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Review

The development of Wilms tumor: From WT1 and microRNA to animal models

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ABSTRACT

Wilms tumor recapitulates the development of the kidney and represents a unique opportunity to understand the relationship between normal and tumor development. This has been illustrated by the findings that mutations of Wnt/β-catenin pathway-related WT1, β-catenin, and WTX together account for about one-third of Wilms tumor cases. While intense efforts are being made to explore the genetic basis of the other two-thirds of tumor cases, it is worth noting that, epigenetic changes, particularly the loss of imprinting of the DNA region encoding the major fetal growth factor IGF_2, which results in its biallelic over-expression, are closely associated with the development of many Wilms tumors. Recent investigations also revealed that mutations of Drosha and Dicer, the RNases required for miRNA generation, and Dis3L2, the 3′–5′ exonuclease that normally degrades miRNAs and mRNAs, could cause predisposition to Wilms tumors, demonstrating that miRNA can play a pivotal role in Wilms tumor development. Interestingly, Lin28, a direct target of miRNA let-7 and potent regulator of stem cell self-renewal and differentiation, is significantly elevated in some Wilms tumors, and enforced expression of Lin28 during kidney development could induce Wilms tumor. With the success in establishing mice nephroblastoma models through over-expressing IGF_2 and deleting WT1, and advances in understanding the ENU-induced rat model, we are now able to explore the molecular and cellular mechanisms induced by these genetic, epigenetic, and miRNA alterations in animal models to understand the development of Wilms tumor. These animal models may also serve as valuable systems to assess new treatment targets and strategies for Wilms tumor.

Contents

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1. Wnt/β-catenin pathway and the development of Wilms tumor

1.1. Introduction

Wilms tumor, also known as nephroblastoma, is the most common childhood kidney tumor. It affects ~1 in 10,000 children and accounts for ~8% of pediatric cancers. The tumor is usually discovered before the age of 6 years old and is regarded as a prototype of tumors originated from abnormal differentiation and development [1]. Though the overall survival rate for Wilms tumor has reached 90%, a significant portion of the patients have survival rate less than 70%, and up to 25% of the survivors suffer from serious chronic health conditions 25 years after diagnosis, underscoring the importance for exploring the molecular and cellular mechanisms of the tumor [2]. The cancer genome project has revealed that, while adult human cancer genomes often contain more than 100 genetic mutations, pediatric cancers such as neuroblastomas and acute lymphocytic leukemia usually have only ~10 mutations [3]. Although the whole genome studies of Wilms tumor remain to be completed, it is conceivable that Wilms tumor might harbor a similar small number of genetic alterations that are responsible for the development of the tumor.

The development of the mammalian kidney depends on the reciprocal interactions between the ureteric bud and metanephric mesenchyme (blastema), which undergoes mesenchymal-to-epithelial transition (MET) to form the epithelial and stromal components of the kidney [4]. It is evident that Wnt9b generated from the ureteric bud and Wnt4 in the mesenchyme play critical roles in the survival, self-renewal, and differentiation of mesenchymal progenitor cells. Recent studies also indicated that signals from the stromal fibroblasts, including Fat4-mediated modulation of the Hippo pathway, cooperate with Wnt9b to promote differentiation of the progenitors in the developing kidney [5]. Most Wilms tumors have a triphasic histology composed of blastema, stroma, and epithelium, recapitulating the kidney development. As a further indication of aberrant differentiation, heterologous elements, such as cartilage and smooth muscle, are observed in some Wilms tumors. Pathological examination also found that clusters of embryonic cells (nephrogenic rests, NR) present in developed kidneys are often precursors of Wilms tumor cells [6]. Microarray analysis found that genes corresponding with the early stage of metanephric development are highly expressed in Wilms tumor, whereas genes corresponding with later kidney development stages are expressed at lower levels [7]. Similar phenomena were observed at protein level using selected markers. A recent study showed that Six2 (a homeodomain transcription factor)-positive progenitor cells, which normally reside adjacent to the ureteric tips in the nephrogenic zone of developing kidney and are largely absent in mature kidney, are extensively present at significantly increased numbers in Wilms tumor tissue [8]. It has also been found that Six2 acts together with the zinc-finger protein Osr1 to maintain nephron progenitor cells in the developing kidney [9]. Notably, it has been shown recently that transient expression of iPS reprogramming transcription factors in mice can induce kidney tumors which consist of undifferentiated dysplastic cells and share a number of characteristics with Wilms tumor [10]. These observations at both morphological and molecular levels led to the general belief that Wilms tumor originates from embryonic mesenchymal progenitors that fail to undergo normal differentiation. Furthermore, it is evident that the tumor may arise from different development stages of the progenitors and manifest as a number of heterogeneous groups [11].

