Lin28 sustains early renal progenitors and induces Wilms tumor

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Wilms Tumor, the most common pediatric kidney cancer, evolves from the failure of terminal differentiation of the embryonic kidney. Here we show that overexpression of the heterochronic regulator *Lin28* during kidney development in mice markedly expands nephrogenic progenitors by blocking their final wave of differentiation, ultimately resulting in a pathology highly reminiscent of Wilms tumor. Using lineage-specific promoters to target *Lin28* to specific cell types, we observed Wilms tumor only when *Lin28* is aberrantly expressed in multiple derivatives of the intermediate mesoderm, implicating the cell of origin as a multipotential renal progenitor. We show that withdrawal of *Lin28* expression reverts tumorigenesis and markedly expands the numbers of glomerulus-like structures and that tumor formation is suppressed by enforced expression of *Let-7* microRNA. Finally, we demonstrate overexpression of the *LIN28B* paralog in a significant percentage of human Wilms tumor. Our data thus implicate the *Lin28/Let-7* pathway in kidney development and tumorigenesis.

[**Keywords:** kidney development; Wilms tumor; *Lin28*]

Supplemental material is available for this article.

Received December 24, 2013; revised version accepted March 25, 2014.

Wilms tumor, a pediatric kidney cancer affecting one in 10,000 children in North America, arises from the failure of embryonic nephrogenic cells to undergo terminal differentiation (Rivera and Haber 2005). The development of the kidney is a complex process that requires reciprocal inductive interactions between the ureteric bud (UB) and metanephric mesenchyme (MM), which leads to proliferation and expansion of the primitive cap mesenchyme (CM) (Grobstein 1955, 1956; Hatini et al. 1996; Davidson 2009). The CM cells differentiate into mature nephrons by a mesenchymal-to-epithelial transition (MET). As they also possess self-renewal capacity, CM cells represent embryonic kidney stem cells (Kobayashi et al. 2008; Pleniceanu et al. 2010). CM cells proliferate and differentiate in the outer nephrogenic zone of the kidney until the second postnatal day in mice (Hartman et al. 2007) and

the 36th week of gestation in humans (Hinchliffe et al. 1991), after which time all remaining CM cells synchronously differentiate to establish the final number of nephrons in the adult kidney (Hartman et al. 2007). Wilms tumor shares histological features with the developing kidney and is frequently associated with persistent areas of embryonic tissue known as nephrogenic rests, which contain blastemal cells with varying degrees of differentiation (Rivera and Haber 2005).

Lin28 is an RNA-binding protein that regulates gene expression via two different mechanisms: one that blocks the processing of the *Let-7* family of microRNAs (miRNAs) (Heo et al. 2008; Newman et al. 2008; Rybak et al. 2008; Viswanathan et al. 2008) and another that involves direct

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Article published online ahead of print. Article and publication date are online at <http://www.genesdev.org/cgi/doi/10.1101/gad.237149.113>.

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binding to a wide array of mRNA targets (for review, see Shyh-Chang and Daley 2013). The *Let-7*-dependent mechanism entails binding to the terminal loop of pri/pre-*Let-7* miRNAs, which prevents their maturation and thus enables the translation of genes that are suppressed by *Let-7* miRNAs (Viswanathan et al. 2008). Oncogenes such as *K-Ras* and *c-Myc* are prominent *Let-7* targets (Viswanathan and Daley 2010). In mammals, *Lin28A* and its closely related paralog, *Lin28B*, are highly expressed in pluripotent cells, where they play an important role in the maintenance of self-renewal and proliferation (Shyh-Chang and Daley 2013). Both *Lin28* proteins are highly expressed in early embryonic development but become down-regulated over time, while levels of mature *Let-7* family members rise as stem cells differentiate into specialized tissue types (Viswanathan and Daley 2010). Overexpression of *LIN28* is common in various tumor types and facilitates cellular transformation (Viswanathan et al. 2009). *Lin28* also promotes reprogramming of somatic cells into induced pluripotent cells (Yu et al. 2007).

Given that *Lin28* is highly active in embryonic tissues and was originally described as a heterochronic gene that regulates developmental timing in *Caenorhabditis elegans* (Ambros and Horvitz 1984; Moss et al. 1997), we hypothesized that *Lin28* overexpression might play a role in pediatric tumor formation by altering the timing of tissue differentiation and organogenesis during embryonic development. Indeed *LIN28A* overexpression has been implicated in type II germ cell tumors (Gillis et al. 2011), which result from a failure of differentiation of primordial germ cells (PGCs) (Oosterhuis and Looijenga 2005), while *LIN28B* has been linked to neuroblastoma (Diskin et al. 2012), a pediatric tumor derived from neural crest tissues that fail to complete their differentiation program (Maris 2010; Molenaar et al. 2012). Last, we reported previously that in rare cases of human Wilms tumor, *LIN28B* overexpression is caused by translocation at the *LIN28B* locus (Viswanathan et al. 2009). Here we describe a novel murine model of Wilms tumor caused by enforced overexpression of *Lin28* during embryonic kidney development and demonstrate by immunohistochemistry that *LIN28B* is overexpressed in up to 30% of cases of human Wilms tumor. These data, together with recent insights from whole-genome sequencing of Wilms tumor, implicate defects in miRNA regulation as a major mechanism of kidney tumorigenesis.

