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—Research Note—

Expression of Amphiregulin During the Pre- and Post-implantation Period in the Mouse Reproductive Tract

Dong-Soo LEE[1)], Yoshiko YANAGIMOTO UETA[1)] and Hiroshi SUZUKI[1,2)]

[1)]Research Unit for Functional Genomics, National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Nishi 2-13, Inada-cho, Obihiro, Hokkaido 080-8555 and [2)]Department of Developmental and Medical Technology, Graduate School of Medicine, The University of Tokyo, 7–3–1, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Abstract. In mammals, embryo implantation is an essential step in reproduction. Implantation is a phenomenon that involves crosstalk between the blastocyst and the maternal endometrium. However, the molecular basis of the connections between the blastocyst and endometrium is not yet fully understood. Amphiregulin is a member of the epidermal growth factor family and is known to be expressed in the luminal epithelium of the mouse uterus on 3.5 days post coitum (dpc). Thus, to clarify the mechanism of amphiregulin at fetomaternal interface, we analyzed the expression pattern of amphiregulin mRNA in the oviducts and uteri of pregnant and psuedopregnant mice by means of real-time PCR. Amphiregulin expression in the pregnant uterus dramatically increased on 2.5 dpc, peaked on 3.5 dpc, and declined by 5.5 dpc. Furthermore, to analyze the effect of the presence of an embryo on amphiregulin expression, we determined the expression pattern of amphiregulin mRNA in the uterus after embryo transfer on 0.5 and 1.5 dpc. A previous study showed that the expression of amphiregulin mRNA depends on the concentration of progesterone. However, our present results indicate that amphiregulin mRNA is upregulated by the presence of fertilized eggs in the lumen of the oviduct on 0.5 dpc.

Key words: Mice, Implantation, Embryo transfer, Amphiregulin, mRNA

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Blastocyst implantation and the successful establishment of pregnancy require delicate interactions between the embryo and maternal environments. Implantation of the mammalian embryo begins with the attachment of a hatched blastocyst to the luminal epithelium of the uterus. Previous studies have indicated that the window of implantation is very narrow and is under strict regulation by ovarian hormones such as estrogen and progesterone [1]. Previous studies have also identified molecules associated with implantation, which appear to be regulated by ovarian hormones [2–5]. Although ovarian hormonal signaling is essential for proper uterine function prior to and during implantation, the changes in the transcripts of these molecules have not been well characterized. Since amphiregulin, a member of the epidermal growth factor family, expresses in the luminal epithelium of mouse uterus on 3.5 dpc, it is believed to be involved in the process of implantation [6]. The expression pattern of amphiregulin receptors in the uterus has not yet been clarified. Amphiregulin is known to participate in the signal transduction of WNT/beta-catenin and is known to be involved with cancer.

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Correspondence: H. Suzuki (e-mail: hisuzuki@obihiro.ac.jp)

Although the physiological significance of amphiregulin in the reproductive tract is not fully understood, it seems that amphiregulin might participate in cellular infiltration in the process of implantation.

To examine the interaction between the embryo and maternal luminal epithelium tissue, it is essential to compare differences between pregnancy and pseudopregnancy. This approach enables clarification of the effect of the presence of an embryo in the reproductive tract on the initiation of a receptive state for implantation. In the present study, the expression pattern of amphiregulin mRNA in the oviduct and uterus of pregnant and psuedopregnant mice was examined by means of real-time quantitative PCR. Furthermore, to analyze the effect of the presence of an embryo in the reproductive tract on the amphiregulin expression, we determined the expression pattern of amphiregulin mRNA in the uterus during the peri-implantation period after embryo transfer on 0.5 and 1.5 dpc.

Materials and Methods

Animals

Female 8–10-week-old ICR mice were purchased from a commercial supplier (CLEA Japan, Tokyo, Japan) and maintained in the animal facility of the National Research Center for Protozoan Diseases at the Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan. All animals were housed in polycarbonate cages and maintained in a specific pathogen-free environment in light-controlled (lights-on from 07:00 to 19:00) and air-conditioned rooms (temperature: 24 ± 1C, humidity: 50 ± 10%). They had free access to standard laboratory chow (CE-2; CLEA Japan) and water *ad libitum*. The animals used in this study were cared for and used under the Guiding Principles for the Care and Use of Research Animals promulgated by the Obihiro University of Agriculture and Veterinary Medicine.

