Involvement of hyaluronan synthesis in ovarian follicle growth in rats

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Abstract

Most of the previous studies on ovarian hyaluronan (HA) have focused on mature antral follicles or corpora lutea, but scarcely on small preantral follicles. Moreover, the origin of follicular HA is unknown. To clarify the localization of HA and its synthases in small growing follicles, involvement of HA in follicle growth, and gonadotropin regulation of HA synthase (Has) gene expression, in this study, perinatal, immature, and adult ovaries of Wistar-Imamichi rats were examined histologically and biochemically and by in vitro follicle culture.

HA was detected in the extracellular matrix of granulosa and theca cell layers of primary follicles and more advanced follicles. Ovarian HA accumulation ontogenetically started in the sex cords of perinatal rats, and its primary site shifted to the intrafollicular region of primary follicles within 5 days of birth. The Has1–3 mRNAs were expressed in the ovaries of perinatal, prepubertal, and adult rats, and the expression levels of Has1 and Has2 genes were modulated during the estrous cycle in adult rats and following administration of exogenous gonadotropins in immature acyclic rats. The Has1 and Has2 mRNAs were predominantly localized in the theca and granulosa cell layers of growing follicles respectively. Treatments with chemicals known to reduce ovarian HA synthesis induced follicular atresia. More directly, the addition of Streptomyces hyaluronidase, which specifically degrades HA, induced the arrest of follicle growth in an in vitro culture system. These results indicate that gonadotropin-regulated HA synthesis is involved in normal follicle growth.

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Introduction

The extracellular matrix (ECM) is composed of sulfated and non-sulfated glycosaminoglycans and structural proteins such as collagens and laminins. Hyaluronan (HA) is the only non-sulfated glycosaminoglycan and consists of N-acetylglucosamine and glucuronic acid. HA is essential for cell migration and proliferation, morphogenesis, wound healing, inflammation, and vascular integrity (Ochsner et al. 2003, Genasetti et al. 2008). High-molecular weight HA accumulates on the cell surface by binding to transmembrane receptors and synthases (HASs) with other hyaladherins (Fries & Kaczmarczyk 2003), and the HA-rich ECM inhibits angiogenesis (West & Kumar 1989, Spicer & Tien 2004). On the other hand, degraded low-molecular weight soluble HA stimulates angiogenesis (Lennon & Singleton 2011). The production and degradation of HA in vertebrates are regulated by at least three types of HASs and several types of hyaluronidase (HYAL) respectively. The expression of each HAS isoform is spatially and temporally controlled in a unique fashion when mammalian cells are stimulated with cytokines (Itano 2008). The disruption of the Has2 gene causes embryonic lethality due to a defect in the formation of the cardiac jelly matrix, which allows the heart to fold during its development (Camenisch et al. 2000, Itano 2008).

The HA content of ovaries is lower than that of other urogenital organs such as the urinary bladder, uterus, vagina, and penis, and the main sites of HA accumulation in ovarian follicles are the ECMs of the theca interna, liquor folliculi, and zona pellucida of large antral follicles, but not those of small preantral follicles (Laurent et al. 1995, Irving-Rodgers & Rodgers 2005). During ovulation, HA accumulation with marked morphological change occurs in the cumulus–oocyte complex; this process is referred to as cumulus expansion. The expansion occurs in cumulus cells of mature preovulatory follicles by a gonadotropin surge that upregulates HAS2 activity via autocrine/paracrine epidermal growth factor signaling (Richards 2005). Cumulus expansion is required for the maintenance of the natural fertilization process by preventing physical damage, dehydration, and polyspermy (Salustri et al. 1999). In assisted reproductive technology, the morphological change has been utilized as a useful indicator of oocyte maturation. After ovulation, HA reduction occurs in the ECM of the remaining mural and membrana granulosa cells of ovulated follicles, i.e. corpora lutea.
Similar HA reduction is observed in the granulosa cell layer of antral follicles under atresia (Salustri et al. 1999, Hatziroodos et al. 2012).

