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The Expression and Roles of Semaphorin Type 3C in Granulosa Cells during The Luteinization Process

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Abstract: Morphological changes are observed during the luteinization process of granulosa cells, however mechanisms and the roles are remained unclear. In the present study, we demonstrated that Sema3C is expressed in granulosa cells and then secreted to extracellular matrix accumulated within cumulus cell and granulosa cell layers. The expression is dependent on EGF-like factors (amphiregulin and neuregulin 1) and EGF receptor dependent manner. The knockdown of *Sema3c* not only significantly decreased the surface area of the granulosa cells, but also significantly suppressed the cell migration of granulosa cells cultured with EGF like factors. The phosphorylation of focal adhesion kinase (FAK) was dramatically suppressed by transfection with *Sema3c* siRNA, with an increasing level of phosphorylated ROCK, indicating that Sema3C regulated actin remodeling in a FAK signaling pathway-dependent manner. Moreover, the expression levels of genes involved in progesterone production, *Star*, *Cyp11a1* were significantly decreased in *Sema3c* siRNA-transfected granulosa cells as compared with control siRNA transfection. From these results, we conclude that Sema3C is secreted and then accumulated within granulosa cell layers and cumulus cell layers. The FAK pathway is activated by Sema3C and induces cell migration and enlarges the area of granulosa cells. These morphological changes are required for luteinization (production of progesterone) of granulosa cells.
Key words: luteinization, Migration, Granulosa cells, Semaphorin, Ovulation

Introduction

The transient and marked secretion of LH from the pituitary gland acts on granulosa cells of preovulatory follicles [1]. This stimulus dramatically changes the expression pattern of genes in the cells to induce the final differentiation known as luteinization [2]. Production of EGF-like factors, amphiregulin (AREG), beta-cellulin (BTC) and epiregulin (EREG) is induced within 1 h after hCG (LH) injection, and then they are released from granulosa cells by ADAM17 [3–5]. The active form, EGF domain, selectively binds to EGF receptor (EGFR, other name is ErbB1) to activate the downstream signaling pathways, such as the Ras-ERK1/2 pathway [4, 6, 7]. Neuregulin 1 (NRG1) that is a member of EGF like factor and acts on ErbB2/ErbB3 heterodimers but not on EGFR is also expressed in granulosa cells [8]. NRG1 enhances EGFR-induced ERK1/2 phosphorylation [9]. Transcription factors are activated by the ERK1/2 signaling pathway and transferred to nucleus, increasing the expression of target genes including the enzymes needed to produce progesterone [2, 10, 11]. Progesterone is converted from cholesterol, and the first step in this conversion is the transition of cholesterol from cytoplasm to mitochondria by StAR that is induced by ERK1/2-regulated transcription factors [12]. The side chain of cholesterol is cut by P450scc, that is encoded by *Cyp11a1,* converting it to pregnenolone, which is then changed to progesterone by HSD3β on the endoplasmic reticulum [13, 14]. Thus, the expression of genes involved in progesterone production, that is induced by EGF-like factors-ErbB family-ERK1/2 pathway, is required for the induction of luteinization.

During the luteinization of granulosa cells, it is known that the cell morphology, as observed under a light microscope, dramatically changes from a circle to a polygon form, and the surface area of luteinized granulosa cells increases, compared with undifferentiated granulosa cells [15]. Recently, Karlsson et al. [16] reported that the morphological changes of granulosa cells were not the result of luteinization, but the source of the differentiation of granulosa cells. They showed that actin remodeling was regulated by cofilin, and the knockdown of coflin significantly suppressed progesterone production of mouse cultured granulosa cells [16]. In our previous study, a focal adhesion component that directly binds to actin filament was degraded in follicular somatic cells during the ovulation process [17]. The degradation induced cell detachment and bleb (lamellipodia) formation, suggesting that dynamic changes of cell cytoskeleton (actin remodeling) are required for the induction of granulosa cell luteinization. However, there is little information about how to induce cell migration and morphological changes in granulosa cells.

