EXPERIMENTAL STUDY

Progestosterone receptor gene expression in preimplantation pig embryos

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Abstract

Objective: It is not known whether progesterone, which plays a key role in establishing and maintaining pregnancy, acts directly on embryos or indirectly through the mother’s reproductive tract. Since the physiological effects of progesterone are mediated by progesterone receptors (PR), the expression of PR during the preimplantation stages of pig embryos was determined.

Design and Methods: Preimplantation pig embryos at different developmental stages were examined using reverse transcription-polymerase chain reaction techniques for the purpose of determining PR gene expression. Immunocytochemistry procedures were used to determine whether PR mRNA is translated into PR protein in preimplantation embryos.

Results: PR mRNA was found in pig embryos at the two-cell stage, but levels started to decline at the four-cell stage; none was detected at the five- to eight-cell stage, nor at any time during the morula and blastocyst stages. Results showed that PR protein was immunostained in pig oocytes and embryos at the 4-cell stage, but that no significant immunostaining occurred during the morula and blastocyst stages.

Conclusion: These results indicate that the effects of PR on early embryogenesis appear to be indirect, perhaps via PR-regulated growth-promoting factors produced in the maternal reproductive tract.

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Introduction

Preimplantation embryonic development is characterized by cell proliferation, differential gene expression and differentiation. Progesterone, an ovarian steroid traditionally regarded as the primary mammalian pregnancy hormone, serves to coordinate a complex series of interactive steps, beginning with the synchronized development of the preimplantation embryo and maternal uterus and ending with implantation (1, 2). The elimination of progesterone from pregnant animals has deleterious effects on embryonic development and implantation (3). The physiological effects of progesterone are known to be mediated initially by a specific intracellular protein referred to as a progesterone receptor (PR) (4). The molecular cloning of avian PR (5) identified it as a member of the nuclear receptor superfamily of transcription factors (6, 7). Researchers using a variety of experimental approaches (including PR localization studies, the use of antiprogestins, and progesterone replacement in ovariec-
tomized animals) have suggested several possible developmental and functional roles for progesterone and PR in the ovaries, mammary glands, and the brain (8–11). Antagonism of progesterone by antibody treatment was able to prevent implantation and normal embryo transport in mice (12). Vinijasanun & Martin reported that the abnormalities in embryo development caused by early ovariec-
tomy of mice were reversed by treatment with progestin (13). However, it is still not clear whether progesterone acts directly on embryos or indirectly through the mother’s reproductive tract.

Using an embryonic stem cell/gene targeting technique with mice carrying null mutations of the PR gene, Lydon et al. showed that male and female embryos that are homozygous for a PR mutation can develop normally to adulthood (8); however, adult female PR mutant mice displayed pleiotropic reproductive abnormalities. They suggested that the primary role of progesterone may lie at the reproductive stage. Progesterone antagonism via antibody treatment was able to prevent embryonic implantation in mice due to uterine rather than embryonic action (12). Other researchers have reported that pig embryo development is dependent on factors which originate in the mother’s reproductive tract (14, 15).
Little evidence has been gathered on the direct effects of progesterone on preimplantation embryonic development. One indication of progesterone’s influence was reported by Hou and Gorski who found that the PR gene is activated in mice embryos at the blastocyst stage (16). The possibility exists that full preimplantation embryogenesis requires the presence of paracrine factors that originate in the mother’s reproductive tract under the influence of progesterone. If progesterone does have such a direct effect on embryonic development, PR must be present; it is therefore important to determine if the PR-encoding gene is activated during early embryogenesis. With this goal in mind, we used (i) reverse transcription (RT)-PCR to detect PR mRNA levels, and (ii) immunocytochemistry techniques to determine the presence of PR protein during preimplantation developmental stages in pig embryos.

