การสร้างเซลล์หัวใจจากเซลล์ต้นกำเนิดตัวอ่อนและเซลล์ชนิดอินดิวซ์พลูริโพเทนท์สเต็มเซลล์ในกระต่าย

นางสาวเพราพิลาส ภักดีดินแดน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Establishment of Cardiac Lineage from Rabbit Embryonic and Induced Pluripotent Stem Cells

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กระต่ายเป็นแบบอย่างที่สำคัญต่อการศึกษาภาวะหัวใจขาดเลือดเพราะมีสรีรวิทยาและพยาธิวิทยาที่ใกล้เคียงกับมนุษย์ อย่างไรก็ตามข้อมูลการสร้างเซลล์ชนิดพลูริโพเทนซ์สเต็มเซลล์และการเปลี่ยนแปลงเป็นเซลล์หัวใจในกระต่ายยังไม่เพียงพอ งานวิจัยนี้ได้ศึกษาการสร้างและทดสอบกระบวนการเกิดเซลล์หัวใจทั้งจากเซลล์ต้นกำเนิดตัวอ่อนและอินดิวซ์พลูริโพเทนซ์สเต็มเซลล์ในห้องปฏิบัติการ

ในการทดลองที่ 1 ทำการเพาะเลี้ยงเซลล์ต้นกำเนิดตัวอ่อนจำนวน 4 เซลล์ไลน์ที่ผลิตจากตัวอ่อนระยะ บลาสโตซิสท์และศึกษาคุณสมบัติพลูริโพเทนซ์ในแง่ของยีนและอิมมูโนพยาธิวิทยา เซลล์เหล่านี้แสดงจำนวนโครโมโซมปกติ (n=44) ทำการชักนำการเกิดเอ็มบริออยด์บอดี้เพื่อศึกษาคุณสมบัติการเปลี่ยนแปลงเป็นเซลล์ชนิดอื่นภายในห้องปฏิบัติการ โดยเซลล์จะเปลี่ยนแปลงเป็นเนื้อเยื่อสามชั้นร่วมทั้งกลุ่มเซลล์เนื้อเยื่อชั้นกลาง (*Brachyury* (*BT*)) และเซลล์ต้นกำเนิดเซลล์หัวใจ (*NKX2.5* และ *GATA4*) การศึกษาความคงตัวของเฮ้าส์คีปปิ้งยีนสองชนิด (*RPL13a* และ *GAPDH*) ในช่วง 7 วันของการเพาะเลี้ยง พบว่ายีนทั้งสองชนิดมีความคงตัวระหว่างการเพาะเลี้ยงไม่แตกต่างกัน ($P>0.05$) ยีน *BT* มีการเพิ่มขึ้นและเพิ่มสูงสุดในวันที่ 2 ของการเพาะเลี้ยงเมื่อเทียบกับเซลล์ต้นกำเนิดตัวอ่อนและเอ็มบริออยด์บอดี้ในวันที่ 3 ($P<0.05$) บีเอ็มพีโฟร์เหนี่ยวนำให้มีการเพิ่มขึ้นของยีน *BT* ในวันที่ 2 และยีนต้นกำเนิดเซลล์หัวใจ *NKX2.5* ในวันที่ 3 และ 5 เมื่อเทียบกับกลุ่มควบคุม (ไม่ใส่ BMP-4) ($P<0.05$) นอกจากนี้บีเอ็มพีโฟร์เหนี่ยวนำให้เซลล์มีการแสดงออกของโปรตีน NKX2.5, Flk1 และ cardiac troponin-T เพิ่มขึ้นในวันที่ 5 และ 7 ตามลำดับ ($P<0.05$) สรุปได้ว่าเซลล์ต้นกำเนิดตัวอ่อนมีศักยภาพในการเปลี่ยนแปลงเป็นเซลล์หัวใจ โดยบีเอ็มพีโฟร์เหนี่ยวนำการเปลี่ยนแปลงเป็นเซลล์หัวใจในแง่การแสดงออกยีนและโปรตีนอย่างมีนัยสำคัญ ($P<0.05$)

การทดลองที่ 2 ใช้ทรานสคริปชั่นแฟคเตอร์ 4 ชนิด (*OCT3/4, SOX2, KLF4, และ c-Myc*) ในการสร้างเซลล์อินดิวซ์พลูริโพเทนซ์สเต็มเซลล์จำนวน 3 เซลล์ไลน์จากเซลล์ผิวหนังของตัวอ่อนกระต่าย โดยทุกเซลล์ไลน์มีการแสดงออกของยีน (*OCT3/4, SOX2, KLF4 และ NANOG*) และโปรตีน (alkaline phosphatase, OCT3/4 และ SSEA-4) ที่บ่งชี้สภาวะพลูริโพเทนซ์ เซลล์ไลน์เหล่านี้แสดงคุณสมบัติการเปลี่ยนแปลงเป็นเซลล์ชนิดอื่นโดยวิธีการฟอร์มในลักษณะที่เรียกว่าเอ็มบริออยด์บอดี้และมีการแสดงออกของยีนและโปรตีนที่เกี่ยวข้องกับเนื้อเยื่อสามชั้น อย่างไรก็ตามความสามารถของแต่ละเซลล์ไลน์และจำนวนเซลล์มีผลต่อการเกิดเอ็มบริออยด์บอดี้ โดยเซลล์ที่มีความหนาแน่น 20,000 เซลล์ต่อหนึ่งเอ็มบริออยด์บอดี้ถูกเลือกมาใช้ในการเปลี่ยนแปลงเซลล์หัวใจ โดยเซลล์ที่เปลี่ยนแปลงจะแสดงเครื่องหมายเซลล์ต้นกำเนิดเซลล์หัวใจชนิด Flk1 (51±1.48%) ในวันที่ 5 แสดงเครื่องหมายเซลล์หัวใจ cardiac troponin-T protein (10.29±1.37%) และยีนเซลล์หัวใจชนิดอื่นๆ (*cardiac ryanodine receptors (RyR2),* α*-actinin* และ *PECAM1*) ในวันที่ 14 นอกจากนี้พบพื้นที่กลุ่มเซลล์หัวใจเริ่มเต้นตั้งแต่วันที่ 11 ของการเพาะเลี้ยง การศึกษานี้สรุปว่าเซลล์อินดิวซ์พลูริโพเทนซ์สเต็มเซลล์ของกระต่ายมีศักยภาพในการเปลี่ยนแปลงเป็นเซลล์หัวใจ อย่างไรก็ตามประสิทธิภาพของการเปลี่ยนแปลงเป็นเซลล์หัวใจมีจำกัด

การศึกษาสรุปได้ว่าเซลล์อินดิวซ์พลูริโพเทนซ์สเต็มเซลล์และเซลล์ต้นกำเนิดตัวอ่อนกระต่ายมีศักยภาพในการเปลี่ยนแปลงไปสู่เซลล์หัวใจโดยชักนำผ่านโครงสร้างสามมิติเอ็มบริออยด์บอดี้ บีเอ็มพีโฟร์เหนี่ยวนำการแสดงออกของยีนเนื้อเยื่อชั้นกลางหัวใจ (*BT*) และยีนของเซลล์ต้นกำเนิดเซลล์หัวใจ (*NKX2.5*) และเพิ่มกลุ่มเซลล์ที่แสดงโปรตีน Flk1 และ cardiac troponin-T เมื่อเทียบกับกลุ่มควบคุม ดังนั้นจึงมีความเป็นไปได้ที่จะสร้างเซลล์หัวใจจากเซลล์อินดิวซ์พลูริโพเทนซ์สเต็มเซลล์และเซลล์ต้นกำเนิดตัวอ่อน อย่างไรก็ตามควรมีการศึกษาปัจจัยที่เกี่ยวข้องเพื่อช่วยเพิ่มประสิทธิภาพในการเปลี่ยนแปลงเซลล์พลูริโพเทนซ์สเต็มเซลล์ของกระต่ายไปสู่เซลล์หัวใจ

บีเอ็มพีโฟร์, การเปลี่ยนแปลงเซลล์เป็นเซลล์หัวใจ, กระต่าย, เซลล์ต้นกำเนิดตัวอ่อน, อินดิวซ์พลูริโพเทนซ์สเต็มเซลล์

ภาควิชา กายวิภาคศาสตร์	ลายมือชื่อนิสิต .....
สาขาวิชา ชีวศาสตร์ทางสัตวแพทย์	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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PRAOPILAS PHAKDEEDINDAN: Establishment of Cardiac Lineage from Rabbit Embryonic and Induced Pluripotent Stem Cells. ADVISOR: ASST. PROF. THEERAWAT THARASANIT, Ph.D., CO-ADVISOR: PROF. MONGKOL TECHAKUMPHU, Ph.D., 89 pp.

Rabbit is a promising model for studying myocardial infarction as several physiological and pathological conditions are resemble to humans. However, the information on establishment of rabbit pluripotent stem cells (PSC) and differentiation potential especially cardiac differentiation in this species has been limited. This research aimed at establishing and examining *in vitro* cardiogenesis of rabbit embryonic stem cells (ESC) and rabbit induced pluripotent stem cells (iPSC).

In experiment 1, four cell lines of ESC derived from blastocyst stage embryos were cultured and characterized for their pluripotency in terms of gene and immunohistochemistry. They exhibited normal karyotypes (n=44). Embryoid body (EB) formation was conducted to study *in vitro* differentiation potential. The EB formation induced cell differentiation into three germ layers including mesodermal lineages (*Brachyury (BT)*) and cardiac progenitor (*NKX2.5* and *GATA4*). Two candidate housekeeping genes (*RPL13a* and *GAPDH*) were examined for its stability during 7 days of culture. The results indicated that these two genes were similar stability during EB culture ($P>0.05$). BT spontaneously upregulated to the highest level on day 2 of EB culture compared with undifferentiated ESC and EB on day 3 ($P<0.05$). BMP-4 significantly upregulated mesoderm marker BT on day 2 and cardiac progenitor marker NKX2.5 on day 3 and 5 of differentiation comparing with control group (without BMP-4) ($P<0.05$). It enhanced a number of NKX2.5, FLK1 and cardiac troponin-T positive cells on day 5 and 7, respectively ($P<0.05$). In conclusion, rabbit ESC retained capability of cardiac differentiation, while BMP-4 significantly promoted cardiac differentiation in terms of gene and protein expressions ($P<0.05$).

In experiment 2, three cell lines rabbit iPSC were generated by reprogramming rabbit embryonic fibroblasts using the 4 transcription factors (OCT3/4, SOX2, KLF4, and c-Myc). All iPSC cell lines expressed genes (*OCT3/4, SOX2, KLF4* and *NANOG*) and proteins (alkaline phosphatase, OCT3/4 and SSEA-4) essentially described for pluripotency. They demonstrated *in vitro* differentiation potential by forming embryoid body (EB) and gene and protein expression related three-germ layer. However, ability of individual cell lines and cell numbers at seeding markedly influenced on EB formation. The cell density at 20,000 cells per EB was selected for cardiac differentiation. The differentiated cells expressed cardiac progenitor marker Flk1 (51±1.48%) on day 5. Cardiac troponin-T protein (10.29±1.37%) and other cardiac marker genes (*cardiac ryanodine receptors* (*RyR2*), α*-actinin* and *PECAM1*) were expressed on day 14. Moreover, cardiac beating areas were observed on day 11 of culture. This study concluded that rabbit iPSC remained their *in vitro* pluripotency with capability of differentiation into mature-phenotype cardiomyocytes. However, the efficiency of cardiac differentiation is still restricted.

In conclusion, these studies demonstrated that rabbit iPSC and ESC have capacity to differentiate toward cardiac fate via 3D-structure embryoid body. BMP-4 enhanced gene expressions of cardiac mesoderm *BT* and cardiac progenitor *NKX2.5*. BMP-4 treated EB upregulated cardiac progenitor marker positive cells (Flk1) and cardiac marker troponin-T positive when compared with controls. These findings highlight the possibility to generate mature cardiomyocytes from rabbit iPSC and ESC for further use. However, other factors of cardiac differentiation should be further examined in order to improve its efficiency.

Department:	Veterinary Anatomy	Student's Signature	.....
Field of Study:	Veterinary Biosciences	Advisor's Signature	.....
Academic Year:	2017	Co-Advisor's Signature	.....

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Chapter I

Introduction

Importance and rationale

Pluripotent cells (embryonic stem cells and induced pluripotent stem cells) can differentiate *in vitro* into specific cells including cardiomyocytes (CMs) by appropriate culture condition (Heins et al., 2004; Kattman et al., 2011; Mummery et al., 2012; Lian et al., 2013). Although beating CMs derived from human embryonic stem cells (ESC) has been reported a decade ago (Kehat et al., 2001), the usage of these cells is limited due to the possibility of tissue rejection after transplantation. In human, CMs can be derived from induced pluripotent stem cells (iPSC) as a patient/disease-specific iPSC (Itzhaki et al., 2011; Yazawa et al., 2011). This technique uses autologous cells for reprogramming and is therefore minimal ethical concern and cell rejection (Takahashi et al., 2007). CMs derived from human iPSC are useful but transplantation study cannot be performed in human. Rabbit pluripotent cells are expected to be a cell source for cardiac regeneration *in vivo* since rabbit is a classical model animal for cardiac disease due to its resemblance to human cardiac physiology. Furthermore prolonged lifespan of rabbit, as compared with mouse, allows long-term follow-up of side effects after cell transplantation. There have been only few reports of rabbit iPSC generation (Lev et al., 2005; Honda et al., 2008; Honda et al., 2013; Osteil et al., 2013) since the first publication of rabbit ESC in 1993 (Graves and Moreadith). However, technique and signaling pathways of cardiac differentiation in rabbit ESC and iPSC are poorly understood. To date, there is no report concerning cardiac differentiation in this species. In human and mouse, small molecules such as Activin A and BMP-4 has been demonstrated to efficient induce cardiac differentiation (Heins et al., 2004; Takei et al., 2009; Kattman et al., 2011; Mummery et al., 2012; Lian et al., 2013; Ye et al., 2013). These shortcomings limit the application of CMs derived rabbit iPSC for transplantation experiment. This thesis was investigated the efficiency of reprograming using retroviral vectors in rabbit embryonic fibroblasts along with the establishment of embryonic stem cells. The ESC and iPSC lines will further be used for study the cardiac

differentiation using several techniques such as hanging drop supplemented with BMP-4. The investigation of fundamental knowledges such as stable housekeeping genes for normalization of rabbit pluripotent stem cells and derivative embryoid bodies were performed.

Objectives of study

1. To establish rabbit ESC and iPSC
2. To BMP-4 effects on cardiac differentiation in rabbit ESC
3. To study stable housekeeping genes in rabbit ESC and derivative embryoid bodies

Hypothesis

Rabbit ESC and iPSC retains differentiation potential towards cardiac lineage.

Literature review

Embryonic stem cells (ESC)

ESC are pluripotent cells generated from an inner cell mass of the blastocyst-stage embryos. They can proliferate unlimitedly and can differentiate into derivatives of all three germ layers including endoderm, mesoderm and ectoderm (Thomson et al., 1998; Odorico et al., 2001). ESC are firstly isolated in mouse in 1981 and the first batch of human ESC is report in 1998 (Evans and Kaufman, 1981; Thomson et al., 1998). The first publication of rabbit ESC was reported in 1993 (Graves and Moreadith). Morphology of ESC is typically compact colonies containing cells with increased nucleus to cytoplasm ratio with 1-2 prominent nuclei. These ESC must have diploid karyotype with high level of telomerase activity. These cells forms benign teratomas containing cells derived from three germ layers after injection into immunodeficient mice (Pera et al., 2000; Reubinoff et al., 2000; Heins et al., 2004). *In vitro* differentiation can also be used to test pluripotent capacity of the ESC. This is performed via either spontaneous or direct differentiation. It has been demonstrated that the ESC can differentiate into cardiomyocytes (CMs), neurons, glia, endothelial cells, hematopoietic progenitors and hepatocyte-like cells *in vitro* (Kaufman et al., 2001; Zhang et al., 2001; Rambhatla et al., 2003; Ko et al., 2007; Medine et al., 2011; Sher et al., 2008; Kattman

et al., 2011; Vanhee et al., 2014; Yao et al., 2014). Of the techniques used for differentiation, removal of feeder and growth factor such as leukemia inhibitory factor (LIF) or basic fibroblast growth factor 2 (bFGF) from culture system, followed by culturing the cell suspension in a small volume of culture medium. This leads to cell aggregation into three-dimension so called embryoid bodies (EB) formation. It is believed that the EB formation itself initiates the signal and spontaneous differentiation of pluripotent cells in three germ layers (Lev et al., 2005).

Table 1 The publications of rabbit ESC, the table shows cells, essential factors for maintain rabbit ESC, pluripotent markers and the level of pluripotency.

Reference	Cell souces	growth factors	Pluripotent markers	Level of Pluripotency
Graves and Moreadith, 1993	*in vivo* blastocyts (day 4 or 5 postcoitus)	LIF	-	Embryoid body formation
				no chimera
Schoonjans et al., 1996	Graves and Moreadith, 1993	LIF	-	alkaline phosphatase activity
				Chimera
Fang et al., 2006	parthenogenetically activated oocytes, fertilized embryos and somatic cell nuclear transfer embryos	bFGF	Expression : Oct3/4, EBAF2, FGF4 and TDGF1	normal karyotype
				alkaline phosphatase activity
			No expression : SSEA-1, SSEA-3, SSEA-4, TRA-1–60 and TRA-1–81.	Embryoid body formation
				Teratoma formation
				Chimera
Wang et al., 2007	Fertilized and parthenogenetic blastocysts	LIF	AP, SSEA-1, SSEA-3, SSEA-4, Oct3/4,Tra-1-60 and TRA-1–81.	normal karyotype
				alkaline phosphatase activity
				Embryoid body formation
				Teratoma formation
Chiang et al., 2008	*in vivo* blastocysts	-	AP and Oct3/4	alkaline phosphatase activity
Honda et al., 2008	*in vivo* blastocysts	bFGF and LIF	NANOG, SSEA-1, SSEA-4, and Oct3/4	normal karyotype
				alkaline phosphatase activity
				Embryoid body formation
				Teratoma formation
				no chimera
Intawicha et al., 2009	*in vivo* embryos	LIF	Expression: Oct-4, NANOG, SSEA-4, TRA-1–60 and TRA-1–81	normal karyotype
				alkaline phosphatase activity
				Embryoid body formation
			No expression: SSEA-1 and SSEA-3	Teratoma formation
				Chimera
Jiang et al., 2014	*in vivo* embryos	bFGF	Klf2, Kl4, Lin28, STAT3, Socs, Oct3/4 and NANOG	normal karyotype
				alkaline phosphatase activity
				active X chromosomes
				Embryoid body formation
				Teratoma formation
Du et al., 2015	vitrified–thawed embryos	bFGF and LIF	Oct3/4, c-Myc, NANOG, Klf-4, Rex1, SOX2 and SSEA-4	normal karyotype
				alkaline phosphatase activity
				Embryoid body formation
				Chimera
				no germline transmission
Honsho et al., 2015	Naïve conversion ESC of Honda et al., 2008	bFGF and LIF	NANOG, SSEA-1, SSEA-4, and Oct3/4	normal karyotype
				alkaline phosphatase activity
				Embryoid body formation
				Teratoma formation
				no chimera
Intawicha et al., 2016	SCNT embryos	LIF	Oct3/4, SSEA-4, TRA-1–60 and TRA-1–81	normal karyotype
				alkaline phosphatase activity
				Embryoid body formation
				Teratoma formation
				no chimera
Wei et al., 2016	*in vivo* blastocysts	bFGF and LIF	Oct3/4, SOX2, NANOG SSEA-1, SSEA-4, TRA-1–60 and TRA-1–81	normal karyotype
				alkaline phosphatase activity
				Embryoid body formation
				Teratoma formation
				no chimera

Induced pluripotent stem cells (iPSC)

The types of stem cells manifest pluripotency such as ESC, embryonic carcinoma cells, and iPSC. While ESC are isolated from the inner cell mass of the blastocyst-stage embryo, iPSC are the pluripotent cells that are generated from reprograming of somatic cells. iPSC was firstly derived from human fibroblasts using ectopic expression of the transcription factors OCT3/4, SOX2, KLF4, and C-MYC (OSKM) which was mediated by retroviral gene transfer. Around 3-4 weeks after reprograming, ESC like colonies were observed (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). The derived human iPSC were similar to human ESC in morphology, karyotype, telomerase activity and protein expression, such as alkaline phosphatase, SSEA-3, SSEA-4, TRA-1-60, TRA-1-80, OCT3/4, SOX2 and NANOG. To date iPSC can be achieved by over expression of 2-6 defined transcription factors, namely, OCT3/4, SOX2, KLF4, C-MYC, NANOG and LIN 28 (Okita et al., 2007; Yu et al., 2007; Lowry et al., 2008; Wang et al., 2010; Muchkaeva et al., 2014; Whitworth et al., 2014).

