In vivo functions of the proprotein convertase PC5/6 during mouse development: Gdf11 is a likely substrate

Rachid Essalmani, Ahmed Zaid, Jadwiga Marcinkiewicz, Ann Chamberland, Antonella Pasquato, Nabil G. Seidah, and Annik Prat*

Laboratory of Biochemical Neuroendocrinology, Clinical Research Institute of Montreal, 110 Pine Avenue West, Montreal, QC, Canada H2W 1R7

Edited by Donald F. Steiner, University of Chicago, Chicago, IL, and approved January 31, 2008 (received for review October 3, 2007)

The proprotein convertase PC5/6 cleaves protein precursors after basic amino acids and is essential for implantation in CD1/129/Sv/C57BL/6 mixed-background mice. Conditional inactivation of *Pcsk5* in the epiblast but not in the extraembryonic tissue bypassed early embryonic lethality but resulted in death at birth. PC5/6-deficient embryos exhibited Gdf11-related phenotypes such as altered anteroposterior patterning with extra vertebrae and lack of tail and kidney agenesis. They also exhibited Gdf11-independent phenotypes, such as a smaller size, multiple hemorrhages, collapsed alveoli, and retarded ossification. *In situ* hybridization revealed overlapping PC5/6 and Gdf11 mRNA expression patterns. *In vitro* and *ex vivo* analyses showed that the selectivity of PC5/6 for Gdf11 essentially resides in the presence of a P1′ Asn in the RSRR↓N cleavage motif. This work identifies Gdf11 as a likely *in vivo* specific substrate of PC5/6 and opens the way to the identification of other key substrates of this convertase.

Furin-like | Meox2-cre | Pcsk5

In mammals, seven proprotein convertases (PCs) cleave protein precursors at basic sites during their transit through the secretory pathway and/or at the cell surface (1, 2). PC-mediated cleavages contribute to generating active products (hormones, neuropeptides, growth factors, receptors, and viral glycoproteins). In some cases, they lead to substrate inactivation (endothelial and lipoprotein lipases) (3, 4). Among the seven basic amino acid-specific PCs, furin, PC5/6, PACE4, and PC7 are ubiquitous or widely distributed, although they exhibit characteristic patterns of expression (5). However, in *ex vivo* coexpression experiments, a given substrate is often cleaved by more than one of these four secretory proteases, which renders difficult the identification of their specific substrates *in vivo*.

PC5/6 is encoded as two splicing isoforms. PC5/6A contains 21 exons and, in PC5/6B, the 21st exon is replaced by 18 additional exons (6, 7). PC5/6A exhibits a C-terminal Cys-rich domain (CRD). In PC5/6B, this domain is extended and followed by a transmembrane domain and a cytosolic tail. Although PC5/6B can be shed and thus released from the membrane, the soluble PC5/6A can interact with cell surface proteoglycans because of its CRD (8). PC5/6B transcripts dominate in the intestine and kidney, whereas PC5/6A is the major isoform in most other tissues assessed, except in the liver, where the transcripts are expressed in equivalent amounts (9).

Because of the redundancy observed *ex vivo* among the basic amino acid-specific PCs, it is important to define their *in vivo* properties. Knockouts (KOs) of the PCs (10, 11) resulted in strong phenotypes, except for PC7, and demonstrated that furin and PC5/6 are essential. Inactivation of the furin gene, *Pcsk3* (PC subtilisin kexin type 3), resulted in embryonic dismorphism with the absence of axial rotation and heart looping and led to embryonic death around embryonic day (E)11 (12). Inactivation of the PACE4 gene, *Pcsk6*, resulted in 25% lethality before E15.5. PACE4$^{-/-}$ embryos frequently exhibit anterior truncations and/or specific laterality defects, such as cyclopia, mislocalization of organs and cardiac malformations (13). Our group showed that *Pcsk5* inactivation, by removal of exon 4 (Δ4) that encodes the catalytic Asp, led to embryonic lethality between E4.5 and E7.5 (9).

To bypass the PC5/6$^{-/-}$ embryonic lethality at the implantation stage and to better define the functions of PC5/6, we decided to develop a conditional approach for *Pcsk5* inactivation. *In situ* hybridization studies indicated that PC5/6 was not significantly expressed in the fetus itself before E7.5 (14). We thus hypothesized that PC5/6 was essential in embryonic cell lineages that give rise to the extraembryonic tissue, which ensures the survival of the embryo in the uterine environment. We show here that using the *Meox2-cre* transgene (Tg), which directs Cre expression in the epiblast at E5 but not in extraembryonic lineages, we were able to bypass early embryonic lethality and identify an essential role of PC5/6 in the regulation of the anteroposterior axial patterning, through the processing of the growth and differentiation factor 11 (Gdf11).

