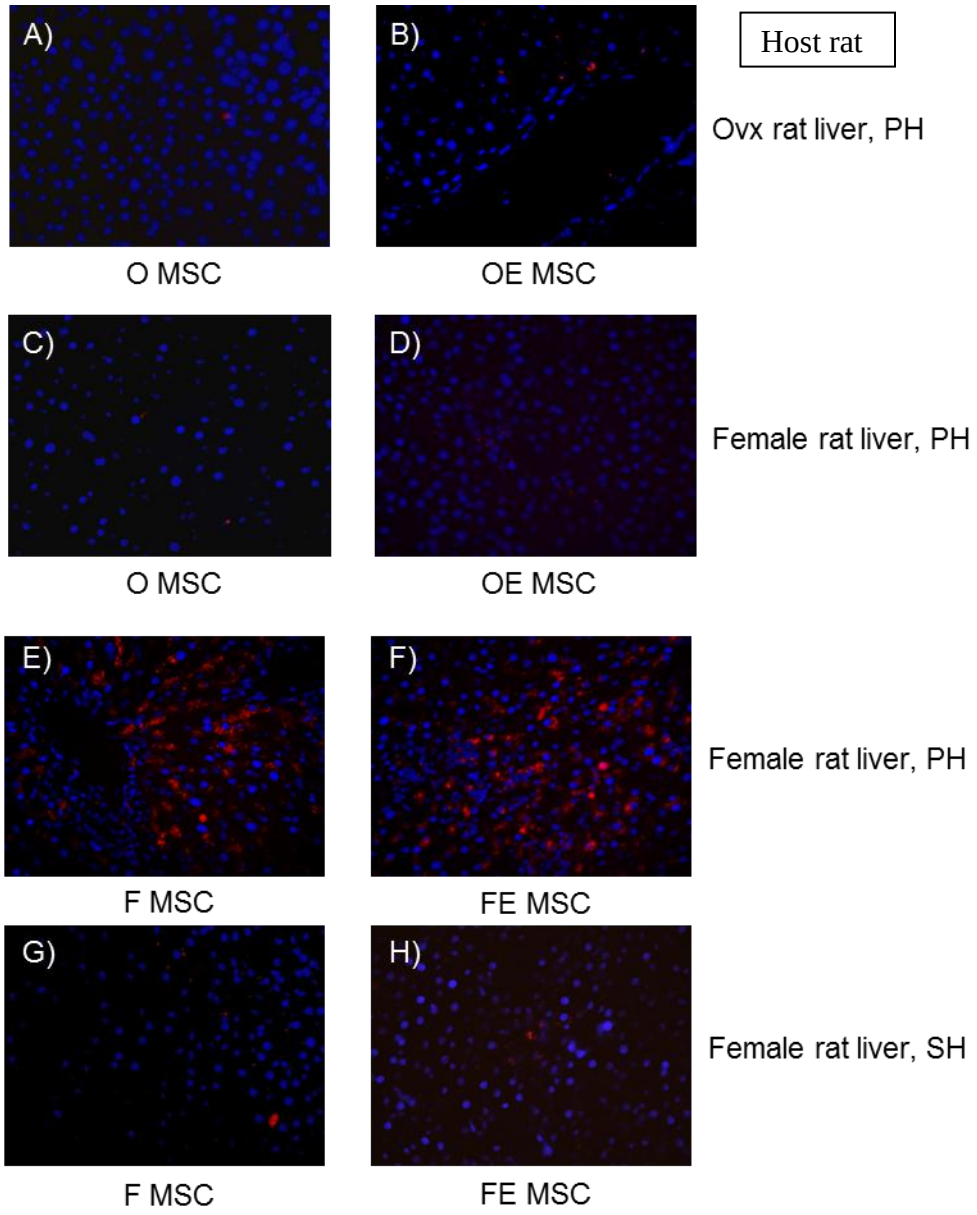


Figure 4.9: Effect of estrogen on the homing capacity of MSCs Liver sections from, A, B) ovx female rats with PH, C-F) normal female rats with PH and G,H) normal female rats with SH. DiI labeled MSCs A-D) from ovx female, and E-H) from normal female in the A,C,E,G) absence and B,D,F,H) presence of estrogen were injected to either female rats with PH, ovx rats with PH or female rat with SH as indicated. (Magnification is 40X).

4.6 MSC MAINTENANCE

4.6.1 APOPTOSIS

To find the mechanism by which estrogen increases the amount of MSCs, apoptotic pathway was searched. It is known that estrogen plays role in apoptosis in different systems. To measure the apoptotic rate of MSCs, TUNEL assay was done. In this assay apoptotic cells are detected according to the fact that apoptotic cells have fragmented internucleosomal DNA. FITC labeled dUTP was observed under fluorescence microscope with 490nm excitation wavelength. To count all of the cells the samples were also stained with DAPI and observed under fluorescence microscope with 359nm excitation wavelength. Figure 4.10A shows representative images of TUNEL staining (Figure 4.10A). Number of TUNEL positive cells were counted and divided to the number of total cells and TUNEL positive percentage was calculated. In our system apoptosis was not induced. We searched for the spontaneous physiological apoptosis rate. Therefore our TUNEL positivity percentage is very low. Estrogen treatment significantly decreased the TUNEL positive percentage both in MSCs isolated from female and MSCs isolated from ovariectomized rat (Figure 4.10B). This suggests a role for estrogen in preventing spontaneous apoptosis in MSCs.

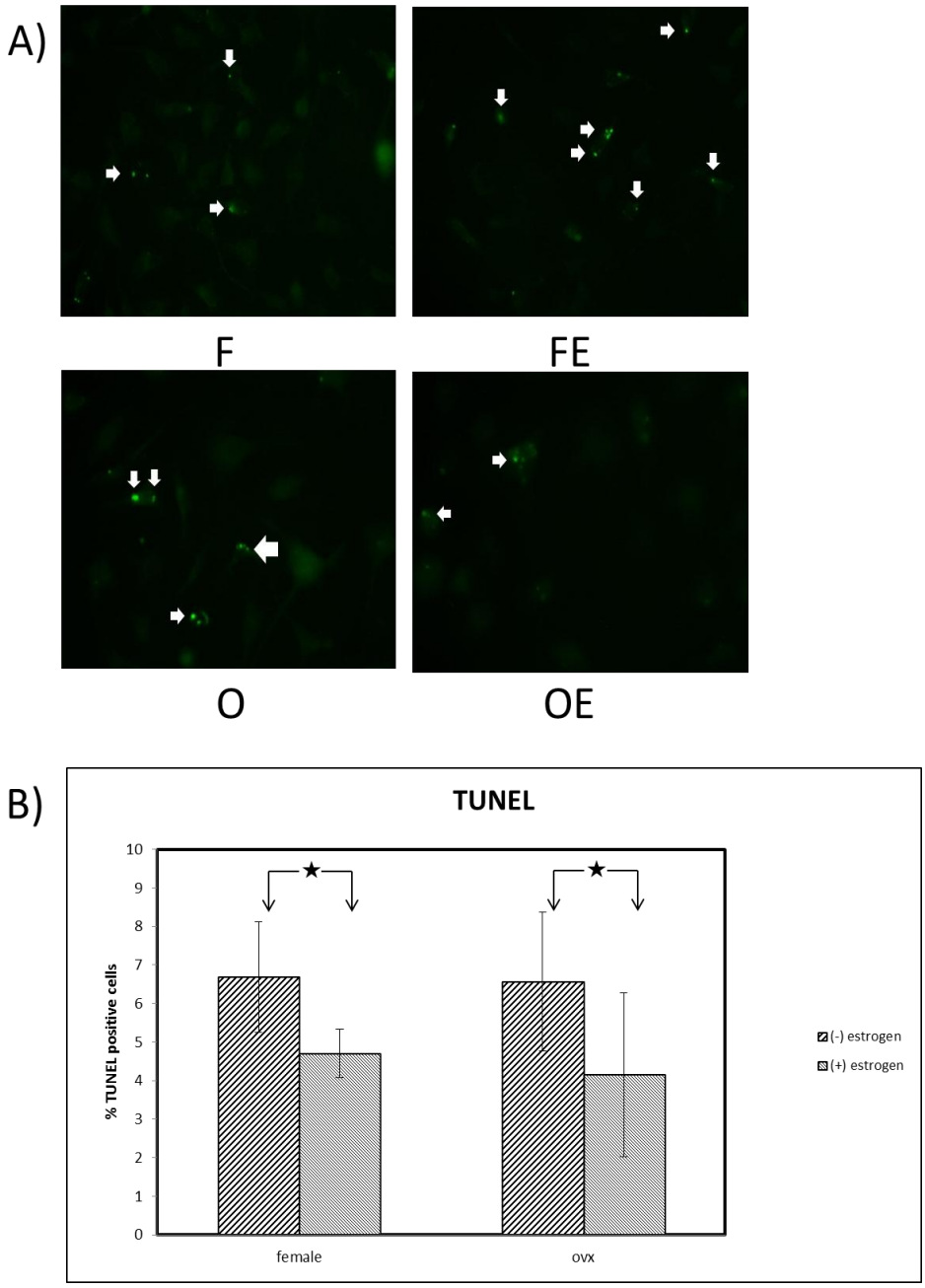


Figure 4.10: Effect of estrogen on MSC apoptosis shown by *in situ* analysis of DNA fragmentation (TUNEL). A) Representative IF images of TUNEL staining of MSCs isolated from normal female and ovx female and cultured in the absence and presence of estrogen Arrow indicates the TUNEL positive cells. B) Percentage of TUNEL positive cells. * indicates $p<0.05$. n=3. Mean ± standart deviations. (Magnification 20X)

4.6.1.1 mRNA EXPRESSION of BCL-2 FAMILY OF GENES

To explore the mechanism by which estrogen might prevent apoptosis, Bcl-2 family of members were searched. These are involved in the intrinsic apoptotic pathway and some members of the family have been shown to be affected by estrogen. The pro-apoptotic members Bak and Bax and the antiapoptotic members Bcl-2 and Bcl-x_L were analyzed in this study.

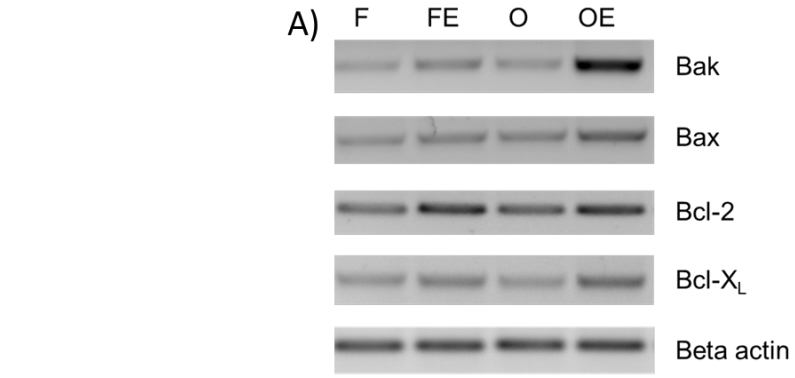
To see whether the MSCs isolated from normal female and ovx female rats express the Bcl-2 family members at mRNA level in the absence and presence of estrogen, RT-PCR was performed. All of the groups tested express *bak, bax, bcl-x_L* and *bcl-2* at the mRNA level (Figure 4.11A) However we could not detect a significant change at the mRNA expression level upon estrogen treatment in these genes (Figure 4.11B).

