Lin28 and *let-7* regulate the timing of cessation of murine nephrogenesis

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In humans and in mice the formation of nephrons during embryonic development reaches completion near the end of gestation, after which no new nephrons are formed. The final nephron complement can vary 10-fold, with reduced nephron number predisposing individuals to hypertension, renal, and cardiovascular diseases in later life. While the heterochronic genes *lin28* and *let-7* are well-established regulators of developmental timing in invertebrates, their role in mammalian organogenesis is not fully understood. Here we report that the Lin28b/*let-7* axis controls the duration of kidney development in mice. Suppression of *let-7* miRNAs, directly or via the transient overexpression of *LIN28B*, can prolong nephrogenesis and enhance kidney function potentially via upregulation of the *Igf2/H19* locus. In contrast, kidney-specific loss of *Lin28b* impairs renal development. Our study reveals mechanisms regulating persistence of nephrogenic mesenchyme and provides a rationale for therapies aimed at increasing nephron mass.
among its many functions, the mammalian kidney removes 
nitrogenous waste, regulates blood volume, and maintains 
bone density. Highly specialized epithelial tubules called 
nephrons serve as the basic functional units of the kidney. Kidney 
development, or nephrogenesis, is a complex process that 
requires reciprocal inductive interactions between two precursor 
tissues derived from the intermediate mesoderm (IM): the 
metanephric mesenchyme (MM) and the ureteric bud (UB). The 
UB gives rise to the branching epithelium of the collecting ducts 
while the MM gives rise to the cap mesenchyme (CM) as well as 
stromal populations\(^1\)–\(^5\). In mouse, the CM has been shown to 
represent a pool of multipotent nephron progenitors which self-
renew and give rise to mature nephrons via a mesenchymal-to-
epithelial transition (MET)\(^6\). Nephron formation continues 
within an outer nephrogenic zone of the kidney until postnatal 
day 2 in mice\(^8\), and the 36th week of gestation in humans\(^9\), after 
which time all remaining nephron progenitors undergo a syn-
chronous wave of differentiation to establish the final number of 
nephrons—the "nephron endowment"—that will persist lifelong in 
the adult. A human kidney contains anywhere from 200,000 to 
over 1.8 million nephrons\(^10\). Children who are born prematurely 
or suffer from intrauterine growth restriction (IUGR) as a 
result of maternal malnutrition have a reduced number of nephrons, 
which negatively affects the filtration function of the kidney. Because new 
nephrons do not form in the extra-uterine environment, children with a compromised nephron endowment are at increased risk of hypertension and development of cardio-
vascular and renal diseases, as well as insulin resistance and type 
2 diabetes as adults\(^11\)–\(^13\). Therefore, there has been interest in 
developing approaches to the treatment and prevention of kidney 
disease.

The RNA-binding protein Lin28 and the let-7 microRNA 
(miRNA) family were originally discovered in Caenorhabditis 
elegans as heterochronic genes regulating developmental 
timing\(^14,15\). In mammals, Lin28a and its paralog Lin28b are 
highly expressed in stem and progenitor cells, where they func-
tion to inhibit biogenesis of the let-7 family of miRNAs. As 
progenitor cells differentiate, Lin28 expression decreases, allowing 
formation of mature let-7 miRNAs\(^16,17\). Members of the let-7 
miRNA family, in turn, bind to the 3' UTR of Lin28 mRNA, 
negatively regulating its expression. Thus, Lin28 proteins and let-
7 miRNAs mutually suppress each other to form a bistable switch 
that is conserved throughout evolution from worms to mam-
mals\(^18\)–\(^20\). Lin28 proteins also bind mRNAs and modulate their 
translation independently of modulation of let-7\(^21\)–\(^23\). Aside from 
their role as developmental regulators, Lin28a/b and let-7 genes 
have been implicated in metabolism\(^24\), wound healing\(^27\), and 
ocrogenesis\(^28,29\). We have recently reported that prolonged 
expression of Lin28 in developing kidneys in mice markedly 
expands nephrogenic progenitors, blocks their final wave of dif-
ferentiation, and ultimately results in neoplastic transformation 
resembling the most common renal neoplasm of childhood, 
Wilms tumor, via misregulation of let-7 miRNAs\(^30\). Wilms tumor 
shares histological features with the developing kidney, and arises 
from inappropriately persisting MM, providing a window into the 
mechanisms of early renal development and into the properties of 
embryonic kidney stem cells\(^31\).

