ADAMTS-1 is involved in normal follicular development, ovulatory process and organization of the medullary vascular network in the ovary

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Abstract

To clarify the role of disintegrin-like and metalloproteinase with thrombospondin type I motifs-1 (ADAMTS-1) in ovarian function, we examined abnormalities in ovulatory processes, folliculogenesis and the vascular system of ADAMTS-1 null ovaries. First, when immature female mice were treated with pregnant mare serum gonadotropin (PMSG)/human chorionic gonadotropin (hCG), the number of ovulated oocytes was markedly decreased in ADAMTS-1 null mice in comparison to ADAMTS-1 (+/-) controls. The proportion of anovulated follicles to total mature follicles was significantly higher in ADAMTS-1 null females when compared with controls. The numbers of growing follicles at each stage were counted. The number of follicles at type 5b (late preantral) and later stages was markedly reduced in ADAMTS-1 null mice, irrespective of gonadotropin treatment (no gonadotropins, PMSG alone or PMSG/hCG). These data demonstrate that impairment of ovarian function to ovulate oocytes in ADAMTS-1 null mice occurs at two different levels: in the development of growing follicles and ovulatory processes. Furthermore, ADAMTS-1 null ovaries included a number of unusual atretic follicles that showed no sign of oocyte degeneration but lost the surrounding granulosa cell layers and were considered to be derived from type 4 or 5a follicles. These results suggest that ADAMTS-1 is important for follicular development beyond the type 4 and/or 5a and for maintaining normal granulosa cell layers in follicles. Finally, the number of large blood vessels in the medullary zone was significantly decreased in ADAMTS-1 null mice ovaries, suggesting that ADAMTS-1 is also involved in the organization of the medullary vascular network.

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Introduction

ADAMTS-1 is the first member of the disintegrin-like and metalloproteinase with thrombospondin type I motifs (ADAMTS) family, and is characterized by having an ADAM metalloproteinase domain and thrombospondin type I motifs (Kuno *et al.* 1997a, 1997b). ADAMTS-1 was originally identified as a gene strongly expressed in cachexigenic tumors (Kuno *et al.* 1997b). ADAMTS-1 consists of proprotein-, metalloproteinase- and disintegrin-like domains, thrombospondin type I motifs and intervening spacer region among them.

As a metalloproteinase associated with the extracellular matrix (ECM) (Kuno & Matsushima 1998, Kuno *et al.* 1999), ADAMTS-1 cleaves aggrecan and versican (Kuno *et al.* 2000, Sandy *et al.* 2001, Rodriguez-Manzanique *et al.* 2002). ADAMTS-1 also possesses anti-angiogenic activity (Vazquez *et al.* 1999, Luque *et al.* 2003) and ADAMTS-1 or its C-terminal half region suppresses tumor growth and experimental metastatic potential (Iruela-Arispe *et al.* 2003, Kuno *et al.* 2004).

The physiological functions of ADAMTS-1 have been investigated using gene knockout mice produced in our laboratories (Shindo *et al.* 2000). ADAMTS-1 null mice display renal anomalies involving enlarged calices and atrophic renal papillae, which resemble ureteropelvic junction (UPJ) obstruction in humans (Shindo *et al.* 2000). This finding indicates that ADAMTS-1 plays an important role in tissue architecture and function in ureteropelvic junction tissue.

We demonstrated that the second major phenotype of ADAMTS-1 null mice is female infertility featuring a markedly low delivery rate and number of pups, indicating that ADAMTS-1 is important in the function of female reproductive organs (Shindo *et al.* 2000). Decreased number of implantation sites was also reported in ADAMTS-1 null mice (Shindo *et al.* 2000, Mittaz *et al.* 2004). ADAMTS-1 mRNA expression is induced in the granulosa cells of the preovulatory follicles after administration of luteinizing hormone (LH) (Espey *et al.* 2000, Robker *et al.* 2000, Boerboom *et al.* 2003), and is sustained in a progesterone-dependent manner (Robker *et al.* 2000). The ADAMTS-1 protein

increases after an LH surge and is localized in the cumulus-oocyte complex (COC) of the preovulatory follicles (Russell *et al.* 2003). Recently, Mittaz *et al.* (2004) reported that ADAMTS-1 null mice trap mature oocytes in ovarian follicles, suggesting that ADAMTS-1 is required for normal ovulation.

