

MicroRNAs control transcription factor NF- κ B (p65) expression in human ovarian cells

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Received: 31 May 2014 / Revised: 11 September 2014 / Accepted: 7 November 2014 / Published online: 18 November 2014
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Abstract MicroRNAs (miRNAs) are known to influence ovarian cell proliferation, apoptosis and hormone release, but it remains unknown whether miRNAs affect ovarian functions via transcription factors. We examined the effect of miRNAs on nuclear factor- κ B (NF- κ B) (p65) expression in human ovarian luteinized granulosa cells. We transfected cultured primary human ovarian luteinized granulosa cells with 80 different constructs encoding human pre-miRNAs and then evaluated NF- κ B (p65) expression (percentage of cells containing p65) by immunocytochemistry. We found that 21 of the constructs stimulated NF- κ B (p65) expression and 18 of the constructs inhibited NF- κ B (p65) expression. This is the first direct demonstration that miRNAs affect NF- κ B (p65) expression and the first genome-scale miRNA screen to identify upregulation and downregulation of NF- κ B accumulation by miRNAs in the ovary. Novel miRNAs that affect the NF- κ B signalling pathway could be useful for the control of NF-

κ B-dependent reproductive processes and the treatment of NF- κ B-dependent reproductive disorders.

Keywords miRNAs · NF- κ B (p65) · Ovary · Reproductive medicine

Introduction

The nuclear factor- κ B (NF- κ B) is a family of regulatory proteins controlling many biological processes such as proliferation, survival, apoptosis, inflammation, stress response, angiogenesis, tissue invasion and metastasis in many normal and cancerous cell types (Karin 2006; Sethi et al. 2008; Wullaert et al. 2011; Lan et al. 2012; Siomek 2012). NF- κ B proteins, especially RelA (p65) and NF- κ B (p50) (Aggarwal 2004; Hayden and Ghosh 2008; Pavlová et al. 2011), can be important regulators of ovarian cell function and pathological transformation. NF- κ B is reported to inhibit apoptosis in normal rat luteal cells (Telleria et al. 2004) and granulosa cells (Xiao et al. 2002; Wang et al. 2002). A transfected NF- κ B (p65) complementary DNA (cDNA) construct reduced nuclear apoptosis, increased mitochondrial apoptosis, stimulated cell proliferation and altered hormone release in porcine granulosa cells (Pavlová et al. 2011). The construct also stimulated cell proliferation and altered hormone release in rat corpus luteum cells (Telleria et al. 2004) but not in human luteal cells (Gonzalez-Navarrete et al. 2007). In addition, NF- κ B has been implicated as an essential factor for survival and apoptosis in human ovarian cancer cells (Zerbini et al. 2011).

NF- κ B could be regulated not only by cDNA but also by microRNAs (miRNAs). miRNAs are a class of small, non-coding RNAs (18–25 nucleotides) that act in post-transcriptional gene regulation. After the precursor miRNAs (pre-miRNAs) are transcribed, they are transported to the cytosol and converted to miRNA-miRNA duplexes. The

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guide strand of a mature miRNA is then incorporated into an RNA-induced silencing complex, which may promote the cleavage or translational repression of target messenger RNAs (mRNAs) (Gammell 2007; Baley and Li 2012; Donadeu et al. 2012). The regulation of NF- κ B signalling by miRNAs in non-ovarian cells was reported previously (Wan et al. 2010; Jiang et al. 2012; Orlarin-George et al. 2013).

RNA interference is involved in the control of basic ovarian cellular functions (Sirotkin et al. 2010a, 2012). Association between the expression of NF- κ B and miR-224 in mouse ovarian granulosa cells (Liang et al. 2013), miR-9 (Guo et al. 2009) and miR-199a (Chen et al. 2008; Yin et al. 2010) in human ovarian cancer cells and miR-199a in human ovarian endometrioma cells (Dai et al. 2012) has been reported. Despite the potential importance of NF- κ B and miRNAs in the control of cellular reproduction, it remains unknown whether miRNAs directly affect ovarian NF- κ B.

