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Apoptosis signal-regulating kinase (ASK1) and transcription factor tumor suppressor protein TP53 suppress rabbit ovarian granulosa cell functions

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ABSTRACT

This study was conducted with the aim to understand the roles of apoptosis signal-regulating kinase (ASK1) and transcription factor tumor suppressor protein TP53, as well as the possible interrelationships, in the control of healthy ovarian cell functions. Rabbit ovarian granulosa cells were transfected with constructs encoding ASK1, TP53, or TP53 + ASK1 and cultured with or without insulin-like growth factor 1 (IGF1). The accumulation of ASK1, the cytoplasmic apoptosis regulators BAX and BCL2, and proliferating cell nuclear antigen (PCNA, a cell proliferation marker), as well as progesterone release, were evaluated by quantitative immunocytochemistry and radioimmunoassay. Results indicate both ASK1 and TP53 promoted the accumulation of BAX, but suppressed that of BCL2 and PCNA. Progesterone release was inhibited by ASK1 and promoted by TP53, while TP53 also stimulated ASK1 accumulation. Additionally, IGF1 stimulated PCNA and reduced progesterone release, but did not affect ASK1. Transfection with ASK1, TP53, or TP53 + ASK1 could modify IGF1 activity, however, there was no cumulative effect with co-transfection of TP53 and ASK1. This is the first results that indicate there is ASK1 suppression of healthy ovarian granulosa cell functions, including promoting apoptosis, inhibiting proliferation, and alter progesterone release. There was also TP53 actions in rabbit ovarian granulosa cells, where it stimulated ASK1, apoptosis, and progesterone release, thus suppressing proliferation and responses to IGF1. The similarity of ASK1 and TP53 effects on apoptosis and proliferation, lack of cumulative action of these molecules, and capacity of TP53 to promote ASK1 accumulation suggest that TP53 can suppress some ovarian granulosa cell functions through ASK1 stimulation.

1. Introduction

Signaling molecules regulating cell proliferation, apoptosis, secretory functions, and the response to extracellular factors have important roles in controlling basic physiological processes, mediating the effects of extracellular factors, and the diagnostics and treatment of cellular disorders. For example, malignant transformation of all cell types is associated with suppression of apoptosis, increased proliferation, secretory activity, and responses to insulin-like growth factor 1 (IGF1), while inhibiting these processes is the

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main approach currently used for the prevention and treatment of cancer (Deng, 2017; Kasprzak et al., 2017; Adamek and Kasprzak, 2018). One such anti-cancer molecule is tumor suppressor protein TP53, a transcription factor that targets several genes related to the cell cycle, repair, apoptosis, insulin-like growth factor reception, and carcinogenesis (Deng, 2017; Conover, 2018; Simabuco et al., 2018). Loss of function of TP53 often results in increased cell proliferation as well as reduced apoptosis and tumorigeneses (Duffy et al., 2017). In addition, loss of TP53 function can also downregulate healthy ovarian cell functions. Transfecting healthy ovarian cells of pigs with cDNA constructs encoding TP53 inhibits cell proliferation, induces apoptosis, and inhibits progesterone release and the response to some upstream hormonal stimulators (Sirotkin et al., 2008, 2012). The functions of TP53 in controlling healthy ovarian cell functions has, however, not been studied in other species.

Another intracellular tumor suppressor is apoptosis signal-regulating kinase 1 (ASK1). Like TP53, ASK1 decreases non-ovarian (Liu et al., 2017) and ovarian (Yin et al., 2017; Hong et al., 2018) cancer cell survival and proliferation and promotes apoptosis in these cells. In cultured chicken ovarian cells, an association has been observed between accumulation of ASK1 and other apoptosis markers (pro-apoptotic BAX and TP53, and anti-apoptotic BCL2), the proliferation marker PCNA, and release of steroid hormones, including progesterone (Sirotkin and Grossmann, 2007). This may indicate that ASK1 is involved in regulating apoptosis, proliferation, and steroidogenesis, as well as the possible functional interrelationships between ASK1 and TP53 in healthy ovarian cells. Nevertheless, the role of ASK1 in the regulation of healthy ovarian cell functions, as well as the functional interrelationships between ASK1 and TP53, remain unknown.

The aim of the present study was to examine the role and possible interrelationships between ASK1 and TP53 in the regulation of basic healthy ovarian cell functions (proliferation, apoptosis, steroidogenesis, and responses to the upstream hormonal regulator IGF1). Granulosa cells are the most popular model for the *in-vitro* studies of ovarian regulators because these cells are easy to isolate, to culture, to transfect and to analyze using immunocytochemical methods. Rabbit ovarian granulosa cells were transfected with cDNA constructs encoding ASK1, TP53, or TP53 + ASK1 and cultured with or without IGF1. There was subsequent analysis of the accumulation of ASK1, the pro-apoptotic peptide BAX, the anti-apoptotic peptide BCL2 (markers of cytoplasmic/mitochondrial/intrinsic apoptosis, Birkinshaw et al., 2017; Deng, 2017; Beyfuss and Hood, 2018), and the proliferation marker PCNA (Wang et al., 2014), as well as progesterone release.

2. Materials and methods

2.1. Animals

Clinically healthy New Zealand White rabbit does (*Oryctolagus cuniculus)* were obtained from the farm of the Research Institute for Animal Production, Nitra, Slovakia and used to conduct this study. The animals were housed in individual cages (103 × 55 × 51 cm) under a constant 16-h light and 8-h dark photoperiod throughout the experiment. The rabbits were fed KV, a commercially-available feed mixture (Tekro, s.r.o., Párovské Háje, Nitra, Slovakia) ad libitum. At 295 d of age, after the second litter had been weaned, the females were euthanized by decapitation. The treatment of animals was approved by the Ministry of Agriculture and Rural Development of the Slovak Republic, No. SK P 28004 and Ro 1488/06-221/3a and the ethical committee of the Research Institute for Animal Production, Nitra, Slovakia.

