SIRT1 DEFICIENCY COMPROMISES MOUSE EMBRYONIC STEM CELL DIFFERENTIATION, AND EMBRYONIC AND ADULT HEMATOPOIESIS IN THE MOUSE

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ABSTRACT

Xuan Ou

SIRT1 DEFICIENCY COMPROMISES MOUSE EMBRYONIC STEM CELL DIFFERENTIATION, AND EMBRYONIC AND ADULT HEMATOPOIESIS IN THE MOUSE

SIRT1 (Sirtuin 1) is a founding member of a family of seven proteins and histone deacetylases. It is involved in cellular resistance to stress, metabolism, differentiation, aging, and tumor suppression. *SIRT1*$^{-/-}$ mice demonstrate embryonic and postnatal development defects. We examined hematopoietic and endothelial cell differentiation of *SIRT1*$^{-/-}$ mouse embryonic stem (mES) cells in *vitro*, and hematopoietic progenitors in *SIRT1*$^{+/+}$, *SIRT1*$^{+/-}$, and *SIRT1*$^{-/-}$ mice. *SIRT1*$^{-/-}$ ES cells exhibited markedly delayed/immature formation of blast colony-forming cells (BL-CFCs). When individual blast colonies were analyzed for hematopoietic and endothelial potential, replated *SIRT1*$^{-/-}$ BL-CFC possessed limited hematopoietic potential, whereas endothelial potential was essentially unaltered. The ability of *SIRT1*$^{-/-}$ ES cells to form primitive erythroid progenitors was not only delayed but greatly decreased. Moreover, after differentiation of *SIRT1*$^{-/-}$ mES cells, there were also significant decreases in granulocyte-macrophage (CFU-GM) and multipotential (CFU-GEMM) progenitor cells. Differentiation delay/defects were associated with delayed capacity to switch off *Oct4*, *Nanog* and *Fgf5*, decreased *β-H1* globin, *β-major* globin, and *Scl* gene expression and reduced activation of the Erk1/2 pathway upon *SIRT1*$^{-/-}$ ES cell commitment. Reintroduction of WT *SIRT1* into *SIRT1*$^{-/-}$ cells partially rescued the primitive erythroid progenitor formation of *SIRT1*$^{-}$

$^{/-}$ cells and the expression of hemoglobin genes, *Hbb-bh1* and *Hbb-b1*, suggesting that the defect of hematopoietic commitment is due to deletion of SIRT1, and not to genetic drifting of *SIRT1*$^{-/-}$ cells. To confirm the requirement for SIRT1 for normal development of hematopoietic progenitor cells, we assessed embryonic and adult hematopoiesis in *SIRT1*$^{+/+}$, *SIRT1*$^{+/-}$ and *SIRT1*$^{-/-}$ mice. Yolk sacs from SIRT1 mutant embryos generated fewer primitive erythroid precursors compared to wild-type (WT) and heterozygous mice. Moreover, knockout of SIRT1 decreased primary bone marrow hematopoietic progenitor cells (HPCs) in 5 week and 12 month old mice, which was especially notable at lower (5%) O_2 tension. In addition these progenitors survived less well *in vitro* under conditions of delayed growth factor addition. Taken together, these results demonstrate that SIRT1 plays a role in ES cell hematopoietic differentiation and mouse hematopoiesis.

Hal E. Broxmeyer Ph.D., Chair

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LIST OF ABBREVIATIONS

AceCs-1	Acetyl CoA synthetase 1
AceCSs	Acetyl-CoA synthetases
Ac-LDL	Acetylated low-density lipoprotein
AGM	Aorta-gonad-mesonephros
ALP	Alkaline phosphatase
ART	ADP-ribosyl transferase
bFGF	basic fibroblast growth factor
BL-CFC	Blast colony-forming cells
CBF	Core binding factor
Cdx-2	Caudal type homeobox 2
CFU-GEMM	Colony-forming unit granulocyte-erythroid- megakaryocyte-macrophage
CFU-GM	Colony-forming-unit-granulocyte-macrophage
CR	Caloric restriction
DAC	Deacetylase
DBC1	Deleted in breast cancer 1
DV	Dorsoventral
E	Embryonic day
EB	Embryoid Body
ECGS	Endothelial cell growth supplement
eNOS	endothelial nitric oxide synthase
EPO	Erythropoietin
ERC	Extrachromosomal rDNA circle

ERK	Extracellular Signal Regulated Kinase
Ery-D	Definitive erythroid progenitors
Ery-P	Primitive erythroid
ES	Embryonic Stem
FBS	Fetal bovine serum
FFA	Free fatty acids
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FOXO1	Forkhead box protein O1
GATA-6	GATA-binding protein 6
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HIC1	Hyper-methylated in cancer-1
HIF-2α	Hypoxia-inducible factor 2 alpha
HNF	Hepatocyte nuclear factor
HSC	Hematopoietic stem cell
hUCB	human umbilical cord blood
ICM	Inner Cell Mass
IGF-1	Insulin growth factor-1
IL-3	Interleukin-3
IL-6	Interleukin- 6
IMDM	Iscove Modified Dulbecco Medium
IP	Immunoprecipitation
JAK	Janus tyrosine associated kinase

Klf-4	Krűppel-like factor 4
KO	Knockout
LIF	Leukemia Inhibitory Factor
LIFR	Leukemia Inhibitory Factor Receptor
LIF-Rβ	Leukemia Inhibitory Factor Receptor Beta
MAPK	Mitogen-Activated Protein Kinase
M-CSF	Macrophage colony-stimulating factor
mES	mouse Embryonic Stem
MTG	monothiglycerol
Myc	Myelocytomatosis oncogene
NAD	Nicotinamide adenine dinucleotide
NAM	Nicotinamide
NAMPT	Nicotinamide phosphoribosyltransferase
NCoR	Nuclear receptor corepressor
NPC	Nuclear progenitor cell
Oct-Sox	Octamer-sox
PFHM	Protein-free hybridoma medium
PGC1-α	Peroxisome proliferator-activated receptor-coactivator
PPARγ	Peroxisome proliferation-activated receptor-γ coactivator-1 (alpha)
P-Sp	Para-aortic splanchnopleura
R	Receptor
Rb	Retinoblastoma protein
RNAi	RNA interference

RSV	Resveratrol
SCF	Stem Cell Factor
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulfate
SHP	Scr Homology Phosphatase
Sir2	Silencing information regulator 2
SIRT	Silent information regulator homologue
SIRT1	Sirtuin1
SMRT	Silencing mediator for retinoid and thyroid hormone receptor
Sox-2	SRY-box containing gene 2
Sp	Somite pair
SSEA-1	Stage specific embryonic antigen-1
STAC	Sirtuin Activating Compounds
STAT-3	Signal Transducer and Activator of Transcription 3
TG	Triglyceride
TGF	Transforming growth factor
UCP	Uncoupling protein
VE	Vascular endothelial
VEGF	Vascular endothelial growth factor
V-P	Ventral-posterior
WAT	White adipose tissues
WT	Wild type

INTRODUCTION

Embryonic Stem (ES) cells are derived from the inner cell mass (ICM) of mouse embryonic day (E) 3.5 blastocysts (Evans and Kaufman 1981; Martin 1981). ES cells have two unique characteristics that distinguish them from all other organ-specific cells. First, they can self-renew, that is they can be maintained and expanded as pure populations of undifferentiated cells for an extended period of time, possibly indefinitely, in culture. Second, they are pluripotent, possessing the capacity to generate every cell type in the body including germ cells. ES cells were initially established and maintained by co-culture with mouse embryonic feeder cells. Subsequently, in the late 1980's a factor in the feeder layer cells was identified that prevented differentiation and promoted stem cell self-renewal. Today this factor is known as Leukemia Inhibitory Factor (LIF), and allows ES cells to grow in the absence of feeder layer cells (Friel, van der Sar et al. 2005). When moved from conditions that maintain them in an undifferentiated state and in suspension culture, ES cells grow into spheres termed embryoid bodies (EBs). Within this context, these cells differentiate into ectoderm, endoderm and mesoderm-derived tissues and give rise to many different somatic cell types (Figure 1). The ability to generate a wide spectrum of differentiated cell types from ES cells in culture offers a powerful approach for studying lineage induction and specification and a promising source of progenitors for cell replacement therapy.

Figure 1

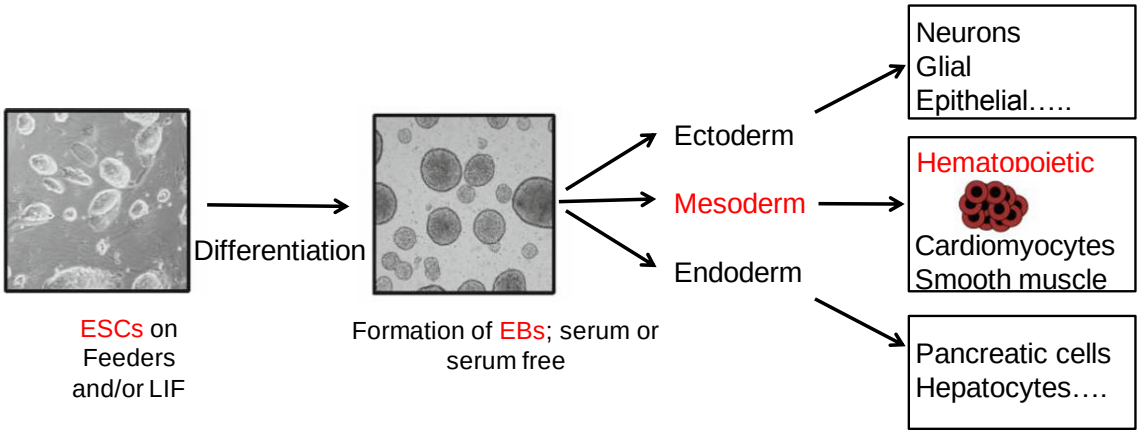

Differentiation
ESCs on Feeders and/or LIF
Formation of EBs; serum or serum free
Ectoderm
Mesoderm
Endoderm
Neurons
Glial
Epithelial…..
Hematopoietic
Cardiomyocytes
Smooth muscle
Pancreatic cells
Hepatocytes….

Figure 1: *In vitro* differentiation of mouse ES cells.

Undifferentiated mouse ES cells develop *in vitro* via three-dimensional aggregates EBs into differentiated cell types of all three primary germ layers. These three germ layers give rise to all organ-specific cell types of our body. Shown are a few examples: neurons, glial, epithelial cells, hematopoietic cells, cardiomyocytes, smooth muscle, pancreatic cells and hepatocytes.

Extracellular signaling that regulates self-renewal of mouse ES (mES) cells

Several soluble factors have been identified that exert either positive or negative effects on ES cell self-renewal. As noted above, LIF, a member of interleukin 6 (IL-6) family is one of those factors and is known to strongly promote self-renewal in ES cells (Smith, Heath et al. 1988). LIF binding to leukemia inhibitory factor receptor (LIFR) on mES cells activates two major pathways, the Janus tyrosine associated kinase (JAK)-signal transducer activator of transcription 3 (STAT3) pathway and the Src homology phosphatase (SHP) 2-Extracellular Signal Regulated Kinase (ERK) pathway (Burdon, Chambers et al. 1999; Burdon, Stracey et al. 1999). LIF binds to LIF-Rβ and activates the JAKs which phosphorylate the SH-2 domain of their downstream target, STAT-3. Once STAT-3 is activated, it undergoes autophosphorylation and forms a STAT-3 homodimer. The STAT-3 dimer then translocates to the nucleus to activate a variety of downstream genes including myelocytomatosis oncogene (Myc, also known as c-Myc) and Krűppel-like factor 4 (Klf-4), which positively control mES cell self-renewal (Niwa, Burdon et al. 1998; Cartwright, McLean et al. 2005).

Another pathway that is activated by LIF is the SHP2-ERK pathway via the gp130 receptor. The LIF-gp130R activates the RAS/mitogen-activated protein kinase (MAPK) pathway. SHP2 associates with Gab1, which causes the activation of Ras, which induces the transactivation of MAPKs and ultimately ERK phosphorylation. This pathway leads to differentiation of murine ES cells (Burdon, Stracey et al. 1999; Burdon, Smith et al. 2002) (Figure 2) (Kristensen, Kalisz et al. 2005).

Besides the LIF-STAT3 signaling pathway, recent studies have implicated BMP-Smad and Wnt-signaling pathways in the maintenance of ES cell pluripotency (Willert and Jones 2006; Wu and Hill 2009).

Figure 2

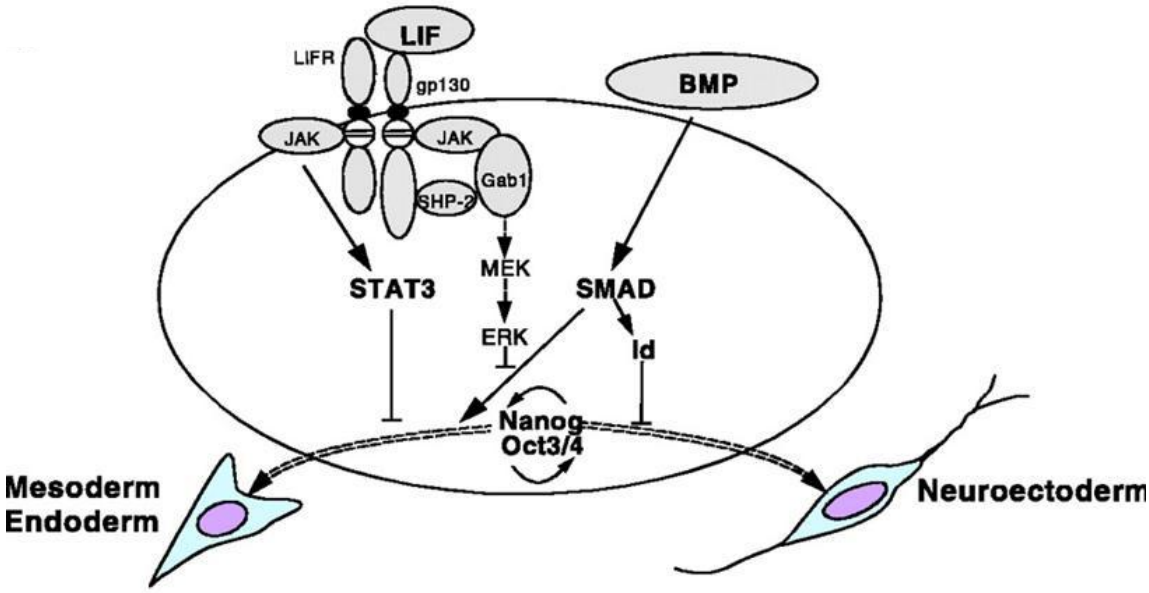

LIF
LIFR
gp130
BMP
JAK
JAK
Gab1
SHP-2
MEK
STAT3
SMAD
ERK
Id
Nanog
Oct3/4
Mesoderm
Endoderm
Neuroectoderm

Figure 2: Regulation of self-renewal in mouse ES cells by Oct3/4, Nanog, BMP-dependent SMAD, and LIF-dependent JAK/STAT3 signaling pathways.

Self-renewal (proliferation) of undifferentiated mouse ES cells is regulated by Nanog, Oct-3/4, and tightly regulated interactions between LIF-dependent JAK/STAT3 pathway(s) and BMP-dependent activation of Id target genes. A MEK-ERK signaling mechanism prevents ES cell self-renewal. Oct-3/4 and Nanog expression prevents differentiation into trophectoderm, primitive endoderm, and mesoderm cells.

Transcription factors controlling self-renewal of mES cells

Extracellular signaling is transduced to the nucleus through transcription factors including STAT3 and MAPK. The factors listed below are part of the transcriptional network and play a central role in maintaining pluripotency.

Oct3/4, also known as Pou5f1, consists of a POU domain as a DNA-binding domain, and two transactivation domains which lie on the N-terminus and the C terminus, respectively (Niwa, Masui et al. 2002). The expression of Oct3/4 is restricted in pluripotent cell lineages such as inner cell mass (ICM) and germ cells *in vivo* and undifferentiated ES cells *in vitro*, where it plays an indispensable role in maintaining pluripotency (Masui 2010). In ES cells, continuous Oct3/4 function at appropriate levels is necessary to maintain pluripotency. A less than two fold decrease in expression causes differentiation into primitive endoderm and mesoderm, while loss of Oct3/4 induces the formation of trophectoderm concomitant with a loss of pluripotency (Niwa, Miyazaki et al. 2000). Oct3/4 regulates a broad range of target genes. From a developmental perspective, the main target is caudal type homeo box2 (Cdx2). Repression of Oct3/4 in ES cells results in differentiation into trophectoderm through upregulation of Cdx2; forced expression of that gene induces differentiation into trophectoderm (Niwa, Toyooka et al. 2005). Oct3/4 has been known to activate downstream genes by binding to enhancers carrying the octamer-sox motif (Oct-Sox enhancer), for synergistic activation with the SRY-box containing gene 2 (Sox2).

Sox2 expression is detected in pluripotent cell lineages and in the nervous system (Avilion, Nicolis et al. 2003). Sox2 consists of a DNA-binding HMG domain and a

transactivation domain that can be divided into three subdomains. The involvement of Sox2 in pluripotency maintenance was first suggested by the occupation of an enhancer consisting of Oct-Sox enhancer in the regulatory region of fibroblast growth factor (FGF) 4. Oct-Sox enhancers are found in the regulatory region of most genes that are specifically expressed in pluripotent stem cells, such as Oct3/4, Sox2, Nanog, Utf1, and Fbx15 (Masui 2010). The primary role of Sox2 in ES cell self-renewal was found to be maintenance of Oct3/4 expression (Masui, Nakatake et al. 2007).

Nanog is also a well-known homeobox transcription factor that is specially expressed in pluripotent cells (Mitsui, Tokuzawa et al. 2003). Nanog consists of three domains: a homeodomain similar to the NK-2 family, which acts as a DNA-binding domain, and the transactivation domains at the N-terminal and C-terminal, through which Nanog dimerizes to exert its full activity (Pan and Pei 2003; Wang, Levasseur et al. 2008). Nanog null embryos at E (embryonic day) 5.5 show disorganization of extraembryonic tissues with no discernible epiblast or extraembryonic ectoderm (Mitsui, Tokuzawa et al. 2003; Okita and Yamanaka 2006). Nanog deficient blastocysts appear normal, but the ICM fails to give rise to epiblasts and only produces parietal endoderm-like cells when cultured *in vitro*. Similarly, mES cells lacking Nanog preferentially differentiate into extraembryonic endoderm lineages even in the presence of LIF, by repression of GATA-binding protein 6 (Gata-6). In the blastocyst, Gata-6 is first expressed in some cells of the ICM, most of which differentiate into extra-embryonic endoderm cells. Interestingly, overexpression of Nanog allows mES cells to self-renew without LIF. Thus, Nanog blocks primitive endoderm differentiation and actively maintains pluripotency (Okita and Yamanaka 2006).

Klf4 was initially identified as a Klf family member expressed in the gut (Shields, Christy et al. 1996). It contains C2H2-type zinc finger motifs. Klf4-null mice die within 15 hours after birth due to a skin barrier defect caused by a perturbation in the differentiation of the epidermis (Segre, Bauer et al. 1999). A differentiation defect is also observed in the goblet cells of the colon in these knockout mice (Katz, Perreault et al. 2002), indicating the indispensable function of Klf4 in the proper development of these organs. In ES cells, Klf4 participates in the activation of an Oct-Sox enhancer in cooperation with Oct3/4 and Sox2 (Nakatake, Fukui et al. 2006). Overexpression of Klf-4 was found to prevent differentiation in embryoid bodies formed in suspension culture, suggesting that Klf4 contributes to ES self renewal (Li, McClintick et al. 2005). Klf4 in combination with Oct3/4, Sox2 and Myc can reprogram somatic cells into pluripotent stem cells (Nakagawa, Koyanagi et al. 2008).

The ES cell property of self-renewal therefore depends on a stoichiometric balance among various signaling molecules. Other molecular markers potentially defining the molecular basis of stem cell identity or "stemness" include Rex-1 (Hosler, Rogers et al. 1993), CD9, and stage specific embryonic antigen-1 (SSEA-1) and mES cells possess enzyme activities for alkaline phosphatase (ALP) (Wobus, Holzhausen et al. 1984) and telomerase (Armstrong, Lako et al. 2000). These markers are summarized in Table 1 (Wobus and Boheler 2005).

Table 1: Markers and properties of mouse embryonic stem cells

Marker	mES cells
SSEA-1	+
SSEA-3/-4	-
TRA-1-60/81	--
TRA-2-54	-
GCTM-2	-
TG 343	?
TG 30	?
CD 9	+
CD 13/prominin	+
Alkaline phosphatase	+
Oct-4	+
Nanog	+
Sox-2	+
FGF4	+
LIF receptor	+
Telomerase activity	+
Regulation of self-renewal	Via gp 130R, MEF feeder layer
Growth characteristics in vitro	Tight, rounded, multiple layers
EB formation	Simple and cystic EBs
Teratoma formation in vitro	+

Table1: Markers and properties of mouse embryonic stem cells.

Identification of cell surface and molecular markers has proven critical to define the molecular basis of stem cell identity or "stemness". It is now well established that undifferentiated mES cells express specific cell surface antigens SSEA-1 and possess enzyme activities for alkaline phosphatase. The fundamental characteristics of murine ES cells such as Oct-4 expression, telomerase activity, and the formation of teratomas containing derivatives of all three primary germ layers in immunodeficient mice are summarized in the table.

In vitro differentiation potential of embryonic stem cells

There is considerable interest in therapeutic applications of pluripotent cells derived from embryos. Much effort is being directed toward understanding the mechanisms for maintenance of the pluripotent state, as well as the pathways leading to lineage specification. During mouse embryogenesis, the primitive endoderm of the epiblast gives rise to three primary germ layers: the ectoderm, the mesoderm and the definitive endoderm. These germ layers interact to form all tissues and organs of the developing embryo. The complex interactions that control the transition of ectoderm to visceral and parietal endoderm in the postimplantation embryo, followed by the formation of mesoderm at the gastrulation stage (days 3 to 7 post coitum), are only beginning to be defined. The *in vitro* differentiation potential of mES cells has facilitated the examination of these processes (Wobus and Boheler 2005).

In the absence of feeder layer cells and removal of LIF, ES cells differentiate into EBs. Within the EBs, it consists of derivatives of the three embryonic germ layers: mesoderm, endoderm and ectoderm (Keller 1995; Smith 2001), similar to those generated during mouse embryogenesis. Three general approaches, outlined in Figure 3, are used to initiate ES cell differentiation. In the first method, ES cells are allowed to aggregate and form three-dimensional colonies known as EBs (Doetschman, Eistetter et al. 1985; Keller 2005). In the second method, ES cells are cultured directly on stromal cells, and differentiation takes place in contact with these cells. The most commonly used stromal cell line for such differentiation studies is OP9, originally isolated from the calvarium of CSF-1-deficient *op/op* mice (Niida, Kondo et al. 2005). The third protocol involves differentiating ES cells in a monolayer on extracellular matrix proteins. The three

differentiation methods have been used to generate a broad spectrum of cell types from ES cells. The first method is the one most used for differentiation of mES cells *in vitro* in our studies. Among three germ layers, mesoderm-derived lineages, including the hematopoietic, vascular, and cardiac, are the easiest to generate from ES cells and have been studied in considerable detail (Keller 2005). Of these, hematopoietic development is the best characterized (Keller 2005). Evidence from several different studies has demonstrated the developmental kinetics of various hematopoietic lineage precursors within EBs. The sequence of events leading to the onset of hematopoiesis within EBs is similar to that found within the mouse embryo. Thus, *in vitro* differentiation of ES cells to hematopoietic cells provides a unique opportunity to study mechanisms involved in the onset of hematopoietic development, and to characterize hematopoietic lineage-specific gene expression.

Figure 3

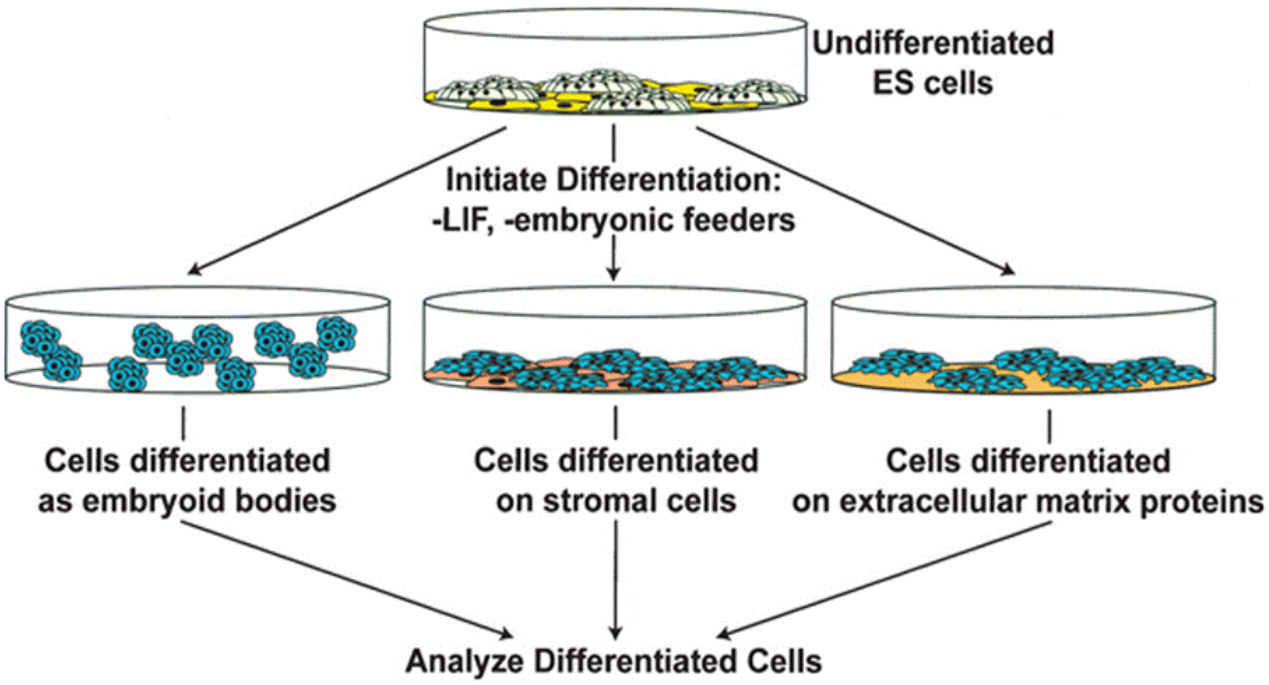

Undifferentiated
ES cells
Initiate Differentiation:
-LIF, -embryonic feeders
Cells differentiated
as embryoid bodies
Cells differentiated
on stromal cells
Cells differentiated
on extracellular matrix proteins
Analyze Differentiated Cells

Figure 3: Three different protocols used for ES cell differentiation.

The three methods have been used to generate a broad spectrum of cell types from ES cells (Keller 1995; Smith 2001). For lineages that have been studied in detail, the first two criteria outlined above have been met as efficient protocols for their differentiation have been established and the sequence of events leading to their development in culture was found to faithfully recapitulate those in the early embryo. The third aim has yet to be fulfilled for most populations.

Hematopoietic development in the mouse embryo

Hematopoiesis in the early mouse embryo initiates at two independent sites, the yolk sac and an intraembryonic region known as the para-aortic splanchnopleura (P-Sp)/aorta-gonad-mesonephros (AGM). Hematopoietic commitment is detected first in the yolk sac, where distinct blood islands appear, shortly following gastrulation (Moore and Metcalf 1970). The yolk sac blood islands consist of two lineages, a population of erythroid cells surrounded by a layer of angioblasts that eventually form the developing vasculature. The parallel temporal development of these lineages in physical proximity provided the basis for the hypothesis that they arise from a common precursor, a cell called the hemangioblast (Keller, Lacaud et al. 1999; Kubo, Chen et al. 2005). The erythroid cells within these blood islands are known as embryonic or primitive erythrocytes (Keller, Lacaud et al. 1999). They are distinct from fetal and adult erythrocytes in that they are large, circulate in the bloodstream as nucleated cells for much of their life span, and contain an embryonic form of hemoglobin (Barker 1968; Brotherton, Chui et al. 1979). The primitive erythroid population represents the major mature hematopoietic component of the yolk sac and is restricted to the yolk sac between the primitive streak and 20 somite pair (sp) stage of development (Haar and Ackerman 1971; Palis, Robertson et al. 1999). One study suggests that these precursors are present in the yolk sac for a relatively short time, possibly no more than 48 hours, indicating that the entire primitive erythroid population could be produced in one rapid and synchronous wave of development (Wong, Chung et al. 1986; Keller, Lacaud et al. 1999). The other mature hematopoietic cells present in the yolk sac are macrophages as well as some megakaryocytes (Cline and Moore 1972; Xu, Matsuoka et al. 2001). The erythroid and megakaryocyte lineages are

tightly associated during development as a primitive bipotential megakaryocyte-erythroid progenitor has been detected in the mouse yolk sac (Klimchenko, Mori et al. 2009). Given the predominance of primitive erythropoiesis, this stage of hematopoietic development is known as primitive hematopoiesis. Primitive hematopoietic progenitors cannot fully recapitulate hematopoiesis in an adult undergoing transplantation. The second wave of hematopoiesis is termed definitive hematopoiesis (Palis, Robertson et al. 1999). Although initiated in this extra-embryonic region, definitive hematopoiesis is most often associated with intra-embryonic sites such as the P-Sp, AGM (Godin, Dieterlen-Lievre et al. 1995; Lacaud, Gore et al. 2002). Definitive hematopoiesis encompasses the development of all lineages other than primitive erythroid, and includes definitive erythroid, myeloid and lymphoid cells. Definitive erythroid cells are distinguished from primitive erythroid cells by the fact that they are small, enucleated and produce adult forms of globin (Barker 1968; Brotherton, Chui et al. 1979). Definitive embryonic hematopoietic progenitors can restore hematopoiesis in an adult undergoing transplantation.

