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GENERATION AND CHARACTERIZATION OF HLA-UNIVERSAL, iPSC-DERIVED PLATELETS

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A Dissertation Submitted in Partial Fulfillment of the Requirements
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Thesis Title	GENERATION AND CHARACTERIZATION OF HLA-UNIVERSAL, iPSC-DERIVED PLATELETS
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Field of Study	Medical Science
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พัชรา นอบนพ : การสร้างและการศึกษาลักษณะของเกล็ดเลือดที่ไม่แสดงเอชแอลเอ ซึ่งพัฒนาจากเซลล์ต้นกำเนิดจากการเหนี่ยวนำ (GENERATION AND CHARACTERIZATION OF HLA-UNIVERSAL, iPSC-DERIVED PLATELETS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. น.พ. วรศักดิ์ โชติเลอศักดิ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ดร. พ.ญ. กัญญา ศุภปีติพร, 88 หน้า.

ความต้องการเกล็ดเลือดมีแนวโน้มที่เพิ่มขึ้นทั่วโลกตั้งแต่อดีตถึงปัจจุบันเนื่องจากการเพิ่มขึ้นของกลุ่มประชากรผู้สูงอายุ อุบัติการณ์เกิดโรคมะเร็งทางเลือด และการปลูกถ่ายเซลล์ต้นกำเนิดของเลือด ในทางตรงข้าม การลดลงของจำนวนผู้บริจาคเกล็ดเลือด ค่าใช้จ่ายที่สูงและเวลาที่ใช้ในการหาผู้บริจาคที่เหมาะสม และปัจจัยอื่นที่เป็นผลเสียของการรับบริจาคเกล็ดเลือด เช่นการติดเชื้อและผลข้างเคียง จำนวนเกล็ดเลือดที่ไม่เพิ่มขึ้นหลังจากได้รับบริจาค เหล่านี้ล้วนเป็นปัจจัยที่ทำให้เกิดปัญหาต่อความสำเร็จของการรับบริจาคเกล็ดเลือด เพื่อแก้ปัญหาเหล่านี้เราจึงเสนอทางเลือกหนึ่งคือการสร้างเกล็ดเลือดในหลอดทดลองที่ไม่กระตุ้นการสร้างภูมิคุ้มกันต่อเอชแอลเอ ซึ่งเป็นสาเหตุหลักทางภูมิคุ้มกันที่ทำให้การได้รับบริจาคเกล็ดเลือดไม่เป็นผล การศึกษานี้เราทำลายยีนเบต้าทูไมโครโกลบูลินในเซลล์ต้นกำเนิดจากการเหนี่ยวนำโดยใช้เทคนิค paired CRISPR/Cas9 nickases แล้วเปลี่ยนไปเป็นเซลล์ต้นกำเนิดของเลือด เมกาคาริโอไซต์ และเกล็ดเลือดตามลำดับด้วยวิธีการสร้างถุงเม็ดเลือด ผลการศึกษาพบว่าเซลล์ต้นกำเนิดจากการเหนี่ยวนำ, เซลล์ต้นกำเนิดของเลือด, เมกาคาริโอไซต์ และเกล็ดเลือดที่เปลี่ยนมาจากเซลล์ต้นกำเนิดจากการเหนี่ยวนำที่ถูกทำลายยีนเบต้าทูไมโครโกลบูลินไม่แสดงออกเอชแอลเอบนผิวเซลล์ นอกจากนี้เมกาคาริโอไซต์ที่เปลี่ยนมาจากเซลล์ต้นกำเนิดจากการเหนี่ยวนำทุกแบบมีลักษณะคล้ายคลึงกับเมกาคาริโอไซต์ปกติ คือขนาดใหญ่ มีนิวเคลียสหลายพู มีจำนวนดีเอ็นเอมากกว่า4ชุด สามารถสร้างขายื่นเพื่อปล่อยเกล็ดเลือดออกมาได้ และสามารถทำงานได้แต่น้อยกว่าเกล็ดเลือดจากเลือดเมื่อถูกกระตุ้นในหลอดทดลอง การศึกษาของเราสนับสนุนว่าการสร้างเกล็ดเลือดที่ไม่แสดงเอชแอลเอบนผิวเซลล์จากเซลล์ต้นกำเนิดจากการเหนี่ยวนำ สามารถทำได้ ในอนาคตการขยายขนาดการผลิตและทดสอบความปลอดภัยของเกล็ดเลือดเป็นสิ่งจำเป็นเพื่อเป็นอีกทางเลือกหนึ่งในการจัดการกับปัญหาการดื้อต่อเกล็ดเลือดและนำไปประยุกต์ใช้กับการรักษาอื่นๆ โดยมีเกล็ดเลือดเป็นพื้นฐานซึ่งใช้ได้กับผู้ป่วยที่มีเอชแอลเอทุกรูปแบบ

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5574910130 : MAJOR MEDICAL SCIENCE
KEYWORDS: PLATELETS, BETA2-MICROGLOBULIN, HLA CLASS I, IPSCS, PAIRED CRISPR/CAS9 NICKASES, KNOCK OUT, ALLOIMMUNITY, PLATELET REFRACTORINESS

PHATCHARA NORBNOP: GENERATION AND CHARACTERIZATION OF HLA-UNIVERSAL, iPSC-DERIVED PLATELETS. ADVISOR: PROF. VORASUK SHOTELERSUK, M.D., CO-ADVISOR: PROF. DR. KANYA SUPHAPEETIPORN, M.D., Ph.D., 88 pp.

Platelet demand has been increased around the world resulting from many factors including the rise of aging population, hematological malignancy incidence, and hematopoietic stem cell transplantation. In addition, inadequacy of donors or matched donors, time consuming, cost, the risk of transfusion-transmitted infections and transfusion-associated reactions, and platelet transfusion refractoriness are limits of this approach. To solve these problems, here we propose to generate platelets *in vitro* which do not induce alloimmunity to HLA class I, a major cause of immune factors in platelet transfusion refractoriness. In this study, we have knocked out β2-microglobulin gene (*β2m*) in iPSCs using paired CRISPR/Cas9 nickases then differentiated to hematopoietic stem cells, megakaryocytes (MKs) and platelets via ES-sac method. Silencing of HLA class I expression on cell surface of *β2m*-knocked out iPSCs, iPSC-derived HSCs, MKs and platelets were observed. Moreover, all lines of iPSC-derived MKs showed large size with multilobed nucleus, polyploidy with higher than 4n of DNA contents, proplatelet formation and they gave rise to functional platelets with lower activity when compared with peripheral blood-derived platelets using platelet activation and platelet aggregation assay based on flow cytometry. In summary, we generated *in vitro* functional iPSC-derived platelets with HLA class I deficiency by knocking out *β2m* gene using paired CRISPR/Cas9nickases. The genome editing system which is highly efficient on target and barely detected off-target, can be applied for regenerative medicine field. Further studies to produce large-scale iPSC-derived platelets and tests about safety profile are needed before the practical use of this approach to prevent or treat the patients of all HLA class I type from risk of life-threatening hemorrhage.

Field of Study: Medical Science
Academic Year: 2017

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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CHAPTER I
INTRODUCTION

Background and Rationale

Platelets were described as a new morphological element in the blood by Giulio Bizzozero in 1882. They are anucleated cells and have important roles in thrombosis and haemostasis [1, 2]. Platelets also are an important component in vascular integrity maintenance; when blood vessel is damaged or injured, platelets interact with several molecules and secrete cytokines to form pack of haemostatic plug tightly for arresting blood leakage [2]. To date, thrombocytopenic bleeding and platelet dysfunction syndromes in patients with major surgical bleeding, hematological or oncological disorders can be prevented or treated commonly by using platelet transfusions [3-6]. However, the risk of life-threatening hemorrhage can be seen in patients with inadequate response to platelet transfusion or platelet refractoriness. It is defined as a less than expected increment of post transfusion platelet count which is calculated based on formulae [7]. In addition, platelet refractoriness also has other disadvantages such as higher costs of inpatient[8], longer hospital stays[8], decreased survival[9], more bleeding and fatal bleeding[10].

The etiology of platelet refractoriness is divided into two main causes. The first are non-immune factors, accounting for more than 80 percents such as fever, sepsis, drugs, splenomegaly, disseminated intravascular coagulation, etc. The second are immune factors, accounting for less than 20 percents involving alloimmunization against human leukocyte antigen (HLA) class I, human platelet antigens (HPA) and ABO antigens [7, 11]. Alloimmunization to HPA caused by generating antibodies against human platelet alloantigens which consist of 33 types to date [12]. HPAs come from single nucleotide polymorphisms in genes encoding for glycoproteins which are important in platelet function [12]. However, alloimmunization to HPA antigens are less common than to the account for most percentage of platelet transfusion refractoriness, HLA class I antigens [7]. The cause of primary immunization against HLA class I antigens is contamination of leucocytes in platelet product [13]. Moreover, platelets present HLA class I antigens itself [14] and absorb soluble HLA class I antigens from plasma [15]. Although HLA-matched

platelet and platelet cross-matching are effective strategies to improve platelet count increments in the patients with HLA alloimmunization, but only two-thirds of the patients can be improved. Moreover, these strategies require financial and significant organizational resources including time-consuming [16, 17].

Since it is difficult, costly, and frequently ineffective to treat HLA class I alloimmune refractory thrombocytopenia, prevention of alloimmunization is recommended. In addition, platelet transfusion can result in viral infections, despite using modern nucleic acid-based screening for viral infection. Therefore, it would be interesting to have *in vitro* platelet units devoid of immune factor inducing alloimmunization. HLA alloimmunization can be avoided by eliminating HLA class I antigens on platelet cell surface. This can be achieved by disruption of one of the three molecules: transporter associated with antigen presentation (TAP) protein [18], HLA-A heavy chain [19], and beta2-microglobulin gene [20, 21]. Since TAP protein is in heterodimer form composed of TAP1 and TAP2 molecules [22] and HLA Class I heavy chain are polymorphic including HLA-A, HLA-B and HLA-C [23], it is difficult to select an appropriate target. However, beta2-microglobulin is a light chain with no polymorphism and necessary to HLA class I assembly and presentation on cell membrane [24]. Therefore, disruption of beta2-microglobulin gene is desirable to generate HLA-deficient platelets.

To date, large-scale *in vitro* production of platelets which are derived from human induced pluripotent stem cells (iPSCs) is available [25]. Human iPSCs can be generated from adult human somatic cells and are similar to human embryonic stem (ES) cells in properties [26]. Since the outstanding properties of iPSCs are self-renewal, proliferation, differentiation, ability *in vitro* culture, and include no ethical concern[27], iPSCs are appropriate cells for generating HLA-deficient platelets.

Targeted genome editing technologies have been developed for research and medical applications. RNA-guided CRISPR/Cas9 nickase has been improved from RNA-guided CRISPR/Cas9 for increasing genome editing specificity and it is also efficient [28]. CRISPR/Cas9 nickase can cleave only one strand of DNA because of mutation at one cleavage domain. DNA double-strand break at the specific site of genomic locus is induced by two Cas9 nickases guided by two gRNA to target each strand of DNA. The DNA double-strand break can be repaired by nonhomologous end-joining and homology-directed repair

[28, 29]. Nonhomologous end-joining can introduce insertion/deletion mutations (indels) in variable lengths at the DNA double-strand break site which can lead to knockout of gene function via frameshift mutations [30].

According to National blood centre Thai Red Cross society, about 200,000 platelet units per year were produced from blood donation, and more than 300 patients per year who frequently receive platelet products encounter with platelet alloimmune. Moreover, the problem of availability of matched donors frequently be seen and the cost of single donor platelet is about 6,000 baths per unit. Nowadays, more than 4.5 million platelet units per year have been transfused to patients with thrombocytopenia around the world. In addition, there are many problems of platelet transfusion such as donor inadequate, transfusion-transmitted infections, transfusion-associated reactions and platelet transfusion refractoriness, etc. To solve these problems there are many solutions, and we suggest a solution that is generation of platelets in a laboratory (*in vitro*) which do not induce alloimmunity (universal platelets).

Here, we propose to generate HLA-universal platelets derived from iPSCs of which two alleles of beta2-microglobulin gene are knocked out by paired CRISPR/Cas9 nickases. Moreover, iPSCs must come from person who has blood group O for preventing ABO incompatibility. The generated HLA-universal platelets can be used for people who need or patients who are in risk of life-threatening hemorrhage from platelet refractoriness. We hope the platelets will be useful in our home "Thailand" and worldwide alike.

Research questions

1. Can the paired CRISPR/Cas9 nickases be used to generate HLA-deficient platelets derived from iPSCs?
2. Can the HLA-deficient platelets derived from iPSCs by paired CRISPR/Cas9 nickases function normally *in vitro*?

Objectives

1. To generate HLA-universal, iPSC-derived platelets by CRISPR/Cas9 nickase.
2. To test platelet functions *in vitro*.

Hypotheses

1. The paired CRISPR/Cas9 nickases can be used to generate HLA-deficient platelets derived from iPSCs.
2. The HLA-deficient platelets derived from iPSCs by paired CRISPR/Cas9 nickases can function normally *in vitro.*

Research Design

In vitro studies

Key words

Platelets, beta2-microglobulin, HLA class I, iPSCs, paired CRISPR/Cas9 nickases, knock out, alloimmunity, platelet refractoriness

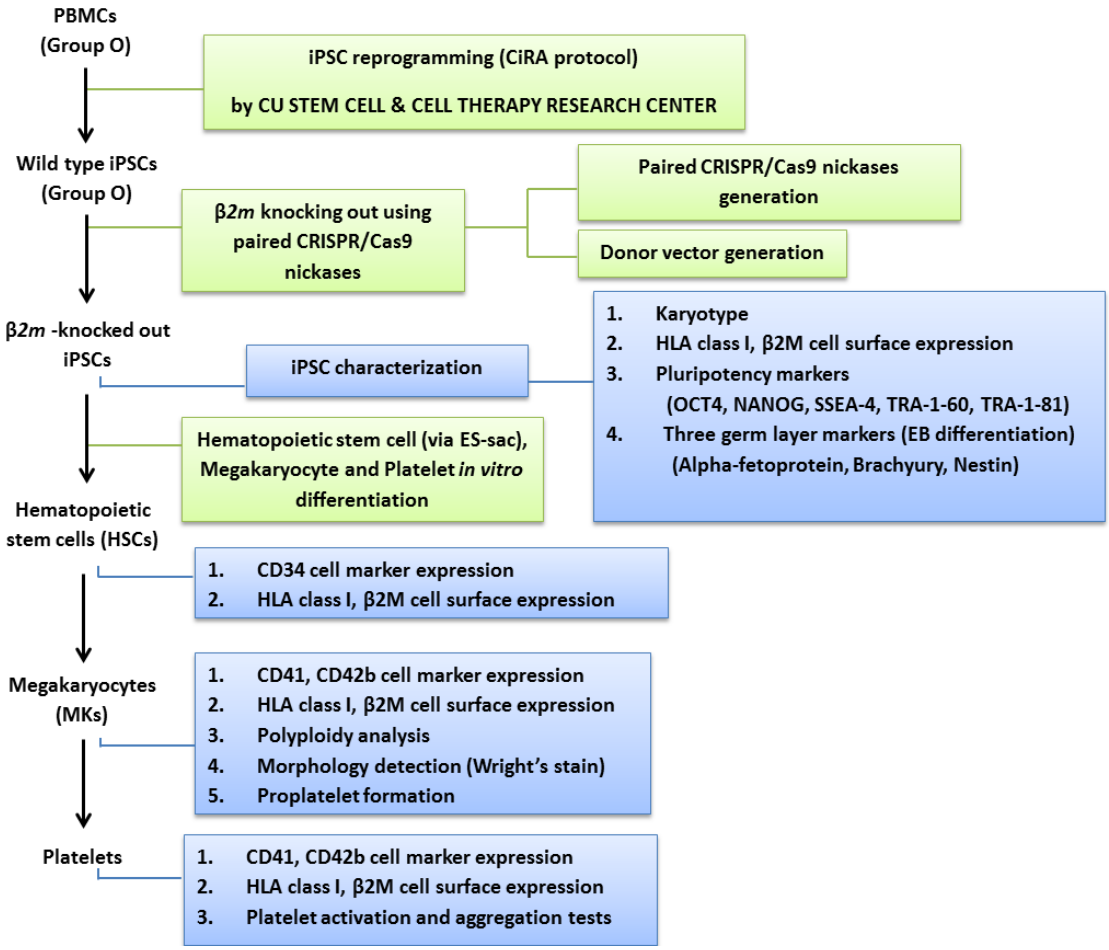
Ethical consideration

Collection of peripheral blood from a healthy volunteer with blood group O for iPSC generation and from a healthy volunteer with any blood group for platelet characterization and functional test was approved by the ethics committee of Institutional Review Board (IRB), Faculty of Medicine, Chulalongkorn University.

Expected benefit

To provide the knowledge about generation and characterization of HLA-universal, iPSC-derived platelets for further study about scaling up HLA-universal, iPSC-derived platelets to test safety profile before preventing or treating the patients of all HLA class I type from risk of life-threatening hemorrhage.

Conceptual Framework



CHAPTER II
REVIEW OF RELATED LITERATURE

Platelet functions in hemostasis

Hemostasis is important in case of bleeding especially in the one who has bleeding disorder. As platelet or thrombocyte is initiator for blood clotting, it is such a main element in this process. The platelet is derived from megakaryocyte in bone marrow and is a circulating anucleate disc shaped cell with 0.5 microns in depth and 3-5 microns in diameter. When blood vessel is injured, the connective tissue matrix such as collagen substratum and other extracellular matrix proteins that lie under vascular endothelial cells will be exposed. Then platelets are activated by interacting with collagen fibrils which serve as a surface for platelet adhesion and strong stimulus. The activated platelets will change their shape, spreading along the collagen and secrete platelet activation mediators such as thromboxane A2 and ADP to stimulate neighboring platelets to be activated. The activated platelets also bind direct to the circulating coagulation protein fibrinogen by GPIIb/IIIa receptors on the platelets. The connection between platelet-fibrinogen-platelet results in platelet aggregation which becomes very large aggregates because of many copies (40,000-80,000 copies) of GPIIb/IIIa on its surface. Moreover, tissue factor which is exposed to the circulating blood due to the protective endothelial layer is disrupted, interact with factor VIIa and finally generate thrombin. The thrombin generation is accelerated by platelets that providing procoagulant phospholipids. The thrombin then activates coagulation cascade and ultimately generates a fibrin deposition which resulting in the packed hemostatic plug that arrests blood loss (Figure 1). After the clot stops bleeding, antithrombotic control mechanisms for clotting termination and fibrinolysis for clotting removal will follow, eventually tissue remodeling. [2, 31, 32]

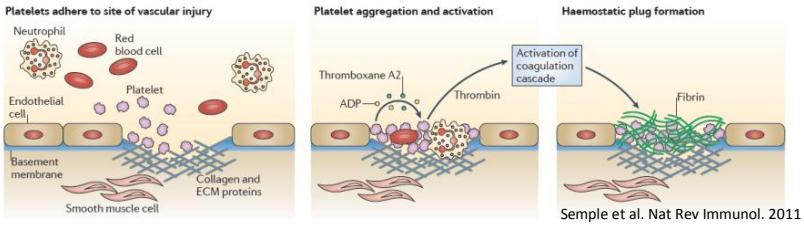


Figure 1 Platelet functions in hemostasis. Three main steps which are platelets adhere to site of vascular injury, platelet activation and aggregation, and hemostatic plug formation.[2]

Platelet activation and aggregation [33] [34]

The initiation of coagulation cascades is primarily involved platelet activity; platelet adhesion, platelet activation, and platelet aggregation contributing to thrombus stabilization (Figure 2). In these steps, there are many mechanisms involved so this review showed brief and some detail that may be useful for the study. The first step, platelet adhesion to extracellular matrix, with high shear in blood circulation, von Willebrand factor (vWF) forms as a bridge between platelet glycoprotein Ib-IX-V (GPIb-IX-V) receptor complex and exposed collagen which binds to platelet GPVI and GPIa/IIa receptors directly as well.

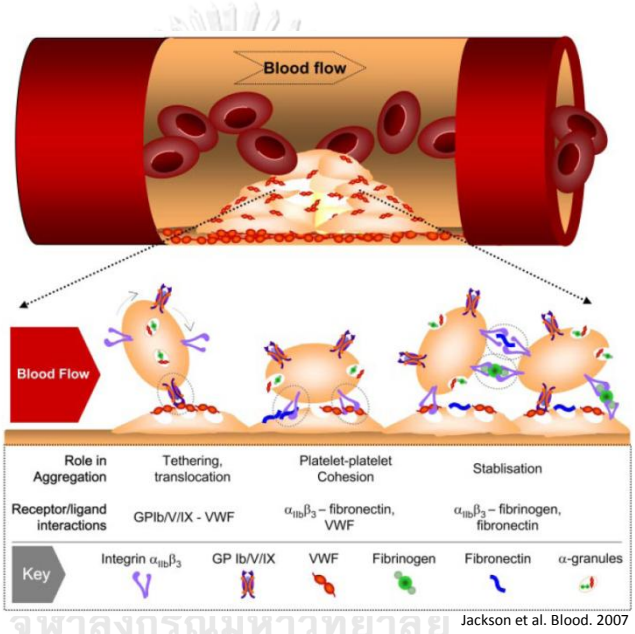
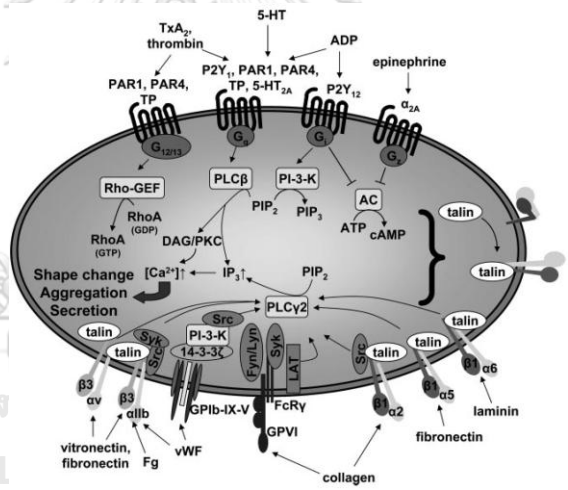


Figure 2 Adhesion receptor-ligand interactions during platelet aggregation under rapid blood flow condition. GPIb and vWF interaction occurs between platelets and surface of immobilized platelets in initial tethering which this adhesive interaction causes platelet translocation because of rapidly reversible and unstable adhesion at the shear rates. During the translocation, platelets stimulated by soluble agonists promote activated integrin $\alpha_{IIb}\beta_3$ (or GPIIbIIIa) bind with vWF and fibronectin resulting in platelet-platelet adhesion sustainable. Finally, the stabilization, aggregation is emerging by the main role of integrin $\alpha_{IIb}\beta_3$ and fibrinogen interaction. [35]

Platelets are activated via signal pathway transduction mediated by platelet membrane glycoproteins, GPIb-IX-V, GPVI, and CLEC-2 (C-type lectin like receptor 2). There are many signaling mechanisms from platelet receptors to activation of integrin for

example in Figure 3. Interaction between GPVI and exposed collagen results in strong platelet activation and platelets release content of dense and alpha-granules. The activated cells mostly release soluble agonists such as ADP, thrombin, thromboxane A2 which induce platelet activation via GPCR increasing concentration of cytosolic calcium and specific signaling pathway activation, activate further platelets until plug formation. The most strong platelet agonist is thrombin which responsible in fibrinogen to fibrin conversion then platelet plugs stabilization. Besides, epinephrine, prostaglanding E2, and serotonin are agonists for platelet activation. Platelet aggregation is caused following platelet activation which up regulates functional integrin adhesion receptors. Activation of GPIIb/IIIa receptors is the most important in platelet aggregation because a number of them expressed on platelets and cross link fibrinogens or vWF between receptors. Then additional platelets will further be recruited to the vascular injury site and thrombus formation subsequently.



Varga-Szabo et al. Arterioscler Thromb Vasc Biol. 2008

Figure 3 Platelet receptors to integrin activation, the linking signaling mechanisms. Platelet stimulation by agonists through platelet receptors or integrin induces platelet function by shape change, secretion, or aggregation. Stimulation of platelet receptor triggers signaling mechanisms within the cells for example, ITAM-signaling pathway activated by GPVI ligation, while G protein-coupled receptors stimulation triggers pathways relevant to adenylyl cyclase and G protein, etc. [34]

In addition of glycoprotein on platelets, platelets also contain at least three granules, dense granules, alpha-granules, and lysosomes, these granules store many mediators including adhesion molecules, coagulation and angiogenic factors, chemokines, and cytokines. Dense granules store active non protein molecules including ADP, ATP,

calcium, serotonin and histamine. Lysosomes contain bactericidal enzymes including glycosidases, cationic proteins, and acid proteases. The most abundant granules are alpha-granules which store mediators important in hemostasis and innate immunity, adhesive glycoproteins including P-selectin (CD62P), vWF, fibrinogen, and GPIIb/IIIa. During platelet activation, P-selectin expressed with large amount from alpha-granules to cell surface, it primarily mediates binding between platelets and other cells such as leukocytes and endothelial cells.

In present, flow cytometry is used to detect two common platelet activation markers, activated GPIIb/IIIa and platelet surface P-selectin staining by the specific antibodies, PAC-1 and CD62P. After platelet stimulated with agonists, inactive GPIIb/IIIa complex will be conformational changed and P-selectin will be exposed on platelet. By this phenomenon, PAC-1 antibody and CD62P antibody can be used to detect this activated GPIIb/IIIa form and the exposed P-selectin [36].

Platelet transfusion and refractoriness

Although the platelets are required for normal hemostatic activity, their lifespan is 10 days and every day approximately 10% of them are removed via the liver and the spleen mainly. To compensate for supporting vascular integrity, about 7.1×10^9 platelets per litter are produced [37] which resulting in normal circulating level in the blood vessel at $150 - 450 \times 10^9$ platelets per litter [7]. In contrast, thrombocytopenia is a condition that platelet count is less than normal and results from ineffective platelet production by bone marrow, accelerated platelet destruction, and platelet splenic pool. Although in ideal, patients with thrombocytopenia should be treated in the etiology, the patients should be treated immediately for a rapid onset response especially in the bleeding thrombocytopenic patients. Therefore, platelet transfusion is one option in emergency situations but it is not usually long-term therapy in thrombocytopenic patients with the real etiologies such as heparin induced thrombocytopenia, ITP (immune thrombocytopenia) and TTP (thrombotic thrombocytopenia purpura) [38].

