Natural and environmental oestrogens induce TGFB1 synthesis in oviduct cells

Barbara P S Cometti^{1,*}, Raghvendra K Dubey^{1,2,3}, Bruno Imthurn¹ and Marinella Rosselli¹

¹Department for Reproductive Endocrinology, Frauenklinik, ²Zurich Center for Integrative Human Physiology (ZIHP) University of Zurich, Zurich, Switzerland and ³Department of Pharmacology & Chemical Biology, University of Pittsburgh, Pittsburgh, Pennsylvania, USA.

Correspondence should be addressed to M Rosselli; Email: Marinella.Rosselli@usz.ch

*(B P S Cometti is now at R&D Scientific Affairs, IBSA Institut Biochimique SA, Pambio-Noranco, Switzerland)

Abstract

Autocrine/paracrine factors generated in response to 17 β -oestradiol (E2), within the oviduct, facilitate early embryo development for implantation. Since transforming growth factor beta 1 (TGFB1) plays a key role in embryo implantation, regulation of its synthesis by E2 may be of biological/pathophysiological relevance. Here, we investigated whether oviduct cells synthesize TGFB1 and whether E2 and environmental oestrogens (EOEs; xenoestrogens and phytoestrogens) modulate its synthesis. Under basal conditions, bovine oviduct cells (OCs; oviduct epithelial cells and oviduct fibroblasts; 1:1 ratio) synthesized TGFB1. E2 concentration-dependent induced TGFB1 levels in OCs and these effects were mimicked by some, but not all EOEs (genistein, biochanin A and 4-hydroxy-2',4',6'-trichlorobiphenyl, 4-hydroxy-2',4',6'-dichlorobiphenyl); moreover, EOEs enhanced ($P < 0.05$) the stimulatory effects of E2 on TGFB1 synthesis. The OCs expressed oestrogen receptors alpha and beta and aryl hydrocarbon; moreover, co-treatment with ER antagonist ICI182780 blocked the stimulatory effects of E2 and EOEs on TGFB1 synthesis. Treatment with non-permeable E2-BSA failed to induce TGFB1, thereby ruling out the involvement of membrane ERs. Cycloheximide (protein synthesis inhibitor) blocked E2-induced TGFB1 synthesis providing evidence for *de novo* synthesis. The stimulatory effects of E2 and EOEs, were inhibited ($P < 0.05$) by MAPK inhibitor (PD98059), whereas intracellular-Ca²⁺ chelator (BAPTA-AM) and adenylyl cyclase inhibitor (SQ22536) abrogated the effects of E2, but not EOEs, suggesting that post-ER effects of E2 and EOEs involve different pathways. Our results provide the first evidence that in OCs, E2 and EOEs stimulate TGFB1 synthesis via an ER-dependent pathway. Exposure of the oviduct to EOEs may result in continuous/sustained induction of TGFB1 levels in a non-cyclic fashion and may induce deleterious effects on reproduction.

Reproduction (2018) 155 233–244

Introduction

The oviduct plays a critical role in reproduction by providing an optimal microenvironment conducive for fertilization and initial stages of embryo development (Li & Winuthayanon 2017). Both rhythmic contraction and relaxation of oviduct smooth muscle cells and ciliary beats of oviduct epithelial cells are critically involved in transporting embryos and gametes (Li & Winuthayanon 2017). Multiple autocrine–paracrine factors generated within the oviduct regulate its function (Li & Winuthayanon 2017). For example, OC produce nitric oxide (Rosselli *et al.* 1998), hydrogen sulphide (Ning *et al.* 2014), prostaglandins (Yousef *et al.* 2016), thromboxane (Huang *et al.* 2004), cyclic AMP (Cometti *et al.* 2003), leukaemia inhibitory factor (LIF1; Reinhart *et al.* 1999, Krishnan *et al.* 2013), inflammatory cytokines, microRNAs (Ibrahim *et al.* 2015) and endothelin (Reinhart *et al.* 2003, Jeoung *et al.* 2010).

TGFB, a 25-kDa homodimer peptide growth factor, plays an important role in regulating growth, differentiation and metabolism in many mammalian cell types. It plays an important role in both adult and embryonic growth and development, inflammation and repair, including angiogenesis and regulation of host resistance mechanisms (Jones *et al.* 2006, Li 2014, Monsivais *et al.* 2017). TGFB induces both autocrine and paracrine effects (Monsivais *et al.* 2017) and plays an important role in reproduction/the reproductive system (Jones *et al.* 2006, Li 2014, Monsivais *et al.* 2017). TGFB has been detected in the human fallopian tube as well as in the human placenta, the human endometrial and embryonic tissues (Zhao *et al.* 1994, Jones *et al.* 2006, Li 2014, Monsivais *et al.* 2017). In human follicles, both theca and granulosa cells produce TGFB1 and TGFB2 (Monsivais *et al.* 2017), suggesting that it may be involved in the regulation of follicular growth and oocyte maturation (Monsivais *et al.* 2017). TGFB1 also

regulates endometrial proliferation and differentiation and is involved in the paracrine regulation of trophoblast–endometrium interaction (Monsivais *et al.* 2017). Importantly, decreased expression of TGFB1 in the uterus, during trophoblast invasion, results in failed embryo implantation (Singh *et al.* 2011, Monsivais *et al.* 2017). Both TGFB1 and TGFB2 produced at the human foetal–maternal interface plays a major regulatory role in the proliferation and differentiation of the trophoblast (Monsivais *et al.* 2017). Moreover, it regulates the local maternal immune response and prevents foetus rejection (Jones *et al.* 2006). Thus, TGFB is postulated to be necessary to maintain pregnancy. However, abnormal increases in TGFB are suggested to be a risk factor for recurrent miscarriages (Ogasawara *et al.* 2000) and in ectopic pregnancy, linked to tubal disorders (Shaw *et al.* 2010, Li *et al.* 2011).

Ovarian hormones like E2 regulate oviduct physiology. E2 influences oviduct function and the fertilization process by controlling the synthesis of autocrine/paracrine factors within the oviduct (Li & Winuthayanon 2017). In this context, we have previously shown that E2 induces the synthesis of LIF1 (Reinhart *et al.* 1999), which plays a key role in implantation. Moreover, we demonstrated that endothelin (ET1), a potent contracting and cell survival factor, is synthesized by bovine oviduct epithelial cells and induces the contraction of bovine oviduct segments (Reinhart *et al.* 2003, Jeoung *et al.* 2010).

Biological actions of E2 are largely mediated via nuclear oestrogen receptors (ER alpha and ER beta). However, non-genomic and non-nuclear receptors/mechanisms may also participate in mediating the pathophysiological actions of E2 (Rosselli *et al.* 2000, Hewitt *et al.* 2016). The endocrine effects of E2 can be mimicked as well as blocked by chemicals that are structurally similar to E2, bind to ERs and possess oestrogenic activity or modulate E2 metabolism by interacting with the aryl hydrocarbon receptor (AhR; Rosselli *et al.* 2000, Matthews & Gustafsson 2006, Shanle & Xu 2011). These oestrogen-like chemicals are termed EOE and are classified into two major categories: phytoestrogens (plant-derived oestrogens) and xenoestrogens (man-made oestrogenic chemicals).

