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LETTER TO THE EDITOR

Piglets cloned from induced pluripotent stem cells

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Dear Editor,

Embryonic stem (ES) cells are powerful tools for generating genetically modified animals that can assist in advancing our knowledge of mammalian physiology and disease. Pigs provide outstanding models of human genetic diseases due to the striking similarities to human anatomy, physiology and genetics, but progress with porcine genetic engineering has been hampered by the lack of germline-competent pig ES cells. To overcome this limitation, genetically modified pigs have been produced using genetically modified somatic cells and nuclear transfer (NT). Yet, somatic cells exhibit limited proliferative capacity and have an extremely low frequency of homologous recombination compared to ES cells. Hence, only a few knockout pig models have been reported thus far using standard gene-targeting approaches.

Remarkably, induced pluripotent stem cells (iPSCs) have been generated by reprogramming somatic cells from multiple mammalian species using defined cocktails of transcription factors [1]. Mouse iPSCs have passed the crucial test of germline contribution and generation of all-iPSC mice through tetraploid embryo complementation [2]. This demonstrated that *bona fide* iPSCs are, if not identical, very similar to ES cells. Likewise, cloned mice have been obtained from mouse iPSCs by the NT method [3]. These studies indicate that iPSCs can substitute ES cells for the purpose of genomic manipulation.

Pig iPSCs (piPSCs) have been reported by several groups [4-6]. These cell lines could be differentiated into the three germ layers *in vitro* and *in vivo*. One group claimed that the piPSCs they generated could pass the crucial test of germ line chimera production [6], but the chimerism was confirmed only by PCR detection. Therefore, whether piPSCs can be generally used as a vehicle to create gene-targeted pigs through chimera approaches, as in mice, remains an open question. Nevertheless, piPSCs are capable of long-term proliferation, which allows lengthier and potentially more sophisticated *in vitro* manipulations. More importantly, they are similar to ES cells in many aspects, suggesting that they may also have a high efficiency of homologous recombination. Hence, if piPSCs were suitable for cloning pigs by NT, gene-targeted pigs could potentially be produced more efficiently than using fibroblasts as nuclear donors. In the present study, we explored the feasibility of generating cloned pigs from piPSCs.

We employed piPSC lines generated by different groups with various strategies. The first two piPSC lines, iPF4-2 and iPM6-11, were induced from porcine ear fibroblasts and porcine bone marrow cells (pBMCs), respectively, of a 10-week-old Danish Landrace pig using a lentiviral vector overexpressing human transcription factors induced with doxycycline (DOX) [4]. The third piPSC line, hsC13, was induced from porcine fetal fibroblasts (PFFs) by retroviral overexpression of human transcription factors [5]. Characterizations of the three iPSC lines referenced above have been previously documented [4, 5]. In addition, we used three piPSC lines (JN2, KSR-4, and 5%O_2-1) produced from pBMCs or PFFs by retroviral overexpression of mouse or porcine transcription factors. The characterization of these piPSC lines is shown in Supplementary information, Table S1.

We then used these piPSCs as donor cells for reconstruction of NT embryos by traditional cloning (TC) [7] or handmade cloning (HMC) methods [8]. For the TC method, a total of 11 923 cloned embryos reconstructed with the 6 piPSC lines were introduced into 71 surrogate mothers. Among these mothers, 25 were pregnant, as detected by ultrasonography on days 24-26 following embryo transfer. As shown in Supplementary information, Table S2, five piPSC lines (except JN-2) could support NT embryos to implant *in vivo*, but none of the embryos was able to develop to term. Some surrogates maintained the pregnancy for as long as 60 days, but most of the fetuses degenerated prior to day 50 after embryo transfer. For the HMC method, 1 585 cloned blastocysts from four piPSC lines (iPF4-2, iPM6-11, KSR-4, 5%O_2-1) were transferred into 15 surrogates, but no cloned piglets were obtained (Supplementary information, Table S3). These results were consistent with embryo transfer data from other groups that were part of our joint initiative to generate piPSC-derived cloned pigs. These researchers followed a similar protocol and transferred a total of 22 260 reconstructed embryos (from 7 additional piPSC lines) to 107 recipient gilts, resulting in 41 pregnancies,

but none developed to term (Supplementary information, Table S4). Altogether, this outcome was disappointing considering that mouse iPSCs have been successfully used as donor cells for creating living cloned mice [3].

