Oocytes prevent cumulus cell apoptosis by maintaining a morphogenic paracrine gradient of bone morphogenetic proteins

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Summary

Paracrine factors secreted by the oocyte regulate a broad range of cumulus cell functions. Characteristically, cumulus cells have a low incidence of apoptosis and we proposed that this is due to oocyte-secreted factors acting in an anti-apoptotic manner. Bovine cumulus-oocyte complexes (COC) were aspirated from abattoir-derived ovaries and oocytectomized (OOX) by microsurgical removal of the oocyte. OOX were treated with doses of either denuded oocytes (DO) or various growth factors for 24 hours (± rFSH; 0.1 IU/ml). Proportions of apoptotic cumulus cells were assessed using TUNEL and laser confocal scanning microscopy followed by image analysis. Quantification of Bcl-2 and Bax proteins in OOX was undertaken by western analysis. Oocyte removal led to a significant increase in cumulus cell apoptosis compared with COC controls (35% versus 9% TUNEL positive, respectively; $P<0.001$). Levels of OOX apoptosis were significantly reversed ($P<0.001$) in a dose-dependent manner when co-cultured with oocytes. Furthermore, the anti-apoptotic effect of oocyte-secreted factors followed a gradient from the site of the oocyte(s). Growth differentiation factor 9 (GDF9) had no significant effect on cumulus cell apoptosis. By contrast, cumulus cell apoptosis was significantly ($P<0.001$) reduced by bone morphogenetic proteins (BMP) 15, 6 or 7. Accordingly, levels of anti-apoptotic Bcl-2 were high in OOX+DO and OOX+BMP15 and low with OOX+GDF9 or OOX alone, whereas the reverse was observed for pro-apoptotic Bax. DO, BMP15 and BMP6 were also able to protect cumulus cells from undergoing apoptosis induced by staurosporine. FSH partially prevented apoptosis in all treatment groups ($P<0.001$). Follistatin and a BMP6 neutralizing antibody, which antagonized the anti-apoptotic effects of BMP15 and BMP6, respectively, whether alone or combined, blocked ~50% of the anti-apoptotic actions of oocytes. These results are the first to demonstrate that oocyte-secreted factors, and particularly BMP15 and BMP6, maintain the low incidence of cumulus cell apoptosis by establishing a localized gradient of bone morphogenetic proteins.

Key words: Oocyte-secreted factors, Bone morphogenetic proteins, Cumulus cell, Apoptosis

Introduction

Mammalian ovarian follicles are highly specialised structures that support the growth and development of oocytes. Folliculogenesis results from a complex balance between proliferation, differentiation and cell death, of both the somatic and germ cell compartments of the follicle. Follicles leave the resting primordial follicle pool and continue to grow, although only very small numbers of follicles ever ovulate. Instead, most ovarian follicles degenerate and die by the process of follicular atresia. In fact, more than 99% of all ovarian follicles in the cow are destined never to ovulate, but undergo atresia at various stages of follicular development (Mariana et al., 1991).

In antral bovine follicles, the earliest and most prominent feature of atresia is death of the granulosa cells, eventually leading to total destruction of the granulosa cell layer lining the inner follicle wall (Irving-Rodgers et al., 2001; Jolly et al., 1994). Jolly et al. demonstrated, using biochemical methods, that granulosa cell death during follicular atresia occurs by the active process of programmed cell death or apoptosis (Jolly et al., 1994). Van Wezel et al. reported that granulosa cells within the middle layers of the membrana granulosa undergo apoptosis, whereas granulosa cells closer to the follicular antrum die via an alternative pathway as a result of terminal differentiation (Van Wezel et al., 1999). At the cellular level, apoptosis is characterized by cytoplasmic and nuclear fragmentation, chromatin condensation, DNA fragmentation and phagocytosis (Hardy, 1999). Nuclear changes typical for early stage apoptosis include DNA fragmentation, which can be detected using terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling (TUNEL).

In the ovary, gonadotrophins and local growth factors have been shown to be regulators of granulosa cell apoptosis. FSH suppresses follicular cell apoptosis in murine pre-antral, early antral and pre-ovulatory follicles (Chun et al., 1994; Chun et al., 1996), and in cultured bovine granulosa cells (Yang and Rajamahendran, 2000). In ovarian antral follicles, there are two major phenotypes of granulosa cells that are anatomically and functionally distinct: mural granulosa cells, which line the wall

of the follicle; and cumulus cells, which surround and are in intimate metabolic contact with the oocyte, forming the cumulus-oocyte complex (COC). Apoptosis can be initiated in at least four different cellular compartments of the antral follicle: theca cells; granulosa cells; cumulus cells; and in the oocyte itself. The first signs of 'classical' or antral atresia of follicles are degeneration of the mural granulosa cells, which lose their aromatase activity and undergo apoptosis (Irving-Rodgers et al., 2001). Later, the theca cells undergo hypertrophy and their androsterone production decreases (Driancourt et al., 1998). The cumulus cells and the oocyte are only affected in the most advanced stages of follicular atresia (Kruip and Dieleman, 1982; Leibfried and First, 1979). As such, during atresia of antral follicles, it is common for mural granulosa cells to be undergoing apoptosis whilst cumulus cells remain healthy within the same follicle (Yang and Rajamahendran, 2000). The mechanism by which oocytes and cumulus cells escape apoptosis is entirely unknown.

Cumulus cells surround and communicate with the oocyte via paracrine factors and through gap junctions (Albertini et al., 2001). Disruption of this communication axis reduces cumulus cell proliferation and induces apoptosis in both cell types (Luciano et al., 2000). The distinct phenotype of cumulus cells is maintained by oocytes via the secretion of paracrine growth factors that regulate a broad range of cumulus cell functions (Eppig et al., 1997; Li et al., 2000). Oocyte-secreted factors promote cumulus cell growth, regulate inhibin synthesis while suppressing steroidogenesis and luteinizing hormone receptor expression (reviewed in Gilchrist et al., 2004a). Currently there is no data available as to whether these oocyte factors may also regulate and maintain the low incidence of cellular apoptosis within cumulus cells.

The mechanisms by which oocytes regulate cumulus cell functions, including the identities of the oocyte-secreted factors, remain largely unknown. Members of the transforming growth factor-β (TGF-β) superfamily are candidate oocyte-secreted molecules due to their ability to mimic the actions of an oocyte on cumulus cells and granulosa cells in vitro (Gilchrist et al., 2004a); in particular growth differentiation factor-9 (GDF-9), bone morphogenetic protein-15 (BMP-15), also referred to as GDF-9B, and BMP-6, all three of which are expressed by the oocyte. BMPs have only recently become recognised as autocrine/paracrine regulators of ovarian follicular development, even though it is well known BMPs regulate growth and differentiation in a broad range of other tissues (Shimasaki et al., 2004). For example, it is known that BMP-7 inhibits apoptosis in several tissues including eye and kidney, and plays a role in skeletal patterning (Dudley et al., 1995; Luo et al., 1995). Recent evidence suggests that BMPs may have a role in regulating ovarian atresia, as a dramatic decrease in the expression of BMP-4, -7, and -3b is a feature of atresia (Erickson and Shimasaki, 2003). As oocytes express BMP-15 and BMP-6, these oocyte-secreted factors may play a role in preventing follicular atresia. Several binding proteins antagonize BMP actions, including follistatin, noggin and gremlin (Balemans and Van Hul, 2002; Canalis et al., 2003). Although evidence is so far lacking, it seems plausible that one mode of regulation of the actions of these oocyte factors on cumulus cells may be by BMP antagonists expressed in the follicle, such as gremlin and follistatin, as well as by interaction with related follicular BMPs, such as BMP-4 and BMP-7, utilising common receptor and signalling pathways.

This study was conducted to investigate the nature of cellular interactions within the ovarian follicle that are responsible for maintaining the distinctive COC microenvironment. We hypothesized that cumulus cells exhibit a low incidence of apoptosis due to their close association with oocytes and their exposure to oocyte-secreted factors. Experiments were designed to determine the effect of oocyte-secreted factors on cumulus cell apoptosis in the presence and absence of FSH, and to examine the nature of the paracrine network of BMP growth factors and their binding proteins regulating cumulus cell apoptosis.

