Gonadotropin regulation and role of ovarian osteopontin in the periovulatory period

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Abstract

Osteopontin (OPN), a secreted glycoprotein, has multiple physiological functions. This study investigated the regulation and roles of OPN in the mouse ovary during the periovulatory stages. Immature female mice were treated with pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) to simulate follicle maturation and ovulation. *In situ* hybridization and real-time RT-PCR were performed to assess expression of *Opn* in the periovulatory ovary. Granulosa cells (GCs) from PMSG-primed immature mice were cultured with or without hCG in the presence or absence of OPN, and effects on expression of *Opn*, progesterone synthesis, and vascular endothelial growth factor (VEGF) signaling were assessed by real-time RT-PCR, ELISA, and western blotting analysis. *Opn* transcripts were significantly upregulated 3 h after hCG treatment, followed by a peak at 16 h, and the transcripts localized to GCs. Incubation with hCG significantly increased quantities of *Opn* transcripts in GCs and OPN levels in the culture medium at 12 and 24 h. Furthermore, OPN treatment caused a significant increase in the levels of *Star* protein, P 450 cholesterol side-chain cleavage enzyme (*p450scc*), 3- β -hydroxysteroid dehydrogenase (*Hsd3b*), and progesterone in the culture medium. OPN treatment promoted *Vegf* expression in GCs, which was significantly suppressed by a phosphoinositide 3-kinase (PI3K) inhibitor. In addition, OPN treatment stimulated phosphorylation of AKT, a downstream PI3K signaling molecule. In conclusion, expression of *Opn* was upregulated in mouse ovarian GCs in response to a gonadotropin surge through epidermal growth factor receptor signaling, which enhances progesterone synthesis and *Vegf* expression during the early-luteal phase.

Key Words

- ▶ gonadotropin
- ▶ epidermal growth factor
- ▶ progesterone
- ▶ corpus luteum

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Introduction

In the periovulatory period, multiple local factors secreted by different ovarian cell compartments mediate or modulate the actions of gonadotropin via the paracrine and/or autocrine pathways (Richards 1994, Christenson & Stouffer 1997). Recently, we have reported that expression

of chemokine CCL11 is upregulated transiently in the ovarian theca–interstitial layer and involved in follicular vascular formation (Kuwabara *et al.* 2012). Identification and characterization of these factors regulated during the periovulatory stages provides understanding of

physiological processes such as follicle transformation to the corpus luteum and oocyte maturation and release.

Osteopontin (OPN) is a secreted glycosylated phosphoprotein, originally isolated from the bone matrix (Giachelli *et al.* 1991). OPN is distributed in a wide variety of tissues, including kidney and epithelial cells of the gastrointestinal tract, gall bladder, pancreas, urinary and reproductive tracts, lungs, breasts, salivary and sweat glands, inner ear, brain, placenta, and arteries (Butler 1989, Giachelli *et al.* 1991, Brown *et al.* 1992). OPN is also present in body fluids such as milk and urine (Denhardt & Guo 1993). A diverse distribution pattern indicates that OPN is multifunctional and involved in angiogenesis and tissue remodeling (Ingber 1992, Giachelli *et al.* 1995, Liaw *et al.* 1995a,b, Corjay *et al.* 1999). OPN promotes cell adhesion, signaling, and migration in the immune response and in neoplasia (Denhardt & Guo 1993, Sodek *et al.* 2000).

In reproductive tissues of females, OPN was detected in the human ovary (Brown *et al.* 1992), human and ovine endometrium (Brown *et al.* 1992, Johnson *et al.* 1999), and oviduct (Gabler *et al.* 2003). Furthermore, some reports have described expression of OPN in ovaries during the periovulatory and luteal phases. According to the results of a microarray analysis of granulosa cells (GCs) from pregnant mare serum gonadotropin (PMSG)-primed immature mice, *Opn* is one of the 419 genes significantly upregulated by human chorionic gonadotropin (hCG) stimulation (McRae *et al.* 2005). Furthermore, a novel ovarian protein, sharing sequence similarity with mammalian and chicken OPNs, was detected in trout periovulatory ovary (Bobe & Goetz 2001). OPN transcripts were also detected in bovine ovarian follicles and corpus lutea (Brunswig-Spickenheier & Mukhopadhyay 2003). Recently, results of a detailed study have indicated that OPN is localized in bovine luteal cells during the luteal phase of the estrous cycle and is involved in the development and regression of the corpus luteum (Poole *et al.* 2013). To our knowledge, normal OPN expression in follicular or luteal cells in adult animals has not been reported, except for expression in the bovine ovary.

Hence, ovarian OPN expression has been demonstrated in some species; however, the regulation, localization, and functions of OPN in the ovary, especially in the periovulatory phase, have yet to be revealed.

Using a PCR array analysis of growth factor transcripts expressed in the mouse ovary, we identified a marked increase in *Opn* transcripts after preovulatory stimulation with hCG. Based on the results, we sought to clarify the detailed localization, regulatory mechanism, and

physiological roles of OPN in the periovulatory ovary using an immature mouse ovarian stimulation model. In this study, we provide experimental evidence that *Opn* expression is markedly upregulated in mouse ovarian GCs in response to a gonadotropin surge through epidermal growth factor receptor (EGFR) signaling, which is involved in corpus luteum formation and function during the early-luteal phase.

