

ESTABLISHMENT OF EMBRYONIC STEM CELL-LIKE CELLS IN PIGS

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ABSTRACT

We generated unique porcine embryonic stem cell-like cells (pESLCs) by using inhibitors of GSK3 β and MEK1. The pESLCs colonies are dome-shape morphology and show the expression of undifferentiated markers including OCT4 and NANOG. Moreover, the self-renewal capacity and morphology of the cell lines are LIF-dependent, consistent with the LIF receptors expression and STAT3 phosphorylation, and continuous culture for over 100 passages was possible without any overt morphological changes and telomerase activity.

Keywords: Pig, Embryonic Stem Cells (ESCs), LIF, Self-renewal, Inhibitor(s)

INTRODUCTION

In 1981, embryonic stem cells (ESCs) were established in mouse (Evans and Kaufman 1981) and many researchers have been attempting to establish ESCs in other species. ESCs are derived from the inner cell mass (ICM) of the blastocyst or pre-blastocyst stage embryos and have self-renewal and pluripotency, including the ability to differentiate into germ cells. Since Evans *et al.* reported regarding pESLCs in 1990, many studies have painstakingly attempted to establish authentic porcine ESCs (Vackova *et al.* 2007, Hall 2008, Brevini *et al.* 2010, Telugu *et al.* 2010). However, the contribution of porcine ESCs to the germ cell lineage remains elusive, not having been able to show the self-renewing capacity of the cell lines. During the process of establishing porcine ESCs, the ICM-derived cells easily differentiate or become extinct after only a limited number of passages in culture. This means that protocols applicable for the establishment of mouse and human ESCs cannot be applied in the case of pig; namely, methodology and culture conditions have not been established. Additionally, the crucial undifferentiated markers are unknown in pig.

Leukemia inhibitory factor (LIF) is a cytokine that is best known for inhibiting the differentiation of mouse ESCs and promoting their self-renewal by means of Stat3 phosphorylation. However, studies on signaling cascades of mouse ESCs led to new findings for the mechanisms of the ground state of ESCs self-renewal. FGF/Erk signaling pathway is also involved in the self-renewal and differentiation of mouse ESCs (Kunath *et al.* 2007), that is, initiation of differentiation is triggered by stimulation of FGF/Erk signaling. Ying *et al.* revealed that blockage of this pathway with the FGF receptor inhibitor SU5402 or the Erk signaling inhibitor PD184352 or PD0325901 in combination with the GSK3 β inhibitor CHIR99021 is sufficient to maintain mouse ESCs in an authentic pluripotent state (Ying *et al.* 2008). These inhibitors efficiently supported the establishment and maintenance of not only mouse ESCs (Ying *et al.* 2008, Ohta *et al.* 2009, Hanna *et al.* 2009) but also rat ESCs (Buehr *et al.* 2008, Li *et al.* 2008), which could contribute to the germline population and genome transmission. On this basis, we examined the effect of these inhibitors in establishing porcine ESCs (Haraguchi *et al.* 2013).

THE ESTABLISHED pESLCs

Effects of inhibitors

The outline of the establishing process is shown in Fig. 1. The cell lines were designated as established cell lines when they were grown satisfactorily after freezing and thawing. To evaluate the effects of GSK3 β and MEK1 inhibition, the respective inhibitors CHIR99021 and PD184352 were added to the culture medium and the pESLCs were cultured. Several remarkable changes were observed in the pESLCs following culture in the medium supplemented with inhibitors. Firstly, the morphology of the colonies changed to a compacted state with high cell density and close cell-cell boundaries, an increase in cell height and smooth colony edges were also observed (Fig. 1). Secondly, the doubling time of the cells was shortened, allowing the colonies to be passaged every 2–3 days as compared to 4–7 days in medium without the inhibitors. The pESLCs could be maintained in continuous culture without cell differentiation or cellular senescence.

Expression of “undifferentiated markers”

In pig, crucial markers for undifferentiated state are unknown. Therefore, we initially examined their morphology and alkaline phosphatase (AP) activity. The isolated colonies began to grow as a monolayer with coarse cell-cell boundaries, in which the cells exhibited polygonal boundaries, high nuclear/cytoplasmic ratios, and abundant lipid-like inclusions, with AP-activity (Fig. 1). We confirmed expression of SSEA1, SSEA4, OCT4, and NANOG proteins in established pESLCs (Fig. 1). As commercially available antibodies are not always cross-reactive with pig proteins, in some cases it was necessary to rely on evaluation by mRNAs expression analysis.

