PULSED INDUCTION, A METHOD TO IDENTIFY GENETIC REGULATORS OF DETERMINATION EVENTS

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PULSED INDUCTION, A METHOD TO IDENTIFY GENETIC REGULATORS OF DETERMINATION EVENTS

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Abstract: Determination is the process in which a stem cell commits to differentiation. The process of how a cell goes through determination is not well understood. Determination is important for proper regulation of cell turn-over in tissue and maintaining the adult stem cell population. Deregulation of determination or differentiation can lead to diseases such as several forms of cancer. In this study I will be using microarrays to identify candidate genes involved in determination by pulse induction of mouse erythroleukemia (MEL) cells with DMSO and looking at gene expression changes as the cells go through the early stages of erythropoiesis. The pulsed induction method I have developed to identify candidate genes is to induce cells for a short time (30 min, 2 hours, etc.) and allow them then to grow for the duration of their differentiation time (8 days). For reference, cells were also harvested at the time when the inducer is removed from the media. Results show high numbers of genes differentially expressed including erythropoiesis specific genes such as GATA1, globin genes and many novel candidate genes that have also been indicated as playing a role in the dynamic early signaling of erythropoiesis. In addition, several genes showed a pendulum effect when allowed to recover, making these interesting candidate genes for maintaining self-renewal of the adult stem cell population.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

Determination is the process in which a stem cell commits to differentiation. The process of how a cell goes through determination is not well understood. Determination is important for proper regulation of cell turn-over in tissues and maintaining the adult stem cell population. Deregulation of determination or differentiation can lead to diseases such as several forms of cancer. Furthermore, in order to use stem cells safely in clinical situations, such as induced pluripotent stem cells (IPSCs), it is critical that all elements of the differentiation and determination mechanisms are fully understood. By taking somatic cells from a patient and turning them into IPSCs then redifferentiating them into the tissue type desired, the tissue can be placed back into the patient with little fear of rejection because the tissue originally came from the patent. To further understand how determination is regulated, I will be focusing on erythropoiesis, which is the formation of red blood cells as a model system.

Hematopoiesis

Hematopoietic stem cells (HSCs) are multipotent adult stem cells that give rise to blood cell types such as erythrocytes (red blood cells). Hematopoietic stem cells are important to maintain and replace dead or damaged cells in the immune system, platelets for clotting of blood and erythrocytes for oxygen transport. Hematopoiesis is broken up into 2 major pathways (Figure 1.1) the leukogenic pathway which gives rise to T-cells and B-cells and the myogenic pathway which gives rise to erythrocytes, platelets, macrophages and other cells associated with the immune system. The hierarchy of hematopoietic stem cells can also be described using molecular markers (Table 1.1) (Seita and Weissman 2010). Hematopoietic stem cells are also plastic, meaning they can be changed into various non-blood cell types such as adipocytes (Sera, LaRue et al. 2009), pancreatic cells (Minamiguchi, Ishikawa et al. 2008), endothelial cells (Elkhafif, El Baz et al. 2011), cardiomyocytes (Pozzobon, Bollini et al. 2010), osteochondrocytes (Dominici, Pritchard et al. 2004; Mehrotra, Rosol et al. 2010), liver cells (Khurana and Mukhopadhyay 2008; Sellamuthu, Manikandan et al. 2011) fibroblasts and myofibroblasts (Ebihara, Masuya et al. 2006). There is still debate whether or not these changes happen naturally in an organism, or if the plasticity of HSCs is limited to the lab.

HSCs arise in the embryo early in its development. While the exact origin of HSCs is still unknown several possibilities have been suggested. One model of HSC origin is the endothelial (Jaffredo, Gautier et al. 1998; Nishikawa, Nishikawa et al. 1998; Eilken 2009; Boisset, van Cappellen et al. 2010; Oberlin, El Hafny et al. 2010). Cells adhering to the ventral side of the aortic endothelium of the embryonic compartment have primitive hematopoietic progenitors cell

surface markers and molecular phenotype CD34+, CD45+, CD31+, CD38-, but do not have Lineage markers GATA-2+, GATA-3+, c-myb+, SCL/TAL1+, c-kit+ and flk1/KDR+ (Tavian, Coulombel et al. 1996; Labastie, Cortés et al. 1998). It has also been suggested that the aorta-gonad mesonephros (AGM) region of the embryo (Murray 1932; Robertson, Kennedy et al. 2000), is the source of hematopoiesis as its generation occurs between embryonic day 10.5-12.0 (E10.5-12.0) and enhanced activity of hematopoietic stem cells occur on E 11 (de Bruijn, Ma et al. 2002; Chen, Yokomizo et al. 2009; Coskun and Hirschi 2010; Taylor, Taoudi et al. 2010). Runx1 (also known as AML1 or CBFA2) is first detectable on E 7.5 before circulation begins from the allantois and the chorion (Zeigler, Sugiyama et al. 2006) and is essential for hematopoietic development (Okuda, van Deursen et al. 1996; Chen, Yokomizo et al. 2009). The placenta has also been proposed as an alternative or extra embryonic source for HSCs (Bárcena, Kapidzic et al. 2009; Robin, Bollerot et al. 2009).

Signaling and regulation of HSCs

Stem cells are highly regulated and the micro environment also called “niches” is important for the regulation of HSC (Schofield 1977; Xie and Spradling 1998). These niches maintain control over functions such as cell survival, self-renewal and cell fate decisions. A common niche for many blood cell types is the bone marrow. Niches in the bone marrow provide micro environment dependent signaling to regulate HSCs. Most HSCs are in a quiescent state (G0/G1 phase of the cell cycle) (Fleming, Alpern et al. 1993). However, when the hematopoietic system is agitated it either turns on or shuts down regulatory pathways. This is accomplished by signaling pathways such as SDF-1 (CXCL12/CXCR4), bone morphogenic proteins, Mpl/Thrombopoietin (TPO), Tie2/Ang1, hedgehog, Notch and wingless (wnt) signaling pathways.

It is thought fetal bone marrow requires stromal derived factor 1 (SDF1), CXCL12 and CXCR4 (Ara, Tokoyoda et al. 2003) to maintain HSC populations and differentiation states. These factors additionally regulate HSC populations in adult bone marrow (Nagasawa, Omatsu et al. 2011). The bone marrow microenvironment also promotes hematopoiesis via jagged1, jagged2, Notch 2, delta 1, delta 4, Hes1, Hes5 and deltax (Chitteti, Cheng et al. 2010). Notch ligands released by endothelial cells promote long term HSC self-renewal and hematopoiesis (Butler, Nolan et al. 2010).

SDF-1 plays an important role in embryonic development by regulating B-cells and lymphopoiesis (Moepps, Braun et al. 2000; Balabanian, Lagane et al. 2005). SDF-1 has also been shown to recruit endothelial progenitor cells from the bone marrow using a CXCR4-dependent mechanism suggesting that SDF-1 and CXCR4 play a role in vascular development (Zheng, Fu et al. 2007). SDF-1/CXCR4

together promote self-renewal of HSCs and regulate attachment of HSCs to the bone marrow. Conversely the release of HSCs from the bone marrow requires matrix metalloproteinase 9 (MMP9) which releases soluble Kit-ligand (Heissig, Hattori et al. 2002). Deletions of CXCR4 in mice cause a reduction in the HSC pool and increase proliferation response to HSC defections (Sugiyama, Kohara et al. 2006; Nie, Han et al. 2008).

Bone morphogenic proteins (BMPs) are growth factors that is part of Transforming Growth Factor Bata (TGF-β) family (Hari Reddi and Reddi 2009). BMP 4 regulates hematopoiesis lineage commitment from mesodermal cells in both embryonic development as well as adult HSCs in the bone marrow (Durand, Robin et al. 2007; Goldman, Bailey et al. 2009). High concentrations of BMP-2, -4 and -7 maintain human cord blood (CB) cells whereas low BMP-4 induced differentiation (Bhatia, Bonnet et al. 1999).

c-Mpl receptor is a cell surface cytokine receptor that responds to Thrombopoietin (TPO). c-Mpl receptor expression occurs mainly on HSCs with an increased cell surface expression in megakaryocytic progenitors, megakaryocytes and leukemic cells (Debili, Wendling et al. 1995). The c-Mpl receptor is also expressed in bone marrow, spleen and fetal liver (Chou and Mulloy 2011). The c-Mpl/TPO singling cascade is responsible for maintaining HSC quiescence and self-renewal (Bersenev, Wu et al. 2008; Rongvaux, Willinger et al. 2011). It has been suggested that TPO starts the c-Mpl signal cascade by binding to c-Mpl and causes it to form a homodimer (Geddis 2010). The cascade activates the downstream JAK-STAT pathway, phosphoniositide 3 kinase (PI3K) and mitogenic activator protein kinases (MAPKs) (Tortolani, Johnston et al. 1995; Tagaya, Burton et al. 1996).

Hedgehog (Hh) signaling is a signaling pathway that plays a role in controlling embryonic and adult stem cell fate, and it has been suggested that Hh signaling is a negative regulator of hematopoiesis

(Trowbridge, Scott et al. 2006). The Hh protein binds to the transmembrane receptor Patched (Ptc), which subsequently activates Smoothened (Smo). Constitutive expression of Ptc in mice causes induction of cell cycling and expansion of primitive bone marrow HSCs (Trowbridge, Scott et al. 2006). While Smo is required for fetal HSC regulation is may be dispensable in adult HSCs (Gao, Graves et al. 2009; Hofmann, Stover et al. 2009). Furthermore it has been shown Gli1, the downstream signal of Hh, plays a role in myeloid progenitor normal and stress proliferation and differentiation (Merchant, Joseph et al. 2010).

The Notch receptors and ligands play a role in proliferation, maintain or enhance HSC self-renewal (Ohishi, Varnum-Finney et al. 2002), differentiation (Stier, Cheng et al. 2002; Varnum-Finney, Brashem-Stein et al. 2003) and cell fate decisions of many cell types including HSCs (Artavanis-Tsakonas, Rand et al. 1999; Lin and Hankenson 2011). Notch has four receptors notch 1-4 and, five ligands, jagged 1 and 2 and delta like 1, 3 and 4 (Ranganathan, Weaver et al. 2011). Notch signaling can respond to intrinsic and extrinsic cues (Artavanis-Tsakonas, Rand et al. 1999). It has been shown in *zebrafish* embryos that the upstream transcription factor Hey2 was essential for HSC differentiation. When Hey2 was knocked down, HSC differentiation was rescued by activation of Notch signaling (Rowlinson and Gering 2010). It has also been shown that up-regulation of Notch and its ligands (Notch 2, Deltex, Jagged 1 and 2 and Delta 1 and 4) promote repopulation and maintenance of osteoblasts *in vitro* (Chitteti, Cheng et al. 2010). However, some studies suggest notch is not required for adult HSCs self-renewal or differentiation (Mancini, Mantei et al. 2005; Maillard, Koch et al. 2008). This may only apply to a subset of notch signaling (Notch1 and Jagged1)

Wingless (Wnt) signaling plays a role in cell fate determination of various cell types including HSCs. Wnt and Notch signaling are involved in cross talk (Clements, Kim et al. 2011), which may provide greater control over self-renewal and cell fate determination. Canonical Wnt signaling is involved in

ESC self-renewal (Sato, Meijer et al. 2003) and HSC differentiation (Woll, Morris et al. 2008; Deng, Zhuang et al. 2013). Conversely, the non-canonical Wnt promotes HSC self-renewal (Fleming, Janzen et al. 2008; Kim, Kang et al. 2009). The canonical Wnt signaling pathway interacts with Frazzled (Fz) receptor and co-receptor LDL receptor related proteins 5 and 6 (LDR 5 and 6) (Wu and Nusse 2002). The typical genes activated downstream are FGF20, DKK1 (Chamorro, Schwartz et al. 2005), WISP1 (Pennica, Swanson et al. 1998), MYC (He, Sparks et al. 1998) and cyclin D1 (Tetsu and McCormick 1999). In the non-canonical Wnt signaling pathway Wnt ligand Wnt3a binds to Fz but has a different downstream effect. The non-canonical pathway causes an increase in intracellular Ca^{2+} activating protein kinase C and calmodulin (Miller, Hocking et al. 1999). The non-canonical Wnt pathway overlaps Notch by stimulating the expressing of Jagged1 in bone marrow thus promoting self-renewal (Kim, Kang et al. 2009)

Erythropoiesis

Erythropoiesis is formation of erythrocytes or red blood cells (RBCs) (Figure 1.2). In differentiation some cells such as myoblasts arrest in G1 (Alemá and Tató 1987; Alema and Tatò 1994) whereas other fibroblasts divide after differentiation begins (Roby, Brumbaugh et al. 1977). While erythropoiesis goes through several divisions (3-4) during differentiation (von Lindern 2006). However, the cells can forgo some of the later divisions (Sankaran, Ludwig et al. 2012). During erythropoiesis, the cell alters its cell cycle shortening the G1 phase from 11 h to 5 h after an initial lag period of 12 h where the cells do not divide (Dolznig, Bartunek et al. 1995; Dolznig, Boulmé et al. 2001; von Lindern, Deiner et al. 2001). While differentiating in cell culture as proerythroblasts, cells synchronize when induced during the initial 12 h lag phase shortening the doubling time from 24 hour to 12 hours (Dolznig, Boulmé et al. 2001). During differentiation, a shortened cell cycle is required for normal-sized erythrocytes to form. The transcription factor E2f4 plays a role in the differentiation cell cycle but not the normal self-renewal cell cycle (von Lindern 2006). However, $E2F4^{-/-}$ mice are not anemic, suggesting that cell divisions do not affect molecular processes other than controlling cell size (Grebien, Dolznig et al. 2005). The switch to differentiation cell cycles is marked by changes in several cell cycle regulators such as cyclin D1 down-regulation and cdk4/cdk6, Cip1 and Kip1 (Murray 1932; Dolznig, Bartunek et al. 1995; Ciemerych, Kenney et al. 2002; Malumbres, Sotillo et al. 2004). Within hematopoiesis some changes are specific to erythropoiesis, include globin synthesis, increase in blood antigens and an enrichment in cation transporters (Novershtern, Subramanian et al. 2011).

Like in many hematopoietic progenitor cells, many signaling pathways regulate erythropoiesis. One cytokine that play a role in erythropoiesis is Stem Cell Factor (SCF) which is a c-kit ligand and promotes self-renewal of erythropoiesis and progenitor stem cells (Huang, Nocka et al. 1990; Zsebo, Williams et al. 1990). p53, ERK and Bcl2/Bcl-XL along with SCF stimulate erythroblast proliferation (Kapur, Chandra et al. 2002; Zeuner, Pedini et al. 2003). Notch signaling also inhibits erythropoiesis and promotes self-renewal of progenitor cells (Ishiko, Matsumura et al. 2005; Tachikawa, Matsushima et al. 2006; Henning, Schroeder et al. 2007). Notch and SCF work together to maintain homeostasis of erythrocytes (Robert-Moreno, Espinosa et al. 2007; Zeuner, Francescangeli et al. 2010).

Once the decision is made to differentiate, erythroid progenitor cells go through the following stages, erythroid burst forming unit (BFU-E), erythroid colony forming unit (CFU-E), proerythroblast, basophilic erythroblast, polychromatic erythroblast, orthochromatic erythroblast, reticulocyte and erythrocyte (Ogawa 1993).

BFU-Es are the first cells committed to the Erythroid lineage and form large clustered burst colonies on semisolid media (Heath, Axelrad et al. 1976). BFU-Es make up approximately 0.03% of bone marrow hematopoietic cells with only about 40% of the population active (Iscove 1977). BFU-Es require interleukin 3 (IL-3) or granulocyte macrophage colony stimulating factor (Sawada, Krantz et al. 1990) as well as Stem Cell Factor (SCF) but no accessory cells are required for self-renewal (Dai, Krantz et al. 1991). However BFU-E have a limited response to erythropoietin (Epo) due to a reduced expression of Epo receptor on the cell surface (Sawada, Krantz et al. 1990).

CFU-Es like BFU-E form colonies on semisolid media but the colonies are much smaller (Heath, Axelrad et al. 1976). CFU-E make up approximately 0.3% of the bone marrow hematopoietic cell population and nearly all CFU-E cells are active (Iscove, Sieber et al. 1974). CFU-Es divide rapidly

with an average doubling time of about 7 hours (Nijhof and Wierenga 1983). However CFU-Es, unlike BFU-Es, respond to erythropoietin and insulin like growth factor 1 (IGF-1) (Noguchi, Fukumoto et al. 1988; Dai, Krantz et al. 1991). CFU-Es resemble proerythroblasts with a size of about 16-20 µm, a large nucleus, irregular chromatin (not much condensed chromatin), basophilic cytoplasm, large mitochondria and a cytoplasm packed with ribosomes (Nijhof and Wierenga 1983) . Late stage CFU-Es may begin to synthesize hemoglobin (Heath, Axelrad et al. 1976).

