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# **Gelatinase A and membrane-type matrix metalloproteinases 1 and 2 are responsible for follicle rupture during ovulation in the medaka**

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**Identification of the hydrolytic enzymes involved in follicle rupture during vertebrate ovulation remains a central challenge for research in reproductive biology. Here we report a novel approach to this problem using an *in vitro* ovulation system in the medaka, *Oryzias latipes*, which is a small freshwater teleost. We found that follicle rupture in the medaka ovary involves the cooperation of at least three matrix metalloproteinases (MMPs), together with the tissue inhibitor of metalloproteinase-2b (TIMP-2b) protein. We determined the discrete roles of each of these proteins during follicle rupture. Our results indicated that gelatinase A induces the hydrolysis of type IV collagen constituting the basement membrane, membrane-type 2 MMP (MT2-MMP) degrades type I collagen present in the theca cell layer, and MT1-MMP and TIMP-2b are involved in the production and regulation of gelatinase A. These findings will help clarify the mechanism of follicle wall degradation during ovulation in mammalian species.**

Keywords: ovulation; follicle rupture; matrix metalloproteinases; medaka fish

Ovulation, which is triggered by a preovulatory surge of luteinizing hormone (LH) released from the pituitary gland, is a dynamic process that results in the liberation of a mature fertilizable ovum from the ovarian follicle. This event, which is known as follicle rupture, has been the subject of intensive investigation over the past century (1-5). Previous studies carried out primarily in mammalian species, including humans, have established that follicle rupture is accomplished by the dissolution of the granulosa cell basement membrane and fragmentation of the collagenous matrix at the apex of the follicular wall, thereby implicating the involvement of proteolytic enzymes. Indeed, a variety of proteases have been proposed as candidates for rupturing the follicle. It is generally believed that follicle rupture during ovulation takes place due to the actions of two proteolytic enzyme systems: the plasminogen activator (PA)/plasmin system (3, 6, 7) and the MMP system (8-11). However, it has been demonstrated that most mouse strains lacking individual proteases retain apparently normal reproductive ability, which is not consistent with this hypothesis (5, 12). A few *MMP*-deficient mice, including those lacking membrane-type 1 (MT1) MMP (13), and a disintegrin and metalloproteinase domain-17 (ADAM-17) (14), die *in utero* or shortly after birth; thus, the roles played by these proteases in ovulation remain to be clarified. In addition, the involvement of cathepsin L, and a disintegrin and MMP domain with thrombospondin-like motifs (ADAMTS-1), has been suggested by studies of mice lacking progesterone receptors (15). Mice null for *ADAMTS-1* have been shown to develop fewer mature follicles (16), although its relationship to follicle rupture is not clear. A recent study suggested that one function of ADAMTS-1 in ovulation is to cleave versican in the matrix of the expanded cumulus-oocyte complex (17). In short, the proteases that are essential for follicle rupture in ovulation have not yet been

identified, despite much effort.

In all vertebrates, the growth and proliferation of oogonia, their development to the oocyte stage, and their eventual release from the ovaries are thought to be under similar endocrine regulation (18-20), although the basic ovarian plan has several morphological variants. When searching for the fundamental mechanisms that are common to vertebrate ovaries, the use of the medaka, *Oryzias latipes*, which is a small freshwater teleost, has several advantages because of its short generation time and the cyclic nature of ovarian activity in mature adults (21, 22): under a constant long photoperiod of 14-h light/10-h dark at 27°C, the medaka usually spawns daily within 1 h of the onset of light for several consecutive days. Thus, we can readily time the successive events of spawning, such as completion of vitellogenesis, breakdown of the germinal vesicle, and ovulation (23). Moreover, using this fish, the process of follicle rupture and oocyte extrusion can be observed *in vitro* in isolated intact follicles (24, 25). We therefore investigated vertebrate ovulatory processes in the medaka, with the particular aim of identifying the enzymes responsible for the proteolytic degradation of the follicle walls.

In the present study, we initially designed an *in vitro* experimental system for the study of ovulation using dissected ovarian follicles, and we then used this system to examine the effects of various protease inhibitors on the rate of ovulation. The finding that inhibitors of MMPs drastically suppressed *in vitro* ovulation prompted us to further explore the spatial and temporal expression patterns of most, if not all, of the MMP genes expressed in the follicular tissue of the medaka at both the mRNA and protein levels. We also examined the relevance of individual MMPs to the ovulatory process.

## **Materials and Methods**

### **Medaka and *In Vitro* Ovulation**

Mature female adults of the orange-red variety of medaka were kept in indoor tanks under reproductive conditions (photoperiod, 10-h dark/14-h light; temperature, 27°C). Except where indicated, ovaries were removed at –6 or –3 h of ovulation and were placed in aseptic saline solution (26). Ovarian follicles were immediately isolated using forceps under a dissecting microscope and were then transferred into 90% medium 199 solution (Earle's medium 199; Dainippon Seiyaku), adjusted to pH 7.4 with NaHCO<sub>3</sub>. At least 20 follicles per culture dish were used in each experiment. The follicles were cultured at 26–27°C in 4 ml of culture medium using a 35 × 10-mm tissue-culture dish. Ovulation was monitored every hour, and the number of oocytes that had successfully ovulated was counted. The ovulation rate was defined as the percentage of ovulated follicles at a given time. The process of ovulation is indicated in hours relative to the beginning of the light period, set at 0 h.

The follicles were incubated with various protease inhibitors, including the MMP inhibitors TAPI-1 (Peptide Institute), TAPI-2 (Peptide Institute), and GM6001 (Chemicon), in order to assess their effects on *in vitro* ovulation. The concentrations of inhibitors were as follows: EDTA, 2 mM; *o*-phenanthroline, 1 mM; TAPI-1, 0.1 mM; TAPI-2, 0.1 mM; GM6001, 10 μM; diisopropyl fluorophosphate (DFP), 0.2 mM; phenylmethanesulfonyl fluoride (PMSF), 0.2 mM; benzamidine, 0.2 mM; antipain, 0.1 mM; chymostatin, 0.1 mM; leupeptin, 0.1 mM; E-64, 0.2 mM; iodoacetic acid, 50 μM; and pepstatin, 10 μM. For the experiments using antibodies, purified antibody fractions (100 μg) were included in the culture system. IgG fractions prepared from pre-immune rabbit antiserum by means of a protein G-Sepharose column were used as controls. In

some experiments, actinomycin D (Sigma), cycloheximide (Sigma), and EDTA were added for various periods of incubation.

### **Isolation of Oocytes and Follicle Layers from Ovulating Follicles**

Follicles that were about to ovulate were isolated as described above. Before spontaneous *in vitro* ovulation, the oocytes were mechanically separated from the follicles using forceps. A follicle devoid of its oocyte is composed of a single inner layer of granulosa cells and a single layer of outer theca cells separated by a basement membrane; the oocyte-free follicle thus prepared was referred to as the “follicle layer”. After ovulation, the oocytes and follicle layers were collected.

### **Preparations of Extracts and Culture Media for Immunoblotting**

Except where indicated, whole ovaries, dissected follicles, isolated oocytes, and follicle layer tissues were homogenized in phosphate-buffered saline (PBS) and centrifuged at 13,000 *g* for 10 min to obtain supernatant fractions and precipitates. The supernatants were used directly for blotting, whereas the precipitate fractions were further boiled in 1% SDS and centrifuged at 13,000 *g* for 10 min, and then the resulting supernatants were used. The *in vitro* follicle culture media were concentrated before use. Samples or immune precipitates were analyzed by Western blot analysis. The primary antibodies were affinity-purified antibodies produced using blot membranes or anti-human TIMP-2 monoclonal antibody (Santa Cruz Biotechnology, Inc.).

### **Immunoprecipitation and Zymography**

Ovarian follicle extracts were prepared as described above. Protein G-Sepharose

beads were treated with buffer A (20 mM Tris·HCl (pH 8.0), 0.15 M NaCl, and 0.05% Tween-20) containing 0.1% BSA for 30 min at 4°C. After washing the beads with buffer A, they were incubated with purified antibodies for 1 h at 4°C and washed with buffer B (50 mM Tris·HCl (pH 8.0), 0.15 M NaCl, 1% Triton X-100, and 0.1% SDS). The beads were then incubated at 4°C for 16 h with the extracts or *in vitro* ovulation culture media that had been added to the same volume of 2× buffer B. The beads were washed four times, incubated with SDS-sample buffer without β-mercaptoethanol for 6 h at room temperature, and analyzed by gelatin zymography.

Cloning, RT-PCR, *in situ* hybridization, recombinant protein preparation, enzyme assays, antibody generation, immunohistochemistry, and the medaka type I collagen isolation procedures are described in *Supporting Methods*.

## **Results**

### **Metalloproteinase Inhibitors Inhibit *In Vitro* Ovulation.**

Follicles isolated at various time points from the ovaries of the medaka were cultured at 26–27°C in order to determine the rate of ovulation. Throughout the study, we defined the time of ovulation as the point at which the fish were expected to ovulate *in vivo*: this corresponded to the start of the light period and was set to 0 h. Follicles that were isolated 2–6 h before the predicted time of ovulation ovulated *in vitro* (Fig. 1A). When follicles were isolated 7 h before the predicted onset of ovulation (that is, at –7 h), the ovulation rate was reduced. Ovulated oocytes were readily recognized by the appearance of freed attaching filaments on their surface (Fig. 1B). Under these conditions, breakdown of the germinal vesicle occurred in almost all of the ovulated oocytes examined. Ovulated oocytes were successfully fertilized and could

subsequently develop into adults; approximately 75% of the fertilized oocytes developed normally to hatching (data not shown). Based on these observations, the *in vitro* ovulation experiments in this study were conducted using follicles isolated 3 or 6 h before the onset of the light phase.

EDTA, *o*-phenanthroline, tumor necrosis factor- $\alpha$  protease inhibitor (TAPI-1), TAPI-2, and GM6001, all of which have been shown to be inhibitors of MMPs, drastically reduced the rate of ovulation (Fig. 1C). Follicles cultured in the presence of the inhibitors had apparently normal morphology (data not shown). When EDTA was included in the tissue cultures between 0 and 4 h, ovulation was completely suppressed (Fig. 1D). In addition, ovulation was almost completely suppressed when actinomycin D or cycloheximide was added between -5 and 0 h (Fig. 1E). These results indicated that the synthesis of mRNA and protein is required for follicle rupture during ovulation. The results also indicate that MMPs play important roles in the proteolytic events associated with the ovulatory process in the medaka.

### **Medaka Ovarian Follicles Express at Least Seven Species of MMPs.**

In order to search for candidate MMPs that are involved in follicle rupture during ovulation in the fish, RT-PCR was performed with two degenerate oligonucleotide primers using total RNA isolated from fish ovaries at the time of ovulation. Subcloning of the amplified cDNA fragments and subsequent analyses identified seven distinct medaka MMPs: *gelatinase A* (AB033754), *gelatinase B* (AB033755), *stromelysin-3* (AB055705), *MT1-MMP* (AB185847), *MT2-MMP* (AB072928), *MT3-MMP* (AB072929) and *MT5-MMP* (AB047650). *In situ* hybridization analysis revealed that *gelatinase A*, *MT1-MMP*, *MT3-MMP*, *MT5-MMP*, and *stromelysin-3* mRNA were

expressed in small, growing oocytes, while *gelatinase B* and *MT2-MMP* mRNA were localized in the tissues ovulated follicles (Fig. 6, *Supporting Information*). The results regarding *gelatinase A*, *gelatinase B*, *MT5-MMP*, and *stromelysin-3* were consistent with the findings of our previous reports (27-29).

