Review Articles

Difficulties in derivation of stable porcine embryonic stem cell lines

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Abstract

To establish stable ES cell lines by using embryos as an isolation source in the pig has not been reported; therefore, the discovery of effective protocols to produce stable ES cells in the pig is of importance in order to further apply this information to contribute to the improvement of human regenerative medicine, biotechnology and agriculture. In this review, it is of a focus to generally provide the information dealing with characterisation of stable ES cells among mammalian species, some obstacles and possible gaps will be proposed and discussed in order to succeed in production of instead of produce stable ES cells in the pig including provision of some results in derivation of farm animal ES-like cells derived by our group.

Keywords: Porcine embryonic stem cells, Self-renew, Differentiation, Mechanisms

ความยากลำบากในการทำสเต็มเซลล์ตัวอ่อนของสุกร

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บทคัดย่อ

การสร้างเซลล์ไลน์ของสเต็มเซลล์ตัวอ่อนโดยใช้ตัวอ่อนเป็นแหล่งในการผลิตสเต็มเซลล์ในสุกรนั้น ยังไม่เคยมีรายงานมาก่อนว่ามีผู้ที่ประสบความสำเร็จในการทำอย่างแท้จริง ดังนั้น การค้นหาวิธีการทำที่มีประสิทธิภาพในการผลิตเซลล์ไลน์ของสเต็มเซลล์ตัวอ่อนในสุกรนั้น จึงมีความสำคัญเป็นอย่างมากในแง่ที่ว่าจะได้นำเอาข้อมูลที่ได้ไปใช้ต่อยอดในการปรับปรุงการรักษาทางด้านการแพทย์ของมนุษย์ทางด้านเทคโนโลยีชีวภาพ และทางด้านการเกษตรต่อไป เนื้อหาของบทความนี้จะเน้นให้ข้อมูลต่างๆ โดยทั่วไปที่เกี่ยวข้องกับการอธิบายลักษณะการสร้างเซลล์ไลน์ของสเต็มเซลล์ตัวอ่อนในสัตว์เลี้ยงลูกด้วยนม อุปสรรคและช่องว่างต่างๆ ที่สามารถพบได้จากการการสร้างเซลล์ไลน์ของสเต็มเซลล์ตัวอ่อนจะถูกนำเสนอและอธิบายเพื่อให้เกิดผลสำเร็จในการสร้างเซลล์ไลน์ของสเต็มเซลล์ตัวอ่อนในสุกรต่อไป รวมทั้งนำเสนอข้อมูลบางส่วนในการสร้างเซลล์ไลน์ที่มีคุณสมบัติเทียบเคียงกับสเต็มเซลล์ของตัวอ่อนในปศุสัตว์ของคณะผู้ทำวิจัยเองด้วย

คำสำคัญ : สเต็มเซลล์ตัวอ่อนของสุกร การคงสภาวะของสเต็มเซลล์ การเปลี่ยนแปลงสภาพของสเต็มเซลล์ไปเป็นเซลล์อื่นๆ ในร่างกาย กลไกการควบคุม

INTRODUCTION

Recently, researching on stem cells seems to be the hottest issue in developmental biology. It is believed that the specific properties of stem cells may provide the great hope in curing a variety of degenerative diseases (Brevini *et al.,* 2012). Generally, stem cells can be isolated from developmental embryos and adult cells. Numbers of embryonic stem (ES) cell research studied in human increase rapidly when compare to adult stem cells. It is because ES cells could differentiate to be certain types of cells in three germ layers e.g. ectoderm, mesoderm and endoderm, but ethical problems are the limitations of ES cell research. Whereas, adult stem cells have some difficulties in isolation and maintenance of undifferentiated adult stem cells in culture system, and have a more definite expansion and restricted potency than ES cells (Nardi, 2005; Pouton and Allsopp, 2005). Therefore, to avoid the difficulties of human rights and political policies of ES cell research isolated from embryos is to use animals as the medical models (Keefer *et al.,* 2007).

Since the first successful isolation and characterisation of ES cells has been reported from mouse blastocysts (Evans and Kaufman, 1981), intensive attempts to derive ES cell lines from other mammals have been studied to find out the most suitable animal models specific to each medical disease. Unfortunately, the establishment of ideal ES cell lines has only been reported only in the mouse and rat so far (Evans and Kaufman, 1981; Buehr *et al.,* 2008; Brevini *et al.,* 2012). These ES cells reach all of the definitions of ES cells, which can be determined using 1) *in vitro* technique examinations e.g. staining and investigation of self-renewing and differentiated gene expressions, embryoid body formation and determination of morphology of undifferentiated and differentiated cells, and 2) *in vivo* evaluation e.g.Formation of teratomas in mice and production of species specific chimeras with germline transmission, which it is the best key to evaluate stable ES cell line establishment, as summarised in Table 1. However, primate ES cells are acceptable to be counted as stable ES cell lines even they do not have all of the ideal ES identity. It is because creating any chimera from primate ES cells especially for human is absolutely prohibited due to the restriction of ethics (Thomson *et al.,* 1995; Thomson *et al.,* 1998).

