Identification of protein kinases that control ovarian hormone release by selective siRNAs

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Abstract

The goal of this study was to identify protein kinases (PKs) that control the secretory activity of human ovarian cells. Cultured ovarian granulosa cells were transfected with 264 siRNA constructs that selectively block the expression of 88 known PKs. The efficiency of transfection and of silencing marker molecules (glyceraldehyde 3-phosphate dehydrogenase, GAPDH and CDC2/p34 PK) was validated by fluorescence microscopy, real-time reverse transcription-PCR, and immunocytochemistry. Release of steroid hormones (progesterone, P4) and IGF1 was determined by RIA. siRNA suppressed the expression of marker molecules by up to 84%. P4 release was suppressed after inhibiting 34 individual PKs and was stimulated after inhibiting 12 PKs. Blocking nine individual PKs inhibited IGF1 release, while the inactivation of 17 others stimulated IGF1 release. Together, these results demonstrate that the release of both steroid and peptide hormones by human ovarian cells is controlled by a large number of PKs, and that siRNA constructs may be useful tools for further defining the role of PKs in controlling ovarian secretory function.

Journal of Molecular Endocrinology (2010) 44, 45–53

Introduction

Reproductive functions are controlled by complex interactions between gonadal and extragonadal hormones, and by intracellular protein kinases (PKs). PKs control both hormone secretion and mediate hormone action on target cells (Berisha & Schams 2005, Sirotkin 2005, Hinzuker-Dunn & Maizels 2006, Peluso 2006), and the presence of many PK family members, such as mitogen-activated PK (MAPK), cyclin-dependent PK (CDK), tyrosine kinase (TK), PI3 kinase (phosphoinositide 3-kinase, PI3K), PKA, and activin receptor-like kinase (ALK) in ovarian granulosa cells, has been well documented (Sirotkin et al. 2000a,b,c, 2008, Shimada et al. 2001, Makarevich et al. 2002, 2004a, Hinzucker-Dunn & Maizels 2006, Kumar et al. 2007, Sawada et al. 2007, Wang & Tsang 2007).

The most well-characterized ovarian hormones include steroids, especially progesterone (P4), and peptide insulin-like growth factor-1 (IGF1). These hormones control a wide array of reproductive events, such as sexual maturation, ovarian folliculogenesis, oocyte maturation, ovulation, and embryogenesis. Furthermore, P4 and IGF1 mediate the action of upstream hormones on ovarian cells (Richards et al. 2002, Berisha & Schams 2005, Peluso 2006). Changes in the levels and interactions between P4 (Maruo et al. 2004, Jamnongjit & Hammes 2006, Jongen et al. 2006) and IGF1 (Maruo et al. 2004, Gadducci et al. 2005) may play a role in the development of certain endocrine-related disorders, such as polycystic ovary syndrome and cancer.

growth factor receptor kinases, heregulin-β1-dependent kinases (HER kinases), and other PKs; Kamath & Buolamwini 2006, Macarulla et al. 2008, Markman 2008) can be expressed in the malignant ovary, but the physiological role of such PKs remains to be studied.

PKs also mediate the effects of hormones on ovarian cells. FSH promotes ovarian folliculogenesis, oocyte maturation, and estrogen release via PKA, MAPK, and PI3K/Akt (Richards et al. 2002, Hinzuku-Dunn & Maizels 2006). GH (Sirotkin & Makarevich 2002, Sirotkin 2005) and oxytocin (Makarevich et al. 2007) can prevent apoptosis and stimulate release of ovarian hormones via PKA and MAPK. Leptin (Sirotkin & Grossmann 2007a,b, Sirotkin et al. 2008) and ghrelin (Sirotkin & Grossmann 2007c) regulate ovarian cell proliferation, apoptosis, and secretory activity through PKA-, MAPK-, and CDK-dependent intracellular mechanisms. EGF can promote ovarian cell proliferation and secretory activity through cAMP-, cGMP-, and MAPK-dependent pathways (Makarevich et al. 2002). IGF1 promotes oocyte proliferation through PKA, PI3K/Akt, and glycogen synthase kinase (Makarevich et al. 2000, Richards et al. 2002), and IGF2 acts through MAPK and TK (Sirotkin & Grossmann 2003, 2006). The stimulatory effects of these growth factors on maturation of the oocyte (Sirotkin et al. 2000a,b,c), of Cumulus oophorus (Sirotkin et al. 2002) and embryo development (Makarevich et al. 2000) are mediated through PKA. P4 prevents apoptosis, maintains survival, and promotes proliferation of ovarian granulosa cells via the PKG-dependent (Peluso 2003) and CDK-dependent (Stouffer et al. 2007) pathways.

