ELIMINATION OF CYTOTOXIC AND GENOTOXIC BARRIERS DURING COURSE OF HSC EXPANSION WITH PIFITHRIN ALPHA TREATMENT

by
Pınar Erkan

Submitted to Graduate School of Natural and Applied Sciences
in Partial Fulfillment of the Requirements
for the Degree of Master of Science in
Biotechnology

Yeditepe University
2017

ELIMINATION OF CYTOTOXIC AND GENOTOXIC BARRIERS DURING COURSE OF HSC EXPANSION WITH PIFITHRIN ALPHA TREATMENT

APPROVED BY:

Assist. Prof. Dr. Fatih Kocabaş .....
(Thesis Supervisor)

Assist. Prof. Dr. Hakan Darıcı .....

Assist. Prof. Dr. Hüseyin Çimen .....

DATE OF APPROVAL: ..../..../2017

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to my thesis supervisor Assist. Prof. Dr. Fatih Kocabaş. Thank you for your encouragement, motivation and confidence through my studies. I have been always admired your attitude in Regenerative Labaratory. Thank you for your guidance through my final project.

I would like to thank Assist. Prof. Dr. Hüseyin Çimen and Assist. Prof. Dr. Hüseyin Çimen for the accompany to my thesis defense. Thank your for your time, devotion and attention.

Most importantly, I would like to thank to my parents for their endless support, their encouragement and also their invaluable faith in me. Thank you all for your support during this period of time. Especially Mehmetcan, you have been always by my side through my life. You have been always by my side and to know that gave me indescribable power. I really appreciate your patience and guidance. And, I would like to thank my all lab partners.

ABSTRACT

ELIMINATION OF CYTOTOXIC AND GENOTOXIC BARRIERS DURING COURSE OF HSC EXPANSION WITH PIFITHRIN ALPHA TREATMENT

Hematopoietic stem cells (HSCs) are used in the treatment of a extensive range of blood diseases. However, the limited number and stagnation feature make it difficult to use in stem cell treatment. Numerous studies have been carried out with small molecules to ensure the expansion and multiplication of HSCs *ex vivo*. As known; Genetic inhibition of P53 has been shown to lead to the proliferation of hematopoietic stem cells (HSCs). The pifithrin-apla is a small molecule compound and the inhibitor of p53 function and is known to be a precaution against genotoxic agents. In this study; The effects of the pifithrin-apla molecule on HSC cells were investigated. For this purpose, mouse HSCs were enriched by magnetic line abstraction. Human umbilical cord blood (UCB) and osseous (BM) mononuclear cells were isolated by histopak intensity gradient centrifugation. When the cells were cultured under appropriate conditions with the prepared HSC media, DMSO treatment was used as a control. After seven days of incubation with the small molecule of pifithrin-apla, mouse HSCs were analyzed for lin (-), sca1, c-kit, MD34 markers. Likewise, for human HSCs, CD34 and CD133 markers were analyzed. Cell line analysis and apoptosis analysis were performed following incubation of FACS-purified mouse LSK cells with the selected optimal dose molecule. At the same time, the effects of Human Umbilical Venous Endothelial Cells (HUVEC) and Human Dermal Fibroblast (HDF) and fat tissue on MSCs were also examined and did not have a negative effect. The small molecule of pifithrin-alpha caused a 3-fold increase in LSK (lin-sca1 + c-kit +) and human UCB HSC cells. Similarly, human BM caused a 2-fold increase in HSC cells. At the same time, chicken HSC was isolated and surface marker secreening was performed. So; Avian influenza and the effects of human HSCs. As a result, it is thought that this molecule can be used to increase *ex vivo* HSC expansion and treatment efficacy.

ÖZET

PİFİTRİN ALPHA TEDAVİSİ İLE HSC GENLEŞME DERECE SİTOTOKSİK VE GENOTOKSİK BARİYERLERİN KALDIRILMASI

Hematopoietik kök hücreler (HSC'ler) çok çeşitli kan hastalıklarının tedavisinde uygulanmaktadır. Bununla birlikte, sınırlı sayıda ve durgunluk özelliği kök hücre tedavisinde kullanılmalarını zorlaştırmaktadır. HSC'lerin *ex vivo* genişlemesi ve çoğalmasını sağlamak adına küçük moleküllerle çeşitli çalışmalar sürdürülmektedir. Bilindiği üzere; P53'un genetik inhibisyonunun, hematopoietik kök hücrelerin (HSC'ler) çoğalmasına yol açtığı gösterilmiştir. Pifithrin-aplha küçük moleküllü bir bileşik ve p53 fonksiyonunun önleyicisidir ve genotoksik ajanlara karşı önlem olduğu bilinmektedir. Bu çalışmada; pifithrin-aplha molekülünün HSC hücreleri üzerindeki etkileri araştırıldı. Bu amaçla, fare HSC'leri, manyetik soy soyutlama yöntemi ile zenginleştirildi. İnsan umbilikal kordon kanı (UCB) ve kemik yapılı (BM) mononukleer hücreler histopak yoğunluk gradient santrifuju ile izole edildi. Hücreler hazırlanan HSC medyası ile uygun koşullarda kültür edilirken kontrol olarak DMSO tedavisi kullanıldı. Pifithrin-aplha küçük molekülü ile yedi günlük inkübasyondan sonrasında fare HSC'leri lin (-), sca1, c-kit, ÇD34 markörleri için analiz edildi. Aynı şekilde insan HSC'leri için ise CD34 ve CD133 markörleri için analiz edildi. FACS ile saflaştırılmış fare LSK hücrelerinin seçilen en uygun doz molekül ile kuluçkaya yatırılmasını takiben hücre döngüsü analizi ve apoptoz analizi yapılmıştır. Aynı zamanda, İnsan Umbilikal Ven Endotel Hücrelerini (HUVEC) ve İnsan Dermal Fibroblast (HDF) ve yağ dokusundan elde edilen MSC'ler üzerinde de etkisi incelenmiş ve olumsuz bir etki göstermemiştir. Pifithrin-aplha küçük molekülü, LSK (lin- sca1 + c-kit +) ve insan UCB HSC hücrelerinde 3 kat artışa neden olmuştur. Benzer şekilde, insan BM HSC hücrelerinde 2 kat artışına neden olmuştur.Aynı zamanda, tavuk HSC izole edilip, yüzey marker taraması gerçekleştirildi. Böylece; kuş gribi gibi hastalıkların araştırılması ve insan HSC'lerinde oluşan etkilerinin önlenmesi için olanaklar araştırıldı.Sonuç olarak, bu molekülün *ex vivo* HSC genişlemesini ve tedavi verimliliğini artırmak için kullanılabileceği düşünülmektedir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/ABBREVIATIONS

AD	Adipose derived
ATM	Ataxia telangiectasia
BM	Bone marrow
CB	Cord blood
CMGF	Chicken macrophage growth factor
CMP	Common myeloid progenitor
CSF	Colony stimulating factor
DC	Dendritic cell
DC	Dentritic cell
DMSO	Dimethyl sulfoxide
DPBS	Dubelco's phosphate buffered saline
EP	Erythrocyte precursor
ES	Embryonic stem cell
FBS	Fetal bovine serum
G-CSF	Granulocyte colony stimulating factor
GMP	Granulocyte / macrophage progenitors
GP	Granulocyte precursor
HDF	Human dermal fibroblast
HSC	Hematapoietic stem cell
CLP	Common lymphoid progenitor
HSPC	Hematapoietic stem and progenitor cell
HUVEC	Human umbilical vein endothelial cells
Lin	Lineage markers
Lin-	Lineage negative cells
LSK	Lin-Sca+c-Kit+ cells
LT	Long term
MACP	Macrophage progenitor
MEP	Megakaryocyte erythroid progenitor
MKP	Megakaryocyte progenitors
MPP	Multi potent progenitor
MSC	Mesenchymal stem cell

NK	Natural killer
NK	Natural killer cell
PB	Peripheral blood
PFT-alpha	Pifithrin alpha
PSA	Penicilin streptomicin amphiciline
RT	Room temperature
RT-PCR	Real time polymerase chain reaction
SCID	Severe combined immunodeficiency
SFEM	Serum free expansion meduim
ST	Short term
UBC	Umbilical cord blood
WBM	Whole bone marrow

1.INTRODUCTION

1.1. HEMATAPOIETIC STEM CELLS (HSC)

Mature blood cells have a limited lifespan and should be constantly changed along life. Blood cells are also produced by a very small population (HSC) proliferation and differentiation of pluripotent hematopoietic stem cells with the self renewal ability. During differentiation, over-matured multi potential and multi lineage processed progenitor cells were first produced and progeny of HSCs were obtained from various intermediate stages as maturation progressed. Bone marrow (BM), peripheral blood (PB), hematopoietic stem and progenitor cells (HSPCs) are the only hematopoiesis center in humans and under normal conditions. The interactive response between a rapid hematopoietic cell and bone marrow stromal cells can be mediated by cytokines especially granulocyte colony stimulating factor, G-CSF, drugs and compounds used in several myeloid suppressive cancer treatments.

It is a potentially important and curative treatment of leukemia disease and for other blood or immune system BM or migratory PB (MPB) cells that are associated with HLA (unrelated transplantation). Autologous transplantation was also identified as specific diseases utilize the patient's own stem cell and progenitor cell. Lymphomas and myelomas are used as a treatment for other malignancies to effectively treat and to transplant an allogeneic stem cell. The umbilical cord blood (CB) has become more and more important than the HSCP source for birth and frozen collection and transport. After transplantation of CB, ratio of platelet and neutrophil recovery inclined rear than that of bone marrow or mobilized peripheral blood transplantation, which is, in part, less than a typical single-strand transplantation stem and progenitor cells [1].

A current research on human HSC is concerned with the detection, isolation, purification and characterization of blood lineage cell types mediated by rapid and permanent hematologic healing after tissue repair and transplantation. For HSPCs non-hematopoietic disorders, cell-based therapies will be examined. To determine this overview; providing information on the current status of HSPC investigations focusing on their experiments and

hematopoietic stem and progenitor cells. As culture media, maturating blood cells, phenotypic markers, and methods used to obtain a multiplicity of transfusions, which are used to support the multiplication or differentiation of stem cell and progenitor cell [2].

McCulloch has offered the stem cell's concept in the first *in vivo* study of the regeneration of the blood system and McCulloch suggests that cell colony forming in the spleen of these recipient mice are observed afterward transplanting the number of syngeneic BM. A very small subpopulation of the analyzing BM cells of these colonies possesses two distinctive features The ability of the myelo-erythroid cell to produce multiple cell types, and the ability of self renewable [3]. These findings suggest that stem cells, are unique lineage cells in the hematopoietic system that have multiple-potency and self-renewal potentials, and the ability to produce the same daughter HSCs without regeneration and self-renewal in multiple activities is differentiated to any functional blood cells the ability.

The area of stem cell research can lead to the emergence of each cell type in which adult stem cells (collectively called tissue-specific stem cells) grow up to include the embryonic stem (ES) and the cells that give birth to a particular organ or tissue and grow up with in the body. A nomenclature system has been developed to indicate the potential for differentiation of various stem cell populations [4].

The first possible purification of mouse BM hematopoietic stem cells was obtained from sorting the stem cells and staining with monoclonal antibodies [10, 11] using a relatively new technology of active color fluorescence. The murine HSCs possess lineage surface markers indicating that phenotype, Lin- Thy-1^{low} Sca-1 +, of the population obtained from enriched murine HSCs representing 0.05% of approximately adult mouse BM cells. Long-term reconstitution was possible to transfer the whole hematopoietic system to BM, [5] that the single cells in this mouse were the subjects of a lethally irradiated mouse later described as 3 months. A reductive approach by researchers demonstrated that, Thy1.1low, however, indicates that there is no Thy1.1high or Thy-1.1 cells caused by a long-term multilineage reconstitution from a donor-hosted donor; as it is true for Sca-1+ cells and Lin-, but only for Lin + cells. These primary studies have limited mouse cells to specific HSCs, and then use additional cell surface markers to seperate them from other cells, among them are only limited to individual cells to renew themselves and arise to long-term fold multilineage maturation [5,6]. After a while, showed that the isolated population contains three different multipotent populations such as Long Term (LT) -HSC, short term

(ST) -HSC and Multi-Potent Progenitor (MPP, a cell population with lost HSC self renewing capability) [6].

HSCs were enriched with adult mouse BM to perform sufficient transplantation of single cell experiments, and they suggest that a single cell was irradiated as lethal to a myelolymphoid long term reconstitution in a CD34 $^{-/\,low}$ c-Kit + Sca-1 + -Lin- line [7,8].

1.2. MOUSE HEMATOPOIETIC STEM CELLS

The mammalian blood system is characterized by the presence of more than ten different types of cells, bearing red blood cells (erythrocytes), megakaryocytes / thrombocytes, myeloid cells (monocytes / macrophages and granulocytes), mast cells, T and B lymphocytes and natural killer cells (NK) and dendritic cells cell type (DCs). These different types of cells are all indicative of a concept derived from a common precursor cell, that is to say, that the HSCs are fascinating and have a remarkable differentiation potential of HSCs. Flow cytometry is used to analyse determination and isolation of several subpopulations of blood cells in laboratory and other cell surface marker phenotypic analysis along with battery wells and to identify highly sensitive reading studies to understand how the more powerful HSC works in these diverse functional cell types and to understand differentiation potentials. While these analyzes are gradually limiting multipotency, a hierarchical structure for hematopoietic development has been proposed. HSCs initially have the ability to replenish them selves, and again lead to MPPs with differentiated differentiation potentials [9,10].

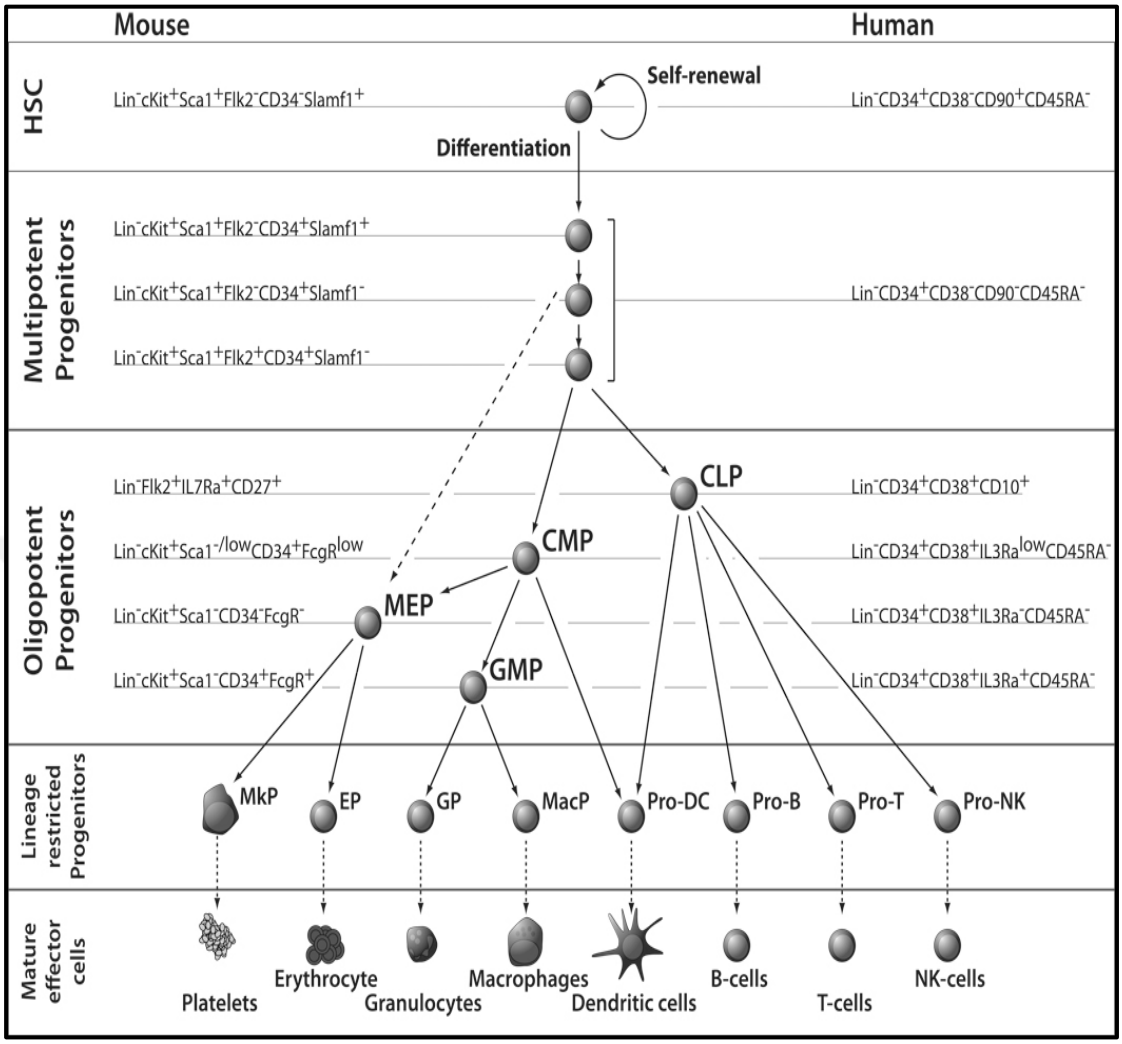

Figure 1.1. Schematic view of hematopoietic hierarchy [10].

It is located at the head of the HSC hierarchy and its described as the potential to achieve all more power hematopoietic cell types, as well as a cell with both self-renewal capacities. During the differentiation HSC loses its first self-renewal capacity, then it gradually loses its genealogical potential as it is determined to growth a mature species cell of a particular lineage. The human and mouse system have their own cell surface marker phenotype of each population as indicated in figure. The first reported progenitor, and the last cell mature and mature B, between the different subgroups and the front of the T cells. In the mouse system, differences in cell surface markers differences of phenotypes and functional underlied in MPPs have revealed their sub-clusters of heterogeneity.

1.3. HUMAN HSC AND PROGENITORS

Human hematapoietic stem cells were isolated and purified using same techniques for murine HSC. Represents the isolated of cells with differentiation levels of cell surface marker phenotype in combination with physiological assay. For hematopoiesis of human, diverse cell types are measured by long-term regenerative properties are assessed in the xenotransplantations model and mice with immunodeficiency are sometimes used in irradiation reconstitution transplanted with fetal human hematolyphoid [11,12]. CD34 was the primary cell surface marker utilized to enrichment of human HSCs. Cord blood, adult BM and human HSCs are only expressed 0.5-5% of adult blood cells by helping of a ligand for CD34, L-selectin, CD34 + cells bring to light that have multi potencial and oligo potencial, but at the same time the population is quite heterogeneous [13].

The first probable human hematapoietic stem cell isolation of represent the CD34 + CD90 + Lin- phenotype with Lin markers including T-, B-, NK and myeloerythroid specific markers [13,14]. Lymphoid and myeloid lineage produced by these cells in *in vivo* SCID (severe combined immunodeficiency) mice *in vitro* colony assay. On the other hand, the rest of the population of CD34 +, CD90-Lin-cells did not produce cell clones capable of producing myeloid and lymphoid cell types [14].

According to CD38, which is differential expression of surface marker, enrichment of human CD34 + HSCs population was obtained. Although CD34 + cells (90-99%) co-express CD38-expressing cells, lymphoid and myeloid cells can lead to multi particulate colonies containing both CD38-negative fraction and low CD90 + fraction [15]. In fact, G-CSF accumulates in the human HSC, co-expresses CD34 and CD90, inducing circulating cells in this way and mediated hematopoietic remodeling and long-term, but stronger contamination of peripheral blood moving, according to increasing the efficiency of human bone marrow transplantation [16]. Human HSC is the CD38 fraction, which leads to the enrichment in the Lin- CD34+ CD38- CD90 + population of human hematopoietic cells resources.

Although following population of Lin-CD34 + CD38-CD90 + human cells is found in terms of high human HSC, retroviral or lentiviral markers, the functional heterogeneity of these population cells possesses immunodeficient mice before xenotransplantation is

detected. Human multi potent progenitor population have identified in human umbilical cord blood, recently described as Lin CD34 + CD38- CD90- CD45RA-, which has very potent the lack of self reneweal capacity and functional properties [17,18]. In the human hematopoietic system, MPP has been detected to attain more potent responses to mouse CMP, GMP, MEP, and CLP than in early handled myeloid and lymphoid progenitors. IL-3RA and CD45RA, which is an isoform of CD45, which regulates selected clasification of negative cytokine signals, have been confirmed in human CMP, GMP, MEP populations *in vitro* and *in vivo* [19,20].