Natural mating and embryo tranfer

Female mice were mated with fertile or vasectomized males. Plug-positive females were sacrificed by cervical dislocation on 0.5–10.5 dpc. Excised oviducts and uteri were immediately immersed into liquid nitrogen and stored at –80 C until use. For total RNA extraction from blastocyst, 200 blastocysts were collected from naturally mated females on 3.5 dpc, immediately immersed in liquid nitrogen and stored at –80 C until use. Embryo transfer was performed as described below. The donor zygotes and embryos were prepared by *in vitro* fertilization and subsequent cultivation *in vitro*. The procedures used for *in vitro* fertilization have been described previously [7]. Zygotes up to the blastocyst stage were cultured in Whitten's medium [8] containing 100 μM EDTA [9] under 5% CO_2 in air at 37 C. Blastocysts (BL) and fertilized eggs at the pronuclear stage (PN) were transferred at 102 h and 6 h after insemination, respectively, into the oviducts of the same recipient. Thus, 7 PN were transferred separately to the right or left oviduct of pseudopregnant recipients on 0.5 or 1.5 dpc, while 7 BL were transferred to the other oviduct, as described previously [10, 11]. Some of the pseudopregnant recipients received only BL embryos into both oviducts on 0.5 dpc. The recipient animals were sacrificed by cervical dislocation on 2.5 and 3.5 dpc. The collected uterine horns were individually frozen and stored until use as described above.

Real-time quantitative PCR

Total RNA was extracted from frozen reproductive tissues and blastocysts by means of a TRI Reagent Kit (Sigma Inc., St. Louis, MO, USA) according to the manufacturer's protocol. The extracted total RNA was then subjected to real-time PCR analysis. Primers and a TaqMan probe for the amphiregulin gene and beta-actin were designed using the primer design software Primer Express version 1.5 (Applied Biosystems, Foster City, CA, USA). The GenBank accession numbers of all the cDNA sequences are summarized in Table 1. Quantification of all gene transcripts was carried out with an ABI PRISM 7900 HT (Applied Biosystems, Foster City, CA, USA). Templates for real-time PCR were obtained by reverse transcriptase reaction of the total RNA. For RT-PCR reactions, a TaqMan One-Step RT-PCR Master Mix Reagents Kit (PE Applied Biosystems, Foster City, CA, USA) was used at 20 μl/tube as follows: the template (20 ng) was mixed with 2 × Master Mix without UNG, 40 × MultiScribe and RNAse Inhibitor Mix, 200 nM TaqMan Probe, and 900 nM of each primer. Reaction conditions were 1 cycle at 48 C for 30 min, 1 cycle at 95 C for 10 min, and then

Table 1. Primer/probe sequences for real-time quantitative PCR

Transcript	Primer/probe sequences (5′ to 3′)	GenBank accession number
Amphiregulin	F: GCGAATGCAGATACATCGAGAA R: TCCACACCGTTCACCAAAGTAA FAM-CATGCAATTGTCATCAAGA-MGB	NM 009704
Beta-actin	F: GCTCTGGCTCCTAGCACCAT R: GCCACCGATCCACACAGAGT FAM-ATCAAGATCATTGCTAATC-MGB	NM 007397

45 cycles of the amplification step (95 C for 15 seconds and 60 C for 1 min). The gene expression levels of amphiregulin were calculated as gene expression rates, as reported previously [12]. Briefly, the amounts of each gene and beta-actin mRNA in samples were estimated with standard curves representing the log of the input amount (log starting with cDNA molecules) as the X axis and the threshold cycle as the Y axis. A relative standard curve (SC) for real-time PCR was used as a common set of samples that linked the experimental PCR plates together and permitted overall analysis of the samples. Preparation and utilization of this SC as a quality control for the efficiency of amplification of PCR plates is described elsewhere [13]. The gene expression rate was obtained by normalizing the amount of amphiregulin cDNA with that of beta-actin. Total RNA from the oviducts and uteri of nonpregnant animals were used as controls because amphiregulin mRNA is stably expressed during the estrous cycle [14].

