Derivation of sheep embryonic stem cells under optimized conditions

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Abstract

Until recently, it has been difficult to derive and maintain stable embryonic stem cells lines from livestock species. Sheep ESCs with characteristics similar to those described for rodents and primates have not been produced. We report the derivation of sheep ESCs under a chemically defined culture system containing fibroblast growth factor 2 (FGF2) and a tankyrase/Wnt inhibitor (IWR1). We also show that several culture conditions used for stabilizing naïve and intermediate pluripotency states in humans and mice were unsuitable to maintain ovine pluripotency in vitro. Sheep ESCs display a smooth dome-shaped colony morphology, and maintain an euploid karyotype and stable expression of pluripotency markers after more than 40 passages. We further demonstrate that IWR1 and FGF2 are essential for the maintenance of an undifferentiated state in *de novo* derived sheep ESCs. The derivation of stable pluripotent cell lines from sheep blastocysts represents a step forward toward understanding pluripotency regulation in livestock species and developing novel biomedical and agricultural applications.

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Introduction

Livestock embryonic stem cells (ESCs) have the potential to transform animal biotechnology by improving traits of agricultural significance, generating biomedical models as well as facilitating the study of pluripotency. Due to their unlimited in vitro proliferative capacity, ESCs could be used to introduce complex genetic modifications that enable the generation of animals with desirable agricultural traits as well as modeling human diseases. Recent advancements in targeted genome editing, especially the CRISPR-Cas9 system, have facilitated the introduction of complex genomic alterations, which when combined with somatic cell nuclear transfer (SCNT), represents a powerful platform for transgenic animal production (Gao *et al.* 2017). Although it remains controversial that success of SCNT is affected by the differentiation state of the donor nucleus (Chung *et al.* 2014), it was reported that a higher proportion of clones derived from ESC nuclei developed to term when compared to clones derived from somatic cell nuclei, suggesting an advantage of using ESCs as nuclei donors for SCNT (Rideout *et al.* 2000). ESCs also represent a robust in vitro model for drug discovery, as well as a

means for testing the effects of teratogens and toxins on development (Tandon & Jyoti 2012). Additionally, ESCs have the ability to generate a variety of lineages in vitro, which makes them a good model for studying cellular differentiation (Ezashi *et al.* 2016). Given the anatomical and physiological similarities that exist between human and livestock species, ESCs are also relevant for expanding their use in regenerative medicine through the creation of animal organ donors (Rashid *et al.* 2014, Wu *et al.* 2016).

As a large livestock species, sheep are docile and share similarity with humans in physiology and size, and have been used as models for vaccine development, asthma pathogenesis and treatment, and optimization of drug delivery, among others (Scheerlinck *et al.* 2008). In addition, sheep have a set of well-established in vitro fertilization procedures.

Until recently, stable ESC lines from livestock species have been difficult to derive and maintain, and ESCs homologous to those described for rodents and primates have not been produced (Ezashi *et al.* 2012, Malaver-Ortega *et al.* 2012, Park & Telugu 2013, Koh & Piedrahita 2014, Soto & Ross 2016). Several reports attribute this failure to the lack of appropriate culture

conditions for derivation and maintenance of ESCs in large animal species (Gandolfi *et al.* 2012, Soto & Ross 2016). The number of reports describing livestock ESCs lines increased drastically in the last 10 years (reviewed by Navarro *et al.* 2019). In sheep, the most successful study was published in 2011 (Zhao *et al.* 2011), where stable sheep ESCs were derived and cultured for more than 30 passages in N2B27 medium supplemented with GSK3 β inhibitor (GSK3 β i) and FGF2. These cell lines pass the pluripotency assays and formed teratoma, however, teratomas did not have the endoderm component, and they were unable to contribute to chimeras when injected in blastocysts.

