Page 1. Running title

Transthyretin in early pregnancy uterus

Page 2. Title page

Progesterone receptor-mediated upregulation of transthyretin (TTR) in pre-implantation mouse uterus

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Page 3. Capsule

Progesterone receptor mediates the dramatic upregulation of transthyretin (*TTR*), a carrier for thyroxine and retinol, in pre-implantation mouse uterus. *TTR* is localized in glandular endometrial epithelium in day 4.5 uterus.

Page 4. Narrative abstract

Transthyretin (TTR), a carrier for thyroxine and retinol, has its mRNA expressed in the glandular endometrial epithelium and its protein detected in the glandular endometrial epithelium and the uterine lumen. *TTR* mRNA is dramatically upregulated in the pre-implantation mouse uterus as well as progesterone-treated ovariectomized mouse uterus, and in both situations the upregulation of *TTR* is blocked by treatment with progesterone receptor antagonist RU486.

Transthyretin (TTR) is a secreted protein that functions as a carrier for thyroid hormone thyroxine (T4) and retinol (1). Numerous investigations have suggested that TTR is associated with female reproduction under both physiological and pathological conditions. The specific localization of TTR protein in the subapical compartment of villous trophoblasts suggests that placental TTR may involve maternal-fetal thyroid hormone and retinol transport (2). The importance of TTR in placenta is supported by the observation that TTR is down-regulated in the placental villous trophoblasts of early pregnancy loss in human (3). In addition, monomeric TTR, an indication of oxidative stress, is significant increased in the amniotic fluids of patients with preeclampsia (4). Although *TTR*-deficient mice were reportedly to have normal fertility (5), the following observations suggest that TTR may play a role in the early pregnancy. TTR was identified as a protein marker in the receptive stage of endometrium in human (6). TTR is one of the major proteins in uterine flushing whose protein content increases dramatically at implantation in mouse (7). TTR is dysregulated in the post-implantation day 5.5 uterus deficient of cytosolic phospholipase A2α (*Pla2g4a*$^{(-/-)}$) (8), which has delayed uterine receptivity (9). Interestingly, our microarray data indicate dysregulation of *TTR* in the pre-implantation day 3.5 mouse uterus deficient of the third receptor for lysophosphatidic acid (LPA), *Lpar3*, which also has delayed uterine receptivity (10). Dysregulation of LPAR3 has also been implicated in the defective uterine receptivity in women with endometriosis (11).

In this study we used mouse as a model to determine the localization and regulation of TTR in the early pregnancy uterus. Wild type (WT) and *Lpar3*-deficient (*Lpar3*$^{(-/-)}$) mice (C57Bl6/129svj mixed background) were derived from Dr. Jerold Chun's mouse colony at The Scripps Research Institute (10). All methods used in this study were approved by the Animal Subjects Programs of the University of Georgia and conform to National Institutes of Health

guidelines and public law. Previously reported procedures were followed: mating (mating night as day 0), uterine tissue collection, and total RNA isolation (10, 12); microarray analysis (13); in situ hybridization (14); 17β-estradiol (E2) and progesterone (P) treatment (15); and estrogen receptor antagonist and progesterone receptor antagonist treatment (16-20). Realtime PCR reactions were performed in 384-well plates using Sybr-Green I intercalating dye on ABI 7900 (Applied Biosystems). Immunohistochemistry was performed using goat-anti-TTR antibody (1:50, Santa Cruz) and ABComplex/HRP method. Statistical analyses were done using two-tail, unequal variance Student's t test. The significant level was set at $p<0.05$.

