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Identification of MicroRNAs Controlling Human Ovarian Cell Proliferation and Apoptosis

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Previous studies have shown that microRNAs (miRNAs) can control steroidogenesis in cultured granulosa cells. In this study we wanted to determine if miRNAs can also affect proliferation and apoptosis in human ovarian cells. The effect of transfection of cultured primary ovarian granulosa cells with 80 different constructs encoding human pre-miRNAs on the expression of the proliferation marker, PCNA, and the apoptosis marker, Bax was evaluated by immunocytochemistry. Eleven out of 80 tested miRNA constructs resulted in stimulation, and 53 miRNAs inhibited expression of PCNA. Furthermore, 11 of the 80 miRNAs tested promoted accumulation of Bax, while 46 miRNAs caused a reduction in Bax in human ovarian cells. In addition, two selected antisense constructs that block the corresponding miRNAs *mir-15a* and *mir-188* were evaluated for their effects on expression of PCNA. An antisense construct inhibiting *mir-15a* (which precursor suppressed PCNA) increased PCNA, whereas an antisense construct for *mir-188* (which precursor did not change PCNA) did not affect PCNA expression. Verification of effects of selected pre-*mir-10a*, *mir-105*, and *mir-182* by using other markers of proliferation (cyclin B1) and apoptosis (TdT and caspase 3) confirmed specificity of miRNAs effects on these processes. This is the first direct demonstration of the involvement of miRNAs in controlling both proliferation and apoptosis by ovarian granulose cells, as well as the identification of miRNAs promoting and suppressing these processes utilizing a genome-wide miRNA screen.

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MicroRNAs (miRNAs) are recently discovered small non-coding RNAs that regulate diverse genetic expression through their control of mRNA stability or translation. miRNA precursors (pre-miRNAs) generated in the nucleus are exported to the cytoplasm, where they are converted to a single-stranded mature miRNA. A mature miRNA, as part of an RNA-induced silencing complex (RISC), can bind 3′-untranslated regions of target mRNAs and induce their degradation, translational repression or both (Silveri et al., 2006; Stefani and Slack, 2006; Mattes et al., 2007; Farazi et al., 2008).

Elucidation of targets of miRNAs by using computer-based prediction based on sequence homology provides indirect evidence concerning the biological role of particular miRNAs (Silveri et al., 2006; Stefani and Slack, 2006; Gammell, 2007; Mattes et al., 2007; Farazi et al., 2008). Direct evidence of the biological functions of some miRNAs has been obtained by studies using transfection-based miRNA overexpression or silencing. Such approaches have indicated involvement of some miRNAs in control of proliferation, apoptosis, and carcinogenesis. The miRNAs that have been shown to inhibit cell proliferation are let-7, mir-17-92, mir-lat, mir-98, mir-125b, mir-133, mir-143, mir-221, mir-222, and mir-224. The inhibitory miRNAs can inhibit cell proliferation by affecting endocrine (IGFBP-5, TGF) and intracellular (RAS/cyclin/cyclin-dependent protein kinase, p27, c-MYC, E2F, SRF, MAP kinase/ERK5, Kit, and PCNA) regulators of the cell cycle (Gammell, 2007; Galardi et al., 2007; Mercatelli et al., 2008; Wang and Lee, 2009). The stimulatory miRNAs are mir-17, mir-372, and mir-373 and have been demonstrated to affect the cell cycle inhibitors, p21, p27, and p53 (Gammell, 2007).

In addition to affecting proliferation some miRNAs can promote apoptosis. The miRNAs (mir-34a,b,c, mir-214) function by activating the production of caspases, p21, p53, and bax (Corney et al., 2007; Gammell, 2007; Hermeking, 2009; Zenz et al., 2009). Alternatively, the miRNAs (mir-15, mir-16, mir-29b, mir-125b, mir-127) can initiate apoptosis by suppression of bmf/bcl, the blocker of the apoptotic inducer Bax (Wang and Lee, 2009; Xia et al., 2009) or certain oncogenes (MCL1, CCND1, and WNT3A, Bonci et al., 2008; Aqeilan et al., 2009). Finally, another miRNA (mir-224) can promote apoptosis by suppressing the apoptosis inhibitor API-6 (Wang and Lee, 2009). Some miRNAs are known inhibitors of apoptosis. Mir-210 and mir-155 decrease production of pro-apoptotic caspases (Wang and Lee, 2009). Mir-21 can reduce apoptosis by activating the anti-apoptotic interleukin/STAT3, PTEN/protein kinase, B/PI3 kinase, and PDCD4 (Yang et al., 2008; Shen et al., 2009; Wang and Lee, 2009; Zhang et al., 2009). Therefore, the involvement of miRNAs in either up- and

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down-regulation of PCNA-associated proliferation and Bax/Bcl-regulated apoptosis has been documented.

The majority of the data concerning the role of miRNAs in controlling proliferation and apoptosis have been obtained using normal and malignant cell lines, but not on primary cells retaining intact miRNA and their targets. Therefore, extrapolation to physiological processes in the intact organism should be interpreted very carefully. Furthermore, the previous comparative studies of the role of different miRNAs (Silveri et al., 2006; Stefani and Slack, 2006; Iorio et al., 2007; Moschos et al., 2007; Pan et al., 2007; Nam et al., 2008; Yang et al., 2008) compared expression/profiling, but not effect of different miRNAs. To our knowledge, only one publication (Ovcharenko et al., 2007) compared the effect of the majority of known miRNAs on apoptosis. Other available publications analyzed the effect of a single or a few miRNAs. No direct genome-wide comparison of the effect of a large number of known miRNAs on both proliferation and apoptosis has been performed.

