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Research Article

Phenotype of Parathyroid-targeted *Cdc73* Deletion in Mice Is Strain-dependent

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Abstract

Hyperparathyroidism jaw-tumor syndrome is an autosomal dominant disorder caused by mutations in the *CDC73/HRPT2* tumor suppressor gene, encoding parafibromin, and manifesting benign or malignant parathyroid tumors, ossifying jaw fibromas, uterine tumors, and kidney lesions. Sporadic parathyroid carcinomas also frequently exhibit inactivating *CDC73* mutations and loss of parafibromin. To study the role of *CDC73* in parathyroid cell proliferation in vivo, we generated mice with a parathyroid-specific deletion of *Cdc73*. Homozygous knockout mice on a mixed B6/129/CD1 background had decreased serum calcium and PTH and smaller parathyroid glands compared with heterozygous or wild-type littermates, whereas homozygous *Cdc73*-null mice on other backgrounds exhibited no abnormalities in parathyroid gland function or development. No hypercalcemia or parathyroid hypercellularity was observed in mice of any background examined at any age. Thus, although postnatally acquired complete loss of *CDC73* causes parathyroid cell proliferation and hyperparathyroidism, such as seen in human hyperparathyroidism jaw-tumor syndrome, our results suggest that earlier, developmentally imposed complete loss of *Cdc73* can cause a primary defect in parathyroid gland structure/function in a strain-dependent manner. This striking disparity in parathyroid phenotype related to genetic background offers a unique opportunity in an in vivo model system to precisely dissect and identify the responsible molecular mechanisms.

Key Words: parathyroid carcinoma, hyperparathyroidism, parathyroid neoplasia, hyperparathyroidism-jaw tumor syndrome, parafibromin

Abbreviations: FIH, familial isolated hyperparathyroidism; HPT-JT, hyperparathyroidism-jaw tumor syndrome.

Germline, inactivating mutations of the *CDC73* tumor suppressor gene (previously called *HRPT2*) in humans lead to the hyperparathyroidism-jaw tumor syndrome (HPT-JT), an autosomal dominant disorder predisposing to multiple primary parathyroid, jaw, uterine, and/or kidney tumors [1]. Familial isolated hyperparathyroidism (FIH) [2, 3] and sporadically presenting parathyroid carcinoma [4-8] have also been associated with germline *CDC73* mutations. Somatic mutation of *CDC73* is common in patients with sporadic parathyroid carcinoma [4-8] and has been described in sporadic renal tumors [9] and ossifying fibromas of the jaws [10].

The mechanisms through which loss of *CDC73*/parafibromin contributes to tumor formation are poorly understood. Parafibromin is a ubiquitously expressed protein bearing sequence homology to Cdc73p, a yeast protein that is a component of the RNA polymerase II-associated Paf1 complex and is an ortholog to Drosophila Hyrax [11]. Parafibromin is an essential component of the human PAF1 complex, which interacts directly with RNA polymerase II [12], and functions in transcriptional regulation through involvement in histone modification, transcriptional initiation, elongation, and post-transcriptional events affecting mRNA stability. Parafibromin can directly interact with β-catenin [11] but its exact role in Wnt/β-catenin signaling is unclear. Parafibromin inhibits cancer cell growth and causes G1-phase arrest in vitro [13], in part through regulation of cyclin D1 [14].

Conventional and conditional transgenic mouse knockouts of *Cdc73* were developed. Homozygous deletion of *Cdc73* is embryonic lethal by embryonic day 6.5 [15]. Controlled germline deletion of *Cdc73*, obtained by crossing *Cdc73*-floxed mice with a tamoxifen-inducible Cre mouse, at later stages of development led to growth retardation, severe cachexia, and death within 20 days of tamoxifen treatment [15]. Initially, no parathyroid gland abnormalities were described in either model [15]. A subsequent study, which followed the conventional heterozygous *Cdc73* knockout mice to 21 months of age, described a subset of mice with increased proliferation in the parathyroid glands, accompanied by histologic abnormalities suggestive of atypical parathyroid adenomas and parathyroid carcinomas in humans, such as

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nuclear pleomorphism and/or fibrous septation but no invasion or metastases [16]. Parathyroid-targeted knockout of *Cdc73*, generated by crossing *Cdc73*-floxed mice with PTH-Cre mice, resulted in similar parathyroid gland abnormalities; both heterozygous and homozygous mice were affected. The kinetics of parathyroid tumorigenesis and biochemical dysfunction were not described but biochemical hyperparathyroidism was seen in heterozygous conventional *Cdc73* knockout mice older than 17 months and heterozygous and homozygous parathyroid-specific *Cdc73* knockout mice older than 20 months [16].

