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REVIEW

Wilms tumor—a renal stem cell malignancy?

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Abstract Wilms' tumor (WT; nephroblastoma) is the most common pediatric renal malignancy and rated fourth in overall incidence among childhood cancers. It is viewed as a prototype of differentiation failure in human neoplasia as it recapitulates the histology of the nephrogenic zone of the growing fetal kidney. The cellular origin of WT is unclear. However, recent genomic, genetic and epigenetic studies point to an early renal stem/progenitor cell that undergoes malignant transformation as the source for WT. In this context, classical WT shares genes and pathways activated in progenitors committed to the renal lineage. However, direct proof and characterization of the WT initiating cell have remained elusive. Novel methodologies recently adopted from the cancer stem cell scientific field, including the analysis of sorted single human tumor cells, have been applied to WT. These have enabled the identification of cell sub-populations that show similarities—in terms of molecular marker expression—to human fetal kidney progenitors and are, therefore, likely to be derivatives of the same lineage. Further elucidation of the WT cancer stem cell or the cell of origin in human tumors and in transgenic mouse models that generate murine tumors may not only provide novel therapeutic targets but also shed light on the normal kidney stem cell.

Keywords Wilms tumor · Nephroblastoma · Embryonic cancer · Stem cells · Cancer stem cells · Renal stem cells · Children

Introduction

Human nephroblastoma or Wilms' tumor (WT) is an embryonic cancer of the developing kidney. It is the most frequent tumor of the genitourinary tract in children and rated fourth in overall incidence among childhood cancers, occurring in 1 in 10,000 children and accounting for 6% of childhood cancers [1, 2]. WT typically arises between the ages of 2 and 5 years, with 95% of cases diagnosed before the age of 10 years [3], although rare cases present in adulthood [4] or in utero [2]. The most common presentation is a palpable abdominal mass, with abdominal pain or hematuria being less frequent. Usually one kidney is affected (unilateral WT), presenting with a single mass, well distinguished from the adjacent kidney tissue by a fibrous capsule [5]; however, bilateral disease occurs in 5% of WT cases and is occasionally manifested in conjunction with other genitourinary malformations [2].

While the past four decades has witnessed a dramatic rise in WT survival rates—up to 90% with a multimodal therapeutic approach—patients in whom a relapse or metastatic disease develops show lower survival rates (around 50%) even with intensive salvage therapy [6]. Moreover, survivors are at increased risk of a broad spectrum of adverse outcomes caused by chemotherapy and radiation therapy, such as late mortality and secondary cancers [7, 8]. The cellular origin of WT is unclear. One

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possibility is that it arises due to the transformation of proliferating renal stem/progenitor cells [9–11]. A number of pieces of indirect evidence exist that support this notion. First, WT typically presents a triphasic histology (blastema, epithelium including immature tubules, glomeruli and stroma) containing multiple undifferentiated and differentiated cell types suggestive of a multipotent cell of origin [1]. Second, the blastema in WT resembles the metanephric mesenchyme of the human fetal kidney where embryonic renal stem cells reside [12–14]. Third, recent molecular data obtained by microarray studies demonstrate that WTs and WT stem-like xenografts steadily over-express genes [15, 16]) that normally regulate the renal stem/progenitor cell pool corresponding to the earliest stages of normal human kidney development. Fourth, recent epigenetic analyses of WT revealed epigenetic alteration in several key embryonic renal stem cell genes [17, 18].

It is becoming apparent that many, if not most, malignancies arise from a cell population that exclusively maintains the ability to self-renew and sustain the tumor via the expression of tumor-progenitor genes [19, 20]. These "cancer stem cells" (CSC) or "tumor initiating cells" (TIC) are often biologically distinct from the differentiated cancer cells that comprise most of the tumor bulk. Since CSC are thought to be primarily responsible for tumor initiation as well as resistance to chemo- and radiotherapy, their persistence after such treatments may account for relapsing disease in WT patients [21–24]. In this review, we discuss genomic, genetic and epigenetic studies as well as the initial analysis of WT carried out within the context of the CSC model, with the aim of shedding light on the WT cell of origin.

