EFFECT OF *JAK2* GENE MUTATIONS ON ERYTHROPOIESIS USING HUMAN INDUCED PLURIPOTENT STEM CELLS

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ผลการกลายพันธุ์ของยีน *JAK2* ต่อกระบวนการสร้างเม็ดเลือดแดงโดยใช้เทคนิคเซลล์ต้นกำเนิด

นางหนึ่งฤทัย นิลศรี

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หนึ่งฤทัย นิลศรี : ผลการกลายพันธุ์ของยีน *JAK2* ต่อกระบวนการสร้างเม็ดเลือดแดงโดยใช้เทคนิคเซลล์ต้นกำเนิด. (EFFECT OF *JAK2* GENE MUTATIONS ON ERYTHROPOIESIS USING HUMAN INDUCED PLURIPOTENT STEM CELLS) อ.ที่ปรึกษาหลัก : ศ. นพ.พลภัทร โรจน์นครินทร์, อ.ที่ปรึกษาร่วม : ผศ. นพ.นิพัญจน์ อิศรเสนา ณ อยุธยา

JAK-STAT pathway มีความสำคัญต่อกระบวนการสร้างเม็ดเลือด และการกลายพันธุ์ของยีน *JAK2* เป็นสาเหตุของการเกิดโรคทางโลหิตวิทยา โดยเฉพาะอย่างยิ่งใน Myeloproliferative neoplasms (MPNs) ซึ่งประกอบไปด้วย polycythemia vera (PV), essential thrombocythemia (ET) และ myelofibrosis โดยสาเหตุส่วนใหญ่เกิดจากการกลายพันธุ์ชนิด JAK2V617F ใน exon 14 และรองลงมาคือ การกลายพันธุ์ของยีน *JAK2* ที่ตำแหน่ง exon 12 จากรายงานพบผู้ป่วยโรคเม็ดเลือดแดงมากผิดปกติ และผู้ป่วยเกล็ดเลือดมากผิดปกติจะมีการกลายพันธุ์ชนิด JAK2V617F ในขณะที่การกลายพันธุ์ของยีน *JAK2* ที่ตำแหน่ง exon 12 พบเฉพาะผู้ป่วยโรคเม็ดเลือดแดงมากผิดปกติเท่านั้น ซึ่งคาดว่าการกลายพันธุ์ทั้งสองชนิดนี้น่าจะมีการสื่อสัญญาณภายในเซลล์ที่แตกต่างกัน และมีผลต่อกระบวนการสร้างเม็ดเลือดแดง ดังนั้น เซลล์ต้นกำเนิดชนิด induced pluripotent stem cells (iPSCs) จึงเหมาะสมที่จะนำมาใช้เป็นโมเดลในการศึกษาพยาธิกำเนิดของโรคดังกล่าว การศึกษานี้ได้ทำการสร้าง iPSCs ที่มีการกลายพันธุ์ของยีน *JAK2* ชนิด JAK2V617F และการกลายพันธุ์ของยีน *JAK2* ที่ตำแหน่ง exon 12 (N542_E543del) โดยใช้ระบบควบคุมการทำงานของยีนด้วยสาร doxycycline ซึ่งเม็ดเลือดแดงที่สร้างได้จาก iPSCs ที่มีการกลายพันธุ์ของยีน *JAK2* ที่ตำแหน่ง exon 12 หลังจากที่ถูกเหนี่ยวนำด้วยสาร doxycycline นั้น มีจำนวนของเซลล์เม็ดเลือดแดงที่มีการแสดงออกของเครื่องหมายซีดีบนผิวเซลล์ที่จำเพาะต่อเม็ดเลือดแดง ($CD71^{+}$ $GlycophorinA^{+}$) เพิ่มสูงขึ้นอย่างมีนัยสำคัญทางสถิติ (p value = 0.007) และลักษณะทางสัณฐานวิทยาของเม็ดเลือดแดงที่อยู่ในระยะตัวแก่มากกว่าเม็ดเลือดแดงที่สร้างได้จาก JAK2V617F รวมถึงมีการแสดงออกของฮีโมโกลบินของผู้ใหญ่ที่มากกว่าด้วย และเมื่อทำการวัดโปรตีนจากการกระตุ้นเซลล์ต้นกำเนิดทางโลหิตวิทยาด้วยฮอร์โมน erythropoietin พบว่า iPSCs ชนิดที่มีการกลายพันธุ์ของ exon 12 มีระดับของ phospho-STAT1 ที่เพิ่มสูงขึ้นมากกว่า แต่มีระดับของ phospho-STAT3 และ phospho-AKT ที่เพิ่มขึ้นน้อยกว่าเมื่อเทียบกับ iPSCs ชนิด JAK2V617F นอกจากนี้ยังพบว่า iPSCs ที่มีการกลายพันธุ์และถูกเหนี่ยวนำด้วยสาร doxycycline นั้น มีการตอบสนองต่อยา arsenic trioxide และ interferon alpha โดยมีผลในการยับยั้งการแบ่งตัวเพิ่มจำนวน และเหนี่ยวนำให้เซลล์มีการตายแบบ apoptosis ซึ่งต่างจากเซลล์ที่ไม่ได้ใส่ doxycycline จะเห็นได้ว่า iPSCs ที่มีการกลายพันธุ์ของยีน *JAK2* ทั้งสองชนิดนี้สามารถนำมาใช้เป็นโมเดลในการศึกษากลไกต่างๆของการเกิดโรค MPNs ได้

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2562 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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Nungruthai Nilsri : EFFECT OF *JAK2* GENE MUTATIONS ON ERYTHROPOIESIS USING HUMAN INDUCED PLURIPOTENT STEM CELLS. Advisor: Prof. PONLAPAT ROJNUCKARIN, M.D.Ph.D Co-advisor: Asst. Prof. NIPAN ISRASENA, M.D.Ph.D

Activating mutations affecting the JAK-STAT signal transduction is the genetic driver of myeloproliferative neoplasms (MPNs) which comprise polycythemia vera (PV), essential thrombocythemia (ET) and myelofibrosis. The *JAK2*p.V617F mutation can produce both erythrocytosis in PV and thrombocytosis in ET, while *JAK2* exon 12 mutations cause only erythrocytosis. We hypothesized that these two mutations activated different intracellular signals. In this study, the induced pluripotent stem cells (iPSCs) were used to model *JAK2*-mutated MPNs. Normal iPSCs underwent lentiviral transduction to overexpress *JAK2*p.V617F or *JAK2*p.N542_E543del (JAK2exon12) under a doxycycline-inducible system. The modified iPSCs were differentiated into erythroid cells. Compared with JAK2V617F-iPSCS, JAK2exon12-iPSCs yielded more total $CD71^{+}GlycophorinA^{+}$ erythroid cells (*p* value = 0.007), displayed more mature morphology and expressed more adult hemoglobin after doxycycline induction. Capillary Western immunoassay revealed significantly higher phospho-STAT1 but lower phospho-STAT3 and lower Phospho-AKT in JAK2exon12-iPSCs compared with those of JAK2V617F-iPSCs in response to erythropoietin. Furthermore, interferon alpha and arsenic trioxide were tested on these modified iPSCs to explore their potentials for MPN therapy. Both agents preferentially inhibited proliferation and promoted apoptosis of the iPSCs expressing mutant *JAK2* compared with those without doxycycline induction. In conclusion, the modified iPSC model can be used to investigate the mechanisms and search for new therapy of MPNs.

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CHAPTER I

INTRODUCTION

Background and rationale

The myeloproliferative neoplasms (MPNs) are clonal disorders of multipotent hematopoietic progenitors. BCR-ABL positivity is the hallmark of chronic myeloid leukemia, while BCR-ABL-negative MPNs clinically manifest as polycythemia vera, essential thrombocythemia or primary myelofribosis (1). Polycythemia vera is a stem cell disorder characterized by panhyperplasia of red blood cells, white blood cells and platelets. Essential thrombocythemia is characterized by an isolated increased number of platelets in peripheral blood and primary myelofibrosis is a condition characterized by the buildup of fibrosis in the bone marrow with splenomegaly from extramedullary hematopoiesis (2). Pathogenesis of BCR-ABL negative MPNs is the over-activation of the JAK/STAT pathway. The most common causes are Janus Kinase 2 gene (*JAK2*) mutations that are JAK2V617F and, less commonly, exon 12 mutations. The frequencies of *JAK2* mutation are approximately 98%, 50-60% and 55-65% in PV, ET and PMF patients, respectively (3). From clinical studies, patients with JAK2V617F present with erythrocytosis and/or thrombocytosis, whereas JAK2 exon 12 mutated patients display only erythrocytosis. The molecular mechanism of this difference is still unclear (4). These 2 mutations are likely to activate different intracellular signals. Human induced pluripotent stem cells (iPSCs) are generated from mature adult cells via induction by four factors, Oct3/4, Sox2, c-Myc, and Klf4. These cells can be derived from normal subjects or patients to be differentiated into various cell types *in vitro*. Furthermore, the cells can be genetically modified and numerically expanded (5). Therefore, iPSCs currently have great potentials to be an important source of hematopoietic cells for cellular therapies or transfusion and to be a useful tool for understanding pathogenesis of genetic and acquired disorders of blood cell development. In this study, I attempt to establish the *JAK2* gene mutations using the viral transduction in human induced pluripotent stem cells and generate erythrocytes from modified iPSCs by using a co-culture system with C3H10T1/2

feeder cells follow according to the Ochi protocol (6). The system will be used as a disease model to understand the molecular pathogenesis of MPNs. Subsequently, it can be used as a model for drug therapy, such as interferon and arsenic trioxide. In the future, the system may also be utilized to generate larger amount of *in vitro*-derived red blood cells for transfusion. The iPSCs will be constructed in two types of *JAK2* gene mutations comprising one point mutation in exon 14 (JAK2V617F) and the small deletion in JAK2 exon 12 (N542_E543 deletion). Viral transfected cells will be selected by drug resistant and determined the *JAK2* expression by using real time PCR and DNA sequencing. All of modified iPSCs will be characterized for the stem cell capacity before using them to produce erythrocytes *in vitro*. Red blood cells derived from culture will be harvested on day 29 and characterized for cell numbers, morphology, erythrocyte specific markers and hemoglobin typing. In addition, iPSC-derived red blood cell progenitors will be evaluated for the JAK/STAT signal transduction and will be tested for drug sensitivity compared with wild-type cells. Modified iPSCs may be a suitable tool for studying *JAK2* mutations in MPNs.

Keywords

JAK2 gene, induced pluripotent stem cells, erythropoiesis

Research design

Experimental design

Research questions

Do iPSCs with mutated *JAK2* possess higher potentials to develop into the erythroid lineage compared with wild-type iPSCs?

Objectives

There are three objectives in this research including;

1. To establish the iPSCs with *JAK2* mutations by using viral transduction
2. To study the efficiency of *JAK2*-mutated iPSC differentiation into erythroid cells

3. To investigate the erythroid produced from modified iPSCs using different types of *JAK2* mutations

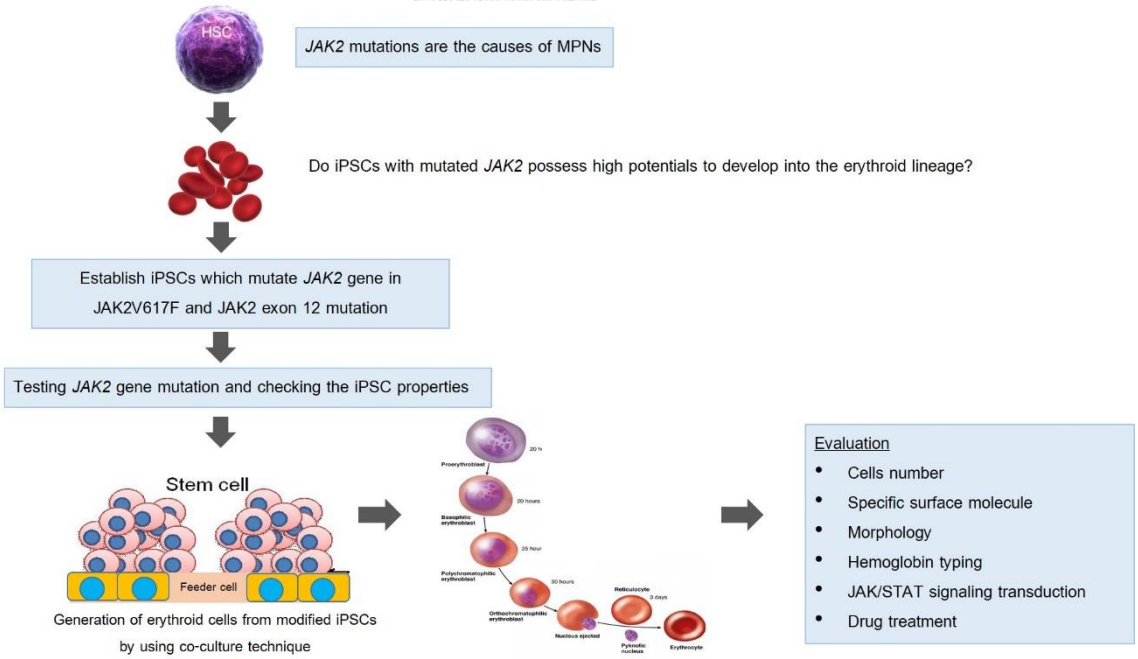
Hypothesis

JAK2 mutations enhance erythroid development and iPSCs with mutated *JAK2* can be used as a MPNs disease model to study erythroid cell development.

Benefits

1. To understand the signaling pathways of erythroid hyperplasia in MPNs from *JAK2* mutations using the iPSCs model
2. To use these *JAK2*-mutated iPSCs as a MPN disease model and drug testing
3. To enhance the production of erythrocytes *in vitro* as a potential for blood transfusion in the future

Conceptual framework



CHAPTER II
REVIEW LITERATURES

Myeloproliferative neoplasms

Myeloproliferative neoplasms (MPNs) are a group of diseases characterized by increased cell proliferation in bone marrow that makes too many erythroid, megakaryocytic and/or granulocytic cells. In 2016, the World Health Organization classified myeloproliferative neoplasms into seven groups (1) composed of

- Chronic myelogenous leukemia, BCR-ABL1–positive (CML)
- Chronic neutrophilic leukemia (CNL)
- Polycythemia vera (PV)
- Essential thrombocythemia (ET)
- Primary myelofibrosis (PMF)
- Chronic eosinophilic leukemia, not otherwise specified (CEL-NOS)
- Myeloproliferative neoplasms, unclassifiable (MPN-U)

CML is the first MPN, of which the pathogenic mutation was found as the Philadelphia chromosome [t(9;22)] causing the BCR-ABL fusion gene. BCR-ABL-negative myeloproliferative neoplasms are the other group of MPNs that are composed of Polycythemia vera, Essential thrombocytosis and Primary myelofibrosis.

Polycythemia vera is a stem cell disorder characterized as pan-hyperplasia of erythroid, megakaryocytic and granulocytic cells in bone marrow (Panmyelosis). PV is most commonly found in men who are over 60 years old and patients often display enlarged spleens and excess red blood cell masses resulting in hyperviscosity of the blood and increased risks for thrombosis. ET is also a disease of the bone marrow hematopoietic stem cells with sustained megakaryocyte proliferation leading to an increase in the number of circulating platelets. It is most prevalent in women over the age of 50 years old and the symptoms show both blood clotting and/or bleeding complications. PMF is a chronic blood cancer which increases fibrous tissue in the bone marrow with loss of normal blood cells production. PMF is most commonly

seen in men and women over the age of 60 and patients often have associated systemic symptoms and a markedly enlarged spleen due to blood formation outside the marrow (extramedullary hematopoiesis). Myelofibrosis (MF) can occur in patients with no prior history of MPNs (primary MF) or as a progression of PV or ET called post-PV or post ET MF, respectively (2).

The pathogenesis of Philadelphia chromosome-negative myeloproliferative neoplasms is originated from the somatic gene mutations. The most common mutated genes are *JAK2*, calreticulin (*CALR*) and myeloproliferative leukemia virus oncogene (*MPL*) which is the thrombopoietin (TPO) receptor. These mutations cause hyper-activation of the signals from erythropoietin receptor (JAK2) and/or TPO receptor (*MPL*, *CALR* and *JAK2*) resulting in erythrocytosis and/or thrombocytosis. Among these 'driver' gene mutations, *JAK2* is the most frequent with mutation frequencies of approximately 98% in PV, 50-60% in ET and 55-65% in PMF (3). Besides these driver mutations, other mutations in epigenetic pathways similar to myelodysplastic syndromes (MDS) have been described in MPNs, e.g. *EZH2, ASXL1* (7). These 'passenger' mutations are not the direct cause of MPNs, but they reflect the progression to MF or acute myeloid leukemia (AML) and confer poorer prognosis.

Myeloproliferative neoplasm patients may present with MPN-characteristic features such as extramedullary hematopoiesis causing splenomegaly, portal or hepatic vein thrombosis, aquagenic pruritus and bone marrow fibrosis. In PV patients, the bone marrow reveals trilineage myeloproliferation with pleomorphic megakaryocytes whereas in ET patients show megakaryocyte proliferation with large and mature morphology and in PMF by megakaryocyte proliferation with atypical in sizes, irregularly folded nuclei and dense clustering accompanied by either reticulin and/or collagen fibrosis (8).

Survival in ET and PV is relatively long, over 10-20 years, and risks for leukemic transformation are low. However, the survival of MF is shorter of approximately 5 years (9). Current first-line treatments are phlebotomy for PV and observation for ET in younger patients. In older patients or ones with high risks for thrombosis, cytoreduction using hydroxyurea or interferon-alpha (younger patients) or analgrelide (for high platelet counts) are treatments of choice. There is currently no

evidence implicating hydroxyurea as being leukemogenic (3). Nowadays, JAK inhibitors which displace ATP from the JH1 domain binding site of JAK2 have been developed for treatment in MPN patients when hydroxyurea or interferon-alfa are intolerant or resistant, however these agents are limited because they also inhibit the wild-type *JAK2* resulting in suppression of normal hematopoiesis and very high costs of JAK2 inhibitors. The role of JAK2 inhibitors on ET is unknown. Moreover, the therapeutic aim of PV and ET is also to prevent thrombosis and alleviate systemic symptoms (10). The contemporary guidelines recommend phlebotomy to control erythrocytosis by maintaining hematocrit less than 45% in patients with PV and low dose aspirin therapy (81mg/dl) in all patients with PV and *JAK2*-mutated ET. In addition, JAK2 inhibitors are able to relieve systemic symptoms and splenomegaly in patients with MF **(Figure 1)** (11-13).

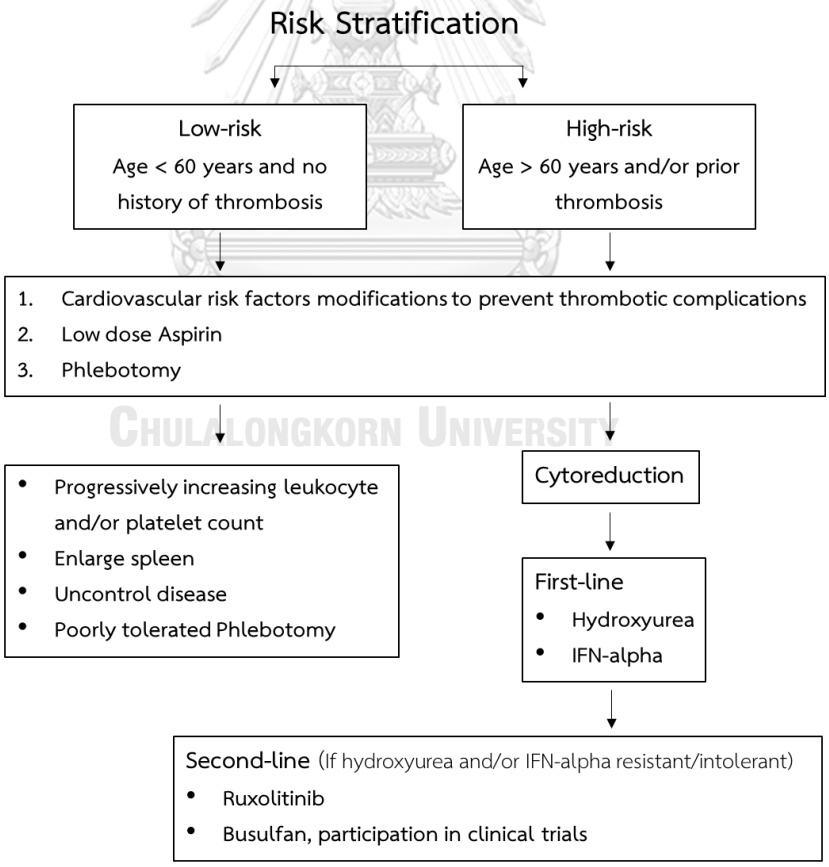


Figure 1 Management algorithms for Philadelphia chromosome-negative myeloproliferative neoplasms (PV and ET)

Interferons (IFNs) are classical anti-proliferative drugs. Many studies reported that IFN-α and IFN-γ induce cycling of hematopoietic stem cells (HSCs). The mechanism starts from IFN-α binding to the interferon α receptor 1 and 2 (IFNAR1 and IFNAR2) and then activates the JAK1 and TYK2 to downstream signal via a complex of STAT1/STAT2/IRF9. The STAT1-STAT2-IRF-9 multimeric complex is called the IFN stimulated gene factor 3 (ISGF3) binds to a specific DNA sequence in IFN-stimulate gene (ISGs). The action of IFN-α have been ascribed to its proapoptotic, antiapoptosis, increase anti-tumor and active NK cell immunity. From the mechanism of IFN-α affecting hematopoiesis, this drug becomes a choice of treatment in the early stage of MPN patients. PV and ET patients with JAK2V617F respond positively to IFN-α treatment around 40% leading to a reduction of JAK2V617F allele burden. However, many patients develop flu-like symptoms, such as headache, tiredness, fatigue and weakness, after treatment with IFN-α. Furthermore, some patients develop symptoms and signs of autoimmune diseases induced by IFN-α (14).