Materials and methods

Animals

Immature female B6D2F1 (21 days old) and adult (16 weeks old) mice were obtained from Charles River Laboratories Japan (Kanagawa, Japan). Immature female mice were treated with 7.5 IU of PMSG (ASKA, Tokyo, Japan) administered by s.c. injection, followed by treatment with 10 IU of hCG (ASKA) by i.p. injection 48 h later to stimulate follicle maturation and ovulation respectively. The Laboratory Animals Ethics Committee of Nippon Medical School reviewed and approved all experimental procedures.

PCR array analyses

Mice ($n=12$) at 21 days of age received injections of PMSG preparation (7.5 IU/animal), containing follicle-stimulating hormone and luteinizing hormone (LH) to stimulate follicular growth. Forty-eight hours later, some animals were treated with an hCG preparation (10 IU/animal, administered i.p.) with LH activity.

The ovaries were dissected from the killed animals 48 h after PMSG treatment ($n=6$ per group) and 6 h after hCG treatment ($n=6$ per group) for total RNA extraction using an RNeasy Mini Kit (Qiagen Sciences). Equal amounts of total RNA from each sample were reverse-transcribed to cDNA using a Superscript First-Strand Synthesis Kit (Invitrogen) and pooled for subsequent PCR array analyses. The expression levels of molecules related to growth factors were evaluated using the RT2 Profiler PCR Array System (SABiosciences, Frederick, MD, USA) and an ABI Prism 7500 Sequence Detection System (Applied Biosystems). The mRNA levels were normalized to the levels of β -actin mRNA using the following equation: relative mRNA expression = $2^{-(Ct \text{ of targeting molecule} - Ct \text{ of } \beta\text{-actin})}$ (Ct , threshold cycle). The change in the expression level was assessed by dividing the relative expression values for hCG-treated animals by those for PMSG-primed animals.

Isolation of ovarian compartments for RT-PCR

Immature female mice were treated with PMSG and hCG as described previously and dissected 6 and 16 h after hCG treatment. Thereafter, GCs were isolated from the whole ovary by puncture of preovulatory follicles with needles and fine forceps under a stereomicroscope. GCs and the residual ovaries (RO) were snap-frozen and stored at -70°C for subsequent analysis. RO represents the tissue remaining after GC collection, heterogeneous tissue comprises theca-interstitial and stromal cells, as well as remaining GCs.

Relative quantification of gene expression

To assess mRNA expression, pre-designed gene-specific primer pairs and probes were selected for the following target genes: *Opn* (ID Mm00436767_m1), *Star* protein (Mm00441558_m1), P450 cholesterol side-chain cleavage enzyme (*p450scc*) (Mm00490735_m1), 3- β -hydroxysteroid dehydrogenase isomerase (*Hsd3b*) (Mm00476184_g1), vascular endothelial growth factor (*Vegf*) (Mm0128-1449_m1), and an endogenous control, β -actin (*Actb*) (Mm00607939_s1) within a list of pre-designed assays (Assays-on-Demand; Applied Biosystems). The whole ovaries or cultured GCs were collected for total RNA extraction after treatment of immature mice with gonadotropin. RNA was extracted using an RNeasy Mini Kit (Qiagen Sciences). Total RNA (2 μg) was reverse-transcribed for subsequent PCR analysis. Real-time PCR was performed in a final reaction volume of 20 μl , containing 10 μl of 2 $\times$ TaqMan Universal PCR Master Mix (Applied Biosystems), 1 μl of 20 $\times$ primer/probe assay mix (Applied Biosystems), and 5 μl of DNA template. PCR was performed in MicroAmp optical 96-well plates with optical adhesive covers (Applied Biosystems). Amplification and detection were performed with an ABI Prism 7500 Sequence Detection System (Applied Biosystems). The amplification conditions were 2 min at 50°C for AmpErase uracil-*N*-glycosylase activity, 10 min at 95°C for AmpliTaq Gold activation, 50 cycles of 15 s at 92°C for denaturation, and 1.5 min at 60°C for annealing and extension. After amplification, the fluorescence end-point was detected at 60°C . The fluorescence data were analyzed with Allelic Discrimination Software for the ABI Prism 7500 instrument. The samples were assayed in duplicate for each gene, and the mean expression was used during subsequent analysis. Relative expression was calculated using the comparative $\Delta\Delta\text{Ct}$ method (Applied Biosystems 1997).

