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Expression of repulsive guidance molecule b (RGMb) in the uterus and ovary during the estrous cycle in rats

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ABSTRACT

Repulsive guidance molecule b (RGMb; a.k.a. Dragon), initially identified in the embryonic dorsal root ganglion, is the first member of the RGM family shown to enhance bone morphogenetic protein (BMP) signaling by acting as a BMP co-receptor. BMP signaling has been demonstrated to play an important role in the reproductive organs. Our previous study found that RGMb was expressed in the reproductive axis, but whether RGMb expression in reproductive organs changes across the estrous cycle remains unknown. Here, we show in the rat that *RGMb* mRNA expression in the uterus was significantly higher during metesterus and diestrus than during proestrus and estrus. Western blotting indicated that RGMb protein was significantly lower during estrus compared with the other three stages. Immunohistochemistry revealed that RGMb protein was mainly localized to the uterine luminal and glandular epithelial cells of the endometrium. *RGMb* mRNA and protein in the ovary remained unchanged during the estrous cycle. RGMb protein was expressed in the oocytes of all follicles. Weak staining for RGMb protein was also found in corpora lutea. RGMb was not detected in granulosa cells and stromal cells. Taken together, RGMb expression in the uterus and ovary across the estrus cycle demonstrate that RGMb may be involved in the regulation of uterine function, follicular development as well as luteal activity.

Introduction

Studies to understand the mechanisms underlying the endometrial cycle are important because of their importance to female fertility and reproduction (Erickson et al., 2004). During the estrous cycle, the uterus and ovary undergo a series of cellular processes including cell proliferation, differentiation and apoptosis. These cellular processes are profoundly influenced by the transforming growth factor β (TGFβ) superfamily, particularly bone morphogenetic proteins (BMPs) (Ma et al., 2011).

Numerous studies have revealed that BMP signaling is involved in multiple developmental and homeostatic processes. BMPs bind to type II and type I serine-threonine kinase receptors, and transduce signals via Smad or non-Smad pathways. Functional loss of the components of the BMP signaling pathway contributes to a wide variety of clinical disorders, including vascular diseases, fibrosis, cancer and reproductive dysfunctions (Corradini et al., 2009; Miyazono et al., 2010). Thus, proper spatiotemporal control of BMP signaling in the appropriate cellular context is required for proper tissue homeostasis (Halbrooks et al., 2007; Tian et al., 2010). Members of the BMP pathway are widely expressed in uterine stroma and glandular epithelium (Erickson et al., 2004). BMP7 contributes to primordial follicle activation and the primordial-to-primary follicle transition, while BMP6 is involved in the dominant follicle selection (Khalaf et al., 2013). Studies with knockout mice have shown that BMP-4 and BMP-8b are necessary for primordial germ cell (PGC) formation (Ying et al., 2001). BMPs also play important roles in folliculogenesis, ovulation, luteinization and luteolysis in rat ovary during the estrous cycle (Erickson and Shimasaki, 2003; Shimasaki et al., 2004).

Recently, the repulsive guidance molecules (RGM) have been found to function as co-receptors that enhance BMP signaling transduction (Samad et al., 2005). RGMb, a member of the repulsive guidance molecule family, was originally found as an adhesion molecule for regulating repulsion of axons (Monnier et al., 2002; Samad et al., 2004). However, RGMb has be found to be expressed in a wide range of tissues and organs including the spinal cord, brain, heart, liver, kidney, lung and reproductive system (Kanomata

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et al., 2009; Severyn et al., 2009; Li et al., 2012). As a co-receptor for BMPs, RGMb binds to BMP receptors and specific BMP ligands (BMP-2, BMP-4), sensitizes cells to low levels of ligand and enhances intracellular Smad phosphorylation in response to BMP ligands (Xia et al., 2010, 2011; Ma et al., 2011), thereby stimulating BMP signaling. It has been shown that RGMb and the functional BMP signaling system are co-expressed and that RGMb in the mouse regulates BMP signaling in cells from reproductive tissues (Xia et al., 2005). However, the role of RGMb in reproductive organs during the normal estrous cycle remains unknown. Thus, as an initial attempt to understand the role of RGMb in reproduction, we first analyzed the expression patterns and localization of RGMb in the rat uterus and ovary during the estrous cycle.

Materials and methods

Animals and sample collection

Female adult Sprague–Dawley rats (200–250 g) were obtained from Qinglong Shan Laboratory Animal Company (Nanjing, China). They were housed under a 12:12 h light: dark cycle with food and water ad libitum. Vaginal smears were taken twice daily. Uteri and ovaries from rats exhibiting at least two consecutive 4-day estrous cycles were collected on proestrus, estrus, metesterus and diestrus phase. One set of the uteri and ovaries was fixed in 4% paraformaldehyde at room temperature for 24 h and then kept in 70% alcohol for immunohistochemistry. The other set of the uteri and ovaries was snap frozen and stored at −80 °C for real-time PCR and Western blotting. The experiments were performed in accordance with the guide for the Care and Use of Laboratory Animals prepared by the Institutional Animal Care and Use Committee of Nanjing Agricultural University.

