A targeted deletion/insertion in the mouse Pcsk1 locus is associated with homozygous embryo preimplantation lethality, mutant allele preferential transmission and heterozygous female susceptibility to dietary fat

Majambu Mbikay\textsuperscript{a,b,*}, Gilles Croissandeau\textsuperscript{a,1}, Francine Sirois\textsuperscript{a,1}, Younes Anini\textsuperscript{a}, Janice Mayne\textsuperscript{a}, Nabil G. Seidah\textsuperscript{c}, Michel Chrétien\textsuperscript{a,b}

\textsuperscript{a} Ottawa Health Research Institute and The Ottawa Hospital, Ottawa, Ontario, Canada
\textsuperscript{b} Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ontario, Canada
\textsuperscript{c} Biochemical Neuroendocrinology Laboratory, Clinical Research Institute of Montreal, Montreal, Quebec, Canada

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Abstract

Proprotein convertase 1 (PC1) is a neuroendocrine proteinase involved in the proteolytic activation of precursors to hormones and neuropeptides. To determine the physiological importance of PC1, we produced a mutant mouse from embryonic stem cells in which its locus (Pcsk1) had been inactivated by homologous recombination. The inactivating mutation consisted of a 32.7-kb internal deletion and a 1.8 kb insertion of the bacterial neomycin resistance gene (neo) under the mouse phosphoglycerate kinase 1 protein (PGKneo). Intercross of Pcsk1\textsuperscript{+/-} mice produced no Pcsk1\textsuperscript{-/-} offspring or blastocysts; in addition, more than 80% of the offspring were Pcsk1\textsuperscript{+/-}. These observations suggested that the mutation caused preimplantation lethality of homozygous embryos and preferential transmission of the mutant allele. Interestingly, RT–PCR analysis on RNA from endocrine tissues from Pcsk1\textsuperscript{+/-} mice revealed the presence of aberrant transcripts specifying the N-terminal half of the PC1 propeptide fused to neo gene product. Mass spectrometric profiles of proopiomelanocortin-derived peptides in the anterior pituitary were similar between Pcsk1\textsuperscript{+/-} and Pcsk1\textsuperscript{+/-} mice, but significantly different between male and female mice of the same genotype. Relative to their wild-type counterparts, female mutant mice exhibited stunted growth under a low fat diet, and catch-up growth under a high-fat diet. The complex phenotype exhibited by this Pcsk1 mutant mouse model may be due to PC1 deficiency aggravated by expression of aberrant gene products from the mutant allele.

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Keywords: Proprotein convertase; PC1; Pcsk1; Genetic deficiency; Preimplantation embryonic lethality; Transmission ratio distortion

Introduction

PC1, also known as PC3 (Smeekens et al., 1991) or SPC3 (Steiner, 1998), belongs to a family of serine proteinases that activate secretory precursor proteins by cleavages after selected pairs of basic residues (Seidah and Chrétien, 1999; Steiner, 1998). In mouse, its Pcsk1 locus maps to chromosome 13 (Seidah et al., 1991). The gene has 15 exons and 14 introns (Ftouhi et al., 1994). It is transcribed into two major mRNA isoforms of 2.8 and 4.4 kb differing in their 3’ untranslated regions (UTR) (Seidah et al., 1991) (Ftouhi et al., 1994). These transcripts are primarily found in neuroendocrine cells (Schäfer et al., 1993; Seidah et al., 1991, 1994; Zheng et al., 1994). They are translated in the endoplasmic reticulum (ER) into a 88-kDa proPC1; the zymogen gets converted in the ER and the Golgi to a 74-kDa form by autocatalytic removal of the prodomain; the mature enzyme is then sorted into secretory granules where it is further processed to its fully active form of 66-kDa by excision of a C-terminal fragment (Benjannet et al., 1993). The prodomain may promote the proper folding of proPC1 and its exit from the ER, as demonstrated for other PCs (Anderson et al.,...
2002; Muller et al., 2000; Rehemtulla et al., 1992); it has also been shown to inhibit PC1 enzymatic activity (Boudreault et al., 1998; Lee et al., 2004). The P domain stabilizes the catalytic domain of the enzyme (Ueda et al., 2003). The C-terminal domain has been implicated in partial inhibition of PC1 activity as well as in its sorting into secretory granules (Jutras et al., 1997, 2000).

The related protease PC2 is often found in the same granules (Kurabuchi and Tanaka, 2002; Malide et al., 1995; Marcinkiewicz et al., 1994; Takumi et al., 1998). The activities of the two convertases are influenced by resident gramin-like proteins: proSAAS, a competitive PC1-specific inhibitor (Basak et al., 2001; Fricker et al., 2000; Qian et al., 2000) and pro7B2, a PC2-specific chaperone and inhibitor (Benjannet et al., 1995; Braks and Martens, 1994; Martens et al., 1994; Mbikay et al., 2001; Muller et al., 1997).

PC1 and PC2 often process the same substrates, but with cleavage site preferences. Their substrates are mostly prohormones and propeptides (Seidah and Chretien, 1999). Proopiomelanocortin (POMC) and proinsulin are the best studied of these substrates. In the anterior lobe (AL) of the pituitary, POMC is processed primarily into adrenocorticotropic hormone (ACTH) and lipoactive hormones (βLPH); in the neurointermediate lobe (NIL), ACTH is further processed to α-melanocyte-stimulating hormone (α-MSH) and CLIP; and βLPH to βMSH and δ-endorphin (END) (Chretien and Seidah, 1991). PC1 is most abundant in the AL; and PC2 most abundant in the NIL (Marcinkiewicz et al., 1993; Seidah et al., 1990, 1991). Accordingly, using co-transfected cells, it was shown that PC1 converts POMC to peptides found in the AL and PC2 to those found in the NIL (Benjannet et al., 1991; Seidah et al., 1990; Zhou et al., 1993). PC1 cleaves proinsulin after KR31-32 pair joining the B and C peptides, while PC2 does it after the KR61-62 pair joining the C and A peptides (Smeekens et al., 1992).

