A critical developmental switch defines the kinetics of kidney cyst formation after loss of Pkd1

Klaus Piontek1, Luis F Menezes1, Miguel A Garcia-Gonzalez1, David L Huso2,3 & Gregory G Germino1,4

Autosomal dominant polycystic kidney disease (ADPKD) is characterized by the gradual replacement of renal tubules by cysts, which results in end-stage renal disease in approximately 50% of affected individuals by the sixth decade of life1. An unanswered question with important clinical implications is whether cyst initiation is an ongoing process throughout the lifetime of an individual, or whether it is restricted to the period of rapid growth during development and early childhood. A related question is whether Pkd1 is required for maintenance of normal tubular structure in adult kidneys. To address these issues, we induced Pkd1 inactivation in mice homozygous for a floxed Pkd1 allele2 at various time points. Inactivation at postnatal day 2 (P2), a time of ongoing cortical nephrogenesis in the mouse, resulted in grossly enlarged, homogenously cystic kidneys within 2 weeks (Fig. 1a). The cortical rim lacked immature or dysplastic elements, indicating that Pkd1 is not required for initial fate decisions during nephrogenesis, which is consistent with previous studies in mice with homozygous germline Pkd1 mutations3. Using nephron segment–specific markers, we found that cysts originated from all tubular segments (Fig. 1a and Supplementary Fig. 1a online).

We next determined whether inactivation of Pkd1 in adult kidneys was sufficient to cause cystic disease by inducing its inactivation in 6-week-old mice (Fig. 1b,c). In contrast to the results of inactivation at P2, we found that the kidneys of these mice, 3 months after inactivation of Pkd1, appeared normal in gross and histopathologic analyses, despite the fact that we had achieved Pkd1 inactivation with >50% efficiency (Fig. 1d). By 6 months after inactivation, however, kidneys were grossly cystic, with cysts derived from all nephron segments (Fig. 1e and Supplementary Fig. 1b). Notably, the proliferation rate of cystic epithelia (as measured by Ki-67 staining) seemed to be slightly higher than that of renal tubular epithelia in age-matched normal control samples, but the difference was not significant, possibly because of the relatively small sample size (Fig. 1f and Table 1a). Hypothesizing that a difference in growth rate between young and adult mice might be the reason for our observations, we determined the effects of Pkd1 inactivation in adolescent mice (~3 weeks old). We achieved rates of Pkd1 inactivation in the mice at this age that were similar to those in adults, and we also obtained identical results to those observed in adults. Kidneys of treated mice did not show cystic change until ~5 months after inactivation (data not shown). These results confirmed that the Pkd1 gene is essential for maintenance of normal tubular structure in adult tissues but, notably, revealed the very different disease progressions that result from early versus late Pkd1 inactivation.

To define the crucial time interval for the differences between the early- and late-inactivation phenotypes and determine the factors responsible for these differences, we induced inactivation at various time points between P2 and P21 with the same protocol used for the P2 inductions (Supplementary Table 1 online). All induced Pkd1 condition mice P12 or younger developed severe cystic disease within 3 weeks, whereas all control mice were free of cysts at the same time point (Fig. 2a and data not shown). Of note, mice induced at ages between P14 and P21, the kidneys of which appeared normal in gross and microscopic examinations 3 months after induction (Fig. 2a and data not shown), reproducibly had late-onset cystic disease (that is, onset of disease by 6 months after Cre induction; data not shown).

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identical to that seen in mice with Cre expression induced later in life (Fig. 1b,c). In contrast, the livers of the mice induced between P14 and P21 were cystic at both early and later time points. P13 induction resulted in an intermediate phenotype; all mice developed renal cysts within 3 weeks, but the number was variable and reduced compared to mice induced at P12.

In our search for possible explanations of the disparate temporal responses to Pkd1 inactivation, we excluded trivial explanations based on differences in the pattern or frequency of the gene’s inactivation. To confirm the efficacy of our inactivation protocol, we examined the pattern of β-galactosidase staining in the kidneys upon Cre recombinase–mediated activation of the ROSA26R reporter transgene at P12 and P14 and found that the staining was similar whether activation occurred at P12 or P14 (Fig. 2b). Southern blotting analysis of kidney samples from Cre-induced mice at P12 and P14 revealed that we had achieved similar frequencies of Pkd1 deletion at these two ages (Fig. 2c). In sum, these studies identified a crucial two-day interval that defines the kidney’s response to Pkd1 inactivation.

