Transforming growth factor-β: its role in ovarian follicle development

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Abstract

Ovarian follicular growth and differentiation in response to transforming growth factor-β (TGFB) was investigated using postnatal and immature ovarian models. TGFB ligand and receptor mRNAs were present in the rat ovary 4–12 days after birth and at day 25. In order to assess the impact of TGFB1 on follicle growth and transition from the primordial through to the primary and preantral stages of development, we established organ cultures with 4-day-old rat ovaries. After 10 days in culture with FSH, TGFB1, or a combination of the two, ovarian follicle numbers were counted and an assessment of atresia was undertaken using TUNEL. Preantral follicle numbers declined significantly when treated with the combination of FSH and TGFB1, consistent with our morphological appraisal suggesting an increase in atretic primary and preantral follicles. To investigate the mechanisms behind the actions of TGFB1, we isolated granulosa cells and treated them with FSH and TGFB1. Markers of proliferative, steroidogenic, and apoptotic capacity were measured by real-time PCR. Cyclin D2 mRNA expression by granulosa cells was significantly increased in response to the combination of FSH and TGFB1. The expression of forkhead homolog in rhabdomyosarcoma (Foxo1) mRNA by granulosa cells was significantly reduced in the presence of both FSH and TGFB1, individually and in combination regimes. By contrast, the expression of steroidogenic enzymes/proteins was largely unaffected by TGFB1. These data suggest an inhibitory role for TGFB1 (in the presence of FSH) in follicle development and progression.


Introduction

Surprisingly little is known about the mechanisms involved in ovarian follicle development and what triggers some follicles to grow, differentiate and ultimately release a matured oocyte while others die. A group of structurally related proteins, known as the transforming growth factor-β (TGFB) superfamily, have been implicated in the local regulation of ovarian function (for review see Knight & Glister 2006). Members of the family exert their effects via serine/threonine kinases that activate SMAD proteins. These receptor–SMAD complexes translocate to the nucleus where changes in nuclear transcription are elicited. Thus, for the effects of TGFB superfamily members to be mediated, all elements of the signalling pathway i.e. ligand, receptors, SMADs and any relevant co-factors must be present. We hypothesize that the combination of these components within a follicle changes during the transition of the follicle from primordial through primary, secondary and tertiary stages of development, and thus determines the biological effect of hormones and growth factors on the growth and differentiation of individual follicles.

TGFB, the index member of the TGFB superfamily, comprises five subtypes, all products of separate genes, three of which, Tgfb1, Tgfb2 and Tgfb3, have been shown to be expressed in mammalian ovarian cells (Hernandez et al. 1990, Mulhern & Schomberg 1990, Derynk et al. 1998). These factors are synthesized in inactive precursor forms that undergo cleavage to produce monomers which can dimerize to 25 kDa forms through the conserved cysteine regions. The active regions of the TGFB monomers share 98–100% identity and functionally, TGFB1, TGFB2 and TGFB3 are indistinguishable in most bioassays (Knecht et al. 1987) giving rise to the suggestion that there is functional redundancy. Despite these findings, null mouse models for each of the TGFB ligands indicate non-overlapping phenotypes (Shull et al. 1992, Proetzel et al. 1995, Sanford et al. 1997) and therefore different physiological functions. In regard to fertility, these issues have been difficult to address given that about half of the TGFB1 null mice die during gestation with the remaining pups dying around the time of weaning and that TGFB2 and TGFB3 null mice exhibit perinatal lethality (for review see Dunker & Krieglstein 2000). However, if inflammatory pathologies can be contained, the lifespan of TGFB1 null mice can be extended so that they reach adulthood. Ingman et al. assessed TGFB1 null mice bred on the immunocompromised severe combined immunodeficiency spontaneous mutation (scid) background and established that their fertility was severely impaired. Irregular ovulation, a reduction in the number of oocytes ovulated and in their developmental competence was reported (Ingman et al. 2006).

