Expression of the endothelial lipase gene in murine embryos and reproductive organs

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Abstract Endothelial lipase (EL) is a recently discovered member of the triglyceride-lipase family that is involved in plasma HDL metabolism. In this study, we investigated the putative role of EL in mouse reproduction by studying EL gene expression in mouse embryos and adult reproductive organs. PCR analysis revealed that EL mRNA is expressed in mouse embryos on embryonic day 8.5 (E8.5) to E11.5, but not later in development. In situ hybridization studies on E10.5 whole embryos and embryonic sections showed expression of EL mRNA in multiple tissues, although of varying intensity. High expression was found in the neuroepithelium of the brain and the neural tube, the mesenchymal cells between organs, the optic lens and cup, and the otocyst. In adult mice, EL mRNA expression was high in ovaries from pregnant mice but low in ovaries from nonpregnant mice. EL mRNA was also highly expressed in placenta and testes. In situ hybridization studies demonstrated intense EL mRNA staining of lutein cells in corpora lutei in ovaries, of spermatocytes in the late pachytene and diplotene stages in testes, and of principal cells in epididymis. These results suggest that EL, in addition to its effects on plasma lipoprotein metabolism, plays a role in murine reproduction.—Lindegaard, M. L. S., J. E. Nielsen, J. Hannibal, and L. B. Nielsen. **Expression of the endothelial lipase gene in murine embryos and reproductive organs. *J. Lipid Res.* 2005.** 46: **439–444.**

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Efficient systems for the delivery of lipids (fatty acids, phospholipids, and cholesterol) to tissues are essential in mammalian reproduction. Endothelial lipase (EL) is a recently discovered member of the triglyceride lipase family, which also comprises LPL, pancreatic lipase, and HL. These lipases are all capable of releasing free fatty acids from triglycerides and/or phospholipids for cellular uptake. LPL and EL can also convey cellular cholesterol uptake (e.g., by facilitating lipoprotein holoparticle internalization) (1–5). The sequence of EL is 45% homologous to that of LPL and 40% to that of HL, and functional domains such as the catalytic residues, heparin binding sites, and residues involved in disulfide bridge formation are conserved. Nevertheless, the substrate specificity of EL is different from that of LPL and HL. EL displays less triglyceride lipase activity than LPL and HL and mainly hydrolyzes phospholipids in HDL particles (conversely, LPL mainly hydrolyzes triglycerides in very low density lipoproteins) (6). Several studies have established a role for EL in plasma HDL metabolism (7–13). These results have led to the notion of an inverse relationship between EL activity and plasma HDL concentration (14). This effect is probably mediated both by lipolysis of phospholipids and by bridging HDL to heparan sulfate proteoglycans, allowing selective cholesterol ester uptake and/or HDL holoparticle internalization (3, 15, 16).

Previous studies on multiple tissues using Northern blotting found that EL mRNA is expressed in placenta, ovaries, and testis (17, 18), but the cellular expression in these tissues was not described. One of these studies (17) also examined mouse embryos at embryonic day 7 (E7), E11, E15, and E17 and did not detect EL mRNA expression. In the present study, we sought to gain insight into the biology of EL in reproduction by performing quantitative EL mRNA analysis and in situ hybridization studies of mouse reproductive organs. Also, we have analyzed EL mRNA expression in mouse embryos from E8.5 to E17.5 and report robust expression at a narrow period around E10.5.

MATERIALS AND METHODS

Tissue sampling

Tissues used for in situ hybridization. For isolation of RNA and subsequent production of a riboprobe, human full-term placen-

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Abbreviations: BCIP, bromochloroindolyl phosphate; DIG, digoxigenin; EL, endothelial lipase; Ex, embryonic day x; NBT, nitroblue tetrazolium; PFA, paraformaldehyde.

