Title:

Thyroid hormone facilitates *in vitro* decidualization of human endometrial stromal cells via thyroid hormone receptors

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Abstract

Endometrial stromal cells differentiate into decidual cells through the process of decidualization. This differentiation is critical for embryo implantation and the successful establishment of pregnancy. Recent epidemiological studies have suggested that thyroid hormone is important in the endometrium during implantation, and it is commonly believed that thyroid hormone is essential for proper development, differentiation, growth, and metabolism. This study aimed to investigate the impact of thyroid hormone on decidualization in human endometrial stromal cells (hESCs) and define its physiological roles *in vitro* by gene targeting. To identify the expression patterns of thyroid hormone, we performed gene expression profiling of hESCs during decidualization after treating them with the thyroid hormone levothyroxine (LT4). A major increase in decidual response was observed after combined treatment with ovarian steroid hormones and thyroid hormone. Moreover, LT4 treatment also affected the regulation of many transcription factors important for decidualization. We found that type 3 deiodinase, which is particularly important in fetal and placental tissues, was up-regulated during decidualization in the presence of thyroid hormone. Further, it was observed that progesterone receptor, an ovarian steroid hormone receptor, was involved in thyroid hormone-induced decidualization. In the absence of thyroid hormone receptor (TR), due to the simultaneous silencing of TRα and TRβ, thyroid hormone expression was unchanged during decidualization. In summary, we demonstrated that thyroid hormone is essential for decidualization in the endometrium. This is the first *in vitro* study to find impaired decidualization as a possible cause of infertility in subclinical hypothyroidism (SCH)

patients.

Introduction

Decidualization is an essential process in the differentiation of endometrial stromal cells that is accompanied by dramatic changes in cell function and is necessary for embryo implantation and pregnancy establishment. The decidualization process in human endometrial stromal cells (hESCs) occurs in response to ovarian steroids including estradiol-17β (E_2) and progesterone. Primary cultured hESCs are commonly used to investigate the mechanisms of decidualization and endometriosis. These cells express functional progesterone receptor (PR) and estrogen receptor (ER), which regulate tissue responsiveness to cognate ligands (1-4).

The expression of various genes is induced or suppressed during decidualization in hESCs (5-7). Among them, prolactin (PRL) and insulin-like growth factor-binding protein 1 (IGFBP-1) are widely used as markers to evaluate the decidualization status of hESCs in cell-culture based studies (8).

Thyroid hormone is closely associated with female reproduction, as it regulates mechanisms in the pregnancy process including ovulation, fertilization, and implantation (9). Thyroid diseases such as autoimmune thyroid disease are common in women of reproductive age (10); even minor thyroid dysfunction has been reported to affect the pregnancy process (11-14). In terms of reproduction, there is a consensus that hypothyroidism has more major adverse effects on menstrual cycles and fertility than hyperthyroidism, or more generally, thyrotoxicosis (15). Subclinical hypothyroidism (SCH) is a mild form of hypothyroidism defined by elevated thyroid-stimulating hormone with normal free thyroxine levels. In general, ovulation is considered to be maintained under a

state of hypothyroidism (16,17). Nevertheless, SCH is associated with decreased fertility and increased miscarriage rates. However, the mechanism surrounding this remains unclear. Clinically, the effectiveness of thyroid hormone replacement for SCH in infertile women has been shown (18-20). The guidelines of the American Thyroid Association, issued in 2017, strongly recommend that women with SCH undergoing *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) should be treated with levothyroxine (LT4) (21).

Considering the effects of thyroid hormone on hESCs, it is essential to understand its interactions with thyroid hormone receptor (TR) and iodothyronine deiodinase (DIO). There are two principal thyroid hormones: thyroxine (T4) and triiodothyronine (T3). T4, a major product of the thyroid gland, exerts its physiological activity by converting to T3 in peripheral tissues to regulate target genes by binding to nuclear receptors (22). Then, deiodinase enzymes regulate the intracellular availability of thyroid hormones and play an important role in regulating the local control of tissue thyroid hormone concentrations (23). There are three DIOs; DIO1, DIO2, and DIO3. DIO1 converts T4 to either T3 or an inactive form of T3, 3,3,5′-triiodothyronine (reverse T3, rT3); DIO2 converts T4 to T3; and DIO3 converts T4 to rT3 (9). The expression of TRs and DIOs in the human endometrium throughout the menstrual cycle has already been reported (24). A recent report demonstrated that DIO3 is strongly expressed while DIO2 is clearly down-regulated during *in vitro* decidualization in mice (25). This evidence suggests that thyroid hormone directly regulates the implantation process. However, few studies have focused on the role of thyroid hormone on decidualization in hESCs.

To date, only one report has demonstrated that progesterone regulates TR expression in the human endometrium via PR (26). Here, we hypothesized that thyroid hormone promotes decidualization by regulating the expression of PR. This study, thus, aimed to investigate the role of thyroid hormone in human endometrial decidualization mechanisms using an *in vitro* model.

Materials & Methods

Reagents

Details of the reagents used are given in a Key Resources Table (Supplementary Table 1) (27).

Tissue collection

Human tissues were obtained with written informed consent from each patient in accordance with the Declaration of Helsinki. Human endometrial tissues were obtained from twenty-four women in the proliferative phase, aged 32–49 years, with regular menstrual cycles and no preoperative hormonal treatment, who underwent hysterectomies due to uterine fibroids. Thyroid function tests were not performed on the subjects during the perioperative period. However, clinical findings associated with thyroid dysfunction, including goiter, were not observed. A portion of each endometrial specimen was analyzed and confirmed to be histologically normal. This study was approved by the institutional review board of Kansai Medical University, Osaka, Japan (project approval number 2006101).

Culture of human ESCs

The hESCs were purified using the standard enzyme digestion method described previously (28). ESCs were cultured in DMEM/F-12 supplemented with 10% fetal bovine serum (Sigma Aldrich, St Louis, MO, USA), 100 IU/mL penicillin, 100 mg/mL streptomycin, and 0.25 μg/mL amphotericin B (Antibiotic-Antimycotic 100×; Gibco, Waltham, MA, USA) at 37 ℃ in a humidified atmosphere of 5% CO_2. The culture medium was replaced 60 min after plating to minimize epithelial cell contamination. The percentage of vimentin-positive cells in confluent ESCs was confirmed to be > 99% according to immunohistochemical staining, as described previously (29). To negate the effect of endogenous steroid hormones, cells were cultured until confluence and then the medium was replaced with phenol red-free DMEM/F-12 supplemented with 10% dextran-coated charcoal stripped (DCS)-fetal calf serum (FCS), 100 IU/mL penicillin, 100 mg/mL streptomycin, 0.25 μg/mL amphotericin B (Antibiotic-Antimycotic 100×; Gibco), and 2 mmol/L L-alanyl-L-glutamine (GlutaMAX; Gibco). After 48 h, ESCs were washed and cultured in DCS-FCS supplemented with E_2 (10^{-8} mol/L; Wako, Osaka, Japan) and medroxyprogesterone acetate (MPA; 10^{-7} mol/ L; Sigma Aldrich) or ethanol as the vehicle control. The hESCs were treated with LT4 (Sigma Aldrich) at various concentrations (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} mol/ L), or left untreated, in addition to treatment with ovarian sex steroid hormones. The culture medium was changed every 3 day for 12 days. Experiments with hESCs from each woman were performed in triplicate.

May-Grunwald Giemsa Staining

For morphology assessments, each sample was stained using the May-Grunwald Giemsa staining technique. Trypsinized hESCs were re-plated on Lab-Tek chamber slides (Thermo Fisher Scientific, Waltham, MA, USA). Cells were stained with a Diff-Quik kit (Sysmex, Hyogo, Japan) according to the manufacturer's instructions.

Cell proliferation assay

Cell proliferation was determined using the Cell Counting Kit-8 (CCK8) assay, which tracks the reduction of the tetrazolium salt WST-8 in live cells to assay relative proliferation rates (Dojindo, Kumamoto, Japan). Briefly, hESCs were seeded at 5000 cells per well in 96-well plates and pre-cultured for 24 h in phenol red-free DMEM containing 10% FCS. The following day, cells were treated with E_2, MPA, and LT4, and incubated for 48 h at 37 °C in a 5% CO_2 incubator. Then, 10 μL CCK8 reagent was added to each well with 100 μL culture medium and ESCs were incubated for 1 h. Absorbance was measured at a wavelength of 450 nm. All values were collected for absorbance read from blank control samples containing medium with no cells.

Semi-quantitative RT-PCR

Total RNA was isolated from cultured ESCs using the RNeasy Minikit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. The first-strand cDNA synthesis kit ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan) was used for cDNA synthesis. Reverse transcription was performed according to the manufacturer’s instructions.