We sought to further study the role of *Cdc73* in parathyroid cell proliferation and function in vivo by generating a parathyroid-specific *Cdc73* knockout mouse.

Materials and Methods

Animals

Mice in which exon 2 of the *Cdc73* gene was flanked by 2 LoxP sites (Hrpt2-floxed) on a mixed C57/129 background were obtained from Dr. Bart Williams [15]. A second line of *Cdc73*-floxed mice was generated by backcrossing *Cdc73*-floxed mice for 10 generations to the FVB strain. PTH-Cre transgenic mice on the FVB background were obtained from Dr. Steven Libutti [17]; a separate line of PTH-Cre mice was generated by backcrossing for 10 generations to CD1 mice. *Cdc73*-floxed mice were crossed with PTH-Cre mice, Cre-positive offspring obtained from this cross were crossed again with *Cdc73*-floxed mice to generate mice homozygous for parathyroid-specific deletion of *Cdc73*, referred to as HPH mice. In total, HPH mice were generated on 3 genetic backgrounds: pure FVB (HPH-FVB), mixed C57/129/CD1 (HPH-M1), and mixed C57/129/FVB (HPH-M2). Mice were fed either Prolab RMH 3000/5P00*, containing 1.09% calcium, 0.78%/0.47% total/available phosphorus, and 2.5 IU/g vitamin D or Teklad 2914, containing 0.7% calcium, 0.6%/0.3% total/available phosphorus, and 0.6 IU/g vitamin D. Genotyping was performed as previously described [15, 17]. All animals were maintained in accordance with guidelines from the Institutional Animal Care Committee and the Association for the Assessment and Accreditation of Laboratory Animal Care. This study was approved by the University of Connecticut Health Center's Institutional Animal Care and Use Committee (Protocol 2009-545).

Serum Biological Chemistry

Blood was collected at 1- to 3-month intervals beginning at 2 months of age via submandibular vein sampling of nonanesthetized mice. A terminal blood collection was performed via cardiac puncture of isoflurane-anesthetized mice. To assess parathyroid function, serum calcium analysis was performed using the Calcium LiquiColor assay (Stanbio/EKF Diagnostics, Boerne, TX, USA); PTH levels were measured using the Mouse Intact PTH ELISA Kit (Immutopics/Quidel, San Clemente, CA, USA; RRID:AB_2941776). To assess kidney function, blood urea nitrogen was measured using the Enzymatic Urea Nitrogen assay (Stanbio). Creatinine was measured using the Creatinine Serum Detection kit (Enzo Life Sciences, Farmingdale, NY, USA) and the Creatinine Detection Kit (Enzo) for urine. Liver function was assessed by measuring serum albumin levels using the Albumin LiquiColor assay (Stanbio).

Histologic Analyses

For analyses from parathyroid tissue, the entire thyroid-parathyroid-trachea complex was dissected en bloc. Additional tissues harvested for analyses included salivary gland, tongue, thymus, heart, lung, liver, spleen, stomach, small intestine, skeletal muscle, kidney, brain, uterus, ovary, testis, and seminal vesicle. Harvested tissues were fixed in 10% formalin, processed, and embedded in paraffin. Hematoxylin and eosin staining on 5-µm sections was used for morphologic examination. Sizing of the parathyroid glands was performed on hematoxylin and eosin-stained slides; slides were photographed and the cross-sectional area was measured from the captured images using ImageJ software. The total parathyroid volume was calculated using the cross-sectional area and section thickness to calculate volume, then summing the serial volumes for each adjacent section.

Statistical Analyses

All statistical analyses were performed using Prism 10 software (GraphPad Software, La Jolla, CA, USA).

Results

Parathyroid-specific *Cdc73* null mice were generated on 3 genetic backgrounds: FVB, mixed C57/129/CD1 (M1), and mixed C57/129/FVB (M2). Mice were viable and fertile; all 4 possible genotypes ($Cdc73^{F/F}$/PTH-$Cre^{+/-}$, $Cdc73^{F/+}$/PTH-$Cre^{+/-}$, $Cdc73^{F/F}$/PTH-$Cre^{-/-}$, $Cdc73^{F/+}$/PTH-$Cre^{-/-}$) were obtained in normal Mendelian ratios on all genetic backgrounds. For simplicity, all PTH-$Cre^{-/-}$ animals, representing wild-type littermates, were grouped together for analyses.