Increased cellular proliferation and apoptosis have previously been described as primary processes involved in the pathogenesis of ADPKD5–7. We therefore stained specimens of littermate-control and cystic mice induced at P7–P12 for markers of these processes.
### Table 1 Proliferation indices of normal and cystic kidneys

(a) Cystic versus noncystic adult kidneys

<table>
<thead>
<tr>
<th>Induced Cre&lt;sup&gt;+&lt;/sup&gt; (cystic)</th>
<th>Induced Cre&lt;sup&gt;-&lt;/sup&gt;/uninduced (noncystic)</th>
</tr>
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<tbody>
<tr>
<td>2.73% (3,414/124,967)</td>
<td>1.62% (1,479/91,497)</td>
</tr>
<tr>
<td>2.21% (2,224/100,741)</td>
<td>2.56% (2,260/88,130)</td>
</tr>
<tr>
<td>4.53% (15,318/337,963)</td>
<td>1.26% (2,468/195,181)</td>
</tr>
<tr>
<td></td>
<td>1.85% (2,743/148,276)</td>
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<td></td>
<td>1.87% (3,390/181,199)</td>
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</table>

(b) Kidneys of Cre<sup>+</sup> and Cre<sup>-</sup> mice induced at P12 and P14

<table>
<thead>
<tr>
<th>P12 Cre&lt;sup&gt;+&lt;/sup&gt;</th>
<th>P12 Cre&lt;sup&gt;-&lt;/sup&gt;</th>
<th>P14 Cre&lt;sup&gt;+&lt;/sup&gt;</th>
<th>P14 Cre&lt;sup&gt;-&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>2.75% (3,837/139,343)</td>
<td>2.18% (3,129/143,341)</td>
<td>2.67% (3,037/113,753)</td>
<td>3.16% (3,687/116,544)</td>
</tr>
<tr>
<td>1.79% (1,001/55,864)</td>
<td>2.17% (2,054/94,480)</td>
<td>2.75% (3,140/114,241)</td>
<td>2.46% (3,104/128,136)</td>
</tr>
<tr>
<td>2.91% (2,256/77,485)</td>
<td>2.67% (3,126/117,041)</td>
<td>3.97% (3,860/97,255)</td>
<td>2.37% (2,974/125,411)</td>
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(c) Kidneys of wild-type young mice

<table>
<thead>
<tr>
<th>P14</th>
<th>P16</th>
<th>P19</th>
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<tbody>
<tr>
<td>4.42% (23,996/54,543)</td>
<td>8.6% (7,339/85,282)</td>
<td>7.31% (7,119/97,393)</td>
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<tr>
<td>38.79% (21,901/54,459)</td>
<td>9.07% (6,260/69,010)</td>
<td>7.52% (6,516/73,326)</td>
</tr>
<tr>
<td>14.8% (8,799/59,396)</td>
<td>9.87% (6,361/64,446)</td>
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Specimens were stained for Ki-67 and DAPI, and the proliferation rate (%) was calculated as the number of Ki-67-positive cells divided by the total number of DAPI-positive cells that were counted for each sample (ratio in parentheses). Each column identifies the class of specimens that was analyzed, and each row lists the data for a single mouse of each class. (a) Proliferation rates in kidneys harvested ~6 months after induction of Pkd1 inactivation in 6-week-old Pkd1<sup>cond/cond</sup> Cre<sup>+</sup> and Pkd1<sup>cond/cond</sup> Cre<sup>-</sup> mice. Pkd1<sup>cond/cond</sup> Cre<sup>-</sup> mice injected with corn oil served as additional negative controls (uninduced). Any differences were not statistically significant (P > 0.05, Mann-Whitney test). (b) Proliferation rates in kidneys harvested ~3 weeks after we induced Pkd1 inactivation in Pkd1<sup>cond/cond</sup> Cre<sup>+</sup> and Pkd1<sup>cond/cond</sup> Cre<sup>-</sup> mice of the indicated ages. The P12-Cre<sup>+</sup> samples were the only ones that were cystic. Any differences were not statistically significant (P > 0.05, Kruskal-Wallis test). (c) Proliferation rates in kidneys of young mice at the ages indicated. The differences in rates are statistically significant (P = 0.0482; Kruskal-Wallis test).

