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The mTOR System Can Affect Basic Ovarian Cell Functions and Mediate the Effect of Ovarian Hormonal Regulators

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ABSTRACT

Both reproductive hormones and the mammalian target of rapamycin (mTOR) intracellular signalling system, including mTOR complex 1 (mTORC1), mTOR complex 2 (mTORC2) and its key enzyme sirtuin 1 (SIRT 1) are involved in the control of ovarian processes but the interrelationship between hormones and mTOR has been studied insufficiently. The aim of our *in vitro* experiments was to elucidate the involvement of mTOR in the control of basic ovarian cell functions and in mediating the action of upstream hormonal stimulators. In the first series of experiments, we examined the effect of the known hormonal regulators of ovarian functions, Follicle-Stimulating Hormone (FSH), oxytocin (OT) and insulin-like growth factor I (IGF-I) (all at 0, 1, 10 and 100 ng mL⁻¹ doses), on the accumulation of SIRT1 in porcine ovarian granulosa cells. In the second series of experiments, we examined the effects of mTOR blockers, PF 046 (an inhibitor of mTORC1) and WYE 687 (inhibitor of both mTORC1 and mTORC2) (both at a dose of 1 µg mL⁻¹) on both basal and FSH-induced (0, 1, 10 and 100 ng FSH mL⁻¹ doses) basic ovarian functions (proliferation, apoptosis and steroidogenesis) of cultured porcine granulosa cells. The accumulation of SIRT1, PCNA (a proliferation-related peptide) and Bax (an apoptosis-related peptide) was detected by immunocytochemistry. The release of progesterone (P4) and testosterone (T) was analysed by EIA. It was observed that either FSH or OT additions increased the SIRT1 accumulation in ovarian cells, whilst IGF-I addition decreased it. The PF 046, when given alone, inhibited ovarian cell proliferation but did not affect apoptosis or the release of P4 and T. The WYE 687, when added alone did not affect proliferation and apoptosis but inhibited the P4 and T release by ovarian cells. The FSH, when given alone, stimulated proliferation did not affect apoptosis and increased the release of both P4 and T. In the presence of PF 046, FSH did not significantly alter proliferation, induced apoptosis and suppressed the P4 and T release, i.e., this inhibitor of mTORC1 prevented the proliferation-stimulating effect induced by the pro-apoptotic action of FSH and inverted the stimulatory action of FSH on steroidogenesis. The WYE 687 prevented the effect of FSH on both proliferation and apoptosis, promoted the FSH-induced P4 release but did not affect the FSH action on the T output, i.e., the inhibition of both mTORC1 and mTORC2 inverted the FSH action on ovarian cell proliferation and apoptosis but not the action on steroidogenesis. Our observations suggest the following: (1) mTORC1 can be involved in the up-regulation of ovarian cell proliferation but not that of apoptosis and steroidogenesis (2) mTORC2 can be involved in the up-regulation of the release of both P4 and T but not the up-regulation of ovarian cell proliferation and apoptosis, (3) FSH is involved in the stimulation of ovarian cell proliferation and P4 and T release and (4) hormones (FSH, OT and IGF-I) can regulate ovarian mTOR and the ability of mTOR to modify FSH effects suggests that mTOR can mediate the actions of hormone on ovarian cells. It is hypothesized that both mTORC1 and mTORC2 may be mediators of the FSH action on ovarian cell proliferation, apoptosis and steroidogenesis.

Key words: mTOR blocker, sirtuin, FSH, ovary, proliferation, apoptosis, steroid hormone

INTRODUCTION

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that regulates cell growth, metabolism and proliferation. It is presented in two distinct multiprotein complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Dowling *et al.*, 2010; Betz and Hall, 2013; Wu and Liu, 2013). Sirtuin 1 (SIRT1) a key enzyme in the mTOR signalling system is a member of the NAD[±]-dependent deacetylase family (Frye, 2000; Jin *et al.*, 2009) and a down-regulator of mTOR activity (Ghosh *et al.*, 2010; Pinton *et al.*, 2013). In non-ovarian cells, SIRT1 regulates metabolism, the cell cycle, cell differentiation, apoptosis and aging (Kwon and Ott, 2008; Song and Surh, 2012).

Some recent studies demonstrated the involvement of the mTOR signalling system in the control of various ovarian functions (Makker *et al.*, 2014). Inhibited proliferation occurred after the administration of rapamycin and everolimus (mTORC1 inhibitors) to healthy mice granulosa cells (Yaba *et al.*, 2008; Yu *et al.*, 2011) and mouse ovarian granulosa cell tumours (Rico *et al.*, 2012), after the addition of NVP-BEZ235 and DS-7423 (inhibitors of PI3K and both mTORC1 and mTORC2) to human ovarian cancer cells (Santiskulvong *et al.*, 2011; Kashiyama *et al.*, 2014) and after the addition of PF 046 (inhibitor of mTORC1) to human ovarian cancer cells (Yuan *et al.*, 2011). Furthermore, treatment with NVP-BEZ235 and DS-7423 increased the apoptosis of human ovarian cancer cells (Santiskulvong *et al.*, 2011; Kashiyama *et al.*, 2014) but the administration of rapamycin, everolimus and PF 046 did not affect the apoptosis of healthy mouse granulosa cells (Yu *et al.*, 2011), mouse ovarian granulosa cell tumours (Rico *et al.*, 2012) and human ovarian cancer cells (Yuan *et al.*, 2011), respectively. Furthermore, rapamycin treatment suppressed the follicular growth and maturation in rodents (Yaba *et al.*, 2008; Zhang *et al.*, 2013) and increased the risk of oligomenorrhea in humans (Braun *et al.*, 2012). Rapamycin did not affect the progesterone (P4) release by bovine corpus luteum cells (Hou *et al.*, 2010) and other investigations of the involvement of mTOR in the control of ovarian secretory activity have not been reported yet.