Increasing evidence suggests that EOE act as endocrine disruptors and interfere with the reproductive process of humans and other species (Rosselli *et al.* 2000, Shanle & Xu 2011). However, the mechanisms involved remain undefined. The fact that some EOE induce oestrogenic effects, whereas others act as anti-oestrogenic (Rosselli *et al.* 2000, Shanle & Xu 2011), has further complicated the issue. Recent findings provide evidence that compared to E2, many EOE known to act as endocrine disruptors have a very low binding affinity for ER alpha and ER beta (Kuiper *et al.* 1998, Rosselli *et al.* 2000, Shanle & Xu 2011). This suggests that EOE may mediate their effects via alternative ER-independent

mechanisms such as modulating E2 metabolism (Rosselli *et al.* 2000, Matthews & Gustafsson 2006).

Locally synthesized factors play a decisive role in regulating oviduct function by providing an optimal microenvironment for the transport of gametes, the fertilization process and the development of an early embryo (Li & Winuthayanon 2017). We previously demonstrated that OCs produce/synthesize factors like LIF1 and ET1, and E2 stimulates their synthesis. Moreover, EOE like phytoestrogens and polychlorinated biphenyls (PCBs) mimicked the effects of E2 on LIF1 (Reinhart *et al.* 1999), whereas contrasting effects were observed on ET1 (Reinhart *et al.* 2003). Whether E2 regulates TGFB1 synthesis in the oviduct and whether EOE mimic the effects of E2 on TGFB1 synthesis remains unknown.

Accordingly, the aims of the present study were to determine the following: (1) whether bovine OC synthesize TGFB1; (2) whether TGFB1 synthesis is regulated by E2; (3) whether the effects of E2 are mediated via *de novo* synthesis; (4) whether the effects of E2 and EOE on TGFB1 synthesis are ER operated; (5) whether EOE modulate the effects of E2 on TGFB1 synthesis and (6) whether E2 and EOE influence TGFB1 synthesis via similar intracellular mechanisms.

Materials and methods

Mixed cultures

Oviducts of young, cyclic, non-pregnant cows were obtained from the local abattoir and the oviduct cells (OCs; mixed population of epithelial cells and fibroblast, 1:1 ratio) were cultured in Ham's F10 (Sigma, Chemie), containing 10% foetal calf serum (FCS, Sigma, steroid-free), according to our previously published method (Reinhart *et al.* 1999). Briefly, we used confluent monolayers of OCs cultured for 6–8 days. The mixed cell cultures of oviduct epithelial cells (OECs) and oviduct fibroblasts (OFCs) were characterized immunohistochemically as previously described (Reinhart *et al.* 1999). Monoclonal antibodies to epithelial cell cytokeratin (anti-cytokeratin AE1/AE3; Dako Diagnostics AG) and antibodies against fibroblast vimentin, (anti-vimentin VIM 3B4; Dako) were used to identify OECs and OFCs in culture. Stained cells were visualized using peroxidase, anti-peroxidase staining (Dako). TGFB1 antibodies (mouse monoclonal IgG; 25 µg/mL; R&D systems) with anti-mouse horseradish immunoperoxidase staining was used for immunostaining of TGFB1 in cultured OCs. Staining of OCs without primary antibodies was negative control.

Cells in primary cultures or first passage were used to assess TGFB1 synthesis. Prior to experiments with mixed cultures, preliminary studies in cultured oviduct fibroblasts (>97% purity) and epithelial cells (>90% purity) were conducted to assess TGFB1 production. Under basal conditions, OECs, oviduct fibroblasts (OFCs) and OCs (OECs+OFCs 1:1 ratio) produced TGFB1. In supernatants collected from cultured cells at 72 h, the TGFB1 levels were 611±95 pg/mg protein in OECs, 845±47 in OFCs and 709±68 in mixed OCs (Supplementary Fig. 1A, see section on supplementary data

given at the end of this article). The production of TGFB1 in different oviduct cell preparations was comparable, and treatment with E2 stimulated its synthesis by $\approx 3.36 \pm 0.5$ and 2.85 ± 0.24 -fold in OECs and OFCs, respectively. In OCs, the basal levels at 72 h ranged between 696 ± 14 and 785 ± 70 pg/mg protein in different oviduct preparations and was induced by 3.2 ± 0.35 -fold in response to E2 (200 ng/mL). Because autocrine/paracrine factors generated by both OECs and OFCs may regulate the physiology and biology of the oviduct, we decided to use the mixed OC culture system to analyse the effects of natural and EOE on TGFB1 synthesis.

Oviduct fibroblast cultures

Bovine OFCs were isolated, characterized and cultured as previously described by us (Reinhart *et al.* 1999). For all assays, we used cells in primary cultures or first passage.

Treatment protocol for TGFB1 synthesis by OCs

Sub-confluent monolayers of bovine OCs were washed with HBSS and fed with Dulbecco's Modified Eagle Medium (DMEM)/Ham's F12 (Gibco), supplemented with 1% charcoal-stripped FCS (Sigma). After 3 days, we collected the culture medium and assayed immunoreactive TGFB1. To investigate whether TGFB1 synthesis is time dependent, we measured the TGFB1 level in conditioned media, collected at 24, 48 and 72 h from OC cultures. To assess the regulation of TGFB1 synthesis by E2 and EOE, OCs were treated for 72 h in DMEM/Ham F12 medium (Sigma), supplemented with 1% steroid-free FCS and containing or lacking E2 (0.2, 2, 20, 100, 200 ng/mL; Sigma); genistein (200 ng/mL; Extrasynthèse, France); biochanin A (200 ng/mL; Extrasynthèse); 4: 2,4,6-trichlorobiphenyl (TCB, 200 ng/mL, AccuStandard); 4-hydroxy-2',5'-dichlorobiphenyl (4-OH-DCB, 200 ng/mL, AccuStandard) or 4-hydroxy-2',4',6'-trichlorobiphenyl (4-OH-TCB, 200 ng/mL, AccuStandard). To study whether basal and E2-stimulated increase in TGFB1 is due to *de novo* synthesis, OCs were treated for 72 h with E2 (200 ng/mL) in the presence or absence of cycloheximide (10 μ M, a protein synthesis inhibitor). To test whether the effects of E2 and EOE are mediated via ERs, OCs were treated with E2, genistein, biochanin A, TCB, 4-OH-DCB and 4-OH-TDB (200 ng/mL) in the presence of 1–10 μ M ICI182720 (ER antagonist; Tocris, Bristol, UK). The rationale for using 1 and 10 μ M of ICI was based on the findings that the relative binding affinities of E2 and ICI182780 for ERs are 1 and 0.89, respectively. Moreover, the inhibitory potency (IC_{50}) is 0.29 nM. Since EOE have very low affinity for ERs as compared to E2 (Kuiper *et al.* 1998), together with the fact that 10-fold higher concentrations of ICI have been shown to completely block the ER-activating effects of E2, we elected to use a concentration of 1 and 10 μ M for the experiments. Moreover, to analyse the possible role of the membrane ERs, OCs were treated with the same concentration of E2 tagged to BSA, which is membrane impermeable (200 ng/mL of E2 is equivalent to 1180 ng/mL E2-BSA, Sigma). Finally, to assess the involvement of intracellular mechanisms in mediating the effects of E2 and EOE on TGFB1 synthesis, we used BAPTA-AM (a membrane-permeable calcium chelator, 1 μ M,

Sigma), SQ22536 (adenylyl cyclase inhibitor, 500 μ M, Sigma) and PD98059 (MAPK inhibitor, 20 μ M, Tocris Cookson LTD, Bristol, UK). For each experiment, the conditioned medium was collected to analyse TGFB1 levels, whereas the remaining cells solubilized in 0.2% SDS and the protein levels were measured using BCA-protein assay kit (Soccochim SA, Lausanne, Switzerland). All experiments were in triplicate using three separate cultures derived from different pools of fresh oviducts.