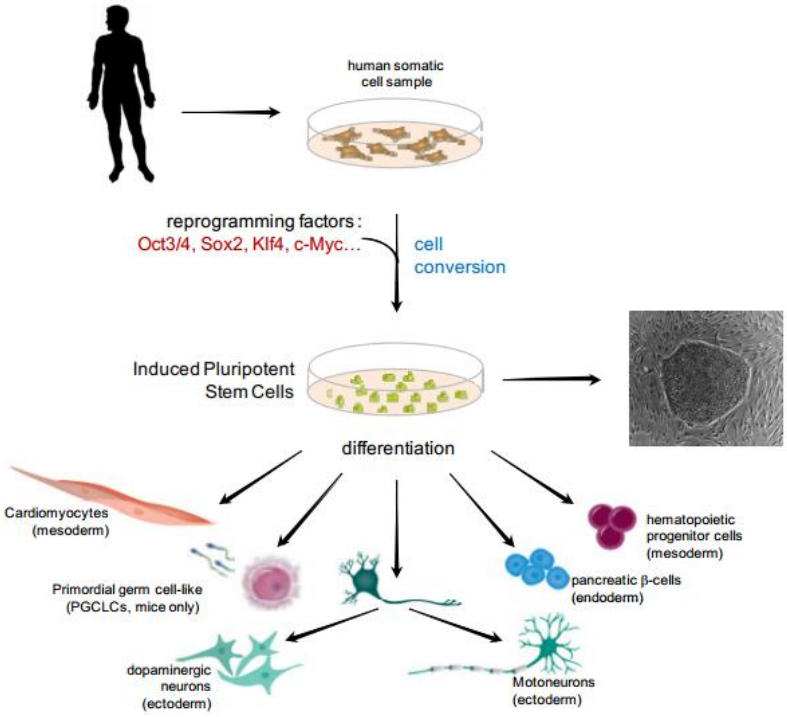

Figure 1 The diagram shows isolation of somatic stem cells which are reprogrammed into iPSC. iPSC have capacity to differentiate into derivatives of all three germ layers (Bayart and Cohen-Haguenauer, 2013).

Similar to ESC, it has been reported that iPSC can also generate into derivatives cells form three germ layers in immunodeficiency mice by mean of the teratoma formation. The iPSC also have potential to differentiate *in vitro*. It is found that both ESC and iPSC spontaneously differentiate into multicellular cell aggregates called embryoid bodies (EB) in an absence of LIF. Recently it is demonstrated that iPSC can differentiate into CMs, motor neuron, dopaminergic neuron, adipocytes and insulin-producing cells (Dimos et al., 2008; Taura et al., 2009; Kawamura et al., 2012; Sanchez-Danes et al., 2012; Shahjalal et al., 2014). In addition it is revealed that CMs derived either iPSC or ESC are highly similar instead of cardio-specific genes (Gupta et al., 2010). This provides the opportunity to generate patient /disease-specific cell lines for disease research or cell replacement therapy. It has been shown that human fibroblasts, oral mucosa fibroblasts, keratinocytes, hematopoietic progenitor cell, mature B lymphocytes, pancreatic beta cells, neural stem cells, liver and stomach cells can be reprogrammed to iPSC (Aoi et al., 2008; Hanna et al., 2008; Stadtfeld et al., 2008; Kim et al., 2009a; Kim et al., 2009b; Aasen and Izpisua Belmonte, 2010; Miyoshi et al., 2010; Tsai et al., 2010; Ohmine et al., 2011; Ohmine et al., 2012).

Methods of reprogramming into iPSC

Several methods have been used to produce iPSC. These methods transduce the pluripotent genes into the target cells. Gene delivery system is a key regulation for iPSC generation. Nowadays the gene delivery system can be performed either integral or non-integral method. The integral iPSC generation require transgene integrations using lentiviral or retroviral. The reprogramming efficiency of integral method is better than non- integral methods but permanent integration of reprogramming factor genes in host cells is concerned. However, establishment of excisable vectors to reduce permanent integration such as transposon constructs or loxP-flanked lentiviral system can be alternatively performed. This vector can be excised by transient expression of

Cre recombinase. The safer reprogramming methods have been developed without genomic integration, including transfection with episomal plasmids, proteins, synthetic modified RNA, microRNA and Sendai virus (Huang and Wu, 2012; Csobonyeiova et al., 2014).

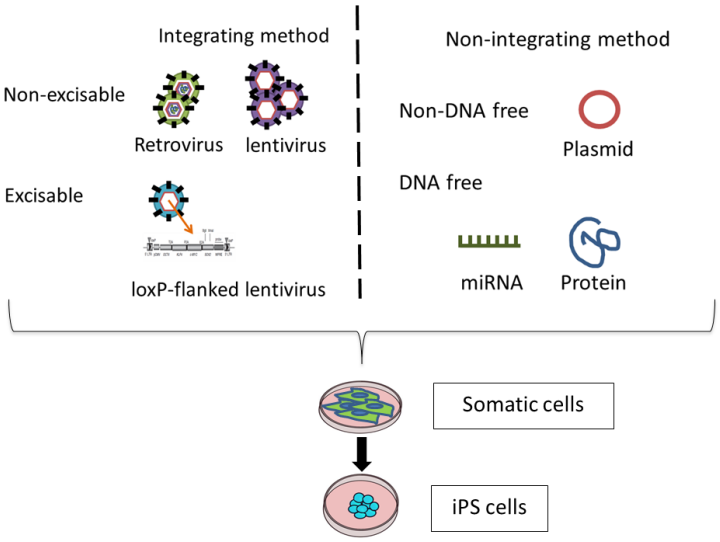

Figure 2 The diagram shows methods of iPSC generation including integrating methods and non- integrating methods (Csobonyeiova et al., 2014).

Table 2 The methods for reprogramming somatic cells to iPSC (Robinton and Daley, 2012; Malik and Rao, 2013)

Vector type		Efficiency (%)	Advantages	Disadvantages
Integrating	Retroviral	~0.001–1	High efficient	Genomic integration, incomplete proviral silencing and slow kinetics
	Lentiviral	~0.1–1.1	High efficient and transduces dividing and non-dividing cells	Genomic integration and incomplete proviral silencing
	Inducible lentiviral	~0.1–2	High efficient and allows controlled expression of factors	Genomic integration and requirement for transactivator expression
Excisable	Transposon	~0.1	Reasonably efficient and no genomic integration	Labour-intensive screening of excised lines
	loxP-flanked lentiviral	~0.1–1	Reasonably efficient and no genomic integration	Labour-intensive screening of excised lines, and loxP sites

				retained in the genome
Non-integrating	Adenoviral	~0.001	No genomic integration	Low efficiency
	Plasmid	~0.001	Only occasional genomic integration	Low efficiency and occasional vector genomic integration
DNA free	Sendai virus	~1	No genomic integration	Sequence-sensitive RNA replicase, and difficulty in purging cells of replicating virus
	Protein	~0.001	No genomic integration, direct delivery of transcription factors and no DNA-related complications	Low efficiency, short half-life, and requirement for large quantities of pure proteins and multiple applications of protein
Vector type		Efficiency (%)	Advantages	Disadvantages
DNA free	Modified mRNA	~1–4.4	No genomic integration, bypasses innate antiviral response, faster reprogramming kinetics, controllable and high efficiency	Requirement for multiple rounds of transfection
	MicroRNA	~0.1	Efficient, faster reprogramming kinetics than commonly used lentiviral or retroviral vectors, no exogenous transcription factors and no risk of integration	Lower efficiency than other commonly used methods

Regulation mechanism of cell reprogramming

OSKM reprogramming factors bind to their targets in a coordinated fashion to initiate the first step of reprogramming. Among these four reprogramming factors, c-Myc has been demonstrated to play a role in initiation of early transition. Only c-Myc expression can downregulate the expression of fibroblast-specific genes and induces the molecular context of the embryonic status within 3 days of transduction. After initiation of reprogramming, mesenchymal-to-epithelial transition (MET) is the next step towards pluripotency. Approximately 5 days after OSKM induction, transformed cells undergo dramatic morphological changes from mesenchymal-like cells to epithelial-like cells. MET is critical step for somatic cells to complete reprogramming (Yang and Rana, 2013).

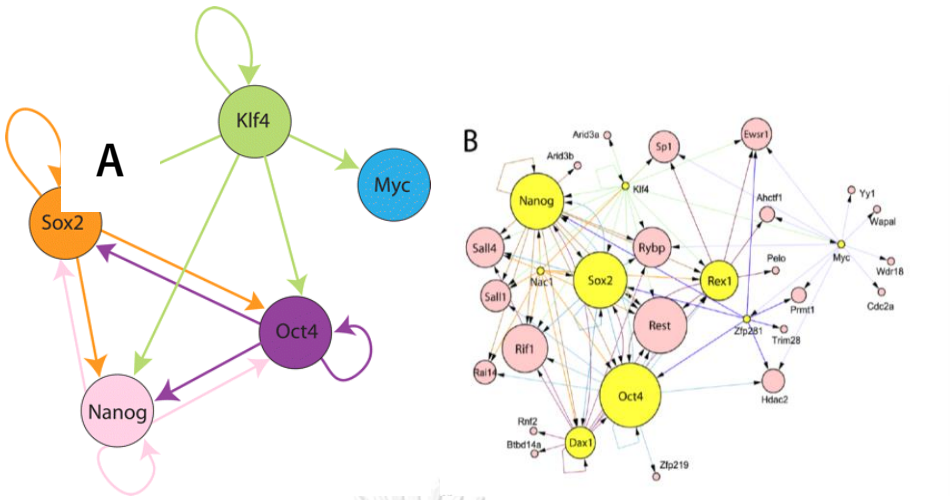

Figure 3 Transcriptional network in pluripotent cells. (A) The figure shows protein interaction in human pluripotent cells. Four transcription factors (*Nanog, Oct3/4, Sox2* and *Klf4*) show auto-regulatory. (B) Expansion of transcriptional regulatory network shows multiple targets. The arrow head indicates the direction of transcriptional regulation (Kim et al., 2008).

The regulatory network is comprised of "core transcriptional factor" (Oct3/4, Sox2 and Nanog) and other transcription factors to maintain pluripotent and self-renewal properties. Oct3/4 is known for maintenance of ESC pluripotency as aforementioned, the level expression of Oct3/4 is crucial for differentiation. The reduction of Oct3/4 expression permits trophoectoderm development, while the enhancement of Oct3/4 expression promotes primitive endoderm differentiation (Niwa et al., 2000; Kim et al., 2008; Kues et al., 2010; Robinton and Daley, 2012). Oct3/4 forms complex with Sox2 and regulates expression of several genes, for example *Utf1, Zfp206, Rex1 and Fgf4*. Sox2 regulates embryonic development and determination of cell fate. Klf-4 binds to GC-rich DNA sequence elements and it can both activate and repress expression of target genes. KLF4 has growth-suppressive ability so the over-expression of KLF4 can suppress proliferation (Kues et al., 2010).

Rabbit pluripotent cells and its pathway

Characteristics comparison of PSC among various species disclosed mechanisms underlying the maintenance of self-renewal and pluripotency as well as epigenetic regulation (Wei et al., 2016). Naive mouse ESC represent the ground (naïve) state of

pluripotency with E-cadherin. Both mouse epiblast stem cells and human ESC represent primed states of pluripotency. They rely on FGF2, activin and Nodal signaling. The primed stem cells can be converted to naive stem cells under appropriate signals such as blocking GSK3 and MAPK signaling pathways, using two inhibitors (2i: CHIR99021 and PD0325901) (Pieters and van Roy, 2014). Basic fibroblast growth factor (bFGF) is necessary to maintain rabbit pluripotency (Fang et al., 2006; Chiang et al., 2008; Honda et al., 2008; Du et al., 2015; Honsho et al., 2015; Wei et al., 2016). Hsieh et al. (2011) demonstrated that a combination of bFGF and LIF in ESC medium generated a normal morphology and exhibited apparently stronger pluripotent marker expressions when compared with only LIF or FGF. It is therefore believed that LIF and bFGF pathways play a central role in maintaining self-renewal and pluripotency. Other molecules such as Noggin (inhibits the bone morphogenetic protein, BMP pathway) and Y-27632 (inhibits apoptosis) can improve maintenance rabbit PSC (Du et al., 2015). In addition, mouse embryonic fibroblasts and rabbit embryonic fibroblasts are the feeder sources to maintain rabbit pluripotent stage (Intawicha et al., 2009). Nevertheless, derivation and culture systems vary among laboratories, contribute various characteristics (i.e. morphology and pluripotency) of rabbit PSC. Therefore, a complete understanding of mechanisms underlying the pluripotent maintenance of rabbit PSC still has a long way to go compared to the knowledge base accumulated for PSC in other animals (Wei et al., 2016).

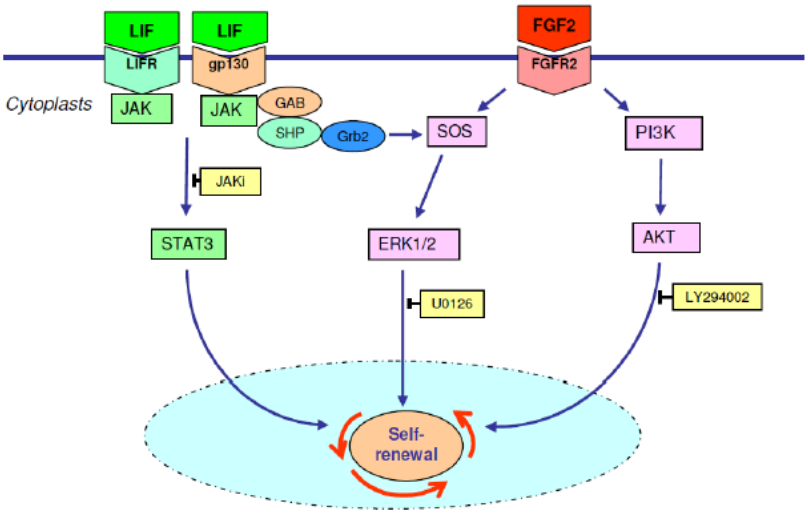

Figure 4 Regulation Pathway of rabbit ESC, the figure shows the combination of LIF and bFGF to maintain the self-renewal properties of rabbit ESC. The downstream regulations are controlled via JAK/STAT pathway, PI3K/AKT pathway and ERK1/2 pathway (Hsieh et al., 2011).

Table 3 The publications of Rabbit induced pluripotent cells, the table shows the methods and reprogrammed cells sources of previous studies. The essential factors for maintain rabbit iPSC, pluripotent markers and the level of pluripotency are demonstrated.

Reference	Cell souces	vectors	growth factors	Pluripotent markers	Level of Pluripotency
Honda et al.,2010	stomach and liver cells	monocystronic lentivirus	LIF, bFGF	AP, SSEA-1,SSEA-4, Oct3/4,Nanog and SSEA-3	normal karyotype
					alkaline phosphatase activity
					Embryoid body formation
					Teratoma formation
					Telomerase activity
					No Chimera
Honda et al., 2013	Naïve conversion iPSC of Honda et al., 2010	monocystronic lentivirus	LIF, bFGF	AP, SSEA-1,SSEA-4, Oct3/4,Nanog and SSEA-3	normal karyotype
					alkaline phosphatase activity
					Embryoid body formation
					Teratoma formation
					Telomerase activity
					No Chimera
Osteil et al., 2013	adult fibroblasts	monocystronic retrovirus	bFGF	AP, SSEA-1,SSEA-4, Oct-4,Nanog,Tra-1-60 and E-cadherin	normal karyotype
					alkaline phosphatase activity
					Embryoid body formation
					Teratoma formation
					No Chimera
Honsho et al., 2015	Naïve conversion iPSC of Honda et al., 2010	monocystronic lentivirus	LIF, bFGF	AP, SSEA-1,SSEA-4, Oct3/4,Nanog and SSEA-3	normal karyotype
					alkaline phosphatase activity
					Embryoid body formation
					Teratoma formation
					Telomerase activity
					No Chimera
Tancos et al., 2017	embryonic fibroblasts	polycistronic lentivirus	LIF, bFGF	Oct3/4, SOX2, and SSEA-4	normal karyotype
					alkaline phosphatase activity
					Embryoid body formation
					Teratoma formation
					No Chimera
Tapponnier et al., 2017	adult fibroblasts	lentivirus	LIF	Nanog, Rex1, Esrrb, Dppa2, Dppa5, Tbx3, Dazl, Fgf4, Piwil2, Cdh1, Otx2, and Lefty2	normal karyotype
					Transcriptome assay
					Epigenetic reconfiguration
					Embryoid body formation
					Teratoma formation
					Integrated ICM

Similarly, there have been some reports of rabbit iPSC (Honda et al., 2010; Tancos et al., 2012; Honda et al., 2013; Osteil et al., 2013; Honsho et al., 2015; Tapponnier et al., 2017). Honda and his colleague (2010) generated iPSC lines derived adult rabbit liver and stomach cells using lentiviral vectors. However, they unsuccessfully reprogrammed rabbit fetal and adult fibroblast cells using lentiviral vector. This suggests that donor cell types are important for iPSC. The properties of generated these cell lines is quite limited as they demonstrated naive conversion of their iPSC to retrieve the capacity of the cells to differentiate into mature oligodendrocytes (Honda et al., 2013). Osteil et al. reported iPSC lines obtained adult rabbit fibroblasts using monocistronic retroviral vectors (2013). They firstly compared molecular and functional properties of rabbit ESC and iPSC. However, the results of their experiment was that the iPSC had closer genetic profiles to rabbit inner cell mass (ICM) than ESC. So they stated that their iPSC were in the state between naïve and primed state with the failure to produce chimera. It is interested that no evidence of cardiomyocytes derived from *in vitro* and *in vivo* differentiation of these iPSC as shown in table 4.

Table 4 Protein and gene expression of three germ layers of previous studies

Reference	Protein or gene expression of three germ layers	
Honda et al., 2010	endoderm	GATA4
	mesoderm	smooth muscle actin
	ectoderm	β-tubulin, glial fibrillary acidic protein
Honda et al., 2013	endoderm	glucagon, FOXA2
	mesoderm	smooth muscle actin
	ectoderm	nestin,β-tubulin, glial fibrillary acidic protein
Osteil et al., 2013	endoderm	FOXA2,glucagon
	mesoderm	Desmin, flK-1
	ectoderm	nestin, glial fibrillary acidic protein
Tancos et al., 2017	endoderm	GATA4
	mesoderm	SMOOTH MUSCLE ACTIN
	ectoderm	βIII-TUBULIN

Embryonic cardiac development and regulation

Cardiac myocytes mainly generate from the mesoderm of three germ layers (endoderm, mesoderm and ectoderm). The cardiac progenitors move through the

primitive steak to create cardiac crescent. Then, they move ventrally and fuse at the midline to form heart tube. The single heart tube undergoes a loop formation and internal division. Then it develops to two atria and two ventricles (Cao et al., 2014).

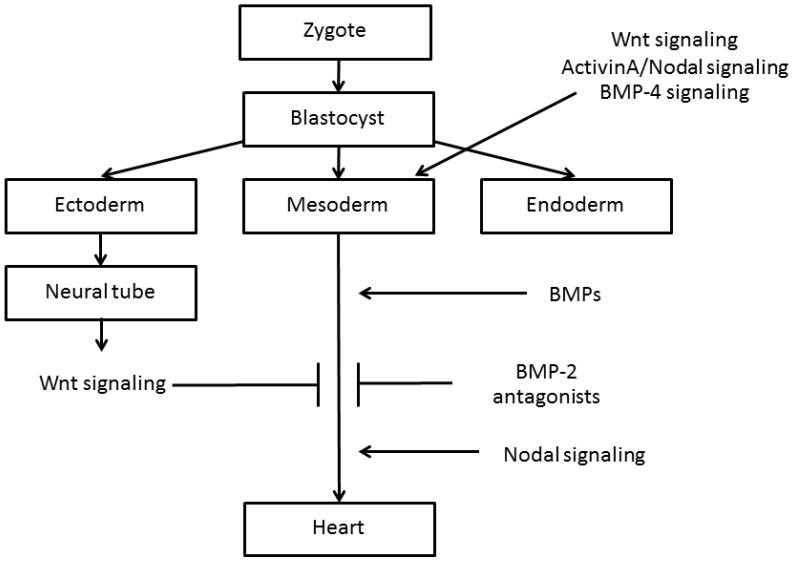

Figure 5 Regulation of cardiac development (modified from Sachinidis et al., 2003)

Molecular signaling of cardiac differentiation and development

Basic regulation of cardiac development is conserved between invertebrates and vertebrates. Heart is the mesoderm-derived embryonic organ. The uncommitted mesodermal cells develop to cardiac cells regulated by growth factors, transcription factors and signaling cascades.

Nodal/activin pathway

Nodal signaling is one of the key regulations of gastrulation, it plays a role to control left-right patterning and induction of mesoderm. Nodal belongs to Transforming Growth factor (TGF) β family and it express through type I or type II TGFβ serine-threonine receptor. The downstream signaling occurs via mediation of epidermal growth factor- cripto1/FRL-1/Cryptic (EGF-CFC) co-receptor which acts to phosphorylate Smad2/3. The phosphorylated Smad signals via Smad 4. The Smad 4

translocates to nucleus and induces the expression of target genes, for example Lefty, pitx2 and BMP-4 (Shen, 2007).