CHALLENGES AND PROBLEMS IN ESTABLISHING STABLE PORCINE EMBRYONIC STEM CELLS

Domesticated farm animals, especially for the pig have highly potentials to be a very good medical model for human diseases contributed to fulfill the suitable strategies for regenerative medicine treatments before any new therapeutic applications provided by those ES cells are applied to human. This is because they share more phylogenic appearances e.g. physiological responses, life span and body size with human than other mammanls, except for non-human primates. Also, they have less serious on critical topics dealing with ethics than those for non-human primates. Moreover, to have some stable ES cell lines isolated from farm animals would benefit in their own agricultural development and biotechnology (Keefer *et al.,* 2007; Brevini *et al.,* 2012).

Timing to establish porcine embryonic stem cells

To succeed in generation of stable porcine embryonic stem (pES) cell lines originated from the early epiblast ES cell origin proposed by the model of mouse embryonic stem (mES) and rat embryonic stem (rES) cells, and the late epiblast ES cell origin proposed by the model of mouse epiblast stem (mEpiSC), rat epiblast stem (rEpiSC) and human embryonic stem (hES) cells (Brons *et al.,* 2007; Tesar *et al.,* 2007; Buehr *et al.,* 2008; Li *et al.,* 2008; Ying *et al.,* 2008), is to use the equivalent conditions manipulated to obtain mES and mEpiSC cells in consideration with the basic knowledge of the pig biology and new drug development as much

Table 1 Self-renewal and pluripotent evaluation methods used to confirm the establishment of embryonic stem cells in mammals.

Evaluation methods	Characteristics	Early epiblast ES cell origin	Late epiblast ES cell origin
Self-renewal evaluation	Morphology	Distinct domed-like colony with small round cells, a high ratio of nuclear/cytoplasm	Distinct flatten colony with large flat cells, a high ratio of nuclear/cytoplasm
	Expression of genes and markers		
	- AP	+	+/-
	- OCT-4, SOX-2	+	+
	- NANOG, REX-1, DPPA-3	+	Low/-
	- CDX-2	-	+
	Cellular signalling path way controls		
	- Positive feedback	LIF, BMP-4, WNT	bFGF, Activin/nodal, WNT
	- Negative feedback	bFGF	BMP-4
Pluripotent evaluation	Pluripotent abilities	Formation of embryonic body, embryoid body, teratomas and chimeras with germline transmittion	Same as the early one, but cannot produce chimeras
	Morphology of differentiated cells	Depend on each type of cells	Depend on each type of cells
	Expression of genes and markers		
	- AP	-	-
	- Ectoderm cells	nestin, SOX-1, PAX-6 and β-III tubulin	Same as the early one
	- Mesoderm cells	brachyury, vimentin, α-actin and α-cardiac myosin	Same as the early one
	- Endoderm cells	AFP, SOX-17 and cytokeratin	Same as the early one
	Functional abilities of differentiated cells	Depend on each type of cells	Depend on each type of cells

as possible. Not only the quality and number of embryos are important to produce stable ES cell lines, but also the embryonic stages may play a crucial role in resolving this problem due to the differences in embryonic development between mice and the pig in terms of the gastrulation controls and their general appearances before implantation (Blomberg *et al.,* 2008a; Arnold and Robertson, 2009; Brevini *et al.,* 2012). Basically, *in vivo* murine pre-implantation blastocysts composed of trophectoderm and inner cell mass (ICMs) are formed around day 3.5. Then, the ICMs of expanded embryos will give rise to epiblasts and primitive endoderm (also known as hypoblast cells) no later than day 4.5, whose epiblasts commonlly give rise to early epiblast ES cells (Brook and Gardner, 1997; Tesar *et al.,* 2007; Arnold and Robertson, 2009). After implantation, a cavity is found in the centre of the epiblasts and the embryo elongates along the proximal-distal axis to yield the late stage called the egg cylinder. This late stage of epiblasts can still be used for production of stable ES cell lines originated from the late epiblast origin (Brons et al., 2007; Tesar *et al.,* 2007). In contrast to the pig, the formation of epiblasts begins at hatching process and is completed around day 12 (Vejlsted *et al.,* 2006). The *in vivo* derived porcine blastocysts develop distinct ICMs on day 5 or 6; then, spherical embryos will hatch from zona pellucida and the formation of undefined epiblasts starts on late day 6 or 7 before ICMs develop to be hypoblasts on days 8 and 9 (Flechon *et al.,* 2004). Naturally, porcine embryos remain detached from the uteri for trophoblastic elongation and increase in their diameter, coincident with the whitish embryonic disc, a source of late epiblast stage producing ES cells, has been developed from early epiblasts in order to await for implantation around day 16 or 17 (Vejlsted *et al.,* 2006). Crucially, vimentin, one of the key markers of mesoderm differentiation, has been found since day 9 of *in vivo*-derived porcine blastocysts (Prelle *et al.,* 2001). This suggests that *in vivo* porcine pre-implantation blastocysts between days 6 and 8, which are theoretically equivalent to murine embryonic stages for deriving ES cells, may be the most suitable stage to derive early epiblast ES cells, and the later stage of porcine embryos could be used to establish the ES cells of late epiblast origin.