### **Effects of the Medaka MMPs on Type I and Type IV Collagens.**

Active recombinant proteins of the seven MMPs were synthesized as fusion proteins, and the MT-MMPs were produced without cytoplasmic and transmembrane domains. All of the MMP preparations yielded a single protein band on SDS-PAGE under reducing conditions (Fig. 7A, *Supporting Information*). The gelatin-hydrolyzing activities of the MMPs were detected using gelatin zymography (Fig. 7B, *Supporting Information*).

Follicle rupture during ovulation is thought to result from dissolution of the collagen fibers in the tunica albuginea and the theca externa, and the major species present in the connective tissue matrix are believed to be type I and type IV collagen. Therefore, we examined the effects of the medaka MMPs on medaka type I collagen. MT2-MMP degraded the collagen most rapidly (Fig. 2A), although hydrolysis was also observed with MT5-MMP. Medaka type I collagen was resistant to gelatinases A and B, MT1-MMP, MT3-MMP, and stromelysin-3. Because we were unable to test medaka type IV collagen due to the difficulty of obtaining this protein substrate in sufficient quantities, collagen of mammalian origin was used. Medaka gelatinases A and B were able to hydrolyze bovine type IV collagen (Fig. 2B).

We also tested whether gelatinases A and B could act as substrates for MT-MMPs. For this purpose, recombinant proenzymes of the gelatinases were prepared. Incubation

of progelatinase A with active MT1-MMP, MT2-MMP, and MT3-MMP resulted in the conversion of the proenzyme (68 kDa) to the mature enzyme (52 kDa) via a 55-kDa intermediate (Fig. 7C, *Supporting Information*). MT5-MMP did not show any detectable activity towards progelatinase A. None of the MT-MMPs tested were able to activate progelatinase B (data not shown).

### **Ovulating Follicles Express MMP Proteins in Distinct Temporal Patterns.**

In order to detect MMPs in the fish ovary, we prepared antibodies against the seven MMPs. Western blot analysis of the fish ovary extracts indicated that the fish ovary expresses gelatinases A and B, MT1-MMP, MT2-MMP, MT5-MMP, and stromelysin-3, whereas MT3-MMP was not detected at any of the levels of significance examined here (Fig. 8, *Supporting Information*). Immunohistochemical staining with antibodies for gelatinase A, MT1-MMP, MT5-MMP, and stromelysin-3 produced signals in the oocyte cytoplasm of small growing follicles (Fig. 9, *Supporting Information*). In contrast, anti-gelatinase B and anti-MT2-MMP antibodies revealed the presence of their respective antigens in the follicular tissue just after oocyte release. These results were consistent with those from the *in situ* hybridization experiments (Fig. 6, *Supporting Information*). Although we attempted to localize MT3-MMP immunohistochemically in the ovary sections, no clear signals were observed. This finding provided further support for the view that MT3-MMP mRNA is not translated sufficiently in the oocyte to permit the detection of the corresponding protein.

In order to gain a better understanding of the relationship between ovulation and MMPs, changes in the expression of the six MMPs during the course of *in vitro* ovulation were investigated using immunoprecipitation/Western blotting or Western

blotting with specific antibodies. Using the extracts of 20 isolated follicles that were ready to ovulate, immunoreactive materials were detected with antibodies to gelatinase A, gelatinase B, MT1-MMP, or MT2-MMP (Fig. 3A), but not with antibodies to MT5-MMP or stromelysin-3 (data not shown). Zymographic analyses of the immunoprecipitates revealed that the oocyte extract and the culture medium contained gelatinase A in two forms: 62- and 55-kDa proteins (Fig. 3B, *Upper Two Panels*). To clarify the relationship between these two forms of gelatinase A, we examined the effects of *p*-aminophenylmercuric acetate (APMA), which is a well-known activator of secreted MMPs (30), on these proteins. Treatment with this compound resulted in a strong reduction in the 62-kDa band, with concomitant appearance of a 59-kDa band in the oocyte extract and culture medium (Fig. 3C, *Upper Panel*). In addition, the 55-kDa protein remained present after APMA treatment. These results demonstrated that the 62-, 59-, and 55-kDa polypeptides are the pro-protein, intermediate protein, and mature active forms of gelatinase A, respectively.

Immunoprecipitation followed by zymographic analyses for gelatinase B showed that the follicular layers expressed the 83-kDa polypeptide alone intracellularly, whereas the follicle culture medium contained an additional 71-kDa polypeptide (Fig. 3B). The results of APMA treatment confirmed that these were the pro- and active mature enzymes, respectively (Fig. 3C).

Based on these results, changes in the intracellular and extracellular levels of pro- and mature active MMPs during the course of ovulation can be summarized as shown in Figure 3D, assuming that the MT-MMPs that are detectable in the tissue extracts are predominantly in the active form. In fact, the sizes of the polypeptides detected using antibodies specific for MT1-MMP and MT2-MMP were in good agreement with the

theoretically predicted sizes. Since gelatinase A, gelatinase B, MT1-MMP, and MT2-MMP were present in an active enzymatic form in the follicles at the time of ovulation, these four MMPs may play a role in follicle rupture during ovulation.

### **Specific Antibodies for Gelatinase A, MT1-MMP, and MT2-MMP Reduce the Rate of *In Vitro* Ovulation.**

We found that the addition of rabbit sera to the system caused the complete suppression of ovulation, regardless of whether antisera or non-immune control sera were used (data not shown). This was presumably due to protease inhibitors that were present in the sera in large quantities. Therefore, we purified IgGs and specific antibodies in order to isolate the serum protease inhibitors for further investigation. We confirmed that IgG fractions containing anti-MMP antibodies inhibited the enzyme activities of the respective antigens by more than 70% *in vitro*, using purified recombinant MMPs and FITC-labeled porcine gelatin type I as a substrate (Fig. 10, *Supporting Information*). Incubation of the follicles with anti-gelatinase A, anti-MT1-MMP, and anti-MT2-MMP antibodies resulted in a marked reduction of ovulation (Fig. 4A). The addition of antibodies together with the respective antigens to the culture nullified the ovulation-suppressing activities of the antibodies. Anti-gelatinase B and anti-MT3-MMP antibodies were found to exert no effect on the rate of ovulation. The combination of two MMP antibodies inhibited *in vitro* ovulation, although the extent of inhibition was similar to that produced by only one type of antibody alone. This was also the case for the combined effect of three MMP antibodies. These results indicate that gelatinase A, MT1-MMP, and MT2-MMP play important roles during *in vitro* ovulation in the medaka.

## **Regulation of Gelatinase A Activity by MT1-MMP and TIMP-2b in Cultured Follicles**

When follicles isolated at –6 h of ovulation were cultured in the presence of GM6001 or anti-MT1-MMP antibody for 5 h, the production of active gelatinase A was markedly reduced (Fig. 4B). The generation of active gelatinase A during the 5-h incubation period was not affected by the addition of anti-MT3-MMP antibody. The results revealed that MT1-MMP plays a role in the activation of progelatinase A in culture.

We next investigated how active gelatinase A generated during the pre-ovulation period was secreted into the medium without leading to marked degradation of the basement membrane. One plausible explanation for the absence of degradation was that intrinsic MMP inhibitors might inhibit the activity of gelatinase A. To test this idea, we isolated three TIMP cDNA clones—TIMP-2a (AB185849), TIMP-2b (AB193468), and TIMP-3 (AB193469)—from medaka ovary mRNAs. Among these clones, TIMP-2b mRNA was found to be the predominant transcript in fully-grown oocytes (Fig. 11, *Supporting Information*). We therefore focused on the role of TIMP-2b in the regulation of gelatinase A activity. The oocyte extracts and the culture medium of ovulating follicles contained TIMP-2b with an Mr of 28 kDa (Fig. 4C, *Upper Panel*; Fig. 12, *Supporting Information*). The maximal TIMP-2b band intensity was detected in the culture medium at –2 h of ovulation, and the signals from subsequent samples examined at 2-h intervals became steadily weaker. These results thus confirmed that the TIMP-2b protein was present in ovulating follicles and was most actively secreted from the oocytes of *in vitro*-cultured follicles during the pre-ovulation period. We further

analyzed the anti-TIMP-2b immunoprecipitates of the culture medium using Western blot analysis and gelatin zymography. A 65-kDa polypeptide was identified (under reducing conditions) using anti-gelatinase-A antibody (Fig. 4C, *Middle Panel*), and two polypeptides of 62 and 55 kDa were detected (under non-reducing conditions) by examining the gelatinolytic activity (Fig. 4C, *Lower Panel*). These results demonstrated the presence of gelatinase A (and progelatinase A) in complex with TIMP-2b in the *in vitro* ovulation culture medium.

In summary, our results demonstrated that TIMP-2b is expressed in ovulating follicles and reaches a maximum concentration during the preovulation period. These findings strongly suggest that this intrinsic inhibitor regulates gelatinase A activity at or around the time of ovulation.

## **DISCUSSION**

In 1916, Schochet (1) first noted that “the rupture of the Graafian follicle is due in part to the digestion of the theca folliculi by a proteolytic ferment or enzyme in the liquor folliculi.” Since then, intense efforts have been made to identify the proteases that are essential for follicle rupture during ovulation, primarily in mammalian species. Although the identification of proteases responsible for follicle rupture remains to be achieved, previous studies have strongly suggested that this process is accomplished by the cooperative effects of several MMPs and their inhibitors (4, 5). Our current findings strongly suggested that this is indeed the case.