178 Semiquantitative RT-PCR (qPCR) was performed using Rotor-Gene Q HRM (Qiagen) and
179 the Thunderbird SYBR qPCR Mix kit (Toyobo), according to the manufacturer's
180 instructions. The RT-PCR efficiency (E%) for the amplification of each gene was calculated
181 using the following formula: $E\% = [-1 + 10(-1/\alpha)] \times 100$, where α is the slope of the
182 corresponding amplification plot (30). For relative quantification, data were normalized
183 against elongation factor-1α (*EF-1α*) as an internal control. The primer sequences were
184 validated and are listed in Table 1.
185
186 **Prolactin and insulin-like growth factor-binding protein 1 expression by ELISA**
187 The release of prolactin (PRL) and insulin-like growth factor-binding protein 1 (IGFBP-1)
188 into the culture supernatant was measured using the relevant DuoSet ELISA kit (R&D
189 systems, Minneapolis, MN, USA) (31, 32) according to the manufacturer's instructions.
190 The intra- and inter-assay coefficients of variation for PRL in cell culture supernatants were
191 3.8% and 4.8%, respectively. Similarly, those for IGFBP-1 in cell culture supernatants were
192 8.8% and 8.1%, respectively.
193
194 **Immunoblot analysis**
195 Whole-cell lysates were prepared using ice-cold lysis buffer containing mammalian
196 protein-extraction reagent from M-PER (Thermo Fisher Scientific) with protease inhibitor
197 cocktail (Millipore, Burlington, MA, USA). Samples were centrifuged at 10,000 × *g* to
198 sediment the cell debris, and the supernatant was used for subsequent immunoblotting
199 experiments. Protein concentrations were determined using the bicinchoninic acid assay

(Thermo Fisher Scientific), according to the manufacture-recommended procedure. 20 μg of protein was loaded onto and resolved using Any kD™ precast SDS-PAGE gel (Bio-Rad Laboratories, Hercules, CA, USA). After electrophoresis, proteins were transferred to membranes using a semi-dry transfer system. The membranes were treated with blocking buffer (Nakarai Tesque, Kyoto, Japan) for 30 min at room temperature (23 ℃). The membranes were then incubated overnight at 4 ℃ with the corresponding primary antibodies: anti-thyroid hormone receptor alpha (1:1000; Abcam, Cambridge, UK) (33), anti-thyroid hormone receptor beta (1:500; Abcam) (34), anti-β-actin (1:5000; Sigma Aldrich) (35). After 1 h of incubation with appropriate horseradish peroxidase-conjugated secondary antibodies: donkey anti-rabbit IgG (GE Healthcare Life Sciences, Little Chalfont, UK) (36) and sheep anti-mouse IgG (GE Healthcare Life Sciences) (37), the signal was developed using enhanced chemiluminescence reagent (GE Healthcare Life Sciences).

Immunoprecipitation analysis

Immunoprecipitation (IP) was carried out using an immunoprecipitation kit (Protein G; Sigma Aldrich) according to the manufacturer's instructions. Briefly, cultured cells were lysed in IP lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, one cocktail). After homogenization, the lysates were centrifuged to sediment the cell debris at 4 °C. Each resulting supernatant sample was exposed to 2 μg mouse monoclonal anti-PR antibody (Santa Cruz Biotechnology, Dallas, TX, USA) (38) and incubated overnight at 4 °C with gentle shaking. A homogenous protein G agarose

suspension was then added to each sample and incubated for 3 h at 4 ℃. Immunocomplexes were collected by centrifugation at 12,000 × *g* for 20 sec and the supernatant was removed. The complexes were then washed five times in wash buffer. Proteins immunoprecipitated from the beads were subjected to 7.5% SDS-PAGE, and then electro-transferred to immuno-blot polyvinylidene difluoride membranes (Bio-Rad Laboratories). Immunoblotting was then performed with anti-PR antibody (1:200; Santa Cruz Biotechnology) (38). Following overnight incubation at 4 °C, immunoblots were washed in Tris-buffered saline with 0.05% Tween 20 (TBST), incubated with horseradish peroxidase-conjugated anti-mouse secondary antibodies (1:5000; GE Healthcare Life Sciences) (37), and visualized using the enhanced chemiluminescence (ECL) Prime Western Blotting detection reagent (GE Healthcare Life Science).

Immunocytochemistry

After incubation with or without each hormone for 12 days, the medium was removed from each sample and the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline solution (PBS) for 15 min at room temperature. After washing three times with PBS, the fixed cells were blocked with 5% bovine serum albumin and 0.3% Triton X-100/PBS for 1 h and incubated with a primary antibody targeting DIO3 (Novus Biologicals, Centennial, CO, USA) (39) overnight at 4 ℃. After washing three times with PBS, cells were incubated for 90 min with Alexa Fluor dye-coupled anti-rabbit secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) (40). The unbound secondary antibody was removed by washing with PBS three times for 15 min each. Next, the samples were

counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Southern Biotech, Birmingham, AL, USA) and analyzed under a Leica AF7000 fluorescence microscope. Fluorescently labeled cells were then quantified by ImageJ and the fold increase was calculated relative to the control group.

Gene silencing of thyroid hormone receptors

The silencing of TR expression was performed with siRNA preparations directed against distinct areas of the gene sequence (Human Stealth Select RNAi; Thermo Fisher Scientific). The transfection of stealth siRNA targeting TRα (THRAHSS 186327) and TRβ (THRBHSS 110745), as well as a negative control scramble siRNA (HSS12935112; Thermo Fisher Scientific) was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

RNA sequencing

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen) and processed using the TruSeq Stranded mRNA Sample Prep Kit (Illumina, San Diego, CA, USA). Poly(A) RNA libraries were then constructed using TruSeq Stranded mRNA Library Preparation Kit (Illumina) and sequenced at 100-bp paired-ends on an Illumina HiSeq 2500 platform. Sequencing data in FASTQ format were deposited in the DDBJ Sequence Read Archive (accession numbers DRR190745 to DRR190750). RNA-seq was performed in duplicate.

Transcriptome analysis

All FASTQ files were analyzed using ikra (41) v1.2.0 RNA-Seq pipeline centered on Salmon workflow (https://github.com/yyoshiaki/ikra). FASTQ files were aligned to the Gencode release 30 (GRCh38.p12) reference genome with Salmon to generate scaled TPM (transcripts per kilobase million) measurements and tximport to load them at the gene level. For differential expression analysis and pathway analysis, the integrated web application iDEP (http://ge-lab.org/idep/) was used. Details of this analysis are given in the Supplementary Information.

Statistical analysis

Data are presented as the mean ± SD. Statistical analyses were performed using one-way ANOVA followed by Wilcoxon signed-rank test or non-parametric Wilcoxon rank sum test within groups, following normalization of the data with JMP 12. $P < 0.05$ was considered statistically significant.

Results

Effects of thyroid hormone on ovarian steroid hormone-induced decidualization in hESCs

To investigate the effects of thyroid hormone treatment on decidualization, the expression of decidual-specific factors and decidual markers, including *PRL* and *IGFBP-1*, was analyzed (Fig. 1A, B). We also examined the mRNA expression of heart and neural crest derivatives-expressed transcript 2 (*HAND2*), a transcription factor expressed in the early

stage of decidualization (Fig. 1C). Treatment with the ovarian hormones E_2 and MPA induced the mRNA expression of *PRL, IGFBP-1*, and *HAND2*. Similarly, LT4 treatment increased the expression of *PRL*, *IGFBP-1*, and *HAND2* to a greater extent than that induced by ovarian hormone treatment alone. However, LT4 treatment alone did not induce the expression of these transcripts. Next, we investigated the protein expression of these factors in the culture supernatant. PRL and IGFBP-1 secretion was enhanced by LT4 (Fig. 1D, E). LT4 treatment facilitated the increased expression of decidualization markers induced by ovarian steroid hormones. Next, we performed a time-course study of changes in the mRNA expression levels of *PRL*, *IGFBP-1*, and *HAND2*. The expression of *PRL* and *HAND2* was increased by ovarian hormone and thyroid hormone treatment group after 3 days of decidualization, and was present at levels usually found after 12 days (Fig. 1F, G). The same tendency was observed for *IGFBP-1*, although there was no significant increase in expression ($P = 0.67$) (Fig. 1H).

Effect of thyroid hormone on cell morphology

Decidualized stromal cells are known to change into a characteristic morphology. The hESCs treated with ovarian steroid hormones for 12 days showed characteristic morphological changes from elongated spindle-shaped cells to enlarged polygonal cells. The decidualized hESCs retained enlarged nuclei and an increased cytoplasm compared to ESCs treated with the vehicle control (Fig. 2A–D). LT4 treatment in addition to ovarian hormones further enhanced these characteristic changes (Fig. 2E, F). LT4 treatment alone did not induce such morphological changes characteristic of decidualization, and showed a

spindle-like shape as in the control (Fig. 2G, H). Thus, the effect of thyroid hormone during decidualization was confirmed in terms of cellular morphology.

Dose-dependent effect of LT4 on expression of decidualization markers

Next, we examined the dose-response effects of LT4 on the expression of decidualization markers. As shown in Fig. 3A, the expression of *PRL* mRNA was enhanced by LT4 in a dose-dependent manner at concentrations of up to 10^{-5} mol/L. It is noteworthy that the physiological concentration of thyroid hormone T4 is 10^{-7} mol/L (42-44).

Effect of thyroid hormone treatment on cell proliferation

We further determined whether thyroid hormone plays a role in the proliferation of hESCs. Even in the absence of each hormone treatment, hESC proliferation increased in a time-dependent manner until 48 h (Supplementary Fig. 1A) (27). Ovarian steroid hormone treatment and combined treatment with thyroid hormone showed no effect on cell proliferation (Supplementary Fig. 1B, C) (27).

