Nephron Progenitor But Not Stromal Progenitor Cells Give Rise to Wilms Tumors in Mouse Models with β-Catenin Activation or Wt1 Ablation and Igf2 Upregulation

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Abstract

Wilms tumor, a common childhood tumor of the kidney, is thought to arise from undifferentiated renal mesenchyme. Variable tumor histology and the identification of tumor subsets displaying different gene expression profiles suggest that tumors may arise at different stages of mesenchyme differentiation and that this ontogenic variability impacts tumor pathology, biology, and clinical outcome. To test the tumorigenic potential of different cell types in the developing kidney, we used kidney progenitor-specific Cre recombinase alleles to introduce Wt1 and Ctnnb1 mutations, two alterations observed in Wilms tumor, into embryonic mouse kidney, with and without biallelic Igf2 expression, another alteration that is observed in a majority of tumors. Use of a Cre allele that targets nephron progenitors to introduce a Ctnnb1 mutation that stabilizes β-catenin resulted in the development of tumors with a predominant epithelial histology and a gene expression profile in which genes characteristic of early renal mesenchyme were not expressed. Nephron progenitors with Wt1 ablation and Igf2 biallelic expression were also tumorigenic but displayed a more triphasic histology and expressed early metanephric mesenchyme genes. In contrast, the targeting of these genetic alterations to stromal progenitors did not result in tumors. These data demonstrate that committed nephron progenitors can give rise to Wilms tumors and that committed stromal progenitors are less tumorigenic, suggesting that human Wilms tumors that display a predominantly stromal histology arise from mesenchyme before commitment to a stromal lineage.

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Introduction

Wilms tumor is a kidney tumor diagnosed primarily in children under the age of five. It is an embryonal tumor and typically exhibits a triphasic histology comprised of cells at differing stages of differentiation that are normally seen in the developing kidney: blastemal cells similar to early, undifferentiated metanephric mesenchyme; epithelial cells arranged in disorganized duct-like structures very similar to nephron ductal epithelial cells; and stromal cells. During normal kidney development, each of these cell types arises from intermediate mesenchyme, and Wilms tumors are generally thought to arise from undifferentiated intermediate and metanephric mesenchyme. However, tumors are very heterogeneous histologically, which has led to the suggestion that variable tumor histology may be a result of mesenchymal cells being mutated and

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transformed at different stages of mesenchymal differentiation. For example, some tumors display predominantly stromal or predominantly epithelial elements, raising the question of whether this distinct histology is the result of transformation of a cell already fated to become stromal or nephron epithelium, respectively.

The kidney is derived from Osr1 expressing intermediate mesoderm [1]. A very small population of cells expressing both Foxd1 and Six2 is present at the onset of metanephric mesenchyme outgrowth and can transiently contribute to nephron epithelium fated cells [2,3]. Subsequently, two major types of progenitor populations exist: nephron progenitors and stromal progenitors [2,4–6]. Nephron progenitors give rise to the majority of the cells in the nephron. The undifferentiated and self-renewing population of uninduced nephron progenitors expresses Cited1 and Six2 [7]. Upon stimulation of BMP7-Smad signaling, these cells lose expression of Cited1 but not Six2 and become sensitive to Wnt/β-catenin signaling from the ureteric bud, resulting in expression of Wnt4 and Left1 and epithelialization [8]. In comparison, stromal progenitors specifically express Foxd1 and give rise to the interstitium, pericytes, and mesangial cells [2,6]. Thus, triphasic Wilms tumors with bladder activity before the specification of nephron or stromal progenitors. Similarly, stromal-predominant tumors have been shown to originate from a stromal progenitor.

Gene expression analysis of a large panel of Wilms tumors resulted in the identification of five subsets of tumors which, in addition to their differing expression profile, displayed differing gene mutation frequencies, histologic features, and clinical outcomes [9]. From this work, a model of Wilms tumor oncogenesis was proposed, but, to date, experimental data regarding the cellular ontogeny of Wilms tumor have been lacking.

We previously successfully generated a genetic endogenous tumor mouse model for Wilms tumor by somatically and mosaically introducing into fetal kidney a combination of alterations observed in human tumors: ablation of Wt1; a transcription factor essential for kidney development; and biallelic expression of Igf2, a fetal mitogen. This was accomplished by use of a ubiquitously expressing, tamoxifen-inducible transgene encoding Cre-recombinase. Tumors were generated using a tamoxifen-inducible transgene encoding Cre-recombinase. Recombination of the Cited1Cre allele results in an in-frame deletion of exon 3 which stabilizes the resulting protein and activates the Wnt/β-catenin pathway [16]. Recombination of the Wt1Cre allele results in ablation of Wt1 function [15]. The H19 allele carries a deletion of H19 and the imprinting control region (ICR1) that is required for silencing of the maternal Igf2 locus. Maternal inheritance of H19 (H19−m) results in biallelic expression of Igf2 [17].

Mice

The Foxd1GCE (B6;129S4-Foxd1tm2(GFP/cre/ERT2)Amj/J), Six2GCE (B6;129–Six2GCE(EUGPlus/EUGGJ/ERT2)Amj/J), Cited1Cre (Cited1-CreERT2GFP, R26tdTomato (B6;129S6-Gt(Rosa)26Sor1tm14(CAG-tdTomato)H2E)), Cnnb1Cre+R26tdTomato, Wt1−/− (B6;129S4-Wt1tm1I1)], Wt1fl/fl, and H19−m mouse strains were used for these studies and have been described previously [5,6,12–17]. In the Foxd1GCE and Six2GCE strains, a GFP-Cre cassette is under the control of the endogenous Foxd1 and Six2 promoters, respectively, whereas the Cited1-CreERT2-GFP strain is a BAC transgenic mouse line in which a GFP-Cre expression construct is under the control of a 190-kb genomic fragment of the Cited1 gene. For all three Cre lines, Cre-recombinase function is inducible in a dose-dependent manner by tamoxifen (TM), and embryos were treated in utero with TM by intraperitoneal injection of the pregnant female to induce Cre activity. Recombination of the Cnnb1Cre ex3(fl) allele results in an in-frame deletion of exon 3 which stabilizes the resulting protein and activates the Wnt/β-catenin pathway [16]. Recombination of the Wt1Cre allele results in ablation of Wt1 function [15]. The H19−m allele carries a deletion of H19 and the imprinting control region (ICR1) that is required for silencing of the maternal Igf2 locus. Maternal inheritance of H19−m (H19−m) results in biallelic expression of Igf2 [17].

