FLI1 IS ESSENTIAL IN ENDOTHELIAL AND HEMATOPOIETIC STEM CELL HOMEOSTASIS

A Dissertation

Presented to the Faculty of the Weill Cornell Graduate School

of Medical Sciences

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Chaitanya R. Badwe

August 2018

FLI1 IS ESSENTIAL IN ENDOTHELIAL AND HEMATOPOIETIC STEM CELL HOMEOSTASIS

Chaitanya R. Badwe

Cornell University 2018

The ETS family of transcription factors is known to play an essential role in hematopoietic and vascular development. One such factor that is widely expressed in all vascular beds and almost all hematopoietic lineages, extending from long term hematopoietic stem cells to terminally differentiated peripheral blood cells, is Fli1. Global deletion of Fli1 leads to embryonic lethality at E11.5 due to dramatic hemorrhaging caused by poor vascular integrity and platelet dysfunction. The role of Fli1 in adults has been explored in several different cells types ranging from megakaryocytes to B and T cells. It has also been implicated in transcriptional regulation of hematopoietic stem and progenitor cells through combinatorial analysis, however its exact contribution to stem cell maintenance and function remains unclear. We hypothesized that Fli1 plays a dual role in regulating both the seed and the soil – specifically hematopoietic stem cell (HSC) function in a cell autonomous manner as well as the niche required for nurturing these cells.

In the first study, we focused our attention on the cell autonomous function of Fli1. We found that global deletion of Fli1 in adults leads to lethality as a result of complete peripheral blood failure in addition to aberrant vasculature. Using the cre-lox system and various transplantation strategies, we identify Fli1 as one of the critical regulators of adult hematopoietic stem cell function. Specific deletion of Fli1 in the hematopoietic compartment alone is sufficient to induce significant reduction in peripheral blood counts, accompanied by hemorrhage, resulting in lethality. On further inspection, $Fli1^{\Delta/\Delta}$ HSCs are unable to expand ex-vivo or engraft in a competitive

setting. Additionally, Fli1 is essential for hematopoietic reconstitution post radiation and its deletion abolishes the ability of stem cells to reconstitute the bone marrow and contribute to peripheral blood lineages. ChIP-seq analysis demonstrates Fli1 occupancy in a majority of essential hematopoietic gene enhancers and promoters. By correlating $Fli1^{\Delta/\Delta}$ HSC RNA-seq analysis with the ChIP occupancy data we can conclude that Fli1 is, in fact, one of the master regulators of the HSC transcriptional program essential to maintain the balance between self-renewal and differentiation. Taken together, our results indicate that Fli1 is required for maintenance of HSC homeostasis and function, making it one of the unique transcription factors to play a critical role in HSC function in adults as well as during development.

In the second set of studies, we interrogated the role of Fli1 in the endothelial niche and more specifically in the maintenance of endothelial homeostasis. We found that Fli1, by itself, is not essential for maintaining endothelial fate during homeostasis or under stress. We hypothesized that this may be due to redundancy between Fli1 and Erg, another ETS factor that shares significant homology with Fli1. Endothelial specific deletion of both Fli1 and Erg results in lethality, 12 days post induction due to disruption of vascular homeostasis and the formation of multiple thrombi in the liver. Fli1 and Erg deletion results in a reduction in a variety of vascular genes in multiple vascular beds in addition to organ specific vascular signatures. The disruption of vascular homeostasis is accompanied by a significant reduction in platelet numbers in peripheral blood. In vitro analysis of the endothelial deletion reveals a complete loss of vascular identity with significant reduction in VE-Cadherin and CD31 expression accompanied by a concomitant increase in mesenchymal marker α-SMA.

Taken together, these studies uncover a critical role for Fli1 in maintaining homeostasis in both endothelial cells as well as hematopoietic stem cells.

BIOGRAPHICAL SKETCH

Chaitanya Badwe was born and raised in Mumbai, India. He obtained his Bachelor of Technology in Pharmaceutical Chemistry and Technology from University Institute of Chemical Technology in May 2008. After that, he moved to the United States of America to pursue his Master's degree at Johns Hopkins University in Baltimore, MD. He completed his degree in Biotechnology with a focus in Drug Discovery and Molecular Targets in May 2010. During his time at Johns Hopkins, he worked on reprogramming of AML cell lines with Dr. Robert J. Arceci. After obtaining his Master's degree, Chaitanya had a short stint in the Stem Cell and Cell Therapies division at Life Technologies under the tutelage of Dr. Soojung Shin and Dr. Mohan Vemuri who were instrumental in guiding him to pursue a PhD. His time at Life Technologies focused on optimizing differentiation and preservation protocols to develop motorneurons and oligodendrocyte for disease modelling and as a cell therapy.

Chaitanya started his PhD in Biochemistry, Cell and Molecular Biology at Weill Cornell Graduate School of Medical Sciences in July, 2011. His first rotation in the summer of 2011, in the lab of Dr. Timothy Chan working under Dr. Logan Walsh yielded his first publication focusing on the role of RECK in breast cancer metastasis. Chaitanya joined the lab of Dr. Shahin Rafii in June 2012 and began investigating the role of Fli1 in endothelial cells. His work on Fli1 diversified into two separate projects that interrogated the role of Fli1 in not only endothelial cells but also hematopoietic stem cells. He has had the opportunity to present his work at multiple conferences in the context of endothelial cell biology as well as hematopoiesis, including the Gordon Research Conference (GRC Angiogenesis) in 2015 and 2017, American Society of

Hematology (ASH 2017) and International Society for Stem Cell Research (ISSCR 2017). Chaitanya's collaborative spirit has seen him co-author multiple peer reviewed articles focused on understanding endothelial cell biology and the interactions between the niche and hematopoietic stem cells. Throughout his Ph.D., Chaitanya has been actively involved in mentoring high school and undergraduate students as well as rotation students who were looking to join the Rafii lab.

His graduate work has resulted in two manuscripts that are close to completion and will be submitted shortly. After his Ph.D., Chaitanya intends to apply his skills to the biopharmaceutical industry and assume a role that will allow him to push research that will create impact.

This work is dedicated to my family.

I stand where I am today, only through their support.

ACKNOWLEDGEMENTS

I want to express my deepest gratitude to my thesis advisor, Dr. Shahin Rafii. His enthusiasm throughout my Ph.D. ensured that there was never a dull moment and his passion for science is something that everyone should strive for. His demanding nature is something that ensures that anyone working under him strives for the best and should never settle for anything less. I will always be grateful for the support he has given me throughout my Ph.D.

I would like to thank Dr. Jason Butler for his invaluable advice and support that assisted me in my pursuit of the hematopoietic aspect of my research. I must acknowledge his lab's generosity in helping me in times of need with reagents for essential experiments. I would also like to thank my committee members Dr. Heidi Stuhlmann and Dr. Vivek Mittal for all their inputs and suggestions to help guide me through my Ph.D.

I want to extend my deepest gratitude to Dr. Michael Poulos for his limitless technical expertise in experimental design and more importantly moral support throughout my Ph.D. I want to take this opportunity to thank the core facilities at Weill Cornell. Jenny Xiang and her team – Wei, Angela, Ryan, Aihong, Adrian, Dong and Tatiana have been instrumental in generating a lot of the data that I present here. I want to thank Dr Raphael Lis and the Starr foundation core for all the flexibility he did afford me for using their equipment. A special thanks to Dr. Lis for all the scientific discussions, technical expertise and moral support that he gave me over the years. Most importantly for being a great friend inside and outside the lab.

I want to thank all the members of the Rafii lab, past and present for all their inputs and discussions that have enabled me to be where I am today. A heartfelt thanks

to Dr. William Schachterle who left the lab a year ago but was instrumental in developing a lot of the ideas presented here today and for all the philosophical discussions that were definitely more frequent than they should have been. I am grateful to Daniel Nolan, Mike Ginsberg and Freddy Reyes for their assistance in a lot of the endothelial cell isolations. I want to thank Tyler, Brian and C.J. Karrasch for always being ready to give a helping hand when I needed, Balvir, Ying and David Redmond for their assistance in the bioinformatic aspect of my thesis. I appreciate all the help from Masa, Peipei, David and Tomer in a lot of the experiments that I present today. I am grateful to Maria, Koji and Sina for their support over the years. A special thanks to Jesus who has been actively involved in the endothelial part of my thesis. I am grateful to Jose Gabriel for all his help in the hematopoietic aspect of my thesis and always being ready to lend me helping hand when I needed.

A majority of one's time in a Ph.D. is spent in lab however the support one receives from outside lab can never be overlooked. A big thank you to all my friends and peers who have supported me over the years through the bad times and the good. Lastly, I would not be where I am today without the support of my family. They have always been there to push me when I needed a nudge and supported me when things seemed hopeless. Their unwavering support has enabled me to achieve what I have today and for that I will be eternally grateful.

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LIST OF ABBREVIATIONS

AGM	Aorta Gonad Mesenephros
BM	Bone Marrow
BMEC	Bone Marrow Endothelial Cell
BMP	Bone Morphogenic Protein
CMP	Common myeloid progenitor
CTA	C-terminal activation domain
DLBCL	Diffuse large B-cell lymphoma
dpc	Days post coitus
EC	Endothelial cell
ETS	E-26 Transformation Specific
Flt3	Fms Related Tyrosine Kinase 3
F-MuLV	Friend murine leukemia virus F-MuLV
FOX	Forkhead box
GMP	granulocyte-monocyte progenitor
GVHD	Graft versus host disease
HDAC	Histone Deacetylase
HGF	Hepatocyte growth factor
HSC	Hematopoietic Stem Cell
HSPC	Human Stem and Progenitor Cells
HUVEC	Human Umbilical Vein Endothelial Cells
KLS	$cKit^{+}Lineage^{-}Sca1^{+}$
KO	Knockout
LT-HSC	Long Term Hematpoietic Stem Cell
MAPK	Mitogen-activated Protein Kinase
MEP	Megakaryocyte–erythroid progenitor cell
MFI	Mean Fluorescent Intensity
MPP	Multipotent progenitor
PCAF	p300/CREB binding protein associated factor
PTM	Post translational modification
SCF	Stem cell factor
SLAM	CD150+CD48-
TF	Transcription Factor
TGFβ	Transforming growth factor beta
TPO	Thrombopoietin
VEGFR	Vascular Endothelial Growth Factor Receptor

CHAPTER 1: INTRODUCTION

1.1 THE SEED AND THE SOIL

The "seed and the soil" is a concept that has transcended generations. First proposed in the 1800s, it emphasized the importance of the environment in outcomes for metastasis (Paget, 1889). The application of this concept is not limited to physiology at a cellular level but transcends society itself. The environment that a child is nurtured in has long term effects on his or her success in society. So be it at the level of a nucleus where a somatic cell is reprogrammed into an omnipotent stem cell or at the socioeconomic level of impacting education and career tracks, the external cues one experiences are as important, if not more important than the internal signals that govern its function.

One of the most tightly regulated microenvironments in the body is the bone marrow (Heissig et al., 2002; Heissig et al., 2005; Morrison and Scadden, 2014; Yu and Scadden, 2016). It is unique in its ability to orchestrate the continuous generation of the hematopoietic system that supports the everyday functions of an organism and generates close to 100 billion cells daily (Dzierzak and de Pater, 2016). The hematopoietic system consists of a wide variety of different lineages with specialized functions (Figure 1.1). These can be broadly classified into three categories – Erythrocytes that are mainly involved with oxygen and carbon dioxide transport, megakaryocytes and platelets involved in the wound healing response and clotting cascade and leukocytes that coordinate immunity. The generation of this wide array of cells is initiated by a single cell at the top of the hierarchy – the hematopoietic stem cell (HSC).

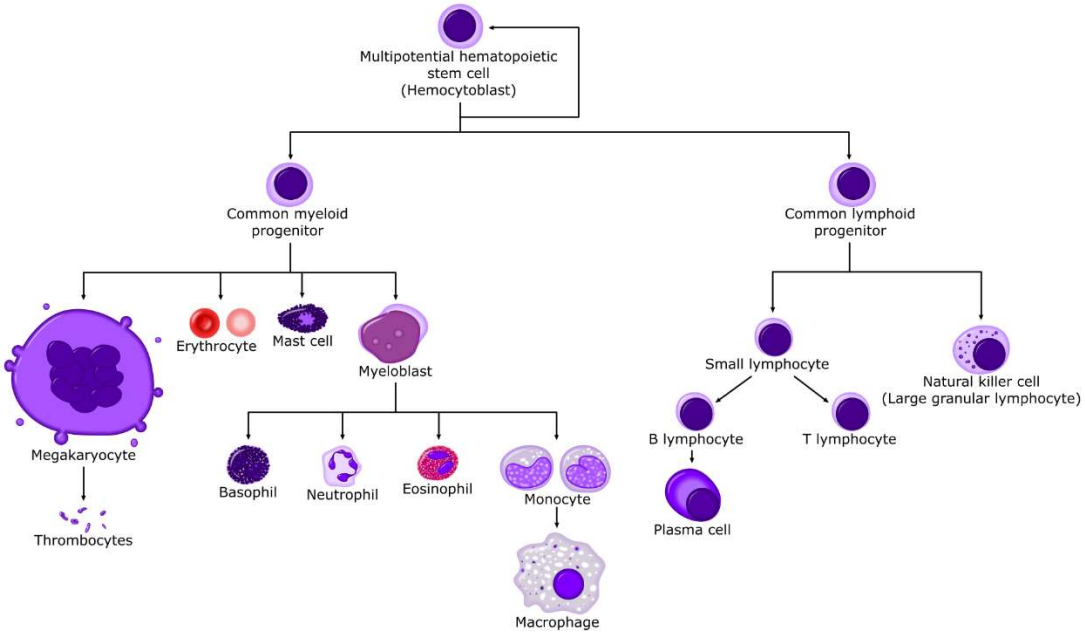


Figure 1.1 Overview of hematopoietic development into various terminally differentiated cell types (H.L. David et al., 2012)

The HSC is unique among other adult stem cells due to the fact that it is easy to isolate from the bone marrow, it is robust in being able to withstand flow sorting added to the fact that transplantation of these cells, even at a clonal level, can reconstitute the entire hematopoietic system of the organism. These properties have enabled extensive studies to understand the mechanisms that govern its capacity to self-renew, expand and differentiate depending on the physiological state and requirement of the organism. The HSC, despite being a cell with such limitless potential, is dependent on the external cues it receives from the niche to maintain the balance between self- renewal and differentiation. This concept was first proposed back in 1978 by R. Schofield when he hypothesized that the surrounding cells determine the fate of the stem cell and hence its location in the tissue is restricted.

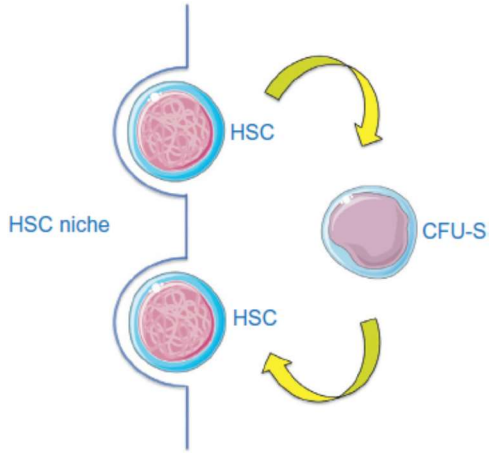


Figure 1.2 A representative mechanism for HSC self-renewal and differentiation from R. Schofield (Yu and Scadden, 2016)

As long as the stem cell is occupying this 'niche' it can maintain its identity and expansion of these cells require the availability of similar niches that can maintain the stem cell in its undifferentiated state (Figure 1.2). Scientists are still interrogating the function of different cell types that encompass the niche as well as the molecular interactions that regulate the crosstalk between each of these cells and the hematopoietic stem cell.

Schofield's hypothesis at the time lacked strong experimental support. Genetic evidence for the role of specific niche cells in hematopoiesis was discovered nearly three decades later when osteoblast cells were found to affect HSC homeostasis through the Jagged-Notch (Calvi et al., 2003) axis as well as through BMP signaling (Zhang et al., 2003). The advent of the cre-lox system (Feil et al., 2009) to spatially control genetic manipulation in specific cell types enabled researchers to interrogate the role of different cell types in the niche through specific pathways involved in HSC regulation.

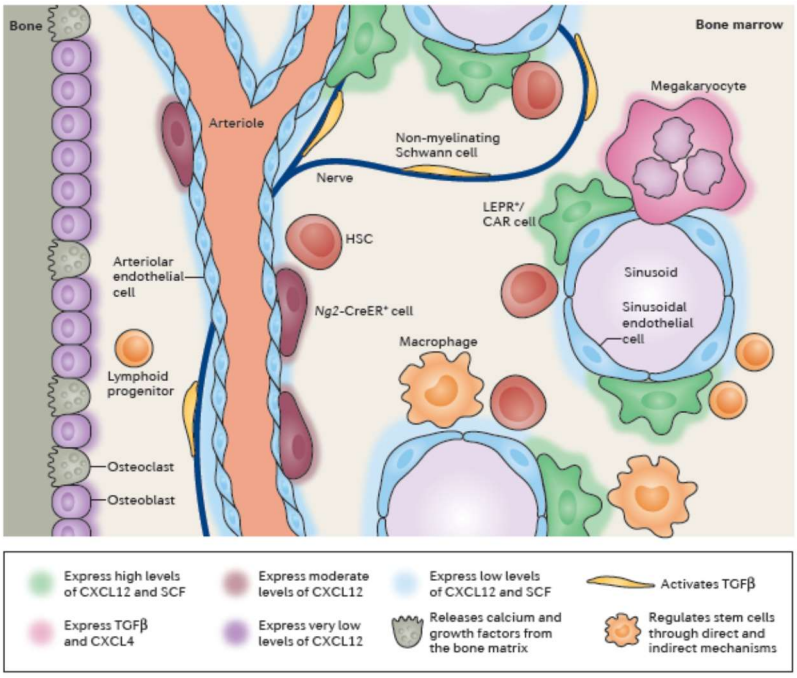


Figure 1.3 The heterogeneity of the HSC niche in the adult bone marrow (Crane et al., 2017)

The osteoblastic niche was implicated in regulating HSC self-renewal as well as differentiation. Osteoblasts were hypothesized to confer quiescence via the Ang-1/Tie2 axis and prevent HSC apoptosis during myelosuppressive stress (Arai et al., 2004). Additionally, BM localization and proximity to the endosteum was implicated to play a role in HSC ageing (Kohler et al., 2009). Increased distance from the endosteum seemed to induce a loss in polarization in aged mouse HSC leading to a concomitant impairment in supporting hematopoiesis. Real time imaging of the BM suggested that HSCs have a tendency to localize closer to the endosteum (Xie et al., 2009). Over the past several years, a variety of different cell types have been implicated to play a critical role in regulating different functions of hematopoiesis (Figure 1.3).

The first niche cell bolstered by elegant and convincing genetic proofs proven to play a role in self-renewal and differentiation of authentic HSC is specific subsets of the endothelial cells within the hematopoietic organs. Studies from our group and others have established an essential role for the vascular niche in maintaining HSC homeostasis in vivo (Avecilla et al., 2004; Heissig et al., 2002; Hooper et al., 2009; Kiel et al., 2005; Kimura et al., 2011). CXCL12, Jagged1, VEGFR2, Jagged2 are just a few of the factors that have been shown to coordinate their effect on the HSC through the vascular niche (Guo et al., 2017; Hooper et al., 2009; Poulos et al., 2013). In addition to their role in vivo, E4ORF1 modified endothelial cells serve as a robust platform to expand and maintain both mouse and human hematopoietic stem and progenitor cells in vitro (Butler et al., 2012; Butler et al., 2010; Kobayashi et al., 2010; Seandel et al., 2008). BM endothelial cells have been shown to have significant potential in maintaining multipotent progenitors in an in vitro setting that are capable of engraftment. Recently, it was shown that BMEC transplant can offer radioprotection post myeloablative radiation revealing a unique angiocrine profile that enables the endothelial cells to support BM recovery (Poulos et al., 2015). All these studies point to a critical role for endothelial cells in regulating HSC biology. A unique cell type that has its origins linked to the hematopoietic stem cell has been a recent addition to the HSC niche. Megakaryocytes have been implicated in maintaining HSC quiescence through the secretion of CXCL4, TGFβ1 as well as Thrombopoietin (Bruns et al., 2014; Nakamura-Ishizu et al., 2014; Nakamura-Ishizu et al., 2015). Megakaryocytes also have a radioprotective role that is critical in mitigating radiation damage for HSCs (Zhao et al., 2014).

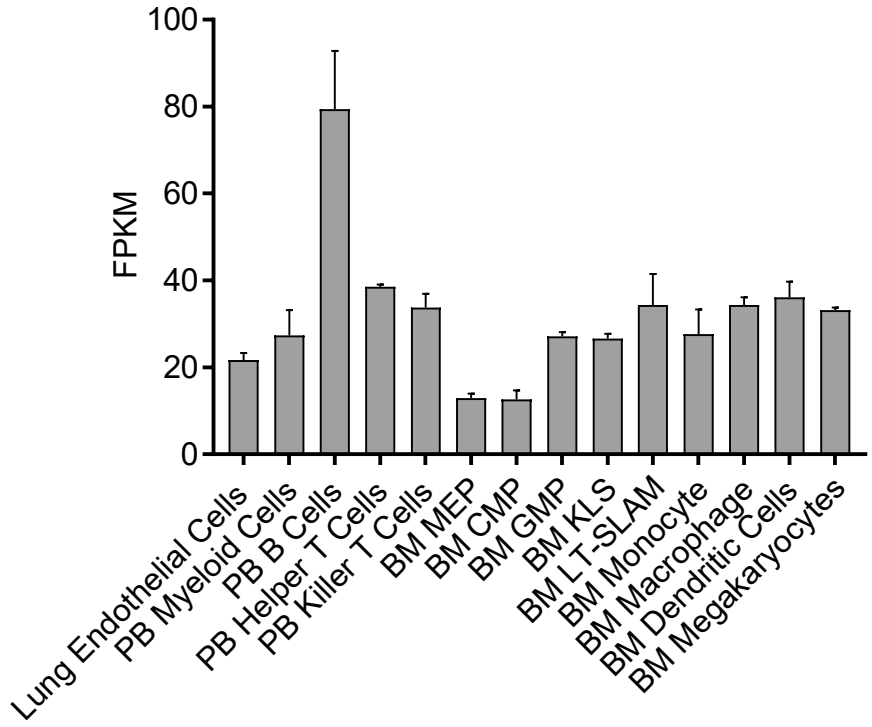


Figure 1.4 Differential Fli1 expression across various cells types – Lung endothelial cells, Peripheral Blood (PB) Differentiated cells, Bone marrow (BM) progenitors (MEP – Megakaryocyte-erythroid progenitor, CMP – common myeloid progenitor, GMP – Granulocyte Monocyte progenitor, KLS – $cKit^{+}Lineage^{neg}Sca1^{+}$, LT-SLAM – KLS $CD150^{+}CD48^{-}$)

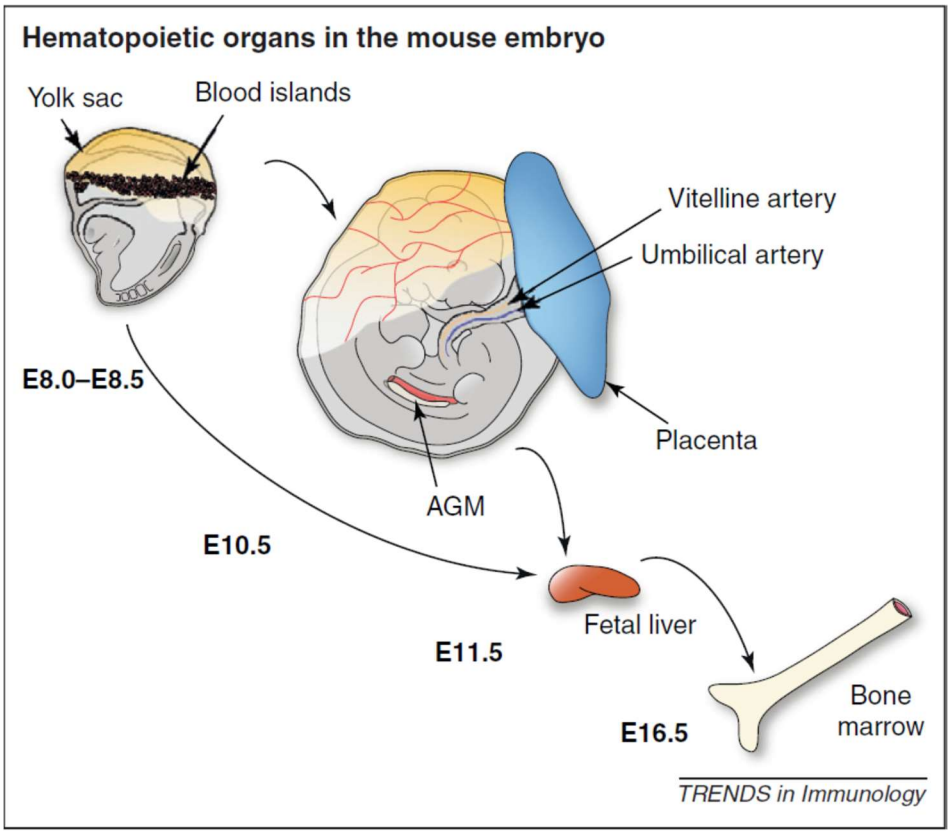
Having observed the importance of different cell types in HSC maintenance, we decided to explore the role of a factor that may be critical in HSC regulation from the cell autonomous perspective as well as from the niche setting. To this end, we interrogated the role of Fli1, which is expressed at high levels in endothelial cells, megakaryocytes as well as HSCs (Figure 1.4).

1.2 EMBRYONIC HEMATOPOIESIS

HSC functions can be broadly classified into three main categories – specification, self-renewal and differentiation. The balance between these however, is temporally and spatially regulated. During development the transcriptional machinery is focused initially on specification followed by self-renewal and differentiation aimed at generating the complete hematopoietic system for the embryo as it grows. During adulthood, this balance shifts towards self-renewal and maintenance. The primary functional difference between an adult HSC and a fetal HSC is that during homeostasis an adult HSC is not required to reconstitute the entire hematopoietic system and is predominantly quiescent which is why a lot of the factors that are essential for HSC function during development do not carry over their effect to adulthood.

During development, hematopoiesis occurs in distinct waves; however, the generated hematopoietic cells can be classified into two categories – primitive hematopoiesis which refers to the hematopoietic cells generated during development which generally do not last throughout the lifespan of the organism and begin to disappear postnatally and definitive hematopoiesis which encompasses the HSC pool that is generated during development which gives rise to the entire hematopoietic system that will maintain homeostasis throughout the lifespan of the organism (Medvinsky et al., 2011). The first hematopoietic cells emerge in the extraembryonic yolk sac even before initiation of circulation (Lux et al., 2008), by about E7.5 days post coitus (dpc) followed by the allantois and the placenta at E9.5 (Figure 1.5B). By

A



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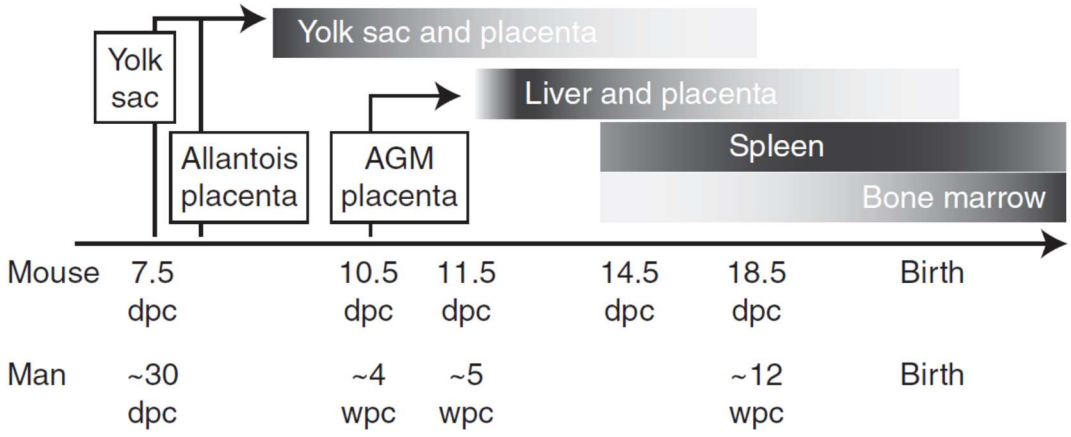


Figure 1.5 A – Different sites of hematopoiesis in the developing embryo, B. Timeline of hematopoietic activity during development in the mouse and man (Costa et al., 2012; Rieger and Schroeder, 2012)

E10.5, hematopoietic cells are detected in the aorta-gonad mesenephros (AGM) region as well as the umbilical and vitelline arteries. There is a steady transition in hematopoietic potential as new sites become active (Cumano and Godin, 2007) however before E10.0 the yolk sac is the predominant site for hematopoiesis giving rise to primitive erythroid, myeloid and lymphoid progenitors(Palis et al., 1999; Yang et al., 2014). The yolk sac was earlier thought to be one of the only embryonic sites of hematopoiesis until clear evidence from chick-quail experiments showed the absence of any donor contribution pre-circulation (Dieterlen-Lievre, 1975). The AGM region is predominantly responsible for de-novo HSC generation (Medvinsky and Dzierzak, 1996; Taoudi and Medvinsky, 2007) however the exact contribution of the other hematopoietic sites to definitive hematopoiesis is still controversial. The expression profile of the HSC lineage has been extensively studied in order to identify markers distinguishing the HSCs at every stage of their development(Chen et al., 2009; Liakhovitskaia et al., 2014; Rybtsov et al., 2011; Taoudi et al., 2008; Taoudi and Medvinsky, 2007). Currently, the profile of $CD45^{+}VE\text{-}Cadherin^{+}cKit^{+}CD34^{+}CD150^{-}$ is well accepted to identify the small HSC pool in the AGM from E10.5-E12.5. Post emergence from the AGM, the HSCs home to the fetal liver where they expand and repopulate extensively, steadily acquiring the more adult KLS phenotype till birth of the mouse (Figure 1.5A, 1.6). Close to birth the HSCs home to the BM where they remain throughout the life of the mouse.

