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Expression of mRNAs for Growth Factor Receptors during Development In Vitro of Porcine Ova Matured and Fertilized In Vitro

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Abstract: *We analyzed the expression of mRNAs for growth factor receptors with receptor tyrosine kinases [ErbB3 (a member of the EGF-R subfamily), insulin-like growth factor-I receptor (IGF-I-R), basic fibroblast growth factor receptor (bFGF-R) and platelet-derived growth factor receptor α (PDGF-Rα)] by reverse transcription-polymerase chain reaction during in vitro development (10 to 168 h of in vitro insemination: zygotes to blastocysts) of porcine ova matured and fertilized in vitro. Transcripts for IGF-I-R, bFGF-R and PDGF-Rα were detectable in ova at all stages of in vitro development. Transcripts for ErbB3 were present in embryos from the late pronucleate stage onward. The results provide evidence that several growth factor receptor mRNAs are present in porcine ova during development in vitro.*

Key words: *Growth factor receptors, mRNA, Development, RT-PCR, Porcine ova.*

Studies on the cellular and molecular factors operating during development provide the basis for the definition of conditions for the production of embryos *in vitro* and for their possible application in basic research and animal breeding. Although the viability to term of *in vitro* matured porcine oocytes following *in vitro* fertilization has been reported [1], the rate of development is low and the factor responsible for development largely remains unknown.

Recently, several lines of evidence suggest that polypeptide growth factor may play a critical role in mammalian embryo development: several growth factors have been shown to be present in the female reproductive tract [2–4], the addition of exogenous growth factor to the culture medium results in a beneficial effect on mammalian embryo development [5-7] and a number of growth factor ligand and/or receptor mRNA transcripts have been detected within bovine [8, 9], ovine [10] and porcine ova [11, 12]. Therefore, the expression of growth factor receptor genes in ova during development has led to considerable interest in providing a basis for the definition of conditions for the production of healthy embryos *in vitro*, but, there is little information about the transcription of growth factor receptor mRNA during *in vitro* development of porcine ova.

The objective of this study is to examine, by the reverse transcription-polymerase chain reaction (RT-PCR) method, whether mRNA for growth factor receptor with receptor tyrosine kinases [ErbB3 (a member of the epidermal growth factor receptor subfamily), insulin-like growth factor-I receptor (IGF-I-R), basic fibroblast growth factor receptor (bFGF-R) and platelet-derived growth factor receptor α (PDGF-Rα)] express in porcine ova matured and fertilized *in vitro* during *in vitro* development.

Materials and Methods

In vitro maturation, fertilization and development of porcine ova

The methods for *in vitro* maturation, fertilization and development of porcine ova were largely based on those described by Yoshida [13]. Briefly, cumulus-oocyte complexes aspirated from the ovarian follicles (2–5 mm in diameter) of slaughtered prepubertal gilts were transferred to a droplet of maturation medium [modified (m) TLP-PVA plus 10% fetal calf serum (FCS) and 10% porcine follicular fluid] under paraffin oil in a polystyrene dish and cultured for 42 h. After incubation, oocytes with an expanded cumulus mass were transferred to fertilization medium [mTALP-PVA plus 2 mM caffeine; 0.1 ml] covered with paraffin oil. The sperm-rich fraction of ejaculates was obtained from a Large White boar

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by the gloved-hand method. Semen samples were washed three times with 0.9% (w/v) NaCl containing 100 mg BSA/l (Sigma Chemical Co., St Louis, MO, USA) and 100 mg kanamycin sulfate/l. Washed spermatozoa were subsequently diluted to 5×10^5 cells/ml in the fertilization medium and used for insemination. The spermatozoa were introduced into the fertilization medium to make a final concentration of $2.5 - 5 \times 10^4$ cells/ml. After 6 h of insemination *in vitro*, oocytes were transferred to a droplet of developmental medium (mNCSU-23; 0.1 ml). After 120 h of insemination *in vitro*, cleaved embryos were transferred to a droplet (0.1 ml) of TC-199 medium supplemented with 10% FCS for further development. *In vitro* maturation and fertilization were performed at 38.5°C in an atmosphere of 5% CO_2 in air. *In vitro* development was performed at 38.5°C in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2. To examine the status of fertilization, some oocytes were removed after *in vitro* fertilization, fixed with acetic alcohol (ethanol and acetic acid, 3:1, v/v) and stained with 1% aceto-orcein. The stages of maturation and fertilization of ova were determined by means of Nomarski differential interference microscopy (Olympus Co., Tokyo, Japan).