The role of PKs in reproduction has largely been examined by observing the association between reproductive events and the accumulation of PK. Some studies have determined the effects of pharmacological PK inhibitors on ovarian cells, but these data remain to be verified by more specific methods because correlative analyses cannot fully describe the biological role of any molecule. Moreover, pharmacological inhibitors usually are not specific for one particular PK, complicating interpretation of any data obtained by their use.

Selective targeting of individual PKs may be achieved using recently developed genomic approaches. PKs can be selectively, specifically, and efficiently silenced by transfecting host cells with corresponding siRNAs. For example, it was recently reported that siRNA- or antisense oligonucleotide-induced knockdown of PI3K/Akt (Ma et al. 2006, Lane et al. 2007, Noske et al. 2007), MAPK/2 murine double minute oncogene (MDM2) (Zeng et al. 2005, Suga et al. 2007), TK/EphB4 (Kumar et al. 2007), TK/c-Met (Sawada et al. 2007), ALK7 (Xu et al. 2006), and embryonic leucine zipper kinase (Melk; Gray et al. 2005) can inhibit proliferation of ovarian carcinoma cells. These knockdowns also induced apoptosis and reduced the growth of ovarian tumors. However, this approach has not been used to explore the role of PKs in reproduction. Therefore, the aim of this study was to examine the involvement of a large number of known PKs in the control of ovarian secretory activity. Specifically, we sought to identify PKs that control the release of key ovarian hormones by performing a genome-scale screen. Cultured primary human ovarian granulosa cells were transfected with siRNA gene constructs that selectively blocked different human PKs. The effects of PK silencing on the release of P4 and IGF1 were evaluated, and we were able to identify a number of PKs, which are involved in control of these hormones.

Materials and methods

Oligonucleotides

The sequences of the siRNAs used here were obtained from the Silencer Select Human Kinase siRNA Library V4 (Ambion, Austin, TX, USA). This library contains 264 unique siRNAs, which target 88 known human kinase genes. These genes were identified using the PANTHER and Gene Ontology Databases. The list and description of genes encoding particular PKs can be found at http://www.ncbi.nlm.nih.gov/sites/entrez?db=gene&cmd=search&term.

Three nonsilencing random sequence siRNAs (Silencer Select Control siRNAs; Ambion), with no homology to any known gene sequence, were used as negative controls. Two positive controls were used at the same concentration as experimental siRNAs; one siRNA targeted GAPDH mRNA (Silencer GAPDH siRNA, Ambion) and the other targeted CDC2 mRNA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), whose presence in the ovary has been previously demonstrated (Sirotkin & Grossmann 2006, Stouffer et al. 2007).

Single-stranded oligonucleotides were synthesized and purified by high performance liquid chromatography to >90% purity, as determined by mass spectroscopy. Oligonucleotides were annealed to generate siRNAs and were analyzed by PAGE (Ambion). Cellular accumulation of randomly selected siRNAs was validated by transfection of HepG2 cells (ATCC, Rockville, MD, USA) using a HepG2 transfection reagent (Altogen Biosystems, Las Vegas, NV, USA) according to the manufacturer’s protocol. Accumulation was quantified by real-time PCR using the TaqMan MicroRNA assay (Applied Biosystems, Foster City, CA, USA). RNAs were placed in 96-well plates (Axygen Scientific, Inc., Union City, CA, USA) at a final concentration of 0.25 nM in 100 μl/well of complete culture medium.
Preparation, transfection, culture, and processing of granulosa cells

