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Stage-Specific Expression of Estrogen Receptor Subtypes and Estrogen Responsive Finger Protein in Preimplantational Mouse Embryos

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Abstract. In hope of understanding possible roles of estrogen during early embryogenesis, we examined the expression of both estrogen receptor α (ERα) and ERβ, a recently cloned novel subtype, in mouse oocytes and preimplantation embryos by means of reverse transcription polymerase chain reaction (RT-PCR). To investigate whether estrogen actually exerts its action, we further determined the expression of efp (estrogen-responsive finger protein), a newly characterized estrogen responsive gene belonging to the RING finger family. ERα mRNA was detected in whole ovaries, cumulus-oocyte complexes, denuded oocytes, 2-cell and 4-cell embryos, whereas it was undetected in 8-cell embryos. Interestingly it reappeared in morulae and blastocysts. ERβ mRNA was detected similarly to ERα except for the absence of ERβ mRNA in morulae. The efp mRNA was detected in whole ovaries, cumulus-oocyte complexes, 4-cell embryos, morulae and blastocysts. The stage specific expression of ERα and ERβ along with detection of the product of the estrogen responsive gene in early preimplantation embryos may indicate the possible physiological roles of estrogen in early embryogenesis.

Key words: Estrogen receptor subtypes, Estrogen-responsive finger protein, Preimplantation embryos, Mouse

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ESTROGEN action is mediated through the estrogen receptor, which is a ligand dependent transcription factor. The estrogen receptor is a member of steroid/thyroid hormone receptor superfamily [1] and specifically binds with estrogen and regulates the estrogen responsive genes. Recently, a novel estrogen receptor (estrogen receptor β; ERβ) was isolated in the rat [2], human [3, 4] and mouse [5], and the classical ER [6–8] was renamed ERα. ERβ has a high degree of sequence homology with ERα and specifically binds to estrogen [2]. The ERβ mRNA was detected mainly in rat ovary, prostate, lung, brain, bladder, uterus and bone [2, 9–13]. Like ERα, ERβ forms homodimers. In addition the transcription activating manner is thought to be similar to ERα [14, 15]. On the other hand the transcription activating function of ERβ is different from that of ERα, which depends on the ligand and responsive element [5, 16, 17].

Estrogen plays a pleiotrophic role in reproduction. Several lines of evidence have indicated the presence of ER in oocytes and embryos. For instance, RT-PCR revealed the presence of ERα mRNA in denuded oocytes and cumulus-oocyte complexes in mice. ERα mRNA decreased in amount in 2-cell embryos and

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disappeared in morulae [18, 19]. Furthermore, the ERα protein was demonstrated in mouse blastocysts by immunocytochemistry [20]. In human, differential expression of ERα and ERβ mRNA in midgestational fetuses was reported [21].

In order to get further insight into the estrogen action during embryogenesis, we examined the presence of mRNA of ERα, ERβ and efp, a newly identified estrogen responsive gene, in mouse eggs at various stage ranging from unfertilized eggs to blastocysts by RT-PCR with specific corresponding primers.

Materials and Methods

Animals

Female ICR mice were maintained in accordance with the institutional guidelines for care and use of laboratory animals. They were injected with PMSG (5 units), followed by hCG injection (5 units) 48 h later. The mice were allowed to mate with male ICR mice. Mating was verified by the presence of a vaginal plug. Denuded oocytes, 2-cell embryos, 4-cell embryos, 8-cell embryos, morulae and blastocysts were obtained 24, 48, 60, 72, 84 and 96 h after the injection of hCG, respectively. The denuded oocytes obtained were treated with hyaluronidase in order to remove the cumulus. Cumulus-oocyte complexes were obtained 24 h after the injection of hCG and were not treated with hyaluronidase. Ovaries were removed 96 h after the injection of hCG. For the extraction of total RNA, ovaries, oocytes and embryos were rapidly frozen in liquid nitrogen and stored at −80 °C until use.