Our understanding of the involvement of miRNAs in the control of gonadal functions are limited to one description of miRNA profiles in normal ovarian cells (Ro et al., 2007), several descriptions of miRNA profiles in ovarian carcinoma cells (Corney et al., 2007; Iorio et al., 2007; Nam et al., 2008; Yang et al., 2008; Wang and Lee, 2009) and two studies on the effects of miRNAs on expression of the HER-2 ovarian tumor protein (Tsuda et al., 2005) and ovarian hormone release (Sirotkin et al., 2009a). Although proliferation and apoptosis of healthy ovarian cells plays a key role in ovarian follicular development, selection, ovulation, and fertility; as well as in the characterization, development, and treatment of reproductive disorders (Hillier, 2001; Macklon and Fauser, 2001; Stouffer et al., 2007), the role of miRNAs in the control of these processes remains unknown, and the miRNAs involved are not identified.

The aim of our studies was to examine the involvement of miRNAs in control of proliferation and apoptosis in human ovarian cells, specifically, to identify miRNAs affecting these processes via genome-scale screen. To this end, the effect of transfection of cultured primary ovarian granulosa cells with gene constructs encoding the majority of identified human pre-miRNAs on the expression of marker of cell proliferation, PCNA and of apoptosis, Bax, was evaluated. The obtained data were verified by evaluation of other markers of proliferation (cyclin B1) and apoptosis (TdT and caspase 3) in granulosa cells transfected with selected pre-miRNAs, and by assessment of PCNA expression in cells transfected with antisense constructs inhibiting selected miRNAs. PCNA and cyclin B1 are both markers and regulators of cell proliferation (Maga and Hubscher, 2003; Galardi et al., 2007; Mercatelli et al., 2008), whilst Bax, caspase 3, and TdT are known markers of apoptosis (Gupta et al., 2009; Zenz et al., 2009; Wang and Lee, 2009).

Materials and Methods

Oligonucleotides

In our experiments, a human/murine Custom Pre-miR™ miRNA Precursor Library (Ambion, Austin, TX) containing 187 individual synthetic miRNA precursors that mimic endogenous precursor miRNAs representing the majority of human miRNAs known at the time and listed in Sanger Institute miRBase:Sequence database (http://microrna.sanger.ac.uk/cgi-bin/sequences/mirna_summary.pl?org=hsa) were used. The majority of these miRNAs was identified in ovarian cells (Corney et al., 2007; Iorio et al., 2007; Ro et al., 2008; Wang and Lee, 2009). Each individual microRNA strand was synthesized according to this database. Two pre-miRNA non-silencing random sequence miRNAs (Ambion), with no homology to any known gene sequence, as well as antisense RNA constructs inhibiting activity of endogenous miRNAs *mir15a* and *mir188* which was synthesized on the basis of Sanger Institute miRBase:Sequence diabase (Ambion) were used as negative controls. Positive controls were the human siRNAs targeting GAPDH mRNA (Silencer® GAPDH siRNA, Ambion) and CREB-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), given at the same concentration as all miRNAs. Single-stranded oligonucleotides were synthesized and purified by high performance liquid chromatography (more than 90% purity as detected by mass spectroscopy), annealed to generate the double-stranded miRNA precursor molecules or siRNAs and analyzed by PAGE (Ambion). Cellular accumulation of 10 randomly selected miRNAs was validated by transfection of HepG2 cells (ATCC, Rockville, MD) using HepG2 transfection reagent (Altogen Biosystems, Las Vegas, NV) according to the manufacturer's recommended transfection protocol, and quantitated by real-time PCR using TaqMan MicroRNA assay (Applied Biosystems, Foster City, CA). RNAs were placed in 96-well plates (Axygen Scientific, Inc., Union City, CA) at a final concentration of 30 nM in 100 μl/well of complete growth media.

Preparation, transfection, culture, and processing of granulosa cells

Experimental design is similar to the design of our previous experiments aimed to identify effects of miRNAs (Sirotkin et al., 2009a) and siRNAs (Sirotkin et al., 2009b) on human ovarian functions. Ovarian granulosa cells were harvested 1–5 days after a spontaneous ovulation from women 36–42 years of age with normal ovarian cycles and morphology who were undergoing ovariectomy because of non-metastatic cancer of the *cervix uteri*. Informed consent of the patients was obtained in accordance with EU and Slovak ethical and medical regulations under supervision of the local ethical committee. Granulosa cells ($30–50 \times 10^6$ per ovary) were isolated and processed as described previously (Sirotkin et al., 2005). Immediately after isolation granulosa cells were suspended in DMEM/Ham's F-12 1:1 mixture (Gibco-Invitrogen, Carlsbad, CA) and the suspension (2×10^6 cells/ml,

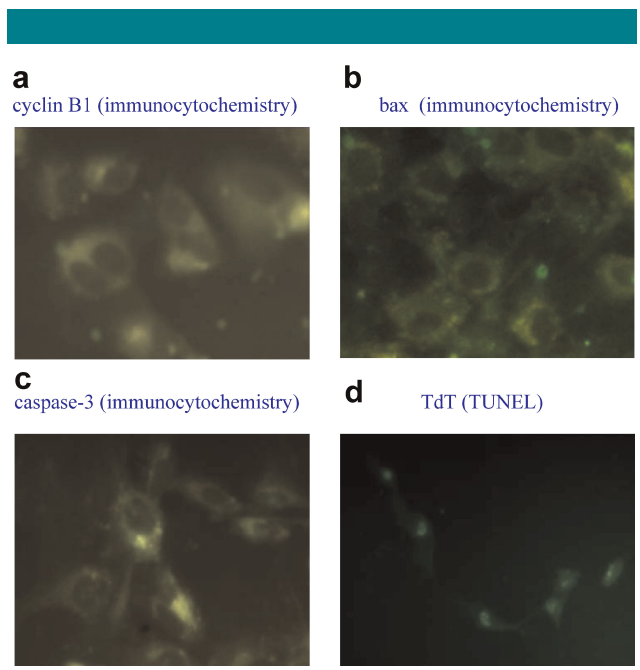

Fig. 1. Presence of proliferation-related protein cyclin B1 (a) and apoptosis-related proteins Bax (b), caspase-3 (c), and TdT (d) in cytoplasm of cultured human ovarian granulose cells. Antigens were detected after 48 h culture by using immunocytochemistry (cyclin B1, Bax, and caspase-3) or TUNEL (TdT) and visualized by FITC and fluorescent microscopy as indicated in the Materials and Methods Section. Magnification 800× (a,b,c) and 200× (d).

