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Unique uterine localization and regulation may differentiate LPA3 from other lysophospholipid receptors for its role in embryo implantation

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Abstract

Objective—To determine factors differentiating LPA3 from other nine lysophospholipid (LP) receptors for its role in embryo implantation.

Design—Experimental mouse models.

Setting—Institute/University research laboratories.

Animal(s)—Wild type, *Lpar3*$^{(-/-)}$, *Lpar1*$^{(-/-)}$*Lpar2*$^{(-/-)}$, and *S1pr2*$^{(-/-)}$*S1pr3*$^{(-/-)}$ mice.

Intervention(s)—Ovariectomy.

Main Outcome Measure(s)—Blue dye injection for determining implantation sites on gestation day 4.5. Realtime PCR for measuring gene expression in whole uterus and separated uterine layers. In situ hybridization for detecting progesterone (P)-induced *Lpar3* expression in the uterine luminal epithelium (LE).

Result(s)—Normal implantation was observed in *Lpar1*$^{(-/-)}$*Lpar2*$^{(-/-)}$ and *S1pr2*$^{(-/-)}$*S1pr3*$^{(-/-)}$ females. Temporal expression showed peak expression of *Lpar3* in the preimplantation uterus and constitutive expression of the other nine LP receptors in the peri-implantation uterus. Spatial localization revealed main expression of *Lpar3* in the LE and broad expression of the remaining LP receptors in all three main uterine layers: LE, stromal, and myometrial layers. Hormonal regulation in ovariectomized uterus indicated upregulation of *Lpar3* but downregulation or no effect of the remaining nine LP receptors by P, and downregulation of most LP receptors, including *Lpar3*, by 17β-estradiol (E2).

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Conclusion(s)—LE localization and upregulation by progesterone differentiates LPA3 from the other nine LP receptors and may underlie its essential role in embryo implantation.

Keywords

lysophospholipid receptors; uterine luminal epithelium; progesterone; embryo implantation

Introduction

Lysophospholipids (LPs) are quantitatively minor lipid species that are well known as components in the biosynthesis of membrane phospholipids and as metabolic intermediates (1). A few LPs, mainly lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P), have been proven to function as extracellular signaling molecules through cell surface G protein-coupled receptors (2), including LPA1-5 (LPA_{1-5}) and S1P1-5 ($S1P_{1-5}$) (3–5). Other potential LP receptors have also been reported (6–9) . Several LP receptor-specific *in vivo* functions have been identified in receptor specific knockout mice, e.g., LPA1 in proper craniofacial formation, neural development, and neuropathic pain (10–12); S1P1 in vascular development (13); S1P2 in auditory and vestibular function (14, 15); S1P4 in shaping the terminal differentiation of megakaryocytes (16); LPA1-3 in spermatogenesis (17); and LPA3 in embryo implantation (18–21).

Embryo implantation is a crucial step for the successful establishment of pregnancy in mammals. It requires effective reciprocal signaling between a competent blastocyst and a receptive uterus during a discrete “implantation window”. The “implantation window” is a limited time during which the uterine environment favors blastocyst growth, attachment, and implantation into the uterine wall (22–25). We have previously shown that deletion of *Lpar3* in mice delays embryo implantation and alters embryo spacing. These defects are caused by uterine rather than embryonic loss of *Lpar3*, indicating uterine defects (18). Defective embryo implantation has not been reported in other available LP receptor-deficient females (10, 13, 16, 26–29) and in this study we confirm normal embryo implantation in mice lacking LPA1, LPA2, S1P2, or S1P3. Some LP receptors, e.g., LPA1, LPA2, and LPA3 (30), share high sequence homology and have comparable expression levels in the preimplantation uterus, yet only deletion of *Lpar3* but not of *Lpar1* and *Lpar2* affects embryo implantation. What factors contribute to LPA3 receptor-specific role in embryo implantation Here we evaluate the ten bona fide LP receptors for their temporal expression patterns in the peri-implantation uterus, their localization in the preimplantation day 3.5 uterus, as well as their uterine regulation by ovarian hormones to identify the uniqueness of *Lpar3* among the LP receptors, which may underscore LPA3 receptor-specificity in embryo implantation, especially in the establishment of uterine receptivity. The information from this study can also provide guidance for identifying candidate genes that are potentially involved in the establishment of uterine receptivity.