Results

PC5/6 Is Expressed in the Extraembryonic Lineage. The inability of mutant embryos to go through the implantation stage often resides in deficiencies in the extraembryonic lineages (15). We thus assessed the PC5/6 mRNA expression in extraembryonic cells at early stages by *in situ* hybridization (Fig. 1). The analysis of embryos *in utero* revealed that, at E4.5, PC5/6 was strongly expressed in decidual cells around the implantation site but not in the luminal epithelium of the uterus or the embryo [data not shown (16)]. At E6.5, PC5/6 expression was detected in large nuclei typical of the trophoblast giant cells, as reported (17). No labeling was observed in the embryo proper. We thus hypothesized that PC5/6 was essential in the extraembryonic tissue.

Epiblast-Specific KO of *Pcsk5*. Because, by *in situ* hybridization, PC5/6 was not strongly expressed at early stages in the embryo proper (data not shown), we decided to perform an epiblast-specific KO of PC5/6 in an attempt to discover PC5/6-related phenotypes later in development or at adulthood. A *Pcsk5* conditional allele was generated by framing the proximal promoter and exon 1 with *loxP* sites [supporting information (SI) Fig. S1]. In the presence of the *CMV-cre* Tg, *Pcsk5*$^{\Delta 1/+}$ mice were

Author contributions: R.E., A.Z., N.G.S., and A. Prat designed research; R.E., A.Z., J.M., A.C., A. Pasquato, and A. Prat performed research; J.M. contributed new reagents/analytic tools; R.E., N.G.S., and A. Prat analyzed data; and N.G.S. and A. Prat wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

*To whom correspondence should be addressed. E-mail: prata@ircm.qc.ca.

This article contains supporting information online at www.pnas.org/cgi/content/full/0709428105/DCSupplemental.

© 2008 by The National Academy of Sciences of the USA

Fig. 1. *In situ* hybridization analysis of PC5/6 expression *in utero* at E6.5. (*A* and *B*) Paraffin sections were hybridized with a ^{35}S-labeled cRNA PC5/6 an antisense (*A*) or sense (*B*) probe and were autoradiographed. (*C* and *D*) Sections were stained with cresyl violet and dipped in an autoradiography emulsion. (*D*) A higher magnification of the squared area in *C* showed a strong labeling on the large nuclei of the embryonic giant trophoblast cells (arrows). ep, epiblast.

generated. Their intercrossing failed to produce Δ1/Δ1 progeny (35% +/+ and 65% Δ1/+ of 101 pups), as observed in Δ4/+ intercrosses (9).

To specifically inactivate the *Pcsk5* gene in the epiblast and not in the extraembryonic lineages (Fig. S2), $Pcsk5^{flox/flox}$ mice were crossed with $Pcsk5^{\Delta 1/+}$ mice that expressed Cre at the *Meox2* locus (*Meox2-cre*) (18). Among the newborn mice, those exhibiting the $Pcsk5^{\Delta 1/flox}$ *Meox2-cre* genotype (absence of + alleles) were invariably *flox*-positive, indicating that the Cre-mediated recombination of the *flox* allele was inefficient. In addition, these mice were underrepresented (13 of 103; 13% vs. the 25% expected), suggesting that, when efficient, Cre recombination, which occurs before E9.5 (Fig. S3), led to embryonic lethality. Indeed, genotyping of embryos at different developmental stages (45 at E9.5 and 150 at E18.5) showed that $Pcsk5^{\Delta 1/flox}$ *Meox2-cre* mice were found in the expected Mendelian proportions (29% and 31%, respectively). At E18.5, only 29 of the 46 embryos isolated (63%) did not exhibit remaining *flox* alleles, as tested by PCR, and corresponded to PC5/6-deficient embryos not expected to survive, hereafter named cKO (for conditional KO) to distinguish them from nonconditional PC5/6$^{-/-}$ embryos. Note that Cre-mediated recombination efficiency was similar in the $Pcsk5^{flox/+}$ *Meox2-cre* progeny (≈70%). Evidence that cKO embryos die at birth came from (*i*) the fact that all of the cKO embryos isolated at E18.5 were alive before being placed on ice; (*ii*) the discovery of two dead pups with a bluish-gray discoloration of the skin (argyria) after careful surveillance of breeding cages; and (*iii*) the birth of a mutant pup that showed an acute respiratory distress and became bluish and died immediately.

Analysis of the PC5/6 cKO Phenotype in E18.5 Embryos. At E18.5, cKO embryos (29/150) were all characterized by a shortened or absent tail and a smaller size (Fig. 2*A*). In addition, most exhibited abdominal herniations. The smallest embryos showed subepidermal hemorrhage on the head and along the spinal chord (Fig. 2*B*). *In situ* hybridization analysis of these embryos revealed PC5/6 labeling close to background, except in the intestine, where a faint signal, likely nonspecific, was sometimes observed (Fig. 2*C*). Indeed, using PCR, no *flox* alleles could be detected in the genomic DNA prepared from their paws, liver, or intestine.