4.6.1.2 PROTEIN EXPRESSION of BCL-2 FAMILY

IF staining and western blot were performed in order to explore the protein expression status of the Bcl-2 family members. The IF staining revealed that MSCs isolated from normal female and ovx female rat and cultured in the absence and presence of estrogen expressed Bak, Bax, Bcl-2 and Bcl-x_L (Figure 4.12).

To evaluate the changes in their expression western blot analysis was done Total proteins were loaded on SDS-PAGE and analyzed for the expression levels of Bak,

Bax, Bcl-2 and Bcl-x_L. Our results showed that the anti-apoptotic members were



B) Relative expression to *β-actin* of the *bak, bax bcl-2* and *bcl-X$_L$*

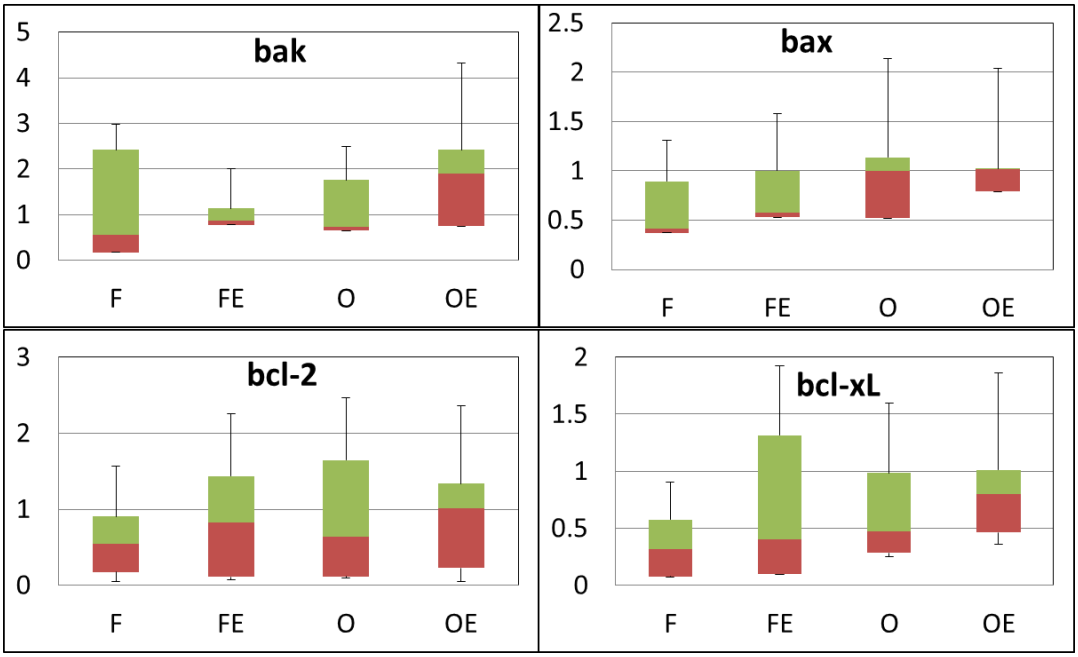


Figure 4.11: Effect of estrogen on the expression profile of the *bcl-2* family of genes in MSCs A) mRNA expression of the Bcl-2 family members, *bak*, *bax*, *bcl-x_L* and *bcl-2 by RT-PCR*. B) Semi quantitative data of RT-PCR shown with box-whisker plot. *Beta actin* was used as a loading control. n=3

affected by estrogen. We observed an increase at the protein levels of Bcl-2 and Bcl-x_L upon estrogen treatment in MSCs isolated from normal female rats. (Figure 4.13A) The pro-apoptotic members of the Bcl-2 family were also affected by estrogen, however this change was not as significant as the change in the anti-apoptotic ones (Figure 4.13B)

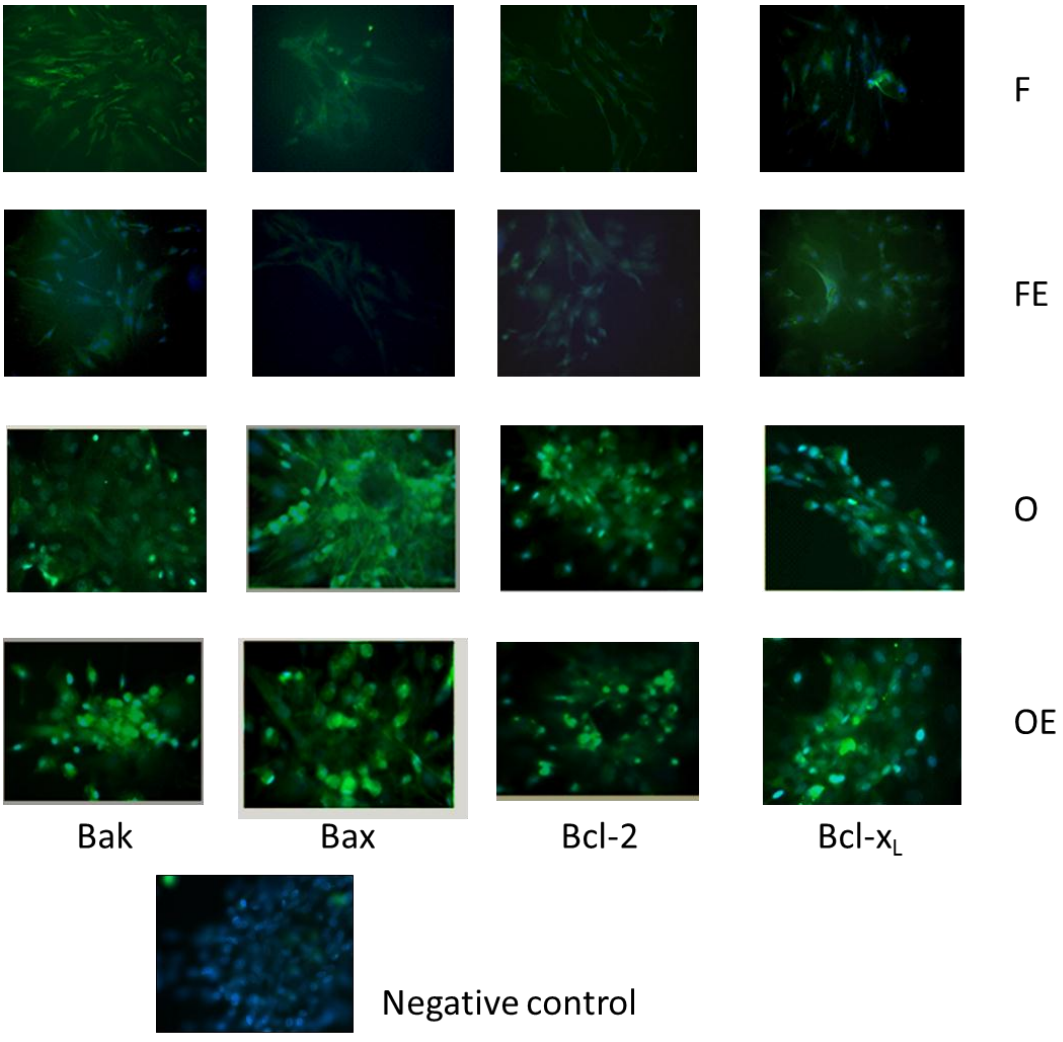


Figure 4.12: Immunofluorescein staining of the Bcl-2 family of proteins on MSCs isolated from normal female and ovx female rat and cultured in the absence and presence of estrogen. (Magnification 20X).

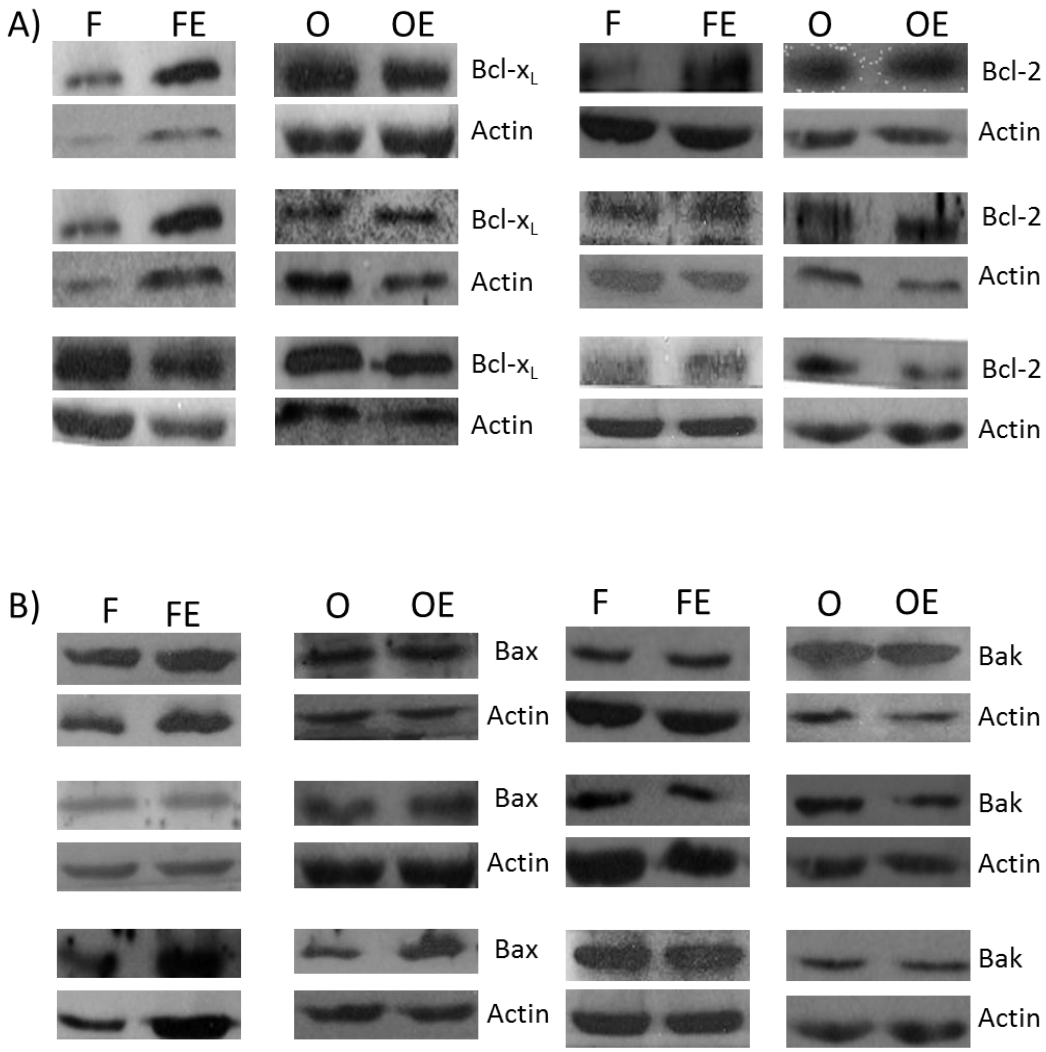


Figure 4.13: Protein expression of the Bcl-2 family of genes in MSCs determined by western blotting. Three sample blots of the A) antiapoptotic Bcl-x_L and Bcl-2, and B) proapoptotic Bak an Bax. Actin was used as a loading control.