We performed a genome-scale screen to identify miRNAs involved in the control of NF- κ B (p65) and determine the effects of these miRNAs on NF- κ B (p65) expression. We transfected cultured primary human ovarian luteinized granulosa cells with 80 pre-miRNA gene constructs and examined the effects of the respective constructs on NF- κ B (p65) expression (percentage of cells containing p65).

Materials and methods

Isolation, transfection and culture of luteinized granulosa cells

We used an experimental design similar to that of our previous experiments aimed at identifying the effects of miRNAs (Sirotkin et al. 2009, 2010b) and siRNAs (Sirotkin 2010) on the functions of human ovarian luteinized granulosa cells. We aspirated follicular fluid with luteinized granulosa cells 1–5 days after spontaneous ovulations from two women 36–42 years of age in each of three experiments, with normal ovarian cycles and morphology who were undergoing ovariectomy because of non-metastatic cancer of the cervix uteri after obtaining the informed consent in accordance with EU and Slovak ethical and medical regulations under the supervision of the local ethical committee governing the Polyclinics and Hospital of Nitra, Nitra where the patients received treatment. We isolated and processed the luteinized granulosa cells as described previously (Sirotkin et al. 2005). Immediately after isolating the luteinized granulosa cells, we suspended them in Dulbecco modified Eagle medium (DMEM)/Ham's F-12 1:1 mixture (Gibco-Invitrogen, Carlsbad, CA, USA). We dispensed portions of the cell suspension (2×10^6 cells/mL, determined by haemocytometer) in 96-well plates (50 mL/well, Axygen Scientific, Inc.) containing miRNAs. miRNA transfections were performed by using cationic transfection reagent (JetSI-ENDO, France) according

to the manufacturer's protocol. In addition, we performed the control transfections with the same transfection reagent conjugated with tetramethylrhodamine (Jet-SI-Endo-FluoR, Polyplus-Transfection). We used two pre-miRNA non-silencing random-sequence miRNAs (Ambion) with no homology to any known genes as negative controls. Human siRNAs targeting glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Silencer 1 GAPDH siRNA, Ambion), CDC2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and CREB-1 mRNA (Santa Cruz Biotechnology, Inc.) were used as positive controls. We have used the same positive controls in our previous experiments (Sirotkin et al. 2009, 2010b, c). After transfection, we diluted the cells with DMEM/Ham's F-12 1:1 mixture supplemented with 10 % bovine foetal serum and 1 % antibiotic–antimycotic solution (Gibco-Invitrogen) to a concentration of 0.5×10^6 cells/mL and cultured them for 48 h in 200 μ L culture medium/well. We then washed the wells in ice-cold PBS, fixed them for 20 min in 4 % paraformaldehyde PBS, washed them in PBS (twice for 5 min), washed them in ethanol (70 % for 5 min, 80 % for 10 min, 96 % twice for 10 min and 100 % for 10 min) and then stored them in 100 % ethanol at -18°C for immunocytochemical analysis. We stained the cells in trypan blue and counted them using a haemocytometer to assess cell concentration and viability in selected wells.

Immunocytochemical analysis

We detected NF- κ B in the luteinized granulosa cells plated on plate wells by immunocytochemistry (Osborn and Isenberg 1994). Primary mouse monoclonal antibodies against human NF- κ B (p65) (dilution 1:100; Santa Cruz Biotechnology, Inc.) were used. The visualization of the binding of primary antibody was performed with secondary goat IgG labelled with fluorescein isothiocyanate (FITC) (dilution 1:500; Sevac, Prague, Czech Republic). We confirmed the specificity of the primary antibodies and the molecular weights of ligands by Western blot (Sirotkin et al. 2006; Pavlová et al. 2011). Cells treated with labelled secondary antibody but lacking the primary antibody were used as negative controls. We then covered the cells with Vectashield anti-fade medium containing 4',6-diamidino-2-phenylindole (DAPI) fluorochrome (H-1200, Vector Laboratories, Inc, Burlingame, CA, USA). We determined the presence of specific immunoreactivity and transfection reagent labelled with tetramethylrhodamine in the cells using a fluorescent microscope (Leica Microsystems, Wetzlar, Germany) equipped with specific wavelength filters for FITC and DAPI channels at $\times 100$ magnification. We determined the total cell number in each well by counting DAPI-stained cells. We calculated the proportion of NF- κ B-positive cells as the number of FITC-stained cells divided by the total cell number, as described previously (Pavlová et al. 2011).