Skeleton analysis of 15 cKO embryos at E18.5 revealed retarded ossification, namely in the mandibles, nasal bone, vertebrae, and limbs (Fig. 3 *A* and *B*). The mutant embryos also showed homeotic transformations (Table S1). Although one embryo had the expected 13 thoracic vertebrae but no tail, 10 had three to four additional thoracic vertebrae (Fig. 3*B*). Only one embryo had seven vertebrosternal ribs, as in WT, whereas the 14 others had one to three additional ribs attached to the sternum (Fig. 3*C*). Also, in most cases, eight lumbar segments

Fig. 2. Epiblast-specific KO of PC5/6 at E18.5. (*A*) Intercrossing of *flox/flox* mice with *Δ1/+* mice harboring a heterozygote knockin of *Cre* at the *Meox2* locus generates ≈25% of *Δ1/flox* and Cre positive embryos. All of the embryos that have a shortened or no tail were characterized by the only presence of *Δ1* alleles at the PC5/6 locus and were Cre positive (cKO). Most of them exhibited abdominal herniations (red arrows). (*B*) In the smallest mutant embryos, subcutaneous hemorrhages were observed (white arrow). (*C*) *In situ* hybridization analysis of PC5/6 mRNA in cryosections of embryos at E18.5 using an antisense cRNA probe revealed a drastic diminution of the signal in cKO embryos, similar to the background obtained with a control sense probe (data not shown).

Fig. 3. Skeletal defects in the PC5/6 mutant embryos at E18.5. (*A*) Note the smaller size, absence of tail, and retarded ossification of mandibles, limbs and vertebrae. (*B*) Up to four additional thoracic segments and (*C*) up to three additional vertebrosternal ribs were present in mutant embryos. Ribs were asymmetrically attached to the sternum that was incompletely fused. (*D*) Up to two additional lumbar segments (L7 and L8) were observed. Note the reduced ossification of vertebrae in the lumbar and sacral regions and the smaller size of the ilium bones.

BIOCHEMISTRY

Fig. 4. Kidney, lung, and liver phenotypes of cKO embryos at E18.5. Paraffin sections were stained with hematoxylin/eosin. (*A*) Twelve of 13 embryos analyzed lacked both kidneys (ki). ad, adrenals; li, liver. (*B*) Note the collapsed alveoli and airways in the lung and (*C*) the multiple liver hemorrhages in mutant embryos.

instead of six were observed (Fig. 3*D*). Finally, the majority of the cKO embryos exhibited only three to nine sacral and caudal segments instead of ≈34 in normal mice. Mutant embryos also differed by an incomplete closure of the sternum and an asymmetric fusion (alternate patterning) of the ribs to the sternum (Fig. 3*C*).

Dissection of six cKO embryos revealed the absence of kidneys in all of them. To better define the PC5/6 cKO phenotype, paraffin sections of E18.5 embryos were stained with hematoxylin/eosin to perform a gross examination of the major organs. Four of nine mutant embryos entirely sectioned lacked both kidneys (Fig. 4*A*). Of the five other half embryos analyzed, only one exhibited a normal kidney (data not shown). In all cases, the adrenal glands were observed, even though their cortex constitutes one of the richest site of PC5/6 expression during development and in the adult (6, 9). Another characteristic feature of the cKO embryos was that the airways and alveoli of their lungs were collapsed (Fig. 4*B*). Taking into account the respiratory distress and bluish coloration of the skin observed in a few pups, it is tempting to hypothesize that lungs of cKO newborns were unable to inhale oxygen and unload CO_2 from the alveolar spaces. Finally, we noticed that the livers of cKO embryos were subject to multiple hemorrhages, although the liver architecture seemed not to be affected (Fig. 4*C*).

Absence of Gdf11 Processing in cKO Embryos. Because several of the above phenotypes, such as the homeotic transformations and absence of kidneys, were reminiscent of those observed in embryos deficient in the growth/differentiation factor 11 (Gdf11) (19), we hypothesized that PC5/6 was implicated in the processing of this TGFβ-like growth factor. To investigate whether PC5/6 and Gdf11 colocalized, their mRNA expression was analyzed at E9.5 and E10.5, when PC5/6 expression increases in the developing embryo (9). PC5/6 and Gdf11 distribution patterns were very similar; the two transcripts were coexpressed in the tail bud, somites, dorsal neural tissue, mandibles, and encephalon. However, PC5/6 expression seemed more widespread (e.g., in the heart ventricle and atrium) and dominated in the caudal part of the trunk and proximal tail, corresponding to the most posterior somites, whereas that of Gdf11 peaked in the tail bud, a continuing source of mesoderm in the after gastrulation embryo (Fig. 5).

To verify whether PC5/6 is the major processing enzyme of Gdf11, we analyzed the processing of Gdf11 both *in vitro* and *ex vivo* (Fig. 6). Precursors of TGFβ-like proteins require a cleavage at site 1 (S1) by basic amino acid-specific PCs to release the mature portion of the growth factor (20). *In vitro* digestion of a

Fig. 5. Localization of PC5/6 and Gdf11 mRNA in WT embryos. Whole-mount *in situ* hybridization (*Left*) was performed at E9.5, whereas *in situ* hybridization on cryosections (*Right*) was carried out in embryos at E9.5 or E10.5 with cRNA PC5/6 and Gdf11 sense (data not shown) and antisense probes. de, diencephalon; ha, heart atrium; hv, heart ventricle; mb, mandibles; me, mesencephalon; psm, presomitic paraxial mesoderm; sca, spinal canal; sch, spinal chord; sfm, somite-forming paraxial mesoderm; tb, tail bud; te, telencephalon.

