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# **Single-chain Tissue-type Plasminogen Activator is a Substrate of Mouse Glandular Kallikrein 24**

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Abbreviated form of title: Effect of glandular kallikrein 24 on tPA

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## ABSTRACT

The Leydig cells of adult mouse testes express at a detectable level three distinct glandular (tissue) kallikrein genes: *mKlk21*, *mKlk24*, and *mKlk27*. Recently, the proteins encoded by these genes were characterized using active recombinant proteases, but their roles in the mouse testis remain to be determined. Among the proteases, mK24 markedly enhanced the activity of tissue-type plasminogen activator when incubated with human recombinant single-chain tissue-type plasminogen activator. This activation was found to be due to the proteolytic conversion of the single-chain enzyme to a two-chain form. The expression of tissue-type plasminogen activator in interstitial Leydig cells was demonstrated by reverse transcriptase-polymerase chain reaction and immunohistochemical analyses. The primary culture medium of adult male testicular Leydig cells contained immunoreactive materials recognized by mK24 antibodies. In addition, the same medium was capable of converting the single-chain plasminogen activator to the two-chain protein. These results suggest that mK24 may play a role in the degradation of extracellular matrix proteins in the interstitial area surrounding the Leydig cells of the adult mouse testis, not only due to its own activity, but also due to that of plasmin, which was eventually produced by the single-chain tissue-type plasminogen activator-converting activity of mK24.

**Key words:** testis, proteolytic processing, mouse spermatogenesis, extracellular matrix, kallikrein, tPA

## INTRODUCTION

The testis is a reproductive organ that serves two crucial functions, namely, the synthesis and secretion of steroid hormones and the production of spermatozoa. Spermatogenesis is classically divided into three major developmental phases: spermatogonial maturation and multiplication, meiosis, and spermiogenesis. Normal testicular development and the maintenance of spermatogenesis are controlled by gonadotropins and testosterone. The importance of Leydig cells in the interstitium and that of Sertoli cells and peritubular cells in the seminiferous tubules has been well documented for these biological processes (Jegou, 1998).

The gene for tissue kallikrein and closely related genes constitute the glandular kallikrein (GK) gene family (Yousef and Diamandis, 2003; Clements, 1997; Bhoola *et al.*, 1992; Diamandis and Yousef, 2002; Richards *et al.*, 1982). Recently, twelve members of this family, *KLK4-KLK15*, were discovered in humans and were assigned to the GK family (Diamandis *et al.*, 2000). A comparison of the human and rodent GK gene loci showed that the location of the tissue kallikrein gene and *KLK4-KLK15* are conserved (Olsson and Lundwall, 2002; Diamandis *et al.*, 2004). In the mouse, there may be at least 37 genes (<http://www.ncbi.nlm.nih.gov/genome/guide/mouse/>), all of which are located in cytogenic region B2 on mouse chromosome 7. Diamandis *et al.* (2004) recently described an update of the genomic organization of mouse GK gene family members. In the present study, we followed the nomenclature indicated by Berg *et al.* (1992).

Existing evidence indicates that 26 of 37 mouse GK genes can code for functional proteins. About half of the 26 genes were characterized by molecular biological or biochemical approaches. They are thought to be involved in many vital functions, such

as kinin production, coagulation and fibrinolysis, activation and/or inactivation of peptide hormones, regulation of growth factors, and extracellular matrix protein turnover (Margolius, 1998). Previous studies have also demonstrated that the mouse testis expresses various GK genes (Carolyn *et al.*, 1999; Penschow *et al.*, 1991; Matsui *et al.*, 2000b). Among them, *mKlk21* (Matsui and Takahashi, 2001), *mKlk24*, which corresponds to *mGK24* in our previous report (Matsui *et al.*, 2005), and *mKlk27* (Matsui *et al.*, 2000b) have been shown to be predominantly expressed in the organ. In addition, *mKlk21* and *mKlk24* are known to encode serine proteases mK21 and mK24, respectively, with trypsin-like specificity, while *mKlk27* encode serine protease mK27 with chymotrypsin-like specificity. Interestingly, expression of these GK genes becomes detectable in association with testicular Leydig cells after 4 weeks of postnatal development. Taken together, these findings strongly suggest that the kallikreins are important for various functions in the adult mouse testes.

In our attempts to further define possible biological roles of the kallikreins, we found that the incubation of single-chain tissue-type plasminogen activator (sctPA) with mK24, but not with mK21 or mK27, resulted in a dramatic increase in tPA activity. In the present study, we report that mK24 proteolytically converts sctPA to a two-chain enzyme (tctPA). These results, together with the finding that this enzyme hydrolyzes certain extracellular matrix (ECM) proteins (Matsui *et al.*, 2005), provide further support for the notion that mK24 plays a role in the turnover of ECM proteins in the adult mouse testis.

## **MATERIALS AND METHODS**

### **Animals**

Mice (C57BL/6NCrj strain) were sacrificed by cervical dislocation, and the testes were rapidly removed and used immediately. In some experiments, the testes were frozen in liquid N<sub>2</sub> and stored at -80°C until use. The experimental procedures used in this study were approved by the Animal Experiment Committee of the Graduate School of Science, Hokkaido University.