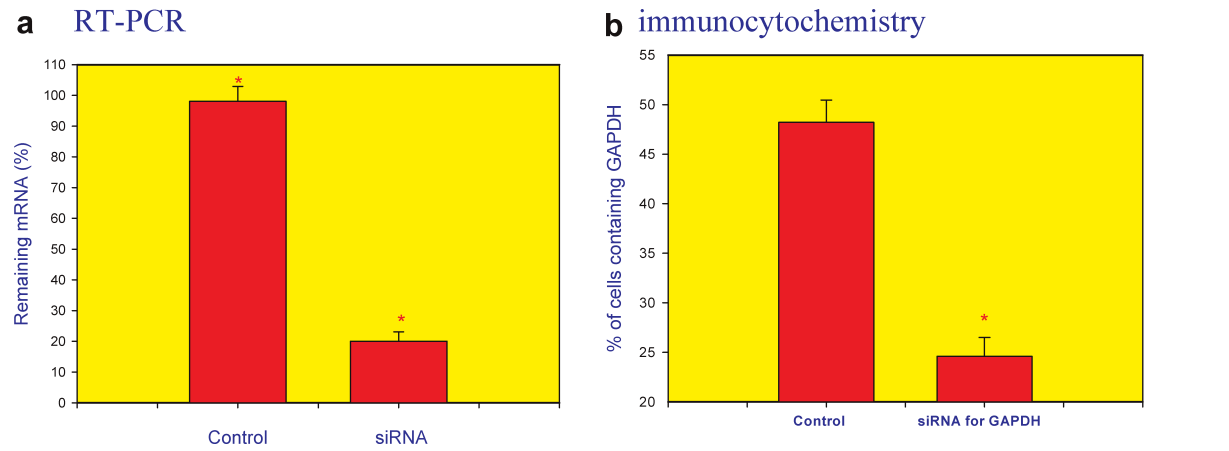

Fig. 2. Silencing of marker protein GAPDH by siRNA against GAPDH in cultured human ovarian granulosa cells. Human granulosa cells were transfected with siRNA construct down-regulating GAPDH. Gene expression levels were determined by real-time PCR (a) and immunocytochemistry (b) 48 h after transfection. By RT-PCR, corrected values were normalized to a sample that was transfected with the non-silencing siRNA. By immunocytochemistry, accumulation of GAPDH protein (percentage of cells containing GAPDH labeled by DAB in relation to total number of cells) was measured. Values represent means ± SEM. *$P < 0.05$ versus control group (cells transfected with non-silencing oligonucleotides).

determined by hemocytometer) was placed in 96-well plates (Axygen Scientific, Inc., 50 μl/well) containing miRNAs and siRNAs. miRNA and siRNA transfections were performed using cationic transfection reagent (JetSI-ENDO, Polyplus-Transfection, Illkirch, France) according to the manufacturer's instructions, and control transfections were performed with (for transfection efficiency) the same transfection reagent conjugated with tetramethylrhodamine (Jet-SI-Endo-FluoR, Polyplus-Transfection). After transfection, cells were diluted with culture medium DMEM/Ham's F-12 1:1 mixture supplemented with 10% bovine fetal serum and 1% antibiotic–antimycotic solution (all from Gibco-Invitrogen) to 0.5×10^6 cells/ml and cultured 48 h in 200 μl of culture medium/well. After culture, the wells were washed three times in ice-cold PBS, fixed for 20 min in 4% paraformaldehyde in PBS, washed in PBS (2×5 min), ethanol (70%: 5 min, 80%: 10 min, 96%: 2×10 min, 100%: 10 min) and kept in 100% ethanol at -18°C for immunocytochemical and TUNEL analysis. In selected wells, cell concentration and viability were performed by Trypan Blue staining and cell counting using a hemocytometer.

Immunocytochemical analysis

Marker proteins in granulosa cells plated on plate wells were detected by immunocytochemistry (Osborn and Isenberg, 1994) by using primary mouse monoclonal antibodies against GAPDH, CREB-1, PCNA, Bax, cyclin B1, and caspase 3 (which cross-react with corresponding human proteins; all from Santa Cruz Biotechnology, Inc.; dilution 1:100), secondary goat IgG labeled with FITC (Sevac, Prague, Czech republic, dilution 1:500). The specificity of primary antibodies and molecular weights of ligands were confirmed by Western blotting (Sirotkin et al., 2006). Cells treated with labeled secondary antibody but omitting the primary antibody were used as negative controls. The presence of specific immunoreactivity and of transfection reagent labeled with tetramethylrhodamine (Jet-SI-Endo-FluoR) in cells was determined by fluorescent microscopy. Presence of cyclin B1, bax, and caspase-3 in human ovarian granulosa cells is illustrated by Figure 1a–c.

