Loss of luteotropic prostaglandin E plays an important role

in the regulation of luteolysis in women

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Running title: Loss of PGE is important for luteolysis in women

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Abstract

Study question: Do intraluteal prostaglandins contribute to luteal regulation in women?

Summary answer: Prostaglandin (PG) E, which is produced in human granulosa-lutein cells stimulated with luteotropic human chorionic gonadotropin (hCG), plays luteotropic roles in the human corpus luteum (CL). hCG promotes the synthesis of PGE in cultured luteinized granulosa cells (LGCs), and PGE exerts similar luteotropic effects to hCG, namely, promoting genes to produce progesterone, enhancing PGE synthesis, suppressing PGF synthesis, and down-regulating the gene expression of luteolytic activin A and bone morphogenetic proteins (BMPs). The expression of PG synthetic and metabolic enzymes in the human CL is driven towards less PGE, but more PGF during luteolysis.

What is known already: Uterine PGF is a major luteolysin in many non-primate species but not in women. Increases in the PGF synthase, AKR1C3, have been observed in the CL of marmoset monkeys during luteolysis. PGE prevents spontaneous or induced luteolysis in domestic animals.

Study design, size, duration: Human CL tissues staged as the early-luteal (n=6), mid-luteal (n=6), late-luteal (n=5), and menstrual (n=3) phases were obtained at the time of hysterectomy for benign gynecological conditions. LGCs were purified from follicular fluids obtained from patients undergoing assisted conception.

Participants/materials, setting, methods: Quantitative RT-PCR was employed to examine changes in the transcript abundance of PG synthetic and metabolic enzymes, steroidogenic enzymes, luteolytic molecules in the staged human CL, and cultured human LGCs treated with hCG, PGE, and PGF. A PGE withdrawal experiment was also conducted in order to reveal the effects of the loss of PGE in LGCs. Progesterone concentration in the culture medium were measured.

Main results and the role of chance: The key enzyme for PGE synthesis, *PTGES* was abundant in the functional CL during the mid-luteal phase, while genes involved in PGF synthesis (*AKR1B1* and *AKR1C1–3*) increased in the CL during the late-luteal phase and menstruation. *PTGES* expression positively correlated with that of *HSD3B1* (r=0.7212, $P<0.001$), while *AKR1C3* expression inversely correlated with that of *HSD3B1* (r=–0.7167, $P<0.001$) and *PTGES* (r=–0.6505, $P<0.01$). PGE exerted similar effects to hCG-promoting genes to produce progesterone and luteotropic PGE, suppress PGF synthetic enzymes, and down-regulate luteolytic molecules such as activin A and BMPs. PGE withdrawal resulted in reductions in the enzymes that produce progesterone and PGE, and the capacity to produce PGE decreased, while that to produce PGF increased during the culture. The addition of PGF did not recapitulate the luteolytic effects of PGE withdrawal.

Limitations, reasons for cautions: Changes in PG synthetic and metabolic enzymes may not represent actual increases in PGF during luteolysis in the CL. The effects of PGF on luteal cells currently remain unclear. The mechanisms responsible for decreases in the synthesis of PGE during a culture and luteolysis have not yet been elucidated in detail.

Wider implications of the findings: The loss of PGE is more important than the effects of PGF during luteolysis in women. This may be accompanied by decreases in the effects of luteinizing hormone/hCG in luteal cells, particularly a decrease in the activation of cAMP/PKA; however, the underlying mechanisms currently remain unknown.

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Key words: corpus luteum / hCG / luteinized granulosa cell / luteolysis / prostaglandin

Introduction

Progesterone is essential for the preparation and maintenance of pregnancy, and produced in the corpus luteum (CL) established from an ovulated dominant follicle in the ovary. The luteinization of granulosa cells and theca interna cells in the ovulated follicle is regulated by luteinizing hormone (LH) secreted from the pituitary gland, and granulosa-lutein and theca-lutein cells produce large amounts of progesterone. The CL undergoes degradation during luteolysis in a non-conception cycle, whereas human chorionic gonadotrophin (hCG) secreted from the developing conceptus rescues the CL from luteolysis, and maintains its function during early pregnancy. LH and hCG bind to the luteinizing hormone/choriogonadotropin receptor (LHCGR) on granulosa-lutein and theca-lutein cells in order to stimulate the expression of the genes responsible for progesterone production, such as the steroidogenic acute regulatory protein (STAR) and 3β-hydroxysteroid dehydrogenase (HSD3B1), by activating the cAMP/PKA pathway (Ascoli *et al.*, 2002). The stimulation of progesterone production through LHCGR by LH or hCG is fundamental to the maintenance of luteal cell integrity and functions. We previously demonstrated that paracrine regulators of CL functions, such as vascular endothelial growth factor, SLIT/ROBO, activin A, and bone morphogenetic proteins (BMPs), are secreted from luteal cells under the control of LH/hCG, and play important roles in luteinization and luteolysis (Dickinson *et al.*, 2008; van den Driesche *et al.*, 2008; Nio-Kobayashi *et al.*, 2015). However, the molecular mechanisms underlying luteolysis and its inhibition by increases in hCG during early pregnancy remain unclear.

The involvement of prostaglandins (PGs) in the regulation of CL functions is well-known, particularly in domestic animals in which PGF is the major driver for luteolysis (Fortier et al., 2008). PGs are eicosanoids derived from arachidonic acids, which are released from cell membrane phospholipids through the actions of phospholipases. The synthesis of PGs begins

with the conversion of arachidonic acid to the unstable PGH_2 through the activity of cyclooxygenases (COX1/PTGS1 and COX2/PTGS2). PGH_2 is then converted to bioactive PGs such as PGE_2 (PGE), $PGF_{2\alpha}$ (PGF), and PGD_2, by specific PG synthases. Three enzymes exhibit PGE synthetic activity, which are involved in the conversion of PGH_2 to PGE: membrane PGES1 (mPGES1/PTGES), mPGES2 (PTGES2), and cytosolic PGES (cPGES/PTGES3). PGF is produced from either PGH_2 or PGE by the actions of aldo-keto reductase (AKR) family members: AKR1B1 and AKR1C3 are known as PGF synthases and directly convert PGH_2 to PGF (Sugimoto *et al.*, 1994; Suzuki-Yamamoto *et al.*, 1999; Kabututu *et al.*, 2009). On the other hand, two closely related enzymes, AKR1C1 and AKR1C2, exhibit weak PGE 9-ketoreductase activity to convert PGE to PGF (Nishizawa *et al.*, 2000). AKR1C1 is the same enzyme as 20α-HSD, which converts progesterone to its inactive form 20α-hydroxyprogesterone and is involved in the regulation of luteolysis in rodents (Nishizawa *et al.*, 2000). PGE and PGF are both converted into inactive metabolites by 15-hydroxy PG dehydrogenase (PGDH) (Ensor and Tai, 1995).

PGF released from the uterus is the signal that initiates luteolysis in many non-primate species (Michael *et al.*, 1994; Okuda *et al.*, 2002). Although uterine PGs are not involved in luteolysis in women, PGs produced by the CL itself may be effecter molecules in luteolysis, because luteolysis may be induced in a small primate, the marmoset monkey by a systemic injection of PGF (Duncan *et al.*, 1998). Bogan *et al.* (2008) previously reported the significant expression of *AKR1B1* and *AKR1C1−3* in the monkey during menses, and an increase in *AKR1C3* expression in the regressing CL at the very-late stage, suggesting the involvement of intraluteal PGF in luteolysis. On the other hand, PGE is generally considered to be a luteotropic factor. The administration of PGE has been shown to prevent spontaneous or induced luteolysis in ewes, and PGE is considered to be involved in maintaining luteal functions during early

pregnancy in domestic animals (Pratt *et al.*, 1977; Henderson *et al.*, 1977; Maqness *et al.*, 1981; Zelinski-Wooten and Stouffer, 1990).