2.2. Granulosa cell isolation, transfection, and culture

Granulosa cells were isolated from rabbit ovaries (Sirotkin et al., 2017) and transfected as previously described (Sirotkin et al., 2008, 2012). Briefly, the ovaries removed from the does after euthanasia were washed in phosphate buffered saline (PBS), placed onto 100-mm diameter culture dishes (Gama, České Budejovice, Czech Republic) containing a 1:1 ratio of basic incubation medium/sterile DMEM/F12 (BioWhittaker™, Verviers, Belgium), supplemented with 10% fetal calf serum (BioWhittaker) and a 1% antibiotic-antimycotic solution (Sigma-Aldrich, St. Louis, MO, USA). Ovaries in this medium were dissected using a blade knife. The granulosa cells released from the dissected follicles were collected by pipette, isolated by centrifugation for 10 min at 200 × *g*, and rinsed in the culture medium that was previously described in this manuscript. Thereafter, the cells were transfected using the lipofection reagent Roti Fect (Carl Roth GmbH + Co., Karlsruhe, Germany) according to the manufacturer's instructions. Granulosa cells were transfected with the following constructs: (1) plasmid for ASK1, pcDNA3 HA-ASK1-WT obtained from Dr. Hidenori Ichijo, Tokyo Medical and Dental University, Tokyo, Japan, and the reporter plasmid pEGFP-N1 for EGFP fluorescence and kanamycin resistance (Clontech laboratories, now Takara Bio USA, Mountain View, CA, USA); (2) plasmid for TP53, pC53-SN3 generated by Dr. B. Vogelstein, Johns Hopkins University School of Medicine, Baltimore, USA, and kindly provided by M. Kaluz, Research Institute of Virology, Bratislava, Slovakia, and the EGFP plasmid previously described in this manuscript; (3) the plasmids for ASK1, TP53, and EGFP that were previously described in this manuscript; and (4) "scramble" control plasmid vector without ASK1 or TP53 insertion as controls, provided by Dr. N. Perkins, University of Dundee, Dundee, UK, and the EGFP plasmid that was previously described in this manuscript. All the constructs were sub-cloned as previously described (Sirotkin et al., 2008). After transfection, granulosa cells (1×10^5 cells/mL) were cultured in basic culture medium (see above) in Falcon 24-well plates (Becton Dickinson, Lincoln Park, NJ, USA), with 2 mL of medium per well, and in chamber slides (Nunc Inc. Naperville, TN, USA), with 200 µL of medium per well, at 38 °C in a humidified environment containing 5% CO_2. The medium was replaced after 2 d of pre-culture. Due to the limited number of granulosa cells that could be harvested from the small rabbit ovaries, several separate experiments were conducted to assess the effect of all the factors on ovarian cell variables.

The first series of experiments were conducted with the aim to examine the effects of the ASK1 construct on the apoptosis markers

BAX and BCL2. The granulosa cells were transfected with the construct encoding EGFP + the scramble construct (control cells), or EGFP + ASK1 (ASK1-transfected cells).

In the second series of experiments, there was examination of the effects of the TP53 construct on the apoptosis markers and ASK1. In this series of experiments, there was comparison of the control granulosa cells mentioned above with the granulosa cells transfected with EGFP + TP53.

In the third series of experiments, there was assessment of the effects of ASK1, TP53, and a combination of both on the expression of the ASK1 gene and the proliferation marker PCNA in granulosa cells cultured with or without IGF1 at doses of 10 and 100 ng/mL. Based on results of previous studies, it was the optimal IGF1 dose affecting cultured rabbit ovarian cells (Makarevich et al., 2000; Sirotkin et al., 2017). In this series of experiments, the granulosa cells were transfected with constructs for EGFP + scramble (control cells), EGFP + ASK1 alone, EGFP + TP53 alone, and a combination of EGFP + TP53 + ASK1. The granulosa cells were then cultured in the presence of biological grade human recombinant IGF1 (Sigma-Aldrich) at doses of 0, 10, or 100 ng/mL. Immediately before experiment, IGF1 was dissolved in culture medium.

In the fourth series of experiments, there was assessment of the effects of ASK1 and TP53, alone or in combination, on progesterone release in granulosa cells cultured with or without IGF1 at doses of 1, 10, or 100 ng/mL. The use of the smaller dose of IGF1 (1 ng/mL) as a result of previous observations of biphasic IGF1 action on progesterone release in cultured rabbit granulosa cells. At the dose of 1 ng/mL, IGF1 inhibited hormone release, while the larger IGF1 doses promoted hormone release (Makarevich et al., 2000). In this series of experiments, there was comparison of progesterone secretion from control granulosa cells and granulosa cells transfected with EGFP + ASK1, EGFP + TP53, EGFP + TP53 + ASK1, and IGF1 at doses of 0, 1, 10, or 100 ng/mL.

In all the experiments, after 2 d of culture, granulosa cell numbers and viability were determined using Trypan blue staining and by counting in a hemocytometer. The medium or granulosa cells were then collected and stored as previously described (Sirotkin et al., 2008, 2012) for quantitative immunocytochemistry or radioimmunoassay (RIA).

2.3. Immunocytochemical analysis

The presence of ASK1, PCNA, BAX, BCL2, and TP53 was demonstrated using immunocytochemistry (Osborn and Isenberg, 1994) with primary mouse monoclonal antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA; dilution 1:500) which cross-reacted with corresponding antigens of human, rat, mouse and yeast origin. Secondary porcine polyclonal antibodies against mouse IgGs, labelled with horseradish peroxidase (Sevac, Prague, Czech Republic; dilution 1:1000) and 3,3′-diaminobenzidine (DAB) reagent (Boehringer Mannheim GmbH, Mannheim, Germany) were used to visualize primary antibodies. The presence of immunoreactivity in the cells was determined by blind observations of one individual using a light microscope (Leica Microsystems, Wetzlar, Germany). The ratio of granulosa cells containing ASK1, PCNA, BAX, and BCL2 to the total granulosa cell number was calculated. The present studies focused predominantly on ASK1, and therefore TP53 gene expression was not quantified. Granulosa cells processed without the primary antibody were used as negative controls. The presence of granulosa cells expressing EGFP (marker of construct integration and expression) was determined using a fluorescence microscope (Leica Microsystems) equipped with specific wave-length filters for green fluorescence.

2.4. Radioimmunoassay

Progesterone concentrations were determined in 25 μL of incubation medium using RIA kits for P4 and E from DSL (Webster, Texas, USA) according to the manufacturer's instructions. The antiserum used had less than 0.01% cross-reactivity with pregnenolone, androstenediol, testosterone, estradiol, cortisol, and IGF1. The sensitivity of the assay was 0,12 ng/mL, the intra- and inter-assay coefficients of variation were 13.1% and 8.0%, respectively. The RIA was validated for use in culture medium samples.