Materials and Methods

Validation of Progenitor Cell-Type Specific Expression of Cre Alleles

To confirm the progenitor specificity of the different Cre alleles and to estimate the proportion of kidney cells resulting from progenitor-specific targeting, Foxd1GCE, Six2GCE, or Cited1Cre/+ mice were crossed with tdTomato reporter mice expressing the loxP-Stop-loxP tdTomato transgene (R26tdTomato). Cre+; R26tdTomato+ embryos were treated with TM (1 or 3 mg/40 g body weight [BW]) at E11.5, E14.5, or E17.5. Embryonic kidneys were harvested at E19.5 and assessed by immunofluorescence (IF) histology. To estimate the frequency of Cre-mediated recombination, kidneys were also harvested at E14.5 and E19.5 and dissociated into single-cell suspensions which were sorted by fluorescence-activated cell sorting analysis (FACS) as described below.

Generation of Tumor-Watch Cohorts

To generate tumor-watch cohorts, Cre+; Wt1fl/fl males were bred with Wt1fl/fl; Cnnb1Cre+R26tdTomato or Wt1fl/fl; H19−m females to generate embryos with the following genotypes: 1) Foxd1GCE, Wt1fl/fl or Wt1fl/fl; Cnnb1Cre+R26tdTomato (C-Wt1-B-cat), 2) Foxd1GCE, Wt1fl/fl; H19−m (F-Wt1-Igf2), 3) Six2GCE, Wt1fl/fl or Wt1fl/fl; Cnnb1Cre+R26tdTomato (S-Wt1-B-cat), 4) Six2GCE, Wt1fl/fl; H19−m (S-Wt1-Igf2), 5) Cited1Cre, Wt1fl/fl; Cnnb1Cre+R26tdTomato (C-Wt1-B-cat), and 6) Cited1Cre, Wt1fl/fl; H19−m (C-Wt1-Igf2). Embryos were treated by intraperitoneal injection of pregnant dames with TM (0.5, 1 or 3 mg/40 g BW) to induce Cre activity in specific fetal kidney progenitors. For Foxd1GCE, TM was administered at E11.5. For Six2GCE, TM was administered at E11.5 or E14.5. In addition to its
expression in fetal kidney nephron progenitors, Cited1Cre is expressed in fetal liver progenitors at E14.5, and β-catenin activation by Cited1Cre at E14.5 in the fetal liver led to the development of hepatocellular carcinoma at an early age in >90% of mice [18]. Because of this competing phenotype, C-Wt1-Cre; Cited1Cre embryos were treated at E17.5, at which point Cited1Cre expression was undetectable in fetal liver [18] but still robust in fetal kidney. Wt1 ablation with Cited1Cre in the context of Igf2 biallelic expression following TM injection at E14.5 did not result in a detectable liver phenotype, and this time point was used to generate the C-Wt1-Igf2 cohort. Mutant mice and littermate controls were monitored for tumor development.

**Histology and Immunohistochemistry**

Tumor and kidney tissue specimens for hematoxylin and eosin (H&E) and immunohistochemistry (IHC) analyses were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5-μm sections. Samples for IF staining were processed in ornithine carbamoyltransferase compound and cut into 12-μm frozen sections. H&E, IHC, and IF analyses of proteins were performed as described previously [10]. Antibodies used were: WT1 (sc-192, Santa Cruz Biotechnology), Ki67 (ab15580, Abcam), pHH3 (06-570, Upstate Biotechnology), β-catenin (610154, BD Biosciences), Dll1 (sc-8624, Santa Cruz Biotechnology), Cited1 (9219, Fisher Scientific), Six2 (11562-1-AP, Proteintech), Pax2 (PRB-276P, Covance), NCAM (C9672, Sigma), E-cadherin (3195, Cell Signaling Technology), K-cadherin (ab79005, Abcam), Vimentin (V2258, Sigma), Collagen IV (AB756P, Chemicon), C-Myc (C099, Santa Cruz Biotechnology), and p21 (ab32138, Abcam). Antibodies used were: WT1 (sc-192, Santa Cruz Biotechnology), Ki67 (ab15580, Abcam), pHH3 (06-570, Upstate Biotechnology), β-catenin (610154, BD Biosciences), Dll1 (sc-8624, Santa Cruz Biotechnology), Cited1 (9219, Fisher Scientific), Six2 (11562-1-AP, Proteintech), Pax2 (PRB-276P, Covance), NCAM (C9672, Sigma), E-cadherin (3195, Cell Signaling Technology), K-cadherin (ab79005, Abcam), Vimentin (V2258, Sigma), Collagen IV (AB756P, Chemicon), C-Myc (C099, Santa Cruz Biotechnology), and p21 (ab32138, Abcam).