Activin proteins are members of the TGF-beta superfamily. Their structures are disulfide-linked dimeric proteins. Activins have a wide range of biological activities, including mesoderm induction, neural cell differentiation, bone remodeling and hematopoiesis regulating in reproductive physiology. Furthermore the activin have been shown to promote self-renewal in human embryonic stem cells (Moore et al., 1998; Barron et al., 2000; Lev et al., 2005; Tsuchida et al., 2009). The structures of Activins are composed of alpha subunit or beta subunits. Four beta subunits have been identified in mammals (βA, βB, βC and βE), whereas only a single alpha-subunit has been reported. Activin A is homodimer of βA- βA (beta A - beta A). Activin βA was the only subunit detected during the initial phase of epithelial–mesenchymal transition (EMT); activin βB was detected at later stages, and inhibin α was not detectable in the heart (Moore et al., 1998; Barron et al., 2000; Lev et al., 2005; Tsuchida et al., 2009)). Activins can bind to two types of transmembrane serine/threonine kinase receptors, type I and type II activin receptors of the target cells. Activin A binds to Activin receptor type II (ActRIIA or ActRIIB) and recuits Activin receptor type IB (ALK-4). Then ALK-4 phosphorylates SMAD family member 2 and 3 (SMAD2 and SMAD3). Phosphorylated SMAD binds to SMAD family member 4 (SMAD4) and forms complex. The complex translocates to the nucleus and activates target genes (Abe et al., 2004; Larsson and Karlsson., 2005; Mathews et al., 1991; Miyazono., 2000).

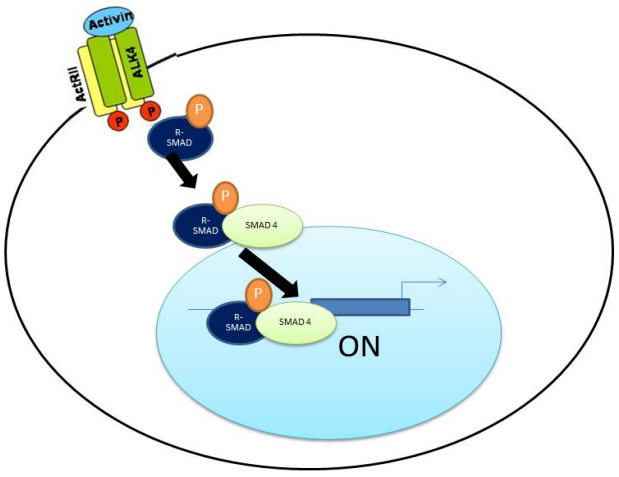

Figure 6 The signaling pathway of activin via Smad family signal transducers. Activin A binds to Activin receptor type II and recuits ALK-4. Then ALK-4 phosphorylates SMAD family member 2 and 3. Phosphorylated SMAD binds to SMAD4 and form complex. The complex translocates to the nucleus and regulates target genes (modified from Tsuchida et al., 2009).

Bone morphogenic proteins/Noggin pathway

The bone morphogenic proteins (BMPs) are secreted protein from Transforming Growth factor (TGF) β family. It is known that BMP signals in the lateral region of the embryos promote heart formation. The evidences show that application of BMPs induced expression of cardiac transcription factor Nkx2.5 and GATA family such as GATA-4, GATA-5 and GATA-6. GATA4 and Nkx2.5 appear to be mediator of BMPs for the expression of cardiac-specific proteins such as α-myosin heavy chain (α-MHC), cardiac troponin C and atrial natriuretic peptide. BMPs induce expression of GATA4 and Nkx2.5 through MAP kinase kinase kinase Tak1 and Smad family (Barron et al., 2000; Lev et al., 2005). Generation of cardiomyocytes from pluripotent cells can be observed as beating area. It has been shown that administration of soluble BMP-2 or BMPr-4 to EB resulted in increased cardiac differentiation with significantly higher beating area and disruption of the TGF-β/BMP pathway by noggin (Behfar et al., 2002; Sachinidis et al., 2003; Kattman et al., 2011). There are three Type-I receptors identified: (1) Activin A receptor type I (ACVR1, activin-like kinase 1, Alk-1), (2) bone morphogenetic protein serine threonine kinase type IA receptor (BRIa, Alk-3) and (3) bone morphogenetic protein serine threonine kinase type IB receptor (BRIb, Alk6) and three Type-II receptors (bone morphogenetic protein serine threonine kinase type II (BRII), Activin receptor type 2A (ActRII) and Activin receptor type 2B (ActRIIB)). BMP signal transmits via Type-I and Type-II receptor which lead to the recrution and phosphorylation of receptor-regulated Smads (R-Smads, Smads1, 5 or 8). The phosphorylated R-Smads are released from the receptor and bound to common mediator Smad (Co-Smad, Smad4) into the complex. The complex migrates into the nucleus and activates the transcription of specific target genes. BMP signaling can also be transduced via MAP3K7 (TAK1)/MAP3K7IP1 and can

be inhibited by secreted inhibitors, like Noggin, chordin, Gremlin and follistatin (Barron et al., 2000; Lev et al., 2005; Nohe et al., 2004; Van et al., 2007).

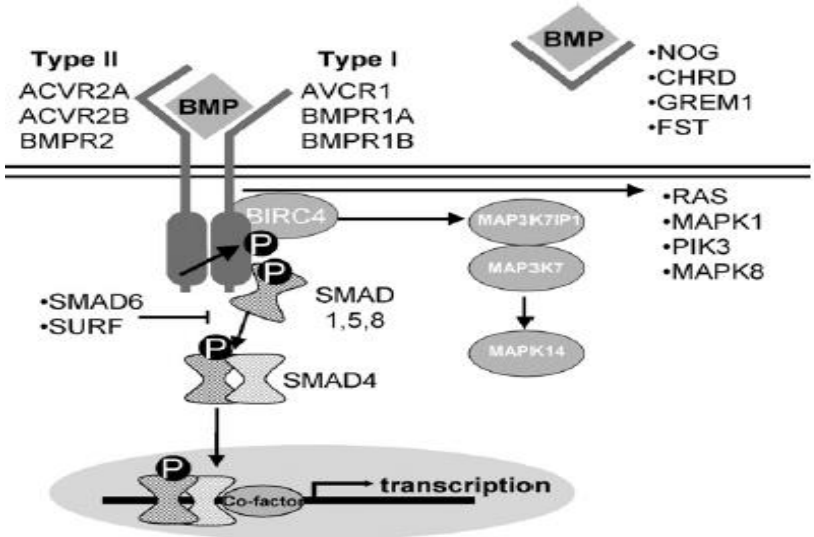

Figure 7 Signaling events of BMPs (Van et al., 2007)

Noggin is a secreted glycoprotein which encoded by the *NOG* gene. Noggin involves cell survival in dorsal development in Xenopus embryos. Nowadays, noggin is known to regulate bone morphogenetic proteins (BMPs). The structure of noggin resembles BMPs in a two-fold axis of symmetry. The BMP-dimer is shaped like a butterfly with wings extending from a core body. Noggin can complex with BMPs called clip segment. This prevents BMPs binding to their receptors (Krause et al., 2011).

Vascular endothelial growth factor (VEGF) pathway

VEGF is an essential growth factor for vascular endothelial cells. As a result of alternative splicing, five VEGF isoforms are derived from a single VEGF gene. The various VEGF forms interact to two tyrosine-kinase receptors, VEGFR-1 (flt-1) and VEGFR-2 (KDR/ Flk1). VEGF is a key regulator of physiological angiogenesis, osteogenesis and reproductive functions. It is reported that VEGF promotes cardiac mesoderm commitment with the evidence of up-regulation Flk-1 and PDGFR-**α**. VEGF significantly enhanced the expression of **α**-myosin heavy chain (**α** -MHC), cardiac troponin I (cTn-I), myosin heavy chain, cardiac troponin and Nkx2.5 in differentiated mouse ESC. Chen et al. reported that VEGF enhances cardiac differentiation by ERK-mediated Flk-1 activation. (Chen et al., 2006; Ye et al., 2013; Neufeld et al., 1999; Ferrara et al., 2003)

Fibroblast growth factors (FGFs) pathway

Fibroblast growth factors (FGFs) provide cell specification. Normally FGF is found in cardiogenic mesoderm, the mutation of FGF-8 in zebrafish leads to decrease of cardiac markers in this population (Reifers et al., 2000). FGF-2 and FGF-4 also is indicated to regulate avian cardiac mesoderm (Antin et al., 1996; Lough et al., 1996; Ladd et al., 1998). In mouse, FGF signaling plays a role in mesoderm differentiation by allowing epithelial-to-mesenchymal (EMT) transition and lead to further patterning (Willems and Leyns, 2008).

WNTs signaling pathway

The wingless/INT proteins (WNTs) are members of a family of cysteine-rich glycosylated ligands. The actions of Wnts are complicated. They either promote or inhibit cardiac differentiation depending on stage and pathway, in addition Wnts relates regulation of cell communication, cell growth and survival process. Wnts binds to its receptor which is a member of frizzled family and the LDL-receptor related protein (LRP5 and LRP6). Then cytoplasmic protein, Dsh is phosphorylated. Activated Dsh inhibits the glycogen synthase kinase-β (GSK-β) and leads to an accumulation of cytoplasmic β-catenin. β-catenin translocates to the nucleus and interacts with T-cell factor (TCF)/Lef transcription factor. This results in expression of genes which promote mesoderm in early stage or inhibits cardiac development of vertebrates in late stage. Moreover, it has been reported that other Wnt signaling such as Wnt-11 in noncanonical pathway promotes mesoderm and cardiogenesis in early stage of avian embryos (Sachinidis et al., 2003; Komiya and Habas, 2008).

Miscellaneous

There are other factors have been reported to promote cardiac differentiation such as retinoic acid, ascorbic acid, dimethylsulfoxide (DMSO) and reactive oxygen species (ROS) (Sachinidis et al., 2003; Lev et al., 2005)

Culture system of cardiac differentiation from pluripotent stem cells

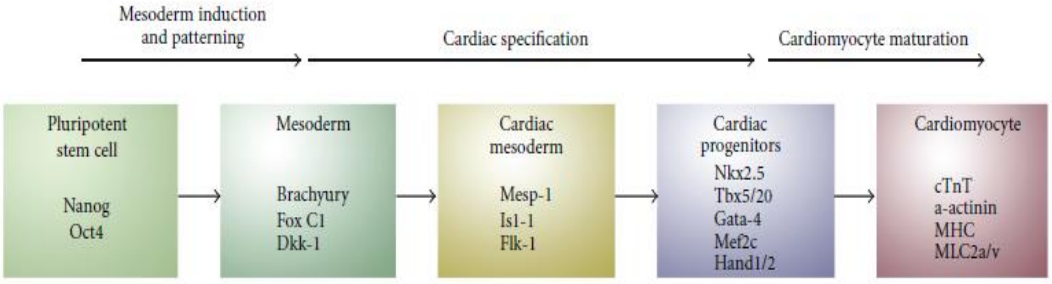

Figure 8 Diagram of sequential steps of cardiac development from pluripotent stem cells to cardiomyocytes. The steps are shown as following; pluripotent cells develop into mesoderm, cardiac mesoderm, and commits to cardiac progenitors. Finally, the cells become beating cardiomyocytes. Typical markers for each step are indicated (Rajala et al., 2011).

Embryoid Body-Based Cardiac Differentiation

After pluripotent cells are dissociated into single cells, they reaggregated in suspension and then develop into three dimensional spheroids which are termed as Embryoid bodies (EB). Cells in EB have a potency to differentiate into derivatives of three germ layers including ectoderm mesoderm and endoderm. These EB, unlike normal embryonic development, generates variable in structure and composition. However, a fraction of the EB may contain cardiomyocytes and demonstrated spontaneous contraction. Traditionally, mouse EB are transferred to 0.1% gelatin-coated dishes and beating areas were first observed 4 days after plating. Previous studies have identified the critical factors for optimization of *in vitro* cardiac differentiaton including the following: (1) the starting cell number for EB formation; (2) medium cultural condition; (3) pluripotent cell lines; and (4) Time of EB plating (Lev et al., 2005; Mummery et al., 2012; Rungarunlert et al., 2013).

Coculture with END-2 Cells

Cardiac differentiation can be performed by coculturing with mouse visceral-endoderm like cells (END-2). Mummery and colleagues indicated that hESC can generate cardiomyocytes by coculture of hESC with END- 2 (Mummery et al., 2003). Endoderm secretes factors, such as bone morphogenetic proteins (BMPs), nodal/activin

A, fibroblast growth factors (FGFs), and repressors of canonical Wnt/β-catenin pathway. However, the efficiency of this protocol is quite low. Switching from 20% fetal calf serum (FBS) to serum-free medium and adding L-ascorbic acid can improve this effieciency. The problem of the protocol is the batch differences of FBS may significant impact on the efficiency of cardiac differentiation. L-ascorbic acid has also been used to enhance cardiac differentiation and promotes cardiac progenitor cell proliferation in mouse pluripotent cells (Cao et al., 2012).

Monolayer and Define Culture Condition

The advantage of monolayer with define culture medium is to limit cell-contamination. Without complex structure, it is easier for small molecule to effect all cells equally and this method do not require replating of EB protocols (Mummery et al., 2012). Human iPSC exposed to activin A and BMP4 in defined RPMI/B-27 minus insulin medium has been shown to improve in cardiac differentiation when compared with serum-based EB differentiation. It was found that insulin in B-27 supplement greatly inhibited cardiac differentiation during the first 5 days (Lian et al., 2013). However, the efficiency of the activin A and BMP4 monolayer is highly variable between cell lines. It is noted that individual pluripotent stem cells require optimization of signaling pathway for efficient cardiac differentiation (Kattman et al., 2011).

Conceptual Framework

The thesis was divided into 2 parts as follows:

Study 1: *In vitro* cardiac differentiation of rabbit embryonic stem cells with emphasizing on reference gene stability and cardiac gene expression

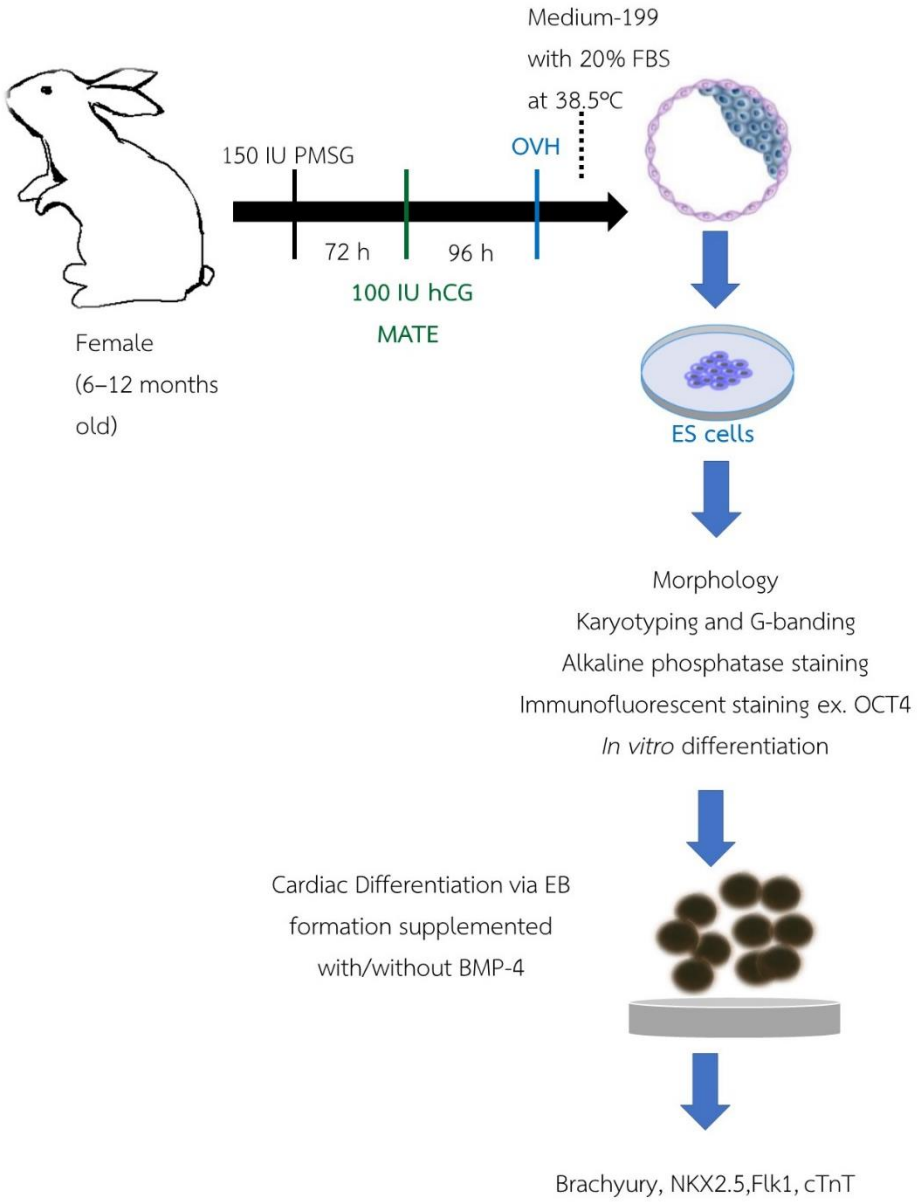

Figure 9 The embryos were collected and cultured for the blastocyst stage embryos and then were explanted on feeder cells for ESC. They were induced into cardiac lineage via EB supplemented with or without BMP-4 and investigated for cardiac related markers.

Study 2: Rabbit induced pluripotent stem cells retain capability of *in vitro* cardiac differentiation

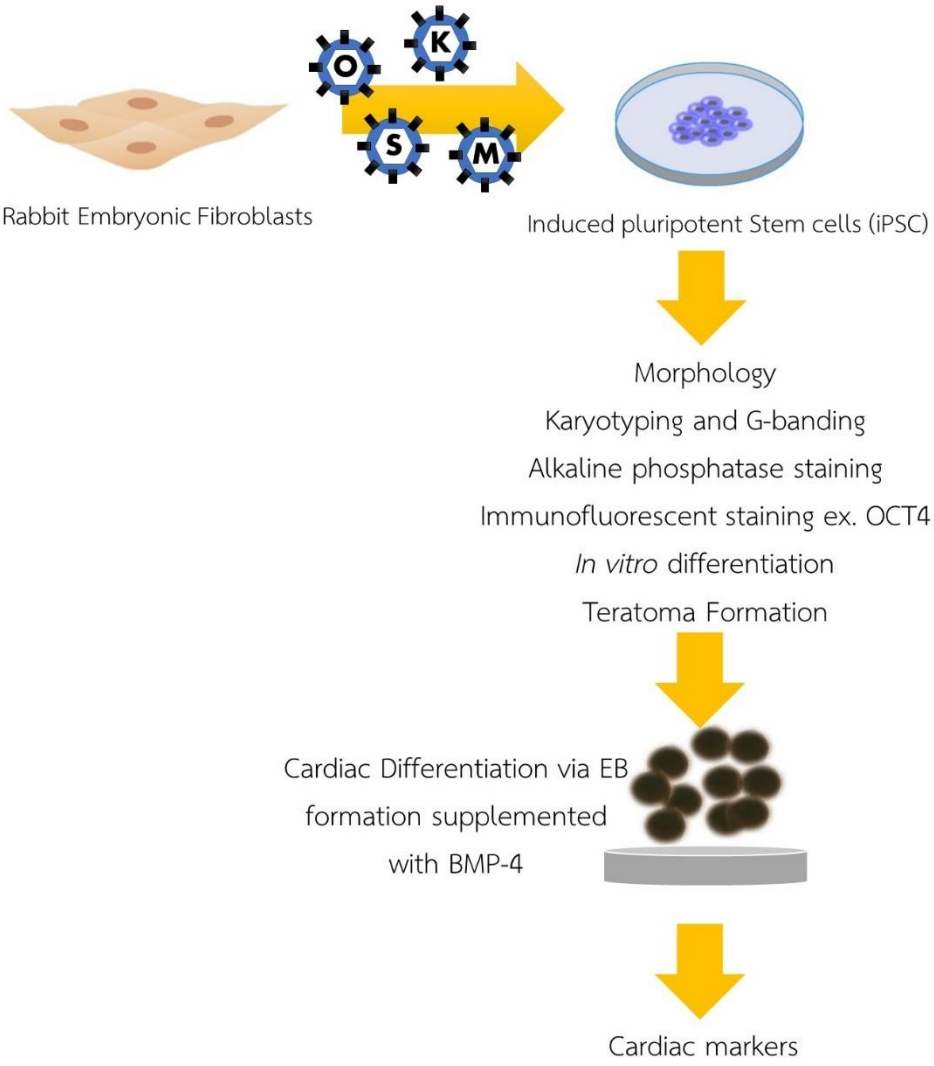

Figure 10 Reprogramming efficiency of rabbit embryonic fibroblasts will be studied using retroviral vector containing reprogramming genes: *OCT3/4, SOX2, KLF4,* and *c-Myc* (OSKM). iPSC were characterized for pluripotency. Then, they were induced into cardiac lineage via 3D structure embryoid bodies (EB) with BMP-4. The differentiating cells were studied for cardiac markers.