1.2. WT1

The association of Wilms tumor with development is further strengthened by the greatly increased risk of developing Wilms tumor in a number of rare abnormal development-associated syndromes, such as WAGR syndrome, Denys–Drash syndrome, Perlman syndrome, and Beckwith–Wiedemann syndrome. The rate of developing Wilms tumor ranges from 95% in WAGR syndrome to 5% in Beckwith–Wiedemann syndrome. WAGR syndrome is characterized by developing Wilms tumors, Aniridia, Genitourinary abnormalities, and mental Retardation. Mutations in the WT1 gene were found to be responsible for the development of Wilms tumor in WAGR patients and also in ~20% of sporadic cases [12]. Further studies revealed that, depending on the cell line used, WT1 may act as both activator and repressor of certain genes, such as c-myc and bcl-2. It appears that these variations in WT1 transcription activities are due to post-translational modifications, such as phosphorylation and interaction with other proteins and RNAs [13]. As suggested by its expression in gonads, spleen, and the lining of the abdominal cavity, loss-of-function mutations of WT1 were also found in Frasier syndrome, mesothelioma, and some adult myeloid leukemia. However, many investigations found that wild type WT1 is highly expressed in a variety of human cancers, including brain tumors, carcinomas, adenocarcinomas, sarcomas, and leukemia, which led to a clinical trial using peptides derived from WT1 as antigens for immunotherapy [14]. It has also been shown that WT1 promotes the survival of cancer cells in culture and may confer resistance on them to chemotherapeutic agents, and increased expression of WT1 in tumors is associated with poor prognosis following chemotherapy [15]. Furthermore, a recent study using primary Wilms tumor cell lines found that mutated WT1 is required for the proliferation of these cells, likely due to its ability to interact and antagonize the suppressive function of p53 [16]. Thus, WT1 has a complicated role in tumorigenesis. It may act as tumor suppressor or oncogene under different circumstances, and mutation of WT1 could result in gain-of-function to promote the growth of tumor.

The expression and function of WT1 during kidney development have been investigated extensively. WT1 is first expressed at low levels in intermediate mesoderm of E9 embryo and later in the metanephric mesenchyme. Its expression level is significantly increased in the condensed mesenchymal cells that surround the ureteric bud, suggesting that it may play an important role in the differentiation of these cells. It is also expressed in the renal vesicle and in the proximal segment of the comma and S-shaped bodies. In the mature kidney, WT1 expression is mainly restricted to the glomerular podocytes, a highly specialized visceral epithelial cell essential for maintaining the glomerular filtration barrier [17]. The importance of WT1 in kidney development has been clearly demonstrated by the finding that WT1 deficient mice fail to develop kidneys [18], and conditional deletion of WT1 after E11.5 caused the disruption of metanephric mesenchyme differentiation and almost no mature nephrons were generated in the mice [17,19]. It was also found that knockdown of WT1 with siRNA in explant culture of E11.5 kidney resulted in blockage of nephron development at the pre-epithelial stage [20]. Therefore, WT1 is required for the survival and differentiation of kidney progenitors. It is worth noting that the expression of WT1 in podocytes of mature kidney is also essential for the function of glomerulus. Altered WT1 expression in mice podocytes, and mutations of WT1 in Denys–Drash syndrome and Frasier syndrome, all lead to glomerulosclerosis and eventually renal failure [21,22].