Characterisation of porcine embryonic stem cells

Recently, pES-like cells could be derived from the ICMs of *in vitro fertilised* embryos at day 7 and the parthenogenetic ones at day 6 when they were cultured in the feeder-dependent culture system at the lower density of mouse embryonic fibroblasts, (MEFs, 25 x 10^4 cells/well in 4-well dishes) commonly used to derive mammalian ES cells, together with either supplement of LIF or bFGF, or both of these two factors in the culture. These ES-like cell lines have reached almost satisfaction of the *in vitro* self-renew characteristics of ES cells and pluripotent abilities (Brevini *et al.,* 2010). In brief, their pES-like cells were small round cells with a high ratio of nucleus to cytoplasm in a distinct colony and they could be passaged more than 45 times. They were also stained positive with alkaline phosphatase (AP), the most common marker used to determine undifferentiated ES and ES-like cells across the mammalian species (Talbot *et al.,* 1993a). Moreover, these pES-like cells expressed some self-renewal proteins e.g. OCT-4, NANOG and SSEA-4, including genes e.g. OCT-4, NANOG, SOX-2 and REX-1. They could form embryoid bodies (EBs) and spontaneously differentiated into three embryonic germ cells including expression of some differentiated protein markers e.g. vimentin, cytokeratin 17 and desmin, and differentiated genes e.g. BMP-4, NF-H and α-amylase.

In our experiments, some ES-like cells were produced from *in vivo-derived* porcine embryos at day 6-8, *in vitro fertilised* bovine blastocysts at day 7-9 and parthenogenetic blastocysts of ovine at day 6-7 while they were cultured in the feeder culture system at a very low density of MEFs (2-4 x 10^4 cells/well in 4-well dishes) supplemented with LIF and bFGF in the culture media (Table 2 and Figure 1). Only epiblast-producing pESB-

like cells exhibit almost the self-renew properties of ES cells in association with pluripotent abilities *in vitro* (Thansa *et al.,* 2007; Thansa *et al.,* 2008; Thansa, 2009).

Generally, our pES-like cell could be derived only from the ICMs isolated by laser technique and the epiblasts. ICMs and epiblasts could attach on MEFs within 24 hours of culture (Table 2), as seen in previous reports (Talbot *et al.,* 1993a; Talbot *et al.,* 1993b; Talbot *et al.,* 2001), while those isolated cells attached on MEFs later than a day were not capable of producing the primary outgrowth of ICMs and epiblasts. These indicate that an ability of porcine embryos that have already developed epiblasts to adhere to the feeders and to form the outgrowth of cells is better than those exhibiting ICMs. It is possibly due to the higher levels of vitronectin and β-integrin-I, which are suggested to play some roles in cell attachment and forming the isolated cell outgrowth in the pig (Brevini *et al,* 2010). However, some bovine embryonic stem (bES)-like and sheep embryonic stem (sES)-like cells could also be derived in our experiments even their developed ICMs embryos attached on MEFs later than 24 hours of culture. Anyway, these ES-like cells could be maintained in the culture for up to 5 passages in the bovine and 3 passages in the sheep, before all of them spontaneously differentiated. These state that culture conditions used in our studies are still not suitable to produce stable ES cell lines in cattle and sheep. Also, it is possible that the shorter time the isolated cells used to attach on feeders, the more possibility to get good quality of ES-like cells would be.

According to the morphology of pES-like cells, two types of pES-like cells; pESA-like and pESB-like cells were observed in the cultures between days 2 and 18, as seen in Figure 1. The outgrowth of epiblast-producing pESA-like cells using intact blastocyst isolation at day 8 of culture (Figure 1A) and pESA-like cells at passage 8 (Figure 1C) showed a number of small round cells with a high ratio of nucleus to cytoplasm spreading individually in the culture. The outgrowth of epiblast-producing pESA-like and pESA-like cells were stained positive with AP (Figure 1B), but only the source producing pESA-like cells at passage 8 expressed AP enzyme (Figure 1D). While the outgrowth of epiblast-producing pESB-like cells isolated by mechanical technique at day 5 of culture (Figure 1E) and the colony of pESB-like cells at passage 5 (Figure 1G) revealed numbers of small round cells with a high ratio of nucleus to cytoplasm in a distinct domed-like colony and AP staining was also positive with the outgrowth of epiblast-producing pESB-like cells (Figure 1F) and pESB-like cells at passage 5 (Figure 1H). In agreement with these results, the outgrowth of ICMs-producing pESB-like cells isolated by laser technique at day 8 of culture had the similar morphology of undifferentiated cells to the epiblast-producing pESB-like cells (Figure 1I) and they were also stained positive with AP (Figure 1J). For bES-like cells, the outgrowth of ICM-producing bES-like cells isolated by mechanical technique at day 11 of culture demonstrated a number of small round cells with a high nucleus to cytoplasmic ratio in a distinct flatten colony (Figure 1K) and they showed positive staining with AP (Figure 1L). In case of sES-like cells, the outgrowth of ICM-producing sES-like cells by using intact blastocyst isolation at day 3 of culture had a distinctive flatten colony containing numbers of small round cells with a high nucleus to cytoplasm ratio (Figure 1M) and they were also stained positive with AP marker (Figure 1N).

