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Physiology

Immunolocalization of estrogen receptor protein in the mouse blastocyst during normal and delayed implantation

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ABSTRACT **We previously showed that estrogen receptor (ER) mRNA is present in preimplantation mouse embryos. The apparent synthesis of ER mRNA by the blastocyst at the time of implantation when estrogen is required was of special interest. A demonstration of the presence of ER protein would support the idea that estrogen can act directly on the embryo. The mouse embryo at the blastocyst stage is differentiated into two cell types, the trophectoderm and the inner cell mass. To determine whether ER mRNA is translated into ER protein and its cell-specific distribution, immunocytochemical analyses were performed in mouse blastocysts. ER protein was detected in all cell types of the normal, dormant, or activated blastocyst. To trace the fate of ER in these cell types, immunocytochemistry was performed in implanting blastocysts and early egg cylinder stage embryos developed in culture. Again, ER was detected in all cells of the implanting blastocyst. At the early egg cylinder stage, continued expression of ER was observed in cells derived from the inner cell mass or the trophoblast. In trophoblast giant cells, ER was concentrated in small regions of the nucleus, possibly the nucleoli, which was similar to that observed in dormant and activated blastocysts. The embryonic expression of ER at such early stages in a broad array of cells suggests that ER may have a general role during early development.**

Estrogen receptor (ER) mRNA is present in preimplantation mouse embryos (1). Interestingly, ER mRNA was detected at the blastocyst stage and was likely of embryonic and not maternal origin. Mouse embryos implant at the blastocyst stage and estrogen is an absolute requirement for this process. In the mouse, blastocysts become dormant and implantation is delayed in ovariectomized mice receiving progesterone only (2). After an estrogen injection, the dormant blastocysts become reactivated and implantation resumes (3, 4). Changes occur in blastocyst metabolism including RNA and protein synthesis in response to estrogen treatment (3–6). There are two possible ways that estrogen can influence the blastocyst. One is to induce changes in the uterus, which in turn produce signals that the blastocyst recognizes. Estrogen-induced uterine changes have been well documented (7). The second possibility is the direct effect(s) of estrogen on the blastocyst. The existence of this latter pathway is controversial. Since estrogen elicits its functions primarily through its receptor protein, the ER, a direct estrogen effect on the blastocyst should require the presence of ER protein in the blastocyst. The presence of ER mRNA in the blastocyst only indicates that the ER gene is being transcribed; it does not guarantee the expression of ER protein. A direct demonstration of ER expression at the protein level is required.

The preimplantation mouse blastocyst contains 32–64 cells (8) and is differentiated into two cell types: the trophectoderm, which contributes to extraembryonic tissues, and the inner cell mass, from which the fetus is derived. On the evening of the third day of pregnancy (vaginal plug = 0.5 day), the blastocyst adheres to the luminal epithelium of the uterus. The trophoblast cells later interdigitate with the epithelial cells and eventually invade the uterus, forming a three-dimensional structure that interlocks with the inner cell mass (9, 10). The inner cell mass continues to proliferate. Determination of the status of ER expression during this period would be informative. If the ER's role is only to prepare for implantation, ER expression should cease at this time. However, if ER has a more general function, its expression should continue.

To examine whether ER is expressed in the implanting blastocyst, we employed *in vitro* culture systems. These systems have been used by others to mimic certain aspects of postimplantation embryonic development (10–13). In contrast to an implanting blastocyst *in utero*, the trophoblast and inner cell mass spread out on the flat plastic substrate during *in vitro* development. Subsequently, a three-dimensional egg cylinder grows out of the inner cell mass without the surrounding trophoblast barrier. Although the development of *in vitro*-cultured embryos is slower compared with its *in vivo* counterpart, light and electron microscopic studies show that the development of the embryo proper is apparently comparable to normal development (10).

