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Expression, Identification, and Bioactivity of Recombinant Human Erythropoietin from LMH/2A (Leghorn Male Hepatoma)Cell Line

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EXPRESSION, IDENTIFICATION, AND BIOACTIVITY OF RECOMBINANT HUMAN ERYTHROPOIETIN FROM LMH/2A (LEGHORN MALE HEPATOMA) CELL LINE

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LIST OF ABBREVIATIONS

EPO- erythropoietin

LMH- Leghorn Male Hepatoma

DNA- Deoxyribonucleic acid

RNA- ribonucleic acid

rDNA- recombinant DNA

nm- nanometers

abs- absorbance

ng- nanogram

µg- microgram

µl- microliter

ml- milliliter

rhEPO- recombinant human erythropoietin

CHO- chinese hamster ovary

HEK- human embryonic kidney

Hu- human

FBS- fetal bovine serum

kD- kilodaltons

CV- coefficient of variation

STD- standard deviation

AVG- average

HIC- hydrophobic interaction chromatography

HPGM- human progenitor growth media

TBS- tris buffered saline

PBS- phosphate buffered saline

C- celcius

AIDS- acquired immune deficiency syndrome

FDA- Food and Drug Administration

EU- European Union

NIH- National Institutes of Health

GMO- genetically modified organisms

tPA- tissue plasminogen activator

DHFR- dihydrofolate reductase

BHK- baby hamster kidney

GM-CSF- Granulocyte-macrophage colony-stimulating factor

G-CSF- Granulocyte colony-stimulating factor

NANA- *N*-acetylneuraminic acid

NGNA- *N*-Glycolylneuraminic acid

PNGaseF- Peptide: *N*-Glycosidase F

D- daltons

k- kilo

HIF-1 hypoxia inducible factor 1

PRCA- pure red cell aplasia

EPO-R- erythropoietin receptor

CFU-E- colony forming unit-erythroid

BFU-E- burst forming unit-erythroid

JAK2- janus kinase 2

SH2- Src-homology2

STAT- signal transducer and activator of transcription

P13K- phosphatidylinositol 3-kinase

BRP- biological reference preparation

NIBSC- National Institute for Biological Standards and Control

HSC- hematopoietic stem cell

GPA- Glycophorin A

HbA- hemoglobin alpha

ELISA- enzyme linked immunosorbant assay

SDS-PAGE- sodium dodecyl sulfate polyacrylamide gel electrophoresis

NIBSC- The National Institue for Biological Standards and Control

ABSTRACT

Erythropoietin (EPO) is the main cytokine regulator for red blood-cell production in the human by binding to erythropoietin receptors to promote erythroid proliferation, survival and differentiation. Administration of recombinant human EPO (rhEPO) as a therapeutic has been shown to improve renal and non-renal anemia and this success has led to a high medical global demand. Chinese hamster ovary (CHO) cells, yeast, and bacteria have been used to produce rhEPO but nevertheless there is still a need for a low-cost and high quality expression system for rhEPO.

LMH/2A cells, as an alternative model, were transfected with the codon optimized human EPO gene for glycosylated rhEPO production. The LMH/2A-rhEPO was detected and purified using a series of chromatographic steps and the purity was determined to be 95%. As expected, LMH/2A-rhEPO was *N*-linked glycosylated indicated by a considerable shift in molecular weight after enzymatic deglycosylation with PNGaseF. The biological activity of purified LMH/2A-rhEPO was investigated *in vitro* using cell based assay (CD34+ cells) and was compared to the biological activities of rhEPO expressed from CHO cells and human cells (HEK293).

In this study, LMH/2A-rhEPO induced cell proliferation which showed a five fold increase in the number of rhEPO-treated CD34+ cells as compared to the number of non-treated CD34+ cells. Furthermore, rhEPO-treated CD34+ cells expressed hemoglobin 2500 fold higher than non-treated CD34+ cells. Growing CD34+ cells supplemented with LMH/2A-rhEPO resulted in an increase in the expression of erythroid markers such as transferrin receptors, glycophorin A, and hemoglobin.

Interestingly, EPO with LMH/2A cell specific glycosylation promotes similar erythroid differentiation of human CD34+ cells, compared to CHO and human cell expressed rhEPO. Therefore, LMH/2A-rhEPO was biologically active *in vitro* by inducing erythroid differentiation which indicates that LMH/2A cells can definitely can be used as a suggested alternative system for rhEPO production.

CHAPTER I

INTRODUCTION

Advances in biotechnology techniques such as recombinant DNA technology have improved the biopharmaceutical industry by utilizing living organisms such as bacteria, fungi, and mammalian cells to produce human recombinant proteins. The successful applications for recombinant DNA technology brought about a rapid growth of biotechnology companies and a number of therapeutic recombinant DNA (rDNA) products such as therapeutic proteins available for human use (Reichert and Paquett, 2003). One particular example of a therapeutic protein of interest in the biopharmaceutical market today is recombinant human Erythropoietin (rhEPO). Human erythropoietin (EPO) is a cytokine that regulates the level of red blood cell production (Richmond et al., 2005) and rhEPO is currently being used to treat patients with anemia, acquired immune deficiency syndrome (AIDS), nonmyeloid malignancies, perioperative surgical, and autologous blood donation (Fisher, 2003).

These advances in biotechnology have created the need for patents that are filed following the discovery and initial characterization of any rDNA product of potential therapeutic application (Bhopale and Nanda, 2005). Patents previously filed on a number of recombinant human therapeutic proteins (e.g. human growth hormone, erythropoietin, interferons , etc.) are starting to expire and as a result have led to the development of alternative versions of biological products called biosimilars or follow-on biologics (Dadashzadeh, 2009). For example, Amgen's United States patent on their multibillion dollar Epogen® (recombinant human EPO) expires in 2013 in the United States and biotechnology companies are hoping to market biosimilars once Epogen® is off-patent (Felcone, 2004). Also, these expiring patents have forced Congress and the Food and Drug Administration (FDA) to enable an expedited approval of biosimilars thus paving the way for the development of a robust competitive United States biosimilar industry

that could drop prices of the biological products and allow consumers better access these biological products (Grabowski et al., 2006). Therefore, biosimilars represent a critical part of the future of biotechnology due to generic companies competing to develop cost-efficient alternative models to produce recombinant human therapeutic proteins. For example, biosimilar rhEPOs have been priced up to 30% lower than the originators in some countries of the European Union (Jelkmann, 2009).

Currently in the biotechnology industry a common production system for rhEPO is the mammalian cell culture system using Chinese hamster ovary (CHO) cells (Inoue et al., 1995). Also, rhEPO has been shown to be produced in bacterial cells (prokaryotic) such as *Escherichia coli* (Huang, 1984) and *Bacillus brevis* (Nagao et al., 1997), in yeast cells (eukaryotic) such as *Saccharomyces cerevisiae* (Elliott et al., 1989) and in insect cells (eukaryotic) such as *Drosophila melanogaster* (Kim et al., 2005). Prokaryotic systems lack the ability to glycosylate proteins and microbial eukaryotes hyperglycosylate proteins, as a result these systems are considered alternatives to CHO cell rhEPO production (Maleki et al., 2010). The CHO cell expression system is the preferred model for rhEPO production because of the ability of CHO cells to glycosylate rhEPO in a similar manner to humans (Hossler et al., 2009) which is important for functional properties such as biological half-life (Takeuchi et al., 1990). However, there have been numerous reports on the effect of CHO cells towards the resulting glycoform profile that is not typically found in humans and was verified to elicit an immune response (Noguchi et al., 1995; Baker et al., 2001).

Large scale CHO-expressed rhEPO production was established to fulfill the current high medical demand for glycosylated rhEPO (Egrie, 1990). However, there is still a high demand for low-cost and high quality glycosylated rhEPO biosimilars. Indeed, a challenge in the

biopharmaceutical industry today is to find the best productive, efficacious, and cost-efficient system for producing properly glycosylated rhEPO. One possible alternative production model involves using an avian cell production system, Leghorn Male Hepatoma (LMH) cells. LMH cells are hepatocellular carcinoma cell line established from a hepatocellular carcinoma induced in a male leghorn chicken by diethylnitrosamine (Kawaguchi et al., 1987) and were successfully used to produce recombinant proteins (Vasudevan et al., 2001; Walzem et al., 1997). Avian cell lines were demonstrated to be effective heterologous gene expression models for recombinant protein production such as rhEPO (Lee et al., 1999).

Therefore, the purposes of this study were 1) to use LMH/2A as an alternative production system for rhEPO, 2) to express rhEPO in LMH/2A cells, 3) to purify and identify rhEPO from LMH/2A cells, and finally 4) to determine the biological activity for the purified LMH/2A-expressed rhEPO by evaluating the ability of this recombinant protein to promote erythroid differentiation, to induce cell proliferation, and to stimulate the expression of erythroid markers such as hemoglobin, transferrin receptors, and glycophorin A. An additional objective was to compare the biological activity of LMH/2A-expressed rhEPO with commercially available mammalian cell lines such as CHO-expressed rhEPO and human-expressed rhEPO (HEK-293).

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CHAPTER II

LITERATURE REVIEW

Introduction to Recombinant DNA Technology

Biotechnology is described as the manipulation of living organisms to produce products that have useful applications for humans. Biotechnology is applied in the biopharmaceutical industry where living organisms such as bacteria, yeast, and fungi are manipulated to produce useful products such as antibiotics, hormones, enzymes, and vaccines. Examples of modern biotechnology being used today in industrial applications are the production of alcohol by yeast and the production of penicillin (an antibiotic) by fungi. Advances in biotechnology have improved the biopharmaceutical industry by utilizing living organisms such as bacteria, fungi and mammalian cells to produce human recombinant proteins. This advancement is due to recombinant DNA technology which uses a series of molecular biotechnology techniques that are used to join two or more different DNA molecules. Recombinant DNA technology has numerous applications in the industrial, agricultural, pharmaceutical, and biomedical fields. Recombinant DNA technology was discovered in 1970 by Paul Berg who produced the first recombinant DNA (Jackson et al., 1972). Then in 1973, Herbert Boyer successfully produced recombinant human insulin in *Escherichia coli* (Cohen et al., 1973). Recombinant DNA technology has made a revolutionary impact in the area of human medicine by enabling the treatment of diseases and genetic disorders with recombinant DNA technology products. According to the National Institutes of Health (NIH) guidelines, recombinant DNA are molecules constructed outside living cells by joining natural or synthetic DNA segments to DNA molecules that can replicate in a living cell or molecules that result from the replication of DNA molecules constructed outside living cells by joining natural or synthetic DNA segments to DNA

molecules. The utilization of this rDNA technology allowed for the development of genetically modified organisms (GMO) which is an organism that has been changed genetically by recombinant DNA (Tolin and Vidaver, 1989).

Biotechnology Industry

The biotechnology industry consists of companies that use biotechnology for manufacturing drugs, gene therapy and genetic testing. Pharmaceutical biotechnology companies use recombinant DNA technology for making products such as drugs, that are widely used in prevention, diagnosis or treatment of many types of diseases. Regulatory agencies around the world require biopharmaceutical companies to both characterized and maintain product quality attributes (Hossler et al., 2009). In biopharmaceutical manufacturing, acceptable criteria for the biopharmaceuticals is based upon the cell line and potency of the drug. The accepted biopharmaceutical is considered property of the biopharmaceutical manufacturer and patents are usually filed on these products. However, a current problem in the biotechnology industry is that there will be major biopharmaceuticals coming off patent that will affect the value of products and create a market for alternative versions of biopharmaceuticals called follow-on biologics or biosimilars. A major challenge for biopharmaceutical companies is that these alternative versions of biopharmaceuticals require extensive comparability studies and testing to meet acceptable criteria by regulatory agencies such as the FDA (Woodcock et al., 2007).

Cell Culture

Over the past one hundred years, the development of techniques in cell culture has led to an increase in the success of culturing cells. Advances in sterile techniques, the addition of antibiotics and understanding the nutrient requirements of cultured cells has led to the ability to

manipulate cultured cells to produce biological products for human use. Animal cell culture was developed in the 1960's (Stoker and Macpherson, 1964) and later in the 1980's cultured animal cells were used to express recombinant proteins (Johnson, 1983). Recently, progresses in gene manipulation and the ability to produce clones has allowed cell culture expression systems to produce high-levels of recombinant proteins (Andersen and Krummen, 2002). Vector systems facilitate the introduction of specific genes into the cells so that transcription can occur for the inserted gene or genes and subsequently get translated into recombinant proteins. A wide variety of techniques and reagents are used to deliver the gene of interest into cells in culture. A common technique for DNA delivery into a cell is through transfection which is a process in which the gene of interest is introduced into a eukaryotic cell by chemical, biological or physical methods. Transformation is the term used to describe the process of inserting a gene of interest into bacteria cells or non-eukaryotic cells.

There are two types of transfection, transient and stable. Transient transfection occurs when DNA is delivered to the cell, transported to the nucleus for transcription but not integrated into the chromosome of the cell. In transient transfections, expression of the transgene can be degraded by nucleases or reduced by cell division. Transient transfections are ideal for comparing regulatory elements and verifying expression of the plasmid (Kaufman, 1997). More recently, this method has demonstrated its ability to produce recombinant proteins in large-scale (Meissner et al., 2001; Pham et al., 2003; Wurm and Bernard 1999). On the other hand, stable transfection occurs when the transgene integrates into the cell's genome and replicates with the chromosomes. Transfected cells that contain the inserted gene can be selected and then amplified to produce a stable cell line. The resulting stable cell line is a source for recombinant protein production that can be used to produce large quantities of recombinant proteins.

Recombinant proteins have been produced in large-scale using stable transfections (Colosimo et al., 2000; Wurm, 1990).

Therapeutic Recombinant Protein Expression Systems

Therapeutic proteins derived from recombinant DNA technology (rDNA) or therapeutic monoclonal antibodies are called biopharmaceuticals (Tsuji and Tsutani, 2008). The successful applications of recombinant DNA technology brought about a rapid growth of biotechnology companies and a number of therapeutic rDNA products such as therapeutic proteins available for human use (Reichert and Paquett, 2003). In 1982, the U.S. Food and Drug Administration (FDA) approved the first genetically engineered drug for human therapeutic use, Genentech's Humulin, a form of human insulin produced by bacteria using recombinant DNA technology (Johnson, 1983). In 1986, Chinese hamster ovary (CHO) was chosen as the host for the production of tissue plasminogen activator (tPA) which was the first recombinant therapeutic protein from mammalian cells to gain regulatory approval (Wurm, 2004). Later additional recombinant DNA products such as hormones, hemopoietic growth factors, blood coagulation products, thrombolytic agents, anticoagulants, interferons, interleukins and therapeutic enzymes gained approval by the FDA and started being used for human therapeutic use (Bhopale and Nanda, 2005).

The majority of therapeutic recombinant proteins are produced in either mammalian cell culture systems (eukaryotic) such CHO cells (most common system) or in bacterial systems (prokaryotic) such as *Escherichia coli* (Chu and Robinson, 2001; Swartz, 2001). Several other mammalian cell lines such as 3T3, BHK, HeLa, and HepG2 have also been used to produce recombinant proteins. However, about half of all recombinant therapeutic proteins currently on the market are produced in mammalian cells, with about 70% of these being generated in suspension culture CHO cells (Walsh, 2006). The most common selection method used to

establish mammalian cells that will stably produce recombinant proteins includes using dihydrofolate reductase (DHFR), an enzyme involved in nucleotide metabolism and glutamate synthetase (Wurm, 2004). Another source of human therapeutic proteins are recombinant glycoproteins produced in yeast, for example, recombinant granulocyte macrophage colony-stimulating factor (GM-CSF), insulin and several vaccines (Wildt and Gerngross, 2005). The cost, quality, and quantity of the final product can depend on the expression system and presents a challenge in the biopharmaceutical industry to find the most suitable alternative production system for human therapeutic proteins. However, care must be taken that the quality of the biopharmaceutical product is not altered when manufactured in a new facility, a new cell line is established, fermentation is altered, new products are introduced into the formulation, or when the formulation is changed (Jelkmann, 2007).

Alternative Expression Systems

Alternative expression systems to produce therapeutic recombinant proteins are currently being utilized in the biopharmaceutical industry and new alternative expression systems are currently being investigated. Alternative expression systems to the popular CHO cell system and bacterial cell system include using human cells, transgenic animals, plant cells, and insect cells (Anderson and Krummen, 2002). Another potential alternative expression system involves using avian cells for producing therapeutic recombinant proteins. Lee et al. (1999) reports using quail fibrosarcoma cells to stably produce rhEPO and suggests that these cells be considered as an alternative to the CHO expressed rhEPO. Furthermore, Brown and Mehtali (2010) propose the avian cell line EB66® to produce vaccines for human therapeutic use due to the fact that recombinant proteins produced in avian cells have a lower fucose content than CHO cell produced recombinant proteins such as in monoclonal antibodies.

LMH/2A Cell Line

LMH cells which are a tumor cell line established from a hepatocellular carcinoma induced in male leghorn chicken by diethylnitrosamine (Kawaguchi et al., 1987). LMH cell lines were minimally responsive to estrogen due to very low levels of the estrogen receptor (Binder et al., 1990). Cell lines, such as the LMH/2A, were derived from the parent LMH cell line by stable transfection of the chicken estrogen receptor to increase the estrogen responsiveness (Sensel et al., 1994). LMH and LMH/2A cells have previously been shown to be capable of transfection with recombinant DNA. Weber et al., (1995) describes some features of the transfection performance of the LMH cell line in the absence of estrogen. Sensel et al. (1994) reports expression of Apolipoprotein II in transfected LMH/2A cells. Furthermore, Vasudevan et al., (2001) described a transient transfection of LMH/2A cells which produced chicken riboflavin carrier protein with a 6 to 12 fold transcriptional induction by a stable estrogen analogue, moxesterol. Walzem et al., (1997) described transfection of LMH/2A cell with a luciferase reporter gene using cationic lipids.

Posttranslational Modifications

Glycosylation is an important posttranslational protein modification occurring in the cytoplasm, the endoplasmic reticulum, and the Golgi apparatus (Jaeken and Carchon, 2009). Prokaryotic hosts such as *Escherichia coli* do not glycosylate proteins and lower eukaryotic expression systems such as yeast cells are typically unable to provide mammalian glycosylation of proteins (Gerngross, 2004). Protein glycosylation is an enzymatic process that attaches glycans (oligosaccharides or carbohydrates) to the protein (Dall'Olio, 1996). Aglycosylated forms of glycoproteins tend to be misfolded, biologically inactive, or rapidly cleared from circulation (Coloma et al., 2000). Yeasts are able to glycosylate proteins, yet the nonhuman

nature of yeast glycans negatively impacts protein half-life because of the affinity of these glycans to high-mannose receptors present on macrophages and endothelial cells (Mistry et al., 1996). Therefore, mammalian cells such as CHO cells have emerged as the most widely used expression system for the production of complex human glycoproteins (Humphreys and Boersig, 2003). CHO cells are typically able to express glycoproteins with humanlike glycosylation patterns, however some glycan structures produced from CHO cell lines differ from those produced in human cells and sometimes have to be modified to meet therapeutic efficacy (Grabowski et al., 1995; Grabenhorst et al., 1999). Although mammalian cell culture systems are the most common recombinant protein production system, there are some disadvantages to using this production system. Mammalian cell culture production systems for recombinant proteins are expensive and time consuming due to slow growth of the cells (Bhopale and Nanda, 2005). This challenge has created a demand in the market for a production system of human therapeutic glycoproteins with reduced costs, increased yields, and more control over the quality of the final product.

Glycosylation of proteins takes on the form of oligosaccharides attached to either the side chain of asparagines (*N*-linked) or serine/threonine (*O*-linked) (Warren, 1993). The addition of oligosaccharides onto a glycoprotein is a complex metabolic pathway that can be controlled through a variety of different conditions such as cell lines (host), bioprocess, and cell culture media used for protein production (Hossler et al., 2009). It is known that mammalian cell lines such as CHO and BHK process recombinant glycoproteins similar to humans (Skibeli et al., 2001). However, in contrast to human cells, CHO cells do not express sialyl-a 2-6 transferase, bisecting *N*-acetyl glucosamine transferase, and a 1-3/4 fucosyl transferase, therefore structural analysis of the sugar groups of natural and recombinant glycoproteins is necessary (Kamerling,

1996; Svensson et al., 1990; Bergwerff et al., 1993). *N*- and *O*- linked glycans have been shown to have a large effect on the immunogenicity, efficacy, solubility, and half-life of commercial biologics (Lis and Sharon, 1993; Van den Steen et al., 1998; Lowe and Marth, 2003). A schematic of *N*-linked glycosylation for a glycoprotein produced in CHO cells is shown in Figure 1 (Hossler et al., 2009).

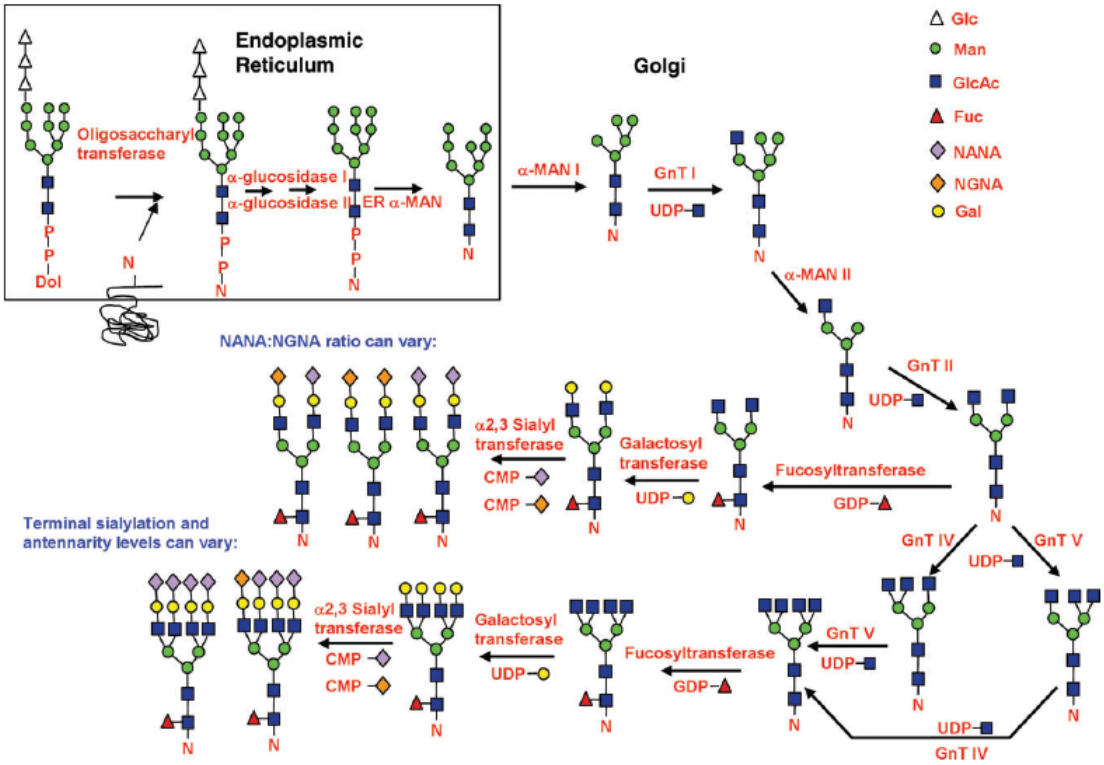

Figure 1. Schematic view of the *N*-linked glycosylation pathway in CHO cells. The precursor Glc3Man9GlcNAc2 is transferred from the dolichol-phosphate donor to an asaparagine on the nascent protein in the endoplasmic reticulum. The final glycoprotein forms can vary depending on the level of sialylation and terminal sugars. The level of antennarity and sialylation will depend on cell culture conditions and expression levels of each enzyme (Hossler et al., 2009).