### **RNA isolation**

Total RNAs were prepared using Isogen (NipponGene, Tokyo, Japan) according to the manufacturer's protocol. Poly(A)<sup>+</sup>RNAs were selected by using an oligo(dT)-cellulose column (Amersham Pharmacia Biotech, Tokyo, Japan).

### **Northern blot analysis of testis tPA**

Total RNAs were isolated from testes at various postnatal stages, and aliquots (30 µg) of the RNA were electrophoresed on formaldehyde/1.2% agarose gel and transferred to a Nytran-plus membrane (Schleicher and Schuell, Kassel, Germany). The blots were hybridized with the <sup>32</sup>P-labeled, 425-bp mouse *tPA* probe (corresponding to nucleotides 1811-2235, GenBank Accession no. NM\_008872) at 37°C in a buffer containing 5 × saline-sodium citrate (SSC), 50 mM sodium acetate (pH 8.0), 0.1% sodium dodecyl sulfate (SDS), 10 × Denhardt's solution, 0.01% sodium pyrophosphate and 100 µg/ml denatured salmon sperm DNA. The membranes were washed in solutions with increasing stringency, with a final wash in 0.1 × SSC/0.1% SDS at 42°C. As a control, 18S rRNA was detected using a specific <sup>32</sup>P-labeled 30-bp cDNA fragment (5'-CGGCATGTATTAGCTCTAGAATTACCACAG-3').

## **RT-PCR analysis**

For the amplification of the *tPA* gene expressed in mouse tissues and in the primary Leydig cell culture, *tPA*-specific primers (sense primer, 5'-TGCCTTCCTCTTCCTCTTCTACAG-3', corresponding to nucleotides 1811-1834 of the GenBank clone NM\_008872 for mouse *tPA*; antisense primer, 5'-AGATACCCCTTCCTTCTCGTGG-3', corresponding to nucleotides 2214-2235 of the same clone) were synthesized. The first-strand cDNA was synthesized from the total RNAs (5 µg) of various tissues and Leydig cells using a SuperScript II Preamplification System (Life Technologies Inc., Rockville, MD). The PCR conditions were 94°C for 5 min, followed by up to 34 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min. The amplified products were electrophoresed on 1.5% agarose gel, and the PCR products were transferred to a Nytran-plus membrane (Schleicher and Schuell). Mouse  $\beta$ -actin mRNA (292-bp, corresponding to nucleotides 943-1234, Accession no. X03672) was amplified as a control.

## **Primary Leydig cell culture**

Testes from 8-week-old mice were decapsulated and digested with 0.25 mg/ml collagenase (Life Technologies, Inc.) After sedimentation, Leydig cells contained in the supernatant were purified on a Percoll gradient (Bernier *et al.*, 1986). Immature Leydig cells were isolated from 2-week-old mouse testes without Percoll gradient purification, as described previously (Matsui and Takahashi, 2001). Leydig cells attached to the surface of the culture dishes were washed three times and recovered for RT-PCR analysis.

## **Recombinant mK preparations**

Using the pET30a expression vector (Novagen) in an *E. coli* expression system, recombinant proteins of mK21, mK24, and mK27 were produced as fusion proteins with an extra amino acid sequence of 51-57 residues (varying with different proteins) at the NH<sub>2</sub>-terminus. Active enzymes were purified as previously described (Matsui *et al.*, 2000b; Matsui and Takahashi, 2001; Matsui *et al.*, 2005).

## **Enzyme activity assay**

Enzyme activity in the presence of various 4-methylcoumaryl-7-amide (MCA)-containing substrates (Peptide Institute, Osaka, Japan) was determined according to a previously reported method (Barrett, 1980).

The sctPA-converting activity of mKs was determined as follows: human sctPA (Biopool AB, Umeå, Sweden) was incubated with enzyme samples in 100 µl of 0.1 M Tris-HCl buffer (pH 8.0) at 37°C for the indicated period of time. After incubation, 50 µg of aprotinin (Sigma) was added to the reaction mixture. The reaction mixture was then adjusted to a 495-µl mixture by adding 0.1 M Tris-HCl buffer (pH 8.0). The enzyme activity of activated tPA in the mixture was determined with L-pyroglutamyl(Pyr)-Gly-Arg-MCA as the substrate. The sctPA-converting activity was also determined by Western blot analysis as previously described (Ohnishi *et al.*, 2004). Briefly, the reaction mixture was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) under reducing conditions, and was transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) (Towbin *et al.*, 1979). The blotted membrane was incubated with goat anti-human melanoma tPA antibody (Biopool AB) at a 1:5,000 dilution and was subsequently

incubated with donkey anti-sheep/goat IgG antibody (Amersham Pharmacia Biotech). Immunoreactive signals were detected using an ECL Western blot detection kit (Amersham Pharmacia Biotech) according to the protocol provided by the manufacturer.

### **NH<sub>2</sub>-terminal amino acid sequence analysis**

Human tPA incubated with or without mK24 was directly analyzed for NH<sub>2</sub>-terminal amino acid sequence using a Perkin-Elmer-Applied Biosystems model 477A sequenator.