Genomic microarray studies

WT gene signature corresponds with early metanephric development

The assumption that the WT cell of origin is a transformed embryonic renal stem/progenitor cell is supported by the results of microarray studies. Global gene profiling during kidney development has provided novel insights into the genetic program that controls murine and human nephrogenesis and sets a reference for abnormal nephrogenesis [25–30]. Accordingly, Dekel et al. demonstrated that the overall gene expression profile of a WT specimen was most similar to that observed for an 8-week human gestation kidney and not later gestation kidneys [29]. Li et al. [15] conducted detailed experiments involving gene expression profiling in WT. They identified 357 genes that were differentially expressed between favorable histology WTs and fetal kidneys (16–22 weeks of gestation). To determine the stage-specific expression of these genes, they compared their data set to that previously obtained for normal rat nephrogenesis [26]. Comparative mapping of the two data sets revealed that WTs consistently over-expressed nephric-progenitor genes corresponding to the earliest stages of kidney development. This result implies that a significant portion of the blastema in WTs has progressed to progenitors committed to nephron formation and mesenchymal–epithelial transition, where it is partially arrested in differentiation.

WT "stemness" gene set elucidated from stem-like WT xenografts Complicating the genomic analysis of WT "stemness" has been the use of whole heterogeneous WT, comprised of mixed populations of immature (blastema) and more differentiated cell types (epithelia, stromal, heterologous elements). To circumvent this problem, we serially propagated WT tissues (originally derived from favorable histology non-syndromic WT) in immunodeficient mice, which resulted in the positive selection of the "stem/progenitor cell" compartment (WT blastema) rather than the primary tumors from which they were derived [16]. We next applied a strategy aimed at identifying normal and tumor-progenitor genes of the developing human kidney based on microarray analysis of these stem-like WT xenografts, human fetal kidneys (FK) and their counterparts, human adult kidney (AK) and renal cell carcinoma (RCC) [16, 31, 32]. Unsupervised clustering of their gene expression revealed that WT and FK cluster together, while AK and RCC form another group with a similar gene expression pattern; this separation allows for the identification of embryonic and adult normal and malignant renal gene sets (Fig. 1). This analysis and subsequent close examination and validation of the target genes in models of renal development, regeneration and tumorigenesis [18] revealed that the WT stem-like xenografts highly over-express a "WT stem/progenitor gene set" comprised of genes that have been shown to specify the normal nephron progenitor cell population (*PAX2*, *LIM1*, *EYA1*, *SIX1*, *SIX2*, *SALL1*, and *WT1*) [33–39], genes of the Wnt/β-catenin signaling pathway (*FZD7*, *FZD2*, *CTNNBIP1*), several imprinted genes and genes of the Polycomb group (*EZH2*, *BMI-1*). Both the Wnt pathway and the Polycomb-group genes are required to maintain stem cell pluripotency and plasticity of human embryonic stem cells (hESCs) during embryonic development [40] and have recently emerged as critical regulators of self-renewal signals of stem and cancer cells [41–49]. The role of the Wnt/β-catenin pathway in kidney development has been thoroughly described [39, 50, 51]. In addition, genes associated with a poor prognosis in WT have been found to be upregulated in the "blastemal-rich" WT xenografts, namely, topoisomerase 2A , *N-MYC* (correlating to a recent

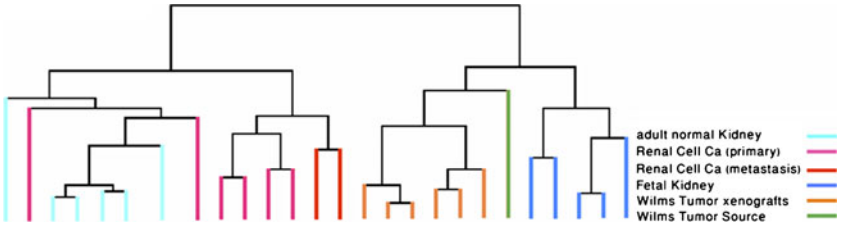

Fig. 1 Microarray analysis of normal and malignant embryonic and adult renal tissues. Unsupervised hierarchical clustering of 9,379 valid genes distinguishes embryonic tissues [fetal kidney tissues, Wilm's tumor (*WT*) source, and WT xenografts] from adult tissues (adult kidney tissues, primary and metastatic renal cell carcinoma) and tumors (primary and metastatic renal cell carcinoma, WT source and WT xenografts) from normal tissues (adult and fetal). Reproduced with permission from Dekel et al. [16]

finding of N-myc upregulation during the propagation of WT Xn, along with the appearance of the blastemal cell stem-like phenotype of the tumor [52]) and *CRAB2P* [53]. Therefore, a cancer stem cell fraction retrieved from primary WTs and residing within the blastema is anticipated to over-express this signature set of genes. In addition, surface antigens, such as neural cell adhesion molecule (NCAM) and FZD7, which are significantly over-expressed in microarrays of stem-like WT xenografts, could potentially serve as initial markers for the prospective isolation of stem/progenitor fractions from primary WTs.