Arsenic trioxide (As_2O_3; ATO) shows efficacy in the treatment of relapsed acute promyelocytic leukemia (APL) via two main mechanisms, inducing of cell differentiation and promoting of apoptosis. ATO at low concentrations induces APL cells differentiation through degradation of PML-RARα which is the protein product from the PML gene fusion with the retinoic acid receptor alpha (RAR alpha) gene. Degradation of PML-RARα allows immature cells to overcome the maturation block (15). PML-RARα expression was necessary for the induction of apoptosis by ATO. High concentrations of ATO induces apoptosis through several points in mitochondrial-induced apoptosis, disruption of mitotic spindle process, chromosomal defect and generation of reactive oxygen species and oxidative stress leading to cell apoptosis and cell cycle arrest (16).

Janus kinases

Janus kinase (JAK) is the non-receptor tyrosine kinase. It is a large protein of more than 1,100 amino acids, with the molecular weight of approximately 120-140 kDa. In mammals, the JAK family is composed of four members that are JAK1, JAK2,

JAK3 and Tyrosine kinase 2 (Tyk2). JAK proteins have seven homology regions (JH1-7). The primary structure of Janus kinases is composed of FERM (amino terminal), Src-homology-2 (SH2), pseudokinase and kinase domains (17). The JH1 domain at the carboxyl terminus is essential for the enzymatic activity, whereas JH2, pseudokinase domain, has a structure similar to a tyrosine kinase but lacking a kinase activity. This domain is related to the regulation of JH1 activity. The JH3 to JH4 domains share homology with SH2 domains. The JH5 to JH7 regions of JAK at FERM domain are associated with cytokine receptors **(Figure 2)** (18).

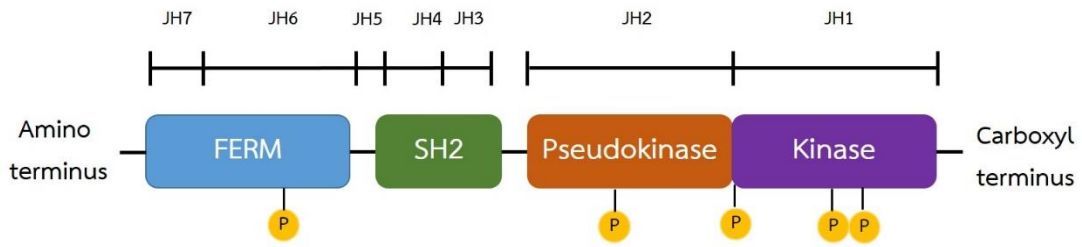


Figure 2 A schematic representation of the primary structure of Janus kinases

JAK1 is a human tyrosine kinase protein which interacts with the common gamma chain (γc) containing cytokine receptors such as interleukin (IL)-2, IL-4, IL-7, IL-9, IL-15 and IL-21 receptors. JAK1 is also essential for certain type I and type II cytokines such as IL-2 and IL-4 receptor family, the gp130 receptor family and IFNs (19). On the other hand, JAK2 is essential for the type II cytokine receptor family, the GM-CSF receptor family and hormone-like cytokines such as growth hormone (GH), erythropoietin (EPO) and thrombopoietin (TPO). Tyk2 is essential for signaling of IL-12 and Toll receptor, which mediates the responses to lipopolysaccharide (LPS) (18).

The JAK/STAT pathway activation plays an important role for promoting cell proliferation, differentiation, migration and anti-apoptosis. The binding of ligands to JAK receptors causes the receptors to dimerize which brings the receptor-associated JAKs into close proximity. JAKs then cross-phosphorylate each other on tyrosine residues and activate the kinase domains. The activated JAK then phosphorylates tyrosine residues on the receptors and signal transducers and activators of

transcription (STATs) which then bind to the phosphorylated tyrosines on the receptor using their SH2 domains. After that, the STATs dissociate from the receptor to form dimerization before translocate to nucleus before binding with specific regulatory DNA sequences to activate transcription of target genes **(Figure 3)** (20).

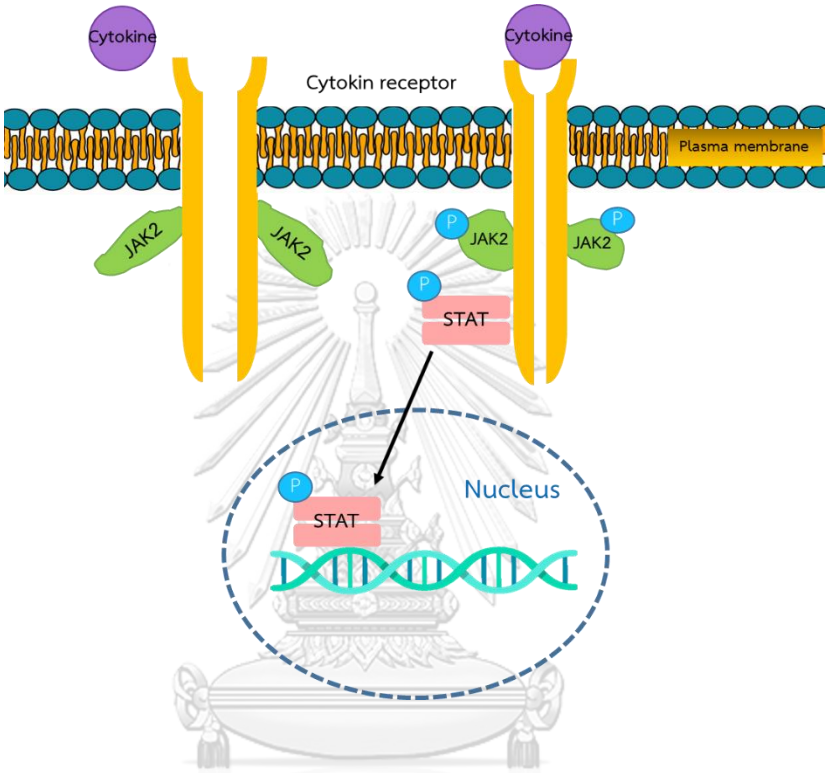


Figure 3 The JAK-STAT intracellular signaling pathway

The JAK-STAT pathway and myeloproliferative neoplasms

JAK-STAT pathway plays a critical role in signal transduction downstream of the EPO, TPO and related receptors which control erythrocyte and megakaryocyte production. Mutations of *JAK2*, most commonly p.V617F, are found in almost all patients with PV and in majority of ET and PMF. The somatic V617F mutation is a G to T alteration in exon 14 of *JAK2* resulting in the substitution of valine to phenylalanine at the codon 617 (JAK2V617F) in the pseudokinase (JH2) domain. The V617F mutation induces a loss of inhibitory activity of the JH2 part on the JH1 kinase part of JAK2, leading to an increased activity of the normal JH1 kinase of JAK2 **(Figure 4)**. This mutation causes hypersensitive hematopoietic stem cells leading to

activation of the JAK-STAT resulting in uncontrolled cell proliferation (21, 22). Approximately 70% of MPN patients can be found JAK2V617F mutation: 95% of PV and 55% and 60% of ET and PMF, respectively. JAK2 exon 12 mutations are also found in MPNs and present only in JAK2V617F-negative PV around 3%. They are not associated with ET and PMF, but may be associated with progression to secondary MF **(Table 1)** (3). JAK2 exon 12 mutations are located in the linker between SH2 and the pseudokinase domains in a region between amino acid 536 and 547 **(Figure 4)** (11). The most frequent mutations are N542-E543del (23%), E543-D544del (11%), and F537-K539delinsL and K539L (10%) (23). Mutations of JAK2 exon12 are almost always heterozygous, while JAK2V617F in PV is usually homozygous **(Figure 5)** (4).

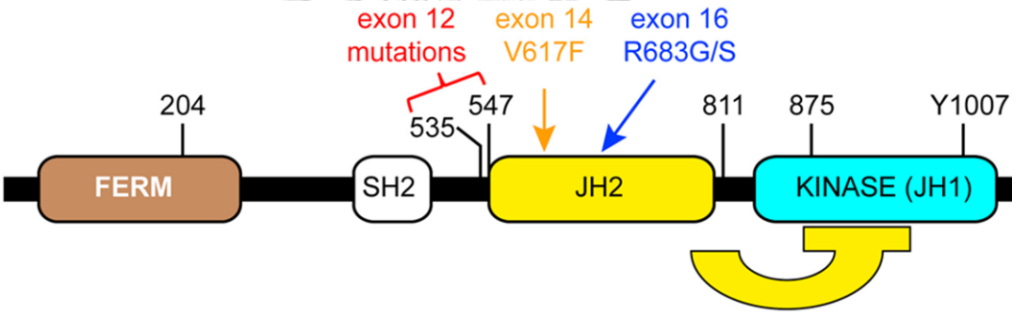


Figure 4 Domain structure of the JAK2 protein and the positions of the most mutated regions

Table 1 Frequency of *JAK2* mutation in BCR-ABL-negative MPNs

Mutation	Mutational Frequency (%)		
	Polycythemia Vera	Essential Thrombocythemia	Primary Myelofibrosis
JAK2 Exon 14 mutation (JAK2V617F)	95	55	60
JAK2 Exon 12 mutations	3	Infrequent	Infrequent

The reasons why a *JAK2* mutation causes PV in one patient and ET in the other patient are unclear. Clinical observations reveal that homozygous JAK2V617F mutation and JAK2 exon12 mutations are usually found in PV, while heterozygous JAK2V617F mutation usually detected in ET (4). The differences in signaling pathways between these mutations are unknown. Currently, the types and allele burdens of *JAK2* mutations can be generated in the iPSC model. Therefore, this model will be used in this study.

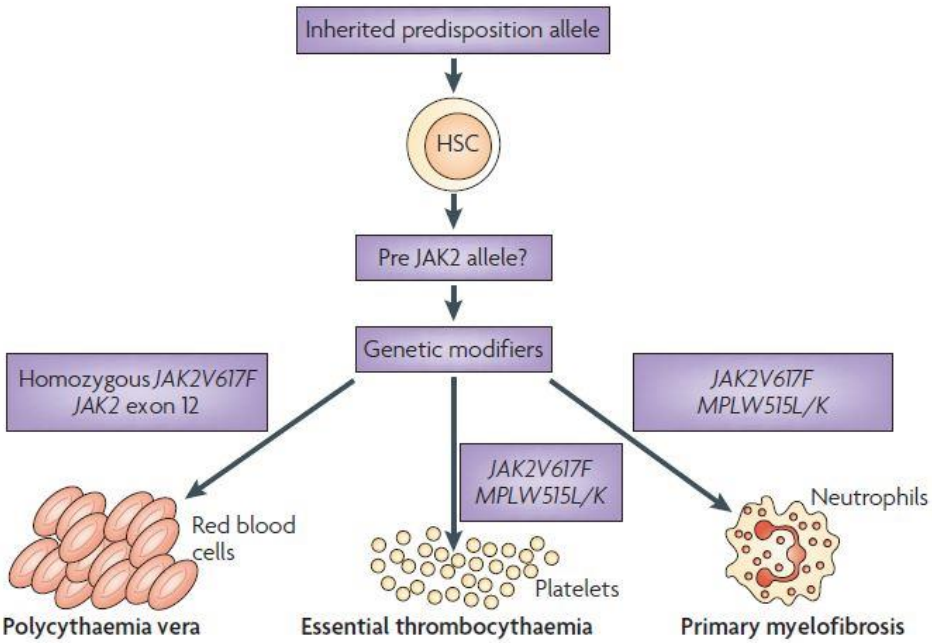


Figure 5 Model of the pathogenesis of PV, ET and PMF

Erythropoiesis

Erythropoiesis is the dynamic process of red blood cells production. Erythrocytes descend in the red bone marrow from pluripotent stem cells which lead to the erythroid progenitors. The earliest committed progenitors are the burst forming unit-erythroid (BFU-E) then divides and differentiates into colony-forming unit-erythroid (CFU-E). After that, CFU-E progenitors divide and differentiate into erythroid cells undergoing many changes including cell sizes, chromatin

condensation, hemoglobinization and enucleation. The stages of erythroid differentiation are composed of proerythroblast, basophilic erythroblast, polychromatic erythroblast, orthochromatic erythroblast, reticulocyte and erythrocyte **(Figure 6)** (24).

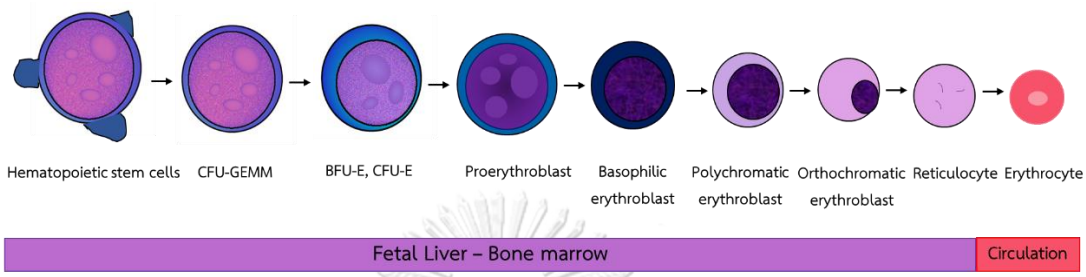


Figure 6 Schematic diagram represents the three major phases of erythropoiesis

Human blood contains erythrocytes 5 x 10^6 cells per microliter (normal range in males 4.7-6.1x 10^6 cells per microliter and 4.2- 5.4 x 10^6 cells per microliter for females). The life span of red blood cells in circulation is 120 days. In the steady state, approximately 1% of erythroid cells are synthesized to replace the number lost daily for senescence but there are higher productions during times of acute or chronic stress. Essential cytokine of erythropoiesis is erythropoietin (EPO) which plays a key role in regulating proliferation, differentiation, and anti-apoptosis. In the first phase of CFU-E, erythroid differentiation is highly EPO dependent whereas later stages are no longer dependent on EPO. Therefore, EPO receptors are lost as erythroid progenitors undergo terminal proliferation and differentiation (25). Binding of EPO to EPO receptor (EpoR) on the surface of erythroid progenitors in bone marrow stimulates activation of multiple intracellular signal transduction pathways including the JAK/STAT pathway, MAPK/ERK pathways and phosphoinositide-3 kinase/AKT pathway (26).

Erythropoiesis and signal transduction

JAK/STAT pathway

When EPO binds EpoR on committed erythroid progenitors, the EPO-EpoR complex induce homo-dimerization of the EpoR leading to downstream activation. The first downstream of EpoR activation is the JAK/STAT5 pathway. STAT5 is membrane bound via the EpoR cytoplasmic tail and upon phosphorylation of EpoR after ligand binding it is phosphorylated by phosphor JAK2. The STAT protein becomes activated through the formation of a homodimer and translocated to the nucleus. Dimerized STAT transcription factors including STAT1, STAT3, and STAT5a/b interact directly with DNA through STAT-specific binding domains and activate genes such as cyclin D1 and BCL-XL resulting in downstream effects on cell cycle control and resistance to apoptosis (27). EPO-mediated activation of JAK2/STAT5 leads to up-regulation of the anti-apoptotic BCL-XL and BCL-2 genes which protect proerythroblasts from apoptosis. STAT5 deficiency was reported to cause a severe effect on fetal erythropoiesis which proceeds at a high rate in the liver during gestation. *STAT5a*$^{-/-}$ *STAT5b*$^{-/-}$ mice exhibit significantly reduced hematocrits compared to wild type. Thus, STAT5 operates an essential role in controlling erythropoiesis in mice (28). The roles of STAT5 in human erythropoiesis remain to be determined.

MAPK/ERK pathways

The MAPK/ERK pathway is activated by EpoR phosphorylation through adaptor proteins. This pathway is initiated by the binding of the SHC/GRB2/SOS complex and bound to the cytoplasmic tail of EpoR to stimulate Ras. Activated Ras then induces a kinase cascade including isoforms of the serine/threonine kinase Raf. The Raf kinase phosphorylates and activates MEKs (MEK1 and MEK2). After that, MEKs phosphorylate and activate mitogen-activated protein kinases (MAPK). Activated MAPKs including extracellular-signal-regulated kinases 1/2 (ERK1/2), c-Jun-amino-terminal kinase (JNK), p38, and ERK5 are translocated into nucleus and promote cell cycle progression and proliferation (29).

PI3K/AKT pathway

The phosphatidylinositol 3-kinase (PI3K) signaling is essential for cell survival, proliferation and maturation of erythroid progenitors. PI3K can be activated by direct binding of the p85 regulatory subunit to a specific tyrosine-phosphorylated site on the EpoR and indirectly through binding to adapter molecules. Lipids phosphorylated by PI3K act as second messengers and directly activate the enzyme AKT (protein kinase B). The PI3K-AKT pathway has been shown to serve a key role in regulation of apoptosis and proliferation in normal erythroid progenitors **(Figure 7)** (30, 31).

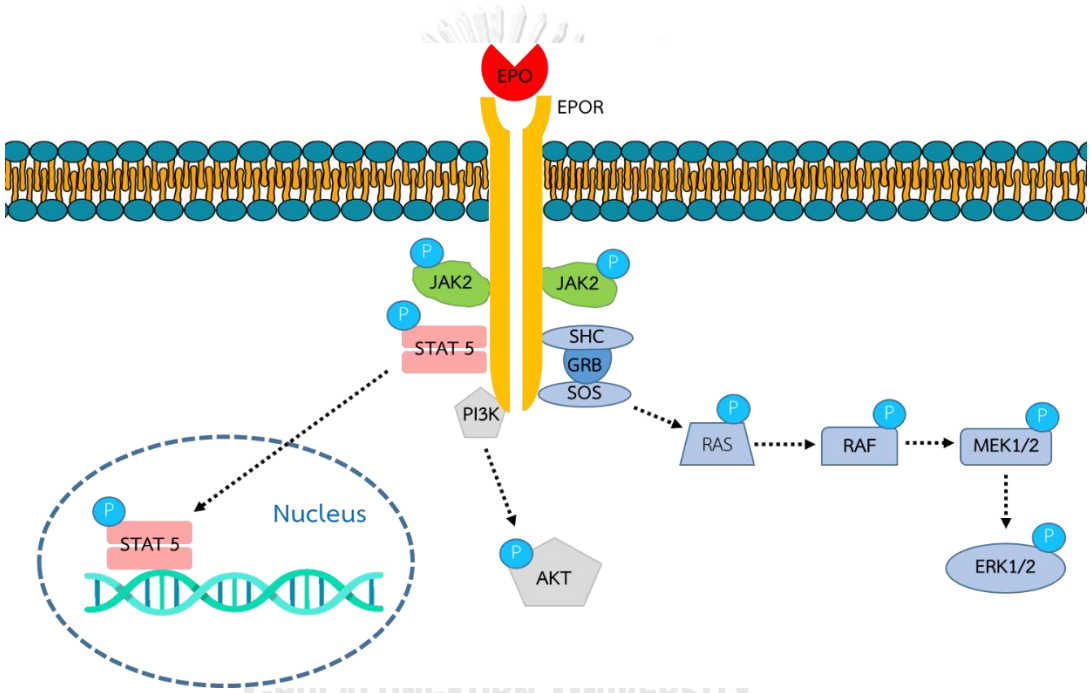


Figure 7 JAK2 activates via multiple downstream signaling molecules in erythropoiesis

Primitive erythrocytes, emerging in the extra embryonic yolk sac, are relatively large cells. In human, the primitive cells are characterized by the expression of embryonic globins (ε, γ and ζ) which form a variety of embryonic hemoglobin tetramers ($\zeta_2\varepsilon_2$; Gower1, $\alpha_2\varepsilon_2$; Gower2, $\zeta_2\gamma_2$; Portland1 and $\zeta_2\beta_2$; Portland2). When erythropoiesis starts in the fetal liver, embryonic globins are immediately switched to fetal globins and expression of a specific fetal β-like globin (γ-globin). Hemoglobin

tetramers consist of α- and γ-globin chains (α2γ2), such as HbF (fetal hemoglobin). Fetal hemoglobin allows the developing fetus to extract oxygen more efficiently from the maternal blood as HbF can bind oxygen better than adult Hb (HbA). At the time of birth, the site of erythropoiesis switches to the bone marrow and the spleen and then fetal globin expression is silenced. Hemoglobin tetramers are composed of α- and β-globin (α2β2, HbA) at approximately 97% of all hemoglobin in adult erythrocytes. HbA2 (α2δ2) and HbF in most adults constitute approximately 2% and 1%, respectively **(Figure 8)** (32, 33).

