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ORIGINAL ARTICLE

Reprogramming of ovine adult fibroblasts to pluripotency via drug-inducible expression of defined factors

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Reprogramming of somatic cells in the enucleated egg made Dolly, the sheep, the first successfully cloned mammal in 1996. However, the mechanism of sheep somatic cell reprogramming has not yet been addressed. Moreover, sheep embryonic stem (ES) cells are still not available, which limits the generation of precise gene-modified sheep. In this study, we report that sheep somatic cells can be directly reprogrammed to induced pluripotent stem (iPS) cells using defined factors (Oct4, Sox2, c-Myc, Klf4, Nanog, Lin28, SV40 large T and hTERT). Our observations indicated that somatic cells from sheep are more difficult to reprogram than somatic cells from other species, in which iPS cells have been reported. We demonstrated that sheep iPS cells express ES cell markers, including alkaline phosphatase, Oct4, Nanog, Sox2, Rex1, stage-specific embryonic antigen-1, TRA-1-60, TRA-1-81 and E-cadherin. Sheep iPS cells exhibited normal karyotypes and were able to differentiate into all three germ layers both *in vitro* and in teratomas. Our study may help to reveal the mechanism of somatic cell reprogramming in sheep and provide a platform to explore the culture conditions for sheep ES cells. Moreover, sheep iPS cells may be directly used to generate precise gene-modified sheep.
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Introduction

Sheep have been an important domesticated animal for a long time. Dolly, the sheep, was the first cloned mammal generated by somatic cell nuclear transfer (SCNT) in 1996 [1]. Gene modification in sheep to improve their production traits, disease resistance ability, and ability to produce valuable, high-quality proteins has been widely applied in agriculture and biomedicine [2-4]. However, traditional transgenic techniques result in the poor con-

trol of exogenous genes and extremely low efficiency, which greatly limits the application of gene-modified sheep.

The gene targeting technique based on embryonic stem (ES) cells, which has been well established in mouse [5-7], allows precise and effective gene knockin and knockout. To date, ES cells have been isolated from a few species, including mouse [5, 8], human [9], monkey [10] and rat [11, 12]. However, it is difficult to isolate and maintain ES cells from sheep embryos and other ungulates, because little is known of the characteristics and culture conditions of the ESCs from these species. Therefore, although many efforts have been made to derive sheep ES cells during the last few decades, no authentic sheep ES cell line has been reported. Although SCNT has been widely used in sheep propagation and the generation of transgenic sheep, the mechanism of so-

matic cell reprogramming in sheep remains unclear.

Somatic cells can be reprogrammed using defined factors to achieve a pluripotent status [13-15]. More importantly, induced pluripotent stem (iPS) cells have been demonstrated to efficiently contribute to the germline to allow precise genetic engineering *in vivo* [16], which means that iPS cells could be used as a substitute for ESCs to generate transgenic animals. In recent years, iPS cells have been generated from many species, including mouse [14, 17], human [13, 15], rat [18, 19] and pig [20-22]. In this study, we reprogrammed somatic cells from sheep to pluripotency using defined factors.