Total RNA preparation and nested reverse transcription-polymerase chain reaction

Total RNA was isolated with ISOGEN (Nippongene, Tokyo) and quantified using spectrophotometricaly at 260 nm. We obtained 1–10 μg total RNA from 100 sets of cumulus-oocyte complexes, denuded oocytes, 2-cell embryos, 4-cell embryos, 8-cell embryos, morulae and blastocysts. The cDNA was synthesized by reverse transcription of 500 ng total RNA with oligo dT primer in RNA PCR kit (Takara, Tokyo). PCR amplification was performed in 50 μl containing 5 pmol of the forward and reverse primers of ERα, ERβ, efp or RPL19, 10 nmol of each dNTP, 1x Taq buffer and 2.5 U of Taq polymerase (Takara, Tokyo). Twenty amplification cycles of PCR were carried out for ERα, ERβ or efp, and thirty amplification cycles of PCR were carried out for RPL19 (30 sec at 94 °C, 30 sec at 55 °C and 1 min at 72 °C). Nested PCR was performed in 50 μl containing 5 μl of PCR products, 100 pmol of nested forward and reverse primers of ERα, ERβ or efp, 10 nmol of each dNTP, 1x Taq buffer and 2.5 U of Taq polymerase. Thirty cycles of PCR were performed (30 sec at 94 °C, 30 sec at 55 °C and 1 min at 72 °C). All PCR products were electrophoresed on a 3% agarose gel. The ampified DNA fragment of ERα is located between the A/B and C domains of mouse ERα. The ampified DNA fragment of ERβ is located between the D and E/F domains of mouse ERβ.

Table 1. Oligonucleotide sequences for PCR primers

ERα forward primer	5′-AATTCTGACAATCGACGCCAG-3′
ERα reverse primer	5′-GTGCTTCAACATTCTCCCTCCTC-3′
ERβ forward primer	5′-ACGAAGTAGGAATGGTCAAGTGT-3′
ERβ reverse primer	5′-ATGCCAAAATTTCCAGAATC-3′
efp forward primer	5′-CCACATTCCCAAAGCCTACCA-3′
efp reverse primer	5′-AATTCCATAAAGACCTGAGAT-3′
RPL19 forward primer (ref. 19)	5′-CTGAAGGTCAAAGGGAATGTG-3′
RPL19 reverse primer (ref. 19)	5′-GGACAGAGTCTTGATGATCTC-3′
ERα forward primer (nest)	5′-GAGAAAGGAAACATGATCATGGA-3′
ERα reverse primer (nest)	5′-TTCATCATGCCCACTTGGTAAC-3′
ERβ forward primer (nest)	5′-AAAGCCAAGAGAACCAGTGGGCAC-3′
ERβ reverse primer (nest)	5′-GCCAATCATGTG-CACCAGTTCCTT-3′
efp forward prime (nest)	5′-CATTCCCAAAGCCTACCATCA-3′
efp reverse primer (nest)	5′-TCCATAAAGACCTGAGATGTG-3′

Results

Nested PCR was utilized to detect the expression of ERα, ERβ and efp mRNA in preimplantation embryos. The mRNA expression of these genes was confirmed by at least two more experiments. PCR with a set of non-RT controls was performed, and indicated that detected signals were not amplified from contaminated genomic DNA (data not shown).

When we used the primer set for ERα, 250 bp bands were detected in whole ovaries, cumulus-oocyte complexes, denuded oocytes, 2-cell embryos, 4-cell embryos, morulae and blastocysts. On the other hand, 8-cell embryos were negative for ERα (Fig. 1). When using the primer set for ERβ, 203 bp bands were observed in whole ovaries, cumulus-oocyte complexes, denuded oocytes, 2-cell embryos, 4-cell embryos and blastocysts, whereas no band was visualized in 8-cell embryos or morulae (Fig. 2). When using the primer set for efp, 585 bp bands were observed in whole ovaries, cumulus-oocyte complexes, 4-cell embryos, morulae and blastocysts, whereas no band was visualized in denuded oocytes, 2-cell embryos or 8-cell embryos (Fig. 3).

In order to ensure that all RNA samples were intact, we performed PCR on the same set of samples with primers that amplified ribosomal protein L19 (RPL19) cDNA [19, 22], which showed 194 bp bands of RPL19 in all the appropriate lanes (Fig. 4). To confirm that the bands observed by PCR were really derived from ERα, ERβ, efp and RPL19, we carried out direct sequencing of these PCR products, and found that each band corresponded to ERα, ERβ, efp and RPL19 as expected. The present data were confirmed by three experiments.

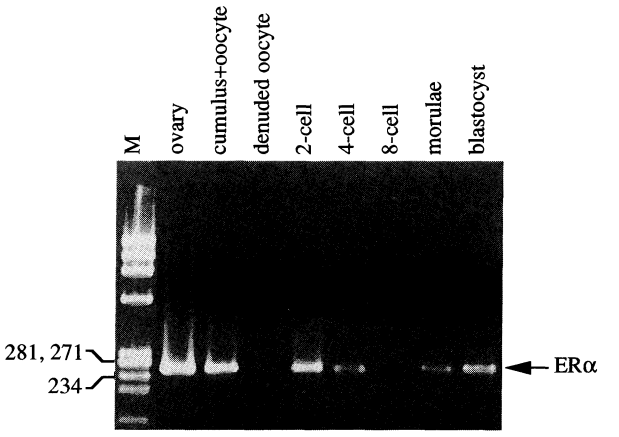

Fig. 1. Expression of ERα mRNA. The PCR products derived from ERα mRNA were used in each sample (250 bp). Total RNA from the ovaries was used as a positive control. Lane M, molecular size markers (lengths at left in nucleotides).

