Hamartin regulates cessation of mouse nephrogenesis independently of Mtor

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Nephrogenesis concludes by the 36th week of gestation in humans and by the third day of postnatal life in mice. Extending the nephrogenic period may reduce the onset of adult renal and cardiovascular disease associated with low nephron numbers. We conditionally deleted either *Mtor* or *Tsc1* (coding for hamartin, an inhibitor of Mtor) in renal progenitor cells. Loss of one *Mtor* allele caused a reduction in nephron numbers; complete deletion led to severe paucity of glomeruli in the kidney resulting in early death after birth. By contrast, loss of one *Tsc1* allele from renal progenitors resulted in a 25% increase in nephron endowment with no adverse effects. Increased progenitor engraftment rates ex vivo relative to controls correlated with prolonged nephrogenesis through the fourth postnatal day. Complete loss of both *Tsc1* alleles in renal progenitors led to a lethal tubular lesion. The hamartin phenotypes are not dependent on the inhibitory effect of TSC on the Mtor complex but are dependent on Raptor.

kidney development | metanephric mesenchyme | Mtor | nephron progenitor cells | Tsc1

Mammalian nephrogenesis progresses through a series of developmental stages during mid to late gestation culminating with the metanephros, which persists as the definitive kidney in the adult. The kidneys contain multiple nephrons, consisting of glomerular capillary tufts surrounded by parietal epithelial capsules and a connecting network of tubules that modify glomerular filtrate composition to aid in fluid and salt homeostasis and perform different metabolic functions, including vitamin D metabolism. The associated juxtaglomerular apparatus contributes to hormonal regulation of blood pressure. Nephrogenesis ends by the 36th week of gestation in humans (1, 2) and by the third day of life in mice (2, 3). The number of nephrons in individual kidneys may vary 10-fold, from ∼200,000 to over 2.5 million. Not all factors contributing to this variation are known, but prematurity, intrauterine growth retardation, and maternal starvation during gestation are strongly associated with low nephron endowment (4). Low nephron numbers contribute to significantly increased risk of chronic kidney disease (CKD), hypertension, and end-stage renal disease (5–8). As the postnatal kidney can repair but not replace nephrons, intervention-free survival depends on the initial nephron number. The burden of renal and cardiovascular disease is immense: More than eight million Americans are affected by CKD, and over 100,000 begin dialysis each year while awaiting renal transplantation, mainly secondary to obesity, diabetes, and hypertension. Thus, a greater understanding of the basic mechanisms regulating nephron endowment and any indication of a path to increasing nephron numbers are of marked scientific and clinical relevance.

Nephron progenitor cells (NPCs) in the metanephric mesenchyme (MM) coalesce into "cups" around ureteric bud (UB) tips. Reciprocal interactions between the UB and NPCs induce differentiation of two nephron primordia, one at each "arm pit" of the new branch (9, 10), via a mesenchymal–epithelial transition in response to UB-derived Wnt9b (11, 12). The NPCs in turn produce GDNF and other signals that induce and maintain UB branching (13). Six2, a transcription factor, maintains the NPC population; Six2 deletion in the NPC leads to the loss of nephrons due to premature differentiation (14–16). Differentiating NPCs extinguish Six2 and create nephrons through formation of an epithelial renal vesicle, which grows into a comma-shaped and then into an S-shaped body. Using an ex vivo transplantation assay, we have demonstrated that postnatal day 0 (P0) progenitors preferentially exit the stem cell niche and differentiate into nephrons relative to young NPCs. Importantly, P0 NPCs can remain in the niche if surrounded by E12.5 $Fgf20^{+}$ progenitors (17) or can self-renew in culture nearly indefinitely (18–20), indicating tunable plasticity. NPC populations are heterogeneous, shifting with embryonic age toward a signature rich with ribosomal components, PolyA-binding proteins, and other transcripts suggestive of an increase in Mtor (mammalian target of rapamycin) activity (17). Mtor, a serine/threonine kinase, serves as a central regulator of cell metabolism, growth, proliferation, autophagy, and survival when in complex with Raptor (forming Mtor complex 1, Mtorc1) or Rictor (forming Mtorc2). Hamartin (coded by the Tuberous sclerosis 1 gene, *Tsc1*), a protein widely expressed in normal tissues, complexes with tuberin (*Tsc2*) (21, 22) and inhibits MtorC1.

Significance

Mammals form the final numbers of nephrons, the functional units of their kidneys, before or within a few days after birth. This initial endowment declines throughout life without the ability to replace nephrons lost to injury. Therefore, humans with low nephron numbers (such as premature infants) have higher rates of kidney disease in adulthood. Here we report that partial reduction in hamartin activity within mouse renal progenitors can lead to a significant increase in nephron numbers in newborns. Nephrogenesis was prolonged by at least a day and correlated with higher engraftment rates in the developing niche in a stem cell engraftment assay. We provide evidence that this phenotype is independent of the inhibitory effect of hamartin on the Mtor complex.

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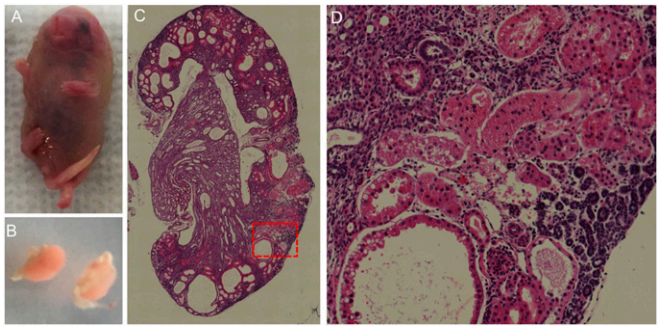

Fig. 1. *Mtor*-deficient kidneys are severely impaired. (*A*) PCD19.5 mice whose kidneys lack *Mtor* appear edematous. (*B*) Mtor-deficient kidneys are small and pale. (*C*) H&E staining of a kidney cross-section (4× objective). (*D*) Magnified view with a 40× objective of the region shown in the dashed red box in *C*. Note multiple cysts and an individual glomerulus.