Blood was collected at 1- to 3-month intervals, from ages 2 to 24 months, for serum biochemical analysis. Homozygous knockout mice on a mixed C57/129/CD1 (M1) genetic background demonstrated decreased serum calcium (mean, 8.2 mg/dL; range, 4.9-9.9) at all timepoints, compared with heterozygous (mean, 9.3 mg/dL; range, 8.0-10.1) or wild-type animals on the same genetic background (mean, 9.3 mg/dL; range, 8.4-10.0) (Fig. 1A); these differences were statistically significant ($P < .0001$). Further analysis of the spread of calcium levels revealed that, although mean serum calcium levels in homozygous knockout animals were decreased compared with wild-type, 54% of homozygous knockout mice were frankly hypocalcemic, with calcium levels of 4.9 to 8.0 mg/dL, whereas the remaining homozygous knockout mice had calcium levels within the normal range (Fig. 1B). Hypocalcemia was accompanied by decreased serum PTH (Fig. 1C), consistent with hypoparathyroidism. Homozygous knockout mice had a mean PTH of 75.6 pg/mL, compared with 110.3 pg/mL in heterozygous mice and 126.3 pg/mL ($P < .03$) (Fig. 1B). Kidney and liver function were normal. Despite hypocalcemia, knockout mice were viable, healthy-appearing, and had no significant differences in growth. Homozygous knockout mice on a pure FVB background (Fig. 1D) or mixed C57/129/FVB (M2), fed either Prolab RMH 3000/5P00* (Fig. 1E) or Teklad 2914 (Fig. 1F) exhibited no biochemical abnormalities.

Histologic examination of the parathyroid glands from homozygous HPH-M1 knockout mice revealed significantly smaller gland sizes ($P < .03$), compared with heterozygous and wild-type littermates (Fig. 2A), often accompanied by abnormal morphology. Homozygous knockout HPH-FVB or

