Deletion of the Gene Encoding Proprotein Convertase 5/6 Causes Early Embryonic Lethality in the Mouse

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PC5 belongs to the proprotein convertase family and activates precursor proteins by cleavage at basic sites during their transit through the secretory pathway and/or at the cell surface. These precursors include prohormones, proreceptors, growth factors, adhesion molecules, and viral glycoproteins. The Pcsk5 gene encodes two alternatively spliced isoforms, the soluble PC5A and transmembrane PC5B. We have carefully analyzed the expression of PC5 in the mouse during development and in adulthood by in situ hybridization, as well as in mouse tissues and various cell lines by quantitative reverse transcription-PCR. The data show that adrenal cortex and intestine are the richest sources of PC5A and PC5B, respectively. To better define the specific physiological roles of PC5, we have generated a mouse Pcsk5Δ4-deficient allele missing exon 4 that encodes the catalytic Asp173. While Δ4/+ heterozygotes were healthy and fertile, genotyping of progeny obtained from Δ4/+ interbreeding indicated that Δ4/Δ4 embryos died between embryonic days 4.5 and 7.5. These data demonstrate that Pcsk5 is an essential gene.

A family of subtilisin-like proteases is responsible for the processing of precursor proteins that transit through the secretory pathway of mammalian cells. This family comprises nine members: PC1/3, PC2, furin, PC4, PACE4, PC5/6, PC7, SKI-1/S1P, and NARC-1/PCSK9 (29, 30, 32). The first seven proprotein convertases (PCs) are more closely related to each other than to SKI-1 and NARC-1. They belong to the kexin subfamily (SKI-1 and NARC-1 belong to the pyrolysin and proteinase K subfamilies, respectively) and share a cleavage specificity for basic motifs of the type (K/R)-(X)n-(K/R) ↓, in which n = 0, 2, 4, or 6. The basic residues present at the C terminus of the smaller product are usually trimmed out by a carboxypeptidase that generates an active molecule, although, in some cases, additional posttranslational modifications may be required (for a review, see reference 13). PCs themselves are synthesized as precursors. Autocatalytic cleavage at one or two intramolecular sites allows the activation of thezymogen by removal of its N-terminal prosegment. The latter acts as an intramolecular chaperone for folding of the enzyme and as a transient in trans inhibitor. PC1/3 and PC2 are present in neuroendocrine tissues, where they contribute to activate hormones or neuropeptides. PC4 is restricted to gonads. Although widespread, the other basic motif-specific PCs exhibit different and contrasting expression patterns (31). In cell lines, coexpression of candidate precursors with these PCs revealed that, in many cases, substrates preferentially processed by PC5 are also efficiently cleaved by furin, PC7, and to a lesser extent by PAC4. This is the case in some α chains of integrins (1); the neural adhesion molecule L1 (17); transforming growth factor β-like proteins (10, 36), including Müllerian inhibiting substance (22); the receptor protein tyrosine phosphatase RPTPμ (5); membrane-bound metalloproteinases such as ADAM-17 (34); and possibly the membrane type-1 matrix metalloproteinase MT1-MMP (39). This ex vivo redundancy probably reflects the ability of these enzymes to compensate for each other in vivo in a tissue-specific manner. In this context, the knockout of these enzymes is very informative. Furin expression is essential during development since its knockout leads to embryonic lethality around embryonic day 11 (E11) (28). However, its liver-specific knockout had no effect on the viability of mice, and in this organ, several candidates for substrates of furin were still well processed (27).

The mouse PC5 gene, Pcsk5, encodes two isoforms generated by alternative splicing, PC5A and PC5B (Fig. 1). They share their first 20 exons, which encode the signal peptide, prosegment, catalytic domain, and N-terminal region of the Cys-rich domain (CRD). A specific additional exon (21A) encodes the last 38 residues of PC5A, while exons 21B to 38 encode the last 1,000 residues of PC5B. PC5A and its closest family member, PACE4, share similar C-terminal CRDs. The latter were recently shown (26) to be responsible for the interaction of these enzymes with cell surface heparan sulfate proteoglycans (HSPGs) via tissue inhibitors of metalloproteinases (TIMPs). This agrees with the cell surface localization of most PC5 candidate substrates.