In this study, we employed immunocytochemistry to examine the cell-type-specific distribution of ER protein in normal, delayed, and activated blastocysts, as well as the fate of ER in the implanting blastocyst *in vitro*. Our finding that ER is present in both the inner cell mass and trophectoderm cells in the blastocyst and that ER expression persists at least until the early egg cylinder stage in all cell types suggests that ER may be important for implantation and/or early embryogenesis. This is paradoxical to the finding that "knockout" mice with disrupted ER genes are viable, which argues that ER may not be needed for early development (14).

MATERIALS AND METHODS

Embryo Recovery. CD-1 female mice (48 days old) were mated with males of the same strain to induce pregnancy (vaginal plug = 0.5 day). Embryos at different stages of development were recovered in Whitten's medium by flushing the reproductive tract (15).

Delayed implantation was induced by ovariectomizing adult mice on day 3 of pregnancy. To maintain delayed implantation, ovariectomized mice were injected with progesterone (2 mg per mouse in 0.1 ml of sesame oil, s.c.) from days 4.5 to 6.5. To terminate delayed implantation, mice were given an injection of 17β-estradiol (25 ng per mouse in 0.1 ml of oil, s.c.) in conjunction with progesterone on the third day of delay.

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Abbreviations: ER, estrogen receptor; BSA, bovine serum albumin; TGF, transforming growth factor; EGFR, epidermal growth factor receptor.

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Blastocysts were collected in Whitten's medium (15). Dormant blastocysts were recovered from ovariectomized mice after 18 h of progesterone injection, whereas activated blastocysts were recovered 18 h after an injection of estradiol and progesterone on day 6.5.

Culturing Fertilized Eggs to Blastocysts. The procedure for culturing fertilized eggs to blastocysts was as described by Hogan *et al.* (8). Briefly, female C57BL/6 mice (Harlan–Sprague–Dawley) were superovulated by injecting 5 international units of pregnant mare's serum gonadotropin (Diosynth, Chicago), followed by 5 international units of human chorionic gonadotropin (Organon) 48 h later. These females were placed with males after human chorionic gonadotropin injection. Mated female mice were sacrificed the following day around noon (day 0.5). Embryos were recovered from the oviducts, stripped of adhering cumulus cells, and cultured in M16 medium under light mineral oil at 37°C in 5% CO_2/95% air in a humidified incubator. The day the embryos were placed in M16 culture medium was defined as day 0. After 4 days of culture, most of the blastocysts were expanded and some were exhibiting zona hatching.

Cultured blastocysts used for immunocytochemistry were collected at noon on day 4 and freed of zona pellucida by a brief exposure to acidified bovine serum albumin (BSA)-free M2 medium (pH 2.5). Zona-free blastocysts were then washed thoroughly in BSA-free M2 medium and attached to chrom alum/gelatin and poly(L-lysine) (Sigma)-coated coverslips. *In utero*-developed normal blastocysts were recovered at 0900 h on day 3 and subjected to a brief Pronase treatment for dissolution of zona pellucida (15). Day 3 normal, day 7 dormant, and day 7 activated blastocysts were cytospun onto poly(L-lysine)-coated slides for immunostaining.

Culturing Embryos Beyond the Blastocyst Stage. The procedure for culturing embryos beyond the blastocyst stage was modified from a combination of protocols (10, 12, 13, 16). CMRL medium 1066 containing 1 mM glutamine (GIBCO/BRL), supplemented with freshly made 1 mM sodium pyruvate (Sigma) and 20% fetal bovine serum was used on the day blastocysts were placed in culture. This complete medium was stored at 4°C for subsequent feeding up to 3 days.

Blastocysts were obtained by culturing fertilized eggs as described above, except the culture medium was phenol red-free. On the morning of day 4 in M16 medium, blastocysts were transferred to four-well chamber plastic slides (Permanox, Nunc) filled with culture medium. The day blastocysts were placed into the serum-containing medium was defined day 0 of transfer. Embryos were fed on the mornings of days 2 and 3 of transfer. During the first day after transfer, most blastocysts had shed their zona pellucidae and adhered to the plastic slides. Between 1 and 2 days after transfer, the mural trophoblast cells migrated out radially on the plastic surface. By the morning of day 4, an egg cylinder could be seen as a raised compact portion pointing to the medium over the trophoblast cells. Trophoblast cells that had transformed to giant cells were distinct by their large size.

