Erythropoiesis from Human Embryonic Stem Cells Through Erythropoietin-Independent AKT Signaling

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Erythropoiesis from Human Embryonic Stem Cells
Through Erythropoietin-Independent AKT Signaling

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Cellular and Molecular Pathology

by

William Sang Kim

2013
ABSTRACT OF THE DISSERTATION

Erythropoiesis from Human Embryonic Stem Cells
Through Erythropoietin-Independent AKT Signaling

by

William Sang Kim
Doctor of Philosophy in Cellular and Molecular Pathology
University of California, Los Angeles, 2013
Professor Gay M. Crooks, Chair

Unlimited self renewal capacity and differentiation potential make human pluripotent stem cells (PSC) a promising source for the ex vivo manufacture of red blood cells (RBC) for safe transfusion. Current methods of erythropoiesis from PSC suffer from low yields of RBCs, most of which contain embryonic and fetal rather than adult hemoglobin. We have previously shown that homo-dimerization of the intracellular component of MPL (ic-MPL) induces erythropoiesis from human cord blood progenitors through unique, EPO-independent mechanisms. The goal of the present study was to investigate the potential of ic-MPL dimerization to induce erythropoiesis from human embryonic stem cells (hESC) and to identify the signaling pathways activated by this strategy. I present here evidence that ic-MPL dimerization induces EPO-independent
erythroid differentiation from hESC through AKT signaling, by generating erythroid progenitors and promoting definitive erythropoiesis with increased RBC enucleation as well as increased gamma:epsilon globin ratio and production of beta-globin protein. ic-MPL dimerization is significantly more potent than EPO in inducing erythropoiesis from hESC and activates a network of key erythroid factors involved in apoptosis, cell cycle, and differentiation. These findings open up potentially new approaches toward the generation of therapeutically relevant RBCs for transfusion.
The dissertation by William Sang Kim is approved.

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2013
DEDICATION

To my wonderful parents Steve and Lisa and my lovely sisters Julie and Carolyn and my crazy dog Snoopy, you are all a precious precious gift from the Lord. And to my Lord and Savior Jesus Christ, who loved me and gave Himself for me, and continues to lead me by the hand in all that I do despite my shortcomings. Thank you.
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9th Annual UCLA Stem Cell Conference. Efficient Erythropoiesis from Human Embryonic Stem Cells through Dimerization of Intracellular MPL. 2013. Los Angeles, CA.


Selected Peer-reviewed Publications


CHAPTER 1:

Introduction
Preface

Red blood cell transfusion is a widely used cellular therapy with approximately 85 million blood components transfused annually worldwide.\textsuperscript{1} Currently, the procedure is exclusively dependent on volunteer donors and is therefore susceptible to limitations in supply and risk for infectious disease transmission.

Human pluripotent stem cells (PSC) offer a potentially unlimited source of cells for cellular therapy because they can be propagated and expanded indefinitely, and maintain the ability to differentiate into cell types representative of all three germ layers. Thus PSC are a potentially ideal source for the \textit{ex vivo} manufacture of large numbers of red blood cell (RBC) units for safe transfusion. Toward this end, it has been shown that PSC can be directed to differentiate into mature enucleated RBCs.\textsuperscript{2,3} Current methods of RBC differentiation from PSC suffer from low yields of RBCs, most of which contain embryonic and fetal rather than adult hemoglobins. Therefore, efficient clinical translation of this strategy is critically dependent on the development of novel methods to enhance the generation of functional RBCs from PSC.

Erythropoietin (EPO) is an essential cytokine for normal erythropoiesis.\textsuperscript{4} Our laboratory has demonstrated an EPO-independent approach for the \textit{ex vivo} expansion and erythroid differentiation of human multilineage hematopoietic progenitors from cord blood utilizing an inducible system in which a fusion protein, consisting of the intracellular domain of the receptor MPL and a drug binding domain F36V (F36V-MPL), is expressed in CD34+ cells via a lentiviral vector.\textsuperscript{5,6} In the F36V-MPL system, homodimerization of intracellular MPL (ic-MPL) is accomplished by the addition of a
small molecule, AP20187 (CID) that binds to F36V, allowing for the induction of MPL signaling in the absence of TPO.

Based on our previous findings from Abdel-Azim et al,⁵ the initial goal of the work presented in this thesis was to study the effect of F36V-MPL on hematopoiesis from hESC and human cord blood progenitors and to understand its mechanism of action. The work by Parekh et al,⁶ came about as part of the above stated goal to further characterize F36V-MPL in cord blood progenitors (included as Chapter 3 of this thesis) and serves as a key stepping stone for Chapter 4 which contains the focal point of the work presented in this thesis: to study the erythropoietic effect of F36V-MPL on hESC and the mechanism of action.

**Human pluripotent stem cells**

PSC research has been a major focus of attention in recent years due to their potentially monumental implications toward cell based therapy, disease modeling, drug screening, and the study of early human development (reviewed in Gonzaelz et al⁷). There are several types of PSC; the naming of which depends on their source of origin. Two main types of PSC are human embryonic stem cells (hESC) and human induced pluripotent stem cells (hiPSC) which will be further discussed below.

*Human embryonic stem cells*

hESC were first derived by Thomson et al, in 1998 from the inner cell mass (ICM) of human blastocysts.⁸ Normally, the ICM represents a transient stage in development and does not persist beyond the blastula stage. However, once isolated and placed
under appropriate culture conditions, the ICM cells begin to undergo unlimited self-renewal while maintaining their pluripotency. It is these ICM-derived cells that are termed hESC. hESC are characterized by tightly packed colonies with well defined edges and a flat appearance and have been shown to express several cell surface antigens in the undifferentiated state, including the keratan sulfate antigens TRA-1-60 and TRA-1-81 along with the stage specific embryonic antigens SSEA3 and SSEA4.

As mentioned above, another hallmark of hESC is their pluripotency, which has been demonstrated through several methods. The first approach is known as embryoid body formation. In this approach, the culture components responsible for maintaining the cells in the undifferentiated state are removed allowing the cells to differentiate into spheres containing cells representative of the three germ layers; mesoderm, endoderm, and ectoderm. The second approach is the directed differentiation of the hESC into specific cell types by either the manipulation of culture conditions or the direct manipulation of transcription factor activity. Lastly, a third approach that has been used to demonstrate hESC pluripotency is the teratoma assay in which hESC are injected into immunodeficient mice. Once injected, the hESC develop into a tumor known as a teratoma composed of cells from mesoderm, endoderm, and ectoderm origin.

Several key transcription factors have been found necessary to the maintenance of the hESC identity. Of these factors, OCT3/4, SOX2, and NANOG are believed to be core master regulatory factors that coordinately work to form and maintain a transcriptional network responsible for maintaining the pluripotent state. Perturbing the expression of these factors has been shown to induce the loss of pluripotency and
subsequent differentiation to various cell types depending on the particular factor that is being affected.\textsuperscript{12-14} hESC also express high levels of telomerase, an enzyme associated with cellular immortality, which accounts for the ability of hESC to proliferate indefinitely without compromising their genomic integrity.\textsuperscript{8}

Although, hESC hold great potential toward the advancement of human health and are considered the gold standard for pluripotency, there are several limitations that hinder their translation to the clinic. One major hurdle is the ethical issues posed by the destruction of embryos during the generation of hESC. Another issue is the need for autologous cells in transplantation therapy to prevent rejection by the patient’s immune system. A potential means to address the need for autologous cells can be found in a technique known as somatic cell nuclear transfer (SCNT) in which a somatic cell nucleus is injected into an enucleated oocyte, effectively reprogramming the donor nucleus and ultimately the resulting cell to pluripotency from which hESC can be subsequently derived.\textsuperscript{15} Unfortunately, this approach is very inefficient and requires the use of donor oocytes which presents major ethical complications of its own.\textsuperscript{15}

\textit{Human induced pluripotent stem cells}

In 1962, Gurdon demonstrated the first successful SCNT by transferring nuclei of intestinal cells from adult frogs into enucleated oocytes which developed into viable tadpoles.\textsuperscript{16} This study demonstrated that pluripotency-inducing factors exist within the oocyte.\textsuperscript{16} About thirty years later, Wlimut et al, performed the first successful mammalian SCNT, in the form of a sheep named Dolly, showing that pluripotency-inducing factors also exist within mammalian oocytes.\textsuperscript{17} These studies helped set the
precedent for a landmark paper published in 2006 by Takahashi and Yamanaka which presented an alternative approach in generating pluripotent stem cells by direct reprogramming of human somatic cells through the ectopic expression of defined pluripotency factors: OCT4, SOX2, KLF4 and MYC (OSKM).18

These induced pluripotent stem cells (iPSCs) were able to form tightly compacted colonies that closely resemble ESCs morphologically, molecularly and phenotypically and are useful for a range of applications, including: autologous cell therapy, disease modelling, drug screening and basic research.7,19-22 Unfortunately, direct reprogramming although conceptually and technically simple, is a very slow and inefficient process, with less than one in a thousand cells being faithfully reprogrammed to pluripotency using conventional approaches.23 Although many different approaches have been developed for direct reprogramming, the majority of studies published have observed that integrating vectors give the best reprogramming efficiencies, which unfortunately presents the issue of insertional mutagenesis and improper silencing of potentially oncogenic factors such as MYC which can lead to tumorigenesis.

Erythropoiesis from pluripotent stem cells

Pluripotent stem cells offer a promising approach to generating an unlimited supply of donor-free RBCs for transfusion to meet the issue of limited supply, especially in alloimmunized patients and patients with rare blood phenotypes. Notably, unlike the majority of stem-cell-derived therapeutics, tumorigenesis is not a concern as RBCs can be irradiated without adversely affecting their in vivo function.24
Significant progress has been made thus far in generating RBCs from PSCs with several methods now available for both hESC and iPSC which can be grouped into two major categories: induction by co-culture with stromal layers (such as OP9 murine bone marrow stroma cells) and induction by embryoid body (EB) formation. However, there are still many hurdles that must be overcome before PSC derived RBCs can be translated to the clinic. For example, although a production efficiency of ~200,000 cells per hESC or iPSC has been achieved,\textsuperscript{24,25} large numbers of mature RBCs are required (2 x 10^{12} RBCs) in a conventional transfusion unit.\textsuperscript{26} Also, the majority of generated cells fail to enucleate and are predominantly composed of embryonic globin which has a poor capacity to carry oxygen and therefore would not be suitable for transfusion.\textsuperscript{24,27,28} Notably, prolonged culture has been shown to increase the production of fetal gamma-globin protein as well as the production of low levels of adult beta-globin mRNA, allowing for a shift from a yolk sac-like primitive phenotype toward a more fetal-like definitive RBC.\textsuperscript{27} In light of the limitations faced by present methodologies, it is of critical importance to identify methods to enhance the generation of functional RBCs from PSCs for efficient clinical translation of this strategy.

**Hematopoietic growth factors and their receptors**

Hematopoietic growth factors (HGF) are required for the growth and survival of hematopoietic cells throughout all stages of development.\textsuperscript{29} Their respective receptors are all structurally related, each consisting of one or two extracellular cytokine-binding domains, a transmembrane domain, and an intracellular signaling domain containing the box 1 and box 2 motifs that recruit kinases of the Janus kinase (JAK) family.\textsuperscript{29}
Activation of the receptor is achieved when binding of the ligand induces homodimerization of two receptor monomers, which induces a major conformational shift in the receptor bringing two tethered JAKs in close proximity, triggering a cascade of phosphorylation events which then go on to activate various signaling molecules that eventually lead to changes in gene expression profile to promote cell survival, proliferation, and differentiation.\textsuperscript{29}

There are two proposed models for the role of growth factors in hematopoietic differentiation: the stochastic vs. the inductive model. In the stochastic model, progenitor commitment to a particular lineage is stochastic with growth factors merely supporting cell survival and proliferation.\textsuperscript{30-34} In the inductive model, growth factors provide an instructive signal to direct progenitor commitment to specific fates.\textsuperscript{34-37} Several hybrid models have also been proposed, where, for example, growth factors direct maturation toward a specific lineage once a committed progenitor has been stochastically generated.\textsuperscript{34,38} It appears that most HGFs follow this hybrid model. For example, fetal livers from Erythropoietin (EPO) receptor (EPOR) knockout mice generated normal numbers of committed erythroid BFU-E and CFU-E progenitors, but still required functional EPOR for BFU-E and CFU-E maturation to erythrocytes.\textsuperscript{39}

Interestingly, several different HGFs and their respective receptors not associated with erythropoiesis have been shown to be able to replace EPO for inducing erythroid maturation. For example, McAurthur et al, demonstrated that retroviral expression and subsequent ligand mediated activation of the macrophage colony-stimulating factor (M-CSF) receptor, \textit{c-fms}, in murine fetal liver cells was able to stimulate the formation of erythroid and megakaryocyte colonies suggesting a functional
conservation of signal transduction mechanisms induced by HGFs between hematopoietic lineages. In further support of the conservation between HGF receptors, we found in Parekh et al, that dimerization of the intracellular domain of MPL (ic-MPL) activates a strong erythropoietic signal although TPO/MPL is the primary regulator of platelet production. In a similar study, Socolovsky et al, demonstrated that activation of a fusion protein containing the extracellular domain of the Prolactin (PRL) receptor fused with the intracellular domain of the Erythropoietin (EPO) receptor as well as the full-length PRL receptor was able to support erythropoiesis, suggesting that the cytoplasmic domains of cytokine receptors are interchangeable for their ability to support terminal differentiation.