4.6.2 KNOCK DOWN OF THE ANTIAPOPTOTIC MEMBERS OF BCL-2 FAMILY

To further confirm the role of the anti-apoptotic members of the Bcl-2 family in estrogen regulated apoptosis of MSCs we used knock down strategy. Since the protein levels of Bcl-2 and Bcl-x_L were increased upon estrogen treatment in MSCs isolated from normal female rat, we knocked down the expression of these members in MSCs isolated from normal female rat by using shRNA plasmids. The transfection was done on the 14^{th} day of cell culture, and the cells were incubated for further 4 days to achieve an optimum silencing. After 4 days total RNA was isolated and the efficiency of knock down was confirmed by RT- PCR (Figure 4.14A and B). After achieving the knock down, TUNEL assay was performed on the cells and TUNEL positive samples were counted as explained before. At least two different observers counted the cells to get accurate results. In the absence of Bcl-x_L or Bcl-2, estrogen addition caused an increase at the percentage of TUNEL positive cells (Figure 4.14C and D) suggesting a role for the anti-apoptotic members in the estrogen regulation of apoptosis in MSCs.

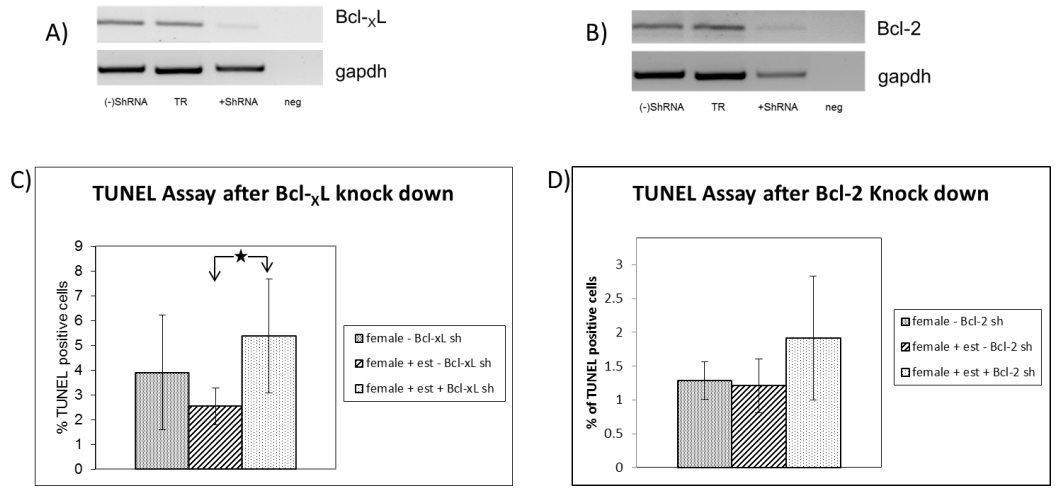
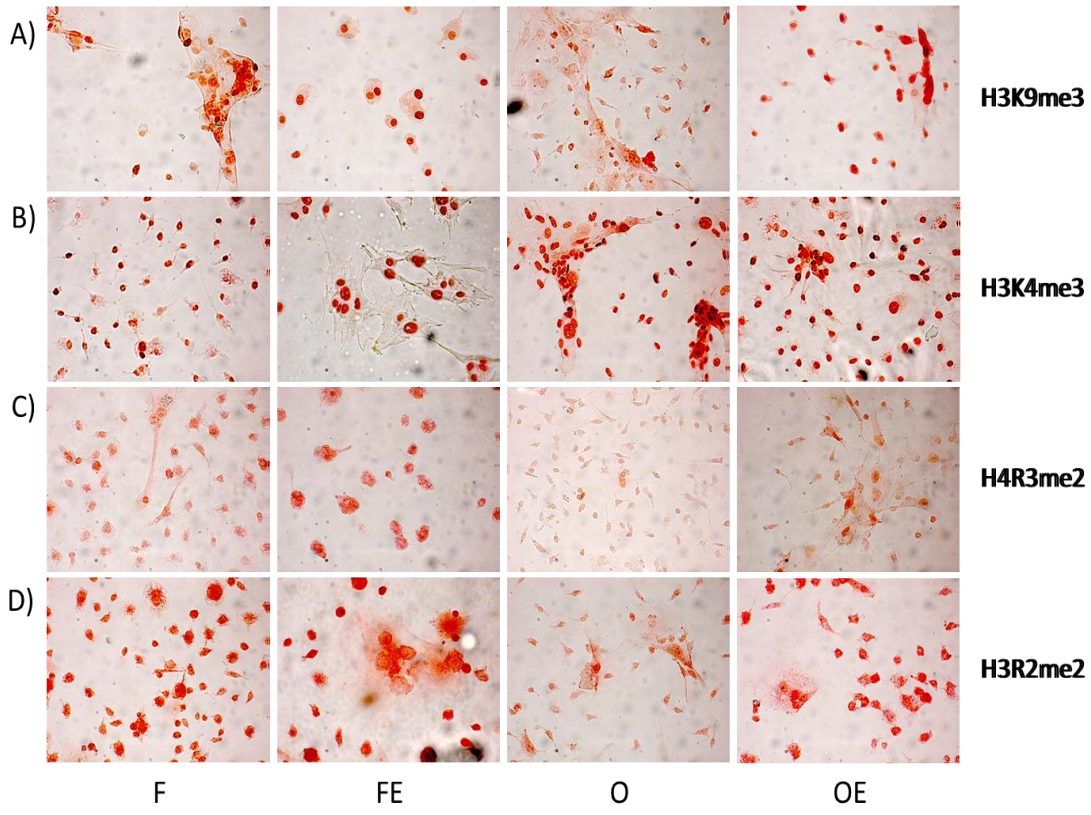


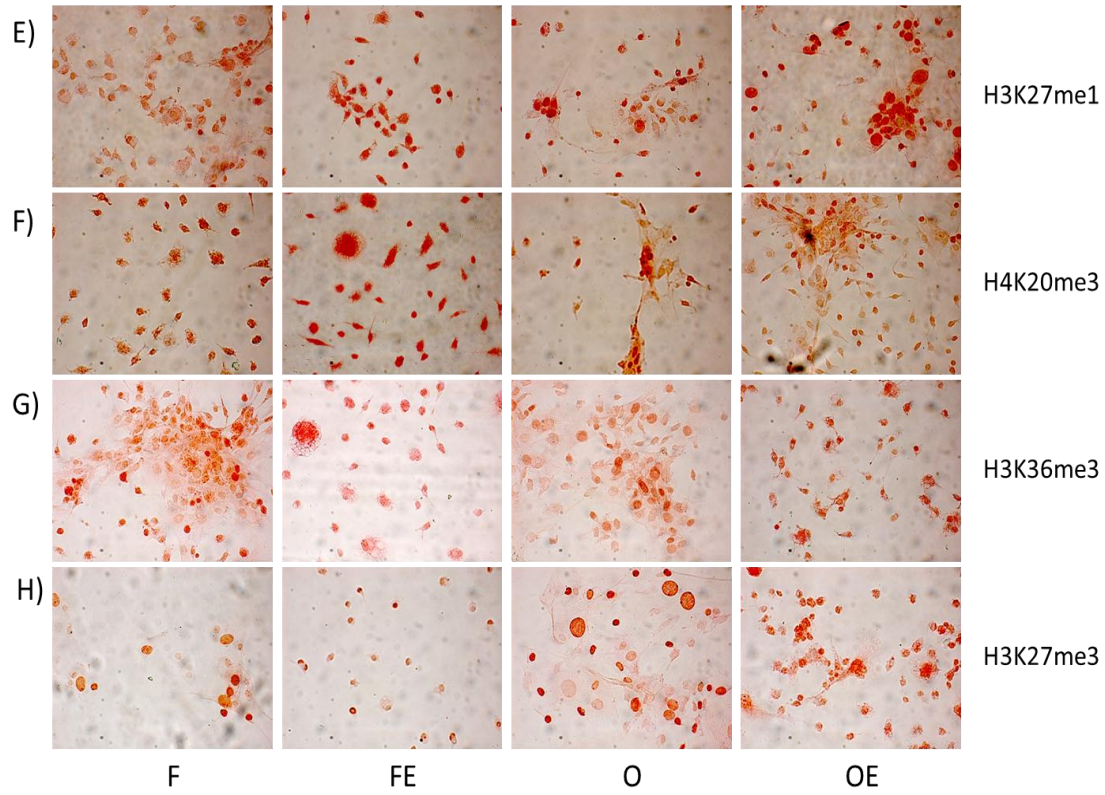
Figure 4.14: Effect of silencing *bcl-x$_L$* and *bcl-2* on the apoptotic rates of MSCs The mRNA expression of A) *bcl-x$_L$* and B) *bcl-2* in MSCs transfected with shRNA of *bcl-x$_L$* and *bcl-2 Gapdh* was used as loading control. Percentage of TUNEL positive cells after MSCs were transfected with C) *bcl-x$_L$* shRNA and D) *bcl-2* shRNA. Mean ± standart deviation TR=Transfection reagent. n=3.

4.7 EFFECT OF ESTROGEN ON HISTONE MODIFICATIONS OF MSCs

Estrogen treatment caused a change in the maintenance of the MSCs. Since estrogen works mainly via the estrogen receptors and regulate thereby the expression of target genes, it was important to search for the possible mechanism. One way is that estrogen could change the modifications on the histone proteins and thereby enable or prevent the expression of genes. Histone protein modifications were compared in estrogen treated and untreated MSC groups. The immunocytochemistry staining revealed that all the samples were positive for H3K9me3, H3K4me3, H4R3me2, H3R2me2, H3K27me1, H4K20me3, H3K36me3 and H3K27me3 (Figure 4.15). Interestingly, we observed a decrease in the staining

density of H3K27me3 in estrogen treated MSCs. Therefore, we performed western blot to evaluate the change of this modification upon estrogen treatment..





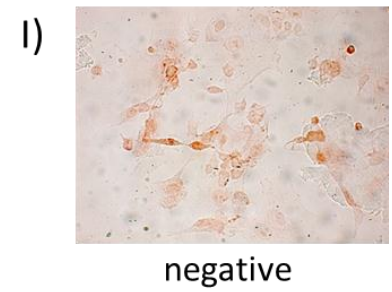


Figure 4.15: The expression of histone proteins by immunocytochemistry. A) H3K9me3, B) H3K4me3, C) H4R3me2, D) H3R2me2, E) H3K27me1, F) H4K20me3, G) H3K36me3 and H) H3K27me3 staining is shown I) Negative sample without any primary antibody. (Magnification 40X).

Acid extracted histone proteins from estrogen treated MSCs and untreated MSCs were loaded on SDS-PAGE and blotted with anti-3mH3K27 antibody. Our results showed that in estrogen treated MSCs 3mH3K27 expression was decreased (Figure 4.16).

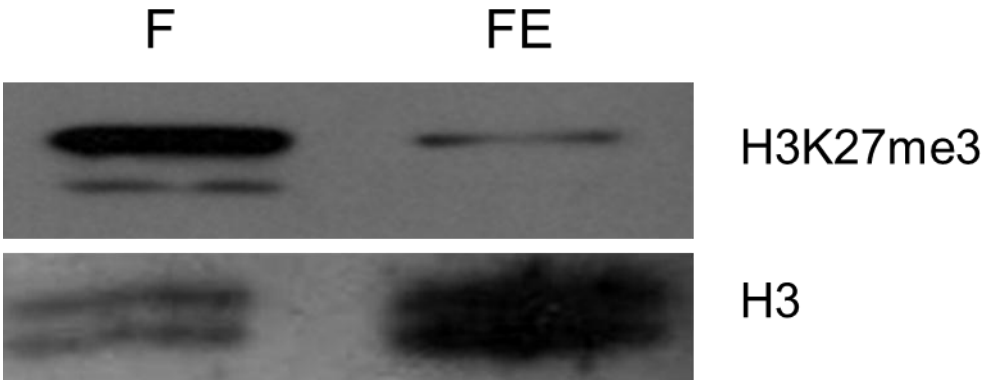


Figure 4.16: Western blotting of modified histone. Histone 3 was used as loading control.

