Efficient derivation of stable primed pluripotent embryonic stem cells from bovine blastocysts

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Embryonic stem cells (ESCs) are derived from the inner cell mass of preimplantation blastocysts. From agricultural and biomedical perspectives, the derivation of stable ESCs from domestic ungulates is important for genomic testing and selection, genome engineering, and modeling human diseases. Cattle are one of the most important domestic ungulates that are commonly used for food and bioreactors. To date, however, it remains a challenge to produce stable pluripotent bovine ESC lines. Employing a culture system containing fibroblast growth factor 2 and an inhibitor of the canonical Wnt–β-catenin signaling pathway, we derived pluripotent bovine ESC lines (bESCs) with stable morphology, transcriptome, karyotype, population-doubling time, pluripotency marker gene expression, and epigenetic features. Under this condition bESC lines were efficiently derived (100% in optimal conditions), were established quickly (3–4 wk), and were simple to propagate (by trypsin treatment). When used as donors for nuclear transfer, bESCs produced normal blastocyst rates, thereby opening the possibility for genomic selection, genome editing, and production of cattle with high genetic value.

Significance

Embryonic stem cells (ESCs) are derived from the inner cell mass of a preimplantation blastocyst, as has been well established in rodents and primates. To date, however, the derivation and stable propagation of pluripotent ESCs from domestic ungulates remain unsuccessful. The production of ESCs from large livestock species is important for genomic testing and selection, genome engineering, and studying human diseases. Here, we report that stable bovine ESCs can be efficiently derived in a culture condition based on Wnt-pathway inhibition. These well-characterized ESC lines not only will enrich our understanding of pluripotency programs in the ungulate species but also will provide a useful resource for the creation of transgenic ungulate models of human diseases.


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applications would lead to a significant reduction of the generational interval and would accelerate genetic progress. Moreover, stable bESCs represent a powerful platform for gaining novel insights into molecular features underpinning the bovine pluripotency program.

**Results**

CTFR Medium Supports Derivation and Stable Long-Term Culture of Pluripotent bESCs. bESCs were derived, propagated, cultured, and subjected to several rounds of freezing and thawing in CTFR medium (CTFR-bESCs). ESC lines could be established by the end of week 3 after ICM or whole-embryo plating and remained stable for more than 50 passages (Fig. 1). Unlike human ESCs and mouse EpiSCs, CTFR-bESCs did not show clearly defined colony margins. CTFR-bESCs stained positive for alkaline phosphatase (AP) (Fig. 1A). To assess the genetic stability of the CTFR-bESCs after long-term culture, we performed karyotyping analysis in two different CTFR-bESC lines at passage 34 (P34). Our results showed a normal chromosome content (2N = 60) in more than 70% of the examined metaphase cells (Fig. S1A). Also, CTFR-bESCs maintained a stable population-doubling time across multiple passages (Fig. S1B).

Immunofluorescence (IF) analysis revealed that long-term cultured CTFR-bESCs expressed the pluripotency transcription factors SOX2 and POU5F1 (also known as “OCT4”) but not the trophoderm (TE) and primitive endoderm (PE) markers CDX2 and GATA6, respectively (Fig. 1B and Fig. S1F). IF analysis of bovine blastocysts showed that SOX2+ cells located exclusively to the ICM, while the CDX2 signal was detected only in TE cells (Fig. 1B). A SOX2+/CDX2− staining pattern was consistently observed in CTFR-bESCs from P4 onwards, indicating that CTFR culture favored the proliferation of the ICM over TE cells.

We performed transcriptome analysis of two independently established CTFR-bESC lines, bovine blastocysts, and bovine fibroblasts via RNA-sequencing (RNA-seq). Our results showed that ICM markers were expressed [reads per kilobase of transcript per million reads mapped (RPKM) ≥0.4] in CTFR-bESCs and bovine blastocysts but not RPKM <0.4 in bovine fibroblasts (Fig. 1C and Fig. S1E). Both TE and PE markers were expressed in the blastocysts but were absent in CTFR-bESCs. These results indicate that the global gene-expression profile of CTFR-bESCs was more similar to the ICM than to TE/PE or fibroblast cells. Of note is that CTFR-bESCs’ transcriptome profile remained stable even after long-term culture (Fig. S1C–E).

Next we performed a teratoma assay to test CTFR-bESCs pluripotency. To this end, we independently injected two CTFR-bESC lines intramuscularly into immunodeficient NOD SCID mice. Both cell lines were able to form teratomas (Fig. S1G) containing tissues from all three primary germ layers: ectoderm, mesoderm, and endoderm, as evidenced by H&E staining (Fig. 1D) and IF analysis: ectoderm (TUJ1), endoderm (FOXA2), and mesoderm (ASM) (Fig. S1H).