Previous studies indicated that LH and hCG promote PGE synthesis by up-regulating the expression of *COX2* and *PTGES* (Kniss, 1999; Duffy and Stouffer, 2001; Sun *et al.*, 2006). Secreted PGE may stimulate angiogenesis in the CL with the involvement of VEGF (Sakurai *et al.*, 2004; Duncan and Nio-Kobayashi, 2013) and also enhances progesterone secretion from luteal cells through prostaglandin E receptor 2 (PTGER2/EP2) (Harris *et al.*, 2001). On the other hand, *AKR1C1−3* expression is also reported to be regulated by hCG in granulosa cells obtained from the periovulatory follicles of monkeys (Dozier *et al.*, 2008). These findings suggest the involvement of intraluteal PGs in the regulation of CL functions and luteolysis; however, the exact functions of intraluteal PGs in the human CL have not yet been elucidated.

This study aimed to investigate 1) the expression profiles of all PG synthetic and metabolic enzymes in the human CL throughout the luteal phase, 2) the roles of PGE, PGF, and PGE withdrawal in the regulation of luteal cell functions, and 3) the regulation of PG synthetic and metabolic enzymes by hCG and PGE.

Materials & Methods

Ethics

The collection of the CL at the time of hysterectomy was approved by the Lothian Medical Research Ethics Committee. The Lothian Medical Research Ethics Committee and Hokkaido University Research Ethics Committee approved the study using cultured human luteinized granulosa cells (LGC) collected during assisted conception. All women gave informed consent.

Reagents

Reagents were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) unless otherwise stated. Insulin-Transferrin-Selenium (ITS)-X and Matrigel were obtained from Life Technologies Inc. (Carlsbad, CA, USA) and BD Biosciences (San Jose, CA, USA), respectively.

Collection of the human CL

Human CLs were collected at the time of surgery from women undergoing hysterectomy for benign conditions such as heavy menstrual bleeding or uterine fibroids. The stages of the CLs were assessed by endometrial morphology and the concentration of LH in daily urine samples, which were obtained prior to surgery as reported previously (Dickinson *et al.*, 2008). The CLs were halved upon collection; one half was snap-frozen and stored at −80°C, and the other half was fixed with formalin and processed in paraffin wax.

Human LGC culture

Follicular aspirates were obtained from patients undergoing assisted conception after ovarian stimulation using a standard procedure (Duncan et al. 2005). Oocytes were removed, the remaining fluid was centrifuged at 1,500 rpm for 10 min, and cells were re-suspended in culture

medium (DMEM/Ham's F-12 containing 2 mM L-glutamine, 10 unit/mL penicillin, 0.1 mg/mL streptomycin, 2.5 μg/mL amphotericin B, and 1× concentration of ITS-X). The cell suspension was layered over 45% Percoll in the culture medium mixture, and centrifuged at 1,200 rpm for 30 min to pellet blood cells. LGCs, visible in the interface, were collected by a pipette and washed three times with Dulbecco's phosphate buffered saline with $MgCl_2$ and $CaCl_2$.

A total of 100,000 viable cells were seeded on Matrigel-coated 24 well plates and cultured with 1 mL of culture medium at 37°C in 5% CO_2 in air. The culture medium was changed every two days, and cells were treated on day six of the culture for 24 hours with either 1) 100 ng/mL hCG or 2) 10 ng/mL PGE and PGF dissolved in ethanol. In the PGE withdrawal experiment, after culturing LGCs in medium containing 1 ng/mL PGE for 7 days, medium was changed without PGE or 10 ng/mL PGE or PGF, and collected 24 hours later on day 8. Some cells were collected on culture day 3 or 7 without any treatments. Each experiment was performed in duplicate and repeated at least three times. Other cells were treated with either 100 ng/mL hCG or 10 ng/mL PGE for 48 hours on culture day 6, and, 48 hours later, the concentration of progesterone in the culture medium was outsourced for measurement using Chemiluminescent immunoassay by SRL Inc. (Sapporo, Japan).

Quantitative RT-PCR (qRT-PCR)

The CLs used for a quantitative gene expression analysis were classified as the early-luteal (1−5 days after the LH surge, n=4), mid-luteal (6−10 days, n=6), late-luteal (11−14 days, n=5), and menstrual (more than 14 days, n=3) phases. Total RNA was extracted from the frozen human CL or cultured LGCs using a RNeasy Mini Kit (Qiagen Ltd., Crawley, UK) following the manufacturer's protocol. RNA (200 ng) was used to prepare cDNA using TaqMan Reverse Transcription regents (Applied Biosystems, Foster City, CA, USA).

The sequences of the primer sets used for this study are listed in Table 1 or previously described (Nio-Kobayashi *et al.*, 2015). Primers were pre-validated by standard PCR and by generating standard curves using qRT-PCR. Each reaction buffer contained 5.0 μL 2×PowerSYBR® Green PCR Master Mix (Applied Biosystems), 0.5 μL primer pair (5 μM), 3.5 μL of nuclease-free H_2O, and 1.0 μL cDNA, and each reaction was conducted in duplicate. The qRT-PCR cycling program consisted of a denaturing step (95°C for 10 min), annealing and extension step (95°C for 15 sec and 60°C for 1 min, repeated for 40 cycles), and a dissociation step (95°C, 60°C, and 95°C for 15 sec each) using a 7900 Sequence Detection System (Applied Biosystems). The relative expression levels of each target to the housekeeping gene (*glucose-6-phosphate dehydrogenase*: *G6PDH*), previously validated using a geNorm analysis (Primerdesign Ltd., Southampton, UK), were quantified using the $^{\Delta}$Ct (for *in vivo* analysis) or $^{\Delta\Delta}$Ct (for *in vitro* analysis) methods.

Statistical analysis

After testing for normality, all statistical analyses were performed using unpaired *t*-tests, a one-way ANOVA with Sidak's multiple comparisons test, or a correlation analysis, using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA), and $P<0.05$ was regarded as significant. Values in the graphs represent the mean ± SEM of relative expression to *G6PDH*.

Immunohistochemistry

Fixed human CLs at the early-luteal (n=6), mid-luteal (n=3), late-luteal (n=3), and menstrual (n=3) phases were used in the immunohistochemical analysis. Tissues were dehydrated through ethanol and embedded into paraffin according to the conventional method. Tissue sections (5

μm thick) were de-waxed and washed twice in distilled water, and antigen retrieval was then performed for 5 minutes in 0.01 M citrate buffer (pH 6.0) using a pressure cooker. After washing twice in phosphate-buffered saline (PBS), sections were incubated with 3% hydrogen peroxide for 20 min followed by Avidin/Biotin blocking solution (Vector Laboratories Inc., Burlingame, CA, USA) for 15 min each. Sections were incubated with normal goat serum at room temperature for 60 min. Thereafter they were incubated with mouse anti-human PTGES (1:200; #160140, Cayman Chemical Company, Ann Arbor, MI, USA) in goat serum at 4°C overnight. Control sections were incubated with non-immune serum and the disappearance of the immunoreaction was confirmed. After washing twice in PBS, sections were incubated with biotinylated anti-mouse IgG (1:500; Vector laboratories Inc.) at room temperature for 60 min. The reaction sites were visualized using a Vectastain ABC Elite kit (Vector Laboratories Inc.) for 60 min followed by an ImmPACT™ DAB Peroxidase Substrate Kit (Vector Laboratories Inc.) for 1 min. Sections were counterstained with hematoxylin and observed under a light microscope (BX51; Olympus corporation, Tokyo, Japan).

