Autotaxin–lysophosphatidic acid–LPA₃ signaling at the embryo-epithelial boundary controls decidualization pathways

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Abstract

During pregnancy, up-regulation of heparin-binding (HB-) EGF and cyclooxygenase-2 (COX-2) in the uterine epithelium contributes to decidualization, a series of uterine morphological changes required for placental formation and fetal development. Here, we report a key role for the lipid mediator lysophosphatidic acid (LPA) in decidualization, acting through its G-protein-coupled receptor LPA₃ in the uterine epithelium. Knockout of Lpar3 or inhibition of the LPA-producing enzyme autotaxin (ATX) in pregnant mice leads to HB-EGF and COX-2 down-regulation near embryos and attenuates decidual reactions. Conversely, selective pharmacological activation of LPA₃ induces decidualization via up-regulation of HB-EGF and COX-2. ATX and its substrate lysophosphatidylcholine can be detected in the uterine epithelium and in pre-implantation-stage embryos, respectively. Our results indicate that ATX–LPA₃ signaling at the embryo-epithelial boundary induces decidualization via the canonical HB-EGF and COX-2 pathways.

Keywords autotaxin; decidualization; embryo implantation; LPA₃; lysophosphatidic acid

Subject Categories Development & Differentiation; Signal Transduction

Introduction

Infertility is a global problem experienced by about 10–15% of couples during their reproductive years. In addition, in spite of recent advances in the artificial reproductive technology (ART), the success rate of pregnancy through ART is still low (~30%; Lim & Wang, 2010; Ramathal et al., 2010; Cha et al., 2012). These pregnancy failures are believed to be mainly due to defects in early pregnancy events including implantation and decidualization.

Decidualization is a series of uterine morphological changes during early pregnancy which is essential for the following placental formation and fetal development (Lim & Wang, 2010; Ramathal et al., 2010; Cha et al., 2012). In mice, decidualization occurs only in the vicinity of the embryos. In humans, this process occurs cyclically, whether or not an embryo is present (pre-decidualization), although it is reinforced by embryo implantation (Cha et al., 2012). In the decidual process, the uterine epithelium breaks down, and extensive proliferation and angiogenesis occur in the subepithelial stroma (decidual reactions). The signaling required for proper decidualization is generated through two types of cell-cell interactions, i.e., embryo-epithelial and epithelial-stromal interactions. Maternal factors such as heparin-binding epidermal growth factor (HB-EGF) and cyclooxygenase-2 (COX-2) are induced in the epithelium surrounding the embryos (embryo-epithelial interaction) and then act on the stroma to induce the expression of bone morphogenetic protein 2 (Bmp2) and wingless-related MMTV integration site 4.
Lysophosphatic acid (LPA) has various roles as a lipid mediator through G-protein-coupled receptors (GPCR). So far, six LPA-specific GPCRs named LPA1-LPA6 have been identified (Aikawa et al., 2015; Sheng et al., 2015). LPA is mainly synthesized from lysophospholipids such as lysophosphatidylcholine (LPC) by a secretory enzyme autotaxin (ATX; Umezu-Goto et al., 2002). Studies of knock-out (KO) mice and human genetic diseases of these LPA-related genes have shown that LPA has various pathophysiological roles including angiogenesis (Yukiura et al., 2011), hair follicle formation (Inoue et al., 2011; Hayashi et al., 2015), bone development (Nishioka et al., 2016), and neural development (Yung et al., 2015). We previously showed that LPA3, which is highly expressed in the uterine epithelium during the peri-implantation period (Ye et al., 2005), has a critical role in the early pregnancy. Lpar3 KO mice show many reproductive defects, including significantly reduced COX-2 (a key enzyme for synthesis of prostaglandins), delayed implantation, aberrant embryo spacing, defects in placental formation and fetal development, and reduced litter size (Ye et al., 2005; Hama et al., 2007). However, the defects in Lpar3 KO were only partially recovered by administration of prostaglandins (Ye et al., 2005), suggesting that other unidentified factors should operate downstream of LPA3. In addition, it is almost unclear what kind of cellular events are affected in Lpar3 KO uteri.

In this study, to gain insights into the signaling and cellular events downstream of LPA3, we administered a potent agonist for LPA3 into the mouse uterine cavity during the peri-implantation period. Unexpectedly, mere activation of the epithelial LPA3 by the agonist induced prominent endometrial morphological changes, which were associated with up-regulation of the above-mentioned decidual factors (HB-EGF, COX-2, Bmp2, and Wnt4). Furthermore, we obtained evidences that endogenously LPA3 signaling was evoked by ATX, an LPA-producing enzyme. These results lead us to propose a novel mechanism for decidualization elicited by embryos; that is, the ATX–LPA3 axis in the embryo-epithelial boundary regulates decidualization by inducing maternal factors such as HB-EGF and COX-2.