Materials and Methods

Collection of bovine oocytes and culture conditions

Unless otherwise specified, all chemicals and reagents were purchased from Sigma (St Louis, MO). Bovine ovaries were collected from local abattoirs and transported to the laboratory in warm saline (30-35°C). COC were aspirated from antral follicles (2-8 mm diameter) using an 18-gauge needle and a 10 ml syringe containing ~2 ml aspiration media (Hepes-buffered Tissue Cultured Medium-199; TCM-199, ICN Biochemicals, Irvine, CA, USA) supplemented with 50 μg/ml kanamycin (Sigma-Aldrish, St Louis, MO), and 4 mg/ml fatty acid-free bovine serum albumin (FAF-BSA; ICPbio Ltd, Aukland, NZ). Intact COC with compact cumulus vestments greater than five cell layers and evenly pigmented cytoplasm were selected under a dissecting microscope and washed twice in Hepes-buffered TCM-199 and once in corresponding culturing media. Complexes were cultured with or without 0.1 IU/ml recombinant human FSH (Organon, Netherlands) in pre-equilibrated 50 μl drops of culture media (bicarbonated-buffered TCM-199 supplemented with 0.23 mmol sodium pyruvate per litre and 0.3 mg/ml polyvinyl alcohol) overlaid with mineral oil and incubated at 39°C with 5% CO_2 in humidified air for 24 hours.

Treatment of cumulus cells

Generation of oocytectomized complexes

The cytoplasm of each oocyte was microsurgically removed from the COC (oocytectomy) using a micromanipulator as described in Buccione et al. (Buccione et al., 1990). The resulting oocytectomized complex (OOX) consists of a hollow zona pellucida surrounded by several layers of intact cumulus cells.

Generation of denuded oocytes

Denuded oocytes (DO) were generated by removing cumulus cells from COC by vortexing for ~4 minutes in 2 ml H-TCM-199/BSA. Any remaining cumulus cells were removed by repeated passage of the oocytes through a fine-bore fire-polished glass pipette in H-TCM-199/BSA.

Growth factors and binding proteins

Recombinant mouse GDF-9 and recombinant ovine BMP-15 were produced in-house as previously described (Kaivo-Oja et al., 2003; McNatty et al., 2005) using transfected 293 human embryonic kidney cell lines (293H), generously donated by Olli Ritvos (Biomedicum, Helsinki, Finland). Recombinant proteins were partially purified using hydrophobic interaction chromatography (HIC), as recently described (Hickey et al., 2005), and their concentrations were then estimated by western blot (Kaivo-Oja et al., 2003; McNatty et al., 2005). Control conditioned medium (293H) was also produced from untransfected 293H cells and purified by HIC. Recombinant human BMP-6,

recombinant human BMP-7, BMP-6 neutralizing antibody, and gremlin were obtained from R&D systems (Minnaepolis, MN). Follistatin-288 was generously donated by S. Shimasaki (University of California, San Diego, USA) and R. Rodgers (The University of Adelaide, Adelaide, Australia).

Determination of DNA damage by TUNEL (assessment of cumulus cell apoptosis)

Cumulus cell apoptotic DNA was detected using TUNEL (Roche Diagnostic, Penzberg, Germany) according to the manufacturer's instructions. Briefly, following culture COC and OOX complexes were washed twice in PBS (pH 7.4) containing 1% BSA, fixed in 4% paraformaldehyde in PBS (pH 7.4) overnight at 4°C and washed twice with PBS/BSA before placing on Cell-Tak-coated coverslips (Beckton Dickinson Biosciences, Franklin Lakes, NJ). Complexes were then permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate for 1 hour at room temperature and washed three times in PBS/BSA. The complexes were then incubated in fluorescein-conjugated dUTP and terminal deoxynucleotide transferase (TUNEL reagents, Roche) for 1 hour at 37°C in the dark. Positive controls were incubated in DNAse 1 (0.005 U/μl), which cleaves all DNA, for 20 minutes at room temperature and washed twice in PBS/BSA before TUNEL. Negative controls were incubated in fluorescein-dUTP in the absence of TdT. After TUNEL, complexes were washed twice in PBS/BSA and counterstained with propidium iodide 0.5 μg/ml (PI) plus RNase A (0.1 mg/ml) for 1 hour at room temperature in the dark to label all nuclei. Complexes were then washed twice in PBS/BSA and mounted with slight coverslip compression in VectaShield anti-bleaching solution (Vector Labs, Burlingame, CA), and stored in the dark at 4°C for confocal analysis.

Confocal microscopy and analysis

Apoptosis in COC and OOX was visualised and quantified using confocal microscopy. Dual fluorescence emission from cumulus cells was detected using a Nikon C1 Confocal Scanning Head and a Nikon TE2000E microscope (Nikon, Tokyo, Japan). Simultaneous emission capture of the apoptotic signal (fluorescein, laser excitation 488 nm, emission 510-530 nm) and the nuclear signal (propidium iodide, excitation laser 532, emission 590-640 nm) was performed.

To generate an accurate representation of the overall apoptotic incidence for all complexes, the depth of each complex was measured through a Z series to divide the construct into three percentiles (optical Z plane sections) at 25%, 50% and 75%. These optical section images were acquired and saved as independent colour channels (green, apoptotic cumulus fluorescence and red, nuclear cumulus fluorescence). The captured images were then processed in Scanalytics IPLab software Version 3.6. (Scanalytics, Fairfax, VA). Quantification of cumulus cell number (for each colour channel) was independently measured using a macro script utilizing an auto segmentation filter for each optical section percentile (three optical 'Z'-plane sections for each complex). A percentage of apoptotic nuclei was generated for each slice and the three percentile values were then averaged to achieve a representation of the total apoptotic nuclei percentage for the whole complex. These processes were repeated separately on each individual complex.

Western blot analysis

Following culture treatments, OOX complexes were lysed in 25 μl RIPA lysis buffer [10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100] and stored at –80°C. Thawed lysates were mixed with 4× loading buffer containing 100 mM dithiothreitol (DTT) and subjected to SDS-PAGE (12% polyacrylamide gel). Proteins were subsequently electrotransferred to nitrocellulose membranes (Hybond-ECL, Amersham Life Science, Ontario, Canada.) in 25 mM Tris, 192 mM glycine containing 20% methanol. Blots were blocked in 20 mM Tris (pH 7.6) containing 13.7 mM NaCl, 1% Tween-20, and 2% blocking agent (provided in ECL Advance Kit) for 1 hour at RT, then incubated overnight with Bcl-2 or Bax rabbit polyclonal antibodies (0.35 μg/ml; Santa Cruz Biotechnology, CA, USA) at 4°C, followed by incubation with horseradish peroxidase-conjugated anti-rabbit antibody (1:200,000; Silenus Laboratories, Melbourne, Australia) and detected using the sensitive Enhanced Chemiluminescence (ECL) Advance system (Amersham Biosciences, Ontario, Canada). Images were then scanned using a flat bed scanner and the intensity of Bcl-2 and Bax bands in each sample was quantitated by the ImageJ Imaging System Software version 1.3 (National Institutes of Health, USA).

Experimental design

Experiment 1: the effect of oocytectomy on cumulus cell apoptosis

The aim of this experiment was to determine whether intact COC have a different level of apoptosis to OOX. Groups of five COC or OOX were cultured in 50 μl drops of culture media for 24 hours before apoptosis was assessed. Six replicate experiments were performed.

Experiment 2: the effect of oocyte-secreted factors on cumulus cell apoptosis

To determine whether oocyte-secreted factors are responsible for the low incidence of apoptosis in cumulus cells of intact COC, OOX were cultured with increasing numbers of denuded oocytes and compared with COC. 5, 25 or 50 denuded oocytes were added to 50 μl culture drops containing 5 OOX. Three replicate experiments were performed.

Experiment 3: pattern of apoptosis in relation to proximity to oocyte-secreted factors origin

The aim of this experiment was to determine the distribution of apoptosis within the cumulus cell complex in relation to the complex's proximity to the oocyte. We quantified the apoptotic incidence in COCs, where the origin of the oocyte-secreted factors is central to the cumulus cell complex, and in contrast, in OOXs co-cultured with denuded oocytes, where the origin of the oocyte-secreted factors is on the outside of the cumulus cell complex. OOXs cultured alone were used as a control, and all complexes were cultured without FSH. Using the confocal microscope, the diameter of complexes was measured after the diameter of the oocyte region was subtracted using Scanalytics IPLab software Version 3.6. This was then divided into three equal layers; inner, middle and outer cumulus cell layers, forming three ring zones around the oocyte. Each layer was equivalent to a proportion of 33% of the total radius. The incidence of apoptosis was then analysed independently in each layer.