Granulosa and theca cells of bovine (Nilsson et al. 2003), human, rat and mouse (Mulheron et al. 1991) ovaries and mouse oocytes (Schmid et al. 1994, Gueripel et al. 2004) express Tgb2 mRNA and protein. Whereas, Tgb3 has been explored in a more limited way, the mRNA having been localized to theca and granulosa cells of follicles of all stages in mice (Schmid et al. 1994) and Tgb3 mRNA and protein to bovine granulosa and theca cells (Nilsson et al. 2003).

Tgbr1 mRNA and protein are expressed by porcine granulosa cells (Goddard et al. 1995), whilst TGFBR1 protein is expressed by mouse luteal cells, granulosa cells, theca cells and oocytes (Juneja et al. 1996, Gueripel et al. 2004). Tgbr2 mRNA is present in rat ovary (Tsuchida et al. 1993), whereas mRNA and protein have been localized to porcine granulosa cells (Goddard et al. 1995) and mouse theca, granulosa cells, oocytes and luteal cells (Schmid et al. 1994, Gueripel et al. 2004). In the human, TGFBR2 protein has been detected in granulosa, theca and interstitial cells (Roy & Kole 1998). Tgbr3 (also known as β-glycan) mRNA and protein are present in porcine granulosa cells (Goddard et al. 1995) and we have previously reported that β-glycan mRNA is expressed by the ovaries of 4, 8 and 12 day old rats with localization of β-glycan protein to oocytes, granulosa cells and theca cells at all stages of folliculogenesis (Drummond et al. 2002).

Despite the presence of TGFβ ligands and receptors in the ovary of a range of species, the direct effects of the TGFβ ligands on follicular development have not received much attention. In a study by Liu et al. (1999), the actions of TGFβ1 on preantral follicular growth were investigated in vitro, on follicles isolated from immature and adult mice. Age-specific effects were recorded for follicle diameter with only preantral follicles from adult mice increasing in size.

In this study, we investigated the role of TGFβ in recruiting follicles into the growth pathway and for its capacity to mediate the transition of follicles from primary to preantral and preantral to antral stages of development. First, we determined the mRNA expression pattern of TGFβ1, TGFβ2, TGFβ3, TGFBR1 and TGFBR2 in the rat ovary during postnatal development. We then took whole ovaries from 4-day-old rats, which contain only primordial and a few primary follicles and cultured them on floating filters for 10 days in the presence or absence of TGFβ1 and FSH. The viability of the follicles was established by TUNEL and follicles at each developmental stage were counted. Since TGFβ1 has been implicated in ovarian steroidogenesis and proliferation, we isolated granulosa cells from diethylstilboestrol (DES)-treated immature rats to investigate its effect on steroidogenic (side chain cleavage (CYP11A1), 3β-hydroxysteroid dehydrogenase (HSD3B) and STAR protein (STAR)) and proliferative (cyclin D2, forkhead homolog in rhabdomyosarcoma (FOXO1 or FOXO1A)) endpoints.

Results

Expression of TGFβ ligand and receptor mRNAs by rat ovaries

TGFβ1, TGFβ2, TGFβ3, TGFBR1 and TGFBR2 were present in rat ovaries as early as 4 days after birth (Figs 1 and 2). Expression of Tgb1 mRNA increased twofold between days 8 and 12 before declining to day 8 levels at day 25. Tgb2 mRNA declined between days 4 and 8, remaining low until day 12, when it increased to day 4 levels at day 25. Tgb3 mRNA levels were similar to that of Tgb2 (Fig. 1), the mRNA declining to a nadir at day 12 before assuming day 4 levels at day 25.

mRNA for the type I and II TGFβ receptors were differentially regulated with Tgbr1 expression high at day 4, declining to a nadir at day 8 before increasing to recover day 4 levels at day 25. By contrast, Tgbr2 appeared to be ubiquitously expressed during the postnatal/immature period of development with no significant change in the levels of its mRNA (Fig. 2).

