

# Mouse Ovarian Granulosa Cells Produce Urokinase-Type Plasminogen Activator, Whereas the Corresponding Rat Cells Produce Tissue-Type Plasminogen Activator

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**Abstract.** It is well established that rat ovarian granulosa cells produce tissue plasminogen activator (tPA). The synthesis and secretion of the enzyme are induced by gonadotropins, and correlate well with the time of follicular rupture in vivo. We have found that in contrast, mouse granulosa cells produce a different form of plasminogen activator, the urokinase-type (uPA). As with tPA synthesis in the rat, uPA production by mouse granulosa cells is induced by gonadotropins, dibutyryl cAMP, and prostaglandin E<sub>2</sub>. However, dexamethasone, a drug which has no effect on tPA synthesis in rat cells inhibits uPA synthesis in the mouse.

Results of these determinations made in cell culture were corroborated by examining follicular fluid, which

is secreted in vivo predominantly by granulosa cells, from stimulated rat and mouse ovarian follicles. Rat follicular fluid contained only tPA, and mouse follicular fluid only uPA, indicating that in vivo, granulosa cells from the two species are secreting different enzymes. The difference in the type of plasminogen activator produced by the rat and mouse granulosa cells was confirmed at the messenger RNA level. After hormone stimulation, only tPA mRNA was present in rat cells, whereas only uPA mRNA was found in mouse cells. Furthermore, the regulation of uPA levels in mouse cells occurs via transient modulation of steady-state levels of mRNA, a pattern similar to that seen with tPA in rat cells.

**I**N mammals, there are two forms of plasminogen activator (PA)<sup>1</sup>: tissue-type PA (tPA) and urokinase-type PA (uPA) (see Danø et al., 1985, for review). Both enzymes catalyze the cleavage of the same peptide bond in plasminogen, but they are antigenically distinct and are the products of different genes. Catalytically, these two PAs differ in one important aspect: the activity of tPA is enhanced by the presence of fibrin, whereas that of uPA is not affected. This difference has been used as a basis to suggest that the physiological function of tPA is primarily fibrinolysis, whereas uPA may be involved in cell migration and tissue remodeling.

It is known that rat granulosa cells, which line the interior of the ovarian follicle, produce copious amounts of tPA around the time of ovulation (Beers et al., 1975; Strickland and Beers, 1976; Canipari and Strickland, 1985; Ny et al., 1985). The synthesis of the enzyme is under the control of gonadotropins, with the mRNA being strongly, but transiently induced by both luteinizing hormone and follicle-stimulating hormone (FSH) (O'Connell et al., 1986). In the course of investigating the roles of PA in ovulation, we have

examined other mammalian species. This paper documents that in the mouse, granulosa cells do not produce tPA, but rather secrete uPA. This synthesis is hormonally regulated in a manner similar to that observed with tPA production by rat granulosa cells: uPA mRNA is strongly induced in the mouse cells by gonadotropins, but the induction is transient. These results document that equivalent ovarian cell types from the rat and mouse produce genetically distinct but functionally similar enzymes.

## Materials and Methods

### Materials

Mice of the CD-1 strain and rats of the Sprague-Dawley strain were obtained from Charles River Breeding Laboratories, Inc., Wilmington, MA. Rat FSH (rFSH-I-6) and luteinizing hormone (rLH-I-6) were obtained from the National Hormone and Pituitary Program of the National Institutes of Health. Pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) were purchased from Organon, West Orange, NJ. Other hormones were obtained as previously described (Canipari and Strickland, 1986). The mouse uPA and tPA cDNA clones were provided by Dr. Dominique Belin and Mr. Richard Rickles, respectively.

1. *Abbreviations used in this paper:* FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; PA, plasminogen activator; PMSG, pregnant mare serum gonadotropin; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator.

## Methods

**Preparation of Cultures.** Female mice, 19 d old, were injected subcutaneously with 2.5 IU of PMSG in 0.02 ml of 0.9% NaCl. Female rats, 26 d old, were injected subcutaneously with 5 IU of PMSG in 0.02 ml of 0.9% NaCl. For mice and rats in which ovulation was to be induced in vivo, 5 IU of hCG in 0.02 ml of 0.9% NaCl was injected 48 h after the administration of PMSG. The animals were killed either 48 h after PMSG injection (in vitro stimulation of cells) or 10 h after the hCG injection, and cells or follicular fluid were collected.