To understand how WT1 regulates kidney progenitor cell survival and differentiation, extensive efforts have been made to find genes regulated by WT1. Based on studies using degenerate oligonucleotides

that bind to WT1, reporter constructs containing multiple binding sequences, and various cell lines, several dozen genes have been identified as potential WT1 targets [23]. They include transcription factors, such as c-Myc and Pax-2, growth factors, such as IGF_2, PDGF, and TGF-β, and growth factor receptors, such as IGF-IR, EGFR, and insulin receptor. However, it is currently unknown whether these genes are indeed WT1 targets that participate in kidney development. To further identify WT1 target genes that regulate nephron progenitor differentiation, chromatin immunoprecipitation with anti-WT1 antibody coupled with a mouse promoter microarray was performed using chromatin prepared from more than 1000 embryonic mouse kidneys [24]. Consistent with its essential role in the survival of kidney progenitor cells, WT1 regulated target genes include Sall1, Pax2, Six2, and FGFR2. The regulatory action of WT1 on multiple components of the TFG-β/BMP system may also contribute to its effect on mesenchymal cell differentiation during nephrogenesis. Significantly, Wnt4 is a critical mediator of ureteric bud-derived Wnt9b, and is required for MET during nephron formation [25,26]. It has been shown that WT1 is a direct transcriptional activator of Wnt4 in mesenchymal cells [17,27]. Further studies found that WT1 affects the chromatin state of Wnt4 locus, and ectopic application of recombinant Wnt4 into WT1-deficient kidneys could rescue the MET defect [28], indicating that Wnt4 is a mediator for WT1-promoted MET.

Wnt signaling plays an important role in many development processes, including cell proliferation, differentiation, adhesion, polarity, pattern formation, and morphogenesis [29]. In the developing kidney, a number of Wnt ligands, such as Wnt2b, Wnt4, Wnt5b, Wnt6, Wnt7b, Wnt9b, and Wnt11, are expressed in a temporally and spatially regulated manner [30]. Studies using genetically engineered mice, particularly conditional or inducible knockout mouse models, have revealed the critical roles of individual Wnt ligand in the development processes. Targeted deletion of Wnt11, which is mainly expressed at the tips of the branching ureteric buds, resulted in ureteric branching defects [31], whereas pretubular aggregates fail to form in Wnt4 knockout mice [25]. Further studies using culture explants of isolated metanephric mesenchymes demonstrated directly that Wnt4 promotes mesenchymal to epithelial transformation [32], indicating that Wnt4 is both required and sufficient for initiating the tubulogenesis process. Interestingly, Wnt9b deleted mice also fail to undergo MET and fail to express Wnt4 [26]. Since Wnt4 is able to induce the differentiation of mesenchymes from Wnt9 deficient mice, these results suggest that ureteric bud-derived Wnt9b promotes Wnt4 production, which in turn orchestrates mesenchymal cell differentiation and nephrogenesis. It is conceivable that a loss-of-function mutation of WT1 results in decreased Wnt4 and blockage of progenitor cell differentiation, which eventually leads to transformation of these cells and the development of Wilms tumor. This is also consistent with the pathological observation that Wilms tumors harboring WT1 mutations are often associated with intralobar nephrogenic rests and contain predominantly stroma [33], reflecting the role of WT1/β-catenin in the differentiation of kidney progenitor cells. Interestingly, muscle cells, and adipose, bone, and cartilage tissue may also be present in these Wilms tumors. Further examination of these stromal cells might provide important clues for understanding the mechanisms of differentiation toward different lineages.

1.3. WTX

Molecular genetics analysis indicated that approximately 20% of sporadic Wilms tumors contain a WT1 mutation or deletion [34]. It has also been found that ~15% of Wilms tumors harbor mutations in β-catenin, a critical signal transducer for Wnt signaling. Since the mutations are often in frame deletions or substitutions, they lead to stabilization of the protein and enhanced signaling [35]. It is worth noting that ~80% Wilms tumors with WT1 mutations also have β-catenin mutations, suggesting that WT1 mutations function together with the canonical Wnt signaling pathway to induce Wilms tumor. WTX (Wilms tumor gene on X chromosome) was first identified through high-resolution comparative genome hybridization analysis, and its loss-of-function mutations were found in ~20% of Wilms tumors [36]. The protein does not contain domains with significant homology to genes of known function. Germline inactivation of WTX results in osteopathia striata with cranial sclerosis (OSCS) syndrome in humans, but does not predispose to any tumors [37]. In mice, global deletion of WTX leads to neonatal death and significant defects in multiple tissues, including the kidney, that are derived from mesenchymal progenitor cells [38]. In an effort to find proteins interacting with β-catenin, WTX was found to regulate β-catenin through promoting its ubiquitination and degradation [39]. It was also shown that mesenchymal cells from WTX-deficient mice have defects in cell fate determination, which is due to aberrant β-catenin activation [38]. These results indicate that the β-catenin-modulating activity of WTX plays an important role in normal development, and likely in the development of Wilms tumors, which is also consistent with the finding that WTX and β-catenin mutations are rarely detected in the same Wilms tumors. Interestingly, it was also found that WTX interacts with p53 and enhances its acetylation by CBP/p300, which was found to be indispensable for p53 activation [40]. It is conceivable that the functional inactivation of p53 may contribute to the observation that only 5% of Wilms tumors were identified having p53 mutations. Since WTX mutations appear equally in tumors with or without WT1/β-catenin mutations, they together account for only about one-third of Wilms tumors, necessitating continued efforts to explore additional genetic, as well as epigenetic and microRNA, changes that may contribute to the development of Wilms tumors.