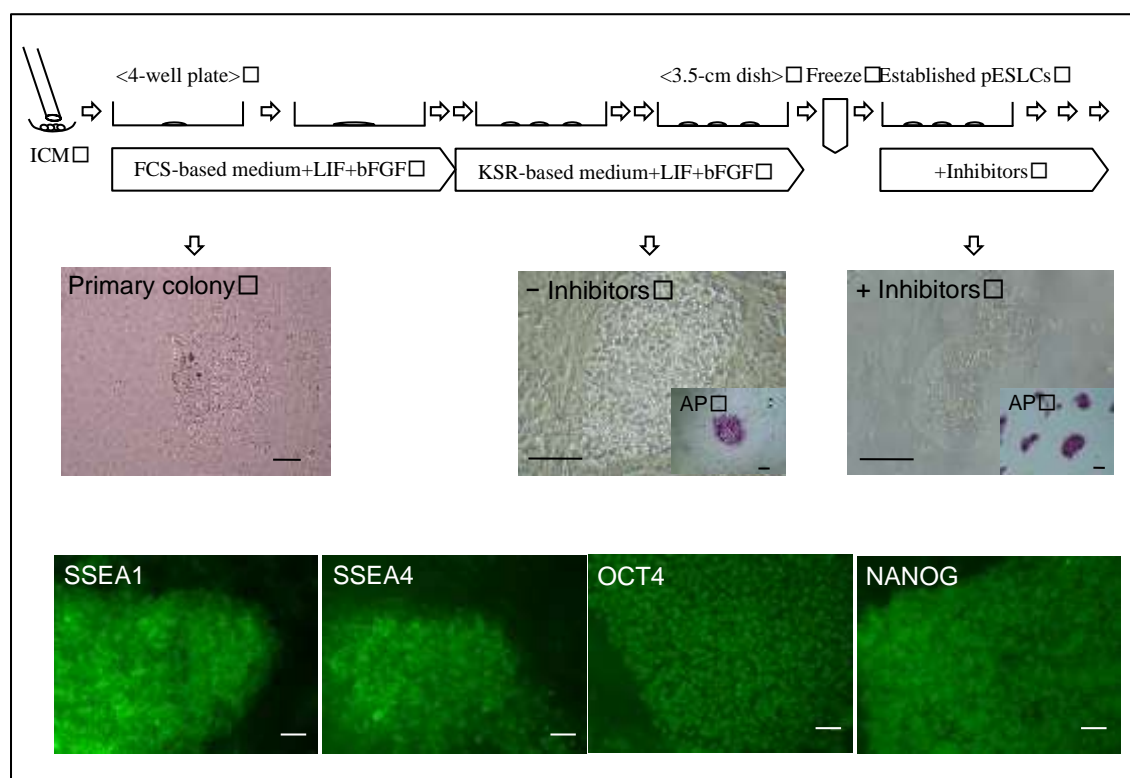


Fig. 1. Outgrowth, isolation of initial stage of pESLCs and establishment of the pESLC lines by using GSK3 β and MEK inhibitors. Adapted from Haraguchi *et al.* J Reprod Dev 2012; 58: 707-716.

LIF-dependent growth

We supplemented both LIF and bFGF into medium for initial stage of outgrowth culture. We confirmed that bFGF is not necessary for self-renewing of pESLCs. On the other hand, the pESLCs cannot maintain the self-renewing capacity in LIF-deprived medium, indicating that the growth of pESLCs is LIF-dependent (Fig. 2). The pESLCs cultured with LIF-supplemented medium show the activation (phosphorylation) of STAT3 and expression of LIF-receptors. Hence, LIF/STAT signaling is functional in the pESLCs similar to mouse ESCs.