Thyroid hormone receptor expression in hESCs

Alternative splicing of the *TRα* gene generates two mature mRNAs encoding two proteins: TRα1 and TRα2. There also are two TRs derived from the *TRβ* gene: TRβ1 and TRβ2 (45). RT-PCR analysis revealed the expression of *TRα1*, *TRα2*, and *TRβ1* mRNA in cultured hESCs (Fig. 3B–D). In contrast, *TRβ2* mRNA was not detected. During the process of decidualization, the treatment with ovarian steroid hormones had no obvious effect on

TRα1, *TRα2*, and *TRβ1* expression. Similarly, additional thyroid hormone treatment did not affect their expression levels. Further analysis of protein levels using anti-TRα antibody and anti-TRβ confirmed the protein expression similar to mRNA in hESCs (Fig. 3E, F).

Ovarian steroid hormone receptors expression in hESCs

Subsequently, we investigated the expression of two isoform of PR (PR-A and PR-B) during decidualization. PR-A and PR-B are transcribed from different promoters of the same gene, and PR-A is a splice variant that lacks the N-terminal region of the full-length PR-B. Combined *PR-A/B* mRNA expression was analyzed, because *PR-A* mRNA levels cannot be directly measured by quantitative RT-PCR. Ovarian hormone treatment induced the expression of *PR-AB*, and LT4 treatment further promoted the up-regulation of *PR-AB* mRNA expression (Fig. 4A). During decidualization, the expression of *PR-B* was also increased, similar to *PR-AB* (Fig. 4B). Both PR-A and PR-B protein expression was also enhanced (Fig. 4C). In contrast, treatment with ovarian hormone treatment and additional LT4 did not significantly affect the expression of ERα or ERβ (Supplementary Fig. 2A, B) (27).

Role of thyroid hormone metabolism during decidualization

The presence and relative mRNA expression of *DIO1*, *DIO2*, and *DIO3* were analyzed in hESCs. The expression of *DIO2* and *DIO3* mRNA was observed in hESCs, whereas *DIO1* was not detected. During decidualization, *DIO2* expression was decreased, while *DIO3* levels increased (Fig. 5A, B). This effect was more pronounced with combined LT4

354 treatment.

355 Immunofluorescence staining confirmed the presence of DIO3 protein in hESCs (Fig. 5C–
356 E). Cytoplasmic staining in decidual cells was observed, and the relative area of
357 fluorescently labeled cells was increased in cells treated with ovarian hormones. Additional
358 thyroid hormone treatment further enhanced this effect. The increased synthesis of DIO3
359 protein was confirmed by immunofluorescence staining.

360

361 **Impact of TR silencing on decidualization**

362 To clarify whether decidualization is more strongly induced by thyroid hormone in cultured
363 hESCs, we performed gene silencing of TRs. Initially, as shown in Fig. 6A–C, we
364 confirmed that the expression of *TRα1*, *TRα2*, and *TRβ1* was suppressed through the
365 simultaneous silencing of TRα and TRβ. Both ovarian hormone treatment and concurrent
366 thyroid hormone treatment did not alter the expression levels of these receptors. The
367 silencing efficacy on the protein level was also confirmed (Fig. 6D, E).

368 Next, we examined the effect of TR silencing on the expression of *PRL* and *IGFBP-1*. The
369 simultaneous silencing of TRα and TRβ significantly reduced the mRNA levels of *PRL* and
370 *IGFBP-1* in the combination treatment group, and the inductive effect of thyroid hormone
371 disappeared (Fig. 6F, G).

372

373 **Effect of TR silencing on PR expression**

374 As described above, TR silencing abolished the induction and enhancement of
375 decidualization by LT4. Thus, we next examined how the activity of thyroid hormone on

decidualization via TR affected the expression of PR. Combined treatment with ovarian hormones and thyroid hormone caused a significant increase in the levels of *PR-AB* mRNA expression during decidualization (Fig. 1C, 6H). Compared to that in the scramble control-treated group, the expression level of *PR-AB* was reduced in the groups treated with TRα and TRβ siRNA (Fig. 6H). In hESCs treated with TRα and TRβ siRNA, the expression of *PR-AB* was increased by ovarian hormone treatment, but no further increase was observed upon additional LT4 treatment. The expression of *PR-B* was not significantly changed by hormone treatments after TRα and TRβ silencing (Fig. 6I).

Effect of thyroid hormone on global gene expression induced by ovarian hormones

The persistent effects of thyroid hormone on the decidualization phenotypes of hESCs are expected to cause significant changes in the gene expression landscape. Genome-wide gene expression patterns were assessed via RNA-Seq using next-generation sequencing. The expression matrix, calculated as scaled TPM and loaded at gene level by tximport (Fig. 7A), was analyzed by the integrated web application iDEP for differential expression and pathway analysis of RNA-Seq data.

Diagnostic plots for read-count data are given in Fig. 7B–D. Hierarchical clustering and principal coordinate analysis (PCA) identified the differential expression of thousands of genes induced by ovarian hormones and thyroid hormone. Notably, thyroid hormone treatment enhanced the expression of genes known to be induced during the decidualization process (Fig. 8A-C). Thyroid hormone also enhanced the expression of both PRs, which are important for decidualization (Fig. 8D, E). Forkhead box O1A (*FOXO1A*) has been

identified as a gene that is up-regulated early in the decidualization process (46,47) (Fig. 8F). In addition, the transcription factor zinc finger and BTB domain containing 16 (*ZBTB16*, also known as *PLZF*) was also up-regulated (Fig. 8G). The expression of downstream genes including decorin (*DCN*), tissue metalloproteinase inhibitor-3 (*TIMP3*), cannabinoid receptor 1 (*CNR1*), and laminin B1 (*LAMB1*) was also investigated (Fig. 8H–K).

Thus, not only specific molecular marker expression but also global gene expression was enhanced by thyroid hormone during decidualization.

Discussion

In this study, we have demonstrated that thyroid hormone facilitated the expression of molecular markers and the induction of morphological changes specific to decidualization in hESCs induced by ovarian hormone treatment. We further confirmed this effect of thyroid hormone through comprehensive transcriptome analysis using next-generation sequencing. There are several protocols used to evaluate the *in vitro* differentiation of hESCs. The duration of treatment with ovarian hormones varies profoundly in different studies, ranging from several hours to 10 days or more (8). We induced *in vitro* decidualization in hESCs using E_2 (10^{-8} mol/L) and MPA (10^{-7} mol/L) over 12 days (48-51). The biologically active form of thyroid hormone is 3,5,3'-triiodothyronine (T3), and T3 has a ten- to fifteen-fold stronger avidity to thyroid hormone receptors than the prohormone thyroxine (T4) (52). *In vivo*, T4 is converted to T3 as needed via DIO and bound to TR. We used LT4 as a physiological thyroid hormone in this study.

We investigated the mRNA expression of the decidualization markers *PRL* and *IGFBP-1*, in addition to *HAND2*. LT4 treatment increased their expression, which was induced by ovarian steroid hormones. However, LT4 monotherapy did not induce the expression of these transcripts. Notably, the addition of thyroid hormone treatment increased the expression of these markers from as early as 3 days. This suggests that thyroid hormone signaling plays an important role in the decidualization process. Thyroid hormone also plays a significant role in diverse processes related to growth, development, differentiation, and metabolism (53). It has been shown that thyroid hormone stimulates cell proliferation in several cell types, including cancer cells (54,55). In mice, the proliferation of subluminal stromal cells stimulated by thyroid hormone during early pregnancy has been demonstrated (25). Mice with a targeted dominant negative mutation in *TRα1* have been reported to have high mortality, reduced fertility, and reduced survival (56,57). A previous report revealed that *TRα1* mRNA expression was increased in the mid-secretory phase, the period in the menstrual cycle when a receptive endometrium suitable for implantation in human endometrium tissue is developed (24). In contrast, our study revealed that thyroid hormone did not affect cell proliferation and the expression of TR in hESCs.

The significant elevation of *PR-AB* mRNA expression was confirmed in cells treated with both ovarian steroid hormones and thyroid hormone in hESCs, and IP demonstrated that PR levels were also increased. This result suggests that thyroid hormone-induced PR signaling is a key pathway for decidualization. PR-A-knockout mice have been reported to display female infertility associated with a lack of decidualization in uterine stromal cells in response to progesterone, as well as defective embryo implantation (58,59). In humans, PR-

B controls a substantially larger cistrome and transcriptome than PR-A during decidualization (60). Although some crosstalk between TR and ovarian steroid hormone receptors—including ER and PR—has been uncovered (26,61), the impact of TR on PR expression has not yet been elucidated.

We then identified that thyroid hormone metabolism was affected by the dysregulation of the expression of DIOs during decidualization. Our analysis of the mRNA expression of DIO subtypes in hESCs during decidualization revealed that *DIO3* expression was increased and *DIO2* expression was decreased. A significant increase in the immunofluorescence staining of DIO3 was also observed; this increase was further up-regulated by thyroid hormone treatment. These results suggested that thyroid hormone homeostasis was maintained during the decidualization process via a feedback mechanism of DIOs. Many reports have confirmed the important role of DIO3 in fetal tissues and placenta (62-64). Evidence in rat models has strongly indicated that DIO3 is highly expressed in the uterus and protects the fetus from premature exposure to maternal thyroid hormone (65). Our findings were consistent with previous observations of DIO3 expression by *in situ* hybridization in human endometrium (66). DIO3-deficient mice were characterized by impaired reproductive function, including low fertility, increased mortality of embryos, and growth retardation (67). A recent study suggested that DIO3 may be essential for mouse decidualization because it is strongly expressed in the decidua (25).