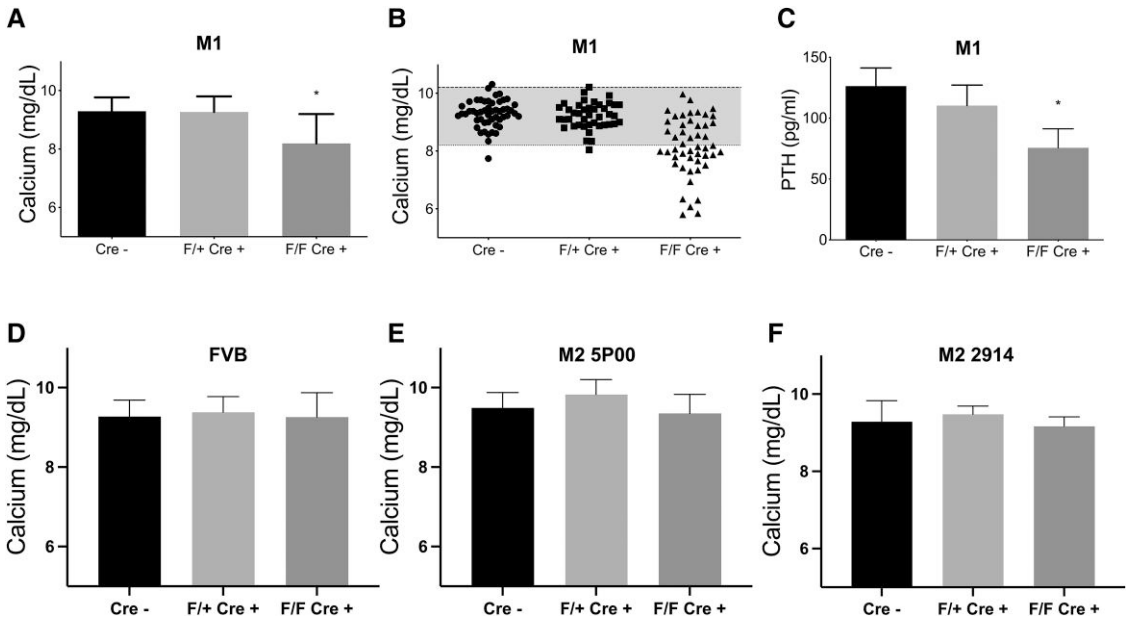

Figure 1. Biochemical abnormalities in parathyroid-specific *Cdc73* knockout mice are background strain dependent. Homozygous knockout mice on a mixed genetic background (M1; C57/129/CD1) demonstrated significantly lower serum calcium levels compared with heterozygous or wild-type animals (A), with 54% of homozygous knockout animals having calcium levels below 8 mg/dL (B). Serum PTH levels were also low (C). Homozygous knockout mice on a pure FVB (D) or mixed C57/129/FVB (M2) strain fed either Prolab RMH 3000/5P00* (E) or the lower calcium Teklad 2914 (F) had serum calcium levels indistinguishable from wild-type mice.

HPH-M2 mice no differences in parathyroid gland size (Fig. 2B) or histologic abnormalities (Fig. 2C). No parathyroid gland enlargement was seen in heterozygous mice at any age.

Discussion

The *CDC73* tumor suppressor gene is a firmly established contributor to parathyroid tumorigenesis, with inactivating germline mutations demonstrated in families with HPT-JT and a subset of families with FIH. Further, germline and somatic inactivating mutations and parafibromin protein expression abnormalities have been identified in sporadic parathyroid tumors. However, an understanding of the precise mechanisms through which loss of *CDC73*/parafibromin drives parathyroid tumor formation remains elusive. We sought to study the role of *CDC73* in parathyroid cell proliferation in vivo by generating a mouse model with a parathyroid-specific deletion of the *Cdc73* gene.

In this model system, the parathyroid gland phenotype was highly dependent on the genetic background of the mice. In a mixed background containing CD1, an outbred strain, biallelic parathyroid-specific deletion of *Cdc73* resulted in hypocalcemia in a majority of animals, accompanied by decreased serum PTH and elevated phosphate levels, consistent with hypoparathyroidism. This hypoparathyroidism was associated with significantly smaller gland sizes, often accompanied by abnormal morphology, suggesting abnormalities of parathyroid gland development. These findings, although surprising, were consistent with initial findings in a previous mouse model in which homozygous germline deletion of *Cdc73* was embryonic lethal, suggesting an important role for *Cdc73* in embryonic development [15].

Although biallelic inactivation of *CDC73* is often seen in parathyroid tumors from patients with HPT-JT or FIH, or in sporadic parathyroid carcinomas, the timing of this biallelic loss is different from that imposed in this model system (ie, early in development). Patients with HPT-JT (and some patients with FIH or sporadic parathyroid carcinoma) inherit 1 abnormal copy of *CDC73*; the second allele is presumed to be normal and functional during development, and only inactivated through acquired mutation or other allelic loss, after development is complete, and only in 1 or a subset of cells. Similarly, in sporadic parathyroid carcinoma, biallelic loss of both copies of *CDC73* can occur somatically, after development, only in 1 or a small number of parathyroid cells.

Our results suggest that, in mice, early, developmentally imposed complete loss of *Cdc73* can cause a primary defect in parathyroid gland development/function, indicating that parafibromin may be required for proper parathyroid gland development, and this novel potential role for parafibromin merits further study. This phenotype is in contrast to the parathyroid cell proliferation and hyperparathyroidism caused by postnatally acquired complete loss of *CDC73* seen in human HPT-JT. Interestingly, the effects of *Cdc73* loss on parathyroid gland phenotype are highly strain specific, both within our own study and when compared with a previously published parathyroid-specific *Cdc73* knockout mouse model [16].

We generated parathyroid-specific *Cdc73*-knockout mice on a pure FVB background with the intent to directly compare our model with other existing mouse models of parathyroid tumorigenesis on the same background, including the PTH-cyclin D1 transgenic model [18] and parathyroid-specific *Men1* deletion [17]. However, although parathyroid specific overexpression of cyclin D1 or loss of *Men1* are capable of producing both biochemical hyperparathyroidism and histologic features of parathyroid neoplasia on an FVB background, we observed a normal biochemical and histologic parathyroid phenotype in HPH-FVB mice. Although genetic background has long been recognized as affecting the phenotype of genetically modified mouse models [19-22], we are