Results

PG synthesis and metabolism in the human CL changes across the luteal phase

An analysis of all PG synthases and degradation enzymes in the human CL throughout the luteal phases by qRT-PCR revealed the differential expression patterns of each gene (Table 2). *COX1* transcripts increased in the regressing CL collected during menstruation ($P<0.01$) whereas *COX2* did not change across the luteal phases. The three PGE synthases were differentially expressed: *PTGES* peaked in the functional CL during the mid-luteal phase ($P<0.01$), whereas *PTGES2* and *PTGES3* peaked in the late-luteal phase ($P<0.05$). The transcripts of all genes involved in PGF synthesis (*AKR1B1* and *AKR1C1–3*) increased in the regressing CL during the late-luteal phase, and increases in *AKR1C3* were maintained in the CL collected during menstruation ($P<0.001$). Regarding PG degradation enzymes, *PGDH* expression did not change during the luteal phase. Changes in the enzymes involved in PG synthesis and metabolism suggest a drive towards less PGE and more PGF with the progression of luteolysis (On-line only Supplementary Fig. 1A).

PGE exerts hCG-like luteotropic effects on steroidogenic cells

As expected, hCG enhanced the expression of *LHCGR* and down-regulated that of *follicular stimulating hormone receptor* (*FSHR*) in human LGCs (Fig. 1A). Similarly, PGE increased *LHCGR* ($P<0.05$) and inhibited *FSHR* ($P<0.0001$) (Fig. 1B). PGE also exerted similar effects to hCG on the steroidogenic enzyme transcripts in LGCs (Fig. 1C, D). They strongly induced *STAR* and enhanced the expression of *CYP11A1* and *HSD3B1*, suggesting that PGE as well as hCG supports steroidogenesis in LGCs. On the other hand, the expression of *CYP21A2* and *HSD17B1* was down-regulated by PGE and hCG (Fig. 1C, D). Unlike hCG, PGE increased transcript abundance for *CYP19A1* ($P<0.05$; Fig. 1C, D). These changes suggest that, similar to

hCG, PGE promotes the production of progesterone in luteal steroidogenic cells (On-line only Supplementary Fig. 1B). The progesterone concentrations were significantly increased in the culture medium of hCG- (143.5 ± 7.42; $P<0.0001$) or PGE-treated cells (106.03 ± 4.44; $P<0.0001$) when compared to that of non-treated (44.18 ± 1.36) or ethanol-treated cells (44.17 ± 0.99), respectively.

PGE exerts hCG-like anti-luteolytic effects on steroidogenic cells

In human LGCs, hCG increased inhibin α-subunit (*INHA*) expression, which will reduce activin, and also decreased the βA- and βB-inhibin subunits (*INHBA* and *INHBB*), which will also decrease activin synthesis. In addition, hCG reduced BMP expression *in vivo* and *in vitro* (Myers *et al.*, 2007; Myers *et al.*, 2008; Nio-Kobayashi *et al.*, 2015). PGE up-regulated *INHA* ($P<0.001$, Fig. 2) and decreased *INHBA* and *INHBB* in LGCs ($P<0.001$, Fig. 2). Furthermore, PGE significantly reduced *BMP2*, *BMP4*, and *BMP6* ($P<0.001$, Fig. 2), suggesting that luteolytic molecules are inhibited by PGE in human luteal steroidogenic cells.

hCG regulates PG synthetic and metabolic enzymes and PGE exerts the same effects

In order to investigate the effects of hCG and PGE on PG synthesis, we treated LGCs with hCG or PGE and analyzed the expression of synthetic and metabolic enzymes for PG by qRT-PCR. hCG and PGE both had similar effects on the transcript abundance of the investigated genes. *COX1* and *COX2* were increased in hCG-treated LGCs, with the expression of the latter being 10-fold higher than that in non-treated controls ($P<0.001$, Fig. 3A). PGE also significantly enhanced the expression of *COX2* ($P<0.01$, Fig. 3B). The three PGE synthetic enzymes were differentially regulated: hCG and PGE markedly increased *PTGES* but decreased *PTGES2* and *PTGES3* ($P<0.001$, Fig. 3A, B). While *AKR1B1* was down-regulated by hCG and PGE

(*P*<0.0001, Fig. 3A, B), *AKR1C1* and *AKR1C2* were both increased in LGCs by hCG alone (*P*<0.001 and *P*<0.05, respectively, Fig. 3A). Neither hCG nor PGE had any effect on the expression of *AKR1C3* (Fig. 3A, B). The expression of *PGDH* was down-regulated by hCG and PGE (*P*<0.001, Fig. 3A, B). Changes in the enzymes involved in PG synthesis and metabolism suggest a drive towards more PGE and less PGF by luteotropic molecules.

PGE withdrawal occurs during luteolysis and exerts luteolytic effects

In the human CL, PTGES immunoreactivity was localized to the cytoplasm of granulosa-lutein cells during the mid-luteal phase, while theca-lutein cells lacked immunoreactivity (Fig. 4A). Non-steroidogenic cells also lacked immunoreactivity (asterisks in Fig. 4A). In the regressing CL collected during menstruation, granulosa-lutein cells were classified into three types as previously reported: intact, apoptotic, and degenerating cells (Nio-Kobayashi *et al.*, 2014). Only intact granulosa-lutein cells were positively immunostained for PTGES (arrow in Fig. 4B), while apoptotic granulosa-lutein cells lacked immunoreactivity (arrowheads in Fig. 4B).

The expression of *PTGES* and *HSD3B1* strongly correlated *in vivo* in the human CL (r=0.7212, *P*<0.001; Fig. 4C), consistent with the luteotropic function of PGE. In contrast, *AKR1C3* strongly and negatively correlated with the expression of *HSD3B1* (r=–0.7167, *P*<0.001; Fig. 4D). An inverse correlation was observed between *PTGES* and *AKR1C3 in vivo* (r=–0.6505, *P*<0.01; Fig. 4E), suggesting a drive towards less PGE and more PGF synthesis during luteolysis.

In the absence of a luteotropic stimulation, *in vitro*, the expression of *STAR*, *COX2*, and *PTGES* significantly decreased, whereas that of *AKR1C3* increased during the culture (Fig. 4F). When PGE was added during the culture and then acutely withdrawn, a marked decrease was observed in the expression of steroidogenic enzymes (*STAR* and *HSD3B1*) and *PTGES* while the

expression of *AKR1C3* was unchanged (Fig. 4G).

Luteolytic effects of PGE withdrawal are not recapitulated by the addition of PGF

We assessed the effects of the addition of PGF to human LGCs on the expression of steroidogenic enzymes, PG synthetic and metabolic enzymes, and activin and BMP luteolytic factors. No effects were observed on the transcript abundance of these genes, as shown by *STAR* and *HSD3B1*, *COX2*, *PTGES*, and *AKR1C3*, *INHA*, *INHBA*, and *INHBB*, and *BMP2*, *BMP4*, and *BMP6* (Table 3). In order to investigate whether PGF was biologically active in these cells, we examined *C-C motif ligand 2* (*CCL2*) ―a gene known to be induced by PGF― in luteal steroidogenic cells (Luo *et al.*, 2011; Nio-Kobayashi *et al.*, 2015). In the human LGC, *CCL2* was increased by PGF ($P<0.05$; Fig. 5A). The addition of PGF did not alter PGE-induced luteotropic *STAR* or *PTGES* transcript abundance (Fig. 5B). Furthermore, PGF did not enhance the reductions in *STAR* or *PTGES* during PGE withdrawal (Fig. 5C).