This complex, multi-step schematic of mouse and human hematopoiesis, while maintaining a accurate homeostasis regulation of stem cell, provides a great boost in a number of terminally differentiated cells.[20]

The generation of cells with peripheral blood or hematopoiesis comes mainly from bone marrow, which is called medullary hematopoiesis. Especially; spleen, liver and lymph nodes, blood column production outside the bone marrow, in other words, high-marrow hematopoiesis. Extra medullary hematopoiesis is common in a many of healthy mammalians like rodents and skunk [21].

Pluripotent-committed cells that differentiate into various cell lines such as; red blood cells, granulocytes, megakaryocytes, monocytes and lymphocytes are produced by stem cells with hematopoiesis begins. Pluripotent stem cells, lymphocytes, routinely analyze modified bone cells as erythrocytes, granulocytic cells and megakaryocyte cell lines, in bone marrow analysis, because the films are similar to stained with Romanowsky blob [22,23].

1.3.1. Mammalian Erythropoiesis

Erythropoiesis, is the production of red blood cells in small exotic mammalians seems to be the similar progress like the native mammals. The erythroid front coarse chromatin and cytoplasm tend to be blue round cores, round cells (large, typically 20-30 micrometers in diameter). These cells and nuclei diminish the size of the nuclear chromatin, and if it is mature cytoplasm, due to hemoglobin production is more blue and reddish. Erythropoiesis

end with the red blood cells maturation; red blood cells in the form of a biconcave disk in the nucleus seems red and orange cytoplasm with stinging and cytology [24].

1.3.2. Mammalian Granulopoiesis

Subsequently, the neutrophils which is known as, heterophils, eosinophils and basophils placed in the granulocytic subpopulations of the stem cell show changes in peripheral blood. Granulocytic leukocytes and irregularly shaped and occasionally eccentric abundant cytoplasm are described as nuclei containing thin chromatin platelets of lavender round cells. Granulocytes characterize the maturation sequence starting with myeloblast, and follow the cell that proceeds along the band, from promyelocyte, myeloid myelocyte and myeloblasts. And the high content of ribosomes that can divide the cell by helping of Romanowsky blue color stains the cytoplasm of these cells. It is not possible to share the myelocytes and tape cells and create the latest stages of maturation. These cells are little in size than the previous ones and their cytoplasm view is less blue [23,25].

1.3.3. Mammalian Platelet Production

The peripheral thrombocytes come from the cytoplasm of the megakaryocytes in the bone marrow. Megakaryoblasts, promegakaryocytes and megakaryocyte presents in mammalian bone marrow containing developmental stages. Usually the megakaryoblasts and promegakaryocytes are much wide than the rubriblast and myeloblasts [25].

1.4. AVIAN HEMATOPOIESIS AND BONE MARROW

Bone marrow is main for late embryonic development in thrombopoesis, erythropoiesis, granulopoiesis and platelet formation. Hematopoiesis begins at the bladder in yolk salc, and when the embryo develops, appears to over the organs like hematopoietic areas, liver and spleen within the predominant activition in the bone marrow of bird.

The formation of erythrocytes mainly occurs in the central skeleton of pigeon puppies, but post two week with this activity is transmission to the axial skeleton, which is six months old, in the third thigh activity of erythropoietic [26]. Granocyte stem cell colony forming

foci of granulopoiesis and other tissues including the spleen, kidney, lung, thymus, gonads, pancreas and bone marrow during the embryo. When compared, Mammalian granulopoesis is more common in mature birds and is found in a many of tissues. Chickens some adult avians, as hematopoietic bone marrow activity, are related with essential erythropoiesis and to be possible thrombopoiesis; just a small percentage seems to be preserved for granulopoietoc maturaion and lymphocytic maturation [27].

Avian bone marrow can also be used for the evaluation of patients for blood cell diseases because birds are the most readily available source of hematopoietic tissue.Non-Regenerative anemia with cytologic examination of BM is execude for patients with birds [28,29].

1.4.1. Avian Erythropoiesis

Avian erythropoietin is required for the proliferation and differentiation of stable progenitor stem cells in a glycoprotein, erythroid array which differs from mammalian erythropoietin. Bird erythropoiesis arise in the lumen of the vascular sinusoids in BM. These sinuses are covered by prolonged endothelial cells linked with immature cells of the erythroid. Erythropoietin is known to be present in anemic birds and the kidney is the production place.

In general, if erythroid cells mature, chromatin becomes increasingly conducive to rounded nuclear shape changes, ellipsoid, cytoplasmic hemoglobin concentration rising leading in increased eosinophilia, and round cell-shaped changes increase as the nucleus size decreases. Unlike the blood cells for the mammalian eritrocytes shape [30].

1.4.2. Avian Granulopoiesis

Bird granulocytes seem to improve parallel in mammals, even though the location of the originating cell is different. For instance, from the stem cells found in the extravascular areas of the BM, bird's heterophills tree is obtained, in contradiction to mammalian neutrophils which improve within vascular spaces. Avian heterophills can also be obtained from non-marrow hematopoiesis [31].

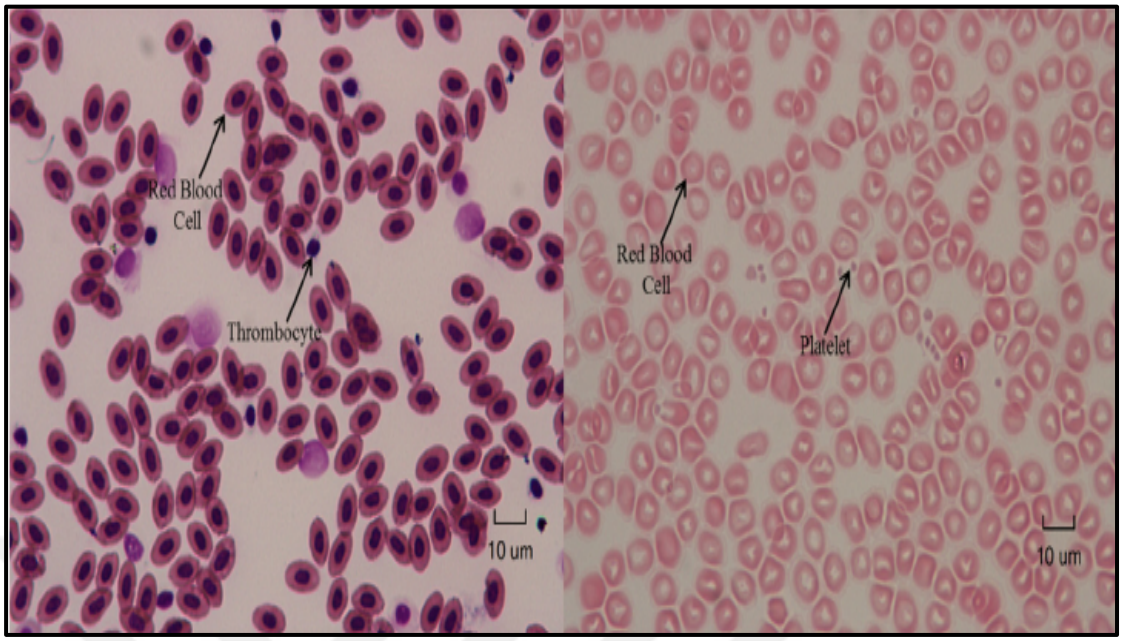

Figure 1.2. Avian and human platelet photomicrographs. Left panel stained with chicken, blood (platelets and red blood cells of the core), a Camco Quick Stain® rod and right panel Wright human blood (platelets and enucleated red blood cells)[30].

Bone marrow immature progenitor cells give rise to mature HSCs in a complex manner. The systems of cell differentiation are arranged in a set of solvable cytokines: colony stimulating factors (CSF) for example, macrophage CSF, granulocyte CSF interleukins, IL-1 for IL7, granulocyte macrophage CSF multiclony SF and; other hematopoietic growth factors like erythropoietin and stem cell factor. *In vitro* clonalization of hematopoietic systems in the natural or recombinant forms of these cytokines is possible in the intermetallic species. In contrast, many birds are not yet available to clean cytokines other than conditioned media derived from cells or tissues in general. Unfortunately, with only two avain cytokines, which are, chicken stem cell factor and chicken macrophage growth factor (CMGF), having mouse STCF sequence homology. For this reason, efforts have been made to identify and describe hematopoietic growth factors and clean bird [32].

1.5. AVIAN INFLUENZA

Avian influenza A knowna as a virus of the H5N1 is a highly pathogenic type and regional to poultry however intersect to human species. As reported, it has been proven that human

population with 60% mortality in more than ten countries is a very deadly infectious disease since World Health Organization 2003. The pathological operation of H5N1 patients is the first submission of fever and respiratory problems with the inclusion of coughing and shortness of breath. [33] In addition, fulminant viral pneumonia, acute respiratory distress syndrome, multiple organ defeat and death are presented in serious cases. When infected, the fetus may spread to other organs from the lungs and lead to a systemic disease causing an extraordinarily high mortality [34]. A fatal result of the H5N1 virus infection has been related with the availability of high viral burden and associated reactive hyper cytokinemia syndrome. Hematological abnormalities were examined in vigorous cases including frequent lymphoid depletion, leukopenia, thrombocytopenia and pancytopenia, possibly bone marrow (BM) suppression results and virus-associated hemophagocytosis. In spite of the certain systemic extanded of the virus, the BM itself did not report viral isolation, but in other cases the viral antigen same autopsy. Virus-mediated BM-repression is fascinating to determine if the apparent cell loss observed is a possible factor contributing to hematological abnormalities and hyper inflammatory cytokine production, so that the suppression observed is a direct invasive consequence of the virus [35].

Bone marrow (BM) is major source of progenitor cells. These progenitor cells contain two types; Hematopoietic stem cells and without blood cells hematopoietic stem cells and mesenchymal lines could be distinguished from cells such as osteoblasts, chondrocytes, adepocytes etc. Hematopoietic stem cells (HSC) have regeneration and differentiation properties. Hematapoietic stem cells can be capable to enter blood circulation system, bone marrow migration. CD34 + cells contain an anterior population of HSC, myeloid, lymphoid and erythroid progenitors. Currently, HSCs have been identified from rich sources for umbilical cord and placental isolation, and have been identified to share similar features, such as the HSC BM.

MSCs have much number of the most important biological properties that constitute the determinants of the criteria for stem cell acceptance above. In the action of the BM, MSCs support for regular hematopoiteic progress cells consist of growing process, maturation, differentiation and survival of HLA regulators. Because damaged cells can differentiate themselves in the phenotype, they can reconstruct damaged tissue among MSC distinguishing capabilities. MSCs also exhibited immune activities by repressing the

proliferation and functionality of NK T, B and monocyte-derived dendritic cells. These features, more importantly, appear to be therapeutic in regulating the immune responsibility against to tissue injury, autoimmunity and transplantation [20,22,36]. BM progenitor cells and H5N1-induced haematological abnormality [20] led to a direct investigation of the infection of the BM H5N1 virus, as a potential target is a rich source [36].

1.6. PIFITHRIN ALPHA (P53 INHIBITOR)

In recent years, small molecules have been developed as tools for stem cell understanding and stem cell regulation and manipulation of stem cell destiny. You can have a wide range of effects from reprogramming, dilation or disease and survival, ablation, or migration of cancer cells without differentiating target stem cells from therapeutic effects in *in vivo* and *in vitro* models [37].

Small molecules have advantages over genetic and other methods. It can perform certain functions of a single protein or more proteins with a good temporal control to change it backwards. This is a useful feature because differentiation is linked to a particular line in a particular order of cellular events. Small molecules can be used in primer cell assays readily adaptable. They can be cell permeable and can affect pathways and processes in the cell and are more stable and growth factors are less expensive. The processes of small molecules are in a manner dependent on the concentration the effects obtained are as flexible as possible. They are potential for development and can be used as chemical probes to advance the understanding of cell destruction control mechanisms [38,39]

Small molecules can alter stem cell renewal and current-specific differentiation. In addition, inducing pluripotent stem cells can have effects on reprogramming they can change certain transcription factors to improve reprogramming efficiency or speed up the reprogramming process.[40] Small molecules can also facilitate transformation of pluripotent stem cells prepared to pure stem cells.

A somatic cell type is a pluripotent bypass, where transdifferentiation can occur when another is transformed. It can be mediated either by serial specific factors or after a limited reprogramming and flow specific degradation transdifferentiation II as follows. A known

small molecule, including potentially reprogramming and differentiation, at the same time, may also affect this second process [41].

The small molecule compound inhibits pifithrin-alpha (PFT-alpha) p53 function and has been reported to protect against a variety of genotoxic agents.

There are important treatments for many types of cancer, such as chemotherapeutic agents or DNA damaging agents like ionizing radiation (IR), termination of cell cycle, or apoptosis. This is a treatment, particularly serious, because p53 is very well tolerated. Because of the very strong damage of normal p53 to tumor suppressor protein p53, which is one of the key players mediating these reactions, accumulates after DNA damage and transcriptionally dependent and independent events of these functions Side effects are caused by a high dose of chemotherapy or radiotherapy in some cases and can be killed p53-deficient tumor, the tumor tissue surrounding, which is very important for tumor suppression[42].

In the treatment of p53-deficient tumors, to reduce side effects associated with others some compounds discovered. A cell-based screen (PFT-α) was developed to identify compounds with the ability to apoptose p53 intervened from a synthetic chemical library of 10 000 synthetic molecules, which is a potent durable water-soluble p53 inhibitor called pifithrin from previos works. It inhibits transactivation of only p53 sensitive genes, but meanwhile suppresses p53 dependent apoptosis [43]. In addition, in some cases, genotoxic stress related with cancer therapy, with non-inducing the formation of tumors, showed that mice retained PFT-α. This compound is shown to be *in vitro* and *in vivo* experiments for prevent cells different from a p53 dependent apoptosis induced differentiation induced by various stimuli, and it is evident that PTF-alpha specifically inhibited p53 and therefore frequently used to distinguish between p53 dependent and independent apoptotic systems. Moreover, in a small number of studies PFT-α was clearly not surprising to observe neuroblastoma cell response to p53 deficiency, which was drug-treated with PFT even at that time. Specific treatment of p53 deficient cells in their system suggests that such treatment is not only in the absence of these cells. Such control is particularly important, while the mechanism of pifithrin alpha induced p53 inhibition at the time of killing is well known, largely unknown to compound has [44].

Somatic cells in exclusive immature tissue stem cells are constantly being subjected to internal and external DNA damage causing variety tension. For protection of the genomic integrity of stem cell and tissue homeostasis, DNA damage repair is critical to active control point mechanisms. For example, ionizing radiation and chemotherapy DNA lesions may cause therapeutic approaches to be present as cytotoxic species of DNA damage between double-strand breaks (DSBs) [45,46].

To reduce the negative effects most of the DSBs caused, cells were exposed to these stresses quickly after DNA damage was activated to the point of control. When activated, the DNA phosphorylates the mutated protein ataxia-telangiectasia (ATM) damage, the multiple downstream target proteins, and the cell cycle control point-induced reaction. After detection of DNA damage, the activated ATM amino-trans-effect transiently phosphorylates with 15 tumor suppressor and p53 directly [47].ATM activates a serine-threonine kinase phosphorylated with CHK2, threonine 18 and serine 20 p53. An E3 ubiquitin ligase targeting MDM2 p53 can be phosphorylated by ATM. Directly or indirectly by ATM, p53 and MDM2 and alterations lead to this phosphorylation, a transcriptional activation and p53 stabilization. Transcription of p53 aggregation with DNA damage, low and repair levels, p21Cip1, block cyclin-dependent kinases (CDKs), leads to a delation either activation of the initiated cell cycle. The pause or control mechanism has the ability to repair DNA damage during the cell cycle arrest continuity induced by the cells. If DNA damage is elevated or cannot be repaired, transcription of pro-apoptotic genes like p53, BAX, and Puma NOXA induces transcription of damaged cells [48,49].

However, p53 dependent apoptosis can occurs in varios organs throughput radiation and chemotherapy. The side effects of cancer or radiation suppressed by p53 mechanisim by treatment of PTF alpha small molecule. The main strategy of this approach is to achieve therapeutic benefit without attacking the major biological mission of p53 such as transcriptional activitya and cell cycle check points. Pifithrin-α has been shown to block the p53 specific transcriptional activity that controls the tumor, and is therefore often suggested to be useful in preventing serious side effects associated with chemotherapy and radiotherapy. Although some studies demonstrated that have preserved different cell types of apoptosis from PFT-α-efficient DNA damage, this effect is presented regardless of its presence or absence in the absence of p53. Interestingly, PFT-α blocked activation of

apoptosis processing and inhibits caspase 9 and caspase 3 without affecting mitochondrial activation. Pifithrin alpha activation not affect the Bax or Bak or mitochondrial membrane potential(cytochrome c) in DNA damage. Instead of that, PFT-alpha is capable of DNA damage-induced caspase activation and apoptosis in the ability of p53-deficient cells to down regulate. Conversely, it may be that this protection does not remove it from other proteins, such as retinoblastoma protein cyclin D3, and cyclin-dependent kinases, which are down-regulation 2, 4 and 6 of other proteins involved in cell cycle progression. These findings suggest that DNA damage apoptosis protects the PFT-α cells with a p53-independent mechanism that involves mitochondria down and contains cyclin D1.

Previous study demonstrate that a p53-independent mechanism protects DNA damage from apoptosis. As a consequence, this mechanism includes a cell cycle regulator protein and because it inhibits apoptosis, cyclin D1-induced caspase-3 activation and apoptosis induced. Conversely, loss of cyclin D3 and CDKs did not affect DNA damage induced apoptosis and did not chanfe the preservation effect of PFT-aplha. However, cyclin D1 could be directly or indirecty involving the process in DNA damage induced apoptosis. The mechanism is still unknown.

On the other hand, changes in the expression level of the infrared source, or post-translational modifications of certain proteins, are not always indicative of their own involvement. For example, the expression of the cyclin D3 by IR is up-regulated, but an event that is effectively ineffective by the PFT-alpha has no effect on the signaling of the cyclin D3 disruption apoptosis.

However, the involvement of other cell cycle regulatory proteins in addition to cyclins may be a multifunctional redundancy between different cyclins and CDKs, as opposed to modulating the expression of the cyclin protein levels of their CDKs. For example, down-regulation leads to an increase in expression of CDK2 and CDK4. Based on this excess, it is not known why Cyclin D1 is present in PFT-alpha to protect apoptotic cells from DNA damage-mediated DNA damage (CDKs), but their expression has been shown to be cyclin-linked modulated, particularly PFT-alpha.

In summary, pifithrin alpha prevent cells from DNA damage and apoptosis by p53 independent mechansim. The PFT-alpha small molecule downregulate caspase 3 and caspase 9 to prevent apoptosis and involved cyclin D1 mechanism to protect DNA

damage. The p53-independent movement is clearly required to solve the underlying mechanism's encryption, further efforts to reduce the serious side effects of chemo and radiotherapy based treatments are initially aimed at threatening the intended target [50].

