The role of IGFs in the regulation of ovarian follicular growth in the brushtail possum (*Trichosurus vulpecula*)

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Abstract

IGFs are known to be key regulators of ovarian follicular growth in eutherian mammals, but little is known regarding their role in marsupials. To better understand the potential role of IGFs in the regulation of follicular growth in marsupials, expression of mRNAs encoding *IGF1*, *IGF2*, *IGF1R*, IGF-binding protein 2 (*IGFBP2*), *IGFBP4* and *IGFBP5* was localized by *in situ* hybridization in developing ovarian follicles of the brushtail possum. In addition, the effects of IGF1 and IGF2 on granulosa cell function were tested *in vitro*. Both granulosa and theca cells synthesize IGF mRNAs, with the theca expressing *IGF1* mRNA and granulosa cell expressing *IGF2* mRNA. Oocytes and granulosa cells express *IGF1R*. Granulosa and theca cells expressed *IGFBP* mRNAs, although the pattern of expression differed between the BPs. *IGFBP5* mRNA was differentially expressed as the follicles developed with granulosa cells of antral follicles no longer expressing *IGFBP5* mRNA, suggesting an increased IGF bioavailability in the antral follicle. The *IGFBP* protease, *PAPPA* mRNA, was also expressed in granulosa cells of growing follicles. Both IGF1 and IGF2 stimulated thymidine incorporation but had no effect on progesterone production. Thus, IGF may be an important regulator of ovarian follicular development in marsupials as has been shown in eutherian mammals.

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Introduction

The common brushtail possum, a marsupial, has an oestrous cycle of around 26 days with a 16–18-day luteal phase and a 9–11-day follicular phase (Fletcher & Selwood 2000). Extensive follicular growth is observed in both juvenile and adult animals with a single follicle of around 5 mm in diameter ovulating each reproductive cycle in adults during the breeding season (Eckery *et al.* 2002a, 2002b). In eutherians, ovarian follicular growth is known to be controlled by both extraovarian and intraovarian factors. The pituitary hormones, FSH and LH, are key extraovarian regulators of follicular growth and selection of the ovulatory follicle in monovular eutherian mammals (Webb & Campbell 2007, Mihm & Evans 2008). Downregulation of GnRH with a long acting agonist causes cessation of reproductive cycles in possums suggesting that FSH and/or LH are regulators of follicular development and ovulation in this species (Eymann *et al.* 2007). However, differences in the expression pattern of *LHCGR* mRNA during ovarian follicular development indicate that the role of LH in follicular growth differs in marsupials compared with

eutherian mammals (Eckery *et al.* 2002b). Granulosa cells of all healthy antral follicles of the brushtail possum express the *LHCGR* mRNA, and are responsive to LH. Therefore, LH cannot be playing a central role in selecting and maintaining a single ovulatory follicle in the possum as postulated in eutherian mammals.

Insulin-like growth factor (IGF) is an intraovarian growth factor that is also involved in the regulation of follicular development in eutherian mammals. IGF is known to stimulate granulosa cell proliferation and progesterone production, although these effects can vary across species and stage of follicular development (Mazerbourg *et al.* 2003, Silva *et al.* 2009). This effect is driven by binding of either IGF1 or IGF2 to the type 1 IGF receptor (Mazerbourg *et al.* 2003, Silva *et al.* 2009). The regulation of IGF actions is complex, and involves a number of binding proteins (BPs) including *IGFBP2*, *IGFBP4* and *IGFBP5* as well as the *IGFBP* protease pregnancy-associated plasma protein-A (*PAPPA*), which have been identified as potential key regulators during ovarian follicular development in eutherian mammals (Spicer 2004, Silva *et al.* 2009). The actions of IGF on

regulation of follicular development in marsupials are unknown. Thus, the objectives of these experiments were to characterize the expression pattern of *IGF1*, *IGF2*, *IGF1R*, *IGFBP2*, *IGFBP4*, *IGFBP5* and *PAPPA* mRNAs using ovarian sections containing follicles in multiple stages of development, and determine the effects of IGF1 and IGF2 on granulosa cell proliferation and progesterone production in the brushtail possum.