When we looked over the characterizations of the piPSC lines used in the above experiments, we noticed that, in contrast to those used in mouse, the foreign transcription factor genes had not been silenced. We thus thought that this may underlie the discrepancy of *in vivo* developmental capacity of NT embryos derived from mouse iPSCs and piPSCs. Previous studies have also demonstrated that the state of histone acetylation in somatic cell NT embryos, significantly impacts the developmental competence in several species [9].

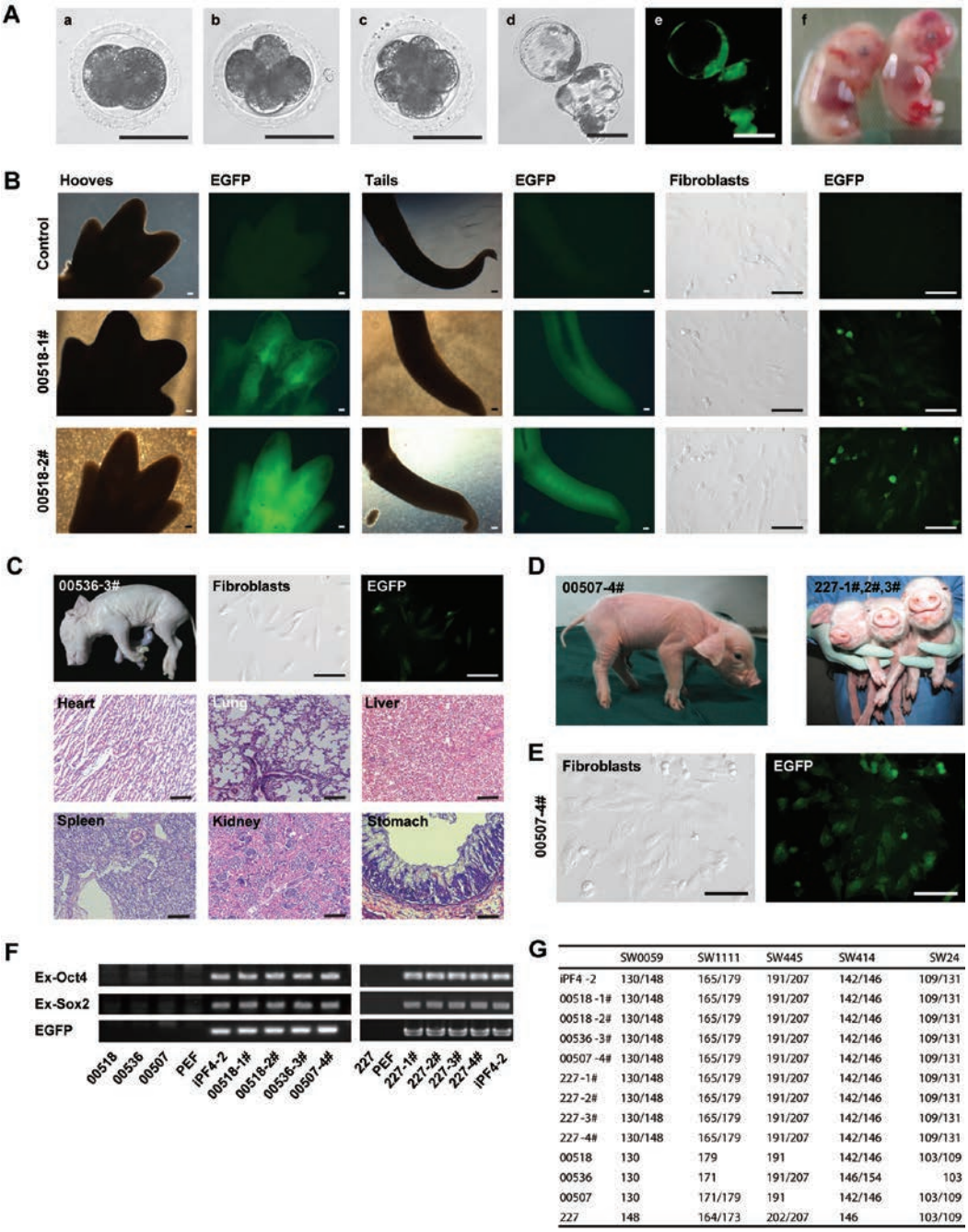
Therefore, in the second round of experiments, we took the following measures to attempt to improve the developmental capacities of cloned embryos: 1) silence the exogenous transcription factors through spontaneous differentiation of piPSCs before they are used as donor cells; 2) increase histone acetylation by treating the constructed embryos with Scriptaid, a novel histone deacetylase inhibitor that can improve cloning efficiency through increasing transcriptional activity [9]. To silence exogenous transcription genes, piPSCs were allowed to spontaneously differentiate for 4-6 days. After that, they became enlarged and flattened, and developed an epithelium-like morphology (Supplementary information, Figure S1A). For iPF4-2 and iPM6-11, the expression of all the exogenous transcription factor genes was reduced significantly after differentiation, as measured by quantitative RT-PCR (Supplementary information, Figure S1B). However, the expression of all four exogenous genes in KSR-4-differentiated cells did not decrease significantly as compare to iPF4-2 and iPM6-11 (Supplementary information, Figure S1C). Some of the exogenous genes in 5% O_2-1-differentiated cells were even expressed at higher levels than in the undifferentiated cells (Supplementary information, Figure S1C). The discrepancy between iPSC lines iPF4-2 and iPM6-11 and the other cell lines is likely due to the use of a DOX inducible system [4].