Statistics

We performed three replicates of each experiment on the effects of each miRNA. For each miRNA, we computed the mean of values of three experiments. We counted at least 1,000 cells per well to compute the ratio of cells containing antigen. We identified the substances that had significant effects on NF-kB expression using a one-way ANOVA. When we identified significant effects, we compared the treated cells with the control cells using a Duncan's multiple range test. We used $P < 0.05$ as the threshold for statistical significance.

Results

Evaluation of the efficiency of transfection and cell viability

The microscopic analysis of the cells treated with transfection reagent labelled with tetramethylrhodamine (Jet-SI-Endo-FluoR) showed that more than 90 % of the cells contained the transfection reagent after culture. The immunocytochemical analysis of the cells transfected with siRNAs targeting GAPDH, CDC2 and CREB-1 showed reductions in the expression of the marker proteins of 3.9-fold, 5.6-fold and 2.5-fold, respectively.

The immunocytochemical analysis of human luteinized granulosa cells from the control group and the miRNA-transfected groups showed that the cells were viable (more than 95 % of the cells remained unstained after the trypan blue exclusion test) and had morphology characteristics of healthy cells. Moreover, the immunocytochemical analysis detected NF-kB (p65) in a substantial number of the cells (Fig. 1).

Identification and quantification of the effects of miRNAs on NF-kB (p65) expression

We found that 18 of the 80 miRNA constructs significantly decreased the proportion of cultured human luteinized

granulosa cells containing NF-kB (p65). The miRNAs that decreased the expression of NF-kB were the following: *let-7g*, *mir-1*, *mir-17-3p*, *mir-18*, *mir-27a*, *mir-29a*, *mir-32*, *mir-134*, *mir-139*, *mir-141*, *mir-142*, *mir-149*, *mir-150*, *mir-152*, *mir-186*, *mir-187*, *mir-188* and *mir-191*. The most potent downregulators of NF-kB were *mir-1*, *mir-27a* and *mir-150*.

We found that 21 of the 80 miRNA constructs significantly increased the proportion of cultured human luteinized granulosa cells containing NF-kB (p65). The miRNAs that increased NF-kB expression were the following: *let-7b*, *let-7c*, *let-7d*, *mir-10a*, *mir-19a*, *mir-23b*, *mir-25*, *mir-31*, *mir-34a*, *mir-99a*, *mir-101*, *mir-107*, *mir-108*, *mir-129*, *mir-133a*, *mir-145*, *mir-153*, *mir-155*, *mir-181a*, *mir-182* and *mir-183*. The most potent upregulators of NF-kB were *mir-10a*, *mir-34a* and *mir-133a* (Fig. 2).

Discussion

Our microscopic and immunocytochemical analyses effectively showed cell viability and the presence of transcription factor NF-kB (p65) in the primary cultures of ovarian luteinized granulosa cells. Furthermore, as in our previous experiments, the analyses demonstrated the ability of the labelled transfection reagent to enter the cells and transfer the marker siRNAs, which efficiently reduced the expression of the marker protein GAPDH (Sirotkin et al. 2009, 2010b, c).

Our experiments demonstrated that the presence of a number of different miRNAs can impact on NF-kB expression in ovarian cells. Previously, the only known associations between miRNAs and NF-kB in ovarian cells were those involving miR-224 in healthy mouse ovarian granulosa cells (Liang et al. 2013), miR-9 (Guo et al. 2009) and miR-199a (Chen et al. 2008; Yin et al. 2010) in human ovarian carcinoma cells and miR-199a in human ovarian endometrioma cells (Dai et al. 2012). Our preliminary observations are the first direct demonstration that miRNAs can both upregulate and downregulate NF-kB (p65) expression in ovarian cells.