The present study indicated that gelatinase A, MT1-MMP, and MT2-MMP play important roles in the process of follicle rupture during ovulation in the medaka. On the basis of our data, we can propose a mechanistic model of this process, implicating these

MMPs together with the intrinsic inhibitor TIMP-2b (Fig. 5). Namely, in the follicle that is destined to ovulate, the synthesis of progelatinase A and MT1-MMP begins in the oocyte at some point prior to ovulation. At the same time, the oocyte produces TIMP-2b. When MT1-MMP has been activated intracellularly and is localized on the surface of the oocyte, this membrane-bound MMP may interact with secreted progelatinase A and TIMP-2b to form the progelatinase A/MT1-MMP/TIMP-2b complex, which is well documented as the unique mechanism for progelatinase A activation in mammals (30-32). Progelatinase A activation in the follicles of the medaka ovary appears to occur in a similar manner. The present model is supported by the finding that the activation of progelatinase A in the culture medium of ovarian follicles was noticeably inhibited by the addition of anti-MT1-MMP antibodies. The expression of furin, which is likely to be involved in the activation of MT1-MMP, has recently been reported in the oocytes of ovarian follicles of the medaka (33). Active gelatinase A produced in this manner may be inhibited until the time of ovulation by the formation of a complex with TIMP-2b; this inhibition is thought to be due to a presumed excess of the inhibitor compared to the amount of enzyme (Fig. 5A). At the time of ovulation, the transcription and translation of MT2-MMP and gelatinase B are initiated in the granulosa cells of the ovulating follicle. The former may undergo furin-catalyzed intracellular activation, and may subsequently be distributed over the surface of the cells, while the latter is secreted from the same cells into the extracellular space in an inactive precursor form. Increased production of active gelatinase A and gelatinase B together with a decrease in TIMP-2b at, or around, the time of ovulation may reverse the molar ratio of MMP/TIMP-2b compared with that of the pre-ovulation period, resulting in the release of MMP from inhibition by TIMP-2b. Unlike MT1-MMP and MT2-MMP, gelatinase A is able to

degrade type VI collagen *in vitro*. Therefore, the basement membrane situated between the granulosa and theca cell layers might be a target substrate for this MMP (Fig. 5B). Although medaka gelatinase B was found to hydrolyze type IV collagen more rapidly than gelatinase A, the results of our *in vitro* ovulation experiments using specific antibodies indicated that the former does not participate in basement-membrane degradation. Basement-membrane degradation resulting from the activity of gelatinase A allows the granulosa cells to come into contact with the ECM in the interstitial space, where the theca cells are localized. As MT2-MMP hydrolyzed medaka collagen type I *in vitro* (Fig. 3), and because this type of collagen is thought to be a major ECM protein in this region, the MT2-MMP expressed on the surface membrane of the granulosa cells is thought to be responsible for the dissolution of the collagen (Fig. 5C). It should be noted that MT2-MMP may play an additional role in the activation of progelatinase A, as we demonstrated here that it was able to activate the precursor gelatinase *in vitro*. Follicle rupture during ovulation in the medaka does not require extensive ECM degradation throughout the layer surrounding the oocyte. As is the case in mammalian ovulation (2), hydrolysis of the ECM proteins at a single restricted site is expected to be sufficient for ovulation in the medaka. Once this local hydrolysis has occurred, the subsequent process appears to proceed spontaneously (Fig. 5D). Clearly, the mechanical force generated by the swelling of the ovulating oocyte facilitates ovulation. However, the mechanism and regulation of this restricted hydrolysis is still unknown. We should also note that externally added active recombinant MMPs, including gelatinase A and MT2-MMP, were found to have no accelerating effect on *in vitro* ovulation. This finding may indicate the importance of well-regulated molecular interactions between the intrinsic MMPs, TIMP-2b, and ECM proteins in follicle rupture. In summary, upon

ovulation, gelatinase A is responsible for the hydrolysis of type IV collagen that forms the basement membrane, while MT2-MMP degrades type I collagen present in the theca cell layer. Moreover, MT1-MMP and TIMP-2b are both involved in the activation of progelatinase A and the regulation of its activity.

We believe that the present study identifies, for the first time, proteases that are critical for follicle rupture during ovulation in vertebrates. The use of similar experimental systems established for mammalian ovaries (34-37) will most likely aid in the determination of the enzymes that facilitate follicle rupture in mammals. The eventual identification of mammalian ovulatory proteases will provide information that is of potential clinical importance. Our current approach and findings are expected to be useful for future studies in this area.

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### **References**

1. Schochet, S. S. (1916) *Anat. Rec.* **10**, 447-457.
2. Espey, L. L. (1967) *Am. J. Physiol.* **212**, 1397-1401.
3. Tsafiriri, A. (1995) *Adv. Exp. Med. Biol.* **377**, 121-140.
4. Richards, J. (2001) *Endocrinology* **142**, 2184-2193.

5. Ny, T., Wahlberg, P. & Brändström, I. J. M. (2002) *Mol. Cell. Endocr.* **187**, 29-38.
6. Beers, W. H. (1975) *Cell* **6**, 379-386.
7. Murdoch, W. J. & McDonnell, A. C. (2002) *Reproduction* **123**, 743-750.
8. Curry, T. E. Jr., Dean, D. D., Woessner, J. F. Jr., & LeMaire, W. J. (1985) *Biol. Reprod.* **33**, 981-991.
9. Liu, K., Wahlberg, P. & Ny, T. (1998) *Endocrinology* **139**, 4735-4738.
10. Hagglund, A. C., Ny, A., Leonardsson, G. & Ny, T. (1999) *Endocrinology* **140**, 4351-4358.
11. Jo, M. & Curry, T. E. Jr. (2004) *Biol. Reprod.* **71**, 1796-1806.
12. Pendás, A. M., Folgueras, A. R., Llano, E., Caterina, J., Frerard, F., Rodríguez, F., Astudillo, A., Noël, A., Birkedal-Hansen, H. & López-Otín, C. (2004) *Mol. Cell Biol.* **24**, 5304-5313.
13. Holmbeck, K., Bianco, P., Caterina, J., Yamada, S., Kromer, M., Kuznetsov, S. A., Mankani, M., Robey, P. G., Poole, A. R., Piodoux, I. *et al.* (1999) *Cell* **99**, 81-92.
14. Peschon, J. J., Slack, J. L., Reddy, P., Stocking, K. L., Sunnarborg, S. W., Lee, D. C., Russell, W. E., Nelson, N., Kozlosky, C. J., Wolfson, M. F. *et al.* (1998) *Science* **282**, 1281-1284.48.
15. Robker, R. L., Russell, D. L., Espey, L. L., Lydon, J. P., O'Malley, B. W. & Richards, J. S. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 4689-4694.
16. Shindo, T., Kurihara, H., Kuno, K., Yokoyama, H., Wada, T., Kurihara, Y., Imai, T., Wang, Y., Ogata, M, Nishimatsu, H. *et al.* (2000) *J. Clin. Invest.* **105**, 1345-1352.
17. Russell, D. L., Doyle, K. M., Ochsner, S. A., Sandy, J. D. & Richards, J. S. (2003) *J. Biol. Chem.* **278**, 42330-42338.
18. Schroeder, P. C. & Talbot, P. (1985) *Gamete Res.* **11**, 191-221.

19. Richards, J. (1994) *Endocr. Rev.* **15**, 725-751.
20. Nagahama, Y., Yoshikuni, M., Yamashita, M. & Tanaka, M. (1994) in *Fish Physiology*, eds. Sherwood, N. M. & Hew, C. L. (Academic, New York), pp. 393-439.
21. Ishikawa, Y. (2000) *BioEssays* **22**, 487-495.
22. Wittbrodt, J., Shima, A. & Scharl, M. (2002) *Nat. Rev. Genet.* **3**, 53-64.
23. Iwamatsu, T. (1978) *J. Exp. Zool.* **206**, 355-364.
24. Hirose, K. & Donaldson, E. (1972) *Bull. Jap. Soc. Scient. Fish.* **38**, 97-100.
25. Pendergrass, P. & Schroeder, P. (1976) *J. Reprod. Fert.* **47**, 229-233.
26. Shibata, Y., Iwamatsu, T., Oba, Y., Kobayashi, D., Tanaka, M., Nagahama, Y., Suzuki, N. & Yoshikuni, M. (2000) *J. Biol. Chem.* **275**, 8349-8354.
27. Matsui, H., Ogiwara, K., Ohkura, R., Yamashita, M. & Takahashi, T. (2000) *Eur. J. Biochem.* **267**, 4658-4667.
28. Kimura, A., Shinohara, M., Ohkura, R. & Takahashi, T. (2001) *Biochim. Biophys. Acta* **1518**, 115-123.
29. Ogiwara, K., Matsui, H., Kimura, A. & Takahashi, T. (2002) *Mol. Reprod. Develop.* **61**, 21-31.
30. Nagase, H. (1997) *Biol. Chem.* **378**, 151-160.
31. Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E. & Seiki, M. (1994) *Nature* **370**, 61-65.
32. Brew, K., Dinakarandian, D. & Nagase, H. (2000) *Biochim. Biophys. Acta* **1477**, 267-283.
33. Ogiwara, K., Shinohara, M. & Takahashi, T. (2004) *Gene* **337**, 79-89.
34. Baranczuk, R. J. & Fainstat, T. (1976) *Am. J. Obstet. Gynecol.* **124**, 517-522.

35. Talbot, P. (1983) *J. Exp. Zool.* **225**, 141-148.
36. Osman, P. & Lieuwma-Noordanus, C. (1980) *J. Reprod. Fert.* **59**, 431-436.
37. Rose, U. M., Hanssen, R. G. J. M. & Kloosterboer, H.J. (1999) *Biol. Reprod.* **61**, 503-511.

## Figure legends

**Fig. 1.** *In vitro* ovulation in medaka and the effects of various chemicals.

(**A**) Follicles obtained from mature medaka ovaries at various pre-ovulation times were incubated. The ovulation rates were determined, and the results are shown as the means of at least five experiments. (**B**) Follicles collected at –6 h were incubated. Photographs were taken at –2 and 4 h of ovulation. (**C**) Follicles collected at –3 h of ovulation were incubated with various protease inhibitors, and the ovulation rates were determined at 4 h. The results are shown as the means ( $\pm$  SD) of at least five experiments. (**D**) Follicles were collected at –3 h for *in vitro* ovulation. The timing of EDTA treatments (2 mM) is indicated by the thick bars in the left panel. The initiation and termination of the treatments were accomplished by rapid exchanges of the culture medium. The ovulation rates were determined at 4 h of ovulation. The results are presented as the means ( $\pm$  SD) of at least five experiments. (**E**) Follicles were collected and incubated at –5 h. Actinomycin D (1  $\mu$ g/ml) or cycloheximide (1  $\mu$ g/ml) was added to the culture. The timing of the treatments is indicated by the thick bars in the left panel. The other details remained as described above.

**Fig. 2.** Hydrolysis of collagens by recombinant medaka MMPs.

(**A**) Type I collagen purified from the whole body of the medaka fish was incubated with purified MMPs, and degradation of the collagen was analyzed by SDS-PAGE (reducing conditions). The results of control experiments without enzymes, but with medaka collagen (None) and bovine type I collagen, are also shown. Asterisks indicate the bands of recombinant enzymes that were added to the incubations. (**B**) FITC-labeled bovine type IV collagen was used as substrate for the enzymatic activity of the purified MMPs.

**Fig. 3.** Expression of MMPs in ovarian follicles.

(A) Mature ovarian follicles were isolated at -6 h of ovulation and cultured *in vitro*. At the indicated times, the oocytes, follicular layers, and medium were obtained separately. The oocyte extracts were immunoprecipitated with rabbit anti-medaka gelatinase A antibodies, and the resulting precipitates were analyzed by Western blot analysis with rat anti-medaka gelatinase A antibodies. Extracts of the oocytes and follicle layer, and the concentrated media were used directly for the Western blot analysis. The sizes (kDa) of the proteins are indicated. (B) The oocyte extracts and media were immunoprecipitated with rabbit anti-gelatinase A antibodies and the immunoprecipitated materials were analyzed using gelatin zymography (*Upper Two Panels*). Extracts of the follicular layers and medium were immunoprecipitated with rabbit anti-gelatinase B antibodies, and the immunoprecipitated materials were analyzed by gelatin zymography (*Lower Two Panels*). (C) The *in vitro* follicle culture medium at 2 h of ovulation was concentrated, treated with 1 mM APMA at 4°C overnight, and immunoprecipitated with anti-gelatinase A antibodies. The immunoprecipitated materials were then subjected to gelatin zymographic analysis (*Upper Panel*). Extracts of the follicular layers at 2 h of ovulation were treated with APMA, as described above, and were then immunoprecipitated with anti-gelatinase B antibodies. The immunoprecipitates were analyzed using gelatin zymography (*Lower Panel*). The sizes of the proteins are indicated. (D) The relative amounts of inactive proenzyme and active mature enzyme at each time point were calculated, based on the results shown in (A) and (B).