These findings motivated us to investigate the role of Mtor and Tsc1 in tuning NPC plasticity.

Herein we report that conditional deletion of *Mtor* in NPC profoundly disrupted nephrogenesis, and hemizygous deletion led to a significant reduction in nephron endowment. By contrast, conditional deletion of *Tsc1* led to a lethal proximal tubular lesion. Surprisingly, hemizygous deletion of *Tsc1* was associated with significantly (25%) increased nephron numbers due to the delayed cessation of nephrogenesis. Accordingly, NPCs hemizygous for *Tsc1* displayed increased stem cell niche engraftment relative to age-matched wild-type cells, which could reflect a slower rate of aging. Finally, epistasis experiments demonstrated that the effects of *Tsc1* deletion were not dependent on its interaction with the Mtor pathway. This suggests that a hamartin target is one variable affecting nephron endowment, representing a potential therapeutic approach to augment nephrogenesis.

Results

Mtor Activity Is Necessary for Normal Development of the Kidney. To study the role of the Mtor pathway in nephron development, we deleted *Mtor* in NPCs using $Six2^{TGC+/tg}$ (14) and the $Mtor^{f/f}$ alleles (23). Reciprocally, we deleted *Tsc1* using a similar strategy. Notably, the $Six2^{TGC+/tg}$ allele is not benign: Fewer nephrons form in hemizygotes relative to wild type (see below). Since a large fraction of gravid dams carrying a litter with *Tsc1* hemizygote kidneys delivered prematurely (before the $Six2^{TGC+/tg}$ controls), we assigned age to all mice in this study as postcoitum days (PCD) based on the plug date.

One-quarter of the pups born to a $Six2^{TGC+/tg}$; $Mtor^{+/f}$ male mated with an $Mtor^{f/f}$ female died within 2 d and displayed severe generalized edema (Fig. 1*A*) not seen in other mice lacking nephrons (e.g., Notch mutants) (24). Genotyping confirmed complete loss of *Mtor* in these pups; no *Mtor*-null mice survived longer than 2 d. The kidneys of *Mtor* mutants were pale and dysplastic (Fig. 1*B*), with tubules in the parenchyma, paucity of glomeruli, and multiple cysts noted in the collecting ducts (Fig. 1 *C* and *D*). These results demonstrate that Mtor activity is essential for proper kidney development.

Although complete loss of *Mtor* led to dysplastic and dysfunctional kidneys, we next explored the hypothesis that Mtor signaling might still affect nephron endowment in hemizygotes. Loss of one *Mtor* allele led to a 24% decrease in the kidney/body weight ratio at PCD19.5 compared with $Six2^{TGC+/tg}$ mice (0.47 ± 0.03 vs. 0.624 ± 0.02, $P < 0.005$), and to a 42% decrease in the ratio compared with wild-type mice (0.47 ± 0.03 vs. 0.9 ± 0.03, $P < 0.005$) (Fig. 2*A*). Accordingly, nephron counts were significantly ($P < 0.005$) lower in $Six2^{TGC+/tg}$; $Mtor^{+/f}$ kidneys (1,140 ± 139) compared with both controls: $Six2^{TGC+/tg}$ (2,850 ± 3) and wild type (5,100 ± 377.5) (Fig. 2*B*), reflective of impaired nephrogenesis. As $Six2^{TGC+/tg}$ itself affects nephrogenesis, $Six2^{TGC+/tg}$ mice serve as controls in this study.

We then assessed the contribution of postnatal nephrogenesis in *Mtor* hemizygotes. $Six2^{TGC+/tg}$; $Mtor^{+/f}$ P30 kidneys had 22.5% fewer nephrons than $Six2^{TGC+/tg}$ controls (13,382 ± 389 vs. 17,250 ± 194, $P < 0.0001$) and 59% fewer nephrons than wild-type kidneys (13,382 ± 194 vs. 27,967 ± 298, $P < 0.0001$) (Fig. 2*C*). Notably, at P200 $Six2^{TGC+/tg}$ animals developed albuminuria (Fig. S1*A*). Despite reduced nephron numbers, glomerular function, assessed in aggregate by serum creatinine (Fig. S1*B*), blood urea nitrogen (BUN) levels (Fig. S1*C*), and albuminuria, was not further compromised in $Six2^{TGC+/tg}$; $Mtor^{+/f}$ ~ P200 mice relative to $Six2TGC^{+/tg}$ controls.

***Tsc1* Loss Is Lethal Due to Severe Renal Tubular Lesion.** While *Mtor* loss may compromise progenitor survival due to metabolic insufficiency, it is possible that elevated Mtor activity would accelerate the cessation of nephrogenesis. To explore this, we first deleted *Tsc1* in NPCs. Although $Six2^{TGC+/tg}$ $Tsc1^{f/f}$ pups (henceforth, "*Tsc1*-null kidneys") were born at the expected Mendelian frequencies and had a normal appearance at birth, they failed to survive longer than 48 h, consistent with renal failure. *Tsc1*-null kidneys produced urine throughout gestation, as demonstrated by amniotic fluid volumes similar to those in hemizygous littermates and the presence of urine in the bladder when pups were killed at PCD19.5 (Fig. S2*A*). Accordingly, and in contrast to *Mtor*-null kidneys, no glomerulopathy was identified in histological sections of *Tsc1*-null kidneys.

