EFFECT OF ESTROGEN ON APOPTOTIC REGULATORY MECHANISMS IN MESENCHYMAL STEM CELL MAINTENANCE

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ABSTRACT

EFFECT OF ESTROGEN ON APOPTOTIC REGULATORY MECHANISMS

IN MESENCHYMAL STEM CELL MAINTENANCE

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Mesenchymal Stem Cells (MSCs) can both self-renew and differentiate into fat, bone, cartilage, and muscle. They have a high therapeutic value due to their differentiation potential and nonimmunogenic characteristics, however their rareness and duration of their culture are the main handicaps in their application in cell-based therapies. Therefore, our aim was to explore the possible mechanisms that are involved in MSC maintenance and proliferation by using rat MSCs as a model. We studied the effect of estrogen on MSCs due to its role in growth regulation, differentiation, and cellular proliferation. In MSCs isolated from both normal and ovariectomized animals, the number and the CFU activity were increased when cultured with estrogen. To reveal the mechanism of the action of estrogen on MSC maintenance, we investigated the apoptotic pathway since estrogen has been shown to have a detrimental effect on apoptosis in other systems. The number of apoptotic cells decreased when MSCs were cultured in the presence of estrogen. To elucidate the molecular mechanism of estrogen's effect on MSC apoptosis, we examined the expression of the *bcl-2* family of genes. The expression of anti- apoptotic Bcl-2 and Bcl-x_L proteins increased in the presence of estrogen, whereas the expression of pro-apoptotic Bak decreased. Our results clearly show that estrogen increases the number of the functional MSCs by differentially regulating the expression of the *bcl-2* family of genes and inhibiting apoptosis. Therefore estrogen treatment of MSCs may offer a potential to increase the number of MSC for treatments.

Keywords: Mesenchymal Stem Cells, estrogen, *bcl-2* family of genes, rat

ÖZET

ÖSTROJEN HORMONUNUN APOPTOZ YOLAKLARI ARACILIĞIYLA MEZENKİMAL KÖK HÜCRELERİNİN MUHAFAZA EDİLMELERİNDEKİ ETKİSİ

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Mezenkimal Kök Hücreler (MKH), kendilerini yenileyebilmelerinin yanı sıra yağ, kemik, kıkırdak, ve kas hücrelerine farklılaşabilmektedirler. Farklı hücre tiplerine dönüşebilme kapasitelerinin yanı sıra alıcıda reddedilme sorunu olmaması, MKH'leri birçok hastalığın tedavisinde bir umut haline getirmiştir. Ancak sayıca az olmaları ve elde edilmelerindeki uzun süreç, MKH kullanılarak yapılacak olan hücre temelli tedavilere engel oluşturmaktadır. Bu sebeple amacımız MKH'lerin çoğalmaları ve muhafaza edilmelerindeki ana mekanizmaları, sıçan MKH'lerini model olarak kullanarak araştırmaktı. Östrojenin farklılaşmadaki, büyümedeki ve çoğalma kapasitesi üzerindeki etkisi göz önüne alınarak, MKH'ler üzerindeki rolü araştırıldı. Normal ve yumurtalıkları alınmış sıçanların MKH'lerinin sayılarının ve koloni oluşturma özelliklerinin bulundukları hücre kültürü ortamına östrojen eklenmesiyle beraber her 2 grupta da arttığı görüldü. Östrojenin MKH'lerin çoğalma katsayılarına etki eden mekanizmasını bulmak amacıyla, diğer sistemlerde östrojenin etkilediği bilinen apoptoz yolakları araştırmaya alındı. Apoptoza uğrayan MKH'lerin hem normal hem de yumurtalıkları alınmış gruplarda östrojen eklenmesiyle azaldığı gözlemlendi. Östrojenin MKH'ler üzerindeki etkisini sağlayan moleküler mekanizmayı açıklamak amacıyla östrojenin etkilediği bir molekül grubu olan *bcl-2* gen ailesi üyelerinin ifadesi incelendi. Anti-apoptotik Bcl-2 ve Bcl-x_L proteinlerinin ifadeleri östrojenin varlığında artarken pro-apoptotik Bak proteininin ifadesi azaldı. Sonuçlarımıza göre östrojen, fonksiyonel MKH'lerin sayılarını *bcl-2* gen ailesi üyelerinin ifadelerini düzenleyerek ve apoptozu engelleyerek artırmaktadır. Bu sebeple MKH'lerin östrojene maruz bırakılması, onların olası tedavilerde kullanılmaları açısından önemli bir potansiyel oluşturmaktadır.

Anahtar Kelimler: Mezenkimal Kök Hücreler, östrojen, *bcl-2* gen ailesi, sıçan

TO MY FAMILY

FOR BEING MY SHOULDER TO CRY AND LAUGH

AND ALWAYS BEING THERE FOR ME,

AND TO DERYA KARA

FOR BELIEVING IN ME AT ALL TIMES

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ABBREVIATIONS

AIF	Apoptosis Inducing Factor
AML	Acute Myeloid Leukemia
AP1	Activator Protein 1
APAF1	Pro-Apoptotic Protease-Activating Factor-1
ASC	Adult Stem Cell
bFGF	Basic Fibroblast Growth Factor
bp	Base Pairs
BH	Bcl-2 homology
BMP4	Bone Morphogenic Protein 4
BSA	Bovine Serum Albumin
cDNA	Complementary Deoxyribonucleic Acid
ddH_2O	Double distilled water
DEPC	Diethylpyrocarbonate
dH_2O	Distilled Water
DIABLO	Direct IAP binding protein with low pI
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
EB	Embryoid Bodies
EGC	Embryonic Germ Cells
EGF	Epidermal Growth Factor
ER	Estrogen Receptor
ERE	Estrogen Response Elements
ESC	Embryonic Stem Cell
GSC	Germ-line Stem Cell
HGF	Hepatocyte Growth Factor
HSC	Hematopoietic Stem Cell
IAP	Inhibitor of Apoptosis

µg	Microgram
µl	Microliter
LIF	Leukemia Inhibitory Factor
mM	miliMolar
M	Molar
MEF	Mouse Embryonic Fibroblast
MetOH	Methyl Alcohol
MSC	Mesenchymal Stem Cell
NaCl	Sodium Chloride
NGF	Nerve Growth Factor
OD	Optical Density
OVX	Ovariectomy
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PGC	Primordial Germ Cell
RNA	Ribonucleic Acid
RT	Room Temperature
RT-PCR	Reverse-Transcriptase Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
Smac	Second Mitochondria-Derived Activator of Caspases
TGF-ß1	Transforming Growth Factor-Beta 1

1. INTRODUCTION

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

In this respect, estrogen which is one of the most crucial hormones influencing the life quality of both men and women is a strong candidate in the maintenance of life standards. It is mainly responsible for female sexual features such as breast development and the menstrual cycle, but it is also involved in the regulation of several other mechanisms. Estrogen has an important effect on blood vessels, neurotransmitter systems and other aspects of the central nervous system (http://www.isoflavones.info/estrogen.php), sexual behaviour, bone structure, cognitive function (Simpson, 2003), and spermatogenesis (Simpson *et al*, 1999). When the estrogen levels decrease and menopause starts in women, osteoporosis and arteriosclerosis become the main problems due to estrogen's role in bone mineralization and prevention of osteoporosis. After menopause, osteoporosis is more common in women than in men. Estrogen also decreases the possibility of becoming an Alzheimer's patient (Yaffe *et al*, 1998). Alzheimer's disease incidence is greater among women than among men mainly due to the decrease in the levels of estrogen after menopause (Simpson *et al*, 1999). Coronary heart disease increases double to threefold in women after menopause and it increases even more in case of the removal of ovaries prior to menopause (Skafar *et al*., 1997). These data drive attention to the importance of estrogen and due to this reason doctors tend to prescribe hormone based products as therapies for menopause

(http://www.isoflavones.info/estrogen.php). This indicates that estrogen has a physiologic significance throughout adult life and our understanding of its importance should not be limited only to the reproduction system.

Stem cells, which are an evolving research topic, are another future hope for therapeutic applications. They are important for living organisms since they carry a potential for differentiating into a wide variety of cells under certain circumstances. Stem cells give rise to multiple specialized cell types which make up the heart, lung, skin, and other tissues; besides replacing cells that are damaged (http://stemcells.nih.gov). Stem cells can also be used in regenerative medicine, gene therapy, tissue repair, and patient specific stem cell therapy (Ivanova *et al*, 2002). There are many disorders, injuries, and diseases which can be cured or at least has the potential to be cured by the use of stem cells; such as Alzheimer's disease, spinal cord injuries, Parkinson's disease, diabetes, Huntington's disease, and different types of cancer (Fuchs *et al*, 2004). Each day new discoveries are being made on the subject.

Estrogen and stem cells, both being key factors in the preservation of life standards, are related with each other. Considering the protective effect of estrogen against severe heart, bone, and neuronal diseases combined with the refining and repairing characteristics of stem cells; they are future promise for many diseases. To understand 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 due to their estrogen responsive genes and their role in stem cell maintenance.

In this context, first stem cells will be explained with their features, types, and importance. Then the next part of the thesis will be based on cell death, apoptosis, and their mechanism of regulation. Finally estrogen will be explained together with its effects on stem cells, apoptotic molecules, and its role in regulating stem cell maintenance. Particularly MSCs which are vital in the formation of several tissues will be focused on, and their regulation through estrogen via pro-apoptotic and anti-apoptotic protein families will be defined.

1.1. Stem Cells

Stem cells are unspecialized cells which are capable of self-renewal and give rise to differentiated cells (Till and Mcculloch 1961). It is still not clear how they remain undifferentiated in their microenvironment. The internal signals which are regulated by genes, and the external signals which consist of secreted factors, and interactions among cells are the main factors stimulating the differentiation of stem cells. They are found in various sources such as the embryo, bone marrow, blood, cornea and retina of the eye, brain, skeletal muscle, dental pulp, liver, skin, lining of the gastrointestinal tract, and pancreas. Although self-renewal and differentiation to other cells are their common features; stem cells vary in their potential to differentiate, duration and pathways of self-renewal, places they are mostly found in, and division properties (Morrison *et al*, 1997).

Stem cells can be classified due to their source of origin and their potential to differentiate to different cell types. There are three main types of stem cells: ESCs, GSCs, and ASCs. When the oocyte is fertilized, it becomes a zygote which has the capacity to give rise to the embryo and extraembryonic membranes. The zygote is the only totipotent cell, which can differentiate to any type of cell and form the embryo. When the embryo becomes 4-5 days old, ESCs are derived from the inner cell mass of the embryo (Brook and Gardner, 1997). They are pluripotent and can give rise to cells from all three germ layers: endoderm, mesoderm, ectoderm (Thomson *et al*, 1998). GSCs are found in the gonadal ridge of the 5 -10 week fetus as primordial germ cells. They are pluripotent when cultured *in vitro* and form EGCs (Xi *et al*, 2005). ASCs on the other hand are found in specific tissues and organs. They are multipotent and give rise to more than a single cell type but are not capable of differentiating into tissues of all germ-layers (Figure 1.1) (Serakinci and Keith, 2006).

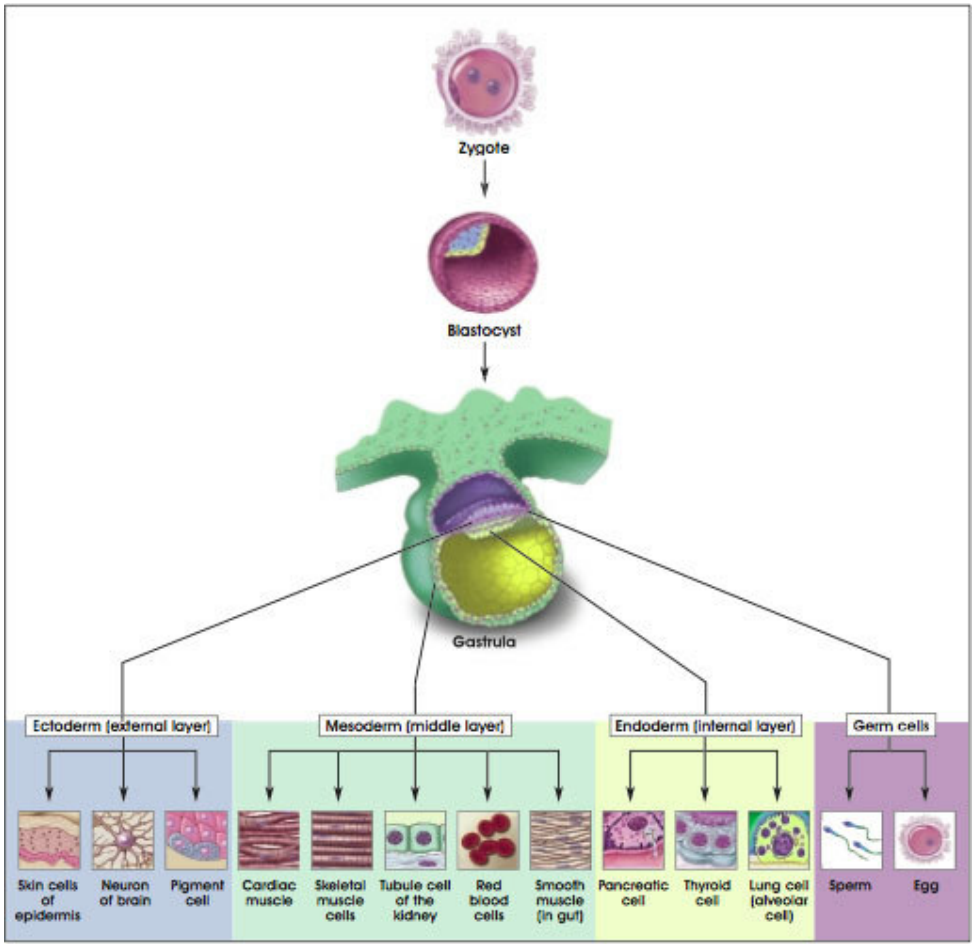


Figure 1.1 The differentiation of cells from the zygote (http://stemcells.nih.gov)

1.1.1. Embryonic Stem Cells

ESCs were first isolated from the inner cell mass of the mouse in 1981 by Kaufman and Martin, and from humans in 1998 by Thomson *et al*. ESCs are not embryos themselves but they can form cells from all three germ-layers and undergo an unlimited number of symmetrical divisions without differentiating *in vitro* (Burdon *et al*, 2002). In order to generate cultures of mouse and human ESCs, the inner cell mass is removed from the trophectoderm and is transferred to the top of MEFs on the culture medium. These MEFs which are called feeder layer are inactivated so that they can not divide, but they provide a sticky surface for the ESCs to attach besides releasing essential factors to the culture medium. Both mouse ESCs

and human ESCs can be grown both with and without a feeder layer (Xu *et al*, 2001; Reunbinoff *et al*, 2000). A single ESC can give rise to a colony of genetically identical cells, or clones under appropriate conditions (Sell, 2003) (Figure 1.2).

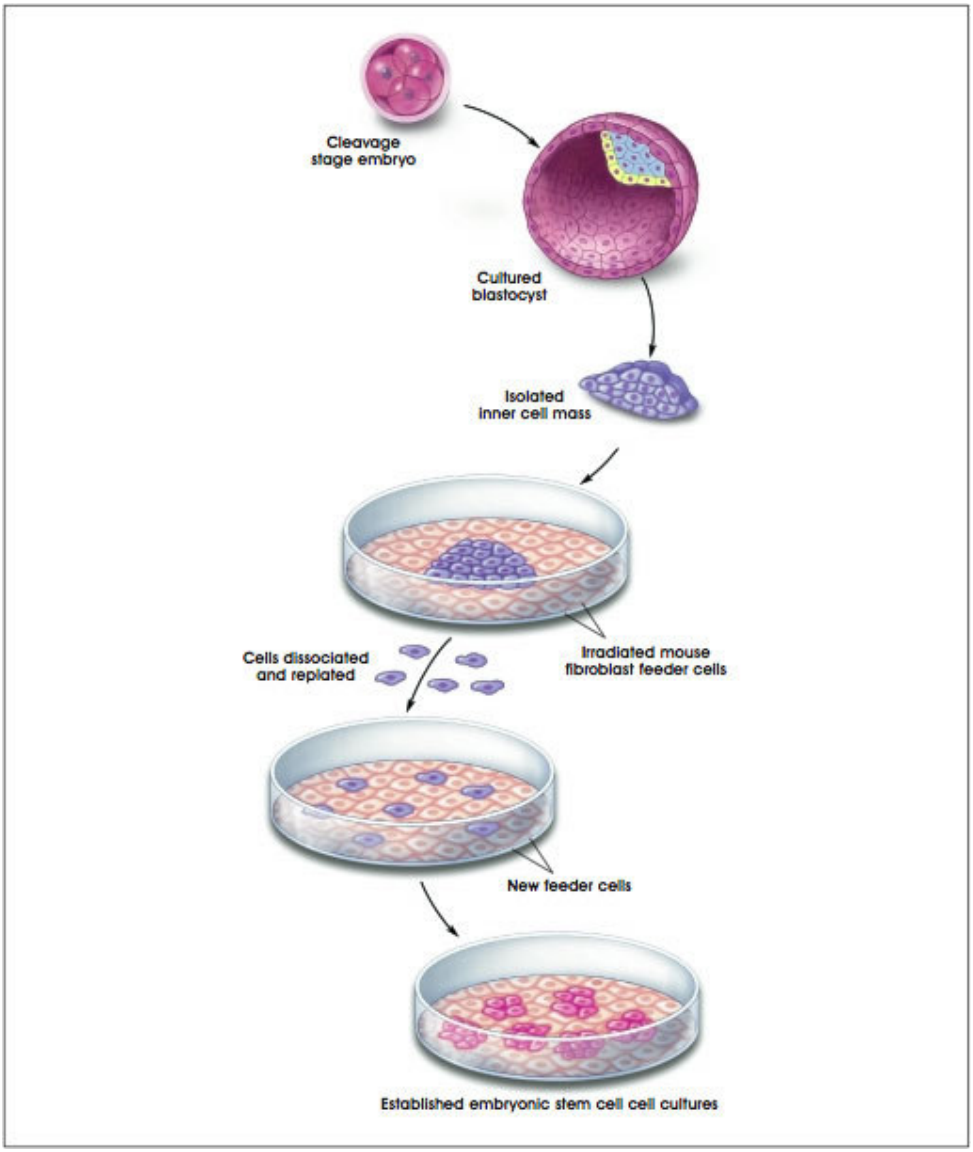


Figure 1.2 Tissue culture methods of ESCs (http://stemcells.nih.gov/**)**

ESCs can stay undifferentiated for a long period of time *in vitro,* and there are several examples to prove the pluripotentiality of ESCs. When mouse ESCs derived from one blastocyst are injected to another blastocyst and is transferred to the uterus of a pseudopregnant mouse *in vivo*, chimeras form which are a mixture of tissues and organs derived both from the host and the donor blastocyst (Prelle *et al*, 1999). Also when ESCs are injected into adult immune-deficient mice, they develop into tumors called teratomas which contain cells from all three germ layers (Martin, 1981). *In vitro* when the culture conditions are changed, ESCs form embryoid bodies (EB) which have a similar structure to teratomas. They form large structures which contain partially differentiated cells from all three germ layers in a disorganized manner (Evans and Kaufman, 1981). Both mouse and human ESCs express *oct-4* which is an important gene in the maintenance of pluripotency. Also with the induction of certain growth factors, ESCs differentiate into different types of cells. In order to induce ectodermally derived cells: retinoic acid, EGF, BMP4, and bFGF are added; to induce mesodermally derived cells: activin-A, and TGF-ß1 are added; and in order to induce differentiation into all three germ layers including the endoderm: HGF, and NGF are added to culture (Bishop *et al*, 2002). These indicate that ESCs are pluripotent and have the potential of contributing to the formation of cells from all germ layers both *in vitro* and *in vivo.*

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

ESCs can be used in several ways in basic and clinical research. Studies on ESCs are very informative on the complex events that occur during human

development. Understanding the genetic and molecular control mechanisms behind the stem cell regulation may provide an understanding on how genetic and growth abnormalities arise and suggest new strategies and methods for their therapy.

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

1.1.2. Embryonic Germ Cells

EGCs were first identified in 1998 by John Gearheart and they were cultured from PGCs obtained from the gonadal ridge and mesentery of the 5th to 9th week of fetal tissue (Figure 1.3). They are pluripotent cells and express *oct-4* besides other pluripotency markers similar to ESCs, and they have the capacity for long-term self-renewal. In addition they have a normal stable karyotype (Shamblott *et al*, 1998). The location and maintenance of germ-line stem cells are among the most studied areas and are clearly identified. Especially *Drosophila* is the best studied model in this area both in male and in female (Gilboa and Lehmann, 2004).

