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Prokineticin ligands and receptors are expressed by germ cells in the human fetal ovary and regulate *COX2* expression

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Abstract

Context: Fetal ovarian development and primordial follicle formation underpin future female fertility. Prokineticin (PROK) ligands regulate cell survival, proliferation and angiogenesis in adult reproductive tissues including the ovary. However, their expression and function during fetal ovarian development remains unclear.

Objective: To investigate expression and localization of the PROK ligands, receptors and their downstream transcriptional targets in the human fetal ovary.

Setting: This study was conducted at the University of Edinburgh.

Participants: Ovaries were collected from 37 morphologically normal human fetuses.

Design and Main Outcome Measures: mRNA and protein expression of PROK ligands and receptors was determined in human fetal ovaries using qRT-PCR, immunoblotting and immunohistochemistry. Functional studies were performed using a human germ cell line stably transfected with PROKR1.

Results: Expression of *PROK1* and *PROKR1* was significantly higher in later gestational ovaries (17-20 weeks) than at earlier gestations (8-11 and 14-16 weeks). *PROK2* also significantly increased across gestational age. *PROKR2* expression remained unchanged. PROK ligand and receptor proteins were predominantly localised to germ cells (including oocytes within primordial follicles) and endothelial cells, indicating these cell types to be the targets of PROK signalling in the human fetal ovary. PROK1 treatment of a germ cell line stably-expressing PROKR1 resulted in ERK phosphorylation, and elevated *COX2* expression.

Conclusions: Developmental changes in expression and regulation of *COX2* and pERK by PROK1 suggest that PROK ligands may be novel regulators of germ cell development in the human fetal

ovary, interacting within a network of growth and survival factors prior to primordial follicle formation.

Introduction

During fetal life, the germ cell complement of the ovary goes through a series of complex processes, initiating with germ cell specification and migration into the gonad, followed by proliferation and entry into meiosis, and culminating in the formation of primordial follicles (1). Proper development of the ovary and primordial follicles defines a female's future reproductive capacity (2), failure of which could lead to premature ovarian insufficiency (POI) or infertility. These dynamic developmental events are regulated by the germ cell niche, a milieu of autocrine, juxtacrine and paracrine factors of both germ cell and somatic cell origin that govern the balance between germ cell proliferation and development, or cell death (3). Although some of the regulators involved in the germ cell niche have been identified (4), the overall mechanisms governing fetal ovarian development remain unclear.

Prokineticin (PROK)1 and PROK2 (85 and 81 aa respectively) are peptide regulators of angiogenesis and inflammation (5). The PROK ligands share 85% homology and signal interchangeably via two G-coupled protein receptors, PROK receptor 1 and 2 (PROKR1 and PROKR2) to activate the MAP kinase and STAT signaling pathways (6,7). PROK ligands promote proliferation, differentiation and survival in both endothelial and immune cells, and have well defined functions in the vascular and gastrointestinal systems (8,9).

PROK ligand expression is highest in endocrine tissues including the reproductive organs, (10,11), however the role(s) of PROK signalling in the ovary and testis remains unclear. PROK ligands are expressed by the interstitial cells of the fetal testis (10), the granulosa cells of primordial and primary

follicles, and in the corpus luteum of the adult ovary (12,13), wherein PROK1 is hypothesized to regulate cellular remodeling. Genome-wide studies of gene expression during human fetal ovarian development have demonstrated increased expression of *PROK1* in the fetal ovary compared to the testis (14), and an increase in *PROK2* in the human fetal ovary with gestation (15), suggesting possible roles for PROK1 and PROK2 during fetal ovarian development. In addition, the PROK ligands regulate the expression of several factors thought to be involved in ovarian development including the interleukin-6 (IL6)-type cytokine Leukaemia Inhibitory Factor (LIF), and cyclooxygenase-2 (COX2), which catalyses the formation of prostaglandins from arachadonic acid (11,16), all of which are expressed by the human fetal ovary. Further investigation of the localization and function of the PROK receptors in the fetal ovary has yet to be performed.

We investigated the expression and function of components of the PROK signalling pathways during human fetal ovarian development. The expression of the PROK ligands and their receptors was characterised during key stages of ovarian development leading up to primordial follicle formation, and functional studies on the role of PROK signalling in regulating gene expression undertaken using a human germ cell tumour line (TCam-2 cells (17)) stably transfected with the PROKR1 receptor (21,24).

Materials and Methods

Tissue collection

Human fetuses (8–20 weeks gestation (by ultrasound and foot length; based on last menstrual period)) were obtained following medical or surgical termination of pregnancy. Informed consent was given and the study approved by the Lothian Research Ethics Committee (LREC 08/S1101/1). All specimens were morphologically normal. Gonads were dissected and mesonephric tissue removed. Sex of 8-12 week specimens was determined by *SRY* PCR genotyping (18). Ovaries were snap frozen at –80° C or fixed in Bouin's solution. Separate specimens were utilized for qRT-PCR, western

blotting, and immunohistochemical staining as described below, with a total of 37 specimens utilized for this study (8-12 week tissues (n=9), 14-16 weeks (n=13), 17-20 weeks (n=15)).

RNA extraction and cDNA synthesis

Total RNA was extracted using RNeasy Micro Kit (Qiagen, Crawley, UK) with on-column DNaseI digestion as per the manufacturer's instructions. First-strand cDNA synthesis was performed using Superscript VILO Master Mix (Life Technologies, Paisley, UK) according to the manufacturer's instructions. Duplicate negative control reactions in which the reverse transcriptase enzyme was omitted were also performed.

Quantitative RT-PCR

Quantification of mRNA expression relative to a housekeeping gene was performed using PowerSYBR Green Master Mix and the ABI7500Fast system (both Life Technologies).

Quantification was performed as described previously (19). Primer sequences utilized for this study are detailed in Table I.

