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Effects of gremlin-2 on the transition of primordial follicles during early folliculogenesis in the human ovary

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ABSTRACT

Objective: To investigate the localization and function of gremlin-2 during human ovarian folliculogenesis.
Study design: Ovarian tissue from a gynecologic cancer patient was cultured in the presence or absence of gremlin-2 and then analyzed histologically. Growing follicles were counted by the microscopic observations of ovarian histological sections. Immunocytochemical staining was carried out to detect the expression of bone morphogenetic protein (BMP) 4 and phosphorylated Smad 1/5/8 (p-Smad 1/5/8).
Results: Gremlin-2 was detected in human primordial, primary, and early growing follicles before culture. By day 4 of culture, the follicle growth rate in the presence of gremlin-2 (13.7%; 24/175) was significantly lower than that of the control (54.8%; 92/175; $p < 0.01$). BMP4 expression was similar in the presence and absence of gremlin-2, whereas the p-Smad 1/5/8 signal was noticeably stronger in the absence of gremlin-2 in primordial and early-stage growing follicles.
Conclusions: Gremlin-2 maintains the follicle store as primordial follicles by suppressing Smad 1/5/8 signaling in the human ovary. The data presented here provide potential insight into reproductive medicine for cases of intractable infertility, such as premature ovarian insufficiency and cancer survivors.

Introduction

Significant recent developments in assisted reproductive medicine have provided effective treatments for many infertile couples. However, more advanced technologies are required to treat conditions that result in intractable infertility, such as premature ovarian insufficiency (POI) [1,2] and ovarian dysfunction due to aggressive chemotherapy and/or radiotherapy in young women with cancer. The techniques of ovarian cryopreservation and autografting have been developing to treat these cancer survivors [3–5]. In addition, a combination of ovarian tissue cryopreservation and in vitro growth of isolated follicles has become an important intervention option for female fertility preservation [6–8]. Numerous factors, including natural hormones, growth factors, and medicines, have been studied as candidate follicle growth promoters in the development of tissue culture systems. However, this technology is not yet available for use in humans.

This study focuses on gremlin-2 because gremlin family members have been reported to promote ovarian follicle survival and maintain the primordial follicle store [9,10]. In particular, gremlin-2 is an attractive target for the regulation of follicular growth [11,12]. Gremlin-2 protein is also called "protein related to differential screening-selected gene aberration in neuroblastoma (DAN) and Cerberus (PRDC)". DAN is a possible tumor-suppressor for neuroblastoma in human. The molecular structure and biochemical interactions of DAN family members have been studied in depth [13,14].

Gremlin 2 is also known to be a potent antagonist of bone morphogenetic proteins (BMPs) 2 and 4. Nilsson and Skinner indicated that BMP-4 promotes primordial follicle development and the primordial-to-primary follicle transition using a rat ovarian culture system [15]. This group subsequently clarified that gremlin-2 inhibits the transition of primordial follicles to a growing stage by binding to BMP4. The BMP signaling pathway is generally mediated by the phosphorylation of Smad/1/5/8 (p-Smad/1/5/8), which plays a key role in the regulation of cell growth and differentiation. Hence, we

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organized this study to examine whether gremlin 2 affects the expression of BMP 4 or p-Smad/1/5/8 using a human ovary culture system.

Materials and methods

Ethics statement

The studies have been approved by the institutional ethical committee of Hyogo College of Medicine (No. 217) and have been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Human ovarian tissues were obtained from a nullipara woman (31 years old) presented with cervical cancer with the International Federation of Gynecology and Obstetrics stage IB1. A cervical tumor 3 cm in diameter was removed by conization, and was diagnosed as glassy cell carcinoma with massive lymphovascular space invasion. Based on written informed consent the patient's ovaries were used for this study.