In situ hybridization analyses

To localize *Opn* in the ovary, paraffin-embedded blocks and ovary sections from PMSG-primed mice before and after hCG treatment were obtained for *in situ* hybridization (ISH) from Genostaff Co. Ltd (Tokyo, Japan). The mouse ovaries were dissected, fixed with Tissue Fixative (Genostaff Co. Ltd), embedded in paraffin according to proprietary procedures, and sectioned at 6 μm . For ISH, tissue sections were deparaffinized with xylene and rehydrated with an ethanol series and PBS. The sections were fixed with 4% paraformaldehyde in PBS for 15 min and then washed with PBS. The sections were treated with 8 $\mu\text{g}/\text{ml}$ proteinase K in PBS for 30 min at 37°C , washed with PBS, re-fixed with 4% paraformaldehyde in PBS, washed again with PBS, and placed in 0.2 mol/l HCl for 10 min. After washing with PBS, the sections were acetylated by incubation for 10 min in 0.1 mol/l tri-ethanolamine-HCl (pH 8.0) with 0.25% acetic anhydride. After washing with PBS, the sections were dehydrated through a series of ethanol solution. Hybridization was performed at 60°C for 16 h with probes at a concentration of 300 ng/ml in Probe Diluent-1 (Genostaff Co. Ltd). After hybridization, the sections were washed with 5 $\times$ HybriWash (Genostaff Co. Ltd, 5 $\times$ saline sodium citrate) at 50°C for 20 min and then with 50% formamide with 2 $\times$ HybriWash at 50°C for 20 min, followed by RNase treatment for 30 min at 37°C with 50 $\mu\text{g}/\text{ml}$ RNaseA in 10 mmol/l Tris-HCl (pH 8.0) with 1 mol/l NaCl and 1 mmol/l EDTA. Then, the sections were washed twice with 2 $\times$ HybriWash at 50°C for 20 min, twice with 0.2 $\times$ HybriWash at 50°C for 20 min, and once with Tris-buffered saline with Tween (TBST), which contains 0.1% Tween 20. After treatment with 0.5% blocking reagent (Roche) in TBST for 30 min, the sections were incubated for 2 h at room temperature with anti-digoxigenin alkaline phosphatase conjugate (Roche) diluted 1:1000 with TBST. The sections were washed twice with TBST and then incubated in 100 mmol/l NaCl, 50 mmol/l MgCl_2 , 0.1% Tween 20, and 100 mmol/l Tris-HCl (pH 9.5). Coloring reactions were carried out overnight in nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt (NBT/BCIP) solution (Sigma), and then samples were washed with PBS. The sections were counterstained with Kernechtrot Stain Solution (Mutoh Chemical Co., Tokyo, Japan), dehydrated, and mounted with Malinol (Mutoh Chemical Co.). The probe sequence used for the assay corresponded to the complementary sequence of *Opn* nucleotides 548–906.

GC preparation and culture

The ovaries were collected from PMSG-primed immature mice and punctured in L-15 Leibovitz medium (Invitrogen). Ovarian debris, oocytes, and small follicles were removed, and the remaining medium containing GCs was collected after low-speed centrifugation at 500 *g* for 10 min. GCs were dispersed by repeated washing and were resuspended into culture medium (McCoy's 5a supplemented with 10^{-7} mol/l androstenedione, 2 mmol/l L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin). For gene expression analyses, GCs were cultured at a density of 250 000 cells/well in a four-well plate (15.5 mm in diameter) with hCG (1 IU/ml) with or without mouse recombinant OPN (0.2 µmol/l; O2260, Sigma). The OPN dose for GC treatment was determined based on the reported EC₅₀ in a cell migration assay using vascular smooth muscle cells to evaluate the effect of OPN via an integrin receptor (Yue *et al.* 1994). GCs were also treated with hCG (1 IU/ml), EGF (5 ng/ml; E9644, Sigma), epiregulin (*Ereg*; 5 ng/ml; 1068-EP, R&D, Minneapolis, MN, USA), or amphiregulin (*Areg*; 50 ng/ml; 989-AR, R&D) with or without AG1478 (50 or 150 nmol/l; Cell Signaling Technology, Waltham, MA, USA), which is a tyrosine kinase inhibitor specifically selective for EGFR. Furthermore, to assess the OPN-induced intracellular signaling, GCs were pretreated for 1 h with LY294002 (Cell Signaling Technology), a phosphoinositide 3-kinase (PI3K) inhibitor (50 µmol/l), and/or PD980592 (100 µmol/l; Cell Signaling Technology), an ERK inhibitor, and cells were incubated with OPN in the culture medium containing hCG. For RNA isolation, incubated GCs were snap-frozen and stored at -80°C . For western blotting analyses, GCs were cultured at a density of 1 000 000 cells/well in a six-well plate (34.8 mm in diameter) with hCG (1 IU/ml) for 12 h. After changing the medium, GCs were incubated with or without OPN (0.2 µmol/l) for 15 min. The cells were scraped into ice-cold PBS and transferred to a microfuge tube followed by centrifugation at 10 000 *g* for 10 min. After aspiration of PBS, cells were lysed by adding 100 µl of $1\times$ SDS sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.1 mol/l dithiothreitol, and 0.01% bromophenol blue) and sonicated for 10–15 s. The lysate was stored at -80°C until analysis and heated to 95–100 $^{\circ}\text{C}$ for 5 min before the assay.

