STUDYING THE EFFECT OF GROWTH FACTORS ON EXPANSION AND ERYTHROPOIETIC DIFFERENTIATION OF HEMATOPOIETIC STEM CELLS

THESIS

Submitted to the Delhi Technological University
for the award of the degree of

DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

Submitted by

ABHISHEK SAINI

Supervisors

Dr. VIMAL KISHOR SINGH
Associate Professor
Amity University, Haryana

Dr. JAI GOPAL SHARMA
Professor
Delhi Technological University, Delhi

Department of Biotechnology, Delhi Technological University
(Formerly Delhi College of Engineering) ShahbadDaulatpur
Main Bawana Road, Delhi-110042, INDIA

January 2019

Dedicated
to
My Parents

DECLARATION

I hereby declare that the thesis entitled **"Studying the effect of growth factors on expansion and erythropoietic differentiation of Hematopoietic stem cells"** submitted by me, for the award of the degree of ***Doctor of Philosophy*** to **Delhi Technological University (Formerly DCE)** is a record of bonafide work carried out by me under the guidance of Dr.Vimal Kishor Singh and Prof. Jai Gopal Sharma.

I further declare that the work reported in this thesis has not been submitted and will not be submitted, either in part or in full, for the award of any other degree or diploma in this Institute or any other Institute or University.

Place: New Delhi

Date: 20.01.2019 **Signature of the Candidate**

CERTIFICATE

This is to certify that the thesis entitled **"Studying the effect of growth factors on expansion and erythropoietic differentiation of Hematopoietic stem cells"** submitted by **Mr. Abhishek Saini** to **Delhi Technological University (Formerly DCE)**, for the award of the degree of "Doctor of Philosophy" in Biotechnology is a record of bonafide work carried out by him. Abhishek Saini has worked under our guidance and supervision and has fulfilled the requirements for the submission of this thesis, which to our knowledge has reached requisite standards.

The results contained in this thesis are original and have not been submitted to any other university or institute for the award of any degree or diploma.

Dr.Vimal Kishor Singh
Supervisor
Associate Professor
Dept. of Biomedical Sciences
Amity University Haryana

Prof. Jai Gopal Sharma
Supervisor & Head
Department of Biotechnology
Delhi Technological
University

ACKNOWLEDGEMENT

Nothing worthwhile comes in life without a struggle. It would not have been possible to write this doctoral thesis without the help and support of the kind people around me who, in their own ways, have helped me overcome difficult situations

I would like to thanks Prof. Yogesh Singh, Vice chancellor, Delhi Technological University, Delhi, for providing me the excellent facilities to carry out this work in this prestigious institute.

I take this opportunity to thankfully acknowledge Prof. Jai Gopal Sharma, Head and DRC Chairman, Department of Biotechnology and Prof. D. Kumar, Former Head and DRC Chairman Department of Biotechnology for their kind support and providing necessary facilities to undertake this study.

There are not enough words in language to express my deepest sense of gratitude to my supervisors Dr. Vimal Kishor Singh and Prof. Jai Gopal Sharma for their constant guidance, inspiration and invaluable suggestions for carrying out this work. I am greatly indebted to them for their constant guidance and valuable suggestion during entire course of this work.

I wish to convey my sincere thanks to Dr. Vimal Kishor Singh, Associate Professor, Department of Biomedical Sciences, Amity University Haryana, for his constant guidance and motivation in the planning and execution of the experiments. I greatly appreciate all his contributions of time, ideas, advices and constructive criticism to make my PhD experience productive and stimulating. It would have been impossible to complete the thesis without his diligence, ideas and guidance.

I gratefully acknowledge Prof. B.D Malhotra, Prof. Pravir Kumar, Dr. Navneeta Bharadvaja, Dr. Yasha Hasija, Dr. Asmita Das, Dr. Saurabh Saxena, Dr. Prakash Chandra and all faculty members for their guidance and useful support during my study period.

I would like to thank my fellow lab mates especially Neeraj, Manisha and Vishwachi for helping and encouraging me throughout my research.

I would like to thank Mr. C.B Singh, Mr. Jitendra, Sh. Mangal Singh Negi, Mr. Sandeep, Mr. Rajesh, Mr.Vijay and all the staff members of the department and University who helped directly or indirectly in achieving this goal.

Thanks are due to the wonderful friends in my life who were always on the stand by to bring me to positivity, hope and smiles when things didn't seem favoring and it seemed a far-fetched journey. Special mention is deserved for all my other peers especially Abhijat, Dr. Satish, Dr. Richa, Dr. Saroj, Dr. RajKumar, Dr. Suveen and Dr. Saurabh who have always been my guiding light when I didn't believe in myself.

Boundless gratitude goes towards my parents and grandparents for their unconditional love, moral support; blessings and never-ending sacrifices that they have made to see me reach so far. I owe my every achievement to them. A great appreciation is expressed to my brother, Rahul, who always encouraged and supported me during the entire journey. I express my love and affection to my ever-loving daughter, Manya, who created a cheerful environment during my most valuable period of my journey. I am also grateful to my parent-in-laws for having faith in me and my grandmother for showering her blessings from heaven.

Now, I am pleased to acknowledge my spouse Monika, for her unconditional support, constant faith, motivation and great patience in all my good and bad times. I am immeasurably grateful to her for every little and big thing she did and continues to do for me.

Above all, I am indebted to Almighty God for being with me always and bestowing his blessings in multifarious ways.

(Abhishek Saini)

ABSTRACT

Red cell production in vertebrates is regulated by several cytokines and factors. The process of erythropoiesis is initiated from the primitive pluripotent stem cell giving rise to mature erythrocyte. This process involves various regulatory factors which induce commitment and further maturation of the cells involved in the red cell lineage. The major growth factors that are involved in Erythropoiesis are granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage (GM)-CSF, interleukin (IL)-3, stem cell factor (SCF), IL-1, IL-6, IL-4, IL-9, IL-11, and insulin growth factor-1 (IGF-1) and Erythropoietin (EPO) (Gregory et al. 1978). EPO comes in action during later stages of maturation of erythroid progenitor cells and primarily on colony-forming unit erythroid (CFU-E) to induce the proliferation and maturation of these cells through the stages of proerythroblast followed by reticulocytes and finally mature erythrocytes (Hillman and Henderson, 1969). CFU-E remains the primary target cell in the bone marrow for EPO but it acts synergistically with other growth factors viz. SCF, GM-CSF, IL-3, IL-4, IL-9, and IGF-1 in order to regulate the maturation and proliferation starting from the stage of the burst-forming unit erythroid (BFU-E) followed by CFU-E to the proerythroblast stage of erythroid cell development (Douay et al., 2005; Yu et al., 2002). SCF, IL-1, IL-3, IL-6, and IL-11 stimulate pluripotent stem cell to differentiate into the CFU granulocyte, erythroid, monocyte, megakaryocyte (GEMM) and the myeloid stem cell. The CFU-GEMM then differentiates into specific CFU for erythroid, granulocytes, monocytes, macrophages, eosinophils, and megakaryocytes cell precursors in the presence of GM-CSF and IL3. These precursors finally differentiate into specific cell types. Besides the role of erythropoiesis EPO also inhibits apoptosis to decrease the rate of cell death in erythroid progenitor cells in the bone marrow and neural cells (Motoyoshi 1992). IL3 encodes an important growth factor/cytokine which supports the proliferation of a broad range of hematopoietic stem cell types and involved in a variety of other cell activities such as cell growth, differentiation and apoptosis. Granulocyte macrophage colony-stimulating factor (GM-CSF) and granulocyte- colony-stimulating factor (G-CSF) are important growth factors which have established roles in hematopoiesis and have an established role as growth factors in clinical practice. G-CSF and GM-CSF regulate myeloid cell production, differentiation and activation (Matthias et al., 1997).

CONTENTS

Page No.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

3D	Three Dimensional
BFU-E	Burst Forming Unit Erythroid
BM	Bone Marrow
CD235a	Cluster of Differentiation 235a (Glycophorin A)
CD34	Cluster of Differentiation 34
CFU-E	Colony Forming Unit Erythroid
EPO	Erythropoietin
EPOR	Erythropoietin receptor
ESCs	Embryonic Stem Cells
Flt3	Fms related Tyrosine Kinase 3
GATA 1	Globlin Transcription Factor 1
GATA 2	Globlin Transcription Factor 2
GCSF	Granulocyte Colony StimulatingFactor
hESCs	human Embryonic Stem Cells
HSCs	Hematopoietic Stem Cells
IGF1	Insulin like Growth Factor 1
IL3	Interleukin-3
iPSCs	induced Pluripotent Stem Cells
JAK2	Janus Kinase 2
Klf1	Kruppel Like Factor1
LDB1	LIM Domain Binding 1
LMO2	LIM Domain Only 2
MCSF	Macrophage Colony StimulatingFactor

miRNAs	micro RNA(Ribose Nucleic Acid)
PB	Peripheral Blood
RBC	Red Blood Cells
SCF	Stem Cell Factor
SCL/Tal1	Stem Cell Leukemia/ T-cell AcuteLymphocytic Leukemia 1
shRNA	short hairpin RNA
Sox2	SRY (Sex determining region Y)-box-2
Stat5	Signal Transducer and Activator ofTranscription 5
UCB	Umbilical Cord Blood
WHO	World Health Organization

Chapter 1

Introduction

1.1 Stem cells

The stem cell can be defined as a cell that posses the self-renewal capability, which enables the cell to go through several cycles of cell division without losing its identity and side by side, having the potential to differentiate in specialized cells.Stem cells can be categorized either on the basis of their potency or their origin. As per their potency, stem cells can be classified as- totipotent, pluripotent, multipotent and unipotent. A totipotent cell can be defined as a cell with the potential to form an entire organism including extra-embryonic tissue. These cells are usually the most primitive cells of the zygote limited to the first couple of divisions. When embryo attains blastocyst stage, the cells of inner mass are pluripotent having two properties: self-renewal and potency. The self-renewal ability allows the cell to divide indefinitely giving rise to identical daughter cells. Bypotency, these cells can be differentiated into all three germ layers- the endoderm, mesoderm, and ectoderm. These germ layers, in turn, give rise to the specific tissues of the adult organism, like internal organs, bones, muscles. Multipotent stem cells, as the name suggest have limited differentiation capacity, and such cells can differentiate only into organ-specific cells. Unipotent stem cells are usually the progenitor cells, which finally differentiate to give rise to the somatic cells. The terminally differentiated cells have a fixed fate; for example, an erythrocyte does not spontaneously become megakaryocyte. However, the concept of induced pluripotent stem cells is altogether different, where any dedifferentiated cell type can be reprogrammed to become an embryonic stem-like cell (Yamanaka and Blau 2010). The iPS cells and human ES cells are similar in several aspects, including their morphology, proliferation pattern, surface marker, and gene expression, telomerase activities, feeder dependence, transcriptional promoter activities, and teratoma formation. hES cell-specific surface antigens expressed by iPS

cells include stage-specific embryonic antigen (SSEA), TRA and NANOG protein. In culture conditions, iPS cells can be indefinitely maintained in their pluripotent state. These studies were validated by transplanting iPS cells in nude mice, where these cells produced teratomas bearing various tissues of all three germ layers. Eventually, iPS cells have also been generated from mouse liver cells, epithelial cells, and neural stem cells using Yamanaka factors (Yamanaka, 2007; Jaenisch and Young, 2008). Human and mouse ES cells differ in many aspects (Rao, 2004), firstly the hEScell make flatter colonies without overriding each other as compared to mouse ES cells. Secondly, the self-renewal property of hES cells is regulated by basic fibroblast growth factor (bFGF) (Amit et al., 2000), and leukemia inhibitor factor (LIF)/Stat-3 pathway in mouse embryonic stem cells (Matsuda et al., 1999; Niwa et al., 1998). Another exciting growth factor Bone Morphogenetic Protein (BMP) in hES cells induces differentiation (Xu et al., 2005), but in case of mouse ES cells have a role in self-renewal (Ying et al., 2003). Irrespective of these differences, Yamanaka et al. demonstrated that the set of four transcription factors could induce pluripotency in somatic cells of both human and mouse origin (Takahashi et al., 2007). The above findings suggest that pluripotency is regulated by the common fundamental transcriptional network in mouse and human. In vertebrates, the cellular organization is in such a way that as the embryo progresses towards the adult organism, the differentiated cells lose their pluripotency. This implies that to convert the somatic cell into the embryonic-like cells, the genes that have been silenced during differentiation have to be reactivated. The exact mechanism by which Yamanaka factors reprogram somatic cells is still debatable, with different research groups currently working to find out the exact mechanism.

Further, extensive studies throughout the globe are being carried out, including the efficiency enhancement of the process and the individual role of each factor. The somatic cell identity is stabilized by various epigenetic factors such as DNA methylation and histone modification and keep cell arrested in the somatic state. Therefore, to attain pluripotency, somatic cells must undergo epigenetic remodeling (Apostolou 2013). However, as per reports, in both murine and human iPS cells may retain their somatic origin epigenetic characters.

Based on the origin of stem cells, they are classified into ES cells and adult stem cells. Adult stem cells are, in general, responsible for the maintenance and repair of the specific tissue where they are located,e.g., Hematopoietic stem cells, Cardiac stem cells, Intestinal stem cells, Neural stem cells, and Mesenchymal stem cells. There is ambiguity over the origin of adult stem cells as per one theory these are embryonic stem cells reserved during tissue development, another says that they possibly are migrating embryonic stem cells that during organogenesis resided in various parts of the body. The last one suggests that they were produced by de-differentiation of a select group of cells within various tissues after embryonic development.

These adult stem cells are multipotent and undergo a series of differentiation before giving rise to the mature cell. Some adult stem cells have been isolated from humans and their potential for cell-based therapies being studied. The cell number of adult stem cells obtained from donors is usually quite low, and for a therapeutic purpose, these cells have to be processed and sometimes expanded before administering to the patient. Most commonly isolated adult stem cells are hematopoietic stem cells aspirated from bone marrow and apheresis. The strict ethical guidelines for using ES cells have helped adult stem cells to gain attention as potential candidates for cell-based therapies. Another added advantage of adult stem cells is that they can be converted into iPS cells, and even if they retain the epigenetic character of their

origin, it will not hamper its functionality. Adult stem cells under the influence/signal of specific cytokines or growth factors from the microenvironment undergo a series of modifications and differentiate into mature cells through a series of progenitor cells. Each step is governed by a specific cytokine that goes and binds to its receptor present on the cell surface of the adult stem cell. These molecules trigger the cascade of signals by certain conformational changes in the cytoplasm region of the receptor molecule. The signal pathway responsible for the specific function is activated, and the desired genes are up-regulated, and undesired is down-regulated. Thus, multipotent stem cell differentiates down the line into progenitor cells which further differentiates into the mature cells. Researchers are exploiting this property of stem cells by mimicking the microenvironment of stem cells in a petri dish and pushing stem cells to differentiate into specialized cell types.

1.2 Hematopoiesis

The blood is connective tissue comprising of more than ten different cell types (lineages), each with specific functions: Leukocytes, Erythrocytes,and megakaryocytes. The common hematopoietic stem cells (HSCs) have their niche in the bone marrow (BM)and can differentiate into each of these cell types, there itself, therefore, bone marrow is termed as the primary site of adult hematopoiesis.Hematopoiesis is the formation of blood cells that occurs in vertebrates in four distinct waves. The most primitive hematopoietic differentiation is marked by the formation of blood islands in the mesodermic region of the extra-embryonic yolk sac during the second week of the fetal period. From ninth week onwards the liver and spleen take over the erythropoiesis, and along with RBCs, myelocyte and platelets are also generated. The postnatal definitive hematopoiesis is stabilized in the thymus and bone marrow.

Organ of erythropoiesis	Fetal period	Kinds of blood cells
Yolk sac	2~9 week	RBC
Liver	9~24 week	RBC, myelocyte, platelet
Spleen	10~24 week	RBC, myelocyte, platelet
Lymph node	8week~after birth	lymphocyte
Bone marrow	10week~afterbirth	RBC, myelocyte, platelet

Table 1.1: The site of erythropoiesis pre and post-fetal period

1.2.1 Wave – 1 Primitive hematopoiesis site-yolk sac

The initial hematopoietic cells are first and foremost observed in the extra-embryonic yolk sac even before the cardiac cells start beating, followed by the allantois and placenta in progression. In mammalian embryo development immediately after the gastrulation occurs in the extraembryonic space, mesoderm cells aggregate to form a network, from where the hematopoietic and vascular systems originate. The peripheral cells of the homogeneous clusters,also start attaining the markers and morphology as same as endothelial cells. The cells present in the internal part of vessels begin to disappear,so the first vessel lumen is opened (Maximov, 1909; Sabin, 1920). The presence of localized thickening that probably represents primordial blood islands in the human embryo has been observed during the 16^{th} day of embryo development (Luckett, 1978). The angioblast, which was subsequently termed as *hemangioblast* is the cluster of mesoderm that gives rise to both hematopoietic and endothelial cells (Sabin, 1920; Murray, 1932). In the yolk sac,red blood cells are predominantly reticulocytes or nucleated erythrocytes, and these cells synthesize primarily embryonic hemoglobin (ζε). The first incidence of cells with functional properties of adult HSCs is generated in the intra-embryonic aorta-gonad-

mesonephros (AGM) region and the placenta (Medvinsky and Dzierzak 1996; Ivanovs et al. 2011, Gekas et al. 2005; Ottersbach and Dzierzak 2005). Once the HSCs are generated in the AGM region, they start migrating to the placenta and fetal liver, the primary sites of their expansion. Followed by the migration to the spleen, and just around the birth time these cells move to the BM. After birth, the leading site of the hematopoiesis is limited to bone marrow and some extent the spleen.

1.2.2 Wave – 2 Colonization of liver

The initiation of cardiac beating in the embryo allow the yolk-sac derived blood cells to access the embryonic tissue, and the very first organ that is colonized is liver. Kelemen and Coll first suggested delivery of yolk sac-derived hematopoietic stem cells to the liver, which there proliferates and differentiates in 1979.

The shift from *primitive* nucleated to *definitive* enucleated erythrocytes reflected by the embryonic, to fetal globin switch in the liver. (Peschle *et al.,* 1984). The primitive nucleated erythrocytes present in the early hepatic rudiment from week 4.5 to week five decrease quickly in number and gradually replaced by definitive nucleated erythrocytes. The hematopoiesis occurs in a monoclonal model; during this phenomenon a single stem-cell pool differentiates to generate primitive yolk sac erythropoiesis; these cells then migrate to the liver to give rise to the definitive erythroblast lineage (Peschle *et al.,* 1985). Second hepatic colonization takes place on day 30 when the first $CD34^{+}$ hematopoietic progenitors could be recognized in the liver. This wave of hematopoietic cells enters the liver at three weeks of human embryo development and constitutes solely of late-stage progenitors. (Tavian *et al.,* 1999).

1.2.3 Wave - 3 Bone-Marrow

The bone marrow is the soft part of the bone that provides the microenvironment for hematopoiesis. It is the main blood-forming tissue in the adult mammal, is the last one that develops in ontogenesis. Bone marrow hematopoiesis starts during the 11^{th} week of human embryo development in specialized mesodermal structures. The earliest blood cells that differentiate within the bone marrow are $CD15^{+}$ myeloid cells, closely followed by glycophorin A^{+}erythrocytes. The hematopoiesis actively occurs in all bones in infants and later restricted to only larger bones like the femur.

1.3 Erythropoiesis

Generation of RBCs, termed as erythropoiesis, is a dynamic process that is governed by specific growth factors in a manner that maintains a steady number of RBCs in the body. This process can be classified into three distinct stages:

- First and foremost the commitment of HSCs into erythropoietic lineage: this stage comprises of the development of erythroid colony-forming units from multipotent hematopoietic progenitors
- The second stage is marked by the division and differentiation of erythroid progenitor cells to increase their population.
- The final stage is the terminal differentiation of these progenitor cells which undergoes enucleation, to produce mature of RBCs

Hematopoietic stem cells (HSC), generated in Yolk sac, differentiates into a common myeloid progenitor, which self renews to maintain its population and when it differentiates two bipotent progenitors are generated namely the granulocyte/macrophage and the erythroid/megakaryocytic pathways. (Suda et al., 1983; Debili et al., 1996; Akashi et al., 2000) The significant difference between these progenitors is the expression of erythropoietin receptor (EPOR), those cells which are

responsive to erythropoietin (EPO) are committed to the erythroid/megakaryocytic pathway. While the progenitor cells deficient of EPOR are committed to the myeloid pathway.

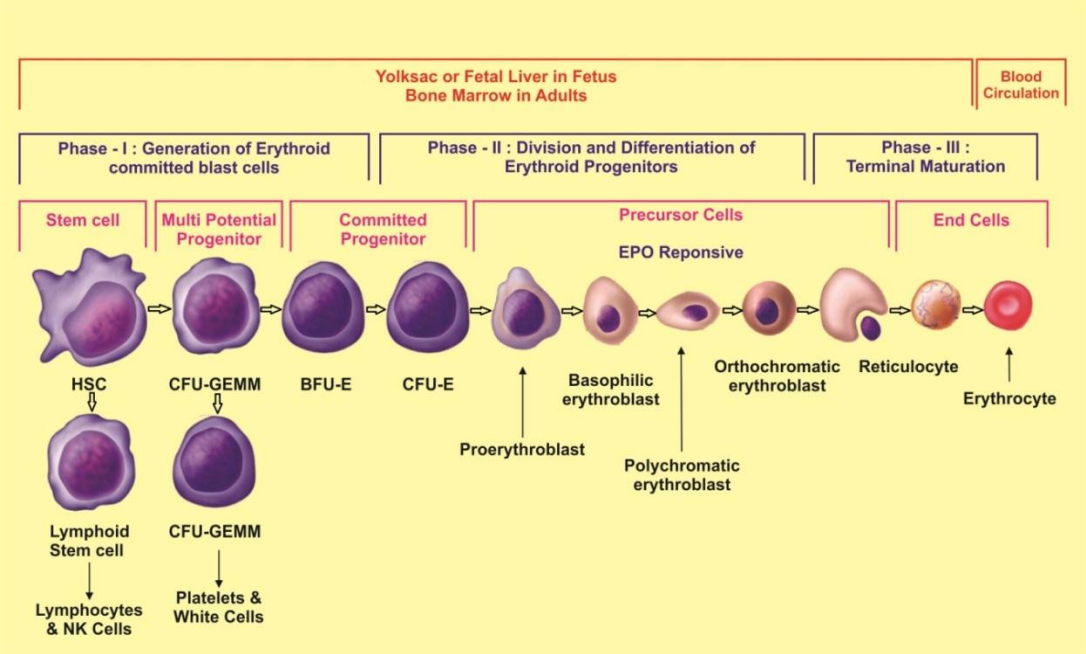


Figure 1.1: ***Erythropoiesis: This schematic diagram represents the three major phases of in-vivo erythropoiesis a) Phase1- Hematopoietic stem cells differentiate into erythroid precursor BFU-E, b) Phase2- Expansion and differentiation of BFU-E into erythroid progenitors CFU-E in the presence of IL3, EPO, c) Phase3- Maturation of erythrocytes through enucleation the reticulocytes are transformed into mature enucleated erythrocytes by the expulsion of the nucleus.***

The burst-forming unit–erythroid (BFU–E)- is known to be the most primitive erythroid-restricted progenitor. Early BFU–Es are highly proliferative and when cultured in semisolid media they can produce up to 20,000 clustered burst colonies. However, these cells express a low level of EPOR, but cells derived from them with maturation attains high expression level of EPOR, therefore responsive to EPO, and begin expressing hemoglobin (Lichtman et al., 2007). BFU-E differentiates into CFU– E, which generate smaller colonies and are highly responsive to EPO as the expression of EPOR increases, CFU-E also expresses many specific gene products

that are required for definitive erythroid development. In tissue culture systems, it has been observed that during the CFU-E phase, the cells start synthesizing hemoglobin and also acquire cytoskeletal proteins.The expression of cellular adhesion molecules in CFU-E distinguished them from other progenitors and termed as nucleated erythroblasts. After that, nucleated erythroid precursors are more prominent, these cells progress from the proerythroblast to basophilic erythroblast and followed by, polychromatophilic erythroblast, and the orthochromatic stage is leading to the reticulocyte. (Lichtman et al., 2007; Jing et al., 2013) (Figure1).

The differentiation process encompasses four distinct major cellular processes:

1) First and most, as the cells mature they accumulate hemoglobin, which drives the basophilic to acidophilic cytoplasmic changes.

2) The cell size continuously decreases with maturation

3) Erythroblast expansion is tightly regulated

4) and nucleus condenses before getting expelled out of the cell make room for the hemoglobin.

The erythropoiesis ends up with the reticulocytes entering the peripheral blood from the marrow, where they get matured into erythrocytes. This stage includes disassembly of cellular components like ribosomes, Golgi bodies, mitochondria, and other organelles. The removal of the nucleus and other organelles changes the cytoskeleton of reticulocytes, which leads to the classic biconcave discoid shape of erythrocytes (Department of Health and Human Services, 2013) The phenomenon of erythrocyte maturation occurs in the erythroblast islands, where macrophages

participate with maturing erythrocytes. (Bessis et al., 1978; Manwani and Bieker, 2008; Rhodes et al., 2008).

The mature erythrocyte has a lifespan of 120 days in blood circulation, and afterward, "senescence" that can be determined by surface antigen expression or physical characteristics of the cell, the senescent cells are removed from the blood by macrophages/reticuloendothelial system. (Gifford et al., 2006).

1.4 Aims and Objectives

The overall work aims to understand the molecular interaction of EPO/EPOR and evaluation of its signification in *in-vitro* erythroid differentiation

The major focus was on the following:

- To study the molecular structure of Erythropoietin and its receptor.

  Since erythropoietin is a vital player of the erythropoiesis,the structure of EPO and its receptor were studied using bioinformatics tools to understand their major interacting sites.

- To design Erythropoietin mimetic for enhanced and regulated ex-vivo erythropoiesis, since the recombinant EPO has a low half-life, the mimetic would serve two purposes at the same time.

- To determine the pluripotency of stem cells by the in-silico method. In our study the source cells play a vital role in the overall experiment so, if we know the pluripotency level of the cells beforehand, it gives us a better understanding of the cells being used for the differentiation in less time with maximal result output.

- To study the interactions between Sox2 and Sox6. The Sox2 primarily responsible for the pluripotency, whereas the Sox6 regulates the differentiation of pluripotent stem cells into the erythroid lineage, so we aimed to identify the underlying mechanism for pluripotency and differentiation into the erythroid lineage.

- To study various phases of erythropoietic development and biological significance of EPO/EPOR. These studies were primarily carried out by ex-vivo culturing of pluripotent stem cells and differentiating them into the erythroid lineage.

Chapter 2

Review of Literature

2.1 History

The medical achievements keep on uncovering new facts now and then; there have been many groundbreaking discoveries that have indeed transformed the way we used to think about the human body. One such discovery was the isolation of pluripotent stem cells from early embryos and culturing them in vitro (Thomson et al., 1998; Reubinoff et al., 2000). However, the foundation for this work was laid by James a Till and Ernest A McCulloch in 1961 by their observation that when an irradiated mouse was given an intravenous injection of bone marrow cells, it led to the formation of proliferating cell colonies in the spleen of those mice. It was only possible due to the presence of blood-forming cells in the bone marrow cells, which restored the blood formation of irradiated mice (Till and McCulloch, 1961). The very next year, Gurdon et al. proved that by somatic cell nuclear transfer (SCNT), somatic cells could be reprogrammed into a pluripotent state. In an interesting experiment, they transferred the nuclei of intestinal cells of an adult frog into oocytes to generate tadpole suggesting the existence of pluripotency-inducing factors (Gurdon 1962). Different research groups kept on working on several aspects of cloning by somatic cell nuclear transfer and cell fusion techniques to generate pluripotent cells. However, after four decades, it was successfully demonstrated that somatic cells could be reprogrammed by fusing the nuclei of a somatic cell with an enucleated ES cell (Tada 2001; Cowan 2005) (Table-1). In 2006 Yamanaka et al. hypothesized that there must be some common factors in the maintenance of pluripotency of ES cells and induction of pluripotency in somatic cells. They started with several transcription factors and finally, were able to generate pluripotent stem cells by reprogramming mouse embryonic or adult fibroblasts cells through the viral insertion of four key transcription factors- *Oct4, c-Myc, Sox2, and Klf4* (Takahashi & Yamanaka, 2006;

Okita et al., 2007; Takahashi et al., 2007). Later on, many other groups reported the similar findings of nuclear reprogramming of both murine and human somatic cells. It clearly, indicates towards the reproducibility and simplicity of the methodology, as stated by Yamanaka. The efficiency of this technique is statistically quite low,i.e., 1%, but it has an immense impact on medicine with great potential to be used in therapeutics. As of now, the technology has evolved, and in place of fibroblast, other cell types like Hepatocytes, Blood mononuclear cells, urine squamous cells are also used for reprogramming with similar results. The methodology has also been refined by using small molecules, and the delivery vector has also seen a shift from viral vectors to various non-viral vectors.

The blood circulation system was first described by Harvey's studies in17th century, followed by the blood transfusion in animals and subsequently in humans. In 1665, Dr. Jean-Baptiste Denys documented the first report on blood transfusion in humans, where he successfully transfused sheep's blood in a 15 years old boy. But, after this attempt, all other blood recipients died soon after blood transfusions.Later in 1818, Dr. James Blundel successfully reported human to human blood transfusion for postpartum bleeding. In 1901, Austrian researcher Karl Landsteiner achieved the breakthrough in human transfusion by describing blood group antigen;he reported that red blood cells got clumped when different blood types were mixed. He concluded that the immunological reactions could be triggered in case the recipient of a blood transfusion had antibodies against the donated blood cells. Since then, many other blood groups have been discovered, which led to the establishment of a large number of blood banks during 1940–1950s, and with time it became an integral part of all the modern clinical modalities (Alter and Klein, 2008).

2.2 Blood transfusion

The global blood collection was reported to be about 103 million units (www.who.int/worldblooddonorday/en/) (Department of Health and Human Services, 2010, 2013;World Health Organization, 2011). As per the WHO report, 82 low income and middle-income countries have only ten donations per 1000 people in the population that would remain highly insufficient to supply a comparable large population residing in these countries (World Health Organization, 2011). Further, the screening facilities are very inefficient in most of the developing countries. As per WHO record, 39 countries are not able to screen all blood donations for one or more of the following transfusion-transmissible infections (TTIs): HIV, hepatitis B, hepatitis C, and syphilis (Department of Health and Human Services, 2013). Again, there are only 106 countries that have national guidelines on the appropriate clinical use of blood. It would be worth noticing that only 13% of low-income countries have a national haemovigilance system to monitor and improve the safety of the transfusion process. Moreover, the blood supply may look sufficient for the time being in developed countries, it likely becomes inefficient to keep supporting a rapidly growing proportion of elderly population (>60 years age) and burgeoning demand for blood transfusions for surgical treatments by the year of 2050 (U.S. Census Bureau, 2004; Ali et al., 2010).

One of the major challenges in clinical settings is to find blood group compatibility for more than 30 blood group systems (308 recognized antigens), including ABO & Rh antigens (Daniels et al.,2009).There are a number of situations such as rare phenotypes, hemoglobinopathies, polytransfusion patients, and polyimmunization that create complications (World Health Organization, 2011). Theoretically, antigen masking and enzymatic cleavage can produce universal donor blood groups (O and

RhD antigens) that can be produced (Bagnis et al., 2011). For safety concerns the donated blood is tested for various kinds of infections before any transfusion [e.g., HIV-1, HIV-2, HTLV-1, HTLV-2, Hepatitis B, Hepatitis C, Syphilis (*T pallidum*), Chagas disease (*T cruzi*), and West Nile Virus, Cytomegalovirus (CMV)], and a number of precautionary measures are followed during transfusion procedures. Even then, blood transfusions are often associated with several complications (Alter and Klein, 2008; World Health Organization, 2011). These complications not only compromise the quality of treatment but may add to the overall cost of the regime also which remains associated with it in any clinical setting (Department of Health and Human Services, 2010). This is true even in the developed countries, where 0.24 % of transfusion has been reported to be associated with adverse reactions (Department of Health and Human Services, 2013). For example, adverse events from transfusions in the US only may account for approximately $17 billion every year.

2.3 **Growth Factors Involved in Erythropoiesis**

The specific lineage of cells is promoted uniquely by chemical signals, also known as cytokines and interleukins. Usually, these substances are glycoproteins, which target specific cell stages. Their major function is to control replication, followed by clonal or lineage selection, and they are also responsible for maturation rate and growth inhibition of stem cells. Now let us discuss each growth factor, first and foremost, erythropoietin. What makes it so important? And why?

2.3.1 Erythropoietin

Over a century ago, Carnot and Deflandre postulated that red blood cell formation is regulated by a humoral factor(Carnot and Deflandre, 1906; Carnot and Deflandre, 1906a). Later on, this factor was named erythropoietin, or EPO, and whose prime

function is to regulate red blood cell production. It binds to dimerize its receptor, EPO-R, present on erythroid progenitor cells.When the EPOR is activated, its cytoplasmic region gets phosphorylated, which activates the JAK-STAT pathway. This, in turn, promotes the proliferation, differentiation, and survival of these cells. EPO is produced primarily in the peritubular fibroblasts in the kidney (Bachmann et al., 1993) with a small contribution from the liver in adults (Bondurant and Koury, 1986). A closed-loop feedback mechanism controls the production of EPO, as a reduced number of erythrocytes results in tissue hypoxia and subsequent activation of EPO gene transcription (Fisher, 1998). The resulting increase in EPO concentration leads to the stimulation of erythropoiesis in the bone marrow,finally resulting in the improvement of oxygen supply and repression of the activated EPO gene (Figure 2.1). Since the cloning of human EPO in 1985 (Lin et al., 1985; Jacobs et al.,1985), recombinant human EPO (rHuEPO) has been used to reverse anemia associated with cancer treatment and chronic renal failure, HIV infection, and in surgery (Adamson and Eschbach, 1990).

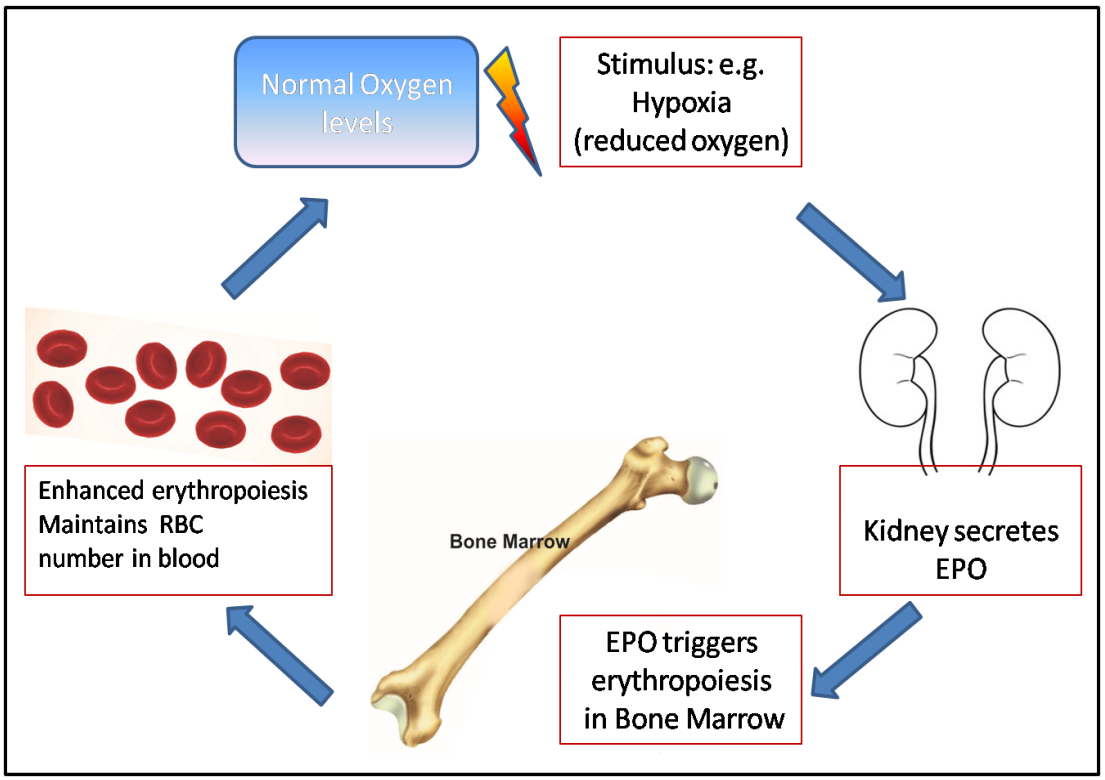


Figure 2.1: Regulation of blood oxygen levels.

When oxygen concentration in the blood is low, the interstitial peritubular cells of the kidney secrete erythropoietin (EPO), which stimulates red blood cell (RBC) production in the bone marrow, the increased erythropoiesis results in an increased number of RBCs in the blood and oxygen content,thereby restoring normal oxygen levels, and the signal for EPO release is repressed. Besides, the role of erythropoiesis EPO also inhibits apoptosis to decrease the rate of cell death in erythroid progenitor cells in the bone marrow and neural cells. Second, most important growth factor/cytokine is IL-3 which is supporting the proliferation of a broad range of hematopoietic stem cell types and involved in a variety of other cell activities such as cell growth, differentiation, and apoptosis followed by granulocyte macrophage colony-stimulating factor (GM-CSF) and granulocyte-colony-stimulating factor (G-CSF) which have established roles in hematopoiesis and have an established role as growth factors in clinical practice. G-CSF and GM-CSF regulate myeloid cell production, differentiation, and activation. The addition of these growth factors is inevitable in *ex vivo* erythropoiesis as to attain desired differentiated product correct growth should be deployed, and the absence of other growth factors will ensure that the progenitor cells will differentiate only into the desired type of cell and make this process more efficient. Besides the media and growth factors, the bone marrow niche remains the major factor in *ex vivo* erythropoiesis, and this is also achievable by using 3D scaffolds. Let us now have a detailed discussion about each growth factor/cytokine. Table 1 summarizes the functions of growth factors along with their protein size, on which receptor they bind, and what are their target cells and their functions.

2.3.2 GM-CSF

Granulocyte macrophage colony-stimulating factor (GM-CSF) is a member of the hematopoietic cytokine family and a 14.6 kDa monomeric protein of 127 amino acids

with two glycosylation sites. Its function is to stimulate the proliferation of granulocyte and/or macrophage progenitor cells, to influence differentiation, induce maturation, and stimulate the functional activity of mature hematopoietic cells. The other name of granulocyte macrophage colony-stimulating factor receptor is CD116 (cluster of differentiation 116), to which granulocyte macrophage colony-stimulating factor binds, and this receptor is not expressed on any erythroid or megakaryocytic lineage cells but on myeloblasts and mature neutrophils. The granulocyte macrophage colony-stimulating factor receptor or CD116 occurs in the form of a heterodimer, which is composed of at least two different subunits: a chain and a chain. The binding site for granulocyte macrophage colony-stimulating factor is present on the subunit , whereas the chain has a role in signal transduction, and finally, an association of the and subunits activates the receptor.

2.3.3 IL-3 (Interleukin-3)

Interleukin-3 originally was discovered by Ihle et al. in mice. In their studies, a T cell-derived factor was responsible for inducing the synthesis of 20-hydroxysteroid dehydrogenase in hematopoietic cells and termed it as interleukin-3. Interleukin gene encodes 152-amino acid long sequence, making a 17 kDa potent growth-promoting cytokine. To determine its function, wide studies have been done like treating different states of bone marrow failure and mobilizing or expanding hematopoietic progenitor cells for transplantation and last but not easy to support engraftment after bone marrow transplantation.

2.3.4 M-CSF

M-CSF is a cytokine, its functional form of the protein is found extracellularly in disulfide-linked homodimer form, and it is produced by proteolytic cleavage of

membrane-bound precursors. It is one of the hematopoietic growth factors that have a major role in the proliferation, differentiation, and survival of monocytes, macrophages, and bone marrow progenitor cells. M-CSF has several different ways to affect macrophages and monocytes, which includes stimulating increased phagocytic and chemotactic activity and increased tumor cell cytotoxicity.

2.3.5 G-CSF

Granulocyte colony-stimulating factor (G-CSF) was first reported and isolated from the mouse in Walter and Eliza Hall Institute, Australia, in 1983, and later in 1986, the human form was cloned by research groups from Japan and the USA/Germany. Granulocyte colony-stimulating factor (G-CSF or GCSF), also called colony-stimulating factor 3 (CSF-3), is a glycoprotein whose major function is to stimulate the bone marrow to produce granulocytes and stem cells and release them into the bloodstream. Therefore, it can be said that, functionally, it is a cytokine and hormone, a type of colony-stimulating factor, which is produced by a number of different tissues. Besides this, it is also known to stimulate the survival, proliferation, differentiation, and function of both neutrophil precursors and mature neutrophils.

2.3.6 BMP4

Another important factor having an essential role in stress erythropoiesis is Bone morphogenetic protein 4 (BMP4). Studies in mice models expressing a dominant-negative Smad5 mutant form that inhibits BMP4 signaling has shown anemic neonatal characteristics. That lasts for two weeks after birth and then resolved (Hegde et al., 2007; Lenox et al., 2005).

This suggests that BMP4 signaling through Smad5 is essential for stress-induced erythropoiesis, and adult or fetal liver hematopoiesis is independent ofBMP4/Smad-4

mediated signaling pathways. (Singbrant et al., 2010; Singbrant et al., 2006). Furthermore, the BMP4 expression is induced by hypoxia in spleen stromal cells (Wu et al. 2010; Millot et al., 2010). BFU-E cells need "priming" with Sonic Hedgehog to be responsive to BMP4, which is a morphogen synthesized and secreted spleen cells (Perry et al., 2009).On their activation with Sonic Hedgehog, the transcription factor Smad5 gets activated through BMP- 4. BMP-4 also induces activities of Scl and Gata2 as well and thus enhances the probability of BFU-E self-renewal. (Lugus et al., 2007; Fuchs et al., 2002; Harandi et al., 2010).

