Transcription factor p53 can regulate proliferation, apoptosis and secretory activity of luteinizing porcine ovarian granulosa cell cultured with and without ghrelin and FSH

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Abstract

The aim of our *in vitro* experiments was to examine the role of transcription factor p53 in controlling the basic functions of ovarian cells and their response to hormonal treatments. Porcine ovarian granulosa cells, transfected and non-transfected with a gene construct encoding p53, were cultured with ghrelin and FSH (all at concentrations of 0, 1, 10, or 100 ng/ml). Accumulation of p53, of apoptosis-related (MAP3K5) and proliferation-related (cyclin B1) substances was evaluated by immunocytochemistry. The secretion of progesterone (P₄), oxytocin (OT), prostaglandin F (PGF), and E (PGE) was measured by RIA. Transfection with the p53 gene construct promoted accumulation of this transcription factor within cells. It also stimulated the expression of a marker of apoptosis (MAP3K5). Over-expression of p53 resulted in reduced accumulation of a marker of proliferation (cyclin B1), P₄, and PGF secretion and increased OT and PGE secretion. Ghrelin, when added alone, did not affect p53 or P₄, but reduced MAP3K5 and increased PGF and PGE secretion. Over-expression of p53 reversed the effect of ghrelin on OT, caused it to be inhibitory to P₄ secretion, but did not modify its action on MAP3K5, PGF, or PGE. FSH promoted the accumulation of p53, MAP3K5, and cyclin B1; these effects were unaffected by p53 transfection. These multiple effects of the p53 gene construct on luteinizing granulosa cells, cultured with and without hormones 1) demonstrate the effects of ghrelin and FSH on porcine ovarian cell apoptosis and secretory activity, 2) confirm the involvement of p53 in promoting apoptosis and inhibiting P₄ secretion in these cells, 3) provide the first evidence that p53 suppress proliferation of ovarian cells, 4) provide the first evidence that p53 is involved in the control of ovarian peptide hormone (OT) and prostaglandin (PGF and PGE) secretion, and 5) suggest that p53 can modulate, but probably not mediate, the effects of ghrelin and FSH on the ovary.

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Introduction

Transcription factor p53 has been shown to be an important inducer of apoptosis and blocker of proliferation in different cell types. Under stress conditions, it induces G1/S cell-cycle arrest (through transcription factors p21 and c-MYC) and G2/M cycle arrests (through cyclin B and CDC2 kinase; Brown *et al.* 2007). It can activate mitochondrial caspases, which cleave MAP kinases (activators of CDC2 kinases and cell cycle at G2/M phase; Marchetti *et al.* 2004) and induce apoptosis-related events (Braithwaite *et al.* 2006, Chowdhury *et al.* 2006). When concerned with the role of p53 in controlling secretory activity, it is able to suppress growth factor secretion (VEGF, Hassan *et al.* 2006) and insulin-like growth factor binding proteins (Grimberg *et al.* 2006), and to promote vasopressin and catecholamine secretion (Chernigovskaya *et al.* 2005).

The presence of p53 was demonstrated in mammalian (Chaffin *et al.* 2003, Herr *et al.* 2004, Hussein *et al.* 2006, Mészáros *et al.* 2007) and avian (Sirotkin *et al.* 2006, Sirotkin & Grossmann 2007b) ovarian cells. In porcine (Hussein *et al.* 2006) and avian (Sirotkin & Grossmann 2007b) ovarian cells, its expression was associated with apoptosis and atresia of ovarian follicles indicating involvement of p53 in the control of these processes. Indeed, in rat and macaque ovarian granulosa cells, it promoted apoptosis and basal progesterone (P₄) and pregnenolone secretion (Amsterdam *et al.* 2003, Cherian-Shaw *et al.* 2004), but not proliferation (Cherian-Shaw *et al.* 2004). Therefore, involvement of p53 might be proposed in the control of ovarian follicle luteinization, atresia, and remodeling. Nevertheless, evidence for the role of p53 in controlling ovarian function is limited to its ability to promote granulosa cell

apoptosis and steroidogenesis. Its involvement in regulating ovarian cell proliferation and secretion of non-steroidal hormones (peptides, prostaglandins) has not yet been studied.