Given that Lin28 and let-7 genes were initially identified as 
heterochronic genes, we hypothesized that the Lin28/let-7 axis 
may control cessation of nephrogenesis and hence perturbation to 
these genes might prolong nephrogenesis and increase nephron 
endowment. In this study, we show an inverse temporal pattern of 
expression for Lin28a/b and let-7 transcripts during kidney 
development implicating Lin28b (and not Lin28a) as playing the 
predominant role in nephrogenesis. A single pulse of LIN28B 
overexpression in the Wt1-expressing MM during kidney 
development results in the formation of a postnatal ectopic 
nephron forming population leading to a substantial increase in 
organ volume, a two-fold increase in the final nephron number 
and a concomitant increase in the filtration function of the kid-
ney. Suppression of let-7 miRNAs during kidney development 
also prolongs nephrogenesis, in this instance restricted to the 
peripheral nephrogenic zone, and enhances kidney function. Conversely, kidney-specific loss of Lin28b impairs renal devel-
opment and function apparently via regulation of let-7 miRNAs. Both 
overexpression of LIN28B and knockout (KO) of let-7 miRNAs 
results in an upregulation of the Igf2/H19 locus that has previously been associated with the persistence of 
metanephric blastema in both Wilms tumor and persistent nephrogenic rests 
persistence of a nephrogenic mesenchyme\(^32\). These data provide a rationale for 
manipulating the Lin28/let-7 pathway to prolong nephrogenesis and suggest that this approach might enable a 
rescue of low nephron endowment and restoration of kidney 
function.

Results

Lin28/let-7 expression during kidney development. Our pre-
vious studies enforced overexpression of Lin28a or LIN28B from 
a variety of kidney specific promoters, including Foxa1, Six2 and 
Cdh16, with no tumor formation, however, overexpression of 
LIN28B driven via a Wt1 promoter surprisingly showed overt 
expansion of a Six2-expressing blastema, reminiscent of Wilms 
tumor\(^33\). This prompted us to explore the expression dynamics of 
Lin28 during normal nephrogenesis in the mouse embryonic 
kidney. Western blot analysis reveals that while both Lin28a and 
Lin28b proteins are expressed at high levels in midgestation, 
Lin28a expression decreases at embryonic day 13.5 (E13.5), whereas 
Lin28b expression is prolonged but exhibits a rapid decline after E16.5 (Fig. 1a), coinciding with the functional 
mature of the first nephrons in the developing kidney\(^31\). 
Quantitative reverse transcription polymerase chain reaction (qRT-PCR) shows that the amount of Lin28b mRNA is sig-
nificantly higher relative to Lin28a at all time points tested 
(Fig. 1b). Along with previous data showing the prevalence of 
LIN28B (but not LIN28A) activation in human Wilms tumor\(^30\), 
this strongly suggests that Lin28b plays the predominant role in 
normal kidney development.

