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Overexpression of Notch Signaling in Renin Cells Leads to a Polycystic Kidney Phenotype

Brian C. Belyea[1,*], **Fang Xu**[1], **Margaret Wiltsie**[1], **Hayes Fountain**[1], **Jennifer Charlton**[1], **Agnes B. Fogo**[2,3], **Maria Luisa S. Sequeira-Lopez**[1], **R. Ariel Gomez**[1,2]

[1]Child Health Research Center, Department of Pediatrics, University of Virginia School of Medicine, Charlottesville, VA 22908, USA

[2]Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center, Nashville, TN 37232, USA

[3]Division of Pediatric Nephrology, Vanderbilt University Medical Center, Nashville, TN 37232, USA

Abstract

Polycystic kidney disease (PKD) is an inherited disorder that results in large kidneys, numerous fluid-filled cysts, and ultimately end stage kidney disease. PKD is either autosomal dominant caused by mutations in *PKD1* or *PKD2* genes or autosomal recessive caused by mutations in the *PKHD1* or *DZIP1L* genes. While the genetic basis of PKD is known, the downstream molecular mechanisms and signaling pathways which lead to deregulation of proliferation, apoptosis, and differentiation are not completely understood. The Notch pathway plays critical roles during kidney development including directing differentiation of various progenitor cells, and aberrant Notch signaling results in gross alternations in cell fate. In this study, we generated and studied transgenic mice which have overexpression of an intracellular fragment of mouse *Notch1* ("NotchIC") in renin-expressing cells. Mice with overexpression of NotchIC in renin-expressing cells developed numerous fluid-filled cysts, enlarged kidneys, anemia, renal insufficiency, and early death. Cysts developed in both glomeruli and proximal tubules, had increased proliferation marks, and had increased levels of Myc. This work implicates the Notch signaling pathway as a central player in PKD pathogenesis, and suggests the Notch-Myc axis may be an important target for therapeutic intervention.

Keywords

Renin; Notch Signaling; Polycystic Kidney Disease

Introduction

Polycystic kidney disease (PKD) is a major cause of end stage kidney disease(1). PKD is characterized by enlarged kidneys containing numerous fluid-filled cysts, hypertension,

[*]Corresponding author: Brian C. Belyea, Tel: 434-924-2878, bcb9e@virginia.edu.

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anemia, and progressive loss of kidney function. PKD is either autosomal dominant (ADPKD) caused by mutations in *PKD1* or *PKD2* genes or autosomal recessive (ARPKD) caused by mutations in the *PKHD1* or DZIP1L genes(2–5). Mutations in these genes lead to low intracellular calcium concentrations within epithelial cells of the kidney, followed by excessive cyclic adenosine monophosphate (cAMP) levels and ultimately deregulation of proliferation, apoptosis, and differentiation pathways(3,6). While the genetic basis of PKD is known, the downstream molecular mechanisms and signaling pathways which lead to deregulation of proliferation, apoptosis, and differentiation are not completely understood.

The Notch pathway is an evolutionarily conserved signaling cascade that is critical for a number of cell processes including differentiation, proliferation, and cell fate specification(7). Notch signaling is initiated when a ligand on one cell binds with a Notch receptor on an adjacent cell leading to a two-step proteolytic cleavage and resulting in liberation of the notch intracellular domain (NICD). NICD then travels to the nucleus where it binds to the transcriptional regulator RBP-J and along with other co-activators, converts RBP-J from a suppressor of gene expression to an activator. Target genes of the Notch pathway include *Hes1*, *Hes5*, *Hey1,* and *c-Myc*(8).

The Notch pathway plays critical roles during kidney development including directing differentiation of various progenitor cells(9). Notch signaling must be tightly regulated, as aberrant signaling can lead to fibrosis(10), tumorigenesis(11,12), and gross alterations in cell fate(13). Previous work from our lab has shown that the Notch pathway is important for regulation of renin expression and renin cell fate determination. Conditional deletion of Notch signaling in renin cells leads to a reduced number of renin-positive JG cells, decreased renin gene expression, and major changes in cell identity(14,15). In addition, others have demonstrated activation of the Notch pathway in mouse models of ADPKD and ARPKD(16). Therefore, we hypothesized that overexpression of Notch signaling during kidney development may play an important role in PKD pathogenesis. To investigate this, we generated and studied transgenic mice which have overexpression of an intracellular fragment of mouse *Notch1* (“NotchIC”) in renin-expressing cells.

