Uterine Expression of cp4 Gene Homolog in the Stripe-faced Dunnart, *Sminthopsis macroura*: Relationship With Conceptus Development and Progesterone Profile

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SUMMARY

The marsupial conceptus is surrounded by a uterine-secreted shell coat for 60–80% of gestation. Coat protein 4 (CP4) is the only marsupial shell coat protein characterized and it has only been identified in one species, the Common Brushtail Possum. In this possum, uterine transcription and secretion of *cp4* during the oestrous cycle is biphasic and associated with the stage of conceptus development. Here we cloned *sm-cp4* from a distantly related species, the Stripe-faced Dunnart (*Sminthopsis macroura*). Transcription of *sm-cp4* and secretion of smCP4 were identified by semi-quantitative RT-PCR and immunohistochemistry, respectively. The effect of reproductive hormones on *sm-cp4* transcription was investigated in vitro by treatment of uterine explant cultures with oestrogen and/or progesterone. In vivo, uterine expression of smCP4 was biphasic and associated with conceptus development. Uterine smCP4 expression (transcription and secretion) began during the pre-ovulatory period and continued post-ovulation during cleavage stages. Transcription of *sm-cp4* continued during the unilaminar blastocyst stage, but smCP4 secretion was reduced during this stage. During the bilaminar blastocyst stage, both transcription of *sm-cp4* and secretion of smCP4 were low before they both resumed during the trilaminar blastocyst stage, and continued during the embryo and fetal stages. In vitro uterine transcription of *sm-cp4* increased after incubation with progesterone.


INTRODUCTION

The marsupial conceptus is surrounded by a shell coat for 60–80% of gestation, from entry of the zygote into the uterus until hatching, which occurs between the late primitive streak stage and limb bud formation, on the day of implantation (Selwood, 2000). The role of the marsupial shell coat is not well understood, but whilst shell-free conceptuses develop normally to 4- and 8-cell stages in vitro, blastocyst formation and maintenance is compromised (Casey and Selwood, 2003).

Coat protein 4 (CP4; Fig. 1) is the first marsupial shell coat protein to be characterized. Indeed, it is the only mammalian uterine-secreted extracellular egg coat identified (Menkhorst and Selwood, 2008). It was first recognised in the Common Brushtail Possum, *Trichosurus vulpecula* (Order Diprotodontia; Cui and Selwood, 2003) and it has been found in the genomic DNA of monotremes, other marsupials and the rabbit, horse and seal (Menkhorst et al., 2008), all mammals known to have uterine additions to post-ovulatory extracellular egg coats (Denker, 2000; Menkhorst and Selwood, 2008). A unique protein with a
molecular weight of 17 kDa, CP4 is 306 amino acids long (Cui and Selwood, 2003). The N-terminus of CP4 shares significant homology with the C-terminus of \( \alpha \)-enolase (Cui and Selwood, 2003), a glycolytic enzyme expressed in a wide variety of tissues (Pancholi, 2001). CP4 is not predicted to be enzymatic as glutamines, which confer enzymatic capability to \( \alpha \)-enolase, are not found in the CP4 amino acid sequence (Fig. 1). The \( \alpha \)-enolase gene also encodes \( \tau \)-crystallin (Wistow et al., 1988), a structural lens protein found in vertebrates, including lampreys, some fish, reptiles, birds (Wistow et al., 1988) and, according to one study, mammals, including the marsupial, the Virginian Opossum (Didelphis virginiana) but not the Tammar Wallaby (Macropus eugenii) (Wistow and Kim, 1991). The vertebrate lens and marsupial shell coat share similar properties: they are both translucent (Caldwell, 1887) with the ability to modulate their form; have little turnover once formed (Wistow and Piigtorsky, 1988; Cui and Selwood, 2003); and are encoded by \( \alpha \)-enolase. Therefore, CP4 is probably a structural protein of the marsupial shell coat (Cui and Selwood, 2003).