Plasma OPN assay

PMSG-primed mice were decapitated at 0, 12, 16, and 24 h after hCG treatment, and blood was collected into the

tubes containing EDTA-2Na (1 mg/ml blood) followed by centrifugation at 3000 *g* for 20 min at 4 $^{\circ}\text{C}$. Plasma aliquots were transferred into 1.5 ml Eppendorf tubes and stored at -80°C for later measurement. Plasma OPN concentrations were measured by ELISA.

ELISA

To analyze progesterone and OPN concentrations, mouse plasma or the GC culture supernatant was examined with the commercial ELISA Kits. OPN levels were assayed with a Mouse OPN ELISA Kit (IBL, Minneapolis, MN, USA; cat #IB3054) according to the manufacturer's instructions. Progesterone levels were determined with a Progesterone ELISA Kit (Cayman Chemical Company, Ann Arbor, MI, USA; cat #582601) according to the manufacturer's instructions.

Western blotting analysis

To analyze AKT phosphorylation, 10 µg of control or OPN-treated cell lysate was separated on a 4–20% SDS–polyacrylamide gel (4–20% Mini-PROTEAN TGX Gel; Bio-Rad) and transferred electrophoretically to a PVDF membrane (Trans-Blot Turbo Mini PVDF Transfer Packs; Bio-Rad). The membrane was blocked in a blocking solution (PVDF Blocking Reagent for Can Get Signal; TOYOBO, Osaka, Japan) and then incubated overnight with a primary antibody. Primary antibodies (1:2000 dilution, Can Get SignalR Immunoreaction Enhancer Solution 1; TOYOBO) were the following: rabbit anti-p-AKT (phospho-Akt (Ser⁴⁷³), 4060; CST), rabbit anti-p-AKT (Thr³⁰⁸, 2965; CST), and rabbit anti-AKT (4691; CST). The unbound primary antibody was removed by washing the membrane at 25 $^{\circ}\text{C}$ with Tris-buffered solution (20 mmol/l Tris–HCl of pH 7.6, 137 mmol/l NaCl, 0.1% Tween 20), followed by incubation with secondary antibody (goat anti-rabbit IgG–HRP; GE Healthcare, Little Chalfont, England) diluted 1:2000 in Can Get SignalR Immunoreaction Enhancer Solution 2 (TOYOBO). The protein was then visualized using ECL Prime Western Blotting Detection Reagents (GE Healthcare) and imaged using a LAS-4000 Luminescent Image Analyzer (FujiFilm Co., Tokyo, Japan). Densitometric analysis was performed using Multi Gauge version 3.1 (FujiFilm Co.), and p-AKT levels were normalized to mean total AKT levels.

Statistical analyses

All data are presented as mean $\pm$ s.e.m. of at least three independent experiments. Each assay was performed

using materials from different mice or GCs individually incubated under specific conditions. The results were analyzed by one-way ANOVA followed by *t*-test with Bonferroni's correction, and $P < 0.05$ was considered statistically significant.

Results

Evaluation of ovarian *Opn* transcripts and plasma OPN levels in mice during the periovulatory period

By using PCR array analyses targeting growth-factor-related molecules of mouse ovarian transcripts, we discovered a marked increase in *Opn* transcripts after hCG stimulation (Supplementary Table 1, see section on supplementary data given at the end of this article). To analyze the regulation in detail, we performed real-time RT-PCR of ovarian *Opn* transcripts in mice treated with PMSG and receiving an ovulatory dose of hCG (1.0 IU/ml) by injection 48 h later. Treatment with hCG increased *Opn* levels within 3 h ($n = 3$, $P < 0.0005$), followed by a peak at 16 h ($n = 3$, $P < 0.0001$; Fig. 1A). Using GCs and RO from the ovaries primed with PMSG for 48 h and subsequently treated with hCG for 6 or 16 h, real-time RT-PCR was carried out to examine *Opn* expression. Expression of *Opn* increased predominantly in the GCs compared with that in the RO at both 6 and 16 h after hCG treatment ($n = 3$; 6 h, $P < 0.005$ and 16 h, $P < 0.00005$; Fig. 1B), indicating that GCs represent a cell type that prominently expresses *Opn* in response to a gonadotropin surge. We also examined the mouse plasma *Opn* levels during gonadotropin treatment. PMSG-primed mice were treated with hCG and killed 0, 12, 16, and 24 h later. Basal plasma *Opn* levels were 3701–4205 ng/ml with a mean of 3987.7 ng/ml (s.d. ± 258.9 ng/ml). Treatment with hCG significantly increased plasma *Opn* levels at 16 h ($n = 4$, 0 h versus 16 h, $P < 0.01$) and 24 h ($n = 4$, 0 h versus 24 h, $P < 0.05$) (Fig. 1C), the kinetics of which are similar to those observed for ovarian *Opn* transcripts.

Opn localization in mouse ovaries during the periovulatory period

We further confirmed the localization of *Opn* by ISH analyses. *Opn* was expressed in cumulus cells of preovulatory follicles 10 h after hCG treatment (Fig. 2A). In addition, *Opn* transcripts were expressed markedly in preovulatory follicle GCs 12 h after hCG treatment (Fig. 2B). In contrast, *Opn* transcripts were distributed sparsely in the ovaries dissected from 16-week-old adult mice (Fig. 2C), which may reflect the transient pattern of

expression of *Opn* in the ovarian cycle. Further evaluations using adult mice at identified estrous stages are required to clarify the normal expression pattern of *Opn* during the ovarian cycle.