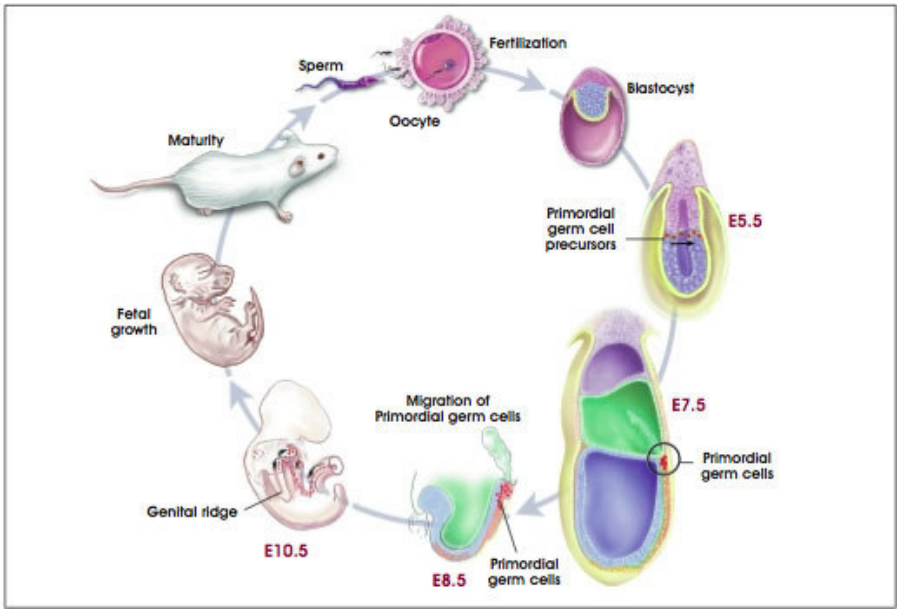


Figure 1.3 The role of primordial germ cells in life cycle
(http://stemcells.nih.gov/info/scireport/appendixA.asp)

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

Although both are pluripotent, EGCs do not have the same proliferation and differentiation capacity as ESCs do, which makes EGCs less appealing for therapeutic applications. Also they do not form teratomas when injected to

immunosuppressed mouse, and they do not participate in the formation of chimeras *in viv0;* which suggest that EGC's differentiation potential is not as wide as ESC's *(*Shamblott *et al,* 1998). In addition there is not much known about their telomerase activity either, which might be a reason why their doublings are shorter than ESCs (http://stemcells.nih.gov/).

1.1.3. Adult Stem Cells

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

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

The number of ASCs is rare compared to other types of cells and they do not divide very often unless there is a stimulus such as tissue injuries or diseases. ASCs are scattered throughout the tissues of the mature organism and have very different roles depending on their local environment. Currently, cell surface markers and observations on stem cell differentiation patterns in test tubes and culture dishes are being used for the characterization of adult stem cells. It is also hard to expand ASCs

in culture in an undifferentiated state. Many factors including growth factors, extracellular matrix contacts, cell-cell interactions, and intrinsic cell kinetics (Sherley, 2002) effect their maintenance and differentiation.

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

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

ASCs can be used in the generation of cells and tissues in cell-based therapies**.** The organs and tissues donated are often used to replace malfunctioning or destroyed tissues, but the need for transplantable tissues and organs is a lot more

compared to the available supply. ASCs offer the possibility of a renewable source for replacement of cells and tissues to treat several diseases. ASCs are potential future treatments for various diseases such as cardiac disorders, diabetes, Alzheimer disease and many others (http://stemcells.nih.gov/).

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

1.1.3.1 Mesenchymal Stem Cells

MSCs are adult stem cells mostly found in the stromal part of the bone marrow. They were first identified in 1966 by Friedenstein and Patrakova by the isolation of bone-forming progenitor cells from the rat bone marrow (Barry and Murphy, 2004). They can self-renew as all stem cells do, and have a multilineage differentiation potential. They can differentiate into osteoblasts, chondrocytes, and adipocytes, myocytes which form the bone, cartilage, tendon, muscle, and fat. They are thought to differentiate into certain types of endodermal and ectodermal tissues as well such as neurons and endothelial cells. In addition they are a part of the hematopoietic stem cell niche (Figure 1.4) (Baksh *et al*, 2004; Caplan and Bruder, 2001).

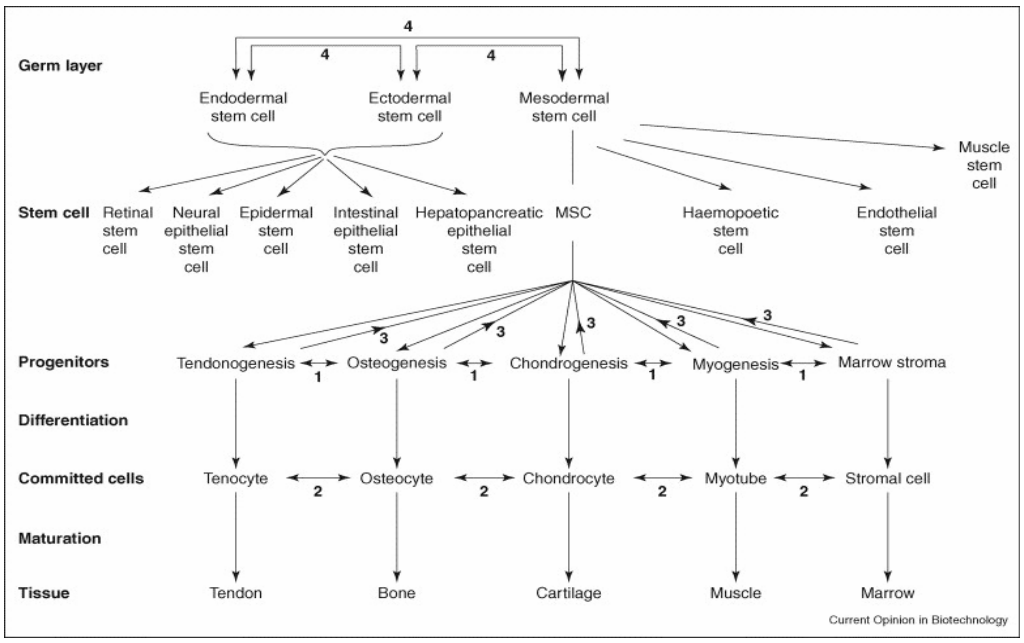


Figure 1.4 The differentiation of MSCs (Raghunath *et al*, 2005)

Although the main source of MSCs is the bone marrow, they can be isolated from other sources as well such as the trabecular bone (Noth *et al*, 2002), adipose tissue, synovium, skeletal muscle, lung, placenta, deciduous teeth (Tuan *et al*, 2003), human umbilical cord perivascular cells (Sarugaser *et al*, 2004), and periosteum (Minguell *et al*, 2001). Among the MSCs obtained from different sources, there is not much difference by means of yield, growth kinetics, cell senescence, multi-lineage differentiation capacity, and gene transduction capacity (De Ugarte *et al*, 2003). Although it is known that MSCs exist in many sources, their niche and the structure of their niche is not clear. HSC and MSC niche can be thought to be the same since both of them are found mostly in the bone marrow, but this is not the case because they are very different from each other and need to remain in different environments with different signals in order to stay undifferentiated. Also MSCs are found in many different sources, so the niches of HSCs and MSCs have to be different (Baksh *et al*, 2004).

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

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

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

There is no *in vivo* marker to distinguish MSCs from other cell types but *in vitro* STRO-1 (Minguell *et al*, 2001), SB-1, SH2, SH3, SH4 (Baksh *et al*, 2004) identify MSCs in humans and CD71, CD90, CD106, and CD117 identify them in rodents (Mangi *et al*, 2003). In general, it is hard to find specific markers for MSCs since they have common characteristics with other types of cells such as endothelia,

epithelial cells, and muscle cells. It is known though that they do not express CD45, CD34, and CD14 which are HSC markers (Baksh *et al*, 2004), and they mostly express a large spectrum of cell adhesion molecules which are important for cell binding and homing such as integrin markers alpha1, alpha5, and beta1 (Barry and Murphy, 2004).

Although MSCs might be thought as a homogeneous population which has a multilineage potential, they are a very heterogeneous population due to the complex regulatory mechanisms which are not fully revealed at the moment. Only a small proportion of MSCs are actively proliferating, and approximately 10% of them are at S + G2 + M stages. However the majority of them are at the Go/G1 phase of the cell cycle, which suggests that MSCs have a high potential to differentiate (Conget and Minguell, 1999). There are many molecules taking part in MSC regulation such as cytokines, growth factors, adhesion molecules, and extracellular matrix components (Kratchmarova *et al*, 2005). The master switches regulating the transitions from stem cells to differentiated cells, and the genes active in specific differentiation patterns are still not known. The reason for the difficulty to find these regulatory genes is their temporal activity, the reaction given to inductive molecules, and the varying differentiation pathways between organisms (Baksh *et al*, 2004; Caplan *et al*, 2001). MSC markers and colony forming assay are accepted currently to be sufficient to identify MSCs.

MSCs are already used in therapies for osteogenesis imperfecta, hematopoietic recovery, bone tissue regeneration (Baksh *et al*, 2004), cardiovascular repair, treatment of lung fibrosis, spinal cord injury, coronary artery disease (Minguell *et al*, 2001), local repair, and regeneration of bone cartilage and tendon. They can form intervertebral disc cartilage, bone, cardiomyocytes, articular cartilage at knee joints, besides neurons, skin epithelia, lung, liver, kidney, intestine, and spleen (Figure 1.5) (Baksh *et al*, 2004).

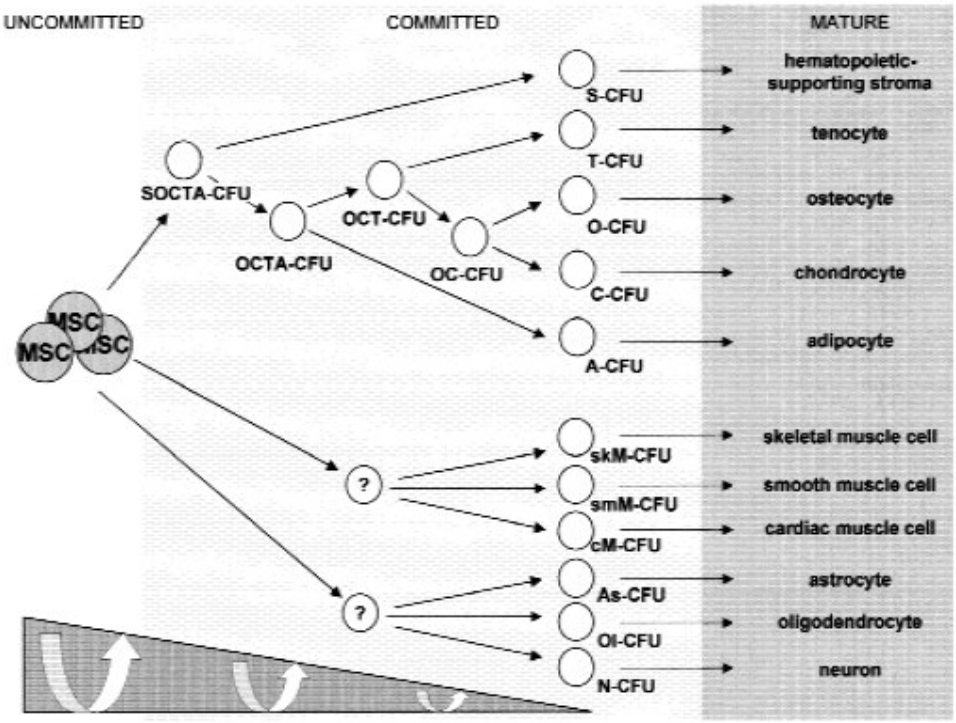


Figure 1.5 The differentiation and plasticity of MSC (Minguell *et al*, 2001**)**

Aside from their differentiation potential and immunesuppression, there are other aspects which make MSCs valuable for therapeutics. MSCs actively inhibit T-cell proliferation and due to this reason they are considered as nonimmunogenic or hypoimmunogenic which is important in host response to allogeneic MSC therapy. The only problem of this aspect is a potential disease transmission from donor to recipient. Another important feature of MSCs is long-term homing and engraftment of implanted cells to various tissues even after the development of immunocompetence. The implanted MSCs can migrate to the specific site of injury by the help of factors that are released from the wound. This has been observed in cases like bone fractures, myocardial infarction, meniscus, and ischemic cerebral injury. Also at specific sites it has been observed that the stem cells injected differentiate according to the local signals at the local site of injury (Miguell *et al*, 2001). MSCs have a high potential for regenerative medicine and tissue engineering besides being used as a gene delivery method, which makes their therapeutic value

very high. Researchers are looking for different ways to use specialized cells derived from MSCs in targeting specific cancerous cells and delivering treatments that will destroy them or make them benign.

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

1.2. Cell Death and Apoptosis

Cell death occurs through two main pathways: necrosis which is accidental cell death and apoptosis which is programmed cell death. These two pathways vary by means of their morphological and biochemical features besides the stimuli to induce them. Necrosis is non-programmed cell death which occurs abruptly when the cell is damaged. The cells swell, organelles dissolve, the plasma membrane ruptures, and the cytoplasmic content gets released which causes an inflammatory response (Desagher and Martinou, 2000). Apoptosis on the other hand, is a gradual and programmed cell death. The cells undergo morphological changes such as nuclear and cytoplasmic hypercondensation, cell shrinkage, plasma membrane blebbing, DNA fragmentation, and cleavage of chromosomes into nucleosomes. Apoptotic bodies form at the end of this process which contains components of dying cell and are removed by phagocytosis preventing the release of cytoplasmic and nuclear components to the extracellular space (Desagher and Martinou, 2000; Shi, 2001). Apoptosis is crucial for homeostasis and is involved in the regulation of several pathways.

1.2.1. Apoptosis and its Evolutionary Conservation

Apoptosis is a cell suicide mechanism which enables organisms to keep their cell number under control and eliminate the cells which have deficiencies such as

genetic defaults or threaten the organism's survival (Ashkenazi and Dixit, 1998). It can be triggered by external and internal death signals and it participates in diverse mechanisms such as embryonic development, tissue morphology determination, and creation of the neural and vascular network. The regulation of apoptosis is complicated and since it is conserved throughout evolution, is preserved very strictly. Any disruption in the apoptotic pathways cause many disorders such as autoimmune diseases, neurodegenerative diseases like Alzheimer's disease, cancer, viral infections, osteoporosis, and AIDS (Green and Martin, 1995; Thompson, 1995).

The apoptotic regulation pathway was first discovered in the nematode *Caenorhabditis Elegans* (Ellis and Horvitz, 1986). The apoptotic interactions cause the death of approximately 131 cells out of 1090 somatic cells at different times and different locations during the development of *C. Elegans* (Steller, 1995). The main apoptotic genes in *C. Elegans* are ced4, ced3, and ced9. CED-4 binds to CED-3 promoting the activation of CED-3, while CED-9 binds to CED-4 inhibiting the activation of CED-3 (Hengartner and Horvitz, 1994). Under normal conditions CED-9 stays bound to CED-4 keeping CED-3 inactive, but apoptotic stimuli cause CED-9 to dissociate by EGL-1 which allows the activation of CED-3 leading to apoptosis (Shi, 2001). A similar system also exists in other organisms. The mammalian homolog of CED-4 is Apaf-1, CED-3 is Caspase 3, Bid and Bcl-2, which are members of Bcl-2 family of proteins are similar to CED-9 and EGL-1 (Figure 1.6) (Zou *et al,* 1997; Shi, 2001).

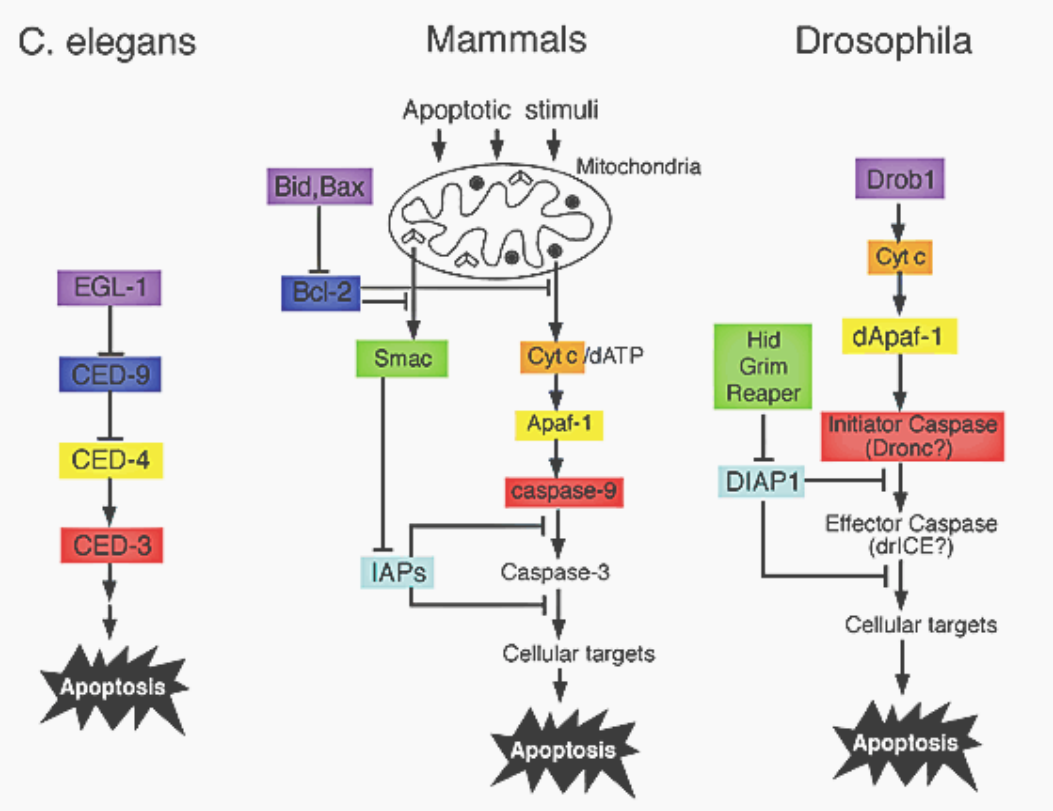


Figure 1.6 The conservation of the apoptotic mechanisms between species (Shi, 2001)

1.2.2. Regulation of Apoptosis

Since apoptosis takes part in such a variety of physiological and non-physiological conditions, understanding the molecular mechanism of the regulation of apoptosis is important. Apoptosis can be induced by either intrinsic pathways which are activated as a result of intracellular stress like DNA damage, or extrinsic pathways which are triggered by extracellular factors acting on the death receptors such as Fas and TNFR-1 (Shi, 2001). In the intrinsic or mitochondrial pathway; cytochrome *c* is released, apoptosome which consists of cytochrome *c* is formed, and APAF1, pro-caspase-9, and caspase-9 are activated. Endoplasmic reticulum stress triggers the ER-specific pathway which results in the activation of caspase-12 in mice or caspase-4, and caspase-3 or caspase-7 in humans. Extrinsic pathways are

activated by extracellular ligand binding to death receptors and results in either caspase activation or need further amplification through the mitochondrial pathway, according to the cell type. Fas and TNFR1 can be given as examples of the most commonly studied death receptors. (Figure 1.7) (Holcik and Sonenberg, 2005; Liston et al, 2003; Gebauer and Hentze, 2004; Ashkenazi and Dixit, 1998)

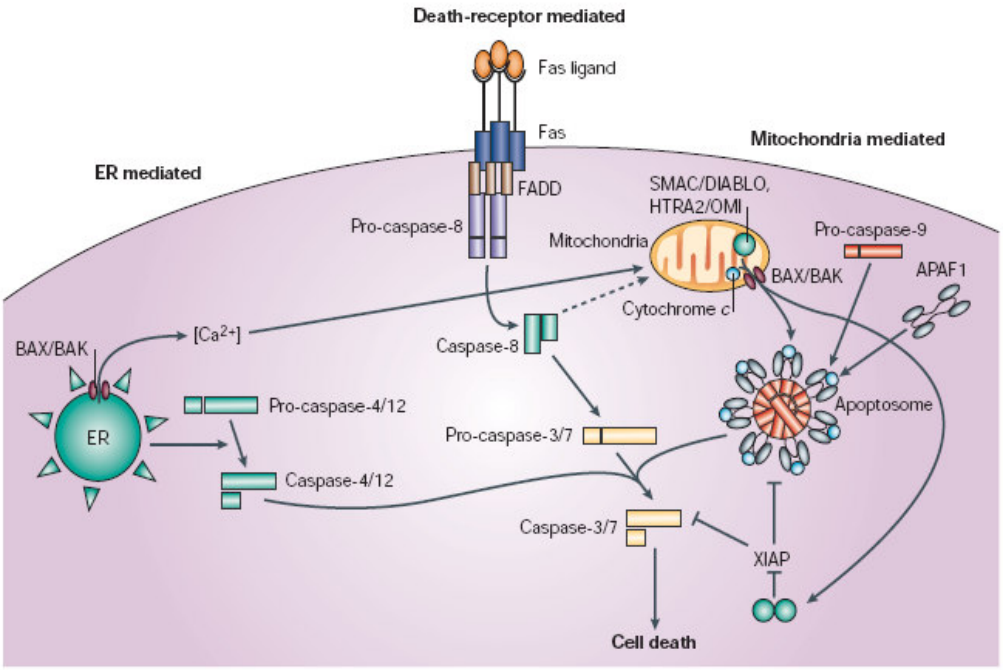


Figure 1.7 Different mediations of the apoptotic pathways (Holcik and Sonenberg, 2005)

1.2.2.1. Mitochondrial Apoptosis

Mitochondrial apoptosis which is also the intrinsic pathway, is crucial for homeostasis and animal development. After receiving apoptotic stimuli, cytochrome *c* is released to the cytoplasm from the intermembrane space of mitochondria. The release of cytochrome *c* triggers the activation of a specific set of proteins called caspases. Caspases are a group of cysteine proteases which cleave selected proteins

at their aspartate residues. Fourteen caspases are identified in mammals and at least eight of them have critical roles in apoptotic regulation (Budihardjo *et al*, 1999). Caspases are categorized as the initiator caspases (caspase-2, caspase-8, caspase-9, caspase-10), and the effector caspases (caspase-3, caspase-6, caspase-7). The effector caspases are activated by initiator caspases while initiator caspases activate themselves. Initiator caspases are strictly regulated requiring an assembly of multicomponent complex since they activate a downstream of caspases. Caspase activity is inhibited by the conserved IAP family of proteins. Eight different mammalian IAPs which include XIAP, c-IAP1, c-IAP2, and ML-IAP/Livin have been identified (Shi, 2002). They target caspase-9, caspase-3 and caspase-7 but not other caspases (Holcik and Sonenberg, 2005).