Sirtuin (SIRT1) can be an important regulator and/or effector of mTOR in the ovary. Rapamycin treatment inhibited the mTOR expression and stimulated the expression of SIRT1 in rat ovaries in vivo (Zhang *et al.*, 2013), supporting the previous observation by Ghosh *et al.* (2010) and Pinton *et al.* (2013) in non-ovarian cells about the negative interrelationship between mTOR and SIRT1. SIRT1 might control reproductive processes through the induction of GnRH expression, the release of LH (Kolthur-Seetharam *et al.*, 2009), the induction of LH receptors (Morita *et al.*, 2012) and direct action on ovarian cell functions. For example, SIRT1 inhibition by sirtinol resulted in the reduced proliferation of a human ovarian granulosa-like tumour cell line (Benayoun *et al.*,

2011). Transfection with a SIRT1 gene construct stimulated or inhibited proliferation, did not affect apoptosis and stimulated the P4 and IGF-1 release by porcine granulosa cells (Pavlova *et al.*, 2013; Sirotkin *et al.*, 2014). Taken together, these data suggest that mTOR/SIRT1 signalling could play an important role in the regulation of ovarian cell proliferation, apoptosis and the resulting ovarian follicular growth, development and atresia. The involvement of this system in the control of ovarian steroidogenesis remains to be examined.

Follicle-Stimulating Hormone (FSH) and other hormones, including oxytocin (OT) and Insulin-like Growth Factor-I (IGF-I) are the best known regulators of ovarian functions. Their effect on ovarian cells could be mediated via various intracellular signalling molecules (Sirotkin, 2011). However, there is insufficient evidence for the role of mTOR/SIRT1 in mediating the action of hormones on ovarian cells. Treatment with FSH resulted in the increased phosphorylation of mTOR (Chen *et al.*, 2007) and its downstream target p70S6K (Alam *et al.*, 2004; Kayampilly and Menon, 2007) in rat granulosa cells. Furthermore, FSH promoted SIRT1 accumulation in porcine ovarian cells (Pavlova *et al.*, 2013). Rapamycin treatment suppressed FSH-stimulated rat granulosa cell proliferation (Kayampilly and Menon, 2007) but not the FSH-stimulated P4 release by rat ovarian granulosa cells (Chen *et al.*, 2007). Transfection-induced overexpression of SIRT1 in cultured porcine ovarian granulosa cells promoted FSH-stimulated proliferation and P4 and IGF-I release. These observations indicate that the action of FSH on ovarian cell proliferation could be at least partly mediated by the ovarian mTOR/SIRT1 system. Nevertheless, the role of mTOR/SIRT1 signalling in the regulation of ovarian cell proliferation and apoptosis requires further confirmation and its involvement in the control of ovarian cell steroidogenesis requires examination. Furthermore, the involvement of mTOR/SIRT1 in mediating the action of FSH on ovarian cell proliferation requires further confirmation and its role in mediating the action of FSH on other ovarian functions (apoptosis, release of steroid hormones) remains unknown. We propose that the hypothetical mediator of hormone action (1) changes under the influence of this hormone, (2) the hypothetical mediator of hormone action affects ovarian functions controlled by this hormone and (3) changes in this hypothetical mediator modify the hormone effects.

The aim of our *in vitro* experiments was to elucidate the involvement of the mTOR system in the control of basic ovarian cell functions and in mediating the action of upstream hormonal stimulators. In the first series of experiments, we examined the effect of the known hormonal regulators of ovarian functions, Follicle-Stimulating Hormone (FSH), oxytocin (OT) and Insulin-like Growth Factor I (IGF-I) on the accumulation of SIRT1 in porcine ovarian granulosa cells. In the second series of experiments, we examined the effects of mTOR blockers, PF 046 (an inhibitor of mTORC1) and WYE 687 (inhibitor of both mTORC1 and mTORC2), on both the basal and FSH-induced proliferation, apoptosis and steroidogenesis of these cells.