Results

Generating sheep iPS cell lines using a Tet-On inducible lentiviral system

We initially tried to use a constitutively expressed lentivirus vector carrying OSCK (Oct4, Sox2, c-Myc and Klf4) or OSCKNL (Oct4, Sox2, c-Myc, Klf4, Nanog and Lin28) to reprogram sheep fibroblasts. The sheep primary fibroblasts were isolated from the ears of a 4-week-old sheep, and were expanded for a few passages before virus transduction. We established a few ES-like cell lines exhibiting ES cell characteristics, including
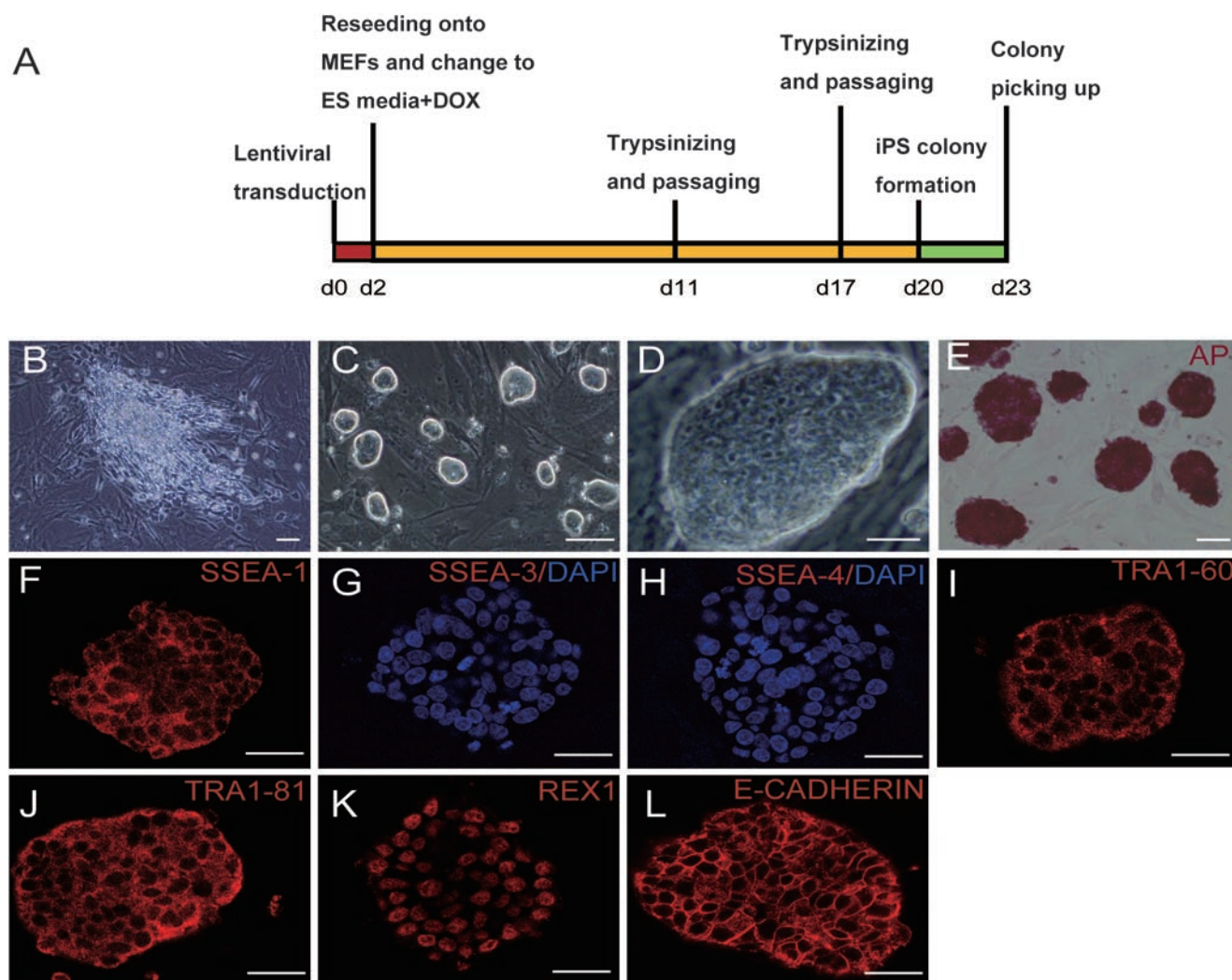
Figure 1 Ovine-induced pluripotent stem cells (oiPSCs) generated by viral transduction. **(A)** Schematic diagram of the reprogramming protocol used. **(B)** Typical non-embryonic stem (ES) cell-like colony. **(C)** Typical oiPSC colony. **(D)** High magnification of the oiPSCs. These iPSCs express the following pluripotency markers: **(E)** alkaline phosphatase (AP), **(F)** SSEA-1, **(I)** TRA-1-60, **(J)** TRA-1-81, **(K)** REX1 and **(L)** E-cadherin. oiPSCs do not express the human ES cell-specific surface antigen SSEA-3 **(G)** or SSEA-4 **(H)**. The images **(F-L)** of immunostaining were acquired by confocal system. Scale bars: 100 μm in **B**, **C** and **E**; 25 μm in **D** and **F-L**.

round, tightly packed morphology, alkaline phosphatase (AP) activity, expression of ES cell markers and the ability to differentiate into three germ layers *in vitro*. The reprogramming efficiency was 10 AP-positive colonies from 1×10^5 fibroblasts. However, these cells could not be stably expanded while maintaining an undifferentiated state. Moreover, these cells failed to differentiate into the three germ layers in teratomas, probably because of the constitutive expression of exogenous genes (data not shown).

To solve this problem, we tried to reprogram sheep fibroblasts with a drug-inducible lentivirus system. The doxycycline (DOX)-inducible transgene expression vectors were constructed, in which Oct4, Sox2, c-Myc, Klf4, Nanog and Lin28 were inserted downstream of a tetracycline operator. The expression of reverse tetracycline transactivator (rtTA) was driven by the EF-1 α promoter in a separate lentiviral vector. The DOX-inducible system has been shown to work very well [20]. The cocktail of lentiviruses was used to transduce sheep fibroblasts. However, the efficiency of generating AP-positive colonies was extremely low (about one AP-positive colony from 1×10^5 fibroblasts). Moreover, these AP-positive colonies could not be stably expanded. Compared with the constitutively expression system, reprogramming sheep somatic cells using the DOX-inducible system was much more difficult, probably because of the low expression levels of exogenous genes. We speculate that the traditional six transcription factors were not sufficient to reprogram sheep adult cells with the DOX-inducible lentiviral system.