The semaphorin family was discovered as a factor inducing nerve system development, but now it is recognized as a multiple regulator of cell to cell adhesion, cell migration, and the inversion of cancer cells [18]. The semaphorin family is comprised of 8 different classes, and classes 3 to 8 have been cloned in mice [19]. Class 3 semaphorin (Sema3) is a unique subtype because Sema3 is secreted by the exocytosis system and then accumulates within the extracellular matrix (ECM) [20]. The release of Sema3 from ECM is induced by ADAMTS-1 [20], and it then acts on heterodimers of plexin and neuropilin (Nrp) to induce cell migration and cytoskeleton remodeling [21–23]. The receptor complex binds to small GTPase proteins, such as Rnd1, which is associated with cell migration and/or cytoskeleton remodeling via focal adhesion kinase (FAK)-dependent mechanisms [24–26]. In granulosa cells, ADAMTS1 is expressed and the exocytosis system is expressed and functional [27–30]. Thus, we hypothesized that the Sema3 family is expressed and secreted from granulosa cells and has an important role in the luteinization of granulosa cells.

In this study, to clear this hypothesis, we examined the expression patterns of the Sema3 family and their receptors in granulosa cells during the ovulation process using the mouse super ovulation model. Additionally, we cultured mouse granulosa cells to investigate how the expression of the Sema3 family is regulated, and its roles in cell migration and morphological changes during the luteinization of granulosa cells.

Materials and Methods

Materials

Equine chorionic gonadotropin, eCG (SEROTROPIN) and hCG (GONATROPIN) were purchased from Asuka Seiyaku (Tokyo, Japan), AREG and NRG1 from R&D systems (Minneapolis, MN, USA). DMEM:F12 medium and penicillin-streptomycin were purchased from Invitrogen (Carlsband, CA, USA). Fetal bovine serum (FBS) was obtained from Life Technologies Inc. (Grand Island, NY, USA). Oligonucleotide poly-(dT) was purchased from Invitrogen, and AMV reverse transcriptase and Taq polymerase were purchased from Promega (Madison, WI, USA). Routine chemicals and reagents were obtained from Nakarai Chemical Co. (Oosaka, Japan), or Sigma Chemical Co. (Sigma; St. Louis, MO, USA).

Animals

Immature female C57Bl/6 mice were obtained from Charles River Japan (Kanagawa, Japan). On day 23 of age, female mice were injected intraperitoneally (IP) with 4 IU of eCG to stimulate follicular growth followed 48 h later with 5 IU hCG to stimulate ovulation and luteinization [28]. Animals were housed under a 16-h light/8-h dark schedule in the Center for Comparative Medicine at Experiment Animal Center of Hiroshima University, and provided food and water *ad libitum*. Animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals, as approved by the Animal Care and Use Committee of Hiroshima University.

Granulosa cell culture

Granulosa cells were harvested by needle puncture from immature mice treated with eCG on day 23 of age as described previously [4]. Briefly, 1×10^6 cells were cultured in 12-well culture plates in 1% serum-containing medium (DMEM:F12 containing penicillin and streptomycin). After 8 h culture, the cells were washed, then cultured for 4 h in fresh, serum-free medium containing forskolin (For) (10 μM) and PMA (20 nM) which mimics LH stimulation of cAMP and diacylglycerol production, or EGF like factors (100 ng/ml of AREG and 10 ng/ml of NRG1), and harvested for RNA analysis [4, 8].

siRNA treatment procedure of cultured mouse granulosa cells

Sema3c pre-designed siRNA was purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, CA, USA). Scrambled control siRNA (Santa Cruz Biotechnology) was used as a negative control. Mouse granulosa cells (1×10^6 cells/well) recovered from eCG-

primed mice were plated in 12-well culture plates for 3 h before transfection. Transfection of siRNA (25 nM) was accomplished with HVJ envelope vector kit GenomONE neo (Ishihara Sangyo, Osaka, Japan) according to the manufacturer's instructions. Cells were incubated at 37°C in a CO_2 incubator, and the culture medium was replaced 5 h after transfection. After transfection, granulosa cells were cultured with 100 ng/ml of AREG and 10 ng/ml of NRG1 for 8 h. The cultured medium (conditioned medium) was collected for use in the cell migration assay. The surface area of the cultured granulosa cells was analyzed using BZ-II application software (Keyence, Tokyo, Japan).

