Bisphenol compounds regulate decidualized stromal cells in modulating trophoblastic spheroid outgrowth and invasion in vitro

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Abstract

Bisphenol A (BPA) is commonly found in epoxy resins used in the manufacture of plastic coatings in food packaging and beverage cans. There is a growing concern about BPA as a weak estrogenic compound that can affect human endocrine function. Chemicals structurally similar to BPA, such as BPF and BPS, have been developed as substitutes in the manufacturing industry. Whether these bisphenol substitutes have adverse effects on human endocrine and reproductive systems remains largely unknown. This study investigated the effects of BPA, BPF, and BPS on regulating the function of decidualized human primary endometrial stromal cells on trophoblast outgrowth and invasion by indirect and direct co-culture models. All three bisphenols did not affect the stromal cell decidualization process. However, BPA- and BPF-treated decidualized stromal cells stimulated trophoblastic spheroid invasion in the indirect co-culture model. The BPA-treated decidualized stromal cells had upregulated expressions of several invasion-related molecules including LIF, whereas both BPA- and BPF-treated decidualized stromal cells had downregulated expressions of anti-invasion molecules including PAI-1 and TNFα . Taken together, BPA and BPF altered the expression of invasive and anti-invasive molecules in decidualized stromal cells modulating its function on trophoblast outgrowth and invasion, which could affect the implantation process and subsequent pregnancy outcome.

1. Introduction

Bisphenol A (BPA) is used in the manufacture of certain plastics and is commonly found in epoxy resins, water bottles, paper bags, baby bottles, coatings in food cans, dental sealants, thermal receipts, and household waste paper [1, 2]. In addition, BPA can be found in the environment, including dust, sewage leachates, and water. It is estimated that 10 billion pounds of BPA are produced worldwide every year [3]. Not surprisingly, humans are exposed to BPA through diet, dust inhalation, and dermal contact [4]. In fact, BPA has been reported in human blood serum (0.2 to 20 ng/mL), breast milk (0.28-3.42 ng/mL), urine (0 -54.3 ng/mL), and follicular fluid (2 ng/mL) [2]. Interestingly, BPA levels in the placenta were significant higher than in maternal and fetal plasma, suggesting BPA accumulates in the maternal-fetal interface [5]. In occupational exposure, cashiers are exposed to daily amounts of BPA estimated to be 50 to 1,000 times greater than in the general population [6-8].

Bisphenol A is an endocrine disruptor and can affect human endocrine functions leading to disorders of the reproductive, endocrine, and immune systems [9-12]. Several countries including Norway, Denmark, Germany, France, and USA have restricted the use of BPA in some consumer products. Several compounds with similar physical and chemical properties to BPA, such as BPF and BPS, have been developed as substitutes in the manufacturing industry. These alternative bisphenol products have also been detected in the environment and in human urine samples [1, 13-17]. However, the effects of these bisphenol products on human reproductive health are still unclear.

Embryo implantation requires highly orchestrated interactions between the

developing blastocyst and the receptive endometrium to enable apposition, adhesion and invasion of the embryo into the uterine wall [18]. At the same time, steroid hormones induce the secretion of glandular and luminal endometrial epithelial cells, as well as decidualization of stromal cells that support the growth of the developing embryo [19]. Decidualized stromal cells also control the invasion of the trophoblast, as well as the anti-oxidant defense response and local immune tolerance [20]. Stromal cells express matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), as well as secrete tumor necrosis factor-β (TNF-β). Matrix metalloproteinases (e.g., MMP2 and MMP9) degrade and reconstruct the extracellular matrix, whereas TIMPs (e.g., TIMP1 and TIMP2) regulate the function of MMPs [19]. Meanwhile, TNF-β produced by stromal cells regulates the balance of MMPs/TIMPs in the local environment [20]. Accumulating evidence suggests that decidualized stromal cells can directly or indirectly interact with the blastocyst or trophoblastic spheroid (blastocyst surrogate) to regulate the migration and invasion of the trophoblast [21-24]. Recent studies have shown that decidualized stromal cells or their spent medium could inhibit trophoblast invasion [25-27]. However, the underlying mechanism of how decidualized stromal cells regulate trophoblast invasion remains largely unknown.

For the past two decades, there has been growing evidence of the adverse effects of BPA on female reproduction, including oocyte quality, endometrial receptivity, embryo implantation and development, and pregnancy outcomes [28]. Supra-physiological doses of BPA have been shown to decrease implantation sites and disrupt

the expression of genes in the endometrium of in vitro and in vivo models [29]. The effect of endocrine disrupting chemicals, such as bisphenol compounds, on trophoblast invasion into the human endometrium is largely unknown. Several recent studies suggest that BPA can alter gene expression in human trophoblastic cells [30] and affect the invasion of human trophoblast HTR-8/SVneo cells in a collagen or Matrigel-coated invasion chamber [31]. Furthermore, BPA at physiological doses can disturb the expression of decidualization markers (e.g., prolactin) and hormone receptors (e.g., ERα, ERβ, PRA, PRB, and hCG/LH-R) in decidualized stromal cells [32]. As the embryo invasion process requires a decidualized endometrium, many studies have used co-culture models of endometrial stromal cells and embryo surrogates (primary trophoblastic cells, trophoblastic cell lines, trophoblastic spheroids, and/or mouse embryo) to investigate endometrium-trophoblast interactions [22, 33-35]. A model using trophoblastic spheroids and human first-trimester decidual tissue showed the invasion pattern was comparable to the invasion pattern in vivo [36]. Embryo implantation research commonly use Jeg-3 human choriocarcinoma cells and Jeg-3 spheroids as an embryo surrogate [37]. The present study aimed to investigate the effects of bisphenol compounds (BPA, BPF, and BPS) on stromal cell decidualization, as well as the subsequent effects on trophoblast invasion using co-culture models with primary decidualized stromal cells and Jeg-3 spheroids.

2. Materials and methods

2.1 Cell culture and reagents

Human choriocarcinoma Jeg-3 (ATCC HTB-36) cells were maintained in DMEM/F12 (Sigma) supplemented with 10% fetal bovine serum (FBS, Invitrogen), L-glutamine, and penicillin/streptomycin at 37° C in a humid atmosphere and 5% CO_2. To generate Jeg-3 spheroids, $3x10^5$ Jeg-3 cells in 3 mL of culture medium were seeded on a 6-well plate and rotated at 88 rpm overnight. Spheroids with a diameter between 100 and 200 μm were selected for the following experiments. Bisphenol A (BPA, purity>99%, CAS 80-05-7; Sigma), bisphenol F (BPF, Bis(4-hydroxyphenyl)methane, purity=98%, CAS 620-92-8; Sigma), and bisphenol S (BPS, 4,4'-Sulfonyldiphenol, purity=98%, CAS 80-09-1; Sigma) were dissolved in DMSO. CYQUANT cell proliferation assay kit (Life Technologies) was used to assess cell proliferation. Measurable serum BPA levels typically range from 0.2 to 20 ng/mL (0.88 to 87.6 nM) [2], and are largely a result of occupational exposure. Previous studies demonstrated that supra-physiological doses of BPA were able to disrupt the function of decidualized stromal cells [32, 38]. In this study, we used BPA levels ranging from physiological dose (1 nM) to non-toxic concentrations (10 μM). Methotrexate (MTX, Sigma) dissolved in DMSO was used as a positive control. An mirVANA PARIS kit (Ambion) was used for RNA and protein extraction.