Statistical analysis

All data are expressed as the mean ± standard error (SE). Statistical comparisons of the relative mRNA expression of each gene between experimental groups were analyzed by analysis of variance followed by a *post hoc* test using StatView 4.5 (Abacus Concepts, Inc., Berkeley, CA, USA). In all statistical tests, differences were considered significant when P was <0.05.

Results

Expression pattern of amphiregulin mRNA in the oviduct and uterus of pregnant and psuedopregnant mice during the pre- and post-implantation periods

Although amphiregulin mRNA expression in the oviducts was significantly different between the pregnant and pseudopregnant animals on 0.5, 6.5, 8.5, and 9.5 dpc, as shown in Fig. 1, their expression levels were not different from the non-pregnant control mice. The changes in amphiregulin expression in the oviducts of both the pregnant and pseudopregnant females ranged by approximately 0.5- to 1.7-fold during observation periods as compared with the non-pregnant control animals. In pregnant animals, the amphiregulin expression of the oviducts on 0.5 dpc was significantly higher than those on 2.5, 3.5, 8.5, and 9.5 dpc. In terms of uterine expression (Fig. 2), however, amphiregulin mRNA in both the pregnant and pseudopregnant females dramatically increased on 2.5 dpc, maximized on 3.5 dpc, and declined by 5.5 dpc. From 3.5 dpc to 8.5 dpc, amphiregulin expression in the uteri of pregnant animals was significantly higher than that of pseudopregnant females. In addition, on 3.5 dpc, amphiregulin mRNA expression in the uteri of pregnant mice was 2.5-fold higher than that of pseudopregnant animals ($P<0.05$, Fig. 2). Uterine expression of amphiregulin on 3.5 dpc was significantly higher than any other experimental group including the non-pregnant control. As shown in Fig. 3, only residual expression of amphiregulin was detected in blastocysts. These results indicate that a large quantity of amphiregulin mRNA in the uteri of pregnant mice on 3.5 dpc was essentially contributed by expression from uterine tissues.

Amphiregulin mRNA expression in the uterus after embryo transfer

The uterine expression of amphiregulin on 2.5 dpc in recipient animals that received both BL and PN or BL only was significantly higher than that of non-pregnant control animals (Fig. 4a). When PN and BL embryos were transferred to pseudopregnant females on 0.5 dpc, amphiregulin

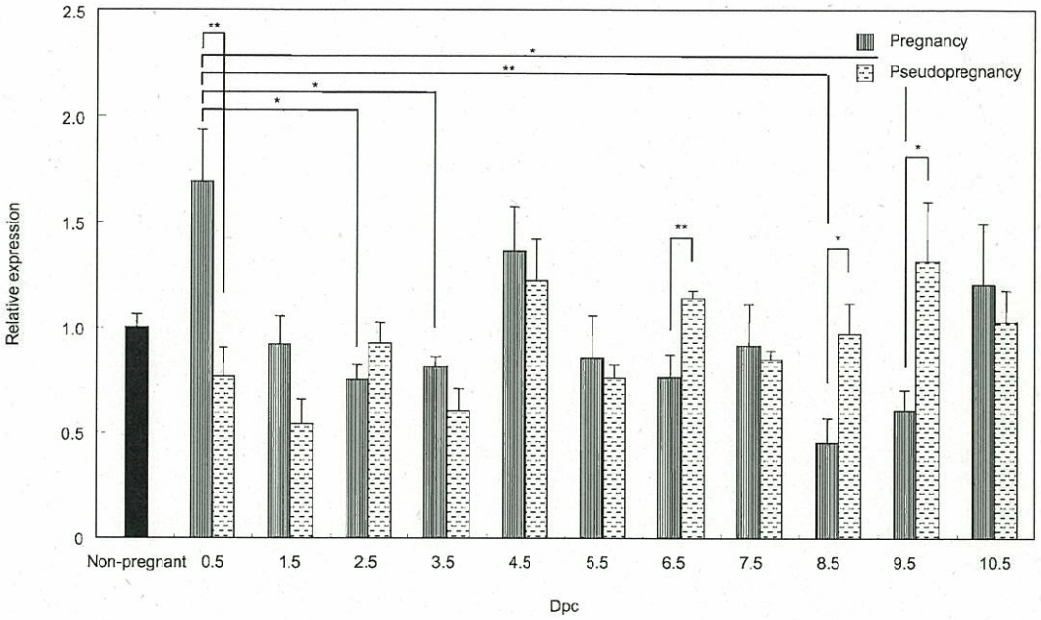

Fig. 1. Expression pattern of amphiregulin mRNA in the oviduct of pregnant and psuedopregnant mice. Total RNA was extracted from the oviducts of pregnant and pseudopregnant mice and subjected to real-time PCR analysis. The mRNA level was expressed relative to the non-pregnant control group. The results are shown as the mean ± SE. Asterisks above the error bar indicate significant differences between the experimental groups. *: P<0.05. **: P<0.01.