Immunohistochemistry

Ovarian sections (5 µm) were mounted on microscope slides (VWR, Radnor, PA, USA), dewaxed and rehydrated. Immunohistochemistry was performed as described previously (19) using rabbit anti-human PROK1 (1:250; Phoenix Pharmaceuticals, CA, USA), PROKR1 (1:100, MBL, MA, USA), PROKR2 (1:50, MBL) or goat anti-human PROK2 (1:50, Santa Cruz, CA, USA). Primary antibody was detected using a goat anti-rabbit biotinylated secondary antibody (1:500, Vector Labs, CA, USA) or a rabbit anti-goat biotinylated secondary antibody (1:500, Dako, Glostrup, Denmark). Antibody was visualised using streptavidin-horseradish peroxidase (Vector) and 3,3-diaminobenzidine (DAB, Dako). The primary antibodies used in this study are not known to cross react, and negative controls in which the primary antibody was omitted were performed in parallel to determine any non-specific

staining. Images were captured using an Olympus Provis microscope (Olympus Optical Co., London, UK) equipped with a Kodak DCS330 camera (Eastman Kodak Co, Rochester, NY).

Production of a hPROKR1 expression vector

The open reading frame of human PROKR1 was amplified from pcDNA2-humPROKR1 (11,16) by PCR using primers attb1 and attb2, to add a Kozak sequence and prolactin signal peptide in-frame upstream of the PROKR1 sequence, creating attb1-Prl-humPROKR1-attb2. Gateway cloning sites within the attb1 and attb2 primers enabled Gateway shuttling of the PCR product into the pLenti6-cppt-CMV-DEST-opre vector, to create pLenti6-cppt-CMV-Prl-hPROKR1-opre (referred to hereafter as pLenti6-hPROKR1). Primer sequences were: attb1: 5'-

ggggacaagtttgtaaaaaagcagggtcaccatggacagcaaaaggttcgtcgcagaaaagggtcccgctgctcctgctgctggtggtgtaaa
tctactcttgccagggtgtggtctccgcgccaatggagaccaccatgggggtcatggatgacaatgccacc-3' and attb2: 5'-
ggggaccactttgtacaagaaagctgggtatcttatttagtctgatgcagtcacctc-3'.

Cell culture, treatments and generation of TCam-2 cells stably expressing PROKR1

TCam-2 cells (a kind gift of Dr. Leendert Looijenga (20)) were cultured as previously described (21). To generate stably transfected cells, TCam-2 cells were plated at ~70% confluency in a 10 cm dish and transfected with 5 µg pLenti6-hPROKR1, using TransIT -LT1 (Mirus Bio, WI, USA) reagent according to manufacturer's instructions. Stable transfectants were selected using Blastacidin (10 µg/ml, Invivogen, CA, USA). Selection was maintained for routine culture, but removed for treatment with PROK1. PROKR1 transfected Ishikawa cells, cultured as previously reported (11), were utilized as a positive control.

For identification of downstream targets, stably transfected PROKR1-TCam-2 cells (PROKR1-TCam-2 cells) were treated with vehicle (dH₂O) or 40nM recombinant human PROK1 (Peprotech, NJ, USA, dissolved in dH₂O) with cells harvested at various timepoints in RLT buffer (Qiagen) prior to RNA extraction.

Western blotting

Protein samples were isolated in RIPA buffer post-treatment and quantified by Bradford Assay (Bio-Rad, Hercules, CA, USA). 15-30µg lysate was denatured in SDS loading buffer containing 2-mercaptoethanol (Sigma-Aldrich, Poole, UK) and separated on 12% Tris-HEPES-SDS gel (for PROKR1 detection) or 4-20% gradient SDS gels ((pERK detection) Thermo Scientific, Loughborough, UK). Proteins were transferred onto Immobilon-FL PVDF membranes (Millipore, Watford, UK) using Semi-Dry Fast Transfer Buffer (Thermo Scientific) on a Fast Semi-Dry Transfer Blotter (Thermo Scientific). Membranes were blocked with Li-Cor blocking buffer (Odyssey, Cambridge, UK) and incubated with primary antibodies for pERK (1:2000, Cell Signalling, MA, USA), PROKR1 (1:2500, MBL) or α -tubulin (1:10,000, Sigma-Aldrich). Corresponding secondary antibody was utilized; anti-rabbit IgG conjugated to Alexa Fluor 680 (1:10000 Invitrogen Life Tech) or anti-mouse IgG conjugated to IRDye 800 (1:10000 Rockland), followed by scanning on a Li-Cor Infra-Red Imaging System (Odyssey, Lincoln, NE, USA). Densitometry was performed using ImageJ software.

Proliferation assay

Sulforhodamine B (SRB, Sigma-Aldrich) colorimetric assay was utilised to determine cell density. PROKR1-TCam-2 cells were plated at 2,000 cells per well in a 96-well plate and fixed at varying timepoints post PROK1 treatment as described with 20% trichloroacetic acid (Sigma-Aldrich). Cellular proteins were stained with 0.04% SRB (vol:vol) in 1% acetic acid (Sigma-Aldrich). After staining SRB was resuspended in 10 mM Tris and cell density quantified at 505 nm.

Statistical Analysis

Gestational qRT-PCR and pERK densitometry data were analysed by ANOVA with the Newman-Keuls post-test for statistical significance. qRT-PCR data post PROK1 treatment of PROKR1-TCam-2 cells were analysed using paired t-tests with GraphPad Prism 5.0 software.

Results

PROK signalling components are developmentally regulated in the human fetal ovary

The presence and pattern of expression of transcripts encoding PROK signalling components during human fetal ovarian development was investigated by qRT-PCR for the PROK ligands (*PROK1* and 2) and receptors (*PROKR1* and 2) across a range of gestations (from 8-20 weeks). Ovarian specimens were grouped into three gestational stages broadly to reflect key developmental events; the proliferation of undifferentiated PGCs (8-11 weeks gestation), the formation of germ cell nests and initial entry of germ cells into meiosis (14-16 weeks) and on-going meiotic entry and the onset of primordial follicle formation (17-20 weeks) (22,23).