As has been described above, most of the ovarian HA research has focused on the accumulation in the cumulus cells of preovulatory antral follicles, but not on the production in small growing preantral follicles or the developing ovary, even though ECM constituents other than HA such as collagens, laminin, proteoglycans, and sulfated glycosaminoglycans are localized in small preantral follicles and play roles in folliculogenesis (Irving-Rodgers et al. 2010, Hatziroodos et al. 2012). A histological study carried out by Dumaresq-Doiron et al. (2011) has illustrated HA expression in the growing preantral follicles of WT and Hyal1-knockout mice for the first time. However, the involvement of HA production in folliculogenesis has not been addressed, as the modulation in HA content could not be induced in the ovaries of the Hyal1-deficient mice. Considering the function of HA along with other ECM components and mechanisms in HA synthesis, it would not be surprising that HA production in the ECMs of immature preantral follicles or developing ovaries is involved in the maintenance of folliculogenesis. It is also important to determine when HA starts to be produced in the ovary and which factor(s) regulate HA synthesis during follicular growth; therefore, in this study, we first examined HA expression in growing preantral follicles of adult rats and in perinatal developing ovaries of rats. We then investigated the expression and localization of Has genes in small preantral follicles. As a mechanistic insight, gonadotropin regulation of Has gene expression in small follicles was examined using acyclic immature rats, the ovaries of which have fewer antral follicles. Furthermore, the effects of HA reduction on follicle growth were examined in vivo and by in vitro follicle culture, to determine the necessity of HA for the maintenance of normal follicular growth.

Materials and methods

Reagents

All the chemicals were purchased from Sigma Chemical Co. or Wako Pure Chemical Industries (Tokyo, Japan), unless otherwise indicated.

Animals

Ovaries obtained from female Wistar-Imamichi rats at various ontogenetic stages were examined (each n = 3–12): untreated rats at embryonic day 19 (E19), rats at postnatal day 1 (P1), P5, and P10 as perinatal samples, and 10-week-old adults rats and 100-week-old senescent rats were used for the examination of ovarian HA expression. Ovarian follicles obtained from P14 rats were used for in vitro follicle culture. P21 rats were also used to examine the effects of exogenous gonadotropins and chemicals such as 4-methylumbelliferone (4-MU) and 6-diazo-5-oxo-1-norleucine (DON). In this study, 10 IU of equine chorionic gonadotropin (eCG) and human CG (hCG) were used. The minimal pharmacological doses needed to reduce P21 rat ovarian HA synthesis in 2 days were preliminarily determined to be 3.85 g/kg body weight for 4-MU by oral administration and 5 mg/kg for DON by i.p. injection. Some of the 4-MU- or DON-treated rats were simultaneously injected with 10 IU eCG. The stage of estrous cycle was determined in adult rats by vaginal cytology. Ovaries dissected immediately after decapitation of the rats were fixed with 4% paraformaldehyde overnight for histological studies or stored at −80°C for RT-PCR. All the experimental protocols were approved by the St Marianna University School of Medicine Animal Care and Use Committee.

RT-PCR

Total RNA (1 μg) was extracted and from the ovaries of P10 and P21 rats treated with either none of the chemicals, saline, eCG, or hCG, 10-week-old rats in each estrous cycle, and 100-week-old rats (n = 4–8 for each animal group). The extracted total RNA was reverse transcribed to obtain cDNA samples that were used as the templates of routine and real-time PCRs. The sequences of primer sets used for the amplification of the Has and β-actin genes by routine PCR were Has1 sense, tggtgctcctcttgctctg; Has1 antisense, ataggtcatccacgcgtggt; Has2 sense, gagacattgcccagaagct; Has2 antisense, ttcttaagggctctggcaa; Has3 sense, tcaagctgacatcgccaat; Has3 antisense, gacgcacatctagacatgtc; β-actin sense, gcaacagctccggctgctgca; and β-actin antisense, tggactacggtctctgctg. Routine PCR was carried out using an RNA PCR Kit (AMV) ver.3.0 (TaKaRa, Shiga, Japan) with the following conditions: 30 s of denaturation at 94°C followed by annealing and extension at 72°C for 30 cycles. The annealing temperatures were 58°C for Has1, 63°C for Has2 and β-actin, and 55°C for Has3. To quantify the expression levels of Has1 and Has2 genes, real-time PCR was carried out using the ABI 7500 Fast Real-Time PCR System and the TaqMan Gene Expression Master Mix and Assays for rat Has1 (Rn00597231_m1) and Has2 (Rn00565774_m1) (Applied Biosystems), according to the manufacturer’s recommendations. The Sequence Detection System software was used for raw data processing and analysis (ver. 1.3.1). The data for each gene as threshold cycle (Ct) values were normalized with those for β-actin (Rn00667869_m1) and with averages of control or initial ΔCt values and then expressed as 2^−1/ΔΔCt values.