In vitro differentiation of mES cells: a model for embryonic hematopoiesis

Although yolk sac blood islands were identified as the earliest site of hematopoietic and endothelial development, attempts to identify, isolate and characterize the precursors representing these initial stages of lineage commitment, including the elusive hemangioblast, have been largely hampered by the inaccessibility of the early mammalian embryo. One promising alternative approach to study early hematopoietic development is the model system based on differentiation of ES cells in culture (Keller 1995; Kyba, Perlingeiro et al. 2003). When induced to differentiate in culture, ES cells

form colonies known as EBs that contain three primary germ cell layers including mesoderm. Primitive erythroid precursors are the first hematopoietic population to develop from mesodermal progenitors and can be detected as early as day 4.0 upon differentiation of mES cells (Keller, Kennedy et al. 1993). Using the ESC/EB system, a precursor has been identified that is generated right after mesodermal precursors that responds to vascular endothelial growth factor (VEGF) and generates colonies consisting of blast cells. These VEGF-responsive blast cell colonies were shown to be able to generate endothelial, primitive erythroid and various definitive hematopoietic precursors. Shortly after the onset of primitive erythropoiesis, definitive precursors were detected in the following order: macrophage by day 4.0-4.5, definitive erythroid by day 4.5-5.0, and mast cell by day 8-9. Detailed analysis of the early stages of hematopoietic cell commitment has shown that both gene expression patterns and the kinetics of the lineage development within EBs are similar, if not identical, to those in the early yolk sac (Choi, Kennedy et al. 1998; Sroczynska, Lancrin et al. 2009). This unique advantage has been fully exploited to investigate the earliest stages of hematopoietic and endothelial cell commitment (Figure 4) (Keller, Lacaud et al. 1999; Keller 2005).

Hemangioblast and hemogenic endothelium

The ES differentiation model has also been exploited to test a long-standing hypothesis that the hematopoietic and endothelial lineages developed from a common progenitor, a cell known as the hemangioblast. Although this hypothesis was proposed many years ago, the identification, isolation and characterization of a precursor with these characteristics has eluded developmental biologists. Nevertheless, the concept of the hemangioblast has gained support from a variety of different experiments. First, analysis of early-stage,

carefully timed EBs led to identification of a progenitor known as the blast-colony-forming cell (BL-CFC), that gives rise to blast-colonies consisting of hematopoietic and vascular progenitors in methylcellulose culture in the presence of VEGF (Kennedy, Firpo et al. 1997) (Figure 3). BL-CFCs represent a transient population that appears within EBs before the establishment of other hematopoietic lineages. The development potential of the BL-CFC strongly suggests that it represents the *in vitro* equivalent of the hemangioblast and, as such, the earliest stage of hematopoietic and endothelial commitment. Second, many studies have demonstrated that developing hematopoietic and endothelial cells express a number of genes in common, including CD34, Flk-1, Flt-1, Tie2, Scl/Tal-1, GATA-2 and PECAM-1(Baron 2003; Cumano and Godin 2007). Third, gene-targeting studies have shown a complete absence of hematopoietic and endothelial development in the yolk sac and P-Sp/AGM of embryos lacking a functional Flk-1 receptor tyrosine kinase (Shalaby, Rossant et al. 1995; Shalaby, Ho et al. 1997; Keller, Lacaud et al. 1999) and a marked defect in the growth and development of both lineages in the yolk sac of embryos lacking transforming growth factor (TGF) β1 (Dickson, Martin et al. 1995; Keller, Lacaud et al. 1999). Fourth, studies on zebrafish have identified a mutation known as *cloche* that affects the development of hematopoietic lineages and endocardium in the embryo (Stainier, Weinstein et al. 1995). Fifth, Nishikawa et al. demonstrated the presence of precursors with hematopoietic and endothelial potential in *Flk-1*$^{+}$ populations isolated from differentiating ES cells (Nishikawa, Nishikawa et al. 1998).

Figure 4

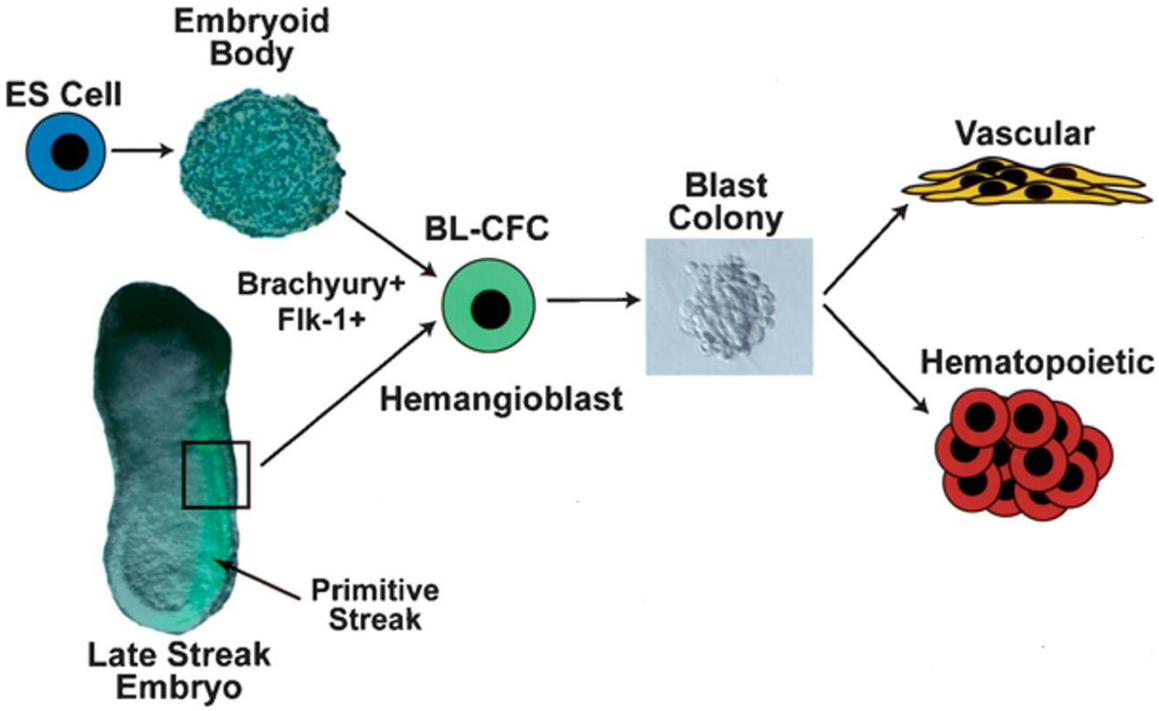

Embryoid
Body
ES Cell
BL-CFC
Brachyury+
Flk-1+
Hemangioblast
Blast
Colony
Vascular
Hematopoietic
Primitive
Streak
Late Streak
Embryo

Figure 4: A comparison of BL-CFC development in EBs to hemangioblast development in the early mouse embryo.

The EB and embryo are derived from an ES cell line in which the GFP cDNA has been targeted to the brachyury locus (Fehling et al. 2003; Huber et al. 2004). The presence of GFP in the EB and the primitive streak of the embryo is indicative of brachyury expression.

On the other hand, a newer theory proposes that hematopoietic stem cells form from a subset of early endothelial cells known as hemogenic endothelium (Figure 5). Nishikawa et al. isolated vascular endothelial (VE)-cadherin^{+} cells from either the yolk-sac or P-Sp/AGM region of the embryo proper and demonstrated multilineage hematopoietic potential in these populations (Nishikawa, Nishikawa et al. 1998). As VE-cadherin is thought to be endothelial specific, these investigators concluded that these hematopoietic cells develop from a population of endothelial cells they referred to as hematogenic endothelium, a population that is presumably the functional equivalent of the hemangioblast. Conditional *runx1* deletion in cells expressing TEK/Tie-2, a receptor for angiopoietin-1 expressed in endothelial cells, resulted in embryonic lethality similar to runx1-deficient embryos (Li, Chen et al. 2006; Yoshimoto, Porayette et al. 2008), supporting a role for hemogenic endothelium in putative HSC emergence. The relationship between hemangioblasts and hemogenic endothelium has not been resolved. Recently, several papers have shed light on the potential relatedness and significance of these cell types. Lancrin et al. analyzed the sequence of cellular events required for the formation of mature blast colonies from cultured *Flk-1*$^{+}$ cells by using time-lapse photography (Lancrin, Sroczynska et al. 2009; Yoshimoto and Yoder 2009). They found that these colonies formed in two stages. First, after 36-48 hours of 'plating' *Flk-1*$^{+}$ cells for growth in culture, the cells form tightly adherent clusters. Subsequently, round, non-adherent cells appear which then proliferate to complete the formation of mature blast colonies. Among the adherent cell clusters at 48 hours, a transient cell population expressing various endothelial (but not mesoderm or BL-CFC) markers appears, displaying potential to form hematopoietic cells. From this population, both primitive and

definitive hematopoietic progenitors formed (Yoshimoto and Yoder 2009). The observations of Lancrin and colleagues (Lancrin, Sroczynska et al. 2009) suggest that hematopoietic progenitor cells arise from hemangioblasts through a hemogenic endothelial intermediate-the first linear pathway to resolve, at least *in vitro*, the relationship between hemangioblasts and hemogenic endothelium (Yoshimoto and Yoder 2009).

By continuous long-term single-cell observation of mouse mesodermal cells generating endothelial cell and blood colonies, Eilken et al. showed that it was possible to detect hemogenic endothelial cells giving rise to blood cells (Eilken, Nishikawa et al. 2009). The authors also directly isolated primary endothelial cells with hemogenic potential to form early mouse embryos. This demonstrates that hemogenic endothelial cells not only can be generated *in vitro* from ES cells but are also naturally present in mouse embryos (Yoshimoto and Yoder 2009). Furthermore, Chen et al. showed that most fetal liver and adult bone-marrow cells originate from the hemogenic endothelium (Chen, Yokomizo et al. 2009). Utilizing combinations of fluorescent reporter transgenes, confocal timelapse microscopy and flow cytometry, Bertrand et al. have directly imaged the birth of HSCs from the ventral wall of the dorsal aorta in the zebrafish embryo (Gerard, Bleyzac et al. 2010). Their results, as well as previous studies in the avian (Jaffredo, Gautier et al. 1998), amphibian (Ciau-Uitz, Walmsley et al. 2000), and mammalian embryo, discussed above suggest that the cellular mechanisms of HSC generation have been highly conserved across vertebrate evolution. The finding that HSC development requires transition through a hemogenic endothelial intermediate should aid efforts to instruct

HSC formation *in vitro* from pluripotent precursors, a necessity for therapies designed to replace adult blood cell lineages.

Figure 5

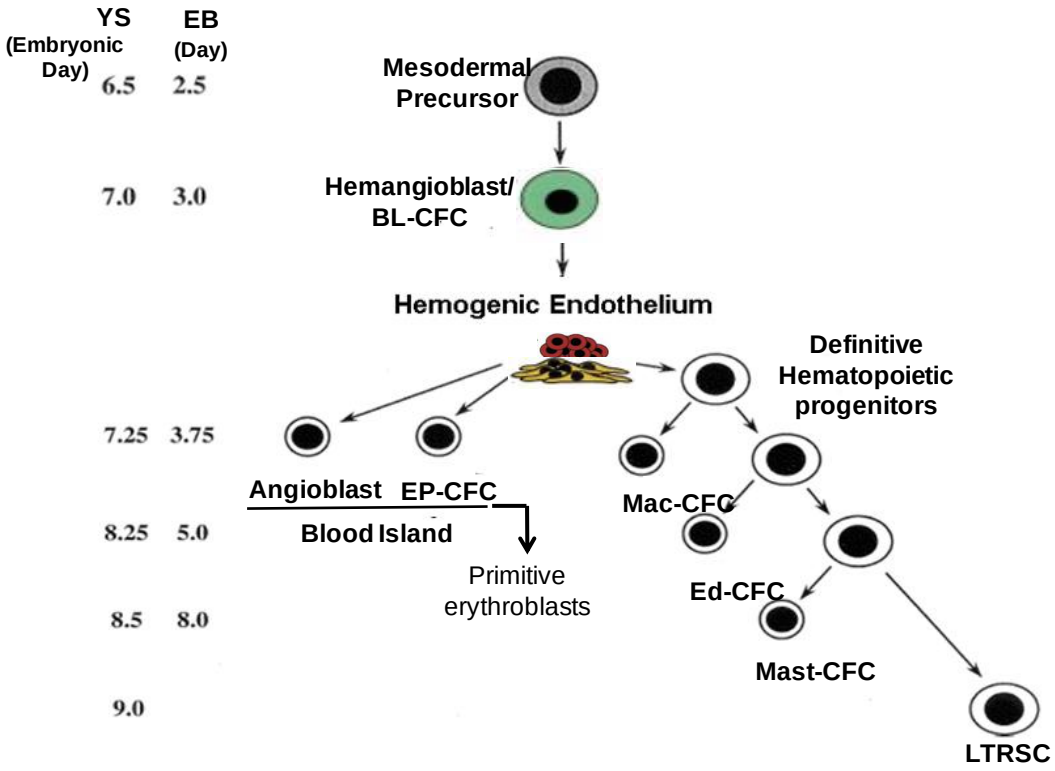

YS
(Embryonic Day)
EB
(Day)
6.5
2.5
7.0
3.0
7.25
3.75
8.25
5.0
8.5
8.0
9.0
Mesodermal Precursor
Hemangioblast/ BL-CFC
Hemogenic Endothelium
Definitive Hematopoietic progenitors
Angioblast
EP-CFC
Blood Island
Primitive erythroblasts
Mac-CFC
Ed-CFC
Mast-CFC
LTRSC

Figure 5: Model of hemangioblast differentiation towards hematopoiesis in the yolk sac.

The first blood cell emerged from mesodermal progenitor cells. Mesodermal progenitor cells further differentiate into hemogenic endothelium, which is likely involved in primitive hematopoiesis and definitive hematopoiesis as well as vasculogenesis. There are two waves of hematopoiesis within the mouse. The first wave of hematopoiesis has been termed as primitive hematopoiesis given the predominance of primitive erythropoiesis in this stage. All lineages other than primitive erythroid, and early macrophage and megakaryocyte will be considered as belonging to the definitive hematopietic system.

Genes important in the development and regulation of the hemangioblast

Although there is evidence that HSC can be generated from hemogenic endothelium, a question still remaining to be answered is whether HSCs emerge directly from hemogenic endothelial cells and whether the hemangioblast exists *in vivo*. As we wait for the answer, the following describes several genes important in the development and regulation of the hemangioblast.

Flk-1

Flk-1 is a receptor tyrosine kinase that binds VEGF. In primitive streak–stage embryos, Flk1 expression is first detectable in cells within and exiting the primitive streak as well as the extraembryonic mesoderm (Yamaguchi, Dumont et al. 1993; Ema, Takahashi et al. 2006). Subsequently, it can be detected in the endothelial cells lining the blood islands of the extraembryonic yolk sac, the first site of embryonic hematopoiesis, by E 7.5 (Lugus, Park et al. 2009). Flk-1 is an important marker required for functional hemangioblast formation and is required for blood and vessel formation. Mice deficient in the Flk-1 receptor tyrosine kinase die *in utero* because of defective vascular and hematopietic development (Shalaby, Rossant et al. 1995). Both primitive and definitive blood cells are derived from *Flk-1*$^{+}$ mesoderm (Lugus, Park et al. 2009). *Flk-1*$^{-/-}$ ES cells are nevertheless able to differentiate into hematopoietic and endothelial cells *in vitro*, but they give rise to a greatly reduced number of blast colonies. In addition, *Flk-1*$^{+}$ but not *Flk-1*$^{-}$ cells derived from ES cells generate T lymphocytes when cocultured with lymphocyte-depleted thymic lobes (de Pooter, Cho et al. 2003), and *Flk-1*$^{+}$ cells differentiated from ES cells *in vitro* are able to reconstitute the hematopoietic systems *in vivo* in severe combined immunodeficient (SCID) mice (Miyagi, Takeno et al. 2002). Cell tracking

studies have indicated that the Flk-1 receptor is initially involved in migration of mesodermal precursor cells to the extraembryonic region of the embryo, the site of yolk sac development. These migrating precursors may be similar to the EB-derived BL-CFCs that have been shown to express Flk-1 and to grow in response to its ligand, VEGF (Irion, Clarke et al. 2010).

Scl/Tal-1

Scl (also known as Tal-1 for T-cell acute leukemia), a member of the helix-loop-helix family of transcription factors, functions at a slightly later developmental stage than Flk-1. The yolk sacs of *Scl*$^{-/-}$ embryos develop a primary vascular network but contain no primitive or definitive hematopoietic cells, indicating a pivotal role for Scl in hematopoietic commitment but not in the early stages of vasculogenesis (Robb, Lyons et al. 1995). Although not essential for establishment of the endothelial lineage, Scl does appear to be required for remodeling and maturation of the vascular system (Visvader, Fujiwara et al. 1998). *In vitro* differentiation studies with *Scl*$^{-/-}$ ES cells show a complete block in primitive and definitive hematopoietic potential, confirming the developmental defect observed *in vivo* (Robertson, Kennedy et al. 2000). Further analysis of the *Scl*$^{-/-}$ EBs reveals a defect as early as the BL-CFC because mutant ES cells were unable to generate blast cell colonies. *Scl*$^{-/-}$ ES cells were, however, able to generate precursors that give rise to transitional colonies that represent a stage of development slightly earlier than the blast cell colony (Robertson, Kennedy et al. 2000). These colonies, named transitional colonies, are also generated by WT ES cells and are easily distinguished by morphological criteria from blast cell colonies and secondary EBs that develop in these replated colonies (Lacaud, Robertson et al. 2001). Functional studies support this

interpretation and demonstrate that BL-CFC, endothelial precursors and primitive and definitive hematopoietic precursors do develop into transitional colonies. Transitional colonies generated from *Scl*$^{-/-}$ EBs have endothelial precursors, but no hematopoietic potential (Lacaud, Robertson et al. 2001). Recently, Lancrin et al. demonstrated that the transcription factor Scl is indispensable for the establishment of hemogenic endothelium population whereas the core binding factor Runx1 (also known as AML1) is critical for generation of definitive hematopoietic cells from hemogenic endothelium (Lancrin, Sroczynska et al. 2009).

Runx1

The AML1 gene (recently renamed Runx1) encoding the DNA-binding subunit of a transcription factor of the core binding factor (CBF) family, is required for establishment of definitive but not primitive hematopoiesis at the hemangioblast stage of development (Lacaud, Gore et al. 2002). *Runx1*$^{-/-}$ embryos develop normal blood islands and progress through the yolk sac phase of hematopoiesis, but die between E11 and E12.5 (Wang, Stacy et al. 1996). Before death, the liver rudiment contains primitive nucleated erythrocytes but lacks all definitive erythroid, myeloid, and megakaryocytic cells, indicating a complete block in development of the definitive hematopoietic program. Analysis of *Runx1*$^{-/-}$ EBs revealed a similar block in definitive hematopoietic potential (Lacaud, Gore et al. 2002). In addition to hematopoietic abnormalities, *Runx1*-deficient animals show extensive central nervous system hemorrhage and necrosis, suggesting an additional vascular defect (Wang, Stacy et al. 1996). Recently, two papers showed that Runx1 functions during the transition from 'hemogenic endothelium' to HSCs and Runx1

function is essential in endothelial cells for hemogenic progenitor and HSC formation from the vasculature (Chen, Yokomizo et al. 2009; Lancrin, Sroczynska et al. 2009).

Background on Sirtuins

Mammalian sirtuins

Sirtuins in mammals were named based on their homology to the *Saccaromyces cerevisiae* gene silencing information regulator 2 (Sir2). The sirtuins consist of seven members, designated silent information regulator homologue (SIRT) 1 through SIRT7 (Blander and Guarente 2004). They share significant sequence homology and contain a nicotinamide adenine dinucleotide (NAD^+)-dependent catalytic core domain, which may act as either a mono-ADP-ribosyl transferase (ART), a NAD^+-dependent deacetylase (DAC), or both (North, Marshall et al. 2003). The N-and/or C-terminal sequences are of variable lengths between different sirtuins. Each sirtuin also has a different localization and function. SIRT1, SIRT6 and SIRT7 are predominately in the nucleus. SIRT2 is predominately cytoplasmic. SIRT3, 4 and 5 are mainly mitochondrial proteins. (Cohen, Miller et al. 2004; Hallows, Lee et al. 2006). Although SIRT1 was initially known as a nuclear protein, it has recently been found to shuttle between the nucleus and the cytoplasm to manifest its function (Tanno, Sakamoto et al. 2007; Iwahara, Hisahara et al. 2009). Of the sirtuin family members, SIRT1 is the most well studied, and has been shown to participate in a number of processes including metabolism, cell survival, chromatin remodeling and differentiation (Anastasiou and Krek 2006; Mantel, Wang et al. 2008; Wang, Sengupta et al. 2008; Finkel, Deng et al. 2009; Haigis and Sinclair 2010). Consistent with its diverse biological functions, SIRT1 regulates several transcription factors including FOXO factors, PGC-1α, p53 and Ku70 (Blander and Guarente 2004; Rodgers, Lerin et al. 2005). Other Sirtuin family members (SIRT2-SIRT7) also play

important roles in cell cycle control, DNA repair, and rDNA transcription (Table 2) (Frye 2000; Haigis and Sinclair 2010).

Table 2: Summary of mammalian sirtuins

Sirtuin	Locations	Interactions	Biology
SIRT1	Nucleus	FOXO, PGC-1α, NF-kβ, Ku70, etc	Metabolism, stress
SIRT2	Cytosol	Tubulin, H4, FOXO	Cell cycle
SIRT3	Mitochondria	AceCS2, GDH	Thermogenesis ATP production
SIRT4	Mitochondria	GDH, IDE, ANT	Insulin secretion
SIRT5	Mitochondria	CPS1	Urea cycle
SIRT6	Nucleus	Histion3, NF-kβ	Base excision repair, metabolism
SIRT7	Nucleus	Pol I	rDNA transcription

Adapted from T Finkel *et al. Nature* **460**, 587-591 (2009) doi:10.1038/nature08197

Table 2: Cellular distribution, target proteins and functions of mammalian sirtuins

The mammalian Sir2 gene family is comprised of seven members. Each is defined by a conserved core domain, and some contain additional N- or C-terminal sequences. SIRT1 interacts with, deacetylases a growing number of proteins, and plays key function in, but not limited to, metabolism, stress and aging. Other family members and their functions are also listed in above table.

SIRT1 Function in Aging

The initial identification of SIRT1 (yeast SIR2) as a lifespan regulator was in a genetic screen to isolate long-living mutants in yeast *Saccharomyces cerevisiae* (Kennedy, Austriaco et al. 1995). Kennedy et al. found that a SIR4 mutant extended yeast lifespan by measuring the number of cell divisions undergone by the yeast mother cell (Kennedy, Austriaco et al. 1995). Later, it was shown that deletion of either SIR3 or SIR4 results in a moderate shortening in lifespan, and SIRT1 (yeast SIR2) mutations severely reduce lifespan (Kaeberlein, McVey et al. 1999). The life span shortening by SIR2 mutants was due to an increase in homologous recombination at the rDNA loci, which results in the formation of an extrachromosomal rDNA circle (ERC) in the nucleolus. Accumulation of ERCs lead to cell cycle arrest, cellular senescence, and aging (Hutchin, Heath et al. 1997). SIR2 represses recombination at the rDNA locus and prevents generation of ERCs, thus slowing down the aging process (Kaeberlein, McVey et al. 1999).

Regulation of lifespan by SIRT1 has been shown to be conserved in a wide range of organisms (Tissenbaum and Guarente 2001; Astrom, Cline et al. 2003; Rogina and Helfand 2004; Hashimoto, Horikawa et al. 2010). Tissenbaum & Guarente reported over-expression of Sir2.1, the worm SIRT1 homolog, significantly extended life span for *Caenorhabditis elegans* (Tissenbaum and Guarente 2001). In *Caenorhabditis elegans*, mutations that reduce the activity of an insulin-like receptor *daf-2* or a phosphatidylinositol-3-OH kinase *age-1* favour entry into the dauer state during larval development and extend lifespan in adults. Downregulation of this pathway activates a forkhead transcription factor *daf-16*, which may regulate targets that promote dauer formation in larvae and stress resistance and longevity in adults. Recently, Hashimoto T

et al. have shown that nicotinamide adenine dinucleotide extends the lifespan of *Caenorhabditis elegans* mediated by *sir-2.1* and *daf-16* (Hashimoto, Horikawa et al. 2010). Similarly, in the fly, *drosophila melanogaster*, an extra copy of the dSir2 gene extends the lifespan of flies by up to 29% through a pathway related to caloric restriction (CR) (Rogina and Helfand 2004). Astrom et al. found that a dSir2 knockout fly has wild type viability, developmental rate, and sex ratio. However, they showed that dSir2 knockout results in shortening of fly life span (Astrom, Cline et al. 2003).

Involvement of mammalian sirtuins in determining organismal life has been extensively investigated. Increasing evidence has suggested that in the complex mammalian system sirtuins may affect aging by regulating multiple independent processes such as glucose homeostasis, insulin secretion, and stress resistance (Luo, Nikolaev et al. 2001; Vaziri, Dessain et al. 2001; Brunet, Sweeney et al. 2004; Cohen, Miller et al. 2004; Motta, Divecha et al. 2004; Picard, Kurtev et al. 2004; Moynihan, Grimm et al. 2005; Rodgers, Lerin et al. 2005; Bordone, Motta et al. 2006; Nayagam, Wang et al. 2006). Some reports have attempted to look at the direct effects of SIRT1 in mammalian tissues and organ longevity (Alcendor, Gao et al. 2007). Using transgenic mice with heart-specific overexpression of SIRT1, it was shown that low to moderate overexpression of SIRT1 attenuates the age-dependent increase in cardiac hypertrophy, apoptosis/fibrosis, cardiac dysfunction, and expression of senescence markers (Alcendor, Gao et al. 2007), suggesting that SIRT1 could retard aging and confer stress resistance to the heart at low to moderate doses (Alcendor, Gao et al. 2007). Numerous studies have shown that caloric restriction promotes life span extension by up to 50% in a wide range of organisms, including yeast, worms, flies, and mice indicating the role of adipose tissue in life span. It

has been documented that decreased adiposity in fat-specific insulin receptor knockout mice leads to increased lifespan. Interestingly, SIRT1 plays a critical role in fat storage and adipogenesis in mammals, thereby linking adipose regulation of longevity to SIRT1 (Ghosh, Spencer et al. 2007). Calorie restriction (CR) refers to a dietary regime, low in calories without nutrition. SIRT1 null mice have been used to provide evidence that SIRT1 mediates aspects of CR. SIRT1 overexpressing transgenic mice displayed some phenotypes similar to mice on a calorie-restricted diet such as leaner body mass, higher metabolic activity, reduced blood cholesterol, adipokines, insulin and fasted glucose levels and higher glucose tolerance than littermate controls. Furthermore, transgenic mice performed better on a rotating rod challenge and also showed a delay in reproduction (Bordone, Cohen et al. 2007). Recently, life span of SIRT1 null mice has been measured. SIRT1 null mice have a shorter life span than do their WT littermates, and CR does not increase the life span of these animals, providing some direct evidence that SIRT1 regulates aging by CR in mammals. However, firm conclusions are complicated by the fact that SIRT1 null mice that survive to adulthood die prematurely and have developmental defects. Life span studies that use SIRT1 tissue-specific knockout mice may ascertain which tissue drives the developmental defects (Haigis and Sinclair 2010).

SIRT1 function in metabolic regulation

Glucose metabolism is a major regulator of cellular energy state and a critical component of the physiology of CR. Organisms with CR mediated longevity show increased insulin sensitivity and corresponding reduction in glucose concentration and insulin levels. SIRT1 has been shown to regulate glucose homeostasis in two tissues (Ghosh, Spencer et al. 2007). One is in the pancreas, associated with a physiological role of SIRT1 in insulin

secretion (Moynihan, Grimm et al. 2005; Bordone, Motta et al. 2006). Another is SIRT1 function in glucose metabolism in the liver (Frescas, Valenti et al. 2005).