RT-PCR analyses

Total RNA was obtained from granulosa cells using the RNeasy mini kit (Qiagen Sciences, Germantown, MD, USA) according to the manufacturer's instructions. Total RNA was reverse transcribed using 500 ng poly-dT and 0.25 U avian myeloblastosis virus-reverse transcriptase (Promega) at 42°C for 75 min and 95°C for 5 min. For the amplification of the cDNA products, specific primers pairs were selected and analyzed as indicated in Table 1. cDNA products were resolved on 1.5% (w/v) agarose gels.

Real-time PCR analyses

cDNAs produced by the above method were also used for real-time PCR analysis. cDNA and primers were added to 15 μl total reaction volume using provided in the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). PCR reactions were then performed using the Step One Real-time PCR systems (Applied Biosystems). The conditions were set to the following parameters: 10 min at 95°C followed by 45 cycles each of 15 sec at 95°C and 1 min at 62 or 64°C. Specific primers pairs were selected and analyzed as indicated in Table 1.

Western blot analyses

On day 23 of age, female mice were injected intraperitoneally (IP) with 4 IU of eCG followed 48 h later with 5 IU hCG. Granulosa cells were recovered from antral follicles, and were then treated with 10 mIU/ml of hyaluronidase (Sigma). Protein samples from granulosa cells or whole ovaries were prepared either by homogenification in whole cell extract buffer. The protein concentration was checked using a DC Protein Assay Kit (Bio-Lad laboratories, Hercules, CA,USA). The extracts (20 μg protein) were diluted by same volume of 2 fold SDS sample buffer, and were then resolved by SDS polyacrylamide

Table 1. Primer List

Gene	Primer sequences	Tm (°C)
Sema3a	F: 5'-AGTGGGTGCCTTACCAAGGA-3' R: 5'-ACTGGCCATCTTCTGCATCC-3'	64
Sema3b	F: 5'-GGGCACAGTGCTGAAAGTGA-3' R: 5'-GAACCGTCTCTTGGCCGTAG-3'	64
Sema3c	F: 5'-TGGCCCAGAGACACATTTTG-3' R: 5'-TGGGAAGTCCTTGGTGGTTC-3'	64
Sema3d	F: 5'-AGGAGCACCGACCTTCAAGA-3' R: 5'-TTGCTGCTTCAGCGACAACT-3'	64
Sema3e	F: 5'-CGGAGGATGGCCAGTATGAC-3' R: 5'-TGCCATACATGTCGCAGTGA-3'	66
Sema3f	F: 5'-ACAAGGAGGGCCCCAACTAT-3' R: 5'-GCACCTCATAGCGTCCATCA-3'	64
PlexinA1	F: 5'-CAGATGCCTGCCTCTAGTG-3' R: 5'-CGTCCTTGTACTTGGGGATG-3'	64
PlexinA2	F: 5'-GCCTTCCACTTCGTTTCTGG-3' R: 5'-ACTAATGGCTGGGAGCTTGG-3'	66
Nrp1	F: 5'-TGGCACAGGTGATGACTTCC-3' R: 5'-CGGGCTACTTTGCCTTTCTG-3'	66
Cyp11a1	F: 5'-GGGAGACATGGCCAAGATGG-3' R: 5'-CAGCCAAAGCCCAAGTACCG-3'	60
Star	F: 5'-GCAGCAGGCAACCTGGTG-3' R: 5'-TGATTGTCTTCGGCAGCC-3'	60
3βHSD	F: 5'-TGGGGAGAGAAGTCCATTCA-3' R: 5'-GGAGCCCCCATTCCTTACTA-3'	60
L19	F: 5'-GGCATAGGGAAGAGGAAGG-3' R: 5'-GGATGTGCTCCATGAGGATGC-3'	60