Experiment 4: dose response of GDF-9, BMP-6 and BMP-15 on cumulus cell apoptosis

In an attempt to examine which of the putative oocyte-secreted factors is contributing to the low incidence of apoptosis observed in COC, OOX were cultured with increasing concentrations of either GDF-9 (0-175 ng/ml), BMP-6 (0-100 ng/ml) or BMP-15 (0-20% v/v), either in the absence or presence of FSH. OOX were also treated with 10% (v/v) 293H, which served as a parent cell line-conditioned media negative control for GDF-9 (equivalent to 132 ng/ml) and 10% (v/v) BMP-15. Three replicates of these experiments were performed using 10 OOX per treatment group per replicate experiment.

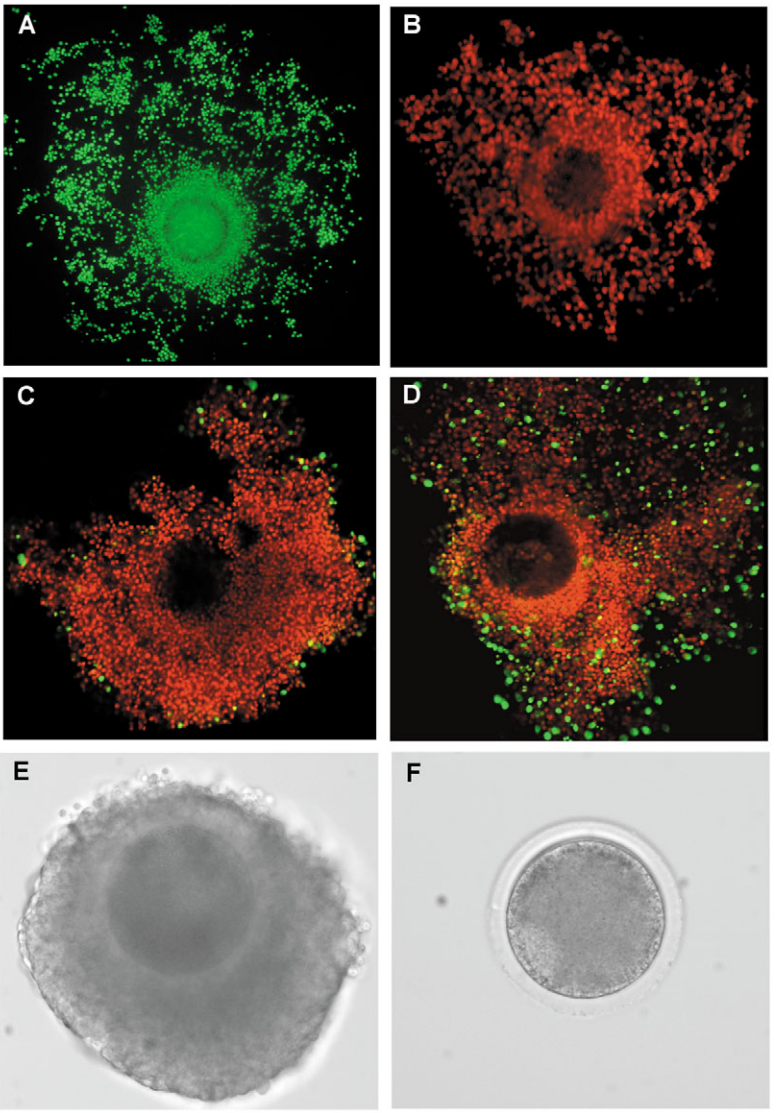

Fig. 1. Representative images of confocal laser scanning microscopy of DNA fragmentation in cumulus cells, as detected by TUNEL (green label). All cell nuclei are also stained with propidium iodide (red). Positive control DNAse 1-treated OOX showed very strong apoptotic staining (99%) (A), negative control did not reveal any apoptotic signals (0%) indicating specific labelling (B), expanded COC after culture with low apoptotic labelling (9%) (C), compared with OOX with higher apoptotic labelling (35%) (D). Light micrograph of an unexpanded intact COC before culture (E) and a denuded oocyte (F), generated by the mechanical removal of cumulus cells from a COC.

Experiment 5: the effect of oocytes, GDF-9 and BMP-15 on cumulus cell expression of Bcl-2 and Bax proteins

This experiment was conducted to confirm that treatment effects on cumulus cell apoptosis, as assessed by TUNEL, are concomitant with changes in expression of key proteins regulating cell death and survival. OOX were cultured for 24 hours untreated, or treated with 132 ng/ml GDF-9, 10% BMP-15, co-cultured with 35 denuded oocytes per well, or 10% 293H (control conditioned medium), and then subjected to western blot for analysis of Bcl-2 and Bax expression.

Experiment 6: effect of oocytes, BMP-6 and BMP-15 on cumulus cell apoptosis induced by staurosporine

A preliminary experiment was conducted to determine the apoptotic effect of staurosporine on bovine cumulus cells, which induced apoptosis in a dose-dependent manner (range; 0.1-100.0 μM, data not shown). The aim of this experiment was to determine whether oocyte-secreted factors could prevent cumulus cells from undergoing apoptosis induced by staurosporine. OOX alone or co-cultured with 35 denuded oocytes, 10 ng/ml BMP-6 or 10% BMP-15, were then exposed to either 0.1 μM or 1.0 μM staurosporine for the last 6 hours of the 24 hour incubation period. Three replicates of this experiment were carried out using 10 OOX per treatment group per replicate experiment.

Experiment 7: effect of BMP antagonists on cumulus cell apoptosis

Follistatin binds to both BMP-15 and activin with high affinity and antagonizes their bioactivity (Lin et al., 2003; Otsuka et al., 2001). Gremlin is expressed in both mural granulosa cells and cumulus cells and selectively blocks BMP-4 and BMP-7 (Merino et al., 1999), and may antagonize BMP-15. The aim of this experiment was to examine the effectiveness of these antagonists against BMP-6 and BMP-15-prevented cumulus cell apoptosis. OOX were cultured with 10% BMP-15 in the presence of increasing doses of follistatin (0-100 μg/ml) or in the presence of increasing doses of gremlin (0-40 μg/ml). In a separate experiment, OOX were treated with 10 ng/ml BMP-6 in the absence or presence of a high dose (20 μg/ml) of a BMP-6 monoclonal neutralizing antibody (NAb). Three replicates of each of these experiments were carried out using 10 OOX per treatment group per replicate experiment.

Experiment 8: role of BMP-15 and BMP-6 in the anti-apoptotic actions of oocytes on cumulus cells

In an attempt to neutralize the anti-apoptotic bioactivity of oocytes on cumulus cells, OOX were co-cultured with 25 denuded oocytes, either in the absence or presence of 50 μg/ml follistatin, 20 μg/ml BMP-6 NAb, or in the presence of both antagonists. A separate experiment was conducted to examine any additive effects of BMP-15 and BMP-6, compared with OOX+oocytes. OOX were treated with denuded oocytes, or with 10 ng/ml BMP-6 and/or 10% BMP-15. Three replicates of these experiments were carried out using 10 OOX per treatment group per replicate experiment.

Experiment 9: effect of BMP-7 and its antagonist, gremlin, on cumulus cell apoptosis

To examine the influence of BMP-7 and its antagonist, gremlin, on cumulus cell apoptosis, OOX were treated with 100 ng/ml BMP-7 and/or 10% BMP-15 in the presence or absence of 2 μg/ml gremlin. Three replicates of this experiment were carried out using 10 OOX per treatment group per replicate experiment.

Statistical analysis

Frequencies of cumulus cell apoptosis were analysed by ANOVA using SigmaStat software (SPSS Inc, Chicago, IL), and significant differences between means were determined using Tukey-Kramer post-hoc test for comparison of multiple means. All cell proportional

data were arc-sine transformed prior to analysis. Differences were considered statistically significant at $P<0.05$.

Results

Experiment 1: the effect of oocytectomy and FSH on cumulus cell apoptosis

TUNEL coupled with confocal scanning microscopy proved a highly effective means of visualising and quantifying cumulus cell apoptosis. TUNEL positive and negative controls (Fig. 1A,B) (99% and 0% apoptosis, respectively) demonstrated specificity. COCs exhibited a low incidence of cumulus cell apoptosis (9%; Fig. 1C), and removal of the oocyte led to a significant increase to 35% in OOXs ($P<0.001$; Fig. 1D). As is expected with bovine cumulus cells, treatment with FSH induced cumulus expansion in both COCs and OOXs (Fig. 1C,D), and significantly decreased the incidence of apoptosis in OOXs (by 10%) and in COCs (by 6%) ($P<0.001$) (Fig. 2).

