STUDY OF PLATELET PRODUCTION FROM MEGAKARYOCYTE BY USING INDUCED PLURIPOTENT STEM CELL

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การศึกษาการสร้างเกล็ดเลือดจากเมกาคาริโอไซต์โดยใช้เซลล์ต้นกำเนิดชนิดไอพีเอส

นายจตุรวัฒน์ ปวีณวงศ์ชัย

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จตุรวัฒน์ ปวีณวงศ์ชัย : การศึกษาการสร้างเกล็ดเลือดจากเมกาคาริโอไซต์โดยใช้เซลล์ต้นกำเนิดชนิดไอพีเอส. (STUDY OF PLATELET PRODUCTION FROM MEGAKARYOCYTE BY USING INDUCED PLURIPOTENT STEM CELL) อ.ที่ปรึกษาหลัก : ศ. นพ.ดร.พลภัทร โรจน์นครินทร์, อ.ที่ปรึกษาร่วม : รศ. นพ.ดร.นิพัญจน์ อิศรเสนา ณ อยุธยา

Glycoproteins (GP) Ib-IX-V ซึ่งพบบนเมกาคาริโอไซต์และเกล็ดเลือด มีบทบาทในการผลิตเกล็ดเลือด โดยจับกับ von Willebrand factor (VWF) และกระตุ้นการสร้างเกล็ดเลือด การขาด GPIb หรือภาวะที่ GPIb ทำงานมากผิดปกติ ส่งผลให้เกิดเกล็ดเลือดที่มีขนาดใหญ่และจำนวนน้อย ซึ่งกลไกที่ก่อให้เกิดความผิดปกตินั้นยังไม่ชัดเจน ในการศึกษาครั้งนี้ เซลล์ต้นกำเนิดชนิดไอพีเอสของมนุษย์ได้ถูกนำมาใช้ในการศึกษาพยาธิกำเนิดของโรค โดยทำการสร้างเซลล์ต้นกำเนิดชนิดไอพีเอสที่มีการกลายพันธุ์ของยีน *GP1BA* ชนิด p.M255V ซึ่งพบได้ในโรค platelet-type von Willebrand disease (PT-VWD) ด้วยเทคนิค CRISPR-Cas9 และเซลล์ต้นกำเนิดชนิดไอพีเอสที่ไม่พบ GPIb ซึ่งได้สร้างมาจากเซลล์ของผู้ป่วย Bernard Soulier (BSS) พบว่า เมกาคาริโอไซต์ของโรคที่มีความผิดปกติของยีน *GP1BA* ทั้ง 2 ชนิดมีการสร้าง proplatelet ขนาดใหญ่ และมีการสร้างเกล็ดเลือดจำนวนน้อยลงและมีขนาดใหญ่เมื่อเทียบกับเกล็ดเลือดปกติ และเมื่อวิเคราะห์โปรตีนด้วยวิธี Capillary Western blot พบว่า เมกาคาริโอไซต์ที่มีความผิดปกติของ GPIb มีการกระตุ้นโปรตีน ERK1/2 ที่ต่ำกว่าเมกาคาริโอไซต์ปกติ และโปรตีน phosphorylated MLC2 (Myosin Light chain 2) มีปริมาณสูงขึ้นในเมกาคาริโอไซต์ที่มีความผิดปกติของ GPIb เมื่อเทียบกับเมกาคาริโอไซต์ปกติ และเมื่อทำการเติมสารยับยั้ง mitogen-activated protein kinase (MAPK) pathway (MEKi) ให้กับเซลล์ต้นกำเนิดชนิดไอพีเอสปกติ พบว่าเกล็ดเลือดที่ได้มีขนาดใหญ่ขึ้น และจำนวนลดน้อยลง รวมถึงมีการเพิ่มของโปรตีน phosphorylated MLC2 เพิ่มมากขึ้น เมื่อเทียบกับภาวะที่ไม่ได้เติมสารยับยั้ง และเมื่อเติม ROCK inhibitor (ROCKi) ซึ่งมีคุณสมบัติยับยั้งการเกิด phosphorylation ของโปรตีน MLC2 ลงในเมกาคาริโอไซต์ที่มีความผิดปกติของยีน *GP1BA* และเมกาคาริโอไซต์ปกติที่ได้รับ MEKi พบว่าเกล็ดเลือดที่ได้มีขนาดเล็กลง และมีจำนวนเพิ่มมากขึ้น เมื่อเปรียบเทียบกับเมกาคาริโอไซต์ที่ผิดปกติที่ไม่ได้เติม ROCKi และเมกาคาริโอไซต์ที่ได้รับ MEKi จะเห็นได้ว่าเซลล์ต้นกำเนิดชนิดไอพีเอสที่มีการดัดแปลงพันธุกรรมสามารถนำมาใช้เป็นโมเดลในการศึกษาความผิดปกติของการสร้างเกล็ดเลือด โดยพบว่าเซลล์ที่มีการหายไปของ GPIb และเซลล์ที่มีการทำงานของ GPIb มากผิดปกติ จะทำให้โปรตีน MAPK/ERK ลดต่ำลง แต่จะเพิ่มการ phosphorylation ของโปรตีน ROCK/MLC2 มากขึ้น ซึ่งส่งผลให้เกิดการสร้างเกล็ดเลือดที่ผิดปกติ

สาขาวิชา	ชีวเวชศาสตร์	ลายมือชื่อนิสิต .....
ปีการศึกษา	2562	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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Jaturawat Pawinwongchai : STUDY OF PLATELET PRODUCTION FROM MEGAKARYOCYTE BY USING INDUCED PLURIPOTENT STEM CELL. Advisor: Prof. PONLAPAT ROJNUCKARIN, M.D. Ph.D. Co-advisor: Assoc. Prof. NIPAN ISRASENA, M.D. Ph.D.

Thrombopoiesis is the process of platelet production from hematopoietic stem cells (HSCs). Glycoproteins (GP) Ib-IX-V that is expressed on the surface of megakaryocytes and platelets binds von Willebrand factor (VWF) plays roles in platelet production. Either the GPIb deficiency or hyper-function can cause macrothrombocytopenia, the molecular mechanisms remain unclear. In this study, the pathogenesis investigations were performed in the human induced pluripotent stem cell (hiPSC) model. CRISPR-Cas9 was used to generate the hiPSCs carrying a gain-of-function *GP1BA* p.M255V mutation which was described in platelet-type von Willebrand disease (PT-VWD). The GPIb deficiency hiPSCs were previously derived from a Bernard Soulier syndrome (BSS) patient. After megakaryocyte differentiation, both hiPSC mutations showed large proplatelet tips and yielded fewer but larger platelets compared with normal hiPSCs. The Capillary Western analyses revealed the lower ERK1/2 activation and higher MLC2 (Myosin light chain 2) phosphorylation in megakaryocytes with mutated GPIb. Adding a mitogen-activated protein kinase (MAPK) pathway inhibitor to normal hiPSCs recapitulated the phenotypes of GPIb mutations and increased MLC2 phosphorylation. Notably, a ROCK inhibitor which could inhibit MLC2 phosphorylation rescued the macrothrombocytopenia phenotypes of both GPIb alterations and normal hiPSCs with a MAPK inhibitor. In conclusion, the genetically-modified hiPSCs can be used to model disorders of proplatelet formation. Both loss- and gain-of-function GPIb reduced MAPK/ ERK activation but enhanced ROCK/ MLC2 phosphorylation resulting in dysregulated platelet generation.

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CHAPTER I

INTRODUCTION

Background and rationale

Platelets are essential for hemostasis. Thrombocytopenia in patients can lead to bleeding symptoms ranging from petechiae and/or bruises to vital organ hemorrhages. Platelet transfusion can be used to obtain an adequate level of circulating functional platelets to reduce the risk of hemorrhagic complications. However, platelets express surface antigens that may induce alloantibody formation decreasing post-transfusion counts and platelet survival. Therefore, platelet transfusion refractoriness is a major complication of long-term platelet supportive care (1). Consequently, the universal platelets lacking human leukocyte antigens (HLA) are the ideal product which can be used in thrombocytopenic patients suffering from platelet refractoriness. On the other hand, thrombocytosis in patients can lead to thrombosis. Cytoreductive therapy can decrease platelet counts but have side effects of anemia and leukopenia. Therefore, deeper insights in mechanisms of platelet production may be applied for the treatments of various platelet disorders.

Platelets are the smallest anucleated blood cells which are approximately 2-4 microns in diameter. Platelets are generated from megakaryocytes which are derived from hematopoietic stem cells in the bone marrow. Platelets have the main function in primary hemostasis (2). When platelets expose to a vascular injury, a platelet plug will be formed by adhesion, activation and secretion, and aggregation to prevent blood loss (3). The process of platelet production is called thrombopoiesis by which hematopoietic stem cells (HSCs) differentiate to megakaryocyte progenitors and megakaryocytes in the presence of the

microenviromental niches and hematopoietic cytokines (4). Thrombopoietin (TPO) is the hematopoietic cytokine that stimulates the proliferation and differentiation of HSCs into the megakaryocytic lineage (5). The megakaryocyte progenitors undergo endomitosis by replicating DNA without cytokinesis resulting in giant polyploid cells (6). Subsequently, large megakaryocytes extend the proplatelets that contains the organelles and granules for platelet formation (7). The molecular mechanisms of endomitosis and proplatelet formation remain unclear. Understanding of thrombopoiesis may be helpful in management of platelet disorders and applicable to *in vitro* platelet production in the future.

TPO activates many signal molecules that regulates the platelet production, such as JAK/STAT, Ras/MAPK, and PI3K pathways (8). Megakaryocytes and platelets express many surface glycoproteins (GP). GPIb-IX-V that is expressed on the surface of megakaryocytes and platelets binds von Willebrand factor (VWF) and plays the critical role in primary hemostasis. The causes of GPIb-IX-V abnormalities are mutations in *GP1BA*, *GP1BB* or *GP9* genes. The *GP1BA* variants result in two bleeding disorders including Bernard Soulier syndrome (BSS) and platelet type von Willebrand disease (PT-VWD). BSS is an autosomal recessive disorder while PT-VWD is an autosomal dominant disease (9). The *GP1BA* gene mutations in BSS are involved in a defect of GPIb-IX-V complex expression on the surface of platelets (10). BSS is an inherited thrombocytopenia with giant platelets and bleeding tendencies. BSS, platelets are not aggregated by ristocetin in the presence of VWF (11, 12). The gain-of-function *GP1BA* gene mutations in PT-VWD result in the excessive GPIbα binding to VWF (13). PT-VWD is characterized by macrothrombocytopenia and low VWF level from abnormal consumption (14). Both activating and inhibitory mutations of *GP1BA* gene can result in macrothrombocytopenia underscoring the importance of GPIb-IX-V in proplatelet formation via unclear mechanisms (15, 16). However, a cell line model for investigating platelet generation *in vitro* is lacking.

Recently, human induced pluripotent stem cells (hiPSCs) have been generated from adult cells by four transcription factors, Oct3/4, Sox2, c-Myc, and Klf4. hiPSCs can become an alternative source of hematopoietic cells that differentiate into megakaryocyte and platelets. They may be used as a versatile tool for understanding of megakaryocyte and platelet biology (17). In the future, generated platelets may be used for clinical transfusion which is currently limited by the low yields of *in vitro* derived platelets.

Previous studies from our group found that hiPSCs from patients with an inactivating mutation of the *GP1BA* gene displayed defective proplatelet formation *in vitro*. In this study, I attempt to generate the activating *GP1BA* mutation by using CRISPR/Cas9 technology in hiPSCs for studies of platelet production compared with normal hiPSCs and cells with the inactivating *GP1BA* mutation. Mutant hiPSCs will be characterized for the pluripotent properties and cultured using a co-culture system to generate megakaryocytes and platelets. The cultures will be observed for proplatelet formation, total number, morphology, surface markers megakaryocytes and platelets. Furthermore, mutant hiPSCs will be studied for the signaling transduction pathways which may be involved in the abnormal platelet production by GPIb defects, such as Ras/MAPK, Rho/ROCK and PI3K pathways.

Keywords

Platelet-type von Willebrand disease, Bernard Soulier syndrome, Induced Pluripotent stem cell, Macrothrombocytopenia, Glycoprotein Ib, Megakaryocyte, Platelet, Proplatelet formation

Research design

Experimental design

Research questions

What are the differences in platelet production and signal transduction between megakaryocytes with over-activating Glycoprotein Ib and normal megakaryocytes generated from hiPSCs?

Objectives

There are three objectives in this research including;

1. To establish hiPSCs with glycoprotein Ib gain-of-function mutation by using CRISPR/Cas9 technique.
2. To study the effects of the GPIb mutation on numbers, sizes and morphology of megakaryocytes, proplatelets and platelets.
3. To compare the protein signaling pathways between hiPSCs with the GPIb mutation and normal hiPSCs.

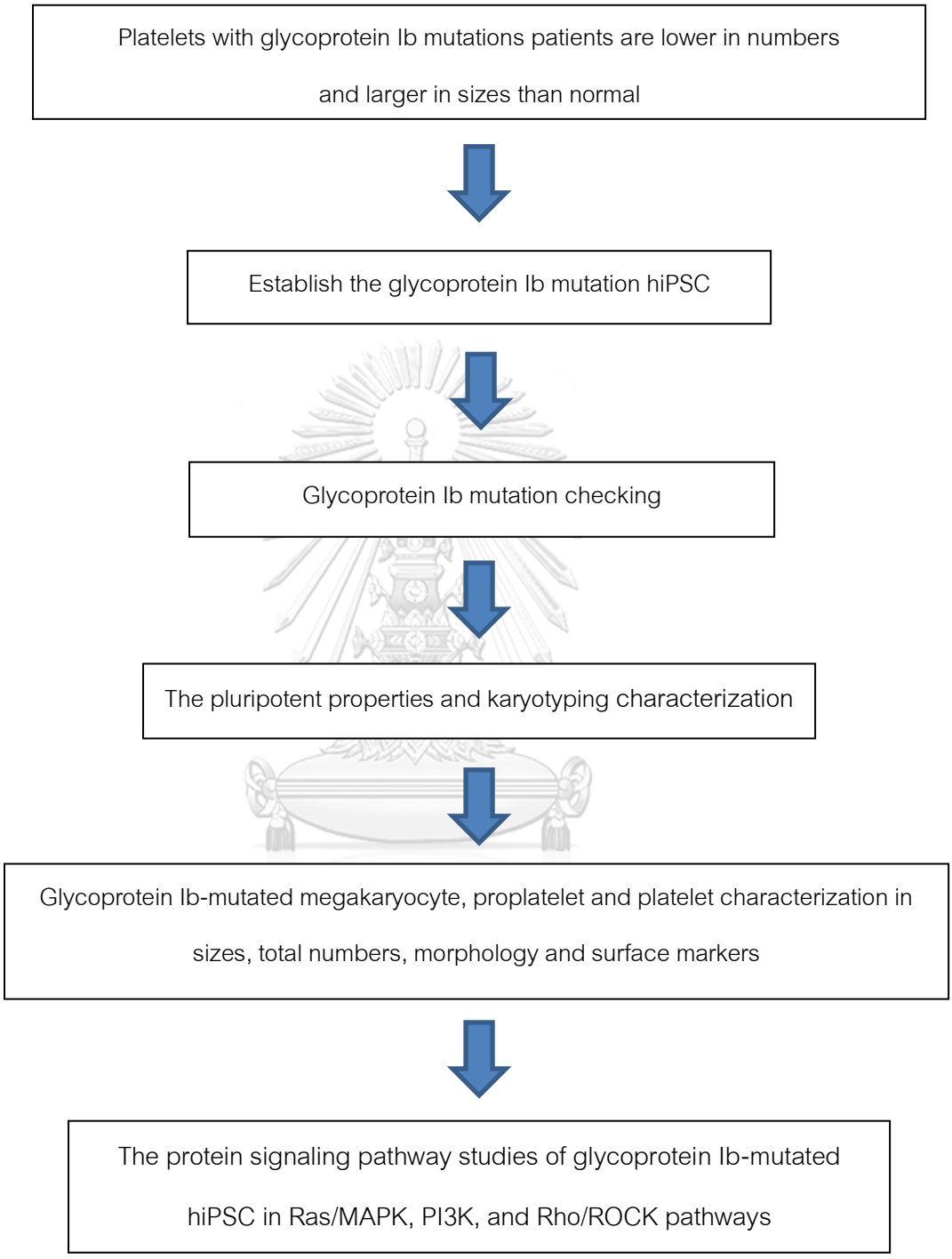
Hypothesis

The hiPSCs with over-activating GP Ib show different platelet production and protein signaling pathways compared with normal hiPSC.

Benefits

1. To establish the disease model of human glycoprotein Ib mutations for understanding of pathogenesis.
2. To study the roles of GPIb on platelet production that may be used to screen for novel therapy.
3. To study the protein signaling pathways critical for platelet generation. This may be applied for *in vitro* platelet production for transfusion in the future.

Conceptual framework



CHAPTER II

REVIEW LITERATURES

Platelets

Platelets or thrombocytes are the smallest blood cells which are anucleate discoid-shaped and approximately 2-4 microns in diameter. Platelets are generated from megakaryocytes which are rare hematopoietic polyploid cells derived from hematopoietic stem cells in the bone marrow. Platelets are released between 140,000 and 400,000 per microliter into blood circulation and approximately one-third is pooled in the spleen. Platelets are circulating in the blood stream for 8-10 days. Primary hemostasis is the main function of platelets which are also involved in other processes including innate immunity, the tumor progression and inflammation (2, 18, 19). When platelets are exposed to the subendothelial matrix following blood vessel injury, they are activated, change shape, adhere to the site of injury and form a hemostatic plug or called aggregation (2). Platelets are also necessary for maintaining vascular integrity. In secondary hemostasis, platelets provide the negatively charged surface for coagulation factor assembly to stop bleeding.

Platelets have the function in primary hemostasis to prevent blood loss. When platelets expose to the vascular injury, the platelet plug will be formed by the processes of adhesion, activation and secretion, and aggregation (Figure 1) (3).

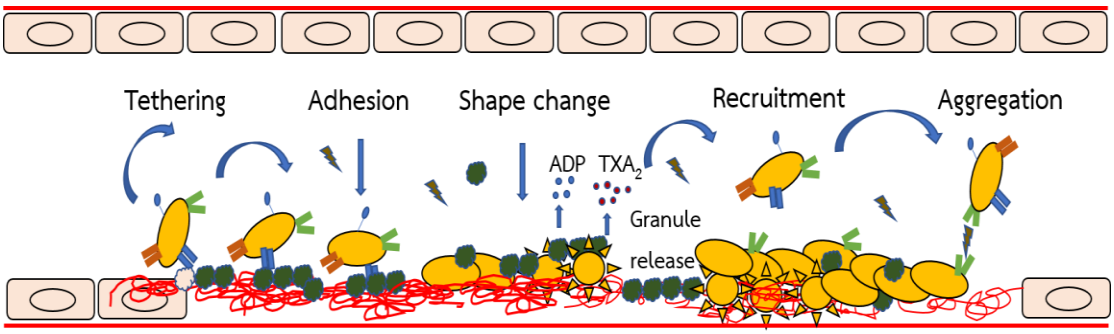


Figure 1 Platelet function in primary hemostasis
Platelets undergo tethering and adhesion at a site of vascular damage, followed by shape change, activation, granule secretion and aggregation (3).

Platelet adhesion is the first step when platelet founds the vascular injury. Platelet adheres to subendothelial extracellular matrix by interaction with specific receptors including glycoprotein Ib-IX-V (GPIb-IX-V) binding to von Willebrand factor (VWF), while GPVI and $\alpha_{II}\beta_1$ interact with collagen. This initial platelet tethering induces firm adhesion between platelet and subendothelial matrix resulting in signal transduction to platelet aggregation (3, 20).

After platelet adhesion, platelet activation and secretion occur to maintain the hemostasis. Platelets produce and release the agonists at the injured site including thromboxane A_2 (TxA_2), adenosine diphosphate (ADP), epinephrine, and thrombin. ADP is secreted from the dense granules of platelets causing elevation of Ca^{2+}, TxA_2 synthesis, protein phosphorylation, shape change, granule release, and activation of intergrin $\alpha_{IIb}\beta_3$ or GPIIb/IIIa. TxA_2 is synthesized from arachidonic acid through the cyclooxygenase-1 (COX-1) pathway causing vasoconstriction, shape change, protein phosphorylation, platelet secretion, and aggregation. Thrombin from coagulation has functions to promote thrombus formation and platelet plug stabilization (Figure 2) (2, 3, 20).