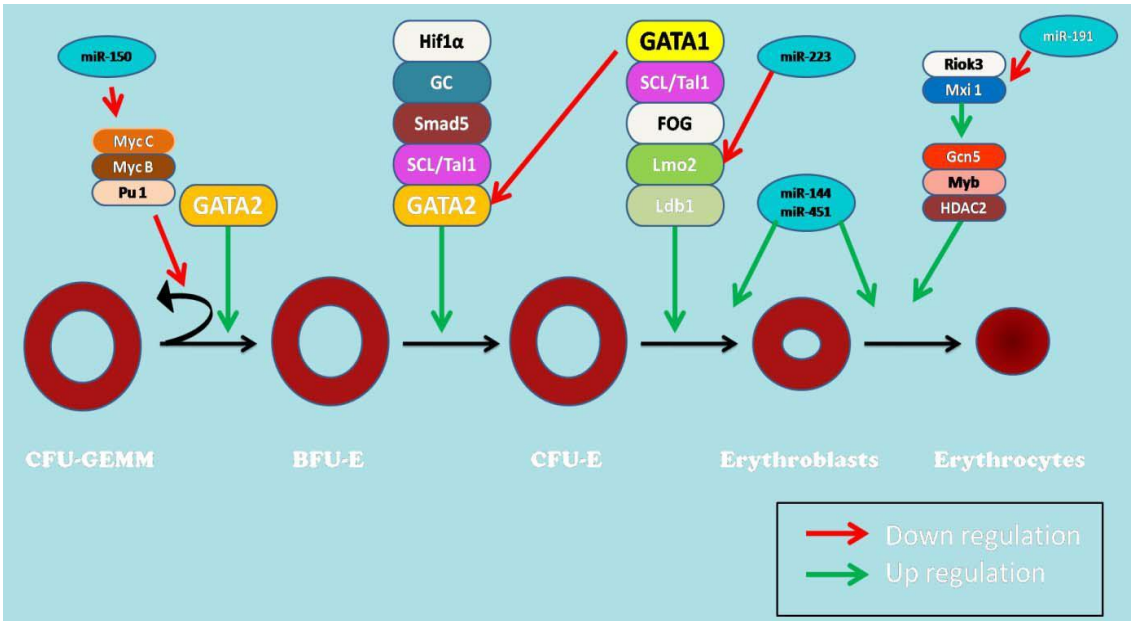


Figure 2.2: Regulation of erythropoiesis by different proteins and miRNAs. The intracellular factors such as transcriptions factors and miRNAs regulate the formation of RBCs. GATA2 activity is downregulated by GATA1 to regulate in erythrocytic determination and terminal differentiation. Other proteins such as SCL/TAL1, Lmo-2, and Ldb1 aid this regulation. miRNAs come into the action starting from the initial stage where miR-150 down-regulates expression of Myb gene to inhibit erythroid lineage differentiation. During terminal maturation, these miRNAs enhance terminal differentiation by downregulating the KIT gene that regulates the proliferation of erythroid progenitors.

2.4 Structure of EPO

2.4.1 Endogenous EPO

The human EPO gene is located on chromosome 7, and it consists of 5 exons and 4introns. The resultant protein has 165 amino acids and a molecular weight of approximately 30 kDa. EPO has 4 glycosylation sites, which affect the biological half-life(activity and stability) in the blood (Lai et al., 1986; Sasaki et al., 1987). It is heavily glycosylated, with approximately 40% of the molecule composed of sialic acid.Desialyation reduces the half-life of EPO in the circulation. Human EPO has 3 N-linked and 1 O-linked glycosylation sites at asparagine residues 24, 38, and 83 and serine 126,respectively (Egrie et al., 1986; Lai et al., 1986; Recny et al., 1987;Bondurant et al., 1986). However, rodent EPO does not contain O-linked sugar, which suggests a lack of significance in EPO stability (Wasley et al., 1991).EPO is a hydrophobic molecule that requires intact disulfide bonds between cysteine 6 and161, and between cysteine 29 and 33, for its activity (Lin et al., 1985; Jacobs et al., 1985;Lai et al., 1986).

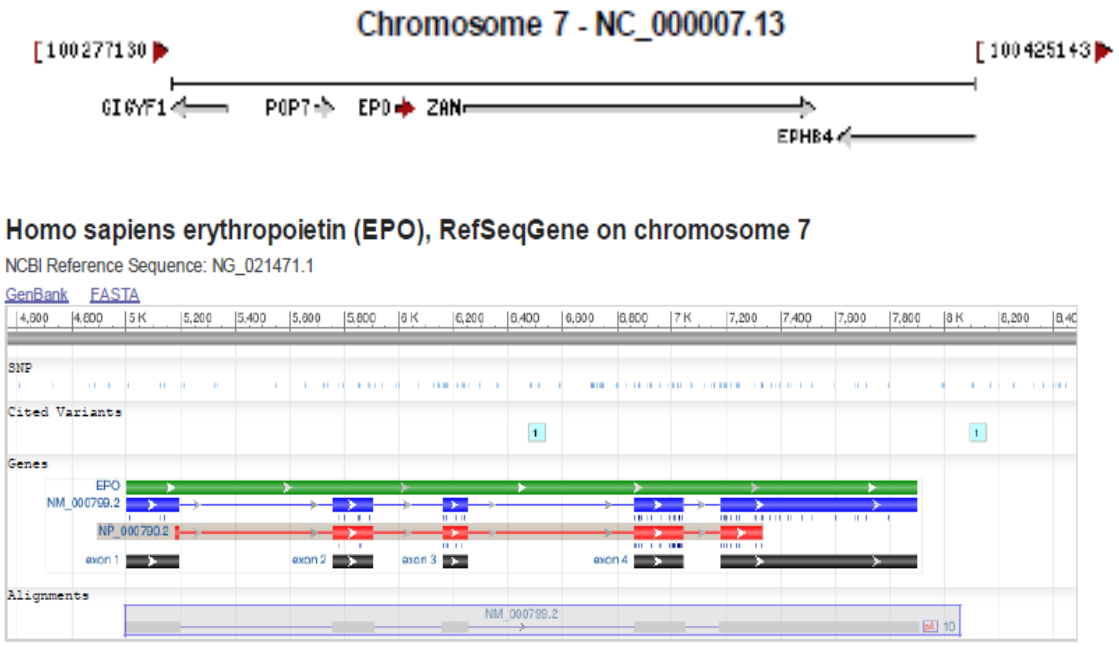


Figure 2.3 Chromosome sequence of Erythropoietin

2.4.2 Exogenous EPO

Exogenous EPO exists in various isoforms, differing mainly in their glycosylation (Storring, 1992). The isoform composition of EPO preparations varies with their source,differing between samples obtained from serum and urine (Tam et al., 1991), between samples obtained from subjects under different pathophysiological conditions (Wide and Bengtsson, 1990), and between human urinary EPOs and different recombinant DNA-derived human EPOs (rHuEPO) (Storring and Gaines Das, 1992;Storring et al., 1996). The differences in isoforms are due to the fact that the glycosylation of EPO is a post-translational process, which is influenced by the type of cell in which the EPO is synthesized and the physiological factors acting upon the cell. Different isoforms of EPO also have different biological activities and immunoreactivities (Takeuchi et al., 1989; Storrings and Gaines Das,1992). Studies on isolated rHuEPO isoforms determined that there is a direct relationship between sialic acid content, circulating half-life, and *in vivo* biological activity, but an inverse relationship with receptor affinity. That is, the molecules with the highest sialic acid content have the longest half-life, and greatest *in vivo* biological activity, and the molecules which are most active *in vitro* and have the highest affinity for the EPO-R are the least active *in vivo* (Egrie et al., 2003).

There are several types of erythropoiesis-stimulating agents (ESAs) that are in clinical use. rHuEPOs include epoetin alfa (Eprex®/Epypo®; Ortho-Biotech UK/Janssen-Cilag, High Wycombe, United Kingdom; Procrit®; Ortho Biotech Products, L.P.;Bridgewater, NJ) and epoetin beta (NeoRecormon®; Hoffman-La Roche, Basel,Switzerland). Epoetin alfa and epoetin beta differ in their pharmacokinetics and pharmacodynamics, but there have been no reports showing differences in their clinical efficacy (Storring et al., 1998). However, compared to epoetin alfa or epoetin

beta,darbepoetin alfa (Aranesp®; Amgen Inc., Thousand Oaks, CA), a rHuEPO analog engineered to contain additional oligosaccharide chains, has been shown to have enhanced biological activity and is around 14-fold more potent compared to rHuEPO (Egrie et al.,2003).

The third-generation exogenous EPO drug, continuous erythropoietin receptor activator (CERA, Mircera®, Roche, Basel, Switzerland), is a pegylated derivative of epoetin alfa. Pegylation involves the attachment of polyethylene glycol (PEG) chains to epoetin alfa, changing its pharmacokinetic and pharmacodynamic properties. In fact, pegylated rHuEPO has a half-life of 130 hours compared to epoetin alfa, which has approximately an 8-hour intravenous half-life (Rastogi and Nissenson, 2006).

2.5 Site of EPO production

During development, erythropoiesis takes place in the fetal liver, and hepatocytes are the major source of EPO (Zanjani et al., 1981). In humans, EPO gene expression appears in the kidney at about 18 weeks of age, and at birth, when the site of hematopoiesis shifts to the bone marrow, the peritubular fibroblasts in the kidney

become the primary EPO-producing cells (Obara et al., 2008; Juul et al., 1998; Koury et al.,1991). Endogenous EPO is maintained in the circulation at a concentration of about 15-30mU/mL of serum, or about 0.01 mM under normal physiological conditions. The half-life of EPO is approximately 7 to 8 hours (Bunn, 2007). However, in case of mice, it has been reported that under severe hypoxic conditions, out of the total EPO production, an adult liver contribution has been reported to be as much as 20-50% (Fandry and Bunn, 1993).

Intriguingly, EPO expression is not confined to the kidney and liver. In fact, EPO mRNA has also been detected in the lungs, testis, uterus, and the brain in rodents

(Chikuma et al., 2000). Overall it can be concluded that in rats under basal conditions, extra-renal organs account for approx 10% of EPO.

2.6 Mechanisms of EPO Action

The EPO-R is a member of the class I cytokine receptor superfamily. The EPO-R mRNA encodes a protein of 508 amino acids that contains a single hydrophobic membrane-spanning domain and has a molecular weight of 60 kDa. Human and mouse EPO receptors share 82% identity in their amino acid sequence (D'Andrea et al.,1993). EPO-R, along with the other members in this cytokine receptor superfamily, have a set of four conserved cysteine residues and a motif located close to the transmembrane domain, Trp-Ser-X-Trp-Ser (WSXWS). This particular motif contributes to EPO-R expression on the cell surface, EPO binding, and EPO-R activation (Quelle et al., 1992;Constantinescu et al., 2001).

EPO signaling starts with the binding of EPO exclusively to EPO-R(Constantinescu et al., 1999; Wojchowski et al., 1999). The EPO-R does not contain a kinase domain, thus depends on associated kinases for its function, such as the Janus family of non-receptor type protein tyrosine kinases (JAKs) (Witthuhn et al., 1993). The JAKfamily has four members, JAK1, JAK2, JAK3, and TYK2. They have a molecular weight in the range of 120-130 kDa. The EPO-R has JAK2 tethered to its cytoplasmic domain, and studies of JAK2 mutations indicate that JAK2 binds to EPO-R at its Box1/Box2 regions (Witthuhn et al., 1993).

EPORhomodimerization by EPO binding leads to a conformational change(Damen and Krystal, 1996), bringing the cytoplasmic domains close,and driving autophosphorylation of JAK2 proteins which activates the JAK-STAT pathway. The requirement of JAK2 in the EPO signaling pathway is evidenced by the finding that

global JAK2 deletion in mice resembles the phenotype seen in mice with EPO or EPO-R deletion. Furthermore, dominant-negative forms of JAK2 block EPO-dependent inhibition of apoptosis in erythroid cells. EPO activation of JAK2 via EPO-R results in the phosphorylation of the cytoplasmic tyrosine residues of the EPO-R to create docking sites for several src homology 2 (SH2) domain-containing adaptor proteins, including STAT5, growth factor receptor-bound protein 2 (Grb2) and the p85 subunit of phosphatidylinositol-3 kinase(PI3K). (Damen and Krystal, 1996; Damen et al., 1995; Quelle et al., 1996; Damen et al., 1995a; Damen et al., 1993; Wojchowski et al., 1999).However, the central pathway in response to EPO is the JAK2/STAT5 activation, whereby its phosphorylation leads to dimerization and translocation of STAT5 to the nucleus, resulting in up-regulation of target genes involved in cell proliferation, differentiation, anti-apoptotic as well as other cellular processes. Some key STAT5 target genes, at least in erythroid progenitor cells, include c-Myc, suppressors of cytokine signaling (SOCS), and D-type cyclins (Bittorf et al., 2000; Brines and Cerami, 2005). Many of these genes are ubiquitous and can provide the anti-apoptotic and proliferative effects of EPO.

2.6.1 Anti-apoptotic effects

Mice express two STAT5 isoforms, STAT5a and STAT5b, which are approximately 95% identical in amino acid sequence. There are conflicting results with regard to the significance of STAT5 in mediating the anti-apoptotic effects of EPO. While one study has shown that there is no abnormality in erythropoiesis in mice deficient in bothSTAT5a and STAT5b isoforms (Teglund et al., 1998), others have shown that STAT5 is necessary inupregulatingBcl-xL expression and mediating the antiapoptotic effects of EPO in erythropoiesis (Socolovsky et al., 2001; Socolovsky et al., 1999).

In a number of hematopoietic cell lines, STAT5 has been shown to induce Bcl-xL expression, an anti-apoptotic Bcl-2 family member (Socolovsky et al., 1999; Dumon et al.,1999; Nosaka et al., 1999; Ariyoshi et al., 2000). Furthermore, EPO administration toEPO-dependent erythroleukemic HCD-57 cells have been shown to increase the expression of Bcl-xL through the binding of EPO-activated STAT5 to a STAT-binding element in the Bcl-xL promoter. More recent studies on STAT5-null fetal liver cells demonstrated apoptosis due to a partial reduction in Bcl-xL expression, but not complete abolishment, indicating that Bcl-xL is not completely dependent on STAT5 activation. However, ectopic expression of Bcl-xL was able to completely prevent apoptosis in the STAT5-null fetal liver cells (Kerenyi et al., 2008). To further support the importance of Bcl-xL in erythropoiesis, erythroid cell-specific Bcl-xL knockout mice exhibit fetal liver hematopoietic defects, and severe anemia during embryogenesis (Wagner et al., 2000).

In addition to inhibition of apoptosis through STAT5, EPO activates PI3-K by phosphorylation, which subsequently activates Akt/PKB (protein kinase B) (Bouscary et al., 2003; Kadri et al., 2005). EPO prevents apoptosis through the PI3-K/Akt pathway by inhibiting Bad, thus maintaining the mitochondrial membrane potential and preventing the release of cytochrome c and activation of caspases (Chong et al., 2003; Chong et al.,2003a).

2.6.2 Anti-inflammatory effects

Previous studies have shown that rHuEPO administration can reduce inflammation particularly in brain injury models where inflammation has a significantly negative impact on disease outcome (Villa et al., 2003). rHuEPO administration was reported to attenuate production of pro-inflammatory cytokines such as TNF-α, which in turn resulted in a much smaller volume of injury. However, the anti-inflammatory effects

of EPO are thought to be indirect, secondary to increased viability of tissue, and a decrease in necrosis, which is often responsible for the secondary inflammatory response, rather than a direct effect of EPO in cytokine regulation (Villa et al., 2003).

2.6.3 Proliferative effects

EPO has been shown to support proliferation in murine erythroid progenitor cells *ex-vivo* as well as induce the entry of erythroid progenitors into the cell cycle. EPO binding to its receptor activates several downstream pathways that may mediate mitogenesis in cells. For instance, EPO treatment given to myoblast C2C12 cells, which express endogenous EPO-R, has been shown to stimulate a proliferative response through the activation of the JAK2/STAT5 pathway. In addition, studies have also shown that the dose-dependent induction of STAT5

DNA-binding activity by EPO correlates with the proliferation rate of HCD-57erythroleukemic cells (Bittorf et al., 1997). EPO has been shown to induce the expression of the proto-oncogene, *c-myc*, a STAT5 target gene (Wittig and Groner, 2005), in normal human erythroid progenitors. Moreover, it was reported that EPOactivates extracellular signal-regulated kinase 2 (ERK2), a MAPK, in the myocardium, and it increased the transcription of D-type cyclins in the intracardiac cells to improve cardiac regeneration. The EPO-mediated activation of the PI3-K/Akt signaling cascade has also been shown to be crucial for mediating signals for survival as well as proliferation (Bouscary, 2003). In fact, inCD34+ progenitor cells, EPO alone was able to induce cell cycle progression as demonstrated by the up-regulation of cyclin D3, E, and A, and these effects were completely abolished by the PI3K inhibitor LY294002.

2.6.4 Angiogenic effects

Angiogenesis is a complex process through which new blood vessels arise from preexisting ones. It takes place in various physiological and pathological settings, ranging from embryonic development to chronic inflammation and tumor formation (Folkman,1995). EPO has been shown to play a role in maintaining the integrity of the microvasculature and stimulating angiogenesis. rHuEPO stimulates both endothelial cell migration and proliferation,which are key steps in the angiogenic process (Heeschen et al., 2003). However, little is known about the mechanisms underlying the vascular effects of EPO. Previous reports have shown that EPO stimulates proliferation and migration of cultured human umbilical vein endothelial cells and human and bovine endothelial cells *in vitro* (Carlini et al., 1995). Interestingly, the angiogenic potential ofrHuEPO on endothelial cells has been reported to be similar to that of VEGF (Ispanovic and Haas, 2008). Recent studies have also shown that rHuEPO increases angiogenesis via increases in VEGF expression in endothelial cells in rats that had undergone ischemic stroke.

2.6.5 Regenerative effects

The role of EPO in the growth and differentiation of erythroid cells have shown that EPO and stem cell factor (SCF) are both required for complete erythropoiesis. SCF promotes erythropoiesis through its cognate receptor c-kit, a receptor tyrosine kinase, which transactivates EPO-R through physical interaction at the cytoplasmic domain leading to phosphorylation of the EPO-R. In addition, it has been shown that SCF induces the expression of STAT5 and EPO-R, resulting in efficient proliferation and survival by EPO (Kapur and Zhang, 2001). c-kit is present in progenitor cells of many tissues and cell types, including most notably the hematopoietic stem cells, as well as

progenitor cells of melanocytes, spermatocytes, myocardiocytes, and the pancreas. Each cell or tissue type appears to have its own unique mechanism of action downstream of c-kit. For example, in spermatogenesis, c-kit plays an essential role in inhibiting apoptosis through downregulation of Fas-mediated apoptosis and by activation of the PI3K/Akt signaling pathway. In melanocytes,c-kit activation reduces p53-mediated apoptosis. During myocardial injury, c-kit positive bone marrow-derived cardiac progenitor cells have been shown to play a critical role in promoting angiogenesis and tissue repair through up-regulation of VEGF. c-kit expression is also observed in the pancreas during pancreatic development and during regeneration following STZ treatment. Finally, c-kit has recently been shown to be important in physiological growth and maintenance of β-cell mass in mice.

2.7 Regulation of EPO

2.7.1 Hypoxia-Inducible Factors

EPO is one of the best-characterized genes that is regulated in an oxygen-dependent manner. In fact, EPO was the first target gene for hypoxia-inducible factor-1 (HIF-1) to be identified (Semenza and Wang, 1992). Cellular adaptation to hypoxia is mediated by hypoxia-inducible factors (HIFs). HIFs belong to the PAS (Per-ARNT (aryl-hydrocarbon-receptor nuclear translocator)-Sim) family of basic helix-loop-helix (bHLH) transcription factors (Wang and Semenza, 1995). They are heterodimeric transcription factors that are composed of a tightly regulated, oxygen-sensitive α-subunit, and a constitutively expressed β-subunit. Three α-subunit isoforms (HIF-1α, HIF-2α, and HIF-3α) and one β-subunit (HIF-1β or ARNT) have been identified to date. As critical regulators of oxygen homeostasis, the HIFs facilitate the delivery of oxygen to tissues and cellular adaptation to oxygen deprivation by modulating the expression of HIF-dependent gene products that are involved in cellular energy

metabolism, angiogenesis, iron metabolism, hematopoiesis, and other processes (Pugh and Ratcliffe, 2003). In addition to regulating oxygen levels, HIFs also have important functions during development. Global deletion of HIF-1αhas been shown to be embryonically lethal at E11 of gestation due to defective development of the heart and blood vessels.

2.7.2 Regulation of Hypoxia-Inducible Genes

Under conditions of adequate oxygen supply, the availability of oxygen enables specific prolyl hydroxylation on proline residues at conserved positions within the oxygen-dependent degradation domain of HIFα. This prolyl hydroxylation becomes a recognition site for the von Hippel-Lindau protein (pVHL), which makes up a part of the E3 ubiquitin ligase complex. Subsequently, pVHL binds to HIFα, leading to ubiquitination and prompt proteasomal degradation (Jaakkola et al., 2001). To date, three isoforms of the HIF prolyl hydroxylases have been identified in mammals termed prolyl hydroxylase domain enzymes (PHD1-3) (Bruick and McKnight, 2001). These enzymes are active during normoxia due to the presence of dioxygen for the hydroxylation reaction. However, during hypoxia, the activity of the prolyl hydroxylases is inhibited, which results in the stabilization of HIF-α. The intact HIF subunits then translocate to the nucleus, where they bind to the HIF-β subunit, ARNT. The HIF-α/ARNT complex binds to DNA, resulting in increased transcription of HIF-target genes. Enhanced expression of HIF-target genes, including *EPO* (Semenza et al., 1991), *VEGF* (Kelly et al., 2003), *glut1* and various glycolytic enzymes (Firth et al., 1994), allow for the survival of the organism, tissue, and cell, respectively.

2.8 Role of EPO in non-erythroid tissues

Accumulating evidence suggests that EPO plays a role in promoting tissue protection in non-erythroid tissues. It has been observed that mice that are a homozygous mutant

in either the *EPO* gene or *EpoR* gene do not survive embryonically by E13.5. just because there is no fetal erythropoiesis. Importantly, although reconstitution of EPO-R in bone marrow cells has been shown to completely rescue mice from embryonic death, these mice still have defects in cardiac morphogenesis during development.

EPO-R has been reported in several non-erythroid tissues, including the small bowel (Juul et al., 1999), uterus, kidney (Westenfelder et al., 1999), as well as the pancreatic β cells (human and rodent) (Fenjves et al., 2003). The observation that the presence of EPO-R and perhaps itself in non-erythroid cells, therefore, extends its putative role beyond hematopoiesis.

2.8.1 Central nervous system

With the wide use of rHuEPO, it was noted that there were other significant benefits outside of the amelioration of hemoglobin levels. It was later found that EPO crossed the blood-brain barrier and had significant beneficial effects in the central nervous system, including amelioration of cognitive function. Both EPO and the EPORare expressed in the central nervous system during development and adulthood (Digicaylioglu et al., 1995), and their expression levels are upregulated after hypoxic injury (Bernaudin et al., 1999). Exogenous EPO treatment has been shown to promote neural stem cell proliferation, survival, and differentiation *in vitro* (Shingo et al., 2001) and increased neuroblast migration to areas exposed to ischemic damage *in vivo*. In addition, exogenous EPO is neuroprotective and neurotrophic in diverse CNS injury models, including hypoxic injury, spinal cord trauma (Kaptanoglu et al., 2004), autoimmune encephalomyelitis (Savino et al., 2006), and amyotrophic lateral sclerosis (Grunfeld et al., 2007). The efficacy of rHuEPO administration was also examined in rats with STZ-induced diabetic neuropathy. Results from this study show that rHuEPO treatment provided recovery from diabetes-induced neuropathy.

Furthermore, a critical neuroprotective role for endogenous EPO in the CNS was also demonstrated through the administration of soluble EPO-R, which neutralizes EPO, which consequently exacerbated ischemic stress and increased tissue injury (Brines et al., 2000). Further confirmation on the importance of EPO signaling in the brain was demonstrated by using conditional EPO-R knockout animals. Brain-specific EPO-R deletion was shown to significantly reduce cell proliferation and impair post-stroke neurogenesis (Tsai et al., 2006).

There has only been one clinical study concerning neural tissue protection following rHuEPO administration. In this study, rHuEPO (100 000 U administered i.v., divided over 3 days) was shown to be effective for improving the short-term clinical outcome of patients with middle cerebral arterial infarction without any acute changes in hemoglobin concentrations (Ehrenreich et al., 2002).

2.8.2 Heart

Several studies have evaluated the role of rHuEPO during cardiac ischemia-reperfusion injury. Similar to findings in neuronal injury models, many studies have shown that exogenous EPO administration before or during injury is highly effective for the prevention and/or treatment against permanent or transient ischemic injury to the heart.

Following ischemia-reperfusion injury, rHuEPO treatment has been shown to significantly improve left ventricular pressure and cardiac function (Parsa et al., 2004).

Early clinical studies of patients with anemia or on hemodialysis have reported that rHuEPO treatment improves cardiac function secondary to the correction of anemia. Clinical trials have shown that patients with mild anemia or diabetes and with severe

congestive heart failure have increased left ventricular ejection fraction and decreased hospitalization days by almost 80% with rHuEPO treatment (Maiese et al., 2005; Silverberg et al., 2003).

Additional investigations involving rHuEPO treatment in patients with moderate to severe chronic heart failure have shown that the peak oxygen consumption and exercise duration of patients are significantly increased following rHuEPO treatment, suggesting that EPO can enhance exercise capacity in patients with heart failure (Mancini and Kunavarapu, 2003). In addition, it has been postulated that rHuEPO can protect the myocardiocytes directly by protecting against apoptosis and increasing cardiac cell proliferation, leading to the reduction in infarct size and in improved left ventricular function (Maiese et al., 2005).

2.8.3 Lungs

Expression of EPO-R was demonstrated in a human alveolar epithelial cell line (A549), normal human bronchial epithelial cells (NHBE), and normal human alveolar epithelium. When rHuEPO treatment was given to A549 and NHBE in a model of acute lung injury by Fas activation, it provided complete protection against cell apoptosis as assessed by PARP (Poly (ADP-ribose) polymerase) cleavage. Specific EPO binding to its receptor was demonstrated using an EPO-blocking antibody and an EPO-R small interfering RNA, which abolished the protective effect of EPO in up-regulating Bcl-xL expression *in vitro* (MacRedmond et al., 2009).

2.8.4 Endothelial Cells

Capillary endothelial cells obtained from a variety of tissues expressed EPO and EPOR and were the first cells implicated experimentally as targets of the extra-hematopoietic activities of EPO (Anagnostou et al., 1990). EPO has been shown to

play a dual role in endothelial cells, in both vascular protection by preserving endothelial cell integrity (Chong et al., 2002a; Chong et al., 2003) and by promoting new capillary formation from preexisting vessels, a process known as angiogenesis (Ribatti et al., 2003; Galeano et al.,2004). Previous studies by Chong et al. (2002) reported that EPO protects vascular endothelium against ischemic injury. In addition, several *in vivo* studies have demonstrated that EPO administration protects endothelial cells against various models of vascular disease (Santhanam et al., 2005; Urao et al., 2006).

EPO has also been reported to prevent blood-brain barrier permeability during injury and maintain cell-cell junctions. EPO has both mitogenic and chemotactic effects that can lead to matrix metalloproteinase-2 (MMP2) production, which is required for endothelial cell migration, cell proliferation, and vessel formation. Angiogenesis was observed in rat aortic rings four days following incubation with rHuEPO (Carlini et al., 1995). Previous studies have suggested that EPO may provide indirect cellular protection through its role in angiogenesis. In brain ischemia injury models where EPO was administered, proliferation and migration of brain capillary endothelial cells may improve blood flow to ischemic cells starved for oxygen and nutrients, decreasing the amount of neuronal damage.

2.8.5 Muscle

Emerging evidence has reported the presence of the EPO-R in adult skeletal muscle cells in humans. In addition, EPO-R mRNA expression and EPOR-associated JAK2 phosphorylation were increased in the muscle tissue after exercise (Rundqvist et al., 2009). This study also found that during rest, there is a small uptake of EPO, but during exercise, there is a small release of EPO from the exercising leg. Transgenic over-expression of EPO in skeletal muscle has been reported to have beneficial effects

against the development of obesity (Hojman et al., 2009). In fact, over-expression of EPO in skeletal muscles was shown to protect against high-fat diet-induced obesity in mice through increased fat oxidation in muscles (Hojman et al., 2009).

2.8.6 Kidney

A potential role for the non-hematopoietic activities of EPO in the kidney was first implied by the identification of EPO-R protein expressed throughout the kidney, including both proximal and distal tubular cells (Westenfelder et al., 1999). A number of groups have shown that EPO or darbepoetin administered in animal models at a high dose (300-5000U/kg b.w. intra-peritoneally) is substantially protective of renal ischemia-reperfusion injury as assessed by residual renal function and histological markers of injury. EPO is also renoprotective against toxins. In these acute experiments, there was no increase in hematocrit. Therefore, the action of EPO was likely to direct on the kidney.

2.8.7 Pancreatic β cells

The definitive role of EPO in pancreatic β cells is currently unknown. However, emerging evidence has demonstrated the potential beneficial role of EPO in the β cells. In addition to the presence of EPO-R in human and rodent islets (Fenjves et al., 2003), studies have shown that rHuEPO in islet cultures (Fenjves et al., 2003) and transfection of the EPO gene in human islets (Fenjves et al., 2004) resulted in maintenance of insulin secretion and increased survival against cytokine-induced cell death *in vitro*.

Furthermore, transgenic mice expressing the dominant-negative mutant of STAT5 specifically in the pancreatic β cells demonstrated no changes inβ-cell homeostasis (Jackerott et al., 2006) however, these mice had increased susceptibility to STZ-

induced β-cell death (Jackerott et al., 2006), supporting the importance of the JAK/STAT pathway in providing cytoprotection to pancreatic β cells during diabetes pathogenesis. Interestingly, mice with STAT5-deficient islets had reduced glucose tolerance and decreased β-cell mass with age. The findings in these studies support the importance of this general class of cytokine receptors and the JAK/STAT pathway in promoting β-cell mass and function.

Table 2.1 Factors that regulate ex-vivo erythropoiesis				
S.No.	**Factors**	**Role/Effect in *Ex-Vivo* Erythropoiesis**	**Receptor**	**References**
1	**Animal/ Human-Derived Growth Factors/Cytokines**			
a)	EPO	Differentiation and proliferation of erythroid	EPOR	Gregory et al., 1978
b)	IL3	Differentiation and proliferation of myeloid progenitor cells	CD123/IL3RA,CD131/IL3 RB	Korpelainen et al.,1996
c)	GM-CSF	White blood cells growth factor	CD116	Rosas et al., 2007
d)	G-CSF	Inducer of HSCs mobilization from the bone marrow into the bloodstream.	CD114	Basu et al., 2002
e)	IGF-1	Stimulate erythroid colony-forming units in the mouse.	IGF1 Receptor	Brox et al.,1996
f)	SCF	Regulates HSCs in the bone marrow.	CD117	Lindern et al., 2004
2	**Synthetic Molecules**			
a)	Dex	Anti inflammation		Leberbauer et al., 2005
b)	Poloxamer 88	Induces enucleation of RBCs		Baek et al., 2009
c)	Cholesterol rich Lipid	Induces proliferation rate enhance the proliferation of EPCs		Leberbauer et al., 2005

d)	Hydrocortisone			Timmins et al., 2011
3	**mi-RNAs**			
a)	miR-150	Differentiation towards the megakaryocytic lineage at the expense of erythroid lineage	MYB	Lu et al., 2008
b)	miR-221 and miR-222	Downregulation of miR-221 and miR222 is required for terminal erythroid progenitor proliferation	KIT	Felli et al., 2005
c)	miR-223	Downregulation of miR-223 is required for the terminal differentiation	ALK4	Felli et al., 2009
d)	miR-24	Downregulation of miR-24 is required for terminal erythroid differentiation	LMO-2	Wang et al., 2008
4	**Transcription Factors**			
a)	GATA1	regulates p21 gene expression during erythroid differentiation	Globin, erythroid-specific membrane proteins, GATA-1, GATA-2	Papetti et al., 2010
b)	GATA2	the cellular proliferation of non-committed and committed erythroid progenitors required for specification of HSCs and	GATA-1 and GATA-2 gene	Orkin et al., 1992 &2000
c)	SCL/TAL1	maturation of the erythroid and megakaryocytic lineages	Glycophorin A, p21 gene	Ravet et al., 2004; Hall et al., 2005
d)	LDB1/LMO2	Ldb1 complexes regulate Klf1		Love et al., 2014
e)	Klf1	Acts as a global regulator of erythroid production and integrity	Globin	Micheal et al., 2010
f)	Gfi1B	represses p21, SOCS1, 3 and its own genes	p21, SOCS1-3, BclxL	Kuo et al., 2007

2.9 Regulation of erythropoiesis

2.9.1 Regulation by Proteins

During erythropoiesis binding of most important growth factors elicit pattern of intracellular proteins. For example, several transcription factors are activated like, Stat5 and EPO-regulated transcriptional regulators present in erythroid cells interact with few lineages restricted transcriptional regulators such as GATA-1 (Globin Transcription Factor 1), SCL/Tal1 (Stem cell leukemia/T-Cell Acute Lymphocytic Leukemia 1), LDB1 (LIM Domain Binding 1), LMO2 (LIM Domain Only 2), Klf1(Kruppel-Like Factor 1), and Gfi-1b (Growth Factor-Independent 1B Transcription Repressor), responsible for the production of mRNAs that are essential for erythropoiesis (Table 2.1, Figure 2.2). GATA1 belongs to the GATA family of transcription factors, which regulates the switch of fetal hemoglobin to adult hemoglobin in erythroid development. While the stem cell leukemia (SCL) gene, which is also known as Tal-1 encodes a critical regulator of both hemopoiesis and vasculogenesis, i.e., a basic helix– loop–helix (bHLH) transcription factor and, therefore, positively regulates erythroid differentiation. Next, LDB1 acts with LMO2 by binding to the LIM domain of a transcription factor in order to regulate red blood cell development by maintaining erythroid precursors in their immature state. LMO2 is a cysteine-rich, two LIM-domain protein that has a central and crucial role in hematopoietic development along with TAL1/SCL and is highly conserved. Klf1 is a hematopoietic-specific transcription factor and induces high expression of adult beta-globin and other erythroid genes. Gfi-1b is an essential proto-oncogenic transcriptional regulator that controls the expression of genes involved in development and differentiation of erythrocytes and megakaryocytes by forming complexes with transcriptional regulatory proteins, including GATA-1, histone deacetylases, and runt-related transcription factor-1.

The expression pattern of specific genes is regulated in a precise time frame at the transcriptional level during the differentiation of erythroid lineage. Functional analysis of erythroid and megakaryocytic specific genes has shown the importance of a sequence, 5' A/T GATA A/G 3', now called the GATA motif, in the lineage-specific expression of these genes. This sequence is associated with a GT or CACC-like sequence in erythroid-specific genes, whereas it is associated with an ETS (E26 transformation-specific) binding site in megakaryocytic specific genes (Orkin et al., 2000; Orkin et al., 1992; Papetti et al., 2010). The GATA family of transcription factors consists of six transcription factors, GATA- 1 to -6 that can bind the consensus sequence 5' A/T GATA A/G 3'. These proteins contain two conserved zinc finger motifs (Cys-X2-Cys-X17-Cys- X2-Cys) peculiar to the GATA family. The carboxyl-terminal zinc finger binds to DNA, whereas the amino-terminal zinc finger stabilizes the DNA/GATA interaction. Outside of the zinc finger regions, the conservation between GATA factors is low, but each factor is conserved between species. GATA-1 and GATA- 2 reported regulating in erythrocytic determination and terminal differentiation.

This is supported by motif analysis reports showing activation of genes enriched consensus sequences for SCL/Tal1 at GATA1-bound sites. These studies suggested a correlation between GATA1 activation and SCL/Tal1 activities. (Fujiwara et al., 2009; Yu et al., 2009; Cheng et al., 2009; Kassouf et al., 2010; Tripic et al., 2009; Ravet et al., 2004; Hall et al., 2003). In erythroid cells, SCL/Tal1 interacts with basic helix loop helix protein E2A (bHLH protein E2A) and makes an activation complex by interacting with LIM domain of LMO2 and Ldb1. Along with GATA1, this complex binds to the GATA1/E-box domains that are spaced ~9 to 11 nucleotides apart (Elliot et al., 2008). Components other than GATA1 in the activation complex

are also necessary for RBC development. This is indicated by studies in Lmo2, GATA1, and SCL/Tal1 knockout mice where all such mice are anemic and die due to lack of primitive erythropoiesis in fetal development (Cantor et al., 2002) . TAL-1 can assemble in a pentameric complex (Wadman et al., 1997) containing the ubiquitously expressed. E-proteins (E2A, HEB, E2-2), LMO-2, Ldb, and GATA-1 in erythroid-committed cells. This complex can lead to activation of erythroid-specific terminal gene expression. GATA-2 variant of this TAL-1 complex is responsible for activation of c-Kit transcription in CD34+ hematopoietic progenitors (Lecuyer et al., 2002; Love et al., 2014; Michael et al., 2010; Kuo et al., 2007). Therefore, to mimic erythropoiesis ex-vivo, a better understanding at molecular level becomes inevitable.

2.9.2 Regulation by miRNAs

In the last few years, a new class of small, endogenous, non-coding RNAs called microRNAs (miRNAs) is reported. miRNAs are important regulators of gene expression at the post-transcriptional level. Thousands of such miRNAs are identified in various organisms, including unicellular microorganisms and complex multicellular organisms showing that regulatory miRNA pathway is conserved in evolution (Listowski et al., 2013). miRNAs are defined as a class of small regulatory RNAs that down-regulates their target genes expression either through degradation of mRNA or inhibiting their translation (Bartel et al., 2009; Guo et al., 2010). Erythropoiesis is also regulated by several miRNAs at various stages, which include lineage determination, progenitor proliferation, terminal differentiation of erythroid, and enucleation of reticulocytes (Table 2.1, Figure-2.5). Out of the miR -24, 144, 150, 191, 221, 222, 223, & 451 is reported to have significant roles in human erythropoiesis. miR-150 comes into the action at the initial stage and down-regulates expression of Myb gene to inhibit

erythroid lineage differentiation (Lu et al., 2008). While miR-24 negatively regulates activin signaling, it down-regulates the mRNA encoding the Activin receptor-like kinase 4 (ALK4) (Wang et al., 2008). LMO2 is a critical transcription factor which gets up-regulated during erythroid differentiation of CD34- progenitor cells and is required for erythroid differentiation, but gets down-regulated due to overexpression of miR-223 (Felli et al., 2009). During the terminal erythroid progenitor proliferation, miR-221 and miR-222 come into action where these miRNAs enhances terminal differentiation by down-regulating the KIT gene that regulates proliferation of erythroid progenitors (Felli et al., 2005)

2.9.3 A regulation under Stress Conditions

As discussed above, the erythropoiesis level is several folds enhanced under stress conditions such as hypoxia, chronic anemia, and acute blood loss. This process is governed by several important factors that tightly regulate survival, proliferation, and terminal differentiation of erythroid progenitors such as CFU-E and produce more erythroblast cells. Since limited proliferative potential (3-5cell divisions) of early EPO-responding CFU-E even in the presence of high level of EPO due to stress conditions remain insufficient to support the extreme demand of mature RBCs in a short span of time. Bone marrow produces number of CFU-E cells from BFU-E cells. This process of generating more number of BFU-/CFU -E is independent of EPO-mediated activities. It is shown that single BFU-E cells can give rise to hundreds of CFU-Es in vitro. BFU-E progenitors may undergo limited self-renewal during stress erythropoiesis, supporting the survival of lethally irradiated mice from anemia. Even more, these BFU-Es can be re-transplanted and protects secondary and tertiary recipients (Harandi et al., 2010).

Under stress conditions, a significant number of cell division, self-renewal, and differentiation activities take place, which is regulated by various factors such as microenvironment growth factor/ Glucocorticoids and Hedgehog signaling along with tissue hypoxia. This is evident from the studies showing the movement of erythropoietic activities from the bone marrow to the spleen during stress conditions until optimal conditions are restored. (McCulloch et al., 1964; Hara et al., 1976). Fetal liver and spleen-derived stromal cell lines are demonstrated to inherit better-supporting properties for in vitro fetal BFU-E proliferation in comparison to bone marrow stromal cells (Slaper- CortenbachIet al., 1987; Yanai et al., 1989; Ohneda et al., 1990). Furthermore, the microenvironment has a significant role in sustaining the erythroid progenitor's self-renewal. This is also evident from the mice studies showing resistance to Friend erythroid leukemia virus-induced erythroid leukemia (Kreja et al., 1985). A number of attempts have been made by various researcher groups to define various regulating factors in spleen and fetal liver.

Another important regulatory factor is the glucocorticoids (GC), which are released from the adrenal glands during conditions of stress erythropoiesis (sepsis/severe trauma). Prednisolone, which is a GC analog, is often used to treat the patients with the RBC progenitor disorder Diamond-Blackfan anemia. (Flygare et al., 2007; Vlachos et al., 2008). Studies in mice lacking GC receptors or expressing a mutated version GC (deficient in DNA binding or transactivation) have shown normal steady-state erythropoiesis but severely impaired stress erythropoiesis. (Reichardt et al., 1998; Bauer et al., 1999). Similarly, GC receptors deficiency resulted in a loss of responsiveness to phenylhydrazine-induced hemolysis and failed to increase CFU-E numbers in the spleen.