Real-time reverse transcription-PCR analysis

Total RNA from cells transfected with siRNA for marker protein GAPDH was isolated using the MagMAX93 Total RNA isolation kit (Ambion). Purified DNase-treated RNA was reverse transcribed using the RETROscript Kit (Ambion). GAPDH gene expression level was determined by real-time PCR on the ABI Prism 7900 Sequence Detection system (Applied Biosystems) using SuperTaq reagents (Ambion) and SYBR Green (Molecular Probes-Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Details of GAPDH PCR analysis were described previously (Ovcharenko et al., 2007).

TUNEL assay

The chamber slides were subjected to the TUNEL (TdT-mediated dUTP nick end labeling) assay by using In Situ Cell Death Detection Kit (Boehringer Mannheim, GmbH, Mannheim, Germany)

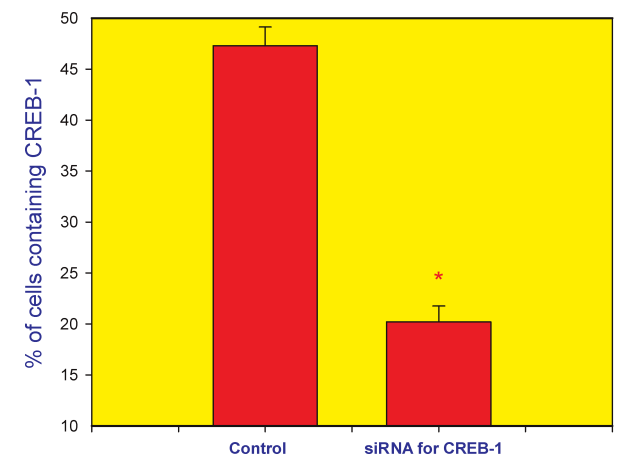

Fig. 3. Silencing of marker protein CREB-1 by siRNA against CREB-1 in cultured human ovarian granulose cells. Human granulosa cells were transfected with siRNA construct down-regulating CREB-1. Accumulation of CREB-1 protein (percentage of cells containing CREB-1 labeled by DAB in relation to total number of cells) was measured 48 h after transfection by immunocytochemistry and light microscopy. Legends as in Figure 2.

according to the instruction of the manufacturer. Cells containing intensive TdT-positive staining in the nuclei were considered apoptotic. Fixed and permeabilized cells incubated without TdT but with secondary FITC-conjugated antibody, were used as negative controls. Permeabilized cells incubated with bovine pancreatic DNAse I (Boehringer Mannheim GmbH, 0.01 mg/ml; 10 min at room temperature) before TdT treatment to induce DNA fragmentation, were used as positive controls. The percentage of TUNEL/TdT-positive cells in each culture was determined by counting of TUNEL-positive and TUNEL-negative cell number using fluorescent microscopy. Presence of TdT in cytoplasm of cultured cells is shown in Figure 1d.

Statistics

Each experimental group was represented by three wells with granulosa cells. The data presented concerning the effects of each substance are means of values obtained in three separate experiments performed on separate days using separate pools of ovaries, obtained from three patients. The proportion of cells containing each analyzed substance was calculated following immunocytochemical analysis by counting at least 1,000 cells per well. All values obtained by PCR were normalized to those obtained for non-silencing control. Significant differences between the experiments were evaluated using one-way ANOVA. When