Fig. 6. *In vitro* and *ex vivo* processing of mouse Gdf11. At the top are shown a schematic of the mGdf11 structure and the percentage *in vitro* cleavage of a 12-mer peptide encompassing the Gdf11 S1 site (WT) and its N297D mutant by purified soluble PC5/6A, furin, PC7, and PACE4. At the bottom are shown Western blot analyses using a C-terminal FLAG (FG) Ab of HEK293 media obtained 24 h after coexpression of recombinant WT mouse Gdf11 cDNA or its empty vector with the full-length forms of the indicated PCs (*Left*) or after coexpression of PC5/6A or PC5/6B with the Gdf11 cleavage site mutants R293A and R296A (*Right*). The migration positions of the uncleaved proGdf11 and its mature Gdf11 forms are emphasized.

12-mer peptide encompassing the predicted S1 of Gdf11 with the four constitutive PCs, furin, PACE4, PC5/6, and PC7 (2) revealed that PC5/6 cleaves the WT sequence better than PACE4, and that furin and PC7 do not process this peptide. The presence of an Asn at the P1′ position (first residue after the cleavage site) is rather unique in PC substrates (2). To assess its importance, a N297D peptide was synthesized, in which Asn was substituted to Asp, which is known to favor furin cleavage (21) and to be present in the S1 processing site of Gdf8, the closest member to Gdf11 in the TGFβ-like protein family with 90% of identity within their TGFβ-like domain. This resulted in the cleavage at S1 by all four convertases and thus in the loss of PC5/6 selectivity (Fig. 6), although PC5/6 remained the fastest-cleaving enzyme (data not shown). We next transiently coexpressed in HEK293 cells mouse Gdf11, tagged with a FLAG epitope at the C terminus, with either PC5/6A, PC5/6B, furin, PC7, or PACE4. Western blot analysis of the media with a FLAG Ab showed that only PC5/6A and PC5/6B efficiently processed proGdf11 into the ≈15-kDa mature Gdf11 (Fig. 6). Evidence that processing by PC5/6 occurred at the canonical S1 site $\mathbf{R}_{293}$xx$\mathbf{R}_{296}$ motif (2) was obtained by the mutagenizing the P1 (R296A) or P4 (R293A) Arg in Gdf11 cDNA. This completely prevented cleavage by PC5/6A or PC5/6B (Fig. 6).

Discussion

We have shown that PC5/6$^{-/-}$ embryos die at the implantation stage (9). We report here that the generation of an epiblast-specific KO, in which the proximal promoter and exon 1 are excised by Cre expressed from the *Meox2* locus, allowed us to bypass early embryonic death but led to death at birth. The rescue of early embryonic death by normal expression of PC5/6 in extraembryonic lineages demonstrated that PC5/6 is essential in these cells. This is in agreement with the high expression of PC5/6 mRNA in cells lining the inner side of the maternal embryonic junction at E6.5 (Fig. 1 and ref. 17) and at E9 in rat (22). The key substrate(s) that necessitate PC5/6 cleavage remain to be identified.

At E18.5, cKO embryos exhibited skeleton alterations and multiple organ defects, including kidney agenesis, collapsed lung alveoli, and multiple hemorrhages. The absence of tail and presence of extra thoracic and lumbar segments in cKO embryos were also observed in embryos deficient in Gdf11 (19) or its main downstream receptors, activin type IIB (23) and ALK5 (24). Gdf11 is a secreted member of the TGFβ superfamily that participates in the establishment of the anterior–posterior axis by controlling the spatiotemporal expression of *Hox* genes. In the absence of Gdf11, the expression pattern of some *Hoxc* genes expanded posteriorly or was caudally displaced (19, 25, 26). Because the activity of Gdf11 but not that of its receptors depends on a PC-mediated cleavage, our data strongly suggest that Gdf11 is a substrate of PC5/6. Gdf11 is a unique secretory protein that specifies the segmental characteristics of vertebrae, likely through a gradual signaling to target cells (19). The combination of the extracellular activities of the membrane-bound PC5/6B and secreted PC5/6A may modulate the gradient of active Gdf11, critical for its patterning functions.