Material and Methods

Animals

Wild type (WT) and *Lpar1*, *Lpar2*, *Lpar3*, *S1pr2*, and *S1pr3* knockout mice (129/SvJ and C57BL/6 mixed background) were generated and genotyped as described (10, 18, 26, 27, 31). The animal facility is on a 12-hour light/dark cycle (6:00 AM to 6:00 PM) at 23±1°C with 30–50 relative humidity. All methods used in this study were approved by the Animal Subjects Programs of The Scripps Research Institute and the University of Georgia and conform to National Institutes of Health guidelines and public law.

Localization of implantation sites and uterine sample collection

Implantation sites were localized as previously described (18). Uterine tissues from gestation days 0.5, 3.5, and 4.5 WT females between 11:00 AM and 12:00 PM were flash-frozen. Pregnancy status was determined by the presence of eggs and sperms in the oviduct (day 0.5), or blastocysts in the uterus (day 3.5), or implantation sites (day 4.5). Uteri from pregnant WT females were included in the study.

Isolation of three uterine layers

Previously reported procedures were modified to separate luminal epithelium (LE), stromal layer, and myometrium from three gestation day 3.5 WT uteri (32, 33). Briefly, sliced open uterine horns were submerged in 0.5% dispase in calcium- and magnesium-free Hanks balanced salt solution (HBSS, Invitrogen) and digested for 2 hours at room temperature. LE sheets were gently scraped. The stromal layer (Str) with glandular epithelium was isolated with a stronger scraping force. LE and stromal layer were collected for RNA isolation. The remaining myometrium was pulverized in liquid nitrogen for RNA isolation.

Hormonal treatment

Hormonal treatment was administered as previously described (34). Each group included 4–6 ovariectomized females. Uterine horns were flash-frozen.

Realtime reverse transcriptase-PCR (RT-PCR)

RNA isolation and realtime PCR were done as previously described (18, 35). To quantify the relative expression levels of each LP receptor, cDNA product from each primer pair (Supplementary Table S1) was subcloned into pGEM vector (Promega). A standard curve was prepared for each primer pair from serial dilution of the pGEM-cDNA plasmid (10^{-1}~10^{-6} fmol). The relative transcript number of each gene was quantified and then normalized to β-actin as a loading control.

In situ hybridization

It was done as previously described (36, 37).

Statistical analysis

Data were expressed as mean ± SD. Statistical analyses were done using Student's unequal variance t-test. The significance level was set at $p<0.05$.

Results

Expression levels of LP receptors in the day 3.5 uterus

Lpar3 shows the highest expression level in the WT uterus at gestation day 3.5 (18). To compare the relative expression of other LP receptors in the uterus, day 3.5 WT uterus was chosen to quantify the expression levels of *Lpar1-5* and *S1pr1-5*. Figure 1 showed that *Lpar1*, *Lpar2*, *S1pr1*, *S1pr2*, and *S1pr4* mRNA levels were comparable to *Lpar3* level in gestation day 3.5 WT uterus. The expression level of *S1pr3* was about 40% of *Lpar3*. The other three LP receptors, *Lpar4*, *Lpar5*, and *S1pr5*, had significantly lower expression levels (Fig. 1).

Embryo implantation in different LP receptor knockout mice

Since other LP receptors are also highly expressed in the preimplantation WT uterus, to identify other LP receptor subtypes (besides LPA3) that might specifically influence implantation, we evaluated embryo implantation on the available and relevant receptor

knockout mice. Of the ten LP receptors examined, seven (*Lpar1-3* and *S1pr1-4*) were expressed at notably higher levels than the other three (*Lpar4-5* and *S1pr5*) in the day 3.5 preimplantation uterus (Fig. 1). These seven highly expressed LPs have been genetically deleted and mice deficient of six of them (except *S1pr1*) have produced viable offspring: *Lpar1*, *Lpar2*, *S1pr2*, *S1pr3*, *S1pr4*and the previously analyzed *Lpar3*. Embryo implantation was evaluated in females deficient of these LP receptors except S1P4.