Granulosa cells were harvested 1–5 days after spontaneous ovulation from women 36–42 years of age with normal ovarian cycles and morphology, who were undergoing ovariectomy due to nonmetastatic cancer of the cervix uteri. Informed consent was obtained from each patient in accordance with EU and Slovakian ethical and medical regulations, under supervision of the local ethical committee. Granulosa cells were isolated and processed as previously described (Sirotkin et al. 2000c, Sirotkin & Grossmann 2007a). Immediately after isolation, they were suspended in DMEM/Ham’s F-12 (1:1) (Gibco-Invitrogen), and cell suspensions (2 × 10⁶ cells/ml) were plated at 50 µl/well into 96-well plates (Axygen Scientific, Inc.) containing the siRNA library. Three siRNAs per PK were tested in these experiments. The PKs targeted by these siRNAs are listed in Figs 1 and 2. Transfections were performed using the cationic transfection reagent JetSI-ENDO (Polypus-Transfection, Illkirch, France) according to the manufacturer’s instructions. Control transfections were performed using the same transfection reagent conjugated with tetramethylrhodamine JetSI-Endo-FluoR (Polypus-Transfection) to assess the incorporation of transfection reagent into the cells via presence of red fluorescence induced by tetramethylrhodamine. After transfection, cells were diluted to a concentration of 0.5 × 10⁶ cells/ml with DMEM/Ham’s F-12 (1:1) supplemented with 10% fetal bovine serum and 1% antibiotic–antimycotic solution (all from Gibco-Invitrogen). The presence of serum allows attachment of cells to the bottom of the wells, which is necessary for immunostaining, but does not affect basal hormone release. Cells were cultured for 48 h in 200 µl of culture medium/well, after which both the medium and cells were processed for RIA and immunocytochemistry as previously described (Sirotkin & Makarevich 2002, Sirotkin et al. 2008). At this time, cell concentration and viability were evaluated by Trypan Blue staining and hemocytometry.

RIA

The concentration of P₄ and IGF1 was measured in 25–50 µl of granulosa cell-conditioned media using RIA kits from DSL (Webster, TX, USA) as previously described (Makarevich et al. 2000, Sirotkin & Makarevich 2002). These kits have been previously validated for use in serum-supplemented culture medium.

Immunocytochemical analysis

After culture, the medium from wells, where cells were cultured, was removed, wells were washed in ice-cold PBS (pH 7.5), fixed for 1 h at room temperature (25°C) in 4% paraformaldehyde, dehydrated in alcohols (70, 80, 96%; 10 min each), and stored in 96% alcohol at −4°C to await immunocytochemical analysis. Marker proteins were detected by immunocytochemistry (Osborn & Isenberg 1994) using primary mouse monoclonal antibody against CDC2/p34 (which cross-reacts with the corresponding human protein; Santa Cruz Biotechnology, Inc.; 1:100), secondary goat IgG labeled with HRP (Santa Cruz Biotechnology, Inc.; 1:1000), and DAB reagent to visualize localization of primary antiserum by brown staining (Boehringer Mannheim GmbH; 10%), or with primary mouse monoclonal antibody against cyclin B1 (Santa Cruz Biotechnology Inc.; 1:100) and FITC-labeled porcine secondary antibody (Sevac, Prague, Czech Republic; 1:1000). Cells treated with only the secondary antibody and DAB, but not the primary antibody, were used as negative controls. The presence of specific CDC2/p34 immunoreactivity in cells was determined by light microscopy. The presence of transfection reagent labeled with tetramethylrhodamine (JetSI-Endo-FluoR) and cyclin B1 in cells was detected by fluorescence microscopy. The specificity of the primary antibodies used here and the molecular weight of their ligands were confirmed by western blotting as described (Sirotkin & Makarevich 2002, Sirotkin & Grossmann 2006, 2007a).