**Fig. 4.** Roles of MMPs and TIMP-2b in follicle rupture.

(A) Follicles were incubated with pre-immune rabbit IgG (control) or specific antibodies or specific antibodies + antigens. The results are presented as the means ( $\pm$  SD) of five separate experiments. (B) Follicles isolated at  $-6$  h were incubated with GM6001, anti-MT1-MMP, or anti-MT3-MMP antibodies. The medium collected at 0 h was immunoprecipitated with anti-gelatinase A antibodies, and the precipitates were analyzed using gelatin zymography (*Upper Panel*). Progelatinase A (62 kDa) and activated gelatinase A (55 kDa) are indicated. The intensities of the bands shown in the upper panel were quantified by densitometry, and the ratio of gelatinase A to progelatinase A in each treatment was determined (*Lower Panel*). The results are presented as the means ( $\pm$  SD) of three experiments. (C) Follicles isolated at  $-6$  h were incubated. Culture media derived from the pre-ovulation period between  $-5$  and 0 h ( $-5/0$ ) and from the post-ovulation period between 0 and 4 h ( $0/4$ ) were obtained. Immunoprecipitation was conducted using anti-medaka TIMP-2b antibodies, and the precipitates were analyzed by Western blotting with mouse anti-human TIMP-2 monoclonal antibody (*Upper Panel*) or anti-gelatinase A antibody (*Middle Ppanel*), or by gelatin zymography (*Lower Ppanel*).

**Fig. 5.** A Model of Follicle Rupture during Ovulation in the Medaka.

(A) In the follicle, a few hours before ovulation, progelatinase A is activated by MT1-MMP on the surface of the oocyte, whereas activated gelatinase A is immediately inactivated by TIMP-2b. (B) At the time of ovulation, the hydrolysis of basement membrane type IV collagen is initiated by active gelatinase A at the follicle–ovarian surface contact site. (C) MT2-MMP, which is now expressed on the surface of the

granulosa cells, can degrade the type I collagen that is present in the theca cell layer. (**D**)  
As a result, the oocyte is exposed at the contact site, leading to ovulation. BM, basement membrane; EC, epithelial cell; GC, granulosa cell; OC, oocyte; TC, theca cell.

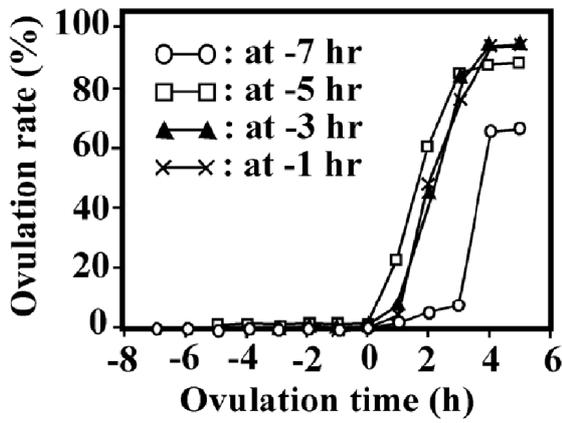
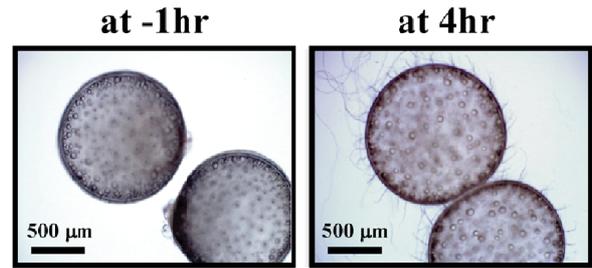
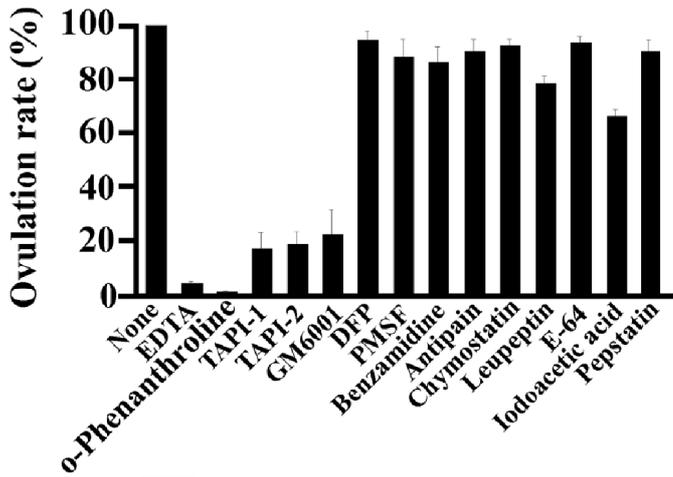
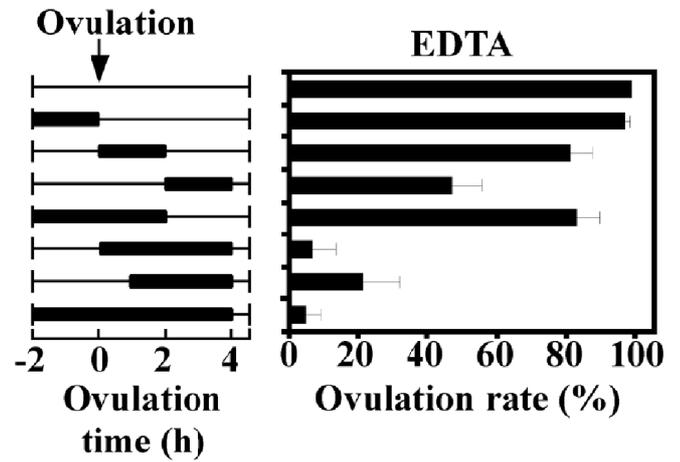
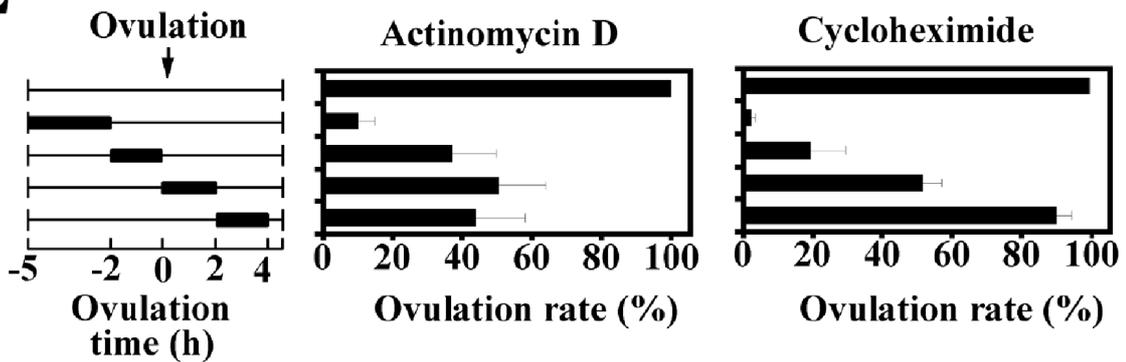
**A****B****C****D****E**