The SV40 large T antigen (T) and the catalytic subunit of human telomerase, hTERT, have been reported to significantly improve the efficiency of generating iPS cells [23, 24]. Therefore, we attempted to establish ovine iPS cell lines by adding SV40 large T and hTERT.

The fibroblasts were simultaneously transduced with a cocktail of lentiviruses expressing reprogramming factors (Oct4, Sox2, c-Myc, Klf4, Nanog, Lin28, SV40 large T and hTERT) and a lentivirus constitutively expressing rtTA. At 24 h after transduction, the cells were trypsinized and plated onto murine embryonic fibroblasts (MEFs) at 2.5×10^4 cells per well in a six-well plate. The next day, the original medium (Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS)) was replaced with medium for ES cell cul-
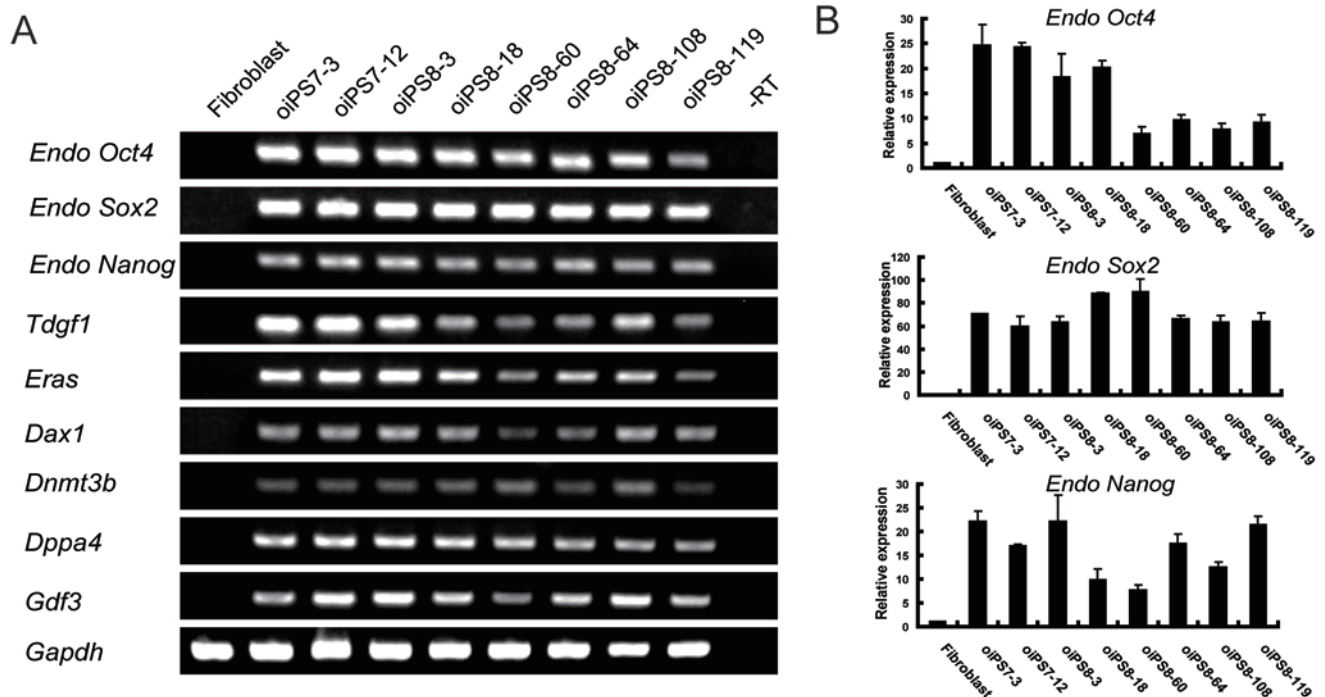
Figure 2 (A) Reverse transcription-polymerase chain reaction (RT-PCR) analysis of the oiPSC lines compared with the gene expression observed in parental sheep primary cells. Abbreviation: -RT, minus reverse transcriptase. **(B)** Quantitative reverse transcription-PCR analyses of endogenous *Oct4*, *Sox2* and *Nanog* expression in oiPSCs relative to parental somatic cell populations. oiPS7-3 and 7-12 represent sheep iPS cell lines generated with seven factors (OSCKNL+T) and oiPS8-3, 8-18, 8-60, 8-64, 8-108, 8-119 represent sheep iPS cell lines generated with eight factors (OSCKNL+T+hTERT).

ture plus DOX (DMEM/F12 + 20% SR + 1 μg/ml DOX).

At 5 days after transduction, infected fibroblasts exhibited changes in morphology and began to proliferate very rapidly. On days 11 and 17, the cells were trypsinized into a single-cell suspension and passaged. We first observed large and round ESC-like colonies with clear boundaries (Figure 1C) on day 20. At 3 days later, we picked the colonies from half of the 6-well plates into a 96-well plate for further expansion. The remaining plates were subjected to AP staining to calculate the efficiency of colony generation. The sheep ESC-like cells (named sheep iPS cells or oiPSCs) were expanded on MEF feeder cells with ES cell medium plus DOX (1 μg/ml).