In mice, pluripotent stem cells (PSC) have been broadly classified into two pluripotency states, naïve and primed, based on their molecular similarity either to the naïve epiblast in pre/peri-implantation inner cell mass (ICM) or the epiblast from peri-gastrulation embryos (Nichols & Smith 2009, De Los Angeles *et al.* 2015). In this regard, mouse ESCs (mESCs) and mouse epiblast stem cells (mEpiSCs) are considered in the naïve and primed pluripotent state, respectively. Although conventional human ESCs are derived from pre-implantation embryos, they resemble mEpiSCs rather than mESCs and are considered to be primed (Tesar *et al.* 2007). Functionally, naïve mESCs can efficiently contribute to high-grade chimeras when introduced into a blastocyst-stage embryo. In contrast, although primed mEpiSCs can differentiate into cells from three germ layers *in vitro*, they do not efficiently generate blastocyst chimeras (Tsukiyama & Ohinata 2014). Naïve and primed PSCs are cultured in distinct culture conditions: mESCs are conventionally cultured in serum-containing medium supplemented with leukemia inhibitory factor (LIF) and mEpiSCs culture contains fibroblast growth factor 2 (FGF2) and activin A. A ground-state culture condition containing LIF and small molecule inhibitors of ERK1/2 and GSK3 β i (2iL) has been developed that enables the derivation of ESCs from non-permissive mouse strains as well as rat blastocysts (Buehr *et al.* 2008, Ying *et al.* 2008). Human PSCs exhibit overt differentiation in 2iL culture and does not support the generation of naïve human ESCs and iPSCs (Ying *et al.* 2008, Hanna *et al.* 2010, Takashima *et al.* 2014). In 2013, the first culture medium (NHSM) for the derivation of naïve-like human ESCs from human blastocyst and iPSCs from human fibroblasts was reported (Gafni *et al.* 2013). Since then, a number of naïve-like culture conditions have been published (Theunissen *et al.* 2014, Irie *et al.* 2015, Guo *et al.* 2016). Furthermore, culture conditions that support the generation of intermediate PSCs with both naïve and primed pluripotency features have also been reported: PSCs cultured in a modified mEpiSC medium containing FGF2, Activin-A and GSK3 β i (Factor *et al.* 2014) showed features characteristic of both mESCs and mEpiSCs (Tsukiyama & Ohinata 2014, Wu *et al.* 2017). In addition to naïve cultures, primed PSC cultures have

also undergone significant improvement over the past decades. For example, the Essential 8™ Medium (E8) is a xeno-free and feeder-free medium specifically developed for primed hPSCs that has demonstrated the ability to maintain pluripotency in multiple PSC lines (Chen *et al.* 2011).

Recently, a novel primed PSC culture was developed for both mouse and human. PSCs grown in this condition display region-selective properties (designated as rsPSCs), and unlike conventional mEpiSCs and primed human PSCs, they selectively engraft to the posterior epiblast of gastrula-stage mouse embryos. The human rsPSC culture consists of the custom mTeSR1 base media, FGF2 and IWR1, a tankyrase and WNT pathway inhibitor (CTFR medium) (Wu *et al.* 2015). Using CTFR medium, we were able to efficiently derive bovine ESCs from blastocysts (CTFR-bESC) (Bogliotti *et al.* 2018). CTFR-bESC displayed stable morphology, karyotype, pluripotency marker expression, epigenetic profiles and were able to form teratoma after injection into immunodeficient mice (Bogliotti *et al.* 2018). In this study, we tested several reported human naïve-like and primed PSC conditions for the derivation and maintenance of sheep ESCs (sESCs).

Materials and methods

Unless otherwise specified, all chemicals used are from SIGMA.

Embryo production

In vitro embryo production was carried out as previously described: ovaries were collected from the slaughterhouse and oocytes were aspirated. Cumulus-oocyte-complexes (COC) were selected and *in vitro* maturation performed in TCM199 supplemented with 10% Ovine Estrus Serum (OES), oFSH (50 ng/mL; National Hormone & Peptide Program, UCLA, CA), bLH (3 mg/mL; Sioux Biochemical), and cysteamine (0.1 mM), for 24 h in 5% CO₂ with humidified atmosphere at 38.5°C (Vilarino *et al.* 2017). Ovine estrus serum was collected from ewes synchronized to be in heat at the time of blood draw. *In vitro* fertilization was performed using fresh semen selected by ascendant migration with a swim-up method using Fertilization Medium (Synthetic oviductal fluid (SOF) supplemented with 2% OES, 10 μ g/mL heparin, and 10 μ g/mL hypotaurine). Mature oocytes and sperm adjusted to a concentration of 1 x 10⁶ sperm/mL were co-incubated for ~14 h in 5% CO₂ with humidified atmosphere at 38.5°C. Embryos were cultured in KSOM (Evolve, Zenith Biotech) with 4 mg/mL of BSA under oil at 38.5°C, 5% CO₂ and 5% O₂. Blastocysts were collected on day 7 post-fertilization.

