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Isolation and characterization of trophoblast-derived stem-like cells from peri-implantation porcine embryos

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ABSTRACT

In mammals, the trophoblast lineage of the embryo is specified before attachment/implantation to become the fetal portion of the placenta. Trophoblast-derived cells were isolated and cultured from day 10 and day 13 porcine embryos and were grown in vitro in a defined, serum-free culture medium for over 2 years without showing any signs of senescence. However, trophoblast-derived cells placed into serum-containing medium rapidly senesce and fail to proliferate. Semiquantitative and quantitative gene expression analyses of cells in culture from 0 to 30 days confirmed the presence (and relative abundance) of mRNA transcripts from genes involved in trophoblast function (*CDX2*, *TEAD4*, *CYP17A1*, *HSD17B1*, *FGFR2*, *PLET*, *HAND1*) as well as some genes known to mediate pluripotency (*POU5F1*, *KLF4*, *CMYC*). Protein immunolocalization demonstrated expression of both trophoblast and mesenchymal cell markers. DNA methylation patterns in promoters of three critical developmental genes (*HAND1*, *KLF4*, *TEAD4*) did not change appreciably over 4 months of culture in vitro. It was demonstrated that these trophoblast-derived cells are easily stably transfected with an exogenous transgene (eGFP) by a variety of methods, and show the ability to survive and to be passaged repeatedly after transfection. In summary, early embryonic porcine trophoblast-derived cells have demonstrated unique characteristics, which means they could be used as valuable tools for laboratory work. Anticipated applications include the study of trophoblast physiology as well as possible solutions for improving efficiency of transgenesis by somatic cell nuclear transfer and for pluripotency reprogramming of cells.

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1. Introduction

In early eutherian mammalian development, trophoblast cells – precursors to the primitive placenta – stand as essential mediators of embryonic growth and survival. Among other functions, the trophoblast provides critical support and direction of embryonic patterning and fetal growth and nutrition (Rielland et al., 2008). Although the trophoblast and other cells of the extra-embryonic membranes are transient in nature, physiological disruption of trophoblast function can cause significant adverse short- and long-term effects in the fetus (Godfrey, 2002).

Although there are basic similarities in placental structure and function among species, there are also very profound and wide-ranging differences in some aspects of the regulation of the trophoblast function and differentiation among placental mammals (Douglas et al., 2009). This diversity of molecular specification and differentiation makes the early trophoblast an intriguing target for

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basic research, but also interesting from very practical and applied perspectives. A better understanding of the trophoblast differentiation and function will lead to a better understanding of early embryo development and potential mechanisms behind early embryonic failure. It may also help to identify new ways to improve biotechnological techniques such as cloning by nuclear transfer.

While much is known about the gross and molecular physiology of trophoblast function and placental differentiation in rodents and humans, much less is known in other eutherian species. Much of the research about trophoblast differentiation has been performed in the murine model, using trophoblast stem (TS) cells, which can self-renew without signs of senescence under specific growth factors in vitro. When these growth factors are removed, however, TS cells change into the different terminal cell types present in the fetal part of the placenta, thus allowing for a characterization of the molecular mechanisms behind the differentiation process. Authenticated TS cells have been isolated in mice (Tanaka et al., 1998; Rielland et al., 2008) and rabbits (Tan et al., 2011), but corresponding information about trophoblast differentiation and function in other model systems, especially large domestic species, is lacking. Here, we report the isolation, culture, and characterization of trophoblast-derived stem-like cells from peri-implantation-stage porcine embryos. These cells could contribute to a more complete understanding of early embryo development – including trophoblast differentiation and function – in large animal model systems. A better understanding of the potential mechanisms behind early embryonic failure may, in turn, help to identify new ways to improve biotechnological techniques such as cloning by somatic cell nuclear transfer.

2. Materials and methods

Experimental reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise noted. All animal procedures were performed with the strictest adherence to animal welfare guidelines and with regulatory oversight by the Institutional Animal Care and Use Committee at Utah State University.