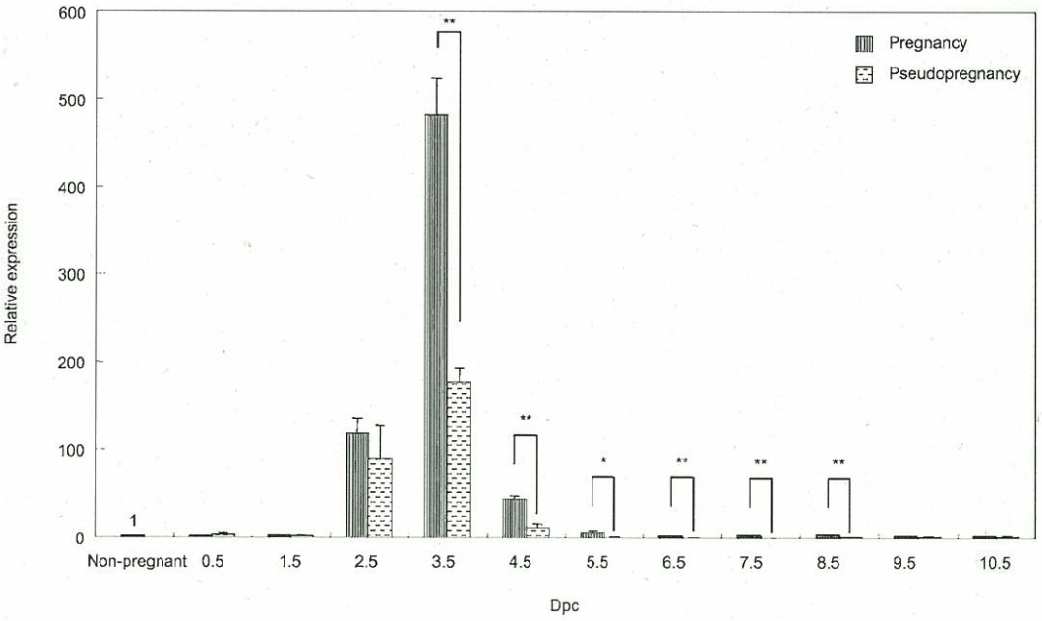

Fig. 2. Expression pattern of amphiregulin mRNA in the uteri of pregnant and psuedopregnant mice. Total RNA was extracted from the uteri of pregnant and pseudopregnant mice and subjected to real-time PCR analysis. The mRNA level was expressed relative to the non-pregnant control group. The results are shown as the mean ± SE. Asterisks indicate significant differences between the experimental groups. *: P<0.05. **: P<0.01. The expression level in pregnant mice on 3.5 dpc was significantly higher than any other pregnant group and the non-pregnant controls at P<0.01. The expression level in pseudopregnant mice on 3.5 dpc was significantly higher than the other pseudopregnant mice (except for 2.5 dpc) and non-pregnant controls at P<0.01. The expression level in pseudopregnant mice on 3.5 dpc was significantly higher than pseudopregnant mice on 2.5 dpc at P<0.05. The expression level in psuedopregnent mice on 2.5 dpc was significantly higher than the other pseudopregnant mice (except for 4.5 dpc) and non-pregnant controls at P<0.05.