Moreover, the outgrowth of epiblast-producing pESA-like cells was not contaminated with any type of cells when they first observed on MEFs. These cells just increased the number and differentiated if they could not sustain their self-renew property. Hatched blastocysts-producing pESA-like cells were manually dissected into 4 pieces when they were passaged to the fresh MEFs every 7-10 days. One from two pESA-like cell lines could be cultured for up to 8 passages at most, together with AP staining positive with the source producing pESA-like cells, not the pESA-like cells themselves (Figure 1C to D). Unfortunately, we did not collect those

Table 2 Summary of farm animal embryonic stem cell-like cells derived from intact blastocysts, isolated ICMs and epiblasts of porcine embryos at day 6-8 of gestation, bovine blastocysts at day 7-9 and ovine blastocysts at day 6-7 cultured in mouse feeder culture system during Feb-August 2007

Species	Source of embryos	Source of ES-producing cells	Isolation technique	Attached to feeder cells	Isolation medium	Culture medium	Number of blastocysts	Primary outgrowth of ICM or epiblast obtained	Maximum passage of ES-like cells obtained
Porcine	*In vivo*	ICMs	Intact blastocysts	No	KO4bh	DM40bh	4	-	-
				No	DM40bh	DM40bh	8	-	-
			Mechanical technique	Day 2-3	KO4bh	DM40bh	4	-	-
				Day 2-3	DM40bh	DM40bh	8	-	-
			Laser application	Day 1	KO4bh	KO4bh	6	1 (16.67%)	-
		Epiblasts	Intact blastocysts	Day 1	KO4bh	DM40bh	10	2 (20%)	17
				Day 1	DM40bh	DM40bh	4	2 (50%)	8
			Mechanical technique	Day 1	KO4bh	DM40bh	8	-	-
				Day 1	DM40bh	DM40bh	8	4 (50%)	5
Bovine	*In vitro fertilisation*	ICMs	Intact blastocysts	Day 1-2	KO4bh	KO4bh	2	1 (50%)	5
				Day 2-4	DM40bh	DM40bh	8	3 (37.50%)	3
			Mechanical technique	Day 1-2	KO4bh	KO4bh	4	-	-
				Day 2-3	DM40bh	DM40bh	7	1 (14.29%)	3
Ovine	Parthenogenetic activation	ICMs	Intact blastocysts	Day 1-2	KO4bh	KO4bh	10	3 (30%)	3

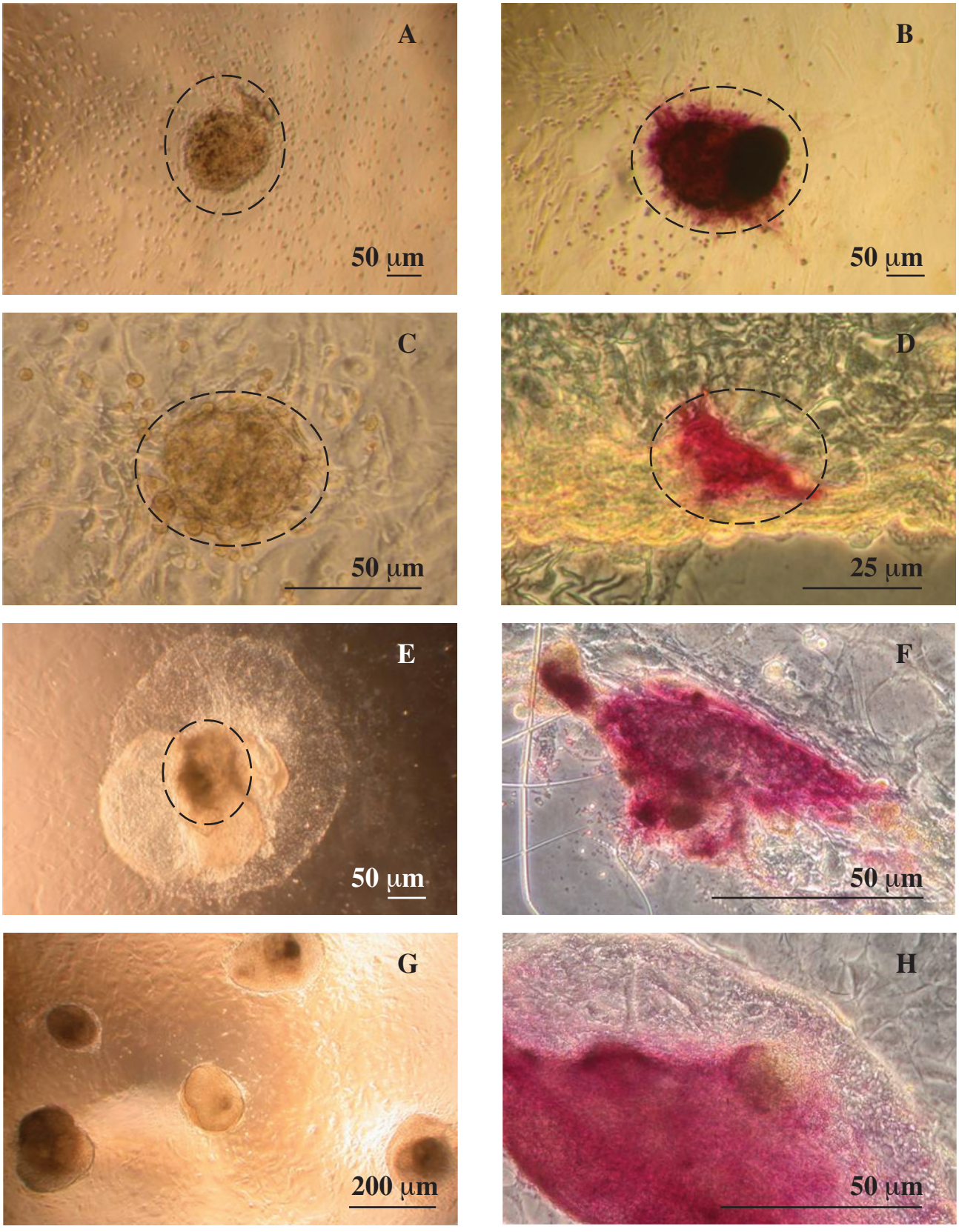

Figure 1 Characteristics and alkaline phosphatase staining of farm animal ES-like cells cultured in mouse feeder culture system in our study.