Figure 1

Evaluation of ovarian *Opn* transcripts and plasma OPN levels in mice during the periovulatory period. (A) Real-time PCR detection of *Opn* transcripts in mouse ovaries during the periovulatory period. Sequential changes in *Opn* expression levels are shown as fold increases relative to expression levels in pregnant mare serum gonadotropin (PMSG)-primed ovaries (0 h after human chorionic gonadotropin (hCG) treatment). PMSG-primed versus 3 h after hCG: $*P < 0.0005$ and PMSG-primed vs 16 h after hCG: $**P < 0.0001$. (B) Real-time RT-PCR analyses of *Opn* transcripts in isolated ovarian compartments (GC, granulosa cells; RO, residual ovaries) obtained from PMSG-primed ovaries (0, 6, and 16 h after hCG treatment). *Opn* levels in each ovarian compartment are shown as fold increases relative to those in GCs 0 h after hCG treatment. GC versus RO, 6 h: $***P < 0.005$ and 16 h: $†P < 0.00005$. (C) Plasma OPN levels during gonadotropin treatment. PMSG-primed mice were treated with hCG and killed 0, 12, 16, and 24 h later. 0 h versus 16 h: $††P < 0.01$ and 0 h versus 24 h: $†††P < 0.05$. All *Opn* transcript values were normalized based on β -actin levels. All data are presented as mean $\pm$ s.e.m. for three independent experiments using material from different mice.

Expression and regulation of *Opn* in cultured GCs

To determine whether the *in vivo* induction of *Opn* expression can be mimicked *in vitro* by activating the LH receptor, immature female mice were treated with PMSG, and GCs were isolated from preovulatory ovaries that were obtained 48 h after treatment. GCs were cultured in the absence or presence of a luteinizing dose of hCG (1.0 IU/ml). Real-time RT-PCR revealed that incubation with hCG significantly induced the expression of *Opn* in cultured GCs at 12 h ($n=3$, $P<0.0005$) and 24 h ($n=3$,

$P<0.00005$; Fig. 3A). To analyze the levels of OPN protein secretion, OPN from GC culture supernatant was measured by ELISA. Incubation with 1 U/ml hCG induced a significant increase in OPN protein levels in the culture media of PMSG-primed GCs at 12 h ($n=3$, $P<0.0005$) and at 24 h ($n=3$, $P<0.0005$) (Fig. 3B).

We further tested whether the upregulation of *Opn* levels is mediated by the activation of LH-induced EGFR signaling, the most prominent mediator of the gonadotropin surge (Park *et al.* 2004). EGF itself is not upregulated by hCG stimulation in mouse ovaries. Therefore, in

Figure 2

In situ hybridization (ISH) of *Opn* in gonadotropin-treated mouse ovaries. ISH was performed using both anti-sense and sense RNA probes for mouse *Opn*. Ovarian sections were obtained from mice pre-treated with PMSG, followed by hCG treatment for 10 h (A) or 12 h (B). *Opn* transcripts were detected in the cumulus cells of preovulatory follicles 10 h after hCG

treatment (A). *Opn* transcripts were markedly expressed in GCs of preovulatory follicles 12 h after hCG treatment (B). In adult mice (16 weeks old), *Opn* transcripts (indicated by arrows) were sparsely distributed in the ovary (C). Scale bars=100 μm.

Figure 3

Expression and regulation of OPN in cultured GCs. Immature female mice were treated with PMSG, and GCs were isolated from preovulatory ovaries obtained 48 h later. GCs were cultured in the absence or presence of a luteinizing dose of hCG (1 IU/ml). (A) Real-time PCR analysis of *Opn* transcripts in cultured GCs. Changes in *Opn* expression are shown as fold increases relative to expression in GCs from PMSG-primed ovaries (cultured for 0 h). 0 h versus 12 h: $*P < 0.0005$ and 0 h versus 24 h: $^{\dagger}P < 0.00005$. (B) OPN levels measured in the supernatants of GC cultures by ELISA. Incubation with 1 IU/ml hCG caused a significant increase in OPN protein levels in the culture media of PMSG-primed GCs at 12 and 24 h. 6 h versus 12 h: $*P < 0.0005$ and 6 h versus 24 h: $*P < 0.0005$. (C) Effect of stimulation and/or inhibition of EGFR on *Opn* expression in cultured GCs. GCs were cultured for 16 h with 1 IU/ml hCG, 5 ng/ml EGF, 5 ng/ml Ereg, or 50 ng/ml Areg. All molecules significantly stimulated *Opn* mRNA expression, compared with those in GCs cultured without any treatment (Cont.). hCG: $***P < 0.005$; EGF: $^{\dagger\dagger\dagger}P < 0.05$; Ereg: $*P < 0.0005$; and Areg: $***P < 0.005$. *Opn* expression was significantly inhibited by the EGFR inhibitor AG1478 added at concentrations of 50 and 150 nmol/l. Non-hormone-treated group: $***P < 0.005$ and $^{\dagger}P < 0.001$; hCG-treated group: $^{\dagger\dagger}P < 0.01$ and $***P < 0.005$; EGF-treated group: not significant and $^{\dagger\dagger\dagger}P < 0.05$; Ereg-treated group: $*P < 0.0005$ and $*P < 0.0005$; and Areg-treated group: $***P < 0.005$ and $***P < 0.005$. Changes in *Opn* expression levels are shown as fold increases relative to expression levels in control GCs (Cont.); cultured without any treatment for 16 h). All data are presented as mean $\pm$ S.E.M. for three independent experiments.