Two independent studies have shown a role for SIRT1 in regulation of insulin secretion in the pancreas. Pancreatic β-cells play a central role in maintaining glucose homeostasis in mammals by secreting insulin in response to elevated blood glucose levels. β-cells have a highly coordinated mechanism that senses glucose and transduces its signal to insulin secretion. In the murine pancreas, SIRT1 is preferentially expressed in β-cells (Moynihan, Grimm et al. 2005). Moyhihan et al. have shown that specific overexpression of SIRT1 in β-cells enhanced glucose-stimulated insulin secretion in transgenic mice and hence improved their glucose tolerance (Moynihan, Grimm et al. 2005). Conversely, knockdown of SIRT1 in β-cell lines compromised insulin secretion. SIRT1 achieved this effect at least partially by repressing transcription of the uncoupling protein (UCP)-2 by directly binding to the UCP-2 promoter (Moynihan, Grimm et al. 2005). Uncoupling protein-2 is a mitochondrial inner-membrane protein that uncouples oxygen consumption during respiration from ATP production by allowing proton leakage down an electrochemical gradient from cytoplasm to mitochondria. Hence, decreased UCP-2 expression mediated by SIRT1 leads to better coupling of mitochondrial respiration and ATP synthesis, which enhances ATP production and thereby sensitizes β-cells to blood glucose level. This is reminiscent of the phenotype of *UCP-2*$^{-/-}$ mice, which exhibited higher ATP levels in pancreatic islets and increased insulin secretion in response to glucose stimulation, whereas over-expression of UCP-2 in cultured β-cell lines reduces ATP levels and glucose-stimulated insulin secretion (Zhang, Baffy et al. 2001). Bordone

et al. have shown that *SIRT1*$^{-/-}$ mice have low level of blood insulin and UCP-2 levels are increased in *SIRT1*$^{-/-}$ mice and SIRT1-knockdown cells (Bordone, Motta et al. 2006).

Liver is another organ that is important in maintaining glucose and lipid homeostasis. SIRT1 has been shown to regulate gluconeogenesis in liver by two mechanisms, one involving the FOXO1 protein and the other through PGC1-α. SIRT1 deacetylases the transcription factor FOXO1. Subsequently, the enhanced expression of FOXO-1 target genes leads to activation of gluconeogenesis and increased glucose release from hepatocytes (Frescas, Valenti et al. 2005). In addition, SIRT1 also interacts with and deacetylases transcriptional co-activator PGC1-α in a ternary complex with hepatocyte nuclear factor (HNF-4) to induce gluconeogenesis following fasting (Rodgers, Lerin et al. 2005).

Overall, the above evidence suggests an impact of SIRT1 function on body glucose homeostasis through its role in insulin secretion in the pancreas and gluconeogenesis in the liver. Thus the activity of SIRT1 may be a relevant therapeutic target for diabetes.

SIRT1 function in lipid metabolism

Another tissue involved in metabolic regulation is adipose tissue, a major site of triglyceride (TG) storage, which is an important energy source when glucose is scarce in the blood. During fasting or starvation, adipose TG is mobilized to produce free fatty acids (FFA), which can be used by other tissues for energy production (Flier 2004). Several studies have been shown that SIRT1 may be one of the potential molecules linking adipose tissue, the effect of food restriction and longevity (Bordone and Guarente 2005; Kloting and Bluher 2005; Fischer-Posovszky, Kukulus et al. 2010). The nuclear

receptor PPARγ (peroxisome proliferation-activated receptor-γ) is a ligand-activated transcription factor which functions as the master regulator of adipogenesis and fat storage in white adipose tissues (WAT). SIRT1 reduces adipogenesis and TG accumulation in the lipid droplets of adipocytes by repressing PPARγ through the transcriptional co-repressor NCoR and silencing mediator for retinoid and thyroid hormone receptor (SMRT) (Picard, Kurtev et al. 2004). Consistently, *SIRT1*$^{+/-}$ mice have low levels of blood FFAs upon fasting or β-adrenergic stimulation and nicotinamide (NAM), a SIRT1 inhibitor, significantly increases fat mass in these mice. Conversely, resveratrol (RSV), a SIRT1 activator, potentially induces mitochondrial activity, through activating PGC1-α by facilitating SIRT1-mediated deacetylation. Furthermore, mice treated with RSV demonstrate a smaller amount of WAT, a reduction in fat storage and smaller adipocytes. In addition, mobilization of FFAs from WAT in response to fasting is impaired in *SIRT1*$^{-/-}$ mice (Howitz, Bitterman et al. 2003; Picard, Kurtev et al. 2004).

Another molecular mechanism of SIRT1 in regulating lipid metabolism is demonstrated by Hallows WC et al. (Hallows, Lee et al. 2006). They showed that mammalian Acetyl-CoA synthetases (AceCSs) are regulated by reversible acetylation and SIRT1 activates the mammalian acetyl CoA synthetase 1 (AceCs-1) via deacetylation. AceCSs catalyzes the synthesis of AceCoA, a key molecule in mitochondrial oxidation and lipid synthesis. Regulation by SIRT1 of AceCS-1 suggests that SIRT1 possibly modulates lipid metabolism by regulating intracellular acetyl-CoA levels (Hallows, Lee et al. 2006; Lomb, Laurent et al. 2010)

SIRT1 and cancer

Many cancers are recognized as age associated diseases because of their prevalence with increasing age. It has been debated whether SIRT1 is a potential oncogene or a potential tumor suppressor. SIRT1 plays a dual role in cell survival and cell death and can be modulated in different directions by a variety of different stimuli. On the one hand, SIRT1 deacetylates and suppresses the tumor suppressor p53 (Luo, Nikolaev et al. 2001; Chua, Mostoslavsky et al. 2005). Deacetylation of lysine 382 on p53 by SIRT1 reduces p53 transactivation, allowing cells to bypass p53-mediated apoptosis. Increased SIRT1 expression in human fibroblasts has been shown to promote cellular proliferation, increase growth rate, reduce cellular senescence, and increase the cellular life span of human embryonic lung fibroblasts 2Bs, while reducing expression of $p16^{INK4A}$, and promoting phosphorylation of Rb (Haigis and Sinclair 2010). Overexpression of SIRT1 has also been shown to repress expression or activity of tumor-suppressor and DNA-repair genes, including FOXO1/2/4, WRN, Rb, p73, MLH1, and NBS1. These data, combined with other studies showing that SIRT1 can suppress apoptosis, imply that SIRT1 might be oncogenic (Haigis and Sinclair 2010).

Two tumor suppressors have been identified as negative regulators of SIRT1. The tumor suppressor HIC1 (hyper-methylated in cancer-1) directly represses SIRT1 to modulate the p53-dependent DNA-damage response (Chen, Wang et al. 2005). Importantly, the authors find that the HIC1 promoter undergoes hypermethylation during aging, which may lead to upregulation of SIRT1 during aging and therefore, susceptibility to cancer. Another tumor suppressor that negatively regulates SIRT1 is DBC1 (deleted in breast cancer 1) (Kim, Chen et al. 2008; Zhao, Kruse et al. 2008). The authors showed that

DBC1 acts as a inhibitor of SIRT1 in human cells. DBC1-mediated repression of SIRT1 leads to increasing levels of p53 acetylation and upregulation of p53-mediated function. In contrast, depletion of endogenous DBC1 by RNA interference (RNAi) stimulates SIRT1-mediated deacetylation of p53 and inhibits p53-dependent apoptosis. Notably, these effects can be reversed in cells by concomitant knockdown of endogenous SIRT1. This indicates that DBC1 may promote breast cancer in part by activating SIRT1, thereby downregulating p53 and/or other tumor-suppressor pathways.

On the other hand, recent work indicates that SIRT1 serves as a tumor suppressor. Firestein et al. demonstrated that overexpression of SIRT1 in $APC^{min/+}$ mice reduces colon cancer formation (Firestein, Blander et al. 2008). Min mice are heterozygous for a germ-line mutation in *Apc*, the mouse homolog of the human *APC* gene. Min mice on the C57BL/6J (B6) background develop numerous adenomatous polyps throughout the small and large intestine (Moser, Pitot et al. 1990). They showed that the reduction in tumor development is caused by the ability of SIRT1 to deacetylate β-catenin and promote cytoplasmic localization of the nuclear-localized oncogenic form of β-catenin. Their further analysis also uncovered a significant inverse correlation between the presence of nuclear SIRT1 and the oncogenic form of β-catenin in 81 human colon tumor specimens analyzed. Ectopic overexpression of SIRT1 also greatly reduces cell proliferation in a human colon cancer cell line whose growth is driven by active β-catenin (Firestein, Blander et al. 2008; Luo and Altieri 2008).

Analyzing published database for SIRT1 expression, Wang et al. found that SIRT1 expression is much lower in the BRCA1-associated breast cancer than BRCA1-wildtype breast cancer in human (Wang, Zheng et al. 2008). They further showed that BRCA1

binds to the SIRT1 promoter and positively regulates SIRT1 gene expression at both the mRNA and protein level, and that BRCA1 deficiency causes reduced SIRT1 levels, which may be responsible for the malignant transformation of BRCA1 mutant cells. Consistently, restoration of SIRT1 levels in BRCA1 mutant cancer cells inhibited proliferation of these cells *in vitro* and tumor formation *in vivo* when the cells were implanted into nude mice. The underlying molecular mechanism is that SIRT1 negatively regulates transcription of survivin, which encodes an anti-apoptotic protein, by deacetylating H3K9 within the promoter of survivin (Wang, Zheng et al. 2008; Deng 2009). Together, these data suggest that SIRT1 mediates BRCA1 signaling and inhibits tumor growth through repressing transcription of oncogenes or activity of oncoproteins.

Wang et al. have shown that $SIRT1^{+/-}$; $p53^{+/-}$ mice develop spontaneous tumors in multiple organs from about 5 months of age, and tumor incidence reaches about 76% by 20 months of age, while only 2 out of 21 $SIRT1^{+/-}$ mice and 3 out of 22 $p53^{+/-}$ mice developed tumors during the same period of time. Chromosome spreads and spectral karyotyping analysis of primary tumors revealed extensive aneuploidy and chromosomal aberrations, notably translocations, chromosome breaks, deletions, end fusions, and dicentric chromosomes. These results suggest that severe genetic instability could be one of the causes for spontaneous tumorigenesis in SIRT1 and p53 double-heterozygous animals. Interestingly, most tumors developed in the $SIRT1^{+/-}$; $p53^{+/-}$ mice lost the remaining WT allele of *p53*, yet retained a functional wild-type *SIRT1* allele. Accordingly, administration of resveratrol to these mice significantly reduced tumor formation (Wang, Sengupta et al. 2008; Deng 2009). These data demonstrate that SIRT1

plays a critical role in maintaining genome integrity and acts as SIRT1 serves as a haploid tumor suppressor gene in mice.

The work summarized above represents a strong body of evidence supporting a role for SIRT1 in cancer development. Yet, the controversy over whether SIRT1 serves as a tumor promoter or a tumor suppressor has not been resolved and discussion will likely continue.

SIRT1 function in vascular endothelial homeostasis

Recent studies point to SIRT1 as a key regulator of vascular endothelial homeostasis controlling angiogenesis, vascular tone and endothelial dysfunction. Using a three-dimensional assay of sprouting angiogenesis assay combined with RNA interference to specifically knock down SIRT1 as a model system, Potente et al. (Potente, Ghaeni et al. 2007) showed that SIRT1 is a critical regulator of sprouting angiogenesis during vascular growth. Furthermore, the authors analyzed SIRT1 mutant mice and found that those mice were impaired in their ability to form new vessels in response to angiogenic signals such as ischemic stress (Potente, Ghaeni et al. 2007). A clue to the mechanism of how SIRT1 modulates endothelial angiogenic function came from time-lapse analysis of segmental vessel formation in transgenic zebrafish embryos with fluorescently labeled endothelial cells in which segmental arteries emanate from the dorsal aorta to form the dorsal longitudinal anastomosing vessel. Comparing the highly organized process of blood vessel formation in control embryos, SIRT1-deficient zebrafish were characterized by vascular patterning defects and hemorrhages due to dysregulated endothelial sprouting and vessel navigation (Potente, Ghaeni et al. 2007; Potente and Dimmeler 2008; Guarani and Potente 2010). By means of gain- and loss-of-function approaches, they

demonstrated that SIRT1 represses FOXO-1 dependent transcriptional activity in endothelial cells, and, thus, point to this transcription factor as an effector in the SIRT1-dependent angiogenic signaling pathway (Potente and Dimmeler 2008; Chen, Peng et al. 2010).

In addition to its function as a regulator of angiogenesis, SIRT1 may be involved in endothelial homeostasis by regulating the endothelial nitric oxide synthase (eNOS). Mattagajasingh et al. demonstrated that SIRT1 promotes endothelium-dependent vasodilation by targeting eNOS for deacetylation, leading to enhanced nitric oxide production (Mattagajasingh, Kim et al. 2007). Resveratrol, a polyphenolic activator of SIRT1, increases the expression of eNOS and the combination of resveratrol with the HMG-CoA reductase (statins) increased activation of eNOS resulting in increased functional recovery in a model of acute myocardial infarction (Wallerath, Li et al. 2005; Penumathsa, Thirunavukkarasu et al. 2007; Rush, Quadrilatero et al. 2007). A recent study by Chen and colleagues further explain the mechanisms underlying post-transcriptional activation of eNOS by SIRT1 (Chen, Peng et al. 2010). SIRT1 deacetylation of eNOD requires AMPK phosphorylation of eNOS. Atheroprotective flow, via AMPK and SIRT1, increases NO bioavailability in endothelium (Chen, Peng et al. 2010). Taken together, these findings suggest SIRT1 as a key regulator of tissue homeostasis by defining the vascular endothelium as an important target tissue for direct or indirect actions of SIRT1.

SIRT1 function in development

SIRT1 is expressed at high levels in mouse embryos with highest mRNA expression is E4.5 embryos. Although expression is down-regulated during subsequent embryogenesis, high level expression remains detectable at E18.5 (Sakamoto, Miura et al. 2004). Many lines of evidence suggest a role of SIRT1 in development. McBurney et al. showed that the protein SIRT1 is important for embryogenesis and gemetogenesis (McBurney, Yang et al. 2003). SIRT1 mutant mice carrying a truncation mutation through targeted replacement of exons 5 and 6 with a hygromycin gene demonstrate 50% mortality at an early postnatal stage (McBurney, Yang et al. 2003). These mice showed developmental defects such as markedly smaller size as compared to their littermates, slower development, defects in eye morphogenesis and cardiac septation. Furthermore, the mice that survive to adulthood were sterile in both sexes, with males having lower sperm count and females failing to ovulate, potentially due to hormonal inefficiency (McBurney, Yang et al. 2003). Cheng et al have demonstrated that up to 90% of SIRT1 mutant mice carrying a deletion of exon 4 die perinatally and those mice exhibit developmental defects of retina and heart, with the remaining 10% of these mutants still surviving at weaning (Cheng, Mostoslavsky et al. 2003; McBurney, Yang et al. 2003; Coussens, Maresh et al. 2008). A recent study (Wang, Sengupta et al. 2008) showed that a majority of *SIRT1*$^{-/-}$ mice died at E9.5-E14.5; whereas about 1% of the mutants on the 129SvEv/FVB background and 9.3% on the 129SvEv/FVB/Black Swiss background survived to adulthood (Wang, Sengupta et al. 2008). In addition, studies showed that SIRT1 influences growth-factor responses and maintenance of embryonic and hematopoietic stem cells (Han, Song et al. 2008; Mantel and Broxmeyer 2008; Narala,

Allsopp et al. 2008). Taken together, these studies suggest that SIRT1 is important in embryonic development. Thus, functions of SIRT1 are worth investigating to better understand mammalian embryogenesis.

SIRT1 activators and inhibitors

The apparent role of SIRT1 and other sirtuin family members in a variety of physiological processes and diseases have prompted searches for compounds that can activate or inhibit SIRT1, since such compounds may potentially provide clinical treatment and therapies. In 2003, the first such molecule, Resveratrol, a compound produced by plants in response to stress was discovered by Howitz et al. and they found that this molecule extend *Saccharomyces cerevisiae* lifespan (Howitz, Bitterman et al. 2003). Since then, many polyphenol activators of SIRT1 have been discovered such as Quercetin, Buetin, SRT1720, Pyrroloquinoxaline and Oxazolopyridine. These compounds are collectively called the Sirtuin Activating Compounds or STACs (Ghosh 2008).

Resveratrol, the activator that has been investigated most extensively, is a polyphenolic compound found naturally in grape skin and a variety of plant products such as fruits and nuts, and was initially reported to increase the catalytic activity of SIRT1 *in vitro*. Resveratrol has also shown effects mimicking SIRT1 activation such as promoting cell survival by deacetylation of p53, inhibiting adipocyte differentiation by PPAR-γ, and sensitizing cells to TNF-α induced apoptosis by NF-κB deacetylation (Picard, Kurtev et al. 2004; Yeung, Hoberg et al. 2004; Parker, Arango et al. 2005). Subsequently, it has been reported to modulate a variety of processes in mammals in a SIRT1-dependent

manner such as neuroprotection, differentiation, tumor suppression and inflammation (Baur, Pearson et al. 2006; Labinskyy, Csiszar et al. 2006; Anekonda and Adamus 2008; Baur 2010). Consistently, resveratrol effects a number of diseases in mammals including cancer, heart diseases, brain damage, hearing loss and injuries to tissues (Baur and Sinclair 2006). Thus, resveratrol may provide us a promising therapy and future research will probably tackle key medical issues like stroke, heart disease, and so on based on their findings concerning resveratrol's therapeutic properties.

Biological functions of SIRT1 have triggered interest in the development of not only SIRT1 activators but also inhibitors. Sir2 enzymes catalyze NAD^+-dependent protein/histone deacetylation, where the acetyl group from the lysine epsilon-amino group is transferred to the ADP-ribose moiety of NAD^+, producing nicotinamide and the novel metabolite O-acetyl-ADP-ribose. Bitterman et al. showed that physiological concentrations of nicotinamide inhibit both Sir2 and SIRT1 *in vitro* (Bitterman, Anderson et al. 2002). Several other chemical inhibitors of SIRT1 have more recently been identified including Sirtinol, EX-527, Cambinol and Tenovin (Napper, Hixon et al. 2005; Haigis and Sinclair 2010). Studies on inhibitors and activators of SIRT1 will likely be ongoing in order to determine if it is possible to safety target SIRT1 and other sirtuin family members to treat “SIRT-associated” diseases.

HYPOTHESIS

We hypothesized that SIRT1 plays a role in embryonic and adult hematopoiesis in the mouse.

AIMS

AIM 1: Determine the role of SIRT1 in self-renewal and differentiation of mouse embryonic stem (mES) cells using the *in vitro* model of differentiation of ES cells into embryoid body (EB) cells, and explore the molecular mechanisms associated with the phenotype.

AIM2: Clarify the role of SIRT1 in embryonic and adult embryogenesis *in vivo* using a SIRT1 mutant mouse model.

MATERIALS AND METHODS

Part I

Cell culture and differentiation

Mouse ES cell line R1 and *SIRT1*$^{-/-}$ mES cells derived from R1 parental cells (A kind gift from Dr. McBurney, Ottawa, Canada) were cultured as reported (Kyba, Perlingeiro et al. 2002) on irradiated mouse embryonic fibroblasts in Dulbecco modified Eagle medium with 15% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT), 1000 U/mL LIF (Chemicon International, Temecula, CA), 10 U/μl penicillin/streptomycin (Invitrogen, Carlsbad, CA), L-Glutamine 200 mM (100×) (Gibco, Carlsbad, CA), 0.1 mM nonessential amino acids (Invitrogen, Carlsbad, CA), and 0.1 mM β-mercaptoethanol (Invitrogen, Carlsbad, CA) at 37℃ / 5% CO_2. Media was changed daily, and cultures passaged every 2 to 3 days.

OP9 stromal cells (ATCC, Manassas, VA) were maintained in α-MEM medium supplemented with 10 U/μl penicillin/streptomycin, L-Glutamine 200 mM (100×) (Gibco), and 20% heat-inactivated fetal calf serum (Invitrogen). Cells were passaged every 4 to 6 days. OP9 coculture was performed as described (Kyba, Perlingeiro et al. 2002).

For primary differentiation assays, ES cells were plated in bacterial grade Petri dishes at 3000 cells/mL in 0.9% methylcellulose-based differentiation media composed of Iscove Modified Dulbecco Medium (IMDM) plus 15% differentiation FBS (StemCell Technologies, Vancouver, BC), 2 mM penicillin/streptomycin (Invitrogen), L-Glutamine

200 mM (100×) (Gibco), 5% protein-free hybridoma medium (PFHM-II) (Gibco), 200 mg/mL iron-saturated holo-transferrin (Sigma, St. Louis, MO), 5 mg/mL ascorbic acid and 450 μM monothioglycerol (Sigma). After withdrawal of ES cells from feeder cells and LIF, cells were incubated for 6 days at 37℃ in 5% CO_2. EBs were viewed by light microscopy and examined for presence of hemoglobin as assessed initially by the red colored EBs.

For secondary plating assays, EBs were digested with 0.25% trypsin (Gibco) followed by passaging cells through a 20-gauge needle 2-3 times. Cells were plated at 25,000 cells/mL with erythropoietin (EPO; 5 U/mL; R&D Systems, Minneapolis, MN), Stem Cell Factor (SCF; 100 ng/mL; R&D system) and Interleukin-3 (IL-3; 1 ng/mL; R&D Systems) for assessment of definitive erythroid progenitor cells, or with EPO (5 U/mL), SCF (100 ng/mL), IL-3 (1 ng/mL), granulocyte-macrophage colony-stimulating factor (GM-CSF; 10 ng/mL; R&D Systems), and macrophage colony-stimulating factor (M-CSF; 5 ng/mL; R&D Systems) for analyses of mixed and granulocyte-macrophage colony assays. For detection of primitive erythroid progenitors, cells were plated with 15% plasma-derived serum (Animal Technologies, Antech, TX) and Epo (5 U/ml) (Palis, Robertson et al. 1999).

Blast colony-forming cell (BL-CFC) assays

EBs were collected at different days post differentiation in liquid suspension culture, washed in PBS, and treated with 0.25% trypsin for 3 minutes at 37℃. EBs were dissociated into single cells by passage two-three times through a 23-gauge needle and plated at 3×10^4 cells in 1ml of methycellulose medium with 10% FCS, 10% FBS, SCF

(100 ng/mL), 25% D4T endothelial cell-conditioned medium (Kennedy, Firpo et al. 1997), 5 ng/ml mouse Vascular Endothelial Growth Factor (VEGF; R&D Systems), 10 ng/ml human IL-6 (R&D Systems) and IMDM. Cells were maintained at 37℃ in a humidified 5% CO_2 incubator. After 3-4 days, developing BL-CFCs were counted.

Generation of Hematopoietic cells from BL-CFCs

BL-CFC were plucked and replated into secondary methycellulose media containing IL-3, IL-6, Epo and SCF. Cultures were maintained as described above and secondary hematopoietic colonies were scored at 7-10 days of growth (Kennedy, D'Souza et al. 2007).

Generation of Endothelial cells from BL-CFCs

Individual BL-CFC were picked and transferred to matrigel-coated microtiter cells containing IMDM with 10% FCS, 10% horse serum (GIBCO), VEGF (5 ng/ml), insulin growth factor-1 (IGF-1, 10 mg/ml), Epo (2 U/ml, R&D Systems), basic fibroblast growth factor (bFGF, 10 ng/ml), IL-11 (50 ng/ml; R&D Systems), SCF (100 ng/ml), endothelial cell growth supplement (ECGS, 100 μg/ml; Collaborative Research), L-glutamine (2 mM), and 4.5×10^{-4} M monothiglycerol (MTG). After 3-4 days in culture, nonadherent cells were removed and adherent cells were cultured for an additional 1-2 weeks in IMDM with 10% FCS, 10% horse serum, VEGF (5 ng/ml), IGF-1 (10 ng/ml), bFGF (10 ng/ml), ECGS (100 μg/ml), L-glutamine (2 mM), and 4.5×10^{-4} M MTG (Kennedy, D'Souza et al. 2007).

Cell Staining

BL-CFCs were plucked onto prewashed glass cover slips that were coated with a thin layer of matrigel and cultured in 12-well dishes in medium containing both hematopoietic and endothelial cytokines, as described above. Four to seven days following the initiation of the cultures, the nonadherent hematopoietic cells were removed and the adherent cells were cultured for an additional 1-2 weeks in medium containing only endothelial growth factors (see last section). For fluorescence analysis, adherent cells were initially cultured in the presence of 10 μg/ml of Dil-Ac-LDL (Biomedical Technologies; Stoughton, MA) at 37℃ for 2 hours. Following this incubation, the cells were washed three times and fixed for 10 minutes in PBS containing 3% paraformaldehyde and 3% sucrose. The fixed cells were washed two to three times and incubated with fluorescein isothiocyanate-mouse anti-CD144 (BD Pharmingen, San Diego, CA) for 1 hour. Following this staining, cells were washed again (five times) and the coverslip with the cells was mounted onto a slide for analysis. The images were acquired with a digital confocal microscope using the Zeiss LSM-510 software program (Oberkochen, Germany).

Vascular sprout formation assay

Aliquots of 200 μL Matrigel (BD Pharmingen) were added to each precooled well of 24-well culture plates and incubated at 37℃ until gelation occurred as described (Feraud, Cao et al. 2001). Then 50, day-6 EBs were suspended in 200 μL medium containing 15% FBS, 0.1 mM β-mercaptoethanol (Invitrogen), 2 mM glutamine, 5 ng/mL recombination murine VEGF, and 50 μgmL endothelial cell growth supplement (BD pharmingen) and cells were seeded on matrigel. EBs were incubated at 37℃ in a 5% CO_2, 95% air

atmosphere. EBs were scored in blinded fashion according to 4 standard classes based on vascular sprout formation: I, no sprout formation; II, few sprouts; III, many sprouts but no network; and IV, many sprouts with network (Feraud, Cao et al. 2001). Another aliquots of day 6 EBs were suspended in IMDM Glutamax and mixed with collage culture medium (Invitrogen) at a final concentration of 50 EBs/mL. Final composition of freshly prepared collagen-based culture medium was as follows: IMDM containing 1.25 mg/mL type I rat tail collagen (BD Biosciences, San Jose, CA), 15% fetal calf serum, 450 mM monothioglycerol, 10 μg/mL insulin, 50 U/mL streptomycin, and 50 U/mL penicillin. After thorough mixing of EBs into collagen-based medium, 1.2 mL were dispensed into 35-mm bacterial Petri dishes. Cultures were then incubated for 8 days for analysis of their vascular development (Feraud, Cao et al. 2001). All Cell cultures were done under both normoxic (~20% O_2) and lower O_2 (5% O_2) conditions.

Flow cytometry analysis for surface markers

Single cell suspensions were prepared from WT and *SIRT1*$^{-/-}$ EBs. Staining was done as described previously (Fehling, Lacaud et al. 2003). Briefly, one million cells were resuspended in FACS staining buffer (PBS +2% FBS) and were stained with c-kit-APC (2B8, eBioscience), and Flk-1-PE (BD Bioscience), incubated for 30 minutes in the dark at 4℃, washed twice with PBS containing 2% BSA, and resuspended in PBS containing 1% paraformaldehyde for analysis by flow cytomery of FACSCalibur (Becton Dickinson, Sunnyvale, CA). Approximately 10,000 events were collected for each sample.

Real-time PCR analysis

Total RNA was extracted from each sample using TRIzol reagent (Invitrogen). RNA was treated with RNase-free DNase for 20 min (Ambion, Austin, TX) at room temperature before reverse transcription with Superscript II RT (Invitrogen, Carlsbad, CA). An RT^2 Profile Custom PCR Array was used to simultaneously examine mRNA levels of 48 genes, including three "housekeeping genes" in 96-well plates according to the manufacturer (SA Biosciences, Frederick, MD). Real-time PCR was performed on an MX30000P Stratagene machine (Stratagene, La Jolla, CA) with SYBR Green PCR Master Mix (SA Biosciences). PCR conditions consisted of a 10 min hot start at 95℃, followed by 40 cycles of 15 seconds at 95℃ and 1 min at 60℃. The average threshold cycle for each gene was determined from triplicate reactions and three independent experiments. Values were exported to a template Excel file for analysis. Analyses of raw data were done through the SA biosciences data analysis web portal (http://www.sabiosciences.com/pcr/arrayanalysis.php).

Western blot analysis

Protein extracts from 2×10^6 mES cells were prepared and immunoblots performed according to standard techniques. Briefly, samples were loaded on a 4-12% sodium dodecyl sulfate (SDS)-polyacrylamide gel (Invitrogen). After transfer of proteins onto polyvinylidene difluoride membrane (Bio-rad, Hercules, CA), nonspecific binding was blocked by incubation with Tris-buffered saline/Tween 20 (25 mM Tris, 25 mM glycine, 0.1% SDS, and 0.1% Tween-20) containing 2% BSA. The membrane was then probed with antibodies against Erk (Cell Signaling Technology, Boston, MA) and β-actin (Cell Signaling Technology). Next, the membrane was incubated with a horseradish-peroxidase-conjuagted secondary antibody and developed with enhanced

chemilluminescence PLUS reagent from (Amersham, Piscataway, NJ). To determine that amounts of protein in each lane were comparable, the membrane was cut at about the expected molecular weight of target proteins and β-actin. The cut membranes were respectively probed with antibodies against target proteins or a rabbit polyclonal antibody against β-actin.

Alkaline Phosphatase Staining

Murine ES cells were cultured with 2-ME and LIF for 3 days to obtain spheroidal colonies. Cells were washed with PBS (BioWhittaker) and fixed with 4% paraformaldehyde (Sigma) in PBS for 5 min. After washing, Cells were stained with an Alkaline Phosphatase Staining Kit (Chemicon, Temucula, CA) at room temperature for 15 min in the dark. Cells were washed with PBS again, and colonies were visualized using an Olympus Microscope.

