Estrogen receptor and progesterone receptor genes are expressed differentially in mouse embryos during preimplantation development

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ABSTRACT Estrogen and progesterone play an important role in the development and implantation of preimplantation embryos. However, it is controversial whether these hormones act directly on the embryos. The effects of these hormones depend on the existence of their specific receptors. To determine whether estrogen receptor (ER) and progesterone receptor genes are expressed in mouse preimplantation embryos, we examined RNA from embryos at different stages of preimplantation development by reverse transcription–polymerase chain reaction techniques. ER mRNA was found in oocytes and fertilized eggs. The message level began to decline at the two-cell stage and reached its lowest level at the five- to eight-cell stage. ER mRNA was not detectable at the morula stage but reappeared at the blastocyst stage. Progesterone receptor mRNA was not detectable until the blastocyst stage. The embryonic expression of ER and progesterone receptor genes in the blastocyst suggests a possible functional requirement for ER and progesterone receptor at this stage of development. These results provide a basis for determining the direct role of estrogen and progesterone in preimplantation embryos.

There are no reports of natural mutations that result in either deficient estrogen synthesis or resistance to estrogen action. This contrasts with testosterone synthesis and action, where single-gene mutations that interfere with both processes have been characterized in many species. This difference has led George and Wilson (1) to suggest that such estrogen-related mutations may be lethal at an early stage of development. Estrogen and progesterone play key roles in the establishment and maintenance of pregnancy. Elimination of these hormones from pregnant animals causes deleterious effects on the development and implantation of the embryos. Hypophysectomy of pregnant rats was shown to result in the delayed entry of eggs into the uterus, expulsion of eggs from the uterus, and retarded development of eggs. Injection of estrone and progesterone significantly reversed this effect (2). Delayed implantation has been observed in ovariectomized mice treated with progesterone only (3). Dormant blastocysts can be reactivated by the injection of estrogen (4, 5). Estrogen and progesterone stimulate the metabolism of delayed implantation mouse embryos (6). It appears to be important to maintain the concentrations of estrogen and progesterone at appropriate levels relative to each other. Elevated ratios of estradiol to progesterone were shown to inhibit blastocyst metabolism and implantation (7). Since these experiments were performed on ovariectomized animals receiving exogenous hormones, it is not clear whether the hormones act directly on the embryo or indirectly through the mother's reproductive tract. Some authors claimed that the effects were indirect, since retardation of embryo development and inhibition of implantation were observed when embryos were grown in oviductal fluid obtained from estrogen-treated, ovariectomized mice, whereas no such effects were observed when embryos were grown in defined medium with estrogen (8). In contrast, studies by Smith (9) showed that embryos treated with estrogen in vitro had significantly higher implantation rates when transferred to foster mothers receiving only progesterone, suggesting that estrogen acts directly on the embryo. To obtain an environment without the complication of maternal effects, some investigators turned to in vitro culturing of embryos. Under these conditions, estrogen was shown to affect the uptake and incorporation of nucleic acid precursors (10, 11) and amino acids (12) by mouse blastocysts. It was also shown that antiestrogen CI 628 and aromatase inhibitor 1,4,6-androstatriene-3,17-dione inhibited the development of mouse embryos (13, 14). This blockade was alleviated by the coadministration of 17β-estradiol. These results support the idea that estrogen affects the embryo directly.

Estrogen and progesterone elicit their functions by binding to their specific receptors. The receptors become activated upon hormone binding and serve as transcription factors that modulate the expression of their target genes (15). For estrogen and progesterone to have any direct effect on the embryo, the receptors for these hormones must be present in the embryo. A demonstration of the expression of estrogen receptor (ER) and progesterone receptor (PR) in preimplantation embryos would provide a basis for the direct effect of estrogen and progesterone on the embryo.

We examined the levels of both ER and PR mRNA during preimplantation development by using reverse transcription (RT)–PCR. We found that ER and PR genes were expressed differentially in the preimplantation mouse embryo. The results for ER contrast with a recent report that preimplantation mouse embryos themselves did not express the ER gene even though maternal message was detected in the oocyte (16). Our results suggest that both maternal and embryonic sources of mRNA are involved.