Figure 1  
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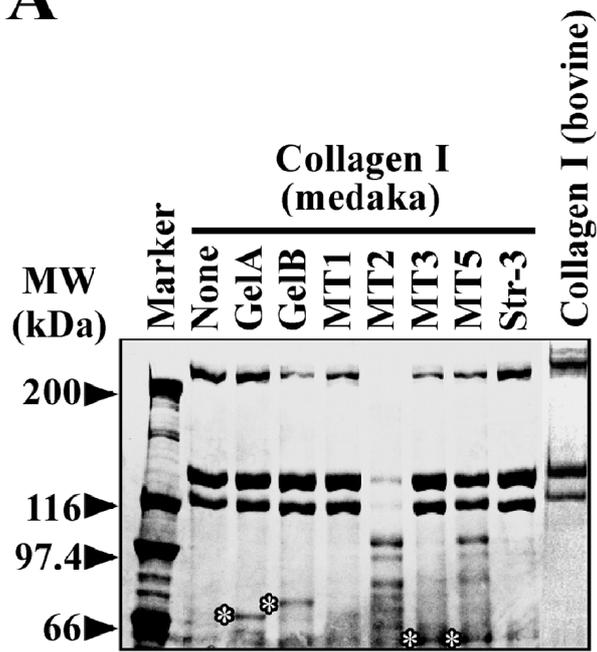
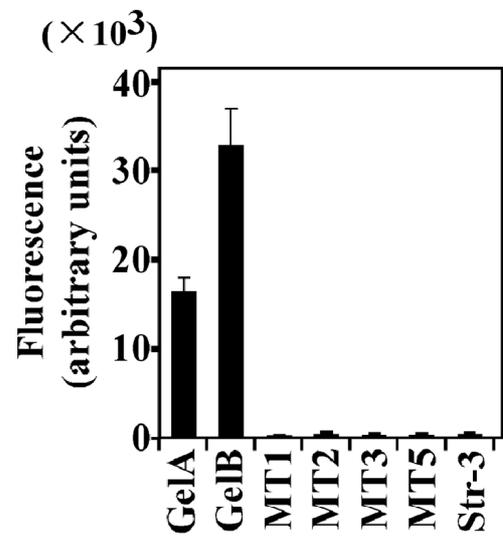
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Figure 2  
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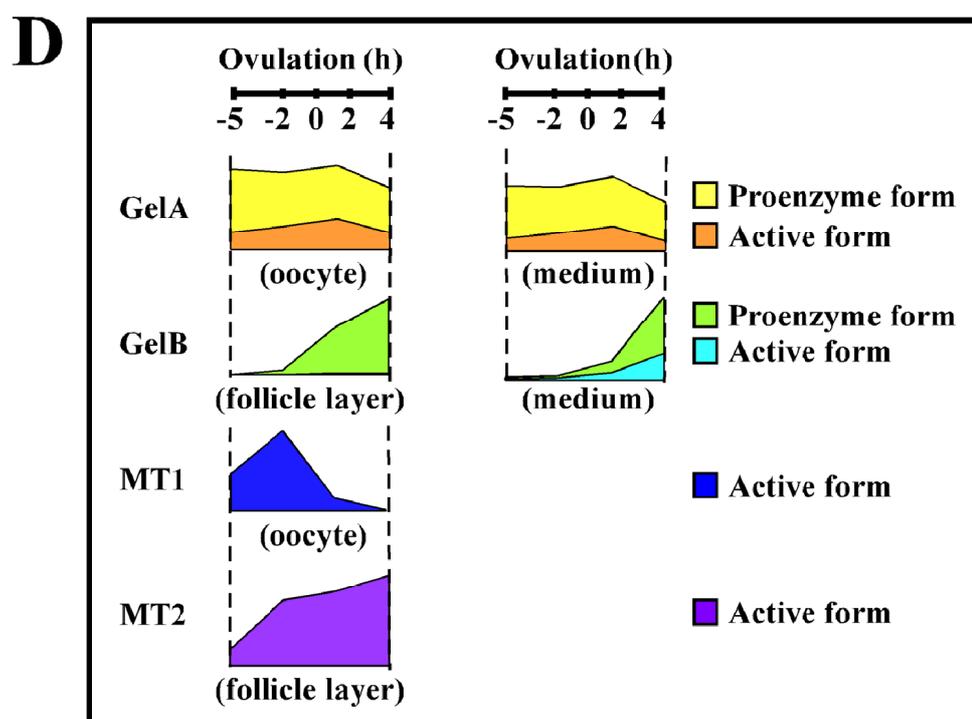
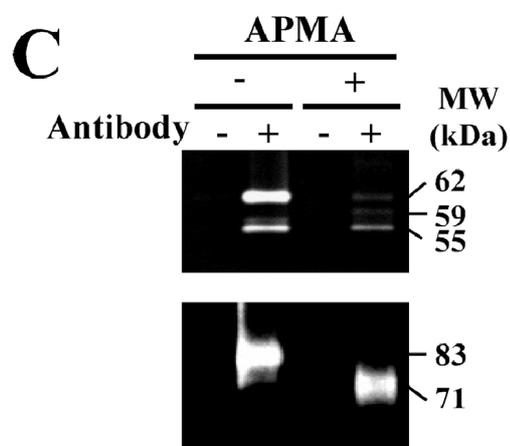
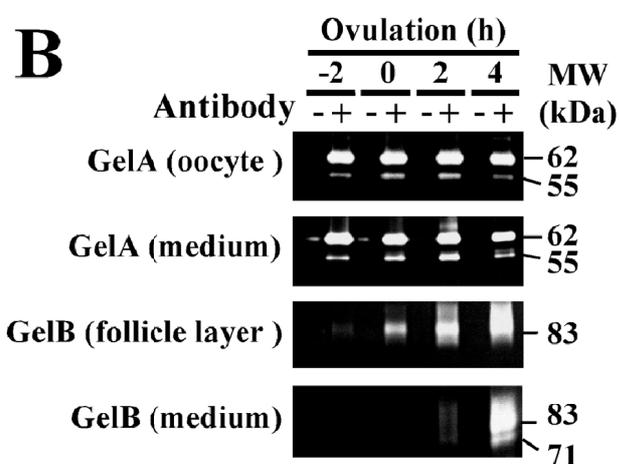
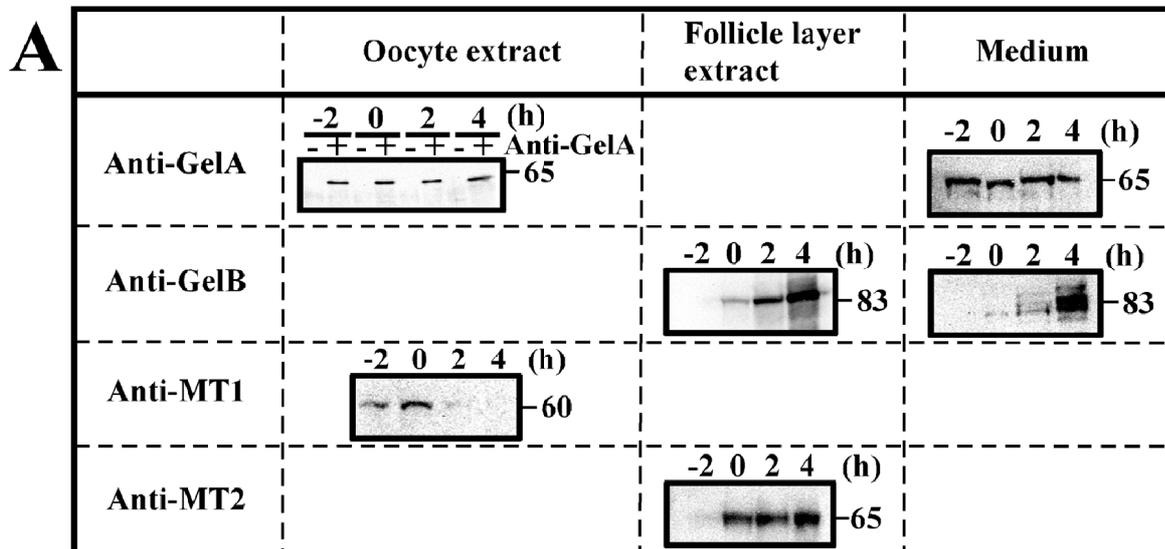


Figure 3  
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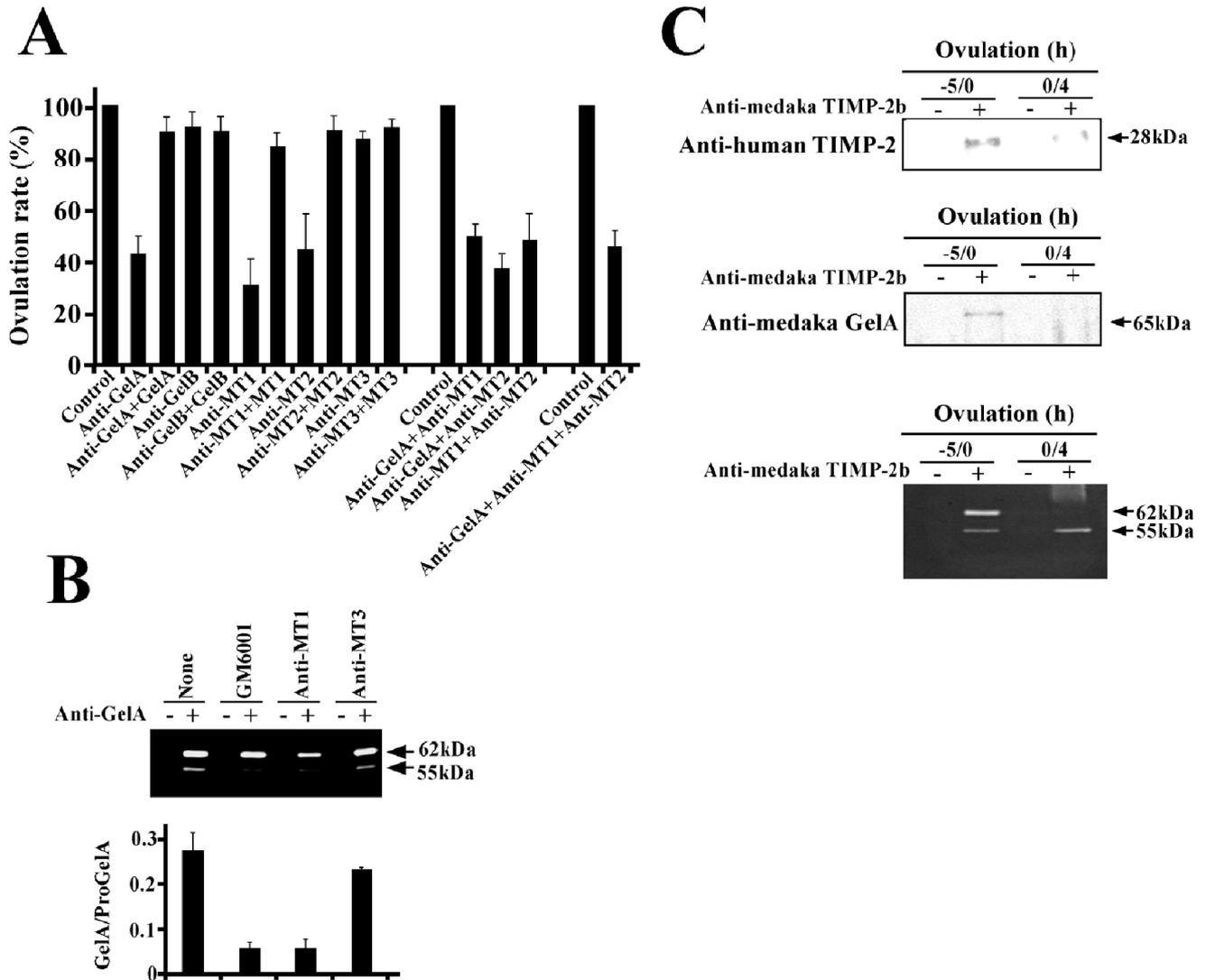


Figure 4  
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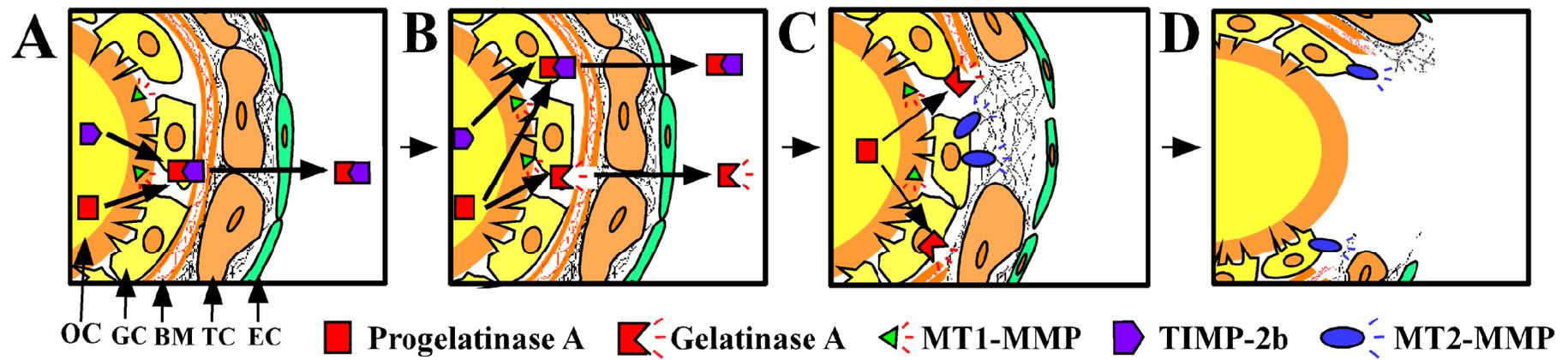


Figure 5  
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## **Supporting Information**

### **Supporting Methods**

#### **Cloning of Various Medaka cDNAs**

RNA was isolated from the medaka ovary (Nippon Gene). The first-strand cDNA synthesis was performed using a SuperScript First-Strand Synthesis System (Life Technologies, Inc.). Full-length cDNAs were obtained by initial PCR amplification of fragments using two degenerate oligonucleotide primers followed by library screening or 5'/3'-RACE. Primers and cloning strategies are described in Tables 1 and 2.

#### **RT-PCR**

Total RNAs were prepared from various tissues and treated with DNase (Roche Molecular Biochemicals). Aliquots of 2  $\mu$ g of the treated RNAs were used for reverse transcription. PCR was performed for 25 or 30 cycles using Ex *Taq* DNA polymerase (Takara). The primers and amplified products are summarized in Table 3.