The history of platelet transfusion comes from the purpose to increase platelet count which start in 1910 that whole blood was used. Then, by the 1950s, donated whole blood was modified by advanced technology to prepare platelet concentrates [39]. In 1959, platelet transfusions first be used and showed that survival rate increase 30 percent

in leukemic patients who usually dye from hemorrhage [40]. Moreover, to date, thrombocytopenic bleeding and platelet dysfunction syndromes in patients with major surgical bleeding, hematological or oncological disorders can be prevented or treated commonly by using platelet transfusions [3-6]. However, the risk of life-threatening hemorrhage can be seen in patients with inadequate response to platelet transfusion or platelet refractoriness. It is defined as a less than expected increment of post transfusion platelet count which is calculated based on formulae [7]. The platelet refractoriness causes higher costs of inpatient [8], longer hospital stays [8], decreased survival [9], more bleeding and fatal bleeding [10].

The etiology of platelet refractoriness

The etiology of platelet refractoriness is divided into two main causes. The first are non-immune factors, accounting for more than 80 percent such as fever, sepsis, drugs, splenomegaly, and disseminated intravascular coagulation, etc. The second are immune factors, accounting for less than 20 percent involving alloimmunization against human leukocyte antigen (HLA) class I, human platelet antigens (HPA) and ABO [7, 11]. For the immune factors, the most common is alloimmune to HLA class I with 80% - 90%, followed by alloimmune to HPA (10% - 20%) and alloimmune to both HLA class I and HPA (5%). ABO mismatch and platelet storage for more than two days reduce the ability of post transfusion platelet recovery [41].

Alloimmunization

Platelet refractoriness that comes from alloantibodies is a main clinical problem especially in chronic thrombocytopenic patients who require blood product transfusion frequently. Alloimmunity is commonly known in the definition that immune response to foreign antigens from members of the same species or alloantigens. Pregnancy, transfusion, or transplantation is involved frequently to the alloimmunization. In addition, platelet reactive antibodies were found in sera of 113 patients from 252 hematology and oncology recipients which anti-HLA specificity was found in sera of 108 patients and anti-HPA specificity was found in 20 patients. Therefore, it supports that antibodies to the HLA are main cause of platelet refractoriness [42]. Although, among hematological patients who receiving chronic platelet transfusion, 20% - 60% of them encounter with

alloimmunization, significant proportion of patients with repeated platelet transfusion do not be alloimmunized [43-45]

The transfused donor platelets are reacted with antibodies to these antigens and can result in survival and *in vivo* functional decrease of the platelets[43]. Moreover, complex immune cellular pathways which regulate recipient B and T cell alloreactivity, antigen processing, NK-cell mediated allorecognition, and IgG anti-platelet immunity have been provided by some studies in trying to explain their mechanisms [46-48].

Alloimmunization to human platelet antigen (HPA)

The pathophysiology of platelet antigen alloimmunization is not clear, even though its detail of phenomenon have been described by clinical studies. [49]

Alloimmunization to human platelet antigen comes from generating antibodies to human platelet alloantigens which consist of 33 types to date. Platelet glycoproteins (GPs) express several types of HPAs which is the genetically polymorphic structures (Figure 4).

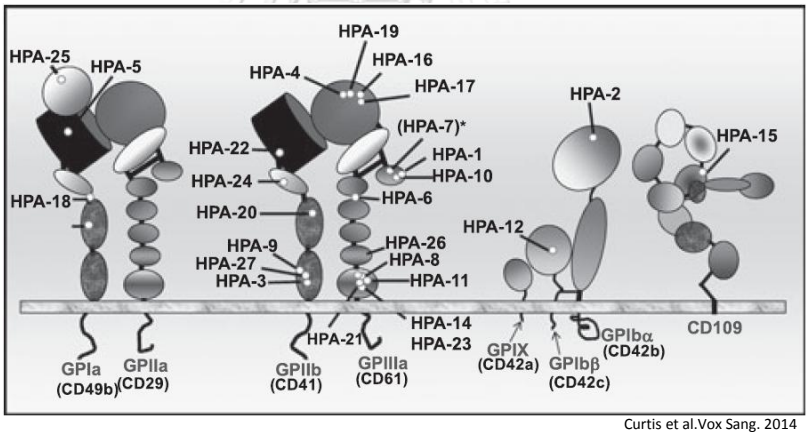
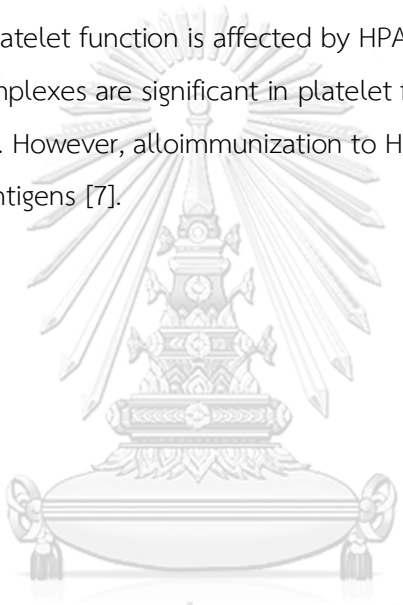


Figure 4 Platelet glycoprotein (GP) structures and human platelet alloantigens (HPAs): GPIa/IIa, GPIIb/IIIa, GPIb/IX and CD 109 with the predicted locations of expressed HPA. [12]

HPAs result from single nucleotide polymorphisms leading to amino acid changes which induce glycoprotein structural changes to form antigens (Table 1). In various populations, the prevalence of HPA polymorphisms is different significantly. Platelet specific antibodies are not commonly frequent with the incident varies from 2% to 11% and they are not associated with reduction in corrected count increment (CCI) statistical

significantly [7]. Moreover, the cases with reported platelet specific antibodies causing transfusion refractoriness are commonly confused by coexisting anti HLA antibodies [29]. Other interesting points are 50% of platelet specific antibodies are autoantibodies and during infected period, 70% of platelet specific antibodies are developed and commonly transient [50]. In addition, transfusion recipients and pregnant woman may develop the alloimmune platelet disorders such as FNIT (fetal and neonatal alloimmune thrombocytopenia), PTP (posttransfusion purpura), and MPR (multitransfusion platelet refractoriness) when they are sensitized. Although some HPAs generally involved in the disorders, others seem unusually involved and to be less immunogenic. There are little evidences suggest that platelet function is affected by HPA alloantibodies, even though all platelet glycoprotein complexes are significant in platelet function which are hemostasis and/or inflammation [12]. However, alloimmunization to HPA antigens are less common than to the HLA class I antigens [7].



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Biallelic HPA

Antigens	Allele frequency[a] Caucasian (%)	African (%)	Asian (%)	Glycoprotein / Amino acid change	Encoding gene / Nucleotide change	Immune platelet disorder reports
HPA-1a	72 a/a	90	100	GPIIIa / L33P	*ITGB3* / T196C	FNAIT, PTP, MPR
HPA-1b	26 a/b	10	0			
	2 b/b					
HPA-2a	85 a/a	71	95	GPIbα / T145M	*GPIBA* / C524T	FNAIT, PTP, MPR
HPA-2b	14 a/b	29	5			
	1 b/b					
HPA-3a	37 a/a	68	59.5	GPIIb / I843S	*ITGA2B* / T2621G	FNAIT, PTP, MPR
HPA-3b	48 a/b	32	40.5			
	15 b/b					
HPA-4a	>99.9 a/a	100	99.5	GPIIIa / R143Q	*ITGB3* / G526A	FNAIT, PTP, MPR
HPA-4b	< 0.1 a/b	0	0.5			
	< 0.1 a/b					
HPA-5a	88 a/a	82	98.6	GPIa / E505K	*ITGA2* / G1648A	FNAIT, PTP, MPR
HPA-5b	20 a/b	1 8	0.4			
	1 b/b					
HPA-15a	35 a/a	65	53	CD109 / Y703S	*CD109* / A2108C	FNAIT, PTP, MPR
HPA-15b	42 a/b	35	47			
	23 b/b					

Low frequency HPA:

Antigens	Phenotypic frequency[a] Caucasian (%)	Glycoprotein / Amino acid change	Encoding gene / Nucleotide change	Immune platelet disorder reports	REF
GPIIb					
HPA-9bw	<1 b/b	GPIIb / V837M	*ITGA2B* / A2603G	FNAIT	
HPA-20bw	<1 b/b	GPIIb / T619M	*ITGA2B* / C1949T	FNAIT	
HPA-22bw	<1 b/b	GPIIb / K164T	*ITGA2B* / A585C	FNAIT	
HPA-24bw	<1 b/b	GPIIb / S472N	*ITGA2B* / G1508A	FNAIT	
HPA-27bw	<1 b/b	GPIIb / L841M	*ITGA2B* / C2614A	FNAIT	
GPIIIa					
HPA-6bw	<1 b/b	GPIIIa / R489Q	*ITGB3* / A1564G	FNAIT	
HPA-7bw	<1 b/b	GPIIIa / P407A	*ITGB3* / G1317C	FNAIT	
HPA-8bw	<1 b/b	GPIIIa / R636C	*ITGB3* / T2004C	FNAIT	
HPA-10bw	<1 b/b	GPIIIa / R62Q	*ITGB3* / A281G	FNAIT	
HPA-11bw	<1 b/b	GPIIIa / R633H	*ITGB3* / A1996G	FNAIT	
HPA-14bw	<1 b/b	GPIIIa / K611del	*ITGB3* / AAG1929-31	FNAIT	
HPA-16bw	<1 b/b	GPIIIa / T140I	*ITGB3* / C517T	FNAIT	
HPA-17bw	<1 b/b	GPIIIa / T195M	*ITGB3* / C622T	FNAIT	
HPA-19bw	<1 b/b	GPIIIa / K137Q	*ITGB3* / A487C	FNAIT	
HPA-21bw	<1 b/b	GPIIIa / E628K	*ITGB3* / G1960A	FNAIT	
HPA-23bw	<1 b/b	GPIIIa/ R622W	*ITGB3* / C1942T	FNAIT	
HPA-26bw	<1 b/b	GPIIIa / K580N	*ITGB3* / G1818T	FNAIT	
GPIb					
HPA-12bw	<1 b/b	GPIbβ / G15E	*GPIBB* / A141G	FNAIT	
GPIa					
HPA-13bw	<1 b/b	GPIa / M799T	*ITGA2* / T2531C	FNAIT	
HPA-18bw	<1 b/b	GPIa / Q716H	*ITGA2* / G2235T	FNAIT	
HPA-25bw	<1 b/b	GPIa / T187M	*ITGA2* / C3347T	FNAIT	

[a]Phenotypic frequencies are Caucasian (North America); African (Benin); Asian (China). HPA frequencies in other races and ethnic groups can be found at: http://www.ebi.ac.uk/ipd/hpa/freqs_1.html.

FNAIT, neonatal alloimmune thrombocytopenia; PTP, post-transfusion purpura; MPR, multi-platelet transfusion refractoriness

Table 1 Human platelet alloantigens: expressed HPAs on six platelet glycoproteins which are GPIIb, GPIIIa, GPIbα, GPIbβ, GPIa, and CD109 have been described. 12 antigens are grouped into 6 biallelic groups which are HPA-1, HPA-2, HPA-3, HPA-4, HPA-5, and HPA-15. The higher frequency antigen indicated 'a' and the 'b' for lower frequency antigen. The frequencies have been defined for different racial groups. 'w' indicates detected only one of the two antigens.

Alloimmunization to human leukocyte antigen (HLA)

Patients who receive previous blood product transfusion with contaminating white blood cells or fetal white blood cells in the case of previous pregnancies will develop HLA antibodies after HLA alloantigen exposure. In addition, the risk of HLA alloimmunization is increased by ABO-mismatched platelet transfusion [51].

The cause of primary immunization against HLA class I antigens is contamination of leucocytes in platelet product [13]. It is important to prevent alloimmunization because of difficultly, cost and often ineffective treatment of alloimmune refractory thrombocytopenia. The transfusion of leukocyte reduced blood components was showed to decrease the rate of alloimmunization to HLA antigens and it is an effective way [52]. Moreover, according to TRAP (Trial to Reduce Alloimmunization to Platelets) study, alloimmunize rate between leucoreduced pooled random-donor platelet concentrates and luecoreduced single-donor such as apheresis is not different and the alloimmunize incidence dropped from 45% to 17-21% [52]. For more examples, the incidence of alloimmunization of AML patients receiving both chemotherapy and leucoreduction dropped from 48% to 16%[53], from 50% to 15%[54]. Reduction of the ability to form antibodies of patients who receiving immunosuppressive treatment seems exists[55]. Although there is the relationship between HLA class I alloantibodies and the platelet refractoriness, more than 50% of patients who develop HLA antibodies did not show platelet transfusion refractoriness[56, 57] and only 30% of the alloimmunized patients develop platelet transfusion refractoriness[58]. It is also no dose response relationship between the incidence of alloimmunization and the number of transfused platelets[59]. Therefore, it is such complicated processes between both recipient and donor or product factors which one will be alloimmunized and alloimmunization will result in transfusion refractoriness.

Major histocompatibility complex (MHC) or Human leukocyte antigen (HLA) [60]

MHC molecule in each species have distinct names, HLA (human leukocyte antigen) is the name for human. MHC class I locus in human consists of HLA-A, HLA-B, and HLA-C. While MHC class II locus consists of HLA-DP, HLA-DQ, and HLA-DR. For type of MHC molecule expression is co-dominantly expression which expressing both 2 alleles. For examples, MHC I genes, in one person have 2 alleles: HLA-A1, HLA-B1, HLA-C1 and HLA-A2,

HLA-B2, HLA-C2, so, this person will have co-dominantly expression in pattern HLA-A1, HLA-A2, HLA-B1, HLA-B2, HLA-C1 and HLA-C2. Moreover MHC is polymorphic which in each HLA-A, B, C or HLA-DP, DQ, DR has many types such as HLA-A has 303 different types, HLA-B has 559 different types. The important function of MHC in immune response is to presenting peptides to T lymphocyte in lymphocyte antigen recognition phase which requires self-MHC restriction (Figure 5). MHC can be divided in to two types which are MHC class I and MHC class II by their structures.

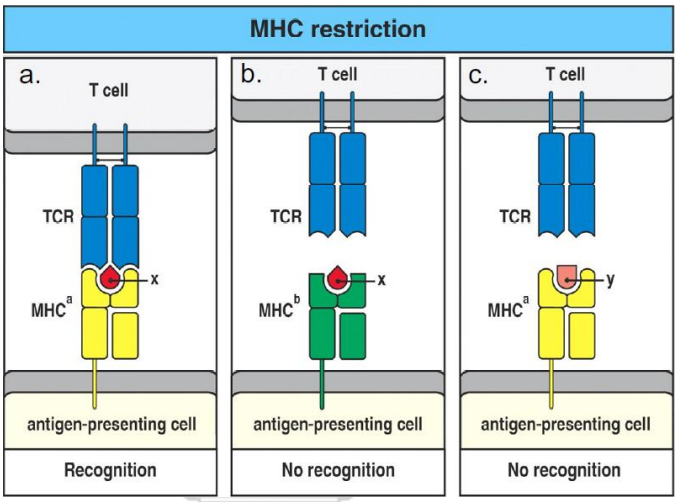

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Figure 5 Dual recognition of T lymphocyte. T lymphocytes recognize antigen by T cell receptor which required recognition in both peptide and self-MHC molecule. a): T lymphocyte recognizes antigen because antigen is digested to be peptide that specific to T cell receptor and the specific peptide is presented by self-MHC molecule. b): no recognition of T lymphocyte because MHC molecule is not self-MHC molecule, even though it is the same peptide in a). b): no recognition because of not specific peptide.

MHC class I or HLA class I

MHC class I is presented on nucleated cells. In each nucleated cell has different amount of MHC class I. Its important immune role is to presenting peptide to CD8+ T lymphocyte which is cytotoxic T cell. The peptides generally come from endogenous proteins including intracellular pathogen products from infection and tumor proteins form tumor cells. MHC class I structure consists of α chain and β2-microglobulin chain which

peptide binding cleft is generated by α1 and α2 domains with close-ended (Figure 6). Then peptide with 8-10 amino acids can bind it.

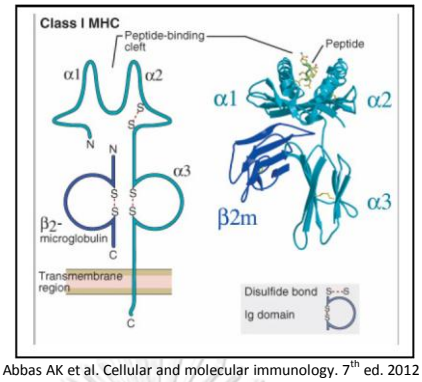

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Figure 6 Structure of MHC class I or HLA class I. It consists of α chain and β2-microglobulin chain which peptide binding cleft is generated by α1 and α2 domains.

Antigen processing and presentation: Endogenous antigen pathway or cytosolic pathway or MHC I pathway (Figure 7)

Protein generation and destruction within cells are continuously happen including viral protein and cancer protein which are generated in cytoplasm. Proteasome which is protease complex consists of cylinder subunits. The proteasome digests proteins into short peptides and their function is increased when activated by IFN-γ. The peptides are sent into endoplasmic reticulum (ER) by TAP-1 and TAP-2 (transporters associated with antigen processing 1/2) on endoplasmic reticulum membrane and their function is increased when activated by IFN-γ too. MHC I molecules which are usually generated in ER will be sent to cell membrane when binding with the peptide. In generally, MHC I molecules bind with TAP through tapasin which is protein nearly the TAP. This binding makes MHC I molecules to being in the position for the peptides. When MHC I molecules bind with the peptides, they will be released from TAP and sent to cell surface for presenting antigens to T lymphocyte. Moreover, MHC still remains on cell membrane when it binds to peptide and MHC I binds with self-peptide which comes from common destruction of protein in normal cell.

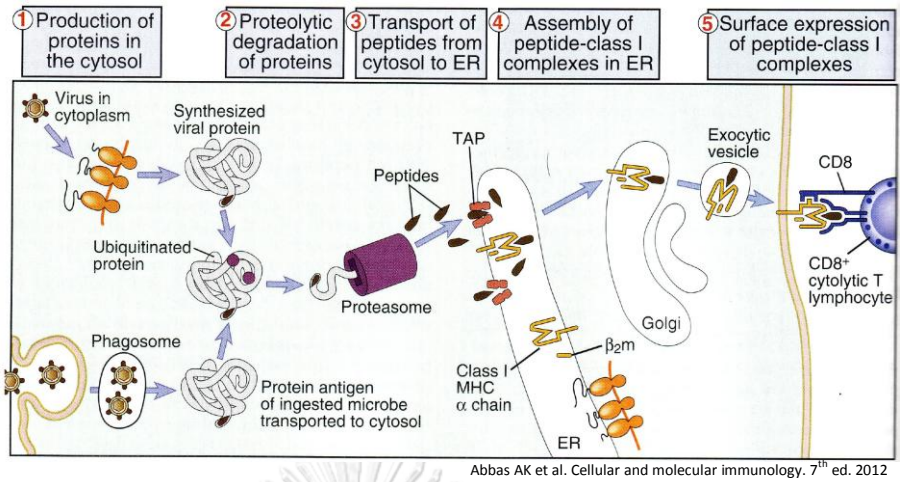

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Figure 7 Endogenous antigen pathway or cytosolic pathway or MHC I pathway. Proteins of pathogen or cancer in cytoplasm are processed by proteasome to be short peptides which are sent to endoplasmic reticulum (ER) through TAP on ER surface. The peptide that binds with MHC I is sent to cell surface for presenting to CD8 + T lymphocyte.

MHC class II or HLA class II

MHC class I is commonly presented on antigen presenting cells (macrophage, dendritic cells and B cells). Its important immune role is to presenting peptide to CD4+ T lymphocyte which is helper T cell. The peptides generally come from exogenous antigens that are phagocytosed by antigen presenting cells. MHC class II structure consists of α chain and β chain which peptide binding groove is generated by $\alpha 1$ and $\beta 1$ domains with open-ended (Figure 8). Then peptide with 13-18 amino acids can bind it.

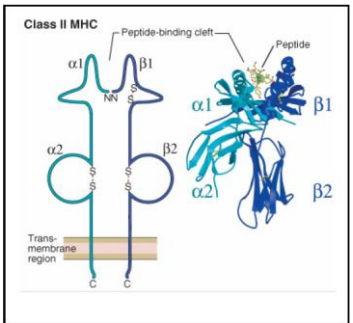

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Figure 8 Structure of MHC class II or HLA class II. It consists of α chain and β chain which peptide binding cleft is generated by $\alpha 1$ and $\beta 1$ domains.

Antigen processing and presentation: Exogenous antigen pathway or endocytic pathway or MHC II pathway (Figure 9)

The antigens come from pathogen increasing in intracellular vesicle, lysosome and extracellular organisms increasing outside cells which are phagocytosed by antigen presenting cells. The phagocytosed antigens are in phagosome or endosome which then fuse to lysosome (phagosome-lysosome fusion) and the antigen are digested by acidic protease. MHC class II molecules are generated in ER and are protected from binding with peptides which are in ER. The protection exists because MHC class II molecules must bind with the peptides that are digested in intracellular vesicle of the antigen presenting cells. The protected mechanism: MHC class II which is generated in ER binds with invariant chain called CLIP (Class II invariant chain peptide) at the same position of peptide binding then moving to endosomal compartment. Then, DM proteins remove CLIP from MHC class II in endosomal compartment and peptides can bind with MHC class II. The peptide-MHC class II complex is sent to cell surface.

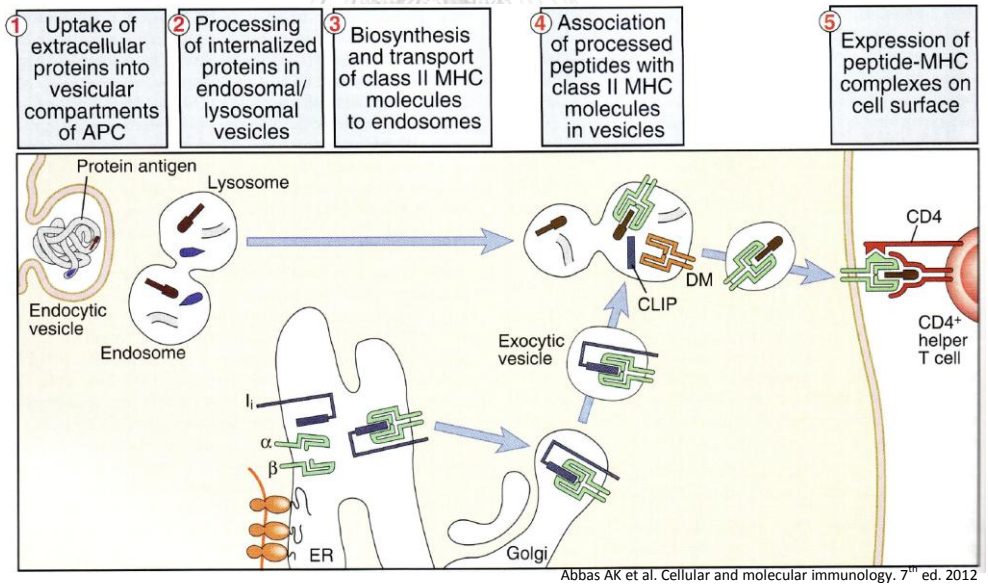


Figure 9 Exogenous antigen pathway or endocytic pathway or MHC II pathway. MHC class II is synthesized in ER which binding with invariant chain for protecting binding of peptide in ER. When they are transported to endosome, invariant chain is digested to be small fraction called CLIP. While phagocytosed antigens are digested to be short peptides within endosome or lysosome, and then bind to MHC class II in the endosome. The binding between the peptide and MHC class II occurs by helping from DM which exchanges the

peptide and the CLIP. Then, peptide-MHC II complex is sent to cell surface for presenting to CD4+ T lymphocyte.

Moreover, cross-presentation of exogenous antigens to be presented to cytotoxic T lymphocyte by MHC class I and cross-presentation of endogenous antigens (autophagosome) to be presented to helper T lymphocyte by MHC class II can happen.

Human leukocyte antigen (HLA) and platelets

Since, platelets present HLA class I antigens on cell surface which consist of HLA-A, HLA-B, and rarely of HLA-C and absorb soluble HLA class I antigens from plasma, HLA-A and HLA-B are the immune factor responsible for the refractoriness [14, 15, 43, 61]. The main antibodies direct against epitopes of the HLA class I with clinically relevant are immunoglobulin G (IgG) [49].

According to one study, platelets express less HLA molecules than peripheral blood mononuclear cells, but platelet surface has been reported that the number of HLA molecules vary in 50,000 – 100,000 which can be evaluated that platelets carry about two thirds of HLA molecules from blood [62]. Other studies suggest that the HLA molecules mainly come from endogenous origin [63] and at the megakaryocyte stage, most of them have been synthesized and loaded [64]. In addition, they also suggested that platelets loss detectable HLA surface molecules at 37°C because peptide dissociating trigger. Consistent with another study that main of HLA molecules on the platelets are composed of heavy chains with trace amounts of β2microglobulin [15] while *in vivo*, HLA molecules are commonly found on platelet surface [64].

Although there is conflict of the finding in where majority of platelet surface HLA class I molecules come from, the platelets absolutely have HLA class I molecules on their surface and involve in HLA alloimmunization and refractoriness. HLA class II is not considered to be immune factor for platelet refractoriness because they are not expressed on platelets [14, 15, 43, 61]. The real function of platelet HLA class I molecules is still unknown, but platelet transfusion refractoriness is related with production of anti-HLA class I alloantibodies.