The terminal *N*-acetylneuraminic acid (NANA) content in a glycoprotein has been shown to be linked to circulatory half-life while *N*-Glycolylneuraminic acid (NGNA), a derivative of NANA that is not typically found in adult humans, (Muchmore et al., 1989) is present in CHO and NS0 cultures (Noguchi et al., 1995; Baker et al., 2001). The expression host is thus very

important toward determining the final glycoform profile of a recombinant glycoprotein, for example CHO cell expressed rhEPO contain low levels of NGNA (1% of total sialic acids) which may elicit a negligible immunogenic response (Noguchi et al., 1995). Furthermore, *N*-acetylglucosamine also plays a principal role in determining *N*-glycan antennarity and the resulting level of terminal glycosylation for example, in rhEPO it was specifically determined that tetrasialylated tetra-antennary *N*-glycans increased *in vivo* bioactivity, rather than the ratio of tetraantennary to biantennary *N*-glycans (Yuen et al. 2003).

The peptide *N*-glycosidase F (PNGaseF) is an enzyme that catalyzes the complete removal of *N*-linked oligosaccharide chains from glycoproteins, a process known as endoglycosidase that results in the cleavage at the asparagine-sugar amide linkage (Norris et al., 1994). PNGaseF is widely used in studies of glycoprotein function and structure (Machado et al., 2011; Skibeli et al., 2001).

Applications

Since the first FDA approval of recombinant human insulin in 1982, approximately 175 biotechnology products are currently being marketed (Walsh, 2005) with more products under development. These recombinant DNA products target more than 200 diseases including cancer, Alzheimer's, cardiovascular diseases, multiple sclerosis, AIDS, and arthritis and are being used to improve therapies through better delivery systems, better diagnostics, safer and more effective medicines, more patient access, and enhanced therapeutic options for physicians and patients (Weintraub, 2006).

An example of a therapeutic recombinant protein is human insulin which has proved to be identical to human pancreatic insulin, safe, efficacious, and less likely to cause immunological reactions during therapeutic use as opposed to animal-derived insulin (Ladisch and Kohlmann,

1992). This recombinant human insulin is used to treat a common human metabolic disease known as diabetes mellitus (McEvoy, 2001). Other examples of therapeutic recombinant proteins produced in mammalian cell lines that have been approved to treat humans include recombinant interferon-β, which is used for the treatment of multiple sclerosis; erythropoietin, which is used for the treatment of anaemia; and recombinant antibodies, such as Herceptin and Rituxan, which are used to treat breast cancer and non-Hodgkin's lymphoma (Wildt and Gerngross, 2005).

Erythropoeitin

Erythropoietin (EPO) is a glycoprotein hormone that is composed of 165 amino acids with an expected molecular weight of 18,398 Daltons (Recny et al., 1987). Erythropoietin consists of a single polypeptide chain of 165 amino acids, two disulphide bonds, three *N*-linked glycosylation sites (Asn 24, Asn 38, and Asn 83), and one *O*-linked glycosylation site (Ser 126) (Su et al., 2010). The average carbohydrate content amounts to 40% (Narhi et al., 1991) which gives erythropoietin an apparent molecular weight of 35-39 kD (Joung et al., 2009) while the expected non-glycosylated molecular weight is 18 kD (Jelkmann, 1992). The carbohydrate moiety is important for the biological activity, stability, and solubility of the protein (Narhi et al., 1991). Erythropoietin is a cytokine that regulates the level of red blood cell production (Richmond et al., 2005). In humans and other mammals, decreased tissue oxygen tension activates the hypoxia inducible factor-1 (HIF-1) which in turn increases EPO production (Guillemin and Krasnow, 1997). Semenza, (1996) discovered that under hypoxic conditions HIF-1 accumulates in the nucleus and binds to a sequence in the enhancer region of the EPO gene and triggers expression of the EPO gene. EPO is produced primarily in the liver during fetal gestation and in the kidney after birth, however in response to hypoxia EPO is produced in

both the kidney and the liver tissue (Noguchi, 2008). EPO is a secreted hormone that binds to receptors on erythroid progenitor cells to promote viability, proliferation, and terminal differentiation of erythroid precursors resulting in increased red blood cell mass (Ebert and Bunn, 1999). The carbohydrate moiety is not required for binding to the EPO receptor (Darling et al., 2002). The mechanisms impairing renal EPO production are not well understood, however, the most well-known cause of anemia is inadequate EPO production, which is often compounded by iron deficiency, and as renal failure progresses so does the contribution of EPO deficiency to anemia (Lankhorst and Wish, 2010). The story of how EPO was discovered dates back to 1878 with Bert and Jourdanet who first established the link between tissue hypoxia and the production of erythrocytes. Carnot and Deflandre, (1906) first formulated the idea of hormonal regulation of erythropoiesis. In 1948, Bonsdorff and Jalavisto (1948) introduced the name "erythropoietin", implying red cell production. In 1949, the erythropoietic activity of exogenous EPO from transfused plasma of normal subjects into patients with pernicous anaemia was first demonstrated by Oliva et al. (1949). In 1977, a major breakthrough was discovered that transformed the anemia therapy field when human erythropoietin was purified from the urine of aplastic anemic patients (Miyake et al., 1977). Later in 1983, this discovery paved the way for the gene for human erythropoietin to be isolated and cloned (Lin et al., 1985). It was discovered in 1986 that rhEPO can correct the anemia of chronic renal disease (Eschbach et al., 1987).

Recombinant Human EPO

The human EPO gene was isolated and used to develop a transfected cell line in CHO cells that produced recombinant human EPO (Jacobs et al., 1985). Since 1985, the CHO cell system became the most common production system for rhEPO (Inoue et al., 1995). Also, rhEPO has been produced in bacterial cells (prokaryotic) such as *Escherichia coli* (Huang, 1984)

and *Bacillus brevis* (Nagao et al., 1997), in yeast cells (eukaryotic) such as *Saccharomyces cerevisiae* (Elliott et al., 1989) and in insect cells (eukaryotic) such as *Drosophila melanogaster* (Kim et al., 2005).

In 1989 rhEPO gained FDA approval for human therapeutic use and the commercially available rhEPO products currently available are epoetin-α, epoetin-β, epoetin-ω, epoetin-δ, and darbepoetin-α (Joyeux-Faure, 2007). These products all have the same amino acid sequence but may differ in glycosylation as a result of type and host cell-specific differences in the production process. Darbepoetin-α is an erythropoietin analog, carrying two additional glycosylation sites, which produces a longer half-life and potency (Joung et al, 2009). The probability of an immunological reaction to rhEPO increases as a consequence of structural differences in the endogenous hEPO and the recombinant form of hEPO (Jelkmann, 2007). Binding of antibodies may influence the pharmacokinetic behavior of rhEPO that can reduce the half-life (Schellekens, 2008). Consequences of the specific interaction of antibodies with rhEPO is dependent on the antibody working in a binding and neutralizing capacity which could result in loss of activity of the protein drug (Schellekens, 2008). The most dramatic side effects occurs when the neutralizing antibodies cross-react with endogenous hEPO that leads to life-threatening pure red cell aplasia (PRCA) (Casadevall et al., 2002).

EPO Receptors

The EPO receptor (EPO-R), which was described initially by D'Andrea et al. (1989), is a member of the cytokine receptor superfamily. The EPO-R is expressed in bone marrow derived erythroid progenitors and several non-hematopoietic tissues including myocytes, cortical neurons, and prostatic, breast and ovarian epithelia (Richmond et al., 2005). The EPO-R is expressed primarily on erythroid cells during differentiation with the highest number of receptors

seen on the colony forming unit erythroid (CFU-E) and burst forming unit erythroid (BFU-E) which decreases in expression as differentiation continues to a mature erythrocyte which does not express the EPO-R (Fisher, 2003). The EPO-R is believed to exist in an unliganded, dimeric state that when bound to EPO undergoes a conformational change which activates the pre-bound, cytoplasmic, tyrosine kinase, janus kinase 2 (JAK2) (Witthuhn et al., 1993). In turn, JAK2 and other tyrosine kinases phosphorylate several cytoplasmic tyrosine residues in the cytoplasmic tail of the EPO-R that act as docking sites for proteins that contain Src-homology2 (SH2) domains which induces several downstream signaling cascades (Richmond et al., 2005). Depending on the cell type, EPO activates tyrosine phosphorylation of the SH2 domain containing transcription factors, signal transducer and activator of transcription (STAT) or phosphatidylinositol 3-kinase (PI3K)/AKT pathway (Rossert and Eckardt, 2005).

Purification

Goldwasser and Kung were the first to purify milligram quantities of >95% pure hEPO in 1977 (Miyake et al., 1977). The purified hEPO led to the successful identification of the amino acid sequence and the isolation of the EPO gene (Jelkmann, 2007). Biologically active rhEPO can be purified from the culture supernatant of genetically engineered mammalian cells such as CHO cells, baby hamster kidney cells BHK-21, or C127 mouse mammary cells (Jelkmann, 2000).

Biopharmaceutical companies producing rhEPO are challenged to find a cost-effective ways to purify rhEPO. New methods for purification of rhEPO have been developed (Broudy et al., 1988; Inoue et al., 1994; Ben Ghanem et al., 1994) and are efficient in terms of yield and specific activity of the final product. Large scale purification procedure using anion exchange, hydrophobic interaction, and gel filtration chromatography have been described (Hu et al., 2004).

Procedures specifically tailored for rhEPO purification at an industrial scale are still in demand. Zanette et al. (2003) describes and ideal system for the capture step in downstream processing that includes both the selectivity of affinity chromatography and the robustness of the so-called 'traditional' chromatography by utilizing phenylboronate agarose in the capture step.

Applications

Recombinant human EPO (rHuEPO) is currently being used to treat patients with anemia associated with chronic renal failure, AIDS patients with anemia due to treatment with zidovudine, nonmyeloid malignancies in patients treated with chemotherapeutic agents, perioperative surgical patients, and autologous blood donation (Fisher, 2003). There has been much clinical success of recombinant human EPO in humans and it continues to be used to treat renal and non-renal anemia (Winearls et al., 1986; Eschbach et al., 1987). Due to the success of rhEPO in treating anaemia in patients with end-stage renal disease, rhEPO is the one of the top-selling biopharmaceutical products in the world, with annual worldwide sales of about $11 billion (Joung et al., 2009). Besides this well-established activity, rhEPO also has therapeutic potential in the treatment of stroke, head trauma, and epilepsy (Cerami, 2001). It has been reported that rhEPO had an effect on angiogenesis which raised considerable interest in the possible application of this rhEPO for cardiovascular protection (George et al., 2005). Joyeux-Faure, (2007) describes that administration rhEPO can confer cerebral and myocardial protection against ischemia-reperfusion injury in terms of reduction in cellular apoptosis and necrosis and also suggests that rhEPO could have beneficial applications in oncology, protecting against chemotherapy cardiotoxicity. Some of the clinical benefits of rhEPO include stimulation of erythropoiesis (increases in the number of reticulocytes and erythrocytes, hematocrit and blood hemoglobin concentration), elimination of the need for – and risks of – transfusion of allogeneic

red blood cells, increase in physical exercise tolerance, prevention of anemia-induced hyperdynamic cardiac state, improvement of cognitive and psychosomatic functions of the brain, and relief of pruritus (Jelkmann, 2000).

Biological Activity of Therapeutic Recombinant Proteins

Much of the early work to determine the biological activity of proteins has come from data obtained from assays which monitored the clearance of radiolabeled proteins. Later, immunoassays became a more common method to measure pharmacokinetics of recombinant therapeutic proteins. The biological activity of recombinant therapeutic proteins is important to show that the molecule can bring about a specific biological change. This can vary from one host cell system to another. The biological activity of a recombinant protein can be measured *in vitro* with bioassays, cell proliferation assays, enzyme assays, or a functional ELISAs. Also, the biological activity can be investigated *in vivo* using animal models.

Recombinant Human EPO Biological Activity

The biological activity of expressed recombinant human EPO can vary from one type of cell to another. The biological activity of recombinant human EPO has been investigated *in vitro* and shows that carbohydrate moiety plays an important role in the biological activity of recombinant human EPO (Takeuchi et al., 1989; and Wasley et al., 1991). The non-glycosylated EPO has a very low *in vivo* bioactivity due to rapid clearance from plasma by the liver (Tsuda et al., 1990). The National Institute for Biological Standards and Control (NIBSC) report notes major differences in reactivities between *in vivo* and *in vitro* biological and immunological assays with the various urinary and rhEPOs tested (Storring and Gaines Das, 1992). Takeuchi et al. (1989) reports that EPO possesses full biological activity only when it is sufficiently sialylated to avoid clearance by the hepatic asialoglycoprotein binding protein. Hayakawa et al.

(1992) investigated the biological activity of four pharmaceutical preparations of rhEPO *in vivo* and showed that their differences in the extent of the activity were not significant and expressed the same biological potencies.

The *in vivo* bioassays to test for biological activity for rhEPO are commonly performed in polycythaemic or normocythaemic mice where endogenous EPO production is suppressed by previous red blood cell transfusion or exposure to hypoxia (Jelkmann, 2003). The assay with normocythaemic mice suffices for routine pharmaceutical quality control of rhEPO and are known to be time consuming and expensive, require many animals, and produce intra- and inter-assay variation of the results (Jelkmann, 2009). According to the European Pharmacopoeia (Ph Eur monograph 1316), the activity of therapeutic rhEPOs is compared to the biological reference preparation (BRP) by either method A (measurement of incorporation of ^{59}Fe into red blood cells 4 days after EPO injection in mice made polycythaemic by exposure to hypobaric hypoxia) or method B (microfluorometrical measurement of reticulocytes in a flow cytometer 4 days after EPO injection in normocythaemic mice) (Jelkmann, 2009).

On the other hand, the *in vitro* bioassays to test for the biological activity for rhEPO include using cell based assays such as rat bone marrow cells (Takeuchi et al., 1989), human UT7/ leukemia cell line (Long et al., 2006), and F-36E/ human leukemia (Joung et al., 2009) and erythroid precursor cells (Akagi et al., 2004; Burns et al., 2002; Macdougall, 2008; Malik et al., 1998; Fibach et al., 1989; Umemura et al., 1990).

CD34+ Cells

All mature blood and immune cells develop from a population of stem cells called hematopoietic stem cells (HSC) such as CD34+ cells that can be isolated from bone marrow, mobilized peripheral blood, and umbilical cord blood (Dimitriou et al., 2003). CD34+ cells

express the protein CD34 but as they differentiate the expression of CD34 gradually decreases (Wojda et al., 2002). The CD34 protein is expressed on virtually all hematopoietic progenitors (Civin et al., 1984). CD34+ cells have the capacity for self-renewal for differentiation into a specialized erythroid cell that is controlled by the cellular micro-environment, growth factors, and cytokines such as EPO (Marks and Pilkington, 2008). CD34+ cells have been used as a model to investigate erythropoiesis for recombinant human EPO (Malik et al., 1998).

Erythroid Differentiation and Erythropoiesis

Erythropoiesis is the process by which multipotential hematopoietic stem cells differentiate into mature, non-nucleated erythrocytes (Richmond et al., 2005). The production of red blood cells involves the terminal differentiation of hematopoietic stem cells in the bone marrow followed by release into the peripheral blood and during the differentiation, erythroid progenitor cells undergo extensive remodeling of their cytoskeleton and loss of nuclei and other organelles like the endoplasmic reticulum (Patterson et al., 2009). A schematic representation of red blood cell production is shown in Figure 2. Red blood cells remain in circulation in the human for approximately 120 days and require the prior production of abundant red cell-specific proteins including hemoglobin, cytoskeletal proteins, and membrane glycoproteins such as glycophorin A (GpA) (Wickrema et al., 1994). Pluripotent hemopoietic stem cells reside in the bone marrow and give rise to all blood cell types through a process of simultaneous lineage commitment, cell proliferation, and differentiation, an ongoing process due to a the blood cells limited life span and an inability to replicate (Socolovsky et al., 1998). In the last two decades, numerous cytokines and extracellular factors such as stem cell factor, interleukin 3, granulocyte–macrophage colony stimulating factor (GM-CSF), and EPO have been identified as essential in the differentiation process of hemopoietic stem cells (Metcalf, 1993). The cytokine EPO is

considered a crucial growth factor for erythropoiesis (Krantz, 1991). As shown in Figure 2, granulocyte colony stimulating factor (G-CSF) acts on the hematopoietic stem cells and EPO acts on both burst forming units-erythriod (BFU-E), colony forming units-erythroid (CFU-E) cells, and erythroblasts to stimulate differentiation into erythrocytes (Yoshimura and Arai, 1996).

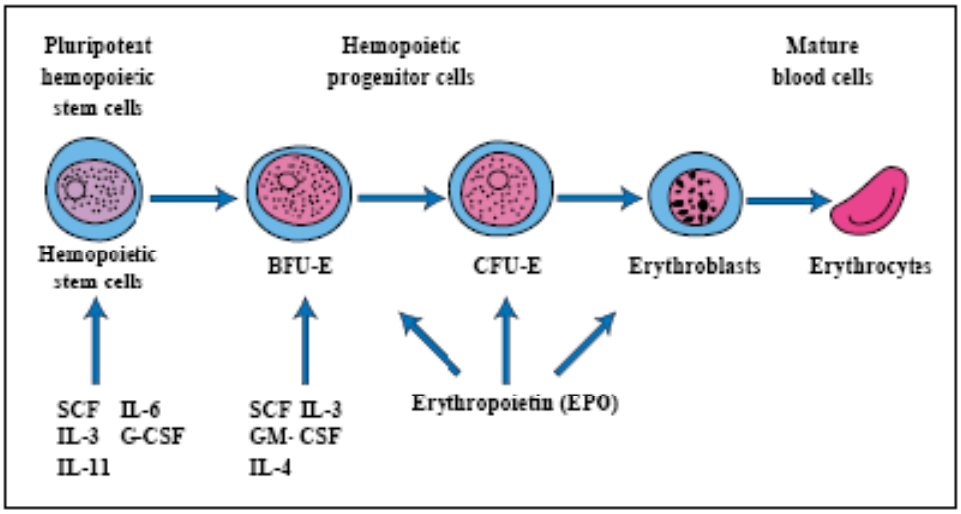

Figure 2. The production of red blood cells. Erythropoietin stimulates differentiation of hemopoietic stem cells into erythrocyte (Yoshimura and Arai, 1996).

Erythroid Markers

Proliferation and differentiation of erythroid precursor cells are modulated by cytokines such as EPO and granulocyte colony stimulating factor (G-CSF) (Sato et al., 2000). Erythroid precursor cells differentiate into hematopoietic cells and express erythroid specific proteins that can be identified and used as markers for differentiation. These erythroid specific proteins such as glycophorins, transferrin receptors, and hemoglobins are used to investigate the differentiation process of erythroid cells.

Hemoglobin (Hb) represents an excellent erythroid marker because hemoglobin synthesis occurs at the early proerythroblast stage of erythrocytic maturation, during the differentiation process, and continues until the final stage of a mature erythrocyte (Pinkus and Said, 1981).

Hemoglobin is a globular protein that transports oxygen (O_2) in human blood from the lungs to the tissues of the body and collects carbon dioxide to bring it back to the lungs. Hemoglobin can bind four oxygen molecules because it contains four subunits (heme groups), each of which contains an iron atom capable to bind to one oxygen molecule (Marengo-Rowe, 2006). Biosynthesis of hemoglobin in humans includes a transition from fetal (γ-globin) to adult (β-globin; α-Hb) during the differentiation process of erythroid cells (Stamatoyannopoulos and Papayannopoulou, 1979). Adult human hemoglobin is a tetrameric protein with a molecular weight of 64.5 kD and contains two polypeptide chains known as the alpha chain (141 amino acids) and the beta chain (146 amino acids) which are similar in length but differ in the amino acid sequence (Perutz et al., 1960). The individual molecular weight of each human hemoglobin alpha and beta chain is 15 kD (Yu et al., 1997).

Another erythroid marker, glycophorin A (GpA), which is the major sialoglycoprotein on human red cells, is one of the best characterized mammalian integral membrane proteins (Marchesi et al., 1972; Tomita and Marchesi 1975). Glycophorin A is an important constituent of the human red blood cell that carries several medically important blood group antigens (Remaley et al., 1991). The protein molecule contains a large hydrophilic portion, carrying the NH-terminal, located on the external surface of the blood cell, and the COOH-terminal is located in the cytoplasm and probably interacts with peripheral proteins (Bretscher, 1975). An unusually large proportion (about 60%) of the GpA is made up of carbohydrates located on the outside of the lipid bilayer, specifically 15 *O*-glycosidic oligosaccharides with the structure of *N*-acetyl galactosamine β(1-4) *N*-acetyl neuraminyl α(2-3) galactosyl β(1-3) *N*-acetyl neuraminyl α(2-6) *N*-acetyl galactosamine and one *N*-glycosidicoligosaccharide located at Asn 26 (Thomas and Winzler, 1969). Non-glycosylated GpA was shown to have an apparent molecular weight of 19

kD and glycosylated GpA was shown to have an apparent molecular weight of 39,000 (Andersson et al., 1981).