There are reports showing antagonistic effects of p53 activation on GC receptor-induced erythropoiesis. p53-/-mice shown the rapid growth of CFU-E and c-Kit/CD34cells that is faster than normal mice in response to hypoxic stress in the spleen.(Ganguli et al., 2002).

It is reported that GCs activity is facilitated through the induction of expression of Myb, Kit, and Lmo2 and inhibition of Gata1 expression (Ganguli et al., 2002; von Lindern et al., 1999). The up-regulated expression of Kit mRNA is detected as early as 4 hours after GC stimulation of BFU-E cells (Flygare et al., 2011). The erythropoietic stress regulation by GCs can be modeled in vitro by growing early erythroid progenitors in the presence of SCF, GCs, and Epo (Bauer et al., 1999; Flygare et al., 2011; Kolbus et al., 2003; Wessely et al., 1997). These studies reported that disruption of GC receptor dimerization severely reduces the in vitro proliferation of fetal liver erythroblasts (Reichardt et al., 1998; Bauer et al., 1999). This suggests a significant role of GC receptor dimerization in GCs induced RBC production. GCs dimerization is required for efficient transactivation of promoters and/or gene repression (Reichardt et al., 1998; Bauer et al., 1999).

GCs induce limited self-renewal of BFU-E cells, and not of CFU-E cells or erythroblasts as reported by studies using mouse fetal liver BFU- E/CFU-E cells grown in serum-free conditions along with SCF and IGF-1. These studies have shown the protection of BFU-E cells from exhaustion and enhanced the formation of CFU-E per BFU-E (Flygare et al., 2011). It is also proposed by Lodish and Flygare that increased levels of GCs likely help in maintaining the earliest erythroid progenitors, increase generation of CFU-E, and may stimulate terminal differentiation (Lodish et al., 2010). That is how it may have positive effects on a rapid and long-lasting increase in RBC production. The presence of Hypoxia-induced factor-1 (HIF-1) binding sites in the promoter regions of most of the genes which are induced through GCs mediated signals indicate a link between Hypoxia and GCs signaling. It suggests that activation of HIF-1 may enhance or replace the effect of GCs on BFU-E self-renewal. This is evident from studies showing enhanced production of CFU-E and then erythroblasts approximately through HIF-1α activation by a prolyl hydroxylase inhibitor (Flygare et al., 2011).

2.10 Clinical Use and Limitations of Hematopoietic Growth Factors

Erythropoietin (EPO) is often used in cancer treatment. But recent studies have shown that EPO can act on non-hematopoietic organs, including solid tumors. The effect of EPO on the survival rate of cancer patients seems to be variable, as it has been observed that EPO decreases the survival of cancer patients with cancer in head and neck and metastatic breast, whereas in case of patients with small-cell lung cancer erythropoiesis-stimulating agents (ESAs), which include EPO, it does not reduce the survival. In a study on 2,500 cases, which included most chronic renal failure cases, only two-thirds of patients were found to be able to predevelop hypertension and seizures as a side effect of rHuEPO. EPO has also been known to be associated with worsening of hypertension and hypertensive encephalopathy, which are less common scenario nowadays. There might be serious problems due to EPO antibodies such as pure red cell aplasia, but fortunately it is rare, and clinicians are still using this drug in a new way to monitor closely for adverse side effects.

Recombinant GM-CSF has revolutionized the supportive care of cancer patients through significant contributions, like enhanced myeloid recovery after cytotoxic chemotherapy. In 1993 Dranoff et al. used GM-CSF as an important adjuvant in cancer vaccine trials, utilizing his observations that, when irradiated tumor cells expressing murine GM-CSF were used, they stimulated potent, long-lasting, and tumor-specific immunity. Similarly, Schmidt et al. demonstrated the treatment of solid tumors (breast, renal cell carcinoma, malignant melanoma, and prostate cancer) by utilizing GM-CSF secreting transformed tumor cells as a potential cure.

However, no condition for the clinical use of IL-3 has been established so far, despite its theoretical advantages as an early-acting cytokine and in contrast to erythropoietin (EPO), G-CSF, or GM-CSF which has been approved for several clinical modalities.

Macrophage colony-stimulating factor (M-CSF), a growth factor stimulating the production of leukocytes, including monocytes and neutrophils, has been clinically used as an agent for leukopenic patients after anticancer therapy. M-CSF improves a survival rate after bone marrow transplantation (BMT) through the reduction of mortality rate associated with BMT, such as bleeding, engraftment failure, and GVHD. M-CSF has been known to accelerate platelet production when administered after anticancer therapy to thrombopenia patients with solid tumors.Granulocyte CSF (G-CSF) is a more powerful agent for various kinds of neutropenia such as neutropenia after anticancer therapy, neutropenia after BMT, aplastic anemia, chronic neutropenia of children, and myelodysplastic syndrome.

2.11 *Ex-vivo* Erythropoiesis

Ex vivo expansion of erythrocytes or manufacturing blood from stem cells is the most attracting approach among the global research community for the last few years. The basis for the principle that *ex vivo* cultivated RBCs (or other blood cells) may be of clinical use was provided by Nakamura and his colleagues who have demonstrated that RBCs derived from the immobilized ESCs cell line can protect mice from lethal anemia (Hiroyama et al., 2008). Recently, the proof-of-concept was described by first-in-human blood transfusion using *ex-vivo* generated RBCs (Giarratana et al., 2011). In a major landmark study, Giarratana et al. show the survival capacity of *ex vivo* cultivated RBCs in humans. As described by them, the $CD34^{+}$ cells obtained from volunteer donors as an aphaeresis product were cultured in a serum-free media (with the addition of Stem cell factor (SCF), Interleukin-3 (IL-3), and Erythropoietin (EPO) resulting into 2 ml of blood RBCs equivalent (10^{10} cells) under GMP conditions. The *ex vivo* expanded RBCs were labeled with ^{51}Cs to trace their fate in patients as approved by FDA U.S. (Giarratana et al.,2011) and demonstrated to be comparable with any native RBCs transfusion. These findings established the proof-of-concept for clinical utilization of *ex vivo* generated RBCs globally. As discussed in

the next section, a number of methods have been demonstrated so far ,which ensure the feasibilities of *ex vivo* RBCs expansion from various source materials (e.g., $CD34^+$ HSCs, Embryonic stem cells, and IPSCs) (Figure2.4).

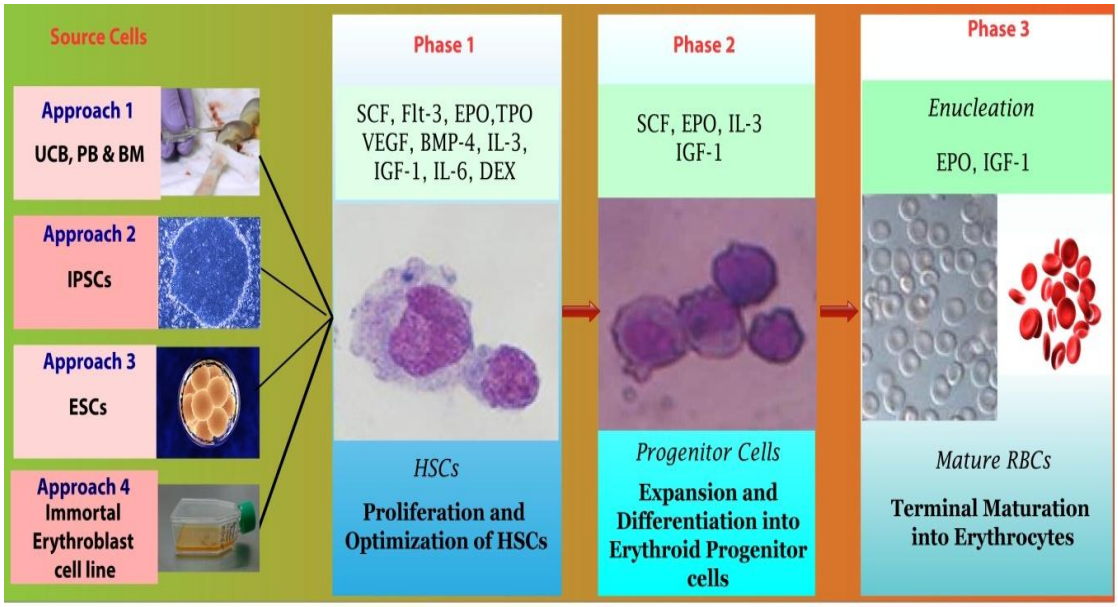


Figure 2.4 | The various methods/protocols described so far for the ex vivo RBCs expansion. As discussed in the main text, manufacturing blood may involve various steps that are to be categorized into three main phases, as depicted in the figure. In phase 1: initial source material is to be collected from a variety of source material(s) on the basis of their availability, suitability, and expansion potential and grown in medium generally supplemented with growth factors to enhance HSCs proliferation:subsequently these cells are cultured in the presence of Erythropoietin to induce their differentiation and maintenance into erythropoietic progenitor stage. Finally, in Phase 3 cultures, these Erythropoietic progenitors may be co-cultured with murine/human stromal cell line support to induce their maturation and enucleation, resulting in mature RBCs. These RBCs are evaluated for their biochemical properties and various antigenic profiles to ensure their nativeness.

There are various factors that essentially regulate the yield and the level of RBCs maturation through these protocols. Reports are there, showing numerical expansion of erythrocyte precursors up to ~0.5 transfusion unit/donation from UCB $CD34^+$ cells, and approximately 2 million fold expansions of enucleated erythrocytes functionally comparable to native RBCs through slight modification of culturing techniques (Neildez-Nguyen et al.,2002). Further, large scale production (up to 10 transfusion unit) from two units of UCB have been demonstrated by technically complex protocols (described in later sections) which presently may appear ill-suited from the commercial-scale production point of view but strengthen the concept that with the advancement of techniques sufficient transfusable units may be produced (Fujimi et al., 2008). Besides that, RBCs precursors, cellular intermediates/nucleated erythroid precursors are generated through these protocols, which may also be of use if transfused. It is expected that these cells are likely to become functionally matured *in vivo* while interacting with internal factors governing their maturation. The same has been reported both in human and animal models (Ende and Ende, 1972; Neildez-Nguyen et al., 2002). Conceptually, the maturing cells, along with functionally matured cells may aid in maintaining the cellular content at the time of transfusion. These maturing cells may proliferate, and each cell may give rise to 4–64 cells before they get enucleated and functionally matured *in vivo*. This hypothesis is supported by the data available from clinical observations in developing countries where 40–80 ml of compatible CB blood (containing 4–7 $\times 10^{10}$ RBCs and 2–8 $\times 10^{9}$ erythroblast cells) is occasionally used for trans-fusion in emergent conditions (Ende and Ende, 1972). Together, these reports strengthen the concept that *ex vivo* RBCs expansion likely support the different clinical short-comes to exist due to limited supply and various rare blood-based disorder.

There has been considerable progress in the field of developing biological control over the expansion of erythrocytes to generate terminally differentiated, fully functional RBCs (Migliaccio et al.,2012). The *in vivo* hematopoietic development process have been studied (Figure 2.3), and in-depth knowledge of the generation of erythroblast regulated through various growth factors have paved the way to mimic the process *in vitro* (Lodish et al., 2010). Altogether, these reports demonstrated the enormous potential inherited by three major cell types, including $CD34^+$ HSPCs, Embryonic stem cells/induced pluripotent stem cells, and immortalized erythroid precursors which can be differentiated into erythroid lineages by using almost similar/overlapping protocols .

Regardless of their types, stem cells are promoted to become erythroid progenitor in three basic steps viz. commitment, expansion, and maturation. There are a number of reports showing the use of various cytokines/growth factors(SCF and EPO are most common) to generate variable yields of immature progenitors or mature RBCs with or without using animal/human-derived feeder coculture system (Figure 2.3). Recently, a new concept of transdifferentiation (directly differentiating human fibroblast cells into erythroblasts) has been shown to bypass the HSPC state (Szabo et al., 2010). Although promising, but the technique remains to be evaluated for their potential to generate large scale RBCs. Most of these protocols suffer from similar problems such as degree of enucleation or erythrocyte formation, predominate form of Hb expressed by them, use of animal-derived products such as serum and feeder cell coculture, and clinical transfusion-related problems such as blood type matching and unavailability of sufficient number of RBCs for transfusion. All these issues can be summarized in the following sections.

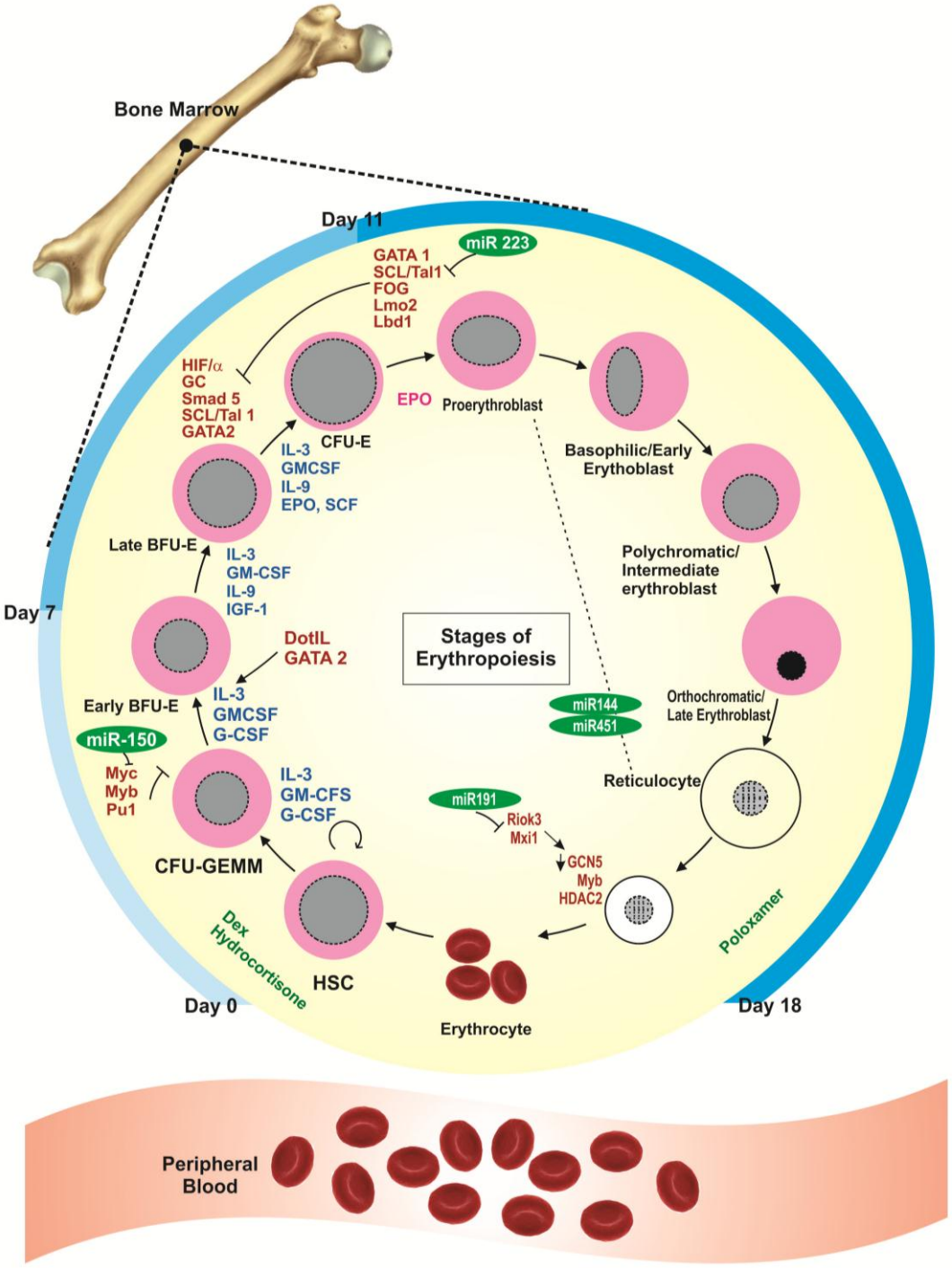


Figure 2.5 | An overview of ex-vivo erythropoiesis.

Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Chemicals and reagents

Common Name	**Company**
Bromophenol blue	Sigma-Aldrich
Cholestrol rich lipid	Sigma-Aldrich
Collagenase	Sigma-Aldrich
CD34 Positive selection kit (Human)	Stem cell Technologies
Dexmethasone	Sigma-Aldrich
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich
Dulbecco's Modified Eagle Medium (DMEM)	Sigma-Aldrich
Ethanol	Sigma-Aldrich
Erythropoieitin	Sigma-Aldrich
Ethylene Diaminetetraacetic acid disodium salt dehydrate (EDTA)	Sigma-Aldrich
Formaldehyde solution	Sigma-Aldrich
Fetal bovine Serum	MP Biomedical
FLT-3	Sigma-Aldrich
Glycerol	Sigma-Aldrich
Glycine	Sigma-Aldrich
Glutraldehyde	Sigma-Aldrich
Hemoglobin powder	Sigma-Aldrich
Histopaque 1077	Sigma-Aldrich
Hydrocortisone	Sigma-Aldrich

Common Name	**Company**
Hydrochloric acid	Sigma-Aldrich
HGDMEM	Sigma-Aldrich
Human AB Plasma	Sigma-Aldrich
IGF-1	Sigma-Aldrich
IL3	Sigma-Aldrich
IMDM	Sigma-Aldrich
Insulin	Sigma-Aldrich
Methanol	Sigma-Aldrich
PBS azide	Sigma-Aldrich
Penicillin streptomycin Mix	Sigma-Aldrich
Potassium chloride	Sigma-Aldrich
Potassium phosphate monobasic	Sigma-Aldrich
RBC lysis buffer	Stem cell technologies
Sodium azide	Sigma -Aldrich
Sodium chloride	Sigma-Aldrich
Sodium Hydroxide	Merck
Sodium Bicarbonate	Merck
Sodium phosphate dibasic	Merck
Sodium phosphate monobasic monohydrate	Sigma-Aldrich

Common Name	Company
Sodium pyrophosphate tetrabasic decahydrate	Sigma-Aldrich
StemSpan Culture media	Stem cell technologies
Transferrin	Sigma-Aldrich
Triton-X 100	Sigma-Aldrich
Tyrpan Blue	Sigma-Aldrich
Thiazolyl Blue Tetrazolium Bromide	Sigma-Aldrich
Trypsin	Sigma-Aldrich

*All other chemicals were of analytical grade.

3.1.2 List of Antibodies

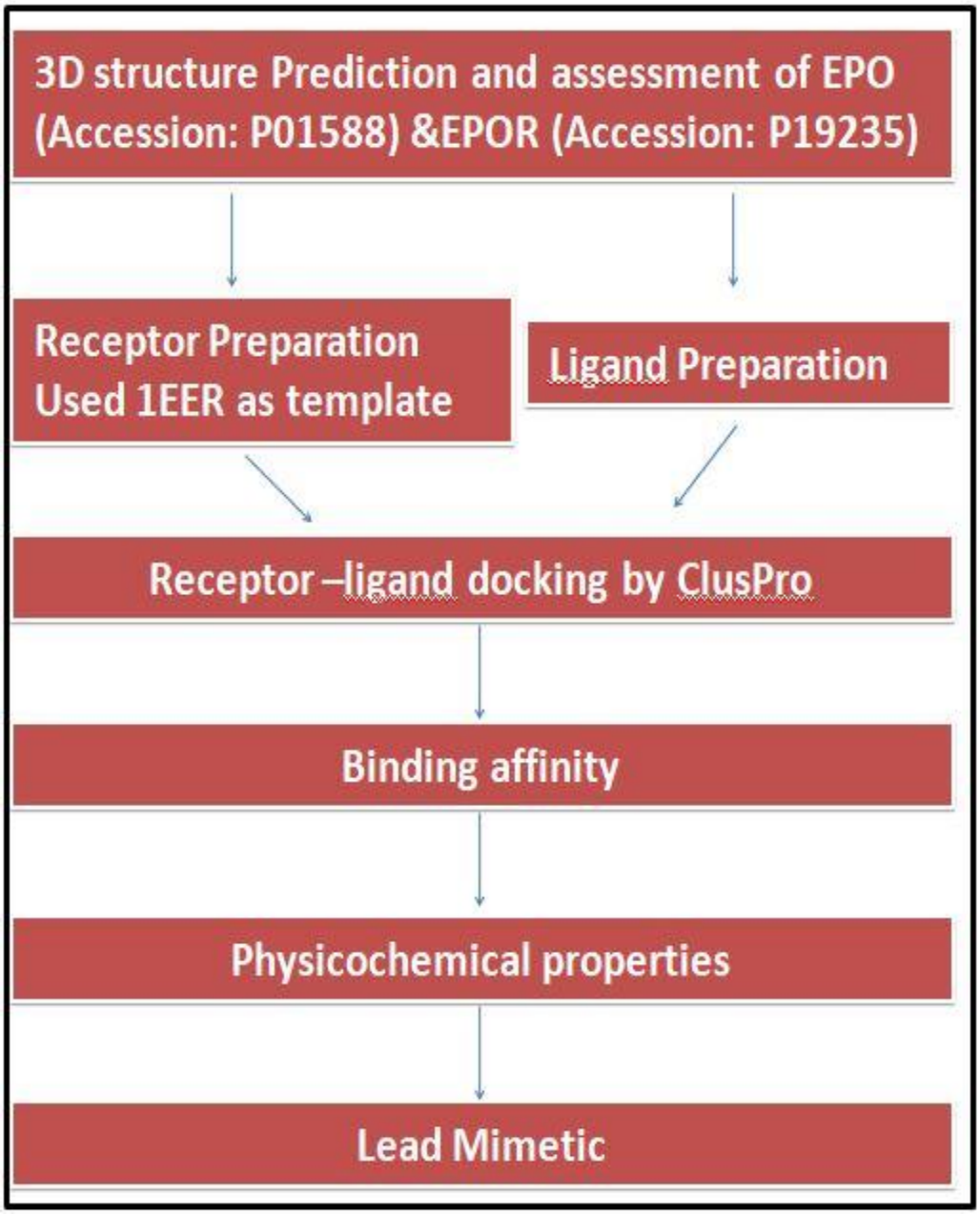
Name of Antibodies	Company
Anti Human CD34+ TUK3 primary Ab	Santacruz
Anti Human CD34+ Qubec end primary Ab	Santacruz
Anti Human CD71+ TUK3 primary Ab	Santacruz
Anti Human CD45+ TUK3 primary Ab	Santacruz
Anti Human CD235a+ primary Ab	Santacruz
Fluorescent Secondary Antibodies (Anti- Mouse IGg)- PE	Santacruz
Fluorescent Secondary Antibodies (Anti- mouseIGg)-FITC	Santacruz

3.1.3 Instruments

Name of instrument	**Company**
Biological safety cabinet	Optics technology
CO2 Incubator	Nuaire
Centrifuge	REMI R8C
Flow cytometer	BD CantoII
Deep freezer (-20C)	Celfrost
Electronic filling device	Borosil
Inverted phase contrast microscope	Magnus
Media filtration assembly	Tarsons
Microcentrifuge	Neutaion
Refrigerator	Samsung
Water bath	Julabo

3.2 Methodology

3.2.1 In-silicotechniques for EPO mimetic designing



3.2.1.1 3D structure Prediction and assessment of EPOR

Erythropoietin receptor binds to EPO from its ECD, so the amino acid sequence of ECD region was retrieved from the UniProt database and searched for 3D structure in protein data bank. In the absence of crystal structure for mammalian EPOR, the 3D structure was generated. The amino acid sequence of EPOR was searched for the

identification of a suitable template through the Basic Local Alignment Search Tool (BLAST). Due to unavailability of a template with higher query coverage and with good identity, the 3D structure was generated through *de novo* method using Iterative Threading ASSEmbly Refinement (I-TASSER) I-TASSER is web-based protein 3D structure prediction tool based on threading approach. It simulates the generated structure and defines its *C*-score, TM-score, and active sites. *C*-score refers to a confidence level of predicted structure of given protein sequences where a high *C*-score (range from −5 to 2) indicates an absolute precise quality of the predicted structure. TM-score is structural assessment parameter in which, a smaller distance between the structures is weighted high. TM-score defines the topology of the structure, and it shows that a score of more than 0.5 ensures a model of absolute topology (Yang et al., 2015). Generated EPOR 3D structure was further evaluated by Swiss model workspace.Swiss server analyzes the various properties viz. solvation energy, torsion angle energy, solventaccessibility, and atom pairwise energy. Estimate of nativeness of these structural properties of the model with comparable features with high-resolution X-ray 3D protein structures is defined by the QMEAN score. Overall the quality of the 3D structure was defined by the QMEAN *Z*-score (Benkert et al., 2008).

3.2.1.2 Mimetic library generation A library was formed on the basis of the interaction sites of EPO-EPOR, known mimetic EMP-EPOR, and by designing analogs of known EPO mimetics through the literature survey. The library was *in silico* matured by changing amino acids at different positions with hydrophobic amino acids and forming various combinations of predicted mimetics. Hydrophobic amino acids were added to the mimetics since they are known to play an important role in EPOR binding and its activation.

3.2.1.3 The receptor Protein Preparation Erythropoietin receptor 3D structure derived through *de novo* method was prepared for molecular docking analysis using the protein preparation wizard (PrepWiz) in the Schrödinger software program (Friesner et al., 2006). Water molecules, unnecessary ligand, unwanted bound ligand, and duplication of any chain were removed, and hydrogen atoms were added to the EPOR protein structure, and the structure is selected for energy minimization and followed by optimization for molecular docking analysis.

3.2.1.4 ligands 3D structure Prediction, The 3D structures of mimetics in the library, were predicted by Pepfold3, a peptide structure prediction server. Pepfold3 is a new 3D structure generation engine. It predicts the structure of peptides based on the Hidden Markov model conformation sampling approach. All mimetics were imported into the workspace of Pepfold3, and the project name was saved and allowed to run. Predicted 3D structures were saved in PDB format.

3.2.1.5 Docking analyses of Mimetic Peptide library with EPOR and Their evaluation Interaction analyses of mimetics with EPOR were done using ClusPro protein-protein molecular docking program. ClusPro server is a rigid docking program based on fast Fourier transformation to generate low energy interaction conformations of a protein-protein complex using the pairwise docking potentials. ClusPro clusters the interaction complexes with low energy and analyzes with semi-definite programming based underestimation program, which identifies the stability of the interaction clusters using the medium-range optimization algorithm. Stable interaction complex is refined using Monte–Carlo simulations (Kozakov et al., 2010). Resulting top docking score mimetic was selected, and its docked complex was analyzed to determine the molecular interactions using the Dimplot program of Ligplot+ (v.1.4.5) module. Dimplot determines the involved molecular interactions

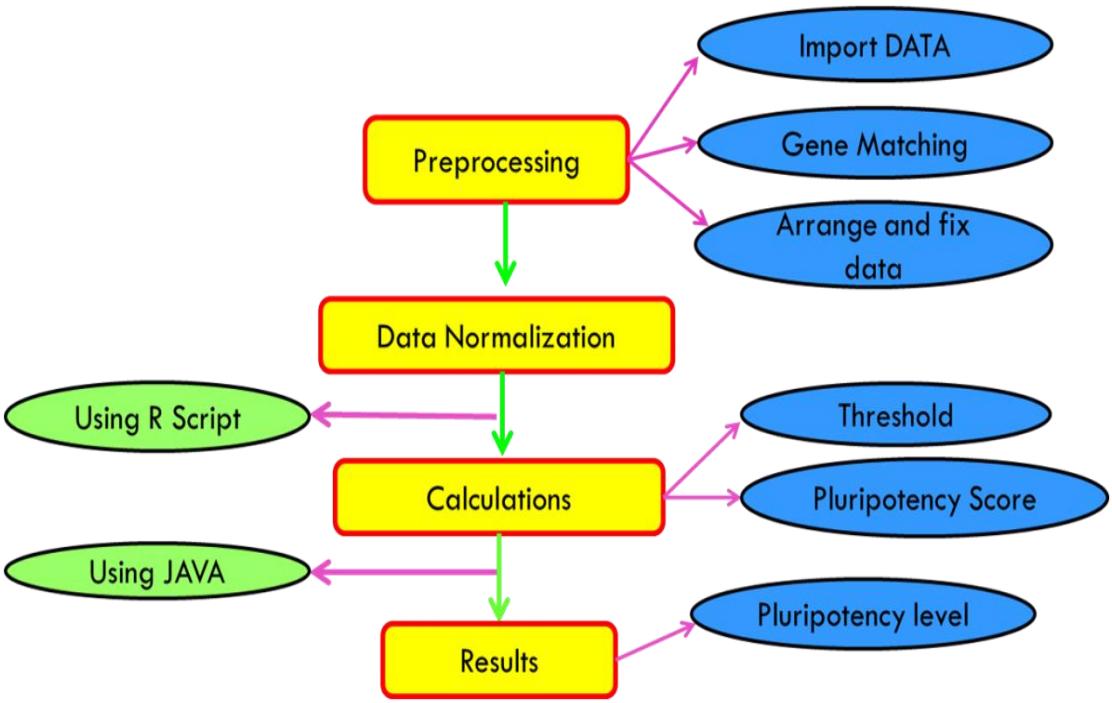
including the hydrogen bonds and hydrophobic interactions, between receptor and ligand interacting complex.

3.2.1.6 Molecular Dynamics simulation of Mimetic-EPOR complex Dynamics and stability of the complex between EPOR and short-listed mimetic were evaluated through the molecular dynamics simulations using MDWeb program. MDWeb is a workspace, which provides standard protocols for molecular dynamicssimulations and to scrutinize the interactions trajectories. The optimized structure of the complex of EPOR and the mimetic shortlisted from the designed mimetic library on the basis of molecular docking was used as input data for molecular simulations. NAMD full MD setup was used for carrying out the simulation. The whole process of simulation includes cleaning of structure, fixing of side chains, the addition of hydrogen atoms, neutralization, the addition of solvent box, and minimization and equilibration of the system to finally achieve the structure prepared by simulation. The equilibration of the system includes heating the solvent to 300 K and reducing the restraints to just protein backbone. These steps are carried out to provide the structure with the necessary conditions to study its dynamics along a span of time. Once the structure is prepared, the water molecules in it are removed to reduce the size of the system to achieve the dry trajectory for plotting various graphs for the analysis of the simulated complex structure. The structural stability, flexibility, and deviations per residues were assessed by the RMSD value.

3.2.1.7 Physicochemical Properties and Biological activity assessment of lead Mimetic Lead mimetic was analyzed for its physicochemical properties and compared with the EMP. Physicochemical properties of mimetic-22 and EMP1including molecular formula, molecular weight, theoretical isoelectric point (pI), the number of negative and positive residues, extinction coefficient, aliphatic index, instability index, and grand average hydropathy (GRAVY) were determined using the Protparam server (Gasteiger et al., 2005).Molecular weight, extinction

coefficient, net charge, and other properties are required to identify the chemical nature of thepeptideand evaluate the binding efficiency of mimetic to a specific receptor. Binding efficiency of mimetic is assessed by potency efficiency index, which is defined by percent inhibition at a given compound concentration divided by its molecular weight. The extinction coefficient illustrates absorbance of light by a protein at a certain wavelength and is used to quantify the formation and concentration of peptides during *in vitro* analyses. Instability index value denotes whether the peptide would be stable *in vitro* or not. The instability index estimates the stability of peptides from a statistical analysis of experimentally defined stable and unstable proteins. Aliphatic index predicts the total volume occupied by aliphatic side chains and indicates the thermostability of protein. Another important parameter that implies the stability of the protein is GRAVY value. It determines hydrophobicity and hydrophilicity of a protein by adding the hydropathy values of each amino acid residue and dividing by the number of amino acids in the protein sequence. A positive value of GRAVY indicates higher hydrophobicity of proteins.

3.2.2 In-silico determination the pluripotency of stem cells



There area total of 175 genes known as marker genes present in any pluripotent cell (Zhao etal. 2012). To validate this conclusion, an interaction network of these marker genes was generated using STRING, and after filtering the resultson the basis ofscore provided by string, top scorer genes were got fetched out.

Next step was to create a method which can identify pluripotency level of any cell. So, for this microarray data files (Control sample) were collected from NCBI's GEO (Gene Expression Omnibus) and *analyzed the results* using GEO2R (a GEO tool). Then, gene expression data file for that particular dataset was downloaded. Aftera preprocessing step which includes Gene matching (Using JAVA) and data arrangement; quantile function was adopted to calculate the Threshold for any cell to be pluripotent. This generated threshold is based upon the Log FC value (Fold Change value) of the particular gene. After getting threshold, another data for test sample was taken and again after repeating the same process (as done for the control sample), after this the log FC value of test sample was checked. Now, our main concern is to check that at what level the cell contains pluripotency? Because the cells which are passing the threshold could either be multipotent or totipotent too. So, to come out of this problem,we have developed a JAVA program. This JAVA program is trained by giving it a particular range for particular key regulator gene in both ESC and iPSC condition. The result of the program is divided into three categories viz. Highly Pluripotent, Partial pluripotent, Low Pluripotent. The Procedure is defined in a stepwise manner below:

Step 1: Extensive literature survey was carried out to identify the genes responsible for the pluripotency of the cell (Total 175 Marker genes were found).

Step 2: To validate our findings STRING tool was used to create an interaction network between all 175 genes, from here we got probability scores which were satisfactory and ensure us their presence.

Step 3: Filtration was done to get the most prominent sets of genes or the key regulators, which later on helpful in determining the level of pluripotency.

Step 4: After validation, our next step is to set up the Threshold value for the cells to pass the pluripotency limit. For this, we have taken microarray data of human's whole genome IPSC's (GSE72078) from GEO datasets of NCBI.

Step 5: Next step is preprocessing of the data by arranging them by matched marker genes with their respective ref IDs and expression values according to the given cell lines(to get matched marker genes JAVA program was developed).

Step 6: After comparison sort the data and normalize it by using any of Median global normalization or Simple Normalization to convert our raw data to appropriate linearly arranged data.

Step 7: R script was used to set threshold by using Quantile function.

Step 8: Now to test our set threshold, a predetermined IPSC test sample (GSE93228) was downloaded,and after preprocessing steps, its pluripotency score was calculated to check whether it is passing the threshold or not (Sample pass).

Step 9: Different graphs were plotted viz. boxplots (to compare normalized and non-normalized data), Clustering graphs, Heatmaps,etc. using R script.

Step 10: Now, for calculating the level of pluripotency we set a range by collecting expression data of various ESCs and IPSCs and further dividing them into different parameters to get promising results.

Step 11: Range was set for key regulators for ESC as well as for IPSCs independently.

Step 12: Three levels were generated,and the ranges were distributed into each level in particular order.

Step 13: Again a JAVA program was developed to determine the level of pluripotency by considering all ranges at different levels.

Step 14: Control and Test sample were analyzed to determine their pluripotency level.

Step 15: To validate our method several other Datasets were checked,and the results were compared with the results of PluriTest (Both results are validating each other).

3.2.3 In-silico techniques for studying the interactions of SOX2 and SOX6

3.2.3.1 Protein interaction pathway analysis using STRING

The protein interaction pathways of Sox2 and Sox6 were predicted using STRING. It is a tool which predicts possible interactions of a protein by data from experiments, text mining, homology, microarray, etc. The interaction pathways help to predict the protein which can interact with specific proteins of interest which in our case are Sox2 and Sox6.

3.2.3.2 Sequence retrieval of Sox2 and Sox6 and their analysis

The sequence of the protein Sox2 and Sox6 were retrieved from NCBI from Accession numbers NP_003097 and AAH47064 respectively. The sequenceswere aligned to protein sequences in the database of PDB using BLAST only to know that there were no suitable templates available to carry homology modeling to predict the 3D structures of both the proteins. So, to predict the tertiary structures of Sox2 and Sox6, modeling was carried out using iTasser (Iterative Threading ASSEmbly Refinement) (Yang etal., 2015)

3.2.3.3 Prediction of 3D structures of Sox2 and Sox6 and their validation

iTasser was used to predict tertiary structures of these proteins; It is an online server to predict the structure and functions of proteins with high quality by using the amino acid sequence. iTasser gives top 5 predicted models from which the top models are the best-predicted model, and hence it was used further. The top models of Sox2 and Sox6 were selected for further study, and their validation was done. Ramachandran plots of both the protein were predicted by RAMPAGE. An overall structure quality of the models was predicted by ERRAT (Colovos et al., 1993).

3.2.3.4 Protein-protein docking

To study the mechanistic interaction of Sox2 and Sox6, the protein-protein interaction of these proteins were carried out using ClusPro. It gives 100 complexes. As a result, the top model of which was further analyzed in our study. The results include the lowest energy of the whole complex and the energy at the center (Comeauet al., 2004).

3.2.3.5Interaction analysis

The interaction of the docked complex retrieved from ClusProwas plotted by DIMPLOT server available in Ligplus (LigPlot+) which is a graphical front-end to LIGPLOT as well. DIMPLOT is used to plots interactions between two proteins across protein-protein or domain-domain interface (Wallace et al., 1995).

3.2.4 Methods for studying various phases of erythropoietic development

3.2.4.1 Isolation of CD34+cells

$CD34^+$ cells were isolated by the use of the Spectra Optia Apheresis System after five days of G-CSF administration i.e. mobilization of CD34+ into peripheral blood. Cells were further purified by density gradient cell separation to remove the traces of Red blood cells, platelets, and plasma.

3.2.4.2 Cultivations of CD34+cells

The cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Sigma) supplemented with Insulin (10 µg/ml), Transferrin (330 µg/ml) and Human serum from male AB plasma (5%) (Sigma).Final pH of media was adjusted to 7.4. The medium was filtered using membrane filter assembly (0.22 µm) syringe. The sterility was routinely checked by incubating the medium at 37ºC for 72h. The medium was thereafter stored at 4ºC.For expansion 10^6/mL $CD34^+$ cells were cultured in above mentioned media in the presence of 10^{-6} M hydrocortisone (Sigma),100 ng/mL SCF (Gene direx), 5 ng/mL IL-3 (Gene direx), and 3 IU/mL EPO (Invitrogen). On day 4, 1 volume of cell culture was diluted in 4 volumes of fresh medium containing SCF, IL-3, Epo, and hydrocortisone. After that, the culture medium containing growth factors was renewed twice a week. Parallel to this in another culture well the same number of CD34+ cells were seeded without any growth factors. The cultures were maintained at 37°C in 5% CO_2 in the air.

3.2.4.3 Cell staining and morphology studies

Cells were stained with *Giemsa Stain*, Modified *Solution* (Sigma) for morphologic analysis. For Giemsa staining, 2-3 × 10^5 cells were washed in PBS (pH 7.4) and incubated with fiveµL of Giemsa in an Eppendorf tube for 5 minutes. The cells were then centrifuged at 1000 rpm for 1 minute,and the pelletwas examined under a microscope.

3.2.4.4Cell viability determination

10µl of 0.4% Trypan blue stain solution was added to 100µl of cells suspension. Cells were loaded on the chamber underneath glass cover slip on the hemocytometer. The solution was taken up by capillary action,and a clean cover slipwas carefully placed.

The live cells were counted as unstained and dead as blue stained cells in four different squares of hemocytometer using a cell counter under the inverted microscope. Finally, cell viability for each ml was calculated by using the formula: % Cell viability= (number of live cells/total number of cells) x100. Similarly, cell viability was determined for each set of the experiment at defined time periods in the triplicate experimental sets.

3.2.4.5 Flow cytometric analysis

The CD34 + cells obtained from apheresis were enumerated by BD CD34+ stem cell enumeration kit. In BD FACSCanto II flow cytometer BD FACS™ 7-color setup beads with BD FACSCanto clinical software v2.4 were used for analysis. An additional optimization for PE–%7- AAD (viaprobe) was done before acquisition.100 µL of the cells obtained were stained for flow enumeration and the dilutions were made as per the WBC count of each sample Low and high process controls with known assayed CD34+ absolute count values were run just before the sample to confirm staining and system integrity. The cells were analyzed for expression CD34 and glycophorin-A prior to differentiation and after differentiation. Each time cells were labeled with unconjugated primary anti–CD34 (TUK3), labeled with FITC conjugated secondary antibody and anti-glycophorin A, labeled with PE-conjugated secondary antibody for phenotyping.

3.3 Statistical Analysis

All experiments were repeated at least three times and data obtained were expressed as mean±standard deviation. The variation between the control and test groups was estimated. Significant variation between these groups was determined by one-way ANOVA (one-way analysis of variance) followed by BonFerroni test. $p<0.05$ was considered significant across the entire experimental groups.

Chapter 4

Results and Discussion

4.1 EPO mimetic designing

4.1.1 Binding Site Identification of EPO-EPOR Complex

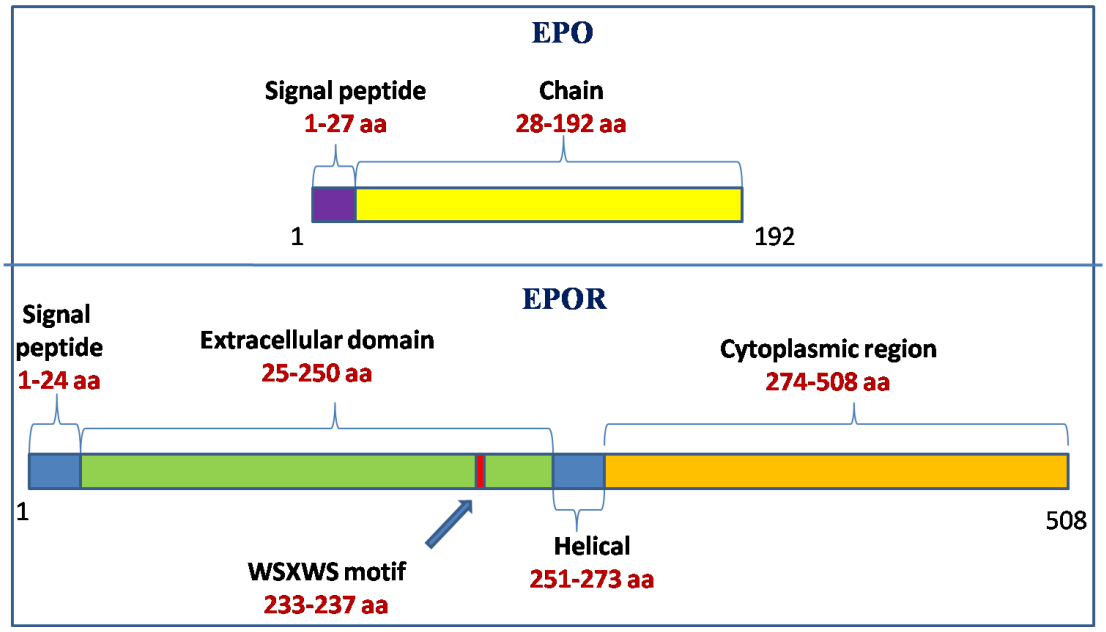


Figure 4.1: Line diagram showing the signal peptide and chain peptide of both EPO and EPOR. The WSXWS motif in the extracellular domain of the EPOR is the key player in binding of EPO.