Results

Lin28 overexpression during embryonic kidney development leads to Wilms tumor

Previously, we and others have shown that *LIN28* plays an important role in germ cell development (West et al. 2009; Shinoda et al. 2013a) and is associated with human germ cell tumors (Gillis et al. 2011; Murray et al. 2013). Thus, we endeavored to overexpress *Lin28* in PGCs by crossing mice containing a *Lox-stop-Lox-Lin28a* cassette (LSL-*Lin28a*) (Supplemental Fig. S1A) with mice carrying a *Vasa-Cre* transgene, which we anticipated would express

Cre in PGCs when transmitted paternally, allowing us to test the potential for *Lin28* to induce germ cell tumors (Gallardo et al. 2007). Contrary to expectations, however, the cross between a LSL-*Lin28a* female and *VasaCre* male did not yield the predicted germ cell phenotype (zero out of 50) but unexpectedly produced renal tumors in 10% of the offspring (five out of 50; four bilateral and one unilateral) (Fig. 1A, top left). Tumors expressed the *Lin28a* transgene, apparently a consequence of aberrant “leaky” activation, whereas normal kidneys showed no transgene expression (Fig. 1B). Crosses of LSL-*Lin28a* males with females carrying the *Vasa-Cre* allele resulted in constitutional overexpression in all tissues by virtue of *Cre* expression in oocytes (Gallardo et al. 2007) and perinatal lethality. Interestingly, the kidneys of transgenic embryonic day 18.5 (E18.5) embryos were larger than the kidneys of their littermate controls and contained fewer mature proximal tubules (Supplemental Fig. S1B). When we harvested the kidneys from E18.5 transgenic and control embryos and transplanted them under the kidney capsule of immunodeficient mice, tumors developed in a high percentage of recipients (seven out of 10) (Fig. 1A, top right panel; Supplemental Fig. S1C). No tumors formed in transplant recipients of control kidneys (zero out of nine). Analysis of tumor gene expression (Fig. 1C) and histology (Fig. 1D) indicated that the *Lin28a*-derived tumors were highly similar to human Wilms tumor.

Previously, we reported two cases of human Wilms tumor in which *LIN28B* was overexpressed as a result of chromosomal translocation (Viswanathan et al. 2009). To determine whether human *LIN28B* overexpression would replicate Wilms tumor formation in mice, we engineered a transgenic strain that afforded spatial and temporal control of human *LIN28B* (or mouse *Lin28a*) overexpression by crossing the *Rosa26-Lox-stop-Lox-rtTA* allele with the *Col1A1-TRE-LIN28B* allele (*Lox-TetOn-LIN28B* mice) (Supplemental Fig. S1D; Zhu et al. 2010). To achieve global *LIN28B* overexpression in the developing and/or adult kidney, we crossed *Lox-TetOn-LIN28B* mice with *Wt1Cre* mice (Zhou et al. 2008), as *Wt1* is expressed in the intermediate mesoderm (Huff 2011), the origin of the metanephric kidney (Davidson 2009). All *Wt1Cre-LIN28B* mice (15 out of 15) developed kidney tumors (Fig. 1A, bottom panel) within the first 2 wk of life when exposed to doxycycline (Dox) induction during embryonic development (E0, E14.5, or even as late as E18.5) (see below). Importantly, the histology of the *LIN28B*-derived tumors was similar to *Lin28a*-derived tumors (Fig. 1D). Taken together, these results establish that overexpression of either murine *Lin28a* or human *LIN28B* during kidney development in transgenic strains of mice leads to kidney tumor formation that is highly reminiscent of human Wilms tumor (Fig. 1E).