However, PC5/6 cKO and Gdf11$^{-/-}$ skeleton defects are not completely identical. Alterations in the patterning of the axial skeleton of Gdf11$^{-/-}$ embryos are more severe with an average of 18 ribs, including 10 vertebrosternal ones, instead of 16–17 ribs, including eight or nine vertebrosternal ones in most cKO embryos. In addition, Gdf11 heterozygotes have an additional thoracic segment not observed in PC5/6 ones. We believe that the PC5/6 cKO is quasicomplete in phenotypically mutant embryos: (*i*) assessment of the presence of *flox* alleles in various tissues of the same embryos failed to detect significant mosaicism [no signal by PCR on genomic DNA (data not shown) and, depending on tissues, 0–3% of residual expression by RT-PCR (Table S2)]; and (*ii*) bilateral kidney agenesis seems as severe, if not more, in PC5/6- than in Gdf11-deficient embryos [> 90% vs. ≈60% (27), respectively]. A more likely explanation for the observed skeletal differences is that, in the absence of PC5/6, another PC, possibly PACE4 (Fig. 6), may exert a minor but not negligible contribution to Gdf11 processing. Finally, the observed phenotypic differences may also be due to different genetic backgrounds, known to affect dramatically some phenotypes (28, 29). As for Gdf11 KO (19), we obtained 129Sv-derived ES cells that were injected in C57BL/6 blastocysts. However, in our case, *in vivo* excision of the *neo* cassette (*flox*) and exon 1 region (Δ*1*) required flipase and Cre (*CMV-cre* and *Meox2-cre*) expressing strains of mixed 129S4/Sv, C57BL/6, and SJL backgrounds. Parallel to this work, we backcrossed our mixed background strains carrying *flox* or Δ*1* alleles nine generations to C57BL/6. Very recently, we were contacted by Shoumo Bhattacharya and colleagues, who isolated a PC5/6 mutant strain obtained by *N*-ethyl *N*-nitrosourea (ENU) mutagenesis and backcrossed to C3H/HeH for several generations (personal communication). When homozygous for this mutation, the embryos exhibited the same phenotypes as our cKO ones. Their ability to go through implantation, possibly because of a PC5/6 residual activity, prompted us to check for the viability of Δ*1*/Δ*1* embryos in an almost pure C57BL/6 background. To our surprise, we detected Δ*1*/Δ*1* embryos in a first litter at E10.5. Further studies will be required to phenotype them at E18.5. If they are as viable as cKO embryos, their characterization will help us understand the PC5/6 contribution to organogenesis and will open the door to the design of new cKOs.

Aside from the axial patterning phenotype, cKO embryos exhibited skeletal phenotypes not reported for Gdf11$^{-/-}$ embryos, such as smaller size, severe retardation of ossification, incomplete and asymmetric fusion of the sternum, and abnormal symphysis of pubic bones. Some of these phenotypes were associated with the lack of platelet-derived growth factor A or B (PDGF A/B) receptors (30) and/or their target genes (31) and the BMP-potentiator crossveinless 2 (32). Because PC5/6 can process PDGFs (33), it is possible that the observed phenotypes are related to the lack of processing of these and/or other TGFβ-like factors, such as BMP4 (20) and Lefty (34). Although the precise cause of death of the cKO pups at birth is unknown, our observations suggest it resides in their inability to breathe. Collapsed alveoli (Fig. 4) are a hallmark of lungs deficient in surfactant. The latter is released into the alveoli by type II pneumocytes to reduce the surface tension of the fluid that lines the alveolar walls. Whether PC5/6 is required in this process needs further investigation. The respiratory distress of the newborns may also be due to hernias in the diaphragm (data not shown). Death may also be due to the absence of kidneys or hemorrhages (Fig. 4). The latter may be related to the absence of PDGF B processing by PC5/6 (33), resulting in fragile blood vessels, as reported in PDGFRβ-deficient mice (35).

Gene inactivation of the other broadly expressed basic amino acid-specific PCs revealed that furin and PACE4 also play important roles in development, likely through cleavage of a network of TGFβ family members (12, 13, 36). However, although PACE4, whose absence leads to 75% of viability, is in most cases accessory, the absence of Furin (12) or PC5/6 during development results in embryonic death. The cKO approach presented here led to the identification of an *in vivo*-specific substrate of PC5/6, namely Gdf11. Whether this enzyme–substrate pair is the result of a quasiabsence of other PCs in Gdf11-expressing cells or its poor processing by other PCs is not yet clear. On the one hand, our *in vitro* data indicate that the presence of the P1′ Asn contributes to make Gdf11 a favorite substrate of PC5/6 (Fig. 6). In agreement, the negative regulation of muscle growth by Gdf8, whose S1 site differs from the Gdf11 one by the presence of a P1′ Asp, does not seem to be affected

in cKO embryos. Gdf8$^{-/-}$ mice are indeed larger and exhibit a 2- to 3-fold increase in their muscle mass. However, Gdf11 may not be exclusively cleaved by PC5/6; a gross examination of cKO pancreata failed to reveal the reported 2-fold reduction of the exocrine pancreas in Gdf11$^{-/-}$ embryos (37). However, this phenotype may also be modulated by the genetic background.

Because no antibodies sensitive and specific enough to detect endogenous levels of Gdf11 or Gdf8 are available, our multiple attempts to evaluate the extent of processing of Gdf11 and Gdf8 in embryo extracts were unsuccessful. Interestingly, database searches revealed that, among mouse secretory proteins, the RSRRN motif is found only in Gdf11.

Our work showed that PC5/6 is vital in the extraembryonic tissue and epiblast. The respective roles of the evolutionary conserved (38) PC5/6A and PC5/6B isoforms remain to be elucidated. The overall more severe phenotype of cKO embryos that die immediately at birth compared with the Gdf11$^{-/-}$ ones that survive up to 24 h should lead to the identification of other key substrates of PC5/6.