To date, only furin (28) and SKI-1/S1P (40) have been shown to be essential genes. Knockout of PC1 (42), PC2 (14), PC4 (21), PAC4 (6), or PC7 (37) genes resulted in viable mice to various degrees, with no or relatively mild phenotypes. To discriminate between substrates specific to PC5 or common to PC5 and other PCs, we set up a strategy aimed at deleting a 4.5-kb genomic fragment containing exon 4 that encodes the catalytic Asp173. Our data show that the absence of catalytically
FIG. 1. Isopforms PCsA and PCsB and their processing. Exons 1 to 20 of the mouse Pcsk5 gene encode the signal peptide (SP; amino acids [aa] 1 to 34), prosegment (aa 35 to 116), catalytic domain (aa 117 to 458), P domain (aa 459 to 600), and N-terminal part of the Cys-rich domain (aa 638 to 877). Exon 21A, specific to PCsA, encodes its last 38 residues, while the 18 additional exons of PCsB (21B to 38) extend the Cys-rich domain up to a transmembrane domain (TMD; aa 1769 to 1790) followed by an 88-aa-long cytosolic tail (CT). The residues of the catalytic triad comprise Asp1910 (boxed D), encoded by exon 4; His1249 (H); and Ser1833 (S). The position of the oxygenation hole Asn1820 (N) is also shown. Arrows in the prosegment indicate autotaxic cleavage sites, and the arrowhead points to the intracellular cleavage site, generating an ~68-kDa PCsA-ΔC form. White ellipses emphasize the putative N-glycosylation sites (Asn227, 383, 667, 754, 804, 854, 951, 1016, 1220, 1317, 1523, 1711, and 1735).

MATERIALS AND METHODS

ISH study. In situ hybridization (ISH) was performed as follows. Ten- to 12-μm-thick cryosections were prepared from embryos at different stages of development or from adult mice (whole-body sections), fixed in 4% formaldehyde, and hybridized as previously described (20) with mouse sense (negative control) and antisense cRNA probes. The latter probes (807 bases) correspond to the mouse PCs coding region for residues 80 to 348 and were synthesized using 35S-UTP and 35S-CTP (>1,000 Ci/mmol; Amersham Biosciences, Piscataway, NJ).

Quantitative analysis of the PCs transcripts in cell lines and tissues. Cells, which were washed three times with phosphate-buffered saline (PBS), and fresh mouse tissues were directly incubated with Trizol reagent (Invitrogen, Burlington, Ontario, Canada). Total RNA was extracted as recommended by the manufacturer. RNA integrity, cDNA synthesis, and quantitative PCR (QPCR) were performed as previously described (11). For each tissue or cell line, one to three RNA samples were analyzed at least twice in triplicates. Specific primers sitting on different exons were used for the simultaneous amplification of the normalizing cDNA for ribosomal protein S16 (11) and the cDNA for either PCsA or PCsB, or PCsB messenger: 5′-GCTACCATGGCTCAATTAAATGCGACA versus 5′-TTTACCAGTACCTTGGTTT CCAAG and 5′-GGCTCTCAGAAGGTTGAGTCCAGA versus 5′-CTACAGAGGCAACAGCAGACT for detection of the WT and Δ4 alleles, respectively.

Animals. PCsC wild-type and heterozygous mice in the C57BL/6 background (minimum of five successive backcrosses) were housed in a 12-h light-dark cycle and fed a standard laboratory diet (Charles River rodent chow 5075; Purina Mills, St. Louis, MO). Mice were genotyped by PCR analysis of tail DNA. The institutional Animal Care and Ethics Committee approved all procedures described below.

PCR genotyping of embryos. PCsC4/- females were superstimulated with 5 IU of pregnant mare serum gonadotropin (PMSG) and superovulated with 5 IU of human chorionic gonadotropin (hCG) 47 h later (23). They were then mated to 10 males, and embryos were either collected the next morning or dissected in the ampulla of the oviduct or dissected at E7.5. After a brief treatment with ~300 mg/ml hyaluronidase, E0.5 embryos were washed in potassium simplex optimized medium (KSMO) containing 20 mM HEPES, pH 7.4, transferred in 20-μl droplets of KSO (25 to 30 embryos/droplet) overlaid with mineral oil, and incubated for 5 days at 37°C (3). Each blastocyst was recovered in 5 μl of PBS and incubated for 1 h at 95°C. Because of the small amount of DNA available, genotypes were analyzed on a nested PCR using the primers used for genotyping (see above) and, for nesting, the following primers: 5′-CACCTGGCTCATAATAGCGACA versus 5′-TTTACCAGTTACCTTGGTTT CCAAG and 5′-GGCTCTCAGAAGGTTGAGTCCAGA versus 5′-CTACAGAGGCAACAGCAGACT for detection of the WT and Δ4 alleles, respectively.

