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PINCH1 Plays an Essential Role in Early Murine Embryonic Development but Is Dispensable in Ventricular Cardiomyocytes

Xingqun Liang, Qiang Zhou, Xiaodong Li, Yunfu Sun, Min Lu, Nancy Dalton, John Ross, Jr., and Ju Chen*

Institute of Molecular Medicine, Department of Medicine, University of California, San Diego, La Jolla, California

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PINCH1, an adaptor protein composed of five LIM domains, mediates protein-protein interactions and functions as a component of the integrin–integrin-linked kinase (ILK) complex. The integrin-ILK signaling complex plays a pivotal role in cell motility, proliferation, and survival during embryonic development of many animal species. To elucidate the physiological function of PINCH1 in mouse embryonic development, we have deleted the mouse *PINCH1* gene by homologous recombination. Mice heterozygous for *PINCH1* are viable and indistinguishable from wild-type littermates. However, no viable homozygous offspring were observed from *PINCH1*$^{+/-}$ intercrosses. Histological analysis of homozygous mutant embryos revealed that they had a disorganized egg cylinder by E5.5, which degenerated by E6.5. Furthermore, E5.5 *PINCH1*$^{-/-}$ embryos exhibited decreased cell proliferation and excessive cell death. We have also generated and analyzed mice in which *PINCH1* has been specifically deleted in ventricular cardiomyocytes. These mice exhibit no basal phenotype, with respect to mouse survival, cardiac histology, or cardiac function as measured by echocardiography. Altogether, these data indicate that PINCH1 plays an essential role in early murine embryonic development but is dispensable in ventricular cardiomyocytes.

Integrins mediate cell-cell and cell-matrix interactions and are required for embryonic development (13, 26, 41). Integrins, which contain a cytoplasmic and extracellular domain, link the cytoplasm to the extracellular matrix and play an important role in cytoskeletal organization and regulation of gene expression, which affects cell adhesion, migration, proliferation, differentiation, and survival (3, 16, 17, 23, 26, 27). Integrins transduce their signals by associating with adaptor proteins that interconnect the integrins to the cytoskeleton, cytoplasmic kinases, and transmembrane growth factor receptors. Proteins associating either directly or indirectly with the cytoplasmic tails of integrins modulates the ligand binding capacity of integrins, altering integrin adhesive function by an inside-out signaling mechanism (25, 30).

An essential binding partner of the integrin cytoplasmic domain is the integrin-linked kinase (ILK). ILK is an ankyrin (ANK) repeat protein containing a serine/threonine kinase domain, which interacts with the cytoplasmic tail of β1, β2, and β3 integrins (21). ILK couples integrins and growth factors to downstream signaling pathways, leading to the regulation of diverse processes, such as cell cycle progression, survival, division, and changes in morphology and spreading (9, 10, 49). Genetic studies of *Drosophila melanogaster*, *Caenorhabditis elegans*, and mice have provided direct evidence that ILK can function as an important mediator of the integrin-dependent signaling pathway (31, 38, 50). ILK is composed of four ANK repeats (21), a pleckstrin homology (PH)-like domain (11), and a C-terminal Ser/Thr kinase domain (21). The first ANK domain has been shown to bind to the two highly related LIM-domain-only proteins PINCH1 and -2 (4, 44, 51).

PINCH, which is composed of five tandemly arrayed LIM domains, has been suggested to play a role in integrin-ILK function (36, 48). LIM domains are double zinc finger structures that serve as protein binding interfaces (33, 39). It has been suggested that PINCH functions as a molecular scaffold that supports the assembly of a multiprotein complex at sites of integrin enrichment (48). Biochemical studies of human PINCH1 have identified ILK as a binding partner for the first LIM domain of PINCH (44) and the SH2-SH3 adaptor protein NCK2 as a partner for the fourth LIM domain (45). Both the colocalization of PINCH1 with integrins and its capacity to bind ILK and NCK2 provided the first hints that PINCH1 might play a role in recruitment of regulatory factors to integrin-rich sites and, therefore, contribute to the integrin signaling cascade (47, 49).

Genetic studies of *C. elegans* and *Drosophila* point to an essential role of PINCH as an adaptor protein in mediating integrin-ILK-dependent signaling (7, 24). The deletion of *UNC-97*, an orthologue of *PINCH1*, in *C. elegans* results in an embryonic-lethal phenotype called PAT (paralyzed and arrested elongation at the twofold stage) (24) resembling that of β-*integrin*/*PAT-3* (15) or *ILK*/*PAT-4* (31). In *Drosophila* muscle, PINCH displays a completely overlapping expression pattern with ILK and βPS integrin, prominently enriched at the muscle attachment sites (7). Flies deficient in *PINCH1* (named *stck* in *Drosophila*) exhibit muscle detachment, similar to the phenotypes of *ILK* and *PS-integrin* (7, 28, 32, 50).