EPOR interacts with EPO at two different sites- site 1 and site 2. First site of EPOR is hydrophobic in nature, mainly due to Phe93 which is responsible for nonpolar interactions; its side chain also consisted of hydrophilic amino acids which were involved in the interaction with the ligand (Figure 4.2). Identified interacting residues from the 3D structure as shown in the Figure 2 were - Leu59, Glu60, Asp61, Thr90, Ser91, Ser92, Phe93, Val94, Pro95, Leu96, Ileu113, His114, Ileu115, Asn116, Ser152, His153, Glu202, Pro203, Ser204, Phe205.

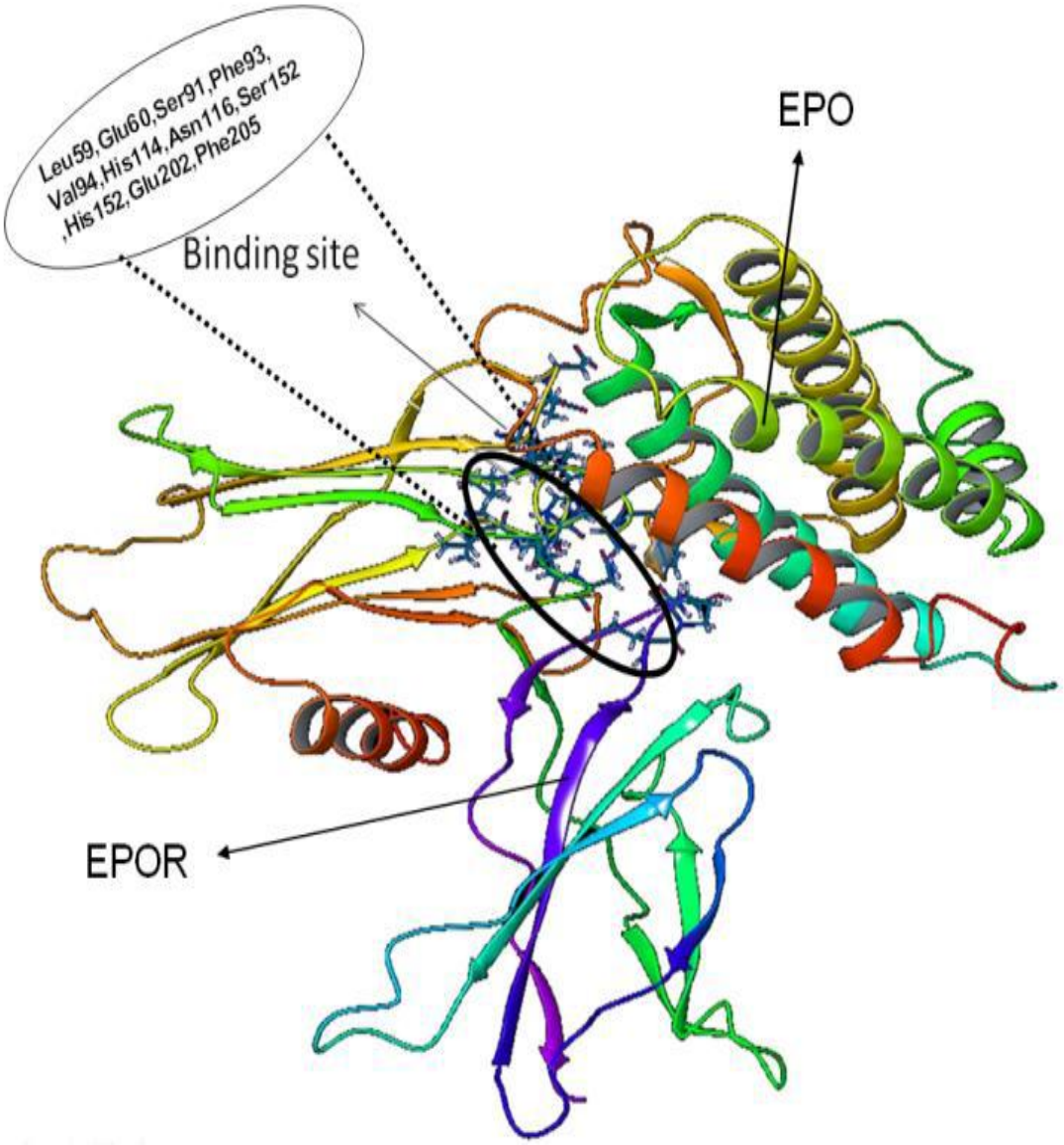


Figure 4.2: ***3D structure of the EPO-EPOR complex. EPOR Chain-A is bound to EPO protein, which is showing the interacting amino acid residues responsible for the formation of complex. Hydrophobic amino acids are responsible for nonpolar interaction in the complex. The side chains are stabilized by other hydrophilic amino acid residues.***

4.1.2 Receptor protein preparation

EPOR had two chains A and chain B. Both chains had similar sequences and for docking analysis duplicated chain was deleted and chain B was deleted and EPOR-

chain A was prepared. ECD region (~25-250) of EPOR is responsible for binding with EPO. EPOR chain A prepared for docking analysis by removing the duplicate chain B, water molecules and any other unwanted ligands from the PDB structure of EPOR.

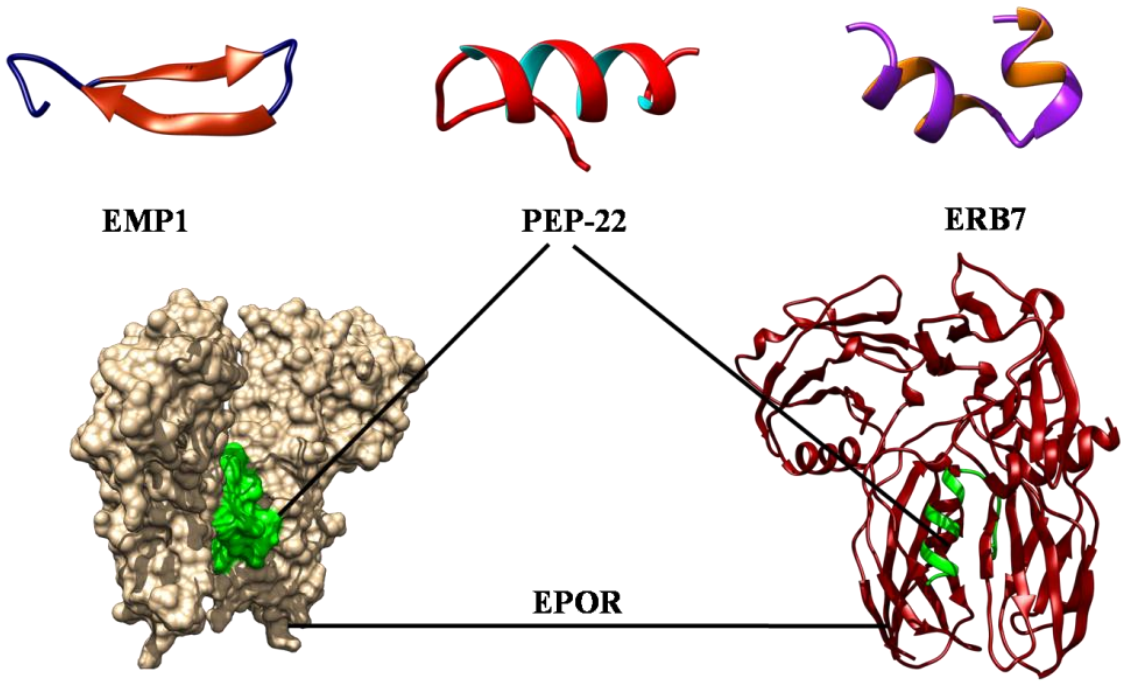
4.1.3 Protein protein Docking

The mimetic library was designed on the basis of binding sites of EPO to EPOR, EMP1 binding sites to EPOR, analogs of reported mimetics (EMP1 and ERB7), and by introducing the hydro-phobic residues at specific positions of mimetics to enhance the binding affinity toward the EPOR. Library was designed for peptides only, and it consisted of more than 40 peptides of all similar structures coordinates with standard amino acids only. Some of the mimetics from designed library with their binding docking score have been shown in **Table 4.1**. For library designing and comparative analyses, EMP1 was docked with EPOR. EPOR receptor was uploaded, and its unstructured terminal residues were removed, and then EMP1 was uploaded as a ligand. ClusPro runs PIPER rigid body docking program and rotates the mimetic ligand with 70,000 rotations at each grid point in *x*, *y*, *z*-axis about the EPOR receptor 3D grid with spacing 1.0 Å. Then using the clustering techniques to find near-native conformations along with eliminating the non-native clusters, the 1000 best energy conformations with the lowest score were clustered and among them, 30 largest clusters were refined by minimizing the Charmm energy of the complexes. The clustering of the poses starts with the lowest energy pose and grouping all poses within 9 Å. ClusPro resulted in a binding score (lowest energy) of −896.0 kcal/mol and center binding energy score of −870.4 kcal/ mol for EMP1.

Sr. No.	*Oligopeptides*	*Size of peptide*	*Binding energy score in kcal/mol (lowest energy)*	*Binding energy score in kcal/mol (center)*
Known mimetic				
EMP1	GGTYSCHFGPLTWVCKPQGG	20	-896.0	-870.4
ERB7	DREGCRRGWVGQCKAWFN	18	-852.6	-750.2
Known3	YSCHFGPLTW	10	-797.0	-775.9
Known4	YSCHFGPLTWVCK	13	-839.0	-743.9
Predicted mimetic				
Pep1	GGCRRNEAEGG	11	-540.0	-451.7
Pep 2	CCRRNEAEC	09	-491.7	-469.5
Pep 3	RRNEYYAYS	09	-705.0	-689.4
Pep 4	GGRREASAHYGG	12	-580.7	-515.1
Pep 5	GGRNEASHYCGG	12	-548.6	-494.0
Pep 6	CRRNEASHY	09	-600.6	-568.3
Pep 7	CRNEAESHY	09	-635.7	-520.6
Pep 8	CYNSYHYLC	09	-640.3	-638.4
Pep 9	CCYNSAHYLC	10	-639.8	-585.5
Pep 10	YYNASYHLL	09	-642.6	-585.1
Pep 11	CYYNNASHL	09	-635.3	-542.9
Pep 12	GGCYSCHLCYGG	12	-622.3	-561.1
Pep 13	GGCCYSCAHLGG	12	-768.7	-652.9
Pep 14	CCYSCNNASHL	11	-656.1	-550.6
Pep 15	CRRNAECLTW	10	-750.6	-620.4
Pep 16	CRRNEAERRNC	12	-610.8	-504.8

Pep 17	RREAERRSHL	10	-569.8	-596.8
Pep 18	CRREAESSYC	10	-573.3	-524.4
Pep 19	CRNEAESHHYCYYNNASHL	19	-782.4	-681.9
Pep 20	CYYNNASHLRREAERRSHL	19	-781.9	-735.3
Pep 21	CRNEAESHYCCYNSAHYLC	19	-769.5	-726.8
Pep 22	ILVGTLIVLIPVLIVLVFLYWQ	22	-994.7	-841.7
Pep 23	GGCRNEAESHHYCYYNNASHLGG	23	-854.8	-651.4

Table 4.1 | ***Known mimetic EMP1 and ERB7 and designed library molecular docking with EPOR results: first EMP1 of size 20 amino acids and EPOR were docked using the clusPro and resulted in energy score of −896.0 kcal/mol and compared with peptides from designed library. Pep-22 showed the high binding energy score −994.7 kcal/mol to EPOR than EMP1 and ERB7***



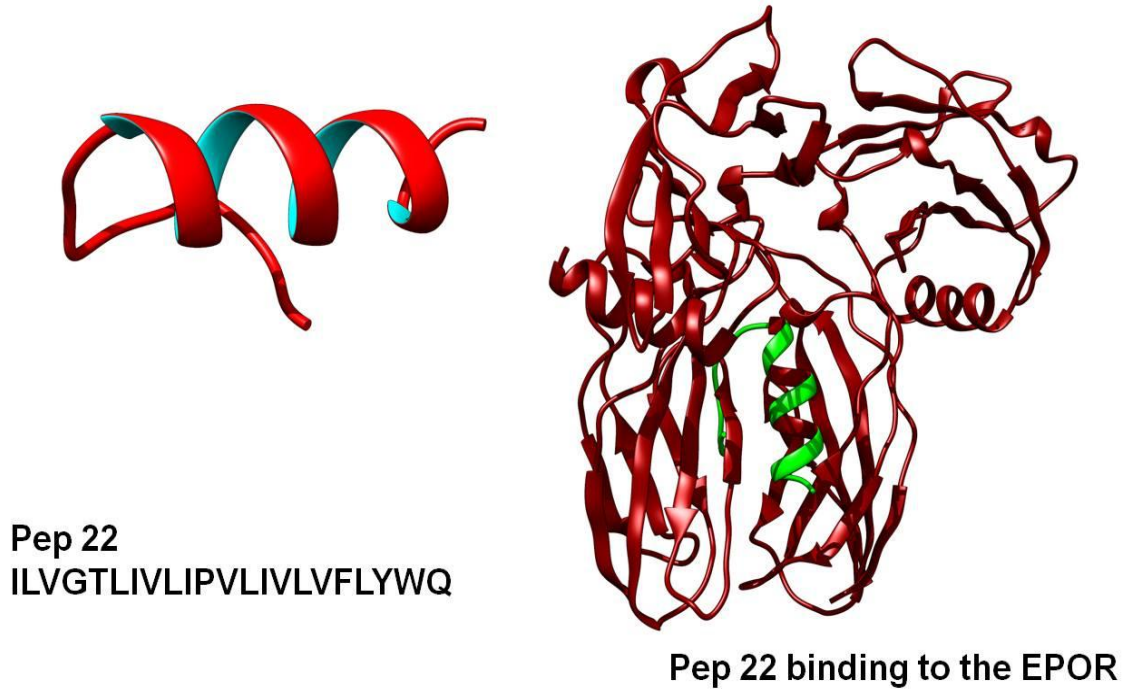


Figure 4.3: Pep-22 showed the high binding energy score −994.7 kcal/mol to EPOR than known mimetics

4.1.4 Molecular dynamics simulation

Dynamic nature of EPOR–pep-9 interactions was studied through the molecular dynamics simulation for 10 ns. During the simulation, the frames of structural deviations were captured of the complex. EPOR–pep-9 complex ran for three frames and showed the stable conformations of interactions. Next, RMSD value was calculated and found to be 0.091 Å, which suggests the less deviation per residue of the interaction complex and stability till the end of the simulation (**Figure 4.4**).

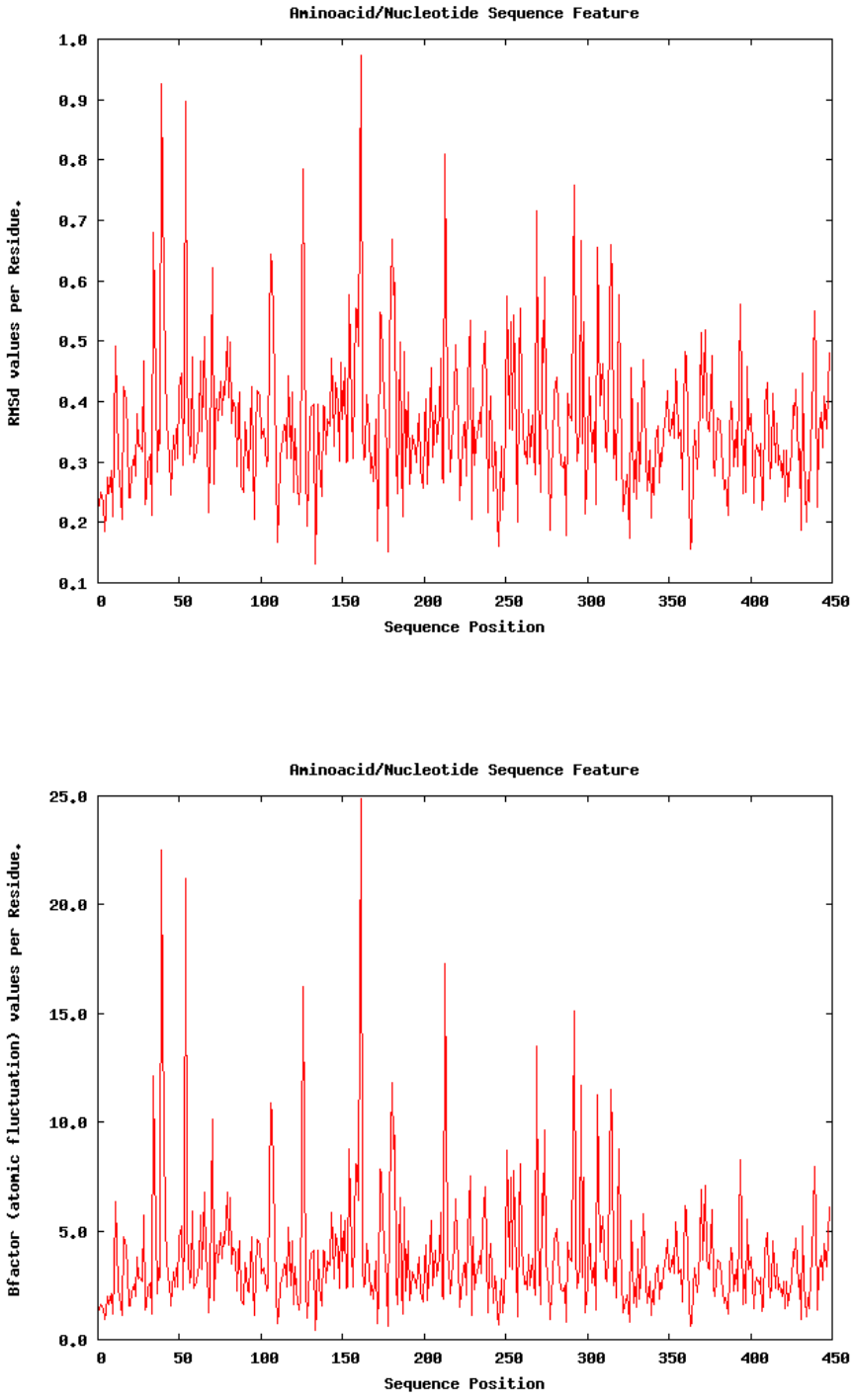


Figure 4.4 Molecular dynamics simulation plot of the EPOR–pep22 complex. The plot depicts the structural deviation per residue, and RMSD value and the B factor B_{eq} (atomic fluctuation) per residue, the value represents isotropic displacement of atoms that were described by anisotropic ADPs during refinement.

4.2Determining the pluripotency of stem cells by in-silico method

The list of marker genes present in any stem cell which decides the pluripotency level of an undifferentiated cell was created. These genes were isolated by using different techniques viz. MACS (Magnetic Cell Sorting Technique) and FCM (flow cytometry) technique which is one of the most effective cell isolating methods.

SUV39H1	SMAD1	LECTINS	KLF4	CD57	CD86	STELLA
SUV39H2	SMAD5	CD133	NANOG	CD58	CD87	TRA-2-54
EHMT2	SMAD8	CD96	REX1	CD59	CD88	CD45
EHMT1	SMAD4	CD34	UTF1	CD60	CD89	CD56
SETDB1	SMAD2	CD38	ZFX	CD61	CD90	CD85
RING1B	SMAD3	CD45	TBN	CD62	CD326	ECSA
EZH2	BETA CATENIN	CD46	FOXD3	CD63	CD9	TM4SF
EED	SSEA1	CD47	HMGA2	CD64	CD55	TRA-2-49
SUZ12	CD15	CD48	NAC1	CD65	CD59	OCT4
DICER1	SSEA3	CD49	GCNF	CD66	CD24	SOX2
DNMT1	SSEA4	CD50	NR6A1	CD67	CD44	CD54
DNMT3a	CD324	CD51	STAT3	CD68	SATA3	CD55
DNMT3b	CD90	DRAP27	LEF1	CD69	NCA1	CD83
DNMT3L	CD117	P24	TCF3	CD70	ALDH1	CD84
CXXC1	CD326	CKIT	SALL4	CD71	MUSASHI-1	DPPA3
BRG1	CD9	SCFR	FBXO15	CD72	LgR5	CD82
SMARCA4	CD29	THY-1	ECAT11	CD73	PSCA	CD53
SMARCA5	CD24	TRA-1-60	FLJ10884	CD74	DCAMKL-1	OCT3
SMARCB1	CD59	TRA-1-81	L1TD1	CD75	TIM3	MRP1
SMARCC1	CD133	FRIZZLED5	ECAT1	CD76	BRCA1	DPPA2
MBD3	CD32	SCF	ECAT9	CD77	SDF1	
HIR A	CD49F	C-KIT	GDF3	CD78	CXCR4	
DPPA5	CD96	TDGF-1	TGF Beta	CD79	PSCA	
ESG1	HAS	CRIPTO	TCF1	CD80	CD96	
DPPA4	PROTECTIN	POU5F1	CD52	CD81	CD44	

Table 4.2: List of 175 signature markers used for identifying Stem cells.

To validate our findings of marker genes, we create an interaction network of all these above genes to check whether they are interaction partners or not. This result assures us that the given marker genes are interacting partners of key regulatory genes, i.e., OCT-4, NANOG, SOX2, KLF4 and are showing great interaction score with each other, which confirms us the presence of all these genes in the pluripotent cell. STRING provided us with the nodes which are commonly interacting with most of the genes and are having highest scores, i.e., POU5F1, NANOG, KLF4, SOX2, SALL4, SMAD2, SMAD4, and DPPA4. Another network was plotted using the resultant common genes which we got from the parent network.

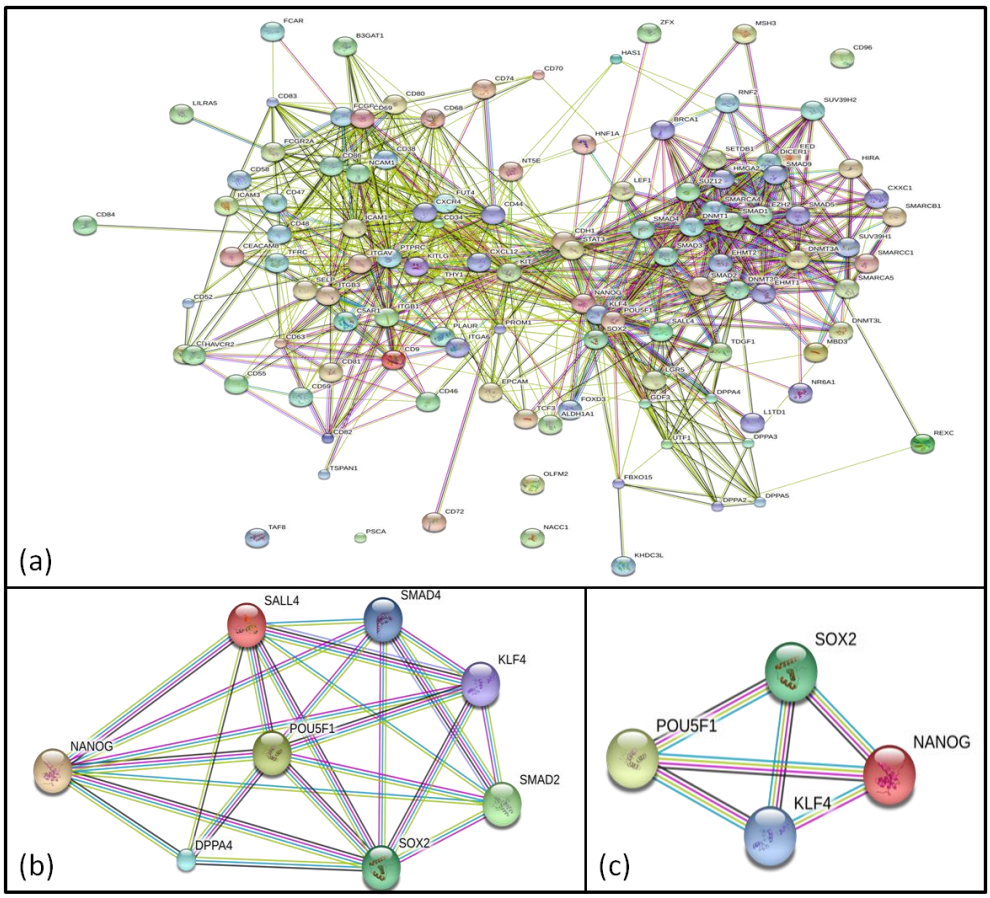
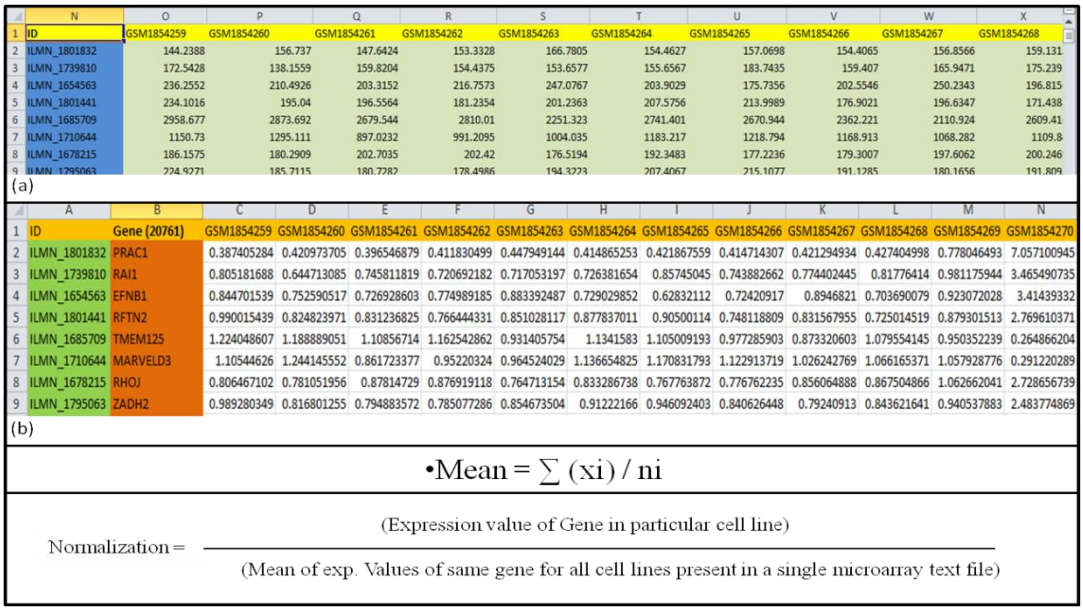


Fig-4.5: (a) Representation of Interaction network between various 175 Pluripotency Marker Genes identified by using STRING, each sphere (node) represents the particular gene. (b) A more specific STRING network is showing important genes responsible for pluripotency. (c) The final network with four key factors namely SOX2, NANOG, POU5F1, and KLF4.

We found that POU5F1, SOX2, NANOG, KLF4 are most commonly interacts. Thusan interaction network using STRING consisting of these four genes was plotted. Finally, genes with the most number of interactions and with highest no. of scores were obtained. By this we inference that the presence of these three genes and their expression values in microarray data could be the defining factor for the level of pluripotency in any cell. Now, our next step is to initially find out the state of any cell, i.e., whether it is pluripotent or not. For this, our first step is to determine the threshold for the genes of the cells. The cell has to pass this threshold before evaluating further. For this, we had taken Microarray data because this is the only way to check the expression value of the genes. The data we take is in TEXT file format, because of its availability. Firstly, the Microarray data containing Reference ID (Different for different types of analysis viz. "ILMN_xxxx" for Illumina analyzed data, "xxxx_s_at" for Affymetrix Data, "xxxxx" for Agilent Data), LogFC (Fold Change value showing Upregulation(+) and downregulation(-) of genes), Gene symbol etc. was downloaded for whole genome of human iPSC and ESC cell lines (GSE72078)from GEO datasets of National Centre for Biotechnology Information (NCBI) by analyzing with GEO2R (A GEO Tool available for visualization of Microarray data along with other relevant calculated statistical parameters). Now downloaded data was pasted into excel sheet with their respective gene expression values taken from series matrix file data. The data was then compared and matched with the expression values of available marker genes.



ID	GSM1854259	GSM1854260	GSM1854261	GSM1854262	GSM1854263	GSM1854264	GSM1854265	GSM1854266	GSM1854267	GSM1854268
ILMN_1801832	144.2388	156.737	147.6424	153.3328	166.7805	154.4627	157.0698	154.4065	156.8566	159.131
ILMN_1739810	172.5428	138.1559	159.8204	154.4375	153.6577	155.6567	183.7435	159.407	165.9471	175.239
ILMN_1654563	236.2552	210.4926	203.3152	216.7573	247.0767	203.9029	175.7356	202.5546	250.2343	196.815
ILMN_1801441	234.1016	195.04	196.5564	181.2354	201.2363	207.5756	213.9989	176.9021	196.6347	171.438
ILMN_1685709	2958.677	2873.692	2679.544	2810.01	2251.323	2741.401	2670.944	2362.221	2110.924	2609.41
ILMN_1710644	1150.73	1295.111	897.0232	991.2095	1004.035	1183.217	1218.794	1168.913	1068.282	1109.8
ILMN_1678215	186.1575	180.2909	202.7035	202.42	176.5194	192.3483	177.2236	179.3007	197.6062	200.246

(a)

ID	Gene (20761)	GSM1854259	GSM1854260	GSM1854261	GSM1854262	GSM1854263	GSM1854264	GSM1854265	GSM1854266	GSM1854267	GSM1854268	GSM1854269	GSM1854270
ILMN_1801832	PRAC1	0.387405284	0.420973705	0.396546879	0.411830499	0.447949144	0.414865253	0.421867559	0.414714307	0.421294934	0.427404998	0.778046493	7.057100945
ILMN_1739810	RAI1	0.805181688	0.644713085	0.745811819	0.720692182	0.717053197	0.726381654	0.85745045	0.743882662	0.774402445	0.81776414	0.981175944	3.465490735
ILMN_1654563	EFNB1	0.844701539	0.752590517	0.726928603	0.774989185	0.883392487	0.729029852	0.62832112	0.72420917	0.8946821	0.703690079	0.923072028	3.41439332
ILMN_1801441	RFTN2	0.990015439	0.824823971	0.831236825	0.766444331	0.851028117	0.877837011	0.90500114	0.748118809	0.831567955	0.725014519	0.879301513	2.769610371
ILMN_1685709	TMEM125	1.224048607	1.188889051	1.10856714	1.162542862	0.931405754	1.1341583	1.105009193	0.977285903	0.873320603	1.079554145	0.950352239	0.264866204
ILMN_1710644	MARVELD3	1.10544626	1.244145552	0.861723377	0.95220324	0.964524029	1.136654825	1.170831793	1.122913719	1.026242769	1.066165371	1.057928776	0.291220289
ILMN_1678215	RHOJ	0.806467102	0.781051956	0.87814729	0.876919118	0.764713154	0.833286738	0.767763872	0.776762235	0.856064888	0.867504866	1.062662041	2.728656739
ILMN_1795063	ZADH2	0.989280349	0.816801255	0.794883572	0.785077286	0.854673504	0.91222166	0.946092403	0.840626448	0.79240913	0.843621641	0.940537883	2.483774869

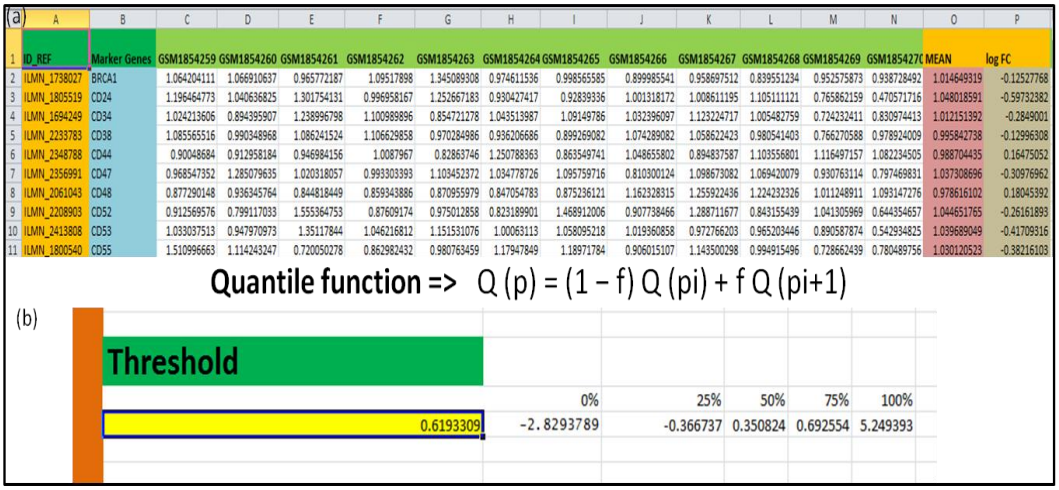
(b)

$$\bullet \text{Mean} = \sum (xi) / ni$$

$$\text{Normalization} = \frac{\text{(Expression value of Gene in particular cell line)}}{\text{(Mean of exp. Values of same gene for all cell lines present in a single microarray text file)}}$$

Fig-4.6 (a) Table is showing Non-Normalized Microarray data (GSE72078) in excel sheet collected from GEO datasets. (b) Normalized microarray data (using Simple normalization function).

After this, we matched our 175 Marker genes with the existing list of NCBI and downloaded expression dataset's gene list by using JAVA program (specially designed for a finding of no. of marker genes present in the microarray data.



(a)

ID_REF	Marker Genes	GSM1854259	GSM1854260	GSM1854261	GSM1854262	GSM1854263	GSM1854264	GSM1854265	GSM1854266	GSM1854267	GSM1854268	GSM1854269	GSM1854270	MEAN	log FC
ILMN_1738027	BRCA1	1.064204111	1.066910637	0.965772187	1.09517898	1.345089308	0.974611536	0.998565585	0.899985541	0.958697512	0.839551234	0.952575873	0.938728492	1.014649319	-0.12527768
ILMN_1805519	CD24	1.196464773	1.040636825	1.301754131	0.996958167	1.252667183	0.930427417	0.92839336	1.001318172	1.008611195	1.105111121	0.765862159	0.470571716	1.048018591	-0.59732382
ILMN_1694249	CD34	1.024213606	0.894395907	1.238996798	1.100989896	0.854721278	1.043513987	1.09149786	1.032396097	1.123224717	1.005482759	0.724232411	0.830974413	1.012151392	-0.2849001
ILMN_2233783	CD38	1.085565516	0.990348968	1.086241524	1.106629858	0.970284986	0.936206686	0.899269082	1.074289082	1.058622423	0.980541403	0.766270588	0.978924009	0.995842738	-0.12996308
ILMN_2348788	CD44	0.90048684	0.912958184	0.946984156	1.0087967	0.82863746	1.250788363	0.863549741	1.048655802	0.894837587	1.103556801	1.116497157	1.082234505	0.988704435	0.16475052
ILMN_2356991	CD47	0.968547352	1.285079635	1.020318057	0.993303393	1.103452372	1.034778726	1.095759716	0.810300124	1.098673082	1.069420079	0.930763114	0.797469831	1.037308696	-0.30976962
ILMN_2061043	CD48	0.877290148	0.936345764	0.844818449	0.859343886	0.870955979	0.847054783	0.875236121	1.162328315	1.255922436	1.224232326	1.011248911	1.093147276	0.978616102	0.18045392
ILMN_2208903	CD52	0.912569576	0.799117033	1.555364753	0.87609174	0.975012858	0.823189901	1.468912006	0.907738466	1.288711677	0.843155439	1.041305969	0.644354657	1.044651765	-0.26161893
ILMN_2413808	CD53	1.033037513	0.947970973	1.35117844	1.046216812	1.151531076	1.00063113	1.058095218	1.019360858	0.972766203	0.965203446	0.890587874	0.542934825	1.039689049	-0.41709316
ILMN_1800540	CD55	1.510996663	1.114243247	0.720050278	0.862982432	0.980763459	1.17947849	1.18971784	0.906015107	1.143500298	0.994915496	0.728662439	0.780489756	1.030120523	-0.38216103

Quantile function => $Q(p) = (1 - f)\,Q(pi) + f\,Q(pi+1)$

(b)

Fig-4.7 (a) Table is showing marker genes with reference IDs and their respective expression values in each cell line. Also, Calculated Mean and log FC values are shown. (b) The calculated Threshold value for detection of pluripotency using Quantile Normalization method through R script (Threshold = 0.6193309).

Total 68 Marker Genes are matched with the Genes of Microarray Data of GSE72078 cell lines. After normalization we took the data for prediction of Threshold using Quantile function through R Studio, this approach gives five different quantile calculated values including min. And max.; after taking the mean for these values, we got our Threshold, i.e., 0.62, which implies that if any cell passes this threshold, then it could say to be pluripotent. Now, the next step is to check the pluripotency status for a sample dataset (Test Sample). For this, we had taken a colon IPSC Cell line. The microarray data was downloaded for Colon IPSC's (GSE93228- Cell lines iPSC CRL1831 (induced pluripotent stem cells) derived from normal colon CRL1831 cells in 3D cell culture conditions and subjected to ionizing radiation doses) and then after arranging and preprocessing the data we check whether the cell lines are Stem cell lines or not. If they all are stem cell lines then we simply check their pluripotency score by taking the quantile normalization of their Log FC value, but if the data consist of both differentiated and undifferentiated cell lines then we have to take mean of each cell line's expression values and then match with the Threshold limit to check whether they are passing the set Threshold value or not. If the resultant score is less than the threshold, then that cell could be either Unipotent, totipotent, multipotent or differentiated somatic cell line.

	ID	GENE_SYMBOL	GSM2448894	GSM2448895	GSM2448896
2	A_23_P34915	ATF3	0.07570648	0.14384651	0.08829689
3	A_23_P155890	NAA11	0.16908455	0.19882202	0.12617302
4	A_24_P246173	MYO9B	0.36398697	0.4689827	0.34682274
5	A_23_P146077	ZNF395	-0.020715714	-0.045152664	-0.020601273
6	A_32_P175739	HK2	0.25235748	0.37810516	0.20498085
7	A_33_P3419785	BNIP3	-0.0845108	-0.082969666	-0.05378151
8	A_33_P3350863	RETN	0.13268661	0.1523037	0.05083847
9	A_23_P214080	EGR1	0.3584175	0.5151024	0.41786766

(a)

	ID_REF	MATCHED GENES	GSM2448894	GSM2448895	GSM2448896	GSM2448897	GSM2448898	GSM2448899	MEAN	S.D.
2	A_23_P207400	BRCA1	1.38351364	1.376838746	0.38318811	0.386125862	1.44940836	1.020925282	1	0.499758794
3	A_23_P34676	CD24	0.744272163	1.607417099	0.894782913	1.011844222	0.592505176	2.29061474	1.190239386	0.641896483
4	A_23_P23829	CD34	-0.487966744	-0.064981111	0.228951986	-0.454359769	-0.21306606	0.714796164	-0.046104256	0.457115519
5	A_23_P167328	CD38	0.081032442	0.110366682	-0.048461156	0.63797343	-0.739991542	0.225116369	0.044339371	0.450460792
6	A_33_P3294509	CD44	0.101691245	0.504888381	0.508086052	0.356673482	-0.466319512	0.434216842	0.239872748	0.377196325
7	A_23_P35230	CD46	0.71376834	0.648747814	1.088147118	0.979391078	0.293287274	0.96256396	0.780984264	0.292074745
8	A_23_P6935	CD47	-0.759760354	-1.632580949	0.288360808	0.522507044	-0.612502401	-2.145987339	-0.723327198	1.043065612
9	A_32_P175934	CD48	0.037592248	0.105475673	-0.092192765	-0.156811571	0.701580185	-0.39111532	0.034088075	0.369822021

(b)

Fig-4.8: (a)Table Showing Test Sample consisting of GENE symbol with their expression values in respective cell lines of Colon IPSC (total 6 IPSC cell lines are taken after neglecting somatic cell lines data). (b)Table after matching our Marker genes with the Genes of Microarray Data of GSE93228 cell lines we obtained 71 matched entries by using JAVA developed program.

