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Faithful expression of imprinted genes in donor cells of SCNT cloned pigs

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ABSTRACT

To understand if the genomic imprinting status of the donor cells is altered during the process of SCNT (somatic cell nuclear transfer), cloned pigs were produced by SCNT using PEF (porcine embryonic fibroblast) and P-PEF (parthenogenetic-PEF) cells as donors. Then, the gene expression and methylation patterns of *H19*, *IGF2*, *NNAT* and *MEST* were compared between PEF *vs.* C-PEF (cloned-PEF), P-PEF *vs.* CP-PEF (cloned-P-PEF), respectively. Taken together, the results revealed that there was no significant difference in the expression of imprinted genes and conserved genomic imprints between the donor and cloned cells.

1. Introduction

Somatic-cell nuclear transfer (SCNT) is a useful technique for the production of embryos in many fields, such as the development of animal models, xenotransplantation and embryonic stem cell research. In this process, the donor cell nucleus is amenable to adequate remodeling and subsequent genetic reprogramming. Genomic imprinting plays critical roles in the regulation and maintenance of parent-specific allele expression [1], with key roles in regulating cellular proliferation, growth and development of both the fetus and placenta. Numerous studies have demonstrated that animals cloned using SCNT suffered placental defects caused by incomplete and aberrant epigenetic reprogramming in the genome of donor cells [2–4]. Furthermore, the expression patterns of imprinted genes were altered in both the clone and parthenogenetic embryos [5–7].

Parthenogenetic (PA) embryos contain exclusively maternal genomes and cannot develop to term in mammals due to the lack of paternal gene expression. Although the developmental defect of PA embryos in mammals has been noted, the PA model has been widely used to study expression patterns of imprinted genes, especially for exploring the establishment of maternal imprinting [8]. Our previous study revealed that abnormal expression of imprinted genes results in retarded development of porcine PA and PSCNT fetuses [9]. Other studies also revealed that abnormal development of cloned and parthenogenetic animals is likely due to the inappropriate expression of imprinted genes, which is controlled by differentially methylated regions (DMRs) [10,11].

DNA methylation is an epigenetic marker that plays roles in the regulation and maintenance of the imprinting control region (ICR). It has also been reported that the establishment or erasure of genomic imprinting is controlled by the methylation or demethylation of DNA [12,13]. In porcine PA embryos, improper DNA methylation patterns have been observed in the DMRs of several imprinted genes, such as *H19*, *IGF2* and *XIST*, all of which were associated with developmental failure [14–16].

Cloned animals produced by SCNT are associated with a high incidence of pregnancy failure, which is often characterized by abnormal placental and fetal development. These abnormalities are thought to be due, in part, to incomplete re-setting of the epigenetic state of DNA in the somatic cell nucleus of the donor [17]. While all of these data are based on fetuses or placentas, the expression and methylation patterns in these tissues may not provide a full representation of the imprinting status of donor cells. In

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Table 1
Primers for qRT-PCR analysis.

Genes	Annealing (°C)	Primer sequences (5′ → 3′)	Size (bp)	Reference/accession
H19	55	F: CTCAAACGACAAGAGATGGT R: AGTGTAGTGGCTCCAGAATG	122	[15]
IGF2	55	F: AAGAGTGCTCTTCCGTAG R: TGTCATAGCGGAAGAACTTG	156	[15]
NNAT	55	F: CCACACCACAGACATCCAGAC R: TAGGCAAGGGACAGTGAGAGG	188	DQ666422
MEST	55	F: GGGCGGCATGGGATAAG R: GCGGGATGTGCAGATAGG	122	XM_005673159.1
GAPDH	55	F: ATTCCACGGCACAGTCAAGG R: ACATACTCAGCACCAGCATCG	120	NM_001206359.1

Table 2
Primers and PCR conditions for BSP analysis.