Real-time PCR

Total RNA was isolated from the frozen uteri and ovaries homogenized in RNAiso Plus (TaKaRa; Dalian, China) according to the supplier's instructions. Remaining DNA was removed by RNase-free DNase treatment (DNaseI, TaKaRa; China). Total RNA was reverse transcribed into cDNA with PrimeScript reverse transcriptase reagent kit (Perfect real time; TaKaRa, Dalian, China). Reverse transcription conditions were as follows: 37 °C for 15 min and 85 °C for 5 s with 0.5 μg total RNA in 10 μl volume. For real-time PCR, cDNA was amplified using a SYBR Premix Ex Taq kit (TaKaRa; Dalian, China) on StepOnePlus Real-Time PCR System (Life Technologies Corporation, v2.2.2). Each sample was tested in triplicate. The PCR reaction (total 20 μl) was amplified containing SYBR® Premix Ex Taq 10 μl, PCR Forward Primer (10 μM) 0.4 μl, PCR Reverse Primer (10 μM) 0.4 μl, ROX Reference Dye (50×) 0.4 μl, DNA template 2 μl and dH_2O 6.8 μl. The amplification program consisted of 95 °C for 30 s, followed by up to 40 cycles of 95 °C for 5 s, 60 °C for 30 s. Primer sequences were as follows: *RGMb* (sense: 5′-CCATGTCCTACGAGGAAAGC-3′, anti-sense 5′-GCAGCAGTAAAGTTGGCATCA-3′), *GAPDH* (sense: 5′-CAAGTTCAACGGCACAGTCAAG-3′, anti-sense: 5′-ACATACTCAGCACCAGCATCAC-3′). The sizes were 262 bp for *RGMb* and 123 bp for *GAPDH*. Samples containing no RNA or RNA without reverse transcriptase were used as negative controls. The gene expression for *RGMb* was normalized to *GAPDH* expression. For real-time PCR results, amplification efficiency was determined for each gene by generating a standard curve using serial dilutions of the cDNA in abscissa and the corresponding threshold cycle Ct in ordinate. The slope of the log-linear phase reflects the amplification efficiency derived from the formula $E = (10^{-\text{slope}} - 1) \times 100$. Amplification efficiency was between 95 and 105%. Amplification was followed by Ct analysis for each primer pair to verify the presence of one gene-specific peak and the absence of primer dimers (Sallon et al., 2010). The threshold cycle Ct was determined for each reaction. *RGMb* mRNA was normalized to the values for the proestrus group.

Western blotting

The frozen uteri and ovaries were homogenized in a Dounce homogenizer (Wheaton, Millville, NJ, USA) using RIPA lysis buffer (Beyotime, Nantong, China) containing 10 mM phenylmethylsulfonyl fluoride (Beyotime). The homogenates were incubated for 30 min on ice and centrifuged at 12,000 × *g* for 10 min at 4 °C. Protein concentration was qualified by BCA Protein Assay Kit (Beyotime). Proteins (45 μg) were resolved on a 10% SDS–PAGE gel (Bio-Rad, Hercules, CA, USA) and transferred onto PVDF (Millipore, Billerica, MA, USA) membranes. After blocking with 5% fat-free milk in TBST (0.1% Tween 20 in TBS) for 1 h at room temperature, the membranes were incubated with antibodies specific to RGMb (1:500, Abcam, Cambridge, MA, USA) and β-actin (ACTB, 1:3000, uBio, China) overnight at 4 °C. After three 10 min washes in TBST, the membranes were incubated with goat anti-rabbit HRP-conjugated IgG antibody (1:2000) for 3 h. After two 5-min washes in TBST followed by one 5-min wash in TBS, the bands were visualized with Super Signal West Pico Chemiluminescent Subs Kits (Pierce Biotechnology, Shanghai, China). Signals were visualized using a Kodak Digital Sciences Image Station 440 (Eastman Kodak, Rochester, NY, USA). Densitometry was performed using Image J software (http://rsbweb.nih.gov/ij/).

Immunohistochemistry

After fixation, uterine and ovarian samples were embedded in paraffin wax and cut into 5 μm thick sections and mounted onto polylysine-coated slides. The slides were then processed for immunohistochemical staining as described previously by us (Zhang et al., 2011). Briefly, the slides were incubated overnight at 4 °C with rabbit polyclonal antibody against RGMb (1:1000, Abcam, USA). The specific immune-reactivity was visualized by the ABC Kit Elite and 0.05% DAB in 10 mM PBS containing 0.01% H_2O_2 for 3 min, then counterstained with hematoxylin. Negative controls were incubated with normal rabbit serum instead of RGMb antibody. We also used the ovary from RGMb knockout mice to verify the specificity of the antibody (see Fig. 4B). Breeding and genotyping of RGMb KO mice (C57/B6/129) have been described previously (Xia et al., 2010). The pictures were taken under the microscope (Nikon YS100, Nikon, Tokyo, Japan).

Statistical analysis

All data are expressed as mean ± SEM. Statistical analyses were performed by GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). The differences in means were analyzed by one-way ANOVA followed by Tukey's multiple comparison tests to evaluate multiple responses. Statistical significant difference was considered to be $P < 0.05$.

Results

Expression of *RGMb* mRNA and protein in rat uterus and ovary during estrous cycle

RGMb mRNA expression in the uteri during the estrous cycle was analyzed by real-time PCR (Fig. 1A). *RGMb* mRNA was readily detected at all stages of the estrous cycle. *RGMb* mRNA levels were significantly higher at metestrus and diestrus than that at

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