Implantation sites were detected at day 4.5 by Evans blue dye in $Lpar1^{(-/-)}$, $Lpar2^{(-/-)}$, $S1pr2^{(-/-)}$, $S1pr3^{(-/-)}$ females, as well as $Lpar1^{(-/-)}Lpar2^{(-/-)}$ and $S1pr2^{(-/-)}S1pr3^{(-/-)}$. On-time implantation was detected in the single knockout mice (data not shown) as well as the double knockout mice (Fig. 2A). The numbers of implantation sites from $Lpar1^{(-/-)}Lpar2^{(-/-)}$ and $S1pr2^{(-/-)}S1pr3^{(-/-)}$ double knockout mice detected at gestation day 4.5 were comparable to that from the WT (Fig. 2B). In addition, normal implantation spacing was observed in these mice, suggesting that these four LP receptors, *Lpar1*, *Lpar2*, *S1pr2*, and *S1pr3*, are not critical for embryo spacing either (Fig. 2A). These observations are contrasting with delayed implantation and embryo crowding in $Lpar3^{(-/-)}$ females (Fig. 2A) (18). These results suggest that LPA1, LPA2, S1P2, and S1P3 are not critical for embryo implantation timing and spacing, in spite of their significant expression levels in the preimplantation day 3.5 uterus.

Receptor mRNA localization in the day 3.5 uterus

Lpar3 mRNA in the uterus is mainly expressed in the LE (18). The LE is the first layer of cells with which a blastocyst communicates prior to implanting in the uterine wall. This LE-specific expression pattern underscores the importance of LPA3 in embryo implantation. To determine if any other LP receptors share this expression pattern, we quantified mRNA levels of the ten LP receptors in the three uterine layers: LE, stromal, and myometrial layers. In addition to *Lpar3*, all of the remaining nine LP receptors were also detectable to some extent in the LE. However, while *Lpar3* was mainly detected in the LE, the remaining nine LP receptors had comparable levels of expression in all three layers (Fig. 3). Transthyretin (TTR) is mainly expressed in the glandular epithelium in the stromal layer (37). *TTR* was used as a marker for stromal layer. Prostaglandin $F_{2\alpha}$ receptor (FP) is mainly expressed in the myometrium of day 3.5 uterus and it served as a marker for myometrium (Fig. 3) (38). These results indicate that *Lpar3* has a unique LE localization that is different from the rest LP receptors examined.

Receptor regulation by ovarian hormones progesterone and estrogen

Ovarian hormones progesterone (P) and estrogen are the master controls of embryo implantation process in mice (39). LP receptor gene expression was therefore assessed for ovarian hormonal influences. It had been demonstrated that *Lpar3* is regulated by both P and 17β-estrodial (E2) (40). To determine the regulation of *Lpar1-5* and *S1pr1-5* by ovarian hormones, prior published hormonal treatment approaches (34) were combined with realtime PCR to quantify the expression levels of LP receptors. *Lpar1* expression was not changed by P (54h) or P (54h)+E2. It was transiently downregulated by E2 only (1h and 6h) (Figs. 4A, S1A). *Lpar2* expression was downregulated by both P and E2 (6h and 24h) (Figs. 4A, S1B). *Lpar3* expression was upregulated by P and downregulated by E2 (6h and 24h). The P-induced upregulation of *Lpar3* could be reversed by E2 treatment for 6h and 24h (Figs. 4A, S1C). *Lpar3* was also upregulated by P after 24h of treatment and this upregulation could be abolished by progesterone receptor (PR) antagonist RU486 (data not shown), indicating the involvement of PR in regulating P-induced uterine *Lpar3* expression. P induced *Lpar3* expression mainly in the LE (Figs. 4B~E). *Lpar4* expression was downregulated by P+E2 (6h) and E2 (6h and 24h), but not P alone (Figs. 4A, S1D). *Lpar5* expression was transiently downregulated by P+E2 and E2 treatments for 6h only (Figs. 4A,

S1E). *S1pr1* expression was decreased only by P+E2 and E2 treatments for 24h, but not affected by P treatment, indicating a main role of E2 in this regulation (Figs. 4A, S2A). *S1pr2* expression was suppressed by E2 treatment at all the time points examined (1h, 6h, and 24h) but not changed by P or P+E2 treatments, indicating that although P did not affect the expression of *S1pr2*, it could block the effect of E2 on *S1pr2* expression (Figs. 4A, S2B). *S1pr3* expression was downregulated by P, P+E2, or E2 (6h and 24h) treatments, indicating that both P and E2 affected *S1pr3* expression (Figs. 4A, S2C). *S1pr4* had a similar response to P and E2 treatments as that of *S1pr3* and *Lpar2* (Figs. 4A, S1B, S2C, S2D). *S1pr5* expression was not affected by either P or E2 (Figs. 4A, S2E). Leukaemia inhibitory factor (*Lif*) was upregulated by E2 and served as a control to demonstrate that the downregulation of LP receptors by E2 was specific (Figs. 4A, S2F) (41). Although LP receptor mRNA levels can be differentially regulated by P and E2, *Lpar3* was the only one upregulated by P treatment (Figs. 4A, S1, S2). The upregulation of uterine *Lpar3* by P is another unique feature among the ten LP receptors.