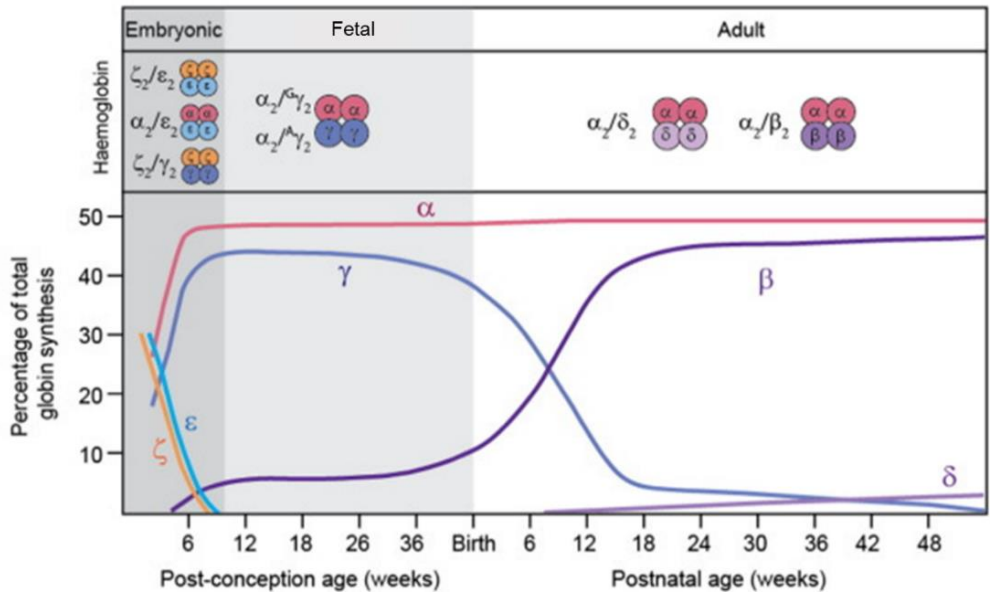


Figure 8 The fetal to adult hemoglobin switching

JAK2V617F and JAK2 exon 12 mutations

The crystal structure of the JH2 domain of JAK2V617F is highly similar to wild-type JH2 but in the ATP binding cleft exhibits some differences. In the resting state without EPO, structures of JAK2 proteins should be kept in an inactive state by the tight contact between the JH2 and JH1 domains. In JAK2V617F mutation, there is the conformation change of JAK2 structure which JH1 disengages from JH2 leading to a

partially active state in allowing some transphosphorylation even in the absence of EPO **(Figure 9)** (11, 34).

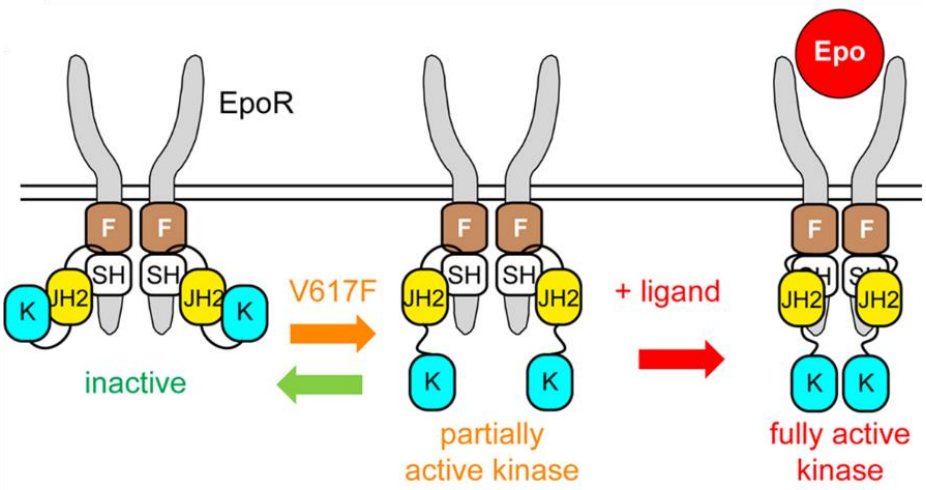


Figure 9 Model of JAK2 activation in JAK2 wild type and JAK2V617F

Comparing the clinical features, there is no difference in gender between JAK2V617F and JAK2 exon 12 mutation but the patients with JAK2 exon 12 mutations present at significantly younger ages than patients with JAK2V617F. Comparing their blood parameters, JAK2 exon 12 mutation patients show the higher levels of hemoglobin and hematocrit than those of patients with a JAK2V617F. On the other hand, most JAK2 exon 12-mutated patients present with normal number of white blood cells and platelets **(Table 2)** (35).

Table 2 Clinical data and hematological parameters of JAK2 Exon 12 and JAK2V617F mutation PV patients

Category of *JAK2* mutation	Exon 12	V617F [10][e]	*p*[e]
Demographics			
Total number	134	86	
Number of females	70 (52%)	51 (59%)	NS
Mean age (range)	52 (14–93)	59 (12–90)	0.006
Hematologic features			
Hemoglobin (g/L)			
Mean ± SD	190 ± 28 (n = 110)	180 ± 23 (n = 86)	<0.0001
Meets current WHO criteria[a]	88 (80%)	55 (69%)	0.002
Hematocrit (%)			
Mean ± SD	61 ± 8 (n = 63)	55 ± 7 (n = 79)	<0.0001
White cells ($\times 10^9$/L)			
Mean ± SD	8.5 ± 3.6	14.1 ± 7.3	<0.0001
Meets previous WHO criteria[b]	17 (13%)	48 (56%)	<0.0001
Meets previous PVSG criteria[c]	34 (26%)	65 (76%)	<0.0001
Platelets ($\times 10^9$/L)			
Mean ± SD	331 ± 200	605 ± 263	<0.0001
Meets previous PVSG/WHO criteria[d]	24 (18%)	64 (80%)	<0.0001

Abbreviations: SD, standard deviation; NS, not significant

[a] >165g/L for females, or >185g/L for males [64].

[b] White cell count $>12 \times 10^9$/L [62].

[c] White cell count $>10 \times 10^9$/L [63]

[d] Platelet count $>400 \times 10^9$/L [62, 63].

[e] Calculated using an unpaired Student's *t*-test

Regarding the mechanism of *JAK2* mutation, heterozygosity for the JAK2V6217F demonstrates a predominantly thrombocytosis phenotype, whereas JAK2V6217F homozygosity or a heterozygous JAK2 exon 12 mutation exhibits erythrocytosis. The molecular basis for this variability is still undefined but is likely influenced by physiologic and/or genetic factors. Interestingly, the erythroid phenotype is shown in transgenic mice with low JAK2N542-E543del expression, whereas in transgenic mice with high JAK2V617F levels express erythrocytosis. This data suggesting that JAK2 exon 12 mutants have a higher erythropoietic activity than JAK2V617F and may have the differences in molecular signaling.

The nature of the mutations are associated with the difference levels of activate JAK2 signaling or the presence of additional MPN-associated mutations (36). *In vitro* mutant JAK2 express the high level of phosphorylated JAK2 leading to the increase levels of phospho-STAT5, phospho-AKT and phospho-ERK1/2. Scott *et al* established the mutant JAK2 exon 12 and V617F in BaF3 cells and found K539L substitution which is exon 12 variant generated the higher levels of phosphorylated JAK2 and equivalent amounts of phospho-STAT5 than JAK2V617F mutation (35). Moreover, the exon 12 mutants also constitutively activate the Ras–ERK signaling pathway, generating levels of phosphorylated ERK1 and ERK2 that are markedly

higher than JAK2 wild-type and JAK2V617F (37). On the other hand, erythroid cells of patients with a JAK2 exon 12 mutation display significantly lower phospho-AKT levels when compare with JAK2V617F mutation. Therefore, the erythroid hyperplasia in both groups is not dependent on PI3K/AKT activation. Nevertheless, JAK2V617F and JAK2 exon 12 mutants have similar tyrosine-phosphorylated SOCS3 activities in BaF3 cells and MPN patients (38). These data suggest that the V617F and exon 12 mutants have overlapping but distinct effects on intracellular signaling pathways downstream of JAK2 and different types and levels of *JAK2* gene mutations are involved in the erythroid phenotypes. Identification of these differences will be very helpful for driving stem cells to erythroid *vs.* megakaryocytic cells as needed.

Human induced pluripotent stem cell

Human induced pluripotent stem cells or iPSCs are generated from human adult tissues by reprogramming adult mature cells into embryonic stem cells. The iPSC technology was discovered in 2006 by Shinya Yamanaka who reported the induction of pluripotent stem cells from mouse embryonic and adult fibroblasts by essential factors including, *Oct3/4*, *Sox2*, *c-Myc* and *Klf4* (5). The generation of iPSC is dependent on the critical transcription factors that are used for the induction. Many transcription factors have been used to increase the efficiency of iPSC production including *OCT3/4*, *SOX* gene family, *KLF* family, *MYC* family, *NANOG*, *LIN28* and *Glis1* (39). *OCT3/4* and the *SOX* gene family that is composed of *SOX 1*, *SOX2*, *SOX3*, and *SOX15* play a key role in maintaining the stem cells pluripotency. The Krüppel-like factor or *KLF* family, including *KLF1*, *KLF2*, *KLF4* and *KLF5*, regulates proliferation, differentiation, growth, development and survival. The *MYC* family factors including *c-MYC*, *L-MYC*, and *N-MYC* are proto-oncogenes implicated in cancer and play a role in regulating cell growth, self-renewal, differentiation and apoptosis of stem cells. *NANOG* is a transcription factor that is involved in the self-renewal of embryonic stem cells and necessary in promoting pluripotency. *LIN28* is an mRNA binding protein that regulates the self-renewal, differentiation and proliferation of embryonic stem cells. In 2007, Thomson reported that human iPSCs are generated by using the

combination of *LIN28*, *OCT4*, *SOX2*, and *NANOG* (40). Finally, *Glis1* is transcription factor that can be used with *OCT3/4*, *SOX2* and *KLF4*.

The iPSC technology is valuable to establish disease models to understand the pathogenesis of genetic diseases caused by both hereditary and acquired mutations, such as cancer. It can also be used for regenerative medicine, generate the human cells or organs for transplantation. There is also the aim to reduce the risk of graft rejection as multi-potential stem cells can be derived from mature cells of the patients and later differentiated into the desired cell types (41). Moreover, it is a very useful tool for testing in personalized drugs and treatments with precision medicine using patient-specific iPSCs (42).

Recently, there are many researchers interested in the generation of erythrocytes and megakaryocytes derived from iPSCs aiming for blood transfusion. In clinical practice, patients with anemia or thrombocytopenia receive packed red cell or platelet transfusions, which are supplied by donors. There is a limitation in patients who receive frequent transfusion may develop antibodies to allogeneic blood cells. Hence, red blood cells from iPSC/ESCs engineered to be compatible with patients with rare blood groups as potential options in these patients. Red blood cells contain no nucleus and therefore cannot be divided after transfused. This would suggest no risk of leukemic transformation and would be an advantage for future clinical trials (43). The main obstacle is the yield of red blood cells derived from iPSCs culture is very low. Enhancement of *in vitro* erythropoiesis by MPN-related genes may be helpful to generate sufficient numbers of red blood cells for transfusion.

Induced pluripotent stem cell and MPNs disease modeling

The underlying genetic defects in BCR/ABL negative MPNs were not well defined and the reason why a JAK2 mutation causes of PV and the other present in essential thrombocythemia is the top question. There have been various hypotheses such as allele burden in heterozygous and homozygous of JAK2V617F may affect the stem cell or progenitor cell behaviors. In addition to *JAK2* mutation, there are many types of mutations that also contribute to the MPNs pathogenesis. Many reports

attempt to study the pathogenesis of MPNs from mouse models which overexpress JAK2V617F mutation. However, it is unlikely that the current animal models are adequate for the identification of pre-JAK2V617F lesions since overexpression of the JAK2V617F mutant results in an acute MPNs disease different from chronic indolent disorders found in humans. There is a limitation as mice do not always demonstrate similar pathological changes as humans. Similarly, overexpressing the JAK2V617F mutated allele in human $CD34^{+}$ cells would unlikely reveal the nature of pre-JAK2 lesions and their relationship to the *JAK2* mutation. Currently, PV patient-specific iPSCs have been generated from blood patients and the result showed enhance erythropoiesis compared with healthy controls when they were placed into erythroid differentiation conditions, recapitulating the major clinical feature of PV (44). The genetic background of the patients and the other co-exiting somatic mutations may effects the study of MPN molecular mechanisms using patient-derived iPSCs. Therefore, normal iPSCs which are modified at the *JAK2* gene will be a suitable tool for studying the specific actions of *JAK2* mutations in MPNs.

CHAPTER III

MATERIALS AND METHODS

Experimental design

Objectives	Experimental plans
1. Establishing the iPSC lines containing inducible *JAK2* mutations	1. Transfect iPSCs with viruses containing JAK2V617F or JAK2 carrying exon 12 mutation (N542_E543 del) under a doxycycline-inducible system
2. Testing for *JAK2* gene mutations in genetically modified iPSCs	1. Conventional PCR and DNA sequencing to detect mutations 2. RQ-PCR to quantify *JAK2* gene expression after doxycycline induction
3. Characterization of modified iPSCs	1. Karyotyping of modified iPSCs 2. RT-PCR to detect the pluripotency gene expression 3. Embryoid body formation and immunofluorescence to prove the pluripotency
4. Generation and characterization of erythroid cells derived from modified iPSCs	1. Determine erythrocyte yields using total cell count and flow cytometry (CD71/GPA) 2. Morphological analysis by Wright stain 3. Hemoglobin analysis using Beta Bio-Rad Variant, RT-PCR and RQ-PCR
5. Using modified iPSCs to model MPNs	Among different *JAK2* constructs 1. Examine the JAK/STAT signal transduction using Capillary Western immunoassay 2. Test for drug sensitivity compared with the wide-type

Research methodology

Normal induced pluripotent stem cells (iPSCs)

This research used a normal human iPSC line named HS4 (A gift from Assist. Prof. Dr. Nipan Israsena, Faculty of Medicine, Chulalongkorn University), which was derived from skin fibroblasts of a healthy subject after informed consent. The transformation was performed in a previous study (45). The experimental designs using human iPSCs were approved by the Institutional Review Board of the Faculty of Medicine at Chulalongkorn University, Bangkok, Thailand (The certificate number 33/2018) and were conducted in accordance with the Declaration of Helsinki.

Induced pluripotent stem cells were cultured on cell culture plates which were coated with matrigel® matrix (Corning, NY, USA) with mTSER medium (STEMCELL Technologies, Vancouver, BC, Canada) containing 1% Antibiotic-Antimycotic. Cells were maintained in a Forma™ Series 3 Water Jacketed CO_2 Incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C in humidified atmosphere with 5% CO_2.

Establishing the iPSC lines with *JAK2* mutations

A normal human iPSC line was modified by overexpressing two types of hyperactive *JAK2* gene which were a point mutation in exon 14 (*JAK2*p.V617F) and a small deletion in exon 12 (*JAK2*p.N542_E543del), using viral transduction.

Firstly, the wild-type *JAK2*-containing plasmid (Addgene, Cambridge, MA, USA) was altered using the Site-directed mutagenesis kit (Thermo Fisher Scientific, Waltham, MA, USA). The constructed plasmids carrying *JAK2*p.V617F (JAK2V617F) and *JAK2*p.N542_E543del (JAK2exon12) were inserted into the complementary site of pLVX-TetOne™-Puro vector by which the mutated *JAK2* were expressed under the Lentiviral Tet-One inducible expression system (Clontech, Palo Alto, CA, USA) **(Figure 10)**.

For the transformation process, purified plasmid DNA were added directly to Stellar™ competent cells (Clontech) and mixed by gentle tapping on ice. Cells were incubated on ice for 30 minutes and then transformed foreign DNA into bacterial

host cells by heat shock. Subsequently, cells were transferred to water bath at 37°C for exactly 45 seconds before put the tubes back on ice for 2 minutes. SOC medium (without antibiotic) was added to the bacteria that were then grown on a shaking incubator (100 rpm) at 37°C for 1 hour. Transformed cell suspensions were plated onto LB agar with ampicillin and incubated at 37°C overnight. Growing colonies which contained the plasmids were isolated.

For lentiviral production, the recombinant vectors containing either JAK2V617F or JAK2exon12 coding sequences were transfected into packaging cells (293 FT cells) and cell-free supernatant were collected and filtered through a 0.45 µm pore size filter after transfection for three days. Virus-containing supernatants were centrifuged at 25,000 rpm for 90 minutes before incubated with normal iPSCs in the presence of 6 µg/ml polybrene for transduction. Transfected iPSCs were cultured for 24 hours and selected by puromycin.

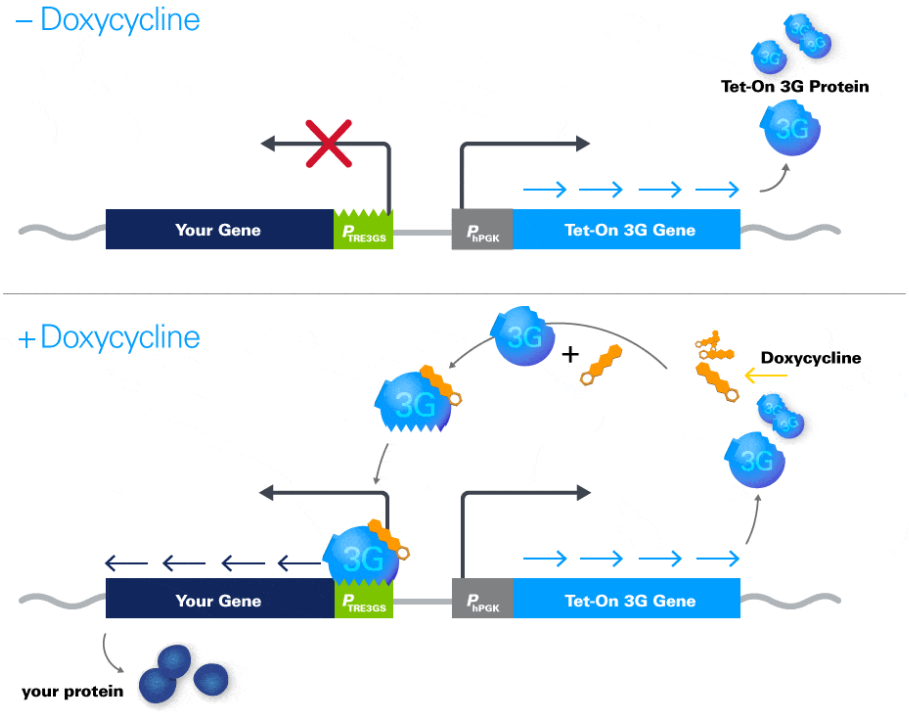


Figure 10 Lentiviral Tet-One Inducible Expression System
(http://www.clontech.com/US/Products/Inducible_Systems/TetSystems_Product_Overview)

Testing *JAK2* gene mutation in the modified iPSCs

The modified iPSCs must be confirmed for *JAK2* gene insertion by using conventional PCR and DNA sequencing. In addition, the expression levels of *JAK2* gene were determined by using quantitative real-time PCR (RQ-PCR).

