

มหาวิทยาลัยมหิดล

รายงานวิจัยฉบับสมบูรณ์

Project: **Nkx2.5-eGFP mouse ESCs for purification of cardiac progenitor cells (CPCs) and cardiomyocytes**

โครงการ: **เซลล์ต้นกำเนิดจากตัวอ่อน NKx2.5-eGFP ในหนูเมาส์สำหรับการแยกเซลล์ตั้งต้นของเซลล์กล้ามเนื้อหัวใจและเซลล์กล้ามเนื้อหัวใจให้บริสุทธิ์**

โดย

Assistant Professor Dr. Sasitorn Rungarunlert

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Full Research Report
รายงานวิจัยฉบับสมบูรณ์

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โดย

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Abstract

Project Code: MRG5680108

Project Title: Nkx2.5-eGFP mouse ESCs for purification of cardiac progenitor cells (CPCs) and cardiomyocytes

Investigator: Assistant Professor Dr. Sasitorn Rungarunlert

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Project Period: 7 years (inclusive of the approved extended period)

Embryonic stem cell (ESC)-derived cardiomyocytes (ESC-CMs) provide an unlimited source of cardiomyocytes for cell replacement therapies and pharmacological testing. However, the obstacle of mature ESC-CM transplantation is the low rate of ESC-CMs, and mature ESC-CM is low survival after cell transplantation. The generation of cardiac progenitor cells from ESCs is a challenge to overcome these problems. This study aimed to establish transgenic mouse ESCs ($Tg^{Nkx2.5\text{-}eGFP}$ ESCs) using Nkx2.5-eGFP-neomycin resistance and differentiate into Nkx2.5-eGFP cardiac progenitor cells (CPCs) and cardiomyocytes to enable tracking and purification of CPCs. Two genotype mouse ESC lines; HM-1 (129/Ola strain derived) and Pzs3 (C57Bl/6 mouse strain derived) were transfected using Nkx2.5-eGFP-SV40-NeoR vector. Then, the neomycin-resistant colonies derived from HM-1 and Pzs3 (31 and 3 colonies, respectively) were picked and propagated for DNA assay and freezing. After DNA analysis, the efficiencies of targeting HM-1 and Pzs3 colonies were 48.4% (15 targeted colonies/31 resistant colonies) and 100% (3 targeted colonies/3 resistant colonies), respectively. Subsequently, the targeted colonies were characterize based on morphology, the ability to form EB, EGFP expression, and in vitro differentiation ability toward cardiomyocytes. Only one $Tg^{Nkx2.5\text{-}eGFP}$ ESC line/ one genotype was chosen for further evaluating differentiation into Nkx2.5-eGFP CPCs and mature cardiomyocytes. The result showed that we could establish $Tg^{Nkx2.5\text{-}eGFP}$ ESC lines derived from two genetic backgrounds. All cell lines can maintain in pluripotent state and exhibited a high efficiency of cardiac differentiation.

Keywords: Nkx2.5-eGFP; purification; progenitor cells; cardiomyocytes; embryonic stem cells

บทคัดย่อ

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ชื่อโครงการ: เซลล์ต้นกำเนิดจากตัวอ่อน NKx2.5-eGFP ในหนูเมาส์ สำหรับการแยกเซลล์ตั้งต้นของเซลล์กล้ามเนื้อหัวใจ และเซลล์กล้ามเนื้อหัวใจให้บริสุทธิ์

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ระยะเวลาโครงการ: 7 ปี

เซลล์กล้ามเนื้อหัวใจที่เปลี่ยนแปลงมาจากเซลล์ต้นกำเนิดจากตัวอ่อน เป็นแหล่งของเซลล์กล้ามเนื้อหัวใจที่ไม่จำกัด สำหรับการรักษาเซลล์บำบัดและการทดสอบทางเภสัชวิทยา อย่างไรก็ตามอุปสรรคของการปลูกถ่ายเซลล์กล้ามเนื้อหัวใจที่โตเต็มที่แล้ว คือ เซลล์ต้นกำเนิดจากตัวอ่อนมีประสิทธิภาพในการเปลี่ยนแปลงเป็นเซลล์กล้ามเนื้อหัวใจที่ต่ำ และเซลล์กล้ามเนื้อหัวใจที่โตเต็มที่แล้วมีอัตราการอยู่รอดต่ำหลังการปลูกถ่ายเซลล์ การสร้างเซลล์ตั้งต้นของเซลล์กล้ามเนื้อหัวใจที่เปลี่ยนแปลงมาจากเซลล์ต้นกำเนิดจากตัวอ่อน เป็นความท้าทายในการเอาชนะปัญหาเหล่านี้ จุดประสงค์ของการศึกษานี้คือเพื่อสร้างเซลล์ต้นกำเนิดจากตัวอ่อนของหนูเมาส์ ที่ดัดแปลงพันธุกรรมด้วย Nkx2.5-eGFP-neomycin resistance และเปลี่ยนแปลงเป็นเซลล์ตั้งต้นของเซลล์กล้ามเนื้อหัวใจและเซลล์กล้ามเนื้อหัวใจ เพื่อติดตามและแยกเซลล์ตั้งต้นของเซลล์กล้ามเนื้อหัวใจให้บริสุทธิ์ เซลล์ต้นกำเนิดจากตัวอ่อน 2 สายพันธุ์ ที่ชื่อว่า HM-1 (สายพันธุ์ 129/Ola) และ Pzs3 (สายพันธุ์ C57Bl/6) ได้รับการติด Nkx2.5-eGFP-SV40-NeoR vector หลังจากนั้นโคโลนีที่ทนต่อนีโอมัยซินที่ได้จาก HM-1 และ Pzs3 (31 และ 3 โคโลนีตามลำดับ) ถูกคัดเลือกและเพิ่มจำนวน สำหรับการทดสอบดีเอ็นเอและการแช่แข็ง การวิเคราะห์ดีเอ็นเอ พบว่า การดัดแปลงพันธุกรรมของ HM-1 และ Pzs3 มีประสิทธิภาพเท่ากับ 48.4% (15 โคโลนีที่ดัดแปลงพันธุกรรม/ 31 โคโลนีที่คัดเลือก) และ 100% (3 โคโลนีที่ดัดแปลงพันธุกรรม/ 3 โคโลนีที่คัดเลือก) ตามลำดับ หลังจากนั้น โคโลนีที่ได้ดัดแปลงพันธุกรรม ถูกตรวจคุณลักษณะตาม รูปร่าง ความสามารถในการสร้าง EB การแสดงออกของโปรตีนเรืองแสงสีเขียว และความสามารถในการเปลี่ยนแปลงเป็นเซลล์กล้ามเนื้อหัวใจ เลือกเซลล์ต้นกำเนิดจากตัวอ่อนที่ดัดแปลงพันธุกรรมด้วย Nkx2.5-eGFP 1 เซลล์ไลน์/ 1 สายพันธุ์ สำหรับการประเมินการเปลี่ยนแปลงเป็นเซลล์ตั้งต้นเซลล์กล้ามเนื้อหัวใจที่แสดงออกของ Nkx2.5-eGFP และ เซลล์กล้ามเนื้อหัวใจ จากการทดลองพบว่า คณะวิจัยสามารถสร้างเซลล์ต้นกำเนิดจากตัวอ่อนของหนูเมาส์ที่ดัดแปลงพันธุกรรมด้วย Nkx2.5-eGFP ได้ทั้ง 2 สายพันธุ์ โดยทุกเซลล์ไลน์สามารถเลี้ยงและเพิ่มจำนวนให้อยู่ในภาวะพลูริโพเทนท์สเต็มเซลล์ได้ และ มีประสิทธิภาพสูงในการเปลี่ยนแปลงเป็นเซลล์กล้ามเนื้อหัวใจ

คำหลัก: Nkx2.5-eGFP การทำให้บริสุทธิ์ เซลล์ตั้งต้น เซลล์กล้ามเนื้อหัวใจ เซลล์ต้นกำเนิดจากตัวอ่อน

Executive summary

Project Title: Nkx2.5-eGFP mouse ESCs for purification of cardiac progenitor cells (CPCs) and cardiomyocytes

Investigator: Assistant Professor Dr. Sasitorn Rungarunlert
Faculty of Veterinary Science, Mahidol University

1. Background and Rationale

Ischemic heart disease, caused by coronary artery stenosis or occlusion, is the most common cause of death and a significant cause of hospital admissions in developing countries such as Thailand, where the mortality of such disease is 4.47% in 2010 (Bureau of Policy and Strategy, 2553). Up to now, cell-based therapies have treated patients. Bone marrow-derived stem cells (BMSCs) and mesenchymal stem cells (MSCs) or adipose-derived stem cells (ADSCs) have conducted for treating ischemic heart disease in clinical trials phase (Mathiasen et al., 2010; 2012; Wollert et al., 2010). These studies have demonstrated improvements in left ventricular systolic function, heart tissue remodeling, myocardial perfusion, and exercise capacity inpatient (Kastrup, 2011). However, difficulties in obtaining sufficient numbers of these cells might constitute a severe impediment for the widespread implementation of intracardiac engraftment.

Up to now, embryonic stem cells (ESCs) are the most promising cell sources for cardiovascular cell therapy due to self-renewal and differentiation into cardiomyocytes (Maherali et al. 2007; Okita et al. 2007; Wernig et al., 2007; Park et al., 2008). The ESC-derived cardiomyocytes (ESC-CMs) display very similar structural and functional properties as heart tissue *in vitro* (Kehat et al., 2001; Mummery et al., 2007; Snir et al., 2003). Besides, transplantation of ESC-CMs into extensive myocardial infarction in rats leads to the formation of stable cardiomyocyte grafts, attenuation of the remodeling process, and the improvement of cardiac function (Caspi et al., 2007; Laflamme et al., 2007).

However, the obstacle of mature ESC-CM transplantation is the low rate of ESC-CMs due to the heterogeneity of ESC differentiation, and mature ESC-CM are sensitive to enzymatic treatment (Desbaillets et al., 2000). The generation of a more uniform population of cardiac progenitor cells (CPCs) *in vitro* before transplantation is a challenge to overcome

these problems. We propose establishing transgenic ESCs by using the Nkx2.5 transcription factor with the enhanced green fluorescent protein (eGFP) reporter ($Tg^{Nkx2.5\text{-}eGFP}$ ESCs or Nkx2.5-eGFP ESCs) for enabling purification and enrichment of CPCs.

2. Objectives

2.1 To generate $Tg^{Nkx2.5\text{-}eGFP}$ ESCs.

2.2 To differentiate $Tg^{Nkx2.5\text{-}eGFP}$ ESCs into Nkx2.5-eGFP CPCs and cardiomyocytes.

3. Methodology

3.1 Construction of Nkx2.5 -promoted eGFP plasmid

An expression vector, pNkx2.5-eGFP, was constructed by cloning a 2.7-kb HindIII–EcoRI fragment of the mouse Nkx2.5 promoter region into the HindIII–EcoRI site of peGFP-1 (Clontech, Palo Alto, CA). The eGFP was expressed under the control of the Nkx2.5 promoter. This plasmid also contains the neomycin- resistance gene to enable selection.

3.2 Transfection of Nkx2.5-eGFP expression plasmid and cell selection

Undifferentiated mouse ESCs, Pzs3 (C57Bl/6-derived ESCs) and HM-1 (129/SvJ-derived ESCs), were combined with the linearized DNA, electroporated, and plated on a fresh layer of iMEFs. ESC medium with neomycin was used for seven days after transfection for selection. Fifty resistant colonies were picked and grown on layers of iMEFs. A selected clone was further amplified and used for the characterization.

3.3 Characterization of $Tg^{Nkx2.5\text{-}eGFP}$ ESCs

- Pluripotent assay
  - ESC morphology
  - Alkaline phosphatase (ALP) activity
- Cardiac differentiation assay
  - The efficiency of EB formation
  - The efficiency of cardiac differentiation and expression of GFP during early cardiac differentiation

3.4 Differentiation of $Tg^{Nkx2.5\text{-}eGFP}$ ESCs into Nkx2.5-eGFP CPCs and mature Cardiomyocytes

4 Results

Establishment and characterization of HM1 (129/Ola) $^{TgNkx2.5/EGFP}$ and Pzs3 (C57Bl/6) $^{TgNkx2.5/EGFP}$ ESCs. Among 15 and 3 targeted colonies (HM1 and Pzs3, respectively) were characterized based on the ability to form EBs, EGFP expression and *in vitro* differentiation ability towards cardiomyocytes. One $Tg^{Nkx2.5\text{-}eGFP}$ ESC line/one genotype mouse ESCs, named as B5-HM1-$Tg^{Nkx2.5\text{-}eGFP}$ and H3-Pzs3-$Tg^{Nkx2.5\text{-}eGFP}$ derived from HM-1 and Pzs3, respectively were chosen for further evaluating differentiation into Nkx2.5-eGFP CPCs and mature Cardiomyocytes.

5 Output

5.1 International Journal Publications

5.1.1 Chakritbudsabong W, Sariya L, Pamonsupornvichit S, Pronarkngver R, Chaiwattanarungruengpaisan S, Ferreira JN, Setthawong P, Phakdeedindan P, Techakumphu M, Tharasanit T, **Rungarunlert S*.** 2017. Generation of a pig induced pluripotent stem cell (piPSC) line from embryonic fibroblasts by incorporating LIN28 to the four transcriptional factor-mediated reprogramming: VSMUi001-D. Stem Cell Res. 24:21-24. (Impact factor: 4.489, year 2019)

5.1.2 **Rungarunlert S**, Ferreira JN, Dinnyes A. 2016. Novel Bioreactor Platform for Scalable Cardiomyogenic Differentiation from Pluripotent Stem Cell-Derived Embryoid Bodies. Methods Mol Biol. 1502:169-79.

5.1.3 Klincumhom N, Tharasanit T, Thongkittidilok C, Tiptanavattana N, **Rungarunlert S**, Dinnyés A, Techakumphu M. 2014. Selective TGF-β1/ALK inhibitor improves neuronal differentiation of mouse embryonic stem cells. Neurosci Lett. 578: 1-6. (Impact factor: 2.274, year 2019)

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I want to express my deep sincere gratitude to all whose support, guidance, and wishes made it possible for me to complete the study successfully.

Prof. Mongkol Techakumphu, my beloved mentor, support and allows me to learn cell-based therapy. I am very honored and grateful to become your student. I am exceedingly obliged for the invaluable experience, encouragement, and plentiful guidance from you. **Prof. Andras Dinnyes**, I am greatly indebted to you for allowing me to work in your lab and learn the fantastic and hot topics of pluripotent stem cells. I am delighted to have all of you as my guidelight. **Dr. Melinda K. Pirity**, my scientific supervisor, is my light angel teacher about pluripotent stem cells and gene targeting. Without your encouragement and numerous enthusiastic discussions, I would not be able to finish my experiment. **Dr. Csilla Nemes** taught me how to make gene targeting and DNA vector. I want to thank you for encouraging me and wipes my tear when I cried from experiment failure. **Dr. Nuttha Klincumhom,** my beloved colleague, help me to experiment without hesitation. We always to spent numerous hours late at night or on weekends together. I am delighted to have you as one of my colleagues. **Dr. Zsuzsanna Polgar** and **Dr. Julianna Kobolak** for giving ESC lines.

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The staff members and all colleagues in (i) BioTalentum Ltd., (ii) Agricultural Biotechnology Center and (iii) Faculty of Veterinary Science, Mahidol University, all my friends who lent their time to keep the lab organized and productive and offered friendship and camaraderie which made the experience that much more fulfilling.

