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EGR2 is a gonadotropin-induced survival factor that controls the expression of IER3 in ovarian granulosa cells

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ABSTRACT

Pituitary gonadotropins are key hormones that orchestrate the growth and development of ovarian follicles. However, limited information is available on intra-ovarian factors that mediate the actions of gonadotropins. In this study, we identified that the early growth response 2 gene (*EGR2*) is a gonadotropin-inducible gene in granulosa cells of rats and humans. Analysis of consensus EGR-binding elements (EBEs) showed that the immediate early response 3 gene (*IER3*) is a novel transcriptional target gene of EGR2 as confirmed by the luciferase assay, electrophoretic mobility-shift assay (EMSA), chromatin immunoprecipitation (ChIP), and western blot analysis. Overexpression of EGR2 promoted survival of KGN human granulosa-derived cells in which IER3 acts as a mediator; knockdown of EGR2 induced death in KGN cells. Additionally, EGR2 was found to regulate the expression of myeloid cell leukemia 1 (MCL-1), which belongs to the BCL-2 family of proteins regulating cell survival. Thus, this study identified a novel signaling axis, comprised of gonadotropins-EGR2-IER3, which is important for the survival of granulosa cells during folliculogenesis.

1. Introduction

Proper growth and development of mammalian ovarian follicles into mature Graafian follicles is critical for successful reproduction. Folliculogenesis is an integrated outcome of complex regulatory networks involving gonadotropins, steroid hormones, and various growth factors [1–3]. Pituitary gonadotropins, such as follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are pivotal factors required for maturation of ovarian follicles [4]. Granulosa cells, which surround oocytes, nourish and support the oocyte by providing signaling molecules and nutrients. Thus, timely proliferation and differentiation of granulosa cells is essential for folliculogenesis [5].

The early growth response (EGR) family is a group of zinc finger transcriptional regulators comprised of *EGR1* (also known as *KROX24*), *EGR2* (*KROX20*), *EGR3*, and *EGR4*, which are upregulated in response to hormones, growth factors, cytokines, neurotransmitters, and environmental stimulants [6–10]. The EGR family of proteins is involved in regulating a diverse spectrum of cellular responses including insulin signaling, bone formation, immune response, fibrogenesis, and memory processes [11–15]. The importance of EGR family in maintaining ovarian function was revealed using the *Egr1*-knockout mouse model, in which $Egr1^{-/-}$ female mice are infertile and lack LH synthesis [16,17]. However, the role of EGR2 in the ovary remains undetermined, although it is one of the differentially expressed genes induced by hCG in mouse periovulatory follicles [18].

In this study, we demonstrated that *EGR2* is a gonadotropin-induced gene that promotes the survival of granulosa cells. Additionally, we identified that the immediate early response 3 gene (*IER3*) is a novel ovarian target of EGR2. Our findings indicate that EGR2 is a potentially important factor that mediates the actions of gonadotropins during folliculogenesis.

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2. Materials and methods

2.1. Animals

Immature female Sprague-Dawley rats (15–18 days old) were purchased from Samtako, (Osan, Korea), housed at CHA University Animal Facility, and maintained in accordance with the guidelines and protocols approved by the Institutional Animal Care and Use Committee of CHA University. For quantitative RT-PCR, female rats (n = 3) were intraperitoneally injected with 10 IU of pregnant mare's serum gonadotropin (PMSG) (Calbiochem, San Diego, CA, USA); 48 h after injection with PMSG, the rats were injected with 10 IU of human chorionic gonadotropin (hCG) (Calbiochem), and granulosa cells were isolated as reported previously [19].

2.2. Cell culture and reagents

Human adult-type granulosa cell tumor-derived KGN cells (Riken, Ibaraki, Japan) were cultured in DMEM-F12 (Caisson, North Logan, UT, USA) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Caisson). Cells were incubated at 37 °C in a 5% CO_2 incubator. Rabbit anti-EGR2 (sc-20690), rabbit anti-IER3 (sc-33171), mouse anti-PARP (SC-74469), rabbit anti-MCL-1 (sc-819), mouse anti-BCL-2 (sc-65392), mouse anti-BCL-xL (sc-8392), rabbit anti-BCL2A1 (sc-8351), rabbit anti-BAX (sc-493), and rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, sc-25778) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Additional antibodies were rabbit anti-BAK (AM03; Calbiochem), rabbit anti-α-tubulin (LF-PA0146; AbFrontier, Seoul, Korea), and rabbit anti-FLAG (2368; Cell Signaling, Danvers, MA, USA); FSH (F4021) and LH (L6420) were purchased from Sigma-Aldrich (St Louis, MO, USA).