In this study, we report the phenotype of *PINCH1* germ line-deficient mice and mice in which *PINCH1* has been spe-

* Corresponding author. Mailing address: Department of Medicine, University of California at San Diego, School of Medicine, 9500 Gilman Dr., La Jolla, CA 92093-0613. Phone: (858) 822-2452. Fax: (858) 534-2069. E-mail: juchen@ucsd.edu.

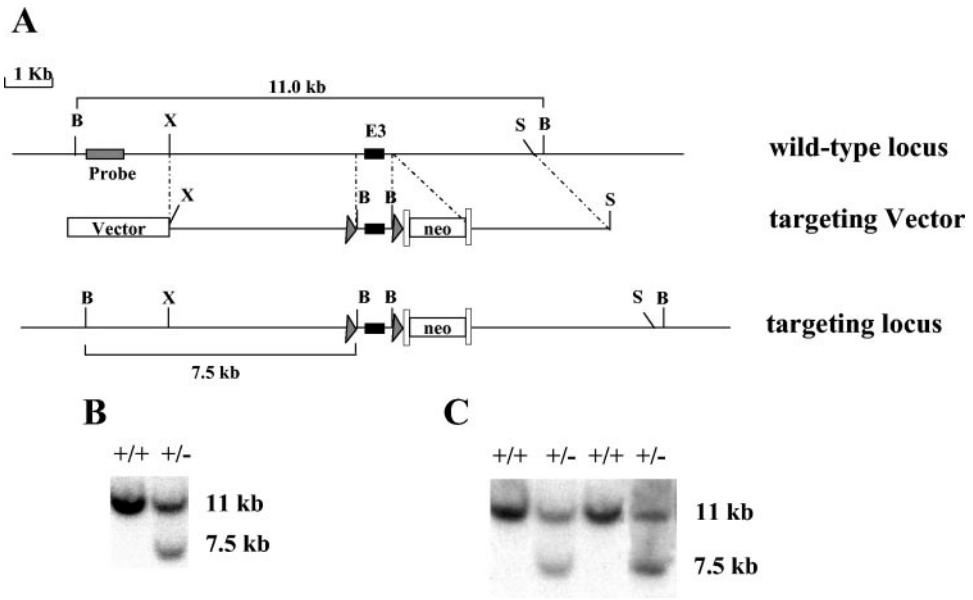

FIG. 1. Targeted generation of *PINCH1*-null mice. (A) Targeting strategy. A restriction map of the relevant genomic region of *PINCH1* is shown on the top, the targeting construct is shown in the center, and the mutated locus after recombination is shown at the bottom. The targeting construct was generated by introducing one *loxP* site into the second intron and another *loxP* site and the *neo* cassette flanked by *frt* sites into the third intron of the *PINCH1* gene. B, BamHI; S, SalI; X, XbaI. neo represents the neomycin resistance gene, arrowheads represent *loxP* sites, and the long boxes represent *frt* sites. (B and C) Detection of wild-type and targeted alleles by Southern blot analysis. DNAs from electroporated ES cells (B) and from the progeny of germ line-transmitted chimera mice (C) were digested with BamHI and analyzed by Southern blot analysis with the probe as shown in panel A. The 11- and 7.5-kb bands represent wild-type and mutant alleles, respectively.

cifically deleted in ventricular cardiomyocytes. We show that *PINCH1* is already detectable in blastocysts at approximately E3.5. *PINCH1*$^{-/-}$ embryos at E5.5 exhibit a disorganized egg cylinder, with decreased cell proliferation and excessive cell death, thus pointing to an important role of PINCH1 in the formation of egg cylinders. We also show that mice in which *PINCH1* is specifically deleted in cardiomyocytes exhibit no basal phenotype with regard to mouse survival, cardiac histology, or cardiac function.

MATERIALS AND METHODS

Gene targeting. A genomic *PINCH1* fragment was isolated from a 129SVJ library (Stratagene) and used to construct the *PINCH1* targeting vector by standard techniques as shown in Fig. 1A. Briefly, one *loxP* site was inserted into the second intron and a second *loxP* site along with the *neo* cassette flanked by *frt* sites was inserted into the third intron of the *PINCH1* gene. The targeting vector was linearized with SalI and electroporated into R1 embryonic stem (ES) cells. Three hundred ninety-four G418-resistant ES clones were screened for homologous recombination by Southern blot analysis as described below.

Southern blot analysis. DNA was extracted from G418-resistant ES cell clones as previously described (34). ES cell DNA was digested with BamHI, electrophoresed on a 0.8% (wt/vol) agarose gel, and subsequently blotted onto nitrocellulose. A 480-bp fragment, corresponding to the 5′ end of the right arm of the target vector, was generated by PCR with mouse genomic DNA and specific *PINCH1* primers (forward primer, 5′-ATATGATAGGAGACACTATTCACG; reverse primer, 5′-AAGCTTCAGAAAGGACCTGT). The PCR product was subsequently radiolabeled using [^{32}P]dATP by random priming (Invitrogen, San Diego, Calif.). DNA blots were hybridized with the radiolabeled probe and visualized by autoradiography. The wild-type allele is represented by a band of 11 kb, whereas a band of 7.5 kb represents the correctly targeted mutant allele.