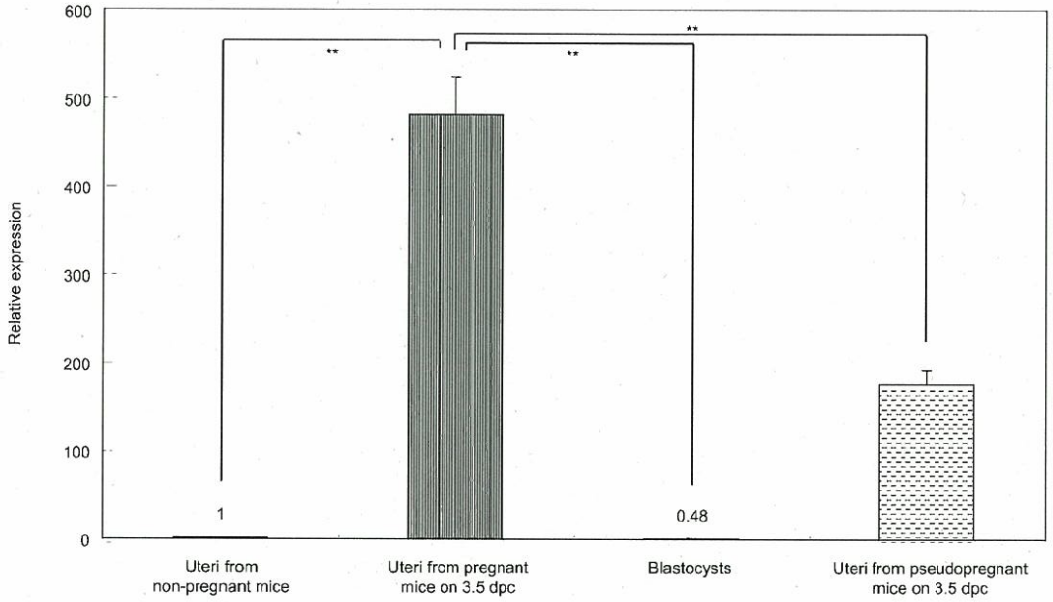

Fig. 3. Expression of amphiregulin mRNA in the uteri of pregnant and pseudopregnant mice on 3.5 dpc and in blastocysts. The mRNA level was expressed relative to the non-pregnant control group. The results are shown as the mean ± SE. Asterisks indicate significant differences between the experimental groups. **: P<0.01.