Flow Cytometry Analysis of Oct-4, CD9 and KLF-4

An aliquot of 1×10^6 cells was washed in ice-cold PBS containing 2% FCS serum (PBS-2% FCS) and incubated with anti-mouse CD16/CD32 receptor monoclonal antibody at 1 μg/100 μl (BD Biosciences) to block non-specific binding of immunoglobulins to the mouse FcIII/II receptors. Cells to be stained for Oct-4 and KLF-4 were fixed with 100 μl of IC fixation buffer (eBioscience, San Diego, CA) for 30 min at room temperature, washed one time with PBS-2% FCS, permeabilized with permeabilization buffer (eBioscience), and incubated with a 1:100 dilution rabbit-mouse Oct3/4 polyclonal antibody (Chemicon) or rabbit-mouse KLF-4 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at room temperature. Cells were washed two

times with 1 ml of PBS-2% FCS followed by staining with 1:100 dilution of fluorescein isothiocyanate (FITC): goat anti-rabbit IgG antibody (Santa Cruz Biotechnology). Finally, the cells were washed two times with PBS-2% FCS and resuspended in PBS-2% FCS for analysis on a FACScan flow cytometer (Becton Dickinson). Cells analyzed for CD9 were incubated first with a 1:100 dilution of anti-mouse CD9 (BD Pharmingen) for 40 min at 4℃, washed two times with ice-cold PBS-2% FCS, and incubated with a 1:500 dilution of FITC: goat-anti rat IgG antibody (Santa Cruz Biotechnology).

Part II

Progenitor assays

Primitive erythroid yolk sac (E8-E8.25) progenitors. Treatment with 0.25% collagenase (Sigma) plus 20% FCS for 10 mintues at 37℃ with vigorous pipetting resulted in single cells which were plated with 15% plasma-derived serum (Animal Technologies, Tyler, TX) and Epo (5 U/mL). Colonies were counted at day 5. (Palis, Robertson et al. 1999) Embryos were generated as described (Wang, Sengupta et al. 2008).

Adult marrow hematopoietic progenitors. Fifty thousand bone marrow nucleated cells were plated onto 35-mm Petri dishes in methylcellulose with 30% FBS (Hyclone, Logan, UT), 1 U/ml hu Epo, 5% pokeweed mitogen mouse spleen cell conditioned medium, and 50 ng/ml murine SCF. (Broxmeyer, Orschell et al. 2005) Colonies were scored after 7 days at ~20% or 5% O_2. Mating of mice was conducted as described (Wang, Sengupta et al. 2008).

Genotyping of Mice

The animals were genotyped by PCR using primer 1, 5'- TCCTTGCCACAGTCACTCA C-3'; primer 2, 5'- ACAGTCCCATTCCCATACC-3'; and primer 3, 5'-CATCTAAACT TTGTTGGCTGC-3'. Primers 1 and 3 are located within intron 4 and amplify the wild-type allele (660 bp). Primer 2 is located within exon 7; the combination of primers 1 and 2 amplifies the deleted allele (716 bp).

Statistical Analysis

Significant differences were determined by student *t*-test comparisons for at least 3 experiments each, with triplicates performed for each experiment.

RESULTS

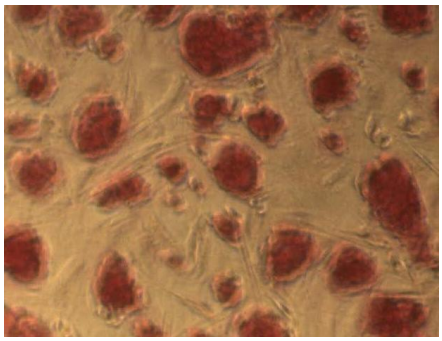
Part I: SIRT1 deficiency compromises hematopoietic differentiation of mouse embryonic stem (mES) cells

SIRT1 is dispensable for self-renewal of mES cells

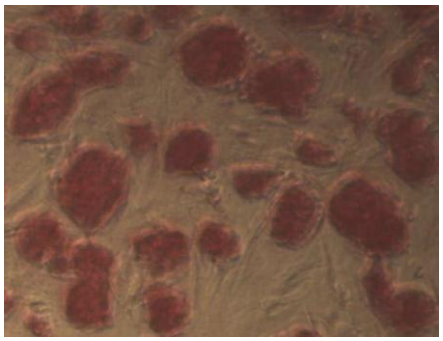
Recently our lab demonstrated that SIRT1 regulates apoptosis and Nanog expression in mES cells in the presence of LIF and 2ME-withdrawal-induced increase in reactive oxygen species by controlling p53 subcellular localization (Han, Song et al. 2008). As a follow on to these studies, we evaluated the effect of *SIRT1*$^{-/-}$ on self-renewal and differentiation of mES cells in the presence of 2-ME, but absence of LIF. First, we explored the role of SIRT1 in the self-renewal of mES cells. ES cell self-renewal depends on a stoichimometric balance among various signaling molecules. There are several molecular markers and transcription factors defining stemness of mES cells including Rex-1 (Hosler, Rogers et al. 1993), CD9, Oct4, Klf-4, SSEA-1 et al (Walker, Chang et al. 2010). Undifferentiated mES cells also possess enzyme activities for alkaline phosphatase (AP) (Wobus, Holzhausen et al. 1984). Therefore, we assessed AP, CD9, Klf-4 and Oct4 expression in WT R1 and *SIRT1*$^{-/-}$ mES cells. Alkaline phosphatase is a hydrolase enzyme responsible for dephosphorylating molecules such as nucleotides, proteins, and alkaloids under alkaline conditions. When fixed ES cells are stained with AP, undifferentiated cells appear red or purple, whereas differentiated cells appear colorless. When maintained with LIF and mouse embryonic fibroblast feeder layer cells, both WT and *SIRT1*$^{-/-}$ mES cells appear red, suggesting AP expression and an

undifferentiated state (Figure 6). Furthermore, we examined the expression of the molecular marker CD9, and pluripotent transcription factors Oct4 and Klf-4. WT and *SIRT1*$^{-/-}$ mES cells showed similar expression of these markers (Figure 7). Taken together, these data suggest that SIRT1 is dispensable for the self-renewal of mES cells.

Figure 6



Phase-contrast 10×



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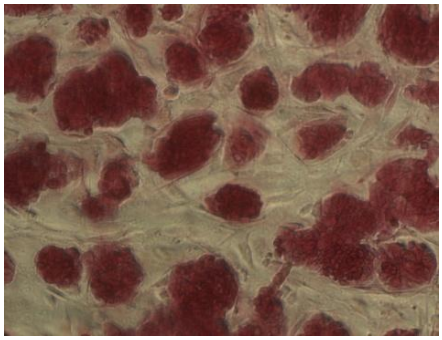
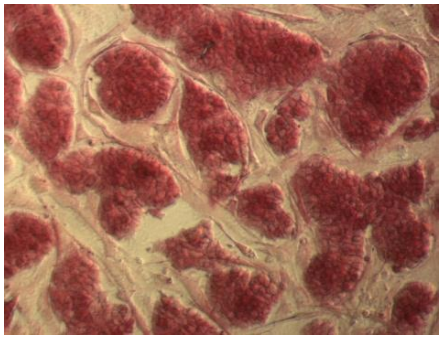


Phase-contrast 20×
WTR1



Phase-contrast 20×
SIRT1-/-

Figure 6: Alkaline phosphatase staining of WT R1 and *SIRT1*$^{-/-}$ cells when cultured with 2-ME and LIF.

One thousand WT and *SIRT1*$^{-/-}$ cells were cultured in medium containing LIF on a MEF feeder layer for 3 days to obtain spheroidal colonies. Then cells were stained with an Alkaline Phosphatase Staining Kit (Chemicon, Temucula, CA) at room temperature and colonies were visualized using an Olympus Microscope.

Figure 7

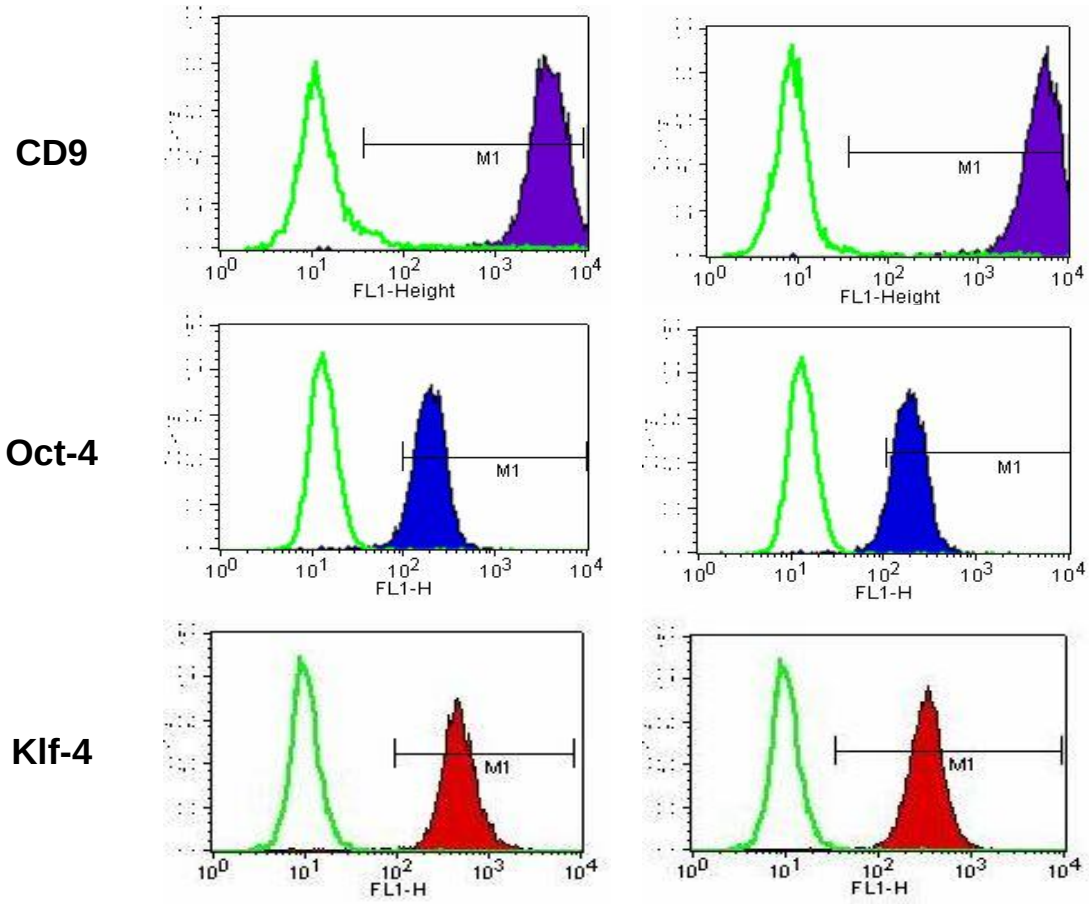


Figure 7: Expression of CD9, Oct-4 and Klf-4 in WT and *SIRT1*$^{-/-}$ mES cells were analyzed by flow cytometry.

WT and *SIRT1*$^{-/-}$ cells were cultured in medium containing LIF on a MEF feeder layer. After 3 days, 1×10^6 of both cells were collected and stained with anti-mouse CD9, Oct4 and Klf-4. One representative of three histograms for each marker is shown in the figure. For CD9, the light grey line is isotype control staining, while the shaded line is the anti-CD9 staining. For Oct-4, the green line is isotype control staining, while the shaded blue line is the anti-Oct-4 staining. For Klf-4, the green line is isotype control staining, while the shaded red line is the anti-Klf-4 staining.

Delayed/Defective hemangioblast development in absence of SIRT1

With removal of LIF and in the absence of feeder layer cells, ES cells grow into EBs, which generate hematopoietic and endothelial progeny, recapitulating development of those populations in the yolk sac (Keller 1995). Endothelial and hematopoietic lineages develop in parallel, and are most intertwined in yolk sac blood islands and the dorsal aorta in the embryo (Jaffredo, Gautier et al. 1998; Lancrin, Sroczynska et al. 2009). In both the yolk sac and embryonic dorsal aorta, blood vessels give rise to the hematopoietic elements via specialized hemogenic endothelium (Chen, Yokomizo et al. 2009). Although SIRT1 regulates vascular endothelial homeostasis controlling angiogenesis, vascular tone and endothelial dysfunction (Mattagajasingh, Kim et al. 2007; Potente, Ghaeni et al. 2007), little information is available on SIRT1 activities in embryonic and adult stages of hematopoietic and endothelial development. A common progenitor of hematopoietic and endothelial cells was identified as the hemangioblast by the BL-CFC assay. To assess whether SIRT1 plays a role in hemangioblast development, we tested the ability of SIRT1 deficient ES cells to give rise to blast colonies by BL-CFC assay. BL-CFCs were analyzed on days 3, 3.5, 4, 5, 5.5, 6 and 7 respectively. As shown in Figure 8A, WT EBs generated BL-CFCs from day 3 and peaked on day 4. *SIRT1*$^{-/-}$ EBs did not generate significant numbers of typical blast colonies until day 4; but were significantly lower than BL-CFCs generated by WT EBs. *SIRT1*$^{-/-}$ BL-CFCs peaked on day 5.5. No colonies were generated in both cell lines on day 7. BL-CFC development in *SIRT1*$^{-/-}$ EBs is thus restricted to a narrower and later window of time, than found in WT EBs. Meanwhile, transitional colonies, which have similar morphology as those developed in

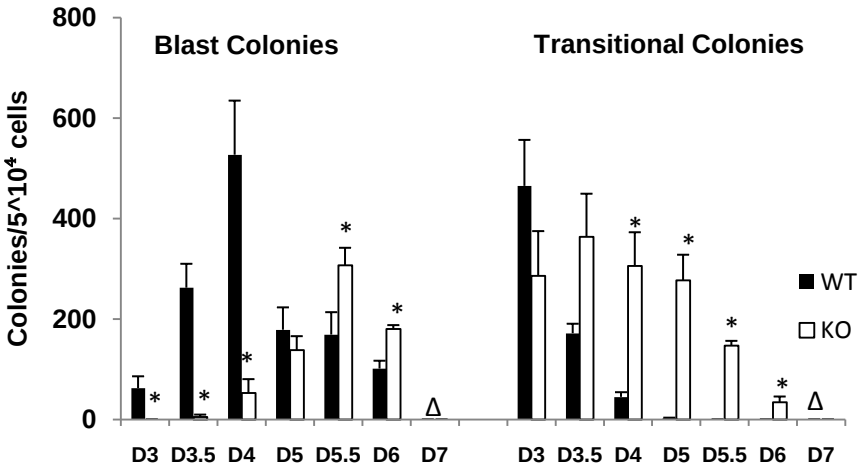
Scl$^{-/-}$ ES cells (Figure 9B) (Robertson, Kennedy et al. 2000) and secondary embryonic bodies were most readily detected in the *SIRT1*$^{-/-}$ cultures (Figure 8A, C).

Flk1 is the receptor 2 for VEGF (VEGF-R2). Others demonstrated that Flk-1 is expressed on BL-CFCs, and onset of Flk1 corresponds to BL-CFC development. c-Kit is expressed on ES cells and populations found at early stages of EB development (Kennedy, Firpo et al. 1997; Choi, Kennedy et al. 1998); its expression is down-regulated following differentiation. Beyond the hemangioblast stage, c-Kit is co-expressed with Flk1 marking establishment of hematopoiesis (Lacaud, Kouskoff et al. 2004). To further assess if *SIRT1*$^{-/-}$ affects *in vitro* developmental potential of ES cells into hemangioblast, we analyzed temporal expression patterns of cell surface Flk1 and c-Kit by flow cytometry. As shown in Figure 8, a distinct *Flk1*$^{+}$, *c-Kit*$^{-}$ cell population (*Flk1*$^{+}$*/c-Kit*$^{-}$*)* emerged in WT EBs by day 3.5 of differentiation, and increased during the next 24 hours. This window defines the hemangioblast stage of development, as demonstrated by presence of BL-CFCs at those times. In *SIRT1*$^{-/-}$ EBs, emergence of a distinct *Flk1*$^{+}$*/c-Kit*$^{-}$ population was reproducibly detected at least 12 hours later than in WT EBs, and the percent of *SIRT1*$^{-/-}$ *Flk*$^{+}$*/c-Kit*$^{-}$ cells was significantly decreased on days 3.5 and 4. The average percentage ± SEM in 3 independent experiments of *Flk1*$^{+}$*/c-kit*$^{-}$ cells was: 27.7±4.6 in *SIRT1*$^{+/+}$ and 2.3±1.3 in *SIRT1*$^{-/-}$ EBs (*p* value=0.007) at day 3.5 of differentiation, 45.7±3.7 in WT and 3.8±0.8 in *SIRT1*$^{-/-}$ EBs (*p* value=0.001) at day 4; by day 5.5, there was a switch with 14.8 ±1.8 in WT and 31.7±3.7 in *SIRT1*$^{-/-}$ EBs (*p* value=0.006). Delayed emergence of *Flk1*$^{+}$*/c-Kit*$^{-}$ cells in *SIRT1-/-* EBs was consistent with delayed BL-CFC development in *SIRT1*$^{-/-}$ EBs (Figure 8A). This delay appears to result from

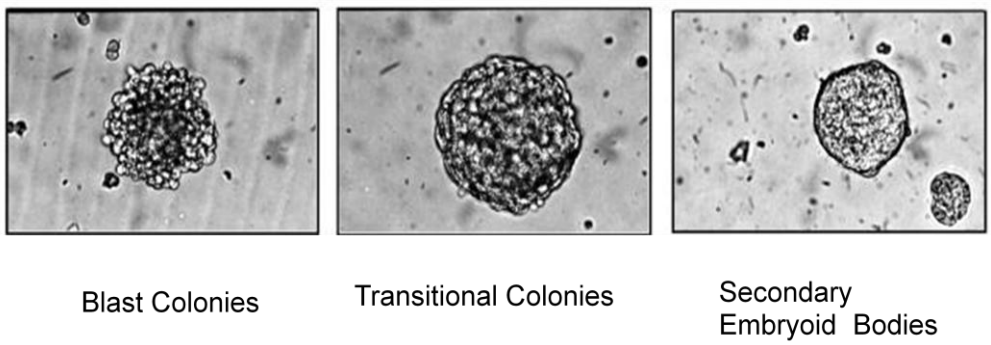
immature differentiation of the *SIRT1*$^{-/-}$ ES cells as kinetics of decline in transitional colonies and secondary EBs, is delayed compared to WT ES cells (Figure 8A, C).

Figure 8

A


Blast Colonies
Transitional Colonies
Colonies/5^10⁴ cells
800
600
400
200
0
WT
KO
D3 D3.5 D4 D5 D5.5 D6 D7
D3 D3.5 D4 D5 D5.5 D6 D7

B


Blast Colonies
Transitional Colonies
Secondary
Embryoid Bodies

C

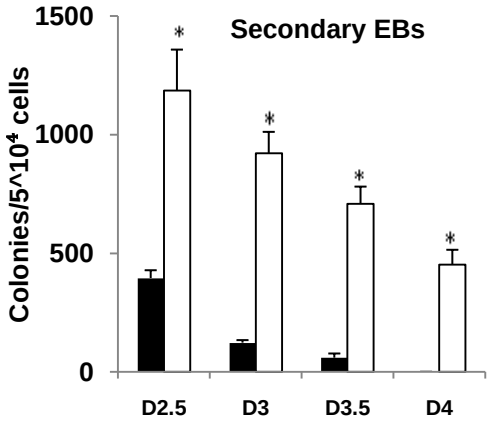

Secondary EBs
Colonies/5^10⁴ cells
1500
1000
500
0
D2.5
D3
D3.5
D4

Figure 8: Blast cell colony development from WT and *SIRT1*$^{-/-}$ ES cells.

(A) Kinetics of blast and transitional colony development in WT and *SIRT1*$^{-/-}$ EBs. Data are shown as mean ± SD. N=3; *, $p<0.01$. Days (D) of differentiation are indicated. Δ= no colony grown. (B) The morphology of blast colonies, transitional colonies and secondary EBs formed in the culture system of BL-CFC assay (Robertson, Kennedy et al. 2000). (C) Secondary EB colonies generated by WT and *SIRT1*$^{-/-}$ EB cells. Days of differentiation are indicated. Data are shown as mean ± SD. N=3; *, $p<0.01$.

Figure 9

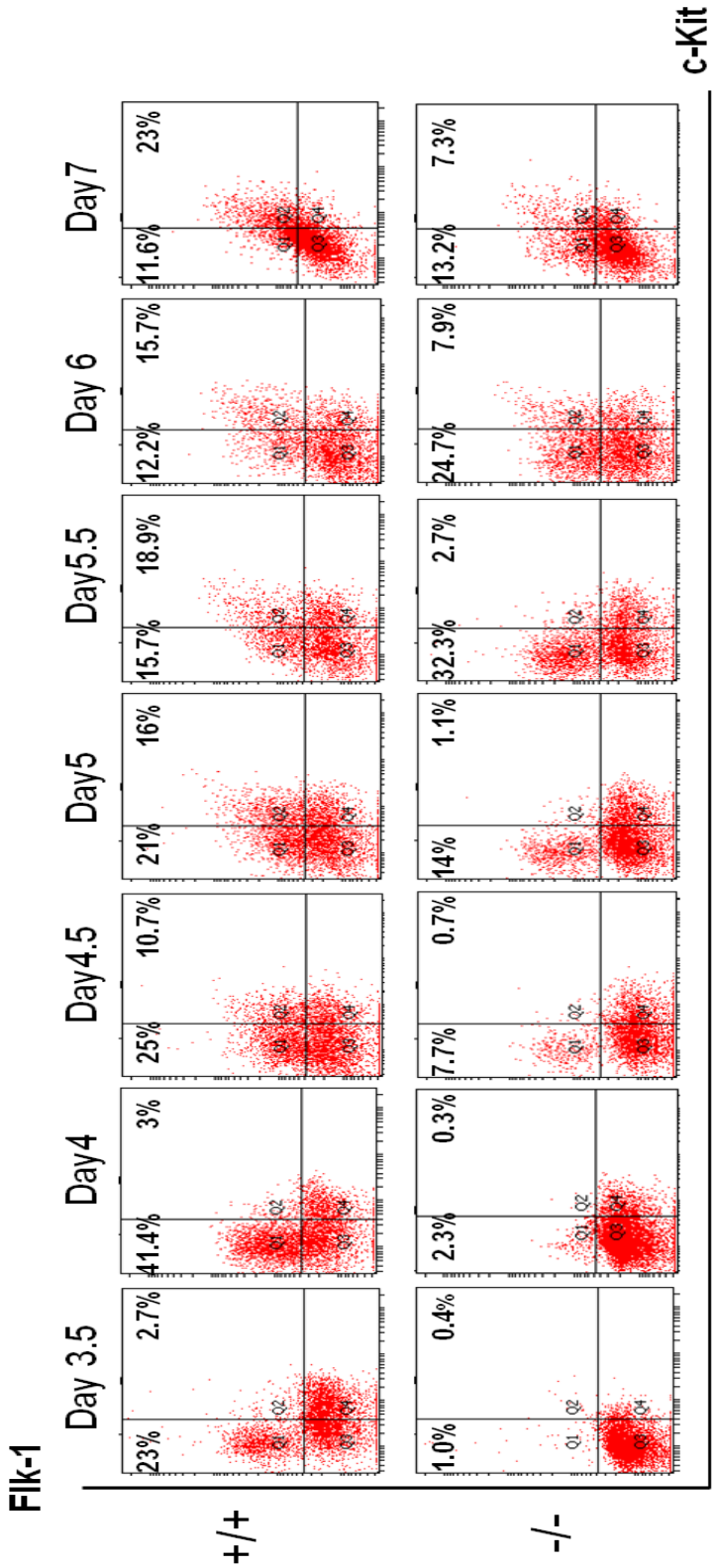

Flk-1
c-Kit
Day 3.5
Day4
Day4.5
Day5
Day5.5
Day 6
Day7
+/+
-/-
23%
2.7%
41.4%
3%
25%
10.7%
21%
16%
15.7%
18.9%
12.2%
15.7%
11.6%
23%
1.0%
0.4%
2.3%
0.3%
7.7%
0.7%
14%
1.1%
32.3%
2.7%
24.7%
7.9%
13.2%
7.3%

Figure 9: FACS analysis of Flk1 and c-Kit expression on WT and *SIRT1*$^{-/-}$ EB-derived cells.

WT and *SIRT1*$^{-/-}$ EBs of day 3.5, 4, 4.5, 5, 5.5, 6 and 7 were collected at the time points and then dissociated into single cells. Viable cell counts were taken by trypan blue exclusion counting. One million of WT and *SIRT1*$^{-/-}$ cells were collected and stained with anti-mouse Flk-1 and c-Kit. After staining, the expression of Flk-1 and c-Kit in WT and *SIRT1*$^{-/-}$ EB-derived cells were analyzed by flow cytometry. Days of differentiation are indicated. Numbers in each quadrant represent the percent of total population in each fraction. Representative of three independent experiments.

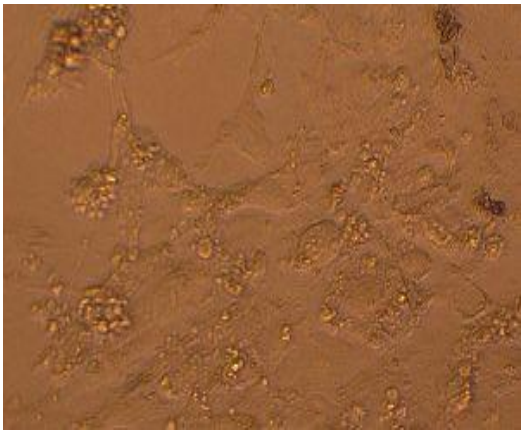
SIRT1-deficient BL-CFCs have reduced hematopoietic, but unaffected endothelial, replating potential

Hemangioblasts generate blast colonies *in vitro* displaying hematopoietic and endothelial potential (Choi, Kennedy et al. 1998). To assess developmental potential of blast colonies, single WT and *SIRT1*$^{-/-}$ BL-CFCs were replated, transferred to microtiter wells, and cultured for 4 days to determine their potential to generate adherent (endothelial) and nonadherent (hematopoietic) cells. Considering the peak of blast colony formation in WT and *SIRT1*$^{-/-}$ ES cells, day 4 of WT and day 5.5 of *SIRT1*$^{-/-}$ blast colonies were used respectively. After expansion (Figure 10A), nonadherent cells of each well were replated in methycellulose to assay hematopoietic potential. Adherent cells were cultured for an additional week and then harvested and analyzed for expression of genes associated with the endothelial lineage. Endothelial cells express vascular endothelial cadherin (VE-cadherin, CD144) and take up acetylated low-density lipoprotein (Ac-LDL) (Perlingeiro, Kyba et al. 2003). Colonies from WT and *SIRT1*$^{-/-}$ cells generated both types of cells. Adherent cells derived from WT and *SIRT1*$^{-/-}$ BL-CFCs, both displayed Dil-Ac-LDL uptake (red) as well as CD144 expression (green) (Figure 10B), indicating that adherent populations derived from WT and *SIRT1*$^{-/-}$ individual blast colonies are of the endothelial lineage. Replating revealed that whereas WT and *SIRT1*$^{-/-}$ generated endothelial cells at a similar frequency, the nonadherent populations from *SIRT1*$^{-/-}$ blast colonies contained fewer secondary hematopoietic precursors with colony-forming potential (Figure 10C). Transitional colonies derived from *SIRT1*$^{-/-}$ ES cells were also replated and analyzed. They were able to give rise to adherent (endothelial) populations (data not shown).

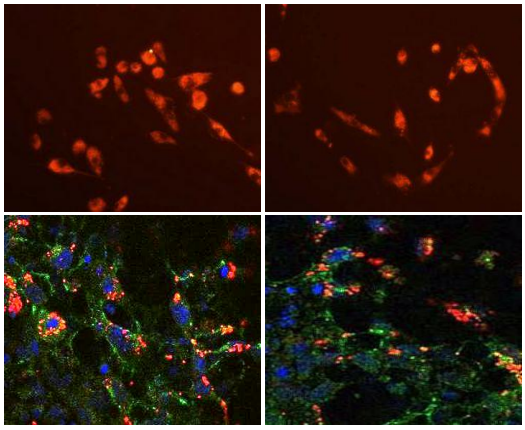
However, they did not generate the round nonadherent (hematopoietic) cells after 4-day culture, which suggests their inability to generate hematopoietic progeny.

Figure 10

A



B



C

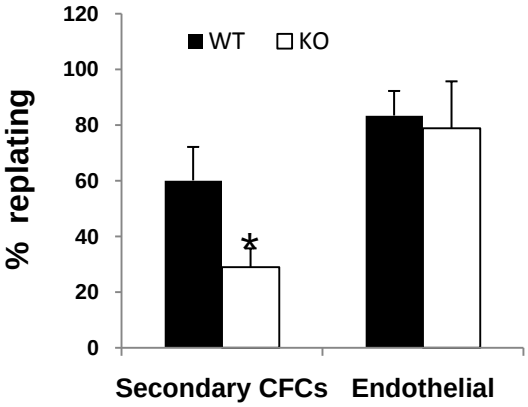


Figure 10: Evaluation of the hematopoietic and endothelial potentials of BL-CFCs derived from WT and *SIRT*$^{-/-}$ cells.