Generation and genotyping of mice. Two independent homologous recombinant clones were microinjected into blastocysts from C57BL/6J mice at the Transgenic Core Facility of the University of California, San Diego. Male chimeras were inbred with female Black Swiss mice to generate germ line-transmitted heterozygous mice with a *neo* cassette (PINCH1$^{+/flox+neo}$). PINCH1$^{+/flox+neo}$ mice were crossed with protamine-Cre (Pro-Cre) mice (35), generating mice which were doubly heterozygous (Pro-Cre/PINCH1$^{+/flox+neo}$). Cre expression in Pro-Cre mice is restricted to male germ cells undergoing spermatogenesis. Therefore, Pro-Cre/PINCH1$^{+/flox+neo}$ males were crossed to female breeders to generate germ line-heterozygous-null mutant offspring. Heterozygous mice were interbred to generate homozygous knockouts. Progeny from these crosses were genotyped by PCR with the wild-type-specific primers (forward primer, P1, CCCAGAAG GACTCTTTTATGAG; reverse primer, P2, CTTGGAGAAGAAGTACTCA GGT) and primers for the mutant allele (*neo*-specific primer, Pneo, AATGGG CTGACCGCTTCCTCGT; reverse primer, P3, CTTGGAGAAGAAGTACTC AGGT). To identify the genotype of the embryos at the peri-implantation stage, uterine deciduas around E5.5 were sectioned and stained with antibody specific for PINCH1 as described below.

PINCH1$^{+/flox+neo}$ mice were crossed with FLPase deleter mice (37), which delete DNA sequences flanked by two *frt* sites in all cell types. This resulted in the generation of mice with a floxed *PINCH1* allele no longer containing the *neo* cassette (PINCH1$^{+/flox}$). PINCH1$^{+/flox}$ mice were subsequently intercrossed and crossed with MLC2v-Cre mice (5, 6) to generate mice which were homozygous floxed (PINCH1$^{flox/flox}$) and doubly heterozygous for *PINCH1* floxed allele and MLC2v-Cre allele (PINCH1$^{+/flox+neo}$ Cre). Interbreeding between both PINCH1$^{flox/flox}$ mice and the PINCH1$^{+/flox+neo}$ Cre mice was used to generate mice in which *PINCH1* is specifically deleted in ventricular cardiomyocytes. To genotype *PINCH1* floxed and MLC2v-Cre-positive alleles, *PINCH1* primers (forward primer, P4, CCCAGAAGGACTCTTTTATGAG; reverse primer, P5, CT TGGAGAAGAAGTACTCAGGT) and Cre primers (forward primer, Pcre1, GTTCGCAAGAACCTGATGGACA; reverse primer, Pcre2, CTAGAGCCTG TTTTGCACGTTC) were used.

Reverse transcription-PCR (RT-PCR) analysis. Total RNA was isolated from ES cells and embryos at E6.5 and E7.5 by using Trizol reagent (GIBCO BRL). First-strand cDNA synthesis was performed with the random primer and Superscript kit (Invitrogen). The cDNA was utilized as a PCR template to perform PCR by standard protocols. Specific primers for *PINCH1* (forward, TCAAGA ATGCTGGCAGACAC; reverse, ACACCAGGCCTTGTTGAGAG) were utilized.

In situ hybridization. Whole-mount in situ hybridization was carried out with digoxigenin-labeled RNA probes as previously described (46). A murine RNA probe spanning the 1,000-bp fragment of *PINCH1* cDNA was utilized.

Dissection and histological analysis of embryos. Timed matings were conducted with interbreeding between *PINCH1*$^{+/-}$ mice. Females with copulation plugs were considered to be at embryonic development day 0.5 (E0.5) of gestation. Pregnant females were sacrificed at different time points of gestation, and the embryos were dissected from maternal tissue, examined, photographed, and genotyped by PCR. For histological preparations, embryos in decidua were fixed in 4% paraformaldehyde overnight at 4°C and destined for paraffin embedding. Serial sagittal sections were cut at 5 μm from paraffin blocks and stained with hematoxylin and eosin.

Immunostaining assays. Five-micrometer sections were treated for 1 h with 5% bovine serum albumin in phosphate-buffered saline and subsequently incubated overnight at 4°C in a humidified chamber with a polyclonal antibody to PINCH1 (provided by A. Rearden, University of California, San Diego). After being washed with 0.25% Triton X-100 in phosphate-buffered saline, the sections were incubated with fluorescently labeled secondary antibodies. The specimens embedded in Vectashield mounting medium (Vector Laboratories) were analyzed under the fluorescence microscope.