When the differentiated piPSCs were used as donor cells in the reconstruction of cloned embryos by the TC method, the resulted NT embryos had a significantly increased rate of blastocyst formation compared to those derived from the undifferentiated ones (Supplementary information, Table S5). Embryos at 2-, 4-, 8-cell, blastocyst and 36-day stages developed normally (Figure 1A). Expression of enhanced green fluorescent protein (EGFP), a transgenic marker in iPF4-2 cells, demonstrated that the blastocyst embryos were derived from piPSCs (Figure 1A). When embryos reconstructed from undifferentiated iPF4-2 by HMC were treated with 0.5 mM Scriptaid for 16 hours, the blastocyst rate was also increased significantly compared to the control group (26.0% vs 17.3%, $P < 0.05$) (Supplementary information, Table S3).

The reconstructed embryos derived from differentiated iPF4-2 and KSR-4 cells and undifferentiated iPF4-2 cell-derived NT embryos treated with Scriptaid were then transferred into surrogate mothers. A total of 545 cloned embryos from KSR-4 differentiated cells were transferred into three surrogates, two of which became pregnant but lost their pregnancies between days 30 and 50. Subsequently, a total of 1135 iPF4-2-differentiated cell-NT embryos were transferred into seven surrogate mothers, three of whom became pregnant. To detect the early development of the cloned fetuses, a pregnant surrogate was euthanized after 36 days of gestation, and two fetuses were retrieved. The two cloned fetuses (00518-1# and 00518-2#) were morphologically normal and the corresponding fetal fibroblasts grew normally and were positive for EGFP (Figure 1A and 1B).

After 113 days of gestation, a pregnant surrogate (00536) that had been implanted with NT embryos derived from differentiated iPF4-2 cells delivered a piglet (00536-3#) (Figure 1C) and a stillbirth naturally. The piglet (00536-3#) was dead when we found it and weighed 639 g. The organs had developed normally and the lungs were inflated, suggesting that it was live at birth and died of an unknown cause (Figure 1C). The other pregnant surrogate with NT embryos derived from differentiated iPF4-2 cells gave cesarean birth to a live and healthy cloned piglet (00507-4#) after 114 days of gestation. The piglet weighed 600 g at birth (Figure 1D). In the Scriptaid-treated group, 107 NT-blastocysts reconstructed from undifferentiated iPF4-2 cells by HMC were transferred to a surrogate. This surrogate was pregnant and gave birth to four live cloned piglets after 110 days of gestation (Figure 1D). The newborn piglets weighed 700, 900, 700 and 400 g.