Experiment 2: the effect of oocyte-secreted factors on cumulus cell apoptosis

To determine if oocyte paracrine factors are responsible for low COC apoptosis, an attempt was made to reduce the incidence of apoptosis in OOX to COC levels, by co-culturing OOXs with increasing concentrations of denuded oocytes. Cumulus cell apoptosis was significantly reduced ($P<0.001$), in a dose-dependent manner, by incubating OOXs with increasing numbers of oocytes. Apoptotic levels in OOXs were completely restored to COC levels at the maximum number of oocytes (50 DO per well), whether in the presence or absence of FSH (Fig. 2). These results indicate that oocyte-secreted factors prevent apoptosis within cumulus cells.

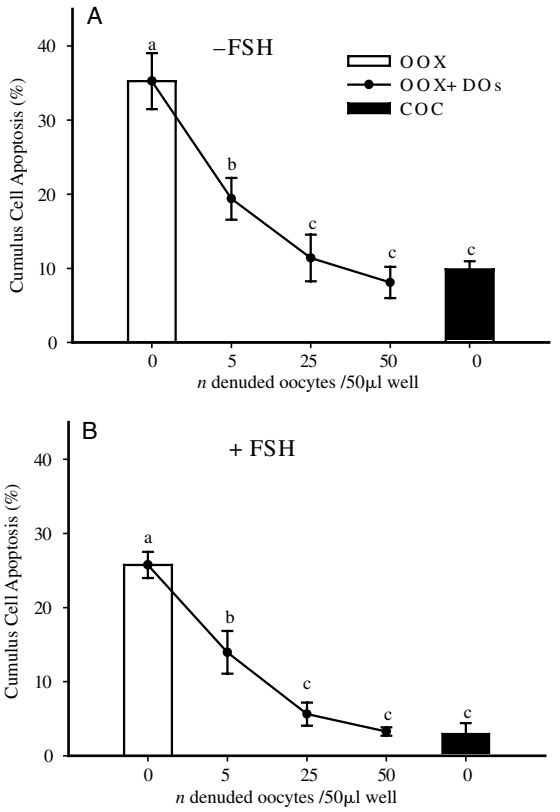

Fig. 2. A dose response of oocyte-secreted factors on cumulus cell apoptosis in the absence (A) or presence (B) of FSH. Oocytectomized complexes (OOX) were cultured with increasing numbers of denuded oocytes (DO) and at the maximum dose were effective at reducing apoptosis to the control COC levels. FSH also significantly reduced apoptosis in COCs and OOXs. Points represent average percentage of apoptotic cumulus cells (mean ±s.e.m.). Values from points with different labels; [a,b,c]differ significantly ($P<0.001$).

Experiment 3: pattern of apoptosis in relation to proximity to oocyte-secreted factor origin

Qualitative observations of confocal images suggested that the apoptotic cells within COCs were mostly distributed to the outer layer of complexes, whereas apoptosis was observed in

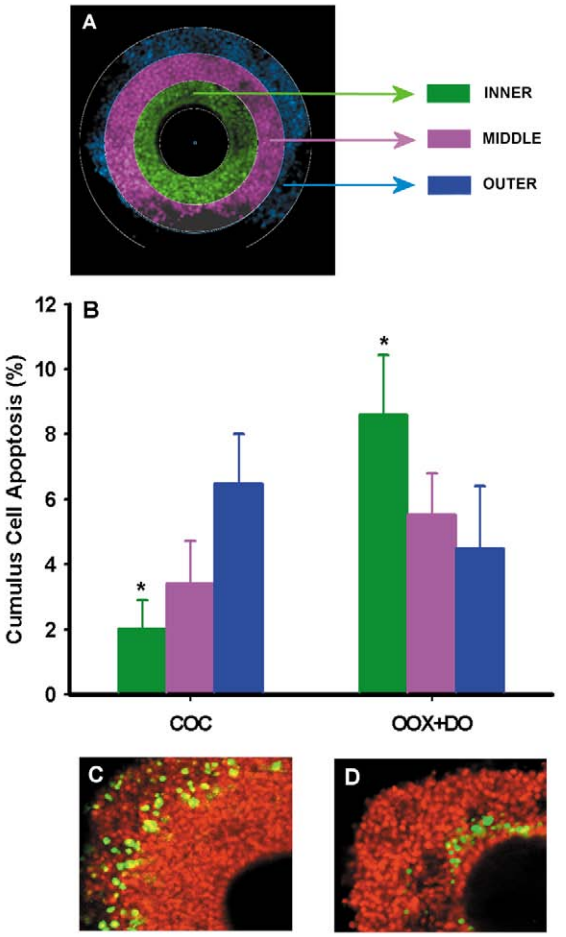

Fig. 3. Pattern of apoptosis within cumulus complexes in relation to proximity to oocyte-secreted factor origin. Diameters of unexpanded COCs and OOXs were measured after culture without FSH, using confocal microscopy and divided into three layers: inner, middle and outer layers. Each layer representing 33% of the radius (A). The incidence of apoptosis was lowest closest to the oocyte, regardless of whether the oocyte was inside the complex [(C), COC inner layer] or oocytes were on the outside of the complex [(D), OOX outer layer], and apoptosis increased with increasing distance from the oocyte (B). Both C and D images are exaggerated examples to illustrate pictorially the morphogenic gradient of apoptosis through the cumulus cell layers. *Significantly different (two-way ANOVA; $P=0.026$).

the inner cumulus layers when OOXs were co-cultured with denuded oocytes. We therefore hypothesized that oocyte-secreted factors establish an anti-apoptotic morphogenic gradient through the cumulus cell layers. To test this hypothesis, we measured the diameter of COC and OOX complexes and then divided them into three layers: inner, middle and outer (Fig. 3A). Within COCs, which contain an intact oocyte, the incidence of apoptosis increased significantly (P<0.026) from the inner layer toward the outer layer (Fig. 3B,C). Conversely, when OOXs were co-cultured with denuded oocytes, the incidence of apoptosis decreased from the inner layer toward the outer layer, which is closest to the source of oocyte-secreted factors (Fig. 3B,D). To illustrate this effect further, the inner layer in COC, which is closest to the oocyte and has the lowest incidence of apoptosis, has a fourfold and significantly (P<0.026) lower incidence of apoptosis, compared with its counterpart inner layer from the OOX+DO group, which has the highest incidence of apoptosis, being the furthest layer from the oocytes (Fig. 3B). To examine the effect of the oocytectomy procedure itself and the associated loss of oocyte-cumulus cell gap junctional communication, apoptosis was analysed in OOX cultured alone for 24 hours. Apoptosis were equally distributed in the three layers; inner, middle and outer (49.5, 49.9 and 45.5%, respectively; P>0.05).

Experiment 4: dose response of GDF-9, BMP-6 and BMP-15 on cumulus cell apoptosis

These experiments were conducted to determine the effect of these putative oocyte-secreted factors on the regulation of cumulus cell apoptosis. OOX complexes were treated with increasing doses of GDF-9, BMP-6 and BMP-15. GDF-9 had no significant effect on the incidence of cumulus cell apoptosis in the presence or absence of FSH, as at the highest dose (175 ng/ml) of GDF-9, apoptosis was not significantly different to the 293H control conditioned