There is evidence that p53 not only regulates basal ovarian function directly but also mediates the effects of hormones. p53 accumulation in ovarian granulosa cells can be induced by glucocorticoids (women; Amsterdam *et al.* 2003), gonadotropins (women; Chaffin *et al.* 2003, Herr *et al.* 2004), leptin (pig; Dineva *et al.* 2007), ghrelin (chicken; Sirotkin *et al.* 2006), and obestatin (pig; Mészáros *et al.* 2007), but inhibited by leptin (chicken; Sirotkin & Grossmann 2007b). Moreover, blockade of p53 prevented hCG-induced progesterone, but not estradiol, secretion by human granulosa cells (Cherian-Shaw *et al.* 2004). It remains unknown whether gonadotropin and ghrelin affect ovarian function through an increase in p53 accumulation. Such a mediating role for p53 would be demonstrated by concomitant hormonal stimulation of p53 and the promotion of hormonal action.

The aim of the present study was to examine the role of p53 in controlling apoptosis and secretory activity of luteinizing porcine ovarian cells cultured with and without peptide hormones. In our experiments, we examined the effects of the well-known (Wathes 1989, Sirotkin *et al.* 1998, 2001, Schams *et al.* 1999, Jiang *et al.* 2003, Maillat *et al.* 2005) gonadotropin follicle-stimulating hormone (FSH) and of the newly discovered hormone ghrelin, whose involvement in the control of avian (Sirotkin *et al.* 2006, Sirotkin & Grossmann 2007a, 2007b) and mammalian (Sirotkin *et al.* 2007, 2008, Tena-Sempere 2008) reproduction has been recently demonstrated. We have also investigated whether p53 may mediate the action of these hormones in the ovary. Using granulosa cells, we have studied a) the influence of transfection with the p53 gene construct encoding p53 on cell apoptosis (accumulation of MAP3K5), proliferation (accumulation of cyclin B1), and secretory activity (secretion of P₄, oxytocin (OT), prostaglandin F (PGF), and E (PGE)), b) the effect of the hormones ghrelin and FSH on p53 accumulation and on selected parameters listed above, and c) the ability of the p53 gene construct to mimic or modify the effects of hormones. Two independent series of experiments were carried out. The main purpose of these experiments was to understand the role of p53, but not of hormones, in control of basic ovarian functions. Therefore, to improve the reliability of the data concerning p53 effects, we have analyzed the samples obtained in different experiments to measure the effect of p53 (but not of hormones) on all possible parameters. The following series of experiments was performed: 1) investigation of the effect of ghrelin and p53 transfection on the expression of p53, MAP3K5, and the secretion of P₄, OT, PGF, and PGE and 2) the investigation of the effect of FSH and p53 transfection on the expression of p53, MAP3K5, and cyclin B1.

Results

Ovarian cells after culture contained markers of apoptosis and proliferation, p53, and secreted the assayed hormones. In addition, after transfection, 40–55% of granulosa cells produced enhanced green fluorescent protein (EGFP), indicative of successful cell transgenesis (Fig. 1).

Series 1: influence of ghrelin on expression of p53 and MAP3K5 and secretion of hormones by cultured porcine granulosa cells transfected or non-transfected with gene construct encoding p53

Immunocytochemical analysis confirmed the presence of p53 in cultured cells (Fig. 2a) and demonstrated the presence of MAP3K5 (Fig. 2b). p53 was located mainly in the nuclei, while MAP3K5 was found mainly in cytoplasm. RIA confirmed the secretion of P₄, OT, PGF, and PGE by these cells (Fig. 3).

Over-expression of p53 in transfected cells was confirmed by immunocytochemistry. Ghrelin did not affect the proportion of p53-positive control cells but decreased it in transfected cells (at all concentrations added; Fig. 2a).

Over-expression of p53 was associated with an increased proportion of cells containing MAP3K5. Ghrelin reduced this index in both control cells (at concentration 100 ng/ml) and transfected cells (at concentrations 10 and 100 ng/ml; Fig. 2b).