Expression studies in T. vulpecula show that cp4 mRNA is expressed by the lung, heart, liver, spleen, kidney, ovary, oviduct and uterus (Cui and Selwood, 2003). Uterine cp4 mRNA expression is biphasic and correlated with the stage of conceptus development (Cui and Selwood, 2003): expression begins during the pre-ovulatory period and continues during cleavage stages before falling to low levels during the unilaminar and bilaminar blastocyst stages. Expression resumes during the trilaminar blastocyst stage and remains high during the embryonic stage of development (Cui and Selwood, 2003).

An important modification of CP4 relative to \( \alpha \)-enolase and \( \tau \)-crystallin is that the marsupial shell coat is extracellularly secreted by nonciliated cells in the luminal and glandular epithelium of the uterus (Casey et al., 2002). In vivo injections of oestradiol during the follicular phase in T. vulpecula result in prolonged shell coat secretion during the luteal phase (Hughes, 1974), suggesting that shell secretion is under hormonal control. Uterine CP4 secretion in T. vulpecula is correlated with cp4 mRNA expression and continues into the fetal stage (Menkhorst et al., 2008).

The Stripe-faced Dunnart, Smíthopsis macroura (order Dasyuromorphia), is a polyoestrous, polyovular, seasonally breeding dasypuid marsupial weighing 20–25 g (Woolley, 1990). The breeding season occurs between June and February (Woolley, 1990) and the 10.7 day long gestation (Selwood and Woolley, 1991) is the shortest of any mammal. Implantation is invasive and the timing of shell loss precise in S. macroura (Selwood and Woolley, 1991), unlike the monovular T. vulpecula where implantation is superficial and the timing of shell loss variable (Hughes and Hall, 1984).

Here we cloned cp4 from S. macroura (sm-cp4), characterized its uterine expression pattern (transcription and secretion) during the follicular and pregnant luteal phases of the oestrous cycle and investigated for the first time the hormonal stimuli regulating the transcription of the uterine secreted protein, smCP4.

**RESULTS**

**Cloning and Identification of sm-cp4 cDNA**

A PCR product of around 900 base pairs from S. macroura uterus (Fig. 2A) was amplified using the outer \( cp4 \) primers (Fig. 1). The sequence of the cloned sm-cp4 cDNA fragment was 874 base pairs long (Fig. 2B; GenBank accession number EF692638). The sm-cp4 was 98% homologous with the T. vulpecula cp4 cDNA sequence (Cui and Selwood, 2003).

The sm-cp4 cDNA sequence encoded 291 amino acid residues (Fig. 3A). The deduced amino acid sequence (with missing C-terminal residues) was 96% homologous with the full length T. vulpecula CP4 sequence (Fig. 3A). Cysteine (C) residues, which have potential binding roles for molecular structure, are well conserved across the two species (Fig. 3A, underlined). The N-terminal of smCP4 had high sequence homology with the C-terminal of \( \alpha \)-enolase (Fig. 3B), with amino acid residues between the 12th and 142nd positions of smCP4 exhibiting high similarity to the residues between the 266th and 403rd positions of the \( \alpha \)-enolase (e.g., human: 85%; Fig. 3B). As in T. vulpecula (Cui and Selwood, 2003), the enzymatically active domains of human \( \alpha \)-enolase, indicated by glutamines at amino acid residues 157, 166 and 209 (Duquerroy et al., 1995), were not found in the smCP4 homologous region (residues 266–403), suggesting that smCP4 is not enzymatically active. In comparison, \( \tau \)-crystallin encodes the glutamines, but has low enzymatic activity due to post-translational monomerization (Wistow et al., 1988).

**Transcription of sm-cp4 in Adult Tissues**

sm-cp4 transcription was found in the lens, lung, heart, liver, spleen, kidney, ovary, oviduct (Fig. 4A) and uterus.
No sm-cp4 transcription was found in the testis (Fig. 4A).

Transcription of sm-cp4 in the Uterus During the Pregnant Oestrous Cycle

Uterine sm-cp4 transcription was biphasic during the pregnant oestrous cycle (Fig. 4B), with transcription of sm-cp4 observed in all stages except the bilaminar blastocyst stage (Fig. 4B).

