การศึกษาหน้าที่ของ ETV2 ในระหว่างที่เกิดกระบวนการสร้างเม็ดเลือดของคนในหลอดทดลอง

นางสาวภัทรวรรณ มีฮาตร์

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Defining the Roles of ETV2 in Human Hematopoiesis *in vitro*

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ภัทรวรรณ มีฮาตร์ : การศึกษาหน้าที่ของ ETV2 ในระหว่างที่เกิดกระบวนการสร้างเม็ดเลือดของคนในหลอดทดลอง (Defining the Roles of ETV2 in Human Hematopoiesis *in vitro*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. นพ. นิพัญจน์ อิศรเสนา ณ อยุธยา, 57 หน้า.

การศึกษากระบวนการสร้างเซลล์เม็ดเลือดโดยละเอียด จะช่วยพัฒนาการสร้างเซลล์เม็ดเลือดจาก pluripotent stem cells (PSCs) ในหลอดทดลองได้ จากการศึกษาในสัตว์ทดลองพบว่า Etv2 (E twenty-six (Ets) variant 2) เป็น transcription factor ที่ทำหน้าที่หลักในการพัฒนาระบบเลือดและหลอดเลือด และยังค้นพบว่า Etv2 อาจทำหน้าที่สำคัญในขั้นตอนการสร้าง hematopoietic stem cells (HSCs) ซึ่งจะเป็นองค์ความรู้ที่สำคัญในการศึกษากระบวนการสร้างเซลล์เม็ดเลือดในคน ดังนั้น ในงานวิจัยนี้ จึงได้นำเอา embryonic stem cell-derived sacs (ES-sac) มาเป็นเครื่องมือในการศึกษาหน้าที่ของ ETV2 ในระหว่างที่มีการพัฒนาระบบเลือดของคนในหลอดทดลอง เนื่องจาก ES-sac มีโครงสร้างที่มองเห็นได้ ทำให้ง่ายต่อการติดตามกระบวนการสร้างเซลล์เม็ดเลือด เราได้ทำการสร้าง ETV2 reporter iPSC line เพื่อใช้ติดตามการแสดงออกของ ETV2 ในระหว่างที่เกิดกระบวนการดังกล่าว ผลการศึกษาพบว่า ETV2 อาจทำหน้าที่ในการกระตุ้น mesodermal progenitors เปลี่ยนไปเป็นเซลล์ในระบบเลือดและหลอดเลือด ในขณะที่ยับยั้งการเปลี่ยนไปเป็นเซลล์หัวใจ (cardiomyocyte) นอกจากนี้ยังพบว่าหลังจากที่ ETV2 มีการแสดงออกในช่วงการพัฒนา mesoderm แล้วจะถูกลดระดับการแสดงออกลง แต่ ETV2 มีการแสดงออกเกิดขึ้นอีกครั้งใน hemogenic endothelium (HE) ในช่วงหลังของการพัฒนาระบบเลือดในคน ซึ่ง HE เป็น endothelial cells ที่สามารถเกิดเป็นเซลล์เม็ดเลือดได้ และการแสดงออกของ *ETV2* mRNA ที่เกิดในช่วงที่ 2 ดังกล่าวจะเกิดขึ้นพร้อมกับการแสดงออกของ *RUNX1C* mRNA ซึ่งเป็นยีนที่ทำหน้าที่เกี่ยวข้องกับการสร้าง HSCs และพบว่าในช่วงเวลาดังกล่าว ETV2:GFP^{+}KIT^{+}CD235a^{-} HE จะมีการแสดงออกของ mRNA ของ *SOX17* ซึ่งเป็นยีนที่ทำหน้าที่เกี่ยวข้องกับ HE ใน aorta-gonad-mesonephros (AGM) ซึ่งจะสูงกว่าในช่วงแรก จากการศึกษาดังกล่าวแสดงให้เห็นว่า ETV2:GFP^{+}KIT^{+}CD235a^{-} HE ที่เกิดขึ้นในช่วงท้ายของกระบวนการสร้างเซลล์เม็ดเลือดในหลอดทดลอง อาจมีคุณสมบัติเป็นเซลล์ใน definitive hematopoietic program ซึ่งเลียนแบบสภาวะใน AGM โดยสรุป ผลจากการศึกษาสนับสนุนว่า ETV2 อาจทำหน้าที่สำคัญในการบวนการสร้าง HSCs

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

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The deep understanding of hematopoiesis would improve the strategy for hematopoietic stem cell (HSC) generation from pluripotent stem cells (PSCs). Several studies in animal model have indicated Etv2 as a master regulator in hematopoietic and vascular development. Moreover, Etv2 might be essential for HSCs generation. To study roles of ETV2 during human hematopoietic development *in vitro*, we used embryonic stem cell-derived sacs (ES-sac) as a model of hematopoietic development in which the steps of hematopoiesis could be visualized. ETV2 reporter iPSC line (ETV2:GFP iPSCs) was generated to track ETV2 expression during the development. We found that hemato-vascular mesoderm (HVM) were generated and given rise to hematopoietic lineage in which might be inducted by ETV2. Moreover, ETV2:GFP expressing mesoderm failed to contribute to cardiac cells, thus implying the inhibition of cardiac fate of mesoderm progenitor. After ETV2 was upregulated during mesodermal specification then downregulated, *ETV2* was upregulated again, the second wave, in hemogenic endothelium (HE) in later ES-sac. On the second wave, expression of *ETV2* correlated with expression of *RUNX1C,* a HSCs markers. Additionally, ETV2 expressing $KIT^{+}CD235a^{-}$ HE population in later ES-sac expressed levels of *SOX17,* a marker of HE in aorta-gonad-mesonephros (AGM), higher than early one, indicating the ETV2:$GFP^{+}KIT^{+}CD235a^{-}$ HE might become more similar to a definitive hematopoietic cell in the AGM. In conclusions, our studies in human hematopoietic development *in vitro* support the notion ETV2 might play an essential role in HSCs generation.

Field of Study: Medical Science Student's Signature .....

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CHAPTER I

Introduction

In vitro generation of hematopoietic stem cells (HSCs) from human pluripotent stem cells (hPSCs), embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), provides a great source of hematopoietic cells for clinical transplantation. However, the generation of long term repopulating HSCs in culture dish has been unsuccessful. It is necessary to clearly understand cellular and molecular pathways in hematopoietic development.

Several studies demonstrated that the hematopoietic development from hPSC *in vitro* recapitulate early embryonic development that progress through mesoderm, hemogenic endothelium (HE) and then hematopoietic cells (Keller 2005, Ackermann, Liebhaber et al. 2015, Ditadi, Sturgeon et al. 2017). Most knowledge of hematopoiesis have been from animal model, which revealed that the long term repopulating HSCs were generated from HE lining up at ventral aspect of the dorsal aorta in the aorta-gonad-mesonephros (AGM) region (Godin, Garcia-Porrero et al. 1993, Medvinsky, Samoylina et al. 1993, Garcia-Porrero, Godin et al. 1995, Cumano, Dieterlen-Lievre et al. 1996, Medvinsky and Dzierzak 1996). In order to derive the hematopoietic cells from hPSCs *ex vivo*, it is important to strongly understand the development in culture dish, including mesoderm patterning, HE formation and the process of hematopoietic formation from HE. Many ETS protein are important in hematopoietic and vascular system. Among these ETS protein, only Etv2 (Ets variant 2) acts as a master regulator in hematopoietic and endothelial development, which Etv2 knockout mouse embryo died because of the lack in hematopoietic and vascular system (Sumanas, Jorniak et al. 2005, Lee, Park et al. 2008). Intriguingly, Etv2 expression were detected in the dorsal aorta (DA) at the time hematopoietic stem cells (HSCs) emerge (Lee, Park et al. 2008). This showed that Etv2 might be required for the generation of HSCs. Moreover, there are several studies supporting the idea that Etv2 has an important function in HSC generation (Ren, Gomez et al. 2010, Lee, Kim et al. 2011, Swiers, Baumann et al. 2013).

To study the roles of ETV2 in human hematopoiesis *in vitro,* we generated *ETV2* reporter iPSC cell line (*ETV2:GFP* iPSCs). Using the reporter line, we found that ETV2:GFP was detected during human hematopoietic development. Consistent with others studies, we showed that ETV2 may play a role in specification of mesodermal progenitor (MP) into hemato-vascular mesoderm (HVM) and inhibit cardiac differentiation. We demonstrated that ES-sac derived HE were generated through ETV2:GFP expressing cells. Importantly, our study support the notion that ETV2 may be necessary for HSC generation that need to be investigated further.

CHAPTER II

Literature review

Pluripotent stem cells (PSCs)

Human pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), provide an invaluable tool for the study of embryonic development and are expected to be an unlimited source of cells for regenerative medicine (Ye, Chou et al. 2012). The PSCs are able to differentiate into cells of the three germ layers after directed differentiation in culture (Doetschman, Eistetter et al. 1985, Thomson, Itskovitz-Eldor et al. 1998). ESCs are pluripotent stem cells originated from the inner cell mass of blastocyst-stage embryos (Evans and Kaufman 1981, Thomson, Itskovitz-Eldor et al. 1998). iPSCs are ESC-like cells generated through reprogramming somatic cells with forced expression of a combination of pluripotent transcription factors, such as Oct3/4, KLF4, c-Myc, and SOX2 (Takahashi and Yamanaka 2006). The advent of iPSCs has helped the scientific community to circumvent the ethical problems faced with the use of ESCs, and also provided cells source for an individual patient. Using gene targeting technology, it is possible to correct genetic defects in the cells, solving the problems of immunological rejection (Togarrati and Suknuntha 2012, Hockemeyer and Jaenisch 2016).

Hematopoiesis

Hematopoiesis is a process of blood production in an organism. The key players in this process are hematopoietic stem cells (HSCs) which are multipotent stem cells with an ability to self-renew and differentiate into all type of blood cells. Clinically, HSCs, with their hematopoietic repopulation potential, are used for transplantation therapies in patients with a variety of hematopoietic disorders. The major limitation of HSC transplantation is the amount of compatible donors. Nonetheless, the discoveries from PSCs research will hopefully lead to successful production of HSCs in a dish for hematopoietic transplantation therapy. Many researchers have developed strategies to

differentiate PSC into hematopoietic lineage *in vitro* (Slukvin 2016). However, multi-lineage engraftment potential from PSCs is unsuccessful. To overcome this limitation, it is necessary to completely understand the process of HSC formation in the embryo.

Embryonic hematopoiesis occurs in sequential waves consisting of the primitive wave, forming erythroid and myeloid cells to help the developing embryo, and the definitive wave, generating erythro-myeloid progenitors and long-term HSCs. During embryogenesis, yolk sac in which located on extra-embryonic area, was thought to be the first place that HSCs are generated before migrating to intra-embryonic organs (Palis, Robertson et al. 1999, Lichanska and Hume 2000, Palis and Yoder 2001, Lux, Yoshimoto et al. 2008). However, the studies in chick-quail chimeras, Xenopus, and mice clearly demonstrated that the intra-embryonic organ, dorsal aorta (DA) of aorta-gonads-mesonephros (AGM), is the origin of HSCs. The specialized endothelial cells at the ventral wall of the DA, namely hemogenic endothelium (HE), are able to form HSCs through a process called the endothelial-to-hematopoietic transition (EHT) (Medvinsky and Dzierzak 1996, Taoudi, Gonneau et al. 2008, Zovein, Hofmann et al. 2008, Eilken, Nishikawa et al. 2009, Yoshimoto and Yoder 2009, Bertrand, Chi et al. 2010, Boisset, van Cappellen et al. 2010, Kissa and Herbomel 2010, Tavian, Biasch et al. 2010, Hirschi 2012, Li, Lan et al. 2012, Rafii, Kloss et al. 2013, Swiers, Baumann et al. 2013). After the HSCs generation in AGM, HSCs migrate to an intermediate niche, fetal liver and placenta, to proliferate and expand and finally colonize at bone marrow where the HSCs will remain throughout adulthood (Zovein, Hofmann et al. 2008, Bertrand, Chi et al. 2010, Boisset, van Cappellen et al. 2010, Kissa and Herbomel 2010, Li, Lan et al. 2012).