To understand the role of let-7 miRNAs in the developing 
kidney, we analyzed mature and precursor let-7 miRNA levels in 
wild-type embryonic kidneys by qRT-PCR. All eight mature 
 precursor let-7 miRNAs were initially identi 
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other members of the let-7 miRNA family were originally discovered in Caenorhabditis elegans as heterochronic genes regulating developmental timing. In mammals, Lin28a and its paralog Lin28b are highly expressed in stem and progenitor cells, where they function to inhibit biogenesis of the let-7 family of miRNAs. As progenitor cells differentiate, Lin28 expression decreases, allowing formation of mature let-7 miRNAs. Members of the let-7 miRNA family, in turn, bind to the 3' UTR of Lin28 mRNA, negatively regulating its expression. Thus, Lin28 proteins and let-7 miRNAs mutually suppress each other to form a bistable switch that is conserved throughout evolution from worms to mammals. Lin28 proteins also bind mRNAs and modulate their translation independently of modulation of let-7. Aside from their role as developmental regulators, Lin28a/b and let-7 genes have been implicated in metabolism, wound healing, and oncogenesis. We have recently reported that prolonged expression of Lin28 in developing kidneys in mice markedly expands nephrogenic progenitors, blocks their final wave of differentiation, and ultimately results in neoplastic transformation resembling the most common renal neoplasm of childhood, Wilms tumor, via misregulation of let-7 miRNAs. Wilms tumor shares histological features with the developing kidney, and arises from inappropriately persisting MM, providing a window into the mechanisms of early renal development and into the properties of embryonic kidney stem cells. Given that Lin28 and let-7 genes were initially identified as heterochronic genes, we hypothesized that the Lin28/let-7 axis may control cessation of nephrogenesis and hence perturbation to these genes might prolong nephrogenesis and increase nephron endowment. In this study, we show an inverse temporal pattern of expression for Lin28a/b and let-7 transcripts during kidney development implicating Lin28b (and not Lin28a) as playing the predominant role in nephrogenesis. A single pulse of LIN28B overexpression in the Wt1-expressing MM during kidney development results in the formation of a postnatal ectopic nephron forming population leading to a substantial increase in organ volume, a two-fold increase in the final nephron number and a concomitant increase in the filtration function of the kidney. Suppression of let-7 miRNAs during kidney development also prolongs nephrogenesis, in this instance restricted to the peripheral nephrogenic zone, and enhances kidney function. Conversely, kidney-specific loss of Lin28b impairs renal development and function apparently via regulation of let-7 miRNAs. Both overexpression of LIN28B and knockout (KO) of let-7 miRNAs results in an upregulation of the Igf2/H19 locus that has previously been associated with the persistence of metanephric blastema in both Wilms tumor and persistent nephrogenic rests persistence of a nephrogenic mesenchyme. These data provide a rationale for manipulating the Lin28/let-7 pathway to prolong nephrogenesis and suggest that this approach might enable a rescue of low nephron endowment and restoration of kidney function.
during embryonic development sustains and expands MM and/or nephron progenitors and blocks the final wave of nephrogenesis, ultimately resulting in oncogenic transformation resembling Wilms tumor. Withdrawal of LIN28B expression reverted tumorigenesis and CM cells eventually underwent terminal differentiation. As lin-28 was initially identified as a heterochronic gene in C. elegans, we hypothesized that Lin28b controlled the timing of cessation of nephrogenesis in mammals. To test this hypothesis, we utilized our previously generated gain-of-function LIN28B mouse model (TRE-LIN28B;lox-STOP-lox-rtTA) with a Wt1-Cre driver, enabling spatial and temporal control of the human LIN28B protein specifically in early kidney progenitors (henceforth referred to as LIN28BWt1 mice) in this model system, LIN28B expression is controlled spatially by the expression of Cre in Wt1-expressing cells and temporally by administration of doxycycline (dox). Wt1 is expressed in the IM, the earliest precursor of the complete metanephric kidney. It is also expressed in the MM within the developing kidney, including both the cortical stroma and the CM, as well as the nephrons formed after induction of this mesenchyme, and ultimately in the podocytes of the glomeruli. Notably, it was only overexpression of LIN28B driven by this promoter that previously revealed that over time these ectopic Six2+ fields

To examine whether we could subtly prolong nephron formation, LIN28B expression was induced using a single 1-day pulse of dox at E16.5, the time point at which endogenous expression of Lin28b protein declines rapidly during kidney development. The induction led to persistence of LIN28B mRNA and protein levels out to postnatal day 5 (P5) in LIN28BWt1 kidneys (Fig. 2a), resulting in a consequent suppression of let-7 miRNAs during this period due to high-level overexpression of ectopic LIN28B (Fig. 2c; Supplementary Figure 2a). We have analyzed the expression of endogenous Lin28a and Lin28b mRNA in LIN28BWt1 kidneys and observe no change in transcript levels between LIN28BWt1 and littermate controls at all the time points tested (Supplementary Figure 2b). This single-day induction resulted in the formation of ectopic fields of nephrogenic mesenchyme through to P14, as demonstrated by the persistent expression of CM-specific transcription factors Six2 and Eya1 (Fig. 2d–f; Supplementary Figure 2c). Indeed, blastema was evident even at P21 ( Supplementary Figure 3 ) with such fields not only evident around the cortical nephrogenic zone but abutting collecting duct epithelium deep within the subcortical parenchyma. Immunohistochemistry staining for Lef1, a marker of the renal vesicles that represent the first epithelial derivatives of the CM, revealed that over time these ectopic Six2+ fields...
underwent a mesenchymal to epithelial transition and subsequently form mature nephrons throughout the renal parenchyma (Fig. 2g).