2.3. Plasmid constructs

The cloning of full-length human IER3 promoter (−1384 to 1) and its truncated promoters (−754 to 1, −283 to 1, and −69 to 1) are described in our previous report [20]. The −157 promoter of IER3 was amplified by PCR using the following primers: 5′-GGGACGCGTGCCTAGGATTGTGCATGTCAA-3′ and 5′-ATCTCTGG-CATGCGAGAATCT-3′. The product of PCR was digested with *Mlu*I and *Sph*I (Enzynomics, Seoul, Korea) and ligated into a basic pGL3 vector (Promega, Madison, WI, USA). The pCDNA3 FLAG-tagged plasmid, encoding EGR2, was a generous gift from Jae-Hwan Kim (CHA University, Seongnam, Korea).

2.4. Immunoblot analysis

The lysates of FSH- or LH-treated KGN cells (1×10^6) were prepared, and immunoblot analyses were performed, as previously described [21]. KGN cells (1×10^6) were transfected with the indicated plasmids or siRNAs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The membranes were detected using Amersham Imager 600 (GE Healthcare Life Sciences, Amersham, Buckinghamshire, UK).

2.5. Luciferase reporter assay

Luciferase reporter assay was performed as previously described [22]. Briefly, KGN cells were transfected with the indicated plasmids or siRNAs. After 24 h of incubation, the cells were lysed, and luciferase activities were measured using the Luciferase Assay System Kit (Promega). Absorbance was measured using FlexStation3 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

2.6. RNA interference

Target sequences of small-interfering RNAs (siRNAs) are as follows: EGR2 #1 (5′-GAAGGCAUAAUCAAUAUU-3′), EGR2 #2 (5′-CUACUGUGGCCGAAAGUUU-3′), and IER3 (5′-CCAGCCAAAAGG-CUUCUCUUU-3′) (Bioneer, Daejeon, Korea). The sequence of control siRNA was 5′-CCUACGCCACCAAUUUCGU-3′. The sense and antisense oligonucleotides were annealed in the presence of annealing buffer. siRNAs were transfected using Lipofectamine 2000 (Invitrogen).

2.7. Subcellular fractionation

Fractionation of nuclear and cytosolic compartments was performed as described previously [23].

2.8. Electrophoretic mobility-shift assay (EMSA)

EMSA assay was performed as described previously [23]. Probes were designed based on predicted EGR2 binding elements (EBEs) found at −124 to −135 (EBE1) and −191 to −202 (EBE2) of the human IER3 promoter. Double-stranded oligonucleotides of EBE1 (sense: 5'-GTCTCCACCCACTCCCTTTG-3′; antisense: 5'-CAAAGG-GAGTGGGTGGAGAC-3′) and EBE2 (sense: 5'-ATCTCACCCACCCC-CACTCACGA-3′; antisense: 5'-TCGTGAGTGGGGGTGGGTGAGAT-3′) were annealed prior to use.

2.9. Chromatin immunoprecipitation (ChIP) analysis

ChIP assays were performed as described previously [23]. DNA fragments were amplified using the following sets of primers flanking EBE1 in the IER3 promoter: −243 forward (5′-CCA-CATGCCTCGACATGT-3′), +20 reverse (5′-GGAGTGTAAGGCCAAGT-3′), and −164 forward (5′-GGGCGCCTAGGATTGT-3′). The PCR products were analyzed using RT-PCR.

2.10. Cell viability assay

KGN cells (2×10^4) were transfected with the indicated plasmids or siRNAs using Lipofectamine 2000. After a 24-h incubation, cell viability was measured using CellTiter-Glo Assay (Promega) according to the manufacturer's instructions.

2.11. Statistical analysis

Multiple comparison analyses of values were performed with Student-Newman-Keuls test using SAS version 9.2 (SAS Institute, Cary, NC, USA). Student's *t*-test was used for comparisons with controls. Data are presented as means ± SEM, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. EGR2 is a gonadotropin-inducible factor in granulosa cells of rat and human

To assess the influence of gonadotropins on the expression of EGR2 in granulosa cells, the expression profile of *Egr2* mRNA was analyzed using real-time RT-PCR in granulosa cells isolated from rats at different time points after injection with PMSG and hCG (Fig. 1A). The expression of *Egr2* mRNA in rats rapidly increased between 2 and 6 h after injection with PMSG and returned to basal level 48 h after administration of PMSG (Fig. 1A). hCG also increased

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