Immunocytochemistry. Essentially similar procedures were used for normal and dormant blastocysts and embryos that were cultured beyond the blastocyst stage *in vitro*. In general, embryos were washed in ice-cold phosphate-buffered saline (PBS). They were then fixed in chilled 4% (vol/vol) formaldehyde in PBS for 10–20 min and permeabilized with PBS containing 0.1 or 0.2% Triton X-100 for 3–5 min. Embryos were incubated first in the blocking solution (PBS/3% BSA/0.2% Tween 20/0.1 M glycine) for 3 h, followed by further incubation in PBS containing 10% (vol/vol) goat serum (Sigma), 0.2% Tween 20, and 0.02% sodium azide at 4°C overnight. After blocking, ER 715 or its controls diluted in the second blocking solution were added and incubated at 4°C overnight. ER 715 is a specific antibody to the ER that was developed in a rabbit against a synthetic peptide corresponding to the hinge region of the ER (17). ER 715 was used at 40–150 ng/ml. The controls included blocking solution without primary antibody, nonimmune rabbit IgG (500 ng/ml) (Sigma), and ER 715 preabsorbed with 6000-fold excess of the peptide. Embryos were washed with PBS containing 3% BSA and 0.2% Tween 20, followed by incubation at 4°C for 2 h or overnight with horseradish peroxidase-conjugated goat anti-rabbit antibody (Vector Laboratories) at 0.5–2 μg/ml in the same buffer or the second blocking solution. Embryos were washed. Embryos older than the blastocyst underwent an additional wash with PBS containing 0.1% Triton X-100 and 0.2% BSA for 20 min. A substrate of 3,3′-diaminobenzidine tetrahydrochloride (Polyscience; 0.5 mg/ml) and 0.02% H_2O_2 (Aldrich) in 50 mM Tris·HCl (pH 7) was added to the embryos. Older embryos were incubated in 3,3′-diaminobenzidine tetrahydrochloride solution for 20 min before the addition of H_2O_2. The reaction was allowed to proceed until color was apparent, which required 1–20 min and was terminated by rinsing in H_2O or PBS/0.1% Triton X-100/0.2% BSA. Blastocysts on coverslips were mounted with Crystal mount (Biomedia, Foster City, CA) and postmounted on slides with Polymount (Polyscience). The chamber slides on which blastocysts were cultured for 2 days were mounted with Crystal mount and coverslipped with Polymount. Early egg cylinder stage embryos, cultured for 4 days from the blastocyst stage, were dehydrated through graded methanol and stored in methanol at −20°C until photographed. Immunostaining of ER in *in utero*-developed day 3 normal, day 7 dormant, and day 7 activated blastocysts was performed by using a Zymed histostain-SP kit specific to rabbit antibodies (Zymed) containing a biotinylated secondary antibody, a horseradish-streptavidin conjugate, and a substrate–chromogen mixture (15). Embryos were poststained with fast green. Reddish-brown deposits indicate sites of immunoreactive ER. Blastocysts were photographed with a Nikon FX-35 DX camera connected to a Nikon microphot-SA microscope. Results of implanting blastocysts and early egg cylinder stage embryos were photographed with a Nikon FX-35 A camera connected to a Nikon inverted microscope. All results were recorded on Kodak color film.

RESULTS

ER Distribution in the Blastocyst. The immunocytochemistry protocol was validated by using ER containing GH3 cells (18) (data not shown). Embryos at different developmental stages around the time of implantation were subjected to such analysis. Table 1 shows the corresponding *in vivo* gestation ages and the time of *in vitro* culturing during these developmental stages. The authenticity of ER immunostaining was confirmed using GH3 cells, which are known to be positive for the ER (18). ER is expressed in GH3 cells at a level of approximately 20,000 receptors per cell. Immunostaining with the ER 715 antibody showed strong nuclear staining in GH3 cells. However, these cells incubated in nonimmune rabbit IgG or ER 715 antibody preneutralized with ER peptide failed to exhibit positive staining (data not shown). To our knowledge, this is the first report of ER detection in GH3 cells by immunocytochemistry.

