Differential expression of the PEA3 subfamily of ETS transcription factors in the mouse ovary and peri-implantation uterus

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Abstract

The objective of the present investigation was to examine the spatio-temporal expression of three members of the ETS family of transcription factors, ERM, ER81, and PEA3, in the peri-implantation mouse uterus and in the ovary. These three factors belong to the PEA3 subfamily and are known to mediate diverse functions ranging from neuronal development to tumor progression. As transcription factors, they regulate the expression of a number of genes with various biological functions. Since several genes with known roles in the reproductive processes have been shown to be under the regulation of one of these factors, we sought to investigate the expression of ERM, ER81, and PEA3 in the mouse ovary and uterus. Quantitative RT-PCR analyses showed that ERM, ER81, and PEA3 were all expressed in the peri-implantation mouse uterus, with higher levels of expression on days 4 and 5 of pregnancy. To determine the cell type-specific expression of these factors, we employed in situ hybridization, the results of which revealed that ERM was expressed in both the epithelium and the stroma on days 4 and 5 of pregnancy. Uterine glands showed a high expression of ERM on those days. ERM was also highly expressed in the corpora lutea of the mouse ovary. Both ER81 and PEA3 were expressed at low levels in the stroma on days 4 and 5. On day 8, while ERM and PEA3 were mainly expressed in the embryo and were at low levels in the maternal decidua in a diffused pattern, ER81 was highly expressed in the vascular bed of the mesometrial deciduum. Both ER81 and PEA3 were undetectable in the mouse ovary. Collectively, these data show that ERM is implicated in the early event of implantation as well as in ovarian functions, while ER81 is involved in the establishment of the maternal vasculature for subsequent placental development. PEA3 is apparently an embryonic factor for early embryogenesis.


Introduction

Embryo implantation is a critical event regulating successful pregnancy. It is a complex process involving intricate interactions between the blastocyst and the uterus. During this process, the uterus undergoes morphological and physiological changes in transforming from a non-receptive to a receptive state (reviewed by Dey 1996). The main driving force for this transformation is the ovarian hormones which act through their intracellular receptors to regulate gene expression and hence influence cellular proliferation and differentiation. The uterus consists of three major cell types, the epithelium, stroma, and myometrium. In mice, the presence of a vaginal plug after mating defines day 1 of pregnancy, and the uterus on this day is under estrogenic influence due to a preovulatory estrogen surge, driving proliferation of the epithelium. From day 3 onward, newly formed corpora lutea in the ovaries secrete progesterone, a major hormone of pregnancy, and this hormone induces stromal cell proliferation. With a small amount of estrogen produced on the morning of day 4 of pregnancy, progesterone prepares the uterus for the attachment reaction that occurs around midnight of day 4 in mice. Following the attachment of the blastocyst onto the uterine epithelium, stromal cells proliferate and differentiate into decidual cells to support the developing conceptus under progesterone (reviewed by Dey 1996, Dey et al. 2004). In the uterus, a unique set of
genes is expressed in a timely manner during the peri-implantation period for the establishment of uterine receptivity and attachment reaction (reviewed by Dey et al. 2004).

The ETS (E26 transformation specific) family of transcription factors is divided into several subfamilies based mainly on sequence homology and location of the ETS domain, an 84 amino acid sequence present in all members of the family (Sharrocks 2001). The PEA3 subfamily is composed of three highly homologous factors, ERM (Etv5), ER81 (Etv1), and PEA3 (Etv4). ETS factors are capable of regulating transcription by binding to $\sim 10$ bp elements in the promoters of target genes, known as ETS-binding sites (EBS; 5'-GGA(A/T)-3'). Individual ETS proteins demonstrate specificity for sequences flanking this core, but it is not uncommon for different ETS factors to bind to the same EBS. All three members of the PEA3 subfamily commonly activate transcription. ERM and PEA3 are also known to be downstream effectors of fibroblast growth factor (FGF) signaling (Roehl & Nusslein-Volhard 2001) and, in several developmental contexts, ERM and PEA3 are present in regions of FGF signaling (Brent & Tabin 2004).