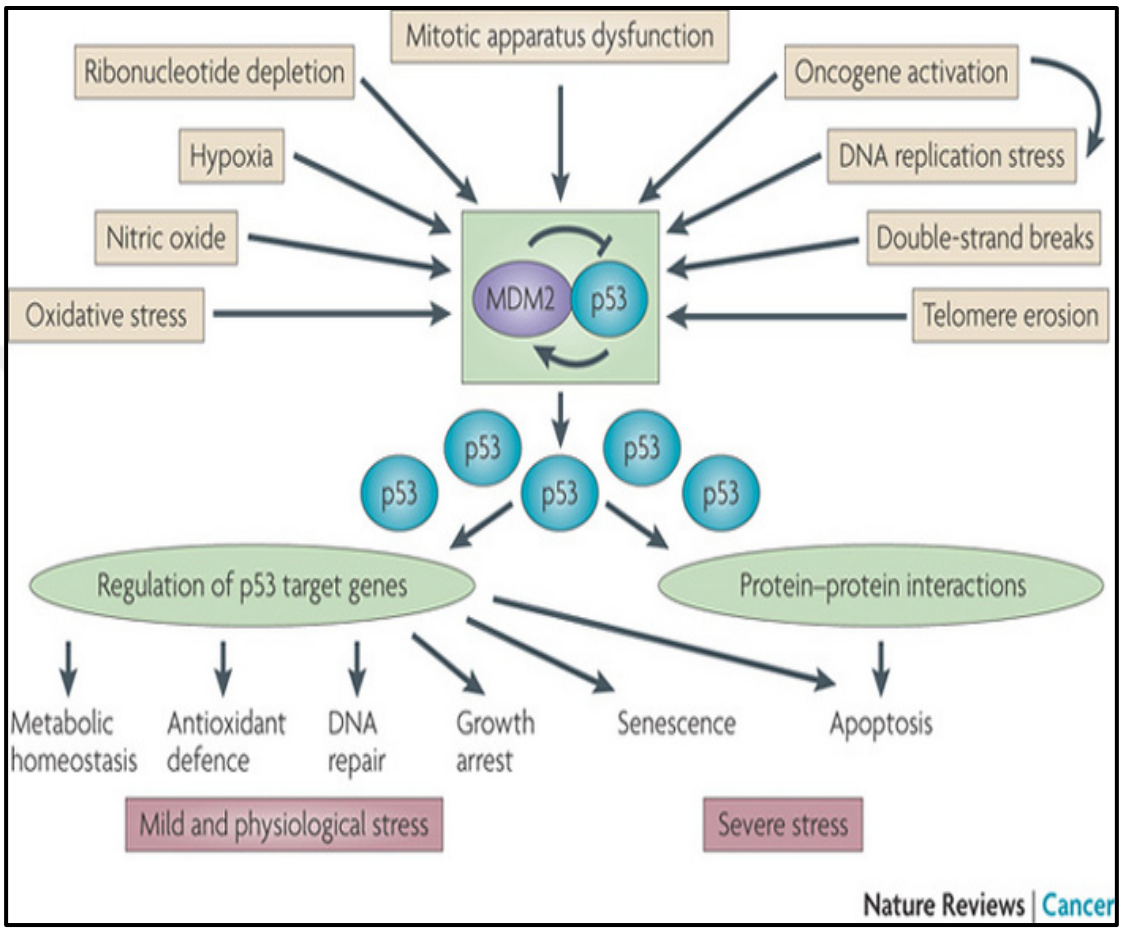

Figure1.3. The shematic figure of P53 pathway [51].

In this study, pifithrin-alpha treatment of HSCs was investigated for the increase the expansion and proliferation of HSCs. The effect of this molecule on mouse HSC, avian HSC, Human Dermal Fibroblast (HDF), Human Umblical Vein Endothelial Cells (HUVEC) and adipose derived Mesenchymal stem cells were studied.

2. MATERIALS AND METHODS

Cells were incubated with the three different doses of pifithrin-α as 0.1μM, 1μM and 10μM concentrations. DMSO (0,5%) was also treated within a cells as a control. All studies about human and animal were commanded by Institutional Clinical Studies Ethical Committee of Yeditpe University and the Institutional Animal Care and Use Committee of Yeditepe University with the decision numbers 547 and 548.

2.1. BONE MARROW COLLECTION AND MAGNETIC LINEAGE DEPLETION PROCESS OF MOUSE DERIVED BONE MARROW

Bone marrow cells were received from femur and tibia of six or eight week Balb/c mouse (Yudetam, Turkey). After euthanasia and sterilize with 70% alcohol, femurs and tibias were flushing with ice-cold dulbecco's phosphate-buffered saline using a syringe and a 26G needle (DPBS, Invitrogen, Gibco, UK, cat no.14190250). The cell suspension was filtered helping of 70μm cell strainer (BD Pharmingen, cat no. 352350). The cells were evaluated by hemocytometer and centrifuged at 1500 rpm for 5 minutes. According to mouse HPSC enrichment set DM (BD Pharmingen, cat no. 558451) protocol, magnetic lineage depletion process was performed. The cells were harvested in ice-cold DPBS supplemented with 2% (v/v) fetal bovine serum (FBS, Sigma Aldrich, USA, cat no. 12103C). The mouse BD Fc block was performed and biotinylated lineage depletion cocktail was added to cell suspension. Following the washing steps, the cells were labeled with streptavidin particles and situated into magnetic field of the IMagnet (BD Pharmingen cat. no. 552311). The lienage negative cells were collected from final depleted fraction.

2.2. BONE MARROW COLLECTION AND MAGNETIC LINEAGE DEPLETION PROCESS OF AVIAN BONE MARROW

Bone marrow cells were obtained from chicken. The cells were flushing with ice-cold dulbecco's phosphate-buffered saline using a syringe and a 26G needle (DPBS, Invitrogen, Gibco, UK, cat no.14190250). The cell suspension was filtered helping of 70µm cell strainer (BD Pharmingen, cat no. 352350). The cells were evaluated by hemocytometer and centrifuged at 1500 rpm for 5 minutes. According to mouse HPSC enrichment set DM (BD Pharmingen, cat no. 558451) protocol, magnetic lineage depletion process was performed. The cells were harvested in ice-cold DPBS supplemented with 2% (v/v) fetal bovine serum (FBS, Sigma Aldrich, USA, cat no. 12103C). The mouse BD Fc block was not performed and directly, biotinylated lineage depletion cocktail was added to cell suspension. Following the washing steps, the cells were labeled with streptavidin particles and situated into magnetic field of the IMagnet (BD Pharmingen cat. no. 552311). The lienage negative cells were collected from final depleted fraction.

2.3. CELL COUNTING AND IMAGING

Lineage negative cells were seeded into StemSpan SFEM media (Stemcell technologies, Vancouver, cat no. 09650) supplemented with Scf (1000 unit/mL), Tpo (1000 unit/mL), Flt-3l (5000 unit/mL) (all from R&D Systems Inc., Minneapolis) and 1% (v/v) PSA (10.000 units/mL penicillin, 10.000 µg/mL streptomycin) (Corning Costar, Sigma Aldrich, USA, cat no. CLS3599) at a intensity of 30.000 cells/well on 96-well plate. The cells were treated with three different doses of pifithrin-α as 0.1µM, 1µM and 10µM concentrations and DMSO (0,5%) treatment used as a control. After four days of treatment process, the cells were stained with Hoechst 33342 (Sigma Aldrich, USA, cat no. 14533) for the screening under fluorescent microscope. The images of cells at day 4,7 and 10 were taken respectively and the cell count was evaluated with Scion image program.

2.4. HSC STAINING OF MURINE BONE MARROW CELLS AND FLOW CYTOMETRY ANALYSIS

Lineage negative cells were seeded at a density of 30,000 cells/well into 96 well plates. The treatment process of pifithrin- α with 0.1, 1 and 10 μM concentrations was applied and cells were stained with HSC markers in accordance to the manufacturer's protocol. Antibody staining with mouse c-Kit (CD117) PE, CD34 FITC, Sca-1 PE-Cy7, lineage cocktail APC (BD StemFlow Cat No 560492) and flow cytometry analysis were performed for the identifications of LSK and LSKCD34^{low} populations. mouse c-Kit (CD117) PE, CD34 FITC, Sca-1 PE-Cy7, lineage cocktail APC (BD StemFlow Cat No 560492).

2.5. UBCB CELL ISOLATION

UCB was obtained from the newborn at the birth time and Onkim Stem Cell Technologies obtained informed consent from the parents. For the isolation UCB mononuclear cells, Ficoll-Paque (Histopaque™, Sigma, Cat.No.10831) density gradient centrifugation (1,083g/ml) was added and cells were seeded at a density of 10,000 cells/well onto 96 well-plate within expansion medium which contains Serum-Free Expansion Medium (StemSpan™ Serum-Free Expansion Medium (SFEM), Stemcell Technologies, Cat.No. 09650) supplemented with 1% PSA (10,000 units/ml penicillin and 10,000 ug/ml streptomycin and 25 μg/mL of Amphotericin B, Gibco, Cat.No.15240062) and human cytokine cocktail (StemSpan™ CC100, Stemcell Technologies, Cat.No. 02690). The cells were treated with pifithrin-α with 0,1μM, 1μM and 10μM concentrations. The cells were also treated with dimethyl sulfoxide (0,5%) (Calbiochem, cat no. 317275) as control.

2.6. FLOW CYTOMETRY ANALYSIS OF HUMAN HSPCS

The UCB mononuclear cells were treated with pifithrin-α for 7 days and antibody staining was performed for flow cytometry analysis. The staining of cells were applied with PE-conjugated anti-human CD34 (Biolegend, Cat.No.343506), APC-conjugated anti-human CD133 (Miltenyibiotec, Order No.130-090-826) antibodies and treated with Aldefluor reagent (ALDEFLUOR™ Kit, Stemcell Technologies, Cat.No. 01700) in accordance to the

manufacturer's manual (Stemcell Technologies). The expression levels of CD34 and CD133 surface markers enzyme activity of UCB mononuclear cells were analyzed.

2.7. CELL CYCLE ANALYSIS (LSK VE HHSPC)

Mouse lineage (-) cell population was stained for obtained murine LSK (Lin^- $Sca1^+C\text{-}kit^+$) by sorted in flow cytometry (FACSARIA III, BD Biosciences, Cat.No. 23-11539-00) to distinguish the cell phases. The human CD34+ cells were also obtained from UCM mononuclear cells that employ flow cytometry. The sorted cells were seeded in 96 well-plate within expansion medium for murine and human at a density 5,000 cell/well for 4 days treatment. The treated cells were stained with Hoechst 33342 (10µg/ml) and Pyronin Y (100µg/ml) and analyzed.

2.8. TOTAL RNA ISOLATION AND CDNA SYNTHESIS

After isolating process of HSC, total RNA isolation was performed from the cells by using High Pure RNA Isolation Kit (Roche, Germany). The isolation procedure was described from the manufacture's instructions. Isolated total RNA samples were used to the synthesis of complementary DNA (cDNA) by using High Fidelity cDNA Synthesis Kit (Roche, Germany). The procedure was applied as a manufacture's instructions.

Table.2.8.The primer sequences were designed to used in Q-PCR

OLIGO ID	FORWARD	REVERSE
mTSC-1	TGAGGTAAACAGCTGAGGGG	ACTGTTCTGGGGACAGATGG
mFOXO-3	TTGTCCCAGATCTACGAGTGG	CTGTGCAGGGACAGGTTGT
mMEIS1	GTTGTCCAAGCCATCACCTT	ATCCACTCGTTCAGGAGGAA
mDNMT3A	GCTTTCTTCTCAGCCTCCCT	CCATGCCAAGACTCACCTTC
mRb1	ACAGATTTGTCCTTCCCGTG	CCATGATTCGATGCTCACAT
mRBl1	AATGGTCCAGGAAACACGAC	GGAAAGTACGGGGTGAGCTA

mRBl2	TGAAGCAATGCCTTCTCC	TTCCGTCGTCCAAGAGAATC
mSlc30-A1	AACACCAGCAATTCCAACG	TCTTCCGCTTCCAGATTGTC
mSIRT	GACGGTATCTATGCTCGCCT	ACACAGAGACGGCTGGAACT
mELF4	AGCCCAGTGATCTGGTCTTC	AGGATAGGGGACCTGTTCCA
mCITED2	GGCTGTCCCTCTATGTGCTG	CATATGGTCTGCCATTTCCA
mGLI1	GAGGTTGGGATGAAGAAGCA	GGAGACAGCATGGCTCACTA
mRUNX1	TTGCCACCTACCATAGAGCC	GGTGGACAGAGGAAGAGGTG
mP15	CAGTTGGGTTCTGCTCCGT	AGATCCCAACGCCCTGAAC
mP19	GTTTTCTTGGTGAAGTTCGTGC	TCATCACCTGGTCCAGGATTC
mAPC	CAGCTTTTACAGTCCCAGGC	TCTGACCACTACTGGAGGCTG

2.9. QUANTITATIVE REAL TIME PCR

After the obtained differentiated cells cDNAs, real time PCR was performed for the observed gene expression levels of these cDNAs. The Maxima SYBR Green/ROX (Fermentas, USA) for the detection of gene levels on real time PCR. The template which are myogenic differentiated cDNAs were mixed with Maxima SYBR Green/ROX qPCR Master Mix (2X). For the normalization of mRNA level glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used. GAPDH was used as a house keeping gene and normalized the result for mRNA. The quantitative Real Time PCR cycles were shown in a table:

Table 2.9. The number of cycles and conditions of QPCR

Polymerase Activation and DNA Denaturation at 95^0C	**Amplification**			**Melt Curve Analysis**
3 minutes	Denaturation at 95^0C	Annealing $55\text{-}60^0$C	Cycles	$55\text{-}95^0$C 0.5^0C 2-5 seconds/step
	10-15 seconds	30-60 seconds	35-40	

2.10. STATISTICAL ANALYSIS

The whole data were shown in the means ± standard errors (S.D.) and graphics were formed by using GraphPad Prism 5 software (GraphPad Prism, USA) . The statistical analysis of results could be performed from ANOVA and Turkey's test using GraphPad Prism 5 software. The result of this experiment were applied from both of them and Statistical significance was determined at $P < 0.05$.

2.11. AD-MSC ISOLATION (CONFIRMATION OF BONA FIDE MSC SURFACE ANTIGENS BY FLOW CYTOMETRY)

MSC cells isolation was performed instantly from adipose tissue after liposuction operation. The adipose tissue (60 ml) was placed into a bottle and mixed with same amounts of collagenase solution for digestion at 37°C for 1 hour by continuous shaking conditions. The digested mixture was centrifuges 2500 rpm for 7 min at RT and supernatant discarded for the remove adipocytes and collagenase solutions; the pellet was resuspended with 2 ml of erythrocyte lysis buffer and incubated at 37°C for 10 min by continuous shaking. After centrifuged at 1400 rpm for 7 min, supernatant discarded and pellet was washed with 1X PBS. The collected cells were filtered through 100 µm cell strainer within a 6-8 ml expansion medium (DMEM, Gibco) and seeded onto T-150cm^2 tissue culture polystyrene flasks (Sigma Aldrich, cat no. CLS3290). The cells were splashed with 1X PBS after 24 hours incubation and refreshed medium.

For the flow cytometry analysis, 10,000 adipose cells per well were seeded into 96 well plate. The treatment of pifithrin-α as 0.1μM, 1μM and 10μM concentrations was performed for 7 days in culture. The cells were labeled with MSC markers for identifying the populations by helping of hCD73 APC, hCD90 FITC, hCD105 PerCP/CY5.5, CD45 PE (Biolegend Cat no: 344006, 328108, 323216, 304008 respectively) antibodies. MSC staining was applied in accordance to the manufacturer's protocol and analyzed.

2.12. BM-MSC ISOLATION

Mouse bone marrow mesenchymal stem cells were isolated according to a protocol modified from Soleimani and Nadri (Soleimani and Nadri 2009). Bone marrow cells were collected as defined before and seeded at a density of $30x10^6$ cells in in T-75cm^2 flasks (Sigma Aldrich, cat no. CLS3290) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% (v/v) FBS (Sigma Aldrich, USA, cat no. 12103C) and 1% (v/v) PSA (10,000 units/ml penicillin and 10,000 ug/ml streptomycin and 25 μg/mL of amphotericin B, Gibco, Cat.No.15240062). After 24 hours, the medium of cells was refreshed and removed from non-adherents cells. The cells were incubated in the humidified incubator at 37°C and 5% CO_2 for 15 days and change the media each 3 -4 days.

2.13. WST1 ANALYSIS

For the analyzing cell viability, hMSC and mMSC were seeded in 96 well plates at a density of 5,000 cell and 10,000 cell per well respectively and treated with pifithrin-α with three different concentrations as 0,1μM, 1μM, 10μM. After 3 days, WST1 reagent was diluted in 1:10 range with culture medium (Cell Proliferation Reagent WST-1, Roche, Cat No. 11644807001) and added 50 μl per well and incubated at 37°C and 5% CO_2. The absorbance values of the samples at the time point of 1 hour, 2 hours and 3 hours measured using a microplate reader at 450 nm wavelength and 630 nm reference wavelengths.

2.14. APOPTOSIS

$Lin^{-}Sca1^{+}Kit^{+}$ (LSK) cells were isolated from mouse lineage (-) cell population by flow cytometry (FACSAriaIII, BD Pharmingen, cat no. 23- 11539-00). Murine LSK cells and human CD34+ UCB cells were seeded at a density of 5000 cells/well in 96-well plates and treated with pifithrin-α and DMSO (0,5%) in three replicates at a three concentrations for 3 days in the humidified incubator at 37°C and 5% CO_2. Following 3 days, cells were collected from 96-well plates and centrifuged at 1500 rpm for 5 minutes and resuspended the pellet in 1X binding buffer (BD Pharmingen, cat no 556570) for the analysing apoptotic cells. The cells were stained with FITC Annexin V and PI according to the manufacturer's manual (BD Pharmingen, cat no 556570) and analyzed by flow cytometry (FACSAriaIII, BD Biosciences, cat no. 23- 11539-00).

2.15. EFFECT OF HSM ON HUVEC

Human umbilical cord endothelial cells (HUVECs, ATCC® CRL1730™) were seeded on 96 well plates at a density of 2,000 cells per well. A day after incubation, the cells were treated with pifithrin-α and DMSO (0,5%) at 0.1, 1 and 10 μM concentrations. Cell proliferation rate was analysed after 3 days teratments by using WST-1 assay (Cell Proliferation Reagent WST-1, Roche, Cat No. 11644807001)

2.16. EFFECT OF HSM ON CARDIAC FIBROBLAST

The hearts dissected from mouse and minced into 3-4 pieces on ice. The samples were placed into regular petri dish with 2-3 ml PBS or HBSS to wash the remaining blood.

For the digested cells, HBSS-trypsin (0.05%) mixture added into the hearts and minced by helping if scalpels. The petri dished were incubated overnight at $+4^{0}$C refrigerator and remaining the minced for the prohibited cluster formation. Medium was added into the flask for restrict the activity of trypsin. The cells were filtered through 50ml falcon tube then centrifuged at 1900 rpm for 10 minutes and pellet was suspended in complete media (10% FBS, 10% Horse serum, 1% PSA) (5-7ml). Cells were seeded in PRIMARIA dish and incubated at 37^{0}C, 5% CO_2 for 2.5-3 hours. Cells taken slowly with pipette and

centrifuged 1900 rpm for 10 minutes and pellet dissolved in 1ml media for further counting analysis.

2.17. EFFECT OF HSM ON HUMAN DERMAL FIBROBLAST

Human dermal fibroblast (HDF) passage 19 cells were obtained from -80 cell storage refrigerants in Yeditepe University Biotechnology Laboratories, Turkey. Cells were dissolved in the room temperature (RT) and DMEM supplemented with 10% (v/v) FBS (Sigma Aldrich, USA, cat no. 12103C) and 1% (v/v) PSA (10.000 units/mL penicillin, 10.000 µg/mL streptomycin, 25 µg/mL amphotericin B) (Invitrogen, Gibco, UK) were added into the cell suspension. The cells were centrifuged at 300 x g for 5 minutes at RT and the pellet was resuspended in fresh medium and transferred into a T-75cm^2flask (Sigma Aldrich, USA, cat no. CLS3289). The cells were incubated in the humidified incubator at 37°C and 5% CO_2. HDF cells were seeded at a density of 5000 cells per well in 96 well plates and treated with 0,1, 1 and 10 µM concentrations for cell viability WST1 assay.

2.18. SURFACE MARKER SCREENING ANALYSIS FROM AVIAN HSCS WITH FLOW CYTOMETRY

Lineage negative cells were seeded into 96 well plates at a density of 50,000 cells/well. Antibody staining were performed by human cell surface marker screening plate (BD Lyoplate Cat No 560747) for 15 minutes at room temperature and c-Kit (CD117) PE, CD34 FITC, Sca-1 PE-Cy7, lineage cocktail APC (BD StemFlow Cat No 560492) were added into the plate and incubated at 15 minutes at room temperature. As a seconder antibody, alexa flour 647 and alexa flour 488(BD Lyoplate Cat No 560747) were stained and incubated for 15 minutes o ice. Flow cytometry analysis was performed for the identifications of LSK and FITC positive populations. The antibody list were shown below from BD Human cell surface marker screening plate kit:

Table 2.18. The human cell surface marker screening plate 1(BD Lyoplate Cat No 560747).