Results
An LPA3 agonist, T13, induces decidualization

To clarify the molecular mechanisms and cellular events induced downstream of LPA3, we injected T13, a potent LPA3 agonist (EC50 ~0.2 nM; Fig EV1A–C; Tamaruya et al., 2004; Kano et al., 2008; Hama & Aoki, 2010), into the uterine cavities of pseudo-pregnant mice at 3.5 days post-coitus (dpc). Interestingly, T13 induced dramatic uterine hypertrophy throughout the uterine horns at 5.5 dpc (Figs 1A and B, and 2A). The T13-induced hypertrophy was completely absent in the uteri of Lpar3 KO uteri mice (Figs 1A and 2A), indicating T13 evokes uterine hypertrophy through the activation of LPA3, T13 induced several cellular changes, which resembled the changes that occur during decidual reactions in normal pregnancy. At 4.5 dpc, stromal proliferation as judged by bromodeoxyuridine (BrdU) labeling was evident in the stromal cells surrounding the embryo (primary decidual zone; PDZ; Fig 2B, upper row). At 5.5 dpc, the proliferative area expanded outside the

![Figure 1](image-url)
**Figure 2. Activation of LPA<sub>3</sub> evokes decidual reactions.**

A-C Immunostaining of CD31 (angiogenesis, A), BrdU (cell proliferation, B), and E-cadherin (LE-breakdown, C) in T13-treated pseudopregnant uteri showed that T13 induced prominent decidual reactions in an LPA<sub>3</sub>-dependent manner. (A) In T13-injected uteri on 5.5 dpc, fine vascular formation was observed in the AM pole. The CD31<sup>+</sup> area was calculated by ImageJ (right, n = 3 for WT with vehicle and Lpar<sub>3</sub> KO with T13, n = 4 for WT with T13). Data are means ± SEM. *P < 0.01 by ANOVA. (B) T13-treated mice were injected with BrdU on the morning of 4.5 or 5.5 dpc. After 2-h chasing, uteri were dissected. Cell proliferation occurred in the PDZ on 4.5 dpc and then SDZ on 5.5 dpc. Higher magnification images of boxed regions are shown in the lower panels. (C) T13 caused LE-breakdown in the AM pole on 5.5 dpc. In each image, nuclei were counterstained with DAPI (blue). M, mesometrial pole. AM, anti-mesometrial pole. Scale bar: 200 µm (A, B and upper row in C) and 50 µm (lower row in C). Each image is a representative from at least three independent experiments.

Source data are available online for this figure.
PDZ (Fig 2B, lower row). In addition, angiogenesis as judged by anti-CD31 staining was prominent in the stromal layer (Fig 2A). At this time, the luminal epithelium collapsed (LE-breakdown) at the antimesometrial (AM) pole, as shown by E-cadherin staining in T13-treated uteri (Fig 2C). We also confirmed that T13-injected uteri showed high alkaline phosphatase activity which is an indicator of decidualized stromal cells (Appendix Fig S1). LPA3 activation seems to induce some factor(s) in the epithelial layer, which then evoke the decidual reactions in the stromal layer. It should be noted that oil-induced decidualization was similarly observed both in wild-type and Lpar3 KO uteri (Fig EV2), confirming that the intrinsic mechanism for decidualization was not affected in Lpar3 KO uteri. This suggests that LPA does not induce decidualization directly but contributes to the induction of decidualization by up-regulating some decidual factors via LPA3. Accordingly, we concluded that all

Table 1. Numbers of genes up- and down-regulated by at least a factor of two in uteri treated with T13.

<table>
<thead>
<tr>
<th>Type of regulation</th>
<th>No. of genes regulated at time after T13 injection (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up-regulated*</td>
<td>10 10 29</td>
</tr>
<tr>
<td>Down-regulated**</td>
<td>7 8 12</td>
</tr>
</tbody>
</table>

The data have been deposited in the NCBI GEO database (accession no. GSE87116). The following genes are up- or down-regulated in 2 h after the T13 injection. Hbegf and Ptgs2 (in bold), responsible genes for uterine functions, were up-regulated.

*Mup1, Cfr, Vmn1r12/Vmn1r14, Gjb2, A2m, Ptgs2, Vmn2r88, P2yr14, Edn1, Vmn1, Ly6a, Cyp4a111, Gpr6a, Hsd3b1, Csn2, Ifi16, Cyp3a25, Rcan1, Ramp3, Lcn2, Crtac1, mir-15, Hbegf, Sc40, Igf5, Cyp4a54, g, Sirpb1, D2, Egr3.

**Lpin2, Tp53i11, Rnasel, Mcpt4, Ccl5, Sult1a1, Tac3, Ceacam1, Cxc10, Cxc19, Igk-u28, H2-t22.

Figure 3. HB-EGF and COX-2 were highly induced on the epithelial layer in the T13-injected uteri.

Temporal and spatial expression of Hbegf and Ptgs2 mRNAs in T13-injected uteri.

A Time course of qRT-PCR quantification of Hbegf and Ptgs2 mRNAs in T13-injected pseudopregnant uteri (n = 8 for 2 h, n = 4 for 6 and 9 h, n = 5 for 0 and 12–48 h of Hbegf, n = 12 for 6 h, n = 4 for 9 h, n = 5 for 0, 2 and 12–48 h of Ptgs2). Both Hbegf and Ptgs2 were transiently up-regulated after the treatment. Data are means ± SEM.