Histology

HA was histochemically detected using the HA-binding protein according to the manufacturer’s instructions. Paraffin sections (6 μm; n = 4–6 for each animal group) were rehydrated and incubated with 2 μg/ml biotinylated HA-binding protein (Seikagaku Biobusiness Corp., Tokyo, Japan) at room temperature for 2 h, followed by incubation with peroxidase-conjugated streptavidin for 5 min (Histone SAB-PO Kit, Nichirei, Tokyo, Japan). The sections pretreated with 100 TRU Streptomyces HYAL were also prepared as negative controls. Signals were detected by development with 3,3′-diaminobenzidine and counterstained with hematoxylin. Signal intensity was determined using ImageJ software (NIH, Bethesda, Maryland).
3,3′-diaminobenzidine, and the sections were counterstained with Mayer's hematoxylin, dehydrated with a graded series of ethanol, and mounted with Entellan (Merck). No signals were detected in sections pretreated with the HYAL. The developmental stages of the follicles were determined as described previously (Pedersen & Peters 1968). Follicles with condensed nuclei in the granulosa cell layer, poorly developed granulosa cell layer itself, and/or expanded theca cell layer with scarce granulosa cell layer were considered to be atretic.

The localization of Has1 and Has2 mRNAs in the ovary (n=3 for each gene) was examined by fluorescent in situ hybridization, as described elsewhere (Takahashi et al. 2008). The primer sequences used to obtain sense and antisense RNA probes were Has1 sense, taatacgactcactatagggtgggactgagt-taccttc; Has1 antisense, aattaaccctcactaaagggcctggtcctataatcc-cact; Has2 sense, taatacgactcactatagggtgtcaatcttctgctgcctc; and Has2 antisense, aattaaccctcactaaagggtctccacacaggg-agagttg.

In vitro follicle culture

We studied the direct effect of HA reduction on ovarian follicles in vitro using previously described mouse single-follicle culture techniques (Tarumi et al. 2012). The established culture system is based on previous research carried out by Romero & Smitz (2009), and oocytes from in vitro-cultured follicles have competence to get matured when treated with hCG/EGF on the 12th day of culture. Growing secondary follicles (100–140 μm in diameter) were isolated from three P14 rats and cultured in 75 μl of α-MEM containing 10% fetal bovine serum, 10 μg/ml insulin, 5.5 μg/ml transferrin, 5 ng/ml sodium selenite, and 100 mIU/ml recombinant follicle-stimulating hormone (FSH; Follistim; Merck & Co., Inc.) in 96-well microplates (n=8–10 per group). In each well, a single follicle was cultured with the following: no treatment; 10 TRU/ml HYAL from Streptomyces hyalurolyticus, which specifically degrades HA but not the other glycosaminoglycans; or 4-MU (0.1, 0.3, or 1 mM) for 6 days. Half of the culture media (40 μl) was changed every other day, and the diameters of the follicles were measured on days 0, 3, and 6.

Statistical analysis

All values are expressed as means ± S.E.M. Differences among the groups at any time point or between different time points were analyzed by a non-parametric ANOVA (Kruskal–Wallis H test) followed by Dunn’s post hoc comparison.