Plate 1								
CD1a	HI149	Ms IgG 1, κ	CD28	L293	Ms IgG 1, κ	CD51/61	23C6	Ms IgG 1, κ
CD1b	M-T101	Ms IgG 1, κ	CD29	HUTS-21	Ms IgG 2a, κ	CD53	HI29	Ms IgG 1, κ
CD1d	CD1d42	Ms IgG 1, κ	CD30	BerH8	Ms IgG 1, κ	CD54	LB- 2	Ms IgG 2b, κ
CD2	RPA-2.10	Ms IgG 1, κ	CD31	WM59	Ms IgG 1, κ	CD55	IA10	Ms IgG 2a, κ
CD3	HIT 3a	Ms IgG 2a, κ	CD32	FL18.26	Ms IgG 2b, κ	CD56	B159	Ms IgG1, κ
CD4	RPA-T4	Ms IgG 1, κ	CD33	HIM3- 4	Ms IgG 1, κ	CD57	NK- 1	Ms IgM, κ
CD4v4	L120	Ms IgG 1, κ	CD34	581	Ms IgG 1, κ	CD58	1C3	Ms IgG 2a, κ
CD5	L17F12	Ms IgG 2a, κ	CD35	E11	Ms IgG 1, κ	CD59	p282 (H19)	Ms IgG 2a, κ
CD6	M-T605	Ms IgG 1, κ	CD36	CB38 (NL07)	Ms IgM, κ	CD61	VI-PL2	Ms IgG 1, κ
CD7	M-T701	Ms IgG 1, κ	CD37	M- B371	Ms IgG 1, κ	CD62E	68- 5H11	Ms IgG 1, κ
CD8a	SK1	Ms IgG 1, κ	CD38	HIT 2	Ms IgG 1, κ	CD62L	Dreg 56	Ms IgG 1, κ
CD8b	2ST 8.5H7	Ms IgG2a, κ	CD39	TU66	Ms IgG2b, κ	CD62P	AK-4	Ms IgG 1, κ
CD9	M-L13	Ms IgG 1, κ	CD40	5C3	Ms IgG 1, κ	CD63	H5C6	Ms IgG 1, κ
CD10	HI10a	Ms IgG	CD41a	HIP8	Ms IgG	CD64	10.1	Ms IgG

		2a, κ			1, κ			1, κ
CD11a	G43-25B	Ms IgG 2a, κ	CD41b	HIP2	Ms IgG 3, κ	CD66 (a,c,d,e)	B1.1/CD66	Ms IgG 2a, κ
CD11b	D12	Ms IgG 2a, κ	CD42a	ALMA.16	Ms IgG 1, κ	CD66b	G10F5	Ms IgM, κ
CD11c	B- ly 6	Ms IgG 1, κ	CD42b	HIP1	Ms IgG 1, κ	CD66f	IID10	Ms IgG 1, κ
CD13	WM15	Ms IgG 1, κ	CD43	1G10	Ms IgG 1, κ	CD69	FN50	Ms IgG 1, κ
CD14	M5E2	Ms IgG 2a, κ	CD44	G44- 26	Ms IgG 2b, κ	CD70	Ki- 24	Ms IgG 3, κ
CD15	HI98	Ms IgM, κ	CD45	HI30	Ms IgG 1, κ	CD71	M-A712	Ms IgG 2a, κ
CD15s	CSLEX1	Ms IgM, κ	CD45RA	HI100	Ms IgG 2b, κ	CD72	J4- 117	Ms IgG 2b, κ
CD16	3G8	Ms IgG 1, κ	CD45RB	MT4	Ms IgG 1, κ	CD73	AD2	Ms IgG 1, κ
CD18	6.7	Ms IgG 1, κ	CD45RO	UCHL1	Ms IgG 2a, κ	CD74	M- B741	Ms IgG 2a, κ
CD19	HIB19	Ms IgG 1, κ	CD46	E4.3	Ms IgG 2a, κ	CD75	LN1	Ms IgM, κ
CD20	2H7	Ms IgG 2b, κ	CD47	B6H12	Ms IgG 1, κ	CD77	5B5	Ms IgM, κ
CD21	B- ly 4	Ms IgG 1, κ	CD48	T U145	Ms IgM, κ	CD79b	CB3- 1	Ms IgG 1, κ
CD22	HIB22	Ms IgG 1, κ	CD49a	SR84	Ms IgG 1, κ	CD80	L307.4	Ms IgG 1, κ
CD23	EBVCS-5	Ms IgG 1, κ	CD49b	AK-7	Ms IgG 1, κ	CD81	JS-81	Ms IgG 1, κ
CD24	ML5	Ms IgG 2a, κ	CD49c	C3 II.1	Ms IgG 1, κ	CD83	HB15e	Ms IgG 1, κ

CD25	M-A251	Ms IgG 1, κ	CD49d	9F10	Ms IgG 1, κ	CD84	2G7	Ms IgG 1, κ
CD26	M-A261	Ms IgG 1, κ	CD49e	VC5	Ms IgG 1, κ	CD85	GHI/75	Ms IgG 2b, κ
CD27	M-T271	Ms IgG 1, κ	CD50	TU41	Ms IgG 2b, κ			

Table 2.19. The human cell surface marker screening plate2 (BD Lyoplate Cat No 560747).

Plate 2								
CD86	2331 (FUN-1)	Ms IgG 1, κ	CD123	9F5	Ms IgG 1, κ	CD172b	B4B6	Ms IgG 1, κ
CD87	VIM5	Ms IgG 1, κ	CD124	hIL4R-M57	Ms IgG 1, κ	CD177	MEM-166	Ms IgG 1, κ
CD88	D53-1473	Ms IgG 1, κ	CD126	M5	Ms IgG1, κ	CD178	NOK- 1	Ms IgG 1
CD89	A59	Ms IgG 1, κ	CD127	hIL- 7R-M21	Ms IgG 1, κ	CD180	G28- 8	Ms IgG 1, κ
CD90	5E10	Ms IgG 1, κ	CD128b	6C6	Ms IgG 1, λ	CD181	5A12	Ms IgG 2b, κ
CD91	A2MR-alpha 2	Ms IgG 1, κ	CD130	AM64	Ms IgG 1, κ	CD183	1C6/CXCR3	Ms IgG 1, κ
CDw93	R139	Ms IgG 2b, κ	CD134	ACT35	Ms IgG 1, κ	CD184	12G5	Ms IgG 2a, κ
CD94	HP- 3D9	Ms IgG 1, κ	CD135	4G8	Ms IgG 1, κ	CD193	5E8	Ms IgG 2b, κ
CD95	DX2	Ms IgG 1, κ	CD137	4B4- 1	Ms IgG 1, κ	CD195	2D7/CCR5	Ms IgG 2a, κ
CD97	VIM3b	Ms IgG 1, κ	CD137 Ligand	C65- 485	Ms IgG 1, κ	CD196	11A9	Ms IgG 1, κ

CD98	UM7F8	Ms IgG 1, κ	CD138	Mi15	Ms IgG 1, κ	CD197	2H4	Ms IgM, κ
CD99	TU12	Ms IgG 2a, κ	CD140a	alpha R1	Ms IgG 2a, κ	CD200	MRC OX-104	Ms IgG 1, κ
CD99R	HIT 4	Ms IgM, κ	CD140b	28D4	Ms IgG 2a, κ	CD205	MG38	Ms IgG 2b
CD100	A8	Ms IgG 1, κ	CD141	1A4	Ms IgG 1, κ	CD206	19.2	Ms IgG 1, κ
CD102	CBR-1C2/2.1	Ms IgG 2a, κ	CD142	HTF-1	Ms IgG 1, κ	CD209	DCN46	Ms IgG 2b, κ
CD103	Ber-ACT8	Ms IgG 1, κ	CD144	55- 7H1	Ms IgG 1, κ	CD220	3B6/IR	Ms IgG 1, κ
CD105	266	Ms IgG 1, κ	CD146	P1H12	Ms IgG 1, κ	CD221	3B7	Ms IgG 1, κ
CD106	51- 10C9	Ms IgG 1, κ	CD147	HIM6	Ms IgG 1, κ	CD226	DX11	Ms IgG 1, κ
CD107a	H4A3	Ms IgG 1, κ	CD150	A12	Ms IgG 1, κ	CD227	HMPV	Ms IgG 1, κ
CD107b	H4B4	Ms IgG 1, κ	CD151	14A2.H1	Ms IgG 1, κ	CD229	HLy9.1.25	Ms IgG 1, κ
CD108	KS-2	Ms IgG 2a, κ	CD152	BNI3	Ms IgG 2a, κ	CD231	M3-3D9 (SN1a)	Ms IgG 1, κ
CD109	TEA 2/16	Ms IgG 1, κ	CD153	D2- 1173	Ms IgG 1, κ	CD235a	GA-R2 (HIR2)	Ms IgG 2b, κ
CD112	R2.525	Ms IgG 1, κ	CD154	TRAP1	Ms IgG 1, κ	CD243	17F9	Ms IgG 2b, κ
CD114	LMM741	Ms IgG 1, κ	CD158a	HP- 3E4	Ms IgM, κ	CD244	2- 69	Ms IgG 2a, κ
CD116	M5D12	Ms IgM, κ	CD158b	CH-L	Ms IgG 2b, κ	CD255	CARL- 1	Ms IgG3

CD117	Y B5.B8	Ms IgG 1, κ	CD161	DX12	Ms IgG 1, κ	CD268	11C1	Ms IgG 1, κ
CD118	12D3	Ms IgG1, κ	CD162	KPL- 1	Ms IgG 1, κ	CD271	C40- 1457	Ms IgG 1, κ
CD119	GIR- 208	Ms IgG 1, κ	CD163	GHI/61	Ms IgG 1, κ	CD273	MIH18	Ms IgG 1, κ
CD120a	MABTN FR1-A1	Ms IgG 1	CD164	N6B6	Ms IgG 2a, κ	CD274	MIH1	Ms IgG 1, κ
CD121a	HIL1R- M1	Ms IgG1, κ	CD165	SN2	Ms IgG 1, κ	CD275	2D3/B7- H2	Ms IgG 2b, κ
CD121b	MNC2	Ms IgG 1, κ	CD166	3A6	Ms IgG 1, κ	CD278		

Table 2.20. The human cell surface marker screening plate 3 (BD Lyoplate Cat No 560747)

Plate 3								
CD279	MIH4	Ms IgG 1, κ	fMLP receptor	5F1	Ms IgG 1, κ	Ms IgG2a IC	G155- 178	Ms IgG2a
CD282	11G7	Ms IgG 1, κ	γδTCR	B1	Ms IgG 1, κ	Ms IgG2b IC	27- 35	Ms IgG2b
CD305	DX26	Ms IgG 1, κ	HPC	BB9	Ms IgG1	Ms IgG3 IC	J606	Ms IgG3
CD309	89106	Ms IgG 1, κ	HLA- A,B,C	G46- 2.6	Ms IgG 1, κ	CD49f	GoH3	Rt IgG 2a, κ
CD314	1D11	Ms IgG 1, κ	HLA-A2	BB7.2	Ms IgG 2b, κ	CD104	439- 9B	Rt IgG2b, κ
CD321	M.AB.F1 1	Ms IgG 1, κ	HLA- DQ	T U169	Ms IgG 2a, κ	CD120b	hTNFR- M1	Rt IgG 2b, κ
CDw327	E20- 1232	Ms IgG1, κ	HLA- DR	G46-6 (L243)	Ms IgG 2a, κ	CD132	T UGh4	Rt IgG 2b, κ

CDw328	F023-420	Ms IgG 1, κ	HLA-DR, DP, DQ	TU39	Ms IgG 2a, κ	CD201	RCR-252	Rt IgG 1, κ
CD329	E10- 286	Ms IgG1, κ	Invariant NK T	6B11	Ms IgG 1, κ	CD210	3F9	Rt IgG 2a, κ
CD335	9E2/NKp46	Ms IgG 1, κ	Disialoganglioside GD2	14.G2a	Ms IgG2a	CD212	2B6/12beta 2	Rt IgG 2a, κ
CD336	P44-8.1	Ms IgG1, κ	MIC A/B	6D4	Ms IgG2a	CD267	1A1-K21-M22	Rt IgG2a, κ
CD337	P30-15	Ms IgG1, κ	NKB1	DX9	Ms IgG 1, κ	CD294	BM16	Rt IgG 2a, κ
CD338	5D3	Ms IgG 2b, κ	SSEA- 1	MC480	Ms IgM, κ	SSEA-3	MC631	Rt IgM
CD304	Neu24.7	Ms IgG1	SSEA- 4	MC813-70	Ms IgG3	CLA	HECA-452	Rt IgM, κ
αβT CR	T10B9.1A-31	Ms IgM, κ	TRA-1-60	TRA-1-60	Ms IgM	Integrin β7	FIB504	Rt IgG 2a, κ
β2- microglobulin	TU99	Ms IgM, κ	TRA-1-81	TRA-1-81	Ms IgM, κ	Rt IgM IC	R4- 22	Rt IgM
BLTR-1	203/14F11	Ms IgG1, κ	Vβ 23	AHUT 7	Ms IgG 1, κ	Rt IgG1 IC	R3- 34	Rt IgG1
CLIP	CerCLIP	Ms IgG 1, κ	Vβ 8	JR2	Ms IgG 2b, κ	Rt IgG2a IC	R35- 95	Rt IgG2a
CMRF-56	CMRF56	Ms IgG1, κ	Ms IgM IC	G155-228	Ms IgM			
EGF Receptor	EGFR1	Ms IgG 2b, κ	Ms IgG1 IC	MOPC-21	Ms IgG1			

2.19. TUNEL ASSAY

According to mouse HPSC enrichment set DM (BD Pharmingen, cat no. 558451) protocol, magnetic lineage depletion process was performed. The cells were harvested in ice-cold DPBS supplemented with 2% (v/v) fetal bovine serum (FBS, Sigma Aldrich, USA, cat no. 12103C). The mouse BD Fc block was performed and biotinylated lineage depletion cocktail was added to cell suspension. Following the washing steps, the cells were labeled with streptavidin particles and situated into magnetic field of the IMagnet (BD Pharmingen cat. no. 552311). The lienage negative cells were collected from final depleted fraction. Mouse LSK cells were seeded at a density of 5000 cells/well in 96-well plates and treated with pifithrin-α and DMSO (0,5%) in three replicates at a three concentrations for 3 days in the humidified incubator at 37°C and 5% CO_2. After 7 days, cell were fixed and permabilized for the Tunel and DAPI labeling. The cells were stained according to *In Situ* Cell Death Detection Kit, Flourescein (Sigma Aldrich, cat no. 11684795910). The cells image were analyzed in Cytell

3. RESULTS

3.1. HDF, HUVEC, HUMAN ADIPOSE AND HUMAN BONE MARROW DERIVED MSCS WST-1 ANALYSIS

The HDF cell were treated with 10 μm , 1 μm and 0.1 μm concentration of pifithrin –α and the viability of cells were measured with WST-1 assay after 5 days after treatments. Relative cell numbers were measuered at OD450 of molecule treated cells to DMSO cells from same day of treatment. The proliferation rates of cells were not increased significantly. On the other hand, pifithrin –α was not decreased the viability of cells. (Figure3.1.A) The HUVEC cell were treated with pifithrin –α and DMSO for the observing cell viability. After 3 days treatments, WST-1 reagent was added into the cells and obtained relative cell numbers. The pifithrin –α affected the proliferation of cells slightly in a positive manner. (Figure3.1.B) WST-1 analysis indicates relative cell proliferation of adipose derived MSCs cells. The cells were treated fro 3 days with pifithrin –α and DMSO was used as control. After measured at OD450, there was no significant contribution. (Figure3.1.C) Human bone marrow HSC cells were treated 3 days with pifithrin –α and DMSO. After completed the treatment process, the cell proliferation rate was analyzed. The molecule was slight increase the proliferation of hBM HSC cells when compared to DMSO. (Figure3.1.D)

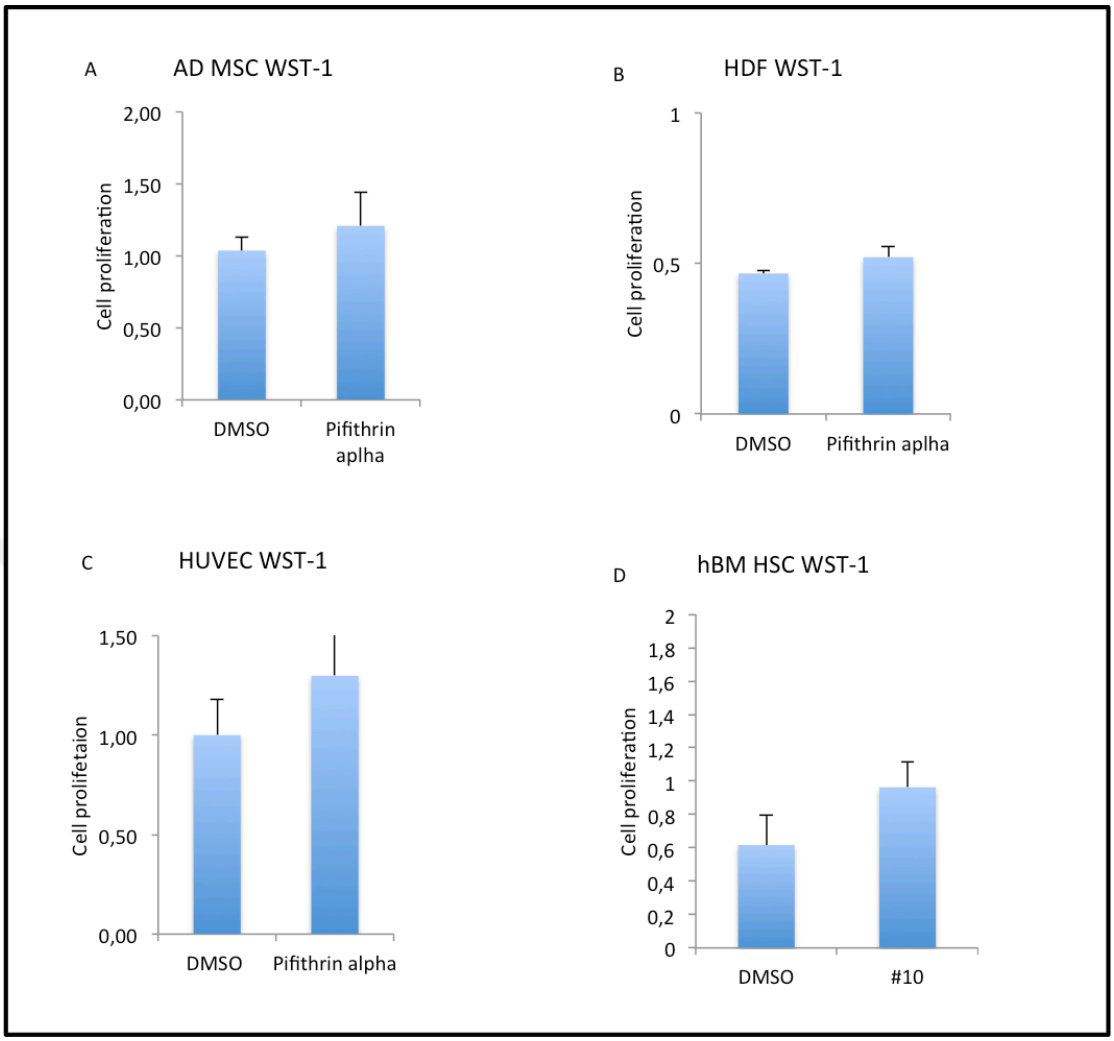

Figure3.1. **(**A) Analysis of cell proliferation of HDF cells by using WST-1 assay with 10 µm , 1 µm and 0.1 µm concentration of pifithrin –α. (B) Analysis of cell proliferation of HUVEC cell derived from mouse by using WST-1 assay. (C) Analysis of cell proliferation of MSCs derived from human adipose tissue by using WST-1 assay. (D) Analysis of cell proliferation of HSCs derived from human bone marrow by using WST-1 assay.

3.2. MOUSE BONE MARROW DERIVED HSCS FLOW CYTOMETRY

The lineage negative cells were isolated and depleted three times from magnetic field of the IMagnet (BD Pharmingen cat. no. 552311). After completed this step, the HSCs were treated with 0,1, 1 and 10 µM dose pifithrin–α and stained with mouse c-Kit (CD117) PE, CD34 FITC, Sca-1 PE-Cy7 for the analyzing LSK cells.