TGFB1 ELISA

The presence of immune-reactive TGFB1 in conditioned medium (200 μ L aliquots) of OCs was analysed using an ELISA Kit (Quantikine, human TGFB1 immunoassay; R&D Systems). Briefly, 200 μ L of activated samples (500 μ L samples treated sequentially for 10 min with 100 μ L 1 M HCl, and 10 min with 100 μ L 1.2 M NaOH/0.5 M Hepes) were added to a microplate coated with recombinant human TGFB sRII and incubated at RT for 3 h. Subsequently, the plate was washed several times and 200 μ L of polyclonal antibody against TGFB1 conjugated to horseradish peroxidase was added and further incubated at RT for 90 min. After washing the plate, 200 μ L of substrate solution containing stabilized hydrogen peroxide and chromogen (tetramethylbenzidine) was added. After incubating at RT for 20 min, the reaction was terminated by adding 50 μ L of 1 M sulphuric acid. The optical density was determined using a microplate reader, set to 450 nm, with a wavelength correction, set to 540 nm. According to the manufacturer's specification, the minimal detectable concentration of TGFB1 was less than 7 pg/mL. TGFB1 concentrations were estimated using a standard curve run under identical conditions. TGFB1 concentrations were normalized against total cell protein and the results are presented as percent of control.

Western blotting

Western blotting was used to further confirm the modulatory effects of E2 and EOE in OCs. Briefly, OCs treated were treated with E2, genistein, biochanin A and 4-OH-TCB (200 ng/mL) in the presence or absence of 10 μ M ICI182720 (ER antagonist). After 72 h, the cells were lysed using 70 μ L of cell lysis buffer and combined with trichloroacetic acid (TCA; 10%) precipitated conditioned medium fraction of respective samples. Sample aliquots containing 25 μ g protein were electrophoretically resolved on a SDS gel under non-reduced conditions and separated proteins were transferred onto nitrocellulose. After blocking with 5% milk in PBS-Tween (Sigma), to prevent unspecific binding, the membranes were incubated with primary antibody for TGFB1 (TGFB1, mouse monoclonal; Santa Cruz Biotechnology; dilution 4 μ g/mL). The membranes were washed and subsequently treated with the corresponding secondary antibody (goat anti-mouse IgG HRP peroxidase (1:5000 dilution)). After washing, the bands were determined using Super signal west dura luminol substrate (Pierce) and Hyperfilm ECL films (Amersham). Changes in TGFB1 were normalized to β -actin (Sigma; 1:5000 dilution), using the LI-COR system. Briefly, membranes were incubated for 5 min with Newblot Nitro Stripping buffer and then washed

3 × 10 min in PBS. Anti-β-actin mouse was obtained from Sigma (1:5000 dilution). IR Dye 800-conjugated Anti-mouse IgG LI-COR was from Biosciences 1:12,500. Changes in protein expression were analysed by measuring optical density using ImageJ software and changes in protein expression presented as optical density ratio between TGFβ1 and β-actin.

To assess whether E2 and EOE activate MAPK pathway, OCs grown for 24 h in medium devoid of serum were treated for 15 min with E2, genistein, biochanin A and 4-OH-TCB (200 ng/mL) in the presence or absence of 10 μM ICI182720 (ER antagonist). The cells were lysed, resolved electrophoretically and probed for phosphorylated and non-phosphorylated MAPK using phosphospecific anti-MAPK mouse monoclonal antibody (phosphor-p44/42 MAPK; Thr202/Tyr204; Cell Signaling) and anti-p44/42 MAPK (ERK1/2) antibody (source rabbit; dilution 1:1000; Cell Signaling). Corresponding HRP-linked secondary antibody was used for detection. Changes in protein expression were analysed by measuring optical density using ImageJ software. Changes in protein expression presented as optical density ratio between phosphorylated MAPK (MAPK-P) and non-phosphorylated MAPK.

Receptor expression studies

The expression of ERs and the AhR in OCs was assessed using Western blotting. Briefly, OC lysates were probed with antibodies to ER alpha, ER beta or AhR, respectively. Antibodies for ER alpha (purified antiserum to human ER alpha), ER beta (purified antiserum to human ER beta) and AhR (rabbit polyclonal IgG against human AhR protein, which recognized AhR at 96 and 122 kDa) were from Alexis, and diluted 1:1000 in buffer containing 1% milk, PBS, 0.2% Tween-20. The secondary biotinylated antibodies (ImmunoPure goat Anti-Rabbit IgG, peroxidase conjugated, Pierce) diluted to 1:25,000 in 1% milk, PBS, Tween 20 0.2% buffer, during 1 h at RT.

Statistical analysis

Data are presented as a mean ± S.E.M. of experiments conducted in triplicates ($n=3$). Statistical analysis was performed using ANOVA or Fisher's least significant test, as appropriate. A value of $P<0.05$ was considered statistically significant.

Results

Effects of E2 on TGFβ1 synthesis

We first established the optimal conditions for TGFβ1 synthesis by the OCs (OECs plus OFCs; 1:1 ratio). They were used for all experiments unless specified otherwise. Figure 1A depicts OFCs positively stained with vimentin (brown staining marked a; negatively stained epithelial cells labelled b) in a representative OC culture. Western blotting of lysates (Fig. 1B) collected from these cultures provides evidence for the presence of ERs alpha and beta, as well as the AhR (Fig. 1B right panel; Supplementary Fig. 1B for original un-cut blot). Top panel of Fig. 1C shows the oviduct fibroblasts

Figure 1 Panel A: Depicts representative photomicrographs (40× mag) of bovine oviduct cells (mixed cultures of oviduct fibroblasts plus oviduct epithelial cells; primary cultures) with positive immunohistochemical labelling of oviduct fibroblasts with monoclonal antibodies against fibroblast vimentin (anti-vimentin VIM 3B4) marked with an arrow (a) and negatively stained epithelial cells (b). Panel B: Left panel depicts representative Western blots depicting the expression of ERs alpha and beta in lysates from bovine oviduct cells. Right panel shows representative Western blot for the expression of aryl hydrocarbon (AhR) receptors in lysates from bovine oviduct cells. Panel C: Top panel shows representative photomicrograph of oviduct fibroblasts (middle panel) and epithelial cells (right panel) with positive immunostaining for TGFβ1, whereas the left panel depicts the negative control. Lower panel depicts bar graph time-dependent synthesis of TGFβ1 by confluent monolayers of bovine oviduct cells. TGFβ1 levels were measured in conditioned medium collected at 0, 24, 48 and 72 h of culture. The graph represents mean of three different experiments. Data (mean ± S.E.M.) are expressed as pg/mg protein (* $P<0.05$ vs 0h).

(middle panel) and epithelial cells (right panel) positively stained for TGFB1. The left panel depicts the negative control (neg-C). In cultured OCs, the level of TGFB1 increased in a time-dependent fashion and was 581.8 ± 13.3 , 720.4 ± 14.3 and 785.7 ± 70.7 pg/mg protein after 24, 48 and 72 h ($P < 0.05$ vs 0h), respectively (Fig. 1C). Under similar culture conditions, we also observed production of immunoreactive TGFB1 in pure OECs and OFCs (Supplementary Fig. 1A). To mimic the physiologic conditions within the oviduct, we used OCs to study the influence of test agents on TGFB1 production.

