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The EST Study of the Peri-implanting Porcine Embryos

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A dramatic morphological change of embryos occurs at peri-implantation. Maternal and embryonic cross-talk during this period, initiated by signals from embryo(s), provides signals for maternal recognition of pregnancy and establishing and maintaining the pregnancy. However, the cellular, biochemical and genetic processes that direct embryo remodeling in mammalian species are not well studied or understood. In order to identify potential genes responsible for morphological change and cross-talk between embryo and uterus, an initial EST analysis was performed. A catalog of expressed genes (Transcriptome) from the d12 peri-implanting porcine embryos was constructed. Six clones were chosen from the initial ESTs for elucidation of their expression patterns during embryogenesis in early pregnancy. A number of these genes demonstrated unique expression profiles in a tissue, cell-type, and temporal fashion, indicating dynamic regulation of embryonic and endometrial gene expressions at different stages of pregnancy. Cross-talk between the embryo and endometrium of the pregnant uterus has provided a suitable micro-environment for the embryo's rapid and dramatic morphological changing process at the peri-implantation stage.

Key words : Embryo, EST, peri-implantation, RT-PCR, uterus

Introduction

The construction and generation of expressed cDNA sequences (cDNA library) and consequent large-scale automated DNA sequencing of such libraries, has led to a revolution in molecular biology as well as animal genetics. The expresses sequences tag (EST) approach and large EST databases have provided a powerful approach for cloning new genes and identifying sequences, as well as analyzing whole gene expression profiles in different cells/tissues and differential developmental stages of living animals. The availability of large EST databases also has provided a way to characterize transcripts which are temporally co-expressed through the study of the parallel gene expression patterns.

Porcine blastocysts exhibit a transient period of dramatic morphological change just before initial attachment of the trophoblast to the uterus. Rapid and dramatic transition of the morphology of blastocysts from spherical to tubular to filamentous forms, up to 100- cm long occurs at peri-implantation period [6,9]. (Fig. 1). Prior to elongation, dynamic changes in gene expression in embryos are occurring that appear to be associated with steroid hormone secretion and a high expression of growth factors in the uterus [12]. However, the cellular and biochemical processes that direct blastocyst morphogenesis are not well understood.

In order to facilitate studies of the molecular mechanisms involved in the unique phenomenon of rapid morphological transition of pig embryos, a complementary DNA (cDNA) library was prepared from day 12 porcine embryo mRNAs. Three distinct stages of the d12 conceptuses (spherical, tubular and filamentous) were used as starting material. Sequence analysis was initiated based on the expressed sequence tag (EST) approach.

Hence, the purpose of the present studies was to test the feasibility of using the EST approach to characterize gene transcripts expressed in peri-implantation stage embryos for subsequent identification of signals responsible for embryo development as well as of unique genes associated with maternal recognition of pregnancy in the pig.

Materials and Methods

Trizol reagent was purchased from Life Technologies (Gaithersburg, MD). Restriction enzymes and Taq polymerase were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). The day 12 cDNA library was constructed using the Zap-cDNA Cloning Kit from Stratagene (La Jolla, CA) and amplified as previously described [4].

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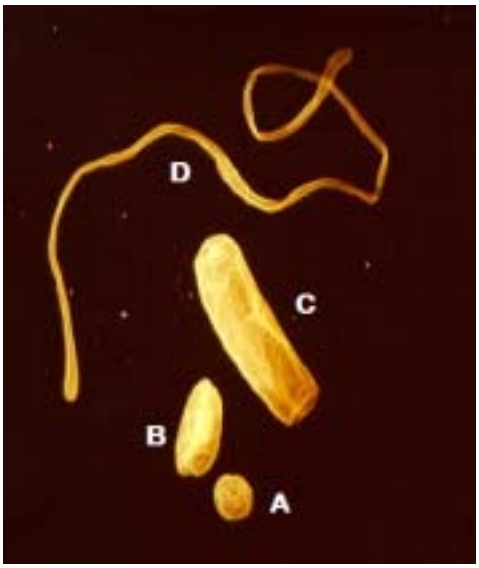

Fig. 1. Dynamic changes in porcine embryo morphology at day 12 (peri- implantation period). Embryos were collected from one pregnant pig at day 12 of pregnancy. A: Spherical (4×2 mm) B: Transitional (6×4 mm) C: Tubular (17×5 mm) D: Filamentous (90×1 mm).