Lin28 overexpression sustains the CM cells in the adult kidney

During kidney development, the nephronogenic progenitor cells of the CM cells differentiate into pretubular epithelial aggregates by a MET at around E12.5 (Rivera

Figure 1. *Lin28* overexpression in embryonic kidneys leads to Wilms tumor. (A) Kidney tumors as a result of *Lin28* overexpression in the kidney. (Top left panel) Renal tumor in a 17-wk-old kidney from the crossing between a LSL-*Lin28a* female and a *VasaCre* male. The smaller kidney is the normal kidney from the opposite side in the same mouse. (Top right panel) Tumor derived by transplantation of a *Lin28a*-overexpressing kidney (from the crossing between a LSL-*Lin28a* male and a *VasaCre* female) under the kidney capsule of an immunodeficient mouse 17 wk post-transplantation. (Bottom panel) *LIN28B*-derived tumors in 3-wk-old kidneys (Dox induction [1g/L] from E0). (B) Western blot analysis of tumors and age-matched normal kidneys with antibodies against *Lin28a*, Flag, and Tubulin. The expression of the Flag tag shows activation of the transgenic *Lin28a* in the tumor. (C) Gene set enrichment analysis (GSEA) of microarray data from *Lin28a*-derived tumors and control kidneys showing statistically significant up-regulation of "Wilms tumor signature genes" in the tumor compared with the control. (D) Typical histology of a human Wilms tumor, a *Lin28a*-derived tumor, and a *LIN28B*-derived tumor. The tumors are triphasic and contain structures of blastema (B), epithelium (E), and mesenchymal stromal (S) cells. Bar, 100 μ m. (E) Schematic representation of the UB and CM cells, which normally exist in the nephrogenic zone of the mouse kidney only until P2. Global *Lin28a* or *LIN28B* overexpression in the developing kidney leads to a pathology similar to human Wilms tumor. However, lineage-specific overexpression did not cause renal tumor formation (see below).

and Haber 2005). Normally, a balance between differentiation and proliferation of CM cells is sustained in the nephrogenic zone of the developing kidney until post-natal day 2 (P2), after which time all CM cells undergo a terminal wave of differentiation (Rumballe et al. 2011). In contrast to normal kidneys, the *Lin28*-derived tumors continue to sustain proliferating CM cells, as evidenced by the expression of CM-specific transcription factors (e.g., *Six2*, *Cited1*, and *Eya1*) and Ki67 staining (Fig. 2A,B; Supplemental Fig. S2A). Moreover, H&E (Fig. 2C) and immunofluorescence (Fig. 2D; Supplemental Fig. S2B,C) staining of the tumors demonstrates that the tumor consists of keratin8-positive UB cells surrounded by Six2-positive CM cells, similar to the structures that normally exist in the nephrogenic zone of the developing kidney. Taken together, these data indicate that *Lin28* overexpres-

sion prolongs the timing of kidney development, sustaining proliferation of the CM cells into adulthood. Interestingly, the CM cells of the tumor retain their differentiation capacity, as evident by gene expression for markers of epithelialization, such as *Wnt4* and *CDH6* (K-cadherin) (Fig. 3A; Supplemental Fig S3A); gross histology (Fig. 3B; Supplemental Fig S3B); and histologic staining for Lotus tetragonolobus lectin (LTL), a specific marker for mature proximal tubules (Fig. 3C). Importantly, the fact that the differentiated epithelial cells are Flag-positive (Supplemental Fig. S3C) indicates that these cells are indeed derived from the *Lin28*-overexpressing cells. Furthermore, the *Lin28*-derived tumors also contain structures resembling differentiated glomeruli (Supplemental Fig. S3D,E). Thus, the CM cells within the tumor retain a differentiation capacity that recapitulates normal kidney development.

Figure 2. *Lin28*-derived tumors harbor proliferating CM cells. (A) Overexpression of CM-specific transcription factors in the tumor. Microarray data (tumor, $n = 4$; control, $n = 4$). (B) *Six2* and Ki67 immunohistochemistry in normal kidneys and *LIN28a*-derived tumors. Bar, 100 μ m. (C) Histology of the E18.5 nephrogenic zone and adult tumors. Note that embryonic structures consisting of a branched UB surrounded by CM cells appears in the adult tumors. Bar, 50 μ m. (D) *Six2* and Keratin8 coimmunostaining in a normal embryonic kidney, a normal adult kidney, and an adult tumor. (Note: Keratin 8 is a marker for UB cells during kidney development and for the collecting duct in the adult kidney). In contrast to normal adult kidneys, in which *Six2* is not expressed and keratin8 expression is restricted to the collecting duct, the expression pattern of *Six2* and Keratin8 in the tumors is similar to the nephrogenic zone during kidney development. Bar, 100 μ m.