Transcripts encoding PROK ligands and receptors were expressed in the human fetal ovary across all gestations investigated. Expression of *PROK1* increased significantly in 17-20 week ovarian tissue compared to both 8-11 (4.9 fold) and 14-16 weeks (2.4 fold, $p=0.02$ and 0.05 respectively, $n=5-7$ samples per group, Figure 1A). *PROK2* expression also increased with increasing gestation, with transcript levels 12- and 24-fold higher in the 14-16 and 17-20 week, compared to 8-11 week fetal ovaries ($p=0.02$ and $p=0.0001$ respectively, Figure 1B). *PROKR1* displayed a similar expression pattern to that of *PROK1*, with a significant increase specifically in 17-20 week tissue in comparison to both 8-11 (2.4 fold) and 14-16 week fetal ovaries (2.3 fold, $p=0.008$ and 0.007 respectively, Figure 1C). Conversely, no gestational change in expression was determined for *PROKR2* (Figure 1D).

PROK ligands localised to the germ cell nests in developing human fetal ovaries

The extent and localisation of PROK ligand protein expression was established using immunohistochemistry on sections of human fetal ovaries isolated from fetuses across a range of gestations. Expression of *PROK1* and *PROK2* was not detected in early tissues (8-12 weeks, data not shown), but was readily detected in fetal ovaries after 14 weeks gestation. *PROK1* expression was germ cell-specific, with pairs or groups of germ cells staining intensely compared to other germ cells within the same nest (Figure 2A-B). This expression pattern was seen throughout later gestational

tissues (14-19 weeks, n=3), with primordial follicles also demonstrating PROK1 expression (Figure 2B inset). PROK2 expression appeared much weaker than that of PROK1, with expression seen in germ cell nests, including both germ and pre-granulosa cells (Figure 2C-D). This expression pattern was consistent across later gestation (14-19 weeks, n=3), although definitive staining in primordial follicles was not seen.

PROK receptors are expressed by germ cells of the fetal ovary

To determine the targets of PROK action in the human fetal ovary, the PROK receptors were also investigated via immunohistochemistry. As with the PROK ligands, the PROK receptors were not at detectable levels in early tissues (8-12 weeks, data not shown). However, both receptors were detectable in later gestations (14-19 weeks, n=3). PROKR1 was strongly expressed by the germ cells of the fetal ovary, with no staining seen in somatic cells surrounding germ cell nests or in negative control tissue (Figure 2E and inset). The endothelial cells of blood vessels in the human fetal ovary also expressed PROKR1 (in keeping with its role in angiogenesis; Figure 2F). PROKR2 protein was also expressed by germ cells, but not in somatic and pre-granulosa cells (Figure 2G-H).

PROKR1-TCam-2 cells as a model for fetal germ cells

TCam-2 cells are derived from a human germ cell tumour and have been previously characterised as a model of human fetal germ cells, as they express numerous markers of early human germ cells (21,24). Parental TCam-2 cells expressed *PROKR1* mRNA at comparable levels to that detected in the human fetal ovary (Figure 3B), however in contrast to the human fetal ovary, PROKR1 protein was almost undetectable in TCam-2 cells (Figure 3A). For this reason, coupled with the fact that TCam-2 cells are refractory to transfection, TCam-2 cells stably transfected with the PROKR1 receptor (PROKR1-TCam-2 cells) were generated to enable functional analysis of PROK ligand signalling onto germ cells. Levels of *PROKR1* mRNA were significantly higher in stably-transfected PROKR1-TCam-2 cells than in parental TCam-2 cells, or in the human fetal ovary (Figure 3B). However, PROKR1 protein levels in the transfected PROKR1-TCam-2 cells were found to be

comparable to those detected in the human fetal ovary (Figure 3A). Ishikawa cells, stably transfected with PROKR1 (PROKR1 ISHI) were utilized as a positive control for mRNA and protein expression of PROKR1. PROKR1 functionality and downstream signal transduction in stable PROKR1-TCam-2 cells was confirmed via induction of phosphorylated ERK (pERK) post PROK1 (40 nM) treatment (Figure 3C), with significant induction after 5 minutes compared to vehicle (dH₂O) treated cells (3.8 ± 1.9 (PROK1) vs. 1.7 ± 0.6 (dH₂O) -fold pERK induction compared to T0, Figure 3D). This induction is comparable to that seen previously post PROK1 treatment in PROKR1 ISHI cells (11).

PROK1 regulation of prostaglandin signalling components

In the endometrium, PROK1 regulates the expression of *COX2* and *LIF* (11,16); both of which have been identified as possible regulators of human ovarian germ cell development (19,25). Whether this regulatory relationship also occurs in human fetal germ cells was investigated by culturing stably transfected PROKR1-TCam-2 cells in the presence or absence of 40nM recombinant human PROK1 for 12 hours. Treatment of PROKR1-TCam-2 cells with PROK1 resulted in a significant 2.9-fold increase in the expression of *COX2* compared to cells treated with vehicle (dH₂O) ($p=0.01$, $n=5$, Figure 4A). *COX2* induction was confirmed to be due to PROK1 signalling via PROKR1, as parental TCam-2 cells expresses relatively little PROKR2 and did not respond to PROK1 treatment (Supplemental Figure 1). *COX2* induction was noted as early as 4 hours post PROK1 treatment (Supplemental Figure 2). No change was detected in the expression of the other prostaglandin precursor enzymes *COX1* and *PTGES* (which encodes the enzyme that catalyses the terminal step of prostaglandin synthesis, from prostaglandin precursor to prostaglandin E₂, Figure 4B and C). Treatment with PROK1 also had no effect on the expression of genes encoding three prostaglandin E receptors (*EP2*, *EP3* and *EP4*, Supplemental Figure 3A) which are known to be expressed by germ cells in the human fetal ovary (19) and have been shown to form positive feedback loops with *COX2* to promote their expression in the renal system (26,27). Additionally, expression of *LIF* nor the other genes that encode related IL6-type cytokines; IL6 and oncostatin-M (OSM) were altered with PROK1 treatment (Figure 4D-F). These data demonstrate that IL6-type cytokines do not respond to PROK1

treatment in this model, in contrast to such regulation in other reproductive tissues. Further, these data confirm that PROK1, acting via PROKR1, is able to regulated *COX2* specifically in an *in vitro* model of human fetal germ cells, and does not initiate a positive feedback loop of prostaglandin E₂ signalling in this system. As prostaglandin signalling has previously been related to enhanced germ cell survival in human fetal ovaries, we examined if PROK1, acting via PROKR1, altered PROKR1-TCam-2 cell number at 1, 2 and 4 days post PROK1 treatment (Supplemental Figure 3B). No change in SRB colorimetric assay was determined suggesting PROK1 is unable to induce changes in PROKR1-TCam-2 cell survival or proliferation.