These results indicate that bESCs derived in CTFR medium are pluripotent and can maintain a stable karyotype and transcriptome after extended in vitro culture.

**Histone Methylation Landscape of CTFR-bESCs.** The lack of culture conditions that support long-term propagation of pluripotent ESCs has impeded our molecular understanding of pluripotency in large livestock species. Taking advantage of the stable pluripotent CTFR-bESCs, we examined the global distribution of H3K4me3 and H3K27me3 marks to gain insights into the epigenetic regulation of the bovine pluripotency program. To this end, we implemented a low-input ChIP-sequencing (ChIP-seq) protocol developed and validated in the P.J.R. laboratory. An average of 31 million uniquely mapped reads were used for peak calling (Fig. S2A), which resulted in 8,816, 2,553, and 3,886 genes associated with H3K4me3, H3K27me3, or both (bivalent domains), respectively. RNA-seq analysis showed that most of the H3K4me3-only genes (94%; RPKM ≥0.4) were expressed, while 47% of the H3K27me3-only and 64% of the bivalent genes were expressed (Fig. 2A). Notably, gene-expression levels were higher for H3K4me3-only genes than for H3K27me3-only and bivalent genes (average RPKM = 34, 5, and 8, respectively).

Gene ontology (GO) terms associated with H3K4me3-only genes included not only housekeeping cellular functions such as protein transport, cell division, transcription, and translation but also specific pluripotency-related functions including ICM proliferation, stem cell population maintenance, and blastocyst development (Fig. 2B). Also, consistent with the use of a small-molecule Wnt inhibitor (IWR1) in CTFR-bESC culture, a noteworthy result was that IF staining for SOX2, POU5F1, GATA6, and CDX2 in bovine blastocysts (Fig. 1A). Notably, gene-expression levels were higher for H3K4me3-only genes than for H3K27me3-only and bivalent genes (average RPKM = 34, 5, and 8, respectively).

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enriched term was the negative regulation of the canonical Wnt-signaling pathway. Enriched GO terms of H3K27me3-only genes related to specific functions that are generally silenced in pluripotent stem cells (PSCs), such as fertilization, startle response, Ig production, and visual perception. Last, enriched GO terms for bivalent genes were mainly related to cell-fate decisions, e.g., cell-fate commitment, neuron migration and differentiation, central nervous system development, and stem cell differentiation (Fig. 2B).

Visual assessment of genes associated with three selected GO terms for H3K4me3, H3K27me3, or bivalent genes are shown in Fig. 2C. We found that all genes containing the H3K4me3 mark presented well-defined peaks in their promoter regions, while H3K27me3 genes displayed broader peaks (some in the promoter region, but most of them covered much of the gene bodies). Bivalent genes showed colocalization of H3K4me3 and H3K27me3 marks near their promoter regions. Notably, the H3K27me3 mark in bivalent genes was more confined to the transcription start site, showing sharp peak shapes similar to the H3K4me3 mark (Fig. 2C).

Although mouse and human ESCs represent distinct pluripotent states, they do share some common transcriptional and epigenetic features characteristic of pluripotent cells. We obtained lists of genes that are known to harbor H3K4me3, H3K27me3, or bivalent domains in human and mouse ESCs and compared them with those in CTFR-bESCs. We found that 62% (n = 4,498) of H3K4me3-only bovine genes also contained the H3K4me3 mark in human and/or mouse ESCs (Fig. S2 B and C). Most of these genes were shared by both species (33%, n = 2,563), but a greater number of genes were shared with human ESCs (25%, n = 1,822) than with mouse ESCs (6%, n = 513). Forty-four percent of the bovine bivalent genes were also shared by mouse and/or human ESCs, and half of these were shared by all three species (n = 757). Similarly, bivalent bovine genes showed more similarity to those of human ESCs than mouse ESCs (13%, n = 447 and 9%, n = 317, respectively). Interestingly, only 4% of the H3K27me3-only bovine genes (n = 1,697) were shared with those in the other two species. This low level of overlap was likely due to the low number of H3K27me3 genes in mouse (n = 137) and human (n = 424) ESCs. Overall, these results suggest that bESCs have an epigenetic landscape similar to ESCs from other mammalian species, further confirming their pluripotent status.