2.2. Stromal cell isolation

Endometrial biopsies were obtained from women visiting the Queen Mary

Hospital for infertility treatment. The study protocol was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority, Hong Kong West Cluster (protocol number UW 13-118). After obtaining written consent, endometrial biopsies (N=15) were collected on day hCG+2 (2 days after hCG injection). The endometrial biopsies were minced by scalpel and digested in phenol red-free DMEM/F12 medium containing 1% BSA, 0.5 mg/mL type I collagenase (Invitrogen) and 0.15 mg/mL DNase I type IV (Worthington Biochemical Corporation) at 37^{o}C for 1 hour. The digestion was repeated for another 30 minutes. Cells from two digestions were pooled and filtered through 100-μm filters to remove undigested tissue. The endometrial glands and stromal cells were separated by filtration through 40-μm filters. Stromal cells passing through the 40-μm filter were maintained in phenol red-free DMEM/F12 medium supplemented with 10% FBS.

2.3. In vitro decidualization

Decidualization of stromal cells was performed as previously described [39]. Briefly, human endometrial stromal cells were seeded at 1.5x10^{5} cells per well in 24-well plates and cultured with 5% charcoal-stripped FBS (csFBS) in phenol red-free DMEM/F12 medium with supplements. After 24 hours, cells were treated with 0.5 mM 8-Bromo-cAMP (Sigma), 10 nM estrogen (E2, 17β-estradiol-acetate; Sigma) and 1 μM progesterone (P4, 4-pregnene-3,20-dione; Sigma) for 9 days. Non-decidualized stromal cells without hormonal treatment were used as the controls. The culture medium was changed every 3 days and bisphenol compounds (1 nM to10 μM BPA, BPF, and BPS)

were added during the decidualization process. Supernatants and cell lysates were collected every 3 days and stored at -80°C for analysis. The culture media of non-decidualized and decidualized stromal cells were also collected at different time points and stored at -80°C for analysis. Morphological changes in these stromal cells were recorded under a phase-contrast microscope. The expression of decidualization markers including prolactin (PRL) and insulin-like growth factor binding protein 1 (IGFBP-1) in both the cell lysates and supernatants were evaluated by qPCR and ELISA.

2.4. RNA extraction, quantitative PCR, and ELISA

Total RNA from stromal cells was isolated using the mirVANA PARIS kit according to the manufacturer's protocol. Total RNA (400 ng) was reverse transcribed using a TaqMan reagents kit (N8080234, Thermo Fisher Scientific) in a 20 μL reaction volume for cDNA synthesis. The cDNA was amplified in a 20 μL reaction volume using TaqMan probes (Applied Biosystems) for decidual markers (PRL: Hs00168730_m1, IGFBP1: Hs00236877_m1), pro-invasion molecules (MMP2: Hs01548727_m1, MMP9: Hs00957562_m1, LIF: Hs01055668_m1, IL-15: Hs01003716_m1, uPA: Hs01547054_m1), and anti-invasion molecules (TIMP1: Hs01092512_g1, TIMP2: Hs00234278_m1, TIMP3: Hs00165949_m1, TIMP4: Hs00162784_m1, PAI-1: Hs00167155_m1, TNFα: Hs00174128_m1, TGFβ1: Hs00998133_m1, TGFβ3: Hs01086000_m1, IL-10: Hs00961622_m1). The relative gene expression was normalized with 18S (4319413E, Applied Biosystems) as the internal control.

The concentrations of PRL and IGFBP1 in the culture media were measured by

ELISA (ab189570, ab100539; Abcam) according to manufacturer's protocol. Briefly, the conditioned samples were diluted in sample dilutent NS at the appropriate dilution ratios (diluted 10 times for PRL and 5 times for IGFBP1). Next, 50 µL of the diluted sample and standard were added into a 96-well plate pre-coated with PRL antibodies or IGFBP1 antibodies, and 50 µL of the antibody cocktail (a mixture of capture and detection antibodies) was also added into each well. The plate was sealed and incubated for 1 hour at room temperature on a plate shaker. After washing thoroughly, 100 µL of TMB substrate was added into each well and incubated for 10 minutes on a shaker protected from light, followed by 100 µL of the stop solution. The absorbance at 450 nm was measured in all samples and standards and performed in duplicate.

2.5. Immunofluorescence staining

Non-decidualized and decidualized stromal cells were fixed in 4% (v/v) paraformaldehyde in PBS for 20 minutes at room temperature. The cells were permeabilized in 0.1% Triton X-100 in PBS for 15 minutes at room temperature. The cells were then blocked with 2% goat serum in PBS for 1 hour at room temperature and incubated with anti-vimentin antibody (1:500, M0725; Dako) diluted in 2% goat serum overnight at 4°C. After washing, cells were incubated with fluorophore-conjugated secondary antibody in PBS containing 2% goat serum. Excess antibodies were washed away and nuclei were stained with DAPI for 10 minutes. Cells were visualized and images were captured under a fluorescence microscope (Nikon).

2.6. Effects of bisphenols on spheroid outgrowth and invasion (Indirect co-culture model)

An indirect co-culture model was used to study if secretory factors from bisphenol-treated decidualized stromal cells could regulate spheroid outgrowth and invasion. Briefly, human primary endometrial stromal cells ($1x10^5$) were decidualized for 9 days in a 25 cm^2 flask. Decidualized cells were seeded onto a 24-well plate in a culture medium containing 5% csFBS with bisphenol treatments. The same number of non-decidualized stromal cells seeded onto a 24-well plate in 5% csFBS culture medium was used as the control, and 5% csFBS culture medium only was used as the negative control. After incubating the stromal cells with different bisphenol treatments for 24 hours, a Matrigel-coated invasion chamber was placed onto the 24-well plate and three embryo-sized Jeg-3 spheroids were added in each chamber in phenol red-free DMEM/F12 medium. The size of each spheroid was recorded as the 'day 0 area'. After 24 hours, outgrowth of spheroids on the upper side of invasion chamber membrane was recorded as the 'day 1 outgrowth area', and invading spheroids in the lower surface of the invasion chamber were stained by crystal violet and recorded as the 'day 1 invasion area'. The spheroid size, outgrowth, and invasion areas were measured by Image J software (NIH Image; https://imagej.nih.gov/ij/) [40]. The relative spheroid outgrowth and invasion were calculated as:

$$relative\ \ outgrowth = \frac{day\ \ 1\ \ outgrowh\ \ area}{day\ \ 0\ \ area}$$

$$relative\ \ invasion = \frac{day\ \ 1\ \ invasion\ \ area}{day\ \ 0\ \ area}$$

2.7. Effect of bisphenols on spheroid outgrowth (direct co-culture model)

A direct co-culture model was used to study if bisphenol-treated decidualized stromal cells directly regulated spheroid outgrowth. Briefly, human primary endometrial stromal cells were decidualized for 9 days as described above. Next, $7x10^4$ decidualized stromal cells were seeded onto 8-well chamber slides in 5% csFBS phenol red-free DMEM/F12 medium with bisphenol treatments. After 24 hours, one Jeg-3 spheroid was added on top of the decidualized stromal cells in each well, and the size of spheroid was recorded as the original growth area. The spheroid outgrowth area at 24 and 48 hours was measured by Image J software [40]. Spheroid outgrowth was calculated as

$$spheroid\ \ outgrowth = \frac{day1/day2\ \ outgrowh\ \ area}{original\ \ area}.$$

2.8. Statistical analysis

All results were expressed as means ± SEM. Statistical comparisons between treatment groups were performed by one-way ANOVA using SPSS 20. Mann-Whitney U test was used when data was not normally distributed. A $p<0.05$ was considered statistically significant.

3. Results

3.1. Effects of bisphenols on the decidualization of primary human endometrial stromal cells

After 9 days of decidualization, the morphology of stromal cells changed from a fibroblast spindle-like shape to round cells with large nuclei (Figure 1A). The stromal cells with or without decidualization were stained positively with stromal cell marker vimentin (Figure 1A).

The expression of prolactin (PRL) and insulin-like growth factor binding protein 1 (IGFBP 1) transcripts were significantly increased in stromal cells on day 3, 6 and 9 of the decidualization process (Figure 1B, N=5) compared with non-decidualized controls. The expression of PRL or IGFBP1 transcripts in the decidualized cells reached a plateau on day 3 and remained stable until day 9. The levels of secreted PRL and IGFBP1 proteins in the culture media on day 3, 6 and 9 were quantified by ELISA. After normalizing with the total RNA in the sample, the amount of secreted PRL in the culture media increased from day 3 to 9, but the amount of IGFBP1 remained steady during the same period (Figure 1B, N=5).

To study the effects of bisphenol compounds on the decidualization of stromal cells, 10 μM of BPA, BPS, or BPF (this concentration is the highest dose that is non-toxic to Jeg-3 cells, see supplementary Figure 1) was added in the decidualization media for 9 days. The bisphenol treatments did not significantly affect the expression levels of PRL and IGFBP1 transcripts in the stromal cells or the secreted proteins in the spent media when compared with the DMSO control (Figure 1C, N=5).

3.2. Effect of bisphenol-treated decidualized stromal cells on trophoblastic spheroid outgrowth and invasion in the indirect co-culture model

To test if lower doses of bisphenol modulated decidualization and suppressed the outgrowth and invasion of Jeg-3 spheroids, stromal cells were cultured and decidualized with or without bisphenol treatments (1 nM to 10 μM BPA, BPS, or BPF), and then co-cultured with untreated Jeg-3 spheroids in the Matrigel-coated invasion chamber for 24 hours (Figure 2A). Non-decidualized stromal cells in the culture medium containing 5% csFBS were used as a control. After 24 hours, the outgrowth and invasion areas were stained with crystal violet for capture and quantification by Image J (Figure 2B).

The outgrowth area of spheroids with or without decidualized stromal cells or with DMSO in the culture media were comparable. The outgrowth of spheroids on decidualized stromal cells treated with 10 μM BPA was significantly increased (Figure 2C). Interestingly, the invasion of spheroids on the lower surface of the chamber was significantly increased with non-decidualized stromal cells relative to the medium control. Treatment with 10 μM BPA or 10 nM and 10 μM BPF, but not BPS, significantly increased the area of spheroid invasion (Figure 2C). The results from the indirect co-culture model suggest that decidualized stromal cells have limited invasion, but either BPA or BPF treatments could stimulate the invasion of trophoblastic cells when compared with non-decidualized stromal cells.

3.3. Effect of bisphenol-treated decidualized stromal cells on trophoblastic

spheroid outgrowth in the direct co-culture model

In the direct co-culture model, Jeg-3 spheroids were directly placed on top of decidualized stromal cells grown on an 8-well chamber slide with or without 10 μM bisphenol treatments (Figure 3A). Spheroid outgrowth on decidualized stromal cells treated with 10 μM BPA or BPF, but not BPS, significantly increased the spheroid outgrowth area on day 1 and 2, whereas no significant difference was found with non-decidualized stromal cells when compared with the control (Figure 3C, N=4).

3.4. Effect of bisphenol treatments on the expression of invasion molecules in the decidualized stromal cells

Decidualization of stromal cells had increased expression of PRL and IGFBP1 transcripts (Figure 1). Decidualized stromal cells treated with 10 μM BPA or BPF did not show increased expression of PRL and IGFBP1 transcripts ($p>0.05$) when compared with the control (Figure 4A); whereas non-decidualized stromal cells had decreased expression of PRL and IGFBP1 transcripts.

We evaluated some important molecules involved in trophoblast invasion including MMPs, uPA, LIF, and IL-15. The expression of MMP2 and LIF transcripts, but not uPA and IL-15, were significantly higher in non-decidualized stromal cells compared with decidualized cells with or without BPA or BPF treatment (Figure 4B), whereas MMP9 transcript was undetectable in stromal cells irrespective of decidualization status. Decidualized stromal cells treated with 10 μM BPA, but not BPF, had increased expression of LIF when compared with the decidualized control (Figure

4B). Bisphenols did not affect the expression of MMP2, uPA, or IL-15.

We evaluated some important anti-invasion molecules including TIMP1-4, PAI-1, TNFα, TGFβ1, TGFβ3, and IL-10. The expressions of TIMP1, TIMP4, TGFβ1 and TGFβ3 were significantly higher, whereas the expressions of TIMP3, PAI-1 and TNFα were significantly lower in non-decidualized stromal cells compared with decidualized stromal cells. No significant change in the expressions of TIMP2 and IL-10 was observed between non-decidualized and decidualized stromal cells (Figure 4C). Decidualized stromal cells treated with 10 μM BPA exhibited higher expression of TIMP4, but lower expressions of PAI-1, TNFα, and IL-10 when compared with untreated decidualized stromal cells (Figure 4C). Similarly, decidualized stromal cells treated with 10 μM BPF exhibited higher expression of TIMP4, but lower expressions of PAI-1 and TNFα when compared with untreated decidualized stromal cells (Figure 4C).