2.5. Statistical analyses

Each series of experiments was performed in triplicate. The data provided are the means of values obtained in these three separate experiments performed on separate days with separate groups of granulosa cells, each obtained from 5 to 8 animals.

2.5.1. Immunocytochemistry

In each chamber (three per group), 500 granulosa cells were scored, i.e., each value represents the mean of nine replicates (4500 cells in total). The percentage of granulosa cells containing antigen in different groups of granulosa cells was calculated.

2.5.2. RIA

Each experimental group was represented by four culture wells, i.e., each value represents the mean of four wells × three experiments, totaling 12 replicates. Assays of progesterone concentration in the incubation medium were performed in duplicate. 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 1×10^6 viable cells/d.

The significance of differences between treatments was evaluated using a two-way analysis of variance (ANOVA) followed by use of the Tukey's test or the chi-squared test using Sigma Plot 11.0 (Systat Software, GmbH, Erkrath, Germany). Differences were considered significant at $P < 0.05$.

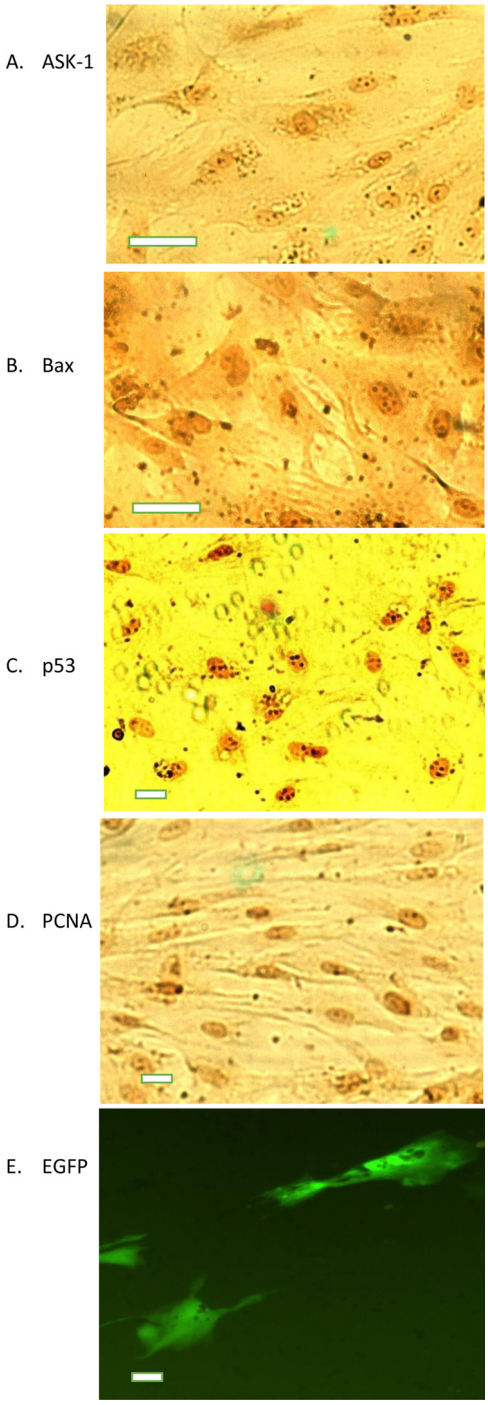

Fig. 1. Cultured control rabbit granulosa cells containing ASK1 (A), BAX (B), TP53 (C), PCNA (D), and EGFP (E) observed using a light (A–D, 3,3'-diaminobenzidine staining; brown) and fluorescent (E, green fluorescence) microscope (Leica Microsystems, Wetzlar, Germany); Scale bars = 10 μm.

3. Results

After culture, ovarian granulosa cells contained ASK1, markers of proliferation (PCNA) and cytoplasmic apoptosis (BAX and BCL2), and TP53 (Fig.1), and secreted substantial amounts of progesterone. After transfection, approximately half the granulosa cells produced EGFP (Fig.1). Cell viability was 95%–98%. No statistically significant differences in granulosa cell number and viability were observed among the control and experimental groups.

Transfection of granulosa cells with the construct encoding ASK1 resulted in a significant increase in the percentage of cells containing ASK1 (Fig. 2A, see differences between transfected and non-transfected cells cultured with IGF1 at a dose of 0 ng/mL). A substantial increase in the percentage of cells containing ASK1 was observed in granulosa cells transfected also with the TP53 construct (Fig. 2B), and with the TP53 + ASK1 constructs (Fig. 2C). Transfection with ASK1 (Fig. 3 A), TP53 (Fig. 3B), and TP53 + ASK1 (Fig. 3C) also decreased the percentage of granulosa cells containing the proliferation marker PCNA. Transfection of granulosa cells with constructs for ASK1 (Fig. 4A) and TP53 + ASK1 (Fig. 4C) also reduced progesterone release. In contrast, transfection with the TP53 construct promoted progesterone output (Fig. 4B).

Transfection of granulosa cells with the ASK1 construct resulted in a substantial increase in the proportion of granulosa cells positive for the pro-apoptotic peptide BAX (Fig. 5A), and a decrease in the percentage of granulosa cells containing the anti-apoptotic peptide BCL2 (Fig. 5B). Transfection of granulosa cells with the TP53 construct also resulted in an increase in the percentage of cells containing BAX (Fig. 6A) and a decrease in BCL2-positive cells (Fig. 6B). In addition, the TP53 construct elicited an increase in the proportion of granulosa cells containing ASK1 (Fig. 6C).

Addition of IGF1 to the control granulosa cells (not transfected with the TP53 or ASK1 constructs) at doses of 10 or 100 ng/mL did not affect ASK1 accumulation. In contrast, in granulosa cells transfected with ASK1, IGF1 promoted ASK1 accumulation when added at the dose of 100 ng/mL (Fig. 2A). In the granulosa cells transfected with either TP53 (Fig. 2B) or TP53 + ASK1 (Fig. 2C), IGF1 reduced ASK1 accumulation at all doses used.