BrdU labeling of embryos. Pregnant females at E5.5 were injected intraperitoneally with bromodeoxyuridine (BrdU; Amersham-Pharmacia, Little Chalfont, Buckinghamshire, United Kingdom) and sacrificed 2 h later. Deciduae were removed and fixed in 4% paraformaldehyde overnight at 4°C. Five-micrometer sections were denatured with 2 N HCl, trypsinized, and incubated with a mouse monoclonal antibody to BrdU (Sigma; B 2531). Detection was performed utilizing a peroxidase ABC kit (Vector Laboratories) and 3,3-diaminobenzidine. BrdU-labeled cells were counted from sections. For quantitative analyses, representative sections from ventral, mid-, and dorsal levels of each embryo were utilized to count the total number of cells and the number that were labeled with BrdU, to give a proliferation index.

Apoptosis assays. Implantation sites (from E5.5 to E6.5) were collected for apoptosis studies. To detect apoptotic cells, the terminal deoxynucleotidyltransferase-mediated biotinylated UTP nick end labeling (TUNEL) assay was performed according to the manufacturer's instructions (fluorescein in situ cell death detection kit; Boehringer Mannheim, Mannheim, Germany). Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) nuclear stain (Vector Laboratories). In addition to TUNEL staining, DNA fragmentation was verified under UV illumination by using the DAPI counterstain.

Echocardiographic analysis. Mice were anesthetized with isofluorane and subjected to echocardiography as previously described (43).

RESULTS

Targeted disruption of *PINCH1* gene in the mouse. To explore the physiological role of PINCH1 in the mouse, we have generated a conditional allele of the *PINCH1* gene by homologous recombination in ES cells. We and others have successfully generated multiple floxed alleles utilizing three *loxP* sites (tri-*loxP*) (5, 18). However, a drawback to the tri-*loxP* approach is that it can require two in vitro manipulations of ES cells, one for the original selection and one to excise the selectable *neo* gene. Removal of the *neo* gene is desirable, as its presence can disrupt expression of the targeted gene, preventing the floxed allele from being phenotypically neutral. However, additional in vitro manipulation of ES cells jeopardizes the likelihood of germ line transmission. To avoid this problem, we and others have capitalized on the recent development of another site-specific recombination system which has been used in transgenic mice, the *frt*-site/FLPase recombinase system, which does not overlap with the *loxP*/*cre*-mediated recombination. A combination of these two systems can be utilized to generate a floxed allele containing the *neo* gene flanked by *frt* sites. In this manner, the *neo* gene can be removed from the floxed allele in vivo by breeding a mouse containing the floxed allele to a mouse which expresses FLPase in all cells (37).

The targeting vector for *PINCH1* was designed as shown in Fig. 1A, in which one *loxP* site was introduced into the second intron and a second *loxP* site as well as the *neo* cassette flanked by *frt* sites was introduced into the third intron of the *PINCH1* gene. This targeting vector was linearized with SalI and introduced into R1 ES cells via electroporation. G418-resistant ES clones were screened for homologous recombination by Southern blot analysis. Of 394 ES cell clones analyzed (Fig. 1B), two had undergone homologous recombination. These two ES cell clones were independently injected into blastocysts and gave rise to chimera mice that were then used to breed mice which would be germ line-transmitting heterozygous mice with the *neo* cassette (PINCH1$^{+/flox+neo}$). The PINCH1$^{+/flox+neo}$ mice were then bred to homozygosity for the conditional allele (PINCH1$^{flox+neo/flox+neo}$). Although the PINCH1$^{flox+neo/flox+neo}$ mice can survive and seem normal, to avoid potential effects of the *neo* cassette on *PINCH1* gene expression, we crossed PINCH1$^{+/flox+neo}$ mice with FLPase deleter mice (37), which deletes DNA sequences flanked by two *frt* sites in all cell types. This resulted in the generation of mice with a floxed *PINCH1* allele no longer containing the *neo* cassette (PINCH1$^{+/flox}$). PINCH1$^{+/flox}$ mice were subsequently intercrossed to generate mice which were homozygous floxed (PINCH1$^{flox/flox}$). The PINCH1$^{flox/flox}$ mice were then crossed with MLC2v-Cre mice to generate mice in which PINCH1 is specifically deleted in ventricular cardiomyocytes.

PINCH1$^{+/flox+neo}$ mice were crossed with Pro-Cre mice (35), generating mice which were doubly heterozygous (Pro-Cre PINCH1$^{+/flox+neo}$). Cre expression in Pro-Cre mice is restricted to male germ cells undergoing spermatogenesis. Therefore Pro-Cre PINCH1$^{+/flox+neo}$ males were crossed to female breeders to generate germ line-heterozygous-null mutant offspring.

TABLE 1. Phenotypes and genotypes of offspring from *PINCH*$^{+/-}$ intercrosses

Stage	No. of offspring				
	Phenotype		Genotype		
	Normal	Abnormal-resorbed	+/+	+/−	−/−
1 mo old	200		60	140	0
E9.5	36	9[a]	12	24	0
E8.5	32	8[a]	10	22	0
E7.5	20	6[a]	10	20	0
E6.5	20	4	7	13	4
E5.5	20[b]	5[b]			

[a] No DNA can be isolated from the resorbed embryos.
[b] Embryo tissue could not be dissected freely from maternal tissue.