Blastocysts grown in culture were made zona-free and attached to coated coverslips to facilitate the immunostaining process. Strong staining was observed when the blastocyst was incubated with the antibody ER 715. No staining was observed when the primary antibody was not included in the incubation or when nonimmune rabbit IgG was used instead of ER 715 (data not shown). Further, staining was much reduced if ER 715 was preabsorbed with an excess of the antigenic ER peptide (Fig. 1). Similar results were obtained from more than 50 blastocysts examined in separate experiments. Representative photographs are shown in Fig. 1. These results strongly argue that the observed staining is specifically due to the

Table 1. Development of mouse embryos around the time of implantation

Developmental stage (Theiler)	Gestation age *in vivo*, days	Principal feature		Total culture time, days	Time after blastocyst transfer, days	ER expression
5	3.5	Blastocyst with zona pellucida		4	0	All cells
5	4.0	Free blastocyst		5	1	—
6	4.5	Implanting blastocyst		6	2	All cells
7–8	5.0–5.5	Early egg cylinder		8	4	Possibly all cells ERs in giant cells cluster to spots in the nucleus

Developmental stages [according to Theiler (19)] and characteristics of embryos at different gestational ages (vaginal plug = 0.5 day) are shown. Total time of *in vitro* culturing or time after transferring blastocysts to serum-containing medium to achieve the corresponding developmental stage *in vivo* are indicated. At the end of *in vitro* culture, development of the embryo is not completely synchronized. The last column summarizes results from this study about the expression of ER during this period.

presence of ER protein in the blastocyst. The staining was concentrated in the nucleus, consistent with the known cellular location of the ER (20, 21).

Nuclear staining was detected in all cell types in the blastocyst. Since blastocysts adhere to the coverslips in random orientation and the blastocoels collapse during the immunocytochemical detection procedure, the three-dimensional structure of blastocysts were lost. Thus, the two cell types of the blastocyst could not always be distinguished. However, repeated detection of ER in all cells of different blastocysts suggests that ER expression is not restricted to certain cells or cell types at this stage of development. The intensity observed in each cell varied somewhat; whether this reflects a real difference in the level of ER expression among different cells is not clear. As shown in Figs. 1 and 2, the staining pattern was similar between *in utero*- or *in vitro*-developed blastocysts. In the progesterone-treated dormant (Fig. 2) and estrogen-treated activated blastocysts (data not shown), immunoreac-