To verify the engineered cell lines, modified iPSCs were harvested and extracted for genomic DNA by using the prepGEM (MicroGEM, Aotearoa, New Zealand). A cell pellet was gently mixed with Buffer blue and prepGEM before incubated in 75°C for 5 minutes and 95°C for 2 minutes. After incubation, DNA was dissolved with Tris-EDTA (TE) buffer and had concentration measured by using a Nanodrop instrument (Thermo fisher scientific).

The DNA from modified iPSCs was assayed for *JAK2* gene insertions using the following methods. Each tube contained cDNA (100 ng/µl final concentration), 10 µM forward and reverse primer mix specific for exogenous *JAK2* (The primer sequences are listed in **Table 3**) and GoTaq® Master Mixes (Promega, Madison, Wisconsin, USA). The PCR condition was initial denaturation in 95°C for 5 minutes, followed by 30 cycles of denaturation in 98°C for 40 seconds, annealing in 63°C for 1 minute and extension in 72°C for 1.30 minutes. Unmodified iPSCs were used as a negative control. DNA templates were amplified in a T gradient Biometra thermal cycler (Biometra GmbH, Göttingen, Germany). Sanger DNA sequencing was used to confirm *JAK2* gene mutations and analyzed by capillary electrophoresis at Macrogen Inc., Korea.

Furthermore, the selected iPSCs were examined for the efficiency of doxycycline inducible system. Cells were cultured in the mTSER medium with 0 and 2 µg/ml of doxycycline (DOX, STEMCELL Technologies) and incubated for 24 hours before harvesting a cell pellet. Cells were extracted for RNA by using RNA purification kit (GeneJet RNA purification kit; Thermo Fisher Scientific). Cells were lysed with lysis buffer and 2-ME and then ethanol was added to the lysate. The lysed cells were transferred into the spin column before centrifuged at 12,000 rpm for 1 minute and then the flow-through was discarded. The first washing buffer was pipetted to the spin column and centrifuged at 12,000 rpm for 1 minute. Flow-through was removed

from the spin column and second washing buffer was added. Columns were centrifuged at 12,000 rpm for 1 minute and the flow-through was discarded before centrifuge again at 12,000 rpm for 1 minute to remove ethanol. The spin columns were transferred into microcentrifuge tubes and nuclease-free water was added. After 1 minute incubation at room temperature, it was centrifuged again at 12,000 rpm for 1 minute to elute the RNA. RNA concentration and purity were determined by using a Nanodrop instrument.

For cDNA synthesis, Oligo (dT) 12-18 primer was added and incubated at 70°C for 5 minutes before adding the mixture of DNA synthesis kit (Thermo Fisher Scientific) composed of Reaction Buffer, RiboLock RNase Inhibitor, 10 mM dNTP Mix and RevertAid M-MuLV RT. After that, the reactions were incubated at 4°C for 5 minutes followed by 37°C for 5 minutes and then incubated at 42°C for 60 minutes. The reactions were terminated by heating at 70°C for 5 minutes before cool down at 4°C. Complementary DNA was analyzed for the amount of *JAK2* gene transcription by using RQ-PCR.

The real time PCR tube 8 strips were used for RQ-PCR. Each of tube contained 1 µl of cDNA (final concentration 100 ng/µl), 1µl of 10 µM forward and reverse primer mix, and 10 µl of CAPITAL™ qPCR Probe Mix (biotechrabbit GmbH, Hennigsdorf, Germany). PCR was performed using exogenous *JAK2*-specific primers and the following protocol: initial denaturation in 95°C for 10 minutes, followed by 40 cycles of denaturation in 95°C for 15 seconds, annealing in 59.5°C for 30 seconds, and extension in 72°C for 45 seconds. PCR was performed using the 7500 Fast Real-time PCR system (Applied Biosystems, Foster City, CA, USA). The relative quantity of each target gene was normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a house keeper gene (The primer sequences are listed in **Table 3**). Fold changes were calculated by evaluating expression using comparative CT ($\Delta\Delta$Ct) method. Normal iPSCs were used as a negative control and all samples were processed in triplicate.

Characterization of genetically modified iPSC properties

The modified iPSCs were required to test for the normal karyotypes, the pluripotency statuses and the capacity to differentiate into cells of the three germ layers.

Karyotyping was used to prove that the numbers and structures of iPSC chromosomes remained normal after the transduction processes. Chromosomal analysis was performed by GTG-banding analysis at the Center for Medical Diagnostic Laboratories, Faculty of Medicine, Chulalongkorn University, Thailand, following the recommendations by the International System for Cytogenetics Nomenclature (ISCN).

The pluripotency of stem cells was evaluated by the presence of stem cell markers including *NANOG, OCT4, SOX2, KLF4* and *MYC*. The expression of these genes was detected in modified iPSCs by using RT-PCR. Messenger RNA was reverse transcribed to cDNA similar to the previously protocol. Amplifications were carried out in a T gradient Biometra Thermal Cycler (Biometra GmbH) in 15 µl reactions containing 1 µl of 10 µM forward and reverse primer mix, 1 µl cDNA (final concentration 100 ng/µl) and 12 µl of GoTaq® Master Mixes. The primer sequences are listed in **table 3** (43, 46-48). PCR conditions included a first step of initial denaturation in 95°C for 7 minutes, followed by 35 cycles of denaturation in 95°C for 1 minute, annealing in 56.5°C for 45 seconds and extension in 72°C for 1 minute. The PCR products were electrophoresed on 2% agarose gel. Human *GAPDH* served as an internal control.

Table 3 List of primer sets for exogenous *JAK2* and pluripotency testing of stem cells

Genes	Forward primers (5'- 3')	Reverse primers (5'- 3')
Exogenous *JAK2*	CCCTCGTAAAGAATTCATGGGAATGGCCTGCCTTACGATG	TCTTTGCTCGAATACATTTTGG
NANOG	ATACCTCAGCCTCCAGCAGA	CAGGACTGGATGTTCTGGGT
OCT4	GAAGGTATTCAGCCAAACGC	GTTACAGAACCACACTCGGA
SOX2	GGGAAATGGGAGGGGTGCAAAAGAGG	TTGCGTGAGTGTGGATGGGATTGGTG
KLF4	ACGATCGTGGCCCCGGAAAAGGACC	TGATTGTAGTGCTTTCTGGCTGGGCTCC
MYC	GCGTCCTGGGAAGGGAGATCCGGAGC	TTGAGGGGCATCGTCGCGGGAGGCTG
GAPDH	AACAGCCTCAAGATCATCAGC	TTGGCAGGTTTTTCTAGACGG

In addition, the ability of stem cell to differentiate into cells of the endoderm, mesoderm and ectoderm were tested via embryoid body (EB) formation and stained by Human Three Germ Layer 3-Color Immunocytochemistry Kit (R&D Systems, Minneapolis, MN, USA). Embryoid bodies were prepared by plating modified iPSCs on an ultra-low attachment tissue culture dish in Iscove's Modified Dulbecco's Medium (IMDM, Fisher scientific, Pittsburg, USA) supplement with 10% FBS and 4 mM L-glutamine (EB medium). The cells clumped called embryoid bodies grew in suspension for 7 days. For differentiation, embryoid bodies were transferred onto 0.1% gelatin coverslips in 24-well plates and cultured in EB medium approximately 14 days prior to performing immunocytochemistry. Differentiated cells were detected with Northern Lights (NL) fluorochrome conjugated antibodies that were specific to cells from ectoderm, mesoderm and endoderm (49). Antibodies to ectodermal cells were anti-human OTX2 conjugated with NL557 and anti-human SOX1 conjugated with NL493. Mesodermal antibodies were anti-human brachyury conjugated with NL557 and anti-human HAND1 conjugated with NL637. Antibodies to endodermal cells were anti-human GATA-4 conjugated with NL493 and anti-human SOX17 conjugated with NL637. All nuclei were counterstained with DAPI. Fluorescence

images were obtained using Axio Observer fluorescence microscopy (Carl Zeiss, Jena, Germany).

Generation and characterization of erythrocytes from modified iPSCs

To compare the abilities of cell differentiation into erythrocytes from normal iPSCs and modified iPSCs, the co-culture system according to the Ochi protocol was used (6).

Feeder cell preparation

Stromal cells, C3H10T1/2 cells, were cultured in Basal Medium Eagle (BME) medium (Biowest, Nuaillé, France) supplemented with 10% Fetal Bovine Serum (FBS), 1% L-glutamine and 1% Antibiotic-Antimycotic termed the C3H10T1/2 medium. C3H10T1/2 cells grew in culture flasks and were harvested for feeder cells at 90-95% confluence. The culture medium was removed and cells were washed twice with Phosphate buffer saline (PBS). C3H10T1/2 cells were dissociated with 0.25% trypsin-5 mM EDTA. The reactions were stopped using BME medium and mixed well to make them into single cells. Cells were centrifuged at 1,200 rpm for 5 minutes before medium removal. After that, cells were treated with 50 Gy gamma irradiation to stop the cell growth and then homogeneously spread onto a 0.1% gelatin-coated dish (15 cm). Irradiated C3H10T1/2 cells or feeder cells were cultured in supplemented BME medium for 24 hours before uses.

ES-Sacs formation

Modified iPSCs were washed with PBS. Cells were dissociated into small pieces (>100 cells) by CTK (Collagenase Type IV, Trypsin and KSR) solutions and incubated at 37°C for 5 minutes. After aspiration of the dissociation buffer, small-size colonies of iPSCs were transferred onto irradiated feeder cells and cultured in a hematopoietic cell differentiation medium (ES medium), Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10 µg/ml human insulin, 5.5 µg/ml human transferrin, 5 ng/ml sodium selenite, 2 mM L-glutamine, 0.45 mM α-

monothioglycerol, 50 µg/ml ascorbic acid and 15% fetal bovine serum (FBS) containing 20 ng/ml recombinant human vascular endothelial growth factor (rhVEGF, R&D Systems) which was refreshed every 2-3 days. On day 14 of culture, embryonic stem cell–derived sacs (ES-Sacs) were emerged.

Differentiation of erythrocytes

Cells from ES-Sacs were gently crushed with a needle and pipette and passed through a 40-µm cell strainer which selected a population of hematopoietic progenitor cells (HPCs) into a 50 ml tube. After that, cells were centrifuged at 1,500 rpm for 10 minutes and counted the HPCs with 0.1% trypan blue. The hematopoietic progenitors at 5×10^4 cells/ml were maintained in hematopoietic cell differentiation medium supplemented with 50 ng/ml human thrombopoietin (TPO, R&D Systems), 50 ng/ml human stem cell factor (SCF, R&D Systems) and 5 IU/ml erythropoietin (EPO, Eprex®, Janssen Pharmaceutical, Beerse, Belgium) and then transferred onto fresh and irradiated C3H10T1/2 cells in a six-well plate for 6 days. After 6 days, cells were transferred to new fresh C3H10T1/2 cells and cultured in hematopoietic cell differentiation medium supplemented only with 5 IU/ml EPO for another 9 days. Culture medium was replaced every 3 days and non-adherent cells were harvested and analyzed on day 29 of culture **(Figure 11).**

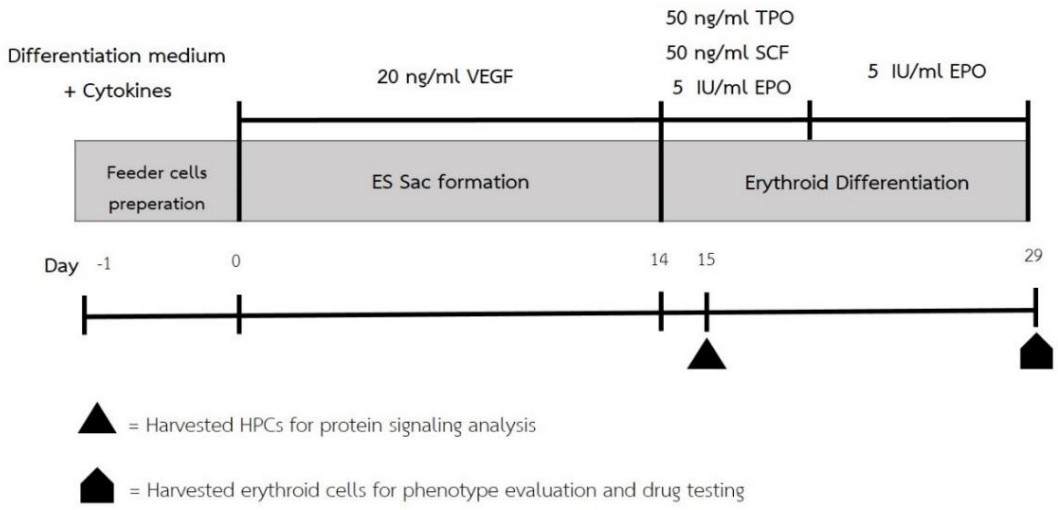


Figure 11 Schematic diagrams of *in vitro* differentiation protocols for erythrocytes production via ES-Sacs formation.
VEGF, Vascular endothelium growth factor; TPO, Thrombopoietin; SCF, Stem cell factor; EPO, Erythropoietin; HPCs, Hematopoietic progenitor cells

Harvested cells were characterized for total cell counts, erythroid-specific surface molecules, morphology and hemoglobin analysis. These erythroid phenotypes were compared in iPSCs with the different mutations.

Surface molecules and numbers of erythrocytes

Erythroid progenitor cells can be identified by the expression of specific cell surface lineage markers. During erythroid differentiation, CD71 (Transferrin receptor) expresses at the early stage of erythrocytes, while Glycophorin A (GPA) expresses at the late stage of differentiation. Cells on day 29 were stained with PE-conjugated anti-human CD71 (Clone CY1G4, BioLegend, San Diego, CA, USA) and FITC-conjugated anti-human Glycophorin A (Clone HI264, BioLegend) to detect cells in the erythroid lineage. Cells were collected and washed with PBS before adding both monoclonal antibodies. Cells were incubated at room temperature in the dark for 30 minutes. After incubation with the antibodies, cell pellets were washed with PBS and flow cytometry was performed by using BD FACSAria II (Becton Dickinson, Franklin Lakes,

NJ, USA). The total erythroid cells were calculated by counting the total numbers of cells and multiplying by the percentages of $CD71^{+}GPA^{+}$ cells.

Morphological analysis

The modified iPSCs on day 29 of culture were classified by morphology into various differentiation stages of the erythroid lineage as proerythroblasts, basophilic erythroblasts, polychromatic erythroblasts, orthochromatic erythroblasts and erythrocytes. Cells were harvested from culture supernatant and were mounted on slides by Cellspin I 1-12 (Tharmac GmbH, Wiesbaden, Germany). Wright-Giemsa stain and phosphate buffer were added to the cells on glass slides and mixed gently for 5 minutes. The slides were then washed thoroughly with deionized water and placed on a rack to dry before observed under Leica DM 1000 microscopy (Leica, Wetzlar, Germany).

Hemoglobin analysis

Generated erythroid cells were analyzed to detect the expression of beta globin subtypes by using Beta Bio-Rad Variant, RT-PCR and RQ-PCR.

The Variant II Beta Thalassemia Short Program utilizes the ion-exchange high-performance liquid chromatography (HPLC) principle (Bio-Rad, California, United States) was applied. The samples were mixed, diluted on the Variant II Sampling Station and injected into the analytical cartridge. The sample report and a chromatogram were generated and showed all hemoglobin fractions eluted, their retention times, the area of the peaks and values of the fractions.

For the expression of beta globin subtypes by RT-PCR, cells were extracted RNA by using RNA purification kit and then synthesized to complementary DNA. DNA concentration and purity were determined by using a Nanodrop instrument. Hemoglobin expression were determined by using specific primers for embryonic hemoglobin (epsilon; ε), fetal hemoglobin (gamma; γ) and adult hemoglobin (beta; β). The primer sequences are listed in **table 4** (50-52). Each PCR tube contained 1 µl of cDNA (final concentration 100 ng/µl), 1 µl of 10 µM forward and reverse primer

mix and 12 µl of GoTaq® Master Mixes (Promega). PCR was performed using the T gradient Biometra Thermal Cycler (Biometra GmbH) by the following protocol: initial denaturation in 98°C for 2 minutes, followed by 30 cycles of denaturation in 95°C for 30 seconds, annealing (in 55.0°C for epsilon and gamma, 53.0°C for beta) for 45 seconds and extension in 72°C for 30 seconds. For beta globin, the annealing temperature was 53°C for 45 seconds.

Moreover, the differences in hemoglobin expression between with and without doxycycline induction were analyzed by using quantitative real-time PCR. Each of real time PCR tube 8 strips contained 1 µl of cDNA (final concentration 100 ng/µl), 1µl of 10 µM forward and reverse primer mix, and 10 µl of CAPITAL™ qPCR Probe Mix (biotechrabbit GmbH). The cDNA templates were amplified by using hemoglobin primers. PCR was performed using the 7500 Fast Real-time PCR system (Applied Biosystems) using the following protocol: initial denaturation in 95°C for 10 minutes followed by 40 cycles of denaturation in 95°C for 15 seconds, annealing (in 55.0°C for epsilon and gamma, 53.0°C for beta) for 30 seconds and extension in 72°C for 45 seconds. The relative quantity of each target gene was normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a house keeper gene. Fold changes were calculated by evaluating expression using comparative CT ($\Delta\Delta$Ct) method. All samples were processed in triplicate.

Table 4 List of primer sets for hemoglobin expression

Genes	Forward primers (5'- 3')	Reverse primers (5'- 3')
Epsilon globin	GCCTGTGGAGCAAGATGAAT	GCGGGCTTGAGGTTGT
Gamma globin	TGAGAACTTCAAGCTCCTGGGAAA	TGCAGAATAAAGCCTATCCTTGAA
Beta globin	TACATTTGCTTCTGACACAAC	ACAGATCCCCAAAGGAC

Using iPSC-derived erythroid cells as a disease model for myeloproliferative neoplasm

The genetically modified erythroid cells generated from culture were examined for cell signaling and drug treatment to investigate for the disease mechanisms and potentially new therapy, respectively.

JAK/STAT signal transduction

To understand the signaling pathways in the mutated JAK2 proteins which lead to erythroid hyperplasia. Cells were investigated for the cell signaling composed of JAK2, STAT1, STAT3, STAT5, ERK1/2 and AKT in both native and phosphorylated forms using Capillary Western immunoassay (ProteinSimple, California, USA).

From ES-Sacs formation, the hematopoietic progenitor cells were harvested and transferred onto fresh irradiated C3H10T1/2 cells and then cultured with a hematopoietic cell differentiation medium supplemented with 50 ng/ml TPO, 50 ng/ml SCF and 5 IU/ml EPO for 24 hours. Stimulated cells were collected and washed twice with PBS before supernatant removal. Cell pellets were homogenized in RIPA lysis buffer containing a protease/phosphatase inhibitor cocktail (MedChemExpress, NJ, USA). The homogenates were centrifuged 12,000 rpm for 20 minutes at 4 °C. The supernatants were measured for protein concentrations using Micro BCA protein assay kit (Thermo Fisher Scientific) and then analyzed by an ELISA reader (Tecan, Männedorf, Switzerland).