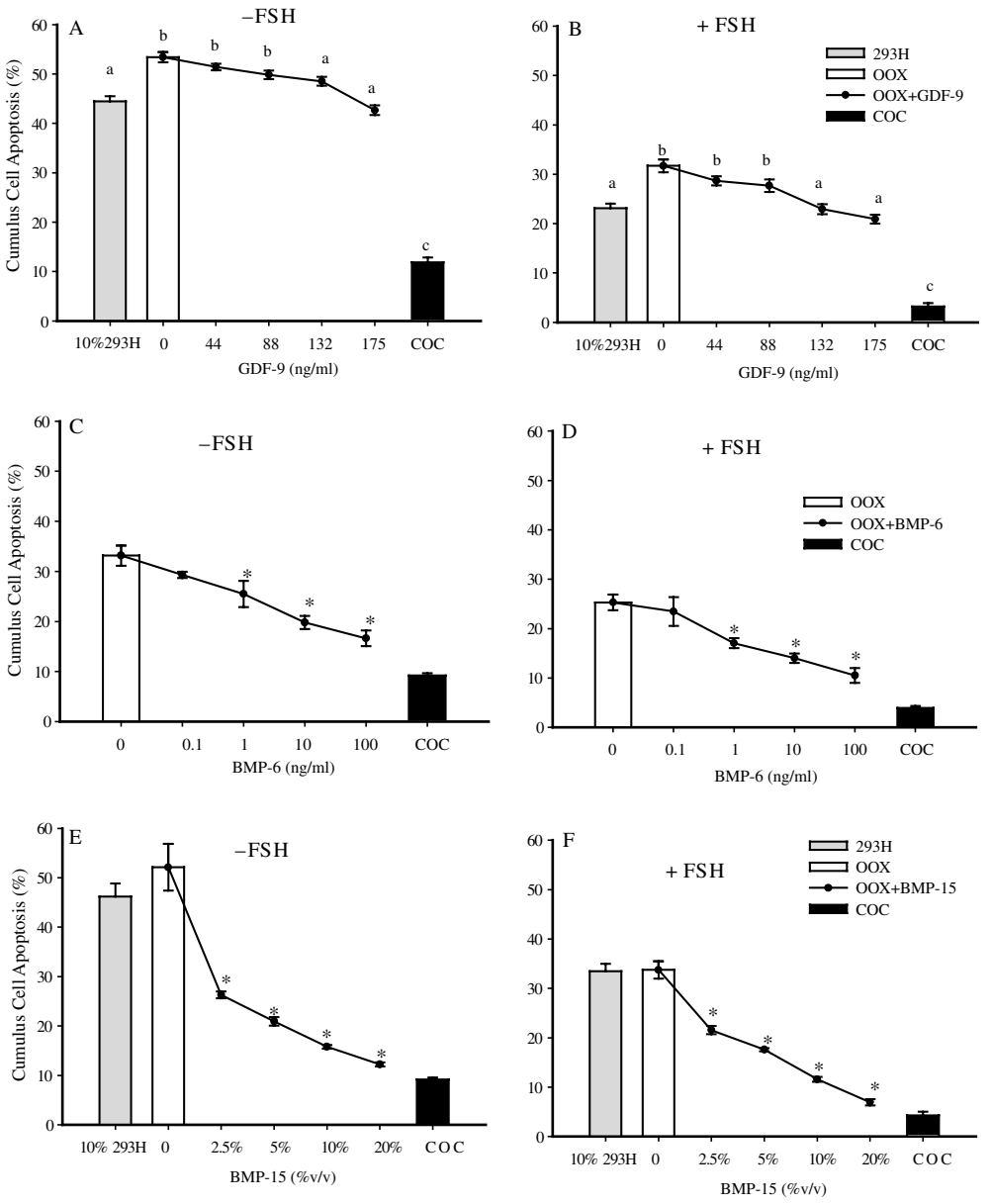

Fig. 4. Dose response of the putative oocyte-secreted factors; GDF-9, BMP-6, BMP-15 on cumulus cell apoptosis. OOX were cultured with increasing concentrations of GDF-9 (0-175 ng/ml), BMP-6 (0-100 ng/ml), and BMP-15 (0-20% v/v), either in the absence (A,C,E) or presence (B,D,F) of FSH. Cumulus cell apoptosis was unaffected by GDF-9 and attenuated in a dose-dependent manner by BMP-6, but more notably by BMP-15. FSH independently attenuated apoptosis regardless of treatment or complex type. Points represent average percentage of apoptotic cumulus cells (mean ±s.e.m.). Values from points with different labels; ^{a,b,c}differ significantly (A,B; P<0.001). Asterisks represent significant difference (P<0.001) relative to the control (OOX) for that factor (C-F).

medium group (Fig. 4A,B). With an increasing dose of BMP-6, cumulus cell apoptosis significantly decreased ($P<0.001$), whether in the presence or absence of FSH (Fig. 4C,D). Cumulus cell apoptosis was significantly reduced ($P<0.001$) in a dose-dependent manner, by treating OOX with an increasing dose of BMP-15, having maximal effect at 20% BMP-15 (Fig. 4E). A similar response was observed when BMP-15 was used in combination with FSH (Fig. 4F). Consistent with experiment 2, FSH had an additive effect in preventing cumulus cell apoptosis, independently decreasing the incidence regardless of growth factor treatment or complex type.

Experiment 5: the effect of oocytes, GDF-9 and BMP-15 on cumulus cell expression of Bcl-2 and Bax proteins

This experiment was conducted to study the pattern of Bcl-2 and Bax expression in cumulus cells of OOX complexes. Expression of Bcl-2 protein was significantly ($P<0.001$) higher in cumulus cells when OOX were co-cultured with denuded oocytes and BMP-15 compared with when untreated or treated with GDF-9 (Fig. 5). By contrast, expression of Bax protein was found to be significantly ($P<0.001$) higher in cumulus cells when OOX were untreated or co-cultured with GDF-9 compared with OOX co-cultured with denuded oocytes or BMP-15, where Bax levels were barely detectable (Fig. 5). These results support our TUNEL results, namely that oocytes and BMP-15 but not GDF-9 are associated with the prevention of cumulus cell apoptosis, and that oocytes and BMP-15 alter the ratio of Bcl-2 to Bax in favour of cell survival (Oltvai et al., 1993), whereas GDF-9 has no effect on the Bcl-2 to Bax ratio.

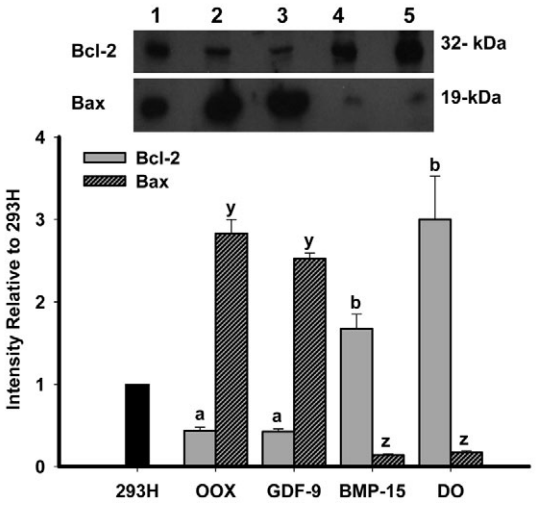

Fig. 5. Effect of denuded oocytes (DO), GDF-9 and BMP-15 on OOX expression of Bcl-2 and Bax proteins as examined by western blot analysis. Groups of 35 OOX were loaded in each lane after the following treatments: lane 1, 10% v/v 293H (control conditioned medium); lane 2, control (OOX alone); lane 3, 132 ng/ml GDF-9; lane 4, 10% v/v BMP-15; lane 5, 0.7 DO/ μl. Band intensities were quantified by densitometry and are expressed relative to the 293H control, from three replicate experiments (mean ±s.e.m.). Bars with different superscripts within a group (^{a,b}Bcl-2, ^{y,z}Bax) are significantly different ($P<0.001$).

Experiment 6: protection of cumulus cells from staurosporine-induced apoptosis by oocytes, BMP-6 and BMP-15

The aim of this experiment was to determine whether oocytes are capable of protecting cumulus cells from an apoptosis-inducing event and whether such an effect can by mimicked by BMP-15 and BMP-6. Staurosporine significantly increased ($P<0.001$) the incidence of cumulus cell apoptosis from 41% to 51% and 74% when treated with 0.1 and 1.0 μM, respectively (Fig. 6). The apoptosis-inducing effects of both doses of staurosporine were completely negated when staurosporine-treated OOXs were co-cultured with denuded oocytes, with apoptosis reduced to COC levels. Also, OOX treated with 10% BMP-15 or 10 ng/ml BMP-6, exposed to the same two doses of staurosporine, showed significantly decreased apoptosis; 17% and 21% (0.1 μM); 25% and 31% (1 μM), respectively ($P<0.001$). These results indicate that the anti-apoptotic actions of oocyte-secreted factors can protect cumulus cells from an apoptotic insult and that both BMP-15 and BMP-6 can mimic this effect.

Experiment 7: effect of BMP antagonists on cumulus cell apoptosis

These experiments were conducted to examine whether BMP-15 and BMP-6 antagonists could neutralize the anti-apoptotic effects of BMP-15 and BMP-6 on cumulus cell apoptosis. There was a significant ($P<0.001$), dose-dependent increase in apoptosis when BMP-15-treated OOXs were cultured with increasing concentrations of follistatin (Fig. 7A). OOX complexes cultured with 10 ng/ml BMP-6 were treated with a high dose (20 μg/ml) of a BMP-6 neutralizing antibody. Fig. 7B illustrates that the BMP-6 antagonist significantly ($P<0.001$) neutralized the anti-apoptotic effect of 10 ng/ml BMP-6.