4. Discussion

We compared the effects of BPA, BPF, and BPS on stromal cell decidualization and the subsequent effects on trophoblast outgrowth and invasion, which are two important processes in the establishment of pregnancy in vivo. Stromal cell decidualization can be induced in vitro using various hormonal cocktails including progesterone, 8-Br-cAMP, and medroxyprogesterone acetate (MPA) [41]; estrogen, 8-Br-cAMP, and MPA [42]; 8-Br-cAMP and MPA [43]; and 0.5 mM 8-Br-cAMP and/or 10 nM estrogen and 1 μM progesterone [39, 44]. In this study, we used the latter method for stromal cell decidualization. Among the three chemicals used to induce stromal cell decidualization, cAMP is the most important, as it induces the expression of two decidual genes (PRL and IGFBP1) and activates the cAMP pathway, followed by progesterone, which helps to maintain the decidualized phenotype of the stromal cells [20]. Estrogen appears to be dispensable, as it is absent in several decidualization protocols [41, 43]. We found bisphenol treatments (BPA, BPF, and BPS) did not affect the expression of decidualization markers during the 9 days of decidualization. This is likely due to the fact that BPA has 100-1000 fold lower estrogenic activity than estrogen [45, 46], and similarly BPS and BPF also have weak estrogenic activity [47]. Therefore, we estimate that 10 μM of bisphenols would be required to produce a decidualization response comparable to that of 10 nM estrogen. Nonetheless, the effects of 10 μM bisphenols on stromal cell decidualization may be minimal compared with the effects of cAMP and progesterone, as observed in this study. Consistent with our findings, it was reported that 5-100 μM BPA did not affect the expression of decidual markers in

stromal cells induced by cAMP [48]. Another study found that BPA at supra-physiological concentrations (10 μg/mL and 20 μg/mL) significantly suppressed the expression of PRL and IGFBP1 in stromal cells decidualized by estrogen, progesterone, and cAMP [49]. Moreover, BPA altered the expression of decidual markers in stromal cells induced by estrogen and progesterone [30, 32].

The temporal and spatial regulation of trophoblast invasion into the endometrium not only relies on the invasion ability of trophoblast cells, but also on the expression of anti-invasive proteins in decidualized stromal cells during the invasion process [20]. In the present study, the invasion ability of trophoblastic spheroids in the co-culture was much higher than in the control medium alone, indicating that secretions from the decidualized stromal cells promote trophoblast invasion. It was reported that trophoblastic cells exposed to conditioned medium collected from decidualized stromal cells had increased invasion ability [24]. Interestingly, the invasion ability of trophoblastic spheroids was even higher when co-cultured with non-decidualized stromal cells. Consistently, the invasion capacity of mouse blastocyst was greater in non-pregnant uterus than in the pregnant decidualized uterus [50], which demonstrates an intrinsic inhibitory nature of decidualized stromal cells to embryo invasion. Furthermore, conditioned media collected from human first-trimester decidual cells suppressed the invasion of trophoblast cells through TGFβ and MMPs/TIMPs pathways in an amnion invasion assay and a type I collagen-coated Transwell invasion assay [51, 52]. The local production of factors such as TIMP1 and TGFβ are known to control trophoblast invasion [53]. In a co-culture model, invasion of extravillous

355 trophoblast HTR8/SVneo-derived spheroids in decidualized stromal cells was significantly lower compared to undifferentiated stromal cells [40]. Another study showed the invasion ability of trophoblast cells (Jeg-3 and ACH-3P) treated with conditioned medium from decidualized stromal cells was stronger than with control conditioned medium from non-decidualized stromal cells [24]. The discrepancies 360 among these studies demonstrate the difficulty in understanding the complex invasion process. More reliable in-vitro models need to be established for future investigations.

Decidualized stromal cells produce molecules that can promote or inhibit trophoblast invasion [24, 54]. The mechanism of how these molecules regulate trophoblast invasion remains largely unknown. High expression levels of gap junction 365 proteins (e.g., Connexin 43) in decidualized stromal cells prevent trophoblast invasion [40]. Likewise, secretory factors from decidualized stromal cells can change the expression of proteins in primary extravillous trophoblasts [26]. In fact, many secretory proteins from decidualized stromal cells are pro-invasion factors including IL-1β, IL-6, IL-8, IL-11, IL-15, IP-10, and LIF; or anti-invasion factors including IL-10, IL-12, 370 VEGF, GCSF, TNFα, and TGFβ [51, 54, 55]. In addition, the two decidualization markers, PRL and IGFBP1, can promote and inhibit trophoblast invasion in vitro, respectively [27, 56]. How the differential expressions of these molecules modulate trophoblast/spheroid invasion needs further investigation.

Decidualized stromal cells treated with BPA or BPF, but not BPS, promoted Jeg-375 3 spheroid invasion and outgrowth, suggesting that the regulatory function of decidualized stromal cells on trophoblast invasion was compromised by the BPA and

BPF treatments. The estrogenic activities of BPS, BPF and BPA were found to be comparable in breast cancer cells, MCF-7 and MELN (MCF-7 cells stably transfected with an estrogen responsive gene), and the relative estrogenic potency of BPF and BPS were 1.07-fold and 0.32-fold, respectively, compared with BPA [47]. The lower estrogenic activity of BPS might explain why it had no effect on regulating spheroid invasion in the present study. In a different co-culture model using first-trimester decidual stromal cells and primary trophoblasts, BPA inhibited the invasion of trophoblast cells by reducing the secretion of CXCL8 in decidual stromal cells [38]. Whether similar changes can be observed in the present co-culture models needs further investigation.

We found that BPA induced the expression of LIF (pro-invasion molecule), and suppressed the expression of PAI-1, TNFα, and IL-10 (anti-invasion molecules) in stromal cells. Interestingly, LIF promoted the adhesion of human first-trimester extravillous trophoblast cells and invasion of Jeg-3 cells through the STAT3 signaling pathway independent of the MMPs/TIMPs system [57, 58]. Also, TNFα suppressed the invasion of first-trimester placental explants by increasing the expression of PAI-1 [55], and inhibited the motility and invasion ability of first-trimester trophoblasts and Jeg-3 in vitro [59, 60]. Meanwhile, IL-10 inhibited cytotrophoblast invasion by suppressing the expression and activity of MMP9 [61, 62]. Most of these changes caused by BPA treatment favor the invasion process. Similarly, BPF suppressed the expression of several anti-invasion molecules (TIMP2, PAI-1, TNFα), which also increased the invasion rate of spheroids.