Two human cases of genetic PC1 deficiency have been reported (Jackson et al., 1997, 2003). The first patient suffered from massive obesity at birth and, as an adult, exhibited hypogonadotrophic hypogonadism. After treatment with gona
dotropin-releasing hormone, resulting in less plasma growth hormone and insulin-like growth factor (IGF-1). POMC and proinsulin processing was also impaired in these mice (Dey et al., 2004; Marzban et al., 2004; Zhu et al., 2002a,b). A chemically induced N22D mutation in the catalytic domain of PC1 was recently described in mouse; homozygous mutants develop obesity and glucose intolerance associated with impaired processing of central and peripheral prohormones and propeptides (Lloyd et al., 2006).

Here, we describe another model of PC1 heritable deficiency, generated by substituting a 32.7-kb internal region of its gene with a 1.8 kb heterologous gene. Homozygotes for this mutation died during preimplantation embryonic development; the mutant allele was preferentially transmitted by heterozygous parents and, in female heterozygotes, weight gain with age was influenced by dietary fat content.

**Materials and methods**

**Pcsk1 disruption vector**

Genomic DNA was extracted from the R1 embryonic stem (ES) cells established from 129Sv mice. Fragments used as Pcsk1 homologous regions in the disruption vector were amplified by PCR from this DNA and cloned into a vector previously used to disrupt the Pcsk4 locus (Mbikay et al., 1997). The 5’ homology amplicon was amplified as a 3.2-kb fragment using the primer pair # 1 (Supplementary Table 1); it extended from nucleotide (nt) 6 of exon 1 to nt 77 of exon 2 (numbering based on Ensembl Pcsk1 locus ID # ENSMUSG00000021587). It was digested at an XhoI site, located at nt 252 in the first intron, and at a 3’-most BamHI site created with the PCR primer; the resulting 2.7-kb fragment was cloned into corresponding sites upstream to the neo gene for bacterial neomycin phosphotransferase II (NPTII) under the mouse phosphoglycerate kinase 1 (PGK) promoter. The 3’ homology amplicons was obtained using primer pair # 2 (see Table 1). It was 6.6 kb long and extended from nt 34 in exon 10 to nt 460 of exon 13. It was digested at the 5’-most SalI site created with the PCR primer and at a KpnI site located 0.56 kb upstream of intron 12 acceptor splice site. The resulting 3.6-kb 5’ fragment was inserted in the corresponding sites downstream to the neo gene and upstream of the Herpes Simplex virus thymidine kinase (tk) gene driven by a PGK promoter. The final vector was named pPC1KO.

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<td>Distorted genotype distribution among offspring of heterozygote intercrosses</td>
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Symblos: ++, female; ++, male.

a Number of litter: 76 total number of mice: 443.
b Genotyping was performed by PCR at weaning.
c Expected distribution: 33% ++/++ and 67% ++/++; p<0.00001, χ² test.
Production of Pcsk1 mutant mice

From the pPC1KO vector, a 10.4-kb insert, extending from the start of the 5′ homology region to the end of the tk gene, was excised by digestion with XhoI and BstBII and purified. The fragment was used to produce Pcsk1+/− (+/−) embryonic stem cells (ES) and mice, as previously described (Zhu et al., 1998).

Briefly, the DNA insert was electroporated into R1 embryonic stem (ES) cells; clones resistant to G418 (due neo integration) and to Gancyclovir (loss of the tk gene) were selected. Pcsk1 recombinant clones were injected into the inner mass of blastocysts derived from C57BL/6J (B6) mice; the blastocysts were implanted into pseudo-pregnant foster mice; male chimeric offspring were mated with B6 females to generate +/− mice. After backcrossing the mutation into the FVB/N genetic backgrounds for 4 generations, a colony of mutant and wild-type mice was maintained by intercrosses of the incipient congenic heterozygotes. Animal manipulations were approved by the institutional Animal Care Committee; they were performed following the guidelines of the Canadian Council on Animal Care.

Blot analyses

Southern blot analysis was performed as previously described (Mbikay et al., 1997): genomic DNA was extracted from ES cells or mouse tail tips by proteinase K digestion followed by phenol chloroform extraction and ethanol precipitation; it was digested with either BanHI or PstI, fractionated on agarose gel, transferred onto a nylon membrane that was radioactively probed for Pcsk1-specific restriction fragments. The probe for BanHI digests was a 0.66-kb DNA fragment derived from the PC1 promoter; for PstI digests, it was a 0.69-bp fragment derived from intron 14.

Western blot analysis was performed with chemiluminescence revelation as previously described (St. Germain et al., 2005). Primary antibodies consisted of rabbit antibodies against the C-terminus of PC1 (Benjannet et al., 1991, 1993), and mouse monoclonal antibody against β-actin (Cederlane, Mississauga, ON). Semi-quantitative densitometry of immunoreactive bands was conducted on the ChemiGenius Bioimaging System using the Genetools software (Syngene, Frederick, MD).

PCRs

PCR genotyping of the mice was conducted using a 3-primer set # 3 (see Supplementary Table 1), consisting of a forward primer derived from the region near the acceptor site of intron 1 and two reverse primers, one derived from the 5′ end of the PGKneo gene (missing in the wild-type allele). DNA from tail tip or ear lobe was used for normalization. The level of the TBP mRNA was used for normalization.