1.3 ADULT HEMATOPOIESIS

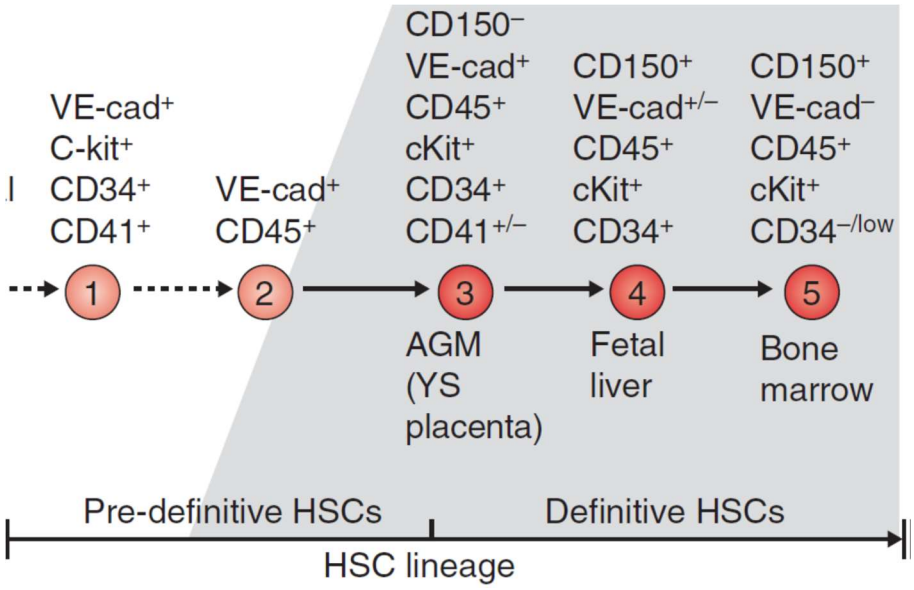


Figure 1.6 A schematic of definitive HSC specification in the developing embryo (Medvinsky et al., 2011)

The adult HSC sits at the top of a hierarchy that enables multiple rounds of amplification before arriving at terminally differentiated peripheral blood cells (Figure 1.7). Every stage of this hierarchy results in a certain degree of lineage restriction (Akashi et al., 2000; Kondo et al., 1997; Miyamoto et al., 2002). The bulk of the expansion of the HSC pool occurs during embryonic development with minimal divisions occurring once the mouse is mature. This applies to both the mouse as well as the human hematopoietic system. The human hematopoietic system bears close resemblance to the mouse however the degree of enrichment in the stem cell pool that can be obtained purely based off the surface expression profile of certain populations is much lower (Majeti et al., 2007; Notta et al., 2011). On the one hand this may be due to an incomplete expression profile for optimal human HSC enrichment; however, on the other hand the read out for enrichment is by xeno transplants into humanized mouse models which may underestimate the degree of stemness harbored by the

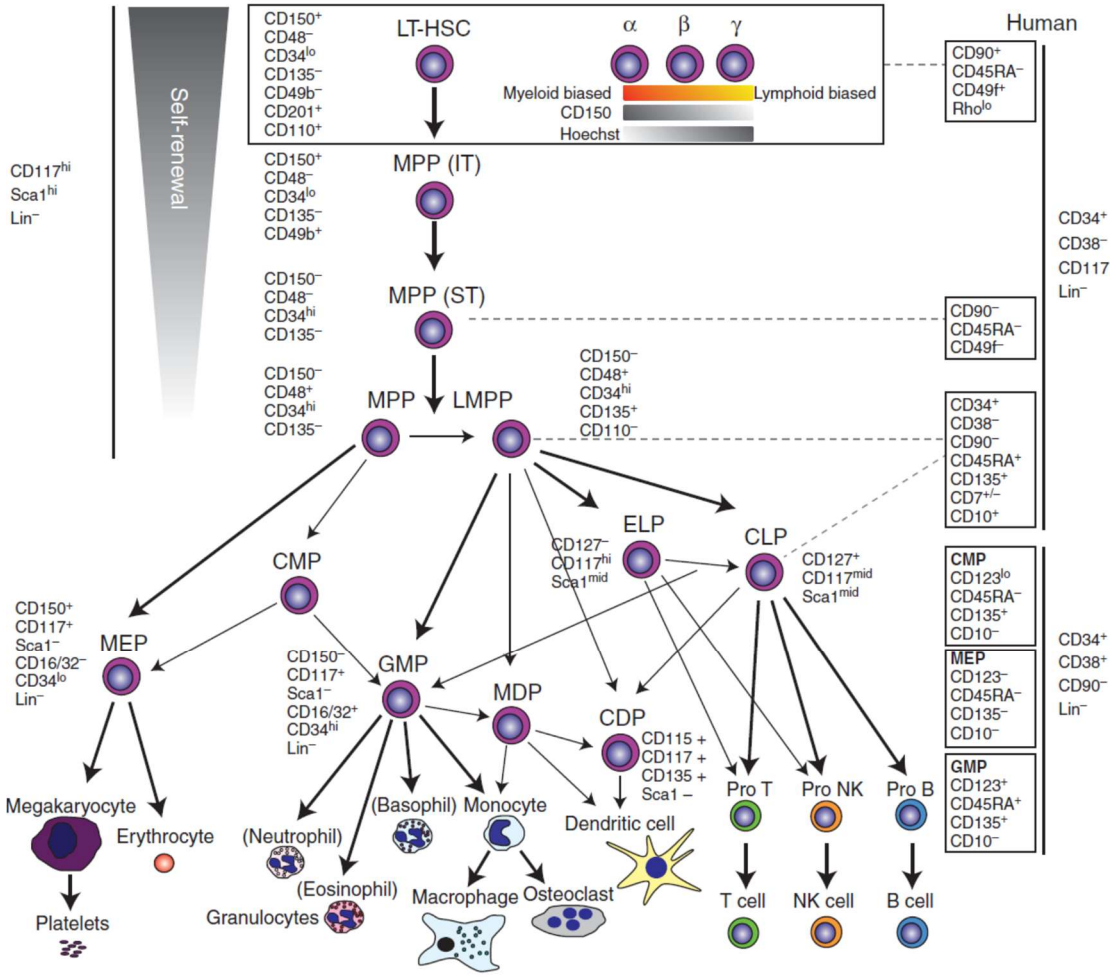


Figure 1.7 Adult hematopoietic hierarchy (Rieger and Schroeder, 2012)

transplanted cells (McDermott et al., 2010; Rongvaux et al., 2011; Strowig et al., 2011).

A large number of factors have been implicated in regulating HSC function. These range from epigenetic modifiers such as Bmi1 (Lessard and Sauvageau, 2003; Park et al., 2003) and cell cycle regulators such as p57 (Matsumoto et al., 2011; Zou et al., 2011) to signaling proteins such as Lnk (Ema et al., 2005) and Stat5 (Wang et al., 2009) but in terms of transcriptional regulation a heptad of Transcription Factors

(Runx1, Gata2, Fli1, Erg, Scl, Lmo2, Lyl1) have been shown to be important for combinatorial interactions necessary for maintaining HSCs in mice as well as humans (Beck et al., 2013; Pimanda et al., 2007; Wilson et al., 2010). Despite having lethal embryonic phenotypes most of these genes do not exhibit any critical role in HSC function during adult homeostasis. There is a degree of redundancy that allows the loss of function of one or more of these factors during adulthood. One drastic example of this in the mouse is Runx1 which during development is only required for specification and not thereafter despite being expressed in a majority of hematopoietic cells throughout the life of the organism (Chen et al., 2009). Of the remaining heptad, Lyl1 and Scl are highly homologous and during adult homeostasis, loss of either of these factors exhibit mild phenotypes however HSCs with both factors absent show significant reduction in self-renewal and overall functionality (Capron et al., 2006; Curtis et al., 2004; Souroullas et al., 2009). Gata2 is one of the factors that has been shown to play a role during development as well as homeostasis (de Pater et al., 2013; Lim et al., 2012) and both Erg and Fli1 have been shown to play a role in endothelial specification and hematopoiesis during development.

Of the 7 TFs, the role of Fli1 has not been explored in the context of HSCs in a cell autonomous manner, developmentally or during adulthood in great detail. Fli1 was first discovered due to its role in erythroleukemia (Ben-David et al., 1990; Ben-David et al., 1991; Watson and Seth, 1992). Studies have shown that dysregulation of Fli1 results in a shift in the balance between mega-erythroid lineage (Starck et al., 2010). Global deletion of Fli1 in mice leads to embryonic lethality at E11.5 (Hart et al., 2000) due to hemorrhage and loss of vascular integrity. Fli1 function has been restricted to endothelial and megakaryocyte lineages developmentally as well as other more differentiated hematopoietic cell types in adults.

1.4 ENDOTHELIAL HOMEOSTASIS AND VASCULAR HETEROGENEITY

The development of the vasculature is one of the earliest events during embryonic development and plays a critical role in the development of the embryo. Vasculogenesis refers to the de novo formation of the first endothelial cells from mesodermal precursors which ultimately form the vascular plexus (Hatzopoulos et al., 1998). The subsequent expansion and remodeling of these primitive blood vessels is termed angiogenesis(Tirziu and Simons, 2009). It is well accepted that the vasculogenesis occurs during development at E7.5 (Coffin et al., 1991). Extra-embryonic mesodermal cells in the yolk sac aggregate to form blood islands that give rise to the first endothelial and hematopoietic lineages expressing CD34, CD31 and QH1 while intra-embryonic endothelial cells form from precursors expressing SCL/TAL1 (Drake et al., 1997). Flk1 or VEGFR2 is one marker that the intra and extra-embryonic endothelial cells have in common. These cells continue to expand and remodel forming the paired dorsal aortas at E8.0 which precedes the start of circulation at E9.0. Endothelial cells and hematopoietic cells are closely related in their ontology (Medvinsky et al., 2011) and share a large number of genes. Definitive hematopoiesis transitions through two basic phases, specification and expansion during development followed by homeostasis and maintenance postnatally while endothelial cells are involved in organ development during development, postnatally until the organism reaches adulthood. In adulthood, most endothelial cells are quiescent (Dejana et al., 2007) due to which a large number of factors that are required during development and postnatally are found to be redundant during adulthood. This factor holds true for HSCs however not for the rest of the hematopoietic system since it is actively generating billions of cells on a daily basis.

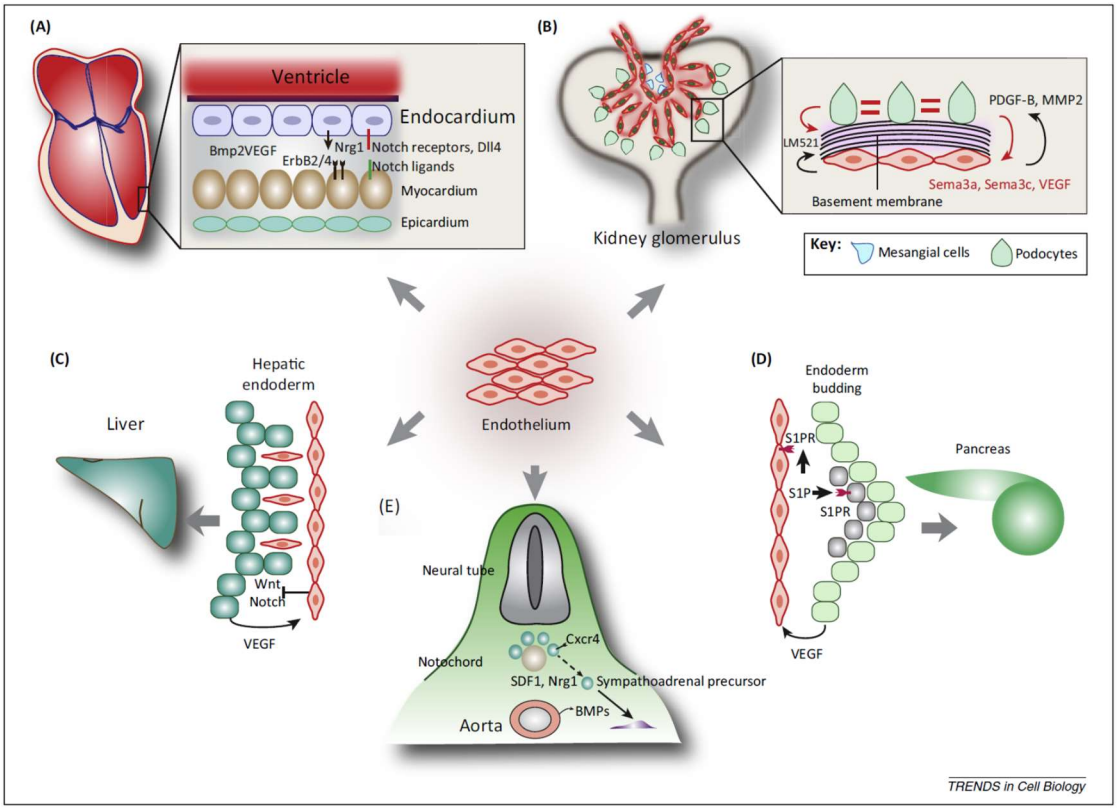


Figure 1.8 The role of endothelial cells during angiogenesis (Ramasamy et al., 2015)

Despite their dormant state, endothelial cells do possess the ability to respond to stimuli and reinitiate angiogenesis if the need arises in the event of an injury (Ding et al., 2010; Ding et al., 2011). There are a few physiological and non-physiological conditions during which endothelial cells are activated and essential. Tumor angiogenesis plays a critical role in progression of cancer be it in solid tumors or leukemias (Carmeliet and Jain, 2000; Jain, 2014). Our understanding of endothelial cell function is always evolving and has come a long way since the classical role that endothelial cells were thought to perform which was carrying blood and nutrients to

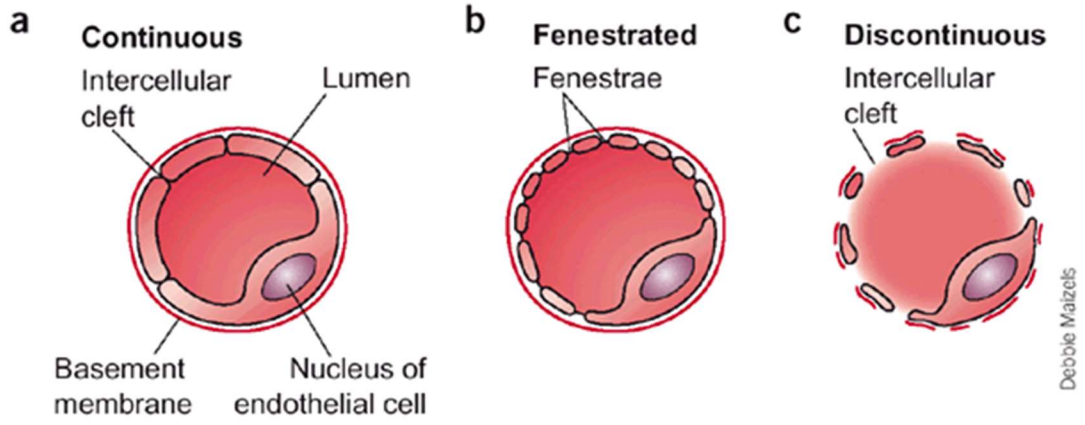


Figure 1.9 Heterogeneity in capillary wall morphology (Cleaver and Melton, 2003)

various organs of the body. There is increasing evidence that ECs not only perform an essential transport network role but are actively involved in growth, differentiation and repair throughout the body. Endothelial cells have been shown to be actively involved in tissue morphogenesis through the secretion of paracrine or 'angiocrine' factors (Figure 1.8). This maybe in the form of secreted factors such as Wnt, BMP and HGF (Ding et al., 2010; Wertheimer et al., 2018) or through surface receptor ligand interactions like Jagged1 and Jagged2 (Poulos et al., 2013).

The above findings revealed a gene expression profile that was distinct depending on the vascular bed involved. The heterogeneity of endothelial cells in inherently not a novel concept (Ribatti et al., 2002) however our understanding of the degree of heterogeneity that exists in the vasculature has changed dramatically. There are multiple levels of heterogeneity that exist in the vasculature. The first being the difference between lymphatic vessels and blood vessels. Within this there is the differentiation between large vessels encompassing arteries and veins and small vessels or capillaries. The most evident heterogeneity is visible from the

morphological perspective. EC capillaries vary from organ to organ in terms of their morphology and may be continuous as seen in fat and muscle or fenestrated in the case of intestinal or kidney glomeruli capillaries or discontinuous as seen in the liver, spleen and BM (Figure 1.9). Another level of heterogeneity is dictated by the spatial positioning of endothelial cells namely organ-based heterogeneity. Functional heterogeneity based on the organ type centers around the gene expression profiles of ECs in different vascular beds (Nolan et al., 2013) and enables them to react differently to external stimuli and orchestrate tissue repair in the case of damage. Understanding these subtle differences in the vasculature is key to being able to exploit the untapped potential of endothelial cells in manipulating disease conditions or making an environment hostile for malignancies and goes back to the concept of the seed and the soil. Recent literature has revealed that tumors hijack the vasculature not only for nutrients and oxygen but to form a malignant niche capable of sustaining growth as well as providing an immune privileged environment protecting the tumor from the host's immune system as well as chemotherapy (Cao et al., 2014; Cao et al., 2017). Most cancer treatments are targeted to mutations and cell intrinsic factors that cripple tumor cells however overlooking the role of the vascular niche from an organ specific perspective in the case of primary tumors as well as metastasis might be one of the biggest reasons for the inefficiency in cancer treatment and the cause for relapse.

Studies over the past two decades have attempted to improve our understanding of the various signaling pathways that orchestrate vascular development, heterogeneity and function. The ETS and FOX family of transcription factors have been shown to be intricately involved in transcriptional regulation of endothelial specification, angiogenesis and homeostasis (Dejana et al., 2007). One of

the earliest factors that has a very specific endothelial expression profile is VEGFR2 or Flk1. Flk1 expression is at its peak during vasculogenesis and angiogenesis and deletion of this receptor is embryonic lethal between E8.5 to E9.5 dpc due to defects in the development of hematopoietic and endothelial cells (Shalaby et al., 1995). Flk1 deletion results in the absence of yolk sac blood islands as well as organized blood vessels at E7.5 along with significantly reduced hematopoietic progenitors. $Flk1^{-/-}$ ES cells were unable to contribute to any endothelial lineages, primitive and definitive hematopoiesis in mouse chimeras demonstrating the essential role of VEGFR2 in endothelial development (Shalaby et al., 1997). Another essential vascular marker is VE-Cadherin or Cdh5 which leads to embryonic lethality at E9.5 similar to VEGFR2, due to impairment of remodeling and maturation in the vascular plexus while also inducing EC apoptosis due to absence of downstream signaling to critical survival signals – Akt and Bcl2 (Carmeliet et al., 1999). Tie2, Flt1, Ang1, Ang2, VEGF-A, Dll4 are just some of the factors that play key roles in endothelial function.

A lot of research has focused on understanding the mechanisms governing endothelial function during development and in adult however given the quiescent nature of the majority of adult vasculature the transcription factors that regulate vascular homeostasis in adults are not known. Most ETS and FOX factors that have drastic vascular permeability or specification defects exhibit mild phenotypes in adult. To this end, we interrogated the role of two ETS factors, Fli1 and Erg, with developmental roles in endothelial stability and that have been shown to be required for direct conversion of amniotic cells to an endothelial fate (Ginsberg et al., 2012; Schachterle et al., 2017) in the context of adult endothelial homeostasis. Identifying the master regulators of endothelial cell fate and vascular homeostasis would give us a tractable platform to interrogate which pathways play key roles in core endothelial fate

regulation as well as understanding the underlying mechanisms involved in vascular organ heterogeneity.

1.5 FLI1 AND THE ETS FAMILY OF TRANSCRIPTION FACTORS

Friend Leukemia virus integration-1 or Fli1 was first identified through its involvement in Friend murine leukemia virus (F-MuLV) induced erythroleukemia. Tumors and cancer is a common theme with respect to the discovery of several genes and Fli1 is no different. It was found that the Fli1 locus is a pro-viral integration site in 75% of the F-MuLV induced erythroleukemia and very specific in terms of its effect on the erythroid lineage since the myeloid and lymphoid tumors did not show any integration bias. This specificity not only extended to the hematopoietic lineage but also the type of virus with other Friend virus complexes, FV-A and FV-P, not showing the same locus rearrangement as seen with the F-MuLV (Ben-David et al., 1990; Ben-David et al., 1991). A year later, Fli1 was also found to be involved in Cas-Br-E MuLV-Induced Non-T, Non-B-Cell Leukemias (Bergeron et al., 1992) and identified in the human genome showing extensive homology to Erg (Watson and Seth, 1992), another gene in the ETS family of transcription factors. Fli1 dysregulation has been detected in a wide variety of non-physiological conditions (Hart et al., 2000; Li et al., 2015). One in particular has contributed to the majority of the research on Fli1 is Ewing Sarcoma. Fli1 is part of a potent translocation t (11;22) (q24; q12) which generates a fusion protein EWS-Fli1 in about 85% of Ewing Sarcomas (Delattre et al., 1992). EWS-Fli1 uses the 3’ ETS DNA binding domain of Fli1 in conjunction with the 5’ transactivation domain of the EWS to enable a gene expression profile that triggers a strong transformation phenotype (May and Denny, 1993).

Fli1 is a member of the E26 transformation specific (ETS) family of transcription factors.(Leprince et al., 1983) The ETS family of TFs play a critical role in several physiological processes specifically in terms of endothelial and hematopoietic lineages (Birdsey et al., 2008; Craig et al., 2015; Kataoka et al., 2011; Lee et al., 2011; Lee et al., 2008; Liu et al., 2012; Loughran et al., 2008; Wei et al., 2009). Ets-1 was the first ETS factor to be discovered as a viral oncogene in E26 avian leukemia (Leprince et al., 1983). Based on homology, 26 ETS genes have been identified in the mouse genome with 27 being identified in humans (Figure 1.10). The ETS factors carry out diverse functions with some family members being exclusively transcriptional activators while others perform primarily repressor function but the majority play a dual role depending on the spatial and temporal context. All members of the ETS family share one common feature – a highly conserved DNA binding domain called the ETS domain that is characterized by a winged helix-turn-helix containing three α-helices and four β-sheets which specifically binds to the DNA sequence 5'-GGA(A/T)-3'. Despite this conserved moiety, structural and conformational differences in ETS factors regulate their DNA binding by recognizing DNA motifs over 11-bases around the GGA site. An additional feature present in a subset of the ETS factors is the pointed domain. The pointed domain is a feature that regulates homotypic and heterotypic interactions between ETS factors and other binding partners that regulate the transcriptional profile of the gene. The pointed domain can also modulate the type of transcriptional activity targeted by the factor switching between repression and activation of genes in different cell types.

Given the large number of ETS factors and their co-expression in several cell types, there are different mechanisms that ensure specificity in terms of their function. The first layer of specificity is through structural differences that change the DNA

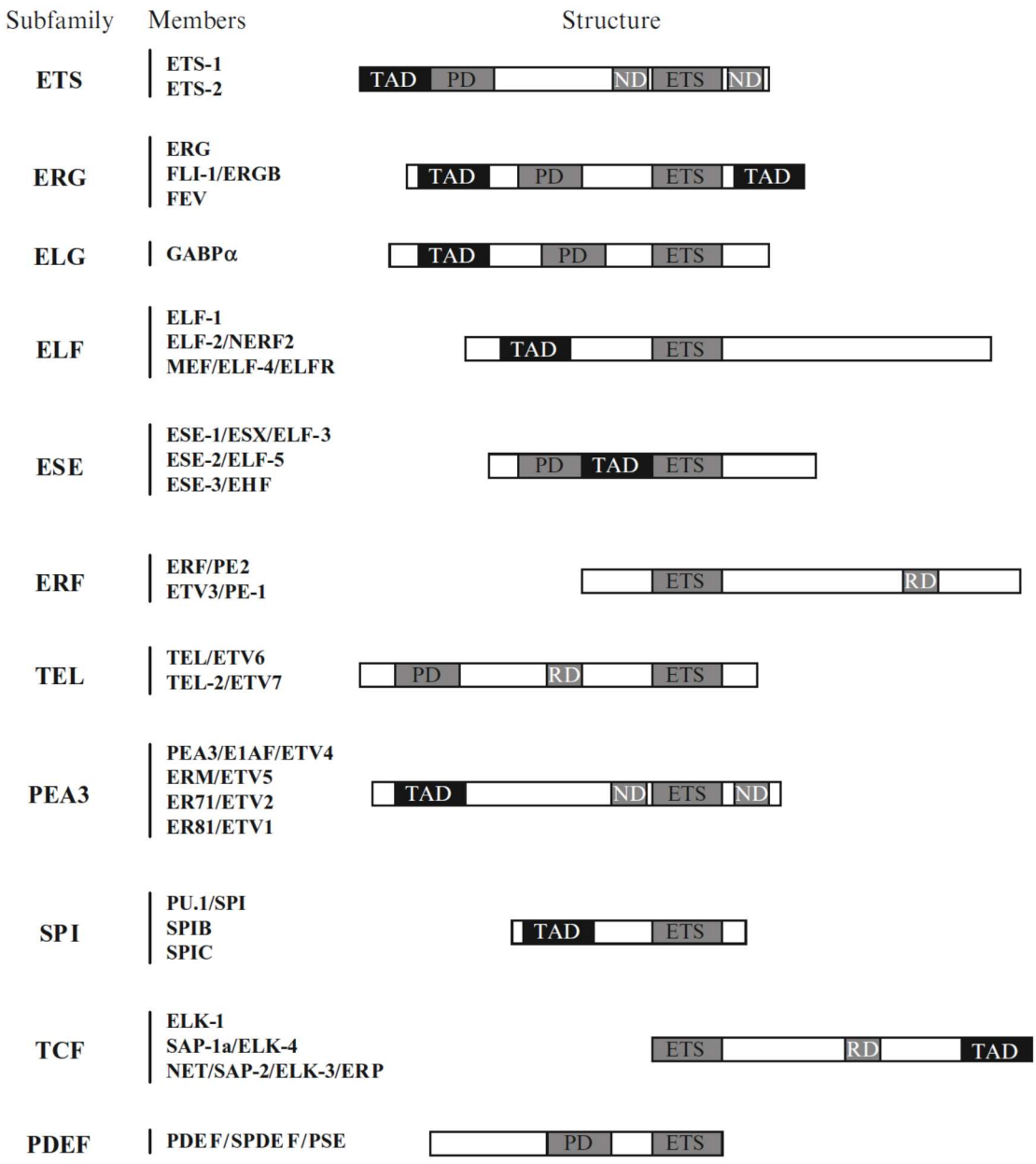


Figure 1.10: The ETS family of transcription factors (PD – pointed domain, TAD – transactivation domain, ND – negative domain, RD – repression domain, ETS – DNA binding domain) (Charlot et al., 2010)

binding profile through differential affinity to various sequences. The second layer is regulated through the binding of co-factors. The pointed domain is one feature that

enables diverse interactions with different binding partners dependent on the cell type and gene expression profile of binding partners within different cells. Both the above mechanisms are further modulated through post translational modifications. Phosphorylation, one of the most common PTM, generally occurs on a serine, threonine or tyrosine through serine/threonine protein kinases or via tyrosine kinases. The MAPK pathway is a predominant player in regulating ETS factor phosphorylation. Other post translational modifications that predominantly target the lysine residue are acetylation, sumoylation and ubiquitination. Acetylation is mediated by acetyltransferases such as p300 which transfer an acetyl group to the specific residue being targeted while histone deacetylases or HDACs carry out acetyl removal. HDACs are generally associated with repressor activity and enable recruitment of cofactors that trigger transcriptional repression directly or through recruitment of subsequent PTM modifying enzymes that enable protein degradation. Sumoylation and ubiquitination involve the addition of a SUMO or ubiquitin moiety respectively and this is carried out by three enzymes. The E1 enzyme which transfers the moiety to the E2 conjugation enzyme which is then attached to the target protein through the E3 ligase. Ubiquitin modulates the transcriptional activity of its attached proteins directly by modulating transcriptional activity and indirectly by modulating the protein stability and half-life through proteasome mediated degradation. The SUMO group, in addition to modifying transcriptional activity and stability, is unique in terms of its ability to modify the cellular localization of its target protein.

Fli1 has two different isoforms in mice while the human variant has 9 different isoforms. The major mouse isoform has a coding DNA sequence of 9 exons covering 1359 nucleotides which translates to a 452 amino acid protein. Fli1 has a large number of annotated post translational modifications throughout the protein however three of

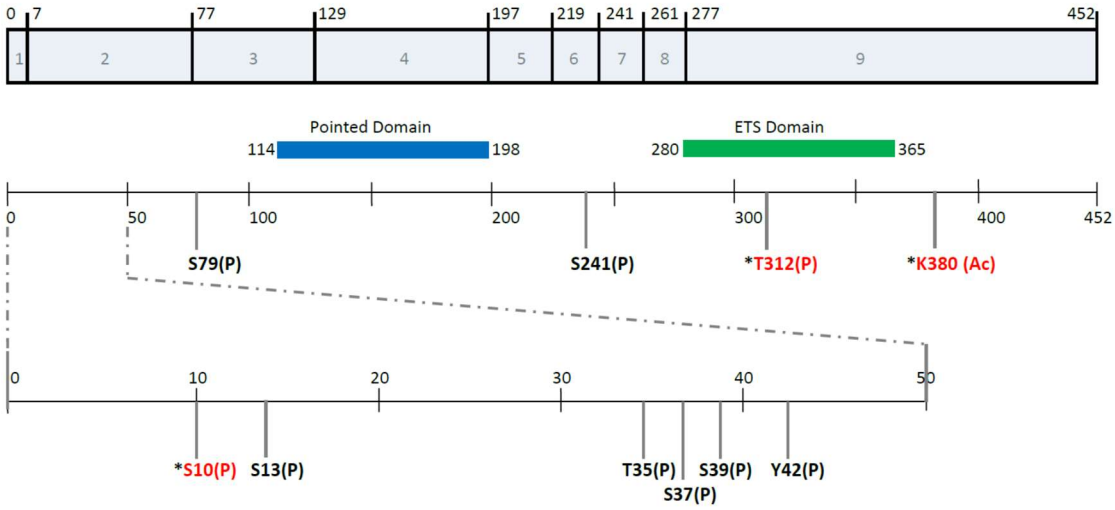


Figure 1.11 Schematic of murine Fli1 gene representing the 9 exons with the location of the pointed domain and ETS DNA binding domain, total protein length of 452 amino acids and various post translational modifications identified for the protein.

those have been implicated so far with functional significance (Figure 1.11). The Serine10 phosphorylation inhibits Runx1 binding essential to switch from an undifferentiated state to a differentiated state in murine megakaryocytic cell line L8057(Huang et al., 2009). This interaction with Runx1 was facilitated purely through amino acids 281-452, essentially exon 9 which covers the bulk of the protein. Replacement of the Serine10 with a phosphomimetic moiety significantly disrupted the Runx1 interaction confirming the requirement of the PTM to facilitate the switch in the cells. The other is a sequential PTM initiated by transforming growth factor β (TGFβ) in fibroblasts. Fli1 is phosphorylated at the threonine 312 by protein kinase C δ (PKC-δ) in response to TGFβ stimulus(Asano and Trojanowska, 2009). This leads to recruitment of p300/CREB binding protein associated factor (PCAF) that acetylates

the Lysine380 residue destabilizing the protein and leading to its degradation(Asano et al., 2007).

1.6 MOUSE MODELS OF FLI1

The first mouse model of Fli1 had a very minor defect in thymic development exhibiting hypocellularity due to dysfunctional prethymic T-cell progenitors (Melet et al., 1996). The design of the intended knockout mouse led to the formation of a mutant Fli1 from an alternate start site further downstream of the excised region and hence the subtle phenotype was not a true reflection of the role of Fli1. In 2000, the first global knockout mouse was designed and Fli1 deletion was found to be embryonic lethal at E11.5 due to a loss of vascular integrity resulting in cerebral hemorrhage with a concomitant reduction in Tie2 (Hart et al., 2000). The Fli1 null embryos were also associated with defects in megakaryopoiesis which corelated with a human condition, Jacobsen or Paris-Trousseau Syndrome where a region containing Fli1 was hemizygously deleted. Fli1 and Erg genes are both characterized by the presence of a pointed domain, the ETS DNA binding domain and an N-terminal and C-terminal transactivation domain. To get a more mechanistic insight into the role of the C-terminal binding domain Spyropoulos et al., 2000 designed a specific deletion targeting the region just upstream of this domain and found that it closely phenocopied the deletion of the entire last exon. Later research expanded on the phenotypes exhibited in these two early papers and observed a significant reduction in fetal liver cellularity in the mouse embryos. Additionally, E10.0 AGM cells were dysfunctional in giving rise to any cells of the megakaryocyte lineage with a concomitant reduction in the levels of c-mpl (Kawada et al., 2001).

Another mouse model was used to specifically target only the carboxy-terminal activation domain (Moussa et al., 2010) and resulted in a embryonic lethal phenotype with reduced penetrance. These mice had reduced survival viability with about 30% dying postnatally. The viable mice have significantly reduced platelet numbers and exhibited defects in platelet aggregation and activation and bleeding times. Some of the genes dysregulated in the BM and megakaryocyte compartment were c-mpl, gpIIb, gpIV and PF4. These mice were a valuable resource given their ability to survive to adulthood. Fli1 was found to regulate B cell development and Fli1$^{\Delta CTA}$ mice had significantly reduced splenic follicular B cells and increased number of transitional and marginal zone B cells compared to controls (Zhang et al., 2008). Fli1 was also correlated to pathogenesis in diffuse large B-cell lymphoma, one of the most common forms of human lymphoma. A gain of function in the Fli1/Ets1 locus was found in about 23% of the DLBCL cases that were analyzed (Bonetti et al., 2013). Fli1 overexpression was also found to dysregulate T-cell development affecting the DN to DP transition and inhibiting CD4 expression while increasing CD8 (Smeets et al., 2013). Given its impact on lymphoid development, the role of Fli1 was explored in the context of lupus and showed strong correlation with hemizygous reduction in Fli1 levels resulting in improved disease outcomes in mouse models for lupus (Mathenia et al., 2010; Molano et al., 2010; Nowling and Gilkeson, 2006; Richard et al., 2013). Fli1 has been shown to play a role in regulating monocyte, macrophage and dendritic cell development. The surviving Fli1$^{\Delta CTA}$ mice had increased numbers of HSC and common dendritic cell precursors compared to controls (Suzuki et al., 2013).