Collection of tissues, cells, and embryos

Pigs were bred at estrus (day 0) and were slaughtered on the indicated days of pregnancy and reproductive tracts were removed immediately for the collection of endometrium, embryos and other tissues. After flushing of the embryos from the uterus, the size of each embryo was measured and embryos were then immediately frozen at -800C until further analysis.

RNA isolation and RT-PCR

Total RNA was isolated from embryos, endometrium at specific days of pregnancy using Trizol reagent according to the manufacturer's instructions. Five micrograms of total RNA were used for first strand cDNA synthesis by utilizing the cDNA CycleTM Kit (Invitrogen, San Diego, CA). Polymerase chain reactions (PCR) (1 minute at 950C, 1 minute at 550C, and 2 minutes at 720C, and 30 cycles) were performed with 0.2 μl of first strand cDNA previously synthesized from total RNA. 10 μl of amplification products from each PCR reaction was analyzed by electrophoresis. The sequences of each oligonucleotide primer used for RT-PCR are shown Table 1.

Results and Discussion

An initial pilot EST analysis of the pig conceptus cDNA library yielded 164 clones that exhibited strong similarity to known sequences in GenBank using the BLAST (Basic Local Alignment Search Tool) computer program search (Data not shown). Six clones did not exhibit any significant relatedness to known sequences and more than thirty other clones exhibited very low similarity to known sequences. Twenty of these initial d 12 porcine conceptus EST clones were chosen and further analyzed by use of the BLAST and ExPaSy Translate Tool programs (http://www.expasy.ch/tools/dna.html), which assign open reading frame. Six of the twenty ESTs were chosen for the subsequent analysis of their corresponding mRNA expression profiles in different stages of embryos and endometrium of different stages of pregnancy.

Table 1. Sequences of the primers used for RT-PCR and size of resultant products

Gene	Primer sequences (5′-3)	Product size
StAR	F: GACGAGGTGCTGAGTAAAGTGA R: AAAGTCCACCTGGGTCTGTG	486 bp
PuF	F: TTCAGGCCTCTGAGGAACTC R: CTCTTATTCATAGATCCAGTC	280 bp
Ubi-RF	F: GTCGAGCCCAGTGATACCAT R: TGACCTTCTTCTTGGGGCGCAG	283 bp
WT-RP	F: TCTGGAGGCTGCTCGTATTT R: CTTTTCTGCCACCATGTTTTC	375 bp
SMP	F: GGAGATACTTTGCTGGAACCA R: CACGTACATTTCAGAGTAATCCT	450 bp
CK-8	F: TGTCACAGTGAACCAGAGCC R: CCATGTCGGTACGCTTTTG	380 bp
GAPDH	F: AAGTGGACATTGTCGCCAT R: TCACAAACATGGGGGCATC	318 bp

Steroidogenic acute regulatory protein (StAR)

The DNA sequence of the porcine StAR cDNA contains the complete open reading frame (ORF) of 285 amino acids (data not shown). The RT-PCR analysis of porcine StAR mRNA in the pregnant uterus revealed that its expression is greater in the endometrium of pigs at mid- and late- stages of pregnancy than at the peri-implantation period (Fig. 2A). However, embryonic expression of StAR mRNA was not detectable by RT-PCR analysis (Fig. 2B). In contrast, StAR mRNA is expressed in the placenta, especially at later stages pregnancy [20]. The expression of StAR gene in the uterus of any species has not been reported yet to our knowledge. This temporal expression of StAR suggests that the uterus

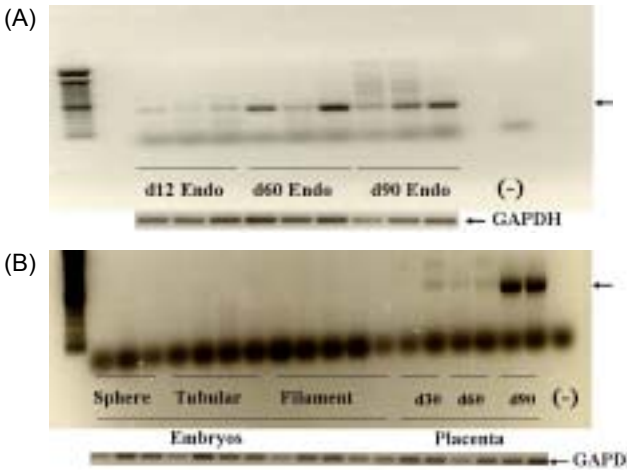

Fig. 2. Expression of porcine StAR mRNA in the uterus (A) and embryos and placenta (B).