Expression vectors and transfection

The 1.1-kb PC1pro-neo cDNA amplified by RT–PCR from pituitary RNA of Pcsk1+/− mice was first subcloned into pCR4TOPO plasmid (Invitrogen, Burlington, ON); it was then excised from this vector with SpeI and NotI and transferred directionally between Nhel and NotI sites into the pCneo eukaryotic expression vector (Promega, Ottawa, ON), under the human cytomegalovirus promoter–enhancer to generate the pCneo/PC1pro-neo vector. Similarly, a 7B2-expression vector was made by inserting the full-length cDNA for rat 7B2 into the same two restriction sites.

For transfection, human embryonic kidney HEK293 cells were seeded into 6-well plates at a density of 7.5×104 per well in DMEM containing 10% fetal bovine serum (FBS); they were cultured to 75% confluence in a 5% CO2–95% air atmosphere at 37 °C; they were then transfected in triplicate with 1 μg of a single or mixed vector DNA using Lipofectamine Reagent liposomes as specified by the manufacturer (Life Technologies, Burlington, ON); they were HCl by sonication three times for 5 s, followed by incubation at 70 °C for 20 min. Extracts were neutralized by addition of 100 μl 0.2 M phosphate buffer, pH 9.5, containing protease inhibitors (Roche Complete Mini Protease Inhibitor Tablets) and centrifuged at 50,000×g for 40 min at 4 °C. The pellets were processed as above twice and supernatants combined. Immunoprecipitation of extracted peptides/proteins were carried out in 1× Tris-buffered saline (pH 7.6) containing 0.1% Tween (TBST). Samples were precleared by incubation with preimmune serum in the presence of Protein A-agarose overnight at 4 °C. They were centrifuged at 14,000×g for 2 min at 4 °C and supernatants incubated with anti-JEND antibody as above. Following immunocapture, the antibody–antigen complexes were washed five times in TBST and eluted from the Protein-A beads by two successive incubations in 150 μl of 0.1 M glycine (pH 2.8) for 10 min at room temperature with shaking. Supernatants were collected, combined and neutralized with 30 μl of 1 M Tris–HCl (pH 9.0). The eluates were equilibrated in 20% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA) with an Amicon Ultra YM3 Centricon (Millipore Corp., Bedford, MA), lyophilized and solubilized in 5 μl of 20% ACN+0.1% TFA. For time-of-flight mass spectral analysis, 2.5 μl of each sample was applied twice to a spot on a gold ProteinChip Array (Ciphergen Biosystems Inc, Palo Alto, CA) and allowed to air dry at room temperature after each application; 1 μl of saturated 3.5-dimethoxy-4-hydroxycinnamic acid (SPA) in 50% ACN+0.5% TFA was added to each spot. Mass spectral analysis was performed by time-of-flight mass spectrometry in a Ciphergen Protein Biology System II (PBS II). Analyses represent an average of 100 shots. Masses were calibrated externally with All-in-1 Peptide Standards (Ciphergen Biosystems Inc.). All data were normalized for total ion current and peak areas calculated using the indirect method (with a bracket height of 0.4 and width expansion factor of 2) contained within Ciphergen’s ProteinChip Software 3.1.
incubated for 48 h before metabolic labeling with radioactive methionine/cysteine.

**Metabolic labeling and immunoprecipitation**

Transfected HEK293 cells were rinsed twice with phosphate-buffered saline (PBS), layered with 1 ml/well of methionine and cysteine-free RPMI medium (ICN Biomedicals, Nepean, ON) and incubated at 37 °C for 30 min to deplete endogenous methionine. RPMI medium containing 250 μCi of 35S-methionine–cysteine/ml/well was substituted and incubation was resumed for 2 h. Spent media were collected and cells were scraped off the dishes into 400 μl of RIPA buffer (50 mM Tris–HCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 150 mM NaCl, 1% SDS). Media and cell lysates were supplemented with 0.04 volumes of a complete protease inhibitor cocktail (Boerhinger Manheim, Laval, QC; 25× stock solution: 1 tablet/2 ml). They were vigorously mixed on a vortex for 2 min, placed on ice for 30 min and centrifuged at 10,000×g for 10 min at 4 °C. Supernatants were collected and each was supplemented with 2 μl of normal rabbit serum and 15 μl of a 50% (w/v) suspension of Protein A–agarose. After a 1-h incubation at 4 °C with rotational mixing, the samples were centrifuged at 3000×g for 5 min at 4 °C. Supernatants were collected and each was supplemented with 5 μl of rabbit anti-7B2 (Paquet et al., 1994) or anti-NPTII antibody (Sigma). They were incubated with mixing as above. The resin with bound immune complexes was then sedimented by centrifugation as above, rinsed three times with RIPA buffer, twice with a buffer containing 1 M NaCl, 10 mM Tris–HCl and 1 mM EDTA, pH 8, and twice with PBS containing 1 mM EDTA. Pellets were suspended in 25 μl of 1× Laemmli buffer each, boiled for 5 min, and sedimented as above. Supernatant was subjected to electrophoresis through polyacrylamide gels (8 or 12%). Gels were fixed for 30 min in a 50% methanol–10% acetic acid solution, treated for 30 min with Amplify fluor solution (Amersham Biosciences, Baie d’Urfe, QC), dried under vacuum and exposed to phosphorimaging screen overnight. Specific radioactive protein bands were visualized and quantified on a Typhoon Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

**Feeding, weight and plasma glucose**

Four-week-old mice were divided into groups (n=6/gender/genotype) that were fed either a low-fat diet (LFD) or a high-fat diet (HFD) (diets no. D12450 or no D12451, respectively, obtained Research Diet, North Brunswick, NJ); the LFD contained 10% kcal and the HFD 45% kcal as fat. Body weight was recorded weekly. After 13 weeks, the mice were fasted for 16 h, anesthetized with isoflurane (Abbott Laboratories, Montreal, QC) and bled by heart puncture. Some mice were i.p. injected with glucose (1.5 mg/g body weight) and blood collected as above 10 or 60 min later. Plasma was separated from blood cells by centrifugation 600×g for 10 min. Plasma glucose was determined using a glucose analyzer (Beckman Coulter, Fullerton, CA).