2. Epigenetic changes associated with the development of Wilms tumor

2.1. Epigenetic changes in cancer

Epigenetic changes, including DNA methylation, post-translational modification of histones, and chromatin remodeling, have profound effects on gene expression and affect cell growth, differentiation, and development. Dysregulation of these epigenetic modifications have been widely implicated in the formation of various cancers [41]. It has been found that aberrant promoter hypermethylation, which could lead to silencing of tumor suppressor genes or miRNAs, such as Rb, p16 (CDKN2a/INK4a), and miR124a, is a common alteration in human neoplasia [42,43]. Cancer cell genomes are also often found to be globally hypomethylated and harbor genome-wide alterations in histone modifications, which have been associated with chromosomal instability, loss of imprinting, and oncogene activation. It is worth noting that epigenetic changes in cancer cells may also result from genetic mutations as illustrated by the frequent mutation of histone-modifying genes in non-Hodgkin's lymphoma [44]. Moreover, it has been reported that a single nucleotide variant within the 5′UTR of DNA mismatch repair gene MLH1 caused promoter DNA methylation and silencing of MLH1, which was linked to youth-onset cancer [45]. Therefore, cancer could be regarded as a disease resulting from genetic as well as epigenetic alterations. These results also led to the development of new therapeutics targeting the epigenetic machinery. For example, DNA methyltransferase inhibitors and histone deacetylase inhibitors have been used effectively as anti-cancer drugs [46].

2.2. IGF_2 and loss of imprinting

Beckwith–Wiedemann syndrome, characterized by multi-organ overgrowth and predisposition to embryonal tumors, including Wilms tumor and hepatoblastoma, is associated with loss of imprinting and abnormal expression of genes, including insulin-like growth factor II (IGF_2) and the non-translated RNA H19, in chromosome 11p15 [47]. It has been found that, in the normal maternal chromosome, the DNA

region between H19 and IGF_2 is bound to the zinc finger protein CTCF and promotes the formation of a chromatin structure that prevents the expression of IGF_2, whereas in the paternal chromosome, the DNA region is methylated, which blocks CTCF binding and allows the expression of IFG_2. In a subgroup of patients with Beckwith–Wiedemann syndrome, a maternally inherited 1.4–2.2 kb deletion in the DNA region results in DNA methylation and loss or reduction of CTCF binding, leading to biallelic expression of IGF_2 [48]. It was also found that ~70% of Wilms tumors have a loss of imprinting at 11p15 and showed biallelic expression of IGF_2 gene [49,50]. Further investigations showed that multiple abnormalities in the region, including abnormal methylation, paternal uniparental disomy, microdeletion, and microinsertion, are associated with the loss of imprinting and Wilms tumor [51,52]. It is conceivable that, similar to the situation in Beckwith–Wiedemann syndrome, these changes alter CTCF binding, resulting in overexpression of IGF_2 that contributes to the development of Wilms tumor. Interestingly, it was found that the loss of imprinting and abnormal methylation in the IGF_2/H19 region can be detected early in Wilms tumor kidney tissues and in kidney tissues adjacent to Wilms tumors, suggesting that the epigenetic changes may represent an early event during the tumor development [53,54]. Interestingly, Wilms tumors with IGF_2 overexpression are mainly associated with perilobar nephrogenic rests and could predominantly consist of blastema differentiated at a lower level [33], indicating that IFG_2-mediated signal suppresses the initiation of differentiation. Notably, the incidence of Wilms tumor in East Asian children is about half of that in Caucasian children. While the mutation rates of WT1, WTX, and β-catenin in Wilms tumors from Japanese children are similar to those of children of other Asian races, the rate of loss of imprinting in IGF_2 region is only ~21%, suggesting that the lower percentage of epigenetic change may be associated with the lower incidence of Wilms tumors [55].