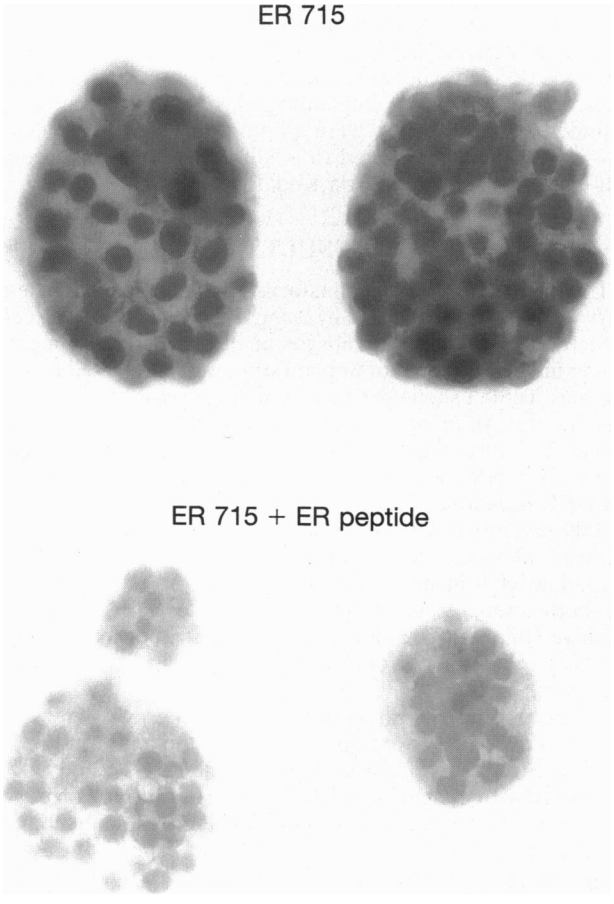

FIG. 1. Immunocytochemical detection of ER in mouse blastocysts developed in culture (×390). Mouse blastocysts were immunostained with antibody ER 715 (150 ng/ml) or ER 715 preabsorbed with ER peptide. Two representative embryos from each group are shown.

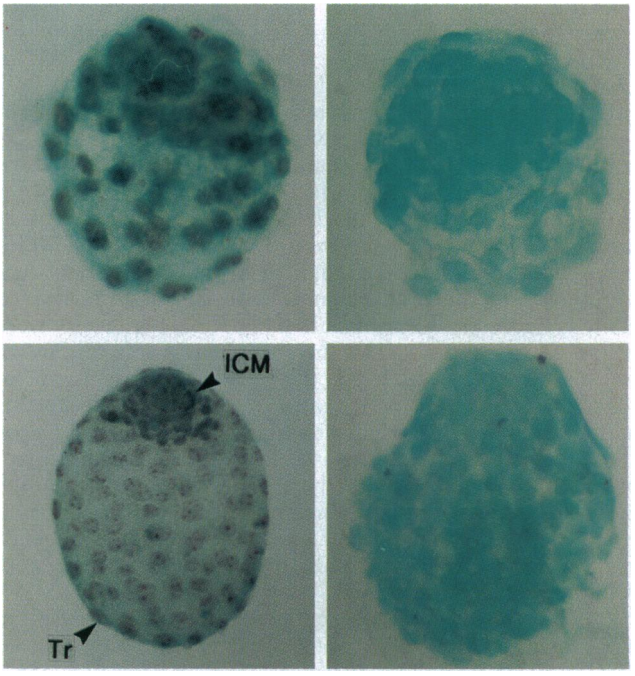

FIG. 2. ER immunostaining in normal, dormant, and activated blastocysts. Reddish-brown deposits indicate the sites of immunoreactive ER. Microphotographs (×140) depict the presence (*Left*) and absence (*Right*) of immunostaining in representative day 3 normal (*Upper*) and day 7 (*Lower*) dormant blastocysts, respectively. Immunostaining pattern in day 7 estrogen-activated blastocysts was similar to that in the progesterone-treated dormant blastocysts (data not shown). Positive immunostaining was observed when blastocysts were incubated with the primary antibody (*Left*), while immunostaining was absent when similar blastocysts were incubated in the primary antibody preneutralized with excess of the antigenic peptide (*Right*). Blastocysts were counterstained with fast green. ICM, inner cell mass; Tr, trophectoderm.

tive ER was present in both the trophectoderm and inner cell mass cells (Fig. 2). In the trophectoderm of dormant blastocysts, the staining was primarily evident in a few areas within the nucleus, suggesting accumulation of ER in the nucleolus. Preabsorption of the antibody with the peptide completely abolished the immunostaining.

ER Expression in Implanting Blastocysts *in Vitro*. After 2 days of culture in the complete serum-containing medium, the blastocyst attached to the bottom of and the trophoblast cells migrated outward on the plastic slides. At this stage, similar to that of the unattached blastocyst, nuclear staining was observed in all cells of both the inner cell mass and the trophoblast cells when immunostaining was performed with the antibody ER 715 (Fig. 3). The staining was specific, since it was undetectable when ER 715 was preabsorbed with the ER peptide. This result indicates that both cell types continue to express ER at this stage.