When added to control granulosa cells, IGF1 promoted the accumulation of the proliferation marker PCNA at 100 ng/mL. In the granulosa cells transfected with either the ASK1 (Fig. 3A) or TP53 + ASK1 (Fig. 3C) constructs, however, IGF1 did not affect amounts of PCNA, while in granulosa cells transfected with the TP53 construct, PCNA accumulation was reduced (Fig. 3B).

In control granulosa cells (not transfected with the ASK1 or TP53 construct), IGF1 addition at doses of 1 or 10 ng/mL (but not 100 ng/mL) substantially reduced progesterone release. In granulosa cells transfected either with the ASK1 (Fig. 4A) or TP53 (Fig. 4B) constructs, IGF1 reduced progesterone output at all doses used. There was no effect on progesterone release in granulosa cells transfected with TP53 + ASK1 (Fig. 4C).

4. Discussion

4.1. Experimental suitability of the cells

Granulosa cell attachment to the well bottom and the creation of cell monolayers, cell viability, presence of the proliferation marker PCNA, EGFP production, progesterone release in both transfected and not transfected cells, and the capacity to respond to exogenous hormone indicated that the ovarian granulosa cells used in the experiments and subjected to transfection were viable and could respond to physiological stimuli. The expression of the EGFP transfection marker, as well as the increased accumulation of ASK1 in granulosa cells transfected with the corresponding gene construct, indicated successful transfection, integration, and expression of the exogenous gene constructs.

4.2. Effects of ASK1

The transfection-induced overexpression of ASK1 was accompanied by an increase in the proportion of granulosa cells positive for the pro-apoptotic peptide BAX, a reduction in the expression of the anti-apoptotic peptide BCL2 and the marker and promoter of proliferation PCNA, and progesterone release. These observations suggest that ASK1 is a potent physiological inhibitor of basic ovarian granulosa cell functions. By suppressing BCL2 and promoting BAX, ASK1 can induce ovarian granulosa cell cytoplasmic/mitochondrial apoptosis, and can block granulosa cell proliferation by inhibiting PCNA accumulation. In addition, ASK1 inhibited the release of progesterone, a known steroid hormonal regulator of reproductive functions and marker of ovarian cell luteinization (Sirotkin, 2014). This suggests that ASK1 can also suppress secretory functions of ovarian granulosa cells and luteinization. The biological significance, fine mechanisms, and possible functional interrelationships between various ASK1 effects require further elucidation. Nevertheless, the present observations are the first indicating the involvement of ASK1 in the regulation of heathy ovarian granulosa cell functions. Similar pro-apoptotic and anti-proliferative ASK1 activity has only been previously reported for ovarian cancer cells (Yin et al., 2017; Hong et al., 2018), implying that ASK1 may be preventing malignant transformation of healthy ovarian granulosa cells. Furthermore, although there was analysis of markers of cytoplasmic/mitochondrial but not nuclear apoptosis, the results of the present study provide additional evidence for ASK1 as a promising target for future anti-cancer drugs.

4.3. Effects of IGF1

In the experiments of the present study, the addition of IGF1 promoted PCNA accumulation and inhibited progesterone release, with these findings being consistent with those of a previous study where there was assessment of IGF1 involvement in the promotion of mammalian ovarian cell proliferation and regulation of ovarian steroidogenesis (Sirotkin, 2014). The results of the present study are also consistent with those from a previous study where there was dose-dependent stimulatory functions of IGF1 on PCNA accumulation and biphasic IGF1 effect on progesterone release by cultured rabbit granulosa cells (Makarevich et al., 2000). Taken together, these observations provide further evidence for the importance of IGF1 in the regulation of rabbit ovarian functions and fecundity (Sirotkin et al., 2014, 2017).