Methods

Construction of the Targeting Vector. Using the Canadian Institutes of Health Research Genomic Resource Facility, a 129/Sv mouse genomic BAC library was screened by using a *Pcsk5* exon 1 probe (E1; 5′-GCGAAGGCGGCGAAGCGTCG vs. 5′-TCCTACGTTGATGAATCCGTAC). The 5′ and 3′ arms of the targeting vector were obtained by digestion of the 56L22-positive clone by using KpnI or BamHI and XbaI, respectively, and subcloned in a pUC19 plasmid whose polylinker exhibits EcoRI, ClaI, KpnI, AscI, PacI, BamHI, SalI, ClaI, and HindIII sites (Fig. S1). The 5′ *loxP* site consisting of two annealed oligonucleotides was inserted into a unique AvrII site of the 5′arm. A 1.9-kb fragment containing the 3′ *loxP* site and a PGK-*neo* cassette flanked with two *frt* sites was subcloned into AscI and PacI sites. Digestion of the targeting vector with ClaI released a 12,951-bp fragment, which was purified on an agarose gel and electroporated into R1 ES cells [(129/Sv x 129/Sv-CP)F1 (39)].

Generation of *Pcsk5* cKO Mice. Six hundred G418-resistant ES clones were screened for homologous recombination by Southern blot analysis of StuI-digested DNA, by using a mixture of 5′A and 3′A external probes (Fig. S1). StuI cleaves in the *neo* cassette and upstream and downstream of the 5′A and 3′A probes, respectively. The DNA of the positive clones was further analyzed by using NheI or EcoRI and the E1 probe. The 4E7 clone revealed a correct insertion of a single copy of the insert at the *Pcsk5* locus. This clone was injected into C57BL/6 blastocysts and transferred to pseudopregnant females to produce chimeras. To eliminate the *neo* cassette, chimeras were mated to expressing the Flp recombinase at the ROSA locus or from a Tg under the control of the β-actin promoter, respectively (The Jackson Laboratory). The latter was found to be more efficient. Resulting heterozygous mice for the floxed allele, *Pcsk5*$^{flox/+}$, were crossed to Tg(*CMV-cre*) mice (40) to delete exon 1 (*Pcsk5*$^{\Delta 1/+}$). The knockin mice expressing Cre at the *Meox2* locus were from The Jackson Laboratory.

PCR Genotyping of Embryos and Mice. Embryos and mice were genotyped by PCR by using liver or tail DNA. *WT*, *neo*, *flox*, and Δ*1* alleles were detected by using the following primers: *WT*, 5′-**GGGATCGGCCAGTAGCCAGACTATACGG** vs. 5′-**CCGAAGCCCTCACATCCTATCCCTCTCC**; *neo*, 5′-GTCCGGTGCCCTGAAT-GAAC vs. 5′-CCGCCAAGCTCTTCAGCAAT; *flox*, 5′-**CAGAATTGCTGTGCTCTGGA** vs. 5′-GTATTGGCATTTCCCTCAGC; and Δ1, 5′-**GGGATCGGCCAGTAGCCAGAC-TATACGG** vs. 5′- **CCCATAAAATGTATTGGCATTTCCCTCAGC**.

Histology. Tissues were collected in BPS and fixed overnight with Bouin's solution at 4°C, washed in ethanol 70%, and embedded in paraffin. Sections were cut at 4 μm thickness and stained with hematoxylin/eosin.

Skeletal Preparations. E18.5 embryos were incubated overnight in tap water at room temperature, immersed for 30 sec in water at 65°C, and then skinned, eviscerated, and dehydrated in 90% ethanol for 1 day. After a 3-day incubation in acetone to remove fat, the skeletons were stained for 36 h in 13% acetic acid, 61.3% ethanol, 0.0046% Alcian blue 8GX (ESBE Laboratory), and 0.002% Alizarin red S (ICN Biochemical). The skeletons were then transferred into a cleaning solution (1% KOH in 20% glycerol) for 24 h at 37°C, further incubated at room temperature until specimens were completely leached, and stored in a 2:2:1 mix of ethanol, glycerol, and benzyl alcohol.

***In Situ* Hybridization.** Twelve-micrometer-thick cryosections of embryos at E9.5, E10.5, or E18.5 were fixed in 4% formaldehyde at 4°C for 1 h. For PC5/6 labeling *in utero*, E6.5 uteri from pregnant females were fixed in 4% formaldehyde overnight at 4°C, embedded in paraffin, and cut into 5-μm sections. After deparaffination and rehydratation, these or the above cryosections were hybridized with mouse PC5/6 antisense and control sense cRNA probe as described (9, 41). To generate a mGdf11 cRNA probe, a cDNA segment covering the sequence encoding amino acids 67–404 was amplified by using sense 5′-GCAGCACAGCCGCGAGCTG and antisense 5′- GATACCGGTGGAG-CAGCCACATCGATCCAC oligonucleotides and subcloned in pDrive (Qiagen).

