NOTE *Theriogenology*

Generation of liver-specific TGF-α/*c*-Myc-overexpressing porcine induced pluripotent stem-like cells and blastocyst formation using nuclear transfer

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ABSTRACT. Transgenic porcine induced pluripotent stem (iPS) cells are attractive cell sources for the development of genetically engineered pig models, because they can be expanded without senescence and have the potential for multiple gene manipulation. They are also useful cell sources for disease modeling and treatment. However, the generation of transgenic porcine iPS cells is rare, and their embryonic development after nuclear transfer (NT) has not yet been reported. We report here the generation of liver-specific oncogenes (TGF-α/*c*-Myc)-overexpressing porcine iPS (T/M iPS)-like cells. They expressed stem cell characteristics and were differentiated into hepatocyte-like cells that express oncogenes. We also confirmed that NT embryos derived from T/M iPS-like cells successfully developed blastocysts *in vitro*. As an initial approach toward porcine transgenic iPS cell generation and their developmental competence after NT, this study provides foundations for the efficient generation of genetically modified porcine iPS cells and animal models.

KEY WORDS: hepatocyte, induced pluripotent stem cell, nuclear transfer, porcine, transgenic

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Transgenic porcine pluripotent stem cells are attractive cell sources for the development of genetically engineered pigs for use in biomedical research on topics, such as cancer development and genetic deformation [7, 14, 26]. One of the major challenges to producing genetically modified pigs is the lack of highly proliferative cells without aging, similar to pluripotent stem cells [14]. Pluripotent stem cells would allow for multiple gene manipulation, selection and screening procedures for multiple gene modifications. Embryonic stem (ES) cells have been used extensively for creating transgenic mice, and the use of ES cells allowed for easy DNA manipulation through the insertion of transgenes [18–20]. Several attempts have been made to generate ES-like cells in porcine, with limited success [9, 10, 23]. Owing to the undefined condition of undifferentiated ES cells and their limited capacity of expansion, the somatic cell nuclear transfer method has been used for generating transgenic pigs [1].

Other application fields of transgenic porcine pluripotent stem cells are disease modeling and drug screening [2, 4, 24]. Previously, such research relied on primary cell culture or immortalized cell lines; however, these cells have limitations to expansion or differentiation [2, 4]. By comparison, transgenic porcine pluripotent stem cells could be practical tools as disease-in-a-dish models, because these cells are an unlimited source of proliferating cells that can be differentiated into most cell types.

In the recent few years, some research teams have generated porcine iPS cells that possess similar characteristics as ES cells [5, 6, 22, 27]. However, the generation of genetically modified porcine iPS cells is rare, and their nuclear transfer (NT) efficiency is unknown. The aims of this study were to generate transgenic porcine iPS cells and investigate their *in vitro* embryonic development after NT.

In order to generate transgenic porcine iPS cells, transgenic porcine fibroblasts that overexpress two proto-oncogenes, TGF-α and *c*-Myc (T/M-fibroblasts), linked with the pig albumin promoter, were obtained according to our established methods [11]. Figure 1A illustrates the process of transgenic porcine iPS cells production. Briefly, a retrovirus containing human, *SOX2*, *Oct4 Klf4* and *Myc* (pE4 vector) was produced, and an MOI 10 of the virus was used to infect 5×10^4 T/M-fibroblasts (passage 17) in a 35-mm cell culture dish. Five days after infection, the 5×10^4 T/M-fibroblasts were transferred to a mitomycin-C-treated mouse embryonic fibroblast feeder (MEF) layer in a 60-mm dish. Next, in order to identify the most efficient condition for generating transgenic TGF-α/*c*-Myc porcine iPS (T/M iPS)-like cells, diverse culture compositions were applied. Ten days after the viral infection, iPS cell-like initial colonies were generated, and alkaline phosphatase (AP) staining was performed to compare the efficiency of colony generation (Fig. 1B and 1B'). These results demonstrated that Knockout DMEM (Gibco, Waltham, MA, U.S.A.) containing 20% Knockout serum replacement (KSR; Gibco) with 10 *n*g/m*l* basic fibroblast growth factor (bFGF; Millipore, Billerica, MA, U.S.A.) was the most effective in generating initial iPS cell-like colonies; however, 10^3 units of leukemia inhibit-