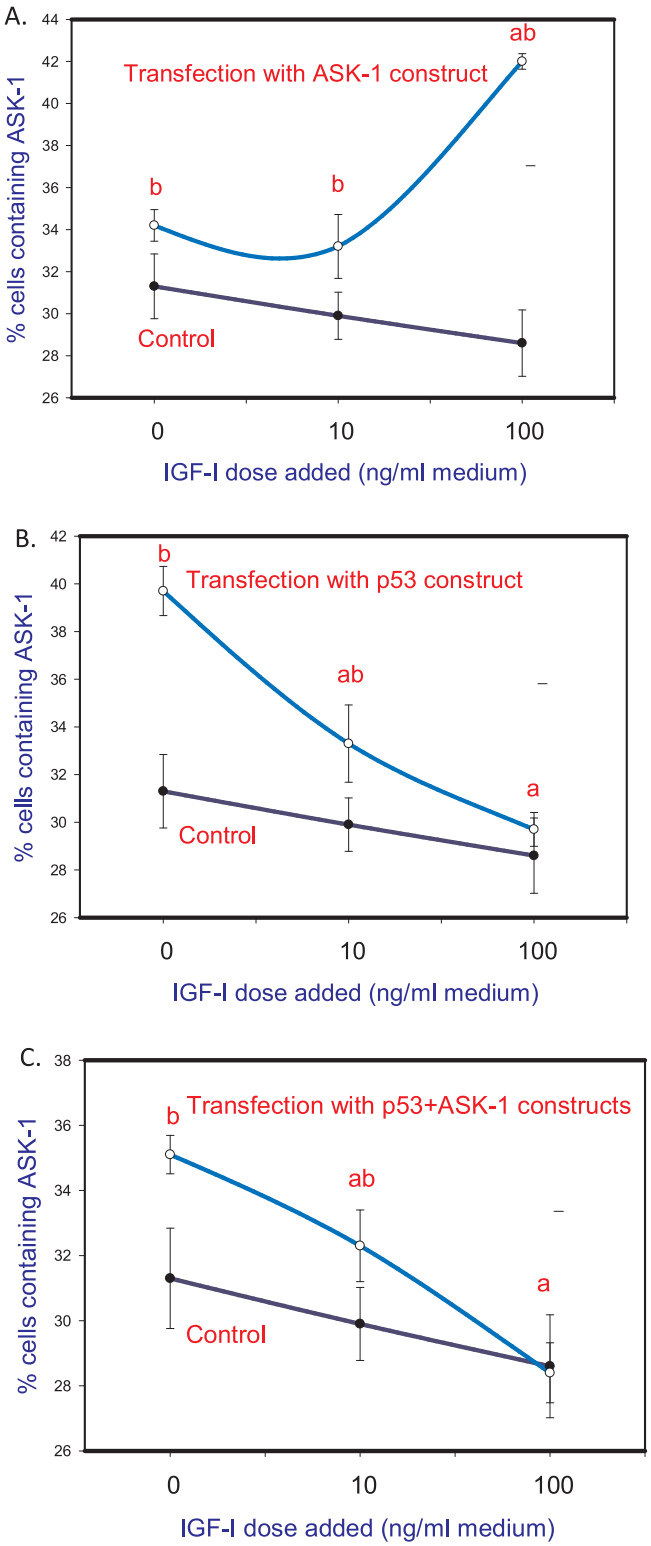

Fig. 2. Effect of adding IGF1 at different concentrations on the accumulation of ASK1 in cultured rabbit ovarian granulosa cells not transfected (control) and transfected with gene constructs encoding ASK1 (A), TP53 (B), and TP53 + ASK1 (C); Data from quantitative immunocytochemistry (Leica Microsystems, Wetzlar, Germany); "a" indicates the effect of IGF1; $P < 0.05$, difference between IGF1 treated (10 or 100 ng/mL) and control (0 ng/mL) cells; "b" indicates the effect of transfection with the gene constructs; $P < 0.05$, indicates there is a difference between corresponding groups of transfected and non-transfected cells.

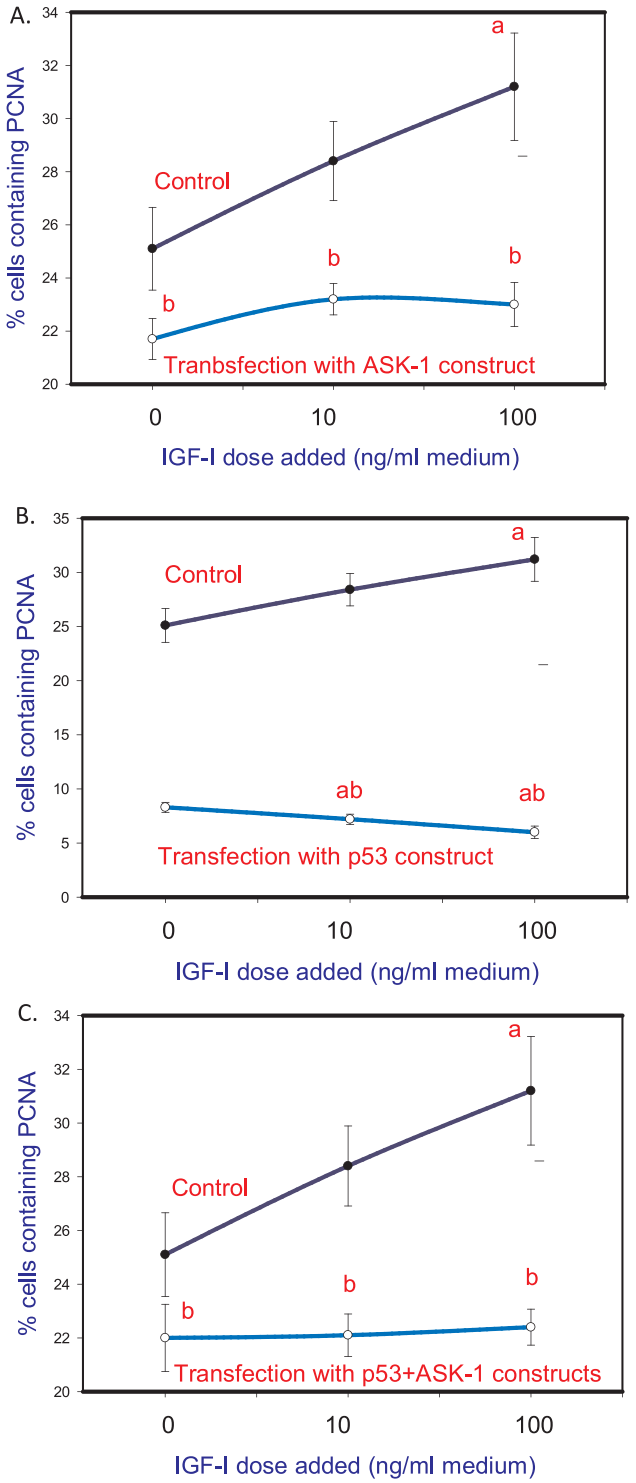

Fig. 3. Effect of adding IGF1 at different concentrations on the accumulation of the marker of proliferation PCNA in cultured rabbit ovarian granulosa cells not transfected (control) and transfected with gene constructs encoding ASK1 (A), TP53 (B), and TP53 + ASK1 (C); Data using quantitative immunocytochemistry (Leica Microsystems, Wetzlar, Germany); "a" indicates the effects of IGF1; $P < 0.05$, difference between IGF1-treated (10 or 100 ng/mL) and control (0 ng/mL) cells; "b" indicates the effects of transfection with the gene constructs; $P < 0.05$, indicates the difference between corresponding groups of transfected and non-transfected cells.