B Representative ISH images of hbegf and Ptgs2 2 h after the injection. T13 strongly induced both transcripts in the epithelial layer. Higher magnification images of boxed regions are shown in each right panel. Each image is a representative from at least three independent experiments. Scale bar: 200 μm (each left panel) and 50 μm (each right panel).
the decidual reactions (LE-breakdown, stromal proliferation, and angiogenesis) could be induced in the absence of embryos solely by activating LPA₃.

LPA₃ evokes decidual reactions through up-regulation of HB-EGF, COX-2, and Bmp2/Wnt4 signalings

To understand the molecular mechanism underlying T13-induced decidualization, we then performed cDNA microarray analyses. Several tens of genes were up-regulated 2 h after the T13 injection (Table 1). Among the genes, we focused on Hbegf and Ptgs2 (encoding COX-2), because they are responsible for decidualization and knockout of these genes interfered with implantation (Lim et al., 1997; Song et al., 2002; Wang et al., 2004; Xie et al., 2007; Large et al., 2014), as was observed in Lpar3 KO mice (Ye et al., 2005). Both Hbegf and Ptgs2 were transiently induced, peaking at 2–9 h after the T13 injection (Fig 3A). Both genes were predominantly up-regulated in the epithelial layer (Fig 3B). Among the EGF family members, only Hbegf was up-regulated by T13 (Fig EV3). In agreement with the up-regulation of Ptgs2, a lipomics analysis confirmed the increase of PGE₂ and PGF₂α in T13-injected uteri 9 h after the injection. The increase of PGE₂ and PGF₂α were markedly suppressed by the COX-2 inhibitor (Fig EV4), suggesting that these PGs are involved in T13-induced decidualization.

The microarray analysis also revealed that the expressions of several thousand genes (3,220 genes at 24 h, 3,365 genes at 30 h, and 3,949 genes at 36 h) differed by a factor of at least two between T13-injected uteri and the control (Table 2). Ingenuity pathway analysis (IPA) revealed that genes involved in the cell cycle and DNA replication were highly affected by T13 injection (Table 3), which is in agreement with the observation that T13 induced the uterine hypertrophy. We also found that downstream of LPA₃, Bmp2/Wnt4 signaling (Table 2 and Fig 4A) was activated. Bmp2 and Wnt4 are well-known decidual factors acting downstream of HB-EGF signaling (Paria et al., 2001; Lee et al., 2007; Franco et al., 2011; Li et al., 2013; Large et al., 2014). Interestingly, a significant negative correlation of gene expression profiles was observed between T13-injected uteri and uteri null for either Egrf (a target of HB-EGF), Bmp2, and Wnt4 (Large et al., 2014; Fig 4B). By upstream analysis using the IPA, we also found a significant positive correlation between genes affected by LPA₃ and E₂ signaling, the latter of which is important for the endometrial proliferation and the establishment of early pregnancy (Lim & Wang, 2010; Ramathal et al., 2010; Cha et al., 2012; Pawar et al., 2015; Table 4). Conversely, there was a negative correlation between genes affected by LPA₃ and an ERα antagonist (fulvestrant; Table 4). These results are consistent with the recent observation that E₂ signaling is down-regulated in pregnant Lpar3 KO uteri (Diao et al., 2015).

We next performed in vivo experiments to evaluate each signal for the development of T13-induced decidualization. Administration of an EGFR inhibitor (AST1306) reduced the levels of T13-induced Bmp2 and Wnt4 (Fig 4B). A COX-2 selective inhibitor (Celecoxib) also down-regulated both Bmp2 and Wnt4 (Fig 4B), suggesting that, in addition to HB-EGF signaling, epithelial COX-2 signaling is required for the induction of Bmp2 and Wnt4 in the stromal layer. By contrast, fulvestrant decreased the Bmp2 level, but not Wnt4 level (Fig 4B). As expected, neither AST1306 nor Celecoxib decreased the expression of Hbegf and Ptgs2 themselves (Appendix Fig S2). Likewise, fulvestrant failed to affect the expression of Hbegf and Ptgs2 (Appendix Fig S2), indicating that LPA₃ signaling in the epithelial layer was not affected by E₂. The signaling pathways identified above can be inhibited by several factors, including an EGFR inhibitor (AST1306), a COX-2 inhibitor (Celecoxib), an ERα antagonist (fulvestrant), a BMPR inhibitor (LDN193189), or a β-catenin inhibitor (XAV939). Each of these factors strongly suppressed T13-induced decidual events including uterine hypertrophy (Fig 5A), stromal cell proliferation (Fig 5B), angiogenesis (Fig 5E), and LE-breakdown (Fig 5E). These results demonstrate that all these signals were essential for T13-induced decidualization.