Ovary organ cultures

An initial time course study was undertaken to determine the most appropriate culture period (Fig. 3A–C and data not shown). Prior to culture, the ovaries of 4-day-old rats (Fig. 3A) contained 67% primordial follicles, 24% primary follicles and 9% preantral follicles (n=8). Follicular development occurred with time in culture. After 5 days in culture (4–5 days), the ovaries contain primordial, primary and many preantral follicles, some with more than three layers of granulosa cells (data not shown). Thereafter, it was evident that centrally located follicles were being lost from the ovary most likely due to hypoxia and a lack of nutrients reaching the interior (Fig. 3B and C). Morphologically abnormal follicles were also present (Fig. 3C). Antral follicles were not observed in ovaries cultured up to 14 days in media alone (Fig. 3B and C). A culture time of 10 days was selected based on the morphological appearance of the ovary (Fig. 3D).

Vasa staining clearly delineated the oocyte cytoplasm making it easier to count follicles (Fig. 3D–G). Primordial to preantral oocytes stained positively, independently of the treatment regimen.
Follicle numbers

The numbers and types of follicles present in ovaries after 10 days of culture in the presence of FSH, TGFβ1 and combined treatment regimens are presented in Fig. 4A. Table 1 shows the proportion of follicles as a percentage of total follicle numbers. Ten days in culture without treatment had no significant effect on the proportion of primordial, primary or preantral follicles compared with freshly isolated 4-day-old ovaries (Fig. 4A and Table 1). Similar numbers of primordial and primary follicles were recorded across all treatment groups. Preantral follicle development, however, was found to be significantly reduced relative to untreated controls in ovaries cultured with FSH and TGFβ1 ($P<0.017$; Fig. 4A and B), but was not different to the number of preantral follicles in 4-day-old fresh (uncultured) ovaries (Fig. 4B).

Apoptosis

To assess the degree to which follicular apoptosis might be affected by our treatment regimen, TUNEL staining was implemented (Fig. 5). Very little apoptosis was observed in day 4 ovaries prior to culture (Fig. 5A). While it was not our intention to undertake a quantitative assessment, we have attempted to gauge the proportion of follicles that were atretic/healthy as determined by TUNEL staining (Fig. 6). After 10 days in culture, there was an increase in the number of apoptotic follicles present in the ovaries of all treatment groups relative to the uncultured 4-day-old fresh (uncultured) ovaries (Fig. 4B).
atresia in the primordial follicle population, whereas FSH alone appeared to reduce the number of healthy preantral follicles (Fig. 6).

**Effects of TGFβ1 on the mRNA expression of steroidogenic proteins and mediators of proliferation**

The expression of cyclin D2 mRNA by cultured granulosa cells was not affected by FSH or TGFβ1 individually, but in combination a significant increase was noted (Fig. 7). Foxo1 mRNA was significantly reduced in the presence of both FSH and TGFβ1, individually and in combination regimes (Fig. 7). Cyp11a1 mRNA expression was essentially unchanged by either FSH or TGFβ1 alone or in combination, whereas Star mRNA expression was enhanced by FSH, but not by TGFβ1 (Fig. 8). Hsd3b mRNA expression was significantly inhibited by FSH and slightly elevated by TGFβ1, whereas the combined FSH/TGFβ1 appeared to mimic the FSH response (Fig. 8).

**Discussion**

Members of the TGFβ superfamily, notably GDF9 and -9B and AMH, have been shown to influence the growth of early stage follicles (for review see Knight & Glister 2006). Few studies, however, have addressed the role that TGFβ itself might play in the growth and development of ovarian follicle populations. In these studies, we have chosen to use our established model of postnatal folliculogenesis (Drummond et al. 2002) to investigate follicle development and transition, as it occurs for the first time, in response to TGFβ. PCR studies...
on whole postnatal/immature ovaries established that the TGFB ligands (TGFB1–3) and receptors (TGFBR1 and 2) were present during the developmental period.