The remaining steps in erythropoiesis cause progressive decreases in cell size and a shift from basophilic to acidophilic cytoplasm do to the accumulation of hemoglobin and loss of organelles (Orlic, Gordon et al. 1965). Proerythroblasts, like CFU-Es, have a large nucleus but with more condensed chromatin and fewer mitochondria (Nijhof and Wierenga 1983). Proerythroblasts have a large diameter of 15-20 µm (Panzenböck, Bartunek et al. 1998).

Basophilic erythroblasts have a diameter of 12 to 18 µm with a large nucleus and more condensed DNA then a Proerythroblast (Orlic, Gordon et al. 1965; Panzenböck, Bartunek et al. 1998).

Polychromatic erythroblasts have a wide range of cell size corresponding with the number of divisions and a smaller nucleus (Panzenböck, Bartunek et al. 1998).

Orthochromatic erythroblasts are about the size of a mature erythrocyte with an acidophilic cytoplasm that is devoid of organelles except for the rare mitochondria, large amount of hemoglobin and fewer free ribosomes (Orlic, Gordon et al. 1965; Panzenböck, Bartunek et al. 1998). Also orthochromatic erythroblasts have a definite loss of self-renewal potential (Tarbutt and Cole 1970).

Reticulocytes undergo enucleation, however reticulocytes still have RNA in the cell, although much is degraded and the cells contain few ribosomes (Koury, Koury et al. 2005). Some reticulocytes stay in the bone marrow until they become mature erythrocytes.

Erythrocytes start to leave the bone marrow at the end of the reticulocyte stage and early mature erythrocyte state. Erythrocytes have a small diameter 7-8 μm with a flat donut shaped membrane for increased surface area and an accumulation of heme causing the red color (Panzenböck, Bartunek et al. 1998).

Activators and regulators of erythropoiesis

Cytokines are important for regulating erythropoiesis, the cytokines thrombopoietin (TPO), IL-3, IL-6, IL-8, IL-9 and IL-11act to increase the number of BUF-Es (Birkmann, Oez et al. 1997). While TPO and the interleukin cytokines are not required for erythropoiesis Stem Cell Factor (SCF) and erythropoietin, (EPO) and their receptors c-kit and Epo-R respectively are required for maintenance of some erythropoiesis progenitors (Russell 1979).

EPO and SCF dependent cells can be isolated from CD34+ peripheral stem cells and bone marrow (Panzenböck, Bartunek et al. 1998). EPO and SCF promote growth of erythroid progenitors in vitro (Muta, Krantz et al. 1994). Alternatively transforming growth factor alpha or steroid hormones in combination with EPO can stimulate self-renewal of some erythroid progenitors (Schroeder, Gibson et al. 1993). SCF is a c-kit ligand that promotes proliferation in embryonic and committed stem cell progenitors (Broudy 1997). However c-Kit expression is only high in early erythroid progenitors, such as BFU-Es and CFU-E sand expression drops off completely by the orthochromatic stage (Broudy 1997).

EPO is a 34 KD glycoprotein that stimulates proliferation, differentiation and survival of erythroid progenitors (Krantz 1991). EPO is primarily produced in the kidney and to a lesser extent in the liver. EPO travels through the blood and binds to the erythropoietin receptor (EPO-R) in erythroid progenitor cells. EPO-R is part of the type 1 cytokine receptor superfamily lacking any kinase domain (Bazan 1990). Upon binding of EPO to the EPO-R it forms a homodimer; then the tyrosine kinase JAK2 phosphorylates the EPO-R and recruits STAT5a/b, SHP1, SHP2, SHIP, p85 and PI-3 kinase

to propagate the signal (Witthuhn, Quelle et al. 1993; Richmond, Chohan et al. 2005). EPO can regulate cell survival and can also regulate cell cycle genes (Fang, Menon et al. 2007; Sathyanarayana, Dev et al. 2008). EPO downstream signals PI3K/Akt regulate cyclin D3 E and A, as well as $p27^{Kip1}$ at the protein level (Sivertsen, Hystad et al. 2006). Protein kinase B (PKB) and Akt also regulate important genes in proliferation and cell survival such as Fox03a and FKHR-L1 at the transcript level (Brunet, Bonni et al. 1999; Kashii, Uchida et al. 2000; Bakker, Blázquez-Domingo et al. 2004). Not only can Akt promote survival and proliferation but Akt can also promote differentiation complementing EPO-R signaling in a JAK2 and IP-3 independent manor (Ghaffari, Kitidis et al. 2006). Akt may modulate differentiation through phosphorylation of GATA-1 (Kadri, Maouche-Chretien et al. 2005).

GATA-1 and GATA-2 are the 2 members of the GATA family of transcription factors that are involved in differentiation of erythrocytes (Shimizu and Yamamoto 2005). GATA-2 expression is higher in early erythroid and other hematopoietic lineages, whereas GATA-1 expression is found in later stages of the erythroid linages (Weiss, Keller et al. 1994; Fujiwara, Chang et al. 2004). While expression of the two transcription factors overlap they also negatively regulate each other on the transcriptional level (Grass, Boyer et al. 2003).

Regulation of GATA-1 is tightly controlled in erythropoiesis. GATA-$1^{-/-}$ mice die at day 10-12 after fertilization with cells arrested at the proerythroblast stage (Keller 1995). Acetylation and phosphorylation also regulate GATA-1's activity (Lamonica, Vakoc et al. 2006). Akt phosphorylates GATA-1 at S310 causing an increase in transcriptional activity of GATA-1 targets (Zhao, Kitidis et al. 2006). GATA-1 is also regulated by direct protein-protein interactions with PU.1 (Rekhtman, Radparvar et al. 1999). GATA-1 can also be enhanced by other interacting proteins like Friend of GATA-1 (FOG-1) (Cantor and Orkin 2005).

Micro RNAs (miRNAs) have been implicated in playing a role in many cellular processes including erythropoiesis. Ago2 is a protein involved in the dicer complex used for gene silencing, and Ago2-/- mice had erythropoiesis arrested with an abundance of basophilic erythroblasts in the bone marrow indicating that miRNA play a critical role in the progression of erythropoiesis (O'Carroll, Mecklenbrauker et al. 2007). When miR-221 and miR-222 are overexpressed in Erythroid progenitor cells, erythropoiesis is blocked, identifying these miRNAs as playing a role in erythropoiesis (Felli, Fontana et al. 2005). Conversely miR-210 and miR- 451 have been implicated in promoting erythropoiesis (Masaki, Ohtsuka et al. 2007; Kosaka, Sugiura et al. 2008) showing a diverse role of miRNA in the regulation of erythropoiesis.

Mouse Erythroleukemia (MEL) cells

MEL cells are virally transformed cells that are maintained at the proerythroblast state by the Friend spleen focus-forming virus (SFFV). This retrovirus integrated into the genome at the *Spi1* locus (Moreau-Gachelin, Tavitian et al. 1988). This caused high expression of the PU.1 transcription factor leading to dephosphorylation of SATA-1 by SHP-1, which is believed to responsible for the arrest in differentiation (Schuetze, Paul et al. 1992). Furthermore PU.1 may also form a complex with histone deacetylase 1 to repress the expression of c-myc (Kihara-Negishi, Yamamoto et al. 2001). MEL cells can be induced to differentiate into erythrocytes by treating with a polar planer solvent, such as DMSO (Friend, Scher et al. 1971).

MEL cells have been shown to have "memory". MEL cells have a lag period of approximately 12 hours when induced, but when the inducer is removed and added back later the cells do not go through the lag phase again (Levenson and Housman 1979). The "memory" of MEL cells lasts anywhere from 18 to 72 hours. Furthermore the commitment is independent of what inducer is used. Changing the inducers partway through differentiation has no effect when compared to cells treated with only one inducer for the same amount of time (Housman, Gusella et al. 1978).

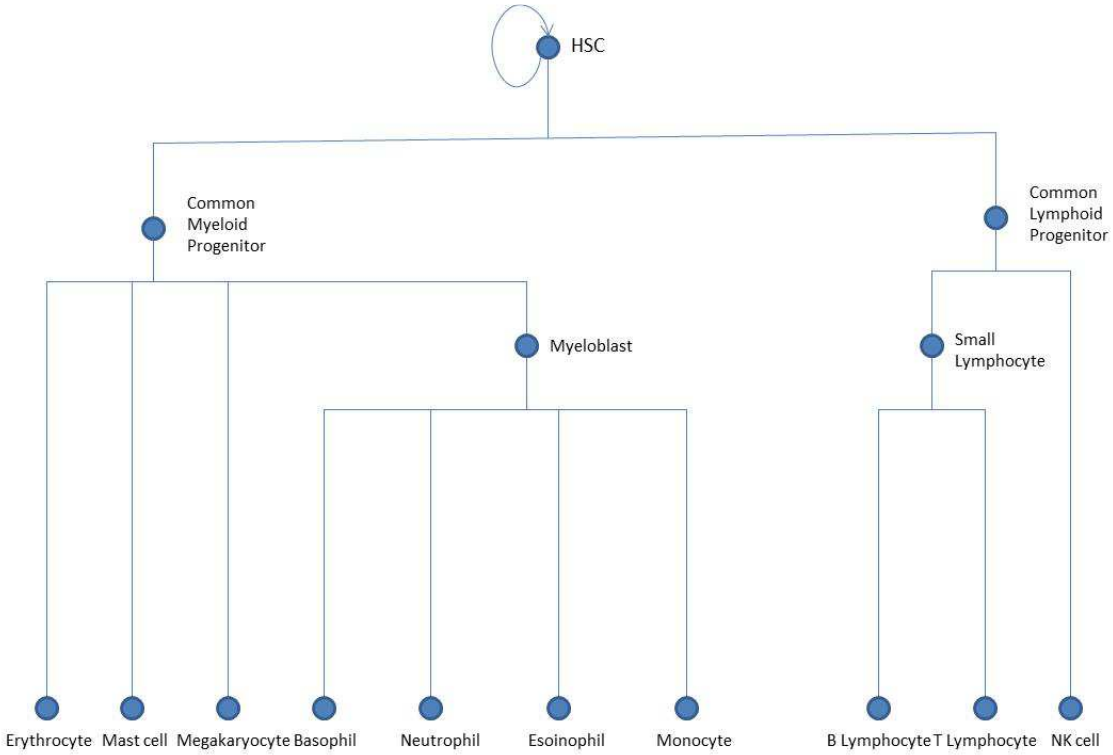


Figure 1.1: Diagram of hematopoiesis, starting with a hematopoietic stem cell (HSC)

Cell type	**Markers**
HSC	Lin^{-} $CD133^{+}$ $CD34^{+}$
Common myeloid progenitor	$CD34^{+}$ $CD38^{+}$ $CD45RA^{-}$ IL-3Ralpha^{+}
Common lymphoid progenitor	$CD34^{+}$ $CD38^{+}$ $CD10^{+}$
Erythrocyte	CD34- CD71- GylA+
Mast cell	$CD3^{-}$ $CD14^{-}$ $CD16^{-}$ $CD19^{-}$ $CD56^{-}$ HLA DR^{+}
Megakaryocyte	$CD3^{4}$- $CD41^{+}$ $CD61^{+}$ $CD45^{-}$
Basophil	$CD22^{+}$ $CD123^{+}$ $CD33^{+/-}$ $CD45^{dim}$
Neutrophil	$CD16^{+}$ $CD11b^{+}$
Eosinophil	IL-3Ralpha^{+} $CD33^{dim}$
Monocyte	$CD14^{+}$ $CD45^{dim}$
B lymphocyte	$CD19^{+}$ $CD27^{+/-}$ $IgD^{+/-}$
T lymphocyte	CD8/$CD4^{+}$ $CD61L^{+/-}$ $CD45Ra^{+/-}$
NK cells	$CD56+^{/-}$ $CD14^{-}$ $CD19^{-}$ $CD16^{+/-}$ $CD3^{+/-}$ $CD1d^{+}$

Table 1.1: Markers for cell types found in the hematopoietic linage.

Figure 1.2: Erythropoiesis: the first committed cell in the Erythroid linage is the burst forming unit erythroblast (BFU-E). BUF-E differentiates into a colony forming unit erythroblast (CFU-E). As the cells continue to differentiate through proerythroblasts to orthochromatic erythroblasts the cells shrink in size, DNA condenses in the nucleus and the cytoplasm becomes more acidic do to the accumulation of hemoglobin. During the reticulocyte stage the cells become enucleated and as the cells become mature erythrocytes they leave the bone marrow and enter blood vesicles.

CHAPTER II

METHODOLOGY

Competent DH5-alpha *E. coli* cells

Competent DH5-alpha *E. coli* cells were made by picking a single colony from an LB (10g tryptone, 5g yeast extract and 10g NaCl in 1 L diH_2O, pH 7.0) agar plate and growing in 5 ml LB broth overnight. 1 ml of LB culture was added to 250 ml SOB (20g tryptone 5g yeast extract, 2 ml 5M NaCl, 2.5 ml 1M KCl, 10 ml 1M $MgCl_2$, 10 ml 1M $MgSO_4$ in 1 L diH_2O, pH 7.0) broth and grown at 18°C to an OD of 0.600. The cells were then centrifuged at 2,500 x g at 4°C for 10 min and the pellet was washed and resuspended in 10 ml ice cold TB(10 mM PIPES, 15 mM $CaCl_2$, 250 mM KCl, 55 mM $MnCl_2$, in 250 ml H_2O, pH 6.7). 1.4 ml of DMSO was added and incubated on ice for 10 min. 0.5 ml aliquots were stored in pre-chilled tubes at -80°C for later use.

Degenerate PCR

The promoter was amplified using 2 µl of 10 mM of each primer, using 10 ng pHippy (Kaykas and Moon 2004) as a template in 20 µl diH_2O and 25 µl 2x master mix (5 µl Taq Polymerase 5 U/µl, 60 µl of $MgCl_2$ 25 mM 6 µl of 100 mM dNTP, 100 µl of 10x PCR buffer, and 329 µl diH_2O). The PCR reaction was pre heated to 94°C for 2 min. The reaction first went through 9 less stringent cycles to allow the degenerate primers to bind. These cycles were denaturing at 94°C for 20 sec, annealing at 60°C for 45 sec and elongation at 72°C for 4 min. Subsequent amplification continued for 19 more cycles at more stringent conditions; denaturing at 94°C for 20 sec, annealing at 55°C for 45 sec and elongation at 72°C for 4 min. A final elongation step of 72°C for 7 min was added to ensure that any fragments were completely amplified.

Plasmid Preparation

10 µl of the ligation reaction were added to 0.1 ml competent DH5-alpha cells prepared as described above and incubated on ice for 30 min. The mixture was then placed in a 42°C heat block for 45 sec and returned to ice for 2 min. The cells were then allowed to recover in low salt LB for 1 hour at 37°C with vigorous shaking. 0.1 ml of recovered cells was added to a low salt LB + Zeocin (Invitrogen Cat. No. R250-01) at 100 µg/ml plate. The plated cells were grown over night at 37°C. To identify transformants the next day 4-10 colonies were picked and put in 5 ml low salt LB + Zeocin (100 µg/ml broth) and grown overnight at 37°C. The cells were centrifuged at 2,000 x g for 10 min at 4°C. The pellet was resuspended in 200 µl ice cold solution 1 (50 mM glucose, 25 mM Tris HCl, 10 mM EDTA) by vortexing. 400 µl of fresh solution 2 (930 µl diH_2O, 20 µl 10 M NaOH, 50 µl 20% SDS) was added and mixed by inverting the tube. The tubes were then put on ice. 300 µl of ice cold solution 3 (294 g potassium acetate, 50 ml glacial acetic acid filled to 1 L with diH_2O) was added and tubes were incubated on ice for 5 min. The tubes were then centrifuged at 16,000 x g for 5 min at room temperature. 600 µl of supernatant was transferred to a fresh microfuge tube and 600 µl isopropanol was added. The tubes were mixed by inverting and incubated at -20°C for 1 hour. The DNA was then centrifuged at 16,000 x g for 5 min at room temperature. The supernatant was removed and the DNA pellet was washed with 1 ml of 70% ethanol then centrifuged at 16,000 x g for 5 min at room temperature. The supernatant was removed and the DNA pellet was air dried for 1 hour. The DNA pellet was then resuspended in 100 µl of 0.1 X TE. RNA was then removed by adding 1 µl of 20 mg/ml RNAse A (Invitrogen Cat. No. 12091-04) and incubated at 42°C for 15 min. The DNA was purified using DNA spin columns (Qiagen Cat. No. 28004).