Immunosurgery of blastocysts

Zona pellucida (ZP)-depleted blastocyst was incubated in KSOM medium with 20% anti-bovine serum (B8270) for 1 h at 38.5°C, followed by five washes with synthetic oviductal

fluid (SOF)-HEPES media. Embryos were incubated in KSOM supplemented with 20% guinea pig complement (Innovative Research) for 1 h at 38.5°C, and washed five times in SOF-HEPES. To isolate the ICM a micro-dispenser was used to gently pipette the ICM and remove the trophoectoderm (TE) cells.

Derivation and culture of sESCs

In vitro produced blastocysts with a well-developed ICM were collected at day 7 of development. Zona pellucida was removed using 1% pronase during the first minute and carefully washed up to ten times. Embryos were plated in separate wells of a 24-well plate onto a gamma-irradiated mice embryonic fibroblasts (MEF) feeder layer and cultured in six different media: 2iL (Ying *et al.* 2008), NHSM (Gafni *et al.* 2013), 4i (Irie *et al.* 2015), FAC (Wu *et al.* 2017), E8 (Chen *et al.* 2011) and CTFR (Wu *et al.* 2017). CTFR medium is a custom basal medium similar to mTeSR medium that contains low fatty acid BSA (MP Biomedicals NZ) (Ludwig *et al.* 2006), and supplemented with 20 ng/mL of human FGF2 (Peprotech) and 2.5 μ M IWR1 (Bogliotti *et al.* 2018). Embryos were cultured at 37°C, 5% CO₂ and humidified atmosphere. After 48 h, embryos that did not attach were pressed onto the bottom of the culture dish using a 22-gauge sterile needle. The media was changed daily for 7 days, outgrowths were dissociated using TrypLE (12563011; Gibco) and passaged to new MEF feeders in the presence of Rho kinase (ROCK) inhibitor Y-27632 (10 μ M). Media was changed daily between passages, and further passages were performed every 4–5 days at a 1:4 split ratio. Feeder-free culture conditions were performed using vitronectin-coated cell culture plates.

Immunofluorescence

Immunofluorescence was performed as previously described with some minor modifications (Bogliotti *et al.* 2018). Sheep ESCs were grown to 70–80% of confluence in four-well dishes that had a coverslip and a MEF feeder layer. Cells were fixed using 4% paraformaldehyde in PBS for 10 min at room temperature and washed three times with PBS. Then, cells were blocked for 30 min with PBST (0.3% Triton X-100 in PBS) supplemented with 3% normal donkey serum (NDS). Cells were incubated in primary antibody at 1:300 dilution in PBST+1% NDS for 1 h, washed three times for 10 min using PBST+1% NDS, and incubated with fluorescent-labeled secondary antibody at 1:500 dilutions for 1 h. Next, cells were washed three times for 10 min each in PBST, and during the last wash, the nuclei were stained with 0.01 μ g/ μ L Hoechst 33342 solution (62249, Gibco). The coverslip with the stained cells was removed from the four-well plate and placed in a microscope slide. Approximately 10 μ L of Prolong (Life Technologies) was added over the cells, and a coverslip was placed on top. Cells were visualized using a Nikon TE2000-U inverted microscope and a Leica TCS SP8 STED 3X Confocal microscope. The primary antibodies used were anti-SOX2 (AN579-5M; BioGenex), anti-OCT3/4 (sc-8628; Santa Cruz Biotechnology), anti-CDX2 (MU392A-UC, BioGenex), anti-GATA6 (sc-9055; Santa Cruz Biotechnology) and anti-NANOG (14-5768; eBioscience).

Alkaline phosphatase (AP) staining

AP staining was performed as previously described: CTFR-sESCs (P36) were grown in a four-well plate to 70–80% confluence and AP Staining was performed using an AP Staining Kit II (00-0055, Stemgent) according to the manufacturer's instructions. CTFR-sESCs were briefly washed with PBST (0.05% Tween 20 in PBS) and fixed for 4 min at room temperature using a fixation solution that was provided with the kit. The fixed cells were washed once with PBST and incubated with AP Substrate solution (provided with the kit) in the dark at room temperature for 15 min. Then, AP Substrate Solution was removed and cells were washed twice with PBS. The stained cells were observed using a Nikon TE2000-U inverted microscope and images were captured using RI Viewer Imaging Software (Bogliotti *et al.* 2018).