Treatment of OCs with E2 (0.2, 2, 20, 100, 200 ng/mL) for 3 days increased TGFB1 levels in a concentration-dependent manner (Fig. 2A). The lowest concentration of E2 that significantly increased TGFB1 levels was 100 ng/mL. At this concentration, E2 increased TGFB1 levels from 100% to $213 \pm 15\%$ ($P < 0.05$ compared to vehicle-treated control; Fig. 2A). To assess whether increases in TGFB1 levels was solely due to *de novo* synthesis and not due to a release of stored intracellular proteins, OCs were treated with E2 in the presence of cycloheximide, a selective protein synthesis inhibitor. Treatment of OCs with cycloheximide significantly inhibited basal and E2 (200 ng/mL)-stimulated synthesis of TGFB1 (Fig. 2B), suggesting that E2-induced TGFB1 production is due to *de novo* synthesis.

The biological effects of E2 are mediated via ERs alpha and beta (Levin *et al.* 2009, Hewitt *et al.* 2016); moreover, its actions can be modulated by ligands (EOEs or endocrine disruptors) for aryl hydrocarbon (AhR) receptors (Matthews & Gustafsson 2006, Shanle & Xu 2011). Western blot analysis of cultured OC lysates provided evidence for the presence of both ERs alpha and beta as well as AhR (Fig. 1B). To assess whether the stimulatory effects of E2 were ER mediated, we tested its effects in the presence and absence of ICI182780, an ER antagonist that binds with equal affinity to both ER alpha and beta (Kuiper *et al.* 1998). As shown in Fig. 3A, ICI182780 abrogated the stimulatory effects of E2 on TGFB1 production in a concentration-dependent manner, suggesting the actions are ER mediated.

Recent studies provide evidence that E2 mediates its action via nuclear as well as membrane ER (Levin 2009). To assess whether E2 stimulates TGFB1 synthesis via membrane ER, we treated OCs with membrane impermeable BSA-tagged E2. As shown in Fig. 3B, equimolar concentrations of E2 tagged to BSA failed to induce TGFB1 synthesis suggesting that E2 mediates its stimulatory actions via nuclear and non-membrane ER.

Effects of phytoestrogens on TGFB1 synthesis

Similar to E2, genistein (200 ng/mL) significantly induced TGFB1 synthesis in OCs ($P < 0.05$ vs control cells treated

Figure 2 Panel A: Bar graph showing the concentration-dependent effects of 17β-oestradiol (0.2–200 ng/mL) on the TGFB1 synthesis by cultured oviduct cells. Data (mean $\pm$ S.E.M.) represent the mean of three different experiments ($n = 3$, in triplicates). All values were normalized to total protein concentration and the amount of TGFB1 synthesized is expressed as percent (%) of control (* $P < 0.05$ vs control; Cont). Panel B: Bar graph showing the modulatory effects of cycloheximide (CHX; 10 μ M) on 17β-oestradiol (E2; 200 ng/mL)-induced synthesis of TGFB1 by cultured bovine oviduct cells treated for 72 h. Data (mean $\pm$ S.E.M.) represents mean of three different experiments ($n = 3$, triplicates). The values were normalized to total protein concentration and expressed as pg/mg protein (§represents significant inhibition, $P < 0.05$ vs control or E2; * $P < 0.05$ vs control).

with vehicle). Treatment with genistein (200 ng/mL) increased TGFB1 synthesis from 100% in controls to $191 \pm 19\%$. Similar to genistein, TGFB1 synthesis was significantly ($P < 0.05$ vs untreated cells) increased in OCs treated with 200 ng/mL biochanin A, a precursor of genistein (Fig. 4A). The stimulatory effects of both genistein and biochanin A on TGFB1 synthesis were completely blocked in the presence of ER antagonist ICI182780 (Fig. 4A), suggesting that their effects were ER mediated.

Figure 3 Panel A: Bar graph showing the concentration-dependent inhibitory effects of ER antagonist ICI182780 (ICI; 1 and 10 μM) on 17β-oestradiol (E2; 200 ng/mL) induced TGFβ1 synthesis in oviduct cells treated for 72 h. Data (mean ± s.e.m.) represents the mean of three different experiments ($n=3$, in triplicates) and values were normalized to total protein concentration. The amount of TGFβ1 synthesized is expressed as percent (%) of control (* $P<0.05$ vs control, Cont; § $P<0.05$ vs E2 alone or -ICI). Panel B: Bar graph comparing the effects of 200 ng/mL of 17β-oestradiol (E2) tagged with or without BSA (E2 or E2-BSA), on TGFβ1 synthesis by cultured bovine oviduct cells. E2, but not E2-BSA, induced TGFβ1 formation. Data (mean ± s.e.m.) represent the mean of three different experiments ($n=3$, triplicates) and values were normalized to total protein concentration. The amount of TGFβ1 synthesized is expressed as percent (%) of control (Con; * $P<0.05$ vs control).

Effects of xenoestrogens/PCBs

To evaluate the effects of PCBs on TGFβ1 synthesis, we treated OCs with 200 ng/mL of TCB, 4-OH-TCB and 4-OH-DCB. Treatment with 4-OH-TCB, but not TCB and 4-OH-DCB, induced TGFβ1 production. The PCB 4-OH-TCB was as potent as E2 and increased TGFβ1 production from 100% in control to 275 ± 25.5% at a

Figure 4 Bar graph showing the modulatory effects of ICI182780 (1 and 10 μM), on: Panel A: 17β-oestradiol (E2, 200 ng/mL), genistein (200 ng/mL), biochanin A (200 ng/mL), and panel B: TCB (200 ng/mL), 4-OH-TCB (200 ng/mL) and 4-OH-DCB (200 ng/mL) stimulated synthesis of TGFβ1 by cultured oviduct cells, treated for 72 h. Data (mean ± s.e.m.) represents the mean of three different experiments ($n=3$, in triplicates) and values were normalized to total protein concentration. The amount of TGFβ1 synthesized is expressed as percent (%) of control (* $P<0.05$ vs control; § $P<0.05$ vs -ICI182780).

concentration of 200 ng/mL (Fig. 4B). Moreover, the effects of 4-OH-TCB (200 ng/mL) on TGFβ1 synthesis were blocked by ICI182780 (Fig. 4B), suggesting that they are ER mediated.

Modulation of TGFβ1 synthesis by E2 and EOE confirmed by Western blotting

Although the ELISA assay is well established to accurately assess TGFβ1 levels, Western blotting was also performed to rule out non-specific interference. Consistent with our observations with ELISA, treatment of OCs with 200 ng/mL E2, 4-OH-TCB, genistein or biochanin A induced TGFβ1 expression, and this stimulatory effect was abrogated in the presence of 10 μM ICI182780 (Fig. 5).

Modulatory effects of EOE plus E2

To investigate whether the effects of E2 on TGFβ1 synthesis were influenced in the presence of phytoestrogens and PCBs, we studied the effects of E2 (200 ng/mL) in the presence and absence of 200 ng/mL of genistein, biochanin A, TCB, 4-OH-DCB and 100 ng/mL 4-OH-TCB. As shown in Fig. 6A and B, EOE

Figure 5 Top panel depicts a representative Western blot showing the modulatory effects of effects 17β-oestradiol (E2, 200 ng/mL), genistein (200 ng/mL), biochanin A (Bio-A; 200 ng/mL), 4-OH-TCB (200 ng/mL) on TGFβ1 synthesis by cultured oviduct cells, treated for 72 h in presence and absence of 10 μM ICI182780. Lower panel shows the changes in optical density from the contrast adjusted blot using ImageJ program. The data depicts change in TGFβ1/β-actin ratio from three separate experiments (* $P < 0.05$ vs control; § $P < 0.05$ vs -ICI182780).

significantly increased the stimulatory effects of E2 on TGFβ1 production.