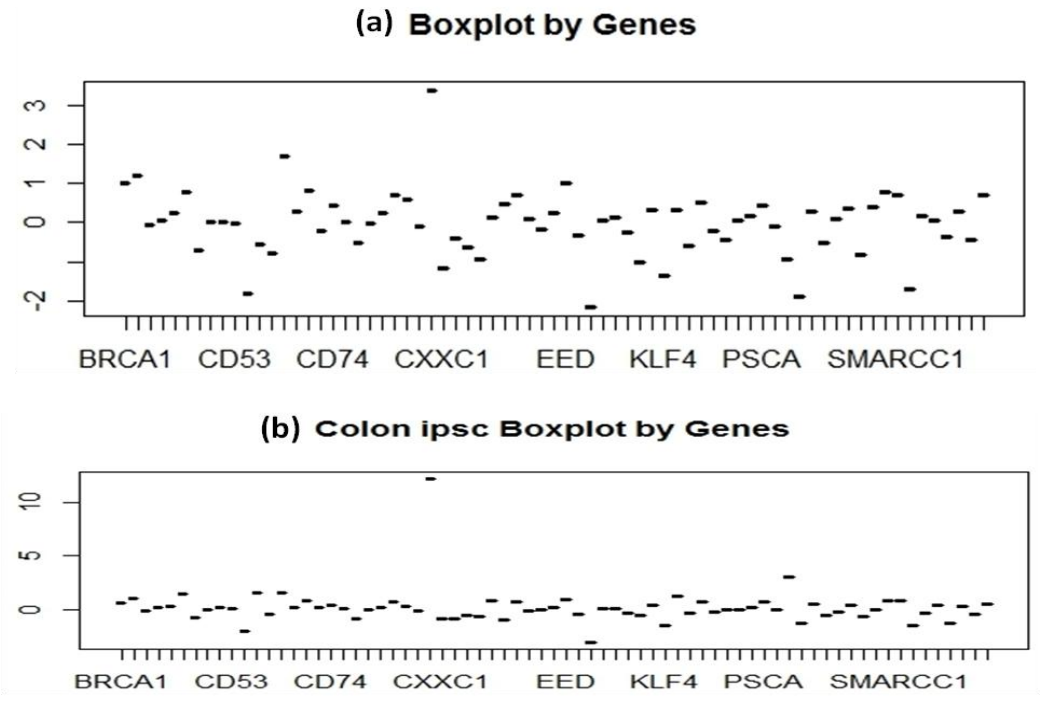


Fig-4.9: (a) Boxplot is showing non-normalized gene expression data of GSE93228, depicting discreteness of the values. (b) Boxplot showing Normalized gene expression values in a linear manner

(a)

5						Threshold
6						
7	0%	25%	50%	75%	100%	
8	-3.0712	-0.47081	0.046304	0.496912	12.16558	1.8333558
9						

(b)

	A	B	C	D	E	F	G
1							Threshold
2							
3	0%	25%	50%	75%	100%		
4	-0.0469868	-0.0469868	-0.0469868	-0.0469868	0.7459761		0.11160578
5							

Fig-4.10: (a) The Pluripotency score is calculated again using R Script as earlier, and it passed the Threshold value. Hence we can say that the Cell line for Microarray data of GSE 93228 sample is pluripotent. (b)After the calculation of pluripotency score using Quantile function we deliberately checked mast cell data which could not pass the Threshold value.

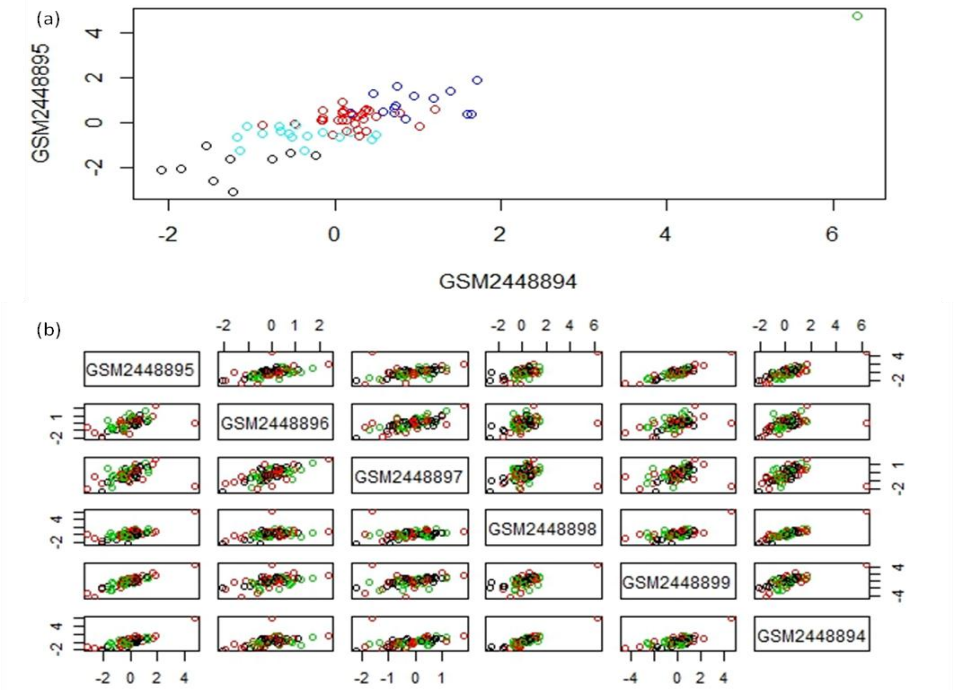


Fig4.11: (a) Graph is showing clustered cell line from test sample by Gene expression data by using K-Means clustering through R Script for the development of Hierarchical clustering in Heatmap. (b)Figure showing K-Means clustered data of all six cell lines of test data sets created using R Script.

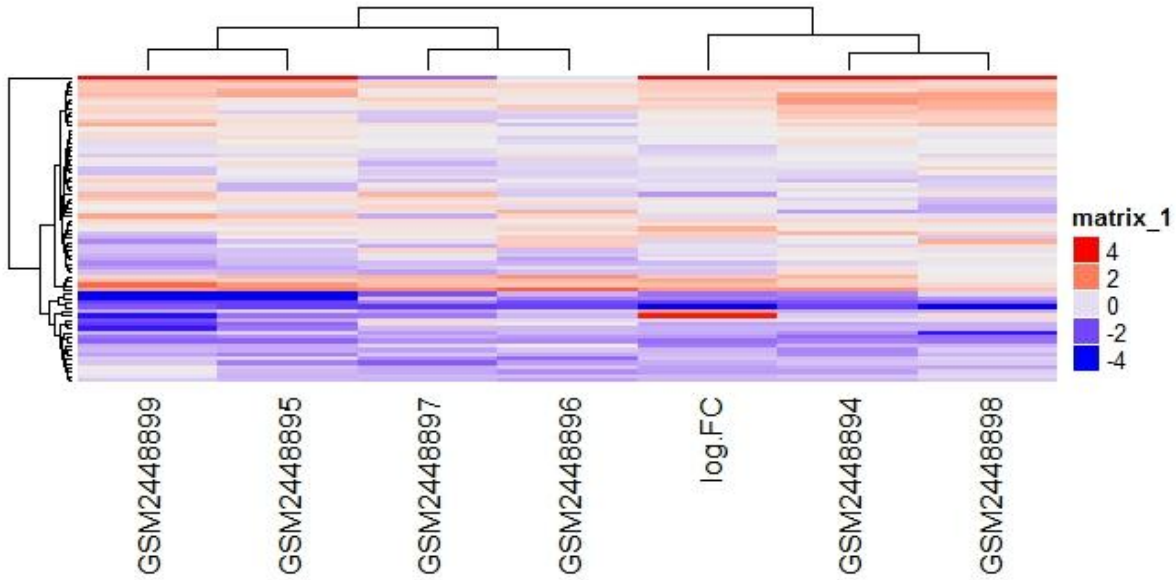


Fig4.12: Heatmap Generated for all test sample's cell line's gene expression data using the R program.

After this, we analyze the level of Pluripotency using JAVA Program. For this, we initially set up a range parameter for all key regulator genes in three different ways viz.

1) Highly Pluripotent cells.

2) Partially Pluripotent cells.

3) Less Pluripotent cells.

	IPSC Range		ESC Range	
	Is Data Manually Normalized or Not			
	YES	NO	YES	NO
1)	5.0 to 9.0	-1.5 to 5.0	2.0 to 3.0	-0.25 to 2.0
2)	2.0 to 4.9	-2.0 to -1.51	1.5 to 1.9	-3.0 to -0.249
3)	-3.0 to 1.9	-3.0 to -2.1	1.2 to 1.49	-15.0 to -2.9

Table 4.3: Pluripotency Score

This Range is based upon the manually compared and calculated expression values of key regulators from Different samples (Microarray Samples) viz. (GSE72078, GSE76282, GSE42445, etc.) present in GEO datasets and depend upon the condition

that either the data is pre-normalized or manually normalized. For both, the conditions the range is individually provided in each case of ESC as well as IPSC.

For Pluripotency level determination we first took the control sample. Our program first checks whether the cell lines are IPSC or ESC. Then after confirming that the cell lines are for IPSC. It asked for whether the microarray data was manually normalized or not and then according to our entries for IPSC cell line with manually normalized data, the program took to consider the range for this condition and gave us the results that in which category or level the given sample is lying.In our control results show that the cells are least pluripotent in normalized IPSC range. Same is the case of test sample our program initially took the same step as done for control and then decides the level of pluripotency. Here, in our test sample, the condition came out for non manually normalized IPSC data and hence, we got the results for that condition range. We also took test sample datasets from different other arrays too like GSE92706 and GSE73330 which were found to be passed and failed respectively. For confirming our results, we cross-check our results with PLURITEST by taking the above IDs data in (.idat*) raw intensity file format, and after analyzing with PLURITEST, the result we got are surprisingly as same as ours. By, this we conclude that the method which we develop to test pluripotency using (.txt) text file format is worth to work with and giving favorable as well as satisfactory results. Here we have shown only results for GSE92706. The comparable results of tested sample with different approaches (i.e., using a text format and .idat format) are shown in figures

Control sample (GSE72078) Pluripotency level determination:

Range: >=-3.0 to <=1.9
Range: GSM1854259
NANOG= 0.88792694
POU5F1= 0.94814897
SOX2= 1.6156561
Less Pluripotent Cell= GSM1854259

Range: >=-3.0 to <=1.9
Range: GSM1854260
NANOG= 0.87607163
POU5F1= 0.9470426
SOX2= 1.1104767
Less Pluripotent Cell= GSM1854260

Range: >=-3.0 to <=1.9
Range: GSM1854261
NANOG= 0.45721972
POU5F1= 0.79864424
SOX2= 0.9874374
Less Pluripotent Cell= GSM1854261

Range: >=-3.0 to <=1.9
Range: GSM1854262
NANOG= 0.70601815
POU5F1= 1.1790252
SOX2= 0.38984066
Less Pluripotent Cell= GSM1854262

Range: >=-3.0 to <=1.9
Range: GSM1854263
NANOG= 0.472952348
POU5F1= 1.056011706
SOX2= 1.505724634
Less Pluripotent Cell= GSM1854263

Range: >=-3.0 to <=1.9
Range: GSM1854264
NANOG= 1.0276734
POU5F1= 0.79453945
SOX2= 1.4517218
Less Pluripotent Cell= GSM1854264

Range: >=-3.0 to <=1.9
Range: GSM1854265
NANOG= 1.022949722
POU5F1= 1.046684461
SOX2= 1.192202724
Less Pluripotent Cell= GSM1854265

Range: >=-3.0 to <=1.9
Range: GSM1854266
NANOG= 1.0564212
POU5F1= 0.88678443
SOX2= 0.42901477
Less Pluripotent Cell= GSM1854266

Range: >=-3.0 to <=1.9
Range: GSM1854267
NANOG= 0.668517
POU5F1= 0.81713855
SOX2= 1.1758825
Less Pluripotent Cell= GSM1854267

Range: >=-3.0 to <=1.9
Range: GSM1854268
NANOG= 0.73580366
POU5F1= 0.9113936
SOX2= 0.42615175
Less Pluripotent Cell= GSM1854268

Range: >=-3.0 to <=1.9
Range: GSM1854269
NANOG= 1.5778602
POU5F1= 1.2110461
SOX2= 0.12783645
Less Pluripotent Cell= GSM1854269

Range: >=-3.0 to <=1.9
Range: GSM1854270
NANOG= 1.45287005
POU5F1= 1.2110461
SOX2= 1.522041676
Less Pluripotent Cell= GSM18542

Test Sample (GSE93228) Pluripotency level determination:

Range: >=-1.5 to <=5.0
Range: GSM2448894
NANOG= 0.053198583
POU5F1= 0.4979063
SOX2= -1.2365668
Highly Pluripotent Cell= GSM2448894

Range: >=-1.5 to <=5.0
Range: GSM2448895
NANOG= -0.661888992
POU5F1= 0.231759788
SOX2= -1.089963819
Highly Pluripotent Cell= GSM2448895

Range: >=-1.5 to <=5.0
Range: GSM2448896
NANOG= -0.089637
POU5F1= -0.07959507
SOX2= -0.59168214
Highly Pluripotent Cell= GSM2448896

Range: >=-1.5 to <=5.0
Range: GSM2448897
NANOG= -0.03093701
POU5F1= 0.239970536
SOX2= -1.397995012
Highly Pluripotent Cell= GSM2448897

Range: >=-1.5 to <=5.0
Range: GSM2448898
NANOG= 0.001854803
POU5F1= 0.20897053
SOX2= -0.087790065
Highly Pluripotent Cell= GSM2448898

Range: >=-1.5 to <=5.0
Range: GSM2448899
NANOG= -1.496865928
POU5F1= -0.03865551
SOX2= -1.2365668
Highly Pluripotent Cell= GSM2448899

Fig.4.13: Result for Pluripotency level determination through JAVA program

From our study, we found that the potency determination is a key factor of gene expression analysis and by considering only the gene effect we could determine various activities of cells including pluripotency. This in vitro approach is competing with other pre available online tools. Such tools are still dealing with some bugs as by considering the only limited number of files and with limited file format reliability. Also, their dependency on online servers are making them not 100% fit for potency determination, but in our case, the approach is working on the text file based logic of creating and arranging raw text file into matched and arranged file format concerning the gene expression values and Log FC. The level of pluripotency which we are calculating through JAVA determines us three levels, where each level gives us an idea about the potency of that particular cell to be differentiated into the basic three lineages. Highest level determines the potency of differentiating into all three lineages, whereas partial and low level determines us the differentiation into either two or one of the three lineages respectively. This approach gave us the way for determining the potency using computer programming language JAVA and statistical method based R Script, which was used in arranging data according to the matched marker genes and finally in the determination of Level of pluripotency using various ranges. Several graphs and plots viz. boxplots, clustering graphs, andheatmap were also developed using an R script. This approach will surely provide open access for identifying pluripotency and understanding the working and expression nature of various genes involved in reprogramming strategies.

4.3 The interactions of SOX2 and SOX6

4.3.1 Protein interaction pathways analysis using STRING

The protein interaction pathways of Sox2 and Sox6 were predicted by using STRING. In the interaction pathway of Sox6, the list of interactants included Sox2 which has been shown in Fig 4.14.

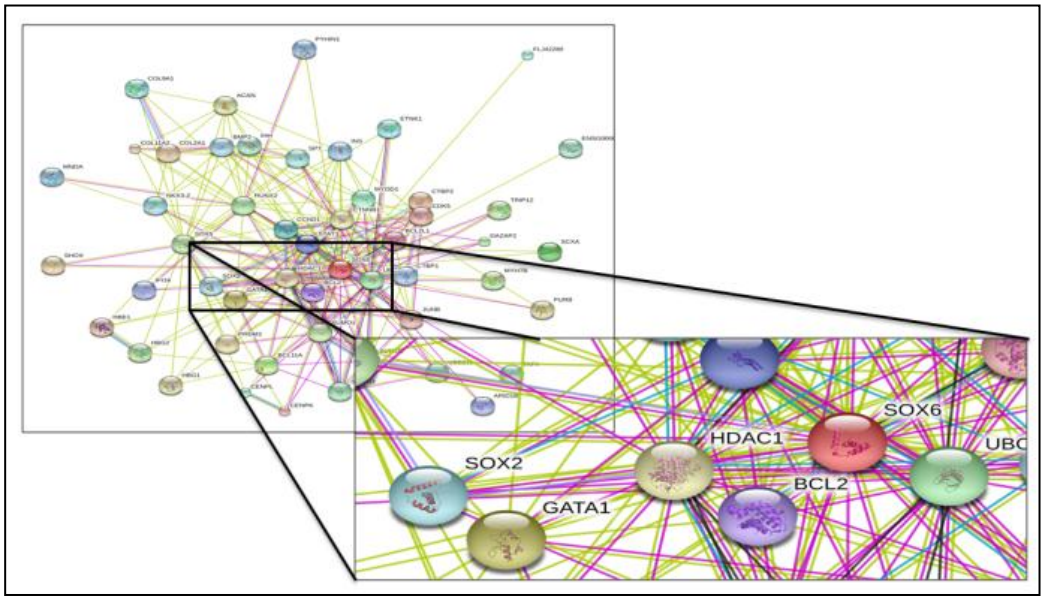


Figure-4.14Interaction pathway as obtained from STRING has been zoomed to highlight the interaction between Sox2 and Sox6.

4.3.2 Structure prediction and validation

The top models from the structures predicted by iTasser (Fig 4.15) were further evaluated with the tools RAMPAGE and ERRAT. RAMPAGE predicts the Ramachandran Plot of the protein whereas ERRAT gives an overall quality of the predicted structure. For Sox2 structure, RAMPAGE predicted 231 (73.3%) residues, 59 (18.7%) residues and 25 (7.9%) residues in favored, allowed and outlier region respectively in the Ramachandran Plot of Sox2 as shown in Fig 4.16 A. An ERRAT score of 93.793 indicates Sox2 structure with very high quality. 3D structure of Sox6

was predicted to be having 523 (63.3%) residues, 204 (24.7%) residues, and 99 (12.0%) residues in favored, allowed and outlier regions respectively as shown in Fig 4.16 B. An ERRAT score of 77.065 indicates a good quality structured Sox6 model.

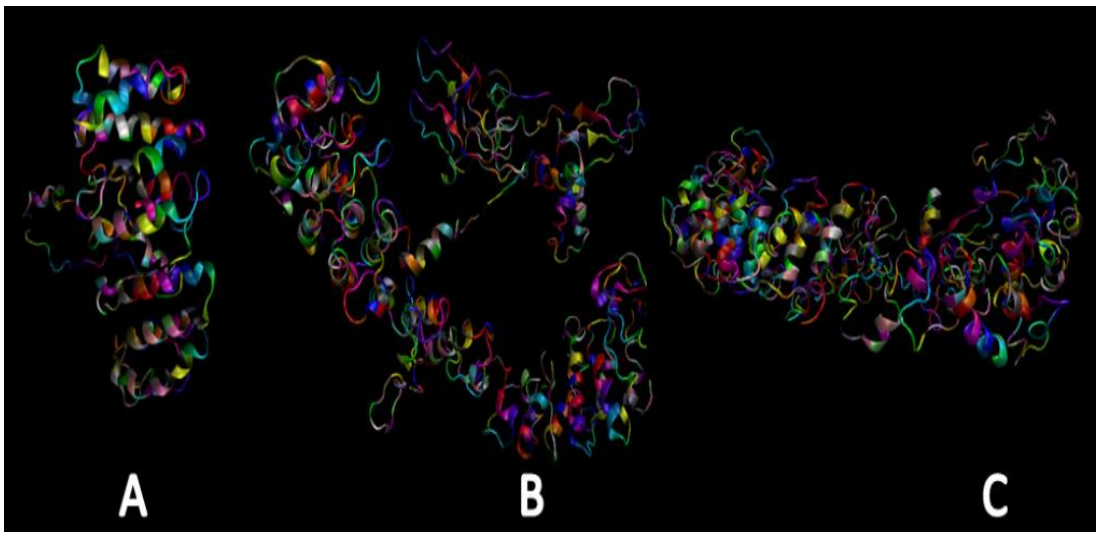


Figure 4.15: A) Predicted structure of Sox2; B) Top view of the predicted structure of Sox6 and C) Side view of Sox6

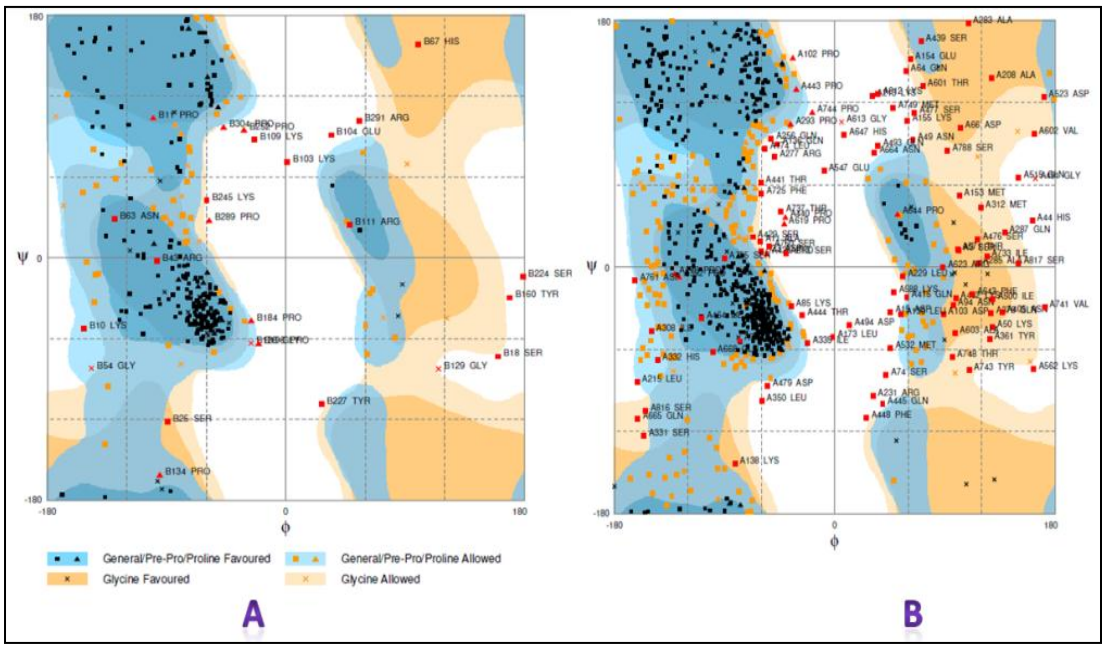


Figure 4.16: A) Ramachandran Plot of Sox2 and B) Ramachandran Plot of Sox6 as predicted from the tool RAMPAGE.

4.3.3 Protein-Protein Docking

The top result of the docking from ClusPro, which is the best-docked confirmation between two proteins, was selected. After docking of Sox2 and Sox6, energy at center and lowest energy of the whole complex was found to be -1218.6 and -1460.4 respectively which represent a docking score with a high binding affinity.

4.3.4 Interaction analysis

The interaction between Sox2 and Sox6 was plotted using DIMPLOT. The data including specific residues forming H-bonds and the residues involved in hydrophobic interactions have been mentioned in the table.The residues involved in the formations of H-bonds have also been labeled in the docked complex and shown as a figure of the interaction site between both the proteins in Fig 4.

The 3D structures of the proteins Sox2 and Sox6 were predicted by iTasser resulting in C-scores of -2.26 and -0.76 respectively. The Ramachandran plots of both structures were predicted by RAMPAGE. The protein-protein docking of Sox2 and Sox6 was carried out by ClusPro which revealed the interactions between both proteins. The lowest energy score of the docked complex was -1460.4 kcal/mol while being -1218.6 kcal/mol at the center of the complex. The interaction between both the proteins as revealed by docking defines the complex to be having a high number of the residues involved in hydrogen bonding and hydrophobic interactions which can further be utilized to explore the underlying mechanism which allows these proteins to regulate the cell fate.

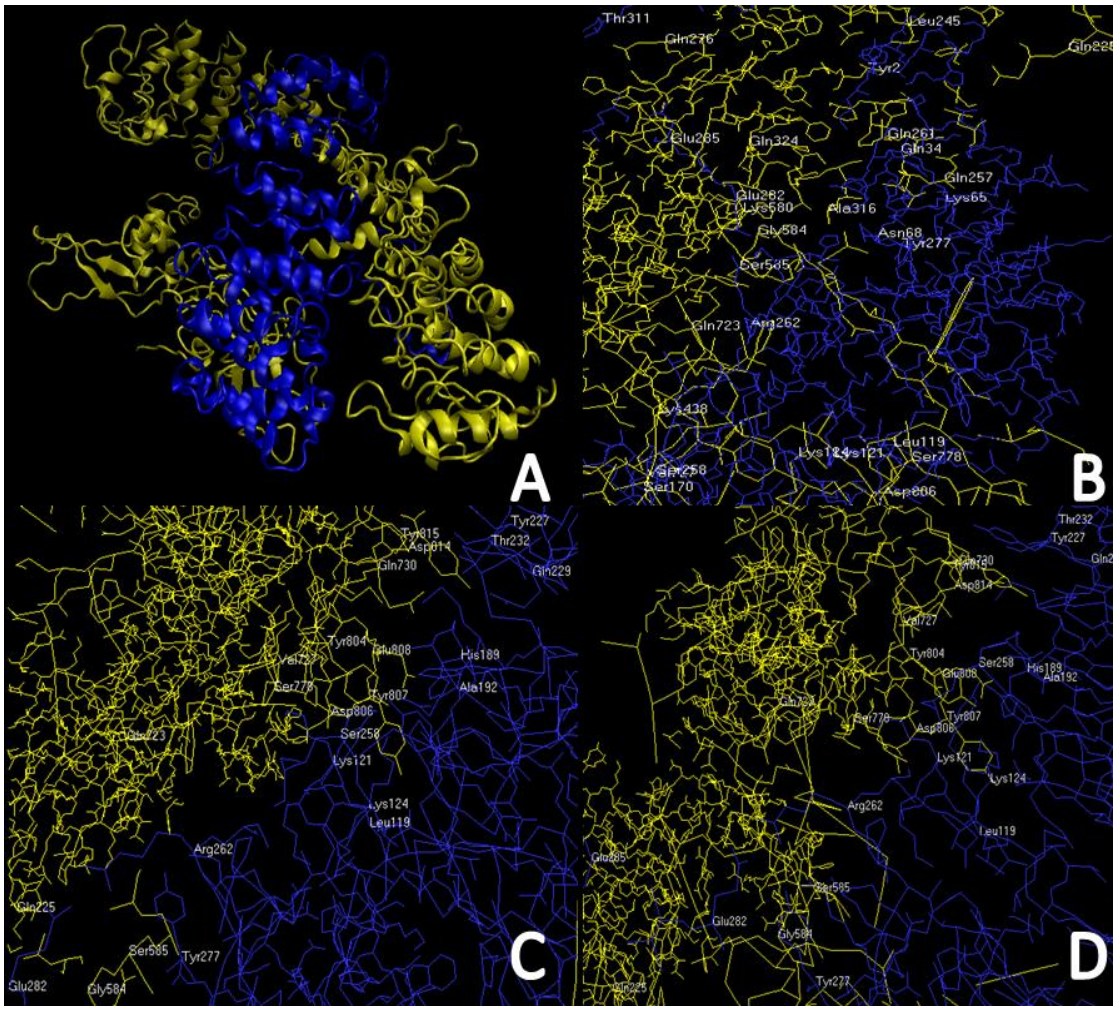


Figure 4.17: The docked complex of Sox2 and Sox6 as given from ClusPro; B), C), and D) the residues from the interface of the complex have been labeled and shown from different views. The Sox2 protein has been shown as blue and Sox6 as yellow.

In the Sox2-Sox6 complex, 28 H-bonds were detected. A total of 21 residues from Sox2 & 22 residues from Sox6 were found to interact with each other via hydrogen bonding, and 29 residues from Sox2 & 33 residues from Sox6 were found to be involved in hydrophobic interactions which have been listed in Table 4.4. Analysis of the interaction between both the proteins indicates a perfect binding between both them by which we can say that Sox2 and Sox6 may also interact with each other in real life scenario.

Residues involved in H-bonding		Residues involved in Hydrophobic Interactions	
Sox2	**Sox6**	**Sox2**	**Sox6**
Gln287, Tyr2, Gln34, Ala30, Lys65, Ser258, Ser170, Thr311, Tyr277, Asn68, Leu119, Lys124, Lys121, His189, Ala192, Tyr227, Gln229, Thr232, Ala281, Glu282, Arg262,	Leu245, Gln261, Gln257, Gln225, Val727, Lys438, Gln276, Gln259, Ala316, Tyr807, Asp806, Ser778, Tyr804, Glu808, Asp814, Tyr815, Gln730, Lys580, Gln324, Gly584, Ser585, Gln723	Met4, Met1, Asn33, Tyr171, Gly32, Ala29, Gly31, His 67, Pro64, Pro134, His101, Met102, Arg96, Ala99, Gly310, Leu205 , Ala248, Gln206, Ser249, Pro 313, Lys35, Asn36, Ser167, Gly136, Met157, Arg156, Gln155, Met120, Tyr160, Ala191, His316, Ala133, Gly190, Ser228, Met194, His198, Val283, Gly280, Ala263	Leu279, Leu244, His249, Met268, Phe307, Ser439, Ile258, Gln256, Ser360, Phe725, Thr726, Tyr361, Met312, Met582, Leu320, Asn582, Asp275, Thr278, His274, Ala313, Ala314, Ala315, Pro440, Met803, Gln240, His359, Pro731, Ala280, Asp575, Ala587, Leu589, Lys588, Ala586

Table 4.4: The specific residues which play a role in the interaction between both the protein have been mentioned in the above table. These specific residues include the ones involved in H-bonding and hydrophobic interactions.

4.4 Studying various phases of erythropoietic development

4.4.1 Selection of CD34+ cells source

CD34+ stem cells were obtained from either the umbilical cord blood or aphaeresis with prior consent of donor. The cells were enriched by Stemcell technologies magnetic assisted cell sorter and compared for their yield, viability, stem cell potency

and aseptic culture conditions. We found that the yield and viability were more or less same for the both sources. But the cells obtained from the umbilical cord blood had abundance of fetal haemoglobin that differs from the adult haemoglobin.

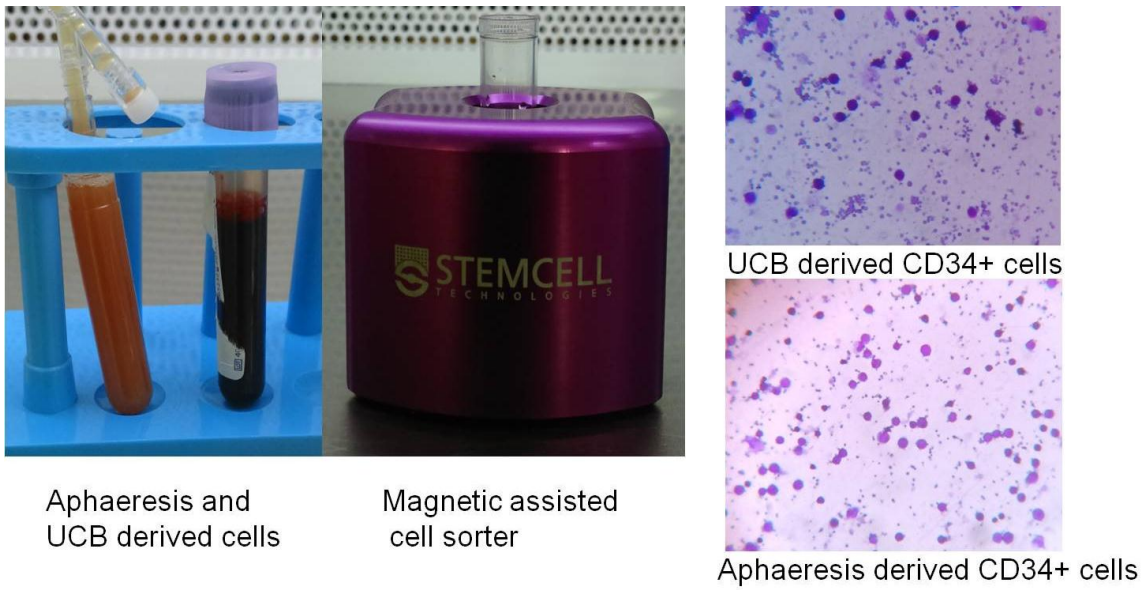


Figure 4.18: The above figure represents the cells obtained from aphaeresis and umbilical cord, on the right hand side the images compare the enriched cells stained with the Giemsa stain observed at 400X magnification under inverted phase contrast microscope.

The cells were seeded at day 0 were obsereved everyday and on day 4 the media was increased to four times the volume to accommodate the new cells.On day 7 the was renewed with only EPO and SCF, on day 11 onwards cells were cultured only in the presence of EPO and media was changed at every third day.

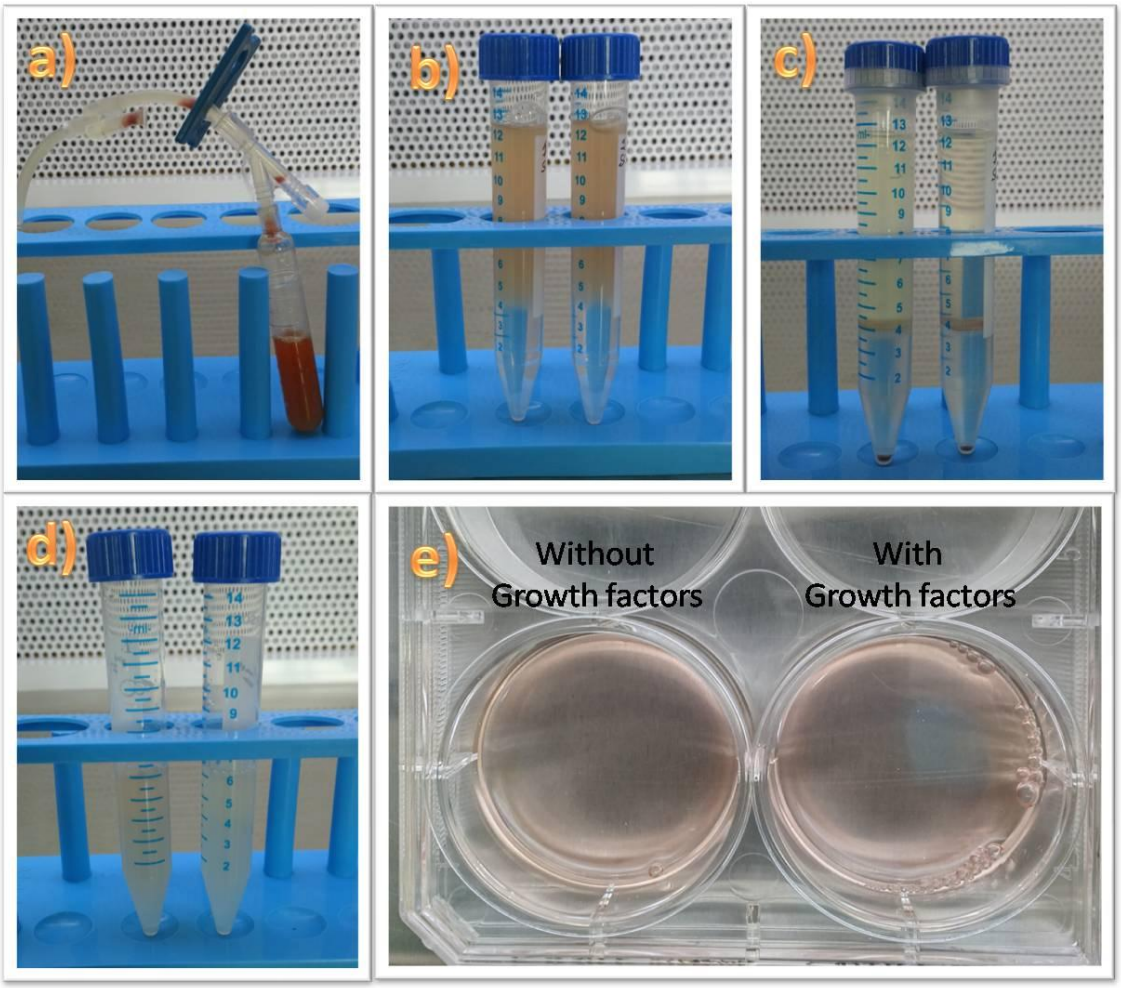


Figure 4.19: Stepwise procedure of culturing CD34+ cells for standardization of expansion of Hematopoietic stem cells and erythroid maturation (a) Tube containing CD34+ cells separated through COBE® Spectra Apheresis System (b) Falcon tube containg cells 4times diltued in PBS layered on Histopaque®-1077 (c) After centrifugation at 400g for 30 minutes RBCs were settled in the bottom and CD34+ MNCs were clearly visible in the interphase (d) Interphase was then washed and resupended in PBS (e) Cluture plate showing the wells seeded with 10^6 cells in both wells with and without growth factors. A total of 2.4 x 10^7 CD34+ cells were obtained from the sample after enrichment.

4.4.2 Cell Staining and Morphology studies

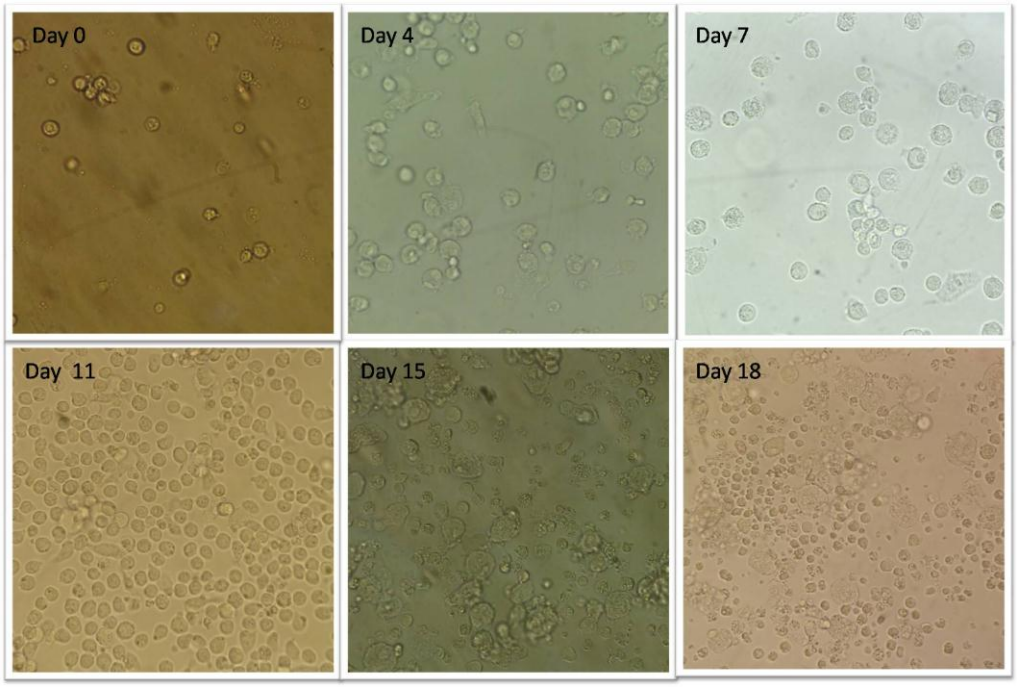


Figure 4.20: Day wise cells morphology as observed at 400x magnification in culture plates. The cells were cultured in the presence of erythroid growth factors and same is being represented by their morphological state.

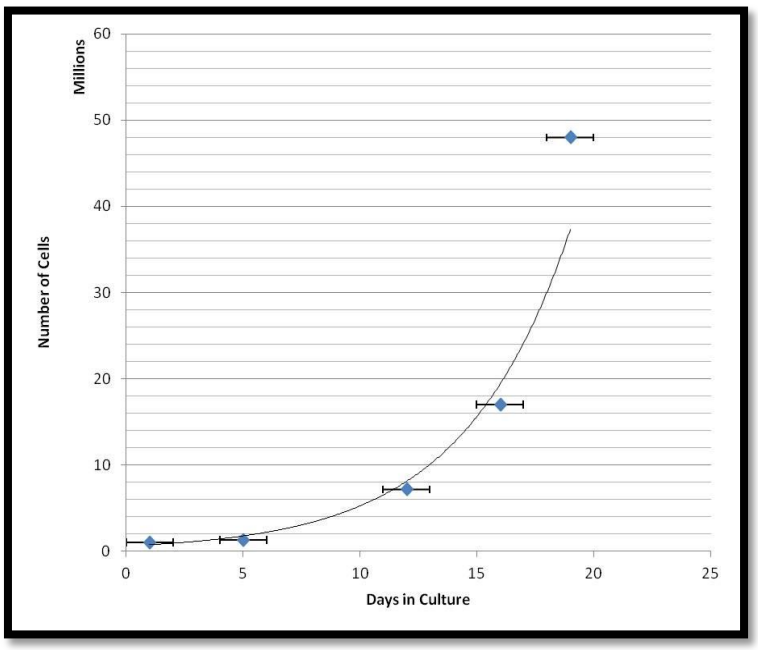


Figure 4.21: Graph showing increase in CD34+ cell population from initial 10^6 cells/ml to 4.8 X 10^6 cells/ml.