Isolation of total RNA

Total RNAs were isolated from embryos at 10 (zygotes), 20 (zygotes), 36 (2- to 4-cell embryos), 84 (8- to 16-cell embryos), 144 (expanded blastocysts) and 168 h (expanded to hatched blastocysts) of *in vitro* fertilization (IVF), respectively. Three samples of ova at each stage were obtained from the different experiments. Only zygotes satisfying the following criteria were subjected to RT-PCR analysis: 10 h of IVF. more than 90% of oocytes penetrated by sperm; 20 h of IVF. more than 90% of zygotes penetrated by sperm and having both male and female pronuclei.

After incubation, ova were separated from surrounding cumulus cells and spermatozoa by agitation in a narrow bore glass pipette in Hepes-TALP-PVA, and the ova were washed 10 times with Hepes-TLP-PVA. Total RNA was isolated from pools of 20–30 ova with a RNeasy Total RNA Kit (Qiagen Inc., Chatsworth, CA, USA) and then eluted in DEPC-treated distilled water. Genomic DNA was degraded by incubating the samples with 1 unit of RQI DNase (Promega, Madison, WI, USA) for 30 min at 37°C. After DNase digestion, the samples were reextracted with a RNeasy Total RNA Kit and total RNA was then eluted in DEPC-treated distilled water.

Oligonucleotide primers of growth factor receptors

The sequences of oligonucleotide primers of growth factors and the sizes of predicted products after amplification are summarized in Table 1. ErbB3 primers were designed according to known regions of the human ErbB3 cDNA sequence [14]: the upstream primer was identical to necleotides 932–952 and the downstream primer was identical to nucleotides 1285–1301. IGF-I-R primers were designed according to the bovine IGF-I-R cDNA sequence [15]: the upstream primer was identical to nucleotides 1389–1408 and the downstream primer represented the reverse complement of nucleotides 1769–1788. bFGF-R primers were designed according to the human bFGF-R cDNA sequence [16]: the upstream primer was identical to nucleotides 1994–2012 and the downstream primer represented the reverse complement of nucleotides 2329–2348. PDGF-Rα primers were designed according to the human PDGF-Rα cDNA sequence [17]: the upstream primer was identical to nucleotides 3033–3056 and the downstream primer

Table 1. Oligonucleotide primers used for RT-PCR analysis

Gene*	Primer sequence	Product length	Restriction enzyme (product size:bp)
ErbB3	5′-CCAAGTATCAGTATGGAGGA-3′ 5′-GACTGGATGTTCAGGTA-3′	370 bp	Taq I (201/169)
IGF-I-R	5′-CACTCACTCTGACGTCTGGT-3′ 5′-GTGTCCTGAGTGTCTGTCGG-3′	400 bp	Fsp I (233/167)
bFGF-R	5′-TGAGAGAAGACGGAATCCTC-3′ 5′-AGAGTGATGTGTGGTCTTT-3′	355 bp	Msl I (199/156)
PDGF-Rα	5′-TTCACCTGGACTTCCTGAAGAGTG-3′ 5′-TGCGAGCTGTGTCTGTTCCTCTTG-3′	235 bp	Hinf I (179/56)

*ErbB3: a member of the EGF-R subfamily; IGF-I-R: Insulin-like growth factor I receptor; bFGF-R: basic Fibroblast growth factor receptor; PDGF-Rα: Platelet-derived growth factor receptor α.

represented the reverse complement of nucleotides 3244–3267.