2.3. Additional epigenetic changes in Wilms tumor

It is evident that there are many other epigenetic changes in Wilms tumors. Polycomb group proteins play an important role in chromatin remodeling and gene silencing during development [56]. It has been shown that polycomb group genes, including EZH2, BMI-1, and SUZ12, are up-regulated in Wilms tumor xenografts, and are down-regulated when tumor cells are induced to differentiate [57]. It was also found that the promoters for PAX2, WT1, and SIX2 are all hypermethylated in adult kidneys, whereas they are hypomethylated in fetal kidneys and Wilms tumors, suggesting that epigenetic changes are closely associated with kidney and Wilms tumor development. The abnormalities of methylation in Wilms tumor were also revealed by the finding that satellite DNA (Sat α and Sat 2) is significantly hypomethylated in Wilms tumors with loss of imprinting in IGF_2/H19 region [58]. Based on the observation that maps of histone H3 trimethylated at lysine 4 (K4me3), lysine 36 (K36me3), or lysine 27 (K27me3) identify promoters, transcripts, or sites of polycomb group protein repression, respectively, chromatin immunoprecipitation with anti-K4me3, K36me3, and K27me3 antibodies and DNA sequencing (ChIP-Seq) were utilized to analyze genomic landscape in Wilms tumors [59]. It was found that Wilms tumors exhibit large active chromatin domains that were found in embryonic stem cells. These domains correspond to genes that are critical for kidney development and provided epigenetic evidence that Wilms tumor cells originate from kidney progenitor cells. It is conceivable that some of these epigenetic changes likely represent critical initiating events in the development of Wilms tumor. Interestingly, it was found that WT1 regulates the transcription of DNA methyltransferase 3A and affects methylation of certain promoters in cells [60] adding another layer of complexity to the relationship between epigenetic modification and Wilms tumors. Finally, it is worth noting that many cancers, including Wilms tumors, have increased methylation variation compared to normal tissues [61], suggesting that both genomic and epigenomic instabilities contribute to tumor heterogeneity.

3. The miRNA system and development of kidney and Wilms tumor

3.1. Introduction

MicroRNAs (miRNAs) are small non-coding RNAs (20–25 nucleotides) that negatively regulate gene expression at the post-transcriptional level. Their biological roles were first revealed during screening for loss of function mutations in *Caenorhabditis elegans* larval development, which revealed that miRNA-coding genes *lin-4* and *let-7* control development by modulating the expression of other genes at the post-transcriptional level [62]. It is now evident that miRNAs exert influences on most, if not all biological processes, including cell growth and differentiation, programmed cell death, tissue and organ development, tumorigenesis, and tumor metastasis. The molecular process of miRNA production has been gradually elucidated in the last decade [63]. It starts with the transcription of genomic sequences coding for miRNAs by RNA polymerase II. This produces the long precursor pri-mRNAs, which are processed by the complex containing RNase Drosha and its cofactor Dgcr8 to form pre-miRNAs. After being transported into the cytoplasm through an exportin 5-dependent process, pre-miRNAs are cleaved by RNase Dicer to generate mature miRNAs that are then incorporated into the RNA-induced silencing complex (RISC) by associating with the Argonaute proteins. A miRNA in the RISC can bind to the 3′ untranslated region of a target mRNA, leading to inhibition of translation through preventing elongation or cleavage of the mRNA. It appears that higher levels of complementarities between the miRNAs and their targets are more likely to induce mRNA degradation, whereas lower levels of complementarities often result in inhibition of translation alone [64].

Studies exploring the roles of miRNA in biological systems have been complicated by the observation that each miRNA has many putative target mRNAs, and it is often quite a challenge to determine which one is important and thus worthy of experimental validation. It has also been shown that multiple miRNAs are often involved in the regulation of a particular protein or cellular process. Additionally, miRNAs are frequently present as families of redundant genes, which make genetic dissections more difficult. Conversely, it has been found that the down-regulation of target proteins by miRNAs is often modest, typically leading to less than 50% reduction. These observations may explain the findings that most *C. elegans* miRNAs are individually not essential for development or viability [65]. Thus, the multi-components and quantitative natures of signal transduction pathways may be primary targets of miRNAs, which would provide effective feedback or feed-forward modulations [66].