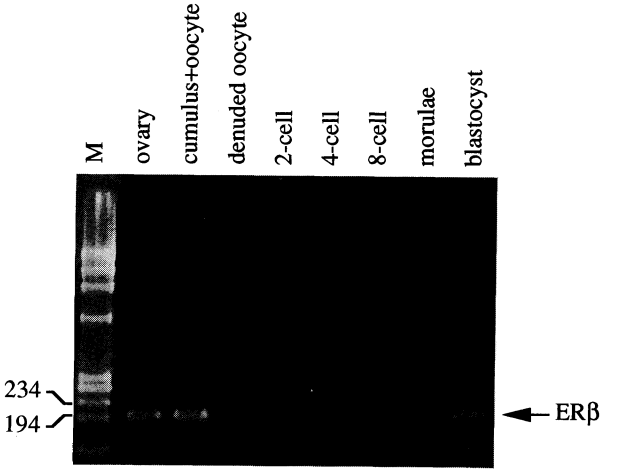

Fig. 2. Expression of ERβ mRNA. The PCR products derived from ERβ mRNA were used in each sample (203 bp). Total RNA from the ovaries was used as a positive control. Lane M, molecular size markers (lengths at left in nucleotides).

Discussion

In the present study we demonstrated the expression of the two subtypes of ERs, ERα and ERβ in preimplantation mouse embryos by RT-PCR (nested PCR). Previously it was reported that ERα mRNA existed in denuded mouse oocytes. Its level was shown to decline with development of the embryo and to be undetected at the morulae stage with subsequent re-expression at the blastocyst stage [19]. In the mouse, maternal RNA, which is synthesized during oogenesis, plays an important role in the development of the embryo before the middle of the 2-cell stage, after which it declines toward the 8-cell stage [23]. It is therefore possible to consider that ERα mRNA detected before the 4-cell stage is of maternal origin. It is also possible that ERα mRNA detected before 4-cell stage is

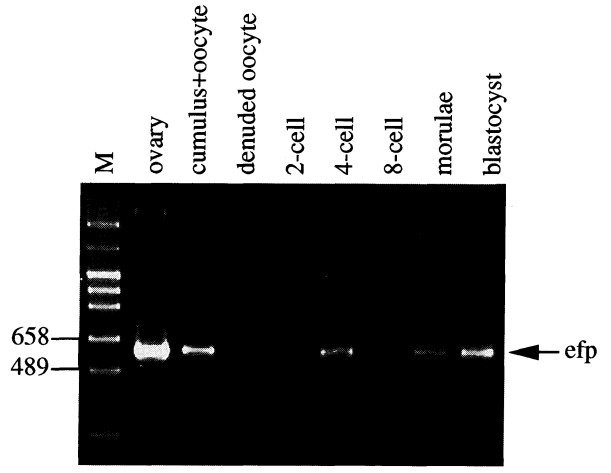

Fig. 3. Expression of efp mRNA. The PCR products derived from efp mRNA were used in each sample (585 bp). Total RNA from the ovaries was used as a positive control. Lane M, molecular size markers (lengths at left in nucleotides).

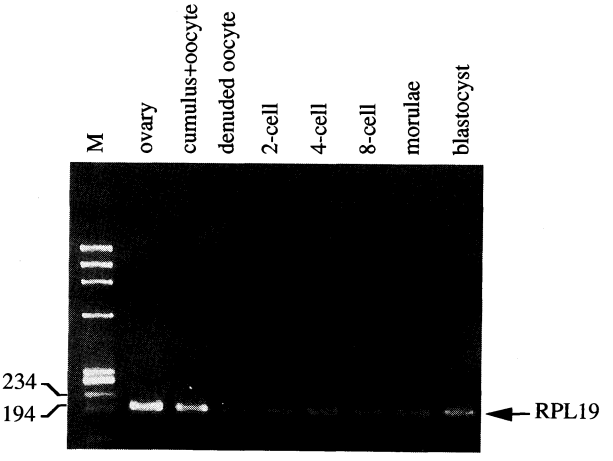

Fig. 4. Expression of RPL19 mRNA. The PCR products derived from RPL19 mRNA were used in each sample (194 bp). Total RNA from the ovaries was used as a positive control. Lane M, molecular size markers (lengths at left in nucleotides).

embryonic RNA, because the synthesis of embryonic RNA begins after the 2 cell stage. On the other hand, the reappearance of ERα mRNA at the blastocyst stage was supposed to reflect the synthesis of embryonic RNA [19]. The present results suggest that the amounts of mRNA of ERα and ERβ are diminished after ovulation and reach their minimum at the 8-cell embryo stage, after which these mRNAs reappear at morulae-blastocyst stage, implying that both ERα mRNA and ERβ mRNA are expressed similarly during the early embryonic stage. But the inadequacy of the RT-PCR (nested PCR) employed in this experiment to quantitate the amounts of mRNA reduces the strength of this contention.