**Fluorescence-Activated Cell Sorting Analysis**

Single-cell suspensions from fetal kidneys harvested at E15.5 or E19.5 from Foxd1GCE/+; R26RtdTomato/+ embryos, Six2GCE/+, R26RtdTomato/+ embryos, and Cited1Cre/+; R26RtdTomato/+ embryos were prepared as previously described [5]. Cells expressing tdTomato as a result of Cre-mediated recombination of the conditionally expressed tdTomato reporter were isolated with the BD FACS Aria high-speed digital cell sorter (BD Biosciences). Cell suspensions from littermate kidneys without a Cre allele served as negative controls.

**Gene Expression Analysis**

Tumor or kidney tissue was flash-frozen in liquid nitrogen. RNA was extracted from specimens or cell pellets using the RNeasy-4PCR kit (Ambion) and converted to cDNA using reverse transcription reagents (Applied Biosystems). Real-time polymerase chain reaction (PC)R was performed using SYBR Green reagent with a 7900HT sequence detection system (Applied Biosystems) using transcript-specific primers (Supplementary Table 2).

**Statistical Analysis**

The Student t test or analysis of variance was used to analyze real-time PCR results. Results were presented as mean values with standard deviation. P values less than .05 were considered statistically significant.

**Results**

**Validation of Progenitor Cell-Type Specific Expression of Cre Alleles**

Lineage tracing of cells derived from targeted progenitors confirmed the specificity of the Cre alleles used (Figure 1A). As expected, use of the Six2GCE and Cited1Cre alleles resulted in tdTomato expression in nephron progenitors and their daughter cells, e.g., comma-shaped bodies, S-shaped bodies, proximal tubules, distal tubules, and glomeruli. Also as expected, as the time between TM injection and harvesting the kidney increased, tdTomato expression shifted more medially as labeled progenitors differentiated and kidney development continued at the periphery. Use of the stromal progenitor-specific Cre (Foxd1Cre) resulted in labeled stromal progenitors and their descendant cells. FACS analysis of kidney cell suspensions for tdTomato cells at E15.5 and E19.5 was also carried out. A representative FACS sorting plot is shown in Figure 1B. tdTomato cells represented 30% to 33% of total kidney cells following Six2GCE-mediated recombination and 5% to 7% following recombination with either the Cited1Cre or Foxd1Cre alleles (Figure 1C and Table 1).

**Targeting of Nephron, But Not Stromal, Progenitors Results in Wilms Tumor Development**

As shown in Table 2, Wt1−/fl; Ctnnb1+/fl; H19+/− alleles carrying either the Six2GCE or Cited1Cre Cre-recombinase-expressing allele developed tumors. The age of onset observed in these two genotypes was similar (Figure 2), and tumor frequency was also similar, despite the increased frequency of recombined cells observed using the Six2GCE allele (Table 1). In contrast, Wt1 ablation and biallelic expression of Igf2 (Wt1−/fl; H19+/− genotype) resulted in tumors only when nephron progenitors were targeted using the Cited1Cre allele (9/18 mice) but not the Six2GCE allele (0/28 mice). When Cited1Cre was used to target nephron progenitors, tumors arising following Wt1 ablation in the context of Igf2 biallelic expression (C-Wt1-Igf2 homozygous) developed at a significantly later age (P = .0058) than when Wt1 was ablated in the context of β-catenin stabilization (C-Wt1-β-catenin−/− mice).

No tumors were observed in animals in which Wt1 ablation in the context of either β-catenin stabilization or Igf2 biallelic expression was targeted to stromal progenitors (F-Wt1-β-catenin−/− and F-Wt1-Igf2 homozygous) (Figure 2A, Table 2).

**β-Catenin Stabilization Is Sufficient for Tumor Development in Nephron-Progenitor Targeted Cells**

Genotyping of tumors confirmed that, as expected, all tumors from Six2GCE−/−; Wt1−/−; Ctnnb1+lox/loxfl mice and Cited1Cre; Wt1−/−; Ctnnb1+lox/loxfl mice carried the recombinated Ctnnb1+lox/loxfl (Ctnnb1−/−) allele. Unexpectedly, many tumors (11/14) retained the unrecombined Wt1−/− allele (Figure 2B), indicating that Wt1 ablation was not required for transformation of the nephron progenitors. Six2GCE−/−; Wt1−/−; Ctnnb1+lox/loxfl mice, Six2GCE−/−; Wt1−/−; Ctnnb1+lox/loxfl mice, and Cited1Cre; Wt1−/−; Ctnnb1+lox/loxfl mice, and Cited1Cre; Wt1−/−; Ctnnb1+lox/loxfl mice developed tumors at a similar rate and at similar ages regardless of differential gene dosages of Wt1 (Figure 2A). To confirm that β-catenin stabilization alone was sufficient for tumorigenesis, we generated a small cohort of Six2GCE−/−; Ctnnb1+lox/loxfl mice. By 35 weeks of age, 2 of 5 animals developed tumors, similar to the observations in the Six2GCE−/−; Wt1−/−; Ctnnb1+lox/loxfl and Six2GCE−/−; Wt1−/−; Ctnnb1+lox/loxfl mice (data not shown).

Of note, Wt1 ablation was observed in all tumors from the Cited1Cre; Wt1−/−; H19+/− mice (Figure 2C), and none of the six Cited1Cre; Wt1−/−; H19+/− littermate control mice developed tumors.

**Tumor Morphology and Histology**

Tumors from the S-Wt1-β-catenin−/− and C-Wt1-β-catenin−/− cohorts were often associated with benign blood-filled cysts, although this occurred less frequently in C-Wt1-β-catenin−/− cohorts. Such cysts were not observed in tumor-bearing C-Wt1-Igf2 mice. In all three genotypic cohorts,
tumors presented as distinct outgrowths from the periphery of the kidney (Figure 3A).