(Ki-67, phospho–histone-3 and caspase-3). Contrary to the published literature, we found few differences in the rate of cell proliferation between control and cystic mice (Table 1b). Epithelial cells lining tubules and cysts of control and cystic kidneys stained similarly for Ki-67 and phospho–histone-3, except for small, widely scattered regions of cystic samples that had increased staining (Fig. 2d and Supplementary Fig. 2a online). Apoptosis was restricted to the renal papilla of wild-type kidneys, whereas cystic specimens had a small number of medullary and cortical cells also undergoing apoptosis (Supplementary Fig. 2b). We then examined whether differences in the rates of proliferation or apoptosis in uninduced P12–P19 Pkd1<sup>cond/cond</sup> mice might explain the age-dependent sensitivity to Pkd1 loss. We found that Ki-67 and phospho–histone-3 expression was similar in the P12 and P14 samples but was markedly different from that observed in the P16 and P19 specimens (Fig. 2e, Table 1c and Supplementary Fig. 3a online), whereas caspase-3 staining was similar at all time points (Supplementary Fig. 3b).

Seeking to explain the sudden global decrease in the proliferation rate in the kidney during the P14–P16 interval, we performed microarray analysis of kidney specimens from P11, P12, P14 and P15 control mice. We found few genes differentially expressed between P11 and P12 or between P14 and P15. In contrast, there was a clear change in the expression pattern between the early (P11 and P12) and late (P14 and P15) samples (Fig. 3 and Supplementary Table 2 online), including 827 genes whose expression differed significantly between these two stages (P < 0.001); of those, 51 had a ≥100% increase in at least one animal, and 21 had a ≥75% decrease in at least one animal, as compared to mean expression at P11 (Supplementary Table 3 online). Grouping the results by gene ontological category, we found that most of the genes have functional roles in physiological processes, particularly in transporter and catalytic activities (Supplementary Table 4 online). In silico analysis of promoter cis-elements showed enrichment for at least one promoter involved in kidney development (Pea3; Supplementary Table 5 online). These data suggest there is an important developmental switch that is activated in this time frame.

Previous studies have convincingly demonstrated that an increase in cell number must occur to account for the increased surface area of macroscopic cysts in human ADPKD. This outcome has been attributed to the high proliferative rate of renal cyst epithelia, and many treatments that are being investigated target this process. Our data challenge this model. Using two independent markers and a semi-automated system to count large numbers of nuclei from >50 images/specimen, we found that proliferation rates in specimens of adult-onset cystic disease were not appreciably higher than in age-matched control kidney samples. Moreover, we found that the proliferation rate in normal P16 kidneys was several-fold higher than that in adult kidneys, yet inactivation of Pkd1 at this early time point resulted in the same slow onset of PKD seen in adult mice. These results suggest that defective growth regulation is probably not the primary defect resulting from loss of Pkd1 and that the relationship between cellular proliferation and cyst formation may be indirect.

There are several potential reasons why our data differ from what has been previously published. First, we used phospho–histone-3 and Ki-67 as markers for proliferation, when most studies of human tissues use proliferating–nuclear cell antigen. The latter may be less reliable and may lead to overestimation of proliferation rates.

Second, differences in the antibody source, in sample preparation and in the cell-counting method may also be factors. It should also be noted that most studies of human samples necessarily use late-stage material, in which secondary effects due to chronic renal failure may be superimposed. Acquired cystic disease is thought to be a pre-malignant lesion with increased proliferative potential. Another possibility, which our study cannot address, is that proliferation actually occurs in bursts rather than in a stochastic, random fashion. If proliferation in cysts really occurs in cyclic bursts, it is possible that previous studies happened to catch cysts undergoing bursts of proliferation by chance, whereas ours did not. Finally, we note that a small increase in the rate of proliferation maintained over a prolonged period of time could account for the gradual enlargement seen in human cystic kidneys. However, it is unlikely that this process can explain the abrupt onset of cyst formation seen in our late-onset model.