Results

Expression of *IGF1*, *IGF2* and *IGF1R* mRNA during follicular development

During follicular development, expression of *IGF1* mRNA (Fig. 1 and Table 1) was limited to the theca of antral follicles. The majority of atretic antral follicles no longer expressed *IGF1* mRNA. Expression was also observed in the area around blood vessels in some animals. In contrast to the relatively sparse expression of *IGF1*, *IGF2* mRNA (Fig. 2 and Table 1) was strongly expressed in granulosa cells of primary, secondary and antral follicles (healthy and atretic). Cells around the blood vessels and some surface epithelial cells also expressed *IGF2* mRNA (data not shown). The *IGF1R*

mRNA (Fig. 3 and Table 1) was expressed in follicles of all developmental stages examined. Oocytes of primordial, primary, secondary and antral follicles expressed *IGF1R* strongly. Secondary and antral (healthy and atretic) follicles also expressed *IGF1R* mRNA in granulosa, but not theca cells. While the interstitial glands did not express *IGF1R* mRNA, expression was observed in some stromal cells.

Expression of *IGFBP2*, *IGFBP4*, *IGFBP5* and *PAPPA* mRNAs during follicular development

The *IGFBP2* mRNA (Fig. 4 and Table 1) was observed in primary, secondary and antral follicles. Both theca and granulosa cells expressed *IGFBP2* mRNA but oocytes did not. Atretic antral follicles also expressed *IGFBP2* mRNA in both granulosa and theca cells. Interstitial tissue as well as cells of the vasculature expressed *IGFBP2* mRNA. *IGFBP4* mRNA (Fig. 5 and Table 1) was primarily expressed in the theca of large preantral and antral follicles and in interstitial tissue. Faint hybridization was also observed in the granulosa cells of some antral follicles. The pattern of expression was similar in atretic antral follicles with the expression observed consistently in the theca cells but only in the granulosa cells of some antral follicles. *IGFBP5* mRNA (Fig. 6 and Table 1) was observed in granulosa cells of primordial, primary and some secondary follicles. However, granulosa cells of antral follicles no longer expressed *IGFBP5* mRNA. Strong expression was also observed in the theca of secondary follicles (when present) and antral follicles (both healthy and atretic). While interstitial glands did not express *IGFBP5* mRNA, expression was observed in some stromal cells close to the follicles, as well as blood vessels and some surface epithelial cells. The mRNA encoding *PAPPA* (Fig. 7 and Table 1) was observed in the granulosa cells of all growing follicles (i.e. starting at the primary stage of development). Atretic antral follicles still expressed *PAPPA* mRNA, although the signal appeared faint. Neither the interstitial cells nor the surface epithelium expressed *PAPPA* mRNA.

Effects of *IGF1* and *IGF2* on granulosa cell function

Treatment of possum granulosa cells with either IGF1 or IGF2 increased cell proliferation. Incorporation of ^3H -thymidine was increased ($P < 0.01$) 1.92 ± 0.27 - and 1.81 ± 0.33 -fold (mean $\pm$ S.E.M.) above control cells after treatment with IGF1 and IGF2 respectively.

Neither IGF1 (1.26 ± 0.43 -fold of controls) nor IGF2 (2.54 ± 1.34 -fold of controls; mean $\pm$ S.E.M.) affected ($P > 0.05$) the concentrations of progesterone in media. The granulosa cells were capable of synthesizing progesterone, although basal concentrations were low, and responding to positive stimuli as treatment with LH increased progesterone concentrations by more than

Figure 1 Corresponding brightfield (A, C and E) and darkfield (B, D and F) views of ovaries from adult brushtail possums following hybridization to *IGF1* antisense (A–D) and sense (E and F) RNA. Expression of *IGF1* mRNA in the theca (t), but not oocyte (o) or granulosa cells (g) of antral follicles (A and B). Expression of *IGF1* mRNA in the cells of the vasculature of the ovary (C and D). Expression was not detectable in the interstitial tissue (i; A and B). Note the lack of specific signal in an ovary hybridized to the *IGF1* sense RNA (E and F).