### **Preparation of antibody**

The antigen used was the recombinant mK24 eluted from a Ni<sup>2+</sup>-column, as described above. Antiserum was raised by injecting female rabbits as described previously (Matsui *et al.*, 2000a). The antibody was purified in the following manner. Purified mK24 was subjected to SDS-PAGE under reducing conditions and was transferred onto a PVDF membrane. The membrane was incubated with the antiserum overnight, and was subsequently washed extensively with phosphate-buffered saline (PBS). The antibody was then eluted from the membrane using 50 mM glycine-HCl (pH 2.5). The eluate was immediately neutralized, concentrated, and dialyzed against PBS for use in the Western blot analysis.

### **Preparation of extracts and Western blot analysis**

Mouse tissues were resuspended at 4°C with lysis buffer containing 25 mM Hepes, 1 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol, aprotinin (10 µg/ml), leupeptin (20 µM), and soybean trypsin inhibitor (20 µg/ml). After homogenization, the insoluble

debris was removed by centrifugation at  $10,000 \times g$  for 15 min at 4°C. After being subjected to SDS-PAGE, the proteins were transferred to a PVDF membrane. The blotted membrane was incubated with or without purified anti-mK24 antibody at a 1:100 dilution and was subsequently incubated with goat anti-rabbit IgG antibody (Amersham Pharmacia Biotech). Immunoreactive signals were detected using an ECL Western blot detection kit.

### **Immunohistochemistry**

Frozen sections (10  $\mu\text{m}$ ) of 8-week-old C57BL/6 mouse testis were dried, fixed in methanol at  $-20^{\circ}\text{C}$  for 20 min, treated with 3%  $\text{H}_2\text{O}_2$  in PBS, and blocked with BlockAce (Dainippon Seiyaku, Osaka, Japan) for 1h at room temperature. The sections were then incubated at 4°C for 12h with goat anti-human melanoma tPA antibody at a 1:500 dilution. After the sections had been washed three times with PBS, they were incubated with horse-radish peroxidase (HRP)-conjugated donkey anti-sheep/goat IgG antibody (for tPA) for 1h at room temperature. Immunocomplexes were detected using a diaminobenzidine detection kit (Vector Laboratories, Burlingame, CA).

## **RESULTS**

### **Effects of recombinant mK21, mK24, and mK27 on sctPA**

When active recombinant mK proteins were incubated with human sctPA, a drastic increase in tPA activity was observed with mK24, but not with mK21 or mK27 (Fig. 1A). sctPA was progressively activated by mK24 treatment, both with increasing amounts of the enzyme and with increases in the duration of incubation (Fig. 1B). The tPA activity activated with 150 ng of mK24 was slightly lower than that with 75 ng of

the protein, but this difference was not significant. The elevation of tPA activity was due to the proteolytic conversion of sctPA (65 kDa) to its two-chain form (33 and 32 kDa) (Fig. 1C). The amino acid sequence analysis of tPA incubated with mK24 for 8h gave two sets of sequences: Ile-Lys-Gly-Gly-Leu- and Ser-Val-Gln-Val-Ile-. The former corresponds to the internal sequence of sctPA (Ile<sup>276</sup> to Leu<sup>280</sup>), and the latter to the NH<sub>2</sub>-terminal sequence (Ser<sup>1</sup> to Ile<sup>5</sup>) (Pennica *et al.*, 1983). These results demonstrated that the Arg<sup>275</sup>-Ile<sup>276</sup> peptide bond in sctPA was selectively cleaved by mK24. tPA incubated with mK24 for 18h retained its activity at a fully activated level, and gave two polypeptides of 33 and 32 kDa upon SDS-PAGE (data not shown), thus indicating the strict cleavage specificity of mK24 on the protein substrate.

Precursor urokinase-type plasminogen activator (pro-uPA) was not activated by mK21, mK24, or mK27 (data not shown).

### **Detection of immunoreactive materials by anti-mK24 antibodies in the testis extract**

Antibodies raised against mK24 were tested by Western blot analysis using a blot loaded with recombinant mK24, mK21, and mK27. The antibodies recognized not only mK24, but also two other mK species (data not shown). When testis extracts obtained at various postnatal ages were examined by Western blotting using the current antibodies, a specific protein band of 35 kDa was detected in tissues from animals that were older than 4 weeks of age (Fig. 2).

### **Expression of tPA in the mouse testis**

After the ability of mK24 to convert sctPA to tctPA was observed *in vitro*, we attempted

to gain a better understanding of its biological relevance. Northern blot analysis of the total RNAs isolated from the mouse testes detected *tPA* mRNA at fairly constant levels in samples from animals ranging from 2 to 8 weeks old (Fig. 3A). However, RT-PCR using the RNA from testicular Leydig cells amplified a specific tPA fragment only in 8-week-old mice (Fig. 3B). Furthermore, immunohistochemical localization of tPA in the testis of adult mice was examined using anti-human tPA antibody. Positive staining was predominantly observed in the seminiferous tubules (Fig. 3C, left panel). At a higher magnification, the staining signals were found to be localized to the area where Sertoli cells reside (Fig. 3C, arrows in the middle panel); this observation was consistent with the notion that tPA is synthesized in and secreted from Sertoli cells in the mouse testis (Lacroix *et al.*, 1977; Vihko *et al.*, 1984a; Vihko *et al.*, 1984b; Liu *et al.*, 1995; Tolli *et al.*, 1995; Zhang *et al.*, 1997; Gunnarsson *et al.*, 1999). In addition, a clear signal was detected in the interstitial space of the testis (Fig. 3C, arrowheads in the middle panel). No staining was observed without the tPA antibody (Fig. 3C, right panel). The results described here strongly suggest that tPA is expressed in the Leydig cells of the adult mouse testis, and these cells also express the *mKlk24* gene. To the best of our knowledge, this is the first study to report the immunohistochemical localization of mouse testis tPA, whereas the morphological data are already available for rat (Vihko *et al.*, 1984b) and human testis (Stubbs *et al.*, 1998).