For the past decade, multiple studies have attempted to developed many protocols for generation of *in vitro* hematopoiestic stem cells from human PSCs. The PSC derived HSCs, however, do not effectively develop into cells of all lineages, fail to produce adult hemoglobin, and have limited long term engraftment potential. Various *in vitro* protocols have been described to generate blood cells from human PSC. There are two major types of strategies for hematopoietic differentiation from PSC - embryoid body (EB) method and co-culture of hPSC with stromal cell lines. The EB method is the aggregation

of PSCs into small clumps which differentiate spontaneously into embryoid bodies in the presence of growth and differentiation factors (Civin, Almeida-Porada et al. 1996, Yahata, Ando et al. 2002, Zambidis, Peault et al. 2005, Ye, Zhan et al. 2009, Bai, Xie et al. 2013). For co-culture of hPSC with stromal cell lines, PSCs are cultured on the irradiated stromal cell lines layer and subsequently differentiate spontaneously (Kaufman, Hanson et al. 2001, Vodyanik, Bork et al. 2005, Tian, Woll et al. 2006, Weisel, Gao et al. 2006, Ledran, Krassowska et al. 2008, Ma, Ebihara et al. 2008, Timmermans, Velghe et al. 2009).

In hematopoietic development process, PSCs are specified into primitive streak (PS), mesodermal cell and hemato-vascular progenitor, respectively (Kennedy, D'Souza et al. 2007, Wang, Tang et al. 2012). As PSCs develop into mesoderm expressing Kdr (Flk1) and platelet-derived growth factor receptor alpha (Pdgfra) (Kataoka, Takakura et al. 1997, Shalaby, Ho et al. 1997).

Hemangioblasts are mesodermal progenitor cells which can form blast colony forming cells (BL-CFCs) consisting of vascular and hematopoietic progenitors. In mouse ESC culture, mouse ESCs differentiate into Kdr^{+} $Pdgfra^{+}$primitive mesodermal cells and then $Kdr^{+}Pdgfra^{-}$lateral plate mesoderm, respectively. The study by Choi revealed that the differentiation of mesodermal cells from hPSCs were defined using expression of APLNR (apelin receptor), PDGFRa, and KDR mesodermal markers which divide mesodermal cells into two phases of development, including primitive mesodermal cells ($^{EMH}lin^{-}$ $APPNR^{+}PDGFRa^{+}$) and hematovascular mesodermal progenitor ($^{EMH}lin^{-}$ $KDR^{bright}APLNR^{+}PDGFRa^{low/-}$), $^{EMH}lin^{-}$ stand for lacking of expression of mesenchymal endothelial and hematopoietic markers (Choi, Vodyanik et al. 2012).

Primitive mesodermal cells have potential to form blast-CFC (BL-CFCs) and act like primitive posterior mesoderm in the embryo. These primitive mesoderm express gene associated with primitive streak (*T, MIXL1, EOMES*) and lateral plate mesoderm (*FOXF1, WNT5a, BMP4*). Following the primitive mesoderm phase, hematovascular mesodermal progenitors were generated in which lack BL-CFC potential. The HVMPs still express lateral plate mesodermal gene and begin upregulating expression of hematopoietic and

vascular genes (*TAL1, HHEX, LMO2, GATA2*, and *ETV2*) and downregulating expression of lateral plate mesodermal gene (Choi, Vodyanik et al. 2012).

Following the mesodermal development, hemogenic endothelium (HE), an endothelial cell in which can generate multilineage HSCs through endothelial to hematopoietic transition (EHT) process, can be detected. Many studies show that only a portion of endothelial cells undergo EHT in hPSC culture. The study of Choi revealed that HE is enriched within VE-cadherin^{+}CD43^{-}endothelial population (Choi, Vodyanik et al. 2012). CD43, which expresses in hematopoietic progenitor, was used to isolate hematopoietic cells from endothelial cells (Vodyanik, Thomson et al. 2006). An expression of CD73 was used to separate HE from non-HE (Choi, Vodyanik et al. 2012, Rafii, Kloss et al. 2013, Uenishi, Theisen et al. 2014). Choi also showed that gene expression profiles of VE-cadherin^{+}CD43^{-}CD73^{-}cells are similar to HE but unlike non-HE. However HE shares many endothelial markers with non-HE. Consistently, Keller group demonstrated that HE were enriched in CD34^{+}CD43^{-}CD73^{-}CD184^{-}cells (Ditadi, Sturgeon et al. 2015).

In human PSC cultures, venous and arterial endothelial cells are the subpopulation within CD73^{+}non-HE. CXCR4 (CD184) expression can be used to identify venous and arterial vascular endothelium in non-HE population. Subcutaneous transplantation of CD184^{+}CD73^{+}cells in mice induced the formation of large vessels expressing EFNB2 (arterial endothelial marker) and lacking EPHB4 expression (venous endothelial marker), which indicated generation of arterial vessels. In contrast, CD184^{-}CD73^{+}cells generated smaller venous vessels with EPHB4 expression representing venous vasculature (Ditadi, Sturgeon et al. 2015).

Hematopoietic progenitor cells (HPCs) with multi-lineage potential derived from hPSC culture are enriched within CD34^{+}population prior to an upregulation of CD45, a pan-hematopoietic marker. However, CD34^{+}cells in hPSC culture are also identified as endothelial and mesenchymal stem cells (Kaiser, Hackanson et al. 2007, Maumus, Peyrafitte et al. 2011, Lin, Ning et al. 2012, Zimmerlin, Donnenberg et al. 2013). Thus, only expression of CD34 is not sufficient to isolate hematopoietic cells from hPSC culture. The study of Vodyanik revealed that CD43 is a marker for emerging hematopoietic progenitor

cells (Vodyanik, Thomson et al. 2006). Time lapse video of hPSC culture showed round hematopoietic progenitor cells gradually acquire expression of CD43 during EHT (Choi, Vodyanik et al. 2012).

Primitive and definitive hematopoiesis are spatially separated during embryonic development *in vivo*. However, hPSC culture systems are not set apart of these waves existing together in culture. So, both hematopoietic programs generate HE and give rise to HPCs ($CD34^+CD43^+$cells) which cannot be separated by surface markers expression. Nevertheless, only definitive HPCs have ability to generate T lymphocyte (Kennedy, Awong et al. 2012). The studies of Kennedy et al and Sturgeon et al showed that Activin/Nodal pathway induced the development of primitive while definitive hematopoietic program was triggered by the Wnt signaling pathway during mesoderm development (Kennedy, Awong et al. 2012, Sturgeon, Ditadi et al. 2014). Glycophorin A (CD235a) was identified as a marker for discrimination of primitive and definitive program which $KDR^+CD235a^+$mesodermal cells develop into primitive hematopoietic cells, whereas $KDR^+CD235a^+$mesodermal cells give rise to definitive hematopoietic cells (Sturgeon, Ditadi et al. 2014). This led to a way to selectively generate definitive hematopoietic progenitors in hPSC culture.

Transcription factors

ETV2

ETV2 (ETS Variant 2) or ER71 is a transcription factor in ETS family that has a conserved winged helix–turn–helix ETS DNA-binding domain which binds to a GGAA/T-binding motif. The studies from various animal models such as mouse, Xenopus, zebrafish and chicken, show that ETV2 is an important transcription factor in embryonic hematopoiesis and vasculogenesis. ETV2 deficient mouse embryos die at embryonic day 11.0 (E11.0) with defects in hematopoiesis and vasculogenesis (Lee, Park et al. 2008, Ferdous, Caprioli et al. 2009). Lineage tracing study of Rasmussen revealed that cardiomyocyte and hematopoietic and vascular cells are derived from the same progenitors, mesodermal progenitors, which were induced into hematopoietic and

endothelial lineages by ETV2 upregulation and guided into cardiomyocytes by ETV2 downregulation (Rasmussen, Kweon et al. 2011). This result is consistent with the study of Kataoka which showed that Etv2 deficient mouse embryo ($Etv2^{-/-}$) cannot generate $Kdr^{+}Pdgfra^{-}$cells, cells in hematopoietic lineage, from $Kdr^{+}Pdgfra^{+}$mesoderm. However, these cells can give rise to $Kdr^{-}Pdgfra^{+}$cells, which are of the cardiac lineage (Kataoka, Hayashi et al. 2011). These results are also supported by the study in zebrafish, which demonstrated that vascular endothelial/endocardial progenitors can differentiate into cardiomyocytes in the absence of etv2 function during zebrafish embryonic development (Palencia-Desai, Kohli et al. 2011). Some studies have also shown Etv2 to be essential during embryonic stage in which HSCs emerged. The study in mouse embryo by Nakagawa shown that Etv2 is expressed in AGM, the first organ of definitive hematopoiesis (Koyano-Nakagawa, Kweon et al. 2012). Therefore, Etv2 may be required for HSC formation from HE through EHT. The study of Swiers suggested that Etv2 was expressed within HE, which was marked by +23 Runx1 enhancer driven GFP expression, where Runx1 is a marker for hematopoietic stem and progenitor cell (HSPC) and HE (Swiers, Baumann et al. 2013). The study in zebrafish by Ren also demonstrated that Etv2 is required for HSCs development from HE (Ren, Gomez et al. 2010). Correspondingly, the study by Lee using conditional deletion of Etv2 in the hematopoietic system of adult mice showed a decrease in the number of HSCs, which was likely a result from an increased cell death (Lee, Kim et al. 2011). Thus, Etv2 was required for HSC maintenance and function. Collectively, Etv2 is an important transcription factor in embryonic hematopoiesis. First, it specifies mesodermal cell into hematopoietic/vascular lineage, while prohibiting cardiac lineage specification. Second, Etv2 is necessary for HE, especially in the process of ETH which generate HSCs. Lastly, Etv2 is an important transcription factor for HSC maintenance.

RUNX1

Runx1 is a key transcription factor for hematopoiesis in vertebrates and is especially important for the emergence of definitive HSCs from HE (Speck and Gilliland

2002). RUNX1 is also known as acute myeloid leukemia 1 due to the discovery of its gene sequence from human patient with acute myeloid leukemia. During embryonic hematopoiesis, HSCs were first detected in aorta-gonad-mesonephros region at 10.5 days post conception (dpc), where the HSCs emerge from the ventral aspect of dosal aorta, and then move to colonize at fetal liver. Runx1 expression can be detected at the two locations, suggesting that Runx1 expression marks earliest hematopoietic precursor cells (North, Gu et al. 1999).

Runx1$^{-/-}$mouse embryo died because of hemorrhage and a complete lack of the definitive hematopoietic system (Okuda, van Deursen et al. 1996, Wang, Stacy et al. 1996, North, Gu et al. 1999, Lacaud, Gore et al. 2002). Recent studies have established that Runx1 is required for the HSC generation from HE through EHT (Yokomizo, Ogawa et al. 2001, Lancrin, Sroczynska et al. 2009, Kissa and Herbomel 2010). During hematopoiesis in mouse and zebrafish, Runx1 expression is regulated by two promotors, proximal P2 and distal P1, which generate 3 isoforms consisting of Runx1a, Runx1b and Runx1c isoform. These three isoforms contain the Runt domain located in the N-terminal region, sharing the same N-terminal region, and are the result of alternative splicing. Runx1c is transcribed from a distal P1 promoter, whereas Runx1a and Runx1b are transcribed from the proximal P2 promoter. Recent studies revealed that the emergence of definitive hematopoietic cells was shown to associate with the expression of Runx1c, and overexpression of Runx1c can accelerate and enhance the production of haemato-endothelial cells *in vitro* (Challen and Goodell 2010, Ran, Lam et al. 2013, Real, Navarro-Montero et al. 2013, Ferrell, Xi et al. 2015). In contrast, Runx1a and Runx1b are expressed throughout both primitive and definitive hematopoietic cells

KIT

c-Kit (CD117) is a growth factor receptor with tyrosine kinase activity that controls intracellular signal transduction pathways including cellular proliferation, maintenance and migration (Marcelo, Goldie et al. 2013). c-Kit mutations lead to embryonic lethality around mid-gestation because of anemia and disrupted HSC development (Chabot,

Stephenson et al. 1988, Sattler and Salgia 2004). Emerging hematopoietic from the aorta endothelium can be discriminated from endothelial cells by c-kit expression, in which hematopoietic clusters were identified in c-Kit^{high}CD31^{+}population, while c-Kit$^{low/-}$CD31^{-} population are circulating hematopoietic cells (Yokomizo and Dzierzak 2010). This reveal that c-Kit is a HSCs marker. The recent studies also showed c-Kit is a marker of HE (Yokomizo and Dzierzak 2010, Tober, Yzaguirre et al. 2013). c-Kit can rescue the development of HE in yolk sac and also activate Runx1 expression, promoting endothelial to hematopoietic transition (EHT) (Marcelo, Sills et al. 2013, Pereira, Chang et al. 2016). Another study revealed that Kit^{low}Runx1^{+}cells with endothelial morphology are in an initial stage of the endothelial-to-hematopoietic transition, in which Kit^{low}Runx1^{+}cells go through Kit^{high}Runx1^{+}cells and subsequently Kit^{high}rounded hematopoietic cells (Frame, Fegan et al. 2016).