Accumulating evidence suggest that BPA is detrimental to female reproductive functions [28], whereas the effects of BPF and BPS are largely unknown. Treatment with BPA, BPS, and BPF had similar effects on the neural differentiations of mouse embryonic stem cells [63]. Neonatal exposure to BPS and BPA in rats delayed puberty onset, altered estrous cyclicity, reduced uterine weight, and increased serum estradiol concentration [64]. Neonatal exposure to BPA, but not BPS, in mouse accelerated onset of puberty, although both bisphenols increased serum estradiol concentration [65]. In mouse, prenatal BPS exposure had similar effect to BPA on female offspring leading to accelerated onset of puberty, abnormal estrous cyclicity, and reduced pregnancy rate [66]. Although BPA, BPS, and BPF share similar structures, they produce different effects in different cells, which requires further investigation. Pharmacokinetic studies suggest different bisphenols have different half-lives in the body [67] and, therefore, could have different effects on cellular responses.

In summary, bisphenol compounds BPA, BPF, and BPS did not affect stromal cell decidualization in vitro. Decidualized stromal cells treated with BPA and BPF, but not BPS, increased the invasion ability of trophoblastic spheroids, partly due to changes in the expression of pro-invasion and anti-invasion molecules. We know that decidualized stromal cells regulate the invasion of trophoblasts, but how bisphenols subsequently influence placental development and pregnancy outcomes requires further investigations.

Competing financial interest declaration

The authors declare no conflicts of interest.

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Figure Legends

Figure 1 Effect of bisphenols on the decidualization of human primary endometrial stromal cells. (A) Immunofluorescence images of endometrial stromal cells on day 9 with or without decidualization (control). Vimentin staining (green) was used to confirm stromal origin. The nuclei were counter-stained with DAPI (blue). (B) Expression of prolactin (PRL) and insulin-like growth factor binding protein 1 (IGFBP1) transcripts (top panel) in stromal cells. Expression of PRL and IGFBP1 proteins (bottom panel) in the culture medium of stromal cells normalized to the total RNA of the sample. (C) Expression levels of PRL and IGFBP1 transcripts (top panel) and proteins (bottom panel) in decidualized stromal cells treated with bisphenols (10 μM BPA, BPF, and BPS) for 3, 6, and 9 days. *statistically different from the control at $p<0.05$, one-way ANOVA.

Figure 2 Effect of bisphenol-treated decidualized stromal cells on spheroid outgrowth and invasion in the indirect co-culture model. (A) Schematic diagram illustrating the invasion chamber set-up for the spheroid outgrowth and invasion assays. (B) Representative images of spheroid outgrowth and invasion in the invasion chamber after 24 hours (Day 1). The outgrowth area is indicated by the yellow line. Spheroid (C) outgrowth and (D) invasion were quantified as the outgrowth area and area of stained cells in (B), respectively. *statistically different from the decidualized stromal cell control at $p<0.05$, one-way ANOVA.

Figure 3 Effect of bisphenol-treated decidualized stromal cells on spheroid outgrowth in the direct co-culture model. (A) Schematic diagram showing the direct co-culture model set-up for the spheroid outgrowth assay. (B) Representative images of spheroid outgrowth on top of the decidualized stromal cells on day 1 and 2. (C) Spheroid outgrowth on top of bisphenol-treated decidualized stromal cells on day 1 and 2 was quantified by analyzing the area of outgrowth (yellow line). *statistically different from the DMSO-treated decidualized stromal cell control at $p<0.05$, one-way ANOVA.

Figure 4 Effects of BPA and BPF on regulating the expression of invasion and anti-invasion factors in stromal cells with or without decidualization. (A) The expression of decidualization markers (PRL and IGFBP1) in stromal cells with or without BPA or BPF treatment were determined by qPCR. (B) The expression of pro-invasion (MMP2, uPA, LIF, and IL-15) transcripts in stromal cells with or without decidualization, and decidualized stromal cells with or without BPA or BPF treatment were determined by qPCR. (C) The expression of anti-invasion (TIMP1-4, PAI-1, TNFα, TGFβ1, TGFβ3, and IL-10) transcripts in stromal cells with or without decidualization, and decidualized stromal cells with or without BPA or BPF treatment were determined by qPCR. *statistically different from the decidualized stromal cell control at $p<0.05$, one-way ANOVA.

Supplementary Figure S1 Bisphenol compounds suppress Jeg-3 cell proliferation. Cell proliferation of Jeg-3 cells exposed to BPA, BPS, and BPF (0.01-100 μM) for 24 hours. One-way ANOVA; *, P<0.05 compared with control; #, P<0.05 compared with 100 μM BPA.

