

Male fertility and protein C inhibitor/plasminogen activator inhibitor-3 (PCI): localization of PCI in mouse testis and failure of single plasminogen activator knockout to restore spermatogenesis in PCI-deficient mice

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Objective: To investigate the mechanisms responsible for the testicular abnormalities and infertility of previously generated male protein C inhibitor (PCI)-deficient mice.

Design: Determination of the localization of PCI in the reproductive organs of wild-type males. Generation of double knockout mice lacking the protease inhibitor PCI and one plasminogen activator, either urokinase (uPA) or tissue plasminogen activator (tPA), both of which are PCI-target proteases.

Setting: Animal research and histologic analysis.

Animal(s): Male mice of desired genotype.

Intervention(s): Fertility testing of double knockout mice.

Main Outcome Measure(s): Infertility of PCI^{-/-}uPA^{-/-} and PCI^{-/-}tPA^{-/-} double knockout mice.

Result(s): In the testes of wild-type males PCI was detected in spermatocytes of prophase I, as well as in late spermatids and mature spermatozoa, but absent from somatic cells. All PCI^{-/-}uPA^{-/-} and PCI^{-/-}tPA^{-/-} male mice were infertile and histologic analysis of testis showed similar alterations as previously described for PCI^{-/-} mice.

Conclusion(s): The abnormal spermatogenesis of PCI (plasminogen activator inhibitor-3)-deficient mice cannot be rescued by single plasminogen activator knockout. (Fertil Steril® 2007;88(Suppl 2):1049–57. ©2007 by American Society for Reproductive Medicine.)

Key Words: PCI, urokinase, tPA, rescue of infertility, mice

Protein C inhibitor (PCI), also designated as plasminogen activator inhibitor 3 or serpin A5 (1, 2), is a member of the serine protease inhibitor (serpin) family that inactivates serine proteases by forming stable, enzymatically inactive 1:1 enzyme inhibitor complexes. PCI inhibits many proteases such as activated protein C (2, 3), thrombin (4), factor Xa (4), factor XIa (4, 5), plasma kallikrein (4, 5), thrombin–thrombomodulin complex (6), urokinase (uPA) (4, 7, 8), tissue plasminogen activator (tPA) (4, 8), the sperm protease acrosin (9, 10), tissue kallikrein (11, 12), and prostate-specific antigen (PSA) (12, 13).

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Previously, we have shown that disruption of the PCI gene, which is highly expressed in the male reproductive tract (10, 14) resulted in infertility of male homozygous PCI-knockout mice (15). Spermatozoa derived from PCI^{-/-} males were malformed and were not able to bind and to fertilize oocytes of PCI^{+/+} females, as shown by in vivo and in vitro fertilization experiments. Histologic analysis of PCI^{-/-} mice revealed abnormal spermatogenesis associated with damage of Sertoli cells, perhaps as a result of unopposed proteolytic activity (15). Such results were consistent with previously accomplished studies showing that assembly and disassembly of Sertoli cells is associated with transient induction of proteases (such as uPA) and protease inhibitors (16, 17), and that their activities must be coordinated to maintain the integrity of the blood–testis barrier (18).

In seminiferous tubules of rats, uPA immunoreactivity was detected at stages VII and VIII in Sertoli cells (19), and recently it has been shown that uPA is also expressed in mouse Leydig cells (20). uPA and tPA, another protease present in the testis (19), convert plasminogen to plasmin, a powerful

matrix-degrading enzyme (21). Immunoreactivity of tPA was found during mid- and late pachytene and diakinesis (stages VII–XIII) in spermatogenic cells, with maximal intensity in stages IX–XIII (19) and in Sertoli cells (22). uPAR (uPA receptor), the cellular receptor of uPA, which dictates the site and extent of proteolysis, is synthesized by germ cells during spermatogenesis and is present on spermatids and mature mouse spermatozoa (23). Both uPA and tPA are known targets of PCI (4, 8).

uPA and tPA have also been described as the major proteases in semen from rodents (24) and they are found also in human semen (25, 26), where they form complexes with PCI (8). By inhibiting uPA and other serine proteases, PCI could regulate the level of proteolytic activity in the seminal fluid. Recently, He et al. (27) have presented clinical evidence that the presence of functionally inactive PCI in seminal plasma, lacking inhibitory activities toward uPA and tPA, may be associated with infertility.

Altogether, these data prompted us to further test the possibility that the observed changes in PCI^{-/-} males were caused by unopposed proteolytic activity of uPA or tPA. We generated double knockout mice lacking PCI and either uPA (28) or tPA (28) and analyzed them in detail. We found that the absence of uPA or tPA did not revert infertility of PCI^{-/-} males. We also determined the localization of PCI antigen in the male reproductive tract of wild-type mice. The pattern of PCI expression suggested involvement in migration of spermatogenic cells in the basal compartment (i.e., where the blood–testis barrier is located) and in maturation of spermatids in the apical compartment of the walls of seminiferous tubules, as specified.