Temporal expression of LP receptors in the peri-implantation uterus

Lpar3 has its peak expression in the preimplantation day 3.5 WT uterus during pregnancy (18). The expression of *Lpar1-5* and *S1pr1-5* at gestation days 0.5, 3.5 (preimplantation), and 4.5 (post-implantation) in WT uterus was also assessed. Interestingly, only *Lpar3* showed a dynamic expression pattern whereas the other nine LP receptors maintained comparable expression levels in the uterus during peri-implantation (Fig. 1).

Discussion

Laser-capture microdissection coupled with realtime PCR and *in situ* hybridization were previously used to localize *Lpar3* in uterus (18). Both methods gave precise gene localization, but were inefficient for screening large number of genes and were not amenable to simultaneously determining their relative expression levels. The simple method used in this study for separating the three main uterine layers has the advantages of roughly localizing uterine gene expression and quantifying relative expression levels of different genes in the same uterine layers. This approach can be validated using *Lpar3*, *TTR*, and *FP* as markers for LE, stromal layer, and myometrium, respectively (Fig. 3) (18, 37, 38). In addition, about 0.5~1.5 μg of total RNA from gestation day 3.5 LE can be obtained, greatly exceeding that from laser-capture microdissection, which is in 10–150 ng range (42). The precise mRNA localization of candidate genes could be further analyzed using the two previously mentioned techniques (18).

Evidence suggests that the LE plays important roles in embryo implantation, especially the establishment of uterine receptivity: it is the first layer of cells that a blastocyst communicates with during the initial stage of implantation; LE cells develop mid-secretory uterodomes or pinopodes, an attribute of receptive uterine tissue; and the LE acts as a transducer of the embryo's presence to elicit underlying stromal responses (33, 42–45). Therefore, the localization of *Lpar3* in the LE underscores its important role in the establishment of uterine receptivity. All ten LP receptors are detectable in the LE, however, only *Lpar3* shows nearly exclusive expression in the LE. Thus, while LP receptors may have roles in the uterus, only LPA3 has the identified role in the establishment of uterine receptivity (18).

Lpar3 is the only LP receptor upregulated by P-PR, and specifically in the LE (Fig. 4 and data not shown), which may be another critical determinant of its function in the establishment of uterine receptivity. P is a master control of embryo implantation and PR-mediated P signaling is indispensable for embryo implantation (39, 46–48). Results from indian hedgehog (*Ihh*, P-PR target gene) uterine epithelium conditional knockout mice

reinforce the critical role of P-PR signaling in LE for embryo implantation (49–51). However, sustained PR expression in the uterine epithelium beyond the expected "implantation window" is detrimental to embryo implantation (46, 47, 52–55). We have demonstrated that PR remains expressed in the LE before decidualization becomes manifested and disappears from LE afterwards (56), suggesting an active role of PR in the LE during the initial implantation process. The observations that *Lpar3* expression peaks in the preimplantation LE and returns to basal level in the post-implantation uterus (Fig. 1) (18) and *Lpar3* is upregulated by P-PR signaling in the LE (Fig. 4 and data not shown) suggest that these two receptors, PR and LPA3, may communicate with each other in the LE for the establishment of uterine receptivity.

P and E2 differentially regulate LP receptors (Figs. 4A, S1, S2). Surprisingly, the downregulation of LP receptors by P and/or E2 seen in ovariectomized uterus was not observed in the peri-implantation uterus, especially at day 3.5 when the P level is high in addition to an estrogen surge (57). One possible explanation is that the uterine local levels and ratios of P and E2 received in the ovariectomized mice did not mimic the physiological conditions in the peri-implantation uterus. However, uterine *Lpar3* peaks at day 3.5 (Fig. 1) (18), which agrees with the upregulation of *Lpar3* by P in the ovariectomized uterus (Fig. 4). The information obtained from this study, e.g., differential spatiotemporal uterine expression and hormonal regulation, can potentially be used as a guide to identify uterine genes critical for embryo implantation.

