The Imprinted Messenger RNA Expression in Cloned Porcine Pre-implantation Embryos

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ABSTRACT

The objective of this study was to determine the mRNA expression patterns of several putative imprinted genes in *in vivo* and *in vitro* fertilized, parthenogenetic, and cloned porcine preimplantation embryos. Both maternally (Dlk1, IGF2, Peg1/Mest and Ndn) and paternally (IGF2r, H19 and Xist) imprinted genes were selected. We have used reverse transcription polymerase chain reaction (RT-PCR) to investigate gene expression patterns in the porcine embryos. IGF2 transcripts were detected in the most of embryos. In nuclear transfer (NT), Peg1/MEST transcripts showed fluctuating pattern. Dlk1 was only expressed partially from the morula and blastocyst stage of NT embryos. Ndn gene expression was started somewhat early for *in vivo* embryos. However, the expressions of maternally imprinted genes were similar in all types of blastocysts (NT, *in vivo* and *in vitro* fertilized, and parthenogenetic embryos). The IGF2R gene expression level was somewhat irregular and varied among samples. However, for the majority samples of all types of embryos, IGF2R expression was diminished after one- to two-cell stages and reappeared at the morulae or blastocyst stage embryos. H19 gene was only expressed early in parthenogenetic and *in vivo* embryos. For NT embryos, H19 was only expressed in blastocysts. Xist expression was detected in all blastocysts with the earliest being *in vivo* 8-cell stage embryos and the last one being NT blastocysts. These putative imprinted genes appeared to have stage specific expression patterns with a fluctuating pattern for some genes (Peg/Mest, IGF2r, H19). These results suggest that stage specific presence of imprinted genes can affect the embryo implantation and fetal development.

(Key words : imprinted gene, gene expression, pre-implantation embryos, porcine)

INTRODUCTION

Despite reliable animal cloning techniques, the production rate for viable offspring via cloning still remains less than 5% (Tamada and Kikyo, 2004). Recent reports on cloned embryos during pre-implantation development suggest that there are certain flaws resulting in inaccurate processing of the many essential early events of normal development by cloned embryos due to the defects in the expression of key regulatory genes (Bioani *et al.*, 2002). An utmost consequence of imprinting is shown by parthenogenetic mouse embryos that have two maternal genomes and no paternal genomes. Those mice are small and die shortly after implantation for absence of expression of paternally imprinted genes (Surani *et al.*, 1984, 1990). Animals cloned by somatic cell nuclear transfer had been showing abnormal phenotypes, possibly caused by abnormal epigenetic changes on genes, especially imprinted ones (Rideout Ⅲ *et al.*, 2001). Expression of imprinted genes is depended on the parent allele that contributed the methylation patterns which are erased during development and reestablished during gametogenesis and at the pre- and post embryo implantation stages (Ross *et al.*, 2003; Ruddock *et al.*, 2004). Changes in expression pattern of imprinted and non-imprinted genes during early development leads to the abnormal fetus and placenta formation (Humpherys *et al*, 2001: Inoue *et al.*, 2002). For imprinted genes are known to regulate fetal growth and normal development, improper imprinting might lead to the defects in large calf syndrome of cloned animals (Ogawa *et al.*, 2003: Shouquan *et al.*, 2004).

Due to their crucial roles in pre-implantation embryo development, it is important to understand the expression patterns of imprinted genes. We investigated the mRNA expression patterns of several putative imprinted genes in both normal and

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cloned porcine pre-implantation embryos in this study.

MATERIALS AND METHODS

1. Recovery and Maturation of Oocytes

Unless otherwise indicated, all chemicals were purchased from Sigma (St. Louis, MO, USA). All procedures used in this experiment were approved by the Animal Care and Use Committee of National Institute of Animal Science (Suwon, Korea). Ovaries were obtained from prepubertal crossbred gilts at a local slaughterhouse and transported to the laboratory at 30~35℃. Cumulus-oocyte complexes (COCs) were collected by the aspiration of the ovary antral follicles (3~6 mm diameter) with 18 gauge needle fixed to a 10 ml disposable syringe. COCs were matured for 40 to 44 h at 38.5℃ under 5% CO_2 in an air. The maturation medium was TCM-199 supplemented with 0.1% (w/v) polyvinyl alcohol (PVA), 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 0.5 ug/ml LH, 0.5 ug/ml FSH, 75 ug/ml penicillin G and 50 ug/ml streptomycin.