Tumors from the S-Wt1-β-cat S and C-Wt1-β-cat S cohorts displayed an epithelial histology, composed mainly of primitive epithelial cells which formed rosettes (Figure 3B, a and b) and more differentiated epithelial cells which formed tubular structures (Figure 3B, e and f). Stromal elements were observed in areas of some tumors but were generally a very minor feature of the tumors. Blastemal elements were even less frequently observed compared with C-Wt1-Igf2 tumors. A similar tumor histology was observed irrespective of whether Wt1 was ablated.

**Targeting Nephron Progenitors with Different Mutations Results in Histologically Different Wilms Tumors**

In contrast to the tumors from S-Wt1-β-cat S and C-Wt1-β-cat S cohorts, tumors from the C-Wt1-Igf2 cohort displayed a triphasic histology comprised of undifferentiated blastemal, and differentiating epithelial and stromal cells, similar to that observed in the original WT mouse model in which somatic, mosaic Wt1 ablation in the context of Igf2 biallelic expression was effected using a TM-inducible Cre that is expressed ubiquitously (Figure 3B, c and d) [10].

**Expression of β-Catenin and Wnt-β-Catenin Pathway Targets in Tumors**

To confirm the stabilization of β-catenin in tumors carrying the Cmmnb1<sup> Nest </sup> allele and to assess the impact of this stabilization on the expression of canonical Wnt-β-catenin target genes, we performed IHC for β-catenin and quantified the expression of downstream targets in tumors. As shown in representative tumors in Figure 4A, robust nuclear β-catenin staining was present in tumors from both **Table 1. Summary of Mouse Mutant Cohorts and TM Injection Conditions**

| Designation | Genotype | Tamoxifen (mg/40 g BW) | Tumor mice/ mutant mice |
|-------------|----------|------------------------|-------------------------|
| S-Wt1-Igf2  | Six2<sup>GCE</sup>, Wt1<sup>−/−</sup>, H19<sup>+/−</sup> | 1 at E11.5 | 0/21 |
|             |          | 2 at E14.5             | 0/7 |
| S-Wt1-β-cat<sup>S</sup> | Six2<sup>GCE</sup>, Wt1<sup>−/−</sup>, β-cat<sup>S</sup>/fl | 3 at E14.5 | 8/11 (73%) |
| S-β-cat<sup>S</sup> | Six2<sup>GCE</sup>, Wt1<sup>−/−</sup>, β-cat<sup>S</sup>/fl | 3 at E14.5 | 6/12 (50%) |
| C-Wt1-Igf2 | Cited1<sup>Cre</sup>, Wt1<sup>+/−</sup>, H19<sup>+/−</sup> | 1 at E14.5 | 9/18 (50%) |
| C-Wt1-β-cat<sup>S</sup> | Cited1<sup>Cre</sup>, Wt1<sup>−/−</sup>, β-cat<sup>S</sup>/fl | 0.5 at E17.5 | 9/20 (45%) |
| F-Wt1-Igf2 | Foxd1<sup>GCE</sup>, Wt1<sup>−/−</sup>, H19<sup>+/−</sup> | 4 at E11.5 | 0/9 |
| F-Wt1-β-cat<sup>S</sup> | Foxd1<sup>GCE</sup>, Wt1<sup>−/−</sup>, β-cat<sup>S</sup>/fl | 3 at E11.5 | 0/17 |
| F-Wt1-β-cat<sup>S</sup> | Foxd1<sup>GCE</sup>, Wt1<sup>+/−</sup>, β-cat<sup>S</sup>/fl | 3 at E11.5 | 0/11 |

**Table 2. Efficiency of Progenitor-Specific Cre-Mediated Recombination by FACS Analysis of tdTomato<sup>+</sup> Cells with Indicated TM Injection Conditions**

| Cre | Time point of TM injection | TM dosage | FACS | tdTomato<sup>+</sup> cells % |
|-----|---------------------------|-----------|------|-----------------------------|
| Six2<sup>GCE</sup> | E11.5 | 1 mg | E15.5 | 33.7 |
|       |       |       | E19.5 | 30.2 |
|       | E14.5 | 3 mg | E19.5 | 30.0 |
| Citd1<sup>Cre</sup> | E14.5 | 1 mg | E19.5 | 6.8 |
|       | E17.5 | 1 mg | E19.5 | 5.1 |
| Foxd1<sup>GCE</sup> | E11.5 | 3 mg | E19.5 | 7.1 |

**Figure 1.** Efficiency of Cre recombination in progenitor-specific Cre lines using R26<sup>TdTomato</sup> reporter mice. (A) Kidney sections from Six2<sup>GCE/+</sup>; R26<sup>TdTomato/+</sup> (a and b), Cited1<sup>Cre</sup>; R26<sup>TdTomato/+</sup> (c and d), and Foxd1<sup>GCE/+</sup>; R26<sup>TdTomato/+</sup> (e) were treated with TM at indicated time points and analyzed for tdTomato reporter expression at E19.5. DAPI was used to counterstain nuclei. Scale bar: 200 μm. (B) Representative dot-plot of tdTomato<sup>+</sup> cells from single cell suspension of Cited1<sup>Cre</sup>; R26<sup>TdTomato/+</sup> kidney at E19.5 after 3 mg/40 g BW TM treatment at E14.5. (C) FACS analysis showing percentage of targeted tdTomato<sup>+</sup> cells from Six2<sup>GCE</sup>, Cited1<sup>Cre</sup>, and Foxd1<sup>GCE</sup>, respectively. Bar labels indicate the embryonic age at the time of TM injection, the embryonic age at time of kidney collection, and the dose of TM used.
Figure 2. Development of WT from nephron progenitors. (A) Kaplan-Meier tumor-free survival analysis showing incidence of WT in Six2\textsuperscript{GCE}, Cited1\textsuperscript{Cre}, and Foxd1\textsuperscript{GCE} mutant mice with Wt1 ablation/haploinsufficiency and \(\beta\)-catenin stabilization (S/C/F-Wt1\textsuperscript{−/−/−}/\(\beta\)-cat\textsuperscript{S} and S/C/F-Wt1\textsuperscript{−/−/−}/\(\beta\)-cat\textsuperscript{S}) and Wt1 ablation and Igf2 upregulation (S/C/F-Wt1\textsuperscript{−/−/−}/Igf2) in comparison to control mice. (B) PCR analysis of DNA from mutant mice showing Ctnnb1 wild-type or exon3 floxed allele and exon3 deleted allele (Ctnnb1\textsuperscript{+} or Ctnnb1\textsuperscript{ex3(fl)} and Ctnnb1\textsuperscript{Δ}) and Wt1 floxed or null alleles (Wt1\textsuperscript{fl} or Wt1\textsuperscript{Δ}).