MATERIALS AND METHODS

Immunohistochemistry to Detect Protein C Inhibitor

Testis and epididymis of sexually nonmature (postnatal days 5, 13, and 22) and adult (2 months) PCI^{+/+} male mice were examined histochemically for the presence of PCI antigen. Organs of PCI^{-/-} mice were used as controls. The tissues were fixed in 4% formaldehyde in phosphate-buffered saline (PBS) and processed for standard paraffin embedding. Antigen retrieval and signal enhancement was performed to increase signal after immunohistochemistry. Histologic sections were pretreated with 0.01 M citrate buffer, pH 6.2 in a steamer for 15 minutes and digested with 0.5 µg/mL proteinase K (in TBS buffer [Tris-buffered saline] supplemented with 2 mM CaCl₂) for 2.5 minutes at 37°C. Slides were then incubated with 10% bovine serum albumin (BSA) in TBS for 30 minutes followed by overnight incubation with purified rabbit polyclonal antibodies against recombinant mouse PCI (15). Secondary detection system was biotinylated goat antirabbit antibody (DakoCytomation, Glostrup, Denmark), avidin-horseradish peroxidase (Vector Laboratories, Burlingame, CA), biotinylated goat antiavidin (Vector Laboratories), and again avidin-horseradish peroxidase. Peroxidase was visualized using diaminobenzidine (Roche Diagnostics

Corp., Vienna, Austria) and tissues were counterstained with methyl green.

Alternatively, some adult testes were fixed with 4% formaldehyde in 0.1 M phosphate buffer supplemented with 10% picric acid. Sections were pretreated with 1% lithium carbonate before antibody staining, as in the study by Odet et al. (20). This method resulted in a superior structural preservation, but in identical label distribution as compared to formaldehyde fixation only.

Sperm smears were obtained from epididymal samples of PCI^{+/+} and PCI^{-/-} mice. Sperms were washed in PBS and smears were air-dried and fixed with methanol/acetone (3:1). After blocking with 3% BSA in PBS, slides were incubated with the anti-PCI antibody as discussed previously. A biotinylated goat antirabbit antibody (DakoCytomation) and avidin-Alexa488 (Invitrogen Corp., Carlsbad, CA) were used for detection. Slides were counterstained with DAPI (4',6-diamidino-2-phenylindole) and mounted with Citifluor (Agar Scientific, Stansted, UK).

Intercrossing of Mice and Fertility Testing

PCI^{-/-} (50% 129S/v : 50% Swiss) females were crossed with uPA^{-/-} (25% 129S/v : 75% C57BL/6) or tPA^{-/-} (25% 129S/v : 75% C57BL/6) males (both kindly provided by Dr. Peter Carmeliet, Center for Transgene Technology and Gene Therapy, Leuven, Belgium) (28) to obtain F₁ generation of heterozygous double knockouts. Subsequently, F₁ generation was crossed to obtain F₂ generation, which was genotyped by Southern blotting on genomic DNA isolated from tail biopsies. Genotyping with respect to uPA allele was done using a 0.24-kb *AccI-HindIII* genomic fragment located 3' of exon 11 of the murine uPA gene designated as probe d (28) in *EcoRI* digested DNA and yielded a 9.7-kb fragment for homologously recombined and a 5.7-kb fragment for wild-type allele. Genotyping on tPA allele was accomplished using a 2.2-kb *XbaI-EcoRI* genomic DNA fragment containing intron VII sequence of the tPA gene and designated as probe b (28) in *EcoRI* digests and yielded a 9.7-kb fragment for homologously recombined and a 6.0-kb fragment for wild-type allele. Six-week-old males of desired genotype (PCI^{-/-} uPA^{-/-}; PCI^{-/-} uPA^{+/-}; PCI^{-/-} uPA^{+/+}; PCI^{-/-} tPA^{-/-}; PCI^{-/-} tPA^{+/-}; PCI^{-/-} tPA^{+/+}) were subsequently mated with wild-type females for at least 3 month. Established double knockout mice and control mice used in these studies were littermates derived from heterozygous breeding pairs.

Determination of the Amidolytic Activity of Testis Extracts

Amidolytic activities of testes extracts derived from six adult mice per genotype (PCI^{-/-}, PCI^{-/-} uPA^{-/-}, PCI^{-/-} tPA^{-/-}, and PCI^{+/+}) were analyzed in six separate experiments. Briefly, both mouse testes were collected in one tube and immediately homogenized in 1.4 mL of 10 mM Tris-buffer, pH 7.4, incubated at 37°C for 1 hour, and subsequently centrifuged twice for 15 minutes at 20,000 x g. Aliquots of the supernatants were normalized with respect to protein concentration (A_{280 nm} = 0.1) and analyzed for amidolytic

activity on Glu-Gly-Arg-pNA (S-2444), H-D-Ile-Pro-Arg-pNA (S-2288), and H-D-Val-Leu-Lys-pNA (S-2251), essentially as suggested by the manufacturer (Chromogenix Instrumentation Laboratory S.p.A., Milano, Italy) and as previously described (15). In the case of S-2251 the assay was run with and without addition of plasminogen (50 $\mu\text{g}/\text{mL}$).

Determination of T Levels in Mouse Plasma

Testosterone levels were determined in plasma samples of 12-week-old $\text{PCI}^{+/+}$ and $\text{PCI}^{-/-}$ mice using Enzymun-Test Testosterone (Roche Diagnostics Corp., Vienna, Austria).

Statistics

Experimental values are expressed as means \pm SEM. Statistical significance of differences in amidolytic activities was analyzed using an unpaired Student's *t*-test.

Ethics

All experiments were performed in compliance with institutional guidelines and with Austrian animal experimental law no. 169/1999 and were approved by the respective university committee.