= Source of blastocysts producing farm animal ES-like cells

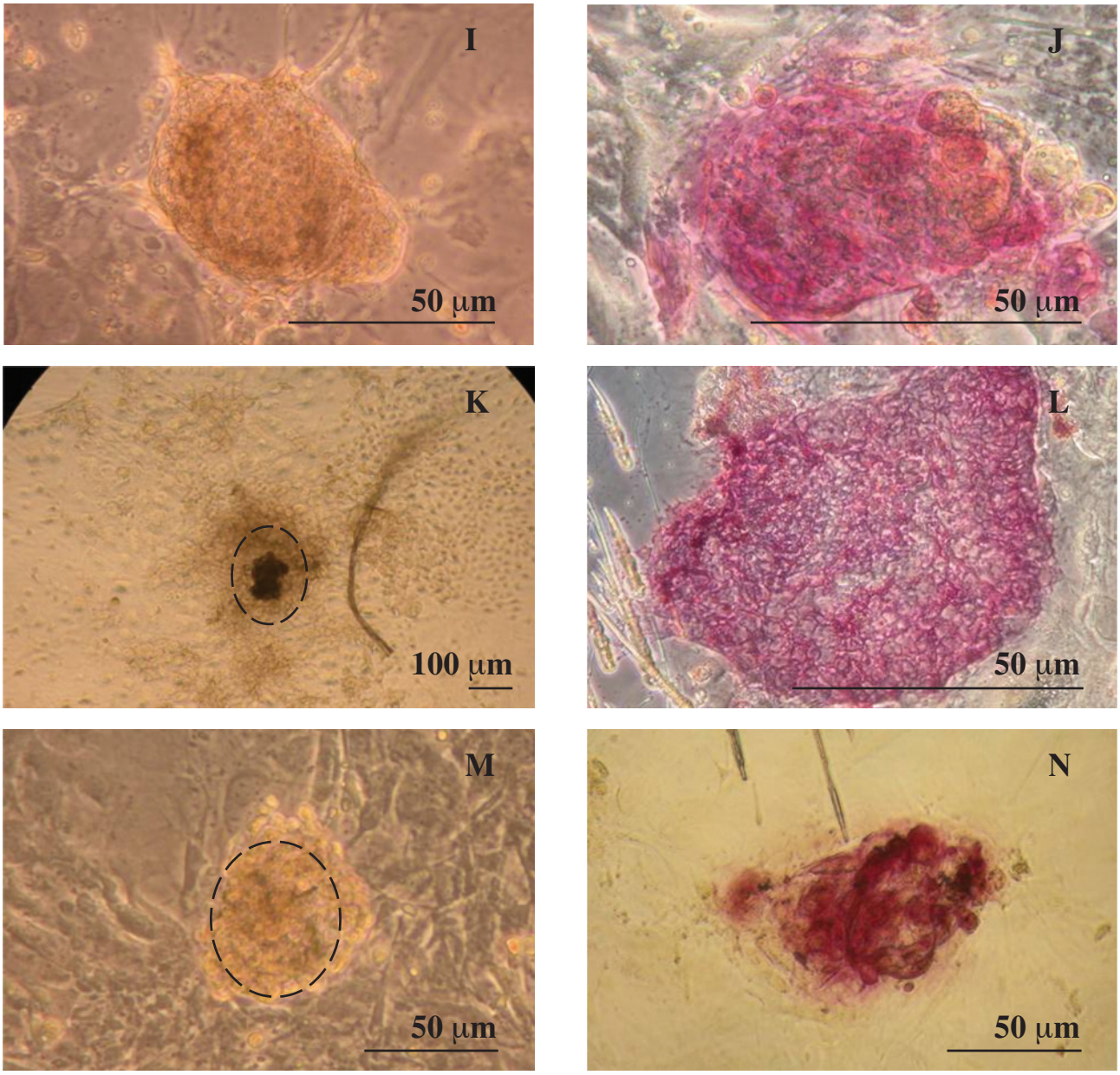

Figure 1 Characteristics and alkaline phosphatase staining of farm animal ES-like cells cultured in mouse feeder culture system in our study (continued).

= Source of blastocysts producing farm animal ES-like cells

pESA-like cells to further test for determining their self-renew characteristics. In contrast to pESA-like cells, the outgrowth of each epiblast producing pESB-like colony was mixed with other cells e.g. epithelial like cells as observed in other studies (Talbot *et al.,* 1995; Talbot and Blomberg, 2008). The pESB-like cells were collected and passaged to the new feeders every 3-5 days. Only the ICMs producing pESB-like cells were not passaged because it seemed their cell numbers did not increase in the culture. So, the colony was used to stain only with AP marker in order to determine their self-renew property. The maximum passage of pESB-like cells in our experiments was 17. In addition, pESB-like cells commonly began to spontaneously differentiate at the rim of the colony to become larger and flatter cells with finally undefined colony when their differentiation was completed. This result is similar to mES cells, but different from primate ES and other ungulate ES-like cells, whose spontaneous differentiation tends to start from the centre of the colony (Thomson *et al.,* 1995; Thomson *et al.,* 1998; Keefer *et al.,* 2007). Not only AP enzyme maker was detected in the epiblast producing pESB-like cells, but some self-renewal genes e.g. OCT-4, NANOG, SOX-2, REX-1 and DPPA-3 were also investigated. Additionally, they could be induced to differentiate into some specific cell types in three embryonic germ layers e.g. neuronal-like, supporting neuronal-like, smooth muscle-like and hepatocyte-like cells. Moreover, the expression of some specific differentiated protein markers e.g. nestin, α-smooth muscle actin and AFP, including genes e.g. nestin, α-smooth muscle actin, smooth muscle myosin, α-cardiac actin, transthyretin, albumin and hepatocyte nuclear factor 1 homeobox B were also found in these induced differentiated cells (Thansa *et al.,* 2008; Thansa, 2009).