Hormonal Regulation of sm-cp4 Transcription In Vitro

Control (C) uterine slices showed sm-cp4 expression (Fig. 4C), probably initiated in vivo. Incubation with the 10× physiological concentration of progesterone at organogenesis (10P) or the physiological concentration of oestradiol and progesterone at ovulation (EP) increased sm-cp4 expression (Fig. 4C). Incubation with the 10× physiological concentration of oestradiol at ovulation (10E, 10EP) decreased sm-cp4 expression (Fig. 4C).

Secretion of smCP4 by the Uterine Epithelium

The binding specificity of the mouse antiserum to CP4 was confirmed by the positive staining of a shell coat surrounding a S. macroura zygote (Fig. 5A), which was not observed in the negative control (Fig. 5B).

Immunohistochemistry against GST-CP4 showed that smCP4 was expressed by the luminal and glandular uterine epithelia in S. macroura (Fig. 5C–H). All stromal staining observed was shown to be nonspecific by the pre-immune
negative controls (Fig. 5I–N). This staining pattern correlates with previous studies where ultramicroscopy, histochemistry and immunocytochemistry using antibodies directed against the whole shell coat identified secretion of the shell coat by the luminal and glandular epithelium in *Sminthopsis crassicaudata* (Roberts et al., 1994; Roberts and Breed, 1996) and *T. vulpecula* (Casey et al., 2002).

SmCP4 secretion, as evidenced by secretory product in the glandular lumen and cytoplasm of luminal and glandular epithelial cells, began during cleavage in the luminal and glandular epithelium and was observed in the luminal and basal glandular epithelium during every stage of the luteal phase (Fig. 5C–H) including the embryonic stage (data not shown). SmCP4 secretion in the apical glandular epithelium was biphasic, ceasing during the unilaminar (Fig. 5E) and bilaminar (data not shown) blastocyst stages. Nonspecific staining in the myometrium (Fig. 5F) may reflect staining of α-enolase; α-enolase is expressed by muscle and activated immune cells in eutherians (Pancholi, 2001).

**DISCUSSION**

This study showed that the cp4 nucleotide and amino acid sequence is highly homologous between *S. macroura* and *T. vulpecula*, providing the first molecular evidence for homology of the marsupial shell coat between marsupial Orders and suggesting that cp4 is conserved in all marsupial species. Further, the biphasic uterine expression pattern found in both species is also likely to be conserved in all marsupial groups. Continued secretion of the marsupial shell coat during late gestation provides strong evidence that a shell coat surrounding the marsupial conceptus is essential during all of the pre-implantation period of gestation, possibly providing an appropriate extracellular matrix and scaffolding for the expanding conceptus, as occurs during blastocyst formation (Casey and Selwood, 2003) when the blastocyst cells attach to the shell coat by a series of cytoplasmic processes (Kress and Selwood, 2006).

This is the first study to investigate the in vitro response of the marsupial endometrium to oestradiol and progesterone. Classically, pre-implantation endometrial epithelial protein synthesis and secretion is described as being regulated by progesterone (Bazer and Slayden, 2008), with limited regulation by pre- and post-ovulatory oestrogen, partly through the up-regulation of progesterone receptors (An et al., 2004). Certainly, in vivo injections of either oestradiol or progesterone induce the production of secretory products by the anoestrous glandular epithelium in *T. vulpecula* (Sizemore et al., 2004) and *M. eugenii* (Renfree, 1973; Shaw and Renfree, 1986), and in vivo injections of oestradiol during the follicular phase prolong shell secretion during the luteal phase in *T. vulpecula* (Hughes, 1974). Here, increased sm-cp4 expression was found following incubation with high levels of progesterone or when basal progesterone was included with the oestradiol culture treatments, suggesting that sm-cp4 expression is regulated by progesterone and
that increasing oestrogen concentration during the follicular phase up-regulates progesterone receptors, allowing basal progesterone levels to stimulate sm-cp4 expression during the follicular phase.