SOX17

Sox17 is a transcription factor expressed in HE in AMG around E8.5–8.75 (Iacovino, Chong et al. 2011). Sox17 is required for generation of fetal and neonatal HSCs, but not in adult HSCs (Kim, Saunders et al. 2007, He, Kim et al. 2011, Clarke, Yzaguirre et al. 2013, Swiers, Baumann et al. 2013). Lizama demonstrated that Sox17 modulates HE by repressing the hematopoietic program through the Sox17/Notch axis, until initiation of EHT (Lizama, Hawkins et al. 2015). Consistently, several studies of HE in murine AGM revealed that the HE showed increasing in Runx1 levels together with decreasing in Sox17 when undergoing endothelium to hemogenic transition (EHT) (Bos, Hawkins et al. 2015). Clark group also demonstrated that SOX17 is essential for definitive hematopoietic commitment (Clarke, Robitaille et al. 2015).

Gene targeting

Gene targeting is a site-specific modification of the gene of interest using DNA repair mechanism triggered double-strand break (DSB) of genomic DNA (Barnes 2001, Lieber 2010). DSB is a critical DNA damage that needs to be repaired immediately to

maintain the integrity of the genome. To repair DBS, there are several DNA repair mechanisms such as non-homologous end joining (NHEJ) and homologous recombination (HR) (van den Bosch, Lohman et al. 2002, Kim and Kim 2014). NHEJ is a DNA repair mechanism in eukaryotic cells that joins the two broken end of DNA, causing an insertion or a deletion (indel) of nucleotide. HR repairs DSBs by using a homologous DNA template endogenous genomic locus or donor vector, which guides the repair process. When properly supplemented with donor vector, HDR will insert the donor molecule at the targeted site. Thus a targeted site specific gene can be modified by inducing DBS and subsequently repaired through NHEJ or HDR using custom-engineered nucleases such as zinc-finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN) or clustered regularly interspaced short palindromic repeat (CRISPR).

ZFN

The zinc-finger DNA recognition domain, which is a common DNA binding module in eukaryotic cells and transcriptional regulation, has been modified to bind to specific DNA sequences of interest and fused with restriction enzyme FokI, the engineered DNA-binding proteins are named zinc-finger nucleases (ZFNs). ZFNs are composed of 6 to 6 zinc finger proteins, each of which can bind 3-bp combination of DNA sequence. The FokI nuclease is a restriction enzyme that can cleave DNA when it forms dimers. Thus, ZFN system requires two ZFNs that bind to the top and bottom strands of targeted DNA sequence to induce a DSB and consequence DNA damage response pathway, either NHEJ or HDR (Kim and Kim 2014).

TALEN

Transcription activator-like effector (TAL effector or TALE) was found in Xanthomonas, a plant pathogen. TALE consists of 10 to 30 tandem repeats of repeat variable di-rescidue or RVD domain. Two amino acid residues of each domain can recognize a single base, guanine (G), adenine (A), cytosine (C), and thymine (T). TALE was engineered by conjugating with a FokI nuclease to form TALE Nucleases (TALENs)

that can induce specific DSBs in target DNA sequences similar to ZFNs (Kim and Kim 2014).

CRISPR

CRISPR or clustered regulatory interspaced short palindromic repeat system is an adaptive defense mechanism in prokaryotic cells that act against viruses and plasmid, whose DNA segment is copied into the genome of the host at CRISPR locus, a genomic memory of invading pathogens. When the host cell is invaded with a pathogen which was memorized, crispr RNA (crRNA) will be transcribed from specific sequences in CRISPR locus and combined with an endogenous CRISPR- associated endonuclease (Cas). This crRNA, together with Cas, subsequently recognizes and binds to DNA of the invading pathogen and induces DBS to obstruct the integration and replication. Type II CRISPR system is one of three CRISPR systems in prokaryotic cells. Type II CRISPR system requires two small RNAs, crRNA and the trans activating crRNA (tracrRNA) that form single guide RNA (sgRNA) which then combines with Cas9 protein. The sgRNA quides endonuclease Cas9 to cleave DNA of invading pathogens. The type II CRISPR also requires a protospacer adjacent motif (PAM) downstream of the target sequence which is recognized by a PAM-binding domain of Cas9. The CRISPR-Cas9 system can be modified and introduced into eukaryotic cells to disrupt and edit a target gene of interest. In contrast to ZFNs and TALENs, the Cas9 endonuclease in CRISPR system induces DSBs as a monomer whereas the FokI in ZFNs and TALENs induces as a dimer. Apart from genome editing capability of CRISPR system which can bind to specific target sequences, Cas9 protein can be engineered into a form lacking nuclease activity (dCas9), to block transcriptional initiation or elongation, a system called CRISPR interference or CRISPRi. To repress or activate transcription, transcriptional repressor or activator domain can be fused to dCas9 (Kim and Kim 2014).

The advancement in iPSC and genome targeting technologies benefit the genomics study of human in understanding gene function, modeling disease, and

generating reporter lines and collections. These technologies are the hope for next generation gene therapy using iPSCs.

CHAPTER III

Material and method

Cell culture and transfection

HS4#2sevtx generated from human skin fibroblast as previously described (Ingrungruanglert et al.,2014) was maintained on Matrigel (BD Biosciences) in a basal growth-factor free mTeSR1 medium (StemCell Technologies). Every 3-5 days, the cells were dissected into small clumps in dissociation solution (CTK; 0.25% trypsin, 20% knockout serum replacement, and 1mM $CaCl_2$ in PBS) and transferred to a new Matrigel-coated plastic ware to maintain undifferentiated state. The VEGF expressing C3H10T1/2 cells were cultured in Eagle basal medium (Invitrogen) containing 10% fetal bovine serum (FBS) and 2 mM L-glutamine.

hiPSCs ware transfected with 5 ug (each) of CRISPR/TALEN and donor vector using P3 Primary Cell 4D-Nucleofector® X (Lonza) according to the manufacturer's instructions. Briefly, iPSCs were passaged two days before transfection. The day of transfection, 5×10^6 cells were dissociated into single cells with Accutase (Thermo Fisher Scientific). Cells were re-suspended in the cocktail solution together with 5 µg each CRISPR/TALEN and 5µg donor plasmid. The transfection was processed using the 4D-Nucleofector X Unit with CB-150 program. Cells were re-plated on a new Matrigel-coated plastic dish with mTeSR1 medium supplemented with 10 µM Y27632. After 24 hours of transfection, medium was changed to mTeSR1 medium without Y27632. After 3 days of transfection, cells were selected by 0.5 µg/ml puromycin or 20 µ/ml hygromycin depend on donor vector.

ES-sac differentiation

hPSCs were cultured on Matrigel-coated dish 4-5 days, followed by ES-sac differentiation, as previously described (Takayama et al.,2008). Briefly, the hPSCs were dissociated with CTK into small clumps (20-30 cells) and transferred into irradiated VEGF expressing C3H10T1/2 feeder and cultured in ES-sac differentiation medium, which was refreshed every 3 days. ES-sac differentiation medium was Iscove modified Dulbecco medium

supplemented with a cocktail of 10 µg/mL human insulin, 5.5 µg/mL human transferrin, 5 ng/mL sodium selenite, 2 mM L-glutamine, 0.45 mM mono-thioglycerol, 50 µg/mL ascorbic acid, and 15% FBS. Cultures were maintained in a 5% CO_2/ 5% O_2/ 90% N_2 environment for the first 7 days and then transferred to a 5% CO_2/ air environment.

Flow cytometry and cell sorting

ES-sac was dissociated to single cells with sequential collagenase IV (1 mg/ml) and trypsin-EDTA (0.25%) treatment. The cells ware stained with CD34-FITC (clone 561), CD43-PE/Cy7 (CD43-10G7), CD73PerCP-Cy5.5 (clone AD2), CD184-Brilliant Violet 421 (clone 12G5), KDR-APC (7D4-6). PDGFRa or CD140a, CD144, CD235a, KIT or CD117 All antibodies were purchased from BD Biosciences. For cell surface markers, staining was carried out in PBS with 10% FCS. The cells were analyzed and sorted using FACSAria II (BD).

Real time quantitative PCR

Total RNA was prepared with TRIzol reagent according to the manufacturer's instructions. Briefly, the pelleted cells were add with 1 mL TRIzol reagent and incubate in 5 minutes at room temperature. Add 0.1 ml of BCP per 1 ml of TRIZOL Reagent. Cap sample tubes securely. Vortex samples vigorously for 15 seconds and incubate the sample at room temperature for 3 to 5 minutes. Centrifuge the samples at 12,000 x g for 10 minutes at 4°C. The mixture was separates and RNA remains in the aqueous phase. Transfer upper aqueous phase carefully without disturbing the interphase into new tube. Add 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL Reagent and Incubate samples overnight at 20°C. Centrifuge at 12,000 x g for 10 minutes at 2 to 4°C. Wash the RNA pellet twice with 75% ethanol. Air-dry RNA pellet and dissolve RNA in water. cDNA was synthesized from total RNA using RevertAid™ H Minus M M-MuLV (Fermentas). Real-time PCR was carried out using Maxiima SYBR Green/ROX qPCR Master Mix (2X) (Fermentas) and Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems). Primer sequences used are listed in Table 1.

Table 1 Real time quantitative PCR primers

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
ETV2	AGGGAACAAGCTGGCAGGGCTTGAA	TCCAGCATGTCTCTGCTGTCGCTGT
RUNX1	ACTCGGCTGAGCTGAGAAATG	GACTTGCGGTGGGTTTGTG
RUNX1a	CTGGTCACTGTGATGGCTGG	CTGCCTTAACATCTCCAGGG
RUNX1b	TGCATGATAAAAGTGGCCTTGT	CGAAGAGTAAAACGATCAGCAAAC
RUNX1c	TGGTTTTCGCTCCGAAGGT	CATGAAGCACTGTGGGTACGA
PDGFRa	TCCTCTGCCTGACATTGACC	TGAAGGTGGAACTGCTGGAAC
BRACHYURY	GGTCTCGGCGCCCTCTTCCTC	GGGCCAACTGCATCATCTCCACA
TAL1/SCL	ATGGTGCAGCTGAGTCCTCC	TCTCATTCTTGCTGAGCTTC
HOXA1	TCCTGGAATACCCCATACTTAGCA	GCCGCCGCAACTGTTG
HOXA2	ACAGCGAAGGGAAATGTAAAAGC	GGGCCCCAGAGACGCTAA
HOXA3	TGCAAAAAGCGACCTACTACGA	CGTCGGCGCCCAAAG
HOXA4	TCCCCATCTGGACCATAATAGG	GCAACCAGCACAGACTCTTAACC
HOXA5	TCTCGTTGCCCTAATTCATCTTTT	CATTCAGGACAAAGAGATGAACAGAA
HOXA6	CCCTCTACCAGGCTGGCTATG	CAGGACCGAGTTGGACTGTTG
HOXA7	CAAAATGCCGAGCCGACTT	TAGCCGGACGCAAAGGG
HOXA9	CCGAGAGGCAGGTCAAGATC	AAATAAGCCCAAATGGCATCA
HOXA10	ACAAGAAATGTCAGCCAGAAAGG	GATGAGCGAGTCGACCAAAAA
HOXA11	ACAGGCTTTCGACCAGTTTTTC	CCTTCTCGGCGCTCTTGTC
HOXB4	TTTTCAGCTTTGGCGAAGATG	ACCGAGGCCCGTCTTCTC
LIN28b	TTGTGAGGGTTGTAAGGG	CAGTAGTAAATAAGGAGGG
GAPDH	CCAGGTGGTCTCCTCTGACTTCAACAG	AGGGTCTCTCTCTTCCTCTTGTGCTCT

Immunofluorescence staining

ES-sac were differentiate on VEGF expressing C3H101/2 feeder cells on glass slide in 24 well plate. At the staining day, the ES-sac were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.3% Triton X-100 in PBS for 45 min. The ES-sac were blocked in blocking buffer (5% normal goat serum and 0.3% Triton X-100 in PBS) for 60 min. Aspirate blocking solution, apply diluted primary antibody and incubate overnight at 4°C. Aspirate primary antibody and wash with washing buffer 3 times, 0.05% tween 20 in PSC. Incubate the ES-sac in fluorochrome-conjugated secondary antibody (Molecular Probes, Invitrogen) for 60 min at room temperature in the dark. Aspirate the secondary antibody and wash 5-7 times. DAPI was added and washed after incubation in 5 min. Fluorescence images were obtained by using Axio Observer fluorescence microscope (Carl Zeiss).

Targeting vector construction

To generate CRISPR-dCas9 vector to target *ETV2* gene (*ETV2* stop codon), gRNAs were designed using Optimized CRISPR Design – MIT web site (http://crispr.mit.edu/). Single-stranded oligonucleotides of gRNA were annealed. The gRNAs were cloned into px461 (provided by Dr. Feng Zhang, and also available through Addgene; 48140) at the BbsI-digested site.