Epigenetic studies

Epigenetic changes are found very early in tumorigenesis and can be even observed in normal tissues before tumors develop. Epigenetic changes have also been linked to normal pluripotent precursor cells from which cancers are thought to arise and may even serve as a predisposing factor [54, 55]. Importantly, early epigenetic changes could explain many of the heterogeneous properties that are commonly associated with the growth, invasive properties, metastasis and resistance to therapy of the tumor cell. Studying the epigenetic state of human cancers poses many hurdles, possibly due to the genetic instability found in most malignancies which masks the true underlying reason for the observed epigenetic alterations. In this regard, analysis of WT tumorigenesis is advantageous as genetic alterations are detected at a rather low frequency [17]. To study the relation of epigenetic changes in WT to the renal progenitor pool, we determined whether over-expression of the nephric progenitor genes observed in primary WT, particularly such over-expression in the stem-like WT-xenografts, is associated with epigenetic alternations [18]. Examination of the DNA methylation profile of such genes (*WT1*, *PAX2*, *SIX2*) disclosed relaxation at promoter regions in human fetal kidney, WT and, especially WT xenografts—compared that found in normal renal cells—that favor transcriptional activation and indicate that early renal stem/progenitor cells are likely to be accumulating in the tumor via disrupted epigenetic mechanisms that drive tumorigenesis ([18]; Fig 2]). Importantly, in progressive WT xenografts we observed repression of *SIX2* transcript levels associated with hypermethylation, which may indicate de-differentiation from the renal lineage. Very recently, Presser Aiden et al. [17]compared genome-wide chromatin profiles of heterogeneous WTs (they selected tumors with pronounced blastemal compartments to achieve more homogeneity), ESCs and normal kidney. They observed that WTs prominently exhibit large active chromatin domains previously observed in ESCs and that these domains frequently correspond to genes that regulate self-renewal of the nephron progenitor pool, such as *SIX2* [56, 57] or to the down-stream target of *SIX2*, *GDNF*, which induces branching of the ureteric bud [58–60]. In addition, WTs and ESCs both exhibited "bivalent" chromatin modifications at silent promoters that may be poised for activation. In WT, a subset of the bivalent promoters were found to correspond to genes expressed at specific renal epithelial structures, but showing low expression levels in WT, supporting the notion that WT cells are aligned with the normal renal lineage differentiation pattern. In addition, early stromal lineage genes (i.e. *FOXD1* and *LHX1*) were also shown to be bivalent, suggesting a source for WT blastemal cells that precedes the differentiation into epithelial or stromal lineages. Accordingly, these authors concluded that WT blastemal cells share an epigenetic landscape with a normal renal stem/progenitor cell that may possess a broader differentiation capacity than was previously suspected.

Wilms' tumor, fetal kidney and stem/progenitor cells—the Wnt/ß-catenin connection

The principles of the WNT signaling pathway are well established (for review see references [10, 24]). Briefly, extra-cellular WNT ligands trigger an intracellular signaling cascade that culminates in the translocation of the cytoplasmic signaling molecule ß-catenin to the nucleus, where it interacts with transcription factors of the TCF/Lef family