The thyroid hormone signaling pathway consists of a complex group of proteins that regulates thyroid hormone synthesis, as well as the activation and transactivation of gene transcription by nuclear receptors; however, growing evidence has suggested the presence

464 of thyroid hormone-triggered non-genomic pathways (68-70). We confirmed that
465 decidualization in hESCs was suppressed by the simultaneous silencing of TRα and TRβ.
466 This result revealed that thyroid hormone-mediated decidualization is induced through
467 genomic alterations. Many non-genomic actions of thyroid hormone have been reported to
468 be mediated via a receptor expressed on a membrane integrin αvβ3 (71), particularly in
469 cancer cells and dividing endothelial cells (72). Other result of our experiments
470 (Supplementary Experiment and Supplementary Fig. 3A, B) (27) have indicated that αvβ3
471 integrins might not be actively involved in our model as a decidual inducer. Further
472 investigation is needed on the involvement of this important signaling pathway.
473 In addition, it must be further examined which TR isoforms play important roles in
474 decidualization. We also found that TRα and TRβ silencing in hESCs suppressed the
475 expression of *PR-AB* and abolished the increase in *PR-AB* expression by thyroid hormone
476 treatment. Thus, we can conclude that thyroid hormone contributes to endometrial
477 decidualization by regulating the expression of PR. However, further investigation
478 regarding the molecular mechanisms of TR and PR is needed.
479 In this study, we performed a global transcriptome expression study using RNA-Seq.
480 Hierarchical clustering and PCA clearly indicated the major difference in gene clusters
481 between the control cells and cells treated with ovarian hormones. These differences were
482 enhanced by further treatment with thyroid hormone. Another interesting finding was that
483 the expression of a series of transcription factors known to be associated with
484 decidualization was enhanced by thyroid hormone treatment. This was further confirmed by
485 RNA-Seq in this study.

There were several limitations to our study that need to be considered. Patients whose tissue samples were used to induce thyroid hormone-mediated decidualization *in vitro* did not have a pre-operative assessment of thyroid function, because they had surgery for uterine fibroids, not for infertility. Thus, their thyroid function could have affected their responsiveness to LT4. In a clinical setting, SCH patients may express anti-thyroid autoantibodies (20,52,73,74), which may be a cause of infertility. Another limitation is the number of samples. If more samples could be collected, stronger statistical results would be verified. Finally, our findings showed that thyroid hormone is important for the decidualization in hESCs, but it has not been proven *in vivo* whether the induction of thyroid hormone-mediated decidualization will promote implantation. We expect our results to lead to further investigations to solve these issues.

In conclusion, we have shown that thyroid hormone plays a critical role in promoting decidualization in hESCs. Further, our results suggest that increased PR expression led to the enhanced induction of thyroid hormone-mediated decidualization. To our knowledge, this is the first *in vitro* finding providing a possible explanation of impaired decidualization as a cause of infertility in SCH patients.

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Data availability

The RNA-Seq data that support the findings of this study are available in the DDBJ Sequence Read Archive (accession numbers DRR190745 to DRR190750). The other datasets that support the findings of this study are available from the corresponding author upon reasonable request.

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33. RRID: AB_882974, https://scicrunch.org/resolver/AB_882974

34. RRID: AB_882978, https://scicrunch.org/resolver/AB_882978

35. RRID: AB_476743, https://scicrunch.org/resolver/AB_476743

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38. RRID: AB_2166687, https://scicrunch.org/resolver/AB_2166687

39. RRID: AB_1290842, https://scicrunch.org/resolver/AB_1290842

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717
718
719 **Figure Legends**
720 **Figure 1. qPCR analysis of the expression of decidual markers, and analysis of PRL**
721 **and IGFBP-1 secretion in human endometrial stromal cells (hESCs).**
722 Human endometrial stromal cells (hESCs) were treated with E_2 (10^{-8} mol/L) + MPA (10^{-7}
723 mol/L), and/or LT4 (10^{-7} mol/L), or vehicle (control) for up to 12 days.
724 (A–C) The mRNA expression of (A) prolactin (*PRL*), (B) insulin-like growth factor-
725 binding protein 1 (*IGFBP-1*), and (C) heart and neural crest derivatives-expressed transcript
726 2 (*HAND2*) was analyzed by RT-PCR after 12 days of culture. (D, E) (D) PRL and (E)
727 IGFBP-1 production in hESC culture supernatants was assayed by ELISA after 12 days of

culture. (F–H) The mRNA expression of (F) *PRL* and (G) *HAND2* was increased from day 3 of decidualization. The same tendency was observed for (H) *IGFBP-1*. Data were normalized to *EF-1α* as a housekeeping gene. Data represent the mean ± SD (n = 5). **P* < 0.05 vs control. #*P* < 0.05 for the indicated comparison.

Figure 2. Effects of ovarian steroid hormones and thyroid hormone on cell morphology during decidualization.

The hESCs were cultured with the following agents for 12 days: (A, B) vehicle (control); (C, D) E_2 (10^{-8} mol/L) + MPA (10^{-7} mol/L); (E, F) E_2 (10^{-8} mol/L) + MPA (10^{-7} mol/L) + LT4 (10^{-7} mol/L); (G, H) LT4 (10^{-7} mol/L). (A, C, E, G): low-power field (×100) images. (B, D, F, H): high-power (×400) images.

Figure 3. Dose-dependent effect of thyroid hormone on induction and enhancement of decidualization and evaluation of thyroid hormone receptor expression during decidualization.

(A) Changes in *PRL* mRNA expression in hESCs treated with various doses of LT4 (10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} mol/ L) as well as E_2 (10^{-8} mol/L) + MPA (10^{-7} mol/L) for 12 days. Data represent the mean ± SD of three independent experiments with different cell preparations (n = 5). (B–D) mRNA levels of (B) thyroid hormone receptor (*TR*) *α1*, (C) *TRα2*, and (D) *TRβ1* in hESCs (n = 5). (E, F) Protein expression levels of (E) TRα, (F) TRβ in hESCs. The hESCs were cultured in the presence of E_2 (10^{-8} mol/L) + MPA (10^{-7} mol/ L) with or without LT4 (10^{-7} mol/L), or vehicle (control), for 12 days.

Figure 4. Progesterone receptor (*PR*) mRNA expression, and PR protein expression by immunoprecipitation (IP) during decidualization.

(A, B) Analysis of mRNA levels of (A) progesterone receptor (*PR*)-*AB* and (B) *PR-B*. Data represent the mean ± SD of five independent experiments with different cell preparations (n = 5). $^{*}P < 0.05$ vs control. $^{\#}P < 0.05$ for the indicated comparison. (C) IP of progesterone receptors in hESCs during decidualization (n = 3). T-47D cell lysate was used as a positive control of PR. The representative image shows the presence of PR-A and PR-B proteins in hESCs treated with E_2 (10^{-8} mol/L), MPA (10^{-7} mol/L), and LT4 (10^{-7} mol/L) for 12 days.

Figure 5. Role of thyroid hormone metabolism in decidualization.

The hESCs were cultured in the presence of E_2 (10^{-8} mol/L) + MPA (10^{-7} mol/ L) with or without LT4 (10^{-7} mol/L), or vehicle (control), for 12 days. (A, B) The mRNA levels of (A) deiodinase (*DIO*) *2* and (B) *DIO3* in hESCs was analyzed each treatment group (n = 5). $^{*}P < 0.05$. (C–E) Immunofluorescence staining was used to confirm DIO3 protein levels in hESCs during decidualization (n = 3) In cells treated with (C) vehicle (control), (D) E_2 (10^{-8} mol/L) + MPA (10^{-7} mol/L), or (E) E2 (10^{-8} mol/L) + MPA (10^{-7} mol/L) and LT4 (10^{-7} mol/L).

Figure 6. Impact of thyroid hormone receptor silencing on decidualization.

(A–C) hESCs were transfected with siRNA targeting TRα and TRβ, or control scramble siRNA. After 48 h, the transfected cells were treated with E_2 (10^{-8} mol/L) + MPA (10^{-7}

mol/L), and LT4 (10^{-7} mol/L), or vehicle (control), for 12 days. The effects of TRα and TRβ silencing on the mRNA levels of (A) *TRα1*, (B) *TRα2*, and (C) *TRβ1* in cultured hESCs were analyzed (n = 3). (D, E) Protein expression levels of (D) TRα, (E) TRβ in hESCs were also analyzed. (F, G) The silencing of TRα and TRβ significantly reduced (F) *PRL* and (G) *IGFBP-1* mRNA levels compared to the scramble siRNA treatment group. (H, I) In TR-silenced hESCs, the expression of (H) *PR-AB* was increased by E_2 + MPA, but no further increase effect was observed by LT4. (I) *PR-B* expression was not significantly altered after TRα and TRβ silencing. $^{*}P < 0.05$ vs control. $^{\#}P < 0.05$ for the indicated comparison.