Whole-Mount *in Situ* Hybridization. This was performed as described (42) except that hybridization with above probes was performed at 70°C and that 2 mM Levamisol (Sigma) was added during the incubation with anti-digoxygenin-alkaline phosphatase Ab (Roche) and subsequent washes.

***In Vitro* Assays, Transfection, and Western Blot Analysis.** The 12-mer peptides were incubated *in vitro* overnight with 2 units of purified PCs [2 pmol amino methyl coumarin (AMC) released per minute from the peptide Pyr-RTKR-MCA) (43)], in 2 mM $CaCl_2$ and 25 mM Tris-Mes, pH 7.5, in a volume of 100 μl. The products were separated by RP-HPLC on a Varian C_{18} column (5 μm, 100 Å, 4.5 × 250 mm), and MS confirmed that cleavage took place exclusively after Arg^{296}. HEK293 cells were grown in DMEM containing 10% heat-inactivated FCS (Canadian Life Technologies) at 37°C in 5% CO_2. Cells (1×10^6 plated on 35-mm plates) were transfected with plain or recombinant vectors (0.6 μg) using Effectene (Qiagen). The medium was replaced 12 h after transfection by a serum-free medium, and cells were grown for an additional 24 h. Media were analyzed by SDS/PAGE, Western blot analysis using the FLAG M2 Ab (1:1,000; Stratagene), and ECL (Amersham Biosciences).

ACKNOWLEDGMENTS. We thank Shoumo Bhattacharya for the exchange of our respective manuscripts before submission. We also thank Gaoxiang Ge for the mGdf11 cDNA, Brigitte Mary for secretarial help, Claudia Toulouse for excellent animal care, and Dominic Fillion and Qinzhang Zhu for solid technical assistance. This work was supported by grants from the Canadian Institutes of Health Research (MGP-44363 and Canada Chair no. 20652).

1. Steiner DF (1998) The proprotein convertases. *Curr Opin Chem Biol* 2:31–39.
2. Seidah NG, Chretien M (1999) Proprotein and prohormone convertases: A family of subtilases generating diverse bioactive polypeptides. *Brain Res* 848:45–62.
3. Jin W, *et al.* (2007) Hepatic proprotein convertases modulate HDL metabolism. *Cell Metab* 6:129–136.
4. Seidah NG, Prat A (2007) The proprotein convertases are potential targets in the treatment of dyslipidemia. *J Mol Med* 85:685–696.
5. Seidah NG, Day R, Marcinkiewicz M, Chretien M (1998) Precursor convertases: An evolutionary ancient, cell-specific, combinatorial mechanism yielding diverse bioactive peptides and proteins. *Ann NY Acad Sci* 839:9–24.
6. Lusson J, *et al.* (1993) cDNA structure of the mouse and rat subtilisin/kexin-like PC5: A candidate proprotein convertase expressed in endocrine and nonendocrine cells. *Proc Natl Acad Sci USA* 90:6691–6695.
7. Nakagawa T, Murakami K, Nakayama K (1993) Identification of an isoform with an extremely large Cys-rich region of PC6, a Kex2-like processing endoprotease. *FEBS Lett* 327:165–171.
8. Nour N, *et al.* (2005) The cysteine-rich domain of the secreted proprotein convertases PC5A and PACE4 functions as a cell surface anchor and interacts with tissue inhibitors of metalloproteinases. *Mol Biol Cell* 16:5215–5226.
9. Essalmani R, *et al.* (2006) Deletion of the gene encoding proprotein convertase 5/6 causes early embryonic lethality in the mouse. *Mol Cell Biol* 26:354–361.
10. Taylor NA, Van de Ven WJ, Creemers JW (2003) Curbing activation: Proprotein convertases in homeostasis and pathology. *FASEB J* 17:1215–1227.
11. Scamuffa N, Calvo F, Chretien M, Seidah NG, Khatib AM (2006) Proprotein convertases: Lessons from knockouts. *FASEB J* 20:1954–1963.
12. Roebroek AJ, *et al.* (1998) Failure of ventral closure and axial rotation in embryos lacking the proprotein convertase Furin. *Development* 125:4863–4876.
13. Constam DB, Robertson EJ (2000) SPC4/PACE4 regulates a TGFbeta signaling network during axis formation. *Genes Dev* 14:1146–1155.
14. Constam DB, Calfon M, Robertson EJ (1996) SPC4, SPC6, and the novel protease SPC7 are coexpressed with bone morphogenetic proteins at distinct sites during embryogenesis. *J Cell Biol* 134:181–191.
15. Rossant J, Cross JC (2001) Placental development: lessons from mouse mutants. *Nat Rev Genet* 2:538–548.
16. Nie GY, Li Y, Minoura H, Findlay JK, Salamonsen LA (2003) Specific and transient up-regulation of proprotein convertase 6 at the site of embryo implantation and identification of a unique transcript in mouse uterus during early pregnancy. *Biol Reprod* 68:439–447.