**Materials and methods**

**Animals**

Gilts \((n = 15)\), aged 12–15 months, were treated with 0.4% altrenogest Regumate porcine (Hoechst UK Ltd, UK) for 18 days and were then superovulated by an initial intraperitoneal injection of 1500 units pregnant mare’s serum gonadotropin followed 72–80 h later by 1000 units human chorionic gonadotrophin (hCG). At 24 h and 30 h after the last intraperitoneal injection, the pigs were artificially inseminated twice. The preimplantation embryos were collected 52 h (1-cell stage), 64 h (2-cell stage), 80 h (4-cell stage), 100 h (five- to eight-cell stage), 120 h (morular stage) or 144 h (blastocyst stage) after the last hCG injection by flushing the oviducts and uterine horns of the pregnant female pigs with 20 ml Dulbecco’s phosphate buffered saline. After flushing, the uterine horns were surgically removed from the female pigs, washed with Dulbecco’s phosphate buffered saline, cut into small pieces and then snap-frozen and stored at \(-70^\circ C\) until use. For immunostaining analysis, fresh embryos were immediately after examination under the microscope. For RNA preparation, pig embryos at the 1-cell stage \((n = 106)\), the 2-cell stage \((n = 60)\), the 4-cell stage \((n = 50)\), the five- to eight-cell stage \((n = 13)\), the morular stage \((n = 7)\) and the blastocyst stage \((n = 4)\) were collected, snap-frozen and stored at \(-70^\circ C\). Pig uterine cells were prepared from the collected uterus for immunostaining by treating the tissue with 0.14 g collagenase and 0.01 g DNase per 100 ml at 37 °C for 30 min twice. The resulting cell suspension was then filtered and centrifuged at 4 °C. Pig uterine cells were examined for their viability under the microscope prior to immunostaining.

**Cell culture conditions**

Rat pituitary cells GH3 were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 1× antibiotic/antimyocytic mix, 5 mmol/l N-(2-hydroxyethyl) piperazine-N’-2-ethanesulfonic acid, 0.37% sodium bicarbonate and supplemented with 10% fetal bovine serum (FBS). Cultures were maintained at 37 °C in a humidified atmosphere of 95% air/5% CO2 and fed every 2 days.

**RNA extraction and RT-PCR**

Unless otherwise stated, all solutions were prepared with water that had been treated with 0.1% diethyl pyrocarbonate (DEPC). Total embryonic RNA was isolated from each pool of embryos at different developmental stages using a modified guanidine/cesium chloride method (16, 17). Briefly, 150 μl Trizol solution containing 15 μg *Escherichia coli* rRNA which was used as a carrier was added to the embryos. Following cell lysis, RNA was precipitated with 3 mol/l potassium acetate (pH 5.2) and 100% ethanol, washed with 75% ethanol and resuspended in 10 μl DEPC-treated water. The concentration of each RNA sample was determined by measuring the absorbance at wavelength 260 (A260). Total uterine RNA was prepared as previously described by Murdoch et al. (18). Briefly, 0.2 g pig uterine tissue was homogenized and the total RNA was then precipitated with sodium acetate and isopropanol. The RNA was resuspended in 50 μl DEPC-treated water and stored at \(-70^\circ C\).

For reverse transcription, 4 μl extracted total RNA which contained approximately 40 cells (or 42.4 embryos at the 1-cell stage, 24 embryos at the 2-cell stage, 20 embryos at the 4-cell stage, 5.2 embryos at the five- to eight-cell stage, 2.8 embryos at the morular stage and 1.6 embryos at the blastocyst stage) were reverse transcribed with Superscript II reverse transcriptase (Gibco-BRL, MD, USA) in a MicroAmp tube (Perkin Elmer, CA, USA) using random primers in a total volume of 20 μl (19). The cDNA RT products were divided into two portions for amplification of PR and β-actin RNA. The PCR reaction mixture consisted of 1/10th RT product, 1× PCR buffer, 2 mmol/l MgCl2, 0.2 mmol/l dNTPs, 1 unit Taq DNA polymerase, and 10 mg/ml PR primers in a total volume of 50 μl. Prior to use in PCR reactions, all solutions were kept on ice to prevent non-specific primer annealing and extension. Reaction mixtures were denatured at 94 °C for 4 min, followed by a total of 35 cycles of denaturation at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. Reactions were post-extended for 10 min at 72 °C. The PR primers used in this study were PR1 \((5’- CCTAGCTCACAGCTTTCTACC-3’)\) and PR2 \((5’- CATCCTGCCAATATCTTTG-3’)\) (5, 20) and the β-actin primers were actin-1 \((5’- AGCACTTGCGGTCCAC-3’)\) and actin-2 \((5’- AGCAGTGGCTGCCACGATG-3’)\) (21). Amplified PR and β-actin product consisting of 179 base pairs (bp) and 410 bp respectively were fractionated on 2% agarose gel stained with ethidium bromide. For control, water was...
substituted for RNA in the PCR reaction. The amplified fragments were confirmed by Southern hybridization with a cloned rat PR cDNA fragment as a probe.