ETV2-GFP vector and RUNX1-RFP vector were designed and generated for gene specific donor templates. ETV2-GFP vector contain *ETV2* homology arms (left and right arms, ~1000 bp for each), 2A-H2B-GFP and PGK-Puromycin flanked by loxp recombination site for PGK-Puromysin removal (Fig 7).

CRISPR/TALEN test, genomic DNA isolation, PCR and T7EI assay

HEK293 $1x10^6$ cells were transfected with 5 ug (each) of CRISPR or TALEN plasmids using Amaxa® Cell Line Nucleofector® Kit V following manufacturer's instructions. The genomic DNA was harvested after 2-3 days after transfection using the QIAamp DNA Mini Kit (QIAGEN) following the manufacturer's instruction. T7 endonuclease I assay was performed for checking double strand break (DSB) as previously described (Shen, Zhang

et al. 2014). PCR was done using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) with gene specific primers. To double check for induced double strand break, PCR products were cloned into pCR8/GW/TOPO vector (Invitrogen) for sequencing analysis.

CHAPTER IV
Results

Evaluation of the suitability of ES-sac differentiation method for the study of early human hematopoiesis

While mouse hematopoiesis is well characterized, experimental limitations make the study of early human hematopoiesis challenging. Pluripotent stem cells (PSC) provide the possibility of a model studying early human hematopoietic development *in vitro*. Several studies demonstrated that *in vitro* hematopoietic differentiation *via* embryoid body (EB) mimics the development cascade *in vivo* (Keller 2005, Ackermann, Liebhaber et al. 2015, Ditadi, Sturgeon et al. 2017). Nevertheless, the structure and density of EB prevents real-time monitoring events occurring within the model. We therefore aim to use a hematopoietic differentiation model called ES-sac, embryonic stem cell-derived sacs (Takayama, Nishikii et al. 2008), to study the roles of ETV2 in hemogenic endothelium generation and hematopoietic stem cell emergence. For ES-sac methods, PSCs were co-cultured with VEGF expressing C3H101/2 feeder cells (Fig 1). The PSCs were induced to generated inflated sac like structure containing hematopoietic like cells emerged from wall of vascular like structure that provides an opportunity to visualize and track the development (Fig 2). Since ES-sac model was much less used than the EB, there are limits of information about cell types generated in the ES-sac model. We therefore first analyzed the components of this structure. Immunofluorescence staining of ES-sacs, CD31 (PECAM-1) and RUNX1 expression were detected in the cells within ES-sac colonies, indicating hematopoietic differentiation (Fig 2). Intriguingly, cells expressing RUNX1 together with CD31 emerged from the wall were observed suggest that this system contain hemogenic endothelium (HE) and allows monitoring of HSC emergence (Fig 2-e and f). Fluorescence activated cell sorting (FACS) analysis was used to study the dynamic change of ES-sac components during the first 14 days of the culture. Mesoderm with hematopoietic potential or hemato-vascular mesoderm (HVM), identified as $KDR^{high}PDGFR^{-}$ cells (Ema, Takahashi et al. 2006, Sakurai, Era et al. 2006, Choi, Vodyanik

et al. 2012), were first detected at Day3 and peaked around Day5-6 of differentiation (Fig 3). HE, which were identified as $CD34^{+}CD43^{-}CD73^{-}CD184^{-}$cells (Ditadi, Sturgeon et al. 2015), were detected following the mesoderm generation. Next, hematopoietic progenitor cells (HPC), $CD34^{+}CD43^{+}$cells, were observed. We also found arterial ($CD34^{+}CD73^{+}CD184^{+}$) and venous ($CD34^{+}CD73^{+}CD184^{-}$) vascular endothelium (VE) (Ditadi, Sturgeon et al. 2015) in the culture started from Day5. Unlike HE and HPC, arterial and venous VE were unable to give rise to hematopoietic colonies in methylcellulose (Fig 4). Taken together, ES-sac method provides useful tool for interrogating early human hematopoiesis in the dish.

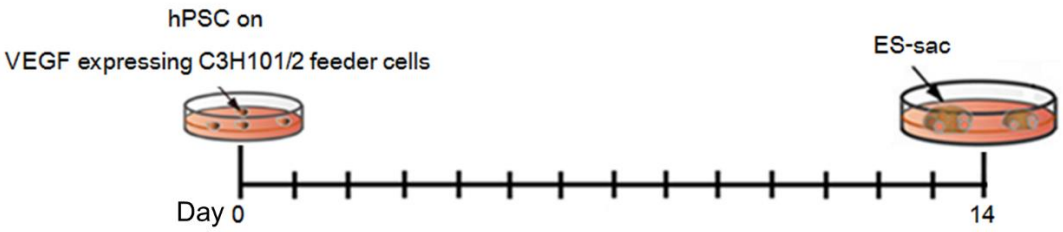

Figure 1 **Schematic of ES-sac differentiation protocol** hPSCs were plated on irradiated VEGF expressing C3H101/2 feeder cells at Day0 in ES-sac differentiation medium that was refreshed every 3 days. VEGF indicates vascular endothelial growth factor.

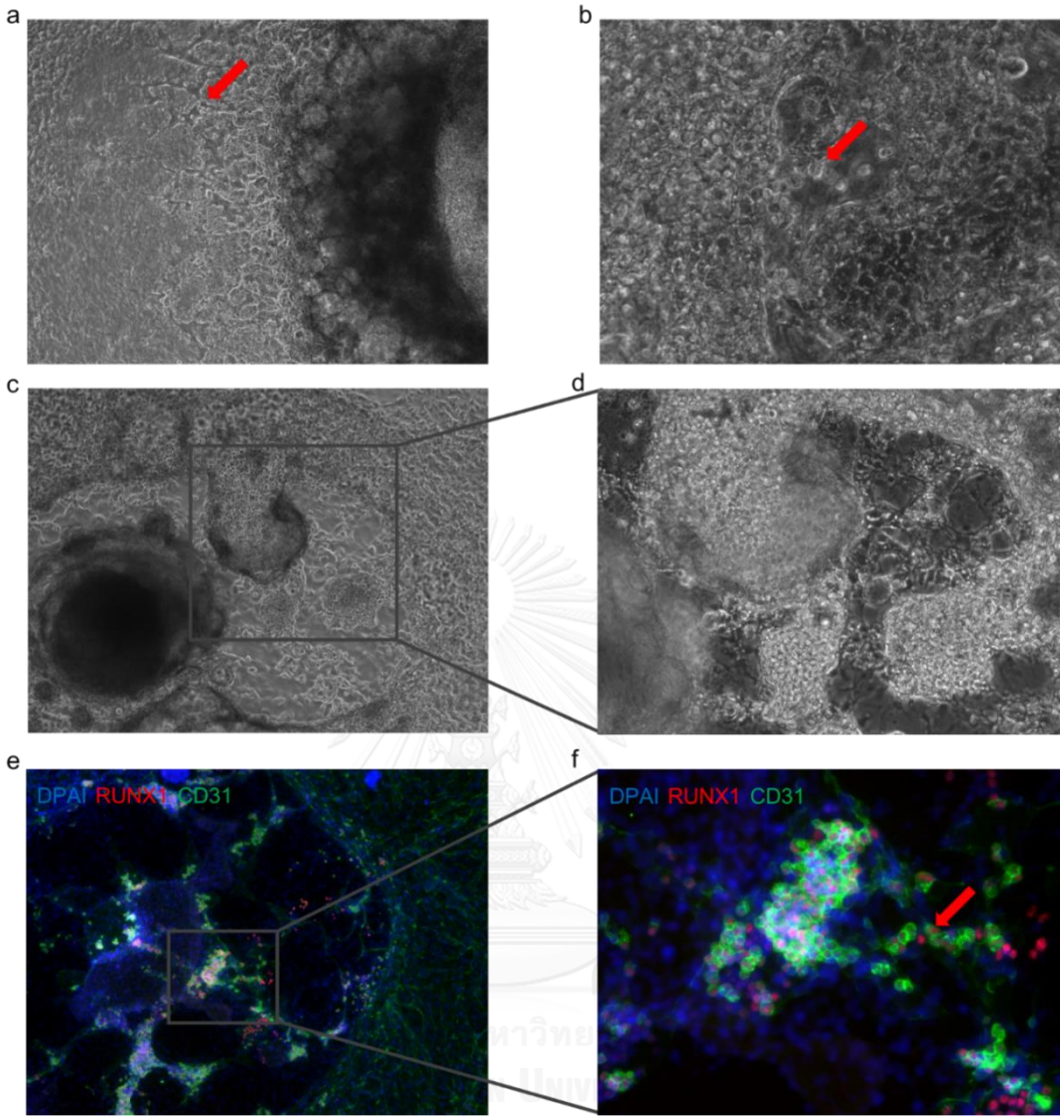

Figure 2 **Human PSC derived ES-sac** (a to d) Day10 ES-sac colony generated vascular like structure spreading out from the middle of colony (red arrow in a) and blood-like cells were observed emerging from the vascular-like structure (red arrow in b). (e and f) Immunofluorescent staining of CD31 and RUNX1 markers in ES-sac was shown. Cells in the ES-sac colony were stained with CD31 (vascular and HE marker) in which form vascular-like structure. RUNX1, a hematopoietic progenitor marker, positive cells also co-stained with CD31, which the cells were located at the vascular wall (f, red arrow). This indicated that hematopoietic cells were likely to emerge from endothelial cells.

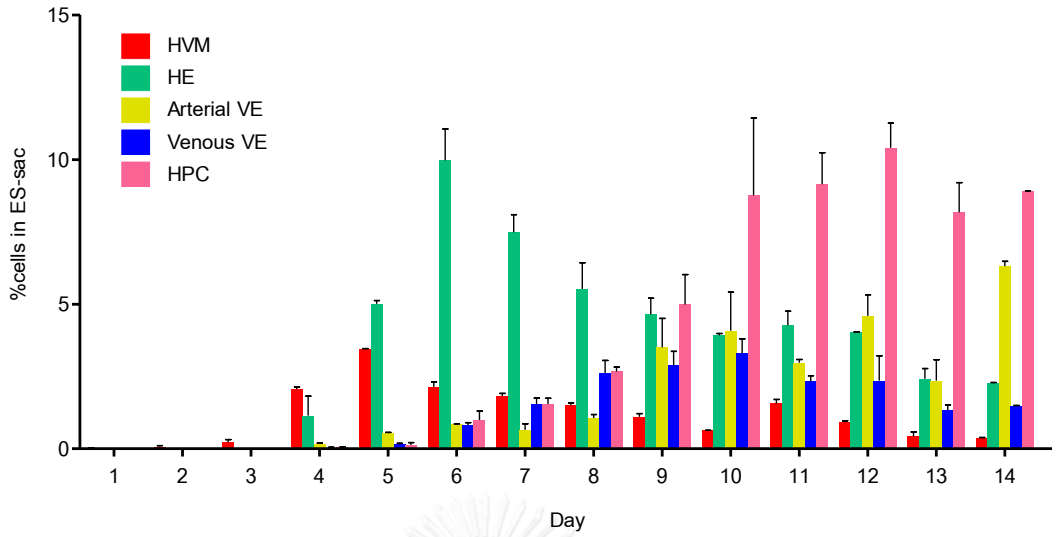

Figure 3 **Time-course analysis of ES-sac differentiation (Day1-14, n=4)** showing the percentages of hemato-vascular mesoderm (HVM, $KDR^{high}PDGFRa^{-}$cells), hemogenic endothelium (HE, $CD34^{+}CD43^{-}CD73^{-}CD184^{-}$cells), arterial vascular endothelium (arterial VE, $CD34^{+}CD43^{-}CD73^{+}CD184^{+}$cells), venous vascular endothelium (venous VE, $CD34^{+}CD43^{-}CD73^{+}CD184^{-}$cells), and hematopoietic progenitor cells (HPCs) in the ES-sac combined with feeder cells. HVM were first detected at Day3 and highest at Day5, and follow by the highest HE generation at Day6. After the formation of HE, HPCs were observed. Additionally, arterial VE and venous VE also were detected in the ES-sac model which indicated that major vessels were existed in the ES-sac model.

