Dicer1 activity in the stromal compartment regulates nephron differentiation and vascular patterning during mammalian kidney organogenesis

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MicroRNAs, activated by the enzyme Dicer1, control post-transcriptional gene expression. *Dicer1* has important roles in the epithelium during nephrogenesis, but its function in stromal cells during kidney development is unknown. To study this, we inactivated *Dicer1* in renal stromal cells. This resulted in hypoplastic kidneys, abnormal differentiation of the nephron tubule and vasculature, and perinatal mortality. In mutant kidneys, genes involved in stromal cell migration and activation were suppressed as were those involved in epithelial and endothelial differentiation and maturation. Consistently, polarity of the proximal tubule was incorrect, distal tubule differentiation was diminished, and elongation of Henle's loop attenuated resulting in lack of inner medulla and papilla in stroma-specific *Dicer1* mutants. Glomerular maturation and capillary loop formation were abnormal, whereas peritubular capillaries, with enhanced branching and increased diameter, formed later. In *Dicer1*-null renal stromal cells, expression of factors associated with migration, proliferation, and morphogenic functions including α-smooth muscle actin, integrin-α8, -β1, and the WNT pathway transcriptional regulator LEF1 were reduced. *Dicer1* mutation in stroma led to loss of expression of distinct microRNAs. Of these, miR-214, -199a-5p, and -199a-3p regulate stromal cell functions *ex vivo*, including WNT pathway activation, migration, and proliferation. Thus, *Dicer1* activity in the renal stromal compartment regulates critical stromal cell functions that, in turn, regulate differentiation of the nephron and vasculature during nephrogenesis.

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Increasing evidence suggests that the renal stroma has critical, instructive roles through tissue interactions during kidney development.[1] Recent cell ablation studies of the renal stroma have provided evidence that it has a role in regulating nephron progenitor cells and vasculature.[2,3] Inactivation of the transcription factor FOXD1 in the cortical stroma results in expansion of nephron progenitor cells and a severe deficit in differentiation.[1,4,5] Inactivation of β-catenin in stromal tissues of the developing kidney leads to loss of elongation of the loop of Henle of the differentiating nephron tubule,[6] and recent studies have also identified Notch signaling in the renal stroma as a regulator of vascular patterning.[7,8]

MicroRNAs (miRNAs) are a family of more than 2000 small noncoding RNAs that function as post-transcriptional regulators and are increasingly recognized as important regulators of gene expression.[9,10] miRNAs are synthesized in the nucleus, processed by the RNase III enzyme Drosha, exported to the cytoplasm, and cleaved for subsequent activation by the RNase III known as Dicer1.[11,12] Therefore, *Dicer1* inactivation results in complete inactivation of miRNA function. Activated miRNAs are loaded into a complex including the Argonaute protein, which enables the miRNA to bind by sequence complementarity to mRNA.[9,13] A single miRNA can bind to 50–100 functionally related mRNA. This binding leads to gene silencing through miRNA-mediated degradation as well as translational suppression by disruption of the ribosomal complex.[9,12,13] Therefore, miRNA activity may regulate sets of genes for specific biological processes during development, metabolism, and homeostasis. Recent studies have identified important roles for post-transcriptional regulators including miRNAs in podocytes,[14,15] juxtaglomerular cells,[16] nephron epithelium and collecting duct system of the developing kidney,[17,18] and in epithelial and stromal cells during adult kidney diseases.[10,19,20] However, the importance of miRNAs in stromal cells has not been explored during kidney development.

Renal stromal cells derive from the cortical stroma overlying the cap mesenchyme.[6,21] This layer of mesenchymal

cells in the zone of nephrogenesis expresses the transcription factor FOXD1. These *Foxd1+* progenitor cells give rise to all the stroma of the developing kidney. Renal stromal cells become vascular smooth muscle cells (VSMCs), glomerular mesangial cells, pericytes, and fibroblasts of the mature kidney.[21] As described above, mice lacking *Foxd1* show severe defects in kidney organogenesis, including markedly reduced kidney volume, longitudinal fusion, ventral rotation, smaller collecting system, and a marked decrease in the number of nephrons. The defects are so severe that it is difficult to understand, from studying these mutants, the functional role of *Foxd1+* mesenchymal progenitors and the stroma they give rise to in nephrogenesis.[1,4]

We therefore tested the hypothesis that deletion of the miRNA-activating enzyme *Dicer1* in *Foxd1+* stromal progenitors may define the importance of post-transcriptional regulation by miRNAs in the stromal tissues during kidney organogenesis. *Dicer1* inactivation in the renal stroma resulted in hypoplastic kidneys with abnormal differentiation of the nephron tubule and vasculature. Three miRNAs—-214, -199a-5p, and -199a-3p—were enriched in the renal stroma and regulate stromal cell functions *ex vivo*. Taken together, these observations suggest that *Dicer1* activity in the renal stromal compartment regulates differentiation of nephron and vascular compartments of the developing kidney.