Lin28 prevents the postnatal wave of differentiation of the CM cells

We documented that *Lin28* overexpression leads to persistent proliferation of CM cells in adult mice. During normal kidney development, *Lin28a* is expressed in CM cells until E13.5, after which expression wanes, while *Lin28b* is not expressed at all (Fig. 4A; Supplemental Fig. S4A). Interestingly, however, the nephrogenic zone of E18.5 transgenic embryos appears normal, without aberrant expansion of the CM cells (Fig. 4B; Supplemental Fig. S4B), suggesting normal proliferation of the CM cells in the nephrogenic zone during embryonic development in the presence of *Lin28*. Importantly, induction of *Lin28* overexpression as late as E18.5 was enough to sustain proliferation of CM cells into adulthood (Supplemental Fig. S4C). To discern whether ectopic *Lin28* expression could reactivate proliferation of nephrogenic cells after the early postnatal period of terminal differentiation, we induced *LIN28B* overexpression in renal tissues by virtue of gene activation via *Wt1*Cre at P10, when no CM cells exist in the normal kidney. When *LIN28B* was

overexpressed at P10, tumors failed to develop, and expansion of *Six2*-positive cells was not detected (Supplemental Fig. S4D). Instead, late *LIN28B* induction resulted in a cystic kidney phenotype (Supplemental Fig. S4E). Collectively, these data suggest that the CM cells retain their differentiation capacity in the presence of *Lin28* expression but that *Lin28* delays the timing of the final postnatal wave of synchronous differentiation, allowing the nephrogenic process to persist, eventually producing Wilms tumor.

Wilms tumor arises when Lin28 is overexpressed in multiple early kidney lineages

We then sought to determine in which cells of the developing kidney overexpression of *Lin28* was required to promote Wilms tumor formation. We overexpressed *LIN28B* or *Lin28a* specifically in CM cells by crossing *Lox-tetOn-LIN28B* and *Lox-TetOn-Lin28a* transgenic mice with *Six2*-Cre mice (Kobayashi et al. 2008). Contrary to expectation, overexpression of *Lin28a* or *LIN28B* in the CM cells failed to induce tumor forma-

Figure 3. Differentiation capacity of the CM cells in the tumor. (A) Overexpression of epithelialization markers Wnt4 and Cdh16 in the tumor. Microarray data (tumor, $n = 4$; normal kidney, $n = 4$). (B) Epithelial structures in a normal embryonic kidney and a *Lin28*-derived tumor. (E) Epithelial structures of comma-shaped/S-shaped bodies differentiated from the CM cells. Bar, 20 μm . (C) Mixture of progenitor cells (Six2-positive) and mature proximal tubule cells (LTL-positive) in the tumor. Bar, 100 μm .

tion (zero out of 15) but instead produced a cystic kidney phenotype. The cystic phenotype appeared in the *Six2*-Cre-*Lin28* mice when *Lin28* overexpression was induced early in embryonic development (Supplemental Figs. S5A,B) or in adult mice (Supplemental Fig. S5C). We crossed *Lox-TetOn-Lin28a/LIN28B* mice with mice carrying *FoxD1Cre* ($n = 10$) to effect stromal cell-specific expression (Humphreys et al. 2010) and with mice carrying *Cdh16Cre* ($n = 7$) to effect UB cell-specific expression (Shao et al. 2002), but neither of these crossings reproduced the tumor phenotype. Interestingly, however, overexpression of *LIN28B* in stromal cells (*FoxD1Cre*) led to hydronephrosis in the adult kidney (Supplemental Fig. S5D), while no

pathology was seen in the *Cdh16Cre* mice. Therefore, in the murine model, *Lin28* activation is required in the earliest renal progenitor that gives rise to the multiple cell types of the developing kidney, implying that *Lin28* promotes a coordinated prolongation of nephrogenesis, which ultimately progresses to Wilms tumor formation (see Fig. 1E).

Lin28 expression is required for the maintenance of CM cells within the tumor

Our data firmly establish a role for *Lin28* overexpression in tumor initiation, but to determine whether continued

Figure 4. *Lin28* overexpression prevents the post-natal wave of differentiation of the CM cells. (A) Analysis of *Lin28a* expression during mouse embryonic kidney development. *Lin28a* is expressed in the CM (Six2-positive) cells of the developing kidney until E13.5. All panels are at the same scale. (B, top panel) *Six2* expression in E18.5 normal and transgenic kidneys. (Bottom panels) *Lin28a* expression in normal and transgenic kidneys. Note that there is no expansion in CM cells of the transgenic kidney compared with the control. Bar, 100 μm .

Lin28 expression is necessary for tumor maintenance, we studied the effect of *Lin28* down-regulation by withdrawal of Dox commencing at P7. While significant numbers of *Six2/Eya1*-positive CM cells persist 2 wk after Dox withdrawal (Fig. 5A; Supplemental Fig S6), at 3 wk after Dox withdrawal, there was a signifi-

cant decrease in CM cells in the kidneys of the transgenic mice, and not overt tumors, but a markedly increased number of well differentiated glomerulus-like structures (Fig. 5B,C). Thus, we conclude that expression of *Lin28* is sufficient for tumor initiation and necessary for tumor maintenance.