The *PINCH1* null mutation results in early embryonic lethality. Heterozygous *PINCH1* mutants survive and have no apparent phenotype. Heterozygous *PINCH1* mice were interbred in an attempt to generate homozygous knockouts. However, the genotypic analysis of mice generated from interbred heterozygous *PINCH1* mice failed to detect any homozygous null offspring in over 200 mice that were analyzed. Of the progeny from heterozygote intercrosses, 30% (n = 60) were wild type and 70% (n = 140) were heterozygous for the *PINCH1* gene (Table 1). Approximately 50 homozygous knockout offspring would have been expected, if homozygous mice were to be viable. These results indicated that loss of PINCH1 function is associated with embryonic lethality.

To determine the time point at which *PINCH1*$^{-/-}$ embryos died, we set up timed matings and analyzed embryos by a

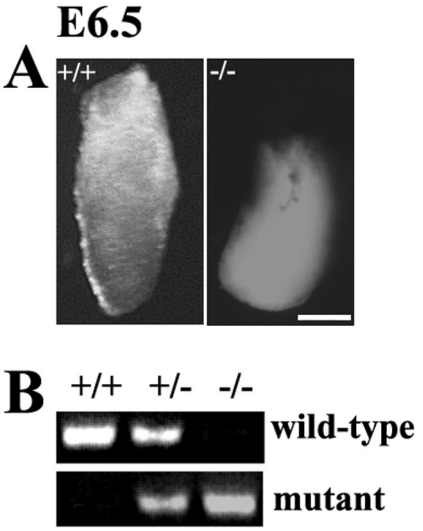

FIG. 2. Phenotype of *PINCH1*$^{-/-}$ embryos at the gross morphological level. (A) At the gross morphological level, a *PINCH1*$^{-/-}$ embryo of E6.5 appears smaller than its wild-type littermate and degenerated. Bar, 50 μm. (B) The genotypes of embryos were identified by PCR.

PCR-based protocol. No homozygous mutant embryos were found between E7.5 and E9.5. However, at E7.5 and E8.5, severely developmentally retarded and resorbed embryos were found, with the percentage of resorption being as high as 25%. At E6.5, 25% of all embryos appeared small and misshapen at a gross morphological level (Fig. 2A). We genotyped four abnormal embryos at E6.5 by PCR and found that they were indeed *PINCH1*$^{-/-}$ mutants (Fig. 2B).

To investigate the time course of lethality, we examined serial sections of embryos in utero between E5.5 and E6.5. To genotype embryos at the peri-implantation stage, uterine decidua at proximately E5.5 was immunostained with antibodies specific for PINCH1. As shown in Fig. 3A and C, both wild-type and *PINCH1*$^{+/-}$ embryos at approximately E5.5 and E6.5 developed into egg cylinders of normal size with a layer of visceral endoderm, which exhibited organized structures with a proximal-distal polarity. In addition, a proamniotic cavity was present in the embryonic region in control embryos at E5.5 (Fig. 3A). In contrast, *PINCH1*$^{-/-}$ embryos at E5.5 that failed to positively stain for PINCH1 antibody appeared significantly smaller and showed abnormal structures, being oval in shape with disorganized inner and outer layers (Fig. 3B). In addition, the proamniotic cavity was missing in most *PINCH1*$^{-/-}$ embryos. At E6.5, about 25% of uterine decidua appeared degenerated and partially resorbed, with no organized cellular structure (Fig. 3D). Extensive resorption as high as 25% was observed in uterine decidua at E7.5 (data not shown). These results indicate that *PINCH1* homozygous mutant mice die shortly after implantation.

***PINCH1* is expressed in embryos during early developmental stages.** Early embryonic lethality resulting from the deletion of the *PINCH1* gene led us to determine the temporal and spatial patterns of *PINCH1* expression during peri-implantation stages. To determine the expression profile of *PINCH1*, we performed both RT-PCR analysis of blastocysts and embryos at E6.5 and E7.5 and whole-mount in situ hybridization of embryos at E6.5 and E7.5. RT-PCR analysis showed that expression of *PINCH1* is already detectable in blastocysts around E3.5 (Fig. 4A). Whole-mount in situ hybridization demonstrated that *PINCH1* is highly expressed in the neuroectoderm at E6.5 and E7.5, with more diffuse staining in the remainder of the embryo (Fig. 4B). These data imply the importance of PINCH1 for early mouse development.