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Chapter 1

Background and Rationale

Ischemic heart disease, caused by coronary artery stenosis or occlusion, is the most common cause of death and a significant cause of hospital admissions in developing countries such as Thailand, where the mortality of such disease is 4.47% in 2010 (Bureau of Policy and Strategy, 2553). Up to now, cell-based therapies have treated patients. Bone marrow-derived stem cells (BMSCs) and mesenchymal stem cells (MSCs) or adipose-derived stem cells (ADSCs) have conducted for treating ischemic heart disease in clinical trials phase (Mathiasen et al., 2010; 2012; Wollert et al., 2010). These studies have demonstrated improvements in left ventricular systolic function, heart tissue remodeling, myocardial perfusion, and exercise capacity inpatient (Kastrup, 2011). However, difficulties in obtaining sufficient numbers of these cells might constitute a severe impediment for the widespread implementation of intracardiac engraftment.

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However, the obstacle of mature ESC-CM transplantation is the low rate of ESC-CMs due to the heterogeneity of ESC differentiation, and mature ESC-CM are sensitive to enzymatic treatment (Desbaillets et al., 2000). The generation of a more uniform population of cardiac progenitor cells (CPCs) *in vitro* before transplantation is a challenge to overcome these problems. We propose establishing transgenic ESCs by using the Nkx2.5 transcription factor with the enhanced green fluorescent protein (eGFP) reporter ($Tg^{Nkx2.5\text{-}eGFP}$ ESCs or Nkx2.5-eGFP ESCs) for enabling purification and enrichment of CPCs.

Chapter 2

Literature Review

Cell-based therapies for the prevention and treatment of cardiac dysfunction offer the potential to modulate cardiac function significantly and improve outcomes in patients with cardiovascular disease (Hassink et al., 2003; White and Claycomb, 2003). Cell-based therapies including fetal cardiomyocytes (Gonzales et al., 2012), skeletal myocytes (Invernici et al., 2008), BMSCs (Barile et al., 2011) and adult cardiac stem cells (CSCs) (Messina et al., 2004) have been demonstrated successful engraftment of cardiomyocytes into the adult heart. However, the inadequate number of donor cells is the main limitation of myocardial transplantation for curing cardiovascular diseases (Laflamme and Murry, 2005). Besides, it is hard to harvest cardiomyocytes derived from CSCs (CSC-CMs) abundantly from the patient's own heart without any harm (Beltrami et al., 2003). On account of ethical reasons, cardiomyoplasty using fetal cardiomyocytes would be impossible for treating heart failure in humans (Penn and Mal, 2006). Hence, these cells are not suitable for a comprehensive application of cell-based therapies.

Up to now, ESCs are the most promising cell sources for cardiovascular cell therapy, avoiding the problems described above. Cardiomyocytes differentiation from ESCs (ESC-CMs) has very similar structural and functional properties as heart tissue *in vitro* (Kehat et al., 2001; Mummery et al., 2007; Snir et al., 2003). Besides, transplantation of ESC-CMs into extensive myocardial infarction in rats leads to the formation of stable cardiomyocyte grafts, attenuation of the remodeling process, and the improvement of cardiac function (Caspi et al., 2007; Laflamme et al., 2007). ESC-CMs usually requires an initial aggregation step to form spherical cell clusters referred to as embryoid bodies (EBs) which recapitulate many aspects of the developing embryo, including differentiation to cells of endoderm, mesoderm, and ectoderm lineages, similar to gastrulation in vivo (Hopfl et al., 2004; Pekkanen-Mattila et al., 2010). Even though, the capability of ESC-CMs has been revealed by numerous research groups; all the differentiation methods have shared problems, which include uncontrolled differentiation and low differentiation rate into cardiac lineage (Boheler et al., 2002; Mummery et al., 2003; Takahashi et al., 2003). Additionally,

the differentiation of ESCs is highly heterogeneous and has the capability of teratoma formation following transplantation, which has obstructed further study of heart disease mechanisms and direct clinical applications using ESC derived cell mixtures. Therefore, there is a significant emphasis on the purification of ESC-CMs such as using discontinuous Percoll gradients (Xu et al., 2002), fluorescence-activated cell sorting (FACS) selection of cardiomyocytes by mitochondrial fluorescent dye labeling (Hattori et al., 2010) and drug selection of genetic modification derived ESCs with cardiomyocytes-promoter-driven selectable markers (Moore et al., 2005).

Abundant research groups have investigated the genetic modification of ESCs to enhance and purify cardiomyocytes separation from other types of cells. For example, ESCs carrying a fusion gene composed of the α-myosin heavy chain (α-MHC) gene promoter and aminoglycoside phosphotransferase (NeoR) cassette is high cardiomyocytes purification (99% pure) during differentiation and a survival period in the recipient heart of at least seven weeks following transplantation (Klug et al., 1996; Zweigerdt et al., 2003; Zandstra et al., 2003). Besides, ESCs carrying the eGFP gene under the control of the myosin light chain 2v (MLC-2v) promoter and cytomegalovirus enhancer can produce a population of cardiomyocytes (97% pure) using Percoll gradient centrifugation and FACS analysis (Muller et al., 2000; Moore et al., 2005).

Although genetic modification of ESCs by mature cardiac genes (α-MHC and MLC-2v) improved cardiac function in post-infarcted mice and rats during transplantation with mature ESC-CMs, it was difficult to purify the cardiomyocytes from this population (Min et al., 2002). For CM transplantation, it is better to purify the transplanted cells as stem/progenitor cells that can differentiate into functional mature cardiomyocytes (Wu et al., 2006). Fetal liver kinase (Flk1+) cells-derived from ESCs (ESC-Flk1), a lateral mesodermal marker, demonstrates that these cells effectively differentiate into cardiomyocytes *in vitro* and *in vivo*. Transplantation of these cells into the hearts of dilated cardiomyopathy (DCM) model mice significantly improves their cardiac function (Kattman et al., 2006). However, ESC-Flk1 also differentiate into mesodermal lineages such as hematopoietic and endothelial cells. Therefore, a better transplantable cell candidate needs to be studied. The Nkx2.5 transcription factor (Nkx2.5) express in early heart progenitor cells and their

myogenic descendants, which is essential for healthy heart morphogenesis, myogenesis, and function. The loss function of Nkx2.5 homeoprotein mutations shows congenital heart disease (Kasahara et al., 2000; 2001; Jay et al., 2004).

A few research groups have studied the genetic modification of ESCs using the Nkx2.5 transcription factor. The NKX2.5 cells-derived from ESCs (ESC-Nkx2.5), Nkx2.5/GFP(+) cardiomyocytes during mouse ESC differentiation, express MHC, MLC-2v, cardiac troponin I (cTnI), and atrial natriuretic peptide (ANP) (Hidaka et al., 2003; Elliott et al., 2011). Hence, Nkx2.5-eGFP ESC-derived CPCs (Nkx2.5-eGFP-CPCs) perhaps the potential for cardiotoxicity testing and pre-clinical cell therapy in myocardial infarction and heart failure. Nevertheless, there are a few reports to purify and enrich Nkx2.5-eGFP ESC-derived CPCs (Nkx2.5-eGFP-CPCs) for drug testing and cell transplantation. Moreover, there is no commercial of these cells for further study. Before cardiomyocyte-based therapy is achieved, a thorough characterization of the cardiomyocytes is needed to ensure their feasibility for these applications. To solve the above problem, we propose establishing transgenic ESCs by using the Nkx2.5 transcription factor with the enhanced green fluorescent protein (eGFP) reporter ($Tg^{Nkx2.5\text{-}eGFP}$ ESCs or Nkx2.5-eGFP ESCs) for enabling purification and enrichment of CPCs.

Chapter 3

Objectives

1. To generate $Tg^{Nkx2.5\text{-}eGFP}$ ESCs.
2. To differentiate $Tg^{Nkx2.5\text{-}eGFP}$ ESCs into Nkx2.5-eGFP CPCs and cardiomyocytes.

Chapter 4

Research methodology and Results

1. Animal ethic

The animal experimental protocol was approved by the committee on Animal Care and Use, Faculty of Veterinary Science, Mahidol University (approval protocol number MUVS 2013-23, attached file 1)

2. Materials and cell culture

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA), and culture reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA), unless otherwise specified. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO2. Media were changed daily on mouse ESC cultures and every two days on cardiac differentiation.

3. Isolation of mouse embryonic fibroblasts (MEFs)

Four pregnant mice (ICR, National Laboratory Animal Center, Mahidol University, Thailand) at 13.5 days post coitus (d.p.c.) were used for preparing MEFs (Nagy, 2006). Mice were anesthesia by isoflurane and then euthanasia by cervical dislocation, as described earlier (AVMA Guidelines for the Euthanasia of Animals 2013) (Figure 2A). After approaching the abdominal midline, the uterine horns were dissected out (Figure 2B-D) and placed into a falcon tube containing Phosphate buffered saline (PBS) without $Ca^{2+}$and Mg^{2+} (Gibco, Invitrogen). Subsequently, the uterine horns were carried out in a tissue culture hood under aseptic conditions and using sterile instruments. The uterine horns were placed into a Petri dish and separated each embryo from its placenta and embryonic sac (Figure 2E-H). Mouse embryos were dissected head (decapitation), and internal organs (red color) were separated by using a sharp surgical scissor, washed in PBS, and place all embryos in a clean Petri dish (Figure 2I-J). The tissue was finely minced using a sterile razor blade until it becomes possible to pipette (Figure 2K). Then, 1 ml of 0.25% trypsin/EDTA (Gibco, Invitrogen), including 100 Kunitz units of DNase I (USB), were added for dissociated tissue.

The dissociated cells were transferred into a 50 ml falcon tube and incubate for 15 min at 37 °C (Figure 2L). After each 5 min of incubation, dissociated cells were pipetted up and down thoroughly and inactivated the trypsin by adding about 1 volume of freshly prepared MEF medium.

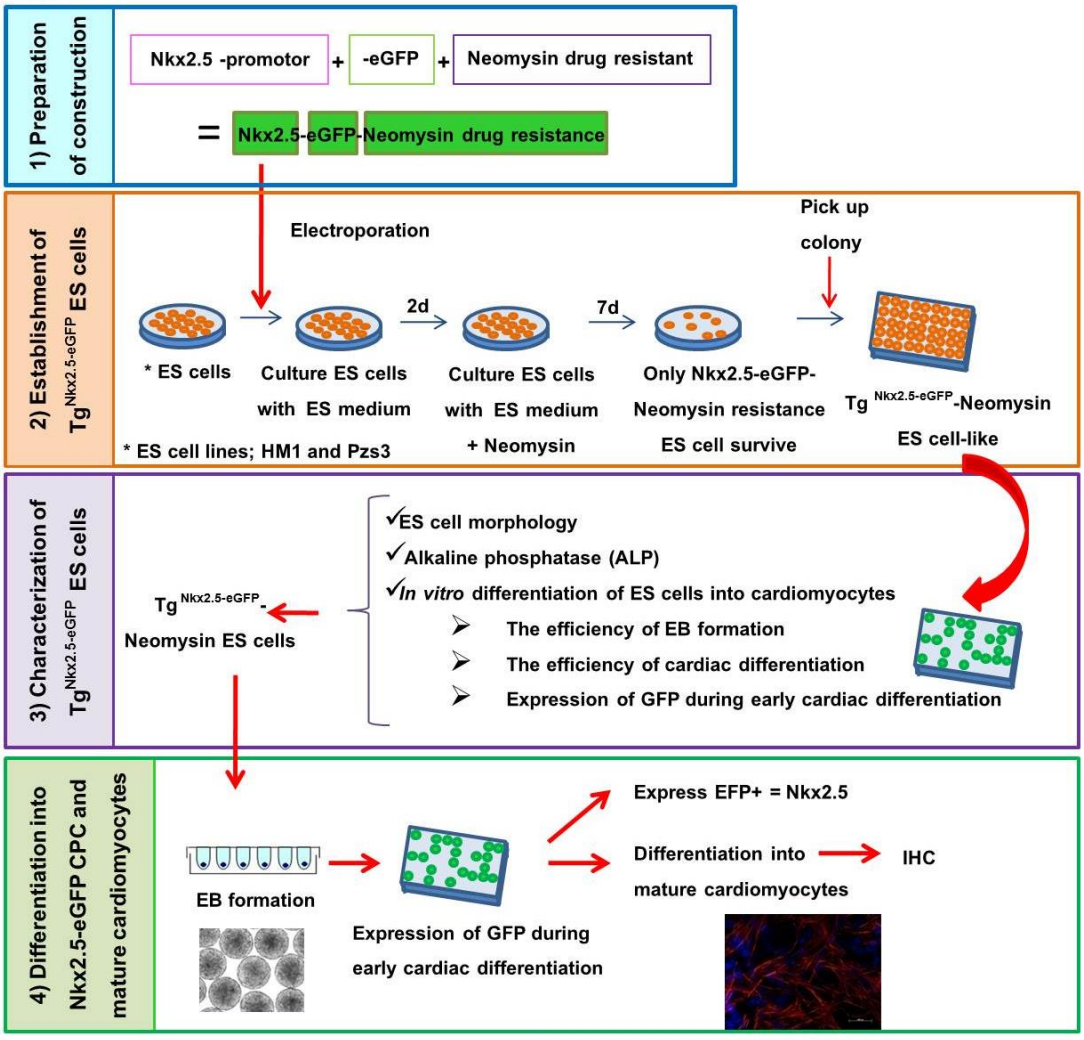

Figure 1. Schematic presentation of generation of $Tg^{Nkx2.5\text{-}eGFP}$ ESCs and differentiation into Nkx2.5-eGFP CPCs and mature cardiomyocytes *in vitro*

Figure 2. Isolation of MEFs. (A) cervical dislocation, (B-C) approach to abdominal midline, (D) uterine horn dissection, (E) a uterine horn, (F) embryo separation from placenta, (G) embryos with embryonic sac, (H) embryos, (I) embryo decapitation and internal organ separation, (J) embryo washing, (K) minced tissue, (L) trypsinization, (M) cell separation by using cell strainer, (N) plating out the cells.

The cell strainer was used for separating the individual cells (Figure 2M). Then, the cells were centrifuged with low-speed (300 x g) for 5 min, removed the supernatant, and resuspend the cell pellet in warm MEF medium. The cells were plated approximately a number of cells, equivalent to 3-4 embryos in each 100 mm tissue culture dish (Figure 2N). The fibroblasts (P0, passage 0) were the only cells attached to the tissue culture dish. After 24-48 h., cells were frozen as stock primary fibroblast (Figure 3 A-B). Twenty-six vials of primary MEFs were stored at liquid nitrogen.