Chapter II

In vitro cardiac differentiation of rabbit embryonic stem cells with emphasizing on reference gene stability and cardiac gene expression

Introduction

Rabbit is a useful animal model for cardiac diseases (Ashida et al., 1997; Ashida et al., 2004; Takato et al., 2010; Wen et al., 2010) due to its similarity of molecular mechanisms and electrophysiology compared with human (Fan and Watanabe, 2003; Pogwizd and Bers, 2008). Although *in vitro* cardiac differentiation has been well established in particular species such as mouse (Jing et al., 2010; Kokkinopoulos et al., 2016) and human (Kehat et al., 2001; Burridge et al., 2007; Lecina et al., 2010) this technology in rabbit has yet to be established (Honda et al., 2008). Indeed, pluripotent cell lines derived from embryos as embryonic stem cells (ESC) or cell reprogramming (induced pluripotent stem cells, iPSC) have been reported in rabbits but overall efficiency of derivation of the cell lines and differentiation potential have been poor (Graves and Moreadith, 1993; Honda et al., 2008; Intawicha et al., 2009; Honda et al., 2010; Honda et al., 2013; Osteil et al., 2013). These shortcomings of pluripotent stem cells in rabbit has been demonstrated to cause by several factors such as poor understanding of pluripotency network and also factors required during culture and differentiation (Hsieh et al., 2011; Honda et al., 2013). It is well characterized that mouse and human ESC essentially require LIF/STAT3 and basic fibroblast growth factor 2 (bFGF2) dependent pathway, respectively (Daheron et al., 2004; Levenstein et al., 2006; Niwa et al., 2009; Onishi and Zandstra, 2015). Although it is not entirely understood, rabbit embryonic stem cells can be maintained via LIF/STAT3 and bFGF2 (MEK-ERK1/2 and PI3K-AKT) signaling (Hsieh et al., 2011). In contrast to pluripotency pathways, molecular networks that drive cellular differentiation into particular cell lineage is poorly understood especially in rabbit model. It has been reported that the rabbit ESC could be differentiated into neural lineage (Honda et al., 2013; Honsho et al., 2015) but there is currently no report on cardiac differentiation. Of several

technique to induce differentiation, cell aggregation with optimal cell density via embryoid body (EB) formation has classically been demonstrated to allow cellular interaction and microenvironment suitable for support differentiation of pluripotent stem cells into three germ layers (Itskovitz-Eldor et al., 2000; Kurosawa, 2007; Zhou et al., 2010) as well as cardiac differentiation (Kehat et al., 2001). To understand changes during differentiation, quantitative polymerase chain reaction (qPCR) has become a technique of choice to rapidly analyze dynamic of gene expression compared to the level of housekeeping gene (HK). These HK genes are regularly constitutive genes to maintain basic cellular function (Butte et al., 2001; Holmgren et al., 2015). The expression of HK is applied as reference point in order to analyze the expression levels of target genes. The HK genes, therefore, need to be stably expressed and must not affect by experimental conditions. However, it has been demonstrated that HK genes can be differently expressed in different cell types and biological treatments (Lee et al., 2002; Lee et al., 2014; Holmgren et al., 2015). Of reference genes frequently used, Ribosomal Protein L13a (*RpL13a*) and *GAPDH* are commonly used to normalize genes of interest during differentiation (Honda et al., 2013; Kim et al., 2015; Lo et al., 2015; Kudova et al., 2016; Lee et al., 2016). Indeed, specific type of HK gene during EB culture has yet to be examined in rabbit in order to standardize the expression patterns of gene of interest during cardiac differentiation. Cardiac differentiation is mediated via initiation of genes associated with cardiac mesoderm and progenitor including *Brachyury* (*BT*) (King et al., 1998; Dixon et al., 2011) and *GATA4 (Charron and Nemer, 1999; Jiang et al., 1999; Watt et al., 2004)* and *NKX2*.5 (Hiroi et al., 2001; Harvey et al., 2002). This regulation of cardiac cell fate has been demonstrated to primarily involve BMP-4 signaling in mouse and human (Lev et al., 2005; van Wijk et al., 2007; Takei et al., 2009; Rajala et al., 2011; Mummery et al., 2012). During cardiac differentiation, the expression of *BT* reached its maximal levels around day 3 and 4 for mouse and in human, respectively (Graichen et al., 2008; Pekkanen-Mattila et al., 2010). This level correlated to the optimal time of EB plating for efficiently cardiac differentiation (Rungarunlert et al., 2013). However, similar study indicating the evidence that the BMP-4 stimulates cardiac gene expression has not been previously demonstrated in rabbit. This study aimed at examining cardiac differentiation of rabbit embryonic stem

cells. Gene profiling of housekeeping genes and the effects of BMP-4 on cardiac differentiation were examined.

Materials and Methods

Reagents and animals

Cell culture reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA), unless otherwise stated. New Zealand White rabbits and ICR mice were purchased from the National Laboratory Animal Center (Mahidol University, Nakhon Pathom, Thailand). BALB/c nude mice were purchased from Nomura Siam International Co, Ltd. (Bangkok, Thailand). Animal maintenance, care, and use procedures were approved by the University Committee (N0.1673036).

Establishment and maintenance of rabbit ESC

Mouse embryonic fibroblasts (MEFs) were derived from 13.5 days post coitum (d.p.c) fetus. They were maintained in high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 1 mM L-glutamine and 1% antibiotic-antimycotic. At passage 3, they were inactivated by mitomycin C and used as feeder cells in this study. Female rabbits (6–12 months old, n=15) were superovulated using 150 IU of pregnant mare serum gonadotrophin (PMSG; Folligon, Intervet, NJ, USA) intramuscularly. At 72 h, they were given 100 IU of human chorionic gonadotropin (hCG; Chorulon, Intervet, NJ, USA) intravenously. Superovulated does were mated with fertile males at the day of hCG administration. The embryos were collected by flushing using Medium-199 (M-199) with 20% FBS, and the embryos were cultured at 38.5ºC in 5% CO_2 and humidified air until becoming blastocyst-stage. Embryos were harvested and their mucin coat and zona pellucida were dissected using 30-gauge needles under a microscope. The inner cell mass was co- cultured with the feeder cells at the density of 25,000-30,000 cells/cm^2 on gelatin-coated dishes in ESC medium which is DMEM/F12 adding 20% knockout serum replacement (KSR), 1% non-essential amino acids (NEAA), 1 mM L-glutamine, 1% antibiotic-antimycotic, 0.1 mM Beta-mercaptoethanol (β-ME), 10 ng/ml, bFGF and 1000IU LIF at 37°C 5% CO_2. The medium was changed consecutively until the outgrowth was clearly present (passage

0). They were mechanically cut into small pieces onto new feeder cells. After passage 3, the cells were passage into small cell clumps using 0.5% TrypLE™ Select. Selective inhibitor of Rho-associated protein coiled-coil containing protein kinase (Rock inhibitor Y-27632, Biovision, CA, USA) was used at a concentration of 10 μM on the day of passage to mitigate cell death.

Alkaline phosphatase (AP)

ESC and differentiated cells were fixed with 4% paraformaldehyde (PFA) for 15 min and washed with in phosphate buffered saline (PBS). For AP activity, they were incubated in a mixture of fast red violet LB, sodium nitrite solution and naphthol AS-BI alkaline solution (Alkaline Phosphatase Kit, Sigma-Aldrich, MO, USA). The positive AP-staining demonstrated red colored colonies.

Immunofluorescence (IF) staining

The expression of pluripotency (OCT3/4 and SSEA-1) and cardiac differentiation (Flk-1, NKX2.5 and Troponin-T) markers were confirmed by IF staining. In brief, cells were fixed with 4% PFA in PBS for 15 min and washed 2 times with PBS. For marker OCT3/4 and troponin, the cells were permeabilized in a mixture of 0.1% (v/v) Triton X-100, 2% (w/v) bovine serum albumin (BSA) and PBS for 10 min. The cells were incubated with primary antibody at 37°C RT or 4°C overnight, and subsequently incubated with secondary antibody in the dark at room temperature (26°C) for 1h. The negative control was performed as described above but the PBS was used instead of the primary antibody. The cells were counter-stained with 4'-6-Diamidino-2-phenylindole (DAPI, 1:200) nuclear marker in mounting medium (VECTASHIELD® Mounting Medium, Vector Laboratories, CA, USA). Antibodies used in this study are shown in table 5.

Karyotype and G banding analysis

The ESC were harvested using 0.5% TrypLE™ Select into single cells. The pellet was resuspended in a warmed 0.075 M KCl hypotonic solution and incubated for 20 min.

Subsequently, they were centrifuged at 201 x *g* 5 min and fixed in a mixture of acetic and methanol (1:3) on ice. The cells were dropped vertically onto a clean slide and left for at least one week prior staining. The cells attached onto the slide were partially digested in 0.05% trypsin, washed in NSS and then stained with 10% (v/v) Giemsa solution. They were rinsed with tap water and air dried overnight. At least 20 metaphases per cell line were examined under a light microscope.

In vitro Differentiation

For *in vitro* differentiation towards three germ layers, cell aggregation via embryoid body (EB) was performed. Briefly, ESC cells were harvested using 0.5% TrypLE™ Select into single cells and formed EB at density of 2,000 cells/well in a NUNC™ Microwell™ 96-well microplate coated with poly 2-hydroxyethyl methacrylate (polyHEMA). The differentiation media was contained DMEM-F12, 15% FBS (SH30070.03, lot AB10163350, HyClone™, Utah, USA), 0.1 mM NEAA, 1 mM L-glutamine, 1% antibiotic-antimycotic, and 0.1 mM β-ME. All cells were maintained at 37°C, in 5% CO_2 in a humidified atmosphere. The media were changed every 2-3 days. For analysis of three-germ layers, the EB were collected on day 7 and the mRNA expression of ectodermal (*PAX6, GBX2*), mesodermal (*PECAM1*) and endodermal (*PITX2)* lineages were determined using reverse transcriptase polymerase chain reaction (PCR). Primers used in this study are listed in Table 8. Further examination of *in vitro* differentiation was performed using 14 days old EB by immunohistochemistry (Leica Microsystems BOND-MAX System). In brief, the EB were fixed with 4% (w/v) paraformaldehyde, embedded in paraffin and cut at a thickness of 4 µm. The slides were incubated with Bond Dewax Solution (Leica Microsystems) for 1 h at 60°C. After that, the antigens retrieval was performed with Bond Epitope Retrieval Solution 2 (Leica Microsystems) for 30 min at 100° C. The slides were separately incubated with primary antibodies (Table 6) at 25° C for 40 min and followed by 3 consecutive rinses with a Bond Wash Solution (Leica Microsystems). Then, hydrogen peroxide (3%) was applied for 5 min and rinsed 3 times. Post primary polymer (Leica Microsystems) were applied for 8 min. The slides were washed, followed with Poly-HRP IgG (Leica Microsystems) for 8 min,

and rinsed 3 times. The diaminobenzidine chromogen was applied for 4 min followed by 3 deionized water rinses. Slides were counterstained with hematoxylin for 5 min. Isotype Mouse IgG1, kappa monoclonal (ab91353, Abcam, Cambridge, UK) were used instead of the primary antibody for the negative control. Brain tumor, appendix and tonsil were used as positive controls for GFAP, vimentin and ß-catenin, respectively.

Normalization of housekeeping genes of Rabbit embryoid body

ESC were cultured and the EB were generated as aforementioned above. Briefly, all cell lines were enzymatically passaged using 0.5X TrypLE™ Select into single cells and EB were generated. Undifferentiated ESC were also collected for qPCR. EB were allowed forming in small wells for 3 days except collecting day 2 samples. For BMP-4 treatment group, EB were formed in differentiating media adding BMP-4 (Biovision, CA, USA) 10 ng/ml for 72 h. Then all EB were plated on 0.1% gelatin coated dish until the samples were collected. Both EB in control (no BMP-4) and BMP-4 treated group were collected at day 2-5. All samples were washed two times with PBS, centrifuged and then stored at -80°C until RNA extraction. Primer of *GAPDH* was used from previous study (Lo et al., 2015) and of *RPL13a* was designed base on reported sequence (XM_002723915.3) using Primer 3 software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer sequences are shown in Table 6. The amplicon was performed using conventional PCR and confirmed by 1st BASE Company, Singapore. For qPCR, RNA was extracted from the cells using RNeasy mini kit (Qiagen, Hilden, Germany). Contaminated DNA was eliminated using DNA treatment (Promega). The cDNA was synthesized with the Superscript III first-strand synthesis system according to the manufacturer's protocol. cDNAs were amplified in a standard 40-cycle with KAPA SYBR® FAST qPCR Kits (KK4600) following manufacturer's instructions using ABI7300 machine. The reaction was performed in duplicate including 3 negative controls and with 3 measuring points (7.5, 13.8 and 15 ng). RefFinder (http://leonxie.esy.es/RefFinder/) was carried out to determine a housekeeping genes which are not affected by cell stages and treatment. RefFinder program integrates results from reliable programs such as geNorm (Vandesompele et al., 2002), Normfinder

(Andersen et al., 2004), BestKeeper (Pfaffl et al., 2004), and the comparative Delta Ct method (Silver et al., 2006) to compare and ranks the tested candidate reference genes using geometric mean of standard deviation.

Cardiac differentiation derived rabbit ESC

The cardiac differentiation was performed as previously described with minor modifications (Takei et al., 2009; Rungarunlert et al., 2013). Briefly, the EB at day 3 were plated on Petri-dish coated with 0.1% gelatin. On day 7 of cardiac differentiation, cells were collected and examined by RT-PCR for mesoderm and cardiac fate (*BT* and *NKX2.5*). To determine mesoderm differentiation in early stage, EB from cell line A B and C were collected on days 2 and 3 for BT expression using qPCR. Undifferentiated ESC were used as a control group. To study the effect of BMP-4 on mesoderm and cardiac differentiation, BMP-4 10 ng/ml were added in differentiation medium for 72 h, then the EB were collected every 24 h. The mesoderm marker BT was investigated at 48 h. The levels of mRNA expression of cardiac progenitor marker *NKX2.5* were investigated comparing to untreated group at day 3 and day 5 respectively by qPCR. The differentiating cells were stained immunelabeling with FlK1 at day 5 and troponin T (cTnT) at day 7. Efficiency BMP-4 induction was evaluated by the number of cells positive to Flk1 and cTnT in relation to total cell numbers, respectively. The examination was randomly performed at least six areas per slide.

Quantitative analysis of gene expression during cardiac differentiation

RNA was extracted from the cells using RNeasy mini kit (Qiagen, Hilden, Germany). Contaminated DNA was eliminated using DNA treatment (Promega, WI, USA). The cDNA was synthesized with the Superscript III first-strand synthesis system according to the manufacturer's protocol. In conventional PCR, cDNA was amplified by GoTaq® green master mix (Promega, WI, USA) and specific primers to detect the expression of each gene. The reactions were performed as follows: 2 min at 95 C for initial denaturation, followed by 35 cycles of 30 sec at 95 C, 30 sec at annealing temperature for each primer. The PCR products were separated using 1% agarose gels

(Bio-Rad, CA, USA) prepared in 100 ml 1×TBE buffer (90 mMTris, 90 mM boric acid, 2 mM EDTA, pH8) containing 5µl RedsafeTM (Intron biotechnology, Kyungki-Do, Korea). For relative quantification of cardiac genes, qPCR were performed in a standard 40-cycle with KAPA SYBR® FAST qPCR Kits (KK4600, Sigma-Aldrich, MO, USA) following manufacturer's instructions using ABI7300 machine. All primer sequences used for cardiac gene expression are shown in Table 7. The PCR products were confirmed by 1st BASE Company, Singapore.

Table 5 Antibodies used in this study

Markers	Primary antibody	Visualization
OCT 3/4	A goat polyclonal IgG, SC8628, Santa Cruz Biotechnology, TX, USA,1:100	A donkey anti-goat IgG-FITC, SC2024, Santa Cruz Biotechnology, TX, USA,1:200
SSEA-1	A mouse monoclonal anti-SSEA-1, MC-480 , ab16285, Abcam, Cambridge, UK, 1:200	A goat anti-mouse IgG-TRITC antibody, T5393, Sigma Aldrich, WI, USA, 1:200
GFAP	A mouse monoclonal anti- glial fibrillary acidic protein , 6F2, DAKO, 1:2400	Poly-HRP anti-mouse IgG (Leica Microsystems)
Vimentin	A mouse monoclonal anti-Vimentin, V9, CellMarque, CA, USA, 1:400	Poly-HRP anti-mouse IgG (Leica Microsystems)
ß-catenin	A mouse monoclonal anti- ß-catenin,14, CellMarque, CA, USA, 1:500	Poly-HRP anti-mouse IgG (Leica Microsystems)
Flk-1	A mouse monoclonal IgG, SC393163, Santa Cruz Biotechnology, TX, USA,1:100	A goat anti-mouse IgG-TRITC antibody, T5393, Sigma Aldrich, WI, USA, 1:200
cTnT (Troponin-T)	A mouse monoclonal IgG, ab33589, Abcam, Cambridge, UK, 1:100	A goat anti-mouse IgG-TRITC antibody, T5393, Sigma Aldrich, WI, USA, 1:200

Table 6 Primers used for normalization

	Forward (5'–3')	Reverse (5'–3')	Product size (bps)	Tm	Accession number or reference
RPL13a	CAAGCGGATGAACACCAACC	ATCCCGTCGAACACCTTGAG	147	55	XM_002723915.3
GAPDH	GGAGCCAAAAGGGTCATCATCTC	GAGGGCCCGTCCACGGTCTTCT	233	60	Lo et al.,2015

Table 7 Primers used in polymerase chain reaction (PCR)

	Forward (5'–3')	Reverse (5'–3')	Product size (bps)	Tm	Accession number or reference
OCT 3/4	CCTTCGCAGGGGGGCCTA	CATGGGGGAGCCCAGAGCA	161	55	Honda et al.,2010
NANOG	CACTGATGCCCGTGGTGCCC	AGCGGAGAGGCGGTGTCTGT	94	60	Osteil et al.,2013
SOX-2	AGCATGATGCAGGAGCAG	GGAGTGGGAGGAAGAGGT	270	55	XM_008266557.2
KLF4	TCCGGCAGGTGCCCCGAATA	CTCCGCCGCTCTCCAGGTCT	131	55	Osteil et al.,2013
GBX2	AACGCGTGAAGGCGGGCAAT	TGCTGGTGCTGGCTCCGAAT	118	55	Osteil et al.,2013
PAX6	GAACAGACACAGCCCTCACA	TCGTAACTCCGCCCATTCAC	160	55	NM_001082217.1
PITX2	AACCTTACAGAAGCCCGAGT	GGAAACTCTTGGTGGACAGC	217	55	XM_008267481.1
CTFR	CACAATTGAAAGCAGGTGGGA	GTTGCTGTGAGGTATGGAGG	224	55	NM_001082716.1
PECAM1	AGAGGAGCTGGAGCAGGTGTTAAT	GCTGATGTGGAACTTCGGAACAGA	145	55	Osteil et al.,2013
BT	GCAAAGGAAAGAAGCGACCAC	TGAGCGGAAGGCAGAGAGAGG	164	55	Thieme et al.,2012
GATA4	CGGCCTCTACCACAAGATGA	AGGTTCTTGGGCTTCCGTTT	252	55	XM_002709438.3
NKX2.5	GCAGATAAGAAAGAGCTGTGCG	GTACCGCTGCTGCTTGAAAC	165	55	XM_002710385.3

Statistical analysis

Data of EB diameters are represented as mean ± standard deviation (SD). The statistical differences among experimental groups were analyzed by one-way Analysis of Variance and Tukey's Multiple Comparison Test. Analysis of cardiac protein expressions was performed by Fisher's exact test. Percentage of positive cells ± SEM was plotted using GraphPad Prism 7 (https://www.graphpad.com/scientific-software/prism/). The difference with $P < 0.05$ was considered statistically significant. To study gene expression related cardiac gene by qPCR, statistical analysis of CT in each group was calculated by Relative Expression Software Tool (REST 2009, www.qiagen.com). P-value (P) < 0.05 was determined as statistical significance. The expression level ± SEM were plotted using GraphPad Prism 7.

Results

Establishment and characterization of rabbit ESC

After plating *in vivo* embryos (n=45) on feeder MEF around 7-14 days, outgrowth colonies (24, 53.3%) were observed and referred to ES-like cells passage 0. The colonies showed clear edge and contained small cells with high nuclear per cytoplasm ratio (Figure 11A). All cell lines (n=4) were positively stained with AP (Figure

14B). The pluripotent genes *KLF-4*, *SOX-2, OCT3/4* and *NANOG* were expressed in all cell lines (Figure 11D). The immunofluorescence also confirmed protein expression of pluripotent markers including OCT3/4 and SSEA-1 (Figure 11C). All ESC differentiated into three germ layers via embryoid body formation as confirmed by gene expression of *GBX2* and *PAX6* (ectoderm), *PECAM1* (mesoderm) and *PITX2* (endoderm) (Figure 11E). The ESC lines had normal karyotypes (n=44, Figure11 F-1 and F-2). They could be maintained in undifferentiated stage for at least 40 passages. In all cell lines, EB day 14 committed into three-germ layer structure as shown in Figure 11G. The immunohistochemical staining of EB presented protein expressions of ectoderm (GFAP), mesoderm (Vimentin) and endoderm (ß-catenin*)* markers.