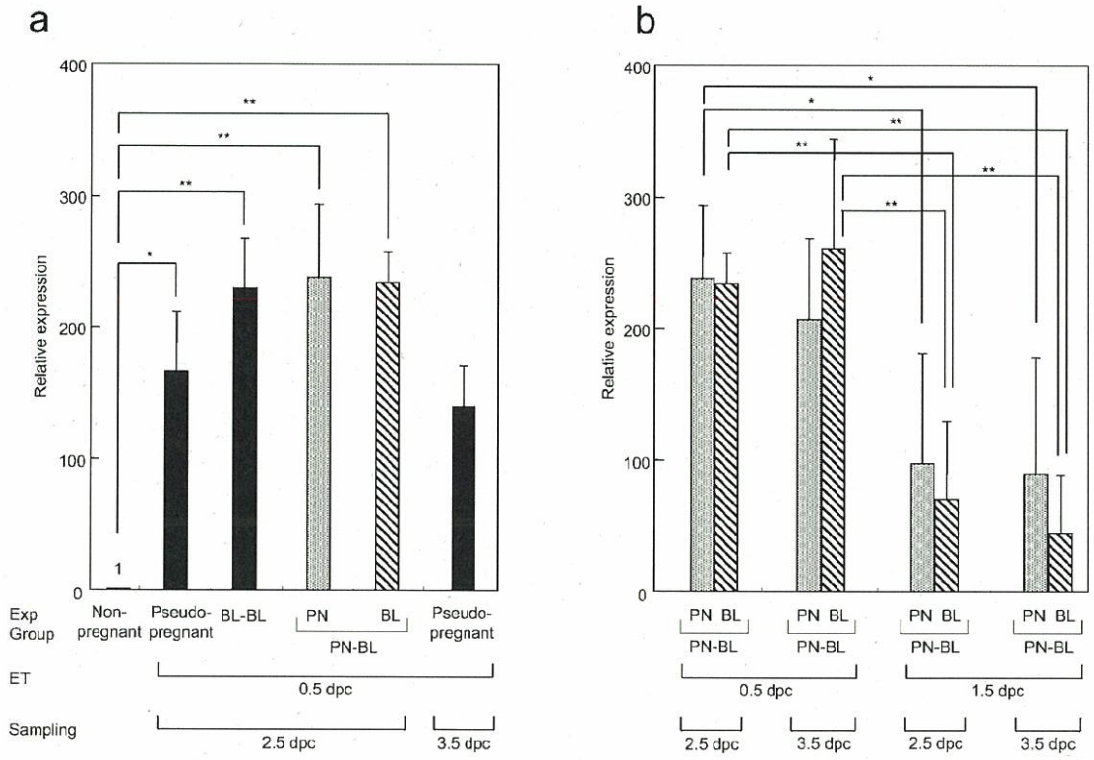

Fig. 4. Expression of amphiregulin mRNA in the uteri of mice after embryo transfer. a: Comparison of mRNA expression among nonpregnant, pseudopregnant and recipient animals. b: Difference of the day of embryo transfer. Blastocysts (BL) and fertilized eggs at the pronuclear stage (PN) were transferred at 102 h and 6 h after insemination, respectively, into the oviducts of the same recipient. Seven PN were transferred separately to the right or left oviduct of pseudopregnant recipients on 0.5 or 1.5 dpc, while seven BL were transferred to the other oviduct. Some of the recipients received only BL embryos into both oviducts. The recipient animals were sacrificed by cervical dislocation on 2.5 and 3.5 dpc. The mRNA level was expressed relative to the control group. The results are shown as the mean ± SE. Asterisks indicate significant differences between the experimental groups. *: P<0.05. **: P<0.01.

mRNA expression in the uteri of the mice on 2.5 and 3.5 dpc was higher than when transfer was conduced on 1.5 dpc (Fig. 4b). There was no difference between PN and BL in the expression levels of amphiregulin in the uteri of mice on 2.5 and 3.5 dpc after transfer.

Discussion

It has been reported that amphiregulin mRNA accumulates in the luminal epithelium of the uterus exclusively at the sites of blastocyst attachment prior to implantation [6]. Among the members of the epidermal growth factor family, HB-EGF, betacellulin, and epiregulin are thought to be locally upregulated in luminal epithelium and stroma underlying implanting blastocysts in response to embryo-derived signals [15], while amphiregulin is known to be regulated by the progesterone and LIF induced by estrogen [15, 16]. On the other hand, it has been shown that amphiregulin mRNA is expressed throughout the estrous cycle in the mouse uterus [14]. Furthermore, the highest expression level of amphiregulin during diestrus is not consistent with the LIF mRNA expression pattern during the estrous cycle [14]. As shown in Fig. 1, in pregnant animals, amphiregulin expression of oviducts on 0.5 dpc was significantly higher than those on 2.5–9.5 dpc and on 0.5 dpc in pseudopregnant animals. The presence of fertilized eggs in the oviducts might upregulate the amphiregulin expression of the oviducts on 0.5 dpc. As the expression of amphiregulin mRNA in the uteri of pregnant animals on 3.5 dpc is significantly higher than that of pseudopregnant animals (Fig. 2), the blastocyst stage seems to be related to explosive expression of amphiregulin around implantation in mice. However, an extremely lowered expression of amphiregulin mRNA was detected in blastocysts (Fig. 3). These results suggest that the presence of a blastocyst might stimulate an increase in amphiregulin expression in the mouse uterus. Since there was no difference in the expression levels of amphiregilin in uteri on 2.5 and 3.5 dpc between the PN and BL transferred embryos (Fig. 4a), embryos at different developmental stages may similarly induce expression of amphiregulin. Furthermore, since lower expression levels of amphiregulin were detected in the uterus on 2.5 or 3.5 dpc in animals that received embryos on 1.5 dpc as compared with animals that received embryos on 0.5 dpc (Fig. 4a), the presence of fertilized eggs in the reproductive tract on 0.5 dpc might influence the strength of the expression levels of amphiregulin during the peri-implantation period. Because mice deficient in amphiregulin are fertile [17], amphiregulin gene expression is evidently not essential for implantation. Other molecules, such as betacellulin, EGF, epigen, HB-EGF, and TGF-alpha, that share ErbB-1 (EGFR) as ErbB ligands, might compensate for the function of amphiregulin required for implantation [18]. Since the expression of amphiregulin mRNA was upregulated in the uteri of pseudopregnant females (Fig. 2), the amphiregulin gene may serve as a molecular marker for the receptive state of the uterus for implantation in mice.

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