Methods

Mice

We generated transgenic mice with overexpression of an intracellular fragment of mouse *Notch1* (“NotchIC”) in renin lineage cells using the Cre-Lox recombination system. Specifically, we crossed mice which express Cre recombinase under the control of the *Renin* locus ($Ren1^{dcre/+}$) with transgenic mice containing a sequence encoding an intracellular portion of the mouse *Notch1* gene (termed “NotchIC”) inserted in the ubiquitously expressed *Rosa26* locus ($Rosa^{Notch/+}$)(17,18). In the absence of Cre recombinase, transcription of *NotchIC* is blocked by a STOP sequence, flanked by Loxp sites. In cells that activate the renin promoter, Cre recombinase is expressed and excises the STOP sequence, resulting in heritable, constitutive expression of *NotchIC* in renin-expressing cells and their descendants. Control mice ($Ren1^{d+/+};Rosa^{Notch1/+}$) and mutant mice ($Ren1^{dcre/+};Rosa^{Notch1/+}$) were studied at 3, 6, and 9 months of age. These studies included a total of 36 female mice (14 control and 22 mutants) and 35 male mice (16 control

and 19 mutants). To trace cells that have activated Cre recombinase, we crossed in the Z/EG reporter which expresses enhanced green fluorescent protein (eGFP) following Cre-mediated recombination(19). Using this reporter, mutant mice (*Ren1*$^{dcre/+}$;*Rosa*$^{Notch1/+}$;*Z/EG*) will express both *NotchIC* and *eGFP* in cells of the renin lineage.

Mice were monitored daily for signs and symptoms of disease. When mice reached study time points or became moribund, they were anesthetized with tribromoethanol (300 mg/kg). All procedures were performed at the University of Virginia Medical Research Building following the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Virginia Animal Care and Use Committee (Protocol Number 2433).

Histology Analysis

Mouse kidneys were harvested, weighed and cut in a midsagittal plane. Kidney halves were fixed in either Bouin's fixative solution or 10% formalin solution overnight, embedded in paraffin, and 5 μm sections cut and stained. H&E stained sections were used to examine overall kidney morphology, and immunohistochemical (IHC) staining with VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA) for renin (rabbit polyclonal anti-mouse antibody, 1:500) was used to determine the distribution of renin-expressing cells, α-smooth muscle actin (1:10,000 Sigma-Aldrich, St. Louis, MO) to check vascular architecture, Ki-67 (1:200 Novus Biologicals, Centennial, CO) to evaluate proliferation, and Lotus Lectin (1:50 Vector Laboratories, Burlingame, CA) to identify proximal tubule epithelial cells. For renin stained sections, the juxtaglomerular apparatus (JGA) index was calculated as the number of renin-positive JGA per total number of glomeruli and expressed as a percentage. Kidney halves from mice with the Z/EG reporter were fixed in 4% PFA at 4^{O}C for 1 hour, embedded in OCT and stored in −80^{O}C. 10 μm frozen sections were stained with Hoechst (1:10,000 Invitrogen, Carlsbad, CA) and observed under fluorescent microscopy. All observations were made unaware of mouse group. All positive and negative controls for IHC worked appropriately.

RNA Extraction and RT-qPCR Analysis

Mouse kidneys were harvested and placed in RNAlater Stabilization Solution (Invitrogen, Carlsbad, CA) at 4^{O}C overnight, then stored in −20^{O}C. Kidney cortex (~1.5 mm3) was minced with Lysing Matrix D and FastPrep-24 (MP Biomedicals, Irvine, CA). Total RNA was isolated using TRIzol Reagent (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions, and further purified with DNA-free kit (Life Technologies, Carlsbad, CA) to remove contaminating DNA. cDNA was prepared from 2 ug RNA using Moloney Murine Leukemia Virus Reverse Transcriptase and Oligo(dT)15 Primer (Promega, Madison, WI) according to the manufacturer's instructions. Quantitative PCR was performed using GoTaq Flexi DNA Polymerase (Promega, Madison, WI) and SYBR Green I (Invitrogen, Carlsbad, CA) for Renin, Notch1, and Myc (Supplementary Table 1).

ELISA Analysis

Mouse whole blood was obtained by cardiac puncture and collected in an EDTA tube, centrifuged at 1,000 g for 15 minutes, and plasma was then stored at −80^{O}C. Renin1 ELISA

was performed using Mouse Renin1 ELISA Kit (RayBiotech, Norcross, GA) according to the manufacturer's directions.

Blood Chemistry and Counts

Blood was collected by cardiac puncture into heparinized and EDTA plasma separator tubes. Complete blood counts (EDTA tubes) and metabolic panels (heparinized tubes) were performed by the University of Virginia Hospital clinical laboratory.

Statistical Analysis

Data are presented as means +/− standard error of the mean. Significance between groups was determined using the Kruskal-Wallis test with Dunn's multiple comparison post-test using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). Differences were considered statistically significant at $*P<0.05$, $**P<0.01$ and $***P<0.001$ levels.

Results

Overexpression of NotchIC in Renin Cell Leads to Large Kidneys with Numerous Cysts

Prior loss-of-function studies have shown that Notch signaling controls renin cell fate. To investigate the effects of Notch activation in renin cells, we used the cre-lox system to heritably activate Notch1 in renin-expressing cells. We compared mutant mice ($Ren1^{dcre/+};Rosa^{NotchIC/+}$) to control mice ($Ren1^{d+/+};Rosa^{NotchIC/+}$) which do not have cre-recombinase at 3 months, 6 months, and 9 months of age. Mutant animals were viable and born at the expected Mendelian ratio and showed no differences in body weight compared to age-matched control mice (Figure 1A and Supplementary Figure 1A). However, on necropsy, mutant mice had large kidneys, and the difference in kidney weight increased with advancing age (Figure 1B,C and Supplementary Figure 1B). These findings were the same in both female and male mice (Supplementary Figure 1A,B). On gross inspection, the kidneys of mutant mice had large cysts on their surface, and this phenotype also worsened with advancing age (Figure 1D). Histologic examination confirmed the presence of numerous cysts throughout the kidney cortex in mutant mice (Figure 1E). High resolution imaging of these cysts demonstrated that many of the cysts are glomerular with residual glomeruli within many of the cysts (Figure 1F). In addition, there were cysts within the proximal tubules as shown by Lotus lectin staining (Supplementary Figure 1C). We did not see cysts within the collecting ducts as shown by Calbindin staining (Supplementary Figure 1D).