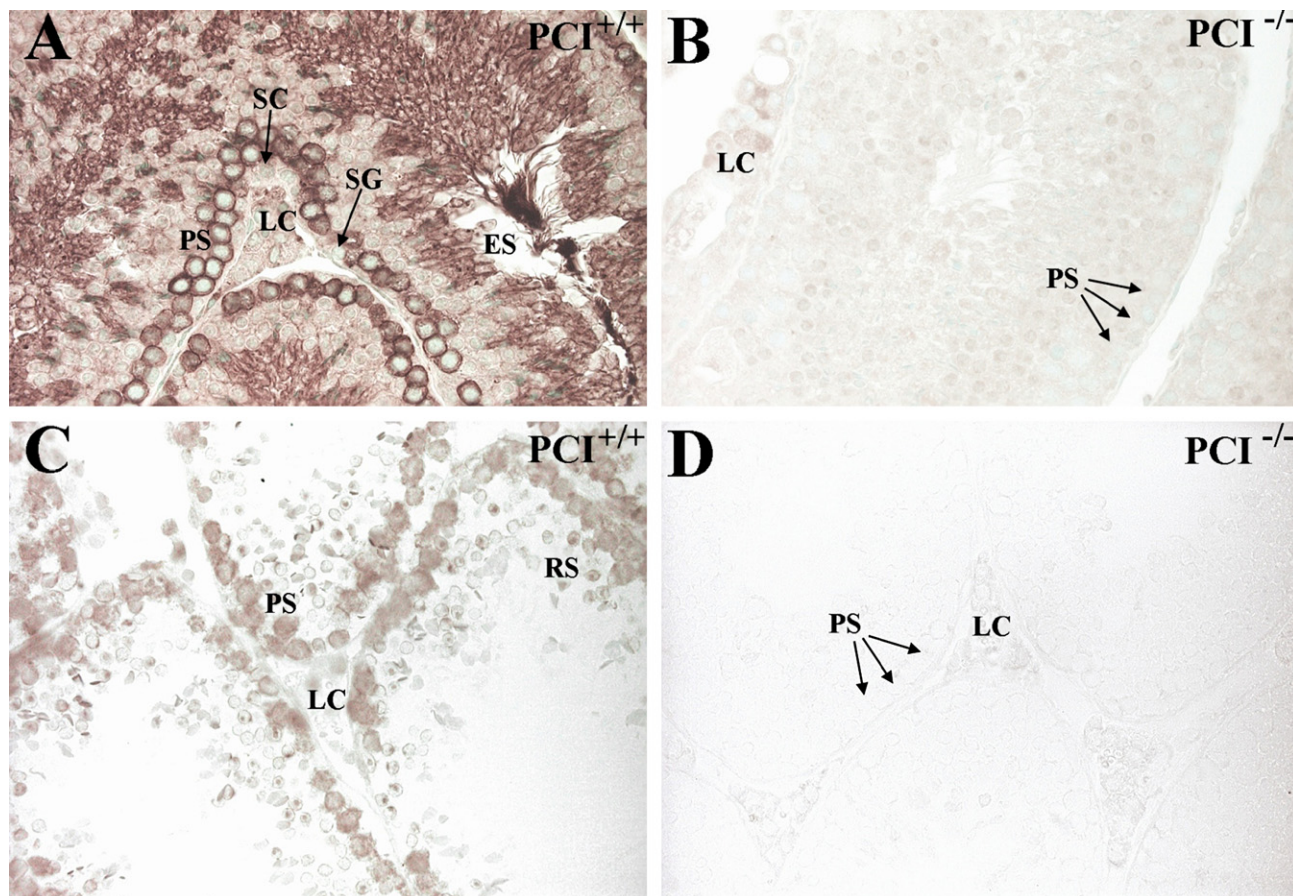
RESULTS

Protein C Inhibitor Localization in Adult Testis and Epididymis

We determined the localization of PCI antigen in reproductive organs of adult mice. Immunohistochemical analysis of testes of wild-type mice revealed signals in two cohorts of spermatogenic and spermiogenic cells (Fig. 1A). The strongest signal was observed in pachytene cells in a stage-dependent manner. Signal in pachytene cells was first detected in stage I, was strongest in the early stages, and was not

FIGURE 1

Protein C inhibitor (PCI) expression in testes of adult mice. (A) In $\text{PCI}^{+/+}$ mice immunohistochemical signal is seen in pachytene spermatocytes and elongated spermatids. Spermatogonia, Sertoli cells, and interstitial Leydig cells are negative. (B) No PCI antigen signal is found in control testis of a $\text{PCI}^{-/-}$ mouse. (C) In situ hybridization to detect PCI mRNA in $\text{PCI}^{+/+}$ testis resulted in strong signal in pachytene cells and in weak signal in round spermatids. (D) No PCI mRNA label on $\text{PCI}^{-/-}$ testis is seen. ES = elongated spermatids; LC = Leydig cells; PS = pachytene spermatocytes; RS = round spermatocytes; SC = Sertoli cells; SG = spermatogonia.