The role of Fli1 so far had been explored in the context of non-cell specific deletion. With the arrival of the cre-lox technology, specific Fli1 deletion in cell types

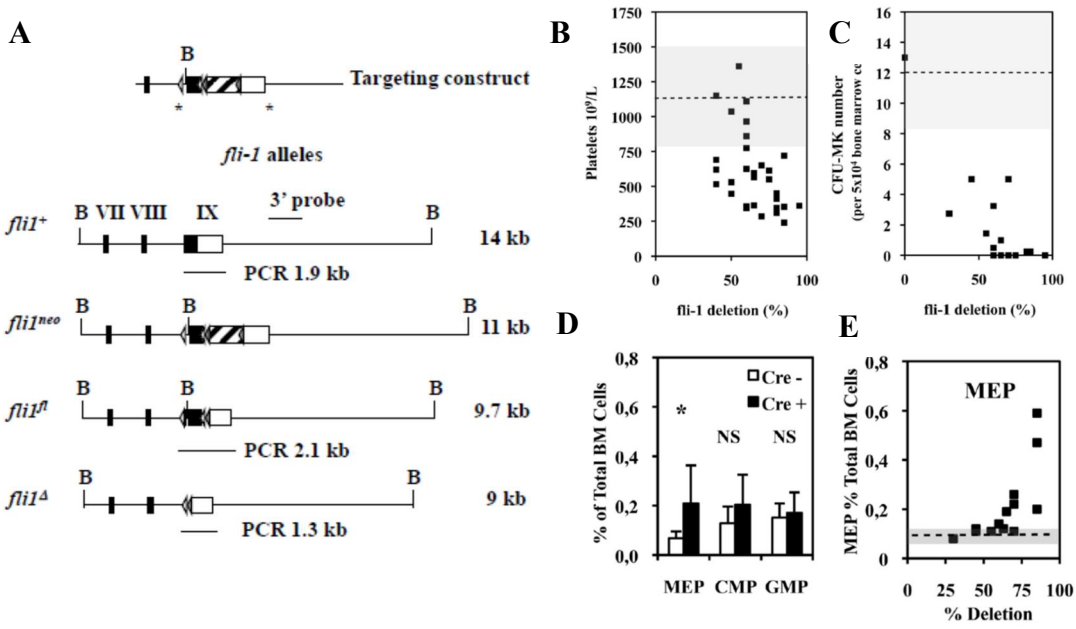


Figure 1.12 : A. Schematic diagram of targeting construct for inducible Fli1 mouse. The region of deletion is in the last exon of Fli1 containing the ETS DNA binding domain. B. Effect of Fli1 deletion on platelet numbers. C. Effect of Fli1 deletion on CFU-Mk numbers for $5x10^4$ BM Cells(Dotted line and gray area represent mean and SD of control mice). D. Histogram representing percentage of multipotent progenitors in the BM (Asterik and NS represent significant $p<0.05$ and not significant). E. Variation in %MEP with Fli1 deletion efficiency (Starck et al., 2010)

of interest could be targeted. Not surprisingly the first two cell types to be targeted were endothelial cells and hematopoietic cells. The conditional knockout of Fli1 in endothelial cells resulted in a very mild vascular defect of increased permeability and a slight reduction in VE-Cadherin and CD31 RNA levels (Asano et al., 2010). The level of knockout in these mice however did not seem to be adequate. The conditional

deletion in the hematopoietic compartment was carried out using the mx1-cre. The effect of deletion in the adult partially phenocopied the global deletion with the mice experiencing mild thrombocytopenia with a drastic decrease in mature megakaryocyte numbers (Figure 1.12). There was an increase in the MEP population by 50% with the CMP and GMP compartments not significantly affected. Fli1 deletion induced a block in differentiation among the mega-erythroid lineage with mature megakaryocyte numbers close to absent. One common observation from both these papers was that the efficiency of Fli1deletion was notably lower than what would be expected from these cre deleters (Asano et al., 2010; Starck et al., 2010). It is difficult to predict if this is to do with genomic accessibility or cre efficiency however it is definitely a point to be noted moving forward with inducible mouse models for Fli1.

Several groups have attempted to elucidate the role of Fli1 in endothelial cells, fibroblasts and various terminally differentiated and multipotent lineages of hematopoietic cells (Asano et al., 2009; Asano et al., 2010; Mathenia et al., 2010; Moussa et al., 2010; Starck et al., 2010; Suzuki et al., 2012; Zhang et al., 2008; Zhang and Watson, 2005); however given the redundancy of factors in adult homeostasis when compared to embryonic development we decided to interrogate the exact function of Fli1 in maintaining hematopoietic homeostasis focusing specifically on the HSC compartment.

We hypothesized that

"Fli1 is essential for maintenance of hematopoietic stem cell and endothelial cell homeostasis."

In Chapter 2 of this dissertation, we investigated the role of Fli1 in HSCs, in vivo and in vitro using various cre lines and animal models so as to ablate Fli1 in both a spatial and temporally controlled manner. We show that Fli1 is unique in its functional relevance in both development as well as adult homeostasis making it indispensable to HSC specification, self-renewal and homeostasis. In Chapter 3, we explored the role of Fli1 in endothelial homeostasis and show that in conjunction with another ETS factor, Erg, Fli1 is essential for maintenance of endothelial fate and is required for survival.

1.7 SIGNIFICANCE

The hematopoietic system has several unique features that have enabled research in this field to progress in leaps and bounds. First, the ability to transplant hematopoietic stem cells that are capable of reconstituting the entire hematopoietic system of the host has pushed new frontiers in terms of regenerative medicine (Kiernan et al., 2017). Stem cell transplantation has become a routine practice for the treatment of hematological disorders as well as malignancies following myeloablative therapies to deplete the host bone marrow. Secondly, the ease of acquiring these precious reconstitution units has aided in researching HSCs. The discovery of factors that trigger the mobilization of HSCs from the BM into circulation such as granulocyte colony-stimulating factor (G-CSF) and AMD3100 (Suárez-Álvarez et al., 2012) has only added to the ease of collecting these cells. Lastly, the ability of these cells to withstand the stress of sorting has allowed them to be studied in great detail and separated extensively based on surface marker expression so as to narrow down the exact expression pattern that distinguishes these cells from their subsequent progeny. Despite having all these advantages, there still are a large number of looming

questions for the successful isolation, maintenance, expansion and transplantation of these cells (Anand et al., 2017; Kiernan et al., 2017; Wagner et al., 2014). Further research is required to minimize engraftment failure as well as post-transplant infections. Understanding the mechanisms that regulate these key features will enable a significant increase in efficiency of transplantation. The processes that are involved in current transplantation settings are

- Isolation and storage of transplantable HSPCs
- Expansion of the isolated HSPCs
- Myeloablation/pre-conditioning of the host for transplantation
- Determination of the quality and quantity of HSPCs to be transplanted
- Post transplantation monitoring to ensure donor engraftment
- Monitoring for post-transplant infections
- Monitoring for donor cell rejection or Graft vs host disease

Each of the steps mentioned above has the potential to be improved from an efficiency point of view. Isolation of a small number of the exact cells required for transplantation with a minimally invasive method without significant side effects on the donor requires a thorough understanding of the mechanisms that govern stem cell mobilization as well as the exact surface marker profile for identifying the number of HSCs present in every isolate. Having the tools for long term storage of these cells without affecting their viability or potency requires an understanding of the mechanisms that govern stem cell maintenance and homeostasis so as to trigger a quiescent state in the cells before storage. Expansion and manipulation of these cell types in situations where cell numbers are not adequate for transplantation requires an in depth understanding of the interactions of the HSCs with the BM niche so that they

can be manipulated to expand the pool of HSCs without triggering differentiation into more lineage restricted progenitors. Understanding the mechanisms governing engraftment and homing would allow a quicker recovery from the myeloablation which itself needs to be optimized. Devising a pre-conditioning regiment that would allow specific homing without rendering the host immunocompromised as well as protecting the host from infections and GVHD would represent a breakthrough in the field of regenerative medicine. What is clear is that despite all the research that has been done thus far, there is still a long way to go in understanding the mechanisms that govern HSC behavior in different scenarios and to this end we intend to interrogate one of the possible master regulators of HSC biology – Fli1 may govern various mechanisms that play a role in each and every aspect of HSC function.

The notion that there exists a single stem cell at the top of the hierarchy in the hematopoietic system was proposed back in 1961 by some pioneering research by Till and McCulloch. The advent of the colony forming units in the spleen as a measure of the stem cell capacity of HSCs marked the beginning of quantitative hematopoietic assays (Becker et al., 1963; Siminovitch et al., 1963; Till and McCulloch, 1961). The establishment of clonal assays in transplantation settings to examine the self-renewal, clonogenicity and lineage contribution enabled researchers to interrogate the 'stemness' of HSCs based on surface marker expression. This triggered an array of publications claiming the ability to enrich the stem cell population based of marker expression (Kiel et al., 2005; Kondo et al., 1997; Morrison and Weissman, 1994; Osawa et al., 1996a; Osawa et al., 1996b). In-vivo transplantation provided functional readouts for each aspect of HSC function. Primary transplants enabled assessment of HSC contribution to multilineage engraftment and self-renewal capacity. Serial transplantation served as a more robust readout to claim HSC self-renewal. The

frequency of HSCs in a given population could be determined by the limiting dilution assay (Szilvassy et al., 1990). The murine system allowed for these robust readouts. Additionally, the generation of genetically modified immunocompromised humanized mice enabled the interrogation of the stem cell functionality of human stem and progenitor cells in a murine system (McDermott et al., 2010).

CHAPTER 2: FLI1 IS ESSENTIAL FOR HEMATOPOIETIC STEM CELL SPECIFICATION, MAINTENANCE AND FUNCTION

2.1 INTRODUCTION

Fli1 has been shown to be essential during development. Global deletion of Fli1 results in embryonic lethality at E11.5 with the mice exhibiting extensive hemorrhage as a result of vascular leakiness and platelet dysfunction (Hart et al., 2000; Spyropoulos et al., 2000). The role of Fli1 has been studied extensively in the context of megakaryocytes differentiation and platelet formation and more recently in the context of other differentiated peripheral blood hematopoietic lineages; however, its role in regulating HSC function is still to be explored (Starck et al., 2010). Genomic occupancy of Fli1 has been explored, in conjunction with a few other genes that have developmental hematopoietic defects, in human cord blood $CD34^{+}$ cells (Beck et al., 2013) as well as in the murine multipotent progenitor cell line, HPC7 (Wilson et al., 2010). This analysis revealed a densely interconnected network of a heptad of transcription factors that overlap extensively in their binding purview – Fli1, Erg, Gata2, Scl, Lyl1, Lmo2 and Runx1; however their analysis implicated four critical genes – Erg, Runx1, Gata2 and Scl to be involved in protein-protein interactions necessary to facilitate formation of a transcriptional complex that can bind to DNA. This genomic binding data provided valuable information into the binding purviews of these genes; however genomic binding does not imply functionality as was seen with a majority of the implicated heptad. The caveat to the binding of multiple factors in the same genomic location was redundancy and this has been shown for four of the seven factors. Gata2 has been shown to be essential in both specification as well as expansion of definitive HSCs during development (de Pater et al., 2013) however its

exact role in adult HSCs has not been explored. Functional relevance of a factor in adult homeostasis has been shown to be quite different compared to development as is seen with Scl, Lyl1 and Lmo2 all of which have developmental endothelial and hematopoietic phenotypes but do not execute similar roles in adult hematopoiesis. Erg is an ETS factor that is closely related to Fli1 and has the most number of binding sites among the heptads. It has been implicated to be one of the essential hematopoietic transcription factors and has shown to be essential for HSC function during development(Kruse et al., 2009; Loughran et al., 2008) as well as in adults (Taoudi et al., 2011). The majority of research implicating a role for Erg in HSC maintenance has used a mutation Mld2 that silences the transcriptional activity of Erg and so overlooks its role in the vascular niche which is essential in maintaining stem cell homeostasis. Recent evidence suggests that Erg may indeed play a dual role in regulating the niche as well as hematopoiesis however the exact mechanisms governing this are still to be explored.

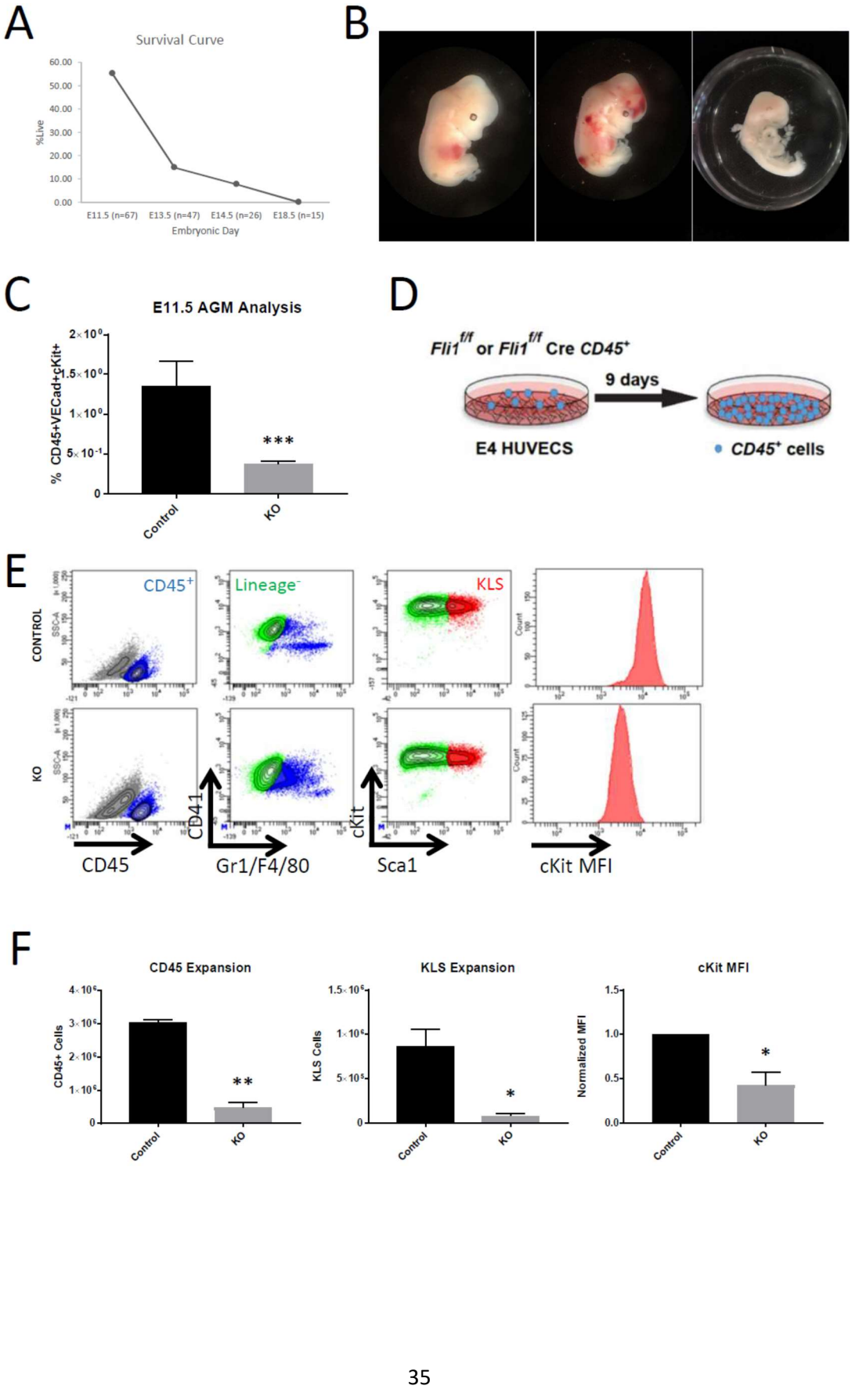
Fli1 is a transcription factor that has been completely overlooked in terms of its contribution to HSCs. Earlier research has demonstrated a role for Fli1 in vascular integrity during development and extensively in megakaryopoiesis. Previous inducible mouse models have revealed difficulties in the efficiency of deleting Fli1 which is why we used strong cre deleters – Cdh5 cre (Chen et al., 2009) and the Rosa CreERT2. In order to better understand the spatial and temporal role of Fli1 in the context of HSCs, we used these cre lines to delete Fli1 at different time points during development as well as in adult. By utilizing well designed transplantation schema in conjunction with the efficient cre deletion we were able to interrogate the function of Fli1 in the context of adult HSC homeostasis as well as its role in hematopoietic reconstitution.

2.2 RESULTS

2.2.1 Fli1 is essential for definitive hematopoiesis during development

In order to investigate the role of Fli1 in the hematopoietic system we used $Fli1^{f/f}$ mice (Starck et al., 2010) and crossed them with Cdh5 (VE-Cadherin) Cre recombinase which deletes in the endothelial cells of AGM region of the developing embryo (Chen et al., 2009). $Fli1^{f/f}$; Cdh5 Cre was embryonic lethal with reduction in mendelian ratios post E11.5 (Figure 2.1A). Very few embryos were observed after E14.5 and most embryos at E13.5 exhibited major hemorrhage or were being resorbed (Figure 2.1B). This closely phenocopied the global deletion of Fli1 which demonstrated that the crux of Fli1 function is focused on hematopoietic and endothelial lineages despite being expressed in a variety of different cell types at much lower levels. In order to assess hematopoietic status of these mice, the AGM region was harvested at E11.5. E11.5 mice did not display any obvious defects visually however on further analysis they exhibited significant reduction in total $CD45^+$ cells as well as $CD45^+VECadherin^+cKit^+$ cell numbers (Figure 2.1C). In order to further evaluate the cell autonomous function of these cells we sorted them for ex-vivo expansion using the robust E4-HUVEC platform (Figure 2.1D) for hematopoietic expansion (Butler et al., 2010; Seandel et al., 2008). The $Fli1^{f/f}$ Cdh5 Cre cells exhibited 6-fold lower expansion potential compared to the same number of $CD45^+$ cells from $Fli1^{f/f}$ littermates. Early definitive hematopoietic cells isolated from the AGM on expansion on the E4 HUVEC platform acquire a more fetal stem cell profile expressing CD41 and Sca1 (Figure 2.1E). Flow analysis of these

Figure 2.1 Fli1 deletion in AGM endothelium results in embryonic lethality and hematopoietic defects (A) Survival curve for $Fli1^{f/f}$ Cdh5 Cre compared to $Fli1^{f/f}$ embryos at time points E11.5 (n=67), E13.5 (n=47), E14.5 (n=26), E18.5 (n=15) **(B)** Representative images of E13.5 embryos with Control $Fli1^{f/f}$ (left) and $Fli1^{f/f}$ Cdh5 Cre **(**Center and Right) showing internal hemorrhage (Center) and absence of heartbeat (Right) **(C)** Quantification of E11.5 AGM $CD45^{+}VECadherin^{+}cKit^{+}$ percentage comparing $Fli1^{f/f}$ to $Fli1^{f/f}$ Cdh5 Cre (*** signifies $p< 0.001$) **(D)** Schematic showing ex-vivo expansion strategy to assay cell autonomous function of AGM $CD45^{+}$ cells **(E)** Representative flow plots showing gating scheme to identify expanded fetal KLS. Histogram to show reduction in cKit levels in Control vs KO expanded $CD45^{+}$ cells **(F)** Quantification of total CD45 and KLS expansion in vitro, cKit mean fluorescent intensity (** signifies $p < 0.01$, * signifies $p < 0.05$)


A
Survival Curve
%Live
60.00
50.00
40.00
30.00
20.00
10.00
0.00
E11.5 (n=67)
E13.5 (n=47)
E14.5 (n=26)
E18.5 (n=15)
Embryonic Day
B
C
E11.5 AGM Analysis
% CD45+VECad+cKit+
2×10⁰
1.5×10⁰
1×10⁰
5×10⁻¹
0
***
Control
KO
D
Fli1f/f or Fli1f/f Cre CD45+
9 days
E4 HUVECS
CD45+ cells
E
CONTROL
KO
CD45+
Lineage-
KLS
SSC-A
Count
CD45
CD41
Gr1/F4/80
cKit
Sca1
cKit MFI
F
CD45 Expansion
CD45+ Cells
4×10⁶
3×10⁶
2×10⁶
1×10⁶
0
**
Control
KO
KLS Expansion
KLS Cells
1.5×10⁶
1×10⁶
5×10⁵
0
*
Control
KO
cKit MFI
Normalized MFI
1.5
1.0
0.5
0.0
*
Control
KO

expanded $CD45^+$ cells revealed defective overall expansion as well as a significant reduction in $cKit^+Lineage^-Sca1^+$ (KLS) expansion (Figure 2.1F). Additionally, the cKit expression levels of these expanded cells had a clear reduction based on mean fluorescence intensity(MFI) (Figure 2.1F). This shift in cKit MFI has been shown before (Kimura et al., 2011) and has been correlated with the absence of cKit signaling which is known to be essential for stem cell survival. These results indicate that Fli1 expression in AGM is essential for embryonic development and survival and that the Fli1-cKit axis is a critical part of definitive hematopoiesis during development.

2.2.2 Fli1 expression in the hematopoietic system is essential to maintain homeostasis

Given previous evidence that Fli1 deletion using temporally inducible cre deletion is not very efficient (Starck et al., 2010) we tested the deletion efficiency of the Rosa CreERT2 in adults (>12 week old) using $Fli1^{f/f}$, $Fli1^{f/f}$ Cre, $Fli1^{f/+}$, $Fli1^{f/+}$ Cre mice. We found that just 3 injections of tamoxifen were sufficient to induce a lethal phenotype in the $Fli1^{f/f}$ Cre mice from Day 7-11 post tamoxifen induction (Figure 2.2A, B). Seeing this drastic phenotype, we designed a transplant setting to assess the cell autonomous effect of Fli1 in the hematopoietic compartment (Figure 2.2C). Lethally irradiated CD45.1 mice were transplanted with $Fli1^{f/f}$ Cre or $Fli1^{f/f}$ whole bone marrow from littermates. Fli1 deletion was induced 4 months post radiation, after confirming engraftment chimerism and peripheral blood counts to ensure homeostatic conditions for the mice. CD45.1; $Fli1^{f/f}$ Cre mice phenocopied the global deletion and died within 11 days of tamoxifen induction compared to the control transplants that were healthy (Figure 2.2D). The Fli1 deleted mice had internal bleeding as well as significant reduction in peripheral blood counts with platelets counts dropping drastically (Figure 2.2E). The importance of Fli1 in megakaryopoiesis and

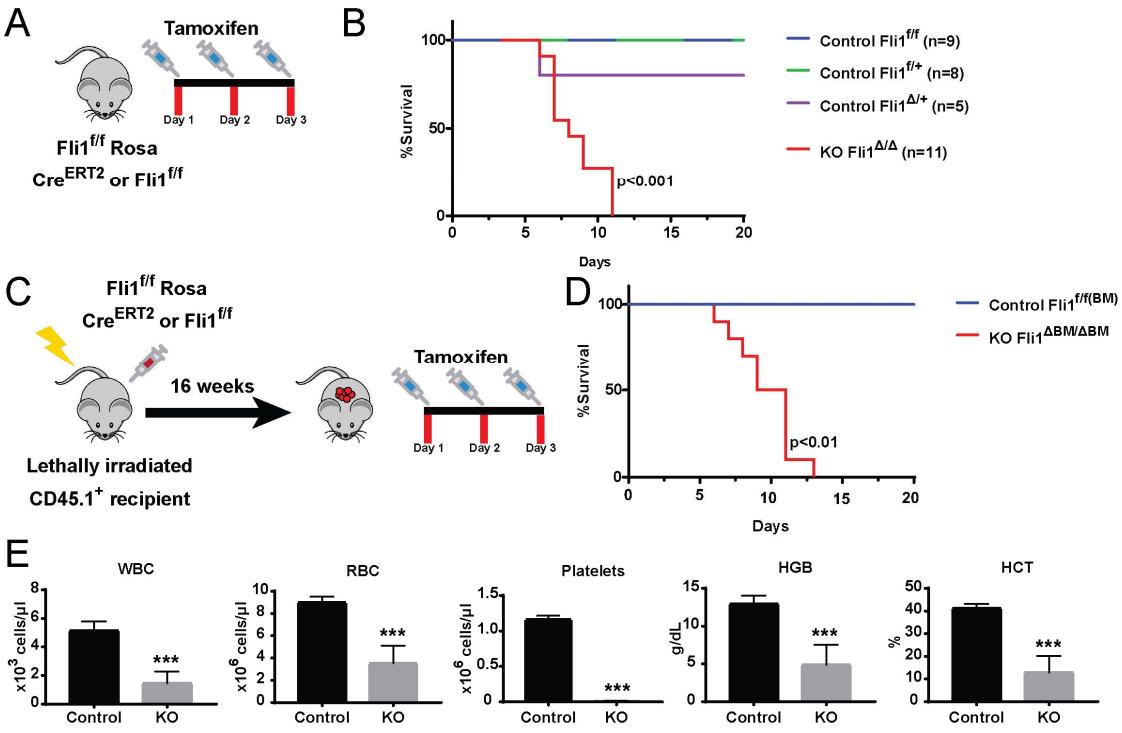


Figure 2.2 Fli1 is required for hematopoietic homeostasis in adults (A) Mouse schematic showing deletion strategy to assess global deletion of Fli1 in adult mice (B) Survival cure for global deletion of Fli1 using Rosa Cre^{ERT2}, experimental cohorts compared $Fli1^{f/f}$, $Fli1^{\Delta/\Delta}$, $Fli1^{f/+}$, $Fli1^{\Delta/+}$ Cre mice (C) Mouse schematic showing transplant strategy to elucidate the role of Fli1 in hematopoietic cells (D) Survival curve for transplanted CD45.1 mice with $Fli1^{f/f}$ or $Fli1^{f/f}$ Cre BM (E) Peripheral blood counts (WBC – White blood cells, RBC – Red blood cells, Platelets, HGB – Hemoglobin, HCT – Hematocrit) of $Fli1^{f/f}$ vs $Fli1^{\Delta/\Delta}$ post deletion (*** signifies $p<0.001$, ** signifies $p<0.01$, * signifies $p<0.05$)

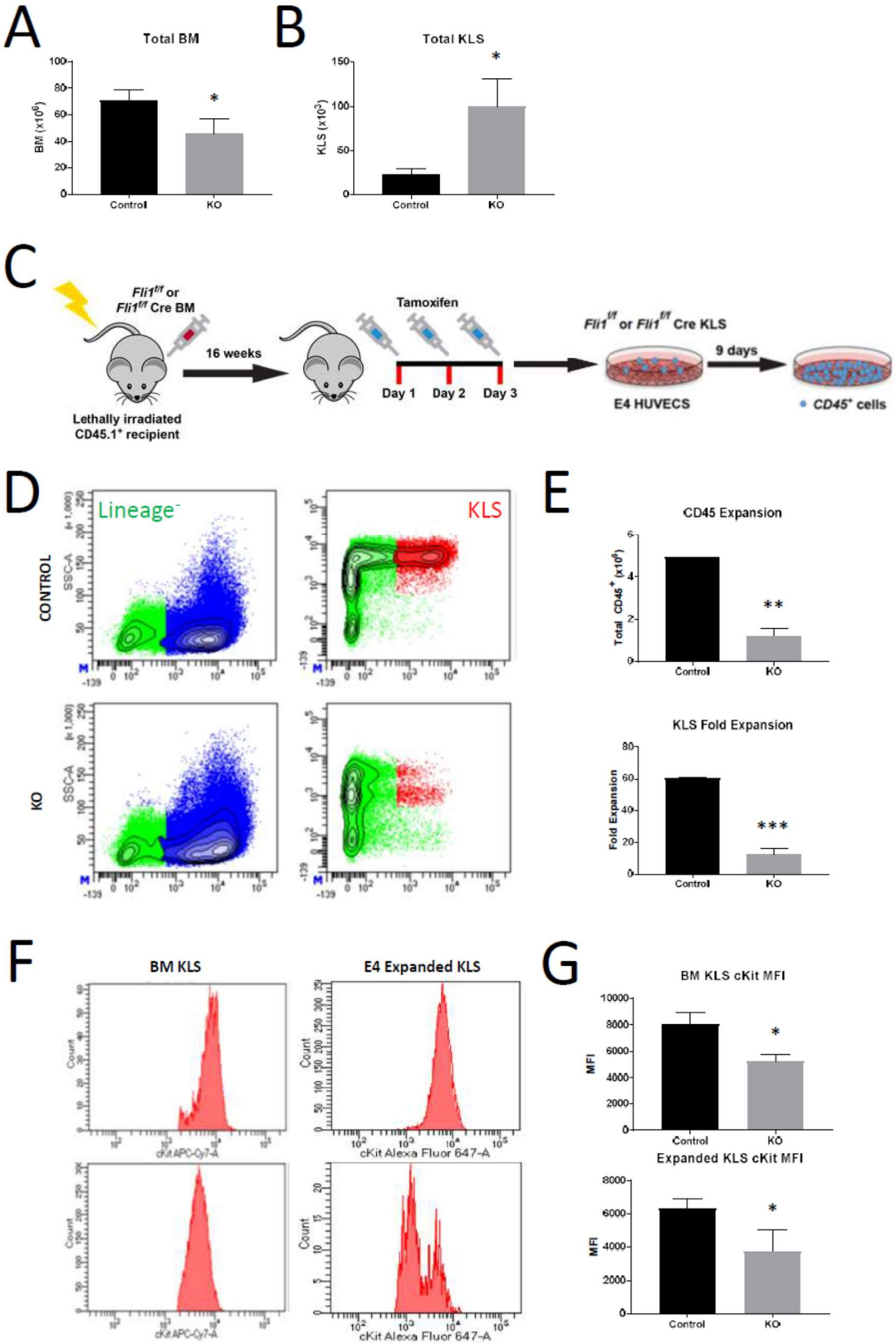
thrombopoiesis has been well studied however the global drop in both WBC and RBC counts has not been shown before. We account this drastic platelet drop to deletion efficiency achieved with the Rosa Cre^{ERT2} mice which compared to the MX1-cre in

the context of this gene is much more effective. Taken together these results show that Fli1 is a critical regulator of hematopoietic homeostasis and essential for survival.