Results

Detection of HA in adult and perinatal ovaries

In the ovaries of 10- to 12-week-old (10-week-old) adult rats, HA was detected in the ECMs of theca and granulosa cell layers, including the basal lamina of primary and antral follicles, but it was present only in low amounts in atretic follicles (Fig. 1A, B, C, D and E). During the estrous cycle, there were no changes in the intensities of HA signals in growing follicles. HA was also detected in the tunica adventitia of ovarian arteries (Fig. 1F), but was scarcely detected in the interstitial region of ovaries of cycling adult rats (Fig. 1A, B, C, D, E and F) and ovaries of 100-week-old senescent rats with
a large majority of interstitial regions, atretic follicles, and cysts (Fig. 1G). No signals were detected in sections pretreated with the HYAL.

To identify the origin of follicular HA, we used developing ovaries obtained from perinatal rats. HA was found in the sex cords of ovaries of E19 rats (Fig. 2A) and in the interstitial region of ovaries of P1 rats (Fig. 2B), but not in naked oocytes or primordial follicles. Follicular HA accumulation began in the granulosa cell layer of primary follicles from P5 (Fig. 2C) and continued to be detected in more mature follicles afterwards (Fig. 2D).

Expression of Has1, Has2, and Has3 in rat ovary during estrous cycle
In the ovaries of proestrus rats, the Has1 and Has2 genes were intensely expressed and the Has3 gene was expressed at lower levels (Fig. 3A, B and C). The ovarian expression of both Has1 and Has2 genes was relatively high during ovulation while being low in diestrus and metestrus of cycling adult rats and in acyclic P10 and 100-week-old rats, whereas the mRNA levels of ovarian Has3 were unregulated by the estrous cycle (Fig. 3D and E). The Has1 mRNA was mainly localized in the extrafollicular theca region of preantral (Fig. 3F) and antral (Fig. 3G) follicles, whereas the Has2 mRNA was found in the granulosa cell layer of ovaries of adult rats (Fig. 3H and I).

Gonadotropin regulation of Has gene expression in immature rat ovaries
The expression levels of Has1 and Has2 genes in the ovaries of P21 rats treated with saline, 10 IU eCG, or 10 IU hCG were examined. Both eCG and hCG acutely induced ovarian Has1 gene expression within 3 h, and the expression levels returned to basal levels 12 h after injection (Fig. 4A). On the other hand, only eCG but not hCG gradually induced Has2 gene expression from 3 to 48 h after treatment (Fig. 4B).

Effects of 4-MU and DON on HA reduction and follicle growth in in vivo-treated rats and in vitro-cultured follicles
The in vivo effects of two chemicals known to reduce HA synthesis and frequently used in the research on ovarian HA reduction were examined. In P21 rats, 2 days after saline injection, HA was abundantly detected in growing follicles (Fig. 5A), whereas treatments with 3.85 g/kg body weight of 4-MU or 5 mg/kg DON diminished ovarian HA synthesis and increased the number of atretic follicles (Fig. 5B and C and Table 1). In addition, the penetration of CD31-immunopositive endothelial cells into the granulosa cell layer was observed in ~10% of total atretic follicles in the 4-MU-treated rats (Supplementary Figure 1, see section on supplementary data given at the end of this article).

To ascertain whether HA reduction induces the arrest of follicle growth and following follicular atresia more directly, we used an in vitro follicle culture system. Preantral follicles (100–140 μm in diameter) obtained from P14 rats progressed to grow in media containing 100 mIU/ml recombinant FSH, whereas the addition of 10 TRU/ml Streptomyces HYAL to the media arrested follicle growth (Fig. 5D). The growth arrest was also observed on the addition of 4-MU at concentrations >0.33 mM (Fig. 5D).