2.1. Embryo production and trophoblast isolation

Porcine embryos were generated by breeding virgin gilts via artificial insemination using semen from boars of proven fertility. On the appropriate day of gestation (day 10 or day 13), the embryos were flushed from the uteri after post-mortem hysterectomy. Flushing was accomplished using a 12-gauge, 8.9 cm gavage needle to inject flush medium (5.55 mM glucose, 0.32 mM sodium pyruvate, 0.68 mM calcium chloride, 0.5 mM magnesium chloride, 40 μg/ml gentamycin, and 3 μg/ml bovine serum albumin [BSA; Thermo Scientific Hyclone; Logan, UT, USA] in calcium- and magnesium-free phosphate-buffered saline [PBS; Hyclone]) inside the porcine uterus. Once the medium was inserted, gentle manual pressure was used to make it circulate through the uterine horn to the cervix. At the cervix, a 50-ml tube was placed into the uterine lumen to collect flushed embryos. Embryos were washed twice in fresh pre-warmed flush medium. Hypodermic needles were then utilized to manually micro-dissect the embryonic disc (ED) away from the surrounding tissue. For day 10 embryos, all the tissue that was left after ED separation was utilized for downstream experiments, whereas for the day 13 embryos, a 1-cm piece of trophectoderm (TE) tissue, which was 6–8 cm distal from the ED, was utilized for our samples. No effort was made to remove any endoderm or mesoderm that might have accompanied the collected trophectoderm, but by collecting tissue from locations somewhat removed from the ED, it was likely that trophoblast cells were the predominant cell type in the collections, and the samples will be referred to as trophectoderm samples. Embryonic disc samples were frozen to utilize for positive controls for PCR.

For TE cell isolation, three to five drops of 0.05% trypsin–EDTA were added to the TE tissue pieces in microcentrifuge tubes and the tubes were placed in the incubator at 39 °C for 5 min. The tubes were then placed in a vortex at medium speed until the tissue started to dissociate. Cell culture medium with 15% (v:v) fetal bovine serum (FBS) was added to inactivate the trypsin, cells were vortexed again for 30 s, and then tubes were centrifuged at a speed of $400 \times g$ for 2 min. After spinning, fibroblast medium was removed completely and cells were placed into standard 6-well cell culture plates in 2.0 ml of culture medium (approximately 1000 cells/35 mm well). Primary cells were cultured in serum (FBS)-free medium called "trophoblast (TE) medium" or in serum-containing medium called "fibroblast (FF) medium" (see Table 1 for formulations), depending on the experimental requirement. The so-called "trophoblast medium" is a repurposed version of a culture medium originally formulated and described for use with porcine skin-derived stem cells (Dyce et al., 2004). Cell culture plates were cultured in a tissue-culture incubator set at 39 °C, 100% humidity, and 6% CO_2 in air. Medium was changed every 6–8 days from the day of cultivation.

Cells were propagated (passaged) by one of three methods: "traditional" trypsin passage of an entire well (essentially as in (Lai and Prather, 2003)), transfer of conditioned culture medium and any associated floating cells/cell debris to a new well, or manual removal of free-floating 'clumps' of cells that periodically break-free from the established cell colonies into fresh medium. Cells from day 10 and day 13 embryos responded similarly to in vitro culture conditions and were used interchangeably for the remainder of the experiments.

2.2. Reverse transcription and PCR

Trophoblast-derived cells cultured in TE and FF media were harvested at day 15 and day 30 after first culture from an embryo. The inner cell mass and trophoblast tissue from a day 13 embryo (snap frozen immediately after collection) were utilized as day 0 controls. RNA was extracted from cells using the Total RNA Kit 1 (Omega Bio-Tek Inc.; Norcross, GA, USA), according to the protocol recommended by the manufacturer. RNA was converted to cDNA utilizing the GoScript Reverse Transcription (RT) System (Promega; Madison, WI, USA). The resulting cDNA was purified using the QIAquick PCR purification kit from