Figure 5. *Lin28* down-regulation leads to differentiation of the CM cells in the tumor. (A) qRT-PCR analysis of *LIN28B*, *Six2*, and *Eya1* (markers form CM cells) in transgenic and control kidneys 1 and 2 wk after Dox withdrawal. (2W) 2-wk-old mice (1 wk without Dox); (3W) 3-wk-old mice (2 wk without Dox). Note that *Six2* and *Eya1* are still expressed 2 wk after Dox is withdrawn. (B) Average glomerulus number per 10× magnification field in the microscope in transgenic and control kidneys 3 wk after Dox withdrawal ($n = 12$). (C) Representative H&E staining of transgenic kidneys 3 wk after Dox withdrawal (transgene 3 wk w/o Dox) compared with a control 4-wk-old kidney and transgenic 1-wk-old and 4-wk-old kidneys maintained on Dox (transgene with Dox). Dox was induced from E0. Note that 3 wk after Dox withdrawal, the transgenic kidneys contain many glomerulus-like structures and do not contain CM cells. Yellow arrows point to glomerulus-like structures. Bar, 100 μm.

Lin28-induced Wilms tumor is suppressed by enforced expression of Let-7

Analysis of miRNA expression in *Lin28*-induced tumors demonstrated significant suppression of mature *Let-7* species (Supplemental Fig. S7A) but no significant change in steady-state levels of pri/pre-*Let-7* (Supplemental Fig. S7B). In accordance with this, *Let-7* target genes were up-regulated in the tumors compared with control kidneys (Supplemental Fig. S7C). To determine whether *Lin28*-induced tumorigenesis could be suppressed by enforced restoration of *Let-7*, we crossed the tumor-prone *LIN28B*

transgenic mice with a strain that expresses a chimeric *Let-7g* (i7s) species whose processing is not inhibited by *Lin28* (Piskounova et al. 2008). We verified by immunohistochemistry staining that the transgenic kidneys overexpressed LIN28B (Supplemental Fig. S7D), that endogenous *Let-7* was down-regulated when the chimeric *Let-7g* transgene was overexpressed in the *LIN28B-i7s* kidneys (Fig. 6A), and that *Let-7* targets were down-regulated in the *Lin28B;i7s* kidneys compared with *Lin28B* kidneys (Supplemental Fig. S7E). We showed previously that the “i7s” mice are smaller than their littermate controls (Zhu

Figure 6. *Lin28* acts through the *Let-7* pathway to prevent normal kidney development. (A) qRT-PCR analysis of mature *Let-7* levels in *LIN28B*, *LIN28B;i7s*, and control kidneys. Dox was provided from E14.5 until the end of the experiment. (B) Morphology of *LIN28B*, *LIN28B;i7s*, and control kidneys. (C) qRT-PCR analysis of the CM markers *Six2* and *Eya1* in *LIN28B*, *LIN28B;i7s*, and control kidneys. (D) H&E staining of *LIN28B*, *LIN28B;i7s*, and control kidneys. Note that the *Lin28B;i7s* kidneys have a normal histology. Bar, 100 μ m.

et al. 2011). Accordingly, kidneys of compound *LIN28B-i7s* mice appeared smaller than normal kidneys (Fig. 6B). Importantly, however, there were no evidence of persistent CM cells in the *LIN28B-i7s* kidneys, as demonstrated by quantitative RT-PCR (qRT-PCR) (Fig. 6C) and histology (Fig. 6D). These results demonstrate that enforced *Let-7* expression can counteract the effect of *LIN28B* overexpression, suggesting that *LIN28B* induces Wilms tumor at least in part by suppressing *Let-7* miRNAs.

LIN28B expression in human Wilms tumor

Cancer-initiating cells (CICs) have recently been isolated from human Wilms tumor (Pode-Shakked et al. 2013). We detected overexpression of *LIN28B* (but not *LIN28A*) in NCAM1⁺ALDH1⁺ wild-type CICs isolated from early-generation Wilms tumor xenografts (harboring a blastemal predominant phenotype) and to a lesser extent in primary Wilms tumor when compared with developing human kidneys (Supplemental Fig. S8A). To determine whether *LIN28* expression is a prominent feature of human Wilms tumor, we analyzed the expression of *LIN28A* and *LIN28B* in human Wilms tumor samples from Boston Children's Hospital (USA, $n = 28$) and an independent set from Great Ormond Street Hospital/University College London Institute of Child Health (UK, $n = 77$). Indeed, immunohistochemical staining of these samples revealed overexpression of *LIN28B* (Fig. 7A) in eight out of 28 and 10 out of 77 samples, respectively, compared with normal kidneys. Conversely, *LIN28A* expression was not detected in

any sample. This observation was further supported by our analysis of published microarray data that indicated frequent expression of *LIN28B* but not *LIN28A* in human Wilms tumor (Fig. 7B; Supplemental Fig. S8B).