Discussion
Most of the studies on ovarian HA have concentrated on the cumulus–oocyte complex of mature antral follicles during ovulation, but have scarcely examined growing preantral follicles. Indeed, cumulus expansion, HA accumulation associated with a dramatic morphological dynamism, is utilized as a marker of human oocyte maturation in assisted reproductive technology and is known to be required for normal in vivo ovulatory and subsequent fertilization processes in mammals and thus its concentration seems to be appropriate. On the other hand, glycosaminoglycans other than HA are known to be expressed in small growing follicles and play roles in folliculogenesis (Irving-Rodgers et al. 2010, Hatzirodos et al. 2012). HA accumulation in small preantral follicles of WT and Hyal1-null mice has recently been revealed.
for the first time (Dumaresq-Doiron et al. 2011), although its detailed localization and origin have not been determined. The expression of HA in small follicles has also been revealed using a biochemical technique (Irving-Rodgers et al. 2010). Furthermore, Orimoto et al. (2008) showed that Hyal1, Hyal2, and Hyal3 are expressed in the preantral/antral follicles of immature mice, which reflects the existence of HA in these follicles. In this study, we demonstrated that HA was localized in the ECM of granulosa and theca cell layers, including the basal lamina, of primary, preantral, and antral follicles of adult cycling ovaries and perinatal developing ovaries. Furthermore, the main site of ovarian HA accumulation shifted from the stromal region to the intrafollicular region at P5, when primary follicles are ontogenetically found. These new histological findings are necessary for further investigations on the involvement of HA production in follicle growth. High-molecular weight HA that accumulates in the ECM has antiangiogenic activity (West & Kumar 1989, Itano 2008) and is found in joints, skin, and cornea (Kakehi et al. 2003), where too much disorderly vascularization is undesirable. The tunica adventitia of arteries is the main site of HA accumulation among blood vessels as well. On the other hand, the capillary vasculature begins to get detected from primary follicles, grows as the follicle grows, and remains on the outside of the follicular basal membrane until ovulation (Geva & Jaffe 2004). Therefore, the transition of the main site of HA accumulation in the perifollicular region may reflect the

Figure 3 Gene expression of Has subtypes in rat ovaries. (A, B and C) Representative electrophoresis of RT-PCR products for Has genes in the ovaries of proestrus rats. Arrowheads indicate the positions of amplified PCR products for Has1 (A, 521 bp), Has2 (B, 692 bp), and Has3 (C, 537 bp). (D) Representative RT-PCR for Has1–3 and β-actin in the ovaries of 10-week-old cyclic and 100-week-old acyclic rats (n = 3–6 for each group). The estrous cycle consisting of diestrus, proestrus, estrus, and metestrus is indicated as d, p, e, and m respectively. (E) Expression levels of Has genes in the ovaries of perinatal, prepubertal, adult, and senescent rats. Values of the diestrus group from 10-week rats were adjusted to 1. Asterisks indicate significant differences in values compared with those of the diestrus group (Dunn’s multiple range test, P < 0.05). (F, G, H, and I) Localization of the Has1 (F and G) and Has2 (H and I) mRNAs in the preantral (F and H) and antral (G and I) follicles of rat ovaries. Fluorescein-labeled antisense RNA probes for each gene were hybridized with ovarian sections followed by Hoechst 33258 staining. Dashed lines indicate the positions of follicular basal lamina. Autofluorescence of erythrocytes is visualized as red, yellow, or white. No signals were found when the sense sequence of Has1 was used as a probe (J and K). Magnifications of objective: ×10 for G and I and ×20 for others.
transition of roles in avascularity from the outermost layer of blood vessels to the basal lamina and granulosa cell layers of primary follicles. As the HA-rich tunica externa of arterial walls does not exist in newly vascularized capillaries, the transition would be required for perifollicular vasculogenesis.