Cell culture, transfections, Western blotting, and antibodies. Human embryonic kidney HEK293 cells were grown in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal calf serum (Canadian Life Technologies, Burlington, Ontario, Canada) at 37°C in 5% CO2. Cells (1 × 106 plated on 35-mm plates) were transfected with plasmid or pCAGGS vectors by electroporation (5). Cells were washed twice with PBS and incubated for 1 h at 95°C. Western blot analysis and activity measurements. Western blot analysis and activity measurements. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters (Hybond-ECL; Amersham Biosciences). After blocking with 5% dry milk in Tris-buffered saline containing 0.05% Tween 20, filters were incubated with primary antibodies (Cell Signaling Technology, Beverly, MA) at 1:1,000 for 1 h at room temperature, followed by 1-h incubation with horseradish peroxidase-conjugated donkey anti-goat (Jackson ImmunoResearch, West Grove, PA) or 1:1,000 horseradish peroxidase-conjugated donkey anti-rabbit (Jackson ImmunoResearch, West Grove, PA) for 1 h before washing extensively with PBS and incubating at room temperature for 1 h with 1:5,000 horseradish peroxidase-conjugated donkey anti-goat (Jackson ImmunoResearch, West Grove, PA) or 1:5,000 horseradish peroxidase-conjugated donkey anti-rabbit (Jackson ImmunoResearch, West Grove, PA) for the determination of specific bands.
In vitro PC5 activity. Supernatants (60 μl) of HEK293 cells transfected with the empty or PC5A- or PC5A4-expressing vectors were incubated in presence of a mixture of 50 μM fluorogenic substrate pyruvic acid-RTKR-7-amido-4-methylcoumarin, 2 mM CaCl₂, 50 mM Tris (pH 7), and 100 μM β-mercaptoethanol in a total volume of 100 μl after a 20-min preincubation of the 40-μl mixture. Fluorescence emission was detected at 460 nm, as described previously (25).

RESULTS

Tissue distribution of PC5 during development and in adulthood. Since the present work deals with inactivation of the Pcsk5 gene, we have characterized by ISH and/or QPCR the expression of mouse PC5 during development and in adulthood (Fig. 2). The first stage that we were able to examine by ISH was E9.5, where labeling was observed in the somites, bulb of umbilical cord, and lung bud. At E10.5 to E11.5, the first bronchial arch, wall of bulbus cordis, and the nasal pit were also labeled, as well as the pancreatic primordium at E11.5. The adrenal primordium, visible at E14.5, is already one of the richest expression sites of PC5/6. By E15.5, the PC5 pattern of expression becomes similar to that of the adult, with strong labeling in small intestine, kidney, and lung. In the developing brain, the future hippocampus (CA3 region) and dorsal raphe are well labeled (E14.5 to E15.5). The strong labeling of incisors, already observed at E19.5 (data not shown), is maintained at P10 and in the adult. After birth, at P4 (data not shown) and at P10, the adrenal cortex is strongly labeled. Note also that several layers of the brain cortex are PC5 positive at P10. Finally, PC5 is rich in vasculature (P10 and adult), including the aorta, which is labeled as early as E14.5.

Many of these data were confirmed and extended by QPCR estimation of the mRNA expression of each PC5 isoform in adult mouse tissues (Fig. 3). Among the tissues examined, the adrenal was the richest source of PC5, with four- to sixfold-higher levels than the small intestine. Interestingly, with the exception of the liver, which contains equal levels of each isoform, the tissues can be distributed in two groups. The first one, in which PC5A dominates (88 to 100%), comprises the adrenal, ovary, uter, lung, aorta, brain cortex, muscle, heart, stomach, cerebellum, pituitary, testis, and spleen. The other
group is characterized by elevated levels of PC5B (74 to 92%) and comprises the jejunum, ileum, kidney, duodenum, and colon. Finally, we estimated PC5 levels in various human and mouse cell lines. While there is no strict correlation between PC5 levels in cell lines and the tissues from which they originate, it is notable that the most highly expressing line, Y1, is derived from a tumor of the adrenal cortex. Two cell lines, HepG2 and HeLa, seem totally devoid of PC5 expression and may thus constitute valuable tools to analyze the requirement of PC5 activity for the processing of candidate substrates.