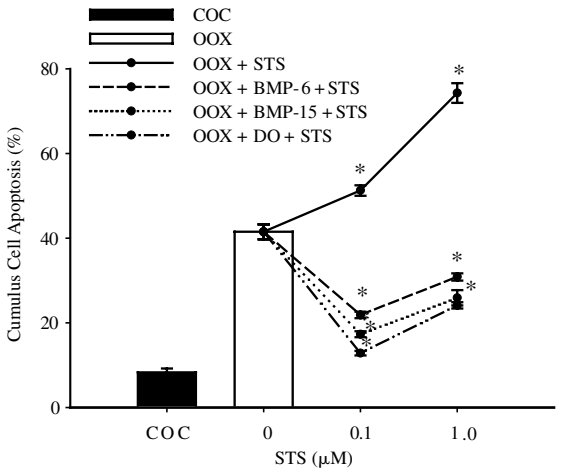

Fig. 6. Protection of cumulus cells from staurosporine-induced apoptosis by denuded oocytes (DO), BMP-6 and BMP-15. OOX alone or co-cultured with 35 DO, 10 ng/ml BMP-6, or 10% v/v BMP-15, were exposed to either 0.1 μM or 1.0 μM staurosporine (STS) in the last 6 hours of incubation. Oocytes, BMP-6 and BMP-15 all prevented staurosporine-induced cumulus cell apoptosis. Asterisks represent OOX means significantly different ($P<0.001$) relative to the OOX control.

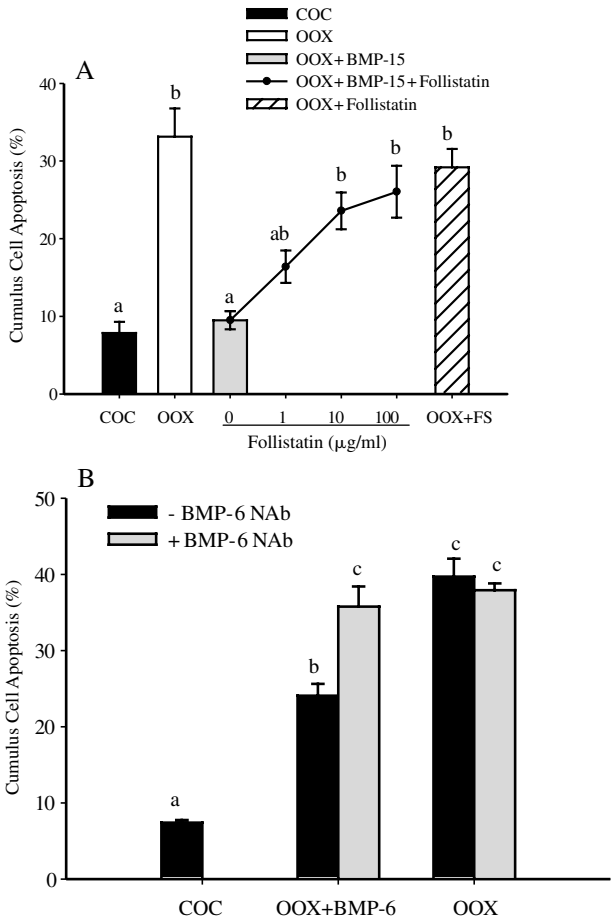

Fig. 7. Effect of BMP antagonists on cumulus cell apoptosis. OOX were cultured with 10% v/v BMP-15 in the presence of increasing doses of follistatin (0-100 μg/ml) (A), and OOX were cultured with 10 ng/ml BMP-6 in the absence or presence of a high neutralizing dose of 20 μg/ml of a BMP-6 neutralizing antibody (NAb) (B). Suppression of cumulus cell apoptosis by BMP-15 was antagonized by follistatin. The NAb effectively antagonized the anti-apoptotic effects of BMP-6. Points and bars represent average percentage of apoptotic cumulus cells (mean ±s.e.m.). Values from points with different labels; [a,b,c]differ significantly (P<0.001).

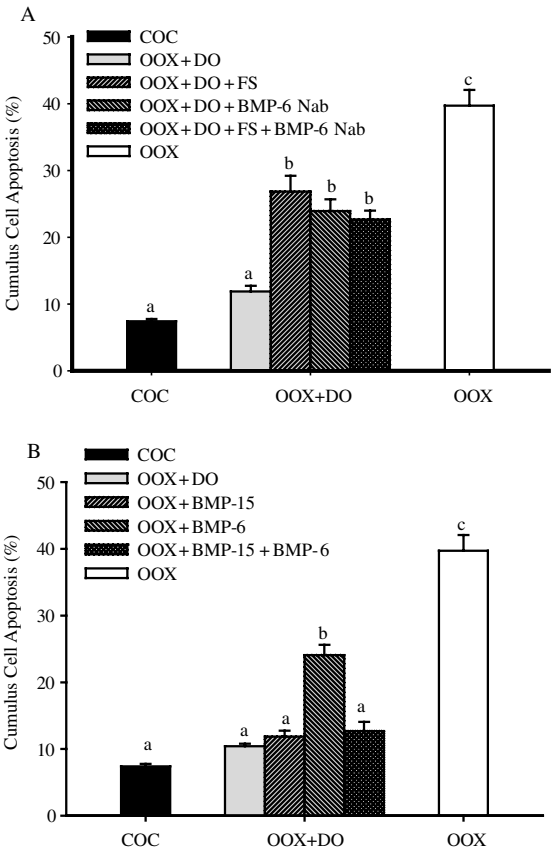

Fig. 8. Role of BMP-15 and BMP-6 in the anti-apoptotic actions of oocytes on cumulus cells. OOX co-cultured with denuded oocyte (25 DOs) were treated with 50 μg/ml follistatin, 20 μg/ml BMP-6 NAb, or a combination of the two (A). Both follistatin and the BMP-6 NAb were effective at partially antagonizing the anti-apoptotic effects of oocytes, however neither completely restored apoptosis to OOX levels, either alone or combined. Co-culturing OOX with DO or treatment with BMP-15 alone or BMP-6 alone decreased cumulus cell apoptosis (B). Combined treatment of OOXs with BMP-6 and BMP-15 did not further decrease apoptosis levels beyond that of BMP-15 alone, suggesting no additive effect of these two BMPs. Bars represent average percentage of apoptotic cumulus cells (mean ±s.e.m.). Values from bars with different labels; [a,b,c]differ significantly (P<0.001).

Experiment 8: role of BMP-15 and BMP-6 in the anti-apoptotic actions of oocytes on cumulus cells

To determine if the anti-apoptotic effects of oocytes on cumulus cells can be attributed to either BMP-15 and/or BMP-6, an attempt was made to neutralize oocyte-secreted factors using follistatin with and without a BMP-6 NAb. Fig. 8A illustrates that OOX co-cultured with oocytes have a reduced incidence of cumulus cell apoptosis compared with OOX alone, which was comparable to the COC control. Either follistatin alone or the BMP-6 NAb alone significantly antagonized ~50% of the anti-apoptotic effects of oocytes on cumulus cells (P<0.001). The effects of follistatin and the BMP-6 NAb were not additive as their combined presence did not further restore apoptosis levels.

The results from Fig. 8A suggested that oocyte-secreted BMP-15 and BMP-6 act redundantly to prevent cumulus cell apoptosis, and as such, should not act in an additive fashion. An experiment was conducted to test this proposal. Co-culturing OOX with denuded oocytes or treatment with BMP-15 alone or BMP-6 alone significantly (P<0.001) decreased cumulus cell apoptosis (Fig. 8B). Combined treatment of OOXs with BMP-6 and BMP-15 did not further decrease apoptosis levels beyond that of BMP-15 alone (P>0.05), suggesting no additive effect of these two BMPs.