Figure 3. Histological analysis of WTs from Six2\textsuperscript{GCE} and Cited1\textsuperscript{Cre} mice. (A) Gross appearance of tumors from Six2\textsuperscript{GCE} mice (a) and Cited1\textsuperscript{Cre} mice (b and c) of the indicated genotypes. (B) H&E staining of kidney tumor sections of S-Wt1-\(\beta\)-cat\textsuperscript{S}, C-Wt1-\(\beta\)-cat\textsuperscript{S}, and C-Wt1-Igf2 mice. Scale bar: 100 \(\mu\)m.
**S-Wt1-β-cat**

S-Wt1-β-cat and C-Wt1-β-cat cohorts. The presence or absence of Wt1 ablation did not affect this robust staining (data not shown). However, β-catenin in the C-Wt1-Igf2 tumors was primarily cytoplasmic (Figure 4A).

Tumors in which β-catenin was stabilized generally displayed increased expression of Axin2, Wif1, and Dkk2, genes previously reported to be upregulated upon Wnt/β-catenin activation, although there was considerable tumor variability in the expression of Axin2 and Dkk2. Despite this variability, there were significant differences in Axin2 expression between Cited1Cre-targeted tumors with β-catenin stabilization versus Cited1Cre-targeted tumors with Wt1 ablation and Igf2 biallelic expression. Upregulation of Wif1 was less variable and was dramatically increased in β-catenin stabilized tumors. Within the same Cited1Cre-targeted progenitor population, increased Wif1 expression was observed in the C-Wt1-β-cat tumors but not the C-Wt1-Igf2 tumors, consistent with Wif1 being a target of the Wnt/β-catenin pathway. Unexpectedly, contrasting data were obtained for CyclinD1 and C-myc, two other Wtβ/β-catenin targets whose expression was significantly higher in tumors not carrying the stabilizing Cmnb1 mutation (Figure 4B).

**Tumors from S-Wt1-β-cat**

Tumors arising following the targeting β-catenin stabilization to committed epithelial progenitors—by either Six2GCE or Cited1Cre—displayed low expression of genes primarily expressed in the intermediate renal mesenchyme and/or later metanephric mesenchyme (Eya1, Osr1, Pax2, and Hoxa11) (Figure 5). However, targeting Wt1 ablation in the context of Igf2 biallelic expression to nephron progenitors using the same Cited1Cre transgene resulted in tumors that robustly expressed these early stage genes. This expression of early mesenchymal genes was similar to that observed in tumors when the same mutations were introduced via the ubiquitously expressed Cre-ER™ allele in the original WT mouse model (Figure 5) [10,19]. Robust expression of Wnt4 and Jag1, markers of induced mesenchyme, and CyclinD1, whose expression is transiently upregulated following induction, was observed only in tumors with Wt1 ablation and Igf2 biallelic expression. Expression of muscle differentiation genes was often variable between tumors within a cohort and also between cohorts (Supplementary Figure 1). Whereas a statistically significant increase in expression was observed for some genes, e.g., Pax3 in Cited1-β-cat and Six1-Wt1-β-cat tumors and Tn in Cited1-Wt1-Igf2 tumors, this was not consistent across the three muscle differentiation genes we assessed.

Assessment of protein expression by IHC staining confirmed that S-Wt1-β-cat and C-Wt1-β-cat tumors did not express PAX2 or different sets of mutations to different cellular compartments of the developing kidney, we were in a position to test whether resulting tumors displayed differential expression of these developmentally regulated genes.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Activity of Wnt/β-catenin signaling in mouse WTs. (A) IHC staining of β-catenin and C-myc for sections from control kidneys and tumors from S-Wt1-β-cat, C-Wt1-β-cat, and C-Wt1-Igf2 mutants. Scale bar: 100 μm. (B) qPCR analysis of Wnt/β-catenin canonical effectors Axin2, CyclinD1, and C-myc and signaling inhibitors Wif1 and Dkk2 in littermate kidneys and tumors from S-Wt1-β-cat, C-Wt1-β-cat, and C-Wt1-Igf2 mutants. The x-axis labels indicate wild-type (Wt1+/m) in littermate kidneys and the presence of progenitor-specific Cre alleles (Six2GCE or Cited1Cre) or Ubiquitous-Cre (U) and the presence of genetic alterations of β-cat, Wt1 ablation (Wt1−/−) or Igf2 upregulation (H19+/−m) in mouse tumors.
CYCLIND1, whereas C-Wt1-Igf2 tumors robustly expressed PAX2. All three tumor types expressed CITED1, a marker of nephron progenitors and, in epithelial elements, expressed E-cadherin, a marker of terminally differentiated epithelium.

In summary, both gene and protein expression data revealed that C-Wt1-Igf2 tumors expressed markers of early mesenchyme and also postinduction genes, whereas S-Wt1-β-catenin and C-Wt1-β-catenin tumors expressed markers of committed nephron progenitors and epithelial differentiation.