In our study, we obtained sheep iPS cell lines with the combination of OSCKNL+T+hTERT, but failed when using the combination of OSCK+T+hTERT. About 41 AP-positive colonies were derived from 1×10^5 initial fibroblasts. To examine whether SV40 large T or hTERT was necessary, the two factors were withdrawn separately. Sheep iPS cells were successfully generated with the combination of OSCKNL+T, but not with the combination of OSCKNL+hTERT. Compared with mouse, human, rat and pig, whose iPS cells have been generated only with OSCK, the reprogramming of sheep somatic cells required additional factors when using the DOX-inducible lentiviral system. The addition of SV40 large T was especially important. Moreover, the efficiency of generating sheep iPS cells using all eight factors (OSCKNL+T+hTERT) was threefold lower than the efficiency of generating pig iPS cells using four factors (OSCK) with a Tet-On lentiviral system (about 170 AP-positive colonies from 1×10^5 fibroblasts [20]). These results indicate that sheep somatic cells are more difficult to reprogram than somatic cells from other species.

Sheep iPS cells exhibit typical ES cell morphology and marker expression

OiPS cells exhibited a round, tightly packed morphology characterized by a high ratio of nucleus to cytoplasm and prominent nucleoli (Figure 1D), which is quite similar to mouse and rat ESCs, but not human ESCs. Two oiPS cell lines we established, oiPS8-108 and oiPS8-119, have already been stably passaged for more than 30 generations (more than 90 days). The doubling time of oiPSCs was about 17.33 h for oiPS8-108 and 16.43 h for oiPS8-119, and the plating efficiency was 28.4% ± 0.17% for oiPS8-108 and 41% ± 0.57% for oiPS8-119.

The oiPS cells exhibited AP activity (Figure 1E) and expressed ES cell makers, including OCT4, SOX2, NANOG (Supplementary information, Figure S1), stage-specific embryonic antigen (SSEA)-1 (Figure 1F), TRA-

1-60 (Figure 1I), TRA-1-81 (Figure 1J), REX1 (Figure 1K) and E-cadherin (CDH1; Figure 1L). The cells showed an absence of the human ESC-specific surface makers, SSEA-3 (Figure 1G) and SSEA-4 (Figure 1H). The expression of E-cadherin suggests that the oiPSCs are reprogrammed beyond the state of 'near pluripotency' (the state of so-called FAB-SCs) to the state of 'full pluripotency' (the state of ES cells) [25-27]. Moreover, RT-polymerase chain reaction (RT-PCR) showed that oiPSCs express many ES cell marker genes, including *Oct4*, *Sox2*, *Nanog*, *Tdgf1*, *Eras*, *Dnmt3b*, *Dax1*, *Dppa4* and *Gdf3* (Figure 2A).

We analyzed the endogenous expression of the pluripotent genes *Oct4*, *Sox2* and *Nanog* by quantitative PCR and found that all three were robustly induced (Figure 2B).

Epigenetic status of sheep iPS cells

The epigenetic status of sheep iPSCs was evaluated using bisulfite genomic sequencing. We analyzed the promoter region of *Nanog* and found that the CpGs were highly unmethylated in sheep iPSCs compared to the parental fibroblasts (Figure 3A). These results indicate that the *Nanog* promoter was reactivated in sheep iPSCs, which is consistent with the robust induction of endogenous *Nanog* during reprogramming.

We also analyzed the karyotype of oiPSCs after passage 15, and the results showed that the sheep iPS clones exhibited a normal karyotype of 54 XY (Figure 3B).

The transgenes were highly expressed in oiPSCs. When DOX was withdrawn, the exogenous genes were markedly downregulated (Figure 3C), and the iPS colonies could not retain normal morphology and consequently underwent differentiation. This suggests that the sheep iPS cell lines we established could not maintain pluripotency in the current culture conditions without DOX.

Sheep iPSCs are able to differentiate into all three germ layers in vitro and in vivo