2. Preparation of Nuclear Donor Cells

Ear skin tissue was collected from 8 month-old miniature pig (Immerge Bio Therapeutics Inc., Cambridge, MA, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibo-BRL, Grand Island, NY, USA) supplemented with 15% (v/v) fetal bovine serum and 75 ug/ml antibiotics. The cells were frozen with DMEM supplemented with 10% dimethylsulfoxide after two passages. Thawed cells were cultured until they reach confluence and were used as donor cells. The ear fibroblast cells were cultured, passaged (3~8 passages) and used as donor cells for NT.

3. Production of Nuclear Transfer

The matured oocytes were stripped by vortexing the COCs in PBS supplemented with 0.1% (w/v) PVA and 0.1% (w/v) hyaluronidase for 4 minutes. Oocytes were enucleated by the aspiration of the first polar body and metaphase-Ⅱ plate in a small amount of surrounding cytoplasm with a glass pipette. All micromanipulation procedures were performed in TCM-199 supplemented with 3 mg/ml BSA and 5 ug/ml cytochalasin B. Enucleation was confirmed by staining the oocytes with 10 ug/ml Hoechst 33342 for 15~20 minutes at 39℃. After enucleation, the oocytes were held in TCM-199 supplemented with 3 mg/ml BSA until injection of donor cells. Reconstructed oocytes were then placed between 0.2 mm diameter wire electrodes (1 mm apart) of a fusion chamber overlaid with 0.3M mannitol solution supplemented with 0.1 mM $MgSO_4$, 1.0 m M $CaCl_2$, and 0.5 m M Hepes. For fusion, two DC pulses of 1.2 k V/cm were applied for 30 microseconds using a BTX Electro Cell Manipulator 2001 (BTX, San Diago, CA, USA). The reconstructed oocytes were divided into two groups and fusion status were checked 1 hour after fusion. NT embryos were cultured in PZM-3 for 6 days.

4. Collection of *In Vivo* Blastocysts

Embryos were collected from synchronized 7 to 11 month-old Landrace gilts. Gilts were artificially inseminated 24 and 36 hours after hCG injection. Immediately after stunning and bleeding of the animals, approximately 168 hours after hCG (120 hours after estimated ovulation), the genital tract was removed and flushed with PBS for embryo collection. Embryos were transported to the laboratory in Tyrode's lactate-HEPES containing 0.1% (w/v) PVA at 37℃ within 30 minutes after collection.

5. Extraction of Total RNA, cDNA Synthesis and RT-PCR

Total RNA samples were prepared from porcine blastocysts of nuclear transfer (NT), Parthenogenesis (PA), *in vitro* fertilization (IVF) and *in vivo* embryos. All embryos were washed twice with DEPC-treated water prior to total RNA extraction. RNA samples were dissolved in 10 ul DEPC-treated water and stored at −70℃ until RT-PCR analysis. First strand cDNA synthesis was achieved by reverse transcription of the RNA by using the oligo (dT) primer and the First strands cDNA Synthesis kit (Roche, Mannheim, Germany). The mRNAs of IGF2, IGF2r, H19, Xist, Peg1/Mest, Dlk1 and Ndn were then detected by RT-PCR with specific primer pairs (Table 1). PCR reactions were performed following PCR machine manufacturer's protocol. The amplification program was as follows: preincubation for HotStart polymerase activation at 95℃ for 15 min, followed by 35 cycles of denaturation at 95℃ for 1 min, annealing at 55~65℃ for 1 min, elongation at 72℃ for 1 sec. The entire PCR products visualized under UV light on 2% agarose gels in 1× TAE buffer containing 2 ul/ml ethidium bromide.