Discussion
Classically, three cell types—blastemal, stromal, and epithelial—are observed in Wilms tumors, and such triphasic tumors have been thought to arise from early undifferentiated metanephric mesenchyme. However, the observation of tumors comprised predominantly of stromal or epithelial elements has led to the suggestion that these tumors arise from fetal kidney cells committed to a stromal or epithelial lineage. The main goals of the current study were to determine if tumors could arise from such committed progenitors and, if they did, whether the resultant tumors displayed a characteristic histology and whether the type of genetic alterations introduced into renal progenitors affected their tumorigenicity and/or the histology of the resulting tumors.

Using a Cre-LoxP approach, we earlier demonstrated that random somatic and mosaic Wt1 ablation in the context of Igf2 biallelic expression, a combination of alterations observed in human Wilms tumors, resulted in mouse tumors with a classic triphasic histology [10]. We have now introduced that same set of mutations and also the combination of Wt1 ablation and β-catenin stabilization, another combination that is also observed in human WTs [11], into specific cellular compartments of the developing kidney using TM-inducible Cre-expressing alleles that target kidney stromal progenitors or nephron progenitors.

Confirmation of Appropriate Targeting of Kidney Progenitors
Our lineage tracing experiments confirmed the expected progenitor-specific expression of each of the Cre alleles employed in the study: kidney stroma was derived from FoxD1GCE-tdTomato–tagged cells, whereas Six2GCE-tdTomato+ cells were present in the cap mesenchyme population at early time points following Cre induction and at later time point in the comma-shaped and S-shaped bodies of developing nephrons and also the tubules and glomeruli of mature nephrons. Interestingly, the tdTomato+ cells present 5 days following targeting by Six2GCE existed in more mature structures than those present 5 days following targeting by Cited1Cre at the same time point. Six2GCE-tdTomato+ cells also represented a larger percentage of the total kidney cell population than Cited1Cre-tdTomato+ cells. Both SIX2 and CITED1 have been identified as markers for committed nephron progenitors, which are ultimately induced to undergo a mesenchyme-epithelial transition in response to WNT9b from the ureteric bud [4,5]. However, recent work suggests a more nuanced compartmentalization in which CITED1+SIX2+ cells, which are nonresponsive to WNT9b induction, lose expression of CITED1, resulting in SIX2+-only cells that do respond to WNT9b-induced epithelialization [8]. Thus, CITED1+SIX2+ cells are proposed to represent the self-renewing nephron progenitor compartment, whereas SIX2+ cells represent cells that can then respond to WNT9b and β-catenin.

Our observation of more differentiated tdTomato+ cells following targeting by Six2GCE is consistent with this model. Unlike the Cited1-
targeted population, the Six2-targeted population would include cells on the cusp of epithelial differentiation, resulting in Six2\(^{\text{GCE}}\)-tdTomato\(^+\) cells being observed at later stages of nephron development. This model could also account for the greater proportion of Six2\(^{\text{GCE}}\)-tdTomato\(^+\) cells than Cited1\(^{\text{Cre}}\)-tdTomato\(^+\) cells 5 days following TM-induced tdTomato expression.

**Tumors Develop Following Mutational Targeting of Nephron Progenitors**

Tumors were observed in both the S-Wt1-\(\beta\)-cat\(^S\) and C-Wt1-\(\beta\)-cat\(^S\) cohorts, demonstrating that Wilms tumors can arise from committed nephron progenitors. Interestingly, a similar frequency of tumor development and age of onset was observed in mice following targeting of Wt1 ablation and \(\beta\)-catenin stabilization with Cited1\(^{\text{Cre}}\) or Six2\(^{\text{GCE}}\), despite the tdTomato-lineage tracing data that indicated a five-fold increase in the percentage of cells following Six2\(^{\text{GCE}}\) versus Cited1\(^{\text{Cre}}\)-induced labeling. This observation could be due to the rapid differentiation of Six2\(^{\text{GCE}}\)-tdTomato\(^+\) cells to a more differentiated cell type that was refractory to transformation by the introduced mutations. A previous study reported development of mouse primitive epithelial tumors with features of Wilms tumors by using Cited1\(^{\text{Cre}}\) to activate \(\beta\)-catenin with or without K-ras activation postnatally [20]. In this study, a proximal tubule specific \(\gamma\)GT-Cre was also applied to activate \(\beta\)-catenin, and K-ras and mutant mice developed metastases to the lungs in addition to the primitive epithelial tumors [20]. These tumors appeared histologically very similar to our S-Wt1-\(\beta\)-cat\(^S\) and C-Wt1-\(\beta\)-cat\(^S\) epithelial Wilms tumors. In our study, Six2\(^{\text{GCE}}\) or Cited1\(^{\text{Cre}}\) was activated at an early stage of kidney development, and nephron progenitors were targeted, which was different from the approach of targeting cells postnatally in their models. Moreover, in S-Wt1-\(\beta\)-cat\(^S\) and C-Wt1-\(\beta\)-cat\(^S\) mutants, a strong association of cystic kidney phenotype was observed with tumor development. Combined, these results suggest that, with \(\beta\)-catenin activation, both nephron progenitors and proximal tubules can give rise to renal epithelial tumors in mice.

