The complex life of WT1

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Summary

The Wilms' tumour gene, *WT1*, encodes a zinc-finger transcription factor that is inactivated in a subset of Wilms' tumours. Mutation analysis in human patients and genetic experiments in mice have revealed that WT1 has a role much wider than just tumour suppression. Alternative splicing, RNA editing, and the use of alternative translation initiation sites generate a multitude of isoforms, which seem to have overlapping but also distinct functions during embryonic development and the maintenance of organ function. Recently, mouse strains lacking the WT1(–KTS) or WT1(+KTS) splice variants of exon 9 were generated. More severe defects of kidneys and gonads are found in mice lacking the WT1(–KTS) variant. Animals lacking the WT1(+KTS) variant show disturbed podocyte function and male-to-female sex reversal. Alternative splicing of exon 5, however, might not modify WT1 function dramatically. Recently, it was also described that reduction of WT1 levels in the kidney results in glomerulosclerosis and upregulation of WT1 in the heart might contribute to neovascularization after infarction.

Key words: Splice-specific functions, Glomerulosclerosis, Knockout mouse models, Frasier syndrome, Denys-Drash syndrome, Sex determination

Introduction

The idea is simple: you clone a gene, employ a variety of biochemical, cellular and genetic assays to understand its function and publish your findings in a scientific journal. You then lean back, take a deep breath and move on to your next challenge. Sometimes, however, life is much more complex and, despite the enormous effort from the scientific community, the precise function of a gene remains elusive. This seems to be – at least in part – the case for *WT1*, a gene mutated in a proportion of embryonic kidney cancers termed Wilms' tumours or nephroblastomas (Haber et al., 1990; Gessler et al., 1990), which are believed to arise from mesenchymal blastema cells that fail to differentiate into metanephric structures and continue to proliferate (Hastie, 1994). The biology of *WT1* is complex and we now know that, in addition to its function as a tumour suppressor, this gene has multiple roles during development and maintenance of body function. Hence, it may not be surprising that mutations in *WT1* are found in a variety of syndromes, including Denys-Drash syndrome (Pelletier et al., 1991; Patek et al., 1999), Frasier syndrome (Barbaux et al., 1997; Klamt et al., 1998), and WAGR (Wilms' tumour, aniridia, genitourinary malformations, mental retardation) syndrome (Gessler et al., 1990). Additional studies have linked *WT1* mutations to malignancies such as leukaemia (Inoue et al., 1994; Tamaki et al., 1999), desmoplastic small round cell tumours (Lae et al., 2002), breast cancer (Silberstein et al., 1997), lung cancer (Oji et al., 2002), and retinoblastoma (Wagner et al., 2002c).

The complexity of WT1 action during development is also reflected on the molecular level. Post-transcriptional modifications of the *Wt1* pre-mRNA lead to the production of up to 24 different isoforms, which seem to serve distinct but also overlapping cellular and developmental functions. Here, we examine the roles of these various isoforms and highlight recent advances in our understanding of WT1 function in development, focusing particularly on gonad formation and sex determination. We also draw attention to open questions, which should be addressed in future experiments. Owing to space limitations, we do not discuss WT1 function in cancer, and interested readers are referred to other more specialized reviews (e.g. Loeb and Sukumar, 2002; Scharnhorst et al., 2001).

WT1 and embryonic development: an update

WT1 is expressed during mammalian embryonic development in many tissues, including the urogenital system, spleen, certain areas of the brain, spinal cord, mesothelial organs, diaphragm, limb, proliferating coelomic epithelium, epicardium and subepicardial mesenchyme (Armstrong et al., 1993; Moore et al., 1998; Moore et al., 1999). Consistent with this expression pattern, targeted disruption of the *Wt1* gene in mice leads to gonadal and renal agenesis, and severe heart, lung, spleen, adrenal and mesothelial abnormalities (Kreidberg et al., 1993; Herzer et al., 1999; Moore et al., 1999). Homozygous $Wt1^{-/-}$ knockout mice develop an incomplete diaphragm, the heart is hypoplastic, and the animals die in utero probably owing to pericardial bleeding and massive oedema. Recent studies suggest that WT1 also plays an important role in neuronal development (Wagner et al., 2002b). Mice with homozygous disruption of *Wt1* displayed severe abnormalities of retinal development, optic nerve hypoplasia, and apoptotic loss of retinal ganglion cell precursors. The class IV POU-domain transcription factor Pou4f2, which is critical for the survival of retinal ganglion cells, is not expressed in $Wt1^{-/-}$ knockout mice, and we have shown that Wt1 can directly transactivate *Pou4f2* at least in cell cultures.