Table 1 Expression pattern of mRNAs encoding insulin-like growth factor 1 (*IGF1*) and *IGF2*, their receptor (R) (*IGF1R*) as well as the binding proteins (BPs) *IGFBP2*, *IGFBP4*, and *IGFBP5* and the protease, pregnancy-associated plasma protein A (*PAPPA*) during follicular development in the brushtail possum.

Follicle type	<i>IGF1</i>	<i>IGF2</i>	<i>IGF1R</i>	<i>IGFBP2</i>	<i>IGFBP4</i>	<i>IGFBP5</i>	<i>PAPPA</i>
Primordial	–	–	o	–	–	g	–
Primary	–	g	o	g	–	g	g
Secondary	–	g	o, g	g, t	t	g, t	g
Antral	t	g	o, g	g, t	g, t	t	g
Atretic antral ^a	–	g	g	g, t	g, t	t	g

–, expression not observed; o, expression observed in oocyte; g, expression observed in granulosa cells; t, expression observed in theca.

^aOocytes not observed in atretic follicles.

tenfold. Both basal concentrations of progesterone secreted into the media and those following treatment were highly variable between different pools of granulosa cells.

Discussion

Both *IGF1* and *IGF2* mRNAs, as well as the mRNAs encoding *IGF1R* and BPs for IGFs, were expressed in ovarian follicles of the brushtail possum. Furthermore, both *IGF1* and *IGF2* were able to regulate granulosa cell proliferation, causing a near doubling of ³H-thymidine incorporation. Thus, it would appear that IGFs have a local role in regulating follicular development in marsupials as has been observed in eutherian mammals.

There were distinct patterns of expression for *IGF1* and *IGF2* mRNAs. The potential role that differential expression of *IGF1* and *IGF2* may play in development of ovarian follicles is unclear. From previous reports, *IGF1* and *IGF2* seem to have similar effects on granulosa cell function (Silva *et al.* 2009), and thus, the actions appear to overlap. However, in other tissues, a group of genes regulated specifically by *IGF1* or *IGF2* as well as those regulated in common by either *IGF1* or *IGF2* have been identified (Pacher *et al.* 2007). Thus, *IGF1* and *IGF2* may have differing roles in regulating ovarian function that have not yet been identified. Given the lack of expression of *IGF1* mRNA in growing preantral follicles, *IGF2* likely is the predominant IGF regulating growth of the follicle during the early stages of development in the brushtail possum. The pattern of expression of IGFs in preantral follicles appears to differ among species. In cattle, neither *IGF1* nor *IGF2* mRNA was detected in preantral follicles (Armstrong *et al.* 2002). In mice, *IGF1*, but not *IGF2*, mRNA was expressed in granulosa cells from the primary stage onward (Wandji *et al.* 1998). In humans, *IGF2*, but not *IGF1*, mRNA was observed in oocytes and granulosa cells of preantral follicles (Zhou & Bondy 1993).

Addition of IGFs (*IGF1* or *IGF2*) to cultures of preantral follicles has been shown to affect follicular growth and function. Preantral follicles of multiple species cultured with *IGF1* or *IGF2* showed increased oocyte and/or follicular diameters when compared with follicles cultured without IGF (Itoh *et al.* 2002, Mao *et al.* 2004,

Zhou & Zhang 2005, Thomas *et al.* 2007, Sharma *et al.* 2009). Addition of IGFs to the media also increased the survival rate of follicles (Zhou & Zhang 2005, Sharma *et al.* 2009) and increased oestradiol production from preantral follicles (Yuan & Giudice 1999, Demeestere *et al.* 2004, Thomas *et al.* 2007). The expression of *IGF2* mRNA in preantral follicles as well as expression of mRNA for *IGF1R* in both granulosa cells and oocytes of preantral follicles in the brushtail possum is consistent with *IGF2* being an important regulator of preantral follicular growth in marsupials.

