การสร้างเซลล์ต้นกำเนิดจากตัวอ่อนที่ผลิตจากการกระตุ้นด้วยสารเคมี และการเปลี่ยนแปลงเซลล์ต้นกำเนิดจากเซลล์พลูริโพเท้นเป็นเซลล์กล้ามเนื้อหัวใจในหนูเม้าส์

นางสาว ศศิธร รุ่งอรุณเลิศ

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ESTABLISHMENT OF PARTHENOGENETIC EMBRYONIC STEM CELLS AND DIFFERENTIATION OF PLURIPOTENT STEM CELLS INTO CARDIAC LINEAGE IN MOUSE

Miss Sasitorn Rungarunlert

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Theriogenology
Department of Obstetrics Gynaecology and Reproduction
Faculty of Veterinary Science
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Thesis Title	ESTABLISHMENT OF PARTHENOGENETIC EMBRYONIC STEM CELLS AND DIFFERENTIATION OF PLURIPOTENT STEM CELLS INTO CARDIAC LINEAGE IN MOUSE
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ศศิธร รุ่งอรุณเลิศ: การสร้างเซลล์ต้นกำเนิดจากตัวอ่อนที่ผลิตจากการกระตุ้นด้วยสารเคมี และการเปลี่ยนแปลงเซลล์ต้นกำเนิดจากเซลล์ พลูริโพเท้นเป็นเซลล์กล้ามเนื้อหัวใจในหนูเม้าส์ (ESTABLISHMENT OF PARTHENOGENETIC EMBRYONIC STEM CELLS AND DIFFERENTIATION OF PLURIPOTENT STEM CELLS INTO CARDIAC LINEAGE IN MOUSE) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ศ.น.สพ.ดร. มงคล เตชะกำพุ อ .ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ.น.สพ.ดร. ธีรวัฒน์ ธาราศานิต , ศ.น.สพ.ดร. แอนดาส ดินนเยส, 115 หน้า

การทดลองที่ 1 เพื่อศึกษาการสร้างเซลล์ต้นกำเนิดจากตัวอ่อนที่ผลิตจากการกระตุ้นด้วยสารเคมี และ ศึกษาความแตกต่างลักษณะคุณสมบัติของแต่ละเซลล์ไลน์ การสร้างเซลล์ต้นกำเนิดจากตัวอ่อนที่ผลิต จากการกระตุ้นด้วยสารเคมี ได้ประสบความสำเร็จจำนวน 4 เซลล์ไลน์ เซลล์ต้นกำเนิดจากตัวอ่อน ทั้ง 4 เซลล์ไลน์ (pES#1-4) แสดงคุณสมบัติ รูปร่างเหมือนเซลล์ต้นกำเนิดจากตัวอ่อน และให้ผลบวกเมื่อทำปฏิกิริยากับอัคคาไลน์ฟอสฟาเตส และ แอนติบอดีที่จำเพาะต่อ Oct-4 Nanog และ SSEA-1 เซลล์ต้นกำเนิดจากตัวอ่อนที่ผลิตจากการกระตุ้นด้วยสารเคมี จำนวน 3 เซลล์ไลน์ (pES#1-3) มีประสิทธิภาพในการเปลี่ยนแปลงเป็นเซลล์เนื้อเยื่อชั้นในและชั้นกลางของตัวอ่อน มากกว่าอีกหนึ่งเซลล์ไลน์ (pES#4) การเปลี่ยนแปลงเซลล์ต้นกำเนิดจากตัวอ่อนเป็นเซลล์กล้ามเนื้อหัวใจมีประสิทธิภาพ 33-100% ของจำนวนการเต้นของเซลล์กล้ามเนื้อหัวใจต่อจำนวนตัวอย่าง ผลการทดลองนี้สรุปได้ว่าเซลล์ต้นกำเนิดจากตัวอ่อนที่ผลิตโดยวิธีการกระตุ้นด้วยสารเคมี มีประสิทธิภาพในการเปลี่ยนแปลงไปเป็นเซลล์ชนิดต่างๆ ในการเพาะเลี้ยงภายนอกร่างกายได้ต่างกัน

การทดลองที่ 2 เพื่อเพิ่มจำนวนของเซลล์ต้นกำเนิดจากตัวอ่อนสำหรับเปลี่ยนแปลงเป็นเซลล์กล้ามเนื้อหัวใจ โดยพัฒนาการเลี้ยงเอ็มบริออยบอดี (embryoid body, EB) ใน slow turning lateral vessel (STLV) bioreactor รวมทั้งศึกษาผลของจำนวนเซลล์ที่ใช้เลี้ยงใน STLV bioreactor รอบหมุนของ STLV bioreactor และ ระยะเวลาการเลี้ยงเอ็มบริออยบอดีก่อนเลี้ยงบนจานเพาะเลี้ยงที่เคลือบด้วยเจลลาติน มีผลต่อการสร้างเอ็มบริออยบอดี และ การเปลี่ยนแปลงเป็นเซลล์กล้ามเนื้อหัวใจ ผลจากการทดลองพบว่า เซลล์จำนวน $3x10^5$ เซลล์ต่อมิลลิลิตร รอบหมุนของ STLV bioreactor เท่ากับ 10 รอบหมุนต่อนาที ระยะเวลาการเลี้ยงเอ็มบริออยบอดีก่อนเลี้ยงบนจานเพาะเลี้ยงที่เคลือบด้วยเจลลาตินเป็นเวลา 3 วันเป็นพารามิเตอร์ที่เหมาะสมสำหรับการเลี้ยงเอ็มบริออยบอดีให้มีขนาดและคุณภาพสำหรับการเปลี่ยนแปลง เป็นเซลล์กล้ามเนื้อหัวใจ พารามิเตอร์ที่เหมาะสมสำหรับการเลี้ยงเอ็มบริออยบอดีใน STLV bioreactor สามารถเพิ่มประสิทธิภาพการเปลี่ยนแปลงเป็นเซลล์กล้ามเนื้อหัวใจได้ดียิ่งขึ้น

การทดลองที่ 3 เพื่อเปรียบเทียบประสิทธิภาพการผลิตเอ็มบริออยบอดี และ การเปลี่ยนแปลงเป็นเซลล์กล้ามเนื้อหัวใจ โดยวิธี STLV bioreactor กับ static suspension culture (SSC) และ hanging drop (HD) หลังจากเลี้ยงเซลล์เป็นเวลา 3 วัน ผลการทดลองพบว่า STLV bioreactor สามารถผลิตเอ็มบริออยบอดีมากกว่า SSC จำนวน 4 เท่า และ เพิ่มจำนวนเซลล์ในเอ็มบริออยบอดีมากกว่า SSC จำนวน 6 เท่า รวมทั้งลดจำนวนเซลล์ที่ไม่สามารถรวมเป็นเอ็มบริออยบอดีได้ 10 เท่าเมื่อเทียบกับวิธี SSC โดยทั่วไป STLV ผลิตเอ็มบริออยบอดีที่มีรูปร่างลักษณะเหมือนกันได้มากกว่าวิธี SSC และ HD เอ็มบริออยบอดีที่เลี้ยงใน STLV มีการแพร่กระจายของ cardiac troponin T (cTnT) สูงกว่าวิธีอื่นในระหว่างการเปลี่ยนแปลงเป็นเซลล์กล้ามเนื้อหัวใจ เมื่อตรวจสอบการแสดงออกของยีนพบว่าเอ็มบริออยบอดีที่เลี้ยงใน STLV และ HD มีการแสดงออกของ ยีนของเซลล์กล้ามเนื้อหัวใจ (Nkx2.5 Tnnt2 Nppa and Myh6) มากกว่าวิธี SSC ดังนั้น เอ็มบริออยบอดีที่เลี้ยงใน STLV เป็นวิธีสำหรับการเพิ่มจำนวนของเซลล์ต้นกำเนิดจากตัวอ่อน และ เปลี่ยนแปลงเป็นเซลล์กล้ามเนื้อหัวใจ

การทดลองที่ 4 เพื่อศึกษาลักษณะคุณสมบัติการสร้าง เอ็มบริออยบอดี และ การเปลี่ยนแปลงเป็นเซลล์กล้ามเนื้อหัวใจของเซลล์อินดิวพลูลิโพเท็นสเต็มเซลล์ใน STLV bioreactor เปรียบเทียบกับวิธี HD ผลการทดลองพบว่า STLV bioreactor สามารถผลิตเอ็มบริออยบอดีที่มีขนาดเท่าๆกัน เหมือนกับวิธี HD เมื่อตรวจสอบการแสดงออกของยีน พบว่าเอ็มบริออยบอดีที่เลี้ยงใน STLV และ HD มีการแสดงออก ของยีนของเซลล์กล้ามเนื้อหัวใจเหมือนกัน เอ็มบริออยบอดีที่เลี้ยงใน STLV bioreactor มีอัตราการเต้นของเซลล์กล้ามเนื้อหัวใจต่อจำนวนตัวอย่าง และ มีพื้นที่ของ cTnT สูงกว่าเอ็มบริออยบอดีที่เลี้ยงใน HD การศึกษาครั้งนี้เป็นการรายงานผลครั้งแรกของการศึกษาการเลี้ยงเซลล์อินดิวพลูลิโพเท็นสเต็มเซลล์ใน STLV bioreactor เพื่อเปลี่ยนแปลงเป็นเซลล์กล้ามเนื้อหัวใจจำนวนมาก

ภาควิชา สูติศาสตร์-เธนุเวชวิทยาและวิทยาการสืบพันธุ์ ลายมือชื่อนิสิต.....

สาขาวิชา วิทยาการสืบพันธุ์สัตว์ ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา 2553 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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SASITORN RUNGARUNLERT: ESTABLISHMENT OF PARTHENOGENETIC EMBRYONIC STEM CELLS AND DIFFERENTIATION OF PLURIPOTENT STEM CELLS INTO CARDIAC LINEAGE IN MOUSE. ADVISOR: PROF. MONGKOL TECHAKUMPHU, D.V.M., DOCTORAT 3e CYCLE, CO-ADVISOR: ASST. PROF. THEERAWAT THARASANIT, D.V.M., Ph.D., PROF. ANDRAS DINNYES, D.V.M., Ph.D., 115 pp.

EXP. 1 aimed to establish embryonic stem (ES) cell lines from parthenogenetic embryos and examine pluripotency differences among the parthenogenetic ES (pES) cell lines. We are able to report the successful establishment of four pluripotent pES cell lines from blastocysts of parthenogenetic origin. Four pES cell lines (pES#1-4) exhibited a typical ES cell morphology and expression of key pluripotency markers (ALP, Oct-4, Nanog and SSEA-1). Three of the four pES cell lines (pES#1-3) exhibited a higher efficiency towards endo-mesoderm differentiation than pES#4. Differentiation towards cardiac cells resulted in all cell lines 33-100% of spontaneous beating cell clusters/well. In conclusion, our results have demonstrated that there are major differences among pES lines in their differentiation ability *in vitro.*

EXP. 2 aimed to enable large-scale culture of ES-derived cells for cardiac differentiation, we developed a scalable bioprocess that directs embryoid body (EB) formation in a fully controlled STLV (slow turning lateral vessel, Synthecon, Inc, Houston, TX, USA) bioreactor following inoculation with a single cell suspension of mouse ES cells. We investigated the effects of inoculating different cell numbers, time of EB adherence to gelatin-coated dishes, and rotation speed for optimal EB formation and cardiac differentiation. Our results showed that $3x10^5$ cells/ml, 10 rpm rotary speed and plating of EBs onto gelatin-coated surfaces three days after culture are the best parameters for optimal size and EB quality on consequent cardiac differentiation. These optimized parameters enrich cardiac differentiation in ES cells when using the STLV method.

EXP. 3 aimed to compare the efficiency of EB formation and cardiac differentiation by using STLV bioreactor to static suspension culture (SSC) and hanging drop (HD) condition. After three days culturing, a 4-fold improvement in the yield of EB formation/ml and a 6-fold enhancement in total cell yield of EBs in STLV conditions vs. SSC conditions were detected. On the other hand, a nearly 10-fold diminishment in total cell yield of free cell which were not incorporation into EBs in STLV condition vs. SSC was observed. Overall, STLV conditions produced more uniform EBs than SSC and HD. During cardiac differentiation, EBs cultured in STLV showed the highest distribution of cardiac troponin T (cTnT). RT-PCR assay demonstrated that EBs cultured in STLV and HD expressed more cardiac markers (Nkx2.5 Tnnt2 Nppa and Myh6) compared with SSC condition. Hence, EBs culture in STLV provides a technological platform for the large-scale generation of ES cell-derived cells and differentiation into cardiomyocytes.

EXP. 4 aimed to characterizes EB formation and subsequent cardiomyocyte differentiation of mouse iPS cells using STLV bioreactor compared to HD. EBs derived from STLV are homogenous in size similar with HD methods. Similar gene expression patterns were observed in both differentiation systems with cardiomyocyte markers by using RT-PCR. Moreover, the percentage of beating cardiomyocytes and the area of cTnT were higher in EBs derived from STLV bioreactor than HD culture. Our study describes, for the first time, a strategy for scalable differentiation of iPS cells into cardiomyocytes in STLV bioreactor culture system.

Department: Obstetrics Gynaecology and Reproduction
Field of Study: Theriogenology
Academic Year: 2010

Student's Signature……
Advisor's Signature……..……
Co-advisor's Signature……..…...
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2PN	two pronuclei
β-ME	β-mercaptoethanol
ALP	alkaline phosphatase
BSA	bovine serum albumin
CB	Cytochalasin B
CD34	a cluster of differentiation molecules 34
COCs	cumulus-oocyte complexes
cTnI	cardiac Troponin I muscle
cTnT	cardiac Troponin T type 2
D	day
DAPI	4´- 6´-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxynucleic acid
E	the day of embryonic development
EBs	embryoid bodies
EGFP	enhanced fluorescent green protein
ERKs	extracellular signal-regulated kinases
ES	embryonic stem
f	fertilized
FBS	fetal bovine serum
GAPDH	glyceraldehyde phosphate dehydrogenase
GATA	GATA -binding protein
GBI	Glass Ball Impeller
GLM	General Linear Model
GVHD	Graft Versus Host Disease
h	hour
HARV	high aspect rotating vessel
hCG	human chorionic gonadotropin
HD	hanging drop
H&E	hematoxyline and eosin
ICM	inner cell mass
IGF2	insulin like growth factor 2
IL	interleukin
i.p.	*intraperitoneal*
iPS	induced pluripotent stem
IU	international unit
IVF	*in vitro* fertilization
KCl	potassium chloride
Klf4	krueppel-like factor 4
KSOM	potassium simplex optimized medium
LIF	leukemia inhibitory factor
M	meiosis
MAP	microtubule-associated protein
MEF	mouse embryonic fibroblast
MHC	major histocompatibility complex
MLC2v	Myosin regulatory light chain 2, ventricular

min	minute
ml	milliliter
MHC	myosin heavy chain
MHC-neo	myosin heavy chain-neomycin resistance
MPC	2-methacryloyloxyethyl phosphorylcholine
mRNA	messenger ribonucleic acid
NASA	National Aeronautics and Space Administration
NEAA	non essential amino acid
Nkx2.5	NK2 transcription factor related, locus 5
nt	nuclear transfer
Oct-4	octamer-binding transcription factor 4
p	parthenogenetic
PBS	phosphate buffered saline
PFA	paraformaldehyde
PI	paddle-impellers
PMSG	pregnant mare serum gonadotropin
poly 2-HEMA	*poly 2*-hydroxyethyl methacrylate
PVP	polyvinylpyrrolidone
Q-RT-PCR	quantitative real time polymerase chain reaction
RCCS	rotating cell culture system
rpm	revolutions per minute
RT-PCR	reverse transcriptase polymerase chain reaction
s	second
SB	sleeping beauty
SCID	Severe Combined Immunodeficiency
SEM	standard error of mean
Sox2	sex determining region Y-box 2
SSC	static suspension culture
SSEA	stage specific embryonic antigen
STAT	signal transducer and activator of transcription
STLV	slow turning lateral vessel
STR	stirred tank bioreactor
TE	trophectoderm
Tnnt2	Troponin T type 2 (cardiac)

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Embryonic stem (ES) cells are capable of unlimited self-renewal *in vitro* and differentiation into cells constituting all three somatic germ layers. ES cells were first isolated from the inner cell mass (ICM) of mouse embryos (Evans and Kaufman, 1981; Martin, 1981; Figure 1A), followed by the derivation of non-human primate and human ES cell lines (Thomson et al., 1996; 1998). Currently, pluripotent cells can also be alternatively obtained by retroviral transduction of a combination of four transcription factors such as octamer-binding transcription factor 4 (Oct-4), sex determining region Y-box 2 (Sox2), c-Myc and Krueppel-like factor 4 (Klf4) into fully differentiated somatic cells known as "induced pluripotent stem (iPS) cells" (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Figure 1B). These cells are equivalent with ES cells in terms of self-renewal and differentiation capacity (Wernig et al., 2007; Park et al., 2008). The unique ability of pluripotent cells to generate a vast range of different cells makes both ES and iPS cells suitable for various cell transplantation, tissue engineering and drug testing applications (Keller, 2005; Lerou and Daley, 2005).

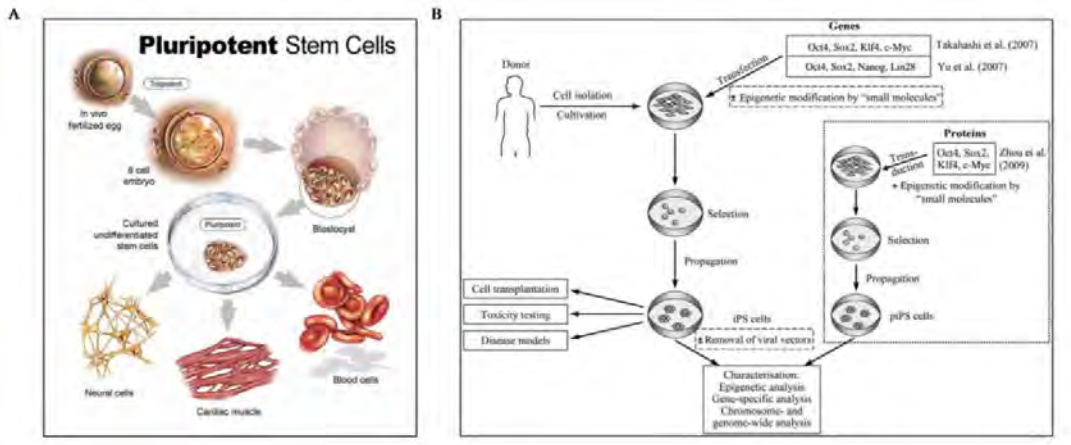


Figure 1. Pluripotent stem cells are comprised of (A) ES cells and (B) iPS cells. (http://www.stemcellresearchfoundation.org/WhatsNew/Pluripotent.htm; Rolletschek and Wobus, 2009)

1.2 Literature Review

1.2.1 Establishment of embryonic stem cell lines

Up to now, ES cells can be derived from 8-cell embryos (Tesar, 2005), morulae, primitive ectoderm of implantation-delayed blastocysts, epiblast of egg cylinder stage of embryos (Brons et al., 2007), single blastomere of 2- to 4-cell embryos (Chung et al., 2006; Wakayama et al, 2007) and tetraploid embryos (Cho et al., 2006). These cells derived from aforementioned types of embryos are usually cultured on mouse embryonic fibroblasts (MEFs) until the expansion of ICM outgrowth occurs. The ICM outgrowth is then dissociated with enzymatic digestion

and plated repeatedly onto a monolayer cell feeder (Nagy et al., 1993).Generally, standard medium for establishment and maintenance of mouse ES cells contain with leukemia inhibitory factor (LIF) because LIF has been shown to play a key role in controlling the stemness. (Ulloa-Montoya, 2005; Nagy and Vintersten, 2006; Rungsiwiwut et al., 2008). LIF is a soluble glycoprotein of the interleukin (IL)-6 family, acting via a membrane bound gp130 signaling complex, that regulates a variety of cell functions through signal transduction and activation of transcription (STAT) signaling (Friel et al., 2005). Several signaling pathways of ES cells have been characterized, and the efficiency of ES cell establishment has been much improved using a defined serum-free medium (Schoonjans et al., 2003; Cheng et al., 2004) or microtubule-associated protein (MAP) - extracellular signal-regulated kinases (ERKs) inhibitor PD 98059 (Buehr and Smith, 2003).

Undifferentiated mouse ES cells are characterized by two unique properties: (i) the unlimited self-renewal capacity and (ii) the capability to differentiate into terminally differentiated cells. Other properties are (iii) a high nuclear:cytoplasmic ratio with prominent nucleoli and (iv) normal chromosome numbers and high levels of telomerase activity. Moreover, they also express pluripotentcy markers such as alkaline phosphatase (ALP), cell surface marker stage specific embryonic antigen-1 (SSEA-1), Oct-4 (Pesce and Schöler, 2001) and Nanog (Yates and Chambers, 2005). In absence of IL-6 family members or removal of MEFs or the inactivation of STAT3, a downstream signaling molecule of the gp130 signaling complex, ES cells differentiate spontaneously *in vitro* (Kurosawa et al., 2003). In suspension culture, they form embryo-like aggregates so-called embryoid bodies (EBs), containing derivatives of the three embryonic germ layers. After ectopic transplantation (e.g. under renal's capsule or into the testis), pluripotent cells give rise to teratocarcinomas, demonstrating their capability to differentiation *in vivo*. When incorporation of the ES cells with normal embryos via blastocyst injection or morula aggregation, these cells contribute to all tissues and organs including the germ-line of chimeric individuals, giving rise to functional gametes (Alex et al., 2005).

ES cell lines or ES-like cells have been derived from a wide range species including rat (Buehr et al., 2003; Iannaccone et al., 1994), rabbit (Wang et al., 2007), porcine (Li et al., 2003), bovine (Wang et al., 2005), canine (Wilcox et al., 2009), feline (Gómez et al., 2010), equine (Guest and Allen, 2007) and non-human primate (Cibelli et al., 2002). Currently, three sources of embryos are used to establish ES cell lines; (i) *in vivo/in vitro* fertilization (IVF) with or without *in vitro* culture (Tielens et al., 2006), (ii) nuclear transferred embryos (Munsie et al., 2000; Wakayama et al., 2005) and (iii) parthenogenetic embryos (Kim et al., 2007). Although a lot of ES cell lines have been established from the fertilized embryos, these differentiated cells derived from ES cells can not be used as cell replacement therapy due to host-recipient immune compatibility. To solve this problem, pluripotent stem cells derived from somatic cells through such processes as somatic cell nuclear transfer (SCNT), iPS and parthenogenesis have been established for autologous transplantation. However, the overall efficiency is very low and the safety issue remains a major concern for iPS cells. An Establishment of ES cell derived from parthenogenetic embryos is attractive as a source of histocompatible cells and tissues for cell therapy (Lee et al., 2008).

1.2.2 Parthenogenetic embryonic stem cells (pES cells)

While human nuclear transferred embryonic stem cells (ntES) cells have yet to be established, ES cells can be alternatively derived from parthenogenesis.

Parthenogenesis is a reproductive mechanism that produces a live birth from an oocyte activated in an absence of sperm especially in lower organism such as insects (Hipp and Atala, 2004). However, this phenomenon cannot occur in mammalian species. To take this advantage form parthenogenesis, a number of preimplantation embryos in a variety of mammalian species such as mouse (Ma et al., 2005; Ibanez et al., 2005), swine (Yi and Park, 2005; Varga, et al., 2008), bovine (Wang et al., 2008), monkey (Mitalipov et al., 2007) and human (Taylor and Braude, 1994) have been produced using parthenogenetic activation. Parthenogenetic activators such as Sr^{2+} and Ca^{2+} can trigger a series of repetitive Ca^{2+} oscillations of the oocytes (Toth et al., 2006) that are resemble to those produced in response to the sperm entry. Consequently, parthenogenetic embryos can be developed directly from the oocytes to blastocysts without sperm contribution (Kline, 1996; Rogers et al., 2006). These embryos contain only maternally derived chromosomes and have genetic material similar to patient's oocytes. Because parthenogenetic embryos cannot develop up to term after transferring into a surrogate uterus due to the poor-developed extraembryonic tissues (Strum et al., 1994; Wu et al., 2006), this technique therefore minimizes the societal and ethical problems (Kiessling, 2005).

The pES cells were first established from mouse followed by non-human primate (Cibelli et al., 2002) and rabbit (Fang et al., 2006; Wang et al., 2007). These pES cells have properties of self-renewal and the capacity to generate cell derivatives from the three germ-layers, confirmed by contributions of germ-line transmission to chimeric animals as well as teratoma formation when injected into Severe Combined Immunodeficiency (SCID) mice (Lengerke et al., 2007). In addition, these pES cells can also be used to study imprinting genes (Allen et al., 1994). For instance, maternal H19 and paternal insulin like growth factor 2 (IGF2) imprinted genes have been shown to express in parthenogenetic embryos and pES cells. IGF2 is downregulated in parthenogenetic embryo. Interestingly, while IGF2 is upregulated when the cells are pluripotent, it is later downregulated when differentiation takes place. H19 is always expressed at higher levels when compared to differentiated cells derived from fertilized embryonic stem cells (fES) cells irrespective the differentiation status of the cells. For cell replacement therapy, a "homozygous ES cells" derived from an meiosis (M) II oocyte can match substantially more unrelated individuals than a heterozygous ES cells. For major histocompatibility complex (MHC) matching, genetic recombination of immature and mature oocyte after parthenogenetic activation has been investigated, and the patterns of recombination have been observed when karyokinesis is interrupted during MI or MII (Kim et al., 2007). In addition, the prime derivations of genetically MHC-matched pluripotent ES cell lines from human blastocysts have been successfully established using parthenogenetic embryos in comparison to nuclear transferred embryos (Revazova et al., 2007). Recent studies on human pES cells have revealed that these ES cells are very similar to the human ES cells derived from IVF or *in vivo* produced blastocysts in gene expression and other characteristics, but full differentiation and development potential of pES cells have to be further investigated before clinical research and therapeutic interventions.

1.2.3 Differentiation of pluripotent stem cells

Efficient and controlled means of directing ES or iPS cell differentiation is crucial for the development of cell replacement therapies (Ulloa-Montoya et al., 2005; Okita et al., 2007). To realize the therapeutic potential of ES cells, it is essential to regulate their differentiation in a reproducible manner. Differentiation of ES cells is performed by two main ways; either by direct differentiation from pluripotent cells or

by the formation of cell aggregates in non-adherent spheroids, called EBs (Doetschman et al., 1985; Höpfl et al., 2004). The molecular and cellular morphogenic signals and events within EBs recapitulate numerous aspects of the embryo development and result in differentiation to cells of three embryonic germ layers (endoderm, mesoderm and ectoderm) similar to the gastrulation of an epiblast-stage embryo *in vivo* (Itskovitz-Eldor et al., 2000). The precise number and spatial coordination of the various cell-cell interactions involved in EB formation are considered to influence the course of ES cell differentiation and, as a result, the control of cell number, size of EBs and quality of EB formation are important step for differentiation strategies (Messana et al., 2008; Mohr et al., 2010).

Methods for inducing EB formation are based on preventing ES cells from attachment to the surface of culture vessels, thereby allowing the non-adherenced ES cells to aggregate and form the EBs. To achieve EB formation, hanging drop (HD) and static suspension culture (SSC) to allow small scale formation of aggregates are commonly used. These culture systems maintain a balance between ES cell aggregation essential for EB formation and prevention of EB agglomeration (Keller, 1995). Even though HD method is commonly used to prepare uniform-sized EBs, this method has several disadvantages in the mass preparation of EBs such as labor-intensive procedure, which hinders the use of differentiated ES cells for therapeutic application (Kurosawa, 2007). Mass EB production is easier from SSC in which ES cells are suspended in a static Petri-dish. One drawback of this method, however, is that the EBs often fuse together to form large aggregates. This has negative effects on cell proliferation and differentiation, as well as causing extensive cell death. Hence, these methods are restricted as far as industrial applications are concerned because of their complication and difficult manageability (Dang and Zandstra, 2005).

Recently, novel bioreactors for large-scale production of ES-derived cells have been developed. A bioreactor is often defined as a device, in which biological processes (cell expansion, differentiation or tissue formation on biomaterial scaffolds) occur in a tightly controlled *in vitro* environment, including the exchange of O_2, nutrients and metabolites (Barron et al., 2003). There are several types of bioreactors. For example, stirred suspension culture (spinner flask) has been successfully employed in some studies for mass scale production of ES-derived cells (Kehoe et al., 2008; 2010). Conventional stirrer vessels may have the disadvantage of generating shear forces although it is manageable. These forces still can damage the cells (Cameron et al., 2006). Another bioreactor that allows agglomeration-free EB formation is the rotating cell culture system (RCCS) developed by the US National Aeronautics and Space Administration (NASA). This system is characterized by EB immobility in space, due to an extremely low fluid shear stress and oxygenation by diffusion (Gerecht-Nir et al., 2004). EBs produced by bioreactors were more uniform in size and had less necrotic centers in comparison to SSC. Furthermore, bioreactors can also be used for culturing iPS cells, which is expected to become a main further application of mass EB production in the near future (Zwi-Dantsis et al., 2011).

1.2.4 Methods for culturing EBs

At the present time, no universally accepted standard exists for measurement of EB formation although characteristics such as EB size, shape, homogeneity and the quality of EB formation, ratio of apoptotic and viable cell are typically used as benchmarks for evaluation (Koike et al., 2007). There are several methods to generate EB formation, as schematically shown in Figure 2. A summary of all techniques used

for generating EBs is presented in Table 1. Traditionally the most common EB culture methods, such as HD and SSC, were used for inducing ES differentiation.

Hanging drop method (HD): The HD method (Figure 2A) provides uniform sizes of EBs by dispensing equal numbers of ES cells in physically separated droplets of media suspended from the lid of a non-coated Petri-dish. This method offers a similar environment for forming individual EBs within each drop via gravity-induced aggregation of the cells. For this reason, this technique has been used to generate plentiful cell types such as neuronal cells (He et al., 2006), hematopoietic cells (Dang et al., 2002), cardiomyocytes (Takahashi et al., 2003), vascular cells (Evans et al., 2007) and chondrocytes (Kramer et al., 2000). The HD method is tremendously useful for appraisal of molecular mechanisms occurring in early embryogenesis in any cell types. However, this technique is mainly used for research purposes and is not suitable for large scale of EB production because of its laborious nature; a typical 100-mm Petri dish can contain no more than 100 drops and each drop usually creates only one EB (Kehoe et al., 2010). Further limitations of this method include major difficulties in exchanging or manipulating the small volume of medium (less than 50 µl which can evaporate easily) without disturbing the EBs. Usually the HD is composed of two steps; the aggregation of ES cells in drops and maturation of aggregates to EBs in suspension culture using low adherence bacterial Petri-dishes. Several elements of the method may be troublesome such as losses of EBs during picking up the formed EBs by pipette and attachment of premature EBs on Petri-dishes (Kurosawa et al., 2007).

Static suspension culture (SSC): The SSC (Figure 2B) is used to produce a large number of EBs by simply inoculating a suspension of ES cells onto a bacteriological grade Petri-dish, ultra-low adherence plate or a Petri-dish coated with cell adhesion inhibitor such as *poly 2*-hydroxyethyl methacrylate (poly 2-HEMA), thereby allowing the cells to spontaneously aggregate into spheroids (Choi et al., 2005). Although it is simple, this method allows little control over the size and shape of EBs. The result is frequent agglomeration of EBs into large, irregular masses because of the probability that ES cells encounter each other accidentally (Dang et al., 2002). An additional limitation of this technique is that EBs may prematurely attach to the plate because of the surface chemistry of the culture vessel, leading to a greater heterogeneity and loss of EBs from the suspension culture. On the other hand, this method is popular for some applications such as differentiation of ES cells into the neuronal lineage (Suslov et al., 2002).

Encapsulation/entrapment: Encapsulation/entrapment of a single cell suspension or small clusters of ES cells in hydrogels (Figure 2C and 2D, respectively), such as methylcellulose (Dang et al., 2002), fibrin (Liu et al., 2006), hyaluronic acid, dextran (Gerecht et al., 2007), alginate (Magyar et al., 2001), or agarose (Kurosawa et al., 2003) represents a transition between HD and SSC approaches by generating individually separated EBs in a semi-solid suspension medium. Entrapment of ES cells in methylcellulose, a temperature sensitive hydrogel, improves the overall synchrony and reproducibility of EB differentiation as it produces EBs of clonal origin. However, the efficiency of EB formation from individual ES cells can be rather low. In addition, soluble factor treatments and retrieval of differentiated cells may be complicated by the presence of the hydrogel material (Dang et al., 2002). Interestingly, this method showed the possibility of designing a single cell culture

system that would mimic the early developmental milieu and allows ES cells to switch between differentiation states within the same culture setting. When human ES cells are encapsulated in a 3D hyaluronic acid hydrogel, the human ES cells can be maintained in an undifferentiated state. On the other hand, when human ES cells are encapsulated in a dextran hydrogel, the human ES cells are induced to differentiate and form EBs. Different types of hydrogels, therefore, act as a unique microenviroment for maintaining ES cells in either undifferentiated or differentiating state (Nonaka et al., 2008).

Multiwell and microfabrication: As an alternative approach for EB formation and culture, multiwell (Figure 2E and 2F) and microfabrication technologies have also been developed recently. Round-bottomed 96-well plates coated with or without reagents (Ezekiel, 2007); 2-methacryloyloxyethyl phosphorylcholine (MPC), glycosaminoglycan (Koike et al., 2005; Konno et al., 2005; Koike et al., 2007) and poly 2-HEMA, have been utilized to prevent cell adhesion to the plastic surface. This technique is among the tools for forming EBs with high uniformity similar to the HD method as a defined number of ES cells is seeded in the separated wells. In contrast to the HD method, this technique has no requirement to exchange or manipulate the medium (approximately 200 µl) and it is easier to observe directly the EB formation with a microscope during cultivation. Because of these advantages, this technique may be used instead of HD method for laboratory research. The forced aggregation system, involving centrifugation of ES cells within round-bottomed (U-shaped) (Ng et al., 2005; Ungrin et al., 2008) and triangle-bottomed (V-shaped) 96-well plates (Burridge et al., 2007), can induce aggregation more rapidly than the HD. This procedure improves the reproducibility of EB production. On the other hand, it still requires individual processing and manipulation of the resulting EBs due to the requirement of one more additional plating step. Microwells fabricated by lithographic methods yielded EBs in an equivalent or at a much higher density than other methods and allow preparation of size-controlled EBs in a scalable manner for reproducible of EB formation (Moeller et al., 2008). Likewise, batches of EBs can be formed in microfluidic chambers and separated from the flowing culture medium by a semi-permeable membrane, allowing for temporal control of the molecular makeup of the medium. The cell patterning method is also useful for high-throughput screening assays, such as the exploration of biochemical agents to direct aggregate-induced differentiation into a specific lineage without plating EBs (Sasaki et al., 2009).

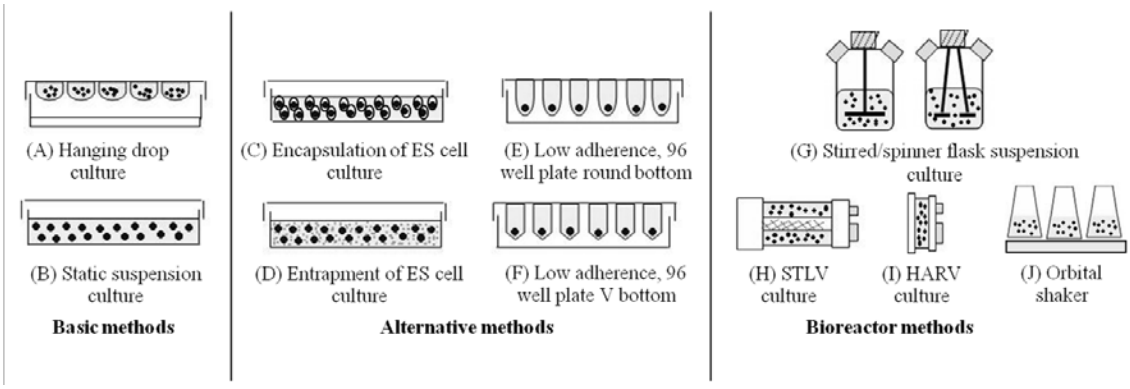


Figure 2. Schematic representation for vessels used in methods to form EBs from ES cells (Rungarunlert et al., 2009).

Table 1. Overview of current *in vitro* cell culture systems for production of EBs and other cell types (Rungarunlert et al., 2009)

Cell culture method	Benefit							Detriment					Propose	Yield	Note	Reference
	a	b	c	d	e	f	g	h	i	j	k	l				
1) HD	x							x	x	x			Differentiation into three germ layers	ND	Mouse ES cells	Dang et al., 2002
2) SSC					x		x						Differentiation into three germ layers and neural lineage	ND	Mouse ES cells	Choi et al., 2005; Nonaka et al., 2008
3) Entrapment of ES cells (methylcellulose)	x							x	x	x			Differentiation into hematopoietic lineage	ND	Mouse ES cells	Dang et al., 2002
4) Multiwell/ Microfibrication																
4.1) 96 Round bottomed well plate	x				x			x					Differentiation into cardiac and neural lineage	94% of wells have a single EB with diameter of 415 µm	Using polyvinyl carbonate PCR plate without coating reagents	Ezekiel et al., 2007
4.2) 96 well plate coated with MPC or CS	x				x			x					Differentiation into cardiac lineage	EB formed MPC and CS was increased cardiac differentiation	Mouse ES cells	Koike et al., 2005

Cell culture method	Benefit							Detriment					Propose	Yield	Note	Reference
	a	b	c	d	e	f	g	h	i	j	k	l				
4.3) 96 Round bottomed well plate	x				x			x					Differentiation into hematopoietic lineage	Single EBs were achieved from PC surface but not from PS surface	Comparison of EB formation derived from various type of 96 well plate; PS and PS coated with MPC	Konno et al., 2005
4.4) 96 Round bottomed well plate	x				x			x					Differentiation into cardiac lineage	Single EB achieved from PS coated with MPC was near 100%	Comparison of EB formation derived from various type of 96 well plate; PS and PS coated with MPC	Koike et al., 2007
4.5) 96 Round bottomed well plate	x				x			x					Differentiation into hematopoietic lineage	Differentiation was achieved with blood cells formed in 90% of EBs	Force aggregation by using centrifugation (human ES cells)	Ng et al., 2007
4.6) 96 V bottomed well plate	x				x			x					Differentiation into cardiac lineage	> 90% EB formation was achieved from this method	Force aggregation by using centrifugation (human ES cells)	Burridge et al., 2007

Cell culture method	Benefit							Detriment					Propose	Yield	Note	Reference
	a	b	c	d	e	f	g	h	i	j	k	l				
5). Bioreactor																
5.1) A 2-L controlled spinner flask		x	x		x	x	x				x	x	Differentiation into cardiac lineage	$4.6x10^9$ cells of cardiomyocytes were produced in a single run	MHC-neo ES cells	Niebruegge et al., 2009
5.2) Stirred		x	x	x	x	x	x				x	x	Expansion and differentiation into three germ layers	ES cells went through thirteen passages over the same 28 day exhibiting higher pluripotency	Comparison of stirred and SSC	zur Nieden et al., 2007
5.3) Stirred		x	x		x	x	x				x	x	Differentiation into vascular lineage	ND	ND	Evans et al., 2007
5.4) Stirred		x	x		x	x	x				x	x	Expansion and differentiation into neural lineage	A ten-fold increase towards neural differentiation	Human EC cells	Serra et al., 2009
5.5) Stirred		x	x	x	x	x	x				x	x	Expansion and differentiation into osteogenic lineage	A ten-fold of calcium per total grams of protein increase over the control culture	Comparison of stirred and SSC Transplantation	Taiani et al., 2010

Cell culture method	Benefit							Detriment					Propose	Yield	Note	Reference
	a	b	c	d	e	f	g	h	i	j	k	l				
5.6) Stirred		x	x		x	x	x				x	x	Differentiation into hepatic lineage	No significant difference in the specific albumin productivity of EB derived from different groups	Comparison of SSC and HD	Yin et al., 2007
5.7) Stirred + encapsulation (HA and dextran)	x	x	x	x	x	x					x	x	Expansion and differentiation into three germ layers	Dextran can induce EB formation from ES cells.	Mouse ES cells	Nonaka et al., 2008
5.8) Stirred + encapsulation (agarose) + perfusion	x	x	x		x	x					x	x	Differentiation into cardiac lineage	The cardiomyocytes production in encapsulated culture was higher than without encapsulation	Comparison of O_2 tension (MHC-neo ES cells)	Bauwens et al., 2005
5.9) Two type of stirred, STLV and SSC		x	x		x		x				x	x	Differentiation into cardiac lineage	EB formed GBI resulted in high EB yield with homogenous in size	Comparison of hydrodynamic condition (shear force)	Yirme et al., 2008

Cell culture method	Benefit						Detriment						Propose	Yield	Note	Reference
	a	b	c	d	e	f	g	h	i	j	k	l				
5.10) RCCS (STLV and HARV)	x	x	x		x	x					x		Differentiation into three germ layers	A three-fold enhancement in generation of EBs compared to SSC	Comparison of different type of bioreactors and SSC (human ES cells)	Gerecht-Nir et al., 2004
5.11) STLV	x	x	x		x	x					x		Differentiation into cardiac lineage	>90% of the ntEBs generated beating area.	Comparison of STLV and SSC	Lü et al., 2008
5.12) HARV+ encapsulation (alginate)	x	x	x		x	x					x		Differentiation into osteogenic lineage	ND	mouse ES cells	Hwang et al., 2009
5.13) HARV+ encapsulation (alginate) + biograss	x	x	x		x	x					x		Differentiation into osteogenic lineage	ND	Using 70s bioglass	Zhang et al., 2009
5.14) Rotary suspension culture using an orbital rotary shaker		x	x		x	x	x					x	Differentiation into three germ layers	A 20-fold enhancement in the number of cells incorporated into primitive EBs in rotary vs. SSC was detected in the first 12 h	Comparison of rotation, SSC and HD	Carpenedo et al., 2007

Cell culture method	Benefit						Detriment						Propose	Yield	Note	Reference
	a	b	c	d	e	f	g	h	i	j	k	l				
5.15) Orbital shaker + microsphere fibrification	x	x	x	x	x	x						x	Differentiation into three germ layers	Degradable PLGA microspheres releasing RA were incorporated within EBs and induced cystic formation earlier than in non microspheres	Degradable PLGA microspheres releasing RA were incorporated within EBs and induced cystic formation	Carpenedo et al., 2009
5.16) Perfused and dialyzed STLV	x	x	x	x	x	x							Differentiation into neural lineage	Perfused STLV can decrease in expression of markers of undifferentiated stage and increase in expression of markers of differentiation	Comparison of perfused and dialyzed STLV, perfused STLV, non-perfused STLV and SSC	Côme et al., 2008

Abbreviations: a: Homogeneity of EB; b: Scalable production of EB; c: Controlled monitoring; d: Integrated single step of culture (expansion and differentiation); e: Easy to manage; f: Flexible culture cells; g: Heterogeneity of EB; h: Small scale production of EB; i: Labor-intensive procedure; j: Difficult to manage; k: Requires a lot of medium; l: Shear force; CS plate: A polystyrene plate coated with a type of glycosaminoglycan; EB: Embryoid body; EC: embryonic carcinoma stem cells; ES: Embryonic stem; h: hours; HARV: A high aspect rotating vessel; HD: Hanging drop; MHC-neo: Myosin heavy chain-neomycin resistance; MPC plate: 96-well polystyrene plate coated with 2-methacryloyloxyethyl phosphorylcholine; ND: No available data; ntEB: EB derived from nuclear transfer ES; PCR: Polymerase chain reaction; PLGA: Poly(lactic-co-glycolic acid)/poly (L-lactic acid); PS plate: Polystyrene plate; O_2: Oxygen; RA: retinoic acid; RCCS: Rotating cell culture system; SSC: Static suspension culture; STLV: A slow turning lateral vessel.