Real-time reverse transcription-PCR analysis

Total RNA was isolated from cells transfected with siRNA using the MagMAX93 Total RNA isolation kit (Ambion). Purified DNase-treated RNA was reverse transcribed using the RETROscript Kit (Ambion). GAPDH and 18S gene expression levels were determined by real-time PCR using the ABI Prism 7900 Sequence Detection system (Applied Biosystems), SuperTaq reagents (Ambion), and SYBR Green (Molecular Probes-Invitrogen), according to the manufacturer’s instructions. GAPDH data were collected using the primer set (forward: gaaggtgaagggcagt, reverse: aagatggtgatgggatttc), and 18S rRNA was also amplified (forward: ttgactcaacaggggaact, reverse: aagagcgtatccatact, probe: 5'-VIC-acccggcccggcagggagt-TAMRA-3') as an endogenous control to adjust for well-to-well variances. Corrected values were normalized to a sample transfected with nonsilencing siRNA. Details of GAPDH PCR analysis have been described previously (Ovcharenko et al. 2007).

Statistical analysis

Data are presented as the mean of values obtained in three separate experiments performed on different days using unique pools of ovaries obtained from three different patients. To downregulate a single PK, three
Figure 1 Changes in the release of P₄ by cultured human ovarian granulosa cells after transfection with siRNA constructs specific for protein kinases. Target protein kinases are indicated on the x-axis. Hormone accumulation was measured 48 h posttransfection by RIA. Values represent mean ± S.E.M. *P < 0.05 versus control group (cells transfected with nonsilencing oligonucleotides).
Figure 2 Changes in the release of IGF1 by cultured human ovarian granulosa cells after transfection with siRNA constructs specific for protein kinases. Target protein kinases are indicated on the x-axis. Values represent mean ± S.E.M. * P < 0.05 versus control group (cells transfected with nonsilencing oligonucleotides).
unique siRNAs specific for this PK were transfected in triplicate. The data represent an average of the triplicate transfections for each of the three different siRNAs, yielding nine wells per gene.

Hormone levels in the incubation medium were assayed in duplicate. For RIAs, the values of blank controls were subtracted from the value determined in cell-conditioned medium to correct for any nonspecific background (<17% of total values). Hormone secretion was calculated per 10^6 cells/day. Following immunocytochemical analysis, the proportion of cells containing CDC2/p34, Jet-SI-Endo-FluoR, and cyclin B1 was calculated by counting at least 1000 cells/well. All PCR results were normalized to a nonsilencing control. Significant differences between the groups were evaluated by paired t-test using Sigma Plot 9.0 statistical software (Systat Software, GmbH, Erkrath, Germany). P values of <0.05 were considered statistically significant.

Results

Efficiency of transfection and silencing

Human granulosa cells were first transfected with a reagent labeled with tetramethylrhodamine (Jet-SI-Endo-FluoR) to test for the ability of this cell type to take up the transfection reagent. Microscopic analysis showed that more than 90% of cells had incorporated the fluorescent transfection reagent after culture for 48 h (data not shown). To confirm that the administration of siRNAs using this method was able to reduce expression of specific mRNAs, cells were transfected with siRNA specific for the marker protein GAPDH, and subsequent real-time RT-PCR analysis showed that the amount of GAPDH mRNA was reduced by 78±6% (Fig. 3). Likewise, immunocytochemical analysis showed that introduction of an siRNA specific for CDC2/p34 resulted in a reduction in 84±8% (Fig. 4). Importantly, cell concentration and viability were not different between treatment groups, as assessed by Trypan Blue staining (data not shown).

Effects of siRNA-induced silencing of PKs on P4 and IGF1 release

Cultured human granulosa cells were transfected with 88 different siRNA constructs targeting a variety of PKs. Inhibition of P4 release was observed after transfection with 34 siRNA constructs. Likewise, P4 release was stimulated after siRNA-mediated inhibition of 12 PKs, whose identity differed from those responsible for P4 inhibition (Fig. 1).

IGF1 release was inhibited by the siRNA-induced inhibition of nine PKs and was stimulated by the inhibition of 17 other PKs (Fig. 2).

Discussion

The present study describes the first large-scale screen of PKs that influence the secretory activity of ovarian cells by using siRNA-mediated gene silencing. We chose to analyze the effect of such silencing on the release of P4 and IGF1, as these hormones are typical and ubiquitous representatives of steroid and peptide ovarian hormones and because they play an important role in control of reproductive functions (Richards et al. 2002, Peluso 2003, 2005, Berisha & Schams 2005).
We observed that knockdown of different PKs was responsible for either augmenting or inhibiting the release of these hormones from ovarian cells.