3.2. miRNA associated with kidney development and Wilms tumor

The potential critical role of miRNAs in developmental processes prompted extensive efforts to examine the expression of miRNAs during kidney development, using both microarrays and next generation sequencing [67]. While miRNAs let7, miR-10a/b, miR-21, miR-30, miR-196a/b, and miR-451 are highly expressed in multiple organ systems during development, some miRNAs were found enriched specifically in the developing kidney, including miR-19a/b, miR-31, miR-146a, miR-192, miR-194, miR-204, miR-215, miR-216, and miR-886 [68]. Compared with mature kidney, miR-192, miR-215, and miR-194 had a significantly lower expression profiles in Wilms tumors regardless of the subtypes, whereas miR-141 and miR-200c showed a significantly lower expression in blastema-type and mixed-type tumors. Meanwhile, their common target, the activin receptor type 2B, a member of the TGF-β receptor family, is highly expressed in the tumors [69], suggesting these miRNAs are modulators of TGF-β pathway during kidney development. Another study found that the expression of a group of miRNAs

(miR-17.5p, miR-18a, miR-19b, miR-92, and miR-20a) is up-regulated in Wilms tumors compared with other kidney tumors or normal kidney tissues, likely due to the increased expression of transcription factor E2F3 [70]. Since they are also highly expressed in multiple cancers and E2F3 is upregulated more significantly in later stages and metastatic Wilms tumors, it is likely that these miRNAs assist in tumor progression. Interestingly, it has been found that specific miRNA families, such as the miR302/367 cluster or a combination of mir-200c, mir-302s, and mir-369s family miRNAs, have the ability to reprogram somatic cells into induced pluripotent cells (iPCs) [71,72]. Whether and how these miRNAs participate in kidney and Wilms tumor development is certainly worth further investigation. With the rapid development and application of new techniques, it is conceivable that more miRNAs will be identified associated with the development of Wilms tumor. However, the more difficult challenges are verifying their cause-effective action in tumor development and identifying their targets.

The critical roles of kidney miRNAs have been explored with various conditional knockouts of Dicer and Drosha. Using the Six2 promoter-controlled Cre, it was shown that deletion of Dicer in nephron epithelium progenitors caused increased apoptosis and reduced nephrogenesis and nephron formation [73,74]. Significantly, increased expression of the pro-apoptotic protein Bim is observed in Dicer-deleted kidney, and a number of miRNAs highly expressed during nephrogenesis are either known or predicted to target the pro-apoptotic protein Bim, suggesting that a lack of Dicer leads to reduction of these miRNAs and increased Bim, which is responsible for kidney progenitor cell apoptosis [74]. Furthermore, removal of Dicer from ureteric bud cells by HoxB7/Cre led to the disrupted branching, and development of renal cysts [73], indicating that miRNAs regulate the growth and differentiation of both ureteric bud and mesenchymal cells. The roles of miRNAs in kidney development have also been examined in *Xenopus pronephros* [75]. Using morpholino antisense oligonucleotides against Dicer and Dgcr8, it was found that miRNAs are required for patterning and terminal differentiation of pronephros. The miR-30 family was identified as an essential player, and the transcription factor Xlim-1/Lhx1 was found to be a major target of miR-30. It is of great interest to know whether miR-30 plays the same role in the development of metanephrons in mammalians. Intriguingly, in mice, conditional knockout of Dicer or Drosha in developing podocytes did not affect the formation of glomeruli, but the mice developed marked proteinuria soon after birth, which rapidly developed to renal failure [76], indicating that miRNAs are also required for maintaining the structure and function of mature podocytes. Similarly, deletion of Dicer in renin-producing juxtaglomerular apparatus resulted in loss of juxtaglomerular cells, leading to decreased plasma renin, reduced blood pressure, and vascular changes, whereas removal of Dicer from the proximal tubule cells did not affect their development and physiological function [77]. Given the involvement of miRNAs in multiple stages of kidney development, particularly in the progenitor cells, it is not unexpected that a subset of Wilms tumor contains the two "hits" Dicer1 mutation [78]. More recently, it was also found that mutations of Drosha were present ~12% of sporadic Wilms tumors [79]. Furthermore, missense mutations of Dicer were found to occur in ~20% of nonepithelial ovarian tumors, further supporting the notion that perturbation of microRNA processing may be oncogenic [80].