Mutant Mice Have Decreased Levels of Renin and Develop Renal Failure

Given our prior work showing that loss of Notch signaling in renin cells leads to decreased renin expression, we wanted to investigate how activation of Notch signaling would affect renin production(14). First, we performed IHC for renin in kidney sections of mutant and control mice. We found a decrease in renin-positive JG cells in mutant mice (Figure 2A). We quantitated the number of renin-positive JG cells per total glomeruli (JG Index), and this showed decreased renin staining in mutant mice at 6 months of age (we were unable to calculate the JG index for mutant animals at 9 months of age because of the severe structural changes and lack of intact renal parenchyma to evaluate) (Figure 2B). Second, we performed

quantitative PCR for renin in the kidney cortex of mutant and control mice, and, while not statistically significant, there was a trend towards decreased renin mRNA in kidneys of mutant mice compared to controls (Figure 2C). Finally, we measured plasma protein levels of renin by ELISA and found that mutant animals have a trend towards decreased circulating renin levels compared to control mice (Figure 2D). In addition, mutant mice developed renal insufficiency with advancing age as measured by blood urea nitrogen (BUN) and serum creatinine (Figure 2E,F).

Mutant Mice Develop Anemia, Splenomegaly, and Early Death

In this study, mutant mice had decreased hemoglobin compared to control mice, and this anemia worsened with age (Figure 3A). There was no difference in the white blood cell count or the platelet count between control and mutant animals (Supplementary Figure 2A). In addition, mutant mice had enlarged spleens compared to control mice (Figure 3B and Supplementary Figure 2B). Mutant spleens had normal anatomy and architecture with no infiltrative, neoplastic, or cystic involvement (Supplementary Figure 2C). Finally, mutant mice demonstrated early death with a median survival of 265 days (Figure 3C).

Overexpression of Notch in Renin Lineage Cells Leads to Increased Proliferation

To investigate the mechanism of cyst formation, we assessed cell proliferation by immunohistochemical staining for Ki67, a cellular marker of proliferation(20). Mutant kidneys showed increased proliferation compared to control kidneys (Figure 4A). This was particularly noticeable in the epithelial cells of the Bowman's capsule, which ultimately became the lining of the glomerular cysts (Figure 4B).

We next performed lineage tracing to identify renin-lineage cells by crossing in a Z/EG reporter which expresses eGFP in cells that have undergone cre-mediated recombination. In this model, eGFP marks the cells which have expressed renin and cre-recombinase and thus have overexpression of NotchIC. Cells lining the cysts expressed eGFP, and these same cells showed increased proliferation (Figure 4C). This suggests that Notch signaling is contributing directly to areas of increased proliferation and cyst formation.

Overexpression of Notch Leads to Increased Myc Expression

We next performed qPCR for *Notch1* from the kidney cortex of control and mutant mice to validate levels of Notch1 expression. As anticipated, there was a significant increase in *Notch1* expression in mutant mice compared to control mice (Figure 5A). Given reports of increased *Myc* expression in models of PKD, as well as its known role as a target of Notch signaling, we also assessed *Myc* by qPCR. We found that, similar to *Notch1* expression, *Myc* was significantly upregulated in mutants compared to control kidneys (Figure 5B). Immunohistochemical staining demonstrated increased protein levels of NOTCH1 and MYC (Figure 5C,D). Staining was most pronounced in the cells lining the cysts, consistent with our lineage tracing results and proliferation studies described above.

Numerous human and mouse studies have confirmed a gene dosage (and subsequent protein dosage) effect of *PKD1*, *PKD2* and *PKHD1* in PKD. In these studies, loss of these genes / proteins leads to alterations in downstream gene networks, including the Notch signaling

pathway(16). In our mouse model, the loss of polycystin or fibrocystin is bypassed, and the Notch pathway is activated directly resulting in a cystic phenotype. To investigate whether activation of Notch signaling affects the expression of these genes, we performed qPCR for *PKD1*, *PKD2* and *PKHD1*. We found a trend towards increased expression of each of these genes in mutant mice with PKD at each of the ages we studied. Indeed, there was significantly increased gene expression of *PKD1* at 3 months and 6 months of age, and there was significantly increased gene expression of *PHKD1* at 9 months of age (Supplementary Figure 2D).

In summary, cells that expressed renin also expressed cre-recombinase which led to constitutive Notch1 expression and eGFP expression in renin-expressing cells and their descendants. These cells comprised the lining of the cysts, had increased MYC protein expression, and had increased proliferation, ultimately leading to cyst formation.