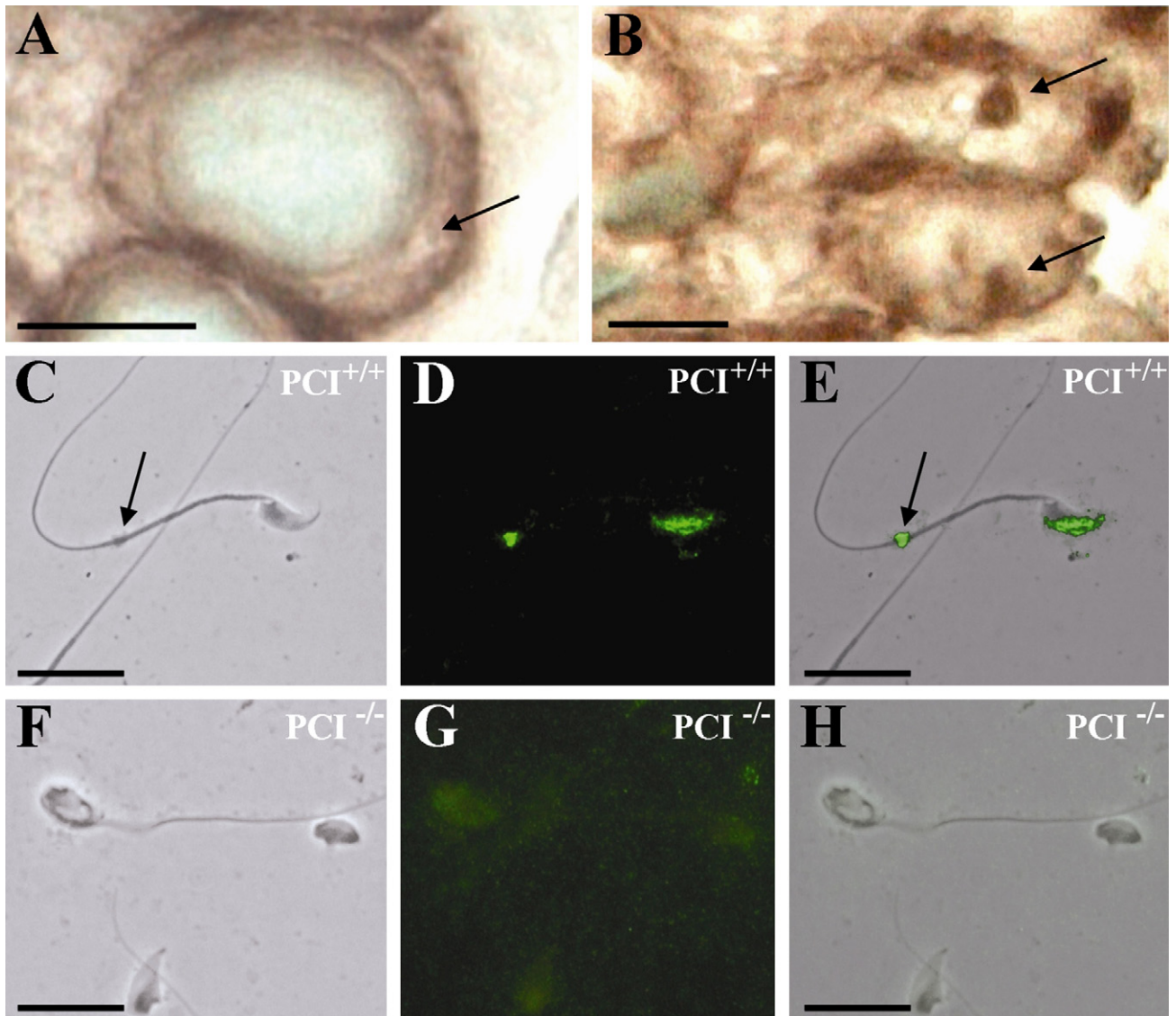
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longer detected from stage IX onward. Signal could also be seen in elongated spermatids where it was detected from step 11 onward until spermiation. Consistent with immunological data, detection of PCI at the mRNA level revealed the strongest signal in pachytene cells, whereas round spermatids were weakly positive. No detectable signal was seen in spermatogonia and Sertoli cells (Fig. 1C). Sections from $PCI^{-/-}$ mice were completely negative (Fig. 1B,D).

We also analyzed the subcellular localization of the PCI signal in different cell types (Fig. 2). In pachytene cells the signal co-localized with the cell membrane and was also present in patchy aggregates in the cytoplasm, possibly corresponding to the Golgi apparatus (Fig. 2A). In elongated spermatids the signal was preferentially found at the cell membrane, in the cytoplasmic droplet, and some label appeared diffusely distributed in the cytoplasm (Fig. 2B). In wild-type epididymal spermatozoa (Fig. 2C–E) the

FIGURE 2

Subcellular distribution of PCI in mouse testis. (A) Signal is predominantly present at the cell membrane in pachytene cells and in patchy aggregates in the cytoplasm (arrow). (B) In elongated spermatids label is preferentially found at the cell membrane and in the cytoplasmic droplets (arrows). (C,D,E) Immunofluorescence on $PCI^{+/+}$ epididymal spermatozoa. Signal is seen in the acrosome and in the cytoplasmic droplet (arrows; C, phase contrast; D, PCI immunofluorescence; E, overlay). (F,G,H) No signal is seen in malformed spermatozoa of $PCI^{-/-}$ mice. Magnification bars, 5 μ m.



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signal was concentrated at acrosomal membranes and in significant amount in the cytoplasmic droplet (Fig. 2C,E). In stage IX, PCI was observed in residual bodies in the process of being incorporated by Sertoli cells (data not shown).