(A) The morphology of adherent and nonadherent cells generated from single blast colony after expansion. (B) Immunostaining and Dil-Ac-Low density lipoprotein (LDL) uptake of adhesive cells generated from a single blast colony from WT and *SIRT1*$^{-/-}$ EB-derived cells. VE-Cadherin expression is indicated by green fluorescence and LDL uptake by red fluorescence. (C) Evaluation of the hematopoietic and endothelial potentials of BL-CFCs. The number of colonies that yielded secondary CFCs or adherent endothelial cells is divided by the total number of replated colonies. Bars represent standard error of the mean number from at least 3 experiments. Data are shown as mean ± SEM. N=3; *, $p<0.05$.

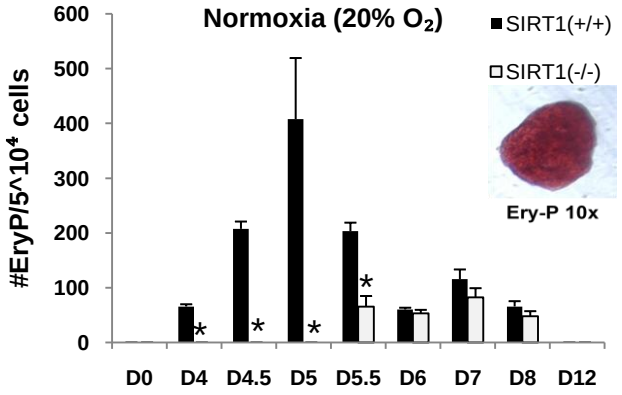
Hematopoietic cell differentiation of *SIRT1*$^{-/-}$ cells is defective

Defective hematopoietic development of blast colonies in *SIRT1*$^{-/-}$ EBs prompted us to investigate their primitive and definitive hematopoietic potential. ES cells were differentiated into EBs for 4-12 days, at which time cells were disrupted and plated for primitive erythroid (EryP) and definitive colonies. In addition to delay of hemangioblast development and limited hematopoietic potential from *SIRT1*$^{-/-}$ blast colonies, *SIRT1*$^{-/-}$ EBs displayed delayed and greatly decreased hematopoietic commitment as demonstrated by later generation of *Flk1*$^{+}$*/c-Kit*$^{+}$ cells (Figure 9) and presence of primitive precursors by day 5.5 of differentiation compared to day 4 for WT cells (Figure 11A). Low oxygen promotes erythropoiesis and other forms of hematopoiesis. Because low oxygen tension affects cellular redox state and SIRT1 is a redox-sensitive molecule (Caito, Rajendrasozhan et al. 2010), we assessed the ability of WT and *SIRT1*$^{-/-}$ EBs to generate erythroid precursors under lowered 5% O_2. Lowered O_2 allowed increased detection of EryP colonies from both WT and *SIRT1*$^{-/-}$ cells. However, even under lowered O_2 no *SIRT1*$^{-/-}$ cell-derived colonies were noticeable until day 5.5, even though the number of WT colonies was greatly enhanced under the lowered O_2. The delay in generation of *SIRT1*$^{-/-}$ EryP colonies was thus readily apparent at both oxygen tensions (Figure 11A, B; note different scales for colony numbers). The morphology of definitive hematopoietic progenitor cells derived from mES cells are demonstrated in Figure 12. We observed somewhat smaller, but still significant decreases in numbers of definitive granulocyte macrophage (CFU-GM) and multipotential (CFU-GEMM) progenitor cells obtained from *SIRT1*$^{-/-}$ compared to WT cells at day 6 and 8 (Figure 13A, B). But the differences of CFU-GM ($1.66_{20\%O2}$ versus $2.56_{5\%O2}$ on day 6, $1.53_{20\%O2}$ versus $1.79_{5\%O2}$ on day 8)

between WT and *SIRT1*$^{-/-}$ EBs were greater at low O_2. Differences in fold changes of cells grown at normoxia and 5% O_2 suggest that *SIRT1*$^{-/-}$ cells are not as responsive as WT cells to the enhancing effect of lowered O_2 tension. In addition, *SIRT1*$^{-/-}$ cells had higher numbers of secondary EBs (Figure 13A, B). We also evaluated mRNA levels of both embryonic and adult globin genes and observed reduction in expression of both genes in *SIRT1*$^{-/-}$ cells (Figure 14A, B). These results suggest a delay/defect in hematopoietic commitment upon *SIRT1*$^{-/-}$ ES cell differentiation.

Figure 11

A


Normoxia (20% O2)
SIRT1(+/+)
SIRT1(-/-)
Ery-P 10x
#EryP/5^10⁴ cells
600
500
400
300
200
100
0
D0
D4
D4.5
D5
D5.5
D6
D7
D8
D12

B

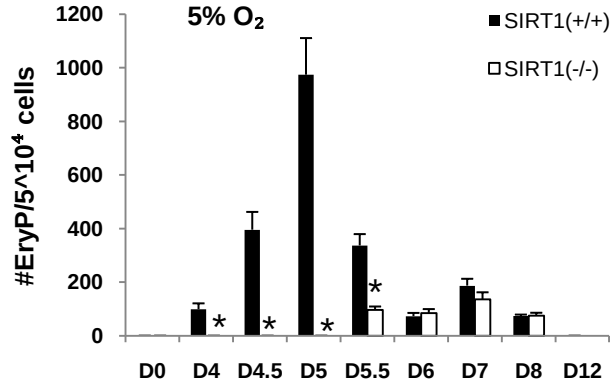
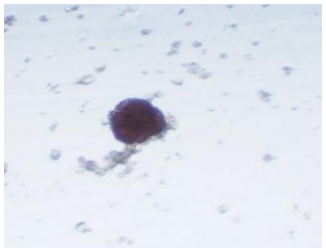

5% O2
SIRT1(+/+)
SIRT1(-/-)
#EryP/5^10⁴ cells
1200
1000
800
600
400
200
0
D0
D4
D4.5
D5
D5.5
D6
D7
D8
D12

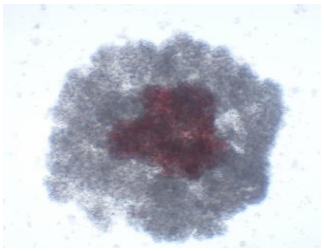
Figure 11: Primitive erythroid colonies generated from WT and *SIRT1*$^{-/-}$ EB-derived cells under normoxia (20% O_2) and lower O_2 (5% O_2) tension.

(A) Primitive erythroid colonies generated from day 0 to day 12 WT and *SIRT1*$^{-/-}$ EB-derived cells under normoxia (20% O_2) tension. Data are shown as mean ± SD. N=3; *, $p<0.05$. Morphology of primitive erythroid colonies formed from WT and *SIRT1*$^{-/-}$ EBs was shown on the right corner. Image acquisition details: Nikon Diaphot microscope, 10×/0.25 numeric aperture objective lens, Nikon F3 camera, NIS-Elements D2.30 software. (B) Primitive erythroid colonies generated from day 0 to day 12 WT and *SIRT1*$^{-/-}$ EB-derived cells under lower (5%) O_2 tension. At each time point, dissociated EB-derived cells were measured by trypan blue exclusion counting, and cells were plated in triplicate in methycellulose colony assays. The type of colonies was evaluated based on their distinguished morphology and bright red color observed under light microscope. Data are shown as mean ± SD. N=3; *, $p<0.05$. Days (D) of differentiation are indicated.

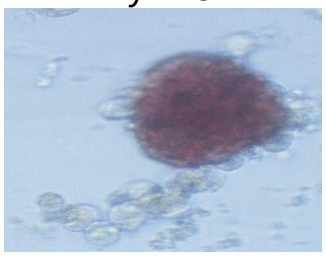
Figure 12



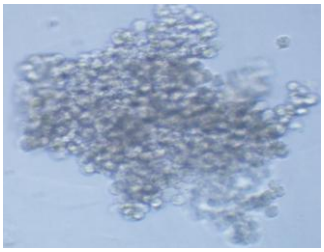
Ery-D 5x



Mixed 5x



Ery-D 10x



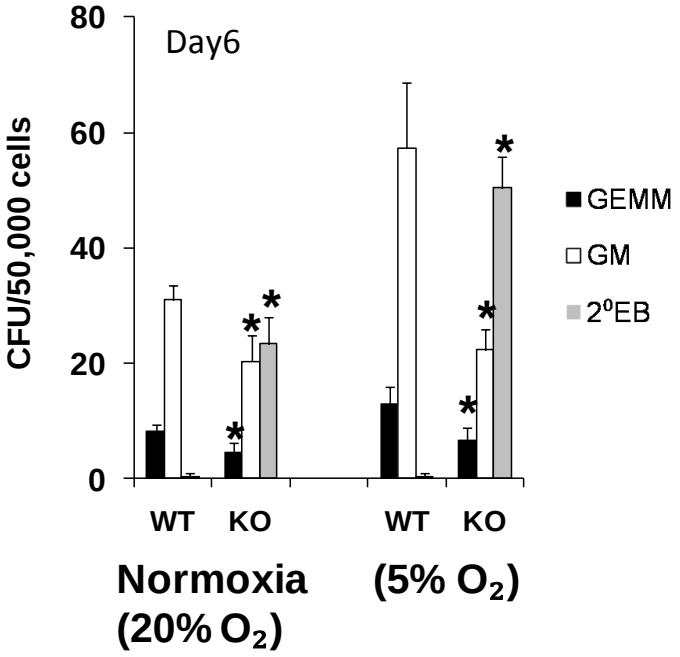
GM 5x

Figure 12: Morphology of definitive erythroid progenitors (Ery-D), definitive GEMM, multilineage progenitors of granulocytes, erythroid cells, macrophages, megakaryocytes; GM, multilineage progenitors of granulocytes and macrophages derived from mouse embryonic stem cells.

Ery-D progenitors were identified as they appear dark red color. GEMM progenitors were evaluated as they are composed of red color cells in the central, and round and refractive cells in the periphery of the colonies. GM progenitors were distinguished by the round and refractive cells. Image acquisition details: Nikon Diaphot microscope, 10×/0.25 numeric aperture objective lens, Nikon F3 camera, NIS-Elements D2.30 software.

Figure 13

A


Day6
CFU/50,000 cells
80
60
40
20
0
GEMM
GM
2⁰EB
WT
KO
WT
KO
Normoxia (20% O2)
(5% O2)

B

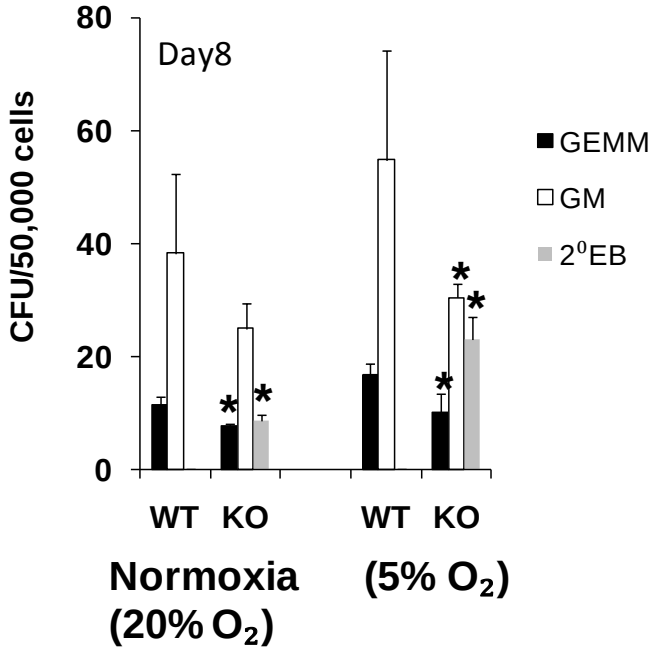
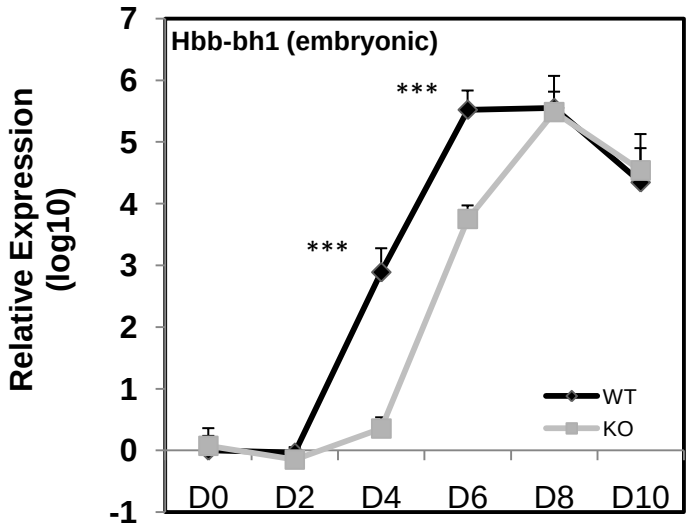

Day8
CFU/50,000 cells
80
60
40
20
0
GEMM
GM
2⁰EB
WT
KO
WT
KO
Normoxia (20% O2)
(5% O2)

Figure 13: Definitive GEMM, multilineage progenitors of granulocytes, erythroid cells, macrophages, megakaryocytes; GM, multilineage progenitors of granulocytes and macrophages; and secondary EBs generated from WT and *SIRT1*$^{-/-}$ EB-derived cells under normoxia condition and lower (5%) O_2 tension at day 6 (A) and day 8 (B). At each time point, dissociated EB-derived cells were measured by trypan blue exclusion counting, and cells were plated in triplicate in methycellulose colony assays. The types of colonies were evaluated based on their distinguished morphology observed under light microscope. Data are shown as mean ± SD. N=3; *, $p<0.05$.

Figure 14

A


Hbb-bh1 (embryonic)
Relative Expression (log10)
7
6
5
4
3
2
1
0
-1
***
***
WT
KO
D0
D2
D4
D6
D8
D10

B

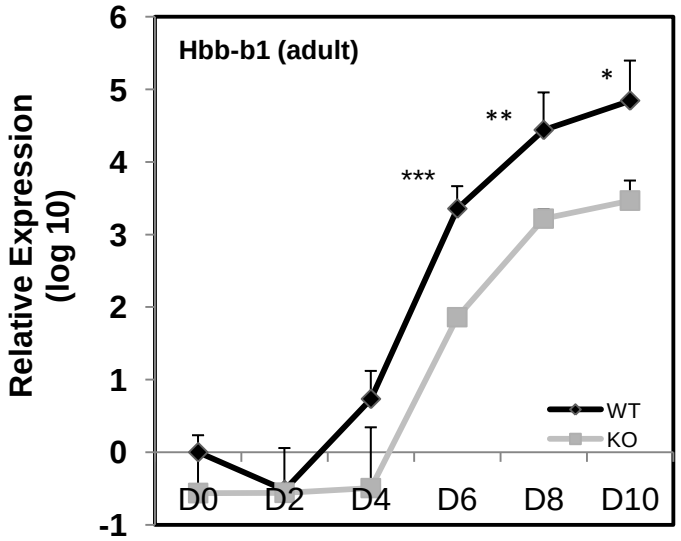

Hbb-b1 (adult)
Relative Expression (log 10)
6
5
4
3
2
1
0
-1
*
**
***
WT
KO
D0
D2
D4
D6
D8
D10

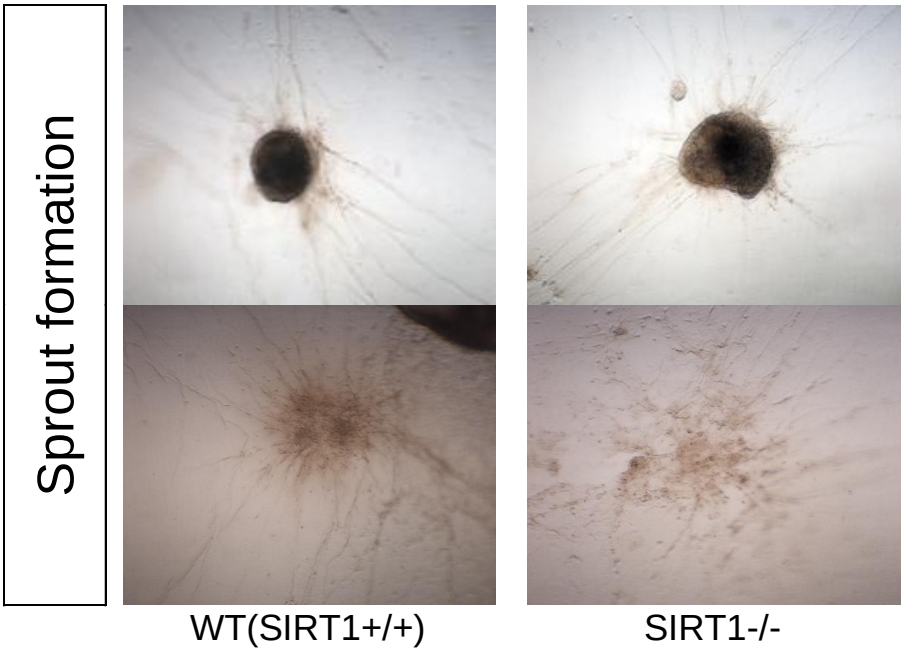
Figure 14: Relative mRNA expression of embryonic globin (marker for Ery-P) (A) and adult globin (marker for Ery-D) (B) genes from WT and *SIRT1*$^{-/-}$ day 0, 2, 4, 6, 8, and 10 EBs by real-time RT-PCR. Error bars indicate standard deviations from the average of three independent experiments, each performed in triplicate; ***, $p<0.001$, **, $p<0.01$, *, $p<0.05$.

Deletion of SIRT1 results in defective formation of a vascular-like network *in vitro*

To assess a role for SIRT1 in angiogenesis, vascular sprout formation developed from day 6 EBs was assessed using a three dimensional spheroidal assay. WT and *SIRT1*$^{-/-}$ EBs developed similar endothelial outgrowths when embedded into collagen gel (Figure 15A). Quantitative analysis on the percentage of angiogenic EBs revealed that loss of SIRT1 expression does not significantly affect endothelial sprout formation (Figure 15B). However, *SIRT1*$^{-/-}$ cells did not form a full vascular-like network *in vitro* (Figure 16A); they had significantly fewer class III (many sprouts but not network) and class IV (abundant networked tubules) vascular sprout formations than WT EBs (Figure 16B).

Figure 15

A


Sprout formation
WT(SIRT1+/+)
SIRT1-/-

B

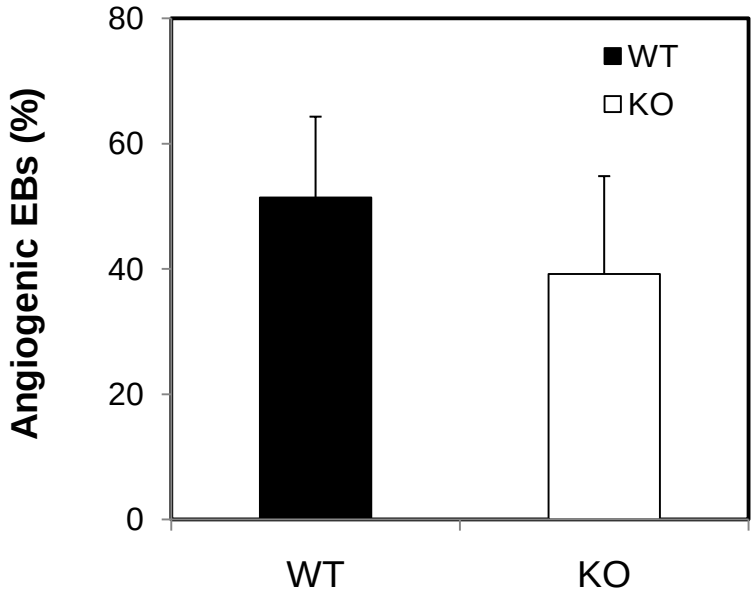

Angiogenic EBs (%)
80
60
40
20
0
WT
KO
WT
KO

Figure 15: Effect of SIRT1 deletion on *in vitro* vascular sprout formation in differentiating EBs.

(A) Representative micrographs of three-dimensional *in vitro* angiogenesis assays with collagen gel-embeded spheroids generated from WT or *SIRT1*$^{-/-}$ day 6 EBs. (B) Analysis of the percentage of angiogenic EBs was performed after 8 days of secondary culture in collagen gel. Data are shown as mean ± SEM; N=3. Image acquisition details: Nikon Diaphot microscope, 10×/0.25 numeric aperture objective lens, Nikon F3 camera, NIS-Elements D2.30 software.

Figure 16

A


Primitive vascular
Network formation
WT (SIRT1+/+)
SIRT1-/-

B

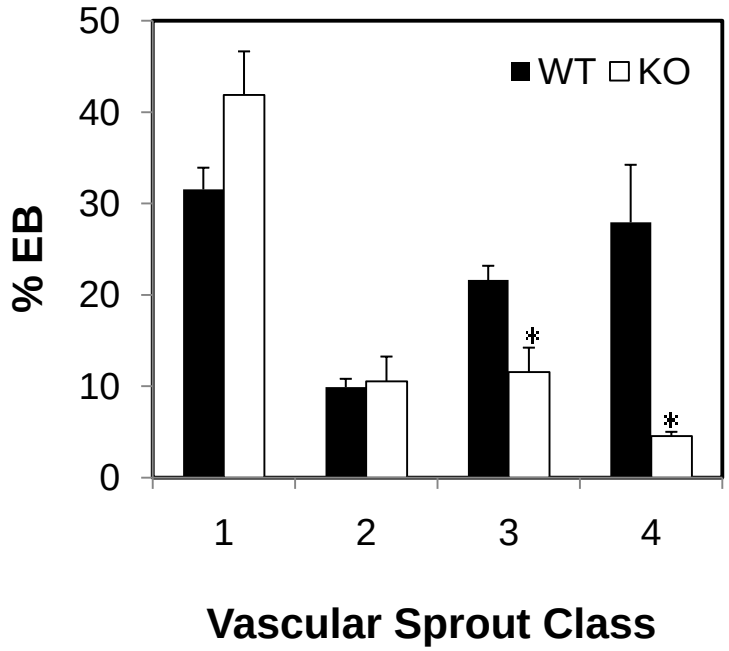

50
40
30
20
10
0
% EB
WT
KO
*
*
1
2
3
4
Vascular Sprout Class

Figure 16: Effect of SIRT1 deletion on *in vitro* vascular sprout formation in differentiating EBs.

(A) Representative micrographs of *in vitro* matrigel assays with WT or *SIRT1*$^{-/-}$ day 6 EBs. Image acquisition details: Nikon Diaphot microscope, 10×/0.25 numeric aperture objective lens, Nikon F3 camera, NIS-Elements D2.30 software. (B) Percentage of each class of vascular sprouting (See methods). Briefly, EBs were incubated EBs were scored in blinded fashion according to 4 standard classes based on vascular sprout formation: I, no sprout formation; II, few sprouts; III, many sprouts but no network; and IV, many sprouts with network (Feraud, Cao et al. 2001). Data are shown as mean ± SEM. N=3; *, p value=0.01-0.04.

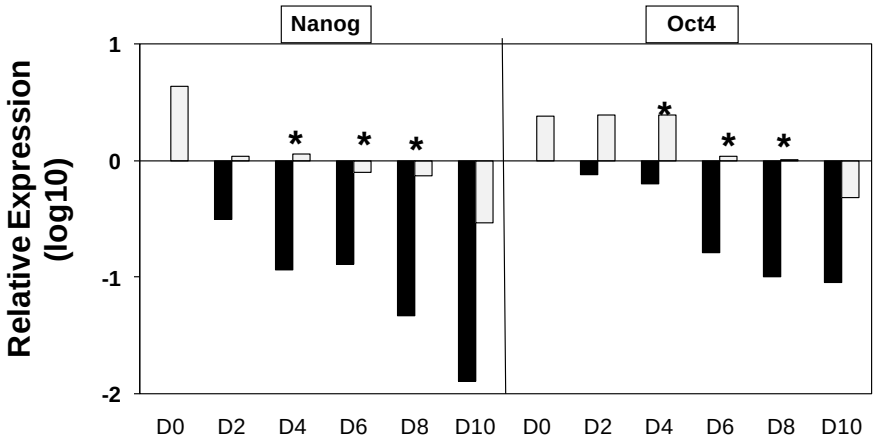
SIRT1 deficiency affects expression of genes involved in mES cell differentiation and hematopoietic commitment

Given the inappropriate differentiation and reduced hematopoietic potential of *SIRT1*$^{-/-}$ ES cells, we collected RNA from EBs and quantified transcript levels of genes that might shed light on loss of ES cell differentiation potential. This includes genes involved in development of epiblast-like cells, onset of mesoderm development, and markers for other germ layers in EBs at different stages. Expression of mRNA for Oct4 (Pou5f1) and Nanog, well known pluripotency transcription factors, were rapidly shut down upon differentiation of WT cells, consistent with rapid loss of potential to generate secondary EBs. However, down-regulation of Oct4 and Nanog was remarkably delayed in *SIRT1*$^{-/-}$ cells upon differentiation (Figure 17A). Notably Fgf5, a gene expressed in the epiblast of early embryos and down-regulated after pre-hemangioblast mesoderm stage (Fehling, Lacaud et al. 2003) was increased and maintained in *SIRT1*$^{-/-}$ EBs. Meanwhile, expression of T and Wnt3, indicative of mesoderm commitment and development, was delayed and sustained (Figure 17B). These gene expression patterns, combined with the results of blast and transitional colony formation in *SIRT1*$^{-/-}$ EBs, indicates the earliest stages of hemangioblast commitment are occurring, but not fully developed in *SIRT1*$^{-/-}$ ES cells at the appropriate time points. We further analyzed expression of Scl/Tal-1, a transcription factor essential for commitment of mesoderm to hematopoiesis (Robertson, Kennedy et al. 2000). It was delayed at least 24 hours and greatly decreased on days 4, 6, and 8 in *SIRT1*$^{-/-}$ EBs (Figure 17B), which is consistent with the interpretation that progression of hematopoietic commitment was compromised in *SIRT1*$^{-/-}$ cultures. Trophectoderm marker Cdx2 (Figure 18B), and molecular markers for

endoderm/mesoderm GATA-4, Sox17, and GATA-6 showed delayed expression in *SIRT1*$^{-/-}$ cells, but at later days, there was no difference between the expression of these genes in WT and *SIRT1*$^{-/-}$ EBs (Figure 18A). Expression of the cardiac lineage marker Nkx2.5 showed no difference (Figure 18A). In addition, the hematopoietic-specific gene GATA-1 was reduced in day 5 *SIRT1*$^{-/-}$ EBs. Notably, GATA-1 at late stage and Runx-1 were non-significantly decreased (Figure 19A, B), suggesting that SIRT1 may largely affect early stages of ES cell commitment to hematopoietic lineage. Together, these data suggest that SIRT1 is critically involved in gene expression for early commitment and differentiation.

Figure 17

A


Nanog
Oct4
Relative Expression (log10)
1
0
-1
-2
D0 D2 D4 D6 D8 D10 D0 D2 D4 D6 D8 D10

B

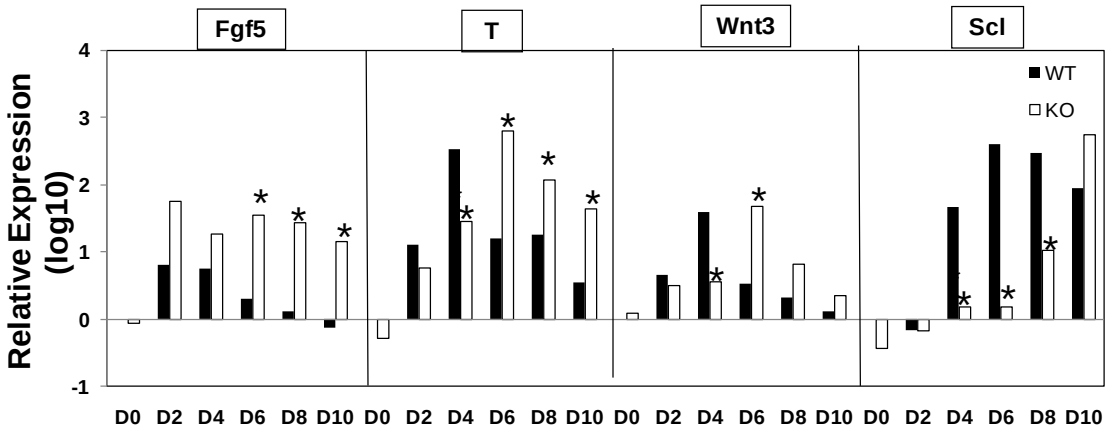
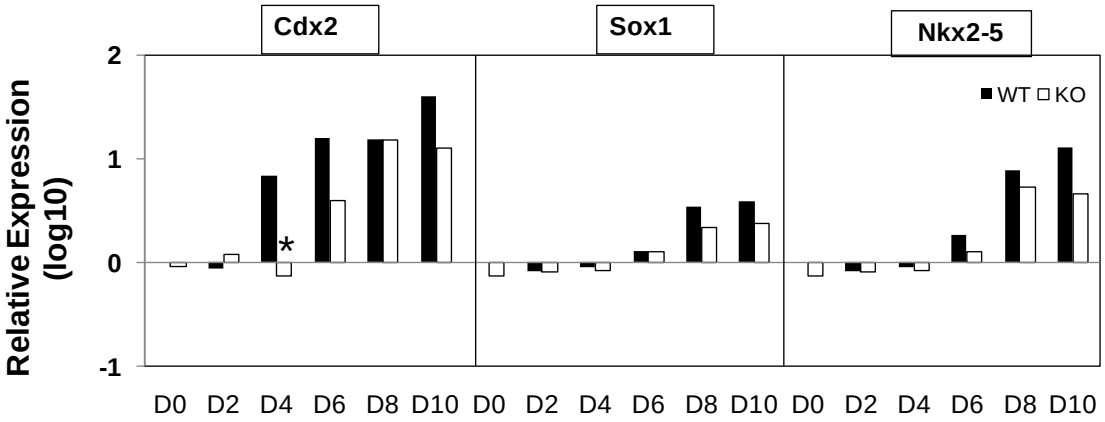

Fgf5
T
Wnt3
Scl
WT
KO
Relative Expression (log10)
4
3
2
1
0
-1
D0 D2 D4 D6 D8 D10 D0 D2 D4 D6 D8 D10 D0 D2 D4 D6 D8 D10 D0 D2 D4 D6 D8 D10

Figure 17: Gene expression analysis of WT and *SIRT1*$^{-/-}$ EBs by real-time PCR analysis.