2.2.3 Fli1 regulates stem cell function in a cell autonomous manner

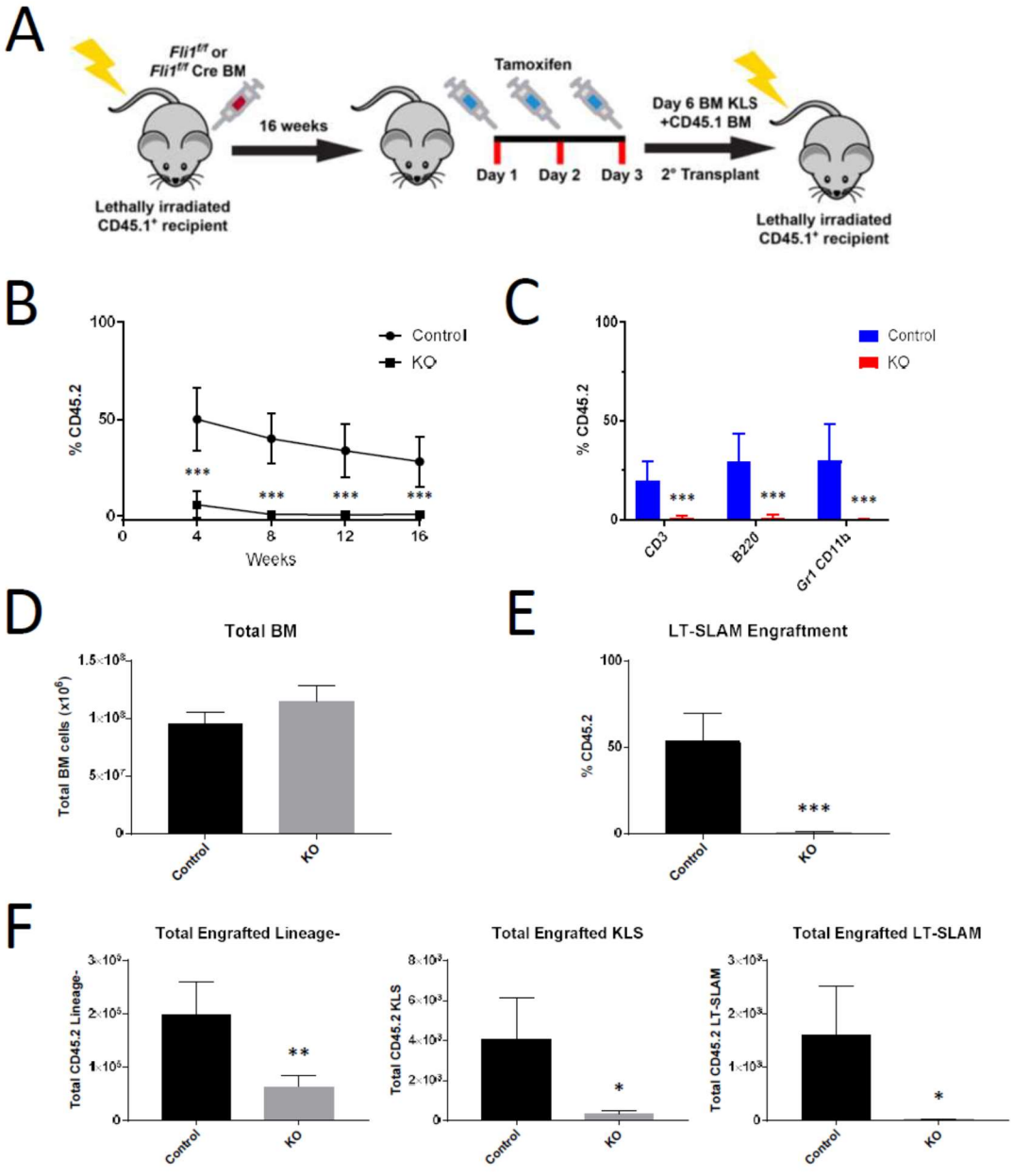
Having observed complete peripheral blood failure in the Fli1 deleted transplanted mice we next analyzed the bone marrow to examine the cause of this hematopoietic effect. There was a significant reduction in bone marrow cellularity that was reflected in bone marrow counts (Figure 2.3A). As expected the drop in peripheral blood levels triggered a spontaneous expansion in the HSC compartment showing a 4-fold increase (Figure 2.3B) in KLS multipotent progenitors ($cKit^{+}Sca1^{+}Lineage^{neg}$). Given the absence of functional data on the role of Fli1 in HSCs we interrogated the functionality of this KLS pool in the BM. We isolated the KLS cells from the deleted mice and expanded them in the E4-HUVEC system (Figure 2.3C) used earlier for the AGM hematopoietic cells. The $Fli1^{\Delta/\Delta}$ cells were severely compromised in terms of their expansion ability showing a 3-fold reduction in total $CD45^{+}$ expansion and a further 8-fold reduction in KLS expansion (Figure 2.3D, E) pointing to defects not only in the differentiation of these progenitors but also their ability to self-renew and maintain their stem cell fate. A common theme that we observed with these KLS cells was the reduction in cKit as was seen in the AGM expansions. cKit levels were significantly reduced both in vivo in the expanded KLS population as well as in vitro post expansion showing two distinct populations of cKit high and cKit mid cells (Figure 2.3F, G). These results demonstrated $Fli1^{\Delta/\Delta}$ mice are functionally compromised in their ability to self-renew, expand and differentiate in an ex-vivo platform. This dysfunction is linked to the Fli1-cKit axis which appears to be essential to maintain homeostasis.

Figure 2.3 Fli1 deletion results in expansion of a dysfunctional stem cell pool that is unable to self-renew, expand and differentiate in an ex-vivo setting (A) BM cellularity of two femurs in transplanted mice comparing Fli1 $^{f/f}$ or Fli1$^{f/f}$ Cre **(B)** Total KLS numbers in two femurs comparing Fli1 $^{f/f}$ or Fli1$^{f/f}$ Cre **(C) S**chematic showing experimental plan for tamoxifen induction followed by in vitro expansion **(D)** Representative flow plots showing gating strategy to assess KLS expansion in vitro **(E)** Quantification of CD45 and KLS expansion between Control and KO cells **(F)** Histograms showing cKit levels in vivo in BM KLS as well as post expansion in vitro in expanded KLS fraction **(G)** Quantification of cKit mean fluorescent intensity in vivo and post expansion in the KLS population (Statistics * signifies $p < 0.05$, ** signifies $p < 0.01$, *** signifies $p < 0.001$)


A
Total BM
BM (x10^6)
Control
KO
B
Total KLS
KLS (x10^3)
Control
KO
C
Fli1^f/f or Fli1^f/f Cre BM
16 weeks
Lethally irradiated CD45.1+ recipient
Tamoxifen
Day 1
Day 2
Day 3
Fli1^f/f or Fli1^f/f Cre KLS
9 days
E4 HUVECS
CD45+ cells
D
CONTROL
KO
Lineage-
KLS
SSC-A
E
CD45 Expansion
Total CD45+ (x10^6)
Control
KO
KLS Fold Expansion
Fold Expansion
Control
KO
F
BM KLS
E4 Expanded KLS
Count
cKit APC-Cy7-A
cKit Alexa Fluor 647-A
G
BM KLS cKit MFI
MFI
Control
KO
Expanded KLS cKit MFI
MFI
Control
KO

Having observed significant expansion and self-renewal defects in the MPPs we decided to further investigate the functionality of the HSCs by performing secondary transplants in conjugation with competitor CD45.1 WBM to ensure survival and BM homeostasis long term (Figure 2.4A). The peripheral blood engraftment of the $Fli1^{\Delta/\Delta}$ cells in the secondary recipients was significantly lower compared to control $Fli1^{f/f}$ and was unchanged for the duration of 16 weeks (Figure 2.4B). This extended to all three peripheral blood lineages – myeloid, B cell and T cells (Figure 2.4C) assessed at Week 16 pointing to an inherent stem cell defect that was funneling down to all peripheral blood lineages. In this context, the presence of competitor ensured that peripheral blood counts between the two transplant cohorts were the same and hematopoietic homeostasis was maintained. After 16 weeks, the BM of the mice was analyzed for engraftment in the stem cell and lineage compartments. The lack of engraftment seen in the peripheral blood was carried over to the BM showing minimal engraftment across the board, in both multipotent progenitors and long-term HSCs (Figure 2.4E, F). The engraftment was least in the LT-SLAM compartment with $Fli1^{\Delta/\Delta}$ cells minimally present (Figure 2.4E). The lineage contribution of the $Fli1^{\Delta/\Delta}$ cells in the BM was also drastically lower but still slightly higher compared to the stem cell compartment (Figure 2.4F). Importantly, the BM cellularity was unchanged between two cohorts due to the presence of CD45.1 cells (Figure 2.4D). This data shows that despite the initial expansion of the stem cell compartment as a result of the peripheral blood failure, the functionality of the LT-HSCs is severely compromised in terms of lineage contribution as well as engraftability and that in the absence of Fli1 the contribution of HSCs to hematopoiesis is minimal.

Figure 2.4 Fli1 regulates stem cell function in a cell autonomous manner (A) Mouse schematic showing experimental plan for secondary transplant to assess cell autonomous role of Fli1 **(B)** Peripheral blood engraftment assessed post-transplant for 16 weeks comparing Control and KO contribution **(C)** Lineage contribution of Control and KO cells to T cells ($CD3^+$), B cells ($B220^+$) and Myeloid cells ($Gr1^+CD11b^+$) in peripheral blood analyzed at Week 16 prior to BM analysis **(D)** BM cellularity from two femurs comparing Control and KO **(E)** Engraftment in the LT-SLAM ($CD45.2^+cKit^+Lineage^-Sca1^+CD150^+CD48^-$) fraction of the BM at 16 weeks **(F)** Quantification of total engrafted $CD45.2^+$ cells in the BM in the $Lineage^-$, KLS and LT-SLAM compartment (Statistics * signifies $p < 0.05$, ** signifies $p < 0.01$, *** signifies $p < 0.001$)


A
Fli1f/f or
Fli1f/f Cre BM
16 weeks
Lethally irradiated
CD45.1+ recipient
Tamoxifen
Day 1
Day 2
Day 3
Day 6 BM KLS
+CD45.1 BM
2° Transplant
Lethally irradiated
CD45.1+ recipient
B
% CD45.2
Control
KO
Weeks
C
% CD45.2
Control
KO
CD3
B220
Gr1 CD11b
D
Total BM
Total BM cells (x10^6)
Control
KO
E
LT-SLAM Engraftment
% CD45.2
Control
KO
F
Total Engrafted Lineage-
Total CD45.2 Lineage-
Total Engrafted KLS
Total CD45.2 KLS
Total Engrafted LT-SLAM
Total CD45.2 LT-SLAM

2.2.4 Fli1 is required for hematopoietic reconstitution

After assessing the dysfunctional engraftment capabilities of Fli1 deleted hematopoietic cells in a secondary transplant setting, we asked if Fli1 is required for hematopoietic reconstitution in primary recipients. Given the initial hematopoietic insult that triggered an expansion in the stem cell pool in the primary transplants it is important to assay the potential of unmanipulated stem cells in their ability to reconstitute hematopoiesis. To this end, we employed two transplant settings, first, the Sca1 depletion assay in which $Fli1^{f/f}$ Cre or $Fli1^{f/f}$ KLS were transplanted with a radiation recovery dose of Sca1 depleted CD45.1 BM into lethally irradiated CD45.1 mice. Deletion in these mice was induced one-week post transplantation so as to ascertain the ability of $Fli1^{\Delta/\Delta}$ HSCs to take over from the radiation recovery dose. There was a significant difference in survival between the Controls and the KO mice with the majority of KO mice dying within 3 weeks (Figure 2.5A) of transplantation implying that $Fli1^{\Delta/\Delta}$ HSCs are unable to sustain complete hematopoietic reconstitution.

In the second setting, we assayed the possible lineage contribution of the Fli1 deleted HSC. We transplanted $Fli1^{f/f}$ Cre or $Fli1^{f/f}$ KLS into lethally irradiated CD45.1 mice with CD45.1 whole bone marrow so as to ensure survival of the transplanted mice (Figure 2.5B). The peripheral blood engraftment in this scenario was comparable to the secondary transplant (Figure 2.4B) and significantly lower than the control over the 16 weeks post-transplant (Figure 2.5D). The deleted Fli1 cells were significantly impaired in reconstituting all three lineages in peripheral blood – Myeloid, B and T cell (Figure 2.5C) once again, confirming that the differentiation of the $Fli1^{\Delta/\Delta}$ HSCs is defective. The engraftment in the bone marrow was also significantly impaired

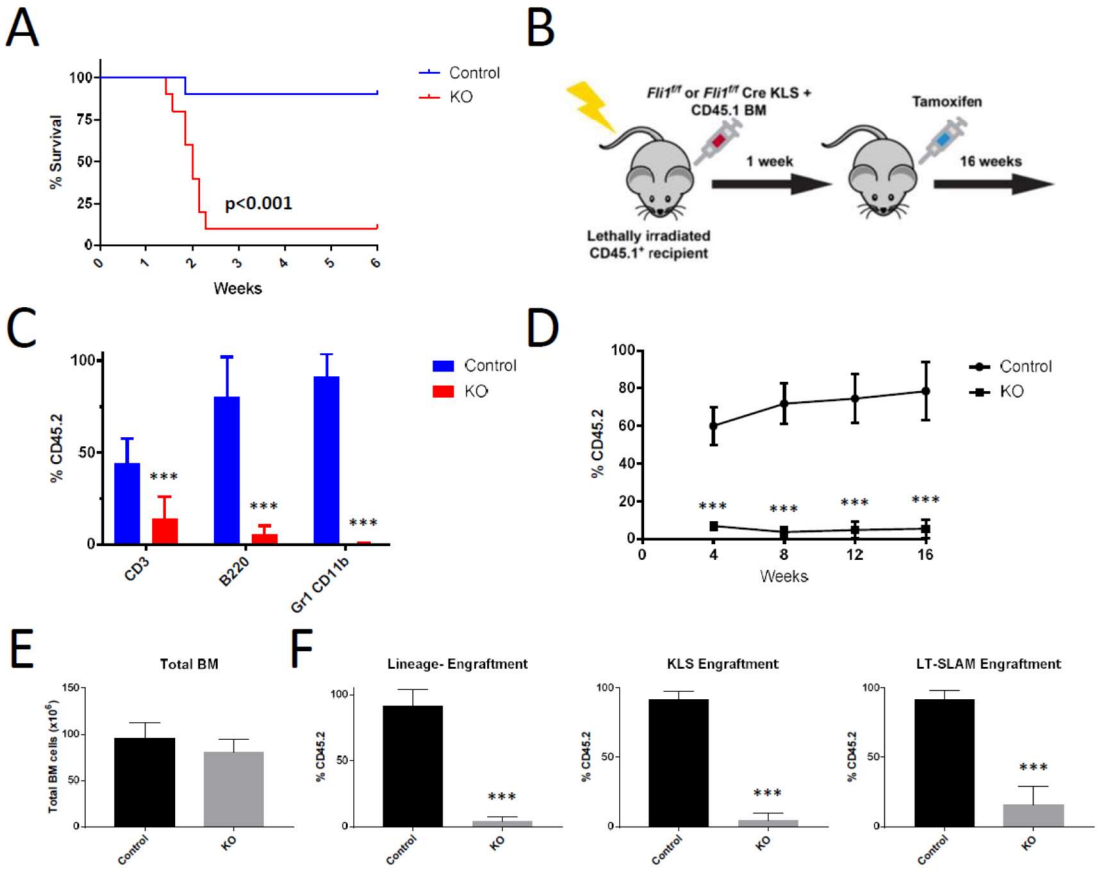


Figure 2.5 Fli1 is required for hematopoietic reconstitution (A) Sca1 depletion curve showing survival between Control and KO mice post-transplant **(B)** Mouse schematic showing experimental plan to assess hematopoietic reconstitution in primary recipients **(C)** Lineage contribution of Control and KO cells to T cells ($CD3^+$), B cells ($B220^+$) and Myeloid cells ($Gr1^+CD11b^+$) in peripheral blood analyzed at Week 16 prior to BM analysis **(D)**Peripheral blood engraftment assessed post-transplant for 16 weeks comparing Control and KO contribution **(E)** BM cellularity for two femurs at Week 18 **(F)** Quantification of % total engrafted $CD45.2^+$ cells in the BM in the $Lineage^-$, KLS and LT-SLAM compartment (Statistics * signifies $p < 0.05$, ** signifies $p < 0.01$, *** signifies $p < 0.001$)

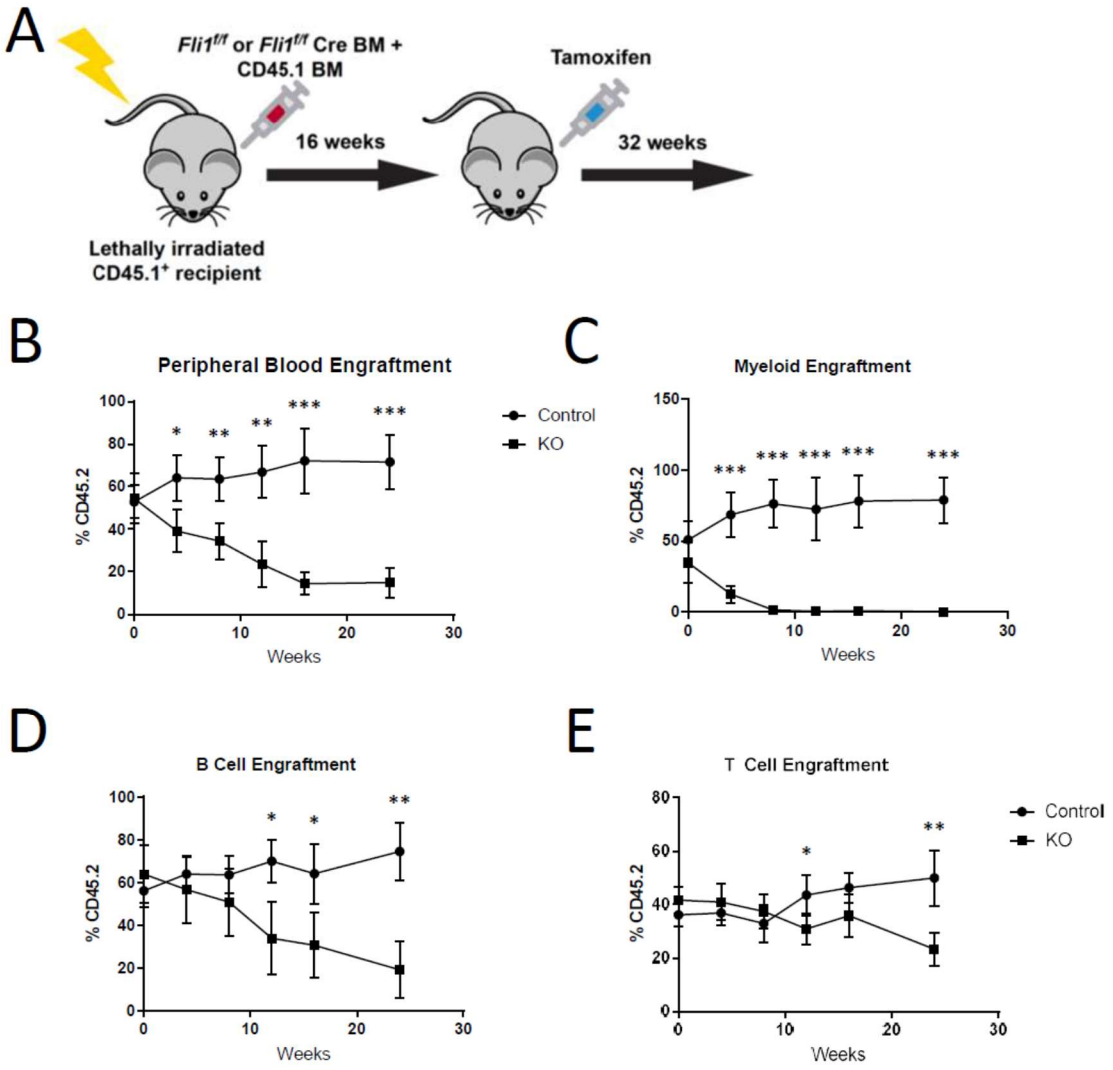


Figure 2.6 Fli1 deletion results in reduction of peripheral blood contribution across all lineages (A) Mouse schematic showing experimental plan to assess effect of Fli1 deletion under homeostatic conditions **(B)** Peripheral blood engraftment assessed post-transplant for 24 weeks comparing Control and KO contribution. Lineage contribution of Control and KO cells to **(C)** Myeloid cells ($Gr1^+CD11b^+$), **(D)** B cells ($B220^+$) and **(E)** T cells ($CD3^+$) in peripheral blood analyzed for 24 weeks post-transplant (Statistics * signifies $p < 0.05$, ** signifies $p < 0.01$, *** signifies $p < 0.001$)

when assessed at 18 weeks post-transplant (Figure 2.5F) affecting the Lineage^{neg}, KLS and LT-SLAM compartments of the BM. The BM cellularity was not affected as were the peripheral blood counts ensuring that these measurements were made under homeostatic conditions post radiation (Figure 2.5E). The functional deficit extended from the HSC compartment all the way to the peripheral blood lineages demonstrating that Fli1 is essential for hematopoietic reconstitution and affects functionality of the LT-HSC in terms of self-renewal, expansion as well as differentiation during reconstitution.

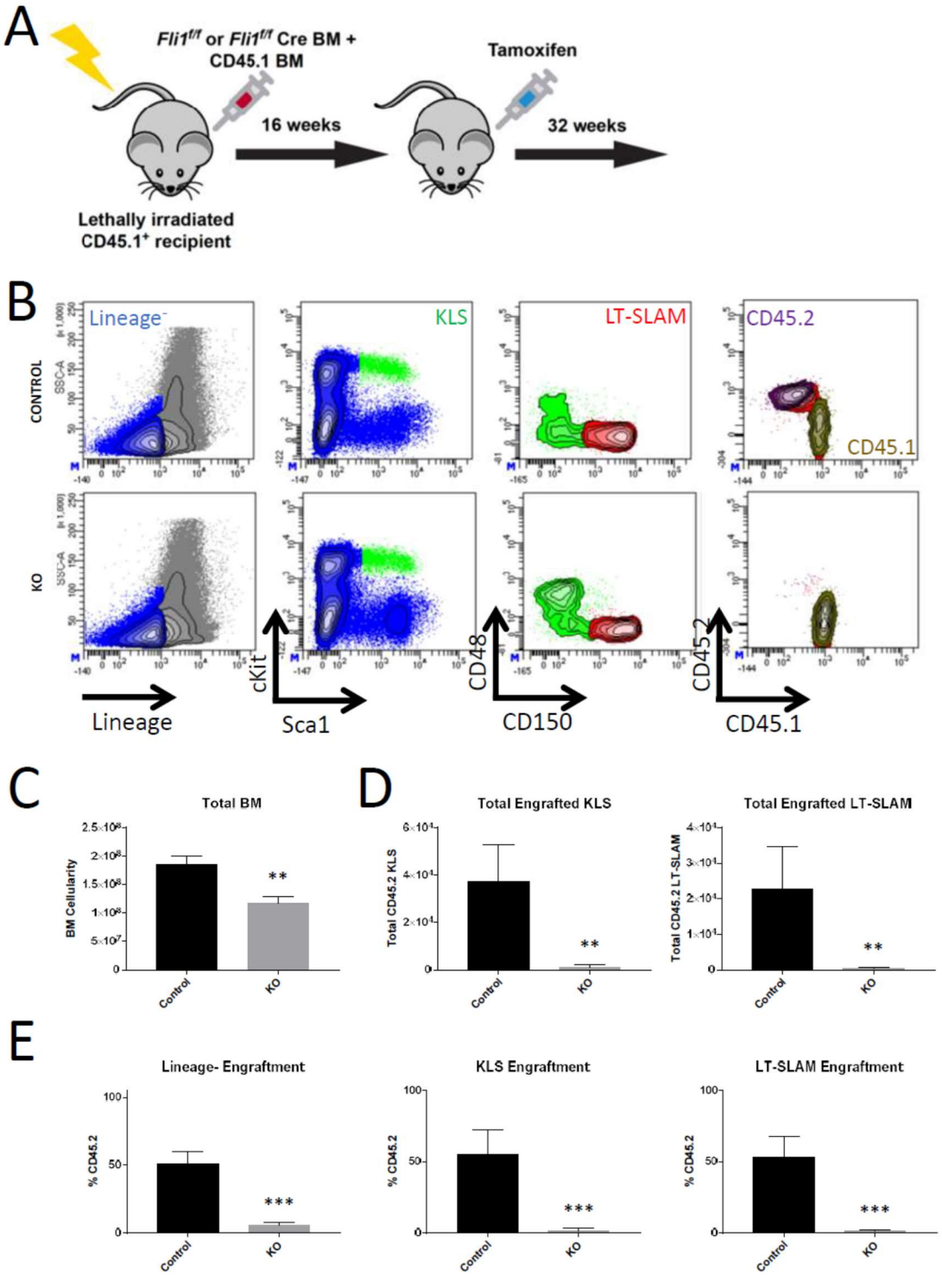
2.2.5 Fli1 is required for stem cell functionality during homeostasis

Having confirmed the role of Fli1 during reconstitution in both a primary and secondary transplant setting, we next asked if Fli1 plays a similar role in the HSC compartment during homeostasis. In order to assess that we designed a transplant setting where the peripheral blood homeostasis is unaffected by deletion. Given the WBM transplant setting was lethal to the animals (Figure 2.2D) we transplanted competitor CD45.1 BM along with Fli1$^{f/f}$ Cre or Fli1$^{f/f}$ BM in equal ratios so as to have equal lineage contribution from the CD45.1 and the experimental cohort of cells. After allowing the transplanted mice to recover for 16 weeks, the deletion was induced with tamoxifen (Figure 2.6A). Post deletion the mice were monitored for 24 weeks to assess peripheral blood lineage contribution from the Fli1$^{\Delta/\Delta}$ or Fli1$^{f/f}$ cells. Every timepoint was also assessed for peripheral blood counts to ensure that the hematopoietic homeostasis of the animal was maintained.

There was a gradual decrease in the peripheral blood contribution (Figure 2.6B) of the Fli1$^{\Delta/\Delta}$ cells to varying degrees depending on the peripheral blood

lineage. The myeloid fraction was the first affected with Fli1$^{\Delta/\Delta}$ contribution dropping significantly by week 8 (Figure 2.6C). The B cell compartment (Figure 2.6D) was slower to respond to the effect of the deletion with the T cells (Figure 2.6E) the last affected. All three compartments did show a significant reduction in engraftment by week 24. Peripheral blood complete blood counts were normal and unchanged between the control and KO cohort throughout the 24 weeks demonstrating the maintenance of peripheral blood homeostasis. The BM of the mice were assayed after 6 months of deletion (Figure 2.7A) and was consistent with the peripheral blood exhibiting significantly reduced or close to absent engraftment from the Fli1$^{\Delta/\Delta}$ cells compared to the controls (Figure 2.7E). BM cellularity was found to be slightly lower in the Fli1$^{\Delta/\Delta}$ mice compared to the controls (Figure 2.7C). The presence of Fli1$^{\Delta/\Delta}$ was not detected in the stem cell compartment in the BM (Figure 2.7D, E) showing minimal engraftment in both KLS as well as LT-SLAM populations (Figure 2.7B). The majority of adult HSCs are more or less dormant throughout the life of an organism. To observe such a drastic depletion in the HSC pool on Fli1 deletion points to an active mechanism that controls HSC fate irrespective of its active functional state suggesting that Fli1 is not only essential for reconstitution but critical for the maintenance and self-renewal of HSCs during adulthood under homeostatic conditions.

Figure 2.7 Fli1 is required for maintenance of HSC homeostasis (A) Mouse **S**chematic showing experimental plan to assess the effect of Fli1 on HSC homeostasis **(B)** Representative flow plots showing gating strategy to assess KLS-SLAM engraftment in 24-week BM **(C)** BM cellularity of two femurs in transplanted mice comparing Control and KO **(D)** Quantification of total engrafted $CD45.2^{+}$ cells in the BM in the KLS and LT-SLAM compartment (Statistics * signifies $p < 0.05$, ** signifies $p < 0.01$, *** signifies $p < 0.001$) **(E)** Quantification of % total engrafted $CD45.2^{+}$ cells in the BM in the $Lineage^{-}$, KLS and LT-SLAM compartment (Statistics * signifies $p < 0.05$, ** signifies $p < 0.01$, *** signifies $p < 0.001$)


A
Fli1f/f or Fli1f/f Cre BM +
CD45.1 BM
16 weeks
Tamoxifen
32 weeks
Lethally irradiated
CD45.1+ recipient
B
CONTROL
KO
Lineage-
KLS
LT-SLAM
CD45.2
CD45.1
Lineage
cKit
Sca1
CD48
CD150
CD45.2
CD45.1
C
Total BM
BM Cellularity
Control
KO
D
Total Engrafted KLS
Total CD45.2 KLS
Total Engrafted LT-SLAM
Total CD45.2 LT-SLAM
E
Lineage- Engraftment
KLS Engraftment
LT-SLAM Engraftment
% CD45.2

2.2.6 Fli1 is essential for Ckit and Mpl expression and occupies critical hematopoietic promoter and enhancer regions in the genome

Having confirmed the functional role of Fli1 in both a developmental setting as well as during adult homeostasis, we performed transcriptional analysis on the KLS compartment of the $Fli1^{\Delta/\Delta}$ in the whole BM transplant setting to better understand the underlying mechanistic changes resulting in this drastic dysfunctionality. Previous ChIP data has shown extensive Fli1 binding in critical hematopoietic loci of transcription factors, epigenetic modulators and signaling cascade proteins; however, Fli1 is not unique in its genomic occupancy (Beck et al., 2013; Gottgens, 2015; Tijssen et al., 2011; Wilson et al., 2016). It shares a large number of binding sites with other genes of the heptad; however, deletion of a majority of these genes (Runx1, Scl, Lyl1, Lmo2) does not result in significant HSC defects suggesting a degree of redundancy and overlap with other transcription factors. Using human $CD34^+$ ChIP data (Beck et al., 2013) we compared the total binding sites for 4 genes, Fli1, Erg, Runx1 and Gata2, and found that Erg and Fli1 have the most binding sites amongst these genes in promoter regions however the bulk of both Fli1 and Erg binding is in enhancer elements (Figure 2.8A). Erg dwarfs the other genes in this category occupying 42837 sites in annotated enhancers in $CD34^+$ cells however Fli1 has the second most binding sites in enhancer and promoter elements. Distribution of binding sites amongst transcriptional start sites, intronic regions and intergenic regions is comparable between the four genes (Figure 2.8B).

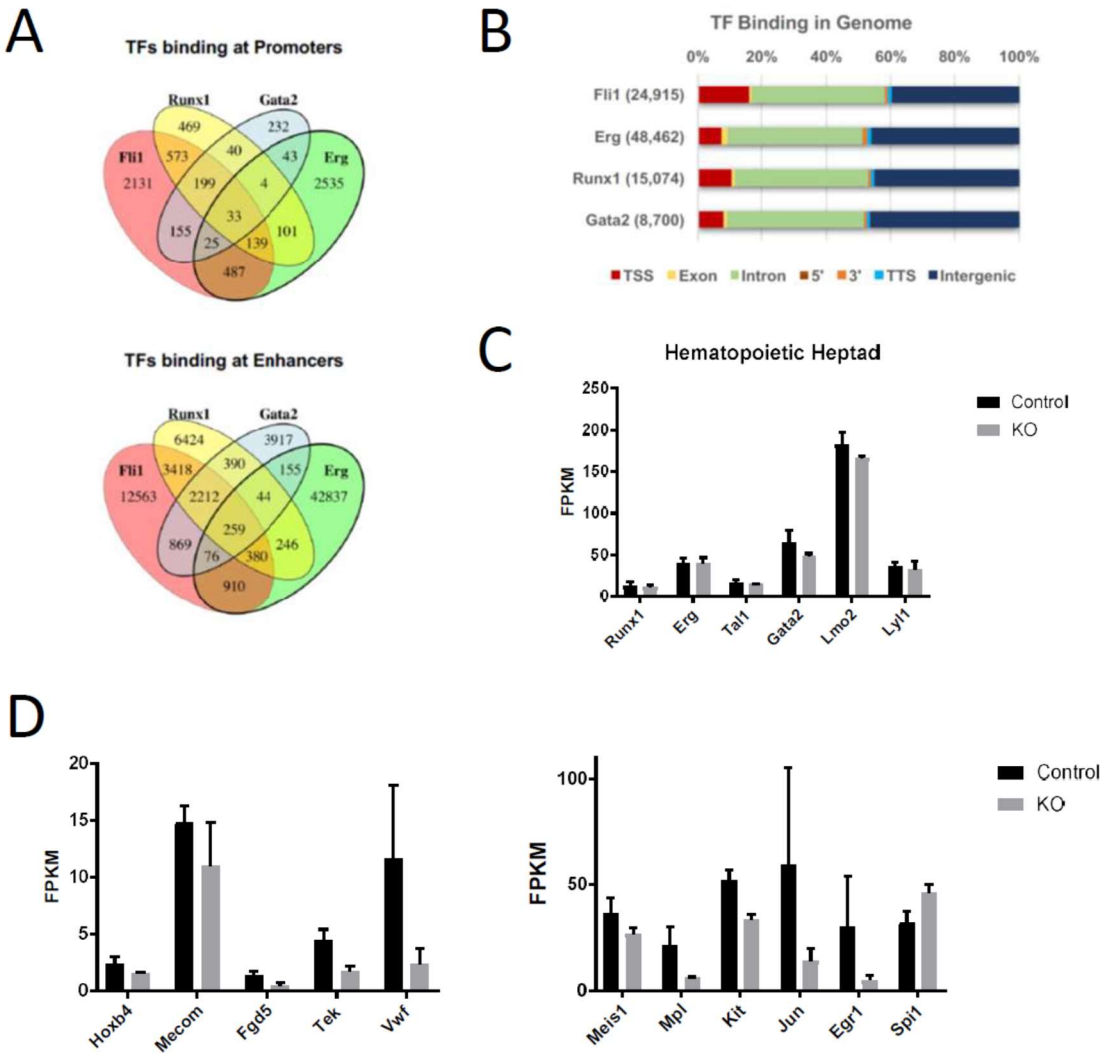


Figure 2.8 Genomic occupancy of Fli1 in human CD34^{+} cells and transcriptional profiling of Fli1$^{\Delta/\Delta}$ cells (A) Venn diagram representing transcription factor binding for Fli1, Runx1, Gata2 and Erg at annotated Promoters and Enhancers **(B)** Distribution of transcription factor binding sites in annotated genomic regions – Transcription start sites (TSS), transcription termination site (TTS), 3'and 5' untranslated regions, exons, introns and intergenic regions. **(C)** RNA expression of the hematopoietic heptad in Fli1$^{\Delta/\Delta}$ KLS cells is not affected **(D)** Genes that are significantly changed in KO cells vs Control

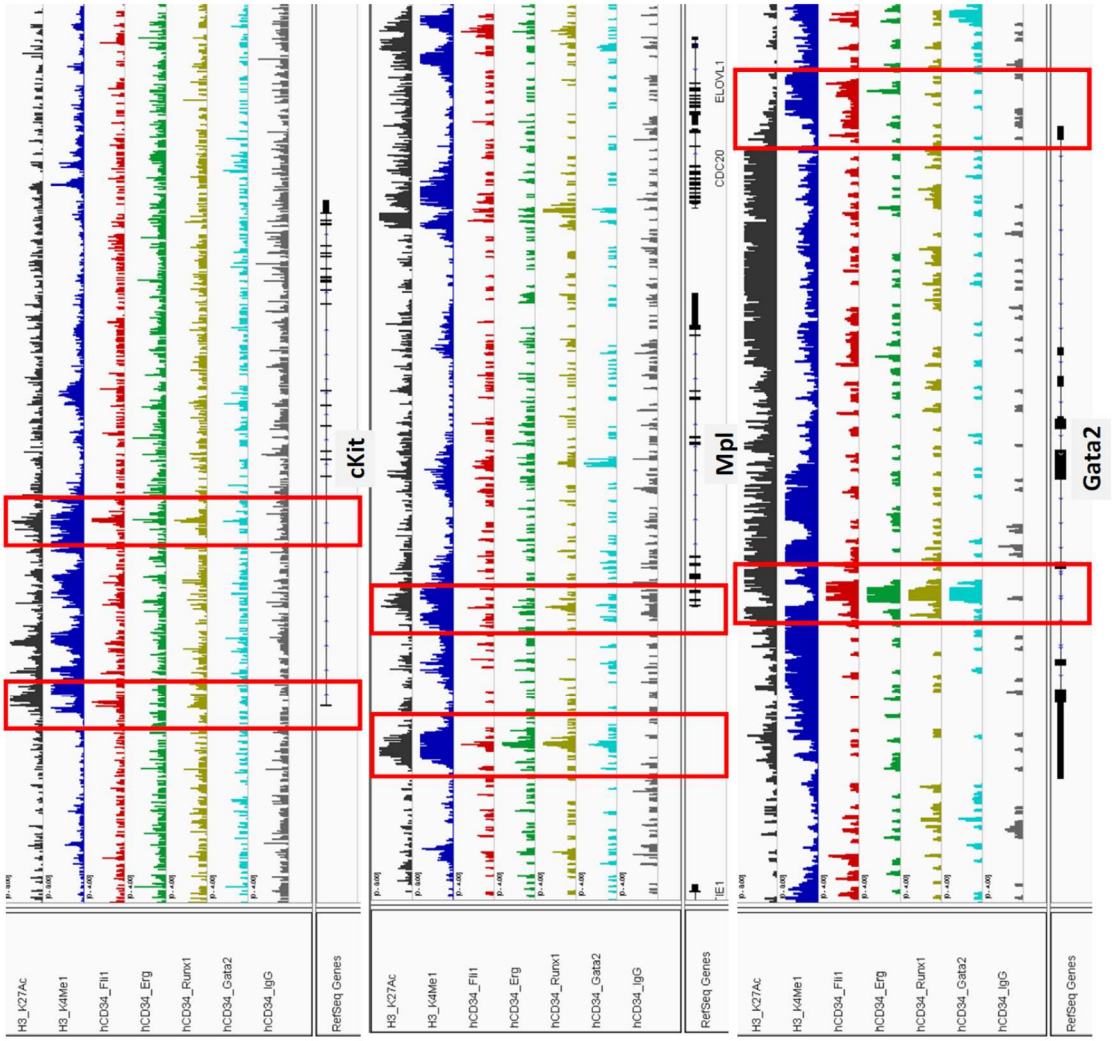


Figure 2.9 Genomic occupancy in hematopoietic loci of interest – cKit, Mpl, Gata2. Human $CD34^{+}$ ChIP data for genomic occupancy of H3K27Ac, H3K4Me1, Fli1, Erg, Runx1, Gata2 and IgG at hematopoietic loci of cKit, Mpl and Gata2. Red boxes highlight binding peaks in promoter and enhancer regions.