Chapter 5

DISCUSSION

The importance of stem cells is rising from day to day. Over 60000 articles have been published is this field in the last 5 years (www.pubmed.com). Also stem cell societies from different parts of the world are gaining more and more members. The number and the participants of the conferences in this field have been increasing. All of this implicates that stem cells are gaining great attention over the years. These special cells are the hope for the treatment of several diseases since they have the ability to differentiate into different kinds of cells of the body. ESCs are pluripotent cells which can differentiate into the cells of the three germ layers. That is why a great deal of work is related to the ESCs. However, besides their amazing differentiation potential there is a huge handicap for these cells, is their source. ESCs are isolated from the blastocysts of embryos whereby the embryo is destroyed. This brings about ethical problems, which makes it difficult if not impossible to work with the ESCs or use these cells in a therapy. The use of ASCs has no ethical issue and is also being used in disease treatment. MSCs are ASCs that can be isolated from various parts of the body like bone marrow or adipose tissue and can be differentiate to the cells of the mesenchymal lineage like osteoblasts, chondrocytes, and adipocytes and also of other lineages like neurons, skin cells and tenocytes. Although MSCs have no ethical or immunogenic problems

there is one draw back of these cells; their rareness. To use these cells in any kind of therapy the number of them has to be increased. Estrogen is known to play role in increasing proliferation and decreasing apoptosis. Estrogen can modulate apoptosis through the Bcl-2 family of proteins in different systems. It can either directly or indirectly affects the expression of these proteins. Therefor we aimed in this study to search for the effects of estrogen on MSC apoptosis and tried to find molecules involved in this regulation.

To test the effects of estrogen on MSCs we added 10^{-7}M estrogen to the culture dishes after the day we harvested MSCs from rat femur and tibia. As a control we used MSCs without adding estrogen. We used MSCs from either untreated or ovariectomized female rats in our experiments. To start with our experiments we first characterized our samples. All four MSC samples were positive for MSC markers CD29, CD90 and CD71 and negative for hematopoietic markers CD34 and CD45 (Figure 4.1). This showed that estrogen did not change the marker expression of our samples which was an indication of not changing the MSC property. Therefore we concluded that it is safe to use estrogen in our studies.

Since we want to see the effects of estrogen we searched for the ER α and β expression. Western blot experiment revealed that MSCs isolated from normal female and ovx female and cultured in the absence and presence of estrogen express both of the receptors (Figure 4.2). In addition to that we tested the estrogen responsiveness of the MSC samples. Semi quantitative PCR was done to evaluate

the expression of the estrogen responsive gene *mmp12* (Figure 4.3). Both in MSCs isolated from normal female rat and from ovx female rat, estrogen treatment increased the expression of *mmp12*. We evaluated also whether this increase is mediated through the ERs. Therefore we treated the MSCs in addition to estrogen also with tamoxifen, since tamoxifen is an ER antagonist. The *mmp12* expression decreased upon tamoxifen treatment suggesting that estrogen is acting through the ERs in mediating *mmp12* expression. As a conclusion we can say that MSCs are estrogen responsive and can react to the action of estrogen and this is mediated via the ERs found in the MSCs.

Another feature of MSCs is formation of colonies. To test whether estrogen had an impact on these characteristics we performed CFU assay. We could observe colonies in all MSC groups tested (Figure 4.4A-D) with an increase in the colony numbers in the presence of estrogen (Figure 4.4E). In addition to that we could observe a significant ($p<0.05$) increase in the number of cells that was present in one colony in estrogen treated samples compared to the untreated ones (Figure 4.4F). As a conclusion we can say that estrogen treatment did not hinder the MSC marker expression or colony formation, but had a positive effect on the number of colonies formed and on the number of cells in a colony. This effect could be attributed to estrogen's proliferative activity. Our results were also parallel to a previous study where it was shown that estrogen increased the number of MSCs obtained from ovariectomized rats [235].

Next we tested the differentiation potential of our MSCs. Upon induction with defined agents we could differentiate our samples into adipogenic and osteogenic lineages as shown by oil red o and alizarin staining, respectively (Figure 4.5A-B). Also the adipogenic markers *lpl* and *pparγ* and osteogenic markers *oc* and *runx*2 expression confirmed the differentiation of our MSCs (Figure 4.5C). It has been shown that estrogen has important role in adipocyte metabolism [236] and that the adipogenic differentiation capacity of MSCs was increased in post menopausal women [237] which could be attributed to the loss of estrogen. We found that estrogen did not prevent the differentiation capacity of any of the samples, however a decrease in the lipid droplets formed and a decrease in the *lpl* and *pparγ* expression was observed when estrogen was added to the differentiation medium of MSCs isolated from normal female. Previously it was reported that MSCs from osteoporotic post menopausal women had an increase in their adipogenic differentiation potential which was shown by increased *pparγ* expression. This could be the result of altered bioavailability of estrogen in these women [238]. Another study showed that the serum from post menopausal in comparison to serum from pre menopausal women, were able to induce adipogenic differentiation in hOP 7 cells which can differentiate into adipocytes and osteocytes like the MSCs [239]. Our result suggested an inhibitory role for estrogen on MSC adipocyte differentiation. Defining the mechanism of this could give new insights to the treatment of obesity and obesity related diseases. It is clearly important to know the relation between estrogen and MSC differentiation, which could explain the

increase of fat tissue in post menopausal women. Another important factor is that we did not see the same scenario in MSCs from ovariectomized rats. This shows that estrogen treatment alone is not responsible for the decrease in adipogenic differentiation capacity of MSCs. Other factors or pathways are involved in this process, which are absent or not responsive after ovariectomization. The molecular mechanism behind this event is not known and should be searched for further clarification.

Another characteristic of MSCs is their homing capacity to injured tissue. For that purpose we performed partial hepatectomy in rats to mimic liver injury. The *in vitro* cultured MSCs were labeled with the fluorescein dye DiI (Figure 4.6), to track them after they were injected to the rats through their tail vein. We administered the MSCs to the rats 1 day post PH, 3 days post PH and 5 days post PH. We observed the highest number of DiI labeled MSCs in the livers of 3 days post PH rats (Figure 4.7). when we injected the MSCs 1 day after the PH we did not see any DiI labeled MSCs in the liver sections. This could be due to loss of signaling molecules to attract the MSCs to the site of injury at that time. We did not observe any MSCs in the liver sections of 5 days post PH rats. A reason for this could be that the native MSCs has reached the injured liver earlier and eliminated the need for the administered MSCs. Determining the optimum timing for the transplantation of the MSCs would increase the benefit of using MSCs in therapies.

We could observe under fluorescein microscope, that both the estrogen treated and the untreated MSCs isolated from normal female migrated to the injured liver of female rats (Figure 4.9E-F) but not to the SH rats' liver (Figure 4.9G-H). However there was a difference in their amount that was present in the liver. The fluorescein microscope images revealed a higher number of estrogen treated MSCs that migrated to the site of injury in contrast to the untreated MSCs. This increase cannot be attributed to the number of MSCs that were initially injected to the rats, because the initial numbers were same in every case. Other mechanisms should be involved in the higher homing capacity of estrogen treated MSCs to the injured liver. The proliferative action of estrogen could have increased the MSCs after injection so that more MSCs were available for migrating. Another possibility could be that estrogen treated MSCs responded faster and more effectively to factors secreted from the injured liver and so reached the site of action earlier. Another important finding here is the absence of DiI labeled ovariectomized MSCs at the site of injury after injection (Figure 4.9A-D). This could be a result of being deprived of estrogen for a long time. Loss of estrogen could have disrupt the homing capacity of MSCs, which would attribute an important role to estrogen in MSCs homing. It is clearly an important issue to reveal the mechanism behind this in order to use the homing capacity of MSCs to the injured tissues in a more efficient way. This would improve the usage of MSCs in therapies by decreasing the treatment time and the need for high numbers of MSCs.

The proliferative effect of estrogen has been shown on rat bone marrow-derived MSCs before [134]. However, the effect of estrogen on the maintenance of MSCs isolated from normal animals have not been reported. To test the effect of estrogen on MSCs maintenance we searched for apoptosis. It is known that estrogen have a dual effect on apoptosis, it can inhibit or promote apoptosis in different cells. In our system we did not induce any kind of apoptosis, but searched for the physiological apoptosis of the MSCs. We compared the apoptotic cells by TUNEL assay. The overall apoptotic rate was less than 10%, which is normal since we did not induce apoptosis in any way. The TUNEL positive cells were significantly ($p<0.05$) decreased in the estrogen treated MSCs. This decrease was observed in both normal female group and ovariectomized female group (Figure 4.10B). From this we conclude that estrogen has an inhibitory role on MSCs spontaneous apoptosis.

The Bcl-2 family of proteins plays a pivotal role in apoptosis. It is also being reported that some of the members are regulated via estrogen like Bcl-2, and Bcl-x_L [123, 199, 205]. In our system we wanted to examine whether estrogen exerts its anti-apoptotic effect via regulation of the Bcl-2 family members. *Bcl-2,*, *bcl-x_L, bak* and *bax* mRNA expression was checked with semi quantitative PCR. At the mRNA level we did not detect any significant difference between the estrogen treated and untreated MSC samples in neither of the groups (Figure 4.11). The Bcl-2 family members are mostly regulated at the protein levels. Therefore we further analyzed the protein levels of these members. IF staining showed that all of the samples

express Bak, Bax, Bcl-2 and Bcl-x_L (Figure 4.12). To test if there is any difference in the expression of these proteins upon estrogen treatment we performed western blotting. Western blot analysis revealed that there was an increase in the Bcl-2 and Bcl-x_L protein levels upon estrogen treatment in MSCs isolated from normal female group (Figure 4.13). This increase in the anti-apoptotic members could explain the decrease in the TUNEL positivity after estrogen treatment. We also observed slight changes in the expression levels of the the pro-apoptotic members upon estrogen treatment. However this change was not as meaningful as the change in the anti-apoptotic ones yet we can not eliminate the role of the pro-apoptotic members in the estrogen regulation of apoptosis. Although the mRNA and protein levels of these proteins did not change, post translational modifications like phosphorylation or compartmentalization could be involved in the regulation of these members and further studies had to be made for the confirmation of this. To test the effect of the anti-apoptotic members on the estrogen regulated apoptosis of MSCs we performed silencing of the anti-apoptotic members in MSCs and treated our samples with estrogen for further four days. We observed a statistical increase in the apoptotic rates of the estrogen treated MSCs in the presence of Bcl-x_L shRNA and Bcl-2 shRNA in comparison to the estrogen treated MSCs in the absence of Bcl-x_L shRNA and Bcl-2 shRNA (Figure 4.14C and D). All together these show that estrogen decreases apoptosis in MSCs via the anti-apoptotic members of the Bcl-2 family. The interaction between estrogen and Bcl-x_L and Bcl-2 is not known.

Estrogen could directly interact with the members or indirectly affect their function. One possible mechanism could be through epigenetic modifications.