Karyotyping

CTFR-sESCs at passage 22 and 38 were grown to 50–60% of confluence and arrested in the metaphase stage by incubating them for 1 h with media containing Demecolcine (200 ng/ μ L). Cells were harvested using TrypLE, washed with PBS, and resuspended in 15 mL of 0.075 M KCL solution pre-warmed to 37°C (KCL solution was added drop by drop). After a 10 min incubation at 37°C, 10 drops of a fixative solution were added (3:1, methanol:acetic acid), and centrifugation was performed at 300 *g* for 5 min. The supernatant was discarded and the pellet was resuspended in 5 mL of the fixative solution, incubated for 15 min and centrifuged. The fixation procedure was repeated three times and cells were resuspended in 100 μ L of the fixative solution and chromosome spreads were prepared by dropping cells suspension over cold slides. After drying for 10–15 min the slides were stained with 3% Giemsa during a 3 min period. Slides were visualized using a Nikon TE2000-U inverted microscope at 400 $\times$ magnification.

Cell cycle analysis by flow cytometry

Cell cycle analysis was performed as previously described: CTFR-sESCs (P23 and P39) and sheep fibroblasts (P11) (used as control) were grown in a 12-well plate to 70–80% confluence and dissociated using TrypLE. After dissociation, 1×10^6 cells were washed twice with PBS supplemented with 2% FBS and 0.1% BSA, and centrifuged at 200 *g* for 5 min. The supernatant was carefully discarded and 1 mL of ice-cold 70% ethanol was added dropwise to resuspend the cell pellet with gentle vortexing. Fixed cells were stored at –20°C until analysis was performed. For flow cytometry analysis, cells were centrifuged at 200 *g* for 10 min, washed in PBS supplemented with 2% FBS and 0.1% BSA, and then in Stain Buffer (554656; BD Pharmingen). A total of 1×10^6 cells were resuspended in 500 μ L of propidium iodide (PI)/RNase Staining Buffer (550825; BD Pharmingen) and filtered through a 5 mL round-bottom polystyrene tube with a cell strainer snap cap (352235; Falcon). Cells were incubated in PI/RNase staining buffer for 15 min, protected from light, and analyzed within 1 h on a FACScan flow cytometer (Becton Dickinson) equipped with a 488 nm excitation laser. Before FACS analysis, cells were

diluted in PBS to 1×10^6 per mL in order to achieve a flow rate of 400 events per second. All data were acquired and analyzed using CellQuest Pro Software (Becton Dickinson). Cell doublets were gated out on a FL2-A/FL2-W dot plot, and a total of 10,000 events were collected per sample.

RNA extraction and reverse transcription

Total RNA was isolated from cells (CTFR-sESCs P15 and P31, and sheep fibroblasts) and tissue (sheep brain and liver) using an RNeasy purification kit (74104; QIAGEN) following the manufacturer's instructions. RNA from 10 blastocysts was extracted using an Arcturus PicoPure RNA Isolation Kit (12204-01, Applied Biosystems) according to the manufacturer's procedures. RNA quantity and quality was analyzed using the NanoDrop 2000C Spectrophotometer (ThermoScientific). cDNA synthesis from cells, tissue and embryos was performed using Superscript II Reverse Transcriptase (18064-014, Invitrogen). Quantitative real-time PCR was performed in QuantStudio 3 (Applied Biosystems) in 20 μ L reactions containing 0.5 μ L of forward and 0.5 μ L of reverse primer (10 μ M stock of each primer), 2 μ L of the cDNA sample, 7 μ L of water and 10 μ L of Fast SYBR Green Master Mix (00575141, Applied Biosystems). Each sample was run in duplicate for each gene and the sequence of the gene-specific primer sets are provided in the Supplementary Table 1 (see section on [supplementary materials](#) given at the end of this article). Relative transcript abundance was calculated using the comparative CT method (Δ CT) with normalization to *GAPDH*.