F36V-MPL and hematopoiesis

For patients suffering from hematological and immunological disorders, human hematopoietic progenitor cell (HPC) transplantation can dramatically improve the quality of life or in severe cases be a life-saving treatment. For example, HPC transplantation has been shown to cure sickle cell disease in selected patients. However, one significant barrier to successful immune reconstitution is failure of donor-derived HPC proliferation and engraftment. Several strategies have been tested such as cytokine treatment and permanent gene modification, but a major hurdle that remains is the ability to regulate expansion of donor cells after transplantation. For example, although TPO mediated activation of its receptor MPL has been shown to support the survival and expansion of HPCs and cells along the megakaryocyte lineage, prolonged
stimulation induces a negative feedback response which causes internalization and deactivation of MPL.\textsuperscript{46}

In order to overcome these issues, our laboratory utilized a lentiviral vector to express a fusion protein F36V-MPL in primitive human cord blood CD34+38-Lin-HPCs.\textsuperscript{5} The fusion protein consists of the ic-MPL and F36V, a mutated FK506 binding protein (FKBP) containing a phenylalanine to valine substitution allowing for specific binding to a synthetic ligand, AP20187 (CID).\textsuperscript{47} Dimerization of ic-MPL is required for signal transduction. CID is able to diffuse across cell membranes and bind to F36V, causing homodimerization of ic-MPL and activation of signaling (Figure 1). This system allows for the control of ic-MPL activation by CID and avoids the issue of negative feedback regulation seen by prolonged TPO activation of its native receptor.\textsuperscript{46} By targeting CD34+38-Lin- cells with this system, we induced a pronounced expansion of CD34+ cells \textit{ex vivo}.\textsuperscript{5} \textit{In vivo} administration of CID after transplantation of cells in immune deficient mice resulted in over 1000-fold expansion of human CD34+Lin- HPC as well as a marked expansion of lymphoid progenitors.\textsuperscript{5} While these data demonstrate that the F36V-MPL dimerization approach allows profound and selectable HPC expansion, the molecular mechanisms of expansion are unknown. This information is essential to assess potential risks and possible alternatives in utilizing this strategy for HPC expansion in the clinic. Also, understanding the mechanism of expansion in this context may have translational importance beyond the hematopoietic system for the induction and control of this intrinsic property in all stem cells.
Specific aims of the dissertation

The work presented in the following three chapters represents my contributions to the understanding of the role of F36V-MPL in erythropoiesis from human cord blood progenitors and hESC and the signaling mechanism of action.

Aim 1. Characterize the ability of F36V-MPL to enhance the generation of hematopoietic stem/progenitor cells from hESC.

In Chapter 2, I present our work demonstrating that F36V-MPL dimerization does not enhance the generation of CD34+ early hematopoietic stem/progenitor cells from
hESC. Conversely, an increase in erythroid cells was observed which served to direct the work presented in Chapter 3 and 4.

Aim 2. Characterize the erythropoietic effect of F36V-MPL on human cord blood progenitors.

In Chapter 3, I present our work demonstrating that F36V-MPL induces erythropoiesis from human cord blood progenitors through a novel pathway. This research was previously published in Stem Cells\(^6\) and is reprinted here in its entirety with permission from John Wiley & Sons, Inc and all co-authors.

Aim 3. Characterize the erythropoietic effect of F36V-MPL from hESC.

Lastly, in Chapter 4, I present my work demonstrating that F36V-MPL induces definitive erythropoiesis from hESC through the AKT pathway and activation of a network of key erythroid factors involved in apoptosis, cell cycle, and differentiation. This work has been submitted for publication to Blood and is incorporated here (with modifications) with permission from all co-authors.

References


CHAPTER 2:
The effect of F36V-MPL on hematopoiesis from human embryonic stem cells
Introduction

Several methods have been utilized for the ex vivo expansion of Hematopoietic stem cells (HSC) such as treatment with key hematopoietic cytokines like TPO, which acts early during hematopoiesis and is critical for the survival and expansion of HSCs.\(^1\) Unfortunately, the in vivo potential of HSCs becomes compromised after ex vivo culture. As mentioned previously, recent work by our laboratory demonstrated that proliferation of adult HSC and lymphoid progenitors can be induced by activation of a fusion protein containing the cytoplasmic domain of the TPO receptor, MPL (F36V-MPL).\(^2\) Provision of a synthetic ligand, CID, either in culture or in vivo after transplantation, induces homodimerization of MPL with resulting proliferation and reduction of apoptosis of human CD34+ HSCs and progenitors that express F36V-MPL. These CD34+ cells retain the ability to engraft immune deficient mice. Unfortunately, this approach does not resolve the issue of immunocompatibility as only one third of patients have a matched related donor.\(^3\)

PSCs provide a potential strategy to circumvent the shortage of HLA compatible HSC donors. Also, PSCs can proliferate indefinitely and present a great potential vehicle for gene therapy. Although PSCs can differentiate toward the hematopoietic lineage, we and others have found that CD34+ cells derived from PSC have limited lymphoid potential and minimal engraftment capacity relative to adult HSCs. Notably, stimulation of the cytoplasmic domain of MPL using a fusion protein consisting of the intracellular and transmembrane domain of MPL and the extracellular domain of the Prolactin receptor was shown to promote hematopoietic commitment of murine PSCs.\(^4\) TPO stimulation was also shown to have a positive effect on the survival and expansion
of CD34+ early hematopoietic progenitors derived from human hESC.\textsuperscript{5} Based on this logical framework, we set out to investigate the possibility of using F36V-MPL to enhance the generation of HSCs and progenitors from hESC that are functionally similar to adult counterparts.

**Materials and Methods**

*Cell lines and culture media*

The hESC line H1 (WiCell, Madison, WI) was maintained and expanded on irradiated primary Mouse Embryonic Fibroblasts (mEFs) (Millipore, Billerica, MA) in 6 well plates (Corning, Lowell, MA) in 3mL/well of hESC medium composed of DMEM-F12 (80\%v/v) with 20\% Knockout Serum Replacement (KSR) in 2 mM L-glutamine, 10 mM nonessential amino acids, 4 ng/ml bFGF (all from Invitrogen), and 50 mM \(\beta\)-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). hESCs between passage numbers 38 and 68 were used for all experiments and were daily supplemented with fresh medium and passaged onto new mEFs every 4-5 days using the StemPro EZ passage tool (Invitrogen). The OP9-M2 cell line, a subclone of the murine BM stromal line OP9 (a generous gift from Dr. H. Mikkola, University of California, Los Angeles, Los Angeles, California) was used as a stromal support for hematopoietic differentiation of hESC. OP9-M2 were maintained in medium consisting of MEM-alpha+GlutaMAX (Invitrogen) supplemented with 20\% Fetal Bovine Serum (FBS) (Thermo Scientific Hyclone, Logan, Utah) and 1X penicillin/streptomycin (Gemini Bio-Products, West Sacramento, CA).
**Generation of stably transduced hESC lines expressing F36V-MPL**

H1 hESC were transferred onto Matrigel (BD Biosciences) in 6 well plates in mTeSR1 Medium (Stem Cell Technologies) for 3-4 days. After cells were transduced for 6 hours in 1mL of mTeSR1 (BD Biosciences) containing virus, the wells were topped off with 1mL fresh virus-free mTsER1 (BD Biosciences) for a subsequent 18 hours prior to complete removal of virus with new medium. A lentiviral vector expressing only F36V and GFP was used in parallel as a negative control. After 48 hours hESCs were passaged onto MEFs to prevent the cells from becoming confluent and subsequently cultured for 5 days in hESC medium as stated in “Cell Lines and Culture Medium.” Cells were then harvested using 0.05% Trypsin (Invitrogen) digestion for 5min at 37C prior to FACS isolation: 1000 SSEA-4+GFP+mCD29-DAPI- cells were sorted and replated per well of a 6 well plate (Corning) containing irradiated mEFs. 10nM of the Rho-Associated Coil Kinase (ROCK) inhibitor, Y-27632 (EMD Millipore, Hayward, CA) was added 24 hours prior to and after sorting as described by Watanabe et al. 2007 to allow survival of dissociated hESCs. The FACS sorted clones were expanded to establish a stably transduced hESC line for subsequent experiments.

**Stromal-based co-culture for hematopoietic differentiation**

Transduced H1 hESC were plated on confluent layers of OP9-M2 stroma in OP9 medium consisting of MEM-alpha+GlutaMAX (Invitrogen) supplemented with 20% Fetal Bovine Serum (FBS) (Thermo Scientific Hyclone, Logan, Utah) and 1X penicillin/streptomycin (Gemini Bio-Products, West Sacramento, CA). 100nM AP20187 (CID) (Clontech, Mountainview, CA) was added every 3-4 days as indicated in each
experiment, from day of plating (day 0) to day 14. After 14 days of culture, cells were harvested using sequential collagenase IV (Stem Cell Technologies, Vancouver, BC) and Trypsin (Invitrogen) digestion, for FACS analysis.

**Cell sorting and flow cytometry**

FACS analysis was performed on LSRII or LSRFortessa and cell sorting on a FACSARia (Becton Dickinson, San Jose, CA) by direct immuno-fluorescence staining with human specific monoclonal antibodies. Non-specific binding was blocked with intravenous immunoglobulin (0.1%) (IVIG; Cutter, Berkley, CA) prior to staining with fluorochrome conjugated antibodies. Cell acquisition used FACSDivaTM (Becton Dickinson) and analysis was performed using FlowJo (Tree Star, Ashland, OR). The following antibodies were used for staining; CD34-PEcy7 (Becton Dickinson) and mCD29-Alexa 647 (AbD Serotec, Raleigh, NC). mCD29-Alexa 647 was used to gate out murine cells. Unstained cells were used as negative controls. FSC/SSC and DAPI (Invitrogen) was used to identify live cells.

**Statistical analysis**

Prism version 5 (GraphPad Software Inc) was used for statistical analysis. Results are presented as the average value of at least three experimental repeats (indicated accordingly) +/- standard deviation. Unpaired Student’s t-test was utilized and P values <0.05 were considered statistically significant.
Results

*Generation of stably transduced hESC line expressing F36V-MPL*

To determine if ligand-inducible signaling of intracellular MPL (ic-MPL) induces hematopoiesis in hESC, the H1 hESC cell line was stably transduced to express either the F36V-MPL fusion protein (Figure 1A) or a control vector expressing only the ligand binding domain, F36V. Each vector co-expressed the marker gene GFP, allowing selection of transduced hESC and monitoring of vector expression in hematopoietic derivatives. Transduced GFP+ hESC were FACS sorted to generate a stably expressing line (Figure 1B,C).

![Diagram of F36V-MPL vector](image)

**Figure 1. Generation of stably transduced H1 hESC line expressing F36V-MPL.** H1 hESCs were transduced with F36V-MPL and isolated by FACS based on FSC/SSC, SSEA-4+, GFP+, mCD29-, DAPI- gating and subsequently cultured to establish a stably expressing line maintaining ~90% GFP+ expression. **A)** Schematic of F36V-MPL vector. **B)** Representative immunophenotype analysis of GFP and mCD29 expression to assess expression of vector in human mCD29- cells through continual cell passaging (25 passages from point of transduction). **C)** Light microscope picture of a stably transduced hESC colony expressing F36V-MPL and GFP.
*F36V-MPL does not effect the generation of total CD34+ cells from hESC*

Once a stable hESC line expressing F36V-MPL was established, the cells were subsequently plated onto the murine stromal line OP9 and cultured without exogenous cytokines for 14 days, in the presence or absence of CID. The level of hematopoietic induction was initially assessed by measuring the output of CD34+ cells. No difference was observed in total CD34+ output between CID treatment and untreated controls (Figure 2). Conversely, an increase in the frequency of GlyA+ cells was observed upon CID treatment (see Chapter 4). Due to the lack of change in total CD34+ output, the attempt to generate HSCs from hESC was not further pursued and attention was given to erythropoietic induction instead which can be found in Chapter 4.

![Figure 2. Dimerization of ic-MPL does not effect the generation of CD34+ cells from hESC.](image)

**Figure 2. Dimerization of ic-MPL does not effect the generation of CD34+ cells from hESC.** H1 hESCs transduced with F36V-MPL were cultured on OP9 stroma for 14 days with or without CID (no cytokines added), and then counted and analyzed by FACS. Summary of Immunophenotype data shown as: % and number of CD34+ cells (n=3).

**Discussion**

Unlike previous reports,\(^4,5\) we find that MPL dimerization does not enhance the output of CD34+ hematopoietic progenitors from embryonic stem cells. A key detail that may account for the difference can be found in the composition of the MPL protein used...
for induction. For example, in Srivastava et al, the wildtype full-length MPL receptor was used to enhance CD34+ output from hESC, while Challier et al, utilized a fusion protein consisting of the intracellular and transmembrane domain of MPL and the extracellular domain of the Prolactin receptor (PRL-R) to induce hematopoiesis from murine embryonic stem cells (mESC). In contrast, our study utilized a fusion protein containing only the intracellular domain of the MPL receptor which specifically promoted erythropoiesis instead of hematopoietic progenitor output. Notably, it has been shown that different domains of the MPL receptor are responsible for regulating different signaling pathways associated with MPL activity, which suggests that the signaling pathways activated by F36V-MPL differs from the activation of the full length MPL receptor which we found to be the case (see Chapter 3 and 4).