Lpar3 expression peaks in the preimplantation day 3.5 mouse uterus and deletion of *Lpar3* leads to delayed uterine receptivity [10]. To understand the molecular mechanism of LPAR3 in uterine receptivity, day 3.5 WT and *Lpar3*$^{(-/-)}$ uteri were screened for differentially expressed genes by microarray analysis, which indicated that *TTR* was down-regulated in day 3.5 *Lpar3*$^{(-/-)}$ uterus (data not shown). To learn more about *TTR* in the early pregnancy, we examined the temporal expression of *TTR* in the whole uterus of both WT and *Lpar3*$^{(-/-)}$ mice during early pregnancy using realtime PCR. *TTR* expression was very low in day 0.5 uterus. It increased a few hundred folds in the uterus from day 0.5 to day 3.5. However, it seemed that from day 3.5 to day 4.5, *TTR* expression dropped slightly but significantly in the WT uterus (Fig. 1A). No significant difference in the *TTR* mRNA levels was detected between WT and *Lpar3*$^{(-/-)}$ uterus at day 0.5 and day 2.5. At day 3.5, *TTR* expression levels were lower in the *Lpar3*$^{(-/-)}$ uterus, which confirmed the microarray results. Interestingly, *TTR* expression levels were higher in the day 4.5 *Lpar3*$^{(-/-)}$ uterus compared to that in the day 4.5 WT uterus and comparable to that of day 3.5 WT uterus (Fig. 1A). In situ hybridization localized *TTR* mRNA expression specifically in the glan-

dular endometrial epithelium (GE) of day 4.5 WT uterus (Fig. 1B). Immunohistochemistry localized TTR protein mainly in the GE and the uterine lumen of day 4.5 WT uterus (Fig. 1C).

It is unknown why *TTR* mRNA levels were decreased in the day 3.5 *Lpar3*$^{(-/-)}$ uterus. Since *Lpar3* is mainly expressed in the luminal endometrial epithelium (LE) while *TTR* is mainly expressed in the GE, the reduced *TTR* mRNA levels in the day 3.5 *Lpar3*$^{(-/-)}$ uterus may not be a direct effect of the lack of *Lpar3* but could be an indirect effect caused by the delayed implantation in *Lpar3*$^{(-/-)}$ uterus [10]. Since reduced or lack of GE are observed in primary decidual zone (Fig. 1B, 1C), the higher *TTR* mRNA levels in the day 4.5 *Lpar3*$^{(-/-)}$ uterus compared to that of day 4.5 WT uterus, which was lower than that in the day 3.5 WT uterus, may reflect reduced GE content in the day 4.5 WT uterus, in which implantation had occurred but not that in day 4.5 *Lpar3*$^{(-/-)}$ uterus [10] or day 3.5 WT uterus. Interestingly, higher TTR expression was also observed in the day 5.5 *Pla2g4a*$^{(-/-)}$ implantation site with deferred implantation (8). These observations raise the possibility that increased TTR expression in the post-implantation uterus may be an indication of delayed uterine receptivity.

The differential temporal expression of *TTR* in the peri-implantation uterus (Fig. 1A) and the previous report that TTR levels increase in the uterine flushing upon embryo implantation (7) suggest hormonal regulation of uterine TTR. In ovariectomized mice, E2 treatment for one hour did not affect *TTR* expression levels, for six hours showed marginal suppression of *TTR* expression, for 24 hours significantly reduced *TTR* levels about 6 folds, and for 74 hours reduced *TTR* levels about 300 folds (data not shown). A positive control *Lif* (Leukemia inhibitory factor), an E2 target gene (21), was dramatically induced by E2 (data not shown), indicating the suppression of *TTR* by E2 was specific. P treatment from 4 to 54 hours significantly induced *TTR* levels (Fig. 1D). In situ hybridization revealed that P-induced *TTR* expression was mainly shown in the GE

(data not shown). However, this induction was abolished upon co-administration of E2 for 6 and 24 hours but not for 1 hour (Fig. 1E). These results demonstrate that *TTR* is coordinately regulated by both E2 and P in the uterus of ovariectomized WT mice.

Fig. 1A showed upregulation of *TTR* in the pre-implantation uterus. To determine the molecular mechanism of *TTR* upregulation in the preimplantation uterus, day 2.5 pregnant WT females were treated with estrogen receptor (ER) antagonist ICI 182 780 or progesterone receptor (PR) antagonist RU486. The uterine tissues were dissected on preimplantation day 3.5. Realtime PCR indicated that ER antagonist ICI 182 780 did not have an effect on *TTR* expression but PR antagonist RU486 reduced *TTR* expression levels about 100 fold in the pre-implantation uterus (Fig. 1F). The dramatic upregulation of *TTR* in the pre-implantation WT uterus (Fig. 1A) is in accordance with the increased secretion of P during the same period of time (22), and this upregulation is PR-dependent but ER-independent (Fig. 1F). Data from another set of ovariectomized mice demonstrated that P-induced uterine *TTR* expression was completely abolished upon pretreatment with RU486 (Fig. 1G), which further confirmed the critical role of PR in P-induced upregulation of uterine *TTR* expression.