Discussion

The results of the present study revealed the expression profiles of the PG synthetic and metabolic enzymes in the human CL and human cultured LGCs. The results obtained strongly support the luteotropic function of PGE in the regulation of the human CL. The production of luteal PGE is enhanced by luteotropic hCG via the up-regulation of key enzymes to produce PGE, namely, *COX2* and *PTGES*. The expression of *PTGES* was the strongest in the functional CL during the mid-luteal stage and positively correlated with that of *HSD3B1*, a key enzyme for progesterone production. The enzymes responsible for luteolytic PGF production, *AKR1B1* and *AKR1C1−3*, significantly increased in the regressing CL during the late-luteal phase and menstruation, and the expression of *AKR1C3* negatively correlated with that of *PTGES* and *HSD3B1*. The capacity to produce PGE decreased, while the expression of *AKR1C3* increased during the culture, and the loss of the PGE stimulation resulted in reductions in the potential to produce progesterone and PGE in cultured LGCs. These results suggest that the main PG produced in luteal tissue changes from PGE to PGF during the maturation and regression of the human CL, and PGE withdrawal is more important than the effects of PGF in the regulation of luteolysis in women. We previously demonstrated that PGE enhances the expression of vascular endothelial growth factor to support angiogenesis (Duncan and Nio-Kobayashi, 2013) and inhibits that of CCL2, a chemokine for macrophage infiltration, in the human CL (Nio-Kobayashi *et al.*, 2015). Loss of PGE synthetic activity in luteal cells results in a destabilization of vasculatures, an enhanced macrophage infiltration, and an increased luteolytic factors such as activin A and BMPs in the CL to promote luteolysis in women (Fig. 6).

It is generally accepted that PGF is a luteolysin, whereas PGE is a luteotropin in most mammals. In ruminants, uterine PGF or PGE circulating in the CL are known to initiate or prevent luteolysis, respectively. This system does not function in women because hysterectomy

has no effect on luteal functions; however, similar to other species, luteal cells themselves possess the ability to produce PGs. There is convincing evidence to demonstrate that locally produced luteal PGs contribute to the regulation of CL functions and life span in cows (Arosh *et al.*, 2004), sheep (Lee *et al.*, 2012), and pigs (Waclawik *et al.*, 2008). We herein showed the differential expression profiles of the genes responsible for PG biosynthesis in the human CL across the luteal phase. A comprehensive analysis of genes involved in PG biosynthesis has been reported by Bogan *et al.* (2008) in the CL of rhesus monkeys showing similar expression patterns for PG synthases, particularly for the expression profiles of a PGE synthase (*PTGES*) and PGF synthase (*AKR1C3*).

A previous study reported that PGE possesses a luteotropic function: PGE increases progesterone production and cAMP accumulation in cultured bovine luteal cells (Rekawiecki *et al.*, 2005) and cultured human granulosa-lutein cells (Chandras *et al.*, 2007). Increases in luteal PGE biosynthesis during the establishment of pregnancy has also been reported in pigs (Waclawik *et al.*, 2008) and sheep (Lee *et al.*, 2012). Consistent with previous findings, the present study using human LGCs revealed that PGE exerts similar luteotropic effects to hCG on the regulation of the genes responsible for progesterone production as well as luteotropic PGE production itself. The production of activin A and several BMPs increases during luteolysis and these molecules exert luteolytic effects on steroidogenic cell functions. PGE also showed similar effects to hCG on the expression of luteolytic activin A and BMPs expression, further supporting the luteotropic functions of PGE.

We also showed the regulation of PG synthetic and metabolic enzymes by luteotropic hCG and PGE in human LGCs. Luteotropic hCG and PGE both up-regulated the expression of *COX2* and *PTGES*, but suppressed that of *AKR1B1*, a major PGF synthase in luteal cells. hCG increased the concentration of PGE in the culture medium of certain cells (data not shown).

However, the effects of hCG on actual PGE synthesis were not consistent among samples, although the expression of *PTGES* was significantly up-regulated by hCG in all samples. We still do not know what influences the effects of hCG on actual PGE synthesis. Further experiments are underway in our laboratory to reveal the mechanisms of differential PGE synthetic ability in LGCs. Luteotropic LH/hCG binds to the common receptor expressed on luteal cells, LHCGR, and promote luteal cell functions mainly via the cAMP/PKA pathway. Human granulosa-lutein cells predominantly express EP2, a receptor for PGE, which acts through the cAMP/PKA pathway, similar to LHCGR (Harris *et al.*, 2001). hCG and PGE may both function mainly through the cAMP/PKA pathway to regulate the expression of PG synthases and degradation enzymes. Luo *et al.* (2015) recently reported that a decline in luteotropic stimuli leads to the induction of COX2 expression and possibly intraluteal PGF production in women. Przygrodzka *et al.* (2016) also noted a decline in intraluteal PGE production and increase in PGF concentrations in the regressing porcine CL; however, the increases observed in PGF did appear to be produced in luteal tissue. This study demonstrated that the loss of the luteotropic signal by PGE leads to a decline in the capacity to produce progesterone and PGE in luteal cells. Although the underlying mechanisms remain unknown, the decline observed in the production of PGE, potentially caused by a reduction in cAMP/PKA activation, may be a key event for spontaneous luteolysis in women.

Other paracrine molecules such as activin A and BMPs also possess the ability to regulate the expression of PG synthetic and metabolic enzymes. Liu *et al.* (2016) recently reported that activin A up-regulates the expression of COX2 in order to promote the synthesis of PGE in immortalized human granulosa lutein cells. Not only luteotropic molecules such as hCG and PGE but also luteolytic molecules including activin A and BMPs are involved in the regulation of PG synthesis in luteal cells. The mechanisms that regulate the production of PG in luteal cells

are complex, and further studies are underway in our laboratory.

Although PGF released from the uterus is a major effector molecule inducing luteolysis in various animals including cows and rodents, it is unlikely that the synthesis of PGF is the major initiator of luteolysis in women. While the luteotropic effects of PGF have been reported in other animals *in vitro* (Kawaguchi *et al.*, 2013), luteolysis was induced in these animals by an intramuscular or intravenous injection of PGF. PGF produced by luteal cells may mainly act on non-steroidogenic cells such as endothelial cells, fibroblasts, and immune cells in the human CL. It is also likely that the effects of PGF are blocked when luteal cells have sufficient potential to synthesize PGE and/or progesterone. Further studies are needed in order to reveal the effects of PGF and its regulation and sites of action in the CL of women.

In conclusion, the LH/hCG responsive steroidogenic cells of the human CL co-ordinate the processes involved in luteolysis and luteal rescue. Our results suggest that the luteotropic functions of PGE produced by LH/hCG in the human CL are important for the maintenance of the CL. PGE produced from luteal cells promotes luteal functions by up-regulating key enzymes to produce progesterone and luteotropic PGE itself. Luteal PGE promotes luteal functions and blocks the expression of luteolytic molecules. While the expression of PGF synthases is increased in the regressing human CL, suggesting the potent contribution of intraluteal PGF in the ongoing luteolytic process, the withdrawal of luteal PGE may be particularly important. Although detailed mechanistic explanations for how the intraluteal PGE/PGF balance is regulated during luteolysis remain unclear, a reduction in cAMP/PKA activation ―as the CL becomes less sensitive to LH― resulting in a reduced capacity for PGE production in luteal cells appears to be a key element underpinning spontaneous luteolysis in women.