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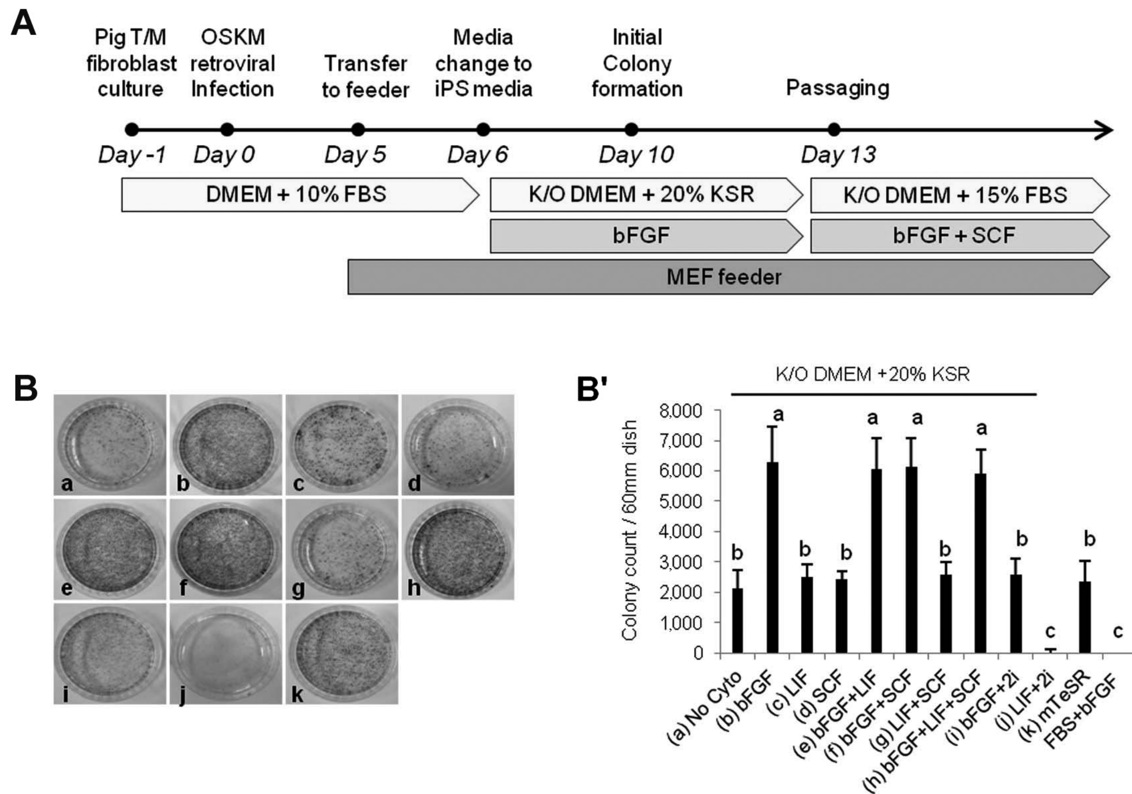

Fig. 1. Generation of transgenic porcine induced pluripotent stem (iPS)-like cells and optimization of their culture condition. A: Scheme for the generation of transgenic porcine iPS-like cells. Liver-specific TGF-α/*c*-Myc (T/M)-overexpressing fibroblasts were infected with retroviruses encoding *Oct4*, *Sox2*, *Klf4* and *c-Myc* (OSKM). Five days post infection, the cell were seeded onto feeder cells. From the following day, the cells were maintained with Knockout (K/O) DMEM containing 20% Knockout serum replacement (KSR) with 10 *n*g/m*l* basic fibroblast growth factor (bFGF). Initial colonies appeared after 10 days of viral infection. After passaging, the cells were maintained with K/O DMEM containing 15% FBS with 10 *n*g/m*l* bFGF and 40 *n*g/m*l* stem cell factor. B-B': AP staining was performed to compare the efficiency of initial colony formation. The number of initial colonies generated in the 60-mm dish was counted. K/O DMEM containing 20% KSR with 10 *n*g/m*l* bFGF was the most effective for colony formation.

ing factor (LIF; Millipore) and 40 *n*g/m*l* stem cell factor (SCF; Prospec, East Brunswick, NJ, U.S.A.) did not induce a synergic effect. Two small molecule inhibitors (2i), 0.8 μM PD0325091 and 3 μM CHIR99021 (Selleck, Houston, TX, U.S.A.), significantly reduced the colony generation rate, even in the bFGF-containing medium. mTeSR medium (Stem Cell Technologies, Vancouver, BC, Canada) was not efficient for generating initial colonies. In our preliminary study, Knockout DMEM containing 15% FBS (ES grade; Hyclone, Logan, UT, U.S.A.) with bFGF was not suitable for initial iPS cell-like colony generation. The initial iPS cell colonies were manually passaged onto the MEF feeder and stably maintained over 35 passages with medium containing Knockout DMEM, 15% FBS, 0.1 mM nonessential amino acid (Gibco), 0.1 mM 2-mercaptoethanol (Gibco), 10 *n*g/m*l* bFGF and 40 *n*g/m*l* SCF.