ER Distribution at the Early Egg Cylinder Stage. The inner cell mass continues to grow after attachment of the blastocyst. On day 4 of culture in the serum-containing medium, a raised portion of compact cells resembling an early egg cylinder was clearly visible. Trophoblast giant cells derived from mural trophoblast cells that spread on the slide were also distinct. Staining was observed with the ER 715 antibody in both portions (Fig. 4) and the specificity was verified with the preabsorbed antibody.

Contrary to the staining observed in the attached and unattached blastocysts, immunostaining in trophoblast giant cells was no longer distributed throughout the nucleus. Instead, a few spots of dense staining were visible, indicating a localization of ER inside the nucleus (Fig. 4). It seems possible that the dense spots correspond to the nucleoli. This pattern of staining was similar to that observed in the dormant blastocyst. It is not known why ER assumes such a distribution in the giant cells. Two possible reasons may lead to such a distribution. (*i*) ER may be devoted to promoting the synthesis of rRNA. It has been shown that the synthesis of rRNA is stimulated by estrogen in delayed blastocysts upon estrogen treatment (22). Changes in the morphology of the nucleoli have also been reported (9). The localization of ER in the nucleoli is consistent with these observations. (*ii*) The giant cells and the dormant blastocyst are losing ER and the small spots of ER are residual ERs that have not been degraded.

The elevated portion of the early egg cylinder also continued to express ER. At this time, the inner cell mass should have differentiated into two layers of cells: the primitive endoderm, which contributes to extraembryonic tissues, and the primitive ectoderm, which gives rise to the embryo proper.

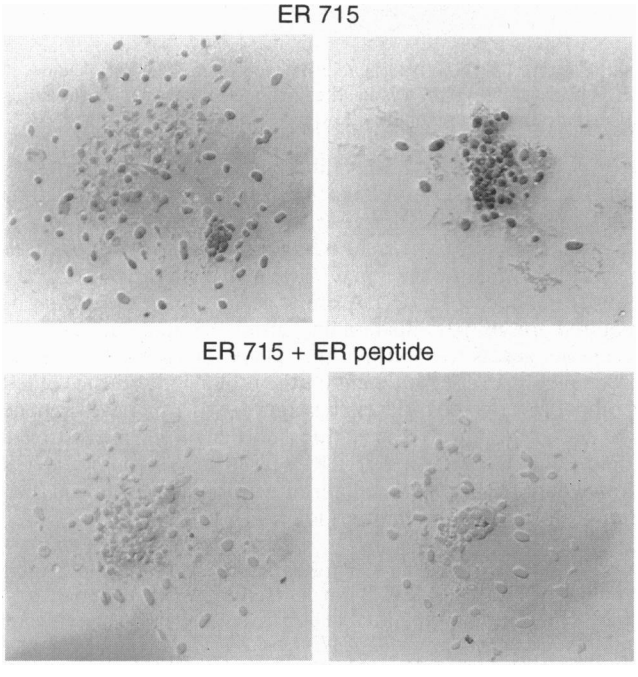

FIG. 3. Immunocytochemical detection of ER in implanting mouse blastocysts (×260). Blastocysts were cultured for 2 days in serum-containing medium. ER antibody ER 715 or ER 715 preabsorbed with ER peptide at 40 ng/ml was used to probe the embryo. The reaction was developed for 5 min. Two representative embryos are shown for each group.

DISCUSSION

We previously demonstrated that ER mRNA is present in the mouse blastocyst. In the present investigation, we demonstrate that ER protein is also present at this stage. Further, we show that ER in the blastocyst resides in the nucleus, which is the normal cellular location of ER, and that ER is present in all cell types of the blastocyst. This expression of ER in all cell types continues in the dormant and implanting blastocyst. By the early egg cylinder stage, cells derived from the inner cell mass and those derived from the trophoblast continue to express ER. However, there is a redistribution of ER in the dormant blastocysts and trophoblast giant cells of the *in vitro* early egg cylinder such that ER clusters to small regions in the nucleus. This possibly reflects a concentration in the nucleoli or degradation of the receptor.