Role of Ca²⁺, PKA and MAPK on E2 and EOE-induced TGFβ1 synthesis

The mechanisms mediating the effects of E2 and EOE on TGFβ1 synthesis remains unclear. To study some of these possible pathways, cells in the first passage were treated with BAPTA-AM (membrane-permeable Ca²⁺ chelator, 1 μM), SQ22536 (adenylyl cyclase inhibitor, 500 μM) and PD98059 (MAPK inhibitor, 20 μM). All the compounds showed a partial but significant ($P < 0.05$) inhibition of E2-induced TGFβ1 synthesis (Fig. 7). However, in contrast to E2, the stimulatory effects of phytoestrogens and PCBs were only inhibited by the MAPK inhibitor PD98059, suggesting that their effects are specifically mediated via the MAPK pathway and not via intracellular calcium release or the PKA pathway (Fig. 8).

Western blotting was performed to confirm the involvement of MAPK phosphorylation in mediating the stimulatory actions of E2 and EOE on TGFβ1 production. As shown in Fig. 9, treatment with 200 ng/

Figure 6 Panel A: Bar graph showing the modulatory effects of 17β-oestradiol (E2; 200 ng/mL), on genistein (200 ng/mL), biochanin A (Bio-A; 200 ng/mL) and (Panel B) on E2 (200 ng/mL), TCB (200 ng/mL), 4-OH-TCB (100 ng/mL) and 4-OH-DCB (200 ng/mL) stimulated synthesis of TGFβ1 by cultured bovine oviduct cells treated for 72 h. Data (mean ± S.E.M.) represent the mean of three different experiments ($n = 3$, triplicates) and values were normalized to total protein concentration. The amount of TGFβ1 synthesized is expressed as percent (%) of control (* $P < 0.05$ vs control). § $P < 0.05$ vs control (Con), § $P < 0.05$ vs respective treatment in absence of E2 (-E2).

mL of E2, 4-OH-TCB, genistein or biochanin A-induced phosphorylated MAPK, and this effect was blocked in OCs co-treated with 10 μM ICI182780 (Fig. 9). These findings provide evidence that E2 and EOE activate MAPK-P via ER-dependent mechanism.

To assess whether modulation of E2-induced TGFβ1 production by various mechanisms (cyclic AMP, intracellular Ca²⁺, MAPK) are associated with or independent of ERs, OCs were treated with BAPTA-AM, SQ22536 or PD98059 in the presence and absence of ICI182780. The attenuating effects of ICI182780 were not further enhanced by BAPTA-AM, SQ22536 and PD98059, suggesting that the stimulatory effects of E2 are mediated via a common pathway involving ER-dependent activation of intracellular calcium release, adenylyl cyclase and the MAPK pathway (Supplementary Fig. 2A).

Figure 7 Bar graph showing the modulatory effects of BAPTA-AM (1 μ M), SQ22.536 (500 μ M) and PD98059 (20 μ M) on 17 β -oestradiol (E2; 200 ng/mL) stimulated synthesis of TGF β 1 by cultured bovine oviduct cells treated for 72 h. Data (mean $\pm$ S.E.M.) represent the mean of three different experiments ($n=3$, triplicates) and values were normalized to total protein concentration. The amount of TGF β 1 synthesized is expressed as percent (%) of control (* $P<0.05$ vs control (C), § $P<0.05$ vs +E2).

Effects in OFCs

Our results from cultured OFCs (>90%) were comparable to those obtained in OCs. As shown in Fig. 10, E2 (200 ng/mL), genistein (200 ng/mL), biochanin A (200 ng/mL), as well as 4-OH-TCB (200 ng/mL), significantly induced ($P<0.05$) TGF β 1 levels. These effects were partially (in cells treated with E2) or completely reversed (in cells treated with all other compounds) by the ER antagonist ICI182780 (1 and 10 μ M; Fig. 10). We also observed

Figure 8 Bar graph showing the modulatory effects of BAPTA-AM (1 μ M), SQ22536 (500 μ M) and PD98059 (20 μ M) on environmental oestrogen's induced TGF β 1 synthesis in cultured bovine oviduct cells. Cells were treated for 72 h with genistein (200 ng/mL) or 4OH-TCB (200 ng/mL) in the presence or absence of BAPTA-AM, SQ22536 or PD98059. Data (mean $\pm$ S.E.M.) represent the mean of three different experiments ($n=3$, triplicates) and values were normalized to total protein concentration. The amount of TGF β 1 synthesized is expressed as percent (%) of control. * $P<0.05$ vs genistein or 4OH-TCB, respectively, in controls, § $P<0.05$ vs +genistein or +4OH-TCB, respectively, in control.

Figure 9 Top panel depicts a representative Western blot, showing the modulatory effects of effects 17 β -oestradiol (E2, 200 ng/mL), genistein (200 ng/mL), biochanin A (200 ng/mL), 4-OH-TCB (200 ng/mL) on MAPK activation (MAPK-P) by cultured oviduct cells, stimulated in presence and absence of 10 μ M ICI182780. Lower panel shows the changes in optical density from contrast adjusted blot using ImageJ program. The graph depicts change in ratio between phosphorylated and non-phosphorylated MAPK-P/MAPK from three separate experiments (* $P<0.05$ vs control; § $P<0.05$ vs -ICI182780).

similar effects in oviduct epithelial cells (Supplementary Fig. 2B).

Discussion

In the present study, we provide evidence that bovine OCs synthesize TGF β 1 under basal conditions. E2 stimulates TGF β 1 production in OCs via ER and involves *de novo* protein synthesis. The stimulatory actions of E2 are mimicked by xenoestrogens (PCBs; TCBS, 4-OH-TCB and 4-OH-DCB) and phytoestrogens (genistein, biochanin A). Moreover, EOs enhance the actions of E2 on TGF β 1 synthesis in an additive fashion. E2 stimulates TGF β 1 production in OCs by regulating intracellular calcium, cyclic AMP and MAPK, whereas EOs induce TGF β 1 solely via MAPK, suggesting that the natural mechanisms and the mechanisms via which EOs induce TGF β 1 are different. Our finding that EOs modulate local synthesis of TGF β 1 within the oviduct suggests that it may play an important role in regulating the biology and physiology of the oviduct associated with early embryo development. More importantly, in contrast to the cyclic effects of E2 on TGF β 1 synthesis, continuous exposure to EOs could induce TGF β 1 levels in a non-cyclic fashion and may induce deleterious effects on the reproductive system.

Figure 10 Top panel: Bar graph showing the modulatory effects of ICI182780 (1 µM and 10 µM) on 17β-oestradiol (E2; 200 ng/mL), genistein (200 ng/mL), biochanin A (Bio-A, 200 ng/mL) and (bottom panel) on TCB (200 ng/mL), 4-OH-TCB (100 ng/mL) and 4-OH-DCB (200 ng/mL) stimulated synthesis of TGFB1 by cultured bovine oviduct fibroblasts. Data (mean ± s.e.m.) represents the mean of three different experiments ($n=3$, triplicates) and values were normalized to total protein concentration. The amount of TGFB1 synthesized is expressed as percent (%) of control. * $P<0.05$ vs control; § $P<0.05$ vs -ICI in respective treatments.