**Immunocytochemistry**

Embryos at different developmental stages were washed twice with buffer M (25% glycerol, 50 mmol/l KCl, 0.5 mmol/l MgCl₂, 0.1 mmol/l EDTA, 50 mmol/l imidazole hydrochloride, 0.5 mmol/l EGTA, 1 mmol/l 2-mercaptoethanol, pH 6.8) and fixed in chilled methanol for 10 min. Next, they were permeabilized with phosphate-buffered saline (PBS) containing 0.1% Triton X-100 for 3–5 min. For immunostaining, embryos were incubated in blocking solution (PBS containing 0.1% Triton X-100 and 3 mg/ml BSA) for 40 min at 37 °C, then incubated in PBS containing 3 mg/ml BSA and diluted PR antibody (SC-20; rabbit polyclone; Santa Cruz Biotechnology, Inc., CA, USA) for another 40 min at 37 °C. Controls included blocking solution without primary antibody, nonimmune purified rabbit immunoglobulin (Ig) G or PR antibody pre-absorbed with competitive peptide (SC-20p). The SC-20 is an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to amino acids 545–564 of the progesterone receptor of human origin which is identical to the corresponding mouse sequence. It reacts with the progesterone receptor of mouse, rat and human origin by Western blotting and immunohistochemistry and does not cross-react with other steroid receptors. When SC-20p was used to pre-absorb PR antibody, the reaction mixture was incubated for 1 h at 37 °C prior to being added to the embryo samples. Embryos were washed with PBS containing 0.1% Triton X-100 and 3 mg/ml BSA and developed with Histostain-Plus according to the manufacturer’s recommendation (Zymed Laboratories Inc., San Francisco, CA, USA).

**Results**

To establish RT-PCR detection limits and specificity, we diluted pig uterine RNA from 1:5⁰ to 1:5⁷ so that each sample contained approximately 102 ng to 2.7 pg total RNA. The reverse transcribed pig uterine cDNA was then amplified with the PR1 and PR2 primers (Fig. 1A). Results indicated that the RT-PCR used in these experiments exhibited satisfactory sensitivity; PR gene expression detection limits were approximately 1.3 pg total pig RNA. E. coli rRNA interference during embryonic RNA preparation was also evaluated as part of the RT-PCR assays (Fig. 1B). It was found that the addition of 0.4 μg E. coli rRNA caused a slight decrease in the amplified PR product intensity in the sample containing 32.5 pg pig uterine RNA.

To measure the presence of PR in each stage of embryogenesis, two methods could be used to normalize the results: one using the same number of embryos (e.g. 38 embryos for each developmental stage), and the second using the same number of cells for each developmental stage (e.g. 38 cells for each stage: 38 embryos for 1-cell, 19 embryos for 2-cell, 9.5 embryos for 4-cell). In this study, the second method was chosen based on the following reasons. First, the amount of total mRNA per embryo is unlikely to be equal, Wu et al. showed that the amount of beta-actin mRNA in oocytes appeared to be higher than that in 2-cell embryos using RNA extracted from 40 mouse embryos at different developmental stages (21). The amount of beta-actin in 40 morulae also appeared to be higher than that in 40 blastocysts. Therefore, although it is difficult to measure directly the amount of mRNA in embryos at various stages, it is possible that the amount of mRNA may be somewhat different per embryo at different developmental stages. Secondly, the sensitivity to detect estrogen receptor mRNA in a few embryos was available in our study. There is no necessity to use large numbers (>10) of embryos for each stage. Finally, the collection of pig embryos at various stages is very time consuming. To obtain the same number of embryos at different stages (e.g. 100 embryos for three independent assays) requires much effort and time.

