Multiple Roles for the Wilms’ Tumor Suppressor, WT1

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Abstract

Wilms’ tumor is a childhood kidney tumor that is a striking example of the way that cancer may arise through development gone awry. A proportion of these tumors develop as a result of the loss of function mutations in the Wilms’ tumor suppressor gene, WT1. Inherited mutations in the WT1 gene can lead to childhood kidney cancer, severe gonadal dysplasia, and life-threatening hypertension. Knockouts show that the gene is essential for the early stages of kidney and gonad formation. These tissues are completely absent in null mice. The WT1 gene encodes numerous protein isoforms, all of which share four zinc fingers. There is a large body of evidence supporting the notion that WT1 is a transcription factor, particularly a transcriptional repressor. Recently, however, we obtained evidence that WT1 colocalizes and is physically associated with splice factors. What is more, one alternative splice isoform of WT1 containing three amino acids, Lys-Thr-Ser (KT3; inserted between zinc fingers 3 and 4) is preferentially associated with splice factors, whereas the other alternative splice version, lacking these three amino acids, preferentially associates with the transcriptional apparatus. Both genetic and evolutionary considerations suggest that these two different forms of the protein have different functions. We will discuss recent evidence to further implicate WT1 in splicing. Our results raise the possibility that regulation of splicing is a crucial factor in the development of the genitourinary system, and that tumors may arise through aberrant splicing. To pursue the regulation and function of WT1 in whole animals, we have been introducing the human gene and large flanking regions cloned in yeast artificial chromosomes directly into mice. These studies have allowed us to dissect the function of WT1 at late as well as at early stages in organogenesis and to identify new sites and surprising new potential functions for the gene.

Introduction

Wilms’ tumor of the kidney is a striking example of the way cancer can arise through aberrant differentiation (1). Wilms’ tumor is a relatively common pediatric cancer, affecting approximately 1 in 10,000 children worldwide during the first few years of life. The tumors arise through inappropriate proliferation of nephric stem cells that should, in normal circumstances, differentiate and stop dividing or perhaps die.

To understand tumorigenesis, it is necessary to consider what we know about the normal—beautifully orchestrated—process of kidney development. The permanent kidney or metanephros arises from two embryonic tissues, the mesonephric duct and metanephric mesenchyme, both of which derive from intermediate mesoderm. Epithelial structures called ureteric buds grow out of the mesonephric duct and invade the mesenchymal cells that condense around the bud to form the blastema. Through a series of reciprocal induction events between the bud and blastema, the nephron and collecting systems form and eventually link up. The bud sends signals to the blastema cells, which first proliferate and then differentiate to form (eventually) the epithelial cells of the proximal and distal tubules, the loop of Henle, and the glomerulus. The ureteric buds, on the other hand, will branch in response to signals from the blastema and go on to form the collecting ducts.

Wilms’ tumors arise from the blastemal cells, probably after induction has been initiated because the tumors often go on to develop tubular and glomerular-like structures. A typical Wilms’ tumor is classified as triphasic, consisting of blastema, epithelial structures, and stromatous components all of which derive from blastema during normal development.

Why Do These Tumors Arise?

In 10–15% of cases, Wilms’ tumors develop through loss of function of the first Wilms’ tumor gene identified, WT1, mapping to chromosome 11p13 (2, 3). In the remaining 85% of cases, WT1 is not mutated, and other genetic lesions are implicated. As yet, no other definite Wilms’ tumor gene has been identified, but a strong candidate is the IGF2 gene on chromosome 11p15. Normally IGF2 is an imprinted gene that is expressed only from the paternal allele. However, in the majority of Wilms’ tumors, there is relaxation of imprinting; therefore, the maternal allele is also expressed (4, 5), which leads (one presumes) to an increased dose that is known to result in developmental abnormalities but is not yet convincingly linked to Wilms’ tumor formation (6, 7).