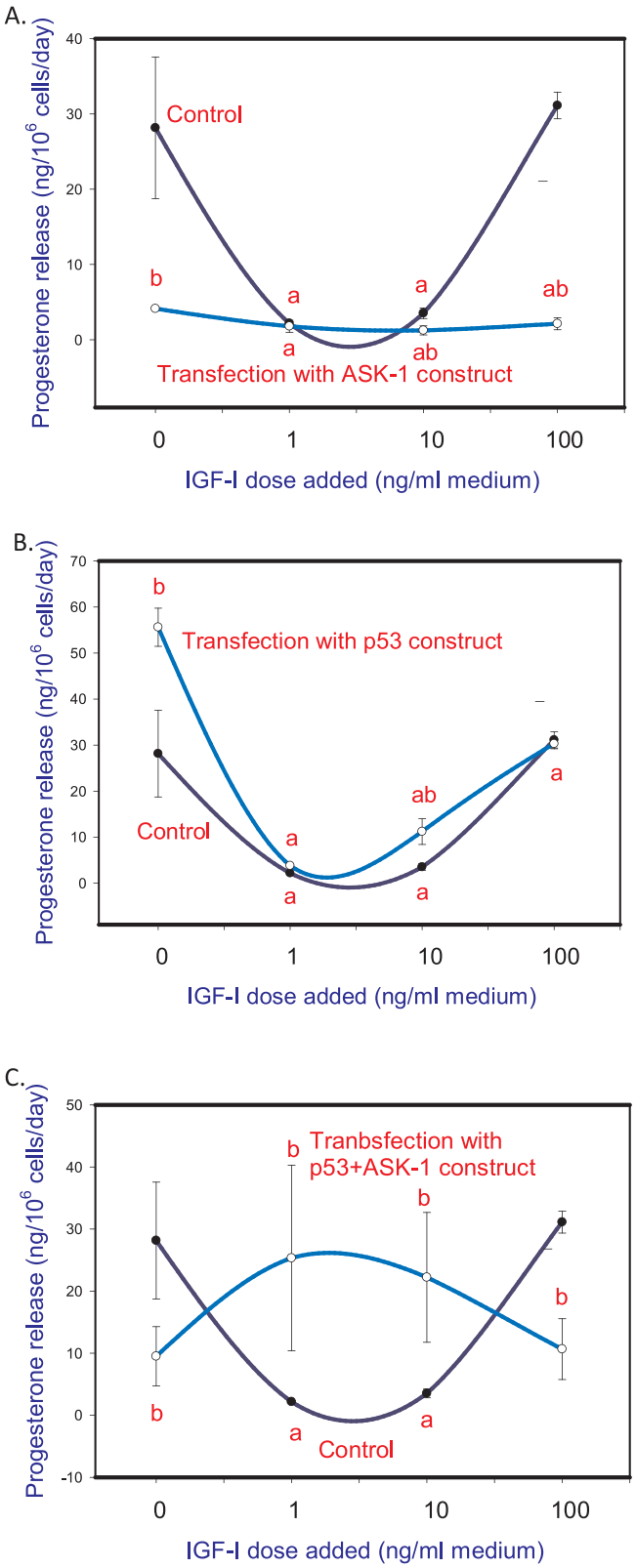

Fig. 4. Effect of adding IGF1 at different concentrations on progesterone release by cultured rabbit ovarian granulosa cells not transfected (control) and transfected with gene construct encoding ASK1 (A), TP53 (B), and TP53 + ASK1 (C); Data from RIA; "a" indicates the effect of IGF1; $P < 0.05$, difference between IGF1-treated (1, 10, or 100 ng/mL) and control (0 ng/mL) cells; "b" indicates the effect of transfection with the gene constructs; $P < 0.05$ indicates the difference between corresponding groups of transfected and non-transfected cells.

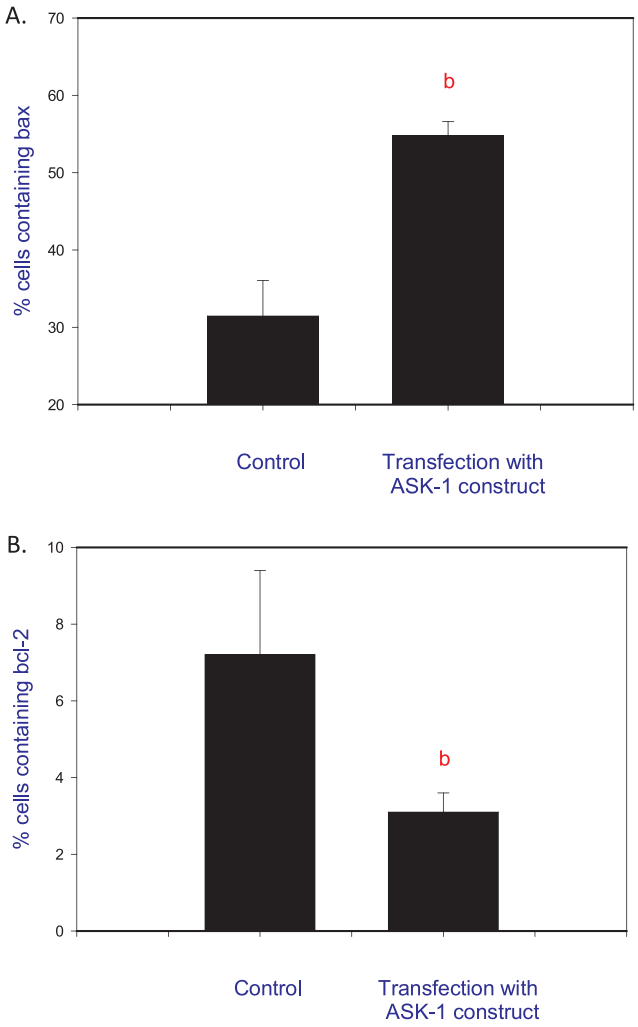

Fig. 5. Effect of transfecting cultured rabbit ovarian granulosa cells with the gene construct encoding ASK1 on the accumulation of the pro-apoptotic peptide BAX (A) and the anti-apoptotic peptide BCL2 (B); Data using quantitative immunocytochemistry (Leica Microsystems, Wetzlar, Germany); "b" indicates the effect of transfection with the gene construct; $P < 0.05$, indicates the difference between corresponding groups of transfected and non-transfected cells.

4.4. Interrelationships between IGF1 and ASK1

Addition of IGF1 did not affect ASK1, and transfection with the ASK1 construct did not substantially alter the effect of IGF1 on PCNA and progesterone. The available data do not indicate, therefore, that functional interrelationships exist between ASK1 and IGF1 within the ovary. Although IGF1 can be an important regulator of healthy ovarian cell functions (Sirotkin, 2014) and a promoter of ovarian cell malignant transformation (Kasprzak et al., 2017; Adamek and Kasprzak, 2018), there is no evidence that ASK1 affects these processes in functional cooperation with IGF1. In contrast, overexpression of the ASK1 gene resulted in stimulation of IGF1-induced ASK1 accumulation, indicating that, with specific conditions, the stimulatory activity of IGF1 on ASK1 and the capacity of ASK1 to modify IGF1 effects cannot be excluded.

4.5. TP53 effects

In the present experiments, transfecting granulosa cells with the TP53 expression construct promoted BAX accumulation, inhibited BCL2 and PCNA accumulation, and also stimulated progesterone release. This suggests that TP53 promotes cytoplasmic/extrinsic apoptosis and had pro-anti-proliferative, and steroidogenic activity in healthy ovarian granulosa cells. Although understanding the functional hierarchical interrelationships between these events requires further investigation, it cannot be excluded that TP53 can suppress granulosa cell proliferation by promoting the release of progesterone, a known inhibitor of ovarian cell mitosis, ovarian follicullogenesis, and fecundity (Peluso and Pru, 2014).