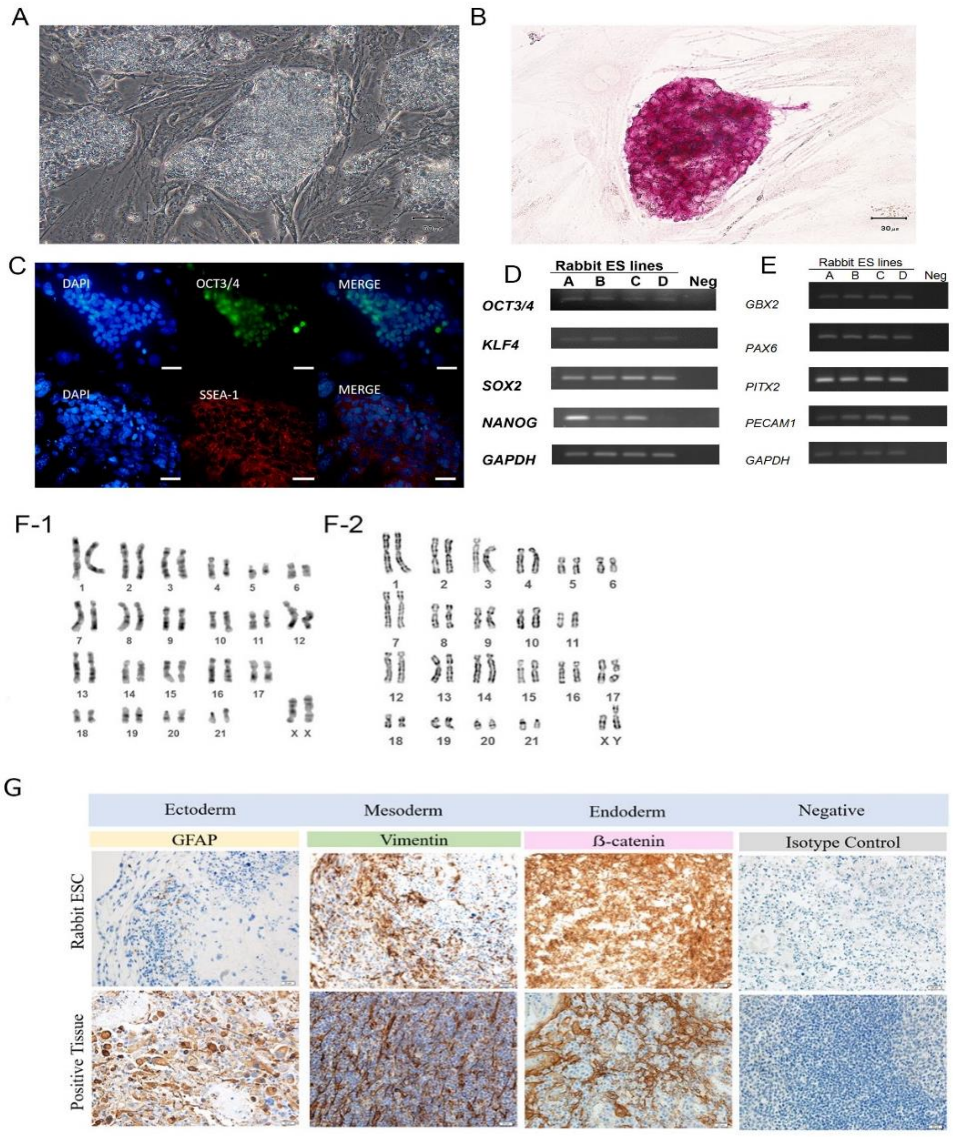

Figure 11 Characterization of rabbit ESC. (A) Rabbit ESC morphological appearance in phase contrast microscopy. ESC exhibits typical morphology of human ESC including a high nuclear per cytoplasmic ratio and flat shape. Scale bar represent 50 µm. (B) ES colonies were positive for alkaline phosphatase (AP) activity. Scale bar represent 30 µm. (C) The Rabbit ESC expressed pluripotency markers including OCT3/4 and SSEA-1. Scale bar represent 40 µm. (D) Expression of pluripotent genes by RT-PCR, *GAPDH* is used as internal control. (E) Expression of three germ layer marker genes, ectoderm (*PAX6, GBX2*), mesoderm (*PECAM1*) and endoderm (*PITX2*). GAPDH is used as internal control. (F-1, F-2) rabbit ESC exhibits a normal karyotype 44, XX and 44, XY. (G) Day 14

EB derived rabbit ESC were stained with antibodies against GFAP, vimentin and ß-catenin for ectoderm, mesoderm and endoderm. Scale bar represent 10 µm

Differentiation of cardiac cell fate via embryoid body formation

In the current study, EB were generated using three different numbers of cells (1,000 cells/EB, 2,000 cells/EB and 3,000 cells/EB). Around two day of culture in microplate coated with polyHEMA, the cells formed spherical EB with a regular shape and uniform size. The diameter were measured at day 3, mean diameter of 1,000 cells/EB in line A, B, C and D at day 3 were 151.41±2.93, 194±3, 183±5.74 and 147.68±2.02. Mean diameter of 2,000 cells/EB are 183.06±6.68, 260.39±3.34, 266.58±7.49 and 182.04±6.8. Mean diameter of 3,000 cells/EB are 203.59±11.65, 323.53±8.97, 337.1±11.77 and 217.49±3.68 in line A, B, C and D respectively. All the cell lines could develop into three-dimensional structure but the ability to form the EB was different among the cell seeding densities and particular cell lines. However, the higher cell seeding (2,000 cells/EB and 3,000 cells/EB) generated larger EB significantly when the diameters were analyzed in the same cell line ($P<0.05$). Comparing diameter difference among cell lines, ESC line B and C gave rise larger EB size than the other two lines ($P<0.05$) at the same seeding cell density. The average of EB diameter in all cell lines was 170.25±3.76, 220.49±7.6 and 259.76±10.62. The results indicated that seeding density and cell lines influenced the formation of size derived rabbit ESC. We selected the seeding cell at 3,000 cell/EB for next study since it generated the largest EB in all cell lines ($P<0.05$). To investigate cardiac fate in rabbit ESC, RT-PCR were used. RNA was extracted from plated EB on day 7. All the cell lines used in this study had ability of cardiac differentiation as cardiac marker genes (*BT, GATA4* and *NKX2.5)* were expressed (Figure 13B). The nucleotide identity of the rabbit cardiac genes was highly similar when compared with other species (Table 9).

Normalization of ESC and differentiating cells

Following analysis of gene expression using different software, Delta CT method resulted in similar averages of standard deviation of both genes (Figure 12B). This

finding was similar for the results obtained from Normfinder and geNorm methods. These methods emonstrated equal stability values of 2.633 and 1.317 for *GAPDH* and *RPL13a, respectively* (Figure 12D and 12E) respectively. However, BestKeeper showed standard deviation of 1.31 and1.37 for *RPL13a* and for *GAPD*H (Figure 12C). From the results obtained from all software, the overall ranking of geometric means of standard deviation or stability values resulting from integrated data using RefFinder software were 1.414 and 1.189 for *RPL13a* and *GAPDH*, respectively (Figure 12A). This indicated that *GAPDH* expression during EB culture was more stable compared with *RPL13a*, thereby indicating the suitability of GAPDH for gene normalization (housekeeping gene) over *RPL13a*.

Table 8 the ranking order from RefFinder program

	Ranking Order (Better--Good--Average)	
Methods	1	2
geNorm	*GAPDH	RPL13a*	
Normfinder	*GAPDH*	*RPL13a*
BestKeeper	*RPL13a*	*GAPDH*
Delta Ct	*GAPDH*	*RPL13a*
Recommended comprehensive ranking	*GAPDH*	*RPL13a*

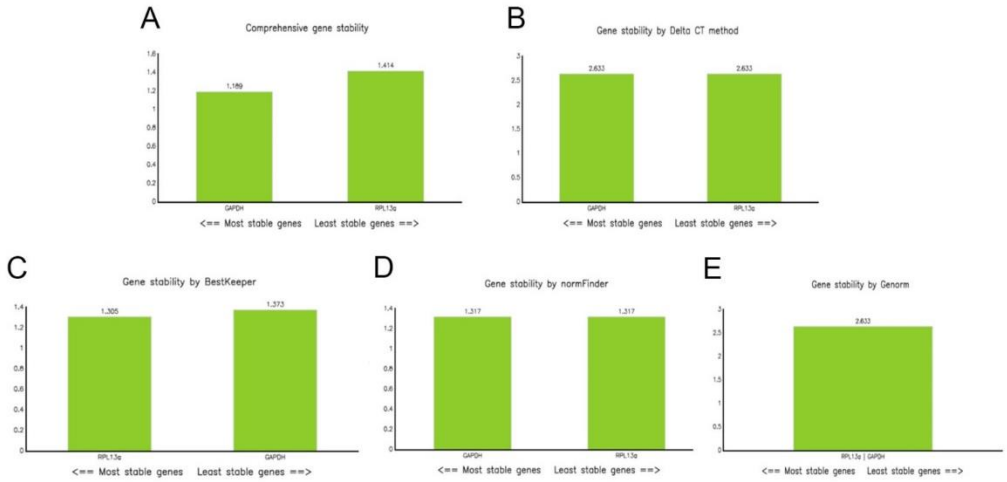

Figure 12 (A) Result of gene expression stability of candidate reference genes analyzed by RefFinder program using geometric mean of standard deviation or stability values from (B) Delta Ct. method (C) BestKeeper (D) Normfinder and (E) geNorm

Quantitative analysis of cardiac gene expression with emphasizing on BMP-4 treatment

Spontaneous cardiac mesoderm differentiation of rabbit embryonic stem cells was examined on day 2 and 3 of EB culture using quantitative RT-PCR (Figure 13A) in order to study the levels of *BT* genes. The results indicated that the expression levels of BT gene were significantly elevated at early stage of differentiation compared with undifferentiated ESC (day 0). This expression remained high on day 3 of differentiation. However, the expression levels decreased when compared with day 2.

To test whether or not BMP-4 promoted cardiac fate in rabbit ESC, BMP-4 at a concentration of 10 ng/ml was supplemented into EB medium. The results demonstrated that mesodermal marker *BT* was significantly increased after treatment BMP-4 for 48 h (Figure 14A, $P<0.05$) when compared with the control (without BMP-4, spontaneous differentiation). Similarly, the cardiac progenitor gene (*Nkx2.5*) was also significantly upregulated in BMP-4 treated EB (Figure 14B, $P<0.05$). The BMP-4 treatment also significantly affected to the numbers of Flk1 51.76±2.74%, Figure 14C), and NKX2.5 (30.1±9.25%, Figure 14D) (cardiac progenitor marker) positive cells compared with the

controls (30.96±1.5% and 8.33±5.89%, respectively). Although we did not observe beating area of cardiomyocytes, cardiac troponin-T positive cells (22.79±3.97%, Figure 14E) significantly increased in BMP-4 treated cells compared with control group (8.63±3.12%, P<0.05).

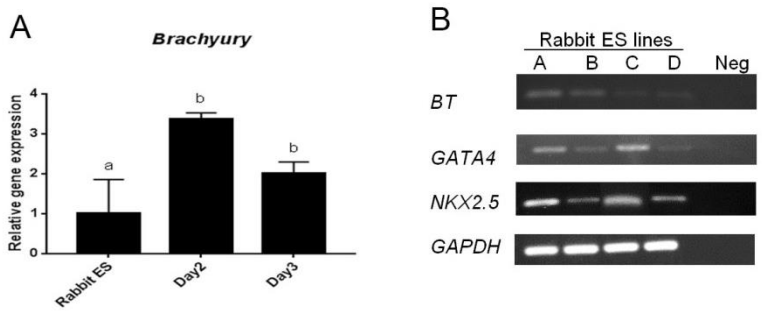

Figure 13 EB formation promotes cardiac differentiation in 15% serum condition (A) Brachyury expression of rabbit EB in vitro at day2-day3. (B) Cardiac marker expression at day 7 of spontaneous differentiating embryoid bodies derived from rabbit ESC by RT-PCR.

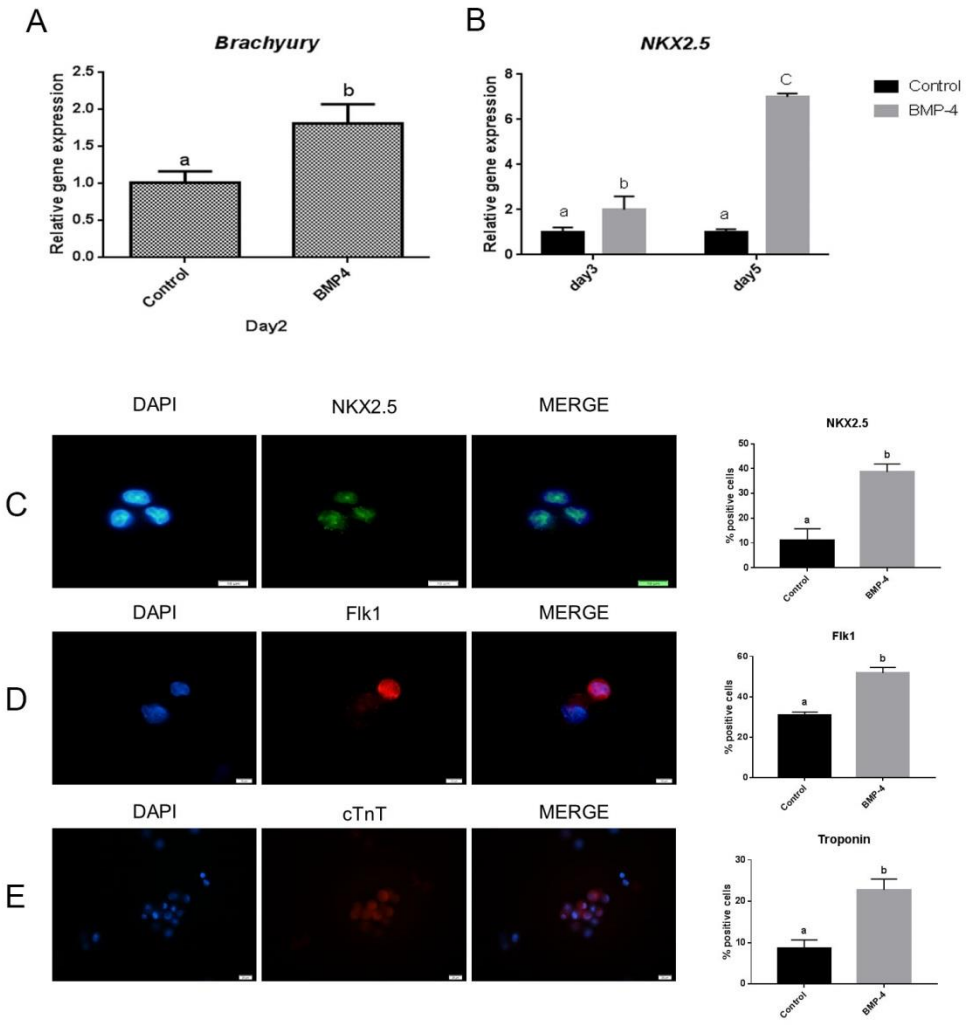

Figure 14 BMP4 promotes cardiac differentiation. BMP-4 up-regulated mRNA level of (A) mesoderm marker *Brachyury* at day2 and (B) cardiac marker *NKX2.5* at day 3 and day 5. It also increased cardiac marker positive cells, NKX2.5 (C) and Flk1 (D) at day 5 and troponin-T (cTnT) at day 7. Scale bars represent (C, D) 10 μm and (E) 20 μm.

Table 9 Sequence alignments of Brachyury, NKX2.5 and GATA4 amplicon products derived mRNA comparing previous reports in GenBank

Species	**Genbank**	**nucleotide identity**
Brachyury		
Rabbit (Oryctolagus cuniculus)	XM_002714985.2	98%
Mouse	XM_004597546.1	91%
Ferret (Mustela putorius)	XM_004769576.1	85%
NKX2.5 **Product length 165 Bps**		
Rabbit (Oryctolagus cuniculus)	XM_002710385.3	90%
Elephant (Loxodonta africana)	XM_003404889.2	90%
Cat (Felis catus)	XM_003981259.3	90%
Human (Homo sapiens)	NM_004387.3	90%
Pig (Sus scrofa)	XM_003134041.1	89%
GATA4 **Product length 252 Bps**		
Rabbit (Oryctolagus cuniculus)	XM_002709438.3	99%
Cat (Felis catus)	XM_011281564.2	95%
Porcine (Sus scrofa)	XM_013990299.1	94%
Leopard (Panthera pardus)	XM_019440945.1	95%

Discussion

In this study, we examined differentiation potentials of rabbit ESC into cardiac cell fates. This development is important for further study in using rabbit as a model for cardiovascular disorders in human. Although several attempts have been made to generate rabbit ESC, the knowledge on factors influencing on *in vitro* cardiac is currently poor. All the rabbit ESC (4 cell lines) were maintained in pluripotent stage using culture medium containing leukemia inhibitory factor and also basic fibroblast growth factors. This culture condition was, however, different from human and mouse (Daheron et al., 2004; Dvorak and Hampl, 2005; Eiselleova et al., 2009; Hirai et al., 2011; Onishi and Zandstra, 2015) suggesting the difference in pluripotency signaling pathways

among species (Daheron et al., 2004; Dvorak and Hampl, 2005; Eiselleova et al., 2009). In fact, several techniques have been used to test pluripotency. In the current study, we used *in vitro* assay primarily by endogenously pluripotent gene expression and embryoid body formation as this technique allows assessing three germ layers formation with less time consuming. Although *in vivo* pluripotency assay by mean of teratoma test has yet to be performed, this study has met a limit assess immunodeficiency mouse. We found in the current study that the ESC spontaneously expressed cardiac mesoderm (*BT)* and progenitor cells *(NKX2.5, GATA4)* indicating that the rabbit ESC used in this study had capability to differentiate toward cardiac lineage. In order to efficiently induce cardiac differentiation via embryoid body, it previously suggested that the day of EB plating should depend on the optimal cell density and expression levels of cardiac mesoderm during early phase of embryoid body culture (Hwang et al., 2009; Choi et al., 2010; Mohr et al., 2010; Rungarunlert et al., 2013). However, it is worth to note that particular species previously reported to have difference in cardiac gene expression during cardiac differentiation (Graichen et al., 2008; Pekkanen-Mattila et al., 2010). It would be therefore necessary to determine the levels of cardiac gene expression in rabbit in order to maximize the possibility of cardiac differentiation. We firstly examined the expression of housekeeping genes since this is essentially critical for determine the level of gene expression. This study firstly identified using different software programs that the two genes (*RpL13a* and *GAPDH*) were stably expressed as similar to other reports (Murphy and Polak, 2002; Barber et al., 2005; Bian et al., 2015; Gentile et al., 2016) . However, the stability values of *GAPDH* during culture of rabbit embryoid body was better compared with *RpL13a.* This study therefore used this housekeeping gene for further study. Our results demonstrated that expression of cardiac mesoderm BT significantly upregulated compared with rabbit ESC and reached its maximal levels on day 2 after spontaneous cardiac differentiation via EB formation. The result obtained was different compared with mouse in that the level of Brachyury was reported to peak on day 3 of cardiac differentiation (Rungarunlert et al., 2013; Lolas et al., 2014). In general, the upregulate expression of BT gene commit at early period of EB development indicated the transition from ground state pluripotency toward mesoderm (Lolas et al., 2014; Beisaw et al., 2017). The EBs in this

study were plated a day later corresponding with the levels of BT expression of rabbit EB decreased by day 3 of differentiation. It is possible that the differentiation proceeded beyond cardiac mesoderm (Kouskoff et al., 2005; Evseenko et al., 2010). In addition, our study also found that supplement of BMP-4 into EB culture medium, significantly increased the levels of BT expression when compared with control (no BMP-4 treatment. BMP-4 plays a central role in regulation of mesoderm formation and cardiogenesis (Lev et al., 2005; Kattman et al., 2011; Rajala et al., 2011; Kim et al., 2015). It induced early cardiac marker *NKX2.5* and *GATA4* via activation of MAP kinase kinase kinase Tak1 and Smad signaling (Monzen et al., 2001; Harvey, 2002; Lev et al., 2005) and improved *in vitro* cardiac differentiation both in human and mouse (Kattman et al., 2011). The increased expression of BT after BMP-4 treatment coincided with the increase in Flk1 and troponin-T positive cells as well as percentage of beating area compared with control group. These findings as well as previous reports strongly indicate the importance of BMP-4 signaling on *in vitro* cardiogenesis. In the current study, although our study firstly demonstrated the possibility to differentiate rabbit ESCs towards cardiac lineages, the numbers of mature cardiomyocytes in terms of cardiac troponin positive cells and beating cells were unfortunately poor. The reason for this remain unclear since several factors have been demonstrated to encounter the success of *in vitro* cardiac differentiation such as culture medium, growth factors, species and types of stem cells (Burridge et al., 2007; Hwang et al., 2009; Mohr et al., 2010; Dierickx et al., 2012; Mummery et al., 2012). These factors should therefore be further studied in order to improve cardiac differentiation in rabbit model.