While it was not our intention to localize ligands and receptors to follicles or cell types, or to quantitate their expression in this context, we can draw some conclusions based on our knowledge of the follicle populations present in the ovary during this developmental period. Tgfb2 and Tgfb1 mRNA expression is high in the ovary 4 days after birth when there are mainly primordial and primary follicles and precursor cells present. Between days 4 and 8, these mRNAs decline as primary and preantral follicles increase in prevalence and granulosa cells multiply. Tgfb2 mRNA remains low at day 12, suggesting that it is the cells of early growing follicles that express this mRNA. Between days 8 and 12, Tgfbr1 mRNA increases coinciding with the differentiation and recruitment of theca cells, the proliferation of granulosa cells and the formation of antral follicles. By contrast, Tgfbr2 mRNA appears to be ubiquitously expressed in the ovary during the postnatal/immature developmental period. We have previously reported that β-glycan (Tgfbr3) and the appropriate SMADs are

Table 1 Proportion of follicle types represented as a percentage of total follicle numbers, before (day 4) and after 10 days in culture with treatment.

<table>
<thead>
<tr>
<th>Whole ovary culture</th>
<th>Day 4 (%)</th>
<th>Control (%)</th>
<th>FSH (%)</th>
<th>TGFB1 (%)</th>
<th>FSH/TGFB1 (%)</th>
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<tr>
<td>Primordial</td>
<td>67</td>
<td>60</td>
<td>67</td>
<td>75</td>
<td>73</td>
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<tr>
<td>Primary</td>
<td>24</td>
<td>31</td>
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<tr>
<td>Preantral</td>
<td>9</td>
<td>10</td>
<td>5</td>
<td>6</td>
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</table>

Figure 5 TUNEL staining in ovaries before and after 10 days of whole ovary culture. (A) Four-day-old ovary, (B) Control, (C) FSH, (D) TGFB1 and (E) combined FSH/TGFB1 after 10 days of culture, immature testis (F) (positive control), (G) and (H) TUNEL negative control sections. The length of the scale bars in B–G is 50 µm, while in A and H it is 100 µm. P, primary; p, primordial; Pr, preantral; St, seminiferous tubule, arrow heads show granulosa cells and arrows positively stained oocytes.
present in the ovary at this time (Drummond et al. 2000). These results indicate that TGFβ signals can be transduced and that it is biologically possible for TGFβ to influence folliculogenesis. Since changes in follicle growth in response to TGFβ1 have been demonstrated previously (Liu et al. 1999), the remaining studies were undertaken with this ligand and not TGFβ2 or TGFβ3.

Follicle development in response to TGFβ1 (in the presence or absence of FSH) was evaluated in an in vitro organ culture system. The results of our initial time course study indicated that a culture period of 10 days was optimal for 4-day-old rat ovaries (age at the start of culture). The ovaries remained morphologically healthy and follicle development occurred spontaneously with primordial, primary and preantral follicles evident in the ovary at the end of the culture period. No antral follicles, however, were observed. Continued culture after day 10 resulted in a loss of follicles as indicated by areas of vacant ovarian interstitial tissue and morphologically atretic follicles predominantly in the central regions of the ovary, indicative of a lack of oxygen and nutrients diffusing through to the interior. With this model, we wanted to determine whether the number of follicles in the ovary changed; if progression between the developmental stages was altered in any way and if the viability of the follicles was affected. We found that combined FSH and TGFβ1 treatment, reduced the number of preantral follicles present in whole ovaries cultured for 10 days. Our TUNEL assessments support this data and suggest an increased apoptosis in primary and preantral follicle populations. Thus, it would appear that fewer follicles successfully make the transition to the preantral follicle stage in the presence of FSH and TGFβ1. TGFβ has been shown to increase the expression of FSH receptor mRNA and to prolong its stability, an effect that is enhanced in the presence of FSH (Inoue et al. 2003), but it is unlikely that this is the mechanism of action given that primordial and primary follicles are unresponsive to FSH stimulation.