Another striking and statistically significant difference between Six2\(^{\text{GCE}}\) versus Cited1\(^{\text{Cre}}\)-targeted cohorts was the tumorigenic effect of the combination of Wt1 ablation and IGF2 biallelic expression. No tumors arose in S-Wt1-Igf2 animals (in which both the CITED1\(^+\) SIX2\(^+\) and the SIX2\(^+\)-only progenitor populations were targeted), whereas 50% (9/18) of C-Wt1-Igf2 animals (in which only the CITED1\(^+\) SIX2\(^+\) population is expected to be targeted) developed tumors (Figure 2A, Table 2). These differences between the Six2\(^{\text{GCE}}\) and Cited1\(^{\text{Cre}}\) cohorts may be due to differences in the cells targeted by these two Cre alleles. In the metanephric mesenchyme surrounding the ureteric bud, two compartments, one CITED1\(^+\) SIX2\(^+\) and one SIX2\(^+\) only, have been delineated [7,8]. Whereas the robust expression of the Cre alleles in the metanephric mesenchyme surrounding the ureteric bud has been demonstrated by immunofluorescence analysis [5], less robust expression of these alleles elsewhere in the developing mesenchyme is possible and perhaps to be expected given that endogenous Six2 and Cited1 expression is observed at low levels at earlier stages of kidney development [12,21,22]. Of note, in contrast to the frequent co-occurrence of Ctnnb1 and WT1 mutations in human Wilms tumors [11], Sanger sequencing revealed that C-Wt1-Igf2 mouse tumors did not spontaneously acquire Ctnnb1 mutations (Huff, unpublished).

Another possible explanation for the difference in the tumorigenic effect of Wt1 ablation and IGF2 biallelic expression in the Six2\(^{\text{GCE}}\) versus the Cited1\(^{\text{Cre}}\) cohort could be related to the experimental approaches used for this study which result in additional differences in Six2\(^{\text{GCE}}\) and Cited1\(^{\text{Cre}}\)-targeted cells. Animals carrying the Six2\(^{\text{GCE}}\) allele in which the Cre-GFP cassette is knocked into the Six2 locus are effectively Six2\(^{\text{Cre}}\). Six2 encodes a transcription factor that plays an important role in nephron progenitor self-renewal [23,5,24]. Whereas Six2\(^{\text{Cre}}\) mice have no discernable kidney phenotype [25], suggesting that nephron progenitor self-renewal is unaffected by Six2 haploinsufficiency, such haploinsufficiency could plausibly impact the ability of Ctnnb1-mutant cells to become tumors. SIX2 and \(\beta\)-catenin are reported to act in both an opposing and a cooperative manner to regulate different subsets of genes during epithelialization of induced nephron progenitors [24]. If Six2 haploinsufficiency is a factor in the differential tumor development in our experimental cohorts, the data would suggest that, in the context of \(\beta\)-catenin stabilization, Six2 haploinsufficiency helps to promote tumorigenesis of nephron progenitors or that Six2 haploinsufficiency is incompatible with tumor development in the context of Wt1 ablation and biallelic IGF2 expression. Heterozygous SIX2 missense mutations are observed in human Wilms tumors and are often coincident with mutations in miRNA processing genes [26,27]. Neither Wt1 nor CTNNB1 mutations were observed in SIX2-mutant tumors. These data are consistent with the model that, in our mice, Six2 haploinsufficiency was not a factor in the differential tumor development we observed between the Six2-Cre and Cited1-Cre cohorts upon the introduction of Wt1 and Ctnnb1 mutations, although further studies will be required to test this experimentally. Of note, in human tumors, IGF2 loss of heterozygosity or loss of imprinting (which results in biallelic expression) is very common and is observed irrespective of SIX2 mutation [26,27].

Previously, we used a ubiquitously expressing Cre transgene to mosaically ablate Wt1 in the genetic background of IGF2 upregulation (U-Wt1-Igf2) [26]. These mice had an earlier onset (10 weeks) and a higher frequency of tumor development (80%) than the C-Wt1-Igf2 tumors in the current study (onset at 25th week and an overall tumor frequency of 50%). Although these two models carry the same genetic mutations, the differences in tumor onset and penetration may be due to differences in the differentiation status of the cells when the mutations are introduced. Many human tumors are thought to arise from intermediate mesoderm which may be the cell of origin for the U-Wt1-Igf2 tumors, as opposed to the more differentiated cells that were targeted by use of the Cited1-Cre transgene.

**Transformation of Nephron Progenitors by \(\beta\)-Catenin Stabilization Does Not Require Wt1 Ablation**

WT1 ablation and CTNNB1 mutation that result in \(\beta\)-catenin stabilization are highly concordant in human Wilms tumors [11,9]. All CTNNB1 mutations reported to date are somatic and those that affect residues encoded in exon 3 that are critical for \(\beta\)-catenin stability almost invariably occur in the presence of Wt1 ablation [11,9,28]. Thus, our observation when we genotyped mouse tumors from Six2\(^{\text{GCE}}\); Wt1\(^{-}\); Ctnnb1\(^{-}\)Igf2\(^{\text{Cre}}\) and Cited1\(^{\text{Cre}}\); Wt1\(^{-}\); Ctnnb1\(^{-}\)Igf2\(^{\text{Cre}}\) mice that only one of six tumors displayed complete Wt1 ablation was highly unexpected. Because this was observed in both the S-Wt1-\(\beta\)-cat\(^S\) and C-Wt1-\(\beta\)-cat\(^S\) cohorts, Six2 haploinsufficiency is likely not a factor in this unexpected finding. In addition, the presence or absence of Wt1 ablation did not result in a statistically significant difference in the frequency or age at which tumors developed. Cohorts with a Wt1\(^{-}\) genotype developed
tumors similar to mice with a WT1\(v^{β}\) genotype, did mice with a WT1\(v^{+/}\) genotype (Figure 2). Although analysis of larger cohorts might, in the future, identify subtle differences in tumor susceptibility between these different genotypes, the current data demonstrate that WT1 ablation in addition to β-catenin stabilization is not required for tumorigenesis of nephron progenitors.