Strikingly, *Tsc1*-null kidneys displayed severe proximal tubular lesions: H&E staining showed that most cortical proximal tubules in *Tsc1*-null kidneys lacked a patent lumen compared with wild-type tubules, and tubular epithelial cells frequently exhibited a random orientation of the nuclei in contrast to the orderly basilar location in wild-type tubules (Fig. 3). To quantify the degree of tubular lesion, we counted the fraction of LTL^+ tubules with a patent lumen in *Tsc1*-null and wild-type kidneys. All control LTL^+ tubules had a patent lumen; ~90% of LTL^+ tissues in the *Tsc1*-null kidneys lacked a lumen (Fig. S2 *B* and *C*). Tubular lesions were detected as early as PCD15.5 (Fig. 3). To further characterize the phenotype, we performed periodic acid Schiff (PAS) staining on *Tsc1*-null and control embryonic kidneys to examine the brush border in cortical proximal tubule cells (Fig. 4*A*). The PAS stain generally showed no well-developed brush border along the apical surface and a lack of intracytoplasmic resorption droplets, both findings divergent from wild-type stains and indicative of loss of tubular cell polarity. Ultrastructural analysis confirmed the lack of a brush border in swollen proximal tubule cells with large eccentric nuclei. The normal number and the distribution of mitochondria implied that some forms of active transport may still exist. Glomerular morphology was unaffected by *Tsc1* loss, consistent with the production of urine. The lesion was limited to the cortical proximal tubules; although it did not obstruct urine flow, it was severe enough to compromise survival (Fig. 4*B*). While we cannot rule out the possibility that loss of *Tsc1* in other Six2-expressing tissues (e.g., cranial nerves and dorsal root ganglia) compromised the

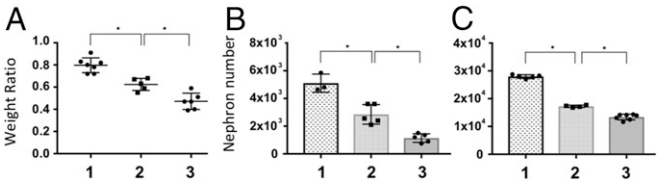

Fig. 2. Kidney size and nephron number are sensitive to Mtor dose. The weight (*A*) and nephron count (*B* and *C*) in controls (1), $Six2^{TGC+/tg}$ (2), and $Six2^{TGC+/tg}$ $Mtor^{+/f}$ (3) kidneys of PCD19.5 pups (*A* and *B*) and adults (*C*). In *B* and *C*, each data point depicts the sum of nephrons in both kidneys from one individual. *$P < 0.05$.

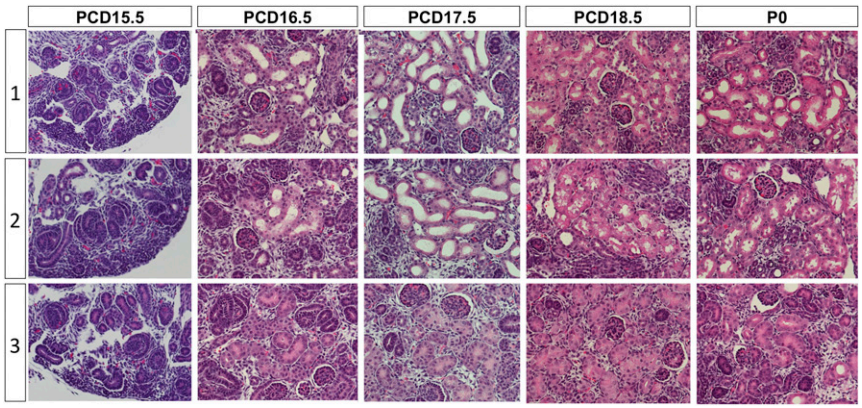

Fig. 3. Severe tubular lesions in kidneys with complete loss of *Tsc1*. H&E staining of kidneys from different embryonic stages (PCD15.5–PCD19.5) and from wild-type (1), *Tsc1*-hemizygous (2), and *Tsc1*-null (3) kidneys. Note the complete occlusion of tubular lumen by large cells that lost cellular polarity as shown by lack of brush borders and atypical nuclear location. Note also that the glomeruli appear normal in all three genotypes. Images were acquired with a 20× objective.

animals, at birth they were viable, motile, and feeding normally. We postulate that the mechanism most likely to impose rapid demise may be impaired tubular function driving the accumulation of electrolytes in the blood and leading to fatal arrhythmia.

***Tsc1*-Hemizygous Kidneys Have More Nephrons than Controls in Two Independent Models.** We next studied the effect of removing one *Tsc1* allele on nephron numbers. We confirmed that hamartin levels were reduced by Western blot analysis of purified NPCs (Fig. S3). Surprisingly, the kidney/body weight ratio of the *Tsc1* hemizygotes (1.5% ± 0.05) was indistinguishable from that of wild-type mice (1.5% ± 0.05, $P = 0.49$) and was 22% higher than that of $Six2^{TGC+/tg}$ mice in adulthood (1.22% ± 0.04, $P < 0.05$) (Fig. 5*A*). Furthermore, deletion of one copy of *Tsc1* in kidney progenitors restored nephron numbers at P30 to the wild-type levels (Fig. 5*B*). The average nephron number in $Six2^{TGC+/tg}$ mice was 17,180 ± 261, significantly lower than that in wild-type mice (25,769 ± 1,756, $P < 0.001$); by contrast, the average nephron number *in Tsc1*-hemizygous kidneys (26,640 ± 514, $P = 0.66$) was indistinguishable from wild-type kidneys and was significantly higher than in $Six2^{TGC+/tg}$ kidneys ($P < 0.0001$). Overall, the loss of one *Tsc1* allele in NPCs increases kidney size and nephron numbers in the Six2-Cre background without any apparent impact on viability or fecundity.