As has been described above, the expression of each HAS isoform is spatially and temporally controlled (Itano 2008). In the ovaries of mammals, it has been revealed that all HAS isoforms are expressed at mRNA levels by RT-PCR and that HAS1 and HAS2 proteins are mainly localized in the theca and granulosa cell regions of mature antral follicles respectively (Richards 2005, Miyake et al. 2009). In this study, by carrying out in situ hybridization, for the first time, we demonstrated that the expression of HAS1 and HAS2 mRNAs occurs not only in antral follicles but also in small preantral follicles. We also demonstrated that the ovarian expression levels of these genes are upregulated at the periovulatory period, indicating that cyclic changes in some factors during the estrous cycle are involved in HA synthesis. In previous studies, it has been revealed that gonadotropin surge, which occurs at proestrus of the estrous cycle, triggers an increase in HA production via HAS2 induction in the cumulus cells of preovulatory follicles (Richards 2005), whereas the gonadotropin regulation of Has gene expression in small preantral follicles has not been examined. To clarify this, we used P21 immature rats and treated them with eCG and hCG as exogenous gonadotropins. The ovaries of prepubertal animals consist of fewer antral follicles but not mature preovulatory follicles and corpus luteum. eCG mainly mimics the action of FSH and weakly acts as a luteinizing hormone (LH), whereas hCG mimics the action of LH in mammals. In this study, we showed that the treatments of P21 rats with these gonadotropins modulated the expression levels of ovarian Has1 and Has2 genes; Has1 mRNA levels were acutely but transiently upregulated by both eCG and hCG treatments, whereas only eCG but not hCG continuously increased ovarian Has2 mRNA levels. These results indicated the gonadotropin regulation of Has1 and Has2 gene expression in immature growing follicles,
considering the main expression sites for these genes in the ovaries of P21 rats.

In this study, the 4-MU treatment inhibited follicle growth in vitro and induced ovarian HA reduction and follicular atresia in vivo. 4-MU is known to inhibit HA synthesis by depletion of UDP-glucuronic acid and downregulation of HAS activity (Kakizaki et al. 2004, Kultti et al. 2009). At doses used in the present study, 4-MU alleviates inflammatory responses caused by excess HA synthesis in the collagen-induced arthritis mouse model in vivo and in vitro (Yoshioka et al. 2013). We also observed that the glutamine analog DON, which inhibits glucosamine and consequent HA synthesis (Tempel et al. 2000), acted on ovaries in the same manner as in vivo 4-MU. Even when eCG was administered in combination with 4-MU or DON, HA production, normal follicle growth, and follicular avoidance of atresia were not observed. These two chemicals have been well known to reduce HA synthesis and have been frequently used for HA reduction studies in various cell lines and organs including ovary (Clark et al. 1987, Tempel et al. 2000, Kakizaki et al. 2004, Kultti et al. 2009, Tzuman et al. 2010, Yoshioka et al. 2013). On the other hand, these chemicals do not specifically exert effects on HASS, and therefore the relationship between follicular HA reduction and follicular atresia is still unclear. To clarify the relationship more directly, we examined the effect of Streptomyces HYAL, which specifically degrades HA but not other glycosaminoglycans such as chondroitin, chondroitin sulfate, dermatan sulfate, keratan sulfate, heparan sulfate, and heparin (Ohya & Kaneko 1970), on the growth of in vitro-cultured preantral follicles and found that HYAL treatment inhibited follicle growth in culture under a condition of relatively high recombinant FSH concentrations. This result indicates that HA reduction in follicular glyocalyx suppresses normal follicle growth and may consequently lead to follicular atresia. Additionally, decreased expression levels of Has1 and Has2 mRNAs and follicular HA were observed in 100-week-old rats, suggesting that the reduction of HA synthesis potentially contributes to the fertility reduction observed in older animals.