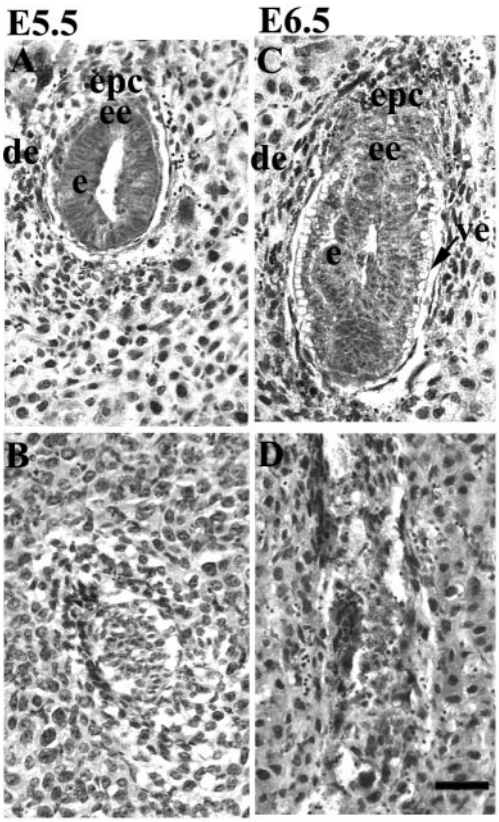

FIG. 3. Histological analysis of embryos generated from *PINCH1* heterozygous intercrosses by hematoxylin and eosin staining. (A and B) Sagittal sections of E5.5 control (A) and mutant (B) embryos. (C and D) Sagittal sections of E6.5 control (C) and mutant (D) embryos. de, decidua; epc, ectoplacental cone; ee, extraembryonic ectoderm; e, ectoderm; ve, visceral endoderm. Bar, 50 μm.

Proliferation and apoptosis in *PINCH1*$^{-/-}$ embryos. At E5.5, *PINCH1*$^{-/-}$ embryos appear smaller than control littermates. Accordingly, we examined the rate of proliferation using BrdU incorporation and the extent of apoptosis by performing TUNEL assays in *PINCH1* mutant and control littermates at E5.5. To genotype embryos, uterine deciduae were immunostained with antibodies specific for PINCH1 (Fig. 5A and B).

BrdU labeling showed that the rate of proliferation in *PINCH1*$^{-/-}$ embryos was significantly reduced in comparison to that of control littermates (Fig. 5C and D). Approximately 85% of cells in visceral endoderm and epiblast of control embryos exhibited BrdU-positive staining, whereas less than 5% of cells in mutants were labeled. As shown by TUNEL staining in Fig. 5E, few apoptotic cells were observed in control embryos where the lumen would be forming. In contrast, ex-

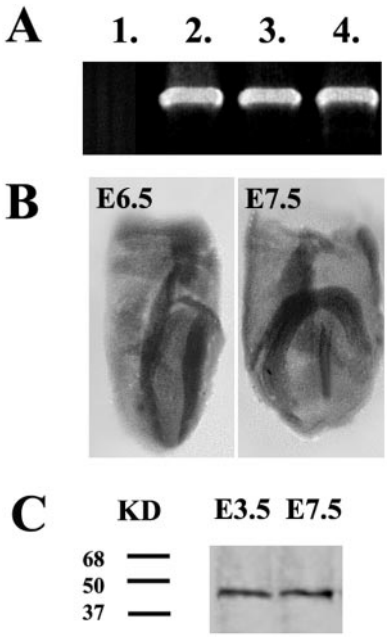

FIG. 4. *PINCH1* expression in mouse embryos at early stages. (A) RT-PCR shows that *PINCH1* expression is already detectable in blastocysts at E3.5. Total RNA was isolated from blastocysts of E3.5 and embryos at E6.5 and E7.5 and analyzed by RT-PCR with specific primers for *PINCH1*. Lane 1, negative control; lane 2, E3.5; lane 3, E6.5; lane 4, E7.5. (B) Whole-mount in situ hybridization with *PINCH1* probe demonstrates that PINCH1 is highly expressed in the neuroectoderm at E6.5 and E7.5, with more diffuse staining in the remainder of the embryo. (C) Western blot analysis shows that PINCH1 is detectable in E3.5 blastocysts. Protein was isolated from E3.5 blastocysts and E7.5 embryos and analyzed with a specific antibody to PINCH1.

cessive TUNEL-positive cells were scattered throughout *PINCH1*$^{-/-}$ embryos at E5.5 (Fig. 5F). Therefore, it is likely that the growth deficit of *PINCH1* homozygous embryos resulted from both decreased cell proliferation and increased cell death.