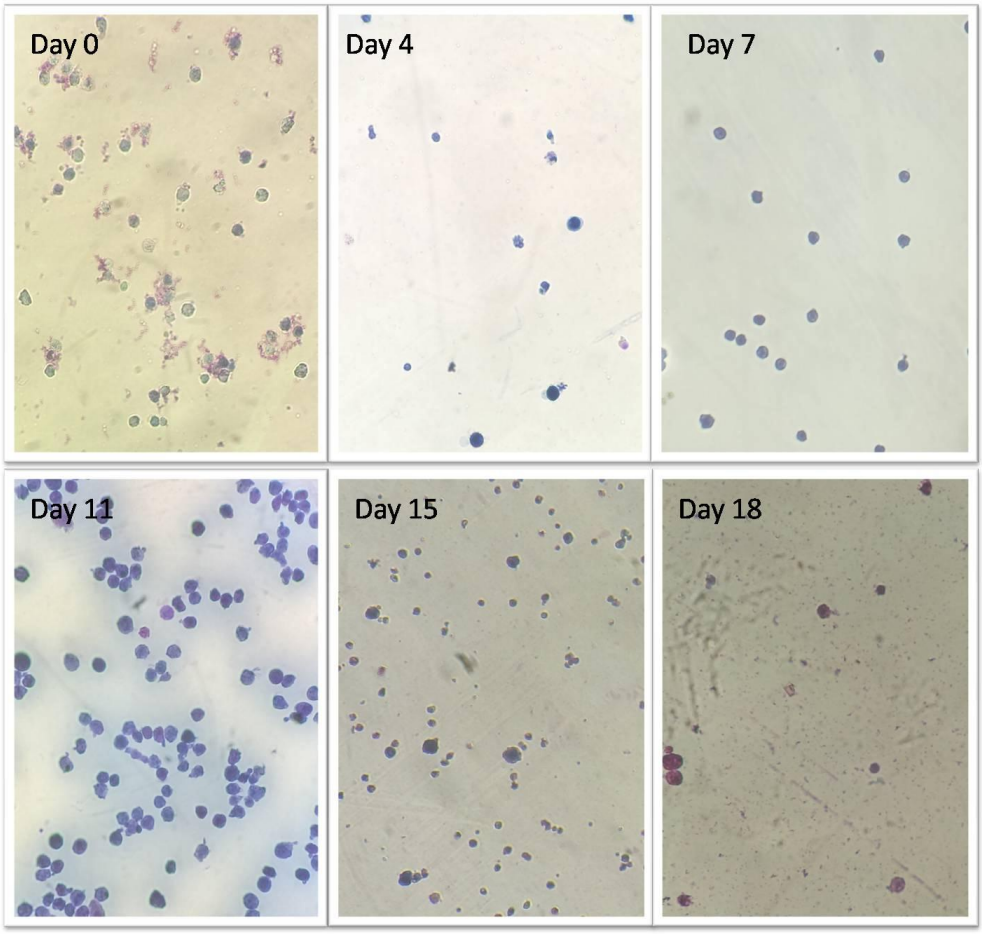


Figure 4.22: Day wise image of cultured cells stained with the Giemsa stain observed at 400X magnification under inverted phase contrast microscope.

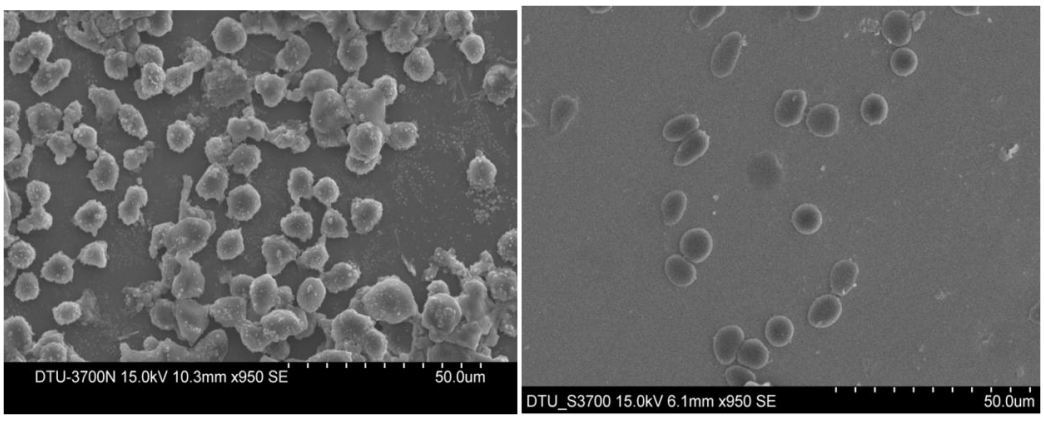


Figure 4.23-SEM images of CD34+ cells at day0 on the left and the differentiated cells obtained after 18 day culture on the right.

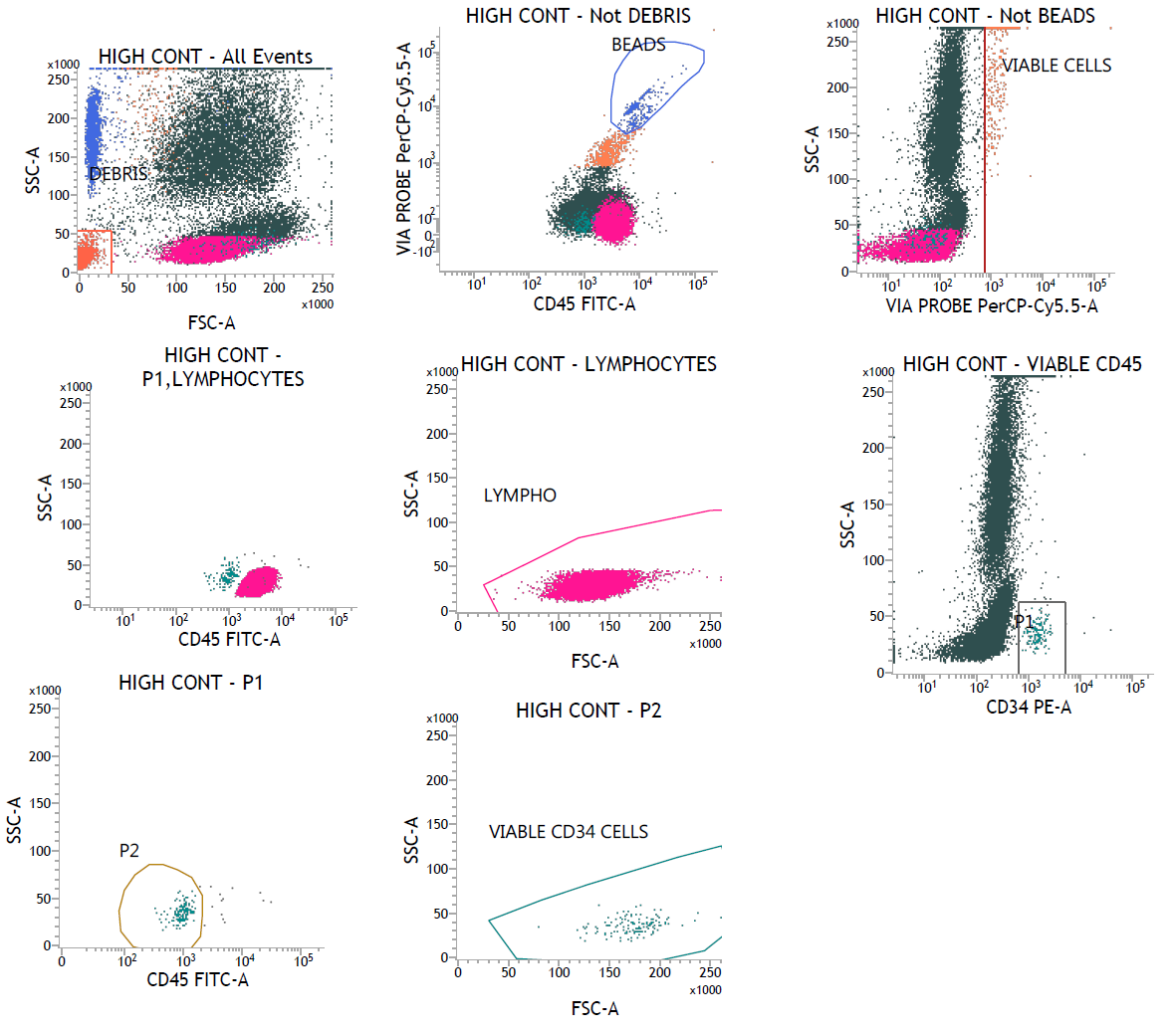


Figure 4.24- Flow cytometric enumeration of High control of CD34 + cells,

The total bead counts were 49400, mean of beads acquired was 3334, and viable CD34+ cells acquired were 204.

The absolute CD34+ count was calculated by formula

Absolute CD34+ cell count = CD34+ cells aquired x Total beads x Dilution/ Beads acquired x sample volume (in ul) The cell count for the high control was 30 CD34+ cells permicrolitre. Similarly cell count was calculated for the low control and final product. The cell count for the low control was 11.08 cells per microlitre as in this case mean of beads acquired was 9005, viable CD34+ cells acquired were 202 and

bead counts were 49400. In the case of final product the mean of beads acquired was 401 and 384, viable CD34+ cells acquired were 200 each and bead counts were 49400, therefore the The mean CD34+ cell count for the product was 2463 and 2572 respectively.

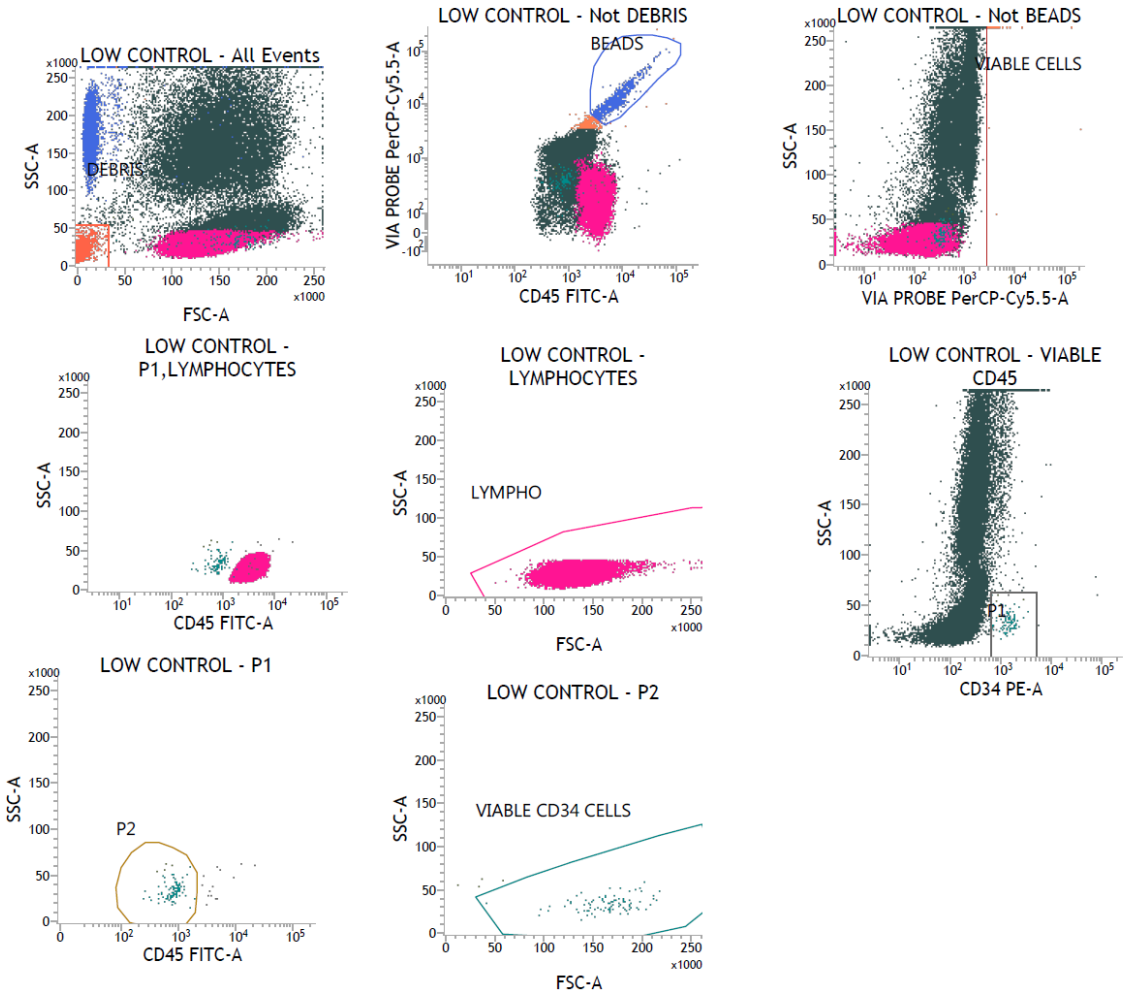


Figure 4.25- Flow cytometric enumeration of low control of CD34 + cells with count of 11.08 cells/ul

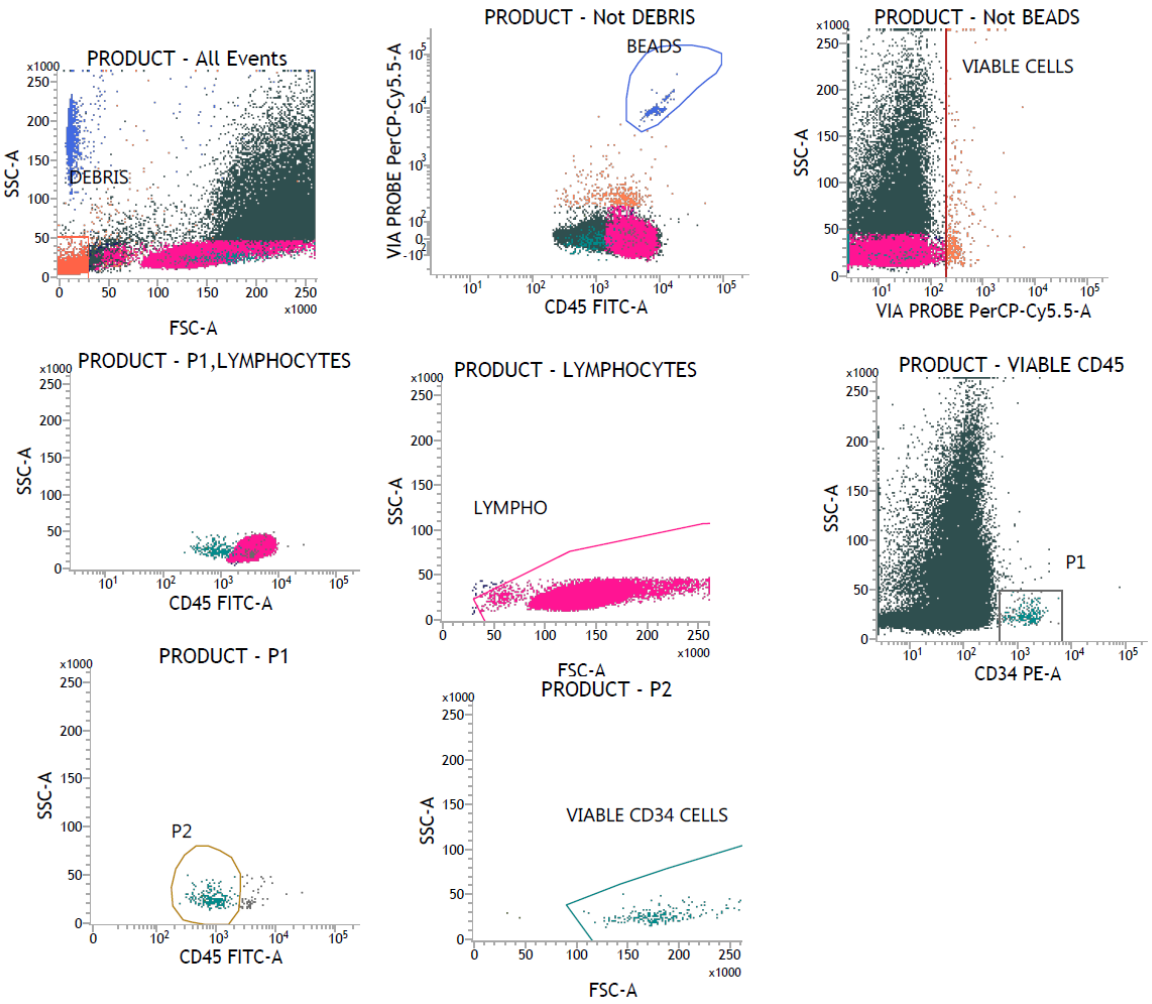


Figure 4.26- Flow cytometric enumeration of final aphaeresis product with Absolute CD34+ count of 2463 cells/ul

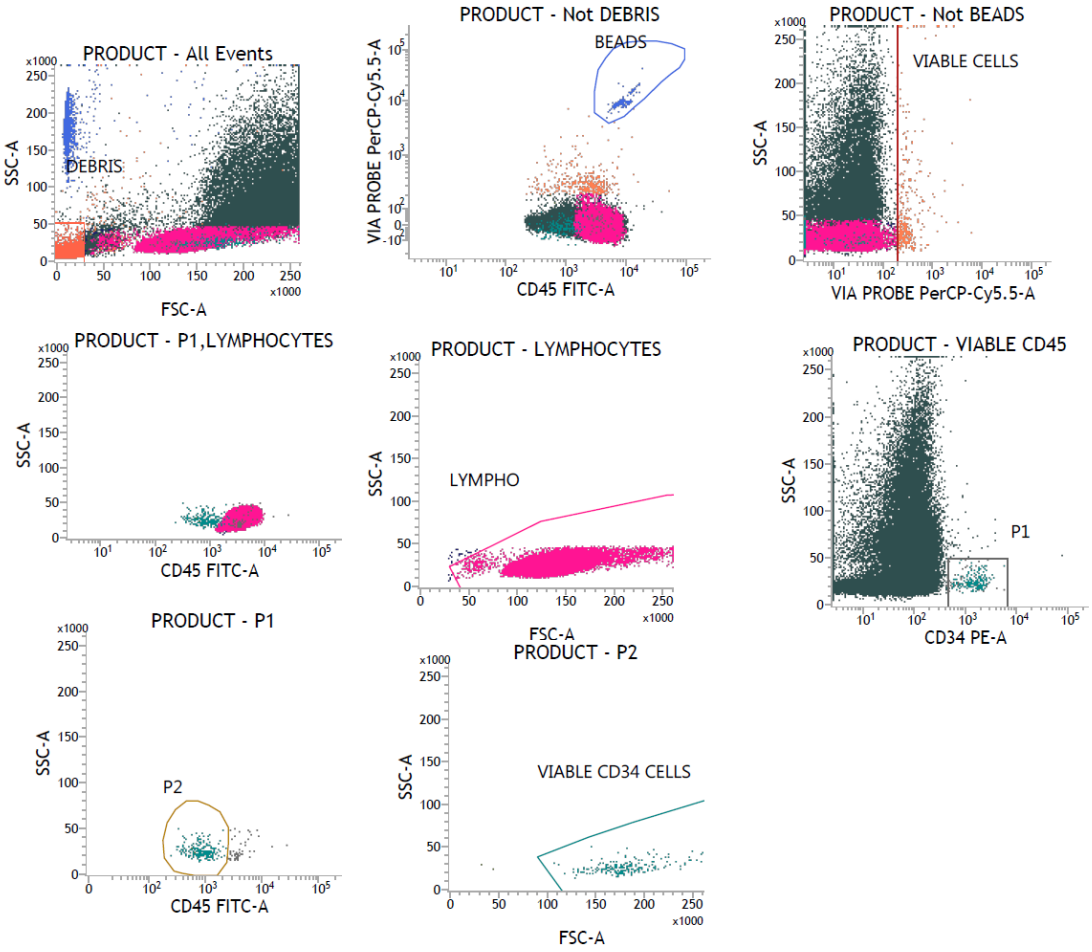


Figure 4.27-Flow cytometric enumeration of final aphaeresis product with Absolute CD34+ count of 2572 cells/ul.

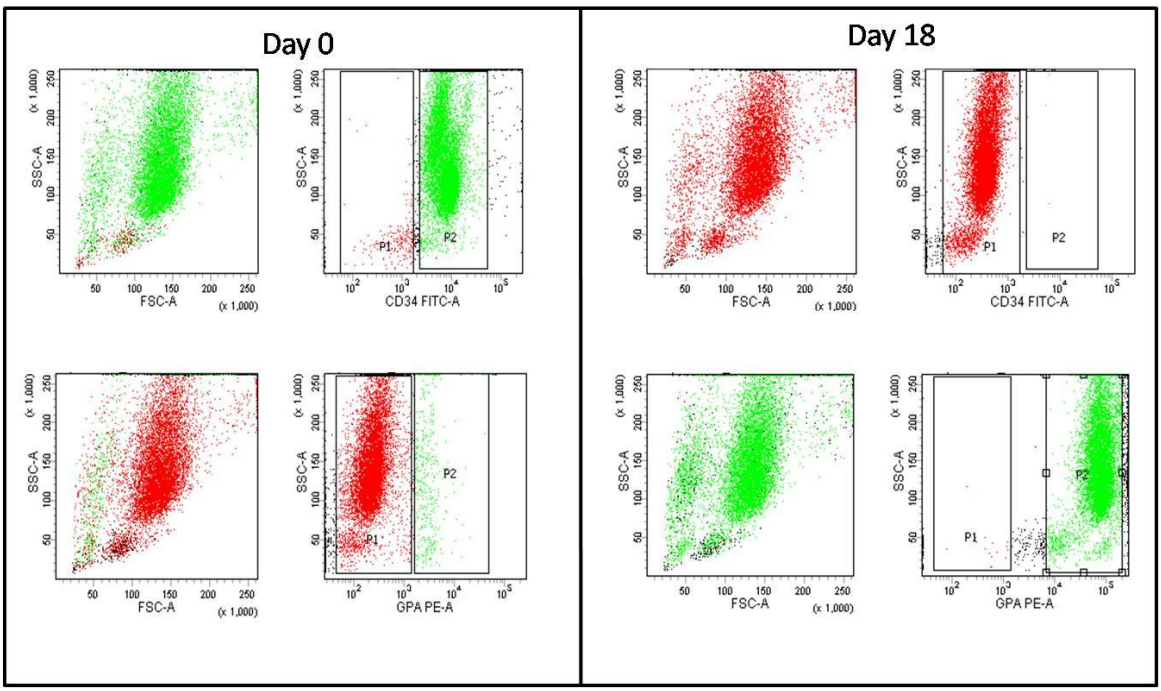


Figure 4.28: Cells were analyzed for their surface markers at day 0 for CD34+ and CD235A (Glycophorin A) expression by using anti CD34+ TUK3 primary antibody alongwith secondary antibody conjugated with PE and anti GlycophorinA primary antibody and secondary antibody conjugated with FITC. As per the surface marker expression the population was CD34+ at day 0 and its expression was lost during the culture and by the day 18 it was totally absent. While the CD235A (Glycophorin A) expression increased to a great extend indicating the differentiation of cells.

.

Chapter 5

Summary and Conclusion

The overall work was to understand the molecular interaction of EPO/EPOR and evaluation of its signification in *in-vitro* erythroid differentiation The first and foremost objective was achieved by literature survey, data mining and bioinformatics tools. A novel EPO mimetic was designed, and it was compared with previously reported EMP1 and ERB7 for interaction with EPOR using the molecular docking simulation analyses and was found to be more potent than EMP1 and ERB7. Novel Pep-22-EPOR complex showed a stable binding with an RMSD value of 0.091 Å through molecular dynamics simulation. Furthermore, physicochemical properties of pep-22 showed that it is a stable protein with high instability index value and good GRAVY score. Moreover, it is predominantly localized to an extracellular region, a prerequisite property for mimetic to interact with cell surface receptor EPOR.

As per the second objective a tool was developed to save the time in searching the best source cells for differentiation into erythroid lineage. It was also compared with other available online tools and found that our tool requires just .txt files. While rest others have their own extentions files for accepting and importing data, their individual way of calculations and presenting results for pluripotency. E.g., PluriTest takes only illumina generated .idat* files, whereas CellNet accepts only Affymetrix based .CEL* file formats of following platforms : Affymetrix Mouse Genome 430 2.0 Array (GPL1261), Affymetrix Mouse Gene 1.0 ST Array, Illumina MouseRef-8 v2.0 expression bead chip, Affymetrix Human Genome U133 Plus 2.0 Array, Affymetrix Human Gene 1.0 ST Array. TeratoScore also accepts the file formats of Affymetrix but it uses only Human Genome U133 plus 2.0 platform. All these tools have different algorithm for the analysis of results as in the case of CellNet, it uses Context Likelihood of Relatedness algo. First, they had generated the complete

training data that would serve to estimate the expression distributions of genes from each platform. Second, they reconstruct gene regulatory networks. Finally, they use the GRNs to train cell and tissue type classifiers.

In PluriTest, our uploaded file is preprocessed following published methods with currently accepted mathematical models. This processed data is then projected against a databased model of pluripotency derived from the transcriptional profiles of hundreds of validated pluripotent and somatic cell lines. This projection allows for the direct comparison of our submitted sample data to hundreds of previously characterized data sets.

In TeratoScore In order to quantitatively estimate the pluripotency of the tumor initiating cells, we calculated the mean gene expression of each lineage and extraembryonic tissue, multiplied them, and divided by 1,000, producing a single score. This analysis, termed TeratoScore, essentially estimates the differentiation potency of the tumor initiating cells—the very goal of Teratoma formation.

The microarray data, which is available on NCBIs GEO Dataset, is present in different forms or we can say that the platforms that were used for cell lines are different for different cells depending upon the requirement of researcher. Different tools accept the files in their default platforms e.g., PluriTest accepts only ILLUMINA microarray data whereas CellNet and TeratoScore accept Affymetrix data.

These tools use different Statistical parameters viz. In CellNet several statistical parameters used for calculation of Gene regulatory network, it uses Z-score analysis and MEAN. Whereas PluriTest involves the use of Quantile Normalization, Loess Normalization (both for normalizing the variance stabilizing transformation values for

the reduction in the spurious variations came during fluorescent dyes color combination analysis.

The results we got from these analyses are in different forms for individual tool e.g. In CellNet the result window shows Heatmap and histograms based on the gene regulatory network analysis. In PluriTest the results consists of pluripotency graph, novelty graph, boxplots, hierarchical clustering and in TeratoScore the results include only the bargraph depicting the TeratoScore outcome. The Bars shows cells score (Teratomas forming score) with respect to that outcome.

All the tools are performing using R script at Backend. PluriTest and CellNet use several parameters on which their working relies i.e. by considering these parameters they actually define the state of cell. These parameters include Telomerase activity, Differentiation, Methylation, Histone Demethylation etc. (as discussed earlier).Whereas TeratoScore paradigm based upon finding of level of Teratomas formation after the IPSCs reprogramming.

The most important thing in any online analysis is the time taken by that particular software. Here, we notice that the Rate of analysis is much faster and frequent in PluriTest as compared to others. In case of CellNet and TeratoScore, the results were sent through mail to the user's given account, but this process at least took 15mins to 2hrs depending upon the net connectivity.

File size of cell line and number of files included in the particular dataset chosen from NCBI is also a great concern in these tools. As for PluriTest, we can choose maximum of 12 files only of the cell lines present in the dataset. In CellNet, it is not mentioned for number of files but the total size of the cell lines to adopt is not more than 128MB. TeratoScore has no such obligations but the chosen file must be of Affymetrix Human Genome U133 plus 2.0 array platform.

First, we got the list of marker genes available in any stem cell whose presence decides the pluripotency level of any undifferentiated cell. These genes got isolated by using different techniques viz. MACS (Magnetic Cell Sorting Technique) and FCM (flow cytometry) technique which are one of the most effective cell isolating methods (Source : Data collected from Embryonic Stem Cell Markers; Wenxiu Zhao, Xiang Ji, Fangfang Zhang, Liang Li and Lan Ma 2012) From our study we found that the potency determination is a key factor of gene expression analysis and by considering only the gene effect we could determine various activities of cells including pluripotency. This in vitro approach is competing with other pre available online tools. As those tools is still dealing with some bugs as by considering only limited number of files and with limited file format reliability. Also, their dependency on online servers are making them not 100% fit for potency determination, but in our case the approach is working on text file based logic of creating and arranging raw text file into matched and arranged file format with respect to the gene expression values and Log FC.

The level of pluripotency which we are calculating through JAVA determines us three levels, where each level gives us an idea about the potency of that particular cell to be differentiated into the basic three lineages. Highest level determines the potency of differentiating into all three lineages, whereas partial and low level determines us the differentiation into either two or one of the three lineages respectively.

This approach gave us the way for determining the potency using computer programming language JAVA and statistical method based R Script, which were used in arranging data according to the matched marker genes and finally in the determination of Level of pluripotency using various ranges. Several graphs and plots viz. boxplots, clustering graphs and heatmap were also developed using R script. This approach will surely provide an open access for identifying pluripotency and

understanding the working and expression nature of various genes involved in reprogramming strategies.

Microarray data proves beneficial in many regards. To get detailed info about any process Related to protein or gene expression or their interaction we do need to take help from it. As in above study we found that to get pluripotency test of any cell sample first we have to access gene expression data of that particular cell and then after grabbing and arranging that data in a proper format, we will be able to fetch pluripotency data after checking whether the log FC value for that test sample either passing the threshold or not. Using this approach we are now in a state to tackle with various problems associated with pre-existing online tools. We can make our own tool based on this approach which will be free from various bugs that are present in existing tools; also we can identify which gene is devoting more in making any cell pluripotent. Through this approach we are able to get Pluripotency state of any type of cell regardless of any particular platform or any file format.

Then in order to find underlying mechanism for pluripotency and differentiation into the erythroid lineage we also studied the interactions of Sox2 and Sox6. The 3D structures of Sox2 and Sox6 after prediction via ab initio modeling and validation were docked with each other. Their docking results indicated a very good binding score and a high number of residues are involved in hydrogen bonding and hydrophobic interactions. This analysis proves our hypothesis of a protein-protein interaction between Sox2 and Sox6 to be true to a certain level, but for further validation of this point, more approaches including wet lab protein-protein interaction studying experiments need to be conducted.

Anemia is commonly prevalent in Indian population. The condition of having less than the normal number of red blood cells or less than the normal quantity of hemoglobin in the blood are known as anemia. Since erythropoietin induces

erythropoiesis / formation of erythrocyte cells in human blood. Our analysis show that EPO mimetic could be a potential therapeutic for treating anemia. Efforts are being made to generate erythrocytes ex-vivo, this mimetic peptide can be used as growth factor for ex-vivo culture of erythrocytes. Hence evaluation of the mimetic peptide may be studied for clinical trials after obtaining relevant permissions. Moreover, with the development of more in depth knowledge about the molecular mechanisms involved in various stages of *ex vivo* erythropoiesis. It would become more efficient to support even larger population's blood demands. The fundamental information shall also be helpful in developing pharmacological alternates for the various stages of erythropoietic development which presently depend upon their interactions with feeder cell support systems in most of the protocols.

References

1. Adamson, J. W., & Eschbach, J. W. (1990). Treatment of the anemia of chronic renal failure with recombinant human erythropoietin. Annual review of medicine, 41(1), 349-360

2. Akashi, K., Traver, D., Miyamoto, T., & Weissman, I. L. (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature, 404(6774), 193.

3. Ali, A., Auvinen, M. K., and Rautonen, J. (2010). The aging population poses a global challenge for blood services. *Transfusion* 50, 584–588. doi: 10.1111/j.1537-2995.2009.02490.x

4. Alter, H. J., and Klein, H. G. (2008). The hazards of blood transfusion in historical perspective. *Blood* 112, 2617–2626. doi: 10.1182/blood-2008-07-077370

5. Amit, M., Carpenter, M. K., Inokuma, M. S., Chiu, C. P., Harris, C. P., Waknitz, M. A., ... & Thomson, J. A. (2000). Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. Developmental biology, 227(2), 271-278.

6. Anagnostou, A., Lee, E. S., Kessimian, N., Levinson, R., & Steiner, M. (1990). Erythropoietin has a mitogenic and positive chemotactic effect on endothelial cells. *Proceedings of the National Academy of Sciences*, *87*(15), 5978-5982.

7. Apostolou, E., & Hochedlinger, K. (2013). Chromatin dynamics during cellular reprogramming. Nature, 502(7472), 462-471.

8. Ariyoshi, K., Nosaka, T., Yamada, K., Onishi, M., Oka, Y., Miyajima, A., & Kitamura, T. (2000). Constitutive activation of STAT5 by a point mutation in the SH2 domain. Journal of Biological Chemistry, 275(32), 24407-24413.

9. Bachmann, S., I., Le Hir, M. I., & Eckardt, K. U. (1993). Co-localization of erythropoietin mRNA and ecto-5'-nucleotidase immunoreactivity in peritubular cells of rat renal cortex indicates that fibroblasts produce erythropoietin. Journal of Histochemistry & Cytochemistry, 41(3), 335-341.

10. Baek, E. J., Kim, H. S., Kim, J. H., Kim, N. J., and Kim, H. O. (2009). Stroma-free mass production of clinical-grade red blood cells (RBCs) by using polox-amer 188 as an RBC survival enhancer. *Transfusion* 49, 2285–2295. doi: 10.1111/j.1537-2995.2009.02303.x

11. Bagnis, C., Chiaroni, J., and Bailly, P. (2011). Elimination of blood group anti-gens: Hope and reality. *Br. J. Haematol.* 152, 392–400. doi: 10.1111/j.1365-2141.2010.08561.x

12. Bartel, D. P. (2009). MicroRNAs: target recognition and regulatory functions. Cell 136(2), 215–233. doi: 10.1016/ j.cell.2009.01.002.

13. Basu, S., Dunn, A., and Ward, A. (2002). G-CSF: function and modes of action (Review). Int J Mol Med.10 (1), 3–10.

14. Bauer, A., Tronche, F., Wessely, O., et al. (1999). The glucocorticoid receptor is required for stress erythropoiesis. Genes Dev. 13(22), 2996–3002.

15. Benkert, P., Tosatto, S. C., & Schomburg, D. (2008). QMEAN: A comprehensive scoring function for model quality assessment. *Proteins: Structure, Function, and Bioinformatics*, *71*(1), 261-277.

16. Bernaudin, M., Marti, H. H., Roussel, S., Divoux, D., Nouvelot, A., MacKenzie, E. T., & Petit, E. (1999). A potential role for erythropoietin in focal permanent cerebral ischemia in mice. *Journal of Cerebral Blood Flow & Metabolism*, *19*(6), 643-651.

17. Bessis, M., Mize, C., and Prenant, M. (1978). Erythropoiesis: comparison of *in vivo* and *in vitro* amplification. *Blood. Cells* 4, 155–174.

18. Bittorf, T., Jaster, R., Lüdtke, B., Kamper, B., & Brock, J. (1997). Requirement for JAK2 inErythropoietin-Induced Signalling Pathways. *Cellular signalling*, *9*(1), 85-89.

19. Bittorf, T., Seiler, J., Lüdtke, B., Büchse, T., Jaster, R., & Brock, J. (2000). Activation of STAT5 during EPO-directed suppression of apoptosis. Cellular signalling, 12(1), 23-30.

20. Bondurant, M. C., & Koury, M. J. (1986). Anemia induces accumulation of erythropoietin mRNA in the kidney and liver. Molecular and cellular biology, 6(7), 2731-2733.

21. Bondurant, M. C., & Koury, M. J. (1986). Anemia induces accumulation of erythropoietin mRNA in the kidney and liver. Molecular and cellular biology, 6(7), 2731-2733.

22. Bouscary, D., Pene, F., Claessens, Y. E., Muller, O., Chrétien, S., Fontenay-Roupie, M., ... & Lacombe, C. (2003). Critical role for PI 3-kinase in the control of erythropoietin-induced erythroid progenitor proliferation. Blood, 101(9), 3436-3443.

23. Brines, M. L., Ghezzi, P., Keenan, S., Agnello, D., De Lanerolle, N. C., Cerami, C., ... & Cerami, A. (2000). Erythropoietin crosses the blood–brain

barrier to protect against experimental brain injury. *Proceedings of the National Academy of Sciences*, *97*(19), 10526-10531.

24. Brines, M., & Cerami, A. (2005). Emerging biological roles for erythropoietin in the nervous system. Nature Reviews Neuroscience, 6(6), 484.

25. Brox, A. G., Zhang, F., Guyda, H., & Gagnon, R. F. (1996). Subtherapeutic erythropoietin and insulin-like growth factor-1 correct the anemia of chronic renal failure in the mouse. *Kidney international*, *50*(3), 937-943.

26. Bruick, R. K., & McKnight, S. L. (2001). A conserved family of prolyl-4-hydroxylases that modify HIF. *Science*, *294*(5545), 1337-1340.

27. Bunn, H. F. (2006). New agents that stimulate erythropoiesis. Blood, 109(3), 868-873.

28. Cantor, A. B., and Orkin, S. H. (2002). Transcriptional regulation of erythropoiesis: an affair involving multiple partners. Oncogene. 21(21), 3368–3376.

29. Carlini, R. G., Reyes, A. A., & Rothstein, M. (1995). Recombinant human erythropoietin stimulates angiogenesis in vitro. *Kidney international*, *47*(3), 740-745.

30. Carnot P, DeFlandre C. Sur l'activite hemopoietique de serum au cours de la regeneration du sang. C R Acad Sci (Paris) **143:**384–386, 1906.

31. Chacko, G. W., Tridandapani, S., Damen, J. E., Liu, L., Krystal, G., & Coggeshall, K. M. (1996). Negative signaling in B lymphocytes induces tyrosine phosphorylation of the 145-kDa inositol polyphosphate 5-phosphatase, SHIP. The Journal of Immunology, 157(6), 2234-2238.

32. Cheng, Y., Wu, W., Kumar, S. A., Yu, D., Deng, W., Tripic, T., et al. (2009). Erythroid GATA1 function revealed by genome-wide analysis of transcription factor occupancy, histone modifications, and mRNA expression. Genome Res. 19(12), 2172–2184. doi: 10.1101/gr.098921.109.

33. Chikuma, M., Masuda, S., Kobayashi, T., Nagao, M., & Sasaki, R. (2000). Tissue-specific regulation of erythropoietin production in the murine kidney, brain, and uterus. American Journal of Physiology-Endocrinology And Metabolism, 279(6), E1242-E1248.

34. Chong, Z. Z., Kang, J. Q., & Maiese, K. (2003). Apaf-1, Bcl-xL, cytochrome c, and caspase-9 form the critical elements for cerebral vascular protection by erythropoietin. Journal of Cerebral Blood Flow & Metabolism, 23(3), 320-330.

35. Chong, Z. Z., Lin, S. H., Kang, J. Q., & Maiese, K. (2003). The tyrosine phosphatase SHP2 modulates MAP kinase p38 and caspase 1 and 3 to foster neuronal survival. Cellular and molecular neurobiology, 23(4-5), 561-578.

36. Colovos, C., & Yeates, T. O. (1993). Verification of protein structures: patterns of nonbonded atomic interactions. *Protein science*, *2*(9), 1511-1519.

37. Comeau, S. R., Gatchell, D. W., Vajda, S., & Camacho, C. J. (2004). ClusPro: an automated docking and discrimination method for the prediction of protein complexes. *Bioinformatics*, *20*(1), 45-50.

38. Constantinescu, S. N., Keren, T., Socolovsky, M., Nam, H. S., Henis, Y. I., & Lodish, H. F. (2001). Ligand-independent oligomerization of cell-surface erythropoietin receptor is mediated by the transmembrane domain. Proceedings of the National Academy of Sciences, 98(8), 4379-4384.

39. Cowan, C. A., Atienza, J., Melton, D. A., & Eggan, K. (2005). Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. Science, 309(5739), 1369-1373.

40. Damen, J. E., Cutler, R. L., Jiao, H., Yi, T., & Krystal, G. (1995). Phosphorylation of tyrosine 503 in the erythropoietin receptor (EpR) is essential for binding the P85 subunit of phosphatidylinositol (PI) 3-kinase and for EpR-associated PI 3-kinase activity. Journal of Biological Chemistry, 270(40), 23402-23408.

41. Damen, J. E., Liu, L., Cutler, R. L., & Krystal, G. (1993). Erythropoietin stimulates the tyrosine phosphorylation of Shc and its association with Grb2 and a 145-Kd tyrosine phosphorylated protein. Blood, 82(8), 2296-2303.

42. Damen, J. E., Wakao, H., Miyajima, A., Krosl, J., Humphries, R. K., Cutler, R. L., & Krystal, G. (1995). Tyrosine 343 in the erythropoietin receptor positively regulates erythropoietin- induced cell proliferation and Stat5 activation. The EMBO Journal, 14(22), 5557-5568.

43. D'andrea, A., Aste-Amezaga, M., Valiante, N. M., Ma, X., Kubin, M., & Trinchieri, G. (1993). Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. Journal of Experimental Medicine, 178(3), 1041-1048.

44. Daniels, G., Castilho, L., Flegel, W. A., Fletcher, A., Garratty, G., Levene, C., et al. (2009). International society of blood transfusion committee on terminology for red blood cell surface antigens: macao report. *Vox. Sang.* 96, 153–156. doi: 10.1111/j.1423-0410.2008.01133.x

45. Debili, N., Coulombel, L., Croisille, L., Katz, A., Guichard, J., Breton-Gorius, J., & Vainchenker, W. (1996). Characterization of a bipotent erythro-megakaryocytic progenitor in human bone marrow. Blood, 88(4), 1284-1296.

46. Department of Health and Human Services. (2010). *The 2009 National BloodCollection and Utilization Survey Report.* Washington, DC: DHHS. Availableonline at: http://www.aabb.org/programs/biovigilance/nbcus/Documents/09-nbcus-report.pdf (Accessed May 4, 2014).

47. Department of Health and Human Services. (2013). *The 2011 National BloodCollection and Utilization Survey Report.* Washington, DC: DHHS. Availableonline at: http://www.aabb.org/programs/biovigilance/nbcus/Documents/11-nbcus-report.pdf (Accessed May 4, 2014).

48. Digicaylioglu, M., Bichet, S., Marti, H. H., Wenger, R. H., Rivas, L. A., Bauer, C., & Gassmann, M. (1995). Localization of specific erythropoietin binding sites in defined areas of the mouse brain. *Proceedings of the National Academy of Sciences*, *92*(9), 3717-3720.

49. Dumon, S., Santos, S. C. R., Debierre-Grockiego, F., Gouilleux-Gruart, V., Cocault, L., Boucheron, C., ... & Gouilleux, F. (1999). IL-3 dependent regulation of Bcl-x L gene expression by STAT5 in a bone marrow derived cell line. Oncogene, 18(29), 4191.

50. Egrie, J. C., Dwyer, E., Browne, J. K., Hitz, A., & Lykos, M. A. (2003). Darbepoetin alfa has a longer circulating half-life and greater in vivo potency

than recombinant human erythropoietin. Experimental hematology, 31(4), 290-299.