Another erythroid marker is human transferrin receptor (CD71). Transferrin receptor is expressed on activated B and T cells, macrophages, reticulocytes, malignant tissues and highly expressed on erythroid precursors which mediates the uptake of transferrin-iron complexes (Marsee et al., 2010). The human transferrin receptor is a transmembrane glycoprotein composed of two identical disulphide-linked monomers with a molecular weight of 90-95 kD each (Sutherland et al., 1981). Transferrin receptor is a type II transmembrane protein that interacts with transferrin and hereditary hemochromatosis protein involved in the cellular transport of iron (Kasibhatla et al., 2005). Transferrin receptor is synthesized in the endoplasmic reticulum and is post translationally modified with phosphate, fatty acyl groups, and oligosaccharides (Omary and Trowbridge, 1981; Schneider et al., 1982). The transferrin receptor uptakes iron via endocytosis which gets released into the cytosol where it is utilized as a cofactor for aconitase, the cytochromes, RNA reductase, and heme, or stored as ferritin (Zhong et al., 2002).

Signal Transduction

EPO signaling involves tyrosine phosphorylation of the homodimeric EPO receptor and subsequently activation of the intracellular anti-apoptotic proteins, kinases, and transcription factors (Jelkmann, 2004). The signal for cellular proliferation and differentiation into erythroblasts is thought to originate at the EPO receptor which is divided into two major regions (Yoshimura and Arai, 1996). The part of the receptor lying nearest the plasma membrane is required for generating the signals for proliferation and differentiation such as the induction of globin synthesis (Ohashi et al., 1994). The remaining half is not required for this signaling and

acts to dampen the signals (Yoshimura and Arai, 1996). The tyrosine kinase called JAK2 associates with the region near the plasma membrane, undergoes autophosphorylation, and phosphorylates the EPO receptor, and the transcription factor called signal transducers and activators of transcription (STAT) (Witthuhn et al., 1993) which plays an important role in promoting cellular proliferation. STAT activated by the phosphorylation translocates to the nucleus and recognizes a specific base sequence in the promoter region of the target gene to initiate transcription (Yoshimura et al., 1995). Tyrosine phosphorylation of the EPO receptor is necessary to initiate a signal transduction pathway mediated by proteins with SH2 domains whereby an activated ras protein can then activate the Raf-MAP kinase cascade, then an enzyme called PI3 kinase binds to the tyrosine phosphorylation site of the receptor (Yoshimura and Arai, 1996). Conversely, the tyrosine phosphatase SH-PTP1 promotes the dephosphorylation of JAK2 to stop generation of the signal (Klingmüller et al., 1995). Another major survival pathway for erythroid cells includes the activation of AKT (protein kinase B), a serine/threonine kinase which is a common mediator of cell survival and proliferation (Ryan et al., 2007). EPO binding to the EPO receptor on erythroid progenitors cells results in enzymatic phosphorylation cascades (signal transduction pathways) that transmit signals from outside the cell to the nucleus and is involved in the activation (phosphorylation by Ser473-Kinase) of AKT which causes general inhibition of pro-apoptotic factors, such as the forkhead transcription factors, Bad and caspase 9, all of which are known to mediate apoptosis (Brunet et al., 1999; Datta et al., 1999; Cardone et al., 2000). A schematic drawing of signal pathways in response to EPO are shown in Figure 3. Two hypotheses have been proposed to explain the requirement for cytokine receptor signaling: 1) the commitment of a progenitor to a particular lineage is a stochastic event, subsequent to which cell differentiation proceeds along a pre-determined program; growth factors are merely

required to ensure the survival and proliferation of committed progenitors or 2) growth factors have a direct role in cell differentiation, predicting that cell fate will be determined by the type of growth factor acting on the cell (Socolovsky et al., 1998).

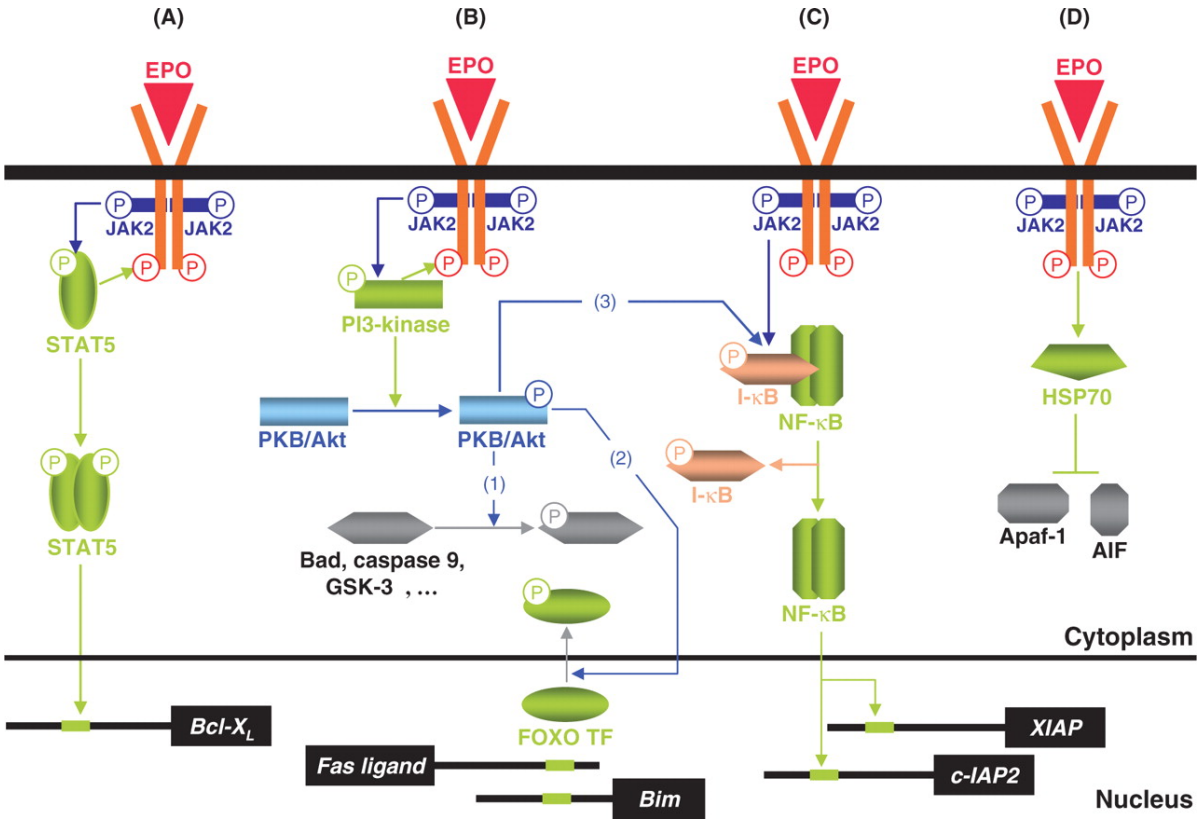

Figure 3. Schematic representation of the erythropoietin (EPO) signaling pathways by which the binding of EPO to its receptor can inhibit apoptosis. Binding of EPO induces (A) phosphorylation of the STAT5 transcription factor that will activate target genes encoding antiapoptotic molecules, (B) phosphorylation of phosphatidylinositol 3-kinase (PI-3 kinase) that, in turn, phosphorylates protein kinase B (PKB)/Akt, which then phosphorylates (1) proapoptotic molecules, such as Bad, caspase 9 or glycogen synthase kinase-3β (GSK-3β), leading to their inactivation, (C) phosphorylation of I-κB, which allows the release of the transcription factor NF-κB, its translocation into the nucleus and activation of target genes encoding antiapoptotic molecules, and (D) activation of Hsp70, which binds to and inactivates proapoptotic molecules, such as apoptosis protease-activating factor-1 (Apaf-1) and apoptosis-inducing factor (AIF) (Rossert and Eckardt, 2005).

CHAPTER III

EXPRESSION AND IDENTIFICATION OF RECOMBINANT HUMAN ERYTHROPOIETIN FROM (LMH/2A) LEGHORN MALE HEPATOMA CELL LINE

Introduction

Human erythropoietin (hEPO) is a hydrophobic acidic glycoprotein (Lee et al., 2009) that is produced by the kidney and the liver in fetuses (Joyeux-Faure, 2007) and is responsible for the regulation of red blood cell production in humans (Lappin et al., 2002). Erythropoietin consists of a single polypeptide chain of 165 amino acids, two disulphide bonds, three *N*-linked glycosylation sites (Asn 24, Asn 38, and Asn 83), and one *O*-linked glycosylation site (Ser 126) (Su et al., 2010). The average carbohydrate content amounts to 40% (Narhi et al., 1991) which gives erythropoietin an apparent molecular weight of 35-39 kD (Joung et al., 2009) while the expected non-glycosylated molecular weight is 18 kD (Jelkmann, 1992). In addition, the carbohydrate moieties of rhEPO contain sialic acids which contribute to the relatively low isoelectric pH of approximately 4.4 (Davis and Arakawa, 1987). De Frutos et al. (2003) showed that preparations of rhEPO were heterogeneous which means that they contain multiple glycoforms, differing in their carbohydrate structure. A schematic presentation of the primary structure of erythropoietin is shown in Figure 4.

In 1983, the gene for hEPO was isolated, cloned and used to produce rhEPO in CHO cells using recombinant DNA technology (Lin et al., 1985). It was discovered in 1986 that rhEPO can correct the anemia of chronic renal disease (Eschbach et al., 1987). This led to large-scale production in CHO cells of rhEPO, used to treat patients with both renal and non-renal anemia (Macdougall and Eckardt, 2006). Success using rhEPO to treat anemia has been shown in patients with chronic kidney disease, cancer, and AIDS (Elliott et al., 2003).

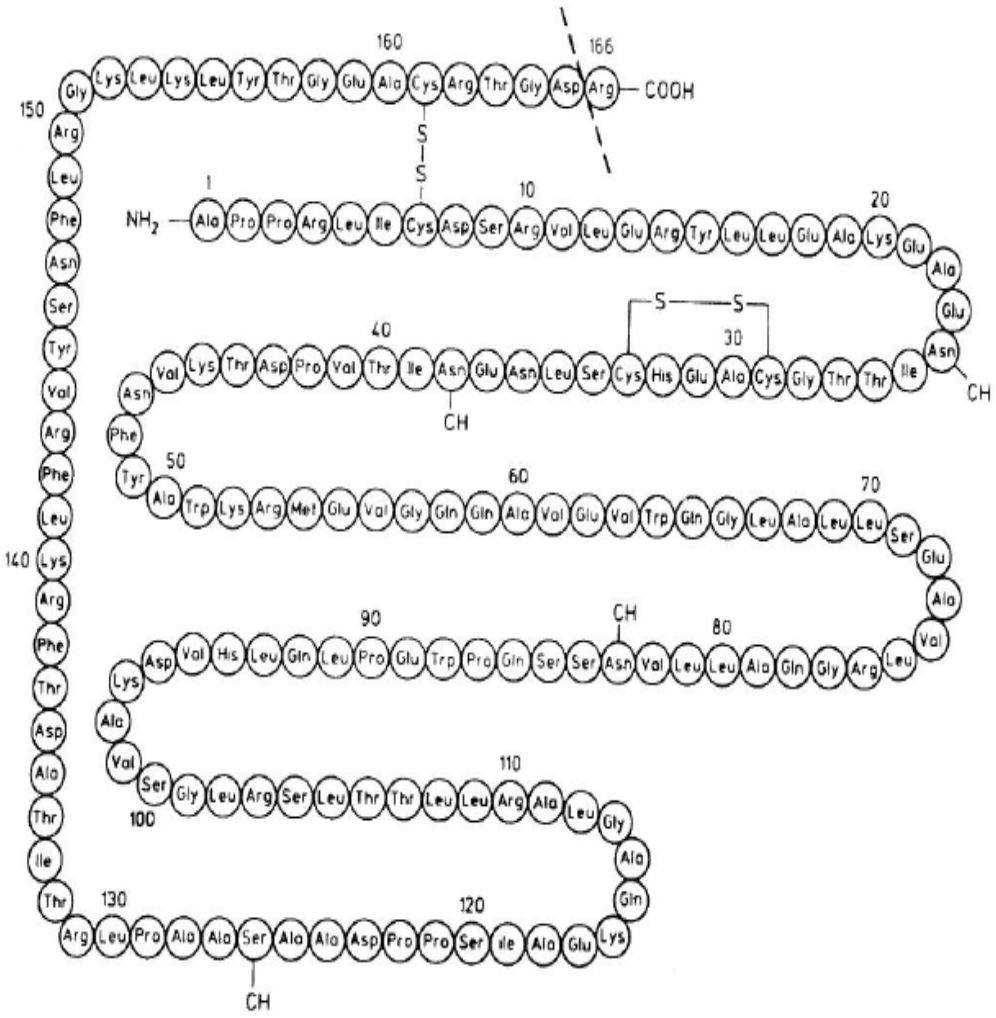

Figure 4. Schematic presentation of the primary structure of human erythropoietin. The mature hormone is composed of 165 amino acids. There are 3 *N*-linked glycosylation sites (CH) at asparagine residues 24, 38, and 83 and one *O*-linked glycosylation site (CH) at the serine residue 126 (Jelkmann, 1992).

Since avian cells such as chicken embryos have already been used for medical products (Fenner et al., 1974) and given that they have the potential to be grown on a large-scale with relatively low costs to produce biosimilars (Lee et al., 1999), the LMH/2A cell line was chosen in this study as a host system for rhEPO expression. Indeed, the LMH cell line has been successfully used to produce recombinant proteins *in vitro* (Binder et al., 1990; Cherentaeva et al., 2008; Vasudevan et al., 2001; Walzem et al., 1997; Weber et al., 1995).

Materials and Methods

Vector Design

- **Materials**

1. Human EPO gene (Integrated DNA Technologies, Coralville, IA, GenBank accession number M113191)
2. Beckman Coulter's CEQ 8000 Genetic Analysis System (Beckman, Brea, CA)
3. Endofree Plasmid Prep Kit (Qiagen, Valencia, CA)
4. Restriction Enzyme SalI-HF (New England Biolabs, Ipswich, MA)
5. Restriction Enzyme AgeI (New England Biolabs, Ipswich, MA)
6. Gene Quant Pro Spectrophotometer (GE, Piscataway, NJ)
7. Quick T4 DNA Ligase kit (New England Biolabs, Ipswich, MA)
8. DNA Ladder supercoiled (Invitrogen, Carlsbad, CA)
9. DNA Ladder (Epicentre, Madison, WI)
10. *E. coli* Top10 cells (Invitrogen, Carlsbad, CA)

- **Procedure**

The human EPO (hEPO) gene was synthesized by Integrated DNA Technologies (GenBank accessioning number M113191.1, see Appendix). The hEPO gene was inserted into plasmid DNA using restriction enzymes SalI-HF and AgeI (New England Biolabs) and ligated using a Quick T4 DNA Ligase Kit (New England Biolabs). The ligated product was transformed into *E. coli* Top10 cells. The plasmid was harvested using the Qiagen EndoFree Plasmid Maxi-Prep kit. The hEPO gene was cloned into a proprietary vector system (TGRX, Inc.). Alignments of DNA sequence, deduced amino acid sequence, and its respective EPO protein were

confirmed. Using the Beckman Coulter's CEQ 8000 Genetic Analysis System, the DNA sequence was verified for the codon optimized vector.

LMH/2A Cell Culture

- **Materials**

1. Biosafety cabinet Level II (Labconco, Kansas City, MO)
2. Incubator (Sanyo, San Diego, CA)
3. Centrifuge (Eppendorf, Germany)
4. 37° C water bath (Cole Palmer, Vernon Hills, IL)
5. Cell culture flasks, T-25 (Corning, Corning, NY)
6. Gelatin (Stemcell Technologies, Vancouver, Canada)
7. LMH/2A cells (ATCC, Manassas, VA)
8. 50 ml conical tube, sterile (Corning, Corning, NY)
9. Waymouth's Media (Gibco, Carlsbad, CA)
10. Fetal bovine serum (FBS), Australian or New Zealand origin (SAFC, Lenexa, KS)
11. FuGENE 6 (Roche Applied Science, Indianapolis, IN)
12. HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Sigma, St. Louis, MO)
13. Puromycin (Invivogen, San Diego, CA)

- **Procedure**

Six T-25 cell culture flasks were coated with gelatin. Gelatin coated flasks were incubated on a flat surface for 30 minutes at room temperature. The excess gelatin solution was aspirated from the T-25 flasks. A vial containing the LMH/2A cells (ATCC) was removed from liquid nitrogen storage and thawed completely in a 37°C water bath. LMH/2A cells were transferred into sterile 50 mL centrifuge tube and 1 ml of Waymouth's media plus 10% FBS was

added to the tube while swirling gently for 1 minute. Slowly 1 ml to 2 mls volume of Waymouth's media plus 10% FBS was added to the tube while swirling gently until there was approximately 30 mls of Waymouth's media plus 10% FBS mixed with the LMH/2A cells. The suspended LMH/2A cells were centrifuged (200 X g), a pellet was formed and the supernatant was gently poured off. The LMH/2A cell pellet was gently resuspended in 20 mls of Waymouth's media plus 10% FBS and the cells were seeded to gelatin-coated T-25 flasks. The LMH/2A cells were grown in Waymouth's media supplemented with 10% FBS and 1 M HEPES and were incubated at 37°C with 5% CO_2. A complex of FuGene 6 transfection reagent and DNA that contained the rhEPO gene were formed at a 1:6 ratio (DNA:FuGene) and were incubated for one hour. One hour before the transfection complex was added to the cells, media on LMH/2A cells was replaced with fresh Waymouth's media plus 10% FBS and 1 M HEPES. The transfection complex was added to the LMH/2A cells at 40-70% confluency for five T-25 flasks. One T-25 flask received no transfection complex and was used as a control. The transfected LMH/2A cells were grown in Waymouth's media supplemented with 10% FBS and 1 M HEPES and 1 μg/mL puromycin. Two days post-transfection, media was collected from the LMH/2A cells to test for rhEPO expression.

Recombinant Human LMH/2A-Expressed EPO

Sandwich Enzyme-Linked Immunosorbent Assay ELISA

- **Materials**

1. Micro-plate reader: Capable of reading the OD (4.0 maximum) of 96-well plate at wavelength 450nm (Bio-Rad, Hercules, CA)
2. Micro-plate Washer: Capable of washing a 96-well plate five times and able to dispense and aspirate 325 ± 25 μL/well (Biotek, Winooski, VT)

3. Immulon 4 HBX flat bottomed, sterile, uncoated, micortiter, polystyrene, 96-well plate (Thermo Electron Corporation, Waltham, MA)
4. Monoclonal antibody (mouse) to human EPO (Abcam, Cambridge, MA)
5. Monoclonal antibody (mouse) to human EPO-HRP (horseradish peroxidase) conjugated (Life Span, Seattle, WA)
6. CHO cell expressed rhEPO (Prospec, Israel)
7. TMB (3,3',5,5' - tetramethylbenzidine) microwell peroxidase substrate system (KPL, Gaithersburg, MD)
8. Albumin Bovine Serum (Sigma, St. Louis, MO)
9. Waymouth's media (Gibco, Carlsbad, CA)
10. Fetal bovine serum (FBS), Australian or New Zealand origin (SAFC, Lenexa, KS)
11. Sulfuric Acid (H_2SO_4, Molecular weight (MW) 98.08, American Chemical Society (ACS) 96.4%)
12. Tromethamine (Tris Base), $(CH_2OH)_3CNH_2$, MW 121.14, Purity >99.9%)
13. Sodium Chloride, (NaCl, MW 58.44, US Parmacopeia (USP)/Food Chemical Codex (FCC))
14. Tris-hydrochloride, (Tris-HCl, MW 157.59, Ultra Pure Grade >99.9%)
15. Potassium Phosphate, Monobasic, (KH_2PO_4, MW 136.09, Guaranteed Reagent (GR) ACS >99.9%)
16. Sodium Phosphate, Dibasic, Heptahydrate, (Na_2HPO_4-$7H_2O$, MW 268.07, Analytical Reagents (AR)(ACS) 99.8%)
17. Potassium Chloride, (KCl, MW 74.56 ACS)
18. Sodium Carbonate, Anhydrous,(Na_2CO_3, MW 105.99, GR ACS >99.5%)

19. Sodium Bicarbonate ($NaHCO_3$, MW 84.01, Certified ACS powder 100.3%)
20. Polyethylene Glycol Sorbitan, Monolaurate (Tween-20) ($C_{58}H_{114}O_{26}$, MW 1227.54, Reagent Grade)
21. Coating buffer: 30mM Na_2CO_3 , 70 mM $NaHCO_3$ [pH 9.6]
22. 1X PBS buffer: 136.9 mM NaCl, 1.4 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl [pH 7.4]
23. Blocking buffer: 1.5% albumin bovine serum, 0.05% Tween 20, 1X PBS
24. TTBS buffer: 0.05% Tween 20, 100mM Tris-HCl, 150 mM NaCl [pH 7.4]
25. Stop solution: 2M H_2SO_4
26. Negative control media: (Waymouth's media, 4.5 % FBS, 1 M HEPES, 100mM Tris-HCl, 150 mM NaCl [pH 7.4])

- **Procedure**

Based upon the ELISA method first established by Engvall and Perlmann (1971), a variation known as the sandwich ELISA was used to quantify the rhEPO in the media from post-transfected LMH/2A cells. The capture antibody anti-mouse monoclonal to human EPO (Abcam) was diluted in coating buffer such that the final working dilution concentration was 5.6 µg/mL. One-hundred µL of the diluted capture antibody was added into to the appropriate wells of the 96-well ELISA plate and incubated for 1 hour at 37° C. After capture antibody incubation, the 96-well ELISA plate was washed one time with TTBS buffer (0.05% Tween 20, 100mM Tris-HCl, 150 mM NaCl [pH 7.4]). Two hundred µL of blocking buffer (1.5% albumin bovine serum, 0.05% Tween 20, 1X PBS) was added to the appropriate wells of the 96-well ELISA plate and incubated for 45 minutes at room temperature. The test samples and CHO-rhEPO standard (Prospec) were diluted in negative control Waymouth's media (4.5 % FBS, 1 M

HEPES, 100mM Tris-HCl, 150 mM NaCl [pH 7.4]). The CHO-rhEPO standard was diluted such that the final working dilution concentrations for the standards were 400, 200, 160, 120, 80, and 40 ng/mL. After standards and test sample preparations, the blocking buffer was removed and 100 µL of the diluted test samples and protein standards were added in triplicates into 96-well plate and incubated at room temperature for 1 hour. After incubation, the 96-well ELISA plate was washed five times with wash buffer (0.05% Tween 20, 100mM Tris-HCl, 150 mM NaCl [pH 7.4]). The detection antibody anti-human EPO with HRP conjugation (Life Span) was diluted in blocking buffer such that the final working dilution concentration was 2 µg/mL. One hundred µL of the diluted detection antibody was added into to the appropriate wells of the 96-well ELISA plate and incubated at room temperature for 1 hour. After the detection antibody dilution, the 96-well ELISA plate was washed five times with wash buffer (0.05% Tween 20, 100mM Tris-HCl, 150 mM NaCl [pH 7.4]) and 100 µL of the TMB Microwell Peroxidase Substrate System (KPL) was added into the wells of the 96-well ELISA plate. The 96-well ELISA plate was incubated at room temperature for 5 minutes and, then, 100 µL of 2M H_2SO_4 was added into the wells of the 96-well ELISA plate. Absorbance readings of the 96-well ELISA plate were taken at 450 nm using an micro-plate reader (Bio-Rad).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis SDS-PAGE

- **Materials**

1. Pre-cast 10-20%Tris-HCl gels (Bio-Rad, Hercules, CA)
2. Bio-Rad Gel Doc (Bio-Rad, Hercules, CA)
3. Electrophoresis Apparatus, Criterion Cell Chamber (Bio-Rad, Hercules, CA)
4. Tris-glycine sodium dodecyl sulfate (SDS) buffer (Bio-Rad, Hercules, CA)
5. Laemmli Sample Buffer (Bio-Rad, Hercules, CA)

6. Molecular Weight Standards, Kaleidoscope Precision Plus Protein Standards (Bio-Rad, Hercules, CA)
7. Dithiothreitol, DTT ($C_4H_{10}O_2S_2$, MW 154.25, ACS 99.4%, Amresco, Solon, OH)
8. Simply blue safe-stain (Invitrogen, Carlsbad, CA)
9. Sodium Chloride, (NaCl, MW 58.44, US Parmacopeia (USP)/Food Chemical Codex (FCC))
10. De-staining buffer: 20% NaCl

- **Procedure**

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was used, as developed by Laemmli (1970), to separate proteins by molecular weight under reducing conditions. Standards were incubated for 10 minutes at 100°C in Laemmli buffer and Dithiothreitol (DTT). These sample mixtures were loaded onto a 10-20% Tris-HCl gel and electrophoresed at 200 Volts for 80 minutes in Tris-Glycine-SDS buffer.