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tal biopsies were collected at the Clinic of Obstetrics, Rigshospitalet, snap frozen in liquid nitrogen, and stored at −141°C. The mothers gave written, informed consent, and the study was approved by the local ethics committee (Ref. KF 01-048/-01). Murine embryos, testes, and ovaries (from 12 day pregnant females) were collected from NMRI mice (M&B, Ry, Denmark). The embryos were separated from placentas and fixed overnight in 4% paraformaldehyde (PFA). Testes were fixed in Stieves fixative, which results in optimal preservation of testis morphology. After overnight fixation of adult mouse testis and of embryos at 4°C, the tissues were subsequently embedded in paraffin and cut into 8 μm and 4 μm sections, respectively. Sections of adult mouse testes were treated with KI/I_2 and sodium thiosulfate pentahydrate to remove Hg from Stieves fixative (19) before further processing. Ovaries were snap frozen in liquid nitrogen, cut into 12 μm thick sections, and stored at −80°C.

Tissues used for PCR analyses. NMRI mouse embryos were collected from at least two mothers at E8.5, E9.5, E10.5, E11.5, E12.5, E13.5, E14.5, and E17.5 (20). Testes were collected from 1–52 day old C3H/He mice (Japan SLC, Shizuoka, Japan) as described (21). Samples from placenta, muscle, testes, duodenum, heart, liver, spleen, brain, kidney, and ovaries from a nonpregnant mouse were collected from adult C57Bl/6 mice (M&B), whereas ovaries from 12 day pregnant mice were collected from NMRI mice (M&B). All tissues were snap frozen and stored at −80°C before RNA isolation.

EL mRNA quantification

Total RNA from CH3/He mouse testes was prepared using the Qiagen RNeasy kit with one-column DNase digest (Qiagen, Hilden, Germany). To obtain sufficient amounts of RNA, several testes from 1-6 day old mice were pooled. From all other tissues used for PCR analysis, total RNA was isolated with Trizol (Invitrogen, Taastrup, Denmark). RNA integrity was ensured by 1% agarose gel electrophoresis, and the RNA concentration was assessed from the absorbance at 260 nm.

First-strand cDNA was synthesized from 1 μg of total RNA with Moloney murine leukemia virus reverse transcriptase (40 units; Roche A/S, Avedore, Denmark) and random hexamer primers in 10 μl reactions. The primers for murine EL (mEL-52, 5′-ACGCACATTCTTTGCATCTG-3′; mEL-32, 5′-ACCCAAGGTGGAAGTCACAG-3′) and human EL (hEL-51, 5′-GTATGCAGGCAACTTCGTGA-3′; hEL-31, 5′-TGATCCCAAGACATCGTTGA-3′) were derived from GenBank sequences NT_082383 and NC_000018, respectively, and purchased from Sigma-Genosys (Pampisford, UK). hEL-51 and hEL-31 span intron 4 in the human EL gene (*LIPG*), whereas mEL-52 and mEL-32 are located in exon 10 of the mouse EL gene. This implies that the mouse EL primers could coamplify contaminating genomic DNA in the cDNA preparations. Nevertheless, we did not see any genomic DNA contamination when quantifying apolipoprotein M gene expression in the same cDNA samples with intron-spanning primers (22). The specificities of the EL PCRs were confirmed by DNA sequencing. The primers for murine β-actin were validated and described previously (23). Real-time PCR analyses were done with a LightCycler and a FAST START DNAmaster SYBR Green kit (Roche A/S). The PCRs (20 μl) contained 2 μl of SYBR Green I mixture, 2–3 mM $MgCl_2$, 10 pmol of each primer, cDNA synthesized from 20 ng of total RNA, and PCR-grade water. The relationship between the time point and the concentration of a mRNA transcript was determined in each run by analyzing the dilution series of cDNA made from 20, 2, 0.2, and 0.02 ng of total RNA from murine placenta (for EL in embryos and EL and β-actin in adult mouse organs) or murine myocardium (for β-actin in murine embryos).