References


CHAPTER 3:

Novel pathways to erythropoiesis induced by dimerization of intracellular MPL

in human hematopoietic progenitors
Preface

In Chapter 2, we observed an erythropoietic effect of F36V-MPL on hESC differentiation (followed up in Chapter 4). In our cell signaling studies, we observed that F36V-MPL activates AKT signaling which is a critical pathway for erythropoiesis (see Chapter 4). The cell signaling studies served as a key logical framework for the work by Parekh et al, of which I was included as an author and is presented below as Chapter 3 of this thesis. My specific role on this paper was in setting up the parameters for the annexin/PI assay and identifying the appropriate house-keeping genes to utilize for the qPCR assay and helping with experimental design.
Novel Pathways to Erythropoiesis Induced by Dimerization of Intracellular c-Mpl in Human Hematopoietic Progenitors

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Key Words: humans • myeloid progenitor cells • erythropoiesis • c-Mpl

ABSTRACT
The cytokine thrombopoietin (Tpo) plays a critical role in hematopoiesis by binding to the extracellular domain of its receptor, c-Mpl. Mpl homodimerization can also be accomplished by binding of a synthetic ligand to a constitutively expressed fusion protein F36VMpl consisting of a ligand binding domain (F36V) and the intracellular signaling domain of Mpl. Unexpectedly, in contrast to Tpo stimulation, robust erythropoiesis is induced after dimerization of F36VMpl in human CD34+ progenitor cells. The goal of this study was to define the hematopoietic progenitor stages at which dimerization of intracellular Mpl induces erythropoiesis and the downstream molecular events that mediate this unexpected effect. Dimerization (in the absence of erythropoietin and other cytokines) in human common myeloid progenitors and megakaryocytic erythroid progenitors caused a significant increase in CD34+ cells (p < .01) and induced all stages of erythropoiesis including production of enucleated red blood cells. In contrast, erythropoiesis was not seen with Tpo stimulation. CD34+ cell expansion was the result of increased cell cycling and survival (p < .05). Microarray profiling of CD34+ cells demonstrated that a unique transcriptional pattern is activated in progenitors by F36VMpl dimerization. Ligand-induced dimerization of intracellular Mpl in human myeloid/erythroid progenitors induces progenitor expansion and erythropoiesis through molecular mechanisms that are not shared by Tpo stimulation of endogenous Mpl.

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION
The cytokine thrombopoietin (Tpo) is a megakaryocytic development factor that also plays a critical role in hematopoietic stem cell (HSC) self-renewal and expansion [1]. The actions of Tpo are mediated through its cell surface receptor c-Mpl, which is expressed by HSC, myeloid/erythroid progenitors, megakaryocytes, and platelets [2-4]. The binding of Tpo to the extracellular domain of c-Mpl results in homodimerization of its intracellular domain leading to activation of proliferation, survival and differentiation pathways [4, 5]. Although Tpo produces little expansion of progenitor cells when used alone, it is commonly used in combination with other cytokines for ex vivo maintenance and expansion of progenitor cells [1]. However, c-Mpl is internalized after binding to Tpo and its expression is downregulated during differentiation, resulting in a limited window of development during which Tpo can act. To directly regulate Mpl signaling, an alternative approach has been used, involving the constitutive intracellular expression of a fusion protein (referred to as F36VMpl) that consists of a ligand-binding domain (F36V) fused to the intracellular signaling domain of c-Mpl. The addition of a synthetic diffusible ligand (chemical inducer of dimerization; CID) to cells results in homodimerization of F36VMpl and signaling is induced.

Our laboratory has previously used a lentiviral vector to express F36VMpl in cord blood (CB) HSC. The addition of

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CID to transduced HSC resulted in significant expansion of CD34+ cells with multilineage potential, making this a potential gene therapy approach for primitive hematopoietic progenitor expansion [2]. The progenitor expansion seen after F36VMpl dimerization of HSC was not seen in a previous study, which targeted a more heterogeneous and differentiated progenitor population [6]. However, both aforementioned studies showed significant erythroid differentiation with F36VMpl dimerization. The unexpected finding of erythroid differentiation with F36VMpl dimerization formed the basis of the studies described here, the goal of which was to define the mechanisms by which F36VMpl dimerization induces erythropoiesis and progenitor expansion. Our approach was to identify the hematopoietic myeloid/erythroid progenitor cell stages at which F36VMpl dimerization acts and to examine the downstream molecular targets of dimerization of intracellular Mpl to understand more clearly how these compared with Tpo stimulation. Defining these mechanisms is critical for understanding how F36VMpl dimerization could be applied for clinical translational uses.

During hematopoiesis, primitive lymphoid progenitor cells give rise to the more lineage-restricted common myeloid [3] and common lymphoid progenitors [7–9]. The myeloid/erythroid lineages arise from a common myeloid progenitor (CMP), which gives rise to the more lineage restricted megakaryocytic erythroid progenitors (MEPs) and granulocytic monocytic progenitors (GMPs). MEPs give rise to megakaryocytes and erythrocytes, whereas GMPs give rise to granulocytes and monocytes [3]. As both CMP and MEP normally express c-Mpl [3], we focused on these specific progenitor stages to directly compare the functional and molecular effects of F36VMpl dimerization with those caused by binding of the natural ligand Tpo to its endogenous receptor. Our data show that ligand-inducible dimerization of intracellular Mpl activates functional and molecular programs in CMP and MEP that are markedly different from those activated by Tpo.

**Materials and Methods**

**Isolation of Human Progenitor Populations**

Umbilical CB was collected from normal deliveries, according to guidelines approved by the University of California Los Angeles Investigational Review Board. Enrichment of CD34+ cells was performed using the magnetic-activated cell sorting system (Miltenyi Biotec, Auburn, CA, http://www.miltenyibiotec.com/en/default.aspx) or RoboSep human CD34 selection system (StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com/). CD34+ enriched cells were incubated with the following anti-human-specific monoclonal antibodies: CD34 PerCP-Cy5.5, CD38 PE-Cy7, CD123 (interleukin-3 receptor alpha) PE, CD 45RA PE-Cy5, FITC-labeled lineage-specific antibodies; CD2, CD7, CD10, and CD20 and the lineage allophycocyanin (APC)-labeled antibodies; CD3, CD4, CD8, CD11b, CD14, CD19, CD56, and glycophorin A (Gly A; all from Becton Dickinson, San Jose, CA, http://www.bd biosciences.com/). An unstained (no antibody) control was used to define negative gates. The following published immunophenotypic definitions were used to isolate myeloid progenitors from thawed CB CD34+ enriched cells by fluorescence-activated cell-sorting (FACS): CD34+CD28−CD45RA−CD123 cyt (CMP), CD34+CD38+CD154RA+CD123−(MEP), CD34+CD38+CD154RA+CD123+ for granulocyte monoctye progenitors (GMP) [3], and CD34+38+38−primitive lymphoid progenitors (HSC) [10]. Sorting was performed on a FACSaria (Becton Dickinson) equipped with five lasers (355, 405, 488, 561, and 633 nm). Isolated populations were analyzed by FACS to assess post sort purity.

**Lentiviral Vector Expression of F36VMpl**

A F36VMpl plasmid (a generous gift of Dr CA Blu, University of Washington, Seattle, WA) was used to produce lentiviral (HIV-1) vectors that express the fusion protein F36VMpl under control of the murine stem cell leukemia virus promoter and a marker gene (green fluorescent protein [GFP]) for in vitro and luciferase (LUC) for in vivo studies expressed under control of the BGK (human phosphoglycerate kinase) promoter (Fig. 1D). Control vectors coexpressed F36V (no c-Mpl) and GFP. Vectors were pseudotyped using vesicular stomatitis virus (VSV) envelope or Gibbon ape leukemia virus (GALV) envelope by transient transfection into 293T cells and concentrated by ultrafiltration and ultracentrifugation [11, 12]. CB progenitors were incubated in 100 μl per well of serum-free transduction medium on recombinant fibroblastin fragment CH-296 (50 μg/ml, Takara Bio Inc, Otsu, Japan, http://www.takara-bio.com) or control non-tissue culture 48-well plates (100,000 cells per well) for 16 hours. The transduction medium consisted of X-vivo15 (BioWhitaker, Walkersville, MD, http://www.lonza.com/resources/product-instructions/bio whittaker-cell-culture/; stem cell factor (50 ng/ml); Flt 3 ligand (50 ng/ml), and Tpo (50 ng/ml), (all cytokines from R&D Systems, Minneapolis, MN, http://www.rndsystems.com/). Viral supernatant (2 × 105 units/ml) was then added along with an additional 100 μl of transduction medium per well to make the total volume 200 μl/well. A second dose of viral supernatant was added 4 hours later. After 30 hours of total exposure to vector, cells were washed with 10 times the volume of Dulbecco phosphate-buffered saline (DPBS; Mediatech, Herdon, VA, http://www.cellgro.com/). Cells were transduced in a common pool and then divided for culture or for transplantation with or without CID.

**Dimerization**

Lyophilized CID (AP2087), (ARIAD Pharmaceuticals, Cambridge, MA, http://www.ariad.com/; regulation kit) was solubilized in 100% ethanol to produce 1 mM stock solution (for in vitro use) or 62.5 mg/ml (for in vivo use) and stored at −30°C. For in vitro experiments, CID was diluted fresh in the culture medium to a final concentration of 100 nM and added to cultures every 48 hours. For in vivo administration, stock solution was diluted fresh on the day of injection. The final solution for injection contained 10% polyethylene glycol 400, 2% Tween 80, and 2.5 mg/ml of CID.

**CMP, MEP, GMP, and Serum Free Cultures**

CMP, MEP, and GMP were transduced with either F35V-Mpl or F36V control vectors and cultured in the presence or absence of CID (100 nM) and in the absence of cytokines. Cells were plated in bulk in 96-well tissue culture plates onto established M5 (murine marrow stromal) cell line in lymphoid medium (RPMI 1,640 [Irvine Scientific, Santa Ana, CA, http://www.iresci.com/], 5% fetal calf serum (FCS) (screened for B-cell cultures), 50 mM 2-ME, 50 U/ml penicillin/50 μg/ml streptomycin, and 200 mM L-glutamine). Cells were recovered for analysis at different time points by trypan blue. Viable cells were counted using trypan blue. Fold increase in the total number of cells compared with the original number of cells plated was calculated. Myeloid lineage potential of progenitors was assessed using a colony-forming unit cell (CFU-C) assay by plating cells in methylcellulose using METHOCELL GF H4435 (StemCell Technologies) and counting colonies after 14 days. Lymphoid potential of progenitors was tested by culturing them (cultures initiated...
Figure 1. Isolation and transduction of progenitors. (A): CD34+Lin− cells were sorted to obtain common myeloid progenitor (CMP), megakaryocyte-erythroid progenitor (MEP), and granulocytic-monocytic progenitor (GMP). (B): Relative colony-forming unit output from CMP, MEP, GMP, and total CD34+ cells (500 cells per population plated). (C): Relative B-lymphoid output in lymphoid cultures initiated with populations shown. (D): Lentiviral F36VMpl vector. Abbreviations: CFU, colony-forming unit; CMP, common myeloid progenitor; F36VMpl, fusion protein; GFP, green fluorescent protein; GMP, granulocytic-monocytic progenitor; HSC, hematopoietic stem cell; MEP, megakaryocyte-erythroid progenitor; MSCV LTR, murine stem cell leukemia virus long-term repeats promoter; FGK, human phosphoglycerate kinase promoter.

with 1,000 cells per progenitor) on MS-5 stroma in lymphoid medium with FLT-3 (5 ng/ml), Tpo (5 ng/ml), and IL-7 (5 ng/ml) for 4 weeks, and using FACs to detect CD19+ B lymphocytes. Erythroid differentiation in serum-free conditions was tested by transducing CB CD34+ cells with F36VMpl and culturing the transduced cells in X-vivo 15 medium on recombinant fibronectin fragment CH-296 coated non-tissue culture 48-well plates in the presence of CID. Tpo (50 ng/ml), Epo (5 units/ml), Epo+CID, or no growth factors.

Immunophenotypic Analysis of Cultured and Transplanted Cells

FACS analysis of cultured cells, as well as bone marrow and spleen cells harvested from mice, was performed on LSR II (Becton Dickinson) by direct immunofluorescence staining with human specific monoclonal antibodies and detection of GFP expression after incubation in 1.2% human intravenous immunoglobulin (IVIG; Cutler, Berkley, CA, http://www.bayer.com). Analysis used FlowJo (Tree Star, Ashland, OR, http://www.flowjo.com). Lineage-specific human antigen expression was determined using the following antibodies: CD45-APC-Cy7, CD24-PE-Cy7, CD41a-PerCP Cy5.5, CD66-PE, Gly A-APC or -PE, CD10-APC or PerCP, CD56-PE, and CD14 FITC (all from Becton Dickinson). The following immunophenotypes were used to identify myeloid progenitors from culture: CMP (CD34+CD38+CD123−CD45RA−), MEPCD34+CD38+CD123−CD45RA−, and GMP (CD34+CD38+CD123+CD45RA−). and double-positive for CD10 and CD34.

Cell Cycle and Apoptosis Analyses

Transduced cells cultured for 5-7 days with or without CID were harvested and stained with Hoechst (Becton Dickinson) for cell cycle analysis and Annexin-APC (Becton Dickinson) for apoptosis analysis. Unstained cells were used to set negative gates. In a separate experiment, transduced cells were cultured for 7 days with or without CID and then incubated with bromodeoxyuridine (BrdU) for 30 or 120 minutes before harvest and analysis. Harvested cells were fixed, permeabilized, and stained with 7-AAD and APC conjugated antibody to BrdU. Unstained cells were used to set negative gates and the BrdU positive cells were scored as cells in the S-phase of cell cycle.

Cell Morphology Analysis

F36VMpl transduced CMP were cultured for 35 days, harvested, and stained with Giemsa-Wright. The stained cells
were scanned with Scanscope XT (Aperio technologies, Vista,
CA, http://www.aperio.com/) to acquire digital images, which
were analyzed using imagescope software (Aperio technolo-
gies, Vista, CA).

Transplantation Assays
Nonobese diabetic/severe combined immunodeficiency inter-
leukin-2 receptor gamma chain knock out (NSG) mice (Jack-
on Laboratories, Har Harb er, ME, http://www.jax.org/) were
used for in vivo experiments according to protocols approved
by the Institutional Animal Care and Use Committee of
University of California Los Angeles. Transduced human cel-
s were suspended in DPBS and inoculated (30-50 microliters
per animal) into the right tibia of sublethally irradiated (250
cGy 4-6 hours before transplantation) mice. Engraftment and
differentiation of human cells were assessed by in vivo biolu-
minescence imaging and/or FACS from harvested organs after
preincubation in 1.2% human IVIG (Cutter) and purified rat
anti-mouse CD16/CD32 (Fc III/I) receptor; Becton Dickinson)
to prevent nonspecific antibody binding. The transplantation
of relatively low numbers of cells (30,000-48,000 MEP cells
per mouse) was designed to provide minimal baseline engraft-
ment from unstimulated cells. C1D of 10 mg/kg (or control
vehicle) was administered by intraperitoneal (IP) injection
daily starting 1 day before transplantation (day 1) until the
time of killing.