gel (7.5%) electrophoresis before being transferred to PVDF membranes (GE Healthcare, Buckinghamshire, UK). The membranes were blocked in Tris-buffered saline and Tween 20 [TBST; 10 mM Tris (pH7.5), 150 mM NaCl and 0.05% Tween 20] containing 5% non-fat Carnation instant milk (skim-milk, Morinaga nyugyo, Tokyo, Japan). Blots were incubated primary antibody (1:1,000 dilution of Anti-Sema3C antibody, R&D systems, Anti-Phospho FAK (Try397) IgG, Cell Signaling Technology, Beverly, MA, USA, Anti-Phospho ROCK2 IgG, abcam, Cambridge, UK or 1:10,000 dilution of Anti-β-Actin antibody, AC74, Sigma) overnight at 4°C. After washing in TBST, enhanced chemiluminescence (ECL) detection was performed by using an ECL prime detection system according the manufacture's specifications (GE Healthcare) and appropriate exposure of the blots to Fuji X-ray film (Tokyo, Japan).

Immunofluorescence

Ovaries were collected and fixed in 4% (w/v) paraformaldehyde (Katayama Kogyo, Osaka, Japan) overnight, dehydrated in 70% ethanol, and embedded in paraffin. The paraffin-embedded fixed sections (7 *μ*m) embedded in paraffin were deparaffinized in xylene washes and quenched with 3% hydrogen peroxide in methanol. The sections were incubated with 20% non-immune goat serum/PBS to block non-specific sites followed by incubation with primary anti-Sema3C antibody (R&D systems) overnight at 4°C. After washing, the sections were further incubated with and secondary FITC-conjugated goat anti-mouse IgG antibodies (Sigma). Slides were mounted using VectaShield with DAPI (Vector Laboratories, Burlingame, CA, USA).

Cell migration assay

Granulosa cells were collected from eCG-primed mouse ovaries as described above, and then stained by Calcein AM (Becton Dickinson and Company, Franklin Lakes, MJ, USA). The granulosa cells were added to the insert chamber of a BD Dalcon 96-Multiwell Insert System (Becton Dickinson and Company). Conditioned medium or fresh medium were added to the lower chamber of each well. After 8 h culture, the cells which had migrated from the insert to lower chamber were detected by fluorescence intensity (494 nm/517 nm, Abs/Em) using a multi-label counter (PerkinElmer, Waltham, MA, USA).

Progesterone assay

Progesterone in the cultured medium was measured by a specific AIA 1800 system (TOSOH, Tokyo, Japan) as described previously [31].

Statistics

Data are presented as mean +/−SEM, and were analyzed using one-way ANOVA (SAS Institute Inc., Cary, NC, USA). All percentage data were subjected to arcsine transformation before ANOVA. When ANOVA revealed a significant effect, the means were compared using Fisher's protected least significant difference post-hoc test, and were considered significant when *P*<0.05.

Results

Expression of genes encoding Sema3 family and their receptors in granulosa cells of preovulatory follicles

We made specific primer sets that recognized each of the *Sema3* family members (type a to f), and found mRNA expression of 2 members of the *Sema3* family (*Sema3b* and *Sema3c*) in granulosa cells recovered from eCG-stimulated mouse ovaries. Real-time RT-PCR analyses showed that *Sema3b* mRNA did not significantly changed; however, *Sema3c* increased markedly within 4 h after hCG administration (Fig. 1A). RT-PCR analyses showed that Sema3C receptors, *Plexina1*, *Plexina2* and *Nrp1* were also expressed in granulosa cells (Fig. 1B).

Protein expression of Sema3C was detected by western blotting and the localization was observed through immunofluorescence using the same antibody. The protein level in whole ovaries dramatically increased after hCG injection and a high level was still detected at 16 h after hCG injection (Fig. 1C). However, when the cells were recovered from pre- or peri-ovulatory follicles and then treated with hyaluronidase, the protein level was much lower than that of whole ovary samples (Fig. 1C). Moreover, positive signals were detected in both cumulus cell layers and among the granulosa cells (Fig. 1D), suggesting that Sema3C was secreted and then accumulated within extracellular matrix within cumulus cell layers and granulosa cell layers.