In our tissue microarray analyses (UK samples), we noted that *LIN28B* expression was restricted to blastemal cells, which are the most undifferentiated tumor component. Clinical outcome data were available for 76 out of 77 UK patients who had been uniformly treated with prenephrectomy chemotherapy. Among the nine *LIN28B*-positive tumors, five patients relapsed, and three died. Among the 67 *LIN28B*-negative tumors, eight patients relapsed, and two died, thus suggesting a significant association of *LIN28B* expression with relapse and death ($P = 0.0059$ and $P = 0.0105$, respectively, two-tailed Fisher's test). Detailed histological and statistical analysis of the *LIN28B*-positive and *LIN28B*-negative tumors can be found in Supplemental Tables 1 and 2. Taken together, these data indicate that expression of *LIN28B* is a feature of a significant minority of cases of human Wilms tumor, which, together with our prior report of activation of *LIN28B* by chromosomal translocation in two cases, implicates *LIN28* in the pathogenesis of human Wilms tumor.

Discussion

It has been argued that pediatric cancer can arise from the failure of embryonic cells to complete their differentiation program (Maris and Denny 2002; Messahel et al. 2005), indicating that the pathogenesis of pediatric tumors is

Figure 7. *LIN28B* expression in human Wilms tumor. (A) *LIN28B* immunohistochemistry in human Wilms tumor. (B) *LIN28B* and *LIN28A* expression levels in diverse types of human renal tumors based on published microarray data (GSE11151).

directly linked to dysregulated embryonic development and organogenesis (Scotting et al. 2005). Here we demonstrate that overexpression of *Lin28*, an RNA-binding protein linked to pluripotency, stem cell self-renewal, and delayed larval development in *C. elegans*, prolongs kidney development and promotes Wilms tumor formation. Reminiscent of its association with heterochronic phenotypes in *C. elegans*, *Lin28* overexpression prevents the synchronous wave of differentiation of CM cells in the developing kidney, which, under normal conditions, is complete by P2 in mice (Hartman et al. 2007) and by 36 wk of gestation in humans (Hinchliffe et al. 1991). In transgenic mice engineered for kidney-specific *Lin28* overexpression, CM cells continue to proliferate into adulthood, resulting in conversion to a tumor highly reminiscent of human Wilms tumor. When activation of *Lin28* was targeted to specific cellular compartments such as UB, stroma, or CM cells, we failed to observe tumor formation; instead, in our model, Cre excision in the intermediate mesoderm, the origin of the entire kidney, was required to induce tumor formation. Theoretically, this observation suggests that *Lin28* activation is required in either a stage-specific manner in the earliest kidney progenitor cells or more than one kidney cell lineage. The fact that tumors formed when *Lin28* overexpression was induced as late as E18.5, when the earliest progenitors no longer exist, suggests that the second explanation is more likely. Our model implies that aberrant *Lin28* expression produces a coordinated expansion of the nephrogenic zone, resulting in proliferating blastema and nephrogenic rests, which are characteristic of human Wilms tumor.

Previously, a murine model of Wilms tumor was generated by *Wt1* ablation and *Igf2* overexpression (Hu et al. 2011), which established that *Wt1* ablation prevents the MET of CM cells that is essential for nephrogenesis. In contrast, in our model, the CM cells persist beyond the period when synchronous differentiation typically occurs and retain their capacity to undergo MET, resulting in a markedly expanded period of nephrogenesis that ultimately progresses to frank tumorigenesis. The differences between these two models demonstrate that pediatric Wilms tumor formation can occur at diverse stages of development and through different molecular mechanisms.

Lin28 has profound effects on both the proliferative and metabolic machinery of tumor cells. By virtue of blocking *Let-7* biogenesis, *Lin28* leads to derepression of known oncogenic targets of *Let-7* such as *Myc* (Sampson et al. 2007), *Ras* (Johnson et al. 2005), *Hmga2* (Lee and Dutta 2007; Mayr et al. 2007), and cyclins (Legesse-Miller et al. 2009; Chang et al. 2012). Moreover, *Lin28* has been shown to promote glycolytic metabolism in tissues and cancer cells and thus is a central regulator of cellular bioenergetics (Shyh-Chang et al. 2013). It appears that *Lin28* functions to balance the proliferative and metabolic needs of rapidly growing cells in the early embryo (Shinoda et al. 2013b) and that this function becomes reactivated in many adult tumors. In cases of pediatric malignancy, *Lin28* appears to prolong the embryonic patterns of tissue growth (as we showed here for Wilms tumor), which is likely the case for germ cell tumors and neuroblastoma.