#### ***In Situ* Hybridization**

*In situ* hybridization was performed using frozen ovary sections (15  $\mu$ m) as described previously (1). RNA probes were prepared by *in vitro* transcription of reverse-transcriptase fragments of cDNAs with T3 or T7 RNA polymerase using a digoxigenin (DIG) RNA-labeling kit (Boehringer-Mannheim). The regions and sizes of the specific probes used are described in Table 3. The hybridization was conducted at 50°C for 18 h in 50% formamide, 5 $\times$  Denhardt's solution, 6 $\times$  SSPE, and 0.5 mg/ml

yeast transfer RNA (for gelatinase A, gelatinase B, MT1-MMP, MT3-MMP, and stromelysin-3) or 50% formamide, 0.5 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10% dextran sulfate (Wako), 1× Denhardt's solution, 0.25% SDS, and 0.2 mg/ml yeast transfer RNA (for MT2-MMP, MT5-MMP, and TIMP-2b). The sections were washed at 50°C in 50% formamide/2× SSC for 30 min, washed once in 2× SSC for 20 min and twice in 0.2× SSC for 20 min at 50°C. The hybridization probes were detected using a Dig Nucleic Acid Detection Kit (Roche Molecular Biochemicals).

### **Preparation of Recombinant Proteins**

Using the pET30a expression vector (Novagen) in the *Escherichia coli* expression system, recombinant proteins were produced as a fusion protein with an extra amino-acid sequence of 37–51 residues (varying with different proteins) at its NH<sub>2</sub>-terminus; the vector-derived amino-terminal stretch contained a His-tag and an S-protein sequence. Harvested cells were lysed and the insoluble materials were dissolved in a solubilization buffer containing 6 M urea, 50 mM Tris-HCl (pH 7.8), and 0.5 M NaCl. Solubilized proteins were subjected to affinity chromatography on Ni<sup>2+</sup>-Sephacel (Amersham Pharmacia Biotech). Eluted recombinant proteins were renatured by dialysis against 50 mM Tris-HCl (pH 8.0). The proteins were further purified in a Mono Q 5/50 GL column (Amersham Pharmacia Biotech). The strategies used for recombinant protein preparation are summarized in Table 4.

The quantities of active enzyme in the purified recombinant MMPs were determined by titrating the samples with GM6001 (Chemicon) according to a previously described method (2). TIMP-2 active-site titration was performed using medaka recombinant gelatinase A, as described previously (3).

## **Enzyme Assays**

Enzyme samples were incubated in a volume of 100  $\mu$ l with FITC-labeled bovine type IV collagen (USBiological) (25  $\mu$ g) in buffer A (50 mM Tris·HCl buffer (pH 7.5) containing 5 mM CaCl<sub>2</sub>, 50  $\mu$ M ZnSO<sub>4</sub>, and 0.02% NaN<sub>3</sub>) for 16 h at 27°C. The reactions were terminated by the addition of 300  $\mu$ l of stopping reagent (42% EtOH, 50 mM Tris·HCl (pH 9.5) and 0.2 M NaCl) and the samples were centrifuged. Aliquots of the resulting supernatants were diluted to a volume of 3.0 ml with distilled water, and the fluorescence was measured at an excitation wavelength of 459 nm and an emission wavelength of 520 nm. Enzyme activities in the presence of native medaka type I collagen were assessed using SDS-PAGE analysis (reducing conditions) of samples incubated with the enzymes in buffer A for 16 h at 27°C. To determine the activity of the FITC-labeled porcine type I gelatin (Molecular Probe), the enzyme samples were incubated at 27°C with the substrate (25  $\mu$ g) in 100  $\mu$ l of buffer A. After 16 h of incubation, the mixtures were filtered using a Viva Spin 0.5-ml Concentrator (molecular weight cutoff <10,000) (Viva Science). Aliquots of 50  $\mu$ l of the filtrate were diluted with 3 ml of distilled water for fluorescence measurement. All incubations were performed at an enzyme:substrate ratio of 1/20–1/100. Gelatin zymography was performed as described previously (1).

## **Isolation of Medaka Type I Collagen**

Type I collagen was isolated from the whole body of a medaka fish according to a previously described method (4). SDS-PAGE of the purified collagen under reducing conditions produced a similar pattern to those of bovine (USBiological) and rainbow trout type I collagens (5), indicating the validity of our isolation procedure.

## **Antibody Generation**

The protein antigens were produced using the bacterial expression system with pET30a. Recombinant proteins eluted from a Ni<sup>2+</sup>-Sepharose column were injected into rabbits or rats. IgGs were purified by affinity chromatography on a protein G-Sepharose column (Amersham Pharmacia Biotech). Specific antibodies were purified by affinity chromatography on a column of antigen coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's protocol. Specific antibodies were also affinity purified using membranes onto which pure antigens were blotted.

## **Immunohistochemistry**

Ovary sections (15  $\mu$ m) were cut on a cryostat and thaw-mounted onto slides coated with silan. Sections on slides that were fixed with 4% paraformaldehyde in PBS or 100% methanol for at least 15 min were treated with 3% H<sub>2</sub>O<sub>2</sub> in PBS. After being blocked with BlockAce (Dainippon Seiyaku) for 1 h at room temperature, each section was incubated with purified primary antibodies for 1 h at room temperature, and was then washed with PBS. Bound antibodies were detected using anti-rabbit IgG (DakoCytomatin EnVision<sup>+</sup> System-labeled polymer-HRP anti-rabbit, Dako), or anti-rat IgG horseradish peroxidase-linked whole antibody (Pharmacia), according to the manufacturer's instructions. Immunocomplexes were detected using an AEC kit (Vector Laboratories).

## **References**

1. Matsui, H., Ogiwara, K., Ohkura, R., Yamashita, M. & Takahashi, T. (2000) *Eur. J. Biochem.* **267**, 4658-4667.

2. Park, H. I., Turk, B. E., Gerkema, F. E., Cantley, L. C. & Sang, Q. X. (2002) *J. Biol. Chem.* **277**, 35168-35175.
3. Willenbrock, F., Crabbe, T., Slocombe, P. M., Sutton, C. W., Docherty, A. J., Cockett, M. I., O'Shea, M., Brocklehurst, K., Phillips, I. R. & Murphy, G. (1993) *Biochemistry* **32**, 4330-4337.
4. Bornstein, P. & Piez, K. A. (1964) *J. Clin. Invest.* **43**, 1813-1823.
5. Saito, M., Sato, K., Kunisaki, N. & Kimura, S. (2000) *Eur. J. Biochem.* **267**, 6943-6950.

**Fig. 6.** *In situ* detection of MMP mRNAs in the medaka ovary. The distributions of seven MMP mRNAs in the medaka ovary were determined by *in situ*-hybridization. Samples were stained with antisense (*Left*) and sense probes (*Right*). The areas or oocytes that showed positive staining are indicated by arrows. Scale bar = 500  $\mu$ m.

**Fig. 7.** Preparation of active recombinant MMPs. (**A**) Purified active MMPs were analyzed by SDS-PAGE under reducing conditions. (**B**) The MMPs were analyzed by gelatin zymography. (**C**) Progelatinase A incubated with MT1-MMP, MT2-MMP, MT3-MMP, and MT5-MMP was analyzed using gelatine zymography. The positions of progelatinase A (68 kDa), the intermediate (55 kDa), and the activated form (52 kDa) are shown. Zymography of progelatinase A and gelatinase A was conducted without incubation in order to determine their mobilities under these conditions (*Right Panel*). Asterisks indicate nonspecifically proteolyzed bands.

**Fig. 8.** Expression of MMP proteins in the medaka ovary. (**A**) Anti-MMP antibodies

were purified and tested for their specificities by Western blot analysis using a membrane loaded with seven recombinant MMP proteins. The S-protein was used to detect all recombinant MMPs. **(B)** Extracts of medaka ovaries taken 1 h after ovulation were analyzed by Western blot analysis using specific MMP antibodies. The sizes of the proteins specifically detected with the antibodies are shown.

**Fig. 9.** Immunohistochemical analysis of MMPs in the medaka ovary. MMPs were immunohistochemically detected in the medaka ovary using purified primary antibodies. The controls were stained with primary antibodies previously treated with the respective antigens (for gelatinase B, MT2-MMP, MT5-MMP, and stromelysin-3), or with diluted preimmune sera (for gelatinase A and MT1-MMP). The areas or oocytes that showed positive staining are indicated by arrows. Scale bar = 500  $\mu\text{m}$ .

**Fig. 10.** Inhibition of recombinant MMP activity by specific antibodies. IgG fractions containing specific antibodies purified through a Protein G-Sepharose 4B column were included in the MMP activity-assay mixtures. The quantity of IgG antibody added amounted to five times (by weight) that of the purified enzymes. The substrate was FITC-labeled porcine type I gelatin. The values given are the average of triplicate assays.

**Fig. 11.** Expression of TIMP-2b mRNA in the medaka ovary. **(A)** RT-PCR for TIMP-2a, TIMP-2b, and TIMP-3 was performed using total RNAs isolated from various tissues of the medaka. Twenty-five PCR cycles were performed. **(B)** RT-PCR for TIMP-2a, TIMP-2b, and TIMP-3 was performed using total RNAs isolated from oocytes of

ovulating follicles at various periovulation time points. Twenty-five PCR cycles were performed. (C) *In situ* hybridization analysis of TIMP-2b mRNA was conducted using the specific probes indicated in Table 3. Staining was performed with antisense (*Left Panel*) and sense probes (*Right Panel*). Specific signals are indicated by arrows. Scale bar = 200  $\mu\text{m}$ .

**Fig. 12.** Detection of TIMP-2b protein using ovulating follicles of the medaka. Follicles isolated at  $-6$  h were incubated in the *in vitro* culture system. At  $-2$ , 0, 2, and 4 h of ovulation, the culture medium was replaced with fresh medium. Oocytes were collected at  $-2$ , 0, 2, and 4 h of ovulation. Oocyte extracts were immunoprecipitated with anti-medaka TIMP-2b antibodies, and the resulting precipitates were analyzed by Western blotting with anti-human TIMP-2 monoclonal antibody (*Upper Panel*). *In vitro* culture media were collected from four samples: those derived from a 3-h incubation between  $-5$  and  $-2$  h ( $-5/-2$  in the *Lower Panel*), and the subsequent 2-h incubations ( $-2/0$ ,  $0/2$ , and  $2/4$  in the *Lower Panel*). Immunoprecipitation and Western blot analyses of the culture media were conducted as described above. Medaka TIMP-2b protein (28 kDa) is shown.