RT-PCR

RT-PCR was carried out with a Gene Amp® RNA PCR Kit (Perkin-Elmer Cetus Co., Nowalk, CT, USA) and a programmable thermal cycler (Astec Co., Fukuoka, Japan). The RT mixture contained aliquots (3 μl) of RNA equivalent to that extractable from 5 oocytes or their surrounding cumulus cells, 4 μl of 25 mM $MgCl_2$ solution, 2 μl of 10 × PCR buffer II, 1 μl of 50 μM Oligo $d(T)_{16}$, 2 μl of 10 mM dATP, 2 μl of 10 mM dCTP, 2 μl of 10 mM dGTP, 2 μl of 10 mM dTTP, 1 μl of 20 unit/μl RNase inhibitor and 1 μl of 50 unit/μl cloned Moloney Murine Leukemia Virus reverse transcriptase to 20 μl. The RT profile comprised the following cycle: at 42°C for 60 min, at 99°C for 5 min and at 5°C for 5 min. After RT the PCR amplification was carried out. The amplification profile comprised 50 cycles: at 95°C for 1 min (dissociation), at 56°C for 1 min (annealing) and at 72°C for 1 min (extension). The final cycle included an additional 7 min at 72°C for complete strand extension. The PCR mixture contained one-fourth of the aliquot of the RT mixture (5 μl), 1 μl of 25 mM $MgCl_2$ solution, 0.2 μl of 100 pmol of upstream primer, 0.2 μl of 100 pmol of downstream primer, 2 μl 10 × PCR buffer, 0.125 μl of 5 unit/μl AmpliTaq^R DNA polymerase and 16.475 μl distilled water (DW) to 25 μl. A defined mRNA (a plasmid pAW109, 10^4 copies, Perkin-Elmer Cetus) was subjected to RT-PCR with interleukin 1-α (IL-1α) primer pair (DM151 and DM152, Perkin-Elmer Cetus) in each experiment and served as an external positive control (308 bp fragment). Detection of β-actin transcript (450 bp fragment) with a β-actin primer pair [11] was served as an internal positive control in each experiment. Reverse transcriptase negative controls were carried out in parallel with the same RNA samples, without reverse transcriptase in the reaction buffer (cDNA RT-). The final washing medium for the oocytes or cumulus cells was subjected to RT-PCR with each primer pair in each experiment and served as a negative control. After PCR amplification, the PCR products were run on 3.5% NuSieve GTG agarose gels (FMC, Rockland, ME, USA) containing 0.5 μg/ml ethidium bromide (Sigma) and photographed.

Verification of RT-PCR products

To confirm the identity of the RT-PCR products, the DNA bands were cut with diagnostic restriction enzymes as shown in Table 1. Ten μl RT-PCR products were incubated at 37°C for 1 h with the appropriate enzyme in a final reaction volume of 20 μl. After the reaction, the mixture was run on 3.5% NuSieve GTG agarose gels (FMC) containing 0.5 μg/ml ethidium bromide (Sigma) and photographed.

Results

Figure 1 shows a gel electrophoresis of RT-PCR products for 4 growth factor receptors, internal (β-actin) and external (IL-1α) positive control groups in ova. Although all RT-PCR amplification gave an amplified product of the predicted size (308 bp) in external (IL-1α) positive control group, transcripts for β-actin were not detected in 8- to 16-cell embryos. On the other hand, no RT-PCR amplification gave an amplified product in either negative control group. With RT-PCR and specific primers, transcripts for IGF-I-R, bFGF-R and PDGF-Rα were detectable in ova at all stages of *in vitro* maturation. Transcripts for ErbB3 were present in ova from the late pronucleate stage onward. The identities of these PCR products were verified by digesting with the restriction enzyme. Restriction enzyme digests of all PCR products yielded fragments of expected size (data not shown).

Temporal patterns of expression of growth factor receptor genes during *in vitro* development of porcine ova are summarized in Table 2.

Discussion

The results of this study showed, for the first time, that 4 different messages for growth factor receptors with receptor tyrosine kinases were detectable in porcine ova during development *in vitro*.

The detection of IGF-I-R and PDGF-Rα and/or bFGF-R transcripts at all stages of *in vitro* development were consistent with the report on bovine ova [8, 9]. Since IGF-I, bFGF and PDGF-A transcripts were detectable in porcine ova during *in vitro* development (unpublished observation), these receptors might mediate various responses in ova by an autocrine circuit. On the other hand, ErbB3 is a member of the EGF-R subfamily of receptor tyrosine kinase and is reported to be a receptor for variety types of EGF agonists [18, 19]. Therefore, ErbB3 in ova during development in this culture system might also mediate various responses to EGF and TGF-α detected as mRNA transcripts in porcine ova (unpublished observation) by the establishment of an autocrine circuit. Three signal transducing transmembrane receptors, which bear significant homology to the EGF-R/ErbB1, are currently known: ErbB2, ErbB3 and ErbB4 [19]. It is therefore necessary to examine whether