MATERIALS AND METHODS

Embryo Culture. Female C57BL/6 mice (Harlan–Sprague–Dawley) were superovulated by intraperitoneal injection of 5 units of pregnant mare's serum gonadotropin (Diosynth, Chicago), followed by 5 units of human chorionic gonadotropin (Organon) 48 hr later. These females were placed with male mice after human chorionic gonadotropin injection. Mated female mice were sacrificed the following day around noon, and eggs [0.5 day postcoitum (dpc)] were released from the excised oviducts. Hyaluronidase (Sigma) at 0.3 mg/ml was used to remove the cumulus cells. The eggs were washed

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; dpc, days postcoitum; RT, reverse transcription; RPL19, ribosomal protein L19.

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through M2 medium (17) and cultured for various times in M16 medium (17) at 37°C in a humidified incubator under 5% CO2/95% air. Embryos were harvested at the desired stage and washed three times through M2 medium. The embryos were either used fresh or frozen on dry ice and stored at −70°C. The final wash of the embryos was also collected to serve as a negative control. In the case of unfertilized oocytes and one-cell embryos, the zona pellucida was removed with acidic Tyrode’s solution (17) before harvesting. The time course of embryo development was approximately as follows: two-cell, 1.5 dpc; three- to four-cell, 2 dpc; five- to eight-cell, 2.5 dpc; compacted morula, 3.5 dpc; blastocyst, 4.5 dpc. Most blastocysts collected were expanded and some were hatching.

RNA Isolation. Total RNA was isolated from a pool of about 100 embryos by a micro adaptation of the guanidine/cesium chloride method (18). One hundred fifty microliters of 4 M guanidine isothiocyanate/25 mM sodium citrate, pH 7/0.5% N-lauroyl sarcosine/10 mM 2-mercaptoethanol containing about 15 µg of Escherichia coli tRNA as a carrier was used to lyse cells. This lysate was then overlaid onto 150 µl of 5.7 M CsCl/0.1 M EDTA, pH 7.0, and centrifuged for 2.5 h at 100,000 rpm, 25°C. The resulting pellet was washed with 70% ethanol and dried. This pellet was suspended, precipitated with ethanol, washed twice, and suspended in water. An aliquot was used to determine RNA concentration by absorbance at 260 nm. The recovery of RNA was calculated according to the recovery of carrier RNA (range, 60–80%). Based on the recovery and the initial number of embryos included, adjustment was made so that each sample contained RNA from the same number of embryos per volume.

RT–PCR. RNA was reverse transcribed at 42°C for 1 h. A 20-µl RT mixture contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C), 0.1% Triton X-100, 5 mM MgCl2, 40 units of RNasin (Promega), 100 pmol of random primer (Pharmacia), 1 mM each dNTP, and 12 units of avian myeloblastosis virus reverse transcriptase (Promega). A portion of the RT product containing RNA from the same amount of embryos was then subjected to PCR in a DNA thermal cycler (model 480, Perkin–Elmer/Cetus). A 100-µl reaction mixture included 50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25°C), 0.1% Triton X-100, 4 mM MgCl2, 200 µM each deoxynucleotide, 2.5 units of Taq DNA polymerase (Promega), and 14 nM each primer. After 15–20 cycles, additional primers were added to reach a final concentration of 190 nM, followed by another 30–35 cycles. The cycling profile was 94°C, 1 min; cool to 55–65°C, 1 min; 55–65°C, 1 min; and 72°C, 1 min. The primer sets used were mERa (positions 1354–1374; 5'-GAGAT-TCTGATGATTGTCTC-3') and mERb (1812–1831; 5'-CATCTCAGGAGGCAAGTCA-3') and mERa (661–682; 5'-TTCGGACATCTGCGGCAAT3') and mERb (934–955; 5'-CATCATGGCCACTCTCGTAAC3') and mPrA (2382–2403; 5'-GCCACAGGGTTGTCAACTAT3') and mPrB (2687–2708; 5'-AACATTCACTGACATTTCCGG-3') and RPL19A (5'-CTCAAGGCTGAAGAAGATGTG-3') and RPL19B (5'-GAGACAGGTTCTGTATGACC-3') (19). The PCR products (15 µl) were fractionated in a 2% agarose gel and visualized by ethidium bromide staining.