Alloimmune pathways

Alloimmunity can be detected by monitoring cytotoxicity T lymphocytes (CTL) or IgG anti-donor antibodies development which both are effector alloimmune responses that primarily responsibility for transfused or transplanted allogeneic cells or tissues.[11] Both CTL and IgG antibody production also require T cells recognition with different pathway. CTL commonly require endogenous pathway, while IgG antibody production commonly require exogenous pathway of CD4+ T-helper cell activation called T-dependent antigen in B lymphocyte activation.

The study suggested that in vitro, HLA class I molecules from allogeneic resting or activated platelets cannot induce CD8+ cytotoxic T lymphocyte response directly. The “directly” word means direct pathway that recipient CD8+ T cells recognize MHC molecules on the donor platelet’s surface[64]. Another study showed more CD8+ T cell function in mediated immunosuppression of IgG antiplatelet immunity[7].

Direct allorecognition and indirect allorecognition[65, 66]

To date, there are two allorecognition mechanisms of recipient T cells that have been shown to initiate alloimmunity, which are in termed direct and indirect pathways. The direct pathway (Figure 10-left) is described by donor antigen presenting cells (APC) present donor peptides through MCH class II molecules to recipient CD4+ T cells with directly interact between T cell receptor (TCR) and MHC class II molecules. And then, the CD4+ T cell activation will help B lymphocytes to be plasma cells to produce anti-MHC class I antibodies. Interestingly, leukoreduced platelet concentrates removes the direct pathway. While indirect pathway (Figure 10-right) is described by recipient APCs uptake donor platelet-derived MHC class I alloantigens. And then, recipient APCs will process and present the peptides from the MHC class I to recipient CD4+ T cells through MHC class II molecules followed by B cell activation to producing anti-MHC class I antibodies by helping of the recipient CD4+ T cells.

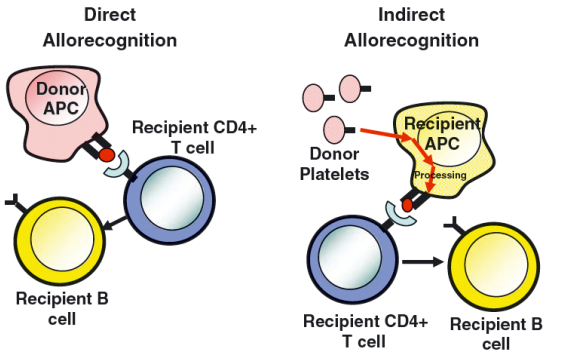


Figure 10 Direct and indirect allorecognition models lead to platelet alloimmunization. Left picture demonstrates direct allorecognition. Right picture demonstrates indirect allorecognition[11].

Moreover, indirect allorecognition involves in various transplantation rejection of cardiac, kidney and skin grafts. And two experiments using murine and rat as animal models to studying platelet immunity in 1995 showed that IgG antidonor immunity is stimulated by allogeneic platelets via the indirect allorecognition[65, 67, 68]. The indirect pathway, recipient APCs which uptake donor platelet antigens use necessary generation of nitric oxide and a noncytosolic with pH-independent processing pathway to stimulate IgG alloantibody production[69].

Human leukocyte antigen reduced platelets

For platelet refractory patients from alloimunization, there are the ways to prevent and treatment them such as leucoreduced platelet concentrates which someone still encounter with platelet refractoriness, the effective ways which are HLA-matched platelet and platelet cross-matching, and acid-treated platelet transfusions.

Although HLA-matched platelet and platelet cross-matching are effective strategies to improve platelet count increments in the patients with HLA alloimmunization, only two-thirds of the patients can be improved. Moreover, these strategies require financial and significant organizational resources including are time-consuming [16, 17]. According to other studies in treatment of the refractory patients who cannot be found their match donors, they are treated with an acid treatment of platelet products for elution of the platelet HLA class I molecules. The treatment results are varying between successful with no adverse effect to fail even in the same person with different time of several transfusions. Although there were successful treatments in some refractory

patients, during processing, the preparing platelets were loss about average 40% and some patient after one transfusion developed a severe transfusion reaction with no platelet increment, and platelet preparation procedure could be critically affects to platelet quality [70, 71]. So, the platelets without HLA class I molecules may survive and function almost normally in the circulation.

According to the concept of survival and functional platelets in circulation without HLA class I molecules has been existed, HLA-deficient platelets were generated in 2010 by using RNA interference-based method to specific target to β2microglobulin expression transducing by lentiviral-based vectors in hematopoietic progenitor cells, and were tested in anti-HLA antibody-mediated cytotoxicity which they efficiently were protected and prevent platelet refractoriness in vivo in 2013 [20, 21]. However, this method is knockdown with up to 85% reduced HLA class I protein expression and up to 95% reduced β2microglobulin mRNA levels in CD34+ progenitor cells. The progenitor cells can proliferate and differentiate to be megakaryocytes and platelets respectively, but them unable to self-renew and are limited cell source. Therefore, if there is need for large amount of the platelet products, they have to produce the CD34+ progenitor cells which are time consuming and where the progenitor cells come from in this study requires stimulation the donors to mobilize the progenitor cells into peripheral blood using recombinant granulocyte colony stimulating factor.

Moreover, HLA-regulated expression for cell-based therapies by several studies which use different kinds of cell and targeted molecules have emerged in the past decade. For example, a lentiviral-based vector system for drug inducible expression of shRNA sequences was used to target either HLA heavy-chain transcripts or β2 microglobulin in HeLa, B-lymphocyte cell lines and peripheral blood monocytes with effectively preventing antibody-mediated cell lysis and CD8+ T cell response [19]. Another study for generating the universal donor cells targeted disruption of both alleles of β2microglobulin gene in embryonic stem cells (ESCs) by using adeno-associated virus (AAV) vectors [72]. In consistent of disruption β2microglobulin gene in embryonic stem cells (ESCs), another study [24] used TALEN technology and showed that β2microglobulin gene null both alleles lead to no presentation of HLA class I molecules on cell surface. Meanwhile, both β2m-/- and β2m+/- hESCs were transplanted into tibialis anterior

muscles of the mice showing hypoimmunogenicity *in vivo*. Other example is to generate universal dendritic cell-like antigen presenting cells from human induced pluripotent stem cells which are unlimited cell source by using zinc finger nuclease (ZFN) to disrupt TAP2 gene. TAP is a heterodimer composed of TAP1 and TAP2 molecules which necessary for HLA class I cell surface expression. Although they suggested the modified cells could avoided recognition of preactivated alloreactive CD8+ T cells, lower levels of HLA class I molecules with reduced diversity of peptides were expressed by the TAP deficient cells [18].

In these studies, no HLA class I molecules on the hESC surfaces showed hypo-immunogenicity *in vivo* and same pluripotency as wildtype hESCs [24] and reduction of HLA class I molecules on platelet surfaces showed efficiently protected against HLA antibody-mediated cytotoxicity including prevented platelet refractoriness *in vivo*, and platelet surface membrane glycoproteins (GPs) including platelet function are not affected [21]. Moreover, natural killer cells which commonly activate their cytotoxicity when interact the cells without HLA class I molecules, have been shown to be relatively weak in resistance to alloengraftment. Consistent to [24] that revealed total output of immune rejection of NK cell was decreased.

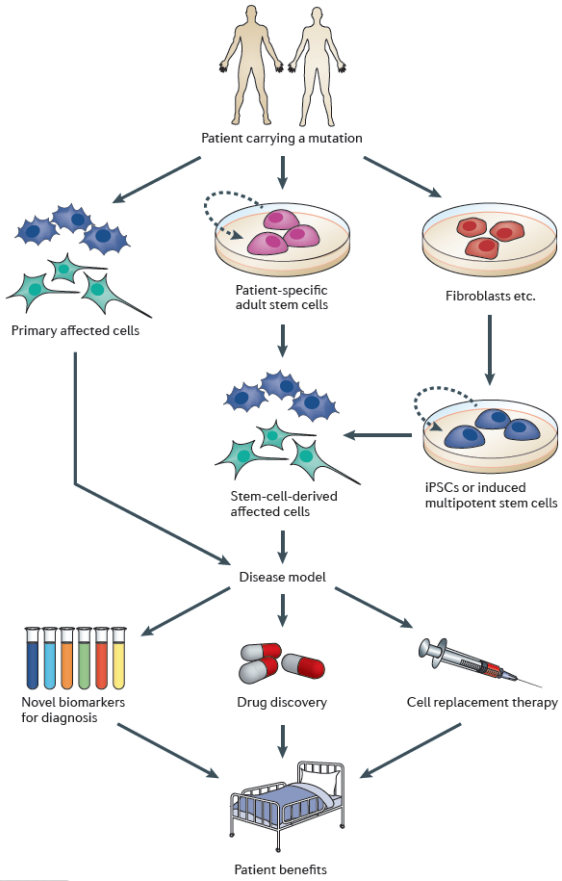
Human leukocyte antigen and platelet functions

In 1988, HLA class I molecules were studied to answer how they involved in platelet function. The researchers suggested that HLA class I molecules on platelets may be involved in platelet release and platelet aggregation. They found that some HLA monoclonal antibodies inhibited both ^{14}C-serotonin release and aggregation which mediated by ADP, collagen and adrenaline, but not thrombin [73]. However, in 1992, the role of HLA class I molecules were suggested that they and FcγRII are cross-linked together by anti HLA class I antibodies to activate platelets [74]. Although the real roles of HLA class I on platelet functions cannot be summarized, recent study revealed that HLA class I-reduced platelets (up to 85%) did not affect the expression of specific membrane glycoproteins (GPs) or even platelet function [20].

Human stem cells [75, 76]

We all were born from fertilization. During next few days before implantation, the fertilized cell is processed by cleavage divisions, the cells called blastomeres (cleavage-stage embryos or totipotent cells). The blastomeres are undifferentiated and not yet committed, so they have the potential to be any cell of the body. Then, about five days of the development, first differentiation event occurs when the outer layer of cells is committed to be part of placenta (trophectoderm) which it separates from inner cell mass (ICM). The inner cell mass cells have the potential to become any cell type of body by three germ layers; ectoderm, mesoderm and endoderm. After that, human body with functional organs will be generated. In many tissues or organs, there are undifferentiated cells surrounded by differentiated cells. These undifferentiated cells are called adult stem cells or somatic stem cells that can divide to remain stem cells and generate many different cell types with more specialized function in only their tissue or organ. For examples, muscle cells, blood cells, nerve cells and brain cells.

The unique properties of all stem cells are capacity in dividing and renewing themselves for long times, unspecialization or no any tissue-specific structures and giving rise to specialized cell types or differentiation. Since these unique capacities, the disease models for understanding disease pathogenesis and new therapeutic development (drug development and transplantation therapies) could be generated (Figure 11). [77]



Sterneckert et al. Nat Rev Genet. 2014

Figure 11 Generation and applications of patient specific disease models. Primary cells, patient specific adult stem cells and iPSCs can be used for disease modeling. Primary cells of patients are not easily available and expanded in culture. Adult stem cells and iPSCs can be expanded in culture and can be differentiated into disease affected cells. Patient specific disease models can be used for identification of new biomarkers in diagnostic procedure improvement, drug discovery and cell replacement therapy [77]. These will be useful for the specific patient or even other patients.

Embryonic stem cells (ESCs)

The embryonic stem cells (ESCs) are inner cell mass-derived cells, which generated by removing inner cell mass from its normal embryonic environment for culturing under appropriate conditions, since after implantation they differentiate to other cell types quickly with more limited potential. The ESCs can continuously proliferate and replicate themselves indefinitely and moreover the developmental potential in forming any cell type of the body still is maintain (Figure 12).

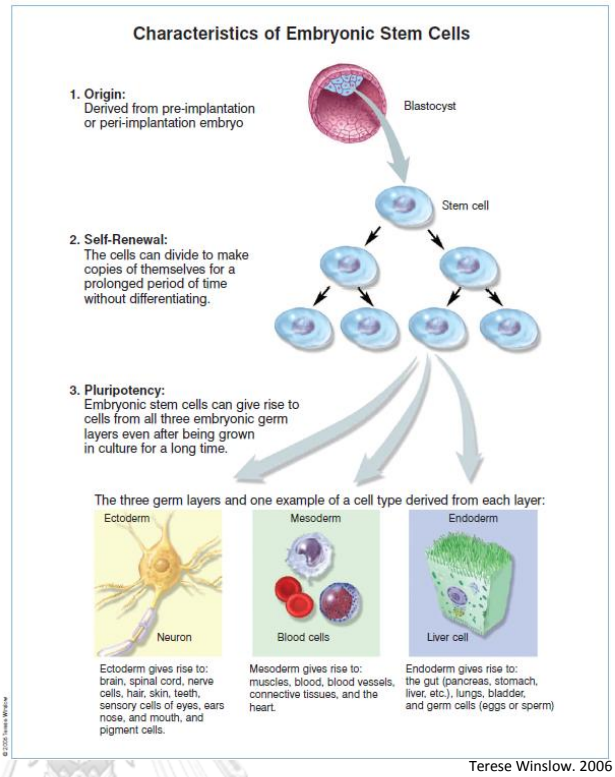


Figure 12 Characteristics of embryonic stem cells (ESCs).

Moreover, human ESCs are derived from embryos which produced by *in vitro* fertilization (IVF) (fertilization by oocytes and sperm to taking place in a culture dish). This process usually is used by clinics to treat infertility of couples which sometimes, IVF produced embryos are no longer required by the couples for producing children. The discarded IVF produced embryos were the sources of the human ESC lines.

Induced pluripotent stem cells (iPSCs)[78]

iPSCs are adult cells are forced to express genes and factors to genetically reprogram for being an embryonic stem cell-like state. The expressed genes and factors are important to maintain the essential properties of embryonic stem cells, so iPSCs have the property of pluripotent stem cells (Figure 13). Human iPSCs were first revealed in late 2007. Although in clinically, the difference of iPSCs and ESCs is not clear, human iPSCs also express the markers of stem cell and can generate cells that characteristic from all three germ layers.

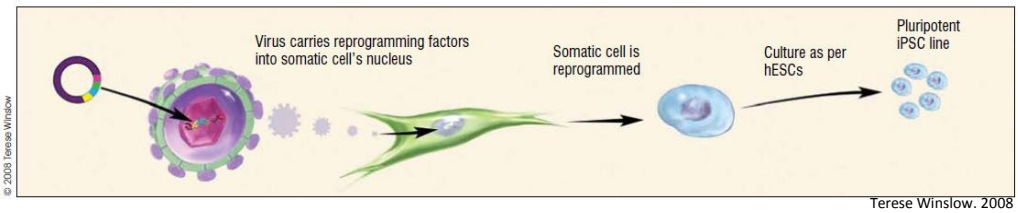


Figure 13 Generation of induced pluripotent stem cells (iPSCs)

The genes which are introduced to adult cells for reprogramming are *Oct4, Sox2, Klf4,* and *c-Myc* [26]. These genes encode important transcription factor proteins which regulate function of other genes critical for embryonic development in early steps.

The genes were known to be associated with the pluripotent maintenance which is the ability of generating all other cell types of body. The created iPSCs could be found in culture after several weeks and to be highly similar to ESCs. In addition, the original four genes or a different combination of *Oct4, Sox2, Nanog,* and *Lin28* were reported to generate iPSCs from human cells. Besides mouse and human, somatic tissues of monkey and rat could be generated to be iPSCs.

Despite more research is required, the potential of iPSCs to be used for drug development, transplantation medicine and disease modeling is beginning to focus by some investigators. In addition, iPSCs can be immune-matched supply for own patients and ethical issues which involve in ESCs production do not apply to iPSCs leading to generating patient specific stem cell lines is a non-controversial strategy.

Hematopoietic stem cells (HSCs), megakaryocytes (MKs) and platelets [60]

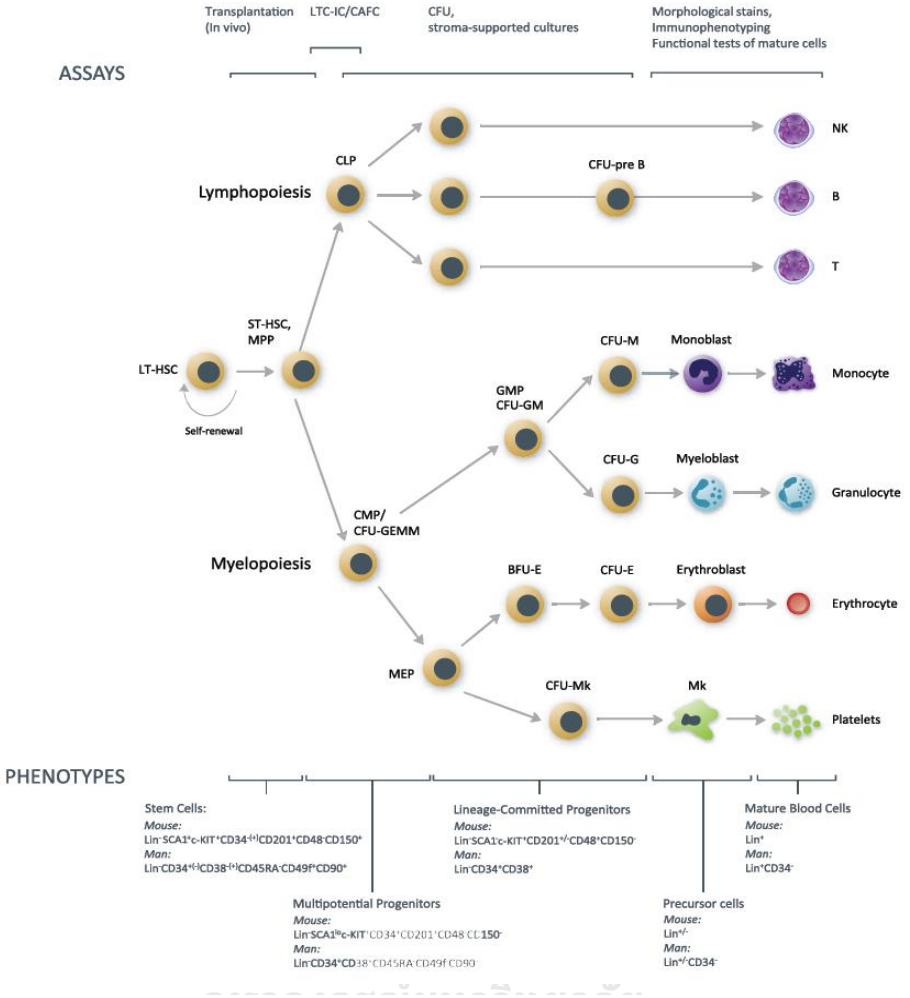
Hematopoiesis is firstly generated in embryonic yolk sac, then liver and spleen in during third month to seven month of pregnancy. After that, hematopoiesis is mostly generated in bone marrow. Hematopoietic stem cells (HSCs) which can self-renew are adult stem cells of all blood cell types. Even though in bone marrow, their exist rate is so small as 1 in 5×10^4 cells, they can rapidly proliferate for replacing loss blood cells. HSCs can generate different blood cells divided into 2 lineage; myeloid progenitor lineage and lymphoid progenitor lineage. Stromal cells in bone marrow involving in blood cell generation and development are fat cells, endothelial cells, macrophage and fibroblast. Under normal conditions, HSCs and progenitor cells can be found in peripheral blood in only small numbers. Large numbers of HSCs and progenitor cells can rapidly mobilized into the circulation by treating with some chemical such as cytokines and compounds which disrupt interaction between bone marrow stromal cells and hematopoietic cells.[79]

Platelets are derived from megakaryocyte (MK) which is in myeloid progenitor lineage (Figure12) and largest hematopoietic cell (approximately 100μm) mainly resides in bone marrow but is also found in lung and peripheral blood. Thrombopoietin (TPO) is main regulator of thrombopoiesis which affects all stages of MK development including endomitosis promotion. Moreover IL-3, IL-6, IL-9, IL-11, BMP-4, Flt-3L, and stem cell factor (SCF) are thought to stimulate MK development, maturation and platelet production. Platelet biogenesis results from cytoplasm and membrane system tailor of megakaryocytes

(MKs). Megakaryocyte features are large size, polyploidy and mutilobed nucleus (from 4n up to 64n in DNA contents). Megakaryocyte cellular enlargement is formed by multiple rounds of endomitosis with unsuccessful in spindle separation, telophase and cytokinesis causes containing of high concentrations of ribosomes which necessary in platelet specific protein production. MKs process proplatelets into sinusoidal blood vessels of bone marrow and with fluid shear forces in this area helps proplatelet fragment separation. In vivo, 2000-10000 platelets are produced from each MK while about 20-400 platelets per each MK which differentiated from iPSCs or ESCs *in vitro* as published by Takayama et al., 2008, and Lu et al., 2011. [80, 81]

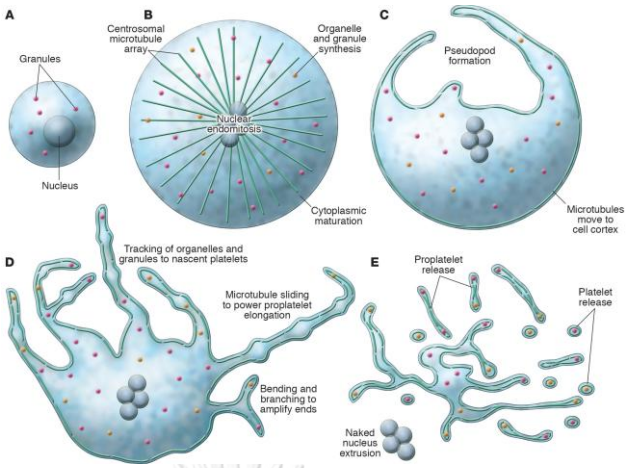
Platelet productions from megakaryocyte are processed as described in the Figure 15 and Figure 16, and the anatomy of proplatelet is shown in Figure 17. Moreover microtubules, α/β-tubulin dimers, are main structural component in proplatelet elongation and actin filaments are spreading throughout proplatelet including swellings and branch points. [82]

Figure 14 Hematopoietic stem cell (HSC) proliferation and differentiation [79]



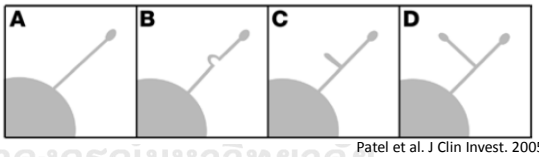
Schematic representation of the production of mature blood cells by the proliferation and differentiation of hematopoietic stem cells. Intermediate stages are also depicted. Transplantation assays identify repopulating stem cells. Assays for Long-Term Culture-Initiating Cells (LTC-IC) and Cobblestone Area-Forming Cells (CAFC) identify very primitive progenitor cells that overlap with stem and progenitors cells. Colony-Forming Unit (CFU) assays identify multipotential and lineage-committed progenitor cells. LT-HSC: Long-Term Hematopoietic Stem Cell; ST-HSC: Short-Term Hematopoietic Stem Cell; MPP: Multipotential Progenitor; CMP: Common Myeloid Progenitor; CLP: Common Lymphoid Progenitor; CFU-GEMM: Colony-Forming Unit - Granulocyte/Erythrocyte/Macrophage/ Megakaryocyte; BFU-E: Burst-Forming Unit - Erythroid; CFU-E: Colony-Forming Unit – Erythroid; CFU-Mk: Colony-Forming Unit - Megakaryocyte; CFU-GM: Colony-Forming Unit - Granulocyte/Macrophage; CFU-G: Colony-Forming Unit – Granulocyte; CFU-M: Colony-Forming Unit – Macrophage. The most definitive markers used to identify the various types of mouse and human hematopoietic cells are shown on the bottom. Additional markers can be used to further distinguish between subsets. Refer to the text for further details. Not shown are the plasmacytoid and myeloid dendritic cell (DC) lineages, which are derived from CLP and CMP, respectively.

Albertus W et al. Stem cell technologies. 2013



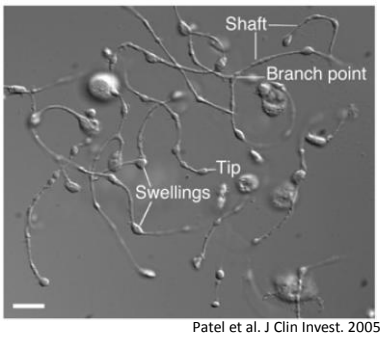
Patel et al. J Clin Invest. 2005

Figure 15 Platelet production from megakaryocytes (MKs). From immature cell (A) develops to mature MK which undergoes endomitosis and cytoplasmic organelle maturation (B). Microtubules translocate to cell cortex with thick pseudopod formation is the starter of proplatelet formation (C). Proplatelet elongated by overlapping microtubules sliding as at the proplatelet ends which finally become platelets, organelles are tracked into (D). A mass of proplatelets are released from the entire MK cytoplasm and the proplatelet ends release individual platelets, eventually nucleus is forced out from mass of proplatelets (E). [82]



Patel et al. J Clin Invest. 2005

Figure 16 Proplatelet amplification. The shaft of proplatelet is bent sharply. The bend folds back to form a loop which elongates to form new proplatelet with a new tip, respectively. [82]



Patel et al. J Clin Invest. 2005

Figure 17 Proplatelet anatomies. From differential interference contrast image of mouse megakaryocyte in vitro show some of hallmark features of proplatelets which are shafts, branch point, swellings and tip. [82]

Genome editing technology and CRISPR-Cas9 nickase [29]

For biological research, manipulation the targeted genomic sequences in living organisms and cells has been a powerful tool and it is also for therapy of genetic diseases. The strategies for efficient induction the precise targeted genomic changes were limited to organisms such as recombineering (recombination mediated genetic engineering) in mice and homologous recombination in yeast, for many years. Including the modification method often need drug selectable markers or left the sequences behind such as residual *loxP* sites from Cre recombinase mediated excision. These traditional methods commonly need additional sequences such as drug resistance marker genes followed by removing them by additional manipulations. However, targeted genome editing by using customized nucleases can avoid these complicated manipulations, and they can supply a general method for inducing precise targeted sequence changes, insertions and deletions in many organisms and cell types.