Granulosa cell cultures were prepared by a modification of the method of Crisp and Denys (1975) as described in detail (Beers et al., 1975). Briefly, the contents of individual follicles were expressed into medium and the cells were collected and cultured at a density of  $1.5 \times 10^5$  cells per 7-mm well in 0.2 ml of DME supplemented with 0.1% BSA at 37°C in a 7% CO<sub>2</sub> atmosphere. For in vitro stimulation, cells were treated with the appropriate hormone for the indicated time, whereas for in vivo-stimulated cells, cultures were maintained for 5 h without further treatment.

Preovulatory follicles were dissected from the ovaries, and the follicular fluid was collected by hemisectioning the isolated follicles in DME (6 follicles/0.2 ml) using a scalpel blade. This hemisection releases the follicular fluid into the medium. The solid tissue was discarded and the medium collected and centrifuged at 1,500 rpm for 10 min. The supernatant containing follicular fluid was collected and stored frozen until assayed for PA activity.

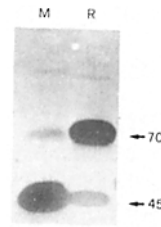
Harvest fluid was assayed by incubating the samples with plasminogen and assaying the plasmin generated using a chromogenic substrate assay (Verheijen et al., 1982; Andrade-Gordon and Strickland, 1986). In this assay, the absorbance generated at 405 nm is related to the PA activity.

**Gel Electrophoresis and Zymography.** For zymography of PA, culture fluids were separated by electrophoresis in 8% polyacrylamide slab gels in the presence of SDS under nonreducing conditions according to the procedure of Laemmli (1970). Molecular weights were calculated from the position of markers that were subjected to electrophoresis in parallel lanes and stained with Coomassie Brilliant Blue R250. The PAs were then visualized by placing the Triton X-100-washed gel on a casein-agar-plasminogen underlay as described previously (Graneli-Piperno and Reich, 1978; Vassalli et al., 1984).

**Immunoprecipitation.** Antiserum against mouse uPA was raised in rabbits against uPA purified from the culture medium of a Lewis lung carcinoma cell line (Marotti et al., 1982). Antiserum against human tPA was prepared in rabbits against enzyme isolated from cultures of HeLa cells. This antiserum cross-reacts strongly with mouse tPA. Both antisera have previously been shown to be specifically neutralizing versus their respective antigens (Marotti et al., 1982). Anti-tPA and anti-uPA antisera were kindly provided by Dr. Edmund Waller and Dr. Dominique Belin, respectively.

Igs were purified by protein A-Sepharose affinity chromatography (Hjelm et al., 1972). Immunoprecipitation was performed by a modification of the method described by Vassalli et al. (1984). Samples (30 µl) were mixed with 3 µl of anti-tPA IgG, 3 µl of anti-uPA IgG, or 3 µl of nonimmune rabbit IgG, incubated overnight at 4°C, and 25 µl of a one-tenth suspension of protein A-Sepharose (Calbiochem-Behring Corp., San Diego, CA) was then added to the samples. After 45 min of incubation at 20°C, the samples were centrifuged for 5 min in an Eppendorf microcentrifuge. Supernatants were removed and pellets washed once with 0.1 ml NET-TS (0.5 M NaCl, 0.001 M EDTA, 0.05 M Tris-HCl, pH 8.1, 1% Triton X-100, 0.2% SDS). The supernatants and washes were combined and lyophilized until 30 µl were left. Samples were mixed with one-third volume of a fourfold electrophoresis sample buffer and subjected to SDS-PAGE. The gels were then analyzed by zymography.