Southern Analysis. PCR products were separated by agarose gel electrophoresis, transferred to nylon membranes (Hybond-N, Amersham) and hybridized to probes that were labeled with 32P by use of a random labeling kit (United States Biochemical). Hybridization was carried out at 42°C overnight in 50% formamide/6× SSPE (20)/5× Denhardt's solution (20)/0.5% SDS containing denatured sheared herring sperm DNA (100 µg/ml) and the probe (106 cpm/ml). The ER probe was a mouse ER cDNA (gift of M. Parker; ref. 21). The PR probe was a 2-kb fragment of mouse PR cDNA (gift of G. Shyamala; ref. 22). The blot was washed sequentially with 2× SSC (20)/0.5% SDS at room temperature, 1× SSC/0.5% SDS at 68°C, and 0.1× SSC/0.1% SDS at 68°C. The blot was used to expose Kodak XAR film (Eastman Kodak) at −70°C.

RESULTS

Previous work used immunocytochemistry and immunoblotting methods to detect ER in mouse embryos as early as 13 days (23) and probably 10 days of gestation age. The sensitivity of these procedures is limited; therefore, to extend our investigation to the preimplantation embryo, the more sensitive RT–PCR technique was used in this study. Since both ER and PR are expressed in the female reproductive tract, it was crucial to avoid maternal cell contamination. In vitro cultured embryos were used to minimize this possible contamination. Cumulus cells, which also express ER, were removed from the eggs and the eggs were washed so that this source of contamination was avoided (data not shown). Further, unfertilized oocytes and one-cell embryos used for analysis were freed of zona pellucida and washed to eliminate any residual cumulus cells that might still have been associated with the zona even after hyaluronidase treatment. RNA from the uterus, in which both ER and PR are expressed, was used as a positive control.

ER mRNA Is Present at Both Early and Late Stages of Preimplantation Development. To determine whether the ER gene is expressed in preimplantation embryos, two sets of primers were used in the PCR analysis. One set of primers, mERa and mERb, is specific for the DNA-binding domain and will yield a DNA product of 295 bp. Since this primer set spans an intron, based on human ER genomic organization (24), amplification of genomic DNA will not occur due to the large size of the intron (>10 kb). Therefore, a 295-bp band indicates the presence of ER mRNA in the original sample. Fig. 1A shows the result from PCR using this set of primers. None of the wash control lanes had any detectable signal, suggesting that no contamination occurred. A bright band of 295 bp was detected in the lane from the unfertilized oocyte. One-cell- and two-cell-embryo lanes also displayed this band, but the signal was less intense than that seen in the oocyte lane. This suggests that one-cell and two-cell embryos also have ER mRNA, though not as much as oocytes. However, there were no 295-bp fragments in the three- to four-cell-embryo, the five- to eight-cell-embryo, or the morula lane.

A most interesting observation was the reoccurrence of the 295-bp fragment in the blastocyst lane, indicating that the ER gene is expressed at this stage. Southern blot analysis confirmed that the 295-bp band detected was indeed of ER origin (Fig. 1B).

To further confirm these findings, another set of primers, mERa and mERb, specific for the steroid-binding domain of the receptor and spanning three introns, was used. A PCR product of 478 bp was expected. Fig. 1C shows the result of Southern analysis of the PCR products. The presence of a 478-bp band which hybridizes to ER cDNA indicates the presence of ER mRNA. A 478-bp band of high intensity is present in the unfertilized oocyte lane. The band intensity appears to decline from the two-cell embryo through the five- to eight-cell embryo. There was no detectable signal in the lane from the morula. This specific band was detected again in blastocysts. We did not detect any signal in the one-cell-embryo lane in this blot. This is not representative of our usual results: similar experiments have consistently shown a specific band exhibiting an intensity close to that shown by the oocyte.