In addition to hCG and EGF, we also induced stimulation of the EGF-like growth factors, Ereg and Areg, both of which are induced by hCG stimulation in mouse ovaries (Shimada *et al.* 2006). We cultured GCs for 16 h with 1 U/ml hCG, 5 ng/ml EGF, 5 ng/ml Ereg, or 50 ng/ml Areg in the presence or absence of AG1478 (50 or 150 nmol/l), which selectively blocks the activation of the EGFR tyrosine kinase. All molecules significantly stimulated *Opn* mRNA expression, compared with those in GCs cultured without any treatment (Cont.) (hCG: $n = 3$, $P < 0.005$; EGF: $n = 3$, $P < 0.05$; Ereg: $n = 3$, $P < 0.0005$; and Areg: $n = 3$, $P < 0.005$) (Fig. 3C). Furthermore, expression was significantly inhibited by the EGFR inhibitor AG1478

in a dose-dependent manner (50 nmol/l AG1478: $n = 3$, $P < 0.005$; 150 nmol/l AG1478: $n = 3$, $P < 0.001$; hCG + 50 nmol/l AG1478: $n = 3$, $P < 0.01$; hCG + 150 nmol/l AG1478: $n = 3$, $P < 0.005$; EGF + 150 nmol/l AG1478: $n = 3$, $P < 0.05$; Ereg + 50 nmol/l AG1478: $n = 3$, $P < 0.0005$; Ereg + 150 nmol/l AG1478: $n = 3$, $P < 0.0005$; Areg + 50 nmol/l AG1478: $n = 3$, $P < 0.005$; and Areg + 150 nmol/l AG1478: $n = 3$, $P < 0.005$). These results indicated that up-regulation of *Opn* expression is mediated by EGFR signaling, which is triggered by an LH surge.

Effect of OPN treatment on progesterone synthesis in cultured GCs

Because *Opn* expression was localized to the early luteinizing GCs, OPN-mediated signaling is probably involved in corpus luteum formation and function. Using the GC culture system, we evaluated the progesterone concentrations in the culture media by ELISA. GCs were collected from PMSG-primed mice and incubated for 12 or 24 h in culture media containing 1 U/ml hCG with or without 0.2 μ mol/l OPN. OPN treatment led to a significant increase in progesterone concentration in the culture medium after 24 h ($n = 4$; $P < 0.05$; Fig. 4A). We also evaluated the effect of OPN treatment on the expression of molecules related to progesterone synthesis. OPN treatment led to a significant increase in *Star* ($n = 4$; 24 h, $P < 0.005$; Fig. 4B), *p450scc* ($n = 4$; 12 h, $P < 0.05$ and 24 h, $P < 0.05$; Fig. 4C), and *Hsd3b* ($n = 4$; 12 h, $P < 0.0005$ and 24 h, $P < 0.005$; Fig. 4D) transcripts. These results indicated that OPN is involved in progesterone synthesis by promoting the expression of molecules related to progesterone synthesis.

Effect of OPN treatment on the signaling pathway to enhance *Vegf* expression in cultured GCs

VEGF is a prominent angiogenic factor that exists in the ovary during follicular development (Kaczmarek *et al.* 2005); therefore, we evaluated the effect of OPN treatment on expression of *Vegf* using the GC culture system. We confirmed that OPN treatment significantly increased *Vegf* transcripts (data not shown). Recently, it has been found that OPN reportedly induces *Vegf* expression through the activation of PI3K/AKT and ERK1/2 in endothelial cells (Dai *et al.* 2009). Therefore, we tested the effects of blocking these pathways on the promotion of expression of *Vegf* by OPN in GCs. GCs were pretreated for 1 h with LY294002 (50 μ mol/l), a PI3K inhibitor, and/or PD980592 (100 μ mol/l), an ERK inhibitor, and cells were incubated for 20 h with 0.2 μ mol/l OPN in the culture medium

Figure 4

Effect of OPN treatment on progesterone synthesis in cultured GCs. GCs were collected from PMSG-primed mice and incubated in culture media containing 1 IU/ml hCG with or without 0.2 μ mol/l OPN for 12 or 24 h. (A) OPN treatment significantly increased progesterone concentration in the culture medium at 24 h, $n=3$; $^{***}P<0.05$. (B, C and D) OPN treatment significantly increased the transcription of molecules associated with progesterone synthesis, $n=4$; (B) *Star* 24 h: $^{***}P<0.005$; (C) *p450scc* 12 h: $^{***}P<0.05$ and 24 h: $^{***}P<0.05$; and (D) *HSD3B* 12 h: $*P<0.0005$ and 24 h: $^{***}P<0.005$. Changes in transcripts levels of each molecule are shown as fold increases relative to levels in GCs cultured without OPN for 12 h. All data are presented as mean $\pm$ S.E.M. for three or four independent experiments.