**Production of PC5-null mice.** A Pcsk5 allele was modified in ES cells by homologous recombination using a targeting vector containing about 12 kb of genomic DNA, in which the exon 4 is flanked with two loxP sites (open triangles) and a PGK-neo cassette. ES cells were transfected with the NotI-KpnI insert (solid line). The expected neo allele is shown. Mating of heterozygotes bearing a Pcsk5<sup>neo</sup> recombinant allele with transgenic mice expressing the Cre recombinase generated progeny in which Cre was expressed and excised the sequences between the loxP sites. The generated Pcsk5<sup><i>Δ4</i></sup>, inactivated allele misses ~4.5 kb of genomic DNA, including exon 4. E, EcoRI; RV, EcoRV; Edl, Ecl136II; H, HindIII; K, KpnI; N, NotI; X, XhoI.

![FIG. 4. Pcsk5 knockout strategy.](http://mcb.asm.org/)

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![FIG. 5. Genomic analysis of 5B10-derived mice.](http://mcb.asm.org/)

Genomic DNA (6 μg) from C57BL/6 (+/+) and BstEII digestion, wild-type and Δ4/+ alleles generate 4.2- and 11-kb fragments, respectively. The lanes marked “1 kb” and “λ/HindIII” contain specific ladders.
The Δ4/Δ4 genotype is embryonic lethal. No homozygous mice were obtained from Δ4/+ intercrosses. Out of 204 mice, 65% were Δ4/+ and 35% were +/+ as expected if the Δ4/Δ4 genotype leads to embryonic death, and no bias was observed between male and female progeny (Table 1, data for 3 weeks). To determine the stage of lethality, heterozygous males were mated with stimulated females and the 35 embryos isolated at E0.5 were genotyped: 77% were Δ4/+ and 23% were Δ4/Δ4, indicating that lethality had occurred before this developmental stage. In a second step, 36 embryos were carefully dissected at E7.5 and their genotyping revealed the absence of any Δ4/Δ4 embryos. Finally, zygotes were collected in the ampulla of the oviduct at E0.5 and cultured for 5 days at 37°C to obtain blastocysts. Their genotyping revealed a Mendelian distribution of the +/+ Δ4/+ and Δ4/Δ4 genotypes (Table 1), indicating that oocyte fertilization and cell division in the early embryo were efficiently achieved, independent of the PC5 genotype. Altogether, our data suggest that embryonic death likely occurs between implantation (~E4.5) and E7.5.

### Table 1. Genotyping of Δ4/+ × Δ4/+ progeny

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cultured at E0.5</th>
<th>E7.5</th>
<th>E14.5</th>
<th>3 wk</th>
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<tbody>
<tr>
<td>+/+</td>
<td>13 (24)</td>
<td>9 (25)</td>
<td>8 (23)</td>
<td>72 (35)</td>
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<tr>
<td>Δ4/+</td>
<td>27 (49)</td>
<td>27 (75)</td>
<td>27 (77)</td>
<td>132 (65)</td>
</tr>
<tr>
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<td>15 (27)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>55 (100)</td>
<td>36 (100)</td>
<td>35 (100)</td>
<td>204 (100)</td>
</tr>
</tbody>
</table>

Analysis of Δ4/+ PCS mRNA and protein. To follow the fate of the PC5 mRNA, QPCR analysis was performed on RNA isolated from either +/+ or Δ4/+ livers. Primers hybridizing to exons 3 and 5 were used since connection of neighboring exons was reported to occur upon exon deletion (4, 14). Indeed, the presence of a lower band (95 bp) in Δ4/+ samples and sequencing of the corresponding DNA revealed that exons 3 and 5 are fused in the mRNA deriving from the recombinant allele (Fig. 6A). In addition, quantitative analysis showed that the Δ4 mRNA is as well produced as the wild-type one (data not shown), indicating that heterozygotes have 50% of mRNA coding for active PC5 compared to that in wild-type mice. Since exons 3 and 5 share the same reading frame, exon 4 deletion may lead to the synthesis of a truncated PC5Δ4 protein lacking 48 residues (from His140 to Tyr187). To check the cellular fate of PC5Δ4, a recombinant pIRESC2-EGFP vector containing a cDNA encoding mouse PC5A C-terminally tagged with V5 was modified to mimic the exon 4 deletion (~144 bp). In transfected HEK293 cells and their media, PC5Δ4 showed a slightly lower apparent molecular mass than PC5A, compatible with the loss of 48 residues (~5 kDa; Fig. 6B). Microsequencing revealed that proPC5A is the major intracellular species (25). Since similar ratios of PC5A to PC5Δ4 (experiment done three times) were revealed by V5 Western blotting, without or with prior immunoprecipitation with a PCS prosequence-specific antibody, we can deduce that proPC5Δ4 is predominant in the cell. In the medium of wild-type PC5A, in addition to the ~110-kDa PC5A, an ~50-kDa C-terminal product (V5 positive) was observed, representing the cleaved CRD as previously reported (26). Taking into account the 10-fold-higher loading for the media.