Activated Notch Signaling Results in Increased Risk of Tumor Development

Aging mutant mice showed a higher-than-expected incidence of tumors. These tumors appeared in the neck and abdomen of mutant mice (Supplementary Figure 2E). In total, 1 out of 16 mutant mice (6.3%) from the 6-month age group developed tumors, and 4 out of 23 mutant mice (17.4%) allowed to age to 9 months developed tumors. No control mice in this study developed tumors.

Discussion

In this work, we demonstrate that overexpression of activated Notch1 in renin-expressing cells leads to the development of a polycystic kidney phenotype. To our knowledge, this is the first model of fulminant PKD resulting from overexpression of Notch1, as opposed to deletion of *PKD1* or *PKD2*, and this work demonstrates the importance of Notch signaling in PKD pathogenesis. This model recapitulates many features of human PKD including enlarged kidneys, numerous cysts, progressive loss of kidney function, and anemia. Mutant mice developed anemia with normal white blood cell and platelet counts. This anemia is likely due to structural kidney damage and declining GFR which is seen in patients with PKD and contributes to a worse prognosis(21). In addition, mutant mice developed splenomegaly which was most likely due to extramedullary hematopoiesis within the spleen as a compensatory response to their anemia. Finally, mutant mice experienced early death, presumably a result of a combination of tumor development, anemia, and kidney failure.

Notch signaling is critically important for nephrogenesis, and activation of Notch signaling within cells of the kidney can lead to cystic changes. In addition to our work, there have been several reports linking the Notch signaling pathway to cyst formation. Fujimura et al. demonstrated that overexpression of Notch2 in Six2-containing nephron progenitor cells resulted in multiple glomerular cysts, dilated renal tubules, and neonatal death(22). Subsequently, Chung et al. demonstrated that overexpression of NICD in developing nephron WNT+ cells resulted in glomerulocystic changes(23). Similarly, overexpression of NICD in Six2+ nephron progenitors resulted in cystic glomeruli(23). In 2008, Chen et al. demonstrated that members of the Notch pathway are differentially expressed in a mouse model of polycystic kidney disease(24). A year later, Song et al. showed that components

of Notch signaling are up-regulated in human PKD samples(25). Most recently, Idowu et al. showed that both AR and AD mouse models of PKD have increased Notch expression – particularly Notch3(16). They showed that protein levels of Notch members are elevated in cyst-lining epithelial cells, Notch expression is correlated with rapidly growing cysts, and inhibition of Notch signaling decreased cyst growth in an *in vitro* PKD model. Our current work advances these prior studies by demonstrating a direct, initiating role of activated Notch signaling in PKD pathogenesis.

In this mouse model of PKD, we found that overexpression of Notch signaling leads to increased Myc signaling and increased cell proliferation. We saw the greatest proliferation in cells lining the cysts, and these same cells also express eGFP (which marks the presence of cre recombinase), NotchIC, and Myc. Aberrant Myc signaling has long been implicated as a cause of increased cell proliferation in PKD(6). Increased Myc expression has been demonstrated in both human and mouse PKD models, Myc has been implicated as an essential factor contributing for cystogenesis, and knockout of Myc in a mouse model of PKD ameliorates cyst development(26–29). Therefore, it is reasonable to conclude that activation of Notch signaling in this model leads to increased Myc signaling, which drives proliferation pathways in cells lining the cysts, ultimately leading to cyst expansion. Proliferation in cells lining the cysts is consistent with other models of PKD(16,28,30,31). More work will be needed to understand how Notch activation specifically in renin-expressing cells leads to cyst formation. Most cases and models of PKD are driven by decreased gene expression levels and protein levels of *PKD1*, *PKD2*, and *PHKD1*, however these gene levels were normal or elevated in our mice. One possibility is that overexpression of Notch in JG cells results in glomerular cysts which lack tubular connections(32). Atubular glomeruli are known to be present in animal models and human cases of PKD and contribute to diminished GFR(33,34).

There are some unique aspects of this PKD model which make it valuable tool to study cystic kidney disease. First, these mice developed both glomerular and tubular cysts which makes them useful to advance our understanding of both PKD and glomerular cystic diseases. Second, the complete penetrance and predictable progression of the kidney disease in this model make it a valuable tool to test potential therapies. In addition, mutant mice developed tumors at a higher rate than control mice. Approximately 17% of mutant mice developed tumors in this model, while in contrast no control mice developed tumors. Mutant mice were between 6 and 9 months of age when tumors were noted. Although the incidence of spontaneous tumor formation has not been reported in wildtype mice at the age of 9 months, there is a report of 9.5% incidence of spontaneous tumors in wildtype mice more than 2 years of age (in that particular study, no wildtype mice developed tumors before 9 months)(35). Thus, the development of tumors in these mutant mice appears to be more than sporadic or coincidence. Presumably, the tumors in these mutant mice are due to cre-recombinase activity outside of the kidney, specifically within B-1 progenitors of the hematopoietic system or within renin-expressing cells in the yolk sac which has been recently reported(36,37). Further work will be needed to determine the origin and identity of these tumors. In summary, we present a unique mouse model of cystic kidney disease which highlights the importance of Notch-Myc signaling in PKD.