Relatively little is known about the expression of *IGFBP* mRNA in preantral follicles in eutherian

Figure 2 Corresponding brightfield (A, C and E) and darkfield (B, D and F) views of ovaries from juvenile and adult brushtail possums following hybridization to *IGF2* antisense (A–D) and sense (E and F) RNA. Expression of *IGF2* mRNA in granulosa cells (g), but not oocyte (o) or theca (t) of secondary (arrowhead) and antral (arrow) follicles (A–D). Expression was not detectable in the interstitial tissue (i; A and B). Note the lack of specific signal in an ovary hybridized to the *IGF2* sense RNA (E and F).

Figure 3 Corresponding brightfield (A, C, E and G) and darkfield (B, D, F and H) views of ovaries from juvenile and adult brushtail possums following hybridization to *IGF1R* antisense (A–F) and sense (G and H) RNA. Oocytes (o) of primordial (#; 2× higher magnification in inset of panel E and F), primary (*), secondary (arrowhead) and antral (arrow) follicles express *IGF1R* (A–F). Expression of *IGF1R* mRNA was also observed in granulosa cells (g) but not theca (t) of secondary (arrowhead) and antral (arrow) follicles (A–F). Expression was not detectable in the interstitial tissue (i; A and B). Note the lack of specific signal in an ovary hybridized to the *IGF1R* sense RNA (G and H).

mammals. In mice, cattle, monkeys and humans, *IGFBP2* mRNA was expressed in growing preantral follicles (Zhou & Bondy 1993, Wandji *et al.* 1998, Arraztoa *et al.* 2002, Thomas *et al.* 2007), and a similar pattern of expression was observed in the brushtail possum. In addition, expression of *IGFBP5* occurred prior to the initiation of follicular growth in the brushtail possum in granulosa cells of primordial follicles as well as growing preantral follicles. This expression pattern is similar to what was observed in mice (Wandji *et al.* 1998); however, *IGFBP5* mRNA was not observed in preantral follicles of monkeys (Arraztoa *et al.* 2002) or humans (Zhou & Bondy 1993). Somewhat surprisingly, strong expression of *PAPPA* mRNA was observed in granulosa cells of growing preantral follicles in possums.

Studies in rats, cows and pigs have linked the expression of *PAPPA* to the development of the dominant, ovulatory follicle (Mazerbourg *et al.* 2001, Hourvitz *et al.* 2002). In rats and humans, using *in situ* hybridization, expression was not observed in preantral follicles (Hourvitz *et al.* 2000, 2002). Collectively, the expression patterns of mRNA encoding *IGFs*, the *IGF1R*, *IGFBPs* as well as an *IGFBP* protease (*PAPPA*) are consistent with a complex regulation of the *IGF* system during preantral follicular growth.

In possums, *IGF2* mRNA was strongly expressed in the granulosa of antral follicles, whereas *IGF1* mRNA was not expressed in granulosa cells and only weakly expressed in the theca cells. Expression of *IGF2* mRNA, with little or no expression of *IGF1* mRNA, has been observed in antral follicles of humans (Zhou & Bondy 1993), sheep (Hastie & Haresign 2006, 2008) and cattle (Perks *et al.* 1995); although in sheep and cattle, expression is limited to the theca cells. In contrast, *IGF1*, but not *IGF2*, mRNA was strongly expressed in granulosa cells of rodents (Wandji *et al.* 1998) and pigs (Liu *et al.* 2000).

Granulosa cells of antral follicles in the brushtail possum were targets for both *IGF1* and *IGF2* actions as shown both by the expression of the *IGF1R* mRNA and stimulation of ^3H -thymidine uptake. *IGF1R* mRNA has

Figure 4 Corresponding brightfield (A, C and E) and darkfield (B, D and F) views of ovaries from juvenile and adult brushtail possums following hybridization to *IGFBP2* antisense (A–D) and sense (E and F) RNA. Expression of *IGFBP2* mRNA in granulosa cells (g) and theca (t), but not oocyte (o) of secondary (arrowhead) and antral (arrow) follicles (A–D). Expression was also detectable in the interstitial tissue (i; C and D). Note the lack of specific signal in an ovary hybridized to the *IGFBP2* sense RNA (E and F).