The roles of Sema3c in the luteinization of cultured mouse granulosa cells

The expression of *Sema3c* was significantly increased by forskolin and PMA treatment when mouse granulosa cells were cultured *in vitro* (Fig. 2A). The induction of *Sema3c* was also observed after the addition of both AREG and NRG1 (AN) (Fig. 2A). The transfection of *Sema3c* siRNA significantly decreased the mRNA level, compared with that of cultured granulosa cells transfected with control siRNA (Fig. 2B).

Using the conditioned medium, granulosa cells were cultured with or without AN. We examined whether the secreted Sema3C affected the migration of granulosa cells or not. Compared with the migration level of fresh medium containing AN, the migration was not changed in the control conditioned medium (Fig. 2C). However, significantly increased the level of fluorescence intensity was significantly increased when the conditioned medium, where granulosa cells were cultured with AN, was added to the lower chamber (Fig. 2C). The induction was not observed by the conditioned medium where granulosa cells transfected with *Sema3c* siRNA were cultured with AN (Fig. 2C).

In the next experiment, we tried to understand the relationship between the expression of *Sema3c* and the morphological changes of cultured granulosa cells with AN. The morphology changed from circular to polygonal forms in the granulosa cells cultured with AN, and these same changes were also observed in the cells transfected with *Sema3c* siRNA (Fig. 3A). However the sur-
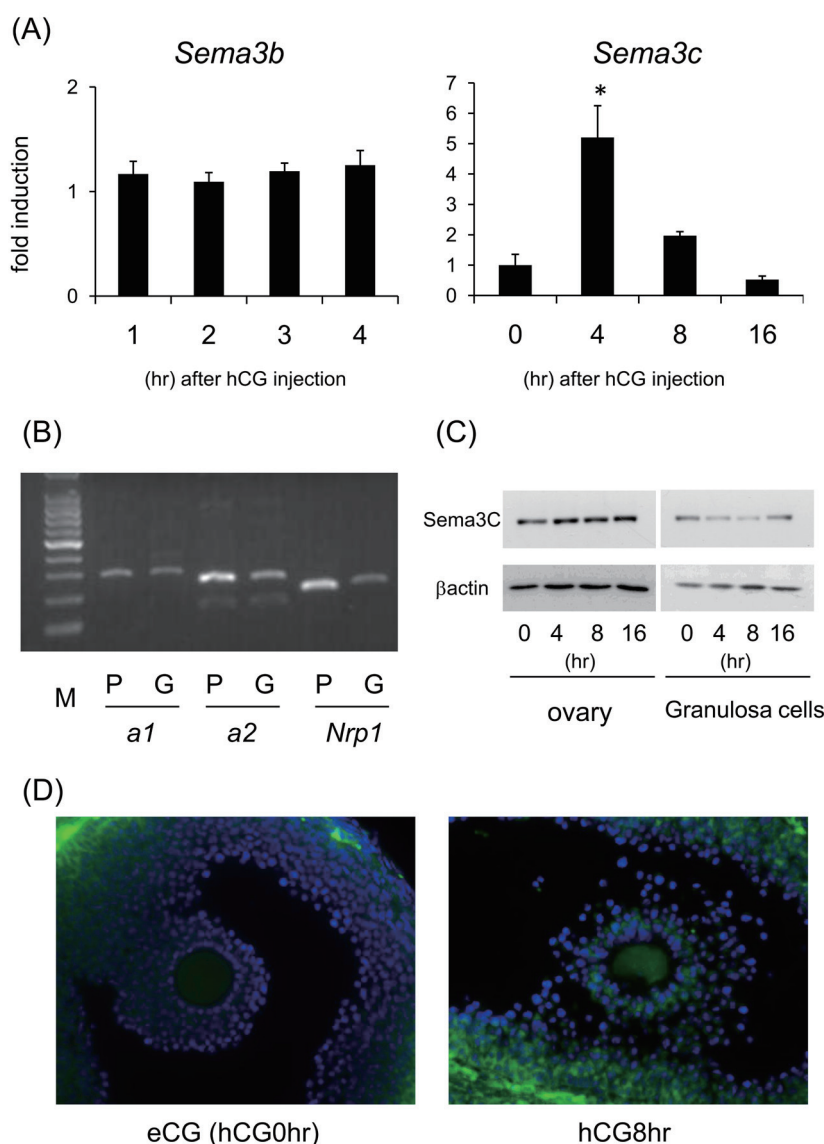
Fig. 1. The expression of Sema3 family members in the ovary during the ovulation process in eCG- and hCG-treated mice.
(A) The temporal changes of *Sema3b* and *Sema3c* mRNA expression in granulosa cells collected from ovaries of mice injected with eCG+hCG. For reference, the 0 hr value is set as 1, and the data are presented as fold increases. Values are mean +/−SEM of three replicates. *, Significant differences were observed as compared with those in granulosa cells recovered from eCG primed mice (hCG 0hr), *, $P<0.05$. (B) The expression of Sema3c receptors, *Plexina1(a1)*, *Plexina2(a2)* and *Nrp1* in granulosa cell of eCG-primed mice. M; bp marker, P; positive control, mRNA purified from adult mouse brain, G; granulosa cells from eCG-primed mice ovary. (C) The kinetic changes of Sema3C protein levels in ovary or granulosa cells of eCG+hCG-injected mice. (D) The localization of Sema3C in preovulatory (eCG) or periovulatory (hCG8hr) follicles. Green is Sema3C, Blue is nuclei (DAPI staining).