ETS factors have been linked to diverse biological processes, but no clear unifying theme has emerged. Gene targeting experiments have provided clues about the function of the members of the PEA3 subfamily. PEA3-deficient male mice exhibit ejaculatory dysfunction possibly stemming from neuronal defects (Laing et al. 2000). ER81-deficient mice exhibit severe motor discoordination and die around 3–5 weeks of age (Arber et al. 2000). These findings thus suggest that the PEA3 subfamily is involved in neuronal pathfinding. Other members of the ETS family are involved in angiogenesis, immune functions, and various developmental processes (Sharrocks 2001). However, it is not known if these transcription factors are expressed in the uterus during early pregnancy. Since PEA3 members are shown to be involved in regulating several genes relevant to reproductive functions, such as cyclo-oxygenase-2 (COX-2) and proteases (Rorth et al. 1990, Crawford et al. 2001, Howe et al. 2001, Levallet et al. 2001, El-Tanani et al. 2004), we sought to examine the spatio-temporal expression of ERM, ER81, and PEA3 in the mouse ovary and peri-implantation uterus by quantitative RT-PCR (qRT-PCR) and in situ hybridization. Our results have shown that these genes exhibit a distinct expression pattern in these tissues, suggesting diverse roles of these factors in ovarian and uterine functions.

Materials and Methods

Mice and tissue preparation

Adult CD-1 mice (Charles River Laboratories, Wilmington, MA, USA) were housed in the Animal Care Facility at the Washington University School of Medicine according to NIH and institutional guidelines for laboratory animals. Ovaries were collected from cycling adult mice. For timed pregnancy, female mice were mated with fertile males of the same strain to induce pregnancy. The morning of finding a vaginal plug was designated day 1 of pregnancy. Mice on days 1, 4, 5, and 8 of pregnancy were killed at 0900 h, and their uteri were collected for RNA preparation and in situ hybridization. Pregnancy on days 1 or 4 was confirmed by recovering embryos from the reproductive tracts. Implantation sites on day 5 were visualized by intravenous injection (0.1 ml/mouse) of Chicago Blue dye solution (1% in saline) and were cut with a sharp scalpel. Day 8 implantation sites containing decidua and embryos were surgically separated from myometrium for RNA preparation.

qRT-PCR

Total RNA was purified using TRI-Reagent (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer’s protocol. Each uterine RNA sample was collected from a single mouse ($n = 4$ or 5 per group). One microgram of RNA was subjected to RT using M-MuLV reverse transcriptase (Roche Applied Science, Indianapolis, IN, USA) for cDNA synthesis. qRT-PCR (qRT-PCR) was performed by monitoring in real time the increase in fluorescence of the SYBR Green dye (Molecular Probes, Eugene, OR, USA) as described (Wittwer et al. 1997, Morrison et al. 1998) using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). For comparison of transcript levels between samples, a standard curve of cycle thresholds for several serial dilutions of a cDNA sample was established and then used to calculate the relative abundance of each gene. Values were then normalized to the relative amounts of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, which were obtained from a similar standard curve. All PCR reactions were performed in duplicate. Sequences of primers used for PCR analysis are given in Table 1.

Hybridization probes

Suitable regions from full-length mouse ERM, ER81, and PEA3 sequences were amplified by PCR and subcloned into pGEM-T Easy Vector (Promega, Madison, WI, USA). Primers used for subcloning are shown in Table 1. Mouse ERM subclone contains nucleotides 826-1048 of NM_023794. ER81 and PEA3 subclones contain 418 bp and 531 bp sequences from untranslated regions respectively. Antisense 35S-labeled cRNA probes were generated using appropriate RNA polymerases. The probes were used at specific activities of $2 \times 10^9$ d.p.m./ml.

In situ hybridization

In situ hybridization was performed as described previously (Das et al. 1994). Uteri from pregnant mice were cut into 4–6 mm pieces and flash frozen in Histo-Freeze (Fisher Scientific, Pittsburgh, PA, USA). Frozen sections
(12 µm) were mounted onto poly-L-lysine-coated slides (Polysciences, Inc., Warrington, PA, USA) and fixed in cold 4% paraformaldehyde in PBS. The sections were prehybridized and hybridized at 45 °C for 4 h in 50% formamide hybridization buffer containing the 35S-labeled antisense cRNA probes for ERM, ER81, and PEA3. After hybridization and washing, the sections were incubated with RNase A (20 µg/ml) at 37 °C for 20 min. RNase A-resistant hybrids were detected by autoradiography using Kodak NTB-2 liquid emulsion (Eastman Kodak Co., Rochester, NY, USA). Sections hybridized with the corresponding antisense probe mixed with a tenfold excess of cold antisense RNA served as negative controls. Slides were post-stained with hematoxylin and eosin. In situ hybridization experiments were repeated at least three times using independent samples.