In the present study, we conducted a detailed examination of the ovarian morphology of ADAMTS-1 null female mice and showed that impairment of ovulation function in ADAMTS-1 null mice can be attributed not only to the ovulatory process of mature follicles but also to changes in the growth process of immature follicles.

Materials and methods

Materials

Pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) were purchased from Teikokuzouki Pharmaceutical Co., (Tokyo, Japan)

Animals and hormone treatment

ADAMTS-1 null mice were generated as described previously (Shindo *et al.* 2000). Littermates obtained by mating heterozygous females (+/-) with homozygous males (-/-) with the genetic background of the 129/Sv x C57BL/6 hybrid were used for phenotypic analysis. Animal experiments complied with the standards stipulated by the Takara-machi Campus of Kanazawa University and adhered to the principles of the UFAW Handbook on the Care and Management of Laboratory Animals, 7th edition.

Measurement of ovulated oocytes

Female mice at 24–26 days old were injected i.p. with 7.5 IU of PMSG followed by 7.5 IU of hCG i.p. 48 h later. Oviducts and ovaries were excised from mice 15 h after hCG administration and were placed into dishes containing PBS. COC was recovered by dissection of both the ampulla and the oviduct, and under a dissecting microscope, the number of ovulated oocytes was determined after treatment with 500 units/ml hyaluronidase (Sigma).

Histological studies and statistical analyses

Excised ovarian tissues were fixed in 10% neutral buffered formalin (Wako Pure Chemical, Osaka, Japan) as described previously (Lydon *et al.* 1995). After the requisite fixation time, ovaries were trimmed, dehydrated with ethanol, cleared in xylene, and infiltrated with paraffin wax.

For analysis of the number of ovarian follicles, each paraffin-embedded ovary was placed with the ovarian hilum at the side and serially sectioned parallel to its longitudinal plane at 2.5 µm using a microtome. Every sixth section was mounted and stained with hematoxylin and eosin. Serial sections of ovaries were photographed and printed at x100 magnification, which enabled clear discrimination of each growing follicle. Each follicle was then numbered on the serial photographs and counted only once when the oocyte was seen. Classification and nomination of ovarian follicles were based on Pederson & Peters (1968). Follicles at the preantral stage were deemed atretic if the oocyte was degenerating (convoluted and condensed or fragmented) (Morita *et al.* 1999).

Area of medullary zone of each ovary was determined on digital microscopic images of the serial sections using MacSCOPE software (Mitani Co., Fukui, Japan). The medullary zone was defined expediently as the internal area of the ovarian cortex which consists of follicles and surrounding ovarian stroma. The section with the largest medullary area among the serial sections (defined as the central plane) was used as the representative section for comparison. The blood vessel area in the medullary zone was also measured on the central plane using MacSCOPE.

The Mann–Whitney test was used for statistical evaluation and $P < 0.05$ was considered to be statistically significant. Fisher's direct probability test was used for statistical evaluations of frequency of unruptured follicles showing COC expansion and frequency of large follicles having two vascular plexus layers.

Immunohistochemical analyses

Cryostat sections of ovary tissue were stained with anti-mouse CD31 monoclonal antibody (Becton-Dickinson, Sunnyvale, CA, USA) with the aid of a HISTOFINE system (Nichirei Co., Tokyo, Japan) as described previously (Kuno *et al.* 2004).

Results

Decreased number of ovulated oocytes in ADAMTS-1 null females

In order to evaluate the total ovarian function of ADAMTS-1 null mice, we first examined the ovulation of oocytes in response to exogenous gonadotropins. Immature mice were used for the study because they ovulate solely in response to exogenous gonadotropins, by which the super-ovulation scheme was applied. We confirmed that ADAMTS-1 +/- females were as fertile as +/+ mice (data not shown), and thus ADAMTS-1 +/- female mice were used as controls for assessment of ovarian function. After treatment with PMSG followed

Figure 1 Reduction in the number of ovulated oocytes in ADAMTS-1 knockout mice under super-ovulatory conditions. ADAMTS-1 $-/-$ ($n=8$) and ADAMTS-1 $+/-$ ($n=9$) mice were treated with PMSG/hCG, 15 h after which oocytes were recovered from both oviducts of mice and were counted. The results are expressed as oocytes per mouse. The data are means $\pm$ S.D. and numerical mean value is also indicated.

by hCG, ovulated oocytes in the oviduct were collected and counted. As shown in Fig. 1, ovaries of the ADAMTS-1 null females ovulated one ninth the

number of oocytes as control mice. These data suggest that the total ovarian function of ADAMTS-1 null female mice is impaired.