ES cells are able to differentiate into all three primary germ layers both *in vitro* and *in vivo*, which is a very important criterion of pluripotency. To test the differentiation capability of oiPSCs *in vitro*, we allowed the cells to form embryoid bodies and undergo differentiation in medium for 7 days, and then analyzed the presence of markers characterizing each germ layer. RT-PCR results showed that sheep iPS cells were able to differentiate into all the three germ layers, as evidenced by the high expression of *Afp* (endoderm), *Dcn* (endoderm), *Foxa2* (endoderm), *Fibronectin* (ectoderm), *Neurod* (ectoderm), *Nfh* (ectoderm), *Actc1* (mesoderm), *Kdr* (mesoderm),
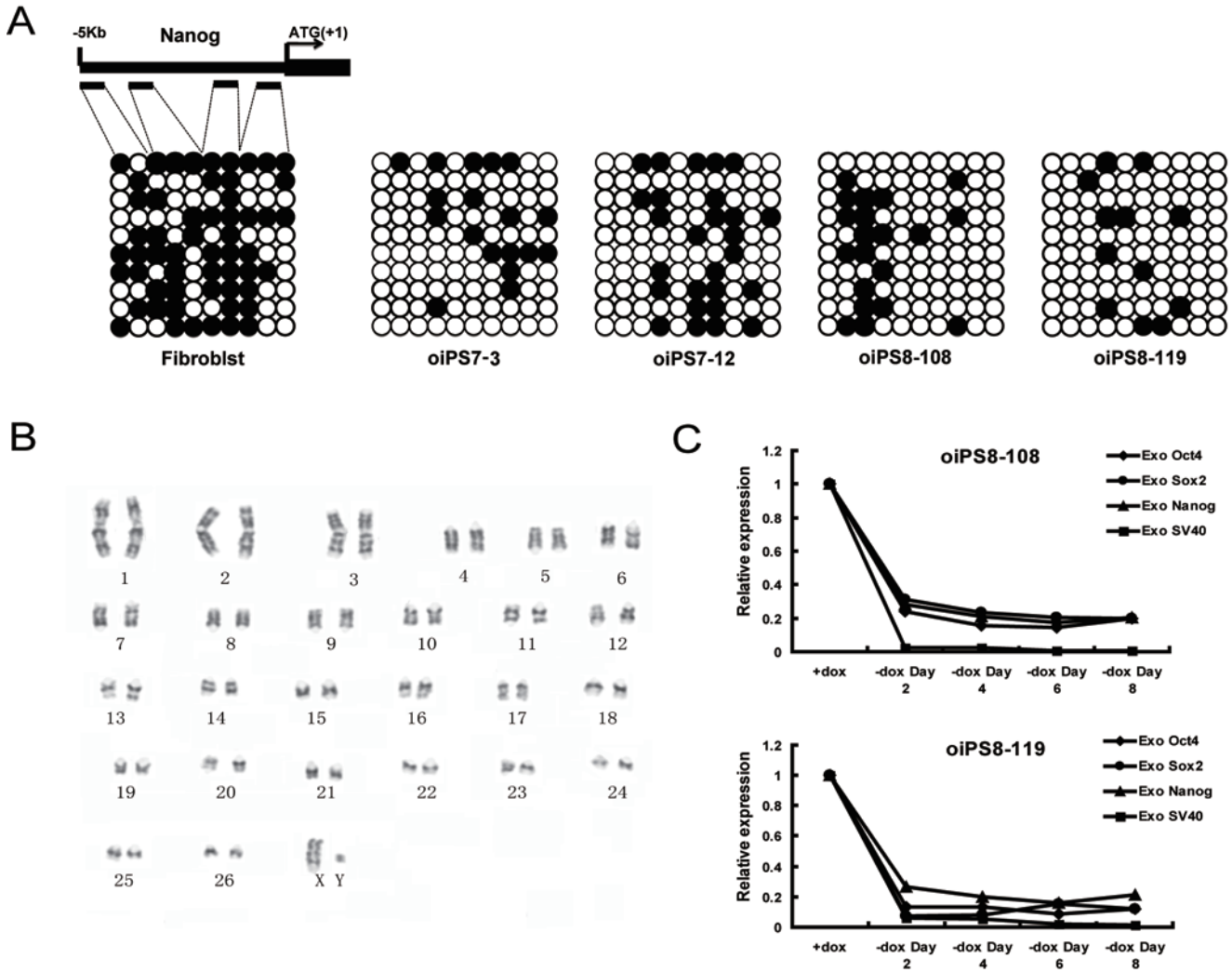
Figure 3 (A) Bisulfite genomic sequencing of the promoter regions of *Nanog*. The open and closed circles indicate unmethylated and methylated CpGs, respectively. **(B)** The oiPSCs at passage 31 showed a normal 54 XY karyotype. **(C)** Quantitative RT-PCR analyses of exogenous gene expression when DOX was withdrawn.

Myf5 (mesoderm) and *Renin* (mesoderm; Figure 4A).

We then examined the *in vitro* differentiation ability of sheep iPS cells by immunocytochemistry. Sheep iPS cells formed EBs and were transferred onto gelatin-coated plates for continued cultivation. Attached cells exhibited various types of morphologies. Immunocytochemistry detected cells positive for SOX17 (endoderm), smooth muscle actin (SMA; mesoderm) and βIII-tubulin (TUJ-1; ectoderm; Figure 4B).