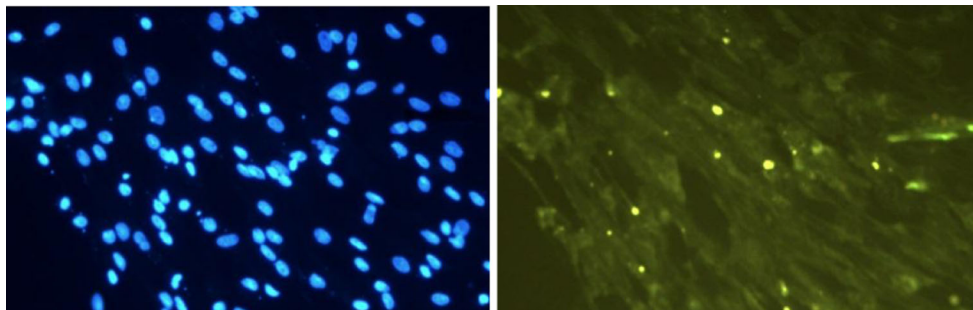


Fig. 1 Presence of DAPI (nuclear marker, *left*) and FITC (marker of NF-kB (p65), *right*) in cultured human ovarian granulosa cells. Antigens were detected after 48-h culture by using immunocytochemistry and visualized

by DAPI and FITC and fluorescent microscopy as indicated in “Materials and methods”. Magnification $\times 400$