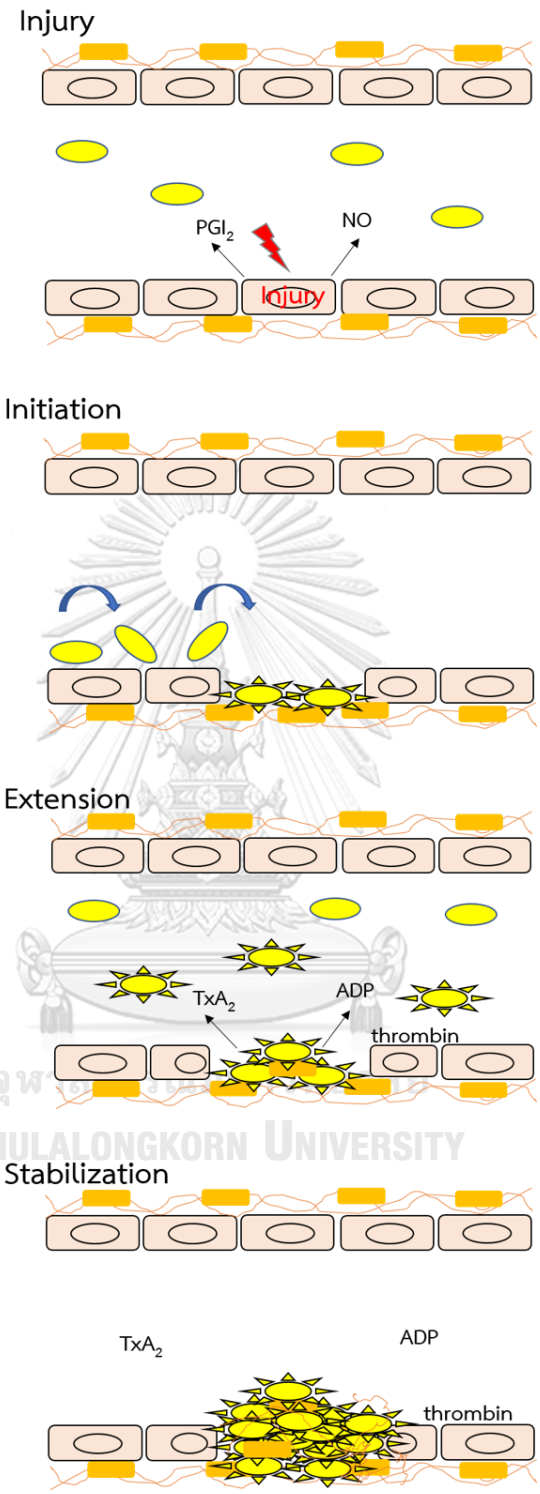


Figure 2 Platelet plug formation

The platelet plug formation at a vascular injury is described in three stages: initiation, extension, and stabilization (2).

Platelet aggregation is the last step of primary hemostasis. Intracellular signals in platelet cause of GPIIb/IIIa shape changes to interact with the ligands (fibrinogen and VWF). The shape change of GPIIb/IIIa is irreversible. Activated GPIIb/IIIa binds to fibrinogen for aggregation and to VWF for structural stabilization (2, 3, 20).

Thrombopoiesis

Thrombopoiesis is the process of platelet production from hematopoietic stem cells (HSCs) (6). The differentiation of HSCs into megakaryocyte progenitors and megakaryocytes depends on the microenviromental niches and hemotopoietic cytokines. HSCs develop into 2 types of megakaryocyte precursors that are burst-forming unit megakaryocytes (BFU-MKs) followed by colony-forming unit megakaryocytes (CFU-MKs) (4, 21). BFU-MK can produce numerous CFU-MKs. CFU-MKs mature into megakaryoblasts, megakaryocytes, and finally platelets (21). BFU-MK and CFU-MK express CD34, c-kit, and HLA-DR surface antigens.

Thrombopoietin (TPO) is the hematopoietic cytokine that supports the self-renewal and expansion of HSCs and stimulates the proliferation and differentiation of HSCs into megakaryocytic lineage (5, 22). The TPO receptor, c-Mpl, is expressed on HSCs, progenitor cells, and megakaryocytes. CFU-MK matures by endomitosis showing increased cell size, increased nuclear content, organelle biogenesis, membrane development, and cytoskeletal rearrangement. The endomitosis is the process that replicates the DNA without cytokinesis. Megakaryocytes enlarge up to 100 μm in size and become polyploid containing the DNA content up to 128N. The ultrastructure of megakaryocytes in culture with TPO shows the demarcation membrane system (DMS) production and granule formation. DMS is the interconnected membranous network which separates cytoplasm for proplatelet formation. The open canalicular system is a channel for releasing granules and specific proteins associated platelets such as

fibrinogen, VWF. The dense tubular system is the megakaryocyte endoplasmic reticulum (Figure 3) (4, 6, 23).

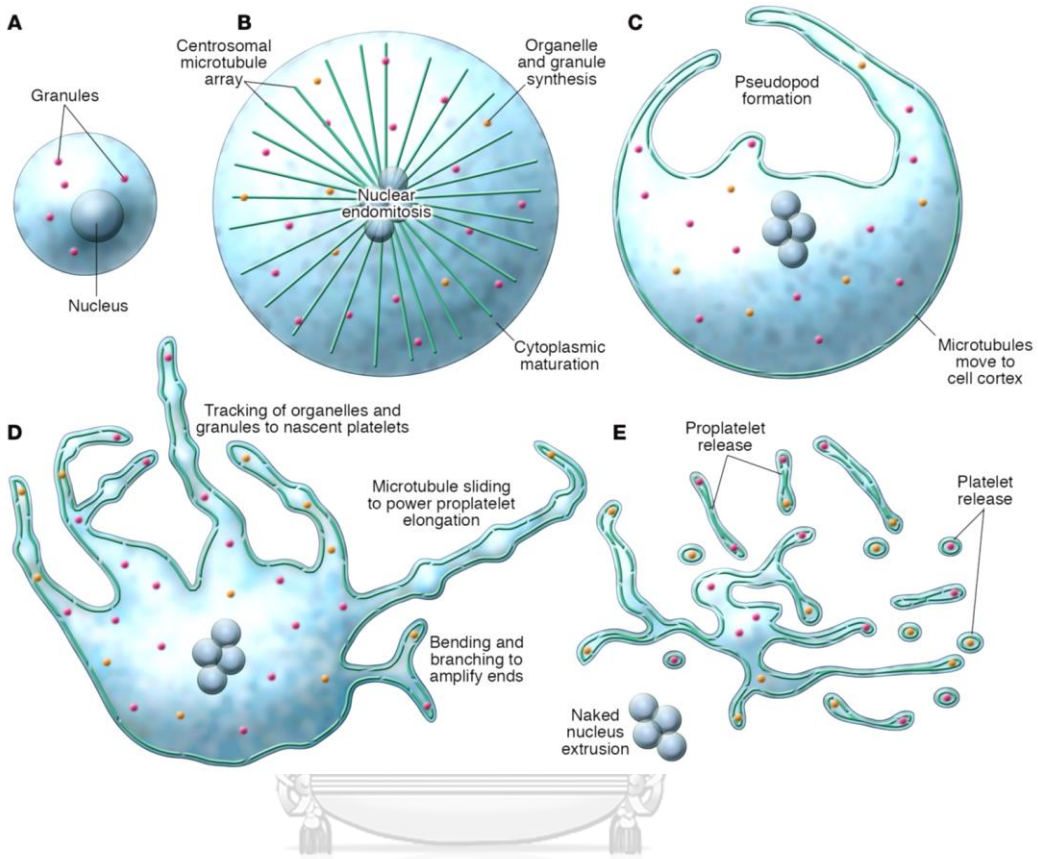


Figure 3 Overview of thrombopoiesis

Megakaryocyte maturation from immature cells is characterized by polyploidization, cellular enlargement and proplatelet extension to released platelets (23).

In megakaryopoiesis, the transcription factors that drive megakaryocyte-erythroid precursor to the megakaryocyte lineage are GATA1, RUNX1, FLI1, NF-E2, and DLX4. GATA1 regulates the erythroid and megakaryocytic differentiation. NF-E2 affects the maturation of megakaryopoiesis accumulating mature megakaryocyte maturation with higher ploidy. DLX4 induces the lineage marker expression and morphologic features of megakaryocytes and represses the erythroid markers and hemoglobin levels. RUNX1 regulates the endomitosis of megakaryocytes. FLI1 is also essential for

megakaryopoiesis as *FLI1* gene mutations cause thrombocytopenia (7, 24-26). Stromal cell-derived factor 1 (SDF1) binds the CXCR4 receptors for megakaryocytes maturation and transportation to the perivascular niche. The perivascular niche is composed of extracellular matrix proteins including VWF, fibrinogen, and fibronectin. Megakaryocyte extends the proplatelets that contains the organelles and granules at this perivascular niche into the blood vessels. VWF is important at the late stage of thrombopoiesis as VWF absence decreases platelet shedding from megakaryocytes. Fibronectin binds to VLA-4 and VLA-5 receptors promoting proplatelet formation (7).

Proplatelet formation is the process by which mature megakaryocyte elongates cytoplasm like pseudopodia with the diameter of approximately 2–4 μm. The proplatelets contain organelles, granules and soluble macromolecules that travel to the distal tip at the final stage of maturation. Proplatelets display numerous platelet bulges (tip) connecting with thin cytoplasmic strings (shaft). A megakaryocyte will continue to extend proplatelets until the cytoplasm is not enough to release and the residue is eventually degraded by macrophage-mediated phagocytosis. Proplatelets extend into the sinusoidal vessels of the bone marrow. Platelets are developed from proplatelet tips and shedding into blood circulation by blood flow at the final stage of platelet production (7, 23, 26).

Thrombopoiesis is regulated by many cytokines especially TPO. Binding of TPO and c-Mpl results in activation of signal molecules including the Janus family of tyrosine kinase and the signal transducers and activators of the transcription (JAK/STAT) pathway, the Ras/mitogen-activated protein kinase (Ras/MAPK) pathway, the phosphoinositide (PI) 3 kinase/Akt pathway, and the Rho/Rho-associated coiled-coil containing protein kinase (Rho/ROCK) pathway. These pathways play important roles in megakaryocyte proliferation and differentiation (27-29).

The JAK/STAT pathway plays an important role in signal transduction in thrombopoiesis. Several tyrosine phosphorylated proteins by TPO, such as JAK2,

STAT3, and STAT5, support proliferation in megakaryocytes and platelets. TPO binding results in JAK2 phosphorylation at tyrosine 112. The phosphorylated JAK2 binds STAT3 and STAT5. Subsequently, STAT3 and STAT5 are phosphorylated to form dimer and translocate into nucleus for transcriptional activation (Figure 4) (5, 28).

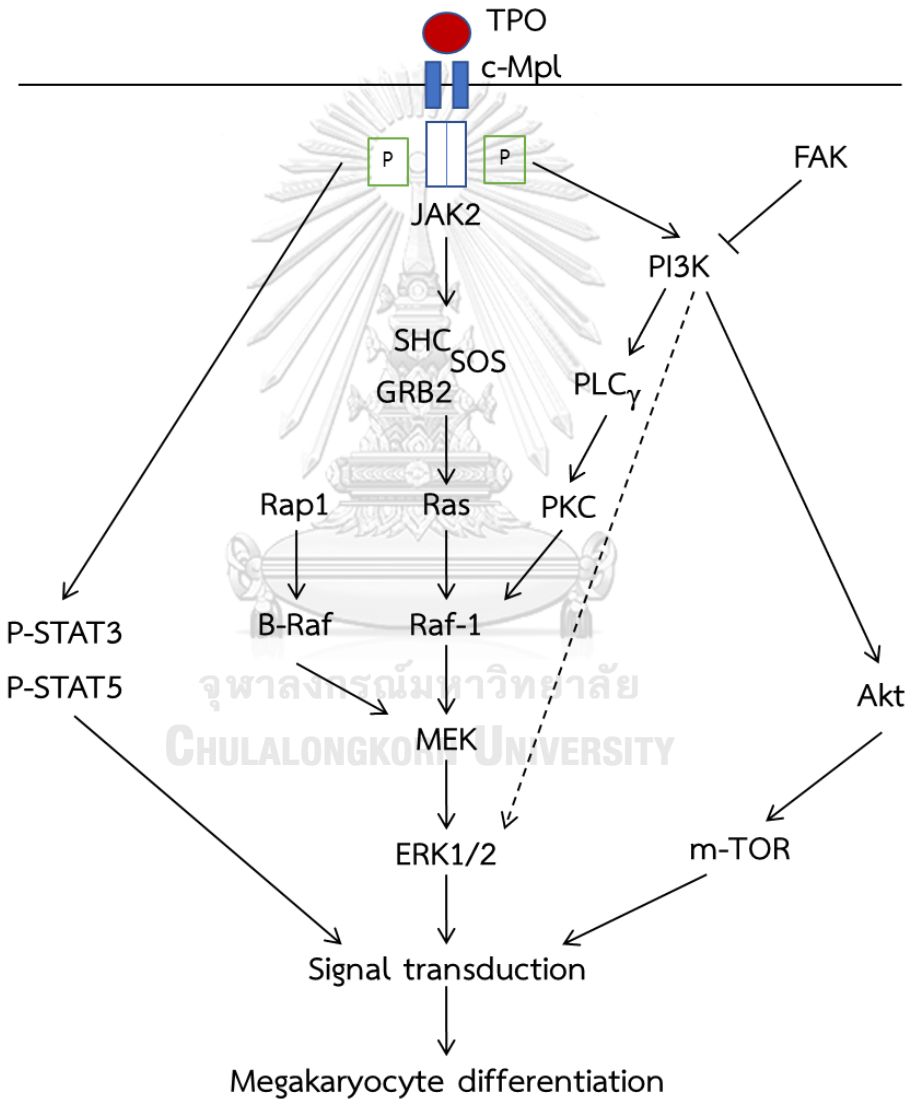


Figure 4 Thrombopoietin (TPO) mediates signaling transduction pathway
TPO is the major cytokine for megakaryocyte proliferation and differentiation (8).

In the Ras/MAPK pathway, MAPK families have four groups of proteins including extracellular signal-related kinases (ERKs) (ERK1/2), ERK5, p38MAPKs and c-Jun amino-terminal kinases (JNKs) which play roles in megakaryocyte proliferation, survival, apoptosis, and differentiation. After TPO stimulation, the phosphorylated JAKs regulate downstream signaling pathways including MAPK and PI3K pathways. The MEK (MAPK kinase)-ERK1/2 pathway induces megakaryocytic differentiation. The MEK-ERK1/2 pathway is involved in proplatelet formation, cell growth arrest, cell size and DNA content accumulation, cell to cell and cell to substrate adhesion increase, and megakaryocyte surface marker expression, such as GPIIb/IIIa and GPIb (8, 28, 30).

The PI3K pathway is important for c-Mpl signaling. PI3K is activated by hematopoietic growth factors, plays an important role in cell survival, drives both mitosis and endomitosis in cells, and promotes platelet formation. The PI3K pathway can induce ERK1/2 phosphorylation. The Akt is the PI3K target which activates the suppression of apoptosis and the growth-factor-mediated survival. The mammalian target of rapamycin (mTOR) is a downstream target of AKT. The mTOR is involved in megakaryocyte polyploidization and differentiation (8, 27, 30).

The Rho/ROCK pathway is a negative regulator of proplatelet formation. The interaction between integrin $\alpha_2\beta_1$ and collagen type I activates the Rho/ROCK pathway which induces the myosin light chain 2 (MLC2) phosphorylation. Phosphor-MLC2 inhibits proplatelet formation. Binding of GPIbα and VWF are regulators of thrombopoiesis by boosting proplatelet formation and platelet production (30, 31).

Glycoprotein Ib-IX-V

Glycoproteins (GPs) are proteins conjugated with oligosaccharide chains by covalent bonds. GPs may serve as structural proteins, immunoglobulins, enzymes, polypeptide hormones, blood group substances, antigens, receptors, and transport proteins (32).

The GP Ib-IX-V complex is encoded by *GP1BA, GP1BB, GP9* and *GP5* genes. *GP1BA* gene is located on chromosome 17 encoding the glycoprotein Ib alpha (GPIbα) subunit (33). GPIbα or cluster of differentiation (CD) 42b is the subunit of GPIb-IX-V complex. The subunits of GPIb-IX-V (GPIbα, GPIbβ, GPIX, and GPV) are type I transmembrane proteins containing leucine-rich repeat (LRR) domains. The GPIbα molecule is linked by disulfide bonds with two GPIbβ in membrane-proximal domain. GPIbβ interacts with GPIX by a non-covalent bond in the extracellular domain. GPIb-IX is a stable complex. GPV associates weakly with GPIX in transmembrane domains.

GPIbα is the extra cellular ligand-binding sites of the complex. GPIb-IX-V is expressed on the surface of megakaryocytes and platelets. Seven LRRs on the N-terminus of GPIbα function as a receptor for A1 domain of von Willebrand factor (VWF) which play role in primary hemostasis by allowing platelets adhesion to subendothelium and signaling the platelets to enhance platelet activation and thrombus formation (33-36). GPIb-IX-V can also bind to other molecules that involved in thrombosis, such as thrombin, P-selectin, and high molecular weight kininogen (HMWK) (36). The GPIb-IX-V links platelet surface to sub-membrane actin filaments for maintaining the platelet shape (Figure 5) (37). Furthermore, the GPIb-IX-V complex is also essential for platelet production from megakaryocytes by promoting proplatelet extension from bone marrow into the vascular space and, subsequently, fragmentation into platelets.

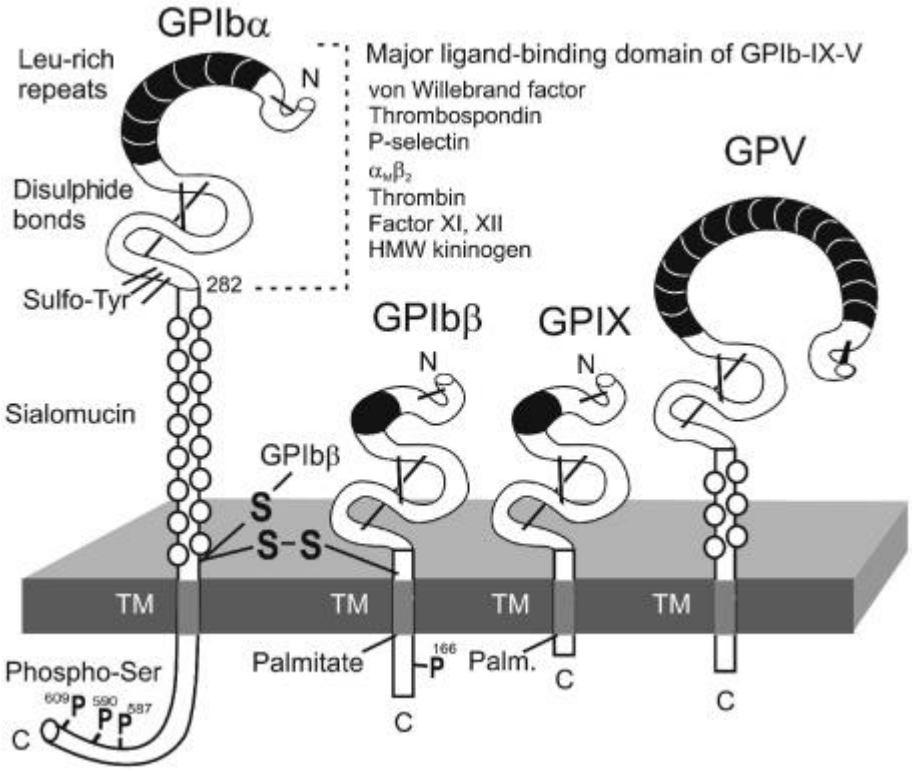


Figure 5 The GPIb-IX-V complex

The GPIb-IX-V complex is composed of GPIbα, GPIbβ, GPIX and GPV (37).