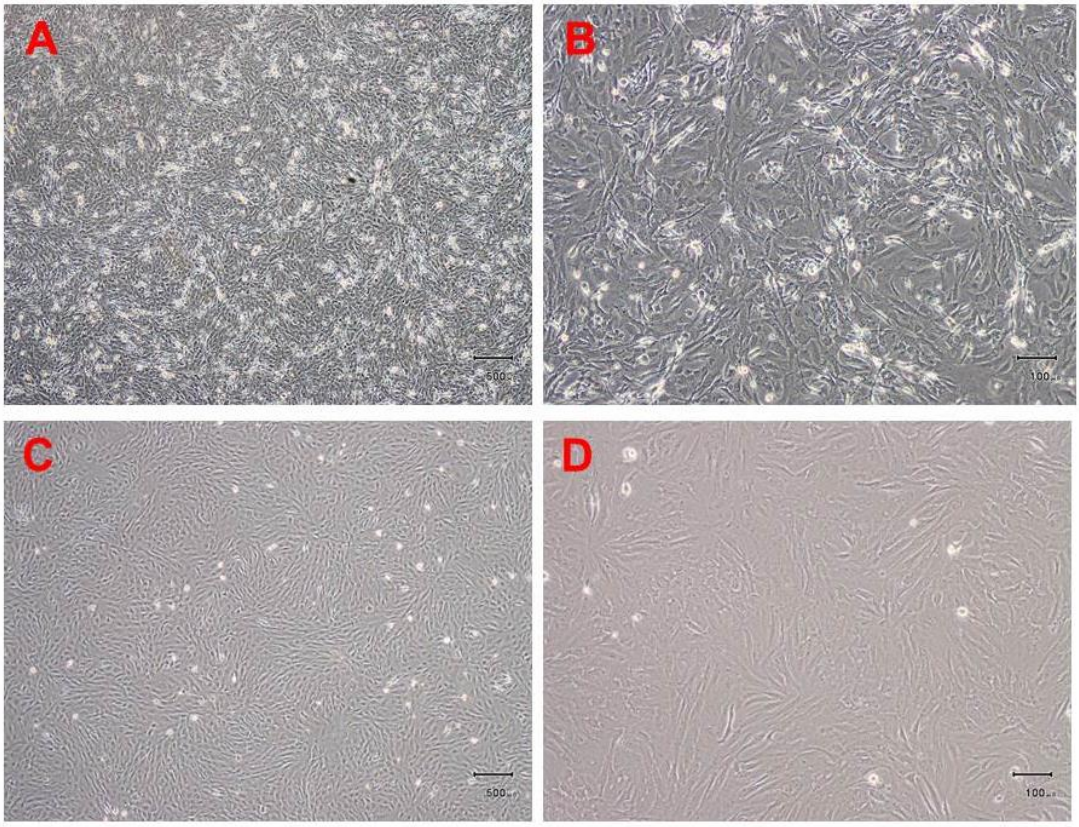

Figure 3 . Morphology of MEFs. (A) Low and (B) high magnification of primary fibroblast (P0) as stock fibroblasts before freezing, (C) Low and (D) high magnification of mitomycin C-inactivated MEFs (P3) as stock feeder cells before freezing

4. Preparation of construction of Nkx2.5 -promoted eGFP plasmid

An expression vector, pNkx2.5-eGFP, was constructed by cloning a 2.7-kb HindIII–EcoRI fragment of the mouse Nkx2.5 promoter region into the HindIII–EcoRI site of peGFP-1 (Clontech, Palo Alto, CA). The eGFP was expressed under the control of the Nkx2.5 promoter. This plasmid also contains the neomycin- resistance gene to enable the selection of permanently transfected clones. Nkx2.5 is specifically expressed in early cardiomyocytes.

5. Mouse ESCs and culture conditions

ESCs were cultured on feeder layers of mitomycin C-inactivated MEF. Those cells were maintained in ESC medium consisting of Dulbecco's Modified Eagle's Medium (DMEM-Glutamax), 15% (v/v) fetal bovine serum (FBS, Sera Laboratories International, West Sussex, RH17 5PB, UK) supplemented with 1,000 U/ml mouse leukemia inhibitory factor (LIF, ESGRO, Chemicon International), 0.1 mM nonessential amino acids (NEAA), 0.1 mM β-mercaptoethanol (β-ME) and 50 U/ml Penicillin, 50 μg/ml Streptomycin. ESCs were passaged every 1-2 days before reaching 70% confluency.

6. Establishment of $Tg^{Nkx2.5\text{-}eGFP}$ ESCs by transfecting with Nkx2.5-eGFP plasmid (Figure 4)

Undifferentiated mESCs (two genetic backgrounds) were grown to confluency on mitomycin C-inactivated MEFs (iMEF) and dissociated with 0.25% trypsin/EDTA (Gibco, Invitrogen) into a single cell suspension and counted. The density of ESCs was 5 x 10^6 cells/ml. Subsequently, ESCs were resuspended in 100 ml of ESC nucleofection solution (VPH-1001) containing 8 mg of linearized Nkx2.5-eGFP-SV40-NeoR plasmid and placed in an electroporation cuvette where the cells were electroporated using the A-023 nucleofection program (Nucleofector II device, Lonza Scientific). Transfected ESCs were plated onto a gelatin-coated dish, and neomycin selection (G418 sulfate, 100 mg/ml, Life Technologies) was initiated seven days following nucleofection lasting. After a further 8-10 days, the visible large undifferentiated ES clonal colonies were detected. The resistant colonies derived from HM-1 and Pzs3 (31 and 3 colonies, respectively) were picked and propagated on 96 well plates for DNA assay and freezing. After DNA assay, our results showed that the efficiencies of targeting ESC colonies were 48.4% (15 targeted colonies/31 resistant colonies) derived from HM-1 and 100% (3 targeted colonies/3 resistant colonies) derived from Pzs3. Subsequently, the targeted colonies were amplified and characterized by morphology, the ability to form EB, EGFP expression, and *in vitro* differentiation ability toward cardiomyocytes.

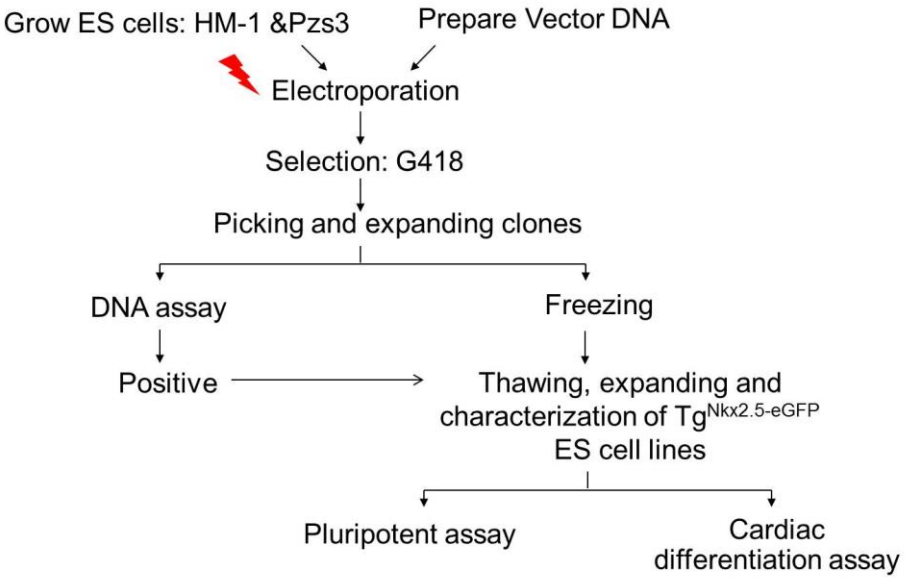

Figure 4. Diagram of establishment and characterization of HM1-Tg$^{Nkx2.5\text{-}eGFP}$ (129/Ola) and Pzs3-Tg$^{Nkx2.5\text{-}eGFP}$ (C57Bl/6) ESC lines

7. Characterization of Tg$^{Nkx2.5\text{-}eGFP}$ ESC lines

7.1 Pluripotent assay

- **ESC morphology:** Tg$^{Nkx2.5\text{-}eGFP}$ ESC lines were maintained in an undifferentiated state on iMEFs. We found that Tg$^{Nkx2.5\text{-}eGFP}$ ESCs showed the typical ESC morphology with round shape, compact, and shiny colonies using phase-contrast light microscopy (Figure 5, upper panel). Moreover, TgNkx2.5-eGFP ESC lines were shown to be invisible of eGPP using a fluorescent microscope (Figure 5, lower panel).
- ***Alkaline phosphatase (ALP) activity:*** Tg$^{Nkx2.5\text{-}eGFP}$ ESC lines were fixed with 4% paraformaldehyde (PFA) in PBS for 15 min and then washed two times with PBS. ALP staining was performed by AST Fast Red TR and a-Naphthol AS-MX Phosphate according to the manufacturer's instructions. The positive ALP colonies were red. We found that Tg$^{Nkx2.5\text{-}eGFP}$ ESC lines (Figure 6, lower panel) were strongly positive for ALP as similar to original cell lines (Figure 6, upper panel)

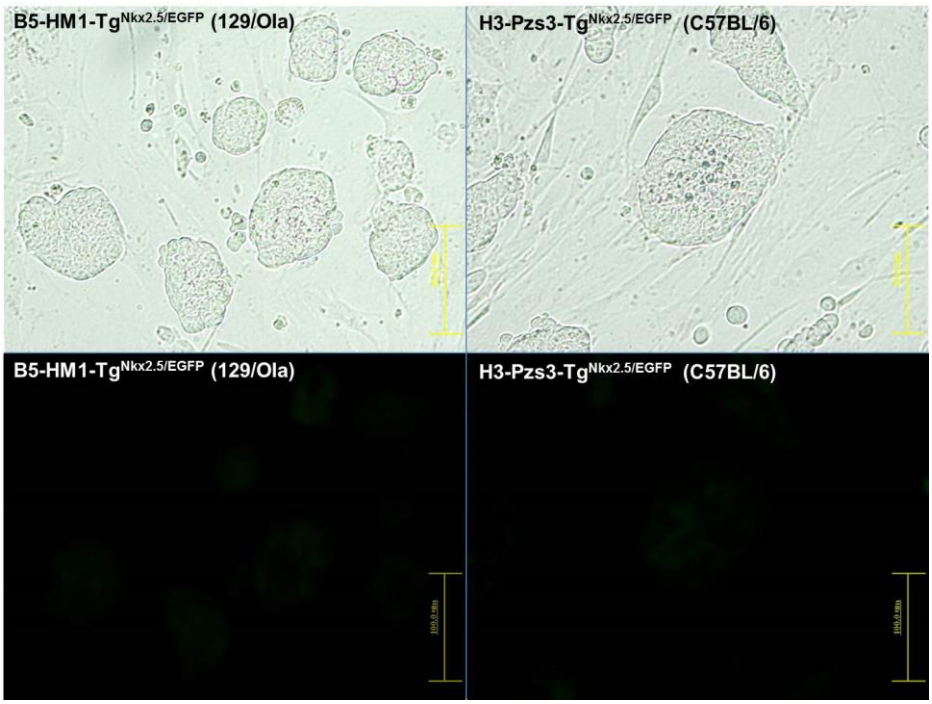

Figure 5. Morphology of B5-HM1-Tg$^{Nkx2.5\text{-}eGFP}$ and H3-Pzs3-Tg$^{Nkx2.5\text{-}eGFP}$ derived from HM-1 and Pzs3, respectively

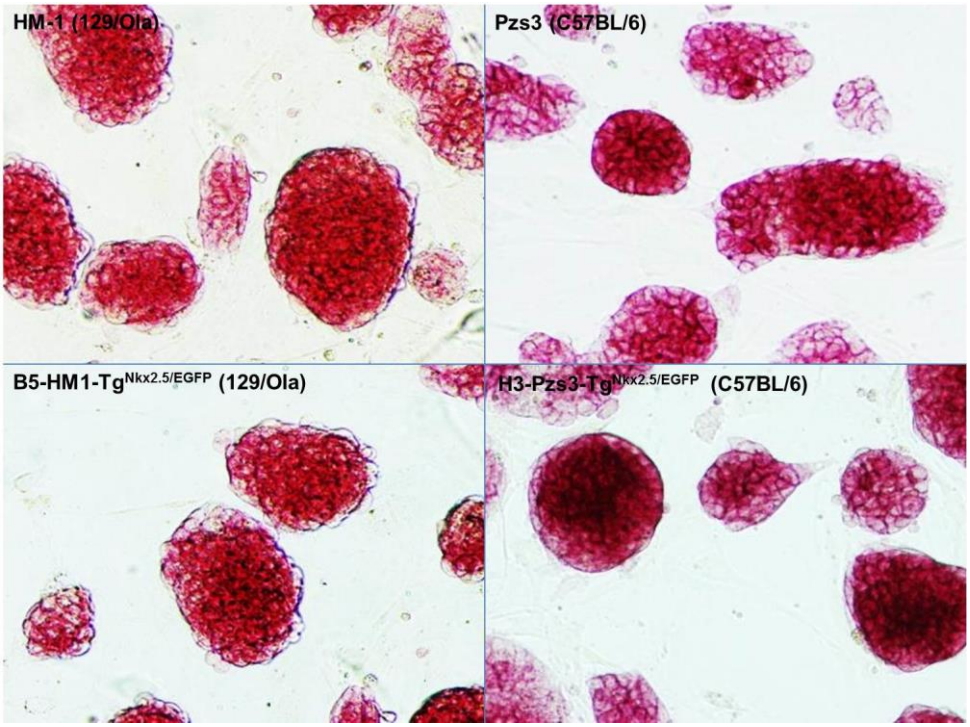

Figure 6. ALP activity. B5-HM1-Tg$^{Nkx2.5\text{-}eGFP}$ and H3-Pzs3-Tg$^{Nkx2.5\text{-}eGFP}$ ESC lines were strongly positive for ALP as similar to original cell lines (HM-1 and Pzs3)

7.2 Cardiac differentiation assay

- **The efficiency of EB formation:** EBs were prepared using the hanging drop (HD) method, as described earlier (Rungarunlert et al., 2013). In brief, $Tg^{Nkx2.5\text{-}eGFP}$ ESCs were dissociated with a 0.05% trypsin-EDTA solution to get a single cell suspension and seeded as $4x10^4$ cells/mL (resulting in 800 cells/drop) suspension in differentiation medium (ES medium without LIF). Three days later, EBs were analysis the morphology of EBs. The results of EB formation showed in Table 1.
- **The efficiency of cardiac differentiation and expression of GFP during early cardiac differentiation:** Individual EBs (day 3) were placed in gelatin-coated 24-well plates and were cultured for 21 days. The cardiac differentiation was observed daily under a fluorescent microscope for evaluating eGFP and beating. Twenty-four EBs were counted for each group, and the rate of beating EBs was evaluated as a percentage of the total number of EBs plated. The efficiency of cardiac differentiation and expression of GFP during early cardiac differentiation showed in Table 1. Moreover, our results showed that eGFP positive cells are visible in all-HM1-$Tg^{Nkx2.5\text{-}eGFP}$ EBs on days 4-7 (Figure 6). H3-Pzs3-$Tg^{Nkx2.5\text{-}eGFP}$ (C57Bl/6) ESC lines are positive eGFP on days 4-8. Interestingly H5 and H7-Pzs3-$Tg^{Nkx2.5\text{-}eGFP}$ (C57Bl/6) ESC lines are also positive eGFP; while, there is no beating derived from both cell lines (Table 1).

8. Differentiation of $Tg^{Nkx2.5\text{-}eGFP}$ ESC lines into Nkx2.5-eGFP CPCs and mature cardiomyocytes:

B5-HM1-$Tg^{Nkx2.5\text{-}eGFP}$ and H3-Pzs3-$Tg^{Nkx2.5\text{-}eGFP}$ ESC lines were used for investigating differentiation into Nkx2.5-eGFP CPCs and mature cardiomyocytes. For cardiac differentiation (as described above), EBs (day 3) were placed in gelatin-coated 24-well plates and were cultured for 14 days. Samples were fixed with 4% PFA, permeabilized with 0.25% Triton-X100 for ten min. Blocking medium were performed in 1% bovine serum albumin (BSA), after which samples were incubated with a primary antibody overnight at 4^{o}C. Antibodies: cardiac troponin T (cTnT, diluted 1:200; Abcam, UK, ab33589), Alexa Fluor 594 (diluted 1:2.000; Invitrogen, A110 05). The EBs were fluorescently imaged and analyzed by using Digital Image Processing Software (AxioVision 4.8.1, Carl Zeiss MicroImaging GmbH, Germany). Our result showed that EGFP positive cells are visible in both $Tg^{Nkx2.5\text{-}eGFP}$ ESC lines and original ESC lines on day 5 (Figure 7). Finally, $Tg^{Nkx2.5\text{-}eGFP}$ ESC lines expressed cTnT and formed a filamentous network similar to the original one (Figure 8).

