Development of Porcine Nuclear Transfer Embryos Reconstituted with Blastocyst-Derived Cells and Enucleated Oocytes

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Recently, production of viable lambs from cells of an established embryonic cell line using nuclear transfer techniques was achieved. Establishment of a similar system in the pig may be useful for the production of transgenic animals in which organs do not contain antigens for xenotransplantation or to analyse functions of isolated human genes. In the present study, the isolation of a porcine cell line from blastocysts is reported. Furthermore, the cells were transferred into enucleated oocytes and the viability and development of the reconstructed embryos was examined.

In vitro-produced hatched blastocysts were cultured in DMEM or BSA-free NCSU-23 in the presence of mitomycin C-inactivated STO cells. Both media were supplemented with 0.1 mM 2-mercaptoethanol, 0.1 mM MEM nonessential amino acids, nucleosides (0.03 mM adenosine, 0.03 mM guanosine, 0.03 mM cytidine, 0.03 mM uridine and 0.01 mM thymidine), 2000 IU/ml LIF, 10 ng/ml bFGF and 20% FCS. Attachment of embryos on feeder cells and formation of colonies of attached embryo-derived cells was observed at 2 and 8 days after culture, respectively. The colonies continued to be subcultured in DMEM supplemented with 60% Buffalo rat liver cell conditioned medium and the same supplements as described above, and a blastocyst-derived cell line was established. The cells were used for nuclear transfer between passages 9 and 30 of culture. In vitro-matured oocytes were activated by applying two direct current (D.C.) pulses of 120 V/mm for a duration of 60 µsec at intervals of 5 sec. Before enucleation, zona pellucidae of all oocytes were cut 10-20% with a fine glass needle in PBS (-) supplemented with 5.55 mM glucose, 1.0 mM glutamine, 7.0 mM taurine, 5.0 mM hypotaurine and 10% FCS (manipulation solution). After cutting, the oocytes were enucleated by pushing out the first polar body and the metaphase II plate in a small amount of surrounding cytoplasm with a glass pipette. The oocytes had been previously cultured in the manipulation solution supplemented with 5 µg/ml Hoechst 33342 and 7.5 µg/ml cytochalasin B for 20 min and confirmation of successful enucleation was achieved by visualising the cytoplasm and removed cytoplasm under ultraviolet light. Immediately prior to injection, a single cell suspension of the donor cells was prepared by trypsinisation and the cell was inserted into the perivitelline space of each enucleated oocyte. Fusion of cells and oocytes was induced at 6 h after activation by applying a single D.C. pulse (100-300 V/mm, 20 µsec). Following the fusion pulse, the complexes were cultured for a period of 1 h in NCSU-23 and fusion was determined. Fused embryos were cultured for an additional 7 days and evaluated for blastocyst formation. At 2 days after culture, the embryos were examined for cleavage. In some experiments, to improve the developmental ability of the reconstructed embryos, they were co-cultured with oocytes activated as described above. In addition, to make sure that the genome of the donor cell is transferred into the cytoplasm, the cells were electroporated with a construct containing the green fluorescent protein (GFP) and fluorescent cells were used as donor cells.

When hatched blastocysts were cultured in DMEM with supplements, no colonies of embryo-derived cells were observed. In contrast, 56% of embryos attached to feeder layers of STO cells formed colonies in NCSU-23 with supplements. When the colonies were subcultured in the absence of feeder cells, a cell line with an epithelial-like cell morphology was obtained. This cell morphology was stable up to at least passage 30. Although no fused embryos were observed when a pulse of 100 V/mm was applied, the fusion rate increased significantly at 150 V/mm (28%) and 200 V/mm (64%). At 200 V/mm, 39% of fused embryos cleaved but no embryos developed beyond the 3-cell stage. When co-cultured with electro-activated oocytes, percentages of reconstructed embryos cleaved (65%) and developed to the 4-cell stage (23%) were significantly higher than those (cleavage: 38%; 4-cell stage: 3%) in the absence of activated oocytes. At 7 days after culture, one reconstructed embryo successfully developed to the blastocyst stage in the presence of activated oocytes. When GFP-expressed cells and enucleated oocytes were fused and the fused embryos were cultured with electro-activated oocytes, 3 of 102 reconstructed embryos developed to the blastocyst stage. All of the blastocysts were positive for fluorescent green under ultraviolet light.

The results of the present study indicate that a porcine cell line can be established from a hatched blastocyst and maintained in vitro for a long period and that reconstructed embryos obtained by transferring the blastocyst-derived cells into enucleated oocytes have ability to develop to the blastocyst stage in vitro.

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