51. Egrie, J. C., Strickland, T. W., Lane, J., Aoki, K., Cohen, A. M., Smalling, R., ... & Hines, D. K. (1986). Characterization and biological effects of recombinant human erythropoietin. Immunobiology, 172(3-5), 213-224.

52. Ehrenreich, H., Aust, C., Krampe, H., Jahn, H., Jacob, S., Herrmann, M., & Sirén, A. L. (2004). Erythropoietin: novel approaches to neuroprotection in human brain disease. *Metabolic brain disease*, *19*(3-4), 195-206.

53. Elliott, S., Pham, E., and Macdougall, I.C. (2008). Erythropoietin: a common mechanism of action. Exp Hematol. 36(12), 1573–1584. doi: 10.1016/j.exphem.2008.08.003.

54. Ende, M., and Ende, N. (1972). Hematopoietic transplantation by means of fetal (cord) blood. A new method. *Va. Med. Mon.* 99, 276–280.

55. Fandrey, J., & Bunn, H. F. (1993). In vivo and in vitro regulation of erythropoietin mRNA: measurement by competitive polymerase chain reaction. Blood, 81(3), 617-623.

56. Felli, N., Fontana, L., Pelosi, E., Botta, R., Bonci, D., Facchiano, F., et al. (2005) MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor downmodulation. Proc Natl Acad Sci U S A. 102(50), 18081–-18086.

57. Felli, N., Pedini, F., Romania, P., Biffoni, M., Morsilli, O., Castelli, G., et al. (2009). MicroRNA 223- dependent expression of LMO2 regulates normal

erythropoiesis. Haematologica. 94(4), 479–486. doi: 10.3324/haema tol.2008.002345.

58. Fenjves, E. S., Ochoa, M. S., Cabrera, O., Mendez, A. J., Kenyon, N. S., Inverardi, L., & Ricordi, C. (2003). Human, nonhuman primate, and rat pancreatic islets express erythropoietin receptors1. *Transplantation*, *75*(8), 1356-1360.

59. Fenjves, E. S., Ochoa, M. S., Gay-Rabinstein, C., Molano, R. D., Pileggi, A., Mendez, A. J., ... & Ricordi, C. (2004). Adenoviral gene transfer of erythropoietin confers cytoprotection to isolated pancreatic islets. *Transplantation*, *77*(1), 13-18.

60. Firth, J. D., Ebert, B. L., Pugh, C. W., & Ratcliffe, P. J. (1994). Oxygen-regulated control elements in the phosphoglycerate kinase 1 and lactate dehydrogenase A genes: similarities with the erythropoietin 3'enhancer. *Proceedings of the National Academy of Sciences*, *91*(14), 6496-6500.

61. Fisher, J. W., Koury, S., Ducey, T., & Mendel, S. (1996). Erythropoietin production by interstitial cells of hypoxic monkey kidneys. British journal of haematology, 95(1), 27-32.

62. Flygare, J., Karlsson, S. (2007). Diamond-Blackfan anemia: erythropoiesis lost in translation. Blood. 109(8), 3152– 3154.

63. Flygare, J., Rayon, Estrada, V., Shin, C., Gupta, S., Lodish, H.F., (2011). HIF-1alpha synergizes with glucocorticoids to promote BFU-E progenitor self-renewal. Blood. 117(12), 3435–3444.

64. Folkman, J. (1995). Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nature medicine*, *1*(1), 27.

65. Friesner, R. A., Murphy, R. B., Repasky, M. P., Frye, L. L., Greenwood, J. R., Halgren, T. A., ... & Mainz, D. T. (2006). Extra precision glide: Docking and scoring incorporating a model of hydrophobic enclosure for protein− ligand complexes. *Journal of medicinal chemistry*, *49*(21), 6177-6196.

66. Fuchs, O., Simakova, O., Klener, P., et al. (2002). Inhibition of Smad5 in human hematopoietic progenitors blocks erythroid differentiation induced by BMP4. Blood Cells Mol Dis. 28(2), 221–233.

67. Fujimi, A., Matsunaga, T., Kobune, M., Kawano, Y., Nagaya, T., Tanaka, I., et al. (2008). *Ex vivo* large-scale generation of human red blood cells from cord blood $CD34^+$ cells by co-culturing with macrophages. *Int. J. Hematol.* 87, 339–350. doi: 10.1007/s12185-008-0062-y

68. Fujiwara, T., O'Geen, H., Keles, S., Blahnik, K., Linnemann, A. K., Kang, Y.A., et al. (2009). Discovering hemato-poietic mechanisms through genomewide analysis of GATA factor chromatin occupancy. Mol Cell. 36(4), 667– 681. doi: 10.1016/j.molcel.2009.11.001.

69. Galeano, M., Altavilla, D., Cucinotta, D., Russo, G. T., Calo, M., Bitto, A., ... & Minutoli, L. (2004). Recombinant human erythropoietin stimulates angiogenesis and wound healing in the genetically diabetic mouse. *Diabetes*, *53*(9), 2509-2517.

70. Ganguli, G., Back, J., Sengupta, S., Wasylyk, B. (2002). The p53 tumour suppressor inhibits glucocorticoidinduced proliferation of erythroid progenitors. EMBO Rep. 3(6), 569–574.

71. Gasteiger, E., Hoogland, C., Gattiker, A., Wilkins, M. R., Appel, R. D., & Bairoch, A. (2005). Protein identification and analysis tools on the ExPASy server. In *The proteomics protocols handbook* (pp. 571-607). Humana press.

72. Gekas, C., Dieterlen-Lièvre, F., Orkin, S. H., & Mikkola, H. K. (2005). The placenta is a niche for hematopoietic stem cells. Developmental cell, 8(3), 365-375.

73. Giarratana, M. C., Kobari, L., Lapillonne, H., Chalmers, D., Kiger, L., Cynober, T., et al. (2005). *Ex vivo* generation of fully mature human red blood cells from hematopoietic stem cells. *Nat. Biotechnol*. 23, 69–74. doi: 10.1038/nbt1047

74. Giarratana, M. C., Rouard, H., Dumont, A., Kiger, L., Safeukui, I., Le Pennec, P. Y., et al. (2011). Proof of principle for transfusion of *in vitro*-generated red blood cells. *Blood* 118, 5071–5079. doi: 10.1182/blood-2011-06-362038

75. Gifford, S. C., Derganc, J., Shevkoplyas, S. S., Yoshida, T., and Bitensky, M. W. (2006). A detailed study of time-dependent changes in human red blood cells: from reticulocyte maturation to erythrocyte senescence. *Br. J. Haematol.* 135, 395–404. doi: 10.1111/j.1365-2141.2006.06279.x

76. Gregory CJ, Eaves AC. Three stages of erythropoietic progenitor cell differentiation distinguished by a number of physical and biologic properties. Blood 1978 Mar; 51(3):527-37.

77. Grunfeld, J. F., Barhum, Y., Blondheim, N., Rabey, J. M., Melamed, E., & Offen, D. (2007). Erythropoietin delays disease onset in an amyotrophic lateral sclerosis model. *Experimental neurology*, *204*(1), 260-263.

78. Guo, H., Ingolia, N. T., Weissman, J. S., and Bartel, D. P. (2010). Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature. 466(7308), 835– 840. doi: 10.1038/nature09267.

79. Gurdon, J. B. (1962). The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles. Development, 10(4), 622-640.

80. Hara, H., Ogawa, M. (1976). Erthropoietic precursors in mice with phenylhydrazine-induced anemia. Am J Hematol. 1(4), 453–458.

81. Harandi, O.F., Hedge, S., Wu, D.C., McKeone, D., Paulson, R.F. (2010). Murine erythroid short-term radioprotection requires a BMP4-dependent, self-renewing population of stress erythroid progenitors. J Clin Invest. 120(12), 4507– 4519.

82. Heeschen, C., Aicher, A., Lehmann, R., Fichtlscherer, S., Vasa, M., Urbich, C., ... & Dimmeler, S. (2003). Erythropoietin is a potent physiologic stimulus for endothelial progenitor cell mobilization. *Blood*, *102*(4), 1340-1346.

83. Hegde, S., Lenox, L.E., Lariviere, A., et al. (2007). An intronic sequence mutated in flexed-tail mice regulates splicing of Smad5. Mamm Genome. 18(12), 852–860.

84. Hiroyama, T., Miharada, K., Sudo, K., Danjo, I., Aoki, N., and Nakamura, Y. (2008). Establishment of mouse embryonic stem cell-derived erythroid progenitor cell lines able to produce functional red blood cells. *PLoS ONE* 6:3. doi: 10.1371/journal.pone.0001544.

85. Hojman, P., Brolin, C., Gissel, H., Brandt, C., Zerahn, B., Pedersen, B. K., & Gehl, J. (2009). Erythropoietin over-expression protects against diet-induced

obesity in mice through increased fat oxidation in muscles. *PloS one*, *4*(6), e5894.

86. Ispanovic, E., Serio, D., & Haas, T. L. (2008). Cdc42 and RhoA have opposing roles in regulating membrane type 1-matrix metalloproteinase localization and matrix metalloproteinase-2 activation. *American Journal of Physiology-Cell Physiology*, *295*(3), C600-C610.

87. Ivanovs, A., Rybtsov, S., Welch, L., Anderson, R. A., Turner, M. L., & Medvinsky, A. (2011). Highly potent human hematopoietic stem cells first emerge in the intraembryonic aorta-gonad-mesonephros region. Journal of Experimental Medicine, 208(12), 2417-2427.

88. Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gielbert, J., Gaskell, S. J., ... & Maxwell, P. H. (2001). Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science*, *292*(5516), 468-472.

89. Jackerott, M., Møldrup, A., Thams, P., Galsgaard, E. D., Knudsen, J., Lee, Y. C., & Nielsen, J. H. (2006). STAT5 activity in pancreatic β-cells influences the severity of diabetes in animal models of type 1 and 2 diabetes. *Diabetes*, *55*(10), 2705-2712.

90. Jacobs, K., Shoemaker, C., Rudersdorf, R., Neill, S. D., Kaufman, R. J., Mufson, A., & Kawakita, M. (1985). Isolation and characterization of genomic and cDNA clones of human erythropoietin. Nature, 313(6005), 806.

91. Jaenisch, R., & Young, R. (2008). Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. Cell, 132(4), 567-582.

92. Jing Hua Zhao and Qihua Tan "Integrated Analysis of Genetic Data with R" 2006 Human Genomics, vol 2, pp.258-265.

93. Juul, S. E., Anderson, D. K., Li, Y., & Christensen, R. D. (1998). Erythropoietin and erythropoietin receptor in the developing human central nervous system. Pediatric research, 43(1), 40.

94. Juul, S. E., Yachnis, A. T., Rojiani, A. M., & Christensen, R. D. (1999). Immunohistochemical localization of erythropoietin and its receptor in the developing human brain. *Pediatric and Developmental Pathology*, *2*(2), 148-158.

95. Kaptanoglu, E., Solaroglu, I., Okutan, O., Surucu, H. S., Akbiyik, F., & Beskonakli, E. (2004). Erythropoietin exerts neuroprotection after acute spinal cord injury in rats: effect on lipid peroxidation and early ultrastructural findings. *Neurosurgical review*, *27*(2), 113-120.

96. Kapur, R., & Zhang, L. (2001). A Novel Mechanism of Cooperation between c-Kit and Erythropoietin Receptor STEM CELL FACTOR INDUCES THE EXPRESSION OF Stat5 AND ERYTHROPOIETIN RECEPTOR, RESULTING IN EFFICIENT PROLIFERATION AND SURVIVAL BY ERYTHROPOIETIN. *Journal of Biological Chemistry*, *276*(2), 1099-1106.

97. Kassouf, M. T., Hughes, J. R., Taylor, S., McGowan, S. J., Soneji, S., Green, A. L., et al. (2010). Genomewide identification of TAL1's functional targets: insights into its mechanisms of action in primary erythroid cells. Genome Res. 20(8), 1064–1083, doi: 10.1101/gr. 104935.110.

98. Kerenyi, M. A., Grebien, F., Gehart, H., Schifrer, M., Artaker, M., Kovacic, B., ... & Müllner, E. W. (2008). Stat5 regulates cellular iron uptake of erythroid cells via IRP-2 and TfR-1. Blood, 112(9), 3878-3888.

99. Kolbus, A., Blazquez-Domingo, M., Carotta, S., et al. (2003). Cooperative signaling between cytokine receptors and the glucocorticoid receptor in the expansion of erythroid progenitors: molecular analysis by expression profiling. Blood. 102(9), 3136–3146.

100. Korpelainen, E. I., Gamble, J. R., Vadas, M. A., and Lopez, A.F. (1996). IL-3 receptor expression, regulation and function in cells of the vasculature. Immunol Cell Biol. 74(1), 1–7.

101. Koury, M.J. and Bondurant, M.C. (1990). Erythropoietin retards DNA breakdown and prevents programmed death in erythroid progenitor cells. Science. 248(4953), 378– 381.

102. Koury, S. T., Bondurant, M. C., Koury, M. J., & Semenza, G. L. (1991). Localization of cells producing erythropoietin in murine liver by in situ hybridization [see comments]. Blood, 77(11), 2497-2503.

103. Kozakov, D., Hall, D. R., Beglov, D., Brenke, R., Comeau, S. R., Shen, Y., ... & Vajda, S. (2010). Achieving reliability and high accuracy in automated protein docking: ClusPro, PIPER, SDU, and stability analysis in CAPRI rounds 13–19. *Proteins: Structure, Function, and Bioinformatics*, *78*(15), 3124-3130.

104. Kreja, L., Seidel, H.J. (1985) . On the role of the spleen in Friend virus (F-MuLV-P) erythroleukemia. Exp Hematol. 13(7), 623–628.

105. Kuo, Y. Y., and Chang, Z. F. (2007). GATA-1 and Gfi-1B interplay to regulate Bcl-xL transcription. Mol Cell Biol. 27, 4261–4272.

106. Lai, P. H., Everett, R., Wang, F. F., Arakawa, T., & Goldwasser, E. (1986). Structural characterization of human erythropoietin. Journal of Biological Chemistry, 261(7), 3116-3121.

107. Leberbauer, C., Boulme, F., Unfried, G., Huber, J., Beug, H., and Mullner, E. W. (2005). Different steroids coregulate long-term expansion versus terminal differentiation in primary human erythroid progenitors. *Blood* 105, 85–94. doi: 10.1182/blood-2004-03-1002

108. Lecuyer, E., Herblot, S., Saint-Denis, M., Martin, R., Begley, C. G., Porcher, C., et al. (2002). The SCL complex regulates c-kit expression in hematopoietic cells through functional interaction with Sp1. Blood 100, 2430–2440.

109. Lenox, L.E., Perry, J.M., Paulson, R.F. (2005). BMP4 and Madh5 regulate the erythroid response to acute anemia. Blood. 105(7), 2741–2748.

110. Lichtman, M. A., Beutler, E., Kipps, T. J., Kaushansky, K., Seligsohn, U., and Prchal, J. (2007). *Williams Hematology*. New York, NY: McGraw-Hill.

111. Lin, F. K., Suggs, S., Lin, C. H., Browne, J. K., Smalling, R., Egrie, J. C., & Stabinsky, Z. (1985). Cloning and expression of the human erythropoietin gene. Proceedings of the National Academy of Sciences, 82(22), 7580-7584.

112. Lindern, M., Schmidt, U., and Beug, H. (2004). Control of erythropoiesis by erythropoietin and stem cell factor: a novel role for Bruton's tyrosine kinase. Cell Cycle. 3(7), 876–879.

113. Listowski, M. A., Heger, E., Bogusł awska, D. M., Machnicka, B., Kuliczkowski, K., Leluk, J., et al. (2013). microRNAs: fine tuning of erythropoiesis. Cell Mol Biol Lett. 18(1), 34–46. doi: 10.2478/s11658-012-0038-z.

114. Lodish, H., Flygare, J., and Chou, S. (2010). From stem cell to erythroblast: regula-tion of red cell production at multiple levels by multiple hormones. *IUBMB Life* 62, 492–496. doi: 10.1002/iub.322

115. Love, P. E., Warzecha, C., and Li, L. (2014). Ldb1 complexes: the new master regulators of erythroid gene transcription. Trends Genet. 30(1), 1–9. doi: 10.1016/j.tig.2013.10.001.

116. Lu, J., Guo, S., and Ebert, B. L., et al. (2008). MicroRNA-mediated control of cell fate in megakaryocyte-erythrocyte progenitors. Dev Cell. 14(6), 843–853. doi: 10.1016/j.devcel.2008.03.012.

117. Luc Douay et al., Ex vivo generation of fully mature human red blood cells from haematopoietic stem cells, Nature Biotechnology 2005 23, 69 - 74.

118. Luckett, W. P. (1978). Origin and differentiation of the yolk sac and extraembryonic mesoderm in presomite human and rhesus monkey embryos. American Journal of Anatomy, 152(1), 59-97.

119. Lugus, J.J., Chung,Y.S., Mills, J.C. et al. (2007). GATA2 functions at multiple steps in hemangioblast development and differentiation. Development. 134(2), 393–405.

120. MacRedmond, R., Singhera, G. K., & Dorscheid, D. R. (2009). Erythropoietin inhibits respiratory epithelial cell apoptosis in a model of acute lung injury. *European Respiratory Journal*, *33*(6), 1403-1414.

121. Maiese, K., Li, F., & Chong, Z. Z. (2005). New avenues of exploration for erythropoietin. *Jama*, *293*(1), 90-95.

122. Mancini, D. M., & Kunavarapu, C. (2003). Effect of erythropoietin on exercise capacity in anemic patients with advanced heart failure. *Kidney International*, *64*(S87), S48.

123. Manwani, D., and Bieker, J. J. (2008). The erythroblastic island. *Curr. Top. Dev. Biol.* 82, 23–53. doi: 10.1016/S0070-2153(07)00002-6

124. Matsuda, T., Nakamura, T., Nakao, K., Arai, T., Katsuki, M., Heike, T., & Yokota, T. (1999). STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. The EMBO journal, 18(15), 4261-4269.

125. Matthias Eder, Georg Geissler, Arnold Ganser, IL-3 in the Clinic Stem Cells 1997;15:327-333.

126. Maximov, A. (1909). Investigations of blood and connective tissue 1. The earliest stages of development of blood and tissue cells in the mammalian embryo until the beginning of the hematopoiesis in the liver [in German]. Arch Mikroskop Anat, 73, 444-561.

127. McCulloch, E.A., Siminovitch, L., Till, J.E. (1964). Spleencolony formation in anemic mice of genotype Ww.Science. 144, 844–846.

128. Medvinsky, A., & Dzierzak, E. (1996). Definitive hematopoiesis is autonomously initiated by the AGM region. Cell, 86(6), 897-906.

129. Michael, R. T., and Perkins, C. A. (2010). KLF1 Directly Coordinates Almost All Aspects of Terminal Erythroid Differentiation. IUBMB Life, 62(12), 886–890. doi: 10.1002/iub.404.

130. Migliaccio, A. R., Masselli, E., Varricchio, L., and Whitsett, C. (2012). Ex-vivo expansion of red blood cells: how real for transfusion in humans? Blood Rev. 26, 81–95. doi: 10.1016/j.blre.2011.11.002.

131. Millot, S., Andrieu, V., Letteron, P. et al. (2010). Erythropoietin stimulates spleen BMP4-dependent stress erythropoiesis and partially corrects anemia in a mouse model of generalized inflammation. Blood. 116(26), 6072–6081.

132. Motoyoshi K, Clinical application of hematopoietic growth factor (IL-3, G-CSF, GM-CSF, and EPO) Nihon Rinsho 1992 Aug;50(8):1967-72.

133. Murray, P. D. F. (1932). The development in vitro of the blood of the early chick embryo. Proceedings of the Royal Society of London. Series B, Containing Papers of a Biological Character, 111(773), 497-521.

134. Neildez-Nguyen, T. M., Wajcman, H., Marden, M. C., Bensidhoum, M., Moncollin, V., Giarratana, M. C., et al. (2002). Human erythroid cells produced cx vivo at large scale differentiate into red blood cells *in vivo*. *Nat. Biotechnol.* 20, 467–472. doi: 10.1038/nbt0502-467

135. Niwa, H., Burdon, T., Chambers, I., & Smith, A. (1998). Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. Genes & development, 12(13), 2048-2060.

136. Nosaka, T., Kawashima, T., Misawa, K., Ikuta, K., Mui, A. L. F., & Kitamura, T. (1999). STAT5 as a molecular regulator of proliferation, differentiation and apoptosis in hematopoietic cells. The EMBO journal, 18(17), 4754-4765.

137. Obara, Y., & Nakahata, N. (2010). The signaling pathway leading to extracellular signal-regulated kinase 5 (ERK5) activation via G-proteins and ERK5-dependent neurotrophic effects. Molecular pharmacology, 77(1), 10-16.

138. Ohneda, O., Yanai, N., Obinata, M. (1990). Microenvironment created by stromal cells is essential for a rapid expansion of erythroid cells in mouse fetal liver. Development. 110(2), 379–384.

139. Okita, K., Ichisaka, T., and Yamanaka, S. (2007). Generation of germlilne-competent induced pluripotent stem cells. *Nature* 448, 313–317. doi: 10.1038/nature05934

140. Orkin, S. H. (1992). GATA-binding transcription factors in hematopoietic cells. Blood 80, 575–581.

141. Orkin, S. H. (2000). Diversification of hematopoietic stem cells to specific lineages. Nat Rev Genet 1, 57–64.

142. Ottersbach, K., & Dzierzak, E. (2005). The murine placenta contains hematopoietic stem cells within the vascular labyrinth region. Developmental cell, 8(3), 377-387.

143. Papetti, M., Wontakal, S.N., Stopka, T., and Skoultchi, A. I. (2010). GATA-1 directly regulates p21 gene expression during erythroid differentiation. Cell Cycle. 9(10), 1972– 80.

144. Parsa, C. J., Kim, J., Riel, R. U., Pascal, L. S., Thompson, R. B., Petrofski, J. A., ... & Koch, W. J. (2004). Cardioprotective Effects of Erythropoietin in the Reperfused Ischemic Heart A POTENTIAL ROLE FOR CARDIAC FIBROBLASTS. *Journal of Biological Chemistry*, *279*(20), 20655-20662.

145. Perry, J.M., Harandi, O.F., Porayette, P., Hegde, S., Kannan, A.K., Paulson, R.F. (2009). Maintenance of the BMP4-dependent stress erythropoiesis pathway in the murine spleen requires hedgehog signaling. Blood. 113(4), 911– 918.

146. Peschle, C., Mavilio, F., Care, A., Migliaccio, G., Migliaccio, A. R., Salvo, G., & Lazzaro, D. (1985). Haemoglobin switching in human embryos: asynchrony of ζ→ α and ε→ γ-globin switches in primitive and definitive erythropoietic lineage. Nature, 313(5999), 235.

147. Peschle, C., Migliaccio, A. R., Migliaccio, G., Petrini, M., Calandrini, M., Russo, G., ... & Comi, P. (1984). Embryonic----Fetal Hb switch in humans: studies on erythroid bursts generated by embryonic progenitors from yolk sac and liver. Proceedings of the National Academy of Sciences, 81(8), 2416-2420.

148. Pugh, C. W., & Ratcliffe, P. J. (2003). Regulation of angiogenesis by hypoxia: role of the HIF system. *Nature medicine*, *9*(6), 677.

149. Quelle, D. E., Quelle, F. W., & Wojchowski, D. M. (1992). Mutations in the WSAWSE and cytosolic domains of the erythropoietin receptor affect signal transduction and ligand binding and internalization. Molecular and Cellular Biology, 12(10), 4553-4561.

150. Rao, R. R., Calhoun, J. D., Qin, X., Rekaya, R., Clark, J. K., & Stice, S. L. (2004). Comparative transcriptional profiling of two human embryonic stem cell lines. Biotechnology and bioengineering, 88(3), 273-286.

151. Rastogi, A., & Nissenson, A. R. (2006). New approaches to the management of anemia of chronic kidney disease: Beyond Epogen and Infed. Kidney International, 70, S14-S16.

152. Ravet, E., Reynaud, D., Titeux, M., Izac, B., Fichelson, S., Roméo, P. H., et al. (2004). Characterization of DNA-binding-dependent and - independent functions of SCL/TAL1 during human erythropoiesis. Blood 103, 3326–3335.

153. Recny, M. A., Scoble, H. A., & Kim, Y. (1987). Structural characterization of natural human urinary and recombinant DNA-derived erythropoietin. Identification of des-arginine 166 erythropoietin. Journal of Biological Chemistry, 262(35), 17156-17163.

154. Reichardt, H.M., Kaestner, K.H., Tuckermann, J. et al. (1998). DNA binding of the glucocorticoid receptor is not essential for survival. Cell. 93(4), 531–541.

155. Reubinoff, B. E., Pera, M. F., Fong, C. Y., Trounson, A., & Bongso, A. (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nature biotechnology, 18(4), 399.

156. Rhodes, M. M., Kopsombut, P., Bondurant, M. C., Price, J. O., and Koury, M. J. (2008). Adherence to macrophages in erythroblastic islands enhances erythroblast proliferation and increases erythrocyte production by a different mechanism than erythropoietin. *Blood* 111, 1700–1708. doi: 10.1182/blood-2007-06-098178

157. Ribatti, D., Vacca, A., Roccaro, A. M., Crivellato, E., & Presta, M. (2003). Erythropoietin as an angiogenic factor. *European journal of clinical investigation*, *33*(10), 891-896.

158. Rosas, M., Gordon, S., and Taylor, P. R. (2007). Characterization of the expression and function of the GM-CSF receptor α-chain in mice. Eur J Immunol. 37(9), 2518–2528.

159. Rundqvist, H., Rullman, E., Sundberg, C. J., Fischer, H., Eisleitner, K., Ståhlberg, M., ... & Gustafsson, T. (2009). Activation of the erythropoietin receptor in human skeletal muscle. *European journal of endocrinology*, *161*(3), 427-434.

160. Sabin, F. R. (1920). Studies on the origin of blood-vessels and of red blood-corpuscles as seen in the living blastoderm of chicks during the second day of incubation. Contrib. Embryol Carneg Inst., 9, 214-262.

161. Santhanam, A. V. R., Smith, L. A., Akiyama, M., Rosales, A. G., Bailey, K. R., & Katusic, Z. S. (2005). Role of endothelial NO synthase phosphorylation in cerebrovascular protective effect of recombinant erythropoietin during subarachnoid hemorrhage–induced cerebral vasospasm. *Stroke*, *36*(12), 2731-2737.

162. Sasaki, H., Bothner, B., Dell, A., & Fukuda, M. (1987). Carbohydrate structure of erythropoietin expressed in Chinese hamster ovary cells by a human erythropoietin cDNA. Journal of Biological Chemistry, 262(25), 12059-12076.

163. Savino, C., Pedotti, R., Baggi, F., Ubiali, F., Gallo, B., Nava, S., ... & Vezzani, A. (2006). Delayed administration of erythropoietin and its non-erythropoietic derivatives ameliorates chronic murine autoimmune encephalomyelitis. *Journal of neuroimmunology*, *172*(1-2), 27-37.

164. Semenza, G. L., & Wang, G. L. (1992). A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer

at a site required for transcriptional activation. *Molecular and cellular biology*, *12*(12), 5447-5454.

165. Semenza, G. L., Nejfelt, M. K., Chi, S. M., & Antonarakis, S. E. (1991). Hypoxia-inducible nuclear factors bind to an enhancer element located 3'to the human erythropoietin gene. *Proceedings of the National Academy of Sciences*, *88*(13), 5680-5684.

166. Shingo, T., Sorokan, S. T., Shimazaki, T., & Weiss, S. (2001). Erythropoietin regulates the in vitro and in vivo production of neuronal progenitors by mammalian forebrain neural stem cells. *Journal of Neuroscience*, *21*(24), 9733-9743.

167. Silverberg, D. S., Wexler, D., Blum, M., Tchebiner, J. Z., Sheps, D., Keren, G., ... & Schwartz, I. (2003). The effect of correction of anaemia in diabetics and non- diabetics with severe resistant congestive heart failure and chronic renal failure by subcutaneous erythropoietin and intravenous iron. *Nephrology Dialysis Transplantation*, *18*(1), 141-146.

168. Singbrant, S., Karlsson, G., Ehinger, M. et al. (2010). Canonical BMP signaling is dispensable for hematopoietic stem cell function in both adult and fetal liver hematopoiesis, but essential to preserve colon architecture. Blood. 115(23), 4689–4698.

169. Singbrant, S., Moody, J.L., Blank, U. et al. (2006). Smad5 is dispensable for adult murine hematopoiesis. Blood. 108(12), 3707–3712.

170. Slaper-Cortenbach, I., Ploemacher, R., Lowenberg, B. (1987). Different stimulative effects of human bone marrow and fetal liver stromal cells on erythropoiesis in long-term culture. Blood. 69(1), 135–139.

171. Socolovsky, M., Fallon, A. E., Brugnara, C., & Lodish, H. F. (1999). Fetal anemia and apoptosis of red cell progenitors in Stat5a−/− 5b−/− mice: a direct role for Stat5 in Bcl-XL induction. Cell, 98(2), 181-191.

172. Socolovsky, M., Nam, H. S., Fleming, M. D., Haase, V. H., Brugnara, C., & Lodish, H. F. (2001). Ineffective erythropoiesis in Stat5a−/− 5b−/− mice due to decreased survival of early erythroblasts. Blood, 98(12), 3261-3273.

173. Storring, P. L., & Das, R. G. (1992). The International Standard for Recombinant DNA-derived Erythropoietin: collaborative study of four recombinant DNA-derived erythropoietins and two highly purified human urinary erythropoietins. Journal of Endocrinology, 134(3), 459-484.

174. Storring, P. L., Tiplady, R. J., Das, R. G., Rafferty, B., & Mistry, Y. G. (1996). Lectin-binding assays for the isoforms of human erythropoietin: comparison of urinary and four recombinant erythropoietins. Journal of endocrinology, 150(3), 401-412.

175. Storring, P. L., Tiplady, R. J., Gaines, R. D., Stenning, B. E., Lamikanra, A., Rafferty, B., & Lee, J. (1998). Epoetin alfa and beta differ in their erythropoietin isoform compositions and biological properties. British journal of haematology, 100(1), 79-89.

176. Suda, T., Suda, J., & Ogawa, M. (1983). Single-cell origin of mouse hemopoietic colonies expressing multiple lineages in variable combinations. Proceedings of the National Academy of Sciences, 80(21), 6689-6693.

177. Szabo, E., Rampalli, S., Risueno, R. M., Schnerch, A., Mitchell, R., Fiebig-Comyn, A., et al. (2010). Direct conversion of human fibroblasts to multilineage blood progenitors. *Nature* 468, 521–526. doi: 10.1038/nature09591

178. Tada, M., Takahama, Y., Abe, K., Nakatsuji, N., & Tada, T. (2001). Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells. Current Biology, 11(19), 1553-1558.

179. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S: Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007, 131:861–872.

180. Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126, 663–676. doi: 10.1016/j.cell.2006.07.024

181. Takeuchi, M., Inoue, N., Strickland, T. W., Kubota, M., Wada, M., Shimizu, R., ... & Kobata, A. (1989). Relationship between sugar chain structure and biological activity of recombinant human erythropoietin produced in Chinese hamster ovary cells. Proceedings of the National Academy of Sciences, 86(20), 7819-7822.

182. Tallack, M. R., & Perkins, A. C. (2010). Megakaryocyte-erythroid lineage promiscuity in EKLF null mouse blood. *haematologica*, *95*(1), 144-147.

183. Tam, R. C., Coleman, S. L., Tiplady, R. J., Storring, P. L., & Cotes, P. M. (1991). Comparisons of human, rat and mouse erythropoietins by isoelectric focusing: differences between serum and urinary erythropoietins. British journal of haematology, 79(3), 504-511.

184. Tavian, M., & Peault, B. (2003). Embryonic development of the human hematopoietic system. International Journal of Developmental Biology, 49(2-3), 243-250.

185. Teglund, S., McKay, C., Schuetz, E., Van Deursen, J. M., Stravopodis, D., Wang, D., ... & Ihle, J. N. (1998). Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. Cell, 93(5), 841-850.

186. Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., et al. (1998). Embryonic stem cell lines derived from human blastocysts. *Science* 282, 1145–1147.

187. Timmins, N. E., Athanasas, S., Günther, M., Buntine, P., and Nielsen, L. K. (2011). Ultrahigh-yield manufacture of red blood cells from hematopoietic stem cells. *Tissue Eng. Part. C Methods* 17, 1131–1137. doi: 10.1089/ten.tec.2011.0207

188. Tripic, T., Deng, W., Cheng, Y., Zhang, Y., Vakoc, C. R., Gregory, G. D., et al. (2009). SCL and associated proteins distinguish active from repressive GATA transcription factor complexes. Blood 113(10), 2191–2201. doi: 10.1182/blood-2008-07-169417.

189. Tsai, P. T., Ohab, J. J., Kertesz, N., Groszer, M., Matter, C., Gao, J., ... & Carmichael, S. T. (2006). A critical role of erythropoietin receptor in neurogenesis and post-stroke recovery. *Journal of Neuroscience*, *26*(4), 1269-1274.

190. Urao, N., Okigaki, M., Yamada, H., Aadachi, Y., Matsuno, K., Matsui, A., ... & Tatsumi, T. (2006). Erythropoietin-mobilized endothelial progenitors enhance reendothelialization via Akt-endothelial nitric oxide synthase activation and prevent neointimal hyperplasia. *Circulation research*, *98*(11), 1405-1413.

191. Villa, P., Van Beek, J., Larsen, A. K., Gerwien, J., Christensen, S., Cerami, A., ... & Torup, L. (2007). Reduced functional deficits, neuroinflammation, and secondary tissue damage after treatment of stroke by nonerythropoietic erythropoietin derivatives. *Journal of Cerebral Blood Flow & Metabolism*, *27*(3), 552-563.

192. Vlachos A., Ball, S., Dahl, N. et al. (2008). Diagnosing and treating Diamond Blackfan anaemia: results of an international clinical consensus conference. Br J Haematol. 142(6), 859–876.

193. Von Lindern, M., Zauner, W., Mellitzer, G., Steinlein, P., Fritsch, G., Huber, K., et al. (1999). The glucocorticoid receptor cooperates with the erythropoietin receptor and c-Kit to enhance and sustain proliferation of erythroid progenitors *in vitro*. *Blood* 94, 550–559.

194. Wadman, I. A., Osada, H., Grutz, G. G., Agulnick, A. D., Westphal, H., Forster, A., et al. (1997). The LIM-only protein Lmo2 is a bridging molecule assembling an erythroid, DNA-binding complex which includes the TAL1, E47, GATA-1 and Ldb1/NLI proteins. EMBO J 16, 3145–3157.

195. Wagner, K. U., Claudio, E., Rucker, E. 3., Riedlinger, G., Broussard, C., Schwartzberg, P. L., ... & Hennighausen, L. (2000). Conditional deletion of the Bcl-x gene from erythroid cells results in hemolytic anemia and profound splenomegaly. Development, 127(22), 4949-4958.

196. Wallace, R. S. (1995). Molecular systematic study of the Cactaceae: using chloroplast DNA variation to elucidate cactus phylogeny. *Bradleya*, *1995*(13), 1-13.

197. Wang, Q., Huang, Z., Xue, H., Jin, C., Ju, X. L., Han, J. D., et al. (2008). MicroRNA miR-24 inhibits erythropoiesis by targeting activin type I receptor ALK4. Blood. 111(2), 588–595.

198. Wasley, L. C., Timony, G., Murtha, P., Stoudemire, J., Dorner, A. J., Caro, J., ... & Kaufman, R. J. (1991). The importance of N-and O-linked oligosaccharides for the biosynthesis and in vitro and in vivo biologic activities of erythropoietin. Blood, 77(12), 2624-2632.

199. Wessely, O., Deiner, E.M., Beug, H., von Lindern, M. (1997). The glucocorticoid receptor is a key regulator of the decision between self-renewal and differentiation in erythroid progenitors. EMBO J. 16(2), 267–280.

200. Westenfelder, C., Biddle, D. L., & Baranowski, R. L. (1999). Human, rat, and mouse kidney cells express functional erythropoietin receptors. *Kidney international*, *55*(3), 808-820.

201. Westenfelder, C., Biddle, D. L., & Baranowski, R. L. (1999). Human, rat, and mouse kidney cells express functional erythropoietin receptors. *Kidney international*, *55*(3), 808-820.

202. Wide, L., & Bengtsson, C. (1990). Molecular charge heterogeneity of human serum erythropoietin. British journal of haematology, 76(1), 121-127.

203. Witthuhn, B. A., Quelle, F. W., Silvennoinen, O., Yi, T., Tang, B., Miura, O., & Ihle, J. N. (1993). JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. Cell, 74(2), 227-236.

204. Wittig, I., & Groner, B. (2005). Signal transducer and activator of transcription 5 (STAT5), a crucial regulator of immune and cancer cells. *Current Drug Targets-Immune, Endocrine & Metabolic Disorders*, *5*(4), 449-463.

205. Wojchowski, D. M., Gregory, R. C., Miller, C. P., Pandit, A. K., & Pircher, T. J. (1999). Signal transduction in the erythropoietin receptor system. Experimental cell research, 253(1), 143-156.

206. Wojchowski, D. M., Gregory, R. C., Miller, C. P., Pandit, A. K., & Pircher, T. J. (1999). Signal transduction in the erythropoietin receptor system. Experimental cell research, 253(1), 143-156.

207. World Health Organization. (2011). Global Database on Blood Safety: Summary Report 2011. Available online at: http://www.who.int/bloodsafety/ global_database/GDBS_ Summary_Report_2011.pdf (Accessed May 4, 2014).

208. Wu , D.C., Paulson, R.F. (2005). Hypoxia regulates BMP4 expression in the murine spleen during the recovery from acute anemia. PLoS One. 5(6), e11303.

209. Xu, R. H., Peck, R. M., Li, D. S., Feng, X., Ludwig, T., & Thomson, J. A. (2005). Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nature methods, 2(3), 185.

210. Yamanaka S, Blau HM. Nuclear reprogramming to a pluripotent state by three approaches. Nature. 2010 Jun;465 (7299):704.

211. Yamanaka S. Strategies and new developments in the generation of patient-specific pluripotent stem cells. Cell stem cell. 2007 Jun 7;1(1):39-49.

212. Yanai, N., Matsuya, Y., Obinata, M. (1989). Spleen stromal cell lines selectively support erythroid colony formation. Blood. 74(7), 2391–2397.

213. Yang, J., & Zhang, Y. (2015). Protein structure and function prediction using I- TASSER. *Current protocols in bioinformatics*, *52*(1), 5-8.

214. Ying, Q. L., Nichols, J., Chambers, I., & Smith, A. (2003). BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. Cell, 115(3), 281-292.

215. Yu X, Shacka JJ, Eells JB et al. Erythropoietin receptor signalling is required for normal brain development. Development 2002; 129: 505–516.

216. Yu, M., Riva, L., Xie, H., Schindler, Y., Moran, T.B., Cheng, y., et al. (2009). Insights into GATA-1- mediated gene activation versus repression via genome - wide chromatin occupancy analysis. Mol Cell. 36(4), 682–695. doi: 10.1016/j.molcel.2009.11.002.

217. Zanjani, E. D., Ascensao, J. L., McGlave, P. B., Banisadre, M., & Ash, R. C. (1981). Studies on the liver to kidney switch of erythropoietin production. The Journal of clinical investigation, 67(4), 1183-1188.

218. Zhao, W., Ji, X., Zhang, F., Li, L., & Ma, L. (2012). Embryonic stem cell markers. *Molecules*, *17*(6), 6196-6236.

Appendix I

REAGENTS AND BUFFERS

(1) 1X Phosphate-buffered saline (PBS; pH-7.4)

NaCl : 8g

KCl : 0.2g

Na_2HpO_4 : 1.44g

KH_2PO_4 : 0.24g

ddH_2O : Adjust the volume to one litre and stored at room temperature

(2) 1X Phosphate-buffered saline (PBS; pH-7.2)

NaCl : 8g

KCl : 0.2g

Na_2HpO_4 : 1.44g

KH_2PO_4 : 0.2g

ddH_2O : Adjust the volume to one litre and stored at room temperature

(3) Typan Blue (0.4%)

Trypan Blue :0.4mg

PBS :1ml

(4) Propidium Iodide (1mg /ml stock)

Propidium Iodide :1mg

PBS :1ml

(5) Paraformaldehyde 2%

Paraformaldehyde :2g

PBS :100ml

APPENDIX II

Program:

Java Program for Matching Marker Genes with the Genes of MicroArray Data:

```
package javaapplication77;

import java.io.FileInputStream;

import java.io.FileNotFoundException;

import java.io.IOException;

import javax.swing.JFileChooser;

import org.apache.poi.xwpf.extractor.XWPFWordExtractor;

import org.apache.poi.xwpf.usermodel.XWPFDocument;

public class GenesComparison {

   String Store;

   String Store1;

   public void GeneList1() throws IOException  {        int i;

   String str1;        String str2;        int count5=0;        int count6=0;

    try {       JFileChooser Chooser = new JFileChooser();

    int returnvalue = Chooser.showOpenDialog(null);

    if(returnvalue==JFileChooser.APPROVE_OPTION){

    XWPFDocument document = new XWPFDocument (new FileInputStream(Chooser.getSelectedFile()));

    XWPFWordExtractor extract = new  XWPFWordExtractor(document);

    Store = extract.getText();                for(i =0; i<=170;i++)      {          str1=Store.split("\n")[i];

    str2= str1;           count5++;           count6=count5;

    }        }       }     catch(FileNotFoundException jk)      {

    }      catch(Exception ml)      {

    }   }       public void GeneList2() throws IOException{

      int j;       int count1=0;       String str3;       String str4;

      try {           JFileChooser Chooser = new JFileChooser();

     int returnvalue = Chooser.showOpenDialog(null);

    if(returnvalue==JFileChooser.APPROVE_OPTION){
```

```
XWPFDocument document = new XWPFDocument (new FileInputStream(Chooser.getSelectedFile()));
XWPFWordExtractor extract = new  XWPFWordExtractor(document);
Store1=extract.getText ();          }      } catch (FileNotFoundException jk) {
 } catch(Exception fg)   {
} }
String Str1;       String Str2;       String Str3;       String Str4;       String Str5;
 int i;        int j;       int Count=0;       int Count1=0;
 int Count2=0;        int y=0;
String CommonGene="A";
int count=0;        int count1=0;       int k;
public void CompareGenes()   {      try{      new Thread()  {            public void run(){
for(;;)   {         for(i=0;i<=170;i++)   {      Str1=Store.split("\n")[i];          Str2=Str1;
for(j=0;j<=34107;j++)    {          Str3=Store1.split("\n")[j];              Str4=Str3;
if(Str2.equals(Str4))     {              Count++;                System.out.println(Str2+" MATCHED "+Str4);
for(k=j+1;k<=34107;k++)     {          Str5=Store1.split("\n")[k];
if(Str4.equals(Str5))      {          count++;                 }  else   {
}    }     }     else       {
}       Count1++;    }          Count2++;
System.out.println(Count+" = Number Of Total matched Gene");
System.out.println(count+" = Number Of Duplicate matched Gene");
}     System.out.println(Count+" = Number Of Total Matched Genes");
      System.out.println(count+" = Number Of Duplicate Matched Genes");  }      }      }.     start();
      }  catch    (Exception hj)      {
  }    }    public static void main(String []args) throws IOException     {     GenesComparison m = new
GenesComparison();
       m.GeneList1();          m.GeneList2();          m.CompareGenes();       }     }
```

Program:

Java Program for Checking Pluripotency Level in a particular cell line.