![PC5A expression and activity](Image)

**FIG. 6.** PC5Δ4 expression and activity. (A) QPCR analysis of PC5 mRNA from +/+ and Δ4/+ livers using primers located in exons 3/5 revealed a unique 239 bp product in +/+ liver and an additional 95-bp one in Δ4/+ liver resulting from an altered splicing connecting exons 3 and 5. (B) HEK293 cells were transfected with the pIRESC2-EGFP vector empty (V) or expressing wild-type PC5A or PC5Δ4, both C-terminally V5 tagged. Cell extracts (20 μg) and media (40 μl out of 1 ml) were analyzed by anti-V5 Western blotting (WB) prior (left panel) or after (right panel) immunoprecipitation (IP) with a prosequence antibody (anti-ppPC5). (C) Online PCS activity in relative fluorescence units (RFU) was measured by incubation of 60 μl of medium with the fluorogenic substrate Pyr-RTKR-AMC. Note the absence of activity in PC5Δ4 and control V media. (D) HEK293 cells were cotransfected with 0.1 μg of a vector expressing PDGF-A carrying a C-terminal V5 tag and 0.9 μg of a vector expressing either no product (V), α1-PDX, the prosequence of furin (ppFur), the prosequence of PC5 (ppPC5), or PC5Δ4. Media (2 μl out of 1 ml) were analyzed by Western blotting using V5 antibodies.
than for the cell lysates, we estimated that while most of PC5A was secreted, only ~5% of PC5Δ4 was found in the medium. Although the same quantity of DNA was used for transfection, the total amount of PC5Δ4 detected was much lower than that of PC5A, suggesting that PC5Δ4 was largely degraded. Interestingly, the secreted fraction of PC5Δ4, which migrated faster than PC5A, underwent zymogen processing since otherwise proPC5Δ4 would have been ~4 kDa heavier than PC5A. This indicates that proPC5Δ4 can exit from the endoplasmic reticulum and be processed in trans. The inactivity of PC5Δ4 in HEK293 media, although expected from the loss of one of the catalytic residues, was assessed using the fluorogenic substrate Pyr-RTKR-AMC. PC5Δ4 activity was not detected since it was found equivalent to that of control medium (empty vector), which was at least 30-fold lower than that obtained from PC5A (Fig. 6C).

Embryonic lethality is expected to result from the loss of PC5 activity. However, since a truncated form of PC5 is produced from the Δ4 allele, the possible in trans inhibitory effect of PC5Δ4 on the activity of the other PCs, namely furin, whose knockout is also embryonic lethal, was assessed. For this, the extent of cleavage of a V5-tagged proPDGF-A, which is efficiently processed by furin in HEK293 cells (33), was estimated in the absence or in presence of overexpressed α1-PDX, the prosegment of furin, or the prosegment of PC5 or PC5Δ4 (Fig. 6D). No inhibitory effect of PC5Δ4 could be detected while the PC inhibitors, α1-PDX and the furin prosegment, efficiently inhibited proPDGF-A processing. In addition, only a very mild inhibitory effect of the PC5 prosegment was detected. Finally, even under overexpression conditions, PC5Δ4 is not inhibitory.