(A) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of stem cell marker Oct4 and Nanog mRNA levels in day 0-10 WT and *SIRT1*$^{-/-}$ EBs. (B) qRT-PCR analysis of mRNA levels of epiblast marker Fgf5, mesoderm markers T, Wnt3 and transcription factor Tal1/Scl in day 0-10 WT and *SIRT1*$^{-/-}$ EBs. Day (D) 0 represents undifferentiated ES cells. Graphs were plotted in logarithmic scale. Data are relative to WT D0 control and mRNA levels of each gene were compared between WT and *SIRT1*$^{-/-}$ EBs at each time point. Results were the average of three independent experiments, each performed in triplicate. *, $p<0.05$.

Figure 18

A


Cdx2
Sox1
Nkx2-5
WT KO
Relative Expression (log10)
2
1
0
-1
*
D0 D2 D4 D6 D8 D10 D0 D2 D4 D6 D8 D10 D0 D2 D4 D6 D8 D10

B

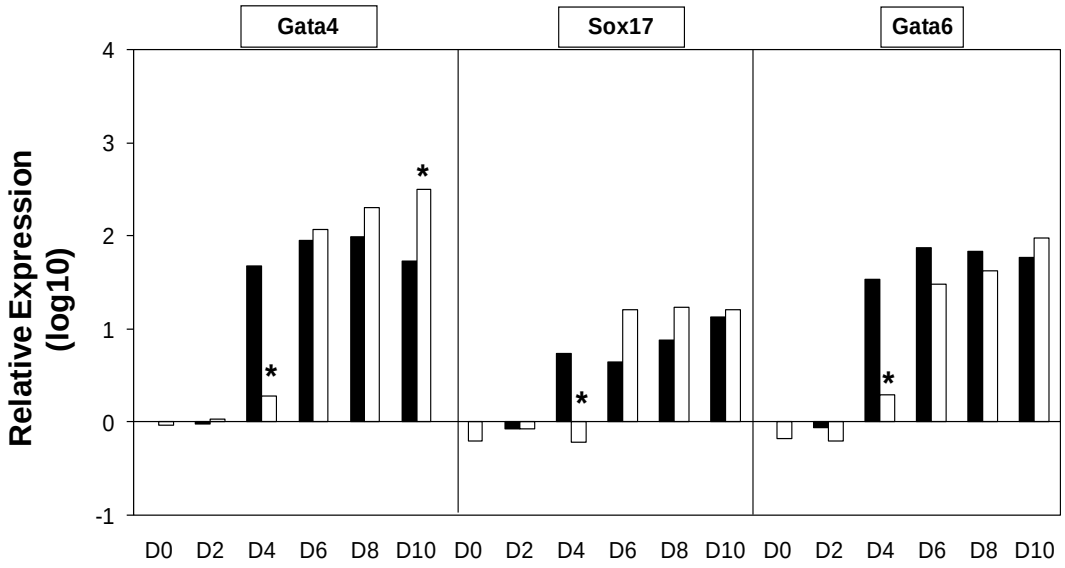
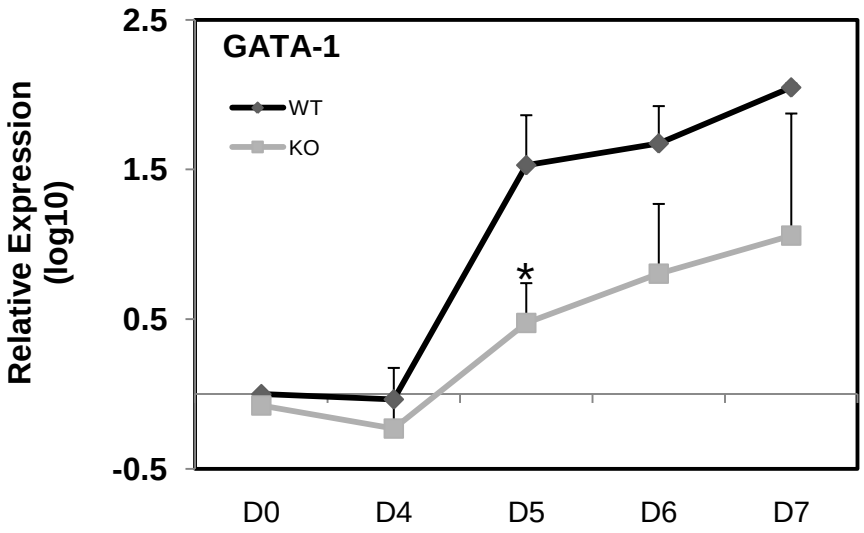

Gata4
Sox17
Gata6
Relative Expression (log10)
4
3
2
1
0
-1
*
D0 D2 D4 D6 D8 D10 D0 D2 D4 D6 D8 D10 D0 D2 D4 D6 D8 D10

Figure 18: Gene expression analysis of WT and *SIRT1*$^{-/-}$ EBs by real-time PCR analysis.

(A) qRT-PCR analysis of mRNA levels of trophectoderm marker Cdx2, neuroectoderm marker Sox1 and cardiac lineage marker Nkx2-5 in day 0-10 WT and *SIRT1*$^{-/-}$ EBs. (B) qRT-PCR analysis of mRNA levels of endoderm/mesoderm markers GATA-4, GATA6 and Sox17 in day 0-10 WT and *SIRT1*$^{-/-}$ EBs. Day (D) 0 represents undifferentiated ES cells. Graphs in (A-D) were plotted in logarithmic scale. Data are relative to WT D0 control and mRNA levels of each gene were compared between WT and *SIRT1*$^{-/-}$ EBs at each time point. Results were the average of three independent experiments, each performed in triplicate. *, $p<0.05$.

Figure 19

A


GATA-1
WT
KO
Relative Expression (log10)
2.5
1.5
0.5
-0.5
*
D0
D4
D5
D6
D7

B

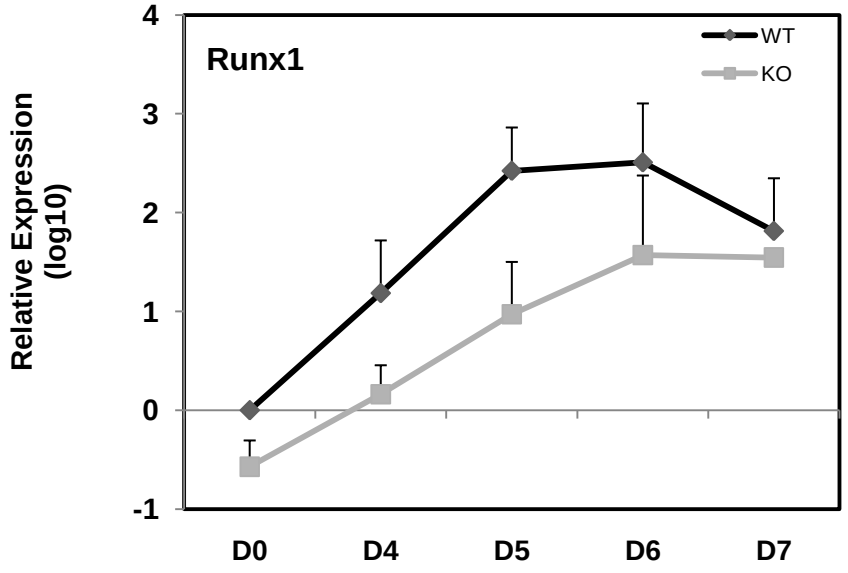

Runx1
WT
KO
Relative Expression (log10)
4
3
2
1
0
-1
D0
D4
D5
D6
D7

Figure 19: qRT-PCR analysis of mRNA levels of genes Gata-1 (A) and Runx-1(B) in day 0, day 4-7 WT and *SIRT1*$^{-/-}$ EBs. Data are relative to WT D0 control and mRNA levels of each gene were compared between WT and *SIRT1*$^{-/-}$ EBs at each time point. Results were the average of three independent experiments, each performed in triplicate. *, $p<0.05$.

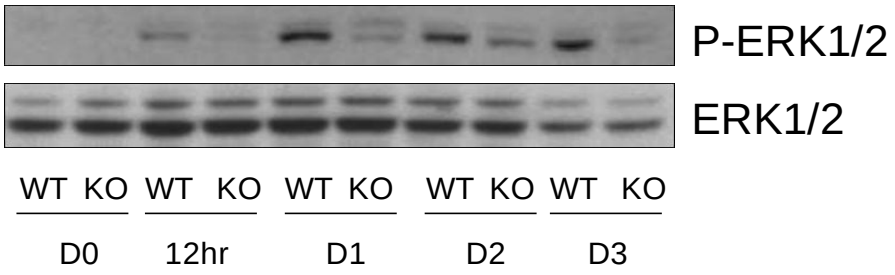
Aberrant signaling pathways during differentiation of *SIRT1*$^{-/-}$ ESCs

Activation of the Ras-Raf-Mek-Erk signaling cascade is important for morphological and gene expression changes indicative of early ES cell differentiation by LIF withdrawal (Lee, McCool et al. 2006). When the Erk pathway is inhibited in LIF withdrawn cells, the pluripotent marker Oct4 shows delayed down-regulation (Lee, McCool et al. 2006). SIRT1 inhibition reduces Erk1/2 signaling in cultured neurons (Li, Xu et al. 2008), so we assessed whether delay in loss of pluripotency markers in *SIRT1*$^{-/-}$ EBs is associated with effects on the Erk pathway during *SIRT1*$^{-/-}$ ES cell commitment. Phosphorylated Erk1/2 was gradually increased during early *SIRT1*$^{+/+}$ ES cell differentiation, consistent with a previous report (Lee, McCool et al. 2006). However, Erk phosphorylation was greatly impaired in *SIRT1*$^{-/-}$ cells (Figure 20A, B). Bone morphogenetic protein *(*BMP), fibroblast growth factor (FGF) and Wnt pathways are there major pathways involved in mesoderm commitment and development. To evaluate signaling pathways for mesodermal differentiation within EBs, high-throughput mRNA expression profiling was used to examine gene expression involved in bone morphogenetic protein *(*BMP), fibroblast growth factor (FGF) and Wnt pathways. Expression of Bmpr, smad8, and smad3 was delayed in *SIRT1*$^{-/-}$ compared with WT EBs. Smads are intracellular signal transducers of the BMP pathway. Among them, Smad7 is an inhibitory molecule for the BMP-SMAD pathway (Casellas and Brivanlou 1998). Smad7 inhibits mesoderm formation and promotes neural cell fate in Xenopus embryos (Bhushan, Chen et al. 1998). Of note, Smad7 was persistently expressed at higher levels in *SIRT1*$^{-/-}$ than in WT EBs (Figure 21). This is interesting in light of Smad7 down-regulation by SIRT1 (Kume, Haneda et al. 2007). Expression of several components of FGF and Wnt signal

transduction pathways were altered in *SIRT1*$^{-/-}$ EBs (Figure 22A, B). FGF and Wnt pathways are implicated in hematopoietic development through protein network interactions (Huelsken and Birchmeier 2001; Thisse and Thisse 2005), suggesting that SIRT1 deficiency may impair embryonic hematopoiesis indirectly through broad signal transduction networks. Our gene-array expression analysis indicates a role for SIRT1 in intracellular signaling during hematopoietic cell development.

Figure 20

A


P-ERK1/2
ERK1/2
WT KO WT KO WT KO WT KO WT KO
D0
12hr
D1
D2
D3

B

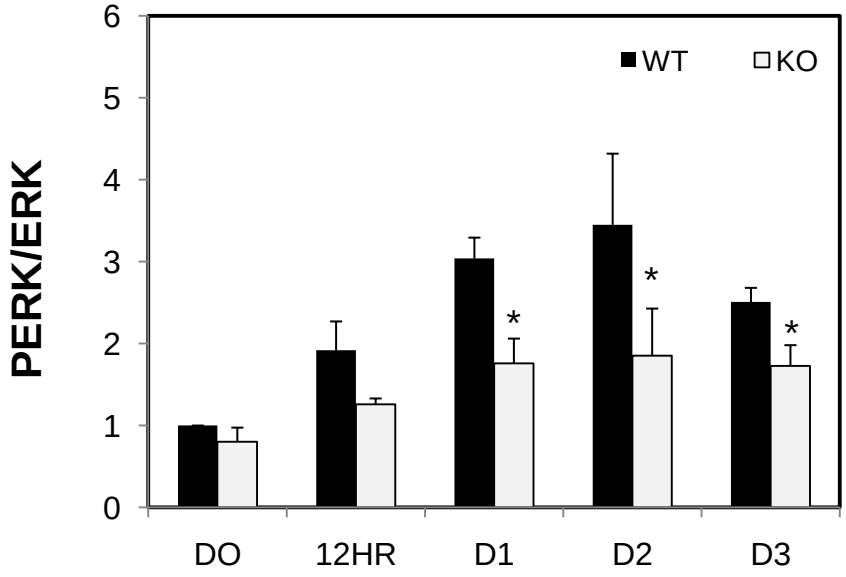

PERK/ERK
WT
KO
0
1
2
3
4
5
6
*
*
*
DO
12HR
D1
D2
D3

Figure 20: Western Blot analysis of Erk signaling pathways.

WT and SIRT1$^{-/-}$ EBs were collected at days 0, 12hr, 1, 2, and 3 upon ES cell differentiation. Cellular lysates were taken and western blot were performed for protein levels. (A) Representative blot and (B) quantification showing phosphorylation of Erk, and Erk1/2 protein expression at different time points during EB development. Data are shown as mean ± SEM. N=3; *, $p<0.05$.

Figure 21

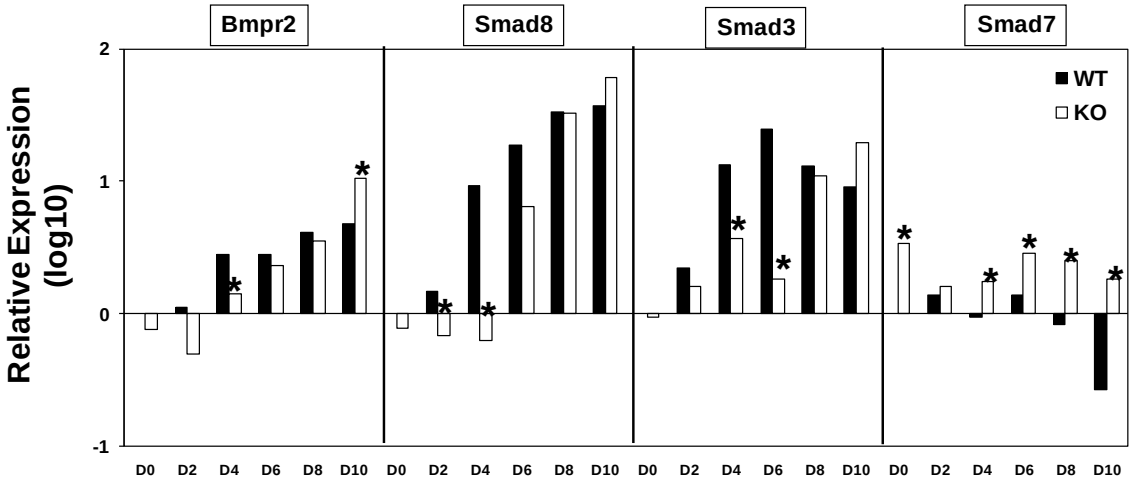
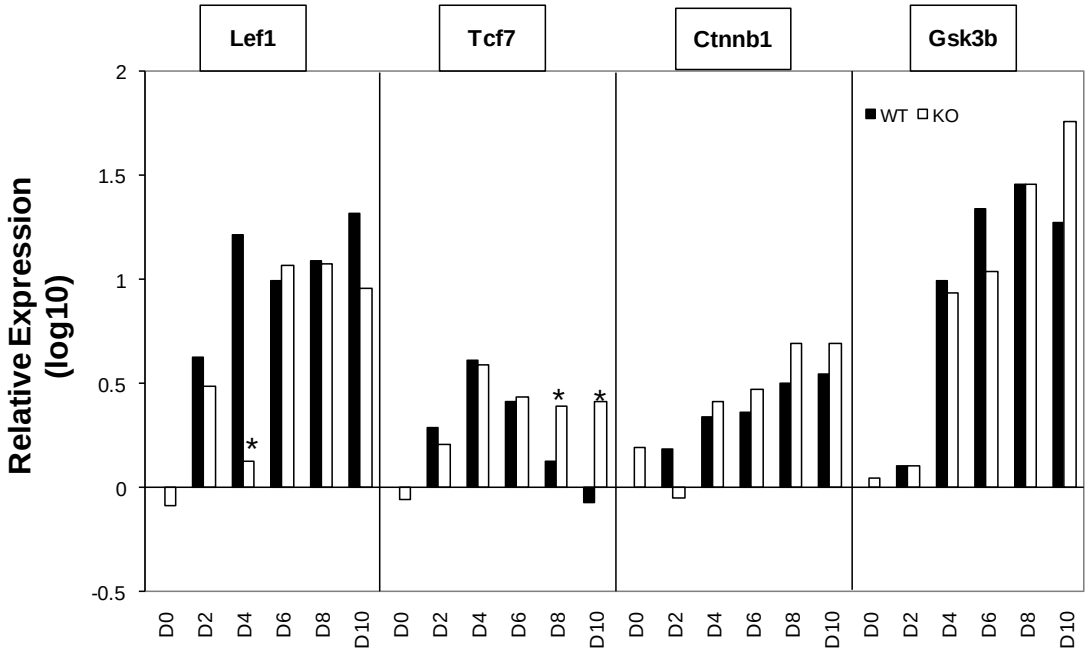

Bmpr2
Smad8
Smad3
Smad7
WT
KO
Relative Expression (log10)
2
1
0
-1
D0 D2 D4 D6 D8 D10

Figure 21: qRT-PCR analysis of mRNA levels of genes (BMPr2, Smad8, Smad3 and Smad7) involved in BMP pathways in day 0-10 WT and *SIRT1*$^{-/-}$ EBs.

D0 represents undifferentiated ES cells. Graphs were plotted in logarithmic scale. Data are relative to WT D0 control and mRNA levels of each gene were compared between WT and *SIRT1*$^{-/-}$ EBs at each time point. Results were of three independent experiments performed in triplicate. *, $p<0.05$.

Figure 22

A


Lef1
Tcf7
Ctnnb1
Gsk3b
WT KO
Relative Expression (log10)
2
1.5
1
0.5
0
-0.5
D0 D2 D4 D6 D8 D10

B

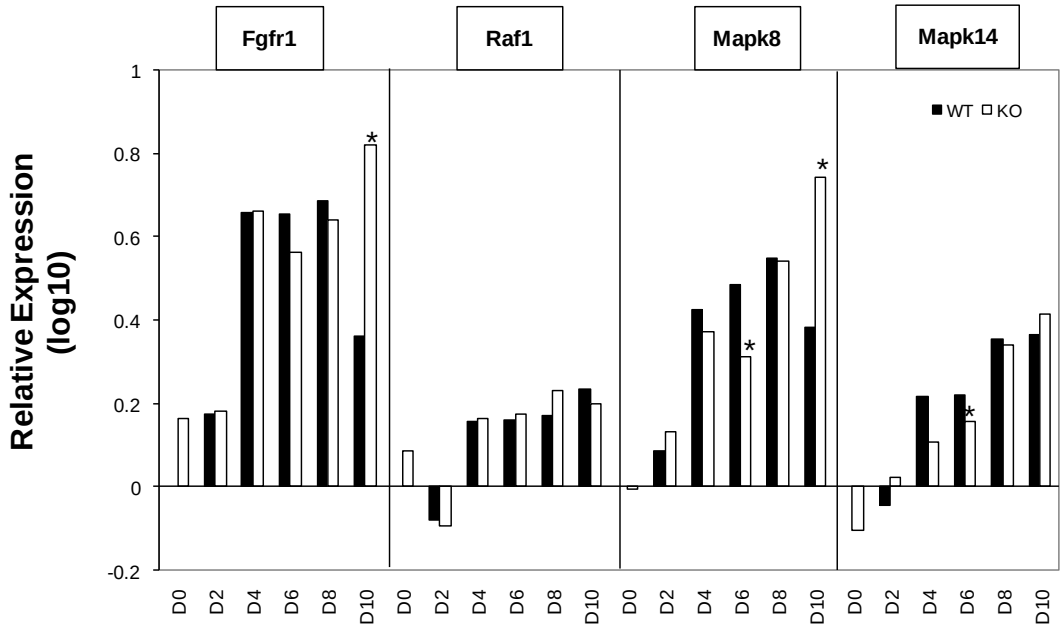

Fgfr1
Raf1
Mapk8
Mapk14
WT KO
Relative Expression (log10)
1
0.8
0.6
0.4
0.2
0
-0.2
D0 D2 D4 D6 D8 D10

Figure 22: (A) qRT-PCR analysis of mRNA levels of genes (Lef1, Tcf7, Ctnnb1 amd Gsk3b) involved in Wnt pathways in day 0-10 WT and *SIRT1*$^{-/-}$ EBs. (B) qRT-PCR analysis of mRNA levels of genes (Fgfr1, Raf1, Mapk8 and Mapk14) involved in FGF pathways in day 0-10 WT and *SIRT1*$^{-/-}$ EBs. Graphs were plotted in logarithmic scale. Data are relative to WT D0 control and mRNA levels of each gene were compared between WT and *SIRT1*$^{-/-}$ EBs at each time point. Results were the average of three independent experiments, each performed in triplicate. *, $p<0.05$.

Reintroduction of SIRT1 gene into *SIRT1*$^{-/-}$ ESCs rescues normal hematopoietic phenotype of *SIRT1*$^{-/-}$ EBs

To examine whether the abnormal differentiation phenotype of *SIRT1*$^{-/-}$ cells can be corrected by adding back a wild-type SIRT1 gene, *in vitro* gene reconstitution studies were performed. Lentiviral vectors were constructed containing EGFP IRES or the SIRT1 gene under control of a Ubc promoter (Figure 23A), giving levels of SIRT1 gene expression proportional to GFP fluorescence intensity. Vector containing EGFP gene alone was used as a control. ES cells were efficiently infected, as shown by flow cytometry 48 hours after infection (Figure 23B). ES cells with high level expression of GFP were sorted and analyzed for SIRT1 expression. Western blot analysis demonstrated that SIRT1 was expressed in *SIRT1*$^{-/-}$ cells transfected with the SIRT1 vector (Figure 23B). *SIRT1*$^{-/-}$ cells generated few EryP on day 4.5, but numbers of primitive progenitors in *SIRT1*$^{-/-}$ cells was significantly increased with reconstitution of SIRT1 to a level comparable to WT cells (Figure 24A). Also, expression of hemoglobin genes, Hbb-bh1 (Figure 24B) and Hbb-b1 (Figure 24C) was recovered in *SIRT1*$^{-/-}$ cells with the SIRT1 gene insert vector, compared with WT cells. This demonstrates that the altered hematopoietic differentiation potential observed for *SIRT1*$^{-/-}$ cells was specific to deletion of SIRT1.

Figure 23

A

pLenti6-Sirt1-Venus

pLenti6-Venus

B

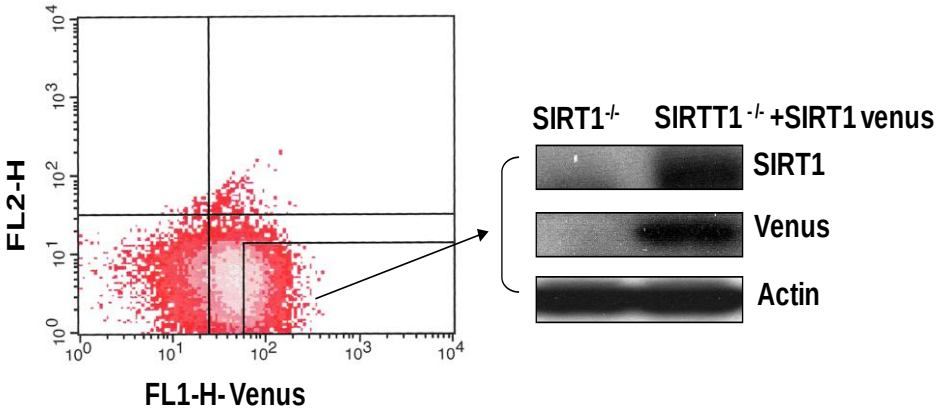
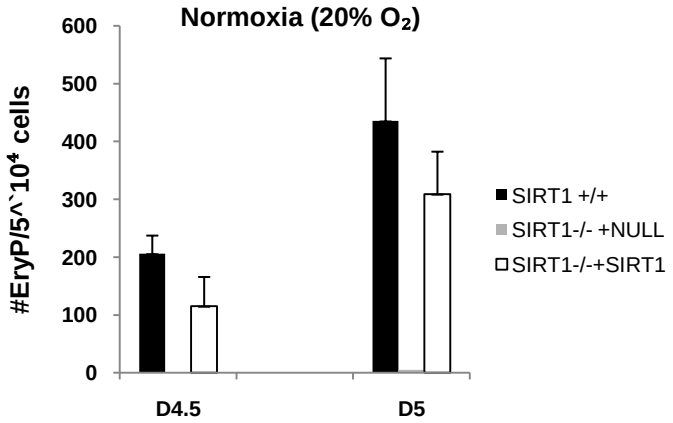


Figure 23: Ectopic expression of exogenous SIRT1 in *SIRT1*$^{-/-}$ ES cells.

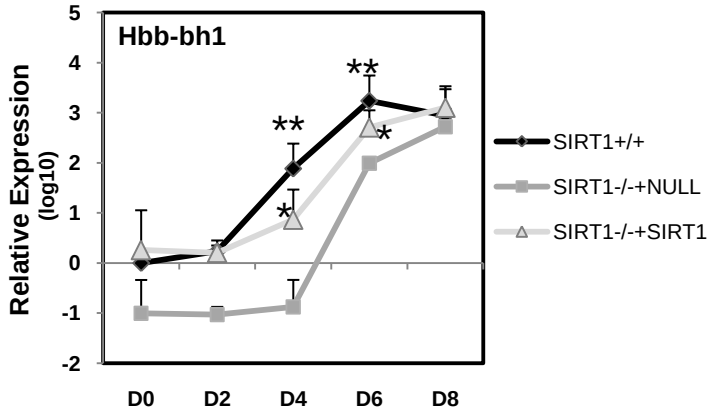
(A) Diagram of pLenti6-SIRT1-Venus lentiviral vector. (B) Transfected *SIRT1*$^{-/-}$ ES cells were sorted and expression of SIRT1 protein was determined by western blotting.

Figure 24

A



B



C

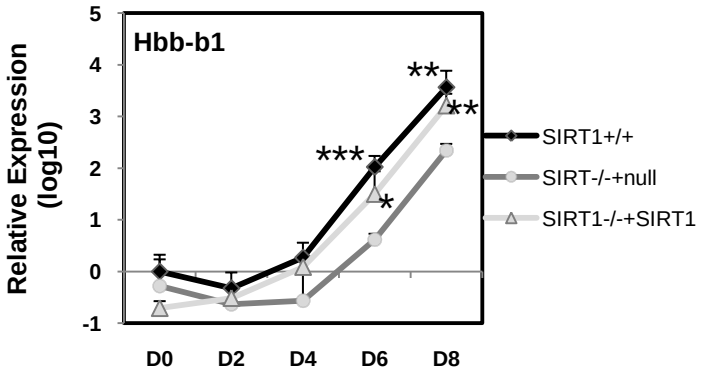


Figure 24: Reconstitution of SIRT1 in *SIRT1*$^{-/-}$ mES cells rescued the differentiation of primitive erythroid progenitors.