Bioluminescence Imaging
In vivo bioluminescence imaging was performed under general
anesthesia with 2% isoflurane. Fifteen minutes before
imaging, each mouse was given 125 mg/kg 1P luciferin
(Promega, Madison, WI, http://www.promega.com/). In vivo
optical imaging was performed with a prototype IVIS 3D bilo-
minescence/fluorescence optical imaging system (Xenogen,
Alameda, CA, http://www.xenogen.com/products/prmcinical-
ingaging/) at different time points. Regions were outlined
around the bodies of the mice to assess signal intensity emi-
ted. Ventral and dorsal images were acquired and total
signal was calculated for each time point [13].

Quantitative RT-PCR
CB CD34+ cells transduced with F36VMpl-GFP or F36V-GFP
were cultured on M5-5 stroma in lymphoid medium with or
without C1D. CD34+GFP+ cells were isolated from culture on
day 7 by FACS and RNA was extracted using a QiaGen
An omnipotent reverse transcriptase kit was used to make
cDNA, which was subjected to quantitative polymerase chain
reaction (qPCR) using TaqMan probe-based gene expression
analysis assays (Applied Biosysstems, Carlsbad, California,
http://www.appliedbiosystems.com/abt/st/ce/en/home.html) for
VEGF (Hs00900055_m1), &-cadherin (Hs01238941_m1),
GATA1 (Hs01085823_m1), Tal1 (Hs01097687_m1), EPOR
(Hs00950427_m1), VEGFR1 (Hs01052561_m1), and VEGFR2
(Hs00911700_m1). The qPCR was done using a 7500 Real
time PCR system Applied Biosystems. To identify appropriate
housekeeping genes, CB CD34+ cells were transduced with
F36VMpl-GFP or F36V-GFP and cultured for 7 days on M5-
5 stroma with or without C1D. RNA was extracted from equal
numbers of CD34+GFP+ cells isolated from culture and sub-
jected to qPCR for 12 housekeeping genes (18sRNA, ACTB,
B2M, GAPDH, GUSB, HPRT1, PBHA, HSP90AB1, RPL13A,
RPLP0, TFB, and UBC) on a human housekeeping gene RT
profiler PCR array (SA Biosciences, Frederick, MD, http://
www.sabiosciences.com/). Based on the results of the house-
keeping array (Supporting Information Table S1); RPL13a
(Hs01926559_g1), HSP90 (Hs00607336_g1), and beta actin
(Hs09999902_m1) were chosen as housekeeping genes
because their expression values were similar in dimerized
and control cells (Supporting Information Table S1). The data
were analyzed using the comparative C (T) method [14].

Microarray Analysis
CB CD34+ cells transduced with F36VMpl-GFP were cultured
in lymphoid medium on M5-5 stroma either with C1D alone,
with Epo (5 international units/ml) alone, or with Tpo
(50 ng/ml) alone. Transduced cells cultured on M5-5 stroma
in the absence of either growth factors or C1D served as
to controls. Three biologically independent experiments were
performed. CD34+GFP+ cells were isolated by FACS after 7
days of culture and subjected to RNA extraction using a Qi-
agen micro kit. Microarray analysis was performed using an
affymetrix U133 plus 2.0 platform (Affymetrix, Santa Clara,
California, http://www.affymetrix.com) and iCHIP software
(Deartment of Bioinformatics, Harvard School of Public Health,
Boston, MA, http://bioinfo1.harvard.edu/complab/dchip). The
quantile method [15] was used to normalize gene expression
values in a PM/MM model [16]. Gene expression values from
control cultures served as a baseline for normalization. Genes
were considered differentially expressed if they were greater
than or equal to twofold upregulated or downregulated
and significant at a p < 0.05 on a paired t-test when compared
with control cells over all three independent experiments. The
Database for Annotation, Visualization and Integrated Discov-
ery (DAVID) (http://david.abcc.ncifcrf.gov/) was used to
drive functional gene class enrichment data. Ingenuity
software (Ingenuity systems, Redwood City, California, http://
www.ingenuity.com/) was used to generate functional gene
network data. The microarray data were deposited in NCBI’s
Gene Expression Omnibus and is accessible through the fol-
token=zk5osqesooms&acc=GSE30583.

Statistical Analysis
Analysis of variance (ANOVA) and linear regression were per-
formed to compare the lymphoid potential of the four different
cell types (CMP, MEP, GMP, and HSC). The Generalized
Cochran-Mantel–Haenszel test was used to determine whether
or not a difference exists in the frequency of colony type (burst
forming unit erythroid (BFU-E), colony-forming unit granulo-
cyte macrophage (CFU-GM), and colony-forming unit granulo-
cyte macrophage (CFU-GEMM)) among the four cell types
(CMP, MEP, GMP, and CD24). The analysis of the differences
in cell numbers between the F36VMpl+C1D, F36Vmp without
C1D, and F36V+C1D arms was based on linear regression that
adjusted for the time of cell counts. A nested ANOVA was per-
formed to examine the association between the proportions of
cyting or apoptotic cells among the three arms. QPCR data
were analyzed with a two-way ANOVA model, which included
a Benjamini and Hochberg adjustment to correct for the false
discovery rates due to multiple comparisons. Statistical analy-
ses were carried out using SAS and R software.

RESULTS
F36VMpl Dimerization in CMP and MEP
Increases Total and CD34+ Cell Numbers and
Induces Erythropoiesis
Lineage potentials of the CMP, GMP, and MEP immunopheno-
dotypes were initially confirmed through in vitro
Figure 2. Fusion protein (F36VMpl) dimerization in megakaryocyte erythroid progenitor (MEP) expands enhanced green fluorescent protein (GFP) positive progenitors and induces erythropoiesis. Transduced MEP cultured ± chemical inducer of dimerization (CID) on stroma without growth factors (starting cell number = 2,303–3,666 cells per arm; n = 5 independent experiments; equal cell numbers were used for each arm in a given experiment) were analyzed for (A) the output of cell types shown and (B) immunophenotype (day 7). (C): Nonobese diabetic/severe combined immunodeficiency interleukin-2 receptor gamma chain knock out (NSG) mice transplanted with transduced MEP and administered CID or control vehicle were imaged (day 14). (D): Longitudinal quantification of bioluminescence. Abbreviations: Gly A, glycophorin A.

myeloid and lymphoid assays of populations isolated by FACS from CR (Fig. 1A). As expected, CMP possessed both granulocyte–monocyte and erythroid clonogenic potential, as shown by robust production of BFU-E, CFU-GM, and CFU-GEMM (Fig. 1B). Consistent with more lineage-restriction, MEP produced predominantly erythroid colonies and GMP produced predominantly granulocyte-monocyte colonies (Fig. 1B, p < .05 for difference in frequency of types of colonies for the four cell types: CMP, MEP, GMP, and unfractionated CD34+ cells, n = 3 independent experiments). In keeping with commitment to the myeloid pathway, CMP, MEP, and GMP were almost devoid of lymphoid potential when compared with control CD34+CD38− progenitor cells (Fig. 1C, p < .05, n = 2 independent experiments).

The effects of F36VMpl dimerization on erythropoiesis were first investigated in MEP, which are progenitors enriched in erythroid potential. MEP cells were transduced to express F36VMpl (Fig. 1D; control vector) and cultured in the presence or absence of CID on the murine stromal line M55 in the absence of exogenous growth factors. CID-treated MEP that expressed F36VMpl produced significantly higher numbers of total CD45+ cells and CD34+ progenitors than cultures from control cells (Fig. 2A, p < .05, n = 5 independent experiments). Of note, Gly A+ (erythroid) cells comprised
Figure 3. Dimerization in common myeloid progenitor (CMP) induces expansion of CD45+; CD34+, and erythroid cells and enhances cell cycling and survival. Transduced CMP cultured ± chemical inducer of dimerization without growth factor (starting cell number = 7,000–53,000 cells per arm, n = 5 independent experiments, equal cell numbers were used for each arm in a given experiment) were analyzed for (A) cell output, (B) immunophenotype (day 7), as well as (C) cell cycle and apoptosis (day 7). (D): Giemsa-Wright staining (×10 magnification; red arrows, erythrocytes; green arrow, erythroid progenitor) and (E) flow cytometry for nucleated erythrocytes in dimerized cultures (day 35). Abbreviations: CID, chemical inducer of dimerization; GFP, green fluorescent protein; G-A, glycoporphin A. 

the predominant cell type produced in dimerized cultures but were not seen in control cultures (Fig. 2A, 2B, p < .01, n = 5 independent experiments). In view of the significant progenitor expansion seen in vitro, we also explored the effects of delivering the dimerizing ligand in vivo after transplantation of MEP into NSG mice. Flow cytometry of bone marrow harvested from mice euthanized on day 14 showed the presence of GFP+ Gly A+ human cells in CID-treated mice and no human cells in control vehicle-treated mice (Supporting Information Fig. S2). In further transplantation experiments, the
Figure 4. Dimerization in common myeloid progenitor (CMP) results in selective generation of vector-expressing megakaryocytic erythroid progenitor (MEP) and CMP. Transduced CMP were cultured ± chemical inducer of dimerization for 7 days (starting cell number = 9,600 cells/ arm) and analyzed for (A) CMP, MEP and granulocytic monocytic progenitor immunophenotypes (gated out of CD45+ lin- cells), (B) cell counts of populations in (A), (C) green fluorescent protein expression of populations in (A). (One representative experiment of three depicted). Abbreviations: CID, chemical inducer of dimerization; CMP, common myeloid progenitor; MEP, megakaryocytic erythroid progenitor; GMP, granulocytic monocytic progenitor; GFP, green fluorescent protein.

GFP marker was replaced in the vector with firefly LUC allowing dynamic tracking of engraftment. Bioluminescent imaging of mice that received CID in vivo from day 1 to day 14 showed higher short-term engraftment than control mice in three of four independent experiments (Figs. 2C, 2D; one representative experiment out of a total of four experiments, and Supporting Information Fig. S2).

In contrast to MEP, no significant effect on cell growth or differentiation was seen when transduced GMP were cultured in the presence of CID (data not shown). The effects of inducing Mpl dimerization in CMP, a more primitive myeloid progenitor that gives rise to MEP and GMP, were next tested. Similar to MEP, transduced CMP stimulated with CID gave rise to significantly higher numbers of total CD45+ (p < .01) and CD34+ (p < .01) cells than non-dimerized cultures (Fig. 3A; n = 5 independent experiments). Gly A expressing cells were again the predominant population (Fig. 3A, 3B, p < .01, n = 5 independent experiments). Morphological analysis of cultures generated from CMP demonstrated that F36VMpl dimerization induced full erythroid differentiation from erythroid progenitors to mature enucleated red blood cells (Fig. 3D, day 35 of culture) despite the absence of Epo. Flow cytometry confirmed that dimerized CMP generated enucleated red blood cells (shown as Gly A- cells that were Hoechst negative, Fig. 3E). In addition to erythroid cells, significantly higher numbers of megakaryocyte (CD41 bright Gly A- ) cells were produced in dimerized cultures than in control cultures (p < .05 for five independent experiments for CMP, Supporting Information Fig. S3A, S3B). Morphologic analysis of cultures also demonstrated generation of monocytes, megakaryocytes, and neutrophils (Supporting Information Fig. S3C).

Most of the CD34+ cells generated during Mpl dimerization expressed relatively low levels of CD34 irrespective of whether the starting population was unselected or CD34+ cells (Supporting Information Fig. S4A). MEP (Fig. 2B), or CMP (Fig. 3B). The CD34 dim population contained clonogenic cells (particularly erythroid colonies; Supporting Information Fig. S4B). Immunophenotypic analyses of cultures generated from CMP showed that dimerized cultures generated all three types of progenitors (CMP, MEP, and GMP). In comparison, control cultures contained GMP and CMP but few or no MEP (Fig. 4A, 4B, one representative experiment of three). These data suggest that F36VMpl dimerization of CMP promotes in vitro generation of MEP but not GMP.

F36VMPL dimerization generated a significantly higher proportion of GFP+ cells when compared with control cells.
c-Mpl Dimerization Induces Novel Erythroid Pathways

(Figs. 2H, 3B, p < .05), indicating that cells expressing the F36VMpl fusion protein were selectively expanded without a bystander effect on nontransduced cells. The selection advantage provided by dimerization was seen specifically in the CMP and MEP (but not GMP) generated from CMP (Fig. 4C) again demonstrating that dimerization results in selective proliferation of CMP and MEP but not GMP.

Figure 5. F36VMpl dimerization induces erythroid gene expression in CD34+ progenitors. Transduced common myeloid progenitor were cultured in a chemical inducer of dimerization (CID) for 7 days. CD34+ GFP+ cells were then sorted and subjected to qPCR. mRNA expression relative to the no CID arm is depicted. Beta actin, RPL13A, and HSP90 were used as housekeeping genes. *p < .05 for F36VMpl + CID versus controls. Abbreviation: CID, chemical inducer of dimerization.

F36VMpl Dimerization Enhances Cell Cycling and Survival

To further elucidate the mechanism underlying the F36VMpl dimerization induced increase in cell output from progenitors, cell cycle status and apoptosis were assessed in cultures initiated with transduced CMP and stimulated for 7 days in the presence or absence of CID F36VMpl dimerization of CMP significantly increased the proportion of CD34+ and total cells in cell cycle by Hoechst uptake (p < .05, n = 3 independent experiments; and confirmed with BrDU uptake; Fig. 3C). In addition, dimerization produced a significantly lower proportion of apoptotic cells (Fig. 3C, p < .05, n = 3 independent experiments).