The phenotype of the \textit{LIN28BWt1} kidneys is unique. There is little clear histological difference with control kidneys at birth, however, within the first few days of postnatal life, there is clear evidence for an expansion and mispositioning of the remaining CM with areas of CM extending deep into the parenchyma (Supplementary Figure 3). A strict occlusion for the initial CM, it is likely that the enforced expression of \textit{LIN28B} under Wt1-Cre promoter is resulting in induction of this gene in mesenchymal populations other than the CM, resulting in the formation of ectopic CM domains surrounding and apparently initiating new lateral buds along the collecting ducts (Supplementary Figure 3). This provides evidence showing that the postnatal collecting duct can respond to an ectopic CM by initiating side branching. Ectopic mesenchyme is still evident as late as P21 although by P14 there is no remaining nephrogenic zone and significant cortical proximal tubular maturation (Supplementary Figure 3). While Six2 expression falls by P14, with initiation of nephron formation within ectopic CM regions, by P21, dilation of the collecting ducts of the papilla is evident, possibly due to increases in urinary filtrate production from the substantially enhanced nephron number (Supplementary Figure 3).

To discern whether transient ectopic \textit{LIN28B} expression could affect kidney development outside of its normal expression

**Fig. 2** \textit{LIN28B} overexpression in embryonic kidney enhances nephrogenesis. \textbf{a, c, d, f} Relative qRT-PCR analysis measuring levels of \textit{LIN28B}, mature \textit{let-7}, \textit{Six2}, and \textit{Eya1} RNAs, respectively, in \textit{LIN28BWt1} and littermate control kidneys at the indicated developmental time points. \textit{n} = 3 for each genotype. Error bars represent mean ± SD. (*) = \textit{p} < 0.05, unpaired, two-tailed Student’s \textit{t} test. \textbf{b, e, g} Representative immunohistochemistry staining against \textit{LIN28B}, \textit{Six2}, and \textit{Lef1} proteins, respectively, in \textit{LIN28BWt1} and littermate control kidneys at the indicated developmental time points. Scale bar, 200 µm.
window (after E16.5), we induced LIN28B at E17.5 and in newborns, time points at which expression of endogenous Lin28b is almost undetectable in the normal kidney (Fig. 1a). Six2 and Eya1 positive cells were not detected after postnatal day 2 in either the E17.5-induced or newborn-induced kidneys (Supplementary Figure 4). Collectively, these data suggest that Lin28b controls timing of kidney development, and that its expression must be precisely timed to ensure proper nephron mass. Transient over-expression of LIN28B can extend the period of nephrogenesis but cannot reactivate it after embryonic day 16.5.