***GP1BA* mutations**

The platelet *GP1BA* gene mutations are the causes of two known bleeding disorders that are Bernard-Soulier syndrome (BSS), and Platelet-type von Willebrand disease (PT-VWD). Both diseases are characterized by thrombocytopenia with large platelets (34, 35). BSS and PT-VWD can be distinguished by clinical features, laboratory, and molecular genetic results (34).

Bernard-Soulier syndrome is a hereditary autosomal recessive bleeding disorder which affects both platelet numbers and function. The underlying causes are mutations in one of the GPIb-IX-V complex encoding genes. BSS is characterized by macrothrombocytopenia, bleeding tendency, reduced ristocetin-induced platelet

agglutination (RIPA), and reduced the GPIb-IX expression on platelet surface (15, 34, 38). Clinical manifestations of BSS are epistaxis, ecchymosis, gingival and cutaneous bleeding, purpura, hemorrhage, and menorrhagia (34, 37, 39). BSS is the results in the defects in one of the three genes (*GP1BA*, *GP1BB*, and *GP9*) which are related with the severity of the bleeding (Figure 6) (37).

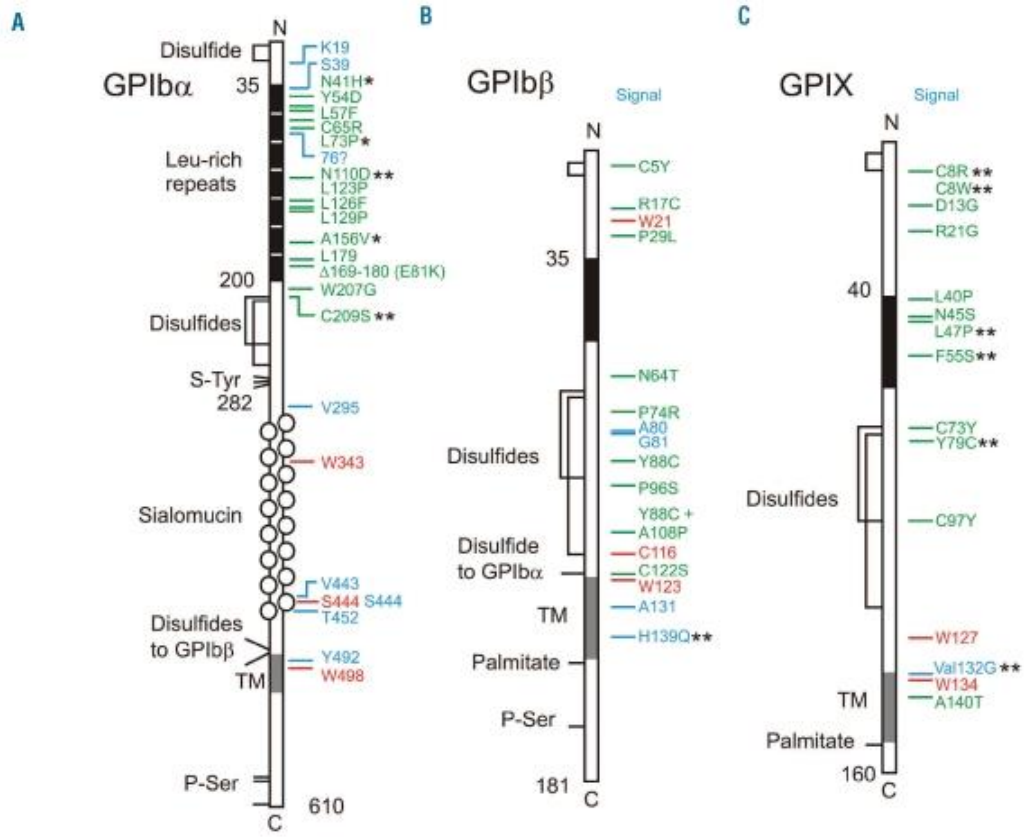


Figure 6 The mutation of GPIbα, GPIbβ, GPIX associated Bernard Soulier syndrome The missense mutations, short deletions, nonsense mutations of GPIbα, GPIbβ and GPIX result in Bernard Soulier syndrome (37).

Platelet-type von Willebrand disease or pseudo-von Willebrand disease is an autosomal dominant bleeding disorders (40). The *GP1BA* mutations in PT-VWD are located in VWF binding region (Table 1) (34). The variants are usually in the

regulatory (R) loop after the leucine-rich repeat of GPIbα. The clinical features of PT-VWD are macrothrombocytopenia, mild to moderate mucocutaneous bleeding, and low VWF levels. The common bleeding features are epistaxis, easy bruising, and dental or gingival bleeding. PT-VWD displays the gain-of-function platelet GPIbα with increased affinity for VWF. PT-VWD platelets can spontaneously bind to high molecular weight multimers of VWF removing of VWF and platelets from blood circulation. PT-VWD and type 2B VWD have similar phenotype but they can distinguish by increased RIPA at low concentration of ristocetin, and absence of high molecular weight VWF (14, 31, 40, 41).

Table 1 *GP1BA* mutations in PT-VWD

Genetic variation	**Protein defect**	**Variation type**
c.736 G>T	Trp 246 Leu	Missense
c.746 G>T	Gly 249 Val	Missense
c.746 G>A	Gly 249 Ser	Missense
c.752 G>T	Asp 251 Tyr	Missense
c.763 A>G	Met 255 Val	Missense
c.765G>A in *GP1BA* gene combined with c.3797C>T in *VWF* gene	Met 255 Ile combined with Malmö/NewYork mutation Pro1266Leu	Missense
c.1383_1410del 27 CCGACCATCCTGGTGTCTGCCACAAGC	in frame_del 9 462 Pro Thr Ile Leu Val Ser Ala Thr Ser 471	Deletion

A previous study from our group generated a BSS model using the patient-derived iPSC technology and found that BSS megakaryocytes did not express surface

GPIb and produced thick proplatelet shafts and tips. BSS platelets that derived from iPSCs exhibited large sizes. The ultrastructure of the iPSC-derived platelets revealed large platelets with dilated DMS (38). Therefore, hiPSC models can be used to mimic pathological platelet production.

Kaur et al. (2017) studied the thrombocytopathy in PT-VWD and found that the interaction between VWF and hyper-responsive GPIbα resulted in bleeding complications. In addition, platelets in PT-VWD showed impairment of thrombus formation (42). Bury et al. (2019) studied the mechanisms of thrombocytopenia in PT-VWD and found that PT-VWD megakaryocytes generated reduced numbers of proplatelets with enlarged tips. Megakaryocytes contacted with collagen in PT-VWD showed the defect in RhoA-MLC2 pathways that preventing proplatelet formation (31). Interestingly, both loss-of-function and gain-of-function mutations of GPIb-IX-V complex resulted in macrothrombocytopenia. The signaling pathways downstream of GPIb-IX-V involving proplatelet formation remain to be determined.

Regarding the signaling transduction, Chang et al. (2007) and Yang et al. (2017) found that the Rho/ROCK pathway was negatively regulated the proplatelet formation (43, 44). Machlus et al. (2016) found that CCL5/CCR5 enhanced megakaryocyte maturation and proplatelet formation by stimulated the AKT signaling pathway (45). Finally, Mazharian et al. (2009) and Ye et al. (2014) indicate that the ERK1/2 pathway was important for the proplatelet formation and the PI3k/Akt pathway increased the megakaryocyte cell survival (29, 46). Therefore, these signaling molecules will be explored in our model.

Hematopoietic stem cells

Stem cells are undifferentiated cells that can differentiate into many cell types. The unique features of stem cell are self-renewal and pluripotency. Stem cells can be divided into two main categories including embryonic stem cells, and adult stem cells. Embryonic stem cells are pluripotent cells derived from the inner cell mass of blastocysts, while adult stem cells or somatic stem cells are multipotent cells that found in many sites of the body such as bone marrow or liver. In addition, the stem cell potencials are divided to totipotent, pluripotent, multipotent, oligopotent, and unipotent cells. (Table 2) (47).

Table 2 Differentiation potentials of stem cell populations

Designation	Differentiation potential implied by designation	Examples of stem/progenitors with these properties
Totipotent	All embryonic and extraembryonic tissues	Zygote
Pluripotent	All embryonic tissues	Inner cell mass, Embryonic stem cell, induced pluripotent stem cell
Multipotent	All lineages of a tissue/organ	Hematopoietic stem cell/ Neutral stem cell
Oligopotent	Several but not all lineages of a tissue/organ	Common myeloid progenitor, Common lymphoid progenitor
Unipotent	Single lineage of a tissue/organ	Macrophage progenitor

Hematopoietic Stem Cells (HSCs) are the cells that can self-renew to give rise to identical daughter HSCs without differentiation and can differentiate into all blood cell lineages through hematopoiesis (Figure 7) (47). HSCs are derived from mesoderm, located in the bone marrow stem cell niches and maintained in a quiescent state.

They are stimulated by intrinsic or extrinsic signals during blood cells disturbances. HSCs are the origin of multipotent progenitors (MPPs). The MPPs lose the self-renewal potential but retain an ability to differentiate into multilineages. Common lymphoid (CLPs) and common myeloid progenitors (CMPs) are derived from MPPs. CMPs can differentiate to megakaryocyte/erythrocyte progenitors (MEPs), granulocyte/macrophage progenitors (GMPs), and dendritic cell progenitors. CLPs can differentiate to T lymphocyte, B lymphocyte, NK cell and dendritic cell progenitors (48).

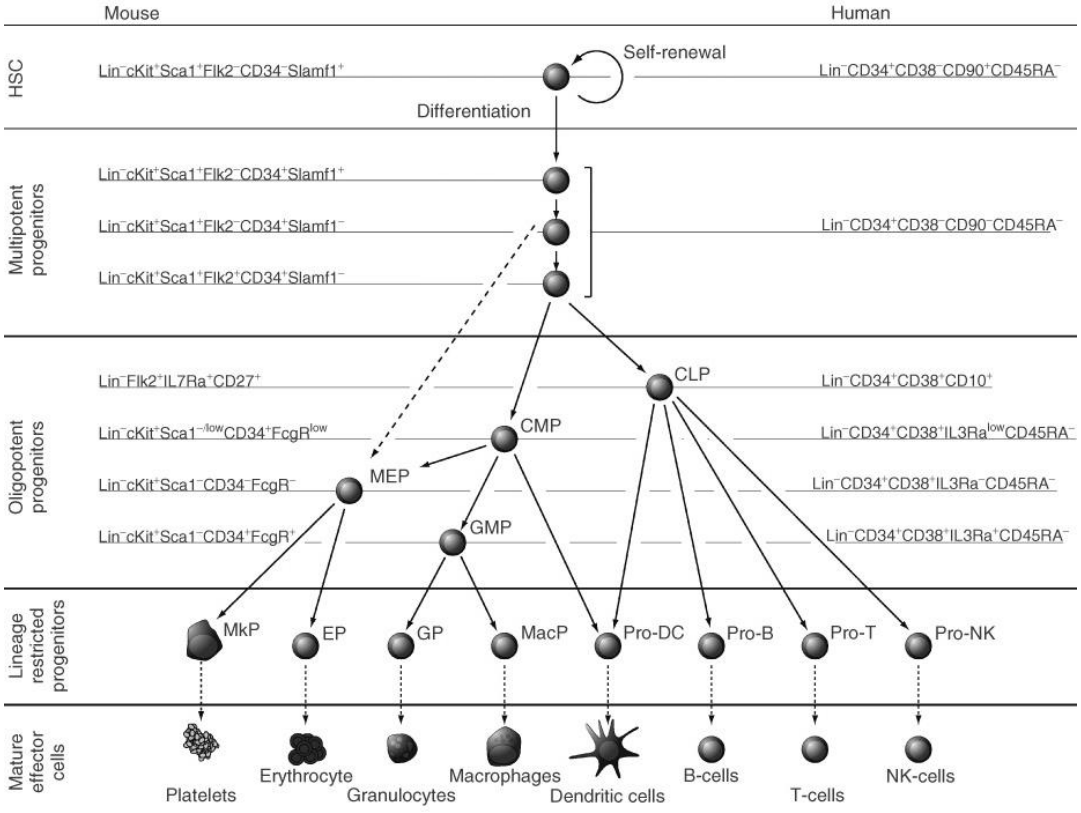


Figure 7 Hierarchy of hematopoiesis

The hematopoietic stem cells start from the top of the hierarchy and differentiate into progenitor and mature blood cells (47).

Human induced pluripotent stem cells

Human induced pluripotent stem cells (hiPSCs) are the stem cells which are reprogramed from human adult cells into embryonic-like stem cells. The iPSC technology was constructed by Shinya Yamanaka in 2006 which reported the induction of pluripotent stem cells from mouse embryonic or adult fibroblasts by transcription factors including, Oct3/4, Sox2, c-Myc, and Klf4. Oct3/4 and Sox2 which are core transcription factors in iPSC pluripotent maintenance and embryonic development. Oct3/4 enhances iPSCs to exhibit the embryonic stem cell characteristics. Klf4 activates the proliferation, differentiation, development, survival, and apoptosis. Klf4 interacts with Oct3/4 and Sox2 to generate iPSCs. c-Myc regulates many gene targets that enhance proliferation, transformation and cell cycle regulation. c-Myc can inhibit the anti-proliferation of Klf4 (17, 49).

The iPSC technology is used for regenerative medicine, disease modeling, drug development and discovery. In transfusion medicine, iPSCs can produce universal red blood cells or platelets for transfusion. In regenerative medicine, iPSCs can generate cells for transplantation without the risk of graft rejection. iPSCs are tools for testing for personalized drugs and using as disease models to investigate the pathogenesis and discover the therapeutic drugs (50).

CHAPTER III

MATERIALS AND METHODS

Experimental design

Objective	Experimental plan
1. Generation the hiPSCs with *GP1BA* mutation	1. CRISPR/Cas9 of the *GP1BA* gene by using ribonucleoprotein system (RNP)
2. Testing of the *GP1BA* mutation in hiPSCs	1. Droplet digital polymerase chain reaction (ddPCR) to detect the mutant clones of hiPSCs 2. DNA sequencing for *GP1BA* gene mutation detection
3. Characterization of *GP1BA* mutated hiPSCs	1. RT-PCR for pluripotent properties checking 2. Karyotyping for chromosome integrity 3. Embryoid body formation to confirm the efficiency of hiPSC differentiation into three germ layers
4. Differentiation and characterization of *GP1BA* mutated hiPSCs	1. Proplatelet formation study of *GP1BA* mutated hiPSCs 2. Culture *GP1BA* mutated hiPSCs for megakaryocytes and platelets 3. Collect megakaryocytes and platelets for total cell counting and CD41/CD42 analysis using flow cytometry 4. Examine the proplatelet formation

	under fluorescence microscopy 5. Determine the sizes of platelets 6. VWF binding study
5. Protein signaling pathway study in glycoprotein Ib mutated hiPSCs	1. Study signaling proteins of thrombopoiesis by using Capillary Western blots of Ras/MAPK, PI3K, and Rho/ROCK pathways.
6. Effects of signaling inhibitors on megakaryocytes	1. Study the effects of MEK inhibitor and ROCK inhibitor on proplatelet formation 2. Study the effects of MEK inhibitor and ROCK inhibitor on platelet sizes and total platelet counts

Research methodology

Generation of the *GP1BA*-mutated hiPSCs by CRISPR-Cas9

Wild-type hiPSCs (the gift from Assoc. Prof. Nipan Israsena, Stem Cell and Cell Therapy Research Unit, Faculty of Medicine, Chulalongkorn University) were derived from skin fibroblasts and the *GP1BA*-null (BSS-A) hiPSCs derived from a Bernard Soulier syndrome patient were constructed in our group as previously reported (38). This experimental study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (The certificate number 010/2020) and complied with the Declaration of Helsinki and ICH-GCP.

The normal hiPSCs were knocked-in with mutated GP1BA-edited gene (p.M255V) using CRISPR/Cas9 ribonucleoprotein (RNP) system. The guide RNAs (gRNAs) or crispr RNA (crRNA) targeting *GP1BA* gene was designed approximately 20 base pairs upstream of PAM (NGG) and an oligo template for genome editing was designed at approximately 100 base pairs using Alt-R HDR Design Tool & Templates (IDT, USA). The gRNAs were combined with Alt-R® CRISPR-Cas9 trans-activating crRNA, ATTO™ 550 (tracrRNA) and Alt-R® S.p. HiFi Cas9 Nuclease V3 to form the gRNA/Cas9 protein ribonucleoprotein complexes. iPSCs were electroporated with RNP complexes by using Amaxa™ P3 Primary Cell 4D-Nucleofactor™ X Kit (Lonza, Germany).

After electroporation, hiPSCs were cultured with mTeSR1 medium (STEMCELL Technologies, Canada) on Matrigel (Corning, USA) for 24 hours and sorted into 96 well plate by BD FACSAria II (Becton Dickinson, USA) for culture on Matrigel with mTeSR medium (Figure 8) (51).

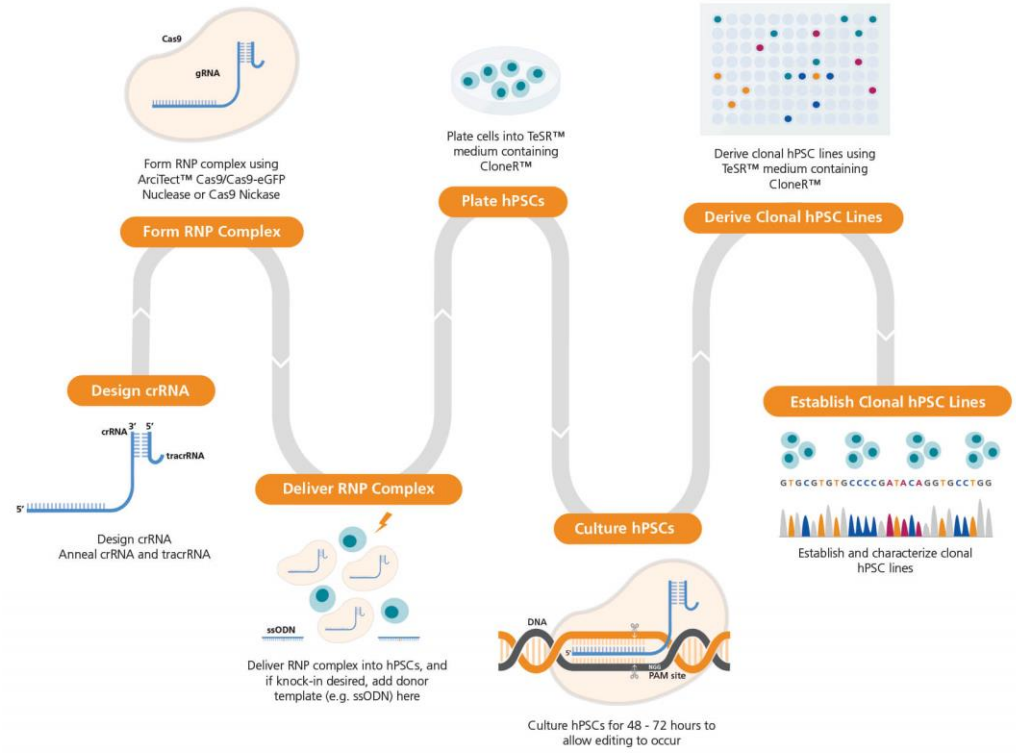


Figure 8 CRISPR/Cas9 ribonucleoprotein complex genome editing workflow
The guide RNA is designed to form CRISPR-Cas9 ribonucleoprotein complex and then delivered into hPSCs with a donor DNA template (51).

Testing of the *GP1BA*-mutated in hiPSCs

After the hiPSCs culture, genomic DNA was extracted from CRISPR/Cas9 hiPSCs using the QIAamp® DNA Mini Kit (QIAGEN, Germany) and detected for the mutation of *GP1BA* gene by using ddPCR™ HDR Genome Edit Detection Assays (BIO-RAD, USA). The positive clone of *GP1BA* mutation hiPSCs or p.M255V mutation was confirmed by DNA Sanger sequencing.