F36VMpl Dimerization Induces Erythroid Gene Expression in CD34+ Progenitors

In view of the expansion of CD34+ progenitor numbers in association with induction of erythropoiesis, we next investigated whether F36VMpl dimerization upregulates erythroid gene expression at the CD34+ progenitor stage. CD34+GFP+ cells generated from CMP were isolated on day 7 from cultures with or without CID, and subjected to quantitative PCR. Mpl dimerization significantly upregulated expression of the erythroid transcription factors SCL and GATA1 [17], E-cadherin (CDH1) (a downstream target of erythropoietin) [18], and the erythropoietin receptor (EPOR) (Fig. 5, p < .05, n = 3 independent experiments). Interestingly, dimerization also significantly upregulated expression of VEGF (p < .05, n = 3 independent experiments), an anti-apoptotic factor for hematopoietic and erythroid progenitors [19, 20].

Erythropoiesis Is Induced by F36VMpl Dimerization but Not Tpo, and Is Independent of Epo

Tpo induces erythropoiesis in serum containing culture systems [21]. This erythroid effect of Tpo is dependent on the erythropoietin present in serum and is not seen in serum-free culture conditions [21]. Since gene expression analysis demonstrated that F36VMpl dimerization results in upregulation of the erythropoietin receptor gene (Fig. 5), we explored whether the erythropoiesis induced by Mpl dimerization was secondary to an increased sensitivity of dimerized cells to erythropoietin present in bovine fetal calf serum. Dimerization in serum-free conditions in the absence of stroma still resulted in erythroid differentiation (Fig. 6), proving that the erythropoiesis induced by F36VMpl dimerization occurs in the absence of erythropoietin. The effects of F36VMpl dimerization on CD34+ progenitors were next directly compared with those of Tpo (the natural ligand of full-length c-Mpl) in serum-free, stroma-free conditions. In contrast to F36VMpl dimerization, Tpo stimulation did not induce erythropoiesis (Fig. 6, n = 2 independent experiments).

When F36VMpl dimerization was compared with stimulation with Epo, no significant difference was seen in the frequency or number of Gly A+ cells in the CID, Epo, and CID+Epo arms (Supporting Information Fig. S3, n = 2 independent experiments).

F36VMpl Dimerization in CD34+ Progenitors Activates Novel Gene Networks Not Seen with Tpo Stimulation

Microarray analysis was performed to explore further the downstream gene networks activated by stimulation of progenitors through F36VMpl dimerization. CD34+GFP+ cells were isolated after 7 days of culture in either CID, Epo, or Tpo. Cells grown in the absence of either CID or growth factors served as negative controls (n = 3 independent experiments). Distinct expression profiles were noted in each of the three experimental arms (Fig. 7A). Specifically, of the genes differentially expressed relative to the negative control (p < .05), 675, 349, and 435 genes were unique to CID-, Epo-, and Tpo-treated cells, respectively (Fig. 7B).

In keeping with the enhanced cycling status of dimerized cells seen in culture (Fig. 3C), dimerization of CD34+ progenitors induced upregulation of cyclin D1 (CCND1). Consistent with the decreased apoptosis of dimerized cells, CID-treated cells showed upregulation of survival genes (Fig. 7A). In concordance with the erythropoiesis seen in culture, Mpl dimerization upregulated several genes associated with erythropoiesis (Fig. 7A), including spectrin genes (SPTB and SPTA1), pyruvate kinase liver and red-cell (PKLR), and hemoglobin subunits beta (HBB), delta (HBD), gamma-1 (HBG1).

Both Epo and CID upregulated genes involved in erythroid development (Fig. 7A). In contrast, CID-treated cells but not Epo-treated cells, showed upregulation of platelet associated genes. Interestingly, CID but not Epo-treated cells showed upregulation of TIMP1. In addition to being a platelet associated gene, TIMP1 is an erythroid promoting factor that acts on early erythroid progenitors and potentiates the erythropoietic effects of Epo [22–24]. Consistent with the
Figure 6. F36VMpl dimerization, and not thrombopoietin, induces erythropoiesis in the absence of erythropoietin. Transduced CD34+ cells were cultured in serum-free medium in the absence of erythropoietin, either with chemical inducer of dimerization or thrombopoietin (Tpo). Untransduced CD34+ cells were cultured in the presence of Tpo. Flow cytometry shows expression of erythroid cells (glycophorin A+) at day 7. Abbreviations: CID, chemical inducer of dimerization; Tpo, thrombopoietin.

lack of erythroid induction seen in culture, Tpo-treated CD34+ cells did not show upregulation of erythroid genes (Fig. 7A). Interestingly, genes associated with megakaryopoiesis, including TMP/1, were also not upregulated by Tpo at the CD34+ progenitor stage.

A network analysis of the differentially expressed genes in the dimerized progenitors showed enrichment for expression of genes involved in myelopoiesis, erythropoiesis, proliferation of blood cells, and red-cell apoptosis (Fig. 7C, p < 0.05). Network analyses of Tpo-stimulated cells did not show enrichment for these networks, providing further evidence that mechanisms underlying the effects of F36VMpl dimerization are distinct from those activated by Tpo (Supporting Information Fig. S6).

**DISCUSSION**

We have previously shown that F36VMpl dimerization of primitive CD34+CD38- cells with lymphoid potential expanded CD34+ progenitor cells and enhanced engraftment [2, 4], making it a potential strategy to improve hematopoietic reconstitution post-HSCT transplantation. Of note, this study also showed erythroid differentiation with F36VMpl dimerization. Studies before our own that used the F36VMpl approach with unfractinated CD34+ cells noted erythroid differentiation with no progenitor expansion [6]. The goal of the current study was to understand at which stage of differentiation F36VMpl dimerization acts to promote progenitor expansion and the mechanisms by which dimerization induces erythropoiesis. We conclude that F36VMpl dimerization in progenitors with myeloid potential (CMP and MEP) induces both CD34+ progenitor expansion and erythropoiesis. Further, we show that the erythropoiesis induced by dimerization is independent of the presence of erythropoietin and reflects molecular mechanisms that are distinct from stimulation of full-length c-Mpl by its natural ligand Tpo.

F36VMpl dimerization studies by Richard et al. [6] demonstrated erythroid differentiation without progenitor expansion. Even though Epo was not added exogenously, these studies [6] were performed in the presence of serum and thus did not show that the erythroid effect of dimerization was independent of Epo. Also, molecular mechanisms induced by dimerization in progenitors were not investigated. The contrasting effects on progenitor expansion in our studies and those by Richard et al. are unlikely to be explained by the stage of differentiation at which dimerization was induced. The former study [6] used the total population of unfractinated CD34+ cells, which contains the CMP and MEP subpopulations. It is more likely that differences in the conditions in which dimerization were induced in the former study, particularly the absence of stroma, reduced progenitor survival, and promoted differentiation.

Unlike Tpo, F36VMpl dimerization does not induce detectable phosphorylation of the signaling molecules STATS (signal transduction and transcriptional activation five) or JAK2 (Janus Kinase two) [25]. We did not detect phosphorylation of STAS or JAK2 in western blot assays of BaF3 (murine pro B cell leukemia cell line) cells that had been transfected with F36VMpl and cultured in the presence of CID (data not shown). In view of functional and signal transduction pathway differences between Tpo stimulation and F36VMpl dimerization, we used microarray to investigate differences in downstream gene expression between CD34+ progenitors undergoing Tpo stimulation or F36VMpl dimerization.

The current study demonstrated that F36VMpl dimerization affects multiple molecular pathways associated with proliferation, survival, and erythropoiesis and that these pathways are activated at the CD34+ progenitor stage. Importantly, there were marked functional and molecular differences between the effects of dimerizing the constitutively expressed intracellular domain of Mpl (through F36VMpl) and exposing cells that express endogenous Mpl to the native ligand Tpo. Several studies have demonstrated that Tpo enhances erythropoiesis [26-28]. However, all such studies examined the effect of Tpo in the presence of Epo or serum, which contains bovine Epo [21]. Consistent with studies from Kobayashi et al. [26], and Liu et al. [21], our experiments did not reveal any functional or molecular evidence that Tpo induces erythroid differentiation in the absence of Epo or in serum free conditions.

Interestingly, although Tpo is well known to be critical for megakaryocytopoiesis, platelet associated genes were upregulated in F36VMpl dimerized, but not Tpo stimulated CD34+ cells. However, it should be noted that studies showing upregulation of megakaryocytic genes in the presence of Tpo involved gene analysis studies in differentiated CD34+ megakaryocytes rather than in their CD34+ progenitors [29, 30]. However, Tpo stimulation of CD34+ progenitors did result in upregulation of
c-Mpl Dimerization Induces Novel Erythroid Pathways

A

B

C

Figure 7. P56V-Mpl dimerization induces a distinct gene expression profile. Transduced CD34+ cells were cultured in the presence of chemical inducer of dimerization (CID), thrombopoietin, Epo, or no treatment. CD34+ GFP+ cells were isolated on day 7 for microarray. (A) Selected genes up-regulated >2-fold with CID. (B) Numbers of genes differentially expressed in each condition compared with no treatment. (C) Network analysis of genes differentially expressed with CID. Abbreviations: CID, chemical inducer of dimerization; Epo, thrombopoietin; Tpo, thrombopoietin.

certain known downstream targets of c-Mpl signaling, for example, PIM1 and lipid synthesis genes [29, 30].

At least two possibilities may explain the disparate effects of Tpo and P56V-Mpl dimerization on erythropoiesis. The constitutive expression of intracellular Mpl in progenitors may allow signaling at progenitor stages that do not normally express the endogenous Mpl receptor and thus cannot respond to Tpo. Retroviral over expression of c-mpl in mouse hematopoietic progenitors led to enhanced erythroid differentiation [31]. However, the expression of c-Mpl in CMP and MEP [3] makes this a less likely explanation.

Alternatively, CID mediated dimerization of the intracellular domain of Mpl may result in conformational changes of the receptor that lead to signaling pathways that are different to those activated by Tpo induced dimerization of full-length Mpl. Native Tpo and Epo receptors contain extracellular, transmembrane and intracellular domains [32]. The intracellular part of c-Mpl and the Epo receptor contain conserved box1 and box2 domains, which are important for signaling and proliferation [5, 33]. The distal region (50 amino acids) of the intracellular part of c-Mpl is different from that of other cytokine receptors, and contains a domain that is important for differentiation effects [3]. In the case of Epo, binding to the two extracellular domains of the receptor induces specific conformational changes, which are then transmitted through the transmembrane domains to the cytosolic domains that mediate signaling pathways [34]. Differences in the efficiency and degree of downstream signaling pathway activation have been noted between the native full-length EpoR and mutant receptors that have an altered extracellular or transmembrane domain conformation [34]. In addition, synthetic extracellular domain binding ligands that induce conformational changes different from those induced by native Epo, result in differing degrees of signal transduction [35]. Similar conformation-dependent signaling mechanisms have been postulated for Tpo [32], but to our knowledge these have not yet been proven. The fusion protein P56V-Mpl contains 121 amino acids from the intracellular domain of c-Mpl, including the box1 and box2 domains, which are important for signaling [3]. However, it does not contain the extracellular or transmembrane domains of Mpl. Thus, we propose that differences between the conformations of P56V-Mpl and the full-length native Tpo receptor may account for the differences between the effects of P56VMpl dimerization and Tpo.

STEM CELLS
SUMMARY

In summary, this study demonstrates that intracellular Mpl dimerization induces progenitor expansion and erythropoiesis in human myeloid progenitors through molecular mechanisms that are not shared by binding of Tpo to native Mpl. The ability to regulate these unique functional effects in human hematopoietic progenitors could be potentially exploited for the enhancement of erythropoiesis in the cell therapy setting. Delineating these effects provides valuable insights into the effects of dimerization in specific hematopoietic progenitor populations. These data also emphasize how constitutive activation of a cytokine signaling domain can produce effects that are not predictable based on stimulation of the endogenous full-length receptor. Defining the mechanisms underlying these differences is essential for the development, characterization, and clinical translation of cytokine signaling domain-based gene therapy strategies.

ACKNOWLEDGMENTS

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REFERENCES


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The authors indicate no potential conflicts of interest.

See www.StemCells.com for supporting information available online.
Figure S1. Unstained samples of (a) untransduced and (b) transduced cells, for flow cytometry experiments described in Figures 2 and 3.
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Figure S2. In vivo dimerization of MEP results in higher short term engraftment. NSG mice were transplanted with transduced MEP and administered CID or control vehicle from day -1 to day 14. Table shows the maximum bioluminescence for each mouse (photons/ second). Flow cytometry plots for bone marrow engraftment of human erythroid (glycophorin A+) cell engraftment are depicted (2 representative mice).
Figure S3. F36VMpl transduced myelo-erythroid progenitors cultured with CID give rise to megakaryocytic cells, neutrophils and monocytes (in addition to erythroid cells).

Megakaryocyte (CD41a+GlyA-) cell counts of cultures (+/- CID) generated from transduced (a) CMP or (b) MEP. (one representative experiment of four shown, p<0.05). (c) Morphology of cells from day 35 of CID stimulated cultures of transduced CMP (Giemsa-Wright staining, 10x magnification).
Figure S4. Functional analysis of in vitro generated cells based on CD34 expression levels. Transduced CD34+ cells were cultured with CID. (a) Three populations of GFP+ cells were seen on day 7 of culture: CD34 negative, CD34 dim and CD34 bright. (b) CFU output from each GFP+ population shown in (a), isolated at day 7 of CID culture.
Figure S5. The erythropoietic effect of F36VMpl dimerization is not synergistic with that of erythropoietin. Transduced CD34+ cells were cultured in serum-free medium with CID or erythropoietin (Epo) or both for 7 days. Flow cytometry plots show generation of erythroid (glycophorin A+) cells.
Figure S6. Functional network analysis of genes differentially expressed in Thrombopoietin and Erythropoietin treated cells. Transduced CD34+ cells were cultured in the presence of CID (see figure 7), (a) Thrombopoietin, (b) Erythropoietin or Control (no growth factors or CID). CD34+GFP+ cells were then sorted at day 7 and subjected to microarray. Shown is Ingenuity Pathway Analysis of hematopoietic functional networks showing genes significantly changed (red up- and green down-regulated) compared with Control arm (p<0.05).
Table S1. Expression of housekeeping genes. CD 34+ cord blood cells were transduced with F36VMpl (or F36V) and cultured on MS-5 stroma +/- CID for 7 days. Equal numbers of CD34+GFP+ cells were sorted from culture and subjected to qPCR for housekeeping genes. C (T) values are depicted.