**Statistics**

Significance was determined by unpaired Student’s t test.

**Results**

**Production and analysis of Pcsk1 mutant mouse**

The disruption vector is shown in Fig. 1A. Homologous recombination between this vector and the Pcsk1 locus should result in the excision of 32.7 kb of internal domain including exon 3 to exon 9. The recombination construct was excised...
from the vector and transfected into R1 ES cells; clones were selected for resistance to G418 and Gancyclovir; they were screened by Western blotting for the anticipated BamH1 and PstI, fragments using probes derived from the promoter and from exon 14, respectively (not shown).

Two clones showing the expected restriction fragment patterns were injected into B6 blastocysts; the latter were implanted into foster female mice; male chimeric mice born from these foster mothers were crossed with B6 females to produce offspring that are heterozygous for the Pcsk1 deletion–insertion (+/−). These were identified either by Southern blotting analysis of BamH1 digests (Fig. 1B) or by triplex PCR (Fig. 1C) of tail-tip genomic DNA.

**Homozygote lethality and transmission ratio distortion**

When +/− mice were intercrossed, no homozygous Pcsk1 mutant (−/−) mice were obtained (Table 1). Genotyping of preimplantation embryos derived from these crosses revealed the presence of a significant percentage of −/− embryos at the 1-cell (31%) and 2-cell (19%) stages; this percentage was below 5% at 4-cell and 8-cell stages and 0% at later stages (Table 2), suggesting that lethality occurred after the second embryonic division. Interestingly, the frequency of the mutant allele among surviving late preimplantation (morula to blastocysts), post-implantation and weanlings was consistently ≥80% instead of the 66.7% expected (p<0.0001, by χ²) (Tables 2 and 3), an indication of preferential transmission of this allele. This preference was still observed in +/+ ×+/− reciprocal crosses (Table 3), excluding any parental origin effect.

To determine whether the lethality of −/− preimplantation embryos was due to intrinsic developmental failure, we cultured fertilized eggs from +/+ ×+/+ and +/− ×+/− crosses and followed their growth for 6 days. We reasoned that death of −/− embryos resulting from heterozygote intercrosses should be reflected by a significant decrease in the number of living embryos at some stage past the second cell division. The results are shown in Fig. 2. Most embryos looked morphologically healthy on day 6 (93%, n=88 for +/+ ×+/+ crosses and 98%, n=83 for +/− ×+/− crosses, hereafter called mixed embryos). However, the growth pattern were distinct: on day 2, all the fertilized eggs reached the 2-cell stage; but on day 3, 45% of the +/+ embryos and only 22% of mixed embryos reached the morula stage; on day 4, the latter caught up with the +/+ as all became morula; but they grew slightly faster on the 2 subsequent days, with 79% reaching the blastocyst stages (vs. 67% for +/+ cell (31%) and 2-cell (19%) stages; this percentage was below 5% at 4-cell and 8-cell stages and 0% at later stages (Table 2), suggesting that lethality occurred after the second embryonic division. Interestingly, the frequency of the mutant allele among surviving late preimplantation (morula to blastocysts), post-implantation and weanlings was consistently ≥80% instead of the 66.7% expected (p<0.0001, by χ²) (Tables 2 and 3), an indication of preferential transmission of this allele. This preference was still observed in +/+ ×+/− reciprocal crosses (Table 3), excluding any parental origin effect.

![Fig. 2. In vitro development of preimplantation embryos from Pcsk1+/+ and Pcsk1+/− intercrosses. Superovulated female mice were mated to males of the same genotype: +/+ ×+/+ are labeled as +/+ and +/− ×+/− as +/−. Fertilized eggs were collected, cultured and scored on a daily basis for developmental stage. d.p.c., day post-coitus; c. morula, compacted morula; blasts, blastocysts.](image-url)
embryos), and 94% (vs. 86% for +/+ embryos) on day 5 and day 6, respectively. All three genotypes were detectable by PCR among blastocysts from heterozygote intercrosses. The survival of −/− embryos in culture suggests that their lethality in vivo may be caused by negative selection in the oviductal environment.

Expression of an aberrant PC1neo fusion mRNA

In the mouse model of targeted inactivation of the Pcsk1 locus reported by Zhu et al. (2002b), the promoter and the first exon were ablated; prenatal and perinatal lethality of homozygous mutants was observed, but nearly 33% of them survived to adulthood. In an attempt to explain the preimplantation lethality of our model, we examined the possibility that the PC1 promoter in the mutant allele could drive the production of an aberrant mRNA and protein that could aggravate the phenotype. A potential primary transcript of the mutant locus would include exon 1, intron 1, a 5′ fragment of exon 2 and the PGKneo sequence down to the polyadenylation signal. After removal of intron 1, this transcript should generate an mRNA with open reading frame (ORF) specifying the signal peptide and an N-terminal propeptide segment of PC1 fused to an extension of indeterminate size (Fig. 3A).

To verify whether such an mRNA was produced, we conducted an RT–PCR on pituitary mRNA from +/+ and +/- mice using primer pair # 4 for wild-type PC1 mRNA or primer pair #5 for the putative PC1neo fusion mRNA. The pituitary was selected as source of RNA because it expresses high levels of PC1 (Seidah et al., 1990, 1994), a reflection of PC1 gene promoter activity. Polyadenylated RNA was specifically reverse-transcribed into cDNA using an oligo-dT primer. The RT–PCR results are shown in Fig. 3B. With primer pair #4, the expected amplicon of 278 bp was detected in both samples (lanes 2 and 3). In contrast, using primer pair #5, the expected amplicon of 354 bp was obtained from +/- pituitaries (lane 5), but not from +/+ pituitaries (lane 4), confirming the existence of the aberrant mRNA. This mRNA was also detectable in pools of unfertilized eggs, fertilized eggs and preimplantation embryos obtained from +/- intercrosses (not shown).