To examine their pluripotency *in vivo*, we injected sheep iPS cells intramuscularly into non-obese diabetic/ severe combined immunodeficient (NOD/SCID) mice. At 4 weeks after injection, tumors became visible. Histological examination showed that the tumors contained various tissues, including neural rosettes (ectoderm),
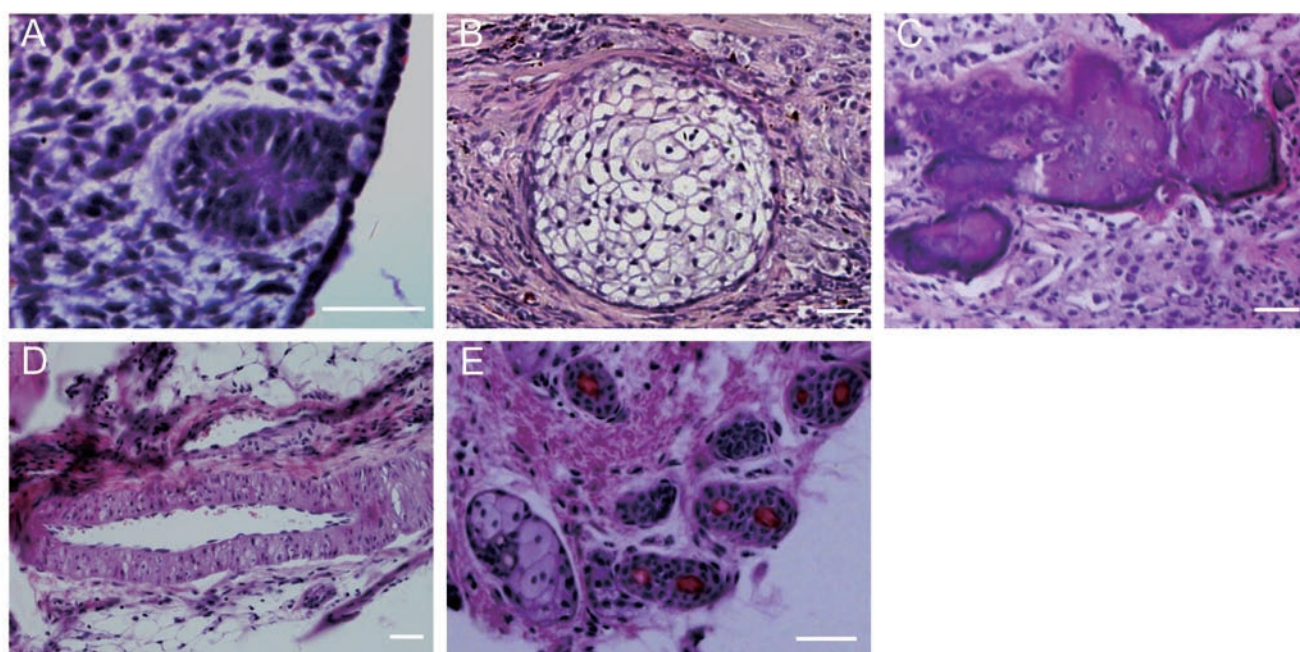
squamous epithelia (ectoderm), bone (mesoderm), gut-like epithelial tissues (endoderm) and glands (endoderm; Figure 5). Thus far, we have observed the complete differentiation ability only of sheep iPS cells generated with all eight factors (OSCKNL+T+hTERT) in the teratomas. For unknown reasons, when the cell lines generated with seven factors (OSCKNL+T) were tested, no differentiation was observed in the teratomas.

Discussion

In this study, we established sheep iPS cell lines with a drug-inducible system to induce the expression of eight defined factors. The sheep iPSCs exhibited ES cell-specific characteristics, including characteristic morphology,