Conclusions

We have demonstrated that rabbit ESC have capacity to differentiate toward cardiac fate. BMP-4 additionally promoted expressions of cardiac mesoderm *BT* and cardiac progenitor *NKX2.5.* The cardiac proteins at cardiac progenitor and mature cells were also increased in BMP-4 treated embyoid body when compared with controls. However, this technology remained inefficiency in rabbit model and further studys to determine factors influencing cardiac cell fate *in vitro* are essentially required.

Chapter III

Rabbit induced pluripotent stem cells retain capability of *in vitro* cardiac differentiation

Introduction

Induced pluripotent stem cells (iPSC) as well as embryonic stem cells (ESC) are pluripotent stem cells (PSC) that have unlimited self-renewal and capability to differentiate into all three germ layers and their derivatives (Takahashi et al., 2007). The iPSC are expected cell source for cell replacement therapy in several diseases including cardiac malfunction. Successful transplantation of cardiomyocyte-like cells derived from iPSC has been demonstrated to improve cardiac structure and electrophysiological functions in small rodent models (Nerbonne et al., 2001; Laflamme et al., 2007). These rat models, however, have short lifespan and different cardio-physiology comparing with human. Therefore, translational knowledge from these species to human application is rather difficult (Peng, 2012). Rabbit is middle-sized animal model that is commonly used to study cardiovascular diseases, especially atherosclerosis and myocardial abnormalities as molecular mechanisms in cardiac diseases are closely similar to human (Fan and Watanabe, 2003; Pogwizd and Bers, 2008). However, information on generation of rabbit iPSC has been limited as only few laboratories have demonstrated the possibility on establishment of rabbit iPSC. In addition, there is no information on *in vitro* cardiac differentiation in rabbit. BMP-4 has been used to promote differentiation of pluripotent stem cells into cardiac cell lineage (Kattman et al., 2011). The BMP-4 induces mesoderm formation via ERK pathway and up-regulates the mesoderm markers (*Brachyury* and *Fetal liver kinase 1*)(Bernardo et al., 2011; Kattman et al., 2011). Fetal liver kinase 1 (Flk1), an early receptor tyrosine kinase, is useful surface marker for determining mesodermal cells (Dumont et al., 1995; Motoike et al., 2003; Hirata et al., 2007; Yang et al., 2008). $Flk1^{+}$ cells derived from pluripotent cells could develop into cardiomyocyte, hematopoietic and endothelial cells(Nishikawa et al., 1998; Ogawa et al., 1999; Iida et al., 2005; Kattman et al., 2006). Furthermore, the BMP-4 also promotes gene expressions of cardiac progenitors (*NKX*

2.5 and *GATA 4*) (Sachinidis et al., 2003) and enhances cardiac differentiation via MAP kinase, Tak1 and Smad family(Takei et al., 2009). The action of BMP-4 to drive mesodermal differentiation of cardiac lineage can be efficiently promoted by three-dimension cell aggregation via embryoid body (EB) formation (Kehat et al., 2001). It has been reported in mouse that relatively large EB size (around 450 μm) promoted cardiac differentiation better than smaller size EB (150 μm)(Hwang et al., 2009). However, the effects of cell seeding density and EB size in relation to cellular aggregation (EB formation) and cardiac differentiation have yet to be studied in rabbit model. In this study, we aimed at establishing induced pluripotent stem cells in rabbit and examined *in vitro* differentiation of rabbit iPSC toward cardiac lineage.

Materials and Methods

Reagents and animals

All chemicals were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA), otherwise indicated. ICR mice and New Zealand White rabbits were purchased from the National Laboratory Animal Center (Mahidol University, Thailand). BALB/c nude mice were purchased from Nomura Siam International Co, Ltd. (Bangkok, Thailand). Animal maintenance, care, and use procedures were performed according to the Animal Ethics Approval of Chulalongkorn University (N0.1673036).

Generation of rabbit induced pluripotent stem cells

The plasmids for retrovirus vectors were purchased from Addgene (www.addgene.com); pMXs-hOct3/4 (Cat# 17217), pMXs-hSox2 (Cat# 17218), pMXs-hKlf4 (Cat# 17219) and pMXs-hc-MYC (Cat# 17220). The virus was produced using pMXs-vector (16 μg) and pVSV-G (4 μg) in 293GP cells by X-tremeGENE Reagents (Roche, Mannheim, Germany) according to manufacturer's instructions. Rabbit embryonic fibroblasts (REFs) were transfected twice with retrovirus in the presence of 4 μg/ml polybrene (Sigma Aldrich, WI, USA). The transfected REFs were dissociated and seeded at a density of 1,000 cells per cm^2 on mitomycin inactivated MEFs (mouse embryonic fibroblast). The iPSC medium was composed of DMEM/F12 containing 20% (v/v) KnockOut serum replacement (KSR), 1 mM L-glutamine, 1% (v/v) non-essential

amino acids (NEAA), 0.1 mM β-mercaptoethanol, 1000 IU/ mL Leukemia inhibitory factor (LIF, Millipore, CA , USA) and 10 ng/ml basic fibroblast growth factor (bFGF, R&D Systems, MN, USA). The induced pluripotent stem (iPS) cell-like colonies were observed on day 7–21 post-transduction. The iPS primary colonies were examined under a phase contrast microscope (Olympus, Shinjuku, Japan). The iPSC were continuously subcultured by enzyme (Tryple ™ Select). In all cases, culture condition was performed at 37℃ in a humidified condition of 5% CO_2 in atmosphere. To determinate of reprogramming efficiency (RE), transfected REFs were passaged and seeded at a density of 600 cells/cm^2. Total primary colonies (larger than 100 μm) were examined for alkaline phosphatase activity (ALP) and counted on day 7 after reprograming. RE was calculated by the following formula.

$$RE = \frac{primary\ colonies\ X\ 100}{total\ of\ transfected\ seeding\ cells}$$

To evaluate the percentage of rabbit iPSC line establishment (% riPSCL). Ten colonies derived transfected REFs were selected randomly for iPSC establishment and characterization. % riPSCL was calculated by the following formula.

$$\%\ riPSCL = \frac{a\ number\ of\ cell\ lines\ X100}{a\ number\ of\ selected\ colonies}$$

Karyotyping and G-banding

Rabbit iPSC were disassociated and centrifuged at 200 *× g* for 5 min. The cell pellet was incubated at 37 ℃ for 20 min in 0.075 M KCl. The cells were washed twice and fixed with a mixture of acetic and methanol (1:3) on ice. They were dropped vertically onto a glass slides and stained with 10% (v/v) Giemsa solution. Numbers of chromosome from at least 20 metaphase spreads were evaluated under a light microscope. For g-banding, the slides containing metaphase spreads were aged for at least 1 week, then the chromosomes were partially digested with 0.05% Trypsin-EDTA, stained with Giemsa and analyzed under a light microscope.

Reverse transcriptase polymerase chain reaction (RT-PCR)

The iPSC and differentiated cells were sampled and stored at -80 ℃ prior to analysis. RNA was extracted using an RNeasy Mini Kit (Qiagen). The amount of RNA and purity were measured by Nanodrop 2000 spectrophotometer (ThermoFisher Scientific, DE, USA). DNase I (Promega, WI, USA) was used to eliminate contaminated DNA. cDNA synthesis (RT+) was performed using SuperScript III Kit (Invitrogen) according to the manufacturer's instructions. Negative control (RT-) was performed as described above without superscript III reagents. cDNA was performed using the specific primers listed in table 10. The PCR cycles were as follows: initialization at 95 ℃ for 2 min, followed by 30 PCR cycles of denaturation at 95 ℃ for 30 s, annealing step at 55-64 ℃ for 30 s and extension step at 72 ℃ for 30 s. To determine the downregulation, the presence of exogenous genes (hOCT3/4, hKlf4, hSOX2 and hc-MyC) was investigated in REF and rabbit iPSC line R1, R2 and R3 at passage 17 using RT-PCR analysis. This was performed simultaneously with the expression of endogenously rabbit pluripotent genes (OCT3/4 and NANOG). Mixture of extracted plasmid (pMXs-hOCT3/4 , pMXs-hSox2, pMXs-hKlf4 and pMXs-hc-MYC) were served as positive control in study of exogenous expression.

Table 10 Primers used in polymerase chain reaction (PCR) in this study

	Forward (5'–3')	Reverse (5'–3')	Product size (bps)	Tm	Accession number or reference
OCT 3/4	CCTTCGCAGGGGGGCCTA	CATGGGGGAGCCCAGAGCA	161	55	Honda et al.,2010
NANOG	CACTGATGCCCGTGGTGCCC	AGCGGAGAGGCGGTGTCTGT	94	60	Osteil et al.,2013
SOX-2	AGCATGATGCAGGAGCAG	GGAGTGGGAGGAAGAGGT	270	55	XM_008266557.2
KLF4	TCCGGCAGGTGCCCCGAATA	CTCCGCCGCTCTCCAGGTCT	131	55	Osteil et al.,2013
GBX2	AACGCGTGAAGGCGGGCAAT	TGCTGGTGCTGGCTCCGAAT	118	55	Osteil et al.,2013
PAX6	GAACAGACACAGCCCTCACA	TCGTAACTCCGCCCATTCAC	160	55	NM_001082217.1
PITX2	AACCTTACAGAAGCCCGAGT	GGAAACTCTTGGTGGACAGC	217	55	XM_008267481.1
CFTR	CACAATTGAAAGCAGGTGGGA	GTTGCTGTGAGGTATGGAGG	225	55	NM_001082716.1
PECAM1	AGAGGAGCTGGAGCAGGTGTTAAT	GCTGATGTGGAACTTCGGAACAGA	145	55	Osteil et al.,2013
α-actinin	CCATATAAGCTGGAAGGACG	GTACTTCTCTGCCACATCAA	139	55	XM_002719521.3
RYR2	GAGCAACGGAGGACTGTTCA	TGACGTAGTCGGAATGGCTG	134	55	NM_001082757.1
hOCT3/4	GTTGCTCTCCACCCCGACTCCTGCTTC	GAGAACCGAGTGAGAGGCAAC	376	60	Chakritbudsabong et al., 2017
hSOX2	CCAGATCCCGCACAAGAGTT	CAAGAGGCGAACACACAACG	264	60	Takahashi et al., 2007
hKlf4	GGCTGATGGGCAAGTTCG	CTGATCGGGCAGGAAGGAT	416	60	Chakritbudsabong et al., 2017
hc-Myc	GCAGCGACTCTGAGGAGGAACAA	TTTTCCTTACGCACAAGAGTTCCGT	581	60	Chakritbudsabong et al., 2018
GAPDH	TGGTGAAGGTCGGAGTGAAC	ATGTAGTGGAGGTCAATGAATGG	121	55	NM_001082253.1

Alkaline phosphatase and immunofluorescent staining

The cells were washed with phosphate buffered saline (PBS) and then fixed with 4% (w/v) paraformaldehyde (PFA) for 15 min. Alkaline phosphatase (ALP) activity was tested using Alkaline Phosphatase Kit (Sigma-Aldrich, MO, USA) following the manufacturer's instructions. The pink-to-red colored colonies were classified as positive to ALP activity. To investigate protein expression, the cells were passaged onto a cover slip and then fixed with 4% (w/v) PFA. The cells were permeabilized if necessary in mixture of 0.1% Triton X-100, 2% bovine serum albumin (BSA) in PBS and the non-specific binding was blocked with 2% BSA. The cells were incubated at 4 °C with primary antibodies overnight. The primary antibodies in this study included OCT-3/4 (SC8628, Santa Cruz Biotechnology, TX, USA, 1:100), SSEA-4 (ab16287, Abcam, Cambridge, UK, 1:50), Flk1 (SC393163, Santa Cruz Biotechnology, 1:100) and cTnT (troponin T, ab33589, Abcam, 1:100). The samples were then stained with secondary antibody corresponding to the primary antibodies used. The 4', 6'-diamidino-2-phenylindole (DAPI) in mounting medium (VECTASHIELD® Mounting Medium, Vector Laboratories, CA, USA) was used to visualize the nucleus. The negative control was performed as described above without primary antibody.

In vitro differentiation

Differentiation was performed using a hanging drop technique in order to promote cell aggregation into three-dimension structure referred as embryoid bodies (EB). The iPSC were dissociated and seeded in each culture drop (20 µl) at the density 20,000 cells in DMEM/F12 medium containing 15% (v/v) FBS. To examine the *in vitro* differentiation, there different techniques were used. Firstly, we investigated the presence of endogenous transduced pluripotent genes (*OCT3/4, NANOG, KLF4* and *SOX2*) in the cell lines after EB formation for 2 and 7 days. Secondly, gene expressions of three-germ differentiation were additionally examined on day 7 of EB plating using RT-PCR. These included the expressions of ectoderm (*PAX6* and *GBX2*), mesoderm (*PECAM1*) and endoderm (*PITX2* and *CFTR*). The presence of proteins associated with three-germ layer differentiation was demonstrated by immunohistochemistry (Leica Microsystems BOND-MAX System). In brief, the EB (day 14 of culture) were fixed with

4% (w/v) paraformaldehyde. They were embedded in paraffin and cut at a thickness of 4 μm. The slides were incubated with Bond Dewax Solution (Leica Microsystems) for 60 min at 60 °C. The epitopes of the antigens were retrieved with Bond Epitope Retrieval Solution 2 (Leica Microsystems) for 30 min at 100° C. The slides were separately incubated with primary antibodies including mouse monoclonal anti- glial fibrillary acidic protein (anti- GFAP, 6F2, DAKO, 1:2400), anti-Vimentin (V9, CellMarque, CA, USA, 1:400) and anti-ß-catenin (14, CellMarque, CA, USA, 1:500) at 25° C for 40 min and followed by 3 consecutive rinses with a Bond Wash Solution (Leica Microsystems). Hydrogen peroxide (3%) was then applied for 5 min and rinsed 3 times. Post primary polymer (Leica Microsystems) were applied for 8 min. The slides were washed, followed with Poly-HRP IgG (Leica Microsystems) for 8 min, and rinsed 3 times. The diaminobenzidine chromogen was applied for 4 min followed by 3 deionized water rinses. Slides were counterstained with hematoxylin for 5 min. Isotype Mouse IgG1, kappa monoclonal (ab91353, Abcam, Cambridge, UK) were used instead of the primary antibody for the negative control. Brain tumor, appendix and tonsil were used as positive controls for GFAP, vimentin and ß-catenin, respectively. . A fluorescent microscope (BXx5, Olympus) and DP2-BSW software were used for visualization and record the samples.

Teratoma formation

To generate teratomas, 5× 10^6 of rabbit iPSC lines (R2 and R3) were subcutaneously injected into six 8-week-old BALB/c nude mice (3 mice per cell line). Around 6-8 weeks after transplantation, the teratomas were observed and dissected. The masses were fixed in 4% (w/v) paraformaldehyde. The samples were embedded in paraffin and cut at a thickness of 4 μm. The samples were deparaffinized and stained with hematoxylin and eosin (HE staining). The slides were examined under a light microscope by an experienced pathologist.

Cardiac differentiation

The protocol for cardiac differentiation via hanging drop technique was performed as previously described (Rungarunlert et al., 2013) with minor modification. Briefly, Briefly, iPSC lines R1, R2 and R3 (passage 22-25) were dissociated and allowed

to aggregate into three-dimension in EB medium which was composed of DMEM/F12 medium supplemented 1 mM L- glutamine, 1% (v/ v) NEAA, 0. 1 mM β-mercaptoethanol, BMP-4 (10 ng/ml) and 15% (v/v) FBS (HyClone™, Utah, USA). To optimize for cardiac differentiation, hanging drop technique was performed using different cell density of 1,000, 3,000, 5,000, 10,000 and 20,000 cells per droplet (day 0). The EB were harvested from hanging drop on day 2 and cultured as suspension. Hanging drop technique was performed for cell density of 1,000, 3,000, 5,000, 10,000 and 20,000 cells per droplet (day 0). An Olympus CKX41 inverted microscope was used for phase-contrast imaging of EB at 72 h post EB culture. The cross-sectional diameters of EB were measured by ImageJ (https://imagej.nih.gov/ij/). For further examination of cardiac differentiation, optimal starting cell seeding density were selected. Hanging drop was performed with EB medium combined BMP-4 treatment. The EB were harvested from hanging drop into suspension with 10 ng/ml BMP-4 treatment on day 2. On day 3, the EB were further plated onto gelatin coated dish or coverslip with EB medium without BMP-4. On day 5 of differentiation, the plating EB on gelatin were digested using TrypLE™ Select (1X) into small clump or single cells, and the cells were counted for cardiovascular progenitor surface marker, Flk1. The proportion of Flk1 positive cells were evaluated by the number of cells positive to Flk1 in relation to the total cell numbers (at least 100 cells, three independent experiments of each cell line). To study mature cardiomyocyte marker (cTnT) plating EB on day 14 of iPSC cell lines R1, R2 and R3 were used. The plating EB on gelatin coated dishes were digested were counted for cardiomyocyte marker cTnT (at least 100 cells, four independent experiments of each cell line). The immunolabeling for Flk1 and cTnT was performed as previously described. The cells positive cTnT on coverslip were photographed using a fluorescent microscope (BXx5, Olympus) and DP2-BSW software. In addition, cardiac gene (cardiac ryanodine receptors (RyR2), α-actinin and PECAM1) were studied in all cell lines using the same protocols. Briefly, the plating EB on day 14 were mechanically harvest form gelatin coated dishes. The mRNA was extracted and RT-PCR analysis were performed as previously described. The differentiated cells were observed daily and the remaining of differentiated cells besides harvested samples in all experiments were

observed for cardiac beating area until day 21. The medium was changed in plating EB every 2-3 days until harvest. Statistical Analysis

Data of EB diameters are represented as mean ± standard deviation (SD). Data for Flk1 and cTnT positive cells are represented as mean ± standard error of the mean (SEM). The statistical differences among experimental groups were analyzed by one-way Analysis of Variance and Tukey's Multiple Comparison Test analysis using GraphPad Prism (www.graphpad.com). *P* value less than 0.05 ($P<0.05$) was considered statistically significant.

Results

Several primary colonies were observed as soon as 4 days post transfection. The reprogramming efficiency calculating from number of transfected fibroblasts that gave rise to ALP positive colonies was 0.191%. Three cell lines were established (referred to as R1, R2 and R3 cell lines) and the percentage of riPSCL was 30%. These cell lines maintained ES-like morphology with positive ALP staining for over 35 passages without losing their proliferative activity. Rabbit iPSC colonies demonstrated distinct boundary between the colonies and feeder cells (Figure 15A). The colonies contained iPSC having high nuclear per cytoplasm ratio and prominent nucleoli. The colonies were strongly positive to ALP (Figure 15B) and to OCT3/4 and SSEA-4 proteins (Figure 15C and 15D). RT-PCR also indicated that they endogenously expressed pluripotent genes (*OCT3/4, SOX-2, KLF-4* and *NANOG*, Figure 15F). Karyotyping and G-banding analysis revealed that the cell lines had normal chromosome numbers (n=44, Figure 15G). Human endogenous genes (*hOCT3/4, hKlf4, hSOX2* and *hc-Myc*) were absent in all rabbit iPSC cell lines (R1, R2 and R3), while the endogenous pluripotent genes *OCT3/4* and *NANOG* were presented (Figure 15E). All rabbit iPSC lines could form 3-dimension structure by mean of embryoid body formation (Figure 16A). This property of the rabbit iPSC cell lines coincided with the down regulation of pluripotent genes (*OCT3/4, NANOG, KLF4* and *SOX2*). *NANOG* expression was completely downregulated by day 2 of EB formation, while *KLF4*, *SOX2* and *OCT3/4* were still expressed (Figure 16C). Although *KLF4 and SOX2* genes were continuously expressed on day 7 of EB culture, the expression of *OCT3/ 4* gene was abolished at this time point.

Simultaneously, the EB culture led to the differentiation of rabbit iPS cells indicating by the expressions of ectodermal (*GBX2, PAX6*), mesodermal (*PECAM1*) and endodermal markers (*PITX2, CFTR*) (Figure 16B). Furthermore, the culture of EB for 14 days also resulted in the differentiation of the iPS cells into three-germ layer structure as shown in Figure 16D. The immunohistochemistry of EB revealed the presences of protein expressions of ectoderm (GFAP), mesoderm (Vimentin) and endoderm (ß-catenin*)* markers in all cell lines (Figure 16D).

Two rabbit iPS cell lines (R2 and R3) were used to demonstrate the capability of *in vivo* differentiation. These two cell lines were capable of *in vivo* differentiation by mean of teratoma formation after cell transplantation into immunocompromised mice. However, the R3 cell line had greater incidence of teratoma formation (2/3, 66.67%) when compared with the R2 cell line (1/3, 33.33%). The histological findings after the haematoxylin and eosin *staining confirmed the structures of teratoma that derived from three-germ layers of origin including epidermis-like (ectoderm), cartilage-like (mesoderm) and gland-like (endoderm) structures* (Figure 16E).