Recent studies reported on the presence of PCI expression in Leydig cells (20, 29) and suggested that PCI might oppose the activity of uPA expressed by Leydig cells (20). In our assays, both at the mRNA and protein level, the majority of Leydig cells, with the exception of a few cells showing weak staining, were devoid of signal, consistent with the absence of morphological abnormalities of Leydig cells in $PCI^{-/-}$ mice (15).

To exclude that the abnormalities observed in the testes of $PCI^{-/-}$ mice are caused by impaired synthesis of testicular hormones, we measured plasma T levels in 12-week-old wild-type and $PCI^{-/-}$ mice. Testosterone levels in $PCI^{-/-}$ mice (646 ± 177 pg/mL; $n = 5$) were not significantly different ($P > .4$) from those determined in $PCI^{+/+}$ mice (853 ± 168 pg/mL; $n = 7$).

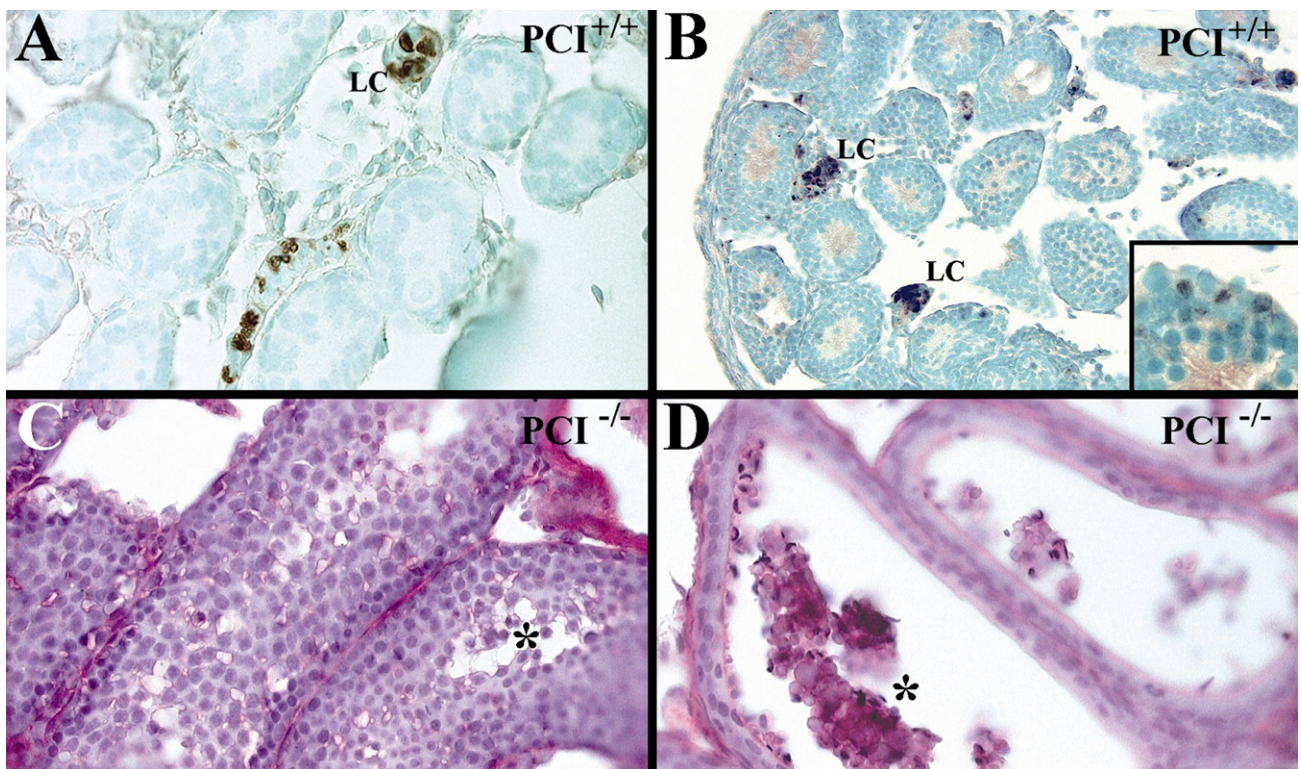
Protein C Inhibitor Distribution in Perinatal and Immature Testis

In newborn mice (day 5) there was no PCI labeling in seminiferous tubules. However, in contrast to adults, Leydig cells were positive at this age (Fig. 3A). The PCI expression in Leydig cells persisted in immature testis with decreasing intensity until puberty. On day 13 the first PCI-positive cells (i.e., pachytene cells) were observed within seminiferous tubules (Fig. 3B) and their number increased at day 22. Therefore, the appearance of PCI in seminiferous tubules seems to occur simultaneously with the onset of spermatogenesis. In mice pachytene cells appear from day 12 onward (30).

Histologic examination of testis and epididymis of $PCI^{-/-}$ mice of 22 days of age revealed the first signs of a similar phenotypic pathology as previously described for adult $PCI^{-/-}$ mice (i.e., presence of partially disrupted seminiferous epithelium; Fig. 3C). Later (34 days postpartum) immature and malformed spermatozoa can be found in the epididymis (Fig. 3D). In contrast, the morphology in 5- and 13-day-old $PCI^{-/-}$ males was normal (data not shown).