ATX–LPA₃ axis endogenously contributes to decidualization

To know the role of endogenous LPA₃ signaling in decidualization, we looked into the decidual reactions in Lpar3 KO uteri. Consistent with the data using the LPA₃ agonist, stromal cell proliferation in pregnant Lpar3 KO uteri was markedly reduced as judged by incorporation of BrdU (Fig 6A and B) and the number of nuclei in the stromal layer (Fig 6C). In addition, Lpar3 KO uteri showed weakened angiogenesis (Fig 6D and E). LE-breakdown was rarely observed in Lpar3 uteri (Fig 6F). All these data suggested that endogenous LPA₃ signaling is important for the development of decidualization. The expressions of Hbegf, Ptgs2, Bmp2, and Wnt4 were also concomitantly reduced (Fig 6G and H), which further explains the reduced decidual reactions in Lpar3 KO uteri.

A remaining question is how LPA is produced in the vicinity of the embryos. It is possible that LPA is derived from embryos and/or uteri. Blastocysts null for either Enpp2 [encoding ATX] or Liph [encoding phosphatidic acid-preferential phospholipase A₁α, (PA-PLA₁α),

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**Table 2. Gene expression profiling of uteri treated with T13.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Time after T13 injection (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24</td>
</tr>
<tr>
<td>No. genes up-regulated &gt; 2 fold</td>
<td>776</td>
</tr>
<tr>
<td>No. genes down-regulated &gt; 2 fold</td>
<td>2,444</td>
</tr>
<tr>
<td>Bmp2 fold increase</td>
<td>2.6↑</td>
</tr>
<tr>
<td>Wnt4 fold increase</td>
<td>1.3↑</td>
</tr>
</tbody>
</table>

The data have been deposited in the NCBI GEO database (accession no. GSE87161).

**Table 3. Top five Molecular and Cellular Functional annotations for differently expressed genes between T13 vs. vehicle-treated uteri 24 h after the injection.**

<table>
<thead>
<tr>
<th>Annotation</th>
<th>No. genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell cycle</td>
<td>282</td>
</tr>
<tr>
<td>Cellular assembly and organization</td>
<td>98</td>
</tr>
<tr>
<td>DNA replication, recombination, and repair</td>
<td>215</td>
</tr>
<tr>
<td>Molecular transport</td>
<td>200</td>
</tr>
<tr>
<td>Cell-to-cell signaling and interaction</td>
<td>247</td>
</tr>
</tbody>
</table>

Functional annotations were analyzed with Ingenuity Pathway Analysis (IPA) software (Qiagen, CA, USA).
another LPA-producing enzyme]) implanted normally (Tanaka et al., 2006; Inoue et al., 2011), suggesting that embryonic ATX and PA-PLA₁₃ are not involved in implantation. In addition, Liph KO female mice showed normal reproductive activity (Inoue et al., 2011). By contrast, the role of maternal ATX remains to be determined, since Enpp2 null mice were embryonic lethal (Tanaka et al., 2006). Anti-ATX staining showed that ATX was predominantly expressed in the uteri and localized throughout the epithelial layer (Fig 7A). Administration of an ATX inhibitor (S15-00826, Fig EV7A–E) into the uterine cavity of pregnant mice resulted in abnormal embryo spacing and implantation failure at 5.5 dpc (Fig 7B–D), as was observed in Lpar3 KO uteri (Ye et al., 2005; Hama et al., 2007). The expression levels of Hbegf, Ptgss2, Bmp2 and Wnt4 were also reduced by the ATX inhibitor (Fig 7E and F). These results indicate that epithelially expressed ATX is responsible for the activation of LPA₃ during the peri-implantation period.

**Discussion**

Our results indicate that LPA is produced in a maternal ATX-dependent manner and present in the vicinity of the embryo, then activates LPA₃ in the epithelial layer (Fig 8). We tried to detect LPA in the eggs as well as in the luminal fluid using LC-MS/MS. While we could not detect LPA in the eggs, small amount of LPA (0.1–0.2 nM) was found in the uterine flushing fluids from the pregnant mice (Appendix Fig S3). Interestingly, LPA with an unsaturated fatty acid (oleic or linoleic acid), a potent ligand for LPA₃ (Bandoh et al., 2000), was detected when the uteri were flushed with the saline containing albumin which is capable of extracting lysophospholipids from outer leaflet of the cells (Okudaira et al., 2014; Appendix Fig S3). LPA was hardly recovered in the albumin-free flushing fluids (Appendix Fig S3), indicating clearly that LPA is present in the extracellular milieu. The estimated egg volume is $\sim 6 \times 10^{-14}$ m³.
Table 4. Upstream analysis 24 h after the T13 injection.