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Authors' roles

Study concept and design of the experiments: JN-K and WCD, Acquisition of data: JN-K, Analysis and interpretation of data: JN-K, Collecting follicular fluids: MK and NS, Drafting of the manuscript: JN-K, WCD, and TI.

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Conflict of interest

None declared.

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Figure legends

Figure 1. hCG-like luteotropic effects of PGE in human LGCs *in vitro*.

hCG (**A**) and PGE (**B**) both enhance the expression of *LHCGR*, but suppress the expression of *FSHR*. hCG (**C**) and PGE (**D**) exert similar effects on the expression of steroidogenic genes. They stimulate the expression of *STAR*, *CYP11A1*, *HSD3B1*, *CYP17A1*, but inhibit that of *CYP21A2* and *HSD17B1*. PGE significantly increases the expression of *CYP19A1* (**D**). CYP: cytochrome P450, FSHR: follicular stimulating hormone receptor, hCG: human chorionic gonadotrophin, HSD: hydroxysteroid dehydrogenase, LHCGR: luteinizing hormone/choriogonadotropin receptor, STAR: steroidogenic acute regulatory protein. No change is indicated as 100% of the control while doubling is 200% and halving is 50% in C and D. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

Figure 2. Effects of PGE on the expression of luteolytic factors in human LGCs *in vitro*.

PGE enhances the expression of inhibin/activin α-subunit (*INHA*), but significantly inhibit that of both inhibin/activin β-subunits (*INHBA* and *INHBB*) as well as three bone morphogenetic proteins (BMPs). ****P*<0.001.

Figure 3. Effects of hCG and PGE on the expression of PG synthetic and metabolic enzymes in human LGCs *in vitro*.

hCG (**A**) and PGE (**B**) both enhance the expression of *COX2* and *PTGES*, while only hCG significantly increases that of *COX1*. *PTGES2* and *PTGES3* are both down-regulated by hCG and PGE. Among the four PGF synthases, *AKR1B1* is significantly decreased by the hCG and PGE treatments, whereas hCG stimulates the expression of *AKR1C1* and *AKR1C2*. *AKR1C3* expression is not altered by these two treatments. The expression of *PGDH* is significantly

reduced by the hCG and PGE treatments. AKR: Aldo-keto-reductase, COX: cyclooxygenase, PTGES: PGE synthase, PGDH: 15-hydroxyprostaglandin dehydrogenase. *P<0.05, **P<0.01, ***P<0.001.

Figure 4. Loss of PGE correlates with reduced steroidogenic activity in luteal cells

Immunoreactivity for PTGES is found in the cytoplasm of granulosa-lutein cells, but not in that of theca-lutein cells (t) or non-steroidogenic cells (*asterisks*) in the human CL during the mid-luteal (ML) stage (**A**). In the regressing CL collected during menstruation (Men), only intact granulosa-lutein cells show positive immunoreactions for PTGES (arrow in **B**), while apoptotic cells (arrowheads in **B**) lack immunoreactivity. The insert in B shows an image of intact and apoptotic granulosa-lutein cells observed at a higher magnification. The mRNA expression of *PTGES* in the human CL positively correlates with that of *HSD3B1* (**C**). *AKR1C3* expression reversely correlates with that of *HSD3B1* (**D**) and *PTGES* (**E**). The expression of *STAR*, *COX2*, and *PTGES* decreases, while that of *AKR1C3* increases during the culture (**F**). The expression of *STAR*, *HSD3B1*, *COX2*, and *PTGES* significantly decreases after PGE withdrawal (**G**). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Figure 5. PGF cannot recapitulate the luteolytic effect of PGE withdrawal

PGF is biologically active because it enhances the expression of *CCL2* (**A**). The addition of PGF cannot block the luteotropic effects of PGE on the expression of *STAR* and *PTGES* (**B**) and recapitulate those of PGE withdrawal (**C**). CCL2: C-C motif ligand 2. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Figure 6. A schema showing how loss of luteal PGE is involved in luteolysis in women

In the functional CL, PGE promote the synthesis of progesterone, PGE, and vascular endothelial growth factor (VEGF) but inhibit that of PGF, CCL2, and luteolytic factors including Activin A and BMPs to maintain luteal function. Loss of PGE synthesis results in an increased macrophage infiltration and luteolytic factors to promote luteolysis. EC: endothelial cell, GLC: granulosa-lutein cell.

Supplementary Figure 1. Schemas showing the change in the expression of PG synthetic and metabolic enzymes during luteolysis *in vivo* (A) and effects of PGE on steroidogenesis *in vitro* (B).

AKR: Aldo-keto-reductase, CBR: carbonyl reductase, COX: cyclooxygenase, CYP: cytochrome P450, HSD: hydroxysteroid dehydrogenase, PTGES: PGE synthase, PGDH: 15-hydroxyprostaglandin dehydrogenase, PGM: prostaglandin metabolite, STAR: steroidogenic acute regulatory protein.

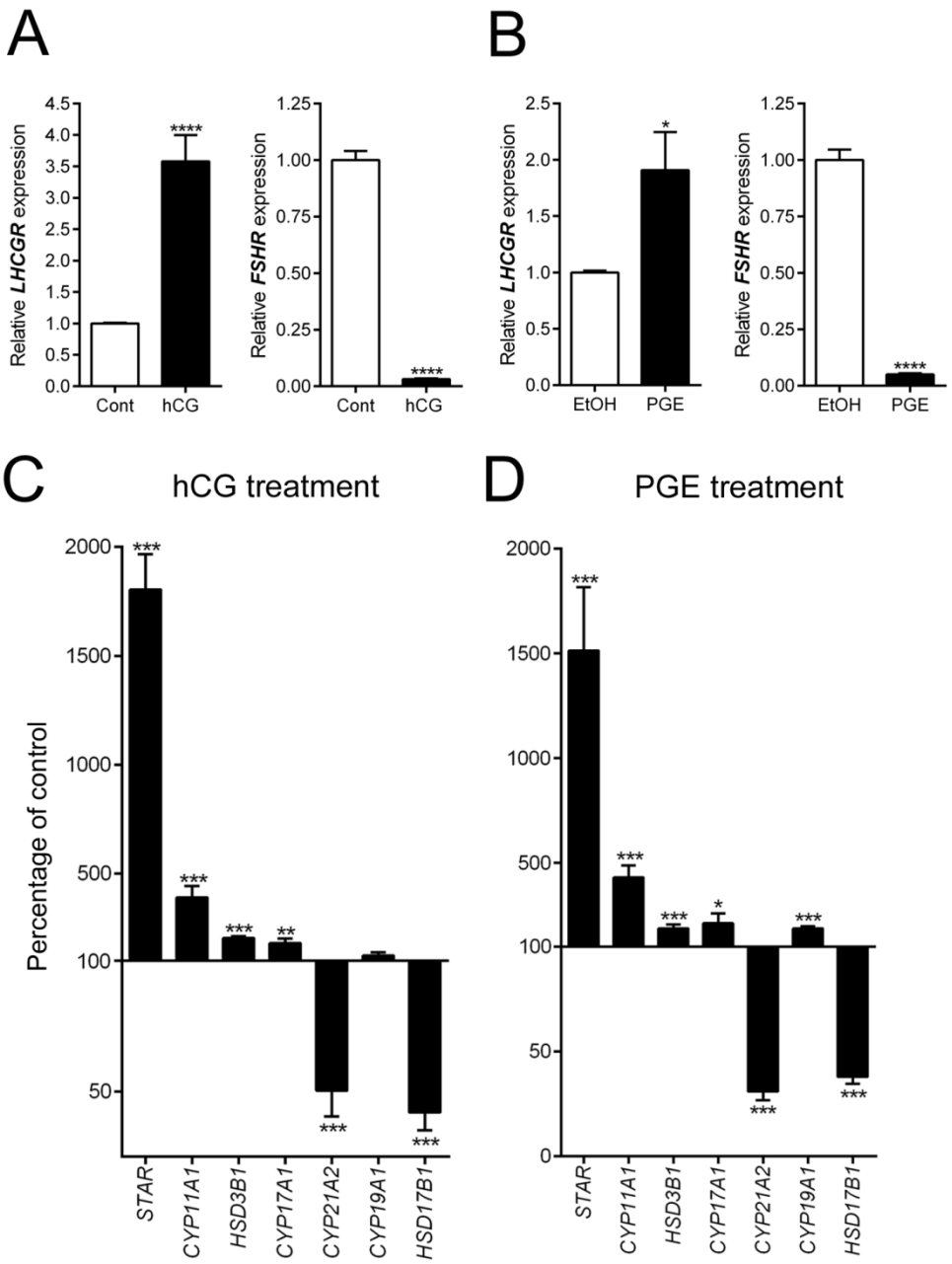


Figure 1 Nio-Kobayashi et al.