How might the high proliferation rate in early cystic disease be linked to the disease process? One possibility is that the high proliferation rate is merely a marker for the developmental status of the organ, and the latter is primarily responsible for the enhanced susceptibility to Pkd1 inactivation. Consistent with this model, the developmental...
The neural tube defect in zebrafish lacking Vangl2, a conserved component of planar cell polarity signaling. We speculate that renal epithelial cells may undergo a similar process during renal development. Neuroblasts undergoing proliferation transiently lose their planar polarity within the kidney. In zebrafish, planar cell polarity signaling couples cell division and morphogenesis during neurulation. Neuroblasts undergoing proliferation transiently lose their planar orientation but reestablish it as daughter cells reorient after cell division. This model may also help to explain the benefits of antiproliferative agents in rodent models.

Figure 2 Susceptibility to rapid-onset cystic disease switches off between P12 and P14. (a) Kidneys of Pkd1cond/cond; tamoxifen-Cre+ mice induced at P12 were cystic within 3 weeks (left), whereas those of mice induced at P14 remained normal 3 months later (right). Scale bar, 2 mm. (b) β-galactosidase staining of kidneys of ROsa26R mice 5 d after induction at P12 (left) and P14 (right), showed that the samples have similar patterns and rates of Cre-mediated induction. Scale bar, 2 mm. (c) Southern blot of kidney DNA isolated from Pkd1cond/cond; tamoxifen-Cre+ mice harvested 5 d after induction at P12 and P14. (d) Ki67-stained sections of Pkd1cond/cond; tamoxifen-Cre+ (left) and Pkd1cond/cond; tamoxifen-Cre+ (right) kidneys 3 weeks after induction at P12. Scale bar, 200 μm. (e) Ki67-stained sections of uninduced Pkd1cond/cond; tamoxifen-Cre+; ROsa26R kidneys harvested on P12–P19. Similar results were obtained for kidneys of uninduced Pkd1cond/cond; tamoxifen-Cre+; ROsa26R mice (data not shown). Scale bar, 100 μm.

By demonstrating that acquired inactivation of Pkd1 in adult tissues leads to cystic disease, we also show that all nephron segments have the same propensity for forming cysts, which is consistent with the results of previous studies of human samples. Collectively, these data suggest that cysts derived from proximal tubules may comprise a substantial fraction of the total cyst burden. This may have implications for any therapy whose activity is restricted to cysts derived from distal nephron segments.

We also show that the response of the renal epithelium to acquired loss of Pkd1 is determined by the developmental state of the organ. These data suggest that early acquired inactivation of Pkd1 may explain the existence of a subset of affected individuals with early, severe ADPKD. The dramatically different response to inactivation in very young mice compared with older mice also suggests that different pathways may be altered in the two groups, which would have important implications for preclinical testing of potential therapies. Most published mouse models of PKD that are used to test therapies, including those for ADPKD, have a relatively rapid onset of disease. Given the very different rate of progression of disease acquired in adult life, it is unclear whether the approaches used for rapid progression would be equally effective in adult models. The gradual onset of cystic disease and the disease’s relatively slow progression in humans suggest that late-onset models, such as the ones identified in this study, may be more predictive of proper treatment of the human condition.

At first glance, the relationship between the developmental status of the kidney and its sensitivity to the effects of Pkd1 loss seems to be consistent with the ‘maturation arrest’ hypothesis of cyst formation. This model suggests that cysts are the result of an arrested developmental process that locks cells in an immature state because of a block either in their normal developmental program or in the regenerative program that follows cellular injury. Although our study was not designed to test this hypothesis, we note that there is a surprising aspect of our data that appear inconsistent with the model. For example, we show that the fully differentiated, mature renal tubular epithelium can undergo cystic change long after the developmental program has completed. In this case, cysts cannot be the result of a maturation arrest. Although we cannot exclude ‘de-differentiation’ in response to acquired Pkd1 loss, there were no signs of renal injury before cyst formation, and cysts formed abruptly and simultaneously throughout the organ. In the case of cyst formation within the first 3 weeks of life, we note that the proliferation rate, which seems to be a very good marker for the incompletely mature state, drops normally in cystic samples. We conclude from these data that kidney cells are particularly susceptible to the loss of Pkd1 in a developmental context, but we have no data to...
suggest that they are developmentally arrested after *Pkd1* inactivation. Further study will be required to test this hypothesis more thoroughly.