Bioreactor: Stem cell-based technologies and tissue engineering possibly permit a wide span of clinical and biotechnology applications in future. Nevertheless, realization of the potential of stem cells will require their large-scale generation in a robust system without any limitation (Placzek et al., 2009). This highlights the requirement for the *in vitro* expansion of stem cells used for therapy prior to their commitment into tissue-specific applications. The potential of bioreactors to address this is demonstrated by their capacity to support a robust and well defined scale-up platform for expansion of ES cells (Krawetz et al., 2010), EB formation (Cormier et al., 2006; zur Nieden et al., 2007) as well as differentiation (Lock et al., 2009). The scaling up of the design, given mass transfer limitations, depends on the type of bioreactor chosen (Pörtner et al., 2005). The theory of selecting bioreactors for stem cell expansion and differentiation beyond bench scale is largely reliant on whether the cells are adherent, suspension grown as single cells or aggregates for EB formation (King and Miller, 2007). Therefore, bioreactor culture systems must be designed according to the application. In addition, bioreactors have a significant advantage over SSC which are as follows: (1) scale up of expansion and differentiation of ES cells; (2) no labor-intensive requirements; (3) no space requirement for available area of ES cell growth; and (4) the ability to monitor and control critical culture parameters (*i.e.* pH, dissolved oxygen, glucose consumption, and lactic acid production) (Niebruegge et al., 2009).

At the present time, EB formation in hydrodynamic conditions has been achieved by using bioreactors. They comprise (i) spinner flasks, (ii) RCCS, (iii) rotary orbital culture, and (iv) complex methods combining these techniques. These techniques generally improve ES cell aggregation and form EB faster and more homogeneously in size compared to typical SSC.

(1) Spinner flasks (Figure 2G) have been pioneered, as promising *in vitro* system for stem cell expansion, EB cultivation and differentiation of ES and iPS cells into specific cell types (Serra et al., 2009). Spinner flasks provide attractive benefits due to their simple design, scalable configuration, the flexible culture of cells as aggregates on microcarriers (Abranches et al., 2007) or scaffolds (Vunjak-Novakovic and Radisic, 2004), and ease of continuous monitoring for tight regulation of the culture environment (e.g. O_2 tension, pH, shear forces, medium exchange rate) (Zandstra et al., 2003). The simpler process in spinner flasks equipped with paddle-impellers (PI) results in the formation of large ES cells agglomerates within a few days (Schroeder et al., 2005). The scaling-up is generally straightforward because of improved mass transport achieved by stirring. However, the flow environment created by the impeller renders them inappropriate, due to the shear stress (Chisti, 2001). Numerous culture parameters for this system have been optimized, including the agitation rate, cell initial concentration, medium compositions, and different culturing approaches have been developed. In addition, a low rate of PI stirring results in cell clumping in aggregation supporting EB cultures (leading to lower mass transport to the cells), while high rates of PI stirring can be harmful for the cells. Consequently, an optimal fluid velocity promoting the suitable shear stress for the cell type being cultured is critical (Fok and Zandstra, 2005).

(2) Rotating cell culture system: Cells in conventional stirrer vessels are exposed to hydrodynamic shear stress resulting in damage to the cells. Another approach for controlling EB agglomeration employs RCCS which is comprised of a slow turning lateral vessel (STLV) (Figure 2H) and a high aspect rotating vessel

(HARV) (Figure 2I), as a milder bioreactor. The advantages of these bioreactors are as follows: (i) horizontal rotation is characterized by extremely low fluid shear stress; (ii) fluid-filled culture vessels are equipped with membrane diffusion gas exchange to optimize oxygen levels; and (iii) membrane area to volume of medium ratio is high, thus enabling efficient gas exchange (Lelkes and Unsworth, 2002). The type of rotating vessel had significant impacted on the process of human EB formation and agglomeration; human EBs formed small aggregates with no necrotic centers in STLV. Conversely, human EBs of extensive cell aggregation with large necrotic center are formed in HARV (Gerecht-Nir et al., 2004). STLV bioreactor was used for cultivating mouse and human ES cells to produce EBs and to compare both the quality and quantity of EBs with those from SSC. ES cells grown in a STLV bioreactor were produced higher quality and yielded a nearly four-fold increase in the number of EB particles. EBs derived from a STLV bioreactor showed enhanced cardiac differentiation in comparison to SSC (Lü et al., 2008).

(3) Rotary orbital culture: Bioreactors may offer a more uniform differentiation environment capable of sustaining increased EB and differentiated cell yield. However, these methods may not be suitable solutions for assessing multiple experimental samples in parallel because of the requirement for larger-volume bioreactors. Orbital rotary shakers (Figure 2J) have been used to produce EBs as the constant circular motion for improving the efficiency of EB formation (Gerlach et al., 2010). The advantages of this technique include accommodation of cell culture dishes on the rotary platform, easily allowing production of numerous parallel samples and allowing comparison of different experimental parameters. EBs formed by using orbital rotary shakers appeared to differentiate more efficiently than those produced in SSC on the basis of morphological appearance and gene expression profile patterns. A 20-fold enhancement in the number of cells incorporated into primitive EBs in rotary versus SSC were detected after the first 12 h, and a four-fold increase in total cell yield was achieved by rotary culture after seven days (Carpenedo et al., 2007).

(4) Complex methods combining these techniques: Recently, complex methods combining the above mentioned techniques have been adopted for solving the problems of these methods and keeping cells floating continuously in the culture medium. For example, the agglomeration of cells was avoided by keeping EBs in Petri-dishes for several days before transferring them into a different kinds of environment; (i) spinner flasks; (ii) rotary suspension culture in dishes on an orbital rotary shaker; (iii) direct seeding ES cells into a spinner flask equipped with a glass ball bulb-shaped impeller (GBI) or (vi) two liters stirred tank bioreactor (STR) equipped with a newly developed pitched-blade turbine impeller (Yirme et al., 2008).

In other cases, the encapsulation of ES cells was combined with transferring them into a bioreactor. For example, encapsulation of ES cells in defined conditions (*i.e.* number of cells per EB and capsule size); alginate (Magyar et al., 2001), agarose (Dang et al., 2004), poly (lactic-co-glycolic acid)/poly (L-lactic acid) microsphere (Fernandes et al., 2007; Carpenedo et al., 2009), hyaluronic acid (Nonaka et al., 2008) and Matrigel (Levenberg et al., 2003) was used to control agglomeration of cells. Then, after the initial period of EB formation, all encapsulated ES cells were transferred to a spinner flask. The encapsulation system allowed a 61-fold expansion in the number of cells, similar to the static control non-stirred culture but significantly higher than the stirred non-encapsulated system. Moreover, combination of the encapsulation of ES cells within alginate hydrogel, with or without 70s bioglass,

followed by culturing cells in an HARV bioreactor directly enhanced both osteogenic differentiation in a functional test and generation of functional 3D mineralized constructs for further application of bone tissue engineering transplantation (Hwang et al., 2009; Zhang et al., 2009). Finally, mouse ES cells expanded as aggregates on microcarriers in stirred vessels retained expression of stem cell markers and could form EBs. Perfusion combined with frequent feeding has been shown to increase the expansion of ES cells and their differentiation into specific lineages, without compromising their stem cell performance (Bauwens et al., 2005). Additionally, the effect of a rotary bioreactor promoted neural differentiation of human ES cells in perfused and dialyzed STLV. The mean time delay for growing to so-called “neural rosette” formations was significantly shortened under STLV conditions compared to conventional SSC. Likewise a perfused STLV bioreactor can decrease the expression of markers of undifferentiated stage and increase the expression of markers of differentiation, especially towards neural lineage commitments (Côme et al., 2008).

Recently, researchers have sought to develop culture systems with integrated bioprocesses, controlling stem cell expansion and differentiation tightly in a fully controlled bioreactor environment. For example, a ten-fold increase in expansion of ES cells as well as consequent neural differentiation was reported while drastically reducing, by 30%, the time required for the differentiation process (Serra et al., 2009). Moreover, microcarrier spinner flasks have been used for the culture of mouse ES and human ES cell expansion and directed differentiation. Mouse ES cells were allowed to proliferate on microporous collagen-coated dextran beads (Cytodex 3), glass microcarriers, and macroporous gelatin-based beads (Cultispher S) in spinner flasks (Akasha et al., 2008). Under different inoculated cell densities and microcarrier concentrations, mouse ES cells on microcarriers showed increased yield of approximate 70-fold (eight days) to about 190-fold (fifteen days). These cultured cells also successfully expressed Oct-4, Nanog, and SSEA-1, and when dissociated from the beads, they formed EBs yielding cells with differentiation markers such as Flk-1, a cluster of differentiation molecules 34 (CD34) and α-myosin heavy chain (α-MHC) (mesoderm), HNF-3b19 (endoderm), and b3-tubulin57 (ectoderm) (Fong et al., 2005).

Computer-controlled bioreactors: As a fore mentioned, the main advantage of computer-controlled bioreactors is a process development by allowing online monitoring and control of specific culture parameters (temperature, pH, PO_2, lactic acid production and glucose consumption), and ensuring a fully controlled environment for stem cell cultivation (Dang and Zandstra, 2005). Oxygen-controlled bioreactors have been used for culturing mouse ES and human ES cell-derived cardiomyocytes. These experiments also assessed the effect of O_2 tension on cardiac differentiation which is a main concern (Bauwens et al., 2005). Moreover, this system was recently applied to culturing cells not only for stem cell expansion but also for differentiation. Expansion of a variety of stem cell types in bioreactors under defined and controlled conditions remains to be addressed. Future challenges also include the combination of expansion and directed differentiation steps in an integrated bioprocess that will ultimately result in scale-up of well differentiated cells to clinically relevant numbers. It is worth mentioning that, although differentiating cells in bioreactors have numerous benefits, these cells have been assessed for functionality by transplantation, and did not always perform well (Akasha et al., 2008; Côme et al., 2008). Ten and twenty days post-implantation ES cells derived chondrogenic and osteogenic bioreactor aggregates showed no obvious influence on the healing process.

In these experiments, all of the bioreactor derived cells showed higher Oct-4 expression in the aggregates, even after 30 days of induced differentiation in a medium without LIF (Taiani et al., 2010). This emphasizes the importance of proper condition set-up and timing during cultivation of cells in bioreactors.

1.2.5 Differentiation of pluripotent stem cells into cardiomyocytes by using bioreactor

Regenerative medicine based on cell transplantation therapy has increasingly attracted an attention as a potentially alternative way for organ transplantation (Wu et al., 2006). Pluripotent stem cells (ES and iPS cells), because of their pluripotency and unlimited self-renewal capacity are promising cell sources to provide sufficient numbers of cells for therapeutic applications. However, the expansion and differentiation of these cells is still limited as a result of their complexity and difficult manageability in scale-up production for industrial purposes (Mummery et al., 2002; Zweigerdt et al., 2003). To solve these problems, bioreactor culture systems offer attractive advantages of ready scalability and relative simplicity (Fong et al., 2005; Bratt-Leal et al., 2009).

Recently, a single-step bioprocess for ES cell-derived cardiomyocyte production has been developed by combining methods to prevent ES cell aggregation (hydrogel encapsulation) and to purify for cardiomyocytes from the heterogeneous cell populations by using genetic selection (myosin heavy chain-neomycin resistance; MHC-neo), with medium perfusion in a controlled bioreactor environment. It has been shown that the cardiomyocytes yield per input ES cells achieved in encapsulated culture were much higher than without encapsulation (3.17 ± 0.90 vs 0.16 ± 0.07). Furthermore, higher cardiomyocyte yields were higher under hypoxic condition (4% O_2 tension) than normoxia condition (20% O_2 tension), when cultured in the stirred culture system (Bauwens et al., 2005). In addition, a 2-L bioreactor process enabling the controlled generation of EBs, derived from MHC-neo ES cell line, has been adopted for enhancing yield of ES-derived cardiomyocyte production. The fill-and-draw feeding protocol will be replaced in a 2-L bioreactor, which allowed constant medium supply and avoided daily fluctuations of medium components. An optimized protocol resulted in more than five times greater cardiomyocyte yield, whereas medium consumption will be 40% less than that in the control system (Serra et al., 2009).

For the controlled large-scale generation for clinical and industrial applications in humans, the efficacy of the dynamic process (Erlenmeyer, STLV, Glass Ball Impeller (GBI) spinner flask and PI spinner flask) were compared to SSC in Petri-dishes by analyzing the quality of EB formation and subsequent differentiation into cardiomyocytes. The EB prearrangement in the static system and EB cultivation in the GBI spinner flask resulted in high EB yield, a round homogenous shape, the fastest growth rate and high contracting EB percentages over all other systems (Yirme et al., 2008).

1.3 Objectives of the thesis

1. To establish mouse pES cell lines from parthenogenetically-derived blastocysts and to examine pluripotency differences among them
2. To compare the effects of, inoculating different cell numbers, rotation speed and time of EB adherence to gelatin-coated dishes on EB formation and cardiac differentiation

3. To compare the effect of EB derivation methods (STLV, SSC and HD) on EB formation and cardiac differentiation
4. To establish a strategy for scalable production of iPS cell derived cardiomyocytes using the STLV bioreactor

1.4 Hypothesis

1. There might be differences among pES cell lines
2. EB formation and cardiac differentiation were not influenced by inoculating different cell numbers, rotation speed and time of EB adherence to gelatin-coated dishes
3. Methods of EB formation (STLV, SSC and HD) would not effect on EB formation and cardiac differentiation
4. The optimized STLV bioreactor parameters were applicable for EB production from different iPS cell lines

1.5 Keywords: Cardiomyocyte, Differentiation, Embryonic stem cells, Induce pluripotent stem cells, Mouse, Parthenogenesis and Pluripotent stem cells

1.6 Research merits:

1. The optimized STLV bioreactor parameters for generating the high quantity of scale-up EBs derived from ES and iPS cells and subsequency differentiation into cardiomyocytes
2. The knowledge about embryogenesis and cardiogenesis by using *in vitro* EB formation
3. The knowledge about the influence of cardiogenesis and cardiomyocyte differentiation *in vitro*
4. The knowledge about the alteration of gene expression pattern during cardiomyocyte differentiation

CHAPTER II

COMPARATIVE CHARACTERIZATION OF MOUSE PARTHENOGENETIC EMBRYONIC STEM (PES) CELL LINES

2.1 Abstract

Derivation of ES cells from parthenogenetic embryos represents a possible alternative approach to create histocompatible cells for regenerative medicine. The objectives of this study were to establish mouse pES cell lines from parthenogenetically-derived blastocysts as a model system for human and animal research and to examine pluripotency differences among the pES cell lines. We are able to report the successful establishment of four pluripotent pES cell lines from blastocysts of parthenogenetic origin (22% efficiency of pES cell line establishment). Four pES cell lines (pES#1 to #4) exhibited a typical ES cell morphology and expression of key pluripotency markers (ALP, Oct-4, Nanog and SSEA-1). Three of the four pES cell lines have shown a high percentage of normal karyotype during long-term culture. Variability in the *in vitro* differentiation potential into cell types of the three germ layers was observed among the different pES cell lines. Three of these (pES#1 to #4) exhibited a higher efficiency towards endo-mesoderm differentiation, strongly expressed differentiation markers towards endo-mesoderm lineage (α-fetoprotein; Flk-1; PECAM and collagen IV) than pES#4. Differentiation towards cardiac cells resulted in all cell lines 33 to 100% of spontaneous beating cell clusters/well. Furthermore, following injection into blastocysts pES#1 cells differentiated successfully *in vivo* into chimeric mice with an efficiency of 75% (three chimeras of four newborns). In conclusion, our results have demonstrated that there are major differences among pES lines in their differentiation ability *in vitro* and that it was possible to generate chimera forming pES cell lines in mouse.

2.2 Introduction

To date, stem cells have been intensively studied in cell replacement therapy for treating several incurable diseases, such as Alzheimer's, Parkinson's and heart diseases. With the creation of ES cells, there is resource with potential to differentiate into all tissues of the body. Interestingly, at the present time this technology is a fast-developing area not only in human but also in companion animals (dogs, cats and horses) because many of the diseases such as diabetes and musculoskeletal injuries of companion animals are similar to those found in human (Schneider et al., 2008; 2010). Furthermore, the establishment of companion animal ES cell lines and the availability of those cell lines for *in vitro* differentiation have already been developed and investigated (Wilcox et al., 2009; Gómez et al., 2010; Guest and Allen, 2007). If the therapeutic possibilities in terms of tissue repair in organs with a limited capacity for functional regeneration, or for generating tissues for reconstructive surgery in human and companion animals, can be exploited, the field of regenerative medicine in human and animal will expand very rapidly. These properties make pluripotent cells an attractive candidate for the cell replacement therapy of various degenerative diseases that are associated with a loss of functional cells. This pluripotent cell technology thus opens the 'window' of cell replacement therapy for both human and veterinary medicines (Tecirlioglu and Trounson, 2007).

Generally, ES cells are derived from the ICM of fertilized embryos. These cells, however, may induce Graft Versus Host Disease (GVHD) after allogenic

transplantation (Lerou and Daley, 2005). To solve this problem, the establishment of ES cell lines from patient derived SCNT-embryos or from female patient's parthenogenetic embryos has recently been developed (Kim et al., 2007) as a potential source of genetically and immunologically "matching" stem cell derivation. The parthenogenetic embryos have the additional advantage of being ethically more acceptable, as such embryos are produced by parthenogenetic activation without fertilization and these embryos could not develop up to term after transfer into a surrogate female (Strum et al., 1994). We must emphasize that generating human embryos intentionally for research purposes is an ethically debated issue, and in many countries legally unacceptable at the moment. Such research would not be financed from the European Union Framework programme. Technically it is also challenging to generate patient-specific ES cells by SCNT in human as the human oocytes needed for this inefficient process are hard to obtain.

Currently, an alternative method known as "iPS cells technology" is gaining popularity, which is based on the derivation of pluripotent stem cells by genetic reprogramming of somatic cell types. This was achieved by retroviral transduction of a combination of four transcription factors, Oct-4, Sox2, c-Myc and Klf4 into somatic cells first in mouse (Takahashi and Yamanaka, 2006) and soon after that in human (Takahashi et al., 2007). Since then the technology has evolved rapidly (Sun and Liu, 2011). However, there are many questions which need to be clarified on the genetic and functional equivalence of these cells compared to human ES cells (Lister et al., 2011), and on the safety evaluation of the iPS technology prior to further applications in human therapy.

Compared to the iPS cells, pES cell lines derived from parthenogenetic embryos have several advantages as they are more close to the "natural" pluripotent cell types (Brevini et al., 2008). The derivation efficiency for pES cell lines is much higher than that for human ntES cell lines where it has never been achieved. Thus the prime method for derivation of MHC matched ES cell lines might be the pES cell line based approach (Revazova et al., 2007). Although an ultimate objective of research in ES cells is undoubtedly for use in human therapy, the religious and ethical considerations of using human embryos have restricted the use of human as a potential model for studying and producing ES cells (Snyder et al., 2006). Therefore, animals such as mouse and rat (Iannaccone et al., 1994) have been used as models for the procedures.

To fully harness the pES method, it is necessary to understand their biology in depth and to clarify technological elements. Here we report the successful establishment of four mouse pES cell lines. We also investigated them by karyotyping for normal chromosomal counts, and by *in vitro* and *in vivo* differentiation assays. The variability among the different pES cell lines in mouse was considerably high and this is an ideal model for human and companion animal research. The potential of *in vitro* differentiation of pES cells into cell types of the three germ layers and into cardiomyocytes have been compared, and chimera embryos have also been created.

2.3 Materials and methods

Chemicals

All chemicals were purchased from Sigma (Sigma Aldrich, St Louis, USA) and culture reagents were purchased from Invitrogen (Invitrogen by Life Technologies, Carlsbad, CA, USA) unless otherwise specified.

***In vitro* cell culture**

Cells were cultured at 37C in a humidified atmosphere containing 5% CO_2. The medium was changed daily for mouse pES cell cultures and every two days during differentiation.

Animals:

Male DBA2, female C57BL/6 and outbred ICR mice were purchased from the National Laboratory Animal Centre, Mahidol University which were raised at the laboratory of the Faculty of Veterinary Sciences, Chulalongkorn University under of controlled lighting (14:10 h light–dark cycle), 20 to 22°C, and 40% to 60% of humidity. F1 mice used in our experiment were derived by mating male DBA2 with female C57BL/6 mice. All procedures for animal management, breeding, and surgery followed the standard operation protocols of the Faculty of Veterinary Sciences, Chulalongkorn University under ethical consideration. Appropriate management of experimental samples and the quality control of the laboratory facilities and equipment were maintained.

2.3.1 Production of embryos

F1 female mice (8 to 10 weeks old) were superovulated by 7.5 international unit (IU) pregnant mare serum gonadotropin (PMSG; Folligon, Intervet) intraperitoneal (i.p.) injection, followed 48 h later by 7.5 IU human chorionic gonadotropin (hCG Chorulon, Intervet) i.p. injection. The cumulus-oocyte complexes (COCs) were recovered from oviducts 15 to 16 h post-hCG injection in M2 medium, and cumulus cells were removed by incubation in 150 IU/ml bovine testicular hyaluronidase in M2 medium. After washing three times in M2 medium, the MII oocytes with clear cytoplasm with uniform texture, homogeneous fine granularity, and intact first polar body were selected. Subsequently, the MII oocytes were activated by using 10 mM/ml Sr^{2+} plus 5 µg/ml Cytochalasin B (CB) in Ca^{2+}-free CZB medium for 6 h (Kishigami et al., 2006). Afterward, oocytes were considered activated when two pronuclei (2PN) are developed. Activated oocytes were cultured for four days in KSOM-AA medium (Speciality Media, Phillipsburg, NJ) at 37C5 under a humidified atmosphere with 5% CO_2 in air until they developed to expanded or hatched blastocysts.

2.3.2 Differential staining of blastocysts

Differential staining of the ICM and the trophectoderm (TE) cells was performed as described earlier by Thouas et al. (2001) with a slight modification. Briefly, intact expanded blastocysts (approximately 96 h after treating with parthenogenetic agent), were incubated with 1% (v/v) Triton X-100, 0.01% polyvinylpyrrolidone (PVP) and 100 µg/ml propidium iodide (Molecular Probes, Invitrogen, Oregon, USA) in phosphate buffered saline (PBS) for up to 15 s. Subsequently, blastocysts were transferred into a fixative solution of 100% ethanol with 25 µg/ml bisbenzimide (Hoechst 33342, Molecular Probes) and maintained in this fixative at room temperature for 10 min. After that, embryos were mounted onto a glass microscopic slide in a droplet of glycerol and sealed with a coverslip. The embryos were counted for ICM and TE cell numbers using an epifluorescent

microscope (BX51 Olympus, Shinjuku, Japan) (Figure 3D). The ICM was classified as a group of embryonic cells stained with only Hoechst 33342 (blue), whilst TE cells were positive to both Hoechst 33342 and propidium iodide. Hence, trophoblast cells appeared pink, whereas ICM cells appeared blue.

2.3.3 Establishment and culture of pES cells

Intact expanded blastocysts (approximately 96 to 102 h after parthenogenetic activation), were cultured on feeder layers of mitomycin C-inactivated confluent MEFs which were obtained from the day of embryonic development (E) 13.5 mouse embryos as described earlier (Nagy et al., 2003). Blastocysts were observed every 24 h for hatching and attachment of trophoblasts to a single MEF layer, while ICM size was also monitored. On day five or six after plating, ICM outgrowths were separated and dissociated from trophoblasts using a glass pipette, followed by trypsinization using 0.25% (w/v) trypsin with ethylenediaminetetraacetic acid (trypsin-EDTA) to promote cell dispersion. After re-plating the ES cell-like colonies were observed, trypsinized and propagated by passaging every two to three days until freezing or characterization. These cells were maintained in ES medium consisting of Dulbecco's Modified Eagle's Medium (DMEM), 20% (v/v) fetal bovine serum (FBS, Hyclone, Logan, UT) supplemented with 2,000 U/ml mouse LIF (ESGRO, Chemicon Int., Temecula, CA, USA), 0.1 mM nonessential amino acids (NEAA), 0.1 mM β-mercaptoethanol (β-ME), 50 U/ml penicillin and 50 µg/ml streptomycin. The medium was changed daily.

2.3.4 Characterizations of pES cells

2.3.4.1 ES cell morphology

Pluripotent mouse pES cells were maintained in an undifferentiated state on MEFs. The pES cells were examined by phase-contrast light microscopy whether their gross morphology correspond to the typical ES cell morphology with round shape, compact, and shiny colonies.

2.3.4.2 Alkaline phosphatase (ALP) activity and Immunohistochemistry (IHC)

The pES cells and EBs were cultured on 1% gelatin-coated coverslips and were fixed with 4% Paraformaldehyde (PFA) in PBS for 15 min. ALP expression was determined using the standard methodology. Samples were permeabilized with 0.25% Triton-X100 for ten min. Blocking was performed in 1% bovine serum albumin (BSA), after which samples were incubated with a primary antibody overnight at 4^{o}C. Primary antibodies for pES cells included goat polyclonal against Oct-4 (sc9081, diluted 1:100; Santa Cruz Biotechnology, Germany), rabbit polyclonal Nanog (sc33760, diluted 1:100 dilution, Santa Cruz Biotechnology, Germany) and mouse monoclonal against SSEA-1 (MC-480, diluted 1:200 dilution; Abcam, UK) and primary antibodies for cardiac differentiation including mouse monoclonal against cardiac troponin T (cTnT, ab33589, diluted 1:200; Abcam, UK). After extensive washing, EBs were exposed to Alexa Fluor 488 donkey anti-goat IgG, Alexa Fluor 594 goat anti-rabbit, Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 594 goat anti-mouse IgG-conjugated secondary antibody (A-11055, A-11012, A-11008 and

A11005, respectively, diluted 1:2,000; Invitrogen, USA) at room temperature for 60 min. The nuclei of cells were counter-stained with 4'-6- Diamidino-2-phenylindole (DAPI) and visualized by fluorescent microscopy.

2.3.4.3 *In vitro* differentiation of pES cells into cells of the three germ layers and cardiomyocytes

EBs were prepared by using the HD method (Adapted from Mummery et al., 2007). In brief, pES cells were dissociated with a 0.05% trypsin-EDTA solution to get a single cell suspension and seeded as $4x10^5$ cells/ml (resulting in 8,000 cells/drop) suspension in differentiation medium (ES medium without LIF). Three days later, EBs were transferred to a 100-mm bacteriological Petri-dish in ten ml of differentiation medium. For differentiation into cell types of the three germ layers, EBs were continuously cultured in suspension for fifteen days. EBs were collected, washed three times with PBS, fixed overnight in 10% neutral-buffered formalin, dehydrated in a series of alcohol gradients (70%–100%), embedded in paraffin and examined for general histological analysis. Sections of 5 µm were stained with hematoxylin and eosin (H&E) for visually assessing morphology. For spontaneous cardiac differentiation, individual EBs on the fifth day were placed in gelatin coated 24-well plates for an additional fifteen days and observed daily under a phase-contrast microscope. 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. At the end point of culturing (twenty-day EBs) EBs were fixed, permeabilized and blocked as described above for IHC.

2.3.4.4 *In vivo* differentiation of pES cells into chimeric offsprings

The ability of pES cells to colonize the germline of a host embryo was tested by injection of pES cells after the fifth passage into host blastocysts and implantation of these chimeric embryos into pseudopregnant foster mothers as described by Nagy et al. (2003). Briefly, blastocyst injection was carried out using day 3.5 blastocysts collected from the uteri of superovulated ICR females by flushing with M2 medium. The collected blastocysts were washed and cultivated at 37°C in KSOM -AA medium under humidified 5% CO_2 in air before being used as recipient blastocysts. The pES cells were dissociated with a 0.05% trypsin-EDTA solution and were resuspended in ES medium. Blastocyst injection was carried out by injecting five pES cells into an ICR blastocyst under microscope equipped with a Narishige-micromanipulator system. After injection, seven to eight chimeric blastocysts were transferred into both uterine horns of a day 2.5 pseudopregnant ICR female mice, which has previously been mated with vasectomized males. The coat-color difference of the pES cell line genetics (dark) in the ICR blastocysts (white) allowed an estimation of chimerism rate (i.e., contribution of ES cell genome to chimeric offspring). Germline transmission of pES cell genome was then tested by crossing high-rate chimeras with ICR mice.

2.3.4.5 Karyotype analysis

Chromosomal numbers of established pES cell lines were performed either every 5th passage of subcultures or before chimera production as described by Nagy et al. (2003) with minor modifications. Briefly, mouse pES cell lines were treated with colcemid in ES medium for 1.5 h at 37°C in an atmosphere of 5% CO_2 in air. Then, the treated pES cell lines were harvested and resuspended for 15 min in 0.075 M KCl at 37°C. The pES cells were placed in hypotonic solution and subsequently fixed in 3:1 mixture of methanol and acetic acid. Chromosome spreads were performed by dropping cell suspension onto slides and stained by the trypsin-Giemsa banding technique. At least 50 metaphases from each cell line were examined in order to count their chromosome numbers.

2.3.4.6 Reverse transcription polymerase chain reaction (RT-PCR) analysis of pluripotent and differentiation expression

RT-PCR reactions to detect pluripotent (Oct-4 and Nanog) and endo-mesoderm differentiation (endoderm lineage: α-fetoprotein and mesoderm lineage: Flk-1, platelet endothelial cellular adhesion molecule (PECAM) and collagen IV) transcripts were performed. Total RNA from pES cells, EBs and fibroblast cells was prepared using the RNeasy Protect Mini Kit (Qiagen) and was reversely transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen). Ex Taq Hot Start Version (Takara, Shiga, Japan) was used in the PCR reaction (Table 2).

2.3.5 Experimental design

After the establishment of pES cell, the new pES cell lines were characterized by morphology of ES cells, chromosome number analysis, IHC for pluripotency markers, and pluripotency *in vitro* by differentiation into the three germ layers (via EB formation and cardiomyocytes then IHC and RT-PCR) and pluripotency *in vivo* by chimera progeny production.

Table 2. Properties of the oligonucleotide primers used in RT-PCR reaction

Primer	Size (bp)	Sequence
Oct-4	293	(Forward) 5'-GGCGTTCTCTTTGGAAAGGTGTTC-3'
		(Reverse) 5'-CTCGAACCACATCCTTCTCT-3'
Nanog	449	(Forward) 5'- TGAGAT–GCTCTGCACAGAGG-3'
		(Reverse) 5'-CAGATGCGTTCACCAGATAG -3'
Flk-1	599	(Forward) 5'-CCTGGTCAAACAGCTCATCA-3'
		(Reverse) 5'-AAGCGTCTGCCTCAATCACT-3'
PECAM	588	(Forward) 5'-AGACTTTAACCAAGGGCGGT-3'
		(Reverse) 5'-TAGCCAGGTTGCGAAGAACT-3'
Collagen IV	463	(Forward) 5'-CAAGCATAGTGGTCCGAGTC-3'
		(Reverse) 5'-AGGCAGGTCAAGTTCTAGCG-3'
α-Fetoprotein	494	(Forward) 5'AGTGCGTGACGGAGAAGAAT-3'
		(Reverse) 5'-TGTCTGGAAGCACTCCTCCT-3'
GAPDH	27	(Forward) 5'-ACCTCAACTACATGGTCTAC-3'
		(Reverse) 5'-TTGTCATTGAGAGCAATGCC -3'

Abbreviation: GAPDH, Glyceraldehyde 3-phosphate dehydrogenase

2.4 Results

2.4.1 Parthenogenetic activation

A total of 130 oocytes were collected (Figure 3A) and activated parthenogenetically as described above. 91.5±6.06% was activated successfully based on the presence of 2PN and one polar body (Figure 3B). 87.7±4.02 and 83.9±5.52 of activated oocytes developed into 2-cell stage embryos and blastocytes, respectively (Figure 3C). In this experiment, we found that the total cell number, ICM cell number and ICM: trophectoderm ratios of parthenogenetic blastocysts were 66.8±2.88, 8.1±0.63 and 1:7.24, respectively (Figure 3D).

2.4.2 Derivation of pES cell lines

Pictures of the blastocysts derived from parthenogenetic activation and plated onto mitomycin-inactivated MEFs (Figure 3C); the hatched blastocysts attached to MEFs (Figure 3E); and the primary ES cell-like colonies on four to five days after trypsinization (Figure 3G) are presented. Four pES cell lines were successfully derived from 18 parthenogenetic blastocysts (22% efficiency, Figure 3H).

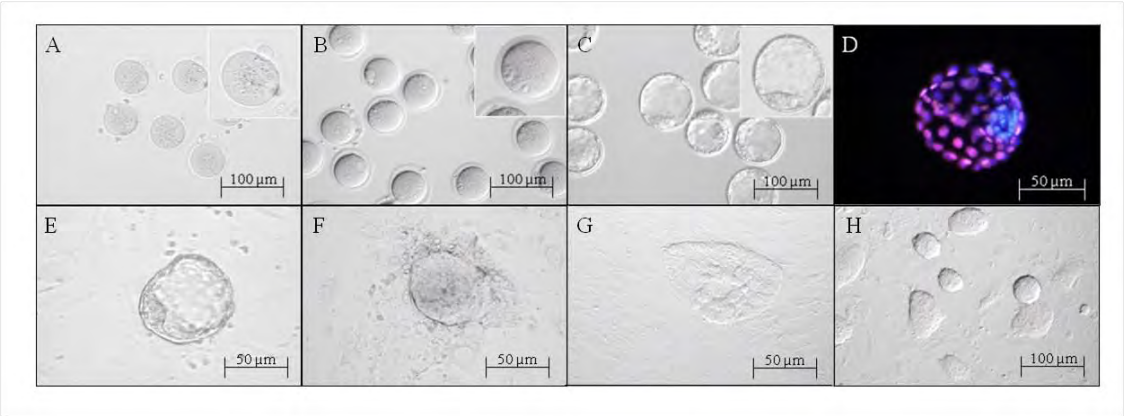


Figure 3. Establishment of mouse pES cells. The procedure of deriving pluripotent stem cells from parthenogenetic activation is shown. (A) MII oocytes exhibit an intact first polar body and homogeneously fine granular and light-colored ooplasm. (B) Oocytes with 2PN and one polar body. (C) Expanded blastocyst with the ICM. (D) Differential staining of blastocyst-stage embryos. (E) The attachment of a hatched blastocyst to feeder layers. (F) ICM-outgrowths. (G) ES-like colony. (H) ES cell-like cell line (1st passage). Scale bars correspond to 100 µm (20x magnification: A to D and H) and 50 µm (4x magnification: E to G).

2.4.3 The effect of different pES cell lines on morphology and proliferation

Four pES cell lines were maintained in culture until passage 20. These cell lines displayed typical morphology of mouse ES cells, including round shape colonies, a high nuclear:cytoplasmic ratio and exhibited vigorous growth rate during long-term culture and

2.4.4 Detection of pluripotency markers in different pES cell lines

Subsequently, we examined if pES cells following long term expansion retain the expression of pluripotency markers. Expression of pluripotency markers was constant during the culture based on ALP activity, IHC (Oct-4, Nanog and SSEA-1) and RT-PCR (Oct-4 and Nanog) examinations. Four pES cells were cultured on MEF forming colonies morphologically similar to that of undifferentiated ES cells and were strongly positive for ALP, Oct-4, Nanog and SSEA-1 by IHC (Figure 4A to 4T) and confirmed by RT-PCR analysis. Our results showed that all pES cell lines expressed both pluripotency markers (Oct-4 and Nanog) and no differences in gene expressions were observed among the pES cell lines (Figure 5). The expression of differentiation-related genes (α-fetoprotein, Flk-1, PECAM and collagen IV) tested was not detected in the pES cell lines, thus confirming the undifferentiated state (Figure 5). Our findings showed that different pES cell lines were able to maintain in long term culture without differentiation and expression (or lack of it) of all pluripotency, and differentiation markers examined was not different among the cell lines.

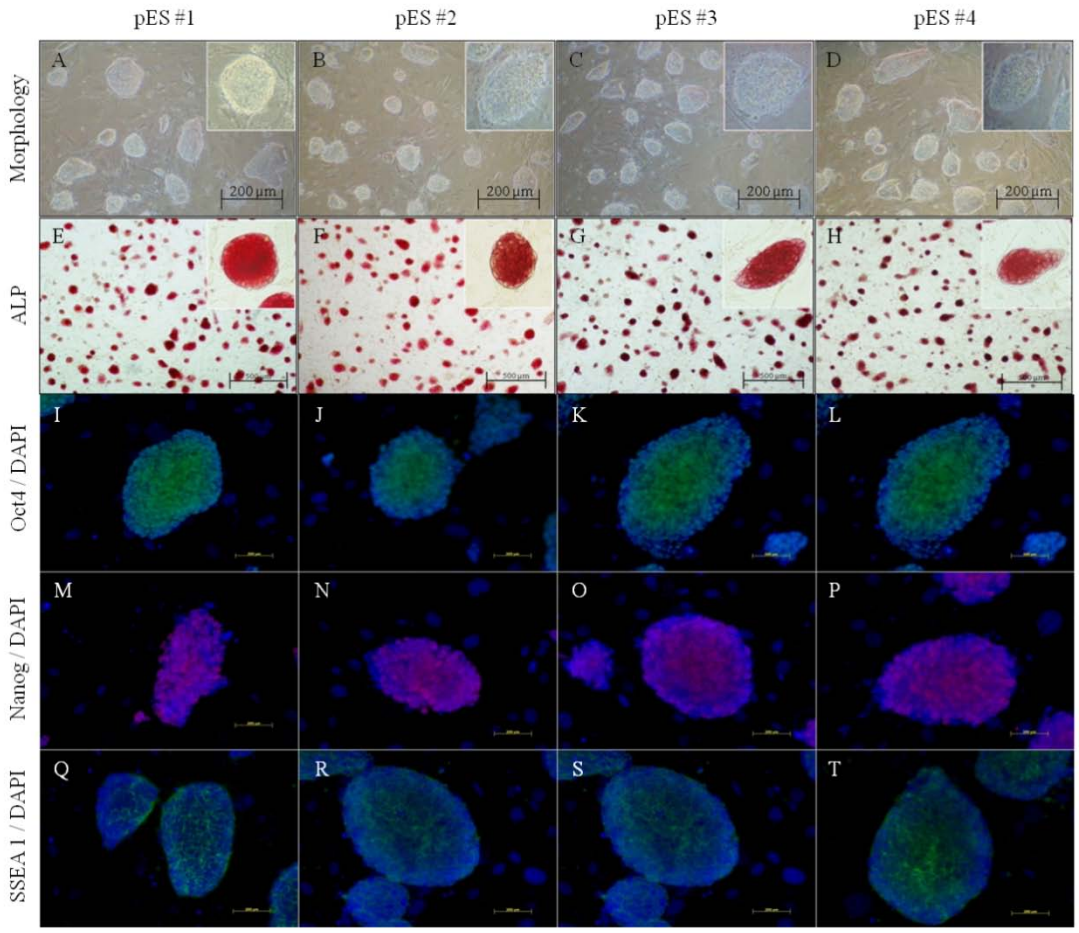


Figure 4. Characterization of mouse pES cell lines (pES #1 to #4). (A to D) all pES cell lines exhibit a typical gross morphology of mouse ES cells with high nuclear/cytoplasmic ratio and round shape. The cell lines strongly expressed ES cell markers (ALP: E to H, Oct-4: I to L, Nanog: M to P and SSEA-1: Q to T counterstaining with or without DAPI).

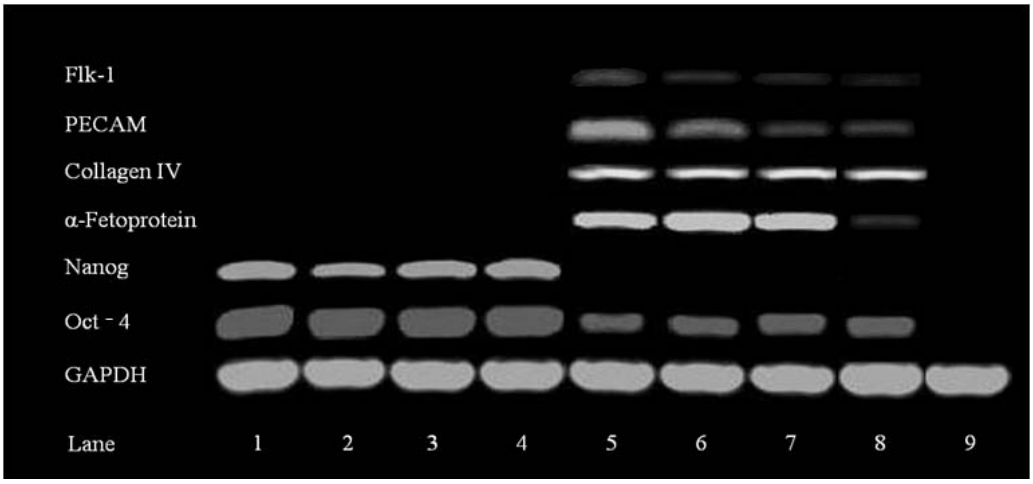


Figure 5. RT-PCR analysis for ES cell marker and differentiation marker gene expression in pES cells; four different pES cell lines and their EBs (day fifteen). ES cell markers: Oct-4 and Nanog; endoderm lineage marker: α-fetoprotein and mesoderm lineage markers: Flk-1, PECAM and collagen IV). RT (-) indicates that the reverse transcriptase was omitted in the cDNA synthesis step, and was subjected to PCR in the same manner with primer sets for GAPDH. Lane 1 to 4: pES #1 to #4: Lane 5 to 8: pEBs #1 to #4 (day fifteen): Lane 9: feeder layer were usually passaged every two days during culture (Figure 4A to 4D; pES#1 to #4). The morphology and proliferation rate were very similar among the four pES cell lines and all of them remained pluripotent following long term expansion.

2.4.5 *In vitro* differentiation of pES cell lines into various cell types

Culturing pES cells in suspension culture without LIF by using the HD method, these cells aggregated, spontaneously differentiated and formed EBs. After day three of culture, a distinctive outer layer of endodermal cells appeared in each droplets containing a spherical EB. In this study, we determined the efficiency of EB formation in HD culture by measuring a percentage of single EB produced from one drop. Our results showed that pES#4 exhibited lower efficiency of EB formation (pES#4; 35% of EB formed vs pES#1 to #3 where 100% of EB formed) and also produced smaller EBs compared to the other pES cell lines (Figure 6A to 6D). Subsequent culture of EBs resulted in EBs displaying foci of pulsatile contractions typical of cardiomyocyte differentiation on day nine in suspension culture and stopped beating on day twelve only in pES#1 to #2 (Supplementary movie 1). There was no beating EBs in pES#3 to #4. PES#1 formed EBs early which became cystic structures by day twelve compared to pES#2 to #3 which formed cystic structures one day later (day thirteen) (Figure 6E to 6G). However, pES#4 did not form cystic EBs at all (Figure 6H). This study also examined the generation of tissues in the differentiating EBs by histology. Overall the pES cell lines were able to differentiate into cell types of all three germ layers: containing neural rosette formations (ectoderm lineage, Figure 6I to 6J and 6L, insert), connective tissue (mesoderm lineage, Figure 6J to 6L, *) and simple squamous epithelium in the periphery and epithelial cells surrounding a lumen in the center, similarly to yolk sac structures (endoderm lineage, Figure 6K, red arrow). However, there were differences in the proportion of the cell types formed among the different cell lines. For example pES#4 formed EBs with low proportion of cells of endodermal lineage and differentiated mainly into neural lineage direction (Figure 6L).