CTFR-bESCs Display Transcriptional and Epigenetic Features Characteristic of the Primed Pluripotent State. To investigate the pluripotency state of CTFR-bESCs, we analyzed the expression of typical naive and primed pluripotency markers identified in mouse and human ESC studies (24, 25). We found that most of the examined primed pluripotency markers were expressed (RPKM ≥ 0.4) in CTFR-bESCs (19/22, 86%), but fewer naive markers were expressed (14/22, 64%). Also, the average RPKM values of all the analyzed markers were higher for the primed than for the naive pluripotency marker genes (RPKM = 25 ± 8.4 and RPKM = 17 ± 13, respectively) (Fig. 3A).

The presence of an H3K4me3 peak in the promoter region of core pluripotency transcription factors, including Pou5f1, Sox2, Sall4, and Nanog genes, is common to both naive and primed ESCS. Consistently, CTFR-bESCs also showed sharp H3K4me3 peaks at the promoter regions of these genes (Fig. 3C). Next, we focused on genes that display distinct histone methylation patterns in naive- and primed-state PSCs in the mouse and human species (26, 27). Consistent with the gene-expression analysis, the epigenetic signatures for all the examined genes in CTFR-bESCs reflect a primed pluripotency state, e.g., bivalent domains are present in HOXA1, FOXA2, GATA6, and TBX3 genes, and there is an accumulation of the H3K27me3 mark in HOXA9 and Nkx2-5 genes (Fig. 3B).

Taken together, these results show that prominent pluripotent epigenetic features are shared among ungulates, rodents, and primates and that CTFR-bESCs harbor a primed pluripotency program.

CTFR-bESCs’ Derivation Is Highly Efficient. CTFR-bESCs’ derivation efficiency was measured as the percentage of embryos successfully establishing a CTFR-bESC line at P3 over the total number of embryos plated. To optimize the CTFR-bESCs’ derivation efficiency, we evaluated three different methods: whole blastocyst, mechanical isolation of the ICM by microdissection, and isolation of the ICM by immunosurgery. We did not find that derivation efficiency differed significantly among the three methods, and the efficiency was comparable to or even higher than that reported for mouse ESCs (Fig. 4A) (23, 28, 29). The initial outgrowths from the three methods were slightly different. The immunosurgery-derived ICM outgrowths were smaller and showed more homogeneous cell morphology than those from the other two methods. By the end of the second week, however, cells showed a similar homogeneous morphology regardless of the derivation method used (Fig. S3A).
Potential applications of CTFR-bESCs for genomic selection. (Fig. S3C).

Discussion

Despite years of research, no stable bESC line that can withstand the rigor of extended passaging in culture while maintaining in vivo pluripotency has been reported. In the current study, we tested a CTFR condition (23) for the derivation of bESCs. Our results show that bESCs can be established efficiently under the CTFR condition by simple whole-blastocyst plating of embryos derived from various sources and genetic backgrounds. CTFR-bESCs are amenable to long-term cultivation and display stable genetic, transcriptional, epigenetic, and functional pluripotency features, including teratoma formation. CTFR-bESCs exhibit molecular features characteristic of primed pluripotency. In addition, we demonstrate the efficacy of using CTFR-bESCs as nuclei donors for the production of NT-cloned blastocysts, a necessary first step toward the generation of live CTFR-bESC NT cattle.

The CTFR culture condition allows robust derivation and long-term propagation of pluripotent bESCs. We consider that the combination of factors, and especially the addition of IWR1, was crucial for the derivation of bESCs. IWR1 blocks the translocation of β-catenin to the nucleus by stabilizing AXIN1/2 (31). The addition of the canonical WNT inhibitor has proven effective in deriving and maintaining mouse EpiSCs and primed human PSCs (23, 32). In bovine species, maintenance of an

displayed stable SOX2 and POU5F1 IF-staining patterns identical to those in the original cells (Fig. S3C).

CTFR-bESCs Can Be Used as Nuclear Donors for NT Cloning. We envision that a potential application of CTFR-bESCs is for genomic selection followed by the production of NT-derived cattle (Fig. 4B). To this end, we tested the possibility of generating NT embryos using CTFR-bESCs (obtained from different embryo sources) and found that all of them were able to produce NT blastocysts at efficiencies ranging from 10 to 20%, a lower blastocyst formation rate than with control fibroblasts (29%) (Fig. 4C). A possible explanation for the lower blastocyst rate is that CTFR-bESCs have a shorter G1 phase of the cell cycle than fibroblasts. Indeed, FACS analysis revealed that CTFR-bESCs had only half as many cells in G1 as fibroblasts (Fig. S3B). Also, we observed a higher percentage of cells in S phase [a cell-cycle stage that is incompatible with NT embryo development (30)] in CTFR-bESCs than in fibroblasts. Finally, the CTFR-bESC NT embryos could again be used to derive secondary CTFR-bESCs that