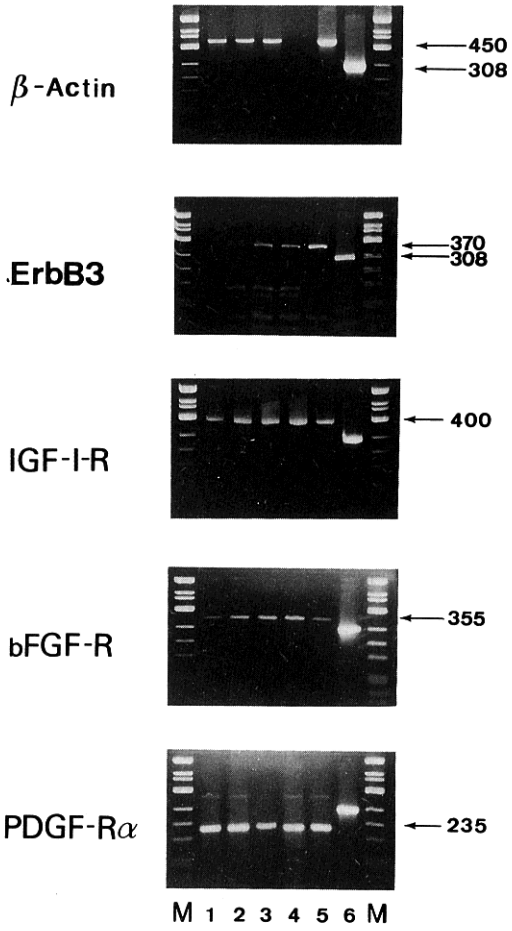

Fig. 1. Detection of β-actin and growth factor receptor mRNA transcripts in porcine ova during *in vitro* development by RT-PCR. Total RNA was isolated from porcine ova, submitted to RT-PCR with primers specific for target sequence and electrophoresed through a 3.5% agarose gel containing ethidium bromide at 10 (lane 1: zygotes), 20 (lane 2: zygotes), 36 (lane 3: 2- to 4-cell embryos),84 (lane 4: 8-to 16-cell embryos) and 144 h (lane 5: expanded blastocyst) of *in vitro* fertilization, respectively. Each lane (1–5) was produced with a cDNA aliquot resulting from RNA from the equivalent of 1/4 of an ovum. IL-1α mRNA served as a positive control RNA (lane 6) which yielded the expected 308 bp product after RT-PCR. Molecular weight markers (M) are φ X174 DNA cut with Hinf I. The size (bp) of the predicted amplified product is indicated.

Table 2. Expression of growth factor receptor mRNAs in porcine ova during *in vitro* development

Hours after *in vitro* insemination (stage)[a]	Growth factor receptor[b]			
	ErbB3	IGF-I-R	bFGF-R	PDGF-Rα
10 (TII-PN)	–	+	+	+
20 (PN-S)	+	+	+	+
36 (2- to 4-cell)	+	+	+	+
84 (8- to16-cell)	+	+	+	+
144 (Exp BL)	+	+	+	+
168 (Exp BL to Ht BL)	+	+	+	+

[a] TII; Telophase II, PN; Pronucleus, S; Syngamy, Exp BL; Expanded blastocyst, Ht BL; Hatched blastocyst. [b] +: detected, –: not detected.

EGF-R and the remaining two members of the EGF-R subfamily of receptor tyrosine kinases mRNA transcripts will be detected during the development of porcine ova.

A switch from maternal to embryonic genome control appears to occur at the 4- to 8-cell stages in porcine embryos [20]. According to this idea IGF-I-R, bFGF-R and PDGF-Rα mRNAs were present as both maternal and embryonic transcripts since they were continuously expressed during development *in vitro* but the temporal pattern of expression of ErbB3 did not occur the case. The transcript for ErbB3 detected in oocytes during maturation *in vitro* (unpublished observation) and in zygotes from the late pronucleate stage onward, but not in zygotes at the early pronucleate stage, support the idea

[21] that the activation of the embryonic genome is actively transcribed earlier than previously thought. Combination with quantitative RT-PCR and transcriptional inhibitors may be necessary to determine the precise timing of the switch from maternal to embryonic transcripts for each gene. Although we used the β-actin gene as an internal control to determine gene expression in this study, the transcript for β-actin was not detected only in 8- to 16-cell embryos, indicating that the maternal message for β-actin was degraded at the point of zygotic genome activation. This is similar to the results obtained in mouse [22] and bovine embryos [23]. Therefore the detection of ß-actin gene expression may not be useful as an internal control during development in these species. Alternatively, this study shows that some growth factor receptor genes are potentially a good candidate for an internal control during development in the pig.

The addition of exogenous growth factor to the medium result in facilitating early mammalian development [5–7], several growth factors have been shown to be present in the female reproductive tract [2–4] and 4 different messages for growth factor receptors were detectable in porcine ova in this study, suggesting that various interactions occur among ova and growth factors during development. Whether these growth factor receptors mRNAs are translated to peptides in ova and the peptides act in mediating growth factor signals, as we have hypothesized, deserves additional investigation.

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