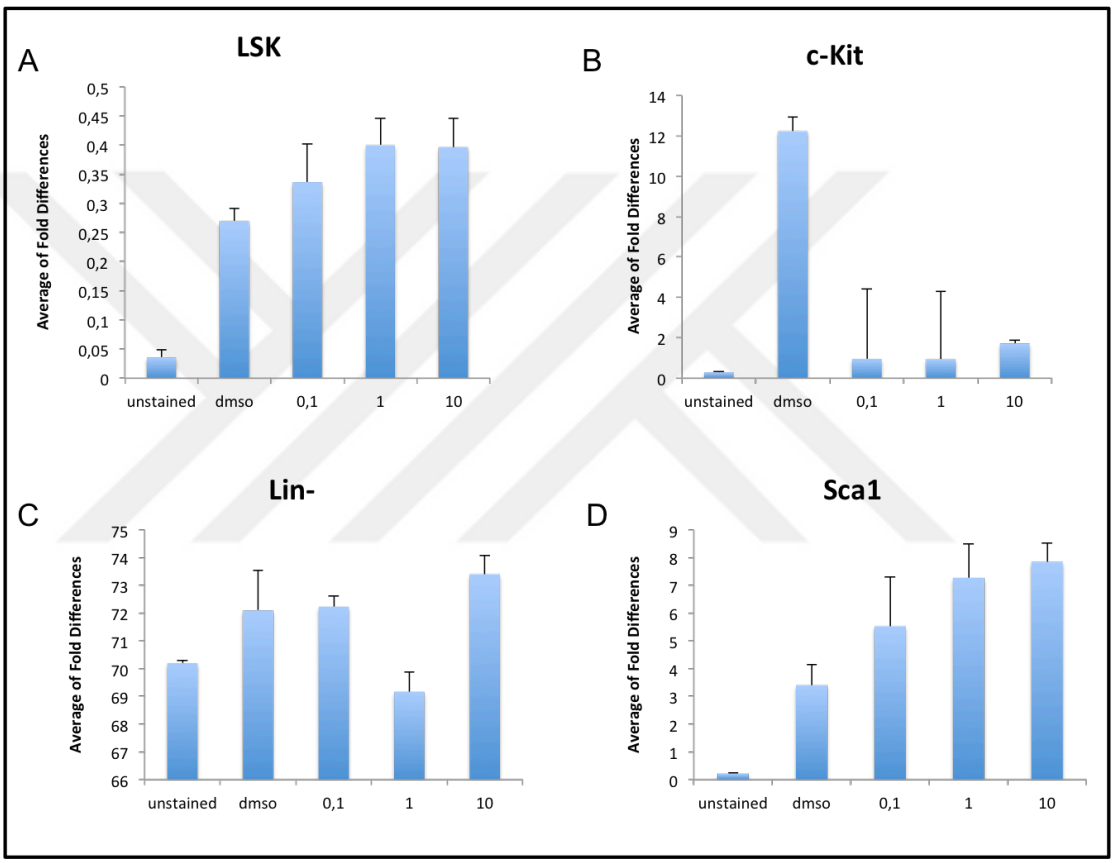

Figure3.2. The HSPCs content (LSK) average fold differences after treatment with pifithrin –α and DMSO by flow analysis. LSK fold differences were increased by dose dependent of pirfithrin- alpha on Figure 3.2.A. C-kit positive cells were increased by dose dependent as LSK result, however DMSO level was much more than pifithrin-alpha on Figure 3.2.B. Lineage negative cells were also showed increased on 10 µM dose of pifithrin alpha on Figure 3.2.C. Sca1 positeve cells were increased by parallel of dose of pifithrin alpha molecule at last figure.

3.3. MOUSE BONE MARROW DERIVED HSCS WERE TREATED WITH PIFITHRIN ALPHA FOR 7 DAYS

The HSPCs were isolated from bone marrow of mouse and after completed depleted procedure, cells were incubated for 7 days treatment with pifithrin–α and DMSO. The number cells were increased significantly by helping of small molecule on LSK cells. The effects of this molecule was investigated and stained for the flow cytometry.

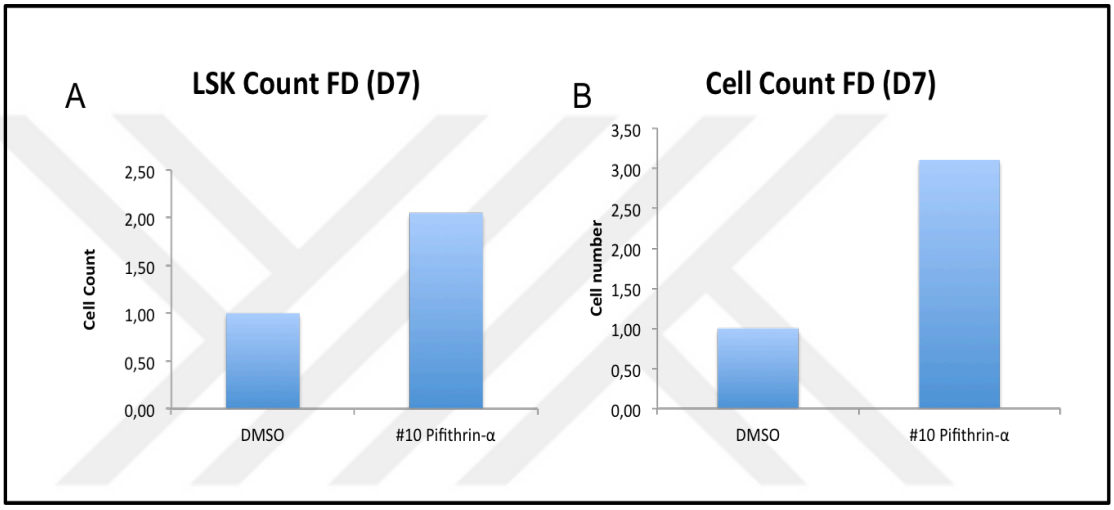

Figure3.3. The cell numbers of LSK cells were observed after treated with pifithrin –α. The HSPCs number 7 days after treatment with pifithrin –α by flow analysis. After incubated and treated cells with different doses of small molecule, the cell number was evaluated. The numbers of LSK cells were increase by helping of pifithrin –α. The cell number also increased significantly.

For the analysis of LSK and long term HSCs count and cell percentage; after competed treatment for 7 days; the cells were analyzed on flow cytometry and measured the cell percentage and number of HSCs.

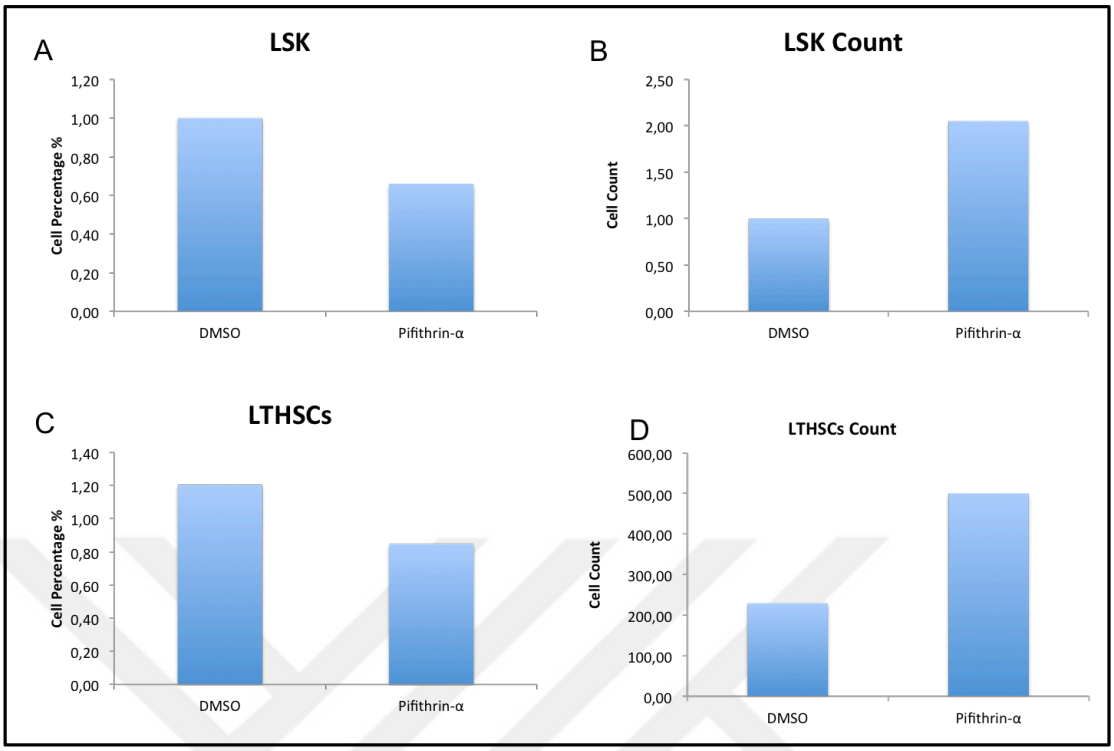

Figure 3.4. The cells were treated with pifithrin –α for 7 days. After stained with antibodies, cell numbers of HSCs were analyzed by flow cytometry. A) The number of cells was increased after treated with small molecule. B) The cell number of long-term HSCs cells was increased approximately two fold comparing to DMSO after treated with pifithrin –α.

3.4. CELL CYCLE ANALYSIS OF MLSK

For the determine cell cycle of HSCs cell, cell cycle analysis was performed. After isolated step, cells were seeded into 96 well plate about 5,000 cells per/well and treated with pifithrin alpha. 3 days later, cells were stained with Hoescht 33342 and Pyronin Y for the analysis of cell cycle. G0 ,G1 and S/G2 phases indicated by flow cytometry.

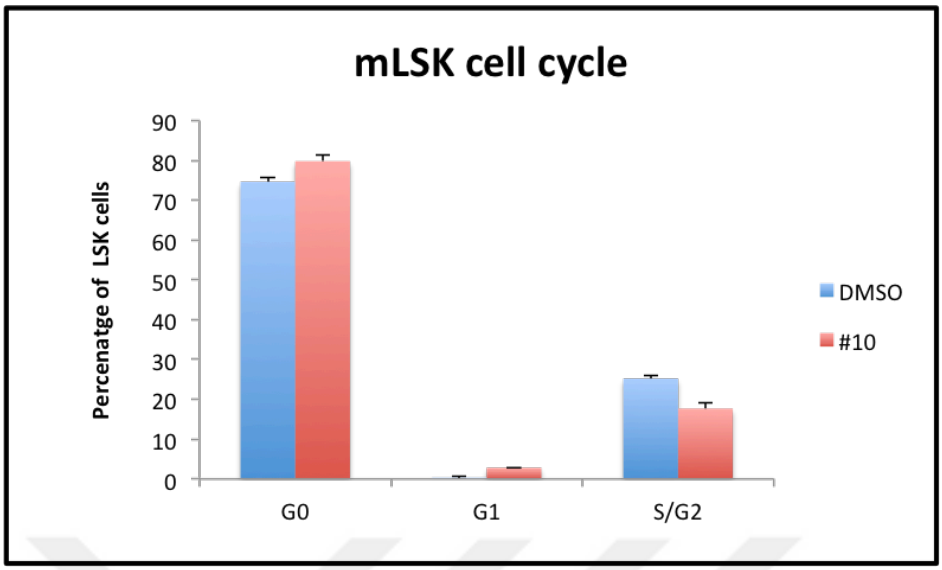

Figure 3.5. The cells were analyzed after staining by flow cytometry. The affects of pifithrin alpha were observed on cell phases. In G0 AND G1 phases the cell percentage were increased and they induced for proliferation. On G2 phase the percentage of LSK cells were decreased.

3.5. APOPTOSIS ANALYSIS OF MLSK

The LSK HSCs were isolated and seeded onto 96 well plate and the treated with pifitrin alpha. On a third day, the measurement was applied. The cells were stained with Annexin V and propidium iodide and analyzed by flow cytometry.

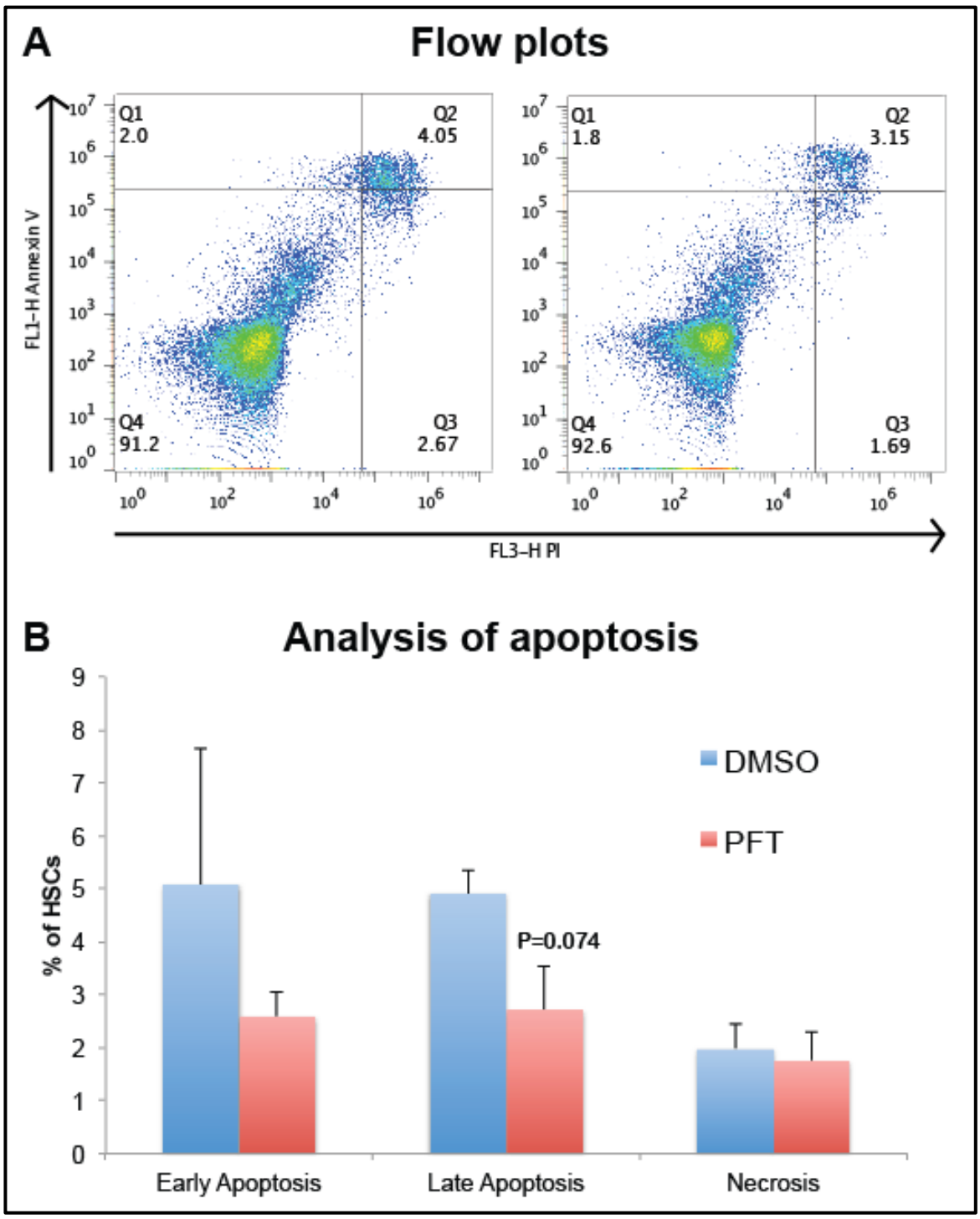

Figure 3.6. The apoptosis analysis was performed and the effect of pifithrin alpha molecule was indicated. The cells were induced for necrosis and early apoptosis, however the late apotosis was decreased.

3.6. MOUSE BONE MARROW DERIVED MSCS FLOW ANALYSIS

The mouse bone marrow derived MSCs were seeded and treated with pifithrin –α. After completed to treatment process, the cells were collected and analyzed by WST-1 assay. The cell viability of MSC was compared with non treated. DMSO and pifithrin –α. There was significant contribution for pifithrin alpha.

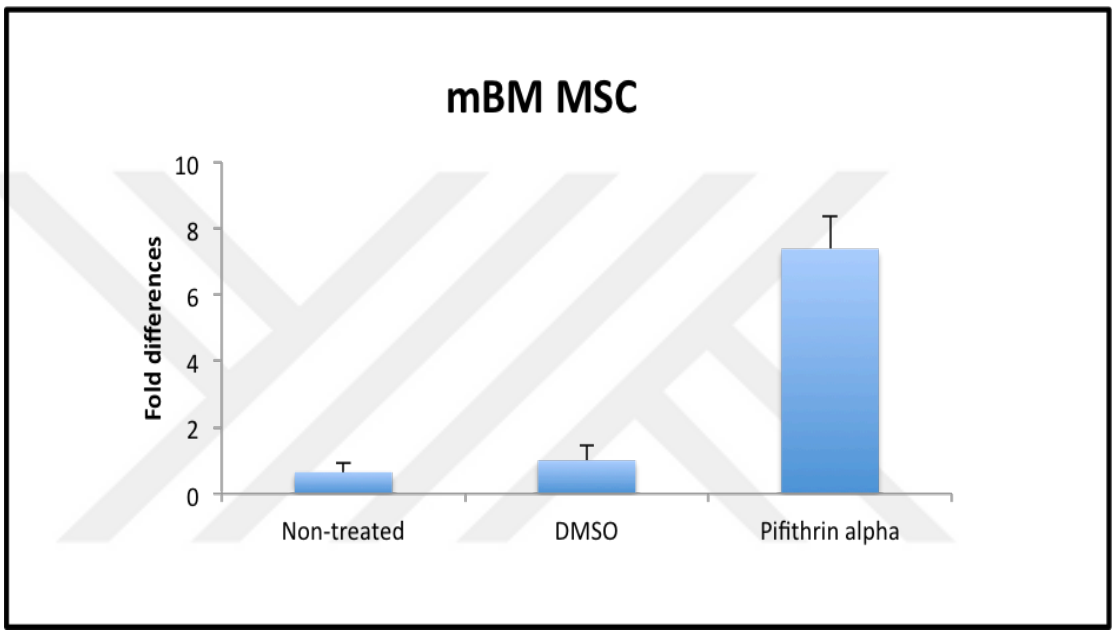

Figure 3.7. WST-1 analysis of mouse BM MSCs. The pifithrin alpha treated cell were significantly increased when compared to DMSO and non treatment cells.

3.7. UCB HSC FLOW ANALYSIS

The UCB HSC cells were obtained from newborn cord blood. The cells were treated with pifithrin –α and the cell numbers were analyzed by flow cytometry. DMSO was decreased the cell number when compared to untreated cells. Furthermore, pifithrin –α increased the cell number significantly.

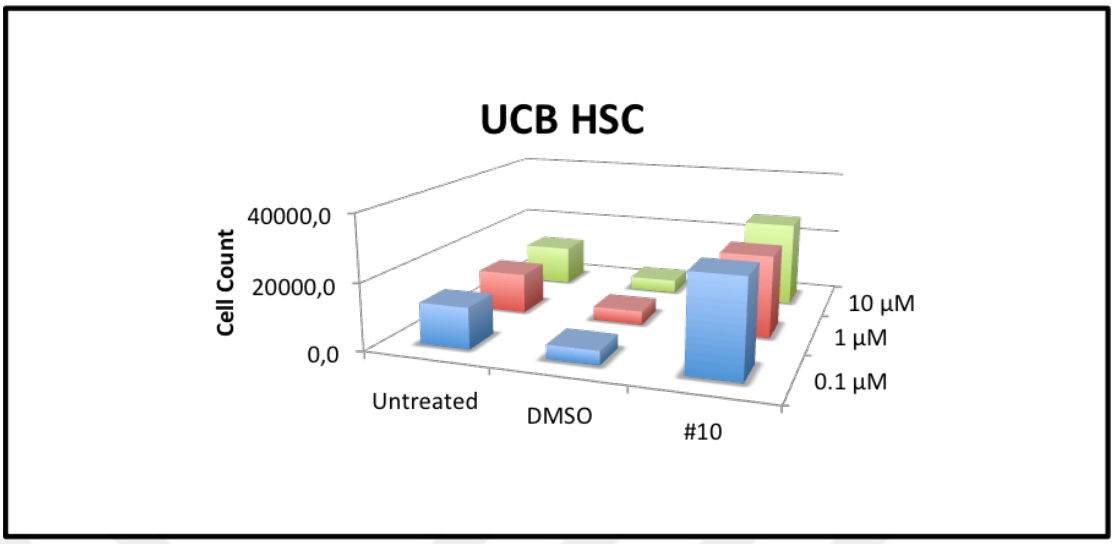

Figure 3.8. The UCB HSC cell counts were measured by flow analysis. The cells were treated with three different doses of pifithrin alpha for comparing cells count. The HSC cells were increased approximately four fold from DMSO.