Regarding bES-like and sES-like cells, the outgrowths of them were derived in the cultures between days 3 and 11 and they were contaminated with other types of cells e.g. epithelial-like and trophectoderm-like cells, as reported in other works (Talbot *et al.,* 1995; Roach *et al*., 2006). The bES-like and sES-like cells were selected to passage to the new feeders every 10-15 days. Additionally, they started differentiating spontaneously from the edge of the colony as similar to pESB-like and mES cells. Unfortunately, we did not collect some bES-like and sES-like cells to further test with other undifferentiated and differentiated methods.

To sum up the results obtained from Brevini *et al.* and our group, it could be concluded that our pES-like cells possibly have a potential to be stable ES cell lines originated from the early epiblast origin if they could further use to produce teratomas in the immuno-suppressive mice and create chimeras with germline transmittion.

Furthermore, one group could establish their porcine epiblast stem cell (pEpiSC)-like cell lines from isolated epiblasts of *in vivo*-derived embryos at day 10.5-12 after insemination cultured in feeder culture system at the normal density of MEFs, (50×10^4 cells/ well in 4-well dishes) commonly used in mammals, supplemented with bFGF in the culture. These pEpiSC-like cell lines demonstrate all key self-renewal characteristics of EpiSC cells and pluripotent capabilities *in vitro* (Alberio *et al.,* 2010). In brief, these pEpiSC-like cells had a distinct flatten colony containing large flat cells with a high ratio of nucleus to cytoplasm. They were stained negative with AP enzyme and expressed some core self-renew proteins e.g. OCT-4 and NANOG. In addition, they had some core pluripotent gene markers e.g. OCT-4, NANOG, SOX-2 and nodal

without the detection of REX-1 expression. These results are in agreement with those reported in the mEpiSC and hES cells (Mitsui *et al.,* 2003; Brons *et al.,* 2007). Moreover, they could form EBs, spontaneously differentiated into three embryonic germ cells and could be induced into neuronal-like cells, together with the expression of some specific differentiated proteins and genes to each embryonic germ layer cell types. These records state that pEpiSC-like cells derived from this group have a high potential to be stable ES cell lines originated from the late epiblast origin if they are able to further produce teratomas in the immunosuppressive mice.

Therefore, these give more data to discover the most suitable culture conditions to establish ES and ES-like cells in the pigs in feeder and non-feeder culture systems. Also, these recent data confirm that ES cells possibly share the similar pattern of biological mechanisms in terms of self-renew and differentiation, but different in terms of genetic sequences and its own pattern of development among mammalian species.

Factors affecting self-renew and differentiation of early and late epiblast embryonic stem cell origins in the pig.

Many recent studies have shown that not only early and late epiblast ES cells have their own extracellular signals controlling their mechanisms of self-renew and differentiation, but they also share the same key transcriptional factors sustaining self-renewal state regulated by three outstanding signalling transduction pathways; 1) receptor tyrosine kinases (RTK) as represented by the effects of LIF and FGF, 2) transforming growth factor-β (TGF-β) as demonstrated by the action of BMP-4, activin and nodal, and 3) wingless (Wnt). Otherwise, transcriptional factors e.g. OCT-4, SOX- 2 and NANOG may be activated directly from some lipid soluble factors that can directly bind to the certain nuclear receptors controlling self-renew and differentiation mechanisms. Also, most extracellular ligands exert their effects via at least one intracellular signalling pathway when the molecules bind to their own transmembrane protein receptors, consequently in additive, synergic or inhibiting effects of the cell functions regulated by a cascade of cell-cell communication (Niwa *et al.,* 2006).

It is a state of the art that OCT-4, SOX-2 and NANOG are essential factors in regulating embryonic development and identity of ES cells (Boyers *et al.,* 2005). Some previous works have reported that porcine blastocysts express OCT-4, while SOX-2 is detected at low levels and NANOG is not determined (Blomberg *et al.,* 2006; Hue *et al.,* 2007; Hall *et al.,* 2009). The collaboration between OCT-4 and SOX2 functions is capable of stimulating NANOGgene activities. So, this propose that the interaction has not been established resulting in no detection of NANOG in porcine blastocysts (Alberio *et al.,* 2010). In the murine blastocysts, OCT-4, SOX-2 and NANOG are investigated in the ICMs. These indicate that ICMs have some pluripotent properties (Boyer *et al.,* 2005; Silva *et al.,* 2009). While, the expression of these self-renewal factors in porcine and ovine embryos is delayed until they are found in the epiblast stage (Guillomot *et al.,* 2004; Alberio *et al.,* 2010) suggesting that the ICM is a transitional stage and does not have the pluripotent properties. These possibly explain why ES cell line establishment from the ICMs of farm animal blastocysts are so difficult. Also, some reports have shown that ICMs derived from porcine blastocysts at day 8 have an epiblast phenotype after culture for 2-3 days, and

then they differentiate in a short period of time (Blomberg *et al.,* 2008b). Therefore, it is possible that using embryos that have already developed epiblast has a better chance to successfully establish stable ES cell lines in domesticated farm animals.