ES and ES$^{TgNkx2.5/EGFP}$ cell lines	Characterization of ES and ES$^{TgNkx2.5/EGFP}$ cell lines into cardiac lineage.										
	The quality of EB formation	The efficiency of cardiac differentiation			EGFP expression						
		Day of EB started beating	% of beating / well	Grade of EB beating	D4	D5	D6	D7	D8	D9	D10
129/Ola HM1	4	D6	100	2-4	-	-	-	-	-	-	-
A1-Tg$^{Nkx2.5/EGFP}$129/Ola	3	D6	67	1-3	+1	+1	-	-	-	-	-
A3-Tg$^{Nkx2.5/EGFP}$129/Ola	4	D7	67	1-2	+2	+2	-	-	-	-	-
A6-Tg$^{Nkx2.5/EGFP}$129/Ola	3	-	0	-	+1	+1	-	-	-	-	-
A11-Tg$^{Nkx2.5/EGFP}$129/Ola	3	D7	83	2-3	+2	+2	-	-	-	-	-
B3-Tg$^{Nkx2.5/EGFP}$129/Ola	4	D6	67	2-3	+1	+1	-	-	-	-	-
B4-Tg$^{Nkx2.5/EGFP}$129/Ola	2	D7	50	2-3	+3	+3	+1	-	-	-	-
B5-Tg$^{Nkx2.5/EGFP}$129/Ola	4	D7	83	3	+3	+4	+1	-	-	-	-
C4-Tg$^{Nkx2.5/EGFP}$129/Ola	3	D7	67	3-4	+1	+2	-	-	-	-	-
C9-Tg$^{Nkx2.5/EGFP}$129/Ola	1	-	0	-	-	-	-	-	-	-	-
E5-Tg$^{Nkx2.5/EGFP}$129/Ola	2	D8	33	1-2	+1	+1	+1	+1	-	-	-
E9-Tg$^{Nkx2.5/EGFP}$129/Ola	3	D6	100	1-3	-	-	-	-	-	-	-
F6-Tg$^{Nkx2.5/EGFP}$129/Ola	3	D7	17	1	-	+1	-	+1	-	-	-
G3-Tg$^{Nkx2.5/EGFP}$129/Ola	4	D6	100	2-4	-	-	-	-	-	-	-
G4-Tg$^{Nkx2.5/EGFP}$129/Ola	4	D7	100	2-3	+2	+2	-	-	-	-	-
G10-Tg$^{Nkx2.5/EGFP}$129/Ola	4	D6	100	3-4	+2	+1	-	-	-	-	-
C57BL/6 (PZS3)	1	D8	33	1	-	-					
H3-Tg$^{Nkx2.5/EGFP}$C57BL/6	2.5	D6	67	1-2	+2	+2	+2	+1	+1	-	-
H5-Tg$^{Nkx2.5/EGFP}$C57BL/6	1	-	0	-	+2	+2	+2	+2	+2	+2	+2
H8-Tg$^{Nkx2.5/EGFP}$C57BL/6	1	-	0	-	+2	+2	+2	+2	+2	+2	+2

Table 1: Establishment and characterization of HM1 (129/Ola) $^{TgNkx2.5/EGFP}$ and Pzs3 (C57Bl/6) $^{TgNkx2.5/EGFP}$ ESC lines. Among 15 and 3 targeted colonies (HM1 and Pzs3, respectively) were characterized based on the ability to form EBs, EGFP expression, and *in vitro* differentiation ability towards cardiomyocytes. One Tg$^{Nkx2.5\text{-}eGFP}$ ESC line/one genotype mouse ESCs, named as B5-HM1-Tg$^{Nkx2.5\text{-}eGFP}$ and H3-Pzs3-Tg$^{Nkx2.5\text{-}eGFP}$ derived from HM-1 (129/Ola strain derived) and Pzs3 (C57Bl/6 mouse strain derived), respectively were chosen for further evaluating differentiation into Nkx2.5-eGFP CPCs and mature cardiomyocytes.

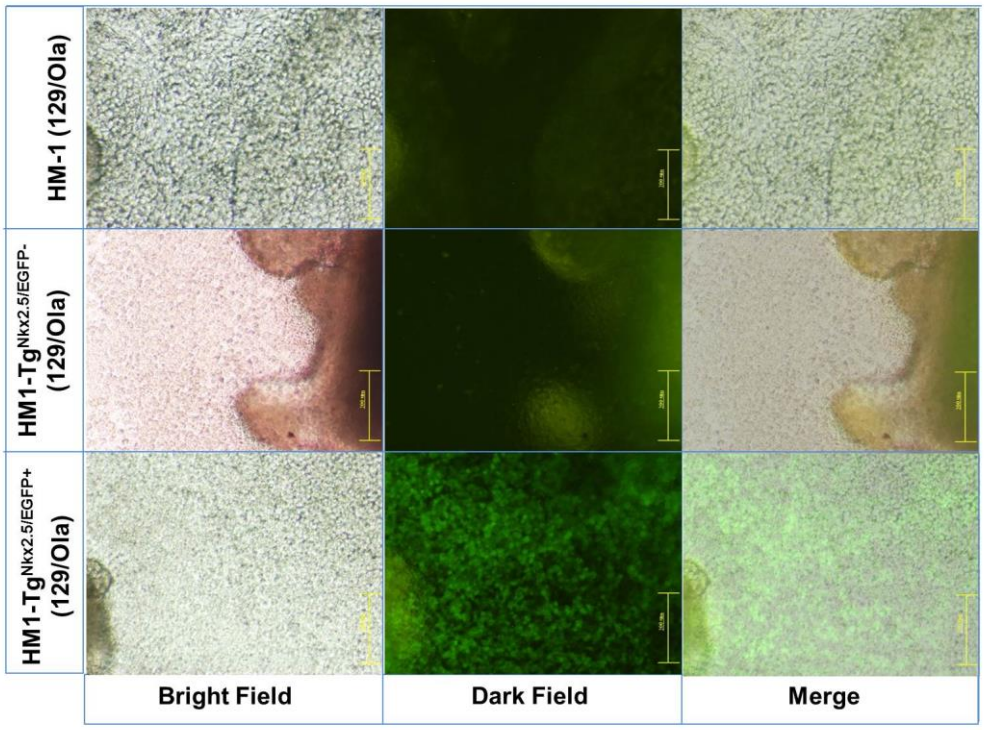

Figure 7. Expression of GFP during early cardiac differentiation. EGFP positive cells are invisible in HM1 and HM1-Tg$^{Nkx2.5\text{-}eGFP-}$ but are visible in HM1$^{TgNkx2.5/EGFP+}$ on cardiac differentiation (day 5).

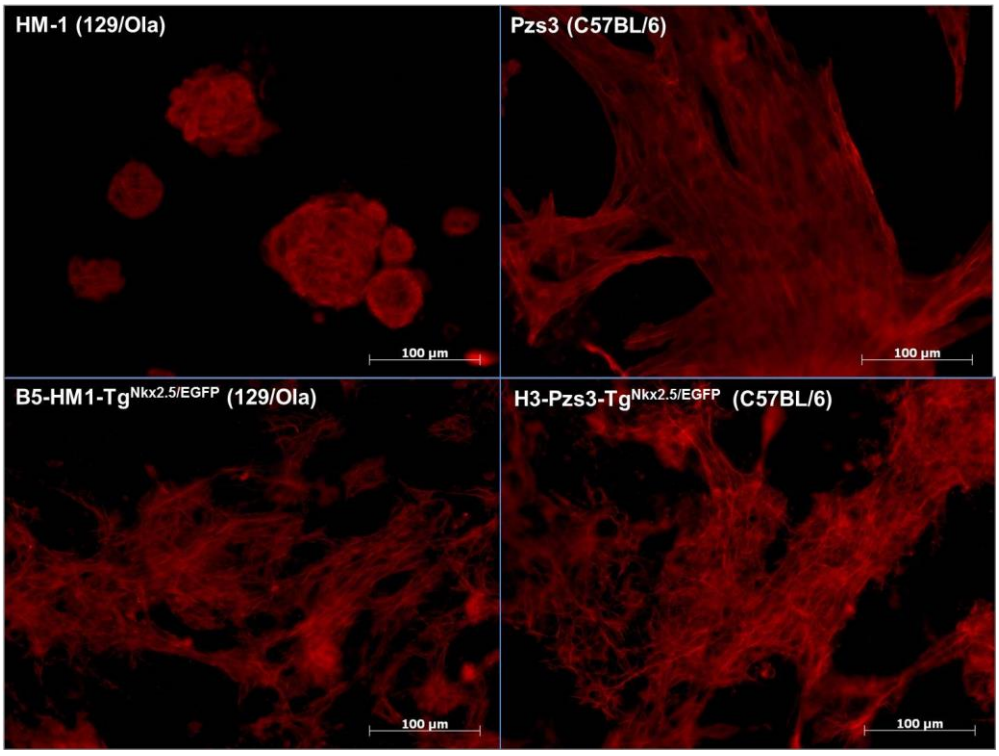

Figure 8. Cardiac differentiation potential of EBs. cTnT were expressed in ESC lines on day 14 during cardiac differentiation (red color).

Chapter 5

Discussion

Track and purification of ESC-CPCs

To track and purify CPCs from a mixed population differentiated from ESCs, we have established an ESC line carrying a GFP as a reporter gene knocked into the *Nkx2.5* locus, $Tg^{Nkx2.5\text{-}eGFP}$ ESCs. Although similar methods have been applied to track EB-derived cardiomyocytes through the *Mlc2v* and *Nkx2.5* gene promoters (Meyer et al., 2000; Hidaka et al., 2003), we focused primarily on programmed towards cardiomyogenic lineage. Moreover, we also established $Tg^{Nkx2.5\text{-}eGFP}$ ESCs (HM1-$Tg^{Nkx2.5\text{-}eGFP}$ and Pzs-$Tg^{Nkx2.5\text{-}eGFP}$ ESC lines), derived from two different genetic backgrounds (129/Ola and C57Bl/6, respectively). We found that the efficacy of cardiac differentiation depends on the line-to-line variability of $Tg^{Nkx2.5\text{-}eGFP}$ ESC lines and genetic backgrounds but is not depend on Nkx2.5-eGFP transfection, consistent with other reports (Hidaka et al., 2003). The selected $Tg^{Nkx2.5\text{-}eGFP}$ ESC lines were differentiated into beating cardiomyocytes and expressed cTnT, specific cellular marker expressed during lineage differentiation. Contrast with some researchers, the loss of one copy of *Nkx2.5* affected cardiac morphogenesis, and the transcription of *Mlc2v* and *Anp* genes was partially reduced in Nkx2.5GFP ES cells. These results suggest that Nkx2.5 strictly controls expression levels of these target genes but that a half dose of Nkx2.5 is adequate to induce cardiac differentiation *in vitro*.

Nkx2.5 is expressed in many non-cardiac tissues, including pharynx, spleen, distal stomach (Kasahara et al., 1998); thus, the GFP(+) cell population perhaps expressed in non-cardiac cells. We demonstrated that $Tg^{Nkx2.5\text{-}eGFP}$ ESC lines did not express GFP(+) during ESC maintenance and Nkx2.5-eGFP CPC differentiation after day 8. We demonstrated that almost all Nkx2.5-eGFP CPC was stained with cTnT antibodies, indicating that most GFP(+) cells we collected are developing mature cardiomyocytes.

Differentiation and diversification of purified Nkx2.5-eGFP CPCs

The cTnT is the tropomyosin-binding subunit of the troponin complex, which plays a crucial role in regulating cardiac muscle contraction. The cTnT is

expressed only in a small proportion of beating foci at the early stages of cardiac differentiation (foci contracting for less than seven days), while it is expressed in most beating foci at later stages (contracting for more than 21 days) (Rungarunlert et al., 2013). We revealed that some Nkx2.5-eGFP CPCs weakly expressed cTnT just after plating, but numerous completely expressed it after several days in culture. We suggest that Nkx2.5-eGFP CPCs can differentiate into mature cardiomyocytes after purifying from mix differentiated cells.

In this study, most Nkx2.5-eGFP CPCs at an early stage of culture (d4+3) were spontaneously beating, as reported by Maltsev *et al.* (1997). However, they are no information on action potential characteristics, including SA node-type, atrial-type, and ventricular-type. Hence, this study should be investigated in the future.

Chapter 6

Conclusion

We have established $Tg^{Nkx2.5\text{-}eGFP}$ ESCs to track and purify the fate of Nkx2.5-eGFP CPCs during cardiomyocyte differentiation. They certainly differentiate into cardiomyocytes. These properties appear to be similarly related to those of cardiomyocytes differentiated *in vivo*. Therefore, this $Tg^{Nkx2.5\text{-}eGFP}$ ESCs are valuable for further understanding the mechanism of cardiomyocyte differentiation and cell fate diversification of subpopulations and will also provide us relevant information to manage and control cardiac differentiation from pluripotent stem cells *in vitro*.

Chapter 7

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Output

International Journal Publications

1. Chakritbudsabong W, Sariya L, Pamonsupornvichit S, Pronarkngver R, Chaiwattanarungruengpaisan S, Ferreira JN, Setthawong P, Phakdeedindan P, Techakumphu M, Tharasanit T, **Rungarunlert S*.** 2017. Generation of a pig induced pluripotent stem cell (piPSC) line from embryonic fibroblasts by incorporating LIN28 to the four transcriptional factor-mediated reprogramming: VSMUi001-D. Stem Cell Res. 24:21-24. (Impact factor: 4.489, year 2019)
2. **Rungarunlert S**, Ferreira JN, Dinnyes A. 2016. Novel Bioreactor Platform for Scalable Cardiomyogenic Differentiation from Pluripotent Stem Cell-Derived Embryoid Bodies. Methods Mol Biol. 1502:169-79.
3. Klincumhom N, Tharasanit T, Thongkittidilok C, Tiptanavattana N, **Rungarunlert S**, Dinnyés A, Techakumphu M. 2014. Selective TGF-β1/ALK inhibitor improves neuronal differentiation of mouse embryonic stem cells. Neurosci Lett. 578: 1-6. (Impact factor: 2.274, year 2019)

Appendix

Order	International Journal Publications
1	Chakritbudsabong W, Sariya L, Pamonsupornvichit S, Pronarkngver R, Chaiwattanarungruengpaisan S, Ferreira JN, Setthawong P, Phakdeedindan P, Techakumphu M, Tharasanit T, **Rungarunlert S*.** 2017. Generation of a pig induced pluripotent stem cell (piPSC) line from embryonic fibroblasts by incorporating LIN28 to the four transcriptional factor-mediated reprogramming: VSMUi001-D. Stem Cell Res. 24:21-24. (Impact factor: 4.489, year 2019)
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Lab Resource: Single Cell Line

Generation of a pig induced pluripotent stem cell (piPSC) line from embryonic fibroblasts by incorporating LIN28 to the four transcriptional factor-mediated reprogramming: VSMUi001-D

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ABSTRACT

Pig induced pluripotent stem cell (piPSC) line was generated from embryonic fibroblast cells using retroviral transduction approaches carrying human transcriptional factors: OCT4, SOX2, KLF4, c-MYC and LIN28. The generated piPSC line, VSMUi001-D, was positive for alkaline phosphatase activity and expressed the pluripotency associated transcription factors including OCT4, SOX2, NANOG and surface markers SSEA-1, all iPSC hallmarks of authenticity. Furthermore, VSMUi001-D exhibited a normal karyotype and formed embryoid bodies *in vitro* and teratomas *in vivo*. Upon cardiac differentiation, VSMUi001-D displayed spontaneous beating and expressed cardiomyocyte markers, like cardiac Troponin T.