Estrogen is needed for implantation in the mouse. The detection of the ER protein in the blastocyst lends further support for a possible direct effect of estrogen during the periimplantation period. The expression pattern of ER in all cell types of the blastocyst is similar to that of platelet-derived growth factor, transforming growth factor α (TGF-α), TGF-β subtypes 1–3, and epidermal growth factor receptor (EGFR) (15, 23, 24). It is speculated that these growth factors and their receptors expressed in the blastocyst are important for embryonic differentiation and implantation. Interestingly, the expression of TGF-β3 and EGFR is downregulated in dormant blastocysts but is upregulated when dormant blastocysts are activated by injecting estradiol into the delayed implanting mice. The universal expression of ER at this stage corresponds well to the universal expression of TGF-β3 and EGFR, suggesting the possibility that TGF-β3 and EGFR expression is under direct regulation by estrogen through ER expressed in the same cells. However, the expression of EGFR in the dormant blastocyst remained unresponsive to estradiol in culture (15).

There are two cell types in the blastocyst that have distinct functions and unique developmental potentials. Although ER was found in both cell types, they may serve very different purposes. During implantation, the trophoblast cells attach to the uterine epithelium and invade the uterus. Mouse blastocysts seem to possess particular properties that enable them to induce decidualization, while inert bodies or younger embryos fail to do so (9). Estrogen-induced changes in the expression of cell surface proteins occur around the time of implantation (6). Such changes might be necessary for correct signaling to the uterus and for establishing proper contact of the trophoblast cells with the uterine luminal epithelial cells. It is possible that such changes are induced by estrogen through ER in the trophoblast cells. It is not clear as to what role ER may play once implantation is initiated. The entire fetus is derived from the inner cell mass. The expression of ER in the inner cell mass suggests that ER may have a general function during early fetal development. ER expression appears to be persistent in cells derived from the inner cell mass at the early egg cylinder stage, suggesting a continued requirement for its presence.

If ER in the embryo is required for implantation or early development, ER mutations that render nonfunctional ER