**LIN28BWt1 mice exhibit enhanced renal function.** Next, we examined the effects of prolonged nephrogenesis in LIN28BWt1 mice (induced with dox at E16.5 for 1 day) on the function of the postnatal kidney. We first analyzed kidneys of these mice and found that in the period following LIN28B induction through P1 there was no significant difference between LIN28BWt1 and littermate control kidneys. However, with the progression of nephrogenesis, LIN28BWt1 kidneys gradually became dramatically and significantly larger, with a three-fold increase in weight at 2–3 months of age (Fig. 3a, b). Modest differences in the weights of other organs (heart and lungs) in 3-month-old LIN28BWt1 mice compared to controls did not reach statistical significance (Supplementary Figure 5a). The body weight of LIN28BWt1 animals was not affected for the first 2 weeks after birth. However, from P14 and until 1 month of age, LIN28BWt1 mice gained significantly less body weight than their littermate controls (Fig. 3c). However, once kidney development was completed in LIN28BWt1 mice, there was a 20% increase in body weight relative to controls at 2–3 months of age (Fig. 3c). These data indicate that LIN28B induction altered the developmental timing and ultimate organ size of the kidney due to prolonged nephrogenesis. To determine whether prolonged nephrogenesis in LIN28BWt1 mice altered nephron endowment, we calculated the nephron number by counting glomeruli in LIN28BWt1 kidneys compared to littermate controls. We found that LIN28BWt1 kidneys had a nearly two-fold increased number of glomeruli compared to 2-month-old controls, indicating increased nephron endowment in LIN28BWt1 animals (Fig. 3d, e). To examine whether the increased nephron number in LIN28BWt1 mice impacts kidney function, we measured glomerular filtration rate (GFR) and serum creatinine at 2 months of age. We found that the GFR was significantly higher in LIN28BWt1 mice, while serum creatinine levels were significantly lower compared to littermate controls, with no sex differences for either test (Fig. 3f, g; Supplementary Figure 5b, c). In addition, a renal function panel revealed that LIN28BWt1 kidneys exhibited normal levels of circulating electrolytes and proteins (Supplementary Figure 5d). These data indicate that LIN28BWt1 kidneys possess enhanced filtration and normal electrolyte handling. However, over a year old LIN28BWt1 mice showed substantive hydronephrosis with consequential loss of renal parenchyma and evidence of tubular casts (Supplementary Figure 5e). This suggested either a ureteropelvic obstruction arising from the formation of ectopic nephrons late in development or that the large volumes of urinary filtrate being produced by such an aberrantly large kidney ultimately caused a hydronephrotic pathology. Given the activity of the Wt1-Cre promoter in the forming nephrons, and particularly in the podocytes of the glomeruli, it is important to note an absence of apparent pathology in the glomeruli or tubular patterning and segmentation.

**Lin28b is required for the normal development of the kidney.** To test whether Lin28b is required for kidney development, we next generated Lin28b1/1; Wt1-Cre KO animals (hereafter referred to as Lin28b KO), in which cells expressing Wt1-Cre lose both endogenous Lin28b alleles (floxed alleles previously described33, Fig. 4a). Using optical projection tomography (OPT) for the CM-restricted transcription factor Six2, we visualized and quantified distinct CM fields or “niches” as well as the number of cells in each individual niche, in E18.5 whole kidney (Fig. 4b)36,37. A niche is defined as a spatially distinct cluster of CM cells and their adjacent epithelial ureteric tip. As such, niche counts also reflect the number of branchpoints of the ureteric tree36,37. While the number of cells in each individual niche was unaltered in kidneys of Lin28b KO animals relative to littermate controls, the niche count in these mice was significantly reduced, resulting in an overall decreased number of progenitor cells in the kidney (Fig. 4c, d). This reflects a decline in CM-driven ureteric branching. It has been previously established that depletion of the progenitor cell population within the MM results in premature cessation of nephrogenesis, small kidneys, low-nephron endowment, and reduced renal function38,39. Consistent with these studies, GFR tests revealed that Lin28b KO mice had significantly impaired kidney function relative to littermate controls (Fig. 4e), indicating that Lin28b activity is required for normal development of the mammalian kidney.