**Northern Analysis.** Total RNA was isolated from granulosa cells by guanidinium thiocyanate extraction and ultracentrifugation through a cushion of CsCl (O'Connell et al., 1986). Hybridization of immobilized RNA to radiolabeled DNA probes was performed according to Thomas (1980). Briefly, 15 µg of total RNA was separated by electrophoresis on a 0.8% agarose gel containing 6% formaldehyde. The gel was then washed twice in  $10 \times$  standard sodium citrate (0.15 M NaCl, 0.015 M Na Citrate, pH 7.0) (SSC) for 10 min at room temperature and the RNA transferred to nitrocellulose by capillary blotting with  $20 \times$  SSC for 20 h. After transfer, the nitrocellulose filter was soaked in  $10 \times$  SSC for 10 min, baked in vacuo for 2 h at 80°C, and placed in a plastic bag with 1 ml/cm prehybridization buffer (6× SSPE, 5× Denhardt's, 0.1% SDS, 0.1 mg/ml salmon sperm DNA, 50% formamide) for 4–24 h at 42°C. The filters were hybridized for 24 h at 42°C in prehybridization buffer containing a mouse tPA cDNA fragment (Ricklefs, R., and S. Strickland, unpublished results) radiolabeled using the random priming method of Feinberg and Vogelstein (1983). The filters were washed at 65°C for 30 min in  $\sim 300$  ml of  $2 \times$  SSC/0.1% SDS (twice),  $1 \times$  SSC/0.1%



**Figure 1.** Zymography of PA secreted by granulosa cell monolayers. PMSG-primed mice (M) and rats (R) were injected with hCG and granulosa cells were collected from ovaries 10 h later. Granulosa cells were plated, and harvest fluid was collected and analyzed by a casein-agar underlay for PA as described in Materials and Methods. All the bands were plasminogen dependent (data not shown).

SDS (once),  $0.2 \times$  SSC/0.1% SDS (once), and exposed to XAR film. Duplicate filters were probed with a mouse uPA cDNA clone (Belin et al., 1985). The filters were then probed a second time with a random primed mouse 18S ribosomal RNA probe (Arnheim and Kuehn, 1979) after having been stripped with 50 mM NaOH at room temperature for 30 min.

## Results

In the rat ovary, tPA is produced by granulosa cells in response to hormonal stimulation at the time of ovulation (Canipari and Strickland, 1985). To evaluate the relevance of the production of this enzyme in ovulation, we have investigated the synthesis of PA in mouse granulosa cells.

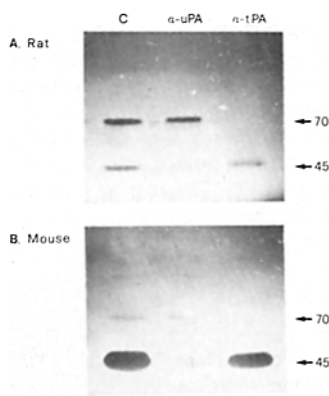
PMSG-primed mice and rats were injected intraperitoneally with hCG. Granulosa cells were collected 10 h after the hCG injection, and maintained in culture for 5 additional h. Conditioned medium was then assayed for the presence of PA by SDS gel electrophoresis followed by visualization of the enzyme by putting the gel on a casein-agar-plasminogen film.

As shown in Fig. 1, when conditioned medium from granulosa cells stimulated in vivo was assayed, there was a significant difference between the rat and mouse. As reported before, rat granulosa cells secrete predominantly tPA.<sup>2</sup> In contrast, mouse granulosa cells produced mainly a PA with a molecular weight similar to that of uPA.

To analyze in detail the type of PA produced by the mouse cells, conditioned medium was treated with antibodies specific for tPA or uPA, and then protein A-Sepharose. As shown in Fig. 2, the high molecular weight PA produced by rat cells is immunoprecipitated with anti-tPA as expected. With the mouse harvest fluid, the predominant band was removed by anti-uPA establishing that these cells produce mainly uPA. The minor band of tPA present in the mouse granulosa conditioned medium appears to be contributed by the oocytes that contaminated the preparation. Mouse oocytes at this stage secrete tPA (Huarte et al., 1985), and in preparations in which oocytes are carefully removed, this band is not present (data not shown, but see also Northern analysis below, Fig. 5).