Impaired follicular development in ADAMTS-1 null mice

To examine whether ADAMTS-1 is involved in the maturation process of follicles, we first compared the number of total mature follicles in immature mice after PMSG/hCG treatment. The numbers of corpus luteum (CL), unruptured follicles (UF, mature follicles that have not undergone ovulation), and luteinized unruptured follicles (LUF, luteinized follicles that trap oocyte inside) per ovary were separately counted for each ovary and the sum of these was defined as the number of total mature follicles (Table 1). As shown in Fig. 2A, the number of total mature follicles in the ovaries of ADAMTS-1 null mice was one-third lower than that of control mice, suggesting that follicular maturation is significantly impaired in ADAMTS-1 knockout mice.

To determine the stages of follicular development affected by disruption of the ADAMTS-1 gene, the number of follicles at follicular stage was compared with controls at three time points, before (no stimulation, Fig. 2B), during (PMSG alone, Fig. 2C) and after PMSG/hCG treatment (Fig. 2D). The results were similar at these time points. The number of type 5b follicles (late preantral follicles with more than four layer of granulosa cells) and higher stage follicles were decreased in ADAMTS-1 null mice, whereas the number of the earlier type 4 follicles (small preantral follicles with only two layers of granulosa cells) were increased in ADAMTS null mice, although the

Table 1 Terms describing the follicular development used in the present study

	Description
Follicle type	
(1) Stages of follicular development based on Pederson & Peter (1968):	
Type 4	Early preantral follicles with two layers of granulosa cells
Type 5a	Middle preantral follicles with three layers of granulosa cells
Type 5b	Late preantral follicles with more than four layers of granulosa cells and without follicle fluid
Type 6	Antral follicles with small scattered antrums
(2) Total mature follicles after injection of hCG	
UF	Follicles that have finished maturation (UF+LUF+CL)
LH	Mature follicles that have not undergone ovulation
CL	Luteinized follicles that have not undergone ovulation and include oocytes
Anovulatory follicles	Luteinized follicles that have undergone ovulation and have stigma
	UF+LUF
(3) General terms showing the follicular development	
Preantral	Follicles with more than two layers of granulosa cells and without antrums (type 4+type 5a+type 5b)
Antral	Follicles with antrums

UF, unruptured follicles; LUF, luteinized unruptured follicles; CL, corpus luteum.

Figure 2 Involvement of ADAMTS-1 in follicular development. (A) Comparison of the number of total mature follicles in super-ovulatory ADAMTS-1 $-/-$ and ADAMTS-1 $+/-$ mice. ADAMTS-1 $-/-$ ($n=7$) and ADAMTS-1 $+/-$ ($n=7$) mice were treated with PMSG/hCG, followed by excision of ovaries, which were then histologically analyzed. Total mature follicles were calculated as the sum of CL, UF, and LUF. The results are expressed as follicles per ovary. The data are means $\pm$ S.D. (B-D) Numbers of ovarian follicles at different stages in ADAMTS-1 $-/-$ and $+/-$ mice treated with PMSG (C), or PMSG/hCG (D), and without gonadotropin (B). ADAMTS-1 $-/-$ and ADAMTS-1 $+/-$ mice were treated with PMSG (48 h) ($n=5$ for each group) (C), or PMSG (48 h) /hCG (15 h) ($n=6$ for each group) (D), and without gonadotropin ($n=5$ for each group) (B), followed by excision of ovaries, which were then histologically analyzed. Staging of ovarian follicles is based on the classification of Pederson and Peters (1968). The results are expressed as follicles per ovary. The data are means $\pm$ S.D.

difference was not sufficient to reach significance in mice treated with PMSG alone. For type 5a follicles (small preantral follicles with three layers of granulosa cells), the difference was barely significant in mice treated

with PMSG alone (Fig. 2C). These results demonstrate that follicular development is significantly impaired at type 5b and thereafter in pre-pubertal ADAMTS-1 null mice, independent of the exogenous gonadotropin

environment. The increases in early-stage follicles (type 4) may be attributable to impairment of follicular growth from the type 4 stage to advanced stages. PMSG-induced follicular growth may explain the relative decrease in the number of type 4 follicles observed in mice treated with PMSG alone (Fig. 2C).