FIGURE 3

(A,B) Localization of PCI in immature mouse testis. (A) 5 days postpartum: Leydig cells (LC) are labeled, whereas seminiferous cords are negative. (B) 13 days postpartum: Leydig cells (LC) are stained and some positive cells can be found within seminiferous tubules (*inset*). (C) Morphological analysis of 22 days postpartum $PCI^{-/-}$ testis shows onset of aberrant sperm cell development. Some immature spermatids (*) are seen in lumina of seminiferous tubules. (D) Later (34 days postpartum), many immature and malformed spermatozoa (*) can be found in the epididymal duct of $PCI^{-/-}$ mice.



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Characterization of Mice Deficient in Protein C Inhibitor and Either uPA or tPA

Having confirmed the expression of uPA and tPA in the testis of adult wild-type mice by reverse transcriptase–polymerase chain reaction (RT-PCR; data not shown), we established double knockout mice lacking PCI and uPA or PCI and tPA, and further tested the fertility of the double knockout males. In spite of their normal sexual activity, as revealed by a normal number of copulation plugs, PCI^{-/-} uPA^{-/-} (13 mice of 13 tested) and PCI^{-/-} tPA^{-/-} (16 mice of 16 tested) male mice were infertile. As expected, all PCI^{-/-} uPA^{+/+} (12/12), PCI^{-/-} uPA^{+/-} (16/16), PCI^{-/-} tPA^{+/+} (14/14), and PCI^{-/-} tPA^{+/-} (13/13) mice were also infertile.

Histologic analysis of testis and epididymis of PCI^{-/-} uPA^{-/-} and PCI^{-/-} tPA^{-/-} (Fig. 4A–D) showed similar alterations as previously described for single knockout PCI^{-/-} mice (15).

Furthermore, we measured amidolytic activities of testis extracts derived from PCI^{-/-} tPA^{-/-} and PCI^{-/-} uPA^{-/-} mice using a nonspecific synthetic substrate (S-2288) and S-2444, a substrate previously used to determine uPA activity

(4). They were not significantly different as compared to those found in PCI^{-/-} mice (Fig. 5). Addition of recombinant mPCI (2 μg/mL) to the testis extracts caused statistically significant quenching of the amidolytic activity ($P < .01$) (Fig. 5). Similarly, amidolytic activities of testes of PCI^{+/+} uPA^{-/-} and PCI^{+/+} tPA^{-/-} were comparable to the levels found in PCI^{+/+} mice (not shown).

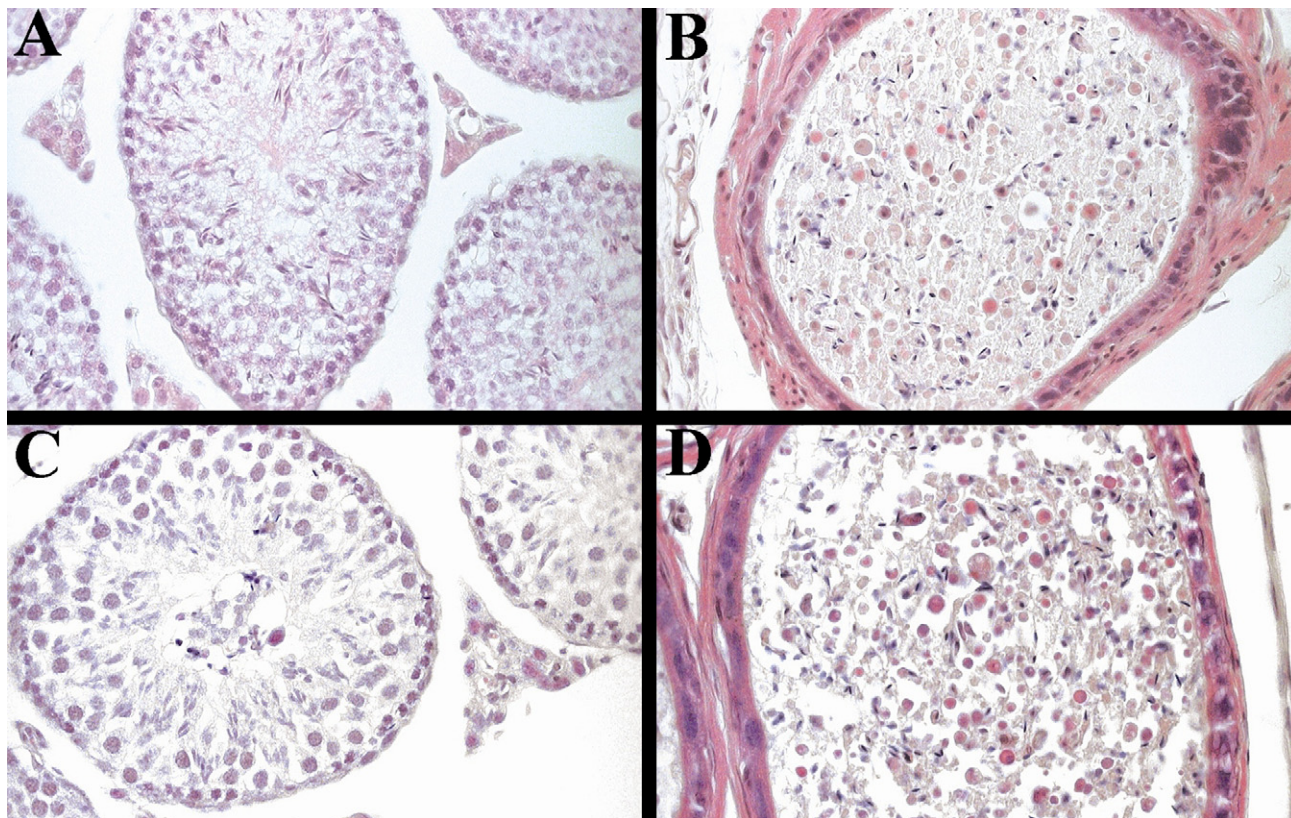
In testis extracts from PCI^{-/-}, PCI^{-/-} tPA^{-/-}, and PCI^{-/-} uPA^{-/-} mice there was no detectable plasmin activity. However, addition of 50 μg/mL of plasminogen caused rapid plasmin formation, as judged from the cleavage of S-2251 (data not shown), indicating residual plasminogen activator activities in the absence of one plasminogen activator and rather low concentration of plasminogen.