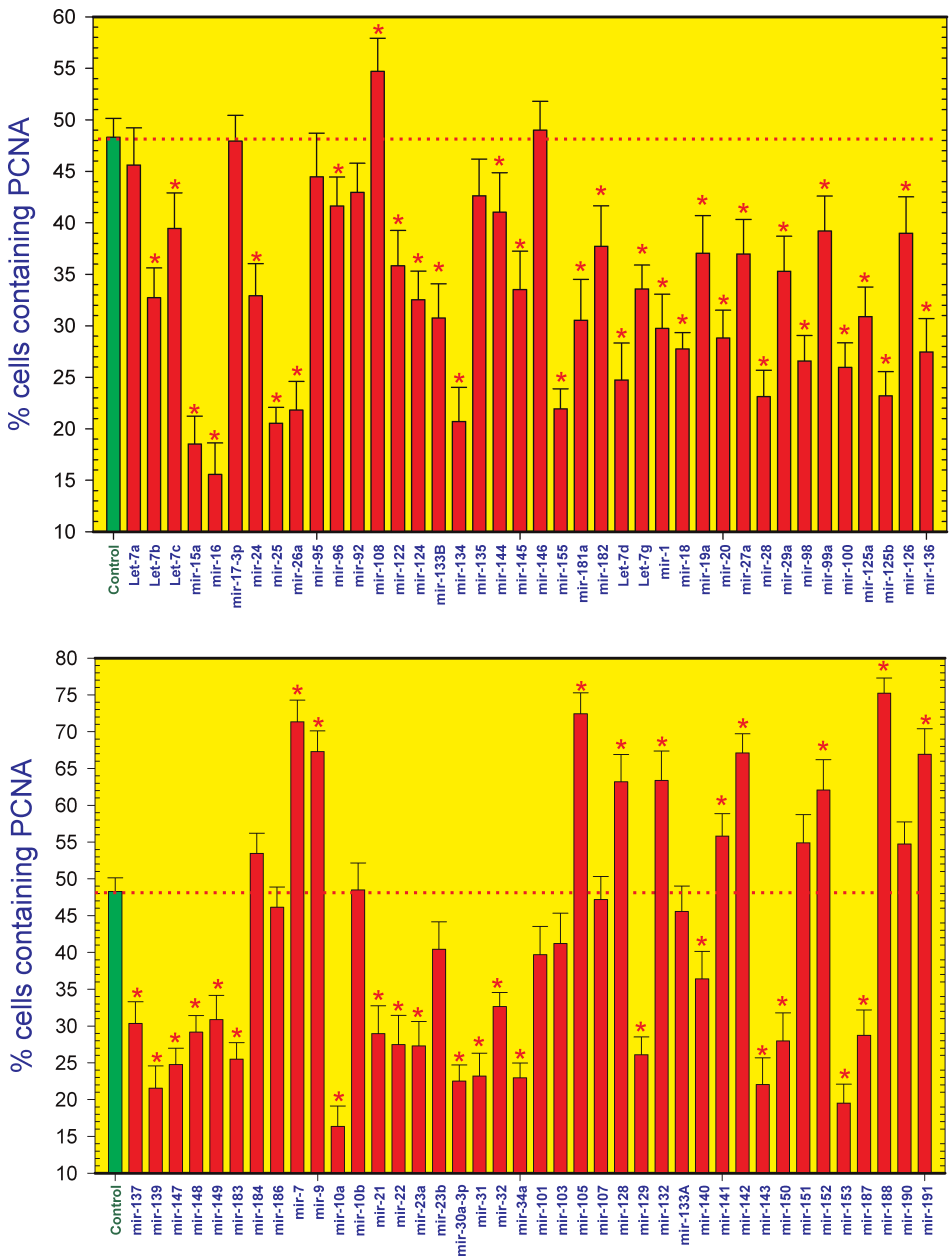

Fig. 4. Changes in the expression of PCNA in cultured human ovarian granulosa cells after transfection with miRNA precursors. Human granulosa cells were transfected with miRNA constructs indicated in the x-axis label. Expression of PCNA (percentage of cells containing PCNA labeled by FITC in relation to total number of cell nuclei stained by DAPI) was measured 48 h after transfection using immunocytochemistry and fluorescent microscopy. Legends as in Figure 2.

effects of treatments were revealed, data from the experimental and control groups were compared by Duncan's multiple range test. Differences from control where $P < 0.05$ were considered as significant.

Results

Evaluation of efficiency of transfection and silencing

To demonstrate that primary human ovarian granulosa cells were amenable to transfection, microscopic analysis, following treatment with the transfection reagent labeled with tetramethylrhodamine (Jet-SI-Endo-FluoR), showed that more than 90% of cells included fluorescent transfection reagent after culture. Real-time RT-PCR analysis of mRNA for marker protein GAPDH after transfection with corresponding siRNA provided reduction in specific mRNA expression for GAPDH (Fig. 2a). Additionally, immunocytochemical analysis showed siRNA-induced reduction in expression of marker proteins GAPDH (Fig. 2b) and CREB-1 (Fig. 3).

Microscopic and immunocytochemical analysis of human granulosa cells from either control, siRNA- and miRNA-transfected groups showed that cells were viable and had morphology characteristic for relatively healthy cells. Moreover, immunocytochemical analysis detected the presence of the proliferation-associated proteins, cyclin B1 (Figs. 1a and 5), PCNA (Figs. 4 and 6) and the apoptosis-related proteins, Bax (Figs. 1b and 7), TdT (Figs. 1d and 8a) and caspase-3 (Fig. 8b). PCNA and TdT (Fig. 1d) were localized in both the cytoplasm and nucleus, whilst cyclin B1 (Fig. 1a), Bax (Fig. 1b), and caspase-3 (Fig. 1c) were detected exclusively in the ovarian cell cytoplasm. Checking cell concentration and viability (75–80%) revealed no significant differences in these indices between the control and miRNA-transfected groups.

Identification and quantification of biological activity of miRNAs affecting expression of proliferation markers

Transfection of cultured human granulosa cells with 11 out of 80 tested miRNA constructs resulted in significant increase in percentage of cells containing PCNA. The miRNAs increasing expression of PCNA 1.2–1.7 times were *mir-108*, *mir-7*, *mir-9*, *mir-105*, *mir-128*, *mir-132*, *mir-141*, *mir-142*, *mir-152*, *mir-188*, and *mir-191*.

PCNA expression was highly reduced (in some cases more than 4.5-fold) after cell transfection with 53 miRNAs—*let-7b*, *let-7c*, *mir-15a*, *mir-16*, *mir-24*, *mir-25*, *mir-26a*, *mir-96*, *mir-122*, *mir-124*, *mir-133b*, *mir-134*, *mir-144*, *mir-145*, *mir-155*, *mir-181a*, *mir-182*, *let-7d*, *let-7g*, *mir-1*, *mir-18*, *mir-19a*, *mir-20*, *mir-27a*, *mir-28*, *mir-29a*, *mir-98*, *mir-99a*, *mir-100*, *mir-125a*, *mir-125b*, *mir-128*, *mir-136*, *mir-137*, *mir-139*, *mir-147*, *mir-148*, *mir-149*, *mir-183*, *mir-10a*, *mir-21*, *mir-22*, *mir-23a*, *mir-30a-3p*, *mir-31*, *mir-32*, *mir-34a*, *mir-129*, *mir-140*, *mir-143*, *mir-150*, *mir-153*, and *mir-187*. Other miRNAs tested did not affect expression of PCNA (Fig. 4).

The cells transfected with miRNA constructs stimulating *mir-10a*, *mir-105* and *mir-182* had reduced expression of cyclin B1 (Fig. 5).

An antisense construct inhibiting *mir-15a*, but not *mir-188*, significantly promoted accumulation of PCNA (Fig. 6).