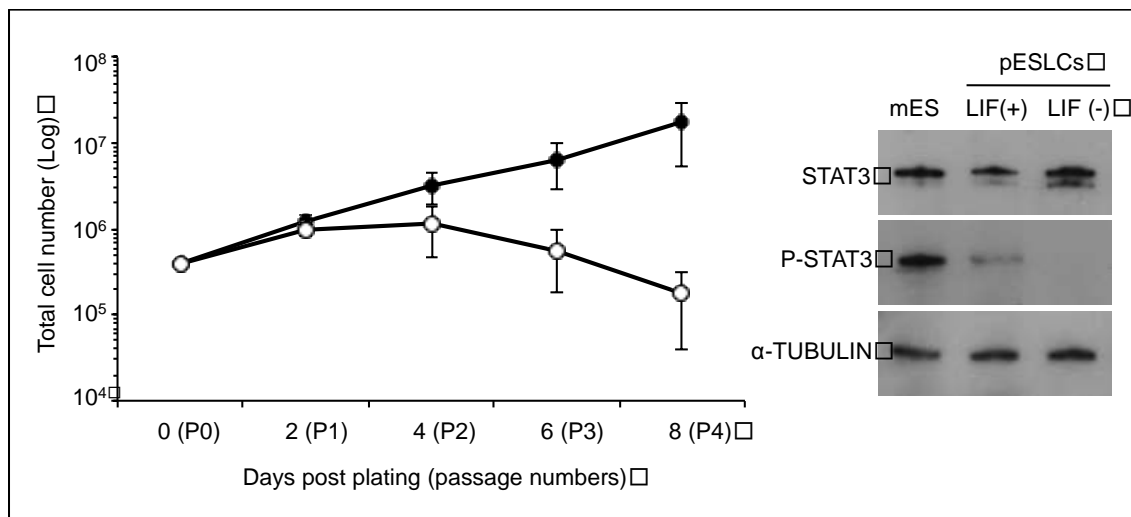


Fig. 2 LIF-dependent growth of the pESLCs. Adapted from Haraguchi *et al.* J Reprod Dev 2012; 58: 707-716.

Cell dispersion

We routinely use CTK (collagenase-trypsin) and TE (trypsin-EDTA) in combination. Alternatively, Accutase® is also available for cell dispersion at single use. However, a Rock inhibitor, such as Y-27632 is also necessary for cell dispersion. Without the Y-27632, the cell viability dramatically reduces and cannot maintain the passage-culture.

Stable cell lines

We attempted to establish the stable cell lines expressing marker genes such as GFP and LacZ. The expression vectors have neomycin resistant gene so that the transfected-cells can be selected by using neomycin analog, G418. Finally, we succeeded to obtain stably transfected cell lines that are useful for lineage analyses of the cells.

Embryoid body and Teratoma formation assay

The pESLCs can form spheres efficiently when they are subjected to suspension culture (Fig. 3). We examined the teratoma formation assay by inoculation of either the pESLCs or the spheres into immunodeficient mice. Unlike mouse ESCs, typical teratoma formation could not be observed either at the macroscopic and tissue levels at 2 months post-innoculation (Fig. 3). Although we expect that muscle tissue (mesoderm) and cartilage tissue (ectoderm) exist in HE stained tissue sections, confirmation by staining with the appropriate antibodies is required. For evaluation of the pluripotent potential of the established cell lines, we consider that teratoma formation assay is necessary before moving to chimera analysis.