A

B
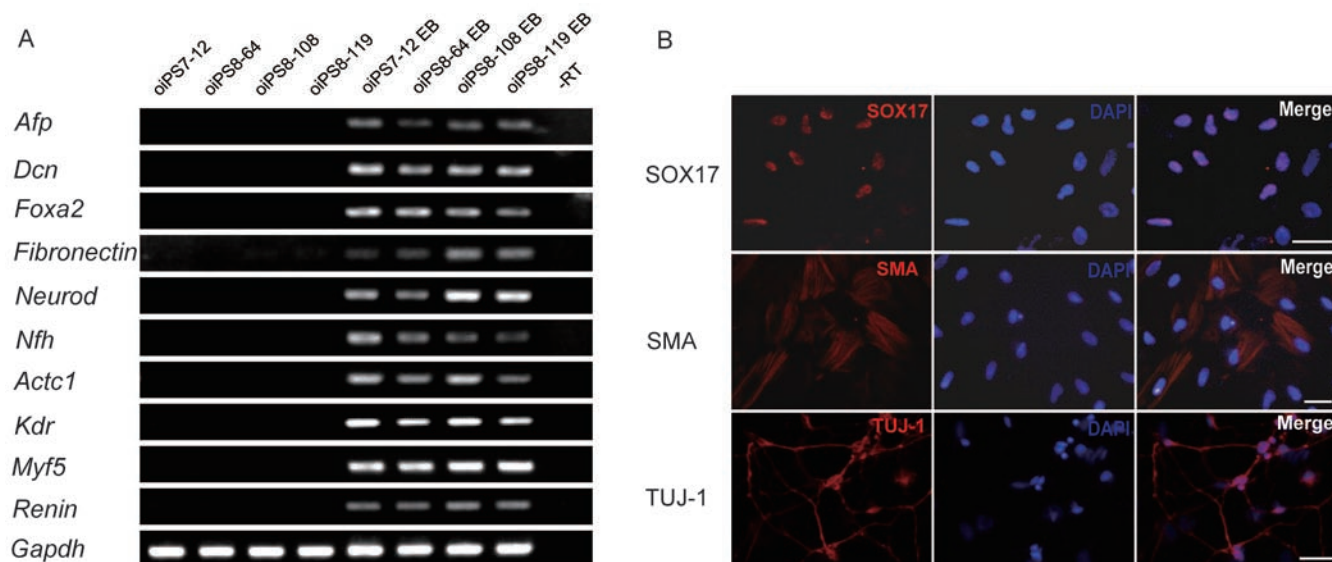
Figure 4 (A) Reverse transcriptase-polymerase chain reaction analyses of various differentiation markers for the three germ layers in the embryoid body. Abbreviation: EB, embryoid body; −RT, minus reverse transcriptase. **(B)** Immunocytochemistry of SOX17, smooth muscle actin (SMA) and βIII-tubulin (TUJ-1). Scale bars: 50 μm.

Figure 5 Hematoxylin-eosin staining of teratomas derived from sheep-induced pluripotent stem cells. The types of tissues that can be found in the teratomas include **(A)** neural rosettes (ectoderm), **(B)** squamous epithelium (ectoderm), **(C)** bones (mesoderm), **(D)** gut-like epithelial tissue (endoderm) and **(E)** glands (endoderm). Scale bars: 50 μm.

AP activity, expression of various ES cell markers and the capability to differentiate into all three germ layers both *in vitro* and *in vivo*.

Sheep are an important domesticated species. Pluri-potent stem cells, which are essential to generate precise gene-modified animals, have not yet been reported in sheep. In this study, we generated pluripotent stem cells from sheep by using iPS technology. Sheep iPS cells

resemble mouse ES cells, rather than human ES cells, in morphology under our current culture conditions. These cells express SSEA-1, rather than SSEA-3 or -4. The properties of sheep iPS cells we revealed should be informative for the identification of sheep pluripotent stem cells and may greatly facilitate the establishment of authentic sheep ES cells.

Sheep iPS cells, whose properties are similar to those of ES cells, provide a unique platform to screen for essential growth factors and/or chemicals that help to maintain self-renewal and pluripotency of sheep ES cells. The most critical problem that has hindered the establishment of sheep ES cells is how to inhibit the differentiation of ES cells. Each of the existing ES cells, including human, monkey, mouse and rat ES cells, requires different culture conditions. It is reasonable to speculate that sheep ES cells need to be maintained under certain unknown culture conditions. Therefore, ES cell culture condition screening based on the sheep iPS cells should be an efficient approach to facilitate the eventual establishment of sheep ES cell lines.

Mouse iPS cells have been shown to be successful in homologous recombination [16] and the generation of chimeric [14, 17, 28] and clonal mice [29, 30]. Sheep iPS cells may allow genetic manipulation by homologous recombination, and may be used as the nuclear donor to generate genetically modified sheep through SCNT.

Using a DOX-inducible lentivirus system, we have generated human (unpublished data) and pig iPS cells with either OSCK (Oct4, Sox2, c-Myc and Klf4) or OSCKNL (Oct4, Sox2, c-Myc, Klf4, Nanog and Lin28) combinations. However, we could not obtain sheep iPS cells with the same procedure, suggesting that somatic cells from sheep are more difficult to reprogram than somatic cells from human or pig.

We observed that adding SV40 large T and hTERT to the OSCKNL combination significantly increased the efficiency of sheep iPS cell production. Moreover, without SV40 large T, we were not able to obtain stable iPS cells from sheep somatic cells by overexpressing six factors (OSCKNL) or seven factors (OSCKNL+hTERT) with the DOX-inducible system. These observations suggest that SV40 large T plays an essential role in reprogramming sheep somatic cells. SV40 large T is an essential protein for SV40 DNA replication and disables the retinoblastoma and p53 tumor suppressor pathways [31-34]. It has been reported that knocking down p53 greatly increases reprogramming efficiency [35-39]. We speculate that SV40 large T probably works by inhibiting p53 to increase reprogramming efficiency. Alternatively, SV40 large T may turn on certain genes or signaling pathways that are critical for sheep pluripotent stem cells or may

cause reprogramming-related chromosome remodeling. hTERT, which has potent anti-apoptotic activity [40], may promote the generation and maintenance of sheep iPS cells by increasing cell proliferation and inhibiting cell death.