Discussion

Identifying novel regulators of fetal ovarian development is essential to enhance our understanding of the processes which lead up to primordial follicle formation, which in turn is critical for female reproduction. The PROK ligands are peptide regulators in various female reproductive tissues, including the endometrium (11,16), placenta (28), and fallopian tube (29). Additionally, studies have identified that PROK ligands are expressed in the granulosa cells of immature follicles and theca of corpora lutea in the adult ovary, where they are hypothesised to regulate differentiation allowing for luteinisation (12,13). Furthermore, PROK receptors are expressed in both steroidogenic cell types of the adult ovary (13). Homozygous ablation of the PROK receptors in murine models revealed loss of *Prokr2*, but not *Prokr1*, resulted in atrophy of the reproductive system (both male and female) (30), however this phenotype was found to be the result of absent GnRH neurons in the hypothalamus, resulting in the loss of hormone production necessary for reproductive development. Within the adult human ovary PROKR1 is abundant (31), and this study reveals this is also the case in the fetal ovary.

This study is the first to examine possible roles for the PROK ligands in the human fetal ovary, after microarray analyses indicated a possible role for these factors during human fetal gonadal development (14,15). We show that all PROK ligands and receptors are expressed in the human fetal ovary and that expression of both *PROK1* and 2 and their shared receptor, *PROKR1*, are up-regulated

with ongoing human ovarian development, with marked increases from the period of germ cell proliferation before meiotic onset to the time of primordial follicle formation. This is in agreement with the data from a previous study, which demonstrated increasing expression of *PROK2* in the human fetal ovary during the second trimester (15). Unlike the adult ovary, where most PROK expression is restricted to the granulosa cells (12,13), PROK ligands and receptors were expressed by the germ cells in the fetal ovary. Additional expression was seen in vascular endothelial cells, in keeping with the well-recognised role of PROK ligands in angiogenesis (32). With primary expression of the PROK components restricted to the fetal germ cells, it is likely that signalling is autocrine or paracrine between adjacent or nearby germ cells. The finding that PROK1 was expressed highly in pairs and small groups of adjacent oocytes is consistent with discrete paracrine signalling and may suggest a role for PROK1 in the synchronous development of germ cells within individual nests as described in mouse (33) and human (34).

Given that PROK signalling is predominately restricted to germ cells, we undertook functional studies of PROK action *in vitro* using TCam-2 cells stably transfected with the human PROKR1 receptor. TCam-2 cells express markers of undifferentiated human fetal germ cells (21,24) and thus may be considered comparable to the germ cells within the earlier gestational ovaries utilised in this study (8-12 weeks)). Using this stably transfected germ cell line, we demonstrated that PROK1, acting through PROKR1, is able to up-regulate phosphorylation of ERK. Induction of pERK downstream of PROK1-PROKR1 signalling has been previously reported and is thought to be downstream of Gq-PKC signalling (11). Phosphorylation of ERK in PROKR1-TCam-2 cells demonstrates active intracellular signalling downstream of stably transfected PROKR1.

PROK1 via PROKR1 signalling was also able to induce expression of *COX2*, encoding a prostaglandin synthesis enzyme, which we have previously shown to be expressed by germ cells in the human fetal ovary (19), the targets of PROK signalling in this organ. Enhanced *COX2* expression in TCam-2 cells in response to PROK1 was specific, as expression of the other prostaglandin synthesis enzymes (*COX1*, *PTGES* [which encodes the enzyme that catalyzes the terminal step of

prostaglandin E2 synthesis from prostaglandin precursor]) and receptors (*EP2–4*), which are known to be expressed by germ cells in the human fetal ovary (19) and have been shown to form positive feedback loops with *COX2* to promote their expression in the renal system (26, 27). The IL6-type cytokines (*IL6*, *LIF*, and *OSM*) also remained unchanged. This is in contrast with studies that report PROK1 regulation of the prostaglandin E2 receptor EP4 in intestinal epithelial cells (9) and *LIF* activation in endometrial epithelial cell (16), and may suggest distinct function for PROK1 in germ cells.

If PROK1 mediates an increase in *COX2* expression in the fetal ovary, this would be likely to result in increased prostaglandin synthesis and action in that tissue. By selectively regulating *COX2* (the inducible cyclooxygenase enzyme) rather than the constitutively-expressed *COX1*, PROK1 is likely to be involved in specific events in the fetal ovary rather than homeostatic function (35). This positions prokineticin signalling upstream of prostaglandin synthesis within the human fetal ovary, a significant finding given that we have previously demonstrated prostaglandin signalling regulates the expression of multiple factors which influence fetal ovarian germ cell development (19). Treatment of human fetal ovaries with prostaglandin E₂ promotes the expression of the genes encoding the neurotrophin brain-derived neurotrophic factor (BDNF), the TGF-beta superfamily member activin A and the anti-apoptotic BCL2 family member MCL1 (19), all of which are involved in the survival, proliferation or differentiation of germ cells. Neurotrophins including BDNF are regulators of germ cell survival and follicle formation in the developing ovary (36,37), activin A promotes germ cell proliferation (38) and may regulate the timing of follicle formation (39) and MCL1 is expressed by oocytes immediately prior to follicle formation (34) and has been implicated in the regulation of mouse oocyte cyst survival and breakdown (40). Therefore, PROK1-mediated up-regulation of *COX2* may be one pathway through which PROK ligands co-ordinately regulate multiple signalling pathways controlling ovarian development. Although there was no change in cell number after PROK1 treatment of PROKR1-TCam-2 cells in this study, PROK1 regulation of germ cell proliferation and survival

should be further investigated in primary ovarian tissue, containing both germ cells and surrounding stromal cells allowing for further elucidation of the signalling cascades downstream of PROK1 action.