To establish the full sequence of these transcripts, oligo-dT-primed cDNA from +/- mouse pituitary RNA was subjected to long-range PCR using a forward primer derived from the region upstream to the initiator ATG in PC1 exon 1 and a reverse primer from the neo mRNA 3′-UTR (primer pair #6). Three cDNA amplicons of 1.6, 1.4 and 1.1 were obtained and sequenced (Fig. 4A). They corresponded to alternate splice variants of the primary transcript. The predictability of the splice sites were confirmed a posteriori using the NNSPLICE 0.9 version algorithm (Reese et al., 1997) online (http://www.fruitfly.org/seq_tools/splice.html). The 1.6-kb and 1.4 amplicons encoded a fusion protein made of the 27-amino acid (aa) signal peptide and the first 58 aa of the propeptide of PC1 followed by a 48-aa extension encoded by the PGK sequence (not shown). The 1.1 amplicons specified a single ORF consisting of the same 85-aa PC1 prepropeptide fused to the full NPTII sequence by a 12-aa joining peptide (Fig. 4B). This amplicon was cloned into the PCIneo expression vector and the resulting vector was transiently transfected into HEK293 cells. Metabolic labeling followed by NPTII-specific immunoprecipitation and SDS–PAGE revealed the presence of the PC1pro-NPTII (∼37 kDa) and a processed NPTII (∼27 kDa) in cell extracts and media (Fig. 4C, lanes 2 and 4). The size of secreted NPTII was smaller than predicted for NPTII (∼32 kDa) if the propeptide was the only segment removed, suggesting that the mature protein underwent other forms of proteolytic processing in the secretory pathway. A 68-kDa protein was co-immunoprecipitated with the NPTII-related proteins from cells expressing the fusion protein (lane 2); its identity is unknown.

Inhibitory activity of aberrant mRNA translational products

The precursor for the neuroendocrine protein 7B2 is a model substrate for testing proprotein convertase activity ex vivo.

Fig. 3. Identification and characterization of aberrant mRNAs transcribed from the mutant allele. (A) Diagramatic representation of the 5′ region of the wild-type (+) and disrupted (−) Pcsk1 allele with below the expected transcript represented below. Intron 1 splicing out is represented with broken lines; primer regions with inverted flags. The prefix “e” in e1, e2 etc stand for exon. (B) Agarose gel analysis of RT–PCR amplicons from pituitary RNA from +/+ and +/- male mice, using primer pairs # 4 and # 5.
To determine whether the PC1-pro-NPTII could affect pro7B2 processing, we transiently co-transfected expression vectors for this aberrant protein and for pro7B2 into HEK293 cells. These cells express furin, PACE4, PC5 and PC7 (Tsuji et al., 2002). The cells were metabolically labeled and 7B2-related proteins were identified following immunoprecipitation, SDS–PAGE and phosphorimaging. 7B2-immunoreactive proteins of estimated molecular weights of 30, 28, 25 and 23 kDa were observed (Fig. 5B); they presumably correspond to 7B2-161, 7B2-161, 7B2-161 and 7B2-161, respectively (see Fig. 5A). 7B2-161 and 7B2-161 are the major secreted forms. The relative abundance of each 7B2 form (% of total) is shown in Fig. 5C. Of all the forms, only the 28-kDa 7B2-161 form was significantly reduced in the presence PC1-Pro-NPTII. PC5A has been shown to generate this form when co-expressed with pro7B2 in monkey kidney BSC40 cells (Benjannet et al., 1997). These results support the possibility that, in +/+ early embryos, aberrant products from the Pcsk1 mutant allele could reduce proteolytic activation of precursors to functional proteins or peptides by broader inhibition of proprotein convertases activities.

**Reduced PC1 expression in the anterior pituitary of Pcsk1+/− mice**

PC1 expression in the anterior lobe of the pituitary of +/+ and +/+−/− mice was compared at the mRNA and protein levels. We chose this lobe because it is the primary site of PC1 expression in this organ (Seidah et al., 1990). Quantitative RT–PCR analysis was conducted using a primer pair that will amplify transcripts from the wild-type Pcsk1 allele only. The anterior pituitary content of PC1 mRNA was comparable between genotypes and between genders. There was a trend towards greater PC1 mRNA levels expression in males than in females of the same genotype (Fig. 6A).

A semi-quantitative immunoblotting of anterior pituitary extracts from +/+ and +/+−/− male mice was conducted using an antibody against a C-terminal fragment of PC1 (identified in Fig. 6B-a). In both genotypes, this antibody recognized a ~80-kDa and a ~20-kDa forms, presumably representing mature PC1-1-643 and the C-terminal peptide PC1-CT-643 (Fig. 6B-b). However, the two PC1 fragments were significantly less abundant in anterior pituitary extracts from +/+−/− mice (p<0.01, n=4) (Figs. 6B-b and c).

Using mass spectrometry (MS), we analyzed the relative abundance of POMC-derived peptides immunoprecipitated with the anti-β-END antibody from acid extracts of anterior pituitaries from males and female mice of both genotypes. Representative MS profiles of these peptides are shown in Fig. 7A. The identities of the peptides specifically immunoprecipitated are provided in Fig. 7B. The identification was based on the close proximity between the expected and the observed molecular masses of the peptides (Supplementary Fig. 1). The MS profiles significantly differed between male and female mice, with males producing more β-END and less βLPH than females. However, within each gender, there was no significant difference between +/+ and +/+−/− mice (Fig. 7C).