Reduction in general atretic pathways in ADAMTS-1 null mice

To examine whether follicular atresia explains the decreased number of growing follicles, we analyzed changes in atretic follicles in ADAMTS-1 null ovaries. As shown in Fig. 3B and C, the number of atretic follicles from preantral follicles (types 4, 5a, and 5b) was markedly lower in ADAMTS-1 null mice treated with PMSG or PMSG/hCG than in control mice. Similarly, under the unstimulated condition, a significant reduction in the number of atretic follicles derived from preantral follicles was observed in ADAMTS-1 null ovaries (Fig. 3A). These data do not support the notion that enhanced atresia causes the reduction in healthy follicles in ADAMTS-1 null ovaries.

Emergence of unusual follicles not associated with granulosa cell layers in ADAMTS-1 null ovaries

Further detailed analysis of ovarian histology revealed that a number of unusual follicles without cytoplasmic fragmentation and not surrounded by layers of normal-looking granulosa cells appeared in unstimulated ADAMTS-1 null ovaries, while such unusual follicles were rarely seen in the controls (Fig. 4A and C). These were not classified as atretic follicles based on the criteria that we used in this study because there were no apparent fragmentation of oocytes, a sign of authentic follicular atresia. These unusual follicles were clearly delineated from usual atresia observed in control ovaries (Fig. 4A and B). The number of these unusual follicles in ADAMTS-1 null mice was not dependent on exogenous gonadotropin stimulation (Fig. 4C). These unusual oocytes were larger than oocytes of healthy type 3 follicles, and approximately equivalent in size to oocytes of type 4 follicles (Fig. 4A). Therefore, such unusual

Figure 3 Reduction in atretic follicles from preantral stages in ADAMTS-1 null mice. (A–C) The numbers of atretic follicles from preantral stages in ovaries of ADAMTS-1 $-/-$ and $+/-$ mice treated with PMSG (B) or PMSG/hCG (C), and without gonadotropin (A). ADAMTS-1 $-/-$ and ADAMTS-1 $+/-$ mice were treated with PMSG (48 h) ($n=5$ for each group) (B), or PMSG (48 h)/hCG (15 h) ($n=6$ for each group) (C), and without gonadotropin ($n=5$ for each group) (A), followed by excision of ovaries, which were then histologically analyzed. The results are expressed as follicles per ovary. The data are means $\pm$ S.D.

Figure 4 Appearance of a number of unusual follicles without granulosa cell layers in ADAMTS-1 null ovaries. (A) Unusual follicles without granulosa cell layers (indicated by arrowheads) formed in ADAMTS-1 null ovaries without stimulation. Healthy follicles (types 3 and 4) are also indicated. Scale bar: 100 μ m. (B) An atretic follicle (arrow) observed in control ovaries without stimulation. Scale bar: 100 μ m. (C) Comparison of the number of unusual follicles in ADAMTS-1 $-/-$ and ADAMTS-1 $+/-$ mice treated with PMSG (48 hr)/hCG (15 h) ($n=6$ for each group), or PMSG (48 h) ($n=5$ for each group), and without gonadotropin ($n=5$ for each group). The results are expressed as follicles per ovary. The data are means $\pm$ S.D.

follicles might be formed from follicles at type 4 or later preantral stages by losing their granulosa cell layers in ADAMTS-1 null ovaries. ADAMTS-1 may be required for maintenance of follicle structure at the preantral stages.

Increased rate of follicles failing to ovulate in ADAMTS-1 null mice

The role of ADAMTS-1 in the ovulatory process was evaluated by determining the proportion of total anovulatory follicles (UF+LUF) as a percentage of the total mature follicles (UF+LUF+CL). As seen in Fig. 5A, the percentage of anovulatory follicles significantly increased in ADAMTS-1 null mice treated with PMSG/hCG when compared with control mice, indicating

that ovulatory ability is significantly compromised in ADAMTS-1 null females. The increase in the anovulatory follicle rate in ADAMTS-1 null mice was attributable to an increase in UF but in LUF (Fig. 5B and C). In 20 of 22 UFs found in ADAMTS-1 null ovaries, the cumulus cells had already expanded around the oocytes (Fig. 6B, D and E), thus suggesting that COC expansion is not affected. In this respect, UF in ADAMTS-1 null mice resemble preovulatory follicles, except for degeneration of the oocyte and the zona pellucida (Fig. 6B and D). In addition, partial luteinizing changes were sporadically observed in the granulosa layer in UF in ADAMTS-1 null ovaries (Fig. 6F) and UF in ADAMTS-1 null mice often retained the thick theca layer despite hCG treatment (Fig. 6E). These findings indicate that thinning and rupture of the follicle wall