**DISCUSSION**

In the present work, we carefully documented PC5 mRNA expression during mouse development, in adulthood, and in human and mouse cell lines (Fig. 2 and 3). The data agree with and extend those obtained in rats, which show that PC5 distribution is distinct and often complementary to those of furin and PACE4 (8, 9, 19, 31, 41). PC5 is richest in the adrenal cortex, small intestine, kidney, ovary, uterus, lung, aorta, and brain cortex. Our data revealed that, except in the liver where both isoforms are equally expressed, PC5A is the major isoform in most tissues (87 to 100%), and only the intestine and kidney show a predominance of PC5B (74 to 92%). The two isoforms (Fig. 1) were shown to have distinct properties: (i) they do not show similar efficiencies toward various substrates in cell lines (2, 16, 18), and (ii) the transmembrane PC5B is restricted to the constitutive secretory pathway (7) and cycles between the Golgi and the cell surface (38), while the soluble PC5A is sorted to both regulated and constitutive secretory pathways (7). However, PC5A can also be associated with the plasma membrane via a ternary complex involving its CRD, TIMPs, and HSPGs (26). Thus, the distinct tissue distribution of the two isoforms may not be fortuitous but rather may reflect isoform-specific functions.

In order to assess the in vivo functions of PC5, we developed a knockout strategy that led to the inactivation of both isoforms involving deletion of the common exon 4 encoding the catalytic Asp173 (Fig. 4). While heterozygote mice harboring a Pcsk5<sup>Δ4</sup> allele (+/Δ4) are healthy and fertile, homozygosity leads to early embryonic lethality that occurs between the blastocyst implantation stage (E4.5) and E7.5 (Table 1), demonstrating that *Pcsk5* is an essential gene.

Deletion of exon 4 resulted in a truncated mRNA in which exons 3 and 5 are fused in frame (Fig. 6A). Cellular overexpression of a cDNA mimicking the exon 4 deletion resulted in low levels of PC5Δ4. In addition, the protein was mostly found in the cell as a zymogen, whereas only a small fraction (~5%) was processed in trans and secreted (Fig. 6B). Thus, the level of the inactive PC5Δ4 protein produced from the *Pcsk5<sup>Δ4</sup>* allele is expected to be negligible. Indeed, the available antibodies directed against PC5 did not detect endogenous PC5Δ4 in heterozygotes (data not shown). Furthermore, media obtained from HEK293 cells overexpressing PC5Δ4 revealed no in vitro activity as compared to that of the wild type (Fig. 6C), and there was no inhibitory effect on the ex vivo processing of pro-platelet-derived growth factor A (proPDGF-A) by endogenous PCs (Fig. 6D). Taken together, these data demonstrate that the residual expression of PC5Δ4 is not responsible for the observed lethality. Similarly, PC2 inactivation through disruption of exon 3 also led to an alternative splicing producing proPC2α3 that remained as an inactive zymogen in vivo (14). Finally, using the FipfloP system, the recent production of a conditional PC5 knockout in which exon 1 was targeted led, in the presence of a CMV-Cre transgene, to the absence of PC5 transcripts and resulted in early lethality of Δ1/Δ1 embryos (Essalmani et al., unpublished data).

Using an antisense morpholino oligonucleotide (24), it was shown that uterine expression of PC5 is required for deciduization at the implantation site in the mouse, but no information is available concerning the requirement for embryonic expression of PC5 at the same stage. Since the litter size does not differ between +/+ and +/Δ4 females (data not shown), we can rule out that the embryonic lethality observed upon intercrosses of +/Δ4 mice is due to a maternal PC5 deficit. This indicates that early de novo synthesis of PC5 in either embryonic or extraembryonic cells is essential. Whether embryonic expression of PC5 is essential for implantation or at later stages remains to be defined. It has been mentioned in a review article (35), but without experimental details, that PC5B inactivation led to embryonic death at E10.5 to E11.5, i.e., at least 3 days after that observed herein upon PC5A+B inactivation. This suggests that PC5B is essential, but the lethality observed in its absence may be delayed by PC5A. Is PC5A also essential? If not, then the key role(s) played by PC5B may reside in extraembryonic giant trophoblasts (41) or unidentified early progenitor cells. In that context, the extensive expression at E9 of PC5, but not furin and PACE4, in the rat maternal-embryonic junction, whose trophoblast giant cells derive from the embryo, is compelling (41). Finally, the elucidation of the critical cognate substrate(s) will require the identification of the exact stage and cell types associated with the observed lethality. Future studies will determine if the essential role of PC5 observed during early development also applies to later stages. The available conditional knockout will allow the induction of PC5 inactivation at different stages as well as in specific tissues, thereby leading to the identification of the substrates and physiological roles of PC5.
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REFERENCES