MATERIALS AND METHODS

Isolation and culture of granulosa cells: Ovaries of non-cycling pubertal gilts, approximately 180 days of age, were obtained after slaughter at a local abattoir. They were washed several times in 95% alcohol and then in sterile 0.9% NaCl. Granulosa cells were aspirated using a syringe and sterile needle from follicles that were 3-5 mm in diameter and they were separated from the follicular fluid by centrifugation for 10 min at 200× g. The cells were then washed in sterile DMEM/F12 1:1 medium (BioWhittakerTM, Verviers, Belgium), resuspended in the same medium supplemented with 10% foetal calf serum (BioWhittakerTM) and 1% antibiotic-antimycotic solution (Sigma, St. Louis, MO, USA) at a final concentration 10^6 cells mL^{-1} medium. The portions of this cell suspension intended for EIA analysis were dispensed to 24-well culture plates (Nunc™, Roskilde, Denmark, 1 mL well⁻¹) and the cells intended for immunocytochemistry were placed in 16-well chamber slides (Nunc Inc., International, Naperville, USA, 200 μL well⁻¹). The plate wells were incubated at 37.5°C in 5% CO₂ in humidified air until a 60-75% confluent monolayer was formed (3-5 days), at which point, the medium was renewed. These cells were used for two series of experiments. In the first series of experiments (examination of the effect of hormones on SIRT1 accumulation), after replacement of the medium, the experimental cells intended for immunocytochemical analysis of SIRT1 expression were cultured in the presence of pFSH (Sigma-Aldrich spols.r.o., St. Louis, MO, USA), oxytocin (Sigma) or IGF-I (Sigma), which was added to the culture medium at concentrations of 0, 1, 10 or 100 ng mL^{-1} . To enable comparison the effects of various hormones, their doses were expressed in ng mL^{-1} but not in IU mL^{-1} as in the 2nd series of experiments. All hormones were of cell culture grade. In the second series of experiments (effect of mTOR blockers on basic and FSH-induced ovarian cell functions), the experimental cells were cultured in the presence of FSH, as mentioned above, at concentrations of 0, 0.01, 0.1 or 1.0 IU mL^{-1} alone or in combination with PF 046 (inhibitor of mTORC1, Selleck Chemicals LLC, Houston, USA, 1 μg mL^{-1}) or WYE 687 (inhibitor of both mTORC1 and mTORC2, Selleck Chemicals LLC, 1 μg mL^{-1}). After two days in culture, the medium from the 24-well plates was gently aspirated and frozen at -24°C until EIA was performed. After the removal of the medium from the chamber slides, the cells were washed in ice-cold PBS (pH 7.5), fixed in paraformaldehyde (4% in PBS, pH 7.2-7.4; 60 min) and kept at 4°C until immunocytochemistry was performed.

Immunocytochemical analysis: The presence of markers of proliferation (PCNA), apoptosis (bax) and mTOR (SIRT1) was detected by immunocytochemistry (Osborn and Isenberg, 1994). After washing and fixation, the cells were incubated in the blocking solution (1% goat serum (from Santa Cruz Biotechnology, Inc., Santa Cruz, USA) in Phosphate-Buffered Saline (PBS) at room temperature for 1 h to block the

nonspecific binding of antiserum. Afterwards, the cells were incubated in the presence of monoclonal antibodies against either PCNA and bax (all from Santa Cruz Biotechnology, Inc., dilution of 1:500 in PBS) for 2 h at room temperature and then overnight at 4°C. For the detection of the binding sites of the primary antibody against PCNA and bax, the cells were incubated in secondary swine antibody against mouse IgG labelled with horseradish peroxidase (Servac, Prague, Czech Republic, dilution of 1:1000) for 1 h. Positive signals were visualized by staining with DAB-substrate (Roche Diagnostics GmbH, Mannheim, Germany). Following DAB-staining, the cells on the chamber slides were washed in PBS and then covered with a drop of glycerol mounting medium (DAKO, Glostrup, Denmark), next, the coverslip was attached to a microslide. The presence and localization of PCNA and bax positivity in cells was proved on the basis of DAB-peroxidase brown staining. Cells processed without the primary antibody were used as the negative control. The ratio of DAB-HRP-stained cells to the total cell number was calculated.

The presence of SIRT1 was detected using primary mouse monoclonal antibodies against SIRT1 (Santa Cruz Biotechnology, Inc., dilution of 1:250). The visualization of the binding of primary antibodies was performed using secondary polyclonal goat antibodies against mouse IgGs labelled with the fluorescent marker fluorescein isothiocyanate (FITC) (Santa Cruz Biotechnology), which was applied at a dilution of 1:1000 and mounted in a Vectashield with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA). The presence of SIRT1 was determined using fluorescence microscopy. Cells processed without the primary antibody were used as the negative control. The cells expressing a signal greater than the background negative control levels were considered positive. The proportion of cells containing visible SIRT1 to the total cell number was calculated.

Immunoassay: The concentrations of P4 and T were determined in 25-100 μL samples of incubation medium by EIA, which was previously validated for use in culture medium, using antisera against steroids produced by the Institute of Animal Science, Neustadt, Germany. The P4 concentrations were measured using EIA as described previously (Prakash *et al.*, 1987). Rabbit antiserum against P4 was obtained from the Research Institute for Animal Production, Schoonoord, Netherlands. It cross-reacted <0.1% with 17 β -estradiol, dihydrotestosterone, testosterone and 17 β -hydroxyprogesterone. The sensitivity was 12.5 pg mL^{-1} . The inter and intra assay coefficients of variation did not exceed 3.3 and 3.0%, respectively. The T was assayed using EIA according to Munster (1989). The sensitivity was 10 pg mL^{-1} . The antiserum cross-reacted <96% with dihydrotestosterone, <3% with androstenedione, <0.01% with progesterone and estradiol, <0.02% with cortisol and <0.001% with corticosterone. The inter- and intra-assay coefficients of variation were 12.3 and 6.8%, respectively.