From total cell count and cell percentage of UCB HSC CD34 cells, the cell numbers of CD34 cells were calculated. The pifithrin –α treated cells number were higher than DMSO treatment. From total cell count and cell percentage of UCB HSC CD133 cells, the cell number of CD34 cells were calculated. The pifithrin –α treated cells number were significantly higher than DMSO treatment. From total cell count and cell percentage of UCB HSC CD34+ CD133+ cells, the cell numbers of CD34 cells were calculated. The DMSO treated cells numbers were higher than pifithrin –α treatment. From total cell count and cell percentage of UCB HSC CD133 cells, the cell number of CD34 cells were calculated. The pifithrin –α treated cells number were significantly higher than DMSO treatment.

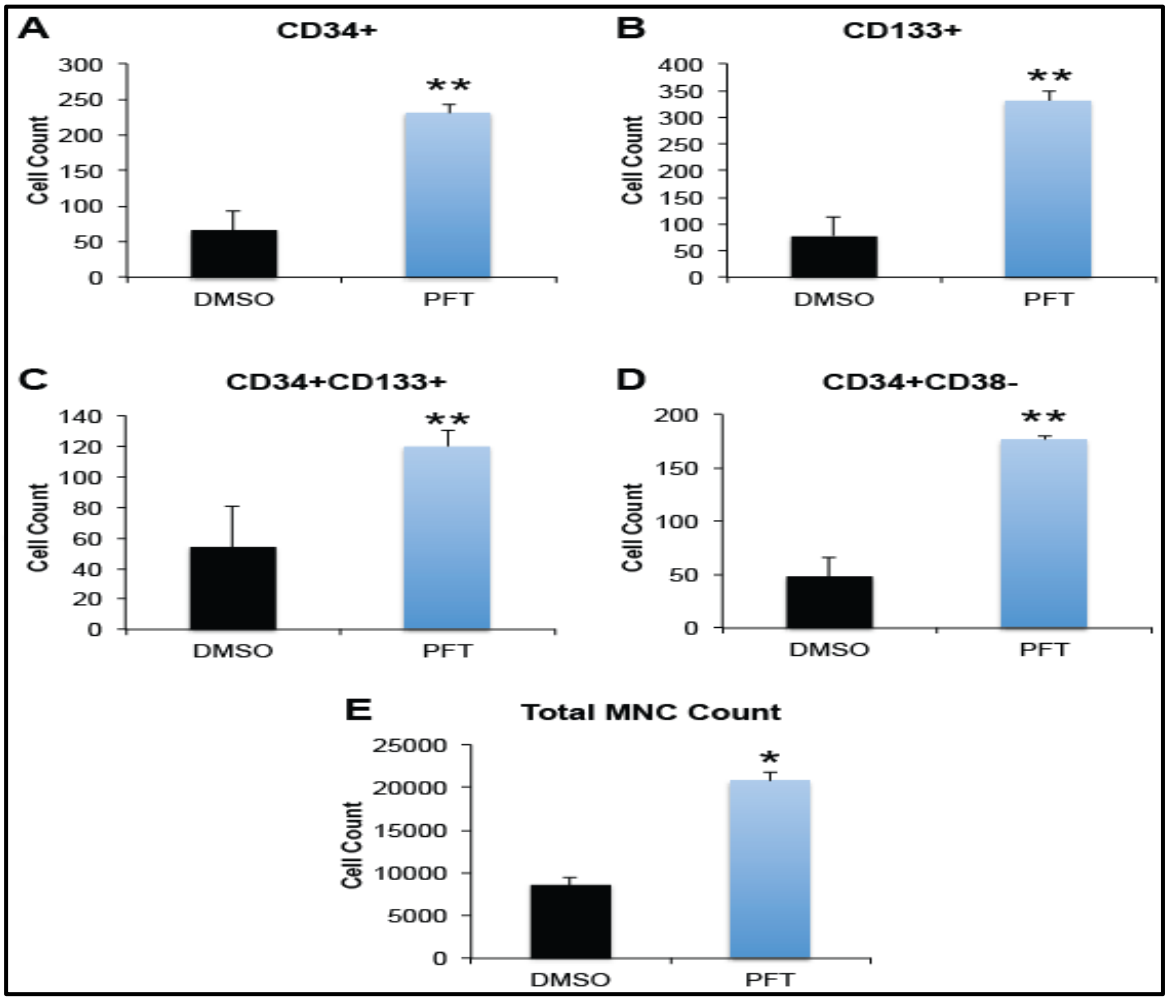

Figure 3.9. The UCB HSCs were treated with pifithrin –α and stained with CD34 antibody. The cell percentage of UCB HSCs was analyzed; DMSO levels of cells was significantly increase. The pifithrin –α levels were increased when compared to untreated cells. However, the DMSO treatment cells level were more than pifithrin –α. (Figure9.A) The UCB HSCs were treated with pifithrin –α and stained with CD133 antibody. The cell percentage of UCB HSCs was analyzed; pifithrin –α levels of cells was significantly increase when compared to untreated cells and DMSO. (Figure9.B) The UCB HSCs were treated with pifithrin –α and stained with CD34 and CD133 antibody. The cell percentage of UCB HSCs was analyzed; DMSO levels of cells was significantly increase when compared to untreated cells and pifithrin –α. (Figure9.C) The UCB HSCs were treated with pifithrin –α and stained with CD34 positive and CD38- antibody. The cell percentage of UCB HSCs was analyzed; DMSO levels of cells was significantly increase when compared to untreated cells and pifithrin –α (Figure9.D). The mononuclear HSC count were also increaed significantly (Figure9.E).

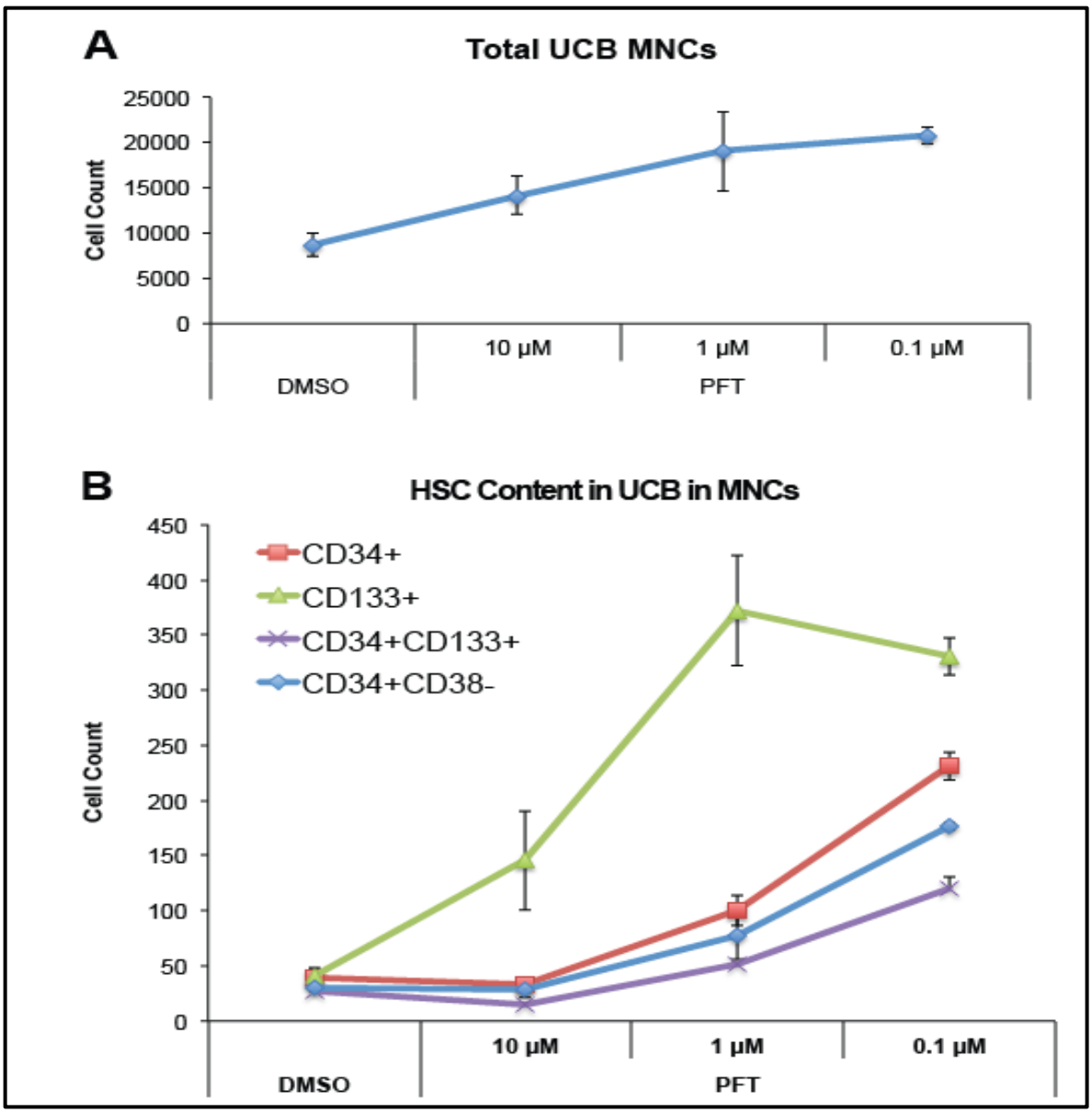

Figure 3.10. (A) Cell numbers of total UCB HSC. The UCB HSCs were treated with different dose of PFT-alpha. The highest cell count was measured on 1 µM dosage. (B) Cell numbers of UCB HSC CD133, UCB HSC CD34+CD133+ and UCB HSC CD34+CD38- were analyzed at three different dosage of pifithin alpha.

3.8. REPOPULATION OF EX VIVO EXPANDED HSCS TREATED WITH PIFITHRIN ALPHA

The HSC stem cells were isolated from 6-8 weeks old balb-c mouse. After harvested mHSCs, the cells were treated with pifithrin alpha for 7 days. The expanded and treated HSCs were injected from SCID mouse eye. After 1 mount later, the blood were taken by helping of hematocrit capillary. The flow cytometry analysis were performed.

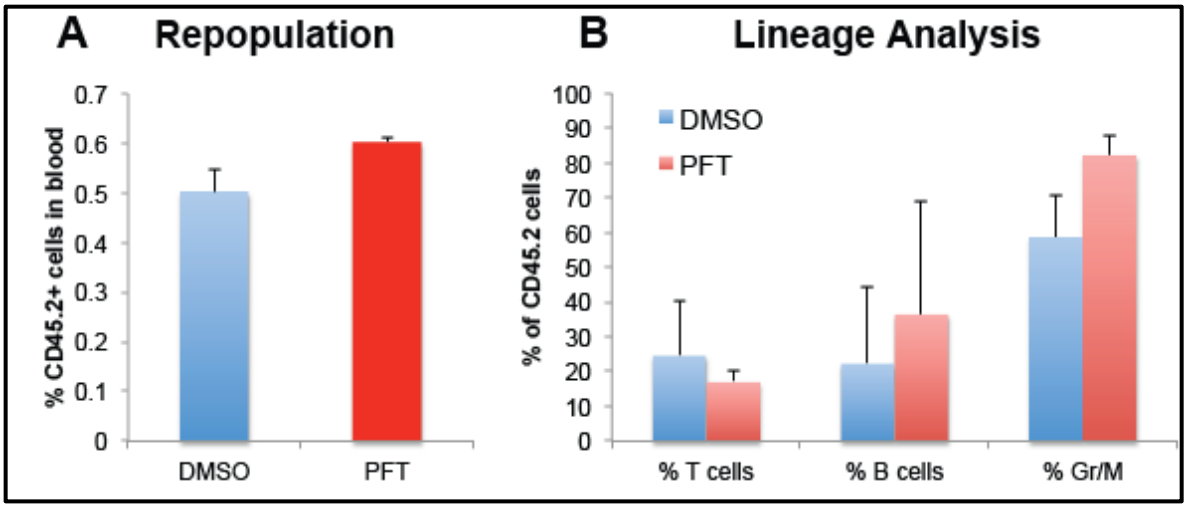

Figure 3.11. The %CD45.2+ cells in blood were analyzed by flow cytometry. The pifithrin alpha expanded and treated HSCs were repopulated succesfully. The percentage of T cell, B cell and granuloyte/macrophage cells were indicated and the small molecule treated cells were increased the cell percentages.

3.9. AVIAN HSC CELLS FLOW CYTOMETRY ANALYSIS

The avian HSC cells were indicated for the observing to be sure isolated and depletion method. Before starting depletion cells were counted by hemocytometry and also after depletion cells number were observed. The cells were stained with same antibody and the number and percentage of cells were compared.

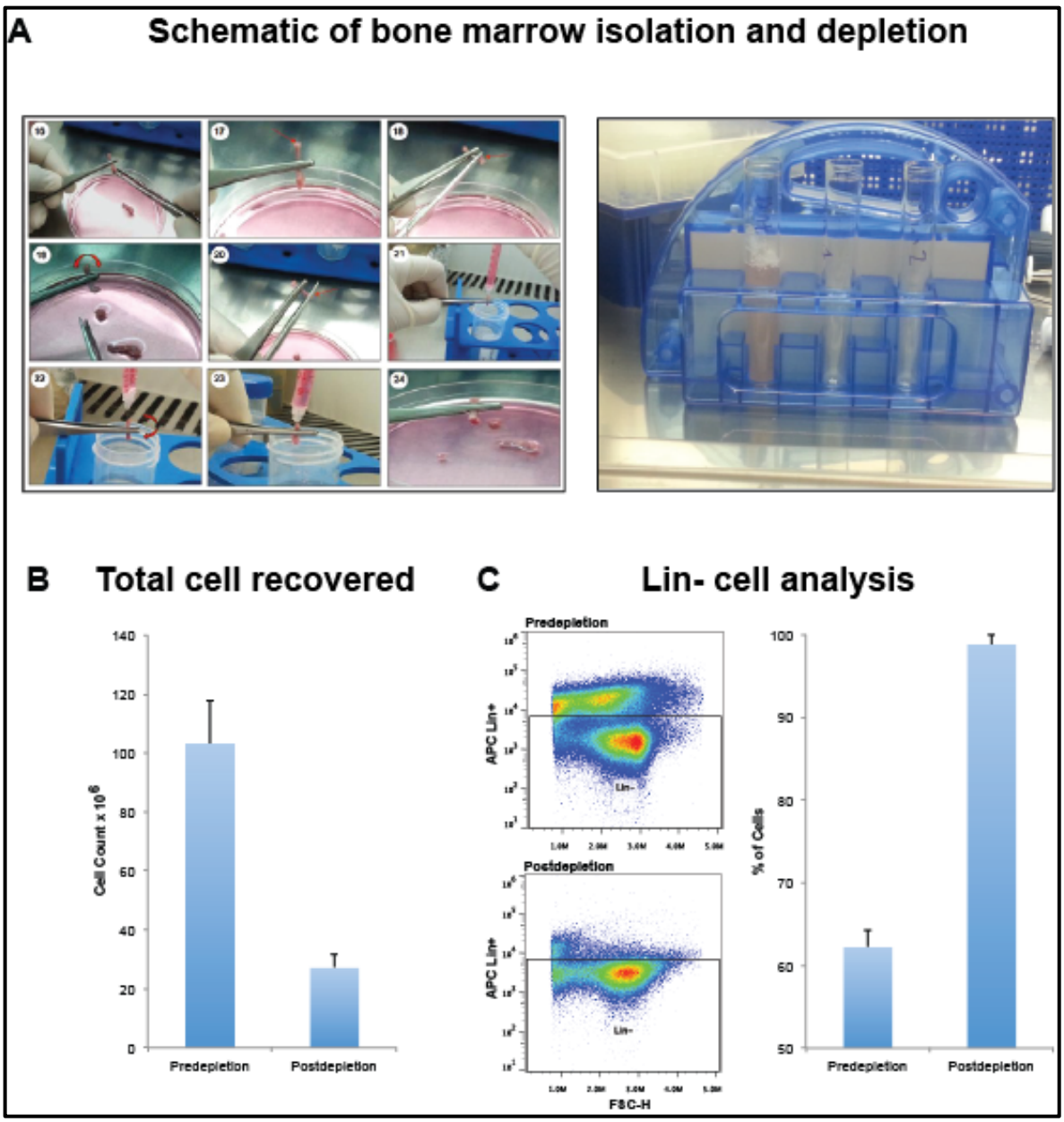

Figure 3.12. The cells were depleted and Lin negative cells were obtained. The LSK cells were stained for flow cytometry analysis. The number of cells was check against before and after depletion method. The number of cells was decreased about five times to WBM. Hematopoietic stem cells markers were stained and examine by flow cytometry. The percentages of cells were compared by whole bone marrow.

The isolated and depleted avaian HSC cells, stained with LSK cell marker such as; mouse c-Kit (CD117) PE, CD34 FITC, Sca-1 PE-Cy7, lineage cocktail APC (BD StemFlow Cat No 560492) and flow cytometry analysis were observed. For the identifications of LSK and LSKCD34^{low} populations the percentage of HSCs were calculated.

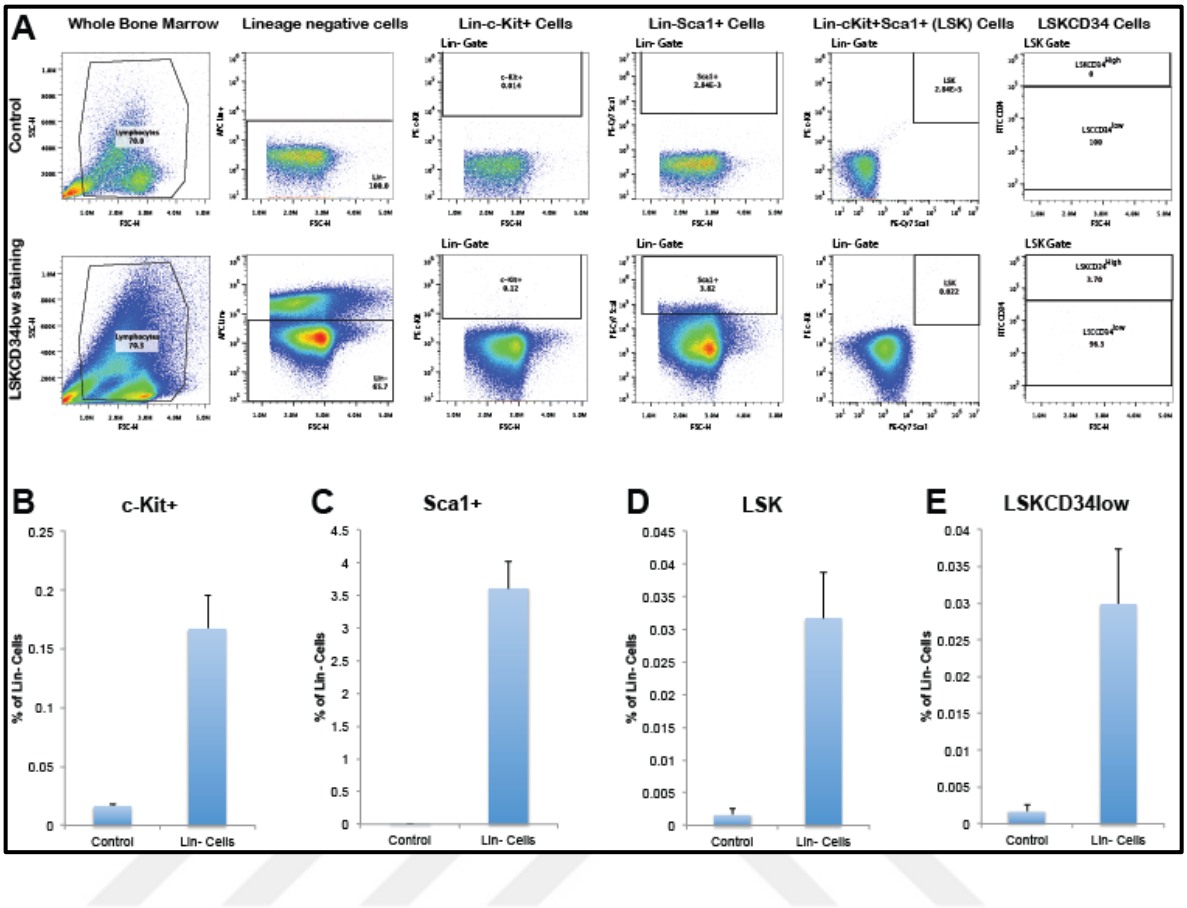

Figure 3.13. A) Hematopoietic stem cells markers were stained and investigated the HSC population by flow cytometry. B) The number of Lin- cells was high for c-kit positive marker.The Lin –cells percentage was 0,1 µM of HSCs. C) Sca1 positive cells were higher for Lin- cells about 4 times compared to control cells. D) The LSK percentage was also higher than control grup. E) The LSKCD34Low cells percentage was near to 0,03 percentage of Lin- cells.