face area of the *Sema3c*-siRNA transfected granulosa cells was significantly lower than that of the control cells (Fig. 3B). To identify the roles of Sema3C in more detail, the downstream pathway of Sema3C-Plexins was examined in granulosa cells. The intensity of the Sema3C positive band (protein level of Sema3C) was dramatically decreased by the *Sema3c* siRNA (Fig. 3C). The phosphorylation of FAK was also dramatically decreased, and
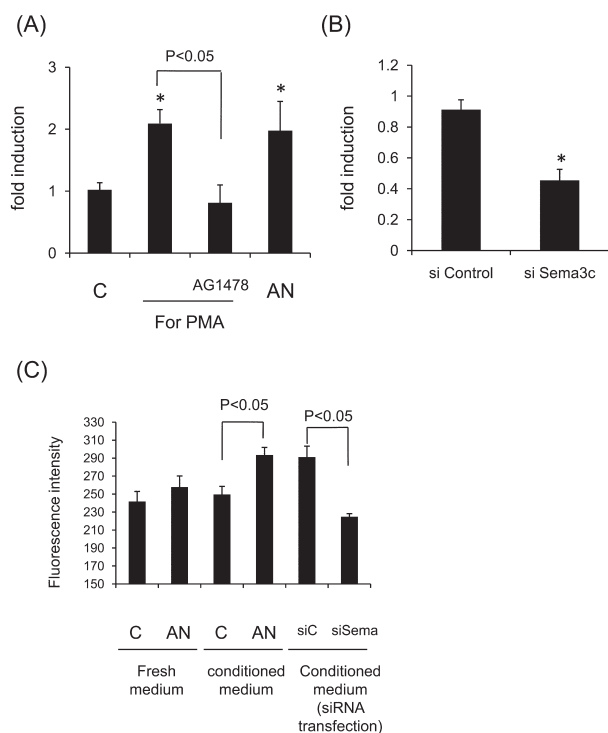
(A)

(B)

(C)

Fig. 2.   The expression and roles of Sema3C in cultured granulosa cells.
(A) The induction of *Sema3c* mRNA in cultured granulosa cells with forskolin+PMA (For PMA) or AREG+NRG1 (AN). C; granulosa cells were cultured without any agonists. (B) The effects of *Sema3c* siRNA on the expression of Sema3c mRNA, *, *P*<0.05. (C) The cell migration of granulosa cells was induced by granulosa cell-secreted Sema3C by AREG and NRG1. AN; AREG and NRG1.

phosphorylation of ROCK was induced in *Sema3c*-siRNA-transfected granulosa cells (Fig. 3C).

The transfection of *Sema3c* siRNA significantly decreased the expression of *Cyp11a1* and *Star*, but not *Hsd3b* mRNA in granulosa cells (Fig. 4). The production of progesterone (secretion level of progesterone in the cultured medium) was also significantly decreased by knockdown of *Sema3c* (Fig. 4).