Current therapies in PKD include supportive measures such as promoting a healthy lifestyle and treatment of hypertension. However, limited disease-altering therapies are available for patients with PKD, due in part to gaps in the knowledge of disease pathogenesis(38). An improved understanding of the genes and signaling pathways that are altered in cyst formation and disease progression will be important for the discovery of targeted, mechanism-based therapies. Our work implicates the Notch signaling pathway as a central player in PKD pathogenesis, and suggests the Notch-Myc axis may be an important target for therapeutic intervention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability Statement:

Data associated with this paper are available by contacting the corresponding author.

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Clinical Perspectives:

- Notch signaling plays important roles in the regulation of fate specification within progenitor cells of the kidney, however the effects of Notch overexpression in renin-expressing progenitors has not been investigated.
- Overexpression of Notch1 within renin-expressing cells leads to a striking polycystic kidney phenotype, accompanied by anemia, splenomegaly, renal insufficiency, and ultimately early death.
- This discovery further implicates the Notch-Myc axis in the pathogenesis of polycystic kidney disease, which may be an important target for therapeutic intervention.

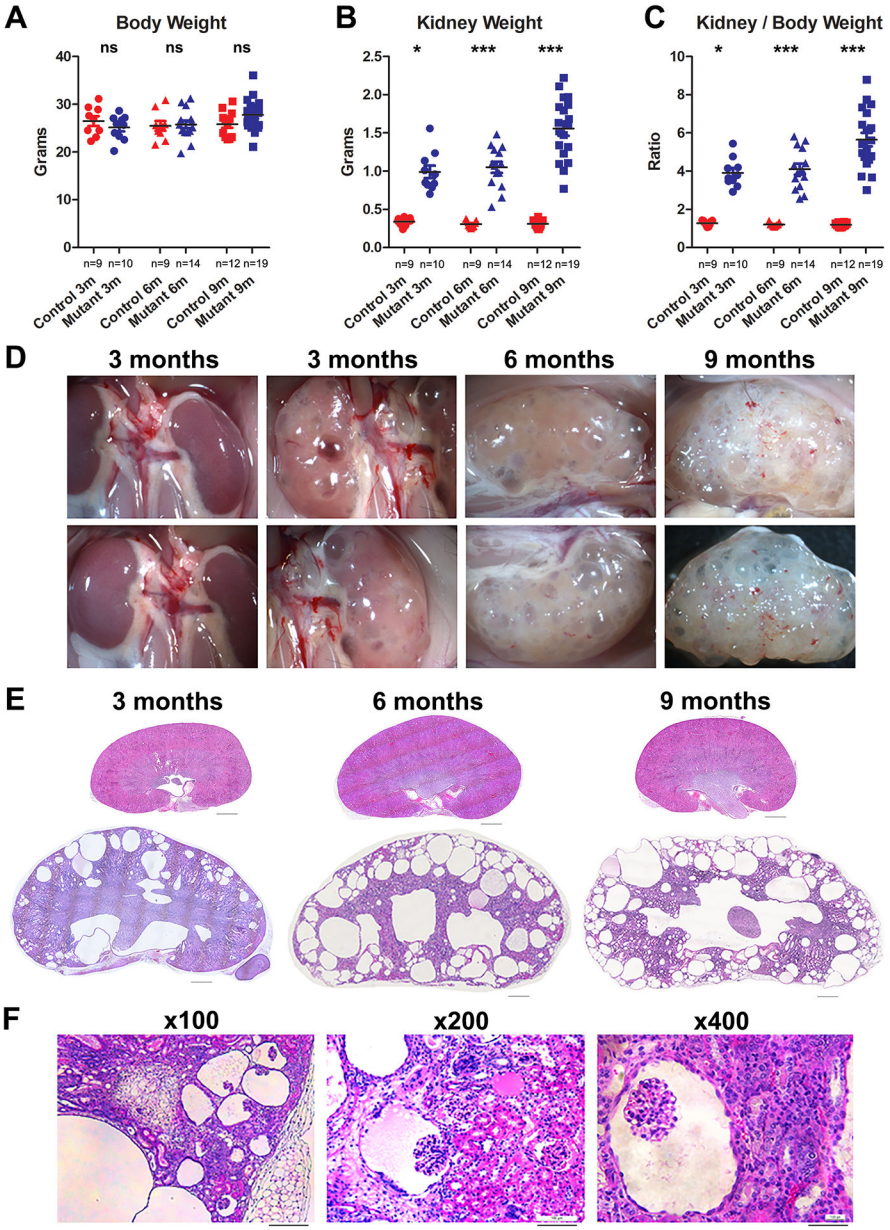

Figure 1. Overexpression of NotchIC in renin cells leads to a polycystic phenotype.