Figure 1

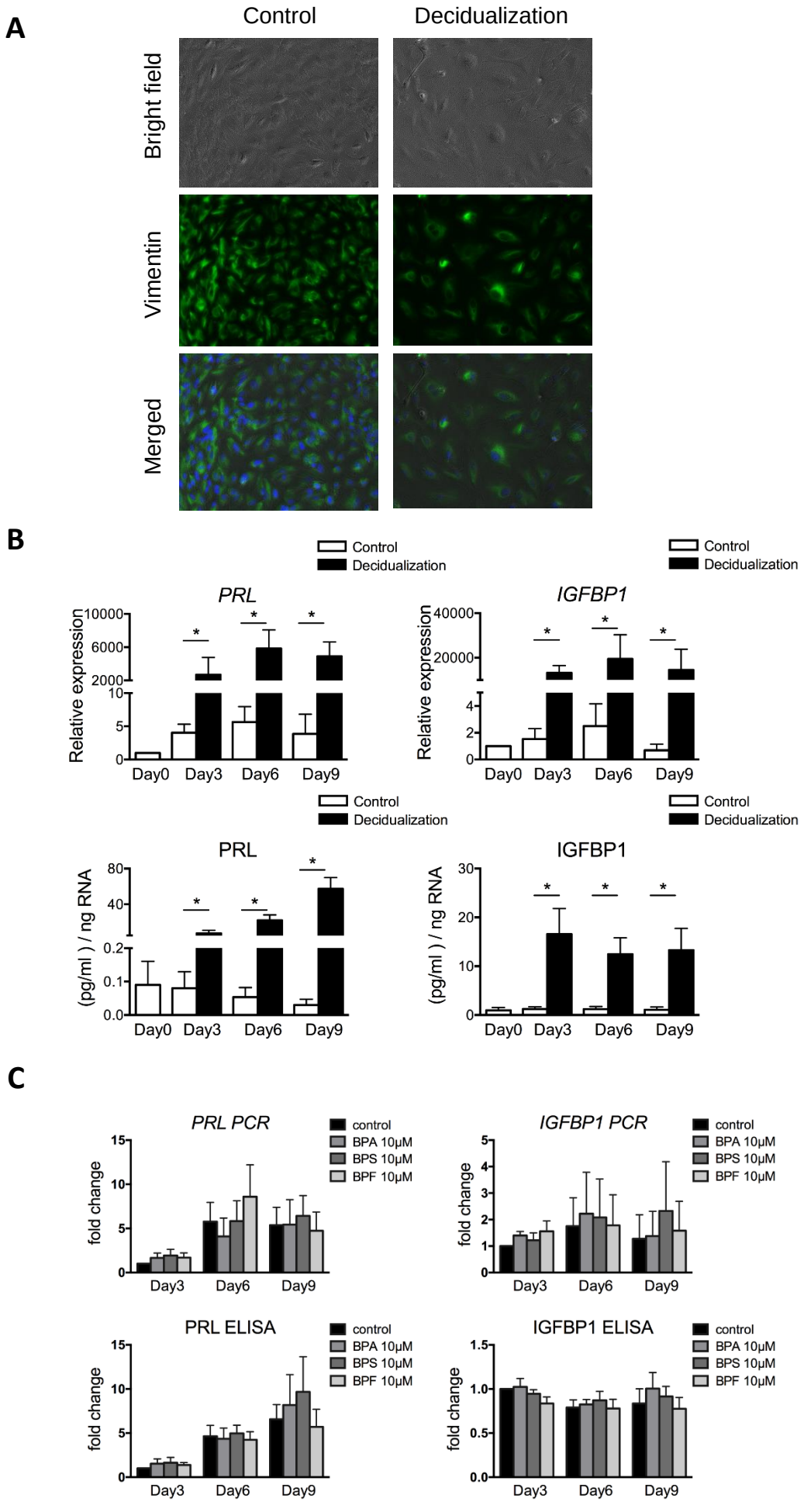
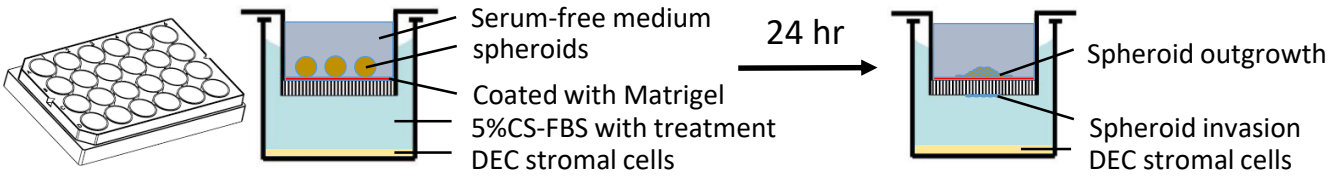
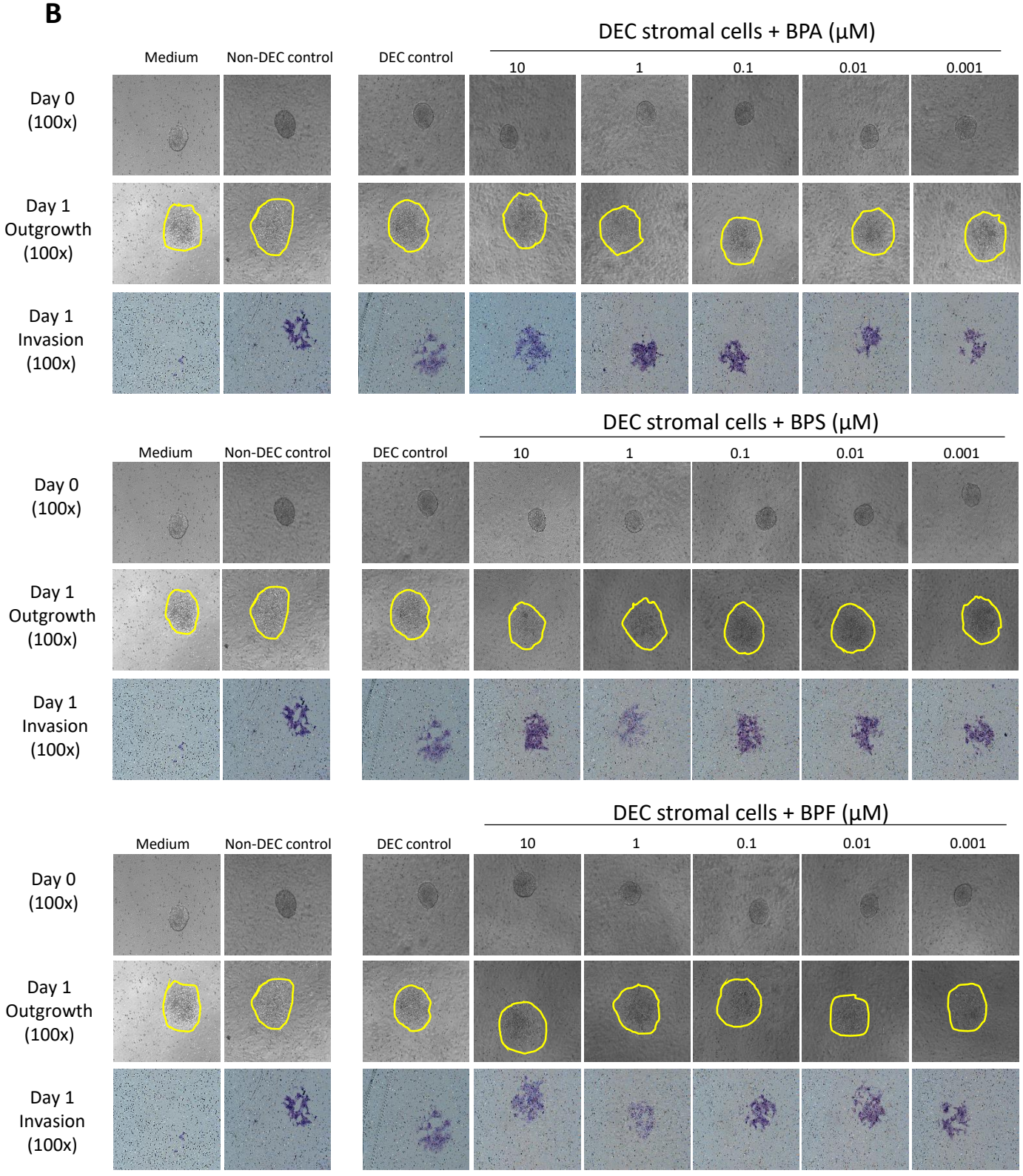

A
Control
Decidualization
Bright field
Vimentin
Merged
B
Control
Decidualization
PRL
IGFBP1
Relative expression
Day0
Day3
Day6
Day9
PRL
IGFBP1
(pg/ml) / ng RNA
C
PRL PCR
IGFBP1 PCR
PRL ELISA
IGFBP1 ELISA
fold change
control
BPA 10μM
BPS 10μM
BPF 10μM