Identification and quantification of biological activity of miRNAs affecting expression of apoptosis markers

Eleven of the 80 miRNAs tested promoted up to twofold accumulation of Bax in human ovarian cells. These were *mir-15a*, *mir-96*, *mir-92*, *mir-124*, *mir-18*, *mir-29a*, *mir-125a*, *mir-136*, *mir-147*, *mir-183*, and *mir-32*. Forty-six miRNAs reduced the expression of Bax in ovarian cells up to fourfold. These were *let-7b*, *let-7c*, *mir-17-3p*, *mir-24*, *mir-25*, *mir-26a*, *mir-95*, *mir-133b*, *mir-134*, *mir-135*, *mir-144*, *mir-145*, *mir-155*, *mir-181a*, *let-7a*, *let-7g*, *mir-1*, *mir-20*, *mir-125b*, *mir-126*, *mir-137*, *mir-139*, *mir-148*, *mir-149*, *mir-186*, *mir-21*, *mir-22*, *mir-23a*, *mir-30a-3p*, *mir-31*, *mir-34a*, *mir-105*, *mir-107*, *mir-128*, *mir-129*, *mir-132*, *mir-133a*, *mir-140*, *mir-14*, *mir-150*, *mir-151*, *mir-152*, *mir-153*, *mir-187*, *mir-190*, and *mir-191* (Fig. 7).

The transfection of cells with miRNA constructs stimulating *mir-105* and *mir-182*, but not *mir-10a* resulted in reduced expression of TdT (Fig. 8a) and caspase-3 (Fig. 8b).

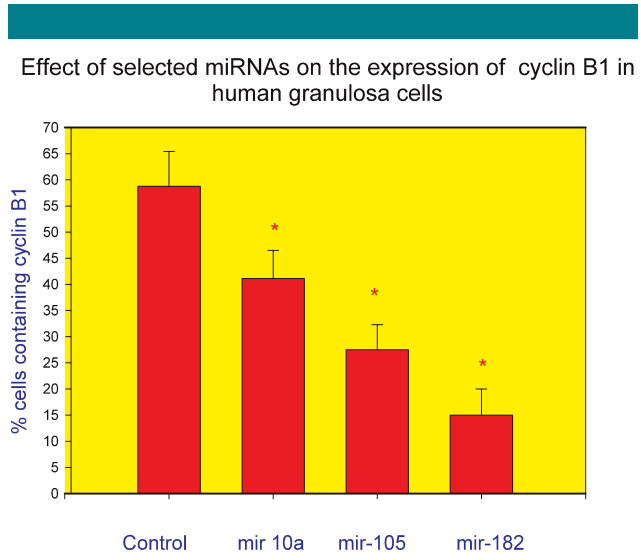

Fig. 5. Changes in the expression of cyclin B1 in cultured human ovarian granulosa cells after transfection with precursors stimulating *mir-10a*, *mir-105*, and *mir-182*. Human granulosa cells were transfected with miRNA constructs indicated in the *x*-axis label. Expression of cyclin B1 (percentage of cells containing cyclin B1 labeled by FITC in relation to total number of cell nuclei stained by DAPI) was measured 48 h after transfection using immunocytochemistry and fluorescent microscopy. Legends as in Figure 2.

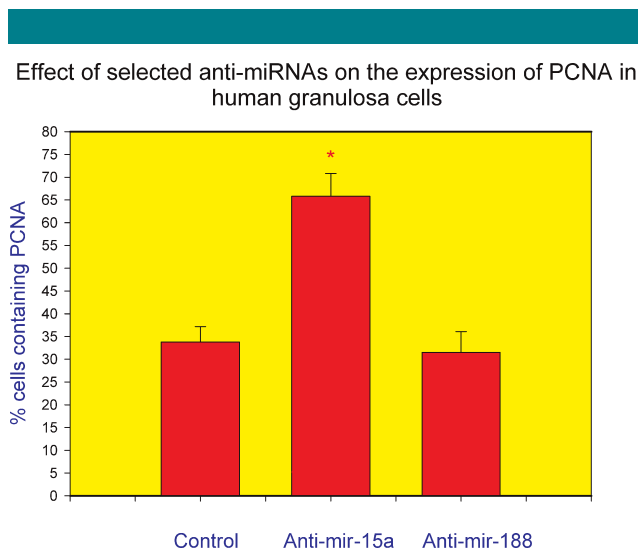

Fig. 6. Changes in the expression of PCNA in cultured human ovarian granulosa cells after transfection with antisense constructs inhibiting *mir-15a* and *mir-188*. Human granulosa cells were transfected with miRNA constructs indicated in the *x*-axis label. Expression of PCNA (percentage of cells containing PCNA labeled by FITC in relation to total number of cell nuclei stained by DAPI) was measured 48 h after transfection using immunocytochemistry and fluorescent microscopy. Legends as in Figure 2.