After SDS-PAGE, the gel was washed with de-ionized H_2O and stained with simply blue safe stain. The gel was de-stained in 20% NaCl. After de-staining, a picture was taken of the gel using the Bio-Rad Gel Doc.

Western Blot

- **Materials**

1. Power Source for the electrophoresis (Bio-Rad, Hercules, CA)
2. Pre-cast 10-20%Tris-HCl gels (Bio-Rad, Hercules, CA)
3. Bio-Rad Gel Doc (Bio-Rad, Hercules, CA)
4. Electrophoresis Apparatus, Criterion Cell Chamber (Bio-Rad, Hercules, CA)
5. Electrophoretic Blotting System (Bio-Rad, Hercules, CA)

6. Alkaline phosphatase (Development Reagent): 5-Bromo-4-chloro-3-indolyl phosphate/ Nitro blue tetrazolium (BCIP/NBT) Liquid Phosphatase Substrate (1-Component) (KPL, Gaithersburg, MD)
7. 10X Tris-Glycine (Bio-Rad, Hercules, CA)
8. Methanol (Mallinckrodt, Phillipsburg, NJ)
9. Sodium Chloride, (NaCl, MW 58.44, US Parmacopeia (USP)/Food Chemical Codex (FCC))
10. Tris-hydrochloride, (Tris-HCl, MW 157.59, Ultra Pure Grade >99.9%)
11. Tris-glycine buffer (Bio-Rad, Hercules, CA)
12. Tris-glycine sodium dodecyl sulfate (SDS) buffer (Bio-Rad, Hercules, CA)
13. Nitrocellulose transfer membrane (0.45 μm) (Whatman, Piscataway, NJ)
14. Non-fat dried milk
15. Gelatin
16. De-ionized H_2O
17. Laemmli Sample Buffer (Bio-Rad, Hercules, CA)
18. Molecular Weight Standards, Kaleidoscope Precision Plus Protein Standards (Bio-Rad, Hercules, CA)
19. Dithiothreitol, DTT (Amresco, Solon, OH)
20. Transfer buffer: 1 X Tris-glycine (Bio-Rad, Hercules, CA), 20 % Methanol
21. TTBS buffer: 0.05% Tween 20, 100mM Tris-HCl, 150 mM NaCl [pH 7.4]
22. Standard rhEPO (Fitzgerald, Acton, MA)
23. Rabbit-anti-EPO (Abcam, Cambridge, MA)
24. Goat-anti-Rabbit IgG AP (Novus, Littleton, CO)

- **Procedure**

SDS-PAGE was used, as developed by Laemmli (1970), to separate proteins by molecular weight under reducing conditions. The standard used was CHO-expressed rhEPO (Fitzgerald). Test samples and standards were incubated for 10 minutes at 100°C in Laemmli buffer and Dithiothreitol (DTT). These sample mixtures were loaded onto a 10-20% Tris-HCl gel and electrophoresed at 200 Volts for 80 minutes in Tris-Glycine-SDS buffer. After SDS-PAGE, the gel was equilibrated in transfer buffer (1 X Tris-Glycine, 20% Methanol) for 2 minutes. The proteins were transferred to 0.45µm-pore-size nitrocellulose at 100 Volts for 50 minutes. The nitrocellulose was removed from the transfer apparatus and blocked with blocking buffer (5.0% Milk, 0.05% Tween 20, 100mM Tris-HCl, 150 mM NaCl [pH 7.4]) overnight at 2-8° C. The following day the nitrocellulose was washed four times for 5 minutes per wash in TTBS buffer (0.05% Tween 20, 100mM Tris-HCl, 150 mM NaCl [pH 7.4]). The nitrocellulose was incubated in primary antibody rabbit-anti-EPO (Abcam) diluted to a final concentration of 1.3 µg/mL in antibody buffer (1% gelatin, 0.05% Tween 20, 100mM Tris-HCl, 150 mM NaCl [pH 7.4]) for 1 hour at room temperature. Then, the nitrocellulose was washed three times for 5 minutes per wash in TTBS buffer (0.05% Tween 20, 100mM Tris-HCl, 150 mM NaCl [pH 7.4]) and incubated in secondary antibody goat-anti-rabbit IgG AP (Novus) diluted to a final concentration of appropriately 120 ng/mL in antibody buffer (1% gelatin, 0.05% Tween 20, 100mM Tris-HCl, 150 mM NaCl [pH 7.4]) for 1 hour at room temperature. The nitrocellose was washed four times for 5 minutes per wash in TTBS (0.05% Tween 20, 100mM Tris-HCl, 150 mM NaCl [pH 7.4]) and one wash for 5 minutes with de-ionized H_2O. Conjugated antibody bound to antigen was developed by using the BCIP/NBT Substrate System (KPL). The Western blot was developed for 5 minutes. Color formation (enzyme reaction) was stopped by rinsing

blots with de-ionized H_2O. The blot was air-dried for 30 minutes and a picture was taken using Bio-Rad Gel Doc.

Purification

- **Materials**

1. Tricorn Columns (GE, Piscataway, NJ)
2. Blue Sepharose™ 6 Fast Flow (GE, Piscataway, NJ)
3. HiTrap™ Q FF, 1ml (GE, Piscataway, NJ)
4. SP Sepharose™ XL (GE, Piscataway, NJ)
5. Cation exchange equilibration buffer: 50 mM Sodium Acetate, 15 mM NaCl [pH 5.0]
6. Anion exchange equilibration buffer: 25 mM Tris, 30 mM Nacl [pH 8.0]
7. Elution buffer: 2 M NaCl
8. Formulation buffer: 20 mM Citrate, 100 mM NaCl [pH 6.9]
9. Tromethamine (Tris Base), $(CH_2OH)_3CNH_2$, MW 121.14, Purity >99.9%)
10. Sodium Chloride, (NaCl, MW 58.44, US Parmacopeia (USP)/Food Chemical Codex (FCC))
11. Sodium Acetate, ($NaC_2H_3O_2$, MW 82.03, ACS)
12. Citrate, ($C_6H_80_7$, MW 192.13, ACS)

- **Procedure**

For LMH/2A-rhEPO purification, the chronological chromatographic steps employed were: Blue Sepharose 6FF, Anion Exchange (Flow-through), Cation-Exchange (Bind and Elute), Hydrophobic Interaction (Flow-through), and Size Exclusion Chromatography.

Media from transfected LMH/2A cells was collected from each T-25 flask and pooled. In the first step, the LMH/2A-rhEPO starting material was captured from the pooled cell culture

harvest media using Blue Sepharose 6FF and eluted with 2M NaCl. Then, the eluted product of LMH/2A-rhEPO was purified using the anion exchange chromatography with anion exchange equilibration buffer (25 mM Tris, 30 mM Nacl [pH 8.0]) in flow-through mode. Further consecutive purification steps were conducted by using cation exchange chromotography with cation exchange equilibration buffer (50 mM Sodium Acetate, 15 mM NaCl [pH 5.0]). Final purification of LMH/2A-rhEPO was produced by using hydrophobic interaction and size exclusion chromatography. The final purified LMH/2A-rhEPO was stored in formulation buffer: (20 mM Citrate, 100 mM Nacl, [pH 6.9]) and stored at -80° C.

Concentration Determination

Bradford Assay

- **Materials**

1. UV/VIS Spectrophotometer (Beckman, Brea, CA , DU® 800)
2. Cuvettes with 1 cm path length (Bio-Rad, Hercules, CA)
3. Protein Assay Dye Reagent Concentrate (Bio-Rad, Hercules, CA)
4. LMH/2A-rhEPO, purified
5. CHO-rhEPO (Prospec, Israel)

- **Procedure**

The purified LMH/2A-rhEPO concentration was analyzed using the Bio-Rad Protein Assay which is based upon the method of Bradford. In this assay, purified CHO-rhEPO (Prospec) was used as the standard for the calibration curve. A standard curve was prepared from dilutions of the protein standard CHO-rhEPO as follows: 10 µg, 7.5 µg, 5 µg, 2.5 µg, and 1 µg. The standards and samples were prepared by mixing 800 µL of diluted standard or sample

with 200 µL of dye reagent concentrate. This mixture was incubated at room temperature 5 minutes, transferred to cuvettes and measured absorbance at 595 nm.

Spectrophotometry

- **Materials**

1. UV/VIS Spectrophotometer (Beckman, Brea, CA , DU® 800)
2. Microcell Quartz Cuvette (10 mm path length, 100 µL)
3. Purified LMH/2A-rhEPO, purified
4. Guanidine HCl, Ultra Pure (CH_5N_3•HCl, MW 95.5, Analytical Reagents (AR)(ACS) 99%)
5. Methanol (CH_4O, F.W. 32.04, Analytical Reagents (AR)(ACS) >=99.8%)
6. Ethanol (C_2H_5OH, F.W. 46.07, Analytical Reagents (AR)(ACS) 99.5%, Anhydrous, 200 Proof)
7. Acetone (CH_3COCH_3, F.W. 58.08, Analytical Reagents (AR)(ACS) 99.6%)
8. Denature buffer: 8 M Guanidine hydrochloride
9. Blank buffer: 20 mM Citrate, 100 mM NaCl [pH 6.9]

- **Procedure**

Purified LMH/2A-rhEPO concentration was determined using a UV spectrophotometer. The blank was prepared by diluting 30 µL of blank buffer (20 mM Citrate, 100 mM NaCl [pH 6.9]) in 90 µL of 8 M Guanidine HCl. Purified LMH/2A-rhEPO was prepared by diluting 30 µL in 90 µL of 8 M Guanidine HCl. The dilutions were incubated for 1 hour. The spectrophotometer was blanked and the absorbance reading for the purified LMH/2A-rhEPO was measured at 280 nm. Methanol, ethanol and acetone were used to clean the cuvette before blanking and before measuring the absorbance of purified LMH/2A-rhEPO.

PNGaseF Deglycosylation

- **Materials**

1. CHO cell expressed rhEPO (CHO-rhEPO) (Prospec, Israel)
2. Human cell expressed rhEPO (Hu-rhEPO) (Humanzyme, Chicago, IL)
3. LMH/2A-expressed rhEPO (LMH/2A-rhEPO), purified
4. PNGaseF Kit (New England Biolabs, Ipswich, MA)
5. Glycoprotein denaturing buffer (New England Biolabs, Ipswich, MA)

- **Procedure**

Glycosylation of CHO-rhEPO, Hu-rhEPO and LMH/2A-rhEPO was investigated using PNGaseF, enzyme which cleaves *N*-linked glycosylation. One μg of purified CHO-rhEPO, Hu-rhEPO and LMH/2A-rhEPO were incubated with glycoprotein denaturing buffer for 10 minutes at 100°C. PNGaseF enzyme (2 Units/ng rhEPO) was added to the mixture and incubated at 37°C for 2 hours. Under the same conditions, 1 μg of the purified CHO-rhEPO, Hu-rhEPO and LMH/2A-rhEPO without PNGaseF enzyme were used as a negative control. The treated and non-treated purified CHO-rhEPO, Hu-rhEPO and LMH/2A-rhEPO were analyzed by Western blot (as described above) except that the standards used in this experiment were CHO-expressed rhEPO (Prospec) and human-expressed rhEPO (Humanzyme).

Results and Discussion

LMH/2A-rhEPO Production

The DNA sequence for the inserted human EPO gene was aligned with EPO DNA sequence (Integrated DNA Technologies) using Clustal X software (data is not shown). The synthesized EPO DNA sequence was 100% identical to the complete DNA coding sequence of EPO from GeneBank accession number M1131.9 (See attachment A). The final vector which

contained the human EPO gene was determined to be 15 kb as seen in Figure 5. Furthermore, by using the inserted DNA sequence, it was verified that the base pair changes made in the vector were the desired mutations and that no other mutations occurred.

Transfection of avian cell lines (EB66®) to produce therapeutic recombinant proteins such as vaccines have been described (Brown and Mehtali, 2010). More interestingly, quail fibrosarcoma fibrosarcoma (QF) cells have been stably transfected and shown to produce

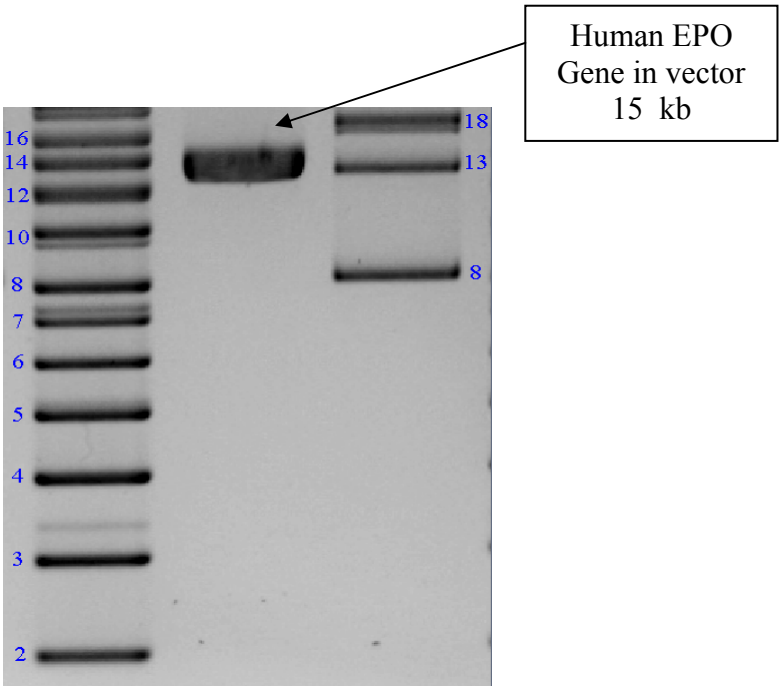

Figure 5. DNA agarose gel of human EPO gene in the vector. The Invitrogen super-coiled DNA ladder was loaded in lane 1 with a range of 16 to 2 kb. The final vector product that contained the human EPO gene was loaded in lane 2. The Epicentre super-coiled DNA ladder was loaded in lane 3 with a range of 18 to 8 kb.

approximately 13 µg/mL rhEPO per $3X10^6$ cells (Lee et al., 1999). The estrogen responsive avian cell LMH/2A have shown their ability to be transfected by expressing recombinant proteins such as chicken riboflavin carrier protein (Vasudevan et al., 2001) and apolipoprotein II (Sensel et al., 1994). Furthermore, LMH/2A cells have shown that they are highly susceptible to transfection with cationic lipids as reported by luciferase activity when compared to other cells,

specifically LMH/2A cells showed 8.5 fold higher luciferase activity than HepG2 cells and and 87.5 fold higher luciferase activity than FTO2B cells (Walzem et al., 1997). Cherentaeva et al. (2008) reports LMH cells were transfected and produced 150 ng/mL of human IFN-β. In this study, LMH/2A cells showed their ability to be transfected and express rhEPO. The transfected LMH/2A cells were healthy and appeared morphologically to be dendritic-like and polygonal with abundant cytoplasm and large round nuclei. Morphology of the LMH/2A cells grown in Waymouth's media supplemented with 10% FBS is shown in Figure 6.

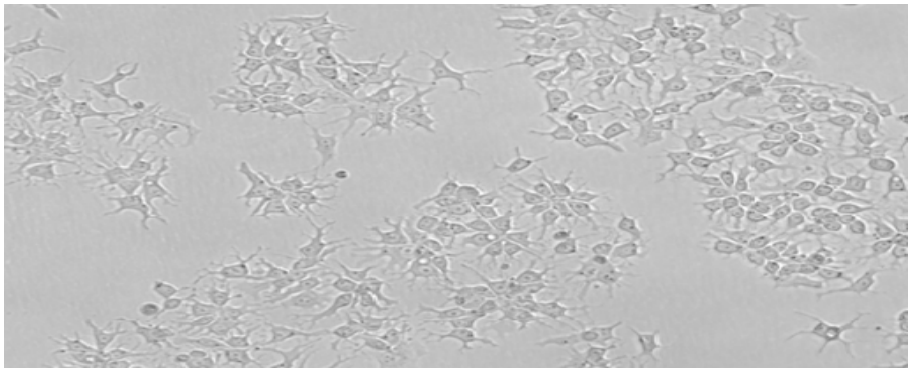

Figure 6. LMH/2A cells grown in Waymouth's Media supplemented with 10% Fetal Bovine Serum.

Recombinant human EPO was detected in the harvested cell culture media from transfected LMH/2A cells as seen in Table 1. Absorbance readings versus concentrations for the calibration curve were fitted to a linear regression model. As indicated in Figure 7, "Goodness of fit" was indicated by coefficient of determination (R^2) of 0.99 from six standard curve points.

The average back-calculated calibration curve concentration (% Accuracy) for each standard was within 90-110% of the known standard solution concentration (Table 1). Descriptive statistics [mean (AVG), standard deviations (STD), % coefficients of variation (CV) and differences from theoretical] is calculated for the calibration curve standards. Descriptive

statistics [AVG, STD, % CV] and estimated EPO concentrations were calculated for the test samples as seen in Table 1.

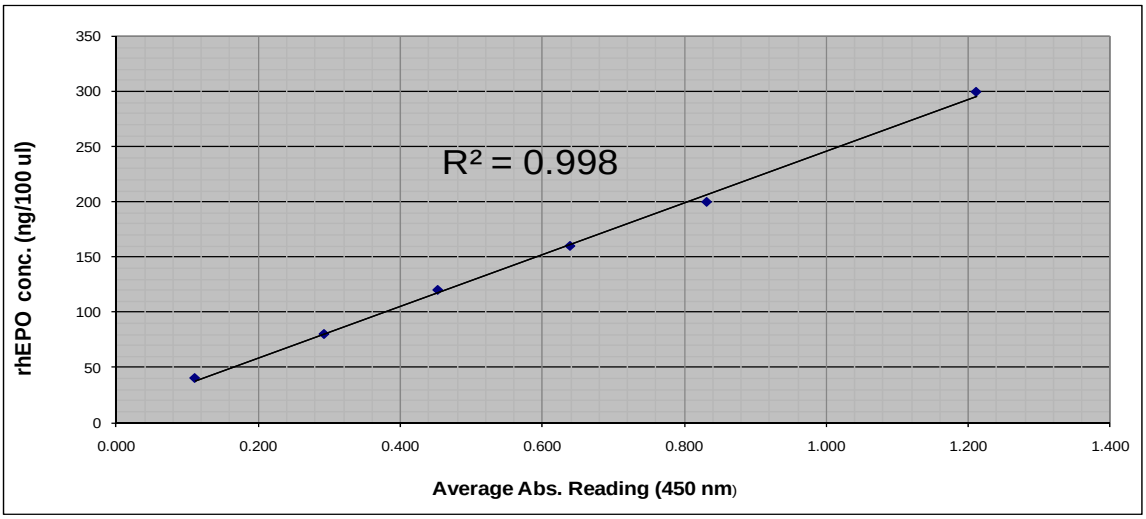

Figure 7. Standard curve for determination of rhEPO concentration in cell culture media by ELISA. Working assays were performed using standards within the range 300-10 ng/mL rhEPO. Values represent the mean of triplicate determinations per concentration.

Table 1. Descriptive statistics for LMH/2A-rhEPO sandwich ELISA[mean (AVG), standard deviations (STD), % coefficients of variation (CV) and accuracy %] is calculated from the absorbance readings of cell culture samples and calibration curve standards. ELISA working assays were performed using EPO standards within the range 300-10 ng/mL rhEPO. Values represent the mean of triplicate determinations per concentration.