After the release of cytochrome *c* from mitochondria, it binds to Apaf-1 and activates it by changing its confirmation (Jiang and Wang, 2000; Purring *et al*, 1999). This complex then binds to dATP or ATP, and forms a multimeric complex which is called apoptosome which activates procaspase-9 (Saleh *et al*, 1999) After being activated, caspase-9 forms a holoenzyme with the apoptosome, targeting primarily procaspase-3 which is one of the most important effector caspases (Jiang and Wang, 2000; Shi, 2001). Active caspase-3 cleaves a wide range of proteins including caspase-2, caspase-6, DFF45, DNA-dependent protein kinase, and nuclear lamin (Thornberry and Lazebnik, 1998). The degradation of DFF45 stops inhibiting DFF40 (CAD), which in return degrades chromatin into nucleosomal fragments, one of the main characteristics of apoptosis.

In cells which have not received any apoptotic stimuli, the enzymatic activity of mature caspases is inhibited by IAPs (Deveraux and Reed, 1999; Miller, 1999). However this inhibition must be supressed in cells which undergo apoptosis and this is regulated by smac also called DIABLO, which is a 25-kD mitochondrial protein (Verhangen *et al*, 2000). It is synthesized in the cytoplasm and is released together with cytochrome *c* to the cytosol upon apoptotic stimuli. While cytochrome *c* activates Apaf-1 and caspase-9, smac interacts with IAP enabling the activation of caspases (Verhangen *et al*, 2000; Chai *et al*, 2000). This process is regulated by the

Bcl-2 family members which effect cytochrome c release and apoptotic regulation (Adams and Cory, 1998; Adams and Cory, 2001). The importance of this pathway is clear by the observation that Apaf-1 is frequently inactivated in cancers such as malignant melanoma (Figure 1.8) (Soengas *et al,* 2001).

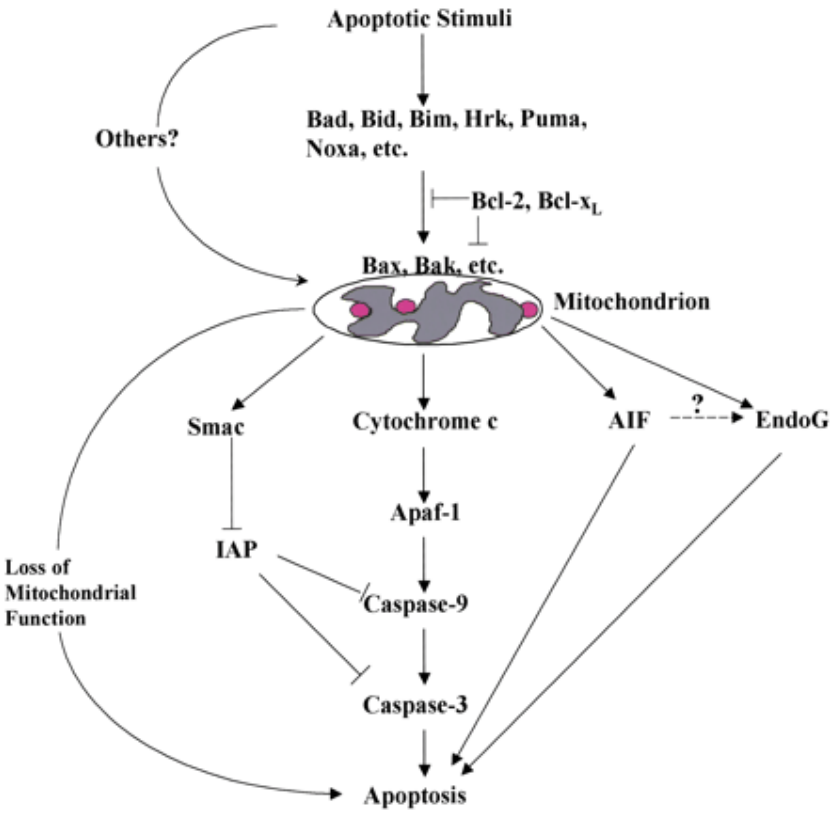


Figure 1.8 Mitochondrial regulation of apoptosis (Wang, 2001)

1.2.2.2. Bcl-2 Family of Proteins

Bcl-2 family of proteins is among the best characterized protein family which takes place in the regulation of apoptosis. It consists of both pro-apoptotic and anti-apoptotic members. They function by forming homo- and heterodimers. The rate of

the pro-apoptotic to anti-apoptotic molecules determines the susceptibility to death stimuli (Chao and Korsmeyer, 1998).

The prototype of this family, Bcl-2, was first identified in B-cell leukemia cell line as an oncogene which inhibited cell death rather than promoting proliferation (Cory and Adams 2002, Bakhshi *et al,* 1985; Cleary and Sklar, 1985). The anti-apoptotic activity of Bcl-2 was proven first by inducing apoptosis with IL-3 deprivation (Vaux *et al*, 1988) and then it was shown that Bcl-2 prevented apoptosis induced by several stimuli such as serum deprivation, chemotherapeutic agents, and heat shock (Tsujimoto, 1989). In higher eukaryotes at least 20 homologs of Bcl-2 have been identified, sharing at least one conserved BH domain (Vaux *et al*, 1992; Hengartner and Horvitz, 1994). These can be categorized into three groups: Bcl-2 like survival factors, Bax-like death factors, and BH-3 only death factors (Figure 1.9).

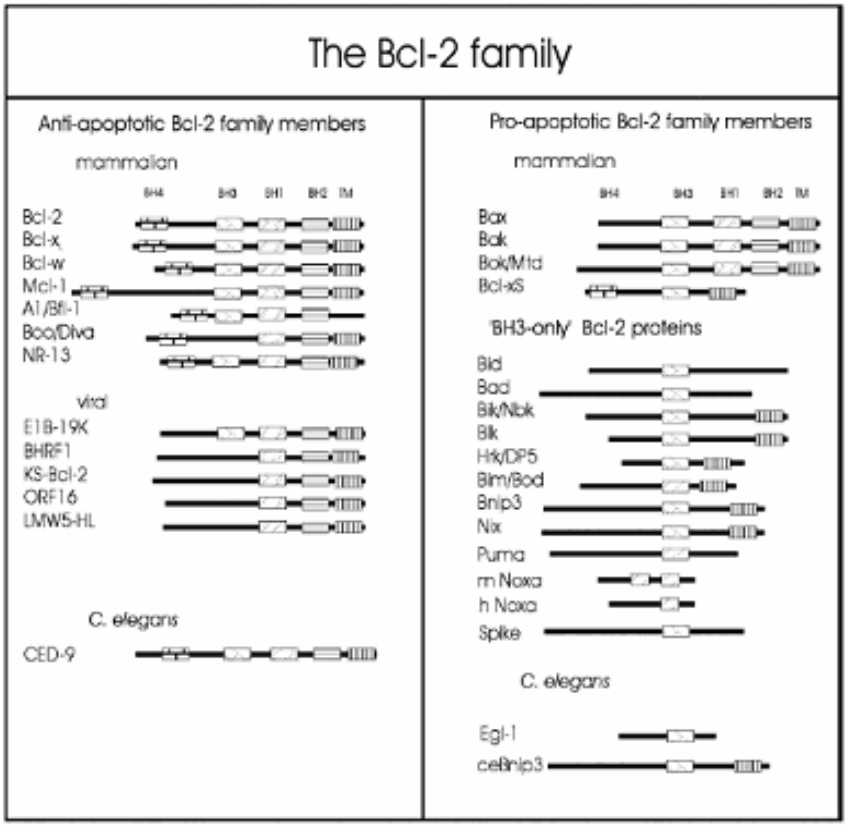


Figure 1.9 Classification of the Bcl-2 family members (Festjens, 2004)

The pro-survival family consists of Bcl-2 and its closest homologs Bcl-x_L, and Bcl-w besides Mcl-1, A1/Bfl-1, and Diva. This is a new class of proto-oncogenes that block cell death without promoting cell proliferation and it also has a role in cell cycle (Conradt and Horvitz, 1998). 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 (Merry and Korsmeyer, 1997). Bcl-2 deficient T cells show increased apoptosis following cell cycle progression. Bcl-2 is widely expressed during embryogenesis but it decreases and becomes much more restricted postnatally in the nervous system (Susin *et al*, 1996). Bcl-2 may be important in maintaining cell survival during interactions between epithelium and mesenchyme (Thornberry and Lazebnik, 1998; Tsujimoto, 1989). In addition, Bcl-2 and Bcl-x_L have a reciprocal pattern of expression in B cell compartments. Bcl-2 itself is required for the survival of kidney and melanocyte stem cells and mature lymphocytes. 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. They contain BH domains BH1, BH2, BH3, BH4, and a hydrophobic C-terminal tail which functions as a membrane anchor (Janiak *et al*, 1994). BH1-3 form a hydrophobic groove, with which BH3-only death factors interact through their BH3 domain, while the BH4 domain stabilizes this pocket (Zha *et al*, 1996; Luo *et al*, 1998). The core three-dimensional structure is well conserved between Bcl-x_L, Bcl-2, and Bcl-w. They are targeted to the outer mitochondrial membrane, endoplasmic reticulum, and nuclear envelope. It is becoming clear that every nucleated cell needs the protection of at least one Bcl-2 homologue, and these homologs regulate tissue homeostasis. Bcl-2 is required for kidney survival, melanocyte stem cells, and mature lymphocytes; Bcl-x_L for neuronal and erythroid cells; and Bcl-w for sperm progenitors in adult mice (Cory and Adams, 2002).

The Bax-like pro-apoptotic death factors consist of Bax, Bak, and Mtd/Bok. These pro-apoptotic members lack the BH4 domain. 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

(Gross *et al*, 1999; Vander Heiden and Thompson, 1999). They stimulate the release of caspases by heterodimerization and also by inducing the release of mitochondrial apoptogenic factors into the cytoplasm by acting on mitochondrial permeability transition pore, which leads to caspase activation (Tsujimoto, 1998). Bax heterodimerizes with Bcl-2, and homodimerizes with itself. When Bax is overexpressed in cells, apoptotic death in response to a death signal accelerates, but when Bcl-2 is overexpressed, it heterodimerizes with Bax and apoptosis is repressed (Kluck *et al*, 1997). Thus, the ratio of Bcl-2 to Bax is important in determining the tendency to go through apoptosis. In healthy cells Bax is a cytosolic monomer and is either loosely attached to the mitochondrial membrane or remains in the cytosol. Upon receiving death stimulus, cytosolic Bax goes through a conformational change and is oligomerized and translocated to the mitochondria where it becomes an integral membrane protein (Wolter *et al*, 1997). Also with the induction of the death stimuli the membrane associated Bax changes the exposition of its N-terminal BH3 domain (Zong *et al*, 2001) whereas in the absence of death signals the N-terminal domain does not allow the transmembrane anchor to function. This explains the reason for Bax failing to insert in the mitochondrial membrane in healthy cells. In addition, when Bax is activated, it binds to and antagonizes anti-apoptotic Bcl-2 proteins (Puthalakath *et al*, 1999). Bak on the other hand is an oligomeric integral mitochondrial membrane protein even in healthy cells, but changes conformation during apoptosis as well to form larger aggregates (Griffiths *et al*, 1999). The Bax-like proteins act in mitochondrial disruption.

The BH-3 only group consists of Bad, Bid, Bik/Biklk, Bim, Hrk, and Bnip3. They have only the BH3 domain which enables dimerization and is sufficient for their killing (Shi, 2001). They induce apoptosis mostly in response to developmental signals or intracellular damage (Huang and Strasser, 2000). They are upstream sensors and mediators of apoptotic cell death. BH3 domains of several BH3-only proteins either directly activate Bax/Bak proteins or bind to the hydrophobic pocket of anti-apoptotic Bcl-2 family members (Letai *et al*, 2002). The programmed death of the somatic cells of *C. Elegans* require a single BH3-only protein, EGL-1. The

eight or more mammalian BH3 proteins homologs allow a more-refined control over cell death.

There are a few models on how Bcl-2 family of proteins work. In one of the most accepted ones Bax and Bak are responsible for the release of mitochondrial proteins and are negatively regulated by Bcl-2 and Bcl-x_L (Korsmeyer, 2000, Antonsson *et al*, 2000). Bax forms a channel either by itself or with the help of other factors and in apoptotic cells this channel is responsible for the release of the proteins from the intermembrane space of mitochondria to the cytosol. However the binding of Bcl-2 to Bax closes this channel. Upon apoptotic stimuli, Bid or PUMA is activated and interacts with the Bcl-2 protein to relieve the inhibition of the Bax-dominated channel. This model is in consistency with many biological and biochemical observations. This model also differentiates the functions of the two pro-apoptotic subfamilies, the BH3-only (Bid or PUMA) and the Bax-like pro-apoptotic members. Bcl-2 family of proteins control the permeability of mitochondria but it is not clear whether they are the primary components of the pores or they regulate the open-close status of the pores. How the three subfamilies of Bcl-2 proteins work *in vivo* is also unclear (Shi, 2001). In the overall picture though, the Bcl-2 family of proteins act as a critical life–death decision point within the common pathway of apoptosis.

1.3. Estrogen

Estrogen is a hormone which is produced by the ovaries and testes. It stimulates the development of secondary sexual characteristics, induces menstruation, and regulates growth, differentiation, cell proliferation, metabolic activities, reproduction, homeostasis, cardiovascular health, bone integrity, cognition, and behavior. It targets many organs like the brain, heart, bone, breast, uterus, and prostate. This wide range of organs, which estrogen is involved in the functioning of, makes estrogen a very important hormone for therapy and therefore it is crucial to understand the imbalances of estrogen, gene networks controlled by estrogen, and regulation of its targets (Deroo and Korach, 2006; Paech *et al*, 1997).

Estrogen regulation occurs through several different mechanisms but mostly it induces cellular changes through estrogen receptor (ER). ER exists in 2 forms: ERα and ERß which have different expression patterns according to the organism and organ they are found in. In this common regulation pathway estrogen diffuses to the cell, binds to an ER and results in the conformational change of the ER. Then this estrogen-ER complex binds to specific DNA sequences called estrogen response elements (ERE) directly or indirectly with AP1. The ligand which binds to ER makes a conformational change, which promotes the binding to ERE. When ERα and ERß form complexes with estrogen from an AP1 site, they signal in different ways depending on the ligand and response element, and they inhibit or activate transcription accordingly (Deroo and Korach, 2006; Smith and O'Maley, 2004). The ligand bound receptor also forms a complex with coactivator proteins which activates target gene expression through chromatin remodeling. This suggests that depending on the complex formed, ERs play different roles in gene regulation. (Weitzmann and Pacifici, 2006; Deroo and Korach, 2006; Paech *et al*, 1997).

Estrogen exerts its effects through its target genes. Estrogen affects hundreds of genes in different tissues and the expression profiles of these genes change with developmental stages and diseases. The coordination of these genes is mainly by estrogen but there are also other factors like type of ER, co-regulators, estrogen exposure time, and estrogen amount (Deroo and Korach, 2006; Tang *et al*, 2004). The regulatory groups which these genes affect can be classified as cytokines/immune response, signal transduction, cell motility/cytoskeleton regulation, growth factors/hormones, apoptosis/cell proliferation, housekeeping, nucleic acid processing, and other/unknown (Stossi *et al*, 2004). Due to these diverse mechanisms, estrogen responsive genes are very crucial for research and clinical purposes.

Estrogen regulation is affected by agonists and antagonists which exhibit tissue-specific estrogenic activity. The same molecule might act both as an antagonist and an agonist depending on the organism and tissue. For example tamoxifen is an agonist in bone and uterus whereas it is an antagonist in breast.

Raloxifene resembles tamoxifen and in addition it is used in the prevention of osteoporosis. These estrogen receptor modulators also affect the ER signaling (Deroo and Korach, 2006; Paech *et al*, 1997).

A very tight correlation has been established between estrogen and several diseases such as breast cancer, ovarian cancer, colorectal cancer, prostate cancer, endometrial cancer, osteoporosis, neurodegenerative diseases, cardiovascular diseases, insulin resistance, lupus erythematosis, endometriosis, and obesity (Deroo and Korach, 2006). However the molecular mechanisms underlying this, is not clear. Both estrogen agonists and antagonists are used for clinical purposes in estrogen regulation.

1.3.1. Estrogen, Apoptosis and Bcl-2 Family of Proteins

Estrogen regulates many mechanisms through its target genes as mentioned before and apoptosis is one of the major ones. Estrogen has a dual role on apoptosis; it can both stimulate and inhibit apoptosis depending on the type of cell. It induces apoptosis in osteoclasts but inhibits in nerve cells, endothelial cells, and epithelial cells (Spyridopoulos *et al*, 1997; Jo *et al*, 1993). The mechanism of estrogen on apoptosis is through the induction of glutathione, Bcl-2 protein, nitric oxide, inhibition of inflammatory cytokine production, and directly as an antioxidant molecule at the biochemical level (Kobayashi *et al*, 1997).

It was noted that apoptotic gradient and Bcl-2 protein which is anti-apoptotic were negatively correlated in the presence of estrogen, attracting the attention to Bcl-2 for estrogen regulation. Estrogen differentially regulates several pro-apoptotic and anti-apoptotic members of the Bcl-2 family of proteins. It increases Bcl-2 protein expression which has several putative estrogen responsive sites, whereas it decreases Bax expression which makes dimers with Bcl-2. This results in the increase in proliferation and decrease in apoptosis, promoting cell survival (Alkayed *et al*, 2001). Another anti-apoptotic family member Bcl-x_L has putative ERE and estrogen increases its expression as well. It is not clear though whether estrogen acts directly

or indirectly on these molecules but Bcl-2 family of proteins have a crucial role in apoptotic regulation of estrogen (Bynoe *et al*, 2000; Pike, 1999).

1.3.2. Estrogen Effects on Osteocytes and MSCs

Among the target tissues and organs of estrogen, bone marrow is one of the most studied ones. It is known that osteoblasts, osteocytes, osteoclasts, bone marrow stromal cells, T cells, B cells and most other cells in human and mouse bone marrow besides MSCs, express both ERα and ERβ (Weitzmann and Pacifici, 2006; Komm *et al*, 1988). Gender, age, and cell type are important determinants of ER isoform expression in the bone marrow. It is known that estrogen stimulates osteoblast differentiation, increases bone marrow proliferation, and is involved in the decrease of apoptosis in osteoblasts. Also it is found that there is an increase in osteocyte apoptosis directly or indirectly in ovx animals and the addition of estrogen decreases the apoptosis rate (Tomkinson *et al*, 1998) In addition, there is an increase in the proliferation rate and ovariectomization decreases proliferation while the addition of estrogen reverses this effect (Fowler *et al*, 2005). In MSCs, estrogen increases the proliferation rate, reduces apoptosis, upregulates ERα expression, and downregulates ERβ expression which is thought to be a repressor (Zhou *et al*, 2001). The exact mechanism which estrogen exerts these effects is not known but osteocyte apoptosis is found to be reversely correlated with the skeletal distribution of Bcl-2 protein (Stevens *et al*, 2000; Tomkinson *et al*, 1998). This suggests that the proliferative and anti-apoptotic effect of estrogen on osteocytes and MSCs might be through the expression of the Bcl-2 family of pro-apoptotic and anti-apoptotic proteins.

2. AIM OF STUDY

MSCs are potential therapeutic agents which are applicable in tissue engineering, gene delivery, carry high potential for regenerative medicine and do not induce any immune reaction. Besides being a future promise for many disease treatments, there are also some handicaps in their applications. Since MSC number is very low *in vivo,* it is important to obtain a certain number of MSCs for their use. The proliferation rate and number of MSCs have to be effectively increased in the shortest time period in order for MSCs to be adequate for therapeutic regiments. Our aim is to examine the role of estrogen for obtaining MSCs in a short period of time. We also attempt to understand the mechanism of the action of estrogen.

It is known that apoptosis is among the main regulatory mechanisms in all organisms and is a conserved system which emphasizes its importance throughout evolution. The members of the Bcl-2 family of proteins are strong candidates in the regulation of MSC since some of the family members have estrogen receptors, and most of them are affected by estrogen evidenced by the presence of putative EREs. In addition, Bcl-2 proteins are among the most important families which serve both as death agonist and antagonists and have a crucial role in apoptotic balance. This makes them potential candidates in the regulation of MSCs. Bcl-2 family members can be manipulated in order to obtain more MSC in a shorter time with higher proliferative capacity and efficiency.

3. MATERIALS AND METHODS

3.1. Animals and Their Treatments

3.1.1. The Maintenance of the Animals

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

3.1.2. Ovariectomization of the Animals

Animals were anesthesized with Ketamin (Ketalar, Park Davis) subcutaneously at a dose of 30mg/kg. The dorsal part was shaved with a razor, and was immobilized. After fully anesthesized, 2 cm of incision were made on both sides of the vertebrate, fat pads were removed to reach the ovaries. Ovaries at the tip of each uteri horn were tied with silk and then removed. Then the remaining fat pads were put back into their location and the skin of the animal was sutured with absorbex 4.0 (75 cm). After the operation the animals were put under a lamp to keep them warm. Animals were kept under supervision until they fully recovered from the anesthesia. Ovx animals were used approximately after 2 months after the operation to ensure the total deprivation of estrogen from their bodies. Each time an ovx animal was sacrificed by cervical dislocation, another female rat of the same age which has not been operated was also sacrificed and called the “normal” group of animal.

3.1.3. Isolation of the Bone Marrow from the Animals

After the animals were sacrificed by cervical dislocation for the isolation of the bone marrow, the gastrocnemius and biceps regions were shaved with a razor. Then the femur and tibia were removed, and the ends of bones were cut and flushed with DMEM (Biochrom FG 0435) which contains 10% Fetal Bovine Serum (Biochrom-S0115) and 1% penicillin/streptomycin solution (Biochrom-A2213), by using a 5ml syringe (Figure 3.1). The mixed media and bone marrow were centrifuged at 4000 rpm for 3 min in order precipitate the cells of the bone marrow. Then the media was removed and the cells were washed three times with 1X PBS buffer to prepare for tissue culturing.