Figure 5 Involvement of ADAMTS-1 in the ovulatory process. (A-C) Comparison of percentages of anovulatory follicles (A), UF (B), and LUF (C) in ADAMTS-1 $-/-$ and ADAMTS-1 $+/-$ mice. ADAMTS-1 $-/-$ ($n=7$) and ADAMTS-1 $+/-$ ($n=7$) mice were treated with PMSG followed by hCG 48 h later, 15 h after which the ovaries were excised and histologically analyzed as described in Materials and methods. Percentages of anovulatory follicles were calculated according to the following formula: anovulatory follicles (UF+LUF)/total-mature follicles (UF+LUF+CL). The results are expressed as follicles per ovary. The data are means $\pm$ S.D.

Figure 6 Ovarian histology of ADAMTS-1 homozygous mutant females. Sections of ovaries from ADAMTS-1 +/- mice (A), and ADAMTS-1 -/- mice (B-F) treated with PMSG/hCG (15 h) were stained with hematoxylin-eosin. Expansion of the cumulus cells was observed in UF of ADAMTS-1 -/- females (B, D and E). (B) UF of ADAMTS-1 -/- mice having a degenerated oocyte (asterisk). (C) typical LUF. (D) UF containing a degenerated zona pellucida. (E) UF surrounded by a thick theca cell layer (arrow). Scale bar: 100 μ m. (F) High-magnification portion of panel (B), depicting partial luteinizing changes (arrow) in the granulosa cell layer of UF in ADAMTS-1 null ovaries.

are compromised in ADAMTS-1 null mice and that luteinization, albeit incomplete, proceeded in the resultant follicles.

Dysplastic vasculature of ADAMTS-1 null ovaries

Alteration of ovarian blood vessels in ADAMTS-1 null mice was examined by immunohistochemical staining with anti-CD31 antibody. In preantral (type 5b) and small antral follicles (type 6), a single-layer capillary network was observed in the theca layer in ADAMTS-1 null ovaries (Fig. 7B and D), as in control mice (Fig. 7A and C). In contrast, the vasculature of large follicles in ADAMTS-1 null mice was somewhat different from that in control mice. Large follicles in ADAMTS-1 null ovaries often had additional layers of capillary network outside the capillary plexus in the theca layer (Fig. 7G and H), while most large follicles in control mice had only a single capillary layer (Fig. 7E). In ADAMTS-1 null ovaries, 10 of 18 large follicles had two layers of

vascular plexus, while in control ovaries, this number was 3 of 28, $P < 0.01$.

Dysplastic vasculature was more striking in the ovarian medulla in ADAMTS-1 null mice (Fig. 8). Under super-ovulatory conditions, various sizes of blood vessels, including a number of large blood vessels (50–150 μ m in diameter), were formed in the medullary zone of control ovaries (Fig. 8A and C). Large blood vessels (more than 50 μ m in diameter) were very few in ovaries of unstimulated control mice, and formation of large blood vessels was induced by administration of gonadotropins in control ovaries (Fig. 8F). In contrast, ADAMTS-1 null ovaries had a small medullary zone (Fig. 8B and E). Formation of large blood vessels of the medullary zone in the presence of gonadotropins (PMSG or PMSG/hCG) was significantly impaired in ADAMTS-1 null mice although smaller vessels were present (Fig. 8B, D, and F). These observations demonstrate that the vascular network of the ovarian medulla is dysplastic or disorganized in ADAMTS-1 null mice.

Figure 7 Follicular microvasculature of ADAMTS-1 null ovaries. Sections of ovaries from ADAMTS-1 $+/+$ (A, C, and E), and ADAMTS-1 $-/-$ mice (B, D, F, G and H) treated with PMSG/hCG (11 h) were stained immunohistochemically with anti-mouse CD31 antibody. (A and B) type 5b follicles; (C and D) type 6 follicles; (E and F) preovulatory follicles; (G) follicles with a single antrum. (H) High-magnification portion of panel (G). In G and H, yellow arrowheads indicate additional capillary layers outside the capillary plexus in the theca layer of follicles in ADAMTS-1 null ovaries. Counterstain: hematoxylin. Scale bar: 200 μ m.