The action of TP53 on ovarian cells has been studied mainly in relation to cancer (Birkinshaw et al., 2017; Deng, 2017; Beyfuss

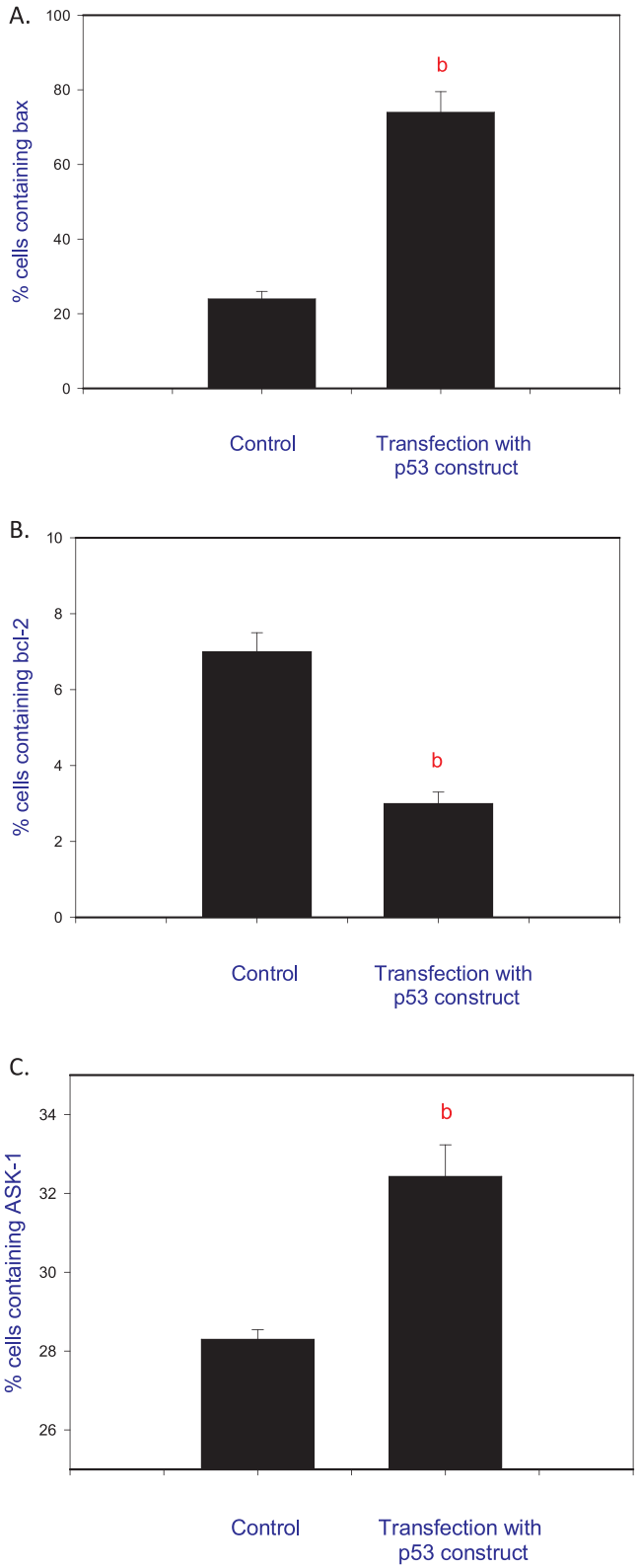

Fig. 6. Effect of transfecting cultured rabbit ovarian granulosa cells with the gene construct encoding TP53-1 on the accumulation of the pro-apoptotic peptide BAX (A), the anti-apoptotic peptide BCL2 (B), and ASK1 (C); Data using quantitative immunocytochemistry (Leica Microsystems, Wetzlar, Germany); "b" indicates the effect of transfection with the gene construct; $P < 0.05$, indicates the difference between corresponding groups of transfected and non-transfected cells.

and Hood, 2018). The suppressive effect of transfecting the TP53 expression construct has previously only been observed in healthy ovarian granulosa cells of pigs (Sirotkin et al., 2008, 2012). The present observations provide the first evidence for TP53 activity in non-porcine healthy ovarian granulosa cell functions, confirming that TP53 may be a physiological inhibitor of healthy ovarian cell functions in different species, and that this transcription factor could be useful for diagnostics and treatment of ovarian functions and dysfunctions including cancer.

4.6. Interrelationships between IGF1 and TP53

In the present experiments, transfection with the TP53 or TP53 + ASK1 constructs inhibited the stimulatory action of IGF1 on PCNA. Although TP53 did not modify the effect of IGF1 on progesterone release, TP53 + ASK1 inhibited this effect. Both TP53 and TP53 + ASK1 induced the inhibitory effects of IGF1 on ASK1. Therefore, TP53 can either prevent or induce IGF1 action on ovarian granulosa cells, and can suppress ovarian functions not only by promoting ovarian granulosa cell apoptosis and suppressing ovarian granulosa cell proliferation and steroidogenesis, but also by modifying the effects of IGF1, a physiological stimulator of ovarian functions.

4.7. Interrelationships between ASK1 and TP53

The results of the present experiments indicate the similarity of the effects of the ASK1 and TP53 constructs on ASK1, proliferation, and cytoplasmic/extrinsic apoptosis. Both the ASK1 and TP53 constructs promoted the accumulation of ASK1 and the cytoplasmic apoptosis-stimulating molecule BAX. Both inhibited the accumulation of the anti-apoptotic protein BCL2 and the proliferation marker PCNA, and neither altered the effect of IGF1 on progesterone release. Co-transfecting cells with the TP53 + ASK1 constructs did not increase the effects of the single constructs. The similar effects of ASK2 and TP53 on proliferation and cytoplasmic/extrinsic apoptosis, lack of cumulative action of these molecules, as well as the capacity of TP53 to stimulate ASK1 accumulation, suggest that TP53 can affect ovarian granulosa cell proliferation and this kind of apoptosis via stimulation of ASK1.