In conclusion, this study has demonstrated that the expression of the PROK ligands, and one of their shared receptors (PROKR1), is up-regulated around the time of the initiation of primordial follicle formation in the human fetal ovary. The finding that PROK1 promotes *COX2* expression in an *in vitro* model of human fetal germ cells suggests a possible role for the PROK ligands in regulating prostaglandin signalling, which itself may influence germ cell survival, proliferation and development (19). Taken together, these data suggest a novel role for prokineticin signalling in the human fetal ovary at a critical time-point in the determination of female fertility.

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Table 1: Primer Sequences for PCR

Gene Target	Forward Primer (5' – 3')	Reverse Primer (5' - 3')
<i>CNTF</i>	CAGGGCCTGAACAAGAACAT	CTAAGAGCCTGGCCAACAAA
<i>COX1</i>	TGTTCCGGTGTCCAGTTCCAATA	ACCTTGAAGGAGTCAGGCATGAG
<i>COX2</i>	CCTTCCTCCTGTGCCTGATG	ACAATCTCATTTGAATCAGGAAGCT
<i>EP1</i>	AGATGGTGGGCCAGCTTGT	GCCACCAACACCAGCATTG
<i>EP2</i>	GACCGCTTACCTGCAGCTGTAC	TGAAGTTGCAGGCGAGCA
<i>EP3</i>	GACGGCCATTTCAGCTTATGG	TTGAAGATCATTTTCAACATCATTATCA
<i>EP4</i>	ACGCCGCCTACTCCTACATG	AGAGGACGGTGGCGAGAAT
<i>GAPDH</i>	GACATCAAGAAGGTGGTGAAGC	GTCCACCACCCTGTTGCTGTAG
<i>IL6</i>	GCCGCCCCACACAGACA	CCGTCGAGGATGTACCGAAT
<i>LIF</i>	TGGTGGAGCTGTACCGCATA	TGGTCCCGGGTGATGTTG
<i>OSM</i>	ACAGAGGACGCTGCTCAGTC	AGGAGTCTGCTGGTGTCTCTG
<i>PROK1</i>	GTGCCACCCCGGCAG	AGCAAGGACAGGTGTGGTGC
<i>PROKR1</i>	TCTTACAATGGCGGTAAGTCCA	CTCTTCGGTGGCAGGCAT
<i>PROK2</i>	TTGGGCGGAGGATGCA	AAATGAAGTCCGTAAACAGGCC
<i>PROKR2</i>	GCTCTGTGCCTCCGTCAACT	CCAGCAAGGCATTGGTGG
<i>PTGES</i>	GAAGAAGGCCTTTGCCAAC	GGGTTAGGACCCAGAAAGGA
<i>RPL32</i>	CATCTCCTTCTCGGCATCA	AACCCTGTTGTCAATGCCTC

Figure 1: PROK ligand and receptor expression is up-regulated during human ovarian development.

Expression of the genes encoding PROK ligands and their shared receptors was analysed by qRT-PCR with the gestation range examined divided into three groups reflecting progression from predominantly germ cell proliferation, entry into meiosis and early primordial follicle formation; 8-11 weeks, 14-16 weeks, and 17-20 weeks.. (A) Expression of *PROK1* increased in 17-20 weeks compared to both 8-12 and 14-16 weeks. (B) *PROK2* expression increased across gestation, with 17-20 weeks significantly increased compared to both 8-12 and 14-16 weeks. (C) *PROKR1* was significantly increased in 17-20 weeks but no change was seen in *PROKR2* expression (D). Expression is relative to the housekeeping gene *GAPDH*. Mean \pm SEM, n=5-7 per group (* $p<0.05$, ** $p<0.01$, and *** $p<0.001$).

Figure 2: PROK ligands and receptors are expressed in germ cell nests in the human fetal ovary.

PROK1 and 2 and their shared receptors *PROKR1* and 2 (brown staining) were immunolocalised in the human fetal ovary (14 week ovarian tissue shown, representative of n=3 14-19 week specimens). (A-B) *PROK1* expression was germ cell (GC) specific in the human fetal ovary, with pairs or multiple closely associated GCs displaying heightened *PROK1* expression compared to others nearby. No staining was seen in stromal cell streams (SC) or pregranulosa cells (PG; i.e. somatic cells interspersed within GC nests). (B inset) *PROK1* was also expressed in primordial follicles (17 week specimen). (C-D) *PROK2* was expressed by GCs and in PG cells within GC nests. No staining was detected in SC streams. (E-F) *PROKR1* was primarily expressed by GCs as well as endothelial cells of blood vessels (BV) in the ovary. (G-H) *PROKR2* was also expressed by GCs but expression was not as

abundant. (E inset). Negative control tissue showed no staining. Scale bars: A, C and E-H : 50µm; B and D: 20µm.

Figure 3: Characterisation of PROKR1-TCam-2 cells. Stable transfection and expression of PROKR1 in TCam-2 cells was validated at both the protein (A) and mRNA (B) level. Expression was investigated in comparison to parental TCam-2 cells (negative control), fetal ovarian tissue (15 weeks) and Ishikawa cells stably expressing PROKR1 (PROKR1 ISHI, positive control). mRNA expression is relative to the housekeeping gene *RPL32* and displayed as mean \pm SEM. (C-D) Functional signalling downstream of transfected PROKR1 was confirmed in PROKR1-TCam-2 cells as determined by significant induction of phosphorylated ERK (pERK) post 40 nM PROK treatment (+PROK) compared to vehicle control (dH₂O, VEH). Significance is determined by ANOVA, ** $p \leq 0.001$).