is a site of synthesis of steroids, as StAR protein is rate-limiting in the acute steroid synthesis step [13]. These results are in agreement with the earlier findings of the expression of a novel form of P450 aromatase in the endometrium during porcine pregnancy (induced immediately post-implantation) [4,10]. The expression of the StAR gene in the placenta at the later stage of pregnancy is in agreement with a previous report of expression of this gene by the pig placenta [18], which is in contrast to human StAR, which is not expressed in this tissue [19].

c-myc

The sequence of the porcine c-myc cDNA clone encodes the entire ORF (152 amino acids) (Fig. 3). The RT-PCR analysis for porcine c-myc mRNA in the endometrium of the pregnant uterus revealed that it is ubiquitously expressed at all stages of pregnancy (Fig. 4). However, mRNA expression of c-myc was not observed in d12 embryos and placenta when examined by RT-PCR analysis (data not shown). The expression of c-myc gene in the uterus of pig has not been reported yet to our knowledge. The c-myc gene family in humans is implicated in cell differentiation and in some tumour cells, including such as endometrial and cervical carcinoma [3,16].

Ubiquitin-ribosome fusion protein

The RT-PCR analysis of porcine ubiquitin-ribosome fusion protein mRNA in the pregnant uterus revealed ubiquitous expression in endometrium at peri-implantation and later pregnancy (Fig. 5A). Embryonic expression of the porcine ubiquitin-ribosome fusion protein mRNA was greater

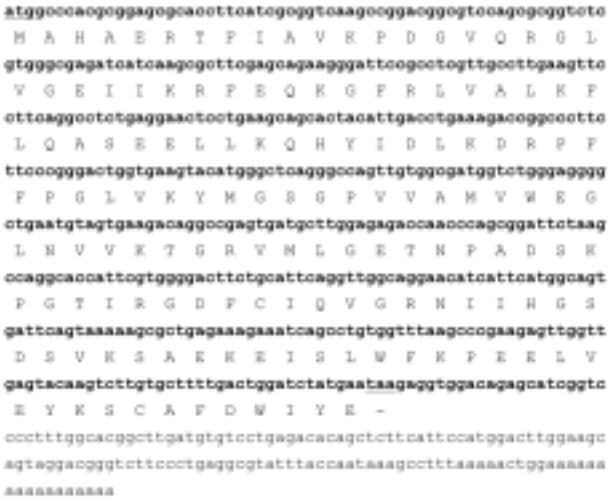

Fig. 3. The DNA and corresponding amino acid sequences of the porcine c-myc transcription factor. (The starting codon atg and non-sense codon taa is underlined, respectively.)

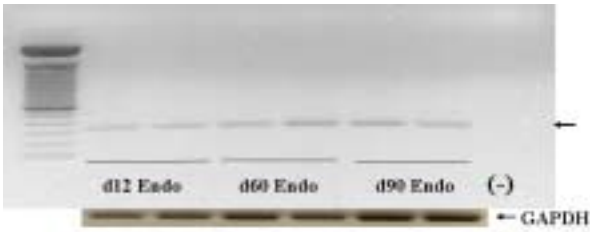

Fig. 4. Expression of porcine c-myc mRNA in the uterus.

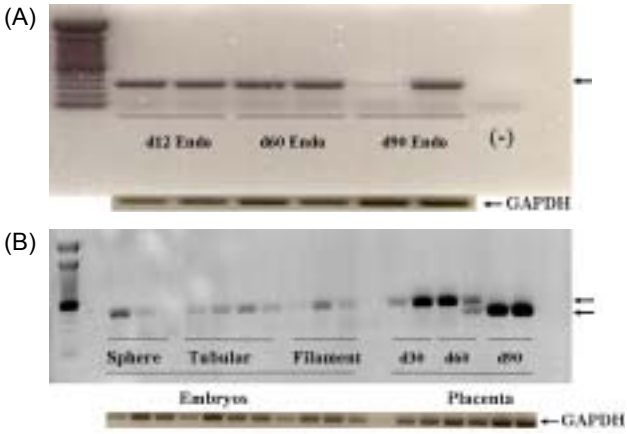

Fig. 5. Expression of porcine ubiquitin-ribosome fusion protein mRNA in the uterus (A) and embryos and placenta (B).