Figure 2

A


Serum-free medium
spheroids
Coated with Matrigel
5%CS-FBS with treatment
DEC stromal cells
24 hr
Spheroid outgrowth
Spheroid invasion
DEC stromal cells

B


DEC stromal cells + BPA (μM)
Medium
Non-DEC control
DEC control
10
1
0.1
0.01
0.001
Day 0 (100x)
Day 1 Outgrowth (100x)
Day 1 Invasion (100x)
DEC stromal cells + BPS (μM)
Medium
Non-DEC control
DEC control
10
1
0.1
0.01
0.001
Day 0 (100x)
Day 1 Outgrowth (100x)
Day 1 Invasion (100x)
DEC stromal cells + BPF (μM)
Medium
Non-DEC control
DEC control
10
1
0.1
0.01
0.001
Day 0 (100x)
Day 1 Outgrowth (100x)
Day 1 Invasion (100x)

C

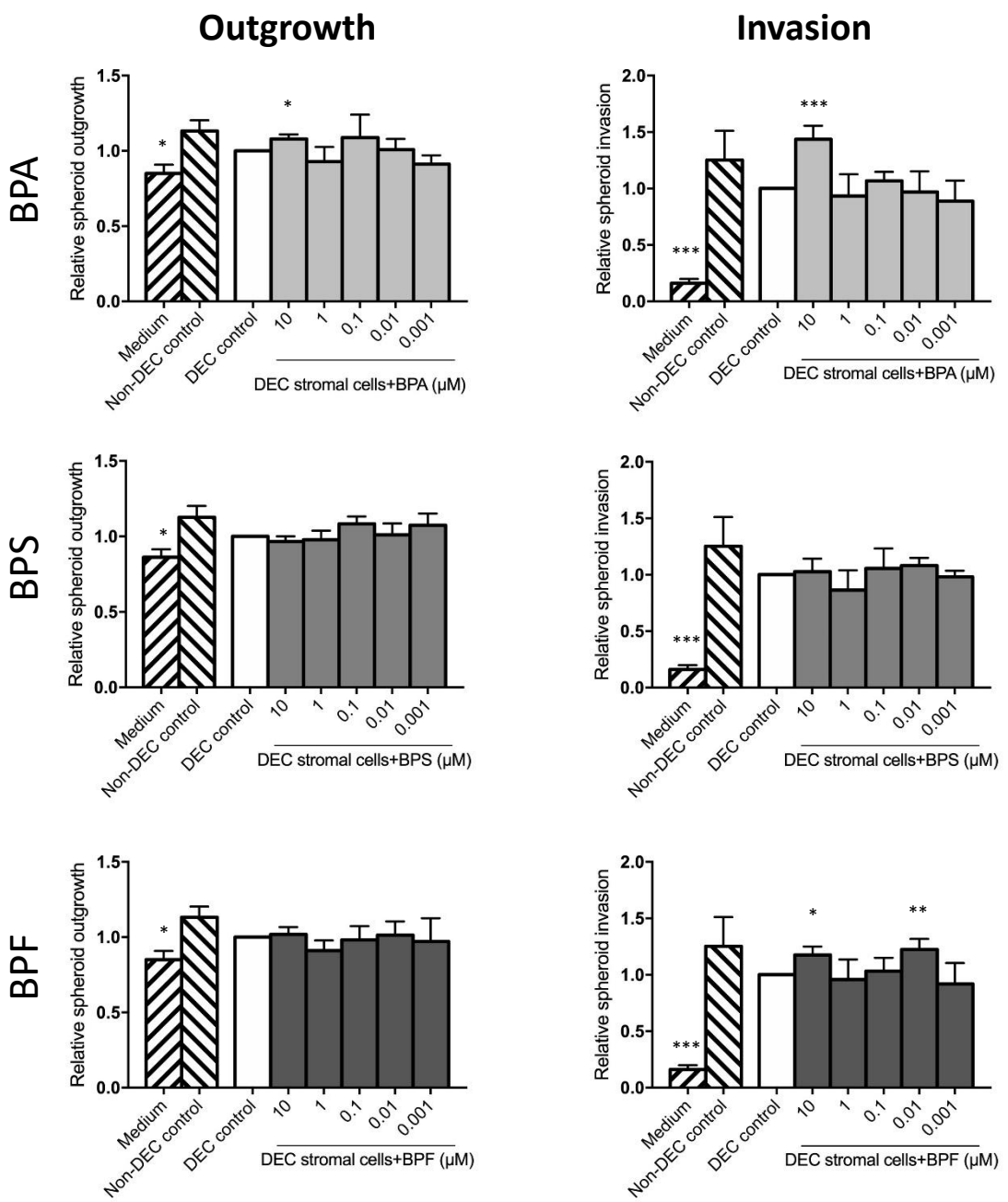

Outgrowth
Invasion
BPA
BPS
BPF
Relative spheroid outgrowth
Relative spheroid invasion
Medium
Non-DEC control
DEC control
10
1
0.1
0.01
0.001
DEC stromal cells+BPA (μM)
DEC stromal cells+BPS (μM)
DEC stromal cells+BPF (μM)