Discussion

The Sema family was demonstrated to be a regulator of axogenesis [32, 33], and it is known to be involved in angiogenesis [34], cancer cell inversion [35], cell detachment [36], migration of cells [23] and actin remodeling [37]. Most of members of the Sema family are transmembrane proteins and act on their specific receptors expressed on neighbor cells [19]. However, the Sema3 family is a secreted protein that is released by the exocytosis system, and then binds to its receptor [20, 38]. During the ovulation process, the secreted factors from granulosa cells are key mediators and potentiators to transfer the ovulation signal to granulosa cells themselves by the autocrine pathway, and to cumulus cells through the paracrine system [3,4]. Both granulosa cells and cumulus cells exhibit cell detachment for the induction of migration of cells [17]. Some of the factors, such as the cytokine family, are released by the exocytosis system [27, 39], suggesting that the Sema3 family is expressed and plays important roles during the ovulation process. We demonstrated the expression of *Sema3b* and *Sema3c* in granulosa cells and the transient increase of *Sema3c* mRNA in granulosa cells during the ovulation process. Sema3C protein was strongly detected in the extracts of hCG-stimulated ovaries but not in granulosa cells, suggesting that Sema3C is immediately secreted in follicular fluid or accumulates within the extracellular matrix among the cumulus cell layers and/or granulosa cell layers.

The expression level of *Sema3c* reached its maximum level at 4 h after hCG injection, and then decreased to the basal level observed in granulosa cells prior to hCG administration. The expression pattern during the ovulation process, and most of the genes are regulated in a ERK1/2-dependent manner [11]. When mouse granulosa cells were cultured with forskolin and PMA, the expression of *Sema3c* significantly increased, compared with granulosa cells without any stimuli. This induction was significantly suppressed by an ErbB family receptor tyrosine kinase inhibitor, AG1478, but was observed in granulosa cells cultured with AREG and NRG1. Both factors are expressed in granulosa cells, and act on the ErbB family to induce the phosphorylation of ERK1/2 [3, 4]. Thus, in granulosa cells, the transcription of *Sema3c* is directly regulated by EGF-like factors-induced ERK1/2 dependent pathway.

Focal adhesion kinase (FAK) activated by Sema3-Plexins pathway induces cell migration and/or cytoskeleton remodeling [24–26]. In the present study, the phosphorylation of FAK was suppressed by the knockdown of *Sema3c*, which resulted in a decreased level of ROCK phosphorylation in granulosa cells cultured with AREG and NRG1. It has been reported that FAK negatively regulates ROCK phosphorylation via a decrease of Rho A activity [40, 41]. The phosphorylated form of ROCK is an inactive type, and the unphosphorylated form (active form) of ROCK binds to cofilin, an actin-depolymerizing factor, and regulates actin dynamics [42]. The binding decreases cofilin activity [43], indicating that FAK induc-
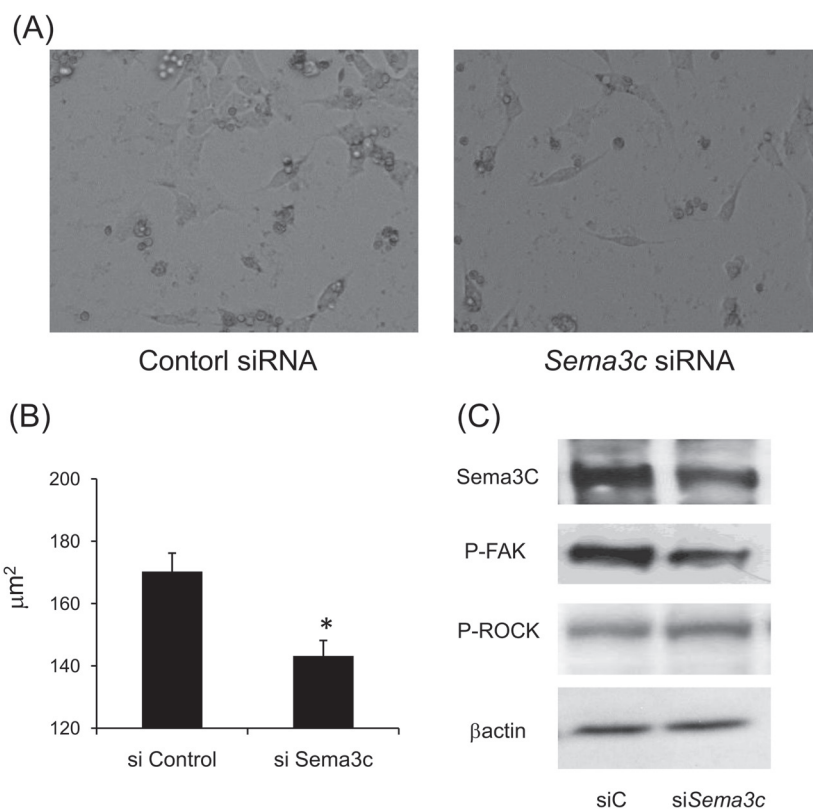
(A)