<table>
<thead>
<tr>
<th>Upstream regulator</th>
<th>Molecule type</th>
<th>Predicted activation state</th>
<th>P-value of overlap</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF2</td>
<td>Cytokine</td>
<td>↑</td>
<td>4.43E-21</td>
</tr>
<tr>
<td>EP400</td>
<td>Other</td>
<td>↑</td>
<td>1.95E-14</td>
</tr>
<tr>
<td>E2F1</td>
<td>Transcription regulator</td>
<td>↑</td>
<td>5.49E-13</td>
</tr>
<tr>
<td>E2f</td>
<td>Group</td>
<td>↑</td>
<td>4.18E-10</td>
</tr>
<tr>
<td>Vegf</td>
<td>Group</td>
<td>↑</td>
<td>2.50E-09</td>
</tr>
<tr>
<td>Estrogen</td>
<td>Chemical drug</td>
<td>↑</td>
<td>4.82E-08</td>
</tr>
<tr>
<td>Cephaloridine</td>
<td>Chemical drug</td>
<td>↑</td>
<td>2.29E-07</td>
</tr>
<tr>
<td>FOXM1</td>
<td>Transcription regulator</td>
<td>↑</td>
<td>8.46E-07</td>
</tr>
<tr>
<td>CDKN1A</td>
<td>Kinase</td>
<td>↓</td>
<td>1.91E-23</td>
</tr>
<tr>
<td>1-alpha, 25-dihydroxy vitamin D3</td>
<td>Chemical drug</td>
<td>↓</td>
<td>3.96E-20</td>
</tr>
<tr>
<td>Rb</td>
<td>Group</td>
<td>↓</td>
<td>2.07E-13</td>
</tr>
<tr>
<td>iRGM</td>
<td>Other</td>
<td>↓</td>
<td>1.17E-12</td>
</tr>
<tr>
<td>Fulvestrant</td>
<td>Chemical drug</td>
<td>↓</td>
<td>2.60E-10</td>
</tr>
<tr>
<td>SMARC81</td>
<td>Transcription regulator</td>
<td>↓</td>
<td>1.13E-08</td>
</tr>
<tr>
<td>PTF1A</td>
<td>Transcription regulator</td>
<td>↑</td>
<td>4.94E-08</td>
</tr>
<tr>
<td>BNIP3L</td>
<td>Other</td>
<td>↓</td>
<td>1.08E-07</td>
</tr>
</tbody>
</table>

Biological networks and pathways were analyzed with Ingenuity Pathway Analysis (IPA) software (Qiagen, CA, USA). Estrogen signaling was significantly affected by T13 injection (in bold).

The expression of LPA₃ in female reproductive tissues is conserved in mammals. Indeed, in mouse, sheep, pig and human, LPA₃ was expressed in the uterine epithelial layer in a female sex hormone-dependent manner (Hama et al., 2006; Kamińska et al., 2008; Liszewska et al., 2012; Guo et al., 2013). In addition, ATX and LPA were detected in the reproductive biological fluids such as follicular fluids and uterine luminal fluids (Liszewska et al., 2009; Seo et al., 2012; Yamamoto et al., 2016). Thus, LPA₃ appears to regulate the female reproductive systems in wide range of mammalian species including human, although there are some slight differences in the process of decidualization between species (Cha et al., 2012).
Figure 5.
Our findings may lead to new treatments for infertility. In *in vitro* fertilization (IVF), endometrial thickness is highly correlated with the success rate of implantation and pregnancy (Noyes et al., 1995; Lim & Wang, 2010). Interestingly, patients with recurrent implantation failure in IVF treatment have reduced LPA₃ levels (Achache et al., 2010). Thus, treatment of human females

![Image](https://example.com/image.png)

**Figure 6.** Endogenous LPA₃ signaling regulates decidual reactions in normal pregnancy.

(A–F) Immunostaining of BrdU (cell proliferation on 4.5 dpc, A), CD31 (angiogenesis on 5.5 dpc, D), and E-cadherin (LE-breakdown on 5.5 dpc, F) in pregnant uteri showed that decidual reactions were poorly occurred in Lpar3 KO mice. For BrdU IHC, pregnant mice were treated with BrdU 2 h before the dissection. In each image, nuclei were counterstained with DAPI (blue). Asterisks indicate the position of embryos. (B) Number of BrdU (+) nuclei in the stromal layer per section in [A] was counted by ImageJ software (n = 5 for each bar). (C) Number of nuclei in the stromal layer per section on 5.5 dpc was counted by ImageJ software (n = 5 for WT and n = 7 for Lpar3 KO uteri). (E) CD31(+) area in the stromal layer per section in (D) was calculated by ImageJ software (n = 5), showing reduced angiogenesis in Lpar3 KO pregnant uteri.

(G, H) The expression of *Hbegf*, *Ptgs2*, *Bmp2*, and *Wnt4* were significantly lower in Lpar3 KO pregnant uteri on 4.0 dpc. Arrowheads indicate the position of embryos. (H) qRT–PCR quantification of *Hbegf*, *Bmp2* and *Wnt4* mRNAs in Lpar3 KO pregnant uteri on 4.0 dpc (*Hbegf*, n = 7 for WT and n = 12 for Lpar3 KO uteri) and 5.5 dpc (*Bmp2* and *Wnt4*, n = 7 for WT and n = 9 for Lpar3 KO uteri).