3.10. AVIAN HSC MARKER SCREENING

The cells were examined for the determine HSC marker. The Avian HSC cells were analyzed by RT-PCR method. The expression levels of HSC marker were observed. The primer list was showed on method, after RNA isolation and cDNA synthesis the HSC marker screening was performed. The results were normalized to GAPDH. Although, lineage depleted cells and whole bone marrow cells were compared.

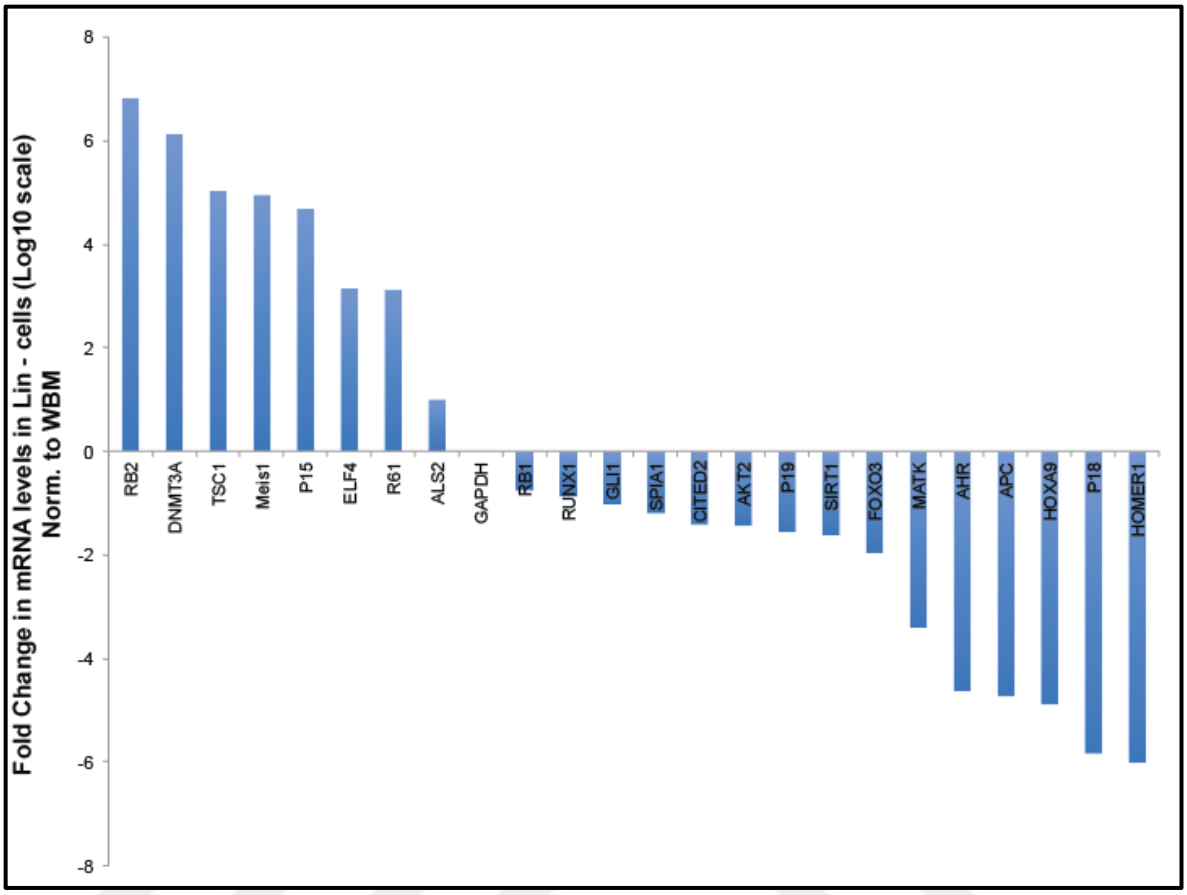

Figure 3.14. The HSC markers were indicated for avian HSCs. Some markers were down regulated and some of was up regulated: TSC1, FOXO3, CITED2, GLI1, RUNX1 and GAPDHV2 were down regulated. MEIS1, RB2, APC, SPIA1, SIRT1, ELF4 P15, P19 were up regulated.

3.11. SURFACE MARKER SCREENING AND DETERMINATION OF AVIAN HSCS

The avian HSC cells were seed onto 96 well plate for the indicated avian surface marker screening. The cells were stained with surface marker antibody (BD Lyoplate Cat No 560747) and c-Kit (CD117) PE, CD34 FITC, Sca-1 PE-Cy7, lineage cocktail APC (BD StemFlow Cat No 560492). For the measuring the wavelength seconder antibody staining was performed. The staining avian HSCs were analyzed by flow cytometry.

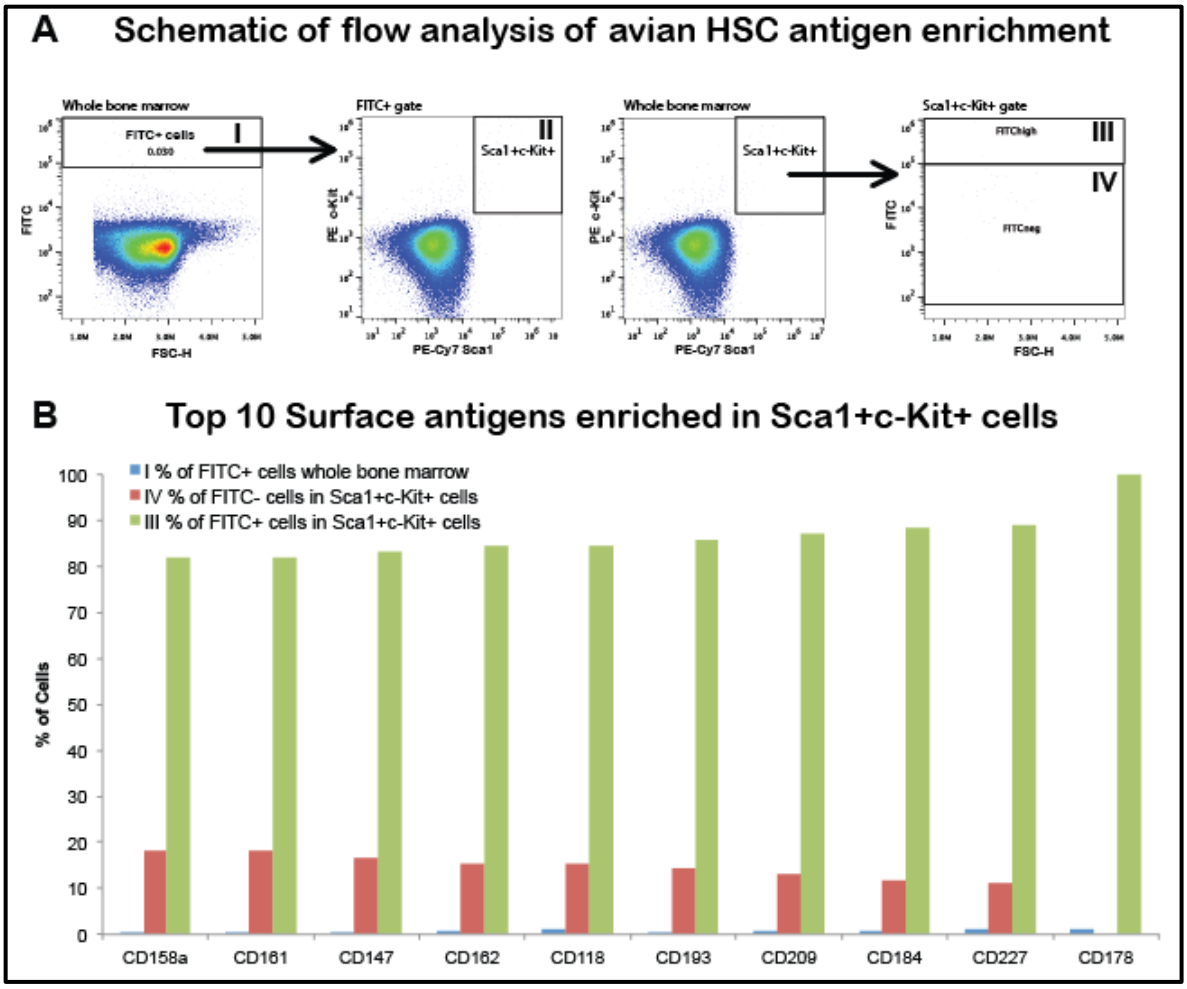

Figure 3.15**.** The LSK percentage and FITC percentage were taken for elimination. The antibodies were chosen by the over of average. The under of average were eliminated. The surface marker of avian HSC was detected according to aligment of FITC+ cells in Sca1+ cKit+ cells.

Table 3.10. The avian surface antigens were determined based on selection criteria. The stem cell selectivity was also calculated.

Gates	Population	Cut offs
I	% of FITC+ cells whole bone marrow	>0.3
II	% of Sca1+c-Kit+ cells in FITC+ cells	>70
III	% of FITC+ cells in Sca1+c-Kit+ cells	None
IV	% of FITC- cells in Sca1+c-Kit+ cells	None
Stem cell selectivity (SCS)	Ratio of III/IV	>2.4

Table 3.11. The avian surface antigens were elimated based on selection criteria. The fold stem cell enrichment factor was calculated and queued.

FITC conjugated Abs	Gates				Stem cell selectivity (SCS)	Stem cell enrichment factor (SCEF)	Fold stem cell enrichment
Surface Antigen	I	II	III	IV	Ratio of III/IV	Ratio of III/I	SCEFxSCS
CD178	1.04	83.3	100	0	15.0	96	1442
CD193	0.38	90.9	85.7	14.3	6.0	226	1352
CD158a	0.33	84	81.8	18.2	4.5	248	1114
CD147	0.4	90.9	83.3	16.7	5.0	208	1039
CD184	0.85	74.5	88.4	11.6	7.6	104	793
CD209	0.76	76.5	87.1	12.9	6.8	115	774
CD158b	0.33	92.3	76.5	23.5	3.3	232	755
CD161	0.51	100	81.8	18.2	4.5	160	721
CD227	1.07	82.4	88.9	11.1	8.0	83	665
CD146	0.32	92.9	74.1	25.9	2.9	232	663
CD162	0.77	71.4	84.6	15.4	5.5	110	604
CD196	0.43	75	76.9	23.1	3.3	179	595
CD220	0.46	85.2	77.1	22.9	3.4	168	564
CD183	0.59	83.9	80	20	4.0	136	542
CD197	0.73	77.8	78.6	21.4	3.7	108	395
CD118	1.19	90.9	84.6	15.4	5.5	71	391
CD56	0.62	83.3	75	25	3.0	121	363
CD97	1.26	71.4	78.9	21.1	3.7	63	234
CD9	1.6	87.5	72.7	27.3	2.7	45	121

3.12. TUNEL ASSAY

The mHSC cells were isolated and seeded into 96 well plate and treated with pifithrin aplha and DMSO for 7 days. After completed treatment process, the cells were stained with Tunel assay for the observing DNA damage cells.

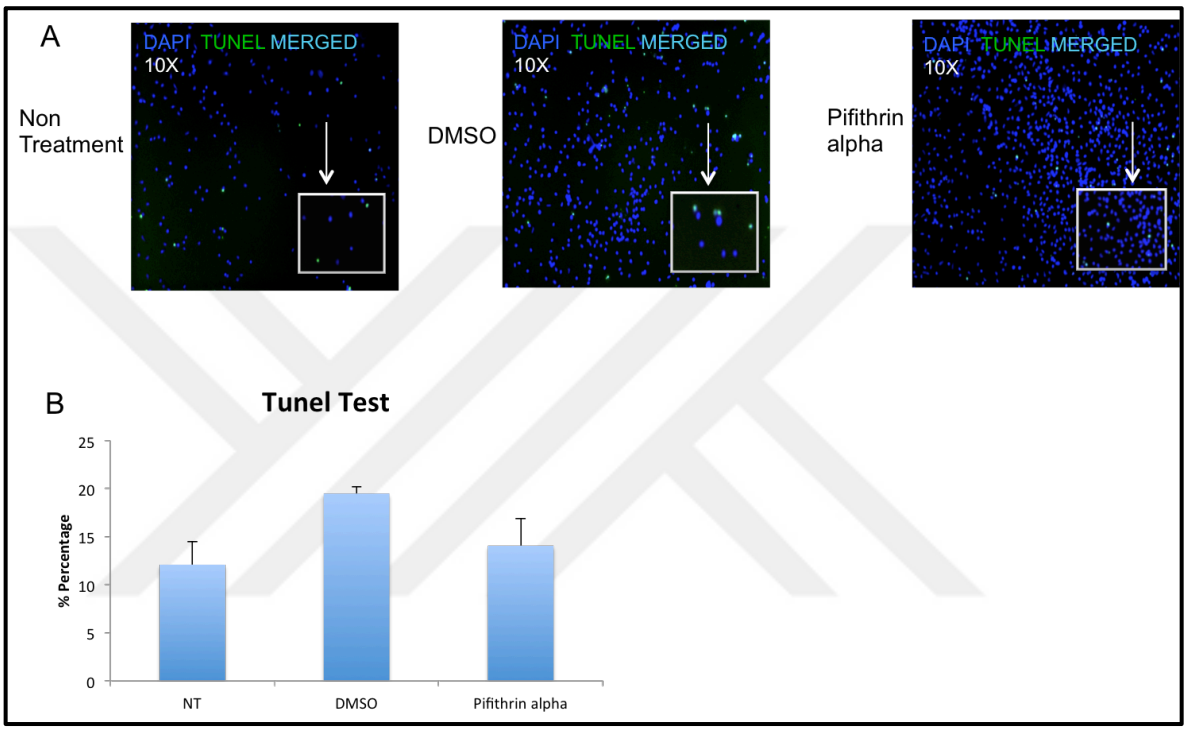

Figure 3.16. The non treatment cell, DMSO and pifithrin alpha treated cells were stained with DAPI and Tunnel assay. DAPI stained cells were indicated with blue color and Tunel stained cells were indicated with green color. The normal DNA and DNA damage images and percentages were evaluated.

4. DISCUSSION

Mouse and human HSPCs can be detected on the basis of many phenotypic markers. Hematopoietic stem cells are characterized by their ability to regenerate and remain LT multilineage hematopoiesis in defined *in vitro* conditions. Progenitor cells have a hematopoietic potential *in vivo* and should be detectable *in vitro*. Current cell-based systems are likely to be more accurate in order to support more accurate *in vivo* microenvironment of hematopietic stem cells as well to promote the growing of lymphoid progenitors. Under suitable conditions, HPSCs can be cultured to encourage renewing itself and to imrpove the quantify of primordial cells or to induce pathogenic expansion and differentiation to matured blood cells. The yield of these cell types are being investigated as cell therapy medicines for their benefit and proposal in the therapy of hereditary and acquired blood cell disorders [6,8].

In previous study; p53 inhibition transcript with a special chemical inhibitor ,whih is cyclic PFT, and anti mitosis agent triggred apoptosis for human tumor cells and the functional wild type p53 were showed. The susceptibility in the integrated treatment was made by testing overall the anti microtubule agents, containig microtubule compounds and stabilized drugs inhibiting tubulin installation. Therefore, the antitumor effect of the p53 inhibitor on the expanded inhibitory impect of the antimycotypic agent has been shown to evidence with striking rising of the response of apoptosis. The precision of paclitaxel was also established in p53 cells afterward suppressing p53 expression. P53 was detected after the regulation of p53 by siRNA, to support of sensitization of P53 was closely related to P53 function, protection and consequences are permanent with the indicatig p53 activation by MDM2 antagonists for the prevent from cytotoxic effect of paclitaxel [16].

The main purpose of this study was detected a small molecule affect on HSCs for the helping increase the proliferation and expansion on HSC. There were lots of works for the increase proliferation rate on HSC to struggle with diseases. Pifithrin alpha known as p53 inhibitor and prevent against genotoxic agents.

The pifithrin alpha was treated onto different cell types like HDF, HUVEC, human Adipose derived MSC, human bone marrow derived MSCs for the observing effect on cells with WST-1 assay. The absorbances of samples were measured and gave some

wavelength. The cell proliferation of these cell types showed increased on treated cells with pifithrin alpha molecule (Figure 3.1). There were not any significant increase on these cell types; however, negative effect of small molecule was not seen. For the observing HSC expansion; mouse bone marrow HSCs were isolated by helping of magnetic field of the IMagnet (BD Pharmingen cat. no. 552311). The lineage negative cells were obtained and incubated with pifithrin alpha molecuele at three different dosages. By this way, the effective dose of pifithrin alpha also could be detected. Mouse bone marrow derived HSCs cells were stained with with mouse c-Kit (CD117) PE, CD34 FITC, Sca-1 PE-Cy7, lineage cocktail APC (BD StemFlow Cat No 560492) and analyzed on flow cytometry. DMSO and non treated cells were used for the comparing treatment of molecule. LSK numbers of cells were increased on pifithrin alpha treated cells (Figure 3.2). For the indicated long term effect of this molecule, isolated mouse HSCs cells were seeded into 96 well plate with StemSpan SFEM media (Stemcell technologies, Vancouver, cat no. 09650) supplemented with Scf (1000 unit/mL), Tpo (1000 unit/mL), Flt-3l (5000 unit/mL) (all from R&D Systems Inc., Minneapolis) and 1% (v/v) PSA (10.000 units/mL penicillin, 10.000 µg/mL streptomycin) (Corning Costar, Sigma Aldrich, USA, cat no. CLS3599) and added pifithrin alpha molecule with three different dose for the investigated cell count and proliferation. SFEM media and supplementers are special media for the culturing HSCs and PSA was used for prevent from contamination. To this end, cells were incubated for seven days with molecule and analyzed with flow cytometry. The mouse HSCs number and total cell counts were increased approximately 3 fold to DMSO (Figure 3.3). The cell cycle analysis of mLSK cells was performed to observe cell cycling. The cells were marked with Hoescht 33342 and Pyronin Y. The treated cells were increaed on G0 and G1 cycles. The results confirmed the cell proliferation of HSCs and preserved the stem cell abilities (Figure 3.5). An apoptosis result was also indicated. The pifithrin alpha decraesed early apoptosis, late apoptosis and necrosis (Figure 3.6). Because of the protection ability from apoptosis of the pifithrin alpha.