Targeted genome editing requires the critical first step which is DNA double stranded break (DSB) generation at the desired genomic region. The double stranded break can be repaired by nonhomologous end-joining (NHEJ) and homology-directed repair (HDR) pathways which operate in nearly all organisms and cell types. NHEJ can results in various lengths of insertion/deletion mutations (indels) which can disrupt translational reading frame of coding sequence. While HDR can use the recombination process in inserting desired sequences or introduce specific point mutations to the target locus through exogenous supplied DNA or donor templates. The desired mutations by the targeted nuclease induced double strand break can be detected by simple screening without drug resistance marker selection because of the typically great frequencies (>1% or >50%in some cases) of alterations.

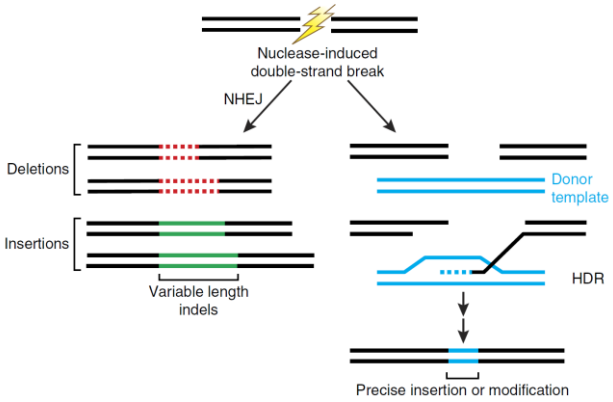


Figure 18 Nuclease induced genome editing and DNA repairing pathways. The DSBs can be repaired by nonhomologous end joining (NHEJ) or homology directed repair (HDR) pathways.[29]

For targeting DSB inducing nuclease, early methods depend on protein based systems with specificity of available designed DNA binding such as meganucleases, zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). In each platform has advantages and disadvantages such as meganucleases which are naturally occurring restriction enzymes commonly need extended DNA recognition sequences (about 14-40 bps) and their DNA recognition and cleavage functions are interlaced in a single domain, so they have been challenging in targeted binding specificity. While ZFNs and TALENs both consist of engineered DNA binding domain fused to cleavage domain of FokI restriction enzyme which is nonspecific nuclease, so their DNA binding domains and FokI cleavage domain are separately (Figure 19). However, ZFNs have limitations in construction of engineered zinc finger arrays because not every nucleotide triplet has corresponding zinc finger and between individual finger domains in an array require accounting for context dependent effects [83] (interaction between individual fingers in an array can reduce their specificity). While TALENs using one to one code between the four possible DNA nucleotides and individual repeats, are much more simpler to design, and can be stronger assemble including have fewer context dependent effects. However, they can require utility of nonstandard molecular biology cloning methods because of their large numbers of highly conserved TALE repeats. In addition, TALEN coding sequences with highly repetitive nature can face the problem in their delivery using some certain viral vectors [84].

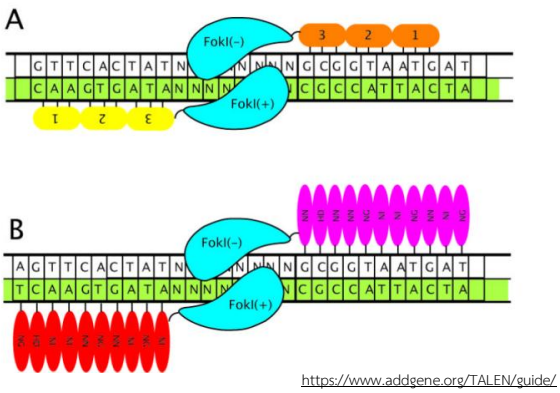


Figure 19 Schematics of ZFNs and TALENs. A: representation of ZFNs B: representation of TALENs https://www.addgene.org/TALEN/guide/

CRISPR/Cas9 systems

CRISPR (clustered, regularly interspaced, short palindromic repeat) systems are adaptive immune mechanisms of many bacteria for protecting from foreign nucleic acids such as plasmids or viruses [85]. For type II CRISPR systems, within the bacterial host genome, CRISPR repeat sequences are incorporated sequences from invading DNA between them which CRISPR RNAs (crRNAs) come from transcription of the CRISPR repeat arrays. So, crRNAs consist of RNA from both invading DNA (known as protospacer sequence) and part of the CRISPR repeat. Each crRNA hybridizes with transactivating CRISPR RNA (tracrRNA), and then they complex with Cas9 (CRISPR-associated protein 9) nuclease [86]. The protospacer RNA of crRNA complementary to target DNA sequence with protospacer adjacent motifs (PAMs) residing nearly, directs Cas9 to cleave that target sequence to be double strand break (DSB) (Figure 20).

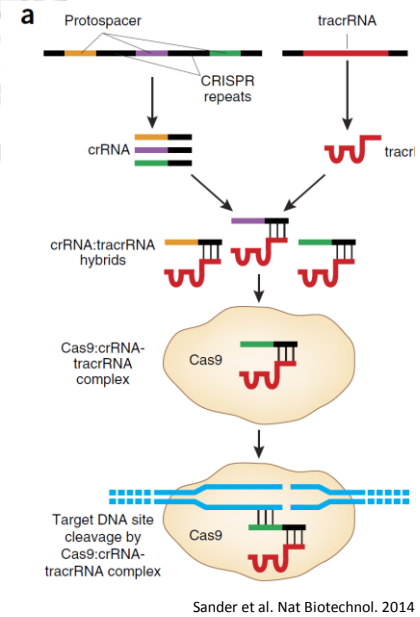


Figure 20 The natural occurring CRISPR/Cas9 system.[29]

For inducing sequence specific double strand breaks and targeted genome editing, type II CRISPR system from *S.pyogenes* has been adapted. The Cas9 nuclease and guide RNA (gRNA consists of crRNA fused with fixed tracrRNA) must be introduced into cells or organism for performing genome editing. Cas9 nuclease can be directed to target DNA sequence in form N_{20}-NGG (N_{20} refers 20 nucleotides) by changing the first 20 nucleotides of gRNA (at 5' end) for targeting the DNA sequence (Figure 21).

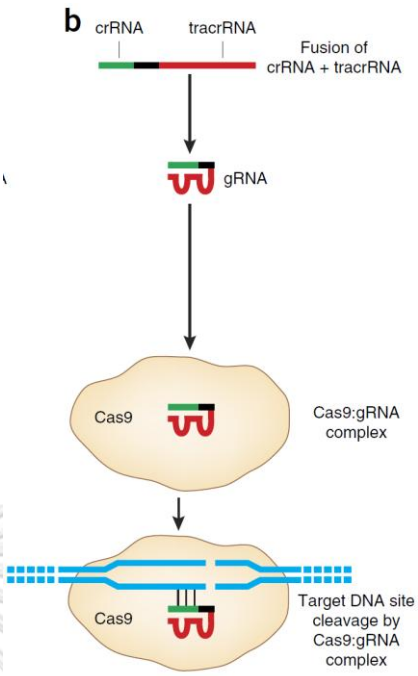


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Figure 21 Engineered CRISPR/Cas9 system. [29]

This system has been demonstrated in 2012 with cutting the various DNA sites *in vitro* (Table2). Moreover, NHEJ and HDR pathways can be induced by using Cas9 and this system can introduce DSBs at multiple sites which are advantage over meganucleases, ZFNs or TALENs. Some study showed not inhibiting by DNA methylation [87].

Table 2 Published examples of using Cas9 nuclease to modify cells and organisms. [29]

Cell type or organism	Cas9 form	Cell type	Reference numbers
Human cells	Cas9 nuclease	HEK293FT, HEK293T, HEK293, K562, iPSC, HUES9, HUES1, BJ-RiPS, HeLa, Jurkat, U2OS	9,13–16,47, 49–51,54,59, 84,85
	Cas9 nickase	HEK293FT, HEK293T	13,14,47,49
	dCas9 (gene regulation)	HEK293FT, HEK293T	70–72,74,82
	dCas9 (imaging)	HEK293T, UMUC3, HeLa	81
Mouse or mouse cells	Cas9 nuclease	Embryos	14,24–26
	Cas9 nickase	Embryos	47
	dCas9 (gene regulation)	NIH3T3	74
Rat	Cas9 nuclease	Embryos	26,36
Rabbit	Cas9 nuclease	Embryos	27
Frog	Cas9 nuclease	Embryos	28
Zebrafish	Cas9 nuclease	Embryos	17,33,37,60,85
Fruit fly	Cas9 nuclease	Embryos	29,30,61
Silkworm	Cas9 nuclease	Embryos	31
Roundworm	Cas9 nuclease	Adult gonads	32,62–67
Rice	Cas9 nuclease	Protoplasts, callus cells	21,23
Wheat	Cas9 nuclease	Protoplasts	21
Sorghum	Cas9 nuclease	Embryos	23
Tobacco	Cas9 nuclease	Protoplasts, leaf tissue	19,20,23
Thale cress	Cas9 nuclease	Protoplasts, seedlings	19,23
Yeast	Cas9 nuclease	*Saccharomyces cerevisiae*	18
Bacteria	Cas9 nuclease	*Streptococcus pneumoniae, E. coli*	8
	dCas9 (gene regulation)	*E. coli*	69,70

HEK, human embryonic kidney; iPSCs, induced pluripotent stem cells; UMUC3, human bladder cancer.

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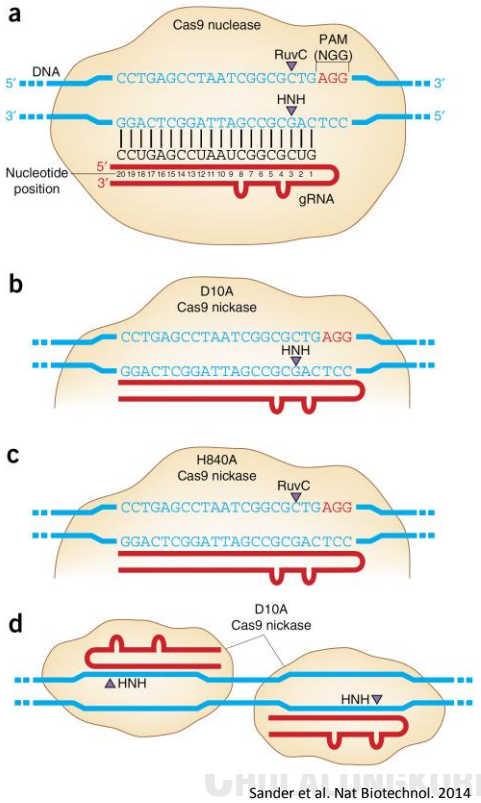
However, off-target from using RNA-guided Cas9 nucleases can be variable in frequency which suggested by many published studies. To improve the specificity, paired nickases approach (Figure 22d) has been proposed which create DSB by targeting to the sites on opposite DNA strands with separated distances 4 – 100 bps. These distances can efficiently introduced HDR and NHEJ [28].

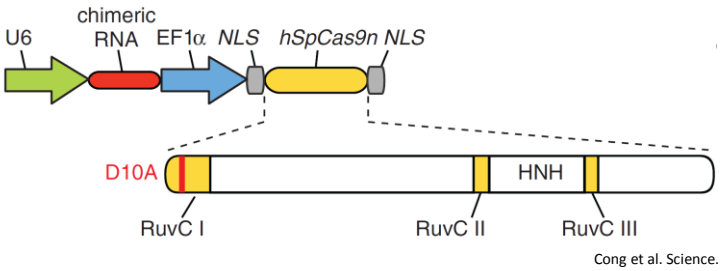


Sander et al. Nat Biotechnol. 2014

Figure 22 Cas9 based systems to change gene sequence or expression. a: Cas9 nuclease creates DSB at target sites which complementary to 5'end of a gRNA. b: Cas9 nickase created by the mutation of RuvC nuclease domain with D10A mutation which cleaves only the complementary DNA strand and recognized by the gRNA. c: Cas9 nickase created by mutation of HNH nuclease domain with H840A mutation which cleaves only DNA strand that does not interact with gRNA. d: Paired nickase strategy for improving Cas9 specificity by two D10A Cas9 nickases with a pair of gRNAs which could generate 5' overhang DSBs. [29]

CRISPR/Cas9 nickases [28, 88]

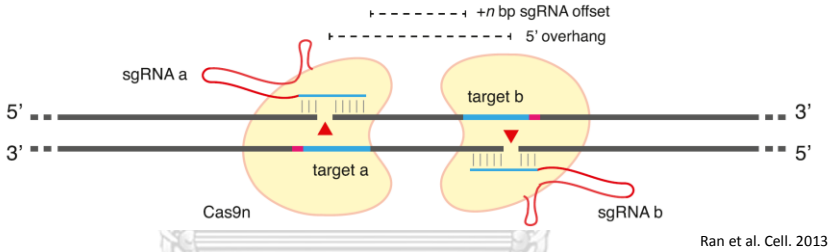
As many studies have found that CRISPR-Cas9 technology can promote mutagenesis via undesired off-target, new strategy with a paired Cas9 nickase was developed to improve the specificity. F.Ann Ran and Feng Zhang et al. proved that the off-target activity reducing by 50 to 1,500 fold in cell line with no changing of on-target rates of modification when compare with wild-type Cas9.



Cong et al. Science. 2013

Figure 23 Schematic shows SpCas9n or Cas9 nicking enzymes by D10A mutation in RuvC I domain. [88]

HNH and RuvC are two conserved nuclease domains of Cas9 enzymes. The HNH domain cleaves DNA strand complementary to the guide RNA while RuvC domain cleaves DNA strand which is not complementary to the guide RNA. Therefore, converting Cas9 enzymes which normally cut both strands of DNA simultaneously to DNA nickases can be done by mutation of H840A in HNH or D10A in RuvC catalytic residues.



Ran et al. Cell. 2013

Figure 24 Schematic showing a pair of sgRNAs guides Cas9 nickases (D10A mutation) creates DNA double-stranded breaks (DSBs). (28)

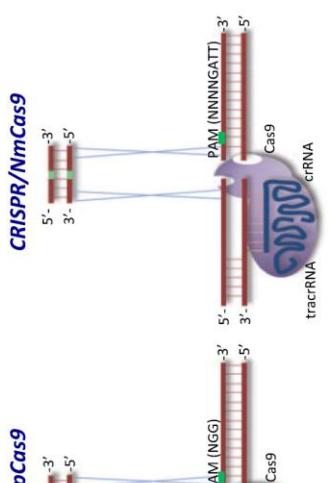
Pair of offset sgRNAs combining with Cas9 nickases (Cas9n) nicks both DNA strands simultaneously making site-specific DSBs in 5'overhang pattern. Moreover, there are several studies in genome editing in human cells and model organisms by using ZFN, TALEN, and CRISPR/Cas (Table 3).

Table 3 Example list of using ZFN, TALEN and CRISPR/Cas-mediated genome editing in human cells and model organisms.

Type of modification	Organism	Genes	Nucleases	Refs
Gene disruption	Human	*CCR5*	ZFN	[65,91,92]
			TALEN	[25,52]
			CRISPR/Cas	[101]
	Human	TCR (T cell receptor)	ZFN	[94,95]
	Zebrafish	*gol* (Golden), *ntl* (No tail), *kra*	ZFN	[66,68]
	Pig	*GGTA1* (α1, 3-galactosyltransferase)	ZFN	[77]
		LDLR (LDL receptor)	TALEN	[76]
	Bovine	*ACAN12, p65*	TALEN	[76]
	Human	*EMX1, PVALB*	CRISPR/Cas	[102]
	Rat	*IgM, Rab38*	ZFN	[70]
	Arabidopsis	*ADH1, TT4*	ZFN	[81]
	C. elegans	*ben-1, rex-1, sdc-2*	ZFN/TALEN	[78]
	Hamster	*DHFR*	ZFN	[37]
	Drosophila	*yellow*	ZFN	[72]
	Rice	*OsSWEET14*	TALEN	[84]
Gene addition	Human	*OCT4, PITX3*	ZFN/TALEN	[45,46]
	Human	*CCR5*	ZFN	[97]
	Human	*F9* (Coagulation Factor IX)	ZFN	[86]
	Mouse	*Rosa26*	ZFN	[57]
	Human	AAVS1	ZFN	[45,96,97]
			TALEN	[46]
			CRISPR/Cas	[103]
	Human	*VEGF-A*	ZFN	[17]
	Zebrafish	*th* (tyrosine hydroxylase), *fam46c, smad5*	TALEN	[80]
	Maize	*IPK1*	ZFN	[82]
Gene correction	Human	*IL2RG*	ZFN	[44,85]
		A1AT (α_1-antitrypsin)	ZFN	[89]
		HBB (β-globin)	ZFN	[87,88]
		SNCA (α-synuclein)	ZFN	[90]
	Tobacco	*SuRA, SurRB* (acetolactate synthase)	ZFN	[83]
	Drosophila	*yellow*	ZFN	[71]

Thomas Gaj et al. Trends Biotechnol. 2013

Table 4 Advantages and disadvantages of conventional gene targeting, ZFNs and TALENs, CRISPR/SpCas9, and CRISPR/NmCas9.[89]



	Conventional Gene Targeting	ZFNs and TALENs	CRISPR/SpCas9	CRISPR/NmCas9
Key features:	- *Introduced construct integrates at target site through homologous recombination*	- *Assembly of pairs of TALE or ZF proteins adjacent to target site from modules recognizing single nucleotides or tripletts, respectively. Dimerized Fok1 nuclease domains introduce DSB.*	- *guideRNA (gRNA): hybrid of 20nt-long sequence-specific crRNA and tracRNA; recruits Cas9 nuclease to genomic target sequence and introduces DSB. Cleavage requires adjacent PAM sequence (5'-NGG-3')*	- *Longer crRNA component (24nt) compared with SpCas9 system. Cleavage requires alternative PAM sequence (5'-NNNNGCTT-3')*
Advantages:	- *Does not require exogenous introduction of cuts into genome* - *Allows various genome modifications (e.g., reporter, knock-out, conditional alleles)*	- *High efficiency* - *No drug selection required* - *DSBs resolved by HDR or NHEJ* - *Can target any genomic sequence (in principle)* - *Biallelic targeting feasible* - *Successful in different cell types and species* - *Lower chance of off-target cleavage than CRISPR*	- *Vey high efficiency* - *No drug selection required* - *DSBs resolved by HDR or NHEJ* - *Rapid construction and easy delivery* - *Efficient biallelic targeting* - *Multiplexing possible (up to 5 genes on both alleles) in vitro and in vivo* - *Successful in different cell types and species*	- *Very high efficiency* - *No drug selection required* - *DSBs resolved by HDR or NHEJ* - *Rapid construction and easy delivery (e.g., single vector system)* - *Longer recognition sequence may reduce off-target effects* - *Variant PAM increases targeting range* - *Successful in different cell types*
Disadvantages:	- *Time consuming & labor-intensive* - *Generally inefficient* - *Requires long homology arms and drug selection* - *Difficult to achieve biallelic targeting* - *Difficult in hESCs and non-pluripotent cell types*	- *More difficult to assemble than CRISPR* - *Remaining possibility of off-target cleavage*	- *Target selection may be limited by requirement for PAM sequence* - *Off-target cleavage possibly more frequent than with TALENs and ZFNs*	- *Target selection may be limited by requirement for PAM sequence* - *Possibility of off-target cleavage*

Ryan M. Walsh et al. Proc Natl Acad Sci USA. 2013

Another method for loss of function experiments is RNAi technology which has become popular because of efficiency, ease of use, efficacy and cost when compared with traditional approaches. However, it has some limitations; first, it is just knockdown method which results in incomplete gene loss of function. It is possible that gene expression that reduced could be enough to fulfill all or some of the role of endogenous genes, and the phenotype can be different from the genetic null phenotype which is important to create or isolate the genetic null mutants. Second, not all situations that introduce dsRNAs/siRNAs/shRNAs into organisms or cells are effective and specific RNAi response. Next there is off-target effect and it can induce unspecific immune response[90].

Gene delivery and targeted genome editing

In order to manipulate the specific regions through genomic editing systems such as ZFNs, TALENs, and CRISPR-Cas which are exogenous elements, they have to be delivered to the targeted cells. Several years, the effective and safe delivery vectors which are viral vectors and non-viral vectors, are developed for gene-based therapy. Although, the viral vectors are used deliver genes for therapy in several cases, they have many limitations such as immunogenicity, carcinogenesis, and difficult vector production (table 6)[91]. While using non-viral vectors to deliver genome editing systems into the precise genomic regions is manipulation without any association of viruses. Therefore, it can avoid getting short-term and long-term adverse effects by viruses. The non-viral vectors can be classified into two groups which are physical methods and chemical methods. The physical method is delivery DNA into target cells by using physical forces to increase the permeability of cell membrane to transgene such as electroporation and nucleofection. The chemical methods is delivery DNA into nucleus by using carrier which prepared by chemical reactions such as lipofection. However, those methods still have limitations. For examples, electroporation can be toxicity to cells which often leading to death of the majority of cells. Lipofection or cationic lipids is limited by low efficacy due to rapid clearance, poor stability and generation of inflammatory or anti-inflammatory responses. Even though lipid-based transfection reagents have high toxicity or low transfection efficiency in ESCs, this method is more successful transgene than using electroporation. Nucleofection method which improved from electroporation is highly efficient to transfer plasmid DNAs into ESCs more than both electroporation and lipid-based transfection. The transfected ESCs still

have their properties. They are the most common methods for non-viral gene transfer to ESCs.[92]

Table 5 The possible gene delivery strategies.[92]

Methods	Cellular context	Length of expression	Relative efficiency	Type of delivered molecule	Suspension vs. adherent	Toxicity	Expertise required	Time required	Cost
Lentivirus	In vitro In vivo	Stable	High	RNA	Adherent and suspension	Low	High	1–3 weeks	$1000
Adenovirus	In vitro In vivo	Transient	High	DNA	Adherent and suspension	Low	High	1–3 weeks	$500–$1000
Adeno-associated virus	In vitro In vivo	Stable	High	DNA	Adherent and suspension	Low	High	1–3 weeks	$500–$1000
Cationic lipids	In vitro In vivo	Transient or stable	Low–moderate	Plasmid Oligos mRNA siRNA Protein	Adherent and suspension	Moderate	Low	1–3 days	$100–$500
Electroporation	In vitro In vivo	Transient or stable	Low–moderate	Plasmid Oligos mRNA siRNA	Suspension	High	Moderate	1–3 days	>$1000
Nucleofection	In vitro In vivo	Transient or stable	High	Plasmid siRNA	Suspension	Moderate	Moderate	1–3 days	>$1000
Microinjection	In vitro In vivo	Transient or stable	High	Plasmid Oligos mRNA siRNA	Adherent	Moderate	Moderate	1–3 days	>$1000
Nuclear transfer	In vitro In vivo	Stable	Moderate	Nuclei	Adherent	Moderate	High	1–3 weeks	>$1000

Kobayashi et al.Birth Defects Res C Embryo Today. 2005

Table 6 Advantages and limitations of viral vectors.[91]

Vector	Genetic material and packaging capacity	Advantages	Limitations
Baculovirus (BVs)[3,11,23]	dsDNA, ~134 kb	• Nonpathogenic to humans; nonreplicating and nontoxic in transduced cells • DNA degradation and lack of integration that improves safety • No pre-existing immunity • Ease of production by infecting insect cells in BSL-1 facilities • Large cloning capacity (at least 38 kb)	• Transient transgene expression • Vulnerable to mechanical force and loss of virus titer during virus concentration and purification • Inactivated by serum complement
Lentivirus (LVs)[64–66]	RNA, 8 kb	• Transduce nondividing and dividing cells • Stable transgene expression • Absence of pre-existing immunity	• Possible insertional mutagenesis • Limited insert size of the transgene • Requiring transfection of multiple plasmids into packaging cells for vector production • LVs should be handled at BSL-2 facilities
Retrovirus (RVs)[64,66]	RNA, 8 kb	• Theoretically stable transgene expression • Low immunogenicity • No (or very low) pre-existing immunity	• Transduce only dividing cells • Possible insertional mutagenesis and induction of leukemia in clinical trials • Limited insert size (8 kb) of the transgene • Requires transfection of multiple plasmids into packaging cells for vector production • RVs should be handled at BSL-2 or BSL-3 facilities • Transgene prone to silencing
Adenovirus (AdVs)[64,66]	dsDNA, 36 kb	• Transduce non-dividing and dividing cells • High-level transgene expression • Easy production with ready-to-use packaging cells • Vector particles produced at high titers (10^{10} pfu/ml) • High cloning capacity (for helper dependent AdV)	• Broad pre-existing immunity • High immunogenicity (yet immunogenicity can be reduced by using helper-dependent AdV) • Transient transgene expression
Adeno-associated virus (AAVs)[64,67]	ssDNA, <5 kb	• Transduce nondividing and dividing cells • Non-pathogenic • Wide cellular tropism • Capable of long-term transgene expression • Low immunogenicity	• Pre-existing immunity • Requiring transfection of multiple plasmids into packaging cells for vector production • Limited insert size (5 kb) of the transgene • Possible transgene integration

Sung et al. Nat Protoc. 2014

CHAPTER III
MATERIALS AND METHODS

Generation of paired CRISPR/Cas9 nickases for beta2-microglobulin gene disruption

To knockout beta2-microglobulin gene, the initiation of exon 2 was chosen for disruption by using a pair of CRISPR/Cas9 nickases and mapped using SnapGene® Viewer 3.1.2. The pair of CRISPR/Cas9 nickases creates DNA double strand break and then DNA repairing mechanism by non-homologous end joining (NHEJ) leads to random insertion or deletion which can produce frameshift mutation or premature stop codon following edited position or homologous directed repair (HDR) leads to precise modification. Target site sequence is used as template to design oligos for guide sequence in single guide RNA, around 20bp needs to be in the rule of CRISPR design followed by NGG PAM sequence (3bp) on 3' end of the target sequence. In addition, Zhang et al. has found that targeting efficiency can be increased by adding guanine nucleotide to 5' end of the guide sequence.