#### **sctPA-converting activity of conditioned medium of cultured Leydig cells**

Leydig cells were isolated from the testes of 2- or 8-week-old mice, and the respective culture media were prepared. The conditioned medium of testicular Leydig cells from 8-week-old mice contained a 35-kDa polypeptide immunoreactive with the

antibody raised in this study (Fig. 4A). No immunoreactive materials were detected when the Leydig cell culture medium of samples from 2-week-old mice were tested (data not shown). Western blot analysis of the culture media using anti-human tPA antibody detected no specific band (data not shown), indicating that the media contained an insufficient amount of tPA for detection. Therefore, the sctPA-converting activity of the media was assayed with human sctPA. The Leydig cell culture medium of 8-week-old mouse testis, but not that of 2-week-old mouse testis, was capable of converting sctPA to tctPA (Fig. 4B). These results indicated that testicular Leydig cells isolated from 8-week-old mice secrete a sctPA-converting enzyme(s).

## DISCUSSION

In the present study, we demonstrated that mK24 converts sctPA to tctPA *in vitro*. This conversion involves the proteolytic cleavage of the peptide bond between Arg<sup>275</sup> and Ile<sup>276</sup> in the human tPA sequence. No further degradation of the two-chain form of tPA was observed, even after the incubation time was elongated for a period of up to 18h. These findings indicate that cleavage by mK24 at the above site is highly specific.

Our attempt to demonstrate the presence of mK24 in the testis unfortunately provided limited information, as the mK24 antibody used in this study was immunoreactive not only with its antigen, but also with mK21 and mK27. However, this result was not surprising, considering that mK24 shares remarkable structural homology (88% identity) with mK21 and mK27. In the future, it will still be necessary to develop an improved method for detecting mK24 alone by raising antibodies with strict specificity for mK24. Despite the difficulties encountered in specifically identifying the entities recognized by the current antibody, we tentatively inferred, from our previous

observations (Matsui *et al.*, 2000b; Matsui and Takahashi, 2001; Matsui *et al.*, 2005) of the testicular expression levels of *mKlk21*, *mKlk24*, and *mKlk27*, that the immunoreactive materials detectable with the antibody may have been mK24 and/or mK21. It should be noted that the size (35-kDa) of the immunoreactive material in testis extracts and Leydig cell conditioned media was greater than those (27 to 30-kDa) of the recombinant mK proteins. Such differences in apparent molecular masses are presumably due to the presence of *N*-glycosylated carbohydrates on the testicular mK proteins.

It has previously been shown that sctPA has only limited plasmin-producing activity unless the cofactor fibrin is present, whereas tctPA itself exhibits full activity in the absence of fibrin (Stubbs *et al.*, 1998). To address the possibility that sctPA could be a substrate of mK24 *in vivo*, the expression of tPA in the mouse testis was investigated. Our present data indicated that the Leydig cells of the adult mouse testis express tPA, and thus that mK24 and tPA are co-expressed in this type of cell. Consistent with this assumption, the conditioned culture medium of Leydig cells contained an immunoreactive material that was recognized by the mK24 antibody. Furthermore, this medium exhibited sctPA-converting activity. Although our mK24 antibody is not specific for the antigen mK24, we tentatively suggest that the sctPA-converting enzyme activity detected in the Leydig cell culture medium was perhaps due to mK24 activity. This line of reasoning is consistent with our present finding that mK24, but neither mK21 nor mK27, has sctPA-converting activity.

The biological significance of the conversion of sctPA to tctPA by mK24 remains to be determined. One plausible explanation for this conversion could be that mK24 serves as a local initiator of the PA/plasmin system in the interstitial tissue of the adult mouse

testis. If this is indeed the case, tctPA produced by mK24 would be expected to be fully active in the absence of the cofactor fibrin, and could readily generate the active serine protease plasmin from its inactive precursor, plasminogen, which is abundantly present in the tissue fluid. The plasmin thus produced may participate in the degradation of ECM proteins. Taking into consideration that mK24 itself is capable of degrading certain ECM proteins, mK24 may play a dual role in the metabolism of ECM proteins in the interstitial area surrounding the Leydig cells of the adult mouse testes—namely, it may carry out direct ECM protein hydrolysis by its own activity, while also exerting an indirect effect on this process through the mK24-mediated activation of the tPA/plasmin system.

Previous studies have established that Sertoli cells are the main source of PA production in the mammalian testes (Lacroix et al., 1977; Canipari and Galdieri, 2000). It has been postulated that the PA/plasmin system is involved in various biological processes that take place within the seminiferous tubules. These processes include the following: the continuous remodeling of the seminiferous epithelium that takes place during the release of the preleptotene spermatocytes from the basement membrane, spermiation, the detachment of residual bodies from the mature spermatids and their phagocytosis by Sertoli cells, and the maintenance and opening of the tight junctions (Sertoli cell barrier) between the neighboring to the adluminal compartment of the seminiferous epithelium (Russel, 1977; Fritz *et al.*, 1989). However, we suspect that mK24 originating from interstitial Leydig cells could come into contact with the sctPA produced by Sertoli cells in the seminiferous tubules.