Furthermore, we also evaluated endo-mesoderm differentiation in EBs on day fifteen by the expression of α- fetoprotein which is initially expressed in the primitive endoderm during early post-implantation stages and is maintained in the visceral and parietal endoderm of the yolk sac during gastrulation. Interestingly, the expression of α- fetoprotein was strongly expressed in pES#1 to #3 but weakly in pES#4 EBs. Expressions of Flk-1, PECAM and collagen IV, all referred as mesoderm specific, were differentially regulated as well. Flk-1 expression, marker of progenitors with vascular potential, was low in all four lines. PECAM, a member of immunoglobulin superfamily expressed by endothelial cells and a subset of hematopoietic cells, was strongly expressed in pES#1 and #2 EBs and at a much lower level in pES#3 and #4. The level of collagen IV expression, a cartilage component, was high is all four lines. The expression of pluripotency markers was not different among cell lines: Oct-4 gradually decreased during differentiation with a still detectable expression level and Nanog expression almost entirely disappeared in EBs on day fifteen (Figure 5).

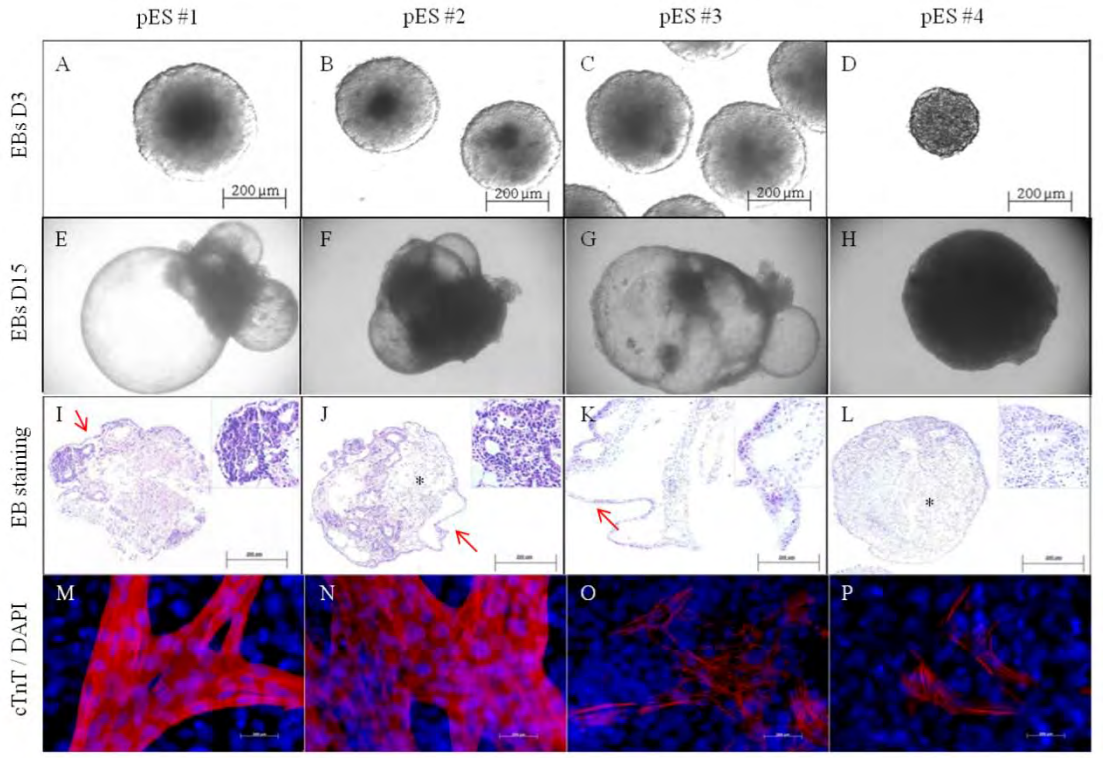


Figure 6. Comparison of *in vitro* and *in vivo* differentiation of mouse pES cells into cell types of the three germ layers. (A to D) Day three EB culture (EBs D3): gross morphology of simple EBs. (E to G) Day fifteen EB culture (EBs D15): gross morphology of typical cystic EBs with visceral yolk-sac-like structures (pES#1 to #3). (H) EBs D15 EB culture: gross morphology of EBs without cavitation structure (pES#4). (I to L) Histological analysis by H&E staining of EBs D15. Note that I to J and L insert: neural rosette formations (ectoderm lineage); J and L (*): connective tissue (mesoderm lineage) and I to K (red arrow): simple squamous epithelium in the periphery and epithelial cells surrounding a lumen in the center, similarity as yolk sac structure (endoderm lineage). (M to P) Detection of typical cardiomyocyte proteins differentiation cultures by IHC staining on day 21 of differentiation. Cells were stained with cTnT (red) and nuclei are stained with DAPI (blue).

These results strongly suggest that although all four pES cell lines were capable of differentiation into cell types of the three germ layers, major variation exists among different pES cell lines in their quantitative and qualitative capacity of differentiation by the EB-method.

2.4.6 *In vitro* differentiation of pES cell lines into cardiomyocytes

Spontaneous cardiomyocyte differentiation was observed in EBs which were plated from day five onto gelatin coated dishes and cultured until day twenty significantly varied among the cell lines. The efficiency of forming contracting EBs after twenty days varied from 33% to 100% of the EBs having contracting regions. Spontaneously beating EBs started to appear at day eight (pES#1), day ten (pES#2 and #3) and day thirteen (pES#4). PES#1 cells showed the greatest efficiency, reaching a maximum of 100% of EBs (Supplementary movie 2), in contrast to the pES#2, 3 and 4, which reached 79%, 83% and 33%, respectively (Figure 7). All contracting EBs kept continuously beating during the long term culture. The above results confirmed that there was not only observable difference in the time course for the development of contraction but also the efficiency of forming contracting EBs varied for the different pES cell lines.

We have evaluated pES cell-derived cardiomyocytes by IHC for cTnT which is a highly cardiac-specific myofilament protein (Figure 6M to 6P). PES#1 and #2 formed an extensive and well-organized cTnT network and showed higher amounts of cTnT positive areas than that in pES#3 and #4 cell-derived cultures. Although, pES#2 and #3 gave a similar ratio of beating EBs, the level of organisation of the cTnT network and the positive areas were different. These results suggested that besides the observable differences in the ratio of cardiac beating, it is important to examine the organization of sarcomeric structures which might reveal important differences among the pES cell-derived cardiomyocytes.

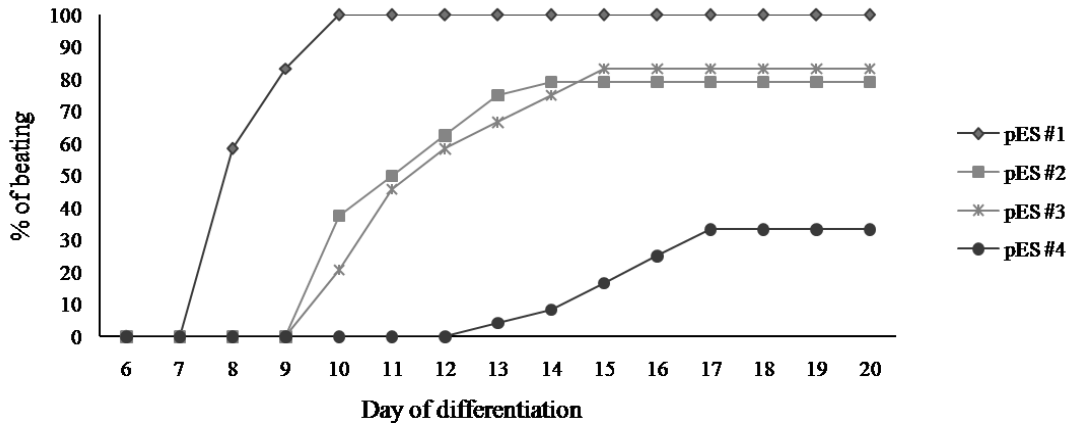


Figure 7. Percentage of contracting EBs formed from different four pES cell lines

2.4.7 *In vivo* developmental potential of pES cell line-derived chimeric embryos

Finally, we chose one pES cell line (pES#1) based on its superior potential of differentiation *in vitro* (EB formation and cardiac differentiation) to test the pluripotency of pES cell lines for *in vivo* differentiation. Figure 8A displays chimeric blastocysts produced by injecting the pES cell into ICR blastocysts. Our result demonstrated that the pES cells were incorporated in the recipient ICM (Figure 8B: insert box with red arrow) and developed further. Chimeric mice with a donor genotype of dark, brown and gray coat color were obtained from the pES cells with an

efficiency of 75% (three chimeric pups from four newborns) (Figure 8C). Unfortunately, only male chimeras have been obtained and they did not give germ-line chimeras. Our result demonstrated that pES cell line can differentiate *in vivo* by contributing to the body formation of the progeny.

Figure 8. *In vivo* developmental potential of pES cell line (A) Blastocyst injection was carried out by injecting ES cells into an ICR blastocyst under microscope attached with micromanipulator. (B) Chimeric blastocysts obtained by blastocyst injection of pES#1. (C) Chimeric offspring from pES #1 cells (mouse with gray coat).

2.4.8 Ploidy analyses of the pES cell lines

The four pES cell lines were analyzed every five passages during long term culture with at least 50 metaphases from each cell line examined for their chromosome numbers (Figure 9A). Our results showed that in three pES cell lines the majority of the cells (above 60 to 70%) possessed a normal diploid chromosomal set of 40 (68, 81 and 71% of the cells in pES#1, 2, and 3, respectively) but in pES#4 the majority (63%) of the cells were aneuploid (Figure 9B).

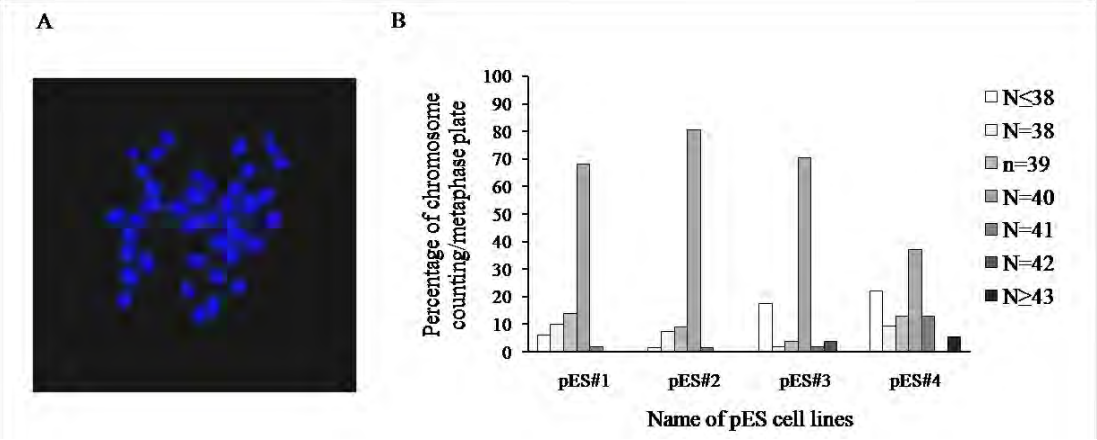


Figure 9. Karyotyping of pES cells. (A) Chromosome complements observed by fluorescent microscopy stained with DAPI. (B) The ploidy ratios of the pES cell lines, pES#1 to #3 cell lines exhibited a mostly (above 60%) normal 40 XX chromosomes ploidy after ten passages, however pES#4 cells showed higher ratio of cells with aneuploid chromosomes.

2.5 Discussion

Establishment of pluripotent stem cells derived from somatic cells via SCNT or iPS cells represent an important model for biomedical research and would provide unlimited resources for cell replacement therapies. On the other hand, the overall efficiency of the SCNT is still very low, and has not been achieved in human and the equivalency and safety issues of iPS cells remains a major concern including activation of potential oncogenes (Hao et al., 2009). Pluripotent stem cells generated from parthenogenetic blastocysts are good candidates as a source of histocompatible cells for transplantation (Drukker, 2008). PES cells have a relatively high efficiency for cell line establishment (around 14%) and the procedures are relatively simple compared to SCNT (Müller and Lengerke, 2009) in human. The pES cells could provide patients with cells for allogeneic (i.e., between genetically unrelated individuals) and even autologous (i.e., within the same individual) transplantation following MHC compatibility profiling and cell banking (De Sousa and Wilmut, 2007). Studies on human pES cells have revealed that these cells were very similar to the human ES cells derived from either IVF or *in vivo* produced blastocysts in their gene expressions and other characteristics (Hao et al., 2009). However, the full differentiation and development potential of these human pES cells have to be further investigated before clinical research and therapeutic interventions.

In this study, four mouse pES cell lines (pES#1 to #4) were established from parthenogenetically activated blastocysts. Subsequently, we have evaluated the differences among individual pES cell lines in mouse as a model for human research. Our result demonstrated that the profile for undifferentiated state of the pES cell lines matched that of other undifferentiated mouse pES cell colonies in previous studies (Shao et al., 2007; Lee et al., 2008). There were no differences detected among the four pES cell lines regarding typical mouse ES morphology, the strongly positive ALP staining, expression of transcription factors (Oct-4 and Nanog), and cell surface antigens (SSEA-1). Our results are in accordance with a study on 59 human ES cell lines from seventeen laboratories worldwide where in spite of diverse genotypes and different techniques used for establishment and maintenance, all ES cell lines exhibited indistinguishable expression patterns for several markers of human ES cells. However, those cell lines were not identical as differences in expression of several lineage markers were evident (Adewumi et al., 2007). Additionally, different gene expression profiles were contributed to different genetic backgrounds of mouse ES cell lines and not the methods (SCNT vs *in vivo* fertilization) used to produce those (Brambrink et al., 2006). In contrast, more detailed microarray analyses can reveal differences among long-term cultured mouse ES cell lines with essentially the same genetic background (Mamo et al., 2010).

Even though the formation of EBs in a suspension culture has been the most popular method to differentiate ES cells derived from cloned and fertilized blastocysts into a wide range of cells (Kurosawa, 2007), not much is known about the characteristics of EBs derived from pES cells. In this study, we compared the EB formation in suspension culture among the four pES cell lines. Three of the pES cell lines (pES#1 to #3) showed higher ratio and size of EBs and the presence of cystic EBs formed compared to that in pES#4. This demonstrates that to some extent the simple test of EB formation is already a useful tool to compare the quality of cell

lines, as the low-performance cell line was also identified as mostly aneuploid (see below).

Cardiomyocyte differentiation potential, by forming beating EBs in suspension, revealed that only pES#1 and #2 produced such EBs, despite that all pES cell lines were able to differentiate into cell types of all three germ layers, containing neural rosette formations (ectoderm lineage), connective tissue (mesoderm lineage) and simple squamous epithelium in the periphery and epithelial cells surrounding a lumen in the center, similar to yolk sac structures (endoderm lineage). These later results were similar to that observed with fES cell lines and EB formation in other studies (Mogi et al., 2009).

Although the functional potential of pES cells to differentiate into ectoderm lineages including dopaminergic and serotonergic neurons were well known and characterized (Sánchez-Pernaute et al., 2009), there have been little reporting regarding the differentiation towards endo-mesodermal lineages. When we have investigated the potential of endo-mesoderm differentiation in EBs the results suggested that three cell lines (pES#1 to #3) were able to differentiate into endoderm lineages and form cystic EBs with a simple squamous epithelium surrounding a lumen in the center by histological analysis and strongly expressing α- fetoprotein. However, pES#4 demonstrated low efficiency of endoderm differentiation without forming cystic EBa and a weak expression of α- fetoprotein. The good results with pES#1 to #3 endoderm differentiation is an interesting observation as in contrast in pathenogenetic embryos differentiation and proliferation of trophectoderm and primitive endoderm is abnormal (Strum et al., 1994). Our pES cell lines were also capable of differentiation into mesoderm lineages, formed connective tissues in EBs and expressed Flk-1, PECAM and collagen IV which are similar to the patterns expected in fES cells (Choi et al., 2005). Our results suggested that lack of paternal imprinting on certain genes (Igf2) did not affect mesoderm differentiation in this case, in contrast with a previous report (Morali et al., 2000).

Recently, pES cell lines demonstrated low efficiency of myogenic differentiation: exclusion of pES cells from the myogenic lineage *in vivo* (Clarke et al., 1988) or delays of myogenin expression *in vitro* (McKarney et al., 1997) because of the impairment in expression of the paternal allele expressed gene Igf2. In that regard, Igf2 is a strong promoting factor of myogenesis thus loss of Igf2 significantly decreased the proliferative capacity of the myoblast population. However, there was no impairment in cardiogenesis *in vivo* (Allen et al., 1994). Most interestingly, our results showed a significant variability in the efficiency of cardiac differentiation among mouse pES cell lines. The efficiency cardiac beating/well derived from pES#1 to #3 cell lines were 80 to 100%, however, a well-organized cTnT network and more positive areas for cTnT were found in pES#1 and #2 cell lines but not in pES#3. This suggested that most of our pES cell lines were able to differentiate into cardiomyocytes similarly as reported (Koh et al., 2009) and the presence of only maternal genomes does not interfere with stem cell function and differentiation (Onodera et al., 2010). Our results showed that differences exist among the individual cell lines for their “favored pathways” and capacity of spontaneous differentiation, which has also been observed in human ES cell lines.

It was demonstrated that prolonged passage in culture decreased the potential for germline transmission in mouse ES cell lines (Nagy et al., 2003). Euploid ES cell lines cultured *in vitro* for more than twenty passages were become mostly aneuploid and the ability of these lines to contribute to the germ line was mostly lost when the proportion of euploid cells dropped under 50% (Longo et al., 1997). Abnormal

chromosome numbers might result in lower efficiency of *in vitro* differentiation, as well. The karyotyping of our pES cell lines revealed that pES#4 cells were mostly abnormal which possibly resulted in low efficiency of *in vitro* differentiation, especially into cardiomyocytes. However, it is encouraging that two third of our pES cell lines maintained a mostly euploid karyotype during long-term culture demonstrating a good genetic stability of pES cell lines.

Finally, one of the pES cell lines was tested positively to differentiate *in vivo* into chimeric mice. This is encouraging, despite the lack of germline transmission demonstrated. After all, this later feature would not be important for human medical application of the cells. Our findings are in harmony with an earlier study on the poor potential of pES cells in chimera studies, which was improved by using a nuclear transfer step (Hikichi et al., 2007). Another report suggested that germline-competent pES cell lines can be produced by using *in vitro* maturation of immature oocytes from adult mouse ovaries and activation instead of using *in vivo* matured MII oocytes (Liu et al., 2011).

In conclusion, according to our results among the four pES cell lines studied all showed similar pluripotency characteristics compared to that of ES cells. The pES cell lines were able to differentiate *in vitro* into cell types of all three germ layers, especially into cardiomyocytes; however, differences in the differentiation process outcomes have been revealed (Table 3). The reason for this variability is not fully understood and may be related to subtle differences in epigenetic state or chromosomal abnormalities present in a proportion of the cells. Nevertheless, one of the pES cell lines has been tested successfully to contribute to *in vivo* chimeric development of pups, generating medium to strong coat-color chimeras. The establishment of pluripotent pES cell lines derived from parthenogenetic embryos demonstrated the feasibility of this approach and its potential to generate patient-specific cell lines. In the mean time the differences observed among pES cell lines make it clear that further basic research and continuous quality control is needed prior to any practical applications in medicine.

Table 3. Comparative mouse ES cells characteristics among the pES cell lines.

pES cell line	Pluripotent of ES cell				*In vitro* differentiation							*In vivo* differentiation	Normal chromosome number (40xx: %)
	ES cell marker				EB formation		Three germ layer			Cardiac			
	ALP	Oct-4	Nanog	SSEA-1	Simple EBs	Cystic EBs	Ectoderm	Mesoderm	Endoderm	% of beating	cTnT		
pES#1	++	++	++	++	++	++	++	++	++	100	++	+	68
pES#2	++	++	++	++	++	++	++	++	++	79	++	NT	81
pES#3	++	++	++	++	++	++	-	+	++	83	+	NT	71
pES#4	++	++	++	++	+	-	+	+	+	33	+	NT	37

Abbreviation: NT**:** no test

ES cell markers: ++: The high expression of ES cell markers
+: The low expression of ES cell markers

EB formation: ++: The high efficiency of EB formation
+: The low efficiency of EB formation
-: None of EB formation

Three germ layers: ++: The high efficiency of differentiation into three germ layers
+: The low efficiency of differentiation into three germ layers
-: None EBs differentiate into three germ layers

Cardiac: ++: The high expression of cTnT
+: The low expression of cTnT

CHAPTER III

ENHANCED CARDIAC DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELLS BY USE OF THE SLOW TURNING LATERAL VESSEL (STLV) BIOREACTOR

3.1 Abstract

EB formation is a common intermediate during *in vitro* differentiation of pluripotent stem cells into specialized cell types. In this study, we optimized the STLV bioreactor for large scale and homogenous EB production from mouse ES cells on consequent cardiac differentiation. We investigated the effects of different inoculating cell numbers, the time of EB adherence to gelatin-coated dishes, and the rotation speed for optimal EB formation and cardiac differentiation. Our results showed that $3x10^5$ cells/ml, 10 rpm rotary speed and plating of EBs onto gelatin-coated surface on the third day after culturing were the best parameters for obtaining optimal EB and size quality on consequent cardiac differentiation. These optimized parameters enriched cardiac differentiation of the ES cells when using the STLV method.

3.2 Introduction

Currently, organ transplantation is the only effective treatment approach for patients suffering from severe heart failure. Nevertheless, adult cardiomyocytes are terminally differentiated cells with limited capacity of cell division. Hence, cardiomyocytes derived from ES cells are anticipated to serve as valuable sources for cell-based cardiovascular drug testing and disease therapy (Boheler et al., 2002). EB formation from ES cells (Mummery et al., 2007) is a common method for producing different cell lineages including cardiomyocytes for further applications. The cells found within EBs are typically heterogeneous and their subsequent differentiation may be unpredictable. This is one of the major drawbacks for further application (Desbaillets et al., 2000). Efforts to manipulate the EB environment and subsequent ES cell differentiation have focused primarily on either controlling medium composition (Schuldiner et al., 2000) or assembly of EBs using different culture methods (Hopfl et al., 2004). The two basic methods for achieving EBs are via: (i) HD (Shamblott et al., 2002; Chen et al., 2011) and (ii) SSC (Dang et al., 2002). The HD culture produce a fairly homogeneous final population of EBs, this culture is inherently incapable of large scale EB production due to laborious-requirement. Whereas, SSC has advantage in that it is easily scalable and requires little expertise, often results in agglomeration of EBs into large and irregular masses as well as causing extensive cell death. Hence, these methods are restricted as far as industrial applications are concerned due to their complication and difficult manageability (Kurosawa, 2007).

Recently, bioreactors have been developed for cell expansion, differentiation or tissue formation on biomaterial scaffolds of ES-derived cells with a tightly controlled environment *in vitro* (Rungarunlert et al., 2009). Stirred suspension culture or spinner flask has the disadvantage of generating shear forces, although it is manageable. These forces still can damage the cells. Another bioreactor that allows agglomeration-free EB formation is the RCCS developed by the NASA. This system is comprised of a STLV and a HARV (Lü et al., 2008; Hwang et al., 2009). STLV bioreactor has been reported to increase the efficiency of EB formation and differentiation of stem cells into the three germ layers of embryonic development

(Gerecht-Nir et al., 2004). However, the influence of dynamic culture parameters (i.e. cell seeding density and speed), specifically for spontaneous cardiac differentiation of ES cells has yet to be reported. In the current study, we used the mouse ES cell line HM1 from 129Sv/Ola genetic background and investigated the effects of inoculating cell number, rotation speed and the time of EB adherence to gelatin-coated dishes for optimal EB formation and subsequent cardiac differentiation in STLV bioreactor. The STLV method confers extremely low fluid shear stress and oxygenation by diffusion. This method also allows creation of a more uniform and homogenous cell population of EBs that is more favorable for subsequent cardiomyocyte differentiation, compared with classical HD method.

3.3 Materials and methods

Chemicals

All chemicals were purchased from Sigma (Sigma Aldrich, St Louis, USA) and culture reagents were purchased from Invitrogen (Life Technologies, Carlsbad, CA, USA), unless otherwise specified.

In vitro cell culture

Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. Medium was changed daily for mouse ES cell culture and every two days during differentiation.

3.3.1 Culture of ES cells

The ES cell line HM1; 129Sv/Ola genetic background was cultured on mitomycin C-inactivated mouse MEFs (ICR genetic background) as described earlier (Magin et al., 1992). These cells were maintained in ES medium consisting of DMEM, 15% (v/v) FBS (Sera Laboratories International, West Sussex, RH17 5PB, UK) supplemented with 1,000 U/ml mouse LIF (ESGRO, Chemicon International, Budapest, Hungary), 0.1 mM NEAA, 0.1 mM β-ME, 50 U/ml penicillin and 50 µg/ml streptomycin. ES cells were cultured on feeder layers at least two passages after thawing and subsequent were cultured without feeder cells on 0.1 % gelatin-coated tissue culture plates in the presence of LIF (2,000 U/ml) in ES medium at least one passages before differentiation procedure. These cells were usually passaged every one to two days prior reaching 70% confluences.

3.3.2 Differentiation methods of ES cells

To induce differentiation, EB produced from the traditional HD method was used (adapted from Mummery et al., 2007). In brief, undifferentiated ES cells were dissociated with 0.05% (w/v) trypsin-EDTA and resuspended in the spontaneous differentiation medium composing of ES medium without LIF supplement. The cells were seeded at $4x10^4$ cells/ml. Then, 20 µl of the spontaneous differentiation medium were dropped on the lids of 10 cm petri-dishes, with ~ 72 drops/plate (resulting in 800 cells/drop). Two days later, EBs were transferred to a 100-mm bacteriological Petri-dish treated with poly-HEMA in ten ml of differentiation medium.

For optimizing STLV bioreactor, undifferentiated ES cells were dissociated with 0.05% (w/v) trypsin-EDTA, resuspended in the spontaneous differentiation medium ($3x10^5$ cells/ml) and inoculated in a 110 ml STLV vessel (The Rotary Cell Culture System™ RCCS-4; Synthecon, Incorporated, Houston, Texas, USA). STLV cultures of EBs were set at 10 revolutions per minute (rpm), except during studies in

which rotation speeds of 15 and 20 rpm were also examined. EBs were maintained for up to 5 days in suspension. Medium was changed every two days by allowing EBs to sediment in the STLV, aspirating the old medium, and re-suspending the EBs with fresh medium.

EBs derived from both groups were collected at different time points (day two to day five) for evaluating gross morphology, size, yield and histological analysis and were plated onto a gelatin-coated culture dishe for evaluating the efficiency of cardiac differentiation.

3.3.3 Morphology, size and yield of EB analysis

Ten ml of STLV culture medium was passed through a 100 µm cell strainer to separate cell aggregates constituting EBs from individual cells. EBs were transferred into Petri-dishes and analyzed using an inverted light microscopy. For size analysis, the diameter average was calculated by measuring the large and small diagonals of twenty representative EBs derived from STLV and HD method. The results of the assessment from three independent experiments are presented. Phase contrast images of EBs were acquired using an Olympus IX71 inverted microscope with an attached Olympus DP 70 digital camera **(**Olympus Europa Holding GmbH, Germany) and analyzed using ImageJ image analysis software (http://rsb.info.nih. gov/ij) in order to measure the cross-sectional area of the EBs. One ml of STLV culture medium was counted for the yield of EB production /ml.

3.3.4 Histological analysis

EBs were collected, washed three times with PBS, fixed with 4% (w/v) PFA for 15 min, embedded in paraffin, and examined for further histological analysis. Sections of 5 µm were stained with H&E, Ki-67 and cleaved-caspase 3 for general morphology, cell proliferation and the apoptosis assessment respectively.

3.3.5 Differentiation of ES cells to cardiomyocytes

At day three, individual EBs were plated onto gelatin coated dishes in separate wells of a 24-well plate for differentiating into cardiomyocytes. Forty-eight EBs were cultured until day 21 and observed daily under a phase-contrast microscope for evaluating the percentage of cardiac beating/well (the number of beating EBs was graphed as the percentage of the total number of EBs plated) and quantification of EB beating activity. Spontaneous cardiac differentiation medium consisted of undifferentiated medium without LIF.

3.3.6 Quantification of EB beating activity

Cardiac beating activity was evaluated by grading the area of beating foci and the beating rate as follows: Grade 1, one area of beating foci with slow beating rate (Supplementary movie 3); Grade 2, two areas of beating foci with slow beating rate (Supplementary movie 4); Grade 3, more than two areas of beating foci with fast beating rate (Supplementary movie 5); Grade 4, whole area of beating foci with fast beating rate (Supplementary movie 6). Forty-eight EBs were counted for each treatment and experiments were performed in triplicate.

3.3.7 Immunohistochemistry

Whole EBs were be plated onto 0.1% gelatin-coated coverslips, fixed with 4% (w/v) PFA for 15 min at room temperature and processed according to the antibody manufacturer’s instructions. Antibodies: cTnT (diluted 1:200; Abcam, UK, ab33589)

and Alexa Fluor 594 (diluted 1:2,000; Invitrogen, A11005). Vectashield mounting medium containing DAPI (Vector Laboratories) was used to counterstain the nuclei. All area of cTnT was fluorescently imaged with a Carl Zeiss (Carl Zeiss MicroImaging GmbH, Germany) microscope and analysed by using Digital Image Processing Software (AxioVision 4.8.1, Carl Zeiss MicroImaging GmbH, Germany) for calculating total area of cTnT/EB.

3.3.8 Statistical analysis

Data are expressed as mean ± SEM and include at least three independent experiments. Statistical analyses were conducted using the Student's *t* test or the General Linear Model (GLM) procedures of SAS. *P*-value $<$ 0.05 will be considered statistically significant.

3.3.9 Experimental design (Figure 10)

Experiment 1: To study the efficiency of EB formation derived from HM1 ES cell line, the HD method was used for EB production. The effect of timing the plating of EBs on gelatin-coated dishes on the efficiency of cardiomyocyte differentiation was investigated. EBs were plated at different days (day two to day five) onto gelatin-coated dishes to study the efficiency of spontaneous cardiomyocyte differentiation. At least three independent replicates were examined.

Experiment 2: To determine the effect of inoculating different cell numbers on the efficiency of EB formation in STLV bioreactor, EBs were generated by inoculating cells at different cell seeding densities: $1x10^5$(STLV1); $2x10^5$ (STLV2); $3x10^5$ (STLV3) and $5x10^5$ (STLV4) cells/ml to STLV culture. In this study, the rotation speed was set to 10 rpm. At least three independent replicates were examined.

Experiment 3: To study the effect of rotation speed on EB formation in STLV bioreactor, ES cells were inoculated in STLV systems using different rotary speeds (10, 15 and 20 rpm). In this study, the cell seeding density was fixed at $3x10^5$ cells/ml and ridded on the best results of experiment 1. At least three independent replicates were examined.

Experiment 4: To study the effect of inoculating different cell numbers and time of EB adherence to gelatin-coated dishes on the efficiency of cardiomyocyte differentiation, EBs were plated at different days (day two to day five) onto gelatin-coated dishes for spontaneous cardiomyocyte differentiation. In this study, the best quality of EBs derived from the best group of inoculating different cell numbers (experiment 1) and rotation speed (experiment 2) were fixed and utilized which based on yields of EBs and the gross morphology of EBs with homogenous in size. At least three independent replicates were examined.

3.4 Results

3.4.1 EB formation using HD method

HM1 ES cells were maintained in an undifferentiated state on MEFs. Their morphology corresponded to typical ES cell morphology including round-shaped, compact, and shiny colonies (Figure 11). Differentiation of these cells into cardiomyocytes was initially achieved, using the HD method, widely used as a conventional protocol for differentiation of mouse ES cells. The ES cells were cultivated in 20 µl/drop (each drop containing 800 cells) for two days..

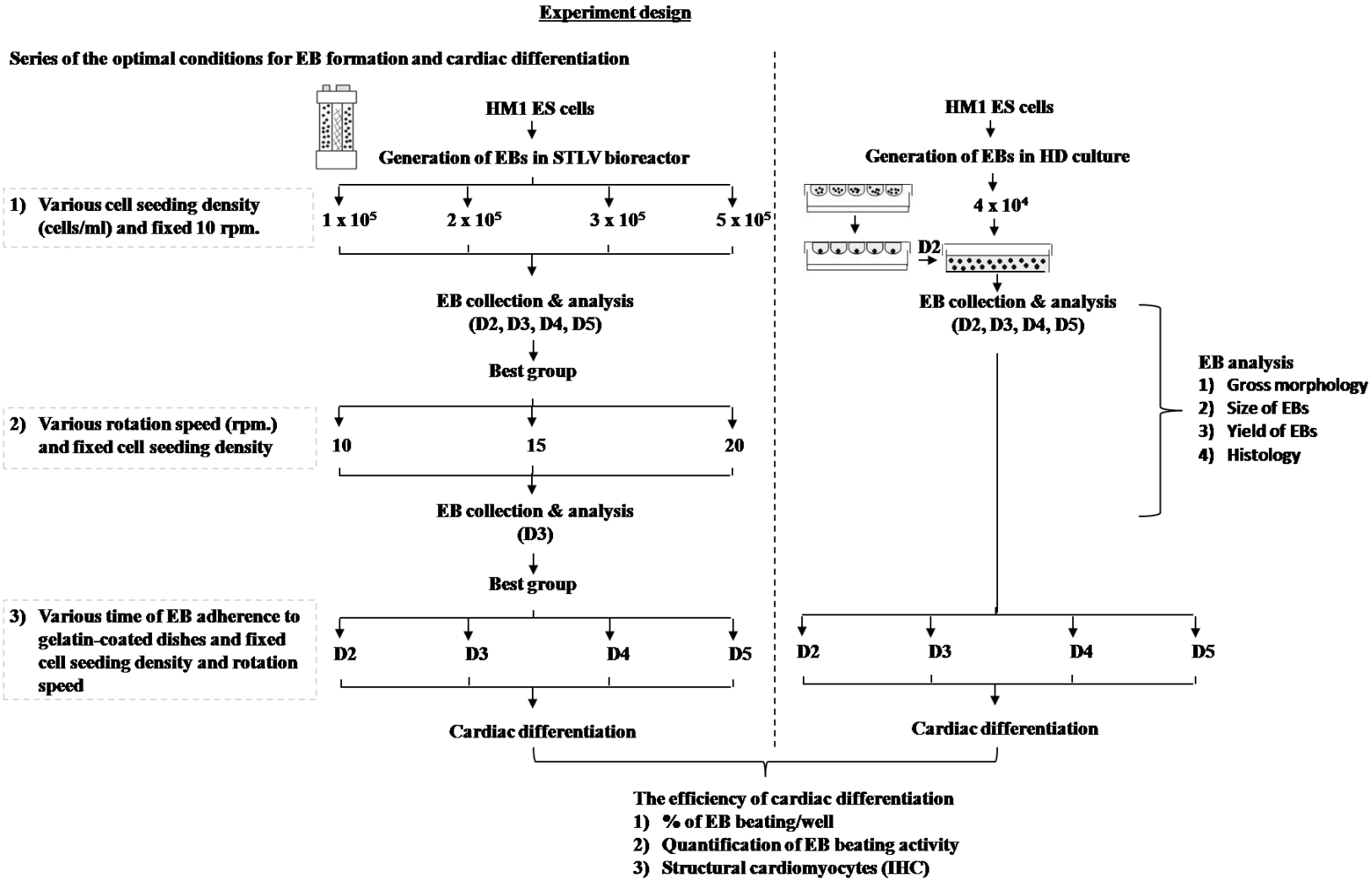


Figure 10. ES cells were maintained in an undifferentiated state on MEFs. HM1 ES cell line exhibits typical gross morphology of mouse ES cells including a high nuclear per cytoplasmic ratio and round shape. Abbrevations: day : D

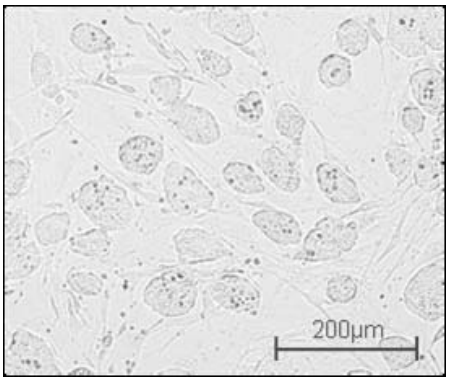


Figure 11. Schematic presentation of *in vitro* differentiation procedure of ES cells into cardiomyocytes in STLV and HD culture.

During this period, three-dimensional differentiating cell aggregates (EBs) were formed. Subsequently, the EBs were transferred to bacterial-grade dishe treated with poly-HEMA for further three days, where they formed larger EBs (Figure 12)The EBs were collected at D2-5 for evaluating the efficiency of EB formation and then plated on gelatin-covered plates for evaluating the efficiency of cardiac differentiation.

During culture, EB derived from HD (HD EBs) are slightly increased in size on D2 (296±7.7 µm) and D3 (370±6.7 µm) but a large increased was found on D4 (500±13.6 µm) and D5 (601±14 µm) (Figure 13). Interestingly, HD EBs have homogenous in size on D2-3 but have heterogonous in size on D4-5. Extensive EB agglomeration led to the formation of two to four large EBs on D4-5 (Figure 12; red arrow).

3.4.2 Effect of cell seeding density on EB formation in STLV

Next, we designed a strategy for scaling-up of the differentiation procedure using STLV cultures. In order to determine the optimal condition for EB formation in the STLV culture system, EBs were generated by inoculating cells at different cell seeding densities: $1x10^5$ (STLV1); $2x10^5$ (STLV2); $3x10^5$ (STLV3) and $5x10^5$ (STLV4) cells/ml to STLV culture. After inoculation (D0), EBs were cultured in STLV systems and samples were collected at different time points (D2 to D5) and analyzed based on their gross morphology (Figure 12), size (Figure 13) and yield (Figure 14). The most uniform size EBs were formed from STLV3 and STLV4 at all examined time points (Figure 12; STLV3, STLV4, at D2, D3, D4, D5). Based on gross morphology, the EBs were consistent in size, morphologically similar and homogeneous (Figure 12). STLV1 and STLV2 cultures appeared to form regular, spherical EBs, only after two days with size of 299 ±12 and 401 ± 16.4 µm (Figure 12; STLV1 and STLV2: D3 and Figure 13 respectively), whereas STLV3 and STLV4 formed them earlier, on day two (464 ± 7.8 and 463 ± 8.2 µm) (Figure 12; STLV3 and STLV4; D2 and Figure 13 respectively).

Noticeably, STLV1 and STLV2 produced less homogenous EBs, with less defined cell aggregates at all examined days (D2 to D5) (Figure 12) and STLV1 exhibited the smallest EBs for all days examined. The yield of EB production/ml in different STLV conditions was determined by counting the number of EBs/ml (Figure 14). The tendency showed that STLV3 culture conditions resulted in the highest yield of EB formation.

Importantly, the STLV1 and STLV 2 bioreactors contained a mixture of small aggregates (STLV1: 37.46, 118.49, 110.54, 143.59 µm on D2, D3, D4 and D5 respectively; STLV2: 163.22, 185.19, 131.20, 253.94 µm on D2, D3, D4 and D5 respectively) and large aggregates (STLV1: 260.33, 502.60, 559.92 and 671.49 µm on D2, D3, D4 and D5 respectively; STLV2: 706.61, 661.52, 745.87 and 768.75 µm on D2, D3, D4 and D5 respectively) (Figure12; STLV1, STLV2 on D2, D3, D4 and D5), whilst STLV3 and STLV4 gave a more homogenous, uniformly sized EB population with little variation (Figure12; STLV3, STLV4 on D2, D3, D4 and D5). Since STLV3 and STLV4 culture conditions produced larger-sized and better yields of EBs and the gross morphology of EBs were the most uniform and homogeneous at almost all examined time points, we selected these two STLV conditions to perform further experiments.

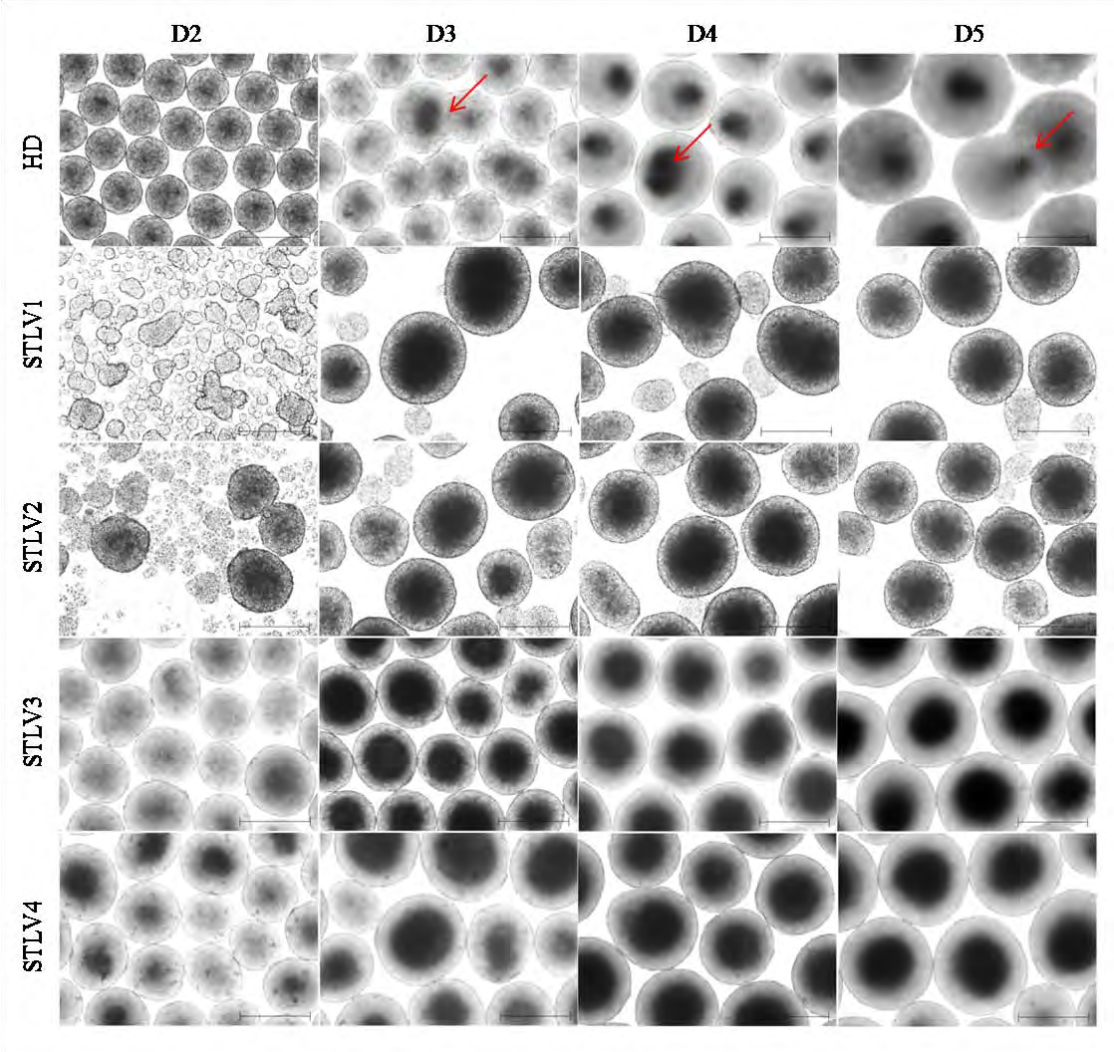


Figure 12. Gross morphology of EBs produced from different cell seeding densities (STLV1-4) in STLV and HD systems during day two to five**.** Scale bars represent 500 µm and x4 magnification.