**Lin28b regulates nephrogenesis in a let-7 dependent manner.** We have shown that transient LIN28B overexpression during kidney development leads to suppression of mature let-7 species (Fig. 2c, Supplementary Figure 2a). Furthermore, we have previously shown that enforced expression of a Lin28-resistant let-7 (i7s) counteracted the effect of LIN28B overexpression by preventing the expansion of the CM (nephron progenitors) in LIN28B-i7s kidneys30. To understand this further, we tested whether let-7 miRNAs are functionally relevant using a genetic loss-of-function (LOF) approach. The mammalian genome encodes twelve let-7 family members expressed from eight distinct genetic loci40, which makes LOF studies of the let-7 miRNA family challenging. Nevertheless, we analyzed a mouse strain harboring viable constitutive combinatorial KO of select let-7 family members: let-7a1; let-7d; let-7f1 (hereafter referred to as let-7 KO) (Fig. 5a; Supplementary Figure 6a). We found that while the expression of endogenous Lin28a and Lin28b mRNAs did not change (Supplementary Figure 6b), let-7 KO kidneys exhibit increased transcript levels of Six2 and Eya1, similar to what was observed in LIN28BWt1 animals, indicating a persistence of the CM population relative to littermate controls (Fig. 5b–d). Furthermore, immunohistochemistry staining against Six2 and Lef1 revealed that progenitor cells were sustained in let-7 KO kidneys until postnatal days P3 and P5, respectively, indicating persistence of this progenitor population one to two days longer than in littermate controls (Fig. 5c, e). While this persistent expression of CM genes is in line with prolonged nephrogenesis, in contrast to the LIN28BWt1 animals, the location of this persistent zone of nephrogenesis is restricted to the periphery of the kidney. Hence, the effect here is subtler and did not result in long term pathology for animals older than 1 year of age (Supplementary Figure 6c). Although the number of progenitor cells per niche was unaltered in the let-7 KO kidneys relative to littermate controls, there was a significant increase in the niche count as shown by Six2 OPT (Fig. 5f–h). This indicates more branching and an overall increase in the number of progenitors in the whole kidney. Interestingly, unlike the LIN28BWt1 animals, no
significantly different was observed in body weight between let-7 KO and littermate controls (Supplementary Figure 7a) despite a significant (more than 50%) increase in kidney weight in let-7 KO animals from birth to adulthood (Fig. 5i) suggesting at least partial specificity of the Lin28/let-7 axis to kidney development. Finally, let-7 KO mice demonstrated a significant increase in GFR and nephron endowment (Fig. 5j, Supplementary Figure 7b), reduction in serum creatinine, and exhibited normal renal panel tests relative to littermate controls, similarly to LIN28BWT1 animals (Fig. 5k; Supplementary Figure 7a). Collectively, these data demonstrate that let-7 KO results in prolonged nephrogenesis and enhanced kidney function, partially phenocopying LIN28B overexpression, but without aberrant pathology noted in LIN28B mice due to the moderate (a day or 2) delay in the cessation. This suggests Lin28b regulates nephrogenesis via suppression of let-7 miRNA biogenesis and that proper timing of cessation of CM is essential for normal kidney function.

**Upregulation of the Igf2/H19 locus.** To identify potential let-7 targets we analyzed RNA sequencing (RNA-seq) data from P3 kidneys of LIN28BWT1 and let-7 KO animals. We discovered 42 genes with overlapping expression between the two mouse models (Fig. 5i, Supplementary Figure 8), among which were two...
Discussion

The ability to form anywhere from 10,000 to 15,000 nephrons per kidney in a mouse, or 200,000 to 1.8 million nephrons per kidney in the human relies upon the self-renewal and survival capacity of renal progenitor cells during prenatal development. By postnatal day 2 in mice and the 36th week of gestation in humans, nephrogenesis terminates with the exhaustion of all remaining CM. Although existing nephrons may be repaired in response to renal injury, new nephrons cannot be formed during adulthood. In mice and humans alike, IUGR correlates strongly with low-nephron endowment, which in turn, predisposes to hypertension, renal and cardiovascular diseases in the adult. Development of regenerative approaches is of particular importance to the treatment and prevention of renal disease. Several studies have attempted to recreate CM populations for in vitro nephron formation using either direct transcriptional reprogramming of somatic cells or directed differentiation of pluripotent stem cells toward a renal progenitor fate. However, the in vitro generation of CM is not likely to help in cases of low nephron endowment, as the anatomically complicated architecture of the kidney poses considerable challenges to the functional integration of a stem cell-derived nephron.