We initially isolated a PCR fragment of the mK24 clone from the mRNA of mouse B16 melanoma cells. Such cancer cells secrete a protease capable of converting human

sctPA to its two-chain enzyme (Matsui *et al.*, 1998). Although the protease secreted from these melanoma cells remains to be purified for detailed characterization, it appears to resemble mK24. For example, in addition to their sctPA-converting activity, both enzymes are trypsin-like serine proteases of a similar molecular size, but also with similar inhibitor profiles. However, in order to determine whether or not these enzymes are identical, further studies will be necessary.

In summary, our data revealed that mK24 converts sctPA to tctPA. Temporal and spatial expression studies of mK24 (Matsui *et al.*, 2005) and tPA (this study) using mouse testis revealed that both genes are co-expressed in the Leydig cells of the adult mouse testis. Conversion of sctPA to tctPA by mK24 is thought to bring about the production of plasmin, an active serine protease capable of hydrolyzing ECM components. In addition, mK24 itself degrades some ECM proteins (Matsui *et al.*, 2005). Based on these considerations, we tentatively suggest that mK24 may be involved in the metabolism of ECM proteins in the interstitial tissue surrounding the Leydig cells in the testis. The current data are expected to be helpful for future physiological studies of this serine protease.

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## Figure Legends

### Fig. 1. Conversion of sctPA to tPA by mK24

(A) Increase in tPA activity as a function of the amount of mK24. sctPA (0.3  $\mu\text{g}$ ) was incubated at 37°C for 2h in 100  $\mu\text{l}$  of 0.1 M Tris-HCl (pH 8.0) with increasing amounts of recombinant mK21 ( $\square$ ), mK24 ( $\bullet$ ), and mK27 ( $\circ$ ), and tPA activity was selectively determined using Pyr-Gly-Arg-MCA in the presence of aprotinin. As a control, mK24 was incubated without sctPA ( $\blacktriangle$ ). (B) Time-course of sctPA activation. sctPA (1.0  $\mu\text{g}$ ) was incubated in 500  $\mu\text{l}$  of 0.1 M Tris-HCl (pH 8.0) with ( $\bullet$ ) or without ( $\blacksquare$ ) mK24 (0.2  $\mu\text{g}$ ). An 80- $\mu\text{l}$  aliquot of the incubation mixture was obtained at the indicated times for an enzymatic activity assay of the activated tPA using Pyr-Gly-Arg-MCA. Incubation of mK24 alone ( $\blacktriangle$ ) was used as another control. (C) Electrophoretic analysis of the conversion. Aliquots of 10  $\mu\text{l}$  were obtained from the reaction mixture at the indicated times in (B), and were used for SDS-PAGE/Western blot analysis using specific antibodies against human tPA.

### Fig. 2. Detection of immunoreactive materials in mouse testes using anti-mK24 antibody

Western blot analysis of testis extract was conducted. Extracts were prepared from the testes of 2-, 4-, 6-, and 8-week-old mice, and were subjected to SDS-PAGE under reducing conditions. The blotted membrane was stained with the antibody raised against mK24. A 35-kDa polypeptide band, indicated by the *arrow*, was detected with the antibody.

### Fig. 3. Expression of tPA in the mouse testis

(A) Northern blot analysis. Total RNA was isolated from 2-, 4-, 6-, and 8-week-old mice, and was used for analysis with the oligonucleotide probe specific for *tPA* or 18S *rRNA*. (B) RT-PCR analysis. Total RNA was isolated from the testicular Leydig cells of 2-week-old and 8-week-old mice and was used as the template for analysis. The numbers on the lane indicate PCR cycles. The lower panel depicts the amplification of *β-actin* mRNA, used as a control. These experiments were conducted twice. (C) Immunohistochemistry. Sections of 8-week-old mouse testis were stained with anti-human tPA antibody (*left and center panel*) or without antibody (*right panel*). The bar represents 50 μm in the *left and right panels*, and 20 μm in the *center panel*. Signals observed in the seminiferous tubule are indicated by *arrows*, and signals observed in the interstitial tissue are indicated by *arrowheads*.

**Fig. 4.** Detection of sctPA-converting enzyme activity in the culture medium of 8-week-old mouse testis Leydig cells

(A) Western blot analysis. Leydig cells were isolated from 8-week-old mouse testes and were cultured at 37°C for 48h. The conditioned medium was concentrated approximately 20 times, and the concentrated sample was subjected to SDS-PAGE/Western blot analysis under reducing conditions using purified anti-mK24 antibody. The blotted membrane was treated with (*right lane*) or without antibody (*left lane*). The *arrow* indicates the 35-kDa band specifically recognized by the current antibody. (B) Proteolytic conversion of sctPA to tctPA by the Leydig cell culture medium. The 48-h culture medium of Leydig cells in (A) was concentrated, and then was incubated with human sctPA (80 ng) in 20 μl 0.1 M Tris-HCl (pH 8.0). After incubation for 12h at 37°C, the reaction mixtures were subjected to reducing

SDS-PAGE/Western blot analysis using anti-human tPA antibody. The positions of sctPA and tctPA are indicated at right. The results were confirmed by three separate experiments.