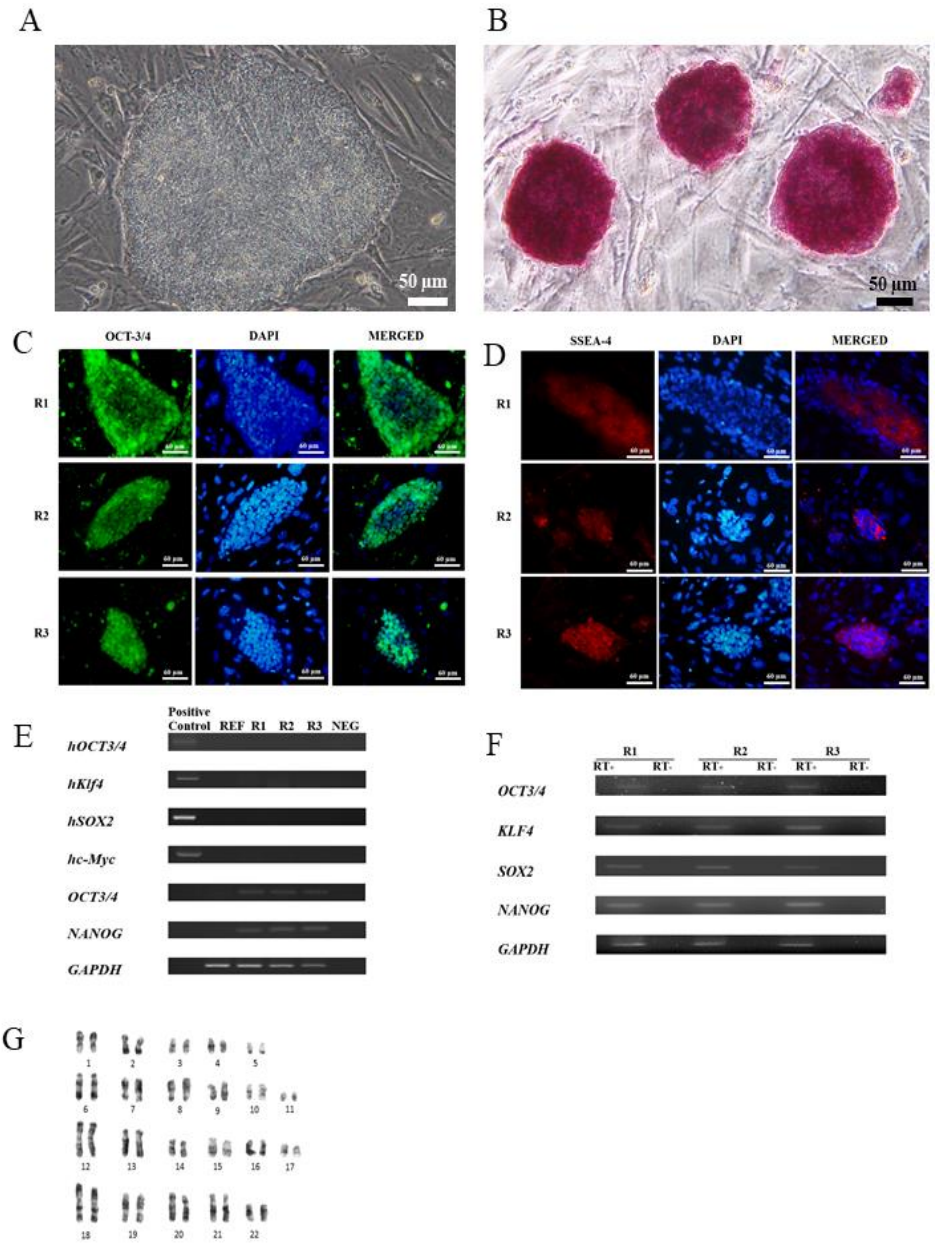

Figure 15 Characterization of rabbit iPSC (A) the colony morphology of rabbit iPSC line R3 at passage 18 (B) ALP staining of rabbit iPSC line R3 at passage 18 (C) The rabbit iPSC were positive stained with OCT-3/4 (green) located in nucleus and co-staining with DAPI (blue) . Scale bar represents 60 µm. (D) The rabbit iPSC were positive stained with stage specific embryonic antigen-4 (SSEA-4) at cell membrane, nucleus were stained with DAPI (blue). Scale bar represents 60 µm. (E) Absence of expression of exogenous pluripotent genes (*hOCT-3/4, hSOX-2, hKLF-4* and *hc-Myc*) in rabbit embryonic fibroblasts (REF) and rabbit iPSC line R1, R2 and R3 at passage 17. Mixture

of extracted plasmid were served as positive control. (F) Expression (RT+) of endogenous pluripotent genes (*OCT-3/4, SOX-2, NANOG* and *KLF-4*) in rabbit iPSC line R1, R2 and R3 at passage 22. PCR without superscript III reagents (RT-) was performed as negative control. (G) G-banding of rabbit iPSC R2 at passage 22

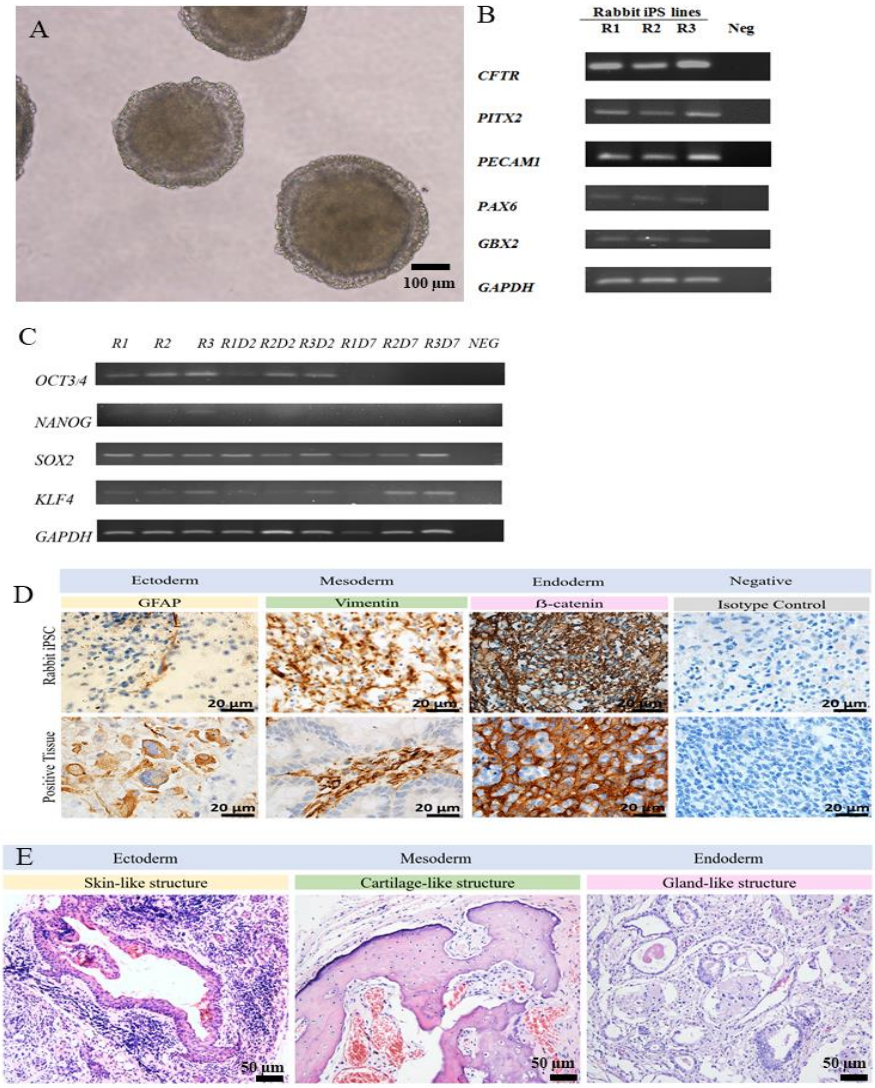

Figure 16 *In vitro* differentiation in rabbit pluripotent cells (A) Representative image of embryoid bodies derived from 20,000 cell density starting at day 3 in DMEM/F-12 containing 15% FBS. Scale bar represents 100 µm. (B) Gene expression of three germ layers; *CFTR* and *PITX2* (endoderm), *PECAM1* (mesoderm) and *PAX6* and *GBX2* (ectoderm) in day 7 EB derived from rabbit iPSC line R1 R2 and R3 at passage 22. (C) Pluripotent genes in EB day 2 and day7. (D) Day 14 EB were fixed and stained with

antibodies against GFAP, vimentin and ß-catenin to identify specific cell lineages. Scale bar represent 20 µm. (E) HE staining of teratoma section generated by rabbit iPSC demonstrated structures derived from three germ layer tissue: epidermis (left panel; ectoderm), cartilage (middle panel; mesoderm) and gland-like structure (right panel; endoderm). Scale bar represent 50 µm.

For cardiac differentiation, all the cell lines could contribute to three-dimensional mass but the ability to form EB was different among the cell seeding densities and particular cell lines. In general, cell seeding density influenced the EB size. Low cell seeding density at 1,000 cells per EB was insufficient to form EB in all cell lines. A cell line (R1) did not form the EB at 3,000 cells/EB (Figure 17A-1). At 5,000 and 10,000 cell density, iPSC line R2 could form EB with larger size compared with R1 and R3 lines ($p<0.05$, Figure 17A-2 and 17A-3). Cell seeding density at 20,000 cells per EB increased EB size to the range of 326 to 467 µm which was previously reported to be optimal EB size for cardiac differentiation (Hwang et al., 2009). This cell density (20,000 cells per EB) was therefore used for cardiac differentiation in this study. The average diameters of EBs obtained for 20,000 cells/EB were 325.8±7.32, 467.4±8.68 and 463.33±18.42 for iPSC line R1, R2 and R3, respectively. After EB were cultured for 72 h in the EB medium with BMP-4, they were harvested and cultured onto gelatin coated dishes. The EB were easily attached to the Petridish and cells were translocated from outermost area of the EB to form multiple cell types and layers. On day 5 of differentiation, a large proportion of cells (51±1.48%) positively expressed with cardiovascular progenitor marker, Flk1 (Figure 17D). There was no significant difference among cell lines. The mean ± SEM of Flk1 positive cells were 53.33±2.3%, 53.17±1.58% and 46.49±2.5% for iPSC line R1, R2 and R3, respectively. Later, the outer layer contained flat elongated cells while the center remained dense darkened area. The elongated cells were seen around day 7 of cardiac differentiation (Figure 17B). They formed filament-like structure and started to spontaneously beat around day 11 to 14 of culture (supplement data 1). In addition, these cells also expressed cardiac marker genes including *RyR2, PECAM1* and *α-actinin* (Figure 17C). For all cell lines, a small proportion of cells were positively stained with cTnT (10.29±1.37%) with striated

structure, indicating morphology of mature cardiomyocytes (Figure 17E). The mean ± SEM of cTnT positive cells in R3 was lowest (4.24±0.16%, $p<0.05$). There was no significant difference between line R1 (14.45±0.54%) and line R2 (12.19±1.13%).

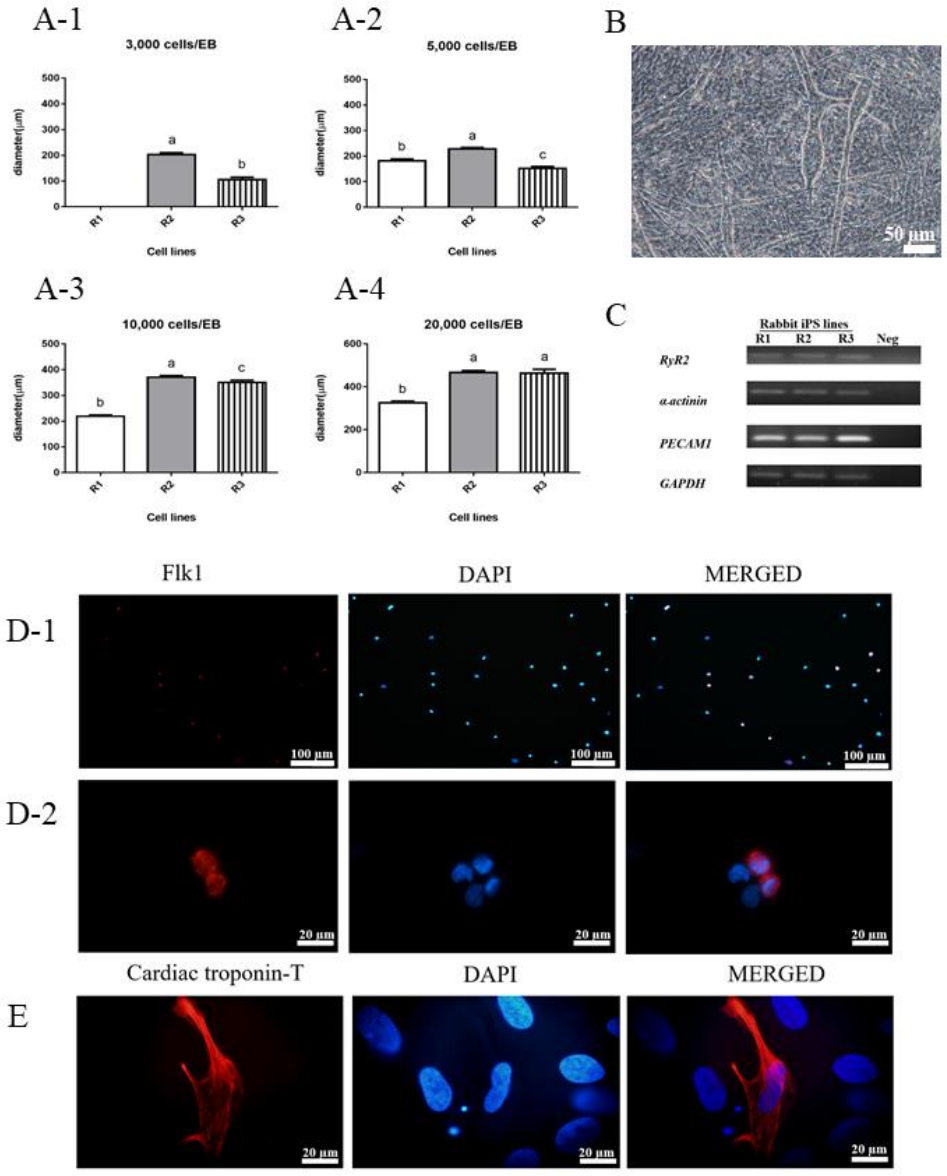

Figure 17 Cardiac differentiation derived rabbit iPSC (A) Analysis of EB diameters at different cell seeding density. P value less than 0.05 ($P<0.05$) was considered statistically significant. The graphs were plotted with letter-coded significant differences (a, b, c). (B) Cardiomyocyte-like cells derived iPSC at day 14. Scale bar represents 50 μm. (C) Gene expression of cardiac markers; *cardiac ryanodine receptors (RyR2),* ***α****-actinin* and *PECAM1* in day 14 EB derived from rabbit iPSC line R1 R2 and R3. (D)

Differentiating cells at day 5 were positively stained with mesodermal surface marker Flk1. Scale bars represents 100 μm (D-1) and 20 μm (D-2) (E) Cardiomyocyte-like cells were positively stained with cardiac troponin-T, cTnT. Scale bar represents 20 μm.

Discussion

In this study we established rabbit iPSC and demonstrated that the iPSC have differentiation potential toward cardiac lineage. Until recently, a limited number of rabbit iPSC lines have been reported (Honda et al., 2010; Honda et al., 2013; Osteil et al., 2013; Honsho et al., 2015). However, information on cardiac differentiation of these iPSC lines has not been demonstrated. Rabbit model was a valuable model for cardiac diseases in human (Pogwizd and Bers, 2008). The establishment of iPSC-based therapy for cardiovascular diseases in rabbit model has not yet been established due to the generation of rabbit iPSC appeared to be difficult and the knowledge on signaling controls of cardiac differentiation is fairly limited. All rabbit iPSC lines including our cell lines were established using viral vectors with ectopic genes *OCT3/4, SOX2, KLF4, and c-Myc* (Honda et al., 2013; Osteil et al., 2013; Tancos et al., 2017). Although this technique may lead to mutational genome integration (Csobonyeiova et al., 2015), this viral transduction is most likely the robust method to introduce ectopic genes into the host genome (Robinton and Daley, 2012). In our study, downregulation of human exogenous genes (hOCT3/4, hKlf4, hSOX2 and hc-MyC) was found in all cell lines, simultaneously with the presence of endogenous pluripotent genes OCT3/4 and NANOG. Although the presence of exogenous genes at differentiation may interfere the differentiation process, the poor efficiency of cardiac differentiation therefore appears to involve other factors rather than the existence of the exogenous genes. Our findings are in an agreement to previous reports that the establishment of rabbit iPSC is very poor (Honda et al., 2010) and its pluripotency is remarkably limited (Honsho et al., 2015). The reason for poor results of viral transduction in this species is still unknown but the poor result is similar to previous reports demonstrating an inefficient viral (human immunodeficiency virus) transduction in rabbit cells. This is likely to involve the process of gene transduction at a post- viral entry and pre-integration step

(Hofmann et al., 1999; Ikeda et al., 2002). This seems to be species specific since gene transduction efficiency with green fluorescent protein expressing viral vectors into rabbit cells was around 5 to 6 times less efficiency compared to human, feline and porcine fibroblasts (supplement data 2). In addition, the maintaining pluripotent factors of rabbit iPSC are poorly understood. This is critical for establishment of pluripotent cell line as particular species requires different signaling to promote and to sustain their pluripotency pathways. For instance, mouse embryonic stem cells needs to be maintained via LIF/STAT-3 pathway (Niwa et al., 2009) while human ESC mainly requires bFGF for pluripotent maintenance (Levenstein et al., 2006; Greber et al., 2010). The rabbit iPSC established in this study demonstrated typical iPSC morphology (flat colony, Figure 15A) which is resembled to human iPSC rather than dome-shaped mouse iPSC. Furthermore, the findings also are in an agreement with other studies that rabbit iPSC are LIF and bFGF dependent (Tancos et al., 2017). The three cell lines established in the current study had potential to develop into all three germ layers and also cardiac lineage, via cellular aggregation using hanging drop technique. However, we found that the EB culture did not completely downregulate entire pluripotent gene as *SOX2* and *KLF4* genes were found to continuously express on day 7 of EB culture, while the pluripotency controlled *NANOG* and *OCT3/4* genes was completely downregulated. The finding is in an agreement with a report of human embryonic stem cells that *KLF4* could still be detected in the two-week cultured EB (Chan et al., 2009). These results suggest that these genes do not only control pluripotency but also balance the cellular homeostasis. For example, the *KLF4* gene has been demonstrated to actively control cellular processes, such as apoptosis (Ghaleb and Yang, 2017). In addition, this study confirmed the capability of retroviral mediated rabbit iPSC lines in *in vitro* and *in vivo* differentiation, in terms of gene and protein expressions in embryoid body and teratoma formation, respectively. The efficient differentiation appears to associate with the downregulation or silence of exogenous genes used during iPSC generation or when the exogenous pluripotent genes were overwhelmed by other pluripotent endogenous genes (Niwa et al., 2000; Ramos-Mejia et al., 2012). In the current study, we differentiated rabbit iPSC into cardiac cell fate via embryoid body formation. This technique is simple and has been reported

to efficiently promote mesodermal transition and also cardiac differentiation (Takei et al., 2009; Mohr et al., 2010), although mature cardiomyocytes can also be generated by other techniques such as monolayer format (Lian et al., 2012; Zhang et al., 2012) and direct transdifferentiation (Ieda et al., 2010; Qian et al., 2012). Using this technique, we demonstrated for the first time that the rabbit iPSC can differentiate toward cardiac lineages (Figure 17). This capability highlights the possibility to use rabbit as a model for treating cardiac disorder in human. Although all established iPSC lines were capable of forming EB, this ability was dependent on cell density (cell number per EB) and cell line (Figure 17A). EB formation was inefficient for low cell density (1,000 and 3,000 cells per EB). This appeared to cause by the sensitivity of rabbit iPSC on enzymatically single cell dissociation similar to human (Beers et al., 2012). We optimized the EB size to around 400 μm since the large EB size (300-450 μm) had been shown to promote cell differentiation into cardiac lineage compared with smaller EB (Hwang et al., 2009; Mohr et al., 2010). The large EB size allowed sufficient cellular interactions and also microenvironments such as oxygen tension suitable for differentiation and proliferation of cardiac progenitor cells (van Oorschot et al., 2011; Van Winkle et al., 2012). Furthermore, a larger size EB tended to preferentially elevate gene expression (*NKX2.5, GATA4, WNT11, TBX5, NFATC1* and *NRG1*) that are responsible for cardiogenic differentiation (Cha et al., 2015). The cardiac differentiation was also promoted by addition of BMP-4 during EB formation (Kattman et al., 2006; Takei et al., 2009) as the BMP-4 is the main regulator for cardiac mesodermal transition and regulates cardiogenesis via NKX2.5 and GATA4 pathways (Barron et al., 2000; Lev et al., 2005). Although these pathways have not been examined in rabbit iPSC, the protocol used in this study efficiently differentiated the iPSC (around 50%) into cardiac progenitor cells by means of Flk1 expression. However, only small population could develop to mature cardiac phenotypes (cTnT positive beating cells). The low efficiency in differentiation of mature cardiac cells may relate to the property of the specific cell lines used. The rabbit iPSC lines used in this study appears to constantly express *SOX2.* The increased expression of SOX2 potentially guides the cell fate generally into neuroectodermal lineage. This condition inhibits mesodermal differentiation and thereby limiting spontaneous cardiac differentiation (Kopp et al., 2008; Thomson et

al., 2011). Although BMP-4 supplement could improve cardiac differentiation of rabbit iPSC, overall efficiency remains poor. This suggests that other factors appear to synergistically interact with cardiac cell fate, rather than BMP-4 alone. It is interesting to examine whether or not other factors such as activin A, FGF2, VEGF, Gsk3 inhibitors and Dickkopf-1 will be needed for cardiac differentiation as previously reported in human (Yang et al., 2008; Lian et al., 2013). Further study for improving cardiac differentiation for rabbit iPSC such as optimization of BMP-4 dosage, identification of molecular networks for cardiac differentiation and cardiac function should be investigated.