LPA1, LPA2, S1P2, and S1P3 are demonstrated to be not individually critical for embryo implantation (Fig. 2). Other reports indicate no implantation defects due to loss of LPA4 and S1P5 (28, 29). There was no mention of implantation defects in the *S1pr4*$^{(-/-)}$ mice either (16). The *in vivo* role of S1P1 in embryo implantation could not be assessed because of embryonic lethality of *S1pr1*$^{(-/-)}$ mice and the lack of uterine-specific *S1pr1*$^{(-/-)}$ mice (13). Although *Lpar5*$^{(-/-)}$mice are not available yet, the lack of distinct spatiotemporal expression patterns in the peri-implantation uterus and its low levels of expression in the peri-implantation uterus (Figs. 1, 3) suggest that LPA5 may not be critical for embryo implantation. Similar implantation defects between *Lpar1*$^{(-/-)}$*Lpar2*$^{(-/-)}$*Lpar3*$^{(-/-)}$ and *Lpar3*$^{(-/-)}$ females (20) reinforces the critical role of LPA3 in the establishment of uterine receptivity. However, these observations do not exclude other potential uterine functions of LP receptors, e.g., S1P1, S1P2, and S1P3 may play a role in decidualization (58).

Two interesting studies suggest that human LPA3 may play a role in the establishment of uterine receptivity (59, 60). Further analyses of human uterine microarray data (identifier GSE6364) (59) indicate that *LPAR3* (*EDG7*) mRNA peaks in the normal early secretory phase endometrium (ESE, preimplantation stage), paralleling data obtained from mouse (18). *LPAR3* mRNA levels are significantly downregulated in the ESE of endometriosis patients, while LPA3 protein levels are marginally downregulated in the ESE and significantly downregulated in the middle and later secretory phase endometrium (59, 60). The differential expression patterns of *LPAR3* in normal and endometriosis patients are not observed in other LP receptors that were included in the database (59). The dysregulation of uterine LPA3 in endometriosis patients suggests that LPA3 may be involved in endometriosis-associated defective uterine receptivity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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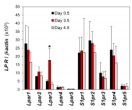

Figure 1.
Expression of LP receptors in gestation days 0.5, 3.5, and 4.5 mouse uterus by realtime PCR. *, P<0.01, compared to day 0.5 or day 4.5. N=3–6. Error bars represent standard deviation.