in the mid-size spherical embryos and filamentous embryos than in the early or later stages of both morphologies (Fig. 5B). Also, a transition in the product size from RT-PCR was observed from earlier to later stage placenta (100 bp less RT-PCR products), which suggests a potential developmental switch of this gene expression in late pregnancy

(Fig. 5B). The ubiquitin-ribosome fusion protein is physiologically induced by interferons in uterus at early pregnancy [5]. The function of this protein is not known, but this protein is over-expressed in colon cancer but not in gastric cancer [2].

Ribosomal protein L10 (RPL10) gene

The porcine ribosomal protein L10 (RPL10) mRNA expression was ubiquitous in the pig endometrium (Fig. 6A) at all stages of pregnancy and in all types of embryos as well as in placenta (Fig. 6B). RPL10 protein is also annotated as Wilm's tumor (WT)-1 QM-Related Protein (WT-QM), which is a novel protein that was originally identified as a putative tumor suppressor gene product elevated in a non-tumorigenic Wilms' tumor relative to the tumorigenic parental cell line. The WT-QM gene encodes a 24 kDa basic protein that associates with the ribosomes in the late step of the 60S subunit assembly (WT-QM shares high sequence homology with ribosomal protein L10) [15]. WT-QM is binding to c-Jun is regulated by zinc ions and phosphorylation by protein kinase C [11]. The WT-QM gene is expressed in a broad range of adult and embryonic tissues [14], which is similar to the RT-PCR results for porcine WT-QM reported in here. Although, the precise role of QM has remained elusive, this novel gene product may be involved in post-translational protein processing which is essential for differentiation of specific tissues during embryogenesis.

Senescence marker protein (SMP)-30

The RT-PCR analysis for porcine Senescence Marker Protein (SMP)-30 in the pregnant uterus revealed that it was much less expressed in the d12 endometrium (early pregnancy) than the d90 endometrium (late pregnancy) (Fig. 7A). Embryonic expression of the porcine SMP-30 mRNAs was apparent for all stages (Fig. 7B). The expression of SMP-30 in embryos and other reproductive tissues (Fig. 7B) has not been previously reported, however, these results suggest the importance of the role of regucalcin in the reproductive tract. Senescence marker protein-30 protein, a calcium-binding protein (regucalcin), is notable for the down-regulation of its gene expression with aging in the livers of rats and its restricted expression to liver and kidney [7,8].

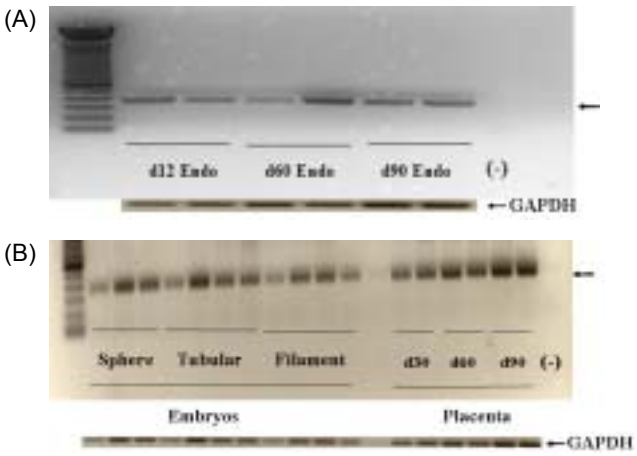

Fig. 6. Expression of porcine ribosomal protein L10 mRNA in the uterus (A) and embryos and placenta (B).