By analyzing histone marks – H3K4me1 and H3 K27Ac, we found enhancer elements that are conserved across species in the hematopoietic stem cell compartment pointing to a specific role for Fli1 in gene regulation for HSCs (Figure 2.9). We first assessed the RNA profile of the hematopoietic heptad in the Control vs KO KLS cells and found that expression of these genes was unchanged (Figure 2.8C). This was not surprising given the overlap in genomic occupancy for most of these essential transcriptional regulators. One enhancer element that corelated well with a reduction in transcript level was Mpl (Figure 2.8D, 2.9). Mpl or thrombopoietin receptor has been shown to be essential for maintaining HSC quiescence as well as preserving HSC homeostasis in the niche (Fleury et al., 2010; Qian et al., 2007; Yoshihara et al., 2007). Both TPO (Thrombopoietin) and Mpl loss of function results in significant thrombocytopenia in addition to affecting HSC homeostasis. This function has not been explored in a cell type specific manner. ETS factor, Erg has also been linked with Mpl regulation before however it appears Fli1 and Erg may have non-redundant roles in Mpl regulation. Another unique binding site for Fli1 is in the promoter of the cKit gene (Figure 2.8D, 2.9). cKit has been shown to be essential for HSC survival and self-renewal however the exact mechanism of cKit regulation in adults is not well understood especially in the context of the hematopoietic stem cell (Heissig et al., 2002; Kimura et al., 2011; Thoren et al., 2008).

Both cKit and Mpl work in conjunction to maintain HSC quiescence and self-renewal. Modulation of cKit expression on a protein level was confirmed during development as well as in adult, pre and post expansion ex vivo (Figure 2.1E, F, 2.3F, G). The steady reduction in peripheral blood engraftment in the BM homeostasis setting lends itself to a mechanism revolving around the disruption of HSC quiescence. Given the role of both these factors in this, they are prime candidates to

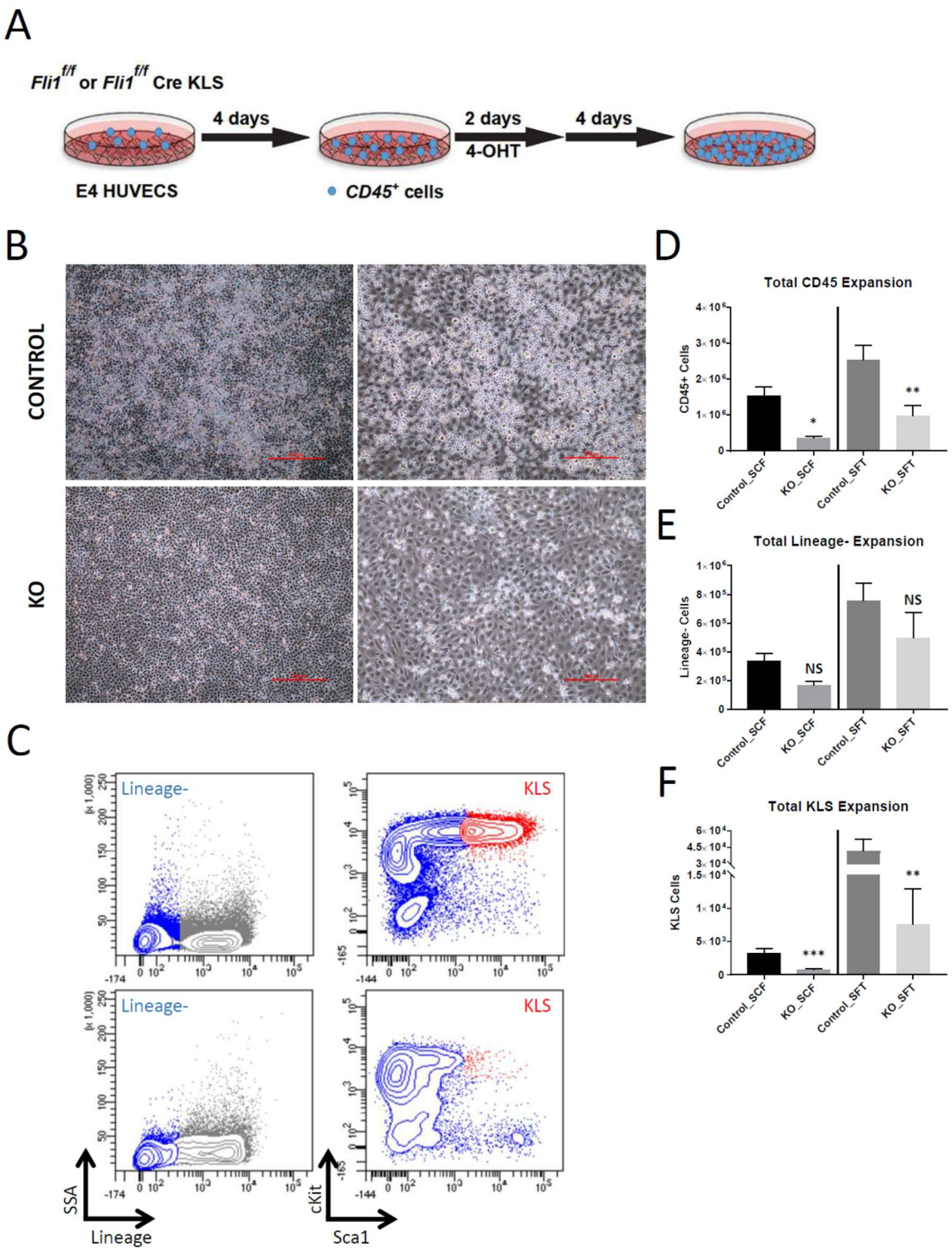
partially rescue the exhaustion phenotype associated with Fli1 deletion. Taken together these results show that Fli1 is a key regulator of the genomic landscape of HSCs and does so through unique binding sites throughout the genome as well as through regulation of cofactor binding in key enhancer elements to regulate specific genomic expression patterns unique to the hematopoietic stem cell compartment.

2.2.7 Fli1 is required for ex-vivo expansion of hematopoietic cells

After gaining a better understanding of the underlying mechanisms controlled by Fli1 in maintaining HSC fate we attempted to rescue the stem cell dysfunctionality of the Fli1 deleted HSCs by specifically overexpressing the two genes of interest identified through the transcriptional analysis of the Fli1 deleted HSCs, Kit and Mpl. In order to achieve this, we utilized the robust ex-vivo expansion platform of E4 HUVECs to expand mouse KLS (Butler et al., 2010; Seandel et al., 2008). The ex-vivo expansion of KLS allows the unique opportunity to manipulate gene expression in an ex-vivo setting and assess its effect on stem cell survival, self-renewal and differentiation. This system functions similar to what would be expected of an HSC in a transplant setting where it reconstitutes the entire hematopoietic system of the mouse.

We first analyzed if Fli1 deleted KLS are able to expand in this ex-vivo setting (Figure 2.10A). A 10-day expansion experiment showed significant differences in the potential of the Fli1 deleted hematopoietic cells to expand. Superficial phase contrast images reflected a large difference in expansion potential between the $Fli1^{\Delta/\Delta}$ and $Fli1^{f/f}$ cells (Figure 2.10B). There was a drastic reduction specifically in the KLS compartment post expansion with both, overall CD45 expansion as well as KLS

Figure 2.10 Fli1 deletion results in significant expansion defects in vitro (A) Schematic showing experimental design for in vitro expansion with deletion induced by 4-hydroxy tamoxifen **(B)** Phase pictures showing differences in expansion between Control and KO **(C)** Representative flow plots showing gating strategy for analysis of KLS expansion demonstrating absence of KLS population in KO expansion. Quantification of invitro expansion across two different conditions of cytokine addition (only SCF (50ng/ml) and SCF, Flt3 and TPO (50ng/ml)) in terms of total **(D)** CD45 expansion, **(E)** total Lineage$^-$ expansion and **(F)** total KLS expansion (Statistics * signifies $p < 0.05$, ** signifies $p < 0.01$, *** signifies $p < 0.001$)


A
Fli1f/f or Fli1f/f Cre KLS
4 days
2 days
4-OHT
4 days
E4 HUVECS
CD45+ cells
B
CONTROL
KO
C
Lineage-
KLS
Lineage-
KLS
SSA
Lineage
cKit
Sca1
D
Total CD45 Expansion
CD45+ Cells
Control_SCF
KO_SCF
Control_SFT
KO_SFT
E
Total Lineage- Expansion
Lineage- Cells
NS
NS
F
Total KLS Expansion
KLS Cells
***
**

expansion significantly reduced (Figure 2.10 D, E, F). Given the two genes of interest, cKit and Mpl, we assessed if the cytokine profile has any effect on the expansion of the $Fli1^{\Delta/\Delta}$.

A large number of cytokine-receptor interactions play a critical role in regulating the balance between HSC homeostasis vs expansion and differentiation. The vascular niche does provide a lot of essential cues for HSC maintenance and expansion however exogenous cytokines can be used to further improve expansion in the invitro setting(Butler et al., 2012; Butler et al., 2010). To this end we used exogenous thrombopoietin (TPO) and fms like tyrosine kinase 3 ligand (Flt3). Given the reduction in Mpl expression we hypothesized that the addition of TPO might modulate the kinetics of Mpl activation and rescue the Mpl axis induced defects in addition to the SCF-cKit defects. We found that the addition of TPO and Flt3 significantly improved the self-renewal and expansion profile of the KO cells (Figure 2.10 D, E, F) hinting at the kinetics of cytokine-receptor binding playing a key role in the deletion defect. Having attempted to improve expansion by the addition of cytokines, we sought to test the effects of removal of cytokines to gauge if Control and KO expansion is comparable without activating cKit and Mpl via their ligands, SCF and TPO (Figure 2.11C). There was no significant difference between total $CD45^{+}$, $Lineage^{-}$ and KLS expansion in the absence (Figure 2.11D) of exogenous cytokines demonstrating that the SCF-cKit and TPO-Mpl axes are critical regulators of expansion and self-renewal in the in vitro expansion system.

Given the recent findings implicating the role of megakaryocytes in HSC (Bruns et al., 2014) maintenance we modified the in vitro system to factor in the absence of megakaryocytes in the $Fli1^{\Delta/\Delta}$ expansions by adding CD45.1 competitor

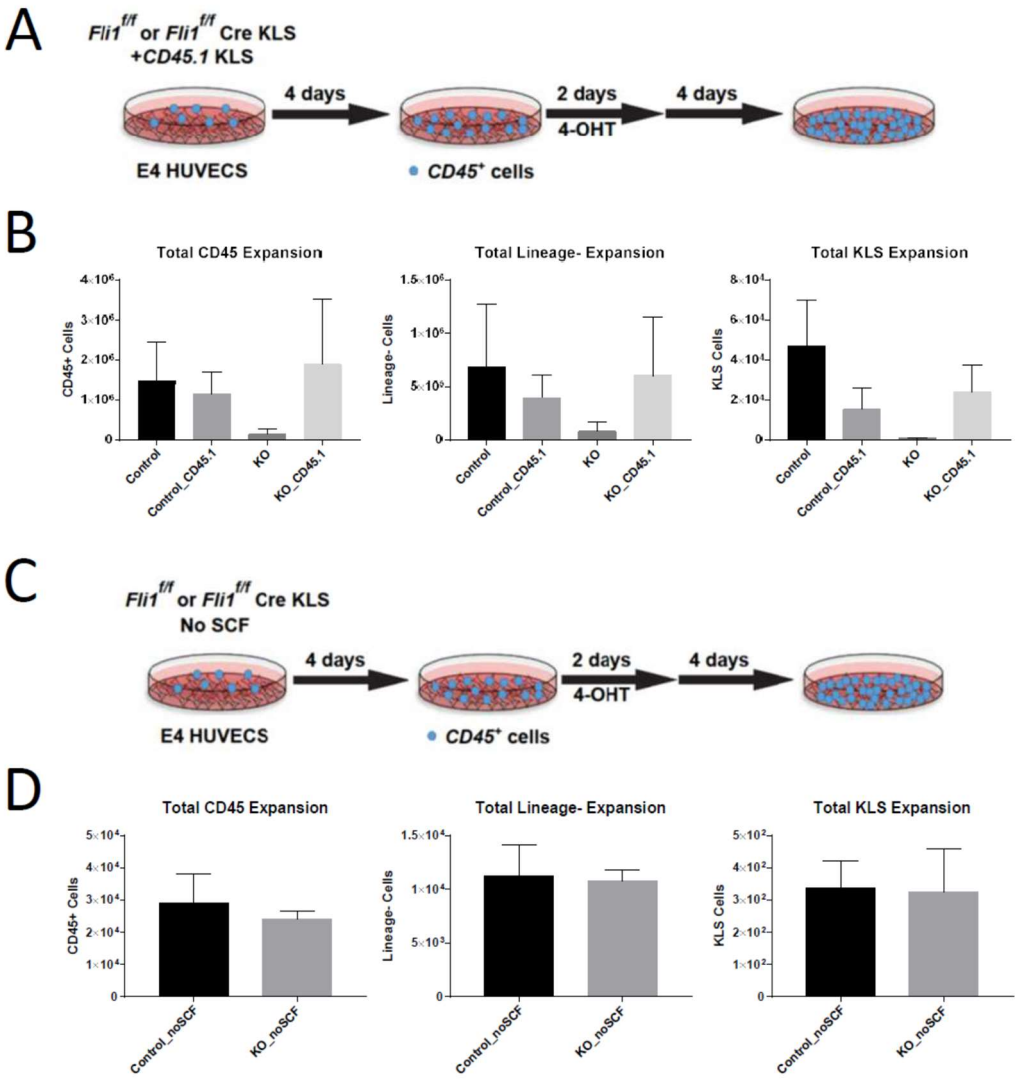


Figure 2.11 In vitro expansion in the presence of a competitor or absence of SCF does not affect Fli1$^{\Delta/\Delta}$ expansion (A) Schematic showing experimental design for in vitro expansion with the addition of competitor **(B)** Quantification of invitro expansion in terms of total CD45 expansion, total Lineage^{-} expansion and total KLS expansion **(C)** Schematic showing experimental design for in vitro expansion without exogenous SCF addition **(D)** Quantification of invitro expansion in terms of total CD45 expansion, total Lineage^{-} expansion and total KLS expansion (Statistics * signifies $p < 0.05$, ** signifies $p < 0.01$, *** signifies $p < 0.001$)

KLS to both Control and KO expansions (Figure 2.11A). Fli1$^{\Delta/\Delta}$ cells have deficient differentiation and the presence of CD45.1 cells would ensure that any supporting differentiated hematopoietic cell is present in the expansion for both Control and KO. Addition of CD45.1 KLS cells to the Control/KO KLS in vitro did not rescue the expansion deficit of the Fli1 deleted cells (Figure 2.11B) validating that megakaryocyte signaling is not involved in the expansion defect of the KO cells.

Having established the baseline with respect to cytokine combinations as well as addition of exogenous competitor KLS, we moved to overexpressing two genes of interest, cKit and Mpl, in the KLS cells using lentiviral constructs so as to rescue the expansion defect of the Fli1 deleted stem cells. An important fact to keep in mind is that HSC homeostasis entails a diverse repertoire of functions – self-renewal, expansion and differentiation. Mpl and cKit target a small part of these various HSC functions. The main target of this rescue would be to maintain the number and percentage of KLS cells in vitro when compared to the control factoring in the starting population of cells. We found that lentiviral overexpression of Mpl resulted in a significant increase in the expansion potential of the Fli1 deleted KLS and was able to override the expansion defect in the stem cell compartment only (Figure 2.12B, C). This translated to a slight increase in total CD45 expansion while giving a significant increase in the KLS fraction which was earlier specifically depleted (Figure 2.12B). Kit overexpression was able to prevent the reduction in KLS numbers while maintaining surface marker expression of the stem cell compartment (Figure 2.10C) which is lost in the Fli1 deleted cells; however, expansion was not significantly affected by its overexpression. This is not completely unexpected as Kit has been linked with HSC self-renewal and survival more so than expansion and differentiation.

Taken together these results show that Fli1 regulates HSC survival, self-renewal and expansion by regulating the expression of Kit and Mpl and through their interaction with SCF and TPO. These rescue experiments provide a mechanistic insight into some of the various pathways regulated by Fli1 in maintaining hematopoietic stem cell homeostasis in the context of stem cell fate.

In conclusion, we demonstrate that Fli1 is essential for definitive hematopoiesis during development. It carries over this function to adult mice where it is required for hematopoietic homeostasis and is critical in maintaining hematopoietic stem cell function in the context of homeostasis as well as reconstitution. Fli1 regulates hematopoietic survival, self-renewal and expansion via two major signaling proteins – cKit and Mpl. Future studies should focus on deciphering the clinical relevance of Fli1 as a target in the context of leukemia and bone marrow transplantation.

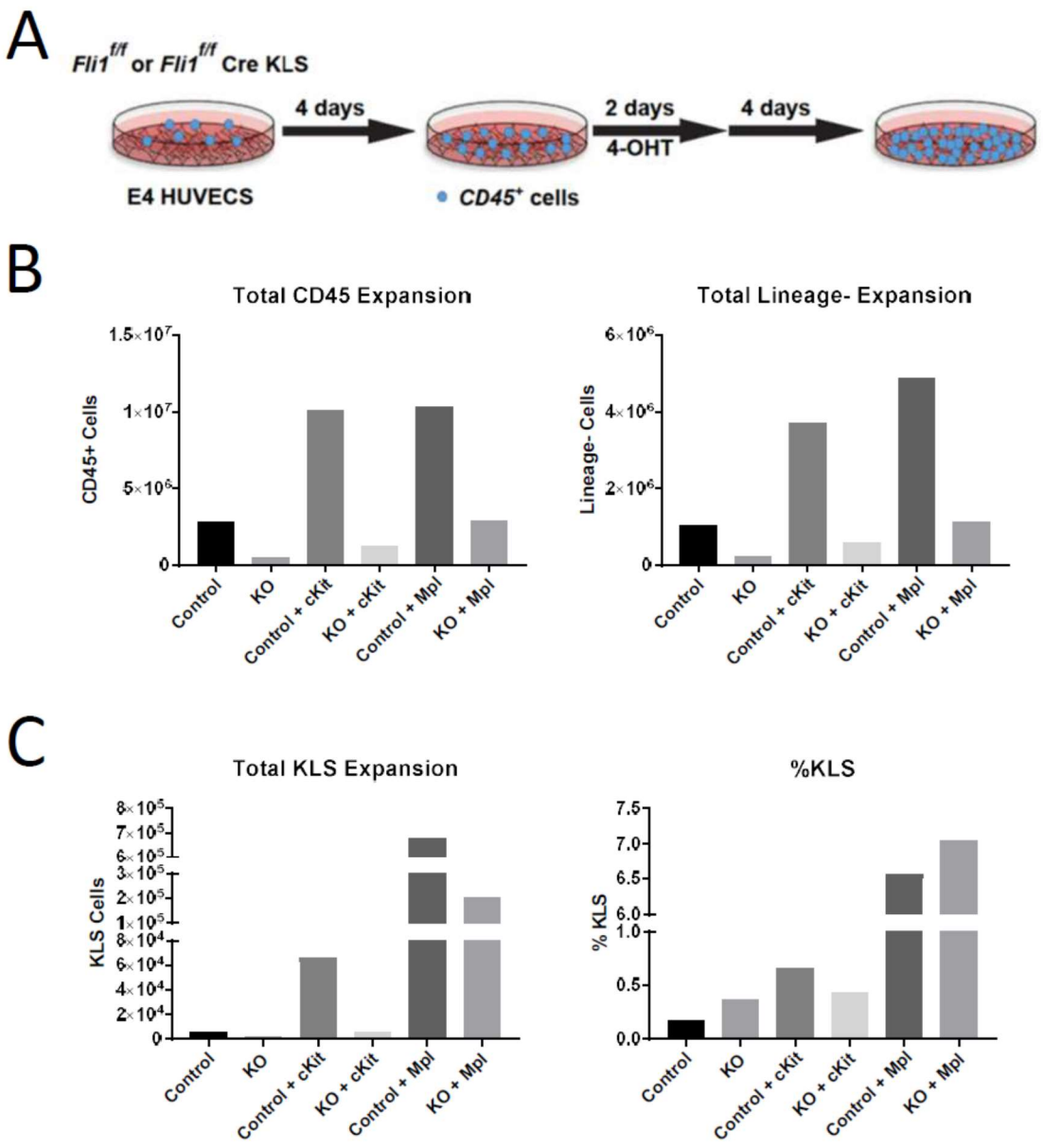


Figure 2.12 Rescue of Fli1 deletion expansion defect by overexpression of cKit and Mpl (A) Schematic showing experimental design for in vitro expansion **(B)** Quantification of invitro expansion in terms of total CD45 expansion and total Lineage$^-$ expansion **(C)** Quantification of invitro expansion in terms of total KLS expansion and %KLS post-expansion

CHAPTER 3: FLI1 AND ERG MAINTAIN A CORE VASCULAR PROGRAM ESSENTIAL FOR ADULT VASCULAR CELL FATE

3.1 INTRODUCTION

The ETS family of factors has been implicated in regulating a wide array of cellular processes in vascular endothelial cell development as well as angiogenesis. While the precise hierarchy of transcription factors that regulate endothelial cell fate has been defined, whether vascular cell fate in adults is dependent on constitutive command of specific ETS transcription factors remain unknown. Our group has previously demonstrated that direct differentiation of mid-gestation human and mouse non-vascular cells to endothelial cells can be achieved through the expression of three of these factors – Etv2, Fli1 and Erg (Ginsberg et al., 2012; Schachterle et al., 2017). This prompted us to hypothesize that there may exist a core transcription factor(s) that are responsible for maintaining the endothelial cell repertoire and fate during adulthood. As is the case with hematopoiesis, there is a significant difference in the requirement of a factor during development as compared to adulthood (Chen et al., 2009). This holds true for the vasculature as well. Etv2 expression is restricted to a short window during development (Kataoka et al., 2011; Meadows et al., 2011) however it is not expressed in endothelial cells after mid-gestation or postnatally. A wide array of factors that are implicated during vasculogenesis and angiogenesis in development are not necessarily essential to maintain vascular homeostasis and endothelial cell fate during adulthood (Hooper et al., 2009; Shalaby et al., 1995). The ability to identify crucial transcription factors that regulate vascular cell fate would offer significant insight into the key pathways required for maintaining ECs in vitro and developing tractable platforms to sustain faithful ECs cell fate in the context of vascular disease and cancer.

Fli1 has been shown to play a role in regulating vascular leakiness during development and to a lesser degree in adult mice (Asano et al., 2010; Hart et al., 2000) and we sought to further explore the role of Fli1 in the context of vascular heterogeneity as well as homeostasis. Erg, a closely related ETS family transcription factor has been shown to play a role both in hematopoiesis and endothelial cell function in HUVECs as well as mice (Birdsey et al., 2008; Birdsey et al., 2012; Birdsey et al., 2015; Dryden et al., 2012; Dufton et al., 2017; Shah et al., 2017). **The close homology and overlapping binding sites (Beck et al., 2013; Tijssen et al., 2011; Wilson et al., 2010) in other cell types led us to hypothesize that there may exist a redundancy in terms of the function of these two factors in maintaining endothelial cell fate and homeostasis.**

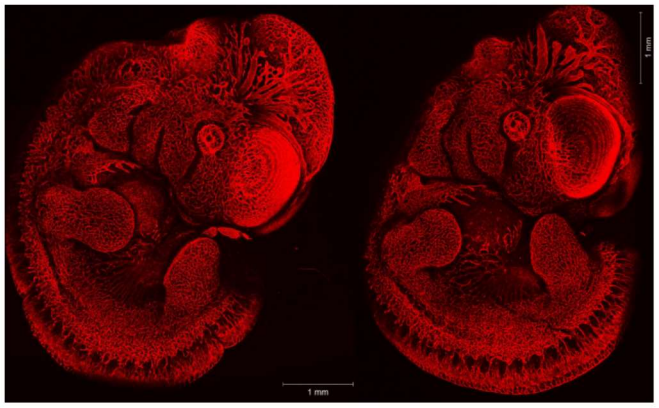
Here we show that Fli1 by itself, is not essential to maintain endothelial cell fate in adult mice and regulate vascular heterogeneity in the context of certain vascular beds. Erg does play an active role in angiogenesis during post-natal development (Birdsey et al., 2015) however in adults it is characterized by mild fibrotic phenotypes (Dufton et al., 2017). Notably, we demonstrate that acting together, Fli1 and Erg are essential in the maintenance of a core vascular program required for homeostasis in adult mice. Deletion of both these factors results in lethality by Day 12 due to hemorrhage and the formation of thrombi in the liver while in vitro, importantly, there is a complete loss in EC identity on the basis of essential marker expression such as VE-Cadherin, CD31 and VEGFR2.

3.2 RESULTS

3.2.1 Fli1 is not essential for endothelial homeostasis during development in the post-natal retina

Fli1 is expressed in every vascular bed in adult mice and given the early vascular implications of Fli1 knockout mice with vascular leakiness leading to embryonic lethality (Hart et al., 2000; Spyropoulos et al., 2000), we hypothesized that it may be one of the key factors involved in maintaining vascular identity. In order to test this hypothesis, we crossed $Fli1^{f/f}$ mice (Starck et al., 2010) with two different Cre lines, the inducible VE-Cadherin (Cdh5) Cre^{ERT2} (Pitulescu et al., 2010)and the developmental Cdh5-cre-1 (Zovein et al., 2008). The late developmental Cdh5-cre-1 (Zovein et al., 2008) induces deletion a day later than the developmental Cdh5-cre due to which hematopoietic deletion and the extent of endothelial deletion is much lower. This is as a result of different sized promoter elements of Cdh5 being used to induce Cre expression in the mice. Cdh5-cre-1 did not phenocopy the embryonic or post-natal lethality seen with the global deletion or with the developmental Cdh5-cre (Chen et al., 2009; Speck and Iruela-Arispe, 2009) that targets the AGM. Mice were born in expected mendelian ratios (Figure 3.1B). Whole mount imaging of E11.5 embryos did not yield any significant changes in endothelial architecture between the Control and Fli1 knockout mice (Figure 3.1A). The focus of our study was to evaluate the effect of Fli1 in adult vascular homeostasis. To this end, we began to explore the role of Fli1 in angiogenesis in post-natal retina. The retina offers a unique system to track vascular growth and regression in a 2D field (Pitulescu et al., 2010). We used both inducible and developmental cre mice to evaluate angiogenic defects in the retina however there

A



B

Fli1$^{f/f}$ Cdh5 cre				
Fli1	Cre	No.	Expected	Actual
f/f	-	61	50%	53.04%
f/f	+	54	50%	46.96%
		115		

C

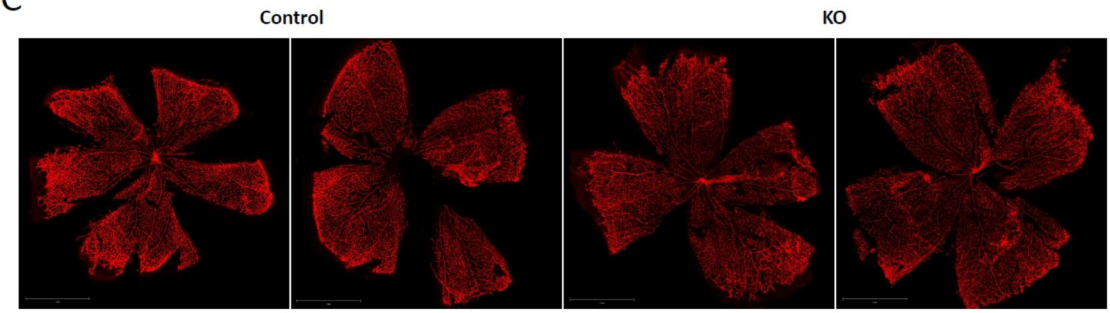


Figure 3.1 Fli1 is not essential for endothelial cell homeostasis during development and in the post-natal retina. (A) Whole mount CD31 staining of endothelial cells of Fli1$^{f/f}$ Cdh5-cre E11.5 embryos. **(B)** Observed and Expected numbers for genotypes from Fli1$^{f/f}$ Cdh5-cre breeders showing number of adult mice > 4wks **(C)** Representative images of isolated P7 retinas of Control and KO pups

were no significant differences between the Fli1$^{f/f}$ and Fli1$^{\Delta EC/\Delta EC}$ in terms of density, patterning and overall vascular architecture (Figure 3.1C). Thus, Fli1 may not be involved with the establishment of vascular structures during development or post-natally.