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Table 1. Degenerate Oligonucleotide Primers Used for Initial PCR Amplification of cDNA Fragments

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Gene		Degenerate primer sequence
<i>MMPs</i>	Sense	5'-CCCAGATG(C/T)GG(A/G)GT(T/G)CCTGA(C/T)-3'
	Antisense	5'-TGGCC(A/C/G)A(A/G)TTCATGAGC(A/C/T)GC(A/C)AC-3'
<i>TIMPs</i>	Sense	5'-CA(C/T)CCICA(A/G)(C/G)AIGCITT(C/T)TG(C/T)AA-3'
	Antisense	5'-C(A/C)(A/T)(A/G)TCC(A/G)TCCAIA(A/G/T)(A/G)CA(C/T)TC-3'

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Table 2. Cloning Strategies for Target Genes

Gene	Regions used for degenerate PCR primer design	Cloning method	GenBank accession number
<i>MT1-MMP(MMP-14)</i>	635-1,098	5'/3'-RACE	AB185847
<i>MT2-MMP (MMP-15)</i>	635-1,089	Screening	AB072928
<i>MT3-MMP (MMP-16)</i>	508-960	Screening	AB072929
<i>Gelatinase A (MMP-2)</i>	398-1,318	Screening, 5'-RACE	AB033754
<i>Gelatinase B (MMP-9)</i>	439-1,369	Screening	AB033755
<i>Stromelysin-3 (MMP-11)</i>	346-817	Screening, 5'/3'-RACE	AB055705
<i>MT5-MMP (MMP-24)</i>	350-807	Screening, 5'/3'-RACE	AB047651
<i>TIMP-2a</i>	276-710	3'-RACE	AB185849
<i>TIMP-2b</i>	248-682	5'/3'-RACE	AB193468
<i>TIMP-3</i>	233-649	5'/3'-RACE	AB193469

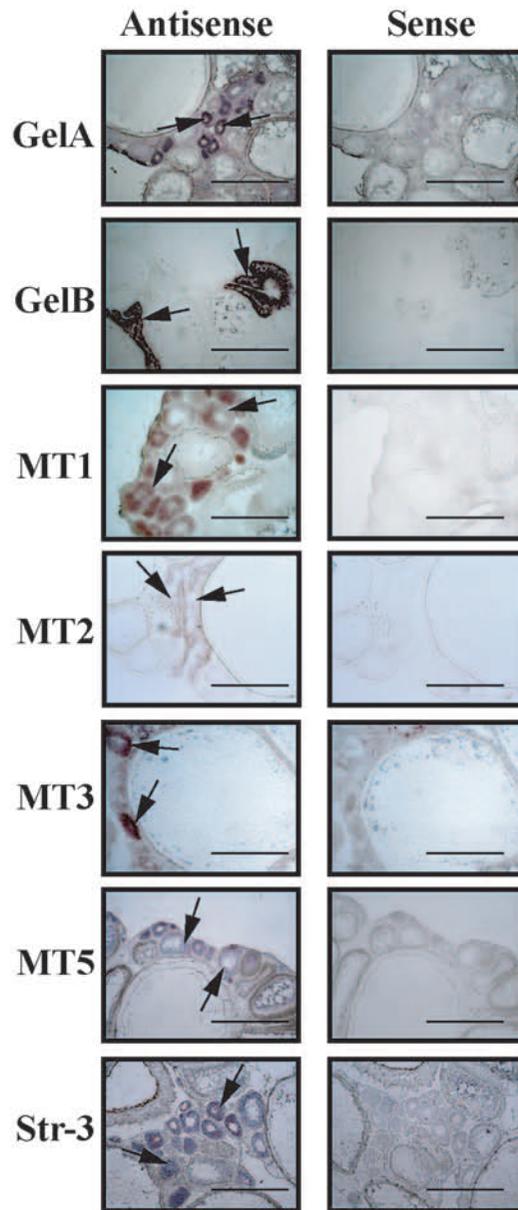
Table 3. PCR Primers Used for the Amplification of Specific DNA Fragments

Gene	Amplified regions in RT-PCR	Probes for in situ hybridization analysis	GenBank accession number
<i>MT1-MMP(MMP-14)</i>	1,827–2,207	2,803–3,211	AB185847
<i>MT2-MMP (MMP-15)</i>	2,036–2,590	1,983–2,409	AB072928
<i>MT3-MMP (MMP-16)</i>	414–972	1,695–2,160	AB072929
<i>Gelatinase A (MMP-2)</i>	2,374–2,683	2,374–2,683	AB033754
<i>Gelatinase B (MMP-9)</i>	2,191–1,482	2,191–2,760	AB033755
<i>Stromelysin-3 (MMP-11)</i>	969–1,482	969–1,482	AB055705
<i>MT5-MMP (MMP-24)</i>	1,228–1,966	1,228–1,966	AB047651
<i>TIMP-2a</i>	1–696	–	AB185849
<i>TIMP-2b</i>	158–670	158–670	AB193468
<i>TIMP-3</i>	322–634	-	AB193469
<i>OLCA-1</i>	1,529–1,840		D89627

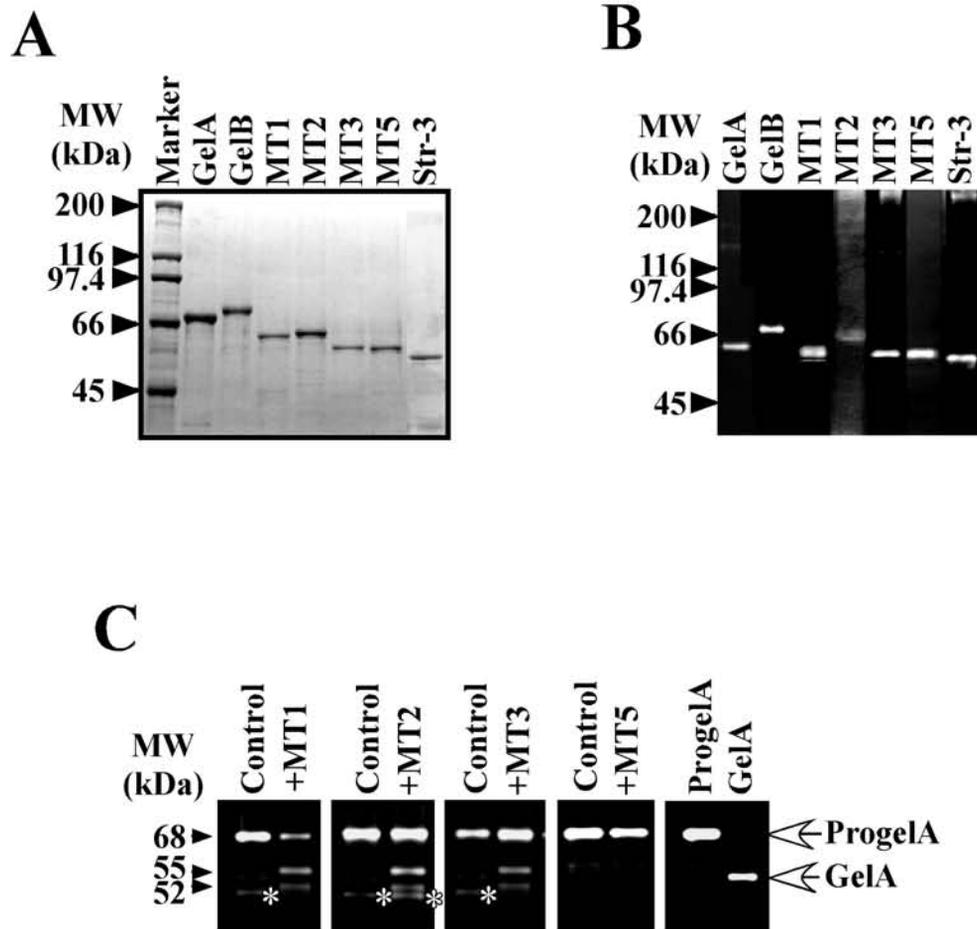
Table 4. Strategy for Recombinant Protein Synthesis

Gene	Expressed regions (nucleotide numbers)	Corresponding amino acids (residue numbers)	Restriction sites and expression vectors	GenBank accession number
<i>MT1-MMP</i>	704–2,080	110–568 (active form*)	<i>EcoRI-HindIII/pET30a</i>	AB185847
<i>MT2-MMP</i>	698–2,188	116–612 (active form*)	<i>EcoRI-HindIII/pET30a</i>	AB072928
<i>MT3-MMP</i>	572–1,903	118–561 (active form*)	<i>EcoRI-HindIII/pET30a</i>	AB072929
<i>Gelatinase A</i>	178–2,070	27–657 (proenzyme form)	<i>EcoRV-HindIII/pET30a</i>	AB033754
<i>Gelatinase A</i>	427–2,070	110–657 (active form)	<i>EcoRV-HindIII/pET30a</i>	AB033754
<i>Gelatinase B</i>	469–2,214	109–690 (active form)	<i>EcoRI-XhoI/pET30a</i>	AB033755
<i>Stromelysin-3</i>	454–1,584	116–492 (active form)	<i>EcoRI-HindIII/pET30a</i>	AB055705
<i>MT5-MMP</i>	407–1,756	50–499 (active form*)	<i>EcoRI-HindIII/pET30a</i>	AB047651
<i>TIMP-2b</i>	230–811	27–220 (active form)	<i>NdeI-XhoI/pET30a</i>	AB193468

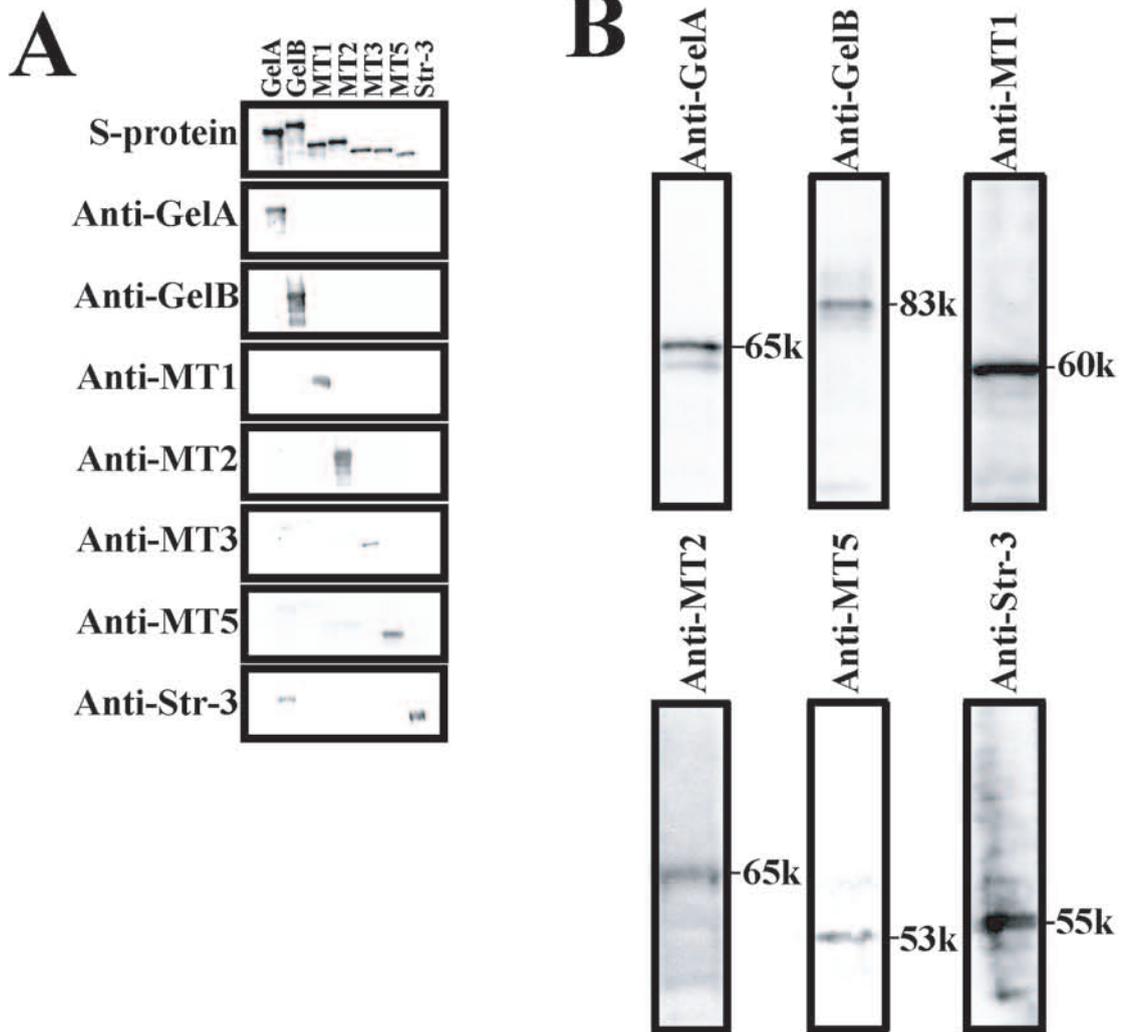
\*Expressed without the transmembrane and cytoplasmic domains.