The loss of a *Tsc1* allele may act only to correct the uncharacterized defect in $Six2^{+/TGC}$ (a Bac insertion transgene). To address this possibility we used $Fgf20^{+/Cre}$ mice (25), a knockin line increasing progenitor exit by a fraction due to reduced Fgf20/Fgf9 signaling (17, 18). Again, we observed a significant increase in nephron numbers in $Fgf20^{+/Cre}$; $Tsc1^{+/f}$ mice, which became statistically indistinguishable from wild type (Fig. 5*D*). This line serves as an independent control confirming that the reduction in hamartin did not act solely by countering the defect introduced by the $Six2^{TGC}$ transgene.

Increased Perdurance of *Tsc1*-Hemizygous Progenitors in the Niche. Hamartin could act by increasing niche numbers, by increasing progenitor proliferation, by prolonging nephrogenesis, or by other mechanisms. Blinded observers quantified niches and progenitors by tomography-based counts on littermates with the genotypes wild type, $Six2^{TGC+/tg}$, and $Six2^{TGC+/tg}$; $Tsc1^{+/f}$ on PCD15.5 and PCD19.5. Cell number per niche was indistinguishable in the three groups at both ages. Although they trended upward at PCD19.5 in $Six2^{TGC+/tg}$; $Tsc1^{+/f}$ mice, niche numbers were statistically indistinguishable in $Six2^{TGC+/tg}$ and $Six2^{TGC+/tg}$; $Tsc1^{+/f}$ mice and were higher in wild-type mice on PCD15.5 and PCD19.5 (Fig. 6).

To determine when NPCs were exhausted (typically by P3 in mice), we stained PCD22.5–PCD25.5 kidneys (P3–P6 in $Six2^{TGC+/tg}$ mice) from the genotypes mentioned above for Six2 (MM) and cytokeratin 8 (UB). For all genotypes, $Six2^+$ NPCs were identified on PCD22.5 but were absent on PCD24.5 (P5 in controls). At PCD23.5, controls (four wild type and two $Six2^{TGC+/tg}$) lacked $Six2^+$ NPCs, while many niches in all seven *Tsc1* hemizygotes contained $Six2^+$ progenitors (Fig. 7*A* and Fig. S4). As an additional control, age-matched wild-type ($Tsc1^{+/f}$) littermates exposed to the same uterine environment as $Six2^{TGC+/tg}$; $Tsc1^{+/f}$ pups were examined; no $Six2^+$ niches were observed. Finally, $Six2^+$ progenitors, and thus nephrogenesis, persisted an additional ∼24 h in the $Fgf20^{+/Cre}$; $Tsc1^{+/f}$ kidney as well (Fig. S4*C*). Importantly, since none of the wild-type littermates had $Six2^+$ progenitors at that age (Fig. 7*B*), the gain in nephrons is the result of the increased lifespan of NPCs hemizygous for *Tsc1*.

***Tsc1*-Hemizygotous Renal Progenitor Cells Have Higher Engraftment Rate.** The above observation suggests a possible shift in the rate of NPC aging. To address this, we investigated how progenitor engraftment was affected by reduced hamartin activity. Deletion of *Tsc1* increases the activity of Mtor, which may be driving the "aged" signature in our single-cell RNA-sequencing (RNA-seq)

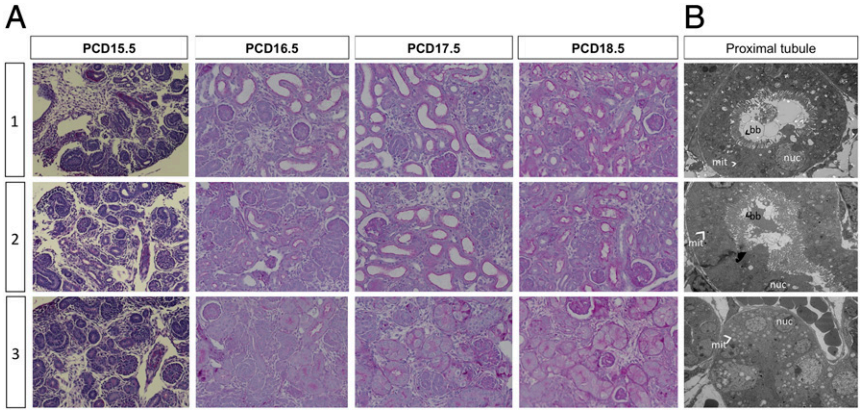

Fig. 4. Complete *Tsc1* loss in the kidneys led to the lack of a tubular brush border. (*A*) PAS staining of the cortical proximal tubules from different embryonic stages and wild type (1), *Tsc1*-hemizygous (2), and *Tsc1*-null (3) kidneys. Images were acquired with a 20× objective. (*B*) Transmission electron microscopy of proximal tubules from $Six2^{TGC+/tg}$ $Tsc1^{f/f}$ PCD19.5 mice. Note lack of a brush border (bb) in *Tsc1*-deficient kidney. Large tubular cells have enlarged nuclei (nuc) and high numbers of mitochondria (mit).