To begin with possible factors affecting the control of self-renewal and differentiation states of the early epiblast ES cell origin in the pig proposed by the model of pES-like cells, it is recently demonstrated that both LIF and bFGF are claimed to be essential to derive the outgrowth and produce pES-like cells. These pES-like cell lines also express some intracellular signalling moleculese.g. STAT3, FGFR-2, AKT, PI3K and PTEN, but LIFR and gp130 are not detected (Brevini *et al.,* 2010). These results are in agreement with no detection of LIFR and gp130 in the ICMs of *in vivo*-derived porcine embryos at day 6 after in semination (Hall *et al.,* 2009). However, only LIFR, not BMP-4, is found in ICMs and epiblasts of *in vivo*-derived porcine blastocysts at day 8 cultured for 24 hours (Blomberg *et al.,* 2008b). These possibly mean that LIF-JAK-STAT3 pathway does not play an important role in self-renewal state of pES-like cells derived from the ICMs of embryos at day 6, but activation through FGF-PI3K-AKT cascade to inhibit the activity of GSK3-β. Consequently, β-catenin and STAT-3 are accumulated resulting in maintenance of self-renewal ground state of pES-like cellsinstead. Then, LIF-JAK-STAT3 pathway probably cooperates with the FGF-PI3K-AKT signalling pathway to support self-renewal mechanisms of pES-like cells derived from the porcine embryos at the later stage, but should not be later than 8 days after insemination, as it is well-explained in mES cells (Niwa *et al.,* 2006).

Regarding the possible chemical molecules affectingthe regulation of self-renew and differentiation mechanisms of the late epiblast ES cell origin in the pig proposed by the model of pEpiSC-like cells, Alberio *et al.* 2010 report that inhibition of activin and nodal mechanisms induces pEpiSC-like cells to differentiate into neuronal cells. These results are similar to those in mEpiSC and hES cells (Mitsui *et al.,* 2003; Brons *et al.,* 2007). It means that the signalling pathway of activin and nodal plays a critical role in self-renewal mechanisms of pEpiSC-like cell lines. On the other hand, inhibition of LIF-JAK-STAT3 cascade does not alter the self renewal and pluripotent abilities of pEpiSC. This confirms that LIF-JAK-STAT3 signalling pathway does not play a master role in maintenance of pEpiSC-like cells, as similar to those in mEpiSC and hES cells (Brons *et al.,* 2007, Tesar *et al.,* 2007). Moreover, administration of BMP-4 could induce pEpiSC-like cells to differentiate to the trophoblastic lineage as seen in mEpiSC (Brons *et al.,* 2007) and hES cells (Xu *et al.,* 2002). Supplement of BMP-4 could also induce pEpiSC-like cells to differentiate togerm cells as similar to the results shown in the mouse (Lawson *et al.,* 1999) and hES cells (Kee *et al.,* 2006). These data indicate that pEpiSC-like cells have some critical pathways controlling the mechanisms of self-renew and pluripotency similar to the mEpiSC and hES cells. However, some more studies are needed to be further investigated in order to complete the story of self-renew and differentiation regulation in pEpiSC-like cells, which the information will be applied to use for derivation of pEpiSC-like cells in non-feeder culture system.

To sum up,if the factors affecting self-renewal and differentiation mechanisms have been clearly drawn in the pig, it can be used to establish true pluripotent

ES cell lines in pigs including other domesticated ungulates, as seen that addition of some inhibitors involving in the regulation of self-renew and differentiation mechanisms of ES cells could generate true mES and rES cell lines (Buehr *et al.,* 2008; Li *et al.,* 2008; Ying *et al.,* 2008).

DISCUSSION

Considering the failure and success of establishing pES-like cell lines previously reported (Prelle *et al.,* 2002; Keefer *et al.,* 2007; Thansa *et al.,* 2008; Thansa, 2009; Brevini *et al.,* 2010) in association with the information described earlier in this review, it is believed that the embryo and culture conditions are the most important factors to provide stable ES cell lines. It is seen that even different embryonic stages, isolation techniques and culture conditions are used, but some pES-like cell lines could be successfully reproduced (Thansa *et al.,* 2008; Thansa, 2009; Brevini *et al.,* 2010). This means that the culture conditions are suitable to some embryos used to derive these pES-like cells, as described in the part of possible factors affecting the self-renew and differentiation controls in the early epiblast ES cell origin. If the complete story of database of intrinsic properties of developing embryos at each stage and their general appearances are set up, it will greatly help in the selection of culture conditions related to their developed receptors and intracellular signalling cascades in order to activate the self-renew mechanism as much as possible resulting in the success of derivation of stable ES cell lines. Not only the source of producing ES-like cell should be considered, but the technique used to produce embryos is also important. To our concerns, *in vivo*-derived embryos are the best choice for deriving ES cells due to having more numbers and better quality of ICMs and epiblasts than any other sources, thereby increasing the chance of establishing ES cell lines (Bavister, 2004). While, the embryo reproduced by IVF technique would be the second choice because polyploidy and polyspermy could be found (Li *et al.,* 2003) resulting in abnormality and low rate of ES cell line establishment. In case of the parthenogenetic embryo, even they are proposed to be an alternative way to use for overcoming the topics on ethics and politics dealing with using fertilised embryos to derive ES cells, especially for human, but it is a suggestion not to use them. This is because they have high incidences of abnormality in polyploidy, the control of insulin growth factor and apoptotic rate (Newman-Smith and Werb, 1995; Hao *et al.,* 2004). Although, the percentage of pES-like cells derived from parthenogenetic embryos is significantly higher than those from the IVF-derived embryos in the pig (Brevini *et al.,* 2010), but it is still in doubt whether anyone would try to confirm them as true pES cell lines by creating chimeras as it is well-known that they are haploidy.

