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Lentivirus Mediated Gene Manipulation in Trophectoderm of Porcine Embryos

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Abstract: Development of tools that can manipulate gene expression specifically and efficiently in the trophectoderm (TE) lineage would greatly aid understanding the roles of different genetic pathways in TE versus embryonic lineages. Here, we showed first time that short-term lentivirus infection of porcine blastocysts could lead to rapid expression of transgene specifically in TE cells. Efficient TE-specific gene knockdown could also be achieved by lentivirus-mediated pol III-driven short hairpin RNA (shRNA) and TE-specific gene expression could be temporal controlled efficiently by combining this system with Tet-On system. This lentivirus lineage-specific infection system would facilitate gene function studies in porcine pre-implatation embryos by specifically knockdown or overexpression of these genes in TE.

Key words: pig, trophectoderm, lentivirus, gene manipulation

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Introduction

Pig is important farm animal and potentially useful in human disease model. However, deficiency of fundamental research in porcine embryonic development and related mechanism severely lagged pace of the adoption of pig as a model in human disease research. Even the essential regulating gene functions are clear during the formation of trophectoderm and inner cell mass in mouse blastocyst, unfortunately, the related knowledge and research in other large animal models is lacking. Up to data, preliminary results indicated that expression patterns such as CDX2 and OCT4 in embryos of livestock including pig, cattle and goat were different from the pattern in mouse embryo. Conventional transgenic approaches such as somatic cell nuclear transfer and pronuclear injection manipulate the genome of whole embryo, so that it is hard to define the roles of genes in a specific cell lineage of embryo. As we all known, porcine blastocysts sustain and continuously develop for at least 3 days *in vitro*, since the cavitation. Thus, porcine embryo during this period is a ready model in the study of trophectoderm (TE) formation and development compared to mouse embryos, which only sustain for 1-2 days *in vitro* before implantation (Kuijk *et al*., 2008). Porcine trophoblastic could provide a powerful model for understanding trophoblast cell biology as well as placental gene expression and proteomics *in vitro*. For this reason, we developed a lentivirus mediated TE specific gene manipulating method in porcine embryo. This method would facilitate gene expression control and the related

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studies on TE developmental process.

Materials and Methods

Unless otherwise stated, all the chemicals were obtained from Sigma-Aldrich Corp (St. Louis, MO, USA).

Preparation of lentivirus vectors

The constitutive and inducible lentivirus vectors were constructed based on commercial vectors (Addgene) FUW-M2rtTA and FUW-tetO-hOCT4. In order to construct FUW-EGFP, FUW-Cdx2DsRed, FUW-miR30-Oct4shRNA-EGFP, using primers flagged by *Xba* I and *Eco* RI, EGFP, Cdx2DsRed and EGFP-miR30-Oct4shRNA were cloned from plasmid pEGFP-C1 and our formerly constructed plasmids pCMV-CDX2DsRed and pGW-EGFP-miR30-Oct4shRNA and replaced the original open reading frame-rtTA behind hUbC promoter in FUW-M2rtTA. In order to construct FUW-tetO-EGFP, FUW-tetO-Cdx2DsRed, and FUW-tetO-miR30-Oct4shRNA-EGFP, those fragments were cloned and used to replace the original open reading frame-hOCT4 behind TRE element in FUW-tetO-hOCT4.

Lentivirus production

In order to obtain high titter virus, those lentivirus plasmids and packing plasmids were transfected into 293T cells using liposome method. After 48 h transfection, virus-containing supernatant was harvested, centrifuged at a low speed (2 000 $r \cdot min^{-1}$ for 10 min), and filter purified with a Millipore Stericup filter unit (Millipore, Billerica, MA). Concentrated virus particles were then aliquoted and stored at –80℃.

IVF procedure

Oocytes were obtained and matured *in vitro* as described previously (Liu *et al*., 2008). The matured COCs was vortexed for 3 min in Hepes-buffered medium with 0.1% hyaluronidase to remove the cumulus cells. Denuded oocytes were washed and held in the modified Tris-buffered medium (mTBM) prior to fertilization (Abeydeera *et al*., 1997). Each 30 oocytes were delivered to 50 μL of mTBM drop under oil and held in 5% CO_2 in air at 39℃. The semen was washed three times in DPBS with 0.1% BSA and after centrifugation for 4 min at 1 900 g the sperm pellet was resuspended in 1 mL mTBM. Following concentration measurement with hemocytometer, resuspension was adjusted to the optimal concentration with additional mTBM. 50 μL of the final sperm dilution was added to the oocytes and incubated for 5 h in 5% CO_2 in air at 39℃. The presumptive zygotes were then washed three times and incubated in PZM3 embryo culture medium (Yoshioka *et al*., 2002) in 5% CO_2 in the air at 39℃.

TE specific lentivirus transduction

On the 5th day of the culture, the cavitated early blastocysts were treated with pronase for 10 min, so that the zona was removed and used to incubate with lentivirus. Forlentivirus mediated TE specific infection, the virus were diluted with CO_2 equilibrated PZM3 to 1×10^7 titer. 2 μL of diluted virus preparation was added to 8-μL droplets under oil containing up to 10 expanded blastocysts. The embryos were cultured with virus for 3 h, then washed by serially transferring using a pipette into fresh PZM3 droplets of at least five times and finally transferred into 500 μL PZM3 medium (for the inducible system, and 2 $\mu g \cdot L^{-1}$ DOX was added in the culture medium).

Immunofluoresence

Embryos were fixed in 4% PFA for 420 min at room temperature and treated for routine immunofluorescence. Briefly, to visualise OCT4, fixed embryos were permeabilised in 0.1% Triton X-100 in PBS for overnight at 4℃ and blocked in 2% BSA in PBS for 1 h. Goat anti OCT4 (SantaCruz) at 1 : 100 and secondary AlexaFluor 488-conjugated anti-goat antibody or secondary AlexaFluor 546-conjugated anti-goat antibody at 1 : 1 000 were used. After antibody incubations and washes, embryos were stained with