Riboprobes

A fragment (253 bp) of the human EL cDNA was amplified from human placental cDNA with hEL-51 and hEL-31 and cloned into a pCR®-Blunt II Topo® plasmid with the Zero Blunt Topo PCR cloning kit (Invitrogen). The sequence in this human EL cDNA fragment is 87% homologous to the corresponding murine EL cDNA segment, and the human probe was found to specifically detect mouse EL mRNA. For studies of ovarian sections, the plasmid with the human EL cDNA fragment was linearized with appropriate restriction enzymes before synthesis of sense or antisense riboprobes with SP6 or a T7 RNA polymerase, respectively (SP6, number 600152; T7, number 600123; Stratagene, AH Diagnostics A/S, Aarhus, Denmark). For the studies of testis, epididymis, and embryos, the hEL-51 and hEL-31 primers were extended with sequences for T7 and T3, respectively (5′-AATTGTAATACGACTCACTATAGGGTGCAGGCAACTTCGTGAAAG-3′ and 5′-GCGCGCAATTAACCCTCACTAAAGGGTGATCCCAAGACATCGTTGAGTCCA-3′) and used to amplify EL cDNA with flanking T7 and T3 sequences. The EL sense or antisense riboprobes were synthesized with a T7 or a T3 RNA polymerase, respectively (SP6, number 600152; T7, number 600123; Stratagene) and labeled with digoxigenin (DIG).

In situ hybridization

Frozen ovarian sections were used for in situ hybridization studies as described by Larsen et al. (24) with slight modifications: before dehydration, the slides were incubated for 12–16 h at room temperature with anti-DIG alkaline phosphatase (Roche A/S), washed three times in phosphate-buffered saline, pH 7.4, with 0.25% (v/v) Triton X-100 for 5 min, once in 0.1 M TBS, pH 8.0, for 30 min, and once in a predetection buffer (0.1 M TBS, 0.05 M $MgCl_2$) for 10 min.

Sections of mouse embryos and adult testis were used for in situ hybridization studies with a slightly different protocol (25) than that used for the ovarian sections. In brief, after deparaffination, the sections were fixed in 4% PFA, treated with proteinase K (2.5–5 μg/ml; Sigma P-2308), postfixed in 4% PFA, prehybridized for 1 h at 50°C, and incubated overnight with DIG-labeled probes (50°C). Cover slides were removed in 2× SSC, and washing continued in 0.1× SSC with 50 mM EDTA for 3 × 30 min at 58°C.

In both protocols, visualization was done with anti-DIG alkaline phosphatase Fab fragments (109274 ENZO; Roche Diagnostics

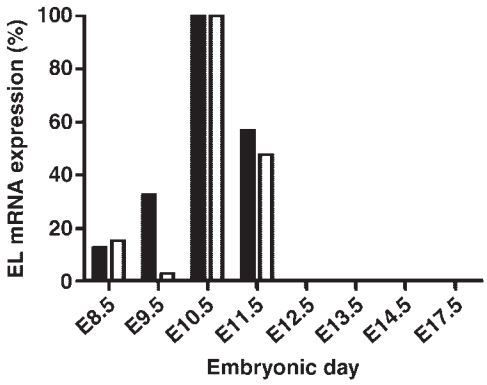

Fig. 1. Time dependence of endothelial lipase (EL) mRNA expression in murine embryos. Values are based on duplicate measurements of EL mRNA from a pool of more than 10 embryos at each time point. Closed bars indicate EL expression when normalized with β-actin; open bars indicate EL expression without normalization. Data are expressed as percentages of expression on embryonic day 10.5 (E10.5).

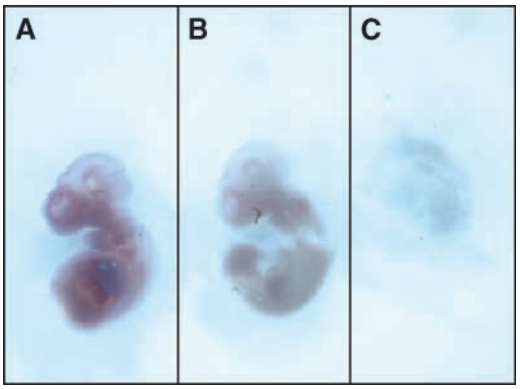

Fig. 2. Whole-mount in situ hybridization of mouse embryos at E10.5. A: EL mRNA visualized with digoxigenin (DIG)-labeled copy RNA antisense probe. B, C: Weak (unspecific) labeling was obtained with the EL sense probe (B), whereas omission of probe showed no signal (C). The whole-mount in situ hybridization studies were done twice on separate occasions with the same result.