In order to design guide sequence, we used CRISPR Design Tool which is available on website http://crispr.mit.edu/ provided by Zhang Lab, MIT 2015. We submitted sequence of exon2, beta2-microglobulin gene into the program, and then the program analyzed double nickase design as we chose for nickase analysis. They gave us all pairs of guide sequence in vary positions ranging by score they predicted, after that we ordered oligos as they recommended for guide sequence from Ward Medic, Sukumvit65, Bangkok, Thailand.

We ordered CRISPR plasmid backbone in third generation, pSpCas9n (BB)-2A-Puro (PX462) from Feng Zhang (Addgene plasmid #48141), Addgene, 75 Sidney St, Suite 550A, Cambridge, MA 02139. This plasmid was used in the 2013 Nature Protocols paper by Ran et al. (Figure 25) and later they improved new version of the plasmid (Plasmid #62987) by correction a point mutation in Puro gene which the older version has less effective of Puro selection in some cell lines (Figure 26).

https://www.addgene.org/48141/

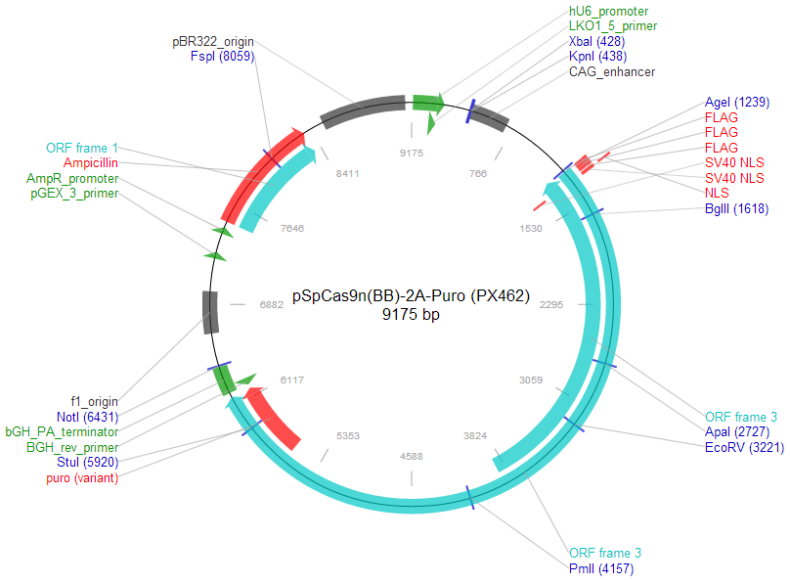
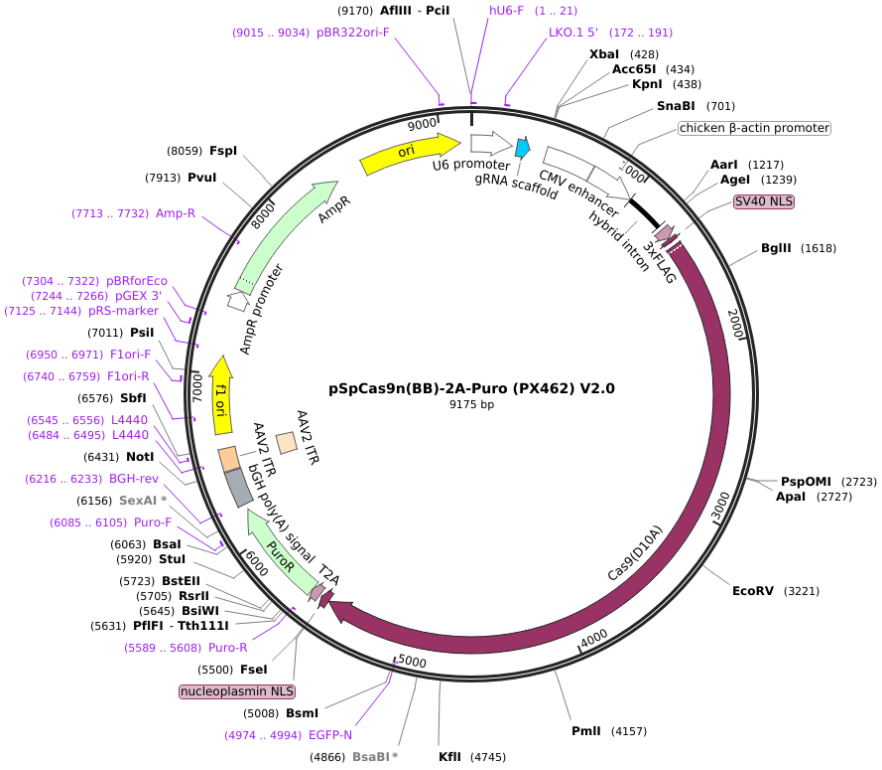


Figure 25 pSpCas9n (BB)-2A-Puro (PX462) (Plasmid #48141).



https://www.addgene.org/62987/

Figure 26 pSpCas9n (BB)-2A-Puro (PX462) V2.0 (Plasmid #62987).

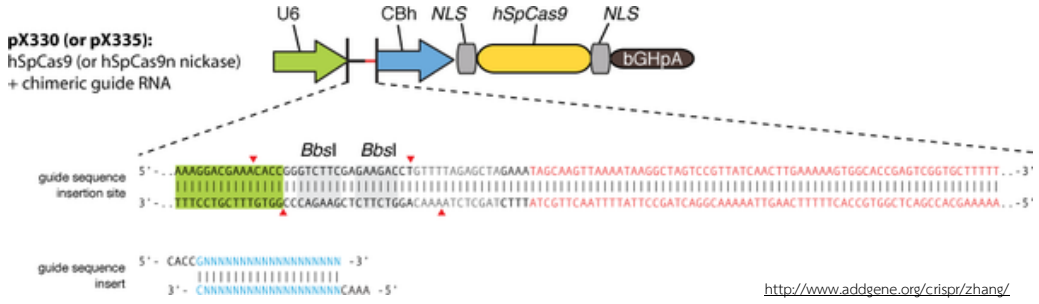


Figure 27 SpCas9 or SpCas9n, D10A nickase + single guide RNA.
http://www.addgene.org/crispr/zhang/

The plasmid contains two expression cassettes of human codon-optimized S. pyogenes Cas9 D10A nickase and single guide RNA. With *BbsI* digestion, we can clone a pair of annealed oligos into the vector seamlessly prior to the sgRNA scaffold (Figure 27). The cloning was done following Target sequence cloning protocol of Zhang Lab.

Efficiency test of the generated CRISPR/Cas9 nickase

To test efficiency of the generated CRISPR/Cas9 nickase, HEK 293T cells were transfected by using 4D-Nucleofector™ X Unit-Transfection in suspension following Amaxa™ 4D-Nucleofector™ Protocol for HEK-293 [ATCC] from Lonza. Cells were harvested by 0.25% trypsin-EDTA and counted for 1×10^6 cells. Then 2.5µg of each generated CRISPR/Cas9 nickase plasmid or 5µg in total of pair generated CRISPR/Cas9 nickase plasmids, were transfected into nucleus of HEK 293T cells and then plasmids encoded for single guide RNA and Cas9 nickase proteins.

The single guided RNA guides Cas9 nickase to cleave genomic DNA at the specific site, then non-homologous end joining DNA repairing pathway leads to random insertion/deletion in vary lengths. By this pathway, genomic DNA was extracted to detect cleavage efficiency of CRISPR/Cas9 nickase by using T7E1 assay.

T7E1assay is a technique for unknown mutation detection. T7E1 is an enzyme used in enzyme mismatch cleavage method because its substrates are double strand DNA with conformational changes to be able to bend further. Heteroduplex dsDNA can be

recognized and cleaved by T7E1 because of its extra helical loop forming and it comes from melting and hybridizing WT and mutant alleles. [93]

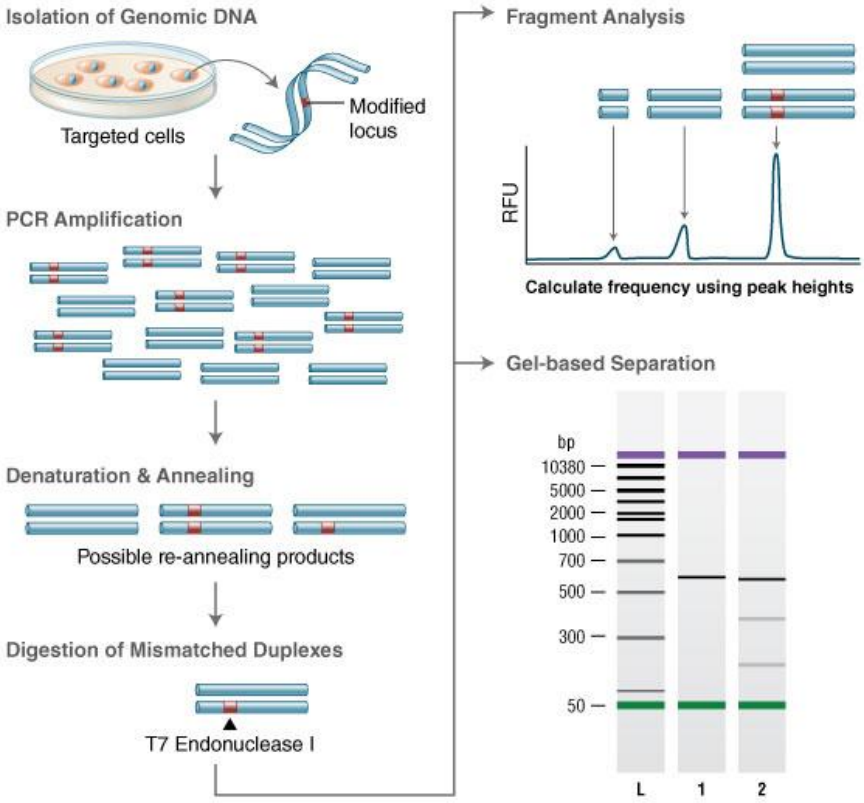


Figure 28 Schematic represents mutation detection workflow of T7E1 https://www.neb.com/products/e3321-engen-mutation-detection-kit

We harvested transfected cells after 72 hour of transfection and then we extracted gDNA using QIAamp DNA blood Mini Kit following DNA purification from blood or body fluids protocol (spin protocol). PCR was performed in proper sizes for band separation after T7E1 cleavage. The PCR products were denatured and re-annealed following Surveyor Program, in this step heteroduplex dsDNA was formed by recombination of DNA strands of wild type and mutant. Next, digestion of the mismatched duplexes was done by using T7E1 enzyme from NEB, incubation at 37°C for 30mins. Then 2% agarose gel electrophoresis was used to separate the DNA fragments.

Generation of Donor vector template for HDR in beta2-microglobulin gene disruption

To generate donor vector template, we clone PCR products of homology arms and hygromycin resistant gene with necessary components hanging restriction enzyme recognition site to pCR-Blunt vector as a backbone vector. The donor vector consists of left homology arm, hygromycin resistant gene and necessary components, and right homology arm, respectively. We used BD2 vector from Addgene as a template for PCR of loxP-PGK-Hyg-loxP.

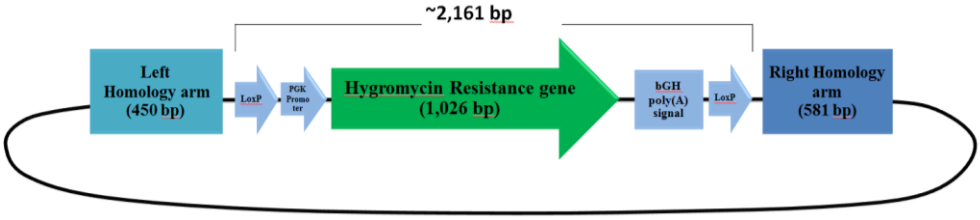


Figure 29 Schematic drawing represents donor vector for knocking out β2-microglobulin gene. Abbreviation; Left homology arm (HL), Right homology arm (HR), PGK promoter (PGK), Hygromycin resistant gene (HygR), bGH poly (A) signal (bGH polyA)

Knocking out beta2-microglobulin gene in human iPSCs (group O)

In order to generate HLA-universal, iPSC-derived platelets, we transfected both paired CRISPR/Cas9 nickases and donor vector to human iPSCs group O. The wild type human iPSCs were provided by Stem cell center of Chulalongkorn University. Human iPS cell culture under feeder-free conditions protocol from Center for iPS cell Research and Application (CiRA), Kyoto University was used to generate human iPSCs from blood-derived mononuclear cells. iPSCs were then cultured in feeder-free condition using Matrigel® Matrix (Corning®) and mTeSR™1 feeder-free maintenance medium for human ES and iPS cells (STEMCELL TECHNOLOGIES) at 37°C in a humidified atmosphere of 5%CO_2. iPSCs were passaged using prepared CTK solutions (0.1 mg/ml collagenase IV, 0.25% (v/v) trypsin in PBS (-) supplemented with 20% (v/v) KSR, and 1 mM calcium chloride).

The transfection was done following Amaxa® 4D-Nucleofector® Basic Protocol for Human Stem Cells, 4D-Nucleofector® X Unit-Transfection in suspension, P3 Primary cell 4D-Nucleofector® X Kit L (Lonza, V4XP-3012) and MEF (DR4) ATCC®SCRC-1045™ was used as

feeder cells. Accutase (StemPro® Accutase®, cell dissociation reagent, gibco by Life Technologies) was used to dissociate iPSCs into single cell for transfection. Media with 20µg/ml Hygromycin was replaced at 72 hours post nucleofection.

PCR-sequencing for β2m^{ko}-iPSCs detection

Genomic DNA was extracted from iPSCs which can survive under Hygromycin drug selection using QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instruction. PCR amplifications were carried out for 796bp of left donor recombination, 858bp of right donor recombination and 548bp of wild type alleles using primers as describe in table 7 and Taq DNA polymerase (Fermentas, Glen Burnie, MD, USA). PCR products were sent for direct sequencing at Macrogen (Seoul, Korea).

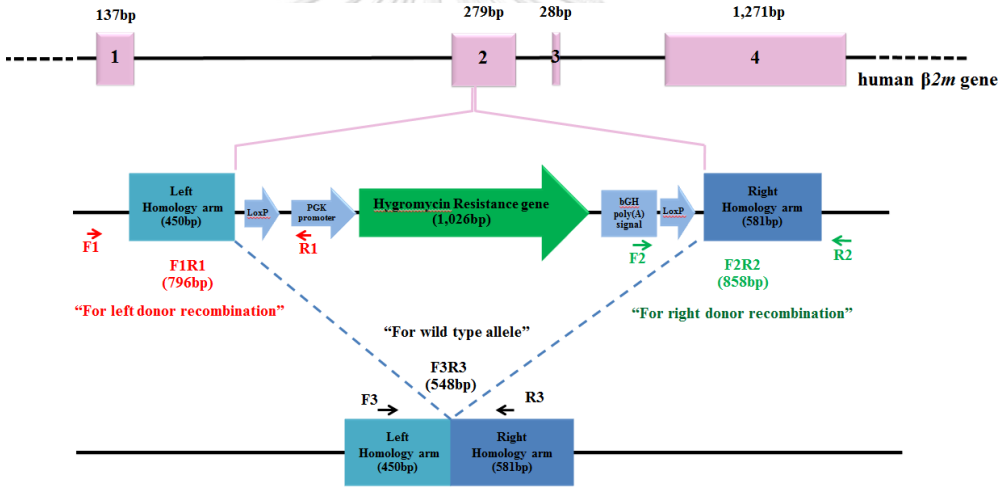


Figure 30 Schematic diagram shows allele of β2m gene with donor recombination and without donor recombination, and primer pairs used to detection. The primer pairs used for left- and right-donor recombination (red and green, respectively) and wild type allele detection.

Alleles	Forward primers	Reverse primers
Left donor recombination	5'-TGGAAGGGGTGGAAACAGAG-3'	5'-ATGTGGAATGTGTGCGAGGC-3'
Right donor recombination	5'-AAGACAATAGCAGGCATGCTG-3'	5'-TGGTTAGAAATAAGGCTGGC-3'
Wild type	5'-GGGAGAAATCGATGACCAAA-3'	5'-CCCTGACAATCCCAATATGC-3'

Table 7 Primer sequences for PCR amplification of donor recombination and wild type allele detection.

Flow cytometry analysis for $\beta 2m^{ko}$ - iPSCs detection

Wild type-iPSCs, Mono- and Bi-allelic $\beta 2m$ knockout iPSCs were harvested using Accutase and flow cytometry was used to detect HLA class I and β2-microglobulin protein cell surface on iPSCs using PerCP-conjugated anti-human HLA-A, B, C (Biolegend) and APC-conjugated anti-human β2-microglobulin (Biolegend). Stained cells were analysed by using BD FACSAria II (Becton Dickinson, Franklin Lakes, NJ, USA).

iPSC characterization

Wild type-iPSCs, Mono- and Bi-allelic $\beta 2m$ knockout iPSCs were characterized for pluripotency markers which are Oct-4 and Nanog (Nuclear marker), SSEA-4, TRA-1-60 and TRA-1-81(Surface marker) and embryoid body differentiation to three germ layers using immunofluorescence (IF) staining and karyotyping. Fluorescence images were obtained by using EVOS™ FL color Imaging Systems, Florescence Microscope (Life technologies, Thermo Fisher Scientific)

iPSC pluripotency markers

Pluripotency Marker	Primary antibody	Secondary antibody
Oct-4A	Anti-Rabbit IgG antibody (Cell Signaling Technology)	Alexa Fluor 488 Goat anti-rabbit IgG antibody (Life technology, Invitrogen)
Nanog		
SSEA-4	Anti-mouse IgG3 antibody (Abcam)	Alexa Fluor 594 Goat anti-mouse IgG antibody (Life technology, Invitrogen)
TRA-1-60	Anti-mouse IgM antibody (Cell Signaling Technology)	FITC Goat anti-mouse IgM antibody (Millipore)
TRA-1-81		

Table 8 Primary and secondary antibodies for iPSC pluripotency markers detection; Oct-4, Nanog, SSEA-4, TRA-1-60 and TRA-1-81.

iPSCs were cultured in Matrigel® Matrix (Corning®) coated 96-well plate around 3-4 days in 37℃, 5% CO2 incubator until they formed packed colonies. Cells were fixed by 4% Paraformaldehyde, permeabilized by 0.3% Triton X-100 (only for Oct-4 and Nanog), blocked by 10% goat serum, stained by primary antibodies and secondary antibodies for detecting pluripotency markers which are showed in the table 8 and stained by DAPI for nuclear staining.

Embryoid body differentiation

iPSCs were dissociated in many big pieces to form embryoid body in ultra-low attachment culture dish and cultured in 37℃, 5% CO2 incubator. On day 7, embryoid bodies were collected and placed on Matrigel® Matrix (Corning®) coated 24-well plate and cultured for 14 days at 37℃ in 5% CO2 incubator. Then cells were fixed by 4% Paraformaldehyde, permeabilized by 0.3% Triton X-100, blocked by 10% goat serum,

stained by primary antibodies and secondary antibodies for detecting three germ layer markers; Nestin (Ectoderm), Brachyury (Mesoderm) and AFP (Endoderm) which are showed in the table 9 and stained by DAPI for nuclear staining.

Marker	Germ layer	Primary antibody	Secondary antibody
Brachyury	Mesoderm	Anti-Rabbit IgG antibody (Abcam)	Alexa Fluor 488 Goat anti-rabbit IgG antibody (Life technology, Invitrogen)
AFP (α-fetoprotein)	Endoderm	Anti-mouse IgG1 antibody (Abcam)	Alexa Fluor 594 Goat anti-mouse IgG antibody (Life technology, Invitrogen)
Nestin	Ectoderm	Anti-mouse IgG1 antibody (Biolegend)	

Table 9 Primary and secondary antibodies for three germ layer markers detection; Brachyury, AFP (α-fetoprotein) and Nestin.

Karyotyping analysis

iPSCs were cultured until they reached to log phase. In this phase, colchicine was added to iPSC culture media and incubated at 37°C in 5% CO2 incubator for 3 hours. Then cells were dissociated by Accutase and were collected by 1XPBS into new tube and centrifuged at 1200 rpm for 8 minutes. Discarded supernatant and flicked the tube to separate single cells then added 3ml of warmed 0.075M KCl or incubated in 37°C, 10 minutes. Centrifuged at 1200 rpm for 5 minutes to collect cell pellet and discarded supernatant. Fixed cells with 3:1 ration of methanol: acetic acid and centrifuged at 1200 rpm for 5 minutes. Repeated fix step for 3 times, but in the second time, fix with 2:1 ration of methanol: acetic acid instead. Then cell pellet was dropped onto slide to observe metaphase stage under microscopy and stained with quinacrine before analysis.

iPSC-derived megakaryocyte and platelet differentiation and characterization

Wild type-iPSCs, Mono- and Bi-allelic β*2m* knockout iPSCs were differentiated to ES-sac like structures which contain a lot of hematopoietic stem cells (HSCs). Then HSCs were harvested for megakaryocyte and platelet differentiation. Then, their expression of cell surface marker CD41, CD42b and HLA class I, β2M were analyzed.

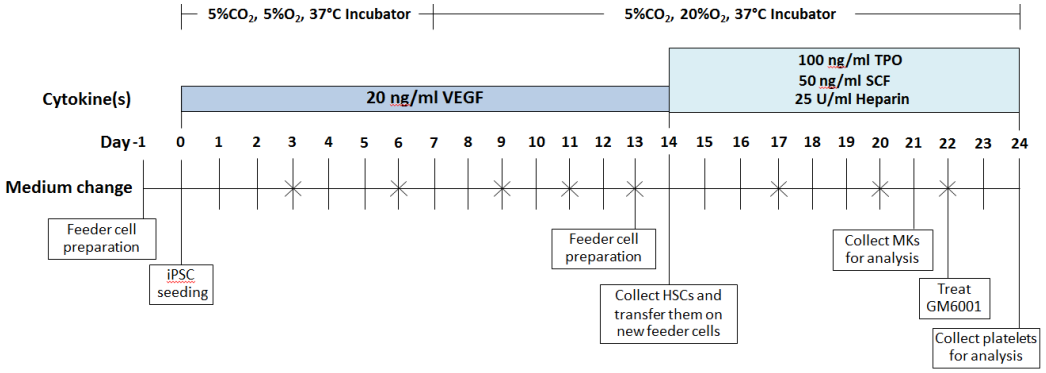


Figure 31 Schematic diagram represents in vitro megakaryocyte (MK) and platelet differentiation protocol via sac-like structure (ES-sac).

The protocol "In vitro generation of megakaryocytes and platelets from human embryonic stem cells and induced pluripotent stem cells" by Naoya Takayama and Koji Eto, 2012 was followed in this study.

Wild type-iPSCs, Mono- and Bi-allelic β*2m* knockout iPSCs were dissociated into small clumps or more than 100 cells per piece by CTK solution and transferred onto irradiated inactivated 10T1/2 cells. Then cultured in hematopoietic cell differentiation medium, IMDM which supplemented with cocktail of 15% FBS, 2mM L-glutamine, 5.5 µg/ml human transferrin, 10 µg/ml human insulin, 5 ng/ml sodium selenite, 0.45 mM α-monothioglycerol, 50 µg/ml ascorbic acid, and 20 ng/ml human vascular endothelial growth factor (VEGF). Media was changed every 2-3 days till day 7 cells were moved from hypoxic condition (5%O_2) to atmosphere condition (20%O_2) and on day 14-15 ES-sacs appeared and hematopoietic stem cells (HSCs) within sacs were collected and crushed with pipette gently passed through 40-µm cell strainer and transferred onto fresh irradiated or mitotically inactivated OP9 cells. Moreover, HSCs were characterized by flow cytometry to detect cell surface marker CD34, CD45, HLA, and β2M. APC/Cy7-conjugated anti-human CD34 (Biolegend), Brilliant Violet421-conjugated anti-human CD45, PerCP-conjugated anti-human HLA-A, B, C (Biolegend) and APC-conjugated anti-human β2-microglobulin

(Biolegend) were used to stain HSCs for hematopoietic cell analysis and HLA, β2M cell surface expression.

HSCs were then cultured in hematopoietic cell differentiation medium supplemented with 25U/ml heparin, 100 ng/ml human thrombopoietin (hTPO; R&D systems), 50 ng/ml human stem cell factor (hSCF; R&D systems) and half media change was performed every 2-3 days after first media change. GM6001, which is broad inhibitor of metalloproteases, was used to treat platelet culture 2 days before analysis without affecting platelet yield to prevent GPIbα and GPV from shedding out of platelets under culture conditions at 37°C by metalloproteinase (ADAM) family protein and disintegrin.

Characterization of iPSC-derived megakaryocytes and platelets

To detect megakaryocyte differentiation on day 21 and platelet-like particles on day 24, loosely adherent cells and floating cells were collected by one-ninth volume of acid citrate dextrose solution adding and centrifuged at 150×g for 10 min to collect megakaryocytes.

For platelet-like particles, one-ninth volume of acid citrate dextrose solution was added and centrifuged at 150×g for 10 min to precipitate any large cells including megakaryocytes. Then supernatant which contains platelets was transferred to new tube and was centrifuged at 400×g for 10 min to precipitate platelet pellet.

Cells were washed by 3%FBS in PBS and stained with FITC-conjugated anti-human CD41a (Biolegend), PE-conjugated anti-human CD42b (Biolegend) and PerCP-conjugated anti-human HLA-A, B, C (Biolegend), APC-conjugated anti-human β2-microglobulin (Biolegend) for both megakaryocytes and platelet-like particles at RT for 20 min. BD FACSAria II (Becton Dickinson, Franklin Lakes, NJ, USA) was used in flow cytometry analysis. In addition, prior to analyse platelets by flow cytometer, platelets were fixed by 1% paraformaldehyde at 4°C for at least 30 min after antibody staining.

iPSC-derived megakaryocyte morphology and functional analysis

Wright's staining

To observe morphology of iPSC-derived megakaryocyte, wright staining was used. On day 21, wild type-MKs, Mono- and Bi-allelic β*2m* knockout MKs were collected and dropped onto glass slide. Whatman filter paper was used to adsorb cell solution and cell pellet was let to dry. Then cell pellet was fixed with methanol and Wright's stain was use to stain cells. Morphology of MKs including size and multilobed nucleus was observed under light microscopy using 100x objective lens with immersion oil.