RNA sequencing

The ICM and TE of two in vitro produced day 7 sheep embryos were mechanically cut using a micro-blade attached to the micromanipulators of an inverted microscope. The TE was washed in PBS, collected in a tube, snap-frozen in liquid nitrogen and stored at -80°C until library preparation. Immunosurgery was performed on the ICM, then snap-frozen and stored at -80°C as previously described. Libraries from sheep ICM and TE were prepared using an Ovation® SoLo RNA-Seq Library Preparation Kit (NuGEN) and sequenced on an Illumina NextSeq instrument producing 75 pb single-end reads. RNA extraction of sESCs (line A: p28, p46; line B: p13, p24) and fibroblasts was performed using an RNeasy Mini Kit (QIAGEN) using DNase treatment (QIAGEN). RNA was analyzed using a 2100 Bioanalyzer (Agilent Technologies) and libraries were prepared using TruSeq® Stranded mRNA Sample Preparation kit (Illumina) and sequenced on a HiSeq 4000 system (Illumina).

All read sets were trimmed (cutadapt/1.16), and individually mapped to the ovine genome Oar_v4.0 (star/2.6.0c). Htseq (version 0.10.0) was used to generate gene counts and data was normalized as Transcripts per Kilobase Million (TPM). A gene was considered expressed when three or more samples had normalized counts ≥ 10 . DESeq (1.24.0) package was used to determine differential gene expression among fibroblasts and sESCs-LCs (lfcThreshold = 1, alpha = 0.05) and the package pheatmap (1.0.12) was used for heatmap. Gene Ontology enrichment analysis was performed using the enrichR tool

(Chen *et al.* 2013) and the Reactome Pathway database (<https://reactome.org/>) with $P < 0.01$ as a cut-off for significant terms. All bioinformatics analysis was performed in R 3.6.0.

Teratoma assay

Immunodeficient NOD mice were injected i.m. with approximately 1×10^6 CTFR-sESCs. The teratoma grew for 14–16 weeks and mice were killed for tissue collection. Teratomas were fixed in 4% paraformaldehyde and subjected to H&E staining to analyze the lineage phenotype.

Data availability

The RNA-seq data that support the findings of this study are openly available in NCBI/SRA at <https://www.ncbi.nlm.nih.gov/sra>, SRA accession: PRJNA609175.

Results

IWR1 supplementation supports derivation and culture of sESCs

We tested different culture conditions for the derivation of sESCs. *In vitro* produced sheep blastocysts were subjected to immunosurgery for ICM isolation, and isolated ICMs plated on MEF under six different culture conditions (Supplementary Fig. 1). ICM outgrowths were passaged once per week until passage three (P3) when colony morphology and pluripotency gene expression were evaluated. Cells cultured in CTFR formed colonies with a round dome-shaped morphology, similar to mESCs. In contrast, cells in other culture conditions appeared differentiated and did not form colonies (Fig. 1A). Immunofluorescence analysis (IF) of P3 cells revealed that only cells cultured in CTFR media expressed pluripotency factors OCT4 and SOX2 (Fig. 1B).

Next, we tested whether CTFR-sESCs can be culture adapted to other conditions that failed to maintain in vivo pluripotent epiblast cells during derivation. P6 CTFR-sESCs were transitioned (50–50% and then 100%) to five other conditions and passaged three times before we performed IF analysis with pluripotency markers (Fig. 2A). IF analysis showed that only the control condition (CTFR) maintained the expression of pluripotency markers SOX2 and OCT4 (Fig. 2B). CTFR-sESCs in other conditions lost dome-shaped morphology after three passages. These findings confirm our derivation results and demonstrate that all tested culture conditions except CTFR medium failed to sustain ovine pluripotency in culture. Because IWR1 is a key component of the CTFR conditions, we tested whether tankyrase inhibition is required for maintenance of pluripotency. We found that withdrawal of IWR1 from CTFR culture resulted in the loss of domed colony morphology after two passages and loss of pluripotency marker expression irrespective of FGF2 concentration (Fig. 3). Additionally,

Figure 1 Derivation of sheep ESCs from inner-cell mass (ICM) of sheep embryos under different culture conditions: CTFR (Wu *et al.* 2017), E8 (Chen *et al.* 2011), FAC (Wu *et al.* 2017), 4i (Irie *et al.* 2015), 2iL (Ying *et al.* 2008), and NHSM (Gafni *et al.* 2013). (A) Brightfield images showing colony morphology of sheep ICM culture under six different ESC media conditions at three different time points (48 h after seeding, P1 and P3). (B) Immunofluorescence staining for OCT4 and SOX2 in different ESCs medium conditions at passage three. Scale bars indicate 200 μ m (magnification: 10 $\times$).

we observed that 4 ng/mL of FGF2 was insufficient for maintaining normal cell proliferation and pluripotency marker expression (OCT4 and SOX2; Fig. 3).