Figure 5 Corresponding brightfield (A, C and E) and darkfield (B, D and F) views of ovaries from juvenile and adult brushtail possums following hybridization to *IGFBP4* antisense (A–D) and sense (E and F) RNA. Expression of *IGFBP4* was first observed in theca of secondary follicles (A and B; arrowhead). Expression of *IGFBP4* mRNA was strongest in the theca (t), with weak signal also seen in the granulosa cells (g) of some but not all antral follicles (A–D, arrow). Expression was not observed in the oocyte (o) of follicles (A and B). Expression was also detectable in the interstitial tissue (i; A and B). Note the lack of specific signal in an ovary hybridized to the *IGFBP4* sense RNA (E and F).

commonly been shown to be expressed in the granulosa cells of antral follicles of eutherian mammals (Zhou & Bondy 1993, Perks *et al.* 1995, Wandji *et al.* 1998, Liu *et al.* 2000, Armstrong *et al.* 2002, Hastie & Haresign 2006). The magnitude of the effects of IGFs on proliferation differs among species and stages of follicular development; however, overall, they seem to promote proliferation and differentiation (Mazerbourg *et al.* 2003, Silva *et al.* 2009). In sheep, follicles of a similar developmental stage (based on similar sized follicles and a similar size of follicle at ovulation) IGF1 stimulated proliferation (Monniaux & Pisselet 1992). Similarly, human, cow and pig granulosa cells responded to IGF1/IGF2 with increased cell proliferation (Savion *et al.* 1981, Baranao & Hammond 1984, Yong *et al.* 1992).

No effects of IGFs were observed on progesterone secretion in the present study. This is similar to what has been observed in granulosa cells of sheep follicles of similar size (Monniaux & Pisselet 1992). However, granulosa cells from more developed follicles respond to IGF1 with increased progesterone production (Monniaux & Pisselet 1992), and granulosa cells from

other species also responded with increased progesterone production (Schams *et al.* 1988, Yong *et al.* 1992, Shaw *et al.* 1993, Armstrong *et al.* 1996, Spicer & Aad 2007). Thus, IGFs could affect progesterone production in preovulatory follicles in the brushtail possum. However, in the two animals with preovulatory follicles present, these follicles did not express *IGFR1* mRNA indicating that preovulatory follicles may lose their ability to respond to IGFs. Moreover, preliminary data obtained with a limited number of animals ($n=1-3$) support this possibility as the granulosa cells showed no response to IGF1 in thymidine incorporation or progesterone production. IGF1 can also augment the effects of FSH on progesterone production in some species (Adashi *et al.* 1984, 1989, Baranao & Hammond 1984, Adashi & Resnick 1987). It is important to note, however,

Figure 6 Corresponding brightfield (A, C, E and G) and darkfield (B, D, F and H) views of ovaries from juvenile brushtail possums following hybridization to *IGFBP5* antisense (A–F) and sense (G and H) RNA. Granulosa cells (g) of primordial (#), primary (*), secondary (arrowhead), but not antral (arrow) follicles express *IGFBP5* (A–F). Theca cells also express *IGFBP5* mRNA (t; C–F) but interstitial tissue did not (i; A and B). Note the lack of specific signal in an ovary hybridized to the *IGFBP5* sense RNA (G and H).