The droplet digital polymerase chain reaction (ddPCR) is the technique that provides high precision and quantification. The ddPCR generate droplet by water-oil emulsion technology. Subsequently, PCR amplification and detection the target PCR product in each droplet use forward and reverse primers that cover the target DNA region, restriction enzyme, reference probe, homology detection repair (HDR) probe

(target probe) and dark probe. The detection the target PCR product in each droplet using two-color detection system (Figure 9) (52).

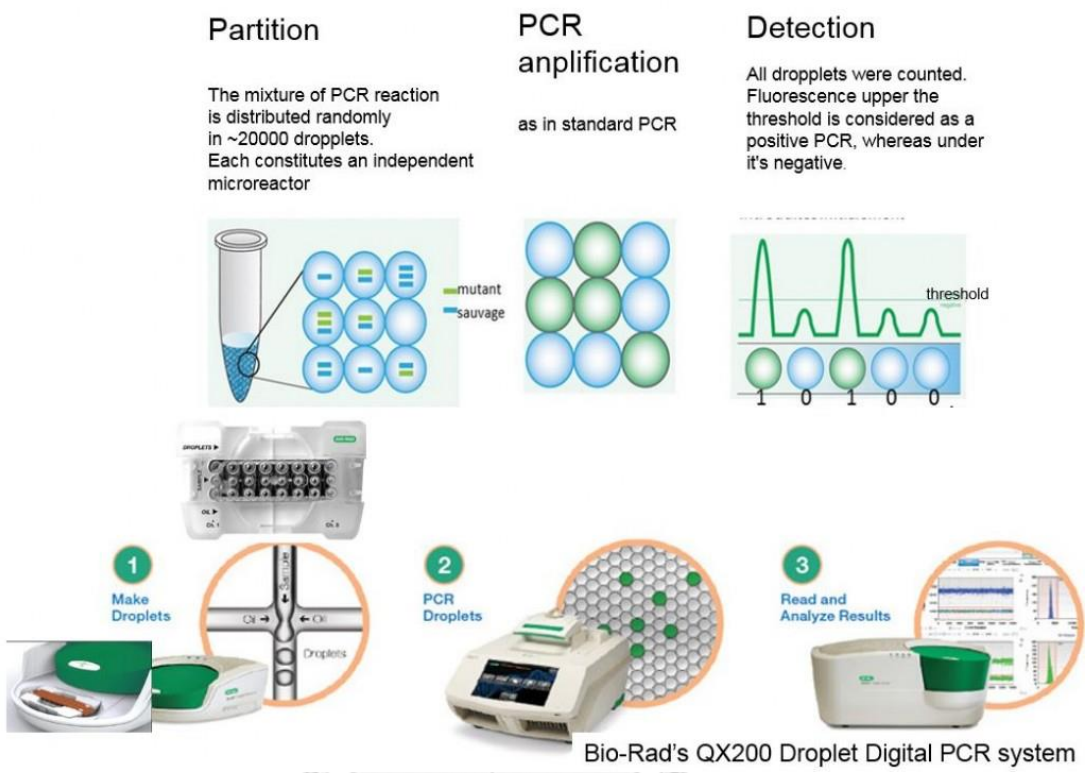


Figure 9 Droplet digital PCR system

The droplet digital PCR workflow is composed of three steps: droplet generation, PCR amplification and droplet detection (52).

Characterization of *GP1BA*-mutated hiPSCs

The *GP1BA*-mutated hiPSCs were tested for pluripotent properties, karyotype, and the efficiency to differentiate into three germ layers.

The pluripotent properties of *GP1BA* mutation hiPSCs were evaluated for the endogenous pluripotency markers detection by reverse transcriptase-PCR (RT-PCR) including Oct4, Sox2, c-Myc, and Klf4, as well as a house keeping gene GAPDH. Messenger RNA was extracted using the GeneJet RNA Purification kit (Thermo Fisher Scientific, USA). Complementary DNA (cDNA) was synthesized from total RNA using

the DNA synthesis kit (Thermo Fisher Scientific, USA). Amplifications for pluripotency markers were carried out in a T gradient Biometra Thermal Cycler (Biometra GmbH). The primers for pluripotency markers are listed in table 3. RT-PCR products were visualized on 2% agarose gel.

Table 3 List of primers for pluripotency markers of stem cells

Genes	Forward primers	Reverse primers
NANOG	ATACCTCAGCCTCCAGCAGA	CAGGACTGGATGTTCTGGGT
OCT4	GAAGGTATTCAGCCAAACGC	GTTACAGAACCACACTCGGA
SOX2	GGGAAATGGGAGGGGTGCAAAAGAGG	TTGCGTGAGTGTGGATGGGATTGGTG
KLF4	TGATTGTAGTGCTTTCTGGCTGGGCTCC	ACGATCGTGGCCCCGGAAAAGGACC
C-Myc	GCGTCCTGGGAAGGGAGATCCGGAGC	TTGAGGGGCATCGTCGCGGGAGGCTG
GAPDH	AACAGCCTCAAGATCATCAGC	TTGGCAGGTTTTTCTAGACGG

Karyotyping of *GP1BA*-mutated hiPSCs was detected for chromosomal numbers and structure compares with normal hiPSCs by GTG-banding analysis at the Center for Medical Diagnostic Laboratories, Faculty of Medicine, Chulalongkorn University, Thailand.

Embryoid body (EB) formation is used for testing the efficiency of *GP1BA*-mutated hiPSCs to differentiate into three germ layers including ectoderm, mesoderm, and endoderm. EB was prepared by culture *GP1BA*-mutated hiPSCs on an ultra-low attachment tissue culture dish with an EB medium consisting of Iscove's Modified Dulbecco's Medium (IMDM) (HyClone, GE Life Sciences, USA) supplemented with 10% fetal bovine serum (FBS), 1% glutamax, and 1% antibiotic-antimycotic (all from Gibco, USA) for 21 days on 0.1% gelatin coated coverslips. Culture media was changed every 2-3 days. Human Three Germ Layer 3-Color Immunocytochemistry Kit (R&D Systems, USA) was used for detection of three germ layer markers: anti-human

OTX2 NL557-conjugated goat IgG and anti-human SOX1 NL493-conjugated goat IgG for ectodermal markers, anti-human Brachyury NL557-conjugated goat IgG and anti-human HAND1 NL637-conjugated goat IgG for mesodermal markers and anti-human GATA-4 NL493-conjugated goat IgG and anti-human SOX17 NL637-conjugated goat IgG for endodermal markers. The 4′,6-Diamidino-2-phenylindole (DAPI) was used for nuclear staining. The images were obtained using Axio Observer fluorescence microscopy (Carl Zeiss, Germany).

Megakaryocytes and platelets differentiation and characterization

The hiPSCs were cultured in the co-culture system for megakaryocytes and platelets differentiation.

Feeder preparation

The mouse embryonic cell line, C3H 10T1/2 cell, was cultured in basal media eagle (BME) (Biowest, France) supplemented with 10% FBS, 1% glutamax, and 1% antibiotic-antimycotic. The marrow stromal cells, OP9, was cultured in minimal essential medium Alpha Modification (MEM-alpha) (HyClone, GE Life Sciences, USA) supplemented with 20% FBS, 1% glutamax, and 1% antibiotic-antimycotic. The C3H 10T1/2 and OP9 cells were dissociated by using 0.25% trypsin-EDTA (Gibco, USA). The C3H 10T1/2 cells were irradiated with 50 Gy X-ray before use for inactivation cells. The inactivated C3H 10T1/2 cells were resuspended in the culture medium and spread into 0.1% gelatin coated plate.

Embryonic stem cell–derived sacs formation method

Embryonic stem cell–derived sac (ES-sac) formation was performed according to ES-Sac method (Figure 10) (53-55). hiPSCs were cultured in mTeSR media for 3-4 days and then washed with phosphate buffer saline (PBS). hiPSCs were dissociated

into small colonies (>100 cells) by combination of collagenase type IV, trypsin, and KnockOut Serum Replacement (KSR) (Thermo Fisher Scientific, USA) or CTK. Small colonies of hiPSCs were transferred onto feeder cells and cultured for 14 days in a hematopoietic cell differentiation medium consisting of IMDM supplemented with 15% FBS, 50 µg/ml ascorbic acid, 10 µg/ml insulin, 5.5 µg/ml transferrin, 5 ng/ml selenite, 2mM L-glutamine, 0.45 mM **α**-monothioglycerol, and 20 ng/ml human vascular endothelial growth factor (VEGF) (R&D Systems, USA). Culture media was changed every 2-3 days. After 14 days, ES-sac was contained hematopoietic progenitor cells (HPCs).

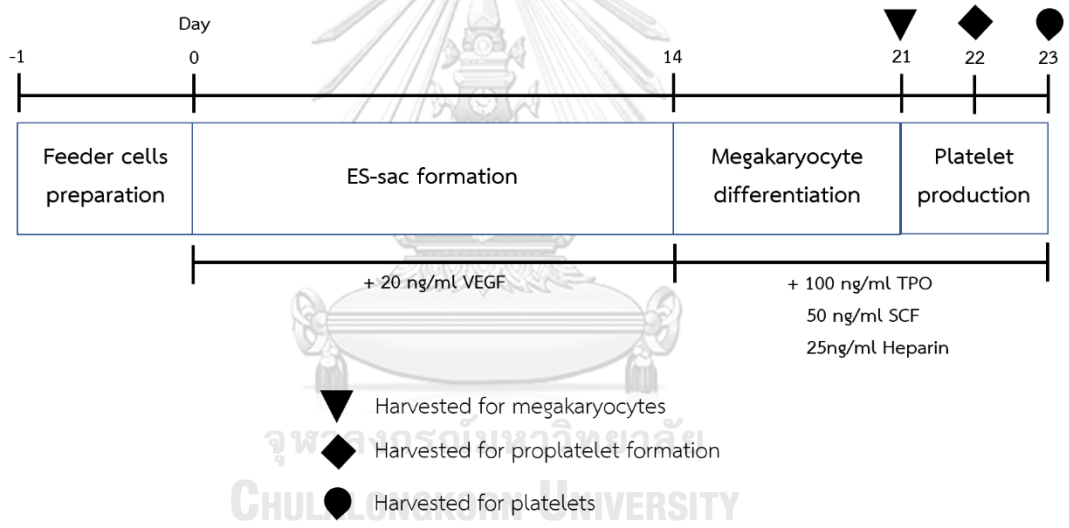


Figure 10 Embryonic stem cell-derived sac (ESSAC) protocol

Megakaryocyte and platelet differentiation

ES-sacs that contain HPCs were torn. The collected cells were passed through a 40-µM cell strainer to obtain HPCs and transferred onto inactivated OP9 cells. The HPCs were cultured with a hematopoietic cell differentiation medium supplemented with 100 ng/ml human thrombopoietin (TPO) (R&D Systems, USA), 50 ng/ml human stem cell factor (SCF) (R&D Systems, USA), and 25 ng/ml heparin (Sigma-Aldrich).

Megakaryocytes were harvested and analyzed on day 21 of culture. Platelets were harvested and analyzed on day 23 of culture.

Proplatelet formation

The hiPSC-derived megakaryocytes were harvested and reseeded onto Matrigel-coated coverslips. The hiPSC-derived megakaryocytes were cultured in a hematopoietic cell differentiation medium supplemented with 100 ng/ml TPO, 50 ng/ml SCF, and 25 ng/ml heparin for 24 hours.

Von Willebrand factor (VWF) binding study

The hiPSC-derived platelets were incubated with VWF (Abcam, UK) at 37ºC (31, 56) and harvested. The hiPSC-derived platelets were seeded on coverslips and centrifuged at 1200 revolutions per minute (RPM) for 5 minutes and had the medium removed. The hiPSC-derived platelets and VWF were stained with fluorescein isothiocyanate (FITC)-conjugated with anti-human CD41 (BioLegend, USA) and Alexa Fluor 647-conjugated anti-VWF (AbCam, UK). The images were obtained using Axio Observer fluorescence microscopy. The percentages of VWF-bound platelets and agglutinated platelets in each sample were enumerated.

Flow cytometry analysis

Megakaryocytes on day 21 and platelets on day 23 were analyzed with anti-CD41a conjugated with FITC and anti-CD42 conjugated with phycoerythrin (PE) (BioLegend, USA) for surface molecules and cell numbers detection. The *GP1BA*-mutated megakaryocytes and platelets surface markers and cell numbers were compared with normal hiPSC-derived megakaryocytes and platelets.

Immunocytochemistry for proplatelets and platelets

For proplatelet staining, culture media was removed from the Matrigel-coated coverslips after reseeding for 24 hours. For platelet staining, the hiPSC-derived platelets were collected in supernatant on day 23 of cultured and centrifuged at 2,000 RPM. The hiPSC-derived platelets were resuspended and smeared onto coverslips and dried.

Coverslips were fixed with 4% formaldehyde for 15 minutes, permeabilized with 0.3% Triton X-100 in PBS for 15 minutes, blocked with the blocking solution for 1 hour, and stained with FITC-conjugated anti-α-tubulin and PE-conjugated anti-actin (BioLegend, USA) for 1 hour. DAPI will be used for nuclear staining. Images of proplatelets and platelets were obtained using Axio Observer fluorescence microscopy. Proplatelet tip sizes and platelet sizes were measured based on the visualized circumferential microtubule coils. The size of *GP1BA*-mutated proplatelet tip and platelet were analyzed by comparison with normal hiPSC-derived proplatelets and platelets.

Signaling transduction pathway studies

For studying the protein signal transduction pathway in platelet production of *GP1BA* mutation, hiPSC-derived megakaryocytes were incubated with megakaryocyte differentiation medium for 24 hours. The hiPSC-derived megakaryocytes were harvested and extracted for protein by RIPA lysis buffer containing protease/phosphatase inhibitor cocktail (MedChemExpress, USA). The protein samples were collected from supernatant after centrifuged at 12,000 RPM for 20 minutes and measured for protein concentrations by the Micro BCA protein assay kit (Thermo Fisher Scientific, USA) then analyzed by an ELISA reader (Tecan, Switzerland).

The protein signaling was performed on the automated Western blots WESTM Simple Western™ assays system (ProteinSimple, USA). Primary antibodies were rabbit anti-ERK1/2 (#4695), anti-phosphorylated ERK1/2 (#4370), anti-AKT (#4691), anti-phosphorylated AKT (#4060), anti-MLC2 (Myosin light chain 2) (#8505) and anti-phosphorylated MLC2 (#3671). All were from Cell Signaling Technology, USA. The HRP-conjugated anti-rabbit antibody was used as secondary antibodies. Sample buffer, fluorescence master mix, secondary antibody, blocking reagent and luminol-peroxide chemiluminescence were obtained from ProteinSimple.

Protein samples were diluted with sample buffer and fluorescence master mix at the final concentration of 3 µg in 5 µl. The samples, biotinylated ladder, antibody diluent, primary antibodies, streptavidin-HRP, secondary antibody, chemiluminescent substrate and wash buffer were pipetted into the plate. The instrument settings were stacking and separation at 375V for 25 minutes, blocking for 5 minutes, incubating of primary and secondary antibodies for 30 minutes each and luminol-peroxide chemiluminescence detection for 15 minutes. The chemiluminescent signals were detected and quantitated by the Compass for SW program. The results were shown as a ratio of the phosphorylated to the total signaling protein.

Effects of signaling inhibitors on megakaryocytes

For studying the signaling inhibitors effects on proplatelet formation, hiPSC-derived megakaryocytes were reseeded on Matrigel-coated coverslips and treated with a MEK inhibitor (MEKi), PD98059 (MedChemExpress, USA) at the concentration of 10 µM for normal hiPSC-derived megakaryocytes (27), a ROCK inhibitor (ROCKi), Y-27632 (MedChemExpress, USA) at 10 µM for normal, BSS-A and M255V hiPSC-derived megakaryocytes (57), and MEKi and ROCKi for normal hiPSC-derived megakaryocytes for 24 hours.

For studying the signaling inhibitors effects on platelet production, normal hiPSC-derived megakaryocytes were treated with MEKi at the concentration of 10 μM, normal, BSS-A and M255V hiPSC-derived megakaryocytes treated with ROCKi at the concentration of 10 μM and normal hiPSC-derived megakaryocytes treated with MEKi plus ROCKi for 2 days. Platelets were collected and analyzed on day 23 of culture.

Statistical analysis

Statistical analysis was performed using SPSS software (version 22.0). The samples were processed in triplicate. Data were presented as mean ± SD. Mean values of three groups were compared using the independent sample t-test. The observed data with P-value < 0.05 was considered as statistical significance.

Chapter IV

RESULTS

The *GP1BA* p.M255V carrying hiPSCs were constructed by CRISPR-Cas9 technique. The M255V and BSS-A hiPSCs were a gain-of-function and loss-of-function *GP1BA* mutations, respectively. The M255V hiPSCs provide a platelet-type von Willebrand disease (PT-VWD) model while BSS-A hiPSCs serve as a Bernard-Soulier syndrome disease model. The M255V and BSS-A hiPSCs were cultured for megakaryocytes and platelets production *in vitro* and compared with normal hiPSCs for studying the molecular functions of *GP1BA* gene in platelet production and study the molecular pathways responsible for these defects.

Generation the *GP1BA*-mutated hiPSCs by CRISPR-Cas9

A normal hiPSCs were knocked-in with the *GP1BA* p.M255V mutation using the CRISPR/Cas9 technique for a gain-of-function *GP1BA* mutation. The gRNA and an oligo template for genome editing which designed by Alt-R HDR Design Tool & Templates were shown in Figure 11.

gRNA for cutting GP1BA mutation

Position	Strand	Sequence	PAM
159	+	CAAGGCCATGACCTCTAACG	TGG

Oligo template for p.M255V GP1BA mutation

ACAATGCTGAAAATGTCTACGTATGGAAGCAAGGTGTGGACGTCAAGGCC**GTG**ACC
TCTAACGTCGCCAGTGTGCAGTGTGACAATTCAGACAAGTTTCC

Figure 11 gRNA and oligo template for *GP1BA* p.M255V mutation genome editing

After CRISPR/Cas9, the genome-edited hiPSCs were tested for *GP1BA* p.M255V mutation by ddPCR. The M255V hiPSCs showed the signals of both the HDR probe (blue dot) and the reference probe (green dot), whereas normal hiPSCs contained

only the reference probe signals (Figure 12A). Sanger DNA sequencing of M255V hiPSCs were used for the *GP1BA* p.M255V mutation confirmation. The *GP1BA* p.M255V mutation was the heterozygous changes of ATG (Met) to GTG (41) codons (Figure 12B).