Polyploidy analysis of iPSC-derived megakaryocytes

To detect polyploidization of iPSC-derived megakaryocytes, propidium iodide (PI) was used to dye DNA content which can also be used for cell cycle analysis. On day 21 wild type-MKs, Mono- and Bi-allelic β*2m* knockout MKs were collected and fixed with 70% cold ethanol. In order to stain DNA, propidium iodide (life technology) was added in staining buffer which contains 4mM Sodium Citrate, 0.2mg/ml RNase A and 0.1% Triton X-100 in 1XPBS. Flow cytometry was used to detect percentage of cells that shows multiples of diploid chromosome content such as 4n, 16n, 32n, 64n. The data was analysed by BD FACSAria II (Becton Dickinson, Franklin Lakes, NJ, and USA) flow cytometer.

Proplatelet formation analysis of iPSC-derived megakaryocytes

To analyse ability of iPSC-derived megakaryocytes in proplatelet formation, proplatelet staining was performed. On day 21, wild type-MKs, Mono- and Bi-allelic β*2m* knockout MKs were collected and were reseeded onto coverslips coated with Matrigel® Matrix (Corning®) and cultured for 24 hour. After that, slides were fixed with 4% paraformaldehyde for 15 min at room temperature (RT). Then permeabilised with 0.3% Triton X-100 in 1XPBS for 15 min at RT and blocked by 10% goat serum for 30 min at RT. Proplatelets were then stained with anti-alpha tubulin antibody (Abcam) and Alexa Fluor™ 488 phalloidin (Molecular Probes, Invitrogen). Fluorescence images were obtained by using EVOS™ FL color Imaging Systems, Florescence Microscope (Life technologies, Thermo Fisher Scientific)

In vitro platelet functional test

Human blood platelet-rich plasma was prepared by centrifugation of Acid citrate dextrose (ACD)-treated human blood at 200×g for 15 min. Then supernatant was collected and added 1μM PGE1 to prevent platelet activation, and centrifuged at 400×g for 10 min to obtain platelet pellet. The pellet was wash with modified Tyrode-HEPES buffer at pH7.4 (10mM HEPES, 12mM $NaHCO_3$, 138mM NaCl, 5.5mM glucose, 2.9mM KCl, 1mM $MgCl_2$, pH7.4) [94] in an appropriate volume and centrifuged at 400×g for 10 min. The washed platelet pellet was resuspended with the modified Tyrode-HEPES buffer at pH7.4 added 1mM $CaCl_2$ and incubated at 37°C for 0.5-1h before functional tests were performed.

iPSC-derived platelets in culture medium were gently collected by added one-ninth volume of acid citrate dextrose solution (85mM sodium citrate, 104mM glucose, and 65mM citric acid) [94] and centrifuged at 150×g for 10 min to eliminate any large cells. Then supernatant was transferred to new tube and added 1μM PGE1 to prevent platelet activation, and centrifuged at 400×g for 10 min to obtained platelet pellet. The pellet was wash with modified Tyrode-HEPES buffer at pH7.4 (10mM HEPES, 12mM $NaHCO_3$, 138mM NaCl, 5.5mM glucose, 2.9mM KCl, 1mM $MgCl_2$, pH7.4) [94] in an appropriate volume and centrifuged at 400×g for 10 min. The washed platelet pellet was resuspended with the modified Tyrode-HEPES buffer at pH7.4 added 1mM $CaCl_2$ and incubated at 37°C for 0.5-1h before functional tests were performed.

Platelet activation assay

Platelets were stimulated by ADP (Adenosine diphosphate, R&D system); 50μM and Thrombin (Sigma); 1U/ml and incubated at 37°C for 1min. Then cells were fixed immediately by 1% paraformaldehyde, at 4°C for at least 30min and washed by staining buffer (3%FBS in PBS). To assess platelet activation by flow cytometry, platelets were stained with PE-conjugated anti-human CD62P antibody (P-selectin, Biolegend) and FITC-conjugated anti-human CD41 (Biolegend) for 20min at room temperature. Non stimulated platelets were used as a control group.

Platelet aggregation assay

Washed FITC-conjugated anti-human CD41 (Biolegend) labeled human blood platelets were mixed with washed PE-conjugated anti-human CD42b (Biolegend) labeled human

blood platelets or washed PE-conjugated anti-human CD42b (Biolegend) labeled iPSC-derived platelets and stimulated with ADP (Adenosine diphosphate, R&D system); 50μM and Thrombin (Sigma); 1U/ml and incubated for 20min at 37°C to trigger platelet aggregation. Then cells were fixed immediately by 1% paraformaldehyde, at 4°C for at least 30min and flow cytometry was used to assess double-colored events represented for platelet aggregation. Non stimulated platelets were used as a control group.

CHAPTER IV

RESULTS

Generation of HLA-universal iPSCs by paired CRISPR/Cas9 nickases

PCR-Sanger sequencing

Paired CRISPR/Cas9 nickases for Beta2-microglobulin gene disruption in exon 2 were constructed with 9 bp sgRNA offset, two sgRNA sequences were designed by the program, 5'-CCATCCGACATTGAAGTTGACTT-3' and 5'-TGGAGAGAGAATTGAAAAAGTGG-3' both were represented as target 6.1 and 6.2, respectively (Figure 32A). T7E1 assay showed efficiency of the paired CRISPR/Cas9 nickases with expected PCR product bands at size around 228 bp and 320 bp in transfected group (Figure 32B) and PCR-Sanger sequencing confirmed donor recombination detection of all PCR bands.

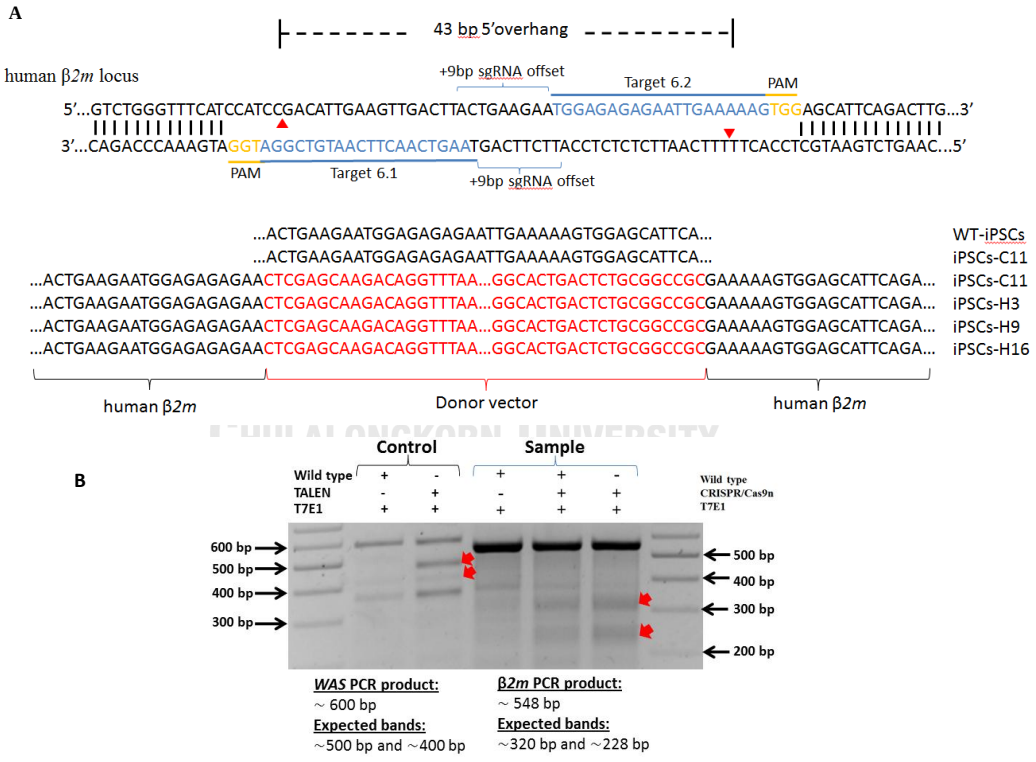


Figure 32 Schematic and agarose gel electrophoresis showed paired gRNAs and T7E1 results. A) two gRNAs with 9 bp offset were designed to target human β2m in exon2, after they induced Cas9 nickase proteins to the target site, double strand DNA breaks and resulting in 43 bp 5'overhang as indicated. Sanger sequencing results revealed β2m sequences of wild type allele of WT-iPSCs and iPSCs-C11, while donor sequences in

between β2m sequences were found in iPSCs-C11, iPSCs-H3, iPSCs-H9, and iPSCs-H16 **B)** CRISPR/Cas9nickase efficiency detected by T7E1 assay which showed the expected PCR product bands at size around 228 bp and 320 bp in transfected groups, positive control from TALEN targeting to *WAS* gene.

After transfection of both paired CRISPR/Cas9 nickases and donor vectors into human wild type- iPSCs, there were many iPSC clones can survive under hygromycin selection condition. Then survived iPSC clones were picked up for proliferation and PCR amplified for donor recombination detection.

In wild type-iPSCs; there was only wild type allele band (548bp). iPSCs-C11 showed left donor recombination band (796bp), right donor recombination band (858bp) and wild type allele band (548bp). While iPSCs-H3, iPSCs-H9, iPSCs-H16 showed left donor recombination band (796bp), right donor recombination band (858bp) but wild type allele band was not found. The PCR amplification results are shown in Figure 33.

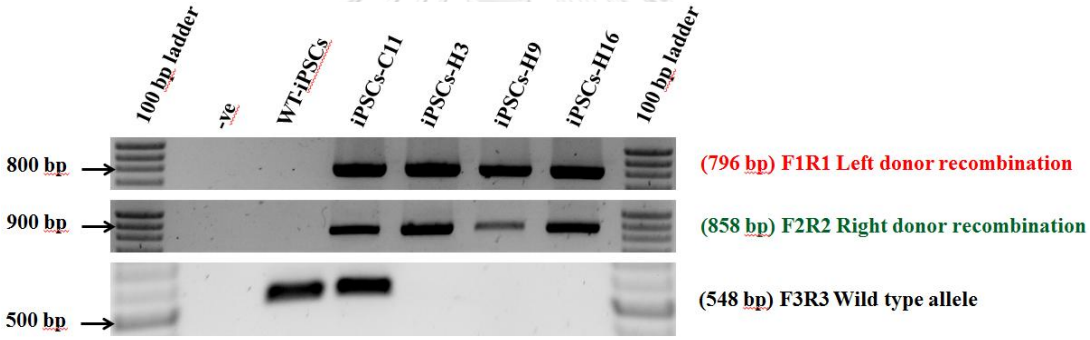


Figure 33 PCR amplification results of donor recombination detection. Gel electrophoresis represents donor recombination detection results of wild type-iPSCs with wild type allele band, iPSCs-C11 with both donor recombination bands and wild type allele band, and iPSCs-H3/H9/H16 with only donor recombination bands. 100-bp ladder was used as base pair measurement.

HLA class I and β2M cell surface expression on β2M^{ko}-iPSCs

Cell surface expression of HLA and β2M of wild type-iPSCs, mono- and bi-allelic β2M^{ko} –iPSCs was measured by flow cytometry. 99.9% of wild type-iPSCs expressed β2M and 96.6% of them expressed HLA-A, B, C while 95.1% of mono-allelic β2M^{ko} –iPSCs did not express either HLA-A, B, C or β2M with shift increasing expression trend when compared with bi-allelic β2M^{ko} –iPSCs. In contrast, 99.5%, 100%, and 99.6% of bi-allelic β2M^{ko} –iPSCs clone 3, clone 9, and clone 16, respectively did not express either HLA-A, B, C or β2M (Figure 34).

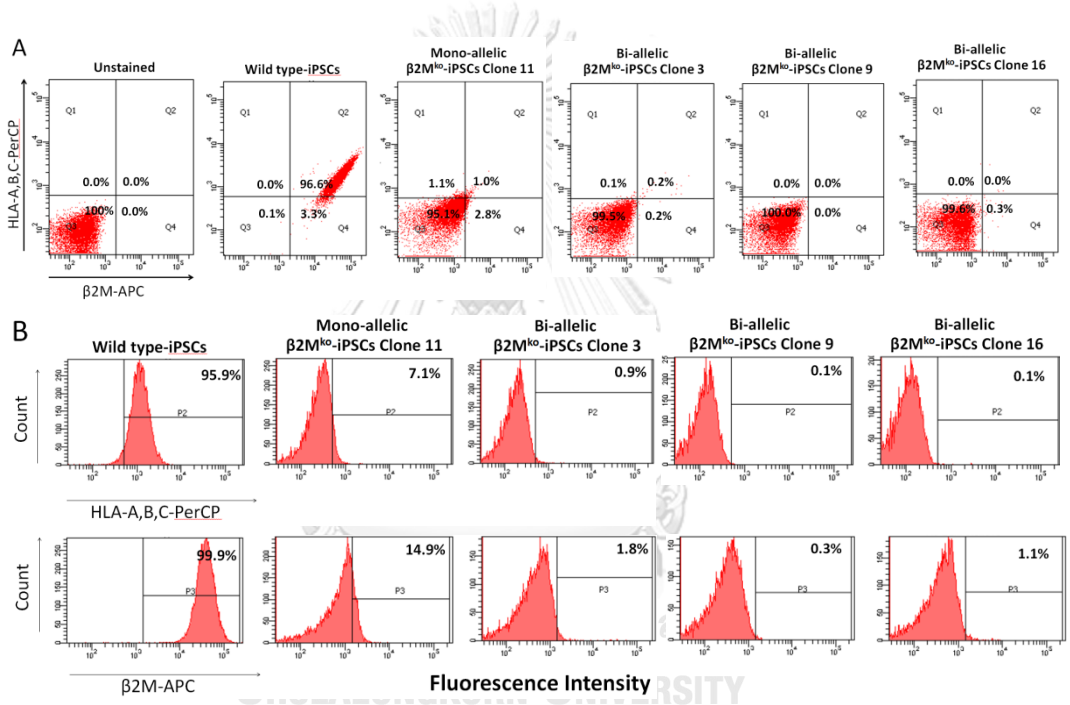


Figure 34 Flow cytometry dot plot of HLA class I and β2M cell surface expression on iPSCs **(A)** and represented in Fluorescence intensity histogram **(B).** Wild type-iPSCs, mono- and bi-allelic β2M^{ko} –iPSCs were stained using PerCP-conjugated anti-human HLA-A, B, C and APC-conjugated anti-human β2-microglobulin when compared with unstained group.

β2M^{ko}-iPSC characterization

All 5 iPSC lines (wild type-iPSCs, mono-allelic β2M^{ko} –iPSCs and 3 lines of bi-allelic β2M^{ko} –iPSCs) sustained embryonic stem cell-like morphology with normal karyotype (Figure 35C). In addition, they expressed pluripotency markers; OCT4, NANOG, SSEA-4, TRA-

1-60 and TRA-1-81(Figure 35A). Their pluripotency were verified by embryoid body differentiation to three germ layers including the marker expression which is α-fetoprotein in endoderm, Brachyury in mesoderm and Nestin in ectoderm, DAPI (blue) was used for nuclear staining (Figure 35B).

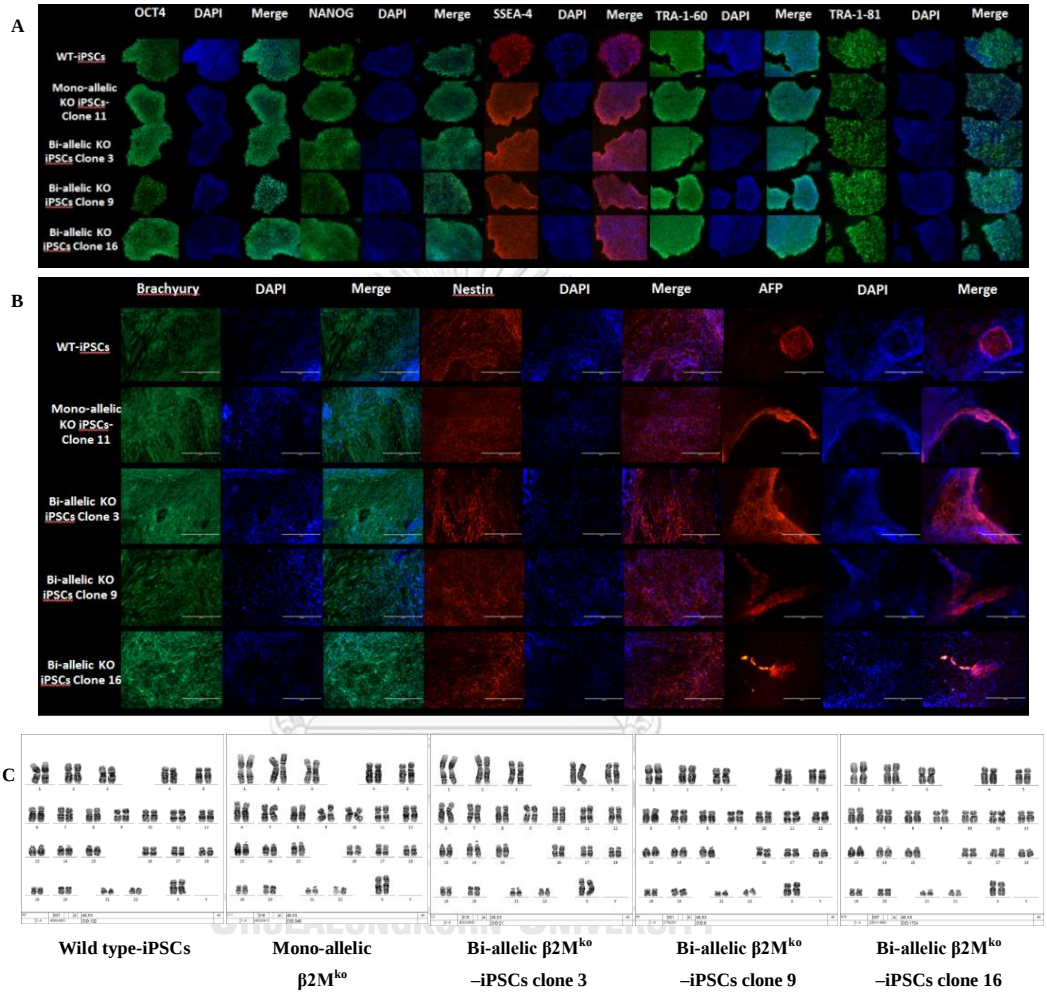


Figure 35 iPSC characterization. A) Immunofluorescence staining showed pluripotency marker expression; OCT4, NANOG, SSEA-4, TRA-1-60, TRA-1-81 and B) α-fetoprotein (endoderm), Brachyury (mesoderm) and Nestin (ectoderm). DAPI (blue) was used for nuclear staining. All iPSC lines were pluripotent. C) Showing normal karyotypes of all iPSC lines.

β2M^{ko}-iPSC-derived hematopoietic stem cell characterization and HLA expression

ES-sac method was used to produce CD34+ hematopoietic stem cells from wild type-iPSCs, mono-allelic β2M^{ko} –iPSCs and 3 lines of bi-allelic β2M^{ko} –iPSCs. ES-sac like structures were observed after two weeks (14 days) of co-culture with 10T1/2 feeder cell and VEGF treatment from all iPSC lines (Figure 36A). Flow cytometry was used to analyze hematopoietic stem cells which obtained from ES-sac using APC/Cy7-conjugated anti-human CD34, Brilliant Violet421-conjugated anti-human CD45, PerCP-conjugated anti-human HLA-A, B, C and APC-conjugated anti-human β2-microglobulin. Hematopoietic stem cells from mono-allelic β2M^{ko} –iPSCs and 3 lines of bi-allelic β2M^{ko} –iPSCs exhibited similar differentiation rate with wild type group (Figure 36B). Moreover 99% of Mono- and bi-allelic β2M^{ko} –HSCs for all lines did not express either HLA-A, B, C or β2M, while 27.9% of wild type-HSCs expressed β2M with 0.7% of them expressed HLA-A, B, C (Figure 36C).

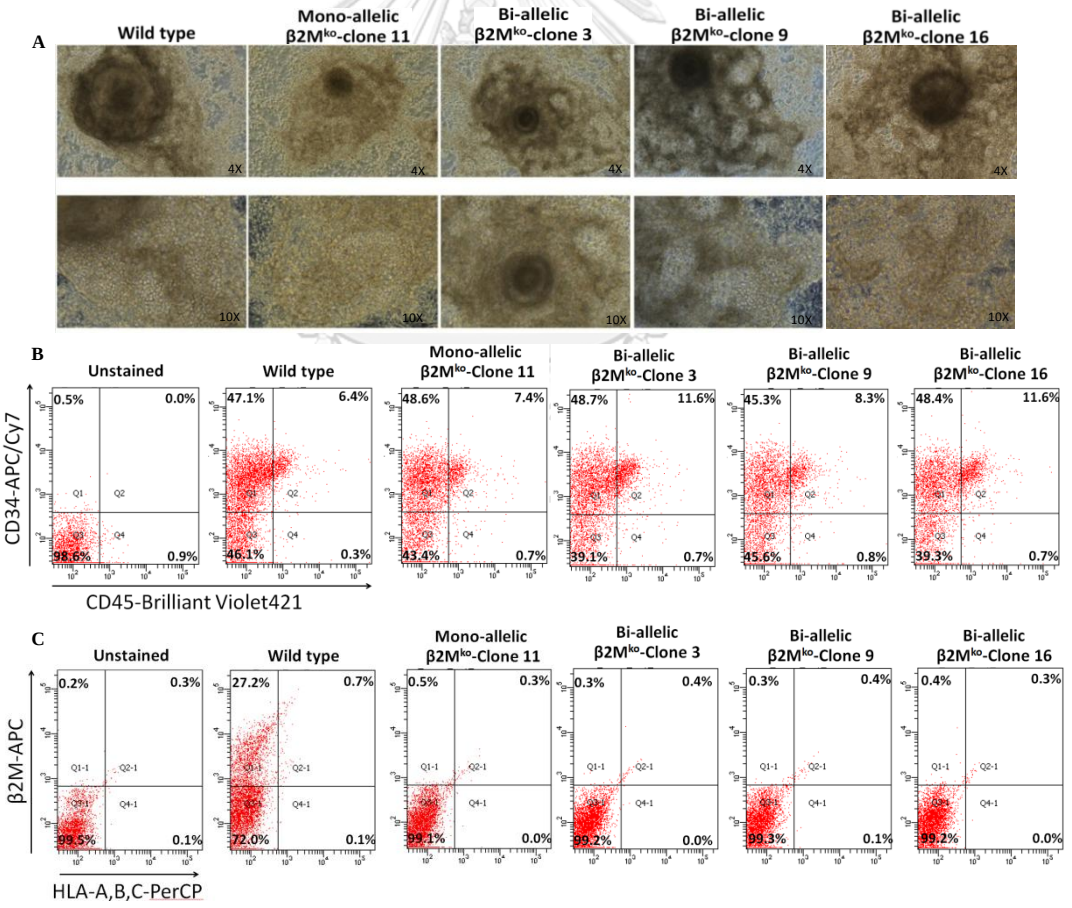


Figure 36 Characterization of Hematopoietic stem cells by flow cytometry. On day14, showing A) phase contrast photomicrographs of ES-sacs which were generated from wild type-iPSCs, mono-allelic β2M^{ko} –iPSCs and 3 lines of bi-allelic β2M^{ko} –iPSCs; magnification 4X and 10X. Flow

cytometry dot plot of progenitor cells which isolated from ES-sacs showing (B) CD34 expression and (C) HLA-A, B, C and β2M expression of all iPSC-derived hematopoietic stem cells lines.

β2M^{ko}-iPSC-derived megakaryocyte characterization and HLA class I expression

CD41/CD42b marker expression and HLA class I expression

In all 5 iPSC lines, Hematopoietic stem cells were isolated from ES-sacs and cultured in hematopoietic cell differentiation medium supplemented with TPO, SCF and Heparin. After 7 days of culture (day21), iPSC-derived MKs of all lines was analyzed by flow cytometry using FITC-conjugated anti-human CD41, PE-conjugated anti-human CD42b, PerCP-conjugated anti-human HLA-A, B, C and APC-conjugated anti-human β2-microglobulin showing in the flow cytometry dot plots. The results revealed that Mono-allelic β2M^{ko}–iPSCs and 3 lines of bi-allelic β2M^{ko}–iPSCs could generate CD41+/CD42b+ megakaryocytes in the similar differentiation rates with wild type group (Figure 37A). Moreover 98.3% of mono-allelic β2M^{ko}–iPSC-derived MKs and 99.6%, 99.8%, and 98.8% of Bi-allelic β2M^{ko}–iPSC-derived MKs clone3, clone9, and clone16 did not express either HLA-A, B, C or β2M when compared with unstained group (98.8% no expression), while 64.1% of wild type-iPSC-derived MKs expressed β2M which 3.1% of them expressed HLA-A, B, C. (Figure 37B)

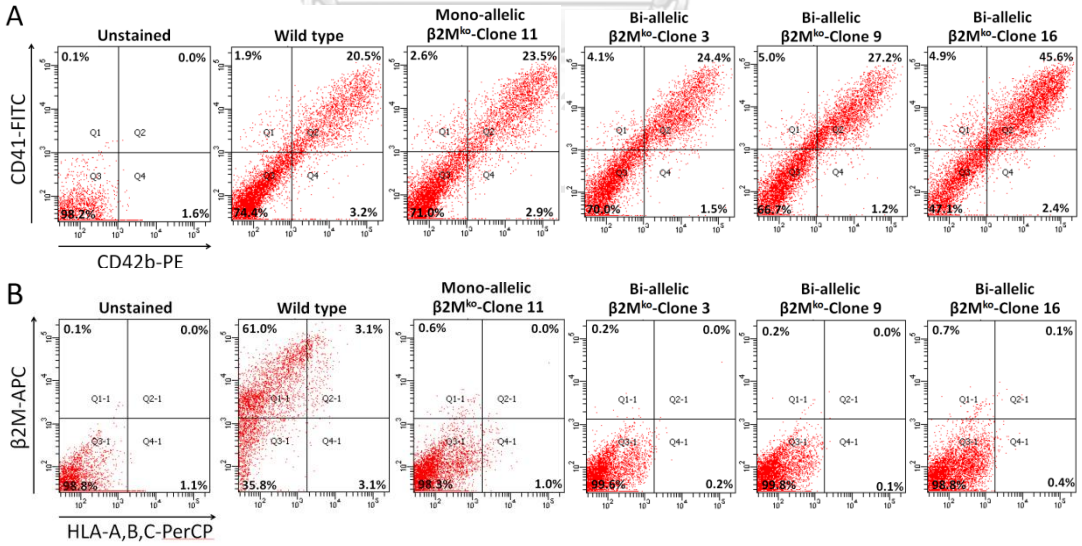


Figure 37 Detection of HLA class I and β2M expression on megakaryocytes by flow cytometry. MKs differentiated from wild type-iPSCs, mono-allelic β2M^{ko}–iPSCs and 3 lines of bi-allelic β2M^{ko}–iPSCs showing in A) flow cytometry dot pots revealed CD41+/CD42b+ expressing iPSC-derived MKs in all iPSC lines and B) HLA-A, B, C and β2M expression.