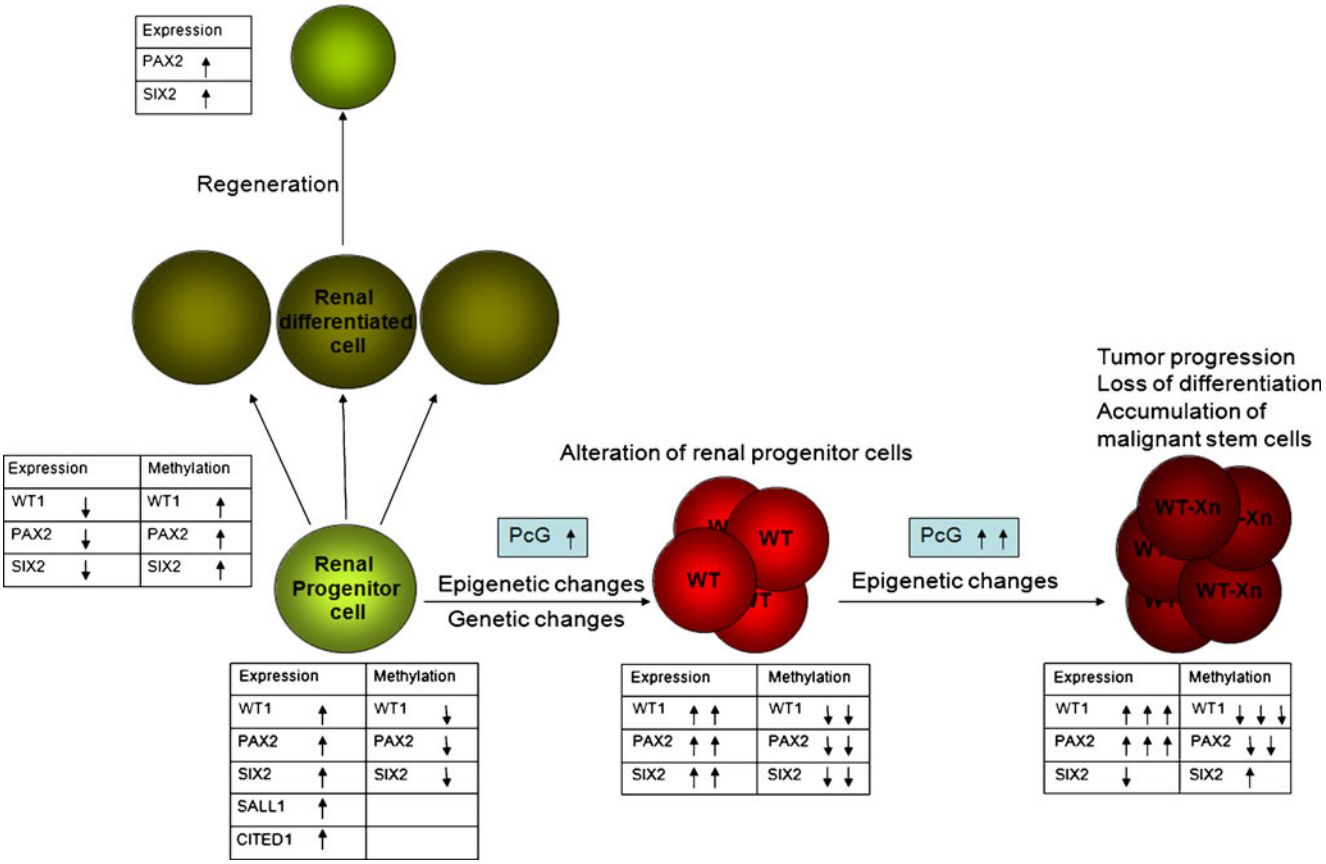

Fig. 2 A hypothetical model of epigenetic changes in renal progenitor cells during renal development and tumorigenesis. *PcG* Polycomb group, *WT* Wilms' tumor, *Xn* xenografts. Reproduced with permission from Metsuyanim et al. [18]