Resource table.

Unique stem cell line identifier	VSMUi001-D
Alternative name(s) of stem cell line	PR39
Institution	Faculty of Veterinary Science, Mahidol University, Thailand
Contact information of distributor	Sasitorn Rungarunlert, sasitorn.run@mahidol.edu
Type of cell line	Induced pluripotent stem cells
Origin	Pig
Additional origin info	N/A
Cell source	Pig embryonic fibroblasts
Method of reprogramming	Retrovirus
Genetic modification	NO
Type of modification	NO
Associated disease	NO
Gene/locus	NO
Method of modification	NO
Name of transgene or resistance	NO
Inducible/constitutive system	NO
Date archived/stock date	July 2016
Cell line repository/bank	N/A
Ethical approval	Institutional Animal Care and Use Committee of Faculty of Veterinary Science, Mahidol University, Nakhon Pathom, Thailand approval obtained (VSMU-2012-57)

Resource utility

LIN28 plays crucial roles in the self-renewal of pluripotent stem cells, growth and cellular metabolism, tissue development, and tumorigenesis. However, its molecular mechanisms regulating cellular growth and differentiation are unknown. Hence, this cell line can be utilized for determining the function of LIN28 on pluripotency and differentiation into three germ layers (Table 1).

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Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Visual record of the line: normal	Fig. 1, panel A
Phenotype	Immunocytochemisty	Expression of pluripotency markers: OCT4, SOX2, and SSEA-1	Fig. 1, panel D
	Flow cytometry	N/A	
Genotype	Karyotype (G-banding) and resolution	Normal karyotype: 38, XY Resolution 450–500	Fig. 1, panel E
Identity	Microsatellite PCR (mPCR)	N/A	
	STR analysis	N/A	
Mutation analysis (IF APPLICABLE)	Sequencing	N/A	
	Southern Blot OR WGS	N/A	
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR: Negative	Fig. 1, panel I
Differentiation potential	EB formation, spontaneous cardiac differentiation and teratoma formation	Spontaneous formation of EBs in suspension culture. Functional beating regions developed after plating the EBs, and these expressed cTnT by IF. Formed teratomas contained the three germ layers. Expression of *SOX17* (endoderm), *NeuroD* (ectoderm), and *Endolase*, *TNNT2* and *TNNI1* (mesoderm and cardiac differentiation markers) in EBs and cardiac differentiation by RT-PCR.	Fig. 1, panels F, G and H
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Not performed	
Genotype additional info (OPTIONAL)	Blood group genotyping	Not performed	
	HLA tissue typing	Not performed	

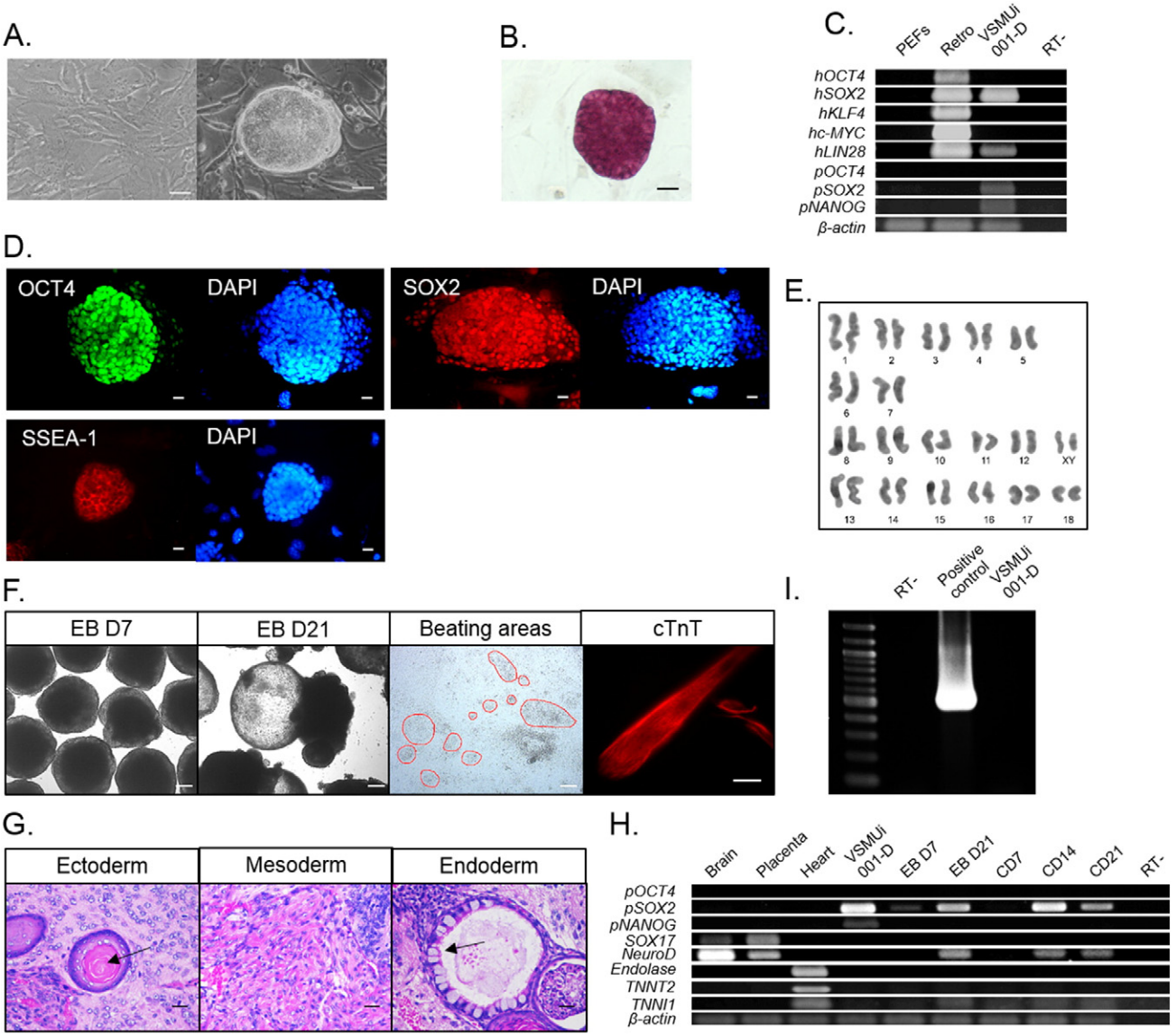

Fig. 1. Characterization of VSMUi001-D.

Resource details

The pig embryonic fibroblasts (PEFs) were isolated from a crossbred pig (Large White/Landrace/Duroc) at embryonic day 28 (E28). To generate VSMUi001-D cell line, five human reprogramming transcription factors (OCT4, SOX2, KLF4, c-MYC and LIN28) were delivered to PEFs by retroviral infection (Esteban et al., 2009). Five days after the infection, pig induced pluripotent stem cell (piPSC)-like colonies were clearly observed and were picked manually after 9–15 days post-reprogramming. Picked colonies were cultured on a feeder layer of mitomycin C inactivated mouse embryonic fibroblasts (iMEFs) in piPSC media and routinely passaged every 2 days by using TrypLE™ Select. VSMUi001-D colonies exhibited a typical mouse embryonic stem cells (mESCs) morphology including compact, dome shape, and clear edge colonies, even after they were propagated over 40 passages. These colonies differed from PEFs (Fig. 1A) (Cheng et al., 2012). VSMUi001-D colonies produced high alkaline phosphatase (AP) activity (Fig. 1B). Moreover, VSMUi001-D also expressed two endogenous pluripotent gene (*pSOX2* and *pNANOG*) as well as two exogenous pluripotent genes (*hSOX2* and *hLIN28*); however, three exogenous pluripotent genes (*hOCT4*, *hKLF4* and *hc-MYC*) were not expressed after transcriptome analysis with reverse transcription polymerase chain reaction (RT-PCR) (Fig. 1C). Further, these colonies were positively immuno-stained with pluripotent transcription factors OCT4 and SOX2, as well as surface marker SSEA-1 (Fig. 1D), indicating that such phenotypic features resemble those of mESCs. The VSMUi001-D cell line exhibited a normal karyotype (38, XY) (Fig. 1E). VSMUi001-D spontaneously generated embryoid bodies (EBs) in suspension culture (day 7 and 21) (Fig. 1F). Moreover, VSMUi001-D formed teratomas containing three germ layers: a keratinized squamous epithelium (ectoderm), skeletal muscles (mesoderm), and a respiratory-like epithelium (endoderm) (Fig. 1G). In addition, VSMUi001-D efficiently formed spontaneously beating functional cardiomyocytes and expressed cardiomyocyte markers including cardiac Troponin T (cTnT) on day 21 of cardiac differentiation (Fig. 1F). Lastly, cardiomyocyte and differentiation genes specific for the three germ layers were confirmed by RT-PCR (Fig. 1H). VSMUi001-D was closely related to *Sus scrofa* with 100% nucleic acid identity by using the nucleotide BLAST (Basic Local Alignment Search Tool) program. This cell line was shown not to be contaminated with mycoplasma (Fig. 1I). Scale bars of Fig. 1 are (A, B, D) 20 μm, (F) 100 μm, (F, cTnT) 20 μm and (G) 25 μm.

Materials and methods

Cell culture

The PEFs were isolated and all cells were maintained using a previous protocol (Cheng et al., 2012).

Generation of retrovirus and VSMUi001-D line

The generation of retroviruses was based on a published protocol (Esteban et al., 2009). All plasmids were purchased from Addgene, pMXs-hOCT4 (Cat# 17217), pMXs-hSOX2 (Cat# 17218), pMXs-hKLF4 (Cat# 17219), pMXs-hc-MYC (Cat# 17220) and pMXs-hLIN28A (Cat#47902). Briefly, the VSV-G-coated retrovirus was produced by co-transfection of pMXs-vector and pVSV-G into GP2–293 cells with X-treme GENE Reagents (Roche). The supernatant containing the virus was collected at 48 h and 72 h, and directly used to infect PEFs. After 2 days, the PEFs were digested by 0.25% trypsin and replaced onto iMEFs. After 9–15 days post-reprogramming, ESC-like colonies were first passaged mechanically using a Pasteur pipette, and by using TrypLE™ Select at later passages.

Alkaline phosphatase and immunofluorescence (IF) staining

Cells were fixed with 4% paraformaldehyde in PBS. For AP activity, piPSCs were incubated in a mixture of naphthol AS-BI alkaline solution with fast red violet LB (Leukocyte Alkaline Phosphatase Kit) for 30 min. The AP-producing piPSC colonies will stain red. The expression of pluripotency and cardiac differentiation markers were confirmed by IF staining according to previous protocol (Cheng et al., 2012). Primary and secondary antibodies displayed in Table 2 were used.

Table 2
Reagents details.

Antibodies used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat# and RRID
Pluripotency markers	Goat anti-OCT4	1:100	Santa Cruz Cat# sc-8628, RRID:AB_653551
	Rabbit anti-SOX2	1:100	Santa Cruz Cat# sc-20,088, RRID:AB_2255358
	Mouse anti-SSEA-1	1:100	Santa Cruz Cat# sc-101,462, RRID:AB_1568826
Differentiation markers	Rabbit anti-cTnT	1:200	Abcam Cat# Ab-33,589, RRID:AB_727035
Secondary antibodies	Alexa Fluor 488 donkey anti-goat IgG	1:2000	Thermo Cat# A-11055, RRID:AB_142672
	Alexa Fluor 594 goat anti-rabbit IgG	1:2000	Thermo Cat# A-11037, RRID:AB_2534095
	Goat anti-mouse IgM-PE	1:1000	Santa Cruz Cat# sc-3768, RRID:AB_649151

Primers		
	Target	Forward/reverse primer (5′–3′)
Exogenous genes (RT-PCR)	*hOCT4*	GTTGCTCTCCACCCCGACTCCTGCTTC/GAGAACCGAGTGAGAGGCAAC
	hSOX2	CCCCTGTGGTTACCTCTTCCTCC/TGCCGTTAATGGCCGTGCC
	hKLF4	GGCTGATGGGCAAGTTCG/CTGATCGGGCAGGAAGGAT
	hc-MYC	GCAGCGACTCTGAGGAGGAACAA/TTTTCCTTACGCACAAGAGTTCCGT
	hLIN28	TCAGCCGACGACCATGGGCT/CCATGTGCAGCTTACTCTGGTGCAC
Pluripotency markers (RT-PCR)	*pOCT4*	ACAAGGAGAAGCTGGAGCCG/CGCGGACCACATCCTTCTCT
	pSOX2	GGTTACCTCTTCTTCCCACTCCA/CAAAAATAGTCCCCCCAAAAGAAG
	pNANOG	TCTGTGTCAGTTTGAGGGACAGG/AACAAGTAAAGCCTCCCTATCCCA
Endoderm genes (RT-PCR)	*SOX17*	CGCACGGAGTTTGAACAATA/CAGACGTCGGGGTAGTTACAG
Ectoderm genes (RT-PCR)	*NeuroD*	GACTTGCGTTCAGGCAAAAGC/GGGCGACTGGTAAGAGTAGG
Mesoderm and cardiac differentiation marker (RT-PCR)	*Endolase*	TCTGTGACTGAATCTATCCAGG/CTTTGGGTTACGGAACTTGCG
	TNNT2	GACGGAGCGTAAGAGTGG/CAGGTCAAACTTCTCCGCC
	TNNI1	AGAGAAAACCCAAGATCACTG/CAGGTAGCGAGCCTTCTCA
House-keeping genes (RT-PCR)	*β-actin*	CGGGACCTGACTGACTACCTC/CCTTAATGTCACGCACGATTTCC
E.g. genotyping		
sequencing (RT-PCR)	*Mitochondrial cytb gene*	ACGGATGAGTTATTCGCTA/ATAGGACGGTAAGTAGTAATGG
Mycoplasma detection (RT-PCR)		CGCCTGAGTAGTACGTWCGC/GCGGTGTGTACAARACCCGA

Karyotype analysis

The piPSCs were treated with 5 μg/ml KaryoMAX® Colcemid™ solution for 1 h and harvested into a single cell solution. Next, 20 G-banded metaphases were used for analysis.

Identification of pig cells

To identify pig cells, primers specific to mitochondrial *cytb* gene (Table 2) were used and the PCR was performed according to the previous protocol (Yang et al., 2014). The PCR product was further confirmed by the di-deoxy dye terminator DNA sequencing method. The nucleotide sequence was analyzed by BLAST.

Differentiation of iPSCs

For differentiation into three-germ layers, suspension culture of EBs was maintained in long-term culture for 21 days. Spontaneous cardiac differentiation was performed according to our established protocol (Rungarunlert et al., 2013). The differentiated cells were collected at day 7, 14 and 21 for RT-PCR-based gene profiling.

Teratoma formation

Approximately 8×10^6 piPSCs combined with a Matrigel substrate (Corning, Inc., USA) were injected into the right flank of nude mice ($n = 2$). After 22 days, tumors were collected for histological analysis to check for their *in vivo* differentiation capacity into derivatives of all three germ layers.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells using the total RNA mini kit (Geneaid Biotech Ltd., Taiwan). The cDNA was synthesized with the Superscript III first-strand synthesis system followed by PCR using GoTaq® green master mix (Promega, USA) to detect the expression of each gene. All PCR products were separated using 2% agarose gels. Primer sequences of PCR product are shown in Table 2.

Mycoplasmas detection

The piPSCs were collected for DNA extraction and the PCR protocol was done according to a previous protocol (Sariya et al., 2015). Primer sequences of PCR product are shown in Table 2.