Collectively, these results show that CTFR condition enabled the derivation of sESCs from sheep ICMs and that IWR1 and high concentrations of FGF2 are required for the maintenance of pluripotency factor expression in sESCs.

CTFR-sESCs exhibit stable morphology and normal karyotype after long term passages

In addition to isolated ICMs, plating whole sheep blastocysts also resulted in the derivation of sESCs under the CTFR condition (Supplementary Fig. 2). sESCs derived in CTFR condition exhibited a stable dome-shaped morphology after long term culture (Fig. 4A and

B). Of note, we observed that CTFR-sESCs colonies were prone to detach when confluent (typically 3–4 days after each passage). Karyotype analysis at passages 21 and 41 indicated that CTFR-sESCs karyotypes were stable with a normal chromosome number of $2n=54$ (Fig. 4B). Alkaline phosphatase staining showed that CTFR-sESCs were positive until at least P36 (Fig. 4B). Analysis of the cell cycle by flow cytometry showed a higher proportion of cells in the S phase compared to sheep fetal fibroblasts (Fig. 4C), a feature associated with highly proliferative pluripotent cells. Immunofluorescence analysis confirmed the expression of the pluripotency markers OCT4 and SOX2 in long-term cultured CTFR-sESCs. Consistent with the epiblast origin of sESCs, the trophoectoderm (TE) and primitive endoderm (PE) markers, CDX2 and GATA6, respectively, were not detected (Fig. 5A). RT-PCR from two late passages of the

Figure 2 Adaptation of CTFR-sESCs to different culture conditions. (A) Brightfield images showing colony morphology of CTFR-sESCs cultured under six different conditions at three different time points (24 h after media change, P2 and P3). CTFR-sESCs P6 were transitioned (50–50% and then 100%) and media was changed daily. (B) Immunofluorescence staining for OCT4 and SOX2 in all the tested ESCs medium conditions at passage three after media change. Scale bars indicate 200 µm in A (magnification: 10x) and 100 µm in B (magnification: 20x).

CTFR-sESCs confirmed the expression of pluripotency markers *OCT4*, *SOX2* and *NANOG*, but not the TE marker *CDX2*, which was expressed in whole blastocysts containing the TE layer. As expected, lineage markers such as *PAX6* (ectoderm), *FOXA2* (endoderm), and *MEOX1* (mesoderm), were not expressed in CTFR-sESCs (Fig. 5B). Moreover, we demonstrated the pluripotency of CTFR-sESC by the formation of a teratoma containing tissues from all three germ layers (Fig. 5C).

Furthermore, we performed RNA-seq using two different CTFR-sESC lines at different passages together with other samples, including ovine fetal fibroblasts, and ICMs and TEs isolated from two sheep blastocysts.

Similar to ICMs, several pluripotency markers were also expressed in CTFR-sESCs, while none of the ICM markers were expressed in fibroblasts (Fig. 6A). Moreover, TE/PE markers were not expressed in the CTFR-sESCs (Fig. 6A). These results confirm the expression of pluripotency markers and the absence of expression of lineage-specific markers in CTFR-sESCs. Notably, the gene expression profiles of the early and late passages of both CTFR-sESC lines clustered closely together, demonstrating the transcriptional stability of CTFR-sESCs over time (Fig. 6B). Still, some variability between cell lines was observed, which could be related to the outbred genetic nature of individual embryos. Additionally, we evaluated

Figure 3 CTFR-sESCs cultured at varying levels of FGF2 with and without IWR1 supplementation. Immunofluorescence staining for OCT4 and SOX2 in the CTFR-sESCs at different FGF concentrations and in the presence or absence of IWR1 (magnification: 10 $\times$).

the expression levels of naïve and prime pluripotency markers in CTFR-sESCs. In agreement with bovine CTFR-ESCs (Bogliotti *et al.* 2018), CTFR-sESC showed primed pluripotency characteristics (Fig. 6C). Among genes differentially expressed between CTFR-sESCs and fibroblast, pluripotency related pathways were the most upregulated gene category in sESCs (Supplementary Fig. 3). Interestingly, *NANOG* expression was low in one of the cell lines and absent in the other, coinciding with low-level expression detected by RT-PCR. Immunofluorescence analysis confirmed the lack of *NANOG* expression in CTFR-sESCs (Supplementary Fig. 4).