Calibration Curve Standards	AVG Abs. (450 nm)	STD DEV	CV %	Back-calculated Conc. (ng/ml)	Accuracy %
1xTBS	0.104	0.010	10.00	0.00	n/a
EPO 300 ng/ml	1.318	0.046	3.40	295.40	1.50
EPO 200 ng/ml	0.939	0.013	1.30	206.60	3.30
EPO 160 ng/ml	0.746	0.013	1.60	161.60	1.00
EPO 120 ng/ml	0.560	0.012	2.20	118.00	1.70
EPO 80 ng/ml	0.400	0.014	3.40	80.50	0.60
EPO 40 ng/ml	0.218	0.022	10.20	37.90	5.40
Negative control Waymouth's Media 4.5 % FBS	0.107	0.016	14.60	0.00	n/a

Cell Culture Harvest Media	AVG Abs. (450 nm)	STD DEV	CV %	Estimated Conc. rhEPO µg/mL
Test sample 1	0.829	0.047	5.68	452.40
Test sample 2	0.580	0.007	1.13	306.50
Test sample 3	0.576	0.015	2.52	304.00
Test sample 4	0.323	0.016	5.03	156.20
Test sample 5	0.248	0.002	0.84	112.20
Negative Control LMH/2A Media	0.094	0.008	8.32	< 0.04

In this study, sandwich ELISA assay showed that the transfected LMH/2A cells produced on average 266 μg/mL LMH/2A-rhEPO per day. Yanagihara et al. (2008), Cointe et al. (2000), and Jeong et al. (2008) have shown that sandwich ELISA is a sensitive method for quantification of rhEPO. *Escherichia coli* produced rhEPO was quantified to be 1.4 mg/mL (Delorme et al., 1992). CHO cell expressed rhEPO was quantified as 1.8 mg/mL of deglycosylated CHO-rhEPO (Yang and Butler, 2002). Yeast cell expressed rhEPO was quantified to be 120 μg/mL glycosylated rhEPO (Maleki et al., 2010). However, these findings in the literature for the quantification of rhEPO cannot be compared because the number of days of incubation of the cells, the number of cells, and the volume of rhEPO product has not been normalized.

LMH/2A-rhEPO Identification

The Western blot data showed that LMH/2A-rhEPO was immunologically detected in the harvested test media at an apparent molecular weight ranging from 25 kD to 38 kD (see Figure 8). This apparent molecular weight showed that LMH/2A-rhEPO is shifted from the theoretical molecular weight of EPO which is 18 kD. Based on sandwich ELISA and Western blot results, it seems that LMH/2A-rhEPO was produced in proper amino acid sequence due to specific cross reactions to the native and denatured forms with different polyclonal and monoclonal antibodies. Machado et al. (2011) successfully detected Hu-rhEPO (SKOV3 cell line) produced *in vitro* and showed a molecular weight of 35 kD. CHO cell expressed rhEPO was detected by Western blot and showed a broad band with an approximate molecular weight of 35 kD (Elliott et al., 2003). On the other hand, *E. coli* expressed rhEPO was detected by Western blot and showed a lower molecular weight of approximately 18 kD (Boissel et al., 1993), the non-glycosylated form. Furthermore, Lee et al. (2000) determined that S2 cells (*Drosophila*) expressed rhEPO and

migrates at a molecular weight of 24 kD. Human cell expressed rhEPO (human myoblasts) showed a molecular weight of approximately 34 kD (Tripathy et al., 1994). These variable

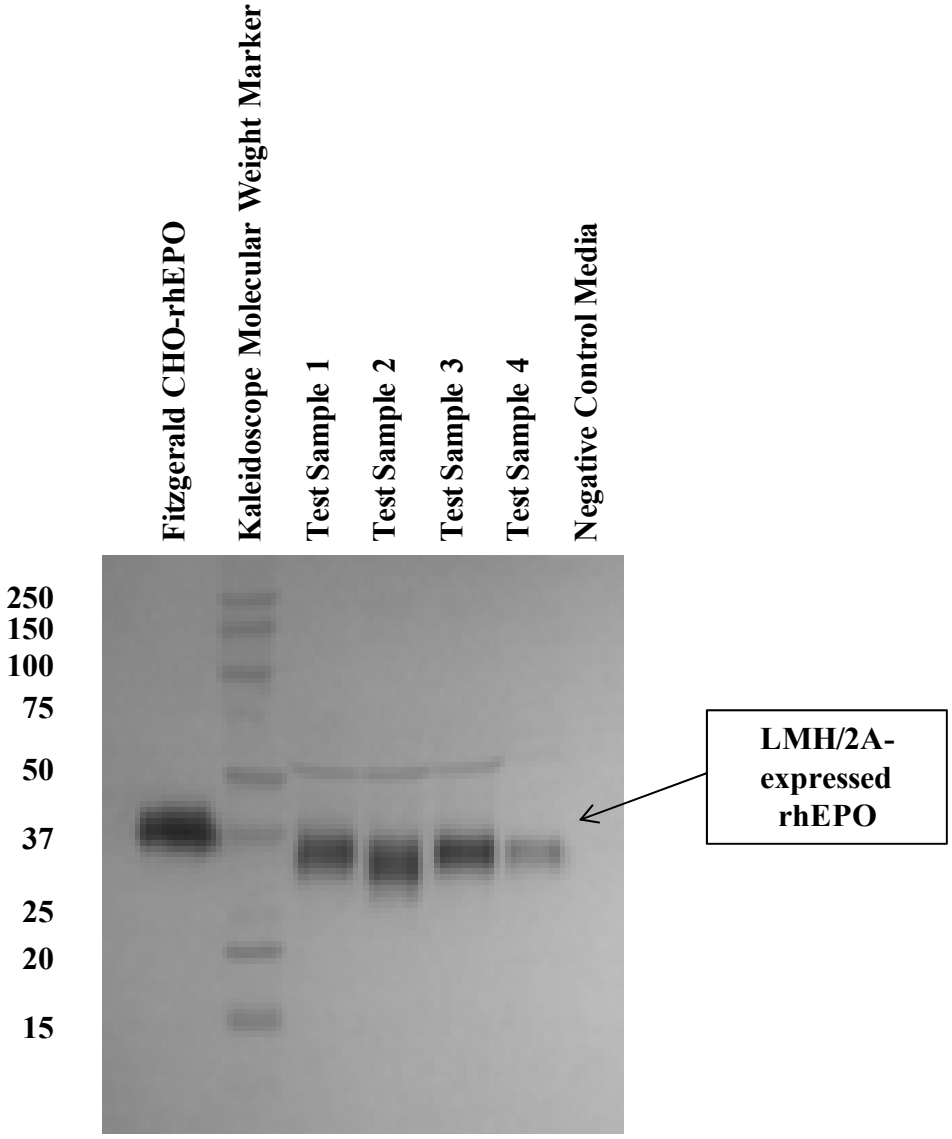

Figure 8. Western blot of LMH/2A-expressed rhEPO from cell culture harvest media. The standard CHO-expressed rhEPO (Fitzgerald) was loaded in lane 1. Kaleidoscope Precision Plus marker with a range of 250 kD to 10 kD was loaded in lane 2. Cell culture harvest media samples were loaded into lanes 3, lane 4, lane 5 and lane 6. Negative Control Waymouth's Media + 10 % FBS was loaded in lane 7.

molecular weight ranges shown by Western blot of rhEPO produced in different cell types is most likely due to variations in glycoforms of rhEPO. To wit, LMH/2A-rhEPO migrates in a

similar range to CHO cell expressed rhEPO and Human cell expressed rhEPO but differs in migration from *Drosophila* expressed rhEPO and *E. coli* expressed rhEPO.

The purity of the purified LMH/2A-rhEPO protein was determined to be $\geq$ 95% and verified by SDS-PAGE (See Figure 9). The SDS-PAGE of purified LMH/2A-rhEPO showed a broad band with an apparent molecular weight ranging from 25 kD to 38 kD.

CHO cell expressed rhEPO has been purified using chromatographic steps including Blue Sepharose which produced a 95% pure product (Garcia del Barco et al., 1995). Zanette et al. (2003) describes that the purification of EPO to a pure and active product that can be achieved by combining high performance of affinity chromatography and an anion exchange chromatography step. Furthermore, EPO has been shown to be purified using ion-exchange chromatography producing a single band, which purity was over 98% determined by SDS-PAGE (Lin et al., 1997). Also, purification of EPO using phenyl sepharose to facilitate hydrophobic interaction chromatography resulted in a 75-85% yield as described by Huang (1980).

EPO, both human and recombinant, have been characterized as heavily glycosylated proteins with several complex-type, *N*-linked carbohydrate side chains that migrate with a molecular mass distribution between 34 kD and 38 kD measured by SDS-PAGE (Dordal et al., 1985; Krystal et al., 1986). The rhEPO band has been described as "ladderlike" and is characteristic of the behavior of heavily glycosylated proteins analyzed by SDS-PAGE and the observed molecular weight of rhEPO is consistent with the presence of several highly branched oligosaccharide side chains attached to the polypeptide backbone (Westphal et al., 1975). Recny et al. (1987) reports that analysis of purified rhEPO by SDS-PAGE demonstrates a broad, diffuse band displaying a molecular mass distribution between 32 kD and 38 kD. Furthermore, Skibeli et al. (2001) showed that human serum EPO emerged as a broad band with

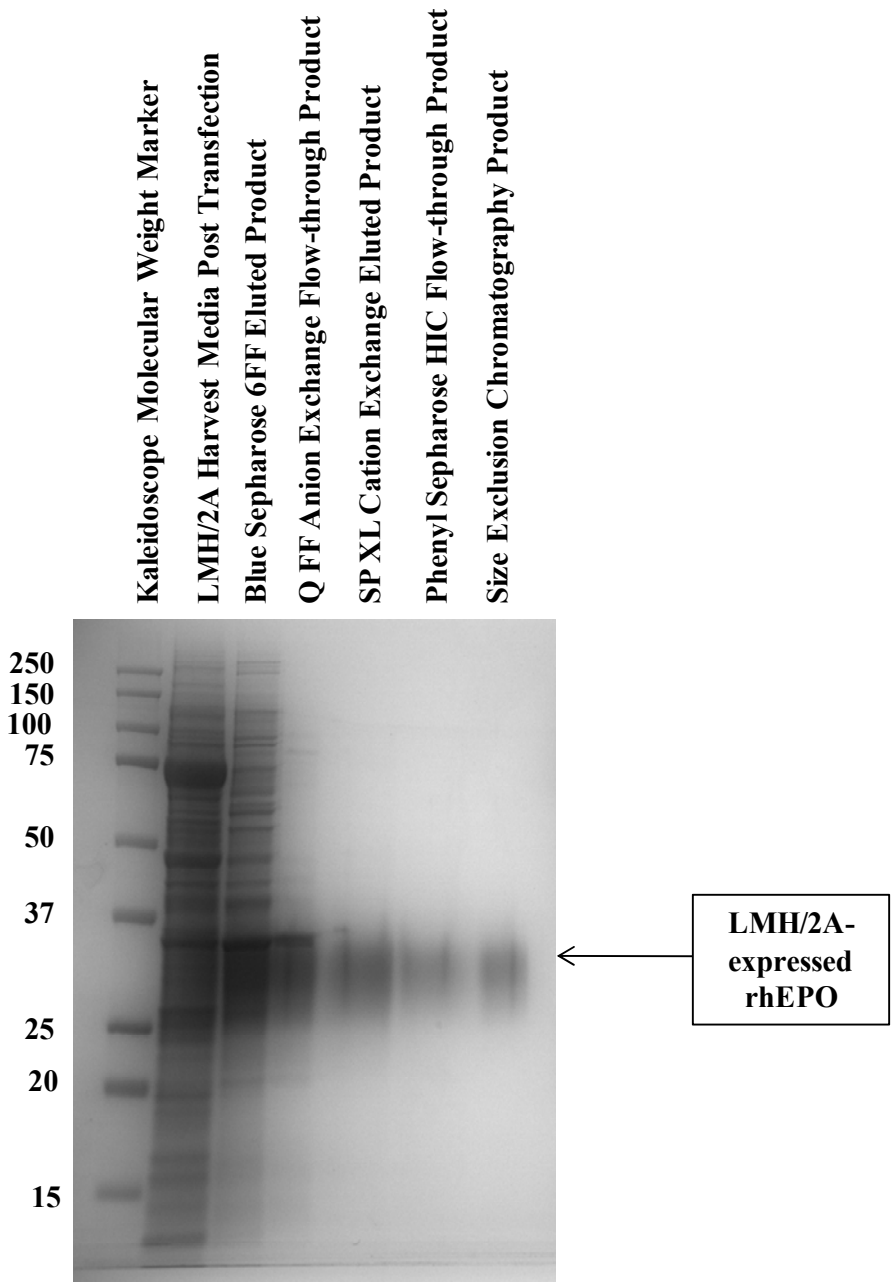

Figure 9. SDS-PAGE analysis of LMH/2A-rhEPO. Gel stained with simply blue safe stain following SDS-PAGE of LMH/2A-rhEPO under reducing conditions. Kaleidoscope Precision Plus marker with a range of 250 kD to 10 kD was loaded in lane 1. LMH/2A harvest media post transfection (cell culture media harvest contains the LMH/2A-rhEPO) was loaded in lane 2. Blue eluted product (elute from the Blue Sepharose 6FF) was loaded in lane 3. QFF Flow-through product (flow-through from the anion exchange) was loaded in lane 4. SP XL Eluted Product (elute from the cation exchange) was loaded in lane 5. Phenyl sepharose FT product (flow-through from the Hydrophobic Interaction Chromatography) was loaded in lane 6. SEC product (size exclusion chromatography final product purified LMH/2A-rhEPO) was loaded in lane 7.

an apparent molecular weight similar to that of rhEPO (34 kd and 40 kd) measured by SDS-PAGE analysis. Also, Inoue et al. (1995) showed that rhEPO migrated to a molecular weight of 38 to 42 kD on SDS-PAGE. The SDS-PAGE data suggests that two dimensional gel electrophoresis reveal several different glycoforms of EPO, as seen by a broad band, which confirms the heterogeneity of rhEPO.

Purified LMH/2A-rhEPO Concentration Determination

Based upon the Bradford method, the concentration of LMH/2A-rhEPO was estimated to be 795 µg/mL as shown in Table 2. Figure 10 is a graph of the standard curve using CHO-rhEPO (Prospec) used to quantify the purified LMH/2A-rhEPO. The concentration of purified

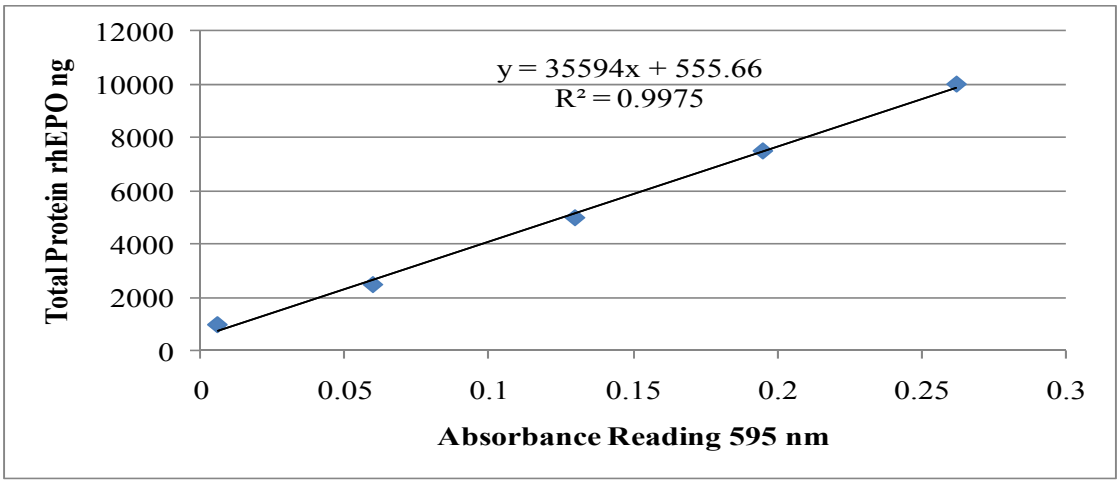

Figure 10. Standard curve of CHO-rhEPO determined by Bradford method. The standard curve is for determination of recombinant human EPO concentration in purified LMH/2A-rhEPO. Working assays were performed using standards within the range 1 µg to 10 µg CHO-rhEPO.

LMH/2A-rhEPO was estimated to be 822 µg/mL based upon the absorbance reading at 280 nm and by using molar absorption coefficient of 22670 M-1 cm-1 for rhEPO (data not shown). Based upon the initial concentrations determined by sandwich ELISA and the final concentrations determined by Bradford method and UV spectrophotometry at 280 nm, the total amount recovered from the cell culture harvest media was 4.8 mg of purified LMH/2A-rhEPO

Table 2. Data from the Bradford method including the absorbance readings of purified LMH/2A-rhEPO and calibration curve standards of CHO-rhEPO (Prospec). Bradford method was performed using CHO-rhEPO standards within the range 1 µg to 10 µg CHO-rhEPO.

Total Protein rhEPO µg/ml	Absorbance Reading 595 nm	Calculated Concentration (µg/ml)	
10 µg CHO-rhEPO	0.262	988.13	
7.5 µg CHO-rhEPO	0.195	749.29	
5 µg CHO-rhEPO	0.130	517.58	
2.5 µg CHO-rhEPO	0.060	268.42	
1 µg CHO-rhEPO	0.006	76.57	
Replicate 1 (LMH/2A-rhEPO)	0.205	785.95	
Replicate 2 (LMH/2A-rhEPO)	0.211	804.82	
	n	Average Concentration (µg/ml)	Standard Deviation (µg/ml)
Purified LMH/2A-rhEPO	2	795.39	13.34

which corresponded to a 72% yield. The final concentration of the purified LMH/2A-rhEPO was used to normalize the amount of LMH/2A-rhEPO used in the PNGaseF deglycosylation experiment and in the experiment to determine its biological activity.

LMH/2A-rhEPO Deglycosylation

Treatment of the purified CHO-rhEPO, Hu-rhEPO and LMH/2A-rhEPO with PNGaseF caused an increase in the electrophoretic mobility of the CHO-rhEPO, Hu-rhEPO and LMH/2A-rhEPO as a result of removal of the *N*-linked glycosylation as shown in Figure 11. PNGaseF is an enzyme that cleaves between the innermost GlcNAc and the Asn residue of high-mannose as well as complex-type *N*-glycans and has been used extensively in investigating the glycosylation characteristics of EPO. The Western blot data showed that LMH/2A-rhEPO expressed in LMH/2A cells were *N*-linked glycosylated. The Western blot data showed broad bands for 1 µg of CHO-rhEPO (molecular weight 28 kD to 40 kD), 1 µg human-rhEPO (molecular weight of 26 kD to 37 kD) and 1 µg LMH/2A-rhEPO (molecular weight of 25 kD to 37 kD). After deglycosylation, bands were detected for each treated rhEPO at a shifted molecular weight of

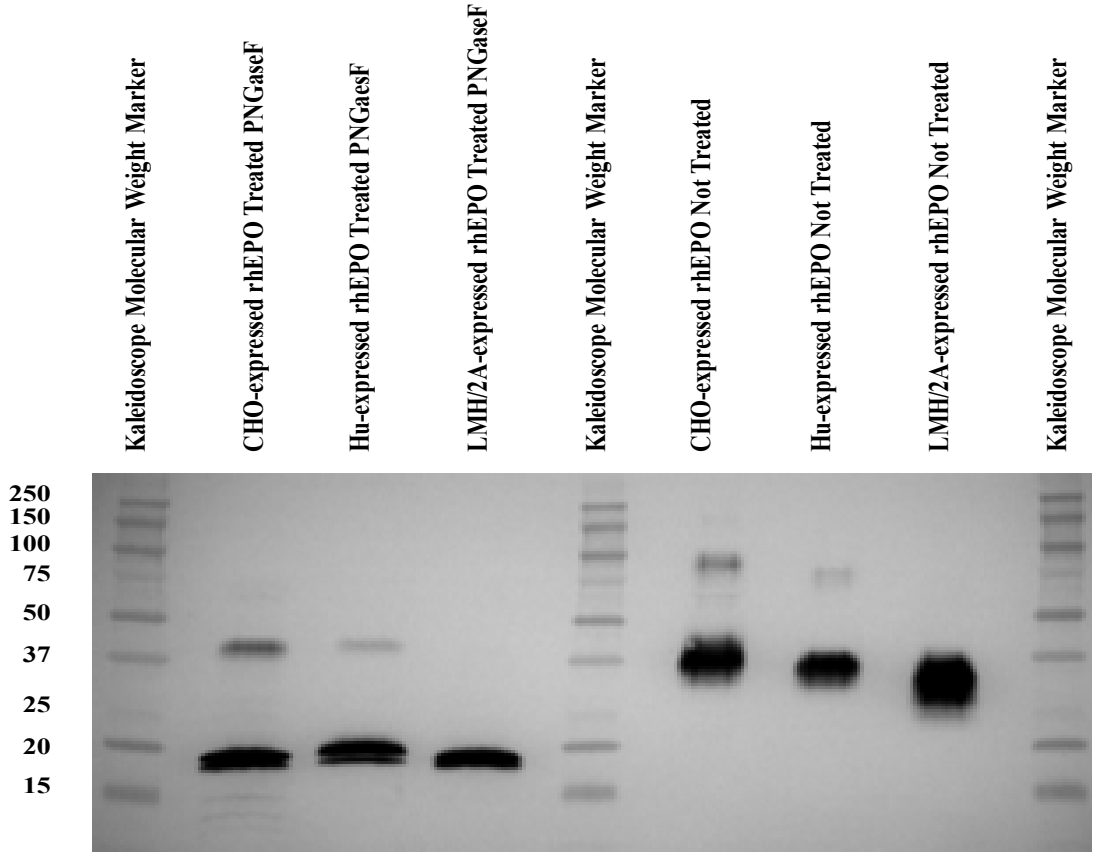

Figure 11. Western blot analysis of rhEPO after deglycosylation wtih PNGaseF treated and non-treated purified, denatured CHO-rhEPO, Hu-rhEPO and LMH/2A-rhEPO. Kaleidoscope Precision Plus marker with a range of 250 kD to 10 kD was loaded in lane 1, 9 and 17. CHO-rhEPO treated with PNGaseF was loaded in lane 3. Hu-rhEPO treated with PNGaseF was loaded in lane 5. LMH/2A-rhEPO treated with PNGaseF was loaded in lane 7. Non-treated CHO-rhEPO was loaded in lane 11. Non-treated Hu-rhEPO was loaded in lane 13. Non-treated LMH/2A-rhEPO was loaded in lane 15.

approximately 18 kD (non-glycosyated form). The bands detected by the de-glycosylated human-rhEPO showed a slightly higher molecular weight due to possible variations in *O*-linked glycosylation. Skibeli et al. (2001) showed that after PNGaseF treatment that the *N*-linked oligosaccharides from human serum EPO were more susceptible to digestion by PNGaseF than the *N*-glycans from CHO-rhEPO. Furthermore, the molecular weight of CHO-rhEPO was estimated to be 40 kD by SDS-PAGE and after complete de–*N*-glycosylation of CHO-rhEPO, the molecular weight shifted to a single band at 21 kD which corresponds to *O*-glycosylated

rhEPO (Skibeli et al., 2001). In another study, digestion with PNGaseF of human-rhEPO (expressed from human ovarian carcinoma SKOV3 cell line) yielded two bands at 18 kD (non-glycosyated form) and 21 kD (*O*-glycosylated glycoform) (Machado et al., 2011). PNGaseF digestion of rhEPO (29 kD) resulted in a band with a molecular weight approximately 19 kD (the *O*-glycosylated form) (Stübiger et al., 2005). These experiments presented evidence that rhEPO exhibits heterogeneity based on the degree of sialylation and additional post-translational modifications of the *O*-glycan.