Figure 7. Global gene expression in hESCs.

(A) Heatmap of differential gene expression in each treatment group. Samples with relatively high expression of a given gene are marked in red, and samples with relatively low expression are marked in green. (B, C) Samples and genes were reordered according to (B) hierarchical clustering and (C) principal component analysis. (D) RNA-Seq showed that thousands of genes were up- or down-regulated by E_2 (10^{-8} mol/L) + MPA (10^{-7} mol/L) and LT4 (10^{-7} mol/L).

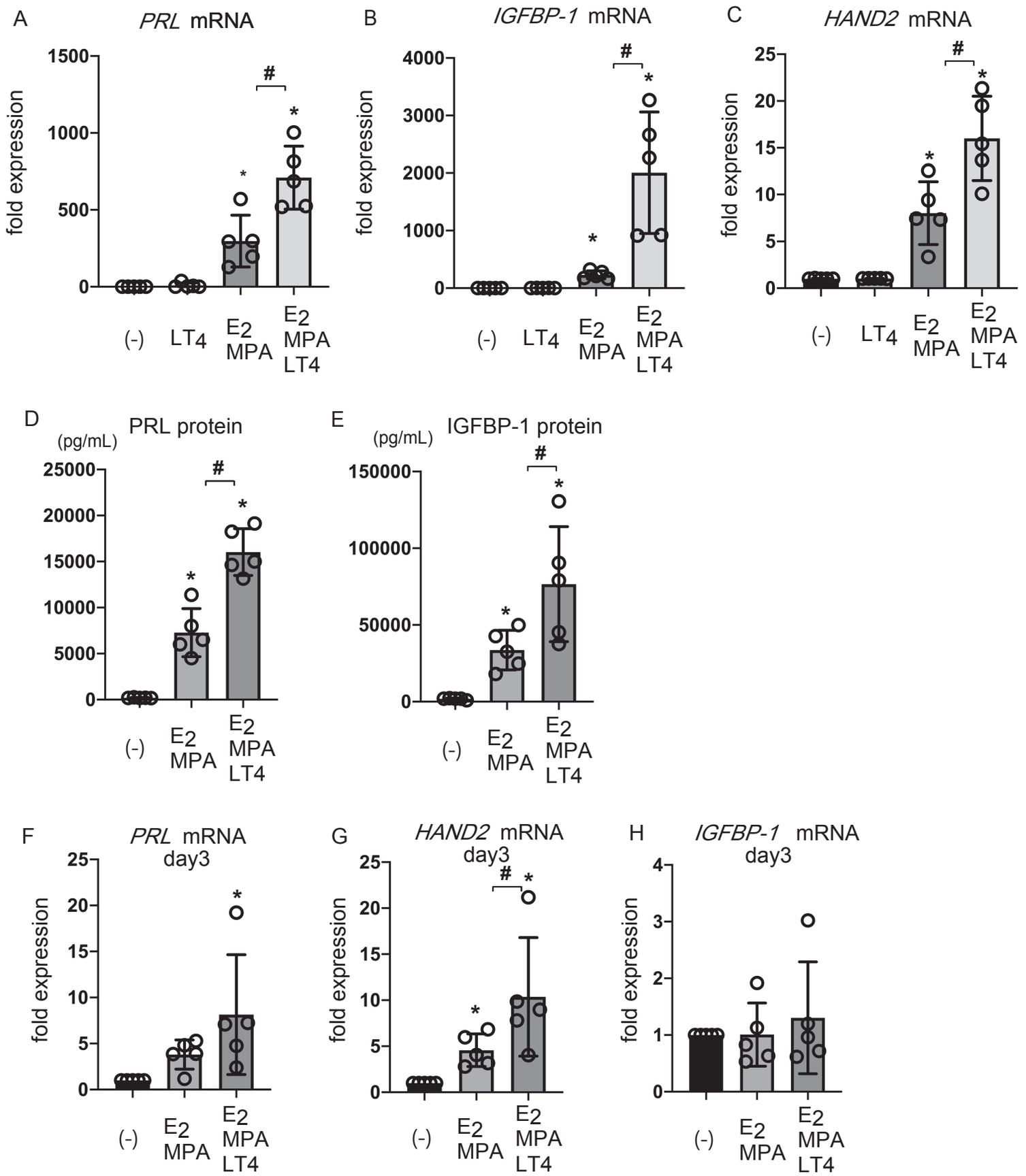
Figure 8. Effect of thyroid hormone on global gene expression during decidualization.

RNA-seq analysis of the expression levels of the following decidual-specific genes: (A) *PRL*, (B) *IGFBP-1*, (C) *HAND2*, (D) *PR-A*, (E) *PR-B*, (F) *FOXO1A*, (G) *ZBTB16*, (H) *DCN*, (I) *TIMP3*, (J) *CNR*, and (K) *LAMB1*.

794 **Table**

795 **Table 1.** Real-time PCR primer sequences.

Figure1


A
PRL mRNA
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LT4
E2
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E2
MPA
LT4
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IGFBP-1 mRNA
fold expression
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HAND2 mRNA
fold expression
25
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15
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5
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D
PRL protein
(pg/mL)
25000
20000
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10000
5000
0
E
IGFBP-1 protein
(pg/mL)
150000
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50000
0
F
PRL mRNA
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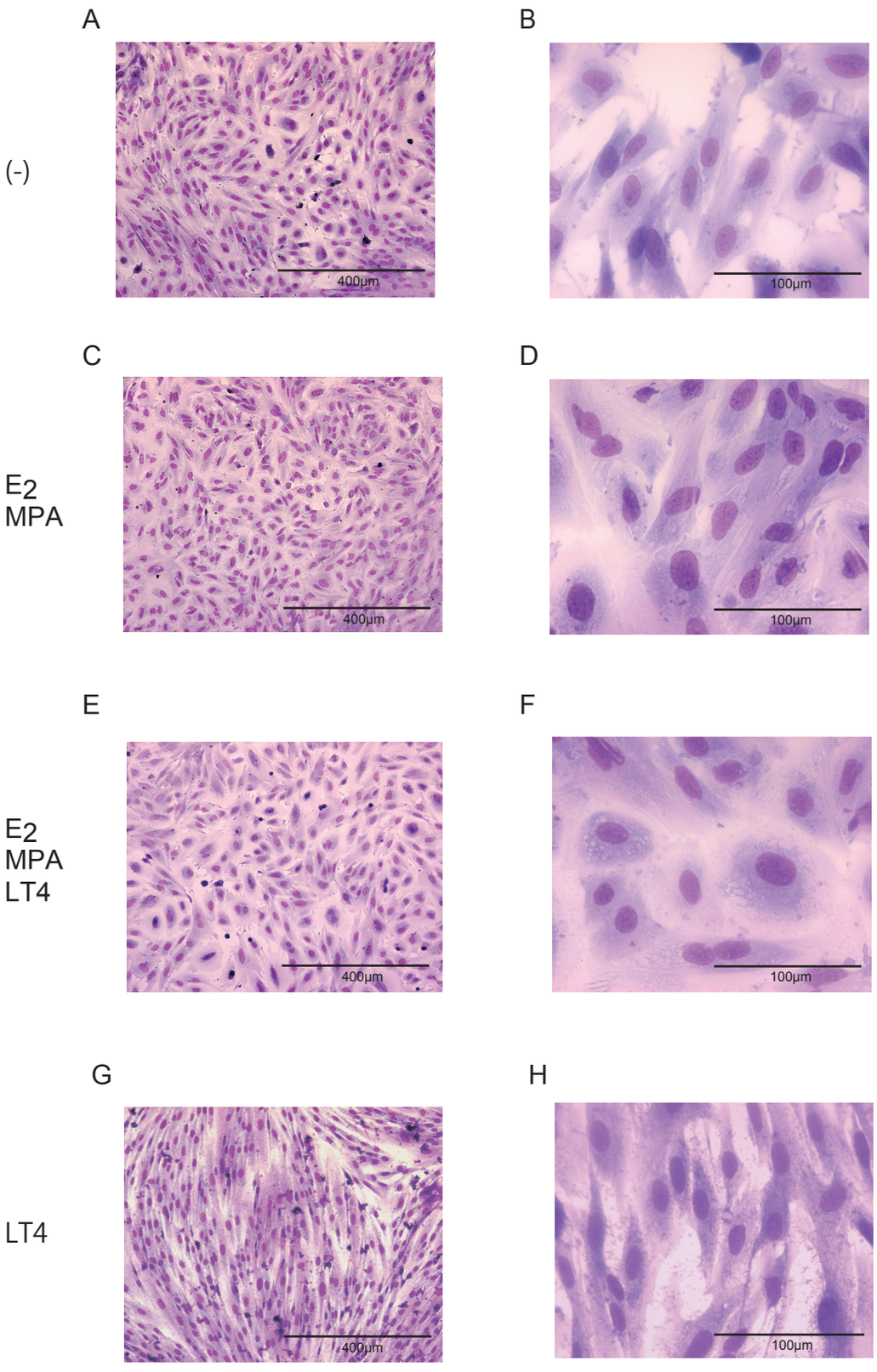
fold expression
G
HAND2 mRNA
day3
fold expression
H
IGFBP-1 mRNA
day3
fold expression