Cell-cell interaction between oviduct epithelial cells and oviduct fibroblasts plays an important role in maintaining the biological and physiological function of the oviduct by generating multiple autocrine/paracrine factors (Singh *et al.* 2011, Li & Winuthayanon 2017). Hence, we employed OCs, a mixed culture of OFCs and OECs (1:1 ratio), to assess TGFB1 synthesis in the oviduct. Our observation that TGFB1 levels increased in the conditioned medium of OCs in a time-dependent fashion suggests that the oviduct continuously synthesizes TGFB1 under basal conditions.

Treatment with E2 stimulated TGFB1 production in OCs in a concentration-dependent manner; moreover, cycloheximide inhibited basal and E2-stimulated TGFB1 production in OCs. This suggests that basal and E2-stimulated TGFB1 production is due to *de novo* synthesis and not simply a release of stored intracellular protein. Moreover, cyclic changes in the levels of E2 may regulate TGFB1 production and importantly, influence the biology and physiology of the oviduct in a timely fashion. TGFB1 plays an important role in embryo implantation, growth and differentiation (Li *et al.* 2011, Li 2014, Monsivais 2017). In this regard, cyclic changes in active TGFB1 expression occur during the menstrual cycle (Arici *et al.* 2003). More importantly, increases in TGFB1 levels in the ovarian follicular fluid, following ovarian stimulation for vitro fertilization correlates

with pregnancy (Fried *et al.* 1998). These observations indicate that E2, together with other cytokines, may stimulate the physiological release of TGFB1 within the oviduct and influence the priming and development of the early embryo for implantation.

Most biological effects of E2 are mediated via ER alpha and/or beta (Hewitt *et al.* 2016). Our observation that pre-treatment with ICI182789, an ER antagonist with equal affinity for ER alpha and beta (Kuiper *et al.* 1998), abrogated the stimulatory effects of E2 on TGFB1, suggests that the effects of E2 are ER mediated. This contention is further supported by our finding that OCs expressed both ER alpha and beta. Since biologically active ER has recently been identified in the membrane (Levin *et al.* 2009, Hewitt *et al.* 2016), we also assessed its role in E2-induced TGFB1 synthesis in OCs. In contrast to E2, equimolar concentrations of E2 tagged to BSA, which is membrane impermeable, failed to induce TGFB1 synthesis. This suggests that E2 stimulates TGFB1 production via nuclear, but not membrane ERs.

Similar to E2, EOE's bind to ERs and possess oestrogenic properties. These characteristics enable EOE's to act as endocrine disruptors and potentially induce pathological affects within the reproductive system (Diamanti-Kandarakis *et al.* 2009, Shanke & Xu 2011). Our findings provide the first evidence that EOE's induce TGFB1 synthesis in bovine OCs. Phytoestrogens, genistein and biochanin A, significantly induced TGFB1 synthesis in OCs. As compared to genistein, biochanin A was less potent in inducing TGFB1 synthesis. Biochanin A is a precursor of genistein, with a 10,000-fold lower binding affinity than genistein for ERs (relative binding affinity of genistein for ER alpha and beta is 4 and 8, respectively, whereas binding for biochanin A is <0.01 for both ER alpha and ER beta; Kuiper *et al.* 1998). This indicates that the potency of EOE's to induce TGFB1 depends on their binding affinity to ERs. This notion is supported by our observation that the stimulatory effects of both genistein and biochanin A were completely blocked by the ER antagonist ICI182780. The fact that genistein and biochanin A are established agonists for ER beta suggests its involvement in mediating the stimulatory effects on TGFB synthesis (Kuiper *et al.* 1998). E2 binds to both ER alpha and beta and is more potent than genistein and biochanin A in stimulating TGFB1 synthesis. This suggests that ER alpha may play a role in stimulating TGFB1 synthesis. Experiments using specific ER alpha and beta agonists and antagonists are required to confirm this contention.

Similar to E2 and phytoestrogens, PCB 4-OH-TCB, but not by TCB and 4-OH-DCB, also induced TGFB1 production. As with phytoestrogens, differences in potency between the PCBs may largely be due to the differences in their binding affinity towards ERs. In fact, 4-OH-TCB is the molecule with the highest affinity (Kuiper *et al.* 1998). Moreover, the affinity for ER increases in ortho-chlorine substituted molecules

and further enhanced in the hydroxylated ones. Thus, 4-OH-DCB has a low affinity for ER (Shanle & Xu 2011) and the non-phenolic but ortho-substituted TCB has very low or no affinity to ER (Shanle & Xu 2011). Our observation that ICI182780 blocked the stimulatory effects of 4-OH-TCB on TGFB1 production suggests that the PCB effects are ER mediated. Since EOs can act as partial agonists or antagonists via nuclear or membrane ERs/GPER, this may, in part, contribute to the differences in action of natural and EOs.

To confirm the stimulatory effects of E2 and EOs on TGFB1 synthesis by OCs were real and not due to non-specific cross-reactivity with some other proteins, we confirmed their actions using Western blotting. Consistent with the results from ELISA assay, we observed the upregulation of TGFB1 protein expression (≈ 26 kDa protein) by E2 and EOs. Moreover, these effects were blocked by ICI182780 (10 μ M), reaffirming the notion that the effects are ER mediated.

The above findings suggest that EOs may induce their deleterious effects in the reproductive system by modulating TGFB1. Indeed, TGFB1 plays an important role in embryo growth and development as well as in the regulation of the local maternal immune response to prevent miscarriage (Singh *et al.* 2011, Li 2014). Importantly, TGFB1 is critical in wound healing and fibrotic scar tissue formation and implicated in tubal abnormalities associated with ectopic pregnancy (Shaw *et al.* 2010). Hence, we assessed and compared the effects of E2 and the EOs on TGFB1 synthesis by OFCs. Similar to the effects in OCs, we found that EOs mimic the stimulatory effects of E2 on TGFB1 synthesis in OFCs. Our findings suggest that EOs can potentially trigger non-cyclic TGFB1 production locally and contribute to pathological remodelling of fallopian tube associated with adhesions.

Our finding that the phytoestrogens and PCBs enhance the stimulatory effects of E2 on TGFB1 production suggests that exposure to EOs can result in non-physiological increase of TGFB1 in the oviduct and induce deleterious actions on the reproductive process. Interestingly, 4OH-DCB, which was ineffective in inducing TGFB1 alone, enhanced the effects of E2, suggesting that EOs with weak oestrogenic activity can also modulate oestrogenic responses in the presence of other endogenous oestrogens. Since cyclic generation of E2 allows events and regulatory feedback to take place at the right moment (Kiyama & Wada-Kiyama 2015), presence of EOs may disrupt this balance. Moreover, in contrast to E2, EOs may accumulate in fatty tissues and be present in the body for a long time, resulting in constant ER activation and adverse health effects (Shanle & Xu 2011).