17. Rancourt SL, Rancourt DE (1997) Murine subtilisin-like proteinase SPC6 is expressed during embryonic implantation, somitogenesis, and skeletal formation. *Dev Genet* 21:75–81.
18. Tallquist MD, Soriano P (2000) Epiblast-restricted Cre expression in MORE mice: A tool to distinguish embryonic vs. extra-embryonic gene function. *Genesis* 26:113–115.
19. McPherron AC, Lawler AM, Lee SJ (1999) Regulation of anterior/posterior patterning of the axial skeleton by growth/differentiation factor 11. *Nat Genet* 22:260–264.
20. Cui Y, *et al.* (2001) The activity and signaling range of mature BMP-4 is regulated by sequential cleavage at two sites within the prodomain of the precursor. *Genes Dev* 15:2797–2802.
21. Henrich S, *et al.* (2003) The crystal structure of the proprotein processing proteinase furin explains its stringent specificity. *Nat Struct Biol* 10:520–526.
22. Zheng M, Seidah NG, Pintar JE (1997) The developmental expression in the rat CNS and peripheral tissues of proteases PC5 and PACE4 mRNAs: Comparison with other proprotein processing enzymes. *Dev Biol* 181:268–283.
23. Oh SP, *et al.* (2002) Activin type IIA and IIB receptors mediate Gdf11 signaling in axial vertebral patterning. *Genes Dev* 16:2749–2754.
24. Andersson O, Reissmann E, Ibanez CF (2006) Growth differentiation factor 11 signals through the transforming growth factor-β receptor ALK5 to regionalize the anterior-posterior axis. *EMBO Rep* 7:831–837.
25. Liu JP, Laufer E, Jessell TM (2001) Assigning the positional identity of spinal motor neurons: Rostrocaudal patterning of Hox-c expression by FGFs, Gdf11, and retinoids. *Neuron* 32:997–1012.
26. Liu JP (2006) The function of growth/differentiation factor 11 (Gdf11) in rostrocaudal patterning of the developing spinal cord. *Development* 133:2865–2874.
27. Esquela AF, Lee SJ (2003) Regulation of metanephric kidney development by growth/differentiation factor 11. *Dev Biol* 257:356–370.
28. Peinado JR, *et al.* (2005) Strain-dependent influences on the hypothalamo-pituitary-adrenal axis profoundly affect the 7B2 and PC2 null phenotypes. *Endocrinology* 146:3438–3444.
29. Bourdeau A, *et al.* (2001) Potential role of modifier genes influencing transforming growth factor-β1 levels in the development of vascular defects in endoglin heterozygous mice with hereditary hemorrhagic telangiectasia. *Am J Pathol* 158:2011–2020.
30. Soriano P (1997) The PDGF α receptor is required for neural crest cell development and for normal patterning of the somites. *Development* 124:2691–2700.
31. Schmahl J, Raymond CS, Soriano P (2007) PDGF signaling specificity is mediated through multiple immediate early genes. *Nat Genet* 39:52–60.
32. Ikeya M, *et al.* (2006) Essential pro-Bmp roles of crossveinless 2 in mouse organogenesis. *Development* 133:4463–4473.
33. Siegfried G, *et al.* (2005) Regulation of the stepwise proteolytic cleavage and secretion of PDGF-B by the proprotein convertases. *Oncogene* 24:6925–6935.
34. Ulloa L, *et al.* (2001) Lefty proteins exhibit unique processing and activate the MAPK pathway. *J Biol Chem* 276:21387–21396.
35. Lindahl P, Johansson BR, Leveen P, Betsholtz C (1997) Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science* 277:242–245.
36. Constam DB, Robertson EJ (2000) Tissue-specific requirements for the proprotein convertase furin/SPC1 during embryonic turning and heart looping. *Development* 127:245–254.
37. Dichmann DS, Yassin H, Serup P (2006) Analysis of pancreatic endocrine development in GDF11-deficient mice. *Dev Dyn* 235:3016–3025.
38. Oliva AA, Jr., Chan SJ, Steiner DF (2000) Evolution of the prohormone convertases: Identification of a homologue of PC6 in the protochordate amphioxus. *Biochim Biophys Acta* 1477:338–348.
39. Nagy A, Gersenstein M, Vintersten K, Behringer R (2003) *Manipulating the Mouse Embryo* (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY).
40. Dupe V, *et al.* (1997) *In vivo* functional analysis of the Hoxa-1 3′ retinoic acid response element (3′RARE). *Development* 124:399–410.
41. Marcinkiewicz M, *et al.* (1999) Nerve growth factor and proprotein convertases furin and PC7 in transected sciatic nerves and in nerve segments cultured in conditioned media: Their presence in Schwann cells, macrophages, and smooth muscle cells. *J Comp Neurol* 403:471–485.
42. Correia KM, Conlon RA (2001) Whole-mount *in situ* hybridization to mouse embryos. *Methods* 23:335–338.
43. Munzer JS, *et al.* (1997) *In vitro* characterization of the novel proprotein convertase PC7. *J Biol Chem* 272:19672–19681.

BIOCHEMISTRY