Transfection of cells decreased the secretion of P₄ secretion. Ghrelin did not affect this index in control cells, but inhibited P₄ secretion by transfected cells at concentrations 1 or 10 ng/ml (Fig. 3a). Transfection induced an increase in OT secretion. Ghrelin (at 10 and

Figure 1 Presence of enhanced green fluorescent protein (EGFP; green fluorescence) as a marker of successful transfection in cultured porcine ovarian granulosa cells. Magnification, 800×. Fluorescence microscopy.

Figure 2 Influence of ghrelin on expression of p53 (a) and MAP3K5 (b) in cultured porcine granulosa cells transfected or non-transfected with gene construct encoding p53. Data from immunocytochemistry; a) significant ($P < 0.05$) difference between hormone-treated (1, 10 or 100 ng/ml) and control (0 ng/ml) cells; b) a significant ($P < 0.05$) difference between corresponding groups of transfected and non-transfected cells.

100 ng/ml) significantly increased OT output in control cells, but decreased it (at all concentrations) in transfected cells (Fig. 3b).

Over-expression of p53 induced a substantial decrease in PGF secretion. Ghrelin stimulated PGF secretion by control (all concentrations) and transfected (10 and 100 ng/ml) cells, although a significant reduction of PGF secretion by ghrelin treated to transfected cells (1 ng/ml) in comparison with control was observed (Fig. 3c).

PGE secretion was increased in transfected cells. Ghrelin promoted PGE secretion in both control (10 or 100 ng/ml) and transfected (1 or 10 ng/ml) cells (Fig. 3d).

Series 2: influence of FSH on expression of p53, MAP3K5, and cyclin B1 in cultured porcine granulosa cells transfected or non-transfected with gene construct encoding p53

This series of experiments confirmed the presence of p53, MAP3K5, and cyclin B1 in cultured cells (Fig. 4). As in the previous experiments, after transfection there was

Figure 3 Influence of ghrelin on progesterone (a), oxytocin (b), prostaglandin F (c), and prostaglandin E (d) secretion by cultured porcine granulosa cells transfected or non-transfected with gene construct encoding p53. RIA data. Legends as in Fig. 2.

Figure 4 Influence of FSH on expression of p53 (a), MAP3K5 (b), and cyclin B1 (c) in cultured porcine granulosa cells transfected or non-transfected with gene construct encoding p53. Data from immunocytochemistry. Legends as in Fig. 2.

an increase in the percentage of cells containing p53. FSH increased the proportion of these cells in both control (all concentrations) and experimental groups (10 and 100 ng/ml), although in transfected cells FSH addition at dose 1 ng/ml resulted in some decrease in the proportion of p53-positive cells (Fig. 4a).

As in previous experiments, over-expression of p53 induced an increase in the proportion of cells containing apoptosis-associated MAP3K5. FSH enhanced this

parameter in control and transfected cells at all concentrations added (Fig. 4b). Transfection also reduced the occurrence of cyclin B1. FSH increased the expression of this marker in control and transfected cells at all concentrations used (Fig. 4c).

Discussion

Immunocytochemistry demonstrated a dramatic increase in accumulation of p53 in cultured luteinizing ovarian granulosa cells transfected with the gene construct encoding p53, indicating the over-expression of this transcription factor in these cells. Comparison of transfected and non-transfected cells cultured without hormones suggest that over-expression of p53 is associated with increased apoptosis (expression of apoptosis-related substance MAP3K5). Our data confirm the role of p53 as the promoter of apoptosis, previously shown in non-ovarian (Braithwaite *et al.* 2006, Chowdhury *et al.* 2006) and ovarian (Amsterdam *et al.* 2003, Cherian-Shaw *et al.* 2004, Hussein *et al.* 2006, Sirotkin & Grossmann 2007b) cells. These data suggest the involvement of p53 in the promotion of ovarian apoptosis and apoptosis-related atresia and the selection of ovarian follicles, although this hypothesis should be confirmed by direct measurement of apoptosis in atretic follicles.