Construction of pHIP-HUT-TR

The vector pHIP-HUT-TR (Figure 2.2 B) was designed using pHippy (Figure 2.2 C. Graciously provided by Dr. Moon) (Kaykas and Moon 2004) which is a bi-directional siRNA producing vector using the human U6 and H1 RNA polymerase 3 promoters. Using degenerate primers (Table 2.2.1) containing a TetR binding site overlapping the TATA box, the promoters were amplified and modified using PCR.

The U6 promoter was amplified to make $U6^{TetO}$ using 2 µl of 10 mM primers U6 UP and U6 DN, using 10 ng pHippy as a template in 20 µl diH_2O and 25 µl 2x master mix. Degenerate primer amplification was the same as described above.

5 µl of the $U6^{TetO}$ PCR fragment 500 ng/µl and 5 µl of pHippy 480 ng/µl were cleaved using restriction enzymes (New England BioLabs Inc.) NheI (1 µl at10 U/µl) and (MluI 1 µl at10 U/µl) in 2 µl 10x NEBuffer 2 and 11 µl diH_2O (total volume 20 µl). The mixture was incubated at 37°C overnight. The pHippy fragments were treated with calf alkaline phosphatase (1 µl at 1U/µl, Promega) for 30 min at 42°C and both fragments were purified using gel extraction by cutting out the 1.8 kb fragment from a 1% agarose gel run in TAE and dissolving the gel slice in 3x volume buffer QG (Qiagen) and isolating the DNA on a DNA spin columns (Qiagen Cat. No. 28004). 1 mg of $U6^{TetO}$ and 400 ng of $pHippy^{-U6}$ fragments were ligated using 1 µl T4 DNA ligase at 400 U/µl (New England BioLabs Inc), 4 µl of 5x T4 DNA ligase buffer and 8 µl diH_2O (total volume 20 µl) and incubated overnight at room temperature.

10 µl of the pHippy^{-U6}/U6^{TetO} ligation reaction were added to 0.1 ml competent DH5-alpha cells and incubated on ice for 30 min. The plasmids were transfected into the competent cells as described above.

The sequence of pHIP-UT (pHIPpy-U6^{TetO}) was confirmed by Sanger di-deoxy sequencing at the Oklahoma State University Recombinant DNA/Protein Resource Facility.

The H1 promoter was amplified to make H1^{TetO} using 2 µl of 10 mM primers "H1 UP" and "H1 DN", using 10 ng of pHippy as a template in 20 µl diH_2O and 25 µl 2x master mix. The PCR reaction was pre heated to 94°C for 2 min. Degenerate primer amplification was the same as described above.

Due to the inverted repeat of the TetR binding site being proximal to the restriction enzyme binding site, the digestion or ligation of H1^{TetO} was ineffective, thus an alternative approach of sewing PCR was used. Two µl of 10 mM primers H1 UP reverse complement shift (H1 UP RC Shift) and U6 DN, using 10 ng pHIP-UT as a template in 20 µl diH_2O and 25 µl 2x master mix. Degenerate primer amplification was the same as described above.

Sewing, also called joining PCR (Yon and Fried 1989), was used to combine the H1 RC shift and H1^{TetO} PCR fragments (Figure 2.3). The fragments are joined together based on overlapping regions that act as primers and templates for the other fragments. One mg of the H1 RC Shift and H1^{TetO} fragments were amplified in 21 µl diH_2O and 25 µl 2x master mix. The PCR reaction was preheated to 94°C for 2 min. The reaction first went through 29 cycles consisting of denaturing at 94°C for 20 sec, annealing at 55°C for 45 sec and elongation at 72°C for 4 min. A final elongation step of 72°C for 7 min was added to ensure that all fragments were completely amplified.

5 µl of the sewing PCR fragment 500 ng/µl and 5 µl of pHippy 480 ng/µl were cleaved using 1 µl NotI at10 U/µl and 1 µl MluI at10 U/µl restriction enzymes (New England BioLabs Inc.) in 2 µl

10x NEBuffer 2 and 11 µl diH_2O (total volume 20 µl) which were incubated at 37°C overnight. The pHippy fragment was then treated with 1 µl calf alkaline phosphatase at 1U/µl (Promega) for 30 min at 42°C. Both fragments were purified using gel extraction by cutting out the 450 bp (sewing) and 1.7 kb (pHippy) fragments from a 1% agarose gel and dissolving the gel slice in 3x volume buffer QG (Qiagen) and isolating the DNA on a DNA spin column (Qiagen Cat. No. 28004). 600 ng of sewing PCR fragment (Figure 2.2 A) and 600 ng pHippy$^{-U6/-H1}$ fragments were ligated in 1 µl T4 DNA ligase 400 at U/µl (New England BioLabs Inc.), 4 µl 5x T4 DNA ligase buffer and 9 µl diH_2O (total volume 20 µl) and incubated overnight at room temperature.

10 µl of the pHippy$^{-U6/-H1}$/sewing PCR fragment ligation reaction were added to 0.1 ml competent DH5-alpha cells and incubated on ice for 30 min. The plasmids were transfected into the competent cells as described above.

The sequence of pHIP-HUT (pHIPpy-H1^{TetO}-U6^{TetO}) was confirmed by Sanger di-deoxy sequencing at the Oklahoma State University Recombinant DNA/Protein Resource Facility.

The tet repressor (Yang, Zubay et al. 1976) was inserted into pHIP-HUT by first isolating it from pCDNA6-TR. 1 mg of pCDNA6-TR and 1 mg of pHIP-HUT were cleaved using 1 µl MluI at 10 U/µl restriction enzyme (New England BioLabs Inc.) in 2 µl 10x NEBuffer (total volume 20 µl) then were incubated at 37°C overnight. The pHIP-HUT digest was then treated with 1 µl calf alkaline phosphatase 1U/µl (Promega) for 30 min at 42°C. The pHIP-HUT 2.1 kb fragment (Figure 2.2A) and the 2.4 kb tet repressor fragment were isolated and purified as described previously. 500 ng of tet repressor and 500 ng of pHIP-HUT fragments were ligated in 1 µl T4 DNA ligase 400 U/µl (New England BioLabs Inc.), 4 µl 5x T4 DNA ligase buffer and 8 µl nanopure filtered H_2O (total volume 20 µl) and incubated overnight at room temperature.

10 μl of pHIP-HUT/tet repressor ligation reaction were added to 0.1 ml competent DH5-alpha cells and incubated on ice for 30 min. The plasmids were transfected into the competent cells as described above.

The sequence of pHIP-HUT-TR was confirmed by Sanger di-deoxy sequencing at the Oklahoma State University Recombinant DNA/Protein Resource Facility.

MDA-MB-231 cells

MDA-MB_231/GFP-RFP cells (Cell BioLabs Inc. Cat. No. AKR-221) were grown in a T-25 flask with high glucose DMEM (HyClone Cat. No. SH30081.01) 10% fetal bovine serum (FBS) (Promega Cat. No. S11550) 0.1 mM non-essential amino acids (NEAA) (BioWhittaker Cat. No. 13-114E) 2% Pen/Strep (Cellgro Cat. No. 30-002-C2) and 2 mM L-glutamine at 37°C and 5% CO_2.

MDA-MB-231 cells were passaged at 70-95% confluence by removing the media and adding 1 ml of 1x Trypsin EDTA (Cellgro Cat. No. 23-452-C1) at 37°C for 1 min while gently shaking by hand. Trypsonization was halted by adding 10 ml of growth media and the cells were removed to a centrifuge tube. The cells were centrifuged at 200 x g for 5 min, resuspended in 10 ml of fresh growth media and placed back into fresh T-25 flasks.

Frozen stocks of MDA-MB-231 cells were made by placing 0.8 ml of 10^6-10^7 cells/ml from growth media into cryovials, then adding 0.1 ml FBS and 0.1 ml DMSO followed by placing the cells in an isopropanol bath in a -80°C freezer overnight. The next day the cryovials were transferred into a liquid nitrogen tank for long term storage.

Mouse Erythroleukemia (MEL) cells

MEL cells (graciously provided by Dr. Robert Matts, Oklahoma State University) were grown in T-25 flasks with high glucose DMEM (HyClone Cat. No. SH30081.01), 10% fetal bovine serum (FBS) (Promega Cat. No. S11550), 0.1 mM non-essential amino acids (NEAA) (BioWhittaker Cat. No. 13-114E), and 2% Pen/Strep (Cellgro Cat. No. 30-002-C2) at 37°C and 5% CO_2.

MEL cells were passaged at 60-80% confluence by removing a 1 ml aliquot of cells and adding 9 ml of growth media to the aliquot to a new T-25 flask.

Frozen stocks of MEL cells were made by placing 0.8 ml of 10^6 cells/ml from growth media into cryovials, then adding 0.1 ml FBS and 0.1 ml DMSO followed by placing the cells in an isopropanol bath in a -80°C freezer overnight. The next day the cryovials were transferred into a liquid nitrogen tank for long term storage.

Validating pHIP-HUT-TR

siRNAs (Table 2.2) were designed by taking 20-24 nt sequences from *copepoud Pontellina plumata* ppluGFP2 copGFP (Shagin, Barsova et al. 2004) DNA sequence (Cell Biolabs Inc.) and aligned using BLASTn with the human genome. 3 sequences with < 100% matches and the reverse complement were designed with an extruding $d(A)_4$ tail at the 5' end.

600 ng of pHIP-HUT-TR was cleaved using 1 µl BsmBI at10 U/µl restriction enzyme (New England BioLabs Inc.) in 2 µl 10x NEBuffer 3 and 11 µl diH_2O (total volume 20 µl). The mixture was incubated at 55°C overnight. The pHIP-HUT-TR digest was then treated with 1 µl calf alkaline phosphatase (1U/µl, Promega) for 30 min at 42°C and the 4.5 kb fragments lacking a siRNA insert were purified by cutting out the gel extraction from a 1% agarose gel run in TAE and dissolving the gel slice in 3x volume buffer QG (Qiagen) and isolating the DNA on a DNA spin columns (Qiagen Cat. No. 28004). 0.1 mM of pHIP-HUT-TR fragment and 10 mM representing a 100 fold excess amount of siRNA were ligated in 1 µl T4 DNA ligase 400 U/µl (New England BioLabs Inc.), 4 µl 5x T4 DNA ligase buffer and 8.5 µl diH_2O (total volume 20 µl) and incubated overnight at room temperature.

10 ml of the pHIP-HUT/siRNA ligation reaction was added to 0.1 ml competent DH5-alpha cells and incubated on ice for 30 min. The plasmids were transfected into the competent cells as described above.

The sequence of pHIP-HUT-TR$^{GFP\ siR\#}$ was confirmed by Sanger di-deoxy sequencing at the Oklahoma State University Recombinant DNA/Protein Resource Facility.

10^4 MDA-MB-231 cells were centrifuged at 200 x g for 5 min and resuspended in 100 µl 1x PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 in 1 L at pH 7.4) and incubated on ice for 5 min. The cells were then electroporated with 1 µg pHIP-HUT-$TR^{GFP\ siR1}$ or pHIP-HUT-$TR^{GFP\ siR3}$ using a Bio-RAD Gene Pulsar set at 960 µFD, 180 V and 200 OHMS. The cells were then incubated on ice for 5 min then 1 ml growth medium was added and eight 100 µl aliquots were placed in a 96 well plate and incubated for 1 hour at 37°C and 5% CO_2. After the recovery period Zeocin (Invitrogen Cat. No. R250-01) was added to a final concentration of 500 µg/ml to each well and the cells were incubated for 24 hours at 37°C and 5% CO_2.

To induce expression of siRNAs, 1 µg/ml of Doxycyclin (Enzo) was added to 4 of the 8 wells and the cells were incubated for 24 hours at 37°C and 5% CO_2. GFP fluorescence was detected using the BioTek Synergy H1 Hybrid Reader using an excitation wavelength of 475 and read wavelength of 505. (Figure 2.4)

Pulse induction of MEL cells

Mouse Erythroleukemia (MEL) cells were separated into seven samples, and each sample was induced to differentiate into erythrocytes by adding 2% DMSO v/v to growth media at 30-40% confluence (Edwards, Harris et al. 1983). At time points 0.5, 2, 4, 8, 18 and 42 hours the MEL cells from separate flasks were removed and centrifuged at 200 x g, washed with fresh growth media and resuspended in 10 ml fresh growth media without DMSO. The pulse-induced MEL cells were then allowed to grow for the duration of the differentiation time (total time 8 days) (Figure 2.1).

As controls, MEL cells were also treated with DMSO for identical times, but the cell RNAs were immediately harvested without allowing the cells to grow until terminal differentiation. These RNAs provide a temporal control of induced gene expression during cell culture following DMSO treatment.

RNA isolation

RNA was extracted from MEL cells essentially as described by (Hoyt and Doktycz 2004). Briefly, MEL cells were centrifuged at 200 x g for 5 min and growth media was decanted. Any remaining media was carefully pipetted off. The cells were then resuspended in 800 µl buffer RLT (Qiagen) with freshly-added β-mercaptoethanol (BME) (15 µl BME for 10 ml RLT). Cells were transferred to a bead milling tube (Bio Plas, Inc., # 4202 and # 4215R) containing seven, 3 mm borosilicate glass beads (Aldrich # Z14392-8, Milwaukee, WI) and placed on ice. Cell disruption and DNA homogenization was achieved by 6 cycles of 2.5 min shaking and 3 min on ice using a 96 well Mini Beadbeater (BioSpec Products Inc.). A 450 µl aliquot of disrupted cells were loaded onto RNeasy spin columns (Quiagen Cat. No. 74106). The disruption mixture was washed and the RNA bound to the columns by centrifugation. Before elution the column was treated with 80 µl of DNAse I solution (10 µl DNAse I and 70 µl RDD) and incubated for 30 min at room temperature. Quality of RNA was verified with a non-denaturing 1% agarose gel and quantified using an ND-1000 spectrophotometer (Nano Drop).

Microarray

The RNA was prepared for labeling using the Target Amp 1 Round Aminoallyl aRNA amplification kit 101 (Epicenter Cat. No TAA1R4924). The aRNA was cleaned up using RNeasy spin columns and cDNA was digested on column using 80 µl of RNase-free DNAse I solution (10 µl DNAse I and 70 µl RDD). The aRNA eluted from the column was then quantified using the ND-1000 spectrophotometer and quality was checked using Agilent RNA 6000 Nano Kit on the Agilent 2100 Bioanalyzer. Approximately 15-20 mg of aRNA was concentrated using a Savant ISS110 SpeedVac Concentrator (Thermo Scientific) on the low temperature setting to a volume of 5 µl. Three µl of labeling buffer (25 mg $NaHCO_3$ in 1 ml nuclease free H_2O) were added to the aRNA sample. One tube each from a Decapack of NHS Alexa Fluor dyes 488, 555 and 647 (Invitrogen) was dissolved in 2 µl of anhydrous DMSO and vortexed for 10 sec to mix. Eight µl of aRNA in labeling buffer were added to the dye, vortexed briefly to mix and incubated at room temperature in the dark for 1 to 1.5 hours.

Unincorporated label and buffers were removed from the labeled aRNA using RNeasy spin columns (Qiagen Cat. No. 74106) and the aRNA and dye concentrations were determined using the ND-1000 Spectrophotometer. For each labeled aRNA, an aliquot representing 60 pMol of dye was dried in the dark using Savant ISS110 SpeedVac Concentrator (Thermo Scientific) on the low temperature setting. For microarray hybridizations, control aRNA labeled with Alexa Fluor 488 was mixed with two aRNA timepoints labeled with Alexa Fluor 555 and 647 in equimolar amounts of fluorescent dyes, and resuspended in 42.5 µl nuclease free H_2O and 42.5 µl 2x hybridization buffer (40-70% deionized formamide, 10x SSC and 0.2% SDS). Experiments were done in triplicate and dyes were swapped between samples of different experiments. The labeled aRNA mixture was denatured at 95°C for 2 min prior to adding the targets to the microarray. The aRNA mixture was pipetted onto a Meebo ReadyArray MM1100 (Microarray Inc.) and a cover slip was placed onto the array. The arrays were hybridized in a Corning

hybridization chamber and incubated at 42°C and 100% humidity overnight. The arrays were then incubated in 10 ml of wash solution 1 (2x SSC and 0.1% SDS) at 42°C for 5 min, transferred to 10 ml of wash solution 2 (0.1x SSC and 0.1% SDS) for 5 min at room temperature, and then washed twice in wash solution 3 (0.1x SSC) at room temperature for 1 min each. Arrays were blown dry with pressurized, filtered air and scanned using Axon GenePix 4400A array scanner (MDS analytical technology) and analyzed using GenePix Pro 7. Normalization of arrays was done using GenePix auto processor (GPAP) v3.2 (http://darwin.biochem.okstate.edu/gpap32/) using Global Loess Normalization and regression background correction.