Our data also challenge a prevailing model that depicts the *Pkd1* gene product as a flow sensor on the primary cilia of renal epithelial cells, acting as a means to maintain tubule morphology\(^5\) in the adult kidney. We find it difficult to understand how it could take several months for the alteration of a highly dynamic process such as flow-sensing to result in an observable phenotype. Our conclusions are supported by a recent study that examined the consequences of induced systemic loss of two ciliogenic genes in adults (Tg737 and Kif3a)\(^26\). Similarly to what we had observed for *Pkd1*, the authors found that the kinetics of cyst formation were dependent on the time at which either gene was inactivated. Given that primary cilia are thought to be the mechano-sensitive organelles responsible for dynamic flow-sensing in the kidney, they concluded that loss of flow-sensing in adult kidney is probably not the primary cause of cyst formation in late-onset cystic disease. Neither our study nor the previous one, however, can exclude a role for cilia-based flow-sensing during renal development, given the rapidity with which cysts form after inactivation of *Pkd1*, Tg737 and Kif3a in the immature organ. The functions of cilia and *Pkd1* in the developing kidney may differ from their respective functions in the adult organ, thus accounting for the different rates at which cysts form after their loss. We also cannot exclude a role for the primary cilium as a flow sensor in the adult organ if ciliary signaling has tonic effects rather than dynamic ones on the maintenance of tubular morphology. Yet another possibility is that *Pkd1* and primary cilia are important in establishing and maintaining planar cell polarity that is independent of mechanosensing that is unchanged over time, but the developmental context in which they are lost explains the different phenotypic consequences. Further studies will be required to distinguish between these possibilities.

Finally, these studies have also identified a previously unrecognized brake point during renal growth that corresponds to significant changes in gene expression. The high rate of proliferation, which is universally distributed throughout the kidney, abruptly decreases at \(\sim P_{15}\) throughout the organ. These observations suggest that there may be a developmental switch that signals the end of the terminal renal maturation process.

**METHODS**

*Pkd1*cond*/cre* mouse lines. We crossed fifth-generation C57BL/6 *Pkd1*^cond*/*cre* mice to fifth-generation C57BL/6 tamoxifen-*Cre* (B6.Cg-Tg(Cre/Esr1)5Amc/J) mice (stock 004682, Jackson Laboratories) and C57BL/6 congenic B6.129S4-Gt(ROSA)26Sortm1Sor/J (stock 003474, Jackson Laboratories) to produce the mice used in these studies. We induced *Cre* recombinase activity in mice \(<3\) weeks of age by intraperitoneally injecting nursing mothers with either tamoxifen (10 mg/40 g) in corn oil (Sigma-Aldrich) or an equal volume of corn oil alone on two consecutive days. Older mice were induced by direct peritoneal injection with similar doses. Mice were killed by isofluorane treatment and kidneys were processed for DNA and RNA extraction and for histological examination (fixed in 4% paraformaldehyde buffered solution, pH 7.4). DNA was digested with EcoRI and probed with a Bgl2-EcoRI *Pkd1* fragment located adjacent to the 5' *loxP* site\(^3\). All studies were performed using protocols approved by the Johns Hopkins University Animal Care and Use Committee, and mice were housed in pathogen-free facilities accredited by the American Association for the Accreditation of Laboratory Animal Care.

**Immunohistochemistry and immunofluorescence**. Kidneys and livers from neonate and adult animals were collected and immediately fixed in 4% paraformaldehyde at 4 °C overnight. To remove excess paraformaldehyde, we washed samples in water for 2 h, dehydrated them in 50% ethanol for 2 h and then 70% ethanol for 2 h, and stored them at room temperature in 70% ethanol. After dehydration in water for 2 h, we dehydrated all samples in a graded alcohol series and embedded them in paraffin for histological analysis.