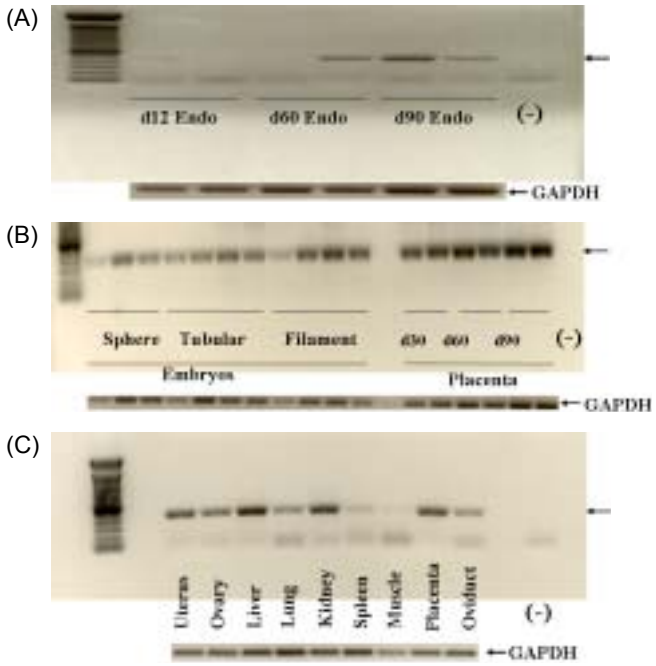

Fig 7. Expression of porcine senescence marker protein-30 mRNA in the uterus (A) and embryos and placenta (B) and other tissues (C).

Cytokeratin-8

The cytokeratin-8 mRNA is ubiquitous expressed in all stages of uterus, including peri-implantation and mid and late pregnancy (data not shown). However, the embryonic expression of the porcine cytokeratin-8 mRNA was very interesting in that it was greater in the early stage embryos than in later the filamentous stage embryos (Fig. 8). Also, the porcine cytokeratin-8 mRNA was observed in later stage placentae, but not in the earlier stage placentae (Fig. 8). Initial EST analysis of the porcine d12 EST embryo library revealed numerous cytokeratin-8 (CK-8) clones, suggesting that the cytokeratin-8 gene is highly expressed. The cytokeratin-8 is major type II keratin and they are commonly used

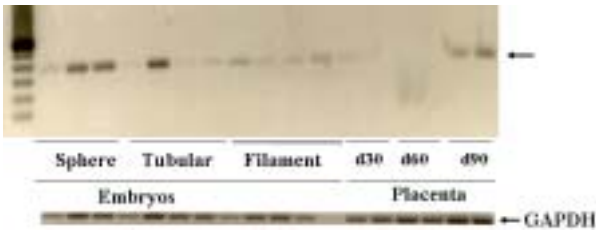

Fig 8. Expression of porcine cytokeratin-8 mRNA in embryos and placenta.

as tumorigenic marker for various types of carcinomas [17]. CK-8 is the first intermediate filament protein expressed during mouse embryogenesis and this expression is found in most embryonic tissues. A targeted null mutation in the CK-8 gene causes mid-gestational lethality and mutant embryos are growth retarded, supporting an essential role for this protein in development [1]. The novel finding was the higher expression of the porcine CK-8 mRNA in the early than later stages of d 12 peri-implantation embryos. This suggests that CK-8 might be involved in the early steps of morphological development of the peri-implanting embryos.

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초록 : Peri-implanting 단계의 돼지배아 EST 연구

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임신초기의 배아는 peri-implantation 단계에서 매우 극적인 형태학적변화가 일어나는데, 이는 임신 인식 인자로 작용하는 배아에서 제공된 signal(s)에 의해서 시작되어지며, 나아가서 모성자궁과 배아의 상호 신호전달이 임신의 시작과 유지에 필수적인 인자로 작용한다. 배아형태의 급격한 리모델링에 관련된 세포학적, 생화학적, 유전학적 연구를 위하여, 또한 자궁과 배아의 상호 신호전달에 관여하는 잠재적 유전자 군을 발굴하기 위하여, peri-implantation 시기의 돼지배아를 이용하여 expresses sequences tag (EST) 분석을 실행하였다. 돼지배아 EST 분석으로 임신초기 특히 전 착상 단계에서 발현되는 유전자들의 카탈로그(Transcriptome)를 작성하였다. 그중에서 6개의 clone을 선택하여 그 발현 양식을 배아 및 자궁 등에서 관찰한 결과, 각각의 유전자들은 조직, 세포 유형 및 임신 시기에 따른 특이적인 발현 현상을 나타내었다. 본 연구결과는, 배아와 자궁 내막에서의 유전자 발현이 임신 시기에 따라서 다이내믹한 상호 조절 작용을 하고 있음을 나타낸다. 이는 전 착상단계의 모성자궁에서 배아와 자궁 내막의 상호 신호전달이 전 착상 단계의 배아의 급격한 형태학적 변화를 가능하게하고 또한 착상에 필요한 적절한 자궁 내부 환경을 제공하고 있음을 보여준다.