Experiment 9: Effect of BMP-7 and its antagonist, gremlin, on cumulus cell apoptosis

This experiment was conducted to determine the effect of adding BMP-7 and its antagonist, gremlin, in the presence of BMP-15, on the regulation of cumulus cell apoptosis. BMP-7 and BMP-15 significantly reduced cumulus cell apoptosis (P<0.001) (Fig. 9). The anti-apoptotic effects of BMP-15 were

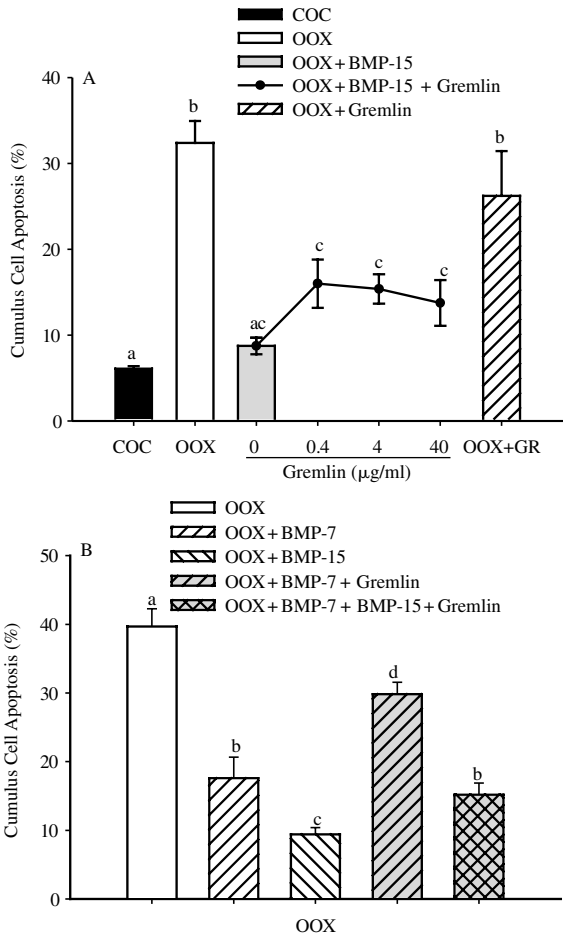

Fig. 9. Effect of BMP-7 and its antagonist gremlin on cumulus cell apoptosis. OOXs were cultured with 10% v/v BMP-15 in the presence of increasing doses of gremlin (0-40 μg/ml) (A). OOX were also co-cultured with 100 ng/ml BMP-7 and/or 10% BMP-15 in the presence or absence of 2 μg/ml gremlin (B). Gremlin did not antagonize the suppressive effect of BMP-15 on cumulus cell apoptosis, whereas it did that of BMP-7. Bars and points represent average percentage of apoptotic cumulus cells (mean ±s.e.m.). Values from bars with different labels; ^{a,b,c}differ significantly (P<0.001).

unaffected by gremlin, including at high doses (Fig. 9A). Conversely, 2 μg/ml gremlin significantly (P<0.001) reversed the inhibitory effect of 100 ng/ml BMP-7, but not when BMP-15 was present (Fig. 9B).

Discussion

The current study was undertaken to test the hypothesis that ovarian cumulus cells exhibit a low incidence of apoptosis due to their close association with oocytes and their exposure to oocyte paracrine factors. This hypothesis was formulated based on the observation that during atresia in antral follicles, the COC is the last compartment of the ovarian follicle to undergo atresia, which is primarily manifested as apoptosis in the mural granulosa cell layer and, at later stages, in the theca cells (Tajima et al., 2002; Yang and Rajamahendran, 2000). The present study demonstrates that removal of the oocyte from the COC by oocytectomy leads to a substantial increase in cumulus cell apoptosis. However, low apoptosis levels can be restored by co-culturing OOX with oocytes, which reduces the incidence of apoptosis in a dose dependent manner, and which is completely restored to COC levels at the maximum concentration of 50 oocytes per well. These findings demonstrate that the low level of cumulus cell apoptosis is largely dependent on the presence of the oocyte. Furthermore, the characteristically low incidence of cumulus cell apoptosis can be specifically attributed to soluble paracrine signals from the oocyte, rather than oocyte gap junctional signalling to cumulus cells, since: (1) the paracrine effects of oocytes were observed in a co-culture environment devoid of direct oocyte-cumulus cell contact; and (2) disrupting oocyte-cumulus cell gap junctional communication by oocytectomy led to a homogenous increase in apoptosis in all cumulus cell layers, not just in the inner layer, which has the highest junctional contact with the oocyte.

The study demonstrates by various means that oocytes actively prevent cumulus cell apoptosis by establishing a morphogenic gradient of oocyte-secreted factors. Firstly, the reduction in cumulus cell apoptosis was assessed by two different methods: we used TUNEL together with quantitative confocal microscopy, and also examined the expression of key proteins regulating apoptosis by western blot. Exposure of OOX to oocytes dramatically induced anti-apoptotic Bcl-2 expression. Conversely, pro-apoptotic Bax expression was high in OOX alone and was notably reduced by oocyte-secreted factors. These results suggest that oocytes prevent apoptosis within cumulus cells by altering the ratio of Bax to Bcl-2 in favour of cell survival. Secondly, the anti-apoptotic actions of oocytes followed a gradient from the site of the oocyte(s). In intact COCs, the incidence of apoptosis was lowest in the inner most layer of cumulus cells and increased with increasing distance from the oocyte. Conversely, in OOX+denuded oocytes, where the oocytes are outside the complex and the OOX is hollow, the outer layer of cumulus cells closest to the oocytes had the lowest level of apoptosis. This is the first direct evidence of a very localised morphogenic gradient of oocyte-secreted factors in the COC, which was proposed some time ago (Eppig, 2001). This gradient of oocyte-secreted factors is likely to play a significant role in the spatial organisation and functional characteristics of cumulus cells within the COC. Thirdly, oocyte-secreted factors were able to protect cumulus cells from an apoptotic insult. Staurosporine induces apoptosis via a cellular signal cascade (to date uncharacterized) as opposed to causing indiscriminate DNA damage (Weil et al., 1996; Yuan et al., 2004). Oocyte-secreted factors prevented apoptosis induced by staurosporine demonstrating that oocytes are able to protect cumulus cells from an apoptosis-inducing event. Together these results demonstrate that oocytes secrete a potent anti-apoptotic factor(s) that acts in a very localised manner.

Supplementation of media with FSH also decreased the incidence of cumulus cell apoptosis in both COCs and OOXs. This is consistent with other studies (Chun et al., 1996; Yang and Rajamahendran, 2000) and with the notion that FSH is an indispensable hormone driving follicular growth and that the primary cause of follicular atresia is inadequate exposure to FSH. It is also noteworthy that the anti-apoptotic effects of FSH were additive, whether in the presence of oocytes, BMP-15 or BMP-6.

Evidence exists, now from many different groups, of the many roles that oocytes play in regulating ovarian follicle development. Oocyte-secreted factors regulate folliculogenesis by modulating a broad range of functions associated with growth and differentiation of granulosa/cumulus cells (reviewed in Eppig, 2001; Gilchrist et al., 2004a). In general, oocyte-secreted factors promote granulosa/cumulus cell proliferation, particularly in synergy with local mitogenic agents such as IGF-I and androgens (Hickey et al., 2005; Li et al., 2000), and simultaneously prevent gonadotrophin-induced differentiation and luteinisation. Adding to this developing model, this study provides the first direct evidence that oocyte-secreted factors also prevent cumulus cell apoptosis. Hence it seems oocytes help stimulate cumulus cell growth by actively preventing differentiation, while simultaneously providing a protective mechanism against cell death and promoting cellular DNA synthesis and proliferation.

Despite the dramatic and critical effects of oocyte-secreted factors on cumulus cell functions, the exact identities of these key oocyte molecules remains largely unknown. To date, the best candidate molecules are members of the TGFβ superfamily, in particular GDF-9 and BMP-15, which can mimic many of the actions of oocytes on granulosa/cumulus cells in vitro (Gilchrist et al., 2004a). It is interesting to note that GDF-9 had no significant effect on the incidence of cumulus cell apoptosis in the present study, despite the fact that GDF-9 is an exceptionally potent granulosa cell mitogen (Gilchrist et al., 2004b; Hayashi et al., 1999; Vitt et al., 2002). Instead, cumulus cell apoptosis was markedly reduced by BMP-15, BMP-6 and BMP-7, which in general are weak mitogens. This study provides multiple lines of evidence that BMP signalling, and not GDF-9 signalling, prevents cumulus cell apoptosis: (1) all three BMPs tested reduced the incidence of cumulus cell apoptosis in a dose-dependent manner; (2) both BMP-6 and BMP-15 protected cumulus cells from apoptosis induced by staurosporine; (3) expression of cumulus cell anti-apoptotic Bcl-2 was stimulated by BMP-15 but not by GDF-9; and in contrast, (4) pro-apoptotic Bax expression was inhibited by BMP-15 but not by GDF-9. These findings support the concept proposed by Oltvai et al. that the ratio of Bcl-2 to Bax determines whether a cell lives or dies (Oltvai et al., 1993), and that BMP-15 and BMP-6 can regulate that ratio.