RESULTS

Dicer1 inactivation in the *Foxd1+* cortical stroma results in multiple defects of nephrogenesis

Foxd1+ nephrogenic progenitors are located in the cortical stroma surrounding the cap mesenchyme in the nephrogenic zone (Supplementary Figure S1A). These progenitors give rise to all of the stromal cells of the developing kidney, including mesangium and vascular smooth muscle (Supplementary Figure S1B).[21,22] Many of these stromal cells are attached to forming capillaries, whereas others are closely associated with the developing tubule (Supplementary Figure S1B).

To inactivate the miRNA processing RNase III gene *Dicer1* in the stromal tissues during kidney development, we crossed the *Foxd1-eGFPCre* (*Foxd1 GC*) allele with the *Dicer1 flox* allele (Figure 1a). In the *Dicer1 flox* allele, exon 23 of the *Dicer1* gene is flanked by two *loxP* sites.[23] This exon encodes most of the second RNase III domain, and therefore removal of the exon results in a null allele.[23] Offspring with the genotype $Foxd1^{+/GC}$; $Dicer1^{fl/fl}$ were born at below the expected Mendelian ratio (expected 12.5%, actual 9.8% ($n = 22/225$)) and survived for a maximum of 2 days after birth (Figure 1b). *Dicer1* is highly expressed in kidney during development (www.genepaint.org), and Cre activity sufficient to cause widespread recombination under the *Foxd1* regulatory sites was confirmed from E10.5 onward (Supplementary Figure S1B).[21] Inactivation of the DICER1 enzyme only in stromal compartment of the kidney was confirmed by immunostaining using an antibody that recognizes an epitope present on full-length protein (Figure 1c and f).[24] Kidneys of these mutant mice were smaller and bladders uniformly empty (Figure 1d and g; Supplementary Table 1), suggesting that the vascular supply and nephrons are sufficiently disrupted that there is no effective production of urine. Although kidney failure is evident from these findings, the uniform death of mice within 2 days of birth, the observation of cyanosis (Supplementary Figure S2A), and the histological abnormalities in the lung at birth, including reduction in branching, and septation resulting in fewer alveolae with smaller diameter (Supplementary Figure S2B), suggest that respiratory failure may also contribute to the high mortality in the early postnatal period. Consistent with such observations, we previously identified that the perivascular stromal lineage in the lung derives from *Foxd1+* lung progenitor cells.[25]

Coronal section of *Dicer1* mutant kidneys revealed the absence of the inner medulla and a shortened outer medulla (Figure 1e and h; Supplementary Figure S1D). Higher magnification revealed that glomeruli were present close to the innermost portion of kidneys and proximal tubules could be detected in this area, suggesting attenuation or the absence of the outer medulla, as well as the inner medulla. Nephron tubules in the cortex were abnormal showing reduced polarization identified by nuclei not restricted to the basolateral position, lack of columnar dimensions, a reduction in the formation of brush border and, in some instances, tubular distension/dilatation, and occasionally frank cyst formation (Figure 1i–n; Supplementary Figure S1D). Glomeruli were also abnormal. Many glomeruli were cystic and some showed dilated vessels. In addition, podocytes retained a cuboidal epithelial morphology (Figure 1k and n), which is normally lost as podocyte differentiation proceeds. Finally, the number of glomeruli in mutant kidneys was reduced (Figure 1o), suggesting that branching morphogenesis or nephrogenesis within the nephrogenic zone was reduced. Consistent with a reduction in nephrogenesis, the nephrogenic zone was reduced in thickness (Figure 1p).

Global transcriptional analysis of mutant kidneys identifies impaired development of stromal cells, epithelial maturation, and vasculogenesis

To explore the global effects of the *Dicer1*-null renal stromal cells on nephrogenesis, we compared the global transcriptome of $Foxd1^{+/GC}$; $Dicer1^{fl/fl}$ kidneys at E15.5 with $Foxd1^{+/GC}$; $Dicer1^{fl/+}$ kidneys at the same time point (Figure 2). This comparison identified 372 differentially regulated genes, with the majority of genes downregulated. One of the most significantly affected pathways was the pathway defined by hepatic stellate cell activation for which all 16 genes of the annotated pathway were downregulated (Figure 2a). Genes that regulate microvascular adhesion and endothelial junction formation were the next most downregulated (agranulocyte adhesion and diapedesis), and epithelial tight junction signaling and vesicle transport were prominently downregulated (tight junction signaling) (Figure 2a). In addition, integrin-like kinase signaling was downregulated.