Taken together, the results with the two sets of primers show an ER mRNA expression pattern demonstrating that the ER mRNA level is high in the oocyte. The level diminishes at the two-cell stage and drops to the lowest level in five- to eight-cell embryos. In the morula, the ER mRNA is
essentially not detectable. The decrease in ER mRNA is not a result of dilution of ER mRNA due to cell division, because total RNA from the same number of embryos was assayed. The ER mRNA reappears at the blastocyst stage. Thus far there is only one known ER gene. Since our primers were chosen both in the DNA-binding domain and the steroid-binding domain, it is unlikely that we have detected forms of ER other than the classical nuclear ER.

**The Pattern of ER mRNA Expression Is Not a Result of Selective Sample Degradation.** To ensure that all RNA samples were intact, we performed PCR on the same set of samples, using primers that amplify ribosomal protein L19 (RPL19) cDNA. Because ribosomal proteins should be expressed universally, failure to detect RPL19 mRNA would suggest that the sample was degraded. A band of the expected size (194 bp) was seen in all the appropriate lanes (Fig. 2), indicating that embryos at all stages had the RPL19 mRNA. The expression of this gene seemed to increase somewhat during development, consistent with the role of ribosomal proteins in protein synthesis. The consistent detection of RPL19 gene expression in preimplantation embryos at all stages suggests that all the RNA samples were intact. A faint band in the wash control of the five- to eight-cell embryo suggested that minor contamination of an unknown source occurred in this sample.

**The PR Gene Is Silent Until the Blastocyst Stage.** The expression of the PR gene was examined by using a set of primers specific for the steroid-binding domain. This set of primers spans two introns, based on chicken PR genomic organization (25), and should give a PCR product of 327 bp. No 327-bp band was detected in the lanes from the oocyte through the morula (Fig. 3A). A band of 327 bp was visible only in the lane from the blastocyst. The specificity of the product was confirmed by Southern analysis (Fig. 3B). Apparently, the PR gene is not expressed until the blastocyst stage during preimplantation development. This pattern of expression differs from that of the ER gene.

**DISCUSSION**

This study examines the expression of two steroid receptor genes in preimplantation mouse embryos by RT–PCR tech-
Expression of the PR gene. (A) Agarose gel showing the PCR product amplified with primers mPRa and mPRb. RNA from 3.6 embryos was included in each sample. Lanes marked (C) are wash controls. Uterine RNA (100 pg) was used as positive control. The annealing temperature for PCR was 62°C, and 20 plus 35 cycles were run. (B) Autoradiogram of Southern blot of the gel shown in A.

Fig. 3. Expression of the PR gene. (A) Agarose gel showing the PCR product amplified with primers mPRa and mPRb. RNA from 3.6 embryos was included in each sample. Lanes marked (C) are wash controls. Uterine RNA (100 pg) was used as positive control. The annealing temperature for PCR was 62°C, and 20 plus 35 cycles were run. (B) Autoradiogram of Southern blot of the gel shown in A.

Fig. 4. Summary of mRNA levels of ER and PR in mouse preimplantation embryos. The ER and PR mRNA levels per embryo are shown in relationship to the age of the embryos. The scale is arbitrary. The corresponding developmental stages are depicted. The drawing of the embryos at various stages of development was adapted from Hogan et al. (17) with permission (Cold Spring Harbor Laboratory Press, copyright 1986). The origin of the mRNA and its possible function are also indicated.

level comparable to that found in one uterine cell. The PR mRNA level in a blastocyst is comparable to that in one uterine cell. Since one blastocyst has 32–64 cells, it is not known if low levels of ER and PR mRNAs are expressed in many cells or if moderate levels are expressed in one or a few cells. This question might be approachable with in situ hybridization or in situ PCR. Since RT–PCR assays only for the mRNA level, we cannot say with certainty that functional proteins are expressed. Attempts to measure ER protein have been limited by the sensitivity of available methods. For example, we have calculated that 1000 or more embryos would be required in order to obtain 20 pg of ER. This could be detected by immunoblotting if the background protein level does not interfere. This is not likely with available methods.