Transduced into cells, it can be cleaved by most PCs after one or more of the paired basic residues located in the C-terminal region, generating a pattern of processed products that differ among cell lines (Benjannet et al., 1997). The expected fragments are diagrammatically represented in Fig. 5A.
Effect of dietary fat on weight gain in wild-type and mutant mice

Genetic PC1 deficiency in human causes congenital obesity (Jackson et al., 1997). A similar phenotype was observed in the recently reported Pcsk1\textsuperscript{N222D/N222D} mouse (Lloyd et al., 2006). In the mouse model lacking the promoter and exon 1 of the Pcsk1 gene (Zhu et al., 2002b), it leads to reduced body mass and weight gain with age, relative to +/+ mice, but causes a trend towards greater weight gain in +/- mice. In our model, no significant difference in these parameters was observed between the two genotypes when mice were fed normal chow (not shown). However, when the effect of low-fat diet (LFD) or high-fat diet (HFD) on weight gain with age was examined, some differences were noted (Fig. 8, n=6 per group). At 4 weeks of age, mice of the same gender were of comparable weight, irrespective of the genotype (26.5±1.1 g for +/- vs. 27.1±1.0 g for +/− males; 21.0±0.4 g for +/- vs. 21.0±0.9 g for +/- females). After 13 weeks of LFD, male mice of both genotypes gained comparable amount of weight (23% for +/- vs +/− mice vs. +25% for +/- mice); but +/- female mice gained significantly less (9%) weight than their +/− counterparts (26%). After 13 weeks of HFD, male mice of both genotypes gained more weight than those under LFD (40% vs. 23% for +/- vs. +/− mice; 33% vs. 25% for +/- mice). In contrast, female mice responded differently to the increase in dietary fat intake: whereas +/- were insensitive to it, gaining the same amount of weight (23%) under either diet, +/− mice, on the other hand, were highly sensitive to it, gaining 43% more weight under HFD compared to 9% more under LFD. Plots of weights with increasing age under the two diets per gender and per genotypes are provided as Supplementary Fig. 3. These results suggested that the mutation at the Pcsk1 locus affected female body response to dietary fat content.

Fig. 5. Inhibition of pro7B2 processing by PC1pro-NPTII. (A) Diagrammatic representation of pro7B2 (top bar) and of known products of its processing by PCs (lower bars). The basic residues preceding the cleavage sites are given on top of the top bar. The number of the first and last residue of each form is indicated at the start and the end of each bar, respectively, taking into account carboxypeptidase (CP)-mediated removal of terminal basic residues after PC cleavage, and amide conversion of the terminal Gly followed CP action. The apparent molecular masses of these forms are given at the right under kDa. (B) Cells were transfected with 1 μg of DNA of pCIneo vector or a mixture of equal amounts of pCIneo and pCIneo-7B2 or of pCIneo-7B2 and pCIneo-PC1pro-neo; 48 h later, they were metabolically labeled with \( ^{35} \text{S} \)-methionine/cysteine. Cell extracts and spent media were analyzed for 7B2 forms by immunoprecipitation and SDS–PAGE and semi-quantitative phosphorimaging. The 7B2\textsuperscript{1–171} processed form affected by PC1pro-NPTII expression is identified with an arrowhead. (C) Histogram representation of the relative abundance of 7B2 forms in the absence (open boxes) or in the presence (filled boxes) of PC1pro-NPTII. Box heights and error bars represent mean percentages of 7B2 forms and standard errors of these means (SEM), respectively. The experiment was conducted in triplicate. *p<0.01.
There were no statistical differences between +/+ and +/- mice \((n=4/\text{gender/genotype})\) in fasting glycemia (g/l) after 13 weeks of LFD \((98.3 \pm 18.6 \text{ vs. } 92 \pm 17.5 \text{ for males and } 98.3 \pm 18.6 \text{ vs. } 92 \pm 17.5 \text{ for females})\) and HFD \((124 \pm 11.9 \text{ vs. } 103 \pm 21.7 \text{ for males and } 98.3 \pm 18.6 \text{ vs. } 92 \pm 17.5 \text{ for females})\) as well as in glycemic response to intraperitoneal glucose injection (not shown), suggesting that the mutation did not induce any significant disturbance of glucose homeostasis.

**Discussion**

In this report, we have described a mouse model of targeted inactivation of the *PC1* gene by a deletion/insertion. The deletion was about 32.7-kb long; it removed about three quarters of the structural gene, from exon 2 to exon 10. The insertion was a 1.8-kb PGKneo gene. This deletion/insertion caused a complex phenotype consisting of preimplantation lethality of homozygous mutants, preferential transmission of the mutant allele and susceptibility of heterozygous female mice to dietary fat content. We examined the possibility that the disrupted *Pcsk1* region contained any nested genes (Yu et al., 2005) or regulatory mRNAs (Alvarez-Garcia and Miska, 2005), the loss of which might be invoked to explain this phenotype. Analysis of the deleted genomic region using the GeneComber algorithm (http://www.bioinformatics.ubc.ca) revealed two short nested genes specifying peptides with no significant homology with any known protein. A TargetScan search (http://www.targetscan.org) also failed to identify any potential microRNA sequence or target in the disrupted region.