Data information: Scale bar = 200 μm (A and D), 50 μm (F) and 100 μm (G). Each image is a representative from at least three independent experiments. Data are means ± SEM, *P < 0.05, **P < 0.01, by Student’s t-test. Source data are available online for this figure.
with an LPA3 agonist may increase the pregnancy rate by promoting the endometrial cell growth and increasing the endometrial thickness in IVF. This idea is supported by the facts that expression of Lpar3 in mice is up- and down-regulated by P4 and E2, respectively (Hama et al, 2006) and that the balance of female sex hormones is also critical for establishment of pregnancy in both species (Lim & Wang, 2010; Ramathal et al, 2010; Cha et al, 2012).
HB-EGF, COX-2, and Wnt4 have been implicated in the progression of endometriosis, the abnormal growth of endometrial tissues outside the uterus (Ota et al., 2001; Uno et al., 2010; Arosh et al., 2015; Miller et al., 2015). Interestingly, in our mouse endometriosis model, endometrial tissues from \textit{Lpar3} KO mice were significantly less developed (Appendix Fig S6). Thus, LPA–LPA3 signaling is critical in the development of endometriosis. Furthermore, HB-EGF, COX-2, and Wnt4 as well as Bmp2 have been shown to be risk factors for sex hormone-dependent diseases, such as prostatic hyperplasia, breast cancer, and ovarian cancer (Bentley et al., 1992; Ferrandina et al., 2002; Levin, 2003; Zong et al., 2012). Since LPA3 is highly expressed in the prostate, mammary gland, and ovary (Bandoh et al., 1999), LPA3 signaling might contribute to the progression of such diseases.

In summary, we propose a novel mechanism for LPA3-mediated decidualization during the peri-implantation period as illustrated in Fig 8. (i) LPA is produced in the vicinity of the embryo in an ATX-dependent manner; (ii) LPA thus generated activates LPA3 in the epithelial layer; (iii) activation of LPA3 up-regulates HB-EGF and COX-2 in the epithelial layer; (iv) Both Bmp2 and Wnt4 are induced in the stromal layer via activation of EGFR, COX-2, and ER\textsubscript{a}, leading to the decidual reactions including LE-breakdown, stromal cell proliferation, and angiogenesis. Because expression of LPA3 and LPA3 signaling is highly dependent on female sex hormones (P\textsubscript{4} and E\textsubscript{2}, respectively; Hama et al., 2006; Diao et al., 2015), this mechanism may operate not only in female reproduction but also in pathological diseases such as endometriosis and female reproductive cancers.

Materials and Methods

Reagents

LPA (1-oleoyl (18:1)) was purchased from Avanti Polar Lipids. A potent LPA3 agonist T13 was synthesized as described previously (Tamaruya et al., 2004; Kano et al., 2008; Hama & Aoki, 2010). LPA and T13 were dried under nitrogen gas and dissolved in 0.01% fatty acid-free bovine serum albumin (Sigma-Aldrich)-PBS using water bath sonication and stocked in −20°C. COX-2 selective inhibitor (Celecoxib) was from TCI. EGFR inhibitor (AST1306), BMPR inhibitor (LDN193189), and Tankyrase inhibitor (XAV939) were obtained from Adooq Bioscience. Estrogen receptor (ER\textsubscript{a}) antagonist (fulvestrant) was obtained from Sigma-Aldrich. All inhibitors and the ER\textsubscript{a} antagonist were dissolved in DMSO and stored at −20°C. ATX inhibitor (S15-00826) was kindly donated by Shionogi pharmaceutical company.

Antibodies

Anti-autotaxin (ATX) polyclonal antibody (pAb) made in guinea pig was a kind gift from Drs Masahiko Watanabe and Masanori Tachikawa (Hokkaido University, Japan). The following antibodies were purchased from distributors: anti-mouse CD31 (PECAM)
monoclonal antibody (mAb) made in rat (BD Pharmingen); anti-BrdU mAb conjugated with fluorescein made in mouse (Roche); anti-mouse E-cadherin mAb made in Rabbit (CST); anti-guinea pig, anti-rat, anti-fluorescein secondary antibodies (biotinylated; Vector); anti-rabbit secondary antibody-Alexa488 (Invitrogen). ATX, CD31, and BrdU were detected using biotinylated secondary antibody followed by TSA Alexa Fluor 488 kit (Invitrogen).

Mice

ICR mice were purchased from CLEA Japan. Lpar3+/− mice (ICR background) were generated by crossing C57BL/6J Lpar3+/− males with ICR wild-type females over eight times.

TGFα shedding assay

TGFα shedding assay was performed as described previously (Inoue et al., 2012). Briefly, HEK293 cells were seeded in 12-well dishes at a density of 2 × 10^5 cells/dish and cultured at 37°C for 24 h. Then, the cells were transfected with cDNAs encoding alkaline phosphatase (AP)-tagged TGFα (AP-TGFα, 0.25 µg), LPA receptors (each 0.1 µg) using Lipofectamine 2000 as transfection reagent. The cells were seeded in 96-well plate at a density of 4 × 10^5 cells/ml (90 µl) in HBSS containing 5 mM HEPES as transfection reagent. The cells were centrifuged at 190 g for 3 min and supernatant and the cells were measured at OD405. TGFα shedding activity was calculated by OD405 of 0 and 1 h.