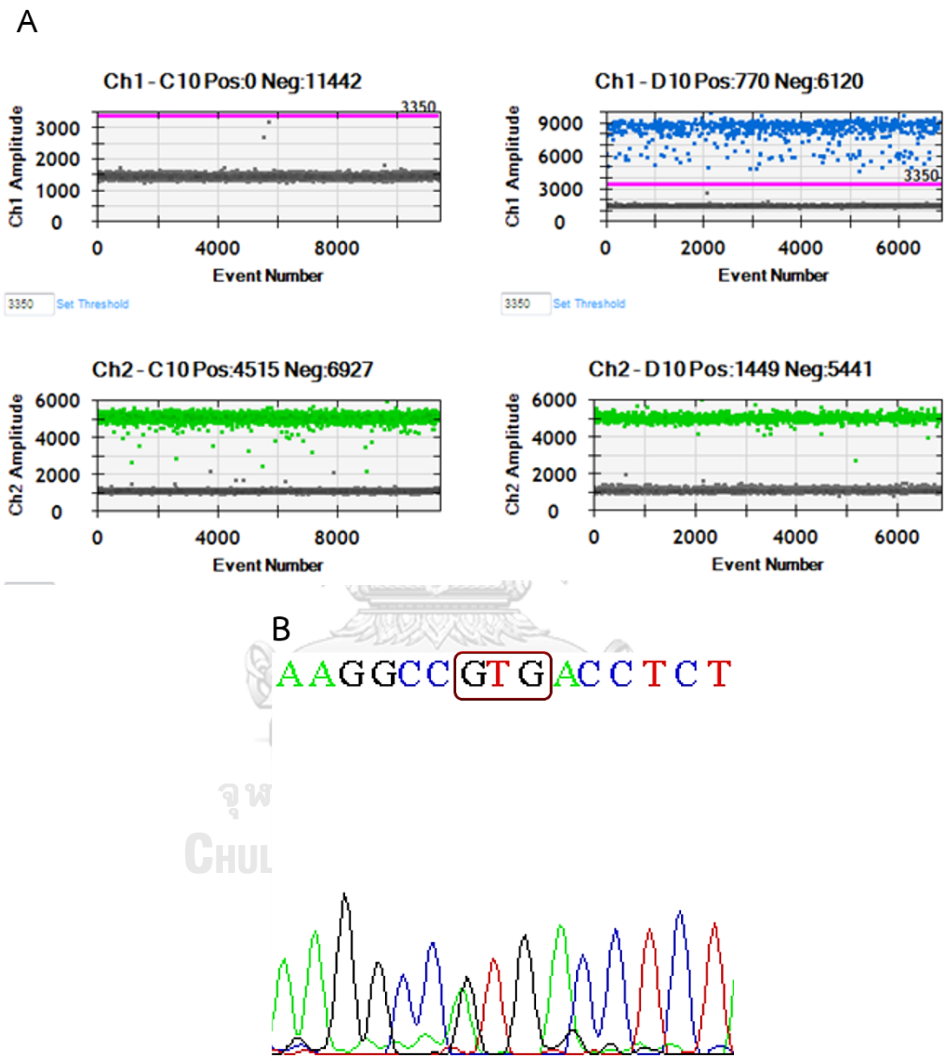


Figure 12 Verification of *GP1BA* p.M255V mutation in M255V hiPSC

A. The droplet digital PCR (ddPCR) results of normal hiPSCs (Left panel) and M255V hiPSCs (Right panel)

B. DNA sequencing confirmed the point mutation of *GP1BA* p.M255V mutation in the M255V hiPSCs.

Characterization of M255V hiPSC

The M255V hiPSCs were examined for pluripotency markers, karyotype and the differentiation capacity. Karyotyping of M255V hiPSCs demonstrated normal male karyotype in numbers (46, XY) and chromosomal structures similar to normal hiPSCs (Figure13).

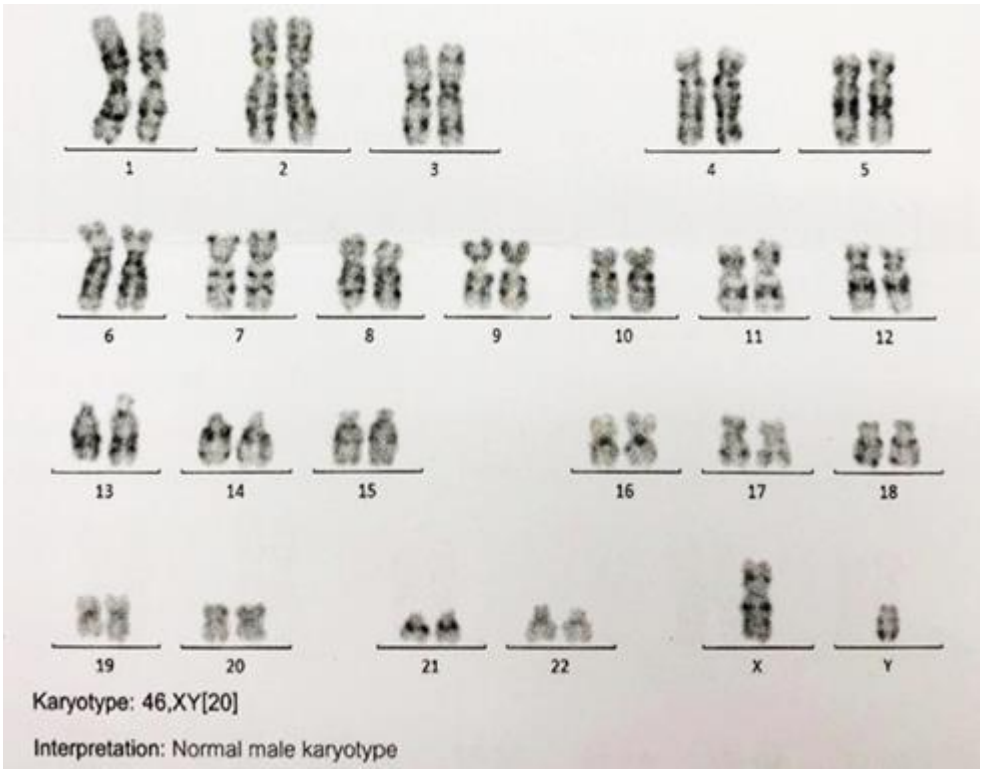


Figure 13 Karyotyping of M255V hiPSCs

The pluripotency stem cell markers of normal, M255V and BSS-A hiPSCs were determined using RT-PCR (Figure 14). The RT-PCR products of *NANOG*, *OCT4*, *SOX2*, *KLF4* and *MYC* were 294, 313, 151, 397 and 328 bp, respectively.

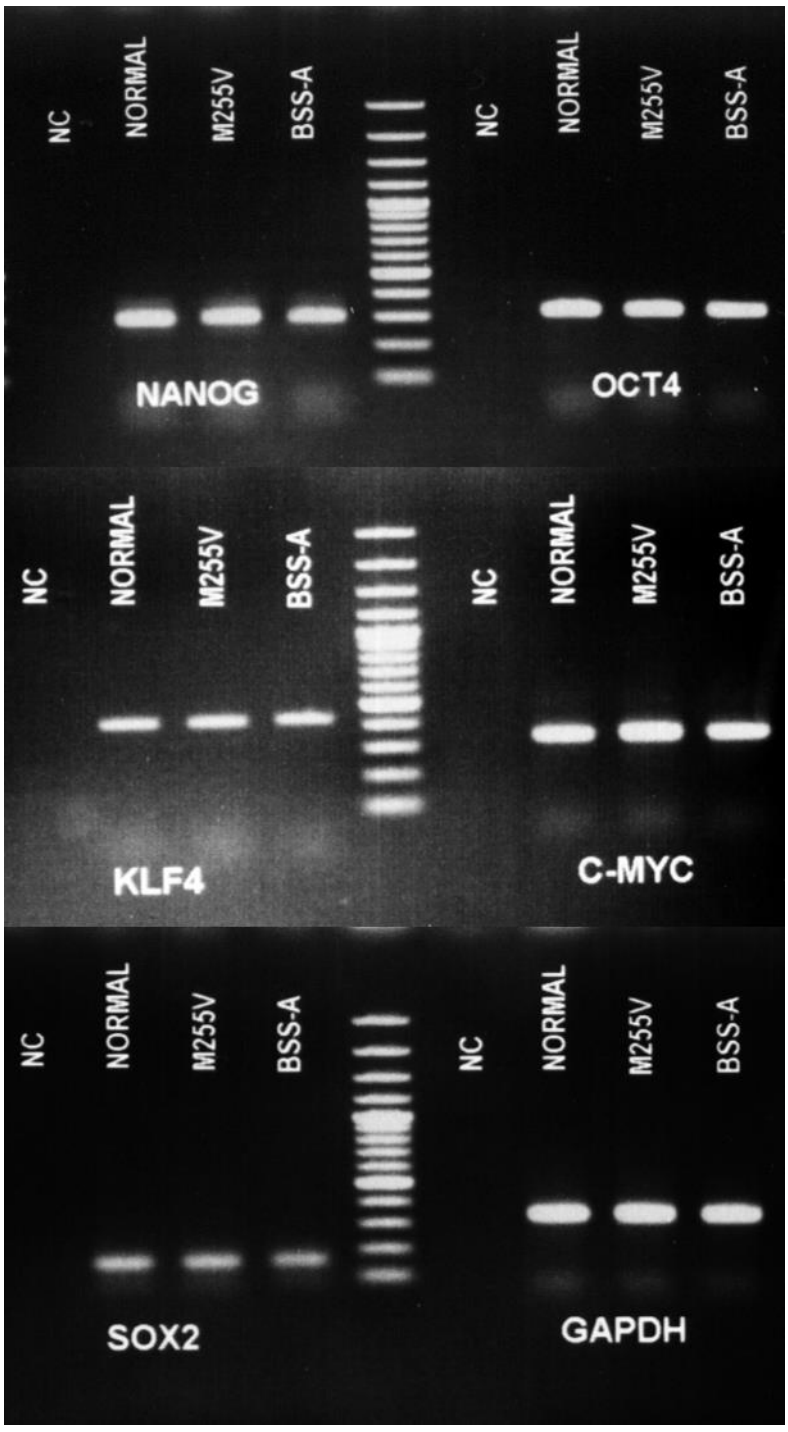


Figure 14 The expression of NANOG, OCT4, SOX2, KLF4 and MYC of M255V hiPSCs using reverse transcriptase polymerase chain reaction (RT-PCR)

The pluripotent capacity to differentiate into three germ layers of M255V hiPSCs was tested by embryoid body formation. Normal, M255V and BSS-A hiPSCs were cultured to embryoid body (Figure 15) and detected for the three germ layers differentiation using Human Three Germ Layer 3-Color Immunocytochemistry Kit. Both of *GP1BA* mutated hiPSCs were simultaneously stained with antibodies for ectoderm, mesoderm and endoderm. Immunofluorescence analysis showed the positive markers for ectoderm (OCT2 and SOX1), mesoderm (Brachyury and HAND1) and endoderm (GATA-4 and SOX17) (Figure 16).

These data demonstrated that M255V and BSS-A hiPSCs showed normal karyotype, pluripotency stem cell properties and the pluripotent capacity of multilineage differentiation which are the stem cell characteristics.

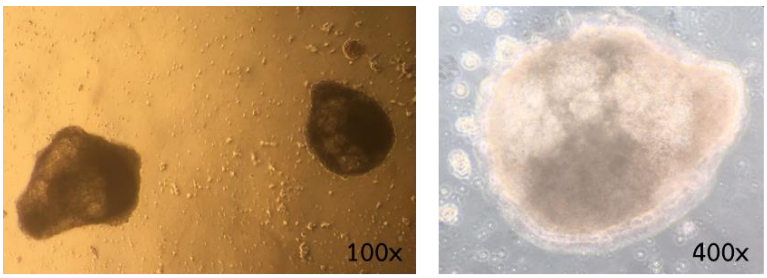


Figure 15 Embryoid body morphology

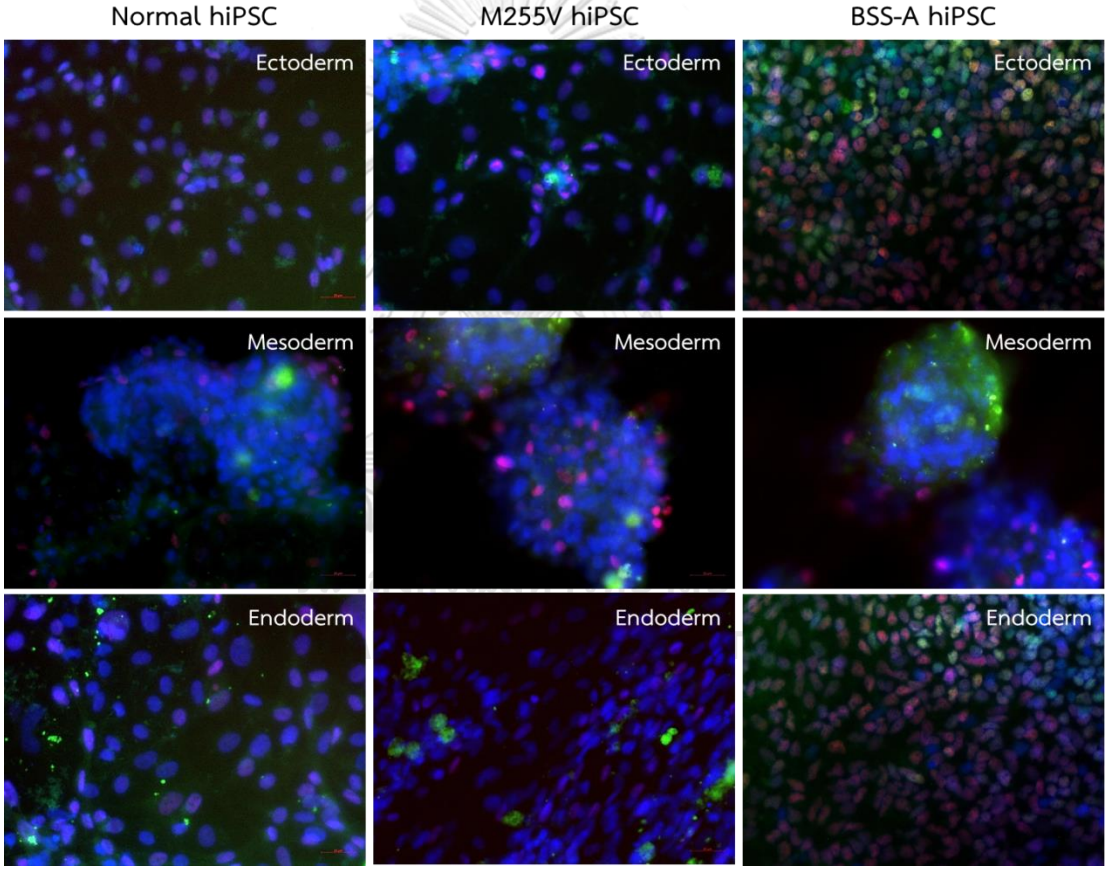


Figure 16 Immunofluorescence staining of normal, M255V and BSS-A hiPSCs for ectoderm, mesoderm and endoderm differentiation

Cells were stained with specific antibodies for ectoderm; anti-OTX2 (red) and anti-SOX1 (green), antibodies for mesoderm; anti- Brachyury (red) and anti- HAND1 (green) and antibodies for endoderm; anti-GATA-4 (green) and anti- SOX17 (red)

Megakaryocytes differentiation and characterization

The ES-sac technique was used for the differentiation of the normal, M255V and BSS-A hiPSCs into megakaryocytes and platelets. Normal, M255V and BSS-A hiPSCs were co-cultured with irradiated C3H10T1/2 feeder cells for 14 days. ES-sacs were torn for HPC harvest. HPCs were reseeded on inactivated OP9 cells for 7 days (day 21). After day 21, the numbers of megakaryocytes were analyzed by flow cytometry. Normal and M255V hiPSC-derived megakaryocytes expressed the intensity of GPIb on the surfaces while BSS-A hiPSC-derived megakaryocytes not expressed (Figure 17). The total numbers of normal, M255V and BSS-A hiPSC-derived megakaryocytes were 44.02±0.75, 42.69±1.96 and 44.43±2.05 cells/µl, respectively, which were not significantly different (p-value > 0.05) (Figure 18).

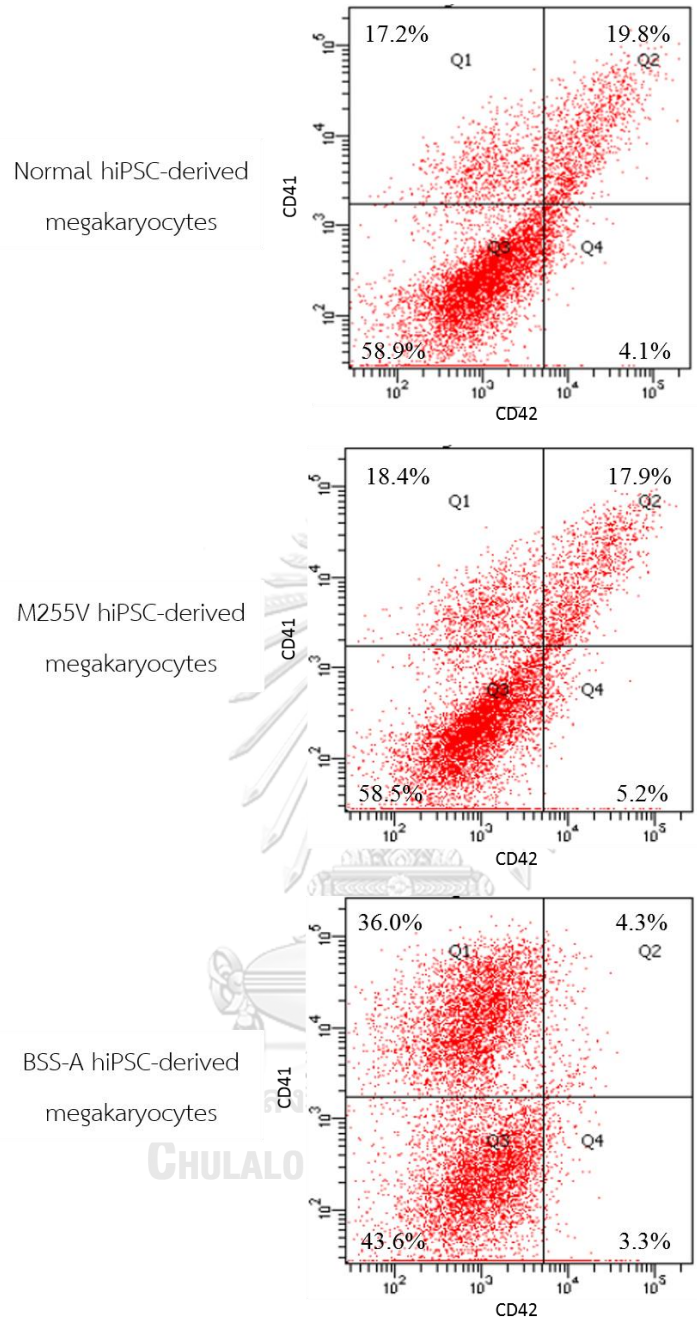


Figure 17 Megakaryocyte differentiation from human induced pluripotent stem cells (hiPSCs)

The hiPSC-derived megakaryocytes were stained with FITC-conjugated anti-CD41a and PE-conjugated anti-CD42. Normal and M255V hiPSC-derived megakaryocytes expressed CD41 and CD42 while BSS-A hiPSC-derived megakaryocytes expressed only CD41.

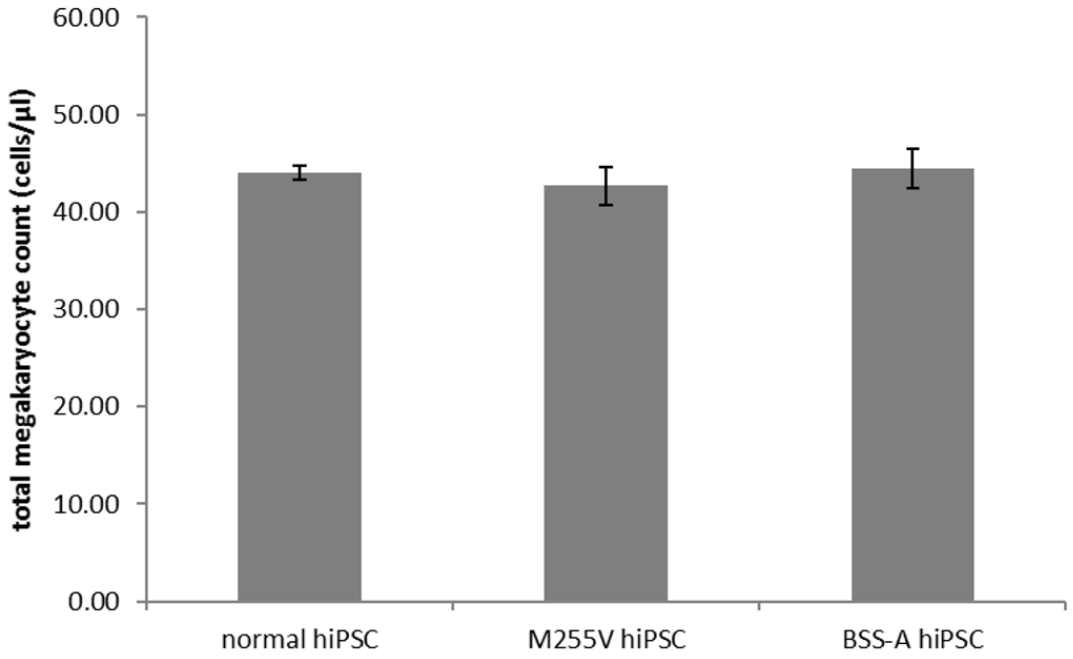


Figure 18 Total CD41+ megakaryocyte cells numbers from normal, M255V and BSS-A hiPSC lines

Total megakaryocyte counts from three hiPSCs were not significantly different.