The biphasic pattern of cp4 transcription found in both species is correlated with their plasma progesterone profiles (Fig. 6; Shorey and Hughes, 1973; Curlewis et al., 1985; Fletcher and Selwood, 2000; Menkhorst et al., 2009). Initiation of cp4 expression during the follicular phase, and the continued expression of cp4 during cleavage, are likely to be regulated by oestriadiol, presumably by the up-regulation of progesterone receptors, which could respond to the basal levels of progesterone present. Supporting this suggestion, there is a pre-ovulatory pulse of progesterone in S. macroura (Fig. 6, Menkhorst et al., 2009), which corresponds to the increase in sm-cp4 transcription found during the late follicular stage and into cleavage (Fig. 4B). Following cleavage, the plasma progesterone profile can directly predict cp4 expression (Fig. 6). In T. vulpecula, low plasma progesterone concentration during the unilaminar and bilaminar blastocyst stages (Shorey and Hughes, 1973; Curlewis et al., 1985; Fletcher and Selwood, 2000) is associated with low levels of cp4 expression (Cui and Selwood, 2003). In S. macroura however, the continued expression of sm-cp4 during the unilaminar blastocyst stage identified in this study is associated with high concentrations of plasma progesterone (Fig. 6, Menkhorst et al., 2009). In both species, cp4 expression decreases during the bilaminar blastocyst stage, a period of low or falling plasma progesterone concentration in both species (Fig. 6). Increasing plasma progesterone concentrations following the formation of the bilaminar blastocyst in both species is associated with increased cp4 expression levels and both the plasma progesterone concentrations and cp4 expression levels remain high until the fetal stage (Fig. 6).

Here, transcription of sm-cp4 and secretion of smCP4 was biphasic and correlated with conceptus development, as previously found in T. vulpecula (Cui and Selwood, 2003). Unlike transcription, smCP4 secretion did not cease in the basal glandular epithelial region during the bilaminar blastocyst stage, suggesting that the high levels of sm-cp4 transcription found during the unilaminar blastocyst stage may allow translation to continue at low levels during the bilaminar blastocyst stage. Further, the presence of granular material, thought to be newly laid shell coat, on the surface of the bilaminar blastocyst in Isodoon macrourus (Hollis and Lyne, 1977), Perameles nasuta (Hollis and Lyne, 1977) and S. crassicaudata (Roberts and Breed, 1996), suggests that secretion of shell coat proteins does not cease during the bilaminar blastocyst stage in these species.

The continued expression of CP4 during the embryonic and fetal stages in both S. macroura and T. vulpecula (Cui and Selwood, 2003), provides strong evidence that a shell coat is essential for conceptus development during all of the pre-implantation period. Shell removal during cleavage and blastocyst stages to stages late in gastrulation (all occur prior to implantation in marsupials) inhibits in vitro blastocyst epithelial maintenance in T. vulpecula (Casey and Selwood, 2003), but later stages have not been examined. The second wave of CP4 secretion began during the trilaminar blastocyst stage in both species, suggesting that the shell coat is critical during later development and since this is a period of rapid conceptus expansion (Tyndale-Biscoe and Renfree, 1987), that a minimum thickness of shell coat is required.

The identification of cystine residues in both CP4 (Cui and Selwood, 2003) and smCP4 (this study) supports Hughes’ (1977) suggestion that the T. vulpecula shell coat contains disulphide bonds. Disulphide bonds were not identified in shell coats from the conceptuses of superovulated S. crassicaudata (Roberts et al., 1994), however, shell coats from conceptuses of superovulated T. vulpecula are abnormal, showing only two bands on SDS–PAGE (Casey et al., 2002), and are not similar in size to CP4. This suggests that in T. vulpecula at least, the shell coat of conceptuses resulting from superovulation does not contain CP4, and therefore may not contain disulphide bonds.

CP4 is the first marsupial shell coat protein characterized, providing a unique opportunity to investigate the expression pattern of the marsupial shell coat and the cues that initiate shell coat protein secretion. This study showed for the first time that synthesis and secretion of CP4 (and possibly other shell coat proteins) is likely to be under the control of...
Figure 5.
progesterone, not oestradiol as previous studies have indicated. The smCP4 sequence and expression pattern identified in this study was highly conserved with *T. vulpecula* CP4, suggesting that it is likely to be conserved in all marsupial species, highlighting the importance of the shell coat for their preimplantation development.