Fig. 3. CTFR-bESCs show molecular signatures characteristic of primed pluripotency. (A) Transcriptome analysis of selected naive and primed pluripotency markers in CTFR-bESCs. RNA-seq was performed, and RPKM values were used to define expressed (RPKM ≥ 0.4; red) and nonexpressed (RPKM < 0.4; green) genes. The means of two biological replicates are shown. (B) Genome browser snapshots of histone methylation profiles of primed and naive pluripotency markers in CTFR-bESCs. (C) Genome browser snapshots of H3K4me3 and H3K27me3 marks in core pluripotency genes (POU5F1, SOX2, NANOG, SALL4) in CTFR-bESCs.
inactive canonical WNT-signaling pathway is important for normal preimplantation and early postimplantation embryo development (33, 34). Activation of the canonical WNT-β-catenin signaling pathway by the addition of 2-amino-4-(3,4-methylenedioxy)benzylamino)-6-(3-methoxyphenyl)pyrimidine (AMBBMP) during in vitro preimplantation development shows detrimental effects for bovine blastocyst development and reduces the total number of cells in the ICM (34). Additionally, in vitro treatment of bovine embryos (from day 5 to day 7 after fertilization) with a protein inhibitor of the canonical WNT-signaling pathway (DKK1), which is naturally secreted by the female reproductive tract and is involved in maternal-to-embryo communication, has been shown to improve embryo survival significantly after transfer to recipients (33). Therefore, it is plausible that the addition of the canonical WNT inhibitor was critical for the successful derivation and propagation of bESCs. Indeed, withdrawal of IWR1 from the culture medium resulted in the loss of pluripotency markers’ expression (Fig. S4). In addition, no bESC line could be established if IWR1 was not present in the medium.

Mouse ESCs are considered the gold standard for naïve PSCs, whose molecular features resemble those of nascent epiblast cells within the murine ICM (35). The primed state of pluripotency is associated with postimplantation epiblast cells and could be stabilized in cocultured ESC EpiSCs. In addition to rodents, naïve-like and primed PSCs, which are mostly defined by respective molecular signatures, have been described in primate species, including humans (2, 20). All reported naïve-like primed PSCs have not passed the stringent germline chimera assay, and genuine primate naïve PSCs remain elusive. It is possible that species differences may account for the difficulty in obtaining cells equivalent to mouse ESCs in nonrodent species. The CTFR-bESCs derived in this study share many defining transcriptional features with mouse EpiSCs and primed human PSCs, for example, the high expression of genes implicated in lineage commitment in cotreatment, e.g., OTX2 and ZIC2 (36), the moderate expression levels of the pluripotency-related transcription factors Homeobox protein NANOG and KLF4, and the negligible expression of many naive pluripotency marker genes such as FGF4, DNM3L, DPPA2, DPPA3, HORMAD1, TFPC2L1, ZFP42 (REX1), and TBX3 (23, 26, 27). Epigenetically, CTFR-bESCs share many features with their mouse and human primed counterparts, such as the presence of bivalent domains in FGF2 and 2.5 μM IWR1 was not present in the medium.

The CTFR-bESCs described in this study were easy to derive from whole blastocysts, fast to obtain, highly efficient to establish, and easy to passage (single-cell dissociation using trypsin). These are all desirable features that will facilitate the creation of genetically superior cattle and the industrial production of valuable pharmaceuticals, as they allow efficient genomic selection through bESC derivation and facile genome editing and are amenable for NT cloning to generate live animals. Another potential use of these cells is the in vitro differentiation to gametes, facilitating in vitro breeding schemes that could result in multiple rounds of genomic selection, gamete production and fertilization, and bESC derivation to achieve genetically superior cattle within a significantly shorter generational interval.

In summary, by using a serum-free culture condition, we have derived stable pluripotent bESC lines that can be propagated over the long term in culture (for more than 70 passages and more than 1 y at the time of writing) while maintaining stable morphology, normal karyotype, pluripotent transcriptome and epigenome signatures, and the ability to generate teratomas containing cells and tissues from all three primary germ lineages. Moreover, CTRF-bESCs were used successfully as nuclear donors to produce cloned blastocysts. The derivation of stable bESCs opens avenues for various agricultural and biotechnological applications. The culture condition and protocol developed in this study can potentially be applied to many other farm animal species for the generation of stable ESCs.

Materials and Methods

Embryo Production and Processing. The IVM-IVF embryos used in this study were handled as previously described (43). In vitro fertilization (IVF)– and SCNT-derived embryos were produced by Trans Ova Genetics using their standard procedures and were shipped overnight to the University of California, Davis for bESC derivation.