(A) There was no difference in body weight between control and mutant mice. (B) Kidneys from mutant mice weighed significantly more than kidneys from control mice, and this difference increased with advancing age. (C) Absolute kidney weights were increased in mutant mice compared to control mice, and also when normalized to body weight. (D) Kidneys from control mice (left panel) were normal in appearance, however, kidneys from mutant mice had numerous large cysts on their surfaces. The number of cysts increased with advancing age. (E) The number and size of cysts increased with advancing age in mutant kidneys (top panel is kidneys from control mice, and bottom panel is kidneys from mutant mice). Scale bar, 1 mm. (F) Many of the cysts are glomerular cysts. Scale bar, 200 μm (left panel), 100 μm (middle panel), 50 μm (right panel).

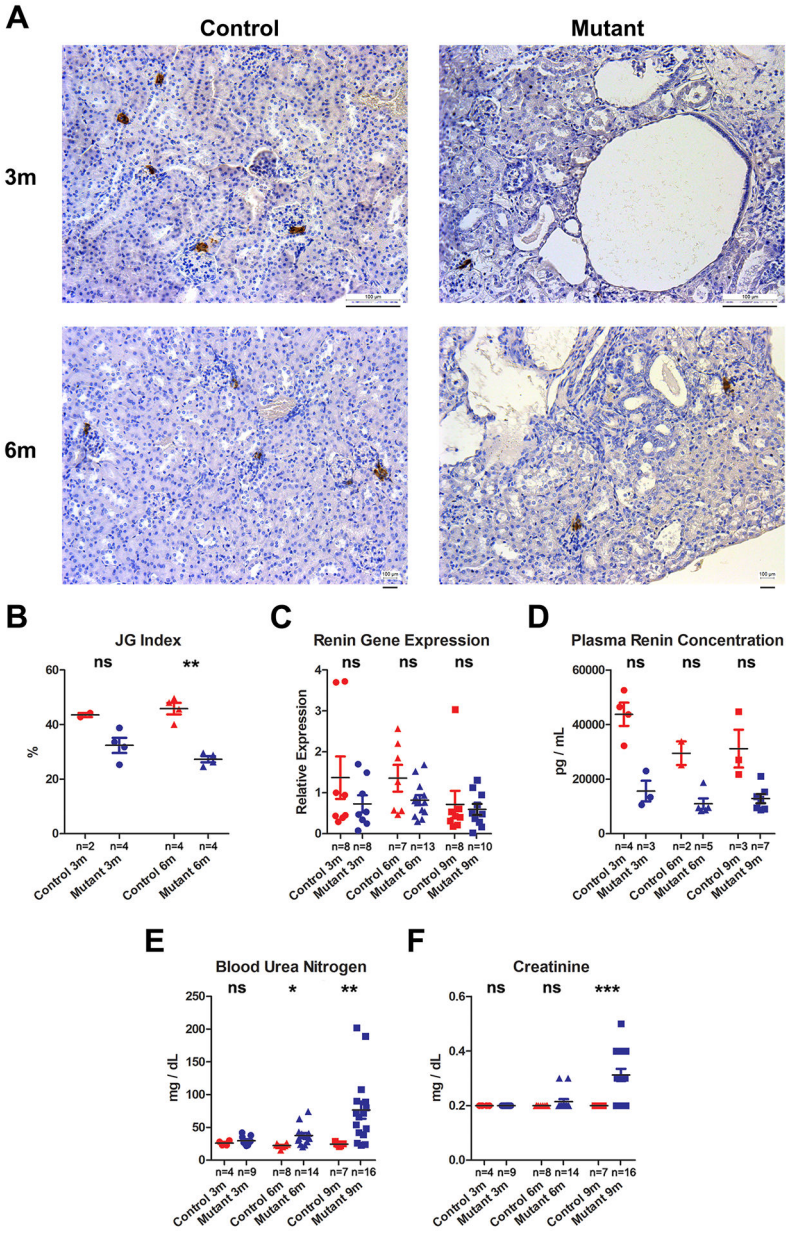

Figure 2. Overexpression of NotchIC in renin-expressing cells leads to decreased renin production and renal insufficiency.

(A) Immunohistochemistry staining for renin in tissue sections from kidney of control and mutant mice at 3 and 6 months of age. There was decreased renin staining in mutant mice compared to controls. Scale bars, 100 μm. (B) Renin staining was quantitated using the JG index. (C) There was a trend towards decreased renin gene expression in the kidneys of mutant mice compared to control mice. (D) There was a trend towards decreased renin protein concentration in the plasma of mutant mice compared to control mice. (E) Mutant animals had elevated blood urea nitrogen compared to control mice. (F) Mutant animals had elevated serum creatinine compared to control mice.