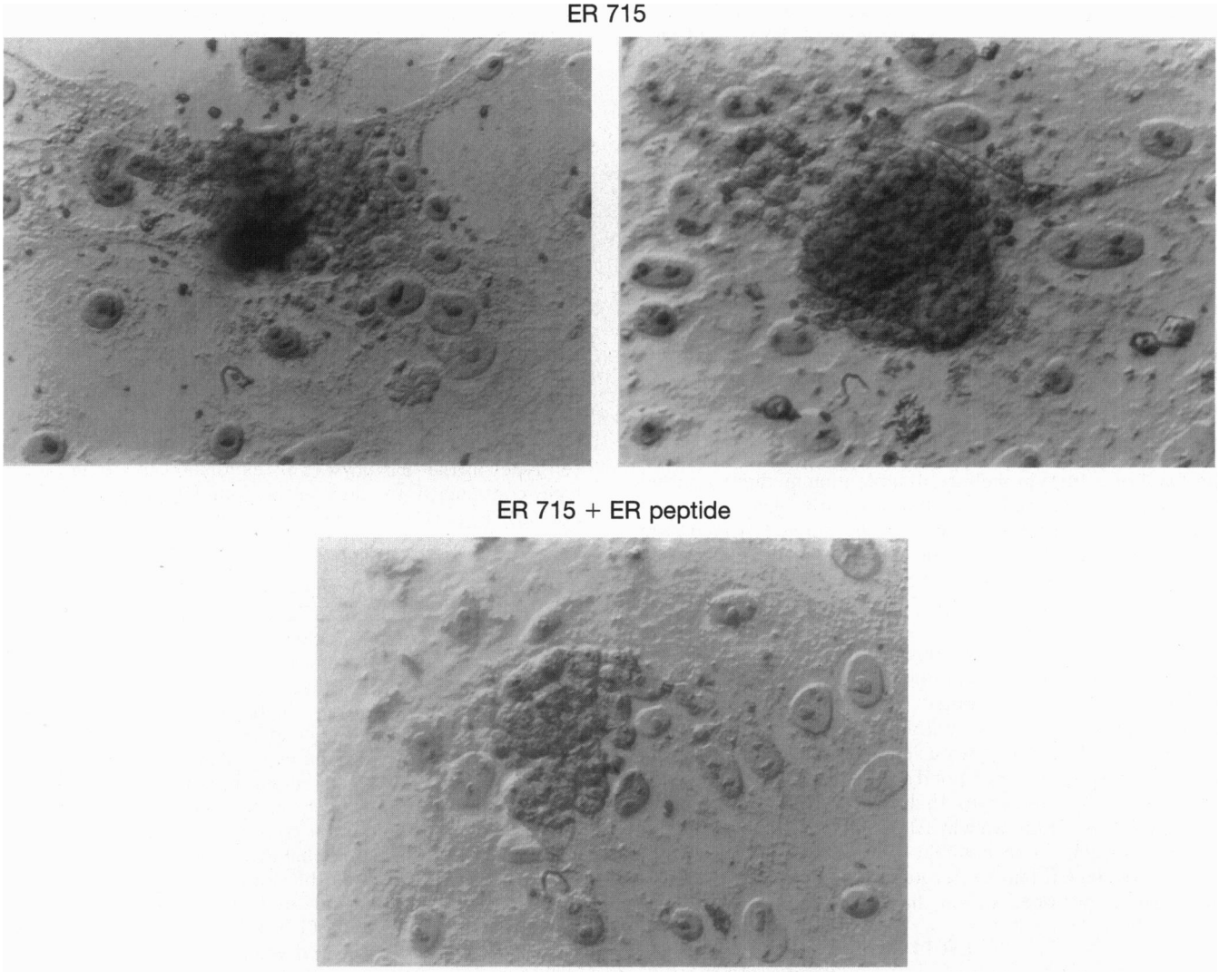

FIG. 4. Immunocytochemical detection of ER in early egg cylinder stage embryos (×300). Blastocysts were cultured for 4 days in serum-containing medium. Antibody ER 715 or ER 715 preabsorbed with ER peptide at 75 ng/ml was used to probe the embryo. The reaction was developed for 20 min. Two representative embryos for ER 715 and one for the peptide control are shown.

would be expected to be embryonic lethal. There is only a single report of a naturally occurring ER mutation (25). The mutation carrier is a male with tall stature associated with incomplete closure of the epiphyses. The extreme rarity of such mutations suggests that ER may be required for embryonic survival. It is therefore surprising that ER-null mice created by insertional mutation were viable (14). These mice are infertile and have abnormal reproductive tracts. Interestingly, the uteri of these mice still contain 5–10% of normal estrogen binding capacity. It is possible that residual ERs exist in these mice. The essential functions of ER for survival may be carried out by these residual receptors. Alternatively, redundant regulatory pathways may exist. In the absence of ER, another protein(s) may substitute for ER.

It is possible that ER expression in early development is only necessary for its continued expression or function in later development but has no role during the pre- or periimplantation period. Perhaps during development, ER is expressed in every cell at early times such as the blastocyst stage, and then some cells gradually lose ER. The changes in ER distribution in dormant blastocysts and the trophoblast giant cells in *in vitro* early egg cylinders may be examples of ER loss in progress. By the time of adulthood, only certain tissues retain ER. This process of expressing ER in all cells initially and restricting receptor expression in a majority of cells later may be necessary if ER is to execute its function at both early and later times. It has been observed that stable transfection, rather than transient transfection, of ER into ER-negative cells is needed for ER to induce certain estrogen-responsive endogenous genes (26). Similar phenomena have also been reported for the progesterone receptor (27). A prolonged existence of ER may be required for its participation in chromatin modeling and the receptor's access to specific target DNA. During the course of development, this requirement for the prolonged presence of ER is probably reflected by its expression very early in development. It will be interesting to see whether a normal response to estrogen could be fully recovered if ER is reintroduced into the ER "knockout" mice sometime later in development or in cell lines derived from the adult mutant mice.

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