In order to confirm the pluripotency of the transgenic porcine iPS-like cells, a characterization of the cells was carried out. As shown in Fig. 2A, insertion of the pCMV-TGF-α and pCMV-*c*-Myc vectors into the genomic DNA of T/M iPS-like cells was confirmed. Figure 2B revealed that T/M iPS-like cells expressed pluripotent genes, including *SOX2*, *Oct4*, *Klf4*, *Nanog*, *Rex1*, *Tdgf* and *Tert.* In Fig. 2C, the cells demonstrated flat and round shapes and were positive for AP. For embryonic body (EB) formation, T/M iPS-like cells were manually picked and transferred to a low attachment dish with differentiation medium (the same as iPS cell maintenance medium without cytokines). At 3–5 days after cultivation, cystic EBs formed. In order to investigate their ability to differentiate into the 3 germ layers, EBs were re-plated onto 0.1% gelatin-coated cell culture plates with differentiation medium for 14 days to induce spontaneous differentiation. In Fig. 2D, immunostaining revealed the expression of 3 germ layer markers; namely, neurofilament for the ectoderm, smooth muscle actin for the mesoderm and keratin7/17 for the endoderm markers. In Fig. 2E, the T/M iPS-like cells stained positively for OCT4, SOX2, Nanog and SSEA-4. Next, to test if the T/M iPS-like cells induce liver formation, hepatocyte differentiation was performed using previous protocols with some modifications [21]. Because