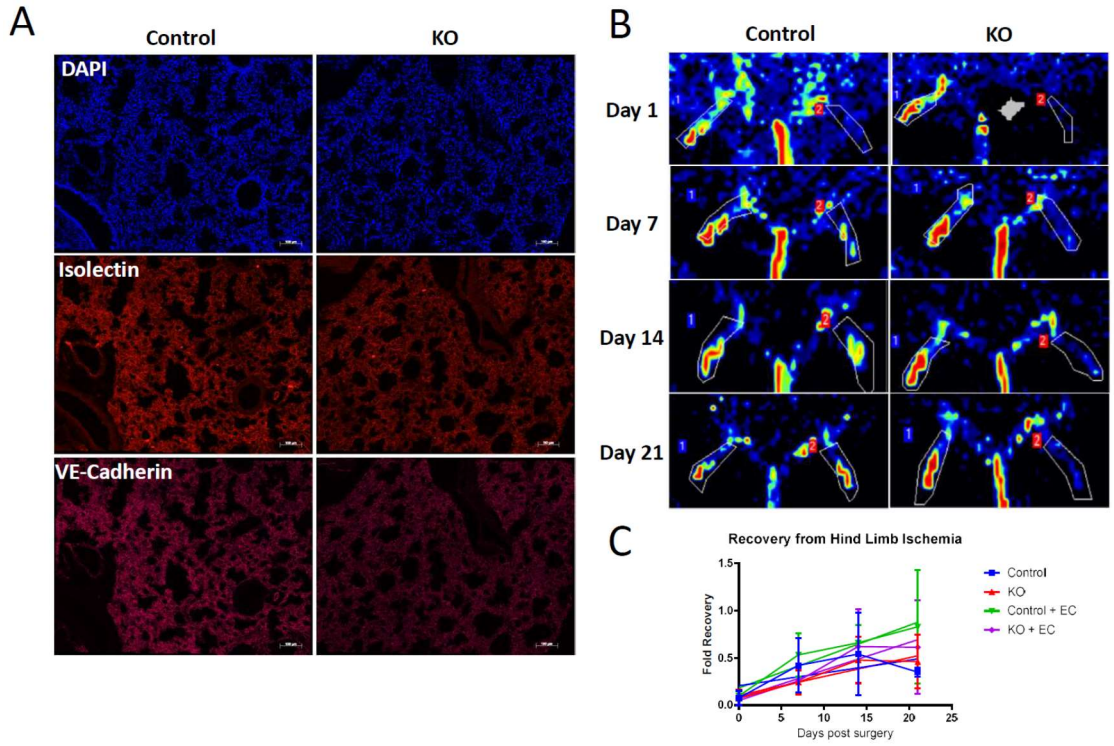


Figure 3.2 Fli1 does not affect EC architecture and is not required for recovery from Hind Limb Ischemia. (A) Representative images of lung vasculature of Control $Fli1^{f/f}$ compared to $Fli1^{f/f}$ Cdh5 cre. Stains are shown for intravital labelling of Isolectin and VE-Cadherin. Nuclei were labeled with DAPI. **(B)** Representative images showing blood flow recovery in HLI model between Control and KO over 12 days. **(C)** Quantification of the fold recovery of ligated leg over control leg in four experimental cohorts – Control, KO, Control + EC, KO + EC

3.2.2 Fli1 deletion does not impair vascular architecture in adult mice and does not regulate the angiocrine repertoire of endothelial cells in the context of bone marrow recovery and hind limb ischemia

After assessing the role of Fli1 during development and in the post-natal retina, we questioned if Fli1 might play a role in regulating the angiogenic and angiocrine profile of endothelial cells in specific organ beds. The heterogeneity of vascular beds has been proposed to be critical in regulating organ development, regeneration and homeostasis (Benedicto et al., 2017; Ding et al., 2010; Ding et al., 2011; Nolan et al., 2013; Rafii et al., 2016; Wertheimer et al., 2018) and so we investigated if Fli1 may play a role in the context of specific organ heterogeneity. We initially assayed the endothelial cell architecture and density in different vascular beds using intravital labelling using fluorescently conjugated VE-Cadherin antibody but found that there was no significant difference between the $Fli1^{f/f}$ and $Fli1^{\Delta EC/\Delta EC}$ mice (Figure 3.2A). This was evaluated across several different deletion conditions ranging from developmental deletion to post-natal deletion at P2-P4 as well as adult deletion post Week 8.

Having assessed the role of Fli1 in regulating the angiogenic repertoire of endothelial cells during homeostasis we questioned if Fli1 may play a role in maintaining specific angiocrine functions of endothelial cells in the context of recovery during physiological stress. Hematopoietic recovery post sublethal radiation is a well-established system where the role of endothelial cells in specific contexts has been explored (Butler et al., 2010; Kobayashi et al., 2010; Poulos et al., 2015). We first assessed if there were any differences in basal counts of the mice with respect to peripheral blood during homeostasis and found there was no difference between the

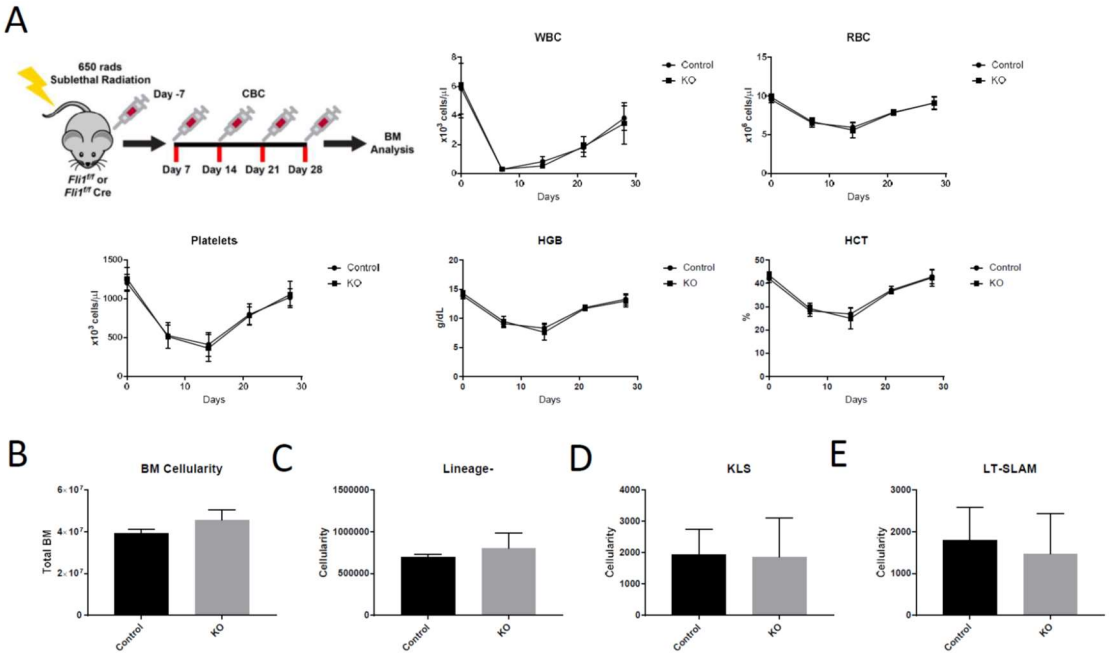


Figure 3.3 Endothelial Fli1 is not required for hematopoietic recovery from sublethal radiation. (A) Schematic for experimental plan to assess peripheral blood recovery from sublethal radiation, peripheral blood counts for WBC (x10^3 cells/µl), RBC (x10^6 cells/µl), Platelets (x10^3 cells/µl), Hemoglobin (g/dL) and Hematocrit (%) assayed at Day 7, 14, 21 and 28 (n=6 mice). **(B)** Bone marrow cellularity measured per femur at Day 28 post radiation. **(C)** Lineage negative cell counts per femur post lineage depletion using Miltenyi lineage depletion kit. **(D)** KLS ($cKit^+Lineage^{neg}Sca1^+$) cells measured per femur in the lineage negative fraction. **(E)** HSC LT-SLAM (KLS $CD150^+CD48^-$) numbers measured per femur in the lineage negative fraction. **(B-D)** BM Analysis was performed on n=3 mice per cohort and all measurements were found to be not significant

$Fli1^{f/f}$ (n=6) and $Fli1^{\Delta EC/\Delta EC}$ (n=7) mice. We next proceeded with determining the response of the mice in the context of radiation damage. To this end, we irradiated the mice with sublethal 650 rads and assessed their recovery in terms of the complete

peripheral blood counts as well as differential WBC counts and distribution (Figure 3.3A). The recovery profile of the peripheral blood for the Controls compared to the knockouts was unchanged showing that Fli1 may not play a significant role in maintaining the angiocrine profile of bone marrow endothelial cells during homeostasis or recovery. Despite the absence of any difference in recovery, we studied if the bone marrrow niche was affected in terms of its ability to sustain stem cell homeostasis. After harvesting bone marrow at Week 4 post radiation, we found that bone marrow cellularity was unchanged by Fli1 deletion (Figure 3.3B). This extended to every subsequent lineage restricted compartment with the lineage negative cellularity unaffected between control and KO mice (Figure 3.3C). The stem cell compartment was not significantly different with KLS numbers as well as LT-SLAM (KLS $CD150^{+}CD48^{-}$) hematopoietic stem cell numbers (Figure 3.3D, E) manifesting no difference between the experimental cohorts.

Having evaluated the bone marrow, we hypothesized that a more rigorous vascular insult might better assess the importance of Fli1 in the context of recovery. The hind limb ischemia model has been shown to be an ideal system to assess angiogenic as well as angiocrine function during revascularization and has proven to be a relevant model to quantify endothelial cell engraftment (Jin et al., 2006; Schachterle et al., 2017). To test the effect of Fli1 in hind limb ischemia model, we performed excision of the femoral artery followed by injection of $5x10^{5}$ Akt transduced endothelial cells (Schachterle et al., 2017) to identify any advantage of exogenously supplied endothelial cells in the $Fli1^{\Delta EC/\Delta EC}$. We found that the four cohorts of Control, KO, Control transplanted and KO transplanted had comparable effect in revascularization (Figure 3.2B, C) from the hind limb ischemia prompting us

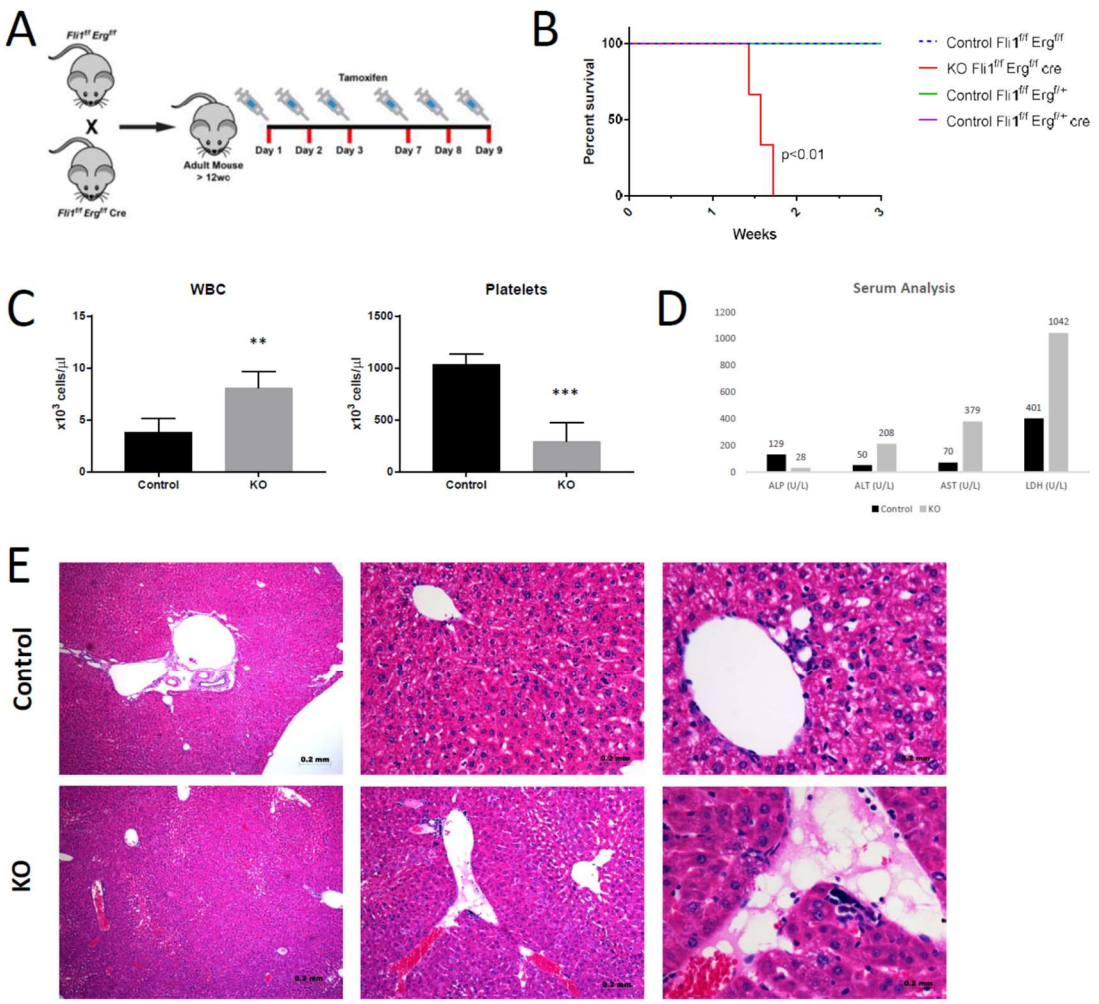


Figure 3.4 Fli1 and Erg maintain endothelial integrity during homeostasis. (A) Mouse schematic showing breeding strategy to obtain adult mice and tamoxifen injection schedule for adult mice. **(B)** Survival curve for post first injection of tamoxifen for four cohorts – $Fli1^{f/f}$ $Erg^{f/f}$ cre (n=6), $Fli1^{f/f}$ $Erg^{f/f}$ (n=4), $Fli1^{f/+}$ $Erg^{f/f}$ cre (n=2), $Fli1^{f/+}$ $Erg^{f/f}$ (n=2). **(C)** Peripheral blood counts comparing WBC ($x10^3$ cells/µl) and Platelets ($x10^3$ cells/µl) from $Fli1^{f/f}$ $Erg^{f/f}$ cre and $Fli1^{f/f}$ $Erg^{f/f}$ mice. **(D)** Serum levels of ALP (U/L), ALT (U/L), AST (U/L) and LDH (U/L) between Control and DKO mice. **(E)** H&E histology sections of the liver comparing Control with DKO mice exhibiting thrombus formation in major vessels.

to conclude that Fli1 by itself, is not responsible for maintaining the angiogenic and angiocrine profile of endothelial cells in the different vascular beds.

3.2.3 Fli1 and Erg maintain endothelial integrity during homeostasis

Having assessed the role of Fli1 in vascular homeostasis and in the context of ischemic revascularization, we hypothesized that Erg may be the more crucial factor for endothelial cell fate. Erg has been shown to regulate several key vascular markers in human umbilical vein endothelial cells (Birdsey et al., 2008; Birdsey et al., 2012; Dryden et al., 2012; Sperone et al., 2011; Starke et al., 2011) in addition to being essential for endothelial cell fate stability during development. Endothelial specific deletion of Erg results in defects in vascular permeability in addition to angiogenic defects in the developing embryo (Birdsey et al., 2015; Dufton et al., 2017; Shah et al., 2017). However, in the context of adult homeostasis, Erg shared a similar fate to Fli1 in exhibiting a minimal functional role. **We hypothesized that Fli1 and Erg may have redundant functions in maintaining endothelial homeostasis in adult mice due to which their single deletions do not show significant impact with respect to endothelial stability.** In order to test this hypothesis, we acquired $Erg^{f/f}$ (Birdsey et al., 2015) mice from the Randi lab and crossed them to our $Fli1^{f/f}$ Cdh5-Cre^{ERT2} mice (Figure 3.4A).

Tamoxifen-mediated cre deletion was induced in adult mice that were at least 12 weeks old. The endothelial cell specific deletion of Fli1 and Erg was lethal and resulted in death between Day 10 and Day 12 post tamoxifen injection (Figure 3.4B). The control mice that were $Erg^{f/+}$ $Fli1^{f/f}$ Cdh5-Cre^{ERT2} were unaffected by the deletion

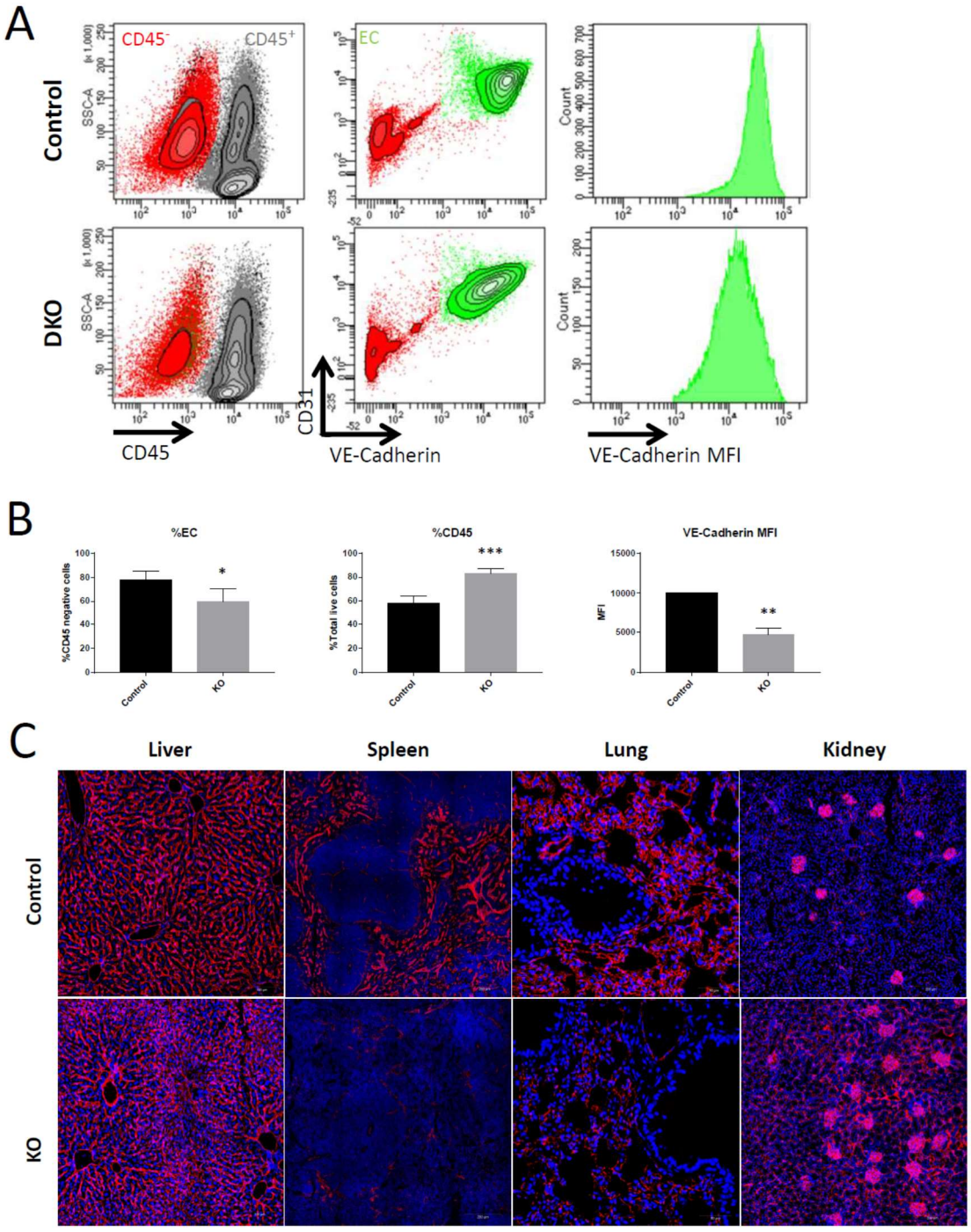
pointing to a very specific dosage requirement with respect to Erg and Fli1 deletion. Upon further inspection, mice at Day 10-12 post tamoxifen had significant hyperemia in the liver pointing to possible vascular leakiness. The peripheral blood counts of these mice were tested to assess for any hematopoietic dysfunction. There was a significant increase in WBC counts in peripheral blood with a significant decrease in platelet numbers (Figure 3.4C) while RBC, Hematocrit and hemoglobin counts were unaffected. One of the critical functions of endothelial cells is to maintain homeostasis in the coagulation cascade. Damaged endothelium can trigger platelet activation through two broad mechanisms – the first being in a cell autonomous manner where the ECs express factors that are prothrombogenic and activate complement system as well as activating platelets to initiate coagulation. The other is through vascular leakiness or detachment which results in the exposure of ECM. Deposited extracellular matrix has prothrombogenic factors such as different collagens that can activate clotting mechanisms and triggering the formation of thrombi. We hypothesize that global dysregulation of endothelial cell homeostasis would result in platelet activation that the body cannot cope with in terms of maintaining platelet numbers adequate to sustain homeostasis. This was confirmed by histological analysis which revealed the formation of large thrombi in both large vessels and sinusoids with significant infiltration of hematopoietic cells which explains the increase in WBC counts in the peripheral blood (Figure 3.4E). In order to ascertain the cause of death, we assayed serum from the mice. Blood was drawn by retroorbital bleed and allowed to coagulate at room temperature for an hour. Serum was obtained by pelleting cells post coagulation and collecting supernatant. The levels of ALT (alanine aminotransferase), AST (aspartate aminotransferase) and LDH (lactate dehydrogenase) in serum were significantly elevated (Figure 3.4D) with respect to control mice pointing to serious liver damage.

Taken together, these results strongly suggest that Fli1 and Erg expression is critical for vascular homeostasis and survival. More importantly, their deletion affects platelet homeostasis and the coagulation cascade that is maintained by the vasculature. The liver appears to be the most affected in terms of vascular dysfunction. Nonetheless, it is plausible that if the mice could survive the initial pro-thrombotic insult other organs could also succumb to vascular thrombosis

3.2.4 Absence of Fli1 and Erg results in significant disruption of endothelial cell architecture and loss of vascular signature

After evaluating the systemic effect of endothelial deletion of Fli1 and Erg, we inquired as to what the impact of Fli1-Erg deletion was on each vascular bed. Our group has shown previously that every vascular bed has a unique signature that enables it to maintain the homeostasis in that organ while at the same time being primed to react to any kind of injury to the specific microenvironment (Nolan et al., 2013). A retroorbital injection of preconjugated VE-Cadherin enables us to intravitally label the endothelium for flow analysis as well as immunofluorescence. Flow analysis revealed a significant reduction in terms of the percentage of ECs in the liver as well as a significant increase in the hematopoietic infiltration through %CD45 cells (Figure 3.5A, B), corroborating the observations of the histological sections of the liver manifesting hematopoietic infiltration in the vicinity of the large vessels in addition to the thrombus formation. Additionally, the regions of the liver most affected were the sinusoids with the VE-Cadherin expression in the large vessels, the least affected. The reduction in VE-Cadherin staining was observed in the lung and the spleen as well with the endothelial cell architecture in the spleen the most severely affected (Figure

Figure 3.5 Fli1 and Erg regulate VE-Cadherin expression in endothelial cells and are essential for maintenance of endothelial architecture in different vascular beds. **(A)** Representative flow plots showing staining schema for analysis of endothelial cell populations in harvested livers from control and double KO mice. After initial CD45 exclusion, endothelial cells were identified by the expression of CD31 and VE-Cadherin. Lymphatic contamination was eliminated based on Podoplanin staining. Histogram shows distribution of VE-Cadherin MFI in the population of ECs. **(B)** Quantification of %EC as a fraction of Live singlets $CD45^-$ cells, %CD45 as a fraction of total Live singlets, Mean Fluorescent Intensity of VE-Cadherin normalized to Control. **(C)** Representative IF images of different vascular beds – Liver, Spleen, Lung and Kidney between Control and KO showing endothelial architecture identified by VE-Cadherin staining from intravital labeling of ECs by VE-Cadherin.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Control
DKO
CD45-
CD45+
EC
SSC-A
Count
CD45
CD31
VE-Cadherin
VE-Cadherin MFI
B
%EC
%CD45
VE-Cadherin MFI
%CD45 negative cells
%Total live cells
MFI
Control
KO
*
***
**
C
Liver
Spleen
Lung
Kidney
Control
KO

3.5C, 3.6). There was a direct correlation with the type of vascular bed in terms of their capillary structure (Cleaver and Melton, 2003) and the degree of vascular disruption with fenestrated capillaries in kidney glomeruli showing significant leakage of the antibody (Figure 3.6). Similarly, the spleen lost the differentiation zone between the red and white pulp.

Given the significant drop in platelet counts, we expected the bone marrow homeostasis to be disturbed. A drop in peripheral blood counts would normally trigger an expansion of the KLS compartment of the bone marrow as seen in the case of Fli1 deletion in the hematopoietic compartment in Chapter 2 however what we observed was a significant decrease in both the KLS and LT-SLAM (Figure 3.7A) HSCs (Kiel et al., 2005) compartment in the double knock out mice compared to the controls (Figure 3.7C). A possible reason for the reduction in the stem cell compartment might have been a broader effect in the bone marrow as a result of disruption of the vasculature. This would have been reflected in overall bone marrow cellularity as well as a drop in the lineage negative fraction of the bone marrow however these counts were not affected by Fli1 Erg deletion (Figure 3.7B). This points to a disruption in the bone marrow niche specifically regulating hematopoietic stem cell homeostasis resulting in decrease in the total number of hematopoietic stem cells in the bone marrow. In order to further evaluate the mechanism behind the hematopoietic stem cell disruption it would be necessary to separate the effect of angiocrine regulation of hematopoietic stem cells and architectural endothelial cell support provided. The invitro platform for hematopoietic expansion will serve as an ideal model to elucidate this mechanism and will be the focus of future studies.

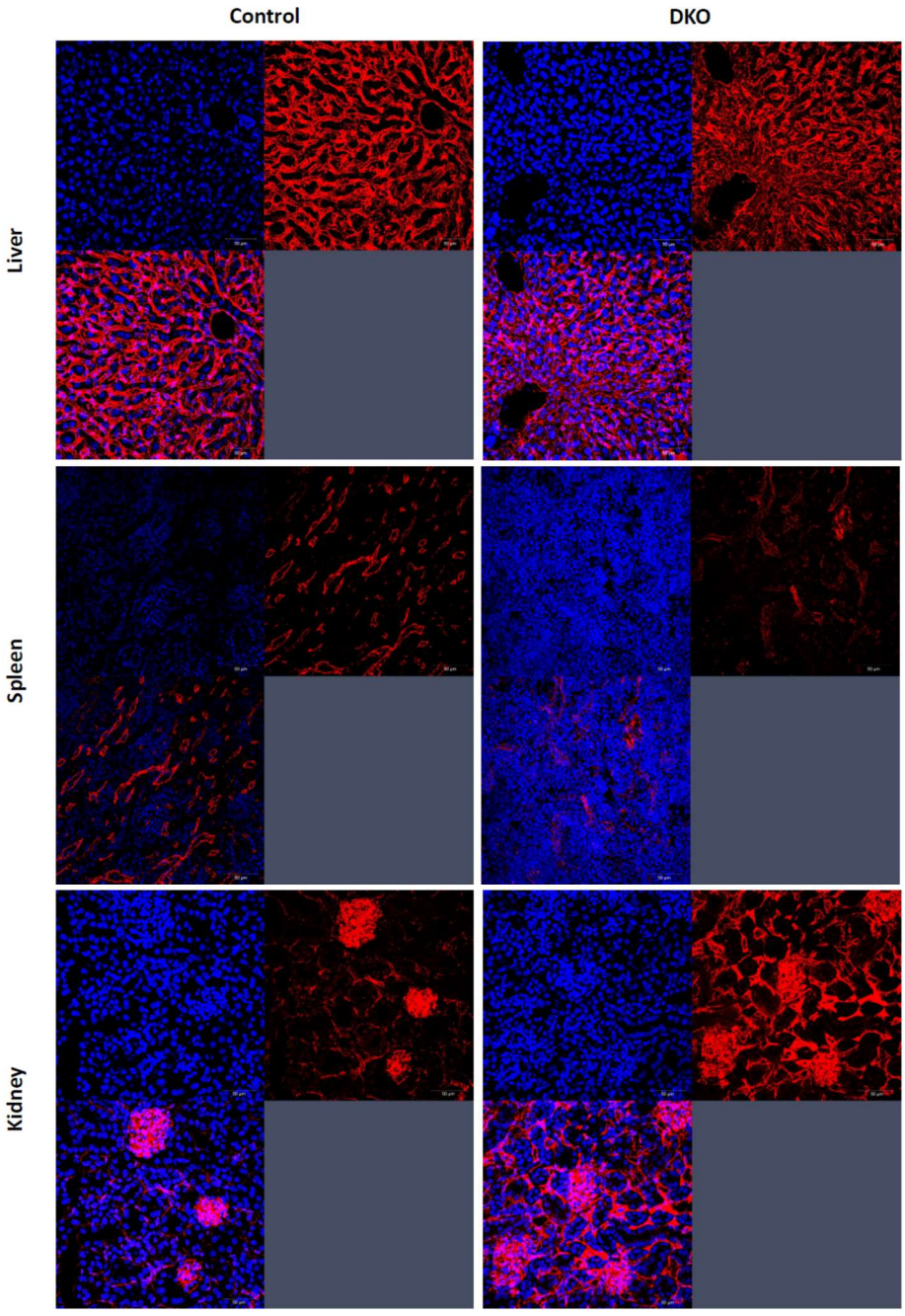
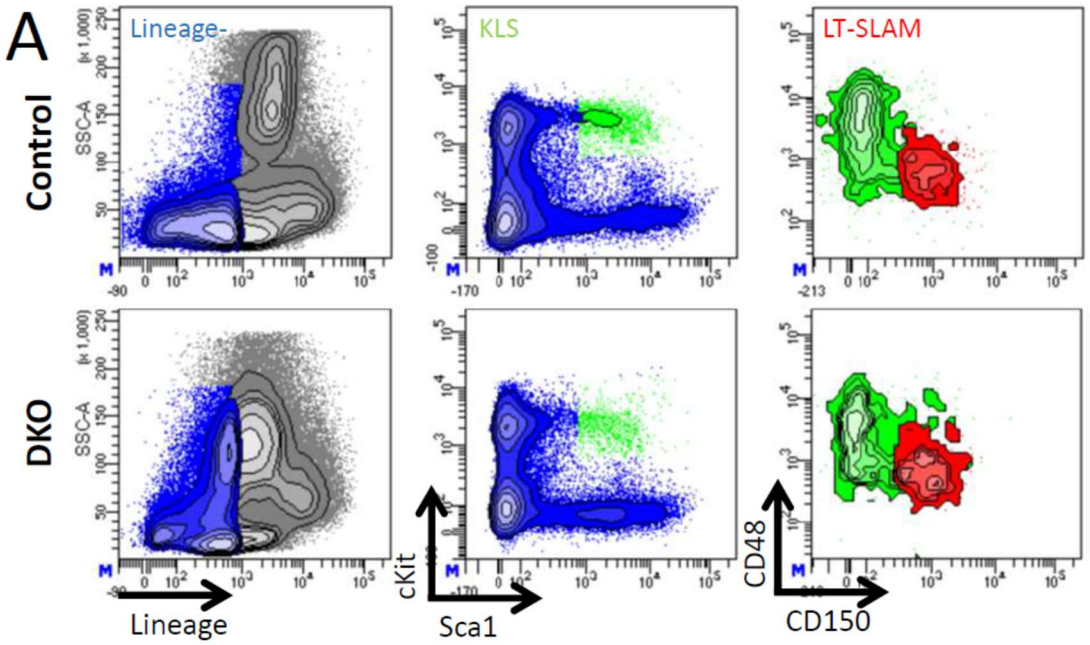


Figure 3.6 Fli1 and Erg are essential for endothelial architecture and control vascular leakiness. Representative images of Liver, Spleen and Kidney emphasizing the disruption of endothelial architecture at higher magnification along with increased antibody leakiness in the kidney glomeruli in the Double KO compared to the Control.