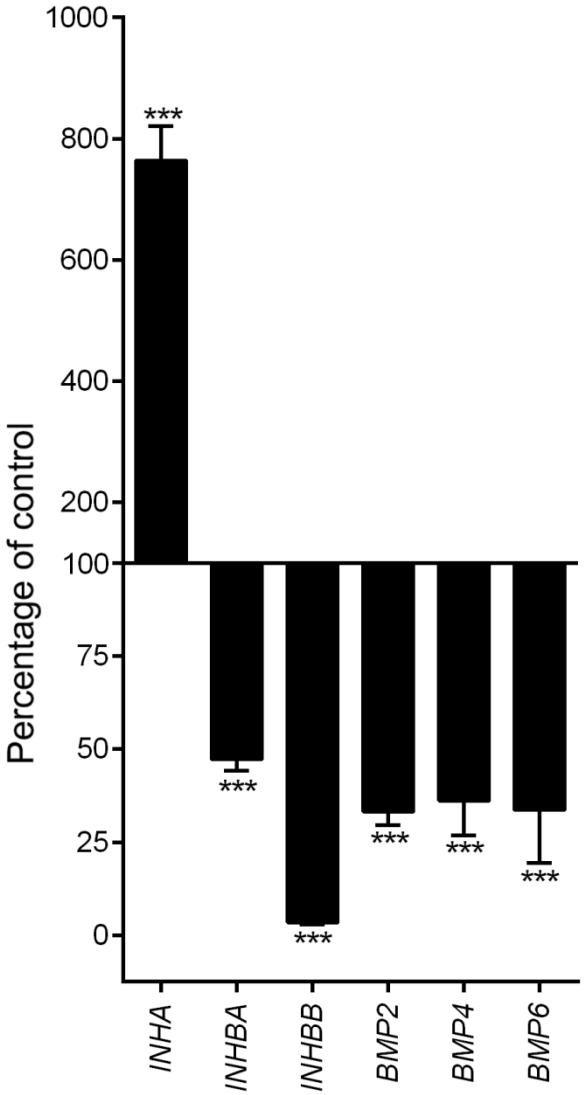


Figure 2 Nio-Kobayashi et al.

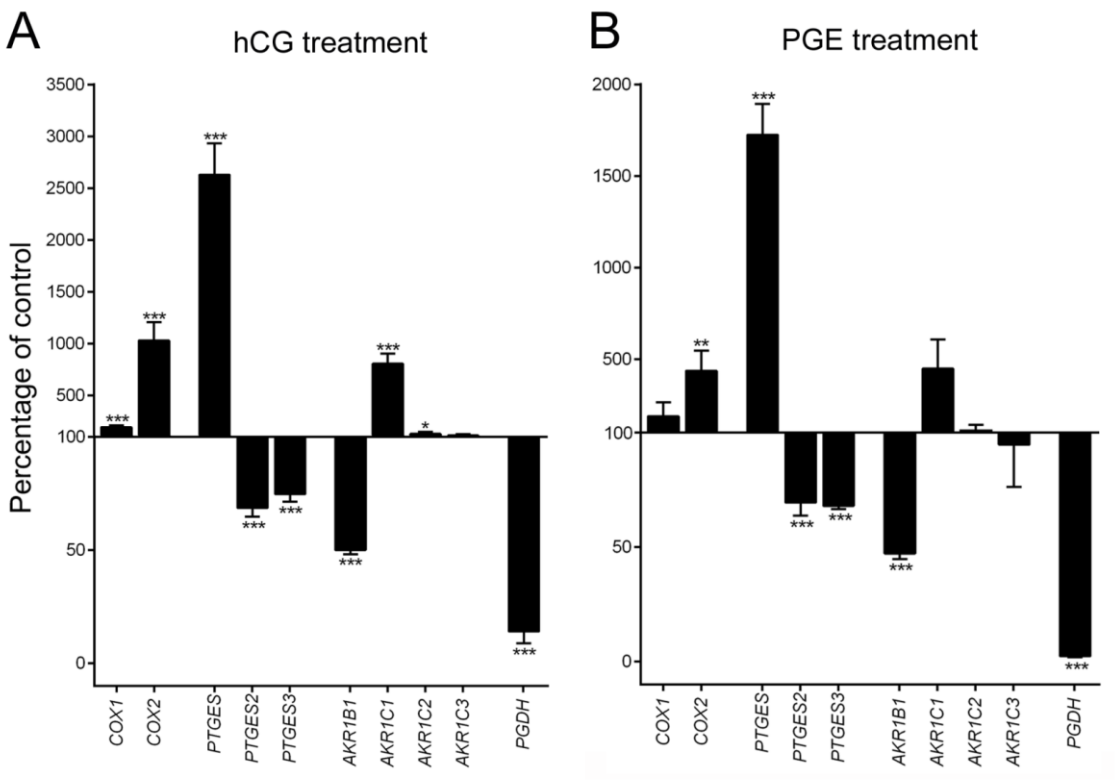


Figure 3 Nio-Kobayashi et al.