Genes	Primer sequences (5′ → 3′)	Size (bp)	Reference/accession
H19 DMR3 (Outer) (Inner)	F: GGTTTTAGGGGGATATTTTTT R: TTAAAAAAACATTACTTCCATATAC F: GATTTTTAGGTTTGTTATTATTT R: CAAATATTCAATAAAAAAACCC 45 cycles of 94 °C 30 s, 55 °C 30 , 72 °C 1 min	208	[16]
NNAT DMR (Outer) (Inner)	F: ATAGTAGGTGTTTAGTGGAGAG R: ATAATCACCGAATATCTACCCTAT F: TGTGTTAGGTAGTTTGTTGGAGAGA R: CTCCCAAACCCTAATAAATCTTCTT 45 cycles of 94 °C 30 s, 55 °C 30 s, 72 °C 1 min	224	[31]
MEST DMR (Outer) (Inner)	F: TGGAGGAATTGTTGTGGGAGGGG R: CAAAAATTTTTCCCTCCACTAC F: GTGGTTGTAGTAGGAGGGGTATT R: CACCCCATTTAAAAACAACGACT 45 cycles of 94 °C 30 s, 53 °C 30 s, 72 °C 1 min	423	XM_005673159.1

the present study, to gain insight into whether the process of SCNT can alter the genomic imprinting status of the donor, we firstly compared gene expression and methylation patterns of the imprinted genes *H19*, *IGF2*, *NNAT* and *MEST* between PEF and C-PEF cells. In addition, as there is no paternal genome and disordered expression of imprinted genes in P-PEF cells, we used qRT-PCR and the BSP assay to determine if the reprogramming process of SCNT can rescue the improper genomic imprinting in P-PEF and CP-PEF.

2. Materials and methods

2.1. Ethics statement

All pig experiments were carried out in accordance with the guidelines on animal care and use of animals in research, which were approved by the Animal Care and Use Committee of Jilin University, Changchun, China (Grant No. 20130302).

2.2. Sample collection

The protocol for SCNT has been previously described [18]. Briefly, the fetuses were collected from artificially inseminated porcine uterine horn at day 28 and digested with collagenase and DNase I after the head, viscera, limbs and tail had been discarded. The isolated PEF were cultured in DMEM supplemented with 20% FBS. After reaching confluence the PEF cells were then frozen and were used as the donor cells for SCNT. To avoid the contamination of parthenogenetic fetuses due to the incomplete enucleation of SCNT, the pIRES-EGFP vector was used to construct a stable donor GFP cell line. For SCNT, porcine ovaries were collected from a local abattoir, placed in a saline solution and transported to the laboratory within 2 h of removal. The cumulus-oocyte complexes (COCs) were aspirated from the ovarian follicles, then the selected COCs were cultured in maturation medium for 42–44 h and mature oocytes exhibiting the first polar body were selected as recipients for SCNT. The single donor PEF cell was introduced into the perivitelline space and fused electrically with 2 DC pulses of 1.2 kV/cm for 30 μs using a BTX Electro Cell Manipulator 2001 (BTX, San Diego, CA) [19]. The embryos were cultured in PZM-3 (Porcine zygote medium-3) medium for 24 h prior to embryo transfer [20]. The C-PEF cells were isolated from the SCNT cloned fetus at day 28 and digested with collagenase and DNase I, following established protocols for the collection of donor PEF cells. To avoid parthenogenesis, GFP positive cells were used for the following study.

Table 3
In vivo development of porcine embryos.

Recipient sows	Donor cell	No of transferred embryo	No. of fetuses	Isolated cell (GFP+)
♀12	PEF	270	12	10 (8+)
♀22	PEF	200	8	8 (8+)
♀18	PEF	200	0	0
♀13	P-PEF	200	0	0
♀7	P-PEF	210	16	14 (13+)
♀14	P-PEF	215	9	8 (6+)

The protocol for harvesting the P-PEF cells has been previously described in detail [6,9]. Briefly, mature eggs were parthenogenetically electrically activated by two DC pulses of 1.2 kV/cm for 30 μs using a BTX Electro Cell Manipulator 2001 (BTX, San Diego, CA), then cultured in cytochalasin B for 4 h to suppress extrusion of