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<sup>a</sup> C (T) value
CHAPTER 4:

Alternative EPO-independent pathways for inducing erythropoiesis from human embryonic stem cells
Introduction

Our laboratory has demonstrated the expansion and erythroid differentiation of human multilineage hematopoietic progenitors from cord blood utilizing an inducible system in which a fusion protein, consisting of the intracellular domain of the receptor MPL and a drug binding domain F36V (F36V-MPL), is expressed in CD34+ cells via a lentiviral vector.\textsuperscript{1,2} Based on these findings, we set out to determine the ability of F36V-MPL to induce/enhance erythropoiesis from hESC and the mechanism of action. As the primary author, my specific role on this project was in the design and execution of all the experiments and subsequent data analysis (except for the western blot and HPLC analysis which were performed by Qiming Deng under Timothy Lane’s supervision and Amanda Grieco under Eric Bouhassira’s supervision, respectively).

Materials and Methods

Cell lines and culture media

The hESC line H1 (WiCell. Madison, WI) was maintained and expanded on irradiated primary Mouse Embryonic Fibroblasts (mEFs) (Millipore, Billerica, MA) in 6 well plates (Corning, Lowell, MA) in 3mL/well of hESC medium composed of DMEM-F12 (80\%v/v) with 20\% Knockout Serum Replacement (KSR) in 2 mM L-glutamine, 10 mM nonessential amino acids, 4 ng/ml bFGF (all from Invitrogen), and 50 mM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). hESCs between passage numbers 38 and 68 were used for all experiments and were daily supplemented with fresh medium and passaged onto new mEFs every 4-5 days using the StemPro EZ passage tool (Invitrogen). The OP9-M2 cell line, a subclone of the murine BM stromal line OP9 (a
generous gift from Dr. H. Mikkola, University of California, Los Angeles, Los Angeles, California) was used as a stromal support for hematopoietic differentiation of hESC. OP9-M2 were maintained in medium consisting of MEM-alpha+GlutaMAX (Invitrogen) supplemented with 20% Fetal Bovine Serum (FBS) (Thermo Scientific Hyclone, Logan, Utah) and 1X penicillin/streptomycin (Gemini Bio-Products, West Sacramento, CA). Ba/F3 cells were cultured in RPMI 1640 (Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum, 2mM L-glutamine, and 100U/mL penicillin/streptomycin (all from Gemini) with 10 ng/mL mIL-3 (Invitrogen) unless otherwise stated.

Stromal-based co-culture for hematopoietic differentiation and erythroblast induction

Transduced H1 hESC were plated on confluent layers of OP9-M2 stroma in OP9 medium consisting of MEM-alpha+GlutaMAX (Invitrogen) supplemented with 20% Fetal Bovine Serum (FBS) (Thermo Scientific Hyclone, Logan, Utah) and 1X penicillin/streptomycin (Gemini Bio-Products, West Sacramento, CA). 100nM AP20187 (CID) (Clontech, Mountainview, CA) was added every 3-4 days as indicated in each experiment, from day of plating (day 0) to day 14. After 14 days of culture, cells were harvested using sequential collagenase IV (Stem Cell Technologies, Vancouver, BC) and Trypsin (Invitrogen) digestion, for FACS analysis or Colony Forming (CFU) assay (Stem Cell Technologies). Megakaryocyte-Erythroid progenitors (MEP) were generated as follows to maximize cell output: 10ng/ml BMP-4 (R&D systems, Minneapolis, MN), 10ng/ml bFGF (Invitrogen), and 10ng/ml VEGF (R&D systems) were added to OP9 medium from Day 0-2 for mesoderm induction. Subsequently, half the medium was removed and replaced with OP9 medium containing Stem Cell Factor (SCF, 50 ng/ml),
Flt3 Ligand (FL, 25 ng/ml), IL-3 (5 ng/ml), Thrombopoietin (TPO, 25 ng/ml), and Erythropoietin (EPO, 2U/ml) (SFITE medium) (all from R&D Systems) for 11 days for hematopoietic induction. After 14 days of culture, cells were harvested using sequential collagenase IV (Stem Cell Technologies) and Trypsin (Invitrogen) digestion. Cells were then FACS sorted to isolate MEP based on GlyA+CD41a/42a+ and then replated on OP9-M2 with various factors as indicated in each experiment. 3 10uM LY294002 (Sigma-Aldrich. St. Louis, MO) and 200nM Akt inhibitor IV (EMD Millipore. Hayward, CA) were added where indicated. For erythroblast induction, transduced H1 hESC plated for 14 days on OP9-M2 (without cytokines) were harvested as above and cultured in suspension in serum-free medium StemSpan SFEM (Stem Cell Technologies) following a 2-step system (modified from Qiu et al 4): In the first step (amplification of progenitors), the cultures were supplemented with SCF (100ng/mL), Flt-3L (33.3ng/mL), IL-3 (13.3ng/mL), BMP4 (13.3ng/mL) and EPO (2.6U/mL) or CID (100nM) every 2-3 days for 7 days. In the second step (differentiation of progenitors to erythroid lineage), the cells were supplemented with SCF (40ng/mL), IGF-1 (40ng/mL), IL-3 (13.3ng/mL), BMP4 (13.3ng/mL) and EPO (3.3U/mL) or CID (100nM) every 2-3 days for 7 days.

Cell sorting and flow cytometry
FACS analysis was performed on LSRII or LSRFortessa and cell sorting on a FACSARia (Becton Dickinson, San Jose, CA) by direct immuno-fluorescence staining with human specific monoclonal antibodies. Non-specific binding was blocked with intravenous immunoglobulin (0.1%) (IVIG; Cutter, Berkley, CA) prior to staining with fluorochrome conjugated antibodies. Cell acquisition used FACSDivaTM (Becton Dickinson) and
analysis was performed using FlowJo (Tree Star, Ashland, OR). The following antibodies were used for staining; CD41a-PE, CD42a-PE, Glycophorin-A-APC, CD43-APC, VE-CAD-PE, CD31-PE, Annexin V-APC, anti-BrdU-APC (all from Becton Dickinson) and mCD29-Alexa 647 (AbD Serotec, Raleigh, NC). mCD29-Alexa 647 was used to gate out murine cells. Unstained cells were used as negative controls. Hoechst 33342 (Invitrogen) was used as a means to distinguish enucleated cells from nucleated cells. For analysis of cultured cells, the following gating strategy was used for all experiments: FSC/SSC, GFP+, with/without DAPI neg, with/without mCD29 neg gating.

*Protein Extraction and Western Blot Analysis*

Ba/F3 cells were starved overnight (~16hrs) in R10 medium without mIL-3. Cells were then treated accordingly for different lengths of time prior to cell lysis and protein extraction. Cells were pelleted and resuspended in RBC lysis buffer (0.5% NP40 150mM NaCl 50mM Tris pH 8.0) with Roche complete protease inhibitor tablets (EMD Millipore), and 1X Phosphatase Inhibitor Cocktail Set III (EMD Millipore). Protein extracts were resolved by SDS-PAGE and transferred onto Immobilon-FL PVDF membranes. Blots were blocked in LiCor Odyssey buffer and incubated with primary antibodies (all from Cell Signaling Technologies, Danvers, MA): p-JAK2 (3771S); B-Actin (3770S); p-AKT-Ser473 (587F11); p-AKT-Thr308 (2965S); pan-AKT (4685S); p-BAD (5284S). Secondary fluorescent antibodies were obtained from LiCor Biosciences, Lincoln, NE (goat anti-mouse IgG IRDye 800CW, goat anti-rabbit IgG IRDye 680LT). Finished blots were scanned with the LiCor Odyssey Imaging System.
Colony forming assay
Total cells from 14 days of hematopoietic induction as mentioned above were sequentially made into a single cell suspension and plated in MethoCult® GF +H4435 (Stem Cell Technologies) in 35 mm cell culture dishes (Nalgene Nunc, Rochester, NY). 150,000 cells (mixture of OP9 and human cells) were plated/culture dish in duplicate. 100nM CID was also supplemented to the assay where indicated. Hematopoietic colonies were counted on day 14 with an inverted Olympus CKX41 microscope.

HPLC for globin protein analysis
MEP (generated as described above in “Stromal-based coculture for hematopoietic differentiation and erythroblast induction”) were sorted and cultured on OP9 stroma for 14 days in OP9 medium +/- 2U/mL EPO or 100nM CID. GlyA+ cells were then collected using a FACSARia III (Becton Dickinson) and washed twice with PBS, and lysed in water by 3 rapid freeze-thaw cycles. Debris was eliminated by centrifugation at 16,000g and the lysates stored in liquid nitrogen before HPLC analysis. HPLC were performed as previously described.5

Quantitative RT-PCR for gene expression analysis
RNA from Erythroblasts generated as mentioned above in “Stromal-based coculture for hematopoietic differentiation and erythroblast induction” was extracted using a Qiagen Micro Kit (Qiagen, Valencia, CA). An omniscript reverse transcriptase kit was used to make cDNA, which was subjected to quantitative polymerase chain reaction (qPCR)
using TaqMan probe-based gene expression analysis assays (Applied Biosystems, Carlsbad, California) for Hs00362216_m1 (HBE), Hs01629437_s1 (HBG), Hs00758889_s1 (HBB), Hs01085823_m1 (GATA1), Hs00355782_m1 (CDKN1A), Hs01078066_m1 (Rb1), Hs00236329_m1 (BCL2L1), Hs00900055_m1 (VEGFA), Hs01060665_g1 (B-actin - VIC). Reactions were loaded onto a MicroAmp Optical 96-Wel Reaction Plate (Applied Biosystems), sealed with MicroAmp Optical Adhesive Film (Applied Biosystems) and amplified in a reaction volume of 20uL on a 7500 Real Time PCR system (Applied Biosystems). The data were analyzed using the comparative C(T) method.\textsuperscript{6}

\textit{Cell cycle and apoptosis assay}

Erythroblasts generated as mentioned above in “Stromal-based coculture for hematopoietic differentiation and erythroblast induction” were harvested and stained with Annexin V-APC and Propidium Iodide (PI) (both from Becton Dickinson) for apoptosis analysis and apoptotic cells scored as positive for either Annexin V or PI or both. Unstained cells were used to set negative gates. Erythroblasts were also incubated with bromodeoxyuridine (BrdU) for 30 or 120 minutes before harvest and analysis for cell cycle analysis. Harvested cells were fixed, permeabilized, and stained with 7-AAD and APC conjugated antibody to BrdU. Unstained cells were used to set negative gates and scored according to manufacture’s guidelines: apoptotic (7-AAD\textsuperscript{−}BrdU\textsuperscript{−}), G0/G1 (7-AAD\textsuperscript{low}BrdU\textsuperscript{−}), S-phase (7-AAD\textsuperscript{+}BrdU\textsuperscript{+}), and G2/M (7-AAD\textsuperscript{hi}BrdU\textsuperscript{−}).

\textit{Statistical analysis}
Prism version 5 (GraphPad Software Inc) was used for statistical analysis. Results are presented as the average value of at least three experimental repeats (indicated accordingly) +/- standard deviation. Unpaired Student’s t-test was utilized and P values <0.05 were considered statistically significant.

Results

*Dimerization of intracellular MPL induces erythropoiesis from human embryonic stem cells*

To determine if dimerization of ic-MPL induces erythropoiesis in human embryonic stem cells (hESC), the H1 cell line was stably transduced to express either the F36V-MPL fusion protein (See Chapter 2) or a control vector expressing only the ligand binding domain, F36V. GFP+ hESC were plated onto the murine stromal line OP9 and cultured without exogenous cytokines for 14 days, in the presence or absence of the synthetic ligand AP20187 (aka chemical inducer of dimerization, CID) which binds to F36V and induces homodimerization of the fusion protein. In the absence of CID, erythroid cells were barely detectable in these conditions. However, CID treated cultures consistently generated a population of erythroid cells, producing significant increases in both frequency (P=0.0001; n=5; Figure 1A,B) and cell number (P=0.003; n=5; Figure 1C) of glycophorin (GlyA)+ cells. CID also induced erythropoiesis in serum-free medium (Supplementary Figure 1 and 2). Thus dimerization of ic-MPL through the F36V-MPL fusion protein was sufficient to induce erythropoiesis from hESC in the absence of any exogenous cytokines, in particular without EPO, a critical regulator of normal erythroid proliferation and differentiation. Notably, erythropoiesis from hESC was significantly greater in the presence of CID than with EPO (P=0.015, n=5, Figure
1D,E) while the addition of CID to EPO further enhanced production of GlyA+ cells (P=0.018; n=5; Figure 1D,E) indicating that ic-MPL dimerization may act through a different pathway to EPO to induce erythropoiesis.