6. Statistical Analysis

The generalized linear model procedure (PROC-GLM) of the Statistical Analysis System (SAS User's Guide, Statistical

Table 1. Primer sequences used for RT-PCR

Genes	Primer sequence	Annealing temp. (℃)	GenBank accession number
Dlk1	Forward 5'-AGG TGA GGT TCG AGT GTC TG-3' Reverse 5'-AGT GCT CTT GGT GAG CTC CT-3'	60	AY172651
IGF2	Forward 5'- CTC GTG CTG CTA TGC TGC TT-3' Reverse 5'-CAG GTG TCA TAG CGG AAG AA-3'	65	NM213883
Peg1/Mest	Forward 5'-GGC CTG CTC AAA TAT GGA AT-3' Reverse 5'-CAC TGG CAT TGT CAT GGA CT-3'	60	AK098397
Ndn	Forward 5'-AAC GTG CTG CGC ATC TTG-3' Reverse 5'-TCA GGT AGT TCT GCT GGA CGA A-3'	58	AY360449
IGF2r	Forward 5'-ATA AAC ACC AAT ATA ACA CT-3' Reverse 5'-GCA CAC GTT AAT ATA AAA CT-3'	55	AF342812
H19	Forward 5'- AAA GAG CAT CTC AAG CGA GT-3' Reverse 5'-GCT CCT GTA CCT GCT ACT AA-3'	55	AY044827
Xist	Forward 5'-ACT AGT GAT GGT TAT GAA AA-3' Reverse 5'-GTA AGA GGA AAG AAA TGA AG-3'	61	AJ429140
β-actin	Forward 5'-CAC TGG CAT TGT CAT GGA CT-3' Reverse 5'-GGC AGC TCG TAG CTC TTC TC-3'	65	U07786

Analysis System, Inc., Cary, NC, USA) was used to analyze data from all experiments. Differences among treatment means were determined by using the Duncan's multiple range-test and *P*-values of <0.05 were considered significant.

RESULTS

1. Expression of Maternally Imprinted Genes

IGF2 transcripts were detected in all but one *in vivo* morula stage and one PA blastocyst stage embryos (Fig. 1, IGF2). In NT, Peg1/MEST transcripts were present between 1-cell and 4-cell stages, disappeared at 8-cell stage and reappeared at the morulae stage (Fig. 1, Peg1/Mest). Dlk1 was only expressed partially from morula and blastocyst stage of NT embryos (Fig. 1, Dlk1). Ndn gene expression started at morula stage for *in vivo* embryos while expression for PA, IVF, and NT embryos started later at blastocyst stage (Fig. 1, Ndn). However, the expression of Ndn, Peg1/MEST and IGF2 were similar in all (NT, *in vivo*, IVF, PA) blastocysts (Fig. 1).

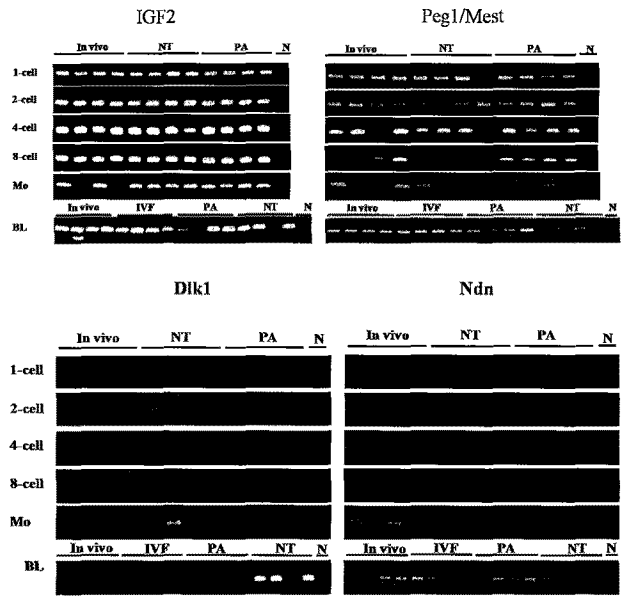

Fig. 1. The expression patterns of maternally imprinted genes in porcine embryos during pre-implantation development. NT: nuclear transfer, PA:parthenogenesis, IVF: *in vitro* fertilization.

2. Expression of the Paternally Imprinted Genes

IGF2R was detected in three of the four PA blastocyst stage embryos and in one of the four PA 1-cell stage embryos (Fig. 2 IGF2R). H19 transcripts were found in three of the four PA 8-cell stage embryos and all blastocyst stage embryos (Fig. 2 H19). Xist expression was detected in all (*in vivo*, NT, PA, IVF) blastocysts with the earliest being *in vivo* 8-cell stage embryos and the last one being NT blastocysts (Fig. 2, Xist).