Origin of ER and PR mRNAs in Preimplantation Embryos. In the mouse, development of the embryo before the middle of the two-cell stage depends largely on protein and RNA synthesized during oogenesis. The embryonic genome is relatively inactive until sometime after the first cleavage. Development of early embryos beyond the two-cell stage requires new mRNA synthesis (17, 26, 27). Since a high level of ER mRNA was detected in the unfertilized oocyte, it is reasonable to argue that the ER mRNA detected in the one-cell embryo was carried over from the oocyte where maternally synthesized mRNA is stored. The ER mRNA level declined from the two-cell to the five- to eight-cell stage. This is compatible with the general observation of loss of maternal RNA in early development (28). Though the embryo is capable of synthesizing RNA from the two-cell to the five- to eight-cell stage, the ER mRNA detected at these stages is
not likely to be synthesized by the embryo, because the amount continues to decline. At the morula stage, ER mRNA is virtually undetectable. The reappearance of ER mRNA and appearance of PR mRNA in the blastocyst must be a result of embryonic mRNA synthesis. At this stage, embryonic transcription is very active.

Implications for the Development and Implantation of Preimplantation Embryos. The differential expression of ER and PR genes during preimplantation development suggests that ER and PR may have different roles during this period. It is known that estrogen stimulates follicular growth in the process of oocyte maturation (29). That both the cumulus cells and the oocyte express ER mRNA suggests that estrogen can influence both cell types. Blastocyst formation is an important period in preimplantation development. The embryo implants at the late blastocyst stage. It is interesting that the embryonic expression of the ER and the PR genes coincides with this time. Blastocyst-specific genes are expressed in the transition from morula to blastocyst, and this expression is required (30, 31). There are also changes in the synthesis and secretion of stage-specific proteins from early to late blastocysts (5, 32). Estrogen apparently is an important regulator in this process, since dormant blastocysts reactivated with estrogen undergo the same changes. The presence of estrogen seems necessary for the development of the embryo because antiestrogen or aromatase inhibitor can block embryo development (13, 14). The function of estrogen might be to stimulate the development of the embryo and prepare it for implantation. This is consistent with the possible lethality of an ER mutation (1). The requirement for ER must go beyond the maternal source, since a homozygous ER mutant from hermaphroditic mice would be viable otherwise. The expression of the ER gene by the embryo itself must be crucial for survival.

Progesterone is also necessary for implantation in mice. Treatment with progesterone antagonists RU486 and ZK98734 prevents normal embryo transport and implantation, but progesterone is apparently not essential for development (33). The timely expression of the PR gene in blastocysts implies that the effect of progesterone can be exerted on the embryo as well.

While this work was in progress, Wu et al. (16) reported that they could detect ER messages in oocytes and two-cell embryos, which is consistent with our findings, but they failed to detect any ER message at the blastocyst stage. This discrepancy may result from the PCR methods, since we used “booster” PCR and a higher number of cycles, which should give greater sensitivity. They concluded that preimplantation embryos do not express ER and that any effect of estrogen on the embryo acts indirectly through the uterus, possibly through the regulation of growth factors and their receptors. Although the expression of growth factors and growth factor receptors (platelet-derived growth factor A, transforming growth factors α and β, and epidermal growth factor receptor) in the embryo has been reported (18, 34, 35), and in some cases, the expression was regulated by estrogen, it is not clear whether the effect was on the embryo directly or mediated through the uterus. Our results clearly demonstrate that the ER gene is expressed by preimplantation embryos. This finding provides a basis for the direct effect of estrogen on the embryo. Since both ER and PR genes are expressed at the blastocyst stage, a new perspective of the mechanism of implantation may be in order. Previous studies have focused largely on uterine response to hormones; our findings suggest an embryonic target for the hormones. Estrogen and progesterone may induce changes both in the uterus and in the embryo that lead to successful implantation.

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