The present data showed the existence of ERα mRNA in morulae, which is in disagreement with the previous report [19]. The reason for this may lie in the different detection methods used. We used the nested PCR method which might enable us to detect smaller amounts of ERα mRNA, but could not detect ERβ mRNA in morulae, which suggests the absence of ERβ mRNA or the presence of an extremely small amount of ERβ mRNA. At any rate, it is intriguing to speculate that ERα acts mainly in morulae, whereas the presence of both of ERα and ERβ may be needed in blastocysts.

It is widely accepted that estrogen in concert with progesterone is a central player in implantation, mainly by modifying endometrial morphology and functions. In this study, ERα and ERβ mRNA were found in morulae-blastocysts after the disappearance of these mRNAs at the 8-cell stage. Since early embryos start to undergo a process of implantation at the late blastocyst stage, it is likely that estrogen plays a role in implantation by acting on embryos as well. The essential role of estrogen in implantation is substantiated by the fact that an anti-estrogen drug or an aromatase inhibitor blocks embryo development [24, 25], but this notion is challenged by the recent finding of successful generation of ERα disrupted mice [26] and identification of a case with ERα mutation [27]. A likely explanation for this is that ERβ can mediate some estrogen actions which are normally elicited through ERα, given the fact that ERβ forms homodimers and its transcription activating manner seems similar to ERα. In the present paper, ERβ mRNA was not detected in morulae, which suggests the absence of ERβ mRNA or the presence of an extremely small amount of ERβ mRNA. Another possible explanation is the existence of unknown estrogen receptors or other factor which might exert estrogen-like biological actions.

In order to examine whether ER observed in early embryos actually mediates the estrogen effects, we determined the expression of one of the estrogen responsive genes, efp [28, 29]. Efp is a member of

the RING finger family, whose members are nuclear proteins and regulate transcription [30]. In a previous report, the co-localization of efp and ERα mRNA in mouse ovaries was shown by *in situ* hybridization [29]. The expression of efp in the periimplantation mouse uterus was also showed by *in situ* hybridization, which suggested the role of efp in amplifying the estrogen effect in preparing of the uterus for implantation [31]. In the present study efp was detected in mouse ovaries and cumulus-oocyte complexes, a finding compatible with the previous study by Orimo *et al.* [29]. Efp mRNA, undetected in denuded oocytes and 2-cell stage embryos, was detected in 4-cell embryos. A possible explanation for the absence of efp mRNA in denuded oocytes and 2-cell stage embryos is that the functional ERs might not exist in these stages. It is also possible that unknown factors affect the trasncription of efp. It was again undetected in 8-cell embryos and found in morulae and blastocysts. The presence of efp mRNA at the 4-cell stage was confirmed by three experiments. It is conceivable that efp mRNA detected at the 4-cell stage might be a reflection of estrogen action mediated through ERs which are expressed at an early embryonic stage. But it is also possible that other unidentified factors could affect the expression of efp mRNA. The absence of efp mRNA in 8-cell embryos seems to be compatible with the present observation that ERs did not exist in embryos at the same stage. Re-expression of efp mRNA in morulae and blastocysts is consistent with ERs being present at these stages. The existence of efp mRNA is interpreted implicitly as demonstrating that estrogen excutes its actions through ERs in each stage, but it is also possible that other unidentified factors could affect the expression of efp mRNA. The progesterone receptor (PR), the generation of which is estrogen-dependent [32], was not detected at early embryonic stage but was first observed at the blastocyst stage by RT-PCR [19]. In view of these findings, it appears that the expression of each estrogen responsive gene may require other unidentified factors, independent of the presence of the estrogen receptor.

The expressions of mRNA of ERα, ERβ and efp were shown in the present paper. In order to understand the estrogen action in early embryos, it is necessary to know the regulation of proteins of these genes. Further studies are required.

In conclusion, the present study shows the existence of mRNA of ER subtypes and one of the estrogen responsive genes, efp, in preimplantation embryos. It is possible that ERβ mediates estrogen actions as well as ERα in early embryogenesis.

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