GOterms were identified using DAVID (http://david.abcc.ncifcrf.gov/).

K-mean clustering by gene was done using Multi Expression Viewer (MeV) v4.9.0 with a Pearson Correlation Matrix of 9 clusters and a maximum iteration of 50.

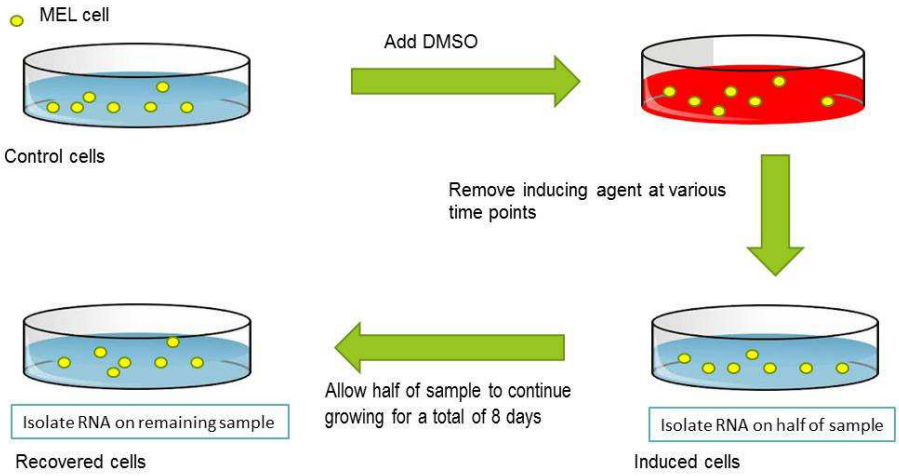


Figure 2.1: Workflow for pulsed induction method

Oligo name	Sequence
H1 DN	5’-CAGGATCCACTTATA**ACTCTATCAATGATAGAGT**CATTTCACGTTTATGGTGATTTCCC-3'
U6 UP	5'-GACGCTAGCCACAAGATATATA**ACTCTATCAATGATAGAGT**CTTTCAAGTTACGG-3'
H1 RC Shift	5'-CGTGAAATGACTCTATCATTGATAGAGTTATAAGTGGATCCTGAGACCG-3'
H1 UP	5’-GCGATATCACTAGGGGGAGGC-3’
U6 DN	5’-CCACGCGTCCCCAGTGGAAAGACGC-3’

Table 2.2.1: Oligonucleotides used for construction of pHIP-HUT. Blue sequence represents the TetR binding site and the underlined sequences are the degenerate portion of the primers

siRNA name	Oligonucleotide pair Sequences
copGFP siRNA 1	5’-AAAACAACAACGGCGGCTACACCAA-3’ 3’-GTTGTTGCCGCCGATGTGGTTAAAA-5’
copGFP siRNA 2	5’-AAAATCCCGCGCTCAGTCGTCCAAT-3’ 3’-AGGGCGCGAGTCATCAGGTTAAAAA-5’
copGFP siRNA 3	5’-AAAAGAGAGCGACGAGAGCGGCCTG-3’ 3’-CTCTCGCTGCTCTCGCCGGACAAAA-5’
No target transfected siRNA	5’-AAAACCAACCTCACCTCAACTTCAT-3’ 3’-GGTTGGAGTGGAGTTGAAGTAAAAA-5’

Table 2.2: siRNA oligos for insert into pHIP-HUT-TR transcription site with 4A 5’ sticky ends.

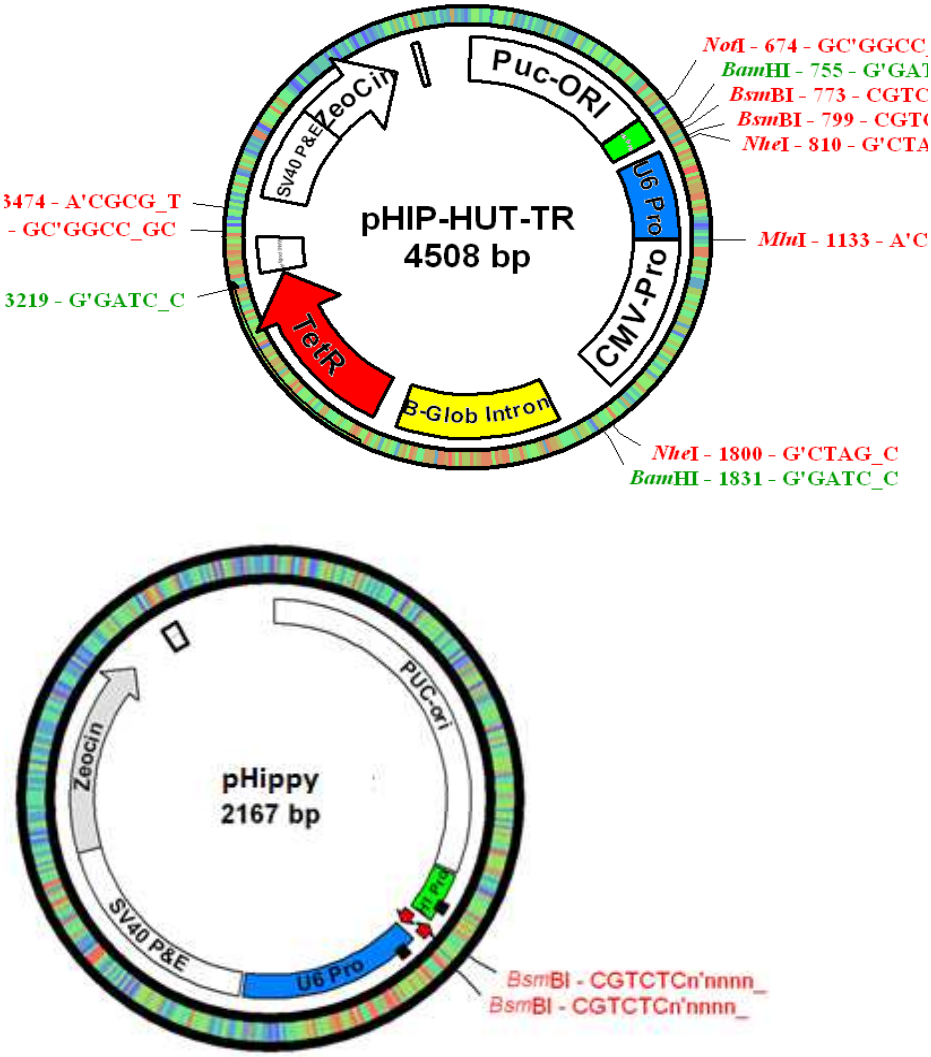


Figure 2.2: pHIP-HUT-TR and pHippy

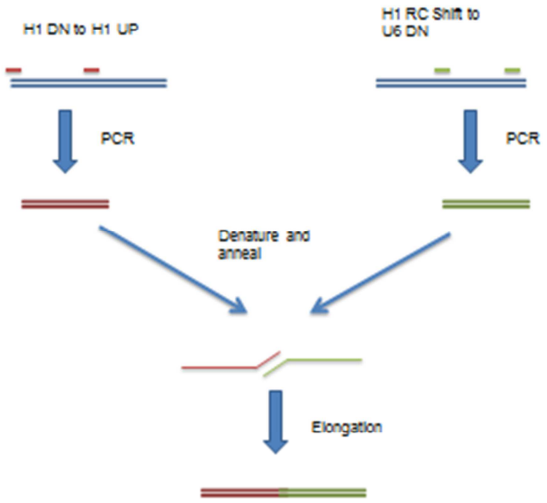


Figure 2.3: Sewing PCR of $H1^{TetO}$ and H1 RC Shift fragments

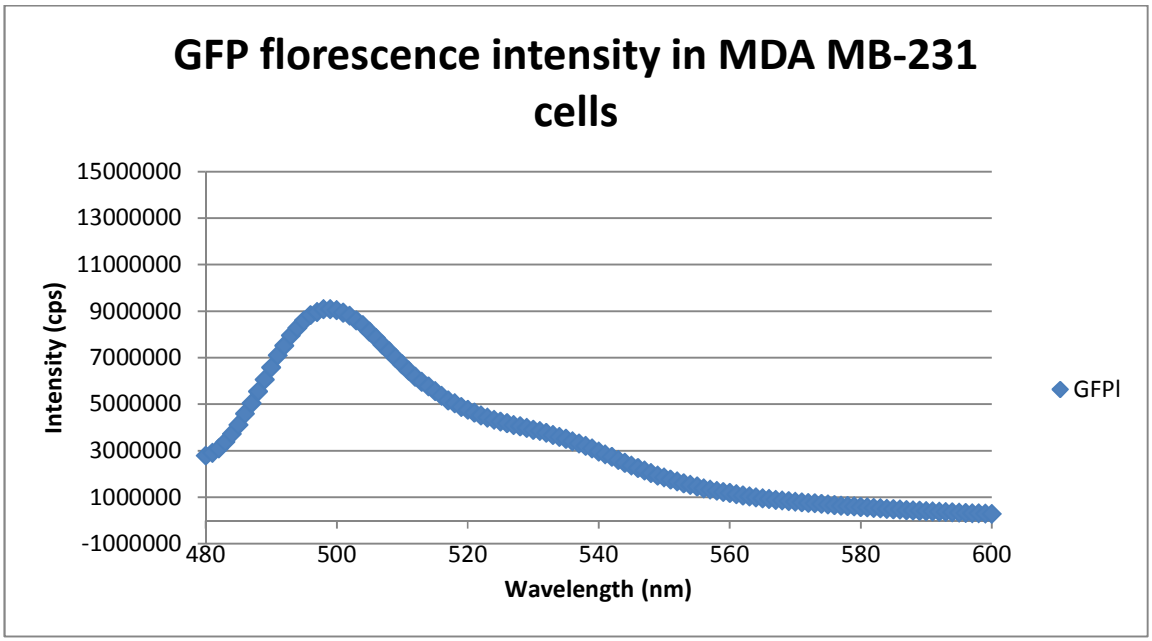


Figure 2.4: GFP emissions in MDA-MD-231 GFP/RFP stable cell line (Cell BioLab Inc.)

CHAPTER III

RESULTS AND DISCUSSION

Cell proliferation

To determine the effects of pulsed induction with 2% DMSO on proliferation and cell growth, I induced cells for 0, 0.5, 2, 12 and 18 hours and compared them to constitutively induced cells (Figure 3.1). There was no significant difference between proliferation of pulsed induced and non-induced (0 hour) cells. This finding indicates that treating cells with 2% DMSO will not adversely affect cell survival or proliferation. However, the constitutively induced cells begin to plateau two days earlier at day 3, whereas pulse-induced and control cells plateau at day 5 and at much higher cell density. The fold change in the constitutively induced cells, > 8 and < 16, indicates that MEL cells go through 4 divisions when undergoing terminal differentiation.

Microarray

Microarrays while limited to the probes on the array generate vast amounts of data. In order to reduce the complexity of the data many genes were filtered by certain criteria. First, genes that are differentially expressed (p-value of < 0.05) in both induced and recovered cells were sorted into two major categories; persistent differentially expressed genes and genes that showed opposed or compensatory expression (Table 3.1). Genes that showed persistent expression or opposed expression are considered candidate genes for further investigation. Genes that showed persistent expression changes are important for determination and/or differentiation. Genes that showed a compensatory change are ether 1) required for self-renewal or 2) only transiently required for determination or differentiation.

The increase in genes that are persistently expressed at the 18 hour time point indicates that some determination event had occurred during the 18 hours of induction. This was further supported by examining the effects on cell cycle genes (Figure 3.2). Seventeen genes known to be involved with cell cycle control showed persistent expression changes with a p-value of < 0.05 after recovery in the 18 hour time points, whereas none show a persistent change in the 2 hour time point and only 4 (*Ccna1*, *Cdk1*, *Cdc25a* and *Cdc25c*) showed changes in the 0.5 hour time point. Genes that showed persistent expression at the 18 hour time point have a greater change when recovered in 89.5% of the genes (Table 3.2), i.e. genes that are up-regulated become more up-regulated and down-regulated genes become more down-regulated after the inducing agent is removed. Contrary to the 18 hour time point, the 0.5 and 2 hour time points only have 16.7% and 26.3% of genes that have greater changes respectively. While at the

earlier time points, the genes showed persistent expression, most seem to be reverting towards the pluripotent state.

After categorizing the genes into the two categories, persistently expressed and opposing expression, four genes showed persistent expression and two genes showed opposing expression at all time points (Figure 3.12). The genes common in the persistent category are *Slc17a6*, *Ifit1*, *Serpine2* and *Zmym4*, the genes common to the opposing category are *Flt4* and *Musk*. *Slc17a6* (solute carrier family 17 member 6) is a vascular glutamate transporter. *Slc17a6* facilitates the uptake of glutamate in synaptic vesicles (Bai, Xu et al. 2001). *Ifit1* (Interferon induced protein 1) with tetratricopeptide receptor 1 plays a role in inhibiting viral translation by binding to single stranded RNA with a 5' triphosphate group (PPP-RNA) (Fensterl, White et al. 2008). *Serpine2* (serpin peptidase inhibitor clade E nexin plasminogen inhibitor type 1 member 2) inhibits enzymes such as thrombin, trypsin, urokinase and promotes neurite extension (Heit, Jackson et al. 2013). *Zmym4* (zing finger MYM type 4) plays a role in cell morphology and cytoskeletal organization (Smedley, Hamoudi et al. 1999). *Flt4* (Fms related tyrosine kinase 4) is a cell surface receptor for vascular endothelial growth factors VEGFC and VEGFD (Galland, Karamysheva et al. 1993). Flt4 plays a role in lymphangiogenesis, development of the vascular network and cardiovascular system. *Flt4* promotes proliferation, cell survival and migration of endothelial cells. *MUSK* (muscle skeletal receptor tyrosine kinase) is involved in MAPK signaling. *MUSK* is involved in the maintenance of neuromuscular junction regulates the actin cytoskeleton and clustering of the acetylcholine receptor (DeChiara, Bowen et al. 1996).

To identify genes involved in self-renewal I looked at genes that were down-regulated when cells were induced and up-regulated when they recovered. Several Rho protein expressions fit this pattern (Figure 3.3) which makes these excellent candidates for self-renewal. *Rhoa* and

Rhot1 are small GTPases that act as secondary signaling molecules to propagate signals within a cell. *RhoBTB1* and *Arhgef5* are Rho guanine nucleotide exchange factors that exchange GDP for GTP in Rho proteins, promoting their activity. *Rtkn* is a scaffolding protein that interacts with the active GTP bound form of Rho proteins. All these proteins have the same expression patterns, when induced: the abundance of transcripts dropped and, when the cells were allowed to recover, their expression increased to help the cell recover to the pluripotent state. This is further supported by the expression of *Arhgap24* which is a Rho GTPase activating protein. This causes the catalysis of GTP to GDP inactivating the Rho protein. *Arhgap24* showed the inverse expression of all the other Rho proteins identified in this study. When induced *Arhgap24* turns off the Rho protein signaling and when the cells are allowed to recover the expression of *Arhgap24* dropped off releasing the repression of the Rho proteins. Furthermore even after a determination event the Rho proteins still play a role in self-renewal of the cells that are in an intermediate state.

Other proteins that showed expression patterns suggesting they are involved in self-renewal are ubiquitin and SUMO. Ubiquitin and SUMO, while it is difficult to determine just how and what they are interacting with, I hypothesize that genes following this pattern of expression (Figure 3.4), regulate differentiation and promote self-renewal. Furthermore SUMO and ubiquitin may play a role in erasing memory of MEL cells. The Ubiquitin C gene *Ubc* was down-regulated when induced and up-regulated during recovery at the 2 and 18 hour time points. *Ubc* may regulate transcription factors through protein degradation or modify proteins, changing their kinase activities. The SUMO genes sumo3 and sae1 showed the same pattern of gene expression at the 18 hour time point. *Sumo3* is a small ubiquitin-like molecule that can post-translationally modify lysines of other peptides possibly altering the function of the proteins or targeting the proteins for degradation. *Sae1* is responsible for catalyzing isopeptide bond formation with

SUMO. *Senp3,* in contrast to *Sae1,* hydrolyses the isopeptide bond of a SUMOlated protein causing the release of SUMO. *Senp3* showed up-regulation when cells were induced and becomes down-regulated when they were allowed to recover indicating that de-SUMOylation of some proteins may be important for progression of commitment or differentiation.