3.4.3 Effect of the rotary speed EB formation in STLV

To study the effect of rotary speed on EB formation, $3x10^5$ (STLV3) cells were inoculated in STLV systems using different rotary speeds (10, 15 and 20 rpm) (Figure 15). The 10 rpm rotary speed was found to facilitate the greatest incorporation

of ES cells within EBs. Normalizing ES cells incorporation to the 110 ml STLV volume demonstrated that EBs formed at other rpms as well, however with lower incorporation rates. Importantly, EBs generated at 10 rpm rotary speed remained similar in size and slightly larger (roughly 442± 6.4μm in diameter) (Figure 16) than EBs formed at other rpm. The rotary speed did not seem to have a major effect on the yield of EB production due to no statistic significant different from all groups (*p*-value >0.05; Figure 17). Therefore, the relatively large number of EBs obtained at 10 rpm, and the more efficient formation of EBs (indicated by fewer individual cells remaining in suspension) were the determining factors for use of this rotary speed for all subsequent experiments performed in this study.

3.4.4 Effect of cell seeding density on cell viability within EBs in STLV and HD

In this study, we used only STLV3 and STLV4 based on the yield, size and homogenous in size of EBs compared to HD method. To elucidate the cause of differences in cell yield between different cell seeding density, cell morphology, proliferation, and apoptosis were performed.

For H&E staining, our showed that all EBs appeared to increase in size of EB cell density as one black spot was observed in center during the long term culture (D3 to D5) compared to D2. Interestingly, only HD EBs began to agglomerate between two EBs at D4 which showed two spot areas of central EB (Figure 18; black arrow). In contrast, STLV EBs did not appear to agglomerate. By D4, HD EBs began to display cystic structures and by D5 more than 50% of HD EBs containing at least one cyst (Figure 18; blue arrow).

Ki67 staining was performed to detect proliferating cells within EBs during differentiation. Higher levels of proliferating cells were detected at D2 and D3 (Ki 67 positive; brown staining). Controversially low levels of proliferating cells were detected at D4 and D5. It is likely due to the relatively long doubling time of cells within EBs, and no differences were noted between cell seeding densities of STLV EBs at same time point (Figure 18). Higher slightly levels of positive Ki67 were found in both of STLV group than HD group, regardless day of EB.

Cleaved-caspase 3 staining also revealed slight differences between different STLV EB groups at the same time point (D2 to D5). STLV4 started to form dense cores of apoptotic cells within EBs earlier than STLV3 due to higher metabolism from higher cells seeding density. In addition, dense cores of apoptotic cells were more pronounced within STLV EB groups after long term EB culture (Figure 18). Positive staining of cleaved-caspase 3 within HD EBs was sparser and appeared to constitute less of the total EB area at D2 and D3 but shown dense cores of apoptotic cells at D4 and D5. These data indicated that long term EB culture reduced cell viability, perhaps because of a greater number of cells undergoing programmed cell death. Importantly, EBs produced in STLVs showed more uniform morphology and a uniform apoptotic hole on the middle whilst EBs derived from HD culture were more cystic (arrows show multiple holes within EBs) and showed less uniform structures inside. In the middle of STLV derived EBs a clearly forming cavity appeared with high apoptotic area. Surrounding this cavity, highly proliferative zone of Ki67 positive cells were visible.

3.4.5 Effects of cell seeding density and time of EB plating on cardiac differentiation in STLV and HD

Individual EBs, produced in STLV3 and STLV4, were plated onto gelatin-coated dishes for spontaneous cardiomyocyte differentiation (Figure 19).

Differentiated EBs were cultured for fourteen days and observed daily under a bright-field microscope. Forty-eight EBs were counted for each treatment group. The number of beating EBs is presented as a percentage in relation to the total number of EBs plated (Figure 20A to 20C).

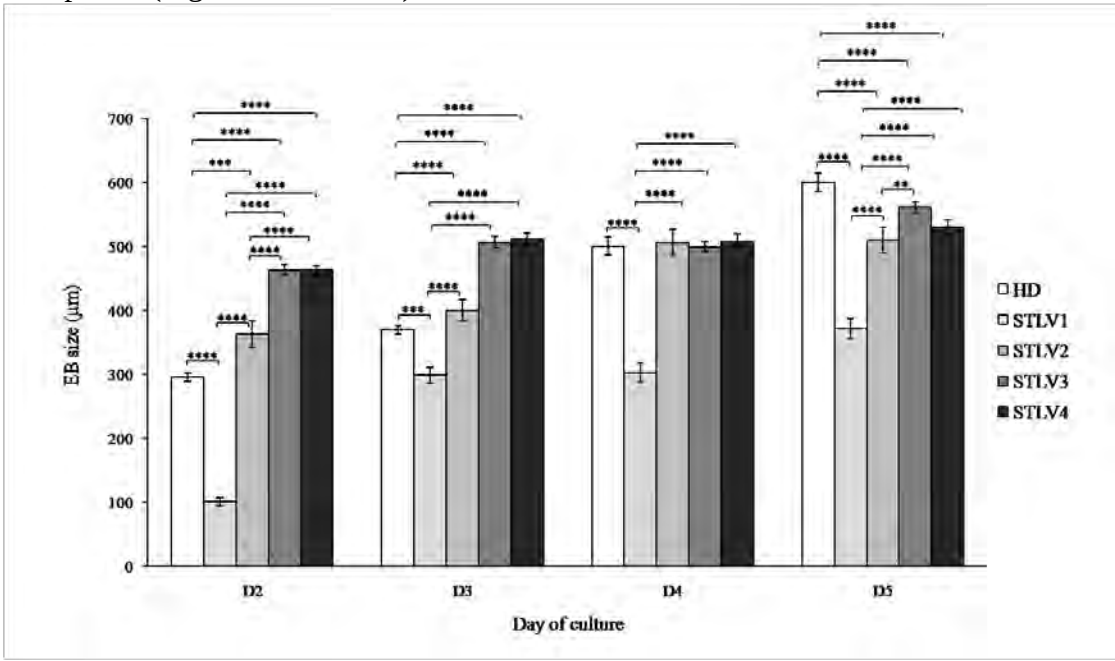


Figure 13. The size of EBs produced from different cell seeding densities (STLV1-4) in STLV and HD systems during day two to five. Data presented as mean ± SEM. The difference was considered significant when a p<0.01; ** p<0.001;*** p<0.0001;****.

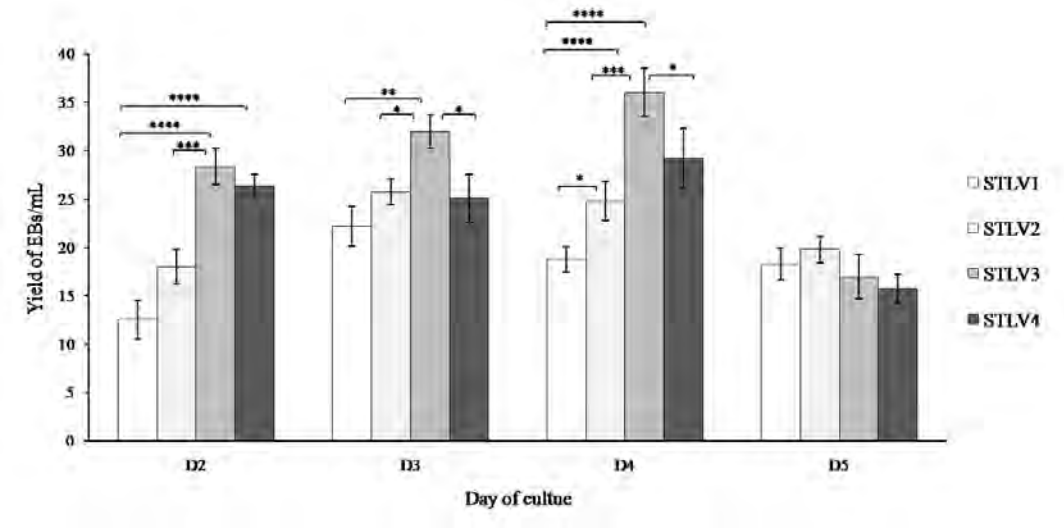


Figure 14. The yield of EBs/ml produced from different cell seeding densities (STLV1-4) in STLV systems during day two to five. Data presented as mean ± SEM. The difference was considered significant when a p <0.05, * p <0.01; ** p <0.001;*** p <0.0001;****.

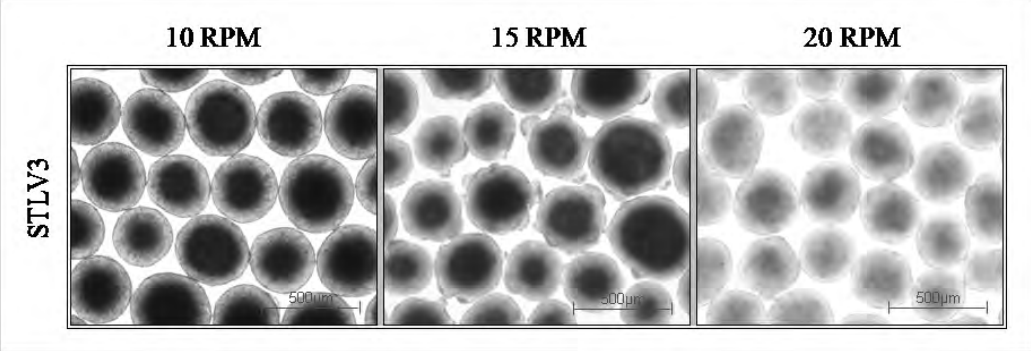


Figure 15. Gross morphology of EBs produced from different rotation speed (10, 15 and 20 rpm.) in STLV systems on day three**.** Scale bars represent 500 µm x4magnification.

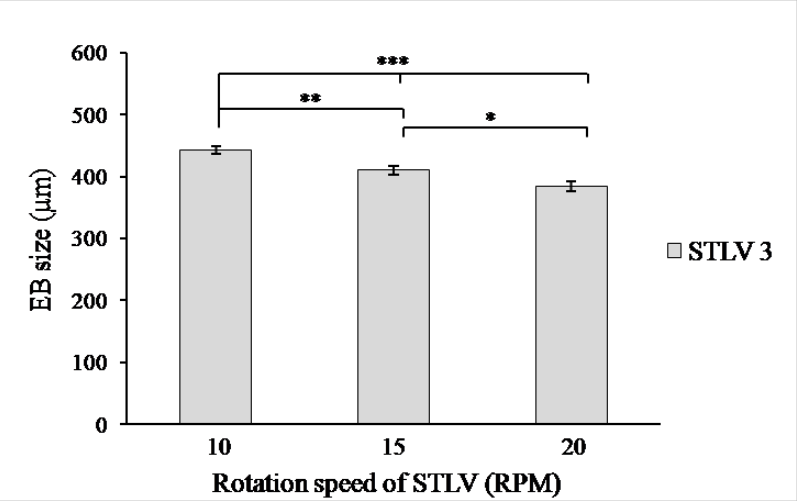


Figure 16. The size of EBs produced from different rotation speed (10, 15 and 20 rpm.) in STLV systems on day three. Data presented as mean ± SEM. The difference was considered significant when a p <0.05, * p <0.01; ** p <0.001;***.

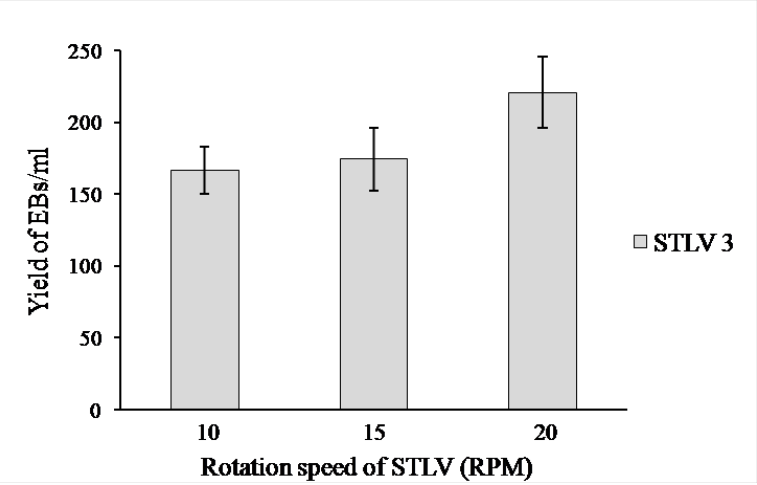


Figure 17. The yield of EBs /ml produced from different rotation speeds (10, 15 and 20 rpm.) in STLV systems on day three. Data presented as mean ± SEM.

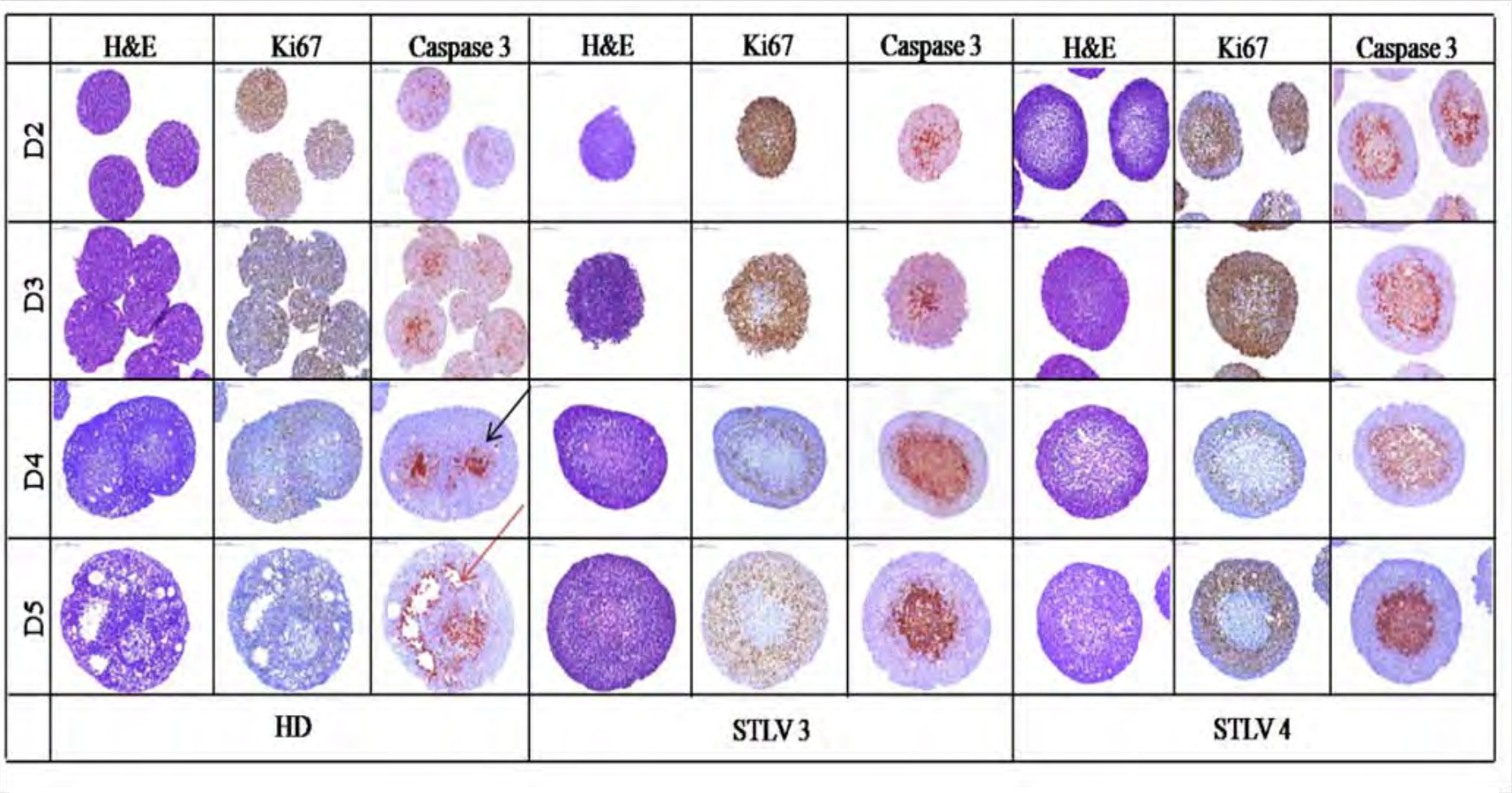


Figure 18. Histological section of EB formation (H&E staining, proliferation marker, Ki-67 and apoptosis marker, caspase 3). Scale bars correspond to 100 μm and magnification, x20.

After plating EB on gelatin plate, EB derived from STLV3, STLV4 and HD plated on D2 and D3 (STLV3/D2, STLV3/D3, STLV4/D2 STLV4/D3, HD/D2, HD/D3) were allowed to attached and spread out quickly than EB derived from those plated on D4 and D5 (STLV3/D4, STLV3/D5, STLV4/D4 STLV4/D5, HD/D4, HD/D5). Moreover, during this culture period, the cell number of early EB plated (D2 and D3) on gelatin were higher than the cell number of later EB plated (D4 and D5) (Figure 19). Interestingly, only HD/D4 and HD/D5 showed cystic EB pattern (Figure 19). Spontaneously contracting EBs began to appear as early as day six in the HD/D3 and HD/D5 groups and day seven in the HD/D2 and HD/D4, STLV3/D2, STLV3/D3 and STLV4/D2 groups (Figure 20A to 20C).

There was no beating EBs in the rest of the STLV groups until day eight (Fig 20B to 20C). Moreover, all plated EBs formed spontaneously beating colonies only in the STLV3/D2, STLV3/D3 and STLV4/D2 groups by day ten (Figure 20B to 20C). However, STLV3/D4, STLV3/D5, STLV4/D4, and STLV4/D5 groups never reached the 100% efficiency: they formed beating cardiomyocytes with maximum 70 to 75% efficiency (Figure 20B to 20C). Besides, none of HD groups is reached the 100% efficiency of cardiac differentiation. EBs plated on D2 and D3 had higher percentages of beating areas, then if they were plated later (D4 and D5) (Figure 20A). This indicated that the day of plating EBs onto gelatinized dishes was critical for this process and that EBs plated on D3 or earlier, is optimal for further applications.

The quantity of cardiac beating was also evaluated by grading the cardiac beating (Figure 20D). Grading was performed based on the size of the beating area and the rate of beating. The grading revealed that STLV3 EBs plated on either D2 or D3 gave the best results (i.e. the highest number and fastest beating contractile areas). The STLV4 EBs could also form cardiomyocytes, however scored lower, according to the grading scale. EBs plated on D2 and D3 had higher percentages of beating areas, then if they were plated later (D4 and D5) (Figure 20D). This suggests that EBs plated on either D2 or D3 from STLV3 generated EB were suitable for producing rhythmically beating cardiomyocytes.

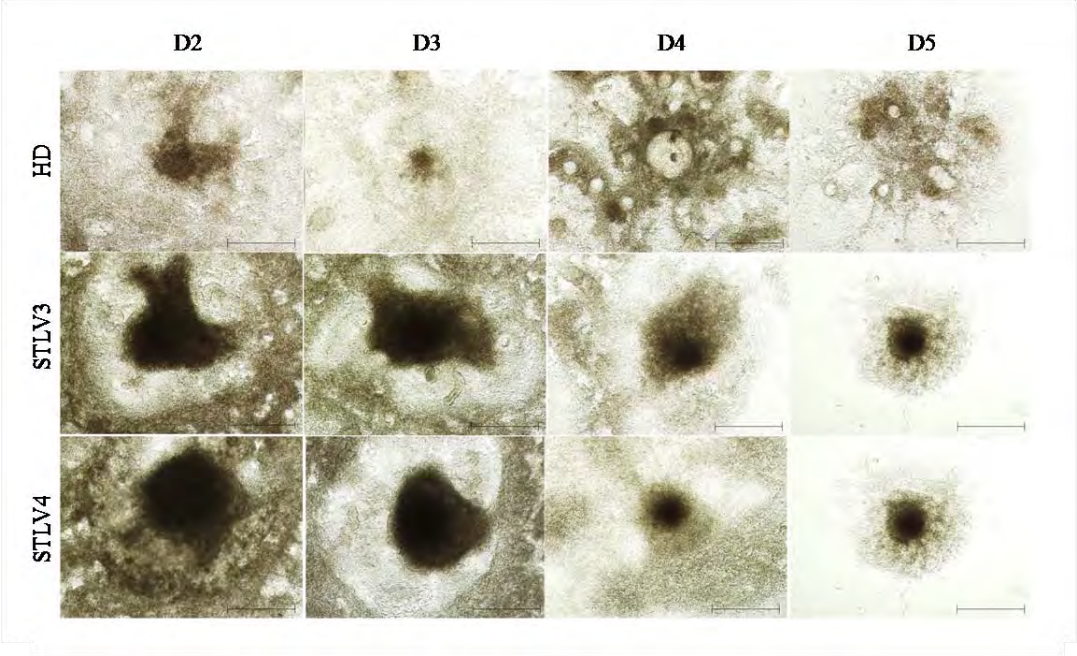


Figure 19. The morphology of EB on day seven after EB plating derived from STLV and HD at different time points.

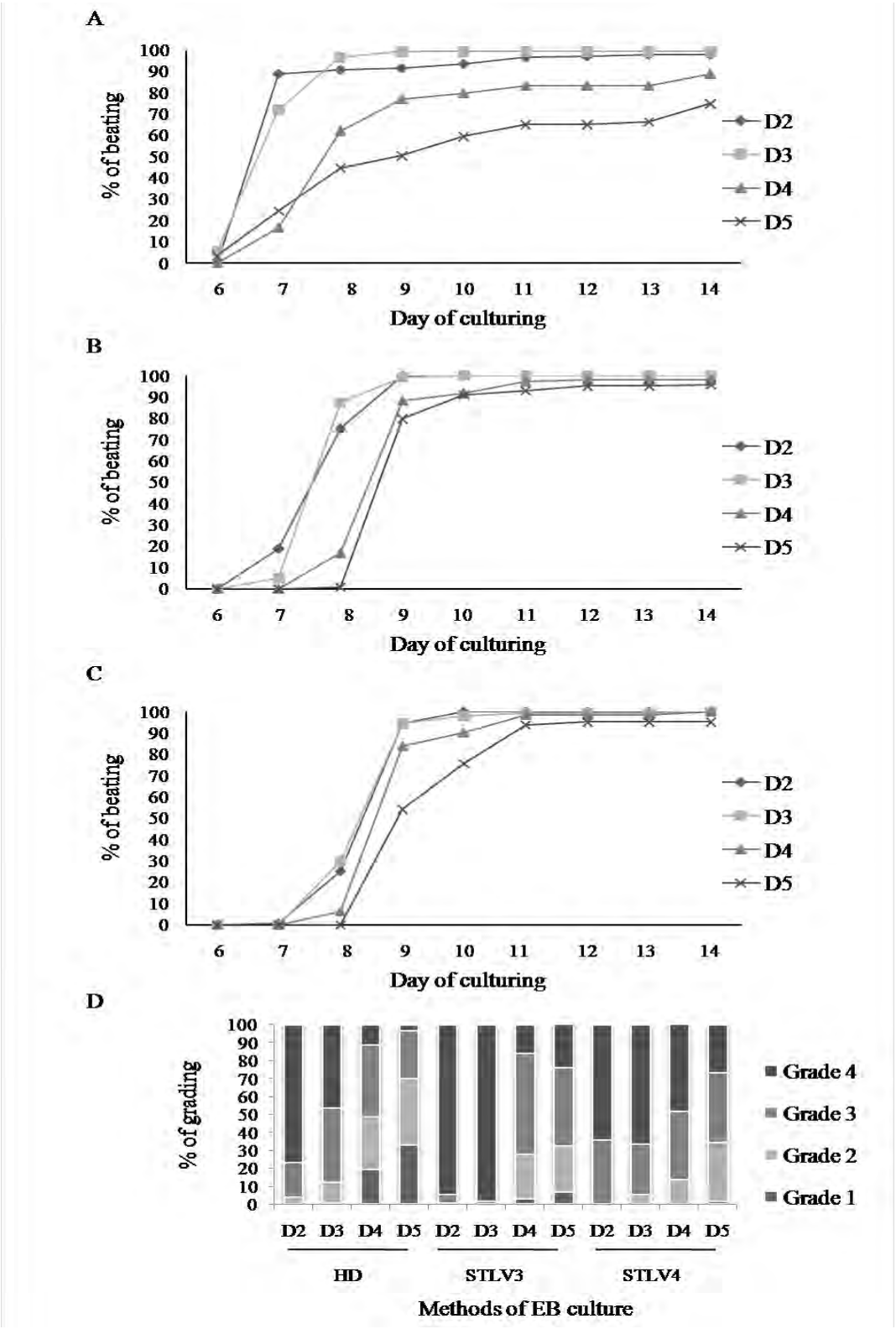


Figure 20. Cardiac differentiation potential of EB derived from different cell seeding density and time of EB plating on cardiac differentiation in STLV and HD. (A-C) Percentage of contracting cardiomyocytes in outgrowth of attached EBs: (A) HD, (B) STLV3 and (C) STLV4. (D) Cardiac beating activity was evaluated by grading the area of beating foci and beating rate.

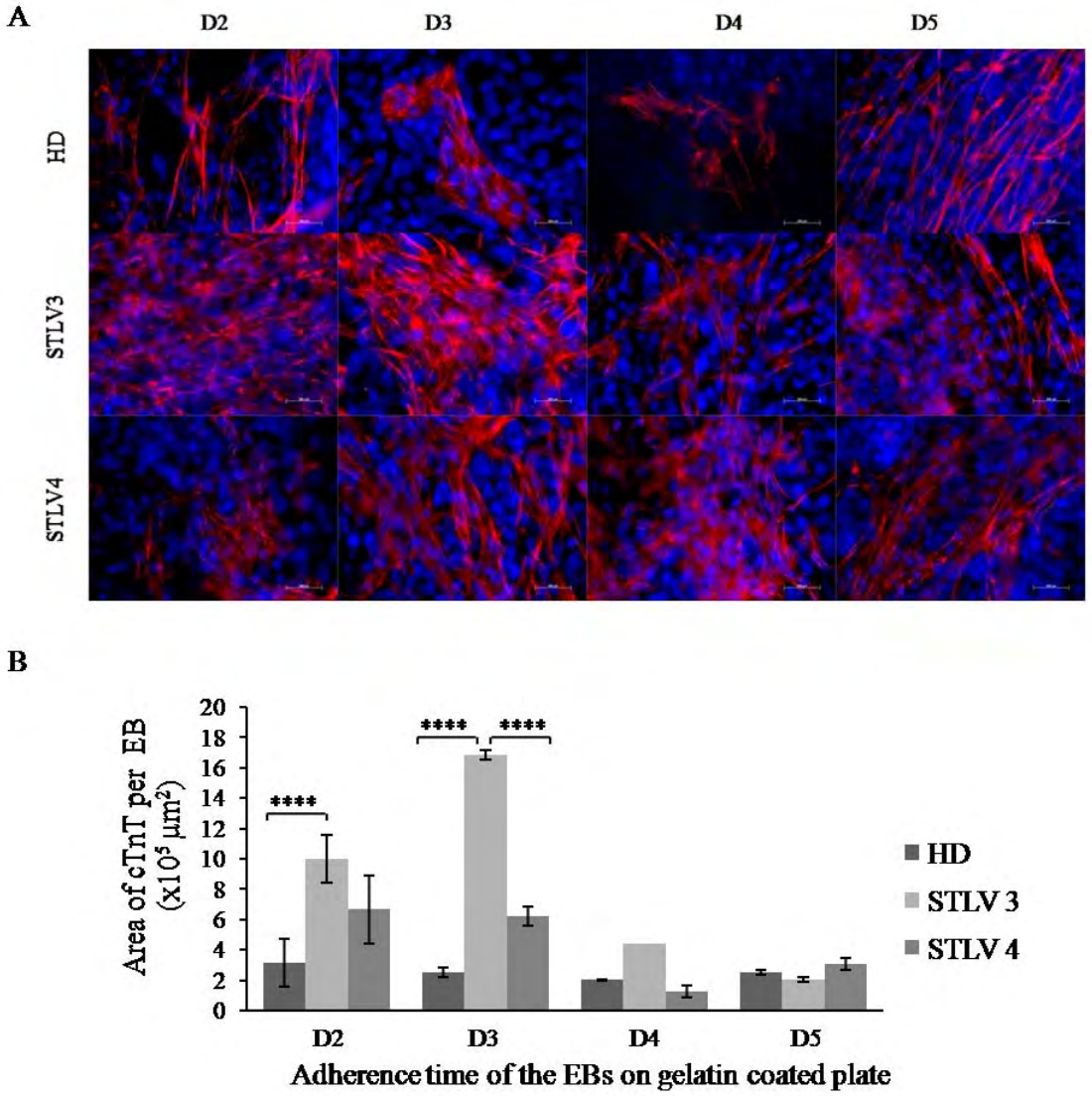


Figure 21. Cardiac differentiation potential of EB derived from different cell seeding density and time of EB plating on cardiac differentiation in STLV and HD. (A) IHC of EBs derived from different cell seeding density was stained with cTnT (red) with DAPI (blue). (B) Area of cTnT per EBs ($x10^5$ μm^2). Scale bars correspond to 200 µm and magnification, x40.

Even thought, expression of cTnT revealed the presence of an extensive and filamentous network at all examined time points (D2 to D5; Figure 21A), the areas of expression of cTnT/an EB in the STLV3 groups was higher than in the other groups, with the STLV3/D3 group showing the highest level of immunostaining intensity (Figure 21B). This confirmed that the STLV3 treatment at D3 produced the most extensive cardiac network.

3.5 Discussion

Although ES cell technology is a promising technique for producing cardiomyocytes for cell based therapy against cardiovascular-diseases, a prerequisite for enabling this technique is the development of bioprocesses for the scale-up cardiac lineage-specific differentiation for clinical and industrial applications (Niebruegge et

al., 2008). Productions of EBs using the classical HD and SSC cultures are a common method for differentiating cardiomyocytes. At the present time, a lot of information has been achieved by these techniques. For example, the several variables (the starting number of cells used to form each EB, growth factors, and the duration of suspension culture prior to EB plating, the particular cell line) affected the extent of cardiogenesis in EBs (Ezekiel 2007; Koike et al., 2007). However, both techniques have limitations including inherently incapable of large scale production and exhibit limited control over cell aggregation during EB formation called agglomeration resulting in the difficulty to control lineage-specific differentiation (Mogi et al., 2009). To solve these problems, bioreactors have been applied for culturing these cells. Recently, bioreactor cultures are increasingly being studied for stem cell expansion and differentiation, much work remains in understanding large-scale lineage-specific differentiation to generate cardiac lineage (Bauwens et al., 2005; zur Nieden et al., 2007; Niebruegge et al., 2009; Serra et al., 2009).

In the current study, we proposed the technique for generating a scalable cardiomyocyte differentiation of ES cells. The EB producing system utilized a scalable STLV and subsequent the cardiomyocyte differentiation was optimized and characterized for their structural and functional properties. Our result showed that HM1 ES cell line was capable of cardiomyocyte differentiation using the traditional HD method, in a similar manner to the similar genetic background of the ES cell lines (Mogi et al., 2009), to myocytes with the appropriate structural and functional properties.

Besides, our major findings demonstrated that the inoculating cell numbers affected not only the efficiency of EB formation but also the efficiency of cardiomyocyte differentiation, as similar to the HD method. When seeded with the low cell seeding density, less than $2x10^5$ cells/ml (STLV1and STLV2), was not able to form EBs due to low cell aggregation. On the other hand, when seeded with the high cell seeding density (more than $5x10^5$cells/ml (STLV4)), the cells were aggregation the large EBs and started to form dense cores of apoptotic cells. These EBs have high apoptotic cells and low proliferation cells within EBs earlier than the low seeding density probably because of the high metabolism and insufficient nutrient and oxygen transfer (Gerecht-Nir et al., 2004; Lü et al., 2008). Hence, when plated EBs derived from $3x10^5$ cells/ml (STLV3), cells were able to spread out and then differentiation into cardiomyocytes with higher rate than another group, regardless time of EB adherence to gelatin-coated dishes. Though, the efficiency of cardiac differentiation was not different between groups when evaluated percentage of cardiac beating/well, the efficiency of cardiac differentiation was different between groups when evaluated the grading of EB beating and area of cTnT. Hence, we suggested that the later two evaluations for cardiomyocyte differentiation are important.

Additionally rotation speeds of STLV did not affect the efficiency of EB formation. This is in agreement with another report (Lü et al., 2008). In contrast, the rotation speed of rotary suspension culture affected the yield and size of EB formation by which the rotary EBs set at 25 rpm formed large EBs by day two, whereas EBs at 40 and 55 rpm rotary conditions formed a greater number of smaller EBs (Carpenedo et al., 2007). Our results strongly suggested that $3x10^5$ cells/ml of cell seeding density and 10 rpm rotary speed improved the quality of EB formation in terms of high yield of EB production with homogenous in size, low apoptotic cells and high proliferation cells compared to other groups (Gerecht-Nir et al., 2004; Lü et al., 2008). On the contrary, when cultured human EBs in STLV were heterogeneous in size and non

round shapes. It is possible that human EBs are difficult to manage when compared with mouse EB because of dissociation process (Yirme et al., 2008).

The morphology of EBs, visualized by H&E staing on sections and parallel marker analysis for proliferation/apoptosis showed that STLV culture condition resulted more uniform EBs with similar inner and outer structures: (i) highly proliferative outher zone (outskirt) and (ii) apoptotic inside (cavity). This mimics mouse embryo development at gastrulation stage when a cavity is being formed in the embryo. Thus EBs derived from STLV culture conditions are more superior quality then HD drived ones.

In addition, we revealed that time of EB adherence to gelatin-coated dishes affected the efficiency of cardiomyocyte differentiation, similar to the HD method. When plated EB derived from HD and STLV on gelatin plate (day two and three), these EBs still had high proliferation cells with low apoptosis cells and high efficience for cell spreading out after EB plating. On the other hand, when plated EB derived from HD and STLV on gelatin plate (day four and five), those EBs still had low proliferation cells with high apoptosis cells and low efficience for cell spreading out after EB plating. Positive staining of cleaved-caspase 3 within all EBs showed reduced cell viability after long term EB culture, perhaps because of a greater number of cells undergoing programmed cell death. This phenomenon was undergone a similar process of normal mouse embryo development and differentiation into three germ layers (Desbaillets et al., 2000). Subsequently, the cavitations formation was the result of both programed cell death and selective cell survival, and that the process depends on signals from visceral endoderm, known as cystic EBs (Karbanová and Mokry, 2002). Our result showed that EB started to form small cystic EB on day four or day five in HD group which pretended to differentiate to endoderm lineage), agreement low efficiency of cardiac differentiation (less cardiac beating per well and low area of cTnT) in HD/D4 and HD/D5 groups. In the contrary, Chen and colleagues (2011) found that cardiac differentiation was the most effective by plating EBs on gelatin coated plates on day four or later (Chen et al., 2011). For cardiac differentiation protocol using HD culture, the initial cell seeding density are usually 400-1,000 ES cells/ml, and the EB were usually plated on gelatin coated dish on day five. Previous study described the development of ES aggregate: firstly, an outer ring of endoderm differentiation is formed surrounding the inner cells which are differentiating into the ectodermal derivative. When plated the third layer, the mesodermal cells develop in the contact zone between endoderm and ectoderm. Hence, our result suggests that plating EB on day three improved the efficiency of cardiac differentiation because mesodermal started to develop earlier than EB plating later. The mean time delay to differentiation into cardiomyocytes was significantly shorter under plating EB on gelatin plate on day three than plating later day (one to two days), regardless the culture condition.

Finally, our resulted demonstrated that the EBs produced in STLV exhibited better quality than traditional HD culture protocol. Moreover, these produced EBs exhibited higher efficience cardiomyocytes differentiation than traditional HD culture protocol. The operating principles of RCCS are as follows: (i) whole-body rotation around a horizontal axis, which is characterized by extremely low fluid shear stress and (ii) oxygenation by active or passive diffusion to the exclusion of all but dissolved gasses from the reactor chamber, yielding a vessel devoid of gas bubbles and gas/fluid interfaces (Lelkes and Unsworth, 2002). Because the rotation speed of STLV is set very slow, the resulting flow pattern in the RCCS turns to be laminar with mild mixing. The settling of the cell clusters, which is associated with oscillations and

tumbling, generates fluid mixing, and an extreme low shear environment is maintained. Another advantage of the RCCS is that they are geometrically designed so that the membrane area to volume of medium ratio is high, thus enabling efficient gas exchange. Our current study has demonstrated that STLV culture can efficiently produce EBs from normal ES cells, and the present results confirmed that the STLV rotation bioreactor could also provide a stable and ideal condition for the pluripotent cells including iPS cells. To our knowledge, this is the first report comparing the effects of STLV and HD culture on the quality and cardiac differentiation efficacy of formed EBs.

In conclusion, we have demonstrated that the optimized STLV bioreactor method provides satisfactory control over the agglomeration process of EBs, thereby enabling cell proliferation and differentiating ES cells towards cardiomyocytes. The starting number of ES cells in EBs and the time of EB adherence onto gelatin-coated plates affected the cardiogenesis. A starting number of $3x10^5$ ES cells/ml, 10 rpm rotary speed and plated on day three following culture onto gelatin-coated surfaces resulted in the highest potential of cardiac differentiation. The ability to produce homogeneous populations of EBs of particular sizes also increases the differentiation potential of ES cells into cardiomyocytes. The establishment of this cultivation system provides a powerful research and clinical tool for several fields such as disease modeling and cardiac tissue engineering, as well as regenerative medicine applications or drug screening tests. This methodology could also be employed to understand the intrinsic EB properties and their effects on cell differentiation and lineage commitment.

CHAPTER IV

COMPARATIVE CARDIAC DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELLS BY USING SLOW TURNING LATERAL VESSEL (STLV) BIOREACTOR VERSUS STATIC SUSPENSION CULTURE (SSC) AND HANGING DROP (HD) CULTURE

4.1 Abstract

Cardiomyocytes derived from ES cells are anticipated to be valuable for cardiovascular drug testing and disease therapy. However, the overall efficiency of cardiomyocytes production by *in vitro* differentiation of ES cells is still low. To enable large-scale culture of ES-derived cells for cardiac differentiation, we developed a scalable bioprocess that directs EB formation in a fully controlled bioreactor following inoculation with a single cell suspension of mouse ES cells. EBs generated by optimized STLV bioreactor were compared to SSC and HD condition for efficiency of EB formation, EB yield, homogeneity of EB size, viability and apoptosis. After further cultivation, it was found that the percentages of beating EBs were much greater compared to conventional method, and the homogeneity of EBs were significantly improved over compared with the other groups. Cardiac specific gene expressions were examined by quantitative (q) RT-PCR (Brachyury, Nkx2.5, Tnnt2 (cTnT), Myh6 (α-MHC), Myl2 (MLV-2v), Nppa (ANP) and Oct-4) and fluorescent immunohistochemistry (e.g. (cTnT). Our results showed that after three days of culture, a four-fold improvement in the yield of EB formation/ml in STLV conditions vs. SSC conditions were detected (170±14.1 and 41±26.8 EBs/ml, respectively). Besides, a six-fold enhancement in total yield of EB/ml was achieved ($39x10^5$ and $7\ x10^5$ cells/ml, respectively). Furthermore, a nearly ten-fold less cells which were not incorporation into EBs in STLV condition vs. SSC conditions were observed ($6x10^4$ and $59x10^4$ cells/ml, respectively). In addition, EBs cultured in STLV conditions exhibited the most uniform size distribution (STLV, 500.9±10.1 and 518.1±10.2; SSC, 443.2±19.6 and 607.9±32.9; HD, 405±5.2 and 472.4±19.9 µm; EBs with minimum and maximum diameters, respectively). The rate of proliferation (Ki67 immunostaining) and the rate of apoptosis (cleaved-caspase 3 immunostaining) in EBs revealed no differences between STLV and SSC groups but were different from HD group. Importantly, STLV conditions produced more uniform EBs than conventional methods. During cardiac differentiation the rate of beating areas/well were similar among all groups at day seven; even though, EBs cultured in STLV showed the highest distribution of cTnT positive areas at day fourteen. qRT-PCR assay demonstrated that EBs cultured in HD condition expressed three-fold higher Brachyury than those generated by STLV or SSC conditions (at day three). EBs cultured in STLV and HD expressed more Tnnt2 - compared with SSC condition (at day fourteen). EBs cultured in STLV not only expressed more Nkx2.5 (at day seven) but also expressed more Nppa and Myh6 (at day fourteen); compared to SSC condition. Myl2 were similar among all groups (at day fourteen). Hence, EBs culture in STLV provides a technological platform for the large-scale generation of ES cell-derived cells and differentiation into cardiomyocytes for clinical and industrial applications.

4.2 Introduction

Congestive heart failure, one of the most frequent cardiovascular diseases in the world, resulted from the inability of the heart to pump enough blood to maintain physiological functions. Heart failure is characterized by the loss of functional cardiomyocytes, known as cardiomyopathy (Sachinidis et al., 2002; 2003). While adult cardiomyocytes are not capable of regeneration after birth, the effective treatment of patients suffering from severe heart failure still remains to be organ transplantation. After ES cell establishment have been established discovery, the functional cardiomyocytes derived from these cells (Kehat et al., 2001; Kehat and Gepstein, 2003), have opened the avenue to valuable cell sources for cell-based therapies, as well as cardiac tissue engineering. Moreover, an unlimited differentiation capacity and indefinite propagation of ES cells represent the strongest advantages for using the ES cells.

Even thought the capability of ES cells to differentiate into cardiomyocytes has been studied by numerous research groups (Doetschman et al., 1985; Wobus et al., 1991), overall efficiency and quantity of differentiation into cardiomyocytes is still low. To realize the therapeutic potential of those cells, it is essential to regulate their differentiation in a reproducible manner. Differentiation of ES cells into cardiomyocytes usually requires an initial aggregation step to form spherical cell clusters referred to EBs which recapitulate many aspects of the developing embryo, including differentiation to cells of endoderm, mesoderm, and ectoderm lineages, resembling gastrulation *in vivo* (Desbaillets et al., 2000). The standard methods for achieving EBs are HD and SSC (Dang et al., 2002; Shamblott et al., 2002; Chen et al., 2011). Nevertheless, these culture systems are limited in their production capacity due to laborious-requirement and are not easily amenable to process-control strategies (Kurosawa, 2007).

To solve these problems, bioreactors (spinner flasks and the RCCS) have been developed for scalable cell expansion and differentiation of ES-derived cells with a tightly controlled *in vitro* environment, including the exchange of oxygen, nutrients and metabolites (Rungarunlert et al., 2009). Spinner flasks, has the disadvantage of generating shear forces and, although manageable, these forces still can damage the cells (Chisti, 2001; Hammond and Hammond, 2001). Another bioreactor, the RCCS, is composed of a STLV and a HARV. EBs produced by using STLV bioreactor were more uniform in size, had less necrotic centers and yielded a nearly four-fold increase in the number of EB particles in comparison to SSC (Gerecht-Nir et al., 2004). Similar to another report, the pluripotent ntEBs formed in STLV bioreactor were homogenous in size, and no large necrotic centers. Subsequently, STLV-produced ntEBs differentiated into cardiomyocytes more efficiently than SSC culture (Lü et al., 2008). Regardless of these advances, there are still some challenges that would need to be addressed before applying this technique including the safe, increasing the scale of cell production and by optimizing culture conditions for the generation of specific cells including cardiomyocytes.

Moreover, many researchers found that protein-based cytokines and growth factors, a number of synthetic chemical compounds were improved the efficiency of cardiomyogenic differentiation *in vitro*. Synthetic chemicals have advantage than others due to a longer active half-life in solution. Hence, these chemicals were stable prolonged *in vitro* cell culture over several days or even weeks (Heng et al., 2004). Ascorbic acid (ASC), one of synthetic chemical compounds (vitamin C) have been shown to promote cardiomyogenic differentiation of ES cells by increasing the expression of cardiac genes, including GATA -binding protein 4 (GATA4), α-MHC,

and β-MHC in ES cells in a developmentally controlled manner (Takahashi et al., 2003).