The WT1 gene was identified because a few percent of children with Wilms’ tumor are born with an eye problem—aniridia—and are often mentally retarded and have genital abnormalities. Such children, said to have the WAGR syndrome, have constitutional hemizygous deletions of chromosome 11p13. Positional cloning in the region led to the isolation of the WT1 gene that encodes a protein with four zinc fingers of the Kruppel type and, therefore, is likely to be a transcription factor (2, 3). Children with the WAGR syndrome have only one copy of WT1 deleted in all their cells. However, when tumors of such individuals were analyzed, the second WT1 copy was found to be mutated or deleted. Thus, the complete loss of function of the WT1 gene is required for tumorigenesis. Hence, WT1 conforms to Knudson’s rule and can be classified as a tumor suppressor gene. However, unlike the majority of tumor suppressor genes such as Rb-1 and p53, WT1 is clearly a developmental gene expressed in, and essential for the production of, only a limited set of mesodermal tissues, particularly the genitourinary system. Here I will discuss our latest findings or approaches to understanding the developmental and biochemical roles of WT1, and how we are using mouse models to help dissect its functions. Although there has been some significant progress of late, we still do not understand why loss of WT1 function in the developing kidney leads to the formation of a Wilms’ tumor.

What Can We Learn about WT1 Function by Studying Tumors?

WT1 is expressed in a dynamic and tissue-specific pattern during development; the major sites of expression during organogenesis are the developing kidney, gonad, and mesothelium (8). All of these tissues experience a mesenchyme-to-epithelial switch as WT1 expres-
sion increases, leading to the hypothesis that WT1 may play a role in this switch. Although direct experimental evidence to prove a role for WT1 in the mesenchyme-to-epithelial switch is awaited, examination of tumors with WT1 mutations is informative.

Wilms’ tumors can be classified into several groups from a histopathological standpoint. The most common are triphasic tumors, containing a mixture of blastema, epithelial structures, and stroma (Fig. 1); another class is mainly stromal in character without epithelial structures. The third—fascinating and perhaps surprising—class comprises Wilms’ tumors with ectopic tissues, particularly muscle (Fig. 1), but more occasionally bone and cartilage; approximately 10% of Wilms’ tumors show these ectopic tissues. Because the WT1 gene is also mutated in only 15% of tumors, it is worthwhile asking whether these show characteristic pathology. In a large survey, Schumacher et al. (9) were able to conclude that tumors with WT mutations are mostly stromal in character, supporting the idea that WT1 is required for epithelial development (9). Miyagawa et al. (10) examined expression of myogenic genes, those essential for muscle formation, in a number of Wilms’ tumors. They found that high expression of genes such as myoD or myf5 could only be detected in the subset of Wilms’ tumors with WT1 mutations. Myogenic gene expression cannot be detected in the kidney during normal development. This leads us to the following model: during normal kidney development, mesenchymal stem cells expressing WT1 can differentiate into epithelial cells (Fig. 2). In tumors, if WT1 is missing, the mesenchymal cells can no longer differentiate into epithelia but can take on different fates, either stromal or myogenic (Fig. 2). The question is whether this is a default pathway or whether WT1 can play a direct role in repressing the myogenic program. Preliminary experiments in cell culture do support the idea that WT1 can repress muscle development (10), but proof of this will await transgenic experiments in mice.

**Does WT1 Play Roles in Both Transcription and Splicing? Significance of Different WT1 Isoforms?**

In all mammals studied, the WT1 gene expresses 16 slightly different protein isoforms through a combination of alternative splicing, RNA editing, and alternative translational start sites (Fig. 3). The significance of these different isoforms remains unclear; however, the fact that they are found in all of the mammals that diverged around 100 million years ago suggests that they all play important functions. It is interesting to consider the situation with the fish WT1 gene, which seems to encode only two different isoforms (Fig. 3; Ref 11). It is noteworthy that these two isoforms—also found in mammals—differ by only three amino acids, KTS, inserted between zinc fingers 3 and 4 through alternative splicing. The fish genitourinary system is more primitive than that found in mammals. We are now trying to determine how the evolution of WT1, both its structural complexity and regulation, may have contributed to the evolution of the genitourinary system.

Because WT1 has four zinc fingers, it was predicted to act as a transcription factor regulating the tissue-specific expression of genes during genitourinary development. There is now a considerable body of evidence supporting this idea (12). WT1 protein binds to guanosine-rich DNA sequences of 9–12 nucleotides long. In transient transfection assays, WT1 represses expression of reporter molecules linked to promoters with such guanosine-rich motifs; in some cases,
however, WT1 can act as a transcriptional activator. Furthermore, WT1 can repress the expression of a number of important growth factor genes in cell culture, including the genes encoding the IGF2, the IGF1 receptor, the platelet-derived growth factor A chain and the epidermal growth factor receptor as well as the developmental gene PAX2 (for review, see Ref. 12). Several of these genes become down-regulated during kidney development concomitant with a rise in WT1 expression supporting physiological significance for the cell culture experiments. However, to this day, there is no conclusive evidence that WT1 acts to repress the expression of these genes in an in vivo chromosomal context during development. Given these findings, we were surprised that WT1—or at least a proportion of WT1—colocalizes with splicing factors in kidney, testes, and mesothelial cells (13). What is more, WT1 was efficiently coimmunoprecipitated in vivo by antibodies to a number of splice factors which suggests that WT1 either directly or indirectly interacts with splicing proteins. We went on to show that the two WT1 isoforms with (+KTS) or without (−KTS) the three-amino-acid insert between zinc fingers 3 and 4 localize differently in transfection experiments. Whereas the −KTS form colocalized mainly with transcription factors, the +KTS form colocalized mainly with the splicing proteins.