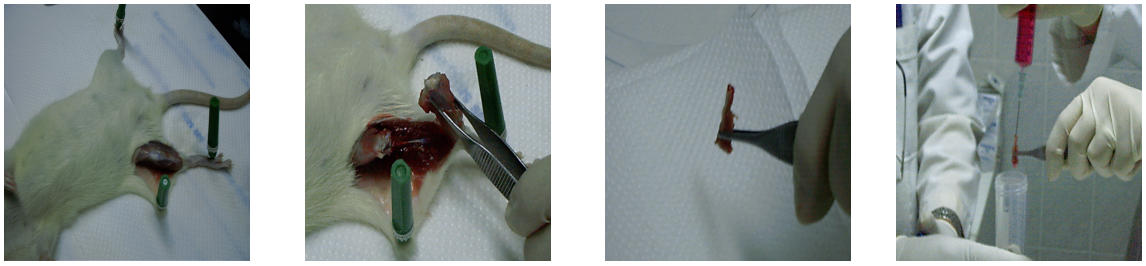


Figure 3.1 The isolation of the bone marrow of the rats

3.2. Cell Culture

3.2.1. Cell Number Detection with Cell Count

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

concentration of cells in original mixture =

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

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

3.2.2. MSC Culture

MSCs were cultured by MesenCult media (Stem Cell technologies) with supplement (Stem Cell Technologies), and 1% penicillin/streptomycin solution (Biochrom-A2213). Next day, the media of the tissue culture plates were changed and the majority of the non-adherent cells were removed. The media of the cells were changed in every 4-5 days, after washing with sterile 1X PBS prior to the change. The adherent cells on the 14th day of the cell culture were mostly MSCs but on the 9th day there were also adherent cells which were not MSCs particularly.

3.2.3. Estrogen Treatment

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

3.3. Standard Solutions and Buffers

DEPC-Treated ddH_2O
1ml DEPC
1lt ddH_2O
Stirred in a hood for 1 hour, autoclaved in order to inactivate the DEPC

10x PBS
80 g NaCl
2 g KCl
8,01g $Na_2HPO_4.2H_2O$
2g KH_2PO_4
1 liter ddH_2O pH: 7,2
Working solution (1XPBS) was prepared by diluting 10XPBS by 10 times

50X TAE Buffer
2M Tris Base (242 g)
57,1 ml Glacial Acetic Acid
50mM EDTA
Add to 1 lt by ddH_2O
Working solution (1XTAE) prepared by diluting 50XTAE by 50 times

10 X Agarose Gel Loading Dye
0,009g BFB
0,009g XC
2,8mL ddH2O
1,2ml 0,5M EDTA.
Total volume brought to 15ml by adding glycerol, dilute 1:10 in sample prior to loading to electrophoresis gel

5M NaOH
10g NaOH
50ml ddH_2O.

4% PFA
4g PFA
100ml 1XPBS
Dissolved by heating, solution prepared freshly

5M NaCl
292.2g NaCl
1lt ddH_2O.

10%SDS
100g SDS
1lt ddH_2O

1M Tris
60.55g Tris
300ml ddH2O
21ml 37% HCl
pH adjusted to 8.0 with HCl, total volume brought to 500ml by adding ddH_2O.

0.5M EDTA
93.05g EDTA
300ml ddH2O
pH adjusted to 8.0 with NaOH, total volume brought to 500ml by adding ddH_2O.

30% Acrylamide Mix
145g Acrylamide
5g bis-acrylamide
500ml ddH_2O
Filtered, stored in dark at 4^0C.

SDS-PAGE Running Buffer (5X stock solution)
15g Tris
73,2g Glycine
5g SDS
1lt ddH2O
1X working solution prepared by diluting the 5X SDS-PAGE running buffer 5 times, stored at 4^0C

Bradford Reagent
100 mg Coomassie Brilliant Blue G-250
100 ml 85%phosphoric acid
50 ml 95%EtOH
850 ml ddH_2O
Filtered through Whatman no:1

Lysis Buffer
150 mM NaCl
500 mM Tris pH:8
1% NP-40
4% Protease Inhibitor Cocktail

10X TBS
12.19 g Tris
87,76 g NaCl
pH adjusted to 8, total volume brought to 1lt by adding ddH_2O

1X TBS-T 0,5%
50 ml 10X TBS
450 ml ddH_2O
2,5 ml Tween-20
Total volume brought to 500ml by adding ddH_2O

Blocking Solution for Western Blot (5%)
2,5 g milk powder
50mL 1X TBS-T

Blocking Solution for Immunofluorescent Staining
1% BSA
3 ml 1XPBS
15 µl tween-20

Cracking Buffer (2X Protein Loading Buffer)
50mM Tris HCl pH: 6,8
2mM EDTA pH: 6,8
1% SDS
20% Glycerol
0,02% BFB
Add 1% ß-mercaptoethanol prior to use

Coomassie Blue Staining Solution:
0,25 g coomassie brilliant blue
45 ml Methanol
45 ml ddH_2O
10 ml glacial acetic acid

5X Running Buffer
15 g Tris base
72 g Glycine
5 g SDS
1 liter ddH_2O
Diluted to 1X prior to use

Semi-dry Transfer Buffer
2,5 g glycine
5,8 g Tris base
0,37 g SDS
200 ml methanol
800 ml ddH_2O

Destaining Solution
100 ml methanol
35 ml acetic acid
365 ml ddH_2O

Permeabilisation solution
0,1 g sodium acetate
100µl Triton X-100
100 ml ddH_2O

3.4. MSC Count

On the 9th and 14th days of the cell culture, the cells were washed with 1X PBS buffer twice and trypsinized (0,25 % trypsin-EDTA solution; Sigma) for 5 minutes at 37^0C. 10ml of MesenCult media was added to trypsinized cells and they were counted on hemocytometer.

3.5. Colony Forming Assay

MSCs were washed with 1X PBS, then they were air dried. MetOH was used for fixation and left for 5 minutes. Then the cells were washed again with 1X PBS buffer and giemsa staining reagent (Carlo Erba code: 453616) was added and left for 5 min. The reaction of giemsa staining was stopped by the addition of tap water and at the end the colonies were dyed in purple.

3.6. Determination of the Gene Expression

3.6.1. Total RNA Isolation from the MSCs

On the 9th and 14th days of the cell culture, total RNAs from the MSCs were isolated. The cells were first washed with 1X PBS buffer in order to eliminate the non-adherent cells in the media. Then the cells were trypsinized and incubated at 37^oC for 5 min. MesenCult media was added to the trypsinized cells and centrifuged at 1500 rpm for 5 min. Then the media was removed and the precipitated cells were washed with cold 1X PBS buffer and centrifuged again at 1500rpm for 5 min. Next the 1X PBS buffer was removed and the total mRNA was isolated from the precipitate by using RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The RNA isolated was stored at -80^oC.

3.6.2. Concentration and Integrity of RNA

1 µl of the total RNA samples were diluted in a ratio of 1:500 with DEPC-treated ddH2O. Then the OD measurements were taken at 260 and 280 nm wavelengths with the spectrophotometer (Beckman, California, USA). The $O.D._{260}$/ $O.D._{280}$ ratios were calculated in order to determine if there is a phenol, protein or DNA contamination in the RNA samples. The expected values are between 1,6-2,0. Then agarose gel electrophoresis was performed to check the integrity of the isolated RNAs. 1% agarose gel was prepared with 1X TAE buffer and 30 ng/ml ethidium bromide solution which was added to visualize the RNA. The samples were prepared by adding 1µl of Agarose Gel Loading Dye, 3µl RNA sample and 6µl DEPC-treated H_2O and were kept at 65 ^oC for 10 minutes in order to denature the RNA. Then all the samples were loaded to the agarose gel and the gel was run at 100V for 30 minutes. It was visualized under the transilluminator (Bio-Rad, California, USA) and MultiAnalyst (Bio-Rad, California, USA) software was used in order to take the photographs of the gels.

3.6.3. cDNA Synthesis and PCR

3.6.3.1. cDNA Synthesis

The cDNAs were synthesized from the total RNA samples with the RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas, Ontario, Canada) according to the manufacturer's protocol. 2µg RNA was mixed with 1µl of oligo(dT) primer and ddH2O to a total volume of 12 µl. They were incubated at 70 ^0C for 5 minutes and then chilled on ice. Then 4µl of 5x reaction buffer, 1µl of Ribonuclease inhibitor, and 2 µl of 10 mM dNTP mix were added to the mixture and incubated at 37 ^0C for 5 minutes. Finally, 1 µl of RevertAid MuLV reverse transcriptase was added and the mixture was incubated at 42 ^{0} C for 60 minutes. The reactions were stopped by heating at 70 ^0C for 10 minutes, and the mixture was put on ice. The cDNAs were stored at -20 ^0C.

3.6.3.2. PCR

3.6.3.2.1. Primer Design

Primers were designed for *gapdh, bcl-2, bcl-x_L , bax,* and *bak.* The cDNA sequences of the rat homologues of these genes are available at the GenBank database. The GenBank Accession numbers of the rat homologues of *bcl-2* is NM_016993; *bcl-x_L* is U34963, *bax* is U49729, and *gapdh is* X02231. For *bak,* the cDNA sequences of mouse and human homologues were aligned by Blast (NCBI), and primers were chosen from the largest conserved regions by preferring the mouse sequence in the points of mismatches. The mice homologue *bak* (Y13231) and human homologue *bak* (U23765) were used (Table 3.1).

Table 3.1 The sequences and the sizes of the primers used

gapdh (210 bp)	Forward Reverse	:5'-CCTCCTCATTGACCTCAACTAC -3' :5'-CATGGTGGTGAAGACGCCAG-3'
bcl-2 (584 bp)	Forward Reverse	: 5'-CCTGGCATCTTCTCCTTC-3' :5'-TGCTGACCTCACTTGTGG-3'
bcl-x_L (346 bp)	Forward Reverse	:5'-TCAATGGCAACCCTTCCTGG-3' :5'-ATCCGACTCACCAATACCTG-3'
bax (578 bp)	Forward Reverse	:5'-AATCATGGACGGGTCC-3' :5'- GCCCATCTTCTTCCAG-3'
bak (536 bp)	Forward Reverse	:5'-CCGGAATTCCAGGACACAGAGGA-3' :5'-CCAAGCTTGCCCAACAGAACCAC-3'

3.6.3.2.2. Semi-Quantitative PCR

In order to compare the mRNA expression levels of the samples, semiquantitative PCR were performed and *gapdh* was used as a control for equal

loading. The cycle number of the RT-PCR reactions of the bcl-2 family members, were optimized in order to detect the expression levels before saturation. In all reactions ddH_2O was added instead of cDNA to the negative control groups. Quantification of the amplicon intensities was performed using MultiAnalyst software. The quantitated values for the bcl-2 family members were normalized with the quantitated values for the gapdh for each sample separately. And the resulting graphs were drawn by these normalized values. The reaction ingredients used in PCR reactions are shown in Table 3.2 and the conditions of different PCR reactions are shown in Figure 3.2.

Table 3.2 The reaction mixture used in PCRs

Reaction Ingredients	Volume
cDNA	1 µl
10X Taq Buffer (MBI Fermentas, Ontario, Canada)	2,5 µl (1X)
25 mM $MgCl_2$ solution ((MBI Fermentas)	1,5 µl (1,5mM)
Forward Primer (Invitrogen)	1 µl (10 pmol)
Reverse Primer (Invitrogen)	1 µl (10 pmol)
2 mM dNTP (MBI Fermentas)	2,5 µl (0,2mM)
Taq Polymerase (MBI Fermentas, Ontario, Canada)	0,2 µl (1U)
ddH_2O	15,3 µl
Total	**25 µl**

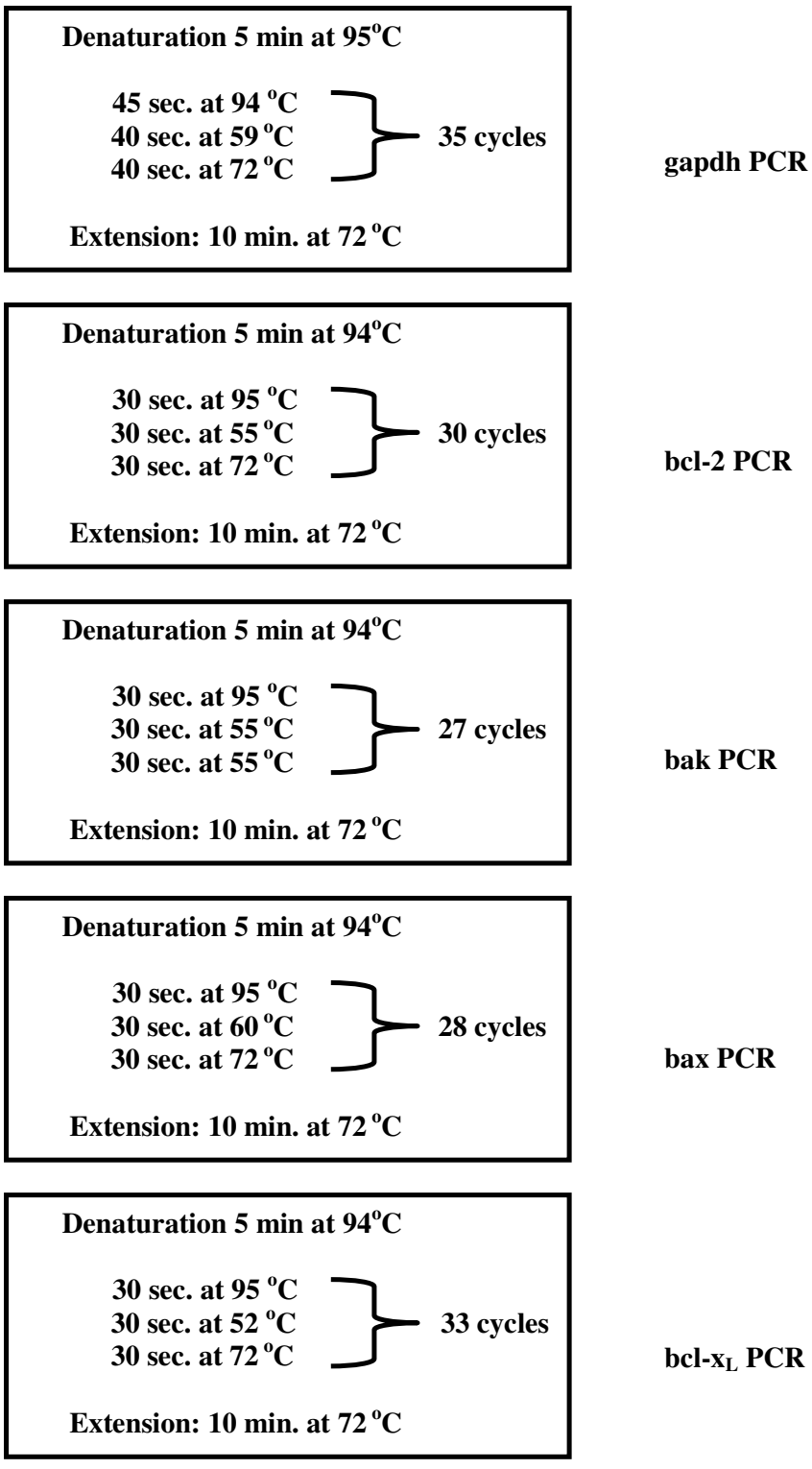


Figure 3.2 The cycle numbers and conditions of the PCRs

3.6.3.3. Agarose Gel Electrophoresis

1% agarose gel was prepared with 1X TAE Buffer and 30 ng/ml ethidium bromide solution. Samples were prepared by addition of 1µl Agarose Gel Loading Dye to 10µl of cDNA sample and loaded to the agarose gel. The gel was run at 120V for 30 minutes and visualized under transilluminator (Bio-Rad, California, USA). MultiAnalyst (Bio-Rad, California, USA) software was used to take photographs of the gels and compare the cDNA band intensities for semi-quantitative analysis. The gene Ruler DNA Ladder Mix (Fermentas, #SM0331) was used as a marker and 5µl was loaded to every gel.

3.7. Protein Expression

3.7.1. Total Protein Isolation from the MSCs

The total protein of the MSCs were isolated on the 9th and 14th days of the cell culture. The cells were washed with 1X PBS buffer twice in order to wash out the non adherent cells. Then the cells were scraped with a scraper and put into a 15 ml falcon tube. Then the mixture was centrifuged at 1500 rpm for 5 minutes. The 1XPBS was removed and the precipitate was suspended in lysis buffer which was prepared freshly as described in section 3.3 and checked against any protein contamination with Bradford reagent prior to use. The mixture was left on ice for 30 min and mixed gently every 5 -10 minutes. Then the mixture was centrifuged at 13000 rpm for 20 minutes. The supernatant which contained the proteins was taken to a separate eppendorf tube. It was first dipped into liquid nitrogen for a few seconds and then stored at -80^0C.

3.7.2. Protein Quantification

Protein quantification was made with Bradford assay. A standard curve was made by using 6 different BSA concentrations as in Table 3.3.

Table 3.3 The standards used for the derivation of the standard curve for protein concentration detection

Standards	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6
BSA(1µg/µl)	0 µl	2,5 µl	5 µl	7,5 µl	10 µl	12,5 µl
0,15 M NaCl	100 µl	97,5 µl	95 µl	92,5 µl	90 µl	87,5 µl
Bradford Reagent	900 µl	900 µl	900 µl	900 µl	900 µl	900 µl

A standard curve was formed by measuring the different BSA concentrations at a fixed wavelength of 595 nm. A formula was derived from the graph derived by putting the O.D. measurements to the y axis and BSA amount to the x axis. Then a second set of measurements was made by taking the lysis buffer sample containing 900µl Bradford reagent+98µl 0,15 M NaCl+2µl lysis buffer as blank. The other samples contained 900µl Bradford reagent+98µl 0,15 M NaCl+2µl protein sample and was measured at 595 nm fixed wavelength as well. The O.D. measurements were put to the formula derived from the standard BSA concentrations, and the concentrations of the proteins were derived.

3.7.3. Western Blotting

3.7.3.1. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The protein samples were separated by SDS-PAGE gel with 10% resolution gel, and 5% stacking gel. The proteins were loaded with a concentration of 20ng for Bcl-2 and Bak experiments and 40ng for Bax and Bcl-x_L experiments. A total volume of 20 µl was loaded to the gel which contained the protein, 1% cracking buffer containing 10% ß-mercaptoethanol, and ddH_2O. The samples were left at 70^0C for 10 minutes and then they were put on ice for 5 minutes. In each experiment, all protein samples were loaded in equal concentrations in order to compare the protein expressions between the groups. The PageRuler Prestained Protein Ladder (MBI Fermentas, Ontario, Canada) was used as a protein molecular weight marker and 5µl was loaded for each gel. The gel was run at 80 volts in 1X cold running buffer until

the proteins passed from the stacking gel to the resolution gel. Then the gel was run at 120 volts until the dyes reached the end of the glass and was stopped. The gel was removed from between the glasses. After the stacking part of the gel was removed and washed with dH_2O, it was put in the semi-dry transfer buffer.

3.7.3.2. Transfer of Proteins to the Membrane

The proteins were transferred onto the membrane by semi-dry transfer since our proteins are within the range of 20kDa-30kDa and semi-dry transfer method is the best for small proteins. 4 Whatman papers (no.1), and the resolution part of the SDS-PAGE gel which are at the sizes of 5,5 cm to 8,5cm were left in the semi-dry transfer buffer for 10 minutes. The membrane was left in MetOH for 30 seconds, in dH_2O for 2 minutes, and then in semi-dry transfer buffer for 10 minutes. Then two of the Whatman papers, the membrane, the gel, and 2 more Whatman papers were put on to the semi-dry transfer apparat (Biorad) in this order. The bubbles between the layers were removed and the transfer was made at 0,16 Ampere for 50 minutes. After the transfer was completed, the membrane was washed 5 minutes with 1X TBS-T buffer before the proteins were immunologically detected.

3.7.3.3. Immunological Detection of Immobilized Proteins

After washing, the membrane was left in the blocking solution for 1 hour at RT on an orbital shaker. Then primary antibodies of Bcl-x_L (H-5 sc-8392 Santa Cruz Biotechnologies), Bak (G-23 sc-832 Santa Cruz Biotechnologies), Bax (P-19 sc-526 Santa Cruz Biotechnologies), or Bcl-2 (N-19 sc-492 Santa Cruz Biotechnologies) were prepared in block solution at a concentration of 1:500 and was left overnight at 4^oC. The following day the membrane was washed with 1X TBS-T 4 times 5 minutes each and incubated with the secondary antibody of Anti-Rabbit-HRP (Sigma, Montana, USA) at a concentration of 1:2500 for 1 hour at RT on an orbital shaker. Then the membrane was washed again 4 times, 5 minutes each. ECL Plus Detection Reagent (Amersham Biosciences, New Jersey, USA) was added on to the membrane and left for 5 minutes. The excess detection reagent was drained off, the membrane

was placed on glass, and covered with stretch film. The wrapped glass was placed in a x-ray film cassette. Films were exposed to x-ray for between 15-30 minutes depending on the intensity of the protein expressions. After exposure, the films were developed.

3.7.3.4. Coomassie Blue Staining of the Gel and Membrane

The gel was stained with coomassie blue staining solution to confirm that the transfer is successful, and contains equal amount of protein. The membrane and gel were left in the staining solution for 5 min, and then they were left in destaining solution overnight, by changing the solution on the membrane and gel each time the colour of the destaining solution turned into blue.