Figure 7 Corresponding brightfield (A, C and E) and darkfield (B, D and F) views of ovaries from juvenile and adult brushtail possums following hybridization to *PAPPa* antisense (A–D) and sense (E and F) RNA. Expression of *PAPPa* was not observed in primordial follicles (A and B; #). The granulosa cells, but not theca (t) or oocytes (o), of growing follicles expressed *PAPPa* mRNA (A–D, arrowhead secondary follicles, arrows antral follicles). Expression was not detectable in the interstitial tissue (i; A and B). Note the lack of specific signal in an ovary hybridized to the *PAPPa* sense RNA (E and F).

that the secretion of progesterone in the cultures in the present study was variable and often at the limit of the detection of the assay. Thus, while factors capable of strongly stimulating progesterone could be identified (i.e. LH), more subtle effects on progesterone secretion may have been below the sensitivity of the assay. Additionally, the interactions between gonadotrophins and IGFs in regulating proliferation and steroidogenesis in marsupials were not examined, and thus, IGFs could synergize with gonadotrophins to stimulate progesterone production as has been observed in other species.

Antral follicles also expressed mRNAs for *IGFBPs*. Similar to what has been observed in multiple eutherian mammals (Nakatani *et al.* 1991, Zhou & Bondy 1993, el-Roeiy *et al.* 1994, Besnard *et al.* 1996, Liu *et al.* 2000, Arraztoa *et al.* 2002, Zhou *et al.* 2003, Canty *et al.* 2006, Hastie & Haresign 2006, Llewellyn *et al.* 2007), theca of antral follicles expressed *IGFBPs*. However, given the inability to detect *IGF1R* mRNA in theca cells, the potential role for IGFs as well as autoregulation of IGF action by theca produced *IGFBPs* is unclear. It is possible that theca produced *IGFBPs* regulate IGF activity in granulosa cells and/or oocytes. Effects of IGFs on granulosa cells of antral follicles are also likely

regulated by locally expressed *IGFBPs* and the *IGFBP* protease *PAPPa*. *IGFBP2* mRNA was strongly expressed in granulosa cells of antral follicles. However, expression of *IGFBP4* mRNA was weak and inconsistent, and *IGFBP5* mRNA was no longer expressed in granulosa cells of antral follicles. In addition, expression of *PAPPa* mRNA remained strong in the granulosa cells. Thus, expression/actions of the BPs may decrease as the follicle develops in the brushtail possum. This is similar to what has been observed in many eutherian mammals (el-Roeiy *et al.* 1994, Besnard *et al.* 1996, Zhou *et al.* 1996, Wandji *et al.* 1998, Hourvitz *et al.* 2000, 2002, Liu *et al.* 2000, Arraztoa *et al.* 2002, Canty *et al.* 2006, Hastie & Haresign 2006, Llewellyn *et al.* 2007). It is important to note that the follicles examined in the brushtail possum did not include any preovulatory follicles (>3 mm diameter), and that further changes in the expression of *IGFBPs* as well as *PAPPa* during the development of the preovulatory follicle are often observed in eutherian mammals (Monget *et al.* 2002, Spicer 2004, Silva *et al.* 2009). Whether similar changes in the expression of *IGFBPs/PAPPa* would also be observed during the development of the preovulatory follicle of the brushtail possum remains to be elucidated.

Atretic antral follicles had similar expression patterns for the *IGFBPs*, *IGF2* and *IGF1R* as healthy follicles. However, expression of *IGF1* in the theca was no longer detectable, and *PAPPa* expression in granulosa cells did not appear to be as strong as was observed in healthy follicles. Taken together, this is consistent with a decreased bioactivity of IGFs in atretic follicle. In eutherian mammals, decreased bioactivity of IGFs through decreased secretion of ligands, decreased receptor expression and/or increased concentrations of BP activity (either through increased expression of BPs or reduced degradation) has also been implicated in atresia (Monget *et al.* 2002, Quirk *et al.* 2004, Spicer 2004, Hastie & Haresign 2006, Webb & Campbell 2007). Thus, IGFs may play an important role in the regulation of follicular health in marsupials as well as in eutherian mammals.