Dimerization of ic-MPL induces the generation of erythroid progenitors

The number of total cells generated from hESC was not significantly different between treated and untreated cultures (Figure 1F). CID treatment also had no detectable effect on proliferation or differentiation of undifferentiated hESC (data not shown). Erythropoiesis proceeds from the undifferentiated hESC stage by progressing through key transition points of increasingly restrictive lineage specification events, beginning with the onset of mesoderm specification prior to hemato-endothelial commitment, further lineage restriction to a bi-potent megakaryocyte-erythroid progenitor (MEP) and ultimately to the generation of erythroid cells. Further analysis of cultures generated from hESC revealed that ic-MPL dimerization increased the frequency of hematopoietic progenitor cells that express CD43 (a marker of hematopoiesis that appears early in culture), but had no significant effect on the generation of endothelial cells (based on VE-cadherin or CD31 expression) (Supplementary Figure 3). To determine if ic-MPL dimerization also induced the generation of clonogenic hematopoietic progenitors, cells from day 14 stromal co-cultures (cultured ±CID) were re-plated onto a standard CFU assay containing hematopoietic cytokines (including EPO but no CID). Cultures that were initially treated with CID prior to CFU plating generated 10-fold more erythroid colonies (BFU-E and CFU-E) compared to untreated controls (P=0.0007; n=15; Figure 2A). In contrast, the
addition of CID did not affect the generation of clonogenic myeloid progenitors (Figure 2B), suggesting that the effect of ic-MPL signaling was specific to the erythroid lineage.

To further explore the stage of differentiation induced with ic-MPL dimerization, Day 14 cultures were analyzed by FACS for the presence of bipotent megakaryocyte-erythroid progenitors (MEP) previously described as co-expressing CD41a and GlyA along with CD34 and CD43. MEP were induced upon CID treatment (P=0.025; n=4) similar to EPO treatment (P=0.0025; n=4), with combined treatment further significantly enhancing MEP output in an additive manner (P=0.003; n=4) (Figure 2E,F; Supplementary Figure 4). The majority of MEP also expressed CD34 (Supplementary Figure 5). These data indicate that ic-MPL dimerization specifically induces the generation of early clonogenic progenitors with erythroid potential from hESC.

*Dimerization of ic-MPL in MEP induces greater terminal erythroid proliferation and differentiation than EPO*

The effect of ic-MPL dimerization on differentiation downstream from the MEP stage was next studied. MEP generated in the absence of CID were isolated at Day 14 by FACS and differentiated in the presence or absence of either Thrombopoietin (TPO), EPO, or CID (Figure 3A-C). Total cell number tended to be higher in CID than either EPO or TPO (Figure 3D). As expected, TPO generated significantly more megakaryocytes (P=0.014; n=3; Figure 3C,E,F) whereas EPO induced more erythroid cells (P=0.026; n=3; Figure 3C,G,H) from MEP than non-treated controls, consistent with the lineage specificity of these cytokines and demonstrating the bipotency of the MEP population. Notably, CID induced a ~40-fold increase in erythroid differentiation
from MEP compared to non-treated controls (P=0.0004; n=3; Figure 3G).

Megakaryocyte frequency and number also tended to be greater with CID, although the increase was not statistically significant (Figure 3E,F). Thus dimerization of ic-MPL produced qualitatively different effects than stimulation of full-length MPL with its natural ligand TPO, primarily inducing erythroid rather than megakaryocytic differentiation.

To further assess the effect of CID on erythroid production from MEP, Day 14 MEP generated without CID were sorted and plated into CFU assay containing EPO or EPO supplemented with CID. As expected, MEP generated mostly erythroid colonies in both conditions; however combined EPO and CID stimulation of MEP generated larger colonies (typical of BFU-E), whereas only small CFU-E were generated in the presence of EPO alone (P=0.0462, n=3; Figure 2G). The number of myeloid colonies (CFU-GM, and CFU-M) generated from MEP was no different between the EPO and CID cultures (Figure 2H) further indicating that ic-MPL induces a strong and specific erythroid effect from the MEP stage.

Next, the ability of CID and EPO to drive terminal erythroid differentiation was compared. CID treatment generated a 4-fold increase in the number of enucleated RBCs (n=4; Figure 4A-C) and more visually prominent hemoglobinization (Figure 4D) compared to EPO. RBCs generated in the presence of CID also gave higher expression of both gamma (fetal) and beta (adult) globin at the transcriptional level (Figure 4E). Globin protein analysis by HPLC demonstrated an increase in alpha-globin expression with CID treatment compared to EPO (Figure 4F; Supplementary Figure 6). Also, although no detectable beta-globin expression was observed with EPO, CID treatment induced the presence of low levels of beta-globin and increased the gamma:epsilon
globin ratio, suggesting a shift toward definitive erythropoiesis (Figure 4G; Supplementary Figure 6). Thus, MPL dimerization is more potent than EPO in the production of erythroid cells and in inducing terminal erythroid differentiation.

*Dimerization of ic-MPL requires AKT signaling for erythroid induction*

Signaling studies require large cell numbers making it challenging to investigate multiple pathways in primary cell populations where availability can be a limiting factor. Ba/F3 cells, a pro-B cell leukemia cell line (Ba/F3), offers a solution to limited cell availability as they can be expanded very easily and remain fairly homogenous throughout many passages. The ease in cell maintenance and homogeneity along with its dependence on IL-3 for growth and survival make the Ba/F3 cell system an ideal model for signaling studies. The IL-3 dependence allows for the introduction of any receptor/ligand of interest along with the subsequent removal of IL-3 and the addition of the respective ligand to measure IL-3 independent growth/survival in culture. Once IL-3 independent growth/survival is detected, the cells can then be processed to identify the signaling pathway(s) responsible.

To identify the signaling mechanism(s) activated by ic-MPL dimerization, phosflow and western blot analysis was performed on Ba/F3 cells transduced with either F36V (control vector), F36V-MPL or wild type full length MPL. IL-3 independent growth/survival was achieved with ic-MPL dimerization in cells expressing F36V-MPL but not the control F36V (data not shown) indicating the ligand specificity and functionality of the system. As expected, TPO stimulation of Ba/F3 cells expressing wild type MPL activated phosphorylation of AKT and STAT5/JAK2 (Figure 5A,B), as well as
p38, and ERK-MAPK (data not shown). Interestingly, unlike the full-length MPL receptor, ic-MPL dimerization with CID activated only the AKT pathway; no phosphorylation of STAT5, JAK2, ERK1/2 or p38 was detected (Figure 5A,B; data not shown). Further evidence that ic-MPL dimerization activates AKT signaling was shown by blocking of AKT phosphorylation in the presence of PI3K inhibitors (LY294002 and Wortmannin) and an AKT specific inhibitor (AKT inhibitor IV) (Figure 5C). In addition, ic-MPL dimerization induced phosphorylation of BAD (Figure 5B), a pro-apoptotic protein which is a downstream target of AKT signaling.

The functional relevance of AKT signaling in hESC-derived erythropoiesis was next explored. Treatment with the PI3K inhibitor LY294002, during 14 days of CID stimulation from hESC significantly reduced the number of GlyA+ cells (P=0.02; n=5; Figure 6A,B) and clonogenic erythroid progenitors (P<0.0001; n=6; Figure 6C). PI3K inhibition also significantly reduced the number of GlyA+ cells generated from isolated MEP (P=0.021; n=4; Figure 6D,E) as did treatment using AKT inhibitor IV (P=0.0009; n=4; Figure 6D,E). In the same conditions, the frequency of CD41a/CD42a+ megakaryocytic cells did not change, indicating that AKT inhibition specifically inhibited erythropoiesis (Figure 6F). Notably, treatment with the JAK2 inhibitor, AG490, reduced megakaryocyte output in the presence of TPO, but had no effect on GlyA+ output in the presence of CID or EPO (data not shown). These results indicate that AKT activation is required for the erythropoiesis induced in hESC by ic-MPL dimerization while JAK2/STAT5 is not.

*ic-MPL induced erythropoiesis is mediated through modulation of cell cycle and survival.*
AKT is a key downstream effector of PI3K and plays a major role in mediating cell cycle, survival, and differentiation.\(^{14}\) To assess the effect of ic-MPL dimerization on apoptosis and cell cycle of erythroid cells, GlyA+CD71+ erythroblasts were generated from hESC for analysis.\(^{10}\) A significantly higher frequency of CID treated erythroblasts were in G0/G1 (P=0.007, n=4) and correspondingly fewer cells were in S-phase (P=0.017, n=4) compared to EPO treatment (Figure 7A). The expression of the cell cycle regulators, \(CDKN1A\)^{15} and \(RB1\)^{16,17}, was also upregulated with CID treatment compared to EPO (Figure 7D). Apoptosis in CID treated erythroblasts was significantly reduced (P=0.005, n=4, Figure 7B; P=0.003, n=4, Figure 7C) compared to EPO treatment. Consistent with reduced apoptosis, CID stimulation phosphorylated BAD\(^{12}\) (Figure 5B) and upregulated expression of \(BCL-xL\) (P=0.004, n=3) and \(VEGFA\) (P=0.008, n=3) (Figure 7D), both of which have been shown to inhibit apoptosis.\(^{18-21}\) Notably, CID treatment upregulated \(GATA1\) expression to significantly higher levels than EPO treatment (Figure 7D). GATA1, which is a key regulator of erythroid development essential for the survival of erythroid precursors and their terminal differentiation, is targeted by PI3K/AKT for phosphorylation and activation.\(^{14}\) Taken together, these results strongly suggest a cell cycle/survival regulatory role for ic-MPL in hESC-derived erythropoiesis through the activation of AKT and GATA1 (Figure 7E).

**DISCUSSION**

In these studies we have shown that ic-MPL dimerization during hESC differentiation induces the generation of MEP and their erythroid progeny in an EPO-independent manner. Unlike either TPO or EPO stimulation, ic-MPL dimerization
induces activation of the AKT signaling pathway, without concurrent activation of JAK2/STAT5 signaling. Furthermore, we show that ic-MPL dimerization activates a network of genes associated with the regulation of cell cycle, apoptosis, and erythroid differentiation, with a corresponding reduction in both apoptosis and cell cycle progression. The consequence of these combined events is to promote RBC production and maturation from hESC significantly more efficiently than EPO.

EPO induced erythropoiesis from hESC is believed to resemble primitive (embryonic yolk sac) rather than definitive (fetal and adult) erythropoiesis, with the majority of erythroid colonies comprising small CFU-E rather than the larger BFU-E and consisting of few if any mature enucleated RBCs.\textsuperscript{3,4} In our studies, MEPs stimulated by ic-MPL dimerization generated large numbers of BFU-Es, and increased the production of enucleated RBCs relative to EPO stimulation, suggesting that ic-MPL induces a shift toward a more mature, definitive state of erythropoiesis. This hypothesis is further strengthened by an increase in fetal (gamma) globin production relative to embryonic (epsilon) globin production, and the detection of adult beta-globin exclusively in CID stimulated cells. The ability of ic-MPL to increase both output and maturation of erythroid cells provides a promising step toward the ultimate goal of generating therapeutically relevant RBCs for transfusion.

Although activation of full length MPL by its ligand TPO has been clearly demonstrated to induce megakaryocytopoiesis, several studies have suggested that TPO also enhances erythropoiesis.\textsuperscript{22-25} However, in all these studies hematopoietic progenitors were stimulated with TPO in the presence of EPO and/or serum (which contains EPO). We and others have found that TPO alone does not stimulate
erythropoiesis in conditions that lack EPO,\textsuperscript{2,26} whereas F36V-MPL is sufficient for erythropoietic induction. Consistent with this dichotomy, we find in this current study that CID induced ic-MPL dimerization does not activate the same set of signaling pathways normally activated by TPO signaling; instead of activating MAPK, JAK/STAT, and AKT signaling, only AKT signaling was detectable with F36V-MPL dimerization. F36V-MPL dimerization in CD34+ cord blood progenitors also induced gene expression profiles distinct from stimulation with either TPO, activating genes associated with erythropoiesis.\textsuperscript{2} These observations further highlight the distinctness and novelty of F36V-MPL signaling.

A fusion protein consisting of the intracellular and transmembrane domain of MPL and the extracellular domain of the Prolactin receptor (PRL-R) has been shown to induce hematopoiesis from murine embryonic stem cells (mESC).\textsuperscript{25} In contrast to our studies, the effect of the PRL-R fusion protein was not erythroid specific as output of both myeloid and erythroid progenitors was increased and the study did not report any effect on erythroid maturation.\textsuperscript{25} A notable difference between the PRL-R and ic-MPL systems is in the method by which MPL is activated: induction of homodimerization through ligand binding to the extracellular PRL-R in contrast to direct homodimerization of the intracellular domain of MPL.

TPO and EPO receptors are both composed of extracellular, transmembrane, and intracellular domains, each component contributing to the ultimate conformational arrangement of the receptor.\textsuperscript{27} For example, upon activation and dimerization of the EPO receptor (EPO-R), the two extracellular domains of EPO-R induce conformational changes that are transmitted through the transmembrane domain to the intracellular
domain which then mediates various signaling pathways. Alterations in the transmembrane or extracellular conformation of EPO-R or replacement with a synthetic extracellular domain has been shown to confer differences in downstream signaling pathway activation. Similarly, changes in tryptophan 515 in the intracellular juxtamembrane boundary of the MPL receptor was shown to influence the angle and dimerization of the transmembrane helix ultimately affecting receptor activation and signal transduction in different ways depending on the amino acid substitution. We postulate that direct dimerization of the intracellular fragment of MPL through the binding of CID to F36V-MPL (which lacks the extracellular and transmembrane domain) results in a conformational arrangement of the signaling domain that is distinct from that induced when activation of the transmembrane and intracellular domains of MPL occurs after ligand binding to an extracellular receptor. These conformational changes still allow AKT activation, but in the context of absent or severely reduced JAK2/STAT5 activation, producing a unique pattern of gene expression and cellular function.