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*Fig. 6. PC1 expression in anterior pituitaries from individual +/+ and +/− mice. (A) The levels of mRNA for PC1 and TBP were determined by Taqman qRT–PCR using primer pairs and fluorogenic probes described in Supplementary Table 2. The forward and reverse primers for PC1 were derived from exon 6 and exon 7, respectively. They should amplify transcripts from the wild-type allele only since the recognized region was ablated in the disrupted allele. The relative levels of PC1 mRNA were derived after normalization for the levels of TBP mRNA. Values represent means of PC1/TBP ratios±SEM. There was no significant difference between same-gender +/+ and +/− mice. Among +/+ mice, however, ♂ mice contained significantly more PC1 mRNA in their anterior pituitary than ♀ mice \((n=6)\). (B) (a) Diagrammatic representation of PC1 domains. The protein is represented as horizontal box, with the prodomain (pro), the fully mature enzyme (PC1) and the C-terminal domain (CT) differentially colored. The P4–P1′ amino acid sequences encompassing the cleavage sites (↓) are shown above the box. The position of the CT antigen used for raise the antibody used in this analysis is shown as a line below the box. (b) Western blot analysis of proteins extracted from anterior pituitaries of individual male mice. Samples were fractionated by 10–20% SDS–PAGE. Membranes were probed with the anti-PC1-CT (upper panel), or an anti-β actin (lower panel) antibody. (c) Comparison of levels of PC1 and PC1-CT peptide. Densities of immunoreactive PC1 bands in (b) were normalized for the cognate densities of the β actin bands. Values represent means of PC1/TBP ratios±SEM \((n=4)\). Compared to +/+ mice, +/− mice contained in 31% and 54% less anterior pituitary PC1 and PC-CT peptide, respectively \((^{*}p<0.01)\).*
Preimplantation lethality of homozygote mutant embryos was observed among N4 incipient congenic mice of either FVB/N or B6 genetic background. Its causes are still uncertain. Homozygote preimplantation lethality in mouse has generally been associated with intrinsic defects, caused by spontaneous or induced genetic lesions. One example of spontaneous lesions is the lethal yellow (A<sup>y</sup>) mutation, a 170-kb deletion at the mouse agouti (a) locus on Chr 2 (Rinchik et al., 1993). Examples of induced lesions include knockout mice for the mixed lineage leukemia (MLL) gene whose product regulates Hox gene expression (Ayton et al., 2001), for the xeroderma pigmento-

sum-associated genes such as the XPD gene (de Boer et al., 1998) and the XAB2 gene (Yonemasu et al., 2005). In some of these genetic lesions, gene ablation was associated with expression of aberrant transcripts that may have contributed to the lethal phenotype. In the A<sup>y</sup> mutant mouse, for example, the deletion ablates the structural gene for Raly, placing the agouti gene under the Raly gene promoter and causing ubiquitous expression of this normally neonatal skin-restricted gene. The Raly gene specifies an RNA binding protein critical for early growth. Thus, the A<sup>y</sup> phenotype results from both Raly gene ablation and ectopic expression of the agouti gene (Duhl et al.,

Fig. 7. Mass spectrometry of POMC-derived peptides from the anterior pituitary of +/+ and +/- mice. Peptides were immunoprecipitated (IP) with anti-βEND antibody (Ab) from acid extracts of individual anterior pituitaries from ♀ and ♂ mice. (A) Typical profiles of time-of-flight mass spectral analyses of the molecular masses of the βEND-specific immunoreactive peptides [(a) and (b)] from ♀ (a) and ♂ (b) mice or non-specific peptides immunoprecipitated by a preimmune serum from ♀/+ mice (c). (B) Schematic representation of major POMC-derived peptides. POMC is represented as a succession of horizontal boxes. Above the bar are provided the P4–P1 tetrapeptide ending with a pair of basic residues. The numbers of P1 residues are indicated. Detected processed peptides are represented as lines below the boxes. Their identities and their expected masses are specified at the right and the left, respectively. (C) Levels of peptides in each sample were calculated relative to the total ion current and peak areas set as 100%. They are presented mean percentages±SEM. The number of mice (n) used was 7 for ♀/+ and ♂+/- mice; 6 for ♀+/- female and ♂+/+ mice). Significant differences between ♂ and ♀ mice are indicated in the upper panel as *<i>p</i>&lt;0.5, **<i>p</i>&lt;0.005 or ***<i>p</i>&lt;0.0005.
In the *MLL*-null mice, the mutant allele expresses truncated in-frame *Mll* transcripts which encode functional domains of the protein (Ayton et al., 2001).

The *Pcsk1* mutation described in this study also causes both gross genetic alterations and production of aberrant transcripts. However, it is unlikely that these structural and expression abnormalities in the mutant allele are the sole determinants of the preimplantation lethality of *−/−* embryos in vivo, since nearly all fertilized eggs from heterozygote intercrosses can develop to the blastocyst stage in vitro (Fig. 2). The growth pattern of these embryos in culture revealed a transient growth delay from the 4-cell to the morula stage, suggesting that this could be the stage of greater vulnerability for the *−/−* embryos. This is partially supported by the genotyping results of in vivo-grown embryos which show a sharp drop in the frequency of *−/−* embryos after the second cell division (Table 3). The lethality of these embryos in vivo may be due to a deficiency for autocrine factors needed to survive under the selective environment of the oviduct. This deficiency may be relieved in vitro through factor sharing among embryos. It is known that low-density culture of mouse fertilized eggs impairs their development to the blastocyst stage. This impairment can be partially corrected by supplementation of bioactive peptide such as epidermal growth factor (EGF), transforming growth factor (TGF) α or β1 or platelet-activating factor (O’Neill, 1998; Paria and Dey, 1990).

We have recently shown that transcripts for all PCs, except PC2, are found in fertilized eggs. Expression of PC1, furin, PACE4 and PC7 is maintained during development to the blastocyst stage, while that for PC4 and PC5 gradually subsides (St. Germain et al., 2005). These enzymes may be involved in the proteolytic activation of a number of precursors to autocrine factors and their receptors. The embryo and the oviduct produce several factors through this process, among them, GnRH (Casan et al., 1999, 2000; Kawamura et al., 2005; Raga et al., 1999), IGF-1 and-2, IGF-1 receptor (IGF-1R) and TGFβ1–3 (Dalton et al., 1994; Doherty et al., 1994; Lighten et al., 1997). GnRH and IGF-1 have been shown to act as anti-apoptotic agents on embryos in culture (Fabian et al., 2004; Jousan and Hansen, 2004; Kawamura et al., 2005).