Statistical analysis: Each experiment was repeated 3 times using different animals (8-14 animals per experiment). Each experimental group was represented by four culture wells or four chamber-slide wells. Differences between groups were evaluated using a two-way ANOVA test followed by a paired Wilcoxon–Mann–Whitney test using the statistics software Sigma Plot 11.0 (Systat Software, GmbH, Erkhart, Germany). Values represent the Mean±SEM. Differences were compared for statistical significance at p-levels less than 0.05 ($p<0.05$).

RESULTS

Cultured porcine granulosa cells were able to create a monolayer in the culture. Immunocytochemical analysis showed the detectable presence of PCNA, Bax and SIRT1 in porcine ovarian granulosa cells after culture. Furthermore, cultured cells were able to produce hormones (P4 and T). These parameters were affected by hormones and mTOR blockers.

Effect of FSH, IGF-I and OT on SIRT1 accumulation in porcine granulosa cells: The percentage of cells containing SIRT1 varied between 3 and 75% and this percentage was

affected by hormonal treatments. The addition of FSH at a dose of 10 ng mL⁻¹ but not at lower (1 ng mL⁻¹) or higher (100 ng mL⁻¹) doses increased the expression of SIRT1 in the cells (Fig. 1a). The OT increased the expression of SIRT1 in ovarian cells at all doses added (1, 10 or 100 ng mL⁻¹) (Fig. 1b). The IGF-I decreased the SIRT1 accumulation when added at the highest dose (100 ng mL⁻¹) but not at lower (1 or 10 ng mL⁻¹) doses (Fig. 1c).

Effect of FSH on the proliferation, apoptosis and secretory activity of porcine granulosa cells: Immunocytochemical analysis revealed that the FSH treatment increased the accumulation of PCNA (at 0.1 IU mL⁻¹ but not at other doses) (Fig. 2a) but did not affect the accumulation of Bax (at all doses added) (Fig. 2b) in porcine ovarian granulosa cells. The EIA analysis showed that the administration of FSH stimulated the release of both P4 (at 0.01 and 1 IU mL⁻¹ but not at 0.1 IU mL⁻¹) (Fig. 2c) and T (at 1 IU mL⁻¹ but not at lower doses) (Fig. 2d) by ovarian cells.

Effect of PF 046 on the proliferation, apoptosis and secretory activity of porcine granulosa cells: Treatment by PF 046 (at dose 1 µg mL⁻¹) resulted in decreased PCNA