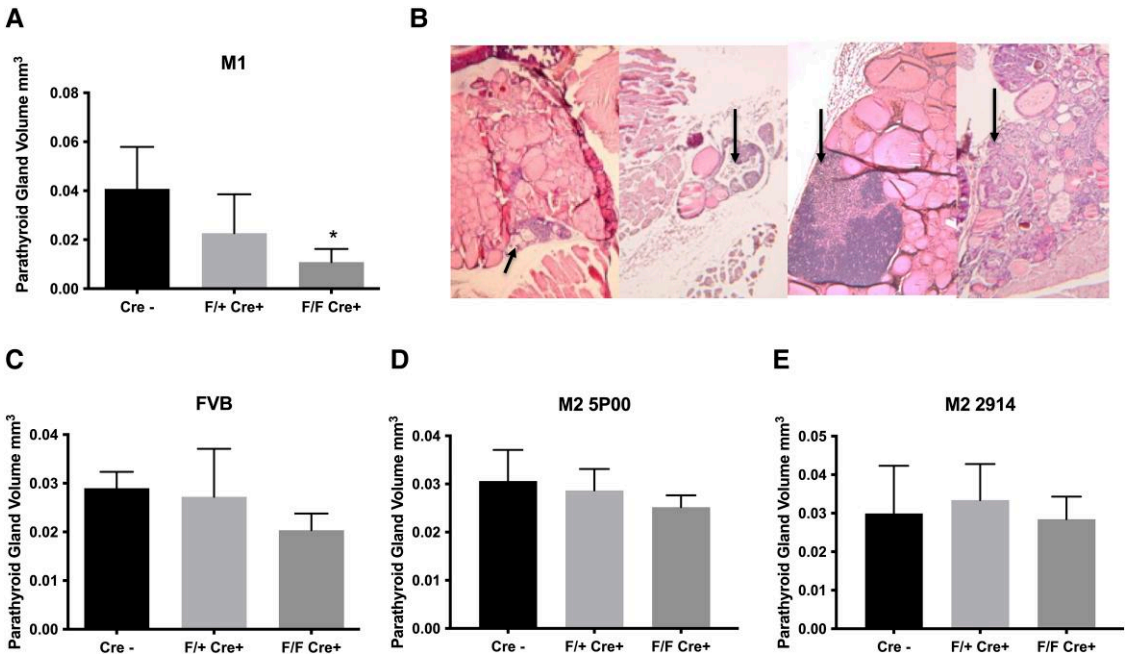

Figure 2. Parathyroid gland abnormalities in parathyroid-specific *Cdc73* knockout mice. Histologic examination of the parathyroid glands from homozygous knockout mice on a mixed genetic background (C57/129/CD1) revealed significantly smaller gland sizes (A), often accompanied by abnormal morphology (B). Parathyroid gland sizes in homozygous knockout mice on FVB (C) or mixed C57/129/FVB (M2) strains fed either Prolab RMH 3000/5P00* (D) or the lower calcium Teklad 2914 (E) were indistinguishable from wild-type mice.

not aware of any other examples of strain-specific differences in parathyroid phenotype.

To further explore the strain dependence of parathyroid specific *Cdc73*-deletion, we generated a mixed background model comparable to the C57/129/FVB background described in Walls et al [16]. Interestingly, our model did not recapitulate that parathyroid phenotype [16]. We hypothesized that dietary differences in the calcium/phosphate/vitamin D content in the mouse chow, which could potentially mask a subtle biochemical phenotype, might be responsible. However, placing either the HPH-M2 mice or HPH-FVB mice on a lower calcium/phosphate/vitamin D diet comparable to that used by Walls et al did not alter the parathyroid phenotype in our mice. The mice used to generate parathyroid-specific *Cdc73* knockouts in both studies were obtained from the same sources and therefore should harbor the same genetic modifications. Further, the HPH-M2 mice, to our knowledge, have the same genetic background described in Walls et al [16] and therefore should be genetically identical. It is possible that subtle differences in background strain composition from breeding schemes or genetic drift may be responsible for the observed phenotypic differences but this remains an open question.

Furthermore, CD1 is an outbred strain, and therefore possesses innate genetic variability not seen in inbred strains. This intrinsic genetic variability might account, at least in part, for the phenotypic dichotomy seen within our HPH-M1 mice, a feature we may otherwise have attributed solely to expected incomplete penetrance. These findings underscore the crucial need for accurate reporting of mouse background strains in genetically engineered models. Importantly, use of the mice reported herein may serve as a key reagent, leading to the discovery of genetic modifiers with relevance to parathyroid development and proliferation, and perhaps to the treatment of human parathyroid disorders.

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Disclosures

The authors declare no conflicts of interest.

Data Availability

Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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