Mechanistic target of rapamycin complex 2 (mTORC2) plays a role in protein synthesis and the PIP3/Akt signaling pathway. The major components of the complex are mtor, Rictor and mist (Figure 3.5). Mist8 is the only major component that showed significant changes in both induced and recovered cells and follows the pattern that suggests the gene is involved in self-renewal. Rictor showed the same pattern and was significantly down-regulated when induced. However, its change in the recovered cells, while elevated, is not significant, indicating that *Mist8* may be a major regulator for the complex during erythropoiesis. Mtor expression was lower in induced and recovered cells but the changes were not significant with a p-value > 0.05. Overall this suggests that mTORC2 may play a role in self-renewal.

Transporters may play a role in bringing signaling molecules into the cell and regulating the pH of the cytoplasm as the cell changes from basophilic to acidophilic (Figure 3.6). Slc10a4 is an orphan of the Slc10a family that transports bile acids. While the substrate of Slc10a4 is not known, its expression indicates that it may play a role in self-renewal. Slc10a4 is most similar to Slc10a1 and is likely to transport an organic acid, which may also regulate the pH in the basophilic cytoplasm of proerythroblasts and basophil erythroblasts. As the cells differentiate and the cytoplasm becomes more acidic, Slc17a6 can sequester glutamate in vesicles; Slc17a6 is also a good candidate gene for commitment because it is persistently expressed at all time points. Not only do the cells sequester acidic amino acids but Slc7a2 transports arginine into the cell to help regulate cytoplasmic pH. Arginine is also the substrate for nitric oxide synthases

(NOS) which will produce NO which may act as a paracrine signaling molecule and also competitively bind heme to prevent developing cells from pulling an over-abundant amount of O_2 out of the blood before they begin circulation.

FLT4 may play a role in signaling changes to the cell cycle during differentiation. *Flt4* was up-regulated during all time points when cells were induced and down-regulated when they recovered (Figure 3.7). Flt4 responds to VEGFC and VEGFD, propagating the signal to RASA1 which in turn can phosphorylate CIP1 and KIP1, causing the switch in the cell cycle. *Rasa1* was up-regulated when cells were induced at all time points, but when they recovered, changes in *Rasa1* were not significant. Because of *Flt4*'s compensatory changes, it is possible that *Flt4* is only transiently activated during differentiation thus its expression is not required to continue when the signal has been passed to downstream targets.

The vasopressin receptor Avpr1B, like Flt4, may also be a transiently activated signal for commitment. Avpr1B is a G-protein coupled receptor and Grk5 is released when Avpr1B binds vasopressin. Similar to *Flt4*, *Avpr1B* only showed a transient activation (Figure 3.8) where it is up-regulated when cells were induced and down-regulated when they recovered. Conversely, Grk5 was persistently expressed and showed a marked increase in recovered cells compared to induced cells. Because both increased at the 2 hour and not at the 0.5 hour time point, it is likely that Avpr1B is not one of the first responders to the induction but may be involved in transmitting the signal for commitment to downstream transcription factors such as NF-kB1

Mitogen activated protein (MAP) kinases *Mapk3*, *Map3k8* and *Map3k14* all showed up-regulation when cells were induced at 2 hours but reverted to normal levels when they recovered. However, at 18 hours, *Map3k8* and *Map3k14* showed opposing expression (Figure 3.9). This indicates that Map3k8 and Map3k14 may only need to be transiently activated during

differentiation, activating other proteins such as transcription factors. Mapk3 induction dropped off from 2 to 18 hours of induction but, when cells were allowed to recover after 18 hours, *Mapk3* showed a strong, persistent up-regulated change. This may allow for persistent activation of many MAP kinase targets to be active causing a permanent change from the original pluripotent state of the control cells.

Many transcription factors or components of a transcription factor complex were differentially expressed, several of which are targets of MAP kinases such as NF-kB1, Myc, Pou2af1 and Mef2c (Figure 3.10a). Not only is nuclear factor kappa B 1 (NF-kB1) activated by MAP kinases it may also be activated by vasopressin stimulation. NF-kB1 is a transcription factor that activates a variety of biological processes. While *NF-kB1* was induced at 2 hours, NF-kB1 didn't show persistent expression until 18 hours. With its early induction and persistent expression after 18 hours, NF-kB1 may transcribe genes required for commitment and maintain the subsequent intermediate state. It is also suspected that NF-kB1 plays a role in the formation of cardiac muscle, so it may be a nonspecific regulator of vascular differentiation. Myc is a transcription factor that targets cell cycle progression genes and has been implicated in various hematopoietic processes including erythropoiesis. *Myc* was up-regulated and showed persistent expression only at the 0.5 hour time point but was up-regulated during all time points in induced cells. Along with Cip1 and Kip1, Myc may regulate the changes in the cell cycle when differentiation is induced. Pou2af1 is a transcriptional coactivator that is known to associate with Oct1 or Oct2 by recognizing the POU domain. *Pou2af1* expression was up-regulated at all time points and was persistently expressed at 18 hours. Based on the expression of Oct1 and Oct2 (Pou2f1 and Pou2f2, respectively) (Figure 3.10b), Pou2af1 interacts with Oct2 and not Oct1 to promote commitment. Mef2c is a transcriptional enhancer that has been reported to play a role in myogenesis and maintaining the differentiated state of muscle cells. *Mef2c* was

persistently expressed at 18 hours and was greatly up-regulated in recovered cells. Given its role in muscles and its expression in erythropoiesis, Mef2c may promote the transcription of commitment genes and help maintain the intermediate state.

During erythropoiesis changes in the cytoskeleton are required to give the erythrocytes their donut shape; however, changes in the cytoskeleton start occurring immediately after induction and some changes persist even before commitment. Cytoskeleton regulatory proteins Zmym4 and Stmn2 may play a role in the reorganization of the cytoskeleton during differentiation of erythrocytes (Figure 3.11). *Stmn2* was persistently up-regulated at the 18 hour time point and is known to be involved in neuronal growth and osteogenesis. Zmym4 also regulates interactions between the cytoskeleton and the membrane and was persistently down-regulated at all time points. The persistent down regulation of Zmym4 indicates that cytoskeletal organization may be important for self-renewal and may be one of the first changes to occur. Stem2, in the absence of Zmym4, may regulate the structural changes of the cytoskeleton in a developing erythrocyte post commitment.

To compare our data to the literature I created a list of 35 genes known to be involved with erythropoiesis (Table 3.3) (Müllner, Dolznig et al. 1996; Trumpp, Refaeli et al. 2001; Dolznig, Grebien et al. 2006; Ghaffari, Kitidis et al. 2006; Heuser, Yap et al. 2009; Harandi, Hedge et al. 2010; Rowlinson and Gering 2010; Hattangadi, Wong et al. 2011; He, Kim et al. 2011; Zhou, Wang et al. 2011; Cai, Langer et al. 2012; Sankaran, Ludwig et al. 2012; Trowbridge and Orkin 2012; Zhang, Prak et al. 2013). I found 63% (22/35) of the genes represented on the list matched our list of candidate genes. Perhaps the genes that were not found in our list of candidate genes were not found because I was only looking at early time points of induction from cells in the proerythroblast stage.

Gene Ontology

In order to elucidate what cellular processes were accruing, gene ontology terms were identified with a shared GO term search with a p-value cut off for over expressed GO terms of < 0.05 (Table 3.4). At the 0.5 hour time point heparan sulfate sulfotransferase activity was a GO term identified. Heparin sulfate plays a role in maintaining the membrane. This is not surprising considering the cells were treated with DMSO which disrupts membranes. Apolipoprotein binding was also identified, indicating that a lot of interactions are occurring at the membrane. This is further supported by calcium ion binding, calcium-dependent phospholipid binding activity and inositol 1, 4, 5 trisphosphate binding. Additional GO categories identified that may play a role in signal transduction are, guanyl nucleotide exchange factor activity, carboxylic acid binding and transport and nitric oxide synthase regulator activity. Carboxylic acid binding and transport activity also indicate transporters playing a role in maintaining pH of the cytoplasm. Arginine is brought into the cell during differentiation, which may regulate the pH as cells become more acidophilic; however, arginine is also the substrate for nitric oxide synthase (NOS) indicating that nitric oxide (NO) may play a role in a developing erythrocyte. NO can act as a paracrine signaling molecule causing the formation of cyclic GMP, or it can competitively bind heme preventing oxygen from the blood being pulled away by non-circulating developing erythrocytes. Also, as expected in developing erythrocytes, heme binding was represented in the GO terms.

Pathway analysis

For the pathway analysis, the expression data of opposing and persistently expressed genes were analyzed separately using reactome.org's (http://www.reactome.org/) analysis tools. The opposing expressed genes showed the same pattern in all time points for signaling of Rho GTPases. The Rho GTPase signaling pathway was down-regulated when cells were induced and when cells were allowed to recover the Rho GTPase pathway was up-regulated. This made the Rho signaling a good starting point for building a model for self-renewal in MEL cells. Interestingly the SUMOylation pathway had the greatest mean difference of all the pathways identified by reactome.org. The mean difference of expression in a pathway is the change in all genes associated in the pathway when comparing induced and recovered cells. This indicates that SUMO could play an important role in self-renewal by posttranslationally regulating proteins. Some other pathways that were well represented and matched some of the GO terms are PIP3 and Akt signaling. The largest pathway for genes that were persistently expressed and up-regulated was the mitogen activating protein kinase (MAPK) cascade. Other pathways that had high mean changes are 'binding and uptake of ligands by scavenging' and 'extracellular matrix organization' which were greater than four fold up-regulated while DNA replication was down-regulated. Similar to gene expression at the 18 hour time point, about 90% all pathways that showed a persistent change had a higher mean change in the recovered cells (Table 3.5).

Protein-protein interactions

To look at protein-protein interactions I used the web tool STRING (http://string-db.org/). Using opposing expressed genes that showed down-regulation when cells were induced and up-regulation when they recovered I was able to link Rho GTPases, GTPase activator proteins (GAPs) and GTPase enhancer proteins (GEFs) to Akt3 and mTORC2. I then added erythropoiesis markers to the list of candidate genes, to identify any interactions between candidate genes and known markers (Figure 3.13). With these interactions I began building a model for self-renewal around the Rho proteins. To look at commitment I used persistently expressed genes that were up-regulated and filled in some of the gaps with genes that were up-regulated when induced in all 3 time points even if they were not persistently expressed. Many of the interactions centered around MAPK proteins. Some of the candidate genes that were linked to the MAPK cascade are the vasopressin G-protein coupled receptor, Flt4 and several transcription factors. I then added the erythropoiesis markers to identify how our candidates may interact with known genes (Figure 3.14).

Model

The self-renewal model (Figure 3.15) was made using primarily genes that were down-regulated when cells were induced and up-regulated when they recovered. This is because the genes with this expression pattern are compensating for the changes that occur during the short induction period, indicating the genes are responsible for maintaining the self-renewal capabilities of the pluripotent cell. By using the information provided by gene ontology, pathway analysis and STRING protein-protein interactions I developed a model in which PIP3 signals mTORC2, which regulates Akt3 and several Rho GTPases which in turn regulate cell cycle control genes Cip1 and Kip1 and the transcription factor GATA1. Rho GTPases along with CDC42 regulate cell survival. mTORC2 can also regulate protein synthesis by regulating eukaryotic initiation factors, several of which are identified as candidates for self-renewal by their expression patterns. Furthermore, protein synthesis decreases as the cells differentiate into erythrocytes (Wang and Proud 2006), which supports the idea that mTORC2 needs to be down-regulated as cells differentiate. Another feature of my model is the transporter Slc10A4, which may bring in an early signal for differentiation or commitment. Slc10A4 shares a similar expression pattern as other members of the model. Other members of the Slc10A family transport bile acids into the cell; however, Slc10A4 is an orphan in the Slc10A family because it does not transport bile acids. Given its similarity to Slc10A1, it may transport an organic acid into the cell which could act as signaling molecule and help regulate the pH as the cell is basophilic during the early stages of differentiation.

The model for commitment (Figure 3.16) was made with persistently expressed genes and genes up-regulated during induction to fill in putative gaps in the protein-protein interactions. Genes that show a persistent change may be associated with commitment because, if the changes are permanent, this indicates the cells did not revert to their original pluripotent phenotype when cells were allowed to recover. Genes that revert to basal levels or become down-regulated may only be transiently required for commitment or differentiation. In the commitment model the amino acid transporter Slc7a2 brings in L-arginine into the cell and Slc17a6 sequesters glutamate in vesicles to help regulate cytoplasmic pH as the cells become more acidophilic. The Vascular Endothelial Growth Factor Receptor 3 (VEGFR3 also called Flt4) can signal the switch to the differentiation cell cycle by activating the CDK inhibitors Cip1 and Kip1. During early time points the vasopressin receptor Avpr1B, along with a MAPK cascade, signals various transcription factors Mef2c, Pou2af1, NF-kB and Rad18 and cytoskeleton/membrane interaction regulator protein Stmn2. Stmn2 and Zmym4 may control the change in shape from a globular cell to the donut shaped erythrocyte.

pHIP-HUT-TR construction and validation.

pHIP-HUT-TR (Figure 3.1 Materials and Methods) was constructed as described in Materials and Methods, and the sequence of the modified promoters was confirmed using Sanger di-deoxy DNA sequencing (Figure 3.17).

Validation of pHIP-HUT-TR was done by knockdown of copGFP using siRNA (Table 3.2 Materials and Methods). First, the limit of detection was determined by taking GFP fluorescence readings of MDA-MB-231 GFP/RFP stably transformed cells (Figure 3.18). For detection above background, it was determined that $6.27x10^4$ cells would be needed. Although some experiments clearly showed a knockdown effect (2 of 4 biological replicates) of the siRNA siR1 and siR3 (Figure 3.19), the combined experiments showed inconsistent knockdown of the GFP expression. Overall, 2 biological replicates showed about a 50% decrease in fluorescence whereas 2 showed no significant decrease. The variations in the biological replicates could be due to inconsistent transformation of cells.

Conclusions

I have developed a novel method that I am calling pulsed induction to identify genes involved in determination events and self-renewal. Using this method I was able to identify many genes that have been identified as being involved with erythropoiesis as well as many genes that may play a novel role in the development of erythrocytes. Using various bioinformatics tools, I have developed a model for self-renewal and commitment. The model illustrates how some of the novel genes may play a role in regulating known markers of erythropoiesis. Future work would involve validating models designed using the pulsed induction method.

I also constructed an inducible bi-directional siRNA expressing vector pHIP-HUT-TR which uses a tetR system to control expression of the inserted siRNA. However, experiments testing its gene silencing ability remain inconclusive as only half of the biological replicates seemed to show a 50% knock down and two showed no significant changes, making the average around 75% knock down with high variation. Further testing of pHIP-HUT-TR needs to be done with various siRNA and cell lines to further validate the vector.

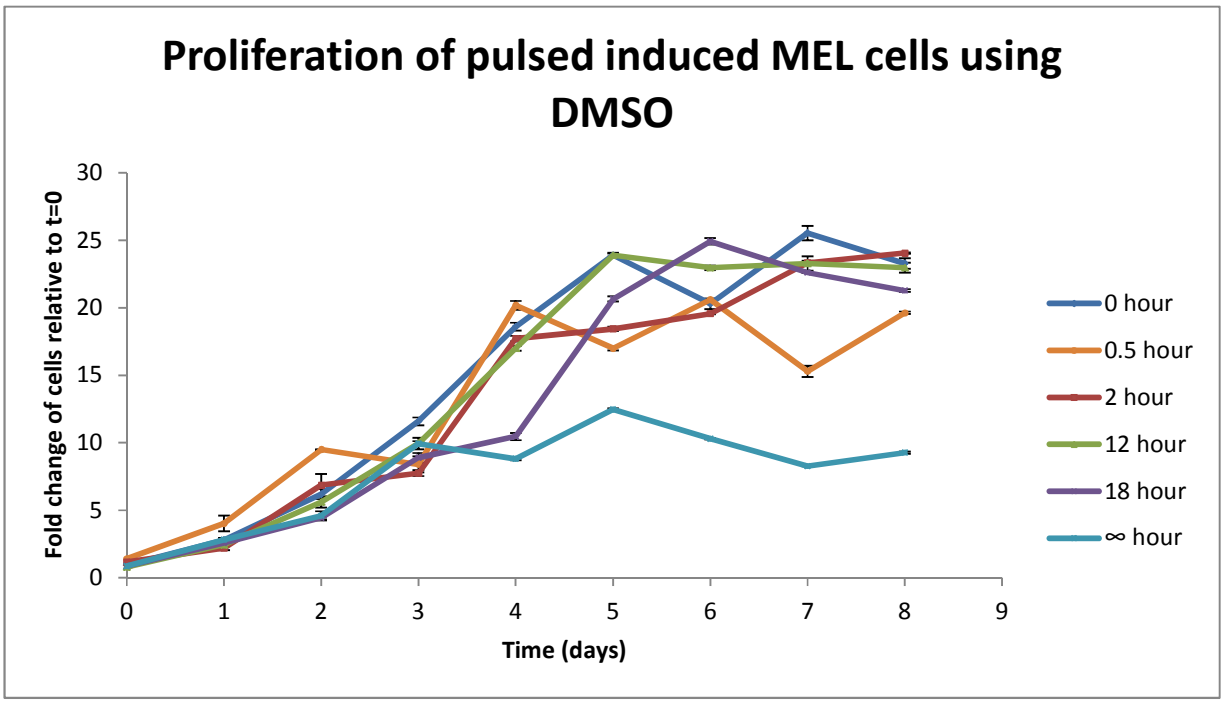


Figure 3.1: Cell proliferation of pulsed induced cells. Cells constitutively induced (light blue line) plateau around day3 whereas 0, 12 and 18 plateau around day 5 and 2 hours never plateaus but its growth does slow.