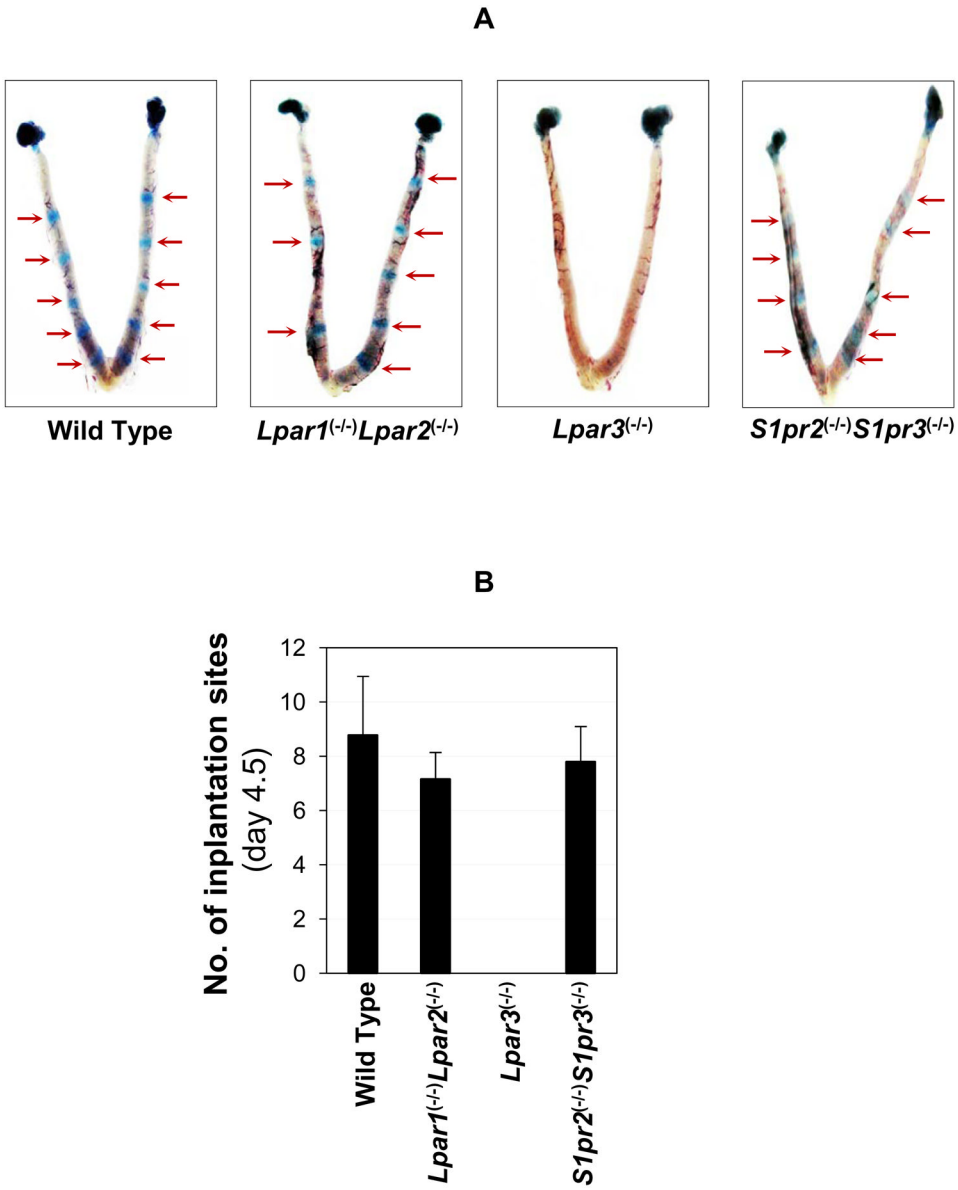

Figure 2.

Detection of implantation sites in wild type, *Lpar1*$^{(-/-)}$*Lpar2*$^{(-/-)}$, *Lpar3*$^{(-/-)}$, and *S1pr2*$^{(-/-)}$*S1pr3*$^{(-/-)}$ females on gestation day 4.5 by Evans blue dye injection. A. Uterine images. Red arrows indicate implantation sites. No implantation sites were detected in the *Lpar3*$^{(-/-)}$ uterus but healthy-looking blastocysts were flushed from the uterine horns (data not shown). B. Number of implantation sites. N=5–9. Error bars represent standard deviation.