As for the isolation techniques used to isolate the source-producing ES cells to derive ES cell lines, the best technique cannot be identified exactly as they have their own advantages and disadvantages. It is because even those isolated ICMs or epiblasts are damaged during the isolation processes, but could positively grow if the culture conditions are suitable for them. Other wise, the isolated cells would die resulting from culturing in improper culture systems. If the differentiated cells have been found nearby the outgrowth of ES-like cells due to contaminated the source-producing ES cells with some somatic cells during the isolation process, the ES-like cells will be selected to further culture in the fresh culture system when they are ready to be passaged.

Regarding culture conditions used in Brevini, Alberio and our groups, it implies that there should be a proper ratio of the outstanding factors controlling both early and late epiblast ES cells. When the ratio is changed, ES cells are driven to differentiate to be other types of cells, as prior described in this review. If a very low density of MEFs is used to derive ES cells, FCS and other ontological molecules that play some important roles in controlling self-renewal mechanisms of ES cells e.g. LIF and bFGF are considered to be supplemented in the culture. If a normal density of MEFs is managed to establish ES cells, KSR and other ontological substances are suggested to be added in the culture system. It is because MEFs is believed to be the main source affecting the balance between self-renew and differentiation states, as they produce numbers of both undifferentiated and differentiated factors in the culture (Prowse *et al.,* 2007). That is why FCS, another well-known source composed of both self-renew and differentiated factors (Freshney, 2005), is suggested to be supplemented in the culture conditions using a very low density of MEFs. While KSR, a modified solution containing some constituents to avoid the unwanted effects as seen in the serum (Freshney, 2005), is recommended to be added in the culture system using a normal density of MEFs. Yet, a combination between dosages of FCS, KSR and other ontological factors to be added in the culture are still needed to be further investigated in order to find out the most suitable culture conditions for deriving stable ES cell lines practically.

As far as our concerns, there should be three ways of choices to derive and improve the protocols for establishing ES and ES-like cell lines in feeder-dependent culture system in the pig, which the knowledge could be further applied to use with other domesticated animals. That is firstly to try to get some ES-like cell lines from such protocols shown that their ES-like cells have reached at least the acceptable keys of *in vitro* self-renew and pluripotent characteristics in order to validate the consistency of ES-like cell production. The second one is to try to use some inhibitors, activators or their combination dealing with the regulation of self-renew and differentiation of the early epiblast ES cell origin proposed by the model of pES-like cells previously explained above to generate ES-like cells in the certain protocol that could produce pES-like cells in order to improve the protocol of derivation and compare the results obtained, as seen that stable mES and rES cell lines could be established by using some inhibitors dealing with self-renew and differentiation controls of ES cells (Buehr *et al.,* 2008; Li *et al.,* 2008; Ying *et al.,* 2008). The last choice is to try to set up new culture conditions based on the information of feeder-dependent and feeder-independent culture system, together with the balance between administration of some known factors affecting self-renew and differentiation mechanisms of ES cells e.g. FCS, KSR, LIF and bFGF in the culture media under suitable environments (Evans and Kaufman, 1981; Thomson *et al.,* 1998; Ludwig *et al.,* 2006; Brons *et al.,* 2007; Brevini *et al.,* 2012). However, it is still a long way to successfully derive ES and ES-like cell lines in non-feeder culture system including using serum-free culture conditions in the pig.

In conclusion, some critical factors are needed to be supplemented to the culture media at the right embryonic stage under suitable conditions in order to succeed in deriving and sustaining ES cell lines. Additionally, immortal ES cells naturally derived theoretically have more than one pattern of ES cell

production, as two patterns producing pES-like cells were observed by our team. Finally, mammals probably share similar pattern of basic ES cell biology in terms of self-renewal and differentiation mechanisms, but different in the sense of species specific evolution. However, it is still a long way to go for establishing some ideal ES cell lines in the pig.

SUMMARIES

To succeed in derivation of some stable pES cell lines originated from the early epiblast ES cell origin, the distinctive epiblasts of *in vivo*-derived porcine blastocyts no later than day 8 after insemination are hightly recommended to be used as the source producing ES cells, while the ICMs of *in vitro fertilised* porcine blastocysts would be considered as the second choice. The isolation techniques e.g. intact and mechanical isolation are favoured to be performed than other methods. Also, mouse feeder culture system is still needed for production of ES cells in a balance between the density of feeders and concentration of some exogenous supplements e.g. FCS, KSR, LIF, bFGF or else into the culture. If the lower density of feeders is prepared to derive ES cells, FCS and some ontological factors are suggested to be added into the culture medium. While, KSR and some ontological molecules are proposed to be supplemented in the medium that normal density of feeders is used. Crucially, culture medium is recommended to be changed daily after ES-like cells are derived at or around the same time in order to keep the proper ratio of the concentration between self-renewal and differentiated factors in the feeder culture system.

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