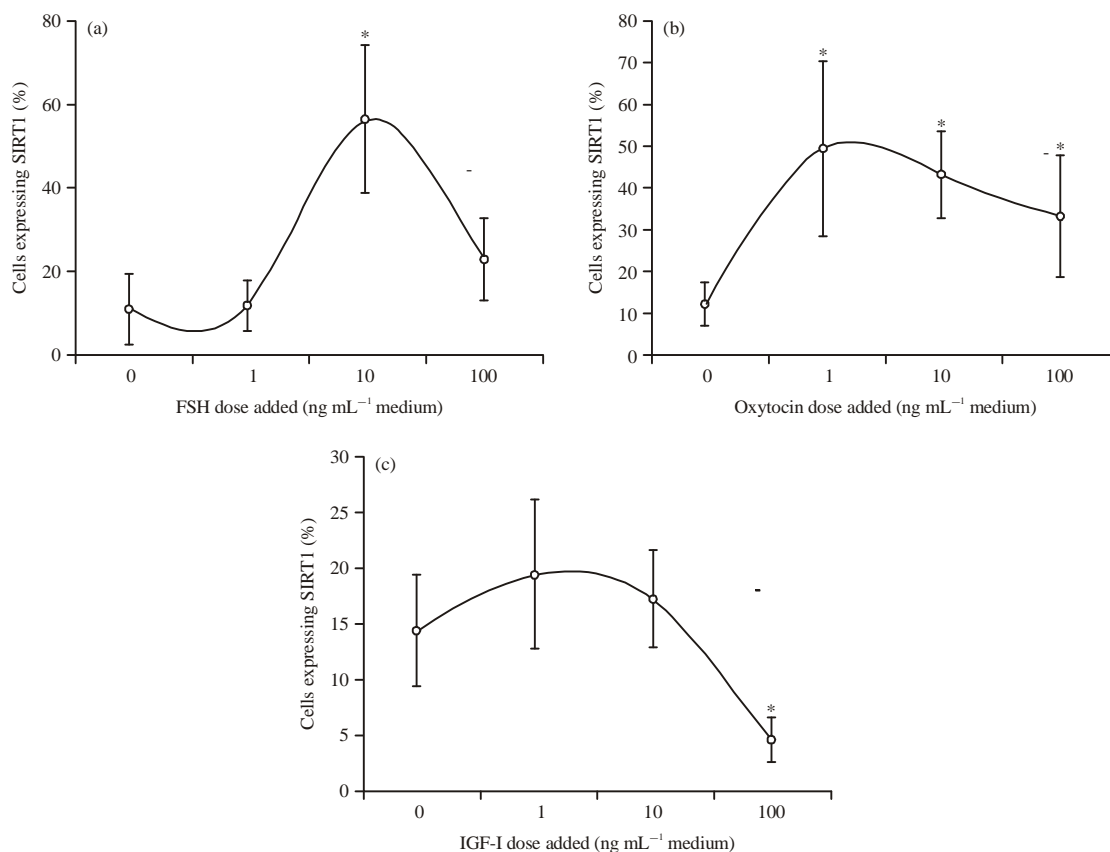


Fig. 1(a-c): (a) Effect of FSH, (b) IGF-I and (c) OT (given at 0, 1, 10 and 100 ng mL⁻¹ medium) on the percentage of porcine granulosa cells expressing the sirtuin SIRT1. Data is the Mean±SEM, *: Effect of hormone addition: significant ($p<0.05$) differences between the cells cultured with (1, 10 or 100 ng mL⁻¹) and without (0 ng mL⁻¹) hormone

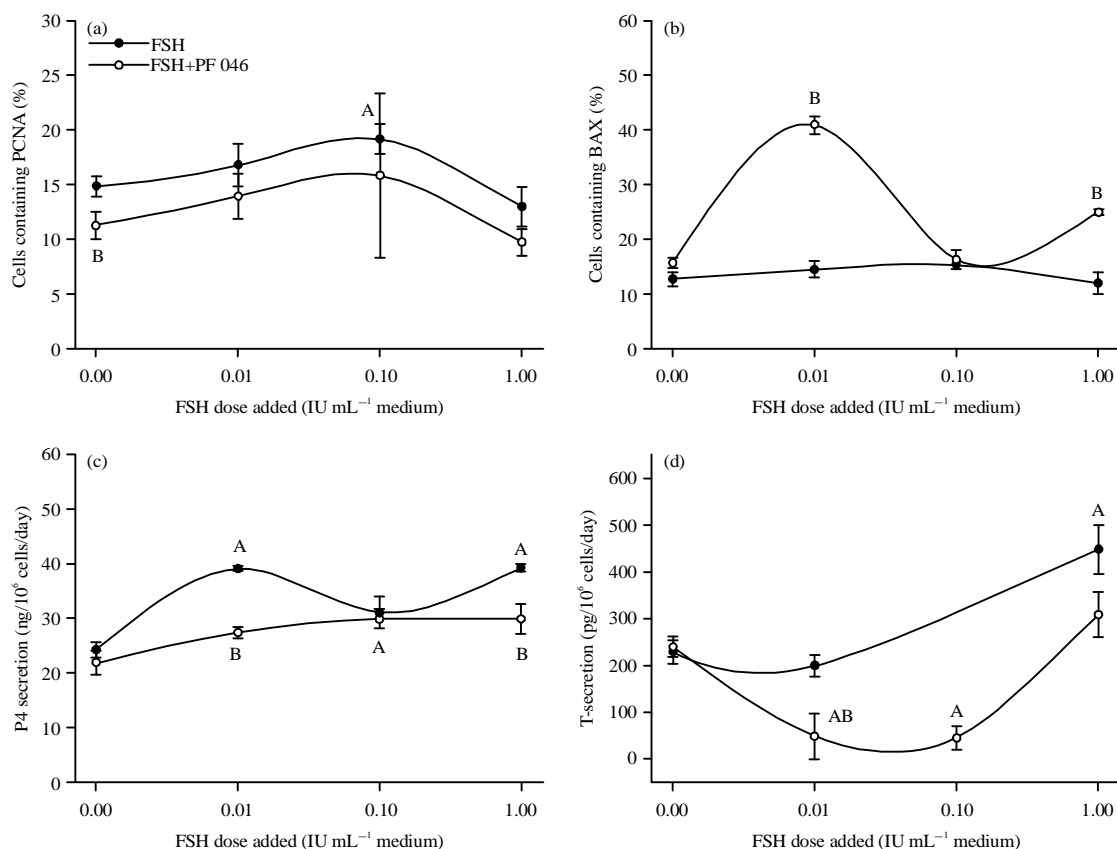


Fig. 2(a-d): (a) Effect of PF 046 (inhibitor of mTORC1) on the proliferation, (b) Apoptosis, (c) Progesterone (P4) release and (d) Testosterone release by porcine granulosa cells cultured with and without FSH. The values represent PCNA (marker of proliferation) accumulation, Bax (marker of apoptosis) accumulation and P4 or T release, as indicated; Means \pm SEM, A: Indicates a significant ($p<0.05$) effect of FSH: differences between cells cultured with FSH (0.01, 0.1 and 1 IU mL $^{-1}$) and control (without FSH addition) cells. B: Indicates a significant ($p<0.05$) modulatory effect of the mTOR blocker (1 μ g mL $^{-1}$) on the action of FSH (0, 0.01; 0.1 and 1 IU mL $^{-1}$): Differences between corresponding groups of cells cultured with and without the mTOR blocker

expression (Fig. 2a) however, no effect of PF 046 on Bax accumulation in ovarian cell was found (Fig. 2b). The administration of PF 046 also did not affect either the P4 (Fig. 2c) or T release (Fig. 2d) by ovarian cells.