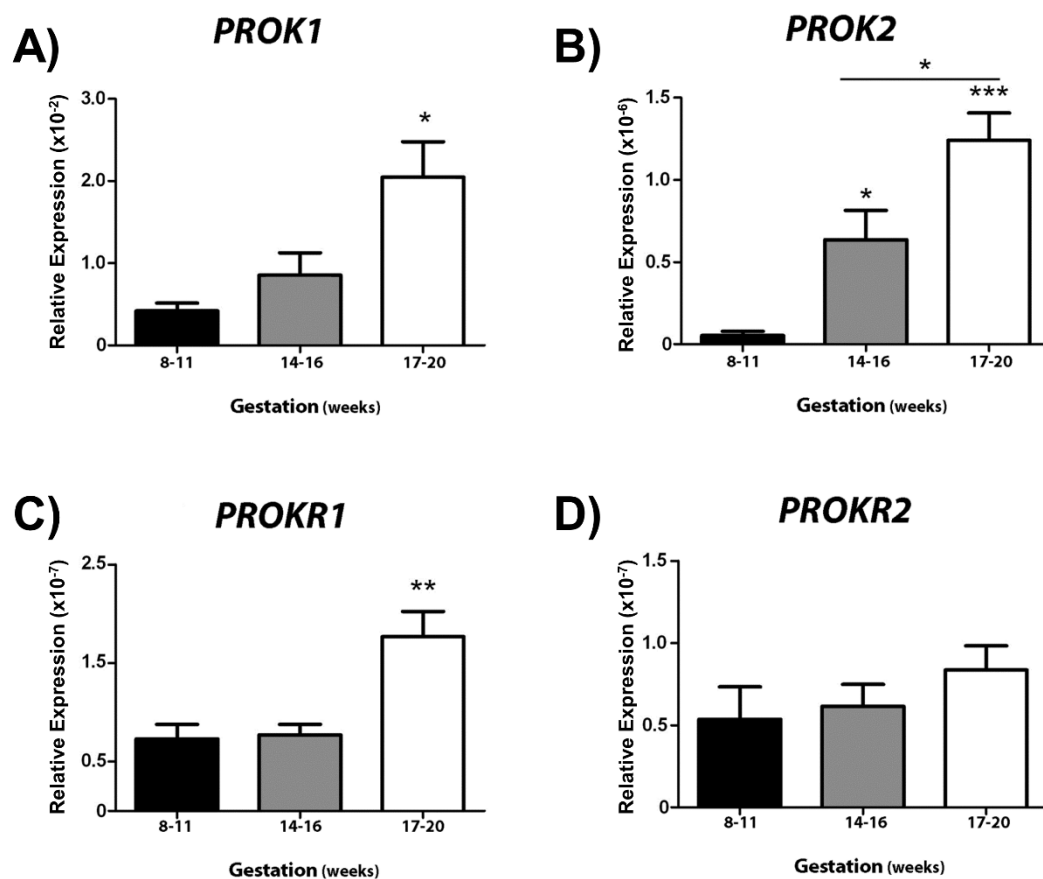
Figure 4: COX2 is regulated by PROK1 in PROKR1-TCam-2 cells. PROKR1-TCam-2 cells were treated for 12h with 40nM PROK1 (+PROK) or vehicle (dH₂O, VEH) and mRNA expression changes evaluated via qRT-PCR. (A) Expression of *COX2*, which encodes a prostaglandin biosynthesis enzyme, was significantly up-regulated in PROK1-treated PROKR1-TCam-2 cells, compared to vehicle-treatment. No significant changes were seen with in expression of the other prostaglandin synthesis enzymes (B) *COX1* or (C) *PTGES*. Additionally, no change is seen in the expression of genes encoding the IL6-type cytokines: (D) *LIF*, (E) *IL6* or (F) *OSM* in PROK1-treated PROKR1-TCam-2 cells. Expression is relative to the housekeeping gene *RPL32*. Mean \pm SEM, n=5 (* $p < 0.05$).