Histone protein modifications determine the on-off state of a gene. They include acetylation, methylation, phosphorylation, sumoylation and ubiqutylation. Several studies have revealed a correlation between estrogen and these modifications. The relation between stem cells and histone modification is that in an undifferentiated state the stem cells mainly possess poised chromatins with silencing and activating chromatin marks together on the chromatin. This means that upon differentiation to a specific lineage the promoter of a gene, specific to that lineage will become open in other words the activating mark will dominate over the repressive mark and the gene will be expressed. If the gene is not expressed in that lineage than the silencing mark will be dominate over the activating mark and the gene won't be expressed [224, 225]. To test whether this could be the case in our system we looked for a global expression of several histone methylation marks. Histone methylation can be activating or silencing depending on the specific location of that modification on the histone tail. Immunostaining revealed the presence of several histone methylations on the MSCs (Figure 4.15). H3K27me3 is an important histone modification in stem cells. It is found on bivalent promoters and has a critical role in determining the transcriptional status of the genes. Previously relation between H3K27me3 and estrogen has been shown [232, 233]. The immunostaining showed a decrease in the intensity of the H3K27me3 mark upon estrogen treatment (Figure

4.15). To further confirm this we performed western blot analysis and detect a decrease in the global H3K27me3 in the estrogen treated MSCs compared to the untreated samples (Figure 4.16). Since the H3K27me3 modification is a silencing mark we concluded that estrogen treatment activated some genes that were not active before. This could also be the case for Bcl-x_L and Bcl-2. However since we did not see any significant difference at the mRNA level of them it is more probable that the anti-apoptotic members are a downstream target of a signaling cascade that is activated by a decrease in the H3K27me3 mark. Chromatin immunoprecipitation should be done on *bcl-x_L* and b*cl-2* to demonstrate the presence or absence of this mark on these genes and if and how it changes upon estrogen treatment. Knocking down the demethylase JMJD3, and observing the change in the protein expression after estrogen treatment would reveal the importance of this histone mark in the regulation of Bcl-x_L and Bcl-2. These histone PTM could also explain the reduced adipogenic differentiation capacity in estrogen treated samples. Estrogen may have an effect on the poised chromatins in the undifferentiated MSCs. It could favor the loss of H3K27me3 in factors that are preventing adipogenic differentiation.

As a conclusion in this study we showed that estrogen does not interfere with the stem cell properties of the rat bone marrow derived MSCs. It increases the number of colonies and decreases the adipogenic differentiation potential of MSCs. Estrogen has a positive effect of MSC homing to injured liver. It decreases the spontaneous apoptosis of MSCs Estrogen exerts its anti-apoptotic effects mainly via

the anti-apoptotic members of the Bcl-2 family. The molecular mechanism behind this is not known but histone PTMs can be the important players in this regulation. Knowing the mechanism behind the maintenance of the MSCs is important in obtaining more MSCs for further clinical investigations.

Chapter 6

FUTURE PERSPECTIVES

In this thesis the effect of estrogen on MSC maintenance was explored. We found that estrogen prevents the simultaneous apoptosis of MSCs through the anti-apoptotic members of the Bcl-2 family. However the molecular mechanism behind this event is not known and should be further analyzed. The expression of b*cl-x*$_L$ and *bcl-2* did not changed at the mRNA level but increased at the protein level upon estrogen treatment, and the reason of this should be searched. Post translational regulation like phosphorylation of the candidate proteins should be studied. We found that global H3K27me3 modification decreases after estrogen treatment in MSCs. Chromatin immunoprecipitation experiments should be performed in order to get a deep look into the histone mark of the Bcl-2 family members and other genes that are known to regulate them. H3K27me3 mark is a repressive mark and the decrease of it upon estrogen treatment could activate some important genes, whose products are increasing the activity of Bcl-x_L and Bcl-2. The enzyme responsible for this histone mark is Ezh2. This enzyme also regulates the transcription of estrogen responsive genes [232]. Also the demethylase JMJD3, which substrate is H3K27me3 seems to be important in Bcl-x_L and Bcl-2

regulation. Detailed relation between estrogen, Ezh2, JMJD3 and Bcl-x_L, Bcl-2 regulation should be investigated.

Estrogen also had an impact on the differentiation potential of MSCs. It decreased the number of lipid vacuoles after adipogenic differentiation as detected by Oil Red O staining. The molecular mechanism is not known and should be analyzed. Epigenetic modifications related to estrogen addition could be the reason of this decrease in differentiation potential, since differentiation is mainly related to the rapid transcriptional regulation, in other words the on or off status of transcription factors and other genes. Detailed inspection of the epigenetic changes of the transcription factors related to the different mesenchymal lineages should be made. The effect of histone methyltransferase Ezh2 and the demethylase JMJD3 on the target genes should be studied. The relation between obesity and MSCs should be inspected. Also the relation between post-menopausal weight gain and MSC differentiation should be searched. Finding the regulatory pathway, could provide new insights into the *in vitro* differentiation of MSCs, which would improve the usage of MSCs for therapeutic purposes.

In this thesis we observed also that much more estrogen treated MSCs migrated to the injured liver compared to the untreated ones. Why this happened is not known and detailed analysis of this phenomenon is important in directing the MSCs through a path *in vivo.* Analyzing the chemoattractant factors secreted from the injured liver could give an insight to the mechanism. The role of estrogen in this

event is not known. Relation between estrogen and the injured liver, the secreted factors and/or the nearby environment should be studied. Other injury models should be checked also to determine whether the effect of estrogen on MSC homing is specific to liver injuries or a general matter. Controlling the path of MSCs would not only make them an ideal candidate for cell based therapies but also make them an ideal vehicle for targeted drug delivery *in vivo*.

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APPENDIX

SOLUTIONS AND BUFFERS

4% Paraformaldehyde (PFA)

4g PFA

Complete volume to 100ml with 1XPBS

Dissolve by heating and constant stirring

10% formaldehyde

2 ml formaldehyde

18 ml ddH_2O

70% Ethanol

70 ml 100% Ethanol

30 ml ddH2O

10x PBS (pH: 7,2)

80 g NaCl

2 g KCl

8,01g Na2HPO4.2H2O

2g KH2PO4

Complete volume to 1Lt with ddH2O

10X Agarose Gel Loading Dye

0,009g bromophenolblue

0,009g XC

2,8mL ddH2O

1,2ml 0,5M EDTA

Complete volume to 15 mL with glycerol

50X TAE Buffer

2M Tris Base (242 g)

57,1 ml Glacial Acetic Acid

50mM EDTA

Complete volume to 1Lt with ddH2O

1M Tris (pH:8)

60.55g Tris

300ml ddH2O

21ml 37% HCl

pH adjusted to 8.0 with HCl, total volume brought to 500ml by adding ddH2O.

Total Protein Lysis Buffer

2M NaCl

1M Tris pH:8.2

0.9% Igepal CA-630 (Sigma, Germany, Germany)

10x Protease Inhibitor Cocktail (Roche, Germany)

778.5µl ddH2O

Bradford Reagent

100 mg Coomassie Brilliant Blue G-250

100 ml 85%phosphoric acid

50 ml 95%EtOH

Complete volume to 1Lt with ddH2O

Filtered through Whatman no:1

10%SDS

10g SDS

Complete volume to 100µ with ddH2O

30% Acrylamide Mix

145g Acrylamide

5g bis-acrylamide

Complete volume to 500ml with ddH2O

Filtered, stored in dark at 40C.

SDS-PAGE Running Buffer (5X stock solution)

15g Tris

73,2g Glycine

5g SDS

Complete volume to 1L with ddH2O

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 10% ß-mercaptoethanol prior to use

10X TBS (pH:8)

12.19 g Tris

87,76 g NaCl

pH adjusted to 8, Complete volume to 1Lt with ddH2O

1X TBS-T 0.3%

50 ml 10X TBS

450 ml ddH2O

1,5 ml Tween-20

1X TBS-T 0.1% (pH: 7.5)

50 ml 10X TBS

450 ml ddH2O

500 µl Tween-20

pH adjusted to 7.5

Blocking Solution for Western Blot (5% BSA) (Histone protein)

0.5g BSA

10mL 1X TBS-T 0.1%

Blocking Solution for Western Blot (4% milk powder)

2 g milk powder

50mL 1X TBS-T 0.3%

Semi-dry Transfer Buffer

2,5 g glycine

5,8 g Tris base

0,37 g SDS

200 ml methanol

Complete volume to 1 Lt with ddH2O

Wet transfer buffer for small proteins

3 g Tris

14.25 g Glycine

200 ml methanol

Complete volume to 1 Lt with ddH2O

Coomassie Blue Staining Solution

0,25 g coomassie brilliant blue

45 ml Methanol

10 ml glacial acetic acid

Complete volume to 100 ml with ddH2O

Destaining Solution

100 ml methanol

35 ml acetic acid

365 ml ddH2O

Blocking Solution for Immunofluorescence (IF) Staining

1 ml 2% BSA

1 ml 1X PBS

5 µl tween-20

2N HCl solution

16.38µl ddH2O

4.3µl HCl

TEB buffer

0.5% triton-100

0.02% NaN3

In 1XPBS

Add 1X protease inhibitor prior to use

Blocking Solution for Immunocytochemistry (histone protein)

2%FBS

0.3% TritonX-100

In 1XPBS

Permeabilization solution for histone protein immunocytochemistry

0.5% saponin

0.3% tritonX-100

In 1XPBS

Lb

10 g Tryptone

10 g NaCl

5 g Yeast extract

Complete volume to 1 Lt with ddH2O and autoclave

Agar

10 g Tryptone

10 g NaCl

5 g Yeast extract

15 g Bacto agar

Complete volume to 1 Lt with ddH2O and autoclave

Adipogenic Induction Media (Prepared in DMEM –LG)

1 µM dexamethasone (Sigma, Germany)

0.5 mM IBMX (Sigma, Germany)

10 µg/mL insulin (Sigma, Germany)

100 µM indomethacine (Sigma, Germany)

10% FBS

1% penicillin/streptomycin antibiotic solution

Osteogenic Induction Media (Prepared in DMEM –LG)

0.1 μM dexamethasone

0.2 mM ascorbic acid ɣ-irradiated (Sigma, Germany)

10 mM glycerol phosphate disodium salt hydrate (Sigma, Germany)

10% FBS

1% penicillin/streptomycin antibiotic solution

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The Effect of Estrogen on Bone Marrow-Derived Rat Mesenchymal Stem Cell Maintenance: Inhibiting Apoptosis Through the Expression of Bcl-x_L and Bcl-2

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Abstract Mesenchymal Stem Cells (MSCs) have high therapeutic value for regenerative medicine and tissue engineering due to their differentiation potential and non-immunogenic characteristics. They are also considered as an effective in vivo delivery agent because of their ability to migrate to the site of injury. A major roadblock in their use for cell-based therapies is their rareness in vivo. Therefore, it is important to obtain increased number of functional MSCs in vitro in order to have adequate numbers for therapeutic regiments. We aimed to investigate the role of estrogen and its mechanism in obtaining more MSCs. MSCs were isolated from female and ovariectomized rats and cultured in the presence and absence of 10^{-7} M estrogen. In the presence of estrogen, not only their CFU-F activity increased but also apoptotic rate decreased as shown by TUNEL staining leading to obtain more MSCs. Also the number of the cells in the colonies increased upon estrogen treatment. To reveal the mechanism of this effect, we focused on Bcl-2 family of proteins. Our immunoblotting experiments combined with knockdown studies suggested a critical role for anti-apoptotic Bcl-x_L and Bcl-2. Estrogen treatment up regulated the expression Bcl-x_L and Bcl-2. When we knocked down the expression of *bcl-x_L* and *bcl-2*, MSCs lacking these genes showed an increase in the apoptotic rate in contrast to normal MSCs following estrogen treatment. Therefore, estrogen treatment will be of great advantage for cell-based therapies in order to get more functional MSCs and may provide opportunities to develop new strategies for debilitating diseases.