Here, we report prolonged nephrogenesis, increased nephron endowment and improved kidney function due to transiently prolonged expression in utero of Lin28b, a heterochronic gene that has been linked to pluripotency, stem cell self-renewal, tissue metabolism, and enhanced wound healing. We show that transient overexpression of LIN28B in a kidney-specific and temporally defined manner delays cessation of nephrogenesis. While this increased nephron endowment yields a substantially larger organ, there is a late onset of hydronephrosis evident in these mice. This may simply result from excessive filtrate formation or represent a pathology arising from a blastemal persistence affecting the ureteropelvic junction. This pathology was not evident in let-7 KO mice, possibly because the loss of let-7 genes only affects the population of cells normally expressing let-7, but does not result in aberrant overexpression of LIN28 in the wider Wt1-expressing populations that include both the CM and the cortical stroma. The discrepancy between let-7 KO and LIN28BWt1 mouse models could also arise from the fact that...
Lin28b regulates other gene expression events by binding to mRNAs and modulating their translation independently of let-7, as well as from the fact that only 3 out of 12 let-7 family members were suppressed during kidney development in the let-7 KO mouse model as opposed to all of them in the LIN28BWT1 animals. The observation of formation of a completely ectopic CM population within the parenchyma of the early postnatal kidney in the LIN28BWT1 overexpression model is intriguing. While this appeared to resolve into nephrons with time, the pathology presented as for nephroblastomatosis/persistent nephrogenic rests, a condition in which there is postnatal persistence of a "blastema" without obligate oncogenic
transformation. This suggests a capacity for LIN28B to convert renal mesenchyme to a CM phenotype. This may have implications in studies looking to recreate or prolong a nephrogenic population in vitro.

Both overexpression of LIN28B and KO of let-7 results in an upregulation of Igf2 and H19 genes at the transcriptional level. While the locus of H19/Igf2 has been shown to be co-regulated by cis-regulatory elements in a tissue-specific manner, recent studies have also indicated that the H19 antisense RNA transcript can also trans-activate the Igf2 promoter. We have demonstrated in our study the up-regulation of both spliced and unspliced Igf2 and H19 transcripts. This observation begs the question of whether this is a direct consequence of the ability of Lin28b or let-7 to bind to the H19 RNA antisense transcript knowing that both Lin28b and let-7 are capable of binding to Igf2 mRNA.

In this study, we show definitively that kidney specific loss of Lin28b results in significantly impaired kidney development, confirming the crucial role of this gene in proper developmental timing of nephrogenesis. We have linked the activity of Lin28b in kidney development to its ability to modulate the production of the let-7 family of miRNAs and shown that Lin28b/let-7 axis regulates the cessation of nephrogenesis possibly via upregulation of the growth-promoting gene Igf2. Modulating cessation of nephrogenesis by delaying it by a day or two (similar to our let-7 KO phenotype) would be an ultimate goal in such studies as it would enhance nephrogenesis and increase the nephron mass without pathological changes (Fig. 6). Indeed, restricting that overactivity to the remaining CM within the peripheral nephrogenic zone, rather than allowing aberrant formation of ectopic nephrons within a wider mesenchymal population, will also be critical to avoid concomitant pathology with such a strategy.

Methods

Animals. All animal work was done in accordance with IACUC guidelines at the ARCH facility in Children’s Hospital Boston. The generation and maintenance of Cella-TR-E-LIN28B and Lin28hflf animals was previously described briefly, flag-tagged human Lin28 open reading frame was cloned into pBS plasmid and target- ing was performed into V6.5 embryonic stem cells. The Wnt1-Cre mice were contributed by the laboratory of Dr. William Pu at the Boston Children’s hospital. For transgene induction, 1 g/1 ml doxycycline (Sigma) was administered to the drinking water at different time points to induce LIN28B. Weanling mice were genotyped via ear clippings processed by Transnetyx. Both males and females were examined throughout this study and no obvious differences were seen.

Quantitative RT-PCR. RNA was isolated by TRIzol from whole kidneys and reverse-transcribed using a mScriptII RT kit (Qagen, #218161). Relative mRNA expression was measured by qPCR using the ∆∆CT method with the following primers: mSix2 (forward primer, 5′-GGAGTGCAGACCTGTTTCA-3′; reverse primer, 5′-CTTCTCATCTGGGACTGCT-3′), mEya1 (forward primer, 5′-TTCCCTGGGACTGACTGTC-3′; reverse primer, 5′-GGAGGCTCTGTG- TCCAA-3′), mLin28a (forward primer, 5′-TACCCTCGTGGCTGATCAGC-3′; reverse primer, 5′-CAGAGGAGGATCTGCTGCCGC-3′); and Lin28b (forward primer, 5′-GGCCCTCTTATCCCTTGGACTGCT-3′; reverse primer, 5′-GACAGCCCTTGGAGG-3′); mLin28b (forward primer, 5′-TTTGGCTGAGGAGGTTAGACTGAC-3′; reverse primer, 5′-ATGGATCAGATGTTGACATGCTGGGA-3′); and Lin28a (forward primer, 5′-AGCATTGGCATCTCTGGGATGTTG-3′; reverse primer, 5′-AGGCGGTTAGGTTCACTTGAGA-3′). Absolute quantification PCR was performed by using DNA standards ordered from IDT for amplicons of mLin28a and Lin28b primers. For qRT-PCR analysis of mature and precursor let-7 miRNAs, we used Qiagen miScript target as described by the manufacturer.