Proplatelet formation, polyploidy analysis and megakaryocyte morphology

On day 21 of cultures, megakaryocyte colonies with proplatelet forming were observed which mono-and bi-allelic $\beta 2M^{ko}$–iPSC-derived MKs performed proplatelet hallmark features including the tip, shafts, a branch point and swellings as same as wild type group (Figure 38A) the features were also found in immunofluorescence (IF) staining results (Figure 38B). In addition of megakaryocyte characterization by phase contrast photomicrographs, IF and flow cytometry, propidium iodide (PI) staining was used to analyze polyploidy nuclei of all iPSC-derived MKs generated from wild type-iPSCs, mono- and bi- allelic $\beta 2M^{ko}$-iPSCs. Mono-and bi- allelic $\beta 2M^{ko}$-iPSC-derived MKs showed DNA contents higher than 8n in similar percentage with wild type–iPSC-derived MKs (Figure 38D). Moreover all lines of iPSC-derived MKs were also dyed by Wright's stain to verify MK morphology which the mono-and bi-allelic $\beta 2M^{ko}$-MKs displayed multilobed nucleus and large size as wild type-MKs (Figure 38C).

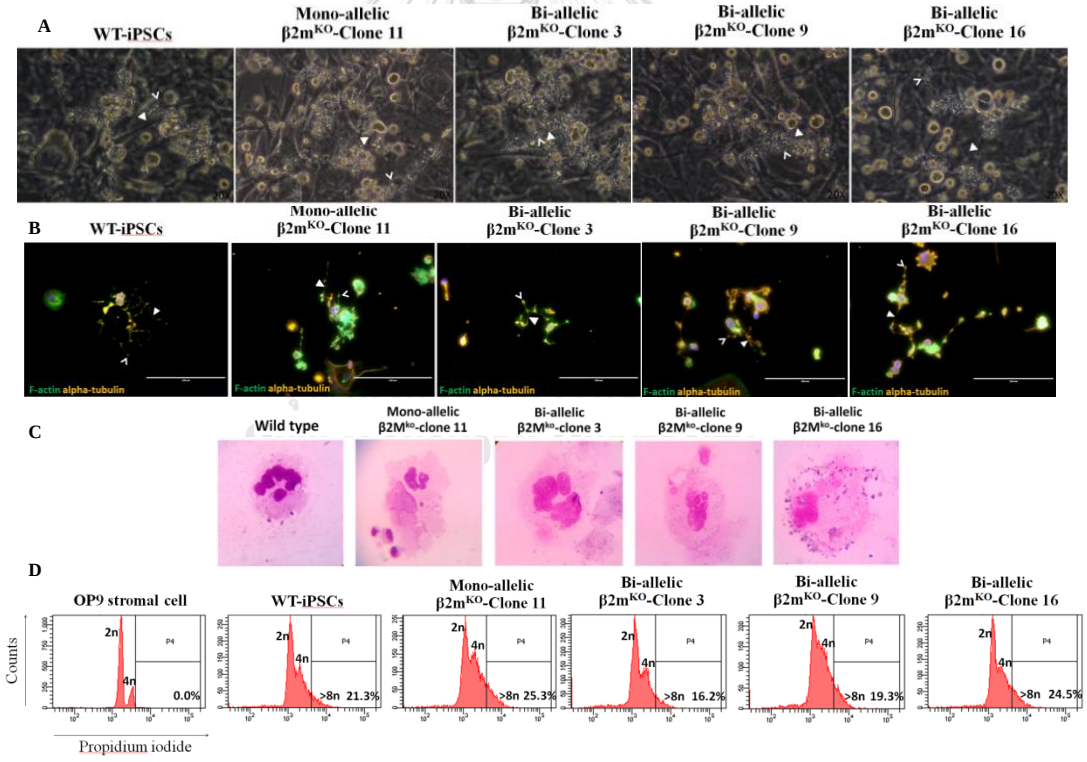


Figure 38 Megakaryocyte characterization by proplatelet formation, MK morphology and polyploidy analysis. iPSC-derived megakaryocytes for all lines showing in **A)** phase contrast photomicrographs represented megakaryocyte colonies with proplatelet forming on day21 of cultures. **B)** Immunofluorescence staining in proplatelet formation assay. **C)** Wright staining and **D)** polyploidy analysis in flow cytometry histogram.

β2M^{ko}-iPSC-derived platelet characterization and HLA expression

Platelet specific antigens CD41 (GPIIb) and CD42b (GPIbα) were used for platelet identification. On Day 24 of culture, platelet-like particles were separated from any larger cells by centrifugation and the number of platelet-like particles was analyzed by flow cytometry staining with FITC-conjugated anti-human CD41, PE-conjugated anti-human CD42b, PerCP-conjugated anti-human HLA-A, B, C and APC-conjugated anti-human β2-microglobulin. The flow cytometry dot plots showed that mono-allelic β2M^{ko}–iPSCs and 3 lines of bi-allelic β2M^{ko}–iPSCs could generate CD41+CD42b+ platelets in the similar differentiation rates with wild type group (Figure 39A). Moreover, they showed that 98.4% of mono-allelic β2M^{ko}-iPSC-derived platelets and 98.3%, 99.4%, 99.3% of bi-allelic β2M^{ko}-iPSC-derived platelets clone3, clone9, and clone16, respectively did not express either HLA-A, B, C or β2M when compared with unstained group (99.5% no expression). While 56.0% of wild type-iPSC-derived platelets expressed β2M which 3.1% of them expressed HLA-A, B, C (Figure 39B).

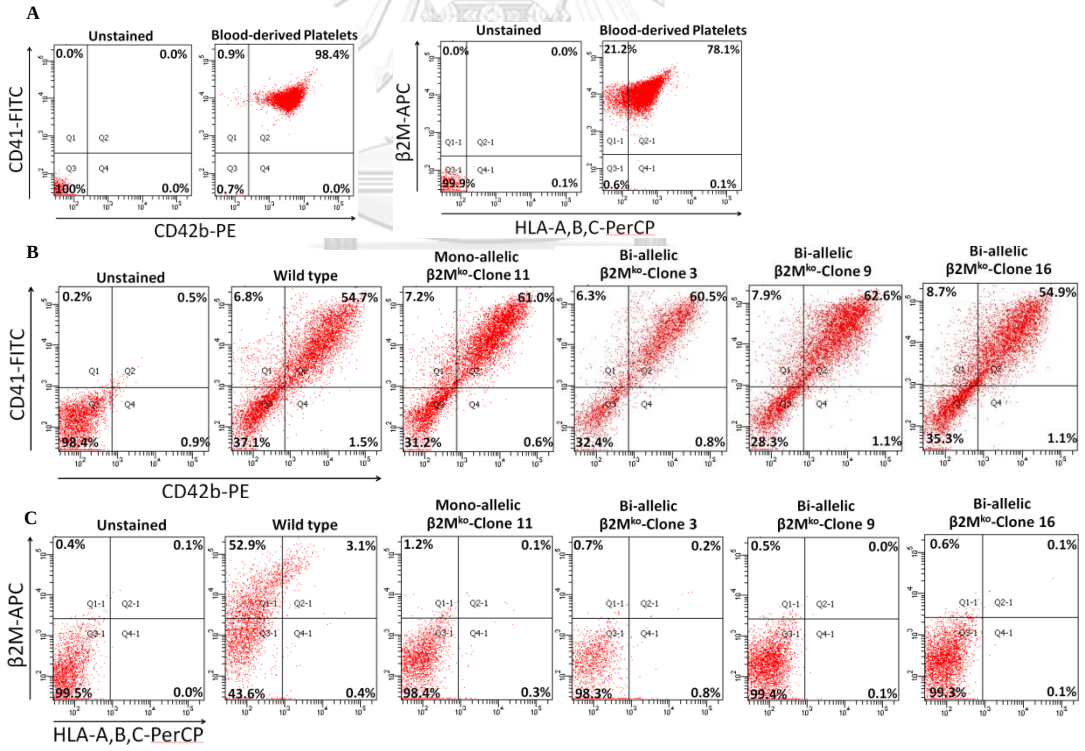


Figure 39 Platelet characterization and HLA-class I, and β2M expression analysis by flow cytometry. Platelets differentiated from blood **A)**. Wild type-iPSCs, mono-allelic β2M^{ko}–iPSCs and 3 lines of bi-allelic β2M^{ko}–iPSCs showing in flow cytometry dot plots revealed expression of **B)** D41, CD42b and **C)** HLA-A, B, C and β2M expression analysis in all iPSC-derived platelet lines.

Functional assessment of β2M^{ko}-iPSC-derived platelets

Platelet activation analysis

On day 24 of culture, iPSC-derived platelets in all lines were collected and stimulated with the classical platelet agonists ADP and thrombin [94] then platelet activation and aggregation results were analyzed using flow cytometry. The results showed that mono-and bi-allelic β2M^{ko}-iPSC-derived platelets were up regulated CD62P expression when compared to the expression frequency of non-stimulated group in which 33.8% from 12.7% of CD41+ expressing mono allelic β2M^{ko}-iPSC-derived platelets, 48.4% from 17.8%, 51.1% from 12.2%, and 36.3% from 10.5% of CD41+ expressing bi-allelic β2M^{ko}-iPSC-derived platelets clone3, clone 9 and clone16, respectively. The expression frequencies were comparable with wild type group where stimulated at 42.5% from 19.3% when non-stimulated. The results were in the same trend with CD41+ expressing peripheral blood-derived platelet group which expressed CD62P when stimulated to 97.3% from 2.1% (Figure 40A). Moreover, fluorescence intensity histogram also showed up regulated CD62P expression of CD41+cells after stimulated with ADP and thrombin when compared with non-stimulated group of all iPSC-derived platelet lines and blood-derived platelets (Figure 40B).

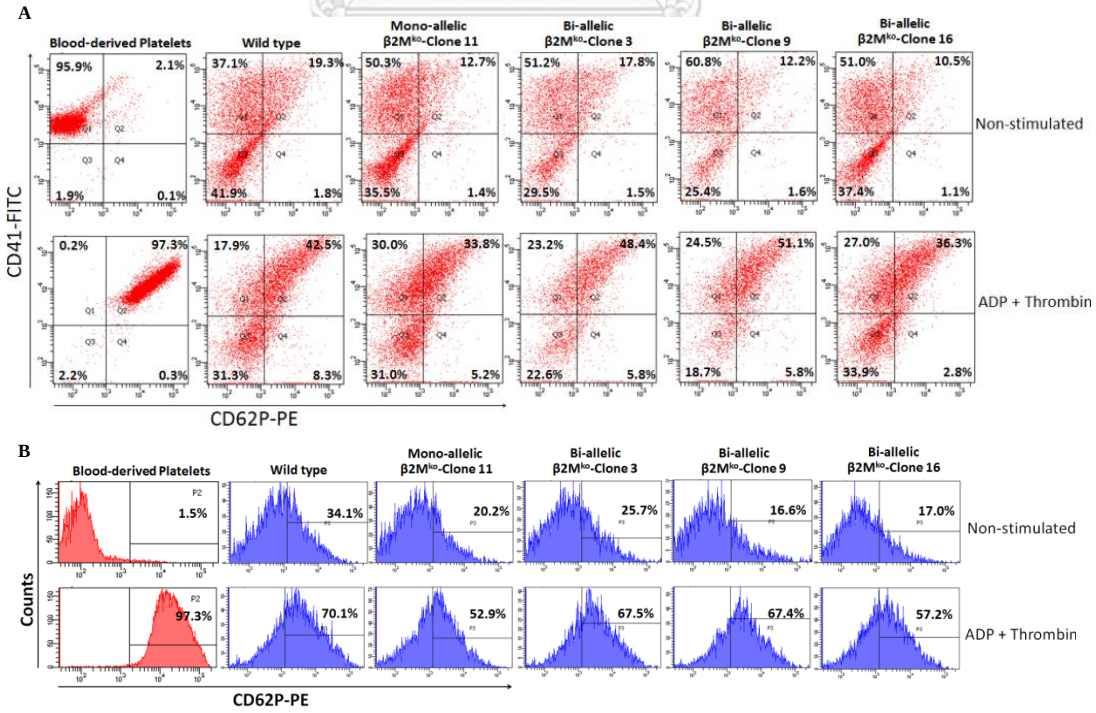


Figure 40 Platelet activation analysis by flow cytometry. CD62P (P-selectin) expression on wild type-iPSC-derived platelets, mono-and bi-allelic β2M^{ko}-iPSC-derived platelets and peripheral blood-

derived platelets were measured in the presence and absence of ADP (50μM) and thrombin (1U/ml) showing in **A)** CD41, CD62P flow cytometry dot plots and **B)** fluorescence intensity histogram of CD41+cells.

Platelet aggregation analysis

In addition to platelet activation assay on day24 of culture, platelet aggregation assay was also performed to assess platelet function. Flow cytometry dot plots showed that double-colored events in quadrant 2 increased after stimulated by ADP and thrombin from 9.5% to 78.1% of blood-derived platelet group, from 9.6% to 16.3% of wild type-iPSC-derived platelet group, from 6.2% to 24.3% of mono-allelic β2M^{ko}-iPSC-derived platelet group, form 6.0% to 19.9%, 9.5% to 24.1%, and 10.7% to 24.2% of bi-allelic β2M^{ko}-iPSC-derived platelet clone 3, clone 9, and clone 16, respectively. (Figure 41)

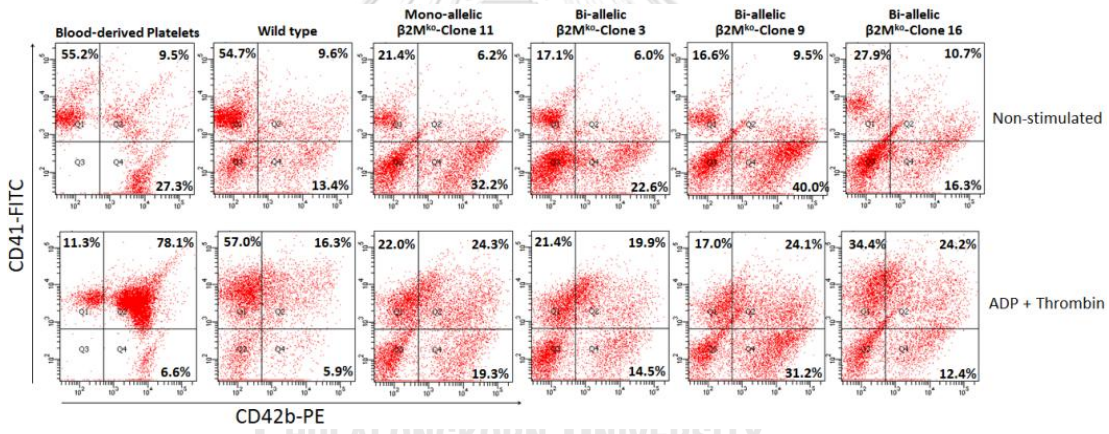


Figure 41 Platelet aggregation analysis by flow cytometry. Dot plots showed platelet aggregation after stimulated with ADP (50μM) and thrombin (1U/ml) when compared with non-stimulated group of blood-derived platelets, wild type-iPSC-derived platelets, mono-allelic β2M^{ko}-iPSC-derived platelets and bi-allelic β2M^{ko}-iPSC-derived platelets in all lines

CHAPTER V
DISCUSSION

Platelet component demand has been reported to be increased over the decade in the United States, Europe, England, and Australia resulting from many factors including the rise of aging population, hematological malignancy incidence, and hematopoietic stem cell transplantation [95]. Even in Thailand, National Blood Centre, Thai Red Cross Society, has been opened for single donor platelets and other blood components to support the hematological patients. In fact, platelet component is directly related with the risk of life-threatening hemorrhage which can be found in the patients with inadequate response to platelet transfusion called platelet transfusion refractoriness. Alloimmune to HLA class I is the main problem (up to 90%) of immune factors as well-known [11]. Although finding of HLA-matched donor or cross-matched platelets is currently management of this problem, but inadequate of donor or matched donors, time consuming, and cost all are limits of this management. Beside those limits, HLA-matched platelets transfusion has been found unsuccessful in 20% to 50% of severe alloimmunized patients [96]. Even large iPSC haplobanks which instituted with hope to most frequent HLA haplotypes covering, cannot fully match minor histocompatibility antigens (mHAs) between recipients and iPSC-derived products which is the risk for immune rejection [97].

Recently, paired cas9 nickases have been found that their on-target efficiency is mostly comparable and sometimes higher than Cas9 nucleases, with their barely detectable off-target effects make them be useful system when specific genome editing and efficiency are required [98]. Therefore paired cas9 nickases system is suitable for cell products for regenerative medicine or transfusion into human which needs both efficient and specific genome editing. Since iPSCs are harder manipulated for genome editing than those human cell lines [99] and platelet generation from iPSCs must be through differentiation stages which need many cell signaling pathway correctly, so using paired cas9 nickases system is the better choice for lower off-target and high efficiency in iPSC genome engineering. We knocked out β*2m* gene in exon2 in order to disrupt HLA class I expression on platelets which this gene also be the target in many previous studies. Since β2m molecule is light chain of dimeric structure of HLA class I molecule, it is necessary for HLA class I presentation on cell surface, and encoded by only one gene, then many

studies have chosen β*2m* gene as a target to disrupt HLA class I cell surface expression. In addition, we chose to target the initiation of exon2 because it is proper for gene disruption, it covers every β*2m* transcript forms and its length is appropriated for paired CRISPR/Cas9 nickases design. The efficiency of the generated CRISPR was seen by T7E1 and we proved knocking out of β*2m* gene by both PCR and Sanger sequence. Then finally we confirmed HLA class I and β2M deficiency on bi-allelic β2M^{ko}-iPSC surface, and a little bit expression on mono-allelic β2M^{ko}-iPSC surface. We used unstained sample to represent cells with no expression because in this sample we did not stain antibody, then it told us how much auto fluorescence was of the cells and is useful in expression gating. It is as expected that paired CRISPR/Cas9 nickases system generated HLA class I-deficient iPSCs completely, while RNAi technology showed up to 82% reduction of HLA class I expression on iPSC surface [100].

In the present, there are protocols to generate platelets from iPSCs as in the previous mentioned works above. In 2008, Takayama et al. developed protocol for in vitro generation of functional platelets from human embryonic stem cells and iPSCs via ES-sacs (sac-like structures derived from ESCs), which concentrated and provided appropriated conditions for hematopoietic progenitor cells to yield MKs that form proplatelets and then release platelets with around $(4.8\pm0.2)\times10^{6}$ platelets from initial 10^{5} hESCs [101]. Besides, the enough amounts of platelets for our study by using the protocol of Takayama et al., our colleague in CU STEM CELL & CELL THERAPY RESEARCH CENTER has been successfully generated functional platelets, so this protocol has been followed for this study. We could generate ES-sacs containing HSCs, MKs, and platelets with similar rate of differentiation and they could function after we assessed their activation and aggregation capacity in all iPSC lines including wild type, mono-and bi-allelic β2M^{ko}-iPSCs. The level of differentiation rate commonly depends on the experiences of the researchers because there are many differentiation factors from the first step including iPSCs, feeder cells, differentiation media, incubators, environments, etc.

Although the previous studies of other research groups with β*2m*-knocking out did not report HLA class I and β2M expression on HSCs, but we found that wild type iPSC-derived HSCs barely expressed HLA class I and expressed β2M in just small number of them, and as expected, bi-allelic β2M^{ko}-iPSC-derived HSCs did not express either HLA class

I or β2M and also in mono-allelic β2M^{ko}-iPSC-derived HSCs. And the previous studies using RNAi technology showed reduction of HLA class I expression on CD34+HSCs up to 85% when compared with nontransduced CD34+HSCs that expressed HLA class I, which is not surprising because of gene knocking down, noted that their CD34+HSCs came from peripheral blood [20]. But it is surprising that HLA class I expression is different between wild type-iPSC derived HSCs and blood-CD34+HSCs. Then we differentiated HSCs to MKs, we noticed increasing number but not hundred percent of wild type-iPSC derived MKs that expressed β2M and HLA class I when compared with their HSC precursor cells. There is no cell surface expression either HLA class I or β2M on both mono-and bi-allelic β2M^{ko}-iPSC-derived MKs while other research group using RNAi technology found 65% reduction of HLA class I expression on MKs when compared with nonmanipulated MKs, they showed in graph of fold HLA class I expression [100]. Our knocked out-MKs for all lines could form proplatelets and exhibited polyploidy nuclei as other study using RNAi technolog [100]. The last step, differentiation of MKs to platelets, we revealed that in blood-derived platelets only 62.3% of them expressed HLA class I cell surface which was very different from wild type-iPSC-derived platelets (3.8%). However, bi-allelic β2M^{ko}-iPSC-derived platelets did not express either HLA class I or β2M on cell surface, mono-allelic β2M^{ko}-iPSC-derived platelets did not express HLA class I but very small number of cells expressed β2M on cell surface when compared with unstained group. About platelet function, our mono-and bi-allelic β2M^{ko}-iPSC-derived platelets showed up regulation of CD62P (P-selectin) which is expressed only on activated platelets, not resting platelets. This CD62P up regulation was also found in HLA class I-knocking down platelets which they showed platelet aggregation as well representing functional platelets [100]. Interestingly, our iPSC-derived platelets around 60-70% of cells showed CD62P up regulation lesser than blood-derived platelets which 97.3% of cells expressed CD62P; exhibiting activation capacity of iPSC-derived platelets was lower than blood-derived platelets, this phenomenon is similar with the other previous study [100].

Platelet aggregation assay based on flow cytometry [102] which has advantage on sample with small amount of platelets; although quadrant 2 (double-colored events) represents platelet aggregation, but some platelet aggregates also come from the same group representing in quadrant 1 and 4 of one-colored events. After stimulation, in all lines of iPSC-derived platelets, percentage of double-colored events was increased, in contrast,

percentage of single-colored events was decreased but not much as in blood-derived platelet group that might be because functional activity of blood-derived platelets is higher than iPSC-derived platelets. Here, it might be CD41 antibody-labeled blood-derived platelets function better than CD42b antibody-labeled iPSC-derived platelets, resulting to intensity of double-colored events shifted to CD41 positive position rather than to the middle of quadrant 2 as shown in the blood-derived platelet group. Labeling platelets with CD41 and CD42b antibodies as we did may be an ease, but it might also get concern in platelet aggregation assay. Since we labeled one antibody per one tube of platelets with HIP8 (CD41 antibody we used in this study) which reported to block GPIIb/IIIa function in firm adhesion of LS174T cells to platelets [103] and HIP1 (CD42b antibodies we used) which reported to inhibit MK colony formation slightly and inhibit proplatelet formation strongly[104], but there is still no evidence about inhibition of platelet-platelet aggregation. However, our result in blood-derived platelet control group showed blood platelet aggregation between two populations (double colored-events) separating from single colored-groups clearly, so it may reflect to aggregation results of all lines of iPSC-derived platelets. It might be explained that not all mixed platelets from two different antibody labeled-groups would be inhibited both GPIIb/IIIa and GPIbα, one group still had normal GPIIb/IIIa and another still had normal GPIbα. Besides, in common pathway of platelet aggregation, GPIIb/IIIa complex is a main adhesion molecule involving in platelet aggregation since it presents on platelets at high density (60,000-80,000 copies per cell) and presents in α-granules of platelets which exposed on cell surface on activation [34]. From this fact, the newly GPIIb/IIIa complex would be exposed on platelet surface after we stimulated with ADP and thrombin, then they could function normally in aggregation process instead if the case of inhibited surface GPIIb/IIIa could not be function. GPIIb/IIIa, this complex will be conformational change after platelet stimulated with agonists, in this point PAC-1 antibody can be used to detect this activated GPIIb/IIIa, and the activated form can bind to fibrinogen as a bridge molecule then form platelet aggregation, besides, the activated GPIIb/IIIa can also bind to fibronectin, von Willebrand factor (vWF), and vitronectin. Moreover, these plasma proteins can bind to other platelet molecules such as GPIb-V-IX, integrins $\alpha_v\beta_3$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, etc [34]. Furthermore, other previous studies revealed HLA class I-deficient platelets could function via activation and aggregation [100] [20]. Accordingly, it is possible that our platelet aggregation results might be real and our

HLA class I-deficient iPSC-derived platelets could function after stimulated with agonists. However, we recommend using other antibodies which do not disturb any platelet function for clearly explanation about your platelet function.

In addition to stop bleeding by clot formation, platelets can also secret cytokines and growth factors which play important role in wound healing process **[105].** The role of platelets does not stop in only hemostasis step, but they involve in later steps which are inflammation, cell proliferation and remodeling because they recruit several cells to the injury site and such alpha granule degranulation causes releasing of many growth factors relevant to wound healing process. The growth factors including PDGF (platelet derived growth factor), EGF (epidermal growth factor), VEGF (vascular endothelial growth factor), and TGFβ (transforming growth factor) have been reported that secreted from platelets, so platelet-rich plasma (PRP) which contains concentration of growth factors is widely applied in clinical applications of promoting wound healing. **[105]** Moreover, anti-aging therapy and platelet-rich plasma relevance has been studied by many research groups but this subject still be debated because of variety of PRP collection or growth factor concentration range. Many growth factors released from activated platelets could stimulate dermal fibroblast proliferation, increase MMPs gene expression and type I collagen, these evidences showed potential in promoting skin remodeling [106]. However, for safety of these applications, the PRP should come from autologous blood that means our generated HLA-deficient platelets might be useful not only hemostasis but wound healing and anti-aging therapy for anyone.