Since we have access to a well-characterized granulosa cell culture system, we decided to investigate granulosa cell functions that might be mediated by FSH and TGFβ1. There are, however, limitations to this model in relation to the whole ovary system. The granulosa cells were isolated from immature rats treated with oestrogen (DES) for 4 days. These granulosa cells have undergone significant proliferation before being cultured and are responsive to FSH. Basically, they are more representative of preantral rather than primary or primordial follicle populations. Using a similar model, Liu et al. (1999) isolated preantral follicles from the ovaries of DES-treated 28-day-old mice and reported that FSH and TGFβ1 individually increased follicular diameter and therefore growth, but in combination follicular diameter was reduced. These observations are consistent with our findings in whole ovary cultures that we report here.

Figure 6 Percentage of atretic (dark histograms) versus healthy (open histograms) follicles in ovaries cultured for 10 days with media, FSH, TGFβ1 and FSH/TGFβ1. The data are presented as mean ± S.D.

Figure 7 The expression of cyclin D2 and Foxo1 mRNA by granulosa cells isolated from DES-treated immature rats, in response to FSH, TGFβ1, or FSH/TGFβ1 (corrected for Gapdh). The data are presented as mean ± S.D., n = 3–4 and are representative of three separate experiments. Different letters denote statistical significance, P < 0.05.
FOXO1 regulates Fas ligand (pro apoptotic factor; Brunet et al. 1999) and p27KIP (cell cycle inhibitor) expression. We were able to confirm the findings of Richards et al. (2002) that FOXO1 expression in granulosa cells is downregulated by FSH, suggesting that apoptosis might be reduced in these cells. Consistent with this observation is the functional luteinization that occurs in vitro when granulosa cells are treated with FSH. Differentiation of granulosa cells is associated with reduced apoptosis (Song et al. 1999). In a seemingly paradoxical situation, FOXO1 levels are elevated in the granulosa cells of growing follicles that express high levels of cyclin D2, an important cell cycle mediator (Ingman et al. 2006). While these observations appear to be at odds, it is likely that FOXO1 is linked to both the proliferative and apoptotic pathways of granulosa cells and that it is the stage of follicle development and their response to hormones that determines the role of FOXO1 at any given time. Primordial and primary follicles do not respond to FSH stimulation and apoptosis in these populations is normally low. These observations suggest that alternative mechanisms regulate growth and differentiation in these follicle populations. Early preantral follicles, however, are just beginning to respond to FSH and contain granulosa cells at the start of a period of exponential growth. These follicles initially may express high levels of FOXO1 but with continued growth these levels are likely to decline consistent with the increase in apoptosis observed in growing follicle populations.

Cyclin D2 mRNA expression by granulosa cells was enhanced by TGFβ1, but only when FSH was present. There is some debate as to whether FSH itself stimulates cyclin D2 expression by granulosa cells. In our own studies, we have found an effect of FSH to be sporadic (this study and unpublished observations), while others (Ogawa et al. 2003) reported no effect and some a stimulation of cyclin D2 expression (Ingman et al. 2006). FSH has been shown to activate the PI3-kinase pathway which leads to inactivation of FOXO1 by AKT-dependent phosphorylation (Richards et al. 2002) effectively releasing the FOXO1-induced repression of cyclin D2 (Park et al. 2005). This, however, does not seem to be enough to stimulate granulosa cell proliferation in response to FSH stimulation in vitro. The combined effects of FSH and activin have been shown to stimulate granulosa cell proliferation in vitro (Ogawa et al. 2003) leading Park et al. (2005) to hypothesize that cross talk in granulosa cells between FOXO1 and the phosphorylated 2/3SMADs results in FOXO1 being antagonized. Since TGFβ also acts via SMADs 2/3, a similar mechanism can be envisaged, although in an inhibitory context. Further studies are required to dissect the mechanisms by which individual follicle populations grow and differentiate, but here we provide evidence to support an inhibitory role for TGFβ1 in preantral follicle growth that most likely involves an increase in apoptosis at the primary and preantral stages of follicle development.