Number of genes that show differential expression in persistent and opposed gene expression categories by time point			
Time point	Total differentially expressed genes	Genes with persistent expression	Genes with opposed expression
0.5 hour Induced	3513	468	479
0.5 hour recovered	7100		
2 hour Induced	4807	133	63
2 hour recovered	1088		
18 hour Induced	5787	1320	1549
18 hour recovered	15354		

Table 3.1: Number of genes differentially expressed at the time points as well as the number of genes that are persistently expressed and have opposing expression all with a p-value of < 0.05.

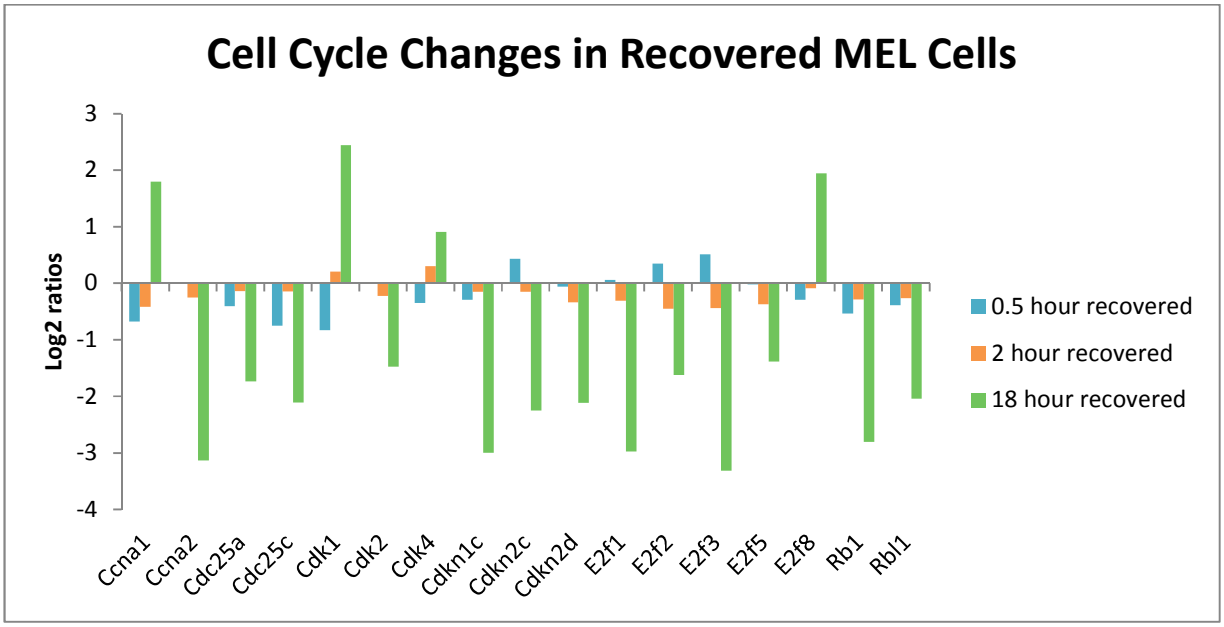
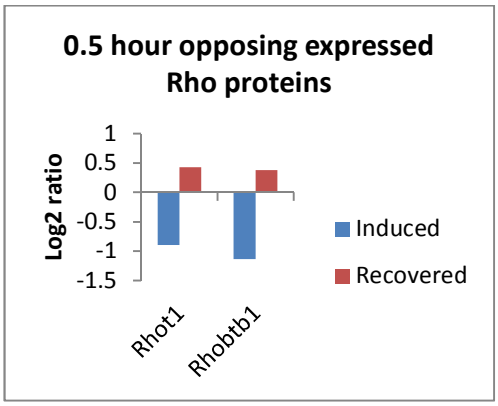


Figure 3.2: Gene expression for 17 cell cycle genes that show persistent changes in the 18 hour recovery cells.

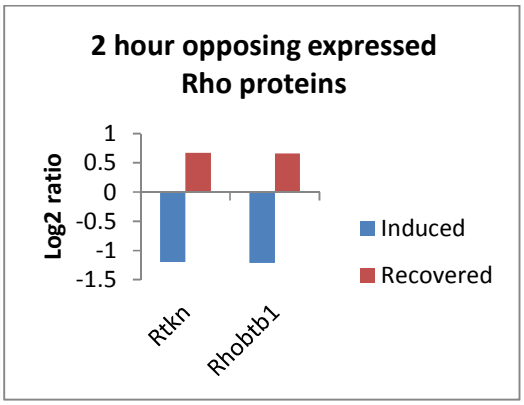
Time point	% of persistent genes with a greater change in recovered cells
0.5 hour	16.7%
2 hour	26.3%
18 hour	89.5%

Table 3.2: Percentage of persistently expressed genes that show a greater change in recovered cells when compared to induced cells, indicating that an epigenetic event has accured between 2 hour and 18 hour.

A



B



C

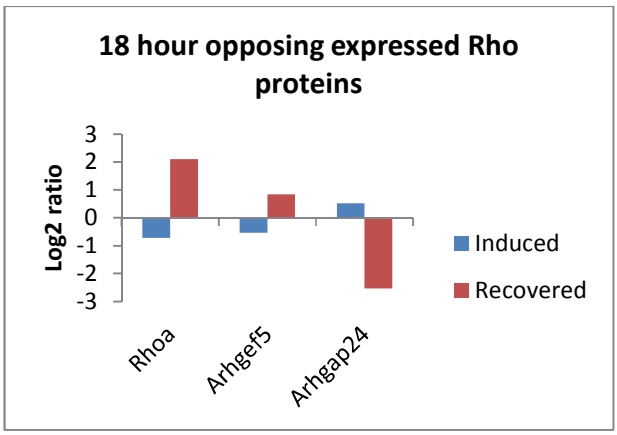


Figure 3.3: Rho proteins and Rho associated proteins that show opposing expression at different time points. A B and C show Rho proteins at the 0.5 hour, 2 hour and 18 hour time points respectively.

A B

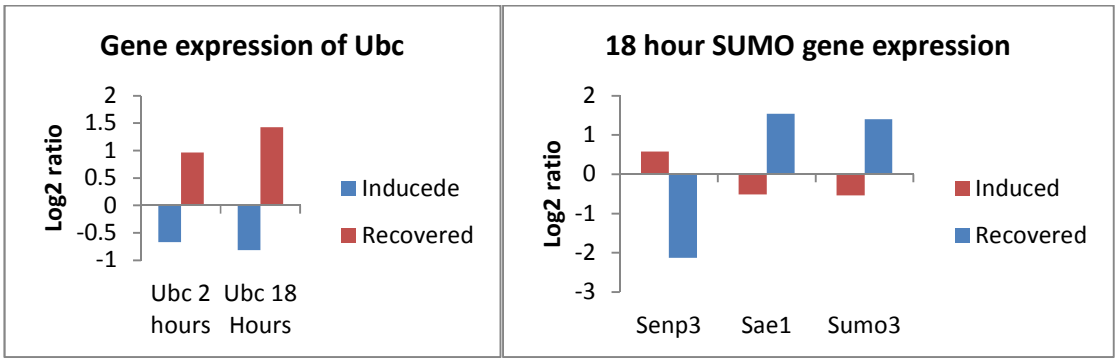


Figure 3.4: A. gene expression for Ubc at 2 hour and 18 hour time points. B. SUMO related genes sumo3, sae1 and senp3 gene expression at the 18 hour time point.

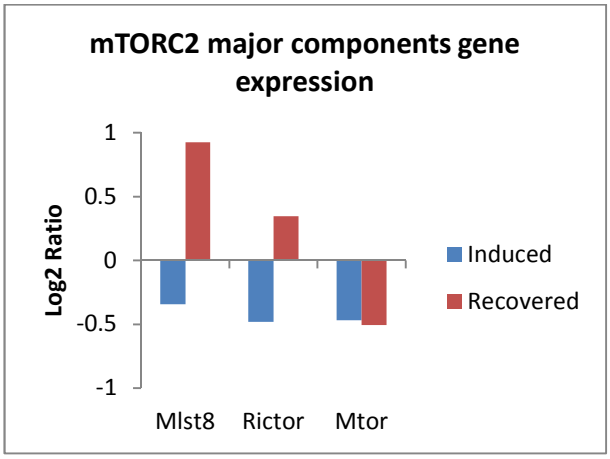


Figure 3.5: mTOR complex 2 major components' gene expression. Mtor expression, while down is not significantly differentially expressed in ether Induced or recovered cells, nor is Rictor in recovered cells.

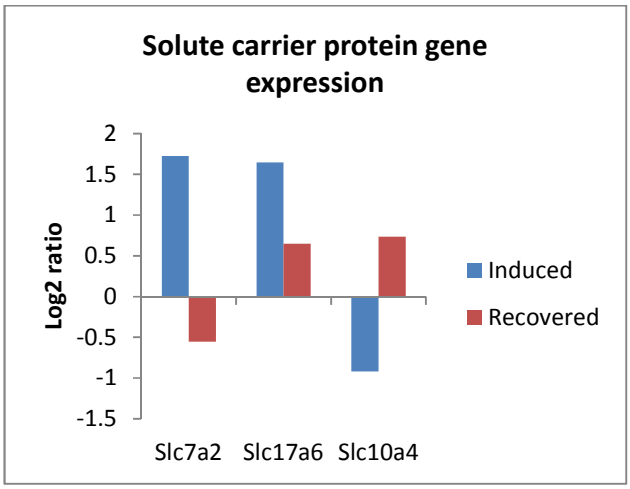


Figure 3.6: Gene expression of solute carrier transport proteins SLC7a2, Slc17a6 and Slc10a4 after 2 hour of induction and recovery.

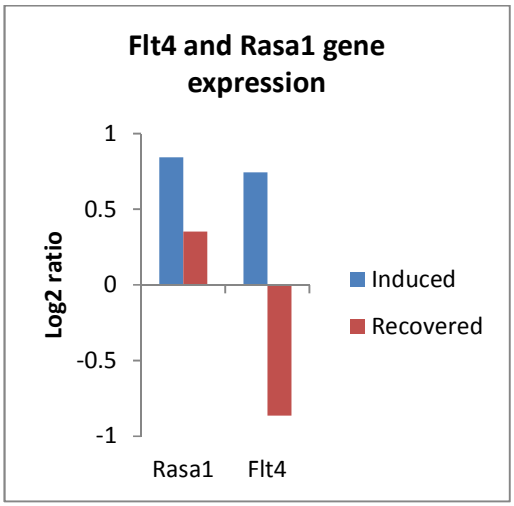


Figure 3.7: Gene expression of Flt4 and Rasa1 at the 18 hour time point.

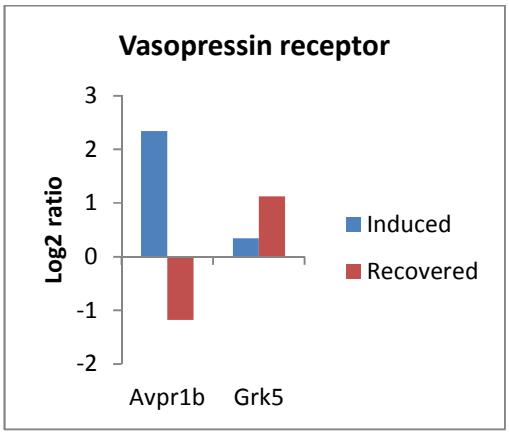


Figure 3.8: Gene expression of the vasopressin receptor Avpr1b and its associated G-protein Grk5 at the 2 hour time point.

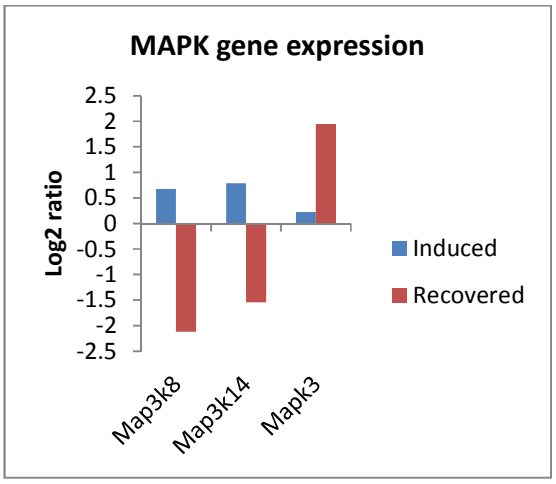
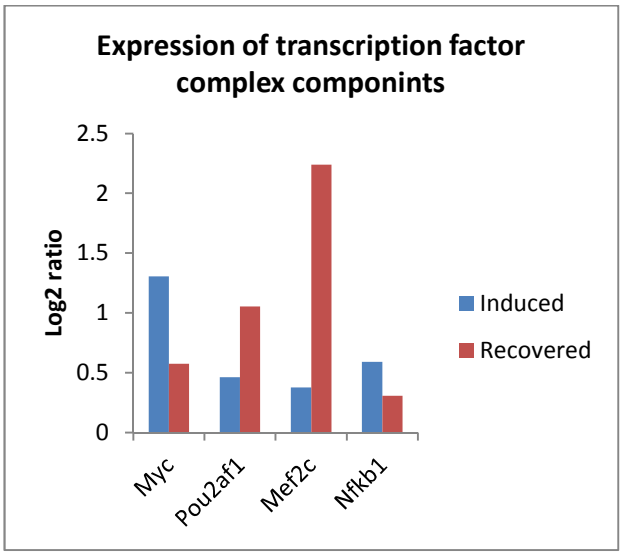


Figure 3.9: Gene expression of mitogen activated protein kinases that have known or predicted interactions with several candidate genes.

A



B

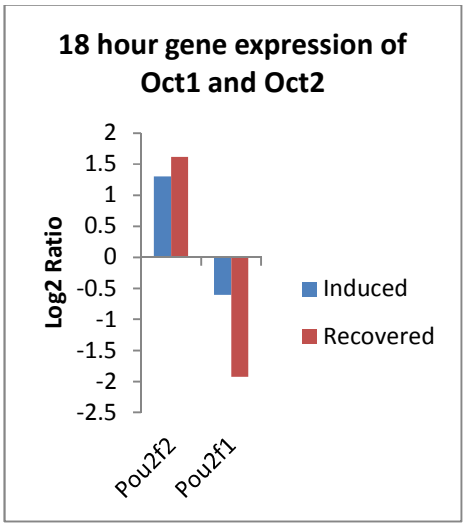


Figure 3.10: A) Gene expression of transcription factors Myc and NF-kB1 at the 2 hour time point and transcription activators/enhancers Pou2af1 and Mef2c at the 18 hour time point. B)Gene expression of Oct1 and Oct2 (Pou2f1 and Pou2f2 respectively).

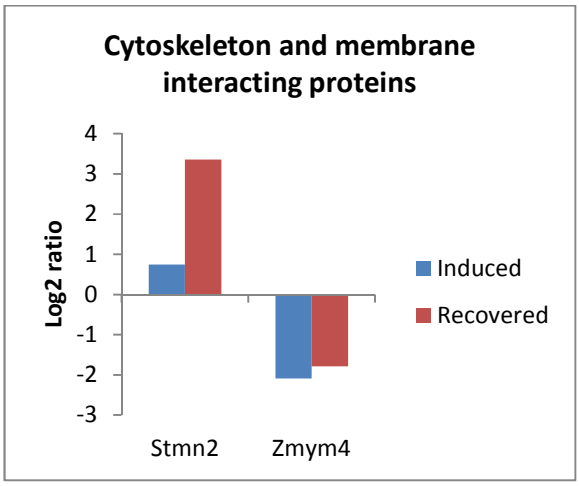


Figure 3.11: Gene expression of cytoskeleton regulator proteins at the 18 hour time point.