Figure 3.7 Endothelial Fli1 and Erg maintain Bone Marrow HSC homeostasis. (A) Representative flow plots showing analysis schematic to assay BM HSC LT-SLAM populations. Gated on Live singlets that were $CD45^{+}$. **(B)** Bone marrow cellularity and Lineage negative cellularity measured per two femurs comparing Control and KO mice. Lineage depletion was carried out using Miltenyi lineage depletion kit **(C)** KLS ($cKit^{+}Lineage^{neg}Sca1^{+}$) cells measured per two femurs in the lineage negative fraction. HSC LT-SLAM (KLS $CD150^{+}CD48^{-}$) numbers measured per two femurs in the lineage negative fraction. (Statistical significance, * for p value < 0.05, *** for p value < 0.001)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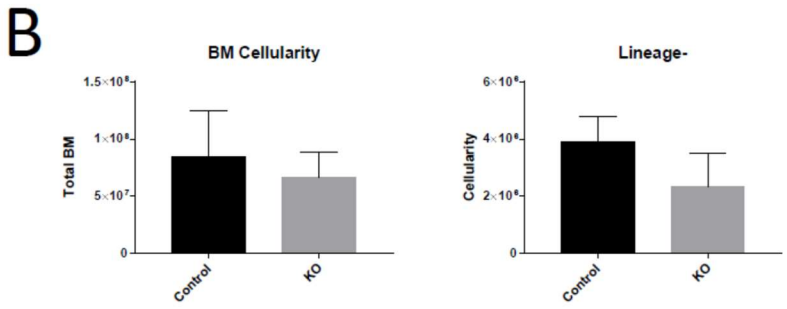
cKit
Sca1
CD48
CD150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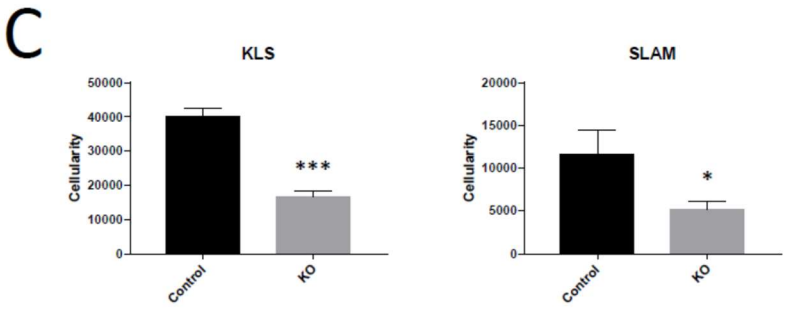
C
KLS
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Control
KO

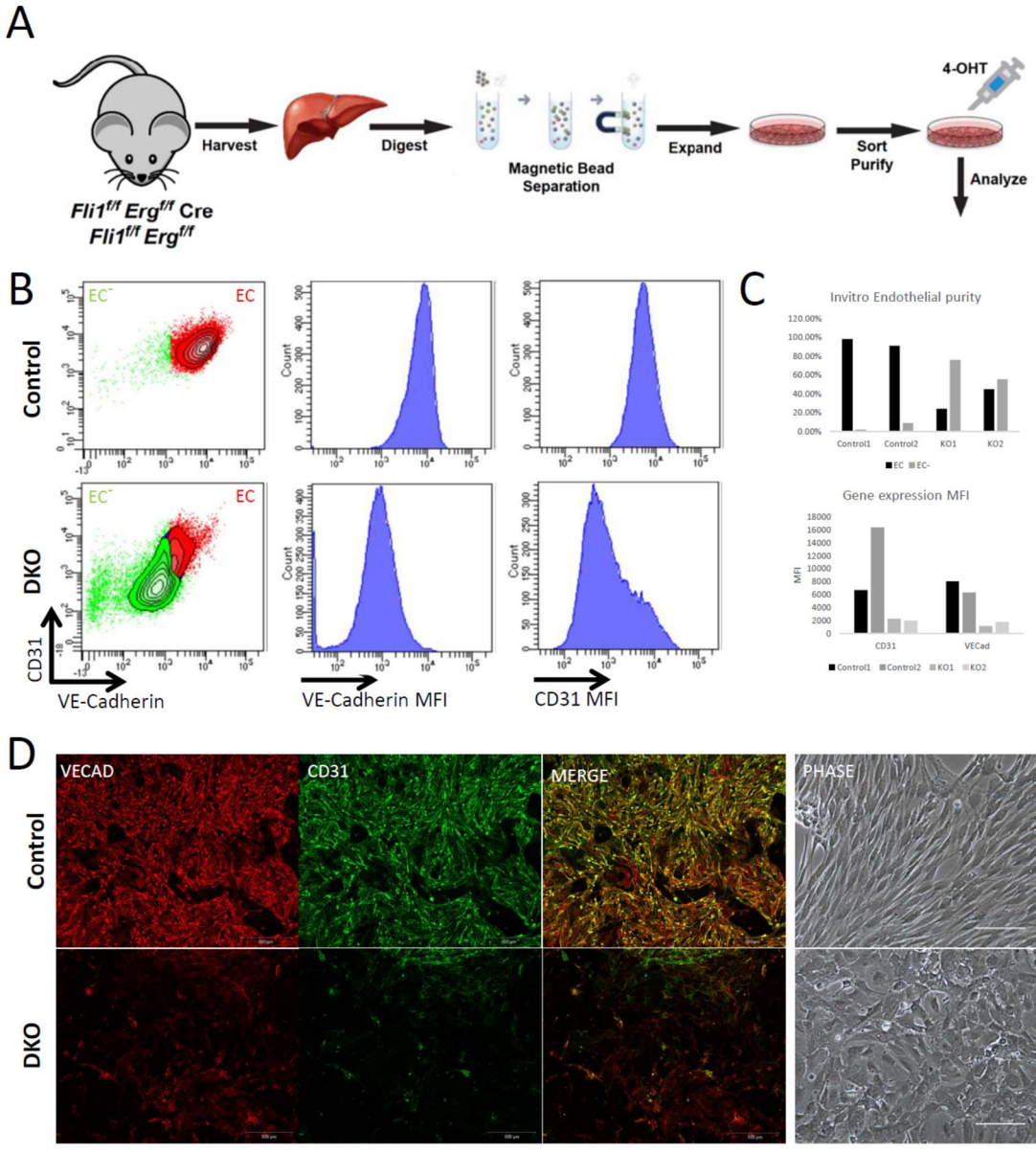
Therefore, Fli1 and Erg are not only involved in maintaining endothelial cell homeostasis and integrity but are also important to enforce the expression of endothelial cell markers essential for regulating vascular structures in addition to the angiocrine profile of endothelial cells critical for bone marrow HSC homeostasis.

3.2.5 In vitro analysis of Fli1-Erg deletion in endothelial cells

One of the caveats of a drastic homeostatic disruption that leads to early lethality is that we cannot assess the full extent of phenotypic and functional impact on the endothelial cell population. A minor loss in junctional integrity can lead to immediate lethality and we hypothesized that since this is rampant on a global scale in the mice we are unable to assess whether there is a loss in endothelial cell fate. In order to further analyze the fate of the endothelial cells we switched to an in vitro system so as to be able to identify changes in the cells over a period of time. Having seen the most drastic effect of Fli1-Erg deletion in the liver we decided to use liver ECs as our primary vascular bed for analysis in vitro. ECs were isolated by CD31 conjugated beads and sorted for purity so as to ensure 100% $CD31^{+}$VE-$Cadherin^{+}CD45^{-}$ authentic endothelial cells as the starting population, before inducing deletion (Figure 3.8A). In order to account for the effects of hydroxy tamoxifen, both Control and KO samples were assessed with and without hydroxy tamoxifen.

After deletion, the knockout endothelial cells began to lose their prototypical morphology and acquired a more mesenchymal appearance by Day 4 (Figure 3.8D). Additionally, as early as Day 4 post deletion there was a reduction in the levels of CD31 and VE-Cadherin when observed by live staining under confocal microscopy.

Figure 3.8 In vitro analysis reveals Fli1 and Erg are essential in maintaining endothelial morphology and endothelial cell fate. (A) Schematic detailing experimental plan for EC isolation for in vitro culture using CD31 conjugated magnetic beads followed by re-purification to ensure pure endothelial cell starting population before inducing deletion **(B)** Representative flow plots showing gating schema for isolation of endothelial cells. Histograms showing mean fluorescent intensity for two critical endothelial cell markers VE-Cadherin and CD31 **(C)** Quantification of endothelial cell purity at Day 7 post deletion based on CD31, VE-Cadherin expression and MFI for CD31 and VE-Cadherin **(D)** Representative images showing live staining for VE-Cadherin (Red) and CD31 (Green) along with Phase to show morphological changes in the endothelial cells at Day 7 post deletion of Fli1 and Erg.


A
Harvest
Digest
Magnetic Bead Separation
Expand
Sort Purify
4-OHT
Analyze
Fli1f/f Ergf/f Cre
Fli1f/f Ergf/f
B
Control
DKO
EC-
EC
CD31
VE-Cadherin
Count
VE-Cadherin MFI
CD31 MFI
C
Invitro Endothelial purity
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
Control1
Control2
KO1
KO2
EC
EC-
Gene expression MFI
MFI
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
CD31
VECad
Control1
Control2
KO1
KO2
D
Control
DKO
VECAD
CD31
MERGE
PHASE

The cells were analyzed at Day 7 post deletion to assess morphology, surface marker expression of CD31 and VE-Cadherin by microscopy as well as flow. Live confocal staining revealed a significant disruption in CD31 and VE-Cadherin staining in the majority of endothelial cells in the deleted primary liver (Figure 3.8D). The majority of cells had lost the junctional staining for VE-Cadherin and CD31, while a few pockets maintained single marker expression. Junctional staining was unaffected in the other three controls – Control $Fli1^{f/f}$ $Erg^{f/f}$, Control with 4-OHT and DKO $Fli1^{f/f}$ $Erg^{f/f}$ Cre. Flow analysis confirmed the loss of endothelial cell markers in vitro showing a drastic reduction in the expression of CD31 as well as VE-Cadherin (Figure 3.8B). Having confirmed the surface marker expression of some essential endothelial cell core genes, we sought to confirm the change in the transcriptional profile of these deleted cells. RNA was collected at Day 7 and compared to Control treated with 4-OHT after normalizing gene expression to β-Actin expression. RNA profiling confirmed the deletion efficiency for both Fli1 and Erg and was consistent with the observed reduction of CD31 and VE-Cadherin at protein level (Figure 3.9). Additionally, other core endothelial genes – VEGFR2, cKit, Tie2 and Sox17 were significantly reduced. One of the hallmark genes of mesenchymal transition, α-SMA was 6.5-fold upregulated. Collectively, these data indicate that Fli1 and Erg are essential for sustaining endothelial cell fate and preventing transitioning to non-vascular cell fate.

It is not surprising that the degree of reduction in marker expression could not be seen in vivo since the mice would not be able to survive such a global loss in endothelial marker expression. The in vitro system gives us a robust platform to decipher the molecular underpinnings governing this drastic fate loss in endothelial

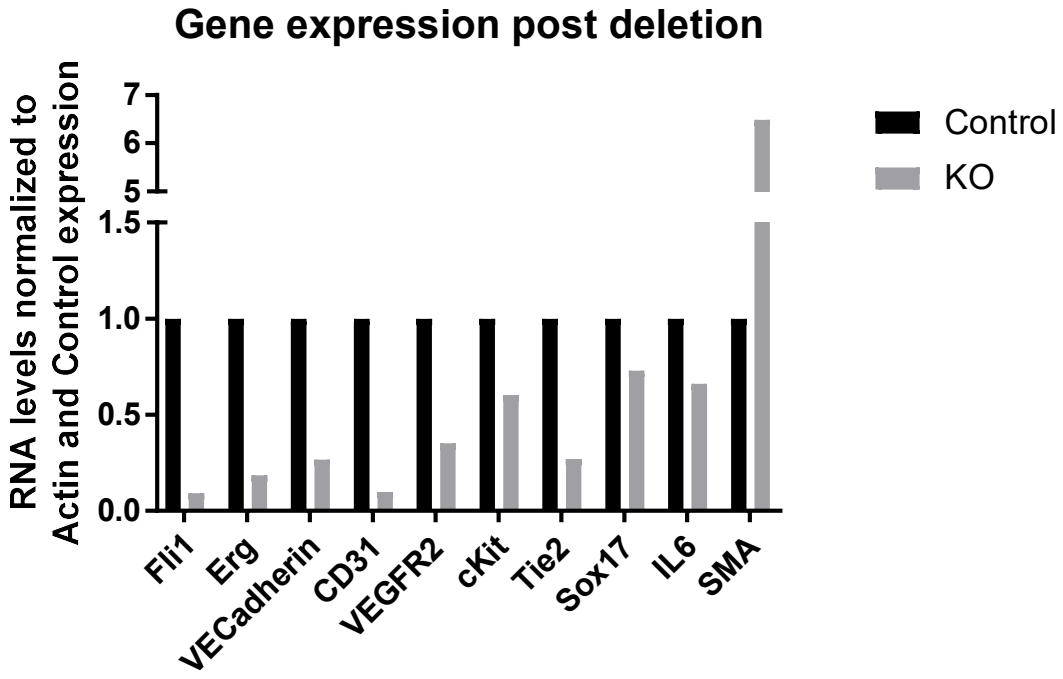


Figure 3.9 Transcriptional analysis of Fli1 Erg deleted cells shows significant reduction in key endothelial genes along with an increase in mesenchymal markers. RNA levels of important endothelial genes analyzed by qPCR and normalized to β Actin levels. Relative gene levels were then normalized to expression in Control cells to calculate fold change in double KO cells

cells. More detailed RNA and protein analysis would give an insight into what pathways could be used to rescue the disruption of endothelial homeostasis.

3.2.6 Genomic occupancy and transcriptional profiling reveal an essential role of Fli1 and Erg as master regulators of a core endothelial cell program

Having explored the phenotypic aspect of the Fli1-Erg deletion on survival, homeostasis and the vasculature we attempted to gain some insight into the underlying mechanisms regulating this drastic endothelial dysfunction. In order to achieve this, we took a two-pronged approach looking at genomic occupancy of Fli1 across different vascular beds using ChIP as well as analyzing changes at the RNA level in the endothelial cells in different vascular beds.

We employed both the mouse and the human system to assess genomic occupancy of Fli1 and Erg in endothelial cells. HUVEC ChIP data was analyzed from available online datasets to compare binding profiles of the two ETS factors. Fli1 and Erg had very similar binding profiles in terms of distribution of binding sites. The total number of binding sites of Fli1 and Erg in HUVECs were 3695 and 3648 respectively with comparable distributions between transcriptional start sites, intron and intergenic binding (Figure 3.10A). Annotated gene promoter binding was higher in Erg with 1664 genes compared to 1447 for Fli1 however only 827 of these genes had overlapping binding sites suggesting a distinct purview for each of these factors in the context of promoter occupancy (Figure 3.10B). Enhancers play an equally important role in regulating gene expression. By using p300 binding in conjunction with H3K27Ac histone marks to identify active enhancers we were able to compare enhancer binding between Fli1 and Erg. Overlapping binding for Fli1 and Erg in enhancers identified 399 peaks with single Fli1 occupancy at 187 peaks and single Erg occupancy at 132 (Figure 3.10C).

After analyzing the human system, we used primary mouse liver endothelial cells that were sorted and pooled to perform a Fli1 ChIP. Fli1 occupancy was assessed

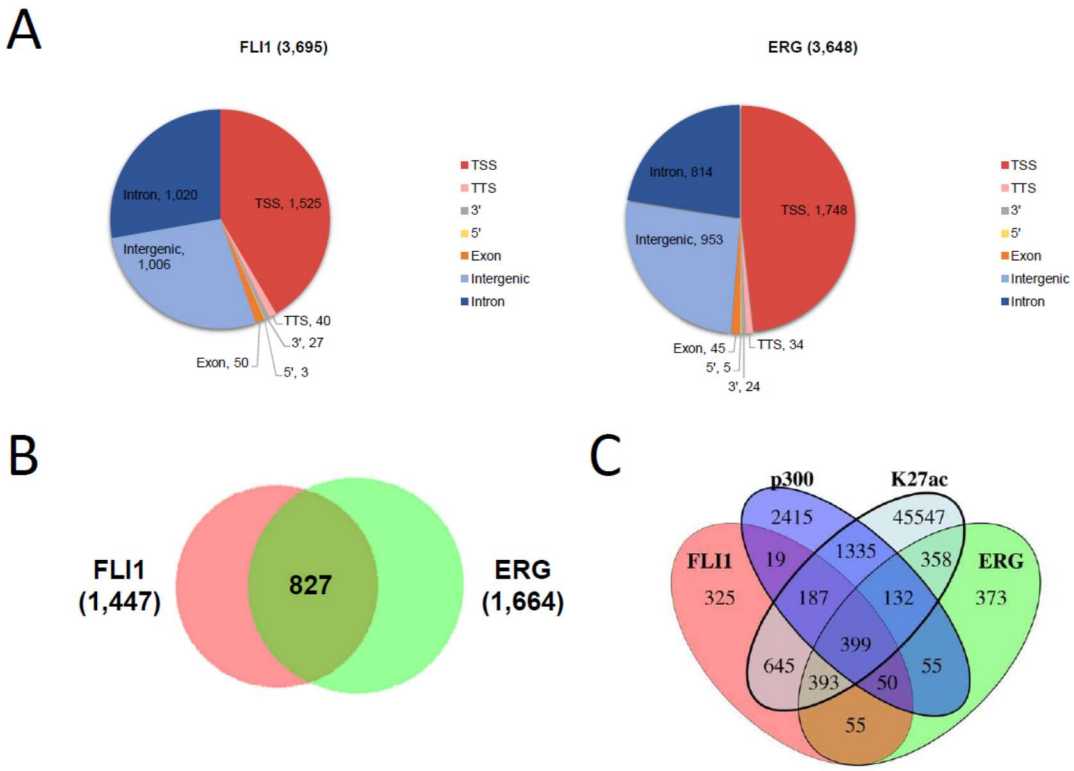


Figure 3.10 Genomic occupancy of Fli1 and Erg in HUVECs reveals similar genomic purviews. (A) Distribution of Fli1 and Erg binding sites in annotated genomic regions – Transcription start sites (TSS), transcription termination site (TTS), 3'and 5' untranslated regions, exons, introns and intergenic regions. **(B)** Venn diagram representing Fli1 an Erg occupancy in promoter regions of genes and the number of overlapping gene promoter regions **(C)** Venn diagram detailing enhancer occupancy of Fli1 and Erg based on p300 and H3K27Ac peaks in HUVECs.

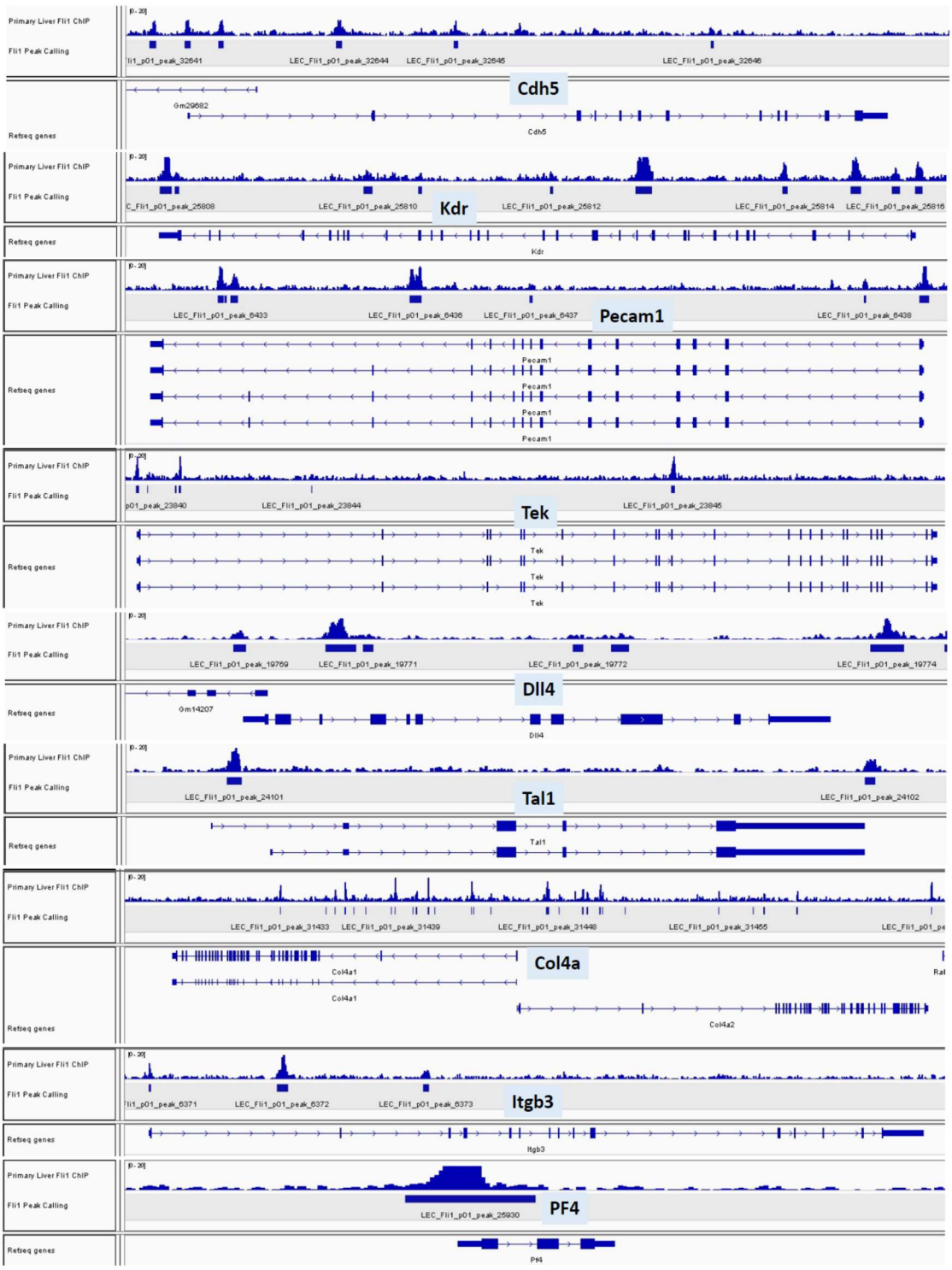


Figure 3.11 Genomic occupancy of Fli1 in essential endothelial genes. Fli1 occupancy in primary mouse liver endothelial cells in endothelial gene loci of interest.

at critical genetic loci to corroborate the drastic endothelial transition that was seen in vitro and in vivo. Fli1 was found to occupy all critical genetic loci of endothelial genes with promoter occupancy in Cdh5 (VE-Cadherin), Kdr (VEGFR2), Pecam1 (CD31), Tek (Tie2), Dll4 and many more (Figure 3.11). Additionally, Fli1 was also found to occupy the promoter region in the PF4 (platelet factor IV) gene which is primarily expressed in megakaryocytes and platelets, hinting at a previously understudied repressive function for Fli1 in endothelial cells.

The genomic purview of both these factors lends to their importance in regulating a core endothelial program essential for vascular homeostasis. Having observed the in vivo and in vitro consequences of Fli1-Erg deletion along with an understanding of the possible targets regulated by these two genes it was important to explore the transcriptional profile of these cells. RNA analysis would complement the genomic purview and unravel the underlying mechanisms governing EC fate loss.

In order to analyze the RNA, we isolated endothelial cells from three different vascular beds – Lung, liver and heart at Day 9 post tamoxifen injection so as to circumvent the issue of platelet contamination as a result of the thrombi formation in the moribund mice. Samples were processed for bulk RNA as well as single cell analysis. The Day 9 samples showed a significant increase in various categories covering extracellular matrix organization, cell migration, integrin signaling, angiogenesis and cell adhesion (Figure 3.12A, 3.13). The main pathways affected were the HIF-1 signaling, TGF-β signaling, MAPK signaling with hematopoietic lineage genes being upregulated as well (Figure 3.12B). **We hypothesize a two-step mechanism in the effect of Fli1-Erg deletion with the first stage being associated with an upregulation of a large number of genes that are regulated by these**

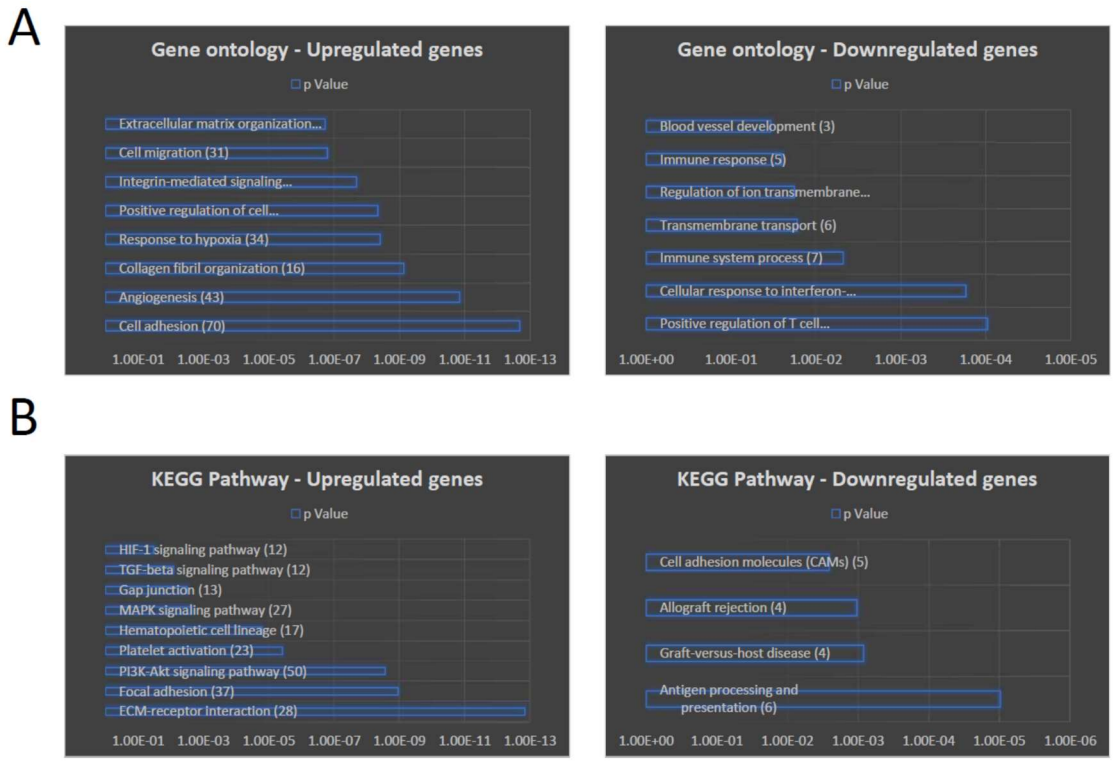


Figure 3.12 Gene ontology and KEGG pathway analysis. (A) Gene ontology analysis of significantly upregulated and downregulated genes across all three vascular beds **(B)** KEGG pathway analysis of significantly upregulated and downregulated genes across all three vascular beds

factors centered around ECM interactions and integrin signaling. This precedes the reduction in endothelial core factor reduction which together triggers an imbalance in the platelet activation pathway along with hematopoietic homeostasis and thrombus formation that causes lethality.

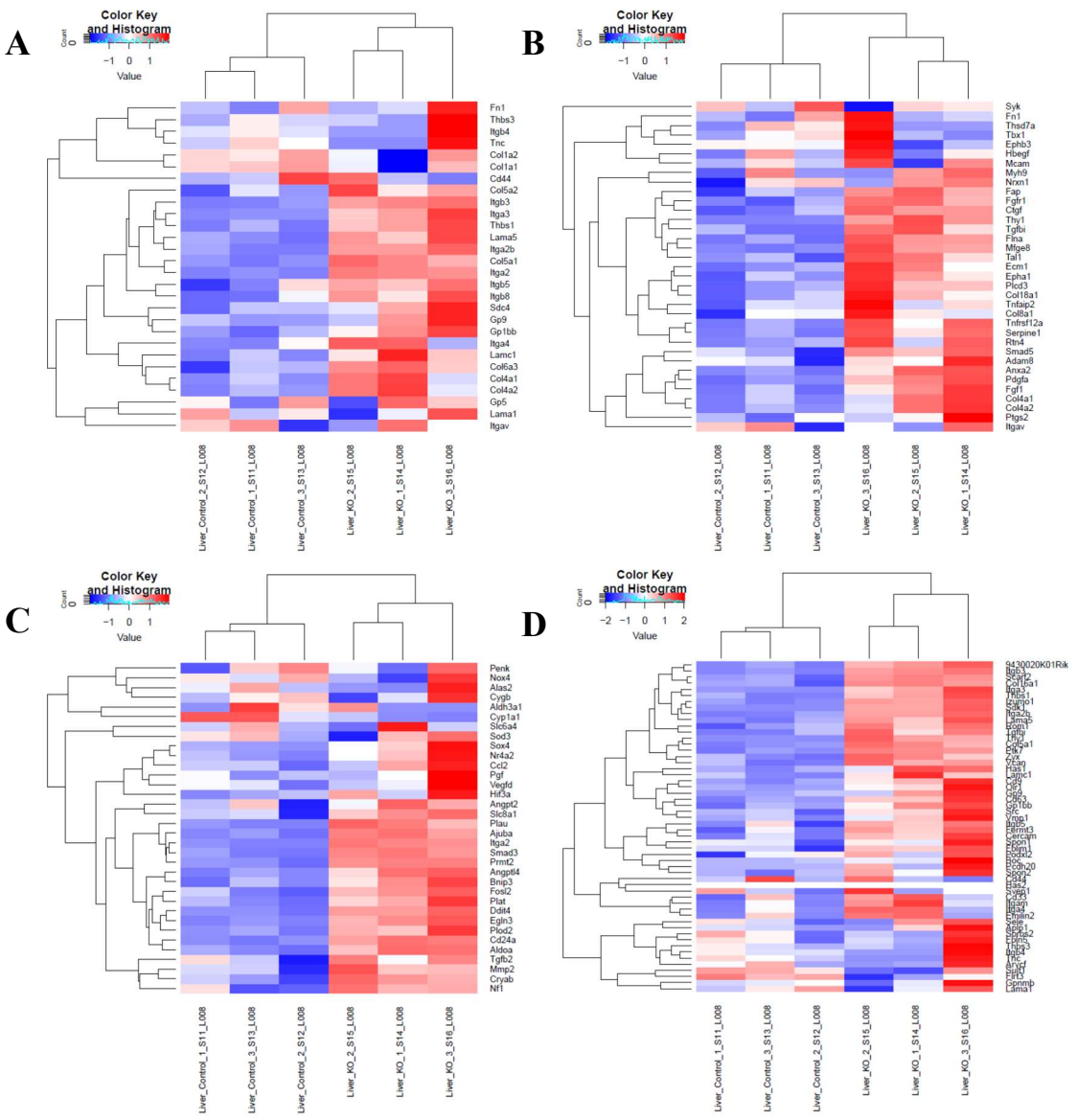


Figure 3.13 RNA Heatmaps reveal consistent changes across ECM receptor interactions (A), Angiogenesis (B), Response to Hypoxia (C) and Cell Adhesion (D) categories

Single cell RNA analysis of the liver endothelial cells was carried out to explore mosaic expression of genes that may lead to endothelial disruption. Two Control and KO livers were analyzed at Day 9 post first injection. An unbiased clustering of endothelial cells revealed 8 clusters in liver ECs (Figure 3.14A). The first 5 clusters 0-4 segregated perfectly based on the sample with majority of Control ECs contributing to Cluster 0, 1 while the KOs clustered mainly in the group 2-4 (Figure 3.14B, C, D). Clusters 5, 6 and 7 had a much lower cell count and were not differentially segregated between Control and KO samples (Figure 3.14C, D). The clear segregation of the Control and KO samples allows for an easy readout of differential gene expression using violin plots (Figure 3.15). Violin plots for specific endothelial genes revealed a significant reduction in the expression pattern in clusters 2, 3, 4 while control clusters had an abundance of the EC specific genes (Figure 3.15A). Some genes that showed clear segregation were Tek (Tie2), Jam2, Lyve1, Sox18, ROBO4 and Dll4 while other genes associated with a more mesenchymal fate or loss in endothelial fate were found to be upregulated in the KO clusters. These were Col4a2, Tgfbr2, Ltbp4 and Itgb3.