Given the variety of organs WT1 seems to be required for,

is there a common theme in the action of WT1 during development? We still don't know the answer to this question. What is striking, however, is that many organs, including the gonads, kidneys, spleen and the retina, show a dramatic increase in apoptotic activity in *Wt1*$^{-/-}$ knockout mice. Whether this is due to a lack of repression of apoptotic genes, lack of activation of anti-apoptotic genes or is simply a reaction of the organ to abnormal cellular differentiation remains to be elucidated.

Additional roles for WT1 in adult life

Genetic analyses have clearly demonstrated the importance of WT1 for a large variety of developmental processes. However, WT1 is also expressed in certain cell types throughout life, including glomerular podocytes in the kidney. Recent observations indicate that WT1 is required for the maintenance of kidney function. Heterozygous *Wt1*$^{+/-}$ mice, which were initially reported to be normal, exhibit increased mortality due to adult onset nephrotic syndrome with the histopathological features of diffuse mesangial sclerosis (Guo et al., 2002). The onset of renal disease depends largely on the degree of reduction in WT1 expression levels. For example, if a transgenic approach using a human-derived yeast artificial chromosome construct is applied, expression levels reach only 60% of those in wild-type mice and, consequently, animals develop massive mesangial sclerosis and renal failure within three weeks of birth. Reduced WT1 levels are associated with reduced expression of the two podocyte-specific markers, nephrin (nphs1) and podocalyxin, which indicates that WT1 might regulate these genes. Indeed, Palmer et al. recently demonstrated that podocalyxin is a direct downstream target of WT1 (Palmer et al., 2001). Whether nephrin is directly downstream of WT1 remains to be investigated.

What are the consequences of these studies for human patients? The finding that heterozygous knockout mice develop renal failure suggests that a similar process may occur in patients who have heterozygous mutations in *WT1*. This has been demonstrated in a large cohort study of patients with Wilms' tumours (Breslow et al., 2000). The risk for development of renal failure 20 years after initial diagnosis is significantly higher in WAGR patients missing one *WT1* allele (38%), than in patients with unilateral Wilms' tumour without *WT1* germ line mutations (1%). Hence, patients who have deletions or mutations should be carefully monitored throughout life. The availability of a mouse model may also allow us to investigate the molecular mechanisms occurring during WT1-dependent mesangial sclerosis and develop therapies for it.

Although expression in kidneys is maintained throughout life, the situation in the heart is somewhat different. During development, WT1 is expressed in the subepicardial layer of this organ, but then gets switched off and is absent in adult heart. Hypoxia and ischemia, however, seem to stimulate WT1 expression in the heart with de novo expression in smooth muscle and endothelial cells of the coronary arteries after myocardial infarction and after normobaric hypoxia (Wagner et al., 2002a). WT1 co-localizes with cell proliferation markers and vascular endothelial growth factor (VEGF) in ischemic and hypoxic hearts. Since proliferation of vascular endothelial and smooth muscle cells is a critical step in the formation of collaterals from pre-existing coronary vessels, WT1 might have a role in the proliferation of coronary vascular cells and thereby in the neovascularization process in the heart. This idea is supported by a study by Natoli et al., which describes a disturbed capillary development in the kidney when WT1 levels are reduced (Natoli et al., 2002a). The molecular signals underlying the activation of WT1 expression during hypoxia and ischemia in the heart remain to be determined.

Multifunctionality through protein variety

WT1 encodes a protein that has four C_2H_2 Zn fingers and has a high degree of structural homology to the early growth response (Egr) family of transcription factors (Rauscher, 1993). The human *WT1* gene spans ~50 kb and consists of ten exons (Call et al., 1990; Gessler et al., 1990) (Fig. 1). Probably the most striking feature of WT1 is the multitude of isoforms that are produced by a combination of alternative splicing, RNA editing and alternative translation start sites. At least 24 different variants can be generated from this locus in mouse and man.