to induce a target gene program [15]. At least two WNT ligands, Wnt9b and Wnt4, are intimately involved in the epithelial differentiation of committed stem/progenitor cells in the metanephric mesenchyme [17, 61–63]. A summary of recent data suggests a model in which a WNT/ß-catenin/TCF/Lef1 signaling axis in the metanephric mesenchyme (MM), i.e. the precursor cells of the nephron, is required for the formation of the pre-tubular aggregate and, consequently, is a prerequisite for nephron development. Interestingly, an ectopic activation of WNT signaling in MM cells induces the formation of ectopic cell aggregates that express markers of the pre-tubular aggregate, including Pax8, Wnt4, Fgf8, and Lhx1 [16]. This finding suggests that the mechanism of ß-catenin stabilization is sufficient to initiate renal epithelial cell lineage progression up to the pre-tubular aggregate stage. Similarly, when ß-catenin is stabilized in isolated MM in organ culture, the cells escape apoptosis, proliferate and initiate pre-tubular aggregate marker expression [16, 17]. Importantly, polarized epithelia are observed in neither setting. In fact, E-cadherin expression is absent in the cell aggregates induced by the stabilization of ß-catenin, and the cellular transition to epithelial structures is completely blocked [16, 17]. Moreover, when ß-catenin is stabilized in the metanephric mesenchyme, the cellular transition to epithelial structures is completely blocked, even in the presumed presence of endogenous ureteric bud-derived inductive signals [28]. Together, these data indicate that ß-catenin/TCF/Lef1 signaling induces a partial program of cell differentiation in the MM, but it blocks progression to terminal nephron differentiation [16–18]. These observations may be relevant not only in the setting of renal organogenesis, but also during tumorigenesis. Notably, stabilization of ß-catenin is frequently observed in WTs. WTs are thought to be initiated in "nephrogenic rests", that is, islands of persistent blastema in the otherwise fully developed kidney [31]. Between 10 and 15% of WTs display oncogenic mutations of ß-catenin [32], while another one third are related to inactivating mutations of the X-linked tumor suppressor gene *WTX*, which normally associates with the ß-catenin degradation complex and promotes ß-catenin disposal [33], although WTX inactivation appears to be a late event in WT without apparent clinical impact [64]. Blastemal regions of WT express a series of genes also expressed in the metanephric mesenchyme [11, 19], suggesting that the response of these cells to an activation of ß-catenin/TCF/Lef1 signaling may be similar. Thus, in analogy to its effects in the metanephric mesenchyme, β-catenin signaling may contribute to the

differentiation arrest in WT and at the same time promote cellular proliferation. In fact, the WNT/β-catenin pathway is likely to be involved, although in different ways, in all WTs, including tumors without mutations. In accordance with this proposal, our previous genomic data showed overexpression of WNT pathway components in WT xenografts derived from tri-phasic classical tumors [1] and, very recently, we also found that primary WT cells obtained from tumors, which lack mutations in WT1/β-catenin, display sensitivity to a blocking antibody against frizzled7 (*FZD7*), a WNT receptor [35]. Application of the FZD7 antibody to the WT cells leads to apoptosis and cell death [35], hence these tumors are likely to depend on extracellular WNT ligand/receptor interactions for survival, which may resemble the molecular framework in normal renal progenitor cells mimicking early stages of fetal kidney development [65]. Clearly, clarification of the functional role of WNT signaling and its molecular downstream signals in specific cellular compartments of WT is required.