Mice in which *PINCH1* is specifically deleted in ventricular cardiomyocytes exhibit no basal phenotype with regard to mouse survival, cardiac histology, or cardiac function. It has been shown that mice which have β1 integrin specifically deleted in ventricular cardiomyocytes display myocardial fibrosis and depressed left ventricular contractility and relaxation (40). In addition, a recent study demonstrated that PINCH1 forms a functional complex with β4 thymosin and ILK. This complex was suggested to play an essential role in promoting cardiomyocyte migration and survival (2). To determine the functional role of PINCH1 in cardiomyocytes, we generated mice in which *PINCH1* has been specifically deleted in ventricular cardiomyocytes by utilizing MLC2v-Cre mice. These mice have been shown to mediate DNA recombination specifically in ventricular cardiomyocytes starting from embryonic day 8.5 (5). Pups in which *PINCH1* has been specifically deleted in ventricular cardiomyocytes (PINCH1$^{flox/flox}$ Cre$^{+/-}$) were born normally, were externally indistinguishable from littermates of other genotypes, were recovered at Mendelian frequency (for MLC2v wild-type mice, 19 offspring were PINCH$^{flox/flox}$ and 18 were PINCH$^{flox/+}$; for MLC2v-Cre/+ mice, 18 offspring were PINCH$^{flox/flox}$ and 23 were PINCH$^{flox/+}$), and grew to adulthood without signs of cardiac malfunction.

DNA analysis of adult animals (1 to 2 months old) confirmed *PINCH1* gene excision only in ventricular tissue derived

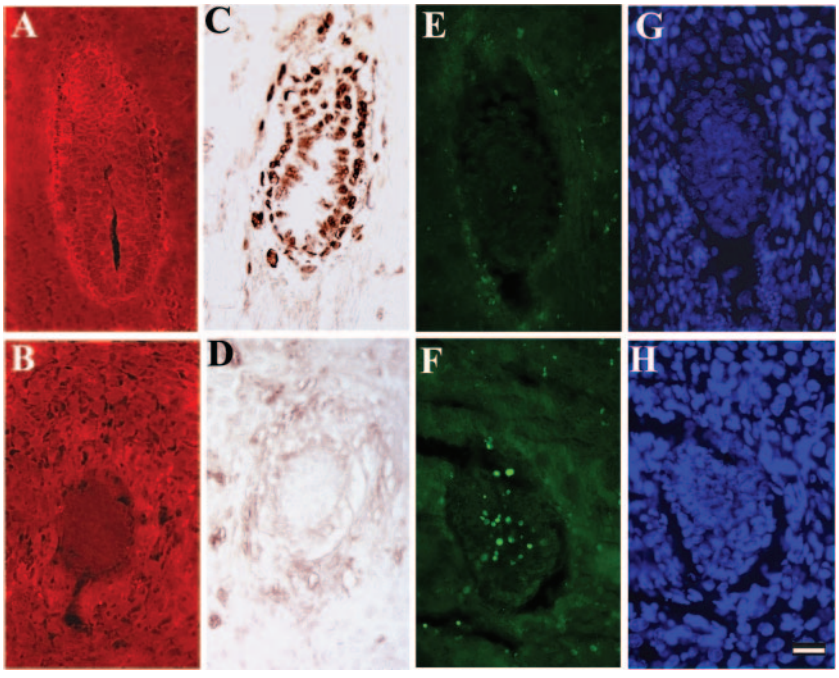

FIG. 5. BrdU labeling and TUNEL analysis of *PINCH1* mutants and control littermates at E5.5. (A and B) Immunostaining with PINCH1 antibody identified control (A) and PINCH1 mutant (B) embryos. (C and D) BrdU labeling showed proliferation indices in control littermates (C) and *PINCH1* mutants (D), showing lower proliferative activity in *PINCH1* mutants than in control littermates. (E to H) TUNEL analysis performed on sections from control littermates (E) and PINCH1 mutants (F); DAPI staining was used to visualize nuclei of control littermates (G) and mutants (H). Bar, 50 μm.

TABLE 2. Echocardiographic measurements under basal conditions[a]

Measurement	PINCH1$^{flox/flox}$ (n = 7)	PINCH1$^{flox/flox}$ Cre$^{+/-}$ (n = 9)
BW (g)	28.1 ± 4.38	30.0 ± 5.27
HR (bpm)	511.6 ± 46.32	471.8 ± 101.7
LVEDD (mm)	3.48 ± 0.38	3.71 ± 0.75
LVESD (mm)	2.00 ± 0.42	2.25 ± 0.65
PWth (mm)	0.63 ± 0.05	0.60 ± 0.03
IVSth (mm)	0.64 ± 0.05	0.61 ± 0.04
LV%FS	43.05 ± 6.31	40.01 ± 6.76
Vcf (circ/s)	8.98 ± 1.70	8.42 ± 2.14

[a] All data are presented as means ± standard errors. BW, body weight; HR, heart rate; LVEDD, end-diastolic left ventricular dimension; LVESD, end-systolic left ventricular dimension; PWth, left ventricular posterior wall thickness; IVSth, interventricular wall thickness; LV%FS, left ventricular percent fractional shortening; Vcf, velocity of circumferential fiber shortening. bpm, beats per minute; circ, circumference.

from PINCH1$^{flox/flox}$ Cre$^{+/-}$ mice, with an efficiency of Cre-mediated recombination of approximately 85% (data not shown), which is in agreement with other studies with MLC2v-Cre-mediated excision (5, 12, 20, 22, 40).