The observation that Cnmb1 mutation targeted to nephron progenitors resulted in tumors in the absence of WT1 mutations is in interesting contrast with human tumors in which CTNNB1 mutations are frequently associated with WT1 mutations [11]. In addition, a subset of human tumors (subset 1) proposed to arise from postinduction nephron progenitors carries neither Cnmb1 nor WT1 mutations [9], although, like the Cnmb1-mutant mouse tumors arising from targeted nephron progenitors, they exhibit a differentiated epithelial histology.

Whether Cnmb1 mutation is sufficient for cell transformation in uncommitted metanephric mesenchyme is not known. Somatic deletion of exon 3 of Cnmb1 using the ubiquitously expressing inducible Cre allele resulted in embryonic lethality even when Cre activity was induced in <5% of cells (Huff, unpublished data). This observation is consistent with the importance of the Wnt/β-catenin pathway and its precise regulation in many developmental processes. Thus, the assessment of the ability of β-catenin stabilization alone to transform uncommitted cells of the embryonic kidney awaits the use of a Cre allele that specifically targets these cells.

### Histologic and Gene Expression Differences between Tumors

Tumors from C-WT1-β-cat\(^v\) and S-WT1-β-cat\(^v\) cohorts were primarily composed of epithelial cells with few stromal and negligible blastemal components. This is consistent with observations that Wnt pathway activation stimulates epithelialization of cells with few stromal elements (Supplementary Figure 1).

In contrast to the histology of tumors from C-WT1-β-cat\(^v\) and S-WT1-β-cat\(^v\) cohorts, stromal and blastemal cells were more frequent in tumors from C-WT1-Igf2 cohorts, although not to the extent observed when a ubiquitously expressed Cre allele was employed to ablate WT1 in the context of Igf2 biallelic expression (Figure 1). In these cases, differences in gene expression were also observed between genotypes rather than between the Cre alleles used to target cells. With few exceptions, no difference in gene expression was observed between tumors from S-WT1-β-cat\(^v\) and tumors from C-WT1-Igf2\(^v\) mice. In contrast, tumors from S-WT1-β-cat\(^v\) and C-WT1-β-cat\(^v\) mice were significantly different from tumors from C-WT1-Igf2\(^v\) mice, and the latter tumors were very similar to U-WT1-Igf2 tumors with respect to the expression of genes expressed during kidney development that are targets of Wnt/β-catenin signaling.

Like human subset 2 and 3 tumors in which WT1 and CTNNB1 mutations are frequently observed (along with IGF2 LOH/LOI), genes normally expressed in intermediate mesoderm and early metanephric mesenchyme were highly expressed in both C-WT1-Igf2 and U-WT1-Igf2 tumors relative to normal kidney and to tumors from the S-WT1-β-cat\(^v\) and C-WT1-β-cat\(^v\) cohorts (Figure 5). This is of particular interest because the expression of intermediate mesoderm genes is more concordant with tumor genotype than it is with the Cre transgene used for introducing gene alterations. Like human S2 and S3 tumors, it has been suggested that the U-WT1-Igf2 tumors arise from intermediate mesoderm or early metanephric mesenchyme, in large part due to the fetal age (~E12) at which the alterations were induced. The observation of tumors with a similar histology and a similar gene expression profile following the introduction of those same alterations at a much later stage of kidney development (~E18) is unexpected. Of note, C-WT1-β-cat\(^v\) tumors did not display a predominant histology of blastemal cells that are most reminiscent of intermediate mesenchyme. These data suggest that WT1 ablation and Igf2 biallelic expression, but not Cnmb1 mutation, in the Cited\(^v\)-targeted committed nephron progenitors result in the reexpression of these early genes and/or results in partial dedifferentiation of these cells which secondarily results in expression of these genes. The fact that the C-WT1-Igf2 and U-WT1-Igf2 tumors (but not tumors from S-WT1-β-cat\(^v\) and C-WT1-β-cat\(^v\) mice) also displayed increased expression of two genes, Wnt4 and Jag1, that characterise nephron progenitors induced to epithelialize and that are subsequently downregulated is consistent with the presence of epithelial elements in the tumors, in addition to blastemal and stromal elements.

### Conclusions

In sum, these data suggest that mutation of nephron progenitors, but not stromal progenitors, results in tumor development, at least with
the sets of alterations introduced and the time points at which they were introduced into the progenitors. Using gene expression data from human tumors, a model for Wilms tumor ontogeny was developed such that tumors expressing genes characteristic of intermediate mesoderm or early metanephrine mesenchyme were proposed to have arisen from cells at these early stages of kidney development. Although this may be the case in general, our experimental data in mice indicate that, even at a later stage, committed nephron progenitors can give rise to tumors. In the case of Ctnnb1 mutation, tumors arising from these progenitors are histologically well differentiated and do not express genes characteristic of earlier stages of kidney development. However, tumors with a more triphasic histology and expression of these early kidney genes are observed when Wt1 ablation and Igf2 biallelic expression are introduced. These data suggest that this combination of alterations can effect a reprogramming of committed nephron progenitors. This model is supported by the observations that ablation of Wt1 results in a block in differentiation of the metanephrine mesenchyme [10] and that, in the testes, Wt1 ablation results in a block in differentiation of Sertoli cells to fetal-like Leydig cells [30].

C-Wt1-Igf2 tumors were similar histologically and with respect to gene expression to tumors rising from the nontargeted introduction of these same alterations into fetal kidney (U-Wt1-Igf2 tumors). Whether this is due to the ability of these alterations to reprogram committed nephron progenitors to an early metanephrine mesenchyme state or because the U-Wt1-Igf2 tumors arise from committed nephron progenitors is not known. The two sets of tumors do differ with respect to the expression of C-myc, suggesting some difference in tumor ontogeny. It will be informative in the future to specifically target early metanephrine mesenchyme and determine the tumorigenicity of these cells and the histologic and gene expression characteristics of resulting tumors.

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