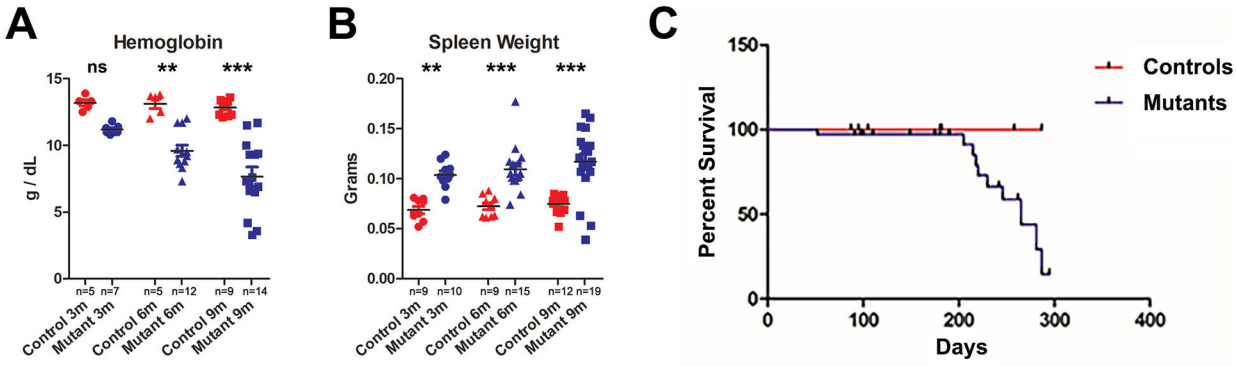

Figure 3. Animals with constitutive Notch signaling in renin-expressing cells develop anemia, splenomegaly, and early death.

(A) Mutant mice developed anemia which worsened with age. (B) Mutant mice developed splenomegaly when compared to control mice, and this also worsened with age. (C) Mutant mice experienced early death with all mutant mice dying before 1 year of age.

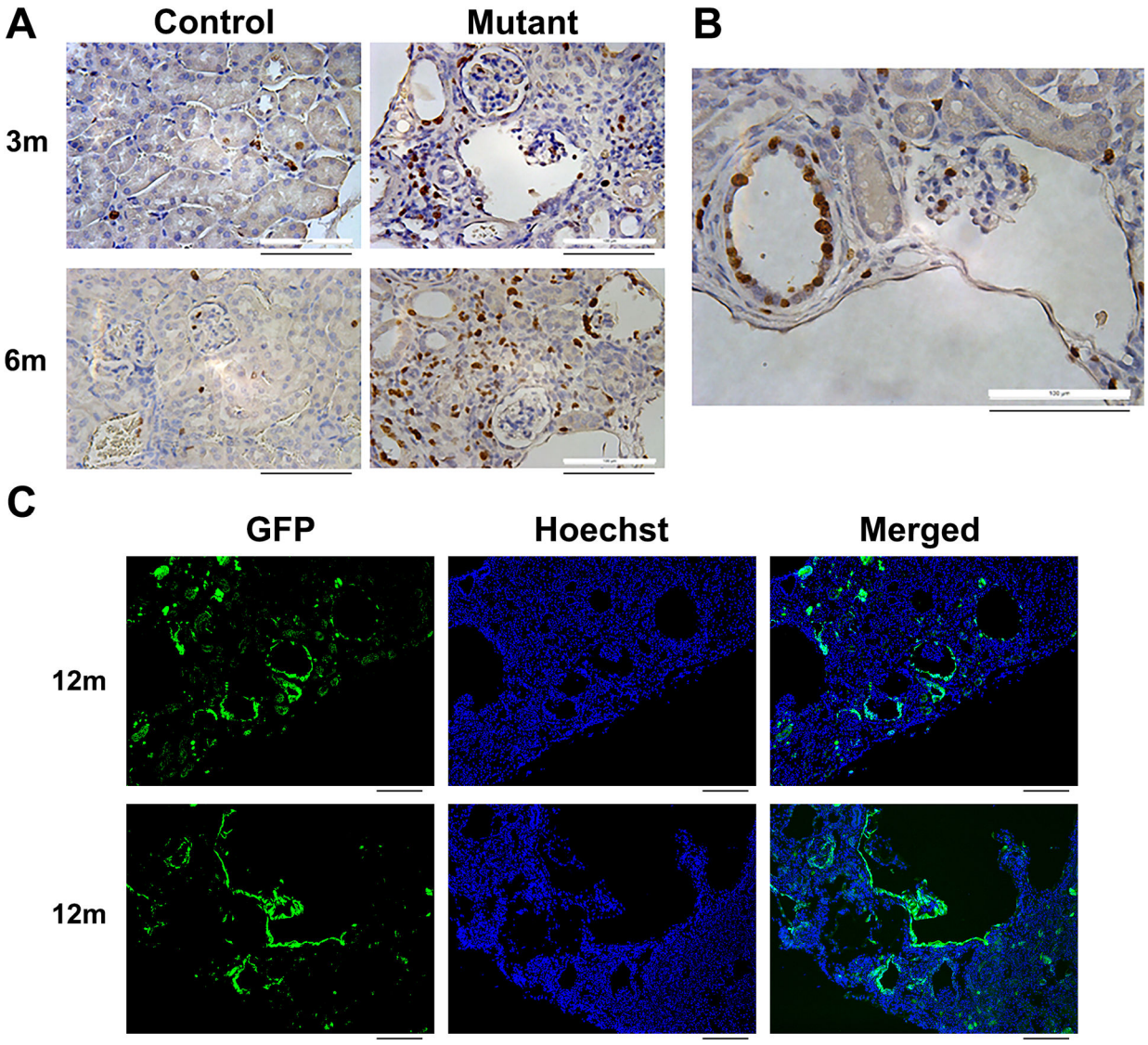

Figure 4. Overexpression of Notch signaling in renin-expressing cells results in increased proliferation.