Let-7 is one the first miRNAs identified in *C. elegans* that regulates larva developmental timing [81]. It functions by directly targeting the translation of other heterochronic genes including Lin28, and is expressed at high levels in various differentiated cells. Further investigations found that the level of Let-7 is mainly regulated by Lin28, which inhibits let-7 precursor processing in undifferentiated cells [82]. Recent studies revealed that Lin28 binds to pre-let-7 RNA and recruits 3′ terminal uridylyl transferase, which uridylates pre-let-7 RNA, leading to its resistance to Dicer processing and quick degradation by the newly identified 3′–5′ exonuclease Dis3L2 [83]. It has been showed that Dis3L2 is also required for mRNA degradation [84,85], indicating it is a critical nuclease in regulating miRNA and mRNA. Intriguingly, germline mutations in Dis3L2 cause Perlman syndrome, characterized by overgrowth at birth and an increased risk to Wilms tumor at an early age [86]. Significantly, Lin28 is highly expressed in undifferentiated embryonic stem cells, its overexpression can facilitate the reprogramming somatic cells, and its activation is presented in about 15% of cancers analyzed [87,88]. Therefore, it is conceivable that the Lin28 let-7 system may play a major role in the development of Wilms tumor in individuals with a Dis3L2 mutation. Interestingly, it has been showed recently that induced expression of Lin28A or Lin28B during kidney development resulted in prolonged proliferation of $Six2^{+}$ progenitor cells and development of Wilms tumor [89]. The phenotype can be suppressed by enforced expression of let-7, demonstrating that the miRNA plays a critical role in the development of these Wilms tumors. Furthermore, increased Lin28B expression was observed in ~17% of human Wilms tumors, and the expression is associated with tumor relapse and mortality [89]. It is worth noting that, while mutations of WT1/β-catenin and alterations of the IGF2 coding region associate with blastemal and stromal types of Wilms tumor respectively, both Dicer mutation and Lin28 overexpression-related Wilms tumors tend to have triphasic histology [78], suggesting that miRNA may act on multiple stages of the differentiation process. These results indicate that further examination of miRNA expression in Wilms tumor might help to understand the pathogenesis and discover new therapeutic targets.

4. Nephroblastoma in animal models

One of the major challenges in understanding Wilms tumor development is a lack of proper cell lines and animal models to explore the molecular mechanisms. SK-NEP-1, a cell line previously thought to represent anaplastic Wilms tumor, is instead related to Ewing sarcoma and contains the EWS-FLI1 gene fusion transcripts characteristic of Ewing sarcoma [90]. Cell lines such as WiT 49 and 17.94 were derived from metastatic Wilms tumors and represent anaplastic tumors, which harbor mutations in p53 and likely many secondary genomic alterations [91]. Since inoculation of these cells under kidney capsule generally does not produce blastema-containing triphase Wilms tumor, they are not very informative in understanding the mechanisms of Wilms tumor development. It is worth noting that, although dispensed Wilms tumor cells generally could not form tumors in immune-deficient mice, xenografted Wilms tumor tissues do survive and grow, and have become a useful model in pediatric preclinical testing program for novel anti-tumor agents studies [92].

It was first observed in the 1970s that nephroblastomas could spontaneously develop in rats, particularly in the Noble strain rat [93]. Subsequent studies demonstrated that transplacental administration of the carcinogen N-ethylnitrosourea (ENU) at E18 induced nephroblastomas in ~50% of Nb rats, whereas similar exposure to another carcinogen (DMN) did not lead to nephroblastoma in these rats [94]. Like Wilms tumors in humans, the ENU-induced tumors contain the triphasic histology that consists of blastemal, stromal, and epithelial components, and the fetal rats are most susceptible to ENU induction on E14–E18 [95], when metanephric differentiation and nephrogenesis are most active in mice. These results indicated that induction of nephroblastoma by ENU in Nb rats could serve as a valuable model to explore the molecular mechanisms of Wilms tumor. Studies in our laboratory also found that the F344 rat, which has been widely used for chemical carcinogenesis studies, rarely developed nephroblastoma when given ENU transplacentally during the final week of gestation, although they did develop ENU-induced neurogenic tumors [96]. To study the inheritance of the susceptibility, fetal Nb and F344 hybrids (F1) and reciprocal backcross hybrids (F2) were exposed to ENU transplacentally and the incidence of nephroblastoma was assessed in these offspring. The result is consistent with the involvement of a major autosomal locus with incomplete penetration [97]. Sequence analysis found that WT1 and WTX are not mutated in these tumors, and microarray analysis of developing Nb and F344 kidney (E14) and nephroblastomas was carried out to examine the