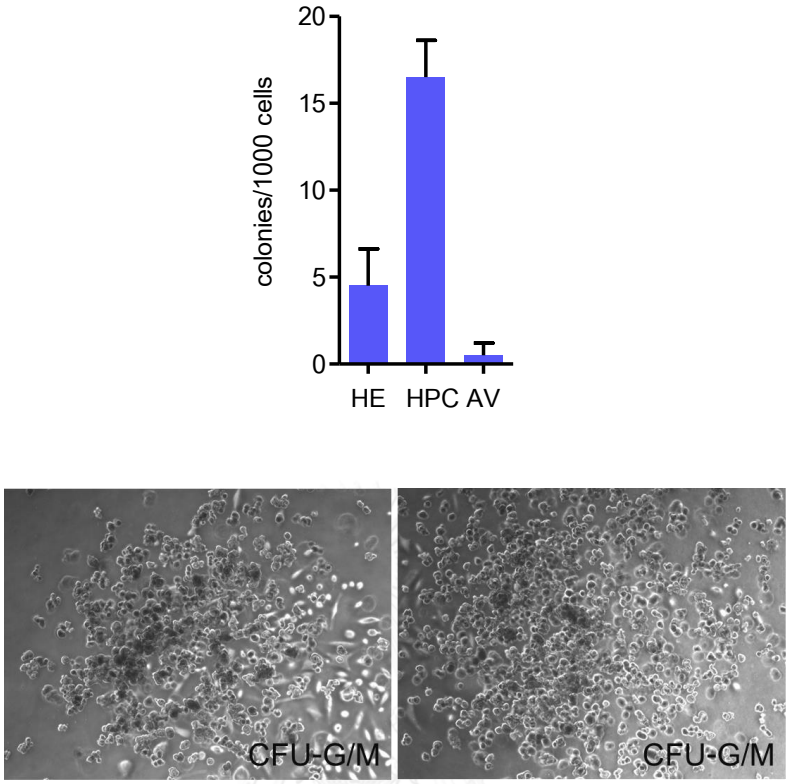

Figure 4 **Clonogenic frequency of HE, HPCs, and AV** A top diagram showed hematopoietic colonies were derived from HE ($CD34^+CD43^-CD73^-CD184^-$cells) and HPC ($CD34^+CD43^+$cells) population, but not AV ($CD34\text{-}CD43^-CD73^+$cells), in methylcelluose. A bottom image showed CFU-G/M colony types. This implied that hematopoietic potential was limited in AV population. (Abbreviations: HE, hemogenic endothelium; HPCs, hematopoietic progenitor cells; AV, arterial and venous vascular endothelium)

Generation and validation of *ETV2-2A-H2B-GFP* iPSC reporter cell line

The study in mouse model revealed that Etv2 is a key master regulator of hematopoietic development that fate mesoderm towards hematopoietic and vascular cells and likely plays a key role in HSC generation (Ren, Gomez et al. 2010, Lee, Kim et al. 2011, Koyano-Nakagawa, Kweon et al. 2012, Swiers, Baumann et al. 2013). Its expression in the mesoderm marks the earliest stage of hemato-vascular commitment. From immunofluorescence staining, we observed ETV2 positive cells within the ES-sac colonies (Fig 5). *ETV2* mRNA was also detected throughout first 14 days of the culture (Fig 6). Importantly, we observed 2 peaks of *ETV2* expression at Day4-10 which might correlates with the separating waves of primitive and definitive hematopoiesis in human. This pattern of temporal expression has not been reported previously and could lead to the new model of human hematopoiesis.

To track the fate of cell expressing ETV2 during human hematopoiesis *in vitro*, we generated *ETV2* reporter iPSC cell line, *ETV2-2A-H2B-GFP* (*ETV2:GFP*). CRISPR/Cas9n system was used to mediate knock in *ETV2* reporter line in which gRNAs were design to target at the last exon of *ETV2* before the stop codon (Fig 7). The *ETV2:GFP* reporter iPSC line retains the pluripotency (Fig 8) and capability to generate blood cell upon co-culture with VEGF expressing C3H101/2 or OP9 feeder cells (Fig 9). To evaluate the accuracy of the *ETV2:GFP* reporter to mark ETV2 expressing cells, dynamic changes of ETV2:GFP^{+}cells were analyzed by FACS. Similar to *ETV2* transcripts, ETV2:GFP^{+}cells were first detected from Day4 of the culture and persist through Day13 (Fig 10). These demonstrated that ETV2:GFP was detected following *ETV2* mRNA expression during ES-sac differentiation. Importantly, the *ETV2* transcripts were detected exclusively within the ETV2:GFP^{+} cell population (Fig 11). Taken together, our data validate the accuracy of the *ETV2:GFP* reporter iPSC lines in marking cells expressing ETV2.

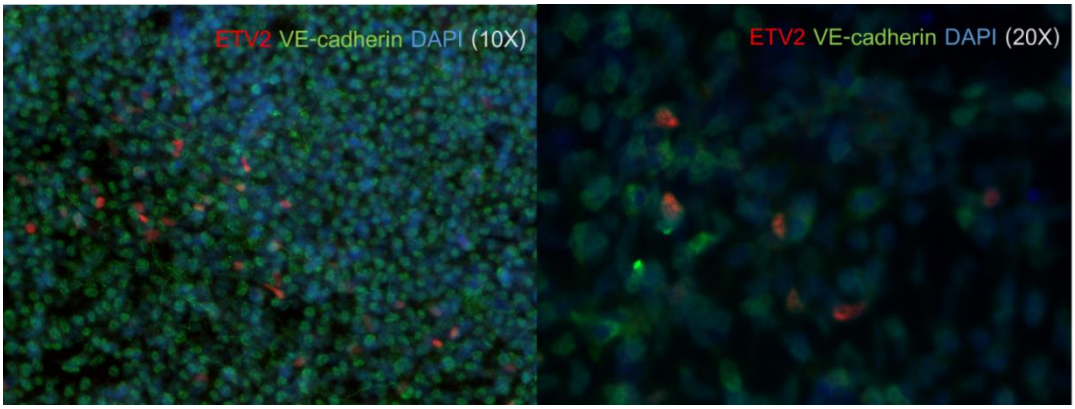

Figure 5 **Immunofluorescent anti-ETV2 and anti-VE-cadherin staining of Day10 ES-sac** ETV2 expressing cells were detected in ES-sac. VE-cadherin is a marker of hematopoietic and vascular cells.

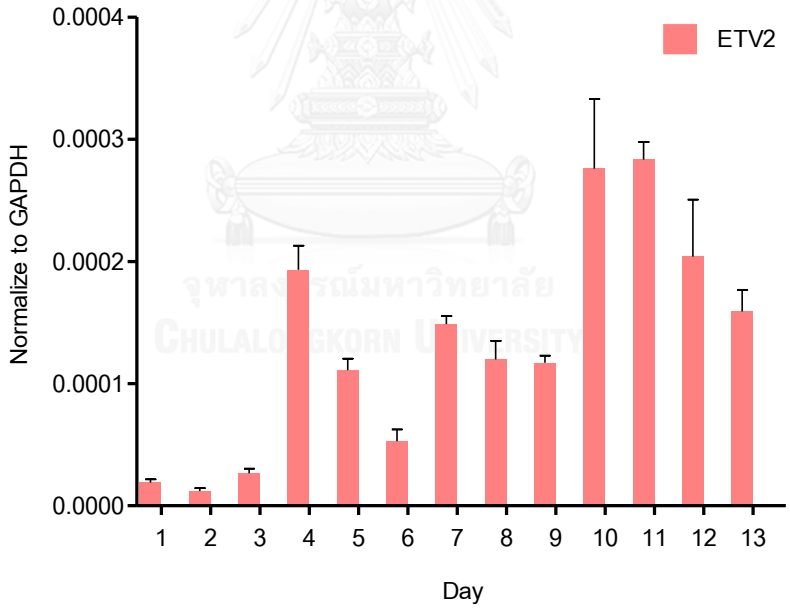

Figure 6 **Quantitative reverse transcription PCR (RT-qPCR) analysis of *ETV2* mRNA expression in ES-sac colonies (Day1-13)** A chart showed that ETV2 function during human hematopoietic differentiation *in vitro.*

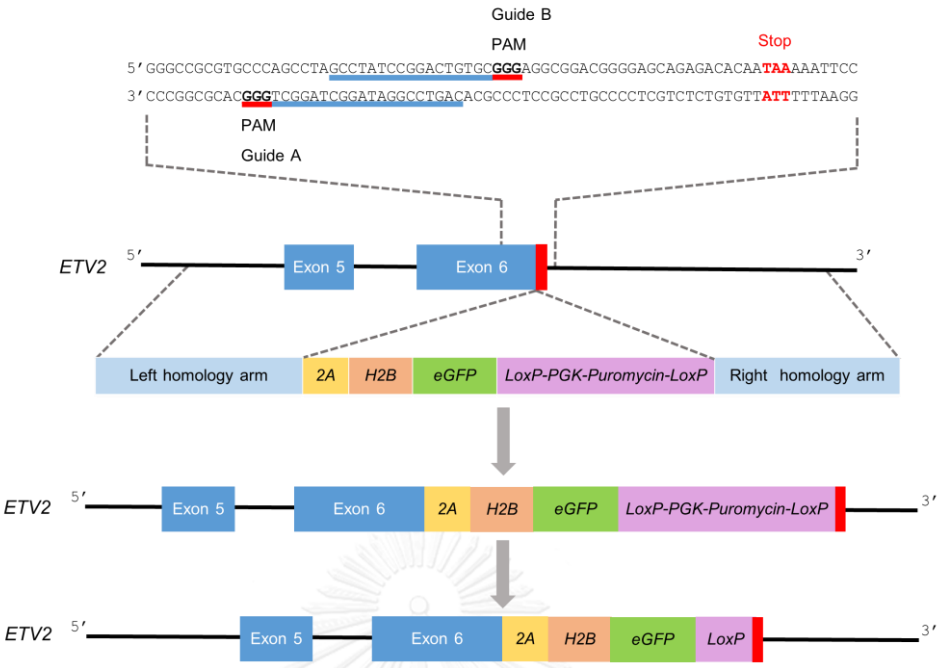

Figure 7 ***ETV2* gene targeting strategy** Using a Cas9 nickase mediated homology recombination technique, a *H2B-2A-GFP* followed by an excisable *LoxP* fragment of selection markers were incorporated into the last exon, before the stop codon of *ETV2* gene.

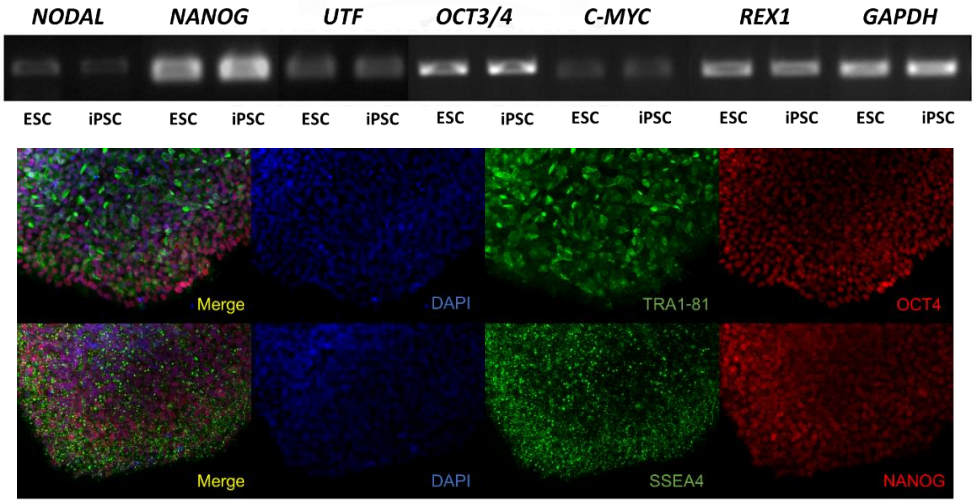

Figure 8 **The *ETV2:GFP* reporter iPSC line retains the pluripotency** that expressed pluripotent markers, mRNA and protein level. A top shows mRNA expression of pluripotent genes in the reporter iPSC line. A bottom is immunofluorescent anti-pluripotent markers staining of the reporter iPSC line.

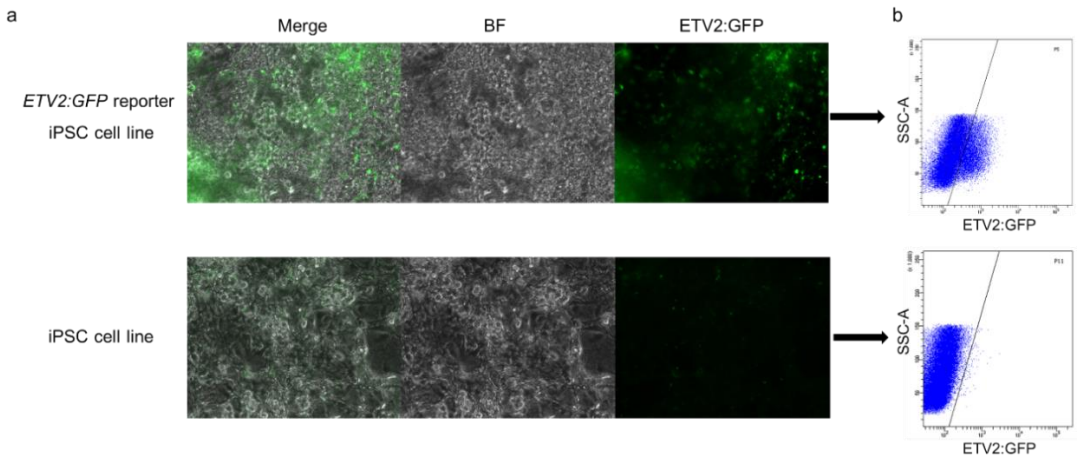

Figure 9 ***ETV2:GFP* iPSCs derived ES-sac (Day10)** (a) *ETV2:GFP* reporter iPSC line derived ES-sac was shown in bright field (BF), fluorescence (ETV2:GFP) and merged images. (b) The cells from ES-sac also was analyzed by FACS. These demonstrated that *ETV2:GFP* iPSCs could differentiate into ES-sac colony and ETV2:GFP expression was observed during the ES-sac development.