Characterization of proplatelet formation

Proplatelets from normal, M255V and BSS-A hiPSC lines were determined by immunofluorescence staining for tubulin and actin after seeded hiPSC-derived megakaryocytes on Matrigel-coated coverslips. M255V and BSS-A hiPSC-derived megakaryocytes produced larger proplatelet tips compared with normal hiPSC-derived megakaryocytes (Figure 19). Proplatelet tip sizes were measured after staining for microtubule coils. The sizes of normal, M255V and BSS-A hiPSC-derived proplatelet tips were 2.23±0.30, 4.39±0.60 and 4.33±0.58 µm, respectively. M255V and BSS-A proplatelet tips were significantly larger than those of normal hiPSC-derived proplatelets (p-value < 0.001, N = 200) (Figure 20).

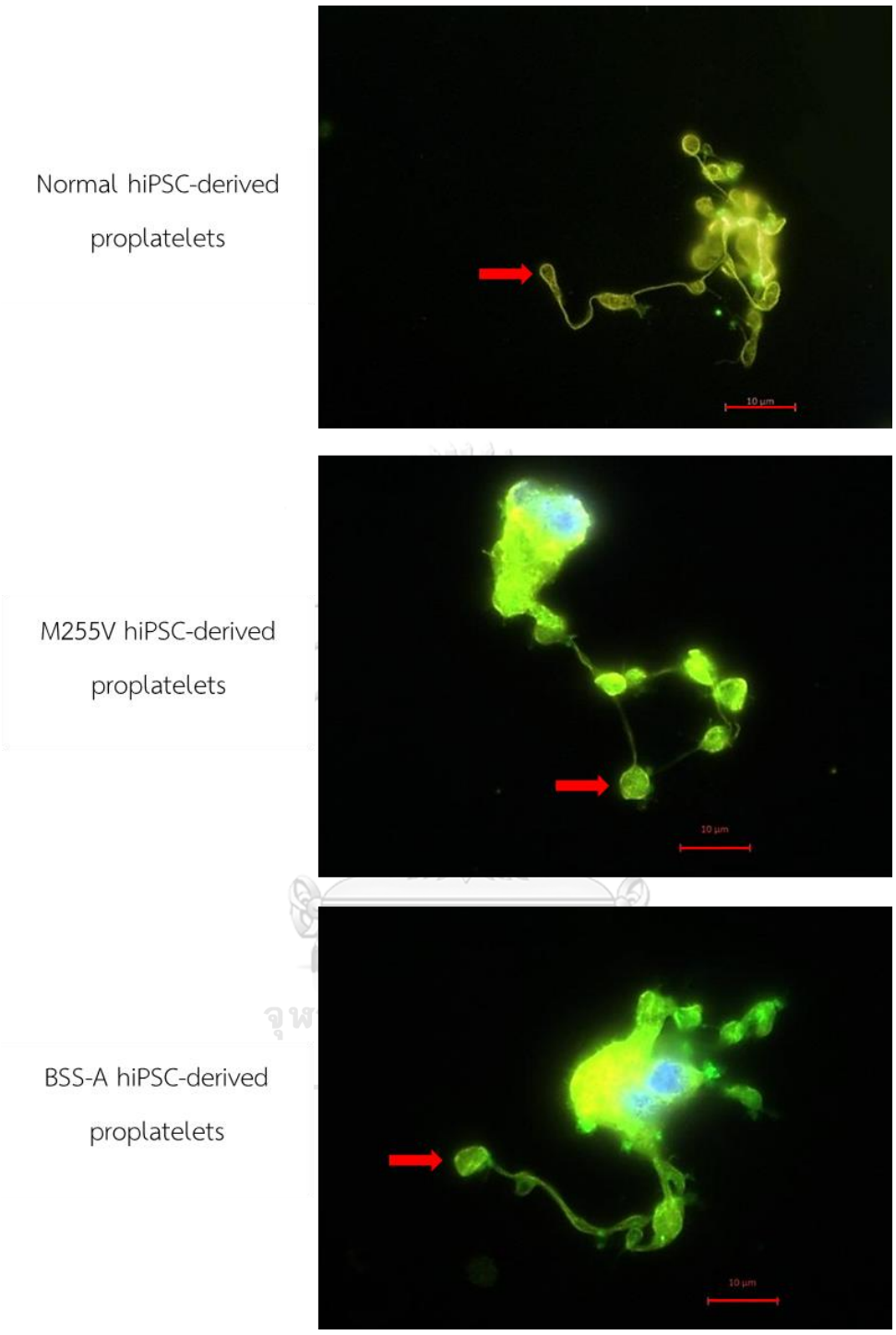


Figure 19 hiPSC-derived megakaryocyte differentiation into proplatelets

Proplatelets were immunofluorescence stained for tubulin (green) and actin (red). M255V and BSS-A hiPSC-derived megakaryocytes produced larger proplatelet tips than normal hiPSC-derived megakaryocytes.

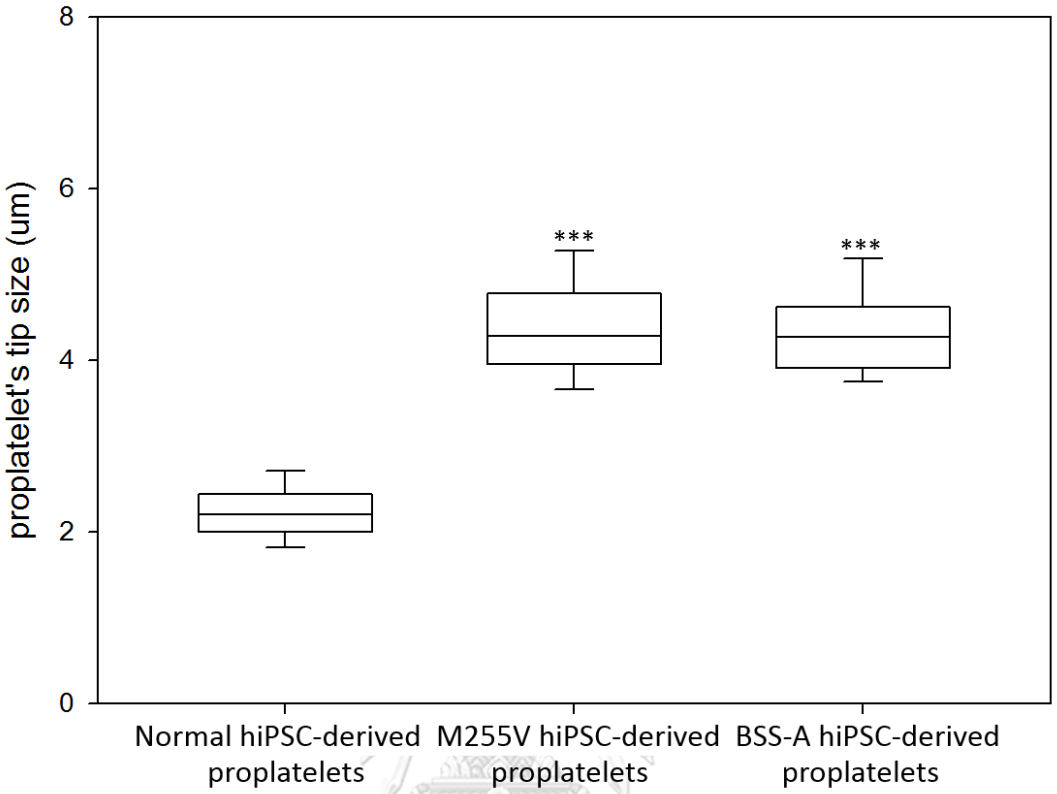


Figure 20 Proplatelet tip sizes of hiPSC-derived megakaryocytes

Sizes of proplatelet tip of normal, M255V and BSS-A hiPSC-derived megakaryocytes were determined by the microtubule coil measuring (N = 200 each). Proplatelet tip sizes derived from M255V and BSS-A hiPSCs were significantly larger than normal hiPSCs (p-value < 0.001).

Characterization of platelets

On day 23, platelets were characterized for the sizes and total counts. The platelet diameters were measured the circumferential microtubule coils after immunofluorescence staining for tubulin and actin. M255V and BSS-A hiPSC-derived megakaryocytes produced larger platelet compared with normal hiPSC-derived megakaryocytes (Figure 21). The sizes of normal, M255V and BSS-A platelets were 2.41±0.53, 4.49±0.66 and 4.41±0.67μm, respectively (p-value < 0.001) (Figure 22).

Total counts of normal, M255V and BSS-A hiPSC-derived platelets were evaluated using flow cytometry (Figure 23). Total platelet counts of normal, M255V and BSS-A hiPSCs were 150.52±3.16, 78.75±3.93 and 76.64±3.12 cells/μl, respectively. M255V and BSS-A platelet yields were significantly lower than those of normal control (p-value < 0.001) (Figure 24).

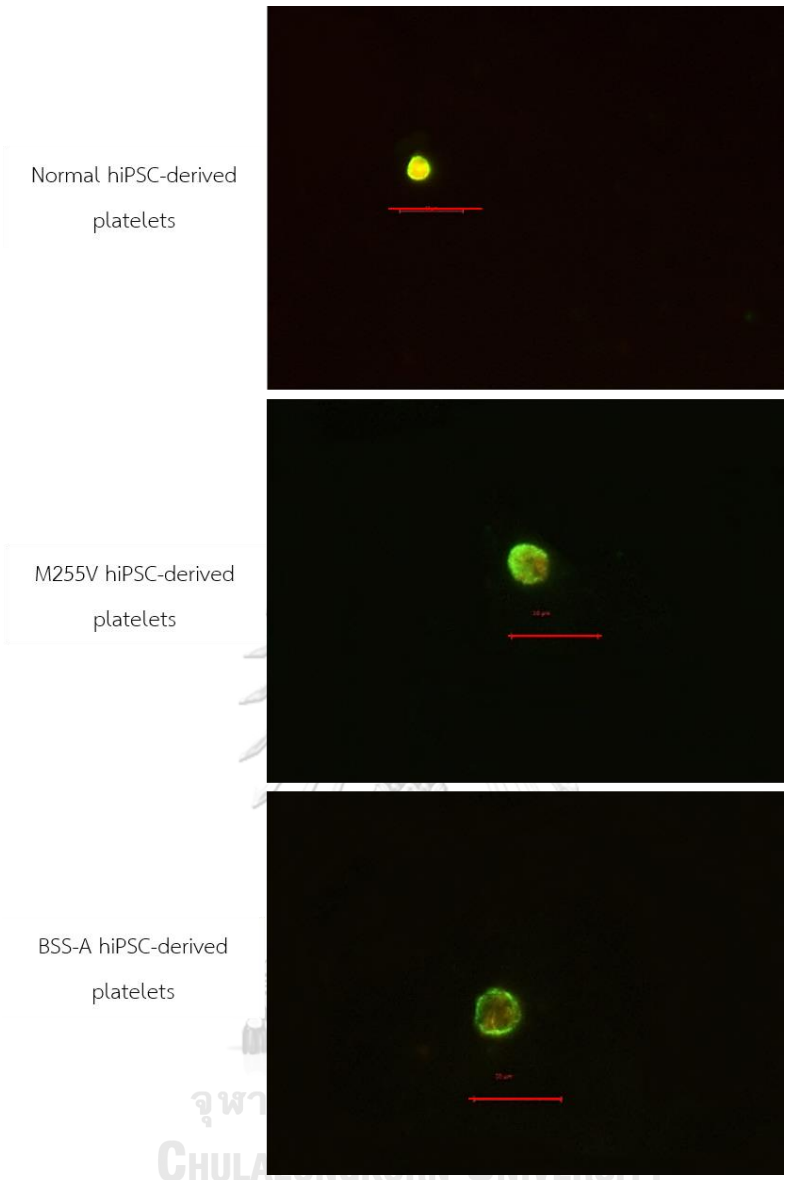


Figure 21 The hiPSC-derived platelets from normal, M255V and BSS-A hiPSCs Platelets were immunofluorescence stained for tubulin (green) and actin (red). M255V and BSS-A hiPSC-derived megakaryocytes produced larger platelets than normal hiPSC-derived megakaryocytes.

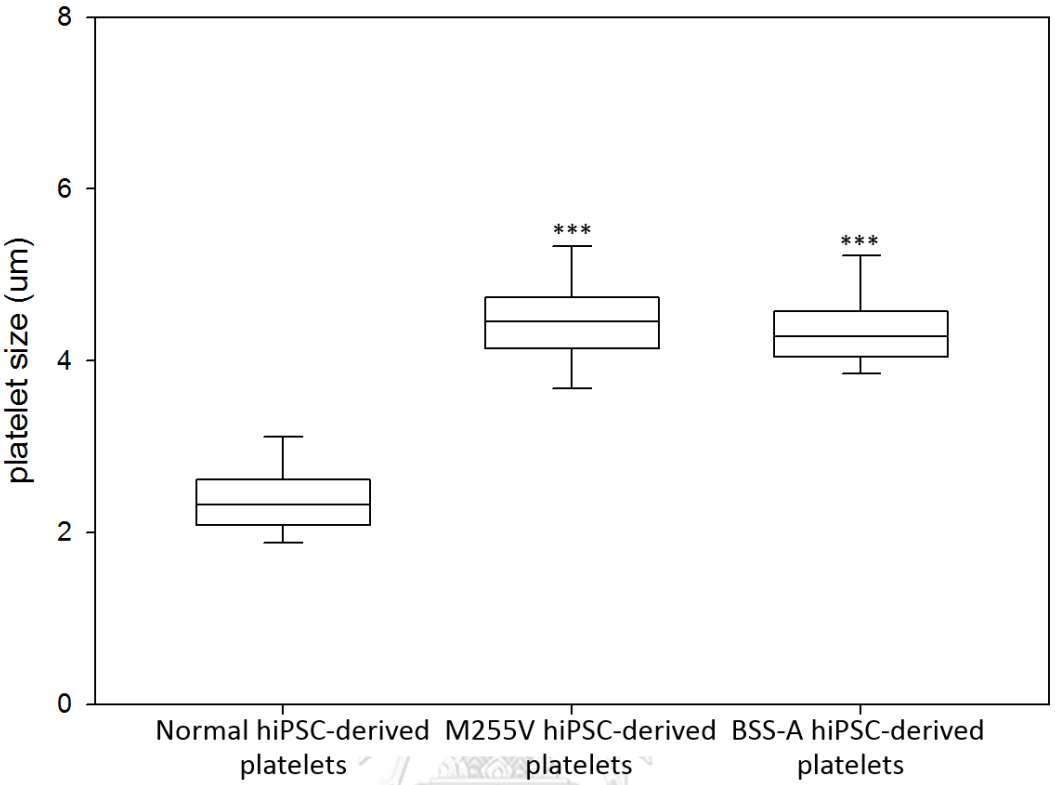


Figure 22 The sizes of platelets derived from normal, M255V and BSS-A hiPSCs
Sizes of hiPSC-derived platelets were determined by the circumferential microtubule coil measuring (N = 200 each). Platelet sizes derived from M255V and BSS-A hiPSCs were significantly larger than normal hiPSCs (p-value < 0.001).

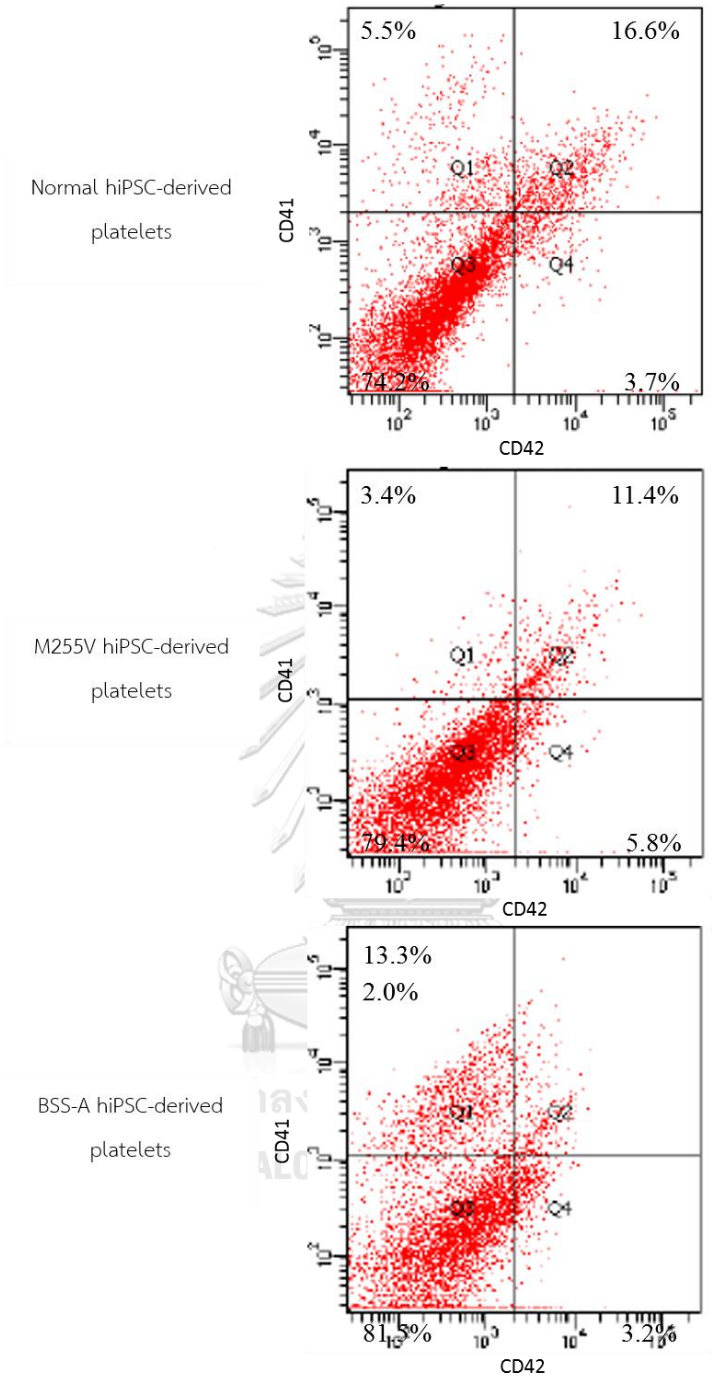


Figure 23 Platelet production from human induced pluripotent stem cells (hiPSCs) The hiPSC-derived platelets were stained with FITC-conjugated anti-CD41a and PE-conjugated anti-CD42. Normal and M255V hiPSC-derived platelets expressed CD41 and CD42 while BSS-A hiPSC-derived platelets expressed only CD41 by flow cytometry.

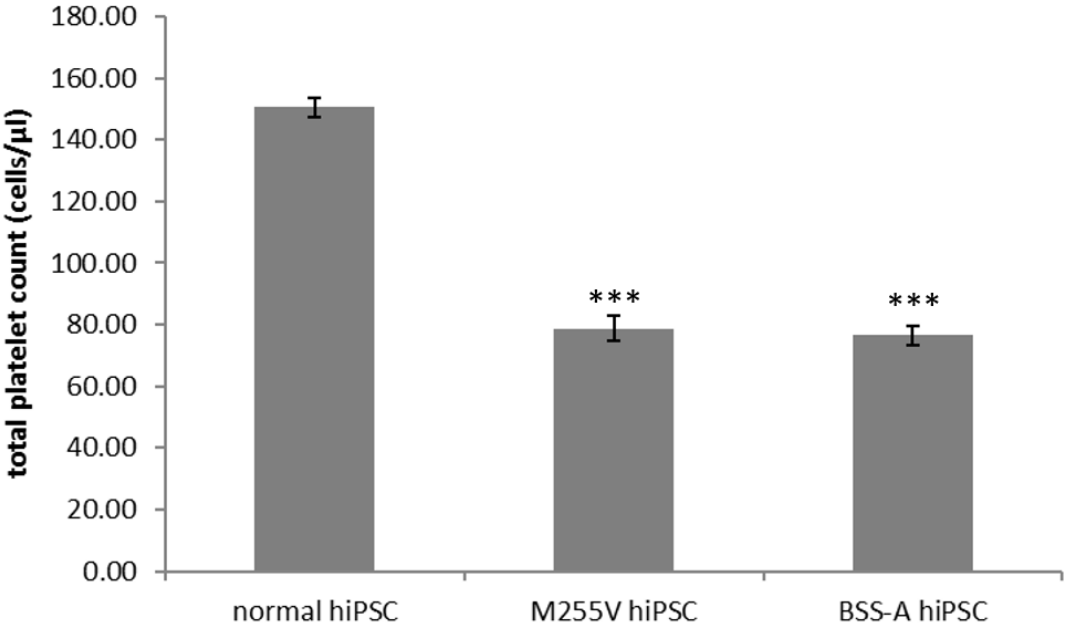


Figure 24 Total CD41+ platelets numbers from normal, M255V and BSS-A hiPSC lines

M255V and BSS-A hiPSC-derived platelet yields were significantly lower than that of normal hiPSC-derived platelets (p-value < 0.001).