$P_4$ release was significantly augmented by siRNA-mediated silencing of PKs related to Akt (AKT1), CDK (CDKN1B), TK (FGFR1, FGFR2, TYRO3), MAPK (MAPK2K6, MAPK3K11, MAPK3K14, MAPK3K6), met proto-oncogene (hepatocyte growth factor receptor) (mesenchymal-epithelial transition factor, MET), and CDK/p21 (PAK1, PAK2). As increased release of $P_4$ is associated with the activation of different reproductive functions, especially proliferation and luteinization of the follicular cells before and after ovulation (Richards et al. 2002, Peluso 2003, 2005, Berisha & Schams 2005), it is possible that the 12 PKs identified here may serve as upregulators of $P_4$-related reproductive processes. This correlates with previous indications that MAPK, PI3K/Akt, CDK, and TK control the release of ovarian $P_4$, as determined by pharmacological inhibition of these PKs (Makarevich et al. 1997, 2004b, Hinzuker-Dunn & Maizels 2006, Sirotkin & Grossmann 2007a). We also identified several PKs whose silencing by siRNA inhibited $P_4$ release by ovarian cells. It could be proposed that these PKs could be physiological inhibitors of ovarian cell proliferation and luteinization induced by $P_4$ (Richards et al. 2002, Peluso 2003, 2005, Berisha & Schams 2005). Moreover, the present observations identified for the first time a number of PKs, belonging to other families (HK, IRAK a.o.), for which involvement in control of reproductive processes has not been demonstrated yet.

The experiments described here also identified PKs that regulate release of ovarian IGF1. IGF1 output was inhibited by the siRNA-induced silencing of nine PKs related to MAPK (MAPK3K7, MAPK3K8, MAPK1, MAPK9), CDK/p21 (PAK1, PAK2), sphingosine kinases (SPHK1), thymidine kinases (TK1), and adenylate kinase. Furthermore, IGF1 output was stimulated by the siRNA-induced inactivation of 17 other distinct PKs. Since IGF1 stimulates most reproductive processes, including promotion of ovarian cell proliferation, inhibition of ovarian cell apoptosis and related atresia of ovarian follicles, stimulation of ovarian steroidogenesis, oocyte maturation etc. (Berisha & Schams 2005), it is possible that the PKs identified here could be involved in their regulation.

A limitation of this study was that it was not able to identify specific mechanisms or sites of action of the PKs we identified. For example, some PKs directly control processes related to $P_4$ synthesis, release, or metabolism, while others affect $P_4$ through feedback mechanisms, including $P_4$ receptors or derivatives. In terms of IGF1, the PKs identified here could play a multitude of roles in a variety of processes, such as IGF1 synthesis, release, metabolism, feedback control mechanisms, or the regulation of other downstream PKs. Moreover, opposing stimulation of $P_4$ and IGF1 under normal (Berisha & Schams 2005) and pathological (Maruo et al. 2004) conditions is possible, suggesting that it is likely that specific mechanisms regarding $P_4$ and IGF1 release in ovarian cells could differ depending on the health of the cell. Finally, the similar action of several siRNAs (specific for MAPK3K7, MAPK9, SPHK1, and AKT1) on both $P_4$ and IGF1 could be explained by the existence of a mutual/opposing stimulation of $P_4$ and IGF1 release within the ovary, which can be controlled by these PKs, though most of our data suggest that thePKs we identified have distinct and independent effects on the regulation of $P_4$ and IGF1.

In conclusion, the use of siRNAs to selectively inhibit specific proteins is likely to be an important tool to further investigate ovarian hormone control. Similar genomic interventions may also serve as important therapies for the control of reproductive processes and the treatment of some hormone-related disorders. Hormone-inhibiting constructs could be useful as new contraceptives, as well as in the development of novel therapies for infertility, hormone-related syndromes, and cancers. The present study is an important first step toward these important clinical goals.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the Ministry of Agriculture of SR (grant number RUVV 07-013).

Acknowledgements

We are grateful to Ing Ž Kuklová and K Tothová for skillful technical assistance.

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Received in final form 6 August 2009
Accepted 27 August 2009
Made available online as an Accepted Preprint 27 August 2009