Effect of WYE 687 on the proliferation, apoptosis and secretory activity of porcine granulosa cells: The WYE 687 (at 1 μ g mL $^{-1}$) did not affect the accumulation of PCNA (Fig. 3a) and Bax (Fig. 3b) in ovarian cells. In contrast, WYE 687 (at 1 μ g mL $^{-1}$) reduced the release of both P4 (Fig. 3c) and T (Fig. 3d) by granulosa cells.

Effect of PF 046 on the FSH-mediated proliferation, apoptosis and secretory activity of porcine granulosa cells: Treatment by PF 046 (at 1 μ g mL $^{-1}$) reduced the PCNA accumulation of cells cultured without FSH, Furthermore in the presence of PF 046, FSH did not exert a statistically significant effect on this parameter (Fig. 2a). The PF 046, when given alone, did not affect the accumulation of bax or the release of P4 or T. Nevertheless, in the

presence of PF 046, FSH increased the accumulation of Bax (at 0.01 and 1 IU mL $^{-1}$ FSH but not at other doses) (Fig. 2b) and suppressed the stimulatory action of FSH on the release of both P4 (at 0.01 and 1 IU mL $^{-1}$ but not at 0.1 IU mL $^{-1}$) (Fig. 2c) and T (at 0.01 IU mL $^{-1}$ but not at other doses) (Fig. 2d) by granulosa cells. Moreover, in the presence of PF 046, FSH (at doses 0.01 and 0.1 IU mL $^{-1}$) reduced the T output.

Effect of WYE 687 on the FSH-mediated proliferation, apoptosis and secretory activity of porcine granulosa cells: The WYE 687, when given alone (at 1 μ g mL $^{-1}$), did not affect the accumulation of PCNA and bax but it reduced the P4 and T release. In presence of WYE 687, the FSH-induced accumulation of PCNA was significantly lower than that in the presence of FSH alone (at doses of 0.01 and 0.1 IU mL $^{-1}$). Moreover, in presence of WYE 687, FSH did not increase PCNA accumulation but did decrease it (at dose 1 IU mL $^{-1}$) (Fig. 3a). WYE 687 significantly reduced the accumulation of bax in cells cultured in the presence of FSH (at 1 μ g mL $^{-1}$) in