**PC1 deficiency in the mutant embryos will undoubtedly lead to impaired processing of precursors to some of these factors. However, considering the overlap in cleavage specificity among PCs (Seidah and Chretien, 1999; Steiner, 1998), furin, PC7 and PACE4 which are expressed throughout preimplantation embryonic development (St. Germain et al., 2005), should be able to partially compensate for this deficiency. The fact that *−/−* preimplantation embryos survive in the mouse model generated by Zhu et al. (2002b) would support such a view. We speculate that, in our model, the deficit of autocrine factors may be aggravated by a more generalized inhibition of PCs caused by secretory truncated PC1 propeptides produced in *−/−* embryos. Propeptides derived from the prodomain of PC1 have been shown to inhibit not only PC1, but also furin, PC5 and PC7 (Basak and Lazure, 2003; Boudreault et al., 1998; Fugere et al., 2002). It is interesting to note that embryonic lethality of *−/−* became noticeable after the 4-cell stage, when degradation of egg-derived mRNAs is virtually complete and activation of the zygotic genome has been initiated (Wang and Dey, 2006). At this time, we have shown that transgenic expression of human PC2 can rescue the early lethality of *−/−* embryos, suggesting that this enzyme is able to compensate for the lack of the wild-type gene (Michaud et al., 1993; Yonemasu et al., 2005).**

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**Fig. 7 (continued).**
stage, activation of both parental mutant alleles would lead to the production and accumulation of the PC1 propeptide fusion proteins with broad inhibitory activity against PCs. This Pcsk1+/− mouse may therefore represent a model of multiple convertase deficiency. The model suggests that drug-mediated broad inhibition of PCs could cause early arrest of pre-implantation embryo development.

Another striking feature of this model is the non-Mendelian, higher frequency of the mutant allele among offspring of all cross combinations. The transmission ratio distortion (TDR) became detectable at the 6- to 8-cell stage of embryonic development and persisted to birth. One of the most examined cases of transmission ratio distortion (TDR) in mouse is the t-complex inversion on Chr 17. In this mouse, the mutant allele is transmitted to up to 95% of the offspring of t/+ males to +/+ female crosses, due to damaging effect of gene products from the t-complex on + sperm during meiosis (Lyon, 2003). Another well-documented case of TDR in mouse is associated with the Om mutation on Chr 11. In this case, TDR was observed in the offspring of reciprocal backcrosses of (B6×DDK)F1 to either parental strain, with preferential transmission of the DDK Om allele when F1 females were mated to B6 males. The distortion has been attributed to meiotic drive leading to preferential segregation of the wild-type chromatids to the polar body during the second meiotic division, leaving the mutant chromatids to contribute to the zygote genome (Wu et al., 2005). The TDR observed with our Pcsk1+/− mouse model cannot be explained through either of these meiotic events since it was absent at the 2-cell stage. It is unclear at present whether the distortion is due to a greater survival of +/+ embryos or a greater lethality of +/+ embryos, under the selective pressure of the oviductal environment.

In humans, total PC1 deficiency is associated with increased body mass at birth (Jackson et al., 1997). The opposite is observed in PC1-null mice generated by Zhu et al. (2002b). In this mouse model, however, +/+ mice gained more weight with age than +/+ mice. In another mouse model of partial PC1 deficiency, a homozygous phenotype of obesity and hyperphagia was reported (Lloyd et al., 2006). In our model, there was no difference between +/+ and +/+ mice of the same gender in weaning weight or age-related weight gain under normal chow. The difference between the three models could be due to differences in genetic backgrounds as well as in the nature of the mutations. The influence of genetic background on the morbidity phenotype was illustrated in a comparative study of mice carrying a targeted inactivation mutation in the Pcsk2 and Sgne1 genes, specifying PC2 and its helper protein 7B2, respectively: both mutations lead to a Cushing-like syndrome in the 129Sv genetic background, but not the B6 one (Peinado et al., 2005).

In the mouse model of PC1 deficiency described in this study, age-induced weight gain is susceptible to dietary fat in female mice: compared to +/+ mice, −/− mice experienced stunted growth under a LFD and catch up growth under HFD. This differential response dietary fat cannot be attributed to differences in appetite since there was no significant difference in food intake between genotypes when mice were fed either a low-fat or a high-fat diet (Supplementary Fig. 3). It was not observed among male mice. The physiological basis for the phenotypic gender dichotomy is unclear. We have observed that the profiles of anterior pituitary POMC-derived peptides were consistently different between male and female mice (Fig. 7). Although these profiles were similar between +/+ and +/+ in this tissue, it remains possible that other precursors in other tissues might be differentially processed. The weak inhibitory activity of the aberrant proteins on PC1-mediated processing of pro7B2 in transfected cells (Fig. 5) is not a compelling argument for an in vivo effect. The putative toxicity of these proteins in early development may be unrelated to this activity. Alternatively, it may be due to derangement of macromolecule...
interactions and dynamics. To explain the greater susceptibility to PC1-deficient female mice to dietary fat, it will be necessary to examine whether the normal levels of PC1 enzymatic activity differs between genders and whether this is associated with differences in PC1-mediated production of central and peripheral peptide hormones and neuropeptides that control nutrient absorption and metabolism.

In summary, we have described a mouse model of heritable PC1 deficiency caused by a deletion/insertion at the Pcsk1 locus and associated with homozygote preimplantation lethality, preferential transmission of the mutant allele and female heterozygote susceptibility to dietary fat.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.03.523.

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