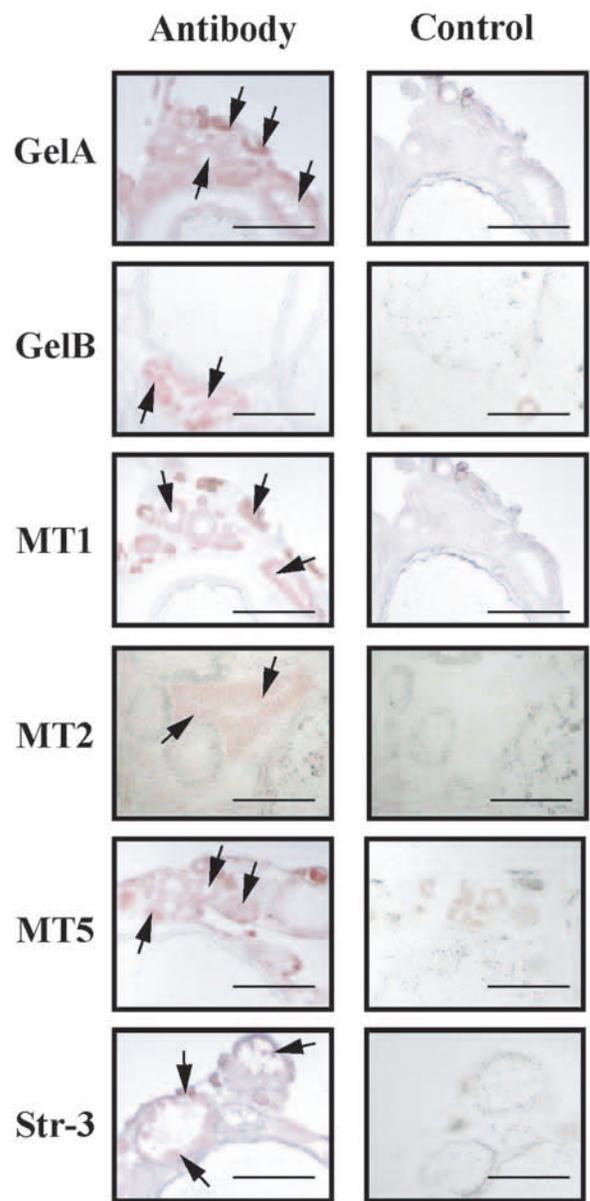
**Fig. 6**  
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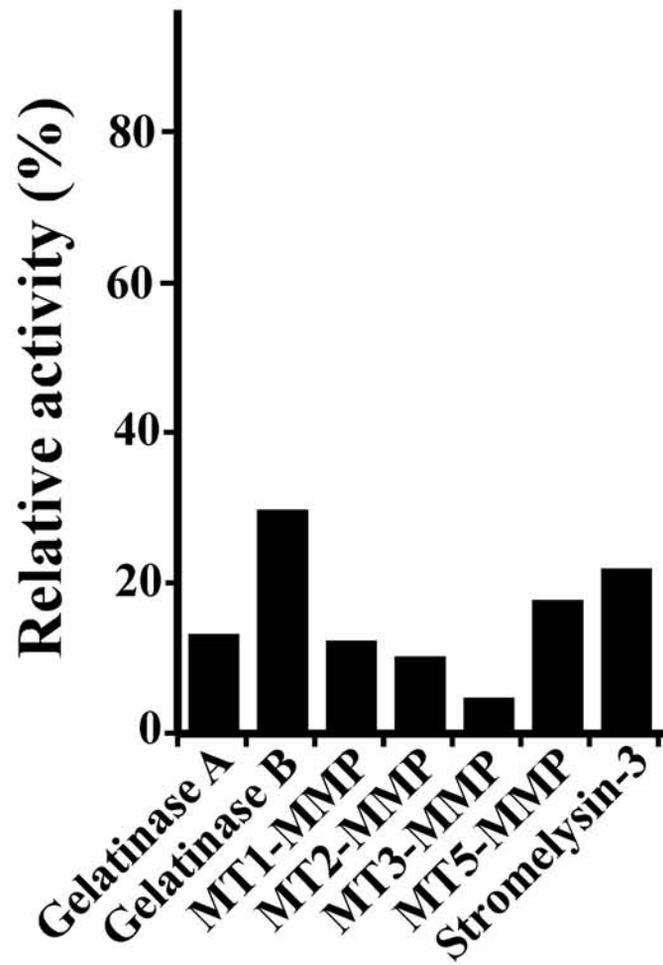
**Fig. 7**  
**Ogiwara et al.**



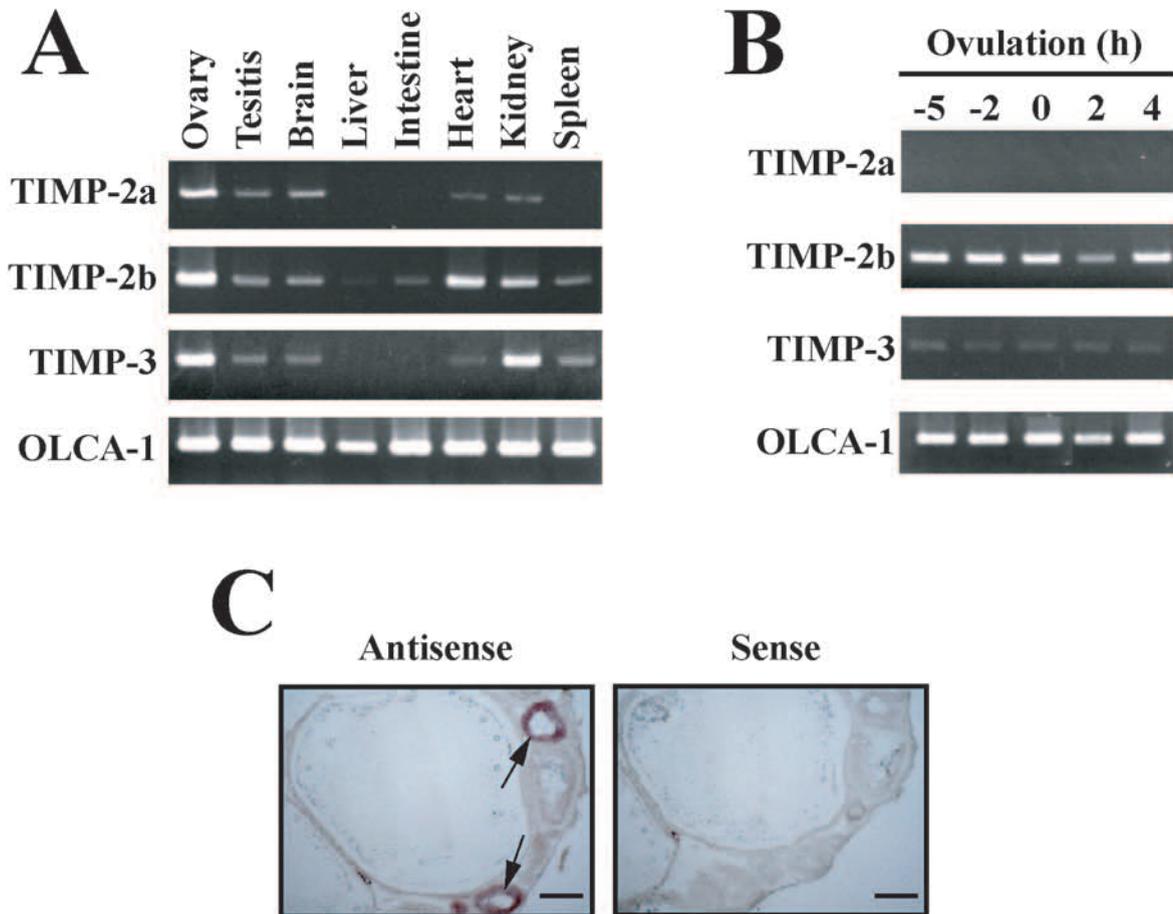
**Fig. 8**  
**Ogiwara et al.**



**Fig. 9**  
**Ogiwara et al.**

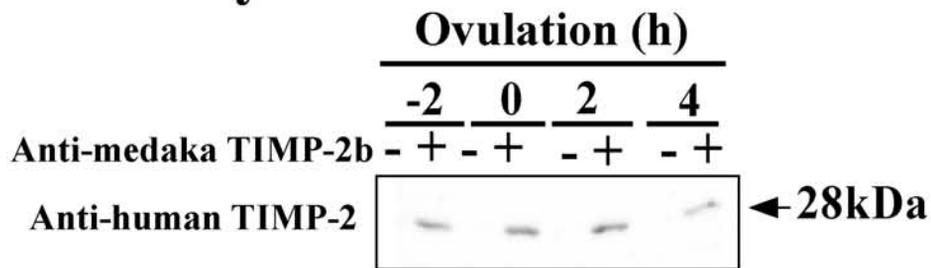


**Fig. 10**  
**Ogiwara et al.**

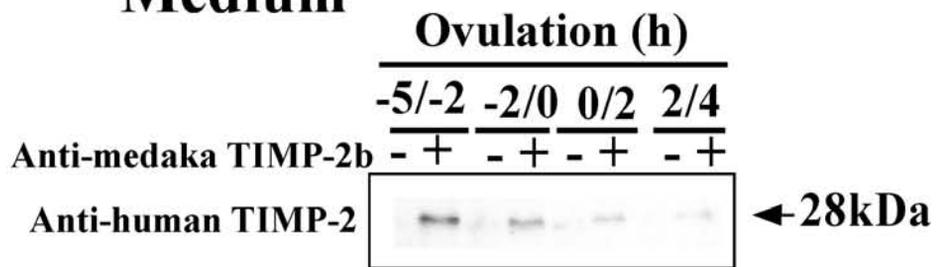


**Fig. 11**  
**Ogiwara et al.**

## Oocyte



## Medium



**Fig. 12**  
**Ogiwara et al.**