Immunoblot analysis. Whole kidneys (from E12.5 to adulthood) were dissected and then lysed in RIPA buffer (Pierce) supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Roche). Lysates were loaded and run on the 12% polyacrylamide gel (Bio-Rad) in 5× Laemmli sample buffer and transferred to a nitrocellulose membrane (GE Healthcare). The membrane was blocked for 1 h in PBS containing 5% milk and subsequently probed with primary antibodies overnight at 4°C. After 1-h incubation with sheep anti-mouse or donkey anti-rabbit HRP-conjugated secondary antibody (GE Healthcare), the protein level was detected with standard ECL reagents (Thermo Scientific). Antibodies used: anti-a-tubulin (1:1000, Cell Signaling, #2148), anti-Lin28a (1:1000, Cell Signaling, #3978), anti-Lin28b (1:1000, mouse preferred) (Cell Signaling, #5422), anti-Six2 (1:1000, Proteintechn Group, #11562-1-AP). Uncropped scans included in Supplementary Figure 10.

Histological analysis. Whole kidneys were fixed in 10% formalin overnight at room temperature, then placed in 70% ethanol and embedded in paraffin. Slides were dewaxed with xylene and rehydrated through a series of washes with decreasing percentages of ethanol. Antigen retrieval was performed in 10 mM sodium citrate buffer (pH 6.0) by placement in decoaching chamber for 45 min at 95°C. Slides were treated with 10% hydrogen peroxide to inhibit endogenous peroxidase activity. After blocking with 5% goat or rabbit serum (VECTASTAIN ABC kit #PK-6101), slides were incubated with primary antibody overnight at 4°C and secondary antibody for 30 min at room temperature. Detection was performed with the VECTASTAIN Elite ABC Kit and DAB Substrate (Vector Laboratories, SK-4100). Sections were counterstained with hematoxylin for 20–30 s then dehydrated in increasing concentrations of ethanol before a 5-min incubation in xylene followed by mounting. Antibodies used: anti-Lin28b (1:250, Cell Signaling, #4196), anti-Six2 (1:250, Proteintechn Group, #11562-1-AP), anti-Lef1 (1:250, Cell Signaling, #2230).

Immunofluorescence and image analysis. Whole kidneys were fixed in 4% PFA for half an hour at 4°C then placed in PBS. Whole mount immunofluorescence, confocal microscopy, and OPT was carried out according to published protocols, briefly, after initial in vivo cell labeling with the nucleoside analog 5-ethyl-2′- deoxyuridine (EdU) and tissue-specific antibodies, OPT and confocal microscopy are used to image the developing kidney. These imaging data then inform a second analysis phase that quantifies (using Imaris and Tree Surveyor software), models and integrates these events at a cell and tissue level in 3D space and across developmental time. Cell counts per niche (confocal) and niche counts (OPT) were performed and reported. Antibodies used: rabbit anti-Six2 (1:1600, Proteintechn Group, #11562-1-AP), anti-rabbit Alexa Fluor-568 conjugated secondary antibody (1:300, Life Technologies).

Blood analysis. Panel renal tests performed on an Abaxis VetScan VS2 chemistry analyzer. Serum creatinine measurement used isotope dilution LC-MS/MS in the O’Brien Core Center for Acute Kidney Injury Research, the University of Alabama at Birmingham School of Medicine.

Gomerul number count. To compare the nephrin number between LIN28BKO and control mice we count the number of glomerulus-like structures in 12 random fields from the kidney cortex of H&E sections under 10x magnification.

Measurement glomerular filtration rate. GFR was measured using a high-throughput method described previously. Fluorescein isothiocyanate (FITC)-
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