Expression of *IGF1R* mRNA in oocytes as well as granulosa cells of preantral and antral follicles indicates that oocytes are also likely targets of IGFs during ovarian follicular development. Expression of *IGF1R* in oocytes has been observed in other species including cattle (Armstrong *et al.* 2002), rats (Zhou *et al.* 1991), humans (Zhou & Bondy 1993) and monkeys (Vendola *et al.* 1999). Addition of *IGF1* or *IGF2* to media during *in vitro* maturation and fertilization increases blastocyst formation indicating that these factors are likely involved in the maturation of the oocyte (Shabankareh & Zandi 2010, Wang *et al.* 2009).

In conclusion, as has been shown in eutherian mammals, IGFs have the potential to be key local regulators in ovarian follicular development in marsupials. *IGF2* may be the dominant IGF regulating follicular

development in the brushtail possum and localization of mRNAs encoding *IGFBPs* and the BP protease, *PAPPA* in a cell and developmental stage-specific manner indicates that complex regulation of the IGFs may be occurring during follicular development in the brushtail possum as has been observed in eutherian mammals.

Materials and Methods

Collection of tissue samples

Ovaries were collected from juveniles (females that were independent of their mothers but not sexually mature as assessed by their body size and pouch development) and adult females following anaesthesia (Juengel *et al.* 2002). Animals were sourced from a captive breeding programme or captured from the wild. All experiments performed were with the approval of the Animals Ethics Committee at Wallaceville Animal Research Centre or Invermay Agricultural Centre and in accordance with the 1999 Animal Protection (Codes of Ethical Conduct) Regulations of New Zealand.

Generation of cDNAs of interest

For the generation of the cDNAs of interest, total cellular RNA from ovarian tissue was reverse transcribed using SuperScript preamplification system (Invitrogen NZ Ltd). cDNAs for the genes of interest were obtained using PCR with standard PCR buffer from Qiagen (Biolab Scientific Ltd, Wellington, New Zealand). Hotstart Taq was used for all genes with the following conditions: 1 cycle at 94 °C for 2 min; 40 cycles of denaturing at 94 °C for 20 s, annealing at 58 °C for 15 s, and extension at 72 °C for 50 s; and final extension at 72 °C for 10 min. Comparisons of known sequences for each gene were used to design primers corresponding to conserved areas of the gene. Primers used for genes are shown in Table 2. Confirmation of isolation of the possum homologue of the gene of interest was obtained by sequencing (Waikato DNA Sequencing Facility; The University of Waikato; Hamilton, New Zealand) of the resulting PCR products, which had been ligated into pGEMTeasy vector (Promega; Dade Diagnostics PTY Ltd, Auckland, New Zealand). Sequences (GenBank accession numbers GU250879–GU250885; Table 2) had ≥89% identity with the corresponding region of the gene in other marsupials (Altschul *et al.* 1990).

In situ hybridization

In situ hybridization was used to determine the localization of mRNAs in ovarian sections (Tisdall *et al.* 1999, Juengel *et al.* 2002). Riboprobes were generated using the Riboprobe Gemini system (Promega). Tissue sections were hybridized overnight at 55 °C with ~45 000 cpm/μl (48 000 dpm) of ³³P-labelled antisense or sense RNA. Tissue sections were viewed and photographed, using both light and dark field illumination, on an Olympus BH-50 microscope comparing hybridization of the antisense and sense riboprobes. As hybridization of the sense RNA over the tissue section was similar or lower in intensity to that observed on the glass of both the sense and antisense hybridized slides, no specific hybridization was considered to have occurred with any of the sense riboprobes.

Specific hybridization for each gene was then determined for specific stages of follicular development (Juengel *et al.* 2002). Follicles were considered to be primordial follicles if they contained an oocyte surrounded by a single layer of flattened or mixed flattened and cuboidal granulosa cells. Primary follicles had an oocyte surrounded by 1–<2 layers of cuboidal granulosa cells. Follicles containing at least two layers of granulosa cells without a formed antrum were considered secondary follicles. Follicles containing an antrum were classified as antral. Follicles with several pyknotic nuclei in the granulosa layer were considered to be atretic. As the majority of atretic follicles observed had developed an antrum, only antral atretic follicles were included in the study. These were limited in number, and often did not include the oocyte in the sections examined, and thus, no data for expression of the genes of interest in oocytes of atretic antral follicles are presented. Follicles from a minimum of three juvenile and five adult animals were examined for each gene.