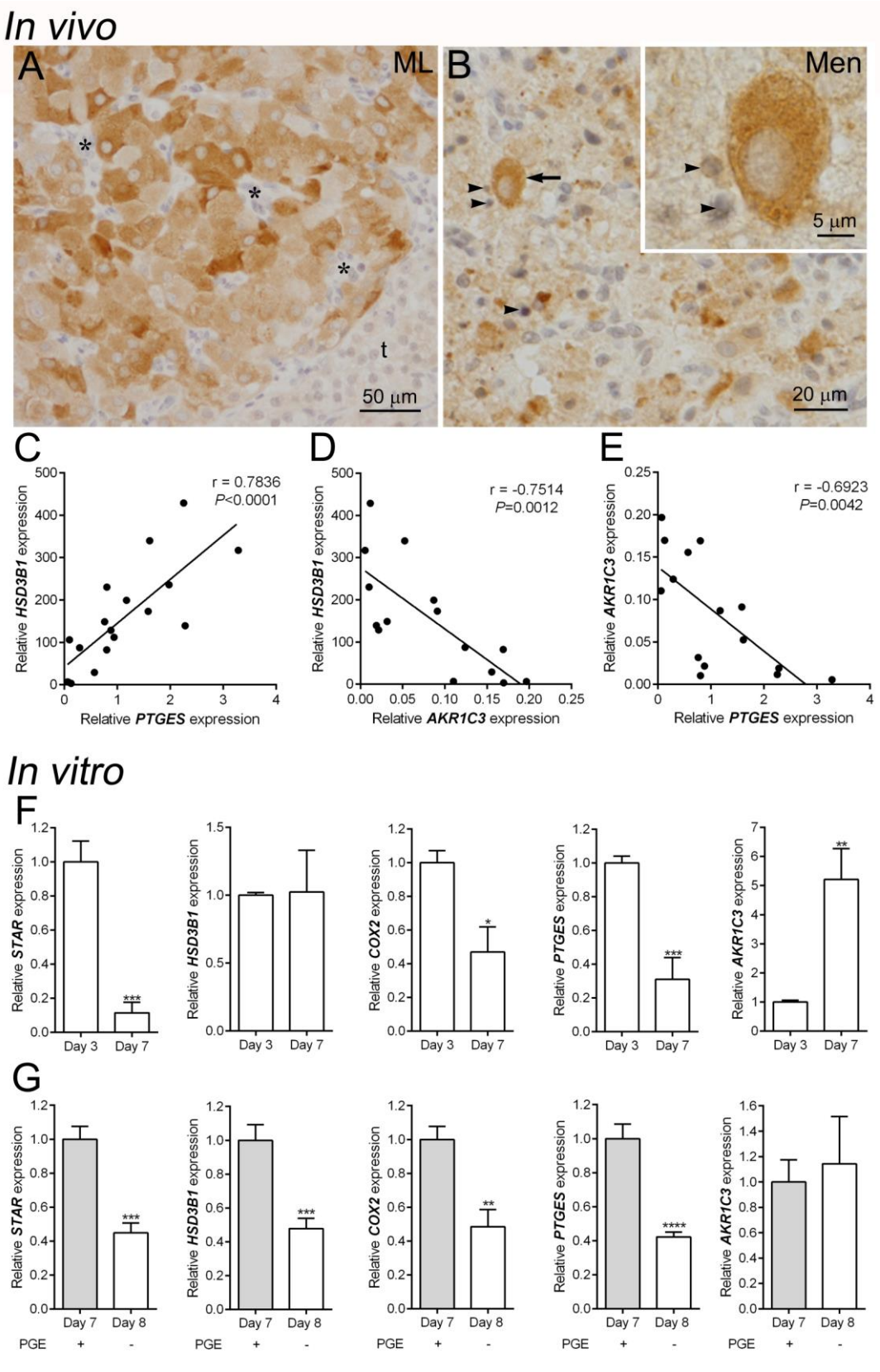


Figure 4 Nio-Kobayashi et al.

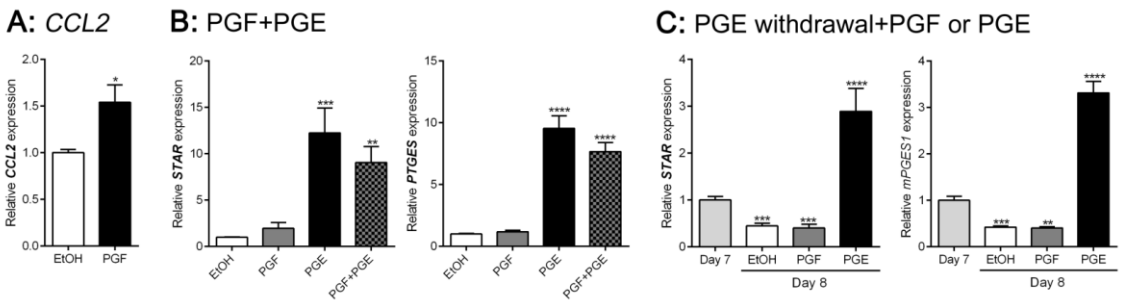


Figure 5 Nio-Kobayashi et al.

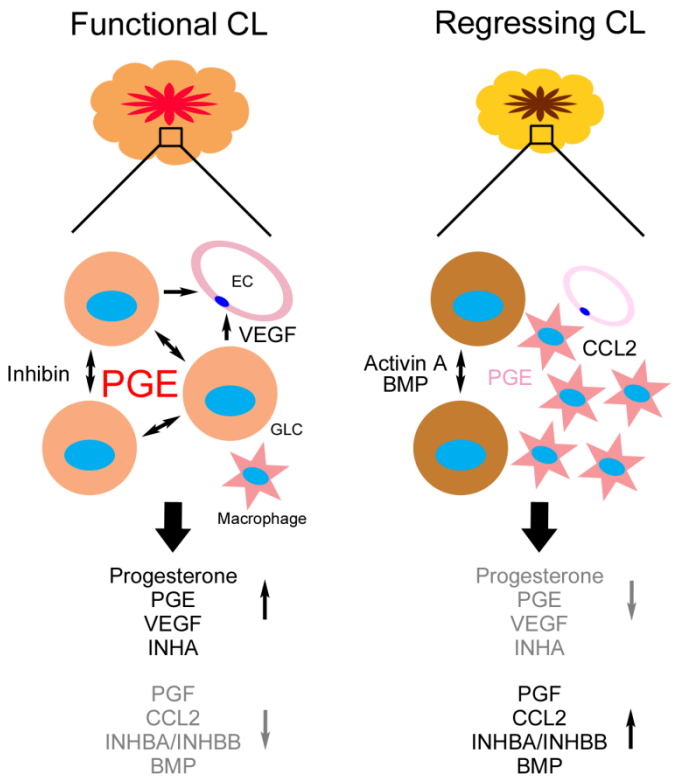
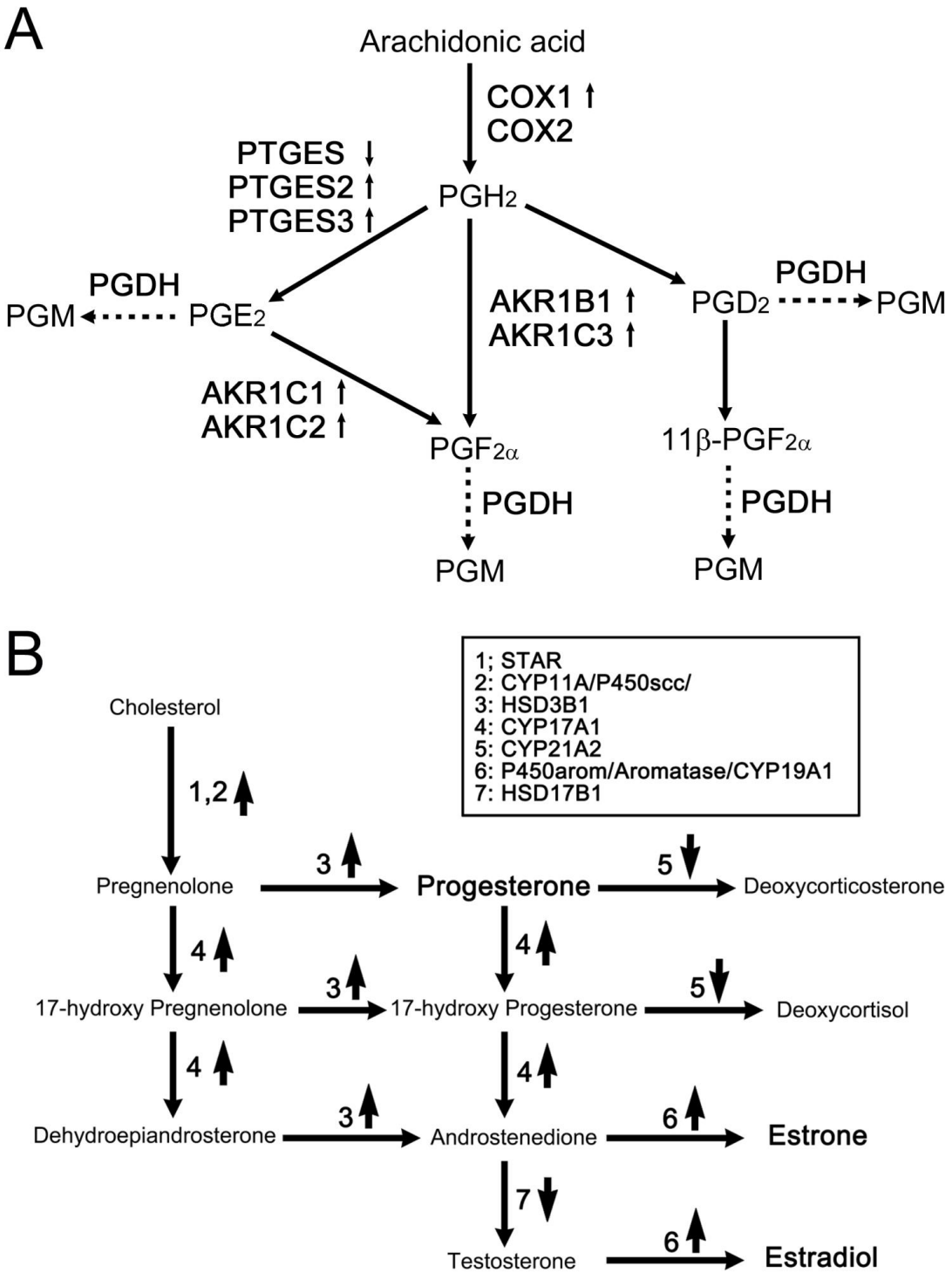