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Novel Bioreactor Platform for Scalable Cardiomyogenic Differentiation from Pluripotent Stem Cell-Derived Embryoid Bodies

Sasitorn Rungarunlert, Joao N. Ferreira, and Andras Dinnyes

Abstract

Generation of cardiomyocytes from pluripotent stem cells (PSCs) is a common and valuable approach to produce large amount of cells for various applications, including assays and models for drug development, cell-based therapies, and tissue engineering. All these applications would benefit from a reliable bioreactor-based methodology to consistently generate homogenous PSC-derived embryoid bodies (EBs) at a large scale, which can further undergo cardiomyogenic differentiation. The goal of this chapter is to describe a scalable method to consistently generate large amount of homogeneous and synchronized EBs from PSCs. This method utilizes a slow-turning lateral vessel bioreactor to direct the EB formation and their subsequent cardiomyogenic lineage differentiation.

Keywords: Bioreactor, Cardiomyocyte, Embryoid body, Pluripotent stem cells, Slow turning lateral vessel

1 Introduction

Approaches involving cardiomyocyte differentiation from pluripotent stem cells (PSCs) provide unique opportunities to produce sufficient cell numbers for cardiac regenerative therapy, tissue engineering, and drug screening for cytotoxicity testing [1, 2]. To establish these approaches for clinical and industrial applications, reliable and reproducible methodologies must be developed to ensure a scalable generation of differentiated PSCs for producing clinically relevant numbers of cardiomyocytes that are safe for human use [3].

Differentiation of PSCs into cardiomyocytes usually needs cell aggregation into spherical-like structures referred to as embryoid bodies (EBs), which are usually composed of three germ layers including the ones generating cardiac-like cells [4]. Although the hanging drop (HD) is able to produce homogeneous EBs (in terms of a regular shape and uniform size), this technique cannot produce EBs on a highly scalable manner, due to a labor-intensive procedure [5]. On the other hand, the static suspension culture (SSC)

technique is easier to perform, but it shows poor reliability in the cell aggregation step, leading to large and irregularly shaped EBs causing cellular apoptosis in their center core [6]. Therefore, the latter two methodologies are currently not suitable for clinical and industrial applications.

The slow-turning lateral vessel (STLV) bioreactor has been developed to overcome the limitations with the previously mentioned culture systems [7]. The STLV bioreactor allowed for direct PSC aggregation under depleted fluid shear stress, high mass transfer, microgravity, and oxygen diffusion in testing studies [8]. Our laboratory has developed a protocol for scalable EB formation and cardiomyogenic differentiation from mouse PSCs by using this STLV bioreactor. The protocol requires an initial seeding density of 3×10^5 PSCs/mL at 10 rotations per minute (rpm) and plating the EBs onto gelatin-coated dishes on the 3rd day of culture. This results in EB production at a large scale and also an increased potential for differentiation into cardiomyocytes [9]. Moreover, the STLV bioreactor is able to produce homogeneous EBs with tunable sizes and shapes [10]. When compared with the SSC technique, the STLV bioreactor system significantly enhanced the efficiency of EB formation by increasing the yield of EB formation/mL and the total cell yield of EB/mL by fourfold and sixfold, respectively; and also by reducing by 1 log the number of cells that were not incorporated into EBs [10]. This has been in agreement with another study [11]. However, this bioreactor system may produce variations in the size and shape of EBs in specific cell types such as human embryonic stem cells (ESCs) [12]. These heterogeneous EBs are generated perhaps due to (1) interruptions in the stirring process, (2) the microgravity within the STLV during media changes causing agglomeration of EBs, or (3) the cell seeding density and rotation speed not being fully optimized [9]. Additionally, STLV-derived EBs showed a larger beating area and a faster beating by enhancing up to 4.2 times the area of cardiac troponin T (cTnT) per EB when compared to SSC- and HD-derived EBs. These observations revealed that the STLV bioreactor methodology delivers scalability, reproducibility, and a relative simplicity in EB production and provides a striking efficiency in cardiomyogenic differentiation over the SSC and HD approaches [10].

2 Materials

2.1 Cell Lines

1. Mouse PSC lines (induced pluripotent stem cell (iPSC) or ESC lines) (*see* **Note 1**).
2. Feeder cells (Mitomycin C-inactivated confluent mouse embryonic fibroblast (MEF)).

2.2 Chemicals and Culture-Grade Reagents

1. High-glucose Dulbecco's modified Eagle medium (DMEM, Invitrogen, cat. no. 11965-092).
2. Fetal bovine serum (FBS, Hyclone, cat. no. SH30070.03) (*see* **Note 2**).
3. GlutaMAX™ Supplement (Invitrogen, cat. no. 35050-061).
4. β-mercaptoethanol (β-ME, Invitrogen, cat. no. 21985-023).
5. Nonessential amino acid solution (NEAA, Invitrogen, cat. no. 11140-050).
6. Penicillin–streptomycin (Invitrogen, cat. no. 15140-122).
7. Mouse leukemia inhibitory factor (mLIF, ESGRO, Chemicon International, cat. no. ESG1107).
8. L-ascorbic acid (Sigma, cat. no. A4403).
9. Trypsin (0.05 %) with ethylenediaminetetraacetic acid (EDTA) (Invitrogen, cat. no. 25300054).
10. Phosphate buffered saline without Ca^{2+}/Mg^{2+} (PBS, Invitrogen, cat. no. 10010-023).
11. Gelatin (Sigma, cat. no. G1890).
12. Trypan blue solution (0.4 % (wt/vol), Sigma Life Science, cat. no. T8154).
13. 70 % ethanol (ethyl alcohol).
14. Distilled water.
15. Labware detergent (e.g., 7× detergent).

2.3 Disposables

1. Cell culture dishes (60 and 100 mm, BD Falcon, cat. nos. 353002 and 353003).
2. Bacteriological petri dish (60 and 150 mm, BD Falcon, cat. nos. 351007 and 351058).
3. 24-well plate (Nunclon®, Sigma, cat. no. D7039).
4. Conical tubes (15 and 50 mL, Falcon, cat. nos. 352097 and 352098).
5. Serological pipettes (5, 10, and 25 mL, Thermo Scientific Nunc, cat. nos. 170355, 170356, and 170357).
6. Syringe (5 and 10 mL).
7. Rapid-Flow™ Sterile Disposable Filter Units (500 mL Bottle Top Filter with 75 mm Supor®machV PES Membrane, Thermo Scientific Nalgene®, cat. no. 595-4520).

2.4 Equipment

1. Slow-turning lateral vessel bioreactor (STLV, RCCS-4H with 110 mL of vessels, Synthecon, Cellon S.A. Bereldange, Luxembourg) (*see* **Note 3**).
2. Inverted phase-contrast microscope (Olympus, IX 71 equipped with an Olympus DP70 digital camera).

3. Stereomicroscope (Nikon, AZ100).
4. Laminar flow hood (Class II, Thermo Scientific).
5. Humidified incubator (37 °C, 5 % CO_2, Thermo Scientific).
6. Water bath (WNB 14, Memmert).
7. Centrifuge (Eppendorf, 5804R).
8. Hemocytometer, Neubauer improved (Carl Roth, cat. no. T728.1).
9. One-way stopcock with female Luer lock port and SPIN-LOCK® connector (B. Braun, cat. no. D100).
10. Laboratory glass bottle (100 and 500 mL, DURAN Group, cat. nos. 21801245, 21801445).
11. Micropipette (1–10, 10–100, and 100–1000 μL, Eppendorf, cat. nos. 3121000023, 3121000074, 3121000120).
12. Pipettor (Thermo Fisher Scientific, Matrix, CellMate II, cat. no. 9501).

2.5 Reagent Setup

1. Incomplete PSC medium: DMEM containing 15 % (vol/vol) FBS, 0.1 mM β-ME, 0.1 mM NEAA, 2 mM GlutaMAX™, and 1 % penicillin–streptomycin (50 μg/mL streptomycin and 50 U/mL penicillin). Filter-sterilize and store at 4 °C for up to 2 weeks (*see* **Note 4**).
2. Complete PSC medium: incomplete PSC medium plus 1000 or 2000 U/mL mLIF. Store at 4 °C for up to 2 weeks (*see* **Note 5**).
3. Cardiac differentiation medium: incomplete PSC medium supplemented with ascorbic acid at 50 μg/mL. Store at 4 °C for up to 2 weeks.
4. L-ascorbic acid: dissolve it in sterile water at 50 mg/mL (1000×). Then filter-sterilize and store temporarily at 4 °C (*see* **Note 6**). For long-term storage, aliquot and store at −20 °C for up to 6 months.
5. Gelatin coating solution 0.1 % (wt/vol): add 0.5 g of tissue culture-grade gelatin into 100 mL of PBS. Autoclave and store at 4 °C.

3 Methods (Fig. 1)

Maintain cell culture at 37 °C in a humidified incubator containing 5 % CO_2 in air. Perform all procedures under a sterilized laminar flow hood (Biological Safety Cabinet Class II). Warm up all mediums to 37 °C in a water bath. Change medium daily on mouse PSC cultures and every 2 days during the PSC differentiation stages.

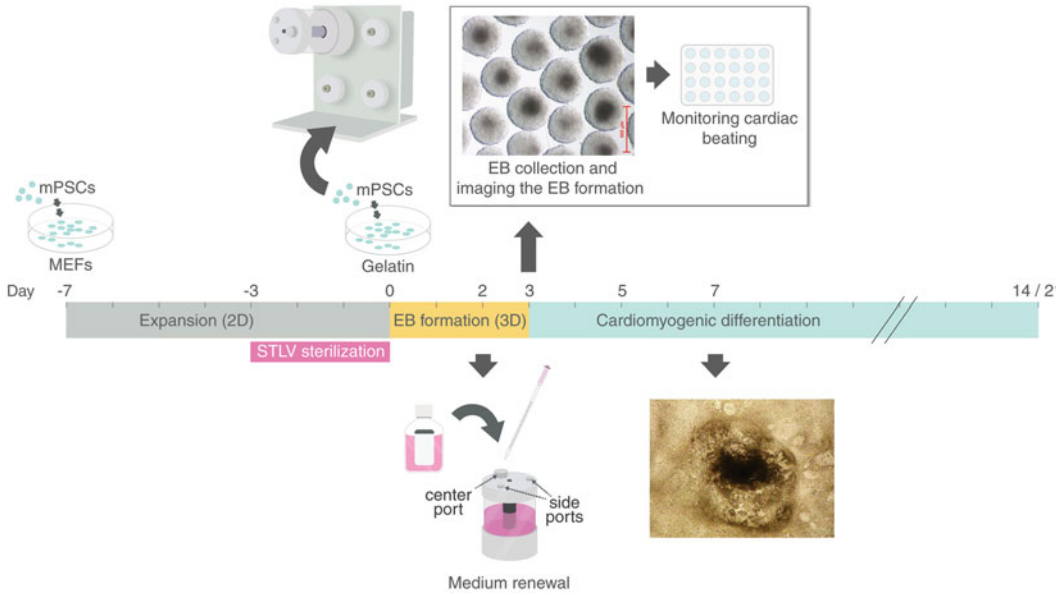

Fig. 1 Schematic illustration of the scalable cardiomyogenic differentiation from PSCs-derived EBs in a STLV bioreactor. PSCs are expanded as monolayers (2D) in 100-mm petri dishes until you obtain $\geq 3.3 \times 10^7$ cells/one vessels. Then, PSCs are passaged and seeded in STLV vessel for generating EB formation. Medium renewal is performed on day 2 and EBs are collected on day 3 for imaging the EB formation and monitoring cardiac beating by plated EBs into 24-well plate. For cardiomyogenic differentiation, beating areas are first observed 4 days after plating and continuously monitored the beating activities on a daily basis for a period of 14–21 days. Scale bar: 500 μm

3.1 Mouse PSC Lines Preparation (Day-7–Day 0)

1. Culture mouse PSCs on feeder cells for at least two passages after thawing in complete PSC medium (LIF 1000 U/mL).
2. Culture mouse PSCs for at least one passage in feeder-free conditions on gelatin-coated 100 mm plates in complete PSC medium (LIF 2000 U/mL) (*see* **Note** 7).

3.2 STLV Bioreactor System Preparation and Sterilization (Day-3)

1. Unscrew and remove the central screws of the STLV by using an Allen wrench.
2. Separate front plate and back plate of STLV. Take of a center port cap and two side port caps.
3. Place all pieces of STLV in plastic or steel bucket for soaking with a labware detergent.
4. Scour plastic parts (both plates of vessel, a center port cap, and two side port caps) with a sponge to remove any remainders.
5. Clean silicone rubber oxygenator transfer membrane which is located along the center axis of the vessel with the tip of your finger using laboratory gloves.
6. Rinse all pieces of STLV thoroughly under continuous flow of distilled water for 20 min and soak them in distilled water

overnight. Then, remove them from distilled water and allow them to dry in laminar flow.

7. Assemble all individual parts of the STLV except a center port cap.
8. With a 25 mL pipette, fill the 110 mL STLV with 70 % ethanol through a center port to soak for 24 h.
9. Open a center port cap and two side port caps of STLV to pour 70 % ethanol.
10. Remove both a center port cap and two side port caps. Then autoclave them separately from the vessel. Cover all port caps with aluminum foil before autoclaving.
11. Loosen the central screws of the STLV (1 turn) by using an Allen wrench.
12. Cover the STLV with aluminum foil and autoclave at 121 °C for 20 min.
13. Remove the STLV and all port caps from autoclave and allow them cool down at room temperature. Keep STLV in a sterile location before use.
14. The rotator base should be cleaned with an alcohol pad or embedded cloth at least 3 times before use. When not in use, rotator should be kept in a sterile place (*see* **Note 8**).

3.3 Culture Dish and Plate Preparation

1. A gelatin-coated 24-well plate and 100-mm dish; add 0.5 and 2 mL of 0.1 % gelatin to a 24-well plate and 100-mm dish, respectively.
2. Rotate plate to evenly distribute the gelatin and cover the entire surface.
3. Place gelatin-coated plate under the laminar flow hood for 30 min.
4. Aspirate off the remaining gelatin solution.
5. Add specific medium such as the incomplete PSC medium or complete PSC medium or cardiac differentiation medium.

3.4 EB Formation in the STLV Bioreactor (Day 0)

3.4.1 Passaging Mouse PSC Lines

1. Aspirate the culture medium and rinse cells once with 5 mL of PBS 1×.
2. Aspirate PBS and add 3 mL of 0.05 % trypsin/EDTA into the plate and place them in the humidified incubator approximately 3–5 min until cell detachment occurs.
3. Gently pipette media solution with cells to further dissociate the cells mechanically.
4. Place the dissociated cells and solution into a 15 mL tube containing 3 mL of incomplete PSC medium. Spin down the cells at 1000 rpm and 4 °C for 5 min by centrifugation.

5. Discard the supernatant and resuspend cells in 10 mL of incomplete PSC medium.
6. Calculate viable cells/mL by trypan blue staining using a hemocytometer.
7. Dilute the cells using incomplete PSC medium so that the final concentration is 3×10^5 cells/mL or 3.3×10^7 cells/110 mL.