In contrast, ASK1 and TP53 had opposite effects on progesterone secretion and on the major effects of IGF1. Progesterone release was inhibited by ASK1, but promoted by TP53.Futhermore, the effects of IGF1 were enhanced by ASK1, but inhibited by TP53. In granulosa cells transfected with the ASK1 construct, IGF1 stimulated PCNA accumulation; but in granulosa cells transfected with TP53, IGF1 inhibited PCNA accumulation. These observations suggest either no, or a negative, interrelationship between ASK1 and TP53 in the control of progesterone release and some IGF1 actions.

Further ASK1 and TP53 loss-of-function studies and analyses of other cellular variables may help to enhance the understanding of the role, mechanisms of action, and interrelationships of ASK1 and TP53 in the ovary. Nevertheless, the present observations are the first indicating the capacity of ASK1 to suppress healthy ovarian granulosa cell functions, namely, to promote apoptosis, inhibit proliferation, and alter progesterone release. Furthermore, these results are the first indicating that there is TP53 activity in rabbit ovarian granulosa cells. In these cells, ASK1 can promote cytoplasmic/mitochondrial/extrinsic apoptosis and progesterone release as well as suppress proliferation and responses to IGF1. The similar effects of ASK1 and TP53 on extrinsic apoptosis and proliferation, the lack of cumulative action of these molecules, and the capacity of TP53 to promote ASK1 accumulation all indicate TP53 can suppress some ovarian granulosa cell functions as a result of ASK1 stimulation. The results of the present study may stimulate further investigation of these molecules, the application of which can be promising for characterization, prediction and regulation of ovarian functions.

Conflict of interest

The authors declare that they have no conflict of interest.

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References

Adamek, A., Kasprzak, A., 2018. Insulin-like growth factor (IGF) system in liver diseases. Intern. J. Mol. Sci. 27https://doi.org/10.3390/ijms19051308. pii: E1308.
Beyfuss, K., Hood, D.A., 2018. A systematic review of p53 regulation of oxidative stress in skeletal muscle. Redox Rep. 23, 100–117.
Birkinshaw, R.W., Czabotar, P.E., 2017. The BCL-2 family of proteins and mitochondrial outer membrane permeabilisation. Semin. Cell Dev. Biol. 72, 152–162.
Conover, C.A., 2018. The IGF-TP53 connection in cancer. Growth Horm. IGF Res. 39, 25–28.
Deng, J., 2017. How to unleash mitochondrial apoptotic blockades to kill cancers? Acta Pharm. Sin. B 7, 18–26.
Duffy, M.J., Synnott, N.C., Crown, J., 2017. Mutant TP53 as a target for cancer treatment. Eur. J. Cancer 83, 258–265.
Hong, L., Peng, S., Li, Y., Fang, Y., Wang, Q., Klausen, C., Yin, C., Wang, S., Leung, P.C.K., Yang, X., 2018. MiR-106a increases granulosa cell viability and is

downregulated in women with diminished ovarian reserve. J. Clin. Endocrinol. Metab. 103, 2157–2166.
Kasprzak, A., Kwasniewski, W., Adamek, A., Gozdzicka-Jozefiak, A., 2017. Insulin-like growth factor (IGF) axis in cancerogenesis. Mutat. Res./Rev. Mutat. Res. 772, 78–104.
Liu, T., Zhou, H.J., Min, W., 2017. ASK family in cardiovascular biology and medicine. Adv. Biol. Regul. 66, 54–62.
Makarevich, A., Sirotkin, A., Chrenek, P., Bulla, J., Hetenyi, L., 2000. The role of IGF1, cAMP/protein kinase A and MAP-kinase in the control of steroid secretion, cyclic nucleotide production, granulosa cell proliferation and preimplantation embryo development in rabbits. J. Steroid Biochem. Mol. Biol. 73, 123–133.
Osborn, M., Isenberg, S., 1994. . Immunocytochemistry of frozen and paraffin tissue sections. In: In: Celis, J.E. (Ed.), Cell Biology. A Laboratory Handbook, vol. 2. Academic Press, New York, pp. 361–367.
Peluso, J.J., Pru, J.K., 2014. Non-canonical progesterone signaling in granulosa cell function. Reproduction 147, R169–R178.
Simabuco, F.M., Morale, M.G., Pavan, I.C.B., Morelli, A.P., Silva, F.R., Tamura, R.E., 2018. TP53 and metabolism: from mechanism to therapeutics. Oncotarget 9, 23780–23823.
Sirotkin, A.V., 2014. Regulators of Ovarian Functions. Nova Publishers, Inc., New York, pp. 194.
Sirotkin, A.V., Grossmann, R., 2007. Leptin directly controls proliferation, apoptosis and secretory activity of cultured chicken ovarian cells. Comp. Biochem. Physiol. Part A: Mol. Integr. Physiol. 148, 422–429.
Sirotkin, A.V., Benco, A., Tandlmajerova, A., Vasícek, D., Kotwica, J., Darlak, K., Valenzuela, F., 2008. Transcription factor TP53 can regulate proliferation, apoptosis and secretory activity of luteinizing porcine ovarian granulosa cell cultured with and without ghrelin and FSH. Reproduction 136, 611–618.
Sirotkin, A.V., Benčo, A., Tandlmajerová, A., Vašíček, D., 2012. Involvement of transcription factor p53 and leptin in control of porcine ovarian granulosa cell functions. Cell Prolif. 45, 9–14.
Sirotkin, A.V., Chrenek, P., Kolesarová, A., Parillo, F., Zerani, M., Boiti, C., 2014. Novel regulators of rabbit reproductive functions. Anim. Reprod. Sci. 148, 188–196.
Sirotkin, A.V., Florkovičová, I.K., Švarcová, O.Ø., Rafay, J., Laurincik, J., Harrath, A.H., 2017. Caloric restriction and IGF1 administration promote rabbit fecundity: possible interrelationships and mechanisms of action. Theriogenology 90, 252–259.
Wang, S.C., 2014. PCNA: a silent housekeeper or a potential therapeutic target? Trends Pharmacol. Sci. 35, 178–186.
Yin, M., Zhou, H.J., Zhang, J., Lin, C., Li, H., Li, X., Li, Y., Zhang, H., Breckenridge, D.G., Ji, W., et al., 2017. ASK1-dependent endothelial cell activation is critical in ovarian cancer growth and metastasis. JCI Insight 21https://doi.org/10.1172/jci.insight.91828. pii: 91828.