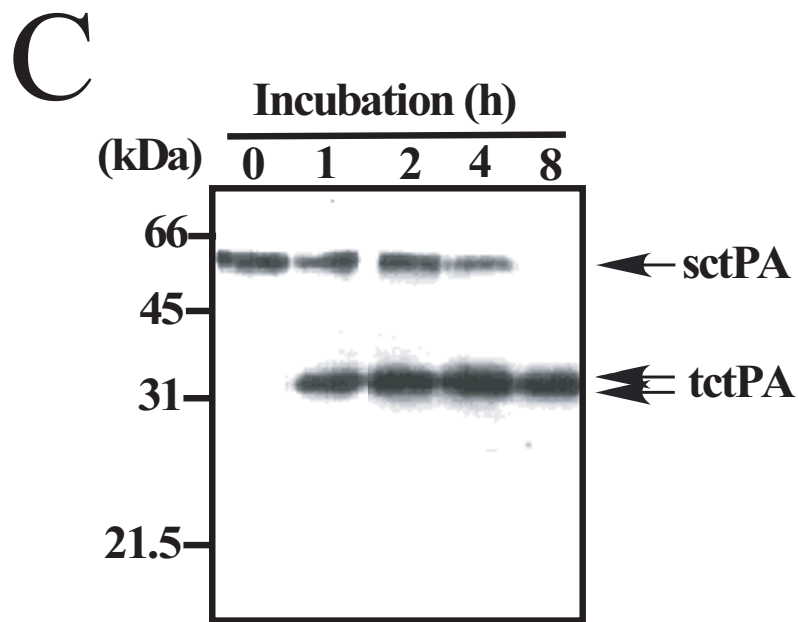
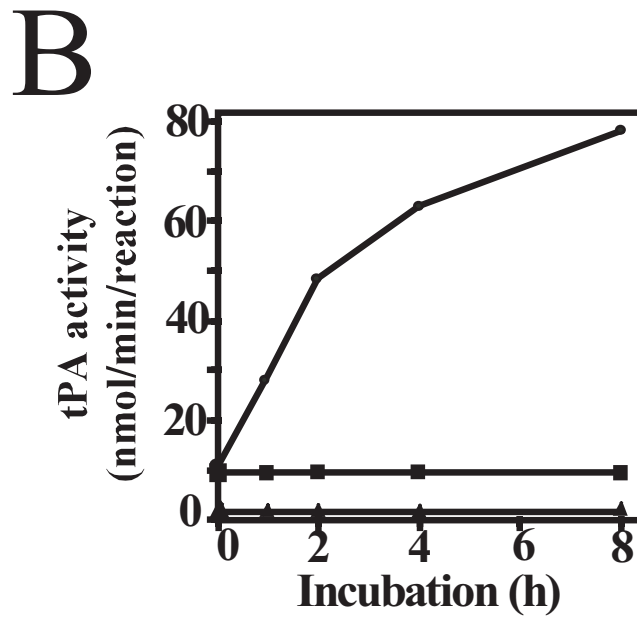
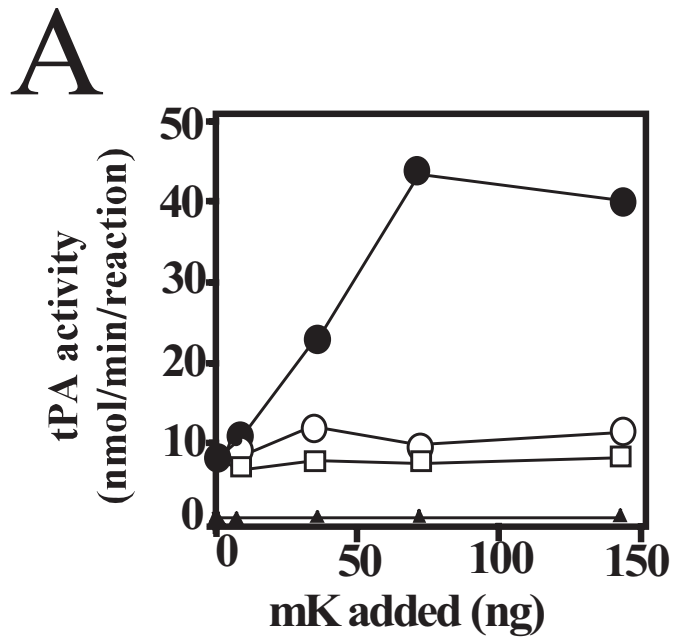


Fig. 1  
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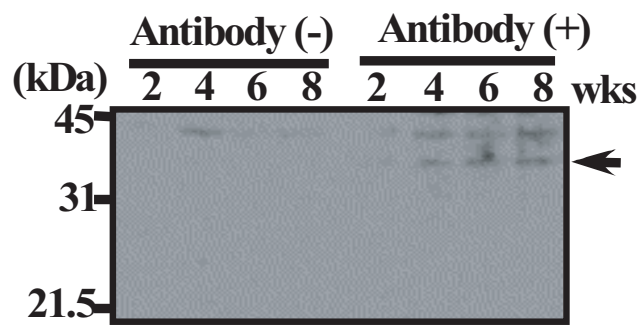