To determine whether the PINCH1$^{flox/flox}$ Cre$^{+/-}$ mice display any histological changes, we performed histological studies. Paraffin sections (10 μm) of heart from PINCH1$^{flox/flox}$ Cre$^{+/-}$ and PINCH1$^{flox/flox}$ littermate controls (6 months old) were stained with hematoxylin and eosin. All specimens appeared normal, exhibiting no sign of hypertrophy, myocardial disarray, infarction, necrosis, fibrosis, calcification, or fat infiltration (data not shown). Cardiac function was evaluated noninvasively by echocardiography at 4 to 6 months of age. There were no significant differences in all of the echocardiographic parameters assessed between wild-type and mutant groups (Table 2).

DISCUSSION

In the present study, we have investigated the functional role of PINCH1 in mouse embryonic development by generating *PINCH1*-deficient mice and analyzing *PINCH1* expression during early embryogenesis. *PINCH1*$^{-/-}$ mice failed to form organized egg cylinders or to cavitate and subsequently died at the peri-implantation period. These results demonstrate a pivotal role for PINCH1 in early mouse embryogenesis. PINCH1$^{-/-}$ embryos exhibited decreased cell proliferation and excessive cell death. However, whether these are the primary effects of lack of PINCH1 or secondary to the overall poor embryonic development remains to be determined.

Morphogenesis of peri-implantation mouse embryos involves extensive cell-cell and cell matrix interactions. During the peri-implantation period, the inner cell mass (ICM) of the blastocyst develops into the primitive endoderm and the epiblast, which form the embryo proper (42). The primitive endoderm forms the surface of the ICM of blastocyst and deposits a basement membrane. The basement membrane is required for adjacent ICM cells to polarize and establish the columnar epiblast (8). The importance of integrin-ILK-mediated cell-cell and cell-matrix interactions during early embryonic development is highlighted by genetic studies in mouse models (1, 13, 29, 38, 41). In β1-integrin-deficient embryos, the primitive endoderm fails to produce laminin α1 and therefore no basement membrane is formed (1, 29). In mouse embryos lacking ILK, the primitive endoderm differentiates and produces a basement membrane but the epiblast fails to polarize or cavitate, and mutants die at the peri-implantation stage (38).

Biochemical studies have demonstrated that PINCH1 functions as an important mediator of integrin- and ILK-dependent signaling pathways (14, 19). Here, we show that *PINCH1*-null mutant mice, like β*1-integrin*- and *ILK*-null mutant mice, die at the peri-implantation stage, providing in vivo evidence that PINCH1 is a critical component of the integrin-ILK pathway in vertebrates.

Our data on *PINCH1* germ line-knockout mice are consistent with genetic studies in *C. elegans* and *Drosophila* (7, 24). Deletion of *PINCH* in *C. elegans* results in an embryonic-lethal phenotype called PAT (24), resembling that of β-*integrin*/*PAT-3* (15) or *ILK*/*PAT-4* (31). *PINCH*-deficient flies exhibit muscle detachment, similar to the phenotypes of *ILK* and *PS-integrin* (7, 28, 32, 50). All these data suggest that the β1-integrin–ILK–PINCH complex is a functional complex that is highly conserved from invertebrates to mammals.

To our surprise, mice in which *PINCH1* has been specifically deleted in ventricular cardiomyocytes exhibit no basal phenotype with regard to mouse survival, cardiac histology, or cardiac function as measured by echocardiography. Although this is a negative result, we think it is a very significant one, for reasons discussed below.

So far all data from *Drosophila*, *C. elegans*, mammalian cells, and our studies with mouse embryos have indicated that PINCH1 is indispensable for β1-integrin function (see discussion above). Mice which have β*1-integrin* specifically deleted in ventricular cardiomyocytes, with the use of the same MLC2v-Cre mouse that we used in our studies, display myocardial fibrosis, depressed left ventricular contractility and relaxation, and development of heart failure by 6 months of age (40). Our data demonstrate that PINCH1 is dispensable for β1-integrin function in ventricular cardiomyocytes.

Additionally, a recent study demonstrated that PINCH1 forms a functional complex with β4 thymosin and ILK. This complex was suggested to play an essential role in promoting cardiomyocyte migration and survival (2). Our data suggest that, if this complex does play an essential role in cardiomyocyte migration and survival, PINCH1 is certainly dispensable for the complex.

Two highly homologous proteins, PINCH1 and PINCH2, are encoded by two distinct genes (48) and have both been shown to interact with the ANK domain of ILK (48). Both proteins are widely expressed, which raised the possibility that they could be functionally redundant (48). Our data demonstrate that PINCH1 is essential for early murine embryonic development and that PINCH2 cannot compensate for the loss of PINCH1 during early embryonic development. However, it is possible that the lack of phenotype in mice in which *PINCH1* is specifically deleted in ventricular cardiomyocytes is due to a redundant role of *PINCH2* in cardiomyocytes.

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