Signaling protein analyses were performed on a Capillary Western immunoassay (ProteinSimple) according to the manufacturer's instructions at the range of 12-230 kDa. Protein samples were diluted to an appropriate concentration, loaded at the amounts of 6 µg in a sample buffer, mixed with Fluorescent Master Mix, and heated at 95°C for 5 minutes. Primary antibodies were rabbit anti-JAK2 (#3230), anti-phosphorylated JAK2 (#3776), rabbit anti-STAT1 (#9175), anti-phosphorylated STAT1 (#7649) antibodies, rabbit anti-STAT3 (#12640), anti-phosphorylated STAT3 (#9145) antibodies, rabbit anti-STAT5 (#9363), anti-phosphorylated STAT5 (#4322), rabbit anti-ERK1/2 (#4695), anti-phosphorylated ERK1/2 (#4370), and rabbit anti-AKT (#4691), anti-phosphorylated AKT (#4060)

antibodies. All primary antibodies purchased from Cell Signaling Technology® were diluted to 1:300 before uses. The secondary antibody was HRP-conjugated anti-rabbit antibody (DM001, ProteinSimple). The samples, blocking reagent, primary antibodies, HRP-conjugated secondary antibodies and chemiluminescent substrate were pipetted into the plate. Instrument default settings were used for stacking and separation at 375 V for 25 minutes, blocking reagent for 5 minutes, primary and secondary antibodies both for 30 minutes and Luminol/peroxide chemiluminescence detection for 15 minutes. The resulting chemiluminescent signal were detected and quantitated by Program Compass for SW.

Effects of drugs on erythroid development from modified iPSCs

The hematopoietic progenitor cells generated from modified iPSCs were cultured in the absence or presence of drugs which have potentials to treat MPN patients, 0.5 μg/ml interferon alpha-2a (Roche, New Jersey, USA) and/or 250 nM arsenic trioxide (M&B, London, United Kingdom) (53, 54). The yields of total erythroid cells were enumerated as above. The percentage and relative changes of cell deaths were determined using FITC-conjugated anti-human Glycophorin A (Clone HI264, BioLegend) and propidium iodide (#421301, BioLegend). The results were compared between with *vs.* without doxycycline to determine the differential effects on mutated *vs.* normal cells, respectively. An ideal drug should affect the mutant cells more than normal cells.

Statistical analysis

Statistical analyses were performed using SPSS software (version 22.0). All the continuous variables were expressed as means ± standard deviations (SD). The statistical differences between groups (doxycycline induction *vs.* no doxycycline induction of mutated *JAK2* transgene expression) were determined using the paired T-test. In addition, one way ANOVA and independent-sample T-test were also used to detect statistical significances. The probability (P) values of less than 0.05 were considered statistically significant.

CHAPTER IV
RESULTS

The normal iPSCs overexpressing JAK2V617F and JAK2 with exon 12 mutation were constructed by viral transduction and erythrocytes were generated *in vitro*. These exogenous genes were controlled by a doxycycline-inducible system. The same lines with *vs*. without doxycycline induction were compared in order to specifically determine the effects of mutated *JAK2*. These modified iPSCs provide a MPNs disease model to understand the molecular mechanisms of *JAK2* gene mutations. Moreover, the *JAK2* mutations which cause overproduction of blood cells may be used to increase the yields of erythrocytes generated in culture for blood transfusion in the future.

Generation of genetically modified iPSCs

A normal human iPSC line (HS4) was modified by overexpressing two types of hyperactive *JAK2* gene mutations which were a point mutation in exon 14 (JAK2V617F) and a small deletion in exon 12 (N542_E543 deletion) (JAK2V617F-iPSCs and JAK2exon12-iPSCs) by using viral transduction. The system used Tet-One inducible expression, in which the insert gene functioned under the doxycycline control. The modified iPSCs were tested for *JAK2* gene insertion by conventional polymerase chain reaction (PCR) using specific primers to *JAK2*-mutated vectors. Only modified iPSCs demonstrated the PCR products representing an inserted *JAK2* gene, whereas they were absent in normal iPSCs **(Figure 12A)**.

The DNA sequencing confirmed the *JAK2*p.V617F mutation, which was a change from GTC (Valine) to TTC (Phenylalanine) in exon 14, and a deletion at the position N542_E543 of *JAK2* gene in the exon 12 mutation line **(Figure 12B)**.

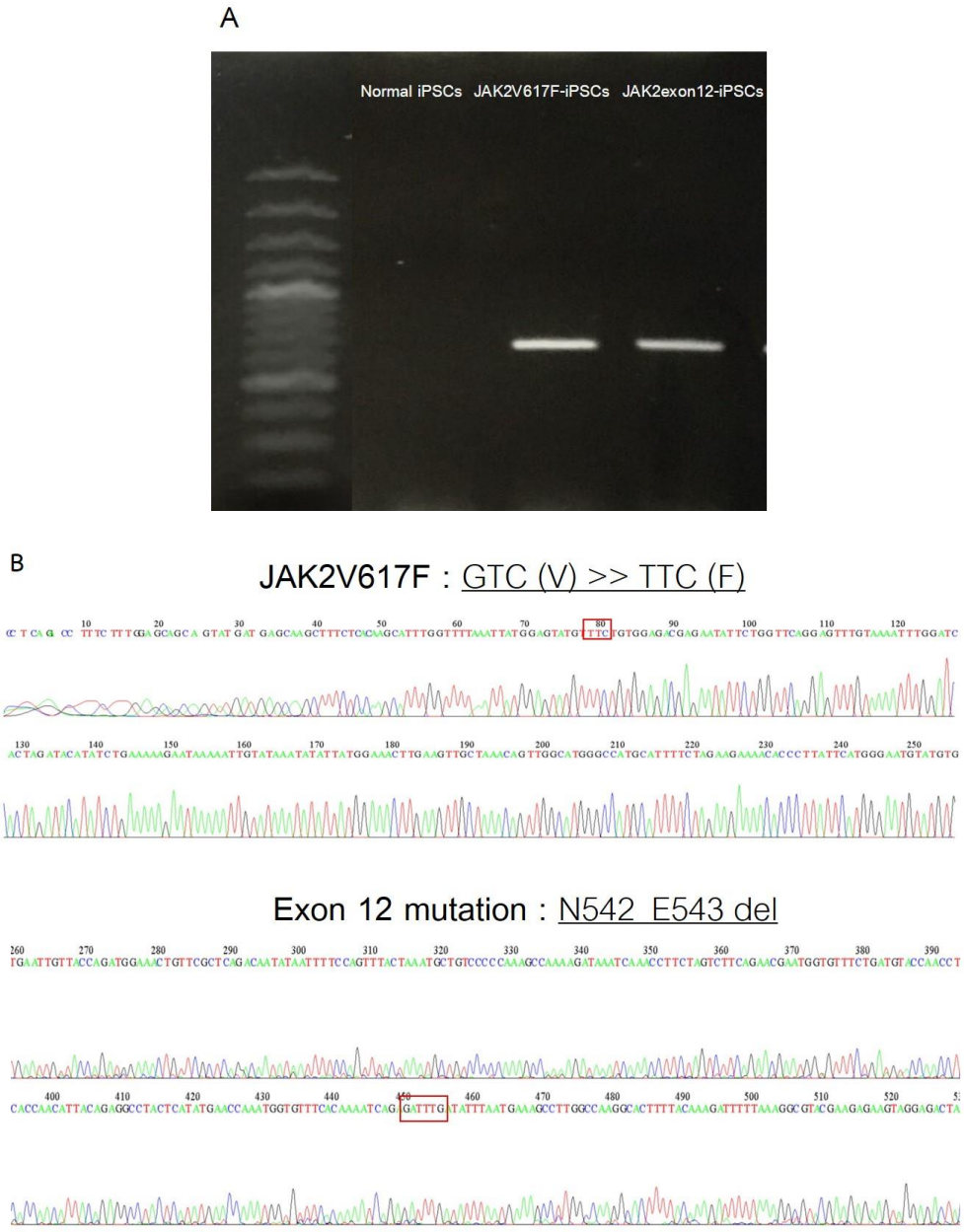


Figure 12 Verification of *JAK2* gene mutations and expression in the modified induced pluripotent stem cells (iPSCs).

A) Conventional polymerase chain reaction (PCR) using transgene-specific primers showed exogenous JAK2 genes in the two modified iPSC lines. The normal iPSC line was used as a negative control. B) DNA sequencing confirmed the point mutation p.V617F in exon 14 and the p.N542_E543del in exon 12 of JAK2 gene in the respective iPSC lines.

Moreover, the selected iPSCs were determined for the efficiency of doxycycline inducible system by evaluating *JAK2* gene expression. After culturing normal iPSCs and modified iPSCs with and without doxycycline for 24 hours, JAK2V617F-iPSCs and JAK2exon12-iPSCs expressed the higher levels of *JAK2* gene after doxycycline exposure at approximately 17.95 ± 1.0 folds (*p* value = 0.008) and 13.7 ± 6.4 folds (*p* value = 0.034), respectively, when compared with cells in the absence of doxycycline **(Figure 13)**.

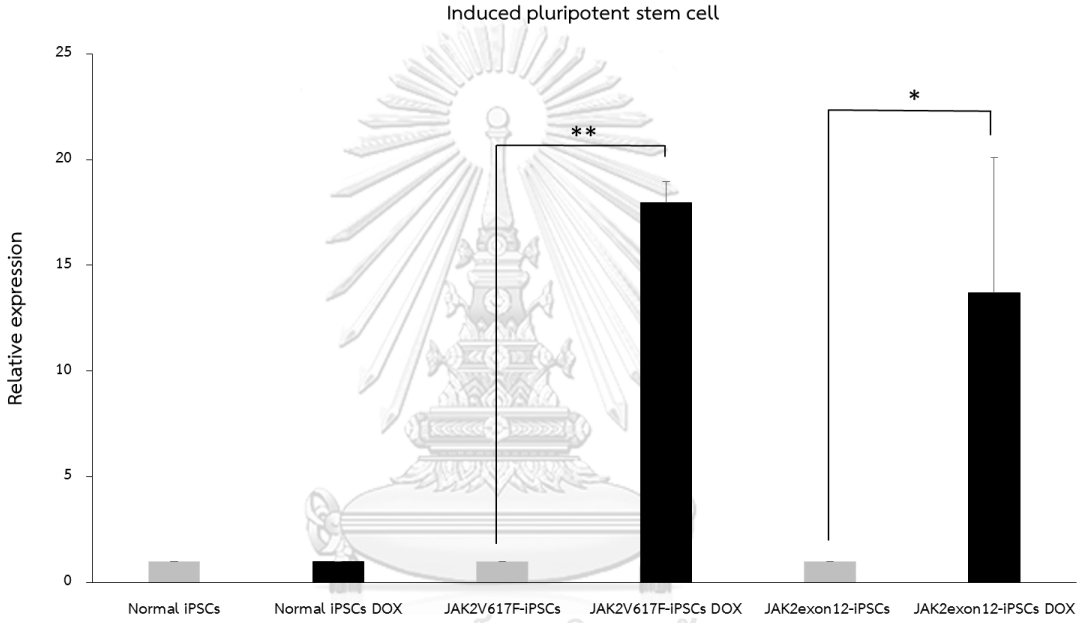


Figure 13 Exogenous *JAK2* gene expression levels in iPSCs after transfection comparing normal, JAK2V617F and JAK2 exon 12 mutation with *vs.* without doxycycline (DOX) induction for 24 hours and analyzed by real-time PCR (RQ-PCR). Data are presented as means ± standard deviations (SD) from three independent experiments. The Pair T-test was used to assess the significant differences between conditions with vs. without doxycycline (DOX). The asterisks (*) and (**) denoted $p<0.05$ and $p<0.01$.

Characteristics of the genetically modified iPSCs

The characteristics of the modified iPSCs were examined regarding karyotype, the pluripotency status and the capacity to differentiation. The GTG-banding of

JAK2V617F-iPSC and JAK2exon12-iPSCs demonstrated normal karyotypes in both numbers (46, XY) and overall structures of chromosomes **(Figure 14)**.

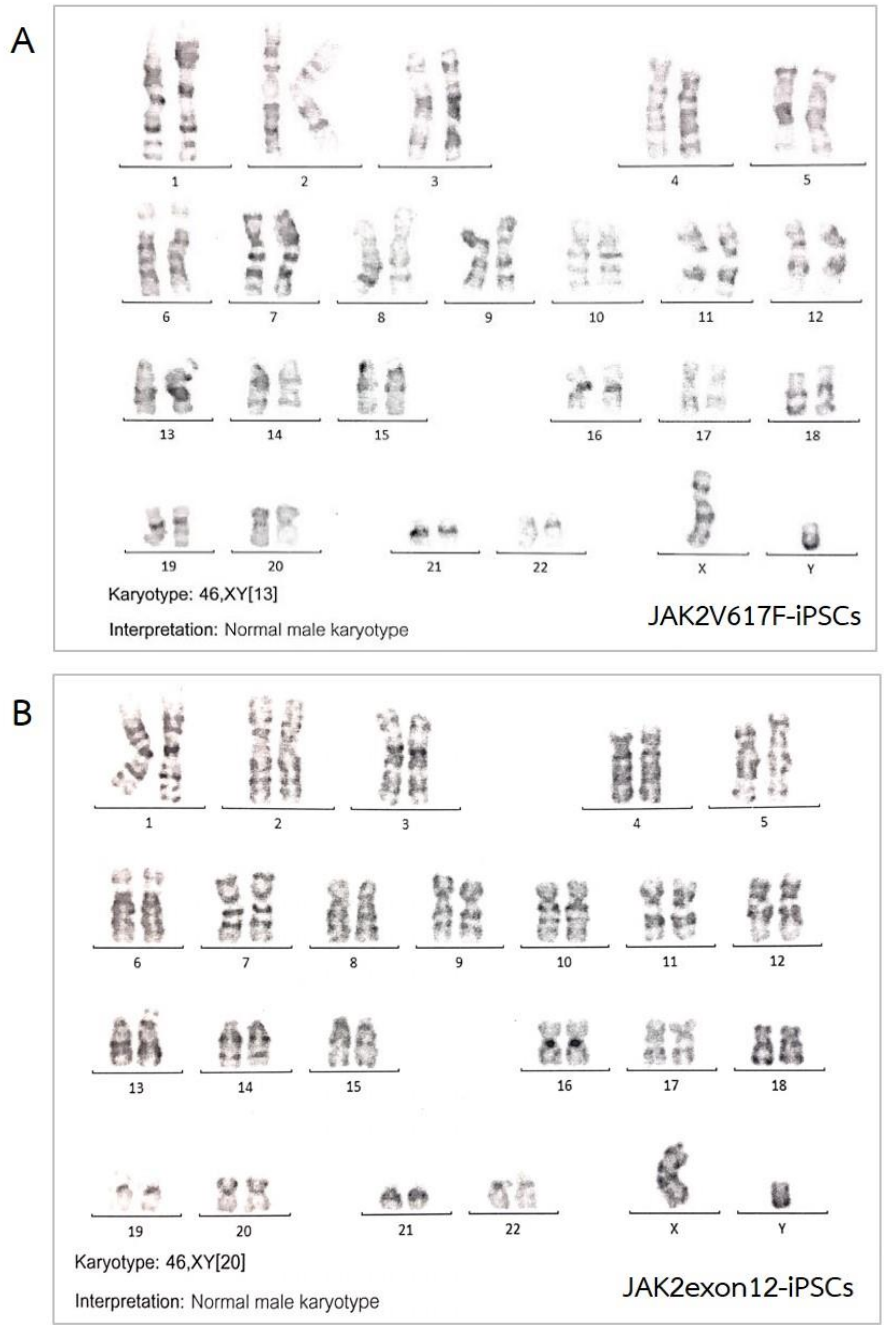


Figure 14 Karyotyping of the genetically modified iPSCs.
JAK2V617F-iPSCs (A) and JAK2N542_E543del-iPSCs (JAK2exon12-iPSCs) (B).

The normal stem cells can continuously divide and self-renew to produce more stem cells. Therefore, the pluripotency of modified iPSCs were determined for the expression of stem cell markers using RT-PCR. **Figure 15** showed the RNA expression of *NANOG* (294 bp), *OCT4* (313 bp), *SOX2* (151 bp), *KLF4* (397 bp) and *MYC* (328 bp) that all represented the pluripotent properties of the modified iPSCs.

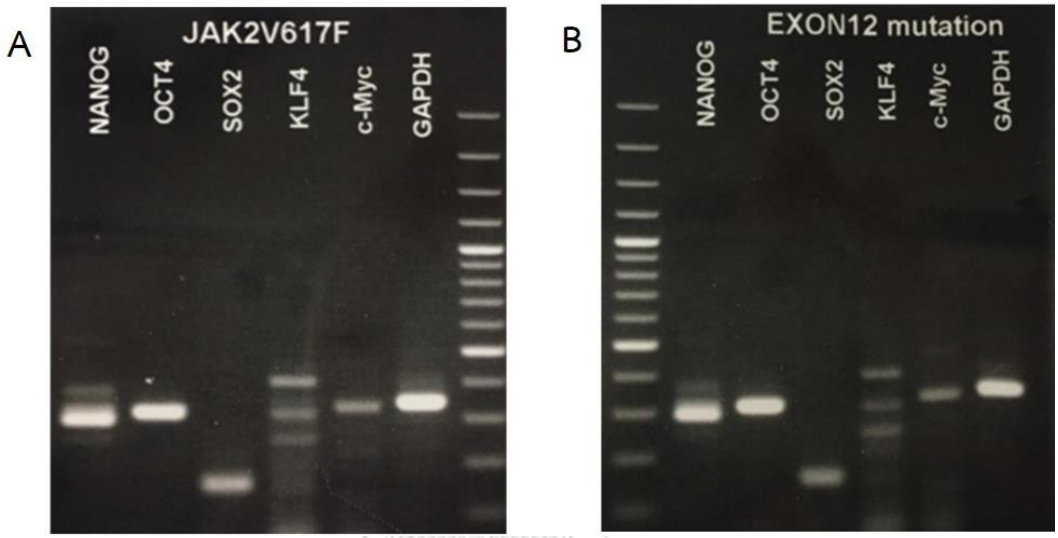


Figure 15 The expression of *NANOG*, *OCT4*, *SOX2*, *KLF4* and *MYC* of JAK2V617F-iPSCs and JAK2exon12-iPSCs using reverse transcriptase polymerase chain reaction (RT-PCR).

Regarding the capacity to differentiate into cells of the three germ layers, modified iPSCs were cultured in the absence of factors that supported pluripotency allowing the cells to aggregate in three dimensional structures to form embryoid bodies **(Figure 16)**. Stem cells within embryoid bodies underwent differentiation and cell specification into the three germ lineages.

Modified iPSC were detected for germ layers from embryoid body differentiation in embryoid medium for 14 days and observed by using the Human Three Germ Layer 3-Color Immunocytochemistry kit. Both of modified iPSCs were simultaneously stained with ectoderm, mesoderm and endoderm antibodies. Immunofluorescence analyses showed that OTX2 and SOX1 which encoded proteins

expressing in ectodermal cells were positive in JAK2V617F-iPSC and JAK2exon12-iPSCs. For mesodermal and endodermal differentiation, both modified iPSCs showed positive staining with anti-HAND1 and anti-GATA-4, respectively **(Figure 17)**.

These data demonstrated that modified iPSCs displayed normal karyotype, maintained pleuripotent properties and had the capacity of multilineage differentiation that were the characteristic of stem cells.

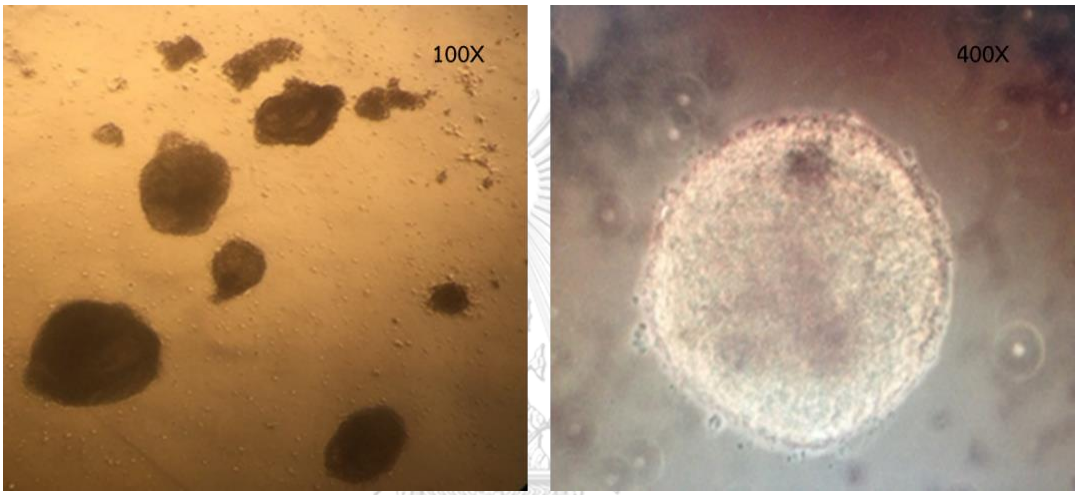


Figure 16 Morphology of cells clumping as embryoid bodies after culturing modified iPSCs under the condition without differentiating cytokines for 7 days.