**MATERIALS AND METHODS**

A colony of *S. macroura* maintained by LS at La Trobe University and The University of Melbourne between 1985 and 2005 provided the 110 animals used in this study. This study followed the Australian National Health and Medical Research Council guidelines for the Care and Use of Animals for Scientific Purposes.

![Figure 6. Plasma progesterone concentration (gray, Thorburn et al., 1971; Curlewis et al., 1985; Fletcher and Selwood, 2000; Menkhorst et al., 2009) and cp4 transcription level (+, Cui and Selwood, 2003 and this study) during the luteal phase in *Trichosurus vulpecula* and *Sminthopsis macroura*. The time in days of each developmental stage is taken post-coitum in *T. vulpecula* (Fletcher and Selwood, 2000) or post-ovulation in *S. macroura* (Selwood and Hickford, 1999). bb, bilaminar blastocyst; c, cleavage stage; e, embryonic; f, fetus; tb, trilaminar blastocyst; ub, unilaminar blastocyst. Illustration modified from Selwood (1980).](image)

![Figure 5. Secretion of smCP4, identified by anti-CP4 mouse anti-serum, in the endometrium in *Sminthopsis macroura* during the follicular and luteal phase of the pregnant oestrous cycle. All inserts are magnified 2.5× greater than the basic figure. A: Zygote showing staining against CP4 on the outer surface of the shell coat (arrowheads), here rolled so that it appears on the inside. B: Pre-ovulatory. No secretion of smCP4 by the luminal (le) or the apical glandular (age) or basal glandular (bge) epithelium was observed. Insert shows a representative gland from the apical glandular epithelium. C: Cleavage stage. Some secretion of smCP4 was found in the luminal and glandular epithelium. Insert shows a representative gland from the basal glandular epithelium. D: Unilaminar blastocyst stage. E: Secretion of smCP4 was found in the luminal epithelium, but no staining was found in the apical glandular epithelium. Insert shows a representative section of the luminal and apical glandular epithelium. F: smCP4 secretion was found in the basal glandular epithelium. Insert shows a gland from the basal glandular epithelium. G: smCP4 secretion was found in the luminal and apical glandular epithelium. Insert shows a representative gland from the apical glandular epithelium. Strong staining of smCP4 at the apical surface of the apical (G) and basal (H) glandular epithelium suggests that smCP4 was being secreted by the entire glandular epithelium. I–N. Equivalent sections to A,C–H incubated with pre-immune negative control anti-serum. All sections stained with DAB and hematoxylin.](image)
Tissue Collection

Daily monitoring (July–December) of animal weight and morning urine samples identified oestrous cycling (Woolley, 1990; Selwood and Woolley, 1991; Selwood and Hickford, 1999). Females were killed by Halothane (Rhone Merieux, West Footscray, VIC, Australia) inhalation followed by cervical dislocation while anaesthetized. All organs removed were washed and stored in sterile warmed (35°C) calcium- and magnesium-free phosphate buffered saline (PBS). The developmental stage of the follicles/corpora lutea and oocytes/conceptuses confirmed the day of the oestrous cycle predicted from daily monitoring.

Total RNA Extraction and Reverse Transcription (RT)

Sm-cp4 was sequenced from uterine tissue collected on day 1 post-ovulation.

Total RNA extraction used the RNeasy® Mini Kit for RNA extraction (Qiagen, Hilden, Germany), according to the manufacturer’s instructions, allowing the use of a small amount of tissue (~10 mg). The SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) was used according to the manufacturer’s instructions to synthesize cDNA using total RNA as a template.

Molecular Cloning of sm-cp4

Two gene specific primers, cp4F (5’-TAGGATCCGATGTTA-TGCTGTGTAC-3’) and cp4R (5’-GCTATCGGGACCTGACATAACA-3’; Fig. 1) which encompass most of the cp4 gene sequence in T. vulpecula, were used for PCR amplification of sm-cp4. The PCR reaction was carried out using the Expand High Fidelity PCR system (Roche, Mannheim, Germany) with a MyCy- cler™ Thermal cycler (Bio-Rad, Regents Park, NSW, Australia). PCR products were analyzed on a 1.0% agarose gel, stained with 0.01% (v/v) SybrSafe™DNA gel stain (Invitrogen) and visualized at 530 nm.