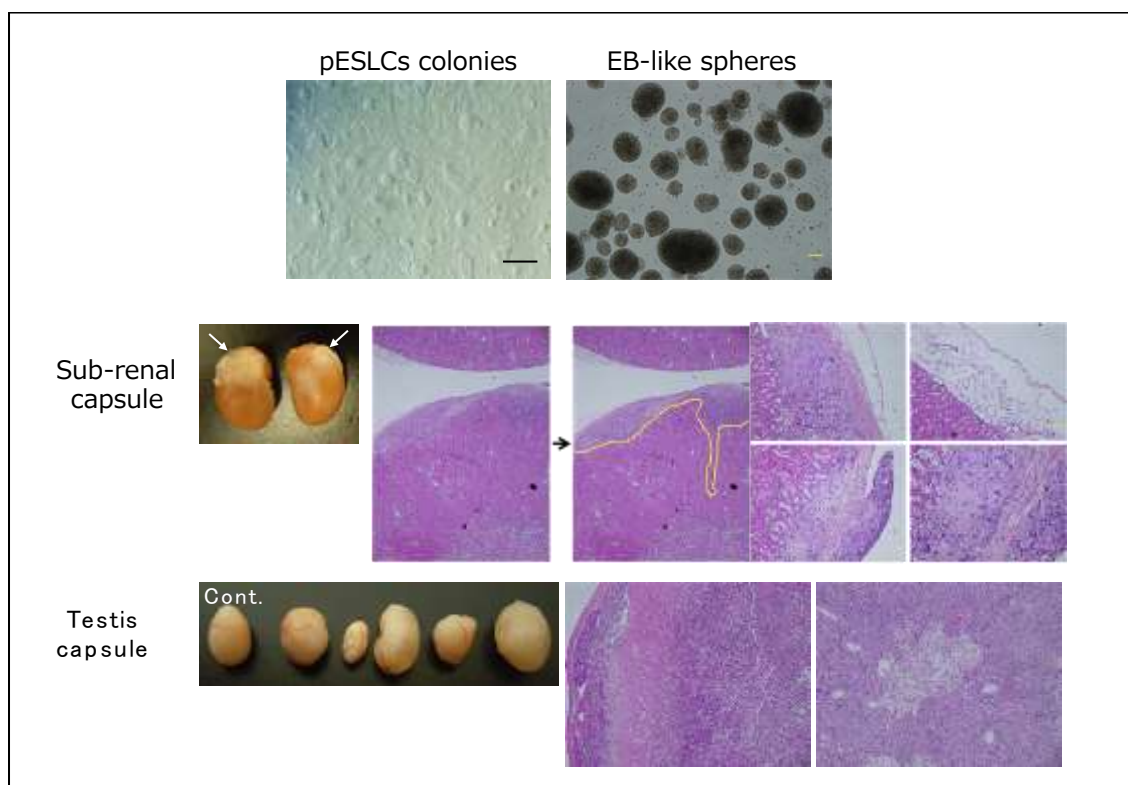


Fig. 3. Teratoma formation assay.

Features of mouse and human ESCs, and pESLCs

Table 1 shows characteristics on mouse and human ESCs, and established pESLCs. In general, ESCs are categorized into two groups from the features, naïve (mouse) and primed (human), respectively. In 2007, pluripotent epiblast stem cells (EpiSCs) are established from late epiblast layer of post-implantation mouse and rat (Brons *et al.* 2007). The features are quite similar in human ESCs; both ESCs and EpiSCs have the ability for teratoma formation but EpiSCs do not contribute to chimera. This may be attributed to differences of developmental stage rather than a species-specific effect. Upon the process of establishing porcine ESCs, we compared the characteristics to that of mouse ESCs. It is not necessarily to declare that putative established porcine ESCs are in the naïve state if the features are agreement with those of mouse ESCs. After all, whether the putative porcine ESCs are “authentic” or “-like”, they need to be confirmed by their contribution to germ-line chimera. The pESLCs established in our laboratory partly indicate the naïve features; the dome-shaped colony, LIF-dependent growth, self-renewal response to inhibitors, and obtainable stable cell lines after drug selection, but still do not show clear pluripotency. X chromosome activation in both alleles (XaXa) may be one of the hallmarks for naïve state as previously reported.

Table 1. Features of mouse ESCs, human ESCs, and pESLCs

Category	Mouse ESCs	Human ESCs	pESLCs
Colony	Dome-shape	Flat	Dome-shape
Growth	○	△	△
Factor	LIF (LIF/STAT3)	bFGF (FGF/ERK)	LIF (LIF/STAT3)
Single cell culture	○	△ (Rock inhibitor)	○ (Rock inhibitor)
Stable clone	○	×	○
X-chromosome	XaXa	XaXi	XaXi
Inhibitors (GSK3 β , MEK)	Self-renewing	Differentiation/ cell death	Self-renewing
Teratoma	○	○	?
Chimera	○	?	?

CONCLUSION

Establishment of true porcine ESCs is no longer necessary if the purpose of the use is to produce a gene modified pig, as it is possible to develop genetically modified pig by nuclear transfer technology (Suzuki *et al.* 2012) and genome editing approaches such as TALEN (Bedell *et al.* 2012) and CRISPR-Cas9 (Wang *et al.* 2013). Particularly, genome-editing technologies will be considered mainstream in the future. Although experimental verification of ESCs contributing to germ cells have only been demonstrated in mouse and rat, the possibility of the authentic ESCs derived from other species is still unknown. There may be an argument that the mouse and rat are rather unique species. Use of domestic animals can be used to verify this by experimental approach and in the case of pig, there is potential for large contribution to medical science and livestock industry.

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