GmbH) and the chromogens bromochloroindolyl phosphate (BCIP; Sigma B-8503) and nitroblue tetrazolium (NBT; Sigma N-6876).

For whole-mount in situ hybridization, embryos were fixed overnight in 4% PFA and processed as previously described (26) using EL sense and antisense probes. The probes were hybridized at 50°C, and embryos were incubated with BCIP (Sigma B-8503) and NBT (Sigma N-6876) for 150 min (26).

RESULTS

EL mRNA expression in mouse embryos

Real-time PCR analysis of pools of murine embryos revealed EL mRNA expression at E8.5–E11.5, with the highest expression at E10.5 (**Fig. 1**). Similar results were obtained with and without β-actin normalization. Whole-mount in situ hybridization using the EL copy RNA probe on murine embryos from E10.5 showed widespread staining of the embryos (**Fig. 2A**). There was a faint background staining with the sense probe (Fig. 2B), which may reflect entrapment of the probe in the embryonic bodies, because there was no hybridization of the sense probe in sectioned embryos (**Fig. 3B**). In situ hybridization on sections of whole embryos at E10.5 extended the findings from whole-mount in situ hybridization. The EL mRNA was seen in multiple tissues, although the tissue expression levels were heterogenous (Fig. 3A). There was intense staining in the neuroepithelium of the brain and the neural tube, the mesenchymal cells between organs, the optic lens and cup, and the otocyst. Strong staining was also seen in the primitive myocardium (Fig. 3C) and in cells lining the primitive vessels. In accordance with the real-time PCR results, EL mRNA expression was absent in most tissues in embryos from E13.5 (Fig. 3E), although there was detectable staining of the neuroepithelium of the lateral ventricles in the brain and in the skin over the snout and hind limb (data not shown).

EL mRNA expression in adult mouse reproductive tissues

When analyzing multiple mouse tissues, the EL mRNA expression was highest in ovaries from pregnant mice but low in ovaries from nonpregnant mice. EL mRNA was also highly expressed in placenta and testis (**Fig. 4**).

In accordance with a previous study (17), in situ hybridization studies showed intense staining of corpora lutei in ovaries from pregnant mice (**Fig. 5A**), which confirmed the specificity of the EL probe. In testis, there was intense EL mRNA staining in spermatocytes. Inspection of the