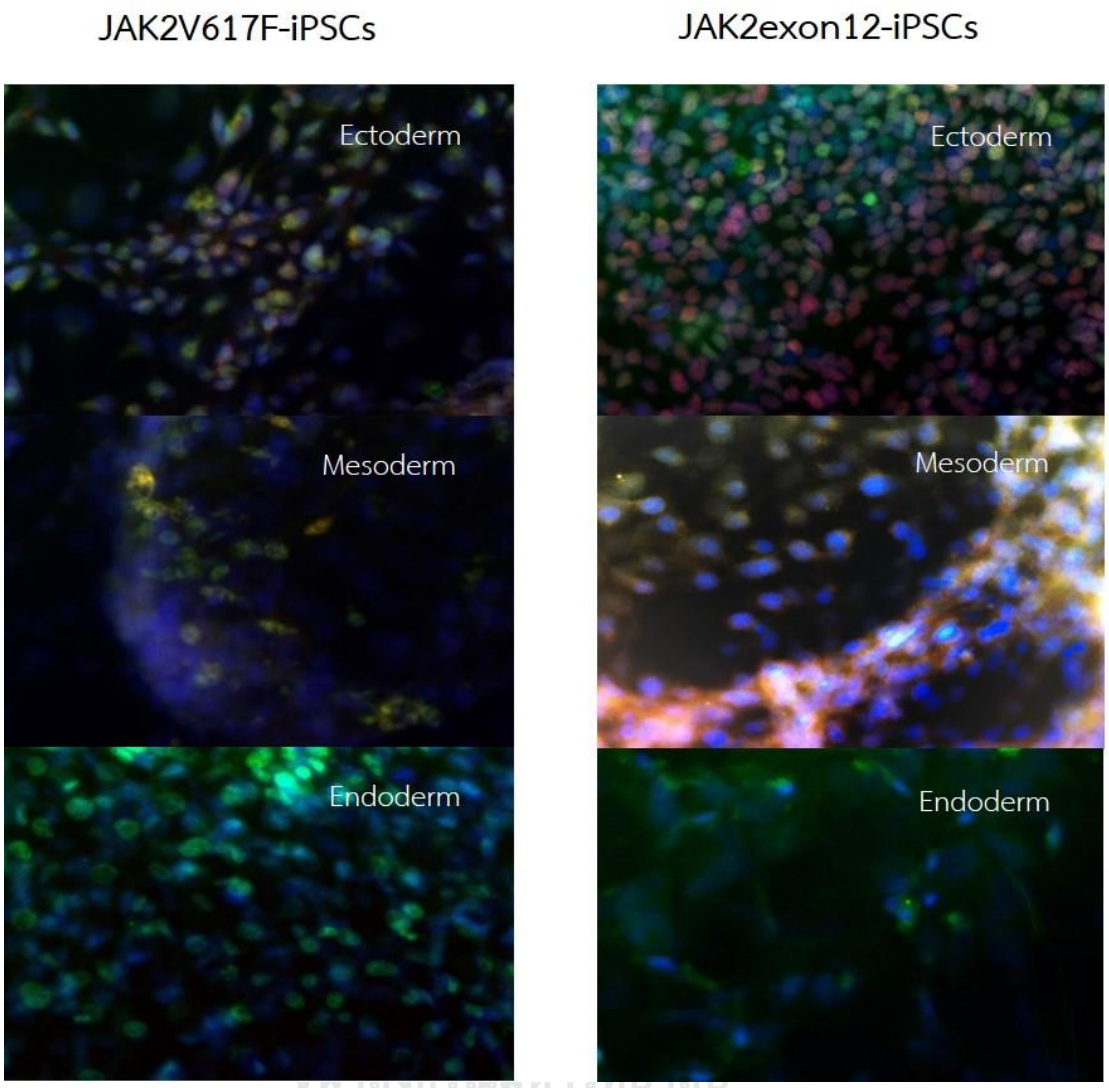


Figure 17 Immunofluorescence of differentiated cells from JAK2V617F-iPSCs and JAK2exon12-iPSCs.

Embryoid bodies were transferred onto 0.1% gelatin coverslips and cultured for 14 days for differentiation. Cells were stained with antibodies specific to ectoderm (red and green), mesoderm (green), endoderm (green) layers and DAPI (blue) for nuclei (400X magnification). The images were captured by Axio Observer fluorescence microscopy.

Erythroid cell differentiation

The generation of erythrocytes used the co-culture system according the Ochi protocol (6). Modified iPSCs were transferred onto irradiated 10T1/2 feeder cells and cultured in ES medium containing rhVEGF until day 14 and ES derived-Sacs which contained hematopoietic progenitor cells were formed **(Figure 18)**. Hematopoietic progenitor cells from ES-Sacs were collected, passed through a 40-micron cell strainer before transferred onto fresh feeder cells and then cultured for 15 days. At that time, round floating cells appeared in culture supernatant. After centrifugation, cell pellets showed the red color suggesting the presence of hemoglobin **(Figure 19)**.

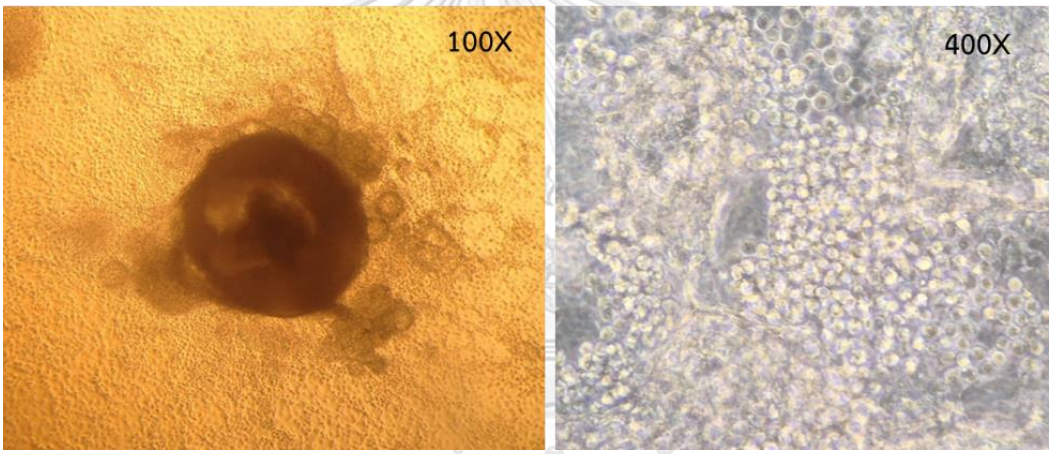


Figure 18 Erythroid cell differentiation from modified induced pluripotent stem cells (iPSCs) via ES-Sacs.

ES-derived sacs containing hematopoietic progenitor cells were generated from modified iPSCs on day 14 at 100X and 400X magnifications.

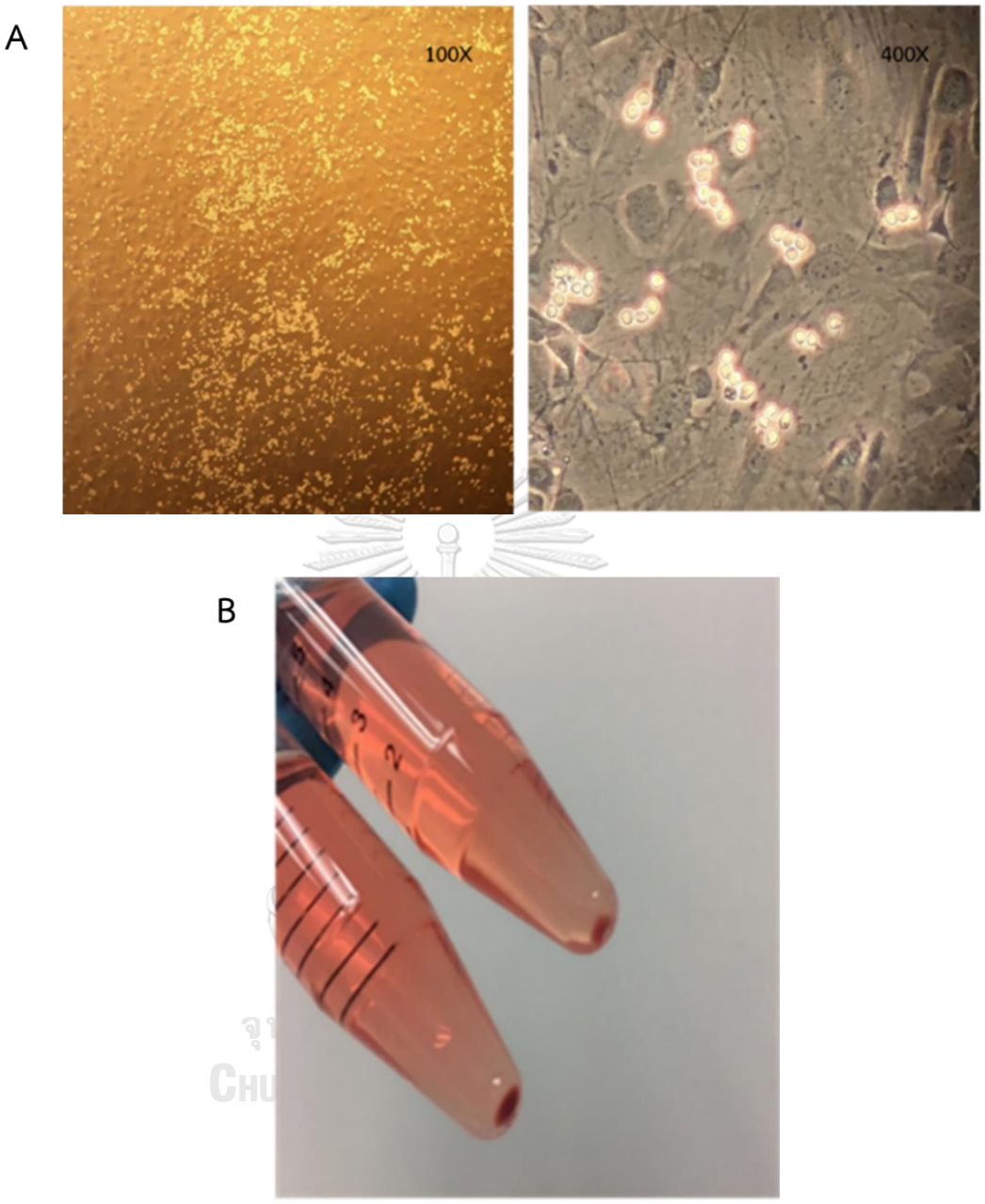


Figure 19 Erythroid cells in a culture plate (A) and red blood cell pellets after centrifugation (B).

The expression of *JAK2* transgenes after doxycycline induction

The inserted gene expression may be different between early and late stages of iPSCs differentiation. Therefore, hematopoietic progenitor cells from ES-Sacs and iPSC-derived erythroid cells were examined for *JAK2* gene expression by RQ-PCR. The

modified iPSCs were differentiated and cultured with *vs*. without doxycycline before harvesting hematopoietic progenitor cells on day 14 and erythroid cells on day 29 to detect *JAK2* mRNA.

At the stage of hematopoietic progenitor cells, JAK2V617F-iPSCs and JAK2exon12-iPSCs after induction with doxycycline expressed *JAK2* mRNA at approximately 24.1 ± 1.3 folds (*p* value = 0.002) and 17.6 ± 5.2 folds (*p* value = 0.046), respectively **(Figure 20A)**. At the erythroid stage, there were *JAK2* gene over-expression levels at 16.9 ± 1.0 folds (*p* value = 0.003) and 9.0 ± 4.9 folds (*p* value = 0.049) in *JAK2*V617F-iPSCs and JAK2exon12-iPSCs, respectively **(Figure 20B)**. There were no differences in *JAK2* gene expression after doxycycline induction among different stages of cellular differentiation. Both hematopoietic progenitor cells and erythroid cells still maintained the high levels of *JAK2* expression.

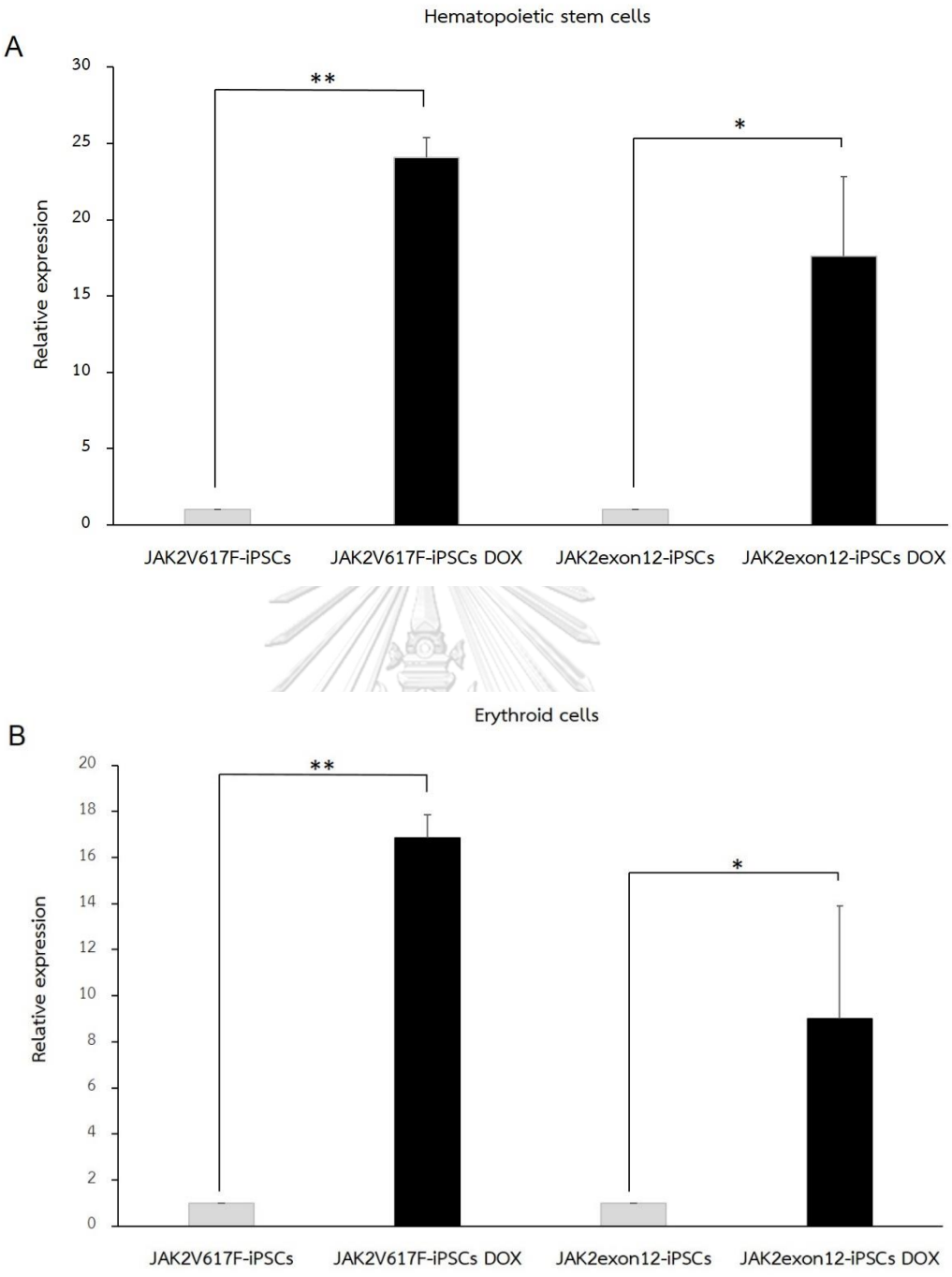


Figure 20 The graph showed relative mRNA levels of exogenous *JAK2* in hematopoietic progenitor cells (A) and erythroid cells (B) determined by RQ-PCR. Data were presented as mean ± SD of three independent experiments. The Pair T-test was used to assess the significant differences between conditions with *vs.* without doxycycline (DOX). The asterisks (*) and (**) denoted $p<0.05$ and $p<0.01$.

Characteristics of erythroid cells derived from the modified iPSCs

During erythroid maturation, the expression of the transferrin receptor (CD71) increases first. Subsequently, the glycophorin A (GPA) is being expressed.

Erythroid cells which were harvested from JAK2V617F-iPSCs and JAK2exon12-iPSCs in the condition without doxycycline on day 29 of differentiation yielded CD71/Glycophorin A (GPA) positivity at approximately 90.1% and 94.1% by flow cytometry, respectively. Cells after doxycycline incubation dually expressed CD71/GPA at 95.4% and 96.2% in JAK2V617F-iPSCs and JAK2exon12-iPSCs, respectively **(Figure 21)**. The percentages appeared to be increased, but there was no statistical significance.

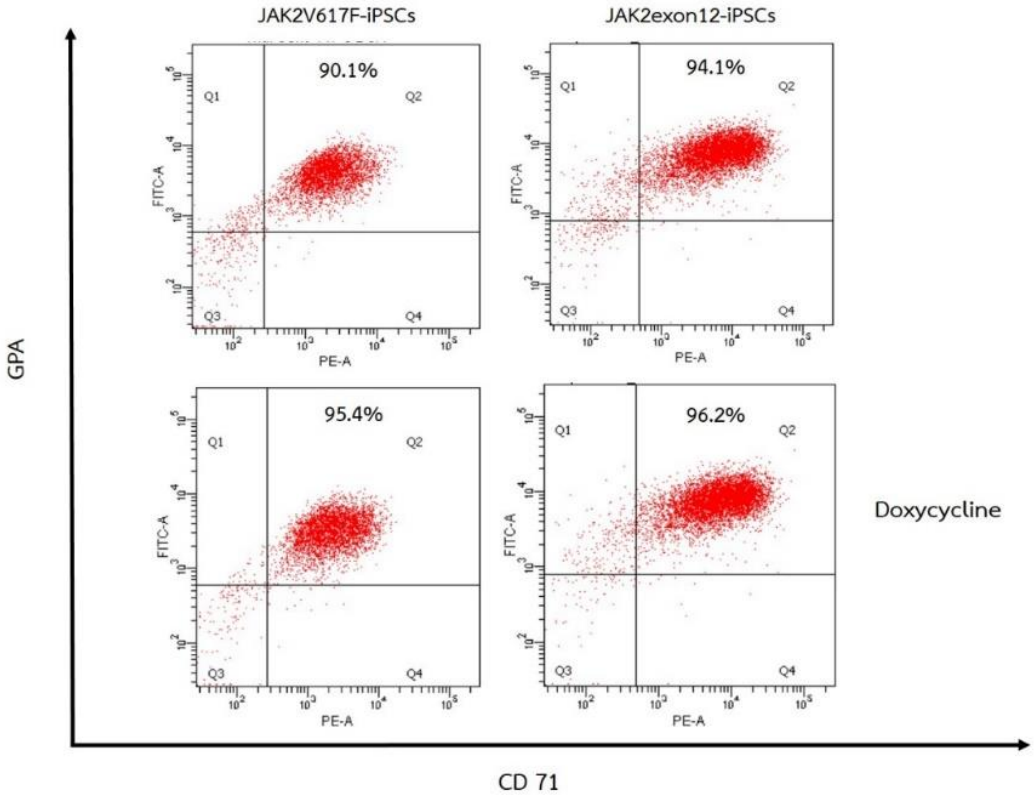


Figure 21 Flow cytometry of erythroid-specific surface molecules on iPSC-derived erythroid cells without *vs*. with doxycycline.

Cells were stained with PE-conjugated anti-human CD71 and FITC-conjugated anti-human Glycophorin A (GPA) antibodies.

Regarding the total numbers of erythroid cells, JAK2V617F-iPSCs without *vs*. with doxycycline yielded approximately 275 ± 19.0 x10^5 cells/ml *vs*. 279 ± 1.4 x10^5 cells/ml, respectively, while JAK2exon12-iPSCs showed a significant increase in the number of erythroid cells upon stimulation with doxycycline from 362 ± 18.3 x10^5 cells/ml (without doxycycline) to 822 ± 17.2 x10^5 cells/ml (*p* value = 0.007). Interestingly, the high potential of erythroid production from JAK2exon12-iPSCs was similar to the pathophysiology of MPN patients with JAK2 exon 12 mutations **(Figure 22)**.