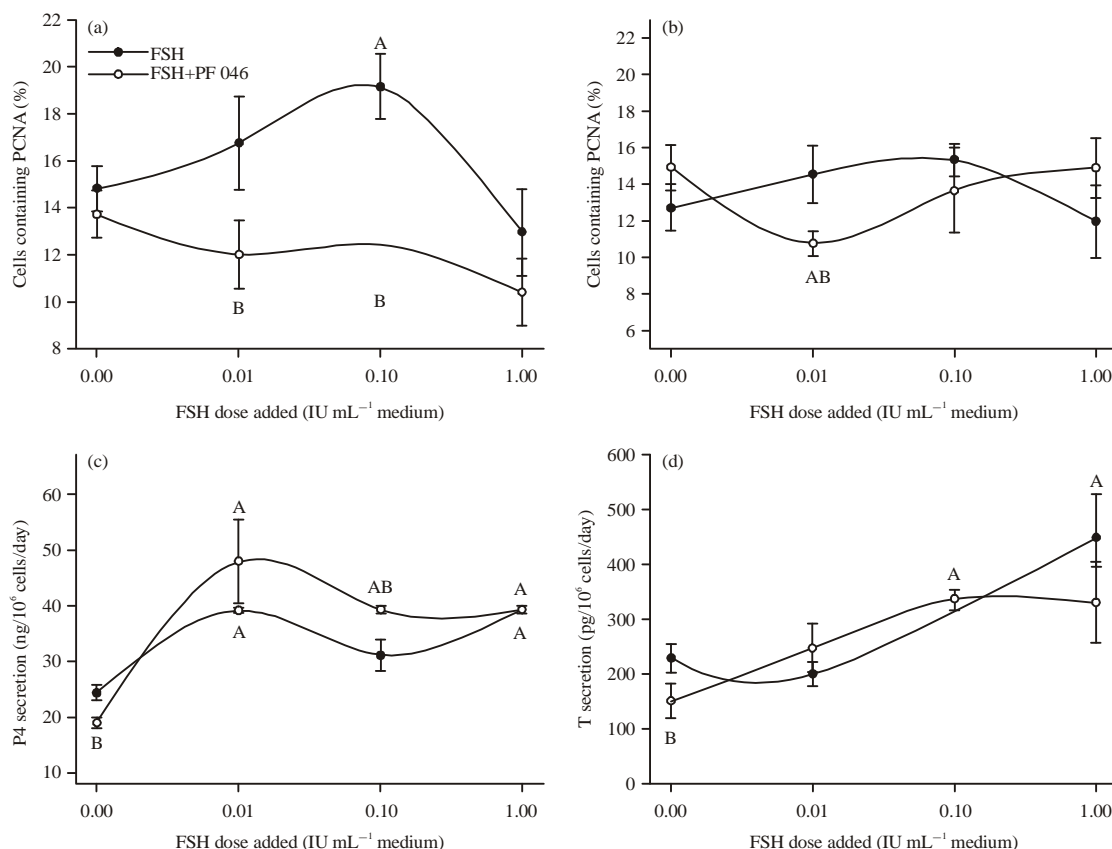


Fig. 3(a-d): (a) Effect of WYE 687 (inhibitor of both mTORC1 and mTORC2) on the proliferation, (b) Apoptosis, (c) Progesterone (P4) release and (d) Testosterone release by porcine granulosa cells cultured with and without FSH. Legends as in Fig. 2

comparison with that in either cells cultured without FSH or in cells cultured with FSH and without WYE 687 (Fig. 3b). WYE 687, when given alone, reduced the release of steroid hormones. However, WYE 687 promoted the FSH-stimulated P4 release (at 0.1 IU mL⁻¹ but not at 0.01 and 1 IU mL⁻¹) (Fig. 3c) but did not substantially affect the stimulatory influence of FSH on the T output (Fig. 3d) by porcine granulosa cells.

DISCUSSION

The creation of a cell monolayer, the presence of PCNA, Bax and SIRT1 in the cells and the release of progesterone and testosterone into the culture medium indicate that the tested porcine granulosa cells were viable and suitable for analysis. Furthermore, the observed changes in these values demonstrate the influence of hormone(s) and mTOR inhibitors (WYE 687 and PF 046) on these indexes.

The role of mTORC1 and mTORC2 in the control of ovarian cell functions: In the current study, decreased ovarian cell proliferation after the administration of PF 046, an inhibitor of mTORC1 was observed. Our results are consistent with the report of Yuan *et al.* (2011) on the anti-proliferative effect of PF 046 in human ovarian cancer cells. This data is

also consistent with the anti-proliferative action of mTORC1 inhibitors: Rapamycin in healthy mouse granulosa cells (Yaba *et al.*, 2008; Yu *et al.*, 2011), everolimus in mouse ovarian granulosa cell tumours (Rico *et al.*, 2012) and mTORC1/2 inhibitors (NVP-BEZ235 and DS-7423) in human ovarian cancer cells (Santiskulvong *et al.*, 2011; Kashiyaama *et al.*, 2014; Yuan *et al.*, 2011). These data suggest that mTORC1 can be involved in the up-regulation of ovarian cell proliferation. However, in our experiments, the granulosa cell proliferation was not affected by WYE 687, an inhibitor of both mTORC1 and mTORC2. The differences between PF 046 and WYE 687 on ovarian cell proliferation can be explained by the opposite actions of mTORC1 and mTORC2 on cell proliferation. The involvement of the mTOR system in both the up- and down-regulation of ovarian cell proliferation is supported by previous experiments that manipulated SIRT1, a physiological inhibitor of the mTOR system. The transfection of porcine granulosa cells with an SIRT1 gene construct was able to either stimulate or inhibit the cell proliferation of these cells (Pavlova *et al.*, 2013; Sirotkin *et al.*, 2014). The SIRT1 inhibition by sirtinol resulted in the reduced proliferation of a human ovarian granulosa-like tumour cell line (Benayoun *et al.*, 2011). Taken together, the available data suggest the involvement of mTOR-SIRT1 signalling in the regulation of ovarian cell proliferation.

In contrast to the results for proliferation, both the present and some previous observations failed to demonstrate the involvement of mTOR-SIRT1 in the control of ovarian cell apoptosis. The administration of rapamycin, everolimus and PF 046 did not affect the apoptosis of healthy mouse granulosa cells (Yu *et al.*, 2011), mouse ovarian granulosa cell tumours (Rico *et al.*, 2012) and human ovarian cancer cells (Yuan *et al.*, 2011), respectively. In the present study, no effect of either PF 046 or WYE 687 on porcine granulosa cell apoptosis was observed as well. Moreover, transfection with SIRT1 gene construct did not alter the apoptosis of porcine granulosa cells in our previous experiment (Sirotkin *et al.*, 2014). However, the inhibition of mTORC1/2 by NVP-BEZ235 and DS-7423 increased the apoptosis of human ovarian cancer cells (Santiskulvong *et al.*, 2011; Kashiyama *et al.*, 2014), indicating the possible involvement of mTOR in the control of at least human ovarian cancer cells. Similarly to proliferation, ovarian cell steroid hormones responded to mTORC1 and mTORC2 blockers in different ways. The PF 046 (mTORC1 blocker) did not affect the basal secretion of either P4 or T. This observation is in line with the report by Hou *et al.* (2010), who failed to find an effect of rapamycin on the P4 secretion by bovine corpus luteum cells. In contrast, WYE 687 (inhibitor of both mTORC1 and mTORC2) in our experiments inhibited the release of both P4 and T. Previously, we observed that the transfection of porcine granulosa cells with an SIRT1 gene construct stimulated their P4 release (Pavlova *et al.*, 2013). Therefore, we presume that ovarian mTORC2/SIRT1 but not mTORC1/SIRT1, can be involved in the up-regulation of ovarian cell steroidogenesis. Taken together, our observations together with previous data suggest the involvement of mTOR in the control of ovarian cell proliferation and steroid hormone release but most likely not in the control of apoptosis. Determining the physiological and practical significance of these findings requires further studies but the possibility that mTOR regulators can be used to control ovarian folliculogenesis and the resulting fecundity-processes that are dependent on ovarian cell proliferation and steroidogenesis (Sirotkin, 2011) should not be excluded. The rapamycin treatment was able to suppress follicular growth and maturation in rodents (Yaba *et al.*, 2008; Zhang *et al.*, 2013) and increased the risk of oligomenorrhea in humans (Braun *et al.*, 2012).