Conclusions

Rabbit iPSC lines can be differentiated into cardiac lineage via 3D-structure embryoid body. The optimization of cardiac differentiation remains to be elucidated in order to improve its efficiency. The findings in this study highlight the possibility to generate mature cardiomyocytes from rabbit iPSC for further use.

Chapter IV

General discussion

Pluripotent stem cells (PSC) have the potential to unlimitedly divide and to differentiate into multiple different tissue types (Takahashi et al., 2007). They can be modified for *in vitro* genetically manipulation by gene insertion or deletion which will serve as a tool for generation of disease models (Hockemeyer and Jaenisch, 2016). Rabbit model is a valuable model for cardiac diseases in human (Fan and Watanabe, 2003; Pogwizd and Bers, 2008). The establishment of PSC-based therapy for cardiovascular diseases in rabbit model has not yet been established due to knowledge on signaling controls of cardiac differentiation is limited. In this study we established rabbit iPSC and ESC and demonstrated that these rabbit PSCs had differentiation potential towards cardiac lineage. The generation of rabbit iPSC appeared to be difficult and a limited number of rabbit iPSC lines have been reported using viral method (Honda et al., 2013; Honda et al., 2010; Honsho et al., 2015; Osteil et al., 2013). In this study, rabbit iPSC lines were also generated using retroviral method. Reprogramming efficiency for rabbit iPSC in this study was 0.191% indicating high efficient method. Previous studies claimed success rate at 0.1-1.5% (Robinton and Daley, 2012; Malik and Rao, 2013) comparing to others method such as nonintegral adenoviral method (0.0001–0.001%) or protein reprogramming (0.001-0.006%) (Malik and Rao, 2013). Although this technique may lead to mutational genome integration (Csobonyeiova et al., 2015), this viral transduction is high efficient method to introduce ectopic genes into the host genome in many cell types (Robinton and Daley, 2012; Malik and Rao, 2013). Lentivirus, unlike retrovirus, can infect both nondividing and proliferating cells and is preferred delivery vector in multiple cell types (Robinton and Daley, 2012; Malik and Rao, 2013). However this is contradictory with the finding in this study that transfection rate of rabbit fibroblast was poor (supplement data 2). The establishment of rabbit ESC were generated from many sources like *in vivo*, *in vitro* fertilized eggs, cryopreserved rabbit embryos, clones and parthenotes (Graves and Moreadith, 1993; Fang et al., 2006; Wang et al., 2007; Honda, 2013; Du et al., 2015;

Intawicha et al., 2016). In our study, *in vivo* fertilized embryos were used. The success rate of ESC establishment in this study (8.89%) which were cultured in high feeder cell density of 35 × 10^3/cm^2 for primary colony was lower than previous study with the same source (12.09%) in low feeder cell density of 6 × 10^3/cm^2 feeder cell density (Honda et al., 2008). Honda and his colleagues mentioned that feeder cell density was critical factor for generate rabbit ESC. There is no reported success rate of ESC establishment from other sources. All reported cell lines shared major pluripotent characteristics with other studies, self-renewal capacity, maintaining of undifferentiated stage in culture, *in vitro* EB formation and teratoma generation. They were maintained at least 40 passages with normal morphology, karyotype and AP positive staining. However, in all rabbit PSC studies, there is no evidence of germline transmission including our studies. At present, germ line–transmitting PSC of mammalian species are available in only mouse and rat, this raises the question of whether ES-like naïve PSC exist in nonrodent species (Du et al., 2015). The maintaining pluripotent factors of rabbit PSC are poorly understood. Several studies reported they maintained rabbit PSC in only bFGF supplemented condition (Fang et al., 2006; Honda et al., 2008; Honda et al., 2010; Osteil et al., 2013). However, in our study we used both LIF and bFGF for maintaining proliferation and potency of rabbit ESC and iPSC. Withdrawal either LIF or bFGF resulted in differentiation. It is elucidated that in rabbit ESC, LIF activates the transcription factor STAT3 and inhibits PSC differentiation and bFGF2 plays downstream signaling (MEK-ERK1/2 and PI3K-AKT) signaling for unlimited proliferation (Hsieh et al., 2011; Lo et al., 2015). However, it is not possible to conclude which main pathway required for rabbit pluripotent stage by present data. There are no study of other factors support pluripotent stages in mice and human such as wnt3a (Singla et al., 2006), IGF-2 (Yuan and Hong, 2017) or BMP-4 (Qi et al., 2004). The rabbit iPSC and ESC established in this study demonstrated typical flat morphology which is resembled to human PSC rather than dome-shaped mouse PSC. In addition, mouse naïve cells require pluripotency maintenance via LIF/STAT-3 pathway (Niwa et al., 2009; Onishi and Zandstra, 2015) while human prime cells need bFGF for pluripotent maintenance (Greber et al., 2010; Levenstein et al., 2006). It is considered that our PSC closed to primed stage than naïve stage since mouse naive state PSC requires mainly LIF and

exhibits dome-like morphology (Nichols and Smith, 2009; Tosolini and Jouneau, 2016). Both naïve and primed pluripotent stem cell are pluripotent, but they demonstrate distinct plasticity potential as evidenced by only naïve PSC contribute chimeras, while primed PSC cannot (Takahashi et al., 2018). To develop rabbit model, further study is needed to optimize factor conditions to maintain its ground state. Although some differentiation potentials were conducted by previous study (Honda et al., 2008; Tancos et al., 2012; Osteil et al., 2013; Honsho et al., 2015), cardiac differentiation of rabbit PSC lines has not been studied. Both our ESC and iPSC demonstrated potential to develop into cardiac lineage, via cellular aggregation using hanging drop technique. However, we found in rabbit iPSC that the EB culture did not completely downregulate entire pluripotent gene as SOX2 and KLF4 genes were continuously expressed on the first week of EB culture, while the pluripotency controlled NANOG and OCT3/4 genes was completely downregulated. The finding is in an agreement with a report of human embryonic stem cells that KLF4 could still be detected in the two-week cultured EB (Chan et al., 2009). These results suggest that these genes do not only control pluripotency but also balance the cellular homeostasis such as apoptosis (Ghaleb and Yang, 2017). In the current study, we differentiated rabbit iPSC and ESC into cardiac fate via simple embryoid body formation. This technique has been reported to promote mesodermal transition and cardiac differentiation (Mohr et al., 2010; Takei et al., 2009). Using this technique, we demonstrated for the first time that the rabbit iPSC can differentiate toward cardiac lineages. The ability to form EB in rabbit PSC were influenced by cell density (cell number per EB) and individual cell line. The cell starting for EB formation in mouse and human normally were around 500 -5,000 cells per EB (Dierickx et al., 2012). In our study we used density of 20,000 cells per EB for iPSC and 3,000 cells per EB for rabbit ESC to generate optimally day 3 diameter (325.8±7.32, 467.4±8.68 and 463.33±18.42 for iPSC line R1, R2 and R3 and 3,000 cells/EB are 203.59±11.65, 323.53±8.97, 337.1±11.77 and 217.49±3.68 for ESC line A, B, C and D respectively). Comparing to mouse, rabbit EB formation was inefficient for low cell density. No iPSC lines can form EB at staring cell density of 1,000 cells per EB. The death of digested pluripotent cells appeared to cause by enzymatical effect on single cell dissociation similarly to human (Beers et al., 2012). This effect was found in both

ESC and iPSC, but higher in iPSC and it can be improved by supplemented rock- ROCK Inhibitor (Y-27632) on day of passage (supplement data3). We optimized the EB size to around 400 μm since the large EB size (300-450 μm) had been shown to promote cell differentiation into cardiac lineage compared with smaller EBs (Hwang et al., 2009; Mohr et al., 2010). The large EB size allowed sufficient cellular interactions and microenvironments such as oxygen tension suitable for differentiation and proliferation of cardiac progenitor cells (van Oorschot et al., 2011; Van Winkle et al., 2012). Furthermore, a larger size EB tended to preferentially elevate gene expression (*NKX2.5, GATA4, WNT11, TBX5, NFATC1* and *NRG1*) that are responsible for cardiogenic differentiation (Cha et al., 2015). Other techniques such as monolayer culture (Lian et al., 2012; Zhang et al., 2012) and direct transdifferentiation (Ieda et al., 2010; Qian et al., 2012) also can enhanced cardiomyocytes. However, we failed to produce cardiomyocytes by monolayer culture (unpublished data). For interpretation of cardiac related gene level, we investigated stable housekeeping genes such as *GAPDH* and *RPL13A* in rabbit ESC and EB. This study firstly identified that the two genes (RpL13a and GAPDH) were stably expressed as similar to other reports (Barber et al., 2005; Bian et al., 2015; Gentile et al., 2016; Murphy and Polak, 2002). They are suitable tools to normalize relative expression level. We elucidated that cardiac mesoderm BT significantly upregulated and reached its maximal levels on day two after spontaneous differentiation via EB formation in differentiating cells derived rabbit ESC. The result obtained was different compared with mouse in that the level of Brachyury was reported to peak on day 3 of cardiac differentiation (Lolas et al., 2014; Rungarunlert et al., 2013). It is suggested that the day of EB plating should depend on the optimal cell density and expression levels of cardiac mesoderm during early phase of embryoid body culture (Choi et al., 2010; Hwang et al., 2009; Mohr et al., 2010; Rungarunlert et al., 2013). The upregulation of BT gene commit at early period of EB development indicated the transition from ground state pluripotency toward mesoderm (Beisaw et al., 2017; Lolas et al., 2014). The EB in this study were plated on day three corresponding with the levels of BT expression and optimal size of EB diameter. The cardiac differentiation was also promoted by addition of BMP-4 during EB formation (Kattman et al., 2006; Takei et al., 2009). BMP-4 plays a central role in regulation of

mesoderm formation and cardiogenesis (Kattman et al., 2011; Kim et al., 2015; Lev et al., 2005; Rajala et al., 2011). It induced early cardiac marker NKX2.5 and GATA4 via activation of MAP kinase kinase kinase Tak1 and Smad signaling (Harvey, 2002; Lev et al., 2005; Monzen et al., 2001) and improved *in vitro* cardiac differentiation both in human and mouse (Kattman et al., 2011). We found that BMP-4 enhances level of cardiac markers in both rabbit PSC and ESC. Studying in rabbit ESC, we found that supplement of BMP-4 into EB culture medium, significantly increased the levels of BT expression when compared with the control. The increased expression of BT after BMP-4 treatment coincided with the increase in Flk1 and troponin-T positive cells as well as percentage of beating area compared with control group. Although BMP-4 supplement could improve cardiac differentiation of rabbit PSC, only small population developed into mature cardiomyocytes (cTnT positive beating cells) in both rabbit ESC and iPSC. This suggests that other factors influence cardiac cell fate, rather than BMP-4 alone. It is necessary to examine other factors related cardiac fate in mice and human such as activin A, FGF2, VEGF, Gsk3 inhibitors and Dickkopf-1 (Laflamme et al., 2007; Yang et al., 2008; Lian et al., 2012; Mummery et al., 2012).The disadvantage of this study is that we used fetal bovine based medium instead of a define medium for cardiac differentiation because we failed to form EB without serum supplemented medium (supplement data 4). Fetal bovine serum was controversial effect to promote cardiac fate (Takei et al., 2009; Mummery et al., 2012) because each batch and source contain different level of molecules. Some molecule such as noggin in FBS can inhibit cardiac differentiation (Mummery et al., 2012). In addition, the poor efficiency of cardiac differentiation in rabbit iPSC may related constant expression of SOX2 which plays role in neuroectodermal fate and inhibits mesodermal differentiation (Kopp et al., 2008; Thomson et al., 2011).

Conclusion

Examination of differentiation potentials of rabbit PSC into cardiac cell fate is important development for further study in conduct cardiovascular disease model in rabbit. We have demonstrated that rabbit ESC and iPSC retains capacity to differentiate toward cardiac fate. Major molecules BMP-4, regulates cardiac differentiation in human and mice, promoted cardiac mesoderm, cardiac progenitor and cardiomyocyte markers

in both rabbit ESC and iPSC. We demonstrated both *GAPDH* and *RPL13A* are stable in ESC and early date of EB formation. They are suitable to normalize level of cardiac gene expression for rabbit differentiation via 3D structure EB. However, the present technology is inefficient to generate cardiomyocytes in large scale, further studies for improving cardiac differentiation such as identification of molecular networks are required.

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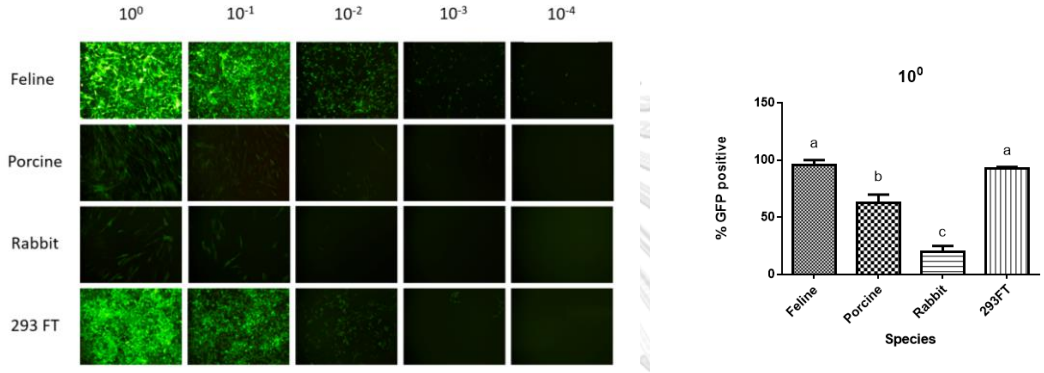
APPENDIX

Supplement Data 1

Video of rhythmic beating area derived rabbit iPSC can be found online at http://dx.doi.org/10.17632/5n3dgm5bs5.1

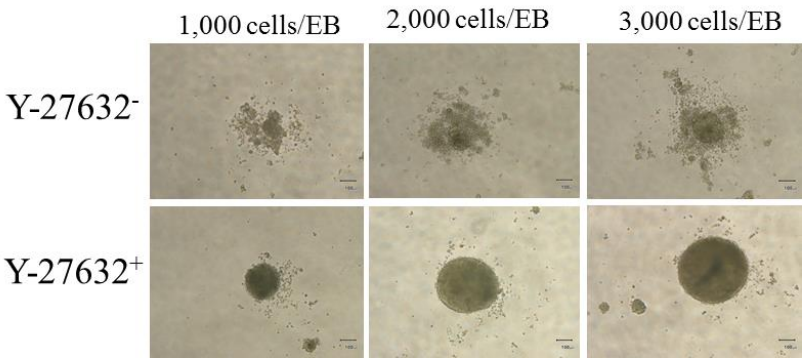
Supplement Data 2



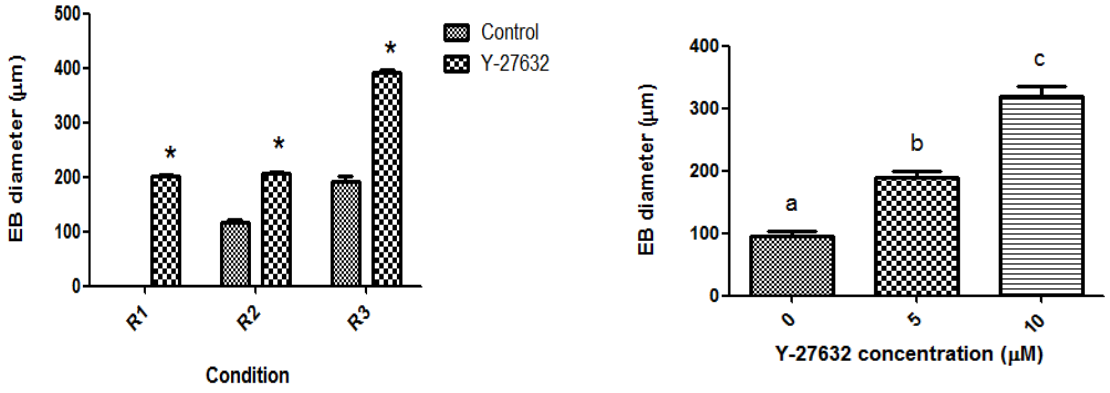
Lentiviral efficiency among species. (left) Lentiviral vector carrying Green fluorescence protein (GFP) transfected in to fibroblast of cats, pig and rabbit. 293FT HEK cells which is susceptible cell line for produce viral vector were used as control. (Right) Rabbit fibroblast was infected by lentivirus at lowest among species ($P<0.05$, Anova, Tukey's Multiple Comparison Test). Letters (a, b and c) indicate significant differences in mean values.

Supplement Data 3

A

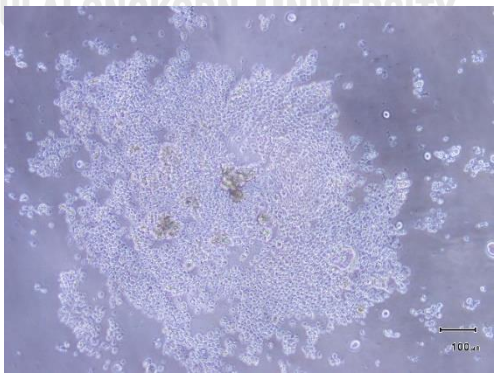


B



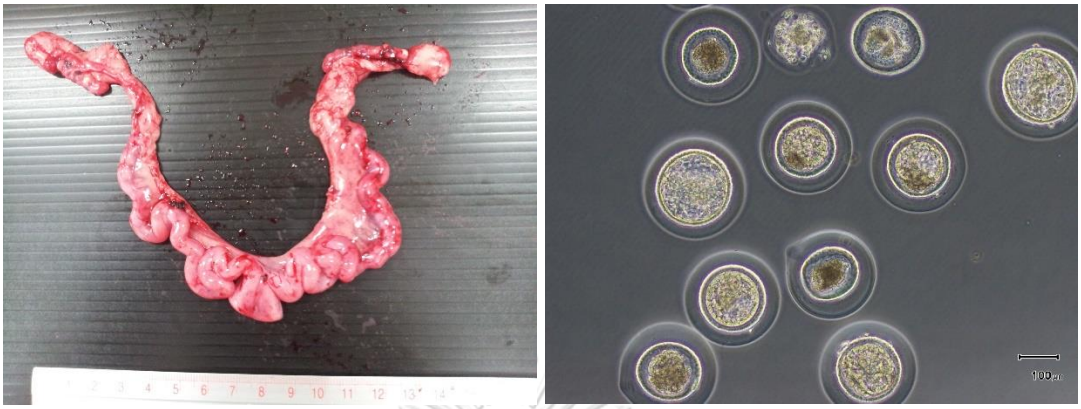
The effect of rock inhibitor on rabbit EB formation (A) Representative EB figures at day 3 of 1,000, 2,000 and 3,000 starting cells seeding per EB in multiple conditions. A lot of dead cells were found without rock inhibitor (Y-27632^{-}) (first row). Scale bars are 100 µm. (B) EB diameter derived 3,000 starting cells seeding per EB with (Y-27632) and without rock inhibitor (control) were analyzed within lines by paired t-test, asterisks representing significance ($P \leq 0.05$).(C) EB diameters derived iPSC R2 are influenced by concentration of rock inhibitor. Values with different superscript within table showed significant difference ($P \leq 0.05$, One-way Anova).

Supplement Data 4



Failure of EB formation in rabbit iPSC without FBS supplement at the bottom of 96 well dish on day 3

Supplement Data 5



(left) Rabbit uterus on day 4 after mating (Right) Rabbit blastocyst stage embryos

VITA

Miss Praopilas Phakdeedindan was born on June 6, 1985 in Bangkok, Thailand. She graduated with Degree of Doctor of Veterinary Medicine (D. V. M) from Chulalongkorn University, Bangkok, Thailand in 2009. She worked as a small animal veterinarian at a private animal hospital for 2 years before enrolled a Master program and received a grant from Chulalongkorn University for Graduate Students. She completed the requirements for the Master of Science Degree field of Veterinary Biosciences in 2013. After that, she received a scholarship from the Doctoral Degree Chulalongkorn University 100th Year Birthday Anniversary and started PhD program at Biochemistry Unit, Department of Veterinary Physiology, Faculty of Veterinary Science, Chulalongkorn University. She is interested in stem cells establishment and culture, in vitro embryos culture, gene transduction, gene expression and gene regulation.