Unfortunately, the carbohydrate structure analysis was not analyzed but the results show that LMH/2A-rhEPO shifted after PNGaseF treatment to a similar molecular weight of the PNGaseF treated CHO-rhEPO and PNGaseF treated hu-rhEPO (approximately 18 kD and 21 kD). Further analysis at the peptide and oligosaccharide level are needed to investigate the individual glycoforms of rhEPOs.

On the other hand, contaminants such as other proteins and growth factors can interfere with the ability of the rhEPO to bind to receptors and demonstrate biological activity, therefore it was essential to remove these contaminants by purification (Hochuli, 1992). *N*-linked glycosylated rhEPO was successfully expressed in LMH/2A cells and purified using a series of chromatography steps to form the final LMH/2A-rhEPO product ($\geq$ 95% purity). The final purified LMH/2A-rhEPO was used in the next experiment to investigate biological activity.

CHAPTER IV

BIOACTIVITY OF LMH/2A EXPESSED RECOMBINANT HUMAN ERYTHROPOIETIN

Introduction

The biological activities of rhEPO produced in either mammalian cells or nonmammalian cells differ from one cell production system to another (Takeuchi et al., 1989). Variation is likely due to the differences in their carbohydrate moieties and have been shown to be important in biological activity (Goldwasser et al.,1974; Goto et al., 1988). For example, experiments with rhEPO glycosylation demonstrated a direct, positive, relationship between number of carbohydrate chains and biological activity *in vivo* (Elliott et al., 2004). However, glycosylation is not required for *in vitro* biological activity of rhEPO (Yamaguchi et al., 1991) or for binding of rhEPO to its receptor (Darling et al., 2002).

The *in vivo* bioassays to test for biological activity for rhEPO are commonly performed in polycythaemic or normocythaemic mice (Jelkmann, 2003). These *in vivo* bioassays are time consuming, expensive, require many animals, and produce intra- and inter-assay variation of the results (Jelkmann, 2009). The *in vitro* bioassays to test for the biological activity for rhEPO include using cell based assays such as rat bone marrow cells (Takeuchi et al., 1989), human UT7/ leukemia cell line (Long et al., 2006), and F-36E/ human leukemia (Joung et al., 2009).

Another *in vitro* assay to investigate the biological activity of rhEPO includes using CD34+ progenitor cells (Malik et al., 1998). CD34+ cells are erythroid progenitors cells collected from the bone marrow, mobilized peripheral blood or umbilical cord blood and they express the protein CD34 (Dimitriou et al., 2003). In the human, the function mechanism of EPO is to circulate in the blood and bind to receptors expressed specifically on erythroid progenitor cells, thereby promoting the viability, proliferation, and terminal differentiation of

erythroid precursors, resulting in an increase in red blood cell mass (Ebert and Bunn, 1999). Shinjo et al. (1997) describes CD34+ cells in normal bone marrow as having the highest number of expressed human EPO receptor (hEPOR) sites, approximately 1600 hEPOR sites per cell. Furthermore, human EPO inhibits the apoptosis of erythroid precursor cells and supports their proliferation and differentiation into erythrocytes, a process known as erythropoiesis (Jelkmann, 1992). As seen in Figure 12, hematopoietic pluripotent stem cells differentiate into erythrocytes and are regulated by cytokines such as GM-CSF, granulocyte macrophage colony stimulating factor and EPO (Socolovsky et al., 1998).

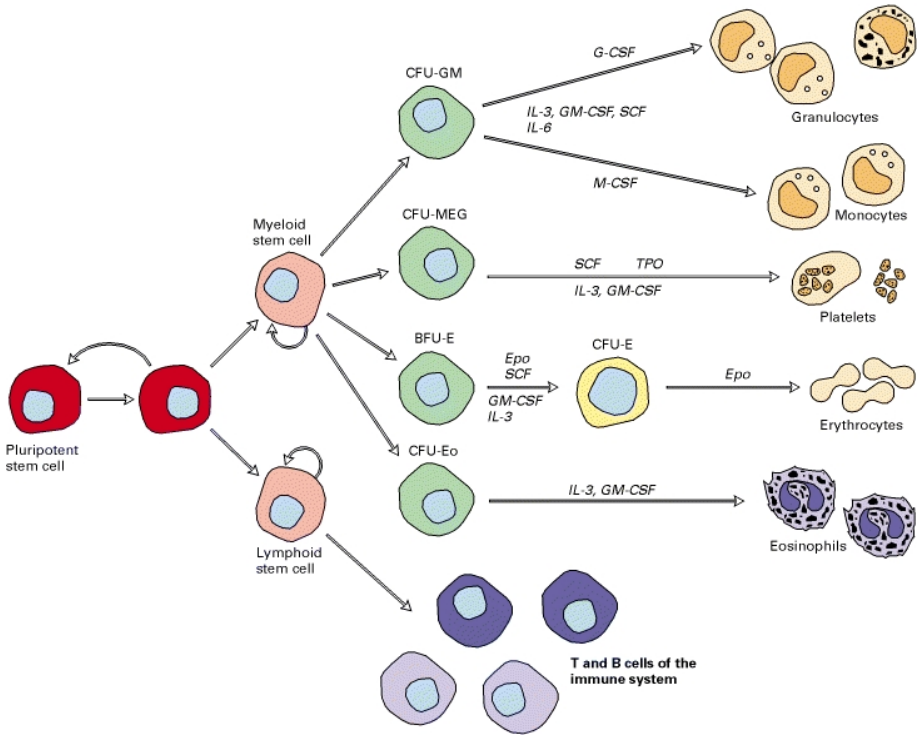

Figure 12. The hematopoietic pathway illustrating bone marrow pluripotent stem cells that can self-renew or differentiate into eight different hematopoietic lineages through a gradual process of commitment and differentiation. Some of the cytokines involved in supporting this process are illustrated such as M-CSF, Macrophage colony stimulating factor; SCF, stem cell factor; EPO, erythropoietin; TPO, thrombopoietin (Socolovsky et al., 1998).

Due to the fact that CD34+ cells (1) resemble *in vivo* erythropoiesis and are described as being the best available *in vitro* model for human erythropoiesis (Fibach et al., 1989; Umemura

et al., 1990); (2) are regulated by EPO for differentiation into hematopoietic cells (Kimbrel and Lu, 2011); (3) have numerous hEPOR sites on their surface (Shinjo et al., 1997); and (4) have previously been used as a model for investigating the ability of rhEPO to induce erythropoiesis (Akagi et al., 2004; Burns et al., 2002; Malik et al., 1998; Macdougall, 2008), these cells were used for this study as the cell line to investigate biological activity of LMH/2A-rhEPO, and compare it to the biological activity of CHO cell expressed rhEPO and human cell (HEK-293) expressed rhEPO.

Materials and Methods

Experimental Design

Twelve T-25 flasks were divided randomly into four different treatment groups as shown in Figure 13, and cultured for seven days as follows:

Treatment 1

Three T-25 flasks of CD34+ cells (~40,000 cells) were grown in HPGM, Human Progenitor Growth Media, (Lonza) supplemented with 10% FBS (SAFC), G-CSF, granulocyte colony-stimulating factor, (20ng/mL; PeproTech), and treated with 55 ng/mL CHO-rhEPO (Prospec) at 37°C and 5% CO_2 for seven days.

Treatment 2

Three T-25 flasks of CD34+ cells (~40,000 cells) were grown in HPGM (Lonza) supplemented with 10% FBS (SAFC), G-CSF, (20ng/mL; PeproTech), and treated with 50 ng/mL Hu-rhEPO (Humanzyme) at 37° C and 5% CO_2 for seven days.

Treatment 3

Three T-25 flasks of CD34+ cells (~40,000 cells) were grown in HPGM (Lonza)

supplemented with 10 % FBS (SAFC), G-CSF, (20 ng/mL; PeproTech), and treated with 50 ng/mL of purified LMH/2A-rhEPO at 37° C and 5% CO_2 for seven days.

Treatment 4

Three T-25 flasks of CD34+ cells (~40,000 cells) were grown in HPGM (Lonza) supplemented with 10% FBS (SAFC), G-CSF, (20ng/mL; PeproTech), and were not treated with rhEPO (negative control) at 37° C and 5% CO_2 for seven days.

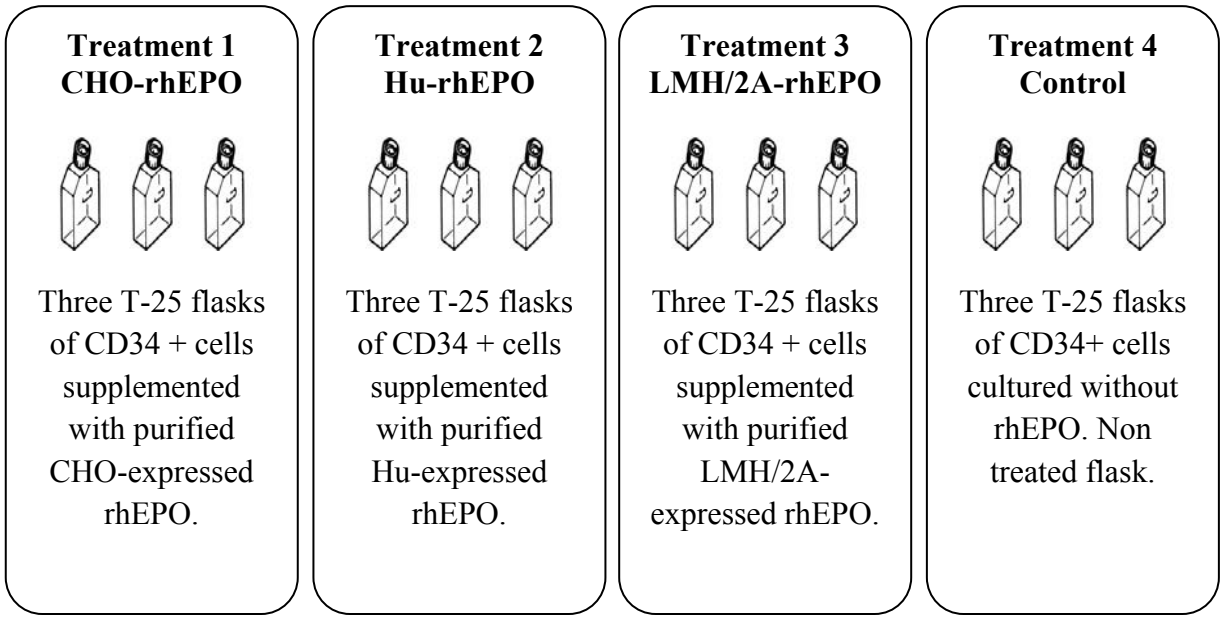

Figure 13. Cell culture set up with three T-25 flasks of CD34+ cells in each treatment group 1, 2, 3 and in treatment group 4 (control). Treatment 1 CD34+ cells were supplemented with CHO-rhEPO. Treatment 2 CD34+ cells were supplemented with Hu-rhEPO. Treatment 3 CD34+ cells were supplemented with LMH/2A-rhEPO. Treatment 4 control CD34+ cells were not supplemented with rhEPO. For each treatment group, the CD34+ cells were grown in HPGM media with 15% FCS and G-CSF (20ng/mL) at 37° C and 5% CO_2 for seven days.

Analysis of Variance (ANOVA)

Data for cell proliferation, hemoglobin, and human transferrin receptor were analyzed for variation between the treatments and within the treatments (within flasks) using General Linear Models (GLM) procedure of SAS® software (SAS Institute, 1995).

In this model, the variations of the effects of rhEPO supplements on CD34+ proliferation and differentiation among the rhEPO-treated groups were tested using the following hypothesis (Ho):

Ho: $\mu_1 = \mu_2 = \mu_3$

Ha: at least one average is not equal, where

μ_1 = average of cell proliferation/differentiation for CHO-rhEPO treated cells, μ_2 = average of cell proliferation/differentiation for Hu-rhEPO treated cells, and μ_3 = average of cell proliferation/differentiation for LMH/2A-rhEPO treated cells. Ha is an alternative hypotheis.

Similarily, the effect of rhEPO supplements for the rhEPO-treated groups on cell proliferation and cell differentiation was compared to the non-treated control group (μ_4) using the following hypothesis (Ho):

Ho: $\mu_1 = \mu_2 = \mu_3 = \mu_4$

Ha: at least one average is not equal, where

μ_1 = average of cell proliferation/differentiation for CHO-rhEPO treated cells, μ_2 = average of cell proliferation/differentiation for Hu-rhEPO treated cells, μ_3 = average of cell proliferation/differentiation for LMH/2A-rhEPO treated cells, and μ_4 = average of cell proliferation/differentiation for non-treated control cells. H_a is an alternative hypotheis. Under the above GLM models, pairwise comparison analyses were also examined between the treated and non-treated control groups. All the multiple pairwise comapriosons were performed at 95% confidence interval (alpha = 0.05).

CD34+ Cell Culture

- **Materials**

1. Biosafety cabinet Level II (Labconco, Kansas City, MO)

2. Incubator (Sanyo, San Diego, CA)
3. Centrifuge (Eppendorf, Germany)
4. 37° C water bath (Cole Palmer, Vernon Hills, IL)
5. Microscope (Zeiss, Germany, Axiovert 40C)
6. Cell culture flasks, T-25 (Corning, Corning, NY)
7. CD34+ cells (Lonza, Switzerland)
8. G-CSF (PeproTech, Rocky Hill, NJ)
9. CHO-rhEPO (Prospec, Israel)
10. Hu-rhEPO (Humanzyme, Chicago, IL)
11. LMH/2A-rhEPO, purified
12. 15 mL conical tube, sterile (Corning, Corning, NY)
13. Human Progenitor Growth Media (HPGM) (Lonza, Switzerland)
14. Fetal bovine serum (FBS), Australian or New Zealand origin (SAFC, Lenexa, KS)
15. Sterile 1X PBS buffer: 136.9 mM NaCl, 1.4 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl [pH 7.4]
16. Sanitizing disposable cloths (PDI, Parsippany, NJ)

- **Procedure**

The CD34+ cells (Lonza, Switzerland) were counted and divided equally into 12 T-25-flasks (~40,000 cells/flask) and grown in suspension. Cell morphology and survivability were inspected in each T-25 flask using an Axiovert 40C microscope (Zeiss) throughout the seven days of culture. After 7 days of culture, media and cells were harvested from the T-25 flasks separately. The cells from each flask were collected by centrifuging the harvested media and cells at 500 X g for 10 minutes in a 15 mL conical tube. The media was gently aspirated off the

cell pellet. All cell culture work with the CD34+ cells was conducted in a Class II biosafety cabinet and procedures were implemented according to biosafety guidelines described by J. L. Caputo (Caputo, 1996). The CD34+ cells were from human source and the materials were treated as potentially infectious and handled with caution. All surfaces and materials were thoroughly sanitized using PDI Germicidal Sani Cloths before and after use.

CD34+ Cell Count

- **Materials**

1. Microscope (Zeiss, Germany, Axiovert 40C)
2. Hemocytometer (Fisher, Pittsburg, PA)
3. Sterile 1X PBS buffer: 136.9 mM NaCl, 1.4 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl [pH 7.4]

- **Procedure**

Cellular proliferation was assessed by cell-counting with a hemocytometer (Fisher). The CD34+ cells from each flask were diluted 1:3 in 1X PBS and mixed gently to make a homogenous mixture of CD34+ cells and 1X PBS. The hemocytometer was used to count the number of cells per unit volume of the suspended CD34+ cells. The total number of cells from each flask were counted from the nine squares of the hemocytometer.

CD34+ Cell Lysates

- **Materials**

1. M-PER lysis buffer (Thermo Scientific, Waltham, MA)
2. CD34+ cell pellets
3. 15 mL conical tube, sterile (Corning, Corning, NY)
4. Centrifuge (Eppendorf, Germany)

- **Procedure**

After cell count samples were taken, the suspended CD34+ cells were centrifuged at 500 X g to pellet the cells. The 1X PBS buffer (supernate) was removed and the pellet of cells were re-suspended in 500 µL M-PER lysis buffer (Thermo Scientific) to dissolve cell membranes and extract total cellular proteins. Three freeze-thaw cycles (20 minutes at -30° C and 5 minutes at 37 °C) were completed on the re-suspended pellet of cells in M-PER lysis buffer. After the last freeze-thaw cycle, the M-PER buffer/cell mixture was centrifuged at 10,000 X g for 20 minutes to pellet the cell debris. The supernate (lysate) for the CD34+ cells were collected and stored at -20° C.

Total Protein Determination

- **Materials**

1. BCA™ (bicinchoninic acid) protein assays (Pierce, Waltham, MA)
   - BCA™ Reagent A
   - BCA™ Reagent B
   - Albumin Standard Ampules, 2 mg/mL
2. Immulon 4 HBX flat bottomed, sterile, uncoated, micortiter, polystyrene, 96-well plate (Thermo Electron Corporation, Waltham, MA)
3. Micro-plate reader: Capable of reading the OD (4.0 maximum) of 96-well plate at wavelength 450nm (Bio-Rad, Hercules, CA)

- **Procedure**

The total protein concentrations for the CD34+ cell lysates were determined by BCA™ (bicinchoninic acid) protein assay (Pierce). The albumin standard was diluted such that the final working dilution concentrations were 2000, 1500, 750, 500, 250, 125, 62.5 µg/ml. The working

reagent was prepared by mixing 50 parts of BCA™ Reagent A with 1 part of BCA™ Reagent B (50:1, Reagent A:B). Twenty five µl of each standard and undiluted sample was loaded into a microplate well. Two hundred µL of the working reagent was added to each well and mixed thoroughly on a plate shaker for 30 seconds. The plate was covered and incubated at 37° C for 30 minutes. After incubation, the plate was cooled to room temperature and the absorbance reading was measured at 550 nm on a plate reader. The total protein for the CD34+ cell lysates were normalized for sandwich ELISA and Western blot experiments.

Erythroid Markers Detection

Erythroid differentiation was assessed by examining the expression of three erythroid specific proteins, glycophorin A (GpA), human transferrin receptor (CD71), and hemoglobin in the CD34 + cell lysates.

Hemoglobin Detection

- **Materials**

1. Hemoglobin ELISA quantification kit (Bethyl, Montgomery, TX)
2. CD34+ lysates

- **Procedure**

The CD34+ cell lysates were tested by sandwich ELISA using a human hemogloblin ELISA quantification kit (Bethyl). The sandwich ELISA for human hemoglobin was conducted using the same materials and procedures as described above in Chapter III with the following modifications: (1) the capture antibody was anti-human hemoglobin (Bethyl) diluted such that the final working dilution concentration was 10 µg/mL, (2) the human hemoglobin standard (Bethyl) was diluted in 1X TBS such that the final working dilution concentrations were 400, 200, 100, 50, 25, and 12.5 ng/mL, (3) the CD34+ cell lysate samples were diluted in 1X TBS and

tested in this sandwich ELISA, and (4) the detection antibody used was anti-human hemoglobin-HRP (Bethyl) and diluted such that the final working dilution concentration was 50 ng/mL.

- **Materials**

27. TMB (3,3',5,5'- tetramethylbenzidine) membrane peroxidase substrate system (KPL, Gaithersburg, MD)
28. Hemoglobin (Bethyl, Montgomery, TX)
29. Anti-human Hemoglobin (Bethyl, Montgomery, TX)
30. CD34+ lysates

- **Procedure**

CD34+ cell lysates were also analyzed by Western blot using an anti-human hemogloblin antibody (Bethyl). The Western blot for human hemoglobin detection was conducted using the same materials and procedures as described above in Chapter III with the following modifications: (1) the standard used in this Western blot was human hemoglobin (Bethyl), (2) the test samples were the CD34+ cell lysates, and (3) detection antibody anti-human hemoglobin-HRP was used to detect human hemoglobin (Bethyl) and diluted to approximately 200 ng/mL in 1 % gelatin/1X TBS/0.05% Tween 20. Antibody bound to antigen was detected by using the TMB membrane substrate system (KPL).

Human Transferrin Receptor Detection

- **Materials**

1. Human Transferrin Receptor ELISA quantification kit (MyBioSource, San Diego, CA)
2. CD34+ lysates

- **Procedure**

The CD34+ cell lysates were tested by sandwich ELISA using a human transferrin receptor ELISA quantification kit (MyBioSource). The sandwich ELSIA for human transferrin receptor was conducted using the same materials and procedures as described above in Chapter III with the following modifications: (1) the pre-coated and blocked 96-well plate was allowed to equilibrate to room temperature, (2) the human transferrin receptor standards were diluted in dilution buffer 1:10 such that the final working dilution concentrations were 2, 1, 0.5, 0.2, 0.1, and 0.05 μg/mL, and (3) the test samples and QC samples were diluted in dilution buffer.

Glycophorin A Detection

- **Materials**

1. Microcon ultracel 4M-10 filter (Millipore, Billerica, MA)
2. Rabbit-anti-human Glycophorin A (AbD Serotec, Raleigh, NC)
3. CD34+ lysates

- **Procedure**

CD34+ cell lysates were concentrated 6X using Microcon ultracel 4M-10 filter (Millipore) then analyzed by Western blot using an anti-human glycophorin A antibody. The Western blot for human glycophorin A detection was conducted using the same materials and procedures as described above in Chapter III with the following modifications: (1) the test samples were the CD34+ cell lysates, and (2) the primary antibody used was rabbit-anti-human glycophorin A (AbD Serotec) and diluted to approximately 5 μg/mL in 1% gelatin/1X TBS/0.05% Tween20.