Discussion

Mouse knockout models have provided critical information on the factors associated with ovarian follicular development (Dong *et al.* 1996, Dierich *et al.* 1998, Couse *et al.* 1999, Soyal *et al.* 2000) and the ovulatory process (Lydon *et al.* 1995, Hizaki *et al.* 1999, Davis *et al.* 1999). We and others have shown that ADAMTS-1 plays important roles in the functions of female genital organs by analyzing ADAMTS-1 null mice (Shindo *et al.* 2000, Mittaz *et al.* 2004). In the present study, we demonstrate that abrogation of ADAMTS-1 impaired

ovulation of oocytes at two different levels; in the development of growing follicles and in the ovulatory process of mature follicles in response to gonadotropins.

Ovarian folliculogenesis starts with the recruitment of nongrowing primordial follicles in the perinatal stage, and proceeds through the primary follicle, preantral, and antral stages (Matzuk *et al.* 2002), under the influence of various factors including FSH, estrogen and growth factors such as c-kit and GDF-9 (Dong *et al.* 1996, Dierich *et al.* 1998, Couse *et al.* 1999, Donovan & de Miguel 2001, Matzuk *et al.* 2002). This study demonstrated that ADAMTS-1 is also required for complete

folliculogenesis. In ADAMTS-1 null mice, the number of type 4 follicles was increased when compared with controls, whereas the number of type 5 b follicles and later stages were decreased. Instead of healthy preantral follicles at type 5b or later, unusual follicles containing enlarged oocytes without fragmentation and losing normal granulosa cells, probably originating from an unusual atretic process in type 4 or 5 follicles, were elevated in the ADAMTS-1 ovaries. Atresia was not accelerated in preantral follicles. These findings are compatible with the notion that ADAMTS-1 is important for follicles to autonomously develop beyond the type 4 or 5a into the next stage and that abrogation of ADAMTS-1 leads to formation of unusual atretic follicles without granulosa cell layers. Granulosa cells rapidly proliferate from the preantral follicle stage (type 4 and 5a), and the ECM in the granulosa cell layer must be reconstituted during follicular development. Thus, ADAMTS-1 may be involved in folliculogenesis as well as the maintenance of follicular structure by participating in the remodeling of the ECM surrounding the granulosa cells of growing follicles.

Ovulation initiated by the LH surge consists of several sequential events involving enlargement of the antrum, expansion of the COC, degradation of the follicle and ovarian wall at the apex of the mature follicles, and release of the COC (Tsafiri *et al.* 1996, Murdoch 2000, Richards *et al.* 2002). We found that the proportion of anovulatory follicles, specifically of UF to total mature follicles, was significantly higher in the ovaries of ADAMTS-1 null mice treated with PMSG/hCG. Significant induction of ADAMTS-1 mRNA expression in rats and in mice has been shown to occur in the granulosa layer of the large follicles after hCG administration (Espey *et al.* 2000, Robker *et al.* 2000). A more recent study demonstrated that ADAMTS-1 protein predominantly accumulates in the ECM of the COC during cumulus cell expansion after hCG administration (Russell *et al.* 2003). Russell *et al.* (2003) also reported that processing of versican is reduced in COC of Progesterone receptor knockout mice in which ADAMTS-1 expression is downregulated (Russell *et al.* 2003). It has therefore been hypothesized that ADAMTS-1 is involved in the re-organization of the

COC matrix by modulating versican prior to hCG-induced ovulation. We observed that cumulus cell expansion occurred in most of the unruptured large follicles of PMSG/hCG-treated ADAMTS-1 null ovaries, thus indicating that ADAMTS-1 is not essential for cumulus cell expansion. However, other ADAMTS family members, such as ADAMTS-4 which has similar substrate specificity to ADAMTS-1, may compensate for the loss of the function of ADAMTS-1 in cumulus expansion; it has been shown that ADAMTS family members including ADAMTS-4 and -5 are expressed in preovulatory follicles in the ovary (Madan *et al.* 2003, Russell *et al.* 2003). Similarly, the incomplete blockage of the ovulatory process observed in ADAMTS-1 knockout mice might be due to the compensatory functions of other ADAMTS family members, such as ADAMTS-4 and -5.