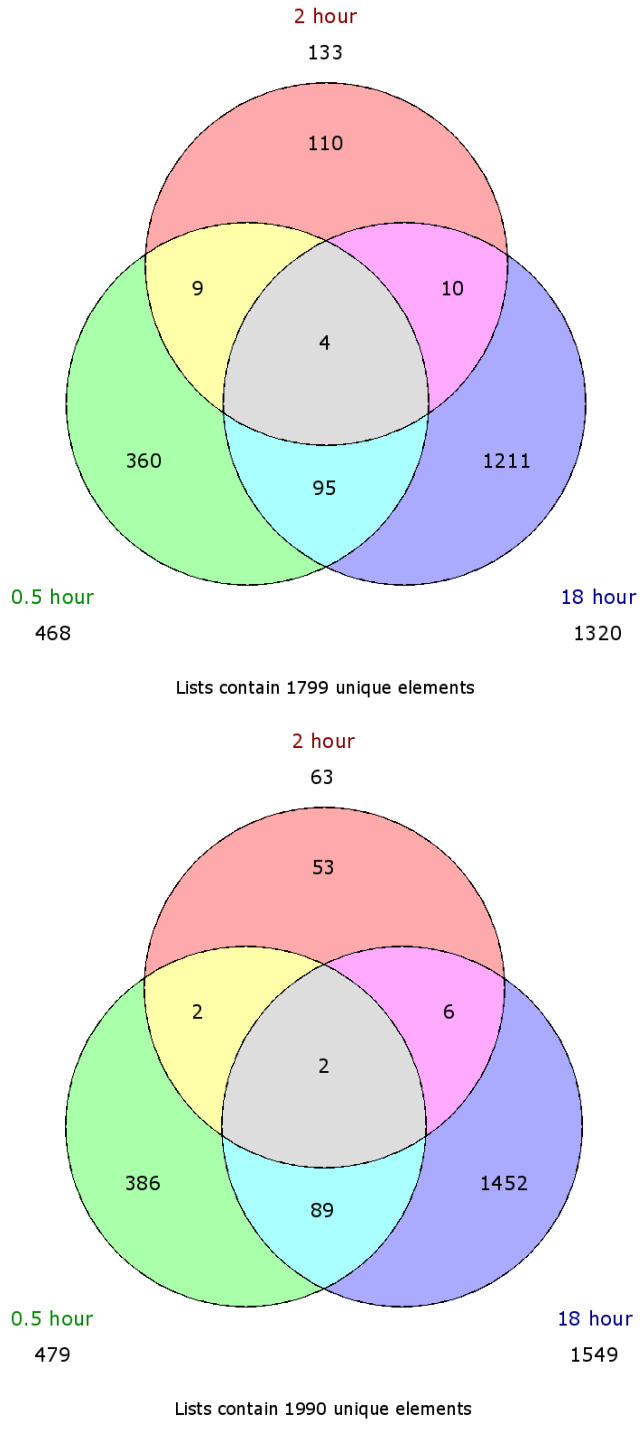


Figure 3.12: The top Venn diagram shows the all persistently expressed genes whereas the bottom Venn diagram show all genes with opposing expression in all time points.

	Gene symbol	Candidate genes identified by pulse induction
1	GATA1	Yes
2	Runx1	Yes
3	Dnmt3a	Yes
4	E2f2	Yes
5	E2f8	Yes
6	Notch2	Yes
7	GATA2	No
8	GATA3	No
9	Cdkn1a (p21)	No
10	Cdkn1b (p27)	Yes
11	Cdc42	No
12	Jagged 2	No
13	Sfpi1	No
14	BMP4	Yes
15	Akt1	No*
16	Myc	Yes
17	Klf1	No*
18	Epor	Yes
19	Smad5	Yes
20	Lmo2	Yes
21	Suz12	Yes
22	Ezh2	Yes
23	P300	Yes
24	Lmo2	Yes
25	Hbb	Yes
26	Sox17	No*
27	Bmi1	Yes
28	ZFP36	Yes
29	Meis1	No
30	MLL5	Yes
31	STAT1	Yes
32	Jak1	Yes
33	STAT5	No*
34	FOG1	No
35	Lsd1	No

Table 3.3: Several genes involved in erythropoiesis and whether or not our pulsed induction method has identified them as candidate genes. * indicate that a member of the same family was identified e.g. akt3 instead of akt1.

GO term
Heparan sulfate sulfotransferase activity
Apolipoprotein binding
Calcium ion binding
Calcium dependent phospholipid binding
Guanyl nucleotide exchange factor activity
Carboxylic acid binding
Carboxylic acid transmembrane transport activity
Heme binding
Nitric oxide synthase regulator activity
Inositol 1, 4, 5 trisphosphate binding

Table 3.4: Gene Ontology categories of candidate genes

Pathway	18h_0d_M	18h_8d_M
Organelle biogenesis and maintenance		
Binding and Uptake of Ligands by Scaveng	1.0	3.3
Extracellular matrix organization	0.9	2.3
Muscle contraction	0.8	1.2
Reproduction	0.5	1.1
Meiosis	0.2	1.0
Transmembrane transport of small molecul	0.2	0.8
Cell-Cell communication	0.3	0.8
Hemostasis	0.2	0.7
Neuronal System	0.2	0.7
Signal Transduction	7.86E-02	0.6
Metabolism	0.1	0.5
Developmental Biology	0.3	0.5
Not assigned	5.14E-02	0.4
Metabolism of proteins	6.64E-02	0.4
Disease	1.12E-02	0.3
Immune System	-0.3	-1.04E-02
Cellular responses to stress	-0.2	-5.33E-02
Circadian Clock	9.10E-02	-0.2
Membrane Trafficking	0.1	-0.3
Cell Cycle	-0.3	-0.3
Gene Expression	-0.2	-0.4
Chromatin organization	-0.1	-0.7
Apoptosis	-0.3	-0.7
DNA Repair	-6.28E-02	-0.7
DNA Replication	-0.6	-1.7

Table 3.5: Pathway analysis of the 18 hour time point, with induced expression on the left and recovered expression on the right.

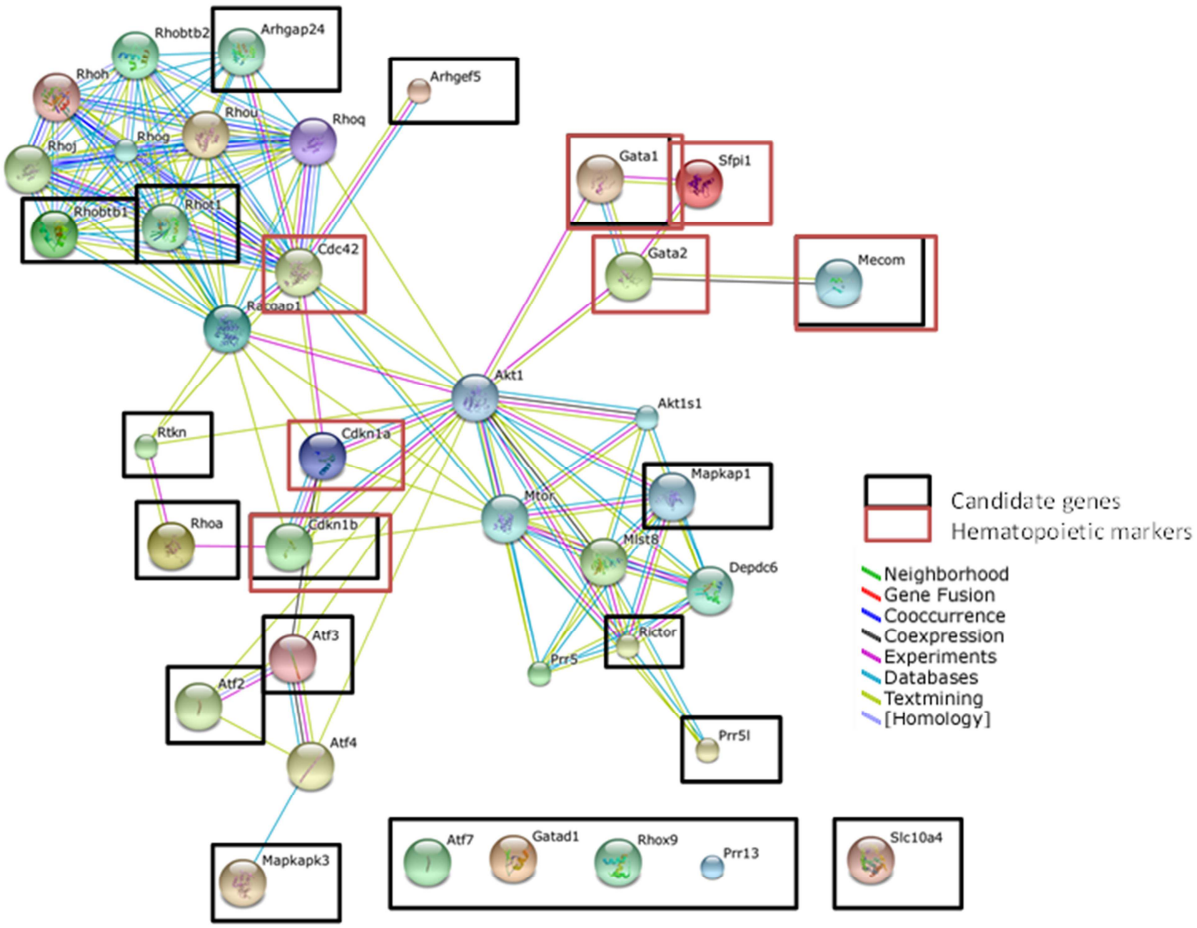


Figure 3.13: STRING protein-protein interactions for self-renewal. Candidate genes are indicated by black boxes and erythropoiesis markers are indicated by red boxes. The lines between proteins indicate how the interaction has been determined from the STRING database.

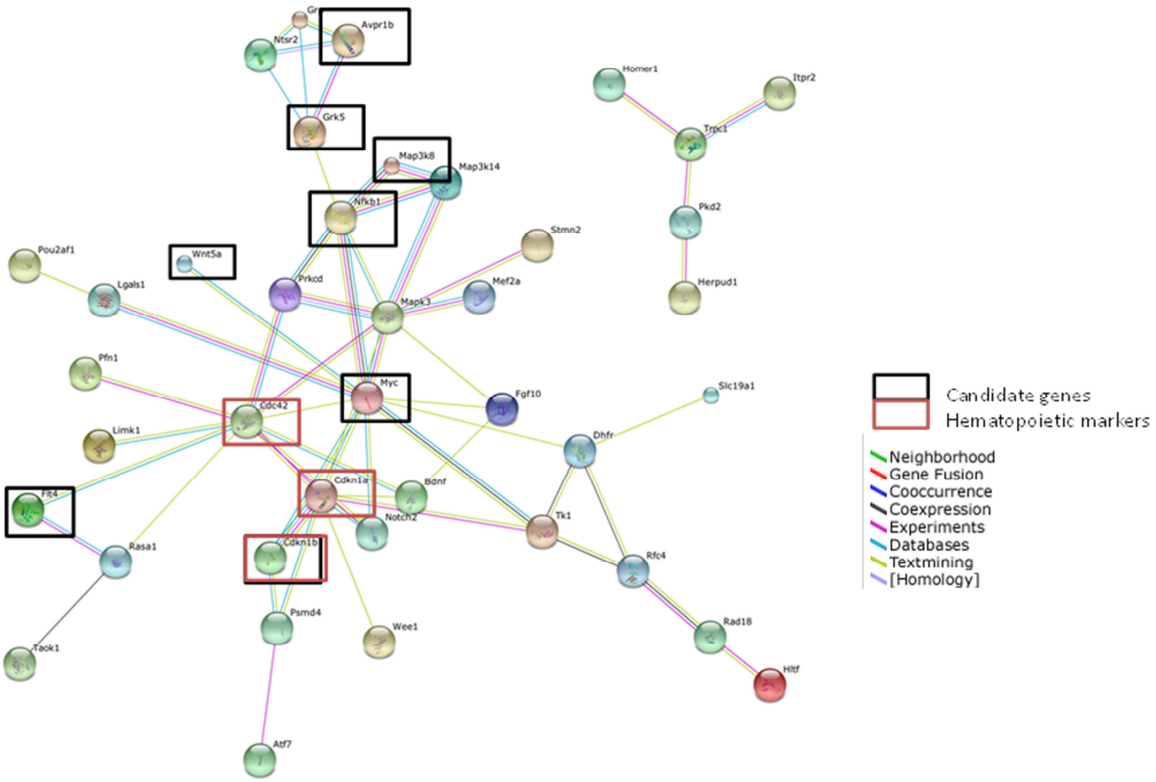
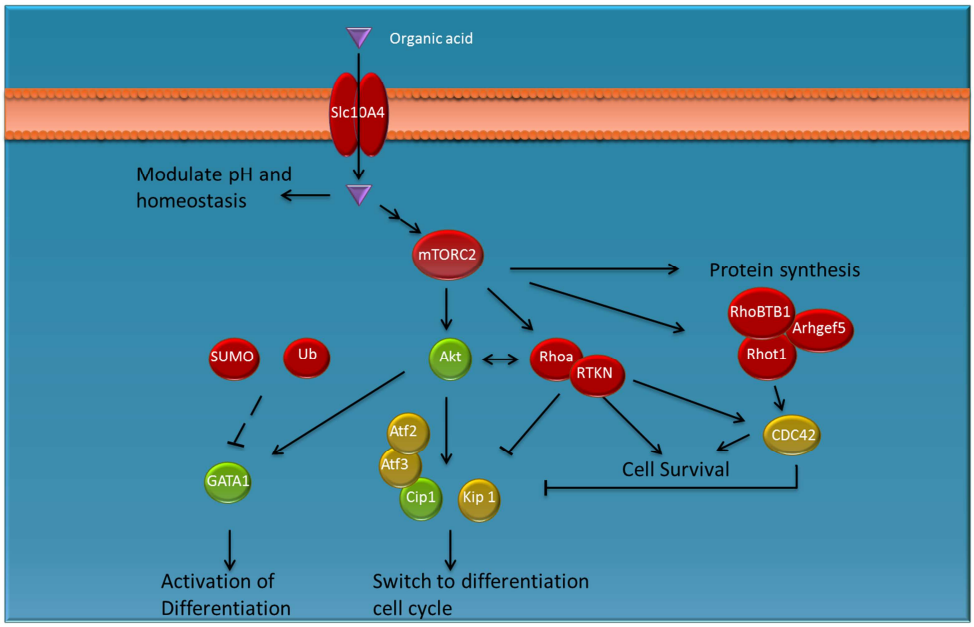


Figure 3.14: STRING protein-protein interactions for commitment. Candidate genes are indicated by black boxes and erythropoiesis markers are indicated by red boxes. The lines between proteins indicate how the interaction has been determined from the STRING database.

A



B

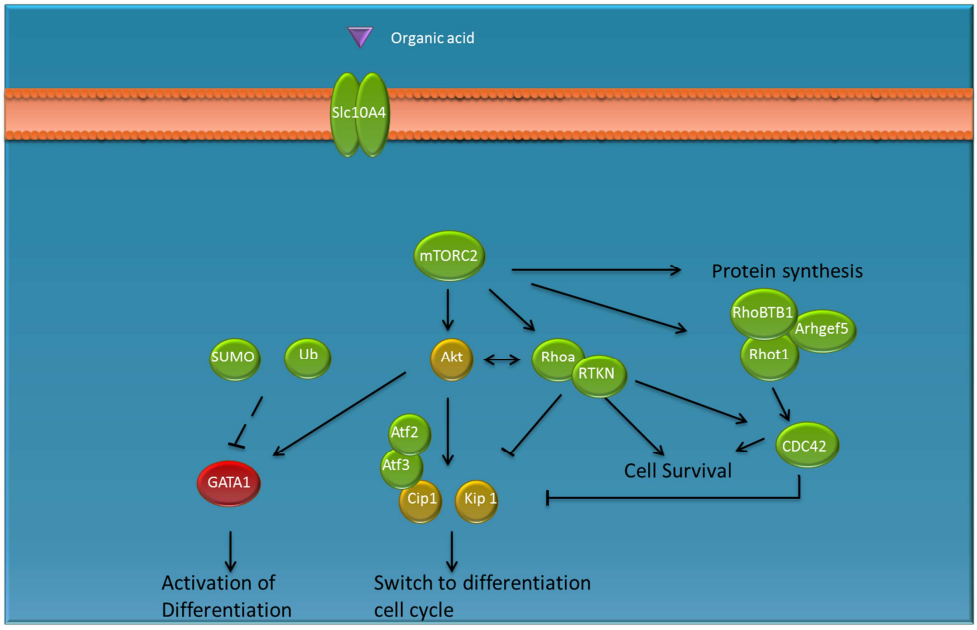


Figure 3.15: Model for self-renewal. Genes in red are > 1.5 fold up-regulated and genes that are green are > 1.5 fold down regulated while genes that are yellow are neither. A. shows gene expression in the pathway for recovered cells. The model illustrates how mTORC2 and Akt3 regulate cell cycle control genes Cip1 and Kip1, GATA1 as well as Rho G-proteins, which can regulate cell cycle control genes and cell survival with CDC42. B. shows the same model during induction.