3.7.4. Immunofluorescent Staining

The cells were grown on cover slips (Deckglaser 100 No.1 12mm) in the cell culture plates. The plates were taken from the 37°C incubator and the media was removed. The cells were washed twice with 1X PBS and fixed with MetOH by incubating for 7 minutes. Then the cells were washed again with 1X PBS twice and left in 3% H_2O_2 for 15 minutes. After washing twice with 1X PBS, the cells were left in blocking solution for 30 minutes at RT. After the completion of the blocking step, the cells were incubated with Bcl-x_L antibody (H-5 sc-8392 Santa Cruz Biotechnologies) at a concentration of 1:100, or Bak antibody (G-23 sc-832 Santa Cruz Biotechnologies) at a concentration of 1:100, or Bax antibody (P-19 sc-526 Santa Cruz Biotechnologies) at a concentration of 1:100, or Bcl-2 (N-19 sc-492 Santa Cruz Biotechnologies) with a concentration of 1:100, or CD90 antibody (Chemicon-MAB1406) at a concentration of 1:500. The samples were left in the antibody solution for 16 hours at 4°C. The sides of the plates were covered with parafilm in order to prevent evaporation. The next day the cells were taken and washed with 1X PBS twice. The secondary antibodies of Anti-Rabbit-FITC (Sigma) with 1:200 dilution for Bak, Bax, and Bcl-x_L antibodies, and Anti-Mouse-FITC (Sigma-F2012-1ML) with 1:100 dilution for Bcl-2 antibody in block solution were

added and incubated for 1 hour at RT in the dark. After the secondary incubation, the cells were washed twice with 1XPBS and DAPI (Roche-10236276001) was added to the slides. After 10 minutes the DAPI staining was washed and the cover slips containing the cells were put on slides with glycerol. The pictures of the slides were taken with the fluorescent microscope (Axio Cam Zeiss) with a total magnification of 400X. The excitation wavelength of FITC was 490nm and the excitation wavelength for DAPI filter was 359nm. After their pictures were taken, the samples were stored in the dark at 4°C.

3.8. In Situ Cell Death Detection (TUNEL ASSAY)

DNA fragmentation was detected in situ by TdT- (terminal deoxynucleotidyl transferase) mediated fluorescein-dUTP labeling kit (Roche Diagnostics, Mannheim, Germany). Cells adherent to the cover slips washed with 1X PBS twice and were fixed in freshly prepared 4% paraformaldehyde in PBS for 1 hour at room temperature. Then the cells were washed with 1X PBS for 3 times and incubated with a freshly prepared permeabilisation solution (0.1 % Triton X-100, 0.1 % sodium citrate) for two minutes on ice. Then the cells were washed again with 1X PBS twice, and 50 µl of TUNEL reaction mixture was added onto each sample and incubated for one hour at 37^{0}C in the dark in a humidified chamber. The samples were washed with 1XPBS and were left in DAPI solution for 10 minutes in dark. Then they were washed again with 1XPBS and put on slides with glycerol. Slides were then analyzed by fluorescent microscopy. For evaluation by the fluorescent 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 slides in the absence of terminal deoxynucleotidyl transferase. For positive controls, the samples were first treated for 10 minutes with DNaseI (Fermentas- EN0521) at a concentration of 0,4u/µl at RT to induce DNA strand breaks prior to labeling procedures, and then incubated with 50 µl of TUNEL reaction mixture. The samples were kept at dark at 4^{0}C.

4. RESULTS

All the experiments were performed with two animal groups: adult female rats (normal group), and ovariectomized adult female rats (ovx group). Cells from both the normal and ovx groups were cultured in the presence and absence of estrogen. The physiological concentration of estrogen used in our experiments was 10^{-7} M, which is widely accepted in literature (Zhou *et al*, 2001). All the experiments were performed on the 9th day and 14th day of the cell culture. MSCs fully mature, and become ready to differentiate on the 14th day of the cell culture. On the other hand, we also included 9th day of cultures where MSCs have not fully matured, are not ready to differentiate, are in the process of forming colonies, and many other single cells adherent to the culture plate are present in the tissue culture besides MSCs. Due to this reason the majority of the cells adherent to the surface of the plate on the 14th day of culture were considered as MSCs, while only the colonies forming on the 9th day of the culture were considered as MSCs as evidenced by the immunostaining with the MSC markers.

4.1. Effect of Estrogen on the Number of MSCs

In order to examine the effect of estrogen on the number of MSCs, the cells of the normal and ovx groups treated with and without estrogen were counted. The cells were trypsinized and counted on the 9th and 14th days of the tissue culture. Since the cells were washed twice before counting them, the non-adherent cells were washed away and only the adherent cells were counted (Table 4.1).

Table 4.1 Cell counts on the 9th day and 14th days of the cell culture Both the number of cells, and the ratio of the 14th day cells compared to the 9th day cells in the same treatment type are seen.

	Normal	**Normal+estrogen**	**Ovx**	**Ovx+estrogen**
9th day	318,750	495,000	116,250	116,250
14th day	348,750	551,250	97,500	163,200

Table 4.2 The ratio of estrogen treated cells to the non-treated cells on the 9th day and 14th day cultures

	Normal+estrogen/normal ratio	**Ovx+estrogen/ovx ratio**
9th day	1,55	1
14th day	1,58	1,67

On the 9th day of the culture, our results showed that the addition of estrogen increased the number of adherent cells in the normal group. The number of the cells in the estrogen treated group was 1,55 times more compared to the group that had not been treated with estrogen. On the other hand in the ovx group the number of cells did not increase with estrogen treatment. (Figure 4.1, Table 4.2).

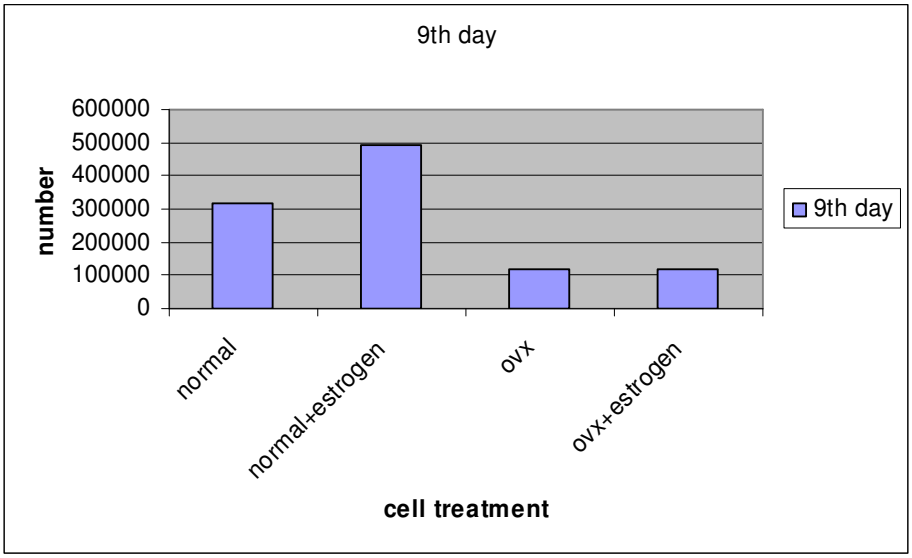


Figure 4.1 Cell number comparisons of the 9th day cell cultures
The number of cells counted in the normal, normal+estrogen, ovx, and ovx+estrogen groups are shown by bars.

However on the 14th day of the cell culture, we found that estrogen treatment increased the number of cells both in the ovx and in the normal group. In the normal group the cell number of estrogen treated cells was 1,58 times more than the group without any treatment, and in the ovx group the estrogen treated cells were 1,67 times more than the group without any treatment (Figure 4.1, Table 4.2).

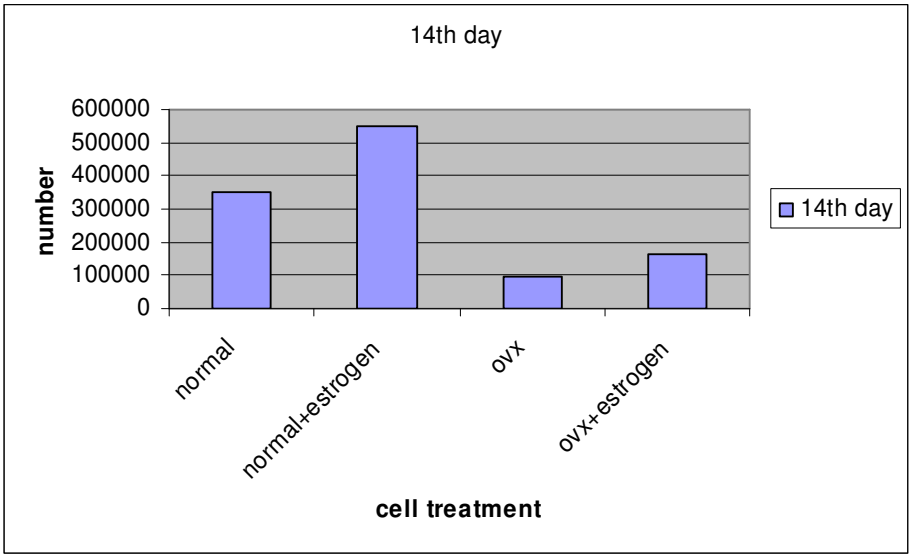


Figure 4.2 Cell number comparisons of the 14th day cell cultures
The number of cells counted in the normal, normal+estrogen, ovx, and ovx+estrogen groups are shown by bars.

These results indicate that the treatment of both the normal and ovx groups with estrogen increases the number of cells on the 14th day, and the normal group number on the 9th day. Estrogen also affects the rate of cell number increase form the 9th day to the 14th day of the cell culture, in both the normal and ovx groups.

4.2. Colony Forming Assay

Colony forming is one of the distinguishing characteristics of the MSCs. In order to see whether the increase in the cell number of the estrogen treated groups are also seen in the colony numbers, colony forming assay was performed in the groups treated with and without estrogen on the 9th and the 14th days of the cell culture. At least two samples from each normal and ovx group treated with and without estrogen were taken, and the colonies were counted (Table 4.3). Then the average number of colonies among each group was calculated and the results were compared among the 9th day groups (Figure 4.3) and 14th day groups (Figure 4.4)

Table 4.3 Average number of colonies counted on the 9th and 14th day culture groups treated with and without estrogen

	Normal	Normal+estrogen	Ovx	Ovx+estrogen
9th day	16	35	12,5	23
14th day	38,5	45	32,5	33

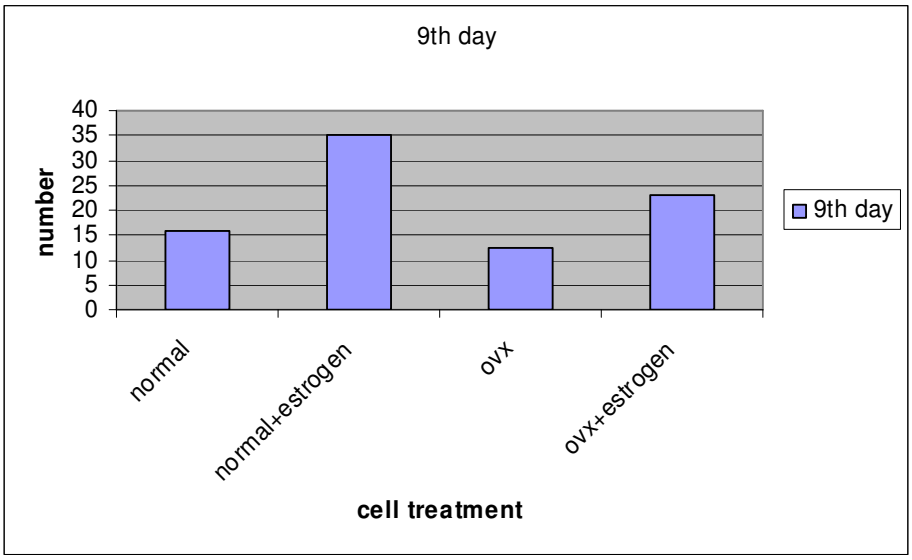


Figure 4.3 The number of colonies on the 9th day of the cell culture
The colonies counted were compared among the 9th day normal, normal+estrogen treated, ovx, and ovx+estrogen treated groups.

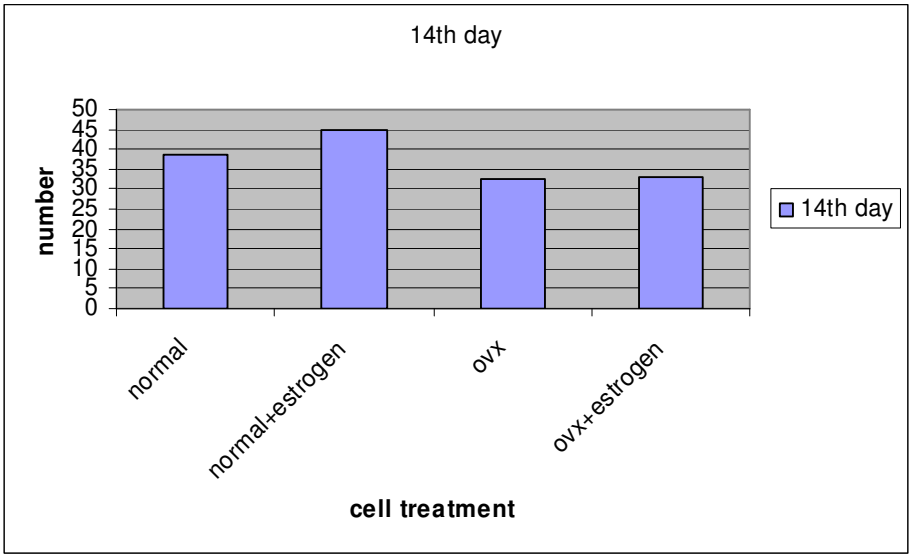
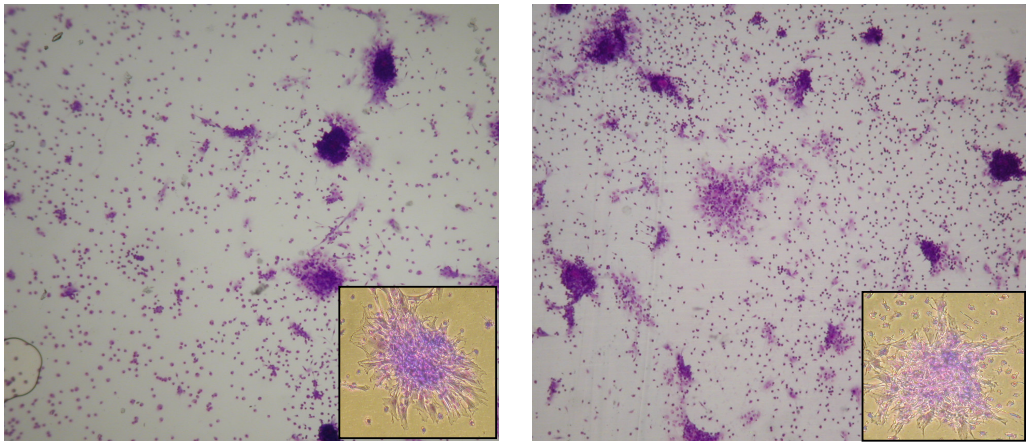


Figure 4.4 The number of colonies on the 14th day of the cell culture
The colonies counted were compared among the 14th day normal, normal+estrogen treated, ovx, and ovx+estrogen treated groups.

On the 9th day of the cell culture, there were very few colonies compared to that of the 14th day. However the addition of estrogen to the culture media, increased the number of colonies approximately two fold both in the normal group and in the ovx group. This suggests that using estrogen enables MSCs to form colonies much more efficiently.

On the 14th day of the cell culture the estrogen treatment of the normal and ovx groups also increased the number of colonies formed (Figure 4.5, Figure 4.6), but to a lesser degree compared to the rate of increase in the 9th day results. These results indicate that estrogen is more effective in the process of MSC colonies being formed. The increase in the number of colonies in both the 9th day and 14th day cell culture groups with the addition of estrogen, are in parallel with the cell count results.

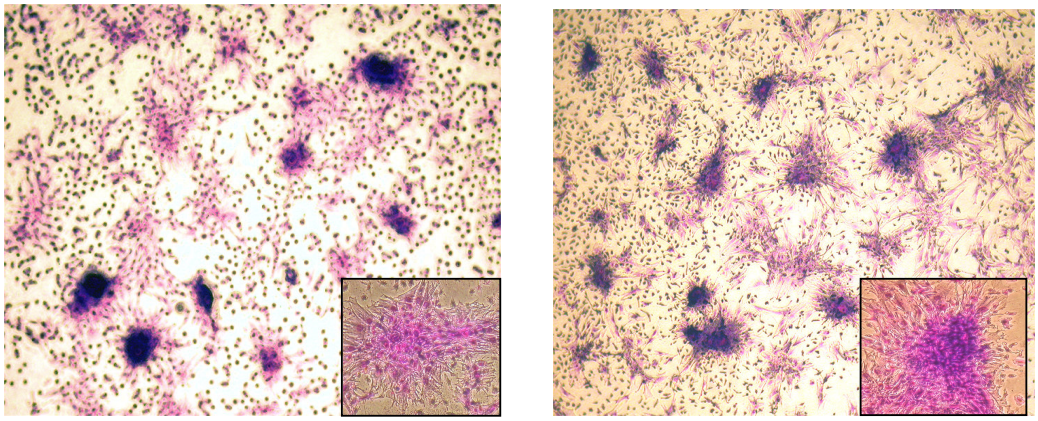
A) B)



14th day normal cells **14th day normal+estrogen cells**

Figure 4.5 Normal and normal+estrogen treated MSC colonies on the 14th day of cell culture The big pictures are viewed with a light microscope with a magnification of 400X and the small pictures are viewed with a magnification of 100x in order to see a more detailed profile of the colonies formed **A)** 14th day colonies formed by normal cells. **B)** 14th day colonies formed by normal cells treated with estrogen.

A) B)



14th day ovx cells **14th day ovx+estrogen cells**

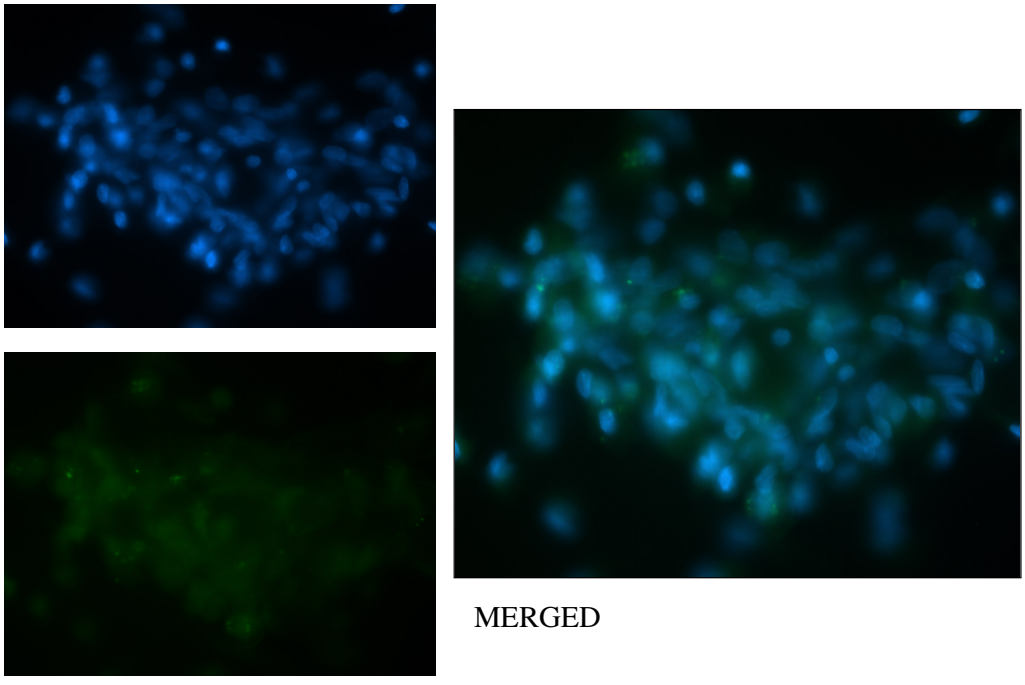
Figure 4.6 Ovx and ovx+estrogen treated MSC colonies on the 14th day of cell culture

The big pictures are viewed with a light microscope with a magnification of 400X and the small pictures are viewed with a magnification of 100X in order to see a more detailed profile of the colonies formed **A)** 14th day colonies formed by ovx cells **B)** 14th day colonies formed by ovx cells treated with estrogen

4.3. Apoptotic Rate of MSCs

Since both the number of cells and CFUs have increased by the addition of estrogen to the culture media in both the normal and ovx groups, a possible explanation was explored for the mechanism of this increase. One possibility was the involvement of the apoptotic pathway since estrogen plays a major role in the apoptotic mechanism of several systems. The MSC groups described in section 4 were checked for the presence of apoptotic cells by the TUNEL assay. We expected to see a decrease in the number of apoptotic cells in the groups cultured with estrogen. The apoptotic cells were counted by using the fluorescent microscope. The FITC filter with an excitation wavelength of 490nm was used to count the apoptotic cells, and the ratio of these cells to the total number of cells were calculated by counting the total number of cells with the DAPI filter with an excitation wavelength of 359nm (Figure 4.7).

DAPI



MERGED

FITC

Figure 4.7 A sample colony from the TUNEL assay. The total number of cells were counted with the DAPI filter, the apoptotic cells were detected with the FITC filter. The fluorescence microscope pictures are taken with a magnification of 400X

At least two samples from each group treated with and without estrogen were counted for both their total number of cells and apoptotic cells on the 9th and the 14th days. Then the ratios of apoptotic cells to total cells were calculated and their averages were taken. The results were compared among the 9th and 14th days of the cell culture (Figure 4.8 and Figure 4.9). While the cells were being counted, only the colonies were considered in order to be sure that the cells counted were MSCs.