RNA editing has been observed in human, rat and mouse WT1 RNA. The proportion of edited RNA observed, however, varied in different studies, and the relevance of this RNA modification remains to be seen. Similarly, we know very little about isoforms produced from alternative translation start sites, which generate proteins with longer or shorter N termini (Bruening and Pelletier, 1996; Scharnhorst et al., 1999). The amount of these variants seems, however, relatively small compared with that produced from the major ATG in all tissues analysed so far. Moreover, the effect of the different N termini on the transcriptional activation/repression activity of WT1 is not dramatic, and it remains to be seen whether these isoforms fulfil an important function in vivo or just represent evolutionary remnants.

Much more data are available on the function of the alternatively spliced isoforms and, given the importance of WT1 in development and disease, they merit a closer examination. There are two alternatively spliced exons in WT1: exon 5 and exon 9. Exon 5, which is mammal specific, encodes 17 amino acids that are included or omitted between the Pro/Glu-rich N-terminus and the Zn finger domain. Exon 9 possesses two alternative splice donor sites. Usage of donor site 1 results in omission, and that of donor site 2 in inclusion, of a KTS sequence between the third and fourth zinc finger. Consequently, isoforms lacking the KTS sequence are often referred to as WT1(–KTS), whereas those containing this are called WT1(+KTS). The ratio between WT1(+KTS) and WT1(–KTS) proteins is nearly constant in all cell types, whereas the ratio of exon 5 splice variants differs between cell types, species and developmental stages (Pritchard Jones and Renshaw, 1997). Interestingly, mutations interfering with the ratio of WT1(+KTS) and WT1(–KTS) proteins lead to Frasier syndrome in humans, indicating the importance of the ratio of these variants (see below).

Biochemical and genetic analysis has given some insights into the functions of the alternatively spliced protein variants. Exon 5 contains a protein-protein interaction domain, which permits association with prostate apoptosis response factor 4 (Par4) (Richard et al., 2001). On a cellular level, this interaction seems to be important, because only exon 5