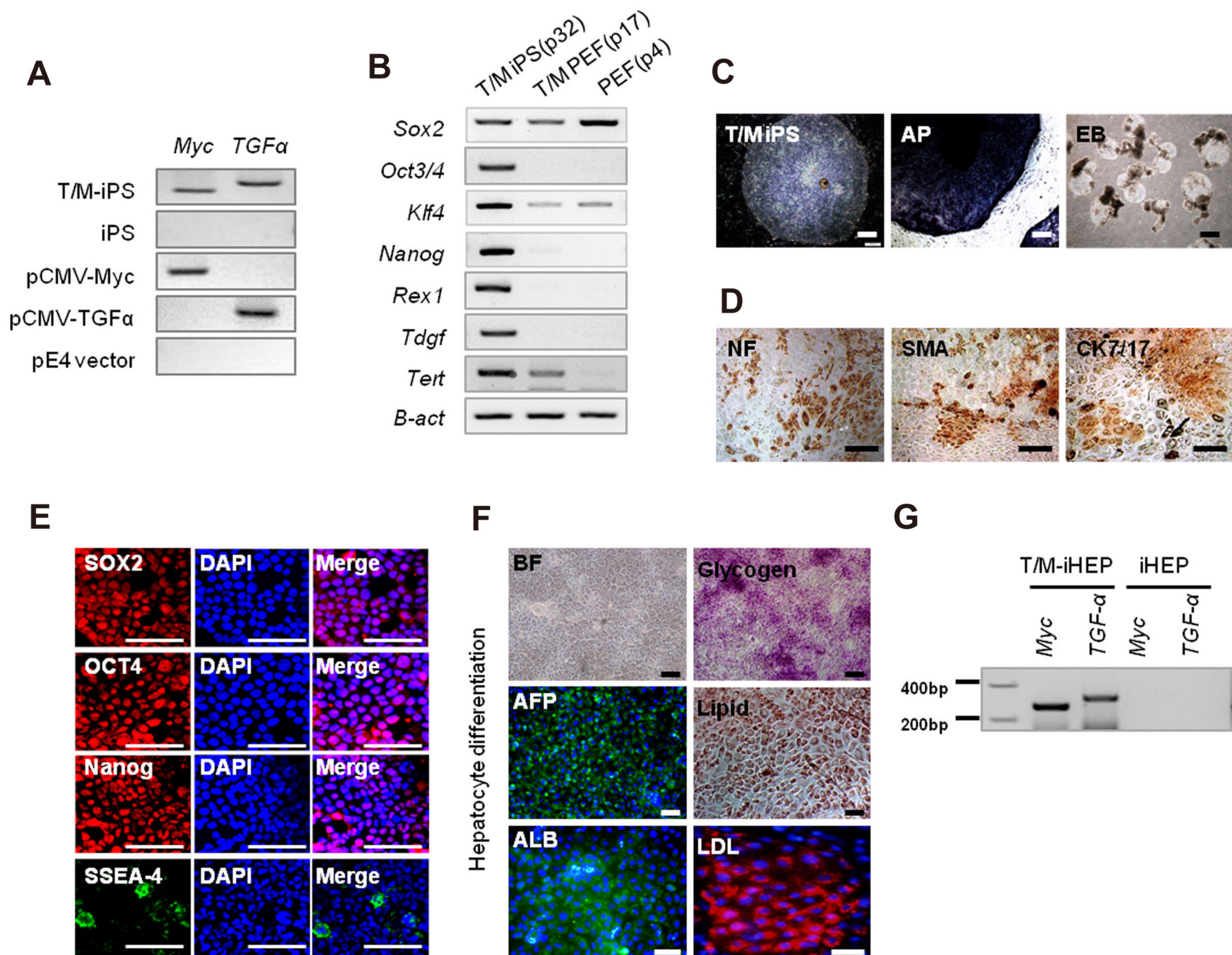

Fig. 2. Characterization of liver-specific TGF-α/*c*-Myc-overexpressing porcine induced pluripotent stem (T/M iPS)-like cells and oncogene expression in the T/M iPS-like cell derived hepatocyte-like cells. A: pCMV-TGF-α and pCMV-*c*-Myc vectors were confirmed in the T/M iPS-like cells, using PCR analysis. B: T/M iPS-like cells expressed pluripotent markers in RT-PCR. C: T/M iPS-like cell colonies showed round and flat shape and were positive for alkaline phosphatase (AP) staining. Cystic embryonic bodies (EB) developed. Bar=200 μm. D: The spontaneously differentiated cells showed various cell types of 3 germ layers, including neurofilament (NF), alpha smooth muscle actin (SMA) and keratin 7/17 (CK7/17), upon immunostaining. Bar=100 μm. E: T/M iPS-like cells were positive for stem cell markers, including SOX2, OCT4, Nanog and SSEA-4, upon immunostaining. Bar=50 μm. F: T/M iPS-like cell derived hepatocyte-like cells demonstrated a polygonal shape under a bright field microscope (BF), and it expressed alpha-fetoprotein (AFP) and albumin (ALB) upon immunostaining. The differentiated hepatocyte-like cells had liver characteristics, such as glycogen and lipid storage and low-density lipoprotein (LDL) uptake. Bar=100 μm. G: T/M iPS-like cell derived hepatocyte-like cells (T/M-iHEP) expressed two oncogenes, Myc and TGFα; however, genetically intact porcine iPS-like cell derived hepatocytes (iHEP) did not.

the T/M-transgenic fibroblast was designed to generate a liver cancer model in pigs, the T/M iPS-like cells derived hepatocytes would be a beneficial cell model to research drug screening and the etiology and pathology of liver cancer. In Fig. 2F, the differentiated hepatocytes demonstrated expression of hepatic markers, including alpha-fetoprotein and albumin. Some liver characteristics, such as glycogen uptake by Periodic acid and Schiff's staining, lipid storage by Oil Red O staining and Dil-labeled low-density lipoprotein uptake, were evident. The RT-PCR results in Fig. 2G showed that T/M iPS-like cells derived hepatocytes (T/M-iHEP) expressed two oncogenes, *c*-Myc and TGF-α, but non-transgenic porcine iPS cell-derived hepatocytes (iHEP) did not.

Next, in order to investigate the development of T/M iPS-like cells NT *in vitro*, matured oocytes *in vitro* were enucleated, and a single cell of porcine skin fibroblasts, porcine iPS-like cells or T/M iPS-like cells was inserted into the perivitelline space of each enucleated oocyte. Membrane fusion and electrical activation were induced according to our previously published protocols [13]. The NT embryos were cultured at 39°C in 5% CO_2, 5% O_2 and 90% N_2 for 7 days. The cleavage and blastocyst formation were evaluated on Days 2 and 7, respectively. After Hoechst 33342 (Sigma,

Table 1. Effect of donor cell type on the *in vitro* development of somatic cell nuclear transfer pig embryos