Von Willebrand factor (VWF) binding study

The hiPSC-derived platelets were incubated with VWF and observed for GPIb-VWF interaction by immunofluorescence staining with FITC-conjugated anti-CD41a and Alexa Fluor 647-conjugated anti-VWF. Normal and BSS-A hiPSC-derived platelets did not bind with VWF, while M255V hiPSC-derived platelets bound to VWF at approximately 87.7±3.1%. Furthermore, only M255V hiPSC-derived platelets were agglutinated in the presence of VWF (Figure 25).

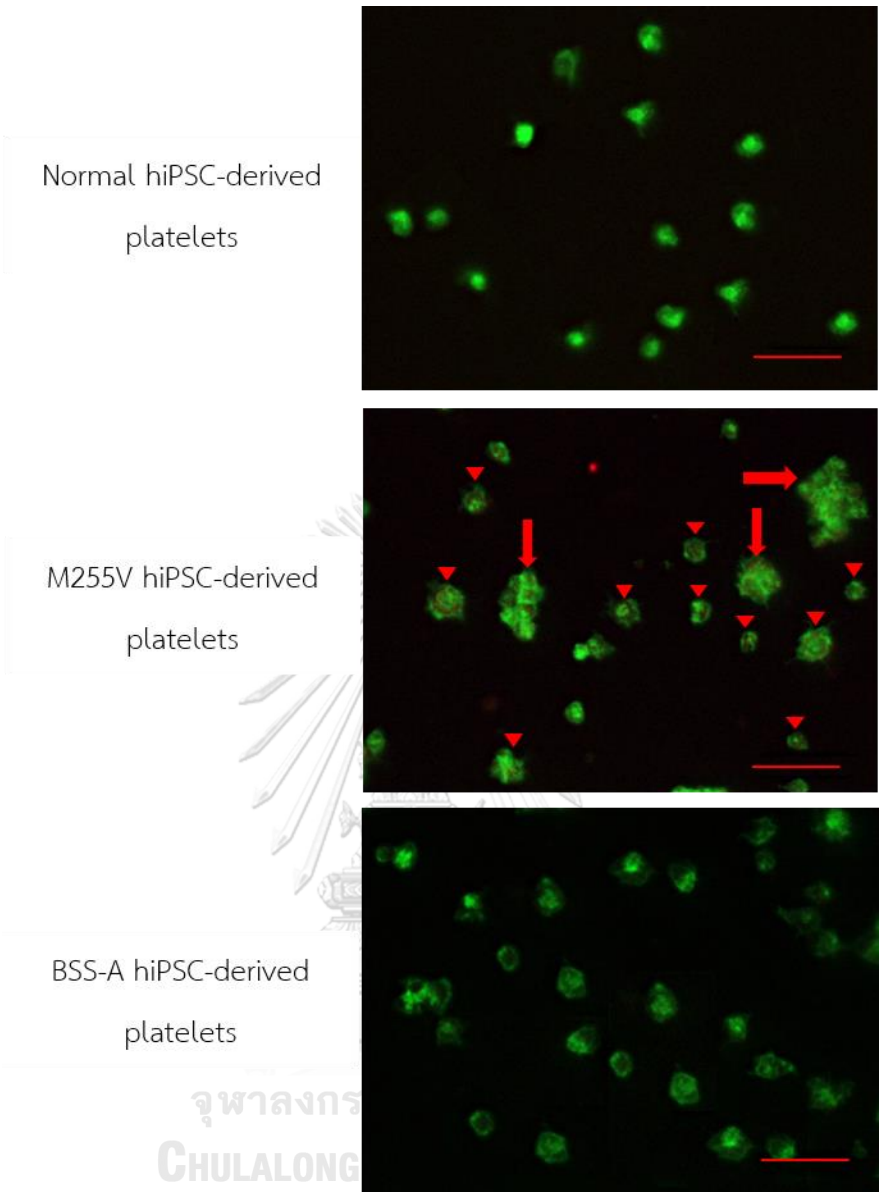


Figure 25 Glycoprotein Ib-von Willebrand factor interaction

After incubation hiPSC-derived platelets with VWF, platelets were stained with FITC-conjugated anti-CD41a (green) and Alexa Fluor 647-conjugated anti-VWF (red). M255V hiPSC-derived platelets can bind VWF (arrow heads) and showed platelet agglutination (arrows), whereas normal and BSS-A hiPSC-derived platelets did not show these phenomena.

Protein signaling pathways

The hiPSC-derived megakaryocytes were cultured with megakaryocytes differentiation medium for 24 hours. Stimulated hiPSC-derived megakaryocytes were collected to study protein signal transduction (MAPK, AKT and Rho/ROCK) pathways regulating the platelet production by investigation for ERK1/2, AKT and MLC2 protein in both total and phosphorylated forms using automated Capillary Western blots (Figure 26).

The ratios of phosphorylated to total ERK1/2 proteins of normal, M255V and BSS-A hiPSC-derived megakaryocytes were 0.85±0.07, 0.50±0.06 and 0.41±0.05, respectively. The ratios of phosphorylated to total AKT in normal, M255V and BSS-A hiPSC-derived megakaryocytes were 0.01±0.00, 0.02±0.01 and 0.01±0.00, respectively. The ratios of phosphorylated to total MLC2 in normal, M255V and BSS-A hiPSC-derived megakaryocytes were 0.02±0.01, 0.08±0.01 and 0.11±0.02, respectively. The ERK1/2 phosphorylation in M255V and BSS-A hiPSC-derived megakaryocytes was significantly lower than normal hiPSC-derived megakaryocytes (p-value < 0.001), while the MLC2 phosphorylation in M255V and BSS-A hiPSC-derived megakaryocytes was significantly greater than normal hiPSC-derived megakaryocytes (p-value < 0.01) (Figure 27).

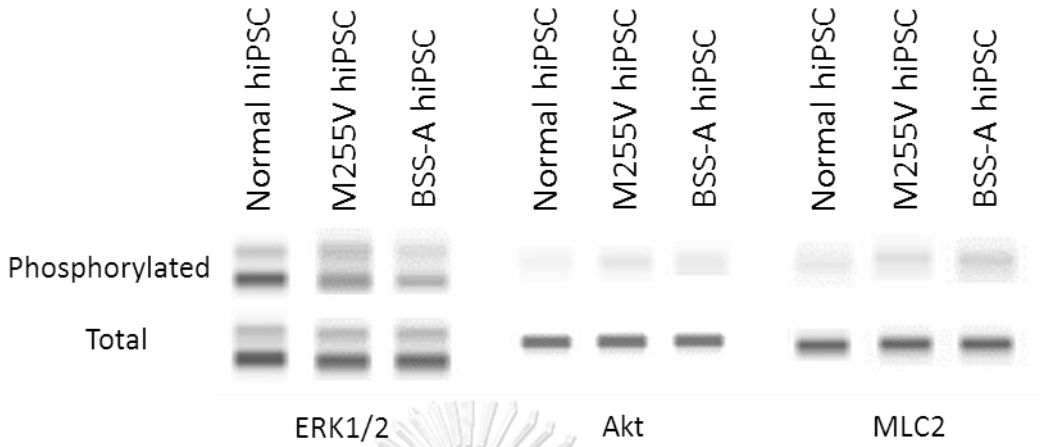


Figure 26 The Western blots of phosphorylated and total signaling proteins from normal, M255V and BSS-A hiPSC-derived megakaryocytes

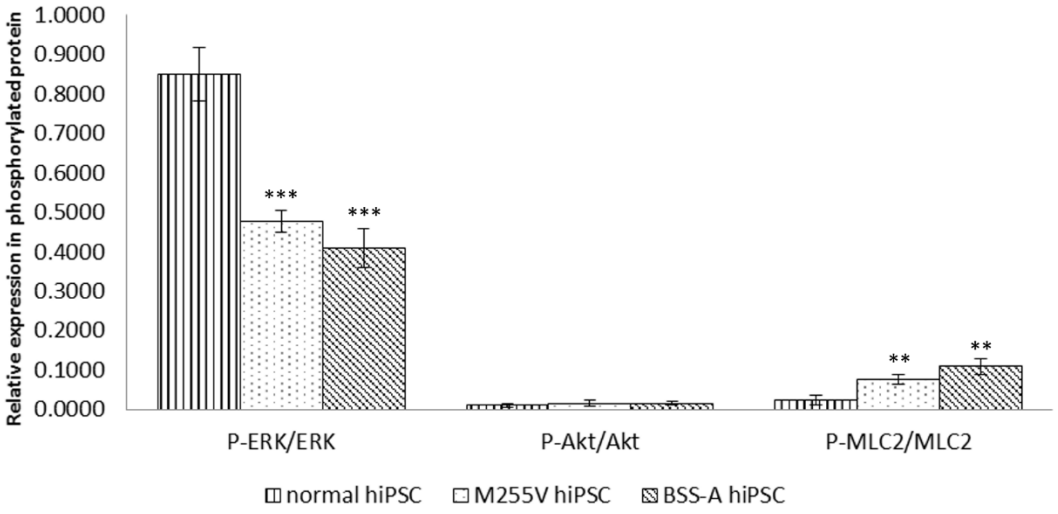


Figure 27 The ratios of phosphorylated to total ERK1/2 (Combined ERK1 and ERK2), AKT and MLC2 proteins of normal, M255V and BSS-A hiPSC-derived megakaryocytes The asterisks (**) and (***) denoted p-value <0.01 and p-value <0.001, respectively.

Effects of signaling inhibitors on megakaryocytes

For studying the signal inhibitors effects on signaling transduction (MAPK, AKT and Rho/ROCK) pathways, normal hiPSC-derived megakaryocytes were treated with a MEK inhibitor (MEKi) and M255V and BSS-A hiPSC-derived megakaryocytes were treated with a ROCK inhibitor. Normal hiPSC-derived megakaryocytes were treated with ROCKi or ROCKi plus MEKi to study the interaction between the MAPK and Rho/ROCK pathways (Figure 28).

The ratios of phosphorylated to total ERK1/2 in normal hiPSC-derived megakaryocytes with MEKi was 0.48±0.07, which was significantly lower than normal hiPSC-derived megakaryocytes without MEKi (p-value < 0.01). The ratios of phosphorylated to total ERK1/2 in M255V and BSS-A hiPSC-derived megakaryocytes with ROCKi were 0.54±0.02 and 0.56±0.02, respectively, which were significantly higher than M255V and BSS-A hiPSC-derived megakaryocytes without ROCKi (p-value < 0.05 and p-value < 0.01, respectively). The ratios of phosphorylated to total MLC2 of normal hiPSC-derived megakaryocytes with MEKi was 0.09±0.01, which was significantly greater than normal hiPSC-derived megakaryocytes without MEKi (p-value < 0.01), while the ratios of phosphorylated to total MLC2 of M255V and BSS-A hiPSC-derived megakaryocytes with ROCKi were undetectable which was significantly lower than M255V and BSS-A hiPSC-derived megakaryocytes without ROCKi (p-value < 0.01). The ERK1/2 and MLC2 phosphorylation in normal hiPSC-derived megakaryocytes with ROCKi were 0.89±0.06 and 0.0, respectively. The ERK1/2 and MLC2 phosphorylation in normal hiPSC-derived megakaryocytes with ROCKi plus MEKi were 0.75±0.08 and 0.0, respectively. The MLC2 of normal hiPSC-derived megakaryocytes with ROCKi only and ROCKi plus MEKi were undetectable which was significantly lower than normal hiPSC-derived megakaryocytes without inhibitor (p-value < 0.05) (Figure 29).

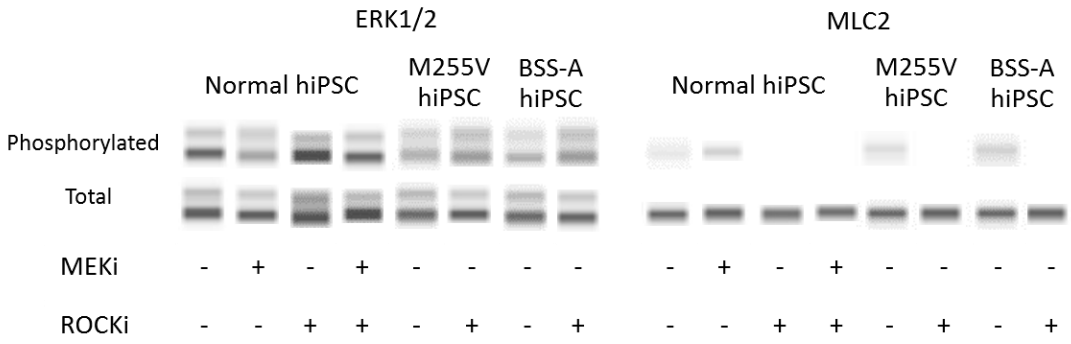


Figure 28 The Western blots of phosphorylated and total signaling proteins from normal hiPSC-derived megakaryocytes with MEK inhibitor (MEKi), ROCK inhibitor (ROCKi) and MEKi plus ROCKi, M255V and BSS-A hiPSC-derived megakaryocytes with ROCKi compared with normal, M255V and BSS-A hiPSc-derived megakaryocytes without inhibitor

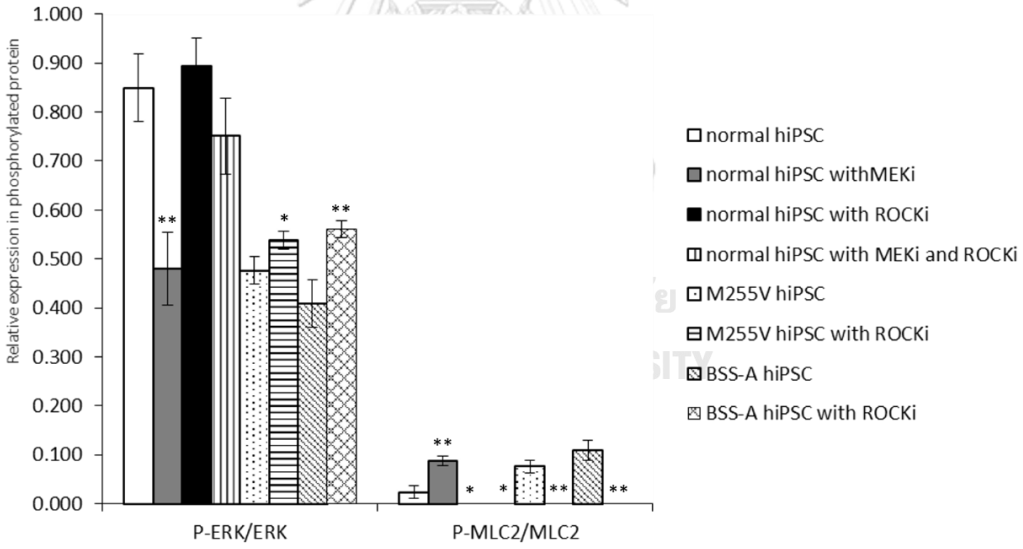


Figure 29 The ratios of phosphorylated to total ERK1/2 (Combined ERK1 plus ERK2), AKT and MLC2 proteins of normal hiPSC-derived megakaryocytes with MEKi, ROCKi and MEKi plus ROCKi, M255V and BSS-A hiPSC-derived megakaryocytes with ROCKi compared with normal, M255V and BSS-A hiPSc-derived megakaryocytes without inhibitor

The asterisks (*) and (**) denoted p-value <0.05 and p-value <0.01, respectively.

Signaling inhibitor effects on proplatelet formation

After adding the inhibitors for 24 hours, proplatelets from normal, M255V and BSS-A hiPSC lines were determined by immunofluorescence staining for tubulin and actin. Normal hiPSC-derived megakaryocytes with MEKi generated significantly larger proplatelet tips compared with normal hiPSC-derived megakaryocytes without MEKi (p-value < 0.001), whereas M255V and BSS-A hiPSC-derived megakaryocytes with ROCKi produced smaller proplatelet tips compared with M255V and BSS-A hiPSC-derived megakaryocytes without ROCKi (p-value < 0.001) (Figure 30).

Proplatelet tip sizes of normal hiPSC-derived megakaryocytes with MEKi, M255V and BSS-A megakaryocytes with ROCKi were 4.20±0.77, 3.01±0.55 and 2.99±0.54 μm, respectively. Proplatelets tip sizes of normal hiPSC-derived megakaryocytes with ROCKi and with ROCKi plus MEKi were 2.23±0.32 and 2.24±0.26 μm, respectively (Figure 31).

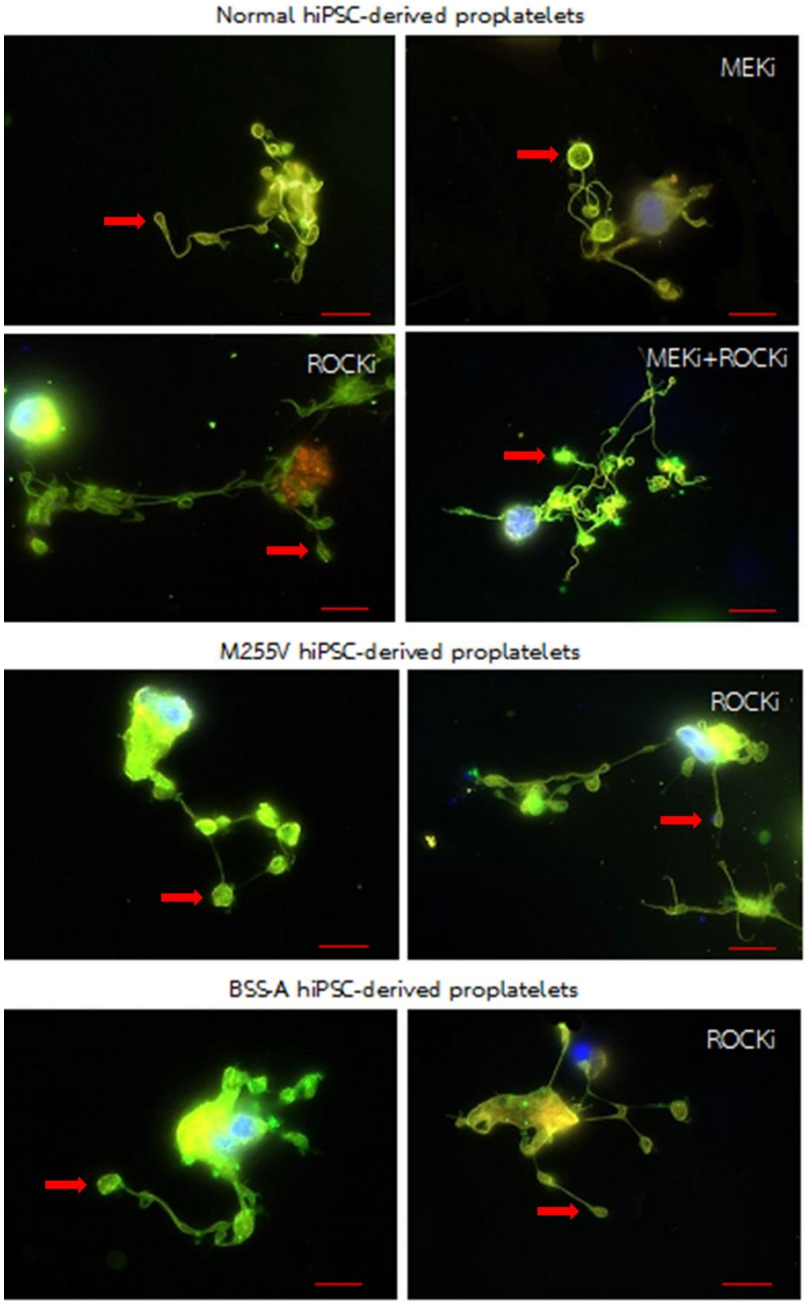


Figure 30 Proplatelet formation in the presence of signaling inhibitors

Proplatelets were immunofluorescence stained for tubulin (green) and actin (red). Normal hiPSC-derived megakaryocytes with MEKi and M255V and BSS-A hiPSC-derived megakaryocytes showed larger proplatelet tips compared with normal hiPSC-derived megakaryocytes without inhibitor. The ROCKi can reverse the proplatelet tips to smaller sizes.