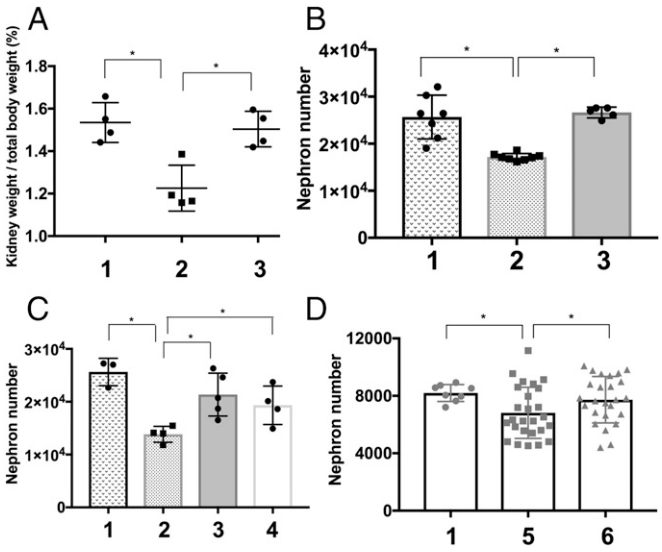

Fig. 5. *Tsc1* deletion increased kidney size and nephron number in an *Mtor*-independent manner. (*A* and *B*) The weight (*A*) and nephron count (*B*) in P30 wild-type (1) and $Six2^{TGC+/tg}$ (2) controls and $Six2^{TGC+/tg}$ $Tsc1^{+/f}$-hemizygous (3) kidneys. (*C*) Reduction in Mtor in $Six2^{TGC+/tg}$ $Tsc1^{+/f}$ $Mtor^{+/f}$ (4) did not impact nephron count relative to $Six2^{TGC+/tg}$ $Tsc1^{+/f}$ (3). In *B* and *C*, each data point shown is the sum of nephrons in both kidneys of one individual. (*D*) Nephron counts in wild-type (1), *Fgf20-Cre* (5), and *Fgf20-Cre* $Tsc1^{+/f}$ (6) kidneys. Glomeruli were counted from a single kidney removed from pups aged between P7 and P11. All litters were born at the same time as controls. $*P < 0.05$.

analysis (17). If elevated Mtor activity caused poor engraftment of older progenitors, we anticipated that *Tsc1*-deficient progenitors would engraft poorly relative to same-age wild-type cells. Alternatively, if Mtor activity was not impeding engraftment, but hamartin activity was, we expected to see improved engraftment.

We used an ex vivo transplantation assay to differentiate between these hypotheses. The engraftment potential of NPCs declines with time, as older NPC tend to exit the niche and differentiate in larger numbers than younger cells. To determine the performance of PCD18.5 $Tsc1^{+/f}$ progenitors engrafted relative to PCD12.5 progenitors, we FACS-purified PCD18.5 $Six2^{TGC+/tg}$; $Tsc1^{+/f}$; $Rosa^{+/tdTomato}$ old progenitors (red+green, due to GFP expressed from the $Six2^{TGC+/tg}$ allele) and PCD12.5 $Six2^{TGC+/tg}$; $CAG\text{-}eCFP^{+/tg}$ young progenitors (blue+green). These cells were injected in a 1:1 ratio into the cap mesenchyme (CM) of a PCD12.5 recipient kidney explant. After 4 d the injected kidneys were fixed and stained for markers of MM and UB (Six2 and cytokeratin 8, respectively); we then quantified the number of red and blue $Six2^+$ cells in z stacks acquired by confocal imaging (17). To normalize the data across experiments, we calculated the percentage of red and blue cells out of the total injected cells remaining in the niche at day 4. IMARIS software enabled analysis of the engraftment rate; the young cells engrafted better than old cells ($86 \pm 3\%$ vs. $13 \pm 3\%$, $P < 0.0001$, 11 sites injected on three dates) (Fig. 7*C*). Therefore, the reduction in *Tsc1* dosage did not alter the negative effect of aging.

We next tested if *Tsc1* loss impacted relative engraftment by comparing same-age cells of different genotypes. We FACS-purified PCD18.5 $Six2^{TGC+/tg}$; $Tsc1^{+/f}$; $Rosa^{+/tdTomato}$ (red+green, *Tsc1*-hemizygote) and $Six2^{TGC+/tg}$; $CAG\text{-}eCFP^{+/tg}$ (blue+green, control) progenitors and mixed them at a 1:1 ratio. When populations of PCD18.5 progenitors were injected into the CM of a PCD12.5 host kidney, *Tsc1*-hemizygote progenitors had a significant engraftment advantage compared with the controls ($85 \pm 5\%$ vs. $15 \pm 4\%$, $P < 0.0001$, 10 sites injected on five dates) (Fig. 7*D*). Therefore, although *Tsc1* loss did not reverse the overall effect of aging on engraftment, decreased hamartin in NPCs does improve the cell engraftment rate.