Total RNA prepared from pig embryos was subjected to RT-PCR and the levels of PR message were determined by the presence of the 179 bp DNA fragment (Fig. 2). PR gene expression was detected in the 2-cell stage of embryonic development, appeared to decline in the 4-cell stage, and became completely undetectable in the 5- to 8-cell, morula and blastocyst

![Figure 1 Establishment of RT-PCR assays. (A) Pig uterine RNA was diluted from 1:5⁰ (101.6 ng) to 1:5⁷ (20.3 ng), 1:5⁵ (4.06 ng), 1:5⁴ (0.8125 pg), 1:5³ (0.1625 pg), 1:5² (0.03125 pg), 1:5¹ (0.00625 pg), and 1:5⁰ (0.00125 pg) as indicated, prior to conducting RT-PCR assays. The position of the amplified PR cDNA fragment was visualized with ethidium bromide and marked with an arrowhead. (B) The effects of carrier rRNA on the RT-PCR results. E. coli rRNA (0.4 μg) was added to the pig uterine RNA prior to RT reaction (lane 2). The amplified PR cDNA product was then used for comparison with the control (lane 1). The experiment was repeated three times. M: molecular weight markers.](image-url)
stages. Corresponding quality control results with the β-actin primer set are shown in Fig. 2B.

The presence of PR protein in preimplantation pig embryos was determined by Western blot analysis (Fig. 3) using pig uterine cytosol and with an immunocytochemistry technique which was established using GH3 cells, and further confirmed with prepared pig uterine cells (Fig. 4). The SC-20 PR antibodies cross-reacted with two proteins present in the pig uterine cytosol. This cross-reaction was diminished when purified IgG which does not specifically recognize PR protein was used as the primary antibody in the Western blot analysis (Fig. 3). It thus appeared that the PR antibodies exhibited an acceptable specificity and were able to recognize the PR proteins in pigs.

GH3 cells originating from rat pituitary cells express PR protein and are responsive to estrogen treatment. Immunostaining was observed in GH3 cells following treatment with SC-20 PR antibodies, and the immunostaining intensity was greatly reduced when purified IgG was used instead of SC-20 antibodies (Fig. 4, upper panel). No staining occurred in the absence of a secondary antibody. The SC-20 PR antibody was then used to immunostain prepared pig uterine cells (Fig. 4, lower panel). Results showed that SC-20 readily stained the uterine cells and the intensity of the stain was significantly reduced when SC-20 antibody was replaced with purified IgG. Similarly, we did not detect any staining when the secondary antibody was omitted from the immunostaining procedure. The absence of strong nuclear staining of PR in GH3 cells or pig uterus was observed in this study. Treatments of samples prior to incubation with SC-20 antibody might have caused some diffusion of PR protein from nucleus into the cytoplasm. Attempts to modify the experimental procedure, such as changing the methods of fixing and permeabilization, did not show significant improvement.

The pig oocytes and 4-cell embryos were immunostained with the same PR antibody SC-20 (Fig. 5). Pig oocytes showed significant staining by SC-20 PR antibodies, and the addition of competitive peptide (SC-20p) significantly diminished immunostaining intensity. Similar results were observed in 4-cell pig embryos: immunostaining occurred in the presence of PR antibodies and successful competition resulted from the addition of the SC-20p peptide, indicating the presence of the PR protein in both cases. In the embryos immunostained positive for PR protein, staining intensity was uniform throughout the embryos and all cells of the embryos exhibited positive staining. However, the staining did not appear to concentrate in the nucleus. All immunostaining experiments were replicated 2–4 times and similar results were obtained in each replication. Immunocytochemistry was also performed on pig embryos at the morula and blastocyst stages; staining results were inconclusive for any definitive conclusion to be drawn.