Contorl siRNA   *Sema3c* siRNA

(B)

(C)

Fig. 3. The effects of *Sema3c* siRNA on the morphology (A) and the surface area (B) of granulosa cells cultured with AREG and NRG1, *, *P<0.05*. (C) The activation of FAK pathway in *Sema3c* siRNA-transfected granulosa cells.
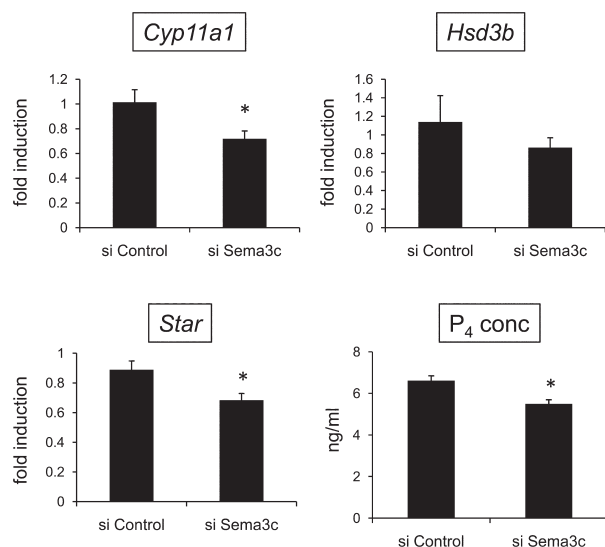

Fig. 4. The expression of genes involved in progesterone production, *Cyp11a1, Hsd3b, Star* and the secretion level in *Sema3c* siRNA-transfected granulosa cells cultured for 8 hr with AREG and NRG1, *, *P<0.05*.

es actin depolymerization via a decrease of ROCK activity leading to cell migration and changes in cell shape. In fact, in the present study, the knockdown of *Sema3c* suppressed cell migration and decreased the surface area of granulosa cells cultured with AREG and NRG1. From above information including the present study, we postulate that during the ovulation process, Sema3C is expressed and activates FAK signaling to reduce the activity of ROCK, which is required for the dramatic changes of granulosa cells during luteinization.

The final step in the differentiation of granulosa cells after ovulation stimuli is the production of progesterone, which is concomitant with the changes in cell shape. Recently, Karlsson et al. [16] reported that the overexpression of a mutant type of cofilin suppressed both the change in cell shape and the production of progesterone. In this study, the knockdown of *Sema3c*, that suppresses cytoskeleton remodeling, significantly reduced not only the expression level of *Cyp11a1* and *Star*, but also the level of progesterone in the cultured medium. Although our data did not reveal the mechanism behind

the change in cell shape, which is induced by granulosa cell differentiation via modification of gene expressions, our data clearly shows that this morphological change is required for luteinization.

In conclusion, Sema3C was expressed at both the mRNA and protein levels in granulosa cells during the ovulation process. Sema3C was secreted and then accumulated within granulosa cell layers and cumulus cell layers. The FAK pathway was activated by Sema3C and induced cell migration and enlarged the area of granulosa cells. These morphological changes are induced by Sema3C through the FAK pathway and are required for luteinization (production of progesterone) of granulosa cells.

Acknowledgements

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