These findings add additional weight to the evidence that +KTS and −KTS WT1 isoforms have different functions. The most compelling evidence comes from an analysis of a human condition known as Frasier’s syndrome. Children with Frasier’s syndrome have a severe nephropathy, in the form of focal mesangial sclerosis, and also have gonadal dysgenesis in males. Remarkably this syndrome arises through a WT1 splicing mutation that prevents the production of the +KTS form from one allele, whereas the other allele is wild type and produces both forms (14, 15). Thus, in these children, one would predict that the ratio of +KTS:−KTS changes from, say, 60:40 to 40:60. Hence the balance of these two different isoforms is critical for normal development and physiological function of the genitourinary system. These findings suggest that the two different WT1 isoforms have different functions, and that the balance of the two forms is critical. In addition, it has been shown that the −KTS WT1 isoform binds to DNA with relatively high affinity and to a 9-nucleotide motif, whereas the +KTS form binds to DNA with much lower affinity and to a motif of approximately 12 nucleotides (16, 17). Moreover, the two proteins behave differently in functional assays. Hence, under certain conditions the −KTS form can induce apoptosis in cell culture, whereas the +KTS form cannot; the −KTS form in some cases promotes tumorigenesis, whereas the +KTS form inhibits it (for review, see Ref. 18).

Thus our findings suggest that WT1 may play roles in splicing as well as in transcription and that the two different isoforms play different roles. This has major implications for the mechanisms underlying normal development and tumorigenesis. However, the difficulty is to prove that WT1 really plays a role in splicing in a physiological context and to understand what that role is.

Several new findings support a role for WT1 in RNA metabolism: (a) we showed with colleagues (19) that WT1 can bind RNA through the zinc fingers, something recently confirmed by Bardesy and Pelletier (20) who were also able to identify specific WT1-binding RNA sequences; and (b) a conserved RNA recognition motif has been identified in the NH2-terminal region of the protein (21). Very recently we have shown that WT1 can be incorporated into active splice complexes (22), and that WT1 sediments with the properties of a ribonucleo-protein complex in nuclear extracts. Furthermore, we have shown that WT1 interacts specifically and directly with a basic splice factor, U2AF65, which is giving us some clues concerning its potential function (22). Remarkably the +KTS form of WT1 interacts much more efficiently with U2AF65 than the −KTS form, correlating with the localization of these isoforms in vivo. Now the challenge is to identify physiological RNA targets and to determine the way in which WT1 influences the processing of these RNA molecules in normal development and during tumorigenesis.

Use of YAC Transgenesis to Explore WT1 Function

We have postulated that WT1 may play a role in the mesenchyme-to-epithelial transition during kidney development, and also that WT1 may act as a suppressor of myogenesis in the mesenchymal stem cell (10). From the perspective of tumorigenesis, it is vital to know whether WT1 plays a role in promoting apoptosis or suppressing the cell cycle. wt1 null mice, although very informative, do not shed any light on these issues. During normal development, WT1 is expressed at low levels in uncondensed mesenchyme; then the levels increase as the cells condense around the mesenchyme-to-epithelial transition during kidney development, and also help to induce terminal differentiation. WT1 levels then stay high in the part of the S-shaped body that will go on to form the glomerulus, and levels continue to be high in the podocyte layer of the glomerulus. The wt1 mutant mice completely lack kidneys; the mesenchymal stem cells form but then die through apoptosis, and the ureteric buds fail to invade the mesenchyme (23). Because these stem cells fail to survive, it is impossible to pursue the role of WT1 in the later stages of nephrogenesis in these mice. wt1 −/− mice also lack gonads and die at 13.5 days of gestation, presumably because of heart failure although this has not been clarified.

Fig. 3. Evolution of WT1 structure.