The Effect of *Tsc1* on Nephron Number and Proximal Tubules Is Agnostic to *Mtor* Dose but Requires both Alleles of Raptor. The increase in nephron numbers could result exclusively from elevated Mtor activity, from reduced hamartin function(s) unrelated to Mtor signaling, or both. To differentiate between these possibilities, we examined nephron numbers in compound hemizygous ($Six2^{TGC+/tg}$; $Mtor^{+/f}$; $Tsc1^{+/f}$) mice. Removal of one *Mtor* allele resulted in reduced nephron numbers (Fig. 2). If this reflected enhanced inhibition of Mtor complex(es) due to an increased Tsc/Mtor ratio, we would expect nephron numbers in $Six2^{TGC+/tg}$; $Mtor^{+/f}$; $Tsc1^{+/f}$ kidneys to return to the levels seen in $Six2^{TGC+/tg}$ kidneys. Instead we found that $Six2^{TGC+/tg}$; $Mtor^{+/f}$; $Tsc1^{+/f}$ and *Tsc1*-hemizygous kidneys were indistinguishable: At P30, the average nephron number in $Six2^{TGC+/tg}$; $Tsc1^{+/f}$ mice ($21{,}355 \pm 1{,}819$) is similar to the average number in $Six2^{TGC+/tg}$; $Mtor^{+/f}$ $Tsc1^{+/f}$ mice ($19{,}325 \pm 1{,}822$, $P = 0.46$). Both were significantly higher than in $Six2^{TGC+/tg}$ control mice ($13{,}838 \pm 755$, $P = 0.01$) (Fig. 5*C*). We concluded that *Mtor*-independent hamartin activity limits nephron number and that the loss of one *Tsc1* allele alleviated this restriction.

To test whether the tubular lesions of *Tsc1*-null kidneys can be reversed by decreasing Mtor activity in progenitors, we mated $Six2^{TGC+/tg}$; $Mtor^{+/f}$; $Tsc1^{+/f}$ males with $Tsc1^{f/f}$ females; 12.5% of the pups had the relevant genotype ($Six2^{TGC+/tg}$; $Tsc1^{f/f}$; $Mtor^{+/f}$), all of which died within the first 2 d of life regardless of their *Mtor* genotype. The fraction of tubules displaying lesions under light microscopy in $Six2^{TGC+/tg}$; $Tsc1^{f/f}$; $Mtor^{+/f}$ kidneys was the same as noted in *Tsc1*-null kidneys (Fig. S2*D*). Thus, the frequency and severity of the tubular lesions caused by complete deletion of *Tsc1* are not modified by the *Mtor* dose.

Finally, we asked whether the scaffold protein Raptor was involved in the *Tsc1* phenotypes. Whereas $Six2TGC^{+/tg}$; $Mtor^{+/f}$ kidneys contained significantly fewer nephrons than $Six2TGC^{+/tg}$ kidneys (Fig. 2), nephron numbers in $Six2TGC^{+/tg}$; $Rap^{+/f}$ ($n = 4$) kidneys were statistically indistinguishable from $Six2TGC^{+/tg}$ ($n = 5$) kidneys (Fig. 6*D*). Although Raptor and the Mtor kinase form MtorC1, Raptor may be in excess such that the loss of free Raptor had no impact whereas the loss of Mtor did. If Raptor

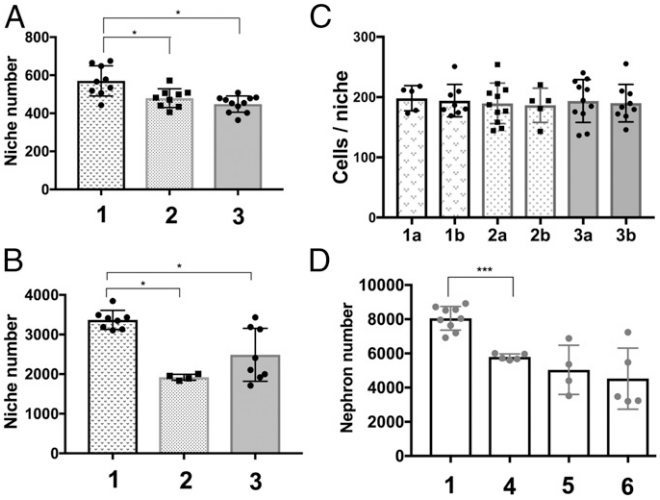

Fig. 6. Hemizygous loss of Tsc1 is not associated with an increase in niche number or cells per niche in early and late gestation. (*A*–*C*) OPT-based niche (*A* and *B*) and cell (*C*) counts derived from confocal images of Six2 antibody-stained kidneys from wild-type (1) and $Six2^{TGC+/tg}$ (2) controls and $Six2^{TGC+/tg}$ $Tsc1^{+/f}$-hemizygous (3) kidneys at PCD15.5 (*A*) and PCD19.5 (*B*). Cell numbers per niche (*C*) were counted at PCD15.5 (1a, 2a, and 3a) and PCD19.5 (1b, 2b, and 3b). (*D*) The *Tsc1*-mediated increase in nephron number requires Raptor. Shown are nephron counts in wild-type controls (1), $Six2^{TGC+/tg}$ (4), $Six2^{TGC+/tg}$ $Rap^{+/f}$ (5), and $Six2^{TGC+/tg}$; $Rap^{+/f}$; $Tsc1^{+/f}$ (6) kidneys. Glomeruli were counted from a single kidney removed from pups aged between P7 and P11. All litters were born at the same time as controls. $*P < 0.05$; *** $P < 0.0001$.