We also succeeded in culturing CTFR-sESC in a feeder-free condition in which plates were coated with vitronectin and Activin-A (20 ng/mL) was added to the CTFR medium (Tomizawa *et al.* 2013). After four passages in the feeder-free condition, CTFR-sESCs maintained their typical dome-shaped morphology and pluripotency markers expression (Supplementary Fig. 5).

Discussion

Stable ESC lines from livestock species have been difficult to derive and maintain, and sheep ESCs homologous to those described for rodents and primates have not been reported (reviewed by Gandolfi *et al.* 2012). Here, we tested multiple culture conditions for their ability to maintain ovine pluripotency in culture. Our results show that among all the culture conditions tested, only CTFR medium could efficiently derive sESC lines from either plating isolated ICMs or whole sheep

blastocysts on MEF feeders. These cell lines maintain a stable morphology, karyotype, and gene expression for more than 40 passages, indicating their potential for self-renewal, and were capable of forming teratomas containing derivatives of the three germ lineages, demonstrating their pluripotency.

Except for CTFR, other naïve, intermediate and primed PSC culture conditions tested, in this study, did not support the maintenance of ovine pluripotency in vitro. Of note, all tested naïve and intermediate media included a GSK3 β i (CHIR99021) which can activate the Wnt/ β -catenin signaling pathway (FAC, 4i, 2iL and NHSM). CTFR culture contains IWR1, a tankyrase inhibitor that stabilizes axin, which then can act as an antagonist for the Wnt/ β -catenin signaling pathway (Huang *et al.* 2009), suggesting that canonical Wnt pathway inhibition is the key for the successful derivation of sESCs. However, it is not clear whether IWR1 enables ESCs derivation because of its effect on tankyrase inhibition, axin stabilization, β -catenin degradation, or a combination of these effects (reviewed by Navarro *et al.* 2019). Also, the inability of E8 medium (which contains FGF2 and Nodal) to stabilize ovine pluripotency in culture further support the indispensable role of the IWR1. Accordingly, removing IWR1 from the CTFR conditions at high and low FGF2 concentrations resulted in cell differentiation and loss of pluripotency. Similar to bovine CTFR-ESCs, suppressing tankyrase/Wnt activity appears to be the key mechanism that promotes stabilization of ovine pluripotency in culture (Bogliotti *et al.* 2018). The Wnt/ β -catenin signaling pathway plays

Figure 4 CTFR-sESCs derivation and characterization. (A) Brightfield images showing whole-embryo derivation of CTFR-sESCs under MEF feeders (magnification: 20x objective, for embryo and outgrowth; scale bars 100 μ m). (B) Brightfield images (P25 and P41) and alkaline phosphatase (AP) staining (P36) showing CTFR-sESCs colony morphology. Scale bars represent 100 μ m. Karyotype analysis revealed normal chromosome number of $2n = 54$ (magnification: 60x objective). (C) Cell cycle analysis by flow cytometry of CTFR-sESCs P23 and P39, and sheep fibroblasts.

Figure 5 Stable expression of pluripotency markers in CTFR-sESCs. (A) Immunofluorescence staining for *OCT4*, *SOX2*, *CDX2* and *GATA6* in sheep blastocysts (magnification: 20× objective) and CTFR-sESCs P22 and P44 (scale bars, 100 μm). (B) Gene expression analysis using RT-qPCR of ICM pluripotency markers (*OCT4*, *SOX2*, *NANOG*), TE markers (*CDX2*), endoderm (*FOXA2*), ectoderm (*PAX6*) and mesoderm (*MEOX1*) from two passages of the CTFR-sESCs (P15 and P31), sheep blastocysts, fibroblasts, liver and brain. Relative transcript abundance was calculated using the comparative CT method ($\Delta\Delta C_T$) normalized to *GAPDH*. Error bars represent s.d. of two technical replicates. Representative images of histological sections of H&E stained teratomas derived from CTFR-sESCs. Tissues of all three germ layers are identified (magnification: 10 ×).