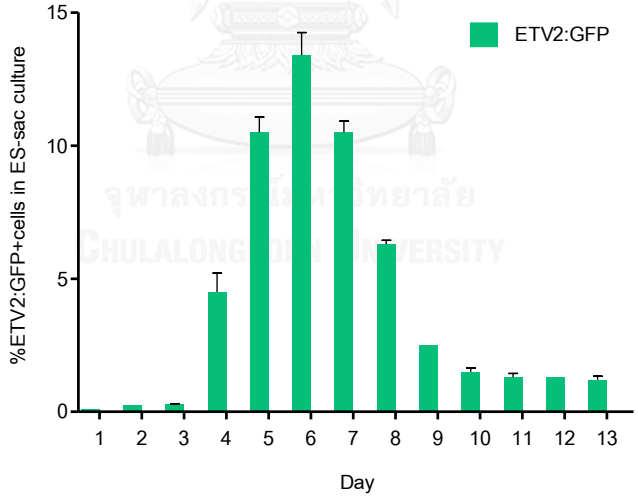

Figure 10 **Time-course differentiation with analysis of ETV2:GFP^{+}cells percentages in ES-sac culture by FACS** (Day1-13 ES-sac, n=4) a chart displayed percentages of ETV2:GFP^{+}cells in ES-sac culture (ES-sac colonies combined with feeder cells). The detection of ETV2:GFP^{+}cells could indicate that ETV2:GFP expression system represents the ETV2 expression during hematopoietic development.

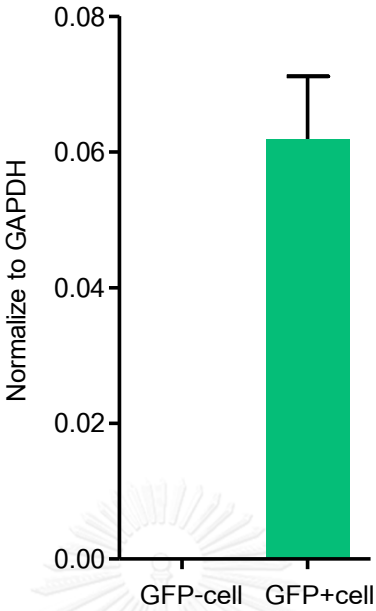

Figure 11 **Analysis of *ETV2* expression in GFP$^+$ and GFP$^-$population by RT-qPCR** GFP$^+$cells and GFP$^-$cells were sorted from Day6 ES-sac and analyzed by real time quantitative PCR for *ETV2* mRNA expression, which is strongly detected in GFP$^+$cell population.

ETV2 is first detected in subgroup of mesoderm then in newly emerge hemogenic endothelium

ETV2:GFP^{+}cells were found 30-40 percent within VE-cadherin^{+}cells (endothelial cells, hemogenic endothelium and some hematopoietic cells) and different subpopulation of mesodermal cells, VE-cadherin^{-}cells. Next, we analyzed ETV2:GFP expression in difference population of mesodermal cells based on previous studies (Fig 12). The VE-cadherin^{-}cells were separated into 6 subgroups based on an expression of PDGFRa and KDR. Primitive mesodermal progenitor (MP), which also contributes to cardiac lineage (Sakurai, Era et al. 2006), was identified as KDR^{+}PDGFR^{+}cells (Fig 13). While hemato-vascular mesoderm (HVM) was identified as KDR^{high}PDGFR^{-}cells, and early hemato-vascular mesoderm (EHVM) as KDR^{high}PDGFR^{+}cells (Wang and Nakayama 2009, Kattman, Witty et al. 2011, Slukvin 2013) (Fig 13). ETV2:GFP^{+}cells were enriched in the population of EHVM and HVM, in which the majority of the population positively expressed ETV2:GFP on Day4-5 of differentiation (Fig 14). ETV2:GFP expression was also detected in ES-sac derived MP, however the percentage was relatively low compared to EHVM and HVM (Fig 14). These results may indicate that MP were likely fated to EHVM and HVM *via* ETV2.

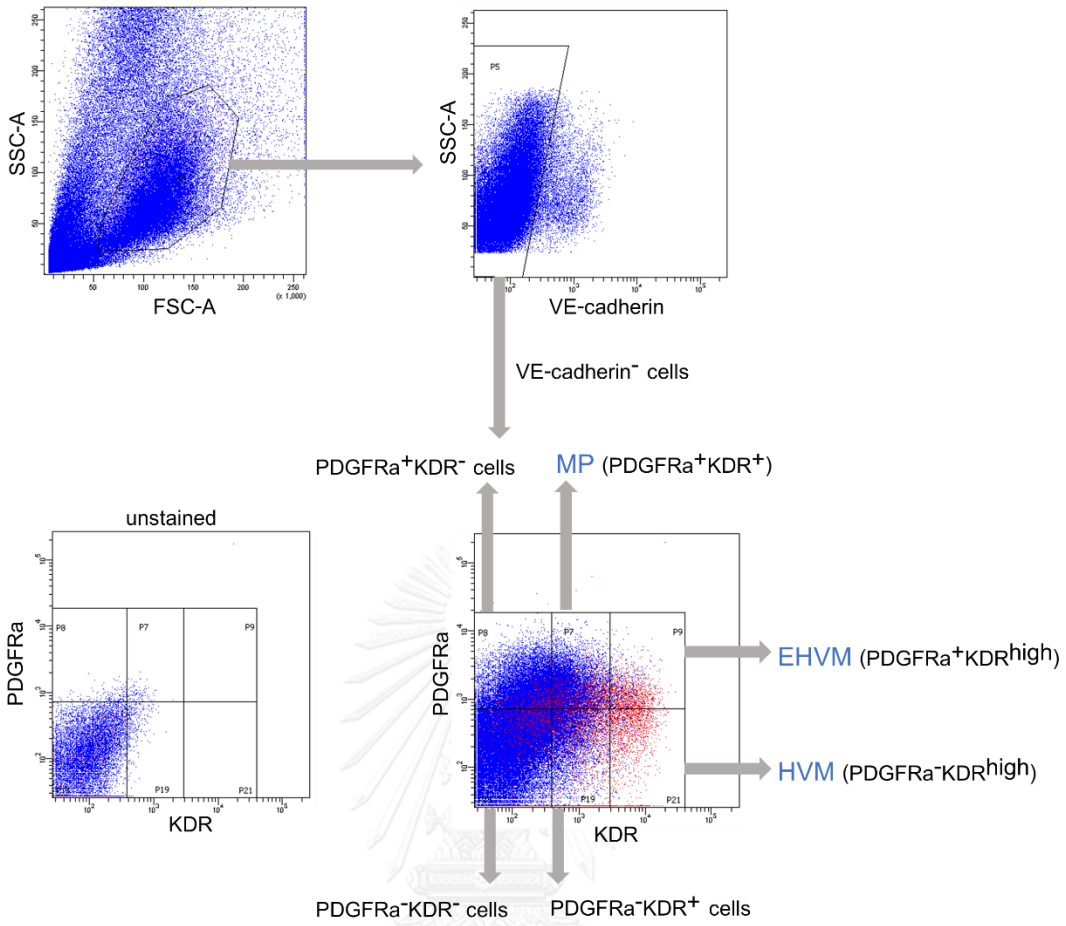

Figure 12 **Schematic diagram for analysis of mesodermal subpopulation in ETV2:GFP^{+}population in ES-sac culture (Day5 ES-sac) by FACS.** Firstly, VE-cadherin^{-} cells were isolated from VE-cadherin^{+}cells, which VE-cadherin^{+}cells committed to hematopoietic and endothelial cells. ES-sac derived VE-cadherin^{-}cells were then divided into 6 subgroup, PDGFR^{+}KDR^{+}cells (mesodermal progenitor or MP), KDR^{high}PDGFR^{+}cells (early hemato-vascular mesoderm or EHVM), KDR^{high}PDGFR^{-}cells (hemato-vascular mesoderm or HVM), PDGFR^{+}KDR^{-}cells (paraxial mesoderm or PM) (Sakurai, Era et al. 2006), PDGFR^{-}KDR^{-}cells and PDGFR^{-}KDR^{+}cells. Finally, the population were analyzed for ETV2:GFP^{+}cell. ETV2:GFP^{+}cell (red dot) were enriched in EHVM and HVM of Day5 ES-sac. ETV2:GFP^{+}cell were also detectable in the MP fractions, at lower levels than in the EHVM and HVM fraction. Unexpectedly, ETV2:GFP^{+}cell also found in other subgroups, indicating that ETV2 probably plays roles in the subgroups.

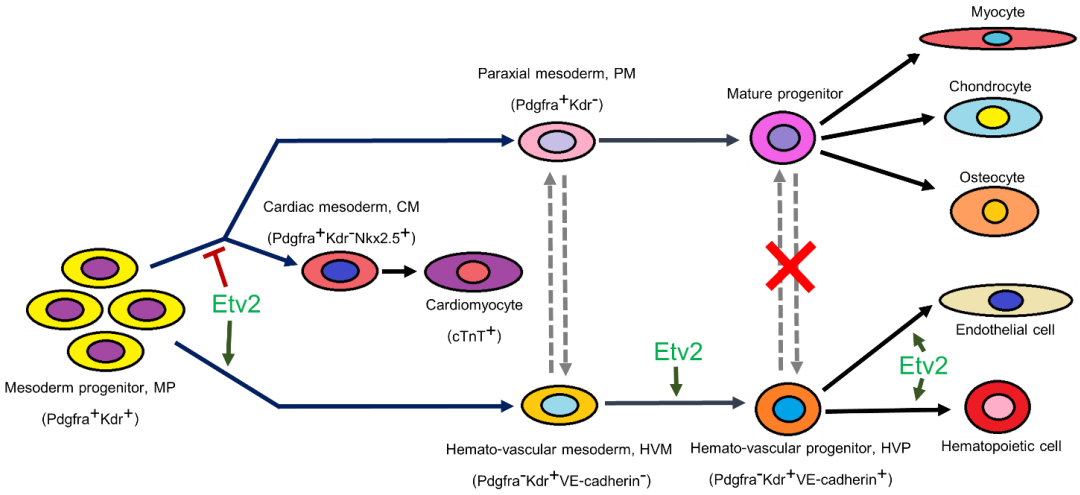

Figure 13 **Schematic representation depicting Etv2 functions in mouse model** Etv2 played the roles in specification of MP ($Pdgfra^{+}Kdr^{+}$cells) into HVM ($Pdgfra^{-}Kdr^{+}VE\text{-}cadherin^{-}$ cells), at the same time, inhibited the MP differentiation into PM ($Pdgfra^{+}Kdr^{-}$) and CM ($Pdgfra^{+}Kdr^{-}Nkx2.5^{+}$). Although the HVM and PM populations exhibit the specific properties of paraxial and lateral mesoderm, respectively, both populations can be converted into each other at an early stage. Additionally, Etv2 promotes the differentiation of HVM into endothelial and hematopoietic lineages (Sakurai, Era et al. 2006, Lammerts van Bueren and Black 2012). (Abbreviations: MP, mesodermal progenitor: CM, cardiac mesoderm: PM, paraxial mesoderm: HVM, hemato-vascular mesoderm: HVP, hemato-vascular progenitor)

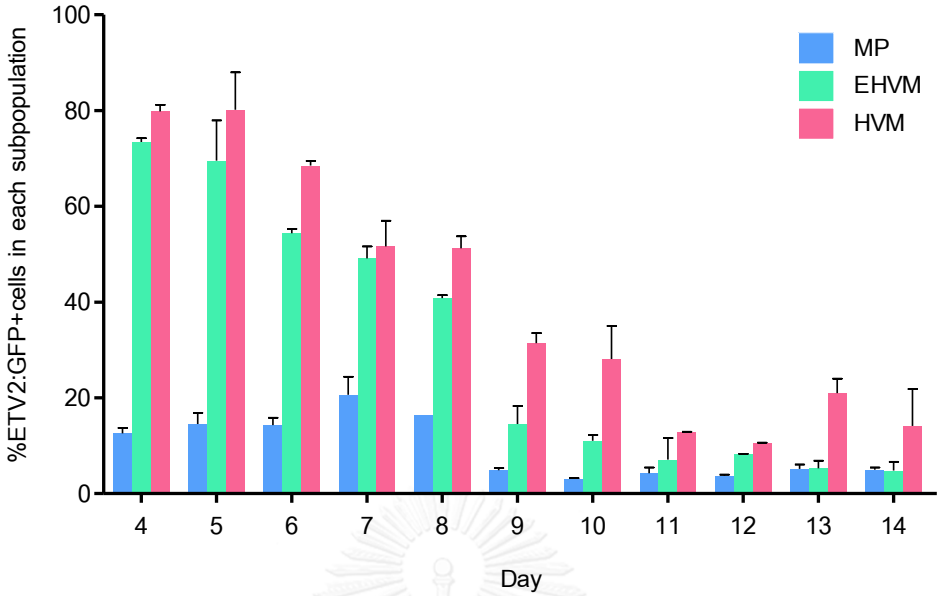

Figure 14 **Percentage of ETV2:GFP^{+}cells in mesodermal subpopulation** Mesodermal progenitor (MP: KDR^{+}PDGFRa^{+}cells), early hemato-vascular mesoderm (EHVM: KDR^{high}PDGFRa^{+}cells), and hemato-vascular mesoderm (HVM: KDR^{high}PDGFRa^{-}cells) were analyzed by FACS for ETV2:GFP^{+}cells during ES-sac differentiation (Day4-14, n=4). ETV2:GFP^{+}cells were enriched in EHVM and HVM especially on Day4-5.