The regulation of *TTR* by P signaling in the uterus has also been indicated in another study, which suggests the role of P-SRC-2 (p160 steroid receptor coactivator 2) in *TTR* regulation. Microarray analysis reveals that *TTR* is among the genes that are upregulated by P treatment in ovariectomized WT mouse uterus (23). P-induced upregulation of *TTR* in uterus is attenuated upon deletion of SRC-2 (23), suggesting that *TTR* is under the control of SRC-2. This suggestion is supported by the upregulation of SRC-2 by P and main localization of SRC-2 in the GE, although SRC-2 is also detected in other uterine compartments, such as LE (23). Our results also demonstrate that co-treatment of E2 plus P for 6 and 24 hours in ovariectomized mice prevents

P-induced uterine *TTR* expression (Fig. 1E). These data suggest that E2 counteracts with P-PR signaling to regulate *TTR* expression in the ovariectomized uterus. It is unknown whether this interaction is ER-dependent or ER-independent. We analyzed the 3kb upstream sequence of *TTR* gene using MatInspector (24). There are three putative progesterone response elements and three putative estrogen response elements in this region. The mechanism of transcriptional regulation of *TTR* by PR and ER has not been established.

The detection of TTR protein in both GE and uterine lumen is in line with TTR as a secreted protein, which is one of the major proteins identified in the uterine flushings (6, 7, 25, 26). The secreted TTR protein functions as a carrier for T4 and retinol. Numerous studies have indicated the importance of T4 and retinol in early pregnancy. Uterus is a site with active thyroid hormone metabolism (27), a process regulated by P and E2 during pregnancy (28). Lack of T4 in thyroparathyroidectomized rats significantly reduces the number of implantation sites (~50%) (29). Retinol is required for female reproduction. The enzymes for retinol synthesis and metabolism are differentially expressed in the uterus during estrous cycle and early pregnancy (30). Microarray analysis has demonstrated that the enzymes involved in retinoic acid synthesis and metabolism, e.g., *ADH5*, *Aldh1a1*, and *Cyp26a1*, are induced by P-PR axis (31). Interestingly, retinol-binding protein synthesis is confined to the GE and under P control in baboon (32), similar as that of TTR in mice (Fig. 1B-G). Since TTR is a carrier for T4 and retinol, both of which are associated with embryo implantation (27, 29-31), understanding the localization and regulation of TTR in the early pregnancy uterus will help understand the roles of T4 and retinol in the early pregnancy.