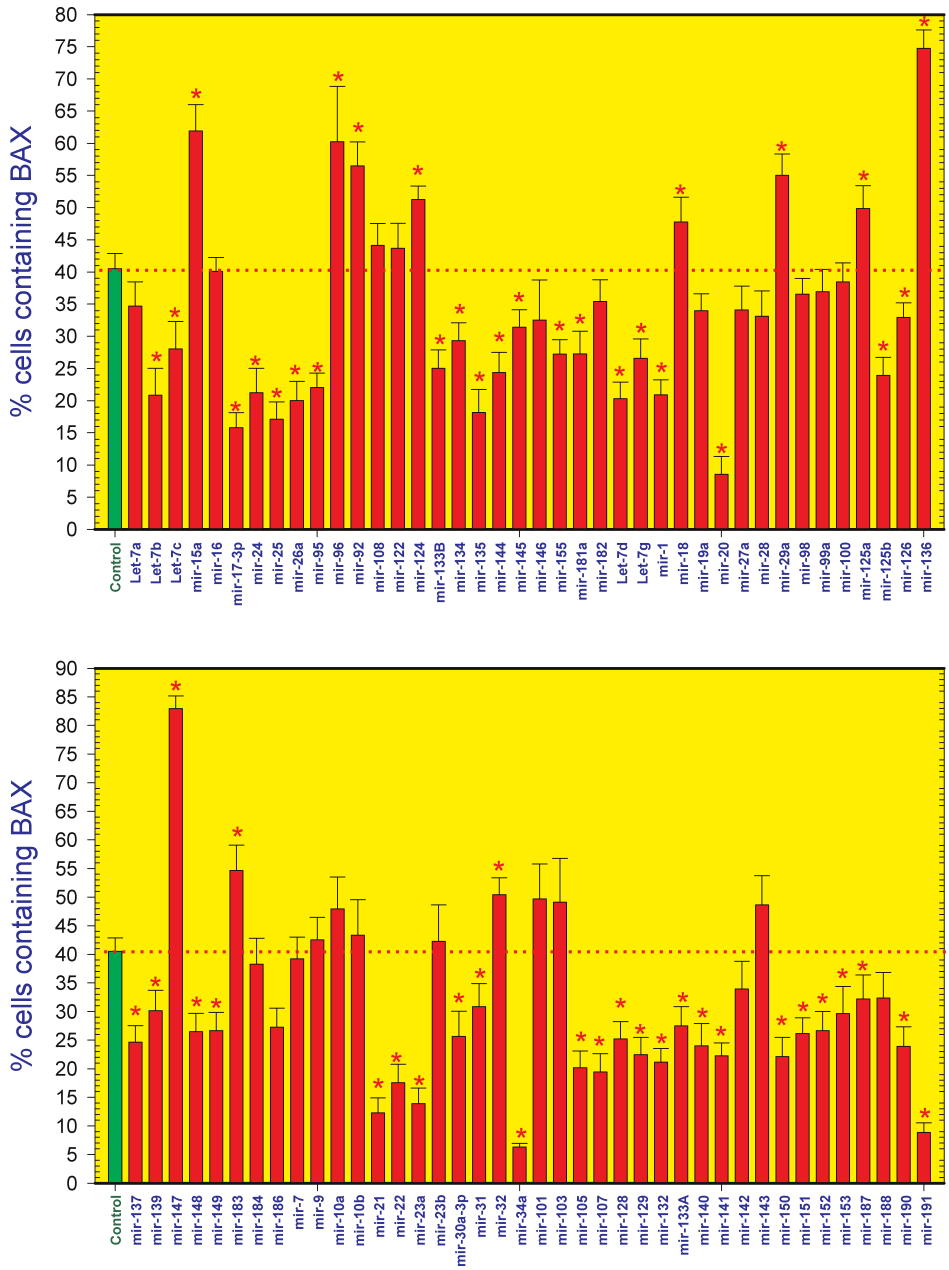

Fig. 7. Changes in the expression of Bax in cultured human ovarian granulosa cells after transfection with miRNA precursors. Human granulosa cells were transfected with miRNA constructs indicated in the *x*-axis label. Expression of Bax (percentage of cells containing Bax labeled by FITC in relation to total number of cell nuclei stained by DAPI) was measured 48 h after transfection using immunocytochemistry and fluorescent microscopy. Legends as in Figure 2.

Discussion

Microscopic and immunocytochemical analysis demonstrated good cell viability and presence of proliferation markers PCNA and cyclin B1 in the cells after culture. On the other hand, the presence of apoptosis markers Bax, TdT, and caspase 3 in the cells was revealed too. Therefore, the primary culture of ovarian granulosa cells could be an adequate model for study of both proliferation and apoptosis and their genomic regulators. Previously this model was successfully used for examination of miRNA (Sirotkin et al., 2009a) and siRNAs (Sirotkin et al., 2009b) effects on ovarian steroid secretory activity. The present results of fluorescent microscopy, real-time RT-PCR analysis and immunocytochemical analysis of transfection and expression markers (tetramethylrhodamine conjugated with transfection reagent, GAPDH mRNA, GAPDH, and CREB-1) confirm our previous data (Ovcharenko et al., 2007; Sirotkin et al., 2009a,b) on the efficiency and reproducibility of the miRNA delivery method used. Previously only the presence of numerous miRNA in ovarian cells was demonstrated, while some miRNAs were specific only for the ovary (Ro et al., 2007). Testing of miRNAs, part of which were homologous to murine ovarian-specific miRNAs (let-7, mir-10, mir-15, mir-16, mir-02, mir-151, mir-30-5, mir-22, see Ro et al., 2007) revealed the