ETV2 might inhibit specification of mesodermal progenitor into cardiac lineage

From previous study in animal model, Etv2 induced MP into hematopoietic and vascular lineages, while inhibited the differentiation of MP into cardiac lineage (Schoenebeck, Keegan et al. 2007, Kataoka, Hayashi et al. 2011, Rasmussen, Kweon et al. 2011, Liu, Kang et al. 2012). To prove whether ETV2 inhibit the generation of cardiac cells, ETV2:GFP^{+}mesoderm (ETV2:GFP^{+}VE-cadherin^{-}) from Day5 ES-sac were sorted and cultured in cardiac differentiation condition (Fig 15). The results demonstrated that ETV2:GFP^{+}mesodermal cells were less proliferative and unable to differentiate into cardiac cells. On the other hand, ETV2:GFP^{-}mesoderm (ETV2:GFP^{-}VE-cadherin^{-}), the number was equal to ETV2:GFP^{+}mesodermal, were able to generate cardiac cells, or cTnT^{+}cells (Fig 16). These results were correlated with others studies in animal models (Schoenebeck, Keegan et al. 2007, Lee, Park et al. 2008, Kataoka, Hayashi et al. 2011, Palencia-Desai, Kohli et al. 2011, Rasmussen, Kweon et al. 2011, Liu, Kang et al. 2012, Liu, Li et al. 2015). Overall, the results could imply the inhibiting roles of ETV2 towards cardiac cell differentiation.

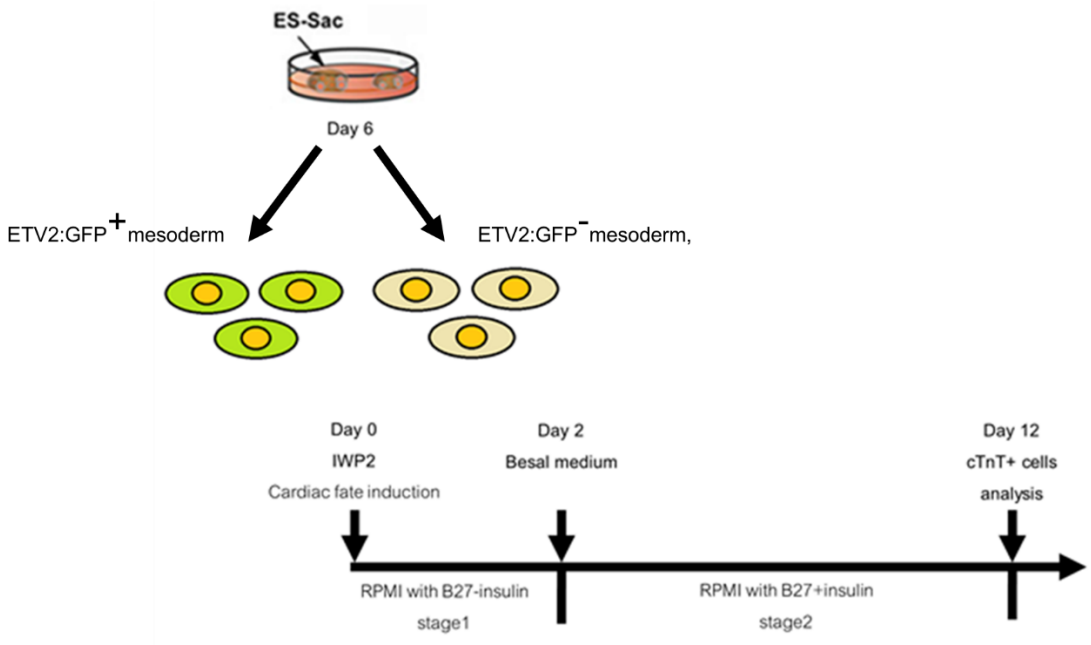

Figure 15 **Schematic strategy for cardiac differentiation from ETV2:GFP^{+} and ETV2:GFP^{-} mesoderm** ES-sac derived ETV2:GFP^{+} and ETV2:GFP^{-}mesodermal cells (30,000 cells) were cultured in cardiac differentiation condition, which 5 μM IWP2 (WNT pathway inhibitor) was added into RPMI/B27 medium (without insulin) during Day0-2 . From Day2, insulin was added into the RPMI/B27 medium, which refreshed every 3 days. Day12 after differentiation, cTnT expressing cells were determined by immunofluorescence staining.

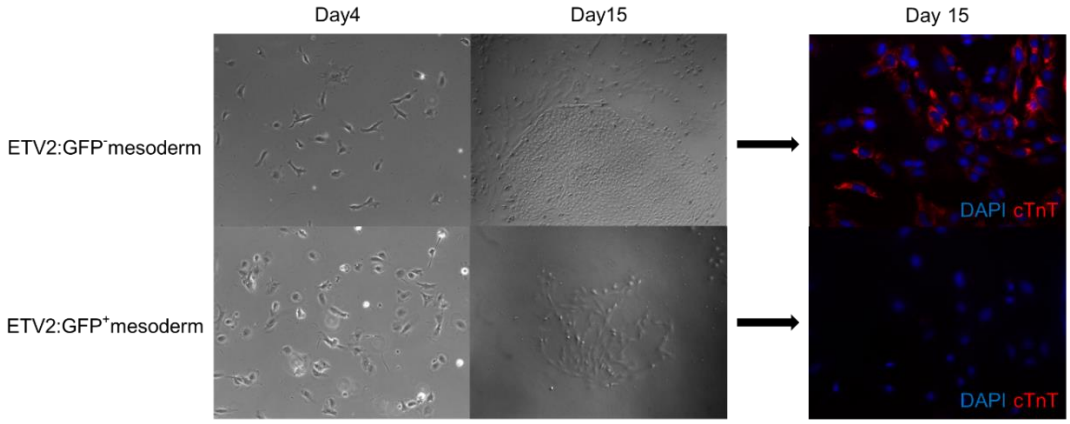

Figure 16 **Cardiac differentiation capability of ETV2:GFP$^-$ and ETV2:GFP$^+$mesodermal cells** ETV2:GFP$^+$mesodermal cells inefficiently proliferated and were unable to differentiate into cardiac cells under cardiac differentiation condition. Conversely, ETV2:GFP$^-$mesodermal cells were able to generate cardiac cell, identified as cTnT$^+$cells.

ETV2 might play a role in definitive hematopoiesis

HSCs with long term engraftment first emerge from HE that line the lumen of dorsal aorta (DA) in aorta-gonad-mesonephros (AGM) (Zovein, Hofmann et al. 2008, Boisset, van Cappellen et al. 2010, Nguyen, Hollway et al. 2014). Interestingly, Etv2 also was detected within DA at the time of HSC emergence (Koyano-Nakagawa, Kweon et al. 2012). This indicated that Etv2 might play a role in HSCs generation. There are several studies supporting the context that ETV2 might be important for HSCs formation (Ren, Gomez et al. 2010, Lee, Kim et al. 2011, Swiers, Baumann et al. 2013). Nevertheless, recent studies suggested that there is heterogeneity of HE defined by expression of CD34 and lack of CD43, CD73 and CD184 expression (CD34$^+$CD43$^-$CD73$^-$CD184$^-$cells). To study definitive HE population in ES-sac model, we identified HE, described by Keller group, in combination with an expression of CD235a and KIT. CD235a can be used to distinguish definitive program (CD235a$^-$cells) from primitive hematopoietic program (CD235a$^+$cells) in early development (Sturgeon, Ditadi et al. 2014). And KIT (CD117) is a marker of HSC and HE in AGM (Goldie, Lucitti et al. 2008, Yokomizo and Dzierzak 2010, Tober, Yzaguirre et al. 2013). Taken together, definitive HE is identified as CD34$^+$CD43$^-$CD73$^-$CD184$^-$CD235a$^-$KIT$^+$cells (KIT$^+$CD235a$^-$HE).

We first analyzed the time-course of HE subpopulation in ES-sac culture. Our data demonstrates that even as early as Day4, primitive subgroup of HE ($CD235^+$) were rare (Fig 17). In contrast, Both KIT^+ and KIT^-HE were able to detect within $CD235a^-$ HE population from Day4-14. We observed ETV2:GFP expressed in most KIT positive fraction of $CD235a^-$HE population whereas the KIT negative fraction contained lower (Fig 18). This result implied that $KIT^+CD235a^-$HE were generated through $ETV2{:}GFP^+$cells.

From previous result, $KIT^+CD235a^-$HE were generated very early of time-course that gave rise the question that whether HE with definitive hematopoietic program were generated in early ES-sac. To prove the question, ES-sac derived HE ($CD34^+CD43^-CD73^-CD184^-$cells) were analyzed for mRNA expression of a definitive hematopoietic marker, *RUNX1C*. RUNX1C, a definitive hematopoietic marker found in HE of the dorsal aorta and associated with the generation of HSCs (Bee, Liddiard et al. 2009). We found that *RUNX1C* was unable to be detected until Day7 of differentiation our data suggests that HE with definitive hematopoietic program were generated in late ES-sac. Surprisingly, expression of *ETV2* mRNA in ES-sac began detected again in the HE population as same time as *RUNX1C* expression in which peaked at Day8 within this time period (Fig 19). Importantly, it seemed that time course expression of *ETV2* was parallel to *RUNX1C* expression, implying that ETV2 might be required for RUNX1C-mediated hematopoiesis which generate more adult like HSC/HPC.

SOX17 is a marker of hemogenic endothelium in the AGM and is required for the generation of HSCs from the AGM (Kim, Saunders et al. 2007, Clarke, Yzaguirre et al. 2013). The recent study showed that the hematopoietic culture mimicking hematopoiesis in aorta-gonad-mesonephros (AGM) generated a network of aorta-like $SOX17^+$ vessels in later culture which $RUNX1C^+$blood cells emerged from the vessels (Ng, Azzola et al. 2016). In our study, $ETV2{:}GFP^+KIT^+CD235a^-$HE found in the later day of culture expresses higher level of *SOX17* (Fig 20). Thus, $ETV2{:}GFP^+KIT^+CD235a^-$HE from the second wave might be similar to HE in AGM. We also found emerging round blood like cells co-expressing SOX17 and RUNX1 (Fig 21) in which might be able to generate HSC. The

properties of cell emerged from late ETV2:GFP^{+}KIT^{+}CD235a^{-}HE need to be clarify in future experiments.