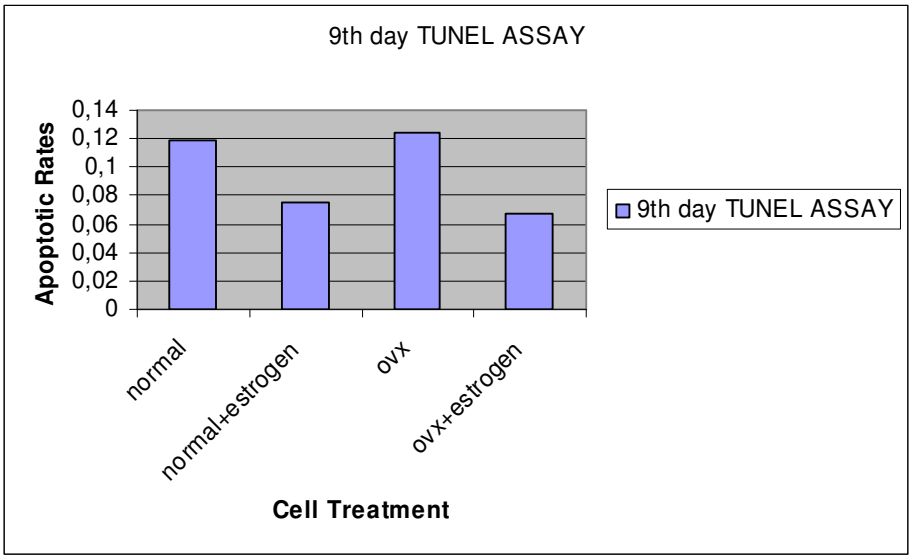


Figure 4.8 The apoptotic cell ratios on the 9th day cell culture
The apoptotic cell ratios of the ovx and normal groups treated with and without estrogen on the 9th day are compared in the figure. The number of TUNEL positive cells were divided to the total number of colonies**.**

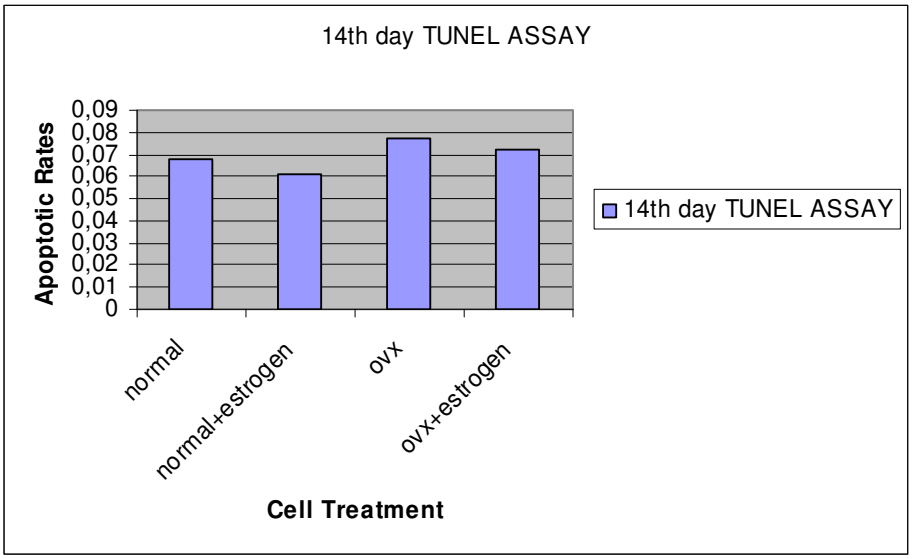


Figure 4.9 The apoptotic cell ratios on the 14th day cell culture
The apoptotic cell ratios of the ovx and normal groups treated with and without estrogen on the 14th day are compared in the figure. The number of TUNEL positive cells were divided to the total number of colonies

Our results showed that both on the 9th day and 14th day of cultures, the rate of apoptotic cells have decreased with the addition of estrogen in the normal and ovx groups. This decrease is more evident in the 9th day groups. The number of apoptotic cells in the estrogen treated groups, are almost half the ratio of the cells without any estrogen treatment. These results are parallel with the colony numbers from the CFU assay in which the increase in the number of colonies were approximately two-fold in the estrogen treated groups on the 9th day of the cell culture. Considering that only MSC colonies were counted in both experiments, throughout the formation of MSC colonies estrogen increases the proliferation rate and formation of colonies while decreasing the apoptotic rate. Estrogen is effective on the 14th day cell culture by increasing the colony numbers and decreasing the apoptotic rates, but it is more effective on the 9th day cultures.

The correlation between the decrease of the apoptotic rates of MSCs with the increase in the colonies with estrogen treatment, suggests that estrogen regulates the number of MSCs through the apoptotic pathway. The most commonly studied

regulators of apoptosis which are also affected by estrogen are the *bcl-2* family of genes. Due to this reason, the effects of estrogen on the expressions of the *bcl-2* family of genes were examined in order to see the whether they are a part of this regulation mechanism of MSCs.

4.4. Gene Expression

The expressions of the *bcl-2* family of genes were investigated both at the mRNA and the protein level. Among the members of the *bcl-2* family of genes; two anti-apoptotic genes; *bcl-2* and *bcl-*x_L, in addition to two pro-apoptotic genes; *bak* and *bax* were chosen as candidates in the regulation of MSCs. These genes are the most intensively studied *bcl-2* family members in mammals and are shown to have ERE. They also have been shown to dimerize in the mammalian systems.

4.4.1. Expression of the *bcl-2* gene family in MSC Maintenance

4.4.1.1. mRNA Isolation and RT-PCR

The total RNAs of the normal and ovx groups treated with and without estrogen were isolated on the 9th and 14th days of the cell culture in order to detect the expression levels of the candidate genes *bak, bax, bcl-*x_L, and *bcl-2*. The concentrations of the total RNAs were measured with the spectrophotometer, and the mRNAs were run on agarose gel in order to check their intensity and purity (Figure 4.10 A). Then cDNAs were synthesized, and the expressions of *bak, bax, bcl-*x_L, *bcl-2* genes were examined with PCR. PCR conditions were optimized before performing further experiments with normal group samples (Figure 4.10 B-E).

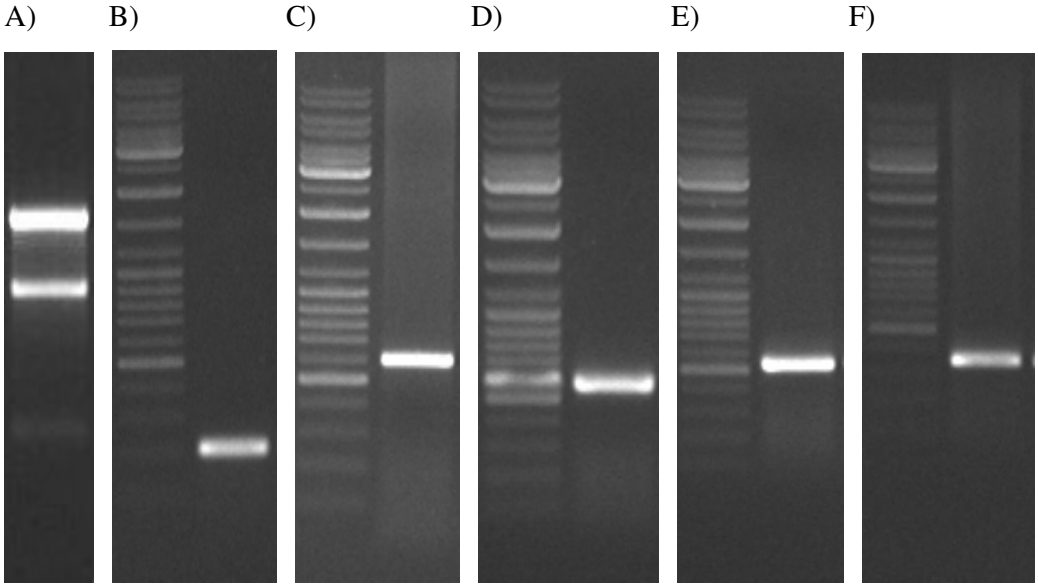


Figure 4.10 The isolation of mRNA and the optimization of PCR cycles
A) In pure mRNA; the 28S, 14S, and 5S bands are observed without any smear. The RNAs isolated all gave clear bands and the measurements of the spectrometer was confirmed by the gel picture. B) The PCR product of *gapdh*, 210 bp C) The PCR product of *bcl-2*, 584 bp D) The PCR product of *bax*, 578 bp E) The PCR product of *bcl-*$x_{L,}$ 346 bp

4.4.1.2. Semi-Quantitative PCR

After the optimization of the cycles, PCR was performed with the normal and ovx groups with and without estrogen treatment on the 9th and 14th days of the cell culture, by using primers for *gapdh, bax, bcl-2, bak,* and *bcl-*x_L. PCR results of the *bcl-2* gene family were normalized according to the *gapdh* PCRs (Figure 4.11).

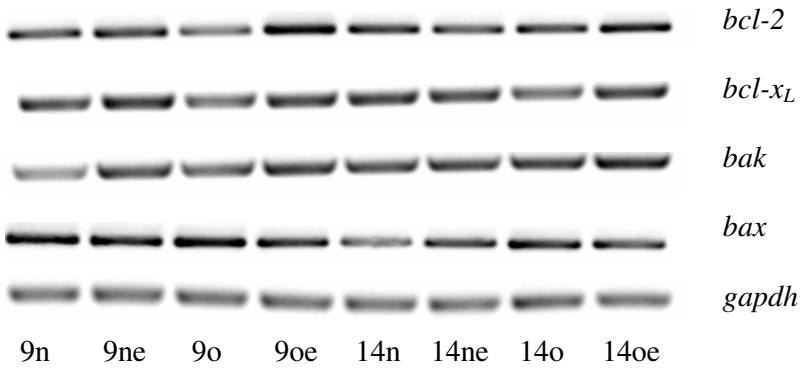
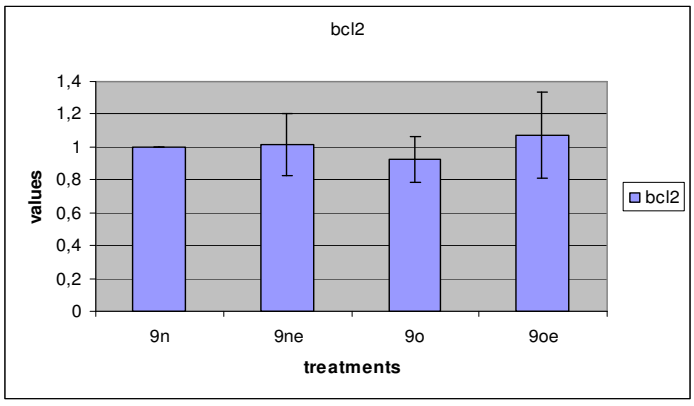


Figure 4.11 The agarose gel picture of the PCR products of the *bcl-2* family of genes For the semi-quantitative analysis of the genes, the expression of the bcl-2 family of genes on the 9th and 14th day in normal, normal+estrogen, ovx (o), and ovx+estrogen (oe) groups were selected for each set of cDNAs and evaluated

Every set was normalized within itself for the evaluation of the gene expression results. After the normalization was made, the 9th day normal values and 14th day normal values were considered as 1 and the ratios of all the other treatment groups were taken according to the normal values of the 9th day and 14th day. This was made to have a standard in the averages, and to evaluate the decrease and increase in the ratios depending on estrogen treatments over the standard 1. The standard deviations were also calculated and the expression results were compared within each gene both on the 9th day (Figure 4.12) and on the 14th day (Figure 4.13).



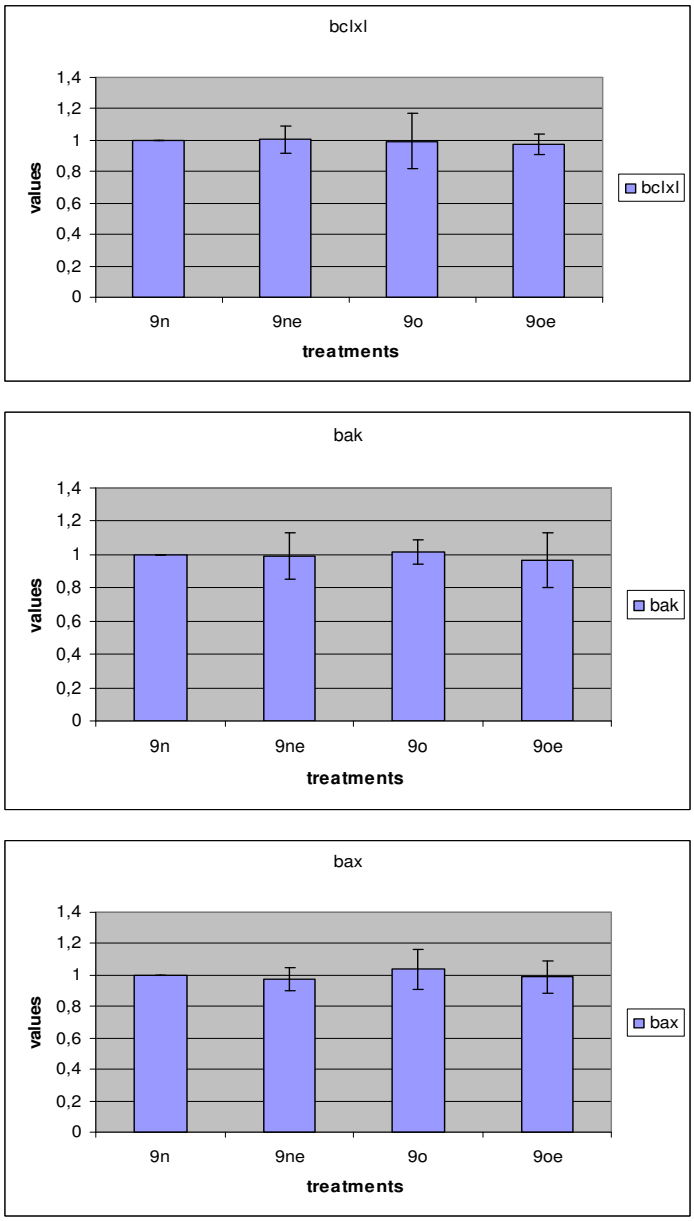
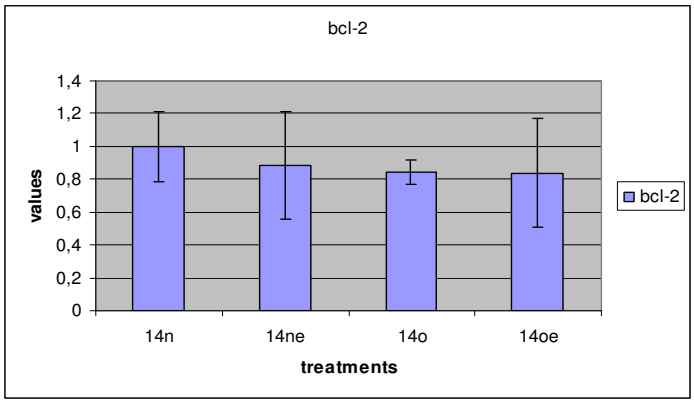
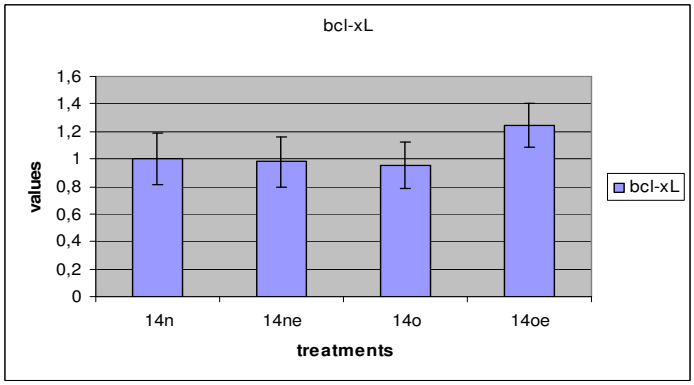
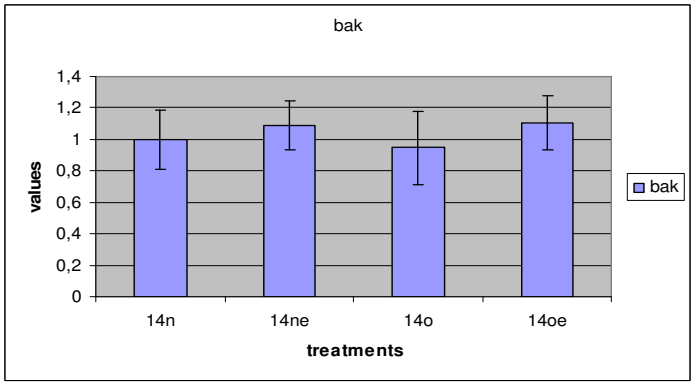


Figure 4.12 The expression graphs of *bcl-2* family of genes on the 9th day cell culture groups A) The expression profile of *bcl-2* B)The expression profile of *bcl-x*$_L$ C) The expression profile of *bak* D)The expression profile of *bax*


bcl-2
1,4
1,2
1
0,8
0,6
0,4
0,2
0
values
14n
14ne
14o
14oe
treatments
bcl-2


bcl-xL
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
values
14n
14ne
14o
14oe
treatments
bcl-xL


bak
1,4
1,2
1
0,8
0,6
0,4
0,2
0
values
14n
14ne
14o
14oe
treatments
bak

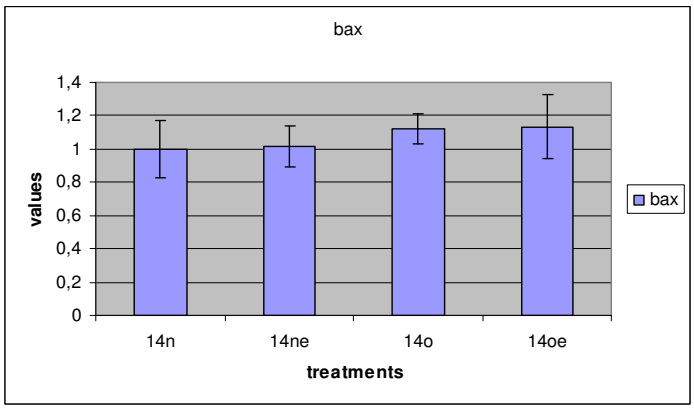

bax
1,4
1,2
1
0,8
0,6
0,4
0,2
0
values
14n
14ne
14o
14oe
treatments
bax

Figure 4.13 The expression graphs of *bcl-2* family of genes on the 14th day cell culture groups A) The expression profile of *bcl-2* B)The expression profile of *bcl-x_L* C) The expression profile of *bak* D)The expression profile of *bax*

Bcl-2 gene expression, which is an anti-apoptotic gene, has increased in the normal and ovx groups on the 9th day with the addition of estrogen (Figure 4.12A). The expression of the *bcl-x_L* gene has slightly increased with the addition of estrogen on the 9th day in the normal group but has slightly decreased in the ovx group (Figure 4.12B). The expression of *bak*, which is a pro-apoptotic gene, has decreased with the addition of estrogen on the 9th day normal and ovx groups (Figure 4.12C). However *bax,* which is also a pro-apoptotic gene, has decreased with the addition of estrogen in both the normal and in the ovx groups on the 9th day (Figure 4.12D). On the 14th day, estrogen has decreased the expression of the *bcl-2* gene both in the normal and ovx groups (Figure 4.13A). The expression of *bcl-x_L* has decreased in the normal group and there was an increase in the ovx group with estrogen treatment on the 14th day (Figure 4.13B). *Bak* expression has increased with the addition of estrogen on the 14th day normal and ovx groups (Figure 4.13C). Also the addition of estrogen has increased the expression of the *bax* gene in the normal and ovx group similar to bak on the 14th day (Figure 4.13D). Although the decreases and increases in the gene expressions are noted by comparing the ratio bars, when the standard deviations are considered, these oscillations are not very crucial in the overall gene expression profiles. This suggests that there is no transcriptional regulation in MSCs by estrogen.

4.4.2. Expression of Bcl-2 family of Proteins

4.4.2.1. Immunofluorescent Staining (IF)

We first performed IF to check the presence of Bcl-2 family of proteins on the 9th and 14th days of normal, normal+estrogen, ovx, and ovx+estrogen groups of MSC cultures (Figure 4.14-4.17). In addition to the Bcl-2 family of proteins, CD90 expression was checked in order to confirm that the cells and colonies are MSC

colonies, since CD90 is a MSC marker (Figure 4.18). Negative controls, in which no primary antibody was added, were included in each group both on the 9th and the 14th day to confirm the specifity of the antibodies (Figure 4.19). Our IF results showed that, all of the Bcl-2 family of proteins that were tested were present in all groups of the 9th and 14th day cell cultures. We also confirmed that these cells were MSCs by detecting the CD90 expression in these colonies.