an important role in human and mouse stem cell renewal and differentiation (Sokol 2011) and stimulation of Wnt by GSK3 inhibition is a key component of mouse naïve ESC culture. The β -catenin destruction complex includes polyposis coli (APC), axin, glycogen synthase kinase (GSK3 β) and casein kinase (CK1a) (Huang *et al.* 2009). The stability of this complex is regulated by tankyrase 1 and 2 (TNKS1/TNKS2). IWR1 is a small molecule that inhibits TNKS1/2 and stabilizes axin, which blocks the translocation of β -catenin to the nucleus (Kirubakaran *et al.* 2014). In addition to the initial reports using IWR1 to stabilize pluripotency in human, mouse and cow ESCs (Wu *et al.* 2015, Bogliotti *et al.* 2018), two recent papers reported that IWR1 (or XAV939) is also needed for stabilizing pig pluripotency in vitro (Choi *et al.* 2019, Gao *et al.* 2019). These findings, combined with the current work, strongly suggest an important role for tankyrase inhibition, axin stabilization, and/or Wnt inhibition for maintaining pluripotency in a great variety of mammalian species. Further research is required to understand the detailed molecular mechanisms that govern livestock pluripotency.

There are several lines of evidence that might explain the positive effect of Wnt/ β -catenin inhibition on sESCs

pluripotency. A recent study, using bovine embryos showed that endogenous Wnt signaling is not required for blastocysts formation. Embryos were exposed to two different Wnt inhibitors (Wnt-C59 or DKK1) and still were able to reach the blastocyst stage. Surprisingly, the presence of Wnt-C59 (non-canonical Wnt pathway inhibitor) increased the number of ICM cells, which suggests that endogenous WNTs regulate ICM proliferation through a signaling pathway independent of β -catenin (Tribulo *et al.* 2017). Another study cultured human embryos in the presence of Cardamonin (a β -catenin inhibitor) and found that transcript levels of pluripotency markers *NANOG*, *OCT4*, *SOX2* and *SALL4* were unaffected, whereas the TE marker *CDX2* was downregulated, indicating that β -catenin degradation or stabilization caused inhibition of TE lineage specification in human blastocysts. Taken together, work in embryos support our results and help explain the positive effect of Wnt inhibition for derivation and maintenance of CTFR-sESCs.

The CTFR-sESCs derived in this study are valuable for addressing basic and translational questions in regenerative medicine (Mahla 2016). Among large animals, the sheep represents an excellent model for cell-based therapeutic approaches because of its similar

Figure 6 Transcription profile of CTFR-sESCs. (A) Expression analysis of specific markers for ICM, TE and PE from two independent CTFR-sESCs (A: P28, P46; B: P13, P24), two embryos where ICM and TE were separated and independently sequenced, and two lines of fibroblasts. Transcriptome analysis was performed with RNA-seq (expressed as TPM). In the color scale, red represents highly expressed genes and green represents low/no expression. (B) Heatmap and hierarchical clustering of differentially expressed genes (adjusted $P < 0.05$) from the two independent CTFR-sESCs (A: P28, P46; B: P13, P24) and the two sheep fibroblast cell lines. (C) Transcriptome analysis of primed and naïve specific gene markers. Data represent the mean of two passages of two independent CTFR-sESCs ($n = 4$) and two independent ICM and TE.

size compared to humans, short gestational interval and well-developed assisted reproductive technologies. Furthermore, stable CTFR-sESC could be harnessed for complex genetic engineering owing to their genetic stability and unlimited proliferative capacity, which could facilitate large-scale genetic modifications for diverse purposes.

In summary, using serum-free medium supplemented with FGF2 and a tankyrase/Wnt inhibitor (IWR1), we were able to *de novo* derive sESCs from ovine blastocysts that maintain stable colony morphology, normal karyotype, a pluripotent transcriptome, and the ability to form teratomas *in vivo* after long-term culture. Additionally, sESC can be cultured in a feeder-free condition while maintaining their characteristics. CTFR-sESCs represent a promising tool for use in agriculture and biotechnology and are likely to contribute novel insights into livestock pluripotency and early development.

Supplementary materials

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-19-0606>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

P J R, J C I B, J W and M V conceived the study. P J R, J W, M V, D A S, Y S B, L Y, C Z, M J, Y Z, C W and E P performed experiments and/or analyzed the data. P J R, J W and M V wrote the manuscript.

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