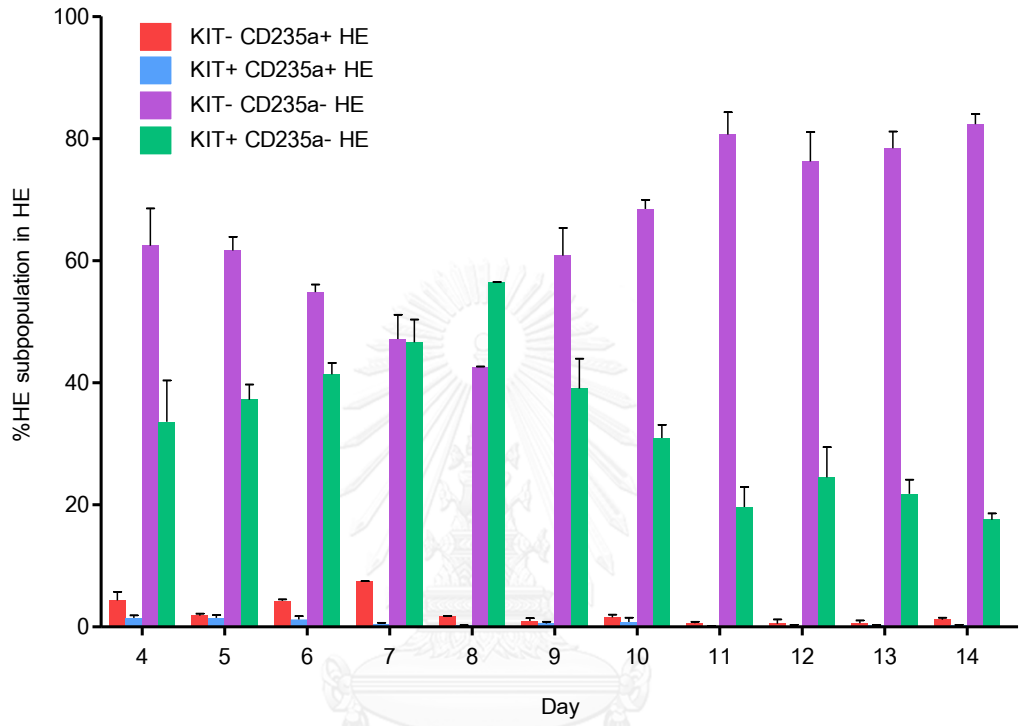

Figure 17 **Time-course analysis of percentage of HE subpopulation in HE (Day4-14 ES-sac, n=4)** The percentages of HE subpopulation in which divided into each subpopulation base on an expression of KIT and CD235a. The most population of HE with primitive hematopoietic program (CD235a^{+}HE) were observed in early ES-sac differentiation. While HE with definitive hematopoietic program (CD235a^{-}HE) were the major population in HE and were observed from early ES-sac development (Day4) throughout the differentiation. The more definitive HE, identified as KIT^{+}CD235a^{-}HE, were also detected in the early phase in which peaked at Day8 then gradually decreased but still detected throughout the differentiation.

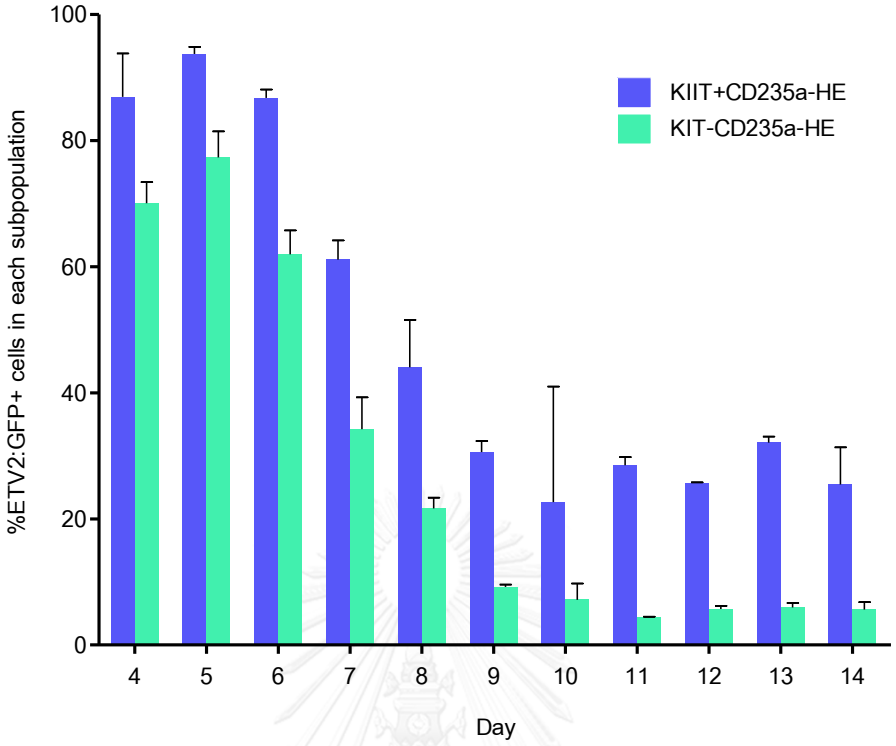

Figure 18 **Percentage of ETV2:GFP^{+}cells in KIT^{+}CD235a^{-}HE and KIT^{-}CD235a^{-}HE** The chart showing all most CD235a^{-}HE were detected for ETV2 on Day5, indicating that these populations were generated through ETV2:GFP^{+}cells.

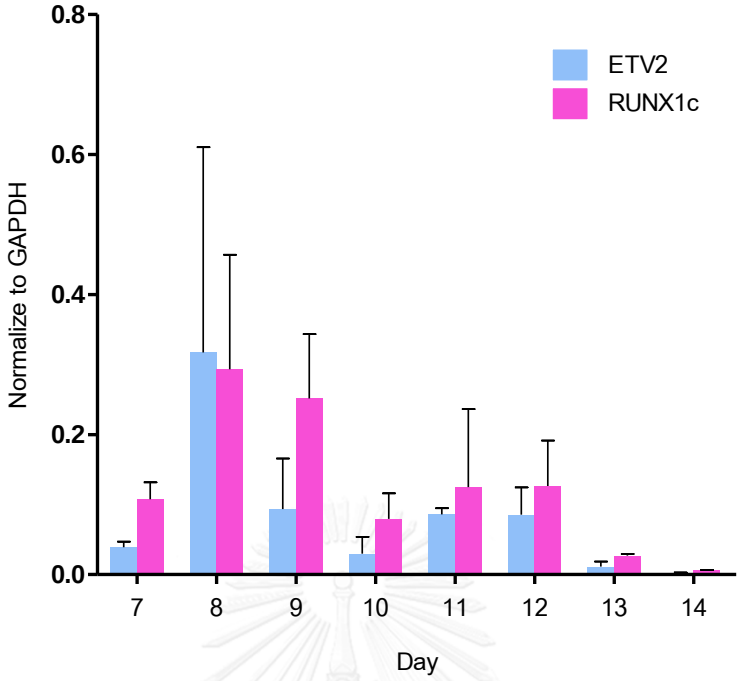

Figure 19 **RT-qPCR of *ETV2* and *RUNX1C* on HE populations ($CD34^{+}CD43^{-}CD73^{-}CD184^{-}$ cells from Day7-14)** *ETV2* expression in ES-sac derived HE was parallel to *RUNX1C* expression.

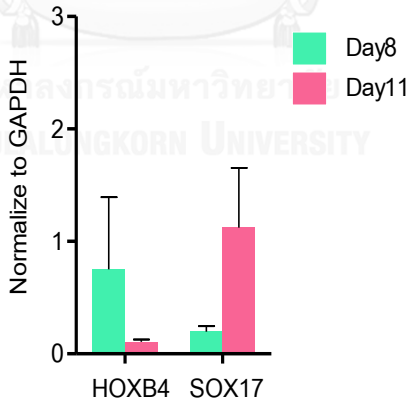

Figure 20 **RT-qPCR of *SOX17* and *HOXB4* in ETV2:$GFP^{+}KIT^{+}CD235a^{-}$HE** of Day8 ES-sac (first wave of *ETV2* expression) compared to Day11 ES-sac (second wave of *ETV2* expression) The second wave (Day11) exhibited higher expression levels of *SOX17* than the first wave. *HOXB4* is a gene associated with mesodermal induction and patterning which was used as negative control.

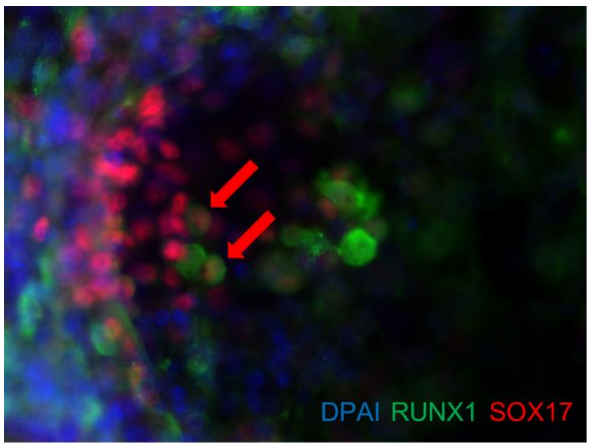

Figure 21 **Immunofluorescent anti RUNX1 and anti SOX17 staining (Day14 ES-sac)** There are emerging round blood like cells co-expressing SOX17 and RUNX1 (red arrow).

CHAPTER V

Discussion

In mouse model, Etv2 plays as an important key master regulator in directing mesodermal fate decision into hematopoietic and vascular lineage. Interestingly, Etv2 showed an important role in HSC generation. In our study in human hematopoietic development *in vitro*, *ETV2* expression was first detected during mesodermal specification. Using *ETV2:GFP* iPSC reporter line, ETV2:GFP expression also was first observed during mesodermal specification in ES-sac model, which around 73 and 80 percent of EHVM and HVM respectively at early ES-sac (Day4) were detected ETV2:GFP expression. Corresponding to the study by Choi that ETV2 was expressed in mesodermal cells with high hematopoietic and vascular potential, $KDR^{bright}APLNR^{+}PDGFRa^{low/-}$cells lacking expression of endothelial, mesenchymal and hematopoietic markers (Choi, Vodyanik et al. 2012). We found that KIT^{+} and $KIT^{-}CD235a^{-}$HE population began to appear from Day4-14 of ES-sac differentiation following HVM generation in which $ETV2:GFP^{+}$cells were detected in $KIT^{+}CD235a^{-}$HE higher than in $KIT^{-}CD235a^{-}$HE population. These studies implied that HVM might be generated and given rise to hematopoietic lineage by ETV2 induction in human hematopoietic development *in vitro*, that were similar to mouse model. However, *ETV2* overexpression, *ETV2* downregulation and *ETV2* knock-out condition need to be investigated to identify essential roles of ETV2 in human hematopoiesis. From Figure 6 (*ETV2* mRNA expression in ES-sac colonies) and Figure 9 ($ETV2:GFP^{+}$cells in ES-sac culture) showed that the levels of *ETV2* mRNA and numbers of $ETV2:GFP^{+}$cells at the later phase of the development were not correlated that might result from $ETV2:GFP^{+}$ cells in later ES-sac were express very high levels of *ETV2* in the cells in which need to be investigated in the future experiment.

We also found that ES-sac derived mesoderm acquired expression of ETV2:GFP were unable to contribute to cardiac cells in cardiac differentiation condition. These results might support the notion that ETV2 inducts mesodermal progenitor fate to hematopoietic and vascular lineage while inhibits cardiac lineage (Lee, Park et al. 2008,

Kataoka, Hayashi et al. 2011, Palencia-Desai, Kohli et al. 2011, Rasmussen, Kweon et al. 2011, Liu, Kang et al. 2012, Liu, Li et al. 2015).

Time course of ES-sac differentiation, there was the second wave of *ETV2* surging again around Day8 coinciding with expression of *RUNX1C,* a key transcription factor associated with the generation of HSC from HE of DA (Bee et al., 2009). Interestingly, *ETV2* expression was parallel to *RUNX1C* expression. Additionally, ETV2:GFP expressing $KIT^{+}CD235a^{-}$HE at late time course of the differentiation express higher level of *SOX17,* which marks HE of the AGM (Ng, Azzola et al. 2016), compared to early time course. Implying that function of ETV2 at the later stage of the differentiation might be associated with the generation of more adult like HSC/HPC from HE that resemble the HE in AGM.

To achieve the goal of generating fully functional, long-term engraftable hematopoietic stem cells (HSCs) from pluripotent stem cells (PSCs), better understanding of the ontogeny of human HSCs is required. Our data suggests that current available HE markers are not sufficient to separate HE that can give rise to HSCs from others. There were two waves of *ETV2* expression, first wave emerged during mesodermal specification or early ES-sac then *ETV2* was downregulated and the second wave emerge at later phase of ES-sac. The two waves of *ETV2* expression suggests the new models that either there are newly form HE which occur late in culture or there is a second pulse of high *ETV2* expression within subpopulation of early generated HE. We are in the process of interrogating which cell population is the source of late $ETV2:GFP^{+}$cells. To prove that late $ET2:GFP^{+}$cells truly contains HSC, transplantation in animal model is required. Nevertheless, *ETV2:GFP* iPSC reporter line we created is a helpful tool for elucidating the mechanism of human blood development and has a potential to be developed into a screening platform for drugs or small molecules that promote hematopoiesis.

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APPENDIX

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