Finally, we generated *in vitro* functional iPSC-derived platelets with HLA class I deficiency by knocking out β*2m* gene using paired CRISPR/Cas9nickases. This idea is not unique, it is competitive filed but we have some niche. Since we started this project a little bit later than other groups, and that gives us the opportunity to use the later technique to knock out gene which is paired CRISPR/Cas9nickases. The genome editing system which is high efficient on target and barely detected off-target, can be applied for regenerative medicine field. Moreover, our data might support the studies about

generation of functional HLA-deficient platelets from iPSCs *in vitro*. Meanwhile, we could generate HLA class I-deficient iPSCs, as a universal donor cells for all HLA class I types because their properties are both self-renewal and differentiation into all cell types of body. The studies about HLA class I-deficient hESCs or human pluripotent stem cells [24, 72] showed hypo-immunogenicity (recognition by $CD8^+$ T cells and NK cells) *in vitro* and *in vivo.* Hopefully, our generated HLA class I-deficient iPSCs could act as a universal donor cells with hypo-immunogenicity. Besides, our study revealed the generated HLA class I-deficient iPSCs capacity in differentiation into three germ layers and to HSCs, MKs, and platelets. However, it should be considered in immune response testing of all differentiated cells from the HLA class I-deficient iPSCs because from previous study [107] bi-allelic β*2m*-knockout hematopoietic donor cells were eliminated by NK cells in mice but this missing-self response was not found in the case of bi-allelic β*2m*-knockout solid organs transplantation in mice and in the case of day 15 bi-allelic β*2m*-knockout EB cells [72]. About tumor formation concern, HLA class I-deficient human and mice are not tumor-prone and for viral infection concerns, bi-allelic β*2m*-knockout mice can survive influenza or Sendai virus infection [72]. These suggest that the HLA class I-deficient iPSCs could be applied with no adverse effects.

Accordingly, many research groups try to generate HLA-universal platelets in vitro as the alternative sources. Since 2010, Constança Figueiredo et. al generated HLA-deficient platelets from hematopoietic progenitor cells (CD34+) using lentiviral-based system to express short-hairpin RNA targeting β*2m* transcripts [20]. In 2013, they showed that the HLA-silenced MKs and platelets could be prevented from anti-HLA antibody mediated cytotoxicity *in vitro* and *in vivo* [21]. Until 2016, they used the same technique to silence HLA expression, but this time in iPSCs, they generated HLA-universal iPSC-derived MKs and platelets which could be survival under refractoriness conditions *in vitro* and *in vivo* [100]. In 2014, Feng et al. scalable generated HLA-universal platelets from human iPSCs using TALEN technology to knock out β*2m* gene [108]. Another research group leading by Daisuke Suzuki and Koji Eto et al., in 2016, reported their study about beta2-microglobulin gene knocked out-MKs and platelets by CRISPR/Cas9 system in iPSCs and NK cell

cytotoxicity activity. They found that the generated HLA class I-knocked out platelets and HLA class I-expressing platelets were immunologically inert for NK cells, while the generated HLA class I-knocked out MKs were attacked potentially by NK cells.

Disruption of HLA expression on platelets was interested by previous research groups as described above, some used RNAi technology to knock down the expression resulting to residual HLA class I expression on cells, some used TALEN and CRISPR/Cas9 system to knock out gene leading to completely HLA-deficient cells. Relevance of HLA class I expression on platelets and platelet transfusion refractoriness was mediated by immune responses which divided into antibody-mediated cytotoxicity and NK cell killing (missing-self theory), these were discussed. Although platelets and MKs with residual of HLA class I expression could escape destruction of antibody-mediated cytotoxicity as reported, but in NK cell cytotoxicity activity has not been tested yet. In contrast, the generated HLA-knocked out platelets and MKs by CRISPR/Cas9 system have not been reported about antibody-mediated cytotoxicity, but NK cell activity was reported. However, in MKs and platelets with residual HLA expression did not induce antibody-mediated cytotoxicity, it is possible that in MKs and platelets with deficient HLA expression would not induce antibody-mediated cytotoxicity as well according to immune response; the specific antibodies will bind with the specific antigens. From Suzuki and Koji Eto et al., in 2016 reports, they suggested that platelets did not express NK cell activating ligands but MKs expressed NK cell activating ligands. Hence for all HLA types including majority of platelet transfusion refractoriness patients, the HLA-knocked out platelets have been immunologically applicable and safety profile enhancement because of contaminating HLA-knocked out MKs rejection by NK cells. From these findings, they might support our study about safety profile and immunologically application in patients with platelet transfusion refractoriness of our generated platelets in the future because we generated HLA-universal iPSC-derived platelets by using paired CRISPR/Cas9nickases which is system for gene knocking out.

For further study, since alloimmune response against HLA class I is the major cause of immune factor in platelet transfusion refractoriness, antibody-mediated cytotoxicity should be tested whether our generated HLA-deficient iPSC-derived platelets can be survived under this refractoriness condition *in vitro* and *in vivo* studies. In addition to antibody-mediated cytotoxicity, NK cell cytotoxicity via missing-self response should also

be tested because our generated platelets lacked of HLA class I expression which is commonly known as inhibitory ligand for NK cell killing inhibition. Moreover, the generated platelets must be demonstrated the ability of survival in peripheral circulation of mouse model with detection of adverse effect and MK contamination is another concern of safety profile so it is necessary to study about contaminated MKs destroying. Since this study might not produce enough amounts of platelets for further studies then large-scale iPSC-derived platelet production is needed. In summary, we need further studies to upscale iPSC-derived platelet generation and test about safety profiles before preventing or treating the patients from risk of life-threatening hemorrhage.

REFERENCES

1. de Gaetano, G., *Historical overview of the role of platelets in hemostasis and thrombosis.* Haematologica, 2001. **86**(4): p. 349-56.
2. Semple, J.W., J.E. Italiano, Jr., and J. Freedman, *Platelets and the immune continuum.* Nat Rev Immunol, 2011. **11**(4): p. 264-74.
3. Blajchman, M.A., et al., *New strategies for the optimal use of platelet transfusions.* Hematology Am Soc Hematol Educ Program, 2008: p. 198-204.
4. Sharma, S., P. Sharma, and L.N. Tyler, *Transfusion of blood and blood products: indications and complications.* Am Fam Physician, 2011. **83**(6): p. 719-24.
5. Blumberg, N., et al., *Platelet transfusion and survival in adults with acute leukemia.* Leukemia, 2008. **22**(3): p. 631-5.
6. Stephan, F., et al., *Thrombocytopenia in critically ill surgical patients: a case-control study evaluating attributable mortality and transfusion requirements.* Crit Care, 1999. **3**(6): p. 151-158.
7. Hod, E. and J. Schwartz, *Platelet transfusion refractoriness.* Br J Haematol, 2008. **142**(3): p. 348-60.
8. Meehan, K.R., et al., *Platelet transfusions: utilization and associated costs in a tertiary care hospital.* Am J Hematol, 2000. **64**(4): p. 251-6.
9. Kerkhoffs, J.L., et al., *The clinical impact of platelet refractoriness: correlation with bleeding and survival.* Transfusion, 2008. **48**(9): p. 1959-65.
10. Toor, A.A., S.Y. Choo, and J.A. Little, *Bleeding risk and platelet transfusion refractoriness in patients with acute myelogenous leukemia who undergo autologous stem cell transplantation.* Bone Marrow Transplant, 2000. **26**(3): p. 315-20.
11. Pavenski, K., J. Freedman, and J.W. Semple, *HLA alloimmunization against platelet transfusions: pathophysiology, significance, prevention and management.* Tissue Antigens, 2012. **79**(4): p. 237-45.

12. Curtis, B.R. and J.G. McFarland, *Human platelet antigens - 2013.* Vox Sang, 2014. **106**(2): p. 93-102.
13. Claas, F.H., et al., *Alloimmunization against the MHC antigens after platelet transfusions is due to contaminating leukocytes in the platelet suspension.* Exp Hematol, 1981. **9**(1): p. 84-9.
14. Santoso, S., et al., *The presence of messenger RNA for HLA class I in human platelets and its capability for protein biosynthesis.* Br J Haematol, 1993. **84**(3): p. 451-6.
15. Lalezari, P. and A.M. Driscoll, *Ability of thrombocytes to acquire HLA specificity from plasma.* Blood, 1982. **59**(1): p. 167-70.
16. Rebulla, P., *Refractoriness to platelet transfusion.* Curr Opin Hematol, 2002. **9**(6): p. 516-20.
17. Wiita, A.P. and A. Nambiar, *Longitudinal management with crossmatch-compatible platelets for refractory patients: alloimmunization, response to transfusion, and clinical outcomes (CME).* Transfusion, 2012. **52**(10): p. 2146-54.
18. Haruta, M., et al., *TAP-deficient human iPS cell-derived myeloid cell lines as unlimited cell source for dendritic cell-like antigen-presenting cells.* Gene Ther, 2013. **20**(5): p. 504-13.
19. Figueiredo, C., et al., *Regulating MHC expression for cellular therapeutics.* Transfusion, 2007. **47**(1): p. 18-27.
20. Figueiredo, C., et al., *Generation of HLA-deficient platelets from hematopoietic progenitor cells.* Transfusion, 2010. **50**(8): p. 1690-701.
21. Gras, C., et al., *HLA-universal platelet transfusions prevent platelet refractoriness in a mouse model.* Hum Gene Ther, 2013. **24**(12): p. 1018-28.
22. Neefjes, J.J., F. Momburg, and G.J. Hammerling, *Selective and ATP-dependent translocation of peptides by the MHC-encoded transporter.* Science, 1993. **261**(5122): p. 769-71.
23. Trowsdale, J., *HLA genomics in the third millennium.* Curr Opin Immunol, 2005. **17**(5): p. 498-504.

24. Lu, P., et al., *Generating hypoimmunogenic human embryonic stem cells by the disruption of beta 2-microglobulin.* Stem Cell Rev, 2013. **9**(6): p. 806-13.
25. Nakamura, S., et al., *Expandable megakaryocyte cell lines enable clinically applicable generation of platelets from human induced pluripotent stem cells.* Cell Stem Cell, 2014. **14**(4): p. 535-48.
26. Takahashi, K., et al., *Induction of pluripotent stem cells from adult human fibroblasts by defined factors.* Cell, 2007. **131**(5): p. 861-72.
27. Domen J, W.A., Weissman IL. , *Bone marrow (hematopoietic) stem cells. In: Regenerative Medicine.* Stem Cell Informatiion from the National Institutes of Health Resource for Stem Cell Research., 2006(United States, Available at: http://stemcells.nih.gov/info/scireport/2006report.htm.Accessed October 28, 2007.).
28. Ran, F.A., et al., *Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity.* Cell, 2013. **154**(6): p. 1380-9.
29. Sander, J.D. and J.K. Joung, *CRISPR-Cas systems for editing, regulating and targeting genomes.* Nat Biotechnol, 2014. **32**(4): p. 347-55.
30. Gaj, T., C.A. Gersbach, and C.F. Barbas, 3rd, *ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering.* Trends Biotechnol, 2013. **31**(7): p. 397-405.
31. Charles S Abrams, M., *Platelet biology.* 2014 UpToDate, 2014. **http://www.uptodate.com/contents/platelet-biology?source=related_link**.
32. Lawrence LK Leung, M., *Overview of hemostasis.* 2014 UpToDate, 2014. **http://www.uptodate.com/contents/overview-of-hemostasis?source=see_link#H1**.
33. Yun, S.H., et al., *Platelet Activation: The Mechanisms and Potential Biomarkers.* Biomed Res Int, 2016. **2016**: p. 9060143.
34. Varga-Szabo, D., I. Pleines, and B. Nieswandt, *Cell adhesion mechanisms in platelets.* Arterioscler Thromb Vasc Biol, 2008. **28**(3): p. 403-12.
35. Jackson, S.P., *The growing complexity of platelet aggregation.* Blood, 2007. **109**(12): p. 5087-95.

36. Lu, Q. and R.A. Malinauskas, *Comparison of two platelet activation markers using flow cytometry after in vitro shear stress exposure of whole human blood.* Artif Organs, 2011. **35**(2): p. 137-44.

37. Hanson, S.R. and S.J. Slichter, *Platelet kinetics in patients with bone marrow hypoplasia: evidence for a fixed platelet requirement.* Blood, 1985. **66**(5): p. 1105-9.

38. Izak, M. and J.B. Bussel, *Management of thrombocytopenia.* F1000Prime Rep, 2014. **6**: p. 45.

39. Blajchman, M.A., *Platelet transfusions: an historical perspective.* Hematology Am Soc Hematol Educ Program, 2008: p. 197.

40. Bayer, W.L., et al., *Use of platelets and other transfusion products in patients with malignancy.* Semin Thromb Hemost, 1992. **18**(4): p. 380-91.

41. Slichter, S.J., et al., *Factors affecting posttransfusion platelet increments, platelet refractoriness, and platelet transfusion intervals in thrombocytopenic patients.* Blood, 2005. **105**(10): p. 4106-14.

42. Bizzozero, G.A., *The importance of the over-jet in full denture construction.* Dent Dig, 1945. **51**: p. 613-6.

43. Rebulla, P., *A mini-review on platelet refractoriness.* Haematologica, 2005. **90**(2): p. 247-53.

44. Seftel, M.D., et al., *Universal prestorage leukoreduction in Canada decreases platelet alloimmunization and refractoriness.* Blood, 2004. **103**(1): p. 333-9.

45. Dutcher, J.P., et al., *Long-term follow-up patients with leukemia receiving platelet transfusions: identification of a large group of patients who do not become alloimmunized.* Blood, 1981. **58**(5): p. 1007-11.

46. Claas, F.H., *Predictive parameters for in vivo alloreactivity.* Transpl Immunol, 2002. **10**(2-3): p. 137-42.

47. Semple, J.W. and J. Freedman, *Recipient antigen-processing pathways of allogeneic platelet antigens: essential mediators of immunity.* Transfusion, 2002. **42**(7): p. 958-61.

48. Sayeh, E., et al., *IgG antiplatelet immunity is dependent on an early innate natural killer cell-derived interferon-gamma response that is regulated by CD8+ T cells.* Blood, 2004. **103**(7): p. 2705-9.

49. *Detection of platelet-reactive antibodies in patients who are refractory to platelet transfusions, and the selection of compatible donors.* Vox Sang, 2003. **84**(1): p. 73-88.

50. McGrath, K., et al., *Transient platelet and HLA antibody formation in multitransfused patients with malignancy.* Br J Haematol, 1988. **68**(3): p. 345-50.

51. Carr, R., et al., *Transfusion of ABO-mismatched platelets leads to early platelet refractoriness.* Br J Haematol, 1990. **75**(3): p. 408-13.

52. *Leukocyte reduction and ultraviolet B irradiation of platelets to prevent alloimmunization and refractoriness to platelet transfusions. The Trial to Reduce Alloimmunization to Platelets Study Group.* N Engl J Med, 1997. **337**(26): p. 1861-9.

53. Murphy, M.F., et al., *Use of leucocyte-poor blood components and HLA-matched-platelet donors to prevent HLA alloimmunization.* Br J Haematol, 1986. **62**(3): p. 529-34.

54. Sniecinski, I., et al., *Prevention of refractoriness and HLA-alloimmunization using filtered blood products.* Blood, 1988. **71**(5): p. 1402-7.

55. van de Watering, L., et al., *HLA and RBC immunization after filtered and buffy coat-depleted blood transfusion in cardiac surgery: a randomized controlled trial.* Transfusion, 2003. **43**(6): p. 765-71.

56. Brand, A., et al., *Alloimmunization after leukocyte-depleted multiple random donor platelet transfusions.* Vox Sang, 1988. **54**(3): p. 160-6.

57. Legler, T.J., et al., *Frequency and causes of refractoriness in multiply transfused patients.* Ann Hematol, 1997. **74**(4): p. 185-9.

58. Hogge, D.E., et al., *Lymphocytotoxic antibody is a predictor of response to random donor platelet transfusion.* Am J Hematol, 1983. **14**(4): p. 363-9.

59. Dutcher, J.P., et al., *Alloimmunization following platelet transfusion: the absence of a dose-response relationship.* Blood, 1981. **57**(3): p. 395-8.

60. Abbas AK, L.A., Pillai Shiv. Cellular and molecular immunology. 7th ed. Philadelphia,W.B. Saunders Company. 2012.
61. Datema, G., et al., *HLA-C expression on platelets: studies with an HLA-Cw1-specific human monoclonal antibody.* Vox Sang, 2000. **79**(2): p. 108-11.
62. Kao, K.J., D.J. Cook, and J.C. Scornik, *Quantitative analysis of platelet surface HLA by W6/32 anti-HLA monoclonal antibody.* Blood, 1986. **68**(3): p. 627-32.
63. Neumuller, J., et al., *Influence of chloroquine or acid treatment of human platelets on the antigenicity of HLA and the 'thrombocyte-specific' glycoproteins Ia/IIa, IIb, and IIb/IIIa.* Vox Sang, 1993. **65**(3): p. 223-31.
64. Gouttefangeas, C., et al., *Thrombocyte HLA molecules retain nonrenewable endogenous peptides of megakaryocyte lineage and do not stimulate direct allocytotoxicity in vitro.* Blood, 2000. **95**(10): p. 3168-75.
65. Semple, J.W., et al., *Indirect allorecognition of platelets by T helper cells during platelet transfusions correlates with anti-major histocompatibility complex antibody and cytotoxic T lymphocyte formation.* Blood, 1995. **86**(2): p. 805-12.
66. Sayeh, E., et al., *Immune responsiveness against allogeneic platelet transfusions is determined by the recipient's major histocompatibility complex class II phenotype.* Transfusion, 2004. **44**(11): p. 1572-8.
67. Reed, E.F., et al., *Monitoring of soluble HLA alloantigens and anti-HLA antibodies identifies heart allograft recipients at risk of transplant-associated coronary artery disease.* Transplantation, 1996. **61**(4): p. 566-72.
68. Bang, A., et al., *Recipient humoral immunity against leukoreduced allogeneic platelets is suppressed by aminoguanidine, a selective inhibitor of inducible nitric oxide synthase.* Blood, 1996. **88**(8): p. 2959-66.
69. Bang, K.W., et al., *Unique processing pathways within recipient antigen-presenting cells determine IgG immunity against donor platelet MHC antigens.* Blood, 2000. **95**(5): p. 1735-42.
70. Castro, E., et al., *Acid elution of platelets HLA-class I antigens in the treatment of a refractory patient.* Br J Haematol, 1998. **100**(1): p. 245-6.

71. Novotny, V.M., et al., *HLA class I-eluted platelets as an alternative to HLA-matched platelets.* Transfusion, 1996. **36**(5): p. 438-44.

72. Riolobos, L., et al., *HLA engineering of human pluripotent stem cells.* Mol Ther, 2013. **21**(6): p. 1232-41.

73. Cosgrove, L.J., et al., *HLA (class I) antigens on platelets are involved in platelet function.* Immunol Cell Biol, 1988. **66 (Pt 1)**: p. 69-77.

74. Rubinstein, E., et al., *Platelet activation by cross-linking HLA class I molecules and Fc receptor.* Blood, 1992. **79**(11): p. 2901-8.

75. Bethesda, M.N.I.o.H., *Stem Cell Basics.* In Stem Cell Information, U.S. Department of Health and Human Services, 2009 **http://stemcells.nih.gov/info/basics/pages/basics2.aspx**.

76. Thomson, J.Y.a.J.A., *EMBRYONIC STEM CELLS.* http://stemcells.nih.gov/info/Regenerative_Medicine/Pages/2006Chapter1.aspx , 2006.

77. Sterneckert, J.L., P. Reinhardt, and H.R. Scholer, *Investigating human disease using stem cell models.* Nat Rev Genet, 2014. **15**(9): p. 625-39.

78. Charles A. Goldthwaite, J., Ph.D., *The Promise of Induced Pluripotent Stem Cells (iPSCs).* In Stem Cell Information [World Wide Web site], 2011. **http://stemcells.nih.gov/info/Regenerative_Medicine/pages/2006chapter10.aspx**.

79. Albertus W. Wognum, P.S.J.S., PhD, *Mini-Review: Hematopoietic Stem and Progenitor cells.* Stem cell technologies, 2013.

80. Machlus, K.R. and J.E. Italiano, Jr., *The incredible journey: From megakaryocyte development to platelet formation.* J Cell Biol, 2013. **201**(6): p. 785-96.

81. Lu, S.J., et al., *Platelets generated from human embryonic stem cells are functional in vitro and in the microcirculation of living mice.* Cell Res, 2011. **21**(3): p. 530-45.

82. Patel, S.R., J.H. Hartwig, and J.E. Italiano, Jr., *The biogenesis of platelets from megakaryocyte proplatelets.* J Clin Invest, 2005. **115**(12): p. 3348-54.

83. Wolfe, S.A., L. Nekludova, and C.O. Pabo, *DNA recognition by Cys2His2 zinc finger proteins.* Annu Rev Biophys Biomol Struct, 2000. **29**: p. 183-212.

84. Holkers, M., et al., *Differential integrity of TALE nuclease genes following adenoviral and lentiviral vector gene transfer into human cells.* Nucleic Acids Res, 2013. **41**(5): p. e63.

85. Wiedenheft, B., S.H. Sternberg, and J.A. Doudna, *RNA-guided genetic silencing systems in bacteria and archaea.* Nature, 2012. **482**(7385): p. 331-8.

86. Jinek, M., et al., *A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity.* Science, 2012. **337**(6096): p. 816-21.

87. Hsu, P.D., et al., *DNA targeting specificity of RNA-guided Cas9 nucleases.* Nat Biotechnol, 2013. **31**(9): p. 827-32.

88. Cong, L., et al., *Multiplex genome engineering using CRISPR/Cas systems.* Science, 2013. **339**(6121): p. 819-23.

89. Walsh, R.M. and K. Hochedlinger, *A variant CRISPR-Cas9 system adds versatility to genome engineering.* Proc Natl Acad Sci U S A, 2013. **110**(39): p. 15514-5.

90. Slack, R.N.a.F.J., *Ins and Outs of RNA Interference*

Analysis. Cell Press: Techniques, 2008.

91. Sung, L.Y., et al., *Efficient gene delivery into cell lines and stem cells using baculovirus.* Nat Protoc, 2014. **9**(8): p. 1882-99.

92. Kobayashi, N., et al., *Gene delivery to embryonic stem cells.* Birth Defects Res C Embryo Today, 2005. **75**(1): p. 10-8.

93. Vouillot, L., A. Thelie, and N. Pollet, *Comparison of T7E1 and surveyor mismatch cleavage assays to detect mutations triggered by engineered nucleases.* G3 (Bethesda), 2015. **5**(3): p. 407-15.

94. Takayama, N., et al., *Generation of functional platelets from human embryonic stem cells in vitro via ES-sacs, VEGF-promoted structures that concentrate hematopoietic progenitors.* Blood, 2008. **111**(11): p. 5298-306.

95. Estcourt, L.J., *Why has demand for platelet components increased? A review.* Transfus Med, 2014. **24**(5): p. 260-8.

96. Cid, J., et al., *Rituximab, plasma exchange and intravenous immunoglobulins as a new treatment strategy for severe HLA alloimmune platelet refractoriness.* Platelets, 2015. **26**(2): p. 190-4.

97. de Rham, C. and J. Villard, *Potential and limitation of HLA-based banking of human pluripotent stem cells for cell therapy.* J Immunol Res, 2014. **2014**: p. 518135.

98. Gopalappa, R., et al., *Paired D10A Cas9 nickases are sometimes more efficient than individual nucleases for gene disruption.* Nucleic Acids Res, 2018.

99. Zhang, Z., et al., *CRISPR/Cas9 Genome-Editing System in Human Stem Cells: Current Status and Future Prospects.* Mol Ther Nucleic Acids, 2017. **9**: p. 230-241.

100. Borger, A.K., et al., *Generation of HLA-universal iPSCs-derived megakaryocytes and platelets for survival under refractoriness conditions.* Mol Med, 2016. **22**.

101. Takayama, N. and K. Eto, *In vitro generation of megakaryocytes and platelets from human embryonic stem cells and induced pluripotent stem cells.* Methods Mol Biol, 2012. **788**: p. 205-17.

102. De Cuyper, I.M., et al., *A novel flow cytometry-based platelet aggregation assay.* Blood, 2013. **121**(10): p. e70-80.

103. McCarty, O.J., et al., *Immobilized platelets support human colon carcinoma cell tethering, rolling, and firm adhesion under dynamic flow conditions.* Blood, 2000. **96**(5): p. 1789-97.

104. Takahashi, R., N. Sekine, and T. Nakatake, *Influence of monoclonal antiplatelet glycoprotein antibodies on in vitro human megakaryocyte colony formation and proplatelet formation.* Blood, 1999. **93**(6): p. 1951-8.

105. Chicharro-Alcantara, D., et al., *Platelet Rich Plasma: New Insights for Cutaneous Wound Healing Management.* J Funct Biomater, 2018. **9**(1).

106. Kim, D.H., et al., *Can Platelet-rich Plasma Be Used for Skin Rejuvenation? Evaluation of Effects of Platelet-rich Plasma on Human Dermal Fibroblast.* Ann Dermatol, 2011. **23**(4): p. 424-31.

107. Bix, M., et al., *Rejection of class I MHC-deficient haemopoietic cells by irradiated MHC-matched mice.* Nature, 1991. **349**(6307): p. 329-31.

108. Feng, Q., et al., *Scalable generation of universal platelets from human induced pluripotent stem cells.* Stem Cell Reports, 2014. **3**(5): p. 817-31.

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