The inhibitory influence of p53 over-expression on the accumulation of cyclin B1 observed in our experiments is the first demonstration of an anti-proliferative role for this transcription factor in the ovary. Previously, the anti-proliferative action of p53 was described only in non-ovarian cells (Marchetti *et al.* 2004, Brown *et al.* 2007), and it was not observed in macaque ovarian cells (Cherian-Shaw *et al.* 2004). The ability of p53 to suppress cyclin B1 (marker and promoter of G2 phase of mitosis) suggests that p53 can block the ovarian cell cycle at this checkpoint. These observations suggest the involvement of p53 in the inhibition of ovarian cell proliferation and the resulting growth of ovarian follicles, although this suggestion needs to be confirmed by direct measurement of cell numbers in growing follicles.

The inhibitory effect of p53 on P_4 and PGF secretion and its stimulatory influence on OT and PGE output demonstrates that p53 can be a potent regulator of ovarian secretory activity. This is the first evidence for its involvement in ovarian peptide hormone and prostaglandin secretion and it corresponds with a previous report (Amsterdam *et al.* 2003) on p53 involvement in rat ovarian steroidogenesis, although there it stimulated rather than inhibited the progestagen secretion. The variety of p53 effects on ovarian steroidogenesis observed in different laboratories could be due to the different experimental models used (animal species and the state of ovarian follicle). Nevertheless, the action of p53 on ovarian P_4 and PGF suggest its involvement in control of ovarian cell luteinization, where P_4 and PGF are important markers and regulators (Wathes 1989, Kotwica & Skarzynski 1993,

Findlay 1994, Schams *et al.* 1999). It is possible that p53 action could be mediated by OT, growth factors, and growth factor-binding proteins, which were shown to control ovarian cell proliferation, apoptosis, and secretory activity (Wathes 1989, Sirotkin *et al.* 1998, 2001, Schams *et al.* 1999) and which could be influenced by p53 in both non-ovarian (Grimberg *et al.* 2006, Hassan *et al.* 2006) and ovarian (Fig. 3b) cells. Taken together, the observed p53-induced promotion of apoptosis, suppression of proliferation, and secretion of hormones – markers and regulators of granulosa cell luteinization – suggest that in physiological conditions this transcription factor can inhibit development of ovarian follicle and/or ovarian cell luteinization and formation of the corpus luteum. This hypothesis requires further experimental confirmation.

The present observations represent the first evidence for involvement of ghrelin in control of porcine ovarian cell functions (proliferation, apoptosis, and hormone release). Inhibitory action of ghrelin on the expression of MAP3K5 suggests possible anti-apoptotic action of ghrelin in porcine ovary. The stimulatory influence of ghrelin on porcine ovarian cell OT, PGF, and PGE, but not P₄, release demonstrates its role as a promoter of ovarian secretory activity. It is possible that the ability of ghrelin to suppress apoptotic markers and PGs is due to its action on OT, whose involvement in the control of PGF, PGE, and anti-apoptotic insulin-like growth factor-1 release by porcine ovarian cells has been demonstrated previously (Sirotkin *et al.* 1998, 2001). They are in line with reports on direct ghrelin action on chicken (Sirotkin *et al.* 2006, Sirotkin & Grossmann 2007a) and rabbit (Sirotkin *et al.* 2008) ovarian cells. The effects of FSH presented here correspond with the ability of FSH to promote both proliferation, apoptosis, luteinization, and the release of peptide and steroid hormones in ovarian cells reported previously (Wathes 1989, Sirotkin *et al.* 1998, 2001, Schams *et al.* 1999, Jiang *et al.* 2003, Mailliet *et al.* 2005). Their pro-apoptotic and pro-proliferative effects suggest the important role of FSH in the promotion of ovarian follicular waves, including the promotion of follicular growth, differentiation, and atresia, as well as in ovarian luteogenesis demonstrated previously (Findlay 1994, Jiang *et al.* 2003). The fine interrelationships between different hormones in control of these ovarian parameters remain for further studies, but differences in the dose–response curves of hormones and pattern of their effects indirectly suggest different physiological roles, as well as the existence of multiple receptors and/or second messenger systems mediating the effects of ghrelin and FSH on the ovary.