The role of FSH in the control of ovarian cell functions:

The role of FSH in the regulation of ovarian processes is well known. The FSH promotes granulosa cell proliferation and steroidogenesis (Yu *et al.*, 2005; Sirotkin *et al.*, 2008; Sirotkin, 2011) and stimulates (Sirotkin *et al.*, 2008; Pavlova *et al.*, 2011) or inhibits (Jiang *et al.*, 2003) apoptosis. The pro-proliferative and pro- or anti-apoptotic effects of FSH suggest that FSH may promote ovarian follicle growth and the development and regulation of follicular atresia (Yacobi *et al.*, 2004; Yu *et al.*, 2005; Sirotkin *et al.*, 2008; Sirotkin, 2011). Moreover, gonadotropins can stimulate the ovarian cell luteinisation associated with increased P4 release (Schams *et al.*, 1999; Murphy *et al.*, 2001). The

present observation that FSH exerts a stimulatory effect on ovarian cell proliferation and steroidogenesis (P4 and T) but not on apoptosis are in agreement with these data on the promotion of ovarian cell functions by FSH.

The role of mTORC1 and mTORC2 in mediating hormone action:

The current study confirmed the previous data on the presence of mTOR-SIRT1 in ovarian granulosa cells and on the ability of FSH to increase the SIRT1 accumulation in porcine granulosa cells (Pavlova *et al.*, 2013; Sirotkin *et al.*, 2014) and the phosphorylation of mTOR (Chen *et al.*, 2007) and its downstream target p70S6K (Alam *et al.*, 2004; Kayampilly and Menon, 2007) in rat granulosa cells. Our present observations are the first demonstration that mTOR-SIRT1 can be controlled by not only FSH but also OT and IGF-I, whilst FSH and OT are down-regulators of this process and IGF-I is an up-regulator. These observations provide indirect evidence for the involvement of mTOR-SIRT1 in mediating the effect of the upstream hormonal regulators of ovarian functions (Sirotkin, 2011). The ability of mTORC1 and mTORC2 blockers to modify and even to invert the observed action of FSH can be considered direct confirmation of this hypothesis.

In present study, FSH when given alone, stimulated proliferation did not affect apoptosis and increased the release of both P4 and T. In the presence of PF 046, FSH did not significantly alter proliferation, induced apoptosis and suppressed P4 and T release, i.e., this inhibitor of mTORC1 prevented the proliferation-stimulating effect of FSH, induced the pro-apoptotic action of FSH and inverted the stimulatory action of FSH on steroidogenesis. The WYE 687 prevented the effect of FSH on both proliferation and apoptosis, promoted the FSH-induced P4 release but did not affect the action of FSH on T output, i.e., the inhibition of both mTORC1 and mTORC2 inverted the action of FSH on ovarian cell proliferation and apoptosis but not steroidogenesis.

The present observations of the ability of mTOR regulators to modify the action of FSH confirm the previous reports concerning the ability of SIRT1 to promote FSH-induced proliferation and P4 release by porcine ovarian cells (Pavlova *et al.*, 2013) and the ability of rapamycin (mTORC1 blocker) to suppress FSH-induced proliferation (Kayampilly and Menon, 2007) but not P4 secretion (Chen *et al.*, 2007) by rat granulosa cells. However, our study is the first to compare mTOR blockers with different mechanisms of action, which enables us to identify the ovarian functions controlled by mTORC1 and mTORC2. Our observations suggest that (1) mTORC1 can be involved in the up-regulation of ovarian cell proliferation but not the up-regulation of apoptosis and steroidogenesis, (2) mTORC2 can be involved in the up-regulation of the release of both P4 and T but not the up-regulation of ovarian cell proliferation and apoptosis, (3) FSH is involved in the stimulation of ovarian cell proliferation and P4 and T release and (4) hormones (FSH, OT and IGF-I) have the ability to regulate ovarian mTOR and mTOR has the ability to modify FSH effects, suggesting that mTOR can mediate the action of hormones on

ovarian cells. It is hypothesized that both mTORC1 and mTORC2 may be mediators of the action of FSH on ovarian cell proliferation, apoptosis and steroidogenesis.

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