JAK2/STAT5 signaling is activated by both EPO and TPO stimulation of their respective receptors and is essential for TPO mediated megakaryocytopenesis. The lack of JAK2/STAT5 in F36V-MPL signaling provides a potential explanation as to why CID induced MPL activation had little effect on megakaryocyte differentiation in our studies. Although CID did induce a moderate increase in total megakaryocyte output from MEP, this was primarily due to an overall increase in cell numbers rather a specific megakaryocyte specific effect by ic-MPL dimerization. Notably, EPO mediated JAK2/STAT5 activity appears to be required primarily for stress induced erythropoiesis as erythroid differentiation was seen in STAT5a−5b− mice during normal
homeostasis,\textsuperscript{32,34,35} Contrary to JAK2/STAT5, the PI3K/AKT pathway is critical for erythropoiesis\textsuperscript{36,37}, mediating erythroid-cell maturation of JAK2-deficient murine fetal liver progenitor cells.\textsuperscript{38}

ic-MPL dimerization resulted in upregulation of \textit{GATA-1}, a key regulator of erythroid development which is essential for terminal differentiation, including globin gene expression\textsuperscript{39}. PI3K/AKT stimulates the phosphorylation and activation of GATA1\textsuperscript{14}, andactivated GATA-1 upregulates its own gene expression through positive feedback regulation\textsuperscript{40}. Thus erythroid maturation produced by ic-MPL induced AKT signaling may be at least partly mediated by both GATA-1 activation and up-regulation. GATA1 has also been shown to upregulate the expression of \textit{CDKN1A} and \textit{RB1} which are cell cycle inhibitors inducing G0/G1 arrest.\textsuperscript{15} RB1 is required for erythroid differentiation in a cell intrinsic manner and forms a complex with GATA-1 to stall cell proliferation and steer erythroid precursors towards terminal differentiation,\textsuperscript{16,17} highlighting the importance of cell cycle regulation in erythroid differentiation. Along with regulation of cell cycle during erythropoiesis, GATA1 has also been shown to be critical for the survival of erythroid precursors, primarily by the upregulation of the anti-apoptotic factor \textit{BCL-xL}.\textsuperscript{18} In our studies, expression of the genes associated with the AKT/GATA-1 dependent cell cycle/survival network was significantly greater upon ic-MPL dimerization than with EPO. Notably, \textit{VEGF} was upregulated by ic-MPL in both hESC in the current studies and our previous report using cord blood progenitors.\textsuperscript{2} \textit{VEGF} is also upregulated by AKT signaling and has also been implicated as an anti-apoptotic factor for erythroid progenitors.\textsuperscript{20,21} Thus, the upregulation of these genes after ic-MPL induced AKT activation, along with a corresponding reduction in cell cycle and apoptosis strongly
suggests a central role for cell cycle/survival mechanisms in the erythroid effects of ic-MPL, and again strongly suggests this regulation is mediated primarily through AKT signaling.

**Figure 1. Dimerization of ic-MPL induces erythropoiesis from hESC.** H1 hESCs were transduced with F36V-MPL or F36V (control vector) and cultured on murine OP9 stroma for 14 days with or without CID or EPO (no other cytokines added). After 14 days of culture, the cells were counted and analyzed by flow cytometry for generation of erythroid (GlyA+) cells. **A**) Representative immunophenotype of F36V-MPL transduced cells cultured only with CID (day 14, gated on GFP+ human [murine CD29-] cells). **B-F**) Summary of FACS data from Day 14 cultures (n=5 experiments). **D-F**) FACS data is shown from F36V-MPL expressing cells only. *P<0.05, **P<0.01, ***P<0.001. GlyA+ cell number was calculated by multiplying the total cell count with the total GlyA+ percentage represented in each experiment. **F**) Total number of DAPI-mCD29-GFP+ cells from Day 14 culture (n=3 experiments).
Figure 2. Dimerization of ic-MPL induces the generation of erythroid progenitors.

H1 hESCs were transduced with F36V-MPL or F36V (control vector) and cultured on OP9 stroma for 14 days with or without CID and/or EPO. After 14 days of culture, the cells were counted and (A-D’) replated in methylcellulose to measure CFU or (E-F) analyzed by FACS. A) number of erythroid colonies (BFU-E and CFU-E) and B) myeloid colonies (CFU-GM and CFU-M) per 150,000 cells (n=15 experiments; ***P<0.001). C) CFU-E and D) BFU-E were both scored as ‘erythroid colonies’ in A. D’) shows GFP expression of BFU-E shown in D. E, F) Summary of FACS data (n=3 experiments), shown as E) frequency of MEP (%GlyA+CD42a/CD42a+) and F) number of MEP; Fold increase is relative to no agent control. G, H) After 14 days of hematopoietic differentiation without CID, GlyA+CD41a/42a+ MEP were FACS sorted and then cultured in CFU assay containing EPO ± CID for 14 days. G) Number of erythroid colonies (BFU-E and CFU-E) and H) myeloid colonies (CFU-GM and CFU-M) per dish per 10,000 cells plated are shown (n=3 experiments; *P<0.05).
Figure 3. Dimerization of ic-MPL induces robust erythroid differentiation from MEP. A) Experimental Schema. Hematopoietic differentiation was induced from F36V-MPL transduced hESC on OP9 stroma Day 0-2 in BMP4, VEGF, and FGF and then Day 3-14 in SCF, Flt-3L, IL-3, TPO, and EPO. GFP+ MEP were isolated by FACS on day 14 and cultured on OP9 stroma for a further 7 days (i.e. total 21 days of culture) in the presence of either TPO, EPO, CID or no agent (Control) and then analyzed by FACS. B) Representative FACS sort gating for isolation of MEP Day 14. C) Representative FACS analysis Day 21. D) Total number of DAPI-GFP+ cells from Day 21 culture (n=3 experiments). E-H) Summary of FACS data at Day 21 shown as: E,F) % and number of megakaryocytes (GlyA-CD41a/CD42a+); G,H) % and number of erythroid (GlyA+CD41a/42a-) cells (n=3); *P<0.05, **P<0.01, ***P<0.001.
Figure 4. Dimerization of ic-MPL induces more efficient erythroid maturation than erythropoietin. A-C) Erythroid enucleation, D) hemoglobinization, and E-F) Globin expression were analyzed after differentiation of isolated MEP in either EPO, CID or neither (Control). MEP were initially generated after 14 days without CID and then cultured in the three conditions shown for a further 7 days (see schema Fig 3A). A) Representative FACS analysis of Hoechst and GFP expression to assess enucleation of erythroid cells (from GlyA+ gated cells). B,C) Summary of FACS data measuring enucleated erythroid cells (% Hoechst\textsuperscript{neg} cells within GlyA+ gate) (n=4). *P<0.05, **P<0.01. D) Light microscopy of Day 21 stromal co-culture showing large, hemoglobinized clones only in CID culture. E) Relative mRNA of globins HBE (embryonic), HBG (fetal), and HBB (adult) by q-PCR. B-actin was used as a housekeeping gene (n=3). ***P<0.001. HPLC results shown as F) % alpha-globin expression relative to total globin (beta, alpha, gamma, epsilon) and G) % expression from beta-locus (beta, gamma, epsilon).
Figure 5. Dimerization of ic-MPL activates p-AKT but not pSTAT5/JAK2 signaling.
Signal transduction analysis of Ba/F3 cells expressing F36V-MPL (FM), wild-type (full length) human MPL (wt-MPL), or F36V (F) transgenes. Cells were cytokine starved overnight prior to stimulation with IL-3, TPO, CID or no agent (control), after which the cells were processed for A) phosphoflow and B) western blot analysis. A) Representative FACS analysis of pAKT (S473) and pSTAT5. The time point of maximal activation is shown for each agent: CID 60min, TPO 60min, and IL-3 30min. B) Representative Western Blot analysis of pAKT (S473 and T308 residues), pan-AKT (control), pJAK2, and pBAD (S112). C) Cells were stimulated with CID for 60min +/- inhibitors as shown. Representative FACS analysis for pAKT (S473).
Figure 6. Induction of erythropoiesis from hESC by ic-MPL dimerization requires PI3K/AKT activation. A-D) F36V-MPL transduced hESC were cultured on OP9 stroma in hematopoietic induction medium from Day 0-14 with or without CID (no cytokines added). From Day 7-14, cells were also treated ± PI3K inhibitor (LY294002). Day 14 cells were processed for A,B) FACS analysis and C) CFU assay. A,B) %GlyA+ and number GlyA+ cells respectively (n=5). C) Total number of erythroid colonies per 150,000 cells plated (n=6). D-F) Day 14 MEP generated from F36V-MPL transduced hESC were isolated by FACS sorting for a further 7 days of culture (i.e. total 21 days of culture) in the presence of SCF, Flt-3L, IL-3, TPO, ± CID along with either LY294002 or AKT inhibitor IV and then analyzed by FACS. D-F) Summary of Day 21 FACS data (n=4) shown as: D) % and E) number of erythroid cells (GlyA+CD41a/CD42a-); F) % megakaryocytes (GlyA-CD41a/CD42a+). *P<0.05, ***P<0.001.
Figure 7. ic-MPL induced erythropoiesis is mediated through modulation of cell cycle and survival. A-D) F36V-MPL transduced hESC were cultured on OP9 stroma in hematopoietic induction medium Day 0-14 without morphogens or cytokines, and then in liquid expansion culture Day 14-28. All analyses are from Day 28. A) Cell cycle analysis: G0/G1 (BrdU\(^{neg}\)7AAD\(^{dim}\)), S-phase (BrdU\(^{pos}\)7AAD\(^{dim}\)), and G2/M cells (BrdU\(^{neg}\)7AAD\(^{hi}\)). B) % Apoptosis based on BrdU\(^{neg}\)7AAD\(^{neg}\). C) % Apoptosis based on Annexin V and PI (n=4). Relative expression of genes shown by q-PCR in CID normalized to EPO. B-actin was used as a housekeeping gene. E) Proposed model of ic-MPL induced erythropoiesis mediated through AKT signaling. *P<0.05, **P<0.01 ***P<0.001.
Supplementary Fig 1. ic-MPL induced erythropoiesis from undifferentiated hESC is serum independent. Hematopoietic differentiation was induced from F36V-MPL transduced hESC by culturing on OP9 stroma Day 0-2 in STEMSPAN (serum-free) supplemented with BMP4, VEGF, and FGF and then from Day 3-14 in SCF, Flt-3L, IL-3, and TPO +/- EPO and/or CID. Immunophenotype analysis of GlyA expression.
Supplementary Fig 2. ic-MPL induced erythropoiesis from MEP is serum independent. Hematopoietic differentiation was induced from F36V-MPL transduced hESC by culturing on OP9 stroma Day 0-2 in BMP4, VEGF, and FGF and day 3-14 in SCF, Flt-3L, IL-3, TPO, and EPO. GFP+ MEP were isolated by FACS sorting on day 14 (see Figure 3) and recultured on retronectin with STEMSPAN (serum-free) medium +/- EPO or CID for 10 more days prior to analysis. A) Light microscope EPO vs CID after 10 days of culture on retronectin with STEMSPAN. B) Immunophenotype analysis of GlyA and CD41a/42a expression. C) Immunophenotype analysis for Hoechst staining of cells in GlyA+ gate.
Supplementary Fig 3. Dimerization of ic-MPL increases generation of early hematopoietic cells but not endothelial cells. H1 hESCs transduced with F36V-MPL were cultured on OP9 stroma for 14 days with or without CID (no cytokines added), and then counted and analyzed by FACS. Summary of Immunophenotype data shown as: A) % and number of CD43+ cells (n=3); B) % and number of VE-cadherin (VE-CAD)+ cells (n=5); C) % and number of CD31+ cells (n=3). % for each represents the frequency from all GFP+ cells. *P<0.05.
Supplementary Fig 4. ic-MPL dimerization induces the generation of MEP. F36V-MPL transduced hESC were cultured on OP9 stroma for 14 days with or without EPO or CID (no other cytokines). After 14 days of culture, the cells were counted and analyzed by FACS. Representative FACS analysis of CD41a/42a and GlyA expression performed on F36V-MPL transduced cells.
Supplementary Fig 5. MEP are predominantly CD34+. Hematopoietic differentiation was induced from F36V-MPL transduced hESC by culturing on OP9 stroma Day 0-2 in BMP4, VEGF, and FGF and Day 3-14 in SCF, Flt-3L, IL-3, TPO, and EPO prior to flow cytometry analysis. **A)** Immunophenotype analysis of GlyA and CD41a/42a expression performed on F36V-MPL transduced cells. **B)** Immunophenotype analysis for CD34 expression of cells in MEP and Eryth gates shown in A.
Figure 6. ic-MPL induces a shift toward fetal and adult globin expression. MEP were initially generated after 14 days of hematopoietic differentiation without CID and then cultured in EPO or CID for a further 7 days (see schema Fig 3A). HPLC results shown for A) EPO vs B) CID treatment.
REFERENCES


CHAPTER 5:

Concluding Remarks
In summary, we have identified a novel EPO-independent approach that is not only more efficient at erythropoiesis but is also able to augment EPO-induced erythropoiesis. The key role for AKT signaling presented here highlights its importance for further study in erythropoiesis and presents AKT as a target for specifically enhancing erythropoiesis, opening up new approaches toward the generation of therapeutically-relevant quantities of RBCs for transfusion. Further work is still required to achieve therapeutic quantities of adult globin expressing, enucleated RBCs that are functionally equivalent to their in vivo counterparts in half-life, membrane surface potential, pliability, gas exchange properties, and immunogenicities before in vitro generated RBCs can make their way into the clinic. It will also be important to test the ability of F36V-MPL to induce erythropoiesis using other established protocols; particularly methods that do not require feeders or other animal products which are unsuitable for clinical applications.

The work presented here also promotes the F36V system as a platform for detailed studies into hematopoietic growth factor signaling mechanisms, namely the importance of different receptor domains for regulation of various downstream signaling molecules and components. In this study, we found that dimerization of ic-MPL is able to induce the activation of AKT signaling without the need for JAK2 activity, which is normally required for WT full-length MPL receptor activity. It would be interesting to identify the precise mechanism of how AKT is being activated in our system which can potentially shed new light into AKT regulation. Such studies would have broad implications beyond erythropoiesis, especially to studies in cancer biology which has
recently found the PI3K/AKT pathway to play a prominent role in cancer cell growth and survival and aid in the effort to develop new AKT inhibitors as cancer therapies.\(^3\)

REFERENCES