3.4.2 Seeding mPSC Lines in STLV Bioreactor

1. Unwrap STLV and gently tighten central screws in the laminar flow.
2. Fill the 90 mL STLV with incomplete PSC medium through a center port using a 25 mL serological pipette.
3. Seed the resuspended cells with incomplete PSC medium through a center port using a 10 mL serological pipette and close all port caps. Wait 5 min to allow cells to settle.
4. Open the two side port caps and screw on pre-sterilized one-way stopcocks to the two side ports.
5. Clean the side ports with an alcohol pad. Fill a 5 mL sterile syringe with incomplete PSC medium and attach this syringe to a side port using a one-way stopcock. Attach an empty 5 mL syringe to another side port using a one-way stopcock.
6. Gently and slowly move the vessel to expel air bubbles from the ports. Gently suction air bubbles by pulling the plunger of the empty syringe and gently replace air bubbles by pressing the plunger of the syringe-containing medium (*see* **Note 9**).
7. After removing air bubbles, close all stopcocks and discard all syringes. Clean the side ports with an alcohol pad and cover the side port caps.
8. Wipe any spilled media immediately from the STLV vessel with an alcohol pad to avoid contamination.
9. Connect the STLV vessel to the rotator base and incubate them inside the 5 % CO_2 incubator.
10. Connect the rotator base to the power supply box, which can be brought out of the incubator to connect to an electrical power outlet (*see* **Note 10**).
11. Set the STLV rotation speed at a speed of 10 rpm and rotate for a minimum of 24 h to monitor for potential leaks (*see* **Note 11**).

3.5 Medium Renewal (Day 2)

1. Turn off bioreactor power and remove the STLV from the rotation base and take it to a laminar flow hood.
2. Let the cells or EBs settle at the bottom for 3 min and open a side port cap and gently aspirate 50 % of medium thought this port (*see* **Note 12**).

3. Fill the 50 mL volume of incomplete PSC medium into STLV through a side port using a 25 mL serological pipette. Wait for 3 min for allowing cells to settle and follow refill medium as described above Section 3.4.2 (**steps 5–12**).

3.6 Cardiomyogenic Differentiation (Day 3–21)

1. Turn off the bioreactor power switch and remove the STLV from the rotation base and take it to a laminar flow hood.
2. Let the cells or EBs settle at the bottom for 3 min and open a side port cap and aspirate 75 % of medium thought this port.
3. For imaging the EB formation stage: gently rotate the STLV and continue to transfer the EBs to a 60-mm dish using a 1000 μL sterile pipette tip (cut ~2 cm from the extremity). Acquire the EBs images by using an Olympus IX71 inverted microscope with an attached Olympus DP 70 digital camera (*see* **Note 13**).
4. For monitoring cardiac beating: gently rotate the STLV and continue transfer the EBs to a 150-mm bacteriological petri dish by using a 25 mL pipette. Carefully observe and select homogenous EBs under stereomicroscope. Place individual EBs into a gelatin-coated 24-well plate containing 500 μL of cardiac differentiation medium. Continuously observe and record the beating activities on a daily basis under an inverted phase-contrast microscope for a period of 14–21 days (*see* **Note 14**). Change medium every 2nd day.

4 Notes

1. The methodology described above was optimized using an HM1 ESC line (deficient in hypoxanthine phosphoribosyl transferase ESC line [9, 10]), A4-iPSC line and B5-iPSC line (mouse Sleeping Beauty transposon-generated iPSC lines [13]). When another PSC line is utilized, the efficiency of cardiac differentiation may be affected and might need further optimization.
2. The same lot of FBS should be used during the complete series of experiments. Changing the FBS may alter the reproducibility of the EB formation and cardiac differentiation.
3. The STLV, RCCS-4H is composed of four culture vessels, a rotator base and a power supply box. The vessels are available in 55, 110, 250, 450, 500, and 1000 mL capacities. We usually use RCCS-4H with 110 mL of culture vessels, which is able to produce approximately 20,000 EBs per vessel [10]. The high capacity vessels require large volume of media, leading to laborious and expensive process. We suggest the use of RCCS-4H with 55 mL or 110 mL of culture vessels.

4. A minimum 500 mL volume of incomplete PSC medium should be prepared for the entire duration of one replication (one 110 mL-culture vessel). To prepare complete PSC medium just add LIF to incomplete PSC medium (basal medium). To prepare cardiac differentiation medium add L-ascorbic acid instead.
5. Culture mouse PSCs in feeder-free conditions. The MEFs feeder cells can be substituted by the addition of LIF supplements in the culture medium (since LIF is secreted by MEFs).
6. L-ascorbic acid should be added in cardiac differentiation medium at the working concentration before use because it may lose efficacy.
7. The mouse PSCs should be expanded until you obtain $\geq 3.3 \times 10^7$ cells/one for 110 mL-culture vessels.
8. Do not autoclave the rotator base. Also, the rotator base should not be placed inside an incubator while not in use since it will rust the rotor/propeller.
9. When replacing air bubbles with medium, do not forcefully pull back the plunger of the empty syringe and push the other syringe-containing medium. Otherwise, this process can damage the oxygenation membrane, leading to poor oxygen diffusion.
10. When the STLV power box is placed inside an incubator it can get damaged. It should be conveniently placed outside, on the top or on the side of the external wall of the incubator.
11. The rotational movements should not be interrupted except when changing medium or collecting EBs; otherwise, it will cause agglomeration of EBs.
12. While changing medium, it is easy to lose floating EBs; thus, do not shake the STLV vessel during that procedure.
13. To make a better image of EBs, move a 60-mm dish anticlockwise until all EBs are located in center. Next, move EBs to get them closer to each other by using a tuberculin syringe.
14. After plating individual EBs into gelatin-coated 24-well plates, EBs should not be disturbed for 48 h (day 4–5 of culture) to allow EBs to attach the plate. Cardiomyogenic differentiation of ESCs and iPSCs should be monitored closely by assessing the beating activity for 14 and 21 days, respectively. The cardiac differentiation efficiency from mouse iPSCs is low and delayed compared with mouse ESCs. This is supported by data showing the percentage of beating EBs and the expression of cardiac-specific messenger RNA (mRNA) for troponin [14].

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Selective TGF-β1/ALK inhibitor improves neuronal differentiation of mouse embryonic stem cells

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HIGHLIGHTS

- Inhibition of TGF-β1/ALK pathway efficiently decrease contamination of pluripotent cells in ESC-derived neuronal population.
- TGF-β1/ALK inhibitor rapidly drives cell fate alteration from pluripotent state toward neuronal lineages.
- TGF-β1/ALK inhibitor significantly suppresses gliogenesis within EBs during neuronal differentiation.

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ABSTRACT

The transforming growth factor-β1 (TGF-β1), a polypeptide member of the TGF-β superfamily, has myriad cellular functions, including cell fate differentiation. We hypothesized that suppression of TGF-β1 signaling would improve the efficacy of neuronal differentiation during embryoid body (EB) development. In this study, mouse embryonic stem cells (ESCs) were allowed to differentiate into their neuronal lineage, both with, and without the TGF-β1 inhibitor (A83-01). After 8 days of EB suspension culture, the samples were examined by morphological analysis, immunocytochemistry and immunohistochemistry with pluripotent (Oct4, Sox2) and neuronal specific markers (Pax6, NeuN). The alteration of gene expressions during EB development was determined by quantitative RT-PCR. Our results revealed that the TGF-β1/ALK inhibitor potentially suppressed pluripotent gene (Oct4) during a rapidly up-regulation of neuronal associated genes including Sox1 and MAP2. Strikingly, during EB development, the expression of GFAP, the astrocyte specific gene, remarkably decreased compared to the non-treated control. This strategy demonstrated the beneficial function of TGF-β1/ALK inhibitor that rapidly and uniformly drives cell fate alteration from pluripotent state toward neuronal lineages.

1. Introduction

Most neurological disorders are considered to be incurable diseases due to the limited capability of the central nervous system (CNS) of adult mammals to regenerate when injured [7]. Novel strategies have been developed in attempts to repair or to replace neuronal cells *via* cell transplantation, treatment with antibodies against myelin-associated neurite growth inhibitors or therapeutic targeting of neuronal-specific receptors [19,26,29].

Pluripotent stem cells have become an alternative source of neuronal cells because theoretically they have the capability to highly proliferate, self-renew and develop into all cell types of the body including neurons [14]. Although embryonic stem cells (ESCs) hold much promise as an *in vitro* model for investigation of mammalian neurogenesis, the generation of ESC-derived neurons for efficient therapeutic study of neurological disorders such

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as stroke, spinal cord injuries, Parkinson's and Alzheimer's diseases remains challenging [17,40]. Many studies demonstrated the robust generation of neuronal cells from mouse ESCs using embryoid body (EB) formation. The 3D-structures of aggregated cells spontaneously differentiate into derivatives of three-embryonic germ layers including ectoderm, mesoderm and endoderm [16,27]. The committed cells within the EB are then induced to differentiate into neuronal lineages by a biologically active form of retinol (retinoic acid; RA) treatment which has been known as an essential factor driving the CNS development during embryogenesis [3,37]. Although the efficiency of neuronal differentiation within the EBs is still limited due to the non-neuronal cell contamination [43], it has been widely used as a cost-effective, simple and potential platform for several studies during neurogenesis [9,24]. Improving the strategy of neuronal differentiation of ESCs is therefore a prerequisite for studying the cellular and molecular mechanisms of neurogenesis and also providing the high-yield generation of homogenous neuronal cell subtypes for cell transplantation and regenerative medicine.

Transforming growth factor beta 1 (TGF-β1) is a polypeptide member of the TGF-β superfamily that is comprised of Bone Morphogenetic Proteins (BMPs), Growth and Differentiation Factors (GDFs), Activin, Nodal and the TGF-β family. These transcription factors have been demonstrated as pleiotropic factors as their effects depend on their concentrations as well as their combinations with other transcription factors. Their signaling transductions are modulated through SMAD phosphorylation [41]. The expression and functions of transcription factors in the TGF-β superfamily are conserved among the species studied and actively involved in cellular homeostasis and differentiation [8,25]. In ESCs, over expression of Nodal induces up-regulation of both mesodermal and endodermal genes, while the neuroectodermal genes were down regulated [33]. Furthermore, proliferation of mouse hippocampal neural progenitor cells and neuronal cells production were inhibited by TGF-β1 treatment [5].

In the current study, the effects of the selective TGF-β1/ALK inhibitors (A83-01 that suppresses activin/nodal signaling pathways including TGF-β type I receptor ALK5 kinase, type I activin/nodal receptor ALK4 and type I nodal receptor ALK7) were examined during neuronal differentiation of mouse ESCs during EB formation [44]. We thus hypothesized that suppression of TGF-β1 signaling would improve the efficacy of neuronal differentiation.

2. Material and methods

2.1. Materials and cell culture condition

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and culture media were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA), unless specified otherwise. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. Medium was changed daily for ESCs culture and on every second day during differentiation.

2.2. Cell culture

Mouse (129S2/SvPas) D3 ESCs (ATCC, Manassas, VA, USA) were cultured on mitomycin C inactivated mouse embryonic fibroblasts (MEFs) as previously described [23]. The pluripotent ESCs were maintained in ESC medium: Dulbecco's modified Eagle's medium (DMEM) containing 15% (v/v) fetal bovine serum (FBS, ES grade Invitrogen), 0.1 mM non-essential amino acids (NEAA), 0.1 mM β-mercaptoethanol (β-ME), 0.1 mM antibiotic–antimycotic (Anti–Anti) and 1000 U/ml mouse leukemia inhibitory factor (LIF, ESGRO, Chemicon International, CA, USA). ESCs were cultured on gelatin-coated dishes in the presence of LIF (2000 U/ml) in ESC medium for at least one passage prior to differentiation, in order to deplete present MEFs from the system.

2.3. TGF-β1 inhibitor treatment during neuronal differentiation

Mouse D3 ESCs were induced to differentiate into neuronal lineage as previously described [15]. Briefly, pluripotent cells were allowed to aggregate in suspension and form EBs for 4 days. All-trans RA (5 μM) was then added to the medium, and the EBs were further cultured for 4 days as shown in Fig. 1A. The 8-day-old EBs were then dissociated into single cell and reseeded in poly-L-ornithine and laminin-coated dish (Roche, CA, USA) at density 2×10^5 cell/cm^2 in neural differentiation medium.

For TGF-β1 inhibitor treatment, the EBs were either cultured with or without 1 μM TGF-β1 inhibitor (A83-01, Biovision, Milpitas, USA) and were harvested for further analysis on days 2, 4, 6 and 8 of culture. The experiments were replicated three times.

2.4. EB morphological analysis

The EB was generated by a hanging drop method as recently described [38]. Two day-old EBs were collected into a new Petridish containing phosphate buffered saline (PBS) supplemented with 5% (v/v) FBS. The EB diameter was calculated after measuring the large and small diagonals of 20 EBs derived from both control and A83-01-treated groups. The measurement was performed under a phase contrast microscope (CKX41, Olympus, Shinjuku, Japan).

2.5. Analysis of alkaline phosphatase (ALP) activity

Mouse ESCs prepared for characterization were cultured for 2–3 days on MEF-coated coverslips. Cells were washed with PBS and then fixed in 4% (v/v) paraformaldehyde (PFA) for 15 min at room temperature (RT). Alkaline phosphatase staining was performed using an ALP histochemistry kit according to the manufacturer's protocol. The ALP-positive ES colonies typified by bright pink/red color under an inverted light microscope.

2.6. Immunocytochemistry

Cultured cells were fixed with 4% PFA for 15 min at RT. Permeabilization was performed using 0.2% (v/v) Triton X-100 (for intracellular staining) for 30 min at RT. Cells were then blocked with 3% (w/v) bovine serum albumin containing 0.5% (v/v) Tween20 in PBS for 30 min. The cells were sequentially incubated at 4 °C with the following primary antibodies diluted in blocking solution overnight: pluripotent marker Oct4 (SC-9081, dilution 1:100, Santa Cruz Biotechnology; mouse) and Sox2 (SC-20088, dilution 1:100, Santa Cruz Biotechnology; rabbit), neuronal marker TUJ1 (PRB-435P, dilution 1:2000, Covance; rabbit) and glial marker GFAP (G6171, dilution 1:200; mouse). The cultures were washed three times with PBS and then incubated with fluorescent-labeled secondary antibodies (Alexa fluor® 488 and Alexa fluor® 647-labeled goat IgG; dilution 1:2000) for 1 h at RT. After three washes with PBS, the cells were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) for 20 min. The examination was performed using a fluorescent microscope (BX51 Olympus, Tokyo, Japan) equipped with a DP72 camera and DP2-BSW software (Olympus, Tokyo, Japan).