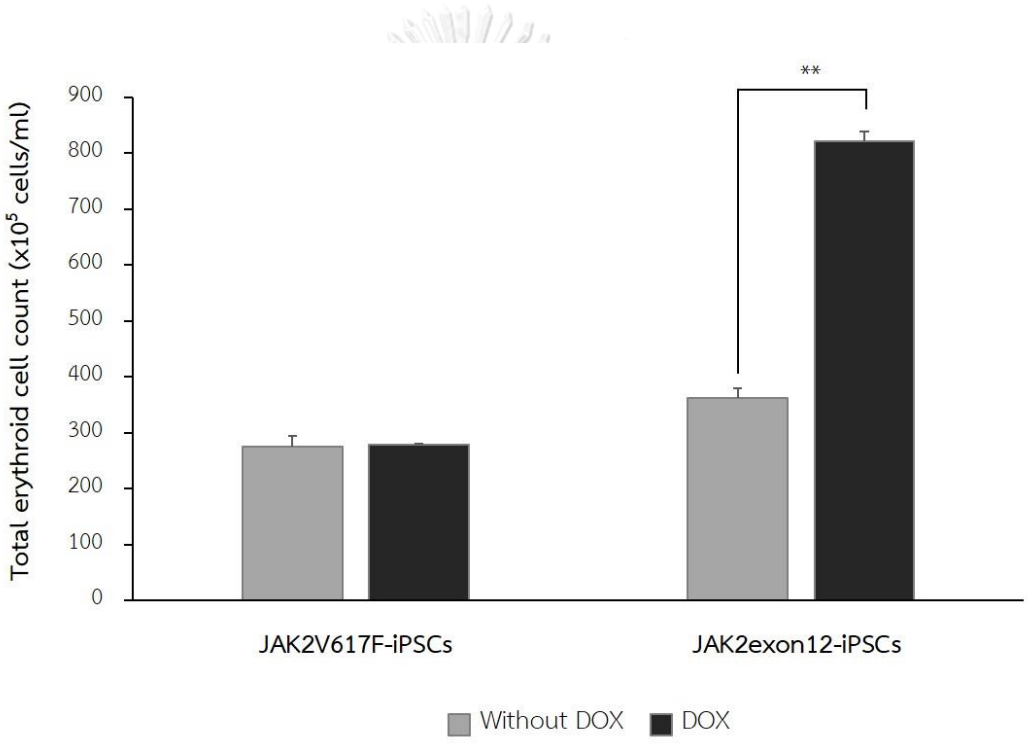


Figure 22 Total $CD71^+GPA^+$ erythoid cell numbers from erythroid differentiation via ES-Sacs with *vs.* without doxycycline.

Data were presented as mean ± SD from three independent experiments. The Pair T-test was used to assess the significance of differences between conditions with *vs.* without doxycycline (DOX). The asterisks (**) denoted $p<0.01$.

Morphology of iPSC-derived erythroid cells

During 29 days of culture, cells from modified iPSCs demonstrated erythroid morphology by Wright-Giemsa staining. Proerythroblasts, basophilic erythroblasts, polychromatic erythroblasts, orthochromatic erythroblasts and erythrocytes were found under light microscopy. The representative series of erythroid cells are shown in **Figure 23**. Proerythroblasts showed the characteristic cell sizes of approximately 15 μm in diameters, round shapes with pseudopodia, basophilic cytoplasm, large nuclei, fine chromatin, visible nucleoli, and high nuclear/cytoplasmic (N/C) ratios. Basophilic erythroblasts exhibited intensely heterochromatic nuclei, cell sizes of approximately 13-15 μm in diameters, deep blue cytoplasm and high N/C ratios. Polychromatic erythroblasts had diameters of approximately 12 μm, round shapes, mixed colors of cytoplasm due to polyribosomes (blue) and hemoglobin (red), condensed nuclei, no nucleolus and low N/C ratios. Orthochromatic erythroblasts displayed the characteristic cell sizes of approximately 8-10 μm in diameters, round cells, pink-orange cytoplasm and pyknotic nuclei. Erythrocytes are anucleate erythroid cells which were 7-8 microns in diameter. They were round in shapes with orange-red cytoplasm and no nucleus **(Figure 23)**.

The percentage of cell in erythrocytic series from *in vitro* erythroid differentiation were demonstrated on **Table 5** and the most prevalent cells in cultures were orthochromatic erythroblasts. JAK2exon12-iPSCs after doxycycline induction showed a significant increase in the subpopulation of more mature orthochromatic erythroblasts when compared with JAK2V617F-iPSCs (p = 0.007) **(Figure 24)**.

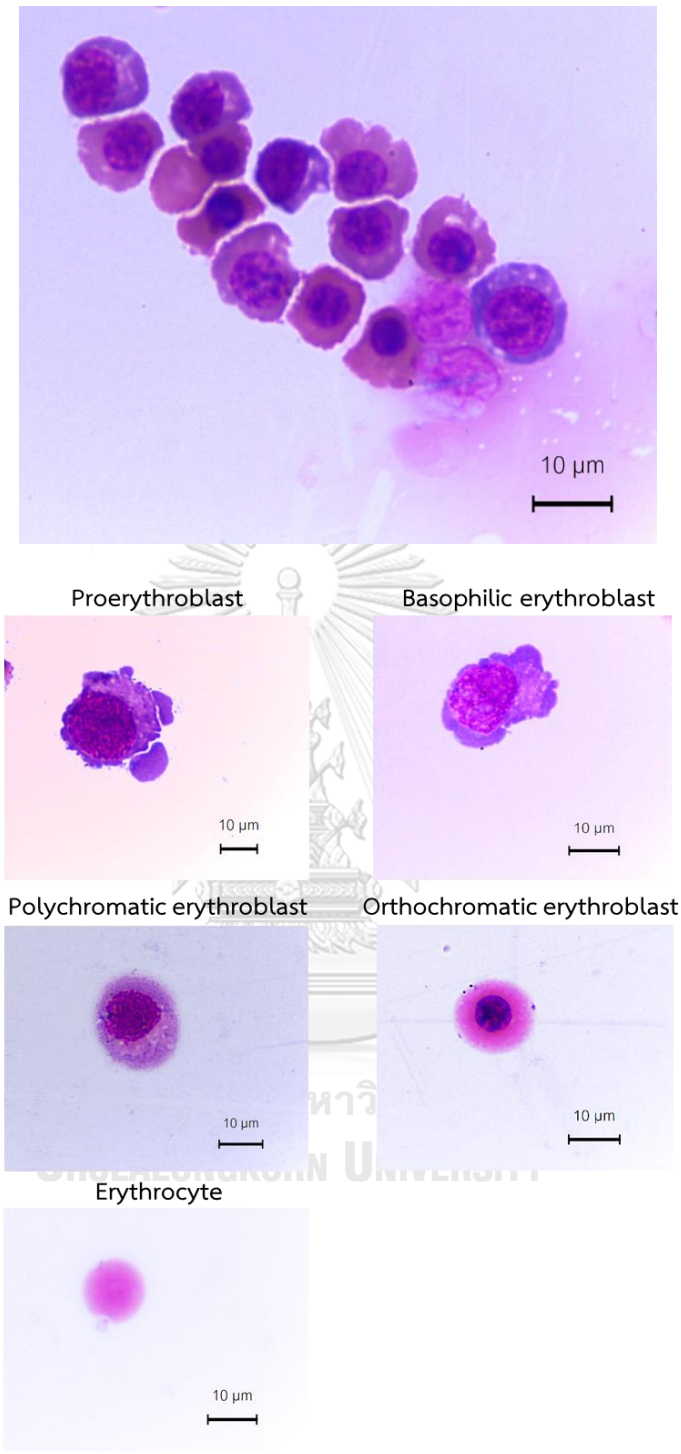


Figure 23 Five stages of erythroid series were classified by Wright-Giemsa stain consisting of proerythroblasts, basophilic erythroblasts, polychromatic erythroblasts, orthochromatic erythroblasts and erythrocytes derived from iPSCs.
The images were captured by Leica DM 1000 microscopy using LAS49 software and the scale bars represented 10 µm for all panels.

Table 5 Erythroid cell differentiation from JAK2V617F-iPSCs and JAK2exon12-iPSCs

Stage	JAK2V617F-iPSCs	JAK2Exon 12 mutation-iPSCs
Proerythroblasts	4.0 ± 1.6	2.0 ± 0.5
Basophilic erythroblasts	6.0 ± 0.5	3.0 ± 1.9
Polychromatic erythroblasts	25.0 ± 6.3	6.0 ± 1.8
Orthochromatic erythroblasts	63.0 ± 5.8	87.0 ± 1.0
Erythrocytes	2.0 ± 0.5	2.0 ± 0.9

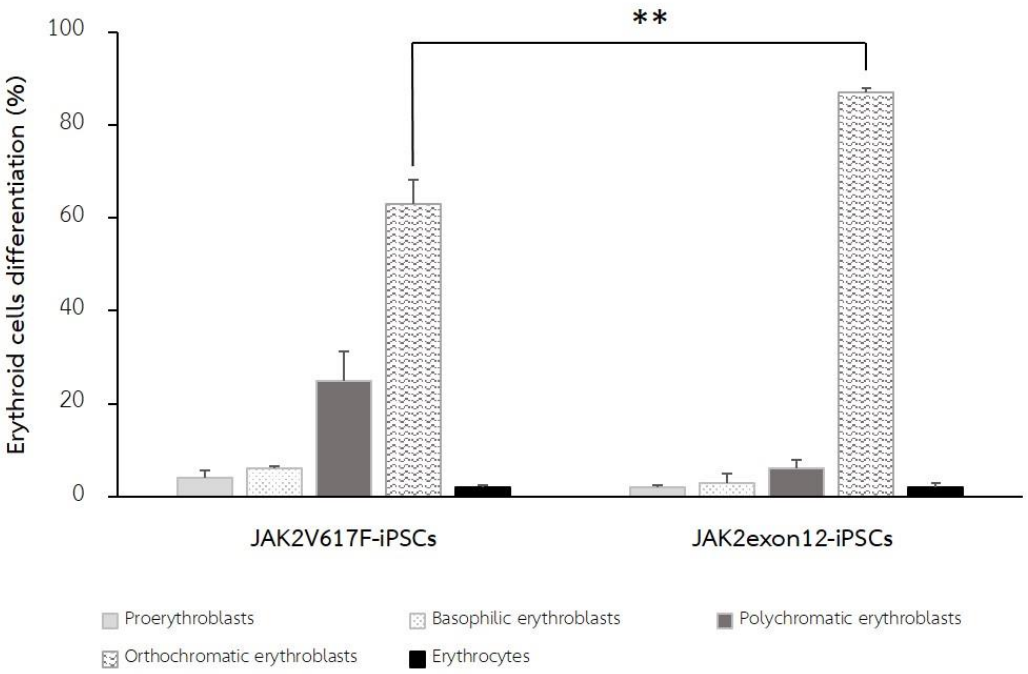


Figure 24 The percentage of erythroid cell differentiation stages.

Data were presented as mean ± SD from three independent experiments. The one way ANOVA was used to assess the significance of differences between JAK2V617F-iPSCs and JAK2exon12-iPSCs. The asterisks (**) denoted $p<0.01$.

Hemoglobin analysis

Hemoglobin types of iPSC-derived red blood cells were detected by using Beta Bio-Rad Variant. In addition, RT-PCR and RQ-PCR were also used to determine the beta-similar globin chains which were embryonic hemoglobin (epsilon; ε), fetal hemoglobin (gamma; γ) and adult hemoglobin (beta; β).

Chromatogram from the Bio-Rad Variant demonstrated the peaks of embryonic hemoglobin at the retention time approximately 0.15 minutes from iPSC-derived red blood cells **(Figure 25)**. The mRNA expression of beta similar globin chains displayed apparent bands of epsilon globin (214 bp), gamma globin (190 bp) and beta globin (199 bp) **(Figure 26)**. This indicates that iPSC-derived erythroids co-expressed embryonic, fetal and adult hemoglobin.

Globin gene expression was also evaluated by real-time PCR (RQ-PCR). After stimulation with doxycycline, JAK2exon12-iPSCs derived erythroid cells showed a significant increase in the beta globin mRNA expression when compared with JAK2V617F-iPSCs (13.35 $\pm$ 0.75 folds *vs.* 7.16 $\pm$ 0.62 folds, respectively, p =0.018) **(Figure 27)**.

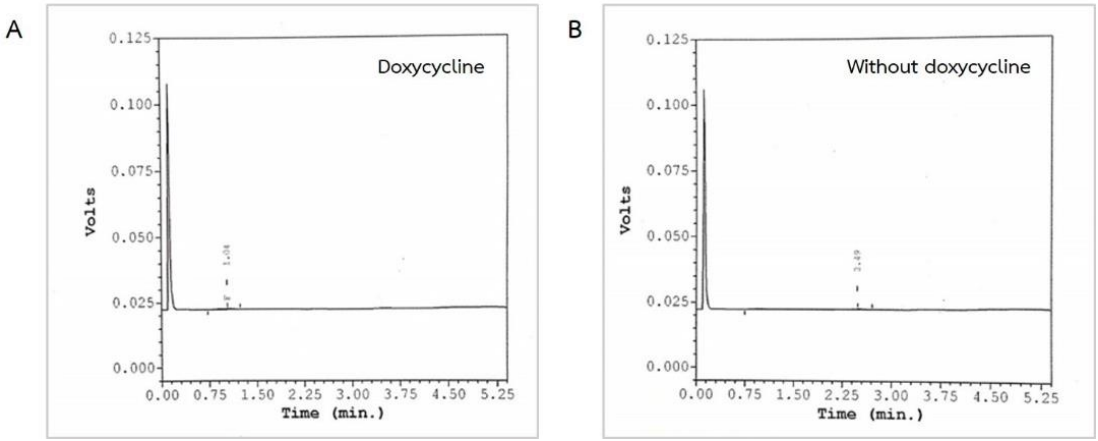


Figure 25 Chromatograms showed embryonic hemoglobin from modified iPSCs in the conditions with (A) *vs*. without doxycycline (B).

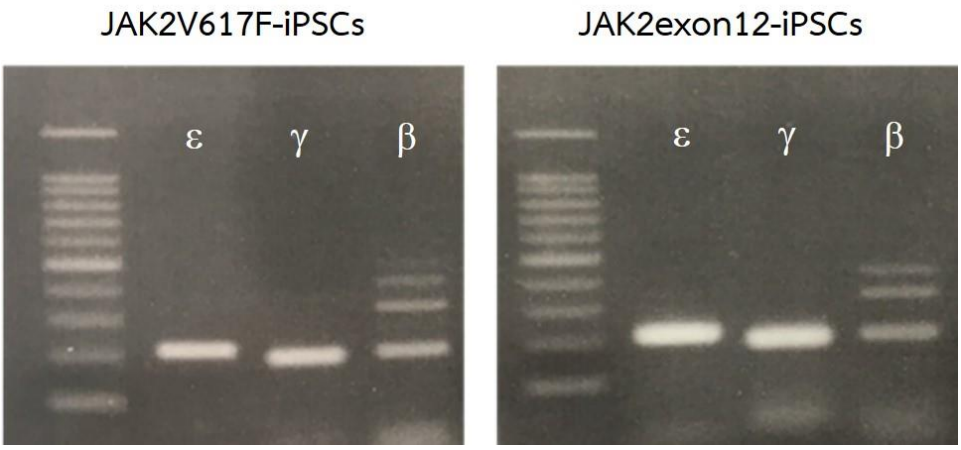


Figure 26 The expression of beta-similar globin chains of iPSC-derived erythroid cells: JAK2V617F-iPSCs and JAK2exon12-iPSCs.

Lane 1-3 are transcripts of epsilon, gamma and beta globin genes, respectively.

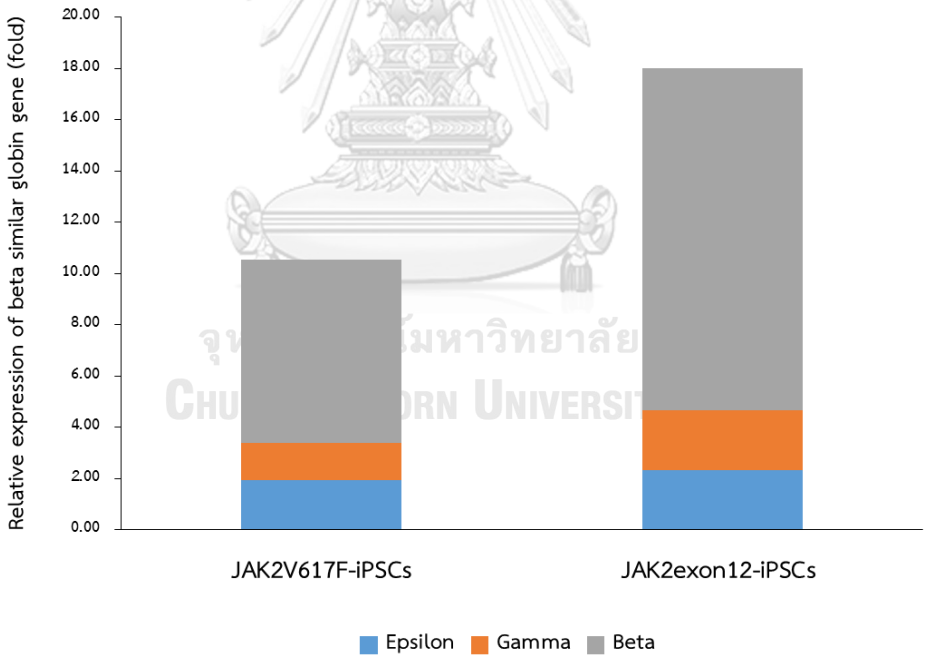


Figure 27 The relative expression of beta-similar globin genes which were epsilon, gamma and beta in iPSC-derived erythroid cells comparing JAK2V617F and JAK2 exon 12 mutation.

Genes were analyzed by RQ-PCR. Data were presented as means of three independent experiments.

JAK/STAT signal transduction

Hematopoietic progenitor cells from ES-Sacs were harvested and cultured with a hematopoietic cell differentiation medium supplemented with 5 IU/ml EPO, 50 ng/ml TPO and 50 ng/ml SCF for 24 hours. Stimulated cells were collected and investigated for the cell signaling composed of JAK2, STAT1, STAT3, STAT5, ERK1/2 and AKT in both native and phosphorylated forms using Capillary Western immunoassay.

The relative changes in phosphorylated JAK2 proteins in the presence of doxycycline in JAK2V617F-iPSCs and JAK2exon12-iPSCs were 2.50 $\pm$ 0.87 and 1.25 $\pm$ 0.29 folds, respectively. Additionally, JAK2V617F-iPSCs and JAK2exon12-iPSCs showed the increases in total JAK2 protein levels of approximately 3 and 1.5 folds, respectively, when compared with cells in the absence of doxycycline.

JAK2V617F-iPSCs showed significant increases in the relative changes of phospho-STAT3 and phospho-AKT proteins when compared with JAK2exon12-iPSCs at 5.60 $\pm$ 0.69 *vs.* 0.20 $\pm$ 0.35 (p value < 0.001) and 1.20 $\pm$ 0.23 *vs.* 0.40 $\pm$ 0.23 (p value = 0.008), respectively. Moreover, the phospho-STAT5 signaling protein level in JAK2V617F-iPSCs was higher than that of JAK2exon12-iPSCs at approximately 0.63 $\pm$ 0.03 *vs.* 0.41 $\pm$ 0.28, but there was no statistical significance.

On the other hand, JAK2exon12-iPSCs expressed a higher level of phospho-STAT1 and phospho-ERK1/2 (1.00 $\pm$ 0.69 and 0.65 $\pm$ 0.00) when compared with JAK2V617F-iPSCs (0.02 $\pm$ 0.14 and 0.51 $\pm$ 0.20). However, only phospho-STAT1 reached a statistical significance (p value = 0.022) **(Figure 28)**.