Fig. 2  
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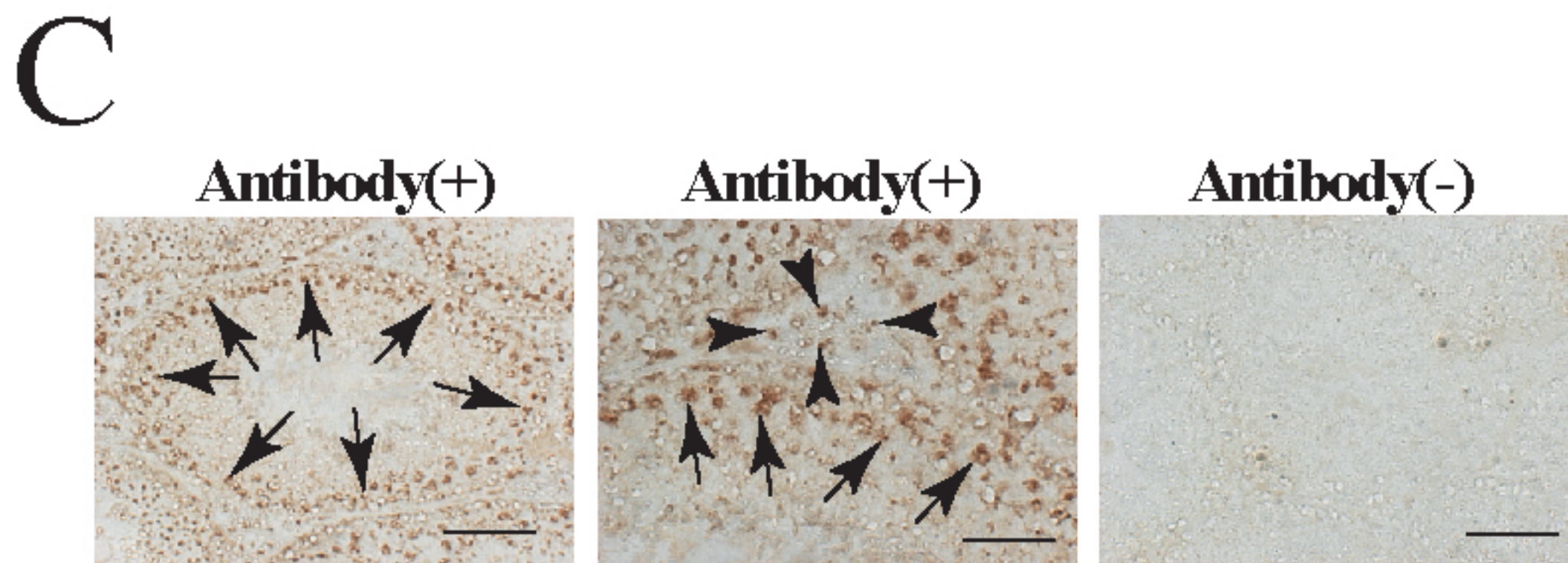
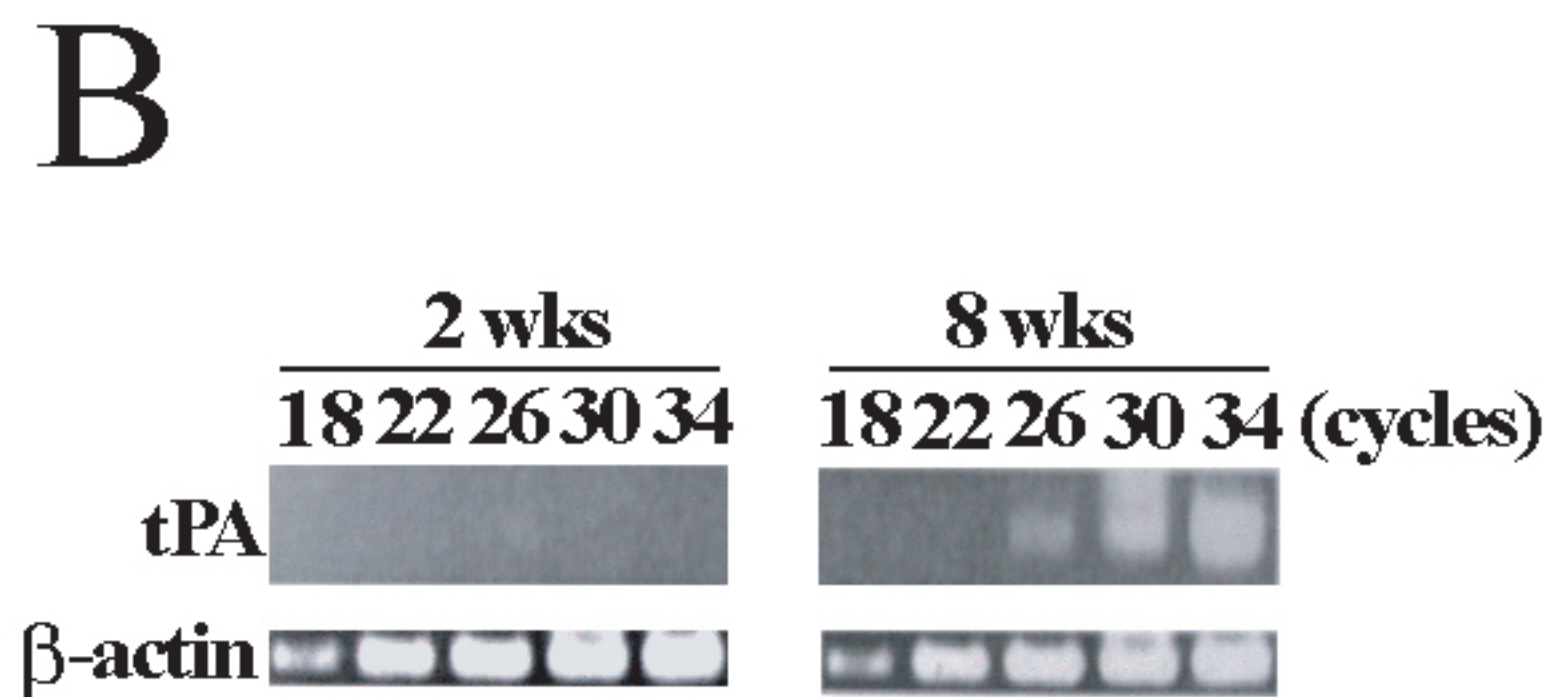
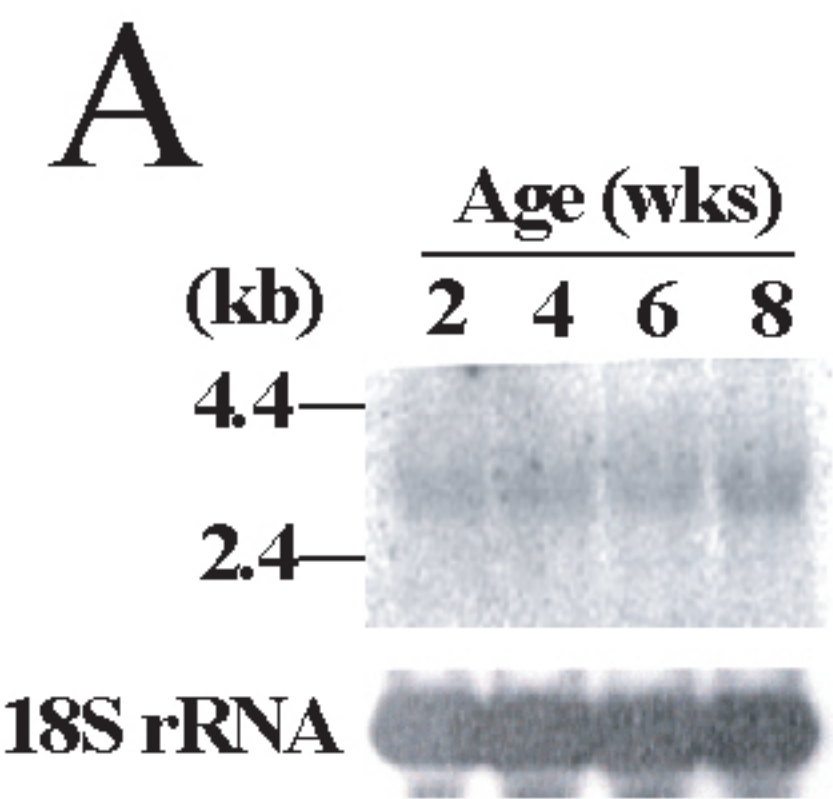