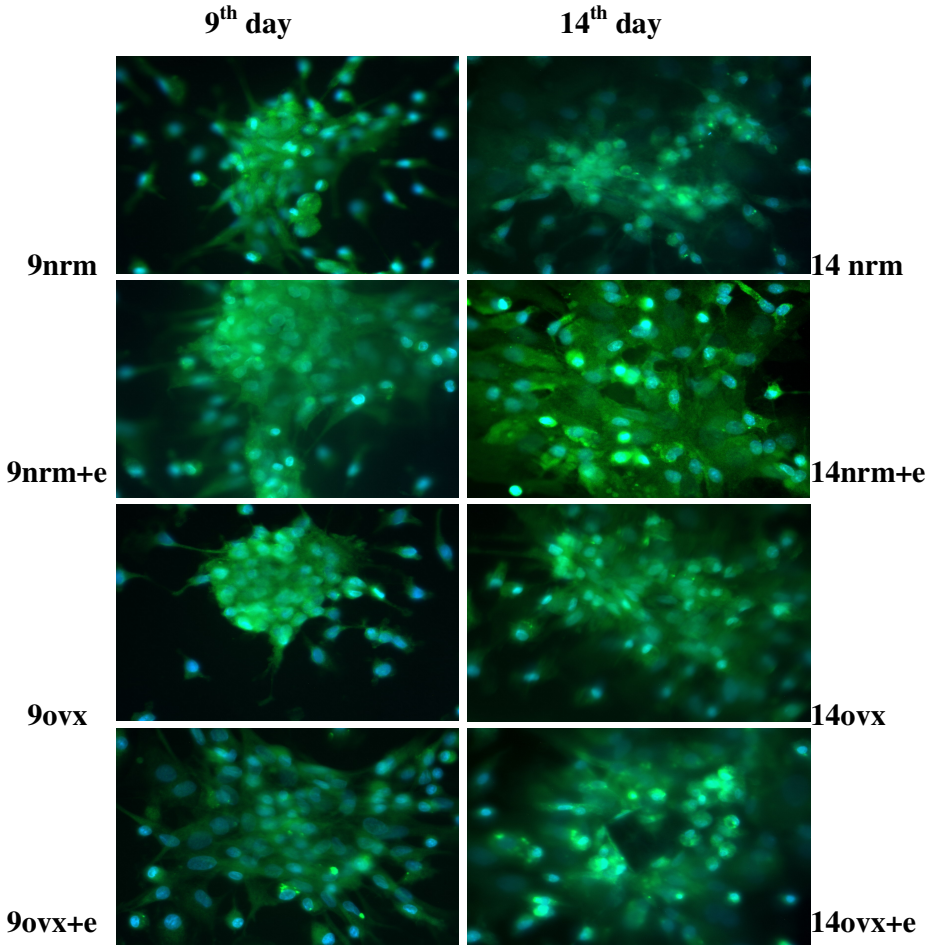


Figure 4.14 Expression of Bcl-2 on 9th day and 14th day culture groups

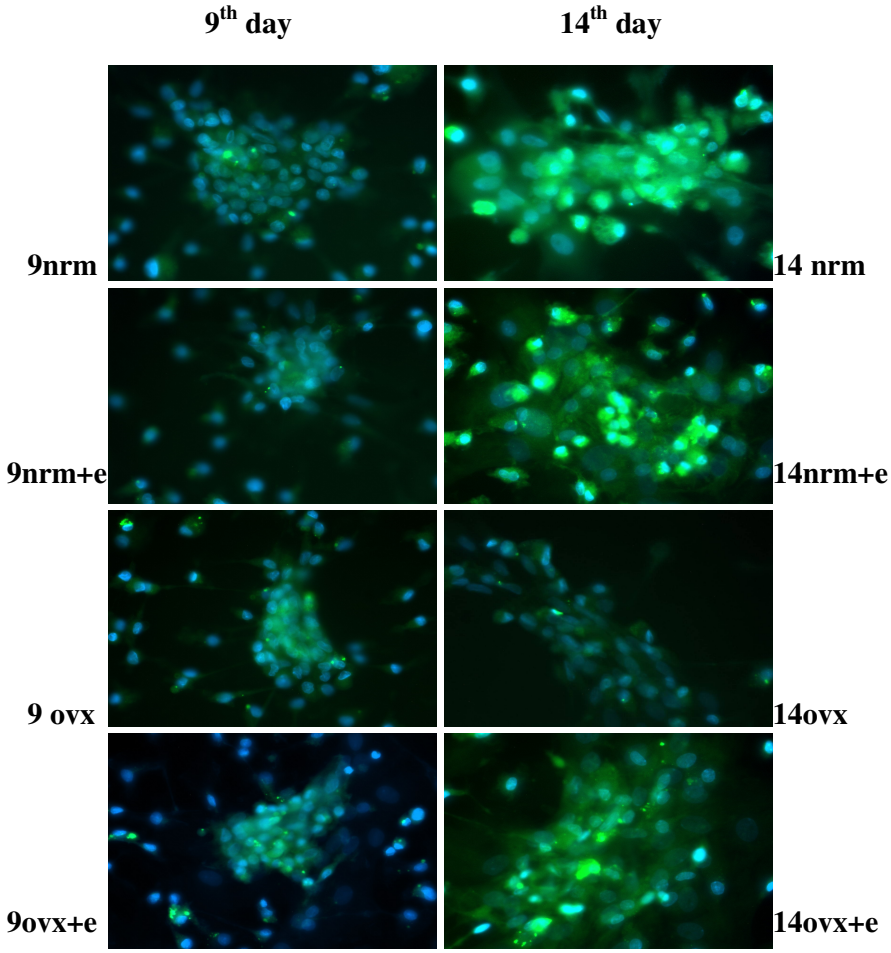


Figure 4.15 Expression of Bcl-x_L on 9th day and 14th day culture groups

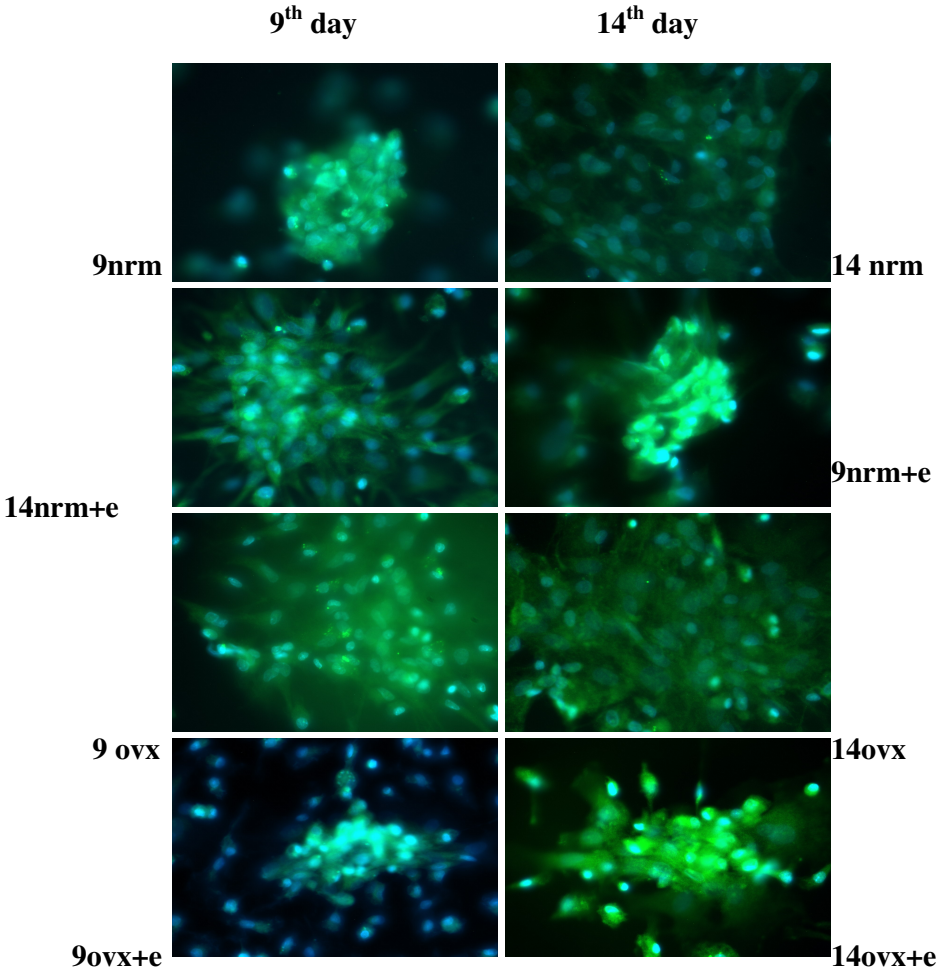


Figure 4.16 Expression of Bak on 9th day and 14th day culture groups

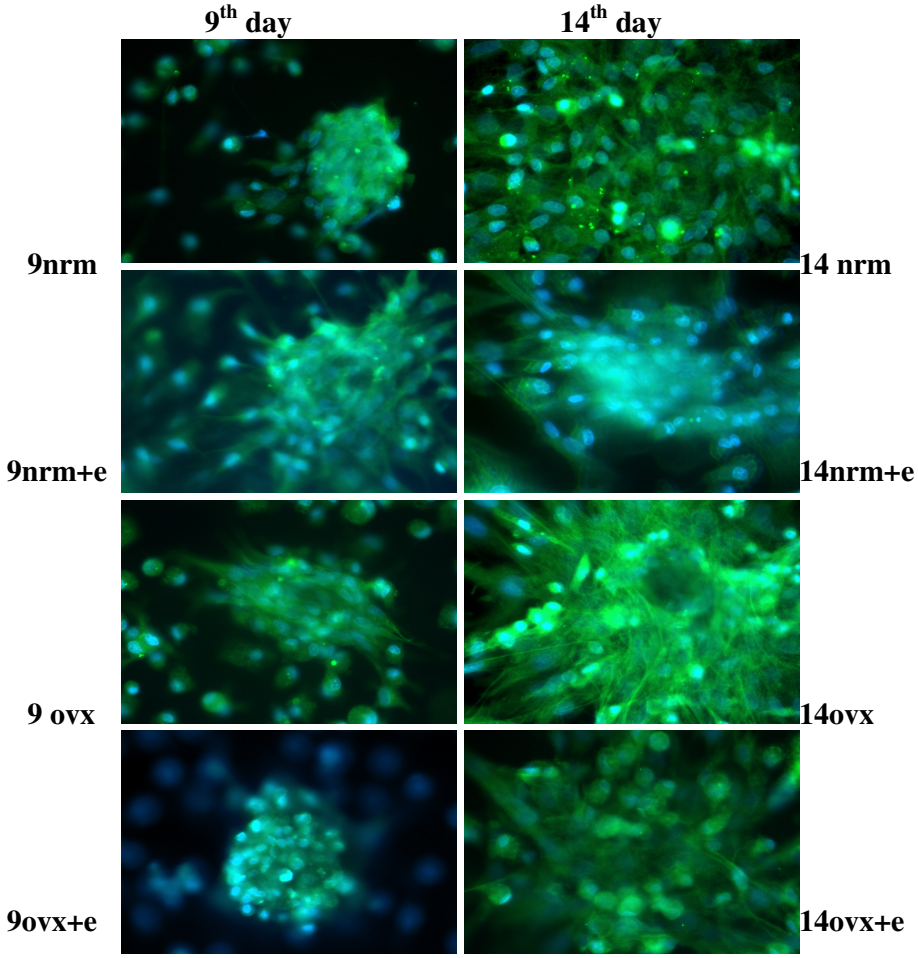
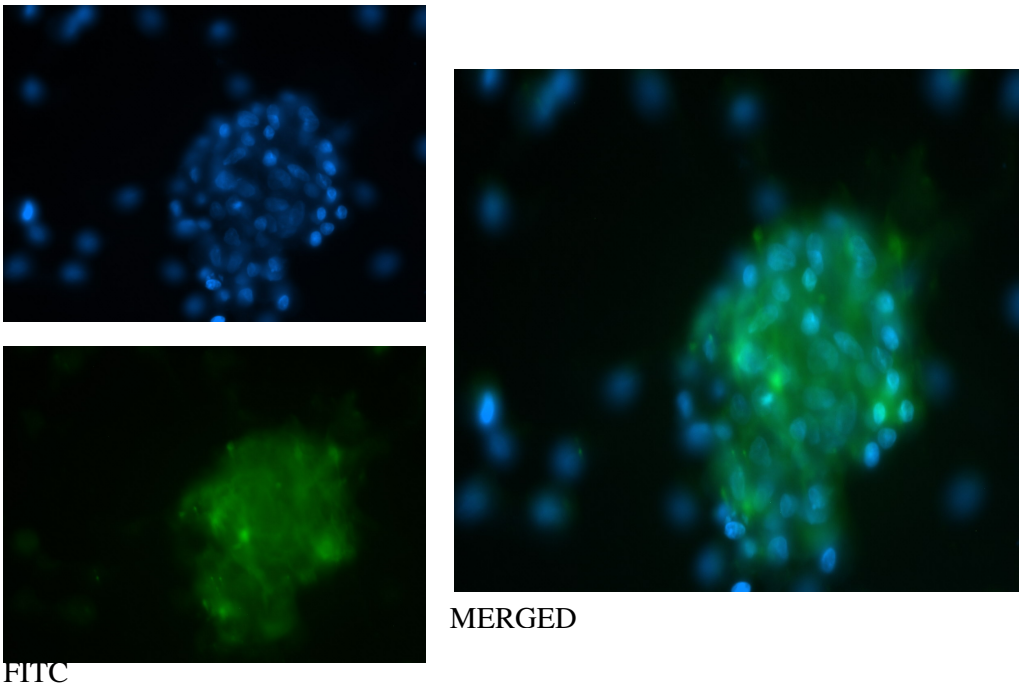


Figure 4.17 Expression of Bax on 9th day and 14th day culture groups

DAPI

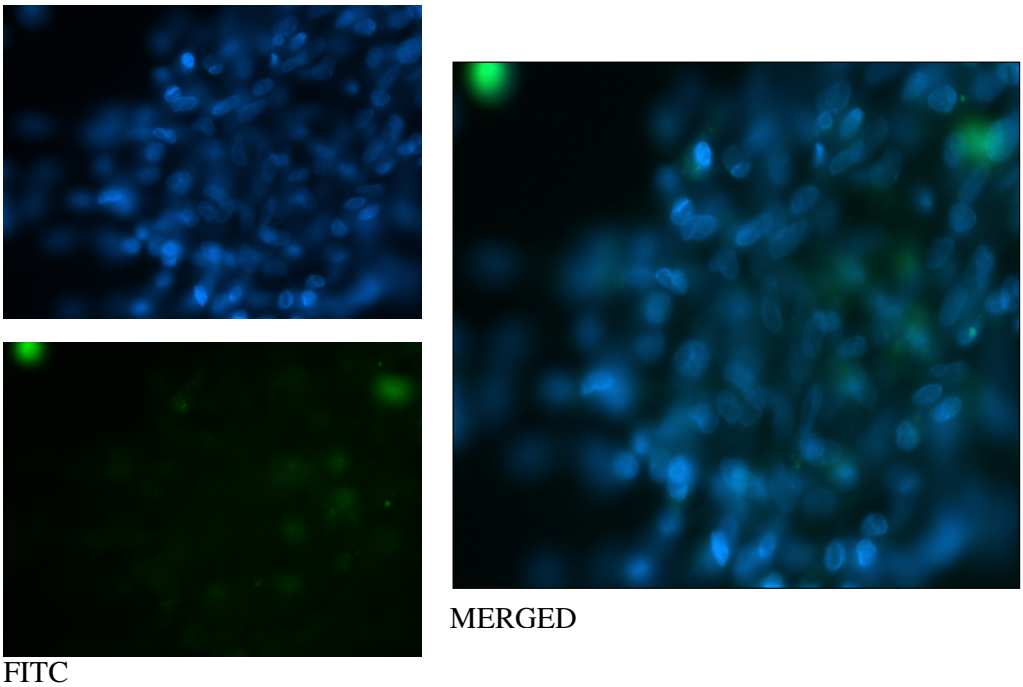


MERGED

FITC

Figure 4.18 CD90 expression in MSC colonies Total magnification of 400X

DAPI



MERGED

FITC

Figure 4.19 Negative sample of MSC colonies Total magnification of 400X

4.4.2.2. Western Blot

We performed western blot analysis in order to quantify the protein expressions that were shown in the IF assays. Proteins isolated from normal, normal+estrogen, ovx, and ovx+estrogen groups of the 9th and 14th day of the culture were loaded to the SDS-PAGE gel and were evaluated separately. Western blot was performed by using antibodies against Bcl-2, Bak, Bax, and Bcl-x_L. Loadings were controlled by staining the membrane with coomassie blue staining after the membranes were developed.

Our results showed that on the 9th day of the culture, the expression of Bcl-2 was approximately the same in the normal group with the addition of estrogen but in the ovx group the expression of Bcl-2 has decreased with the addition of estrogen (Figure 4.20). On the other hand Bcl-x_L expression was the same with the addition of estrogen both in the normal group and ovx group on the 9th day of cell culture (Figure 4.21). Bak has increased in the normal group of the 9th day with estrogen treatment but was approximately the same in the ovx group (Figure 4.22). The expression of Bax has increased both in the normal and ovx group with the addition of estrogen (Figure 4.23). Our data on the expression of Bcl-2 family of proteins on the 9th day of MSC culture with and without estrogen treatment did not reveal a correlation as we expected. This might be due to other cells and their signals interfering with MSCs, or the difference in the signals throughout the formation period of the MSC colonies.

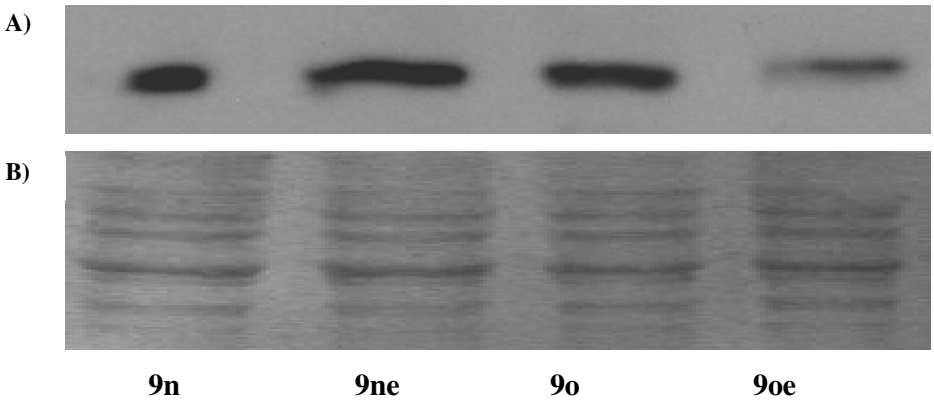


Figure 4.20 Bcl-2 expression on the 9th day of cell culture
A) Western blot results of the Bcl-2 protein expression on the 9th day n, n+e, ovx, ovx+e groups B) Equal loading of 20ng of protein from 9th day groups

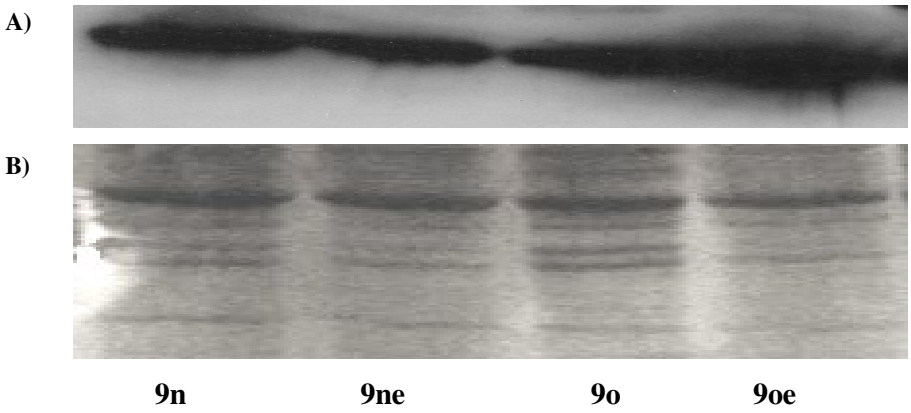


Figure 4.21 Bcl-x_L expression on the 9th day of cell culture
A) Western blot results of the Bcl-x_L protein expression on the 9th day n, n+e, ovx, ovx+e groups B) Equal loading of 40ng of protein from 9th day groups

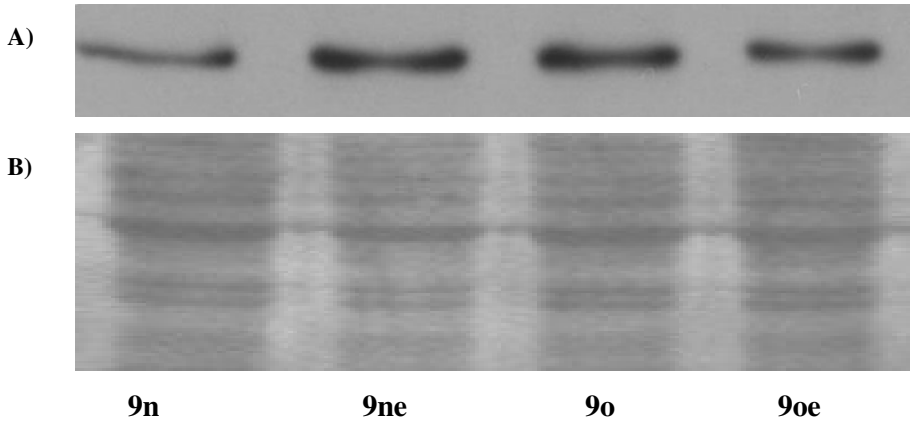


Figure 4.22 Bak expression on the 9th day of cell culture
A) Western blot results of the Bak protein expression on the 9th day n, n+e, ovx, ovx+e groups B) Equal loading of 20ng of protein from 9th day groups

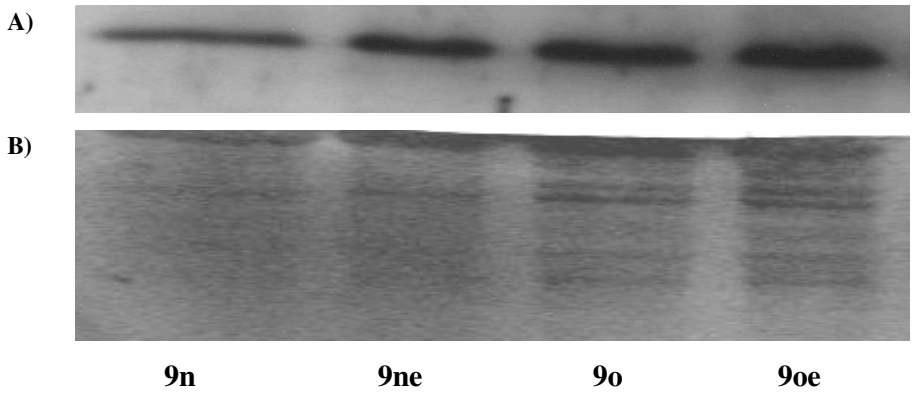


Figure 4.23 Bax expression on the 9th day of cell culture
A) Western blot results of the Bax protein expression on the 9th day n, n+e, ovx, ovx+e groups B) Equal loading of 40ng of protein from 9th day groups

On the 14th day of cell culture, we observed an expression pattern of the Bcl-2 family of proteins that supported our hypothesis. Among these proteins, the expressions of Bcl-2 and Bcl-$x_{L,}$ have increased when the MSCs were cultured with estrogen in the normal groups (Figure 4.24, 4.25). This suggests that these genes are candidates for increasing the proliferation and decreasing the apoptotic rate in MSCs. In addition the expression of Bak, decreases with the addition of estrogen in the normal group (Figure 4.26). This result also assigns a role as a candidate gene in MSC regulation. In the ovx groups; Bak expression decreases (Figure 4.26) and Bcl-x_L decreases with the addition of estrogen (Figure 4.25) which is in correlation with their normal groups. However, Bcl-2 protein expression decreases in the ovx group

with the addition of estrogen on the 14th day, but since the ovx group is deprived of estrogen for a certain period, this might affect the estrogen susceptibility. Bax expression on the other hand has not changed much in the normal group and has increased in the ovx group with estrogen treatment (Figure 4.27). These results suggest that Bcl-2, Bcl-$x_{L,}$ and Bak are potential candidates for the regulation of MSCs by estrogen, while Bax is not affected by estrogen in our system as expected, so it is not considered as a candidate gene in MSC regulation.