Intrauterine injection of T13 and inhibitors

Adult females (8–12 weeks) were mated with vasectomized males. The day a plug was found after mating was designed as 0.5 dpc of pseudopregnancy. At 3.5 dpc, females were anesthetized with three types of reagent cocktail (M/M/B; 0.3 mg/kg of medetomidine, 0.01% BSA-PBS was injected into the uterine cavity adjacent to the ovary. Inhibitors for EGFR, BMPR, or Tankyrase (100 µg/g) were added to the supernatant and the cells, and measured at optical density at 405 nm (OD405). After incubation at 37°C for 30 min, the cells were stimulated with LPA or T13 and incubated at 37°C for 1 h. After 1 h, the cells centrifuged at 190 g for 3 min and supernatant (80 µl) was moved into a new 96-well plate. An amount of 80 µl of 10 mM p-nitrophényl phosphate (pNPP) in 2× pNPP buffer [40 mM Tris–HCl (pH 9.5)], 40 mM NaCl and 10 mM MgCl2]/well was added to the supernatant and the cells, and measured at optical density at 405 nm (OD405). After incubation at 37°C for 1 h, both the supernatant and the cells were measured at OD405. TGFα shedding activity was calculated by OD405 of 0 and 1 h.

Evans Blue solution to detect deciduoma, and uterine weights were recorded to assess the extent of decidualization.

Evaluation of implantation in mice treated with autotaxin inhibitor

Adult female mice were mated with fertile WT males. At 3.5 dpc, females were injected with 5 µl/horn of autotaxin inhibitor (1 mM) or vehicle (3.3% DMSO in PBS) in a similar manner as T13 injection. On 5.5 dpc, mice were injected with 1% Evans Blue solution to visualize embryo implantation sites. The distance between each blastocyst was calculated by National Institutes of Health Image software. The coefficient of evaluation (= the degree of equidis- tance) was calculated by dividing the standard deviation by the mean distance between blastocysts in a horn.

Immunohistochemistry

Uteri were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) at 4°C overnight and embedded in O.C.T. compound (Sakura Finetek). Tissues were cryosectioned at 8 µm. The cryosections were plated on MAS-coated glass slides (Matsunami Glass), blocked in IgG from the same animal species of the secondary antibody for 30 min at room temperature, incubated at 4°C overnight with primary antibodies, incubated with biotinylated secondary antibody and TSA Alexa Fluor 488 kit (Invitrogen) for 5 min, counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) to reveal the nuclei and photographed with a Zeiss LSM700 confocal fluorescence microscope. Number of nuclei and CD31-positive area were calculated by ImageJ (National Institutes of Health). For Figs 2A–C (upper panel), 5B, 6A and D, the brightness and contrast was changed using ImageJ (National Institutes of Health).

Bromo-deoxyuridin (BrdU) in vivo incorporation assay

Females were injected with BrdU (100 µg per kg body weight) on the morning of 3.5 dpc (10:00). The mice were killed 2 h after injection, and the uteri were freshly embedded in O.C.T. compound (Sakura Finetek). The embedded uteri were cryosectioned, and the sections were fixed in methanol for 10 min at room temperature and immersed in 2N HCl at 37°C to denature DNA for immunohistochemical detection.

Quantitative RT–PCR analysis

Total RNA from whole uterine tissues was isolated with a GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich) and then reverse-transcribed with a High-Capacity cDNA RT Kits (Applied Biosystems) according to the manufacturer’s instructions. PCRs were performed with SYBR Premix Ex Taq II (Takara Bio) and were monitored by ABI Prism 7300 (Applied Biosystems). Standard plasmids ranging from 10^3 to 10^8 copies per well were used to quantify the absolute number of transcripts of cDNA samples. The numbers of transcripts were normalized to the number of a house-keeping gene, Gapdh, in the same sample. PCR was performed using the following primers: 5′-TGACCCCAGCTCAGGGAAAG-3′ and 5′-GATCTGTACC; for Areg; 5′-GATCTGTACC; for Bmp2;
RNA in situ hybridization

Uteri were fixed overnight in 4% (w/v) paraformaldehyde at 4°C and then embedded in O.C.T. compound (Sakura Finetek). The slides (Matsunami Glass) and processed for RNA in situ detection using the RNAscope 2.0 High Definition RED kit according to manufacturer’s instructions (ACDbio).

LC-MS/MS analysis of prostaglandins and related lipids

T13-injected uteri were collected 9 h after the injection and processed for LC-MS/MS-based lipidomics analyses as previously described (Arita, 2012). MS/MS analyses were performed in negative ion mode. Arachidonic acid-derived metabolites were identified and quantified by multiple reaction monitoring.

LC-MS/MS analysis of LPA

To obtain a plasma sample, a blood sample was collected 1 h after the administration of ATX inhibitor (20 mg/kg, i.p.) and then centrifuged at 1,500 g for 5 min. Quantification of LPA was performed as previously described (Kato et al, 2016) using the highly sensitive LC-MS/MS system.