To unmask antigens, we boiled 5-μm sections in Target Retrieval solution (Dako USA) in a pressure cooker. Ki-67 was stained as described in the manufacturer’s protocol (Dako USA) with biotinylated secondary antibody to rat IgG (Dako USA). We used the ApopTag Apoptosis Detection Kit for TUNEL staining according to the manufacturer’s protocol (Chemicon). Cleaved–caspase-3 staining was performed as described in the manufacturer’s protocol (Cell Signaling Technology). *Dolichos biflorus* agglutinin (DBA) and *Lotus tetragonolobus* lectin (LTL) (Vector Laboratories) were used at a dilution of 1:100. Antibodies to aquaporin-2 and Tamm-Horsfall glycoprotein were gifts (see Acknowledgments) and were each used at a 1:500 dilution. Secondary antibody (Jackson Immunoresearch Laboratories) conjugated to Cy3 was used at a 1:400 dilution. We captured images with a Nikon Eclipse E600 microscope and either a SPOT-RT monochromatic (SPOT Diagnostic Instruments) or a Nikon DXM1200 digital camera.
Proliferation studies. Sections were scanned on an Olympus IX81 microscope with a Prior Proscan II controller and Hamamatsu EM-CCD C9100 digital camera and were analyzed with Slidebook Pro 4.1 software using the automated stitching function. We exported stitched images composed of Cy3 and DAPI captures as individual 16-bit channels in TIFF format. For sections too large to be assembled into one file (because of memory limitations), we performed two or three nonoverlapping scans instead. Individual TIFF files were opened in ImageJ 1.39a, and the nucleus-counter function of the WCIF plug-in was used to count the number of labeled nuclei (Cy3 for Ki-67–positive nuclei, DAPI for total number of nuclei). For each sample, we captured a set of 50–100 images at ×10 magnification for analysis.

Statistical analyses. We calculated the statistical significance of differences in proliferation rates using the Mann-Whitney (Table 1a) or Kruskal-Wallis (Table 1b,c) nonparametric tests and considered a value of $P < 0.05$ significant.

Microarrays. We isolated total RNA from kidneys with the Qiagen RNA extraction kit. We determined RNA concentration by spectrophotometry (NanoDrop) and then used 10 μg of total RNA for each hybridization. We analyzed 16 kidneys of uninduced Pkd1cond/cond, tamoxifen-Cre−, and ROSA26R+ mice (four samples from each time point: P11, P12, P14 and P15) with Mouse RefS equation 8 (8) Illumina Sentrix BeadChip microarray chips. The P11 and P14 animals were littermates, as were the P12 and P15 animals. Two samples from each time point were randomly selected for technical replication with the same platform. The results were analyzed with implementations provided by the open-source bioconductor project (http://www.bioconductor.org)27. Briefly, we used the log of expression values, followed by quantile normalization and linear model and empirical Bayes methods for differential gene-expression analysis28. The comparisons included (P11 and P12) versus (P14 and P15), (P11 and P14) versus (P12 and P15), and (P11 and P15) versus (P12 and P14). We considered genes with an fdr-adjusted $P$-value less than 1 × 10−5 in the first contrast, but not in the other two, as significantly different. Multidimensional scaling analysis was performed by using the correlation matrix of the expression values. Identification of overrepresented promoter cis elements was done with PRIMO (http://gastro.imbg.ku.dk/primoweb/)29. Gene ontological classification30 of the differentially expressed genes was performed by using the DAVID Bioinformatics Database functional-annotation tools (http://david.abcc.ncifcrf.gov/)30. The microarray data have been deposited in the Gene Expression Omnibus maintained by the US National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo/).

Accession numbers. Gene Expression Omnibus series GSE9167.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

K.P. designed and performed the experiments and contributed to the writing of the manuscript. L.F.M. assisted in the experimental design, helped to analyze the histopathology, performed the microarray analyses and assisted in the writing of the manuscript. M.A.G.-G. helped perform the experiments and contributed conceptually to the project. D.L.H. helped with the histopathology. G.G.G. directed the project and wrote the manuscript.

Note: Supplementary information is available on the Nature Medicine website.

LETTERS