Culture of granulosa cells

Granulosa cells were collected from healthy follicles between 0.5 and 2.5 mm in diameter from adult female possums during the breeding season. Oocytes were removed before cells were cultured in a humidified incubator with 5% CO₂ in air at 37 °C. Viability of cells was determined by trypan blue exclusion and averaged 65% at the time of collection. Granulosa cells (quadruplicate wells) were cultured in DMEM:F12 (Invitrogen) supplemented with 15 mM HEPES, 3.15 g/l glucose, 0.1% BSA (Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin, insulin (10 ng/ml; Sigma), holo-transferrin (5 ng/ml; Invitrogen) and sodium selenite (10 ng/ml; Sigma) containing 0 or 100 ng/ml of

Table 2 Primers used for obtaining partial cDNAs for genes of interest and the GenBank accession number of the resulting brushtail possum sequences.

Genes	Forward primer	Reverse primer	GenBank accession numbers
<i>IGFI</i>	AAA AAT CAG CAG TCT TCC AAC	TTG GGC ATG TCG GTG TG	GU250879
<i>IGF2</i>	CCT TTG CCT CGT GCT GC	GGG ACG GTG ACG CTT GG	GU250880
<i>IGFIR</i>	GAG TTC AAY TGT CAC CAY GTG G	GGG TTR TAC TGC CAG CAC	GU250881
<i>IGFBP2</i>	CTC AAG TCA GGC ATG AAG GA	GTA GAA GAG ATG RCA CTC GG	GU250882
<i>IGFBP4</i>	CCT GGC TGT GGC TGC TG	TTG GGG TGG AAG TTG CC	GU250883
<i>IGFBP5</i>	CCT TGC GAC GAG AAA GC	TTC ATC CCR TAC TTG TCC AC	GU250884
<i>PAPPA</i>	CCT TAC AGA GCC TAC TTG GAT G	GAT TTG GCG TGA AGG AGT C	GU250885

IGF1 (Long-R3, Gro-Pep, Adelaide, SA, Australia) or IGF2 (R&D Systems Inc., Minneapolis, MN, USA). Doses of IGF1 and IGF2 were chosen following preliminary experiments testing doses between 0.1 and 100 ng/ml of IGF1 or IGF2 as the dose with maximum effect (data not shown). For determination of proliferation potential, 20 000 cells per well were cultured in a total volume of 125 µl in 96-well microtitre plates. After 18 h of culture, methyl ³H-thymidine (0.4 µCi/well; PerkinElmer, Boston, MA, USA; 20 Ci/ml) was added, and cells were cultured for an additional 6 h. Cells were harvested onto a thin filtermat, and incorporation of ³H-thymidine was determined using a liquid scintillation counter (Wallac Trilux MicroBeta 1450; Biolab). Effects of growth factors on steroid production were determined following culture of 40 000 cells per well in a total volume of 250 µl. A positive control, 1 ng/ml ovine LH (purified in-house), was included in each culture. Every 48 h, 200 µl of medium was removed from each well and replaced with 200 µl of fresh medium containing the respective treatments. Media samples from the last 48 h of treatment were collected on day 6. At the time of collection, media samples were frozen at -20 °C until concentrations of progesterone could be determined by RIA (Asher 1990). The sensitivity of the progesterone assay was 17 pg/ml, and the intra- and inter-assay coefficients of variation were 11.4 and 12.2% respectively. A minimum of three replicate experiments were undertaken for determination of effect of IGF1 or IGF2 on thymidine incorporation and progesterone production. The effects of IGF1 or IGF2 on granulosa cell function were analyzed using a paired *t*-test comparing untreated control cells to those treated with IGF1 or IGF2.

Declaration of interest

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

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