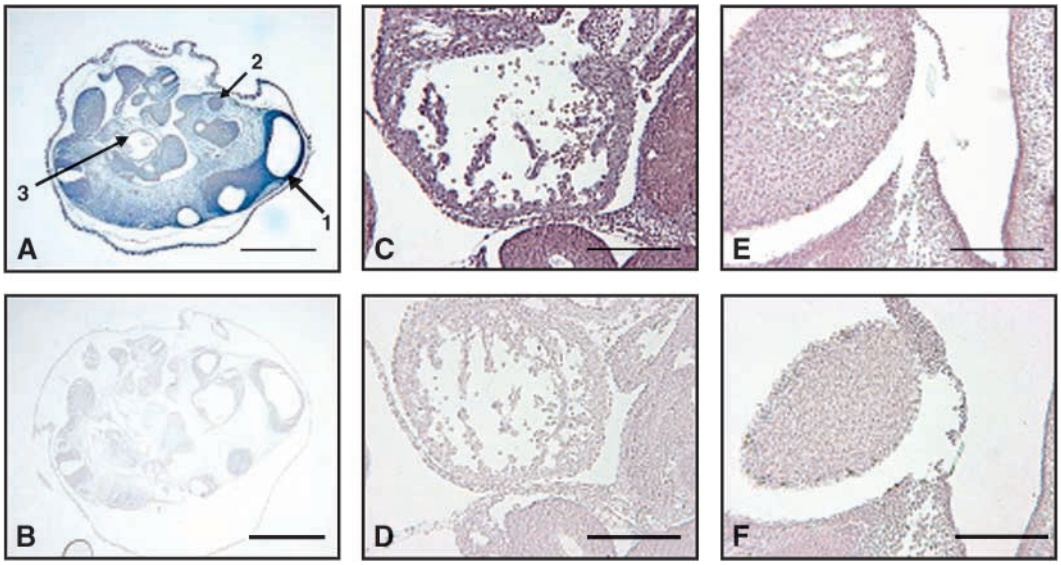

Fig. 3. In situ hybridization studies on sections of whole mouse embryos at E10.5. A and B show consecutive sections treated with DIG-labeled EL antisense and sense copy RNA probes, respectively (bars = 1 mm). Arrows point at neuroepithelium (1), optic cup (2), and myocardium (3). C–F show the primitive myocardium after hybridization of EL antisense (C, E) and sense (D, F) probes on embryos at E10.5 (C, D) and E13.5 (E, F) (bars = 0.2 mm). The in situ hybridization studies were done three times with the same result.

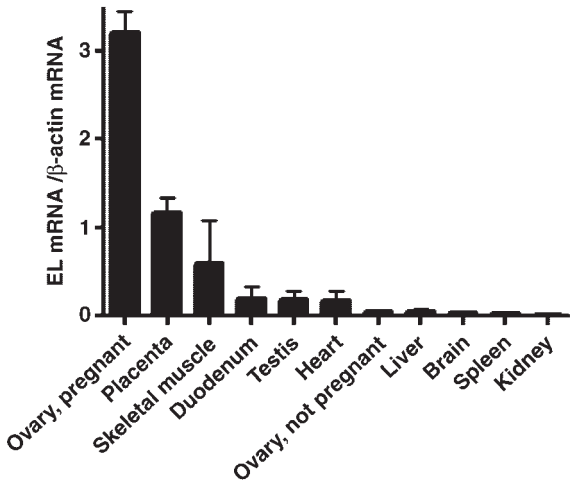

Fig. 4. Relative EL mRNA expression in adult murine tissues. Values are based on duplicate measurements of EL mRNA. In each sample, mRNA expression was normalized with that of β-actin. Values are means ± SEM; n = 3 (liver, kidney, heart, duodenum, skeletal muscle, and spleen from two males and one female), 1 (ovaries), 2 (testis), or 5 (placenta). The overall results were similar without β-actin normalization.

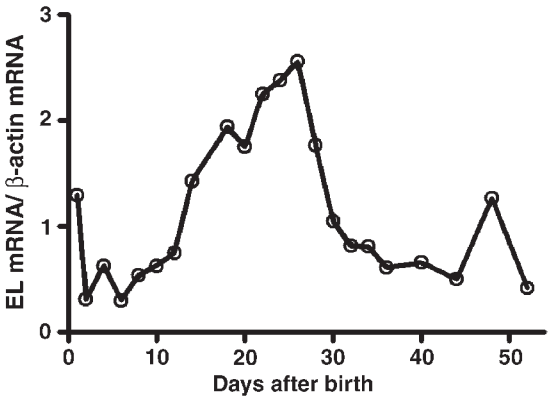

Fig. 6. Testicular expression of EL mRNA from day 1–52 postnatally. Values are based on duplicate measurements of EL mRNA. In each sample, mRNA expression was normalized with that of β-actin. The overall result was similar without β-actin normalization.

stained sections suggested that EL mRNA expression was confined to spermatocytes in developmental stages IX–XI as defined by Russel et al. (27) (i.e., the late pachytene and diplotene stages of meiosis) (Fig. 5E). To verify this result, we analyzed EL expression in mouse testes from 0 to 52 day old mice (21). EL mRNA expression was upregulated from days 16 to 30, with the highest expression at day 26 (**Fig. 6**). Based on previous extensive analysis of stage-dependent gene expression in spermatocytes, this finding also supports the conclusion that EL expression in spermatocytes primarily occurs from the late pachytene stage (21). In addition to spermatocytes, there was expression in principal cells of the epididymal ducts. Interestingly, there was no expression in scattered isolated cells (probably clear cells) in the epididymal epithelium (Fig. 5C).