![Fig. 3. Evolution of WT1 structure.](image)
To pursue WT1 function at different stages of development and in different tissues, we are using a transgenic approach using very large human fragments containing the WT1 gene cloned in YACs. There are several types of experiment that we are trying to perform: (a) The first of these is to express WT1 ectopically in different tissues at different stages of development. For example, if we ectopically express WT1 in muscle precursor cells, will myogenesis be prevented? On the other hand, if WT1 is expressed in a range of mesenchymal cells inappropriately, will these convert to epithelial cells? Does ectopic expression of WT1 influence the proliferation of stem cells or induce cell death? Often in this type of experiment, tissue-specific promoters are used to drive the expression of individual cDNAs. In the case of WT1, we do not think that this is desirable because 16 different forms of the protein are produced, and the ratios of these are more or less constant. As an alternative strategy, we are replacing the WT1 promoter in the YAC with other promoters that will drive expression in all of the WT1 isoforms simultaneously and hopefully in the correct ratio (Fig. 4). If an interesting phenotype is observed, we can then make mutations of the YAC in the yeast cell; therefore, particular WT1 isoforms would be disrupted or structural motifs would be altered. So far, we have replaced the WT1 promoter with the cytomegalo-virus promoter that should drive expression in all cells. When the YAC was injected into 1000 or more fertilized eggs, no transgenic mice were recovered. In fact, no transgenic mice have been obtained up until 13 days of embryogenesis. Thus, expression of WT1 everywhere causes lethality at an early stage of development (24). More recently, we have replaced the WT1 promoter with an actin promoter and hematoepoietic promoter. The latter is interesting because WT1 mutations have been identified in some myeloid leukemias, and the proteins are underexpressed in a number of leukemias.

(b) We are using transgenesis to follow expression of the WT1 gene in mice and in order to allow dissection of the elements necessary for correct tissue-specific and developmental expression of WT1. To do this, the WT1 coding region was fused to a β-galactosidase reporter in the YAC, which was then purified and used to make transgenic lines (Fig. 4). The β-galactosidase is expressed in all of the known WT1-expressing tissues as well as in some interesting new sites of expression (25, 26). This reporter is proving to be very useful for studying the early stages of intermediate mesoderm and kidney development and the subsequent formation of the genitourinary system. We are now making deletion constructs and mutations of specific promoter motifs in the WT1 YAC and testing the ability of these to be expressed in a normal pattern.

(c) The final type of YAC transgene experiment is to attempt rescues of the null phenotype in mice (Fig. 4). If the human WT1 YAC can rescue development of the WT1 −/− mice, it will be possible to use this as a system to test the importance of different WT1 protein domains and isoforms. Thus, mutations in splice sites, dimerization domains, and RNA recognition motif (RRM) can be made, and the YACs can be tested for their ability to rescue. Although the first rescue experiments with the nonmutated YAC have not worked completely, they have given new insights into the function of WT1 during the development of the kidney and other tissues (26). Hence, our partial rescues show that WT1 function is required at several distinct stages of nephrogenesis as predicted by the expression pattern. Ultimately it will be necessary to make specific WT1 mutations in the endogenous mouse gene using homologous recombination in ES cells and to use the conditional cre/lox system to detect the normal function of WT1 in different tissues at different stages of development.

References
Discussion

*Speaker*: Could you say something about why you find only partial rescue by the human transgene?

*Dr. Hastie*: We think we only get partial rescue because we find the level of expression of the human transgene is lower than that of the endogenous mouse gene at later stages of kidney development. Whereas, it is expressed at the right levels in the developing epicardium. So we think it’s a dosage effect. But we are now trying to rescue with a mouse transgene to try to work out what’s the difference.

*Dr. Phillip Sharp*: When you find the KTS form in the splicing bodies, have you expressed some parts of the protein and asked what is actually targeting it to these speckled regions?

*Dr. Hastie*: Yes, we’ve tried that. It’s, of course, more complicated than we would like. Certainly you require some zinc fingers normally when you do that sort of experiment. But, if we impair DNA binding in different ways, we still often get localization with speckles. It seems two different WT1 domains are involved. Then also it seems two domains are involved in interaction with U2AF65. It’s not simple.

*Dr. Bruce Ponder*: I asked him if there was any question he wished you’d ask, he could ask it now.

*Dr. Hastie*: One thing I should say we’re doing is to try to put fish genes back into mice. We’ve done this already to try to work out what it is that’s different in evolution between the two.
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