Ovarian tissue culture

The ovarian tissue treatment process is shown in Fig. 1. The ovarian cortex was isolated from excised tissues, and sectioned into 260 strips sized 2 mm × 10 mm. Before culture, 20 strips were selected randomly and fixed in formalin for morphological examination and detection of gremlin 2. When organizing this study, we estimated that about 20 strips would be necessary for each experimental group. From our experience, just a few of the strips actually contain sufficient numbers of follicles but it is impossible to recognize which strips those are before histological examination. On average, 5 and 15 strips from 20 were needed for immunocytochemical study and Hematoxilin/Eosin staining, respectively. In total, 240 strips were used for culture. In practice, 5 strips were put into each well equipped with collagen-coated membrane inserts (Transwell-COL: Corning Incorporated Life Science, Tewksbury, NY) after fragmentation as shown in Fig. 1C and D. The 240 strips distributed into 48 wells were incubated for 24 h in 5% CO2 in air. The culture medium consisted of α-minimum essential medium (α-MEM: Invitrogen, MA) supplemented with 5% of fetal calf serum (FCS), 10 μg/ml insulin, 5.5 μg/ml transferrin, 5 ng/ml sodium selenite (ITS; Sigma–Aldrich Japan, Tokyo Japan), antibiotic-antimycotic solution (10 IU/ml penicillin, 10 mg/ml streptomycin, and 25 ng/ml amphotericin B), 0.001% 2-mercaptoethanol (Millipore, MA), 1% GlutaMAX™ (Gibco, Life Technologies, CA). Additionally, 3 μM phosphatase and tensin homolog (PTEN) inhibitor (pbV-OHpic: Merk, Darmstadt, Germany), and 740Y-P (R&D Systems MN) were supplemented for transition of resting primordial follicles to primary follicles according to previous reports [16,17]. The medium was first added to the wells (500 μL/well); then, the ovarian fragments were put onto collagen-coated membrane inserts; finally, 150 μL medium was added to slightly cover the ovarian fragments on each well. Next day, termed day 1, the 240 strips (5 strip each) were transferred to 48 wells of new plates and divided into two experimental groups: one group received no addition and the other received an addition of 100 ng/ml recombinant gremlin-2 (BioVision CA). The culture medium of both groups contained additional supplements of 1 ng/ml epidermal growth factor (EGF; R&D Systems MN), 10 ng/ml insulin-like growth factor 1 (IGF1; R&D Systems MN), 10 ng/ml recombinant human activin A (HumanZyme, IL), 1 mU/ml recombinant human FSH (follistim; MSD, NJ), and 1 ng/ml stem cell factor (SCF; R&D Systems MN) but did not contain PTEN inhibitor or 740Y-P. In each group present or absent gremlin 2, 20 strips distributed into 4 wells were sampled on days 2, 4, and 6 for analysis.

Morphologic analysis

On days 0, 2, 4, and 6, the ovarian tissue fragments were fixed with 10% formalin in neutral buffer and embedded in paraffin blocks. Ten fragments each were used to compare follicular growth in the absence or presence of exogenous gremlin-2. Each block was cut into serial 4-μm-thick sections using a microtome (Leica RM2125). Sections were deparaffinized, rehydrated through a graded ethanol series, and subjected to hematoxylin and eosin (HE) staining. Growing and non-growing follicles were examined by microscopic observation at a magnification of 200× (Olympus AX80, Tokyo, Japan). Follicles containing an oocyte covered with more than one layer of cuboidal granulosa cells were assessed as growing follicles; and follicles with flat epithelial cells were assessed as non-growing follicles. Follicles that included degenerated oocytes and granulosa cells with nuclear pyknosis were excluded from the analysis.

Statistical analysis

The numbers of living and growing follicles were estimated by counting in ovarian sections prepared from cultured ovaries on days 0, 2, 4, and 6. The percentage of growing follicles was

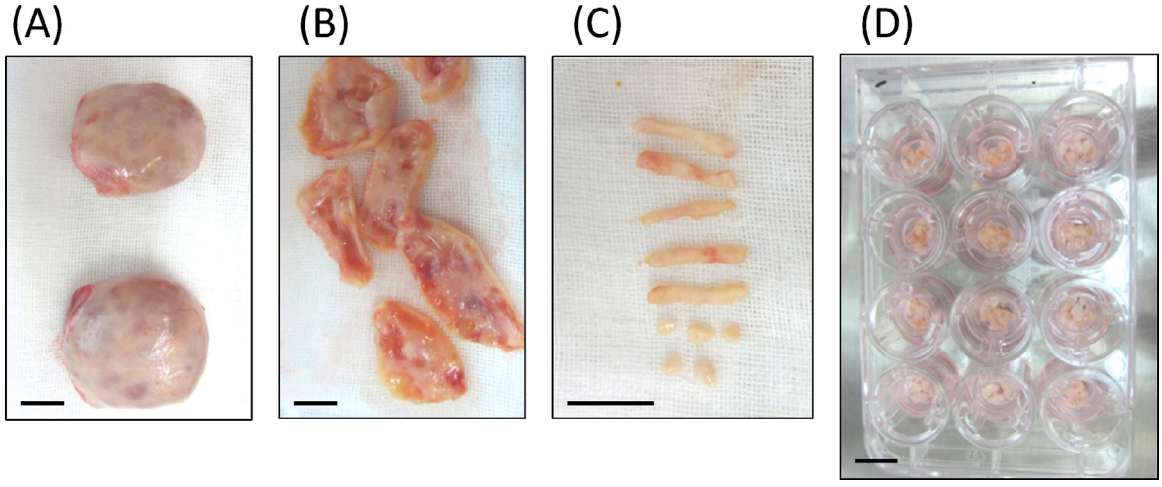

Fig. 1. Ovarian tissue treatment before culture. (A) Ovarian tissue was excised from a patient after obtaining informed consent. (B) The cortex regions were isolated from ovarian tissues. In all, 240 strips sized 2 mm × 10 mm were prepared for culture. Each strip was further fragmented into five pieces (C) then put onto a collagen-coated membrane insert attached to each well (D). Scale bars: 10 mm.