Although the granulosa cell preparations are greater than 90% pure, we considered the possibility that the uPA in the mouse cultures was originating from a contaminating cell type. For this reason, we analyzed the PA production from the granulosa cells that surround the oocyte, the cumulus

2. The lower molecular weight form produced by rat granulosa cells is in fact uPA (see Fig. 3). However, the following facts about its appearance should be noted. (a) This form is not regulated by gonadotropins. (b) The casein-underlay technique is more sensitive for uPA than tPA for unknown reasons, and hence the relative size of the lytic zones is misleading. (c) Rat granulosa cells at this stage contain very little if any uPA mRNA (see Fig. 5).



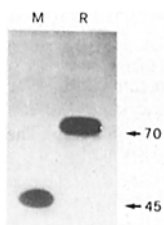
**Figure 2.** Characterization of the granulosa cell PA. Harvest fluids from the *in vivo*-stimulated rat (A) and mouse (B) granulosa cell monolayers (see Fig. 1) were analyzed by zymography before (C) and after immunoabsorption with anti-uPA IgG or anti-tPA IgG.

cells. These granulosa cells can be obtained as a pure population, and, since the assay for PA is so sensitive, can be readily assayed for PA-type. This experiment revealed that mouse cumulus cells also produced exclusively uPA (data not shown).

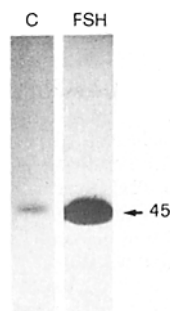
To establish the type of PA secreted by granulosa cells *in vivo*, we investigated PA activity in follicular fluid after *in vivo* stimulation with hCG. PMSG-primed animals were injected with hCG, and follicular fluid was collected 10 h later and assayed for PA activity. In this case the PA present would be the type secreted by the granulosa cells *in vivo*. The results in Fig. 3 show that, indeed, in the follicular fluid there is only one type of PA, and it corresponds to the predominant form found in each specific granulosa cell culture: tPA for the rat, uPA for the mouse.

In the rat, the production of tPA by granulosa cells is under hormonal regulation and several substances can induce the production of this enzyme *in vitro* (Canipari and Strickland, 1986; Ny et al., 1985). It was of interest to study if the production of uPA in mouse granulosa cells was also stimulated by hormones *in vitro*. Granulosa cells were prepared from PMSG-primed animals and cultured in medium alone or in the presence of FSH. As shown in Fig. 4, the production of uPA by mouse granulosa cells is induced by FSH. The spectrum of hormones which regulates uPA in the mouse granulosa cells was similar to that for tPA in rat cells. Table I shows that uPA was also induced by dibutyryl cAMP and prostaglandin E<sub>2</sub>, but only minimally by prostaglandin F<sub>2a</sub>. One notable difference in regulation is the effect of dexamethasone. This glucocorticoid is known to suppress uPA synthesis in many instances (see, e.g., Vassalli et al., 1976; Busso et al., 1986), and it significantly inhibits the FSH-stimulated uPA secretion by mouse cells but does not affect the FSH-stimulated tPA secretion by rat cells.

Since the harvest fluid from mouse granulosa cells contained so little tPA activity, we investigated if this was due to a lack of the enzyme or a difficulty in detecting it because



**Figure 3.** Zymography of PA present in follicular fluid. PMSG-primed mice (M) and rats (R) were injected with hCG and follicles were dissected from ovaries 10 h later. Follicular fluid was collected and analyzed for PA activity as described in Fig. 1.



**Figure 4.** Zymography of PA produced by granulosa cells stimulated in monolayer. Granulosa cells from PMSG-primed mice were plated and treated with (FSH) or without (C) 100 ng/ml FSH. Harvest fluid was collected 10 h later and analyzed for PA activity as described in Fig. 1.