Degradation of ECM within the theca layer and tunica albuginea is necessary for follicle rupture. Matrix metalloproteinases (MMPs), such as MMP13, have been suggested to contribute to the breakdown of the follicle wall (Curry *et al.* 2001). We found that ADAMTS-1 null mice exhibited impairment of theca layer thinning and the ovulatory response to PMSG/hCG treatment (Fig. 6). Although ADAMTS-1 is not capable of degrading fibrillar collagens, such as collagens I and III (Rodriguez-Manzanique *et al.* 2002), it is possible that ADAMTS-1 indirectly promotes degradation of the collagenous layers of the follicle wall by triggering signals that activate MMPs. Our previous study showed that ADAMTS-1 null mice exhibit renal phenotypes that resemble human UPJ stenosis, accumulating excessive collagen fibers in UPJ tissue (Shindo *et al.* 2000). It is likely that ADAMTS-1 plays a similar role in regulation of ECM remodeling in both UPJ tissue and ovaries.

Vascular endothelial growth factor (VEGF)-mediated follicular angiogenesis is involved in gonadotropin-dependent follicle development (Zimmermann *et al.* 2003). As ADAMTS-1 generally functions as an anti-angiogenic factor through inhibition of VEGF action, it can be expected that loss of ADAMTS-1 may affect vasculature in the ovary. In the present study, we found that the follicular angiogenesis of large follicles was somehow upregulated in ADAMTS-1 null ovaries. This

Figure 8 Medullary vascular structure of ADAMTS-1 null ovaries. (A and B) Histology of the medullar zone. Sections of ovaries from ADAMTS-1 +/- (A) and ADAMTS-1 -/- mice (B) treated with PMSG/hCG (15 h) were stained with hematoxylin-eosin. Arrowheads indicate large blood vessels (more than 50 µm in diameter). Boundaries between the medullar zone (m) and the cortical zone (c) are indicated by the gray lines. Scale bar: 100 µm. (C and D) Immunohistochemistry for CD31 in the medullar zone. Sections of ovaries from ADAMTS-1 +/- (C) and ADAMTS-1 -/- mice (D) treated with PMSG/hCG (11 h) were stained immunohistochemically with anti-mouse CD31 antibody. Counterstaining: hematoxylin. Scale bar: 100 µm. (E) Reduction in the medullary zone area in ADAMTS-1 null ovaries treated with PMSG/hCG (15 h). The areas of the central medullary zones of ADAMTS-1 +/- (n=6) and ADAMTS-1 -/- (n=6) ovaries, which are shown in panels (A) and (B), were measured as described in Materials and methods. (F) Reduction in the area occupied by large blood vessels (more than 50 µm in diameter) in ADAMTS-1 null ovaries treated with PMSG (48 h) (n=5 for each group), or PMSG/hCG (15 h) (n=6 for each group), and without gonadotropin (n=5 for each group). Total area occupied by large vessels in the central medullary zones was measured as described in Materials and methods.

might be due to loss of anti-angiogenic activity of ADAMTS-1. In contrast, more significant changes in the vasculature network in ADAMTS-1 null ovaries were observed in the medullary zone. We found that gonadotropin-induced formation of large blood vessels in the medullary zone was significantly impaired in ADAMTS-1 null mice. This shows that ADAMTS-1 does not function merely as an anti-angiogenic factor in the ovary, but positively regulates the formation of the blood vessel network, although the mechanisms remain to be investigated. ADAMTS-1 might participate in branching and dilation processes of blood vessel formation, although we cannot exclude the possibility that vascular changes in the medullary zone are the result of a decreased number of mature follicles.

As we found that types 5b and 6 follicles in ADAMTS-1 null mice had normal vascular plexus, it is unlikely that the decreased follicular development of ADAMTS-1 null mice can be attributed to alteration in follicular angiogenesis. However, dysplasia of large blood vessels in the medullary zone may affect blood supply to growing follicles and, therefore retard gonadotropin-dependent follicular development in ADAMTS-1 null mice.

In summary, our results provide evidence that ADAMTS-1 is involved in the ovulatory response of mature follicles, follicular development, and the organization of the medullary vascular network. An interesting question regarding the human ovary that arises based on the present results is whether defective ADAMTS-1 expression is a model for any ovarian dysfunction observed in human patients. Further studies on the functions of ADAMTS family members, including ADAMTS-1, in the ovary can be expected to advance the understanding of the molecular mechanisms of infertility.

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