DISCUSSION

The present data show that EL is expressed in the murine embryo as well as in adult mouse reproductive organs. Hirata et al. (17) did not report EL mRNA using in situ hybridization on mouse embryos at E7 or with Northern blotting of total RNA from mouse embryos at E11. Those results thus supported the idea that EL is not

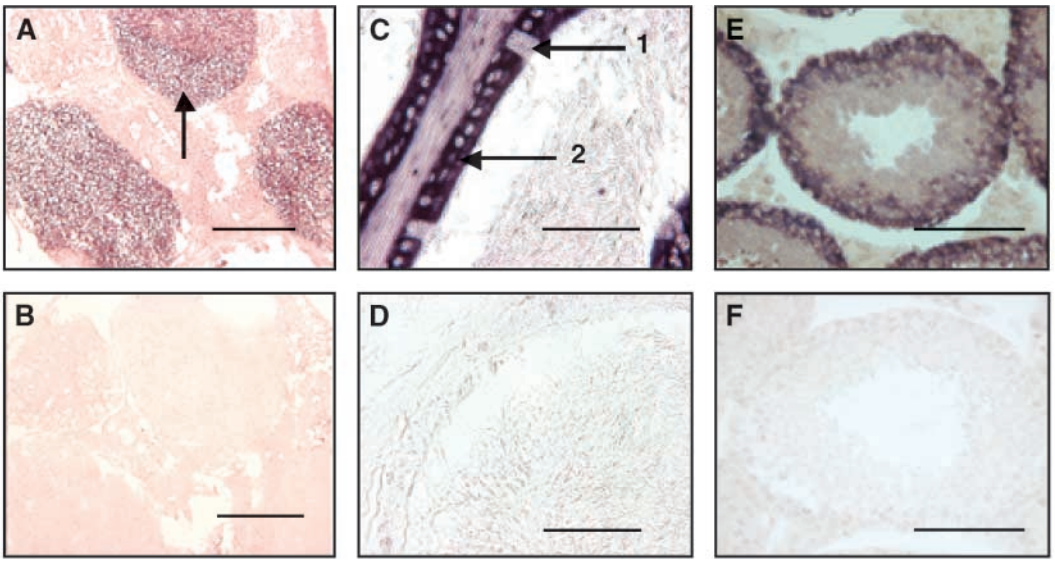

Fig. 5. In situ hybridization studies on sections of ovary, epididymis, and testis. A and B show an ovary from a 12 day pregnant mouse. The arrow points at a corpus luteum (bars = 0.25 mm). C and D show epididymal cells lining the lumen. Arrows point at a clear cell (1) and a principal cell (2) (bars = 0.05 mm). E and F show a testicular tubulus seminiferous at stage IX–XI (bars = 0.2 mm). A, C, and E were treated with the EL antisense probe; B, D, and F were treated with the EL sense probe. The in situ hybridization studies were done three times with the same results.

expressed during embryonic development. However, the present quantitative PCR analyses of systematically collected embryos showed that EL is indeed expressed at a very high level between E8.5 and E11.5. The whole embryo EL mRNA content was actually larger than that in ovaries from pregnant mice. Because whole embryo EL mRNA expression was essentially absent already at E12.5, it is possible that the lack of EL expression at E11 reported previously may be attributable to differences in the determination of conception time or the strain of mouse used [NMRI mice in the present study and Swiss Webster mice in the former (17)]. Importantly, in situ hybridization studies, both on whole-mounted embryos and on embryo sections at E10.5, confirmed the real-time PCR results and showed widespread EL mRNA expression, including in the neuroepithelium, the mesenchyme surrounding internal organs, primitive large vessels, and the myocardium.