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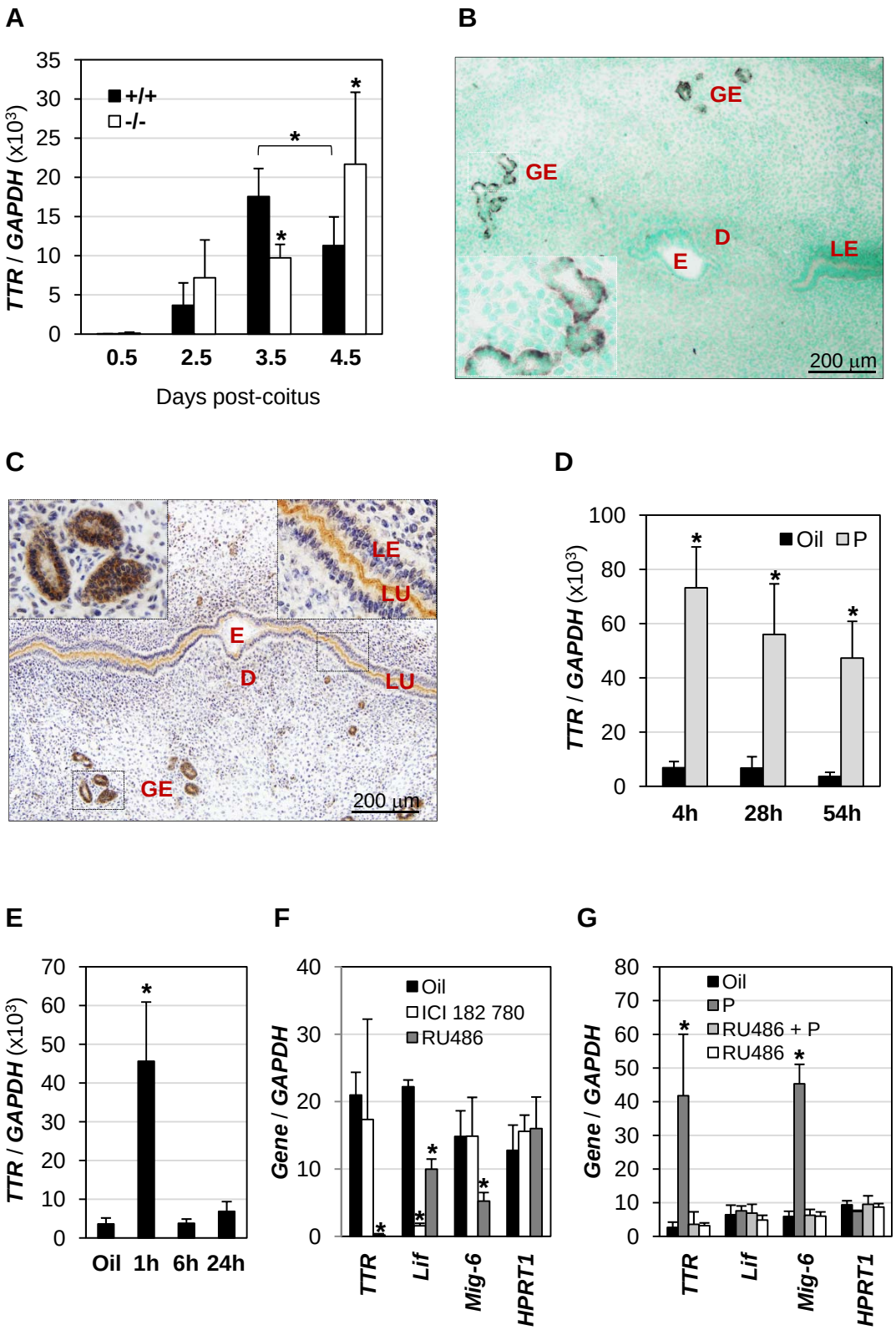
FIGURE LEGEND

Figure 1. A. Expression levels of *TTR* in WT (+/+) and *Lpar3*$^{(-/-)}$ (-/-) peri-implantation uterus using realtime PCR. N=3-10. B. Localization of *TTR* mRNA in day 4.5 WT uterus by in situ hybridization using a *TT*R anti-sense probe. The area in the dotted white rectangle is shown in the left bottom corner. Specific signal (dark brown) is detected in the glandular endometrial epithelium (GE). No specific signals were detected using a sense probe (data not shown). The section was counterstained with 1% methyl green (14). C. Localization of TTR protein in day 4.5 WT uterus by immunohistochemistry. Signals (brown) in the two dotted black rectangles are shown in the left up corner (for GE) and right up corner (for uterine lumen), respectively. No specific signals were detected in the negative control without the primary anti-TTR antibody (data not shown). The section was counterstained with Hematoxylin. B & C: GE, glandular endometrial epithelium; LU, uterine lumen; E, embryo; LE, luminal endometrial epithelium; D, primary decidual zone; scale bars, 200 μm. D. Regulation of *TTR* by progesterone (P) in the uterus of WT ovariectomized mice determined by realtime PCR. E. Effects of P and 17β-estradiol (E2) cotreatment on *TTR* expression in the uterus of WT ovariectomized mice determined by realtime PCR. P was given once each day for three times, then E2 was given once; the uterine tissues were dissected hour(s) post-E2 treatment as indicated. D & E, N=4-6. F. Expression of *TTR* in the pre-implantation WT uterus of mice treated with estrogen receptor antagonist ICI 182 780 or progesterone receptor antagonist RU486 determined by realtime PCR. N=3-4. G. Effect of RU486 on uterine *TTR* expression in ovariectomized mice. N=4-5. F & G, arbitrary scale; controls include (21, 33): *Lif* (Leukemia inhibitory factor), *Mig-6* (Mitogen-inducible gene 6), and *HPRT1* (Hypoxanthine guanine phosphoribosyl transferase 1). A, D, E, F, G: *GAPDH* as a loading control; error bars representing standard deviation; * $P<0.05$.



Diao et al, Figure 1