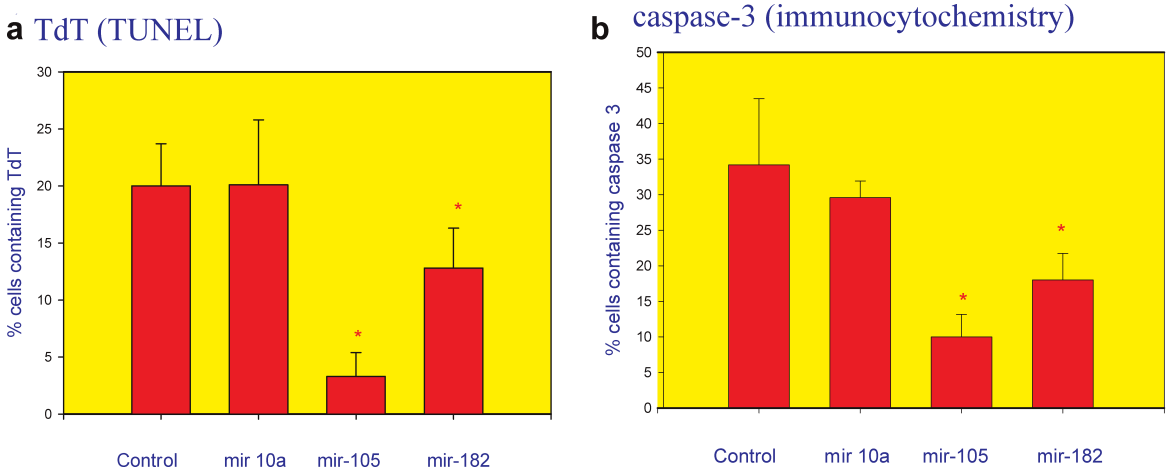

Fig. 8. Changes in the expression of markers of apoptosis, TdT (a) (TUNEL) and caspase-3 (immunocytochemistry) in cultured human ovarian granulosa cells after transfection with precursors stimulating *mir-10a*, *mir-105*, and *mir-182*. Human granulosa cells were transfected with miRNA constructs indicated in the *x*-axis label. Expression of TdT and caspase-3 (percentage of cells containing antigen labeled by FITC in relation to total number of cell nuclei stained by DAPI) was measured 48 h after transfection using fluorescent microscopy. Legends as in Figure 2.

influence of some miRNAs on ovarian cell proliferation and apoptosis. The potential involvement of miRNAs in the control of ovarian cell proliferation and apoptosis was indicated previously only indirectly, by different miRNAs profiles in normal and malignant ovarian cells (Nam et al., 2008; Yang et al., 2008; Wang and Lee, 2009). These are the first direct demonstration, that miRNAs control ovarian cell proliferation and apoptosis. The finding of both inhibitory and stimulatory effects of miRNAs in this study confirms the previous finding in non-ovarian cells that miRNA can either up- and down-regulate expression of some genes related to proliferation (Gammell, 2007; Galardi et al., 2007; Mercatelli et al., 2008; Wang and Lee, 2009) and apoptosis (Corney et al., 2007; Hermeking, 2009; Shen et al., 2009; Wang and Lee, 2009; Xia et al., 2009; Zenz et al., 2009), probably via suppression of transcription repressors (Mattes et al., 2007; Wang and Lee, 2009). The opposite effects of tested *mir-15a* antagonist and precursor on PCNA, as well as the similarity of effect of some selected miRNAs on the expression of two markers of cell proliferation (PCNA and cyclin B1) and of three markers of apoptosis (Bax, TdT, and caspase 3) observed in our experiments confirm the specificity of miRNAs effect on both proliferation and apoptosis of human ovarian cells. On the other hand, different effects of *mir-105* on the expression of PCNA and cyclin B1 observed in our experiments, suggest, that this miRNA can have different action on different phases of the cell cycle because increased accumulation of PCNA occurs during S-phase, and cyclin B1- during G2-phase of mitotic cycle (Jones and Kazlauskas, 2001; Maga and Hubscher, 2003).

Noteworthy, there was previous report of the involvement of miRNAs in control of ovarian cell proliferation and apoptosis. Therefore, the present observations, together with our previous report on the involvement of miRNAs in the control of ovarian hormone release (Sirotkin et al., 2009a) demonstrates the existence of new, miRNA-dependent, mechanism of control of ovarian functions.

Similarity of effect of some miRNAs (let-7, mir-98, mir-125b) on ovarian cell proliferation observed here and previously on non-ovarian cells (Gammell, 2007; Galardi et al., 2007; Mercatelli et al., 2008; Wang and Lee, 2009) suggest universal role of these miRNAs in control of proliferation of different cell types. Nevertheless, this is the first demonstration of involvement of a number of new mRNAs in ether inhibition and stimulation of cell proliferation.

The ability of mir-15a to promote apoptosis in ovarian cells corroborates the previous reports (Bonci et al., 2008; Aqeilan et al., 2009; Wang and Lee, 2009; Xia et al., 2009) on the pro-apoptotic role of this miRNA in other cell types. On the other hand, the opposite action of mir-34a on ovarian and non-ovarian cells (Corney et al., 2007; Gammell, 2007; Hermeking, 2009; Zenz et al., 2009) suggest a different role of this miRNA in different cell types. Moreover, this is the first demonstration of the involvement of some miRNAs in up- and down-regulation of apoptosis in any cell type.

The comparison of miRNAs showed, that some very close paralogs had some very disparate effects. This suggests a very high specificity of miRNA in the control of ovarian cell proliferation and apoptosis, when only slight changes in their composition can dramatically have diverse effects. Furthermore, this comparison suggests that some miRNAs could be involved in the control of both proliferation and apoptosis.

The association of ovarian follicle growth/cell proliferation and atresia/apoptosis with the production of estrogens and androgens (Hillier, 2001; Jamnongjit and Hammes, 2006; Stouffer et al., 2007) and the ability of these hormones to promote cell proliferation and malignant transformation of reproductive organs (Jamnongjit and Hammes, 2006; Jongen et al., 2006), as well as the action of a number of miRNAs on ovarian testosterone and estradiol release (Sirotkin et al., 2009a) suggest that some effects of miRNAs on ovarian cell proliferation and apoptosis can be mediated by changes in steroid hormones. In support of this idea, our two studies using primary granulosa cells have enumerated a number of miRNAs that affect both ovarian steroid release and proliferation and apoptosis. Practically all studied miRNAs were able to affect both steroid hormone and proliferation or apoptosis.

Understanding the biological role and mechanisms of action of different miRNA in the ovary requires further studies. Nevertheless, the observed effects of miRNAs on proliferation, apoptosis, and steroidogenesis within the ovary, as well as the association of miRNA expression with malignant transformation of ovarian cells suggest importance and

potential usefulness of miRNAs in the control of reproduction and treatment of reproductive disorders.

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