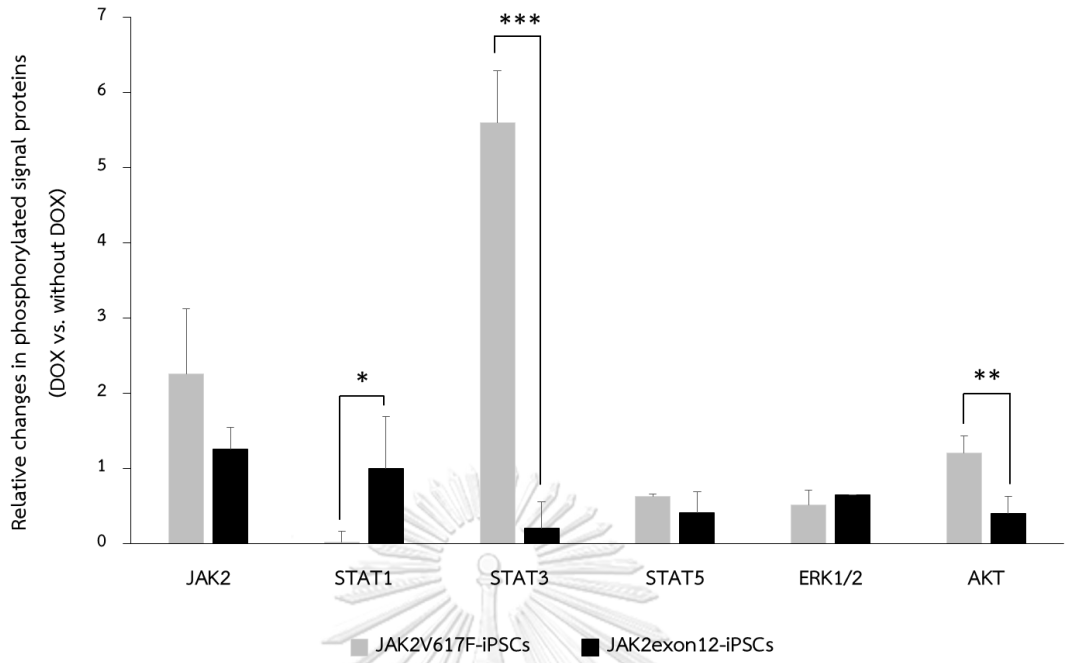


Figure 28 The relative changes of phosphorylated signaling proteins that were JAK2, STAT1, STAT3, STAT5, ERK1/2 and AKT after doxycycline induction in JAK2V617F-iPSCs and JAK2exon12-iPSCs compared with those without doxycycline.

The levels of phosphoproteins were corrected by the amounts of respective total proteins. Data were presented as means ± SD from three independent experiments. The Independent-sample T-test was used to assess the significance of differences between JAK2V617F-iPSCs *vs.* JAK2exon12-iPSCs. The asterisks (*), (**) and (***) denoted $p<0.05$, $p<0.01$ and $p<0.001$, respectively.

Interferon alpha and arsenic trioxide treatments

Interferon alpha and arsenic trioxide were tested on modified iPSCs with *vs.* without doxycycline induction to examine the relative sensitivity of cells expressing mutant *JAK2* compared with wild-type cells. The hematopoietic progenitor cells generated from JAK2V617F-iPSCs and JAK2exon12-iPSCs were cultured in the absence or presence of 0.5 μg/ml interferon alpha and/or 250 nM arsenic trioxide for 15 days. The total erythroid cells and percentage of cell deaths were determined by cell counting and immunocytochemistry.

The JAK2V617F-iPSCs showed a significant decrease in the number of erythroid cells after treatment with arsenic trioxide, interferon alpha and the combination of both drugs from 412 ± 6.35 x10^5 cells/ml (untreated) to 250 ± 6.35, 261 ± 6.35 and 172 ± 57.4 x10^5 cells/ml, respectively. Statistical analyses revealed the significant *p*-values of 0.008, 0.010 and 0.002, respectively.

The JAK2exon12-iPSCs displayed a significant decrease in cell numbers from 686 ± 32.33 x10^5 cells/ml (untreated) to 305 ± 12.7 x10^5 cells/ml with arsenic trioxide (*p* value = 0.003), 300 ± 38.68 x10^5 cells/ml with interferon alpha (*p* value = 0.003) and 189 ± 19.05 x10^5 cells/ml with interferon alpha plus arsenic trioxide (*p* value = 0.002). Notably, these agents did not affect the cell numbers of modified iPSCs without doxycycline induction **(Figure 29)**.

Concerning the relative changes of apoptotic cells when compared with the untreated control, JAK2V617F-iPSCs after treatments with arsenic trioxide, interferon alpha and the combination of both drugs showed the increases of 3.50 ± 2.10, 2.19 ± 0.42 and 4.00 ± 0.98 folds, respectively, while JAK2exon12-iPSCs displayed the relative changes of approximately 2.40 ± 0.57, 2.67 ± 1.04 and 2.50 ± 0.51 folds, respectively. There was no apoptotic rate difference in modified iPSCs after incubation with either one or both drugs without doxycycline stimulation **(Figure 30)**.

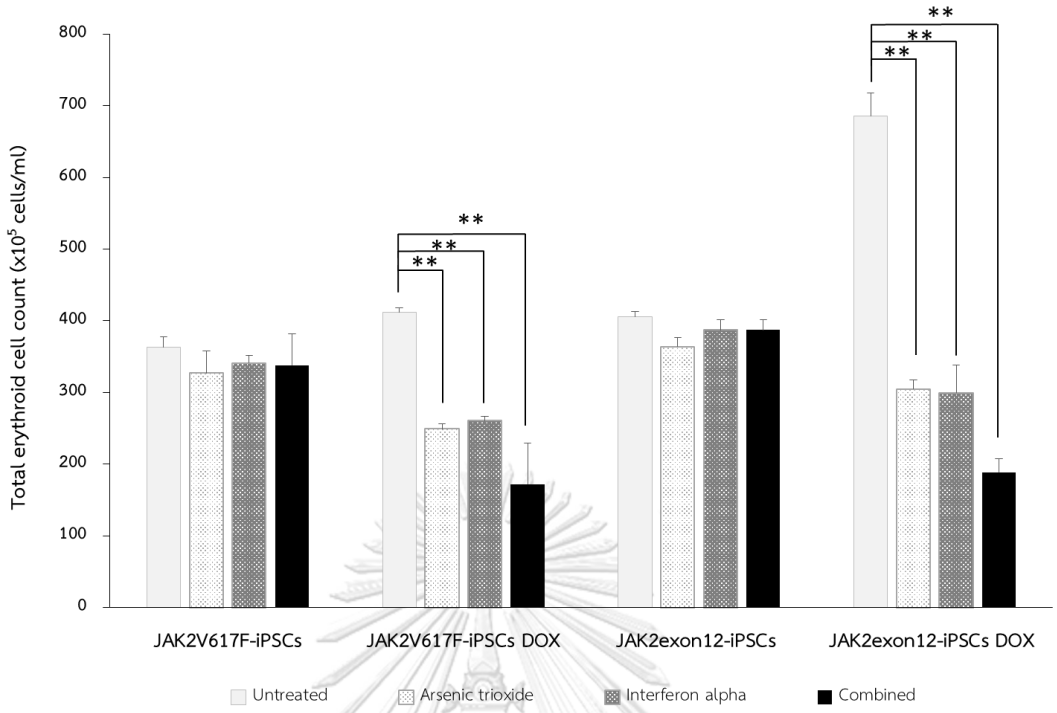


Figure 29 Erythroid cell numbers after incubations without (untreated) *vs*. with arsenic trioxide, interferon alpha and the combination of both drugs.

Data were presented as means ± SD from three independent experiments. The one way ANOVA was used to assess the significance of different groups of drugs. The asterisks (**) denoted $p<0.01$.

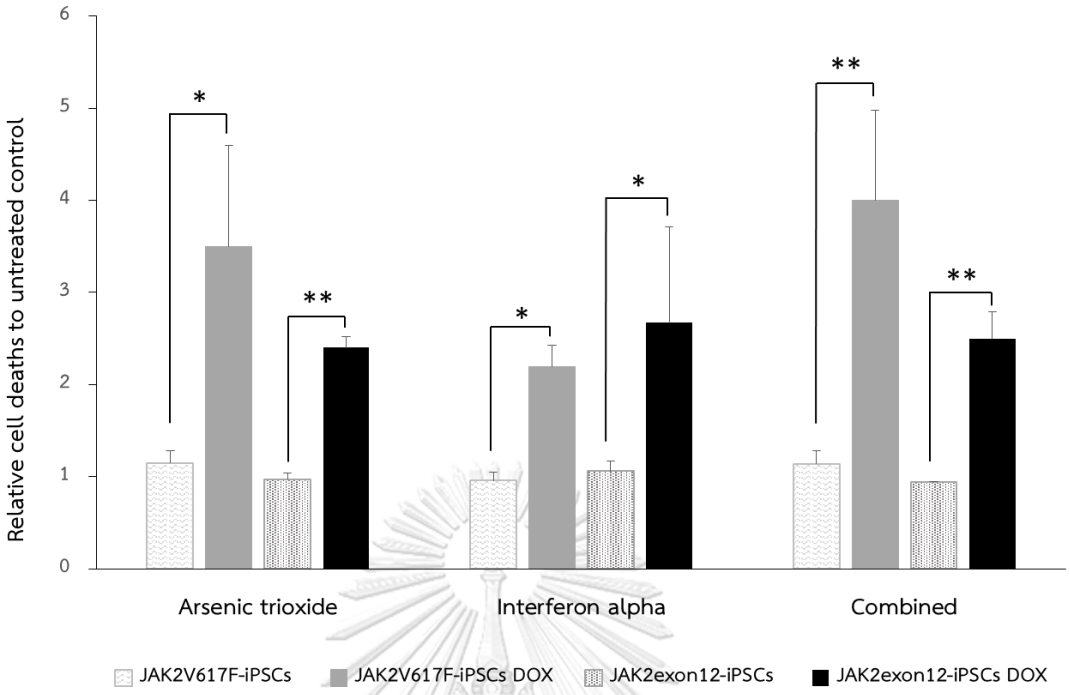


Figure 30 The relative increases in numbers of apoptotic cells in modified iPSCs in the presence of arsenic trioxide, interferon alpha and the combination of both drugs. Data were presented as means ± SD from three independent experiments. The Pair T-test was used to assess the significant difference between JAK2V617F-iPSCs and JAK2exon12-iPSCs. The asterisks (*) and (**) denoted $p<0.05$ and $p<0.01$, respectively.

CHAPTER V

DISCUSSION

The most common mechanisms of myeloproliferation are *JAK2* gene mutations. From clinical studies, patients with *JAK2* gene mutations present with various phenotypes, *i.e.* erythrocytosis, thrombocytosis and/or granulocytosis. The molecular mechanism of this disparity is still unclear and may be from differences in signal transduction mediated by mutated *JAK2*. Mouse models have been widely used to investigate the pathophysiology underlying human genetic diseases because mice have almost similar set of genes which can be modified for studies. However, there is a limitation as mice do not always demonstrate similar pathological changes as humans (55). In order to study hematological diseases, human hematopoietic stem cells must be expanded and maintained *ex vivo* which is a complicated process. Peripheral blood mononuclear cells are an easier alternative source, but they have a limited lifespan in culture (56, 57).

Human induced pluripotent stem cells (iPSCs) are generated from mature cells *via* induction by four factors, *OCT3/4*, *SOX2*, *c-MYC* and *KLF4*. These stem cells can be derived from either normal subjects or patients to be differentiated into various cell types *in vitro*. Furthermore, the cells can be genetically modified and greatly expanded to overcome the ethical restriction from using embryonic stem cells (55, 58). Therefore, iPSCs have potentials to be blood disease models which are probably closer to human physiology than cancer cell lines or animals. Moreover, they may become cell sources for transfusion or immunotherapy in the future.

In 2012, iPSCs containing heterozygous and homozygous JAK2V617F were generated from MPN patients and studied for molecular mechanisms. However, MPN patient samples usually co-carried other genetic defects including 20q deletion, *ASXL1, FBXO15* and *MATN2* (59) mutations that can affect iPSC phenotypes. Clonal subpopulation may vary among samples depending on disease progression and treatment processes. Furthermore, different clones of iPSCs may display distinct intracellular signaling and growth potentials. In this study, the iPSC lines with

doxycycline-inducible *JAK2* mutations were constructed from normal iPSCs. The *in vitro* erythrocyte generation was observed comparing between cultures with *vs.* without doxycycline. Therefore, the phenotype differences were attributed solely to the overexpressed mutated *JAK2* genes without interferences by other genetic and/or epigenetic background.

From our study, the lentivirally-modified iPSCs retained normal karyotypes, stemness properties and multi-lineage potentials. The modified iPSC-derived red blood cells displayed erythroid morphology by Wright-Giemsa stain, erythroid surface CD markers by flow cytometry and embryonic hemoglobin expression similar to normal iPSC-derived cells. Interestingly, JAK2exon12-iPSCs significantly enhanced erythroid cell proliferation mimicking the pathophysiology of JAK2 exon 12 mutations in patients with PV. In addition, *JAK2* with an exon 12 mutation resulted in enhanced erythroid differentiation as shown by more mature morphology and higher expression of adult globin as determined by RQ-PCR. In contrast, JAK2V617F-iPSCs did not showed a significant increase in erythroid cell proliferation as enumerated by cell counting and differentiation as evaluated by morphology and hemoglobin analysis despite the overexpression of *JAK2* transgene on RQ-PCR assay and JAK2 protein by Western immunoassay. This disparity may be explained by that JAK2V617F expression in our study was more consistent with heterozygous JAK2V617F mutation in essential thrombocythemia (ET) patients because endogenous *JAK2* gene was still present. The polycythemia vera (PV) patients usually carry homozygous JAK2V617F mutation and, less frequently, heterozygous JAK2 exon 12 mutation as modeled in our study (60). Therefore, modified iPSCs from this study can be used as an experimental model to investigate the molecular pathogenesis of MPN patients and possibly answer the questions why the JAK2V617F and JAK2 exon 12 mutations attributed to different erythroid phenotypes. Moreover, JAK2V617F-iPSCs carrying doxycycline-inducible gene expression which reversibly turned on in the presence of doxycycline mimicking the heterozygous JAK2V617F mutation in essential thrombocythemia is a better tool for differentiation into megakaryocytes and study in disease mechanism.

To our knowledge, this is the first report comparing JAK2V617F and JAK2 exon 12 mutation (*JAK2*p.N542_E543del) effects on erythroid development from modified

iPSCs and exploring their signaling pathways. Notably, JAK2V617F-iPSCs showed significantly higher relative changes of phospho-STAT3 and phospho-AKT signaling proteins, whereas JAK2exon12-iPSCs exhibited higher amounts of phospho-STAT1.

This result did not according with the studied from cells line in 2007, Scott *et al* established the mutant JAK2 exon 12 and JAK2V617F in BaF3 cells line using retrovirus transduction the result found K539L substitution which is exon 12 variant generated the higher levels of JAK2 than JAK2V617F mutation but showed the equivalent levels of STAT5 (36). In the other hand, the study from human showed the conflicting result, for example, Teofili *et al* (2007) studied the phosphorylation of STAT-3 and STAT-5 in patients with Ph-negative myeloproliferative diseases. Bone marrow biopsies of ET patients who had JAK2V617F mutation showed an increase in phospho-STAT3 by immunohistochemical analysis and immunoblotting (61). Additionally, bone marrow trephine biopsy sections of MPN patients, JAK2V617F mutation was associated with significantly increased levels of phospho-STAT5 and phospho-AKT in hematopoietic cells, which were most prominent in megakaryocytes (62). Furthermore, cells reprogrammed from heterozygous JAK2V617F patients showed a high level of phospho-STAT5 and displayed TPO-independent formation of megakaryocytic colonies but not EPO-independent erythroid colony (63). Supporting these data, the study in BaF3/EPOR cells transduced with various types of *JAK2* gene mutations including JAK2V617F, N542_E543del, H538QK539L, K539L and F537_K539delinsL showed that these exon 12 mutations activated the RAS-ERK signaling pathway. The levels of phospho-ERK1 and ERK2 were markedly higher than JAK2V617F mutation and there were variable levels of phospho-ERK in different types of exon 12 mutations (36). Moreover, iPSCs from homozygous JAK2V617F patients which usually display the polycythemia vera phenotype showed a higher level of phospho-ERK1/2 when compared with heterozygous JAK2V617F-iPSCs (63).

In addition, the STAT1 knockout mice showed reduced bone marrow-derived erythroid colony forming units and less differentiated phenotypes associated with increased apoptosis of early erythroblasts. These data demonstrated that STAT1 played a critical role in the regulation of erythropoiesis (64). On the other hand, STAT3 is probably a minor signaling molecule for EPO-independent growth but may

play an important role in megakaryopoiesis. Therefore, dissimilar signals may explain the different phenotypes of patients with heterozygous JAK2V617F and those with *JAK2* exon 12 mutations.

In the past, potentially new drugs were screened in immortalized cancer cell lines and animal models which cannot always predict efficacy and safety in humans (65). The iPSCs can be differentiated into disease specific cell types and demonstrate the phenotypes similar to primary cells. Drug screening on these iPSC-derived cells may be helpful for discoveries of novel treatments. Interferon alpha that signals through the JAK/STAT pathway has been used for the treatments of ET or PV. The mechanisms of action of interferon-alpha2 have been ascribed to its anti-proliferative, pro-apoptotic, anti-angiogenic, and immunomodulatory effects (66). Interestingly, interferon can decrease the mutated *JAK2* allele burdens in MPN patients. In addition, the combination with the other drugs may be more efficacious for advanced and transformed diseases (67). Arsenic trioxide is the standard treatment for relapsed acute promyelocytic leukemia (APL) through promoting apoptosis which is involved intracellular glutathione and hydrogen peroxide (15). Recent researches showed that hematological malignancies other than APL also responded to combination therapy containing arsenic trioxide. JAK2V617F-UT7 cell lines were generated and revealed the synergistic effects of interferon alpha and arsenic trioxide combination (68). The mechanism of IFN alpha may affect JAK2V617F-iPSCs and JAK2exon12-iPSCs through p38 MAP kinase pathway. In 2010, Lu *et al* demonstrated that low doses of interferon alpha induced apoptosis of JAK2V617F positive hematopoietic progenitor cells from PV patients through p38 MAP kinase pathway (69). Whereas, arsenic trioxide may act via p73 supporting by data from Lunghi *et al* (2004) reported the combined treatment with ATO and MEK1 inhibitors enhances the affinity of phosphoacetylated p73 for the p53 AIP1 promoter leading to p53AIP1 up-regulation and increased apoptosis in NB4 and K562 cell lines (54).

According to our experiments, JAK2V617F-iPSCs and JAK2exon12-iPSCs showed a significant decrease in the number of erythroid cells and an increase in apoptotic cells after treatment with arsenic trioxide, interferon alpha and the

combined regimen. The additive effect of these two agents was observed in our model. Interestingly, arsenic trioxide and interferon alpha treatments showed the specific effects on mutated iPSCs but did not in the condition without doxycycline induction. This disease model of JAK2V617F and JAK2 exon 12 mutation suggests the potential roles of arsenic trioxide and interferon alpha in therapy of MPN patients as they can preferentially suppress malignant clones. In the future, this modified iPSCs can be used to test for other new therapeutic agents.

Derivation of red blood cells from iPSCs may become blood products for transfusion. Genetic engineering can be applied to generate erythrocytes with very rare blood groups without requirement for exceptional donors. The proteome analysis of erythroid cells differentiated from iPSC lines revealed a similar pattern to that of normal adult erythroid cells (70). However, the challenges of erythroid production are inefficient enucleation, low expression of the adult β hemoglobin and scalable production (71). From our result, JAK2exon12-iPSCs enhanced red blood cell production with a greater number of the late-stage erythrocytes and produced more adult hemoglobin ($\alpha_2\beta_2$, HbA) at the mRNA level. Overexpression of *JAK2* with exon 12 mutations may be one of the factors to improve the blood cell production for transfusion.

In conclusion, our study used the iPSC technology to obtain deeper understanding of the *JAK2* mutation effects on erythropoiesis. The *JAK2* exon 12 mutation strongly promoted erythroid cell proliferation and differentiation correlating with STAT1 and ERK activation. Modified iPSCs provided a model to study the mechanisms of mutated *JAK2*, screen for novel therapeutic agents and possibly offer a potential source for red blood cell transfusion in the future.

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