Enabling large-scale culture of ES-derived cells for cardiac differentiation, we developed a scalable bioprocess that directs EB formation in a fully controlled STLV bioreactor following inoculation with a single cell suspension of mouse ES cells. EBs generated by optimized STLV bioreactor was compared to SSC and HD condition for efficiency of EB formation and subsequently cardiac differentiation. Moreover, we also investigated the effect of ASC supplement on cardiomyocyte differentiation. The STLV method also allows creation of a more uniform and homogenous cell population of EBs that is more favorable for subsequent cardiomyocyte differentiation, compared with classical SSC and HD methods.

4.3 Materials and methods

Materials and methods of this study were followed as materials and methods of chapter III (pg 33 to 35) except specific details which were described below.

4.3.1 Differentiation methods of ES cells

The optimized STLV protocol for optimal size and EB quality consequent cardiac differentiation was followed as chapter III. In brief, $3x10^5$ cells/ml of inoculating cell seeding density and 10 rpm rotary speed. Generation of EBs by using SSC method, ES cells were seed directly into a 150-mm bacteriological Petri-dish treated with poly-HEMA in 55 ml of differentiation medium. The initial cell seeding density of SSC method was similar to STLV method. Generation of EBs by using HD method, suspension cultures of EBs were initiated by resuspending $4x10^4$ cells/ml. Then, 20 µl of the spontaneous differentiation medium were dropped on the lids of 10 cm petri-dishes (resulting in 800 cells). All EBs were maintained for up to three days in same culture without interruption. Then, all EBs were collected at day three for evaluating gross morphology, size, yield and histological analysis and were plated on 0.1%-gelatin-coated culture dishes for evaluating the efficiency of cardiac differentiation.

4.3.2 Yield of EB, total cell yield of EBs/ml and total free cells/ml analysis

One ml of STLV and SSC culture medium which was not passed through a 100 µm cell strainer was counted for investigating the yield of EB production. Subsequently, those EBs were trypsin and counted for evaluating the total cell yield of EBs/ml. On the other hand, one ml of STLV and SSC culture medium which was passed through a 100 µm cell strainer was trypsin and counted for total free cells/ml. These free cells referred cells which were not incorporation into EBs were passed filter.

4.3.3 Histological analysis

For detecting three germ layers in EB formation, EB sections were stained with Oct-4 (pluripotency ES cell marker), α-Fetoprotein (early endoderm lineage marker), Brachyury (early mesoderm lineage marker) and Pax6 (early ectoderm lineage marker).

4.3.4 Differentiation of ES cells to cardiomyocytes

At day three, individual EBs were plated onto gelatin coated dishes into separate wells of a 24-well plate for cardiomyocyte differentiation. Forty-eight EBs were cultured for 21 days and observed daily under a phase-contrast microscope for

evaluation of the percentage of cardiac beating/well (the number of beating EBs was graphed as the percentage of the total number of EBs plated) and quantification of EB beating activity. Spontaneous cardiac differentiation medium consisted of ES undifferentiated medium without LIF. ASC was added in this medium, in case of induced cardiac differentiation.

4.3.5 Quantitative real-time polymerase chain reaction (qRT-PCR)

Undifferentiated ES cells (day 0) and differentiating EBs (day 3, 7, 14, and 21) were frozen in -80 ºC. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. For each sample, 500 ng RNA was reverse transcribed using the SuperScript VILO cDNA Synthesis, as per the manufacturer's instructions. After the reverse transcription, the cDNA samples were diluted to 2.5 ng/µl concentration and dispensed to once-use aliquots. qRT-PCR reactions were performed as previously described (Kobolak et al. 2009). GAPDH was used as a reference gene in the study. The PCR-related primers and reaction conditions were described in Table 4 and were carried out on a Rotor-Gene Q real-time PCR machine (Qiagen, Hilden, Germany). The Relative Expression Software Tool 2008 V2.0.7 was applied to determine differences in expression of the studied genes.

4.3.6 Statistical analysis

Data are expressed as mean ± SEM and include at least three independent experiments. Statistical analyses were conducted using the Student's *t* test or the General Linear Model (GLM) procedures of SAS. *P*-value $<$ 0.05 will be considered statistically significant.

4.3.7 Experimental design (Figure 22)

Experiment 1: To study the effect of EB culture methods on EB formation and the efficiency of cardiomyocyte differentiation. STLV, SSC and HD condition, were compared. STLV and SSC cultures were equally initiated with the cell seeding density of ES cells ($3x10^5$ cells/ml) and HD EBs were performed with 800 cells per drop ($4x10^4$ cells/ml). After 3 day cuture, EBs were collected for EB analysis and were plated on gelatin coated dish for the efficiency of cardiomyocyte differentiation. At least three independent replicates were examined.

Experiment 2: To study the effect of ASC on the efficiency of cardiomyocyte differentiation in STLV, SSC and HD culture. After three day cuture, EBs were plated on gelatin coated dish cultured and divided into two groups: (i) culture in spontanous cardiac differentiation (without ASC supplement) or (ii) culture in induced cardiac differentiation (with ASC supplement) for the efficiency of cardiomyocyte differentiation. At least three independent replicates were examined.

4.4 Results

4.4.1 Effect of EB culture condition on EB formation

To study the effect of EB culture condition on time course of EB formation, three culture condition; STLV, SSC and HD condition, were compared. STLV and SSC cultures

were equally initiated with the cell seeding density of ES cells ($3x10^5$ cells/ml), and HD EBs were performed with 800 cells/drop ($4x10^4$ cells/ml). Inoculation cells in SSC cultures were started to aggregate at 12 h and formed EBs at 24 h earlier than

inoculation cells in STLV culture which were started to aggregate at 18 h and formed EBs at 36 h. However, agglomerations of SSC EBs were appeared at 48 h which did not achieve in STLV (Figure 23).

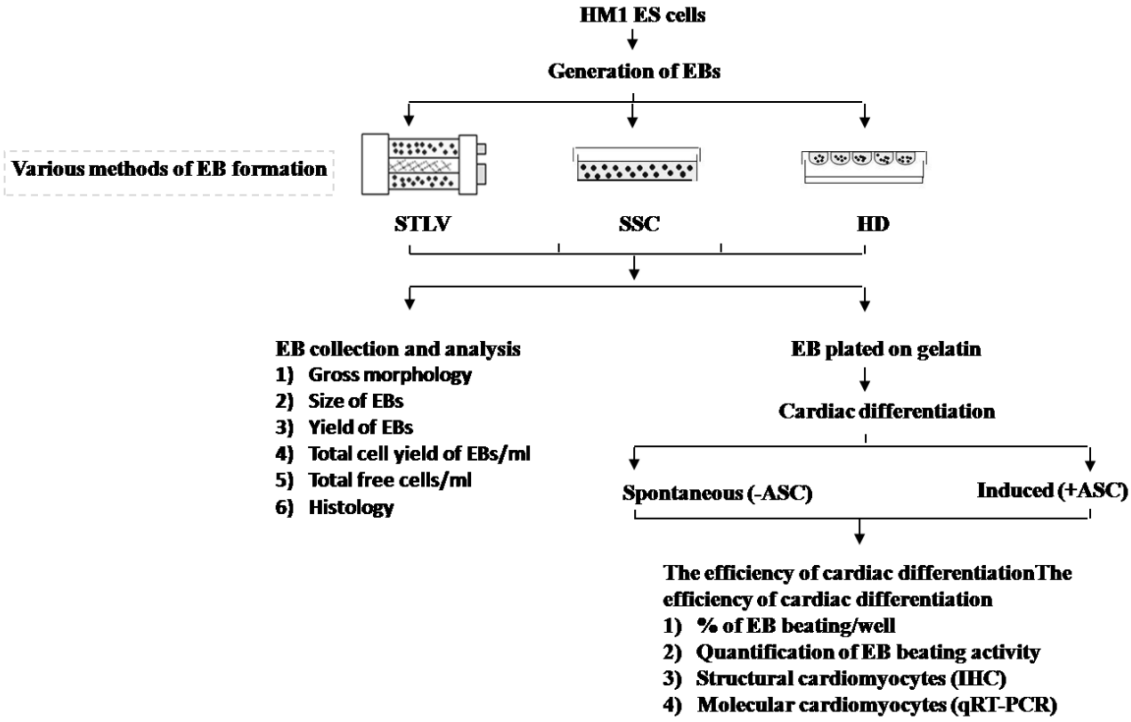


Figure 22. Schematic presentation of *in vitro* differentiation procedure of ES cells into cardiomyocytes in STLV, SSC and HD culture system.

Interestingly, 36 h after culturing EBs in STLV, these EB appeared to form bigger and higher uniform in size than EBs in SSC. On day two of culturing EBs, SSC EBs started to form large agglomerates of multiple EBs and then formed irregular and heterogeneous in size on day three. In contrast, STLV EBs on day three increased slightly in size from day two and still remained regular and spherical with no appearance of agglomeration. In HD group, EBs started to aggregate by 12 h and form EBs by 24 h. However, three-four EBs were present in one drop. One EB/one drop was appeared by 48 h (Figure 23). Besides, STLV EBs revealed relatively homogenous, round and uniform in size EBs at day 3 of cultivation with minimum and maximum diameters ranging between 500.9±10.1 and 518.1±10.2µm, respectively. In contrast, EBs grown in SSC and HD condition were heterogeneous and agglomeration with minimum and maximum diameters ranging between 443.2±19.6, 607.9±32.9, 405±5.2 and 472.4±19.9 µm, respectively (Figure 23 and 24).

4.4.2 Effect of EB culture condition on yield, total cell yield of EBs/ml and total free cells/ml

To evaluate the effect of EB culture condition on the yield of EB production, one ml of STLV and SSC culture medium was passed through a 100 µm cell strainer in order to separate cell aggregates constituting EBs from individual cells. The size of EBs bigger than 100 µm that did not pass the strainer was counted. Our results showed that after three days of culture, a four-fold improvement in generation of EBs

in STLV conditions vs SSC was detected (170±14.1 and 41±26.8 EBs/ml, respectively, Figure 25A). Consequency, these EBs were counted for evaluating the total cell number which are able to aggregate and form EBs. Our result demonstrated that a six-fold enhancement in total cell yield of EB per ml was achieved in STLV compared to in SSC ($39x10^5$ and $7 x10^5$ cells/ml, respectively, Figure 25B). On the other hand, the medium which passed strainer, referred to the total free cells which were not incorporation into EBs, were counted. A nearly ten-fold diminishment in total cell yield of free cell in STLV condition vs SSC was observed ($6x10^4$ and $59x10^4$ cells/ml, respectively, Figure 25C).

4.4.3 Effect of EB culture condition on cell viability of within EBs

Staining of day three EBs with H&E revealed no difference in the presence of cell morphology between STLV and HD EBs but different from SSC EBs which appeared to have irregular shape (Figure 26). Ki 67 staining of proliferation cells and caspase 3 staining of apoptosis cells revealed different between STLV and SSC with HD group (Figure 26). Interestingly, HD EBs contained sparse, individual not only proliferative cells but also apoptosis cells. Both EBs derived from STLV and HD groups contained with EB proliferative cells but also apoptosis cells in center of EBs (Figure 26).

Table 4. Properties of the oligonucleotide primers used in qRT-PCR reaction

Primer	Size (bp)	Sequence
Oct-4	110	(Forward) 5'- AGCCGACAACAATGAGAACC -3'
		(Reverse) 5'- TCTCCAGACTCCACCTCACA -3'
Brachyury	161	(Forward) 5'- TACACCTCTAATGTCCTCCCTTG -3'
		(Reverse) 5'- CCATACAGTTGACTTCCCAACAC -3'
Nkx2.5	74	(Forward) 5'- ACACCCACGCCTTTCTCAGT -3'
		(Reverse) 5'- AGGTCCCCAGACGCCA -3'
Tnnt2	117	(Forward) 5'- CGGATCAATGACAACCAGA -3'
		(Reverse) 5'- GGGCAAGGACACAAGCAG -3'
Myh6	80	(Forward) 5'- CTACGCCTTCGTCTCTCAGG -3'
		(Reverse) 5'- AGGCACTATCAGTGGCCAAG -3'
Myl2	90	(Forward) 5'- TGACCTAAGGGACACATTTGC -3'
		(Reverse) 5'- ATTGGACCTGGAGCCTCTTT -3'
Nppa	101	(Forward) 5'- GTCTTGCCTCTCCCACTCTG -3'
		(Reverse) 5'- TTTCGTCCTTGGTGCTGAA -3'
GAPDH	79	(Forward) 5'- AATGTGTCCGTCGTGGATCT -3'
		(Reverse) 5'- CCTGCTTCACCACCTTCTTG -3'

Abbreviation: Nkx2.5, NK2 transcription factor related, locus 5: Tnnt2, Troponin T type 2 (cardiac): Myh6, Myosin, heavy chain 6, cardiac muscle, alpha: Mly2, Myosin regulatory light chain 2, ventricular/cardiac muscle isoform: Nppn, natriuretic peptide type A

4.4.4 Effect of EB culture condition on pluripotency and germ layer marker expression during EB differentiation

To evaluate different protein expressions during EB formation among different culture methods, EB were collected on day three.

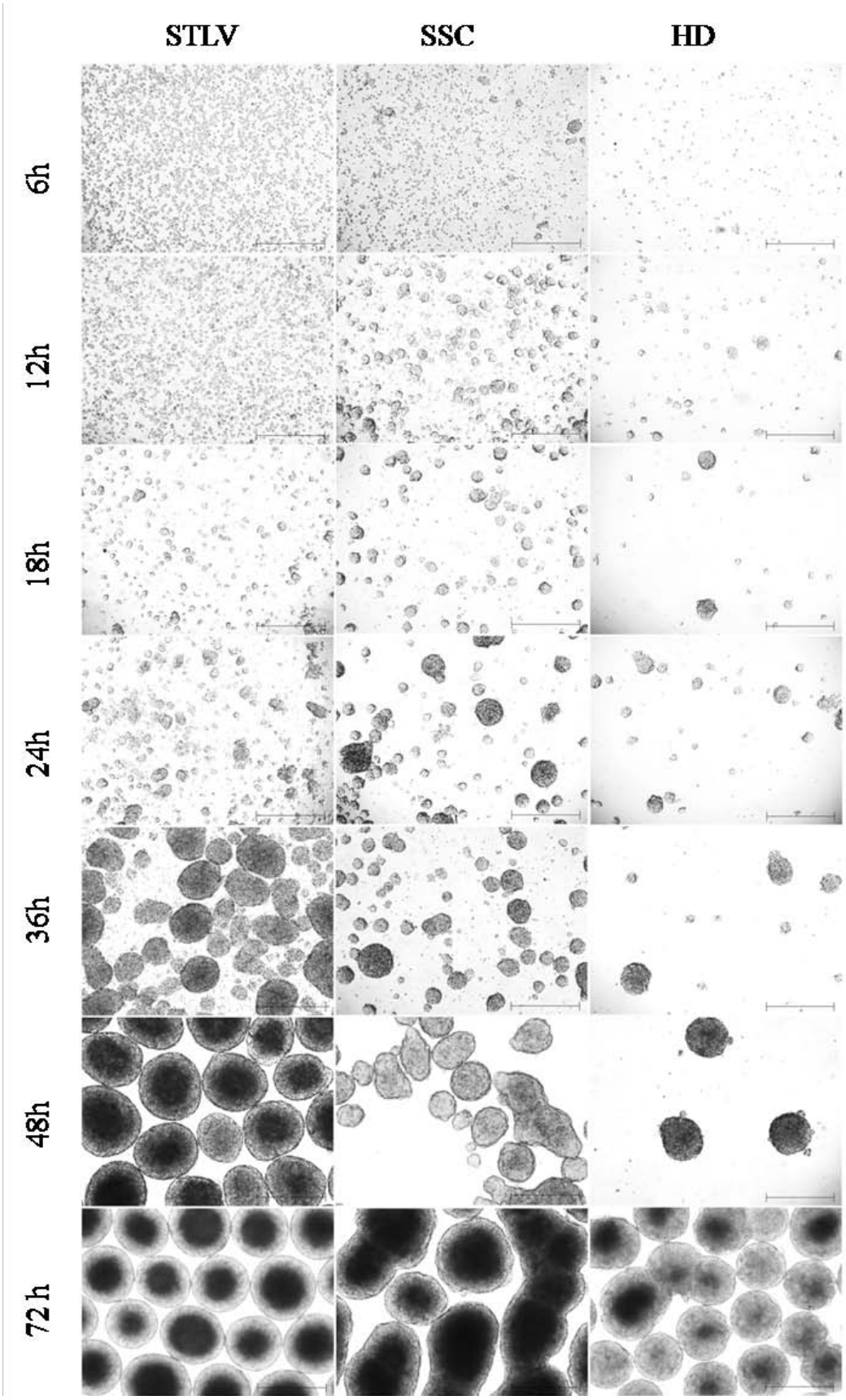


Figure 23. Gross morphology of EBs produced from EB culture conditions (STLV, SSC and HD)**.** Scale bars represent 500 µm x4 magnification.

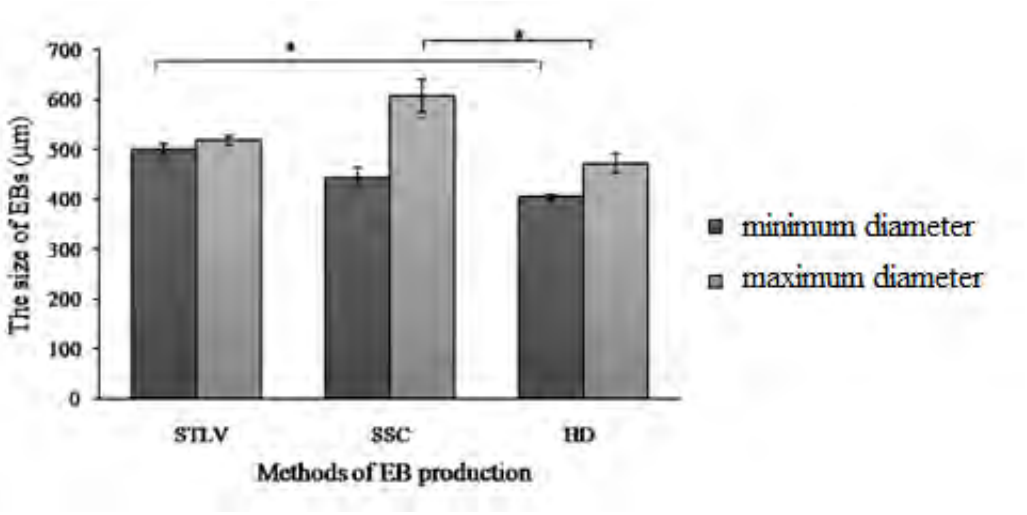


Figure 24. The size of EBs produced from different culture condition (STLV, SSC and HD) on day three. Data presented as mean ± SEM. The difference was considered significant for a $p<0.05$, *.

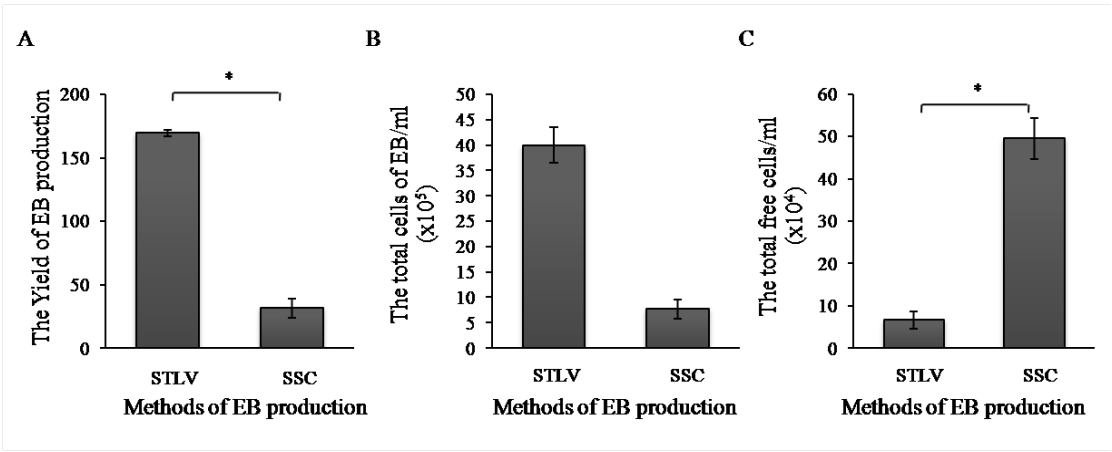


Figure 25. The efficiency of EBs from different culture condition (STLV and SSC) on day three. (A) The yield of EB production/ml. (B) The total cells of EB/ml ($x10^5$). (C) The total free cells/ml ($x10^4$). Data presented as mean ± SEM. The difference was considered significant for a $p<0.05$, *.

Protein expressions from the differentiating EBs were assessed by IHC staining for pluripotency marker Oct-4, and with germ layer markers (mesodermal: Brachyury, endodermal: α-fetoprotein and ectodermal: Pax6) (Figure 26). Additionally, qRT-PCR was used to assess the mRNA expression levels of the corresponding markers and to confirm the immunocytochemical data. Moreover, cardiac lineage markers Nkx2.5 (early cardiac marker), Tnnt2, Myh6, Myl2 and Nppa (late cardiac markers) were also evaluated (Figure 30).

Although, the mRNA expression level for Oct-4 started to decrease after the initiation of EB differentiation (Figure 30), the protein expression for Oct-4 was already invisible by D3 (Figure 26). There was no difference in mRNA expression level for Oct-4 among different culture method. Unexpectedly, the mRNA expression level for Brachyury started to increase after the initiation of EB differentiation derived from HD group (Figure 30). Nevertheless, the protein expression for Brachyury was

visible in all EBs on day three. The expression could be seen abundant in the all of the EBs derived from STLV and HD group but was only sporadic in SSC group (Figure 26). The protein expression of Pax 6 was notably low in all the EB sections (Figure 26). The mRNA expression level for α-fetoprotein showed low expression in EBs derived from different culture methods (Figure 30). Interestingly, the protein expression for α-fetoprotein was already visible in the center of EBs derived from SSC group and remained sporadic in EBs derived from other group. The mRNA expression level of the cardiac differentiation including Nkx2.5, Tnnt2, Myh6, Myl2 and Nppa was not expressed in all EBs on this day (Figure 30).

4.4.5 Effect of EB culture condition on efficiency of cardiac differentiation

One day after plating EB on gelatin coated plate, EBs were able to attach to the plate. Then, the cells started to spread from the EB to form a monolayer at periphery (Figure 27). During the culture periods, the cell numbers of STLV EBs increased continuously and the colonies almost covered the entire area of the wells of a 24 well plate on day twelve. None of EBs derived from SSC and HD group spreaded and almost covered the entire area of the wells of a 24 well plate until end of culturing. In addition, all EBs showed similar morphology, whether or not they were culture with ASC.

Figure 28 A to 28B illustrates the cumulative percentage of EBs containing contracting area derived from STLV, SSC and HD EBs. Even though, HD EBs appeared higher percentage earlier rhythmically contracting areas of EB (on day six) than STLV and SSC EBs (on day seven), HD EBs were not reached 100% of beating during culture. STLV EBs showed similar percentage of cardiac beating with SSC EBs. All group of EB started to stop beating on day seventeen. Moreover, alls EBs showed similar percentage of cardiac beating, whether they were culture with or without ASC. The quantity of cardiac beating was also evaluated by grading the cardiac beating (Figure 28C). Grading was performed based on the size of the beating area and the rate of beating. The grading revealed that STLV EBs showed higher the highest number and fastest beating contractile areas (Grade 4) higher than other EB groups. Adding ASC for inducing cardiac differentiation was also increased the highest number and fastest beating contractile areas, whether they were produced from by different culture methods. This suggests that EBs derived from STLV generated EB were superior for producing rhythmically beating cardiomyocytes. Moreover, ASC supplement also improved differentiation of ES cells into cardiomyocytes.

To evaluate further the ability of cardiac outgrowth to form EBs among different culture condition, STLV and SSC and HD outgrowths were immunostained with cTnT antibody which marks cardiac lineage commitment. Our results demonstrated that STLV EBs were superior for producing morphology and structural of cardiac differentiation based on the presence of an extensive and filamentous network of cTnT in central and peripheral of EBs (Figure 29A) and highest the area of cTnT (Figure 29B). While, SSC and HD EBs staining indicated the distribution of cTnT most in central EB and rare in peripheral of EB (Figure 29A). Moreover, these EBs showed low the expression of cTnT which were calculated cTnT area of an EB (Figure 29B).

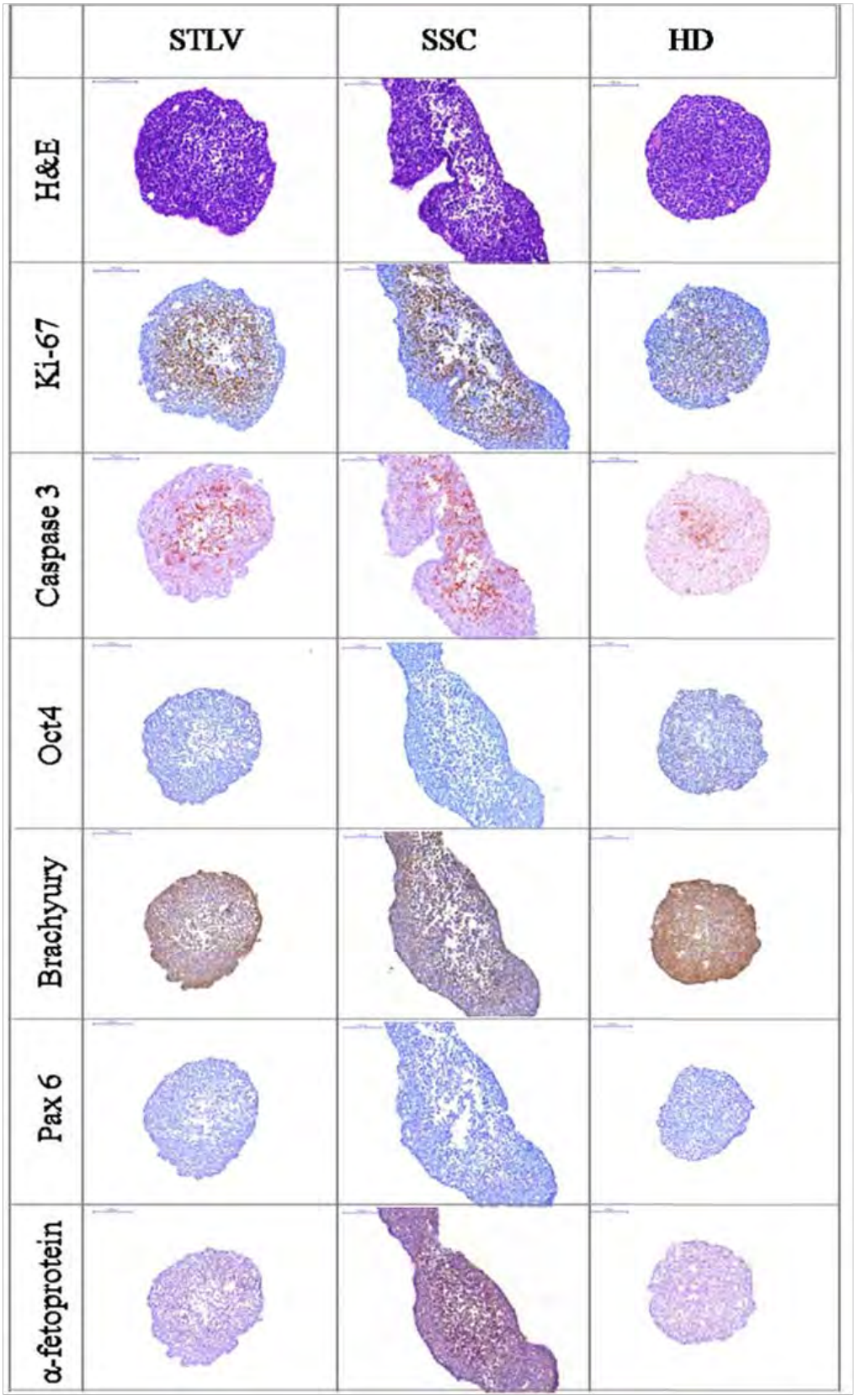


Figure 26. Histological section of EB formation (H&E staining and stainings: for the proliferation marker Ki-67; apoptosis marker cleaved-caspase 3; pluripotency marker Oct -4; mesoderm marker Brachyury; ectoderm marker Pax6 and endoderm marker α-fetoprotein). Scale bars represent 200 µm x20 magnification.

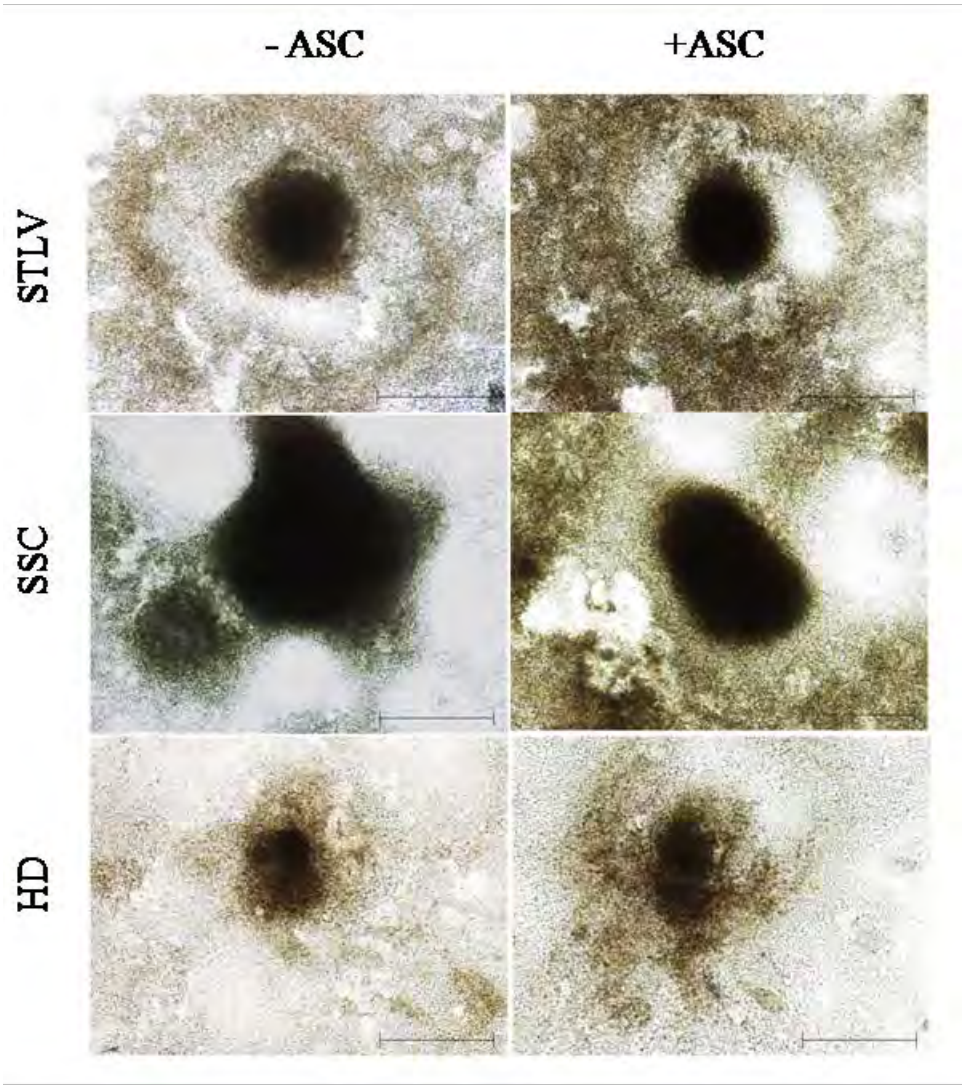


Figure 27. The morphology of EB on day seven which plated EBs derived from STLV, SSC and HD cultured with or without ASC.

4.4.6 Effect of EB culture condition on gene expression during cardiac differentiation

The induction of cardiac differentiation was further analyzed by qRT-PCR (Figure 30). RNA was extracted from EBs derived from STLV, SSC and HD at days 3, 7, 14 and 21 culture with or without ASC. Gene expressions of standard pluripotency (Oct-4; Figure 30A), early mesoderm (Brachyury; Figure 30B), early (Nkx2.5; Figure 30C) and late (Tnnt2, Myh6, Myl2 and Nppa (Figure 30D to 30G) cardiac markers were assayed. Moreover, α-fetoprotein which is endoderm differentiation marker was also investigated (Figure 30H).

Figure 30 shows transcripts from the different culture conditions at day 3, 7, 14 and 21. The level of Oct-4 (gene expressed by cells in the ICM as well as the epiblast) was high in undifferentiated cells and by day seven, diminished to almost undetectable levels at all examined samples (Figure 30A). In parallel, many of the up-regulated genes in EBs were phenotypic markers of cardiac gene expression: Nkx2.5, Tnnt2, Myh6, Myl2 and Nppa (Figure 30C to 30G).

As expected, the level of Brachyury mRNA was elevated at the beginning of differentiation (day three to day seven; Figure 30B) and showed delaying expression

thereafter. However, Brachyury mRNA was expressed only in EBs derived from HD group whether they were culture with or without ASC. In other group, the level of Brachyury decreased gradually without any peak, suggesting there were no subsets of cells in our STLV and SSC-derived EBs entering the primitive streak-stage but rather, cells within EBs gradually became engaged towards different lineages (e.g. cardiac).

Beside, Nkx2.5 gene expression, early cardiac precursors, peaked by day seven and stayed high until day fourteen in EBs cultured with ASC supplement; they were produced from by different culture methods. However, Nkx2.5 gene expression seem to be delay peaked by day fourteen and stayed high until day 21 in EBs cultured in an absence of ascorbic. Hence, ASC supplement induced cardiac differentiation by increasing Nkx2.5 mRNA. In addition, STLV EBs expressed higher Nkx2.5 mRNA than other EBs. Our results demonstrated that STLV EBs cultured in ascorbic acid supplement showed highest Nkx2.5 mRNA expression Figure 30C).

At the later time point of spontaneous cardiac differentiation, cardiac markers became prominent. The expression of Tnnt2, Myh6 and Myl2 (Figure 30E to 30F) (which are cardiomyocyte structural constituents) peaked by day fourteen and down-regulation by day 21 in all groups. No statistically different of Tnnt2 mRNA expression was observed between all groups except STLV EBs cultured in medium with ASC supplement. SSC EBs cultured in medium without ASC supplement showed lower Tnnt2 mRNA expression than other groups. No pattern of Tnnt2 mRNA expression was revealed between either different culture methods or culturing with or without ASC supplement (Figure 30D). Moreover, STLV EBs cultured with ASC supplement showed highest Myh6 mRNA expression. However these genes were low expression in STLV EBs cultured without ASC. Our result showed that there was no difference of Myl2 mRNA expression among different culture methods cultured either with or without ASC except the STLV EBs that cultured in medium with ASC supplement. Nppa regulates multiple ion channels in atrial cardiomyocytes. In our study Nppn was also highly expressed and peaked by day fourteen (Figure 30G). These observations confirmed that cardiac differentiation towards terminal cell lineage commitments was successful from STLV the culture-derived EBs. Unexpectedly, α-fetoprotein gene expression was also detected on day fourteen and highly up-regulated on day 21 in all groups. Interestingly, the α-fetoprotein mRNA was highly expressed in HD EB (a six time and a three time in HD EB cultured with and without ASC by day 21). This result suggested that culture condition method affected the mRNA expressions of cardiac differentiation.

4.5 Discussion

To prove hypothesis that the optimized STLV culture system can also provide a stable and ideal condition for the mouse ES cells to form highly EB production and retain good quality subsequence highly efficient differentiation into cardiomyocytes. EB formation in STLV was directly compared to SSC and HD. Our result showed that STLV culture system produced higher EBs with higher homogenous in size than SSC and HD culture system. In case of number of EB formation, seven days post-seeding of human ES cells, revealed nearly four times more EBs in STLV compared to SSC (Gerecht-Nir et al., 2004).

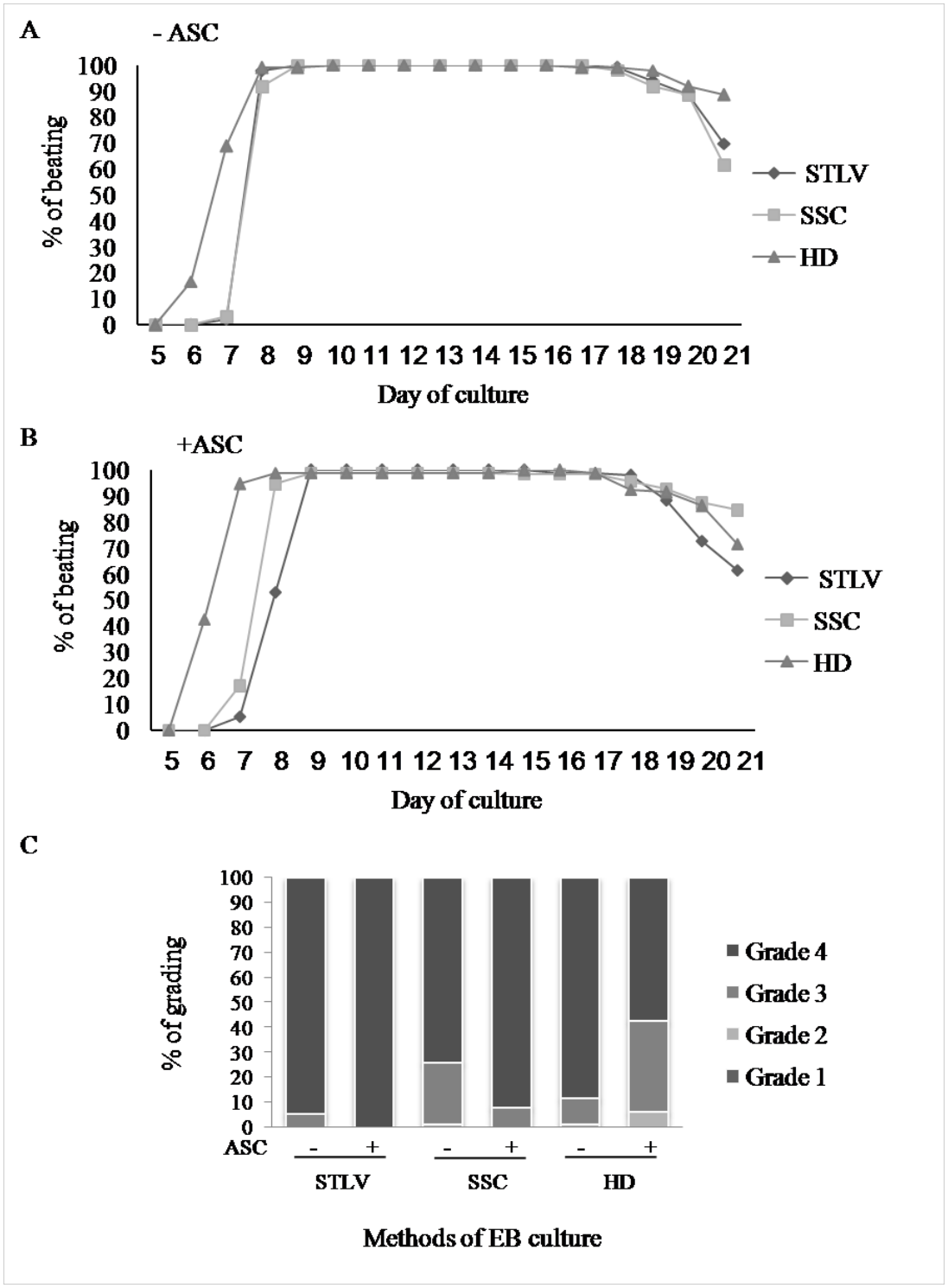


Figure 28. The efficiency of cardiac differentiation derived from different culture method. (A to B) Percentage of contracting cardiomyocytes in outgrowth of attached EBs: A, without ASC supplement and B, with ASC supplement. (C) Cardiac beating activity was evaluated by grading the area of beating foci and the beating rate.