It remains unknown whether p53 can mediate the effects of these hormones on ovarian cells. As postulated above, p53 could be considered as a mediator of hormone action if 1) the hormone affects p53 and 2) p53 mimics or modifies the effect of the hormone. Regarding the first requirement, previous studies have

demonstrated the stimulatory action of gonadotropin (women; Chaffin *et al.* 2003, Herr *et al.* 2004), leptin (pig; Dineva *et al.* 2007), ghrelin (chicken; Sirotkin *et al.* 2006), and obestatin (pig; Mészárosóvá *et al.* 2007) on p53 accumulation in ovarian cells in different species, although in some cases it was inhibited by leptin (chicken; Sirotkin & Grossmann 2007b). A stimulatory effect of gonadotropin, but not of ghrelin, was observed in our experiments as well, although the present methods do not definitely show whether the hormone affected the number of cells containing p53 or p53 accumulation in each cell. On the other hand, ghrelin, in contrast to FSH, reduced over-expression of p53 in transfected cells. This could suggest potential direct or feedback inhibitory influence of ghrelin on p53, expressed in conditions of p53 overproduction. Concerning the second requirement, the effects of p53 and a hormone have not previously been studied in the same experiment, although the ability of a p53 blocker to prevent gonadotropin function has been described (Cherian-Shaw *et al.* 2004). In our experiments, p53 had some similar effects to those of ghrelin (on OT, and PGE, but not on MAP3K5, P₄, and PGF) and FSH (on MAP3K5, but not on cyclin B1). Furthermore, p53 was able to promote, suppress, and invert some effects of ghrelin (on MAP3K5, P₄, OT, but not on PGF and PGE). This fact can indirectly indicate that some effects of ghrelin could be mediated by p53. Over-expression of p53 did not substantially modify FSH action on MAP3K5 and cyclin B1). On the other hand, FSH treatment increased both p53 and MAP3K5 in both control and p53-transfected cells. Thus, p53 could be the mediator of FSH stimulation of MAP3K5 expression in cells. In contrast, the opposing effects of p53 and FSH on cyclin B1 expression seem to be independent, and p53 expression does not seem to interfere with the ability of FSH to induce cyclin B1 expression.

Evaluation of these results according to the two criteria listed above shows that none of the hormones tested meets all the requirements for describing p53 as mediator in all aspects of ovarian function. Therefore, the present data do not provide decisive evidence for a mediator role of p53 in the actions of ghrelin and FSH. Probably, such evidence could provide studies of the consequences of p53 deficiency *in vivo* or *in vitro*. Nevertheless, the ability of p53 to modify ghrelin action observed in our experiments suggest the functional interrelationships between this transcription factor and metabolic hormone in control of ovarian cell apoptosis, proliferation, secretory activity, and related events (follicular growth, differentiation, selection, luteinization, and atresia).

Taken together, these observations are an effect of the p53 gene construct on nine parameters of luteinizing granulosa cell activity, in the presence and absence of hormones to 1) demonstrate the influence of ghrelin and FSH on porcine ovarian cell proliferation, apoptosis, and

secretory activity, 2) provide the first demonstration that p53 suppresses ovarian cell cycle, 3) confirm the involvement of p53 in promoting apoptosis and in control of P₄ secretion by these cells, 4) provide the first evidence that p53 may control ovarian peptide hormone (OT) and prostaglandin (PGF and PGE) secretion, and 5) suggest that p53 is a modulator, but probably not a mediator, of ghrelin and FSH action on the ovary.