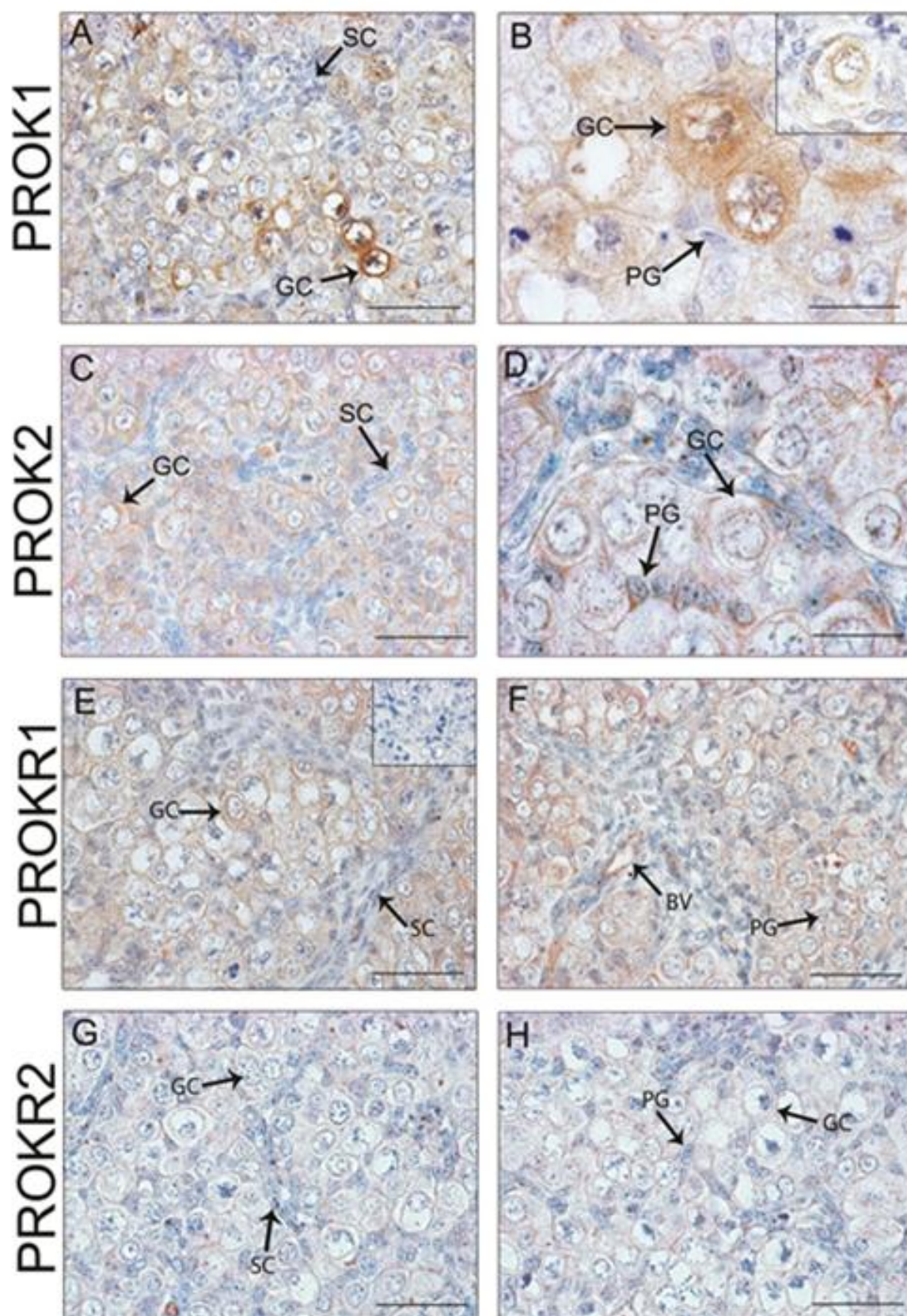
Supplemental Figure 1: Validation of PROK1 via PROKR1 induction of COX2. (A)

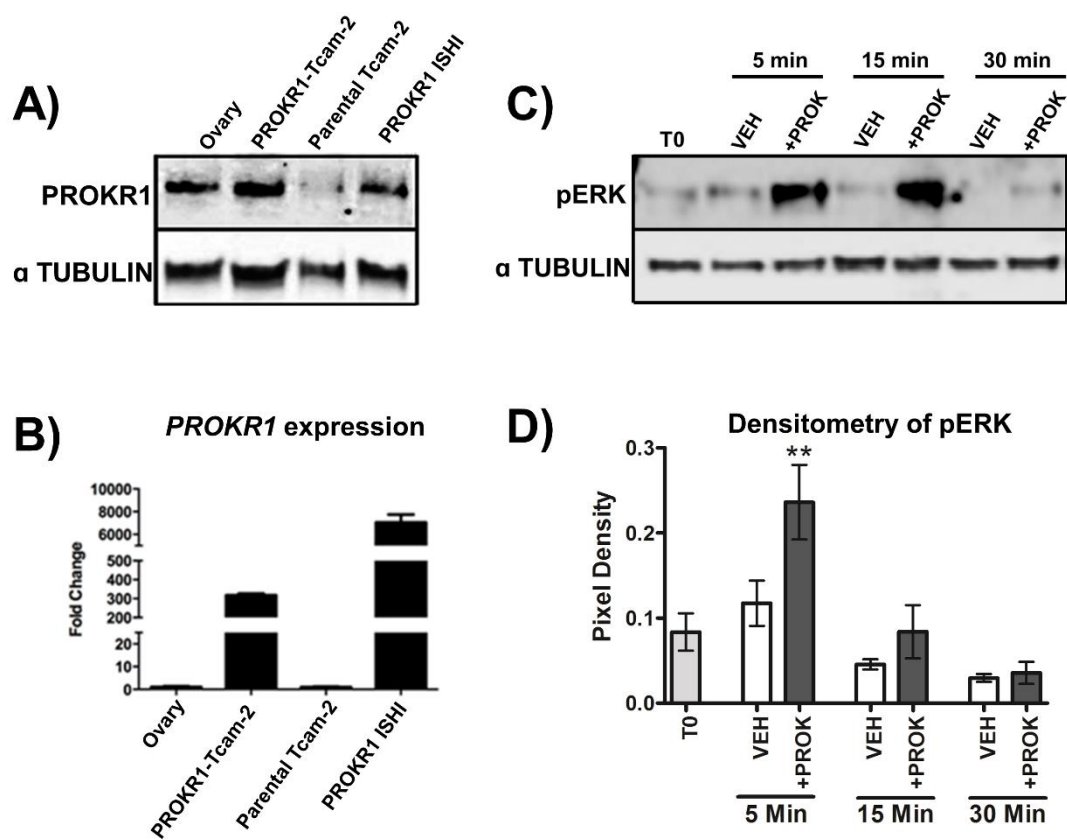
Further characterisation of the parental TCam-2 cells demonstrates they express significantly less PROKR2 transcript than human fetal ovarian tissue (15-18 weeks). (B) Further, treatment of parental TCam-2 cells with 40 nM PROK1 (+PROK) did not induce *COX2* expression compared to vehicle control (VEH), demonstrating that mRNA induction in PROKR1-TCam-2 cells is the result of PROK1 via PROKR1 downstream signalling. Data shown is relative to housekeeping gene *RPL32* and is mean \pm SEM, * $p \leq 0.05$.