Microarray analysis

The quantity and quality of total RNA were determined with an Agilent 2100 Bioanalyzer (RNA Nano 6000) and Nanodrop, respectively. For each of six time points (0.5, 1, 2, 24, 30, and 36 h after the T13 injection) and two conditions (vehicle control and T13-injected), total RNA was prepared from three individuals and pooled. DNA microarray analyses were carried out using Whole mouse genome oligo DNA microarray kit ver2.0 (Agilent Technology, for time points 0.5, 1, and 2 h) and GeneChip Mouse Gene 2.0 ST Array (Affymetrix, for time points 24, 30, and 36 h) according to the manufacturer’s protocol. Up-regulated and down-regulated genes were defined as those whose expression levels were increased and decreased, respectively, by a factor of at least 2. Biological networks and pathways were analyzed with Ingenuity Pathway Analysis (IPA) software (Qiagen, CA, USA). The data have been deposited to the NCBI GEO database, with accession no. GSE87116 (0.5–2 h after the T13 injection) and GSE87161 (24–36 h after the T13 injection). The raw data of uterine-specific Egfr or Wnt4 KO were kindly disclosed by the corresponding author of the previous article (Large et al, 2014). The data of Bmp2 (Lee et al, 2007; accession no. GSE10193), Egr, or Wnt4 KO were analyzed using R (https://www.R-project.org/). Venn diagrams were created by Venny 2.1.0 (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

Pharmacokinetics of T13 and ATX inhibitor

T13 (10 nmol) was injected into uteri, and then, uteri were collected 0, 3, and 6 h after the injection. 20–30 mg of uterine tissues was homogenized in methanol. For the analysis of the ATX inhibitor, the inhibitor was injected to mice p.o. (10 or 30 mg/kg), and then, bloods were collected. Collected samples were processed for LC-MS/MS as previously performed (Okudaira et al, 2014).

Phosphodiesterase activity assay

Phosphodiesterase activity was measured colorimetrically as previously described (Sakagami et al, 2005) with some modifications. Each mouse ENPP protein was transiently expressed in HEK293A cells using Lipofectamine 2000 as transfection reagent. For the analysis of the specificity of ATX inhibitor to ENPP proteins, culture supernatants were incubated with substrates, p-nitrophenyl thymidine monophosphate (4 mM, pNP-TMP, for ENPP1-5) or p-nitrophenylphosphorylcholine (4 mM, pNP-PC, for ENPP6, 7), in the presence or absence of the ATX inhibitor (1 mM) in a 96-well microplate at 37°C for 30 min. The amount of p-nitrophenolate (p-NP) was determined by the absorbance at 405 nm with a SpectraMax190 microplate reader (Molecular Devices). The phosphodiesterase activity was calculated assuming the inhibitor-free absorbance as 100% activity. For the determination of $IC_{50}$ value, ENPP2 protein was incubated with 1 mM pNP-TMP in the presence of ATX inhibitor for 40 min. A four-parameter sigmoid curve for $IC_{50}$ value was fitted to concentration–response plots using GraphPad Prism 6 (GraphPad, USA).

Statistical analysis

The significance of differences between groups was determined by Student’s t-test or a multiway analysis of variance (ANOVA) followed by a Bonferroni post hoc analysis using GraphPad Prism 6 (GraphPad).

Study approval

Mice were maintained according to the Guidelines for Animal Experimentation of Tohoku University, and the protocol was approved by the Institutional Animal Care and Use Committee at Tohoku University (Approval number: 2014PhLMO-018).

Acknowledgements

We thank Shionogi pharmaceutical Co., Ltd for the gift of the ATX inhibitor. We also thank Dr Yasumasa Nishito (Core Technology and Research Center, Tokyo Metropolitan Institute of Medical Science) for DNA microarray analysis. The present work was supported partly by AMED-CREST (Japan Agency for Medical Research and Development, Core Research for Evolutional Science and Technology) for J.A and M.M., PRESTO (Japan Science and Technology Agency, Precursory Research for Embryonic Science and Technology) for A.I., Ministry of Education, Culture, Sports, Science and Technology (MEXT) Grant-in-Aid for Scientific Research for J.A. and M.M.
Author contributions
SA and KK designed the study and carried out most of the experiments. SA wrote the draft of the manuscript. JW, KK, and DS performed the LC-MS/MS experiments. TN, TF, MK, HI, and YY designed the endometriosis experiment and gave many useful comments. YH designed the experiments on decidua reactions. ST, YT, and MM designed and carried out the experiment on DNA microarray. MA designed and performed mediator lipidomics experiment. JC provided the LPA4 KO mice and gave many useful comments. JA supervised all aspects of the study, including experimental design, discussion, data interpretation, and modified the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

References


Noma T, Lemaire A, Naga Prasad SV, Paria BC, Bagchi IC, Taylor RN, Bagchi MK (2001) Cytosolic phospholipase A{alpha} deficiency is crucial for implantation in any medium, provided the original work is distributed under the terms of the Creative Commons Attribution 4.0 License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.