AKT/Phospho-AKT Detection

- **Materials**

1. Rabbit-anti-phospho-AKT (Cell Signaling, Danvers, MA)

2. Rabbit-anti-AKT (Cell Signaling, Danvers, MA)
3. CD34+ lysates

- **Procedure**

EPO signaling was assessed by examining the intracellular signaling AKT/AKT-phospho pathways. The Western blots for total AKT and phophorylated AKT detections were conducted using the same materials and procedures as described above in Chapter III with the following modifications: (1) the test samples were the CD34+ cell lysates, (2) rabbit-anti-AKT (Cell Signaling) was diluted to approximately 1:33 in 1% gelatin/1X TBS/0.05% Tween20 for Total AKT detection, and (3) rabbit-anti-phospho-AKT (Cell Signaling) was diluted to approximately 1:33 in 1% gelatin/1X TBS/0.05% Tween20 for Phospho-AKT detection.

Results and Discussion

CD34+ Cell Culture

Cell morphology of the CD34+ cells grown in HPGM media is shown in Figure 14. The CD34+ cells initially placed in culture were small and round, similar in morphology to small blasts. Larger erythroid blasts, referred to as preproerythroblasts, in the CD34+ cultured cells were observed on Day 2. By Day 7, proerythroblasts became the predominant population in the CD34+ culture. The CD34+ nuclei were round and broad-based cytoplasmic projects were observed. Over the 7 days of culture, it became visible that the CD34+ cells cultured with LMH/2A cell expressed rhEPO (LMH/2A-rhEPO) were morphologically more mature than cells cultured without rhEPO (treatment 4, negative control) (Data not shown).

After the media was removed, the appearance of CD34+ cell pellets isolated from the CD34+ cells cultured with CHO-rhEPO, Hu-rhEPO, and LMH/2A-rhEPO was red while the appearance of CD34+ cell pellets collected from non-treated control group was white (see Figure

15). Zhang D et al. (2007) demonstrated that a red cell pellet is evidence of an elevation in hemoglobin synthesis.

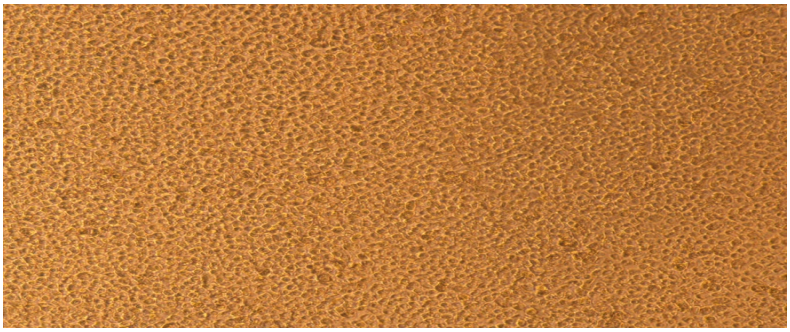

Figure 14. CD34+ cells cultured in Human Progenitor Growth Media (HPGM).

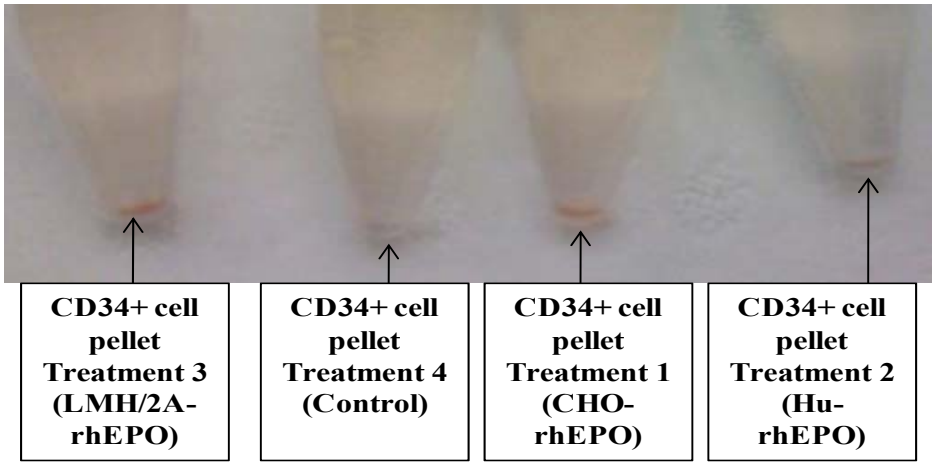

Figure 15. CD34+ stem cell pellet isolated after centrifuging harvested media and cells from CD34+ cells that were cultured in T-25 flasks with Human Progenitor Growth Media (HPGM) + 15% FCS and G-CSF (20ng/mL) at 37° C and 5% CO_2 for seven days. CD34+ stem cells were cultured with CHO cell expressed rhEPO (CHO-rhEPO), human Hek293 cell expressed rhEPO (Hu-rhEPO), LMH2A cell expressed rhEPO (LMH/2A-rhEPO), and no rhEPO (Control).

In this study, the accumulation of hemoglobin in CD34+ cells in response to LMH/2A-rhEPO supplement is likely contributing toward current medical research aims that have focused on finding alternative means to conventional blood transfusions that rely on donor blood by establishing *in vitro* method for red blood cell production, a process termed *ex vivo* erythropoiesis (Fujimi et al., 2008). The expansion of cord blood cells such as CD34+ cells

using a variety of cytokine combinations including rhEPO have been reported (Boehm et al., 2009; Douay and Andreu, 2007; Munugalavadla et al., 2005).

Furthermore, other medical research has focused on treating patients with anemia with a combination of GM-CSF and rhEPO. *In vitro,* rhEPO along with GM-CSF stimulates the the proliferation and differentiation of hematopoietic progenitor cells (Malik et al., 1998). Bernell et al. (1996) reports success using *in vivo* stimulation of hematopoietic progenitor cells using a combination therapy of GM-CSF/rhEPO in patients with anemia from myelodysplastic syndromes measured by an increase in hemoglobin levels in treated patients.

Proliferation of CD34+ Cells

As seen in Figure 16, the number of cells counted in each of the treated groups was approximately five fold higher than the non-treated groups ($P<0.05$). There were no significant differences in the averages of number of cells between treated groups ($P>0.05$).

Wojda et al. (2002) demonstrated a rapid increase in the cell numbers of cultured CD34+ cells supplemented with rhEPO in the first 7 days and subsequently showed that this proliferation corresponded to a rise in hemoglobin expression. In another study, erythroid proliferation and differentiation of CD34+ cells supplemented with rhEPO was observed (Harashima et al., 2002). Furthermore, Flores-Guzmán et al. (2002) showed that rhEPO is involved in the control of *in vitro* growth of hematopoietic progenitor cells and induces proliferation and expansion of CD34+ cells.

Total Protein in CD34+ Cell Lysates

The total protein in the CD34+ lysates was estimated using BCA assay, data shown in Table 3. The total protein concentrations were used for the sandwich ELISA and Western blot experiments on the CD34+ cell lysates to normalize the total protein loaded for each sample.

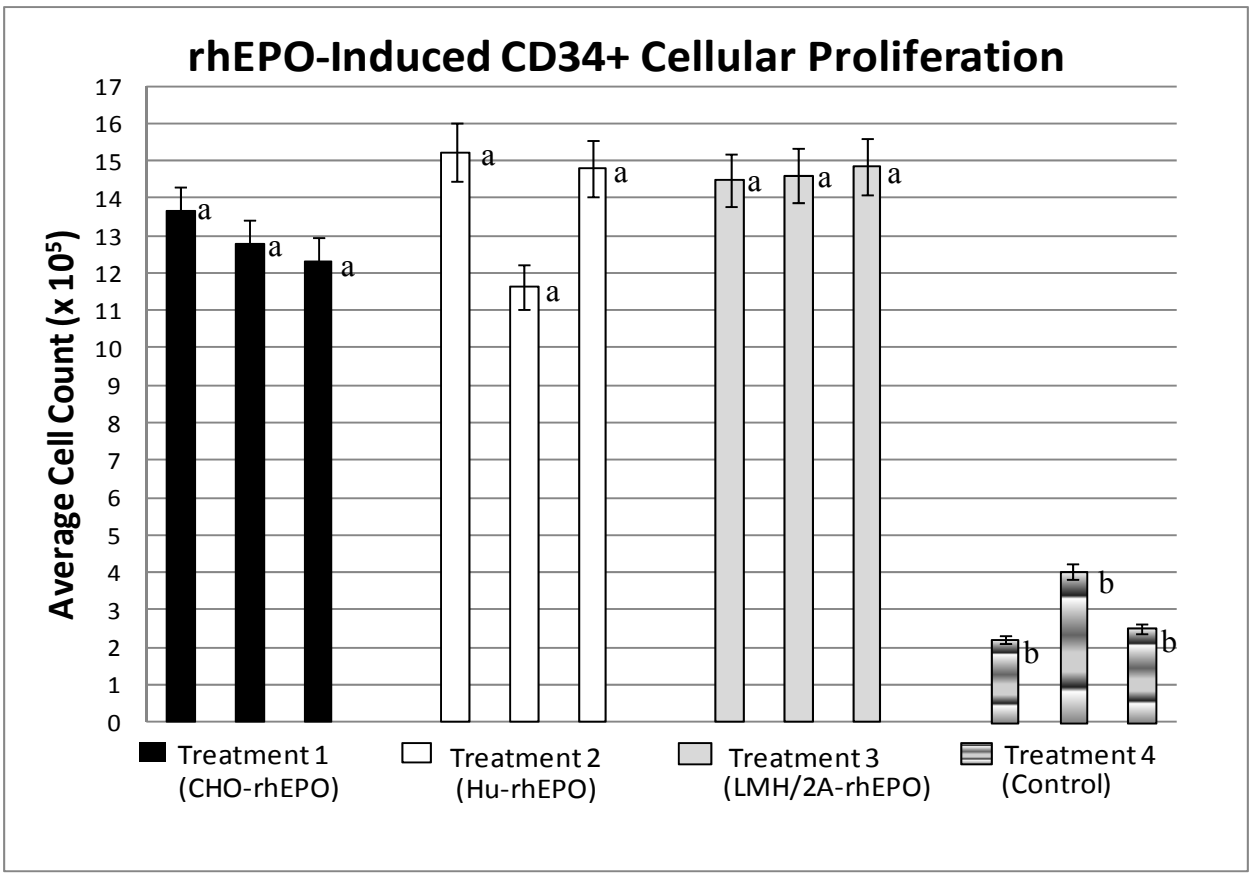

Figure 16. Data shown indicates the average of cell count of CD34+ cells (x 10^5) from 12 independent experiments after seven days of culture. CD34+ stem cells were cultured with CHO cell expressed rhEPO (CHO-rhEPO Treatment 1), human (HEK293) cell expressed rhEPO (Hu-rhEPO Treatment 2), LMH2A cell expressed rhEPO (LMH/2A-rhEPO Treatment 3), and no rhEPO treatment (Treatment 4) for 7 days. Each T-25 flask was seeded with approximately 0.4 x 10^5 CD34+ cells.
[a,b] Averages with no common superscripts are different (P<0.05). Error bars are the standard deviations, n=3.

Human Hemoglobin Detection

Human hemoglobin (HbA) was detected in the CD34+ cell lysates from Treatment 1, Treatment 2, and Treatment 3 by sandwich ELISA and Western blot. HbA concentrations in the CD34+ cell lysates estimated by sandwich ELISA are shown in Figure 17. As expected, the HbA concentrations in each of the treated groups were approximately 2500 fold higher than the HbA concentrations in non-treated control group (P<0.05). There were no significant differences in average HbA concentrations between or within the treated groups (P>0.05).

Table 3. Total protein concentrations for the CD34+ cell lysates determined by BCA (bicinchoninic acid) protein assay. CD34+ stem cells were cultured with CHO cell expressed rhEPO (CHO-rhEPO Treatment 1), human (HEK293) cell expressed rhEPO (Hu-rhEPO Treatment 2), LMH2A cell expressed rhEPO (LMH/2A-rhEPO Treatment 3), and no rhEPO treatment (Treatment 4) for 7 days.

CD34+ Cell Lysate	Flask	Total Protein Concentration ug/ml
Treatment 1 (CHO-rhEPO)	1	309.5
Treatment 1 (CHO-rhEPO)	2	336.6
Treatment 1 (CHO-rhEPO)	3	366.6
Treatment 2 (Hu-rhEPO)	1	257.4
Treatment 2 (Hu-rhEPO)	2	232.4
Treatment 2 (Hu-rhEPO)	3	318.9
Treatment 3 (LMH/2A-rhEPO)	1	239.7
Treatment 3 (LMH/2A-rhEPO)	2	249.3
Treatment 3 (LMH/2A-rhEPO)	3	324.2
Treatment 4 (Control)	1	253.9
Treatment 4 (Control)	2	312.5
Treatment 4 (Control)	3	347.5

The sandwich ELISA absorbance readings versus concentrations of hemoglobin standard for the calibration curve were fitted to a Four Parameter Logistic Regression Model. "Goodness of fit" was indicated by an average correlation coefficient (R^2) of 1.00 from six standard curve points. The average back-calculated calibration curve concentration for each standard was within 90-110% of the known standard solution concentration.

The Western blot, seen in Figure 18, showed that HbA was detected at approximately 14 kD (monomer) for each treatment groups (1, 2, and 3) in the CD34+ cell lysates. HbA could not be detected in the control group (treatment 4) CD34+ cell lysates because the concentration of HbA in the control CD34+ lysates were below the detection limit of this assay. Hemoglobin levels have been measured in cultured CD34+ cells supplemented with rhEPO which showed a direct relationship of hemoglobin accumulation and differentiation (Wojda et al., 2002).

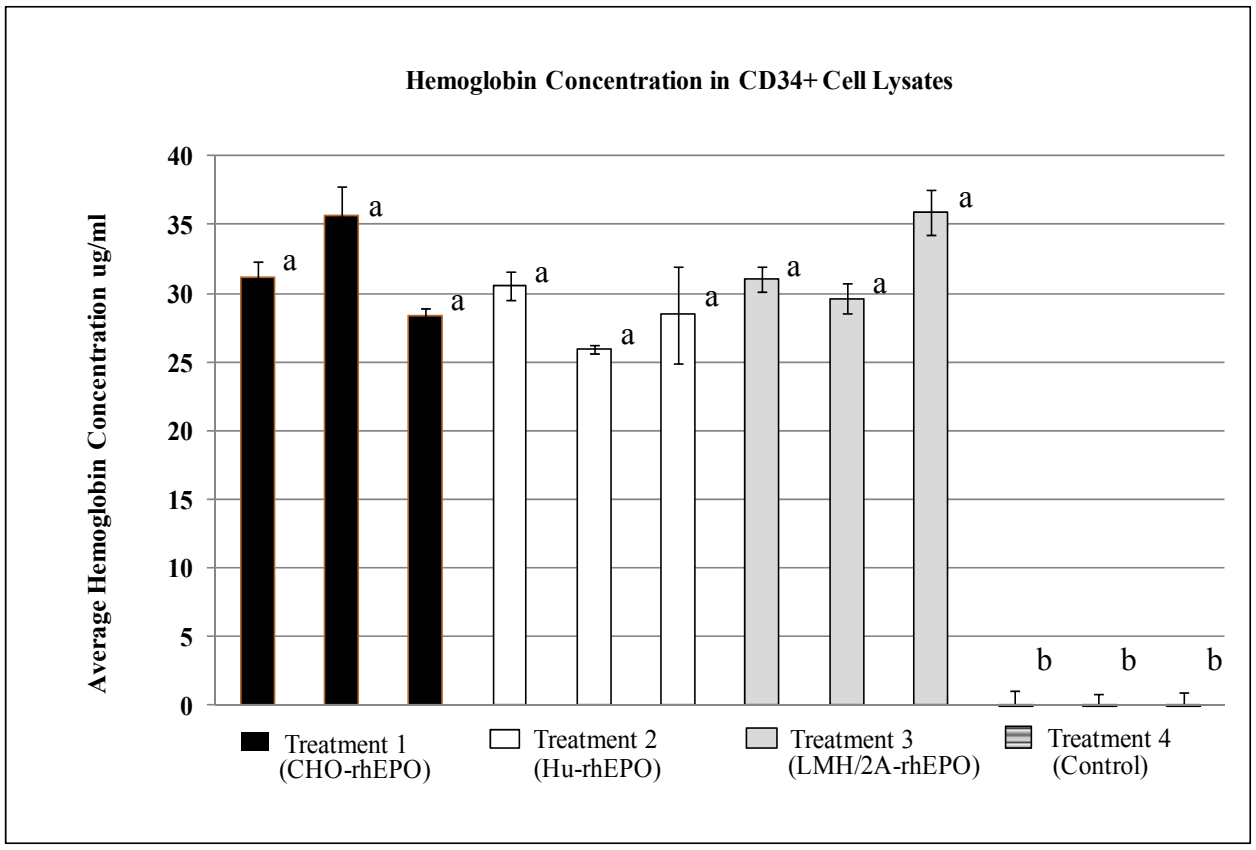

Figure 17. Average human hemoglobin concentration in CD34+ cell lysates determined by sandwich ELISA. CD34+ stem cells were cultured with CHO cell expressed rhEPO (CHO-rhEPO Treatment 1), human (HEK293) cell expressed rhEPO (Hu-rhEPO Treatment 2), LMH2A cell expressed rhEPO (LMH/2A-rhEPO Treatment 3), and no rhEPO treatment (Treatment 4) for 7 days.
[a,b] Averages with no common superscripts are different ($P<0.05$). Error bars are the standard deviations, n=3.

Also, CD34+ cells supplemented with rhEPO were used as an *in vitro* model for erythropoiesis and showed production of hemoglobin (Malik et al., 1998). Ishikawa et al. (2010) and Denicola et al. (1998) detected hemoglobin monomer at 15 kD. Wojda et al. (2002) quantified hemoglobin by HPLC in CD34+ cell pellets as 20 to 25 pg/cell. In another study, CD34+ cell lysates showed approximately 350 ng/mL of hemoglobin (Moutouh-de Parseval et al., 2007). The concentrations of hemoglobin cannot be compared because the number of days of incubation of the cells, the number of cells, and the volume of lysate has not been normalized.

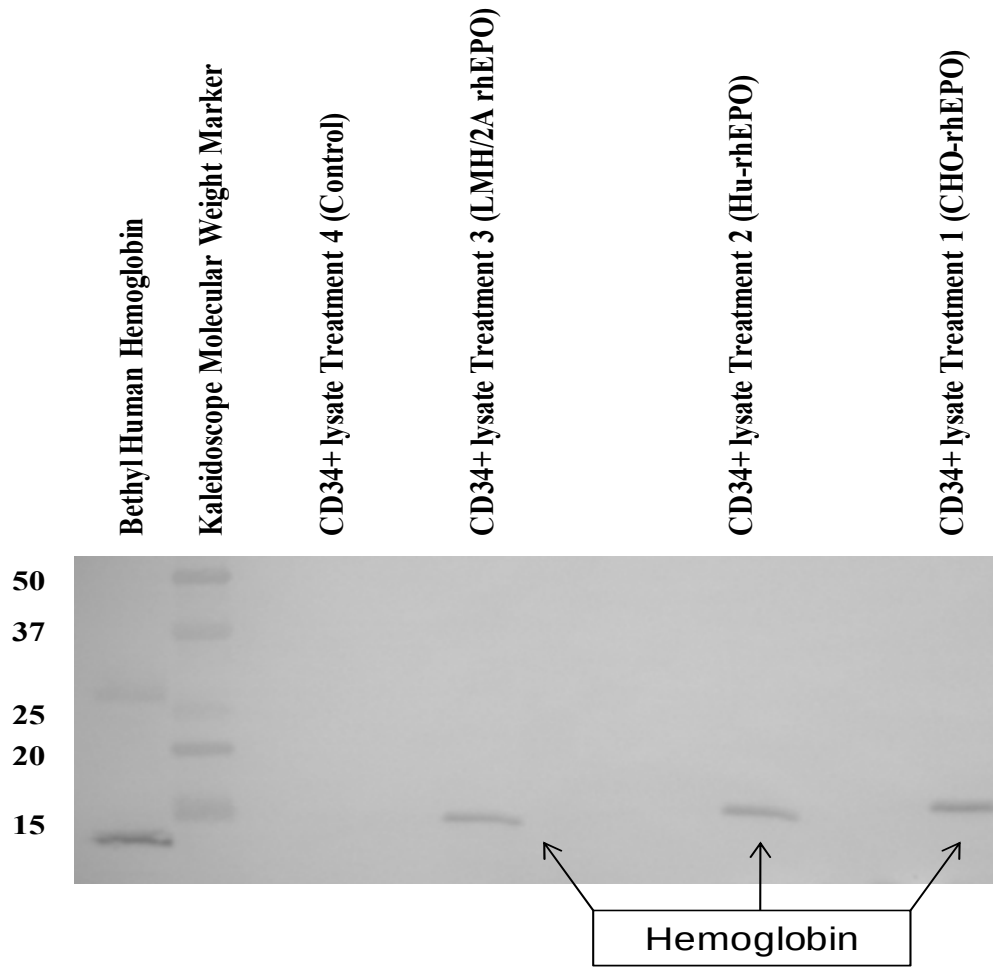

Figure 18. Western blot analysis for human hemoglobin detection in CD34+ cell lysates from CD34+ stem cells that were cultured with CHO cell expressed rhEPO (CHO-rhEPO), human (HEK293) cell expressed rhEPO (Hu-rhEPO), LMH2A cell expressed rhEPO (LMH/2A-rhEPO), and no rhEPO treatment (Control) for 7 days. The standard human hemoglobin (Bethyl) was loaded in lane 1. Kaleidoscope Precision Plus marker with a range of 50 kD to 15 kD was loaded in lane 2. CD34+ cell lysate from the control group was loaded in lane 4, 8, and 12. CD34+ cell lysate from the LMH/2A-rhEPO treatment group was loaded in lane 6. CD34+ cell lysate from the Hu-rhEPO treatment group was loaded in lane 10. CD34+ cell lysate from CHO-rhEPO treatment group was loaded in lane 13.

Based upon these results, it is promising that LMH/2A-rhEPO could have therapeutic applications such as in the treatments of patients with anemia or used in differentiation of erythroid progenitors for *ex vivo* erythropoiesis. Treatment of patients with rhEPO has been shown to increase hemoglobin levels (Jelkmann, 2004; Porter et al., 1996; Stasi et al., 2002).

Furthermore, Harousseau et al., (2005) showed that rhEPO increased hemoglobin levels in anemic cancer patients receiving chemotherapy by increasing the average hemoglobin concentration from 9.6 g/dL to a final average concentration of 12.0 g/dL hemoglobin. Anemia is defined as a hemoglobin level of <12 g/dL (Dicato, 2003).