All of the cloned piglets had the same white coat as that of the pig (Danish Landrace) from which iPF4-2 piPSCs were derived (Figure 1C and 1D), and the fibroblasts isolated from the six piglets were positive for EGFP (Figure 1C and 1E). To further ensure that the lineages of the fetuses and piglets were derived from iPF4-2 piPSCs, we analyzed Oct4, Sox2, and EGFP transgene integration, and also microsatellite DNA. Tissues from the two fetuses and delivered piglets contained the exogenous transgenes, as demonstrated by PCR (Figure 1F). Analysis of microsatellite DNA also showed that the genomes of the fetuses and piglets were the same as those of the iPF4-2 cells, but they were different from the surrogates (Figure 1G). These findings firmly demonstrated



	SW0059	SW1111	SW445	SW414	SW24
iPF4 -2	130/148	165/179	191/207	142/146	109/131
00518 -1#	130/148	165/179	191/207	142/146	109/131
00518 -2#	130/148	165/179	191/207	142/146	109/131
00536 -3#	130/148	165/179	191/207	142/146	109/131
00507 -4#	130/148	165/179	191/207	142/146	109/131
227 -1#	130/148	165/179	191/207	142/146	109/131
227 -2#	130/148	165/179	191/207	142/146	109/131
227 -3#	130/148	165/179	191/207	142/146	109/131
227 -4#	130/148	165/179	191/207	142/146	109/131
00518	130	179	191	142/146	103/109
00536	130	171	191/207	146/154	103
00507	130	171/179	191	142/146	103/109
227	148	164/173	202/207	146	103/109

Figure 1 Derivation and characterization of cloned piglets from piPSCs. **(A)** Preimplantation and post-implantation development of the cloned embryos from piPSCs. Embryos at two-cell (a), four-cell (b), eight-cell (c), blastocyst stages (d, e) and two 36 day-old cloned fetuses (f) are shown. Scale bars are 100 μm. **(B)** The morphology and fluorescence of the hooves (left), tails (middle) and fibroblasts (right) of the 36 day-old embryos. Scale bars are 100 μm. **(C)** The morphology, fluorescence and hematoxylin/eosin-stained sections of tissues from piglet 00536-3#. Scale bars are 100 μm. **(D)** Cloned piglets. 00507-4# from differentiated iPF4-2 cell, 4 days old; 227-1#, 2#, 3# from undifferentiated iPF4-2, 2 days old. **(E)** Porcine ear fibroblasts (PEFs) from 00507-4#, EGFP positive. Scale bars are 100 μm. **(F)** PCR demonstrating genomic integration of Oct4, Sox2, and EGFP using tissues of the cloned fetuses and piglets. PEF, the original fibroblasts used to create iPF4-2. 00518, 00536, 00507, 227, foster mothers. 00518-1#, 00518-2#, the cloned fetuses derived from differentiated iPF4-2 cells. 00536-3# , 00507-4#, the cloned piglets derived from iPF4-2-differentiated cells. 227-1#~4#, the HMC piglets derived from iPF4-2. **(G)** Microsatellite analysis of the donor piPSC line iPF4-2, cloned fetuses and piglets. 00518, 00536, 00507, 227, foster mothers; 00518-1# and 00518-2#, cloned fetuses; 00536-3# and 00507-4#, the cloned piglets from differentiated iPF4-2 cells; 227-1#~4#, the cloned piglets derived from the Scriptaid-treated NT embryos from iPF4-2 cells.

that the fetuses and piglets were cloned from the piPSCs.

The piglet 00507-4# died 32 days after birth. Myeloencephalitis was found (Supplementary information, Figure S2), which indicated the death was due to infection and likely unrelated to the NT procedure. One of the four piglets generated from NT embryos treated with Scriptaid died soon after birth. The others survived for 4, 14 and 16 days. It is well known that a high incidence with unknown cause of death occurs among cloned pigs made by NT [10]. Therefore, it is likely that the death of at least some piPSC-derived piglets was caused by the NT itself. Therefore, we expect that healthy pigs can be produced from piPSCs through NT if the procedure is optimized and/or more embryos are transferred to surrogate mothers.

In conclusion, our study shows that piPSCs can be used as donors for NT if the exogenous transcription factors are silenced through differentiation or by adding a histone deacetylase inhibitor to the embryos. According to our knowledge, this is the first report about a live mammal other than the mouse derived from iPSCs with nuclear transfer. In the future, this discovery may allow the generation of genetically modified pigs after gene targeting of the piPSCs. The latter could represent an efficient way to produce genetically engineered pigs.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)