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Introduction

Increased human lifespan has brought the concept of a better life quality, since the body functions and health conditions decline with time. Identifying with the pioneering studies of Friedenstein [1], mesenchymal stem cells (MSCs) have demonstrated great potential to tackle these problems. Multipotent MSCs have potential to differentiate into tissue-specific cell types such as osteocytes, adipocytes, chondrocytes, myoblasts, cardiomyocytes [1–3]. MSCs emerge as an important player of cellular-based therapies in wide variety of pathologies such as neurodegenerative and cardiovascular diseases, diabetes, and cancer due to their ability to migrate in vivo to promote regeneration of damaged tissue, treat inflammation, and promote angiogenesis without inducing immune reaction [4–7]. Besides being a future promise for many disease treatments, there are also some handicaps in their applications. One major roadblock is their rareness and the fact that they constitute a very small population of nucleated cells in the bone marrow [8]. Their number has to be increased before they can be used for therapeutic purposes.

Similar to stem cells, estrogen hormone can also be accepted as a key factor in the preservation of life standards. Estrogen is mainly responsible for female sexual features but also influences many physiological processes in mammals including cellular proliferation and apoptosis [9, 10]. In humans, increased incidence of osteoporosis and coronary heart diseases after menopause suggests the involvement of estrogen in these disorders [11, 12]. Since MSCs differentiate into bone and muscle cells, the effect of

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estrogen on MSCs is also critical. Understanding the mechanism of their interaction is vital for obtaining the maximum efficiency from this collaboration. Apoptotic pathways, which are conserved throughout evolution, are strong candidates for this cooperation. Estrogen has a dual role on apoptosis; it can both stimulate and inhibit apoptosis depending on the type of cell, the duration and amount of estrogen and the downstream target signaling pathway. Estrogen promotes apoptosis in osteoclasts, thymocytes and neurons [13–16]. On the other hand, it has antiapoptotic effect on osteocyte, umbilical vein endothelial cells, hippocampal neurons and breast cancer cell lines [17–20].

Although the mechanism of the apoptotic or antiapoptotic function of estrogen is not always through the same pathway, regulation through the *bcl-2* family of genes is a major candidate in this process. The members of the Bcl-2 family of proteins determine the life-or-death of a cell by controlling the release of mitochondrial apoptogenic factors, cytochrome c and apoptosis inducing factors (AIF), which activate the downstream executional phases, including the activation of the caspases [21, 22]. Estrogen increases the expression of anti-apoptotic but decreases the expression of pro-apoptotic *bcl-2* family of genes [19, 23, 24]. The effect of estrogen on the expression of *bcl-x*$_L$ and *bcl-2* is mediated through estrogen response element (ERE) sites of these genes [19, 23]. In addition, estrogen can also affect the expression of Bcl-2 family of genes through the non genomic pathway which involves the phosphorylation of the proteins [24]. Regardless of the mechanism, it is evident that estrogen has a crucial role in apoptotic regulation.

The present study was undertaken to investigate the molecular mechanisms of estrogen during MSC maintenance. In order to explain the mechanism of the action of estrogen, we hypothesize that estrogen differentially regulates the expression of *bcl-2* family of genes and this control results in obtaining more MSC by inhibiting apoptosis.

Material and Methods

Isolation and Culture of MSCs

MSCs were obtained from female and ovariectomized female 9-week old, 280–300 g Sprague–Dawley rats. The animals were permitted unlimited access to food and water at all times and were housed under controlled environmental conditions (22°C) with a 12-hour light and 12 h dark cycle in the animal holding facility of the Department of Molecular Biology and Genetics at the Bilkent University. This study protocol complied with Bilkent University's guidelines on humane care and use of laboratory animals. Bone marrow heterogeneous cell population was collected from the femur and tibia by flushing with a 5 mL syringe containing 10% FBS (Hyclone, Logan, UT, USA) in DMEM (Invitrogen, Paisley, UK) after the rats were sacrificed by cervical dislocation. The cells were cultured in plastic culture dishes with Mesencult Media (StemCell Technology, Vancouver, Canada) with 20% supplement (StemCell Technology) and a 1% penicillin/streptomycin solution (Hyclone) in a 5% CO_2 incubator at 37°C. The next day, the media of the tissue culture plates were changed and the non-adherent cells were removed. The media of the cells were changed every 4 days, after washing with sterile 1x PBS prior to the change. For the estrogen treatment, MSCs were cultured with 10^{-7} M estrogen (17 ß-estradiol, Sigma, MO, USA).

Colony-Forming Assay

After washed in 1x PBS and air dried, MSCs were fixed with cold methanol for 5 min. The cells were washed again in a 1x PBS buffer and stained with giemsa (Carlo Erba, Milano, Italy) for 5 min. The reaction of the giemsa staining was stopped by the addition of tap water. The number of colonies was counted under light microscope.

In Situ Analysis of DNA Fragmentation (TUNEL)

DNA fragmentation was detected in situ by TdT- (terminal deoxynucleotidyl transferase) mediated fluorescein-dUTP labeling kit (Roche Diagnostics, Mannheim, Germany). Cells were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature and then washed in PBS for 30 min. After incubating with a permeabilisation solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 min on ice, 50 µl of TUNEL reaction mixture was put into each sample and incubated for 1 h at 37°C in the dark in a humidified chamber. Slides were then directly analyzed by fluorescence microscopy. For evaluation by fluorescence microscopy, we used an excitation wavelength in the range of 450–500 nm and detection in the range of 515–565 nm. As negative control, we incubated the samples in the absence of terminal deoxynucleotidyl transferase. For positive controls, the samples were first treated with DNase I (1,000 U/ml in 50 mM Tris–HCl, pH 7.5, 1 mg/ml BSA) for 10 min at 20°C to induce DNA strand breaks prior to labeling procedures and then incubated with 50 µl of TUNEL reaction mixture.

Total RNA Isolation and Reverse Transcription

MSCs were trypsinized and the total cellular RNA was isolated from the precipitate by using the RNeasy Mini kit

(Qiagen, Hilden, Germany) according to the manufacturer's protocol, with additional DNase treatment. The cDNAs were synthesized from the total RNA samples with the DyNAmo cDNA synthesis kit (Finnzymes, Espoo, Finland) according to the manufacturer's protocol.

RT-PCR

cDNA amplification for *bcl2, bcl-x_L, CD90, CD71, CD45, CD34, CD29, MMP12, GAPDH,* and β-Actin were performed by using DyNAzyme II (Finnzymes). The primers and conditions of amplification indicated in Table 1 and 2 respectively.

Protein Isolation and Quantification

MSCs were scraped from the cell culture plates in 1x PBS and the precipitate was treated with a lysis buffer containing 0.05 M Tris HCl, 1x protease inhibitor, 0.25 M NaCl and 1% (*v*/*v*) IGEPAL for 30 min on ice. Then the lysate was centrifugated for 20 min at 13,000 rpm and 4°C. Protein concentrations of supernatants were determined with Bradford protein assay as described [25].

Western Blotting

The proteins were separated on 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The membrane was blocked with blocking solution for 2 h at room temperature and was incubated in antibody solution at the concentration of 1:500 (for Bcl-x_L, Bak, Bcl-2, Bax); 1:200 (for ERα, ERß) and 1:1000 for Actin for o/n at 4°C. Then the secondary antibody, anti-Rabbit-HRP were applied for 1 h in blocking solution at a concentration of 1:1000. Finally, Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific, IL, USA) was applied to the membrane for 4 min and placed in an X-ray film cassette and developed. All antibodies, except for anti-Rabbit-HRP, were purchased from Santa Cruz (Santa Cruz, CA, USA).

Transfection of *bcl-2* and *bcl-x_L* shRNA

shRNA of *bcl-2* and *bcl-x_L* were purchased from Openbiosystems, (AL,USA) and prepared according to the manufacturer's protocol. 1 μg Bcl-2 and Bcl-x_L shRNA was added to 100 μL serum free, antibiotic free DMEM-LG (HyClone). In another tube 4 μL and 3 μL transfection reagent Dharmafect (Thermo Scientific) was diluted with 196 μL and 197 μL serum free antibiotic free DMEM-LG for Bcl-2 and Bcl-x_L respectively. After 5 min of incubation, the contents of shRNA and transfection reagent containing tubes were mixed and incubated at room temperature for 20 min. 1,200 μL antibiotic free complete DMEM-LG was added to the mixture. The cells were washed with 1x PBS and then the transfection mixture was added. DMEM-LG and transfection reagent only was given to the cells as two types of control. Next day the medium

Table 1 Primers and the product size of the genes

gapdh	forward	5′-CCTCCTCATTGACCTCAACTAC -3′	210 bp
	reverse	5′-CATGGTGGTGAAGACGCCAG-3′	
bcl-2	forward	5′-CCTGGCATCTTCTCCTTC-3′	584 bp
	reverse	5′-TGCTGACCTCACTTGTGG-3′	
bcl-x_L	forward	5′-TCAATGGCAACCCTTCCTGG-3′	346 bp
	reverse	5′-ATCCGACTCACCAATACCTG-3′	
beta actin	forward	5'-CTGGCCTCACTGTCCACCTT- 3'	65 bp
	reverse	5'-GGGCCGGACTCATCGTACT- 3'	
CD 90	forward	5′- CCAGTCATCAGCATCACTCT- 3′	374 bp
	reverse	5′- AGCTTGTCTCTGATCACATT- 3′	
CD 34	forward	5′- TGTCTGCTCCTTGAATCT -3′	281 bp
	reverse	5′- CCTGTGGGACTCCAACT- 3′	
CD 71	forward	5′- ATGGTTCGTACAGCAGCAGA- 3′	182 bp
	reverse	5′- CGAGCAGAATACAGCCATTG- 3′	
CD 29	forward	5′-ACTTCAGACTTCCGCATTGG -3′	190 bp
	reverse	5′- GCTGCTGACCAACAAGTTCA- 3′	
CD 45	forward	5′- ATGTTATTGGGAGGGTGCAA-3′	175 bp
	reverse	5′- AAAATGTAACGCGCTTCAGG-3′	
Mmp 12	forward	5′- GGCAACTGGACACCTCAACT -3′	382 bp
	reverse	5′- GTCCAGGTTTCTGCCTCATC -3′	

Table 2 PCR conditions for the genes

Genes	Initial denaturation	Denaturation	Annealing	Extension	Cycles	Final extension
gapdh	95°C, 5 min	94°C 45 sec	55°C 40 sec	72°C 40 sec	23	72°C, 5 min
bcl-2	95°C, 5 min	94°C 40 sec	63°C 35 sec	72°C 40 sec	36	72°C, 5 min
bcl-x_L	95°C, 5 min	94°C 30 sec	52°C 30 sec	72°C 30 sec	31	72°C, 5 min
beta actin	95°C, 5 min	94°C 40 sec	60°C 35 sec	72°C 40 sec	25	72°C, 5 min
CD 90	95°C, 5 min	94°C 30 sec	55°C 30 sec	72°C 30 sec	30	72°C, 5 min
CD 34	95°C, 5 min	94°C 30 sec	55°C 30 sec	72°C 30 sec	30	72°C, 5 min
CD 71	95°C, 5 min	94°C 40 sec	66°C 60 sec	72°C 40 sec	35	72°C, 5 min
CD 29	95°C, 5 min	94°C 30 sec	60°C 30 sec	72°C 30 sec	29	72°C, 5 min
CD 45	95°C, 5 min	94°C 30 sec	60°C 30 sec	72°C 30 sec	23	72°C, 5 min
Mmp 12	95°C, 10 min	94°C 40 sec	48°C 60 sec	72°C 40 sec	28	72°C, 5 min

was changed to their normal media. 4 days after transfection, RNA isolation and TUNEL assays were performed.