Whether non-cyclic, abnormally high levels of TGFB1 may play a role in tubal infertility can only be speculated. Increased incidences of ectopic pregnancies have been observed after recurrent surgery for pelvic

adhesions, presence of adenomas, fibrotic scar tissues following wound healing, ovarian hyper-stimulation and local infections; moreover, these conditions are also associated with an increase in TGFB1 levels (Tonello & Polli 2007, Shaw *et al.* 2010, Xiong *et al.* 2013, Li *et al.* 2014). For example, Li *et al.* (2011) reported high expression of TGFB1 in occluded fallopian tubes. Since TGFB1 is a master regulator of fibrosis (Meng *et al.* 2016), it is feasible that increased or abnormal exposure to EOs may induce TGFB1 levels, which could adversely influence oviduct function and biology leading to tubal occlusion and dysfunction. Indeed, exposure to exogenous oestrogens like DES has been associated with unfavourable pregnancy outcomes, including ectopic pregnancy (Palmer *et al.* 2001). Although multiple factors may contribute to tubal disorders, the autocrine/paracrine role of TGFB1 in response to oestrogens and ER activation may be critical in early embryo priming and transport. It is feasible that continuous presence of abnormally high TGFB1 levels, in response to high E2 or EOs exposure, triggers implantation like conditions within the oviduct leading to ectopic pregnancies.

E2 activates intracellular calcium, adenylyl cyclase and MAPK via non-genomic mechanisms (Nilsson *et al.* 2001). Since these pathways actively induce TGFB1 formation, they may in part mediate the stimulatory effects of E2 on TGFB1. Indeed, similar to ICI182780, intracellular calcium chelator BAPTA-AM, adenylyl cyclase inhibitor SQ22536 and the MAPK inhibitor PD98059, abrogated the stimulatory effects of E2. Since the inhibitory effects of BAPTA-AM, SQ22536 and PD98059 were not additive, nor did they modulate the effects of ICI182780, suggests that they inhibit via a common mechanism linked to ER. This contention is supported by our finding that ICI12780 blocked the stimulatory effects of E2 and EOs on MAPK phosphorylation. Interestingly, MAPK inhibitor PD98059, but not Ca^{2+} and adenylyl cyclase inhibitors, abrogated the stimulatory effects of EOs (phytoestrogen and PCBs) on TGFB1, suggesting that E2 and EOs mediate their stimulatory actions on TGFB1 via MAPK activation.

The ER antagonist blocked the effects of phytoestrogens and PCBs suggesting that they are ER mediated. However, we cannot rule out the participation of other mechanisms. Both phytoestrogens and PCBs are AhR ligands and cross-talk between ERs and AhRs influence ER expressions and transcriptional activation. Silencing of AhR lowers/inhibits TGFB1 production (Gramatzki *et al.* 2009) and the OCs express AhR. Interestingly, PD98056, which blocked the stimulatory effects of EOs, is also an AhR antagonist (Reiners *et al.* 1998) and may have blocked the effects of EOs on TGFB1 via this mechanism. Further in-depth studies are required to test these possibilities.

Almost every cell in the body produces some form of TGFB and expresses TGFB receptors, suggesting

an important role in preventing disease. Within the female reproductive system, TGFβ1 is involved in embryo implantation, growth and differentiation, placental differentiation, endometrium proliferation and differentiation, trophoblast–endometrium interaction during trophoblast invasion into the uterus, cytokine network regulation during pregnancy to maintain a healthy foetus, immune-suppressor to regulate the maternal immune response and avoid miscarriage (Jones *et al.* 2006, Li 2014). TGFβ levels peak during the window of implantation, which prepares the uterus for embryo implantation by modulating immune responses and localized/controlled apoptosis of endometrial stromal cells and tissue remodelling (Li & Winuthayanon 2017). TGFβ regulates angiogenesis and its expression at the embryo–uterine interphase, thought to play a critical role in placenta development (Li 2014, Li & Winuthayanon 2017). Although an increase in TGFβ1 in follicular fluid following ovarian stimulation and in vitro fertilization correlates with pregnancy (Fried & Wramsby 1998), abnormally high TGFβ1 levels are associated with miscarriages (Ogasawara *et al.* 2000).

In conclusion, our findings provide evidence that E2 and EOE regulate TGFβ1 synthesis in OC. EOE-mediated non-physiologic stimulation of TGFβ1 synthesis within the oviduct may play an important role in governing the role of the oviduct in the biology and pathophysiology of reproduction.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-17-0425>.

Declaration of interest

The authors declare that there is no conflict of interest, which could be perceived as prejudicing the impartiality of the research reported.

Funding

Supported by the Swiss National Sciences Foundation grant 32-55738.98 and 31003A-138067.

Acknowledgements

The data presented in this manuscript was, in part, the PhD dissertation (Diss., Naturwissenschaften ETH Zürich, Nr. 14994, 2003) work submitted by Barbara Cometti to ETH Zurich, Switzerland (<http://dx.doi.org/10.3929/ethz-a-004540868>).

References

Arici A & Sozen I 2003 Expression, menstrual cycle-dependent activation, and bimodal mitogenic effect of transforming growth factor-β1 in human

- myometrium and leiomyoma. *American Journal of Obstetrics and Gynecology* **188** 76–83. (<https://doi.org/10.1067/mob.2003.118>)
- Cometti B, Dubey RK, Imthurn B, Jackson EK & Rosselli M 2003 Oviduct cells express the cyclic AMP-adenosine pathway. *Biology of Reproduction* **69** 868–875. (<https://doi.org/10.1095/biolreprod.103.017962>)
- Diamanti-Kandarakis E, Bourguignon J-P, Giudice LC, Hauser R, Prins GS, Soto AM, Zoeller RT & Gore AC 2009 Endocrine-disrupting chemicals: an endocrine society scientific statement. *Endocrine Reviews* **30** 293–342. (<https://doi.org/10.1210/er.2009-0002>)
- Fried G & Wramsby H 1998 Increase in transforming growth factor beta1 in ovarian follicular fluid following ovarian stimulation and in-vitro fertilization correlates to pregnancy. *Human Reproduction* **13** 656–659. (<https://doi.org/10.1093/humrep/13.3.656>)
- Gramatzki D, Pantazis G, Schittenhelm J, Tabatabai G, Köhle C, Wick W, Schwarz M, Weller M & Tritschler I 2009 Aryl hydrocarbon receptor inhibition downregulates the TGF-β/Smad pathway in human glioblastoma cells. *Oncogene* **28** 2593–2605. (<https://doi.org/10.1038/onc.2009.104>)
- Hewitt SC, Winuthayanon W & Korach KS 2016 What's new in estrogen receptor action in the female reproductive tract. *Journal of Molecular Endocrinology* **56** R55–R71. (<https://doi.org/10.1530/JME-15-0254>)
- Huang J-C, Goldsby JS, Arbab F, Melhem N, Aleksic N & Wu KK 2004 Oviduct prostacyclin functions as a paracrine factor to augment the development of embryos. *Human Reproduction* **19** 2907–2912. (<https://doi.org/10.1093/humrep/deh520>)
- Ibrahim S, Salilew-Wondim D, Rings F, Hoelker M, Neuhoff C, Tholen E, Looft G, Schellander K & Tesfaye D 2015 Expression pattern of inflammatory response genes and their regulatory microRNAs in bovine oviductal cells in response to liposaccharide: implication for early embryonic development. *PLoS ONE* **10** e0119388. (<https://doi.org/10.1371/journal.pone.0119388>)
- Jeoung M, Lee S, Hawng H-K, Cheon Y-P, Jeong YK, Gye MC, Iglarz M, Ko C & Bridges PJ 2010 Identification of a novel role of endothelins within the oviduct. *Endocrinology* **151** 2858–2867. (<https://doi.org/10.1210/en.2009-1155>)
- Jones RL, Stoikos C, Findlay JK & Salamonsen LA 2006 TGF-β superfamily expression and actions in the endometrium and placenta. *Reproduction* **132** 217–232. (<https://doi.org/10.1530/rep.1.01076>)
- Kiyama R & Wada-Kiyama Y 2015 Estrogenic endocrine disruptors: molecular mechanism of action. *Environmental International* **83** 11–40. (<https://doi.org/10.1016/j.envint.2015.05.012>)
- Krishnan T, Winship A, Sonderegger S, Menkhurst E, Horne AW, Brown J, Zhang J-G, Nicola NA, Tong S & Dimitriadis E 2013 The role of leukemia inhibitory factor in tubal ectopic pregnancy. *Placenta* **34** 1014–1019. (<https://doi.org/10.1016/j.placenta.2013.09.003>)
- Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B & Gustafsson JA 1998 Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology* **139** 4252–4263. (<https://doi.org/10.1210/endo.139.10.6216>)
- Levin ER 2009 Plasma membrane estrogen receptors *Trends in Endocrinology and Metabolism* **20** 477–482. (<https://doi.org/10.1016/j.tem.2009.06.009>)
- Li Q 2014 Transforming growth factor β signaling in uterine development and function. *Journal of Animal Science and Biotechnology* **5** 52. (<https://doi.org/10.1186/2049-1891-5-52>)
- Li S & Winuthayanon W 2017 Oviduct: roles in fertilization and early embryo development. *Journal of Endocrinology* **232** R1–R26 (<https://doi.org/10.1530/JOE-16-0302>)
- Li Z, Sun Y, Min W & Zhang D 2011 Correlation between overexpression of transforming growth factor-beta 1 in occluded fallopian tubes and postsurgical pregnancy among infertile women. *International Journal of Gynecology and Obstetrics* **112** 11–14. (<https://doi.org/10.1016/j.ijgo.2010.07.016>)
- Matthews J & Gustafsson J-A 2006 Estrogen receptor and aryl hydrocarbon receptor signaling pathways. *Nuclear Receptor Signaling* **4** e016.
- Meng X-m, Nikolic-Paterson DJ & Lan HY 2016 TGF-β, the master regulator of fibrosis. *Nature Reviews Nephrology* **12** 325–338. (<https://doi.org/10.1038/nrneph.2016.48>)
- Monsivais D, Matzuk MM & Pangas SA 2017 The TGF-β family in the reproductive tract. *Cold Spring Harbor Perspectives in Biology* **9** a022251. (<https://doi.org/10.1101/cshperspect.a022251>)