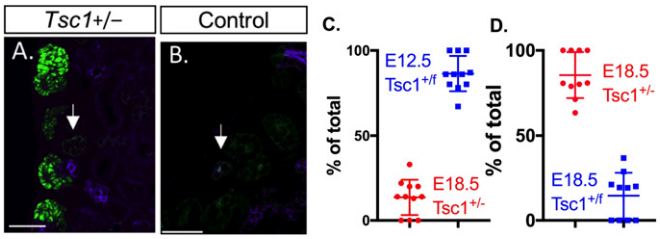

Fig. 7. *Tsc1* loss in renal progenitors leads to prolonged nephrogenesis and improved engraftment. (*A*) Magnified view of the nephron progenitor cells (arrow) in the periphery identified with anti-Six2 antibody in PCD23.5 (P4.5) $Six2^{TGC+/tg}$ $Tsc1^{+/f}$ kidneys. (*B*) Magnified view of kidney's periphery from $Tsc1^{f/f}$ (wild-type) littermates in which nephrogenesis has ended (the arrow points to background staining). Purple: cytokeratin 8; green: Six2. (Scale bars represent 50 μM.) (*C* and *D*) Niche engraftment success presented as the percentage of $Six2^{TGC+/tg}$; $Tsc1^{+/f}$ $Rosa^{+/tdTomato}$ and $Six2^{TGC+/tg}$; $CAG\text{-}eCFP^{+/tg}$ cells in the total number of cells detected at the end of 4-d culture. (*C*) Comparison between $Tsc1^{+/f}$ and $Tsc1^{+/-}$ cells of different ages. (*D*) Comparison between $Tsc1^{+/f}$ and $Tsc1^{+/-}$ cells of the same age.

activities were mediated via MtorC1, we expected that reducing hamartin levels would elevate nephron number in *Rap* hemizygotes as it did in *Mtor* hemizygotes. Surprisingly, loss of *Tsc1* in $Six2TGC^{+/tg}$; $Tsc1^{+/f}$; $Rap^{+/f}$ kidneys ($n = 5$) had no impact on nephron numbers (Fig. 6*D*). These series of genetic experiments suggest a complex mechanism by which hamartin regulates nephrogenesis duration in mice, proposed in Fig. S6.

Discussion

Here we describe a role for hamartin in regulating NPC exhaustion during kidney development. Complete deletion of either *Mtor* or *Tsc1* in mice NPCs led to postnatal lethality but apparently by different mechanisms. *Mtor*-deficient NPC failed to develop a functional kidney, reminiscent of the loss of *Mtor* in other stem cell compartments (26–31). The lethal phenotype reflects the vital role of the kidney in neonates: In utero, the filtering function of the kidney is replaced by the maternal placenta.

Complete deletion of *Tsc1* spared the glomerulus but disrupted cortical proximal tubule morphology with loss of the brush border and minimal luminal space. The proximal tubules act in resorption and homeostasis of glucose, ions, and water; the loss of Tsc1 must have rendered them sufficiently impaired to cause lethality. This phenotype is reminiscent of one described previously (32) in which a later, mosaic deletion of *Tsc1* in proximal tubules, glomeruli, and ducts resulted in severe, MtorC1-dependent polycystic kidney. Our results reflect an earlier, fully penetrant effect of hamartin that is Mtor independent.

Importantly, hemizygous deletion of *Tsc1* increased nephron numbers in $Six2^{TGC+/tg}$ $Tsc1^{+/f}$ kidneys, restoring them to the level seen in the wild type. While environmental factors such as a high-protein diet (33) can increase nephron endowment in rats, few genetic manipulations can cause the same effect (34). We found that the number of nephron progenitors and niches were the same in $Tsc1^{+/-}$ and controls at PCD15.5 and PCD19.5. However, $Tsc1^{+/-}$ kidneys displayed prolonged nephrogenesis, with $Six2^{+}$ NPCs detected at PCD23.5 in a fraction of the niches, while there was no detectable Six2 staining past P25.5/PCD22.5 in control mice. Extended nephrogenesis by even a fraction of a day can account for the observed increase in nephron number.

Extended nephrogenesis in *Tsc1* hemizygotes was consistent with the effect of losing one *Tsc1* allele on the behavior of individual NPCs. Our explant system permits simultaneous interrogation of genetically defined cells engrafted in genetically defined environments. Importantly, we found that older NPCs lacking one *Tsc1* allele have significantly higher engraftment rates than wild-type NPCs of the same age, as is consistent with a prolonged nephrogenic period and, presumably, a slower rate of aging. Note that not all NPCs have to display these properties for a gain of 25% in nephron numbers. Because hamartin loss impacted both $Six2^{TGC+/tg}$ and $Fgf20^{+/Cre}$ NPCs, its impact implies a more general mechanism that compensates indirectly for the deficiency in these models.

In humans, tuberous sclerosis disease is caused by loss of function of *TSC1* or *TSC2*. Kidney involvement manifests with angiomyolipoma (AML) and cysts, which contribute to bleeding and CKD, respectively. It is not known whether newborns with *TSC1* loss have more nephrons at birth, as any gain may be obscured by the secondary disease complications. It is possible that an increased nephron number offers a physiological advantage, as cysts promote nephron loss. In AML, in addition to germline loss of *TSC1* or *2*, somatic loss of heterozygosity is frequently identified (35, 36). We found the complete deletion of *Tsc1* in NPCs leads to severe and lethal tubular lesions, and others have demonstrated severe cysts in *Tsc1*-null kidneys (32). Therefore, the loss of heterozygosity may lead to similar, mTOR-independent tubular lesions and eventually, in an mTORC1-dependent manner, to cystogenesis.

Inhibition of mTOR by rapamycin revolutionized tuberous sclerosis management and was particularly helpful in treating hamartomas. However, we have shown that viability was not restored and that the severity of the tubular lesions in *Tsc1*-null kidneys was not ameliorated by *Mtor* deletion. In addition, it did not modify nephron numbers in $Six2^{TGC+/tg}$ $Tsc^{+/-}$ kidneys. It should be noted that Mtor deletion could affect MtorC1, MtorC2, and other Mtor-containing complexes (37). Since reduced hamartin levels increase nephron number in *Mtor*, *Fgf20*, and $Six2^{TGC}$ hemizygotes but not in Raptor hemizygotes, we conclude that hamartin activities are Mtor independent but Raptor dependent. This is reminiscent of recent reports that free Raptor has mTOR-independent activity (38), which hamartin may be able to modulate (Fig. S6).