Adipogenic Differentiation

An adipogenic induction medium was prepared freshly by adding 1 μM dexamethasone (Sigma), 10 μg/ml insulin (Sigma), 100 μM indomethacin (Sigma) and 0.5 mM IBMX to LG-DMEM (HyClone), containing 1x penicillin-streptomycin (HyClone) and 10% FBS (HyClone). The MSCs were cultured for 21 days, changing the medium every 2 days with freshly prepared medium. After 21 days the cells were fixed with 4% paraformaldehyde and stained with Oil Red O (Sigma).

Osteogenic Differentiation

An osteogenic induction medium was prepared by freshly adding 0.1 μM dexamethasone (Sigma), 0.2 mM ascorbic acid 2-phosphate (Sigma) and 10 mM glycerol-2-phosphate (Sigma) to LG-DMEM (HyClone), containing 1x penicillin-streptomycin (HyClone) and 10% FBS (HyClone). The MSCs were cultured for 21 days, changing the medium every 2 days with freshly prepared medium. After 21 days the cells were fixed with 10% formaldehyde and stained with Von Kossa (Sigma).

Statistical Analysis

All data are expressed as means ± SD. Data were analyzed by performing paired *t*-test using Minitab Statistical Software ® (State College, Pennsylvania, USA). A value of $P<0.05$ was considered to be statistically significant.

Results

We first examined the characteristics of MSCs isolated from normal and ovariectomized rats and cultured in the absence or presence of 10^{-7} M estrogen (Fig. 1). We repeated our experiments three times with three animals per group. By using RT-PCR, we showed that all of the experimental groups were positive for mesenchymal stem cell markers (CD29, CD71 and CD90) and negative for hematopoietic cell markers (CD34, CD45) (Fig. 1a). We also examined the expression levels of ER α and ER β in our experimental groups. Our western blot data showed that both ER α and ER β were expressed in MSCs isolated from normal and ovariectomized rats (Fig. 1b). We also showed that upon estrogen addition, the expression of an estrogen responsive gene, MMP12 increased both in normal and ovariectomized animals (Fig. 1c).

We then investigated the effect of estrogen treatment on MSCs' CFU-F activities and differentiation capabilities (Fig. 2). The results of our CFU-F assay showed that estrogen treatment caused an increase in the colony numbers of MSCs that were isolated from both normal and ovariectomized rats (Fig. 2a). Moreover, the number of the cells in the colonies also increased when MSCs were treated with estrogen (Fig. 2a). When MSCs isolated from both normal and ovariectomized animals were put into adipogenic and osteogenic differentiation medium, they

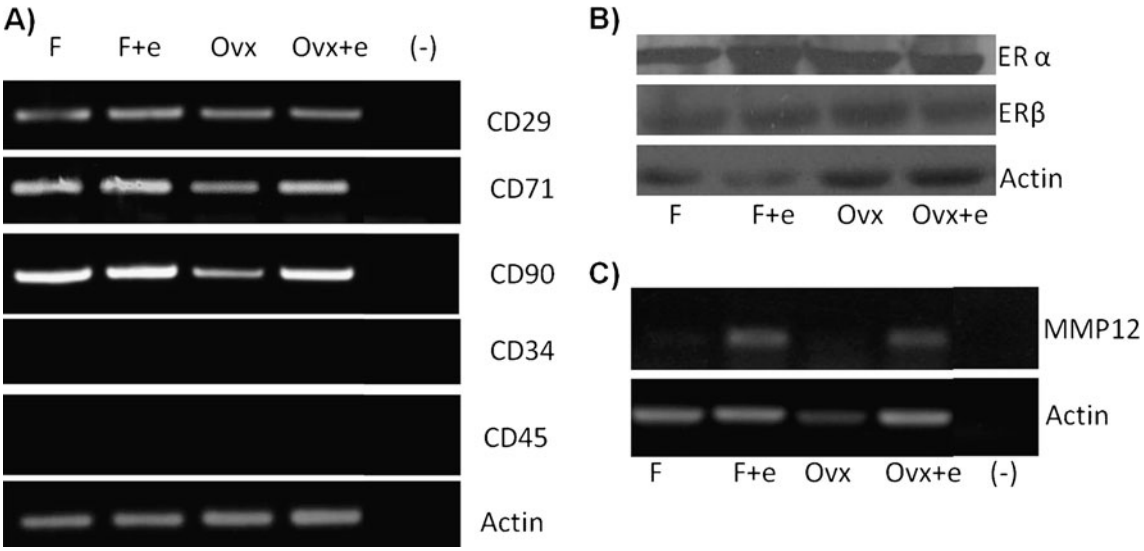

Fig. 1 Characterization of bone marrow-derived mesenchymal stem cells. **a** The expression of the markers of MSCs (CD90, CD29 and CD71) and hematopoietic cells (CD34, CD45) by RT-PCR. **b** Protein expression of ERα and ERβ determined by Western blot. Actin expression was used as loading control. **c** mRNA expression estrogen regulatory gene, MMP12 determined by RT-PCR. Actin expression was used as loading control. (F: MSCs obtained from female rats; F +e: MSCs obtained from female rats treated with estrogen; Ovx: MSCs obtained from ovariectomized rats; Ovx+e: MSCs obtained from ovariectomized rats treated with estrogen.)

were able to form adipocytes and osteocytes (Fig. 2b). Our results revealed that the capacity of differentiation into adipocyte lineage was to a lesser degree when MSCs were incubated in the presence of estrogen (Fig. 2b (*ii* vs *iv*)).

Since the cell number may be directly related to the involvement of apoptosis, we then examined the effect of estrogen on the apoptotic rate of MSCs (Fig. 3). Apoptotic rate was calculated as the ratio of TUNEL (+) cells to the

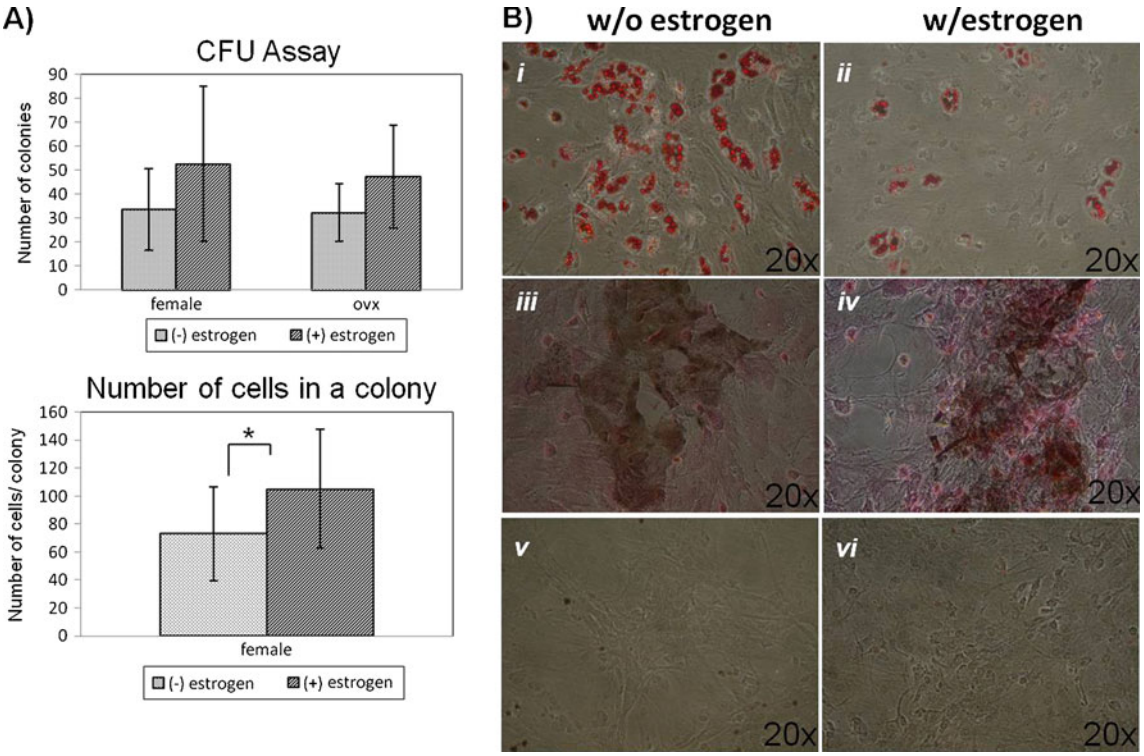

Fig. 2 Effect of estrogen on the bone marrow-derived mesenchymal stem cells. **a** CFU activities and the number of the cells in a colony * indicates $p \leq 0.05$; **b** Differentiation capacities of MSCs in the absence (*i, iii*) and presence (*ii, iv*) of estrogen. MSCs were stained with Oil Red O to determine adipogenic differentiation (*i, ii*), with Von Kossa staining to determine osteogenic differentiation (*iii, iv*)

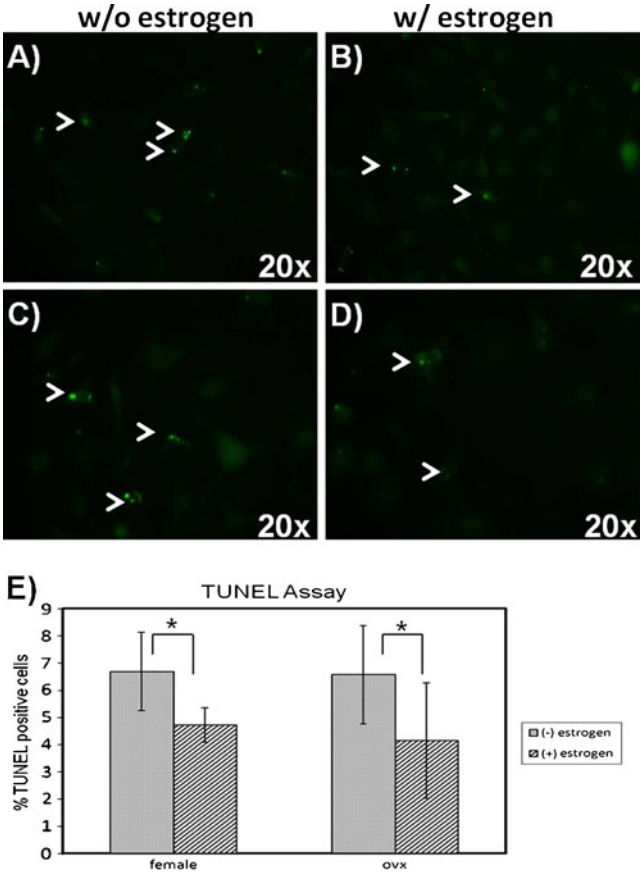

Fig. 3 In situ analysis of DNA fragmentation (TUNEL) of MSCs. MSCs were isolated from normal (**a**, **b**) and ovariectomized (**c**, **d**) rats. Cells were cultured in the absence (**a**, **c**) and presence (**b**, **d**) of estrogen. **e** Percentage of apoptotic cells. * indicates $p \leq 0.05$

total number of cells. Addition of estrogen decreased the number of TUNEL (+) MSCs isolated both from normal (Fig. 3a and b) and ovariectomized rats (Fig. 3c and d). Our results suggested that estrogen protects MSCs from apoptosis (Fig. 3e).