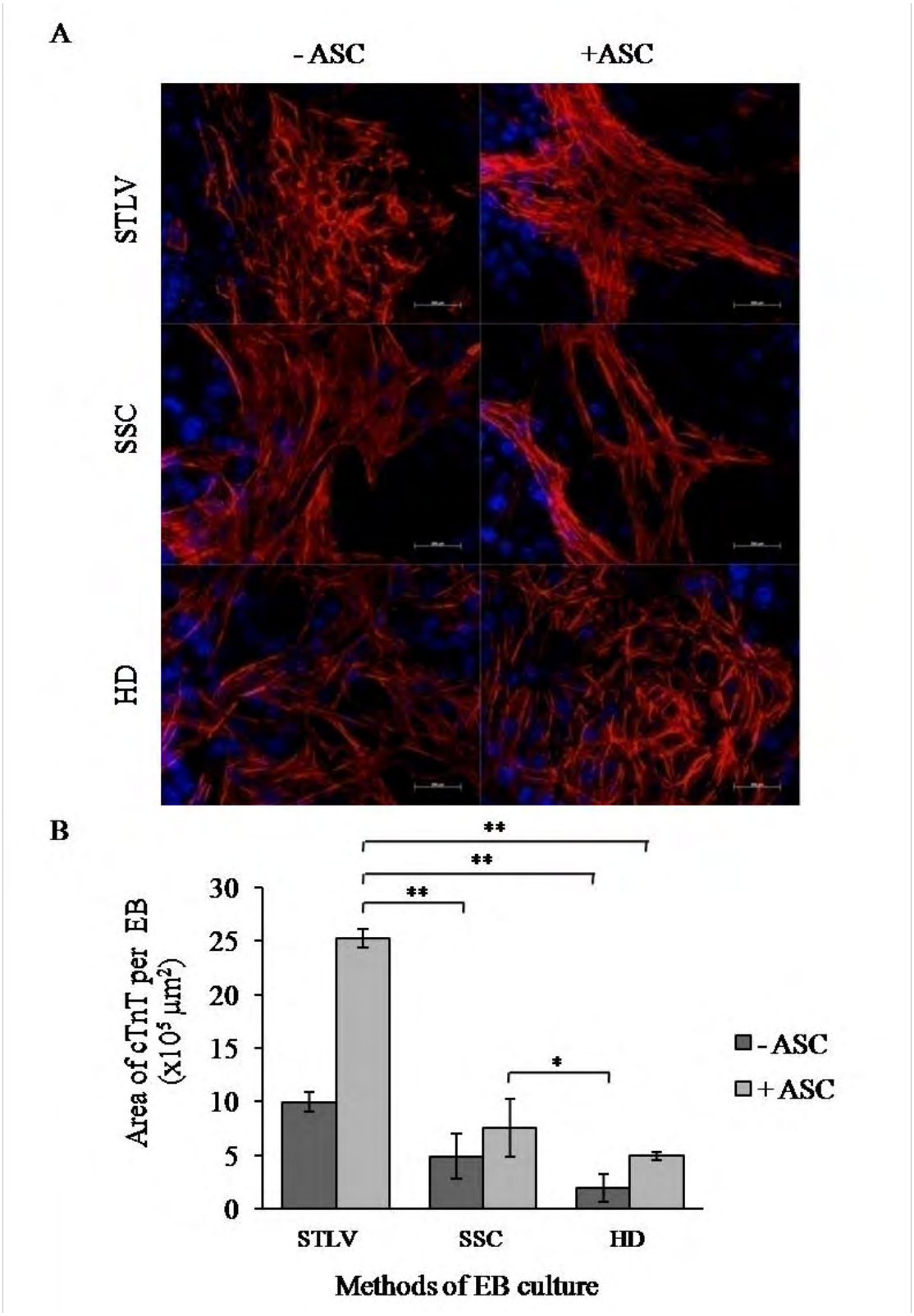
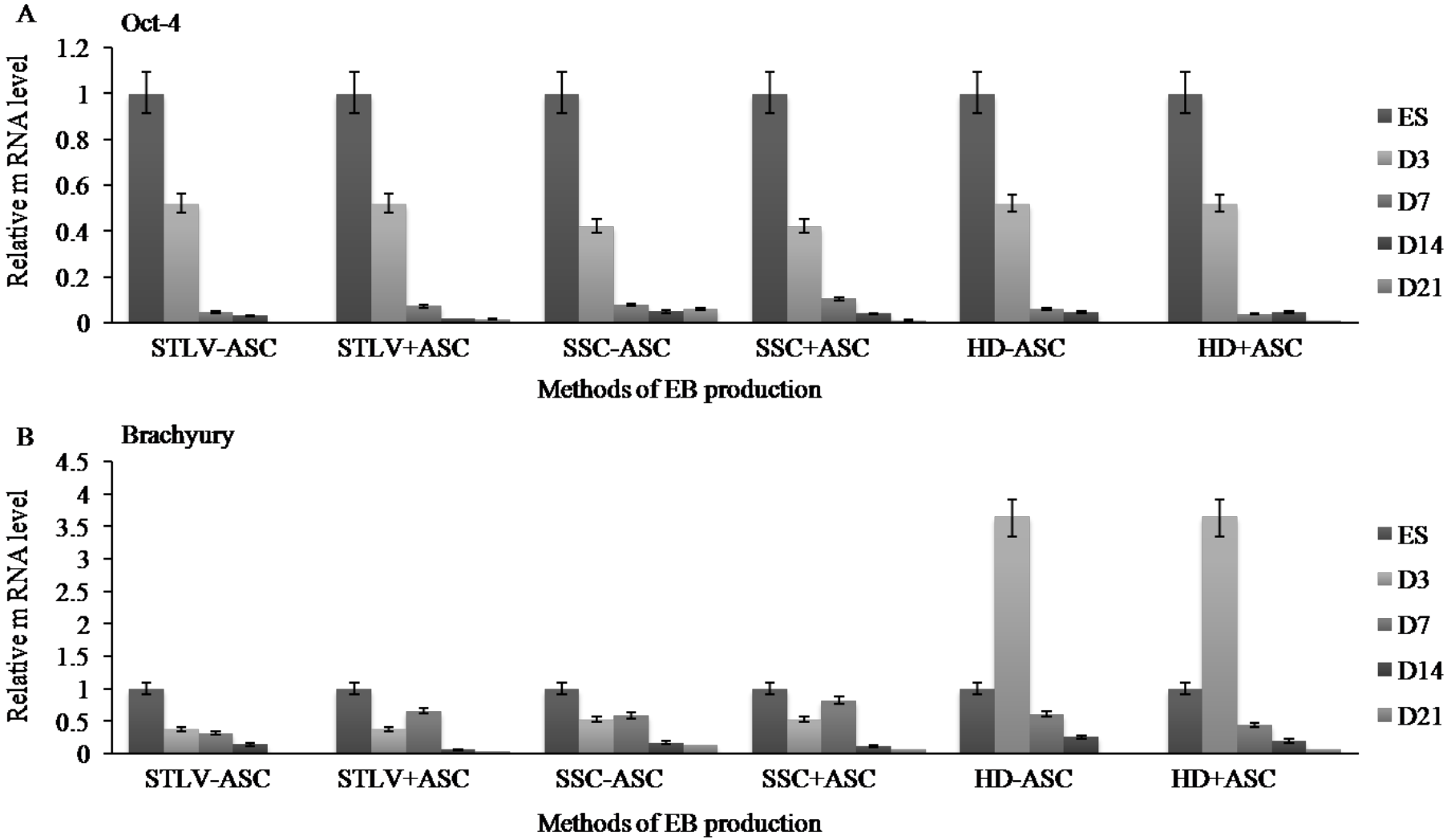
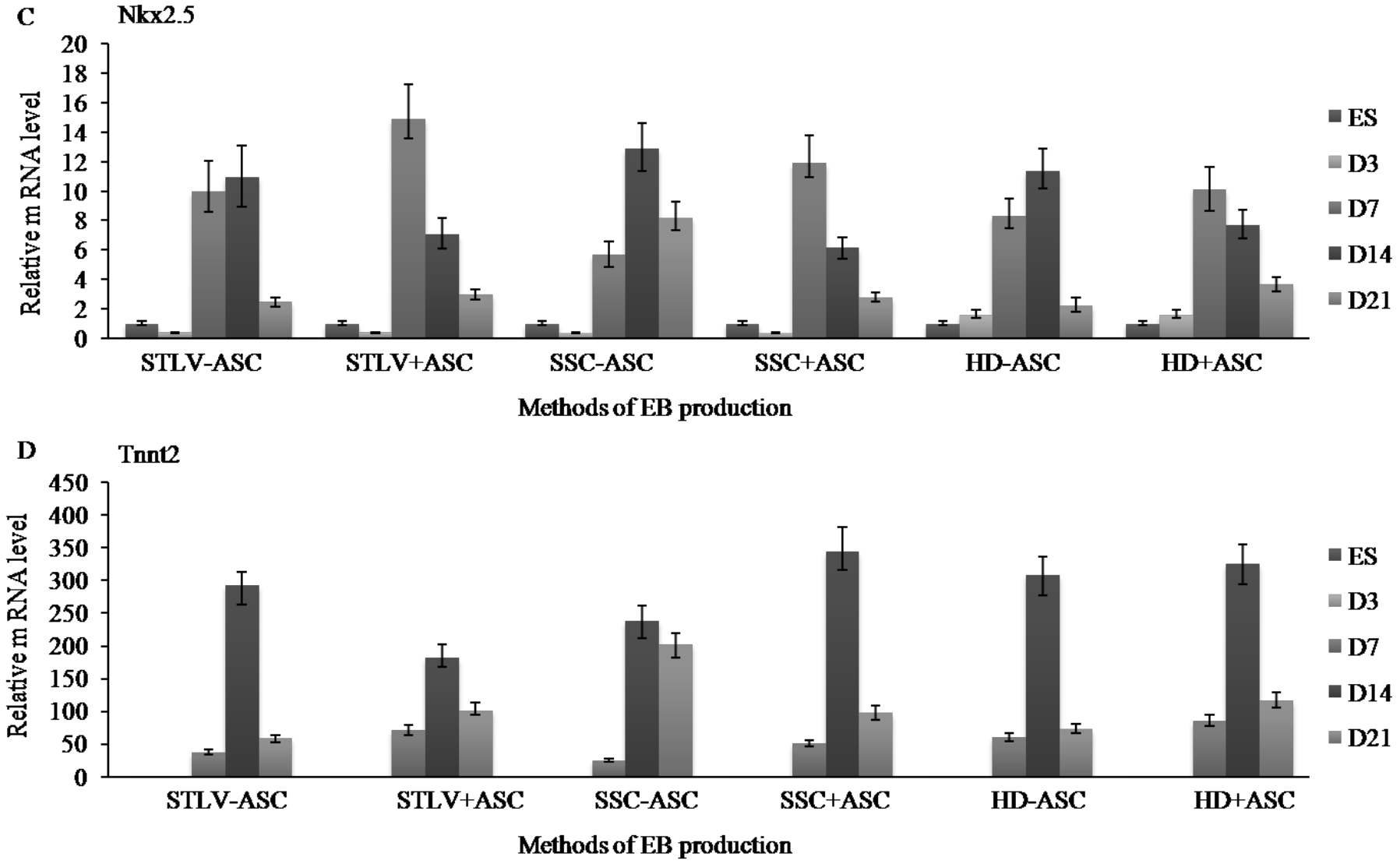
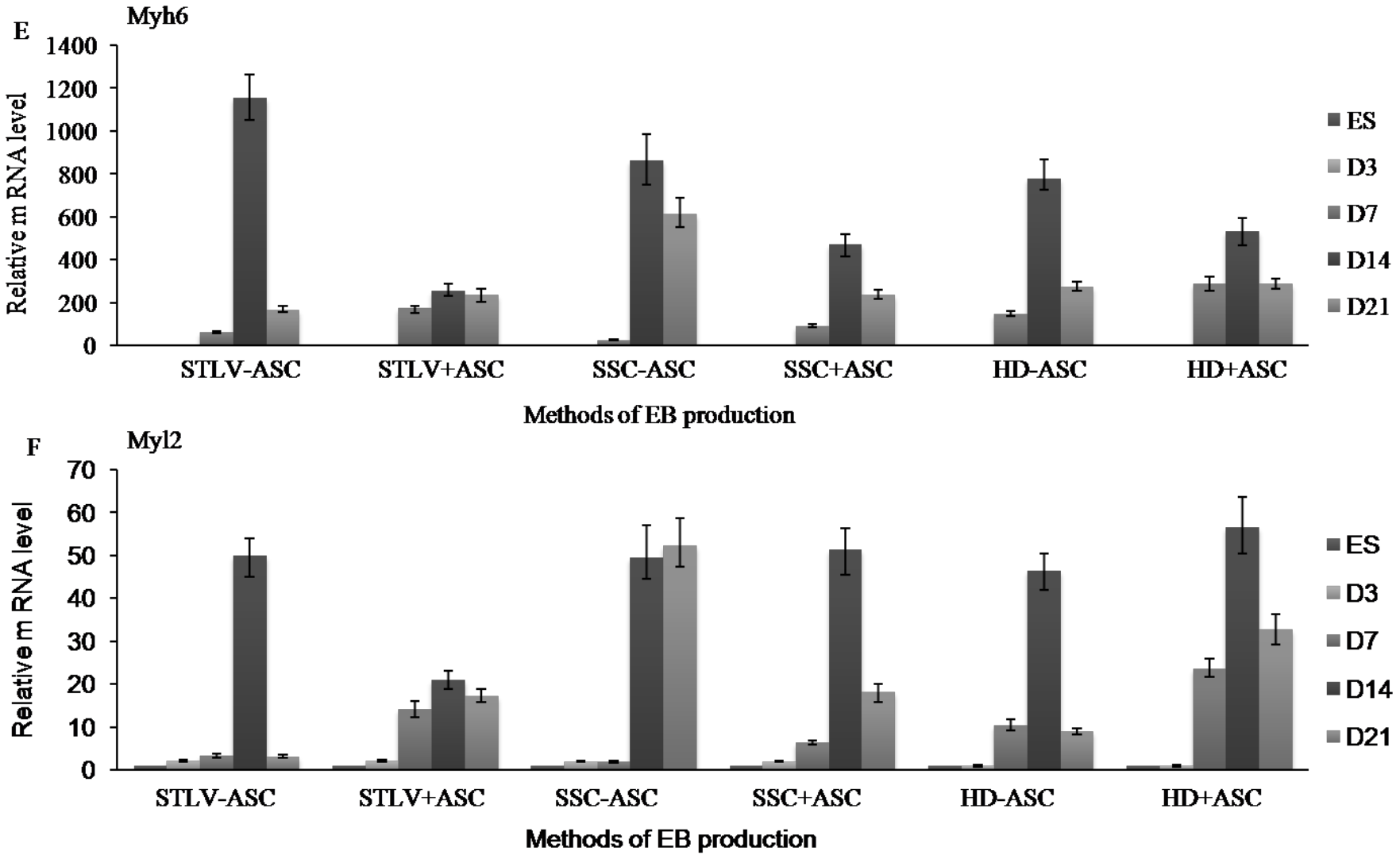


Figure 29. Cardiac differentiation potential of EB derived from different culture method (STLV, SSC and HD) supplement with or without ASC (A) Immunocytochemistry of EBs was stained with cTnT (red) with DAPI (blue). (B) Area of cTnT per EBs ($x10^5$ μm^2). Scale bars represent 200 µm x40magnification.


A
Oct-4
Relative m RNA level
1.2
1
0.8
0.6
0.4
0.2
0
STLV-ASC
STLV+ASC
SSC-ASC
SSC+ASC
HD-ASC
HD+ASC
Methods of EB production
ES
D3
D7
D14
D21
B
Brachyury
Relative m RNA level
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
STLV-ASC
STLV+ASC
SSC-ASC
SSC+ASC
HD-ASC
HD+ASC
Methods of EB production
ES
D3
D7
D14
D21


C
Nkx2.5
Relative m RNA level
20
18
16
14
12
10
8
6
4
2
0
STLV-ASC
STLV+ASC
SSC-ASC
SSC+ASC
HD-ASC
HD+ASC
Methods of EB production
ES
D3
D7
D14
D21
D
Tnnt2
Relative m RNA level
450
400
350
300
250
200
150
100
50
0
STLV-ASC
STLV+ASC
SSC-ASC
SSC+ASC
HD-ASC
HD+ASC
Methods of EB production
ES
D3
D7
D14
D21


E
Myh6
Relative m RNA level
1400
1200
1000
800
600
400
200
0
STLV-ASC
STLV+ASC
SSC-ASC
SSC+ASC
HD-ASC
HD+ASC
Methods of EB production
ES
D3
D7
D14
D21
F
Myl2
Relative m RNA level
70
60
50
40
30
20
10
0
STLV-ASC
STLV+ASC
SSC-ASC
SSC+ASC
HD-ASC
HD+ASC
Methods of EB production
ES
D3
D7
D14
D21

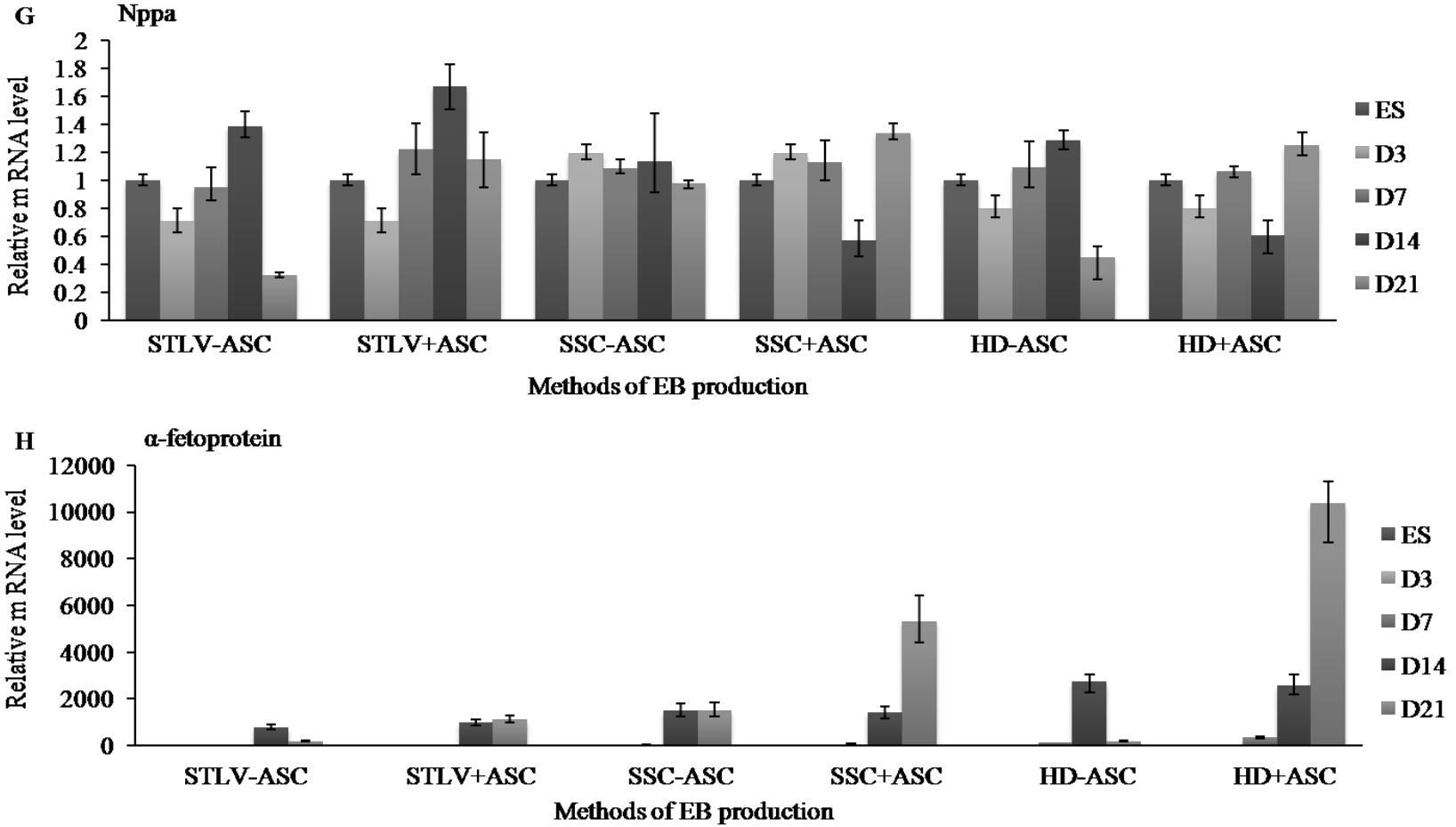


Figure 30. qRT-PCR analysis of gene expressions in STLV, SSC and HD EBs cultured with or without ASC. (A) Oct-4, the pluripotent marker (B) Brachyury, mesodermal marker (C) Nkx2.5, early cardiac marker (D to G) late cardiac marker (D) Tnnt2 (E) Myh6 (F) Myl2 and (G) Nppa (H) α-fetoprotein.

These results further confirmed that three days post-seeding of mouse ES cells, the STLV culture system have higher efficiently for EB production approximately produce EBs from four times more than SSC. Furthermore, in case of homogenous size of EBs, our result was similar as Lü et al. (2008) that STLV EBs had higher homogenous in size than SSC and HD, regardless species of ES cells (mouse or human), source of ES cells (fES or ntES), cell seeding density, rotation speed and the duration of suspension culture. STLV culture provide a stable and ideal condition for mouse ES cells to form highly EB production and retain good quality than other cultures because this culture provides as follows: (i) low fluid shear stress due to whole-body rotation around a horizontal axis, (ii) yielding a vessel devoid of gas bubbles and gas/fluid interfaces due to oxygenation by active or passive diffusion to the exclusion of all but dissolved gasses from the reactor chamber, (iii) mild mixing of cells due to very low rotation speed of STLV, (iv) enabling efficient gas exchange due to the high membrane area to volume of medium ratio. Controversially, Yirme et al. (2008) demonstrated that human STLV EBs had heterogeneous in size as similar as SSC EBs which was different from our results. It appreaded that interrupting stirring and microgravity in STLV when manipulating STLV vessel including changing medium may lead to agglomeration of EBs and formation of aggregates that are too large for optimal growth and differentiation, thereby disturbing the evolution of the early stages of the gastrulation or method of harvest human ES cells. Although proliferating cells in EBs did not difference between groups, STLV and SSC EBs had higher proportion of apoptotic cells in the center of EBs than obtained in HD EBs. Our result supported that high cell seeding density of ES cells inoculated in STLV and SSC culture resulted in a greater number of cells within the EB which were higher than those obtained in HD EB.

Even though HD EBs had rhythmically contracting areas of EB one day earlier than STLV and SSC EBs, the overall ability of cardiac differentiation; lower expression of cTnT and limitation distribute of cTnT detecting by IHC assay were observed in this group. In addition, percentage of cardiac beating colony/well was similarly between STLV EBs and SSC EBs. However, STLV EBs showed a higher efficiency of cardiac differentiation more than SSC EBs including, higher whole area beating and fast beating. Besides, measurement of cTnT area per EB showed that STLV expressed cTnT three to five times higher than SSC and HD, regardless the culture medium. Furthermore, ASC supplementation increased cTnT area per EB in all groups (2.2 times in STLV, 3.4 times in SSC and 1.3 times in HD group). Moreover, ASC supplementation was also induced cardiac differentiation of ES, as similar with Takahashi et al. (2003). Our results were in an agreement with Burridge et al. (2007) who demonstrated that a high degree of heterogeneity in human EB size cultured in SSC. This has been proposed to limit the cardiomyocyte differentiation in all cell lines tested. Interestingly, the beating outgrowth (day seven) from STLV had readily identifiable cellular morphology, even before the onset of beating, and were most commonly located in a round ring shape at the EB perimeter (Burridge et al., 2007).

For further investigate, the aim of the current work was also to study whether the EB formation as well as the temporal and spatial organisation of germ layers takes place in a similar manner during early EB differentiation. Furthermore, we studied whether the differences in EB characteristics, especially mRNA and protein expression patterns, would correlate with cardiac differentiation potential among different culture condition. Our results showed that the level of Oct-4 was high in undifferentiated cells and by day seven, diminished to almost undetectable levels at all

examined samples, agreement with other report (Pekkanen-Mattila, et al., 2010). In contrast, they reported that the Oct-4 mRNA expression still also detected until day 28 (Adewumi, et al., 2007). In parallel, many of the genes that were up-regulated in EBs were phenotypic markers of cardiac gene expression: Nkx2.5, Tnnt2, Myh6, Myl2, Nppa. Thus, Nkx2.5 gene expression, which marks early cardiac precursors (Schwartz et al., 1999; Ranganayakulu et al., 1998) peaked by day seven and stayed high until day fourteen. During mouse embryogenesis Nkx2.5 is expressed abundantly when the heart forms and in standard *in vitro* differentiation assays, peaks at day five to day eight. In our STLV system, the expression of Nkx2.5 seems to be prolonged (i.e. the level is still detectable even at day fourteen), suggesting that cardiac precursors are present even during late time points. One explanation might be that the optimized STLV conditions ensure a gradual and permanent microenvironment and equalized nutrient support achieved by continuous rotation. This may accelerate and stabilize the formation of more stable EBs, with no significant decrease in constituent ES cell viability. Brachyury is a gene crucial to induce early mesoderm formation and is up-regulated in the primitive streak of the developing embryo (Dobrovolskaia-Zavadskaia et al., 1927). As expected, the levels of Brachyury mRNA were elevated at the beginning of differentiation (day three to day seven; Figure 30B) and showed decaying expression thereafter. The transcript level of Brachyury usually peaks at day three to day four in *in vitro* cardiac differentiation assays, then decreases thereafter. In our assays, the level of Brachyury decreased gradually without any peak derived from STLV and SSC EBs, suggesting there were no subsets of cells in our STLV and HD derived EBs entering the primitive streak-stage but rather, cells within EBs gradually became engaged towards different lineages (e.g. cardiac). On the other hand, Brachyury reached its peak around day three similar to earlier studies in HD method (Bettiol et al., 2007; Graichen et al., 2008; Pekkanen-Mattila et al., 2010). Our results confirmed that the culture condition affect the Brachyury mRNA expression.

In agreement with this, at the later time point of spontaneous cardiac differentiation, cardiac markers became prominent. The expression of Tnnt2, Myh6 and Myl2 (which are cardiomyocyte structural constituents) peaked by day fourteen. Atrial natriuretic peptide Nppa, encoded by Nppa, regulates multiple ion channels in atrial cardiomyocytes and in our study, was also highly expressed and peaked by day fourteen. These observations were found to be similar as previously reported (Boheler et al., 2002). At the mRNA level of these genes, we did not see any correlation with the cardiac differentiation, irrespective the different culture methods and the present of ASC.

In conclusion, we have demonstrated that the optimized STLV bioreactor method provides satisfactory control over the agglomeration process of EBs, therefore enabling cell proliferation and differentiating ES cells towards cardiomyocytes. The culture condition methods of EB production and quality of EBs affected the cardiogenesis. The ability to produce homogeneous populations of EBs of particular sizes also increases the differentiation potential of ES cells into cardiomyocytes. The establishment of this cultivation system provides a powerful research and clinical tool for several fields such as disease modeling and cardiac tissue engineering, as well as regenerative medicine applications or drug screening tests. This methodology could also be employed to expand our understanding of intrinsic EB properties and their effect on cell differentiation and lineage commitment.

CHAPTER V

SCALABLE PRODUCTION OF CARDIOMYOCYTES DERIVED FROM MOUSE INDUCED PLURIPOTENT STEM CELLS BY USE OF THE SLOW TURNING LATERAL VESSEL (STLV) BIOREACTOR

5.1 Abstract

Patient-specific cardiomyocytes derived from iPS cells hold great promise as an unlimited cell source for autologous cardiovascular cell-replacement therapies and drug screening. Before applying this technique for clinical and industrial application; however, reproducible methods must be developed to establish efficient, safe and scale-up iPS cell differentiation to produce clinically relevant numbers of cardiomyocytes. Here, we describe and characterize a scenario method of EB formation in hydrodynamic conditions using STLV bioreactor and subsequent differentiation of different iPS cells into cardiomyocytes. The efficiency of the dynamic process compared to ones derived by the well established HD was analyzed with respect to the morphology, size of EB formation and the structural and molecular properties of the cardiomyocytes. EBs derived from STLV are homogenous in size and they show a proliferative outer zone and an apoptotic inner cell mass which corresponds to the embryonic development, respectively. Similar gene expression pattern was observed by the two methods: both STLV and HD produced. EBs were proven to express pluripotent markers, mesoderm markers, cardiac transcription factors, and cardiomyocyte structural genes based on qRT-PCR analysis of RNA samples from different timepoints of cardiac differentiation. Interestingly, the percentage of rhythmically beating cardiomyocyes and the corresponding area of cTnT were higher in EBs derived from STLV bioreactor than HD culture. Our study describes, for the first time, a strategy for scalable differentiation of iPS cells into cardiomyocytes in STLV bioreactor culture system. Our study defines that STLV bioreactor can be potentially used for large-scale production of cardiomyocytes for drug testing and for further clinical and industrial applications.

5.2 Introduction

Heart disease such as myocardial infarction and cardiomyopathy are irreversible and incurable by current therapeutic as a result of the lack of proliferative and terminal of cardiac differentiation soon after birth (MacLellan and Schneider, 2000). One of novel therapeutic strategy is therapeutic cell transplantation and replacement by using the emerging technologies of stem cells, cell therapy, and tissue engineering to repopulate the injured heart and to restore cardiac function (Hassink et al., 2003). Even though, these types of transplantation are promising including fetal and neonatal cardiomyocytes and bone marrow-derived cells; the cell-based cardiac repair strategy has been completely hampered by the lack of suitable cell sources for human cardiomyocytes (Xu et al., 2002).

Currently, an alternative method named as “iPS cells technology” is gaining popularity, which is based on the derivation of pluripotent stem cells by genetic reprogramming of somatic cell types. This was achieved by the exogenous expression of a combination of four transcription factors, namely Oct-4, Sox2, c-Myc and Klf4, into adult somatic cells (Takahashi and Yamanaka, 2006; Takahashi et al., 2007; Hochedlinger and Plath, 2009). The main advantages of iPS cells are: (i) their pluripotency resembles that of ES cells and (ii) they are autologous, therefore,

histocompatible, obviating the need for prolonged immunosuppressive therapy in the context of cell transplantation (Kiskinis and Eggan, 2010).

In spite of the robust gene transfer with retroviral and lentiviral vectors, an alternative approach would still be desirable for safer generation of iPS cells. A specific risk associated with the use of viral vectors for generating iPS cells relates to the ectopic expression of the delivered transcription factors in the progeny of the reprogrammed cells. Hence, the tumorigenic potential occurrence in offspring associated with oncogene reactivation derived from potentially oncogenic transcription factors such as c-Myc or Klf4 reprogramming factors is a main drawback of therapeutic applications. In addition, insertional mutagenesis and the alterable expression pattern of nearby genes because of retroviral integration itself, might limit clinical application of iPS cells generated by introducing with integrating viruses (Stadtfeld and Hochedlinger, 2010). Hence, it is necessary to establish transgene integration–free iPS cells for their future therapeutic application. To solve this problem, one of methods, namely sleeping beauty (SB) transposon, is paving the way toward novel clinically relevant gene and cell-base therapy strategies (Wilber et al., 2007). The SB transposition, a cut-and-paste procedure, is the element ‘‘jumps’’ from one DNA molecule to another. This transposon is able to integrate into chromosomes and afterwards can be excised by a subsequent transposon system. Therefore, the generation of iPS cells by using the SB transposon is safer compared to retroviral and lentiviral vectors as only a few base pair is left behind the integration site in the genome (VandenDriessche et al., 2009). Despite these advances, there are still some challenges that would need to be addressed as well as the safe, and the efficient differentiation into different cell lineages. Furthermore, it will be important to analyze and develop the scale-up protocol for the efficient and high throughput differentiation into all cell lineages (Zwi-Dantsis et al., 2011). Hence, iPS cell lines derived from non-viral transfection of a SB transposon, which comprises the coding sequences of Oct4, Sox2, c-Myc and Klf4, were utilized in this current study.

At the present time, patient-specific cardiomyocytes derived from the iPS technology provides unique opportunities for an unlimited cell source for autologous cardiovascular cell-replacement therapies, individualized drug screening and toxicity testing and disease modeling in human. Before applying this technique for clinical and industrial application, reproducible methods must be developed to improve efficient, safety and scale-up iPS cell differentiation to produce clinically relevant numbers of cardiomyocytes. Here, we describe and characterize a scenario method of EB formation in hydrodynamic conditions using STLV bioreactor and subsequent differentiation of mouse iPS cells into cardiomyocytes. The aimed of this study were: (i) to establish a strategy for scalable production of iPS cell derived cardiomyocytes using the STLV bioreactor (ii) to characterize the structural and molecular properties of the cardiomyocyte derived from STLV vs. well-established HD culture methods; (iii) to compare the differentiation ability of various mouse iPS cell lines (generated by using SB transposon) to cardiomyocytes and (iv) to compare the effect supplementary ASC in the culture medium on cardiac differentiation of iPS cells.

5.3 Materials and methods

Materials and methods of this study were followed as materials and methods of chapter III (pg. 33 to 35) except specific details which were described below.

5.3.1 Pluripotent cell lines

The following mouse iPS cell lines were used: A4-iPS, B3-iPS and B5-iPS cell lines from C57BL/6 genetic background; passages 8-12, kindly provided by Suchitra Muenthaisong) which were generated by SB mediated transoposon sytem harboring pluripotency gene cassette of Oct-4, Sox2, c-Myc and Klf4 genes.(Muenthaisong et al., 2011). ES cell line ($H3^{TgNkx2.5/\ EGFP}$-ES) was used as a reference cell line for the experiments which were targeted with the cardiac specific mouse Nkx2.5 promoter driven enhanced fluorescent green protein (EGFP) (C57BL/6 genetic background; passages 10-12) which were used for control (Rungarunlert et al., 2011).

5.3.2 Differentiation methods of pluripotent cells

The optimized STLV protocol for optimal size and EB quality consequent cardiac differentiation was followed as chapter III. In brief, $3x10^5$ cells/ml of inoculating cell seeding density and 10 rpm rotary speed and plating of EBs onto gelatin-coated surfaces three days after culture were utilized. At day three of differentiation, EBs derived from HD and STLV groups were collected for evaluating gross morphology, size, yield and histological analysis and then were plated on 0.1%-gelatin-coated culture dishes for evaluating the efficiency of cardiac differentiation.

5.3.3 Differentiation of pluripotent cells into cardiomyocytes

Pluripotent cells differentiation into cardiomyocytes was describe as chapter IV (pg. 51).

5.3.4 Quantitative real-time polymerase chain reaction (qRT-PCR)

QRT-PCR was evaluated as chapter IV (pg. 51). However, PCR-related primers and reaction conditions were described in Table 5.

5.3.5 Statistical analysis

Data are expressed as mean ± SEM and include at least three independent experiments. Statistical analyses were conducted using the Student's *t* test or the General Linear Model (GLM) procedures of SAS. *P*-value $<$ 0.05 will be considered statistically significant.

5.3.6 Experimental design (Figure 31)

Experiment 1: To establish a strategy for scalable production of iPS derived cardiomyocytes using a STLV bioreactor and characterize the structural and molecular properties of the cardiomyocytes generated in STLV in comparison to the well-established static HD culture method. Three mouse iPS cell lines (A4-iPS, B3-iPS and B5-iPS; generated by using SB transposon) and an ES cell line ($H3^{TgNkx2.5/\ EGFP}$-ES) were utilized. The efficiency of EB formation and cardiomyocyte differentiation was evaluated. At least three independent replicates were examined.

Experiment 2: To compare differentiation of different iPS cell lines (A4-iPS, B3-iPS and B5-iPS; generated by using SB transposon) and ES cell line ($H3^{TgNkx2.5/\ EGFP}$-ES). HD method was used for producing EB. The efficiency of EB formation and cardiomyocyte differentiation was evaluated. At least three independent replicates were examined.

Experiment 3: To compare the effect of culture medium supplemented by ASC on cardiac differentiation of iPS cells. Three mouse iPS cell lines (A4-iPS, B3-iPS and B5-iPS; generated by using SB transposon) and an ES cell line ($H3^{TgNkx2.5/\ EGFP}$-ES) were utilized. The cells were cultured and differentiated either spontaneous or induced manner in order to produce cardiomyocytes. The efficiency of EB formation and cardiomyocyte differentiation was evaluated. At least three independent replicates were examined.

5.4 Results

5.4.1 Differentiation of iPS cells by using the HD method

In this study, three iPS cell line (A4-iPS, B3-iPS and B5-iPS) were generated by the SB transposon of four transcription factors (Oct-4, Sox2, c-Myc and Klf4) and an ES cell line ($H3^{TgNkx2.5/\ EGFP}$-ES) were targeted with the cardiac specific mouse Nkx2.5 promoter driven EGFP which were utilized (Muenthaisong, et al., 2011; Rungarunlert et al., 2011). Differentiation of these cell lines into cardiomyocytes was initially achieved, using the HD method, traditionally used for differentiation of mouse ES cells. The iPS cells were cultivated in 20 µl/drop (each drop containing 800 cells) for two days. During this period, three-dimensional differentiating cell aggregates (EBs) were formed. Subsequently, the EBs were transferred to bacterial-grade dishes for another one day in suspension, where they formed larger EBs (Figure 32). The EBs were then plated on gelatin-covered plates. Spontaneously beating areas appeared in EBs beginning from day two to four post-plating.

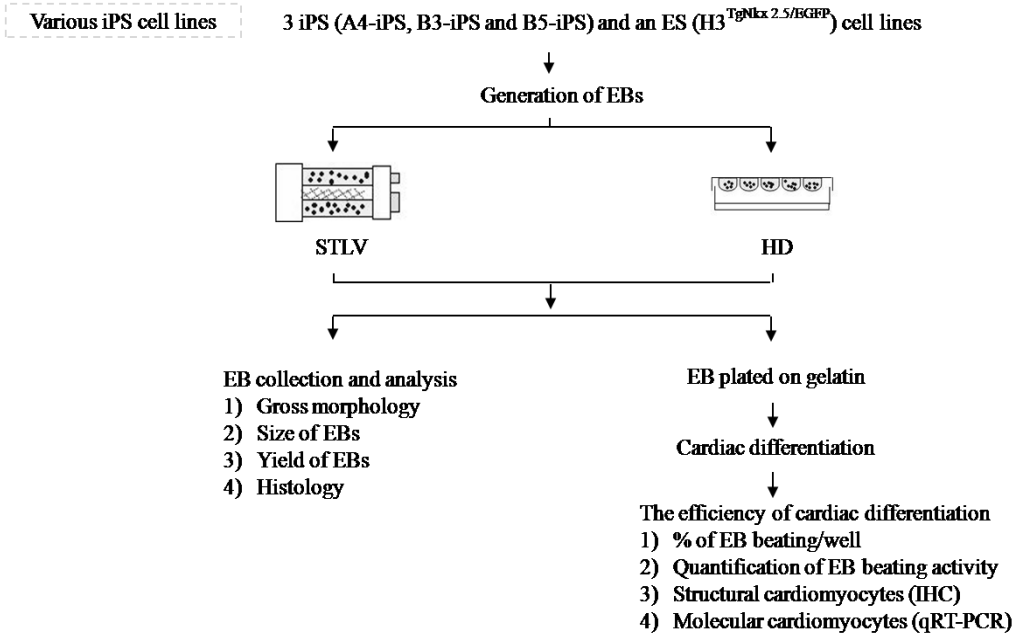


Figure 31. Schematic presentation of *in vitro* differentiation procedure of pluripotent cell lines into cardiomyocytes in STLV bioreactor culture and HD culture

Table 5. Properties of the oligonucleotide primers used in qRT-PCR reaction

Primer	Size (bp)	Sequence
Oct-4	110	(Forward) 5'- AGCCGACAACAATGAGAACC -3'
		(Reverse) 5'- TCTCCAGACTCCACCTCACA -3'
Klf4	140	(Forward) 5'- ACTCACACAGGCGAGAAACC -3'
		(Reverse) 5'- AAGGCCCTGTCACACTTCTG -3'
c-Myc	152	(Forward) 5'- GAATTCGGCTGGATTTCCTTTGG-3'
		(Reverse) 5'- ATTGGTCCTCAAGAGACAGGTT-3'
Sox2	112	(Forward) 5'- ACCAGCTCGCAGACCTACAT -3'
		(Reverse) 5'- CCTCGGACTTGACCACAGAG -3'
Brachyury-T	161	(Forward) 5'- TACACCTCTAATGTCCTCCCTTG -3'
		(Reverse) 5'- CCATACAGTTGACTTCCCAACAC -3'
Nkx2-5	74	(Forward) 5'- ACACCCACGCCTTTCTCAGT -3'
		(Reverse) 5'- AGGTCCCCAGACGCCA -3'
Gata4	134	(Forward) 5'- CAGCAGCAGTGAAGAGATGC -3'
		(Reverse) 5'- CAGACAGCACTGGATGGATG -3'
Gata6	126	(Forward) 5'- ACAGCCCACTTCTGTGTTCC -3'
		(Reverse) 5'- GGGACTGTGTTGGTGTTCTTG -3'
Tnnt2	117	(Forward) 5'- CGGATCAATGACAACCAGA -3'
		(Reverse) 5'- GGGCAAGGACACAAGCAG -3'
Tnni3	121	(Forward) 5'- GGTGGACAAAGTGGATGAAGAG -3'
		(Reverse) 5'- GGTGGGCCGCTTAAACTTG -3'
Myh6	80	(Forward) 5'- CTACGCCTTCGTCTCTCAGG -3'
		(Reverse) 5'- AGGCACTATCAGTGGCCAAG -3'
Myl2	90	(Forward) 5'- TGACCTAAGGGACACATTTGC -3'
		(Reverse) 5'- ATTGGACCTGGAGCCTCTTT -3'
α-fetoprotein	129	(Forward) 5'- AACAAGGAGGAGTGCTTCCA -3'
		(Reverse) 5'- GGTTGTTGCCTGGAGGTTT -3'
Nestin	176	(Forward) 5'- GGTCTTCCCCTCGAATCTCTC-3'
		(Reverse) 5'- GATCCAGGCAGCTCCCATT-3'
Gapdh	79	(Forward) 5'- AATGTGTCCGTCGTGGATCT -3'
		(Reverse) 5'- CCTGCTTCACCACCTTCTTG -3'

5.4.2 Effect of EB culture condition on *in vitro* EB formation: comparison of multiple cell lines

Next, we designed a strategy for scaling-up the differentiation procedure using STLV cultures. Undifferentiated iPS and ES cells were removed from the MEF feeder, trypsinized into single cells, and cultivated in STLV at a concentration of $3x10^5$ cells/ml. The cultures were stirred at 10 rpm. Inoculation A4-iPS, B5-iPS and $H3^{TgNkx2.5/\ EGFP}$-ES cells in STLV cultures were started to aggregate at 18 h and formed EBs at 36 h early than inoculation B3-iPS cells in STLV culture which were started to aggregate at 36 h and formed EBs at 48 h. All STLV EBs on day three increased slightly in size from day two and still remained regular and spherical with no appearance of agglomeration. In HD group, all EBs were started to aggregate at 12 h and formed EBs at 24 h; however, three to four EBs were present in one drop. One EB/one drop was appeared at 48 h (Figure 32). Besides, these EBs derived from STLV bioreactor (STLV EBs) revealed relatively homogenous and round EBs at day three of cultivation. Additionally, EB size were not different from HD EBs (STLV

EBs; H3$^{TgNkx2.5/\ EGFP}$-ES, 356±47; A4-iPS, 328±37; B5-iPS, 312±69 µm vs. HD EBs; H3$^{TgNkx2.5/\ EGFP}$-ES, 371±42; A4-iPS, 269±22; B5-iPS, 256±31 µm) (Figure 32 and 33). To provide some quantitative information regarding the efficiency of this process, we counted the number of generated EBs. We found that the average number of EBs that can be obtained in STLV is 22±1.5, 19±3.5 and 17±3 EBs/ml for an initial dose of $3x10^5$ cells/ml (H3$^{TgNkx2.5/\ EGFP}$-ES A4-iPS and B5-iPS, respectively). There were no different of EB production between two iPS cell lines; namely A4-iPS and B5-iPS. Moreover, those iPS cell lines showed the efficiency of EB formation similar to the ES cell lines (H3$^{TgNkx2.5/\ EGFP}$-ES) (Figure 34).

Interestingly, an iPS cell lines, namely B3-iPS was different from A4-iPS, B5-iPS and H3$^{TgNkx2.5/\ EGFP}$-ES in case of EB formation. Even thought B3 iPS cell line started to form EB similar to A4-iPS and B5-iPS cell lines in HD culture. However, this cell line started to form EB later than these cell lines in STLV bioreactor (48 h after inoculation). In addition, B3-iPS formed EBs in STLV bioreactor with smaller size than that in HD culture (STLV EBs, 148.2±20.83 µm vs. HD EBs 269±18 µm) (Figure 33). Inoculation B3-iPS cells in STLV were produced higher EB formation (62±11.5 EBs/ml) with smaller size compared to other cell lines (Figure 34).

Our result demonstrated that STLV bioreactor can product homogenous in size of iPS cells, similarly to the HD method and that B3 iPS cell line behaved differently from the rest of iPS and ES cell lines.

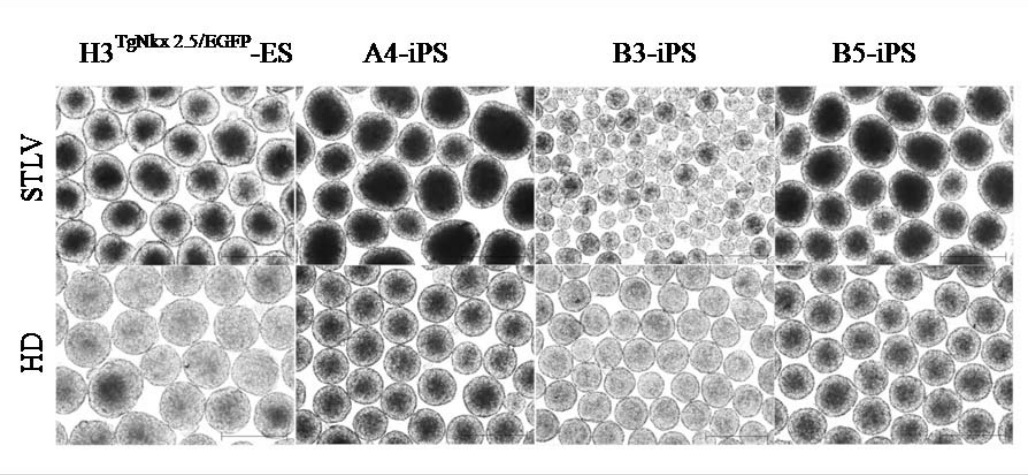


Figure 32. Gross morphology of EBs derived from STLV and HD culture conditions. Scale bars represent 500 µm x 4magnification.

5.4.3 Effect of EB culture condition on cell viability within EBs: comparison of multiple cell lines

To elucidate possible causatives of differences in cell yield among different pluripotent cell lines of STLV and HD EBs, cell morphology, proliferation, and apoptosis assays were performed. Gross morphology and H&E staining of serially sectioned of EBs revealed that there were no obvious morphologic differences among all examined cell lines (STLV vs HD) at day three except B3-iPS cell line (Figure 35).