Human Transferrin Receptor (CD71) Detection

As expected, CD71 was detected in the CD34+ cell lysates from Treatment 1, Treatment 2 and Treatment 3. In the control treatment 4, CD71 concentrations were below the detection limit and estimated to be less than 250 ng/mL. CD71 concentrations in the CD34+ cell lysates are shown in Figure 19. CD71 concentrations in each of the treated groups were approximately 12 fold higher than the CD71 concentrations in the non-treated control group ($P<0.05$). There were no significant differences in human transferrin receptor concentrations between or within rhEPO treated groups 1, 2, and 3 ($P>0.05$).

The sandwich ELISA absorbance readings versus concentrations for the calibration curve were fitted to a Four Parameter Logistic Regression Model. "Goodness of fit" was indicated by an average correlation coefficient (R^2) 1.00 from six standard curve points. The average back-calculated calibration curve concentration for each standard was within 90-110% of the known standard solution concentration.

CD71 was detected by flow cytometry in CD34+ cells supplemented with rhEPO (Wojda et al., 2002; D'Arena et al., 1996; Moutouh-de Parseval et al., 2007; Patterson et al., 2009). Moutouh-de Parseval et al. (2007) describes CD71 as an early marker of erythroid differentiation. Similar to endogenous human EPO, rhEPO interacts with the precursor erythroid cells (BFU-E, CFU-E) causing proliferation and differentiation of these cells and expression of erythroid markers such as CD71 (Parisotto et al., 2000).

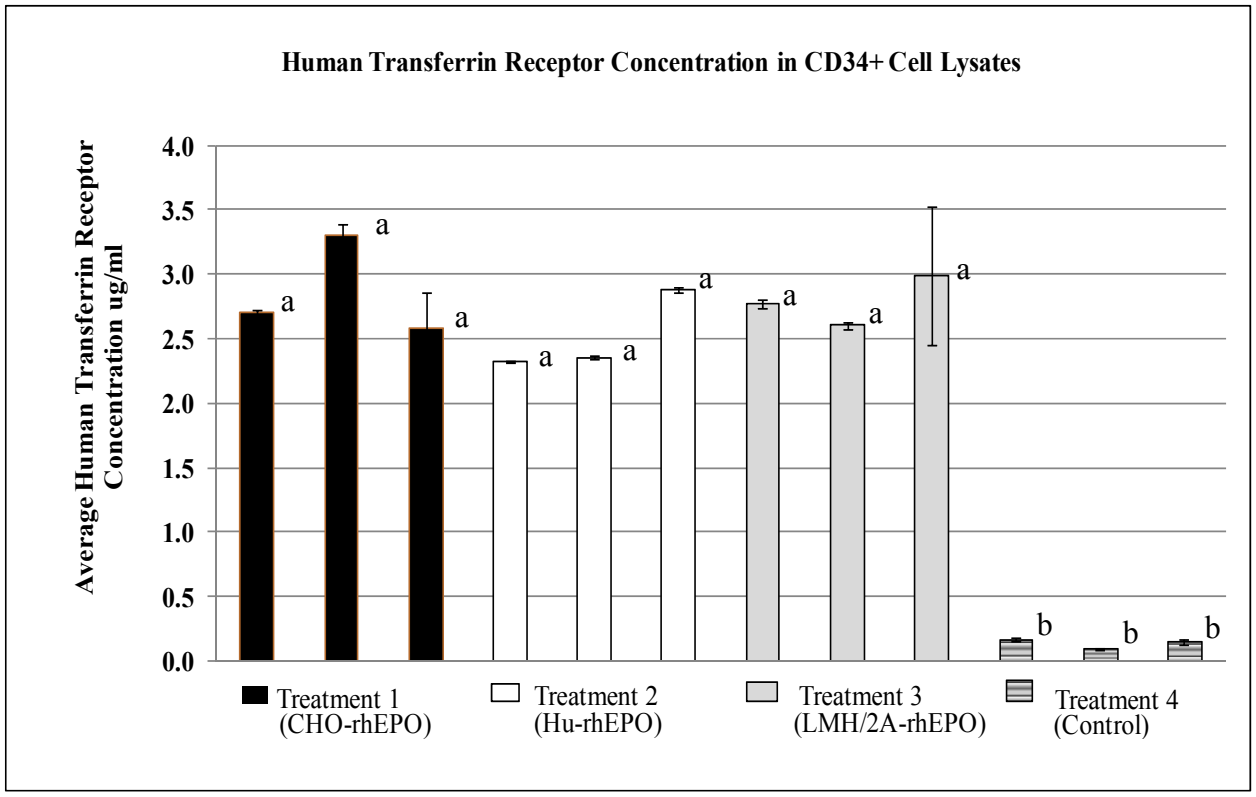

Figure 19. Data indicates the average human transferrin receptor concentrations in CD34+ cell lysates determined by sandwich ELISA. Cord blood CD34+ stem cells were cultured with CHO cell expressed rhEPO (CHO-rhEPO Treatment 1), human Hek293 cell expressed rhEPO (Hu-rhEPO Treatment 2), LMH2A cell expressed rhEPO (LMH/2A-rhEPO Treatment 3), and no rhEPO treatment (Treatment 4) for 7 days.
[a,b] Averages with no common superscripts are different ($P<0.05$). Error bars are the standard deviations, n=3.

CD71 is a key protein for the cellular uptake of transferrin iron in erythroid cells and is very useful tool for the diagnosis of anemia (Vernet, 1999). In fact, the use of rhEPO has shown to cause a change in iron metabolism which leads to an increase in the level of the serum CD71 in the human (Beguin et al., 1993). Also, treatment of anemic patients who had peripheral blood stem cell transplantation (PBSCT) with rhEPO showed an increase in serum CD71 concentrations when compared to non-treated group (Vanstraelen et al., 2005). Baron et al. (2003) showed that rhEPO therapy in anemic patients who had PBSCT expanded the

erythropoietic activity through increased serum CD71 concentrations which was also correlated with an increase in HbA. The ability of LMH/2A-rhEPO to increase concentrations of CD71 in erythroid cells suggests that LMH/2A-rhEPO could have therapeutic applications in the treatments of patients with anemia who have had PBSCT.

Glycophorin A Detection

The Western blot showed that bands were detected in each treatment group (1, 2 and 3) at approximately 39 kD (glycosylated form glycophorin A) in the CD34+ cell lysates (Figure 20) but glycophorin A could not be detected in the control group (treatment 4) CD34+ cell lysates. Glycophorin A (CD235a) is heavily glycosylated, approximately 60 % of its weight is attributable to carbohydrates, and has an apparent molecular weight of 36 kD to 39 kD.

McHale et al. (1999) reports that rhEPO causes an increase in the expression of cell surface glycophorin A (GpA) on erythroid cells. Expression of glycophorin A was detected in the CD34+ cells supplemented with rhEPO by flow cytometry (Moutouh-de Parseval et al., 2007; Wojda et al., 2002). Glycophorin A was detected in erythroid lysates (Perelman et al., 2003), in CD34+ lysates (Patterson et al., 2009). Remaley et al. (1991) showed that the apparent molecular weight of glycophorin A was approximately 38 kD. Andersson et al. (1981) demonstrates that glycophorin A is a useful marker of late erythroid differentiation.

Current human cancer chemotherapy formulations include using recombinant tumor necrosis factor-alpha (rTNFα) as a potent antitumor agent, however, the administration of rTNFα is accompanied by severe side-effects such as anemia (Lejeune et al., 1994). The treatment with rTNFα causes anemia in cancer patients due to inhibitory mechanisms that interfere with normal erythrocyte production (Dicato, 2003). Specifically rTNFα has been

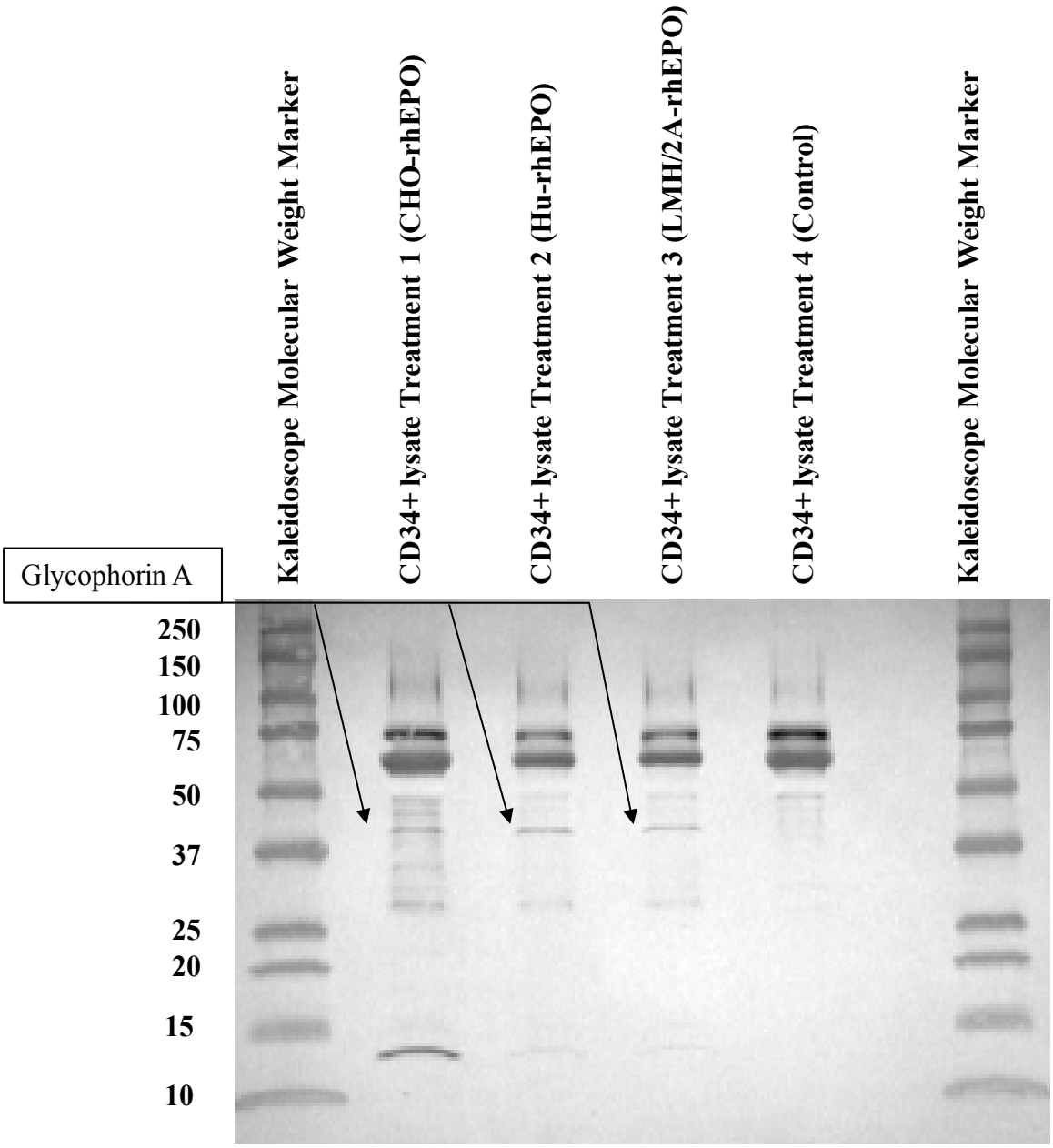

Figure 20. Western blot analysis for glycophorin A detection in concentrated (6x) CD34+ cell lysates from cord blood CD34+ stem cells that were cultured with CHO cell expressed rhEPO (CHO-rhEPO), human Hek293 cell expressed rhEPO (Hu-rhEPO), LMH2A cell expressed rhEPO (LMH/2A-rhEPO), and no rhEPO treatment (Control) for 7 days. Kaleidoscope Precision Plus marker with a range of 250 kD to 15 kD was loaded in lane 1and 12. CD34+ cell lysate from the CHO-rhEPO treatment group control group was loaded in lane 3. CD34+ cell lysate from the Hu-rhEPO treatment group was loaded in lane 5. CD34+ cell lysate from the LMH/2A-rhEPO treatment group was loaded in lane 7. CD34+ cell lysate from the control group was loaded in lane 9.

reported to inhibit the synthesis of glycophorin A in CD34+ cells (Xiao et al., 2002). Glycophorin A is a important constituent of the human red blood cell membrane and carries medically important blood group antigens such as Mi.I (Remaley et al., 1991). Treatment of anemic patients with rhEPO is known to reverse anemic symptoms and increase glycophorin A expression in erythroid progenitor cells (Ghaffari et al., 2006). Therefore, based upon the results of this study, LMH/2A-rhEPO could be considered as a therapeutic to treat anemic patients in chemotherapy.

The detection of hemoglobin, glycophorin A and transferrin receptor in LMH/2A-rhEPO treated CD34+ cells are consistent with the findings by Wojda et al. (2002) who showed expression of hemoglobin and surface membrane proteins such as the CD71 and glycophorin A in response to stimulation by rhEPO which induces proliferation and differentiation in CD34+ cells.

AKT/Phospo-AKT Detection

The Western blot data showed that LMH/2A-rhEPO activated AKT to similar extent for HEK293-expressed rhEPO and CHO-expressed rhEPO proteins (Figure 21). The AKT/Phospo-AKT Western blot results showed bands detected in the CD34+ lysates similar to the Western blot results for AKT/Phospho-AKT detection as described by Dao et al. (2007), Metzner et al. (2009), and Sivertsen et al. (2006). Furthermore, Metzner et al. (2009) also demonstrated that using an AKT inhibitor (triciribine) caused significant reduction of in the proliferation of CD34+ cells. Binding of EPO to its receptor initiates AKT intracellular signaling cascades, which promote cell survival, proliferation and differentiation (Nteliopoulos et al., 2009). EPO promotes *in vitro* proliferation of progenitor cells in an AKT-dependent manner (George et al.,

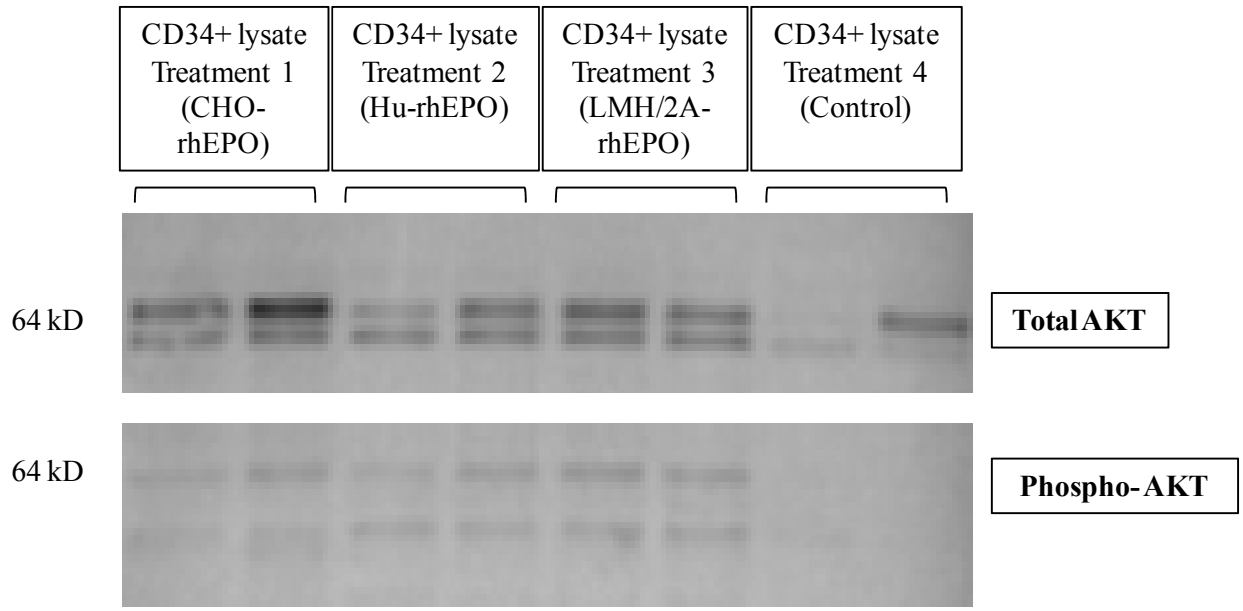

Figure 21. Western blots analysis of phosphorylated forms of phospho-serine specific AKT (lower blot) and total AKT proteins (upper blot). Cord blood CD34+ stem cells were cultured with CHO cell expressed rhEPO (CHO-rhEPO Treatment 1), human Hek293 cell expressed rhEPO (Hu-rhEPO Treatment 2), LMH2A cell expressed rhEPO (LMH/2A-rhEPO Treatment 3), and no rhEPO treatment (Treatment 4) for 7 days.

2005). Erythroid differentiation and cell survival in response to rhEPO has been shown that the JAK2/PI 3-kinase/AKT/Bad pathway is involved in the signaling cascade that leads to erythroid cell survival (Lambert et al., 2003). The PI3K-AKT pathway has been shown to play a central role in regulation of apoptosis and proliferation in several systems, including normal erythroid progenitors (Haseyama et al., 1999; Uddin et al., 2000). Sivertsen et al. (2006) demonstrated that the induced differentiation of CD34+ cells using rhEPO is dependent on PI3K/AKT signaling pathway.

The ability of LMH/2A cells to express *N*-linked glycosylated rhEPO which showed biological activity *in vitro* by promoting erythroid proliferation, differentiation and activation of signaling cascades in erythroid progenitor cells makes them a candidate for an alternative expression system for rhEPO. The findings of this study suggests that LMH/2A-rhEPO is a potential therapeutic for treatment of anemic patients, e. g., patients in chemotherapy or stem

cell transplant patients. Also, LMH/2A-rhEPO could used in differentiation of erythroid progenitors for *ex vivo* erythropoiesis.

CHAPTER V

CONCLUSION

Therapy with rhEPO has become the standard treatment for patients with renal and non-renal anemia. Currently in the biopharmaceutical industry, patents on original rhEPO products are starting to expire. This allows an opportunity to develop biosimilars for rhEPO with advantages such as an altered carbohydrate content that can increase *in vivo* survival and serum half-life (Sinclair and Elliott, 2005). For example, Darbepoietin-α is an erythropoietin analog that carries two additional glycosylation sites that gives a longer half-life and potency (Joyeux-Faure, 2007). A current challenge in rhEPO therapy involves the use of rhEPO associated with antibody-mediated pure red cell anemia (PRCA) which develops when neutralizing antibodies cross-react to endogenous hEPO (Macdougall, 2004). Therefore, biosimilars are need to be developed to minimize this potential to cause immune reactions.

In this study, LMH/2A cells were successfully used to produce glycosylated rhEPO on average 266 µg/mL per day. LMH/2A-expressed rhEPO was purified using a series of chromatograph steps and purity was > 95%. The concentration of the purified LMH/2A-rhEPO was estimated to be approximately 800 µg/ml. Treatment of the purified LMH/2A-rhEPO with deglycosylation enzyme showed that it is *N*-linked glycosylated in accordance with CHO-rhEPO and Hu-rhEPO which are also *N*-linked glycosylated (Shahrokh et al., 2010).

The biological activity of the purified LMH/2A-rhEPO was investigated *in vitro* using a cell based assay (CD34+ cells) and showed that LMH/2A-rhEPO induced cell proliferation (cell count) and stimulated the expression of erythroid markers such as hemoglobin, transferrin receptors, and glycophorin A. Interestingly, LMH/2A-rhEPO, with LMH2A cell specific glycosylation, promotes similar erythroid proliferation, differentiation and activation of signaling

cascades of human CD34+ progenitor cells when compared to non-human cell expressed CHO-rhEPO and to human cell expressed Hu-rhEPO.

Therefore, LMH/2A-rhEPO is considered a potential biosimilar and promising candidate to commercially available rhEPO expressed from CHO and human cell lines. Therefore, these results suggest that LMH/2A-rhEPO is biologically active *in vitro* by inducing erythroid differentiation and proliferation. It is intuitive that the enhanced activity of LMH/2A-rhEPO *in vitro* suggests that it may have a more effective *in vivo* activity, and subsequently, improve current therapeutic applications. Future studies for LMH/2A-expressed rhEPO will include 1) investigating the ability of LMH/2A to glycosylate rhEPO with a human-like glycosylation pattern and 2) examining the role of glycosylated LMH/2A-expressed rhEPO *in vivo* and *in vitro* for stability, bioactivity, half-life, folding, targeting, and function.

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APPENDIX

Human erythropoietin gene, complete cds
GenBank: M11319.1

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LOCUS       HUMERPA              582 bp    DNA     linear   PRI 08-NOV-1994
DEFINITION  Human erythropoietin gene, complete cds.
ACCESSION   M11319 REGION:
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VERSION     M11319.1  GI:182197
KEYWORDS    erythropoietin.
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;
            Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 582)
  AUTHORS   Lin,F.-K., Suggs,S., Lin,C.-H., Browne,J.K., Smalling,R.,
            Egrie,J.C., Chen,K., Fox,G.M., Martin,F., Stabinsky,Z.,
            Badrawi,S.M., Lai,P.-H. and Goldwasser,E.
  TITLE     Cloning and expression of the human erythropoietin gene
  JOURNAL   Proc. Natl. Acad. Sci. U.S.A. 82 (22), 7580-7584 (1985)
   PUBMED   3865178
COMMENT     Original source text: Human fetal liver DNA (T. Maniatis library),
            clone lambda HE1.
            Draft entry and sequence for [1] were kindly provided in
                computer-readable form by F.-K.Lin, 18-FEB-1986. The erythropoietin
            gene, when introduced into Chinese hamster ovary cells, produces
            the biologically active protein.
FEATURES             Location/Qualifiers
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ORIGIN 1 bp upstream of HindIII site.

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  121 aggtacctct tggaggccaa ggaggccgag aatatcacga cgggctgtgc tgaacactgc
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VITA

Amanda Cecile Ranzino Broussard was the third child born from Thomas Ranzino and Cacky Ranzino in Baton Rouge, Louisiana, in 1980. She attended St. Thomas More Elementary School and Bishop Sullivan High School. After graduating she attended Louisiana State University from August 1998 to May 2003, where she earned her bachelor's degree in biological sciences. In 2004, she married Ronald Paul Broussard Jr.

After graduation she worked at Pennington Biomedical Research Center for 3 years under Dr. Andy Deutsch. In 2006, she began work at the Louisiana State University AgCenter in the Veterinary Science department under the direction of Dr. Richard Cooper, and is now a candidate for the degree of Master of Natural Sciences in the Department of Natural Sciences at Louisiana State University, Baton Rouge, Louisiana.