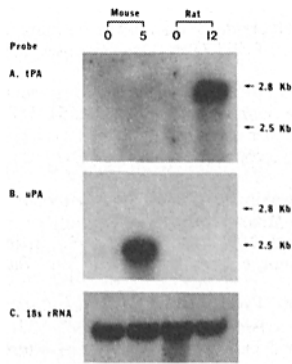
of the presence of an inhibitor. RNA was isolated from both rat and mouse granulosa cells before and after induction with hCG *in vivo*, and then hybridized with specific probes to mouse tPA mRNA and uPA mRNA. When granulosa cells were collected all the oocytes were carefully removed to avoid contamination of the RNA with any tPA mRNA that might be contained in the oocytes (Huarte et al., 1985). As shown in Fig. 5, rat granulosa cells contained an RNA that hybridized to the tPA probe after hormone induction, but no detectable band was observed with the uPA probe. Conversely, hybridization with mouse cell RNA was found only with the uPA probe after hormone treatment. Since both the tPA and uPA probes used in this experiment were of murine origin, the lack of a signal for tPA mRNA in the mouse cells conclusively shows that these cells do not contain detectable levels of this species. It should be noted that control experiments performed with RNA from rat kidney, a rich source of rat uPA mRNA, demonstrated as expected that the mouse uPA cDNA detects rat uPA mRNA under the conditions used (data not shown). Therefore, the rat cells do not contain detectable uPA mRNA. Thus, these results correlate well with the zymographic analyses, and confirm that the absence of secretion of tPA by the mouse cells is due to lack of tPA mRNA.

FSH and luteinizing hormone regulate tPA activity in rat granulosa cells by modulating the steady-state concentration of tPA mRNA. Since in mouse granulosa cells the enzyme being produced is uPA, we examined at what level the production of this enzyme was regulated. The time course of the induction of uPA *in vivo* after hCG was studied by monitor-

**Table I. Hormonal Induction of PA in Rat and Mouse Granulosa Cells\***

	PA activity (absorbance at 405 nm)	
	Rat (tPA)	Mouse (uPA)
Control	0.035 ± 0.004	0.060 ± 0.015
FSH (1 µg/ml)	0.318 ± 0.004	0.295 ± 0.031
dibutyryl cAMP (10 <sup>-3</sup> M)	0.350 ± 0.005	0.483 ± 0.058
Dexamethasone (10 <sup>-8</sup> M)	0.025 ± 0.005	0.010 ± 0.002
FSH + dexamethasone	0.420 ± 0.025	0.152 ± 0.025
Prostaglandin E <sub>2</sub> (10 <sup>-6</sup> M)	0.188 ± 0.017	0.258 ± 0.040
Prostaglandin F <sub>2a</sub> (10 <sup>-6</sup> M)	0.070 ± 0.024	0.112 ± 0.014

\* Rat and mouse granulosa cells from PMSG-primed animals were cultured for 8 h (rat) or 3 h (mouse) in the presence of the indicated hormones (see explanation for times chosen in legend to Fig. 5), and the harvest fluid assayed for PA activity for 2 h. Control experiments with specific antibodies for tPA or uPA (see Fig. 2) indicated that all the stimulated PA activity in the rat was tPA, and in the mouse was uPA.



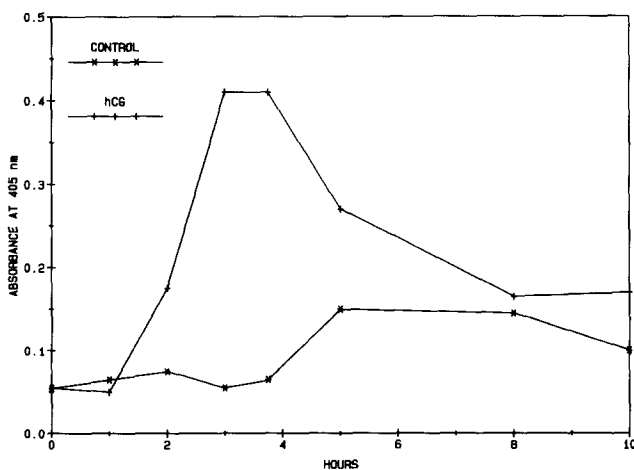
**Figure 5.** Northern blot analysis of mRNA for PAs present in mouse and rat granulosa cells. Granulosa cells were collected from PMSG-primed mice and rats at time zero and at 5 h (*mouse*) or 12 h (*rat*) after hCG injection, and the RNA was extracted. The different lengths of in vivo stimulation for the two species were chosen because the highest enzyme activity had been observed at these times (see Fig. 6 and Beers et al., 1975). Total cellular RNA

(15  $\mu$ g) was subjected to electrophoresis on an agarose gel, transferred to nitrocellulose, and the filter was probed with random-primed cDNA clones for mouse tPA, mouse uPA, or 18 s rRNA. It should be noted that control experiments indicated that the mouse uPA probe hybridizes well to rat uPA mRNA, and therefore that the lack of uPA mRNA in the rat granulosa cell sample is not due to lack of detection with a heterologous probe.