Point mutations in *WT1*, *WTX*, β -catenin, and abnormalities involving translocations in chromosome 6 have been linked to Wilms tumor (Knudson and Strong 1972; Rivera and Haber 2005; Hu et al. 2011), and several susceptibility loci for Wilms tumor were recently identified by a genome-wide association study (Turnbull et al. 2012). However, the underlying genetic basis of most cases of Wilms tumor remains unknown (Turnbull et al. 2012). Here we report that up to a third of human Wilms tumors overexpress *LIN28B*. Moreover, our data suggest an association of *LIN28B* expression with a “high-risk” subtype of Wilms tumor called “blastemal type” (Vujanec et al. 2002) that is defined by the persistence of a large proportion of blastemal cells in the viable tumor component after pre-nephrectomy chemotherapy. Therefore, *LIN28B* expression may be a marker of such therapy-resistant blastemal cells, which currently cannot be identified in chemo-naïve tumors treated according to the North American approach of immediate nephrectomy, but a much larger unselected cohort of tumors would be required to investigate this properly. Prior studies have implicated aberrant expression of the *HACE1* locus on chromosome 6 in Wilms tumor (Slade et al. 2010), but *LIN28B* is tightly linked to this locus, and our detection of aberrant overexpression of *LIN28B* in a significant minority of human Wilms tumors suggests the possibility of coordinate dysregulation. Previously, we reported that rare cases of Wilms tumor result from activation of *LIN28B* by chromosomal translocation and that amplification of the *LIN28B* locus occurs in only ~2% of tumors (Viswanathan et al. 2009); thus, the mechanism of *LIN28B* activation remains unexplained in most cases. Based on our demonstration that both murine *Lin28a* and human *LIN28B* are competent to induce Wilms tumor in our mouse model, we speculate that the prevalence of *LIN28B* activation and the absence of aberrant *LIN28A* expression in human Wilms tumor is likely due to a specific mechanism of *LIN28B* up-regulation and not differences in the transforming potential of the genes. In support of this hypothesis, it has been shown recently (Aiden et al. 2010) that the promoter of *LIN28A* in human Wilms tumors is enriched with histone K27me3.

The miRNA pathway is a common target for dysregulation in different types of tumors (Merritt et al. 2008; Bahubeshi et al. 2011). Recently, it was demonstrated that *DIS3L2*, the gene responsible for Perlman syndrome, which entails a predisposition to Wilms tumor, is a nuclease responsible for degrading *Let-7* miRNAs that have become polyuridylated due to *Lin28*-mediated recruitment of a terminal uridylyl transferase (Chang et al. 2013). Taken together with the observation of *LIN28B* overexpression in up to 30% of human Wilms tumors and our demonstration that enforced expression of *Let-7* abrogates *Lin28*-induced kidney tumorigenesis, these data suggest that a common mechanism of Wilms tumor pathophysiology is dysregulation of *LET-7* miRNA biogenesis or function. This hypothesis would be reinforced if indeed *Drosha* and *Dicer1* mutations or other mutation in the miRNA processing machinery are likewise found in Wilms tumor. The *LIN28/LET-7* pathway represents an appealing therapeutic target

for Wilms tumor through either inhibition of *LIN28* function or delivery of *LET-7* to tumor cells.

Materials and methods

Mice

All animal procedures were conducted according to animal care guidelines approved by the Institutional Animal Care and Use Committee at Boston Children's Hospital.

LSL-*Lin28a* mice

A Lox-stop (four PGK-polyA and three sv40 polyA) Lox cassette and a Flag-tagged murine *Lin28a* ORF were cloned into pEF6 plasmid (Invitrogen, catalog no. V962-20) downstream from a PEF-1 α promoter, and targeting was performed into V6.5 embryonic stem cells. Chimeric mice were generated by injection of embryonic stem cells into BALB/c blastocysts and then bred to CD-1 females to generate germline-transmitted pups.

Lox-TetOn-*Lin28* mice were previously generated in our laboratory as described in Zhu et al. (2010, 2011).

The following Cre mice were obtained from Jackson laboratory: *Vasa*Cre (stock no. 006954), *Six2*Cre (stock no. 009606), *FoxD1*Cre (stock no. 012463), and *Cdh16*Cre (stock no. 012237). The *Wt1*Cre mice were contributed by the laboratory of Dr. William Pu at Boston Children's Hospital. Cre mice were crossed to the Lox-TetOn-*Lin28* mice, and 1 mg/mL Dox was administered to the drinking water at different time points to induce *LIN28B/Lin28a* expression. To achieve co-overexpression of *Lin28* and *Let7*, we crossed *Wt1*Cre mice with TRE-7S21L ("i7s" mice) mice and then crossed the *Wt1*Cre;i7s mice with Lox-TetOn-*Lin28* mice.