(A) Mutant animals had increased proliferation in their kidneys as measured by Ki67 staining compared to control animals. Scale bars, 100 μm. (B) Proliferation was especially pronounced in the epithelial cells lining the glomerular cysts. Scale bar, 100 μm. (C) Lineage tracing with the Z/EG reporter demonstrates that cells which express NotchIC lined the cysts. Scale bars, 200 μm.

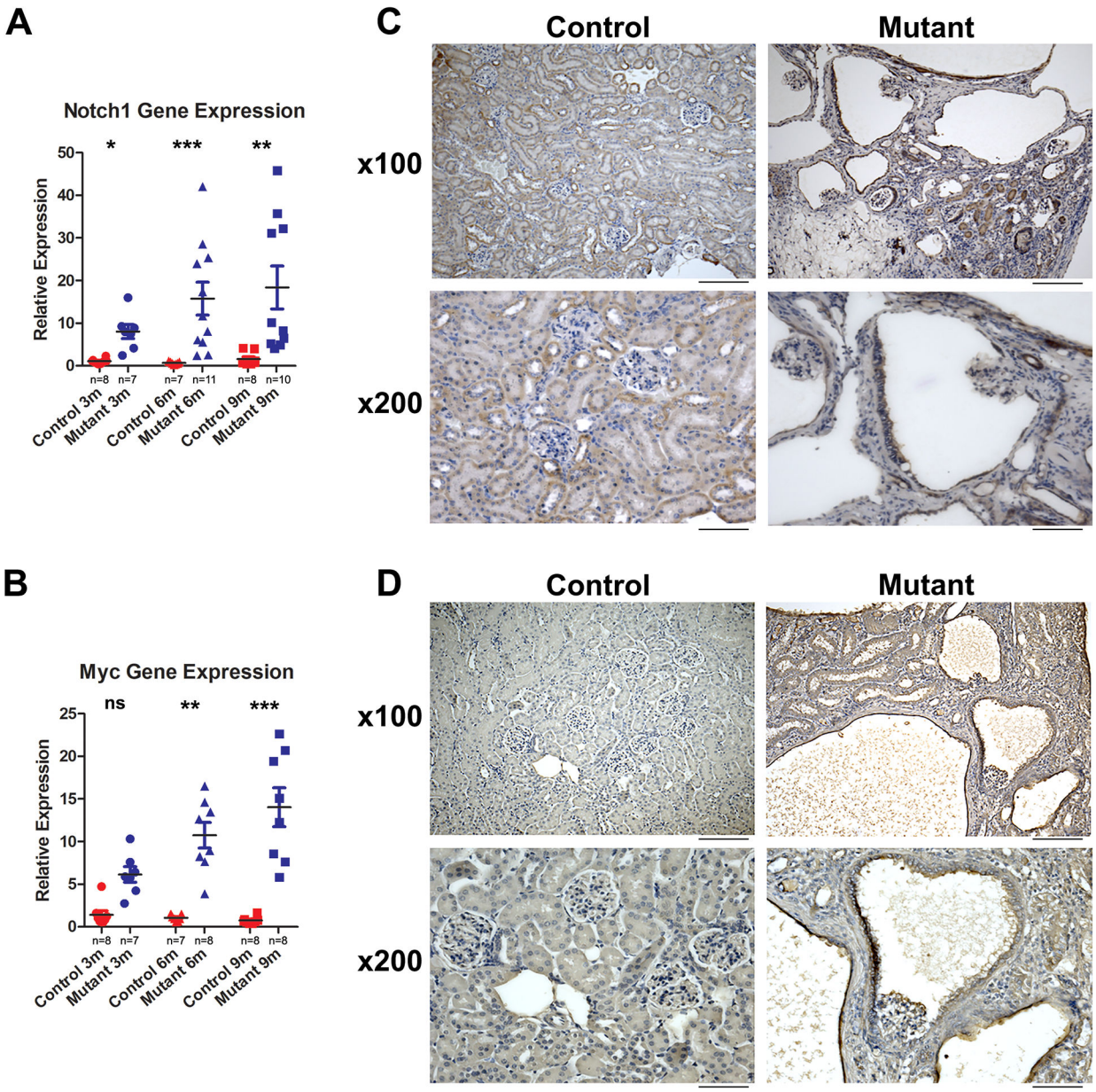

Figure 5. Overexpression of NotchIC leads to increased *Myc* expression.

(A) Mutant mice had increased *Notch1* expression compared to control mice. (B) Mutant mice had increased *Myc* expression compared to control mice. (C) Mutant mice had increased Notch1 protein in kidneys compared to control mice. Notch1 protein was pronounced in the cells lining the cysts. Scale bars, 200 μm (top panel), 100 μm (bottom panel). (D) Mutant mice had increased Myc protein in kidneys compared to control mice. Scale bars, 200 μm (top panel), 100 μm (bottom panel).