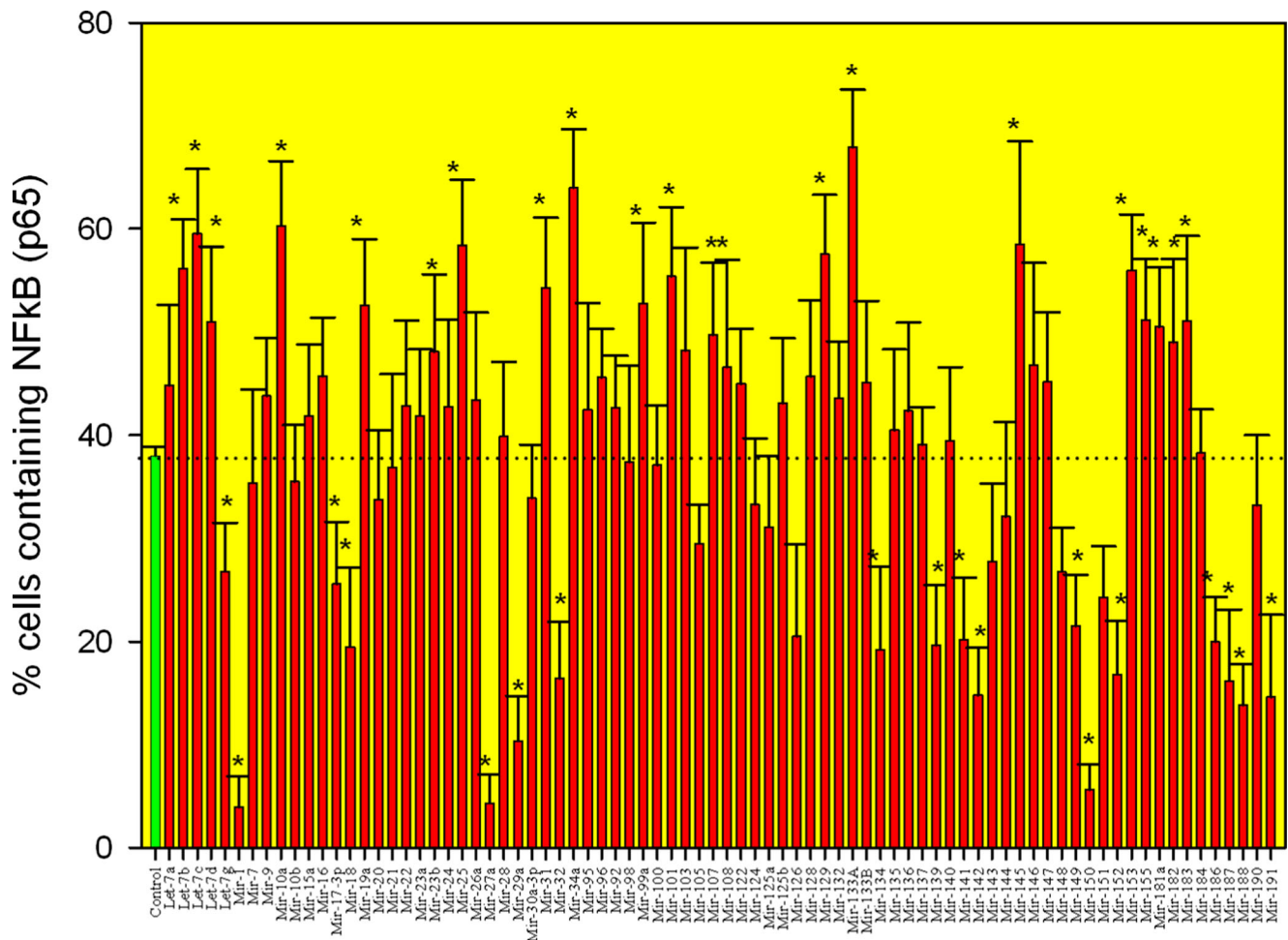


Fig. 2 Effect of miRNA transfection on NF- κ B (p65) accumulation in human granulosa cells. We transfected primary human granulosa cells with miRNA constructs, indicated on the x-axis. We measured the expression of NF- κ B (percentage of cells containing NF- κ B labelled

with FITC in relation to the total number of cells stained by DAPI) 48 h after transfection and fluorescent microscopy. Values are means \pm SEM. * P < 0.05 vs control group. (The first column is cells transfected with non-silencing oligonucleotides)

miRNAs can be used as tools to regulate ovarian NF- κ B and NF- κ B-dependent processes including proliferation, apoptosis and secretion. The ability of NF- κ B overexpression to affect these functions has been demonstrated previously (Pavlová et al. 2011). Our observations will now enable us to identify some of the processes regulated by the miRNA-NF- κ B axis. For example, most of the miRNAs that increased NF- κ B expression in the present experiments (except for *mir-108*) inhibited cell proliferation in previous experiments (Sirotkin et al. 2010b). Therefore, we hypothesize that these miRNAs could suppress the proliferation of human ovarian cells by interacting with NF- κ B. Some of the miRNAs (for example, *mir-182*) that increased NF- κ B expression in the present experiments inhibited nuclear apoptosis in previous experiments (Sirotkin et al. 2010b). Therefore, we hypothesize that the miRNA-NF- κ B axis is involved in the control of this nuclear apoptosis. Some of the miRNAs (*mir-1*, *mir-27a* and *mir-134*) that inhibited NF- κ B expression in the present

experiments were previously shown to inhibit both proliferation (Sirotkin et al. 2010b) and progesterone release (Sirotkin et al. 2009) in human ovarian cells. Progesterone is a physiological stimulator of ovarian cell proliferation and luteinization (Spitz et al. 2000; Sirotkin 2014). Therefore, we hypothesize that miRNAs that interact with NF- κ B to inhibit proliferation and progesterone release could regulate ovarian follicle development and luteinization and thus could be potential contraceptive agents. The mechanisms of effects of miRNAs, examined in our experiment on NF- κ B expression, activation and translocation into nucleus in ovarian cells remain to be elucidated in further studies.

Acknowledgments The authors express their deep gratitude to Mrs. K. Tothová and Ž. Kuklová for skilful technical assistance. The present studies were supported by the Ministry of Agriculture of the Slovak Republic (projects RVVU 07-02 and RVVU 07-013). A.H. Harrath, S.H. Alwasel and A.V. Sirotkin extend their appreciation to the Deanship

of Scientific Research at King Saud University for funding this work through the research group project NoRGP-VPP-164.

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