Trypsinization and passaging of transduced sheep fibroblasts during the reprogramming progress were critical. Since adding SV40 large T and hTERT increased the proliferation of cells, the transduced cells quickly formed very large colonies and became confluent. Therefore, we trypsinized the colonies into single cells, and passaged them onto new dishes to release the well-reprogrammed cells. By being trypsinized and passaged once or twice, the AP-positive colonies appeared about 20 days after viral transduction. Without trypsinizing and passaging, no ESC-like colonies could be obtained.

In the future, sheep iPS cells may be used to study the mechanisms of reprogramming and maintenance of pluripotency. These cells may also be used to generate precise gene-modified sheep by SCNT.

Materials and Methods

Cell culture

The sheep primary fibroblasts were derived from the ear of a young sheep and were cultured in α-DMEM culture medium (Invitrogen) supplemented with 10% FBS (Hyclone). The sheep iPSCs were maintained on irradiated CF-1 MEFs in DMEM/F12 supplemented with 20% KnockOut serum replacer, 0.1 mM non-essential amino acids, 1 mM L-glutamine and 0.1 mM β-mercaptoethanol (ES medium; all from Invitrogen, Carlsbad, CA, USA). The medium was also supplemented with 1 µg/ml DOX (Chemicon). The sheep iPSCs were split with TrypLE (Invitrogen) at a ratio of 1:10 every 3 days. To form embryoid bodies, the sheep iPSCs were dissociated with TrypLE and transferred to a petri dish with differentiation medium consisting of DMEM (Invitrogen) supplemented with 10% of FBS (Hyclone). The doubling time of oiPSCs was determined as described by Kim [41]. The plating efficiency was determined as described by Amit et al. [42].

Lentiviral transduction and reprogramming culture

Human cDNAs were inserted downstream of the tet operator in a lenti-vector. On day 0, 0.1 million sheep fibroblast cells were transduced with a lentivirus carrying GFP (negative control) or a cocktail of lentiviruses carrying reprogramming factors. At 1 day after transduction, the cells were harvested by trypsinization and plated onto MEFs at 2.5×10^4 cells per well in a six-well plate. The next day, the medium (DMEM containing 10% FBS) was replaced with ES medium plus DOX (1 µg/ml). On days 11 and 17, the cells were trypsinized into a single-cell suspension and passaged twice. ESC-like colonies were picked on day 23 and plated onto new culture dishes with feeder cells.

Immunostaining

Immunostaining was carried out similarly to previously described methods [43]. The primary antibodies used were anti-Oct4

(1:100, Santa Cruz), anti-Sox2 (1:1 000, Millipore), anti-Nanog (1:150, Santa Cruz), anti-SSEA1 (Ascites, 1:500, Developmental Studies Hybridoma Bank), anti-SSEA3 (Ascites, 1:400, Developmental Studies Hybridoma Bank), anti-SSEA4 (Ascites, 1:400, Developmental Studies Hybridoma Bank), anti-Tra-1-60 (1:150, Chemicon), anti-Tra-1-81 (1:150, Chemicon), anti-Rex1 (1:200, Santa Cruz), anti-E-cadherin (1:100, BD), anti-βIII-tubulin (1:100, Santa Cruz), anti-SMA (1:200, Santa Cruz) and anti-Sox17 (1:100, R&D).

Real-time PCR

Total RNA was prepared using an RNeasy kit (Qiagen) and reverse transcribed to synthesize first-strand DNA. The cDNA was used as a template for RT-PCR. Real-time PCR was performed in an Eppendorf Mastercycler real-time PCR system using a SYBR Green-based PCR Master mix (TOBOYO). The PCR primers are listed in Supplementary information, Table S1. The CT data for the gene of interest of each sample that were obtained from the real-time PCR were normalized to the internal control (GAPDH), and the fold changes of iPS samples relative to fibroblasts were calculated.

Bisulphite genomic sequencing

Bisulphite treatment was performed using a CpGenome modification kit (Chemicon) according to the manufacturer's recommendations. The PCR primers are listed in Supplementary information, Table S2. The amplified products were cloned into the T-vector, and at least 10 randomly selected clones were sequenced.

Karyotype analysis

Karyotyping was performed at the Xiangtan Center Hospital using standard protocols for high-resolution G-banding.

Teratoma formation

The oiPSCs were injected intramuscularly into NOD/SCID mice (approximately 5×10^6 cells per site). After 4-6 weeks, tumors were processed for hematoxylin and eosin staining. All animal experiments were conducted in accordance with the Guide of SIBS for the Care and Use of Animals for Research Purposes and were approved by the SIBS Animal Care Committee.

Acknowledgments

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