For the transplantation experiments, embryos were harvested by cesarean section at E18.5. Kidneys were harvested from transgenic and control embryos, dissected to smaller pieces, and then transplanted under the kidney capsules of NSG mice.

qRT-PCR

RNA was isolated by TRIzol and reverse-transcribed using SuperScript III (Invitrogen, catalog no. 18080-051) or miScript II RT kit (Qiagen, catalog no. 218161). mRNA expression was measured by qPCR using the $\Delta\Delta C_T$ method with the following primers: *mLin28a*: *mSix2* (forward primer, GCAAGTCAGCA ACTGGTTC; reverse primer, CTTCTCATCCTCGGAATC GC), *mEya1* (forward primer, TTTCCCTGGGACTACGAATG; reverse primer, GGAAAGCCATCTGTTCCAAA), *mGapdh* (forward primer, GCAGTGGCAAAGTGGAGATTG; reverse primer, AATTTGCCGTGAGTGAGTCATC), and *mbActin* (forward primer, TACTCCTGCTTGCTGATCCAC; reverse primer, CA GAAGGAGATTACTGCTCTGGCT); and *hLIN28B* (forward primer, GCCCCTTGGATATTCAGTC; reverse primer, TGA CT CAAGGCCTTTGGAAG). For qRT-PCR of mature *Let7* miRNA and pre/pri-*Let7*, we used Qiagen miScript target as described in the miScript protocol.

Microarray

RNA from four *Lin28a*-derived Wilms tumor samples and four control kidneys was harvested and processed using TRIzol. The Illumina Ref-8 microarray platform was used by the Boston Children's Hospital Intellectual and Developmental Disabilities Research Center (IDDRC) Molecular Genetics Core Facility. The microarray data have been deposited in Gene Expression Omnibus

(GEO) and given the series accession number GSE56323. Gene set enrichment analysis (GSEA) was used to identify gene sets and pathways associated with a set of up-regulated or down-regulated genes. Published microarray data from GEO (GSE3822, GSE6890, and GSE12588) were used for the analysis of *Lin28a* and *Lin28b* during mouse kidney development. Data from Oncomine (<https://www.oncomine.org>) were used for the analysis of *LIN28A* and *LIN28B* expression in human Wilms tumor samples.

Histological analysis

Tissue samples were fixed in 10% buffered formalin and embedded in paraffin. Immunostaining was performed using the following antibodies: *LIN28A* (1:250; Cell Signaling, catalog no. 8641S), *LIN28B* (1:250; Cell Signaling, catalog no. 4196S), *SIX2* (1:400; Proteintech Group, catalog no. 11562-1-AP), *LTL* (1:500; Vector Laboratories, catalog no. FL-1321), and *Keratin 8* (1:50; Developmental Studies Hybridoma Bank [DSHB], catalog no. TROMA-I-s [TROMA-I-s]).

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 a decloaking chamber for 30 min on high temperature.

Immunohistochemistry was performed with Elite ABC kit and DAB substrate (Vector Laboratories) according to the manufacturer's protocol. For immunofluorescence, Alexa 488- or Alexa568-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies were used. *Lin28B* immunohistochemistry was scored as positive versus absent staining. In all cases, positive staining was seen in groups of blastemal cells.

To compare the nephron number between transgenic mice and controls, we count the number of glomerulus-like structures in 12 random fields (from the kidney cortex) under 10 $\times$ magnification.

Statistical analysis

Data are presented as mean $\pm$ SEM, and Student's *t*-test (two-tailed distribution, two-sample unequal variance) was used to calculate *P*-values. Statistical significance is displayed as *P* < 0.05 (*) or *P* < 0.01 (**) unless specified otherwise. The tests were performed using Microsoft Excel, with the test type always set to two-sample equal variance.

Acknowledgments

We thank all of the clinicians at Boston Children's Hospital and the UK Children's Cancer and Leukaemia Group (CCLG) Centres who managed the care of the children entered into clinical and biology studies. We especially thank Dr. Richard Williams and Mr. Tasnim Chagtai (University College London [UCL] Institute of Child Health) for access to clinical and microarray expression data, and Dr. Sergey Popov (Institute of Cancer Research, University of London) and Professor Gordan Vujanic (University of Wales Medical School, Cardiff University) for assistance with construction of TMAs from patients treated in the SIOP WT 2001 trial in the UK. We thank the CCLG Tissue Bank for access to samples. G.Q.D. is an investigator of the Howard Hughes Medical Institute and the Manton Center for Orphan Disease Research and an affiliate member of the Broad Institute. This work was funded by the Ellison Medical Foundation and private funds of the Children's Hospital. K.P.-J. receives support from Cancer Research UK, Great Ormond Street Children's Charity, Children with Cancer Charity, and the National Institute for Health Research, Great Ormond Street Hospital, UCL Biomedical Research Centre award. The CCLG Tissue Bank is funded by Cancer Research UK and CCLG.

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Genes Dev. 2014, **28**: originally published online April 14, 2014
Access the most recent version at doi:[10.1101/gad.237149.113](https://doi.org/10.1101/gad.237149.113)

Supplemental Material <http://genesdev.cshlp.org/content/suppl/2014/04/07/gad.237149.113.DC1>

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