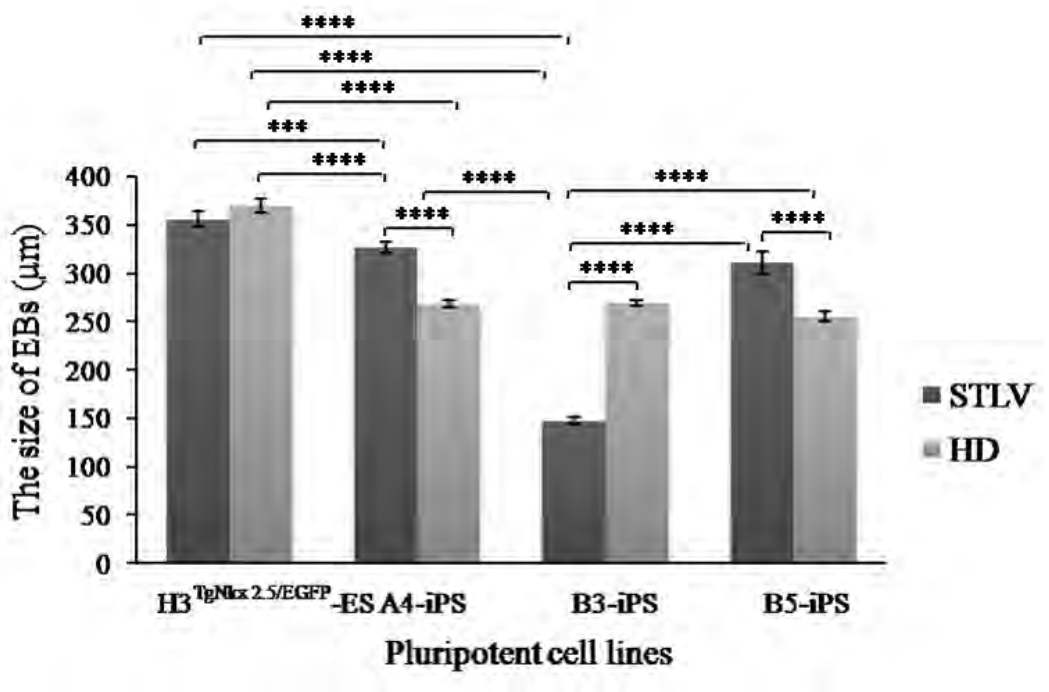


Figure 33. The size of EBs derived from STLV and HD culture conditions (the mean of the average diagonals with SEM). The difference was considered significant when $p<0.001$;***: $p<0.0001$;****.

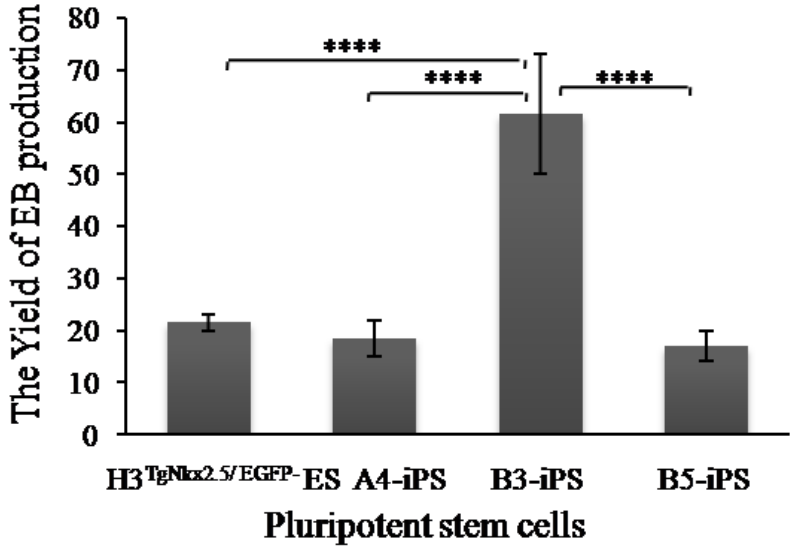


Figure 34. The yield of EB production is given in number of EBs/ml derived from different iPS cell lines culture in STLV. Data presented as mean ± SEM. The difference was considered significant when a $p<0.0001$;****.

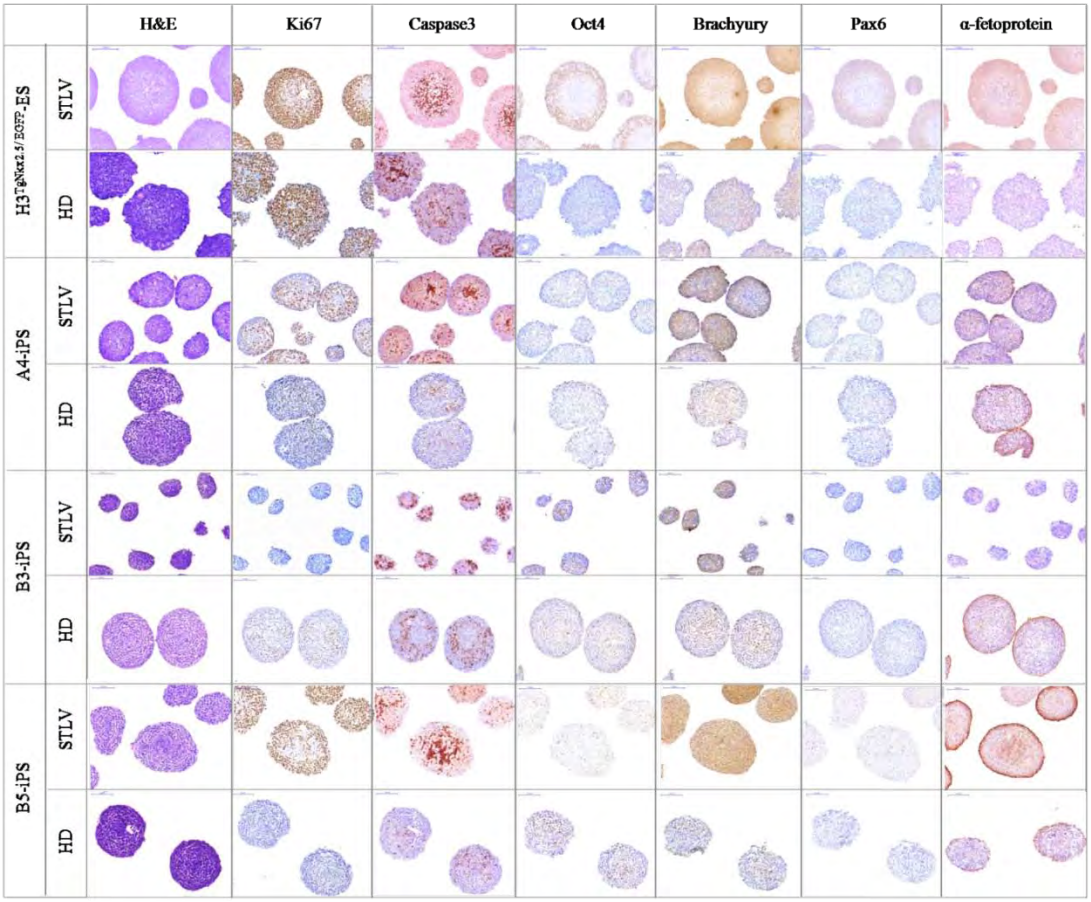


Figure 35. Histological section of EB formation (H&E staining and stainings: for the proliferation marker Ki-67; apoptosis marker cleaved-caspase 3; pluripotency marker Oct- 4; mesoderm marker Brachyury-T; ectoderm marker Pax6 and endoderm marker α-fetoprotein). Scale bars represent 200 µm x20 magnification.

Ki67 staining was performed to detect proliferating cells within EBs during the differentiation process. Our results revealed that high levels of proliferating cells (Ki 67 positive; brown stain) were detected in $H3^{TgNkx2.5/\ EGFP}$-ES culturing in both STLV and HD culture. Besides, A4-iPS and B5-IPS cultured in STLV showed higher levels of proliferating cells than those one cultured in HD condition. However, B3-iPS cell line cultured in HD showed higher levels of proliferating cells than those one cultured in STLV condition. Furthermore, iPS EBs contained less proliferating cells than EBs from control ES cell line (Figure 35).

Cleaved-caspase 3 staining (Caspase 3 positive; red stain) for detection of apoptotic cells revealed slight differences between STLV and HD produced EBs. STLV EBS started to form a dense apoptotic core in the middle of EBs and earlier than HD. HD EBs contained sparse apoptotic zones within EBs. Both EBs derived from STLV and HD groups had proliferative cells but also apoptosis cells in center of EBs (Figure 35). No different pattern were observed between different culture condition (STLV vs HD), different pluripotent stem cell lines (iPS vs ES) EBs and different iPS cell lines (A4-iPSand B5-iPS except specially B3-iPS cell line (Figure 35).

5.4.4 Effect of EB culture condition on pluripotency and germ layer marker expression during differentiation within EBs: comparison of multiple cell lines

To evaluate different protein expression during EB formation among different culture method and different cell lines, EB were collected on day three. Protein expression in the sections from the differentiating EBs was assessed by IHC staining with pluripotency marker: Oct-4, and with different germ layer markers (mesodermal: Brachyury, endodermal: α-fetoprotein and ectodermal: Pax6) (Figure 35). Additionally, qRT-PCR was used to assess the mRNA expression levels of the same markers and to confirm the IHC data. Moreover, the expression of cardiac lineage markers was also evaluated (Figure 38).

Our result showed that the Oct-4 mRNA expression level of $H3^{TgNkx2.5/\ EGFP}$- ES cells started to decrease after the initiation of EB differentiation in HD but not in STLV group (Figure 38A). This was also confirmed by detecting the protein expression for Oct-4 which was invisible in HD EB section but visible in STLV EB section (Figure 35). As expected, the Oct-4 mRNA expression level of all iPS ES cells was higher than those of $H3^{TgNkx2.5/\ EGFP}$- ES cells due to exogenous expression of the pluripotency cassette harboring Oct-4. The Oct-4 mRNA expression level of A4-iPS and B5-iPS cells started to decrease after the initiation of EB differentiation. Our results also revealed that Oct-4 mRNA expression of A4-iPS and B5-iPS cells in HD was lower than those one in STLV. Again, B3-iPS cell line showed a unique pattern: higher Oct-4 mRNA level of EB on day three than iPS cells (Figure 38A). However, no difference in Oct-4 protein expression of EB section was detected among different iPS EBs on day three (Figure 35).

The expression level for Brachyury mRNA started to increase after the initiation of EB differentiation at line A4-iPS and B5-iPS in the HD group (Figure 38). Nonetheless, the protein expression for Brachyury was occasionally visible in all EB section on day three. The highest expression level was seen in all of the EBs derived from STLV groups (Brachyury positive; brown color, Figure 35). No difference in the protein expression for Brachyury was observed among different pluripotent cell lines (Figure 35). The protein expression of Pax 6 was notably low in all the EB sections (Figure 35). The α-fetoprotein mRNA expression level of $H3^{TgNkx2.5/\ EGFP}$- ES cells cultured in STLV was higher than in HD (Figure 38M) which is in an agreement with the protein expression of the gene (Figure 35). In contrast, B5-iPS ES cells cultured in HD exhibited higher α-fetoprotein mRNA expression level than these cultured in STLV (Figure 38M). The mRNA expression level of the cardiac marker genes including Nkx2.5, Tnnt2, Myh6 and Myl2 were not detectable at any EBs on this day except B5-iPS cells (Figure 38 F, 38I, 38K and 38L).

5.4.5 Effect of EB culture condition on *in vitro* cardiac differentiation: comparison of multiple cell lines

Individual EBs, produced in STLV and HD, were plated onto gelatin-coated dishes and assayed for in spontaneous cardiomyocyte differentiation. Differentiated EBs were cultured for 21 days and observed daily under a bright-field microscope. Forty-eight EBs were counted for each treatment group and three replicates were analyzed. The number of beating EBs is presented as a percentage of the total number of EBs plated (Figure 36A to 36E).

Similarly to the HD method, following plating of the EBs on gelatin-coated plates most of the EBs did not retain their round three-dimensional shape, but rather spread out on the culture plate. Beating areas appeared mainly in the outgrowths of

the aggregates but also occasionally at the center of the mass as well. The percentage of plated EBs containing beating areas continued to increase with time until reaching a maximal number at day ten to day fourteen post-plating.

Contracting EBs of the $H3^{TgNkx2.5/\ EGFP}$- ES cell line began to appear as early as day six when cultured in HD and as early as day seven when cultured in STLV (Figure 36A). However, the $H3^{TgNkx2.5/\ EGFP}$- ES cells culturing in HD never reached the 100% efficiency for beating: they formed beating cardiomyocytes with maximum 90% efficiency and only cardiomyocytes derived from STLV reached the 100% efficiency (Figure 36A). Contracting EBs of all iPS cell lines began later than $H3^{TgNkx2.5/\ EGFP}$- ES cells (Figure 36A to 36D). The pattern for A4-iPS and B5-iPS cell lines were similar and independent whether they whether derived from HD or STLV culture conditions (Figure 36B to 36C). In contrast, B3-iPS cell lines showed delay of contracting EB (no beating EBs until day thirteen) and exhibited low efficiency of cardiac differentiation (38% of EB were beating) (Figure 36D).

The quantity of cardiac beating was also evaluated by grading of cardiac beating (Figure 36E). Grading was performed based on the size of the beating area and the rate of beating. The grading revealed that $H3^{TgNkx2.5/\ EGFP}$- ES cells cultured in STLV had the highest number and fastest beating contractile areas (Grade 4), higher than the ones cultured in HD EBs, in contrast to iPS cells. Notably, B3-iPS EBs could also form cardiomyocytes, however scored lower, according to the grading scale, compared to other iPS cell lines.

To evaluate further the ability of control ES and different iPS cells in order to form cardiomyocytes, outgrowths were immunostained with cTnT antibody. This marker is used to visualize late cardiac lineage commitment in cell culture. Our results demonstrated that the $H3^{TgNkx2.5/\ EGFP}$- ES cell line cultured in STLV is superior for producing cardiomyocytes based on the presence of an extensive and filamentous cTnT network in both central and peripheral parts of the attached EBs (Figure 37A) and has the highest area of cTnT positive cells (Figure 37B). Our result also revealed that there is a strong variability among different iPS cell lines. A4-iPS showed the highest the presence of an extensive and filamentous network of cTnT (Figure 37A), confirming by cTnT positive areas (Figure 37B). Additionally, B3-iPS cell line which cultured in STLV wasinferior for producing structural cardiomyocytes (no positive cTnT area was present) (Figure 37A). Beside, our result demonstrated that differentiation of all iPS cells into cardiomyocytes was lower in efficiency then compared to ES cells. Oct-4 expression was still observed in all iPS cardiac outgrowth on day fourteen but was already absent in $H3^{TgNkx2.5/\ EGFP}$- EBs. B3- iPS EBs showed strong expression of Oct-4 in all area of the outgrowth. B5- iPS EBs had higher of Oct-4 expression than A4-iPS EBs, related with the low efficiency of cardiac differentiation and cTnT expression than another one.

5.4.6 Effect of ascorbic acid supplement on *in vitro* cardiac differentiation: comparison of multiple cell lines

To investigate the effect of ASC supplement for inducing cardiac differentiation, individual EBs, produced in STLV and HD, were plated onto gelatin-coated dishes and cultured in either spontaneous (without ASC supplement) or in induced cardiomyocyte differentiation medium (with ASC supplement). We found that ASC supplementation improved cardiac differentiation in all EB groups, regardless the method of EB production (HD vs. STLV) (Figure 36A to 36E and 37A and 37B). Induced cardiac differentiation by adding ASC increased the percentage of cardiac beating. Cells induced by ASC resulted the highest number and fastest beating

contractile areas and increased the presence of an extensive and filamentous network of cTnT in central and peripheral of EBs areas.

5.4.7 Effect of EB culture condition on gene expression during cardiac differentiation: comparison of multiple cell lines

To further characterize the differentiation process of undifferentiated iPS cells and of ES cells in both the STLV and HD methods qRT-PCR were performed (Figure 38). The choice of genes was dependent on the fact that early mesendodermal progenitors generate mesoderm, cardiac mesoderm, and cardiomyocytes. Genes mark endodermal lineages help to visualize vital molecular signals for mesoderm formation which is critical step for cardiac lineage commitment. Ectodermal marker genes help to investigate whether those cell were differentiated into ectoderm lineage.

To control for the presence of different cellular intermediates and key regulators and to identify mature cardiomyocytes, we included a series of marker genes in the study: Oct-4, Klf4, Sox2 and c-Myc expression indicates the presence of undifferentiated cells and these factors were exogenously introduced to all iPS cell lines. Brachyury was chosen as mesodermal marker. GATA4, GATA6 and Nkx2.5 were included as markers for early cardiac mesoderm. In addition, cTnT (encoding by TNNT2 gene), cardiac Troponin I muscle (cTnI; encoding by Tnni3 gene), α-MHC (encoding by Myh6 gene), MLC2v (encoding by Myl2 gene), and served as markers for cardiomyocytes. Moreover, α-fetoprotein (endoderm lineage markers) and Nestin (ectoderm lineage markers) were also investigated.

The expression of the pluripotency genes; Oct-4, Klf4, Sox2 and c-Myc was greatly decreased in $H3^{TgNkx2.5/\ EGFP}$- ES cells, A4-iPS and B5-iPS during differentiation. In contrast, the expression of these pluripotency genes was up-regulated in B3-iPS cells during differentiation. The Oct-4 and Klf4 mRNA expression of B3-iPS was up-regulated on day three and day seven during culture and started to be down-regulated on day fourteen. At later, on day 21, it was still detectable (Figure 38A to 38B). In addition, Sox2 and cMyc mRNA expression of B3-iPS cell line was up-regulated on day three and started to be down-regulated on day seven, then was still detectable on day 21 (Figure 38C to 38D).

These show that different culture conditions may affect pluripotency gene expression. The Oct-4 and Sox2 mRNA expression of $H3^{TgNkx2.5/\ EGFP}$- ES cells was up-regulation in STLV culture but was down-regulated in HD culture. In the case of iPS cells, Oct-4 and Sox2 mRNA expression was up-regulated in HD but down-regulated in STLV culture conditions of A4-iPS and B5-iPS cells (Figure 38A to38C).

The first event related to cardiomyogenesis is the up-regulation of mesoderm marker Brachyury which was up-regulated on the day three of differentiation, then subsequently decreased (Figure 38E). Our result suggested that different culture conditions may affect the kinetic of Brachyury expression. Brachyury mRNA expression was early detected at day three in HD culture vs day seven in STLV culture. Moreover, ASC supplement improved the efficiency of cardiac differentiation by increasing the Brachyury mRNA expression (Figure 38E).

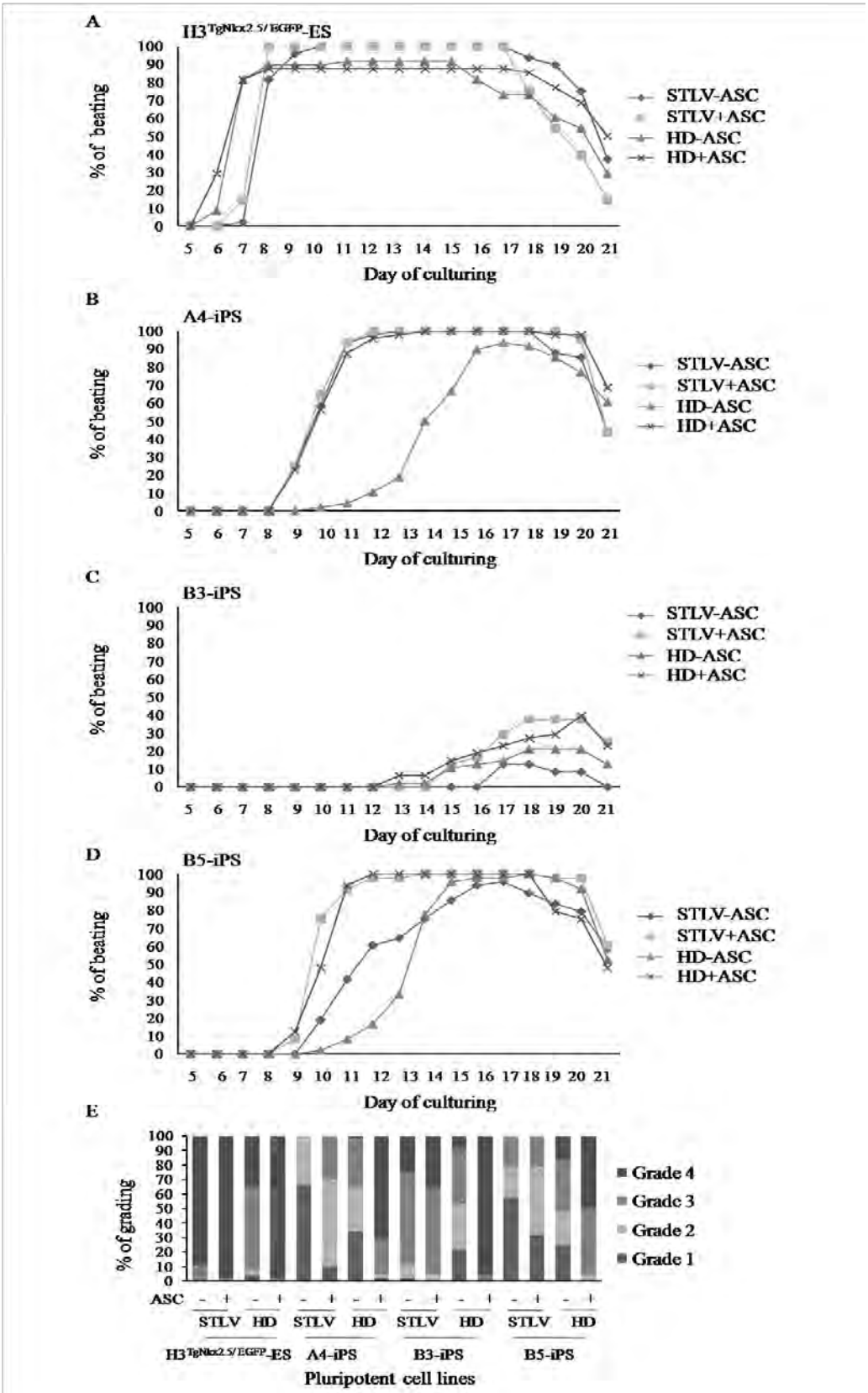


Figure 36. The efficiency of cardiac differentiation derived from different pluripotent cell lines. Percentage of contracting cardiomyocytes in outgrowth of attached EBs with or without ASC supplement: (A) H3-ES (B) A4-iPS, (C) B3-iPS and (D) B5-iPS. (E) Cardiac beating activity was evaluated by grading the area of beating foci and the beating rate.

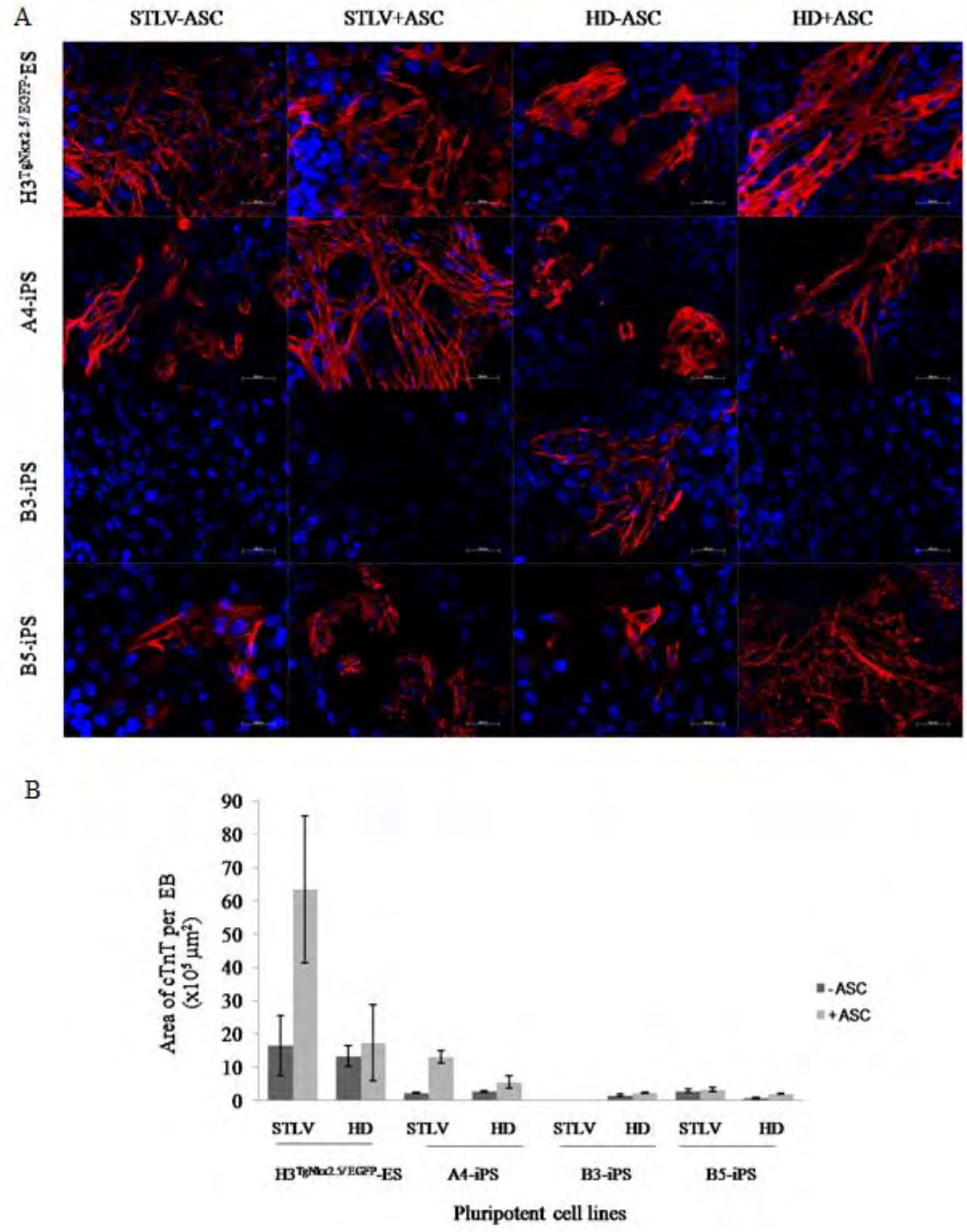
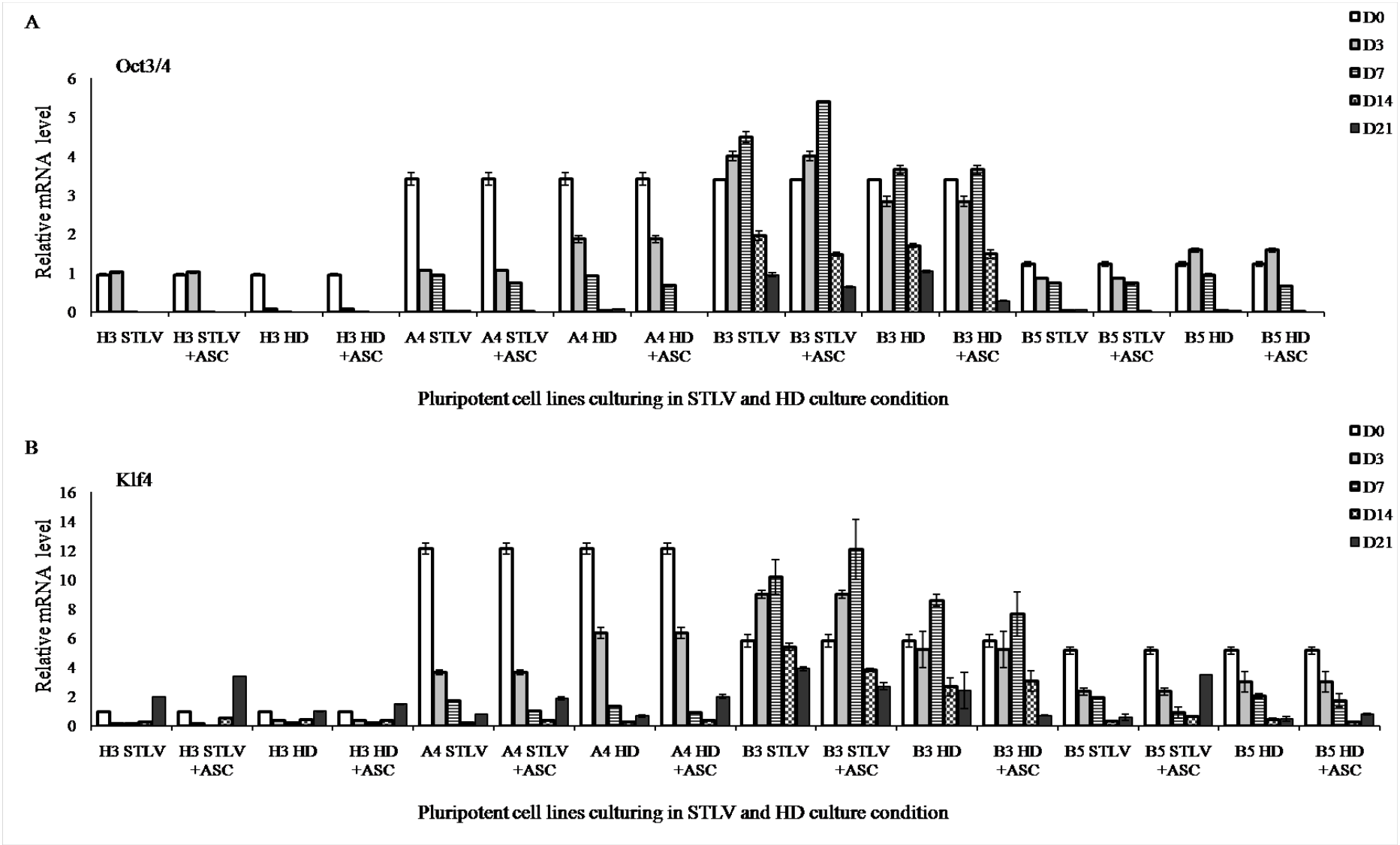
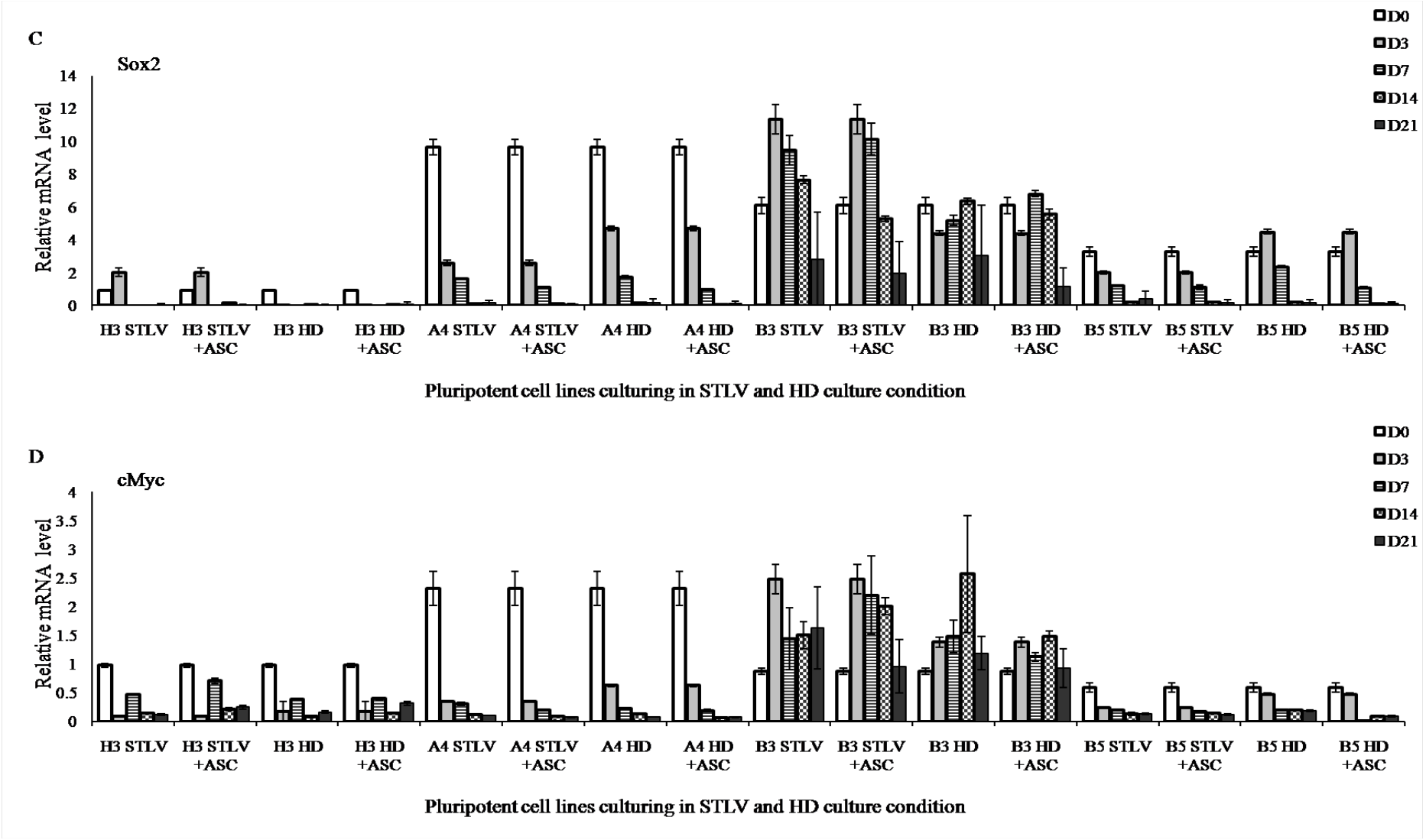
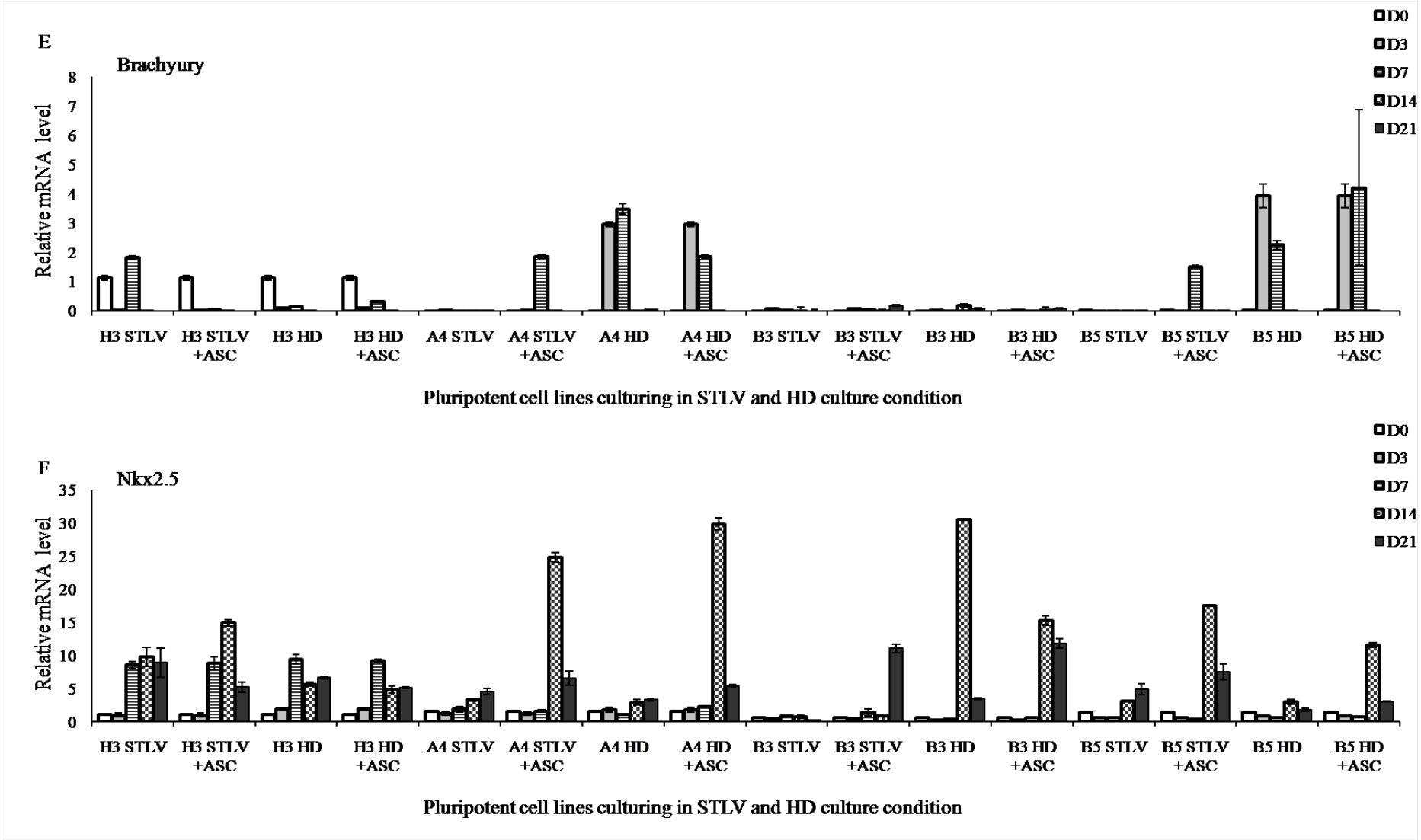
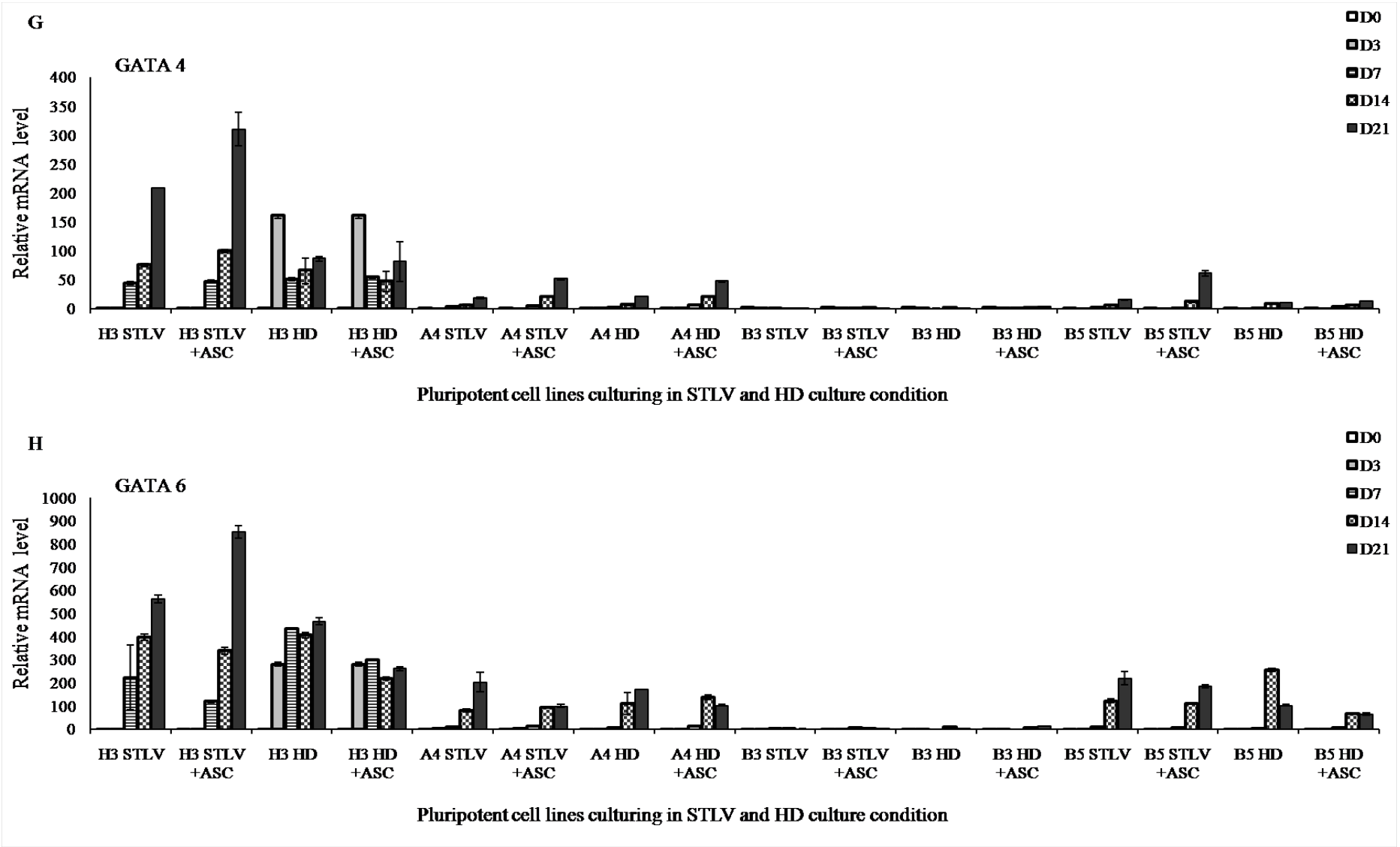


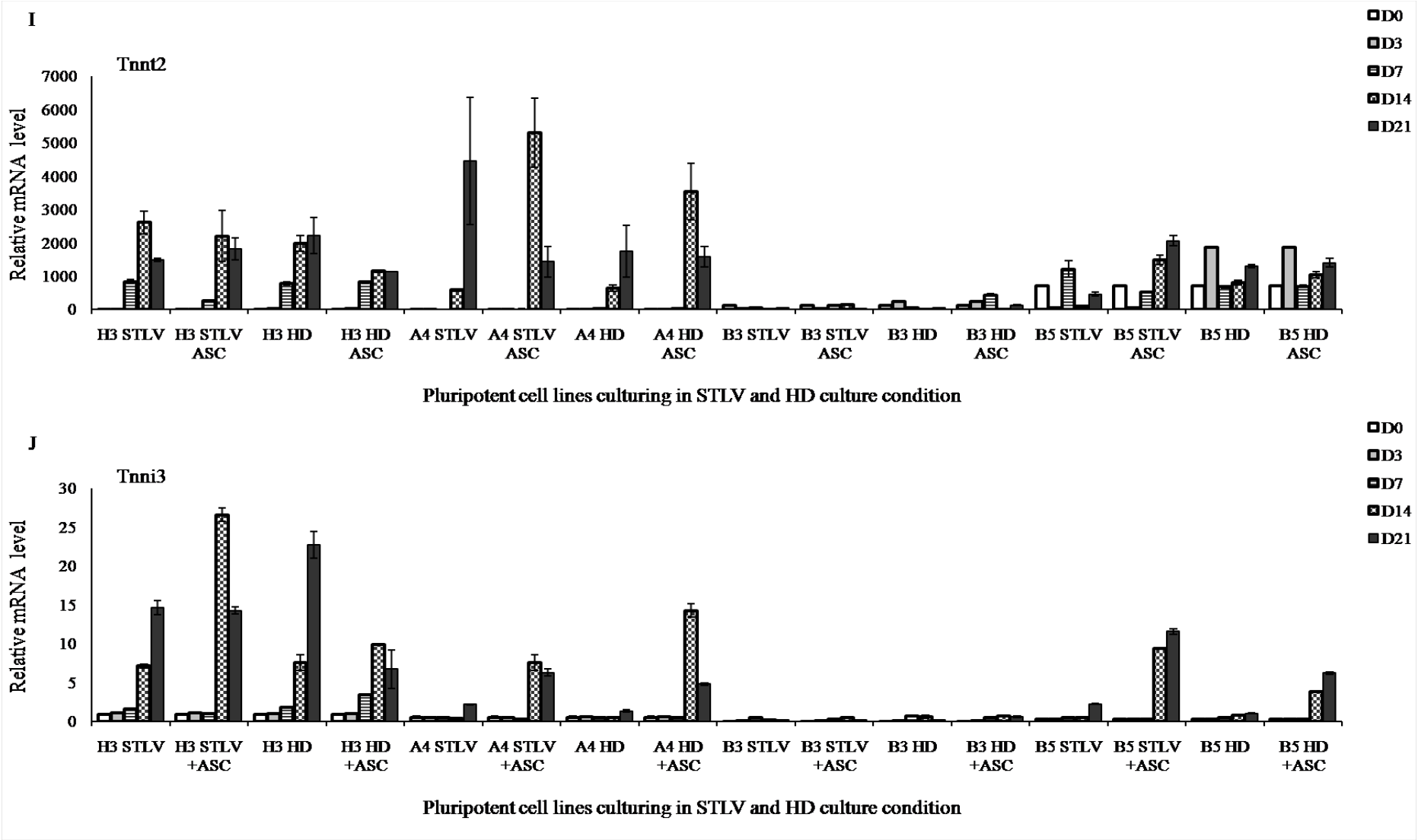
Figure 37. Cardiac differentiation potential of EB derived from different pluripotent cell lines cultured in STLV and HD supplement with or without ASC (A) Immunocytochemistry of EBs was stained with cTnT (red) with DAPI (blue). (B) Area of cTnT per EBs ($x10^5$ μm^2). Scale bars represent 200 µm x40 magnification.