containing hCG. OPN treatment significantly increased *Vegf* transcripts ($n=3$, $P<0.005$), and *Vegf* expression was inhibited significantly by incubation with LY294002 ($n=3$; LY294002: $P<0.005$ and LY294002+PD80592: $P<0.0005$), but not by PD80592 alone (Fig. 5A). These results indicated that OPN primarily enhances expression of *Vegf* through the PI3K/AKT pathway, but not through ERK. Next, we confirmed whether OPN stimulation of GCs promotes the activation of AKT, a downstream PI3K signaling molecule. GCs from PMSG-primed immature mice were pre-treated with 1 IU/ml hCG for 12 h and incubated with or without 0.2 μ mol/l OPN for 15 min. GCs were collected, and cell lysates were subjected to western blotting analysis for detection of AKT phosphorylation using the following AKT-specific antibodies: rabbit anti-AKT, rabbit anti-p-AKT (Ser⁴⁷³), and rabbit anti-p-AKT

(Thr³⁰⁸). Treatment with OPN for 15 min significantly stimulated the phosphorylation of AKT at its regulatory residues Thr³⁰⁸ ($n=3$, $P<0.005$) and Ser⁴⁷³ ($n=3$, $P<0.05$) (Fig. 5B).

Discussion

The periovulatory period is characterized by a surge of gonadotropins that brings about dramatic functional and structural changes in the ovary, leading to oocyte maturation, follicle rupture, and corpus luteum formation. Several studies have shown that the gonadotropin surge induces rapid but transient expression of specific genes associated with this process (Richards *et al.* 1998, Tsafiri & Reich 1999). On the basis of the results obtained using the PCR array and real-time RT-PCR analyses of mouse ovarian gene expression during the periovulatory period, we found a marked increase in *Opn* levels. *In vitro* analyses using cultured GCs also provided evidence of OPN protein secretion in response to hCG treatment.

Because *Opn* expression, stimulated by gonadotropin, was clearly localized on ovarian mural GCs as observed by ISH, we focused on expression of *Opn* in the mural GCs during the periovulatory period. According to the results from the immature mouse ovarian stimulation model, there was a considerable time lag (16 h) before levels of *Opn* reached a peak value after hCG injection. Therefore, we speculated that molecular signaling might mediate the gonadotropin surge to promote expression of *Opn*. Recently, results from several studies have indicated that EGF-like growth factors (e.g. amphiregulin and epiregulin) are prominent mediators of LH action in the ovulatory follicle (Park *et al.* 2004). Furthermore, up-regulation of the expression of *Opn* by EGF has been reported for several cultured cell types, including breast cancer, colon cancer, rat kidney epithelial, HL60, and HepG2 cells (Atkins *et al.* 1997, Malyankar *et al.* 1997, Zhang *et al.* 2003). We investigated whether EGFR signaling is also involved in the promotion of the expression of *Opn* in mouse GCs. On the basis of *in vitro* analyses using cultured GCs, EGF and EGF-like growth factors stimulated expression of *Opn*, while an EGFR antagonist completely inhibited the effects of hCG on the expression of *Opn*. These findings indicate that EGFR signaling is a prominent pathway for mediating the LH surge, thus enhancing the expression of *Opn*. Because EGF-like growth factors are involved in several ovarian physiological processes, including ovulation, steroidogenesis, oocyte maturation, and cumulus expansion (Hsieh *et al.* 2009), it is interesting to consider that these physiological processes may be

Figure 5

Effect of treatment with OPN on signaling pathway enhancing expression of *Vegf* expression in cultured GCs. (A) GCs were pretreated for 1 h with LY294002, a PI3K inhibitor (50 $\mu\text{mol/l}$), and/or PD980592, an ERK inhibitor (100 $\mu\text{mol/l}$), and then GCs were incubated for 20 h with 0.2 $\mu\text{mol/l}$ OPN in culture media containing 1.0 IU/ml hCG. Incubation with OPN significantly increased *Vegf* transcripts. *** $P < 0.005$. The increase was inhibited significantly by incubation with LY294002, but not by PD80592 alone. LY294002: *** $P < 0.005$ and LY294002 + PD80592: * $P < 0.0005$. Changes in *Vegf* levels are shown as fold increases relative to levels in GCs cultured without OPN, LY294002, or PD80592. (B) Detection of AKT phosphorylation in OPN-treated GCs. GCs from PMSG-primed immature mice were pre-treated with 1 IU/ml hCG for 12 h and incubated with or without 0.2 $\mu\text{mol/l}$ OPN for 15 min. GCs were collected, and cell lysates were subjected to western blotting to detect AKT phosphorylation using the following AKT-specific antibodies: rabbit anti-AKT (Pan), rabbit anti-phospho-AKT (anti-p-AKT Ser⁴⁷³), and rabbit anti-p-AKT (Thr³⁰⁸). Densitometric analysis of p-AKT levels normalized to total AKT levels. Thr³⁰⁸: *** $P < 0.005$ and Ser⁴⁷³: *** $P < 0.005$. Changes in p-AKT levels are shown as fold increases relative to those in controls (GCs before OPN treatment). All data are presented as mean $\pm$ S.E.M. for three independent experiments.