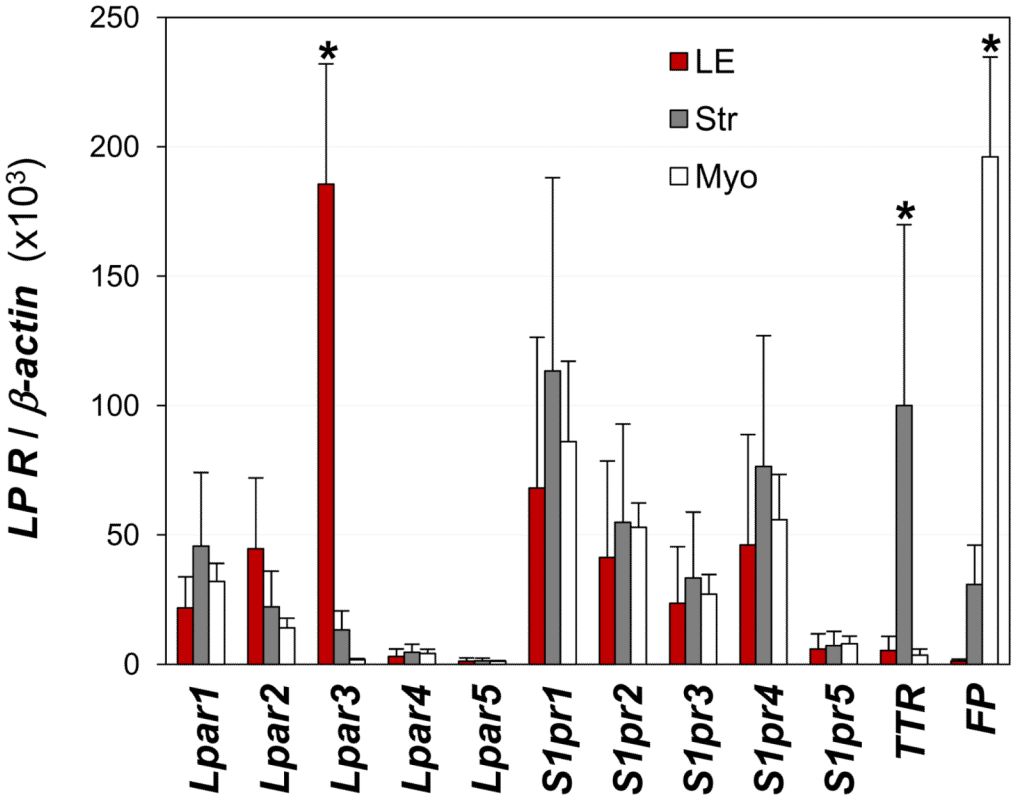

Figure 3.

Localization of LP receptors in isolated uterine luminal epithelium (LE), stromal layer (Str) with glandular epithelium, and myometrium (Myo) from preimplantation day 3.5 wild type uterus by realtime PCR. LE-specific *Lpar3* (18), glandular epithelium-specific *TTR* (transthyretin) (37), and myometrium-specific FP (prostaglandin $F_{2\alpha}$ receptor) (38) were served as a marker for LE, stromal layer, and myometrium, respectively. * $P<0.05$ compared to "Str" and "Myo" for *Lpar3*, "LE" and "Myo" for *TTR*, "LE" and "Str" for *FP*. N=3. Error bars represent standard deviation.

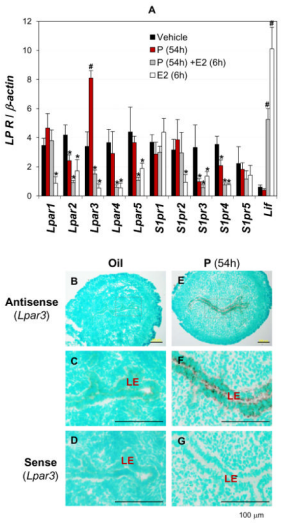

Figure 4.

Regulation of LP receptors by progesterone (P) and 17β-estrodiol (E2) in ovariectomized mouse uterus. A. Effects of P and E2 on LP receptor expression determined by realtime PCR. Wild type virgin females (6 weeks old) were ovariectomized and allowed to recover for two weeks. “Oil”: daily injection of vehicle (0.1 ml sesame oil) for three days, sacrificed 6h after the final injection (54h from the first injection). “P”: daily injection of P (2 mg/mouse, Sigma) for 3 days, sacrificed 6h after the final injection (54h from the first injection). “P+E2”: daily injection of P for 3 days, plus a single dose of E2 (100 ng/mouse, Sigma) on day 3, sacrificed 1h, 6h, and 24h later. “E2”: daily injection of vehicle for two days, plus a single dose of E2 on day three, sacrificed 1h, 6h, and 24h later. A complete set of data is in Supplementary Figures S1 and S2. Arbitrary scale. Leukaemia inhibitory factor (*Lif*), as a control for regulation of LP receptors by E2; β-actin as a loading control. * $P<0.05$ (downregulation) and # $P<0.05$ (upregulation), compared to control “oil”. N=4–6. Error bars represent standard deviation. “54h” indicates the duration of P treatment (54 hours); “6h” indicates 6 hours post E2 injection. In situ hybridization on ovariectomized uteri are shown in B~G. B. *Lpar3* antisense probe, oil-injected. C. Enlarged view of the rectangle area in B. D. *Lpar3* sense probe, oil-injected. E. *Lpar3* antisense probe, P-injected. F. Enlarged view of the rectangle area in E. G. *Lpar3* sense probe, P-injected. The duration of treatment was 54h. The sections were counterstained with 1% methyl green. Specific signal (dark brown) is detected in the uterine luminal epithelium (LE). No specific signals were detected in the negative control using a sense *Lpar3* probe (D, G). Positive control (day 3.5 uterus, *Lpar3* antisense probe) is shown in Supplementary Figure S3. N=3. Scale bar: 100 μm.