Materials and Methods

Preparation, culture, and processing of ovarian cells

Ovaries of non-cycling pubertal gilts, about 180 days of age, were obtained after slaughter at a local abattoir. They were washed several times (5 s each) in sterile 0.9% NaCl and 95% alcohol. Granulosa cells were aspirated by syringe and sterile needle from follicles 3–5 mm in diameter, suspended in Dulbecco's modified Eagle's medium (DMEM)/F-12 1:1 + 2% fetal calf serum (all from Sigma), and washed twice by centrifugation at 200 *g* for 10 min and resuspended in DMEM/F-12 + 2% FCS. Three kinds of plasmids were subcloned from plasmid pcDNA3/pc53-SN3 and used for transfection of granulosa cells: an expression vector for p53 construct containing an insertion for resistance to ampicillin (University of Dundee, Dundee, UK), a 'scramble' control plasmid vector without p53 insertion (University of Dundee), and a marker reporter plasmid pEGFP-N1 for EGFP and resistance to kanamycin (Clontech). The wild-type pc53-SN3 construct containing a 1.8 kb insert of 13 copies of p53 RE 5'-CCTGCCTGGACTTGCCTGG-3' was as described previously (Baker *et al.* 1990). These plasmids were multiplied by the following procedures: bacteria *Escherichia coli* stem DH5 α were cultured in LB medium consisting of 1 l H₂O, 10 g tryptone, 5 g yeast autolysate, and 10 g NaCl; pH 7.0 with NaOH; 1 ml of LB medium was inoculated with one colony of *E. coli* stem DH5 α and incubated at 37 °C, 200 *g*; 10 ml of LB medium was inoculated with 100 μ l night bacterial culture and incubated for 3 h at 37 °C, 200 *g*. The cell suspension was cooled for 10 min and centrifuged at 200 *g* for 10 min at 4 °C. Cells were resuspended in 10 ml ice-cold 100 mM CaCl₂. The cell suspension was centrifuged at 200 *g* for 10 min at 4 °C. Sedimented cells were resuspended in 2 ml ice-cold 100 mM CaCl₂. Aliquots (200 μ l) of competent cells resuspended in cold

Eppendorf tubes (Greiner bio-one, Longwood, FL, USA) were placed in a refrigerator at 4 °C.

To 10 aliquots (200 μ l) of competent cells in each tube, 50 ng DNA (10 μ l) was added to be cloned, mixed, and incubated on ice for 30 min. Tubes were preheated in a water bath at 42 °C for 90 s and quickly cooled in ice for 1–2 min. Thereafter, 0.8 ml SOC medium was added to each tube and incubated for 45 min at 37 °C. A proportionate quantity of bacteria was inoculated in selective LB medium with agar (15 g of agar in 1 l LB medium) in a Petri dish (Gama, a. s. České Budějovice, Czech Republic) and incubated at 37 °C for 16 h. Five milliliters of LB medium with ampicillin (Gibco BRL) for p53-related plasmids and kanamycin (Gibco) for EGFP-N1 was inoculated with one colony of transformed cells and incubated overnight at 37 °C and 200 *g*. The cell suspension was centrifuged at 200 *g* at 4 °C for 10 min. Recombinant plasmids were isolated using a NucleoSpin Plasmid kit (Macherey Nagel, Düren, Germany) according to the manufacturer's instructions.

An experimental group of cells was transfected with a gene construct encoding EGFP and a gene construct for transcription factor p53. A control group of granulosa cells was transfected by a gene construct encoding EGFP and the same, but insertless plasmid vector. Transfection was performed using transfection reagent Roti Fect (Carl Roth, Karlsruhe, Germany) according to the manufacturer's instructions.

After transfection, granulosa cells (1 $\times$ 10⁶ cells/ml) were cultured in DMEM/F-12 supplemented with 10% calf fetal serum and 1% antibiotic-antimycotic solution (all from Sigma) in Falcon 24-well plates (Becton Dickinson, Lincoln Park, NJ, USA), 2 ml medium per well, and in chamber slides (Nunc Inc., Naperville, TN, USA), 200 μ l medium per well, at 38 °C and 5% CO₂ in humidified air. After 2 d pre-culture, the medium was replaced with medium of the same composition. In addition, cells transfected either with gene construct encoding EGFP + construct without p53 (control) and with that encoding EGFP + p53 were treated with 0, 1, 10, or 100 ng/ml of human recombinant ghrelin (Peptides International Inc., Louisville, KY, USA) and porcine FSH (NHPP). All hormones were of biological grade. They were dissolved in culture medium immediately before experiment. After 2 d of culture, the medium from 24-well plates was aspirated and frozen at –18 °C to await RIA. Luteinizing cells in chamber slides were washed in 700 μ l well ice-cold PBS (5 min), fixed in paraformaldehyde (4% in PBS, pH 7.2–7.4), and held at 4 °C to await immunocytochemistry.