Java Program
/*

```
 * To change this license header, choose License Headers in Project Properties.
 * To change this template file, choose Tools | Templates
 * and open the template in the editor.
 */
package javaapplication77;

import java.io.FileInputStream;
import java.io.FileNotFoundException;
import java.io.IOException;
import java.sql.Connection;
import java.sql.DriverManager;
import java.sql.PreparedStatement;
import java.sql.ResultSet;
import javax.swing.JFileChooser;
import javax.swing.JOptionPane;
import org.apache.poi.xssf.usermodel.XSSFCell;
import org.apache.poi.xssf.usermodel.XSSFRow;
import org.apache.poi.xssf.usermodel.XSSFSheet;
import org.apache.poi.xssf.usermodel.XSSFWorkbook;
public class Pluritest {
        String value=null;          String value1=null;         String value2=null;         String value3=null;          String value4=null;
        String value5=null;         String value6=null;         String value7=null;         String value8=null;
        String value9=null;         String value10=null;        String value11=null;        String value12=null;
        String Svalue1=null;        String Svalue2=null;        String Svalue3=null;        String Svalue4=null;
        String Svalue5=null;        String Svalue6=null;        String Svalue7=null;        String Svalue8=null;
        String Svalue9=null;        String Svalue10=null;       String Svalue11=null;       String Svalue12=null;
        String Nvalue1=null;        String Nvalue2=null;        String Nvalue3=null;        String Nvalue4=null;
        String Nvalue5=null;        String Nvalue6=null;        String Nvalue7=null;        String Nvalue8=null;
        String Nvalue9=null;        String Nvalue10=null;       String Nvalue11=null;       String Nvalue12=null;
        String Value1=null;         String Value2=null;         String Value3=null;         String Value4=null;
        String Value5=null;         String Value6=null;         String Value7=null;         String Value8=null;
        String Value9=null;         String Value10=null;        String Value11=null;        String Value12=null;
    public void getFile()throws IOException  {
        int Count=0;
        try {
            JFileChooser Chooser = new JFileChooser();
            int returnvalue = Chooser.showOpenDialog(null);
            if(returnvalue==JFileChooser.APPROVE_OPTION)
{          int reply= JOptionPane.showConfirmDialog(null,"Is Data Normalized or Not?","Question
Message",JOptionPane.YES_NO_OPTION);
           if(reply==JOptionPane.YES_OPTION){
           XSSFWorkbook workbook = new XSSFWorkbook  (new FileInputStream(Chooser.getSelectedFile()));
           XSSFSheet sheet = workbook.getSheet("Sheet1");
            XSSFRow row = sheet.getRow(0);
            int colnum= row.getLastCellNum();
            int rownum = sheet.getLastRowNum()+1;
             System.out.println(String.valueOf(colnum));
             System.out.println(String.valueOf(rownum));
             for(int i=1; i<=rownum;i++)    {   XSSFRow Row = sheet.getRow(i);    for(int j=1;j<2;j++)      {
             XSSFCell cell = Row.getCell(j);    value= cell.toString();       if(value.equalsIgnoreCase("POU5F1")){
              System.out.println(value);        int number = cell.getRowIndex()+1;
              XSSFRow Row1 = sheet.getRow(number-1);                XSSFRow Row2 = sheet.getRow(0);
              for(int z=2;z<3;z++ )      { XSSFCell cell1=Row1.getCell(z);           value1 = cell1.toString();
              XSSFCell cell2=Row2.getCell(z);         Value1 = cell2.toString();
    }
                        for(int a=3;a<4;a++)           {  XSSFCell cell1=Row1.getCell(a);           value2 = cell1.toString();
                        XSSFCell cell2=Row2.getCell(a);                             Value2 = cell2.toString();
                    }
                        for(int a=4;a<5;a++)           {  XSSFCell cell1=Row1.getCell(a);           value3 = cell1.toString();
                        XSSFCell cell2=Row2.getCell(a);                             Value3 = cell2.toString();
                    }
                        for(int a=5;a<6;a++)          {    XSSFCell cell1=Row1.getCell(a);           value4 = cell1.toString();
                        XSSFCell cell2=Row2.getCell(a);                             Value4 = cell2.toString();
                    }
                        for(int a=6;a<7;a++)        {  XSSFCell cell1=Row1.getCell(a);              value5 = cell1.toString();
                        XSSFCell cell2=Row2.getCell(a);                                   Value5 = cell2.toString();
                    }
                        for(int a=7;a<8;a++)         {  XSSFCell cell1=Row1.getCell(a);              value6 = cell1.toString();
                        XSSFCell cell2=Row2.getCell(a);                              Value6 = cell2.toString();
                    }
                        for(int a=8;a<9;a++)          {   XSSFCell cell1=Row1.getCell(a);           value7 = cell1.toString();
                        XSSFCell cell2=Row2.getCell(a);                             Value7 = cell2.toString();
                }
                        for(int a=9;a<10;a++)        {    XSSFCell cell1=Row1.getCell(a);           value8 = cell1.toString();
```

```
                XSSFCell cell2=Row2.getCell(a);                                    Value8 = cell2.toString();
            }
                for(int a=10;a<11;a++)      {   XSSFCell cell1=Row1.getCell(a);          value9 = cell1.toString();
                XSSFCell cell2=Row2.getCell(a);                                    Value9 = cell2.toString();
            }
                for(int a=11;a<12;a++)      {   XSSFCell cell1=Row1.getCell(a);          value10 = cell1.toString();
                XSSFCell cell2=Row2.getCell(a);                          Value10 = cell2.toString();
            }
                for(int a=12;a<13;a++)      {   XSSFCell cell1=Row1.getCell(a);          value11 = cell1.toString();
                XSSFCell cell2=Row2.getCell(a);                                    Value11 = cell2.toString();
           }
                for(int a=13;a<14;a++)      {  XSSFCell cell1=Row1.getCell(a);          value12 = cell1.toString();
                XSSFCell cell2=Row2.getCell(a);                                    Value12 = cell2.toString();

        System.out.println(Value12);    }     System.out.println(number);       }
        if(value.equalsIgnoreCase("SOX2"))    {     System.out.println(value);
        int number = cell.getRowIndex()+1;     XSSFRow Row1 = sheet.getRow(number-1);

              for(int z=2;z<3;z++)          {    XSSFCell cell1=Row1.getCell(z);        Svalue1 = cell1.toString();
            }
              for(int a=3;a<4;a++)          {   XSSFCell cell1=Row1.getCell(a);         Svalue2 = cell1.toString();
        }
              for(int a=4;a<5;a++)          {  XSSFCell cell1=Row1.getCell(a);          Svalue3 = cell1.toString();
        }
              for(int a=5;a<6;a++)          {  XSSFCell cell1=Row1.getCell(a);          Svalue4 = cell1.toString();
        }
              for(int a=6;a<7;a++)          {  XSSFCell cell1=Row1.getCell(a);          Svalue5 = cell1.toString();
        }
              for(int a=7;a<8;a++)          {  XSSFCell cell1=Row1.getCell(a);          Svalue6 = cell1.toString();
        }
              for(int a=8;a<9;a++)          {   XSSFCell cell1=Row1.getCell(a);         Svalue7 = cell1.toString();
        }
              for(int a=9;a<10;a++)         {   XSSFCell cell1=Row1.getCell(a);         Svalue8 = cell1.toString();
        }
              for(int a=10;a<11;a++)        {   XSSFCell cell1=Row1.getCell(a);         Svalue9 = cell1.toString();
        }
              for(int a=11;a<12;a++)        {   XSSFCell cell1=Row1.getCell(a);         Svalue10 = cell1.toString();
        }
              for(int a=12;a<13;a++)        {   XSSFCell cell1=Row1.getCell(a);         Svalue11 = cell1.toString();

        }      for(int a=13;a<14;a++)       {   XSSFCell cell1=Row1.getCell(a);         Svalue12 = cell1.toString();

for(int z=2;z<3;z++)                {   XSSFCell cell1=Row1.getCell(z);          Nvalue1 = cell1.toString();
}   for(int a=3;a<4;a++)            {    XSSFCell cell1=Row1.getCell(a);         Nvalue2 = cell1.toString();
}   for(int a=4;a<5;a++)            {   XSSFCell cell1=Row1.getCell(a);          Nvalue3 = cell1.toString();
}   for(int a=5;a<6;a++)            {   XSSFCell cell1=Row1.getCell(a);          Nvalue4 = cell1.toString();
}   for(int a=6;a<7;a++)            {   XSSFCell cell1=Row1.getCell(a);          Nvalue5 = cell1.toString();
}   for(int a=7;a<8;a++)            {  XSSFCell cell1=Row1.getCell(a);           Nvalue6 = cell1.toString();
}    for(int a=8;a<9;a++)           {   XSSFCell cell1=Row1.getCell(a);          Nvalue7 = cell1.toString();
}    for(int a=9;a<10;a++ )         {   XSSFCell cell1=Row1.getCell(a);          Nvalue8 = cell1.toString();
}   for(int a=10;a<11;a++)          {   XSSFCell cell1=Row1.getCell(a);          Nvalue9 = cell1.toString();
}    for(int a=11;a<12;a++)         {   XSSFCell cell1=Row1.getCell(a);          Nvalue10 = cell1.toString();
}    for(int a=12;a<13;a++)         {   XSSFCell cell1=Row1.getCell(a);          Nvalue11 = cell1.toString();
}    for(int a=13;a<14;a++)                  {   XSSFCell cell1=Row1.getCell(a);          Nvalue12 = cell1.toString();
}    System.out.println(number);      }     }
          } }         else if(reply==JOptionPane.NO_OPTION )        {
          } } }       catch(FileNotFoundException jk)   {    }     catch(Exception lk)     { }
        }
                float NanogGSM1854259;      float NanogGSM1854260;      float NanogGSM1854261;      float
NanogGSM1854262;
                float NanogGSM1854263;      float NanogGSM1854264;      float NanogGSM1854265;      float
NanogGSM1854267;
                float NanogGSM1854268;      float NanogGSM1854269;      float NanogGSM1854270;      float
NanogGSM1854266;
                float Pou5f1GSM1854259;     float Pou5f1GSM1854260;     float Pou5f1GSM1854261;     float
            Pou5f1GSM1854262;
                float Pou5f1GSM1854263;     float Pou5f1GSM1854264;     float Pou5f1GSM1854265;     float
Pou5f1GSM1854266;
              float Pou5f1GSM1854267;     float Pou5f1GSM1854268;    float Pou5f1GSM1854269;    float
Pou5f1GSM1854270;
              float Sox2GSM1854259;       float Sox2GSM1854260;       float Sox2GSM1854261;       float
Sox2GSM1854262;
              float Sox2GSM1854263;       float Sox2GSM1854264;       float Sox2GSM1854265;       float
Sox2GSM1854266;
```

```
            float Sox2GSM1854267;        float Sox2GSM1854268;        float Sox2GSM1854269;        float
Sox2GSM1854270;
 public void Fetchdata()
 {     Pou5f1GSM1854259 = Float.valueOf(value1);
Pou5f1GSM1854260= Float.valueOf(value2);          Pou5f1GSM1854261= Float.valueOf(value3);
Pou5f1GSM1854262= Float.valueOf(value4);          Pou5f1GSM1854263= Float.valueOf(value5);
Pou5f1GSM1854264= Float.valueOf(value6);          Pou5f1GSM1854265= Float.valueOf(value7);
Pou5f1GSM1854266= Float.valueOf(value8);          Pou5f1GSM1854267= Float.valueOf(value9);
Pou5f1GSM1854268= Float.valueOf(value10);                 Pou5f1GSM1854269= Float.valueOf(value11);
Pou5f1GSM1854270= Float.valueOf(value12);         NanogGSM1854259 =Float.valueOf(Nvalue1);
NanogGSM1854260 =Float.valueOf(Nvalue2);                  NanogGSM1854261 =Float.valueOf(Nvalue3);
NanogGSM1854262 =Float.valueOf(Nvalue4);                  NanogGSM1854263 =Float.valueOf(Nvalue5);
NanogGSM1854264 =Float.valueOf(Nvalue6);                  NanogGSM1854265 =Float.valueOf(Nvalue7);
NanogGSM1854266 =Float.valueOf(Nvalue8);          NanogGSM1854267 =Float.valueOf(Nvalue9);
NanogGSM1854268 =Float.valueOf(Nvalue10);         NanogGSM1854269 =Float.valueOf(Nvalue11);
NanogGSM1854270 =Float.valueOf(Nvalue12);           Sox2GSM1854259= Float.valueOf(Svalue1);
Sox2GSM1854260= Float.valueOf(Svalue2);           Sox2GSM1854261= Float.valueOf(Svalue3);
Sox2GSM1854262= Float.valueOf(Svalue4);                   Sox2GSM1854263= Float.valueOf(Svalue5);
Sox2GSM1854264= Float.valueOf(Svalue6);             Sox2GSM1854265= Float.valueOf(Svalue7);
Sox2GSM1854266= Float.valueOf(Svalue8);           Sox2GSM1854267= Float.valueOf(Svalue9);
Sox2GSM1854268= Float.valueOf(Svalue10);          Sox2GSM1854269= Float.valueOf(Svalue11);
Sox2GSM1854270= Float.valueOf(Svalue12);
 }  public void CompareExpression()
{
if(Value1.contains("GSM")&&Value2.contains("GSM")&&Value3.contains("GSM")&&Value4.contains("GSM")&&Value5.cont
ains("GSM")&&Value6.contains("GSM")&&Value7.contains("GSM")&&Value8.contains("GSM")&&Value9.contains("GSM")
&&Value10.contains("GSM")&&Value11.contains("GSM")){
      if(NanogGSM1854259>=5.0&&NanogGSM1854259<=9.0){
       if(Pou5f1GSM1854259>=5.0&&Pou5f1GSM1854259<=9.0){
          if(Sox2GSM1854259>=5.0&&Sox2GSM1854259<=9.0){
             System.out.println("Range: >=0.3 to <=3.0");
             System.out.println("Range: GSM1854259\nNANOG= "+NanogGSM1854259);
             System.out.println("POU5F1= "+Pou5f1GSM1854259);
             System.out.println("SOX2= "+Sox2GSM1854259);
             System.out.println("Highly Pluripotent Cell= "+"GSM1854259");
          }
       }
      }else if(NanogGSM1854259>=2.0&&NanogGSM1854259<=4.9){
       if(Pou5f1GSM1854259>=2.0&&Pou5f1GSM1854259<=4.9){
          if(Sox2GSM1854259>=2.0&&Sox2GSM1854259<=4.9){
             System.out.println("Partial Pluripotent Cell= "+"GSM1854259");
          }
       }
      }else if(NanogGSM1854259>=-3.0&&NanogGSM1854259<=1.9){
       if(Pou5f1GSM1854259>=-3.0&&Pou5f1GSM1854259<=1.9){
          if(Sox2GSM1854259>=-3.0&&Sox2GSM1854259<=1.9){
             System.out.println("Range: >=-3.0 to <=1.9");
             System.out.println("Range: GSM1854259\nNANOG= "+NanogGSM1854259);
             System.out.println("POU5F1= "+Pou5f1GSM1854259);
             System.out.println("SOX2= "+Sox2GSM1854259);
             System.out.println("Less Pluripotent Cell= "+"GSM1854259");
          }
       }
      }
       if(NanogGSM1854260>=5.0&&NanogGSM1854260<=9.0){
       if(Pou5f1GSM1854260>=5.0&&Pou5f1GSM1854260<=9.0){
          if(Sox2GSM1854260>=5.0&&Sox2GSM1854260<=9.0){
              System.out.println("Range: >=0.3 to <=3.0");
             System.out.println("Range: GSM1854260\nNANOG= "+NanogGSM1854260);
             System.out.println("POU5F1= "+Pou5f1GSM1854260);
             System.out.println("SOX2= "+Sox2GSM1854260);
             System.out.println("Highly Pluripotent Cell= "+"GSM1854260");
          }
       }
      }else if(NanogGSM1854260>=2.0&&NanogGSM1854260<=4.9){
       if(Pou5f1GSM1854260>=2.0&&Pou5f1GSM1854260<=4.9){
          if(Sox2GSM1854260>=2.0&&Sox2GSM1854260<=4.9){
             System.out.println("Partial Pluripotent Cell= "+"GSM1854260");
          }      }
      }     else if(NanogGSM1854260>=-3.0&&NanogGSM1854260<=1.9)   {
          if(Pou5f1GSM1854260>=-3.0&&Pou5f1GSM1854260<=1.9)    {
          if(Sox2GSM1854260>=-3.0&&Sox2GSM1854260<=1.9)   {
             System.out.println("Range: >=-3.0 to <=1.9");
             System.out.println("Range: GSM1854260\nNANOG= "+NanogGSM1854260);
```

```
            System.out.println("POU5F1= "+Pou5f1GSM1854260);
            System.out.println("SOX2= "+Sox2GSM1854260);
            System.out.println("Less Pluripotent Cell= "+"GSM1854260");
        }
    }
}
    if(NanogGSM1854261>=5.0&&NanogGSM1854261<=9.0){
    if(Pou5f1GSM1854261>=5.0&&Pou5f1GSM1854261<=9.0){
        if(Sox2GSM1854261>=5.0&&Sox2GSM1854261<=9.0){
             System.out.println("Range: >=0.3 to <=3.0");
            System.out.println("Range: GSM1854261\nNANOG= "+NanogGSM1854261);
            System.out.println("POU5F1= "+Pou5f1GSM1854261);
            System.out.println("SOX2= "+Sox2GSM1854261);
            System.out.println("Highly Pluripotent Cell= "+"GSM1854261");
        }
    }
}else if(NanogGSM1854261>=2.0&&NanogGSM1854261<=4.9){
    if(Pou5f1GSM1854261>=2.0&&Pou5f1GSM1854261<=4.9){
        if(Sox2GSM1854261>=2.0&&Sox2GSM1854261<=4.9){
            System.out.println("Partial Pluripotent Cell= "+"GSM1854261");
        }
    }
}else if(NanogGSM1854261>=-3.0&&NanogGSM1854261<=1.9){
    if(Pou5f1GSM1854261>=-3.0&&Pou5f1GSM1854261<=1.9){
        if(Sox2GSM1854261>=-3.0&&Sox2GSM1854261<=1.9){
             System.out.println("Range: >=-3.0 to <=1.9");
            System.out.println("Range: GSM1854261\nNANOG= "+NanogGSM1854261);
            System.out.println("POU5F1= "+Pou5f1GSM1854261);
            System.out.println("SOX2= "+Sox2GSM1854261);
            System.out.println("Less Pluripotent Cell= "+"GSM1854261");
        }
    }
}
     if(NanogGSM1854262>=5.0&&NanogGSM1854262<=9.0){
    if(Pou5f1GSM1854262>=5.0&&Pou5f1GSM1854262<=9.0){
        if(Sox2GSM1854262>=5.0&&Sox2GSM1854262<=9.0){
             System.out.println("Range: >=0.3 to <=3.0");
            System.out.println("Range: GSM1854262\nNANOG= "+NanogGSM1854262);
            System.out.println("POU5F1= "+Pou5f1GSM1854262);
            System.out.println("SOX2= "+Sox2GSM1854262);
            System.out.println("Highly Pluripotent Cell= "+"GSM1854262");
        }
    }
}else if(NanogGSM1854262>=2.0&&NanogGSM1854262<=4.9){
    if(Pou5f1GSM1854262>=2.0&&Pou5f1GSM1854262<=4.9){
        if(Sox2GSM1854262>=2.0&&Sox2GSM1854262<=4.9){
            System.out.println("Partial Pluripotent Cell= "+"GSM1854262");
        }
    }
}else if(NanogGSM1854262>=-3.0&&NanogGSM1854262<=1.9){
    if(Pou5f1GSM1854262>=-3.0&&Pou5f1GSM1854262<=1.9){
        if(Sox2GSM1854262>=-3.0&&Sox2GSM1854262<=1.9){
             System.out.println("Range: >=-3.0 to <=1.9");
            System.out.println("Range: GSM1854262\nNANOG= "+NanogGSM1854262);
            System.out.println("POU5F1= "+Pou5f1GSM1854262);
        }
    }
}
     if(NanogGSM1854263>=5.0&&NanogGSM1854263<=9.0){
    if(Pou5f1GSM1854263>=5.0&&Pou5f1GSM1854263<=9.0){
        if(Sox2GSM1854263>=5.0&&Sox2GSM1854263<=9.0){
             System.out.println("Range: >=0.3 to <=3.0");
            System.out.println("Range: GSM1854263\nNANOG= "+NanogGSM1854263);
            System.out.println("POU5F1= "+Pou5f1GSM1854263);
        }
    }
}else if(NanogGSM1854263>=2.0&&NanogGSM1854263<=4.9){
    if(Pou5f1GSM1854263>=2.0&&Pou5f1GSM1854263<=4.9){
        if(Sox2GSM1854263>=2.0&&Sox2GSM1854263<=4.9){
            System.out.println("Partial Pluripotent Cell= "+"GSM1854263");
        }
    }
}else if(NanogGSM1854263>=-3.0&&NanogGSM1854263<=1.9){
    if(Pou5f1GSM1854263>=-3.0&&Pou5f1GSM1854263<=1.9){
        if(Sox2GSM1854263>=-3.0&&Sox2GSM1854263<=1.9){
```

```
          System.out.println("Range: >=-3.0 to <=1.9");
          System.out.println("Range: GSM1854263\nNANOG= "+NanogGSM1854263);
          System.out.println("POU5F1= "+Pou5f1GSM1854263);
          System.out.println("SOX2= "+Sox2GSM1854263);
          System.out.println("Less Pluripotent Cell= "+"GSM1854263");
      }
   }
}
    if(NanogGSM1854264>=5.0&&NanogGSM1854264<=9.0){
  if(Pou5f1GSM1854264>=5.0&&Pou5f1GSM1854264<=9.0){
     if(Sox2GSM1854264>=5.0&&Sox2GSM1854264<=9.0){
          System.out.println("Range: >=0.3 to <=3.0");
          System.out.println("Range: GSM1854264\nNANOG= "+NanogGSM1854264);
          System.out.println("POU5F1= "+Pou5f1GSM1854264);
          System.out.println("SOX2= "+Sox2GSM1854264);
          System.out.println("Highly Pluripotent Cell= "+"GSM1854264");
      }
   }
}else if(NanogGSM1854264>=2.0&&NanogGSM1854264<=4.9){
  if(Pou5f1GSM1854264>=2.0&&Pou5f1GSM1854264<=4.9){
     if(Sox2GSM1854264>=2.0&&Sox2GSM1854264<=4.9){
          System.out.println("Partial Pluripotent Cell= "+"GSM1854264");
      }
   }
}else if(NanogGSM1854264>=-3.0&&NanogGSM1854264<=1.9){
  if(Pou5f1GSM1854264>=-3.0&&Pou5f1GSM1854264<=1.9){
     if(Sox2GSM1854264>=-3.0&&Sox2GSM1854264<=1.9){
          System.out.println("Range: >=-3.0 to <=1.9");
          System.out.println("Range: GSM1854264\nNANOG= "+NanogGSM1854264);
          System.out.println("POU5F1= "+Pou5f1GSM1854264);
          System.out.println("SOX2= "+Sox2GSM1854264);
          System.out.println("Less Pluripotent Cell= "+"GSM1854264");
      }
   }
}
    if(NanogGSM1854265>=5.0&&NanogGSM1854265<=9.0){
  if(Pou5f1GSM1854265>=5.0&&Pou5f1GSM1854265<=9.0){
     if(Sox2GSM1854265>=5.0&&Sox2GSM1854265<=9.0){
          System.out.println("Range: >=0.3 to <=3.0");
          System.out.println("Range: GSM1854265\nNANOG= "+NanogGSM1854265);
          System.out.println("POU5F1= "+Pou5f1GSM1854265);
          System.out.println("SOX2= "+Sox2GSM1854265);
          System.out.println("Highly Pluripotent Cell= "+"GSM1854265");
      }
   }
}else if(NanogGSM1854265>=2.0&&NanogGSM1854265<=4.9){
  if(Pou5f1GSM1854265>=2.0&&Pou5f1GSM1854265<=4.9){
     if(Sox2GSM1854265>=2.0&&Sox2GSM1854265<=4.9){
          System.out.println("Partial Pluripotent Cell= "+"GSM1854265");
      }
   }
}else if(NanogGSM1854265>=-3.0&&NanogGSM1854265<=1.9){
  if(Pou5f1GSM1854265>=-3.0&&Pou5f1GSM1854265<=1.9){
     if(Sox2GSM1854265>=-3.0&&Sox2GSM1854265<=1.9){
          System.out.println("Range: >=-3.0 to <=1.9");
          System.out.println("Range: GSM1854265\nNANOG= "+NanogGSM1854265);
          System.out.println("POU5F1= "+Pou5f1GSM1854265);
          System.out.println("SOX2= "+Sox2GSM1854265);
          System.out.println("Less Pluripotent Cell= "+"GSM1854265");
      }
   }
}
     if(NanogGSM1854266>=5.0&&NanogGSM1854266<=9.0){
  if(Pou5f1GSM1854266>=5.0&&Pou5f1GSM1854266<=9.0){
     if(Sox2GSM1854266>=5.0&&Sox2GSM1854266<=9.0){
          System.out.println("Range: >=0.3 to <=3.0");
          System.out.println("Range: GSM1854266\nNANOG= "+NanogGSM1854266);
          System.out.println("POU5F1= "+Pou5f1GSM1854266);
          System.out.println("SOX2= "+Sox2GSM1854266);
          System.out.println("Highly Pluripotent Cell= "+"GSM1854266");
      }
   }
}else if(NanogGSM1854266>=2.0&&NanogGSM1854266<=4.9){
  if(Pou5f1GSM1854266>=2.0&&Pou5f1GSM1854266<=4.9){
```

```
    if(Sox2GSM1854266>=2.0&&Sox2GSM1854266<=4.9){
        System.out.println("Partial Pluripotent Cell= "+"GSM1854266");
    }
  }
}else if(NanogGSM1854266>=-3.0&&NanogGSM1854266<=1.9){
  if(Pou5f1GSM1854266>=-3.0&&Pou5f1GSM1854266<=1.9){
    if(Sox2GSM1854266>=-3.0&&Sox2GSM1854266<=1.9){
        System.out.println("Range: >=-3.0 to <=1.9");
        System.out.println("Range: GSM1854266\nNANOG= "+NanogGSM1854266);
        System.out.println("POU5F1= "+Pou5f1GSM1854266);
        System.out.println("SOX2= "+Sox2GSM1854266);
        System.out.println("Less Pluripotent Cell= "+"GSM1854266");
    }
  }
}
      if(NanogGSM1854267>=5.0&&NanogGSM1854267<=9.0){
  if(Pou5f1GSM1854267>=5.0&&Pou5f1GSM1854267<=9.0){
    if(Sox2GSM1854267>=5.0&&Sox2GSM1854267<=9.0){
        System.out.println("Range: >=0.3 to <=3.0");
        System.out.println("Range: GSM1854267\nNANOG= "+NanogGSM1854267);
        System.out.println("POU5F1= "+Pou5f1GSM1854267);
        System.out.println("SOX2= "+Sox2GSM1854267);
        System.out.println("Highly Pluripotent Cell= "+"GSM1854267");
    }
  }
}else if(NanogGSM1854267>=2.0&&NanogGSM1854267<=4.9){
  if(Pou5f1GSM1854267>=2.0&&Pou5f1GSM1854267<=4.9){
    if(Sox2GSM1854267>=2.0&&Sox2GSM1854267<=4.9){
        System.out.println("Partial Pluripotent Cell= "+"GSM1854267");
    }
  }
}else if(NanogGSM1854267>=-3.0&&NanogGSM1854267<=1.9){
  if(Pou5f1GSM1854267>=-3.0&&Pou5f1GSM1854267<=1.9){
    if(Sox2GSM1854267>=-3.0&&Sox2GSM1854267<=1.9){
        System.out.println("Range: >=-3.0 to <=1.9");
        System.out.println("Range: GSM1854267\nNANOG= "+NanogGSM1854267);
        System.out.println("POU5F1= "+Pou5f1GSM1854267);
        System.out.println("SOX2= "+Sox2GSM1854267);
        System.out.println("Less Pluripotent Cell= "+"GSM1854267");
    }
  }
}
      if(NanogGSM1854268>=5.0&&NanogGSM1854268<=9.0){
  if(Pou5f1GSM1854268>=5.0&&Pou5f1GSM1854268<=9.0){
    if(Sox2GSM1854268>=5.0&&Sox2GSM1854268<=9.0){
        System.out.println("Range: >=0.3 to <=3.0");
        System.out.println("Range: GSM1854268\nNANOG= "+NanogGSM1854268);
        System.out.println("POU5F1= "+Pou5f1GSM1854268);
    }
  }
}else if(NanogGSM1854268>=2.0&&NanogGSM1854268<=4.9){
  if(Pou5f1GSM1854268>=2.0&&Pou5f1GSM1854268<=4.9){
    if(Sox2GSM1854268>=2.0&&Sox2GSM1854268<=4.9){
        System.out.println("Partial Pluripotent Cell= "+"GSM1854268");
    }
  }
}else if(NanogGSM1854268>=-3.0&&NanogGSM1854268<=1.9){
  if(Pou5f1GSM1854268>=-3.0&&Pou5f1GSM1854268<=1.9){
    if(Sox2GSM1854268>=-3.0&&Sox2GSM1854268<=1.9){
        System.out.println("Range: >=-3.0 to <=1.9");
        System.out.println("Range: GSM1854268\nNANOG= "+NanogGSM1854268);
        System.out.println("POU5F1= "+Pou5f1GSM1854268);
        System.out.println("SOX2= "+Sox2GSM1854268);
        System.out.println("Less Pluripotent Cell= "+"GSM1854268");
    }
  }
}
      if(NanogGSM1854269>=5.0&&NanogGSM1854269<=9.0){
  if(Pou5f1GSM1854269>=5.0&&Pou5f1GSM1854269<=9.0){
    if(Sox2GSM1854269>=5.0&&Sox2GSM1854269<=9.0){
        System.out.println("Range: >=0.3 to <=3.0");
        System.out.println("Range: GSM1854269\nNANOG= "+NanogGSM1854269);
        System.out.println("POU5F1= "+Pou5f1GSM1854269);
        System.out.println("SOX2= "+Sox2GSM1854269);
        System.out.println("Highly Pluripotent Cell= "+"GSM1854269");
```

```
            }
          }
        }else if(NanogGSM1854269>=2.0&&NanogGSM1854269<=4.9){
          if(Pou5f1GSM1854269>=2.0&&Pou5f1GSM1854269<=4.9){
             if(Sox2GSM1854269>=2.0&&Sox2GSM1854269<=4.9){
                System.out.println("Partial Pluripotent Cell= "+"GSM1854269");
             }
          }
        }else if(NanogGSM1854269>=-3.0&&NanogGSM1854269<=1.9){
          if(Pou5f1GSM1854269>=-3.0&&Pou5f1GSM1854269<=1.9){
             if(Sox2GSM1854269>=-3.0&&Sox2GSM1854269<=1.9){
                  System.out.println("Range: >=-3.0 to <=1.9");
                System.out.println("Range: GSM1854269\nNANOG= "+NanogGSM1854269);
                System.out.println("POU5F1= "+Pou5f1GSM1854269);
                System.out.println("SOX2= "+Sox2GSM1854269);
                System.out.println("Less Pluripotent Cell= "+"GSM1854269");
             }
          }
        }

        }
        if(Value12.contains("EGSM")){
           //System.out.println("Ani");
           if(NanogGSM1854270>=2.0&&NanogGSM1854270<=3.0){
          if(Pou5f1GSM1854270>=2.0&&Pou5f1GSM1854270<=3.0){
             if(Sox2GSM1854270>=2.0&&Sox2GSM1854270<=3.0){
                  System.out.println("Range: >=0.3 to <=3.0");
                System.out.println("Range: GSM1854270\nNANOG= "+NanogGSM1854270);
                System.out.println("POU5F1= "+Pou5f1GSM1854270);
                System.out.println("SOX2= "+Sox2GSM1854270);
                System.out.println("Highly Pluripotent Cell= "+"GSM1854270");
             }    }
        }else if(NanogGSM1854270>=1.5&&NanogGSM1854270<=1.9){
          if(Pou5f1GSM1854270>=1.5&&Pou5f1GSM1854270<=1.9){
             if(Sox2GSM1854270>=1.5&&Sox2GSM1854270<=1.9){
                System.out.println("Partial Pluripotent Cell= "+"GSM1854270");
             }      }
        }else if(NanogGSM1854270>=1.2&&NanogGSM1854270<=1.49){
          if(Pou5f1GSM1854270>=1.2&&Pou5f1GSM1854270<=1.49){
             if(Sox2GSM1854270>=1.2&&Sox2GSM1854270<=1.49){
                System.out.println("Less Pluripotent Cell= "+"GSM1854270");
             }      }      }      }      }
    public static void main(String[]args) throws IOException{
       Pluritest test= new Pluritest();
       test.getFile();
      test.Fetchdata();
       test.CompareExpression();
    }
}
```

PUBLICATIONS FROM THESIS

1. **Abhishek Saini**, Jai Gopal Sharma, Vimal Kishor Singh, "*Determining pluripotency of a cell by an in silico method"* RJLBPCS (2018), Vol. 4(4), pp.130-145.

2. **Abhishek Saini**, Jai Gopal Sharma, Vimal Kishor Singh, "*Structural analysis and protein-protein interaction of sox2 and sox6 to study the regulatory mechanism of pluripotency and differentiation: a bioinformatics approach*.", IJCRT (2018), Vol.6, (2), pp.726-730.

3. Vimal Kishor Singh, **Abhishek Saini**, Manisha Kalsan, Neeraj Kumar, Ramesh K Chandra. *Describing the Stem Cell Potency: The Various Methods of Functional Assessment and In silico Diagnostics*. Frontiers in Cell and Developmental Biology (2016) Vol 4(134)DOI: 10.3389/fcell.2016.00134

4. Vimal Kishor Singh, **Abhishek Saini**, Manisha Kalsan, Neeraj Kumar, Ramesh Chandra. *Stage-Specific Regulation of Erythropoiesis and Its Implications in Ex - Vivo RBCs Generation*. Journal of Stem Cells (2016)11:3 pp.149-169.

5. Vimal Kishor Singh, **Abhishek Saini**, Neeraj Kumar and Manisha Kalsan. *Blood Generation from Stem Cells: An Overview*. International Journal of Science and Research (2016) 5:3, 1880-1884.

6. Vimal Kishor Singh, Neeraj Kumar, Manisha Kalsan, **Abhishek Saini**. *In silico Designing and Optimization of EPO Mimetic Using Combinatorial Library*. International Journal of Science and Research (2015) 4(1), 1110-1117.

7. Vimal Kishor Singh, **Abhishek Saini**, Ramesh Chandra. *Review Article Role of Erythropoietin and Other Growth Factors in Ex Vivo Erythropoiesis*. Advances in Regenerative Medicine. (2014)1,1-8., DOI:10.1155/2014/426520.

8. Vimal Kishor Singh, **Abhishek Saini**, Kohichiro Tsuji, P B Sharma, Ramesh Chandra. *Manufacturing blood ex vivo: a futuristic approach to deal with the supply and safety concerns*. Frontiers in Cell and Developmental Biology (2014) 2 (26), 1-18 doi: 10.3389/fcell.2014.00026.

Other Publications

1. Vimal Kishor Singh, **Abhishek Saini**, Ramesh K Chandra. *The Implications and Future Perspectives of Nanomedicine for Cancer Stem Cell Targeted Therapies*. Frontiers in Molecular Biosciences (2017) DOI: 10.3389/fmolb.2017.00052.

2. Singh VK, Goyal I, **Abhishek Saini** and Chandra R *Studying the Effect of Carica papaya Leaf Extract on the Shelf Life of Platelets*, International Journal of Science and Research (2017) Vol 6:5.

3. Vimal Kishor Singh, Neeraj Kumar, Manisha Kalsan, **Abhishek Saini**, Ramesh Chandra. *A Novel Peptide Thrombopoietin Mimetic Designing and Optimization Using Computational Approach*. Frontiers in Bioengineering and Biotechnology (2016) 4, 1-11 doi:10.3389/fbioe.2016.00069.

4. Vimal Kishor Singh, Ishita Goyal, **Abhishek Saini**, Neeraj Kumar, Manisha Kalsan, and Ramesh Chandra. *Designing an In-Silico Mimetic for Thrombopoietin Using Combinatorial Library*. International Journal of Science and Research, (2016) 5:4, 2427-2432.

5. Vimal Kishor Singh, Neeraj Kumar, Manisha Kalsan, **Abhishek Saini**, Ramesh Chandra. *Mechanism of Induction: Induced Pluripotent Stem Cells (iPSC)*. Journal of stem cells (2015) 10(1) pp-43-62.

6. Vimal Kishor Singh, Manisha Kalsan, Neeraj Kumar, **Abhishek Saini**, Ramesh Chandra. *Induced Pluripotent Stem Cells: Applications in regenerative medicine, disease modeling, and drug discovery*. Frontiers in Cell and Developmental Biology (2015) 3:2. doi: 10.3389/fcell.2015.00002.

Book Chapter

1. Abhishek Saini. Four Transcription factors are necessary for generating iPSCs form adult cells. Submitted for publication as Chapter in book, Molecular Medicine and Stem Cell Therapy-2018, (in process)

Poster Presentation in International/National conferences

1. **Abhishek Saini**, JaiGopal Sharma, Vimal Kishor Singh "Generation of erythroid cell line by chemically induced erythroleukemia cell line" during NCOBE held at Jamia Millia Islamia (April, 2017).

2. Vishwachi Tripathi, **Abhishek Saini**, Vimal Kishor Singh "Syntropic interaction between *Shewanella* and *E.coli* in microbial fuel cells" during NCOBE held at Jamia Millia Islamia (April, 2017).

3. **Abhishek Saini**, JaiGopal Sharma, Vimal Kishor Singh "Analysis of Hematopoietic progenitors cells isolated from UCB, PB and mobilized PB as source cells for ex-vivo generation of erythrocytes" during RTBDD at Shri Mata Vaishno Devi University (30-31, March, 2017).

4. **Abhishek Saini**, Vimal Kishor Singh, Manisha Kalsan , Neeraj Kumar, Ramesh Chandra "Ex-vivo expansion of CD34+ peripheral blood mononuclear cells isolated by Aphaeresis system and significance of Erythropoietin, SCF, and IL-3 growth factors" at International Conference on Translational Medicine Emerging Trends in Biomedicine, Biotechnology and Stem cells present status and future prospects, Amity University, Gurgaon (Feb, 2016).

5. Vimal Kishor Singh, **Abhishek Saini**, Manisha Kalsan, Neeraj Kumar, Ramesh Chandra. "Blood generation from stem cell; An overview." at International Conference on Translational Medicine Emerging Trends in Biomedicine, Biotechnology and Stem cells present status and future prospects, Amity University, Gurgaon (Feb, 2016).

6. **Abhishek Saini**, Vimal Kishor Singh, "*Comparison of hematopoietic stem cells expansion with EPO and its mimetic*" at International Conference on Translational Medicine in 21st Century Barkatullah University, Bhopal (2015).

Workshops and Courses

1. **Abhishek Saini** (2018) Advanced Diploma in Flow Cytometry Techniques by BDJH FACS Academy
2. **Abhishek Saini** (2018) Workshop on "Raising Awareness on Plagiarism and Copyrights" on 20th March, 2018 at DTU.
3. **Abhishek Saini** (2018) TEQIP-III sponsored FDP on "RDTM" from 12 to 16 March, 2018 at Department of Biotechnology, DTU.

4. **Abhishek Saini** (2018) Elsevier Author workshop on 22.3.2018 at Central Library, DTU.
5. **Abhishek Saini** (2018) Hands on training on HPLC by Application Scientist (ThermoFisher) Mr. Sandeep in Department of Biotechnology, DTU.
6. **Abhishek Saini** (2017) Certificate Course in Molecular Medicine during June 2017 at Amity University, Haryana.
7. **Abhishek Saini** (2014) FACSOrient course conducted by BD Biosciences, India from 24th to 26th March, 2014 at Jamia Hamdard.