Figure2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C
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LT4
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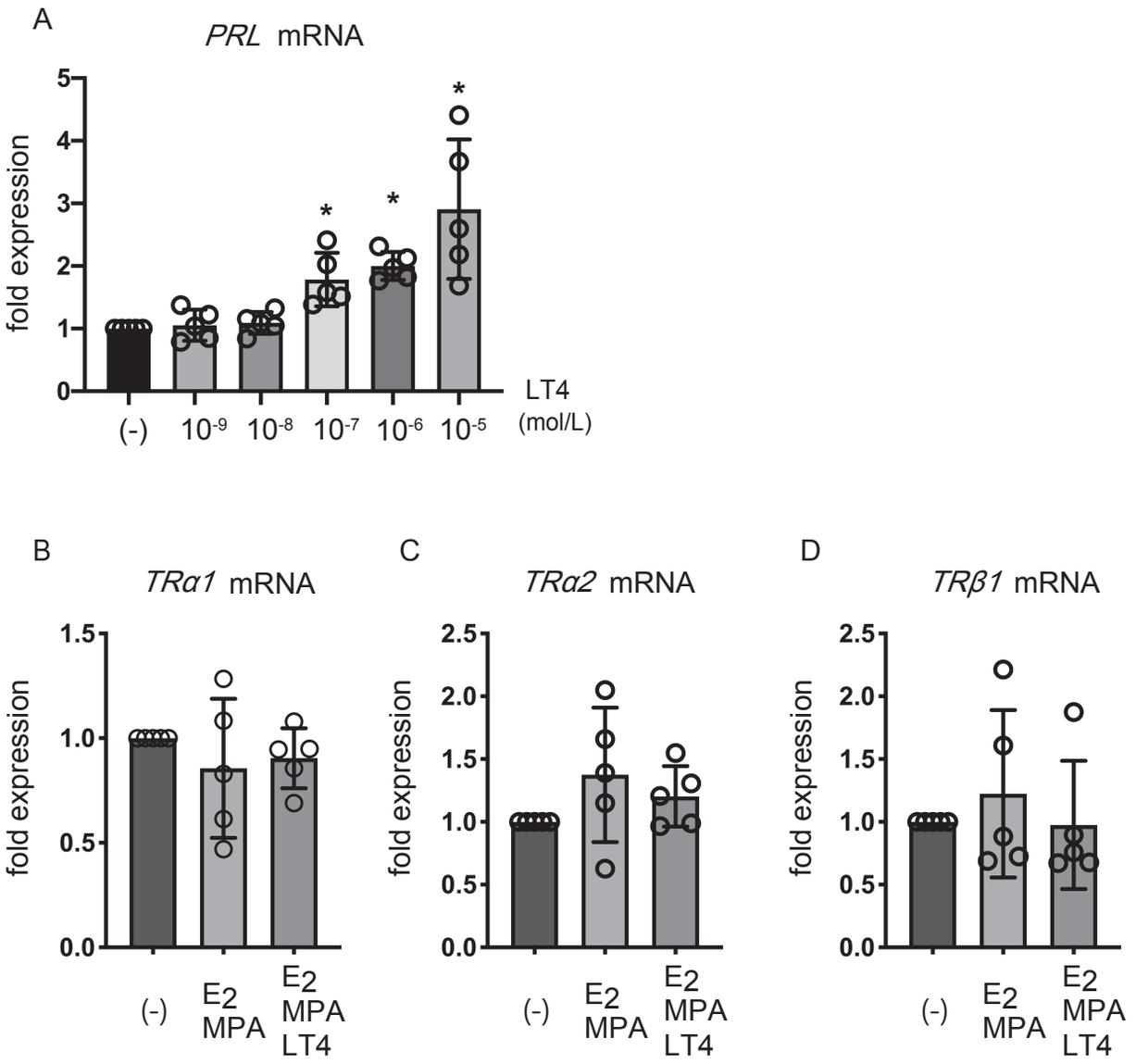
Figure3


A
PRL mRNA
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MPA
LT4

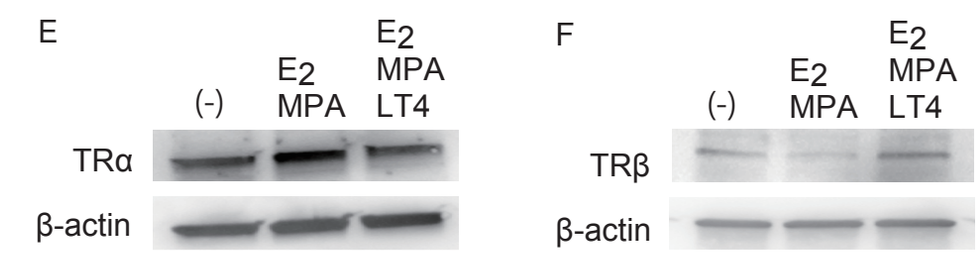
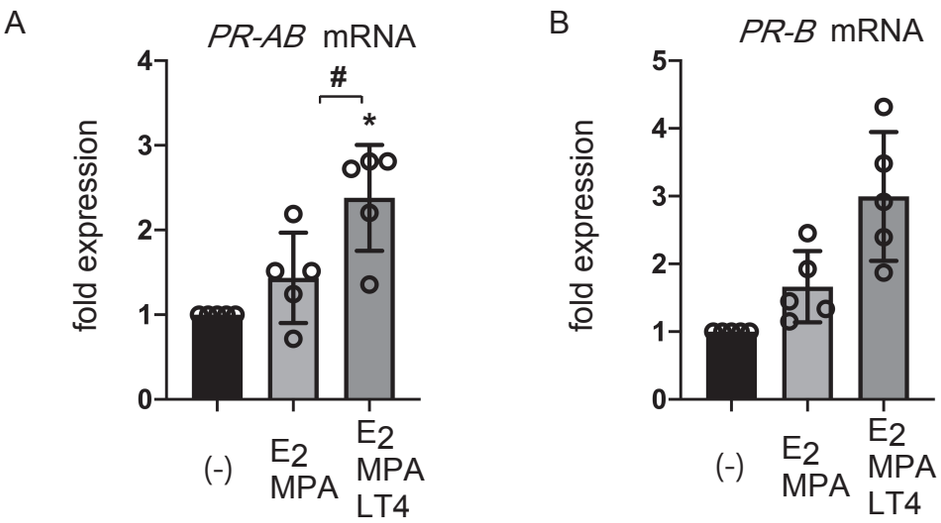

E
(-)
E2
MPA
E2
MPA
LT4
TRα
β-actin
F
(-)
E2
MPA
E2
MPA
LT4
TRβ
β-actin

Figure4


A
PR-AB mRNA
fold expression
#
*
(-)
E2
MPA
E2
MPA
LT4
B
PR-B mRNA
fold expression
(-)
E2
MPA
E2
MPA
LT4