Results

**qRT-PCR of the PEA3 family of transcription factors in the mouse uterus**

We set out to determine the steady-state levels of ERM, ER81, and PEA3 in the peri-implantation mouse uterus by qRT-PCR. Gene specific primers were designed as shown in Table 1. Values were normalized to the relative amounts of GAPDH cDNA. The results showed that all three genes showed higher mRNA expression on days 4 and 5 of pregnancy than other samples (Fig. 1). There was no significant difference between RNA samples from day 5 implantation sites and inter-implantation sites. ERM subclone (223 bp)

<table>
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<tr>
<th>Gene</th>
<th>Up primer sequence</th>
<th>Down primer sequence</th>
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<tr>
<td>GAPDH</td>
<td>TGCCCCCATGGTTGAGTG</td>
<td>TGTGGTCATGACCCCCCTCC</td>
</tr>
<tr>
<td>18S</td>
<td>CGCCGCTAGAGGTGAATT</td>
<td>CGAACCTCCGACTTTCGTT</td>
</tr>
<tr>
<td>ERM</td>
<td>AAGAGGTGGCTCGGCCTG</td>
<td>CTTCTGATGAGCCCTTCTT</td>
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<td>ER81</td>
<td>TGAAAATGGAGCCCCCAAT</td>
<td>GCCTGTGTCCTGCAAGGGCT</td>
</tr>
<tr>
<td>PEA3</td>
<td>ATCATGCAGAAGGTGGCTG</td>
<td>CCGGTCAAACTCAGCCTCC</td>
</tr>
<tr>
<td>ERM subclone (223 bp)</td>
<td>CAGTCAGGATGTCGAGAAG</td>
<td>CTCAGTTGAGACCAGAGTCC</td>
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<tr>
<td>ER81 subclone (418 bp)</td>
<td>TTTTGGCTGGCTGGAATT</td>
<td>GCCAGGCAGAATTTGTC</td>
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<tr>
<td>PEA3 subclone (531 bp)</td>
<td>CAGTCCTTTCCTGTCCCCACT</td>
<td></td>
</tr>
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</table>

**ERM was expressed in the mouse ovary and the peri-implantation uterus**

The objective of this experiment was to determine cell type-specific expression of ERM in the peri-implantation (days 1, 4, 5, and 8; day 1 = vaginal plug) mouse uterus. As shown in Fig. 2, ERM was expressed at low levels in the uterus on day 1 of pregnancy. On day 4, heightened expression of ERM was noted both in the epithelium and stroma, suggesting that increasing levels of progesterone is an inducing factor. This was consistent with qRT-PCR results in Fig. 1. During the early phase of implantation on day 5, ERM expression was sustained in the stroma, and endometrial glands showed high expression of ERM. Notably, mesometrial stromal cells exhibited intense ERM expression. On day 8, endometrial expression of ERM decreased, while the developing embryo showed strong expression of ERM mRNA. In cycling mice, ERM mRNA was highly expressed in the ovary with concentrated accumulation in the corpora lutea (Fig. 2). This result suggests a potential role for ERM in ovarian function.

**ER81 was expressed in the vasculature in the maternal decidua**

Expression of ER81 on day 1 of pregnancy was very low to undetectable (Fig. 3). On days 4 and 5 of pregnancy, very low levels of ER81 expression were noted in the uterine stroma, while epithelial cells were negative. On day 8, the accumulation of ER81 mRNA was predominantly evident in the developing vasculature of the mesometrial bed. This result suggests that ER81 is a marker of endometrial angiogenesis. Thus, ER81 may be implicated in subsequent placental development. ER81 expression in the ovary was undetectable by in situ hybridization (data not shown).

**PEA3 was expressed in the developing embryo**

PEA3 mRNA was undetectable on day 1, but was induced at low levels in the stromal cells on days 4 and 5 of pregnancy (Fig. 4). PEA3 expression on day 5 was especially concentrated around the implanting embryo. It was also noted in the blastocyst. On day 8, basal levels of PEA3 expression were observed in the maternal decidua, while it was highly expressed in the embryo proper. ER81 expression in the ovary was undetectable by in situ hybridization (data not shown). This result shows that PEA3 is an embryonic factor from the very early stage of development. Overall, in situ hybridization showed that three members of PEA3 subfamily of transcription factors exhibit distinct cell type-specific expression in the ovary and peri-implantation uterus and embryo during early pregnancy.