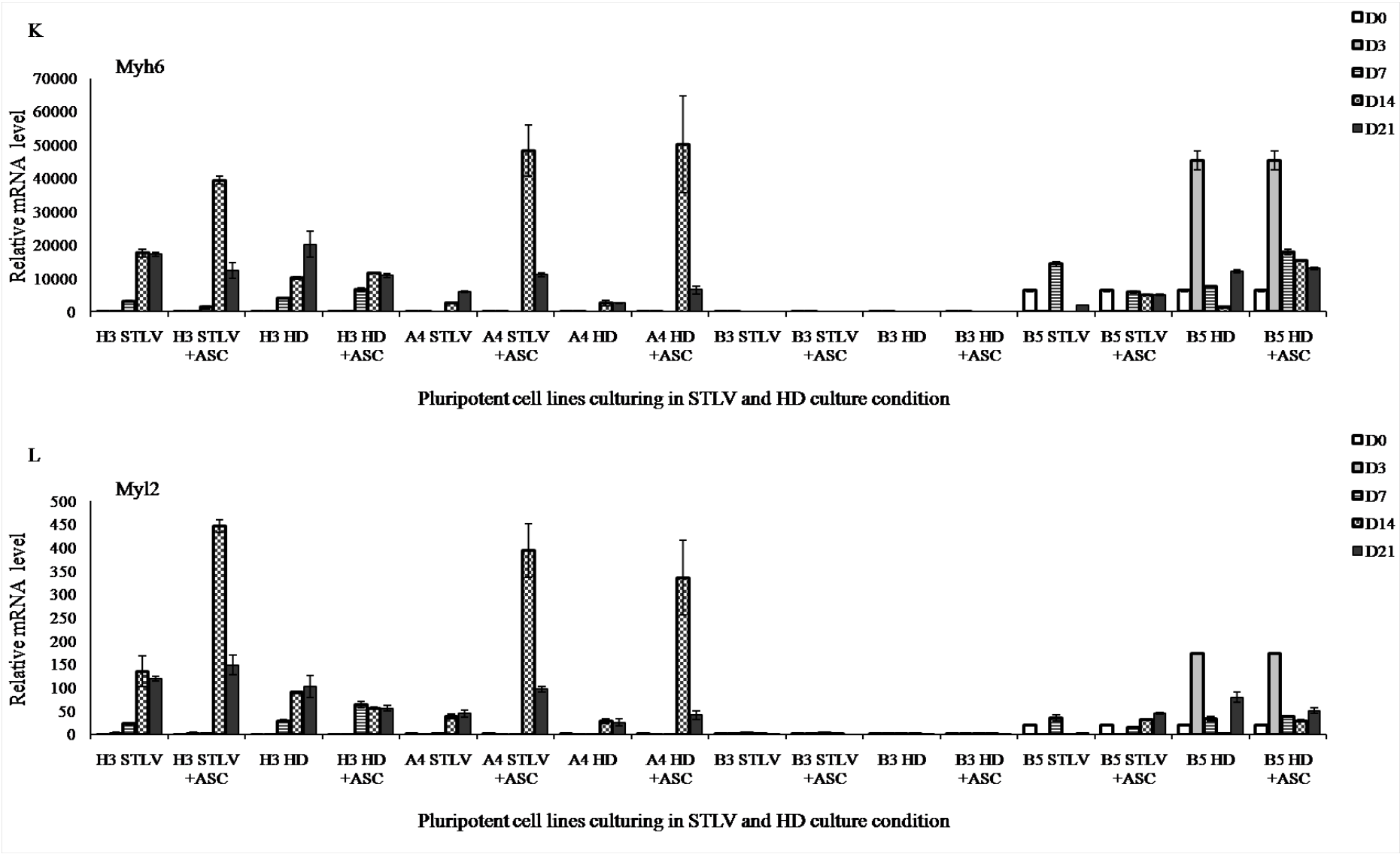

A
Oct3/4
Relative mRNA level
0
1
2
3
4
5
6
D0
D3
D7
D14
D21
H3 STLV
H3 STLV +ASC
H3 HD
H3 HD +ASC
A4 STLV
A4 STLV +ASC
A4 HD
A4 HD +ASC
B3 STLV
B3 STLV +ASC
B3 HD
B3 HD +ASC
B5 STLV
B5 STLV +ASC
B5 HD
B5 HD +ASC
Pluripotent cell lines culturing in STLV and HD culture condition
B
Klf4
Relative mRNA level
0
2
4
6
8
10
12
14
16
D0
D3
D7
D14
D21
H3 STLV
H3 STLV +ASC
H3 HD
H3 HD +ASC
A4 STLV
A4 STLV +ASC
A4 HD
A4 HD +ASC
B3 STLV
B3 STLV +ASC
B3 HD
B3 HD +ASC
B5 STLV
B5 STLV +ASC
B5 HD
B5 HD +ASC
Pluripotent cell lines culturing in STLV and HD culture condition


C
Sox2
Relative mRNA level
14
12
10
8
6
4
2
0
D0
D3
D7
D14
D21
H3 STLV
H3 STLV +ASC
H3 HD
H3 HD +ASC
A4 STLV
A4 STLV +ASC
A4 HD
A4 HD +ASC
B3 STLV
B3 STLV +ASC
B3 HD
B3 HD +ASC
B5 STLV
B5 STLV +ASC
B5 HD
B5 HD +ASC
Pluripotent cell lines culturing in STLV and HD culture condition
D
cMyc
Relative mRNA level
4
3.5
3
2.5
2
1.5
1
0.5
0
D0
D3
D7
D14
D21
H3 STLV
H3 STLV +ASC
H3 HD
H3 HD +ASC
A4 STLV
A4 STLV +ASC
A4 HD
A4 HD +ASC
B3 STLV
B3 STLV +ASC
B3 HD
B3 HD +ASC
B5 STLV
B5 STLV +ASC
B5 HD
B5 HD +ASC
Pluripotent cell lines culturing in STLV and HD culture condition


E
Brachyury
Relative mRNA level
0
1
2
3
4
5
6
7
8
D0
D3
D7
D14
D21
H3 STLV
H3 STLV +ASC
H3 HD
H3 HD +ASC
A4 STLV
A4 STLV +ASC
A4 HD
A4 HD +ASC
B3 STLV
B3 STLV +ASC
B3 HD
B3 HD +ASC
B5 STLV
B5 STLV +ASC
B5 HD
B5 HD +ASC
Pluripotent cell lines culturing in STLV and HD culture condition
F
Nkx2.5
Relative mRNA level
0
5
10
15
20
25
30
35
D0
D3
D7
D14
D21
H3 STLV
H3 STLV +ASC
H3 HD
H3 HD +ASC
A4 STLV
A4 STLV +ASC
A4 HD
A4 HD +ASC
B3 STLV
B3 STLV +ASC
B3 HD
B3 HD +ASC
B5 STLV
B5 STLV +ASC
B5 HD
B5 HD +ASC
Pluripotent cell lines culturing in STLV and HD culture condition


G
GATA 4
Relative mRNA level
400
350
300
250
200
150
100
50
0
D0
D3
D7
D14
D21
H3 STLV
H3 STLV +ASC
H3 HD
H3 HD +ASC
A4 STLV
A4 STLV +ASC
A4 HD
A4 HD +ASC
B3 STLV
B3 STLV +ASC
B3 HD
B3 HD +ASC
B5 STLV
B5 STLV +ASC
B5 HD
B5 HD +ASC
Pluripotent cell lines culturing in STLV and HD culture condition
H
GATA 6
Relative mRNA level
1000
900
800
700
600
500
400
300
200
100
0
D0
D3
D7
D14
D21
H3 STLV
H3 STLV +ASC
H3 HD
H3 HD +ASC
A4 STLV
A4 STLV +ASC
A4 HD
A4 HD +ASC
B3 STLV
B3 STLV +ASC
B3 HD
B3 HD +ASC
B5 STLV
B5 STLV +ASC
B5 HD
B5 HD +ASC
Pluripotent cell lines culturing in STLV and HD culture condition


I
Tnnt2
Relative mRNA level
7000
6000
5000
4000
3000
2000
1000
0
D0
D3
D7
D14
D21
H3 STLV
H3 STLV ASC
H3 HD
H3 HD ASC
A4 STLV
A4 STLV ASC
A4 HD
A4 HD ASC
B3 STLV
B3 STLV ASC
B3 HD
B3 HD ASC
B5 STLV
B5 STLV ASC
B5 HD
B5 HD ASC
Pluripotent cell lines culturing in STLV and HD culture condition
J
Tnni3
Relative mRNA level
30
25
20
15
10
5
0
D0
D3
D7
D14
D21
H3 STLV
H3 STLV +ASC
H3 HD
H3 HD +ASC
A4 STLV
A4 STLV +ASC
A4 HD
A4 HD +ASC
B3 STLV
B3 STLV +ASC
B3 HD
B3 HD +ASC
B5 STLV
B5 STLV +ASC
B5 HD
B5 HD +ASC
Pluripotent cell lines culturing in STLV and HD culture condition


K
Myh6
Relative mRNA level
70000
60000
50000
40000
30000
20000
10000
0
D0
D3
D7
D14
D21
H3 STLV
H3 STLV +ASC
H3 HD
H3 HD +ASC
A4 STLV
A4 STLV +ASC
A4 HD
A4 HD +ASC
B3 STLV
B3 STLV +ASC
B3 HD
B3 HD +ASC
B5 STLV
B5 STLV +ASC
B5 HD
B5 HD +ASC
Pluripotent cell lines culturing in STLV and HD culture condition
L
Myl2
Relative mRNA level
500
450
400
350
300
250
200
150
100
50
0
D0
D3
D7
D14
D21
H3 STLV
H3 STLV +ASC
H3 HD
H3 HD +ASC
A4 STLV
A4 STLV +ASC
A4 HD
A4 HD +ASC
B3 STLV
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Pluripotent cell lines culturing in STLV and HD culture condition

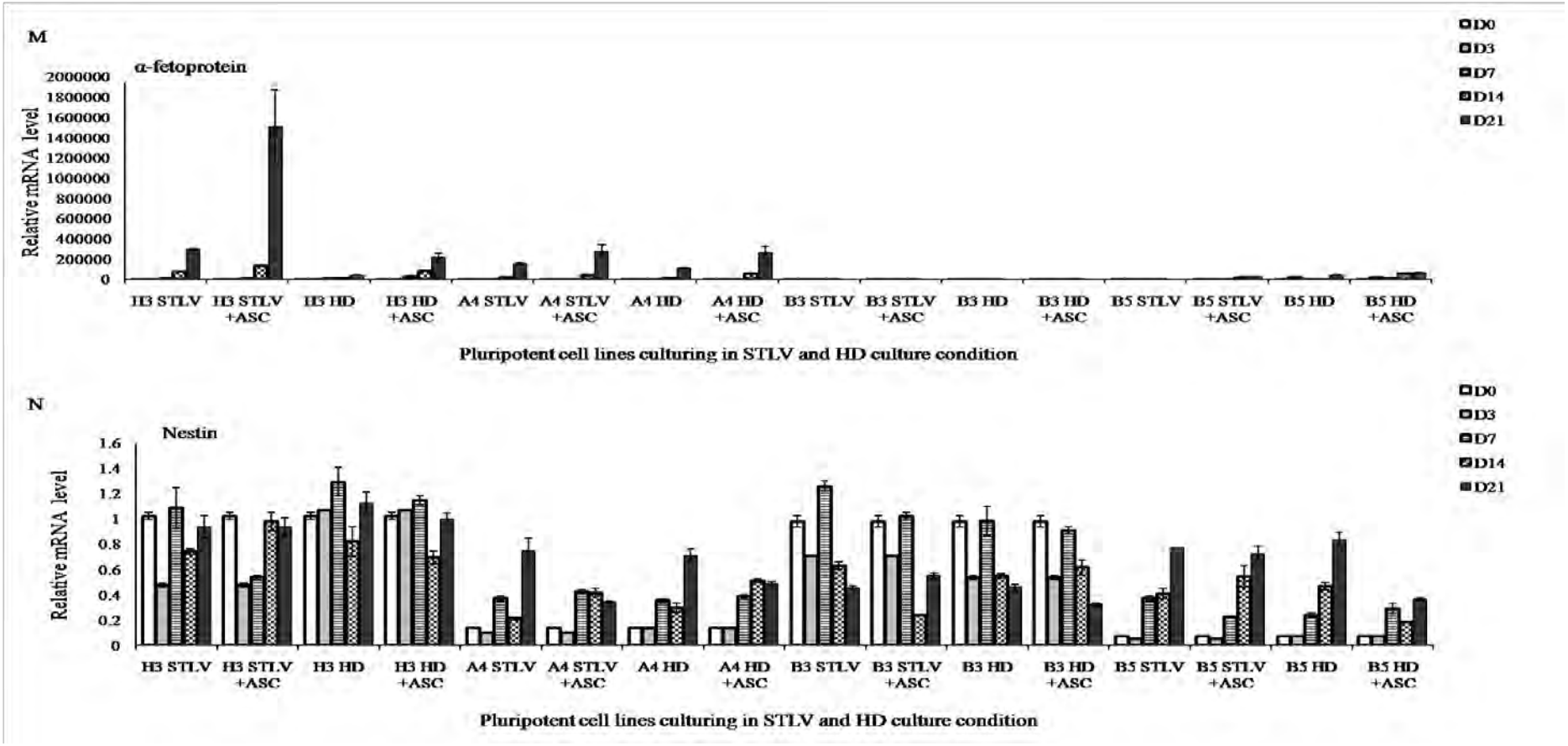

Figure 38. qRT-PCR analysis of gene expression of different pluripotent stem cells in STLV and HD EBs. RNA extracted from EBs at days 0, 3, 7, 14 and 21. (A) Oct3/4, (B) Klf4, (C) Sox2, (D) cMyc expression indicates the presence of undifferentiated cells. (E) Brachyury-T was chosen as mesodermal markers. (F) Nkx2.5, (G) GATA4 and (H) (GATA6) were included as markers for cardiac mesoderm. In addition, (I) TNNT2, (J) Tnni3, (K) MYH6 and (L) MYL2 and served as markers for cardiomyocytes. (M) α-fetoprotein and (N) Nestin were detected for investigate endoderm and ectoderm differentiation.

Increased expression of the early cardiac mesodermal marker gene Nkx2.5, was shown by day seven, then stayed high until day fourteen afterwards in EBs culturing with ASC supplement and was down-regulated by day 21 at $H3^{TgNkx2.5/\ EGFP}$- ES cells derived cardiac outgrowths. Our result showed that the Nkx2.5 mRNA of all iPS cell lines expressed with a delay compare to the one of $H3^{TgNkx2.5/\ EGFP}$- ES cells which peaked by day fourteen and down-regulated by day 21. No difference of the Nkx2.5 mRNA expression was detected between different culture conditions; however ASC supplement improved the efficiency of cardiac differentiation. This was visible by the increase of the Nkx2.5 mRNA expression level (maximum ten times) at all lines except B3-iPS line cultured in HD (Figure 38F). GATA4 and GATA6 were expressed earliest in the differentiating $H3^{TgNkx2.5/\ EGFP}$- ES cells cultured in HD on day three and in STLV on day seven. In differentiating iPS cells it was expressed first on day fourteen. Interestingly, the GATA4 and GATA6 mRNA expression of $H3^{TgNkx2.5/\ EGFP}$- ES cells culturing in STLV were expressed the highest by day 21 (Figure 38G to 38H).

At later time points of spontaneous cardiac differentiation, cardiac markers became prominent. The expression of Tnnt2, Tnni3, Myh6 and Myl2 (Figure 38I to 38L) (which are cardiomyocyte structural constituents) were detected by day seven, peaked by day fourteen and down-regulated by day 21 in $H3^{TgNkx2.5/\ EGFP}$- ES cells. However, iPS cell lines showed delayed expression of these genes as they were detected and peaked by only for day fourteen and down-regulated afterword by day 21. $H3^{TgNkx2.5/\ EGFP}$- ES cells cultured in STLV and supplemented with ASC showed the best result of cardiac differentiation due to highest express the Tnni3 and Myh6 and Myl2 mRNA expression. The expression of those genes were different among different iPS cell lines (A4-iPS> B5-iPS>B3-iPS (Figure 38I to 38L), in agreement with the different efficiency of cardiac differentiation (Figure 36 and 37). Besides, ASC supplement improved the efficiency of cardiac differentiation by increasing Tnni3 and Myh6 and Myl2 mRNA expression in all EBs (Figure 38I to 38L).

5.4 Discussions

While, the traditional HD method has been the most widely used strategy to generate differentiating EBs in both the mouse ES and iPS cells (Takahashi et al., 2003), this technique is unsuitable to produce a lot of EBs and cannot meet the demands for therapeutic applications because of its complexity and difficult manageability. STLV culture is a great promise for generating clinically relevant number of iPS-derived cardiomyocytes as it is amenable to scale up, facilitate process control, and simplify cell production processes. This is the first report demonstrating that optimized STLV culture is suitable for the production of scalable EBs derived from difference iPS cell lines. Our result also first showed that EBs derived from STLV were uniform and homogenous in size which is similar to the homogeneity of EBs derived from the HD method. Moreover, EBs derived from STLV showed the efficiency of cardiac differentiation similar to that EBs derived from HD base on the percentage of beating cardiomyocyes and the areas of cTnT. Similar gene expression patterns were observed in both differentiation systems with the sequential expression of pluripotency markers, mesoderm markers, cardiac transcription factors, and cardiomyocyte structural genes by using qRT-PCR. Moreover, our result showed that STLV is able not only to produce mass amount of EBs but also it can also produce good quality EBs from iPS cells, similarly to ES cells. Controversial, Yirme et al. (2008) demonstrated that human STLV EBs had heterogeneous in size as similar as SSC EBs which was different from our results, Gerecht-Nir et al. (2004) and Côme et

al (2008). It is perhaps that interrupting stirring and microgravity in STLV when manipulating STLV vessel including changing medium leads to agglomeration of EBs and formation of aggregates that are too large for optimal growth and differentiation and thus disturb the evolution of the early stages of the gastrulation or method of harvest human ES cells.

Moreover, we also compared the cardiac differentiation potential among different iPS cell lines. Moreover, the cardiac differentiation potential of iPS cell lines was compared to the potential of a reference ES cell line. We compared various iPS cell lines in terms of the relative efficiency of their EB formation, the percentage of contracting EBs they form, the upregulation of cardiac gene expression and concomitant downregulation of pluripotency genes. Subsequently, the optimized STLV condition was also applied to produce mass EBs derived from difference iPS cell lines and ASC was supplemented to increase cardiac differentiation. Our findings suggest that iPS cells are a viable alternative to ES cells as an autologous cell source for cardiac cell transplantation. Our result confirmed that iPS cells have similar properties as ES cells, such as in quality of EB formation (size, homogeneity, proliferation and apoptosis within EBs) when cultured in STLV and HD culture. Interestingly, STLV method improved the efficiency of EB formation due to higher presence of proliferating cells in EBs compared to the HD method. However, the quality of EBs potential may vary between individual cell lines, related with small EB formation derived from B3-iPS cell line derived from STLV. The cause of this perhaps relate to subtle differences in basal gene expression or epigenetic state which we demonstrated the variation of the expression of pluripotent genes and cardiac markers.

Although, a previous study indicated that the differences between differentiation of iPS cells and ES cells into cardiac lineage are no greater than the differences already noted between ES cell lines (Zhang et al., 2009), our result suggest that all iPS cell lines showed delayed and less efficient cardiac differentiation compared with the ES cell line. The time course of spontaneous cardiac differentiation, ES cell-derived EBs started early cardiac beating than iPS cell – derived EBs in HD method (day six vs day ten to day thirteen, respectively). Similar with, one of the first studies to examine the time course of mouse iPS cells differentiation into cardiomyocytes noted a substantial delay in cardiogenesis compared to mouse ES cells, (Mauritz et al., 2008) whilst an another study using different iPS cell lines found no difference between the iPS and ES cell lines in cardiac differentiation (Zhang et al., 2009). In addition, beating areas in iPS cell – derived EBs were usually smaller than those in ES cell EBs. These observed differences between the three analyzed cell lines were in agreement with the evaluated positive area of cTnT. The efficiency of spontaneous cardiac differentiation were variable among iPS cell lines; 50% of A4, 2% of B3 and 77% of B5 on day fourteen. Similar differences in differentiation potential and efficiency are frequently observed among individual ES cell lines, and the observed differences between the analyzed ES and iPS cell lines may reflect similar cell line differences. As well, the different cardiac differentiation potential may be attributed to the fact that the transgenic ES cell line ($H3^{TgNkx2.5/\ EGFP}$- ES cells) was preselected for both robust *in vitro* proliferation and efficient cardiac differentiation which shown early cardiac beating and higher percentage of EB beating than maternal cell line (data not shown). This ES cell line can be spontaneous differentiated to give 90% beating EBs. Previous report suggested that delayed differentiation of all iPS cell lines might be in relationship to the sustained expression of the pluripotency marker genes Oct-4 during

differentiation. This is in an agreement with our study as well. Our study also confirmed that all iPS cells showed delayed cardiac differentiation perhaps due to the presence of the exogenous pluripotency cassette harboring Oct-4, Klf4, Sox2 and c-Myc pluripotency genes. On the other hand the gene expression of early mesendodermal progenitors which is essential for the induction of mesoderm, and further cardiac mesoderm were also delayed in all iPS cell lines compared to ES cell line ($H3^{TgNkx2.5/\ EGFP}$- ES cells). Moreover, we observed high contribution of Oct-4 positive cells in all iPS-derived EBs and outgrowths but not in the ES- derived ones. Notably, B3-iPS cell line behaved different from the rest of iPS and the reference ES cell line. All area of B3-iPS derived EBs showed high Oct-4 and consequent low cTnT expression on day 21 of EB culture. In addition, this cell line did not perform well in cardiac differentiation assays as it was measured by the efficiency of cardiac beating (only 38% of EBs were beating and beating areas were usually smaller than those for other iPS cells) It may be that this cell line has incomplete transgene silencing. This result was also confirmed by detecting the highest expression of the pluripotency genes; Oct-4, Klf4, Sox2 and c-Myc. In agreement with this, the gene expressions of early mesendodermal progenitors, cardiac mesoderm, and cardiomyocytes were also low in the B3-iPS cell lines.

Cardiac differentiation of ES cells can be also further enhanced by adding culture supplements in cell medium such as retinoic acid (Wobus et al., 1997), bone morphogenetic proteins or ASC, serum omission, or co-culture with endoderm- like cells (Mummery et al., 2007). In current study, we also investigated effect on ASC supplement on cardiac differentiation of different iPS cell lines in STLV and HD culture conditions. Our result confirmed ASC enhances differentiation of iPS and ES cell into cardiomyocytes relied on improving the percentage of cardiac beating and quantity of EB beating. Moreover, ASC increased the appearance of an extensive and filamentous network of cTnT and in agreement with previous reports (Takahashi et al., 2003). Our study demonstrated that ASC induced cardiac differentiation by increasing the expression of cardiac mesoderm (Nkx2.5, GATA4 and GATA6 genes) and cardiomyocytes (Tnnt2, Tnni3, Myh6 and Myl2 genes), in agreement with previous reports (Takahashi et al., 2003). Interestingly, treatment with ASC also induced iPS and ES cells into endoderm lineage by increasing α-fetoprotein but were not induced those cells into ectoderm lineage.

In conclusion, our study describes, for the first time, a strategy for scalable differentiation of iPS cells into cardiomyocytes with the appropriate molecular and structural properties using STLV bioreactor. Our study defines that STLV bioreactor can be used to provide means for large-scale production of transplantable therapeutic cardiomyocytes for clinical and industrial application.

CHAPTER VI

GENERAL DISCUSSION AND CONCLUSIONS

Current strategy: (i) Differentiation of autologous pluripotent stem cells into cardiomyocytes

Although ES cells hold the promise as a universal resource for cellular therapy, these cells are incapable of allogenic transplantation due to immune rejection (Lerou and Daley, 2005). To solve this problem, autologous pluripotent stem cells have been established as known as pES cells (Kim et al., 2007) and iPS cells (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Patient-specific cardiomyocytes derived from these cells provides unique opportunity for an unlimited cell source for autologous cell-replacement therapy. The capability of fES cells to differentiate into cardiomyocytes has been studied by numerous research groups. However, little is still unknown about the capacity and molecular biology of cardiomyocyte differentiation derived from the pES and iPS cells. Before using these techniques for clinical therapy, many questions are needed to be clarified on the genetic and functional equivalence of these cells compared to fES cells (Lister et al., 2011). Here, we investigated the efficiency of cardiomyocyte differentiation derived from pES and iPS cells. Moreover, the variability of cardiomyocyte differentiation among the different cell lines in comparison to different types of ES cells was investigated.

In case of pES cells, all four pES cell lines showed similar pluripotency characteristics compared to that of ES cells. The pES cell lines were able to differentiate *in vitro* into cell types of all three germ layers, especially into cardiomyocytes. However, differences in the differentiation outcomes were revealed. The reason for this variability is not fully understood and may be related to the subtle differences in epigenetic state or chromosomal abnormalities presented in a proportion of cells.

For iPS cells, our study found that there was a significant variability in the efficiency of cardiac differentiation among the iPS cell lines. At this point, it is still not clear whether our results revealed general differences between ES and iPS cells. Similar differences in differentiation potential and efficiency are frequently observed among individual ES cell lines, and the observed differences between the analyzed ES and iPS cell lines may reflect similar cell line differences. The explanation may be the different of the gene expressions among different cell lines. Our study found that iPS cells (A4-iPS) which showed a high efficiency of cardiac differentiation, showed early down-regulation of the expressed pluripotency genes and up-regulation of the gene for early mesendodermal progenitors generate mesoderm, cardiac mesoderm, and cardiomyocytes. On the other hand, iPS (B3-iPS) cells, which showed low efficiency of cardiomyocyte differentiation, showed high expressed the pluripotency genes during differentiation (up-regulated by day three and almost peaked by day seven and down-regulated by day fourteen). These genes were still high and long-term expressed until day 21. It may be that this cell line was incompletely transgene silencing as the basic cause for the delayed and less efficient cardiac differentiation of the iPS cell lines. Moreover, iPS cells showed lower in the efficiency of cardiac differentiation when compared to ES cells. Our study suggested that delayed differentiation of all iPS cell lines, perhaps in relationship to the sustained expression of the pluripotency marker genes Oct-4, Klf4, Sox2 and cMyc during differentiation,

was in an agreement with previous study. We found that all iPS cell lines showed higher the expression of the pluripotent marker genes (Oct-4, Klf4, Sox2 and cMyc) compared to ES cell line. On the other hand, the gene expressions of early mesendodermal progenitors were delayed to detect in all iPS cell lines compared to ES cell line.

Current strategy: (ii) Enhance cardiac differentiation of pluripotent stem cells by STLV bioreactor

In the current study, it describes and characterizes a scenario method of EB formation in hydrodynamic conditions using STLV bioreactor and subsequent differentiation of mouse pluripotent stem cells including ES and iPS cells into cardiomyocytes. It was demonstrated that the optimized STLV bioreactor method provided a satisfactory control over the agglomeration process of EBs, thereby enabling cell proliferation and differentiation of the pluripotent stem cells towards cardiomyocytes. The initial numbers of pluripotent stem cells in EBs and the time of EB adhered onto gelatin-coated plate affected the cardiogenesis. A starting number of $3x10^5$ pluripotent stem cells/ml, 10 rpm rotary speed and plated on day three following culture onto gelatin-coated surface resulted in the highest potential of cardiac differentiation. The ability to produce homogeneous populations of EBs of particular sizes also increased the differentiation potential of pluripotent stem cells into cardiomyocytes. Moreover, the optimized STLV bioreactor was compared to SSC and HD conditions. Overall, the efficiency of STLV condition for generating EBs showed higher efficiency than SSC condition based on uniformity of EBs, the number of EB/ml generation and total cell yield of EB/ml. Besides, EB derived from STLV showed higher the efficiency of cardiac differentiation than SSC and HD based on the area of cTnT. STLV culture provide a stable and ideal condition for the mouse pluripotent stem cells to form highly EB production and retain good quality than other culture system because this culture provides as follows: (i) low fluid shear stress due to whole-body rotation around a horizontal axis, (ii) yielding a vessel devoid of gas bubbles and gas per fluid interfaces due to oxygenation by active or passive diffusion to the exclusion of all but dissolved gasses from the reactor chamber, (iii) mild mixing of cells due to very low rotation speed of STLV and (iv) enabling efficient gas exchange due to the high membrane area to volume of medium ratio.

Moreover, we also found that ASC supplement affected on cardiac differentiation of pluripotent cell lines. Our result confirmed that ASC enhanced differentiation of pluripotent cell lines into cardiomyocytes as improving the percentage of cardiac beating and quantity of EB beating. Moreover, ASC also increased the presence of an extensive and filamentous network of cTnT in central and peripheral areas of the EBs and also improved morphology and structure and morphology of cardiac differentiation as previously reported (Takahashi et al., 2003). Our study demonstrated that ASC induced cardiac differentiation by increasing the expression of cardiac mesoderm (Nkx2.5, GATA4 and GATA6 genes) and cardiomyocytes (TNNT2, TNNI3, MYH6 and MYL2 genes), similar to a previous report (Takahashi et al., 2003). Interestingly, treatment with ASC was capable of inducing the iPS and ES cells into endoderm lineage by increasing the α-fetoprotein.

Future direction:

Bioprocessing and commercialization of pluripotent stem cells and tissue engineering products in cell replacement therapy have potential to transform breakthrough from the research bench to patient bedside. This is expected to be a long

process, however, as there are many key practical issues to be addressed before moving ahead from the laboratory-scale. Laboratory-scale suspension cultures in HD or Petri-dishes are useful tools for development and initial optimization. Encapsulation/ entrapment of ES cells, multi-well and microfabrication methods can improve high-throughput EB production. However, these approaches are not suitable for further therapeutic because of their labor intensive, time consuming and culture-to-culture variability and the lack of repetitive monitoring. Bioreactor culture systems can solve many of these problems, and offers several advantages over the conventional technique for expanding and differentiating ES cells into specific lineages, without compromising their properties. Future challenges in bioreactor development will include the design of advanced and sophisticated monitoring platforms that allow monitoring at the cellular level including temperature, pH and O_2 levels. With respect to pluripotent stem cells, we envision of a scenario, where a complete bioprocess would exist in the bioreactor for the expansion and subsequent differentiation of the pluripotent stem cells to generate the specialized cell type of interest. For example, the current achievements of cardiomyocytes derived from ES cells would be developed into cardiovascular grafts tissue engineering, with an emphasis on its possibility for clinical use in cardiovascular surgery. The engineering of human cardiac tissue patch would be used to illustrate the biological requirements and engineering approaches for human applications. For future therapeutic application, the specialized cells differentiated from pluripotent stem cells could then be used for cell therapies or combined with scaffolds to produce tissue construct and transplant to specific patients.

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APPENDICES

APPENDIX A

Detail of medium preparation

1. Mouse ES medium

	Reagent	Company	Catalog #	Storage (C°)	Volume (ml)
1	DMEM (High Glucose)	Invitrogen	31966-047	4	500
2	ES qualified FBS/ ES qualified FBS	Sera Laboratories Internatinal/ Hyclone	100-125/ SH30898.03	-20	90
3	β-ME	Sigma	M7522	4	6
4	NEAA (1:100)	Sigma	M7145	4	6
5	Penicillin/steptomycin	Gibco	15140-122	-20	6
6	Glutamax	Invitrogen	35050	-20	6
7	LIF (1,000 /2,000IU)	Chemicon	ESG 1107	4	0.0006
	Total volume			4	615

2. Spontaneous cardiac differentiation medium

Mouse ES medium without LIF

3. Induce cardiac differentiation medium

	Reagent	Company	Catalog #	Storage (C°)	Volume (ml)
1	Mouse ES medium without LIF				500
2	ASC (dilution 10x)	Sigma	A4403	Room tempera ture	5
	Total volume			4	550

4. Fibroblast medium

	Reagent	Company	Catalog #	Storage (C°)	Volume (ml)
1	DMEM (High Glucose)	Invitrogen	31966-047	4	500
2	FBS	Gibco	10108-165	-20	55
3	Penicillin/steptomycin	Gibco	15140-122	-20	5.5
	Total volume			4	555.5

APPENDIX B

Paper publications

1. **Rungarunlert, S.**, Klincumhom, N., Ujhelly, O., Techakumphu, M., Pirity, M.K. and Dinnyes, A. Enhanced cardiac differentiation of mouse pluripotent (ES and iPS) stem cells by use of the slow-turning, lateral vessel (STLV) bioreactor. Manuscript preparation.
2. **Rungarunlert, S.**, Rungsiwiwut, R., Suphankong, S., Panasophonkul, S., Pruksananonda, K., Virutamasen, P., Pirity, M.K., Dinnyes, A., Tharasanit, T. and Techakumphu, M. 2011. Comparative characterization of four mouse parthenogenetic embryonic stem (pES) cell lines. Thai J. Vet. Med. In press.
3. **Rungarunlert, S.**, Klincumhom, N., Bock, I., Nemes, C., Techakumphu, M., Pirity, M.K. and Dinnyes, A. 2011. Enhanced cardiac differentiation of mouse embryonic stem cells by use of the slow-turning, lateral vessel (STLV) bioreactor. Biotechnol Lett. In press.
4. **Rungarunlert, S.**, Techakumphu, M., Pirity, M.K. and Dinnyes, A. 2009. Embryoid body formation from mouse pluripotent (ES and iPS) stem cells: benefits of bioreactor. World J. Stem Cells. 1(1): 11-21.
5. Rungsiwiwut, R., **Rungarunlert, S.**, Numchaisrika, P., Pruksananonda, K., Techakumphu, M. and Virutamasen, P. 2008. Effect of leukemia inhibitory factor (LIF) on the quality of *in vitro* produced mouse blastocysts and subsequent Derivation of embryonic stem (ES) cells. J. Med. Assoc. Thai. 91(5): 608-614.
6. Tharasanit, T., **Rungarunlert, S.** and Techakumphu, M. 2009. Cryopreservation of immature porcine oocytes using open pulled straw vitrification. Thai J. Vet. Med. 39(2): 163-171.

Proceeding and posters/oral presentations

1. **Rungarunlert, S.**, Tharasanit, T. and Techakumphu, M. 2006. Cryopreservation of immature porcine oocytes using open pulled straw vitrification. 2nd Symposium on Animal Reproduction: Trend of biotechnology for Animal Reproduction From basic to advance. Faculty of Veterinary Science, Chulalongkorn University, Bangkok, March 27-28th, 17. Oral.
2. **Rungarunlert, S.**, Tharasanit, T. and Techakumphu, M. 2006. Cryopreservation of immature porcine oocytes using open pulled straw vitrification. RGJ Seminar Series XLVII "Reproductive Biotechnology for Improving Animal Breeding Stretegies", Nan, Thailand, October 20th, 61. Poster.
3. **Rungarunlert, S.**, Tharasanit, T. and Techakumphu, M. 2006. Cryopreservation of immature porcine oocytes using open pulled straw vitrification. The 3rd Asian Reproductive Biotechnology Conference, Hanoi, Vietnam, November 29th - December 1st, 159. Poster.
4. **Rungarunlert, S.**, Rungsiwiwut, R., Suphankong, S., Panasophonkul, S., Thongphakdee, A., Dinnyes, A., Tharasanit, T. and Techakumphu, M. 2008. Establishment of parthenogenetic embryonic stem (ES) cell lines in mouse.

16th International Congress on Animal Reproduction, Budapest, Hungary, July 13-17th, 198. Poster.

5. **Rungarunlert, S.**, Tar, K., Muenthaisong S., Feher, A., Bock, I., Techakumphu, M., Pirity, M.K. and Dinnyes, A. 2009. Differentiation of embryonic stem (ES) cells into cardiac lineage. 15th Annual Conference of the Hungarian Society of Cell- and Developmental Biology, Hungary, April 17-19th. Poster.
6. Pogar, Z., Tar, K., **Rungarunlert, S.**, Muenthaisong, S., Bock, I., Pirity, M.K. and Dinnyes, A. 2009. Improved derivation of embryonic stem cell lines from inbred C57BL/6J mouse strain. The 7th Annual meeting of International Society of Stem Cell Research (ISSCR), Barcelona, Spain, July 8-11th, 104. Poster.
7. **Rungarunlert, S.**, Klincumhom, N., Muenthaisong, S., Techakumphu, M., Tar, K., Pirity, M.K. and Dinnyes, A. 2009. Differentiation of mouse embryonic stem (ES) cells into cardiomyocytes by using slow turning lateral vessel (STLV/Bioreactor) Commission on Higher Education Congress II: University Staff Development Consortium (CHE-USDC Congress II), Chonburi, Thailand, August 27-29th. Oral.
8. **Rungarunlert, S.**, Tar, K., Muenthaisong, S., M. Techakumphu, Pirity, M.K. and Dinnyes, A. 2010. Differentiation of mouse embryonic stem (ES) cells into cardiomyocytes by using slow turning lateral vessel (STLV/Bioreactor). 36th Annual Conference of the IETS/23rd Annual meeting SBTE, Cordoba, Argentina, January 9-12th. Reproduction, Fertility and Development. 50 (1): 355. Poster.
9. **Rungarunlert, S.**, Klincumhom, N., Nemes, C., Techakumphu, M. Pirity, M.K. and Dinnyes, A. 2011. Mass production of Nkx 2.5 positive cardiac progenitor cells derived from mouse embryonic stem (ES) cells in slow turning lateral vessel (STLV) for cell transplantation and drug testing. The 37th Annual Conference of the International Embryo Transfer Society (IETS) 2011, Wyndham Orlando Resort, Orlando, Florida, United States, January 8-12th. Reproduction, Fertility and Development. 23 (1): 244. Poster.
10. **Rungarunlert, S.**, Klincumhom, N., Ujhelly, O., Techakumphu, M., Pirity, M.K. and Dinnyes, A. 2011. Differentiation of Mouse Pluripotent Stem Cells into Cardiomyocytes by Using Slow Turning Lateral Vessel (STLV) Bioreactor. The 10th Chulalongkorn University Veterinary Anual conference. Faculty of Veterinary Science, Chulalongkorn University, Thailand, 21st-22rd April. Oral.

Training courses

1. Training course of microarray and whole embryo in situ hybridization (WISH) at Institute of Experimental Genetics, Helmholtz Zentrum münchen, German Research Center for Environmental Health (GmbH), Germany, February 23-27th, 2009.
2. Training course of immunostainting, Agricultural Biotechnology Center Institute, Szent Isvant University, Hungary, April 15-16th, 2009.
3. Training course of real time PCR, Agricultural Biotechnology Center Institute, Szent Isvant University, Hungary, September 24-25th, 2009.
4. Training course of western blot, Agricultural Biotechnology Center Institute, Szent Isvant University, Hungary, Jaunary 14-15th, 2010.
5. Training course of molecular biology (XChIP and MeDIP), Epigenetic course at INRA Domaine de Vilvert UMR 1198 Biologie du Développement et

Reproduction Bâtiment 212 78352 Jouy-en-Josas, FRANCE, 28th March-2nd April, 2010.

6. Training course of FASC analysis, Agricultural Biotechnology Center Institute, Szent Isvant University, Hungary, May 5-7th, 2010.
7. Training course of detection of unknown mitochondrial SNPs by High-Resolution Melting (HRM) Agricultural Biotechnology Center Institute, Szent Isvant University, Hungary, October 25th, 2010.
8. The induced pluripotent stem cells: The Roslin Institute, The University of Edinburgh, Roslin Biocentre, Roslin, Midlothian, EH25 9PS, Scotland, UK, October 09-10th, 2010.

Conferences, meetings and seminars

1. The 3rd Asian Reproductive Biotechnology Conference, Hanoi, Vietnam, November 29th - December 1st, 2006.
2. The World Association of Young Scientists, The third World Science Forum-Budapest, The Hungarian Academy of Sciences, Budapest, Hungary, November 8-10th, 2007.
3. Pathways to an Accepted Manuscript: Workshop for Authors Publishing Scientific Papers in English, Szent Istvan University, Faculty of Veterinary Science, Department and Clinic for Obstetrics and Reproduction, Section for Reproductive Pathology and Clinical Endocrinology H-1078 Budapest VII., Istvan u. 2, Hungary, November 20-22th, 2007.
4. The 16th International Congress on Animal Reproduction, Budapest, Hungary, July 13-17th, 2008.
5. The 7th Annual meeting of International Society of Stem Cell Research (ISSCR), Barcelona, Spain, July 8-11th, 2009.
6. The 3rd International Congress on Stem Cells and Tissue Formation, Maritim Hotel and Internationale Congress Center Dresden, Germany, July 11-14th, 2010.
7. The induced pluripotent stem cells: production and utility in regenerative medicine meeting at The BioPark Hertfordshire, Broadwater Road, Welwyn Garden City, Hertfordshire, AL7 3AX. United Kingdom, October 07th, 2010.
8. The 4th International Symposium "Stem Cells, Development and Regulation at Grand Hotel Krasnapolsky, Dam, 9. 1012 JS, Amsterdam, Netherlands from October 11- 15th, 2010.
9. The Yearly Open Workshop of the PartnErS ‘Comparative embryonic stem cell research in mammalians’ FP7 project, coordinated by the BioTalentum Ltd., September 15th, 2010.
10. Clonet Annual meeting, Municipal Library, Godollo, Hungary, October 25th, 2010.
11. InduStem: The Yearly Open Workshop Stem cells imaging technologies and cardiac differentiation, October 26th, 2010.

VITAE

Miss Sasitorn Rungarunlert has been working on embryology, embryonic stem cell biology and animal laboratory manipulations since 2005. After finishing Doctor of Veterinary Science (DVM) with the 2nd honor from Faculty of Veterinary Science, Mahidol University, Thailand on 2004, she was teacher assistance of that University. At the present time, she is studying in the sixth years of PhD student at the department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University. She has been supporting by grant under the program Strategic Scholarships for Frontier Research Network for the Joint Ph.D. Program Thai Doctoral degree from the Office of the Higher Education Commission, Thailand and the Clonet project. She also participarted in a lot of training programs integrating different disciplines needed for biomedical research: embryology, cell biology, developmental biology, bioimaging, molecular biology and bioinformatics, established by the Clonet project such as training course of microarray and whole embryo in situ hybridization (WISH) at Institute of Experimental Genetics, Helmholtz Zentrum münchen, German Research Center for Environmental Health (GmbH), Germany and training course of molecular biology (XChIP and MeDIP), Epigenetic course at INRA, Jouy-en-Josas, France. Her thesis is focus primarily on establishment of ES cells and differentiation in to cardiomyocytes.