Figure 3

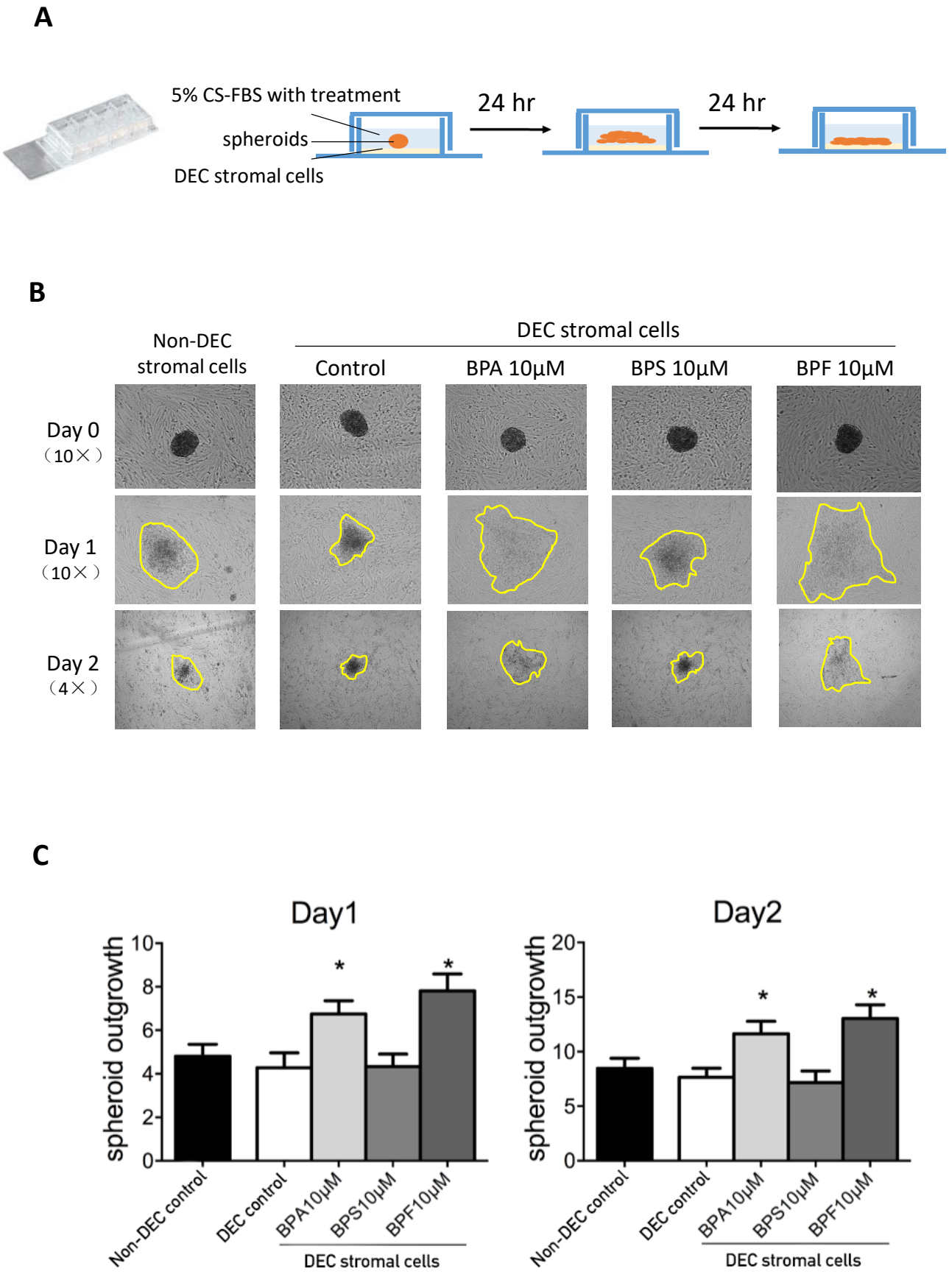
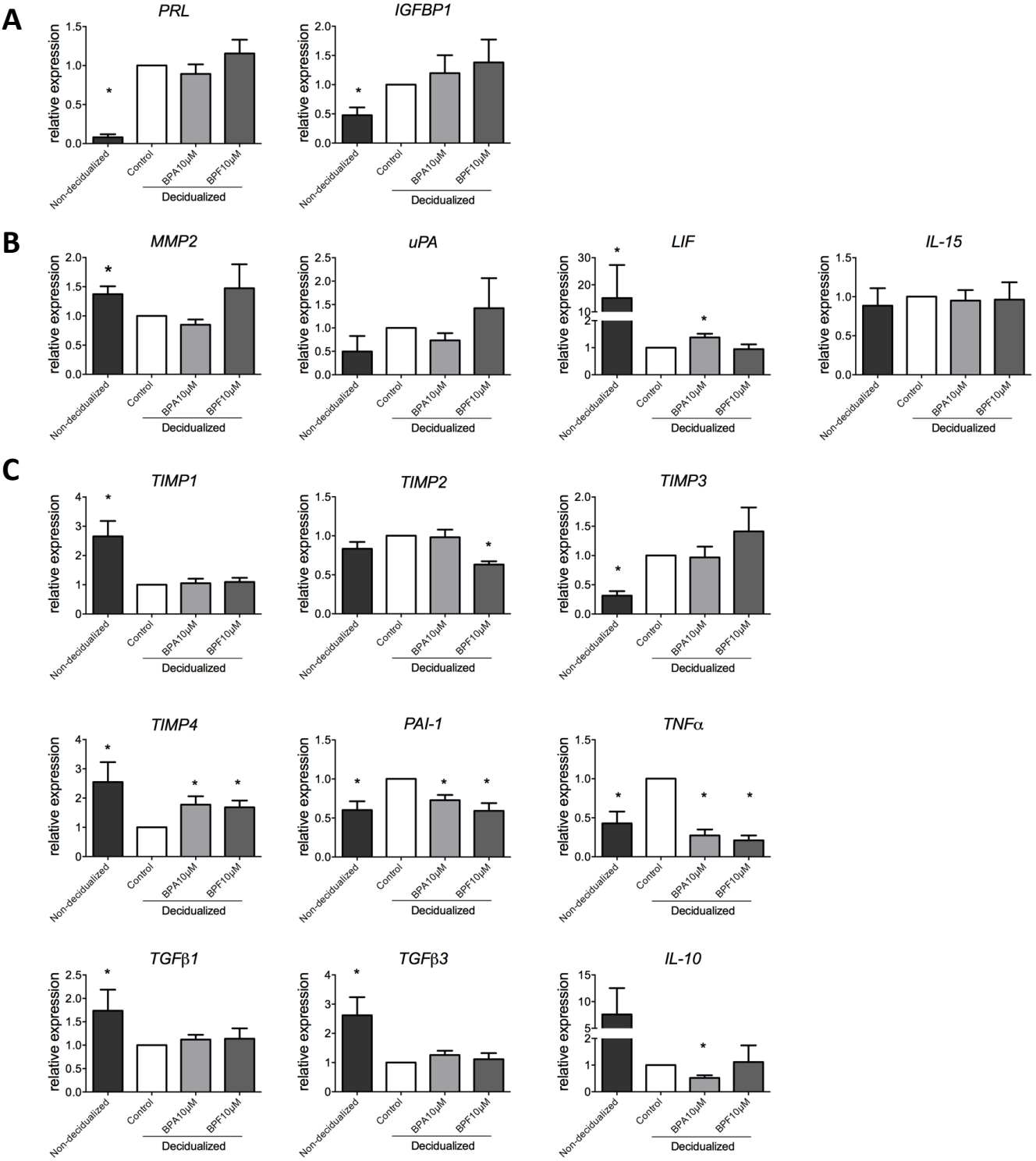

A
5% CS-FBS with treatment
spheroids
DEC stromal cells
24 hr
24 hr
B
Non-DEC stromal cells
DEC stromal cells
Control
BPA 10μM
BPS 10μM
BPF 10μM
Day 0 (10×)
Day 1 (10×)
Day 2 (4×)
C
Day1
Day2
spheroid outgrowth
Non-DEC control
DEC control
BPA10μM
BPS10μM
BPF10μM
DEC stromal cells

Figure 4


A
PRL
IGFBP1
B
MMP2
uPA
LIF
IL-15
C
TIMP1
TIMP2
TIMP3
TIMP4
PAI-1
TNFα
TGFβ1
TGFβ3
IL-10
relative expression
Non-decidualized
Control
BPA10μM
BPF10μM
Decidualized

Supplemental Figure 1