DISCUSSION

In a previous study, we analyzed male reproductive organs of PCI-deficient mice (15). These mice were infertile and histologic analysis of their testes revealed premature release of immature germ cells into the lumen of the seminiferous tubules. A significant number of Sertoli cells were clearly damaged.

FIGURE 4

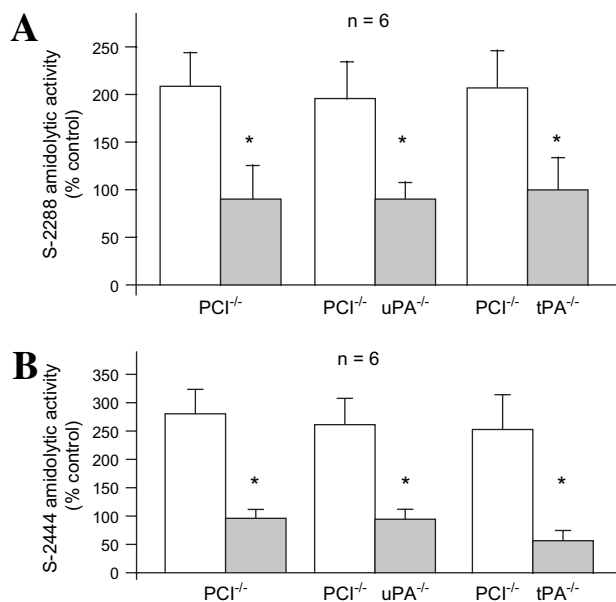
Morphological analysis of reproductive organs of adult double knockout mice lacking PCI and one of its target proteases. (A,C) testis, (B,D) cauda epididymidis, (A,B) combined PCI–tPA deficiency, (C,D) combined PCI–uPA deficiency.



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FIGURE 5

Amidolytic activities of testis extracts prepared from adult $PCI^{-/-}$ and double knockout mice lacking PCI and uPA or tPA . The values are expressed as percentage of the amidolytic activity of testis extracts of $PCI^{+/+}$ mice, in the absence of additional components (*open bars*) or in the presence of $2 \mu\text{g/mL}$ recombinant $mPCI$ (*gray bars*). Amidolytic activity was determined using the substrate S-2288 (**A**) or the substrate S-2444 (**B**). Data are presented as means \pm SEM. The absence of uPA or tPA did not result in significant change of the amidolytic activity in these extracts. Addition of $2 \mu\text{g/mL}$ recombinant $mPCI$ to the testis extracts caused statistically significant ($P < .01$) quenching of the amidolytic activity (*).



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In the epididymis we found an accumulation of immature and malformed spermatozoa.

To analyze further possible causes of such changes, we determined PCI localization in the testis of wild-type mice. Several attempts have recently been made to pinpoint the exact sources of PCI expression in rodent testis; however, the obtained results were controversial (20, 29, 31, 32). In this study we demonstrate expression of PCI in spermatogenic and spermiogenic cells using in situ detection of PCI mRNA or protein. Sertoli cells were not expressing PCI , which indicates that the damage seen in Sertoli cells of $PCI^{-/-}$ mice might be a secondary effect.

The expression of PCI in the basal and apical compartments of the seminiferous tubule wall (i.e., in pachytene and spermiogenic cells) raised the possibility that PCI is involved in the migration of spermatogenic cells from the

basal toward the apical compartment until spermiation. These migration requires proteolysis of extracellular matrix components and reorganization of junctional complexes (for a review, see Siu and Cheng; 33). The involved junctional complexes play crucial roles both in the basal and apical compartments of the seminiferous tubules. Leptotene cells must traverse junctional complexes formed by neighboring Sertoli cells, the blood–testis barrier. In the apical compartments special junctional complexes (apical ectoplasmic specialization and the tubulobulbar complex) are formed that are involved in cell signaling processes before sperm release.

A main function of the blood–testis barrier is to limit the passage of substances to germ cells in such a way that only spermatogonia and preleptotene cells are freely accessible for blood derived or interstitial testicular molecules; all other spermatogenic cells lie within the blood–testis barrier and are supported through Sertoli cells only.

The cellular localization of PCI expression in adult testis and the time point of the onset of PCI expression in immature testis suggest an involvement of PCI in migration events in the basal compartment of the seminiferous tubule wall. The expression in early pachytene stages may indicate a protease-inhibiting effect, which could be necessary after transgression of the germ cells through the blood–testis barrier. However, combined homozygous deficiency of PCI and either uPA or tPA did not rescue fertility. Interestingly, uPA - or tPA -deficient mice were shown to have normal fertility. Reduced fertility was detected only in case of uPA – tPA double knockout mice, possibly because of the overall poor condition of these animals (28) and impaired ovulation in the double knockout females (34).