Table 1 Characteristics of immunoassays used in experiments.

Substance assayed	Specificity of assay (cross-reactivity of antiserum)	Sensitivity of assay (ng/ml)	Coefficient of variation	
			Intra-assay	Inter-assay
Progesterone	<0.001% to cortisol, corticosterone, cortisol, androstenediol, pregnenolone, estradiol, testosterone	0.12	13.1	8.0
PGF	<0.01% to PGA-1, PGA-2, PGB-1, PGB-2, <0.1% to PGE-1, PGE-2, 66% to PGF-1, 100% to PGF-2	0.003	10.5	5.6
PGE	<28.0% to PGA-1, <7.0% to PGA-2, <0.6% to PGB-1, <1.4% to PGB-2, <5.0% to PGF-1, <1.5% to PGF-2, 165% to PGE-1, 100% to PGE-2	0.015	7.5	4.0
Oxytocin	<0.01% to arginine-vasopressin, lysine-vasopressin, arginine-vasotocin, ghrelin, leptin and FSH	0.12	13.1	8.0

At this time, after finishing the culture, cell numbers and viability were determined by trypan blue staining and counting in a hemocytometer. Viability was 70–80%. No statistically significant differences in these indices between control and experimental groups were observed.

Immunoassays

Concentrations of P₄, OT, PGF, and PGE were determined by RIA in 25–100 µl samples of incubation medium. P₄ was assayed using RIA/IRMA kits from DSL (Webster, MA, USA) according to the manufacturer's instructions. OT, PGE-2, and PGF metabolites (PGFM) were assayed by our own RIA/EIA systems (Kotwica & Skarzynski 1993, Skarzynski *et al.* 1999, Duras *et al.* 2005) respectively. Determination of PGFM in medium reliably reflects PGF-2a secretion ($r=0.92$; $P<0.001$) from cultured cells (Skarzynski *et al.* 1999). The characteristics of these assays are presented in Table 1.

Immunocytochemical analysis

The presence of p53, MAP3K5, and cyclin B1 was demonstrated by immunocytochemistry (Osborn & Isenberg 1994) using primary mouse monoclonal antibodies against these substances (Chemicon, San Diego, CA, USA; dilution 1:500) cross-reacted with corresponding antigens of human, rat, mouse, and yeast origin. Secondary porcine polyclonal antibodies against mouse IgGs, labeled with horseradish peroxidase (Sevac, Prague, Czech Republic; dilution 1:1000) and 3,3' diaminobenzidine (DAB) reagent (Boehringer Mannheim GmbH) were used for the visualization of primary antibody. The presence of immunoreactivity in the cells was determined by one observer by using light microscopy. The percentage of cells containing visible antigen was counted. Cells processed without the primary antibody were used as negative control. The presence of cells containing EGFP was detected using a fluorescence microscope.

Statistical analysis

Each series of experiments was performed thrice. The data shown are the means of values obtained in these three separate experiments performed on separate days with separate groups of granulosa cells, each obtained from 15–20 animals.

RIA

Each experimental group was represented by four culture wells, i.e., each value represents means of 4 wells × 3 experiments = 12 replicates. Assays of hormone concentration in the incubation medium were performed in duplicates. The values of blank controls (serum-supplemented medium incubated without cells) were subtracted from the specific values determined by RIA in cell-conditioned medium to exclude any non-specific background (less than 10% of total values). Rates of secretion were calculated per 10⁶ viable cells/day.

Immunocytochemistry

In each chamber (three per group), 1000 cells were scored, i.e., each value representing the means of nine replicates (9000

cells in total). The percentage of cells containing antigen in different groups of cells was calculated.

Significant differences between the experiments were evaluated using two-way ANOVA. When the effects of treatments were revealed, data from the experimental and control groups were compared by Student's *t*-test using Sigma Plot 9.0 software (Systat Software, GmbH, Erkrhart, Germany). Differences from control at $P<0.05$ were considered as 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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