Wilms' tumor lineage hierarchy

The majority of WT are tri-phasic and composed of the three main tissues seen in the human embryonic kidney: undifferentiated blastema (mimicking the normal metanephric mesenchyme and normal kidney stem/progenitor cells), immature tubular epithelia (thought to arise by an attempt of the blastemal cell to differentiate toward renal tubules) and stromal elements (that may be analogous to the interstitium of the embryonic kidney). However, while the metanephric mesenchymal cells give rise to all cell types in the kidney, within the induced MM there exists a nephron epithelial progenitor (*Six2*+) that fulfils all of the criteria of a committed stem cell in that it is capable of self-renewing and differentiating to all nephron epithelia [56] and a separate stromal progenitor (*FoxD1*+) that gives rise to interstitial cells. There is no evidence to date that these lineages cross boundaries. Therefore, one can speculate on the existence of a common progenitor cell that is present early on in the intermediate mesoderm and which gives rise to stromal and epithelial lineages, such as the WT cell of origin. However, this cell type remains elusive. Mugford et al. [66] have recently shown that *osr1*+ cells give rise to all lineages of the kidney, but that the different lineages are distinct from one another at the earliest time points they examined [Embryonic day (E) 9.5 onward]; therefore, a common renal stem/progenitor might only exist earlier in the mesoderm. An alternative explanation for the WT lineage hierarchy is transformation of the *Six2*+ nephron stem/progenitor cell or of a yet to be identified epithelial renal stem cell that predominantly accumulates in blastema, partially differentiates to tubular elements, but also de-differentiates/trans-differentiates to take on a stromal fate which appears in the tumor. In fact, we have observed methylation and repression of *Six2* in the progressive WT xenografts, indicating de-differentiation into an earlier lineage [18]. Complicating the WT lineage hierarchy even further is the appearance of tumors that are not tri-phasic but rather have a stromal appearance and/or contain ectopic mesenchymal elements (e.g. rhabdomyoblasts, cartilage, osteoid tissue and fat). In these cases, the WT lineage hierarchy has been linked to genetic alternations. For instance, two-hit inactivation mutations in the *WT1* gene are often found in stromal predominant WTs, suggesting that cells lacking nuclear WT1 protein function begin a faulty differentiation program that does not follow mesenchymal–epithelial transition. It has also been suggested the E-cadherin, a distinct epithelialization marker, is a target of *WT1*, which might provide an explanation for the stromal appearance in tumors with *WT1* mutations [67]. Along these lines, a recent report evaluating WT cells with *WT1* mutations in vitro showed them to adopt a mesenchymal stem cell phenotype (although the in vitro culturing conditions possibly influence cells' gene expression and surface antigens) and express molecular markers of the paraxial mesoderm, a potential source of kidney stromal cells [68]. Tumors with *WT1* mutations and concomitant loss of the wild-type allele are frequently associated with mutations in β-catenin. Importantly, tumors harboring β-catenin mutations and constitutive canonical Wnt pathway activation similarly show mesenchymal lineage specificity (including myogenic lineages) via the induction of expression target genes [69]. In this context, tumors with *WT1*/β-catenin alterations may resemble sarcomas that arise from the embryonic mesoderm. Therefore, at this point in time, we cannot categorize all WTs under the heading of a "differentiation disease" caused by mutations hampering terminal differentiation of a renal stem cell; rather, we indicate a nephrogenic versus mesenchymal lineage in classical versus genetically altered WT. These potentially correspond to a differentiation stage of committed renal epithelial or non-committed mesenchymal stem/progenitor cells at the time of oncogenic transformation. Clearly, an understanding of a bona fide renal stem cell that is capable of generating all lineages in the kidney is needed. Only when we have acquired this understanding will it be possible to determine whether all WTs originate from a renal stem cell and to prove the hypothesis by generating transgenic mice for specific WT subtypes.

Cancer stem cells in WT

A growing body of evidence support the notion that many, if not most, malignancies arise from a relatively small