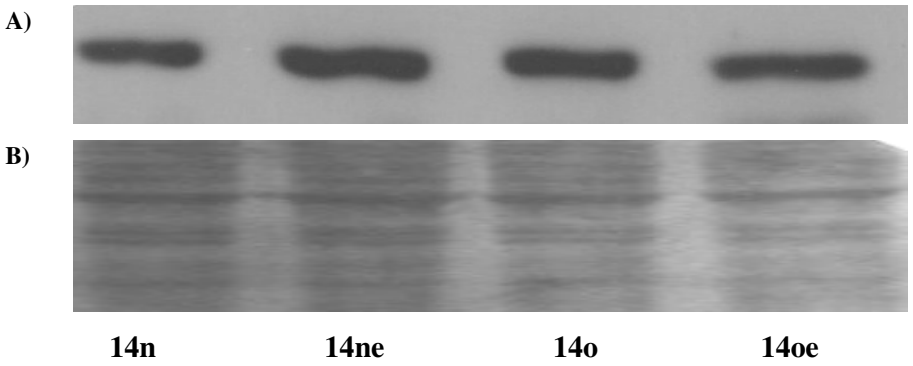


Figure 4.24 Bcl-2 expression on the 14th day of cell culture
A) Western blot results of the Bcl-2 protein expression on the 14th day n, n+e, ovx, ovx+e groups B) Equal loading of 20ng of protein from 14th day groups

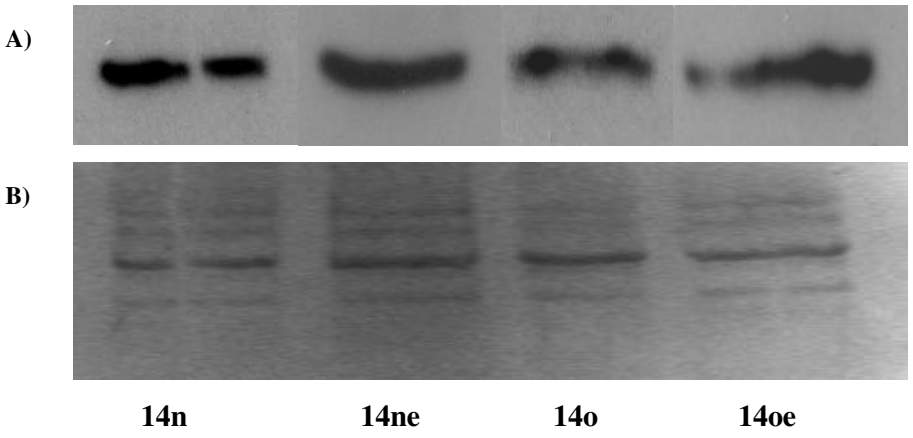


Figure 4.25 Bcl-x_L expression on the 14th day of cell culture
A) Western blot results of the Bcl-x_L protein expression on the 14th day n, n+e, ovx, ovx+e groups B) Equal loading of 40ng of protein from 14th day groups

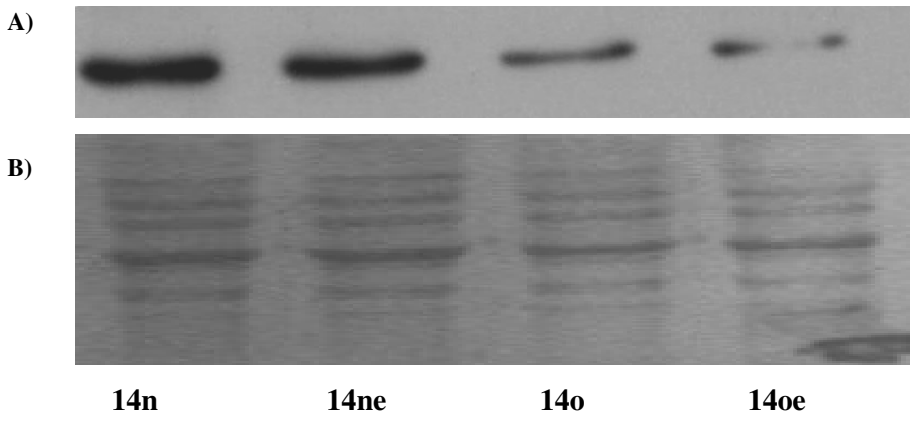


Figure 4.26 Bak expression on the 14th day of cell culture
A) Western blot results of the Bak protein expression on the 14th day n, n+e, ovx, ovx+e groups B) Equal loading of 20ng of protein from 14th day groups

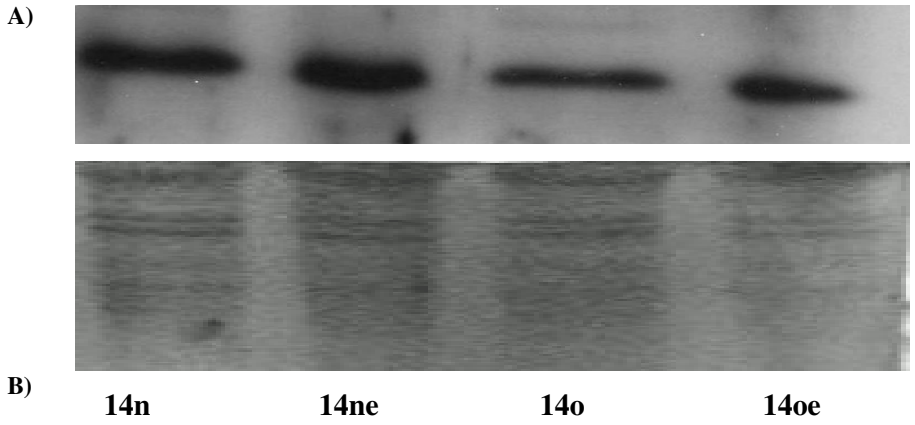


Figure 4.27 Bax expression on the 14th day of cell culture
A) Western blot results of the Bax protein expression on the 14th day n, n+e, ovx, ovx+e groups B) Equal loading of 40ng of protein from 14th day groups

5. DISCUSSION

Stem cells are the new hope for the century since they have the potential to differentiate to several types of cells and tissues. They are future candidates for many therapeutic applications and can be the cure for many diseases such as neurodegenerative diseases, diabetes, and cancer (Fuchs and Segre, 2000). MSCs are ASCs which can differentiate to osteoblasts, chondrocytes, adipocytes, myocytes, cartilage, tendon, muscle, ad fat tissue (Baksh *et al*, 2004; Caplan and Bruder, 2001). One of the major issues is that they form a very small population of nucleated cells in the bone marrow and their number has to be increased before they can be used for therapeutic purposes. There are several factors increasing cellular proliferation, and estrogen is one of them.

Estrogen regulates many pathways vital for the homeostasis of the body and its loss or decrease results in serious health problems such as osteoporosis, arteriosclerosis, increase in coronary heart diseases and neurodegenerative diseases. It has also been shown that estrogen reduces apoptosis when apoptosis is induced, and increases the proliferation rate in MSCs in other systems (Zhou *et al*, 2001).

Apoptosis is a conserved mechanism throughout evolution and any disruption in the apoptotic pathway results in severe problems such as neurodegenerative diseases, cancer, autoimmune disorders, and viral infections. Mitochondrion is one of the key regulators of the apoptotic pathway and cytochrome *c* is one of the most important apoptotic factors which activate the caspase cascade. When cytochrome *c* is released, it leads to apoptosis and the members of the *bcl-2* family of genes are the main molecules that regulate its release from the mitochondria. Estrogen differentially affects the expression of bcl-2 family members. It increases Bcl-2 and Bcl-x_L expression which are anti-apoptotic members whereas it decreases Bax and Bak expression (Alkayed *et al,* 2001). It is not clear though whether estrogen affects the *bcl-2* family of genes directly or indirectly, but estrogen is crucial in the regulation of their expression (Dubal *et al*, 1999; Pike, 1999).

The effect of estrogen on the *bcl-2* family of genes and MSC cell proliferation makes apoptosis a strong candidate in MSC maintenance. Therefore our main aim was to find out the effects of estrogen on MSC maintenance, that is on the proliferation rate and apoptosis. We also hypothesize that estrogen regulates the expression of *bcl-2* family of genes and this controls apoptosis.

In order to reach our aim we took the following approach; bone marrow cells were isolated and cultured for 14 days with MSC media. According to this protocol, culturing for 14 days makes these cells ready for differentiation. However we performed all our experiments on day 9 and day 14 of the cell culture. The reason is that the 9th day was considered as a step in the process of MSC colony formation and we wanted to check the regulation throughout the formation of MSCs. It is also important to note that there is not any well-defined marker to identify MSCs in the bone marrow cells at the time of isolation. 9th day cell cultures contain not only MSCs but also other adherent fibroblastic cells. Therefore, in the experiments where all cells are isolated such as cell counting, mRNA expression, and Western blot on the 9th day cell cultures, we can not rule out the possibility of other adherent single cells interfering with the results. The cells on the 14th day, however, are mainly composed of MSC colonies and the data obtained from the 14th day culture give a more accurate picture regarding to the nature of MSCs.

In order to detect the effect of estrogen on MSC number, the adherent cells in the tissue culture were counted on the 9th day and 14th days of the tissue culture. On the 14th day, estrogen increased the number of both the normal and ovx group which suggests that estrogen has an effect on MSC number both in estrogen deprived conditions, which is the ovx group, and the normal group. On the 9th day, estrogen treatment increased the number of the normal cells but the ovx group was not affected by estrogen. This might indicate that in the process of MSC formation, cells from ovx animals are not affected by estrogen since they are deprived of estrogen completely for at least 2 months. A 9 day period might not be enough for them to increase their proliferation rate since their estrogen level was almost zero for a long time. On the other hand, normal cells on the 9th day were more susceptible to the

effects of estrogen since they have a basal amount of estrogen and it is easier for them to increase their proliferation rate. Also it should be considered that other types of cells in addition to MSCs are isolated on the 9th day due to other single cells adherent to the culture plate existed on the 9th day, so they might affect the numbers counted, since all the cells in the cell culture plate have to be isolated for cell counting.

To see if the increasing number of cells are MSCs, colony forming assay was made on the 9th and 14th days. Although on the 9th day there were only a few colonies with few number of cells, it was observed that both on the 9th day and the 14th day the colony number increased with the addition of estrogen both in the normal and ovx groups. In some samples the colony number of the normal group treated with estrogen increased compared to the untreated group on the 14th day. On the 9th day ovx group only, the addition of estrogen did not seem to affect the cell number, but there was an increase in the colony number, suggesting the induction of MSC formation. Also it is possible that estrogen increased the MSC cell number but since there were other cells in the culture, the increase in MSCs could not be seen in the total cell number increase. In general these results were in correlation with the cell numbers counted, so we can conclude that estrogen increases the MSC number and the colonies it forms throughout MSC maintenance. These results are also in accordance with Zhou *et al*, 2001, which has shown that MSCs increase in number with estrogen treatment.

To check estrogen's role in MSCs maintenance, 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, both on the 9th day and 14th day cell cultures, the addition of estrogen decreased the death ratios in the normal and ovx groups. Especially on the 9th day the apoptotic decrease rates were the same as the increase on the 9th day colony forming rates, this suggested that estrogen is effective in the process of MSC colony formation both by increasing proliferation and decreasing apoptosis. It is important to note that in our system, we

did not induce apoptosis with any factor. Therefore our apoptotic rate of 10% seems to be the physiological effect of estrogen on MSCs.

It is observed that the 9th day cell culture colonies are more susceptible to estrogen and the number of colonies increase with a higher rate compared to the 14th day cell cultures. Similarly the apoptotic rates decrease with a higher rate in the 9th day culture cells compared to the 14th day culture cells with the addition of estrogen. On the 9th day MSCs are in a tendency of increasing their number and on the 14th day they are ready to differentiate to other cell types, suggesting that MSCs perform more symmetrical divisions throughout the process of colony forming on the 9th day and more asymmetrical divisions on the 14th day cell cultures. From these results, it is tempting to speculate that estrogen has a more profound affect on symmetrical divisions and is less effective in the process of asymmetrical division in MSC maintenance. The correlation between these is a future aspect to be checked.

After analyzing the effect of estrogen on MSC proliferation and apoptosis, the next step was to see whether this was via the *bcl-2* family of genes. First of all the gene expressions of *bcl-2, bcl-x$_L$, bak,* and *bax* were checked on the mRNA level. All groups expressed the *bcl-2* family of genes both on the 9th day and on the 14th day in variable levels. The average of at least 3 different groups were taken for semi-quantitative PCR analysis, and although there were slight oscillations in the gene expression levels on different days and groups, when the standard deviations were considered, the increases and decreases were not very significant. This suggested that the *bcl-2* family of genes were expressed but they were not affected much by estrogen on the transcriptional level.

In addition to the mRNA expression profile of *bcl-2* family of genes, we also examined the expression of Bcl-2, Bcl-x$_L$, Bak, Bax proteins on the groups treated with and without estrogen. In the IF experiments all the groups both on the 9th and 14th days of the cell culture expressed these proteins so western blot was performed to quantitate their expression levels. The 9th day was again used as a group to see the expression profile throughout the formation of MSCs but our main aim was to see the 14th day cell culture results in which MSCs have fully formed. Our western blot

results showed that all the proteins were differentially expressed in all groups both on the 9th and 14th days of the cell culture. However we did not observe a stable pattern of expression where anti-apoptotic members increased and pro-apoptotic members decreased with estrogen addition on the 9th day. During the isolation of both mRNAs and proteins on the 9th day, many other cells adherent to the tissue culture which were not MSCs were isolated together with MSCs as mentioned before. The signals from the other cells might have affected the protein and gene expression, so the results are not very dependable. The 14th day expressions were more dependable and important since the majority of the cells were MSCs.

On the 14th day cell culture, the normal + estrogen expressions of Bcl-2, and Bcl-x_L which are anti-apoptotic members of the *bcl-2* family of genes, have increased compared to the normal expressions, while the expression of Bak which is a pro-apoptotic member has decreased with the addition of estrogen. The addition of estrogen had a similar affect on the ovx group compared to the normal group in the Bcl-x_L and Bak expressions on the 14th day culture. The Bcl-2 protein expression did not increase in the ovx group on the 14th day, which might be because of the loss of estrogen susceptibility due to a long period in an estrogen deprived environment. The expression of our 4th candidate protein Bax, was approximately the same with or without the addition of estrogen in the normal groups, and has increased in the ovx group with the addition of estrogen. Bax, different from the other candidate members, is cytosolic and does not go to the mitochondria unless apoptosis is induced. In our experiments there was no stimulus to induce apoptosis, so Bax might be present in the cells but not active (Wolter *et al*, 1997). In cases of starvation or other stresses, the expression levels of Bax might be different (Zhu *et al*, 2006). As a result; Bcl-2, Bcl-$x_{L,}$ and Bak seem to be strong candidates of the Bcl-2 family of proteins which are affected by estrogen on the translational level in MSC regulation, and estrogen might be regulating the apoptotic and proliferative activity by increasing the level of Bcl-2 and Bcl-x_L proteins whereas decreasing the level of Bak. Since the Bcl-2 family of proteins function as homo- and heterodimers, these results suggest that these anti-apoptotic and pro-apoptotic candidates might be forming dimers among themselves. 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 some members of the *bcl-2* family of genes are shown to be important in the differentiation and regulation pathways (Potten *et al*, 1997; Polakowska *et al*, 2005; Domen *et al*, 2000).

The reason for observing differential expression at the protein level but not at the mRNA level might be post-transcriptional and transcriptional modifications on the *bcl-2* family of genes such as phosphorylation, dephosphorylation, dimerization, translocation, cleavage, chromatin remodeling, etc. (Tsujimoto and Shimizu, 2000). These may be caused either by estrogen or other regulatory pathways.

The effect of estrogen on some other genes has also been studied in MSCs such as BMP-2, ALP, type I collagen, cbfa1, and TGFβ-1 on the mRNA level. These are mostly osteogenic markers and the addition of estrogen has increased the expression of those genes both in the normal and ovx groups, suggesting that estrogen might have a role not only in the decrease of apoptosis and increase in proliferation, but also differentiation and osteogenic potential (Zhou *et al*, 2001). It should be noted though, that these results are at the transcriptional level and the protein expressions should also be checked because they might not be parallel to mRNA results as in our experiments. These results might be important in the treatment of osteoporosis and bone fractures.

In general we can conclude that apoptosis is one of the main regulatory mechanisms in the maintenance and proliferation rate of MSCs. Bcl-2, Bak, and Bcl-x_L are strong candidates in this regulation and estrogen affects the expression rates of these proteins, increasing the colonies and decreasing the apoptotic rate.

6. FUTURE STUDIES

Further studies can be made on the regulation of *bcl-2* family of genes on MSC maintenance and proliferation. Our candidate genes *bcl-x_L*, *bcl-2*, and *bak* can both be silenced and over expressed in order to confirm that the regulation of estrogen is via these genes. If the MSC colony numbers change according to the silencing and over expression of these genes, this might suggest that with estrogen treatment the anti-apoptotic candidate genes can be increased and MSC number can become sufficient for treatments. Our lab is performing these experiments. In addition, the differentiation capacity of the MSCs obtained by the over expression of anti-apoptotic genes or silencing of the pro-apoptotic genes can be tested to see whether the efficiency and differentiation potential has changed or not. Other than Bcl-2 family members, the effects of estrogen on ERα and ERβ expressions in MSCs can also be tested in addition to the Bcl-2 family of proteins. In other studies it has been shown that estrogen has an effect of ER expressions in the bone marrow and MSCs, so the coordination of the ER expressions with the Bcl-2 family of proteins can be tested (Zhou *et al*, 2001).

Since we hypothesize that our candidate genes are regulated on the translational level, the possible mechanisms behind this regulation might be studied in detail. If estrogen does not change the expressions of these genes on the mRNA level significantly, there might be post-transcriptional modifications effecting the protein expressions. These possible modifications can be studied and checked on whether they are effective in the expression of *bcl-2* family of genes in the presence and absence of estrogen.

Besides the expressions of the members of *bcl-2* family of genes, the expressions of upstream and downstream molecules can be checked. For example the tumor suppressor p53 gene can be checked which is known to be a regulator of bcl-2 and bax both *in vitro* and *in vivo* (Miyashita *et al*, 1994). Also the expressions

of the downstream molecules of the *bcl-2* family of genes might be checked such as initiator and effector caspases.

The MSC number in cell cultures can be increased for a higher efficiency in the experiments. The main problem especially on the 9th day experiments was having very few colonies with small number of cells, and unidentified single cells being also adherent to the plate. Therefore especially in the colony forming assay, IF experiment, and TUNEL assay, only the colonies were considered as MSCs but it was not possible to eliminate the other cells. Due to this reason the 9th day experiments and results were not dependable in these experiments. There is no marker to identify MSC *in vivo*, the markers which MSCs express are common with the other cells adhering to the plates such as fibroblasts and due to this reason are not very helpful in their selection. Specific markers such as cd90 are not expressed in MSCs until 4 or 5 days have passed after culturing them, so it is really difficult to plate pure MSCs at the very beginning of the cell culture. Maybe with dynabeads HSCs, with negative selection HSCs can be removed at the very beginning of the cell culture by using specific markers. Purifying MSC at the very beginning would increase the preciseness of the experiments.

Also the effects of estrogen on MSC number and its efficiency in treatment should be tested *in vivo*. The MSCs, in which the anti-apoptotic genes are overexpressed and pro-apoptotic genes are silenced, can be labeled and injected to rats. Since MSCs help in the forming of bone and muscle cells, the rats which have muscle injuries, and bone fractures might be used to find out the differences between the recovery rates and the contribution of the injected MSCs to the injury sites compared to the control groups. This can be done both in normal and ovx animals.

In addition to the effects of estrogen, the effects of estrogen antagonists such as tamoxifen, raloxifene, and ICI can be tested both in normal and ovx groups in the presence and absence of estrogen in order to see the changes in expression. Some antagonists also act like agonists depending on the presence of absence of estrogen

so these effects can also be observed to see if a higher yield of cells can be obtained with these combinations.

Other experimental conditions and their affects on MSC efficiency with estrogen addition can be observed by testing the effect of isolating MSCs at different passage numbers since it is known that passaging MSCs changes their differentiation potential. In addition MSCs isolated from different sources such as adipose tissue can be tested to see if their efficiency is the same as the ones isolated from the bone marrow.

In general if the increase of MSC number can be accomplished *in vitro* and *in vivo* in shorter time periods, it would be very useful for MSC based treatments. The best way to accomplish this is to figure out the mechanism which regulates their proliferation and interfere with it in order to increase this rate. Although further studies have to be done and the results have to be confirmed, the apoptotic pathway and especially some members of the bcl-2 family of genes seem to be strong candidates in the regulatory pathway which can be manipulated by estrogen treatment.

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