**Materials and Methods**

**Animals**

Sprague–Dawley rats were obtained from Central Animal Services, Monash University (Melbourne, Australia). Ovaries were collected from untreated rats at 4, 8, 12 and 25 days of age. Ovaries were used either for organ culture, RNA extraction or for the preparation of formalin-fixed, paraffin-embedded tissue blocks. In addition, some animals at 21 days of age received a DES implant for 96 h, prior to ovary collection and the isolation of granulosa cells (Drummond et al. 1999). Animals were maintained under standard conditions of lighting and temperature and received laboratory feed pellets and water ad libitum. The project was approved by the institutional Animal Experimentation and Ethics Committee as conforming to the guidelines of the National Health and Medical Research Council of Australia.
**RNA extraction**

Ovaries were dissected free of fat and adhering tissue and homogenized in 1 ml Ultraspec RNA reagent (Biotec: Fisher Biotec, Melbourne, Australia). After 5 min on ice, 0.2 ml chloroform per ml of Ultraspec RNA reagent was added to the samples, which were then shaken vigorously and stored at 4°C for 5 min prior to centrifuging for 15 min at 12,000 g. RNA was precipitated from the aqueous phase with 1 vol of isopropanol, after which the pellet was washed twice with ethanol, air-dried and resuspended in sterile water. To ensure that the RNA was completely dissolved, the samples were incubated for 10 min at 60°C. The samples were then treated with DNA-free (Ambion: Austin, TX, USA) to remove DNAses. At least three independent pools of RNA were prepared for each age/treatment group. The number of ovaries/pool ranged from 24 to 40, for postnatal animals and 2/pool for immature animals.

**Reverse transcription**

RNA (1 μg) was reverse transcribed with 50 units MMLV (Expand) reverse transcriptase (Roche) and final concentrations of 1 × cDNA synthesis buffer (supplied with enzyme), 1 mM NTPs (Roche), 20 units Rnasin (Promega) 10 mM dithiothreitol and 25 pmol oligo dT15 (Roche), as previously described (Drummond et al. 1999).

**Real time PCR**

mRNA expression was analysed using the Roche LightCycler (Roche) as previously described (Drummond et al. 2000). Briefly, an ovarian cDNA pool diluted 1:2–1:2000 was used as the standard for the analyses. Sample cDNAs diluted 1:2–1:10 in sterile water were added to individual capillaries. Taq enzyme, dNTPs, reaction buffer and SYBR GREEN I dye were supplied in the FastStart DNA Master SYBR Green I kit (Roche); after mixing the reagents 2 μl/capillary was added. Primer concentrations of 10 pmol and 3 mM Mg were added to each capillary. Primer sequences are given in Table 2. The capillary volume was made up to 20 μl with sterile water. Forty cycles of PCR were programmed to ensure that the threshold crossing point (cycle number) was attained. Fluorescence emission was monitored continuously during cycling. At the completion of cycling, melting curve analysis was carried out to establish the specificity of the amplicons produced. In addition, each amplicon was sequenced to verify the identity of the amplified product (data not shown). The level of expression of each mRNA and their estimated crossing points in each sample were determined relative to the standard preparation using the LightCycler computer software. A ratio of specific mRNA/housekeeper gene amplification was then calculated. In most instances, individual pools for each age group or treatment with each primer set were performed in a single PCR experiment. The intra-assay variation was never more than 5% (n = 7) regardless of the primer set. The nature of Lightcycler PCR diminishes issues such as assay sensitivity and, at the concentrations of standard and sample utilized in these studies, the sensitivity threshold (picograms) was never approached.