(A) Number of primitive erythroid colonies generated by day 4.5 and day 5 WT (*SIRT1*$^{+/+}$), *SIRT1*$^{-/-}$ + null, and *SIRT1*$^{-/-}$ + SIRT1 ES cells. (B, C) qRT-PCR analysis of mRNA levels of embryonic globin (Hbb-bh1) and adult globin (Hbb-b1) in Day (D) 0-8 WT (*SIRT1*$^{+/+}$), *SIRT1*$^{-/-}$ + null, and *SIRT1*$^{-/-}$ + SIRT1 EBs. Data represent fold changes in EBs, relative to *SIRT1*$^{+/+}$ D0 control and comparison between WT (*SIRT1*$^{+/+}$), *SIRT1*$^{-/-}$ + null, and *SIRT1*$^{-/-}$ + SIRT1 EBs at each time point. Results are average of three independent experiments. Each data point denotes six biological replicates; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. Data are shown as mean ± SEM.

Part II: SIRT1 deficiency compromises embryonic and adult hematopoiesis in the mouse

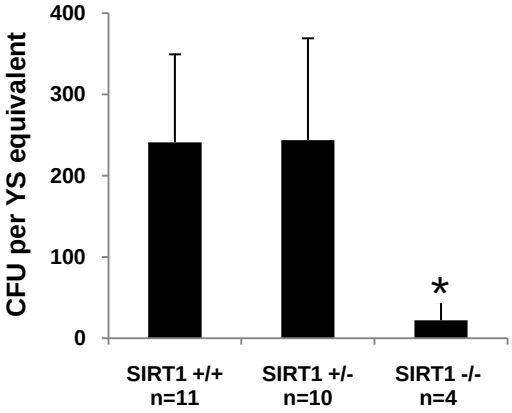
Defective primitive hematopoiesis during early development and decreased adult hematopoiesis in *SIRT1*$^{-/-}$ mice

Based on our above studies we evaluated embryo and adult bone marrow hematopoiesis in *SIRT1*$^{+/+}$, *SIRT1*$^{+/-}$ and *SIRT1*$^{-/-}$ mice (Wang, Sengupta et al. 2008). We performed *in vitro* colony-forming assays on yolk sac cells from E8 to E8.25 embryos. We observed significant decreases in absolute numbers of primitive erythroid progenitors from *SIRT1*$^{-/-}$, but not *SIRT1*$^{+/-}$, E8 to E8.25 yolk sacs under normoxic conditions compared to *SIRT1* $^{+/+}$ yolk sacs (Figure 25A). Genotypes of the yolk sacs (Figure 25B) were confirmed and the targeting vectors for deletion of SIRT1 were listed in Figure 25C. Less than 10% of *SIRT1*$^{-/-}$ mice survive to birth (Wang, Sengupta et al. 2008), but *SIRT1*$^{+/-}$ mice are born with normal Mendelian genetics. We assessed absolute numbers of hematopoietic progenitor cells (CFU-GM, BFU-E, and CFU-GEMM) per femur of 5 week old *SIRT1*$^{+/-}$ vs. *SIRT1*$^{+/+}$ mice detected via culturing bone marrow cells in vitro at both ~20% and 5% O_2 tension. In that way, we could determine if there were differences in progenitor cell numbers in *SIRT1*$^{+/-}$ and *SIRT1*$^{+/+}$ cells, and whether or not the growth of these cells could be enhanced by growth in lowered O_2. While we detected no significant differences in colony numbers of *SIRT1*$^{+/+}$ and *SIRT1*$^{+/-}$ cells grown *in vitro* under normoxic conditions from bone marrow of 5 week old mice, we found that *SIRT1*$^{+/+}$, but not *SIRT1*$^{+/-}$ cells were enhanced in numbers at lowered O_2 (Figure 26). Thus, *SIRT1*$^{+/-}$ cells do not sense enhanced growth conditions of lowered O_2. We were also able to evaluate *SIRT1*$^{+/+}$, *SIRT1*+/- and *SIRT1*$^{-/-}$ mice that were over one year old. As shown in Figure

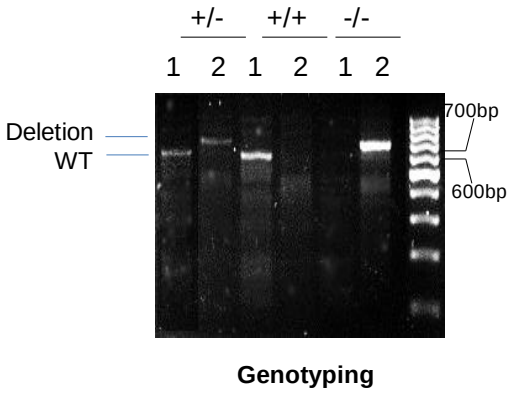
27A, absolute numbers of *SIRT1*$^{+/-}$ and *SIRT1*$^{-/-}$ progenitors were significantly decreased by almost half when cells were cultured under normoxic conditions, suggesting an age-related decrease in *SIRT*$^{+/-}$ numbers. Consistent with the *SIRT1*$^{+/-}$ data from 5 week old mice (Figure 26), neither *SIRT1*$^{+/-}$ nor *SIRT1*$^{-/-}$ progenitors from the more aged mice responded to the enhancing effects of lowered O_2 that the *SIRT1*$^{+/+}$ cells responded to, confirming that *SIRT1*$^{+/-}$, and also *SIRT1*$^{-/-}$ progenitors from adult bone marrow fail to respond to lowered O_2. At lowered O_2, the differences detected in numbers of progenitor cells in S-phase from *SIRT1*$^{+/+}$ mice to that of either *SIRT1*$^{+/-}$ or *SIRT1*$^{-/-}$, was substantially greater than that seen at normoxia (Figure 27B). Nucleated bone marrow cellularities/femur did not show significant differences between *SIRT1*$^{+/+}$, *SIRT1*$^{+/-}$, or *SIRT1*$^{-/-}$ mice (Table 3). We also assessed the *in vitro* survival of hematopoietic progenitor cells (CFU-GM) from 5 week and over 1 year old mice growing at 5% O_2 in response to growth factor deprivation and delayed addition of Epo, PWMSCM and TPO at day 1 after culture in the presence of FBS. Seven days after addition of cytokines there was a significantly greater decrease in colony formation for 5 week old bone marrow of *SIRT1*$^{+/-}$ mice and *SIRT1*$^{-/-}$ marrow of >1 year old mice compared to their comparable aged matched *SIRT1*$^{+/+}$ mice (Figure 28). This demonstrates that *SIRT1*$^{+/-}$ and *SIRT1*$^{-/-}$ progenitors survive less well than the *SIRT1*$^{+/+}$ progenitors, under these conditions of growth factor deprivation. Also, there was a significantly greater decrease in progenitor cells from older compared to younger *SIRT1*$^{+/+}$ mice.

Figure 25

A



B



C

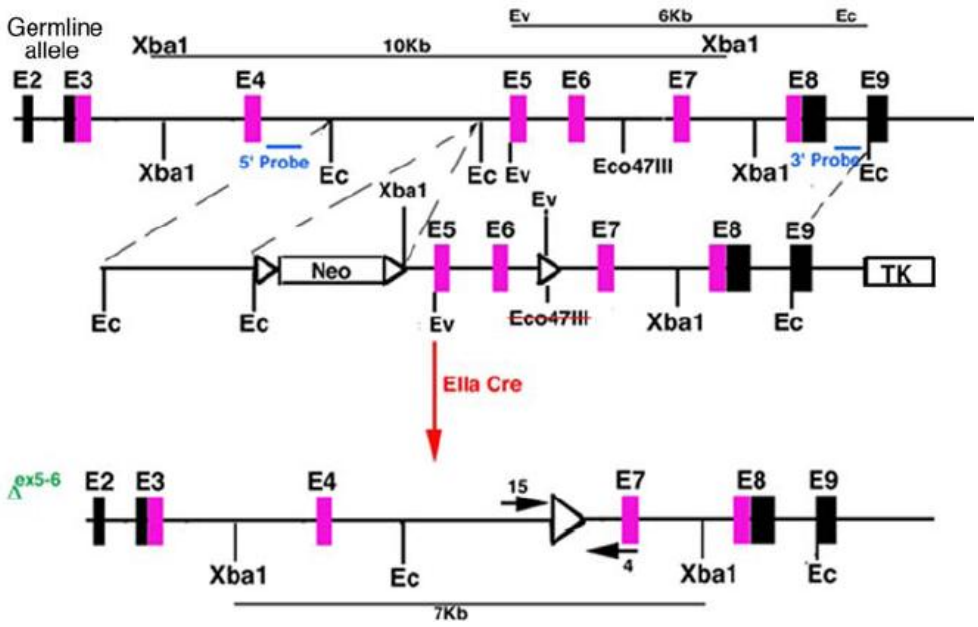


Figure 25: Hematopoietic progenitor cell colony forming assay from yolk sac of WT (*SIRT1*$^{+/+}$), *SIRT1*$^{+/-}$ and *SIRT1*$^{-/-}$ mice.

(A) Primitive erythroid colony formation from yolk sac cells generated from E8 to E8.25 *SIRT1*$^{+/+}$, *SIRT*$^{+/-}$ and *SIRT1*$^{-/-}$ embryos (*SIRT1*$^{+/+}$, n=11; heterozygous, n=10; *SIRT1*$^{-/-}$, n=4) were grown in methylcellulose-based medium. Data are shown as mean ± SD; *, $p<0.01$. (B) PCR shows the genotyping of SIRT1 mutant embryos. The primers are: forward-5'-TCCTTGCCACAGTCACTCAC-3', reverse for wild type 5'-CATCTAAACTTTGTTGGCTGC-3', reverse for deletion 5'-ACAGTCCCATTCCCAT ACC-3'. Primers 1 and 3 are located within intron4 and amplify the WT allele (600bp). Primer 2 located within exon7, the combination of primer1 and 2 amplifies the deleted allele (716bp). (C) Targeted disruption of SIRT1 in mouse. Figure C is adapted from (Wang, Sengupta et al. 2008).

Figure 26

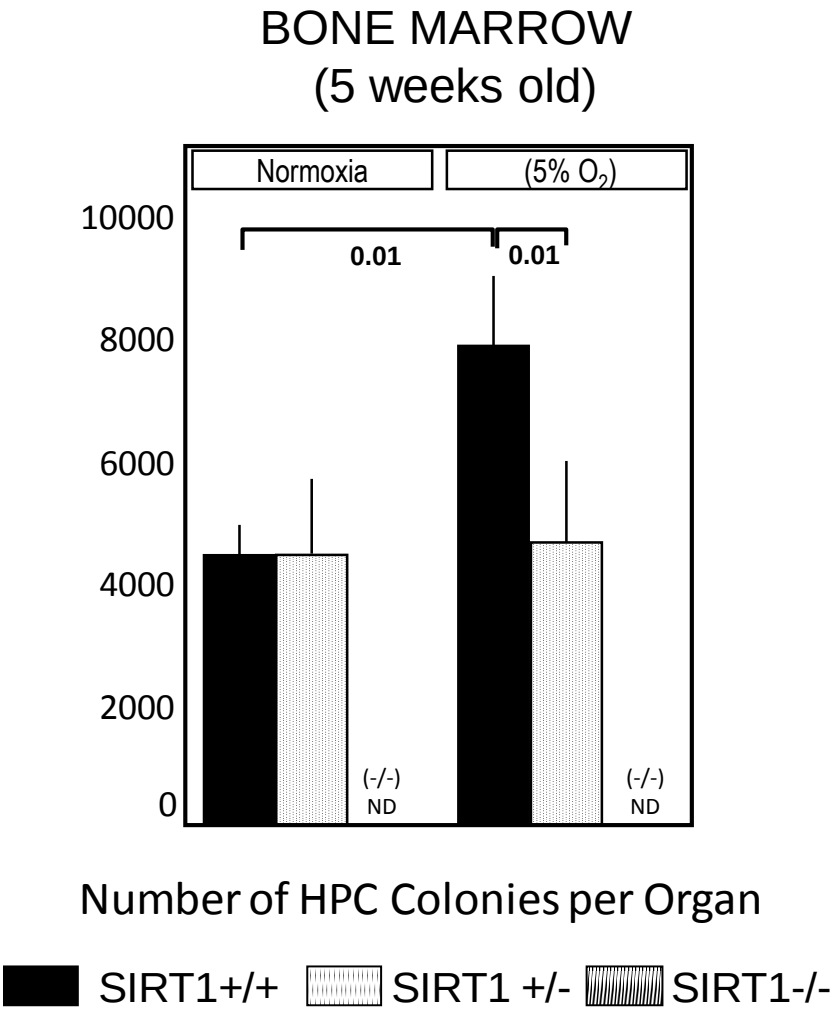
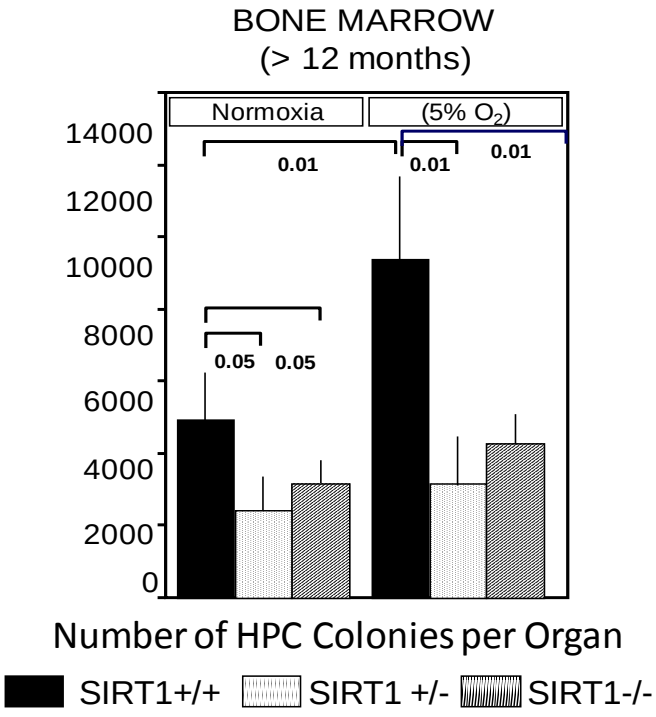

BONE MARROW
(5 weeks old)
Normoxia
(5% O_2)
10000
8000
6000
4000
2000
0
0.01
0.01
(-/-)
ND
(-/-)
ND
Number of HPC Colonies per Organ
SIRT1+/+
SIRT1 +/-
SIRT1-/-

Figure 26: Hematopoietic progenitor cell colony formation from bone marrow of 5 week old SIRT1 heterozygote (*SIRT1*$^{+/-}$) or WT controls under normoxia (20%O_2) and 5% O_2 conditions. Data are shown as mean ± SD; ND=*SIRT1*$^{-/-}$ cells were not available for use and were not done. Data are the average of 3-5 mice/group. Numbers of HPC colonies were compared between WT mice under normoxia and 5% O_2 and WT and SIRT1+/- mice under normoxia and 5% O_2. P values for each group comparison were notified in the figure.

Figure 27

A


BONE MARROW
(> 12 months)
Normoxia
(5% O2)
14000
12000
10000
8000
6000
4000
2000
0
0.01
0.01
0.01
0.05
0.05
Number of HPC Colonies per Organ
SIRT1+/+
SIRT1 +/-
SIRT1-/-

B

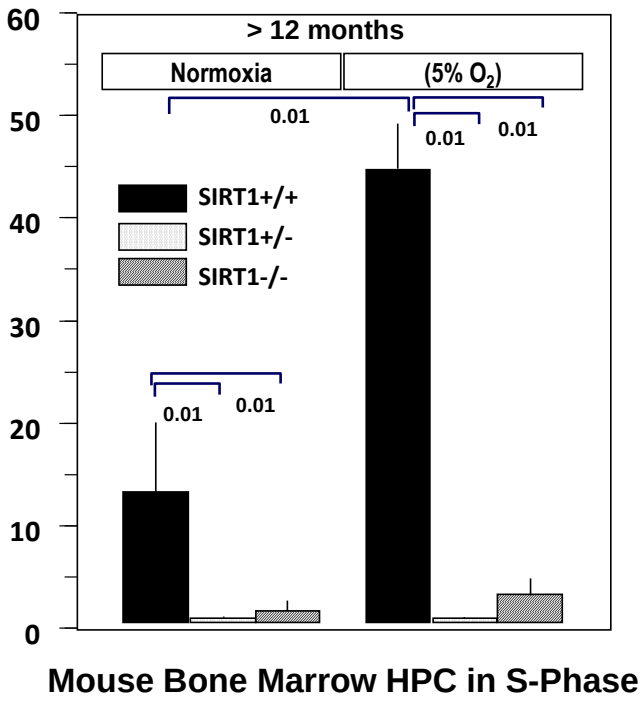

60
50
40
30
20
10
0
> 12 months
Normoxia
(5% O2)
0.01
0.01
0.01
SIRT1+/+
SIRT1+/-
SIRT1-/-
0.01
0.01
Mouse Bone Marrow HPC in S-Phase

Figure 27: (A) Hematopoietic progenitor cell colony formation from bone marrow of 12 month old SIRT1 heterozygote (*SIRT1*$^{+/-}$) or homozygote (*SIRT1*$^{-/-}$) null mice or from WT controls under normoxia (20%O_2) or 5% O_2 conditions. (B) Cycling status of mouse bone marrow progenitor cells generated from 12 months old WT (*SIRT1*$^{+/+}$), *SIRT1*$^{+/-}$ and *SIRT1*$^{-/-}$ mice under normoxia (20%O_2) and 5% O_2 conditions. This shows percent progenitors in DNA synthesis (S-phase) as determined by high specific activity tritiated thymidine kill technique (Broxmeyer, Orschell et al. 2005). P values were calculated with Student's t test. ND= not done. Data are the average of 3-5 mice/group.

Figure 28

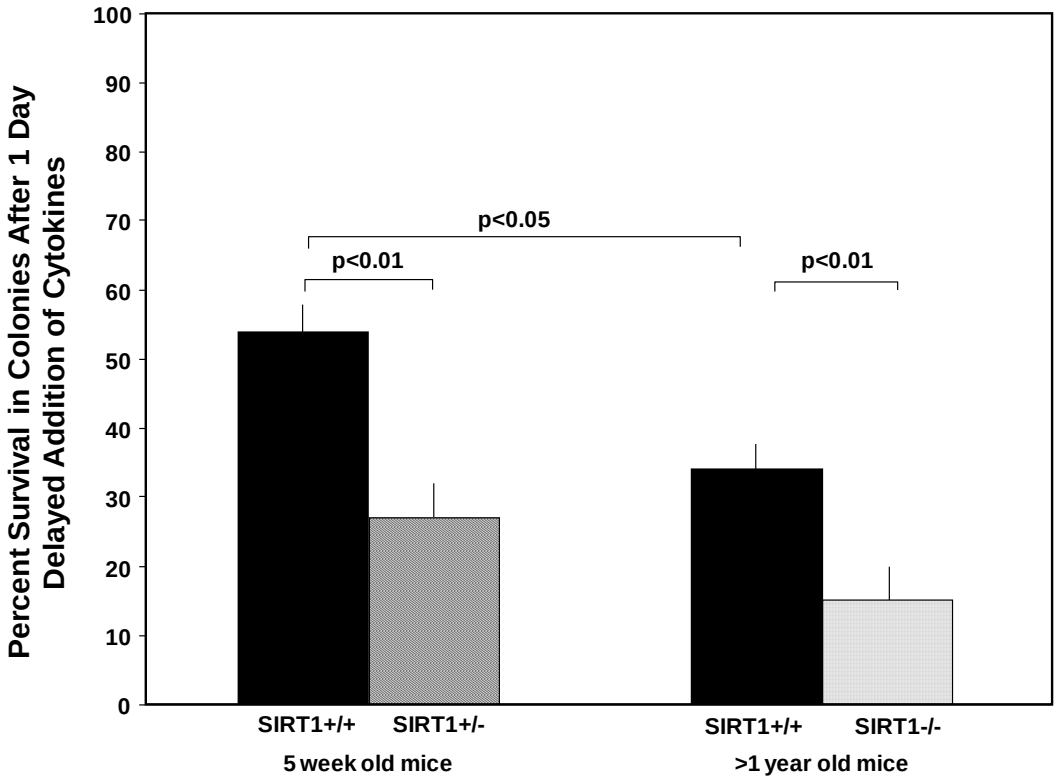

Percent Survival in Colonies After 1 Day Delayed Addition of Cytokines
100
90
80
70
60
50
40
30
20
10
0
p<0.05
p<0.01
p<0.01
SIRT1+/+
SIRT1+/-
SIRT1+/+
SIRT1-/-
5 week old mice
>1 year old mice

Figure 28: Survival of hematopoietic progenitor cells *in vitro* from bone marrow of 5 week old SIRT1 heterozygote ($SIRT1^{+/-}$) or WT controls ($SIRT1^{+/+}$), and from bone marrow of 12 month old homozygote ($SIRT1^{-/-}$) null mice or from WT controls ($SIRT1^{+/+}$) after 1 day delayed addition of cytokines as denoted by decreased colony formation. Results are expressed as mean ±ISEM for 4-5 mice per group.

Table 3: Nucleated bone marrow cellularity of WT, *SRIT1*$^{+/-}$ and *SIRT1*$^{-/-}$ mice

	NUCLEATED CELLULARITY (BONE MARROW)					
	5 WEEKS OLD			>12 MONTHS		
	MEAN	SEM	P	MEAN	SEM	P
WT (+/+)	15.4	1.0	CONTROL	20.4	2.4	CONTROL
HET(+/-)	15.3	3.0	0.490	14.4	4.4	0.230
KO (-/-)	ND	ND	ND	15.1	2.7	0.105

Table 3: Nucleated cellularity/femur for WT, *SIRT1*$^{+/+}$ and *SIRT1*$^{-/-}$ mice of 5 weeks and over a year old. ND=not done. Data are the average of 3-5 mice/group.

DISCUSSION

Although roles of SIRT1 in cell differentiation, self-renewal and corresponding signal transduction pathways have been investigated previously in different cell types, roles of SIRT1 during ES cell differentiation commitment and for ES cell self-renewal are poorly understood. In this study, using an ES cell/EB *in vitro* differentiation system, our results demonstrated that loss of SIRT1 delayed the timing of ES cells to exit the pluripotent stem cell program upon LIF withdrawal, and SIRT1 deficiency compromises hematopoietic lineage differentiation of mouse ES cells, while SIRT1 is dispensable for self-renewal of ES cells.

With regard to differentiation, SIRT1 has been shown to regulate skeletal muscle gene expression and differentiation as a potential sensor of the redox state (Fulco, Schiltz et al. 2003). SIRT1 forms a complex with the acetyltransferase PCAF and MyoD and, when overexpressed, retards muscle differentiation. Conversely, cells with decreased SIRT1 differentiate prematurely (Fulco, Schiltz et al. 2003). SIRT1 also regulates cell differentiation in pig preadipocytes and normal human keratinocytes (Bai, Pang et al. 2008; Blander, Bhimavarapu et al. 2009). SIRT1 mRNA was widely expressed in various pig tissues from different developmental stages and throughout the entire differentiation process of pig preadipocytes. Resveratrol (SIRT1 activator) significantly inhibited pig preadipocyte proliferation and differentiation, while nicotinamide (SIRT1 inhibitor) greatly stimulated the proliferation and differentiation of pig preadipocytes (Bai, Pang et al. 2008). In keratinocytes, nicotinamide inhibited expression of keratinocyte differentiation markers, whereas resveratrol enhanced expression of keratinocyte differentiation markers. Similar results were obtained in keratinocytes manipulated to

overexpress or underexpress SIRT1 (Blander, Bhimavarapu et al. 2009). Most notably, under oxidative conditions, SIRT1 participates in suppression of neurogenesis. Direct activation of SIRT1 suppressed proliferation of nuclear progenitor cells (NPCs), whereas reducing conditions had the opposite effect (Prozorovski, Schulze-Topphoff et al. 2008). In contrast, under normal conditions, overexpression of SIRT1 enhances neuronal differentiation. The number of neurospheres was significantly decreased by pharmacological inhibitors of SIRT1, dominant-negative SIRT1 and SIRT1-siRNA, whereas overexpression of SIRT1 enhanced neuronal differentiation and decreased Hes1 expression (Hisahara, Chiba et al. 2008; Prozorovski, Schulze-Topphoff et al. 2008). Based on these previous reports, it is possible that a role for SIRT1 in promoting or suppressing differentiation may be condition-related and cell-type specific.

In our study, *SIRT1*$^{-/-}$ ES cells did not show marked differences in morphology and expression of mES-specific markers compared to WT R1 mES cells (Figure 6A, B), suggesting that SIRT1 is dispensable for maintain pluripotency of mES cells. We recently reported that under oxidative stress, in the absence of 2-ME, SIRT1 is important for maintenance of ESCs by inhibiting p53-mediated suppression of Nanog expression (Han, Song et al. 2008). However, in this present study with LIF-withdrawal and in the presence of 2-ME, SIRT1 clearly plays a role in differentiation from mesoderm to specific hematopoietic lineage commitment. We compared the expression of acetylated-p53 upon ES cell differentiation between WT and *SIRT1*$^{-/-}$ cells with LIF-withdrawal and in the presence of 2-ME. However, we did not find significant difference of expression of acetylated-p53 between WT and *SIRT1*$^{-/-}$ ES cells (data not shown), demonstrating that SIRT1 is a condition-sensitive protein and might play distinct roles on differentiation

under specific conditions via different pathways. Recently, Calvanese et al. (Calvanese, Lara et al. 2010) published that elimination of SIRT1 in mouse TC1 ES cells has little impact on expression of pluripotency factors and SIRT1 regulation of developmental genes during differentiation of mouse and human ES cells. The impact of SIRT1 silencing on the expression of pluripotency and differentiation markers are much greater after induction of differentiation, which is consistent with our work that SIRT1 is dispensable for self-renewal of ES cells, but regulates the genes involved in differentiation.

Although functional requirements for SIRT1 during embryogenesis and gametogenesis have been demonstrated in mammals (McBurney, Yang et al. 2003), roles for SIRT1 during ES cell differentiation commitment are poorly understood. In this study, using an ES cell/EB *in vitro* differentiation system, we demonstrate that loss of SIRT1 affects the potential and timing of ES cells to exit the pluripotent stem cell program upon LIF withdrawal. The most apparent defect identified is likely delayed shut off of Oct4 and Nanog (Figure 17A) and persistent Fgf5 (Figure 17B) expression, which could account for diminished mesoderm commitment. Inactivation of Oct4 occurs concomitantly with deacetylation of its promoter region shortly after differentiation begins (Feldman, Gerson et al. 2006) and the level of Nanog decreases rapidly following LIF withdrawal (Torres and Watt 2008). Upon removal of LIF or induction of differentiation by addition of external signals, *SIRT1*$^{+/+}$ ES cells are able to rapidly down-regulate these genes in response to external signals. However, as demonstrated herein, the ability to switch off Oct4 and Nanog expression is delayed by loss of SIRT1. Early differentiation of ES cells into primitive ectoderm, key to formation of the three germ layers, was unaffected, as

suggested by the presence of Oct4 and Fgf5 expression in day 2 EBs. However, *SIRT1*$^{-/-}$ EBs persistently express high levels of Fgf5 from day 6 to 10. Further analysis of EB transcriptional profiles indicates delayed and sustained expression of specific mesoderm markers (T and Wnt3) in *SIRT1*$^{-/-}$ EBs. The delayed/sustained Fgf5/T/Wnt3 (Figure 17B) gene expression supports functional data regarding delays/defects of development of BL-CFCs and generation of *Flk-1*$^{+}$ *c-Kit*$^{-}$ cells in *SIRT1*$^{-/-}$ EBs. *SIRT1*$^{-/-}$ ES cells did not show marked differences in morphology and expression of mES-specific markers, indicating that in unperturbed conditions, SIRT1 is not required to maintain the undifferentiated phenotype state. This is consistent with previous findings (McBurney, Yang et al. 2003) and studies suggesting that elimination of SIRT1 in mouse TC1 ES cells has little impact on expression of pluripotency factors (Calvanese, Lara et al. 2010). However, our present study, in light of the latter work indicating SIRT1 regulation of developmental genes during differentiation of mouse and human ES cells, strongly suggests that SIRT1 depletion most dramatically affected target gene expression profiles during early stage ES cell commitment to differentiation.