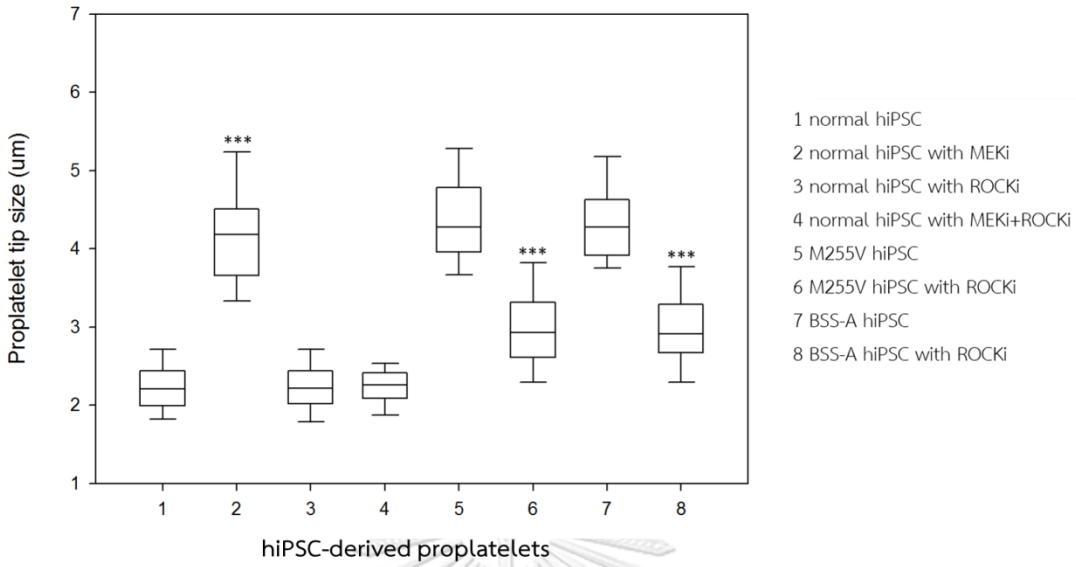


Figure 31 Sizes of proplatelet tip of hiPSC-derived megakaryocytes in the presence of inhibitors

Proplatelet tips from normal hiPSC-derived megakaryocytes with a MEKi were significantly larger than normal hiPSC-derived megakaryocytes without inhibitor, whereas M255V and BSS-A hiPSC-derived proplatelet tips with a ROCKi were significantly closer to normal. The asterisks (***) denoted p-values <0.001.

Signaling inhibitor effects on platelet production

Normal hiPSC-derived platelets with MEKi were significantly larger compared with normal hiPSC-derived platelets without MEKi (p-value < 0.001), whereas M255V and BSS-A hiPSC-derived platelets with ROCKi were smaller compared with M255V and BSS-A hiPSC-derived platelets without ROCKi (p-value < 0.001) (Figure 32). The sizes of M255V and BSS-A hiPSC-derived platelets with ROCKi were 3.01±0.56 and 3.00±0.56 μm, respectively. Normal hiPSC-derived platelets size with MEKi, ROCKi and MEKi plus ROCKi were 4.31±0.82, 2.43±0.33 and 2.43±0.32 μm, respectively (Figure 33).

Total platelet counts of hiPSC-derived platelets were enumerated by flow cytometry. Total platelet counts from M255V and BSS-A hiPSC-derived platelets with ROCKi were 133.66±5.37 and 134.54±3.47 cells/μl, respectively. Total platelet counts of normal hiPSC-derived platelets with MEKi, ROCKi and MEKi plus ROCKi were 58.39±4.90, 154.33±5.56 and 146.35±4.33 cells/μl, respectively. The MEKi significantly decreased the platelet yields of normal hiPSCs, whereas ROCKi increases the platelet counts derived from M255V and BSS-A hiPSCs (p-value < 0.001) (Figure 34).

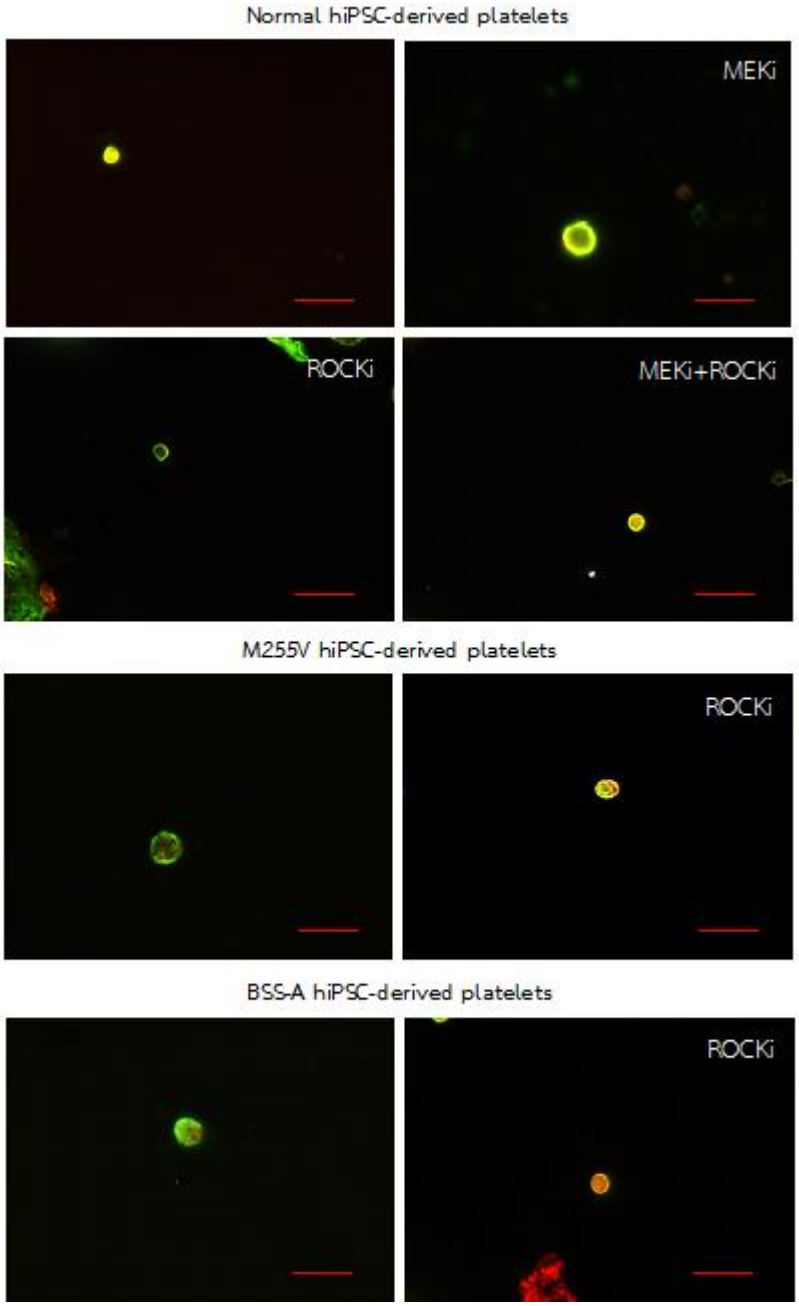


Figure 32 Platelet production in the presence of signaling inhibitors

Normal hiPSC-derived platelets with a MEKi became larger compared with those without inhibitor, whereas M255V and BSS-A hiPSC-derived platelets with a ROCKi were smaller compared with those without an inhibitor.

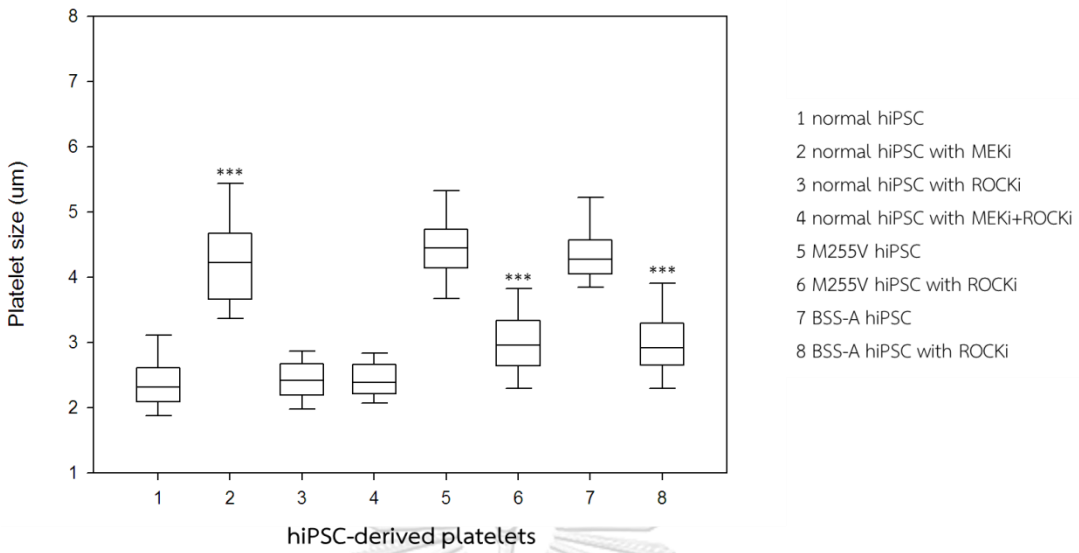


Figure 33 Sizes of hiPSC-derived platelets in the presence of signaling inhibitors Normal hiPSC-derived platelets with a MEKi were significantly larger than normal hiPSC-derived platelets without an inhibitor, whereas M255V and BSS-A hiPSC-derived platelets with a ROCKi were significantly closer to normal. The asterisks (***) denoted p-values <0.001.

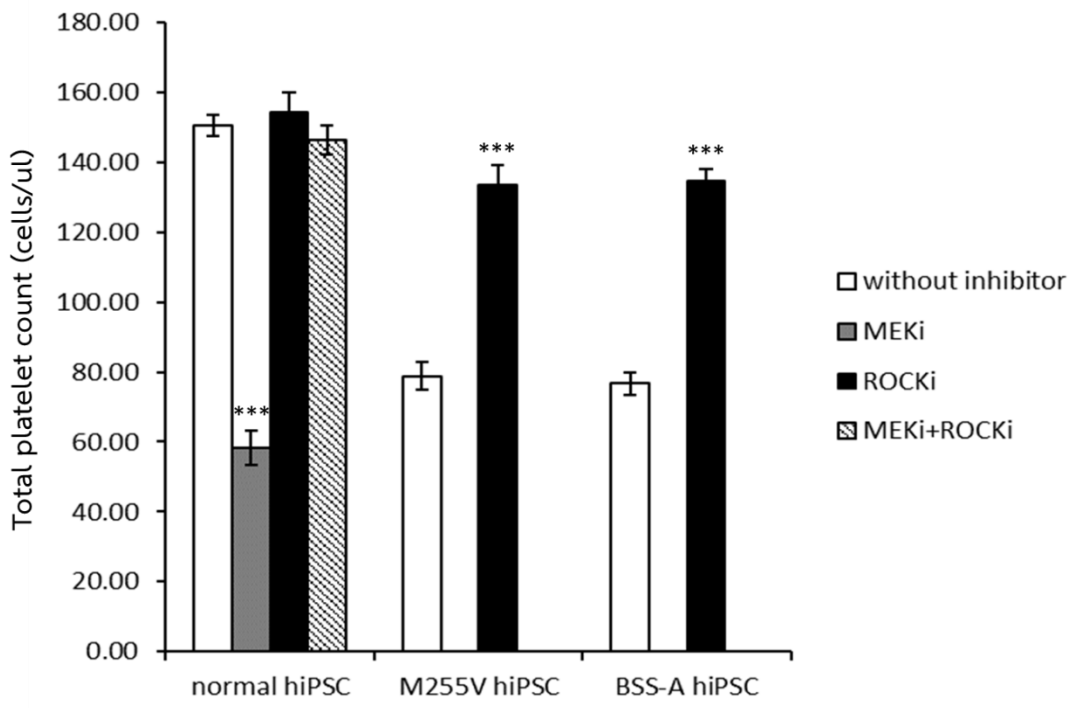


Figure 34 Total CD41+ platelets numbers from normal, M255V and BSS-A hiPSC lines in the presence of inhibitors

Normal hiPSCs with a MEKi, M255V and BSS-A hiPSCs yielded lower platelet counts than those of normal cells without the inhibitor. This defect was corrected by the ROCKi (p-value < 0.001).

CHAPTER V

DISSCUSSION

Thrombopoiesis is the process of hematopoietic stem cell (HSC) differentiation into megakaryocyte progenitors, megakaryocytes and platelets. It depends on the microenviromental niches and hemotopoietic cytokines. Glycoproteins (GP) Ib-IX-V that is expressed on the surface of megakaryocytes and platelets binds von Willebrand factor (VWF) and plays roles in platelet production but the molecular mechanisms remain unclear. The human induced pluripotent stem cells (hiPSCs) are used as the model for pathogenesis investigation. The hiPSCs are reprogramed from adult cells *via* induction by *OCT3/4*, *SOX2*, *c-MYC* and *KLF4*. The hiPSCs can be derived into various cell types *in vitro*. Therefore, hiPSCs have potentials to be disease models which are probably closer to human physiology.

From our study, the hiPSCs with the gain-of-function GPIb (*GP1BA* p.M255V) were generated using CRISPR/Cas9 technique as a disease model for platelet generation in PT-VWD. In this study, M255V hiPSC-derived platelets with the gain-of-function GPIb mutation retained the normal karyotypes, pluripotenet stem cell properties and multi-lineage potentials and bound exogenous VWF resulting in agglutination similar to PT-VWD (31). The M255V megakaryocytes produced abnormally large proplatelet tips and lower numbers of larger platelets consistent with the macrothrombocytopenia phenotype of the PT-VWD patients (14). Therefore, hiPSCs can be used to study the molecular mechanisms of thrombopoiesis in the presence of GPIb mutations.

From our signaling transduction pathway experiments, both gain-of-function and loss-of-function mutations of *GP1BA* resulted in significantly decreased ERK1/2 phosphorylation and increased MLC2 phosphorylation. Notably, the ERK activation in

mutant cells was reduced but not completely abolished. The ERK1/2 is one of the signaling proteins in Ras/MAPK pathway which plays important roles in megakaryocyte proliferation, differentiation and proplatelet formation (8, 30, 58). ERK1/2 phosphorylation is also required for the proplatelet formation process (46). The MAPKs are activated by several hematopoietic growth factors including TPO via the c-MPL receptor and also by extracellular matrix proteins via integrins (59, 60). The mechanism of GPIb induction of ERK phosphorylation remains undefined. On the other hand, MLC2 is a negative regulator of proplatelet formation downstream of the Rho/ROCK pathway. The Rho/ROCK down-modulation is necessary to promote proplatelet formation (31, 43, 44). GPIb-IX-V binding to VWF can stimulate Cdc42 which in-turn inhibits ROCK signaling as shown in Figure 35 (61). On contrary, there was no significant change in the PI3K/AKT pathway in the presence of GPIb mutations. The PI3K/AKT pathway has been shown to increase the megakaryocyte survival, but probably plays minor or no role in GPIb-induced proplatelet formation (29, 46).

After adding a chemical MEK inhibitor into normal hiPSC-derived megakaryocytes, ERK1/2 phosphorylation was reduced, while MLC2 signaling was enhanced suggesting that the MAPK/ERK pathway is a negative regulator of the MLC2. Interestingly, proplatelet formation of normal hiPSCs exhibited larger proplatelet tips, larger platelet sizes and lower platelet yields in the presence of a MEK inhibitor. The degrees of changes of ERK phosphorylation and macrothrombocytopenia phenotypes of normal hiPSC-derived megakaryocytes under MAPK inhibition were similar to those with gain-of-function and loss-of-function GPIb. Therefore, MAPK/ERK pathway is likely responsible for the abnormal proplatelet formation in the GPIb-mutated megakaryocytes (28).

In the presence of a ROCK inhibitor, M255V and BSS-A hiPSCs showed the reduced MLC2 phosphorylation and mildly increased ERK1/2 signals suggesting that

the Rho/ROCK pathway was also a negative regulator of the MAPK pathway. Interestingly, the ROCK inhibitor could reverse the macrothrombocytopenia phenotypes of both M255V and BSS-A megakaryocytes. These results confirmed that Rho/ROCK pathway negatively regulated proplatelet formation (62) downstream of MEK because it could reverse the effects at least partially despite the MAPK pathway inhibition.

To confirm the interaction between Rho/ROCK and MAPK pathways, normal hiPSCs were incubated with MEKi alone, ROCKi alone or MEKi plus ROCKi. The ERK1/2 phosphorylation was similar to normal hiPSCs without inhibitor while the MLC2 phosphorylation was undetectable. These results suggest that the ROCKi can rescue the effects of the MEKi. This may be mediated by MLC2 down-modulation and MAPK activation. The balance between the opposing Rho/ROCK and MAPK pathways is important for normal platelet formation as summarized in Figure 35.

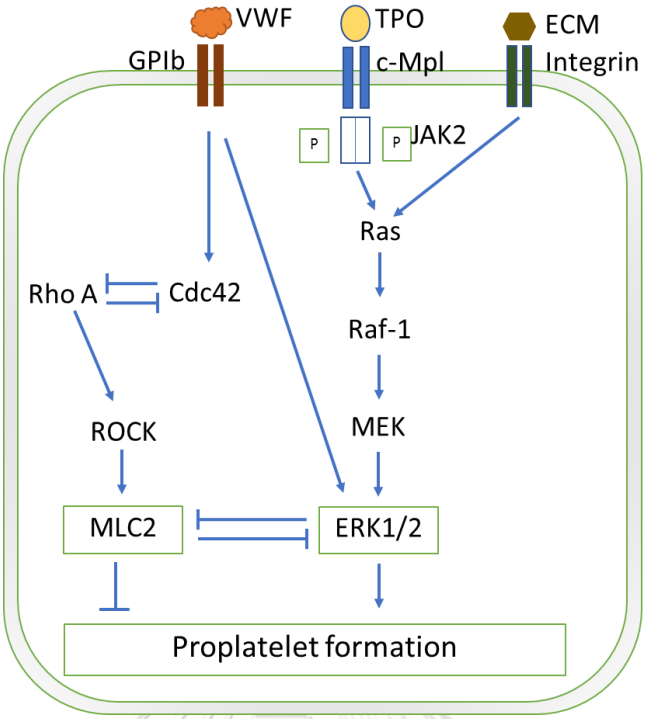


Figure 35 The proposed schematic diagram shows the regulation of proplatelet formation by GPIb-mediated signal transduction.

Furthermore, the gain-of-function and loss-of-function *GP1BA* mutation hiPSCs showed the similar phenotypes and signal transduction patterns. The interaction between GPIb and VWF can stimulate Cdc42 resulting MLC2 phosphorylation that inhibits proplatelet formation (61). In BSS, Cdc42 is not activated, therefore, impeding proplatelet formation. In the presence of gain-of-function mutation or PT-VWD, the downstream signals of GPIb showed the similar pattern resulting in inhibition of proplatelet formation. Therefore, optimal levels of GPIb activation are required for normal platelet production. Additionally, the signal transduction pathways controlling proplatelet formation are probably involved other molecules and pathways that should be explored in the future. From our results, a potential therapy for macrothrombocytopenia might be the inhibition of Rho/ROCK signaling pathway.

In conclusion, our study successfully generated the hiPSC disease models for PT-VWD and BSS using gene editing and patient-derived hiPSCs, respectively. Our data demonstrated the opposite functions of MAPK and Rho/ROCK pathways in controlling the numbers and sizes of generated platelets. This model may be used to screen for novel therapy and agents stimulating *in vitro* platelet production in the future.

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