2.7. Immunohistochemistry

Eight-day-old EBs were fixed with 4% PFA and cryosectioned at 7-μm thickness. Antibodies used for EB immunolabeling included:

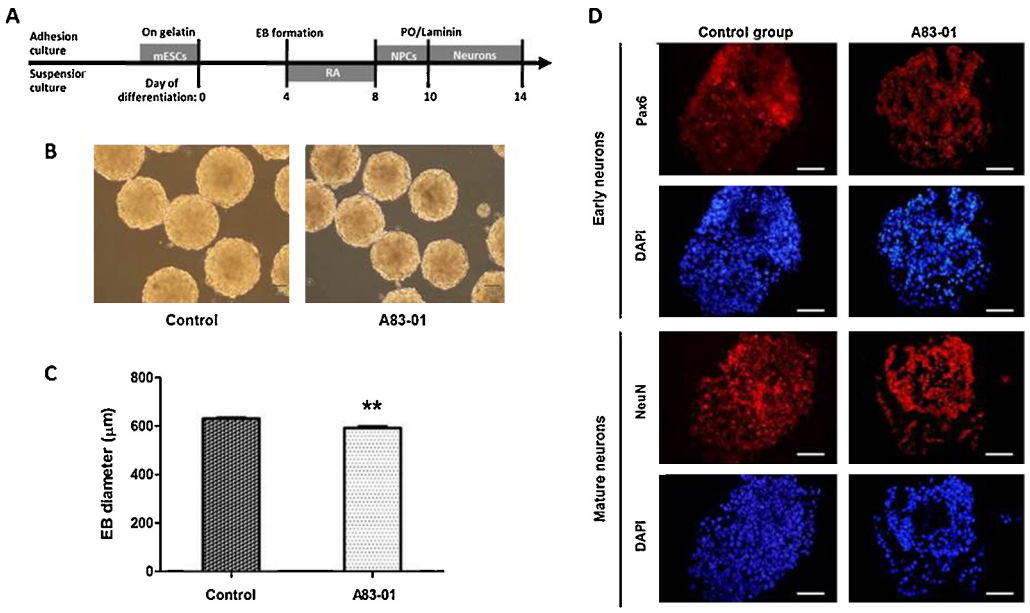


Fig. 1. Differentiation of mouse ESCs into neuronal lineages. (A) Schematic of neuronal differentiation of ESCs using EB formation method. (B) To evaluate the size of ESC-derived EBs, ESCs were allowed to aggregate into 3D-structure by a hanging drop method for 2 days. (C) The diameters of two-day old EBs from control and A83-01-treated groups were measured. Data represented as mean ± S.E.M. ($N = 20$ EBs from each group). The results were considered significant when $^{*}P < 0.01$. (D) To characterize the early and mature stage of neuron during formation of RA-treated EBs, the expression of Pax6 and NeuN was examined by immunohistochemistry. Note that the expression of these markers in both groups is distributed throughout the EB. Scale bar: 100 mm.

neuroepithelial cell (NEPC) marker Pax6 (Pax6, dilution 1:200, DSHB; mouse) and mature neuronal cell marker NeuN (MAB 377, dilution 1:100, Millipore; mouse). Primary antibodies were detected by anti-mouse TritC secondary antibody (T2402, dilution 1:100). Slides were counterstained with DAPI. The sections were analyzed with a fluorescent microscope.

2.8. Quantitative RT-PCR assays

Total RNA was extracted from EBs day 2, 4, 6 and 8 by using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. For each sample, 500 ng RNA were reversely transcribed using the First-Strand cDNA SynthesisKit (SuperScript III Kit, Invitrogen, CA, USA), as per the manufacturer's instructions. After the reverse transcription, the cDNA samples were diluted to 2.5 ng/μl and dispensed to once-use aliquots. Quantitative real-time RT-PCR was performed on the ABI PRISM 7300 Real-time cycler (Applied Biosystems, CA, USA) with Power SYBR Green PCR Master Mix (Applied Biosystems, WA, UK) and specific primer pairs listed in supplementary method. The melting curves were plotted to confirm the specificity of the product. Relative expression was calibrated to the undifferentiated ESCs (day 0) equaling 1. Expression levels of individual transcripts were normalized to GAPDH expression.

2.9. Statistical analysis

The statistics generated in this study were assessed by Student's *t*-test using SPSS software (version17.0 SPSS Inc., IL, USA). Data were expressed as a mean ± standard error of the mean (SEM), of at least three independent replications. The results were considered significant difference when *P*-values were less than 0.05 (*) and 0.01 (**).

3. Results

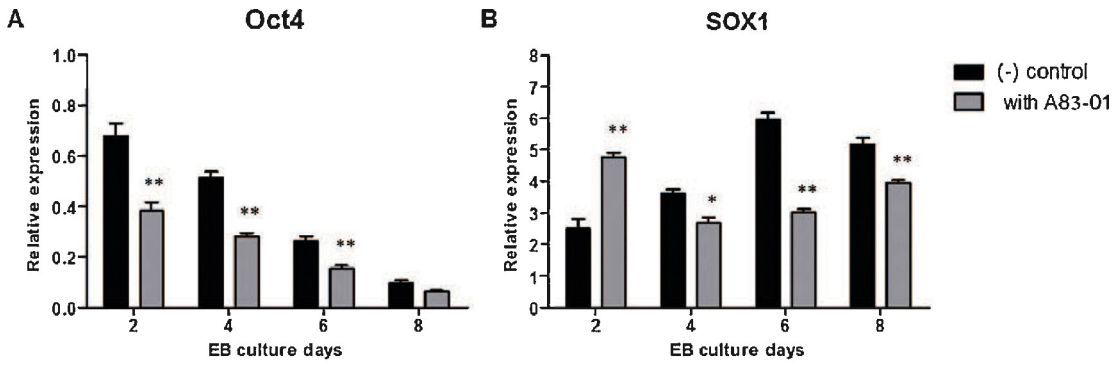
3.1. Effect of selective TGF-β1/ALK inhibitor on EB formation

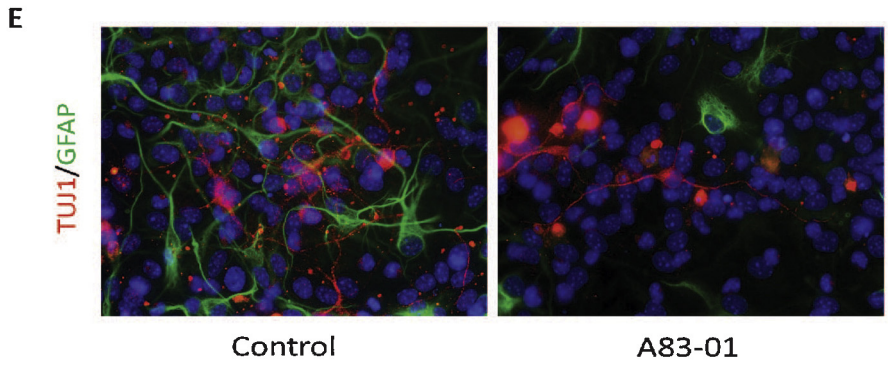
Prior to neuronal differentiation, mouse ESCs were maintained in ground state of pluripotency (see Supplementary Fig. 1). They strongly expressed alkaline phosphate (ALP, bright pink–red colony) and also undifferentiated ESC markers (Oct4 and Sox2). Following EB formation using a hanging drop method, ESCs spontaneously aggregated and formed as floating EBs in suspension without LIF. A total of 20 EBs in each group were then collected and examined on day 2 after cell seeding. The EB morphology was a spherical shape of uniform size, which was similar between the two experimental groups (Fig. 1B). However, the diameters of EB-treated with A83-01 (592.6 ± 27 μm) were significantly smaller than those observed in a control group (631.7 ± 14.1 μm, $P < 0.01$; Fig. 1C).

3.2. Effect of selective TGF-β1/ALK inhibitor on neuronal differentiation

To characterize the neuronal fate alteration during EB culture, the EBs were cryo-sectioned and immunostained with Pax6 antibody, a NEPC marker. The NeuN antibody was also used (Fig. 1D) to reveal the differentiation ability of NEPCs toward mature neurons within the EBs. Immunohistochemistry analysis revealed that the EBs derived from A83-01-treated and control groups contained a complex network of a mixed population of both early and mature neurons.

To examine whether TGF-β1/ALK inhibitor alters the gene expression during neuronal differentiation, the EBs were sequentially collected and analyzed by quantitative RT-PCR assay. Total RNA was extracted from EBs derived from both groups at days 2, 4, 6 and 8 of differentiation for pluripotent state (Oct4), early or neuronal progenitor cell fate (Sox1), mature neuron (MAP2) and glia fate (GFAP) markers. The results showed that the mRNA





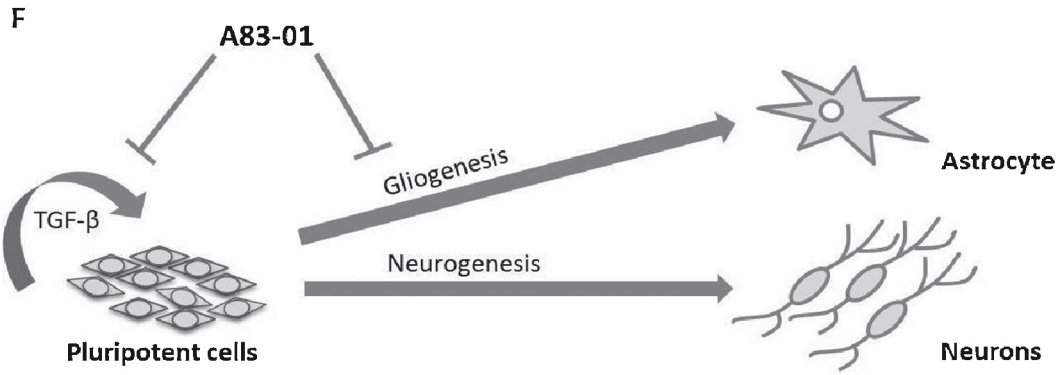


Fig. 2. Quantitative RT-PCR analysis of mRNA expression levels during neuronal differentiation. Mouse ESCs were cultured in non-adhesive dishes for 8 days in differentiated medium supplemented with 1 μM A83-01 (grey bars). RNA was isolated from undifferentiated ES cell colonies (referred to as day 0) and from cell spheres at day 2, 4, 6 and 8. Expression of Oct4 (A), Sox1 (B), MAP2 (C), and GFAP (D) was normalized to the house keeping gene, GAPDH. Data shown as mean ± S.E.M. of three independent experiments. * ($P < 0.05$) and ** ($P < 0.01$) indicate the values that differ significantly. (E) Mature neurons derived from control and A83-01-treated groups were characterized by immunofluorescent staining with neuronal markers (TUJ1 and GFAP for beta-III tubulin and gial cell marker, respectively). (F) Model of proposed pathway that contributes to the action of A83-01 on cell fate during neuronal differentiation.

expression encoding the undifferentiated ESC marker Oct4 in A83-01-treated EBs was pronouncedly down-regulated over 8 days of culture than the control group (Fig. 2A). This sequential down-regulation of the pluripotent gene occurred simultaneously with the dramatically up-regulation of Sox1 and MAP2 mRNA levels. Selective TGF-β1/ALK inhibitor significantly increased Sox1 and MAP2 levels after 2 days of differentiation and a similar pattern of these neuronal mRNA expression levels was observed during the first 4 days of EB culture. However, Sox1 was later expressed at a lower level compared with EBs in non-treated medium (Fig. 2B). There was no significant difference in mRNA expression of MAP2 in A83-01-treated or non-treated EBs after 4 days of differentiation (Fig. 2C).

Interestingly, the mRNA level of GFAP was dramatically decreased in the A83-01-treated EBs and markedly exhibited a contrary dynamic of GFAP expression, compared with those in the control, as shown in Fig. 2D. Treatment of the EBs with selective TGF-β1/ALK inhibitor has significantly down-regulated the GFAP expression throughout EB suspension period. Likewise, the expression of neuronal marker TUJ1 and glial marker GFAP was observed in differentiated cells derived from EBs at 14 days of differentiation by immunostaining. The result demonstrated the A83-01-treated EBs has a lower in number of GFAP-positive cells comparing to the control (Fig. 2E).

4. Discussion

In the present study, we determined the effect of selective TGF-β1/ALK inhibitor (A83-01) on ESC-derived EB development toward neuronal lineages *via* EB formation strategy. Several studies revealed that the TGF-β1 signaling pathway is important for *in vitro* and *in vivo* neurogenesis [11]. For example, TGF-β1 stimulation appears to negatively affect neuronal differentiation and development [42] and over-expression of TGF-β1 in transgenic mice hampered *in vivo* neurogenesis, in terms of the production of early neurons in hippocampus [5]. Recently, the maintenance of TGF-β1 signaling by inhibitor 5 of protein phosphatase 1 or IPP5 markedly hampered the neurite outgrowth of rat dorsal root ganglion [12]. On the other hand, the TGF-β1 inhibitors such as SB431542 or A83-01 appeared to transform the pluripotent ESC fate into neuronal lineages including those of motor neuron progenitors [31].

By following the supplementation of A83-01 in EB culture medium, the day 2-EBs derived from each group by hanging drop method were formed into homogenous spheres of uniform size as previously reported [6,38]. However, with the same number of cells seeded, the A83-01-treated EBs were significantly smaller in size than the controls. However, since we observed the marked down-regulation of pluripotent gene expression (Oct4) along with the up-regulation of neuronal markers (Sox1 and MAP2) of day 2-EBs treated with TGF-β1 inhibitor (Fig. 2A–C), it is possible that the inhibition of this pathway could rapidly commit the ESCs toward neuronal lineages. This may also coincide with the finding that the EB-mediated differentiation efficiency was dependent on the EB size. Although the smaller size may reflect the poor proliferation activity, our objective was to improve neuronal efficiency by A83-01 rather than to increase the number of particular neurons. Previous studies indicated that the larger sizes of EB tended to differentiate toward mesoderm and endoderm, while the smaller EBs hinted their differentiation fate toward ectoderm lineage [30,32]. This result supports our hypothesis that suppression of TGF-β1 signaling would improve the efficacy of neuronal differentiation.

A series of studies revealed that RA plays a significant role during embryogenesis and CNS development, which has been an essential inductive factor for neuronal differentiation of ESCs in a concentration and time dependent manner [22,39]. In this study, EBs were cultured in medium containing 5 μM of RA which were added after 4 days of EB suspension culture, which aimed to induce ESCs to generate high yields of Pax6-positive NPCs as demonstrated earlier [2]. Our results showed the consecutive up-regulation of the Sox1 and MAP2 mRNA levels after supplementation of RA on day 4 resembled the progression of early neuronal differentiation in embryos [20]. Although cell fate within the EBs can be successfully directed into neuronal lineage in the presence of RA [10], the EBs were inevitably contaminated with residual pluripotent cells [1,4]. In order to reduce the contamination of the ESCs within the EB, the ground state of ESCs needs to be depleted. Here, the selective TGF-β1/ALK inhibitor, A83-01, rapidly reduced Oct4 expression similar to that observed in mouse embryos treated with TGF-β1 inhibitor, SB431542 [13]. This inhibitor also markedly increased the rate of neuronal cell fate alteration detected by the expression levels of early neuronal markers including of Nestin, Olig2, and Sox1 mRNA [28,36].

In adult brains, TGF-β1 and other cytokines, interleukin-1β (IL-1β) can activate and are involved in the modulation of GFAP expression as a neural response for cell regeneration and inflammation upon neurodegenerative diseases or brain/spinal cord injury [21,35]. In *in vitro* study, twofold up-regulation of the GFAP level has been reported in astrocyte culture treated with TGF-β1 [18]. In this report, we demonstrated a neuronal differentiation strategy *via* EB formation that treats the inhibitor A83-01 to block the TGF-β1 pathway. This inhibitor not only promotes neuronal differentiation, but also significantly suppressed the gliogenic fate of pluripotent cells (Fig. 2F). Given that a homogenous neuronal population derived from the ESCs is a prerequisite for clinical application, inhibition of TGF-β1/ALK pathways during neuronal differentiation using A83-01 may become an alternative strategy for reducing the risk of pluripotent cell contamination and creating a more uniformity neuronal cell production.

In addition, previous study demonstrated that the TGF-β1 inhibitor significantly promoted neurogenesis in aged and irradiated adult mouse brain [34]. Therefore, *in vivo* study of A83-01 would reveal its exact role and underlying mechanisms during neurogenesis. Although our finding suggests that the selective TGF-β1 inhibitor, A83-01, improves the neuronal differentiation during EB culture system, future work including the study of neuroprotective roles against disease-associated toxicity may provide an alternative approach for preventing the initiation of several neurological diseases.

5. Conclusion

In this study, we demonstrate that a selective TGF-β1/ALK inhibitor rapidly stimulates the cell fate alteration of ESCs from an undifferentiated state toward neuronal lineages within EBs. Moreover, the hindered effect of A83-01 on gliogenesis would offer an alternative strategy for generating a uniform neuronal population and further developing neuronal differentiation strategies for future therapeutic applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2014.06.001.

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