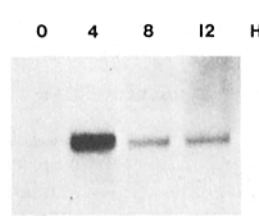
ing enzyme secretion and the steady-state concentration of uPA mRNA. As shown in Figs. 6 and 7, both enzyme secretion and mRNA levels peak at 3–5 h and there is then a rapid decrease. This regulatory pattern is similar to that observed with tPA in rat granulosa cells.

## Discussion

In terms of anatomical structure, endocrinology, and physiology, the rat and mouse ovary are virtually identical (Mossman and Duke, 1973). Therefore, it is surprising that their granulosa cells produce different forms of PA. These different forms, although having distinct catalytic properties which may indicate distinct functions, ultimately catalyze the cleavage of the same bond in plasminogen. Furthermore, the tPA production by rat cells and the uPA production by mouse cells are induced by gonadotropins, dibutyryl cAMP, and PGE<sub>2</sub>; in both rat and mouse cells, the induction is tran-



**Figure 6.** Time course of PA produced by mouse granulosa cells after induction by hCG. PMSG-primed mice were injected with hCG or with solvent alone (*CONTROL*). At the indicated times, granulosa cells were placed in culture, and harvest fluid was collected 2 h later. The samples were assayed for PA activity for 2 h.



**Figure 7.** Time course of uPA mRNA levels after induction by hCG. Granulosa cells were collected from PMSG-primed mice at different times after hCG injection and the RNA was extracted. Total RNA was processed as in Fig. 5 and probed with a random primed uPA cDNA clone.

sient and synthesis returns to basal levels 24 h after hormone treatment, and in both the regulation of enzyme synthesis is controlled at the level of steady-state mRNA concentrations.

The difference in PA synthesis in mouse and rat cells has several possible explanations. In the interior of the rodent follicle, tPA and uPA may be interchangeable, with both being equally capable of producing plasmin. In this case, it is possible that the stimulation of the activity of tPA by fibrin is not relevant. Alternatively, there may be some function required in the rat ovary that is better provided by tPA, or a function in the mouse for which uPA is better suited.

It is noteworthy that in addition to stimulation by fibrin, the activity of tPA can also be enhanced by heparin and follicular fluid proteoglycans (Andrade-Gordon and Strickland, 1986). It is of interest in this regard that the preovulatory rat follicle contains a high concentration of mucopolysaccharides. Furthermore, these high molecular weight proteoglycans are degraded before ovulation in the rat, reducing the viscosity of the follicular fluid and allowing the egg to move freely in escaping (Jensen and Zachariae, 1958). The proteoglycan composition of the rat follicular fluid, the proteolysis of the fluid that occurs, and the demonstrated interaction of heparin-like molecules with fibrinolytic components, all suggest that the interaction of tPA with mucopolysaccharides may be important in the process of rat ovulation. It will be of interest to determine if the properties of rat and mouse follicular fluid differ significantly in these aspects, and whether such a difference might partially account for the production of different PAs by the rat and mouse granulosa cells.

Many examples exist of large discrepancies in the amount of a gene product found in the same organ of different species (Wilson et al., 1977); whereas some of these differences can be rationally explained, for others there is no obvious explanation. To our knowledge, no other example exists in comparative mammalian cell biology that is analogous to the situation reported here: that equivalent cells from closely related species achieve a similar function by expressing related but genetically different enzymes.

Information on the expression of tPA or uPA in the granulosa cells of various species may ultimately prove useful in determining the function of these enzymes in ovarian physiology. Furthermore, as molecular analysis of the regulatory elements of the PA genes proceeds, it will be of interest to examine how dibutyryl cAMP mediation of expression of tPA in the rat granulosa cell has been changed to mediation of uPA in the mouse.

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