The band containing the cDNA fragment was excised from the gel and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Annadale, NSW, Australia). The purified cDNA fragment (~100 ng) was ligated into a pGEM-T Easy plasmid vector (Promega) according to the manufacturer’s instructions. The pGEM-T Easy-cp4 construct was transformed into Escherichia coli bacterial strain JM109 Competent Cells (Promega) according to the manufacturer’s instructions. A single positive colony grown in Luria-Broth (1% Bacto-Tryptone, 0.5% Bacto-Yeast, 0.5% NaCl, pH 7.5) overnight allowed plasmid DNA isolation using the Genelute Plasmid Mini Prep kit (Sigma–Aldrich, Castle Hill, NSW, Australia).

Nucleotide sequencing was performed by a commercial company (Gandel Charitable Trust Sequencing Centre; Monash University, Melbourne, Australia) using the Dye Primer Sequenase Kit (Perkin-Elmer, Foster City, CA) and electrophoresed on an ABI 373 A DNA sequencer (Perkin-Elmer).

DNA sequences were browsed and edited by Microsoft® Word-pad and submitted for analysis using a variety of computer programs either locally or via the Internet. The putative amino acid sequence of smCP4 was converted from sm-cp4 cDNA sequence using “BioPHP-Translate DNA to protein” protocol on line (http://biophp.org/minitools/dna_to_protein/demo.php). Both nucleotide sequence and amino acid sequence were used for homology search against databases, such as GenBank, EMBL, etc., using the computer program BLASTN and BLASTP (http://www.ncbi.nlm.nih.gov/BLAST/). The smCP4 protein and α-enzolase sequences used for multiple alignments were retrieved from GenBank or EMBL, their access numbers are given in brackets: sm-cp4 (GenBank accession number EF692638), α-enzolase from Homo sapiens (BAD96912), Bos taurus (NP_776474), Mus musculus (AAH04017), Gallus gallus (NP_990451) and Xenopus laevis (NP_001080606). The aligned sequences were displayed and edited using the computer software GeneDoc (Nicholas and Nicholas, 1997).

Analysis of sm-cp4 Expression in Adult Tissues

The lens, lung, heart, liver, spleen, kidney, ovary, oviduct, uterus, and testis were collected from adult S. macroura. Uterine tissue was collected from nine stages of the pregnant oestrous cycle: early pre-ovulatory, late pre-ovulatory, early cleavage, late cleavage, unilaminar blastocyst, bilaminar blastocyst, trilaminar blastocyst, embryonic and fetal stages. Each tissue sample was collected from three different animals.

Three replicates of cDNA were synthesized from each sample of total RNA. The quantity of cDNA converted in each sample was estimated using house-keeping gene PCR, Glyceraldehyde-3-phosphate dehydrogenase (gapdh) was used for all tissues except uterus for which 18s ribosomal RNA (18s) was used. All PCR reactions were carried out using GoTaq® Green Master Mix (Promega), according to the manufacturer’s instructions.

PCR products for sm-cp4 were amplified from each sample using the two gene specific primers for the novel, 3’ end of cp4 (Fig. 1): p4f2 (5’-GTGCCAGTCACCTCTG-3’) and cp4R, analyzed by agarose gel electrophoresis and confirmed as homologous to T. vulpecula by Southern hybridization using a T. vulpecula cp4 cDNA probe as described previously (Cui and Selwood, 2000; Cui and Selwood, 2003) except that the AlkPhos Direct Labelling Kit (Amersham, Buckinghamshire, UK) was used according to the manufacturer’s instructions.