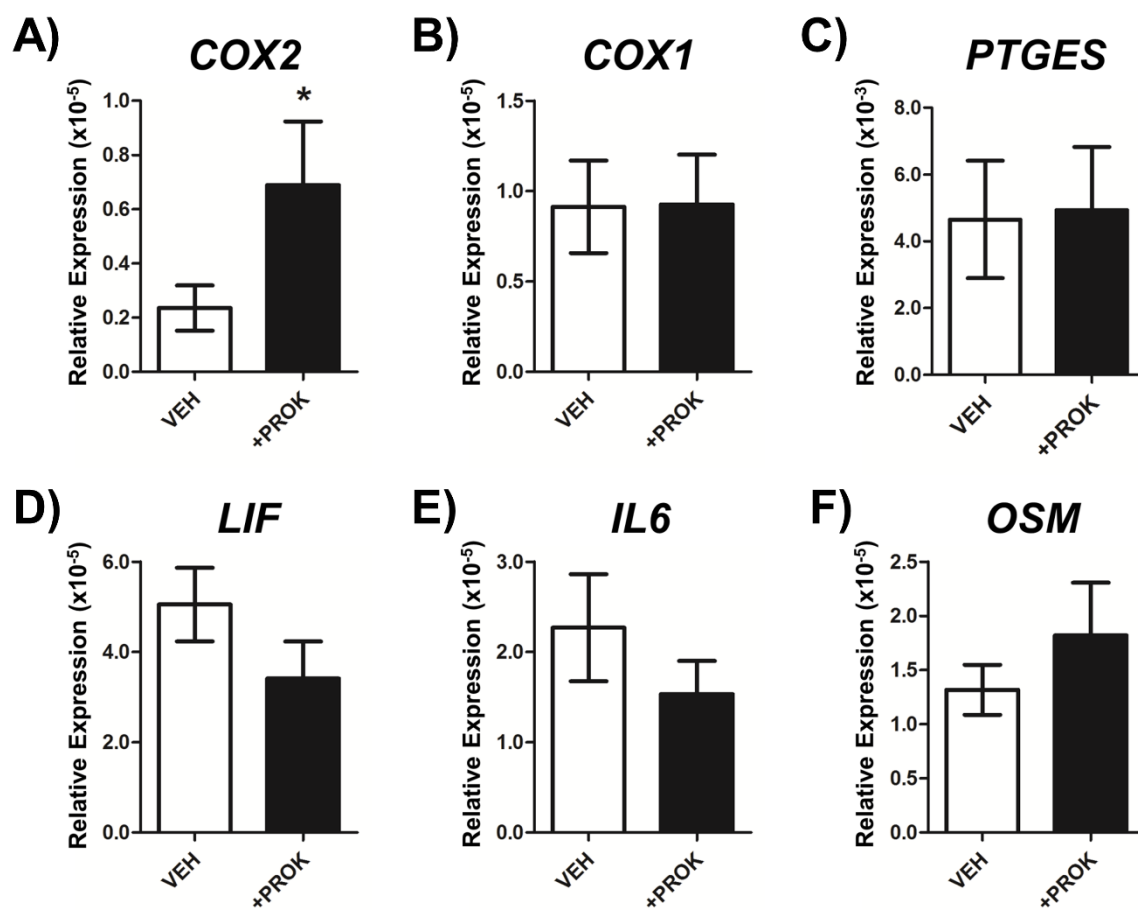
Supplemental Figure 2: *COX2* induction over time. *COX2* induction by PROK1 via PROKR1 was examined over a timecourse at 2, 4, 8, 12 and 24 hours (H). Enhanced *COX2* mRNA was seen as early as 4 hours post PROK1 treatment (+PROK) compared to vehicle control (VEH).

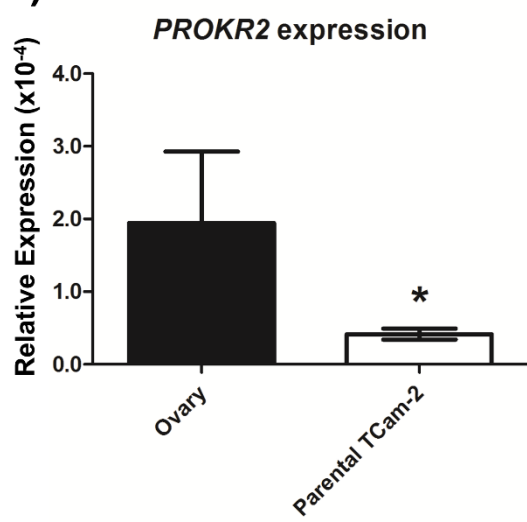
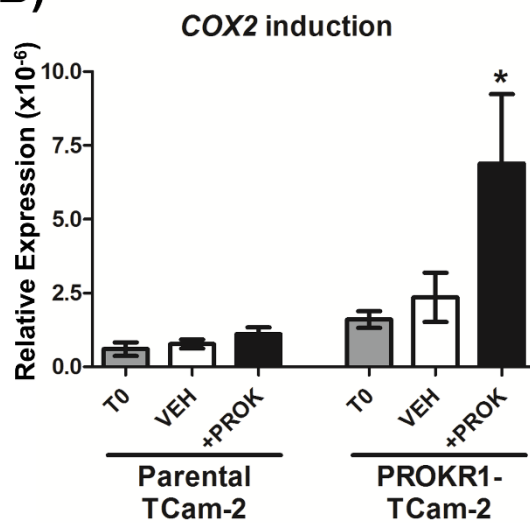
Supplemental Figure 3: Investigation of PROK1 functionality in PROKR1-TCam-2 cells. (A) As *COX2*, a prostaglandin synthesis enzyme, was induced by PROK1 treatment (+PROK) compared to vehicle treated PROKR1-TCam-2 cells (VEH), the prostaglandin E_2 receptors (EP1-4) were also investigated for changes in mRNA expression via qRT-PCR after 12 hours of treatment, as they form a positive feedback loop in other tissues. No change in expression was determined. (B) Changes in proliferation and survival were investigated downstream of PROK1 treatment of PROKR1-TCam2 cells treated with PROK or vehicle over 24, 48, and 96 hours. No significant difference was determined via colorimetric SRB assay suggesting PROK1 does not function to alter cell turnover in these cells.









A)**B)**

COX2 induction