Figure 6 Nio-Kobayashi et al.



Supplementary figure 1 Nio-Kobayashi et al.

Table 1. Primers used for quantitative PCR analysis

Genes name	Protein name	Accession no.	Forward 5' to 3'	Reverse 5' to 3'	PCR Product size (bp)
G6PDH	G6PDH	NM_000402	CGGAAACGGTCGTACACTTC	CCGACTGATGGAAGGCATC	155
COX1/PTGS1	COX1	NM_000962	GAGTACCGCAAGAGGTTTGG	GGAGACCCTTGAGGGAAAAG	211
COX2/PTGS2	COX2	NM_000963	CGCTTTATGCTGAAGCCCTA	GCTTCCAGTAGGCAGGAGAA	235
mPGES1/PTGES	mPGES1	NM_0004878	AGTGAGGCTGCGGAAGAAG	AGGGTTAGGACCCAGAAAGG	181
mPGES2/PTGES2	mPGES2	NM_025072	CTCATCAGCAAGCGACTCAA	GTGTGCTGCATCAGGTCATC	209
cPGES/PTGES3	cPGES	NM_006601	CAGTTGTCTCGGAGGAAGTGA	AAGCTTTGCCCTTTCTTTTG	175
AKR1B1	AKR1B1	NM_001628	AGCCATGGCAAGCCGTCTC	GCACCACAGCTTGCTGACG	247
AKR1C1	AKR1C1	NM_001353	ACCAAATTGGCAATTGAAGCT	TGGGATCACTTCCTCACCTG	279
AKR1C2	AKR1C2	NM_001354	GGAAGAAACATTTGCTAACC	CAGTCCAACCTGCTCCTCA	224
AKR1C3	AKR1C3	NM_003739	CTGGGATCTCAACGAGACAA	GCTTTCATGTCCTCTGCAGTC	230
PGDH	PGDH	NM_000860	CAGCCGGTTTATTGTGCTTC	GGTGGGTCCAAAATTCCATAG	227
HSD3B1	3β-HSD	NM_000862	CCATGAAGAAGAGCCTCTGG	GTTGTTCAGGGCCTCGTTTA	202
STAR	STAR	NM_000349	GGCTACTCAGCATCGACCTC	CATCCCACTGTCACCAGATG	250

AKR; aldo-keto reductase, COX; cyclo-oxygenase, G6PDH; glucose-6-phosphate dehydrogenase, HSD; hydroxysteroid dehydrogenase, PGDH; 15-hydroxy PG dehydrogenase, PGES/PTGES; prostaglandin E synthase, STAR; steroidogenic acute regulatory protein

Table 2. Changes in the mRNA expression of prostaglandin synthetic and metabolic enzymes in the human corpus luteum across luteal phases

	EL n=4	ML n=6	LL n=5	Men n=3	P value
COX1	0.02 ± 0.012[a]	0.02 ± 0.004[a]	0.03 ± 0.011[a]	0.09 ± 0.041[b]	*P*<0.01
COX2	0.01 ± 0.007	0.02 ± 0.008	0.02 ± 0.007	0.02 ± 0.013	NS
PTGES	0.97 ± 0.394	1.88 ± 0.378[a]	0.83 ± 0.217	0.09 ± 0.028[b]	*P*<0.01
PTGES2	0.49 ± 0.070[a]	0.70 ± 0.077	0.93 ± 0.226[b]	0.59 ± 0.092	*P*<0.05
PTGES3	3.23 ± 0.408[a]	3.36 ± 0.429[a]	6.11 ± 1.166[b]	4.82 ± 1.821	*P*<0.05
AKR1B1	1.44 ± 0.392[a]	1.91 ± 0.301	2.66 ± 0.589[b]	1.28 ± 0.612[a]	*P*<0.05
AKR1C1	0.04 ± 0.011[a]	0.03 ± 0.008[a]	0.11 ± 0.028[b]	0.07 ± 0.028	*P*<0.05
AKR1C2	0.33 ± 0.153	0.27 ± 0.088[a]	0.65 ± 0.188[b]	0.46 ± 0.224	*P*<0.05
AKR1C3	0.02 ± 0.007[a]	0.03 ± 0.012[a]	0.12 ± 0.015[b]	0.16 ± 0.031[b]	*P*<0.001
PGDH	0.07 ± 0.027	0.06 ± 0.015	0.08 ± 0.020	0.07 ± 0.038	NS

Values represent the mean ± SEM of relative expression to *G6PDH*. Statistical analyses were performed using a one-way ANOVA with Sidak's multiple comparisons test, using GraphPad Prism 6 software. Values with different superscript letters indicate significant difference (P<0.05). NS: not significant. EL; early-luteal, LL; late-luteal, Men; menstrual, ML; mid-luteal.

Table 3. Effect of prostaglandin (PG)F on the mRNA expression of steroidogenic enzymes, PG synthases and metabolic enzymes, and luteolytic factors

Genes	Control (n=6)	PGF (n=6)	*P* value
STAR	2.18 ± 0.669	2.75 ± 0.505	NS
HSD3B1	32.06 ± 2.337	31.42 ± 1.706	NS
COX2	0.04 ± 0.006	0.04 ± 0.006	NS
PTGES	0.12 ± 0.015	0.11 ± 0.021	NS
AKR1C3	0.0039 ± 0.0002	0.0035 ± 0.0002	NS
INHA	0.54 ± 0.121	0.54 ± 0.057	NS
INHBA	0.74 ± 0.146	0.76 ± 0.103	NS
INHBB	0.11 ± 0.064	0.09 ± 0.052	NS
BMP2	0.11 ± 0.064	0.09 ± 0.052	NS
BMP4	0.07 ± 0.034	0.06 ± 0.025	NS
BMP6	0.05 ± 0.024	0.05 ± 0.016	NS

Values represent the mean ± SEM of relative expression to *G6PDH*. Statistical analyses were performed by unpaired Student's t-tests using GraphPad Prism 6 software, and $P<0.05$ was regarded as significant. NS: not significant.