The theca interna is the site of steroid hormone synthesis and follicular vasculature and therefore consists of steroid-producing endocrine cells, capillary endothelial cells, and fibroblasts. Just inside of the theca interna, the basal lamina defines follicle structure by itself and acts as a barrier between layers of extrafollicular theca cells and intrafollicular granulosa cells. Capillary intrusion into the granulosa cell layer occurs only in mature follicles in response to the gonadotropin surge during ovulation, but it does not occur in growing or atretic follicles (Geva & Jaffe 2004). Although the fate of the thecal layer during atresia varies among species, a sharp reduction of perifollicular vascularity is commonly observed in mammals (Hsueh et al. 1994). Naturally, the intrusion does not occur during follicle growth, as is critical for the maintenance of follicular structure. In this study, we observed that HA synthesis was reduced in the ovaries of the 4-MU- and DON-treated rats and that CD31-immunopositive cells intruded into the granulosa cell layer of part of immature preantral follicles in the 4-MU-treated rats. As the accumulation of high-molecular weight HA in the ECM has antiangiogenic activity (West & Kumar 1989, Spicer & Tien 2004), vascular integrity around growing follicles may be impaired by HA reduction and the degraded follicular glyocalyx may permit endothelial cells to intrude. Indeed, Tempel et al. (2000) found the capillary intrusion

**Table 1 In vivo effects of HA synthesis inhibitor 4-MU and gonadotropin treatments on follicular atresia.**

P21 immature rats were treated with saline, 10 IU eCG, LD50 dose of 4-MU, or 4-MU + eCG. After a couple of days, ovaries were histologically examined. One ovary was obtained from each rat and total and atretic follicles in the primary and preantral secondary (class 3a–5b) and antral stage (≥6) were counted as described previously (Takahashi et al. 2008). The data are given as means ± S.E.M. with values rounded to the first decimal place.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Class of follicle</th>
<th>Total</th>
<th>Atretic</th>
<th>Ratio (%)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>3a–5b</td>
<td>92.5 ± 8.3</td>
<td>17.1 ± 1.3</td>
<td>18.3</td>
<td>12</td>
</tr>
<tr>
<td>eCG</td>
<td>≥6</td>
<td>0.2 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.1</td>
<td>6</td>
</tr>
<tr>
<td>4-MU</td>
<td>3a–5b</td>
<td>82.8 ± 9.4</td>
<td>8.4 ± 1.2</td>
<td>9.1</td>
<td>6</td>
</tr>
<tr>
<td>4-MU + eCG</td>
<td>≥6</td>
<td>15.3 ± 1.2</td>
<td>1.4 ± 0.0</td>
<td>9.2</td>
<td>6</td>
</tr>
<tr>
<td>DON</td>
<td>3a–5b</td>
<td>96.3 ± 9.3</td>
<td>88.0 ± 5.1</td>
<td>91.4</td>
<td>4</td>
</tr>
<tr>
<td>DON + eCG</td>
<td>≥6</td>
<td>0.8 ± 0.3</td>
<td>0.8 ± 0.3</td>
<td>100</td>
<td>4</td>
</tr>
</tbody>
</table>

*Percentages of atretic follicles per total follicles in an individual rat. Significant differences compared with the values of the saline- or eCG-treated groups at the 5% level (Dunn’s multiple comparison test).
into rat preovulatory follicles during DON treatment and, therefore, intrafollicular avascularity maintained by HA accumulation would exist in growing preantral follicles as well as preovulatory ones.

In this study, we confirmed HA to be present in the ECM of primary and more growing preantral and antral follicles, found Has1 and Has2 mRNAs to be localized in small growing follicles, and illustrated the transition of the main site of HA accumulation from the stromal region to follicular region at P5, all of which suggest the involvement of HA production in follicle growth. The gonadotropin regulation of Has gene expression in immature ovaries, which mainly contain small preantral follicles, was also revealed. HA reduction by Streptomyces HYAL caused the arrest of follicle growth in vitro, even under sufficient levels of gonadotropins for growth. Furthermore, both 4-MU and DON induced ovarian HA reduction and follicular atresia in vivo. Endothelial cells were detected in the granulosa cell layer of part of atretic follicles in rats treated with these chemicals. In conclusion, our results indicate that gonadotropin-regulated ovarian HA synthesis is involved in normal follicle growth.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-13-0464.

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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Author contribution statement
N Takahashi contributed to the study concept and design; the acquisition, analysis, and interpretation of data; and the drafting of this manuscript. W Tammi provided technical support for the study, especially for in vitro follicle culture. B Ishizuka managed the funding and supervised the study. All authors critically reviewed the manuscript for intellectual content.

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