partially mediated by OPN signaling. The up-regulation of *Opn* expression during the early-corpus luteum phase and the effect of OPN on GCs promoting progesterone synthesis strongly support the enhancement of corpus luteum function by OPN during this period. As levels of

OPN also increased with hCG treatment, similar to *Opn* transcripts, it is likely that locally secreted OPN from GCs can leak into blood circulation. Consequently, it would be fascinating to evaluate plasma OPN as a potential biomarker in infertile patients in a future study.

In the ovaries, there is intense angiogenesis throughout follicular development, allowing adequate nutritional and hormonal supply for ovarian follicular growth, oocyte development, ovulation, and subsequent corpus luteum formation. Several pro-angiogenic factors exist in the ovary during follicular development, such as VEGF, fibroblast growth factor 2, angiotensin 2, insulin-like growth factor 1, EGF, angiotensin, and endothelin 1 (Bruno *et al.* 2009). OPN reportedly enhances the expression of *Vegf* and induces angiogenesis in endothelial cells (Dai *et al.* 2009). In this study, we showed that OPN treatment in the GC culture system promotes the expression of *Vegf*, which encodes a prominent angiogenic factor in the early luteinizing period. We also showed that treatment with a PI3K inhibitor in the GC culture system significantly suppresses the promotion of *Vegf* expression, and that OPN treatment stimulates the phosphorylation of AKT, a downstream PI3K signaling molecule. Taking these results together, OPN is considered to serve as a pro-angiogenic factor in the ovary during the early-luteal phase by promoting expression of *Vegf* through PI3K/AKT signaling, which enhances corpus luteum formation by promoting vascular formation.

According to the results from ISH experiments, a gonadotropin surge primarily stimulates *Opn* expression in cumulus cells in advance of mural GCs, indicating that OPN is also implicated in some physiological roles in cumulus-oocyte complexes. Interestingly, there are some similarities between OPN and hyaluronic acid (HA), because both are categorized as extracellular matrix components, and both are present on the surfaces of cumulus cells in response to a gonadotropin surge. Furthermore, OPN and HA can be degraded into smaller biologically active fragments by specific enzymes and bind to the same cell-surface receptor, CD44 antigen. As HA is known to play crucial roles in cumulus cell expansion, oocyte maturation, and further embryonic development (Marei *et al.* 2012), it can be speculated that these physiological processes are also regulated by OPN. *In vitro* oocyte culture experiments to assess the effects of OPN on oocyte nuclear maturation rates, embryo development to blastocyst stage, and blastocyst quality may provide additional insights into the role of OPN. This new understanding might allow future use of OPN during follicle culture and *in vitro* oocyte maturation in clinical practice.

Along with the CD44 antigen, OPN binds several different integrin receptors, including $\alpha v \beta 1$, $\alpha v \beta 3$, $\alpha v \beta 5$, $\alpha 4 \beta 1$, and $\alpha 9 \beta 1$, through its conserved cell-binding arginine–glycine–aspartic acid domain (Hu *et al.* 1995, Liaw *et al.* 1995a,b, Smith *et al.* 1996, Bayless *et al.* 1998). Interestingly, binding of OPN to these receptors has distinct functional consequences (Tuck *et al.* 2000). To our knowledge, this is the first study to demonstrate molecular evidence that OPN transcriptionally enhances progesterone synthesis and follicular angiogenesis. As OPN is a multifunctional molecule, it might also be involved in other physiological processes such as tissue remodeling after ovarian injury caused by ovulation and/or survival of early-luteal cells. Further investigation, specifically a blocking assay using cell-surface-receptor-specific blockers, might provide an in-depth understanding of OPN function in the ovary, especially ligand–receptor interactions and distinct biological functions.

In a previous investigation using OPN-knockout mice, a specific ovarian phenotype was not mentioned, and the mice were reportedly fertile (Liaw *et al.* 1995a,b). Therefore, up-regulation of *Opn* in the periovulatory phase appears not to be essential for the maintenance of ovarian function. During OPN deficiency, other molecules induced by the gonadotropin surge may be acting in a compensatory manner during the periovulatory period. Accordingly, OPN is considered to be a contributor to maintaining proper periovulatory ovarian function. This contribution is important because stable ovarian physiology is achieved by multi-component molecular processes, which is essential for maintaining female reproductive capacity and for species preservation.

In conclusion, *Opn* expression is markedly up-regulated in mouse ovarian GCs in response to a gonadotropin surge through EGFR signaling, which enhances progesterone synthesis and expression of *Vegf* during the early-luteal phase.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JOE-14-0203>.

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.

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