Inactivation of either the uPA or the tPA gene in PCI -deficient mice did not result in a significant decrease in the amidolytic activity of testis extracts, suggesting that neither plasminogen activator substantially contributes to the overall proteolytic activity in testis. A separate study, where we determined amidolytic activities of testis extracts from acrosin knockout ($acrosin^{-/-}$) mice, revealed that these extracts exhibited less than 10% activity on S-2444 and ~50% activity on S-2288 as compared to extracts from $acrosin^{+/+}$ mice. It is therefore not unlikely that PCI opposes other proteases in the basal compartment. Many serine proteases have recently been identified in human testis and thus far nonidentified orthologues may act in mice as well. These proteases include leydin, expressed by human Leydig cells (35), and members of the TESP family (testicular serine proteases). TESP5 (36) (formerly testisin) is expressed by premeiotic testicular germ cells (37) and in mice in the cytoplasm and on the plasma membrane of round and elongating spermatids (38), TESP1 and TESP2 are expressed in haploid round spermatids (39), TESP3 is localized solely in spermatogenic cells of the testis (40), TESP4 is present in the haploid stages of male germ cells (41). The latter proteases were suggested, at least in the mouse, to participate in the proteolysis of the zona pellucida (ZP) of the oocyte to create a penetration pathway for

spermatozoa (36). Very recently it was shown that the type II transmembrane serine proteases (TTSPs) DESC1 (42) and matriptase-3 (43) are present in testis and epididymis. Furthermore, PCI has been shown to form stable inhibitory complexes with these proteases in vitro. However, the functions of these proteases in the reproductive organs are currently unknown.

The other main site of PCI localization is the apical compartment of the seminiferous tubules. Here, another prominent target serine protease for PCI, acrosin, is present in the acrosome. PCI has been shown to be localized to the acrosome of intact hamster, human (44), and mouse (this study) spermatozoa. PCI is known to inhibit acrosin in vitro (9, 10). Currently, studies are under way to examine whether the observed abnormalities in PCI-deficient mice are caused by unopposed acrosin activity.

Apart from the importance of PCI in spermatogenesis and spermiogenesis, immunohistochemistry results together with the malformation of spermatozoa observed in PCI-deficient mice indicate that PCI may play an important role in further sperm cell maturation during the passage through the epididymis as inferred from the presence of PCI in the cytoplasmic droplet (CD). After completion of acrosome formation, the Golgi apparatus moves to the proximal part of the middle piece and the cytoplasmic protrusion containing the remnant Golgi stacks forms the CD. During the passage of spermatozoa to the cauda epididymidis, the CD shows a typical migration from the proximal middle piece of spermatozoa toward the tail. The CD is finally shed and thought to be digested by epithelial cells of the epididymidal duct. Spermatozoa that fail to shed CDs display immotile, bent flagella as in PCI^{-/-} mice (15). In fact, the presence of CDs in ejaculated spermatozoa is used as a marker of infertility, underlining the fact that the CD is highly important for maturation of spermatozoa. Nevertheless, presently the function of the CD remains enigmatic. The Golgi stacks deteriorate during epididymal migration and markers for protein transport functions are lost from saccules (45). However, markers for glycosylating activities persist and some saccules are in contact with the cell membrane (45). This suggests involvement in plasma membrane modification events, which are known to occur during sperm migration/maturation in the epididymis (46). These membrane modifications are thought to enhance motility and fertility of spermatozoa. The CD has also been suggested to be involved in volume maintenance of spermatozoa during passage through female genital tract (47). Very recently the serine protease TESP5 (36) has been identified in the CD. It remains to be elucidated whether PCI acts as an antagonist to TESP5 or other, so far not identified proteases in the CD, or whether it is directly involved in membrane remodeling or volume regulation.

Our results on PCI expression in Leydig cells are in disagreement with previous reports where PCI expression was found in adult mouse Leydig cells (20, 29). In our study we could find an age-dependent expression of PCI in Leydig cells of immature testis only. Two generations of Leydig cells

have been reported to occur (for a review, see Habert et al. 48). The first generation serves to establish the male identity of the reproductive tract, whereas the second generation is thought to be involved in determination of secondary male sex characteristics and in maintenance of spermatogenesis. A tempting conclusion might be that PCI-positive cells correspond to the “immature” and PCI-negative to the “mature” Leydig cell population. Whatever the role of PCI in Leydig cells may be, it appears that the loss of PCI in Leydig cells can be compensated for because PCI^{-/-} males show apparently undisturbed testis development until the onset of meiosis. Moreover, we show here that PCI-deficient mice exhibit plasma T levels comparable to those of wild-type mice.

In conclusion, we have demonstrated that the spermatogenic cells of the seminiferous tubules are the main source of PCI in mouse testis and the expression pattern of PCI suggests involvement in multiple processes of sperm cell differentiation and maturation in both testis and epididymis. Our results—using double gene-deficient mice—suggest that PCI may have additional functions than just opposing uPA or tPA, perhaps by inhibition of acrosin or additional known or so far unidentified proteases. Future studies are needed to address these questions.

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