In Vitro Hormone Incubation

Uterine tissue was collected from cyclic animals (n = 6) at ovulation (~±3 days), thus, it had been stimulated by hormones in vivo. Using anoestrous or ovariectomised animals, which would not have been stimulated by in vivo hormones, was unfeasible as the unstimulated uterus is very small in S. macroura and would have made the number of animals required for this study prohibitively large. The uterus was cut into thin slices (~2 mm³), containing myometrium and endometrium. Each slice was placed on top of a piece of siliconized lens tissue paper (AquaSil Siliconising Fluid, Pierce, Selby Biolab, Clayton, VIC, Australia; Whatman lens cleaning tissue #105, Whatman International, Maidstone, England; Simpson et al., 2000) floating in 1 ml of Dulbecco’s Modified Eagles Medium (DMEM; Sigma–Aldrich) containing 1% antibiotics (penicillin, streptomycin), 1% l-glutamine, 10% heat-inactivated Fetal Calf Serum (all from Sigma–Aldrich) and treatments reflecting plasma oestradiol or progesterone concentrations at ovulation and organogenesis included and cultured for 96 hr at 35°C with 6% carbon dioxide, with the medium replenished after 48 hr. Explant culture on the siliconized lens paper facilitated both the absorption of required nutrients from the medium and gas exchange (Simpson et al., 2000).

The plasma oestradiol concentration at ovulation (24 pg/ml) in S. macroura was estimated from D. virginiana (Harder and Fleming, 1981), a marsupial with an appropriate life history. The plasma progesterone concentrations–basal concentration at ovulation (4 ng/ml) and the peak concentration during organogenesis (18 ng/ml), were from S. macroura (Menkhorst et al., 2005).

Uterine explants were treated with oestradiol alone (24 pg/ml), oestradiol plus basal progesterone (24 pg/ml oestradiol – 4 ng/ml progesterone), peak progesterone (18 ng/ml), or control. Given that the tissue was collected from animals around the time of ovulation and would already have been stimulated by oestrogen, oestradiol and progesterone treatments were included together. Both physiological and 10 times physiological hormonal concentrations were tested.
After culture, the uterine slices were snap frozen for RNA extraction. The amount of cDNA in the sample was adjusted using 18s RNA house-keeping gene RT-PCR, and the transcription level of sm-cp4 after treatment incubation was estimated using RT-PCR and Southern hybridization as described above.

Immunohistochemistry

Uterine samples were obtained from the pre-ovulatory (n = 2), cleavage (n = 4), unilaminar blastocyst (n = 3), bilaminar blastocyst (n = 3), trilaminar blastocyst (n = 1) and embryonic (n = 2) stages of development.

Uterine CP4 was identified using a polyclonal antiserum against recombinant Glutathione S-Transferase-Coat Protein 4 (GST-CP4), as previously described (Menkhorst et al., 2008). Briefly, sections of paraformaldehyde-fixed, paraffin-embedded uteri were placed onto Superfrost slides (Menzel-Glaser, Braunschweig, Germany) and incubated at 45°C for no longer than 24 hr, before being stored at 4°C for no longer than 1 month. Tissue sections were deparaffinized in Histolene (Grale Scientific, Ringwood, VIC, Australia) and rehydrated in a succession of ethanol washes. Incubation in a detergent, 1.0% Triton X-100 (Promega) for 15 min improved antibody penetration and reduced background staining. Endogenous peroxidase and endogenous avidin and biotin activity were blocked by incubation with commercial products ( Peroxidase blocking reagent, DAKO, Carpinteria, CA; Avidin/Biotin Blocking Kit, Zymed, Mt Waverly, VIC, Australia) according to the manufacturer’s instructions. The Histostain Plus Broad Spectrum (DAB) kit (Zymed) was used according to the manufacturer’s instructions, before the sections were counterstained with hematoxylin, dehydrated and mounted using DPX (BDH, Laboratory Supplies, Poole, England) and allowed to dry for a minimum of 48 hr at 45°C.

The primary antibody incubation was for 16 hr at 4°C using a mouse anti-GST-CP4 antiserum diluted to 1:750 in PBS containing 1% w/v Bovine Serum Albumin (BSA) and 10% (v/v) inactivated goat serum (Zymed). Negative control slides were incubated in 1:750 pre-immune mouse serum, also diluted in PBS containing BSA and inactivated goat serum.

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