**Discussion**

The highlight of the present investigation was that three ETS transcription factors belonging to the PEA3 subfamily exhibited differential cellular localization in the adult mouse ovary and uterus, and the developing embryo...
during early pregnancy. Our expression analyses showed that ERM was expressed in the epithelium and stroma on days 4 and 5 of pregnancy, suggesting that this factor is implicated in the early events of uterine preparation and implantation. The expression of ERM in the corpora lutea suggests its role in ovarian functions. The distinct expression of ER81 in the mesometrial bed on day 8 of pregnancy suggested that it is involved in the establishment of the maternal vasculature for subsequent placental development. PEA3 is apparently an embryonic factor for early embryogenesis. A previous report showed that ERM, ER81, and PEA3 are expressed in many developing organs of different cellular origins during mouse embryonic development (Chotteau-Lelievre et al. 2001). Our results showed two points of overlapping expression: ERM and PEA3 were expressed in the stroma on day 5 and in the embryo proper on day 8 of pregnancy. Expression did not overlap on other days during early pregnancy, suggesting differential roles for these factors in female reproductive functions.

Except for PEA3 in male fertility (Laing et al. 2000), no other member of the ETS transcription factor is implicated directly in the reproductive processes. Previous studies revealed that ERM, ER81, and PEA3 are differentially regulated during mouse embryogenesis, possibly to provide diverse mechanisms of cellular control (Chotteau-Lelievre et al. 2001). ETS-1, another member of the ETS family of transcription factors, has been shown to be expressed in the human endometrium during the menstrual cycle and is implicated in uterine angiogenesis (Fujimoto et al. 2003). ETS-1, along with ETS-2, also exhibit differential expression patterns during early embryogenesis and organogenesis (Maroulakou et al. 1994). Our result supplements these reports that members of the PEA3 subfamily function differentially in early developmental and reproductive events.

ERM, ER81, and PEA3 are highly homologous factors and are known as transcriptional activators. Regulation of these factors at the gene expression level is important for their availability in controlling target genes, while their activity may also be regulated by post-translational modification. Some of the known target genes of members of the PEA3 subfamily are follicle-stimulating hormone receptor, COX-2, stromelysin, osteopontin, matrilysin, and urokinase plasminogen activator (Rorth et al. 1990, Crawford et al. 2001, Howe et al. 2001, Levallet et al. 2001, El-Tanani et al. 2004). ERM, ER81, and PEA3 are highly conserved in their protein sequences and are thus capable of inducing significant activation of target genes via PEA3 consensus on promoters (Howe et al. 2001). It is thus plausible that the PEA3 subfamily of ETS factors function as transcriptional activators of the above-mentioned signaling molecules in reproductive events. Indeed, COX-2 is a critical factor for both ovulation and implantation (Lim et al. 1997). Likewise, stromelysin and other proteases are implicated in female reproductive events (Osteen et al. 1994).
Schatz et al. 1999). It is therefore plausible that genes involved in prostaglandin production and extracellular matrix remodeling are regulated by these transcription factors. We have also described herein that only ERM is detectable in the mouse ovary. Along with previous reports showing that ERM is also expressed in the mouse testis (Hsia & Cornwall 2004), it is possible that ERM is involved in gonadal functions.

Several reports have shown that members of the PEA3 subfamily are dysregulated in mammary tumors (Trimble et al. 1993, Shepherd et al. 2001). All three members show increased expression in mammary tumors, suggesting their roles in breast cancer progression (Shepherd et al. 2001). PEA3 is also associated in tumor progression in ovarian carcinoma (Davidson et al. 2003). In several tumor tissues, PEA3 regulates the expression of matrilysin and metallo-
proteinase-1 (Horiuchi et al. 2003, Yamamoto et al. 2004), suggesting that this factor is involved in tumor invasion by regulating extracellular matrix remodeling.

Our present study has shown that, in the adult mouse ovary and peri-implantation uterus, ERM is a predominant factor in both tissues. We have preliminary findings that ERM-deficient female mice suffer from infertility with yet to be identified reason(s). While specific role(s) of ERM in female reproduction remains to be investigated, our data have provided information about the possible targets of ERM functions and will help in revealing the mechanism of infertility in ERM-deficient mice.

Acknowledgements

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Figure 4 Cell type-specific expression of PEA3 in the peri-implantation mouse uterus. Gene expression in the peri-implantation mouse uterus was assessed by in situ hybridization of paraformaldehyde-fixed frozen sections with 35S-labeled cRNA probes. Darkfield photomicrographs of representative uterine sections are shown at 40 ×, except for day 8 uterine sections which are shown at 20 ×. le, luminal epithelium; s, stroma; myo, myometrium; bl, blastocyst; em, embryo.


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