**Granulosa cell cultures**

Granulosa cells were released from 25-day-old, DES-treated rat ovaries by repeated puncture with fine gauge needles, as previously described (Xiao et al. 1992). After washing and counting, the granulosa cells (5 × 10⁵/well) were plated and incubated for 24 h at 37°C in McCoy’s 5C (containing glutamine 2 mM, transferrin 100 μg/ml and penicillin 100 U/ml, 8% FBS). Cells were preincubated in the presence or absence of 1 μM R 1881 (R 1881, Sigma, St Louis, MO, USA) for 20 min at 37°C. Following 20 min, the medium was changed to fresh medium containing 1 μM R 1881 and the incubation was continued for the appropriate time. The medium was then changed to fresh medium containing 50 U/ml penicillin, 100 μg/ml streptomycin (both from Sigma), 100 × 10⁻⁶ M androstenedione (Sigma), 20% FBS, and R 1881, and incubated for 48 h. Cell viability was confirmed by trypan blue exclusion. RNA extraction was performed as above and the RNA was treated as described for the ovaries.

### Table 2

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streptomycin 100 µg/ml, and fungizone 250 ng/ml). The next day the media was changed and treatments were added for 2 h; FSH (100 ng/ml); rFSH-rIβ, obtained from the National Hormone and Pituitary Distribution Program and the NIADDK, NIH, Baltimore, MD, USA) and or TGFβ1 (1 and 10 ng/ml). The doses were based on previous studies undertaken in our laboratory. At the end of the incubation period, RNA was extracted as described.

**Whole ovary cultures**

Whole ovaries were cultured using a protocol similar to that described by Nilsson et al. (2001). Ovaries from 4-day-old rats were dissected free of the ovarian bursa and other extraneous tissue. Whole ovaries were cultured for 10 days on floating filters (0.4 µm Millicell-CM; Millipore Corp., Bedford, MA, USA) in 0.5 ml Dulbecco-modified Eagle medium (DMEM–Ham F-12 medium (1:1, v/v) containing 0.1% BSA (Sigma), 0.1% Albumax II (Gibco BRL), 5 × ITS-X (supplement containing insulin, sodium, transferrin, sodium selenite, ethanolamine; Life Technologies, Inc.), and 0.05 mg/ml l-ascorbic acid (Sigma) in a 24-well culture plate, each with one control and one treatment group (Nunc plate, Applied Scientific, South San Francisco, CA, USA). Medium was supplemented with penicillin and streptomycin to prevent bacterial contamination. Ovaries were randomly assigned to control or treatment groups with three ovaries (from three litters) per treatment group. DMEM/Ham’s F-12 medium was the cultured medium used in the control group, while the experimental group was cultured in DMEM/Ham’s F-12 media supplemented with 1% blocking reagent (Roche), after which the primary antibody of the appropriate concentration was added to the selected sections for 60 min at room temperature. After washing in PBS, the sections were incubated with a peroxidase conjugated avidin–biotin complex (Vector Elite, Vector Labs, Burlingame, CA, USA) for 60 min at room temperature after which the reaction product was developed using 3,3’ diaminobenzidine tetrahydrochloride (DAB; DAKO) and hydrogen peroxide in PBS. The sections were counterstained with haematoxylin, dehydrated in ethanol, cleared in histosol and coverslips mounted using DPX (BDH, VWR International Ltd, Poole, UK). Control sections received buffer in place of primary antibody. Follicles were classified as previously described (Drummond et al. 2002).

**Follicle counting**

Cultured ovaries were fixed, paraffin embedded and sectioned at 5 µm. Three serial sections were mounted/slide and every alternate slide (first and third sections) was used for Vasa staining and subsequent counting. Primordial, primary and preantral follicles were counted. Images were captured using a ×20 objective and only Vasa-positive oocytes (follicles) in which a nucleolus could be visualized, were counted using analySIS Professional Imaging software, version 5.0 (Imaging Research Inc., Ontario, Canada). Percentage distribution was determined for each follicle class in a given treatment group.