The role of EL in embryogenesis is unexplored. The finding of EL mRNA expression in multiple tissues around E10.5 may reflect a high cellular demand for lipids when the embryo enters its exponential growth phase. EL mediates cellular uptake of lipids by lipolysis of phospholipids and by binding lipoproteins to heparan sulfate proteoglycans, allowing selective cholesteryl ester uptake and lipoprotein holoparticle internalization (3). The importance of fatty acids and cholesterol for the development of the embryo in terms of cell membrane synthesis, bile acid formation, development of the central nervous system, and energy requirements is evident and makes the present finding of embryonic EL expression of little surprise. It is interesting, however, that the tissue distribution of EL mRNA in embryos at E10.5 to some extent is similar to that of Sonic hedgehog protein (28, 29). Cholesterol and phospholipids are used in the lipidation of hedgehog proteins (30), which is required for hedgehog signaling and normal development of the fetus. Nevertheless, EL knockout mice survive in utero, implying that EL expression is not crucial for development. A clue to an explanation may come from the observation that adult EL knockout mice have increased expression of LPL in skeletal muscle and of HL in the liver (10). These findings suggest that upregulation of LPL and HL can compensate for a lack of EL. Both lipases are expressed in rodent embryos (31, 32), and it is conceivable that one or both substitute for EL in the EL-deficient embryos.

By quantitative PCR analyses of various tissues, we were able to confirm and complement previously published qualitative data demonstrating EL mRNA expression in adult murine reproductive organs (17, 18) (i.e., placenta, ovary, and testis). These results were obtained in several different mouse strains, and we recently observed EL protein and mRNA expression in human placenta and testicular tubuli (M. L. S. Lindegaard and L. B. Nielsen, unpublished observation), supporting the notion that EL might be biologically important in reproductive organs. The present data reveal that the expression of EL mRNA in ovaries increases dramatically during pregnancy, and in situ hybridization specified that it was located in corpora lutei. After conception, the ovaries supply progesterone to maintain the pregnancy (33). EL could play a role by supplying the lutein cells in corpus luteum with cholesterol for progesterone production. The placenta also displays substantial amounts of EL mRNA (17, 18). In addition to its lipolytic activity, the role of EL in placenta could also involve the uptake of cholesterol for progesterone production. In mice, the giant cells of the placenta become steroidogenic in mid pregnancy (33).

The stage-dependent expression pattern of EL mRNA in mouse testes suggested that mouse testis EL mRNA expression is confined to spermatocytes in the late pachytene and diplotene stages. In mice, the spermatogenic cycle is divided into 12 stages (27). During development from spermatogonia to mature spermatozoa, the cells pass through several mitotic divisions as well as meiosis. The mitotic divisions are incomplete, and cytoplasmic bridges connect sister cells that originate from the same spermatogonia until they finally separate during the pachytene stage of meiosis. It is conceivable that EL activity could be involved in completing the mitotic division of the adjoining sister cells. However, it should be noted that the finding of EL mRNA in spermatocytes does not necessarily reflect EL protein expression, because mRNA translation is extensively repressed during spermatogenesis (34).

EL mRNA was also highly expressed in principal cells in the epididymal ducts. The principal cells are the major source of glycerylphosphoryl choline, which is abundant in semen and believed to be involved in modifying sperm cell motility (35, 36). It has been proposed that phosphatidylcholine from plasma lipoproteins serves as a precursor for glycerylphosphoryl choline production (37). A possible role of EL in the epididymis could involve bridging the phospholipid-containing lipoproteins on the surface of the epididymal principal cells.

In conclusion, the present studies revealed that EL mRNA is highly and widely expressed in murine embryos at a narrow time window (E8.5–E11.5) and that EL mRNA is expressed in substantial amounts in both male and female murine reproductive organs. These findings suggest that EL may play important roles in several aspects of reproduction.

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