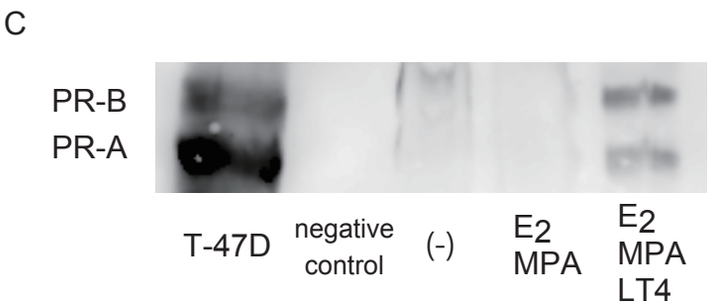

C
PR-B
PR-A
T-47D
negative
control
(-)
E2
MPA
E2
MPA
LT4

Figure5

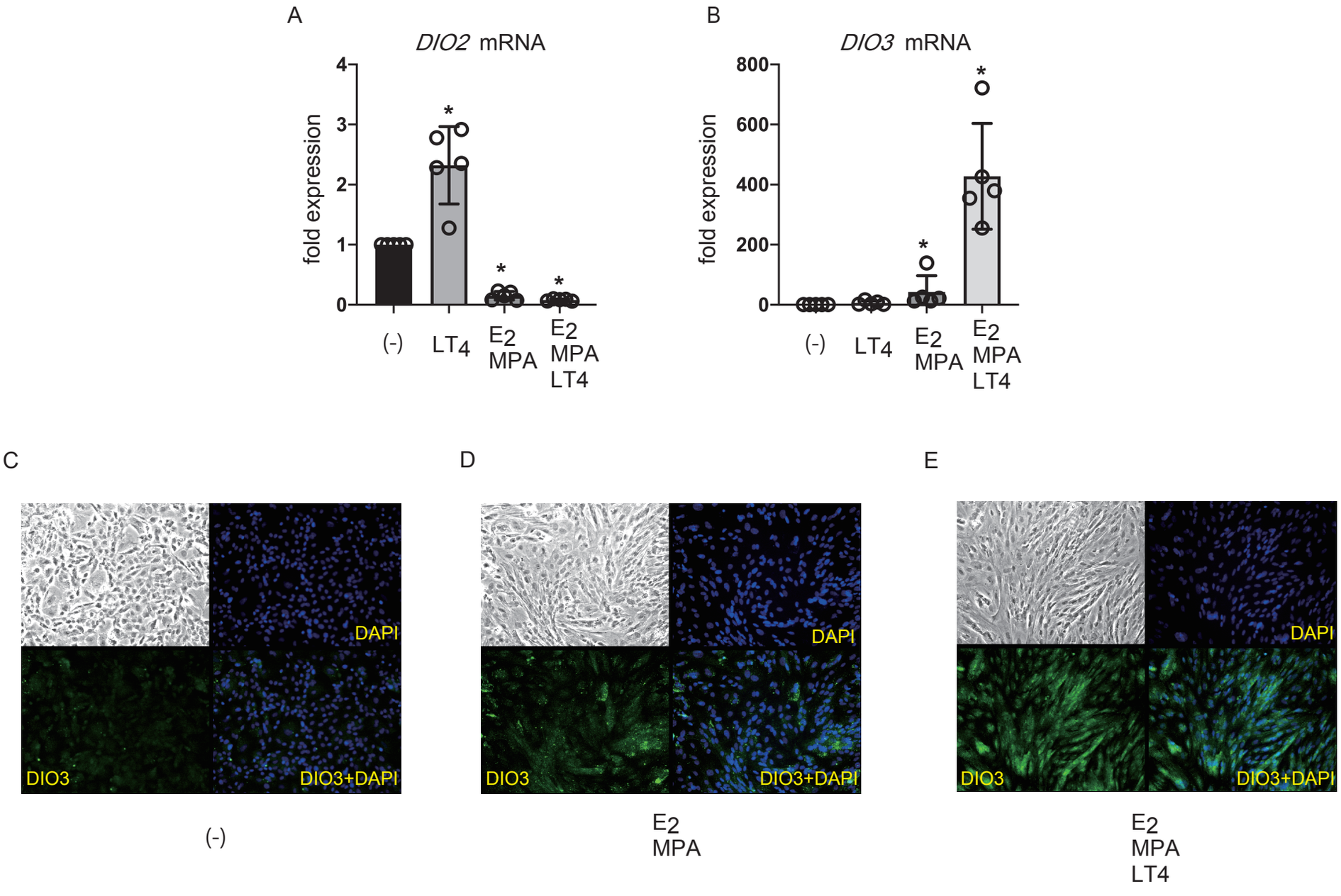

A
DIO2 mRNA
fold expression
4
3
2
1
0
*
*
*
(-)
LT4
E2
MPA
E2
MPA
LT4
B
DIO3 mRNA
fold expression
800
600
400
200
0
*
*
(-)
LT4
E2
MPA
E2
MPA
LT4
C
D
E
DAPI
DIO3
DIO3+DAPI
(-)
E2
MPA
E2
MPA
LT4

Figure6

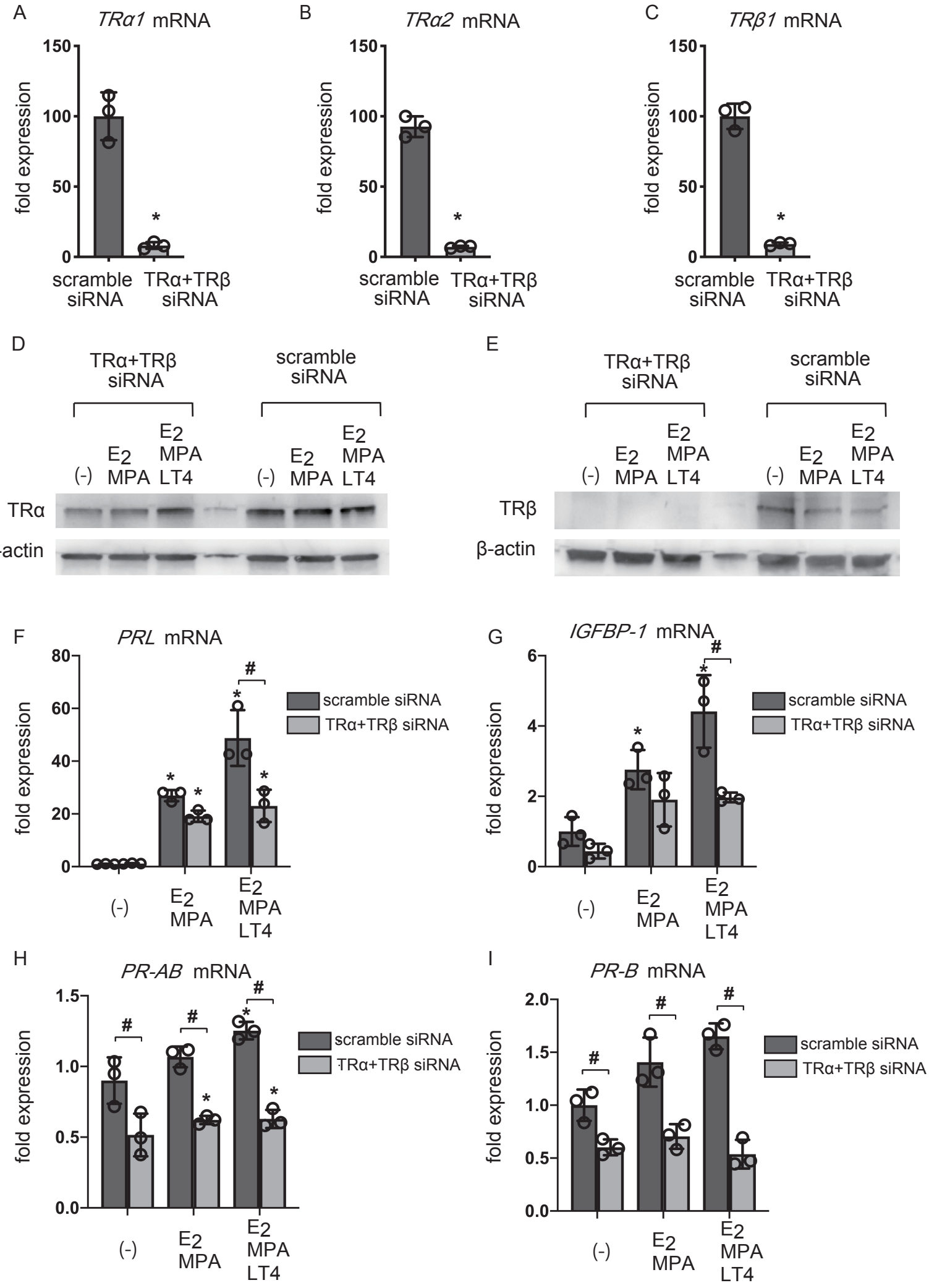
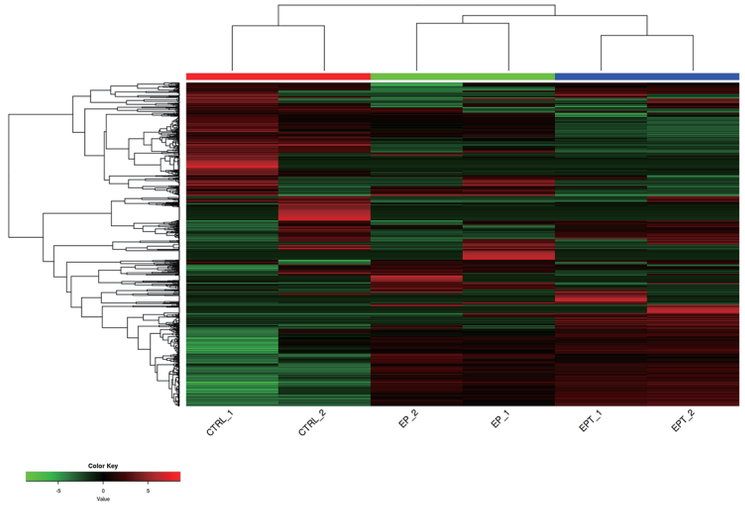
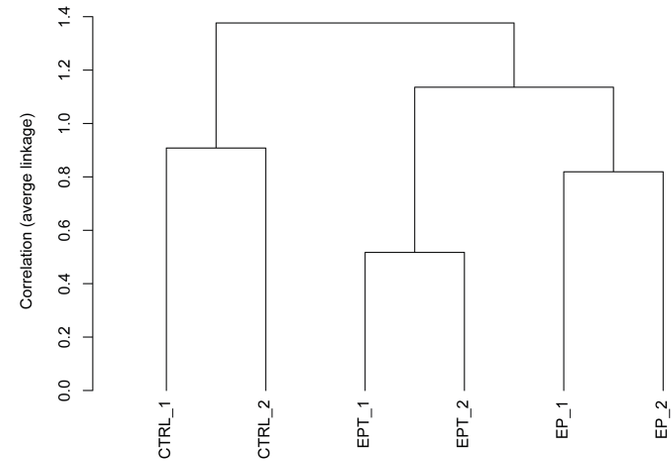