Several questions remain to be addressed by future investigation. The molecular mechanisms behind our observed $Tsc1^{+/-}$ NPC niche retention and higher engraftment rates remain unknown, as are the hamartin targets involved. Hamartin and tuberin form a complex that negatively regulates the Rheb (Ras homolog enriched in brain) GTPase, the main regulator of mTORC1. Hamartin also interacts with other pathways that affect nephrogenesis: For example, it can modulate TGF-β/Smad2/3 signaling independently from tuberin (39). Smad signaling has an important role in kidney development via regulation of NPC exhaustion (20). However, we could not detect changes in the level of phospho-Smad in *Tsc1* hemizygotes (Fig. S5). Our genetic analysis suggested that hamartin impacts nephron number independently of Mtor or the dosage-sensitive Fgf20 but that its activity does require Raptor. We speculate that hamartin may participate in balancing the impact of free Raptor on nephrogenesis. When hamartin levels are reduced, a net positive effect is gained (Fig. S6). The pathogenesis of $Tsc1^{-/-}$-associated tubular lesions remains to be fully characterized, and, last, the implications for human health need to be explored. At present, the field is focused on modulating Mtor; our study suggests Mtor-independent targets should also be considered.

In conclusion, we find that a reduction in hamartin has a net positive effect on nephron number by enabling progenitor expansion, acting independently of the mTor complex but requiring Raptor. This suggests distinct targets and offers a therapeutic path toward increasing nephron numbers in premature newborns. Over the long run, augmenting nephron numbers could also help address the high rates of adult renal and cardiovascular disease. These findings may also provide a model that will enhance our understanding of the initial steps in the process leading to AML and cyst formation in tuberous sclerosis patients.

Materials and Methods

Animals. All mice were maintained in the Cincinnati Children's Hospital Medical Center (CCHMC) animal facility according to the animal care regulations. The Animal Studies Committee of CCHMC approved the experimental protocols (IACUC2016-0022/0032). The following lines were used: $Tg(Six2\text{-}EGFPIcre)1^{Amc}$ (herein $Six2Cre^{tg/+}$) (14), $Rosa^{tom/tom}$ (40), $CAG\text{-}eCFP^{tg/tg}$ (herein $ECFP^{tg/tg}$), $Mtor^{f/f}$ (23), $Fgf20^{+/Cre}$ (25), $Tsc1^{f/f}$ (41), and $Rptor^{tm1.1Dmsa}$ (herein $Rap^{f/f}$) (42).

For NPCs in niche engraftment we crossed males of the genotypes *Six2 Cre*$^{tg/+}$ *Rosa*$^{tom/tom}$ and *Six2 Cre*$^{tg/+}$ *CAG-eCFP*$^{tg/tg}$, *Six2 Cre*$^{tg/+}$ *Mtor*$^{f/+}$ or *Six2 Cre*$^{tg/+}$ *Tsc1*$^{f/+}$ with *Mtor*$^{f/f}$, *Tsc1*$^{f/f}$ or CD1 females.

Nephron Count. Nephron count was performed as described in ref. 18 and in *Supporting Information*. Two individuals, blinded to the genotypes of kidneys being scored, performed the counts independently, and results were averaged.

Histology. Embryonic and adult kidneys were dissected in ice-cold PBS and fixed overnight in fresh 4% paraformaldehyde in PBS. Kidneys were embedded in paraffin. For overall morphology, tissue was stained in hematoxylin for 4 min and eosin for 1 min. Automated PAS staining was performed by the pathology core of CCHMC. Electron microscopy methods are provided in *Supporting Information*.

FACS Sorting of Renal Progenitor Cells. Progenitors were sorted as described by Chen et al. (17). Detailed protocol can be found in *Supporting Information*.

Kidney Organ Culture. Mouse metanephric organ cultures were performed as detailed in *Supporting Information*.

Transplantation of Renal Progenitor Cells. The injection procedure is detailed elsewhere (17). Six recipient PCD12.5 CD1 kidneys were incubated in two Transwell chambers (Falcon) for 1–2 h before transplantation. The 1:1 (red/blue) cell mixture was injected in one or two niches in each kidney. Injected kidneys were then cultured for 4 d. Twenty-two niches were injected in this study.

Immunostaining, Confocal Imaging, and Image Analysis. Detailed protocols and a list of the antibodies we used can be found in *Supporting Information*.

BUN Measurement. BUN measurement was carried out by the Cincinnati Veterinary Laboratory (Cincinnati, OH).

Niche and Progenitor Cell Count. Whole-mount and confocal microscopy and optical projection tomography (OPT) were carried out according to published protocols (43). Cell counts per niche (confocal microscopy) and niche counts (OPT) were performed as reported (43).

Statistical Analysis. Two-tailed unpaired *t* tests were performed for the analysis of kidney size, nephron counts, and BUN levels. The percentages of red or blue cells out of the total number of cells coinjected in multiple sites on different days were combined to derive the average percentage and SD. A two-tailed Student *t* test was used to calculate the *P* value. Error estimates of all pooled data were calculated as SEM. The percentage of single cells vs. groups was calculated separately for cells of each color in individual injected niches. The data are presented using the GraphPad Prism version 7.

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