Mouse bone marrow cells could be derived for mesenchymal stem cells or hematopoietic stem cells. The mBM MSC cells were also explored. The proliferation rate of pifithrin alpha molecule was increased on MSC. The cells were treated for 5 days with molecule and DMSO as a controlled. Afterwards, the WST-1 assay was exerted. The measurement of absorbance values was collected (Figure 3.7.) UCB HSC cells were acquired by

newborn cord blood. The cells were isolated with Ficoll-Paque (Histopaque™, Sigma, Cat.No.10831). And mononuclear cells were seeded into 96 well plate within Serum-Free Expansion Medium (StemSpan™ Serum-Free Expansion Medium (SFEM), Stemcell Technologies, Cat.No. 09650) supplemented with 1% PSA (10,000 units/ml penicillin and 10,000 ug/ml streptomycin and 25 µg/mL of Amphotericin B, Gibco, Cat.No.15240062) and human cytokine cocktail (StemSpan™ CC100, Stemcell Technologies, Cat.No. 02690). The cells were incubated in the humidified incubator at 37°C and 5% CO_2 with pifithrin alpha molecule on different dosages. The UCB HSCs were stained to indicated cell number and proliferation by flow cytometry(Figure 3.8). Thereafter, UCB HSC cells were stained with CD34 antibody and the percentage of treated cell were increased significantly according to DMSO. Likewise, the cell percentage of pifithrin alpha molecule was significantly increased on CD133 staining. The UCB HSCs were also stained with CD34+CD133+ antibody for the observing double positive cell count. The monunuclear cells count was increased approximately 3 times to DMSO. (Figure 3.9). The PFT-alpha moleluces expanded the UCB HSCs siginificantly. Nowadays, the umbilical cord is very important source for harvesting HSCs. The effective dosage of PFT-alpha were also observed (Figure 3.10). The 0,1 µM was showed the highest cell count for UCB HSCs.

The repopulation of *ex vivo* expanded HSC were treated with pifithrin alpha. The HSCs were isolated from balb-c mouse for the comparing repopulated HSCs. The cells were injected to SCID mouse eye. Because of the cleanest bloold flow is circulated from eye vein and the HSCs could be resided to the HSC niches well. The percentage of CD45.2 were analyzed and PFT alpha treated HSCs were repopulated and expanded succesfully. For the observing quality and differentiation ability of repopulated HSCs; T cells, B cells and Gr/M cells were investigated. The results indicated that, the expanded HSCs were have an effect on immunu sytesm (Figure 3.11).

Avian HSC cells were isolated with mouse antibodies because, there was not specific antibody were detected for avian HSCs before. This challenge achieved by after many trials. The isolated and depletion method were completed successfully and analyzed cells after stained with mouse antibodies. The population of HSCs was indicated on flow plots (Figure 3.10). The cell counts of WBM and Linage depleted cells were calculated, by this way aspects of isolation avian HSC cells could be appeared. Generally, 5 to 1 ratio of cells was depleted as lineage negative cells. The cell percentage of CD34 positive, Sca1 positive

and c kit positive cells were executed (Figure 3.12). The hematopoietic stem cell marker expression levels was also investigated for avian HSCs. As a primer TSC-1, FOXO-3, P19, APC, DNMT3A, Slc30-A1, Slc30-A1, GLI1, MEIS1, Slc30-A1, SIRT, RUNX1, P15, Rb1, RBl1, ELF4, CITED2 etc. were used for the observing regulations of HSCs. Some of these marker were detected for the up regulated and down regulated (Figure 3.14). These genes were selected from HSC gene pool. The main purpose was the determine or had an idea of behind the mechansim of avian HSCs. The MEIS1 is transcrioptional factor for HSCs [52]. The Rb2 is key regulator of entry into cell division(tumor supressor). Dnmt3a is essential for hematopoietic stem cell differentiation [53]. Tsc1 its regulation of diverse cellular processes, particularly cell growth and related with mtor pathway; regulator of cell metabolism, growth, proliferation and survival. P15 inhibit cell cycle arrest and tumor suppresor. Elf 4 is involved in natural killer cell development and function, innate immunity. Runx important for hematopoises(CLP AND CMP). SIRT1 regulates aging process [54]. Homer1 is chemical homeostasis within tissue. P18 inhibits HSC cycling. Hoxa9 increased apoptosis and decreased proliferation; induced differentiation. APC leads to ineffective hematopoiesis [55]. AHR leads to HSC exhaustion and diseases. These are the roles of genes in HSCs gene pool. The mechanism of avian HSCs have a role in mTOR pathway and AKT2 / AKT signalling pathway [56,57].

The human surface markers were researched for the avian HSCs, in order to determine surface antigens. Roughly 240 antibody screening were performed and some of them were selected. This study has a crucial role for the avian influenza. In avian influenza human HSC and MSC were discontinue, however the avian HSC and MSC cells were not affected. To the illuminate this topic, the antibody screening and HSC isolation were performed, successfully. Some antigens were selected according to selection criteria by helping of previous studies[58]. The cut offs were determined based on LSK percentage in FITC+ plots [59]. To be sure that these markers were stained HSCs, the percentage of Sca1+cKit+ cells as far highest level like 70%. The stem cell selectivity ratio selected also to eliminate and selected best of surface antigens. The potential avian HSCs markers were determined firstly (Figure 3.15).

The Tunel assay was performed to evaluated DNA damage on mouse HSC cells. The enzymatic labeling of free 3’ DNA ends for DNA damage was performed with Tunnel assay. The nuclear marker (DAPI) stained mHSC cells and compared normal DNA and

DNA-damage. The Tunel positive cells ratio was calculated for non treatment, DMSO and pifithrin alpha treated mHSC. The pifithrin alpha molecule was not exhibited significant differences with non treatment cell. On the other hands, DMSO treated cells were showed DNA damage increased compared with non treatment cells meaning that pifithrin alpha does not induce bax or bak on DNA damage, instead of them it gives ability to protect p53 deficent cells and down regulate cyclin d1 regulation [43]. PFT-alpha protects cells from DNA damage-induced apoptosis also by a p53-independent mechanism [50].

In conclusion, this study assessed; pifithrin alpha effectives on mosue HSC, mouse MSC, human MSC, HDF, HUVEC and UB HSC. The major purpose was screened the higher cell percentage and proliferation of pifithrin alpha treated cells according to DMSO treated cells. The expanded HSCs is very crucail for the blood diseases and transplantaions. The HSCs is most commonly performed in primary immunodeficiency diseases such as severe combined immunodeficiency (SCID), Wiskott-Aldrich syndrome (WAS) syndrome, Hämophagocytische lymphohistiocytosis (HLH), and X-linked lymphoproliferative disease (XLP) [60].

As a novel study, avian HSC cells were isolated and obtained LSK lineage. The surface antibodies and potential HSC markers were screening in triumph. The avian influenza virus effects could be analyzed inwards. Thence, studies of other influenza like swine influenza virus etc. could be investiagted. The mechanism behind the animal virus infection could be determined and drugs to prevent from these disease could be designed.

While using small molecules to investigate and solve stem cell biology problems, there are some approaches like hypothesis-based signal pathways and small molecule modulators which are used to incorporate them into key regulatory domains in previous knowledge approaches. The pheniotype based invention is controlled by using small independent molecular libraries, independent high-throughput data browsing, in order to replace the regulatory pathway with a particular phenotype. The active molecules cellular targets may then be determined. Continue to develop understanding of the complex biology of stem cells and for individual tandem with other cell biological and genomic techniques. These approaches can be investigating in the following studies such as, adult stem cells, somatic cell dedifferentiation, stem cell directely differentiation and stem cell niches and reprogramming[61]. Small molecules have also helped to define the different semi stable states of the pluripotency and self-renewal, thus facilitating transitions from one state to

another. The small molecules and their impact on humanity has been and continues to be, enormous.

REFERENCES

1. L. T. Christopher and M. C. Yoder. Novel Methods for Determining Hematopoietic Stem and Progenitor Cell Emergence in the Murine Yolk Sac. *The International Journal of Developmental Biology,* 54:6-7, 2010.

2. E. J. Connie. Hematopoietic Stem Cells: Concepts, Definitions, and the New Reality. *Blood*, 125:2605-2613, 2015.

3. J.Nayan, A. Takeda and N. R. Yaseen. Colony Forming Cell (CFC) Assay for Human Hematopoietic Cells. *Journal of Visualized Experiments : JoVE*, 46:2195, 2010.

4. M.Ogawa. Differentiation and Proliferation of Hematopoietic Stem Cells. *Blood,* 81:2844-2853, 1993.

5. N. Uchida and IL. Weismann. Searching for Hematopoietic Stem Cells: Evidence That Thy-1.1lo Lin- Sca-1+ Cells Are the Only Stem Cells in C57BL/Ka-Thy-1.1 Bone Marrow. *The Journal of Experimental Medicine*, 175:175-184,1992.

6. K. Ikuta, and I. L. Weissman. Evidence That Hematopoietic Stem Cells Express Mouse c-Kit but Do Not Depend on Steel Factor for Their Generation. *Proceedings of the National Academy of Sciences of the United States of America*, 89:1502–1506,1992.

7. L. G. Smith, I. L. Weissman and S. Heimfeld. Clonal Analysis of Hematopoietic Stem-Cell Differentiation in Vivo. *Proceedings of the National Academy of Sciences of the United States of America*, 88:2788–2792, 1991.

8. N.F. Mina. SCA-1 Expression Level Identifies Quiescent Hematopoietic Stem and Progenitor Cells. *Stem Cell Reports*, 8:1472–1478, 2017.

9. O. Hideyuki, L. Ding and S. J. Morrison. SLAM Family Markers Resolve Functionally Distinct Subpopulations of Hematopoietic Stem Cells and Multipotent Progenitors. *Cell Stem Cell*, 13:102–116, 2013

10. S. Jun and I. L. Weissman. Hematopoietic Stem Cell: Self-Renewal versus Differentiation. *Wiley Interdisciplinary Reviews. Systems Biology and Medicine*, 2:640-653, 2010.

11. G. Markus. Dendritic Cell Potentials of Early Lymphoid and Myeloid Progenitors. *Blood*, 97:3333-3341, 2001.

12. C. M. Baum. Isolation of a Candidate Human Hematopoietic Stem-Cell Population. *Proceedings of the National Academy of Sciences of the United States of America*, 89:2804–2808, 1992.

13. D. DiGiusto. Human Fetal Bone Marrow Early Progenitors for T, B, and Myeloid Cells are Found Exclusively in the Population Expressing High Levels of CD34. *Blood* , 84:421-432,1994.

14. L. Murray. Enrichment of Human Hematopoietic Stem Cell Activity in the CD34+Thy-1+Lin- Subpopulation from Mobilized Peripheral Blood. *Blood* , 85:368-378, 1995.

15. U. Nobuko. HIV, but Not Murine Leukemia Virus, Vectors Mediate High Efficiency Gene Transfer into Freshly Isolated G_0/G_1 Human Hematopoietic Stem Cells. *Proceedings of the National Academy of Sciences of the United States of America*, 95:11939–11944, 1998.

16. U. Naoya. Kinetics of Lentiviral Vector Transduction in Human $CD34^+$ Cells. *Experimental Hematology*, 44:106-115, 2016.

17. B. Mickie. Purification of Primitive Human Hematopoietic Cells Capable of Repopulating Immune-Deficient Mice. *Proceedings of the National Academy of Sciences of the United States of America*, 94:5320–5325, 1997.

18. G. Fritsch. Rapid Discrimination of Early CD34+ Myeloid Progenitors Using CD45-RA Analysis. *Blood*, 81:2301-2309, 1993.

19. M. Ravindra, Y. P. Christopher and I. L. Weissman. Identification of a Hierarchy of Multipotent Hematopoietic Progenitors in Human Cord Blood. *Cell Stem Cell*, 1:635–645, 2007.

20. A. H. Galy. Delineation of T-progenitor Cell Activity within the CD34+ Compartment of Adult Bone Marrow. *Blood,* 85:2770-2778, 1995.

21. G. Markus. Prospective Isolation of Human Clonogenic Common Myeloid Progenitors. *Proceedings of the National Academy of Sciences of the United States of America,*99:11872–11877, 2002.

22. K. Atsushi. Stem Cell Leukemia Protein Directs Hematopoietic Stem Cell Fate. *Blood*, 103:3336-3341, 2004.

24. C. Mario, F. Mercuriali, and C. Brugnara. Use of Recombinant Human Erythropoietin Outside the Setting of Uremia. *Blood*, 89:4248-4267, 1997.

25. M. Cazzola. Recombinant Human Erythropoietin in the Anemia Associated with Multiple Myeloma or non-Hodgkin's Lymphoma: Dose Finding and Identification of Predictors of Response. *Blood*, 86:4446-4453, 1995.

26. J. S. Malik, J. D. Menno and Y. Guan. Avian Influenza Virus (H5N1): A Threat to Human Health. *Clinical Microbiology Reviews*, 20:243–267, 2007.

27. G. Robert. H5N1 Outbreaks and Enzootic Influenza. *Emerging Infectious Diseases*, 12:3-8, 2006.

28. D. Shailesh. Avian Influenza Surveillance Reveals Presence of Low Pathogenic Avian Influenza Viruses in Poultry during 2009-2011 in the West Bengal State, India.*Virology Journal,* 9:151, 2012.

29. T. Maytawan. Tropism of Avian Influenza A (H5N1) Virus to Mesenchymal Stem Cells and $CD34^{+}$ Hematopoietic Stem Cells. Ed. Kevan L. Hartshorn. *PLoS ONE* 8:e81805, 2013.

30. P. Birgit. Growth and Differentiation of Human Stem Cell Factor/Erythropoietin-Dependent Erythroid Progenitor Cells In Vitro. *Blood*, 92:3658-3668,1998.

31. C. Y. W. Patrick, T. K. Edward, C. Kwok-Hung , C. Y. Lau, K. P. Susanna and Y. Kwok-Yung. Cytokine Profiles Induced by the Novel Swine-Origin Influenza A/H1N1 Virus: Implications for Treatment Strategies. *Journel Infect Diseases*, 201:346-353, 2010.

32. C. Nicolas-Bolnet, M. A. Qureshi, J. A. Cieszynski and R. L. Taylor. Avian hematopoiesis in response to avian cytokines. Poult Science, 74: 1970–1976, 1995.

33. R. Kirsty. Influenza Virus and Endothelial Cells: A Species Specific

Relationship. *Frontiers in Microbiology,* 5:653, 2014.

34. C. Ilaria, and D. J. Alexander. Avian Influenza Infections in Birds – a Moving Target. *Influenza and Other Respiratory Viruses*, 1:11-18, 2007.

35. A. Sudeepta and M. F. Pittenger. Human Mesenchymal Stem Cells Modulate Allogeneic Immune Cell Responses. *Blood*, 105:1815-1822, 2005.

36. C. Tawee. Human Disease from Influenza A (H5N1), Thailand, 2004. *Emerging Infectious Diseases*, 11:201–209, 2005.

37. Oren M. Decision Making by p53: Life, Death and Cancer. *Cell Death Differ, 10:431-442, 20*03.

38. C. Culmsee, X. Zhu, Q. S. Yu, S. L. Chan, S. Camandola and Z. Guo. A Synthetic Inhibitor of p53 Protects Neurons Against Death Induced by Ischemic and Excitotoxic Insults, and Amyloid beta-Peptide. *Journel Neurochem*, 77:220-228, 2001.

39. T. Pecere, F. Sarinella, C. Salata, B. Gatto, A. Bet and D. F. Vecchia. Involvement of p53 in Specific Anti-neuroectodermal Tumor Activity of Aoe-emodin. *Inernational Journal Cancer*, 106:836-847, 2003.

40. T. Schafer, C. Scheuer, K. Roemer, D. Menger and B. Vollmar. Inhibition of p53 Protects Liver Tissue Against Endotoxin-induced Apoptotic and Necrotic Cell Death. *FASEB Journel,* 17:660-667, 2003.

41. P. J. Murphy, M. D. Galigniana, Y. Morishima, J. M. Harrell, R. M. Kwok and M. Ljungman. Pifithrin-alpha Inhibits p53 Signaling after Interaction of the Tumor Suppressor Protein with Hsp90 and its Nuclear Translocation. *Journal Biology Chemistry*, 279:30195-30201, 2004.

42. F. Bunz, P. M. Hwang, C. Torrance, T. Waldman, Y. Zhang, L. Dillehay, J. Williams, C. Lengauer, K. W. Kinzlerand and B. Vegelstein. Disruption of p53 in Human Cancer Cells Alters the Responses to Therapeutic Agents. *Journal Clinical Investigation*, 104:263–269, 1999.

43. P. Perego, M. Giarola, S. C. Righetti, R. Supino, C. Caserini, D. Delia, M. A. Pierotti, T. Miyashita, J. C. Reed and F. Zunino. Association Between Cisplatin Resistance and

Mutation of *p53* Gene and Reduced *bax* Expression in Ovarian Carcinoma Cell Systems. *Cancer Research*, 56:556–562, 1996.

44. D. Carvajal, C. Tovar, H. Yang, B. T. Vu, D. C. Heimbrook, and L. T. Vassilev. Activation of p53 by MDM2 Antagonists can Protect Proliferating Cells from Mitotic Inhibitors. *Cancer Research*, 65:1918–1924, 2005.

45. C. H. Westphal, S. Rowan, C. Schmaltz, A. Elson, D. E. Fisher and P. Leder. Atm and p53 Cooperate in Apoptosis and Suppression of Tumorigenesis, but not in Resistance to Acute Radiation Toxicity. *Nature Geneicst,* 16:397–401, 1997.

46. S. Loft and H. E. Poulsen. Cancer Risk and Oxidative DNA Damage in Man. *Journal of Molecular Medicine*, 74:297–312, 1996.

47. M.Valko, C. J. Rhodes, J. Moncol, M. Izakovic, and M. Mazur. Free Radicals, Metals and Antioxidants in Oxidative Stress-induced Cancer. *Chemico-Biological Interactions*, 160:1–40, 2006 .

48. K. V. Pajcini, N. A. Speck and W. S. Pear. Notch Signaling in Mammalian Hematopoietic Stem Cells. *Leukemia*, 25:1525–1532, 2011.

49. L. J. Beverly, D. W. Felsher and A. J. Capobianco. Suppression of p53 by Notch in lymphomagenesis: Implications for Initiation and Regression. *Cancer Research*, 65:7159–7168, 2005.

50. D. Sohn, V. Graupner, D. Neise, F. Essmann, K. Schulze and R. U. Jänicke. Pifithrin-alpha Protects Against DNA Damage-Induced Apoptosis Downstream of Mitochondria Independent of p53. *Cell Death and Differentiation* , 16:869–878, 2009.

51. A. J. Levine and M. Oren. The first 30 years of p53: Growing Ever More Complex. *Nature Reviews Cancer*, 9:749-758, 2009.

52. M. Michelle Erin. Meis1 Is Required for Adult Mouse Erythropoiesis, Megakaryopoiesis and Hematopoietic Stem Cell Expansion. *PLoS ONE*, 11:3, 2016.

53. M. Okano, D. W. Bell, D. A. Haber and E. Li. DNA Methyltransferases Dnmt3a and Dnmt3b Are Essential for De Novo Methylation and Mammalian Development. *Cell*, 99: 247-257, 1999.

54. L. Kentson, and D. Zhang. RUNX1 and RUNX1-ETO: Roles in Hematopoiesis and Leukemogenesis. *Frontiers in bioscience : a Journal and Virtual Library* 17,2012.

55. N. Kawamata, C. W. Miller and H. P Koeffler. Molecular Analysis of a Family of Cyclin-Dependent Kinase Inhibitor Genes (*p15/MTS2/INK4b* and *p18/INK4c*) in Non-Small Cell Lung Cancers. *Molecular Carcinogenesis*. 14: 263–268, 1995.

56. L. Mathieu, and M. S. David. mTOR Signaling in Growth Control and Disease. *Cell*, 149: 274–293, 2012.

57. D. Brendan, and C. Lewis . AKT/PKB Signaling: Navigating Downstream. *Cell,* 129:1261–1274, 2007.

58. A. Giuseppe. Recommendations for Managing the Donation of Hematopoietic Stem Cells from Related and Unrelated Donors for Allogeneic Transplantation. *Blood Transfusion,* 11:296–304, 2013.

59. C. Geoff. Antigen-Specific T Lymphocyte Function Following Cord Blood Transplantation. *Biology of Blood and Marrow Transplantation : Journal of the American Society for Blood and Marrow Transplantation,* 12:1335–1342, 2012.

60. M. Laura and D. C. Link. The Hematopoietic Stem Cell Niche in Homeostasis and Disease, *Blood,* 126: 2443–2451, 2015.

61. J. A. Efe and Sheng Ding. The Evolving Biology of Small Molecules: Controlling Cell Fate and Identity. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 366: 2208–2221, 2011.