subpopulation of tumor cells termed cancer stem cells or tumor-initiating cells [21, 22, 55, 70]. Studies on CSC/TICs have shown that they are exclusively responsible for tumor formation and progression and, interestingly, that they are endowed with stem cell properties; in particular, tumor-stem cells share with normal stem cells their key features of self-renewal, differentiation capabilities, cell signaling pathways and drug resistance [22, 71–73]. In addition, a similarity in cell surface markers suggests that normal tissue stem cells may be the targets of oncogenic transformation and give rise to CSC [22]. In this context, prospective isolation of tissue-specific stem cells has been typically done based on surface marker expression, enabling the isolation of highly purified populations of stem/progenitor cells by flow cytometry [22]. The lack of stem cell markers in organs such as the kidney has hampered progress in identifying stem cells, emphasizing the importance of such markers for the characterization of normal and cancer stem/progenitor populations [18]. Over the past few years, progress has been made towards identifying normal and malignant human renal stem/progenitor cells utilizing phenotypic markers. Microarray analysis of stem-like WT xenografts and developing human kidneys have identified developmental surface antigens relevant for sorting out cell fractions from either the human fetal kidney or Wilms' tumor that may function as normal and cancer stem/progenitor cells, respectively. Sorting experiments have linked surface marker expression on human fetal kidney (hFK) or WT cells to the expression of the renal "stemness" genes [21, 22]: immunoselection based on NCAM1 (CD56) and the renal epithelial differentiation marker EpCAM (CD227) [74] pinpointed putative MM stem/progenitors (NCAM+EpCAM-) that over-expressed the renal progenitor genes *Six2*/*Cited1*/*Sall1*/*Wt1*, *vimentin* and *E-cadherin*, indicating the presence of early progenitors that have not yet differentiated to more mature ones (NCAM+EpCAM+) [21]. Similarly, working in vitro with primary cultures of classical WT, we were able to define an $NCAM^+$ cell population that is a strong candidate for the tumor stem/progenitor cell population [22]. Phenotypically, these primary WT cultures mostly lacked mesenchymal or hematopoietic stem cell markers. The sorted $NCAM^+$ tumor cell fraction from cultures, and not $CD133^+$ cells, was shown to be highly clonogenic, sphere-forming and over-expressing the renal "stemness" signature set predicted by our previous microarray experiments [19, 22], i.e. renal progenitor genes (*Wt1, Six2, Osr1, Pax2*), Polycomb-group members (*Ezh2*, *Bmi1*) and the Wnt pathway member ß-catenin (*CTNNB1*). In addition, the $NCAM^+$ fraction highly expressed vimentin and showed low E-cadherin expression, which resembles the early MM stages of kidney development [22]. We specifically demonstrated high levels of the poor prognostic marker TOP2A in the $NCAM^+$ cell fraction and the down-regulation of both TOP2A and NCAM following etoposide or irinotecan treatment of WT cells, suggesting that the NCAM cell population is likely to be especially susceptible to topoisomerase inhibitors. In terms of molecular marker expression, these data show similarities between $NCAM^+$ progenitors from hFK and WT; it therefore appears likely that these cell populations are derivatives of the same lineage. However, the in vitro studies were unable to functionally define human CSCs, a definition based on the enriched capacity of prospectively isolated single cells to regenerate cancers and recapitulate the heterogeneous phenotypes of the parental cancer from which they were derived using xenograft mouse models. Moreover, one has to show that the CSCs are able to reproduce themselves through the process of self-renewal, which can be studied in serial mouse transplantation assays [21, 22]. Importantly, while WT xenografts are readily formed via implantation of fresh surgical samples into immunodeficient hosts [16] (80% take rate), WT is notorious for its inability to form xenografts from single-cell suspensions of fresh primary WT and especially after culture and in vitro growth [31] (Fig. 3). This lack of in vivo tumorigenicity might be related to rapid differentiation of the stem/progenitor cell fraction in culture [18, 22, 74]. In fact, tumorigenic favorable histology WT cell lines are not available. Interestingly, the progenitor population of the condensed MM that gives rise to nephron epithelia has yet

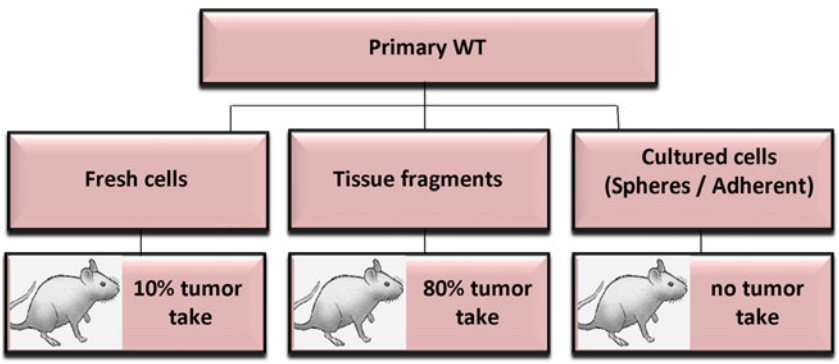

Fig. 3 Hurdles in the establishment of WT xenografts from single cells. WT xenografts are readily established from tissue fragment implantation (80% graft take) but are rarely formed by the injection of single fresh primary (10% graft take) or cultured (0% graft take) WT cells, respectively

to be cultured, probably as result of lack of appropriate niche factors, hypothetically drawing a line between the WT CSCs and the renal epithelial stem cells.

Altogether, taking into account these inherent limitations of WT and the fact that cell sorting and heterotransplantation in immune-deficient host animals require large numbers of the rather rare human Wilms' tumors, model systems that allow for in vivo studies of WT at the single cell level are warranted.

Conclusions

Data accumulating from molecular studies of classical WT indirectly suggest an embryonic renal stem/progenitor cell origin for this malignancy. Initial application of the cancer stem cell model to WT and subsequent functional analysis of individual WT subpopulations that express molecular renal "stemness" markers reflect the first steps in determining the relationship of WT CSCs/TICs and renal stem cells. NCAM has been highlighted as a putative target for future therapies. Further in vivo studies assessing the functional properties of single xeno-transplanted WT cells are crucial for the identification of WT stem cells and for definitely pinpointing novel therapeutic targets for this disease.

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