**Immunohistochemistry**

Vasa-mouse homologue (Abcam, Cambridge, UK), an oocyte-specific marker, was localized to rat oocytes in the primordial stage of development, to aid follicle counting. Sections (5 µm) of organ-cultured, formalin-fixed paraffin-embedded rat ovary were stained using standard immunohistochemical protocols. Briefly, sections were dewaxed in histosol (Australian Biostain, Traralgon, VIC, Australia), dehydrated in ethanol and washed in water. Sections were placed in citrate buffer (0.1 M, pH 6) and microwaved (900 W) for 10 min to retrieve antigens. The sections were cooled and then equilibrated in 0.1 M PBS pH 7.4. Endogenous peroxidase activity was blocked by incubating the sections for 30 min in 3% hydrogen peroxide followed by three washes in distilled water. The sections were blocked for 30 min in 1% blocking reagent (Roche), after which the primary antibody diluted 1:800 was added and the sections incubated for 18 h at 4 °C. Following extensive washing in PBS, the biotinylated second antibody dilute 1:200 (Dako, Sydney, Australia) was added to the sections for a 60 min incubation at room temperature. After washing in PBS, the sections were incubated with a peroxidase conjugated avidin–biotin complex (Vector Elite, Vector Labs, Burlingame, CA, USA) for 60 min at room temperature after which the reaction product was developed using 3,3’ diaminobenzidine tetrahydrochloride (DAB; DAKO) and hydrogen peroxide in PBS. The sections were counterstained with haematoxylin, dehydrated in ethanol, cleared in histosol and coverslips mounted using DPX (BDH, VWR International Ltd, Poole, UK). Control sections received buffer in place of primary antibody. Follicles were classified as previously described (Drummond et al. 2002).

**TUNEL staining**

Apoptotic cells in ovarian sections were detected using terminal deoxynucleotidyl transferase (TdT) mediated dUTP-biotin nick-end labelling (TUNEL). The ApoTag Peroxidase in situ apoptosis detection kit (Chemicon International, Melbourne, Australia) was utilized. Ovarian sections mounted on superfrost slides, were deparaffinized and rehydrated prior to commencing. The slides were washed (5 min) in PBS (1.0 mM, pH 7.4), then transferred into equilibration buffer. After 10 min at room temperature in a humidified chamber, working strength TdT enzyme was added to positive sections while negative control sections, received PBS. Plastic coverslips were added and slides were placed in humidified chamber at 37 °C for 1 h. The slides were then washed with stop/wash buffer at 37 °C for 30 min, agitating after 10 min followed by a 5 min PBS wash. The slides were then blocked for endogenous peroxidase activity by immersion in 3% hydrogen peroxide. After two washes in PBS, the slides were placed in CAS blocking solution (Dako) for 30 min at room temperature. Excess blocking solution was tapped off and the antigenic conjugate was applied to all sections. Plastic coverslips were placed on top of sections and incubated for 30 min at room temperature in a humidified chamber, followed by two PBS washes (5 min each). Apoptotic cells were visualized with the addition of 0.05% (w/v) DAB chromagen (Sigma) for ~1–2 min. The reaction was stopped by placing the slides into distilled H₂O. The sections were lightly counterstained using neat Harris haematoxylin, dehydrated through a graded series of alcohols and mounted under glass with DPX (Sigma). TUNEL was applied to three to four randomly selected slides/whole cultured ovary, in order to reveal the extent of...
apoptosis. On each slide, one section was used for TUNEL analysis, while the other acted as a negative control. Images were captured with a ×20 objective and TdT-positively stained follicles were counted and categorized according to follicle (primordial, primary and preantral) class using analyseSIS Professional Imaging software, version 5.0. Follicles that stained positively for TdT enzyme were categorized as ‘atretic’ and represented as a percentage of the total number of follicles.

**Statistical analysis**

Statistical significance was determined using SPSS software for Windows, version 14.0 (SPSS GmbH Software, Munich, Germany) by ANOVA in conjunction with a *post hoc* multiple comparison test (either Newman Keul’s: PCR data or Tukey’s: follicle counts). Each experiment was repeated at least three times, with *n* equal to a minimum of 3 per age group or treatment, in each experiment. The data are presented as the mean ± s.n. *P* values of <0.05 compared with the appropriate control were regarded as statistically significant.

**Declaration of interest**

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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