- Nilsson S, Mäkelä S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M & Gustafsson J-A 2001 Mechanism of estrogen action. *Physiological Reviews* **81** 1535–1565. (<https://doi.org/10.1152/physrev.2001.81.4.1535>)
- Ning N, Zhu J, Du Y, Gao X, Liu C & Li J 2014 Dysregulation of hydrogen sulphide metabolism impairs oviductal transport of embryos. *Nature Communications* **5** 4107. (<https://doi.org/10.1038/ncomms5107>)
- Ogasawara MS, Aoki K, Aoyama T, Katano K, Iinuma Y, Ozaki Y & Suzumori K 2000 Elevation of transforming growth factor-beta1 is associated with recurrent miscarriage. *Journal of Clinical Immunology* **20** 453–457. (<https://doi.org/10.1023/A:1026459800016>)
- Palmer JR, Hatch EE, Rao RS, Kaufman RH, Herbst AL, Noller KL, Titus-Ernstoff L & Hoover RN 2001 Infertility among women exposed prenatally to diethylstilbestrol. *American Journal of Epidemiology* **154** 316–321. (<https://doi.org/10.1093/aje/154.4.316>)
- Reiners JJ Jr, Lee JY, Clift RE, Dudley DT & Myrand SP 1998 PD98059 is an equipotent antagonist of the aryl hydrocarbon receptor and inhibitor of mitogen-activated protein kinase kinase. *Molecular Pharmacology* **53** 438–445.
- Reinhart KC, Dubey RK, Keller PJ, Lauper U & Rosselli M 1999 Xenoestrogens and Phytoestrogens induce the synthesis of leukemia inhibitory factor by human and bovine oviduct cells. *Molecular Human Reproduction* **5** 899–907. (<https://doi.org/10.1093/molehr/5.10.899>)
- Reinhart KC, Dubey RK, Cometti B, Keller PJ & Rosselli M 2003 Differential effects of natural and environmental estrogens on endothelin synthesis in bovine oviduct cells. *Biology of Reproduction* **68** 1430–1436. (<https://doi.org/10.1095/biolreprod.102.006569>)
- Rosselli M, Dubey RK, Rosselli MA, Macas E, Fink D, Lauper U, Keller PJ & Imthurn B 1998 Role of nitric oxide in the biology, physiology and pathophysiology of reproduction. *Human Reproduction Update* **4** 3–24. (<https://doi.org/10.1093/humupd/4.1.3>)
- Rosselli M, Reinhart K, Imthurn B, Keller PJ & Dubey RK 2000 Cellular and biochemical mechanisms by which environmental estrogens influence reproductive function. *Human Reproduction Update* **6** 332–350. (<https://doi.org/10.1093/humupd/6.4.332>)
- Shanle EK & Xu W 2011 Endocrine disrupting chemicals targeting estrogen receptor signaling: identification and mechanisms of action. *Chemical Research in Toxicology* **14** 6–19. (<https://doi.org/10.1021/tx100231n>)
- Shaw JLV, Dey SK, Critchley HOD & Horne AW 2010 Current knowledge of the aetiology of human tubal ectopic pregnancy. *Human Reproduction Update* **16** 432–444. (<https://doi.org/10.1093/humupd/dmp057>)
- Singh M, Chaudhry P & Asselin E 2011 Bridging endometrial receptivity and implantation: network of hormone, cytokines, and growth factors. *Journal of Endocrinology* **210** 5–14. (<https://doi.org/10.1530/JOE-10-0461>)
- Tonello A & Poli G 2007 Tubal ectopic pregnancy: macrophages under the microscope. *Human Reproduction* **22** 2577–2584. (<https://doi.org/10.1093/humrep/dem246>)
- Xiong W, Frasch SC, Thomas SM, Bratton DL & Henson PM 2013 Induction of TGF-β1 synthesis by macrophages in response to apoptotic cells requires activation of scavenger receptor CD36. *PLoS ONE* **8** e72772. (<https://doi.org/10.1371/journal.pone.0072772>)
- Yousef MA, Marey MA, Hambruch N, Hayakawa H, Shimizu T, Hussien HA, Abdel-Razek A-RK, Pfarrer C & Miyamoto A 2016 Sperm binding to oviduct epithelial cells enhances TGFβ1 and IL10 expressions in epithelial cells as well as neutrophils in vitro: prostaglandin E2 as a main regulator of anti-inflammatory response in the bovine oviduct. *PLoS ONE* **11** e0162309. (<https://doi.org/10.1371/journal.pone.0162309>)
- Zhao Y, Chegini N & Flanders KC 1994 Human fallopian tube expresses transforming growth factor (TGF beta) isoforms, TGF beta type I-III receptor messenger ribonucleic acid and protein, and contains [125I] TGF beta – binding sites. *The Journal of Clinical Endocrinology & Metabolism* **79** 1177–1184. (<https://doi.org/10.1210/jcem.79.4.7962292>)

Received 12 July 2017

First decision 8 August 2017

Revised manuscript received 23 November 2017

Accepted 15 December 2017