In order to understand the molecular mechanism of estrogen action on apoptosis, we focused on Bcl-2 family of proteins. We examined the expressions of both anti-apoptotic (Bcl-x_L, Bcl-2) and pro-apoptotic (Bak and Bax) members of this family (Fig. 4). Our western blot experiments revealed that addition of estrogen caused an increase in the expression of anti-apoptotic members of Bcl-2 family of proteins (Fig. 4a). On the other hand, we did not observe any change in the expression of pro-apoptotic members (Fig. 4b). We knocked down the expression of *bcl-2* and *bcl-x_L* by using shRNA to reveal that the anti-apoptotic effect of estrogen on MSCs was through these genes (Fig. 5a) and checked the effect of this silencing on the apoptotic rates of MSCs determined by TUNEL assay (Fig. 5b,c). Our results showed that the decreasing effect of estrogen on apoptosis in non-transfected MSCs reversed after transfecting the cells with shRNA of either *bcl-2*or *bcl-x_L*. The percentage of the apoptotic cells increased when *bcl-x_L* or *bcl-2* shRNA transfected cells cultured even in the presence of estrogen (Fig. 5b and c respectively).

Discussion

Stem cells are the new hope for the humans 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 [26]. Estrogen also influences the quality of life both in men and women and is a strong candidate in the maintenance of life standards. Estrogen has important effects on blood vessels, neurotransmitter release, sexual behaviour, bone structure, cognitive function and spermatogenesis and regulates many pathways vital for the homeostasis [27, 28]. Therefore our understanding of its importance should not be limited only to the reproductive system. In this study, we aimed to understand the role of estrogen on the MSCs which emerge as an important player of cellular-based therapies due to lack of immunogenicity and ethical problems.

Since we focused on the role of estrogen on MSCs, we first examined the expression status and functionality of ER α and ER β on these cells (Fig. 1b and c). Bone marrow is one of the target tissues of estrogen and osteoblasts,

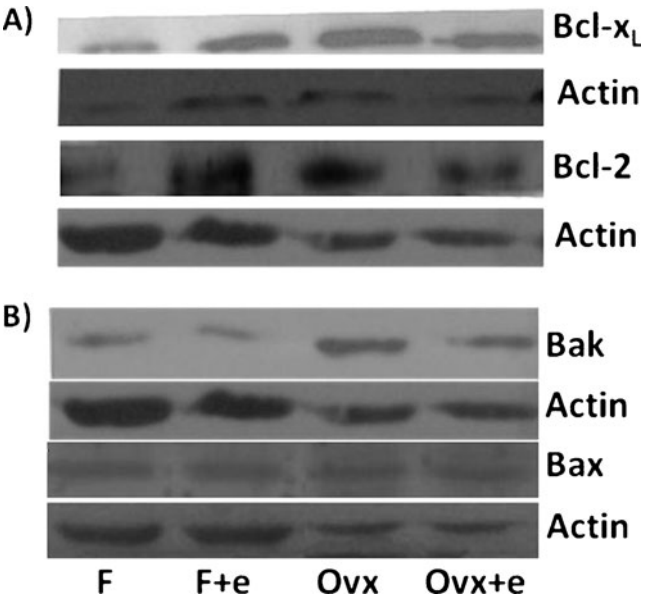

Fig. 4 Expression of **a** anti-apoptotic and **b** pro-apoptotic members of Bcl-2 family of proteins in MSCs determined by Western blot. Actin expression was used as loading control (F: MSCs obtained from female rats; F+e: MSCs obtained from female rats treated with estrogen; Ovx: MSCs obtained from ovariectomized rats; Ovx+e: MSCs obtained from ovariectomized rats treated with estrogen)

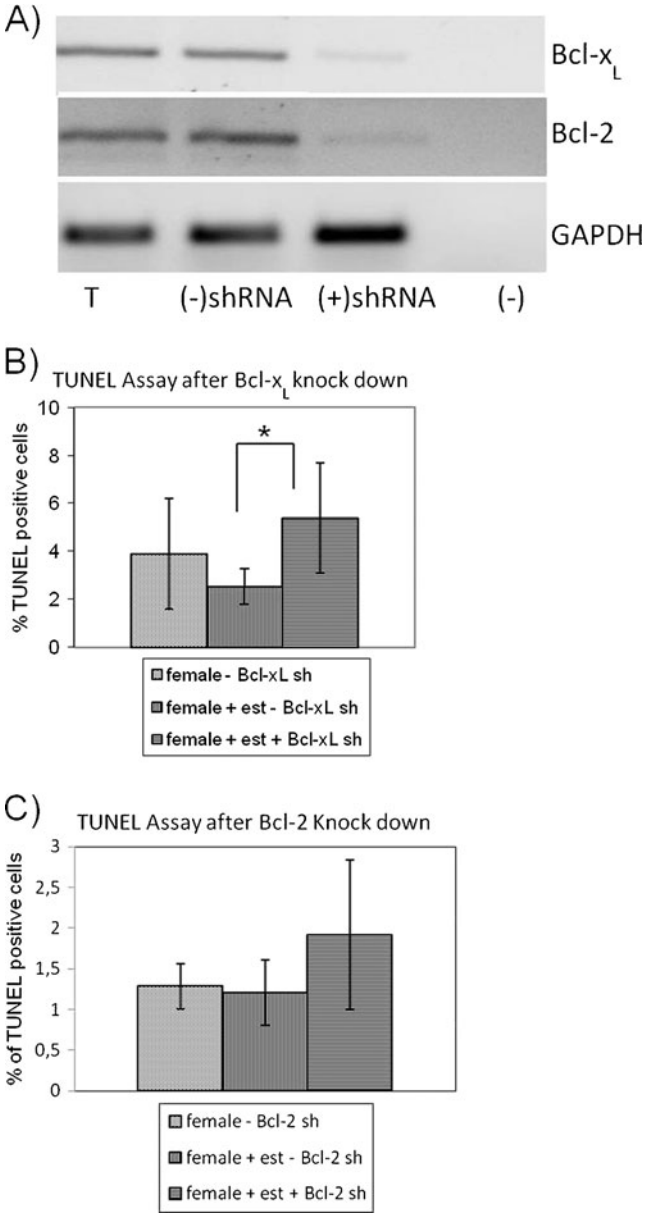

Fig. 5 Effect of silencing the expression of *bcl-x_L and bcl-2* on the apoptotic rates. **a** Expression of *bcl-2 and bcl-x_L* determined by RT-PCR in MSCs transfected with and without shRNA of *bcl-2 and bcl-x_L*. T: control vector transfection. Expression of GAPDH was used as loading control. **b** Percentage of apoptotic cells after MSCs were transfected with *bcl-x_L* shRNA determined by TUNEL assay. * indicates $p \leq 0.05$. **c** Percentage of apoptotic cells after MSCs were transfected with *bcl-2* shRNA determined by TUNEL assay

osteocytes, osteoclasts, T cells and B cells in human and mouse bone marrow express both ERα and ERβ [29]. Our results showed that rat bone marrow-derived MSCs also express ERα and ERβ (Fig. 1b). Our data regarding to the expression of estrogen regulated gene, MMP12 [30], suggest that these receptors are functional. MSCs isolated both from normal and ovariectomized rats increased the expression of MMP12 upon estrogen addition (Fig. 1c).

Estrogen addition to the culture media increased the number of colonies both in the normal and ovariectomized group which suggests that estrogen has an increasing effect on the number of functional MSCs both in normal and estrogen deprived conditions (Fig. 2). In addition the number of cells in a colony was more when cells were treated with estrogen. These results are also in accordance with a previous study which showed that the number of MSCs obtained from ovariectomized animals was increased upon estrogen addition [31].

In addition, we observed that the differentiation potency of MSCs into adipocyte but not to osteocytes could be affected with estrogen. Estrogen has been shown to have an important role on adipocyte metabolism [32] and our results provided support that this effect might also be at the level of differentiation. It has been reported that MSCs from osteoporotic post-menopausal women showed an increase in PPARγ expression and thus an increase in adipocytes [33]. In addition, serum from post menopausal in comparison to serum from pre-menopausal women, were able to induce adipogenic differentiation in a human bone progenitor cell clone (hOP 7) derived from bone marrow [34]. Therefore, in the absence of estrogen, MSCs' adipocyte differentiation capacity increased suggesting that estrogen influences adipogenesis and this may explain why post-menopausal women gain weight and develop diseases associated with obesity. Molecular mechanism of this effect in MSCs is not known and clearly warranted new experiments.

The proliferative effect of estrogen has been shown on rat bone marrow-derived MSCs before [35]. In contrast to proliferation, the effect of estrogen on the maintenance of rat bone marrow-derived MSCs from normal animals have not been reported. We examined the effect of estrogen in the apoptotic rates of MSC by in situ cell death assay. When the ratios of apoptotic cells to total cells in the colonies were compared, the addition of estrogen decreased the death ratios in the normal and ovariectomized groups suggesting that estrogen is effective in the process of MSC colony formation both by decreasing apoptosis in addition to increasing proliferation. It is important to note that in our system, we did not induce apoptosis with any factor therefore we evaluated the spontaneous physiological apoptosis of bone marrow-derived MSCs.

In order to understand the molecular mechanism of the decrease in apoptosis upon estrogen administration, we focused on the Bcl-2 family of proteins. The Bcl-2 protein family consists of both pro- and anti-apoptotic members. The founder of the family, Bcl-2, was first identified in 1985 as the part of the most common translocation in human B-cell lymphoma [36]. After the identification of Bcl-2 as a survival factor, many different homologues were identified and characterized [37]. Recent reports established the effect of estrogen on the expression of Bcl-2 family of

proteins in other systems [19, 23, 24]. Our immunoblotting and knock down data also suggested that estrogen controls the apoptosis by regulating the expression of Bcl-2 family of proteins in rat MSCs (Fig. 4). These results are in correlation with other studies performed on other stem cells like skin stem cells, HSCs, and epithelial stem cells where apoptosis and members of the *bcl-2* family of genes were shown to be important in the differentiation and regulatory pathways [38–40]. When we cultured MSCs in the presence of estrogen, the expression of anti-apoptotic Bcl-2 and Bcl-x_L were increased. This effect was more evident when MSCs were isolated from normal female rats than the ovariectomized rats. The Bcl-2 protein expression did not increase in the ovariectomized group, which might be explained by the loss of estrogen susceptibility due to a long period in an estrogen deprived environment. On the other hand, the expression of proapoptotic Bak and Bax proteins did not change with estrogen treatment. To confirm the role of anti-apoptotic Bcl-2 family of proteins on MSCs' maintenance, we silenced the expression of Bcl-2 and Bcl-x_L. Our data showed that upon knocking down the expression of these genes, we observed the inhibition of apoptosis (Fig. 5) and CFU-F capacity of (data not shown) in MSCs isolated from normal and ovariectomized rats, suggesting a critical role for these genes in the estrogen regulation of apoptosis.

In conclusion, we have shown that apoptosis is one of the main regulatory mechanisms in the maintenance of MSCs. Bcl-2 and Bcl-x_L are important proteins in this regulation and estrogen affects the expression rates of these proteins, increasing the colonies and decreasing the apoptotic rate. Modulation of with estrogen may help to obtain more MSCs which might be important for clinical applications.

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Conflict of Interest The authors declare no potential conflicts of interest

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