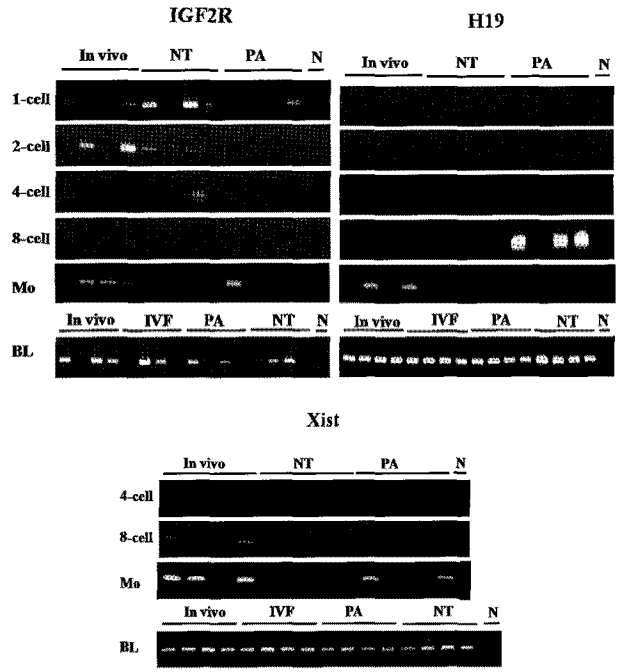

Fig. 2. The expression patterns of paternally imprinted genes in porcine embryos during pre-implantation development. NT: nuclear transfer, PA: parthenogenesis, IVF: *in vitro* fertilization.

DISCUSSION

During the implantation, mammalian embryo undergoes many crucial episodes that affect the genome (Latham and Schult, 2001). In the present study, we examined the expression of seven putative imprinted genes: H19, IGF2r, Xist, Dlk1, IGF2, Peg1/Mest and Ndn in porcine embryos from 1 cell to blastocyst stage. Our results showed that putative imprinted genes appear to have cleavage stage specific expression patterns during pre-implantation, including inappropriate expression of imprinted genes such as biallelic expression or silencing in NT embryos. Of the maternally imprinted genes, IGF2 encodes a growth factor that regulates tissue differentiation, fetal growth and placental development (Gebert *et al.*, 2006). Dlk1 mRNA is reported to be expressed in mouse embryos during the post-implantation development (Schmidt *et al.*, 2000) but not in any of the bovine oocytes or embryos (Ruddock *et al.*, 2004). In our porcine samples, Dlk1 transcripts were detected in one of four morulae and three of four blastocysts stage NT embryos. Ndn, encoding necdin protein, was reported to be expressed only in one-fourth of human morulae and human blastocysts tested (Salpekar *et al.*, 2001) and similarly expressed for porcine embryos in this study. For paternally imprinted genes, H19 encodes for an untranslated RNA that is highly expressed in human placenta (Brannan *et al.*, 1990; Goshen *et al.*, 1993). In the present study, H19 transcripts were found in three of the four PA 8-cell stage embryos and all blastocyst stage embryos (Fig. 2), suggesting that H19 gene is imprinted paternally. In cattle, only IGF2R is proven to be imprinted (Killian *et al.*, 2001).

Of the seven putatively imprinted genes investigated during porcine preimplantation development, two putative maternally imprinted genes (IGF2 and Peg1/Mest) showed an almost ubiquitous expression pattern, excluding them from being imprinted genes in pigs. Rest five of the genes examined (Dlk1, Ndn, IGF2R, H19, Xist) showed characteristic of maternally (Dlk1 and Ndn) and paternally (IGF2R, H19 and Xist) imprinted genes. For most of these five imprinted genes, transcripts appeared to be expressed early and then shut off, indicating a potential role in the nuclear transfer, fertilization, or *in vivo* embryo development events. There was an apparent difference in expression patterns of imprinted genes between *in vivo*, NT and PA embryos. These results suggest to stage specific presence of imprinted genes that affect the embryo implantation and fetal development. Still there is very little information on imprinted genes of pigs. To gain an explanation for the role of imprinted gene on the porcine implantation mechanism, further investigation of the system of imprinted gene regulation is required.

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