Microsurgery and Immunosuppression of Bovine Blastocysts. Isolated ICMS were obtained by microsurgery. Briefly, zona pellucida (ZP)-depleted blastocysts were placed in a Petri dish containing PBS, and microsurgery was performed using a microblade connected to micromanipulation equipment (NT88-V3; Nikon/Narisghie) attached to an inverted microscope (TE2000-U; Nikon). Immunosuppression was carried out by incubating the embryos in KSOM medium with 20% anti-bovine serum (Jackson ImmunoResearch) for 1 h at 37 °C followed by repeated washes with synthetic oviductal fluid (SOF) Hepes and incubation in KSOM medium supplemented with 20% guinea pig complement (Innovative Research) for 1 h at 37 °C.

Derivation and Culture of CTRF-bESCs. Individual whole blastocysts or isolated ICMS were placed in separate wells of a 12-well dish seeded with a monolayer of gamma-irradiated mouse embryonic fibroblasts (MEFs), were cultured in CTRF medium [a custom basal medium similar to mTeSR medium (STEMCELL Technologies)] that is completely devoid of growth factors FGF2 and TGFβ and contains low fatty acid BSA (MP Biomedicals N2), similar to basal medium in the published recipe (44), and supplemented with 20 ng/mL human FGF2 and 2.5 μM IWR1, and were incubated at 37 °C and 5% CO2 (23). After 48 h, blastocysts/ICMS that failed to adhere to the feeder layer were physically pressed against the bottom of the culture dish with a 22-gauge needle under a microscope to facilitate attachment. Thereafter, the medium was changed daily. Outgrowths (after 6–7 d in culture) were dissociated and passaged using TrypLE (12563011; Gibco) and the early outgrowths were arrayed into the 96-well plates containing MEFs and were passaged every 4 d at a 1:10 split ratio. To increase cell survival, optionally, the ROCK inhibitor Y-27632 (10 μM) was added to the wells 1 h before passage and was also added to the newly prepared wells containing MEFs and fresh culture medium during the 24 h until the next medium change. Medium was changed daily between passages. The ROCK inhibitor is dispensable for routine maintenance and passaging of CTRF-bESCs.

Cells and embryos were immunostained and imaged as previously described (45) using the following primary antibodies: anti-GATA6 (sc-7244, Santa Cruz Biotechnology). 

RNA-Seq. A confluent well of a six-well plate of two CTRF-bESC lines (A: cells P13, P23, P35, P45; B: cells at P12, P24, P35, and P46) were used for RNA-seq. Whole blastocysts and fibroblasts were used as controls. Total RNA was isolated using the Qiagen RNeasy Mini Kit and then was reverse-transcribed using with SuperScript III RT (Invitrogen). Libraries were constructed using the TruSeq RNA Sample Prep Kit (Illumina) and were sequenced on an Illumina HiSeq 2500 system according to the manufacturer’s instructions. Sequenced reads were mapped to the bovine UMD3.1 genome assembly and Ensembl
78 genebuild annotation using CLC Genomics Workbench 7.0 (CLC bio). RPKM values were calculated for each gene.

ChIP-seq. Two lines of CTFR-bESCs at P12 were separated from the MEFs using 9227. Biological factors. Sequencing libraries were generated following the manufacturer’s instructions using the ThruPLEX DNA-Seq Kit (R00406; Rubicon) with 16 cycles in the library amplification step. Libraries were sequenced at the Vincent J. Coates Genomics Sequencing Laboratory at the University of California, Berkeley in an Illumina HiSeq 4000 platform where sequencing was performed as 100 bp paired-end. Raw reads were checked for sequencing quality using FastQC and then aligned to the annotated bovine genome (UMD 3.1 assembly) using bwa aln (46, 47). Peak calling was done using MACS2 (48) with narrow settings for H3K4me3 (-g 2.67e-9 0.01 -m 2 100 –B) and broad settings for H3K27me3 (-g 2.67e-9 -0.05 -m 2 100 broad –B) and visualized using the Golden Helix GenomeBrowse tool (Golden Helix, Inc., available at www.goldenhelix.com). Peaks were further analyzed using Hypergeometric Optimization of Motif Enrichment (HOMER) (49) to find peak associations with gene features, and GO analysis was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (50, 51).

Somatic Cell NT. For testing the capacity of established CTFR-bESC lines to make NT blastocysts, several CTFR-bESC lines were used as nuclear donors for reconstructing enucleated oocytes using standard SCNT methodology (45, 52). Primary fibroblasts were used as controls.

Karyotyping, the teratoma formation assay, AP staining, population-doubling time, and cell counting analysis by flow cytometry are described in SI Materials and Methods.

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