Our results also suggest that SIRT1 deficiency impairs proper differentiation of blast cell colonies, the hematopoietic developmental potential of blast cells *in vitro*, and to a lesser extent of endothelial cells where primitive vascular network formation is impaired. Hematopoietic progenitor differentiation of *SIRT1*$^{-/-}$cells is defective. Low level mRNA expression of genes associated with hematopoietic development such as Scl, β-H1 globin and β-major globin was detected at days 4, 6, 8 in *SIRT1*$^{-/-}$ EBs (Figure 17B), consistent with the early defect in primitive and definitive hematopoiesis. Basic helix-loop-helix transcription factor Scl is necessary for the early stages of *in vitro* differentiation of

mesoderm to hematopoietic lineages and acts during a limited time window (D'Souza, Elefanty et al. 2005). *Scl*$^{-/-}$ ES cells were not able to form blast colonies, but did form transitional colonies (Robertson, Kennedy et al. 2000). Transitional colonies derived from *Scl*$^{-/-}$ ES cells are unable to generate hematopoietic precursors but do form cells with endothelial characteristics (Robertson, Kennedy et al. 2000). Induction of Scl at later than day 4 could not restore competence to differentiate to hematopoietic progenitor cell fate (D'Souza, Elefanty et al. 2005). Mesodermal cells that failed to express Scl at the right stage appear to be irreversibly excluded from a hematopoietic progenitor fate. In our study, we found that *SIRT1*$^{-/-}$ EBs has the same phenotype as *Scl*$^{-/-}$ EBs. *SIRT1*$^{-/-}$ ES cells generated a greater number of transitional colonies instead of blast colonies. *SIRT1*$^{-/-}$ ES cells were able to form endothelial lineages, but were defective in formation of a full vascular-like network. The progression of hematopoiesis of blast cells was defective in *SIRT1*$^{-/-}$ EBs. Thus, decreased expression of Scl, combined with sustained expression of Fgf5/T, at the mesoderm stage in *SIRT1*$^{-/-}$ EBs may explain its inability to adequately generate mature blast colonies and progress into primitive hematopoietic commitment. In addition, the transitional colonies formed during *SIRT1*$^{-/-}$ BL-CFC culture system had the similar morphology as those formed from *Scl*$^{-/-}$ EBs. Whether the defect in the formation of mature blast colonies from *SIRT1*$^{-/-}$ ES cells would be rescued by the reconstitution of Scl at the early time points and whether SIRT1 regulates Scl directly at the post-translational level or indirectly at the transcription level will be an interesting avenue of further study.

ES cell pluripotency and cell fate determination are profoundly affected by MAPK, BMP, FGF and Wnt signaling pathways (Loebel, Watson et al. 2003). Ras/Erk pathway

promotes differentiation of and suppresses self-renewal of mouse ES cells. Lee et al. showed that when the Erk pathway is inhibited in LIF withdrawn cells, the pluripotent marker Oct4 shows delayed downregulation (Lee, McCool et al. 2006). As shown in Figure 17A, pluripotent markers Oct and Nanog both showed delayed down-regulation in *SIRT1*$^{-/-}$ EBs. Phosphorylated Erk1/2 was gradually increased during WT ES cell differentiation, consistent with previous report (Lee, McCool et al. 2006). As noted, phosphorylation of Erk is less activated in *SIRT1*$^{-/-}$ cells. The delayed down-regulation of Oct4 and Nanog in *SIRT1*$^{-/-}$ cells can be explained by the aberrant Erk signaling pathways. Taken together, we proposed a mole for SIRT1-mediated regulation of hematopoietic differentiation of mESCs as shown in Figure 29. In our model, the hemangioblast was listed as the progenitor give rise to an intermediate-hemogenic endothelium and further differentiate into both hematopoiesis and vasculogenesis. Recent studies provided evidence for the clonal origin of hematopoietic cells from a single hemogenic endothelial cell in vitro and for the generation of hematopoietic stem cells from hemogenic endothelium in vivo directly, which challenged the old concept of the hemangioblast and existence of the hemangioblast in vivo (Boisset, van Cappellen et al. 2010; Lancrin, Sroczynska et al. 2010; Swiers, Speck et al. 2010). However, this study result has been refuted by a recent study demonstrating that the existence of a population of hemangioblasts in the adult mouse uterus, adding substance to the long-lasting debate on the persistence of these ancestral progenitor cells in the developed organism (Peault 2010; Sun, Zhang et al. 2010). These intriguing observations revive a continuing debate and should stimulate further experimentation to confirm and document the presence of

hemangioblast and angio-hematopoietic stem cells, therefore understanding how blood cells are generated.

The formation of blood in the blood in the embryo is dependent on BMP. In Xenopus, formation of primitive and definitive blood requires BMP signaling (Walmsley, Ciau-Uitz et al. 2002). BMPs promote blood formation by specifying mesoderm during early dorsoventral (DV) formation. BMP induction during early gastrulation causes excess ventral-posterior (V-P) mesoderm and enhanced hematopoiesis. Moreover, specific involvement of the BMP signaling transducers Smad1, Smad5 and Smad7 has been demonstrated in blood development (Lengerke, Schmitt et al. 2008). The inhibitory Smad for BMP signaling, Smad7, is involved in early patterning events. Low levels of Smad7 block activation of dorsal mesoderm genes, and high levels block all mesoderm gene expression in Xenopus embryos (Bhushan, Chen et al. 1998). Kume et al. showed that in mesangial cells, SIRT1 directly interacts with the N-terminus of Smad 7. Furthermore, SIRT1 reversed acetyl-transferase (p300) mediated acetylation of two lysine residues (Lys-64 and 70) on Smad7. The Smad7 expression level was increased by SIRT1 knock-down and reduced by SIRT1 overexpression. SIRT1-mediated deacetylation of Smad7 enhanced Smad ubiquitination regulatory factor 1 (Smurf1) mediated ubiquitin proteasome degradation, which contributed to the low expression of Smad7 in SIRT1 overexpression mesangial cells. (Kume, Haneda et al. 2007). We found by qRT-PCR, that Smad7 is persistently expressed in *SIRT1*$^{-/-}$ EBs. This persistence may account for defective hematopoietic differentiation in *SIRT1*$^{-/-}$ EBs. Based on the above study in mesangial cells, it will be interesting to explore further whether SIRT1 interacts with

Smad7 at the protein level and reverses acetyl-transferase (p300) mediated acetylation of two lysine residues (Lys-64 and 70) on Smad7 in ES cells.

Evidence from mouse models indicates that SIRT1 is a central regulator of embryonic and somatic stem cell function (Han, Song et al. 2008; Prozorovski, Schulze-Topphoff et al. 2008). We found that lack of SIRT1 expression led to decreases in primitive erythroid progenitor cells in E8 to E8.25 yolk sacs. Since the majority of SIRT1 null embryos die between E9.5 and E14.5, we did not have access to *SIRT1*$^{-/-}$ embryos at later time points. There is ample evidence that *SIRT1*$^{-/-}$ mice have developmental defects such as markedly smaller size as compared to their littermates and slower development. We cannot exclude the possibility the decrease in Ery-P is due to a developmental delay in *SIRT1*$^{-/-}$ embryos. Consistent with studies of Wang et al. (Wang, Sengupta et al. 2008), *SIRT1*$^{-/-}$ embryos were abnormally small. In addition, these embryos show nuclear fragmentation and cell death. We also found that hematopoietic progenitor cells from *SIRT1*$^{+/+}$ and *SIRT1*$^{-/-}$ mice survive less well *in vitro* at lowered O_2 tension after the stress of delayed addition of growth factors. Decreased survival is directly associated with enhanced apoptosis (Broxmeyer, Cooper et al. 2003). This could be another reason for differences in numbers of Ery-P in *SIRT1*$^{-/-}$ embryos. Analysis of bone marrow of *SIRT1*$^{-/-}$ adult mice demonstrated decreased hematopoietic progenitor cell numbers and cell cycle status. The decrease was more apparent when cells were cultured under lowered O_2 tension. This is of interest since during hypoxia, activated SIRT1 augments hypoxia-inducible factor 2 alpha (HIF-2α) signaling and consequently participates in regulation of the HIF-2α target gene, Epo (Dioum, Chen et al. 2009). Congenital SIRT1 deficiency affects fetal and adult Epo gene expression in mice (Dioum, Chen et al. 2009). Epo, generally considered an

erythropoietic growth factor, is also a potent prosurvival factor that protects developing stem and progenitor cells in a variety of organs (Noguchi, Asavaritikrai et al. 2007). It would be very interesting to explore further whether SIRT1, HIF-2α and Epo signaling would be associated with the defective phenotype of *SIRT1*$^{-/-}$ mice we demonstrated. Renal Epo gene expression differs between *SIRT1*$^{+/-}$ and *SIRT1*$^{+/+}$ mice exposed to 6% O_2, while both groups of mice have similar amounts of renal Epo mRNA under ambient O_2 (Dioum, Chen et al. 2009). Similarly, our data showed that hematopoietic progenitor colony formation from adult mice significantly differed between *SIRT1*$^{+/+}$ and *SIRT1*$^{+/-}$ mice under 5% O_2 while both groups of mice had similar numbers under normoxia.

Sirtuins are implicated in aging (Donmez and Guarente 2010). *SIRT1*$^{+/-}$ mice are born with apparently normal mendelian frequency, while less than 10% of *SIRT1*$^{-/-}$ mice live to birth. It is not yet clear why some *SIRT1*$^{-/-}$ mice live to birth while others don't. We detected no significant differences in primitive erythroid progenitors generated between *SIRT1*$^{+/-}$ and *SIRT1*$^{-/-}$ embryos, similar to HPC numbers from 5 week old *SIRT1*$^{+/-}$ and *SIRT1*$^{+/+}$ mice when cells were cultured under normoxia. That *SIRT1*$^{+/-}$ cells from adult mice (>12 months) demonstrate decreased hematopoietic progenitor cell numbers at normoxia, similar to that of *SIRT1*$^{-/-}$ cells and that *SIRT1*$^{+/-}$ and *SIRT1*$^{-/-}$ cells in the aged mice show even greater differences compared with *SIRT1*$^{+/+}$ mice when cells are cultured at lowered O_2 allows us to evaluate a role for SIRT1 in the hematopoietic aging process, as there will be more *SIRT1*$^{+/-}$ than *SIRT1*$^{-/-}$ mice surviving to older ages.

Analyses of *SIRT1*$^{-/-}$ mice revealed that this protein serves essential functions during embryonic and postnatal development, as well as for several homeostatic programs during adulthood (Cheng, Mostoslavsky et al. 2003; McBurney, Yang et al. 2003;

Coussens, Maresh et al. 2008; Narala, Allsopp et al. 2008). Our results demonstrate that SIRT1 is an important regulator of mES cell differentiation and hematopoiesis, and suggest its role in the earliest stages of hematopoietic and endothelial development. Since hematopoietic and endothelial development is not extinguished by knock-out of SIRT1, and definitive hematopoiesis is less affected compared with primitive hematopoiesis, there might be distinct pathways of differentiation for primitive and definitive hematopoiesis, or compensation by other sirtuin, or non-sirtuin family members.

Figure 29

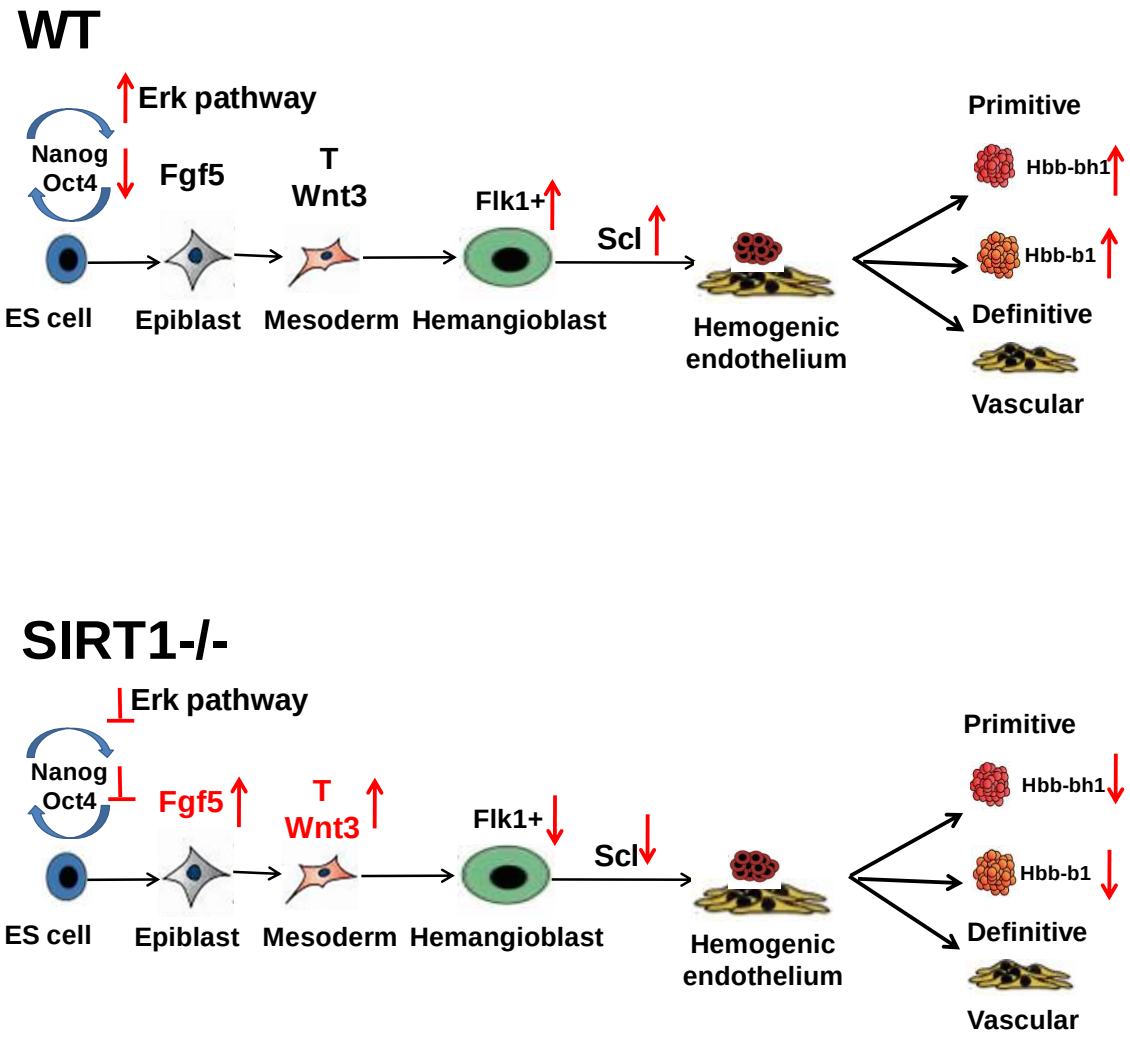

WT
Erk pathway
Nanog
Oct4
Fgf5
T
Wnt3
Flk1+
Scl
Primitive
Hbb-bh1
Hbb-b1
Definitive
Vascular
ES cell
Epiblast
Mesoderm
Hemangioblast
Hemogenic endothelium
SIRT1-/-
Erk pathway
Nanog
Oct4
Fgf5
T
Wnt3
Flk1+
Scl
Primitive
Hbb-bh1
Hbb-b1
Definitive
Vascular
ES cell
Epiblast
Mesoderm
Hemangioblast
Hemogenic endothelium

Figure 29: Proposed model for SIRT1–mediated regulation of hematopoietic differentiation of mES cells.

Upon removal of LIF or induction of differentiation by addition of external signals, WT ES cells are able to rapidly upregulate of phosphorylation of Erk1/2 and downregulate the expression of Oct4 and Nanog in response to external signals. Next, Fgf5, a gene expressed in the epiblast of early embryos, down-regulated after pre-hemangioblast mesoderm stage in WT ES cells. Then T, Wnt3 and Scl were upregulated for mesoderm commitment and progression into hematopoietic differentiation of WT ES cells. However, in *SIRT1-/-* ES cells, the ability to switch off Oct4 and Nanog expression and activation of Erk pathway is delayed by loss of SIRT1. *SIRT1*$^{-/-}$ EBs persistently express high levels of Fgf5 from day 6 to 10. The delayed/sustained Fgf5/T/Wnt3 gene expression and decreased Scl expression supports functional data regarding delays/defects of development of BL-CFCs, generation of *Flk-1*$^{+}$*/c-Kit*$^{-}$ cells in *SIRT1*$^{-/-}$ EBs, and generation of primitive and definitive hematopoietic progenitor cells.

FUTURE DIRECTIONS

Testing SIRT1 activators and inhibitors in mES cell hematopoietic differentiation

As discussed in the Introduction and stated in the Discussion, SIRT1 activators and inhibitors have been investigated extensively and reported to modulate a variety of processes in mammals in a SIRT1-dependent manner such as differentiation, tumor suppression and inflammation (Picard, Kurtev et al. 2004; Yeung, Hoberg et al. 2004; Parker, Arango et al. 2005). We hypothesize that the SIRT1 activator (resveratrol) promotes hematopoietic differentiation of mES cells, while SIRT1 inhibitors (NAM, Sirtinol) compromise hematopoietic differentiation of mES cells, resulting in the phenotype similar to *SIRT1*$^{-/-}$ mES cells. To test this, first we will determine the optimal concentration of resveratrol/NAM/Sirtinol. We will further evaluate the formation of blast colony, primitive progenitor cells, and definitive progenitor cells derived from mES cells treated with resveratrol/NAM/Sirtinol by *in vitro/in vivo* hematopoietic differentiation assay compared WT ES cells without treatment. We would expect that treatment with resveratrol increases the formation of blast colonies and primitive and definitive progenitor cells. However, treatment with inhibitors of SIRT1 perturbs the formation of those progenitor cells.

SIRT1 localization in mES cells

SIRT1 modulates neuronal differentiation through its nuclear translocation. SIRT1 was found in the cytoplasm of embryonic and adult NPCs. However, in response to differentiation stimulus, SIRT1 was transiently localized in the nucleus (Hisahara, Chiba et al. 2008). Furthermore, to explore further how SIRT1 regulates mES cell

differentiation, we could investigate its localization in mES cells and after cell differentiation by immunostaining. We can also determine its subcellular distribution by western blot analysis of cytoplasmic and nuclear fraction of cells under un-differentiation or differentiation conditions.

SIRT1 regulation of Scl in mES cells

As we stated in the Discussion, decreased expression of Scl at the mesoderm stage in *SIRT1*$^{-/-}$ EBs may explain its inability to adequately generate mature blast colonies and progress into primitive hematopoietic commitment. In addition, the transitional colonies formed during *SIRT1*$^{-/-}$ BL-CFC culture system had a similar morphology to those formed from *Scl*$^{-/-}$ EBs. To further explore the relationship between Scl and SIRT1, we can determine whether the defect in the formation of mature blast colonies from *SIRT1*$^{-/-}$ ES cells would be rescued by the reconstitution of Scl at the early time points. *SIRT1*$^{-/-}$ ES cells will be transfected with a lentiviral vector overexpressing Scl and then performed in vitro blast-colony forming cell assay upon *SIRT1*$^{-/-}$ ES cells. We would expect the increase of mature blast colony formation and the appropriate progression into hematopoietic lineage.

SIRT1 regulation on Smad7 in mES cells

By qRT-PCR we found that Smad7 is persistently expressed in *SIRT1*$^{-/-}$ EBs. Persistent expression of Smad7 in *SIRT1*$^{-/-}$ EBs may account for defective hematopoietic differentiation in *SIRT1*$^{-/-}$ EBs. Therefore, it will be interesting to determine whether SIRT1 interacts with Smad7 at the protein level in mES cells under undifferentiated or differentiated conditions. To investigate the interaction between SIRT1 and Smad7, we

can perform co-immunoprecipitation (IP) analysis. We can also determine which part of Smad7 interacts with SIRT1 by using deletion mutants of Smad7 vectors, Smad7 (WT), Smad7 (ΔCT), and Smad7 (ΔNT). We can further explore whether SIRT1 deacetylates the lysine residues of Smad7 based on SIRT1 is a deacetylase.

SIRT1 regulation of survivin in endothelial differentiation of mES cells

Our results showed that vascular sprout formation developed from day 6 WT and *SIRT1*$^{-/-}$ EBs developed similar endothelial outgrowths when embedded into collagen gel (Figure 15A). However, *SIRT1*$^{-/-}$ cells did not form a full vascular-like network *in vitro* (Figure 16A); they had significantly fewer class III (many sprouts but not network) and class IV (abundant networked tubules) vascular sprout formations than WT EBs (Figure 16B). Survivin plays a key role in maintenance of vascular integrity and is crucial for normal embryonic angiogenesis (Zwerts, Lupu et al. 2007). It has been shown that SIRT1 interacts with survivin by deacetylating H3K9 within the promoter of survivin (Wang, Zheng et al. 2008; Deng 2009). We hypothesize that the role of SIRT1 during vascular growth and maturation derived from mES cells could be mediated, at least in part, by a deacetylation-dependent regulation of survivin. To test whether SIRT1 could regulate survivin acetylation in endothelial cells, we will perform coimmunoprecipitation experiments in human umbilical vein endothelial cells transfected with Myc-tagged SIRT1 and Flag-tagged survivin. We would expect to detect an interaction between SIRT1 and survivin when immunoprecipitation is performed with either an anti-Flag or anti-Myc antibody. We will next test the possibility that SIRT1 might interact with survivin to regulate its deacetylation. In order to address whether SIRT1-mediated regulation of survivin is capable of regulating the angiogenic activity of survivin, we will

transfect endothelial cells with siRNAs targeting SIRT1 and survivin, and will assess the sprout-forming activity of the transfected cells in a spheroid assay. We would expect silencing of survivin gene expression lead to decreased basal angiogenic activity. If surviving is overexpressed, it might partially rescue the inhibitory effects of SIRT1 gene silencing on the sprout-forming activity of endothelial cells, which would suggest that SIRT1 regulates endothelial angiogenic functions, at least in part, by modulating the transcriptional activity of survivin.

SIRT1 regulation on HIF-2α signaling in mES cells under hypoxia

Hypoxia changes cellular metabolism and thus activates redox-sensitive as well as oxygen-dependent signal transducers. Under hypoxia, in cultured human embryonic kidney (HEK) 293 cells and mice, SIRT1 has been shown to selectively stimulate activity of the transcription factor HIF-2α during hypoxia and to augment WT HIF-2α-activated transcription of isolated mouse *vegfA* and *Epo* regulatory regions (Dioum, Chen et al. 2009). Epo, generally considered an erythropoietic growth factor, is also a potent prosurvival factor that protects developing stem cell and progenitor cells in a variety of organs (Noguchi, Asavaritikrai et al. 2007). We observed that, in contrast to *SIRT1*$^{+/+}$ cells, neither *SIRT1*$^{+/-}$ nor *SIRT1*$^{-/-}$ progenitors from aged mice responded to the enhancing effects of lowered O_2. We might expect that under hypoxia stress, *SIRT1*$^{+/-}$ and *SIRT1*$^{-/-}$ progenitors still do not respond to lowered O_2. We could investigate if SIRT1 knock-out perturbs the HIF-2α-Epo pathway. Whether HIF-responsive and HIF-2α-activated transcription of mouse *VegfA* and *Epo* regulatory regions respond to SIRT1 overexpression will be evaluated. Chromatin immunoprecipitation will be performed to evaluate whether there is a increase of recruitment of SIRT1and HIF-2α to the *Epo*

enhancer region in hematopoietic stem cells during hypoxia. Pharmacological manipulations of SIRT1 activity will be performed. We could expect increased abundance of *Epo* mRNA with agents that stimulate SIRT1 deacetylase activity, and decreased *Epo* mRNA with agents that inhibit SIRT1 deacetylase activity.

Role of SIRT1 on hematopoiesis

Vitamin B3 (niacin) is a water-soluble vitamin that occurs in two forms: nicotinamide and its acid form, nicotinic acid. Although nicotinamide leads to inhibition of sirtuins, both nicotinic acid and nicotinamide lead to increased cellular NAD^+ production. Whether Vitamin B3 treatment directly inhibits sirtuins or, alternatively, upregulates these enzymes via increased NAD^+ production remains to be answered (Denu 2005). Skokowa et al. (Skokowa, Lan et al. 2009) reported that G-CSF stimulates nicotinamide phosphoribosyltransferase (NAMPT), with subsequent upregulation of NAD^+ and SIRT1 synthesis in myeloid progenitors and neutrophils from subjects with congenital neutropenia. Notably, treatment of healthy individuals with 10-20 mg kg^{-1} d^{-1} of vitamin B3 led to a marked increase in the neutrophilic granulocyte content (up to twofold within 2 day) in peripheral blood without affecting any other blood cell lineages (Skokowa, Lan et al. 2009). G-CSF is the most common mobilizing agent used clinically (Broxmeyer, Hangoc et al. 2007; Pelus and Fukuda 2008). The mechanisms of action of G-CSF in mobilizing HSCs is complicated and not fully understood. Based of a clinical model, an essential role of the nicotinamide-NAMPT-NAD^+-SIRT1 pathway in G-CSF-triggered myelopoiesis has been demonstrated (Skokowa, Lan et al. 2009). Therefore, it will be worthwhile to explore further the role of SIRT1 in self-renewal, differentiation, homing and engraftment of HSCs, and whether a SIRT1-dependent pathway is involved in G-

CSF-triggered mobilization. To test this, first we need to determine whether SIRT1 plays a role in proliferation, mobilization, homing, or engraftment of HSCs.

As demonstrated in the Results section, absolute numbers of HPCs were significantly decreased by almost half in *SIRT1*$^{+/-}$ and *SIRT1*$^{-/-}$ one year old mice. To assess role of *SIRT1*$^{-/-}$ and *SIRT1*$^{+/-}$ HSC for engraftment and homing. First, we will breed the *SIRT1*$^{+/-}$ and *SIRT1*$^{-/+}$ mice, currently on a mixed strain background, onto a C57BL/6 mouse strain background so that the competitive repopulating cells can be assessed in a CD45 congenic system. Donor cells will be CD45.2, competitor cells will be CD45.1 (Boy J), and lethally irradiated recipients will be F1 (Dual CD45.2/CD45.1). This will allow us detection of donor, competitor and host cells. Then the repopulating ability will be evaluated between *SIRT1*$^{+/+}$ and *SIRT1*$^{-/+}$ mice to determine whether SIRT1 plays a role in engraftment of HSC.

We will test whether overexpression of SIRT1, using a lentiviral vector system, enhances HPC and HSC growth. Next we will determine the effect of overexpressing SIRT1 on the colony-forming ability of hematopoietic cells. Primary mouse BM cells will be transduced with SIRT1-lentiviral vectors and GFP^{+} cells will be sorted and then plated in semi-solid methycellulose colony assays containing growth factors conducive to progenitor cell growth. We will also evaluate whether SIRT1 overexpression enhances the survival of primary hematopoietic progenitors in delayed growth factor addition colony assays. To determine the effect of SIRT1 overexpression on mouse HSC differentiation and self-renewal, we will perform *in vivo* competitive HSC repopulation assays. To further elucidate the effect of SIRT1 overexpression *in vivo*, the absolute numbers of GFP^{+}sca-1^{+}lineage^{-} cells in transplanted animals at 1, 2 and 4 weeks (short-

term) after transplantation will also evaluated. Engraftment is a complex process involving many physiological processes. The ability of transplanted cells to move from PB to the BM shortly after transplantation is called homing and is crucial for establishing stable engraftment of donor cells. We will transplant transduced donor cells into irradiated recipient mice without competitor cells and analyze the BM of the recipient animals 24 hours after transplantation by FACS for chimerism of donor cells. The long-term engraftment ability of HSCs after overexpression of SIRT1 and multilineage differentiation will also be examined and measured. Furthermore, G-CSF-induced HPC mobilization will be examined in *SIRT1*$^{-/-}$ mice to determine whether SIRT1 is involved in the migration in the BM. *SIRT1*$^{-/-}$ mice will be breed onto a C57BL/6 mouse strain. Then C57BL/6 *SIRT1*$^{+/-}$ and the control C57BL/6 mice will be treated with G-CSF. The HSC/HPCs in peripheral blood, spleen and bone marrow will be collected, examined by *in vitro* colony assay and compared between C57BL/6 *SIRT1*$^{+/-}$ and the control C57BL/6 mice.

The CD34^{+} fraction of human umbilical cord blood (hUCB) contains HSCs capable of engrafting NOD/SCID mice *in vivo*. First, the expression of SIRT1 will be evaluated in CD34^{+} fraction. After sorting CD34^{+} fraction hUCB, cell will be treated with activators and inhibitors of SIRT1. The engraftment assay will be performed to evaluate the effect of activators and inhibitors of SIRT1 on the engraftment. After 6 weeks, human cell engraftment will be assessed by measuring human CD45 positive cells in recipient marrow. We would expect treatment of SIRT1 activators resveratrol would enhance hUCB CD34^{+} *in vivo* engraftment of immunodeficient NOD/SCID mice.

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Teaching Experience

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Honors, Awards, Fellowships

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Indiana University Graduate Travel Grant, 2006
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Outstanding Student Award for Graduate Research, 2003-2005
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Publications

Ou X, Chae HD, Wang RH, Shelley WC, Deng CX, Yoder MC, Broxmeyer HE. SIRT1 deficiency compromises mouse embryonic stem cell hematopoietic differentiation, and embryonic and adult hematopoiesis in the mouse. *Blood*, 2010 (Prepublished online).

Talyor T, Kim YJ, Ou X, Derbigny W, Broxmeyer HE. Toll like receptor 2 mediates proliferation, survival, NF-κβ translocation, and cytokine mRNA expression in LIF-maintained undifferentiated mouse embryonic stem cells. *Stem Cells Dev*. 2010 Feb 4. [Epub ahead of print]

Han MK, Song EK, Guo Y, Ou X, Mantel C, Broxmeyer, HE. SIRT1 regulates apoptosis and Nanog expression in mouse embryonic stem cells by controlling p53 subcellular localization. *Cell Stem Cell*.

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Conference Attended

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37th Annual Scientific Meeting: ISEH/ International Society for Experimental Hematology, Boston, MA, July, 2008.
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Meeting Abstracts

Ou X, Chae HD, Han MK, Taylor T, Kim YJ, Broxmeyer HE. SIRT1 deficiency compromises mouse embryonic stem cell hematopoietic differentiation, and embryonic and adult hematopoiesis in the mouse. 52st Annual American Society of Hematology Conference; Blood. 2010

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