Discussion

The detailed mechanisms which drive growth and differentiation in preimplantation embryos are not well understood. Mammalian embryos such as hamster embryos show developmental block and growth delay when cultured in vitro, suggesting that full preimplantation development requires additional factors from maternal sources (22). Since PR activation in most of the examined tissues was estrogen induced and estrogen receptors were reported to be present in the embryos during the early implantation stage (23, 24), it may be that estrogen is involved in progesterone activation via receptors in the maternal reproductive tract and/or embryos and thus is required for successful implantation. In mice, Vinijisanun et al. reported that antagonism of progesterone by antibody treatment
resulted in complete implantation blockage without significant effects on embryo transport or development, suggesting that implantation blockage resulting from a lack of progesterone is due to uterine rather than embryonic mechanisms (12). Lydon et al. found that mutant mouse strains carrying a germ-line mutation of the PR gene underwent apparently normal embryogenesis and developed to adulthood (8), again implying that influence of progesterone comes from the maternal reproductive tract. The absence of the expression of pig PR in the report of Yelich et al. when they studied the gene expression in early developing pig conceptus provided further evidence for the importance of maternal PR during preimplantation (25). However, contrary results with mouse embryos have been reported by other researchers. For example, Hou and Gorski failed to detect the presence of PR mRNA prior to the blastocyst stage when they used RT-PCR assays to examine mouse embryos (16). Therefore, they hypothesized that PR expression is primarily embryonic, and that progesterone plays a direct role in the process of mouse embryonic development by binding with its receptor in mouse embryos.

The renewed incorporation of uridine as an indication of new mRNA synthesis was first detected in 4-cell pig embryos. This suggests that early pig embryo development is directed by the maternal genome prior to the activation of the embryonic genome at this stage (26, 27). A decrease in total uptake and the incorporation of radiolabeled methionine into protein was also observed in pig embryos from the oocyte to

Figure 4 Immunostaining of PR protein in GH3 and pig uterine cells. GH3 cells (upper panel) or prepared pig uterine cells (lower panel) were immunostained with SC-20 PR antibody (+SC-20) or with purified IgG (-SC-20). Each experiment was repeated at least three times. The bar represents 10 μm.

Figure 5 Immunocytochemistry assay of PR protein in oocyte and the 4-cell pig embryo. Pig oocytes (A, C, E) and 4-cell embryos (B, D, F) were immunostained with either PR antibody (SC-20) or the PR antibody pre-absorbed with SC-20p competing peptide (SC-20+SC-20p). Pig oocytes and 4-cell embryos prior to immunostaining (A, B) are shown for reference. Approximately 3–6 embryos were stained for each developmental stage and each experiment was repeated at least twice. The bar represents 100 μm.
4-cell stage: this may be a reflection of the diminishing pool of maternally derived mRNA available for translation (28). The developmental stage during which an embryo makes the transition from dependence on transcripts derived from the maternal genome to those derived from the zygotic genome is species-specific. In mice, a 70% decrease in RNA has been observed between fertilization and the 2-cell stage (29, 30), and a similar decrease between fertilization and the 8-cell stage has been reported for cattle and sheep (31, 32). In the present study, the decline in PR mRNA in the 4-cell pig embryos apparently correlates with the degradation of maternally derived RNA. Activation of the embryonic expression of PR did not resume until a later stage of embryogenesis in pigs.

The results of this study indicate the presence of PR protein in the oocyte and early stage of preimplantation pig embryos, with PR gene expression undetectable beyond the 4-cell stage. We interpret these results as indicating that PR protein at these developmental stages is maternally directed, and that the physiological function of progesterone in early gestation is most likely mediated through the maternal reproductive tract and not through direct binding to embryonic receptors.

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