gene expression profiles. It is worth noting that the expression of a number of genes, including epidermal growth factor receptor, inhibitor of DNA binding 2, and Notch ligand Jagged 1, are increased in Nb rat and further elevated in nephroblastomas [97]. Since many of these genes play essential roles in development and differentiation, these results further demonstrate the value of this model for non-WT1-associated nephroblastoma. We have also performed genome-wide association studies to investigate the locus involved in the susceptibility to ENU-induced nephroblastoma in Nb rat. It is conceivable that, together with the whole genome sequencing of Nb rat and ENU-induced nephroblastoma, these data will provide significant insight into the mechanisms of Wilms tumor development.

In contrast with rats, mice did not develop nephroblastomas when exposed to ENU intraplacentally, which prevented the use of many genetically engineered mice to explore the molecular mechanisms. Investigations in *Drosophila* and a number of mammalian systems have found that the JAK/STAT system plays an important role in the regulation of tissue progenitor cells [98]. Previous studies found that STAT1 activation regulates proliferation and differentiation of renal progenitors in an explant culture system [99], and is associated with Wilms tumors and tumor cell survival and growth [100]. Furthermore, it was shown that conditional expression of IFN-γ in astrocytes can induce medulloblastoma [101], which is the most common malignant brain tumor in children and likely arises from cerebellar stem cells that have been prevented from differentiating into their normal cell types [102]. These results propelled us to ask whether expression of IFN-γ or STAT1 using Cre-mediated removal of stop codons from upstream of the transgenes could facilitate or induce the development of Wilms tumor in mice. Preliminary data indicated that expression of STAT1 in metanephric mesenchymal cells did not lead to Wilms tumor development, even in the p53 deficient background, whereas expression of IFN-γ resulted in embryo death [Yang et al., unpublished observation]. Therefore, the combination of STAT1 expression with additional tumor-promoting factors, such as exposure to ENU and elevated IGF_2 expression, might be needed to induce the development of Wilms tumor. Interestingly, it was shown that conditional WT1 ablation in metanephric mesenchyme and constitutive IGF_2 upregulation in mice induce Wilms tumors at an early age [19]. As human WT1 and IGF_2 are both localized on the short arm of chromosome 11 and close to half of Wilms tumor patients with WT1 mutations also contain a loss of heterozygosity or loss of imprinting at IGF_2 region [103], it is interesting to explore whether alterations of WT1 and IGF_2 together lead to Wilms tumor formation. Nevertheless, the first Wilms tumor model in mice provided a powerful system to investigate the initiation and progression of Wilms tumor. It could also be a valuable alternative to xenografted Wilms tumor tissue or cells to evaluate various anti-tumor strategies and therapies.

5. Summary

Significant progress has been made over the last decade in understanding kidney development and the mechanisms of Wilms tumor formation. While initiator events in the majority of sporadic cases of Wilms tumor remain to be elucidated, it is evident that genetic, epigenetic, and miRNA alterations all can contribute to this process. The success in inducing mice nephroblastoma with IGF_2 over-expression clearly underlined the importance of a loss of IGF_2/H19 imprinting in the development of Wilms tumors and is instructive in generating additional mice models to further analyze and validate the critical events in tumor development. The recent discoveries that enzymes critical for miRNA production and degradation are both associated with Wilms tumor argue strongly that miRNAs play an important role in kidney and Wilms tumor development. Interestingly, WT1, WTX, β-catenin, IGF_2, and miRNAs all affect the early growth and differentiation of mesoderm and kidney progenitor cells. Whether they act on a common cell program or signal transduction pathway to induce Wilms tumor remains to be known. It is conceivable that on-going efforts in analyzing the Wilms tumor genome and transcriptome will shed new light on genetic and epigenetic changes critical for tumor development. Furthermore, with the development of mice and rat nephroblastoma models, it is possible to use new genetic engineering tools to evaluate the roles of the genetic, epigenetic, and miRNA changes in tumor development efficiently, and provide foundations for novel target therapies, especially for bilateral and relapsed Wilms tumors.

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