Fig. 3  
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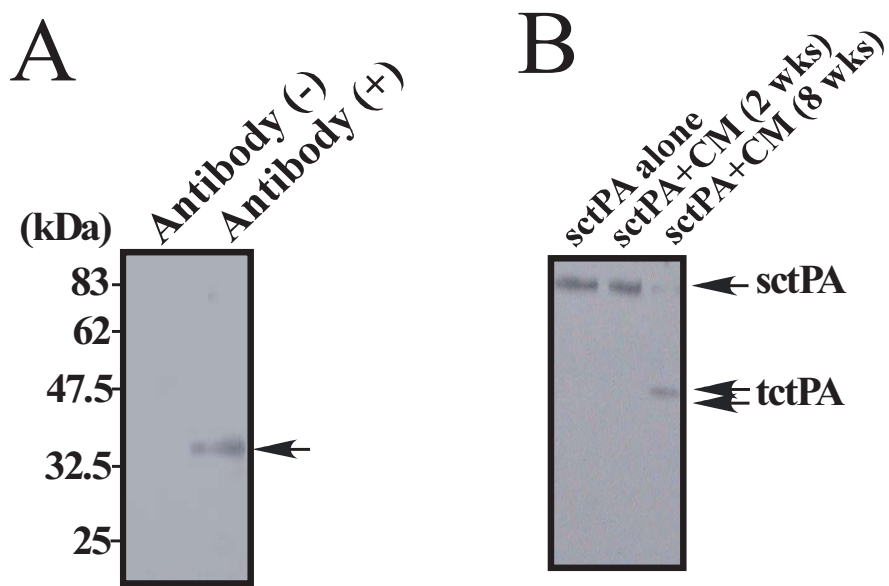


Fig. 4  
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