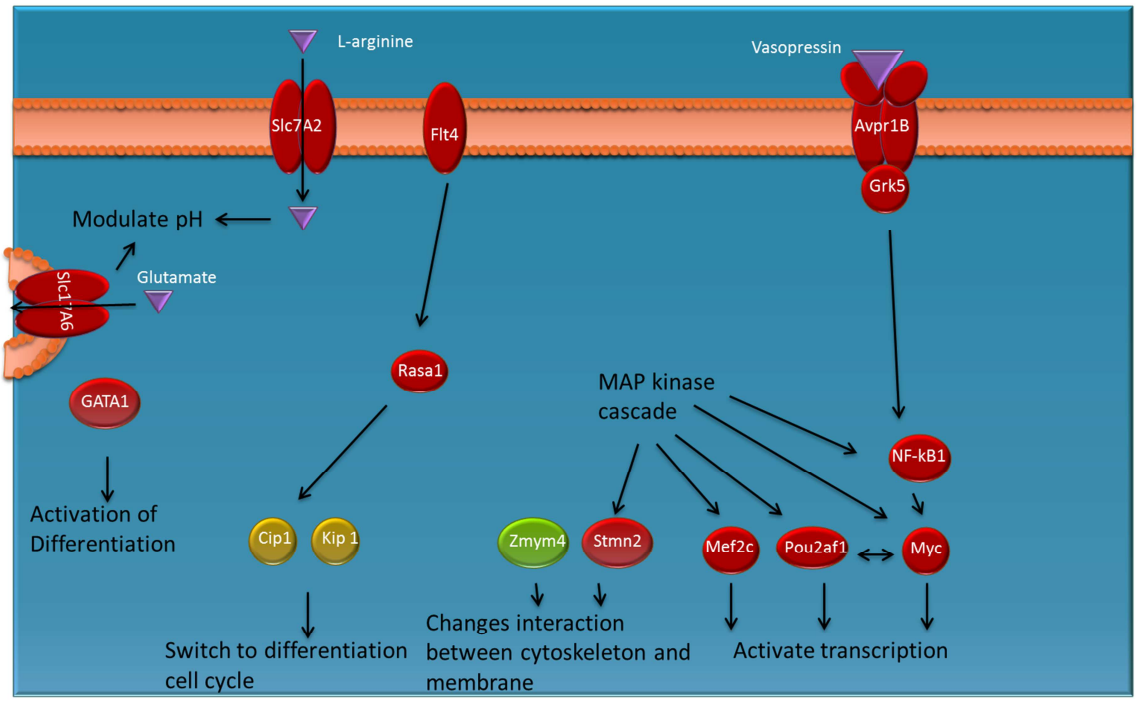


Figure 3.16: Model for commitment. Genes in red are > 1.5 fold up-regulated and genes that are green are > 1.5 fold down regulated while genes that are yellow are neither. This model illustrates how the MAPK cascade regulates transcription factors identified with the pulsed induction method as well as how two receptors, Avpr1B and the Flt4 receptor, signal other molecules to promote commitment and/or differentiation. Amino acid transporters may also play a role in maintaining cytoplasmic pH as the cells become more acidophilic.

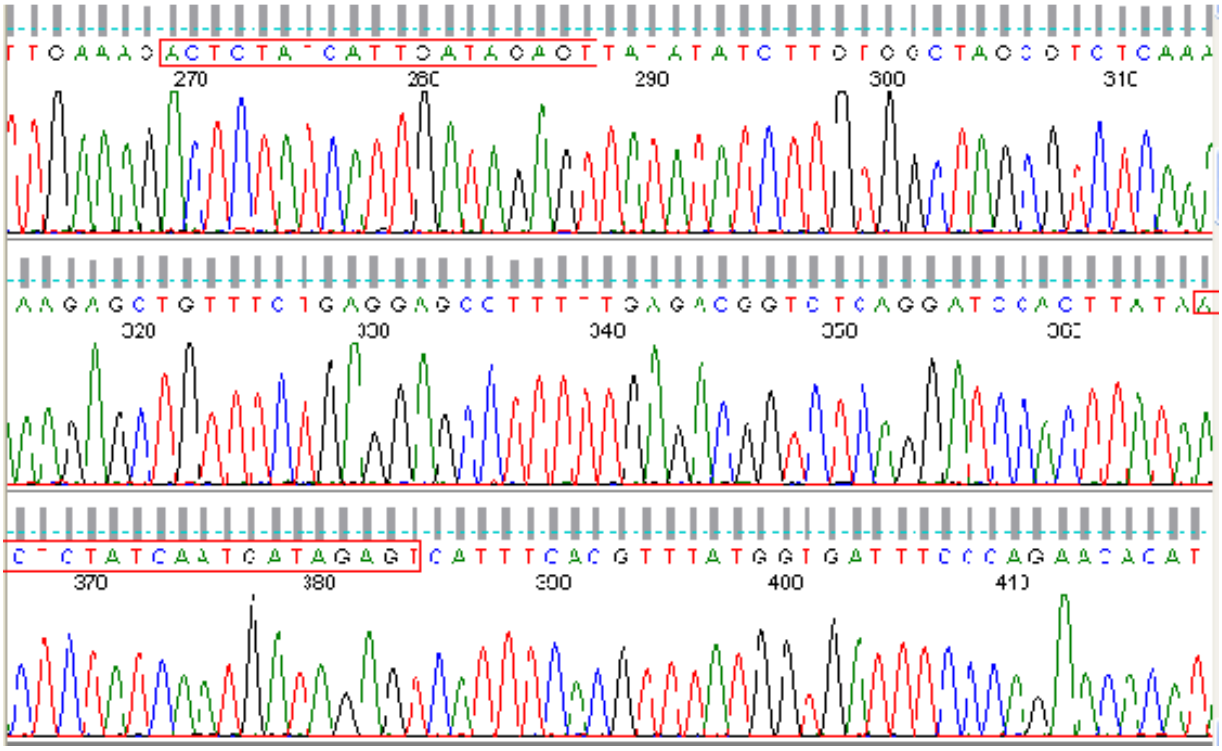


Figure 3.17: TetR binding sequence in U6 (Top) and H1 (bottom) in pHIP-HUT-TR are highlighted in the red box.

Dilution	250/0	200/50	150/100	100/150	50/200	10/240	1/149	0/250
Cells/well	3.13×10^5	2.51×10^5	1.88×10^5	1.25×10^5	6.27×10^4	1.25×10^4	1.25×10^3	0

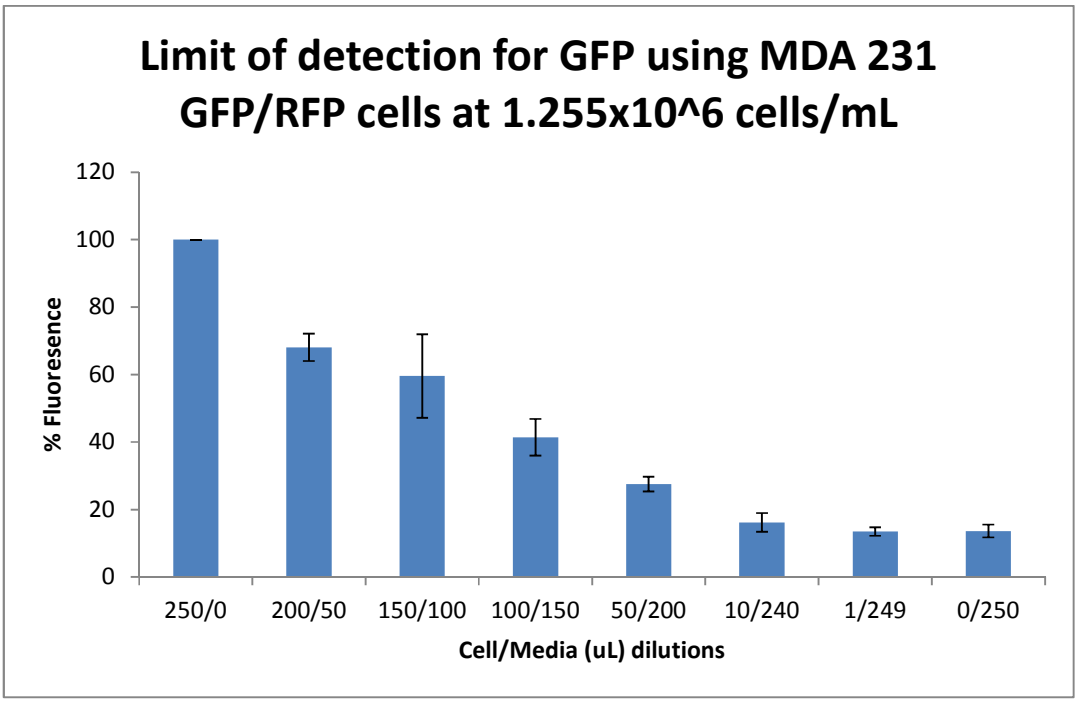


Figure 3.18: Detection limit of fluorescence of copGFP in MDA MB-231 GFP/RFP cells (Cell BioLabs Inc.) using 1.255×10^6 cells/ml in the undiluted sample (250/0)

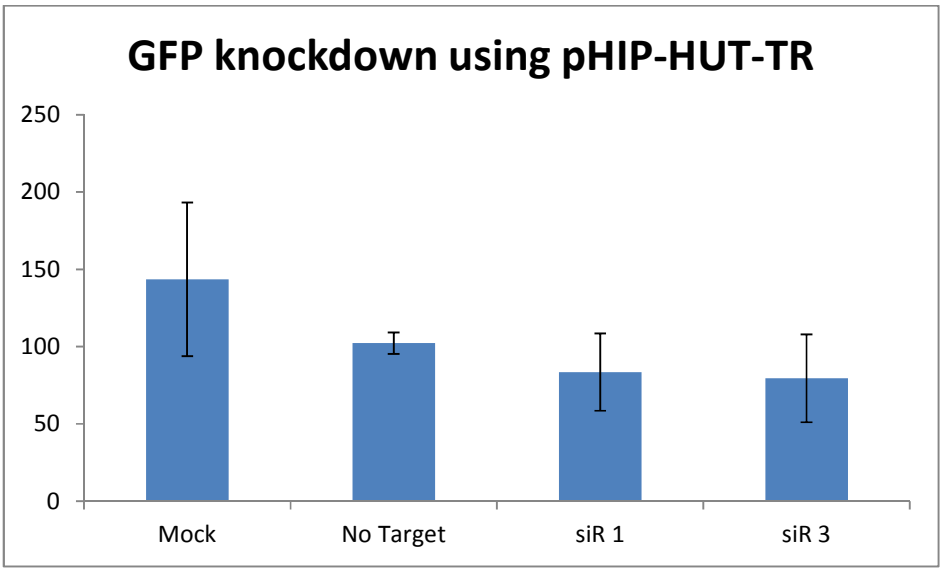


Figure 3.19: Knockdown of GFP in MDA 231 GFP/RFP cells using Mock transfected, No target siRNA, siRNA 1 and siRNA3 when induced with Doxycycline. All fluorescence is relative to the non-induced control that was transformed or mock treated.

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APPENDICES

pHIP-HUT-TR sequence

1 CAATTCGCCA GGAACCGTAA AAAGGCCGCG TTGCTGGCGT TTTTCCATAG

51 GCTCCGCCCC CCTGACGAGC ATCACAAAAA TCGACGCTCA AGTCAGAGGT

101 GGCGAAACCC GACAGGACTA TAAAGATACC AGGCGTTTCC CCCTGGAAGC

151 TCCCTCGTGC GCTCTCCTGT TCCGACCCTG CCGCTTACCG GATACCTGTC

201 CGCCTTTCTC CCTTCGGGAA GCGTGGCGCT TTCTCATAGC TCACGCTGTA

251 GGTATCTCAG TTCGGTGTAG GTCGTTCGCT CCAAGCTGGG CTGTGTGCAC

301 GAACCCCCCG TTCAGCCCGA CCGCTGCGCC TTATCCGGTA ACTATCGTCT

351 TGAGTCCAAC CCGGTAAGAC ACGACTTATC GCCACTGGCA GCAGCCACTG

401 GTAACAGGAT TAGCAGAGCG AGGTATGTAG GCGGTGCTAC AGAGTTCTTG

451 AAGTGGTGGC CTAACTACGG CTACACTAGA AGGACAGTAT TTGGTATCTG

501 CGCTCTGCTG AAGCCAGTTA CCTTCGGAAA AAGAGTTGGT AGCTCTTGAT

551 CCGGCAAACA AACCACCGCT GGTAGCGGTG GTTTTTTTGT TTGCAAGCAG

601 CAGATTACGC GCAGAAAAAA AGGATCTCAA GAAGATCCTT TGATCTTTTC

651 TACGGGGTCT GACGCTCAGT GGGCGGCCGC ATTTGCATGT CGCTATGTGT

701 TCTGGGAAAT CACCATAAAC GTGAAATGAC TCTATCATTG ATAGAGTTAT

751 AAGTGGATCC TGAGACCGTC TCAAAAAGCA AGCTGACCCT GAAGTTCATT

801 TTTTGAGACG CTAGCCACAA GATATATAAC TCTATCAATG ATAGAGTCTT

851 TCAAGTTACG GTAAGCATAT GATAGTCCAT TTTAAAACAT AATTTTAAAA

901 CTGCAAACTA CCCAAGAAAT TATTACTTTC TACGTCACGT ATTTTGTACT

951 AATATCTTTG TGTTTACAGT CAAATTAATT CTAATTATCT CTCTAACAGC

1001 CTTGTATCGT ATATGCAAAT ATGAAGGAAT CATGGGAAAT AGGCCCTCTT

1051 CCTGCCCGAC CTTGGCGCGC GCTCGGCGCG CGGTCACGCT CCGTCACGTG

1101 GTGCGTTTTG CCTGCGCGTC TTTCCACTGG GGACGCGTTG ACATTGATTA

1151 TTGACTAGTT ATTAATAGTA ATCAATTACG GGGTCATTAG TTCATAGCCC

1201 ATATATGGAG TTCCGCGTTA CATAACTTAC GGTAAATGGC CCGCCTGGCT

1251 GACCGCCCAA CGACCCCCGC CCATTGACGT CAATAATGAC GTATGTTCCC

1301 ATAGTAACGC CAATAGGGAC TTTCCATTGA CGTCAATGGG TGGAGTATTT

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1751 TACTGGCTTA TCGAAATTAA TACGACTCAC TATAGGGAGA CCCAAGCTGG

1801 CTAGCGTTTA AACTTAAGCT TGGTACCCGG GGATCCTCTA GGGCCTCTGA

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2151 CTTTAGCTTG CATTTGTAAC GAATTTTTAA ATTCACTTTT GTTTATTTGT

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2601 GATAAAAGTA AAGTGATTAA CAGCGCATTA GAGCTGCTTA ATGAGGTCGG

2651 AATCGAAGGT TTAACAACCC GTAAACTCGC CCAGAAGCTA GGTGTAGAGC

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2951 CCAACAAGGT TTTTCACTAG AGAATGCATT ATATGCACTC AGCGCTGTGG

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4351 GTTCATAAAC GCGGGGTTCG GTCCCAGGGC TGGCACTCTG TCGATACCCC

4401 ACCGAGACCC CATTGGGGCC AATACGCCCG CGTTTCTTCC TTTTCCCCAC

4451 CCCACCCCCC AAGTTCGGGT GAAGGCCCAG GGCTCGCAGC CAACGTCGGG

4501 GCGGCAGG

pHIPPY sequence

1 CAATTCGCCA GGAACCGTAA AAAGGCCGCG TTGCTGGCGT TTTTCCATAG

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2101 TTTCCCCACC CCACCCCCCA AGTTCGGGTG AAGGCCCAGG GCTCGCAGCC

2151 AACGTCGGGG CGGCAGG

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