Henceforth, Fli1 and Erg are actively enforcing a majority of critical endothelial cell functions. Their combined genomic purview encompasses a core transcriptional program that ensures endothelial cell fate and homeostasis not just in the context of endothelial cells but the supporting cells that are dependent on endothelial angiocrine signals to maintain homeostasis. Having assessed the genomic locations of these ETS factors and combining them with transcriptional profiling of ECs in bulk as well as at single cell resolution confirms these two factors to not just be passive players in endothelial homeostasis but master regulators of endothelial cell fate from a global perspective as well as in terms of organ heterogeneity. This global

dysregulation offers us a unique opportunity to tease out the multitude of different pathways that are regulated so as to maintain the multifaceted functionality that endothelial cells perform on a day to day basis.

In conclusion, the unexpected finding that a combination of two transcription factors regulate endothelial cell fate in the adult mice has major implications for vascular biology. Given the catastrophic consequence of loss of both Erg and Fli1 in vascular integrity indicates that numerous pathophysiological conditions could emerge as a result of dysregulation of expression of these transcription factors. For example, post ischemic fibrosis, and endothelial to mesenchymal transitions could be driven in aberrant and maladapted downregulation of Fli1 and Erg.

Figure 3.14 Single cell analysis of Liver endothelial cells reveals complete segregation of Control and DKO ECs. (A) Resolution of single cell analysis into 8 clusters **(B)** Segregation of samples into specific clusters reveals dominant control and KO clusters **(C)** Contribution of each sample to different clusters **(D)** Cluster distribution among the samples

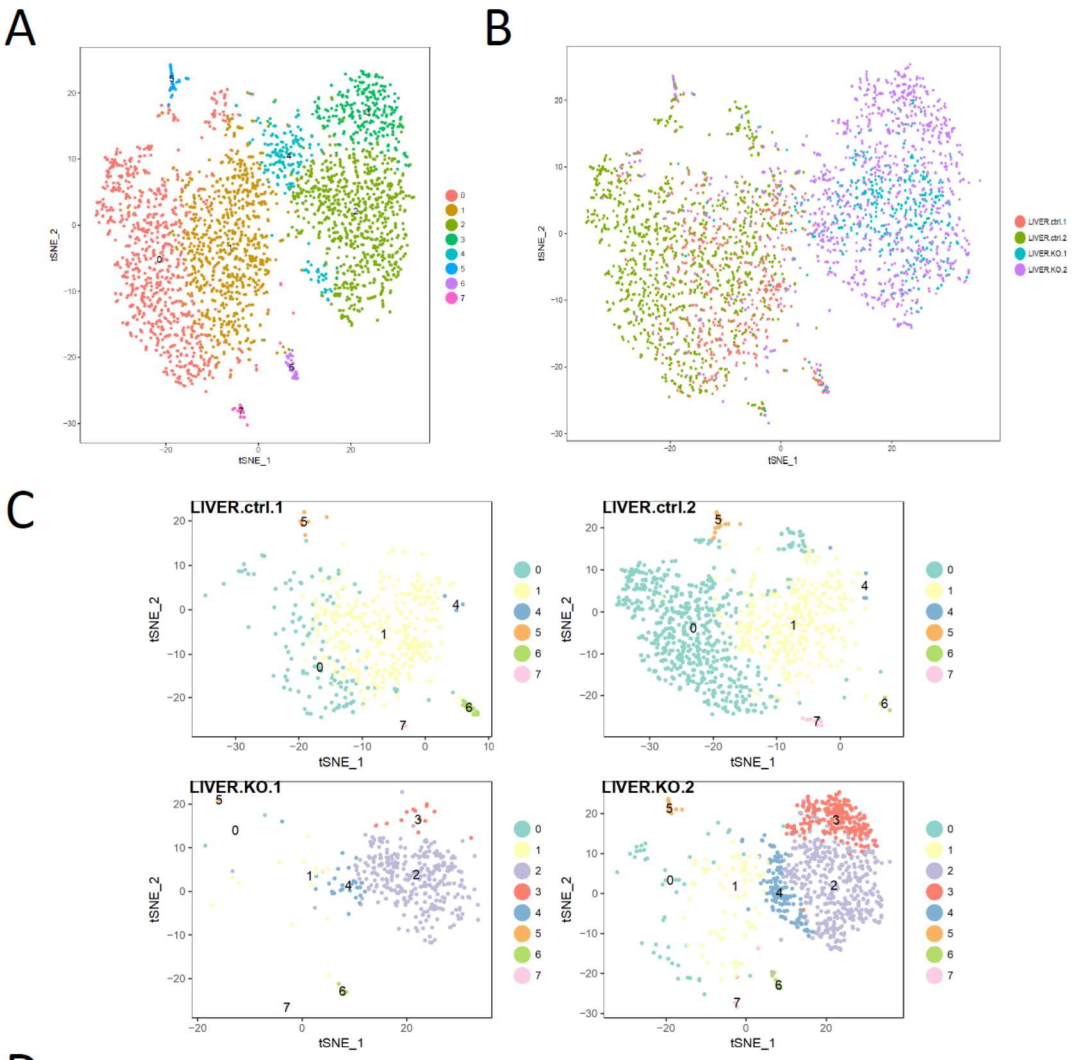
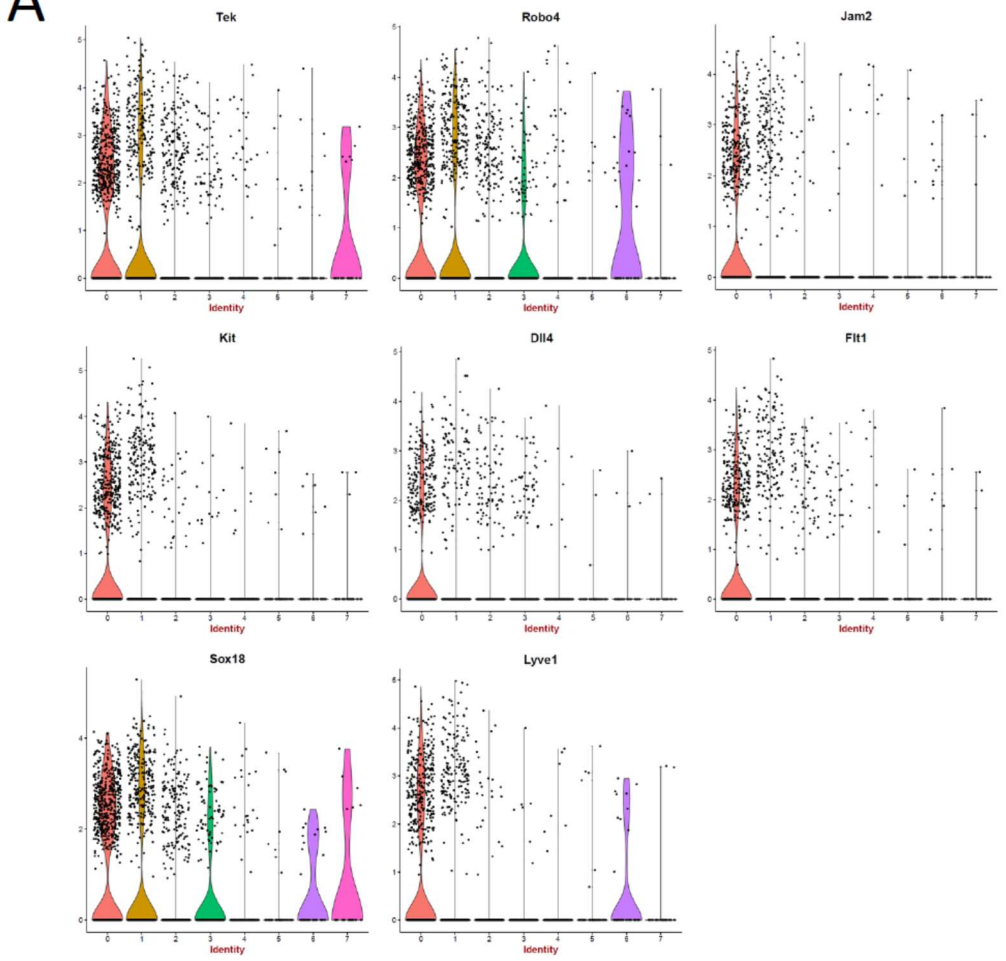
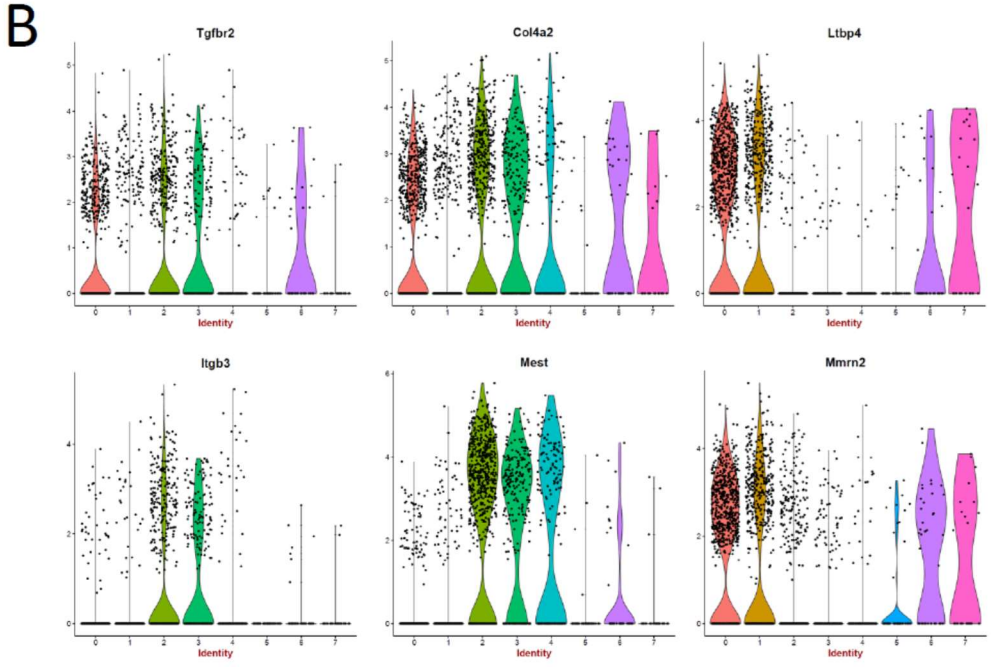

A
B
C
D
tSNE_1
tSNE_2
LIVER.ctrl.1
LIVER.ctrl.2
LIVER.KO.1
LIVER.KO.2

Figure 3.15 Violin plots reveal specific genes segregate specifically to Control (0,1) and KO clusters (2,3,4). (A) Dominant expression of endothelial specific genes in Control cluster are downregulated in the DKO clusters **(B)** Specific genes related to mesenchymal fate and implicated in loss of endothelial cell fate are differentially expressed between the Control and KO clusters.


A
Tek
Robo4
Jam2
Kit
Dll4
Flt1
Sox18
Lyve1
Identity


B
Tgfbr2
Col4a2
Ltbp4
Itgb3
Mest
Mmrn2
Identity

CHAPTER 4: CONCLUSIONS AND DISCUSSION

The ETS family of transcription factors have been implicated extensively to play a role in endothelial and hematopoietic regulation (Randi et al., 2009; Sharrocks, 2001). Here we investigated the role of Fli1, one of the key ETS factors that is expressed in both these lineages and showed that it is essential in maintaining key functions of both these cell types that are necessary for homeostasis. In Chapter 2, we focused on the understudied role of Fli1 in the hematopoietic stem cells and demonstrated that Fli1 is involved in critical mechanisms that regulate Hematopoietic and HSC homeostasis and function in adult mice. In Chapter 3, we elucidated the role of Fli1 in endothelial cells and showed that on its own Fli1 is not critical to maintain endothelial identity and function however it shares a significant overlap with one of its closely related ETS factors, Erg. We demonstrated that Fli1 and Erg synergize to maintain endothelial cell homeostasis and endothelial cell fate in adult mice. The biggest differentiating factor in these studies is the ability of Fli1 to regulate essential homeostatic functions in adult mice. A large number of genes have well defined roles during development but most of them are not actively involved in adult homeostasis and have redundancy with other factors due to which they are not essential in adults. We propose that Fli1 is a master regulator that directly regulates homeostatic processes in these two cell types which are overlooked in the context of regular physiology.

4.1 FLI1 AND HEMATOPOIESIS

Early mouse models implicated a role for Fli1 in vascular integrity as well as platelet function due to developmental defects that were observed in global knockout

models. We demonstrated that this effect is mediated through expression of Fli1 in hemogenic endothelium and concurrently in the arising pre-HSCs that give rise to the hematopoietic system. Fli1 deletion in early AGM endothelial cells resulted in massive hemorrhage and defective hematopoiesis. AGM hematopoietic cells lacking Fli1 are unable to expand in vitro and are significantly reduced in overall numbers in $Fli1^{\Delta EC/\Delta EC}$ embryos. This effect carried over in the adult setting where inducing Fli1 deletion caused complete bone marrow failure with peripheral blood counts going down in all three categories of WBCs, RBCs and platelets while platelet counts were most severely affected. Fli1 is unique in having an effect at every stage of hematopoietic differentiation from the HSC all the way down to terminally differentiated cell types such as erythrocytes and platelets.

Another aspect that differentiates Fli1 in its function is its ability to orchestrate hematopoietic reconstitution. Through the various transplant experiments assessing lineage contribution and bone marrow engraftment, we demonstrated that absence of Fli1 is a significant impediment in achieving effective engraftment and subsequent differentiation to peripheral blood lineages. $Fli1^{\Delta/\Delta}$ HSCs were defective in both primary and secondary transplant settings being unable to contribute to any peripheral blood lineage – B cell, T cell or myeloid lineages. Most importantly, we demonstrate that Fli1 is not only required in the context of hematopoietic reconstitution but for HSC homeostasis. During homeostasis, HSC contribution to hematopoiesis is minimal. The reduction in HSC numbers under homeostasis due to Fli1 deletion demonstrates the importance of the transcriptional activity of this ETS factor in HSCs.

Given that this role for Fli1 has not been studied before in this context it might be a factor that is applicable in the context of bone marrow transplants from a clinical

perspective. Heterozygote deletion of Fli1 in Paris Trousseau syndrome suggests the possibility of a more advanced role of Fli1 in humans. Keeping that in mind, it would be interesting to assess Fli1 levels in clinical trials of cord blood or bone marrow transplants to see, on a very basic level if Fli1 confers an engraftment advantage. An ideal scenario correlating Fli1 expression with better probability of engraftment would enable early screening of transplants to ascertain successful engraftment even before administration. Fli1 is unique in being a transcription factor that regulates HSC activity during homeostasis. The most obvious implication for this is from the perspective of cancer or leukemia. If Fli1 downregulation can be used to modulate the expansion of leukemias the clinical applicability of this is limitless but this is where the omnipresent nature of this gene is a detriment. Clinical targeting of a factor that may lead to thrombocytopenia is less than ideal however there is always a work around for such situations. In this study, we have described two pathways – cKit and Mpl that address only a certain aspect of Fli1 function and so further research in understanding the other pathways involved in regulating leukemic expansion would be necessary to use Fli1 in a clinical setting. Additionally, screening small molecules that target specific functions of Fli1 are another alternative and a few compounds have already been identified that are able to downregulate Fli1 activity (Li et al., 2015; Li et al., 2012)

In conclusion, Fli1 is an essential factor in hematopoietic regulation during development and more importantly in adults. This study has merely scratched the surface of the various functions that Fli1 executes in the context of hematopoietic stem cell homeostasis. Further studies translating the role of Fli1 from the murine model to humans will allow us to tap into the unlimited potential of this master regulator of hematopoiesis. Identifying the functional aspect of Fli1 in HSC maintenance and self-

renewal adds to the body of work implicating diverse functions of Fli1 in different hematopoietic cell types. The question remains as to how does Fli1 orchestrate this intricate transcriptional regulation in different cell types from HSCs to platelets to endothelial cells. Three aspects that may regulate this specificity may be – different isoforms, different binding partners and post translational modifications by signaling cascades in a cell type specific manner. The first aspect – isoforms though the easiest to identify through deep sequencing of Fli1 across various cell types is one of the hardest to prove in terms of cause and effect. Identification of different expression levels of isoforms need not imply functionality and will require both invitro overexpression studies as well as in vivo loss of function studies to understand the significance of this differential expression. This can prove difficult given functional overlap between isoforms and hence presents a significant challenge.

The second aspect focused on binding partners can be addressed through Co-immunoprecipitation in a cell type specific manner to identify differences in the binding profile of Fli1. Furthermore, identifying important binding partners opens the possibility of studying the type of interaction between Fli1 and its partners, necessary to facilitate the cell specific transcriptional regulation. Lastly, post translational modifications (PTMs) can be identified by looking specifically at immunoprecipitated Fli1 and carrying out mass spectrometry to compare phosphorylation and other PTMs that may be cell type specific. All three of these aspects are interlinked and most likely contribute to the specificity of not just Fli1 but a number of different transcription factors. In conjunction with the genomic purview and the transcriptomic analysis for various cell types, Fli1 offers a unique opportunity to take a glimpse into the mechanisms governing transcriptional specificity for TFs.

4.2 FLI1 AND ENDOTHELIAL IDENTITY

"One is only as young as one's endothelium"

-Anonymous

Our understanding of the various functions that endothelial cells perform has grown exponentially over the past few years. These cells are no longer just considered to be basic plumbing for the body, circulating essential nutrients and oxygen to various organs but orchestrate organ development, regeneration and are involved with regulation of different stem cell niches. Dysregulation of the endothelial cells can lead to aberrant fibrosis, malignancies and other non-physiological conditions. We sought to identify a factor that is essential in regulating endothelial cell fate. Endothelial cell fate is a term that encompasses all the functions of endothelial cells but at its core represents the basic functionality of endothelial cells and that is to maintain homeostasis through the expression of key surface markers such as VE-Cadherin, CD31 and VEGFR2. Our results here showed that Fli1, by itself is not essential for maintenance of this endothelial cell fate in the post AGM development, or the post-natal retina or in adults. We demonstrate that this was due to redundancy with its closely related ETS family gene, Erg. Deletion of Fli1 and Erg resulted in lethality and we demonstrated that these two factors are essential for the maintenance of endothelial homeostasis and endothelial cell fate in adult mice. These observations are counter intuitive to our current understanding of endothelial biology since the majority of endothelial cells in adult vascular beds are quiescent and do not proliferate unless they are given a physiological insult or injury.

We identify two transcription factors that together regulate endothelial homeostasis and whose dysregulation is lethal to mice due to the complete loss of vascular integrity resulting in the formation of thrombi in the liver. Being able to observe this phenomenon in an in vitro setting gave us a better idea of the extent of cell fate loss in the endothelial cells. In vitro deletion demonstrated a drastic downregulation in VE-Cadherin, CD31 and VEGFR2 that can never be observed in vivo given the essential nature of their expression however the robust invitro system provides us with a platform to interrogate the various pathways that govern endothelial cell fate. The invitro system shows a consistent timeline for loss of key endothelial markers and enables us to test various pathways that may be involved in regulating key aspects of endothelial cell biology. The ability to live stain for markers such as VE-Cadherin and CD31 can allow us to conduct high content high throughput screens for multiple factors and pathways that may regulate endothelial cell fate. The recovery of these critical markers is just the first step in the level of functionality that Fli1 and Erg execute in endothelial cells. The second level would involve organ heterogeneity and being able to identify organ specific factors that enable endothelium in organ beds to respond appropriately so as to maintain homeostasis or facilitate repair and recovery in an organ. From a clinical standpoint, being able to address endothelial cell repertoire especially in the context of organ heterogeneity would enable us to decipher vascular cues that may regulate metastasis in specific vascular beds. Additionally, being able to understand the mechanisms that govern endothelial fate may improve our ability to culture endothelial cells which is currently challenging. Robust techniques for endothelial cell culture would allow us to step into the field of cell therapy. Endothelial cells by themselves, have been shown to provide radioprotection (Poulos et al., 2015) in transplant settings however the plasticity of endothelial cells allowing for direct differentiation to other cell types (Lis et al., 2017; Sandler et al.,

2014) would open up infinite possibilities in terms of clinical applications for cell therapy.

4.3 CONCLUDING REMARKS

Genomic analysis is now available that allows us to compare the purview of Fli1 across multiple cells types from endothelial cells to megakaryocytes to hematopoietic stem and progenitors. Needless to say, the genomic accessibility of Fli1 is different in different cell types and understanding the underlying mechanisms that govern this change is important to further enhance our understanding of what defines a certain cell type. Master regulators is a term that has been used to describe genes such as MyoD or Oct4 since their expression can be used to influence cell fate choices however this is a simplistic view of looking at gene regulation and does not factor in other environmental or cell autonomous cues that play a role in facilitating such transitions. If these factors were such effective master regulators then the efficiency of say iPS generation would be much higher compared to the low percentage conversion that is seen right now. It is here that the other factors influencing transcriptional regulation need to be studied in more detail. Leaving aside the obvious gene isoforms with different structure, there are two mechanisms that define context driven expression and those are binding partners and post translational modifications. Understanding these mechanisms in a cell type specific manner would open up the possibilities of efficient and effective direct conversion between cell types. What makes Fli1 an ideal candidate for these studies is its broad expression pattern with cell type specific function. Fli1 is expressed in fibroblasts, endothelial cells and almost all hematopoietic cell lineage except erythrocytes and it performs very different roles in

each of these cell types. Leveraging this to understand the mechanisms controlling Fli1 specificity is definitely an area that needs to be pursued in the future.

The studies presented here demonstrate take the first steps in establishing the importance of Fli1 in the context of hematopoiesis and endothelial cell identity. In hematopoietic cells, we uncovered a novel mechanism implicating Fli1 with the regulation of hematopoietic stem cell homeostasis through two critical surface proteins – cKit and Mpl. In endothelial cells, we propose a novel concept that two transcription factors – Fli1 and Erg are involved in maintaining the fate of endothelial cells during homeostasis and their dysregulation results in a complete loss in identity of the endothelial cells. Future studies will be able to decipher the different mechanisms that govern this drastic loss in identity and disruption of homeostasis and its application in clinical settings as well as understanding the upstream signals that govern the specificity of the effect of these transcription factors in a cell type specific manner.

MATERIALS AND METHODS

Mice

VE-Cadherin cre mice were obtained from the labs of Dr Arispe and Dr Speck and were maintained on a C57Bl6 background. Fli1$^{f/f}$ mice were obtained from EMMA (ID:05992) as frozen embryos and rederived at the MSKCC Mouse Genetics core and maintained on a C57Bl6 background. C57BL/6J (CD45.2), B6.SJL-Ptprca Pepcb/BoyJ (CD45.1) and Rosa Cre^{ERT2} mice were obtained from Jackson laboratories. The breeding, maintenance and experimental procedures were performed in accordance with the guidelines of Institutional Animal Care and Use Committee of Weill Cornell Medical College. Genotyping was confirmed in the lab using genoptyping primers (Fli11-F CAGAGCCCTGGCTGTCTTGAACTCCTAGACCAG, Fli1-wt-R GGCGCTGTCGGACAGTAGTTCCAGGAGAAACTG, Fli1-del-R GTAGGGAATCCTACAATGATGAGACCCGTGTGCTG) and also sent to Transnetyx for offsite genotyping.

Whole Mount staining

Embryos were fixed in 4% PFA overnight and then washed twice in PBS before staining. Embryos were blocked in PBS with 5% milk, 5% serum and 0.1% Triton-X twice for 30' at room temperature. Staining was carried out with Anti CD31(Biolegend MEC13.3) overnight followed by 5X washes with block solution for 20' at room temperature. Secondary staining was carried out overnight followed by 5X washes. Embryos were maintained in PBS before imaging on an LSM 710 Confocal Microscope (Zeiss).

Post-natal retina

Retinas were collected at P6 and fixed overnight in 4% PFA. They were processed as described (Pitulescu et al., 2010)

Hindlimb Ischemia

The proximal part of the femoral artery and the bifurcation point between the popliteal artery and the saphenous artery of $Fli1^{f/f}$ mice were ligated and all side branches were dissected. The femoral artery was excised out via ligated points. With the muscle still exposed, $5x10^5$ cells suspended in PBS were injected into the gracilis muscle. Hindlimb reperfusion was measured at indicated time points with laser Doppler perfusion imager (Persican PM3, Perimed).

Isolation of early definitive pre-HSCs from AGM

Embryos at E11.5 were used for AGM isolation and subsequent processing was carried out as described (Morgan et al., 2008)

In vitro expansion of hematopoietic cells on E4-HUVEC platform

Human umbilical vein endothelial cells (HUVECs) were isolated as described (Rafii et al., 1994) and cultured in endothelial cell growth medium – M199 (Sigma), 10% FBS (Omega Scientific), 50 μ g ml^{-1} endothelial mitogen (Alfa Aesar), and 100 μ g ml^{-1} heparin (Sigma). Once endothelial cells were transduced with lentiviral vectors expressing E4ORF1 gene they were selected in Ex-vivo medium for two weeks and then cultured in human endothelial cell media and plated for expansion. Isolated $cKit^+Sca1^+Lineage^-$ (KLS) cells were plated on confluent 6-well for expansion in hematopoietic stem cell expansion medium – StemSpan SFEM (STEMCELL

Technologies) with 10% KnockOut Serum Replacement (Invitrogen), Glutamax and 20mM HEPES (Invitrogen) 50 ng ml^{-1} mouse c-Kit ligand (SCF, Peprotech).

Bone marrow transplantation and peripheral blood analysis

For all transplant experiments, mice were irradiated with 950 cGy and transplanted with experiment specific bone marrow recovery dose. Peripheral blood and bone marrow analysis was performed at 4-week intervals with antibodies against c-Kit/CD117 (2B8), Sca-1/Ly-6A (D7), CD48 (HM48-1), CD150 (mShad150), CD45.1(A20) and CD45.2 (104). Lineage antibody cocktail included: CD41 (MWReg30), TER119 (TER119), B220 (RA3-6B2), CD11b (M1/70), Gr1 (RB6-8C5) and CD3 (17A2). DAPI was used to discriminate and eliminate dead cells from analysis. All antibodies were obtained from Biolegend unless mentioned. Peripheral blood was collected using retro-orbital bleeds in 1:1 ratio with PBS-EDTA (10mM). Blood was lysed using RBC lysis (Biolegend) and then analyzed for multilineage engraftment using above antibodies.

Histology

H&E staining of 12-mm paraffin sections was performed by Histoserv and imaged on a BX51 (Olympus) light microscope.

Serum Analysis

Retro-orbital bleeds were used to obtain 250µl of blood from mice before sacrificing. Blood was allowed to coagulate for an hour at room temperature. Cells were separated by a high-speed spin at 10,000 rpm for 1'. Supernatant was collected and submitted for serum analysis at the Histology Core at MSKCC.

Immunohistochemistry

Mice were injected retro-orbitally with fluorescently labeled anti-VE-Cadherin antibody (BV13, Biolegend), 8 minutes prior to sacrifice. Harvested organs were fixed overnight in 4% PFA followed by 72 hours in 30% sucrose. Organs were embedded in O.C.T. (VWR). 16µm sections were counter stained with DAPI (Biolegend) and imaged on an LSM 710 Confocal Microscope (Zeiss).

Peripheral Blood analysis

Retro-orbital blood was collected on indicated days using heparinized capillary pipettes. A volume of 65µl blood was diluted in 195µl of PBS-EDTA (10µM) to prevent clotting. Complete blood counts and Differential blood counts were obtained with an automated Advia 120 (Siemens).

Isolation of organ specific adult mouse endothelial cells

Mice were injected retro-orbitally with 25µg of anti-VE-cadherin-AF647 antibody (BV13, Biolegend) 8 min prior to sacrifice and the organs were harvested. For flow cytometry and cell sorting, organs, including lungs, liver and heart were minced and incubated with collagenase A (25 mg ml^{-1}), dispase II (25 mg ml^{-1}), and DNase (250 µg ml^{-1}) (Roche) at 37 °C for 20–30 min to create a single-cell suspension. Cells were filtered through a 70µm filter immediately before counter staining. The single-cell suspension was blocked with anti-mouse CD16/32 (2.4G2) before antibody staining with anti-mouse CD31 PE-Cy7 (390, Biolegend), anti-mouse CD45 (30-F11) and anti-mouse Podoplanin (8.1.1. Biolegend). Resulting adult mouse endothelial cell cultures were cultured on fibronectin-coated (Sigma-Aldrich) plates in mouse endothelial cell (mEC) media – DMEM:Ham's F-12 (Sigma) supplemented with 20% FBS (Omega Scientific), 20 mM HEPES (Invitrogen), 100µg ml^{-1} heparin (Sigma), 50µg ml^{-1}

endothelial mitogen (Alfa Aesar) and 5μM SB431542 (R&D, 1614). Purity of mECs and absence of contaminating hematopoietic cells were confirmed by flow cytometry and confocal microscopy. The same panel of antibodies was used for in vitro purification of primary endothelial cells that were isolated using CD31-captured (Biolegend MEC13.3) magnetic beads (Life Technologies).

RNA isolation and sequencing

At least 100 ng of total RNA from both freshly harvested and cultured cells was isolated (phenol-chloroform separation of TRIzol LS) and purified using Qiagen RNeasy Mini Kit. RNA quality was verified using an Agilent Technologies 2100 Bioanalyzer. RNA library preps were prepared and multiplexed using Illumina TruSeq RNA Library Preparation Kit v2 (non-stranded and poly-A selection) and 10 nM of cDNA was used as input for high-throughput sequencing via Illumina's HiSeq 4000 producing 51 bp paired-end reads. Sequencing reads were demultiplexed (bcl2fastq v2.17), checked for quality (FastQC v0.11.5). Paired end reads were mapped against Mus Musculus reference genome GRCm38 v87 using Star aligner after the read quality control and trimming performed using Trimmomatic. Reads were counted to the reference gene annotations using htseq and the counts were analyzed and tested for differential using edgeR with plots and heatmaps made in R.

ddSeq and Single Cell analysis

A single cell suspension was loaded into the Bio-Rad ddSEQ Single-Cell Isolator on which cells were isolated, lysed and barcoded in droplets. Droplets were then disrupted and cDNA was pooled for second strand synthesis. Libraries were generated with direct tagmentation followed by 3' enrichment and sample indexing using Illumina Nextera library prep kit. Pooled libraries were sequenced on the Illumina

NextSeq500 sequencer. Sequencing data were primarily analyzed using the SureCell RNA Single-Cell App in Illumina BaseSpace Sequence Hub. In particular, sequencing reads were aligned to reference genome using STAR aligner; cell barcodes were used to separate reads from different cells, and unique moledular identifiers (UMI) were used to remove duplicate reads that were actually derived from the same mRNA molecule. A knee plot was generated based on the number of UMI counts per cell barcode in order to identify "good" cells separating from empty beads or noise. A raw UMI counts table for each gene in each cell was then prepared. Seurat version 2.0.1 was used for clustering analysis. We excluded cells with an outlier number of genes (4000) detected as potential multiplets. To mitigate the effects of cell cycle heterogeneity in data, we followed a previously published approach by assigning each cell a score based on its expression of canonical cell phase markers and then regressing these out. We also regressed out effects associated with number of UMIs and ribosomal gene content. A principle component analysis (PCA) was performed, where the top 16 principal components were selected for cell clustering and t-SNE projection.

ChIP Analysis

Anti-Fli1 or control Rabbit IgG (Ab46540) were used to identify Fli1-bound regions using a method based on detailed protocol report (Nolan et al., 2013). Cells ($2–5X10^7$) were fixed in 1% paraformaldehyde diluted in EC media. Fixation was quenched with 125mM glycine and the cells were washed three times with PBS. After nuclei isolation and sonication using a Bioruptor, chromatin-protein complexes were incubated with 10 mg antibody bound to Dynabeads (Invitrogen) overnight at 4°C under gentle agitation. Complexes were washed with PBS containing 0.5% BSA and 5mM EDTA

using magnetic separation and then DNA purified by phenol-chloroform extraction. Single read (50/50 bp) libraries were produced and then sequenced at the MSKCC Integrated Genomics Operation on Illumina HiSeq 4000.

Statistical Analyses

All data are presented as mean ± s.d. The data presented in the figures reflect multiple independent experiments performed on different days using different mice. The significance of differences was determined using a two-tailed Student's t-test, unless otherwise stated. $p > 0.05$ was considered not significant; * $p < 0.05$; * * $p < 0.01$; * * * $p < 0.001$. In all the figures, n refers to the number of mice when applicable. All statistical analyses were performed using Graphpad Prism software. No animals were excluded from analyses.

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