Type of cells	Passage	Fusion	Cleavage	Blastocyst	No. of cells in blastocyst
		Number (%)	Number (%)	Number (%)	
Fibroblasts	5–6	206/243 (85.0 ± 2.9)	177/205 (86.3 ± 2.2)	57/205 (27.9 ± 2.5) [a)]	40.6 ± 2.5
iPS-like cells	23–37	192/248 (77.6 ± 3.8)	140/188 (73.9 ± 6.4)	20/188 (10.1 ± 3.6) [b)]	33.0 ± 2.9
T/M iPS-like cells	23–28	182/240 (76.4 ± 5.5)	126/175 (73.1 ± 7.6)	18/175 (11.1 ± 2.8) [b)]	34.1 ± 4.3

*Five replicates. Data are presented as mean ± SEM. a, b) Within a column, values with different superscripts are different (P<0.01).

St. Louis, MO, U.S.A.) staining, the total blastocyst cell count was obtained using an epifluorescence microscope (TE300, Nikon, Tokyo, Japan). As shown in Table 1, NT embryos that were derived from oocytes fused with porcine fibroblasts showed a higher cleavage rate (86.3% vs. 73.1%) and blastocyst formation level (27.9% vs. 11.1%) than embryos derived from oocytes fused with T/M iPS-like cells. The proportion of oocytes successfully fused with donor cells (76.4–85.0%) and the cell number in the blastocyst (34.1–40.6 cells per blastocyst) after NT were not altered by the donor cell type.

In this report, we developed porcine transgenic iPS-like cells by optimizing their culture condition, and we confirmed their blastocyst formation using NT. The T/M iPS-like cells demonstrated stem cell characteristics and expressed pluripotent markers. Previous reports have shown that supplementation with bFGF or LIF was required for porcine iPS cell culture [3, 5, 17, 22, 25]. Similarly, we found that bFGF was critical for iPS cell generation, but LIF and 2i were not, suggesting that our T/M iPS-like cells possess more similar characteristics with primed human iPS cells than with naïve mouse iPS cells. Some previously reported porcine iPS cell lines could be maintained in a serum-free condition [14, 27]; however, in this study, our T/M iPS-like cells were maintained in a serum-containing condition. Similar to our T/M iPS-like cells, many putative porcine ES cell lines required serum and bFGF for long-term maintenance [10, 12, 16]. To investigate *in vivo* differentiation ability of the T/M iPS-like cells, we performed teratoma formation assay using non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice. The cells (1 to 5 × 10^6) were injected into the mice, however, teratoma was not produced. In some previous reports, pig pluripotent stem-like cells did not produce teratoma [12, 14, 16]. Incompletely silenced transgenes of the stem cells and not well-optimized *in vivo* injection condition might interrupt teratoma formation of porcine iPS-like cells.

In this study, our T/M iPS-like cells could be differentiated into oncogene-expressing hepatocyte-like cells, and the differentiated cells showed functional liver markers, making them beneficial for studies on liver cancer and treatment.

The iPS or ES cells have been used for NT to generate cloned offspring in mice, and they demonstrated higher blastocyst efficiency than fibroblasts (33–43% vs. 5.8%) [28]. By comparison, our results presented that porcine iPS cells did not show improved blastocyst formation rate compare to the fibroblasts. We speculate that the incompletely down regulated exogenous transgenes of porcine iPS cells might be one of the causes for interrupting blastocyst development. In a previous report, *in vitro* blastocysts development rate of nuclear transfer embryo derived from porcine iPS cells using traditional cloning method was 5.3 to 14.8% [8]. Similar to our results, that study also speculated that incompletely silenced foreign transcription factors interrupted blastocyst formation and cloned piglet production. The insertion of transgenes, using a virus or vector, into genomic DNA for reprogramming has been used as a general approach for porcine iPS cell generation [5, 14, 15]. Regulating stem cell differentiation or histone modification using chemicals, such as Scriptaid, histone deacetylase inhibitor, was beneficial to improve cloning efficiency of porcine iPS cells [8]. And also, novel strategies to make transgene-free porcine iPS cells might be more beneficial for increasing the NT efficiency of porcine iPS cells.

In conclusion, our study offers the optimal generation and culture conditions for transgenic porcine iPS-like cells and shows blastocyst formation using NT *in vitro*. Although there remain some challenges for increasing the cells' practical value, this study nevertheless offers a foundation for the practical use of transgenic porcine iPS cells and the efficient development of genetically engineered pigs.

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