There are two divergent signalling pathways activated by the TGFβ superfamily; the BMP pathway and the TGFβ/activin pathway. GDF-9 was recently shown to signal through the ALK5 (Mazerbourg et al., 2004) and BMPRII (Vitt et al., 2002) receptors, activating SMAD 2/3 molecules and hence eliciting a TGFβ-like intracellular response (Kaivo-Oja et al., 2003; Roh et al., 2003). Conversely, BMP-15, BMP-6 and BMP-7 have been shown to signal through ALK6 and BMPRII receptors, thereby activating the alternate SMAD 1/5/8 pathway (reviewed by Shimasaki et al., 2004). Hence it seems likely that bovine oocyte-secreted factors stimulate both signalling pathways simultaneously in cumulus cells; activation of the SMAD 1/5/8 pathway by BMP-15 and BMP-6 transmitting the anti-apoptotic actions of the oocyte, and activation of the alternate SMAD 2/3 pathway by GDF-9 conveying the oocyte's mitogenic signal.

A further objective of the current study was to attempt to at least partially identify the native oocyte-secreted factors preventing cumulus cell apoptosis. This is most easily achieved through experimental neutralization of the effects of oocytes on cumulus cells, as actual purification of oocyte-secreted factors has so far proved unfeasible. Several high-affinity binding proteins antagonize BMP actions, including follistatin and gremlin. Follistatin, which is highly expressed by granulosa cells in developing follicles, inhibits the biological activities of activins and BMP-15 by forming inactive complexes (Otsuka et al., 2001; Shimasaki et al., 2004). In the current study, follistatin and a BMP-6 neutralizing antibody were able to antagonize the anti-apoptotic effects of BMP-15 and BMP-6, respectively. Whereas gremlin, which is expressed in granulosa cells and cumulus cells and is a known BMP-2, BMP-4 and BMP-7 antagonist (Sudo et al., 2004), did not antagonize the anti-apoptotic actions of BMP-15.

We next went on to examine the capacity of the BMP antagonists to neutralise the anti-apoptotic effects of the oocyte. Follistatin or the BMP-6 neutralizing antibody alone were able to partially antagonize the anti-apoptotic actions of the oocyte, suggesting that this action by bovine oocytes can be attributed in part to BMP-15 and/or BMP-6. These findings describe an entirely novel function for these oocyte-secreted molecules. BMP-15 and BMP-6 appear to act redundantly to prevent cumulus cell apoptosis. The recombinant proteins did not have an additive effect on apoptosis when added together, and simultaneous neutralization of native oocyte BMP-15 and BMP-6 did not increase the effect of neutralizing either alone. These data provide the first direct evidence that endogenous BMP-15 and BMP-6 oocyte proteins are important anti-apoptotic oocyte-secreted factors, but also that these molecules account for only part of the total anti-apoptotic activity of the oocyte (~50%), the remaining portion of which is yet to be determined. Candidate molecules may include other members of the TGFβ superfamily, however it seems unlikely to include the subfamily of TGFβs and activins, as stimulation of their SMAD 2/3 signalling pathway, as exemplified here by GDF-9, has no effect on cumulus cell apoptosis. A putative oocyte-secreted GDF-9/BMP-15 heterodimer may play some role in regulating cumulus cell apoptosis, however at this stage, little is known of this molecule or of its signalling pathway (Juengel and McNatty, 2005).

BMPR-II is an indispensable receptor for the transmission of the paracrine actions of oocytes to cumulus/granulosa cells, as it is the sole type-II receptor for BMP-15 and GDF-9 and an important receptor for BMP-6 (Shimasaki et al., 2004). Efficacy of oocyte-secreted factors on cumulus cells would be reduced if alternate non-oocyte-secreted factors ligands expressed in the follicle that bind BMPR-II, such as BMP-7, BMP-4 or BMP-2, competed for BMPR-II binding thereby reducing its availability. In the present study, we show that BMP-7 can mimic the action of oocyte-secreted BMP-15 or BMP-6 in preventing cumulus cell apoptosis, even though it is not an oocyte-secreted factor and is only expressed by theca in the follicle (Lee et al., 2001). Gremlin is a BMP-binding protein expressed by granulosa cells in response to GDF-9 and BMP-4 which selectively inhibits certain BMPs without affecting GDF9 (Pangas et al., 2004). Gremlin, which is known to be highly effective at antagonizing BMP-2 and BMP-4 actions (Sudo et al., 2004), neutralized the anti-apoptotic effect of BMP-7 but was ineffective against BMP-15 (present study). As such, the anti-apoptotic actions on cumulus cells of the

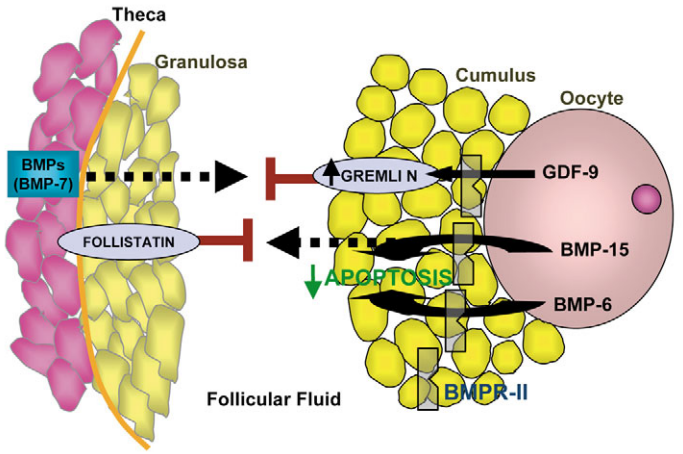

Fig. 10. Proposed model by which the paracrine network of BMP/GDF-9 growth factors and their binding proteins interact to regulate apoptosis in the COC microenvironment. Oocyte-secreted BMP-15 and BMP-6 signal through the cumulus cell receptor BMPR-II to actively prevent cumulus cell death. Oocyte-secreted GDF-9, also acting through BMPR-II but using a different type-I receptor to the BMPs, does not prevent cumulus cell apoptosis but induces cumulus cell gremlin expression. GDF-9-stimulated gremlin expression may in turn block theca and granulosa cell-derived BMPs from competing with BMP-15, BMP-6 and GDF-9 for BMPR-II binding. In addition, follistatin produced by mural granulosa cells and present in follicular fluid, may limit the anti-apoptotic effects of oocyte BMP-15 specifically to the COC microenvironment.

endogenous oocyte-product, BMP-15, were unaffected by the combined presence of BMP-7 and gremlin. Such interactions suggest an additional level of complexity of mechanisms regulating communication between the oocyte and its surrounding cumulus cells.

These results support and extend the model proposed by Pangas et al. whereby gremlin modulates GDF9 and BMP interactions (Pangas et al., 2004), which we propose are crucial for maintenance of the highly specialized COC microenvironment (see model) (Fig. 10). This extended model hypothesizes that oocyte-GDF9 induces cumulus cell gremlin expression, which in turn blocks theca and granulosa cell-derived BMPs from competing with BMP-15 and GDF-9 for BMPR-II binding. In addition, follistatin produced by mural granulosa cells and present in follicular fluid, may limit the anti-apoptotic effects of BMP-15 specifically to the COC. Apoptosis and subsequent atresia of the mural granulosa cell layer may be able to proceed despite the apparent presence of BMP-15 in follicular fluid (McNatty et al., 2004), due to follistatin neutralisation of BMP-15 at this site. This model illustrates our hypothesis that oocytes actively prevent death of cells in their immediate microenvironment only, by establishing a morphogenic gradient of BMP-15 and BMP-6 emanating from the oocyte. This gradient of BMPs is either sufficiently dilute and/or adequately neutralized outside the COC microenvironment, such that mural granulosa cell apoptosis and follicular atresia can proceed whilst the COC remains relatively healthy. We hypothesize that this is the mechanism whereby, in large mammals, the cumulus cells and oocyte are the last cell types to die during advanced follicular atresia.

Collectively, the evidence presented in this paper demonstrates for the first time that oocytes, in particular the oocyte-secreted factors BMP-6 and BMP-15, are responsible for the low incidence of apoptosis within cumulus cells, through the establishment of a paracrine network of BMP growth factors and their binding proteins. Thus prevention of cumulus cell apoptosis can be added to the growing compendium of follicular cell functions regulated by oocytes. This adds further support to the emerging doctrine that oocytes secrete these paracrine factors to establish and maintain an immediate microenvironment, which is distinct from that of the rest of the follicle (Eppig, 2001; Gilchrist et al., 2004a). Specific knowledge of GDF-9 and BMP interactions, and about the spatial and temporal regulation of their native antagonists is required, as regulation of this oocyte-cumulus cell communication network will have physiological implications for oocyte growth and development, oocyte maturation, ovulation and developmental competence of the ensuing embryo.

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