A
TRα1 mRNA
fold expression
150
100
50
0
*
scramble siRNA
TRα+TRβ siRNA
B
TRα2 mRNA
fold expression
150
100
50
0
*
scramble siRNA
TRα+TRβ siRNA
C
TRβ1 mRNA
fold expression
150
100
50
0
*
scramble siRNA
TRα+TRβ siRNA
D
TRα+TRβ siRNA
scramble siRNA
(-)
E2
MPA
E2 MPA LT4
TRα
β-actin
E
TRα+TRβ siRNA
scramble siRNA
(-)
E2
MPA
E2 MPA LT4
TRβ
β-actin
F
PRL mRNA
fold expression
80
60
40
20
0
#
*
scramble siRNA
TRα+TRβ siRNA
(-)
E2
MPA
E2 MPA LT4
G
IGFBP-1 mRNA
fold expression
6
4
2
0
#
*
scramble siRNA
TRα+TRβ siRNA
(-)
E2
MPA
E2 MPA LT4
H
PR-AB mRNA
fold expression
1.5
1.0
0.5
0.0
#
*
scramble siRNA
TRα+TRβ siRNA
(-)
E2
MPA
E2 MPA LT4
I
PR-B mRNA
fold expression
2.0
1.5
1.0
0.5
0.0
#
scramble siRNA
TRα+TRβ siRNA
(-)
E2
MPA
E2 MPA LT4

Figure7

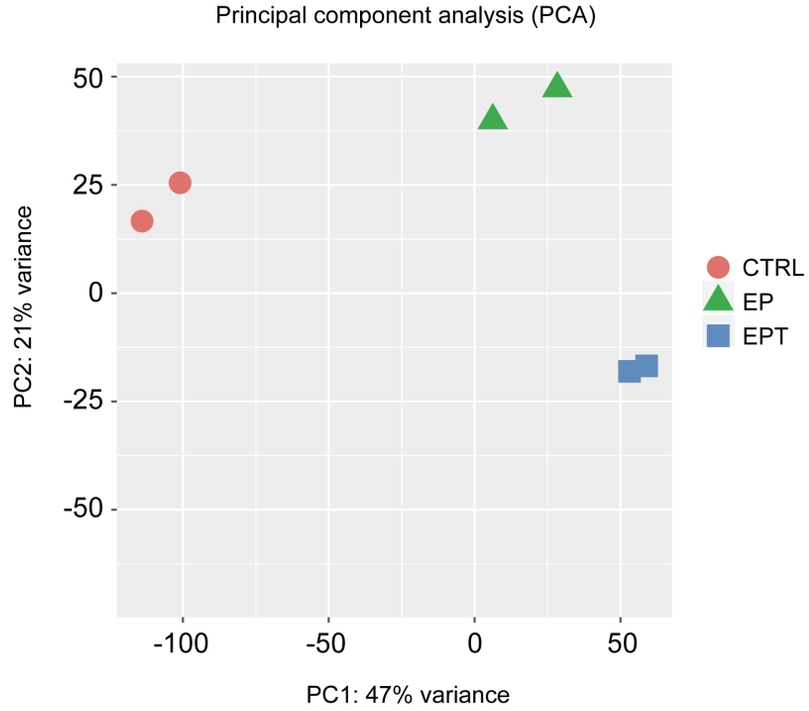
A



B



C



D

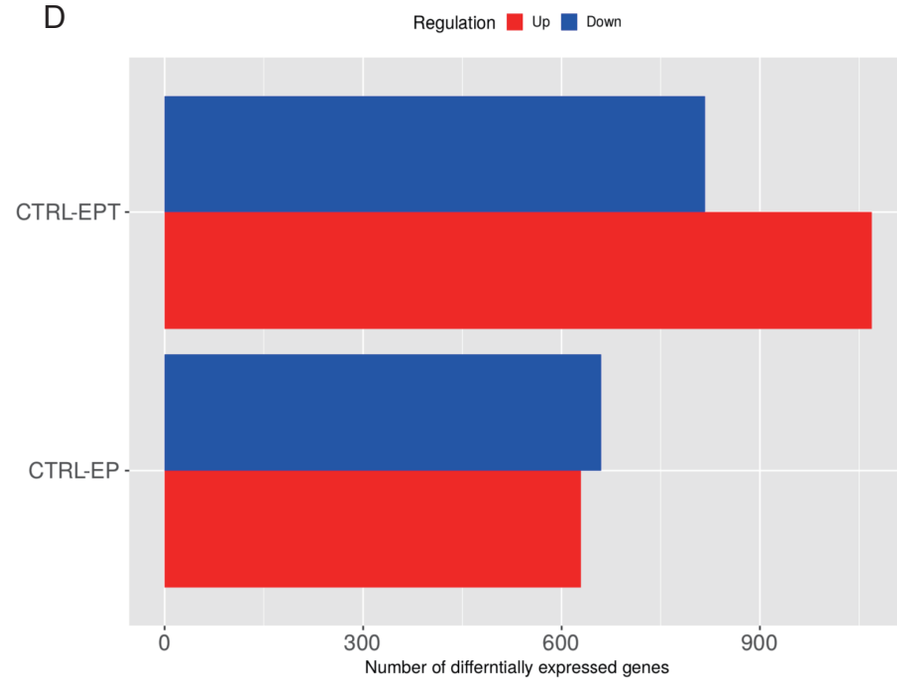
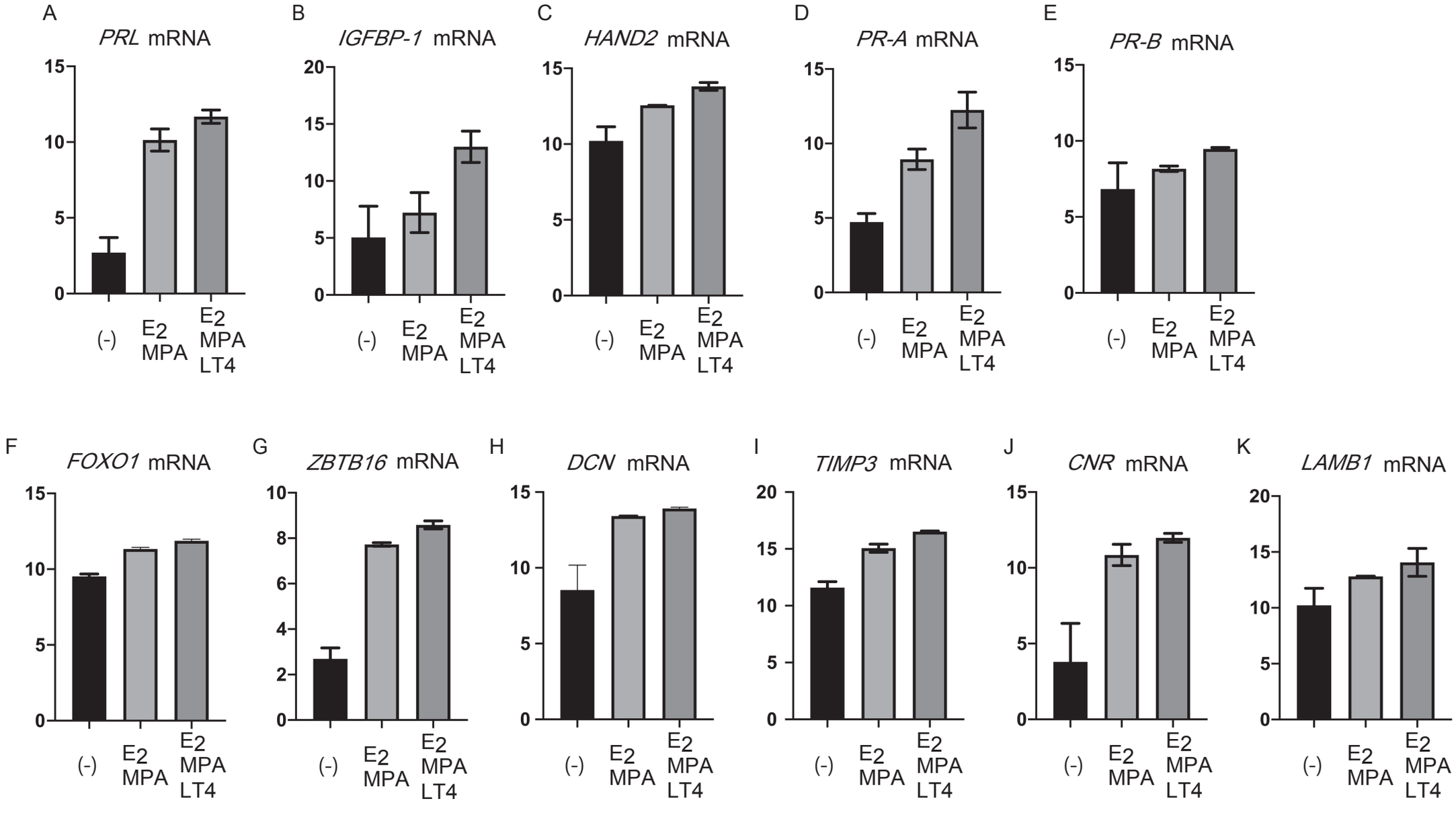


Figure8


A
PRL mRNA
B
IGFBP-1 mRNA
C
HAND2 mRNA
D
PR-A mRNA
E
PR-B mRNA
F
FOXO1 mRNA
G
ZBTB16 mRNA
H
DCN mRNA
I
TIMP3 mRNA
J
CNR mRNA
K
LAMB1 mRNA
(-)
E2
MPA
E2 MPA LT4