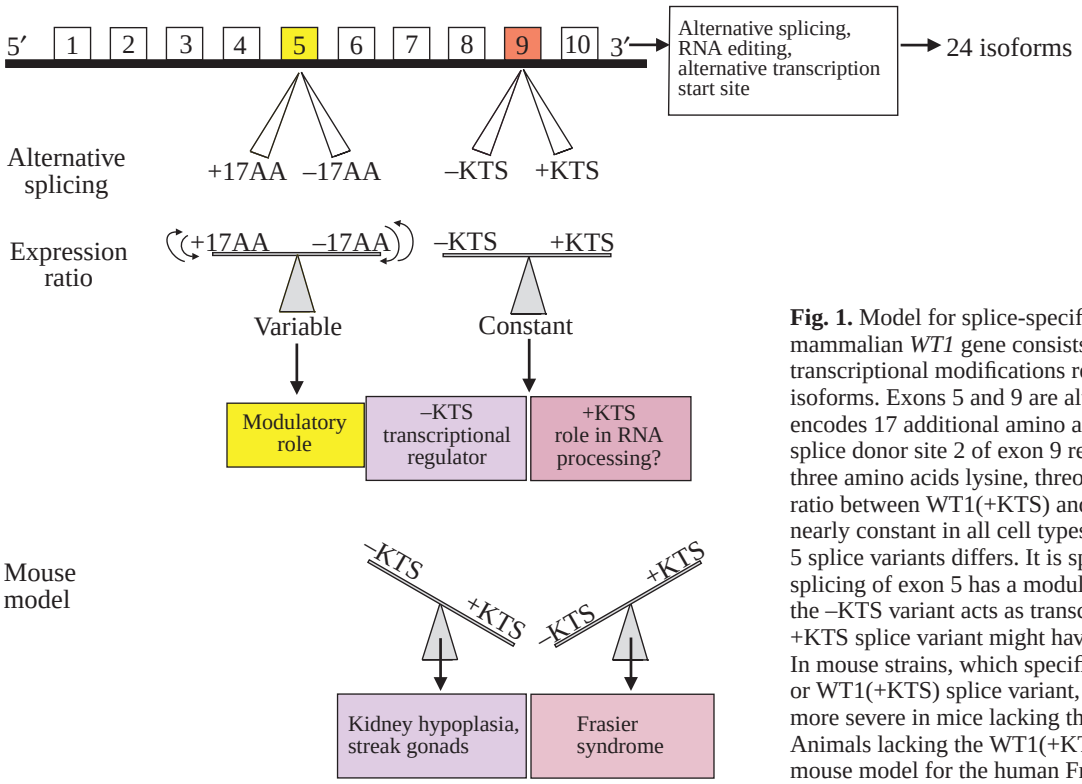

Fig. 1. Model for splice-specific functions of WT1. The mammalian *WT1* gene consists of 10 exons. Post-transcriptional modifications result in the generation of 24 isoforms. Exons 5 and 9 are alternatively spliced. Exon 5 encodes 17 additional amino acids; the use of alternative splice donor site 2 of exon 9 results in inclusion of the three amino acids lysine, threonine and serine (+KTS). The ratio between WT1(+KTS) and WT1(–KTS) proteins is nearly constant in all cell types, whereas the ratio of exon 5 splice variants differs. It is speculated that alternative splicing of exon 5 has a modulatory role on WT1 function; the –KTS variant acts as transcriptional regulator; and the +KTS splice variant might have a role in RNA processing. In mouse strains, which specifically lack the WT1(–KTS) or WT1(+KTS) splice variant, the urogenital phenotype is more severe in mice lacking the WT1(–KTS) variant. Animals lacking the WT1(+KTS) variant represent a mouse model for the human Frasier syndrome.

containing isoforms of WT1 can overcome a UV-induced apoptotic signal, when transfected into HEK293 cells. The ratio between WT1 variants containing and lacking exon 5 seems to vary, which further suggests a modulating role of this alternative exon. The presence of exon 5 only in mammals, upregulation in the uterine embryonic implantation site (Zhou et al., 1993), and expression in the adult mammary glands (Silberstein et al., 1997) suggested this isoform has a role in mammal-specific functions (i.e. embryonic implantation and lactation). Furthermore, the presence/absence of exon 5 might modify the function of the WT1 protein when additional mutations exist. Natoli et al. expressed various variants of WT1 in the developing nephron ectopically, by using the nephrin promoter (Natoli et al., 2002a). In mice expressing a *Wt1* cDNA with a deletion of the third and fourth zinc finger, no influence on mouse renal development and function could be detected when the construct contained exon 5. However, when exon 5 was also missing, the mice showed poor postnatal survival, and glomerular abnormalities with a reduced number of glomerular capillaries that were dilated. In these animals, expression of platelet endothelial cell adhesion molecule 1 (PECAM-1) is greatly reduced on glomerular endothelial cells, suggesting a role of WT1 in vasculogenesis during kidney development. Surprisingly, exon 5 function during normal development seems to be much more obscure: Natoli et al. found out that mice carrying a deletion of exon 5 have no developmental defects and are fertile (Natoli et al., 2002b). Unfortunately, their study did not include mutations that interfere with the production of WT1 +exon 5 variants only, which may have given additional clues to the function of this alternative splicing event. At the moment, we can only conclude that exon 5 does not seem to represent a major modifier of WT1 function. More subtle defects might be discovered in long-term studies of genetically modified mice or its function might become apparent only in a diseased state.

Much more studied, but in many ways even more mysterious, are the WT1 (KTS) splice variants produced by alternative splicing of exon 9. A myriad of studies have demonstrated that WT1 (–KTS) variants act as transcriptional regulators that have activating and repressing capabilities depending on promoter, cell type and cell cycle stage. WT1 (+KTS) products, however, show a distinct nuclear staining (Larsson et al., 1995; Englert et al., 1995) and co-purify and interact with splicing machinery (Davies et al., 1998). The distinct nuclear localization of WT1 (+KTS) variants was recently confirmed in vivo in mice producing only one of the alternatively spliced products (Hammes et al., 2001). Since WT1(+KTS) also seems to have a much higher affinity for RNA than DNA (Caricasole et al., 1996; Laity et al., 2000), this variant might play a role in RNA processing. Indeed, WT1(+KTS) isoforms co-purify with the active component of splicing extracts (Ladomery et al., 1999), and WTAP, a protein that interacts with WT1, has a homologue in *Drosophila* that functions in splicing during sex determination (Hastie, 2001; Ortega et al., 2003). Unfortunately, apart from this circumstantial evidence, we do not have any direct proof for a role of WT1 in splicing.

Recent work has described specific in vivo functions of the different WT1 splice variants in embryonic development. Hammes et al. generated mouse strains that specifically lack the WT1(–KTS) or WT1(+KTS) splice variant (Hammes et al., 2001). Homozygous mice of both strains survive until birth, indicating that these different splice variants are functionally redundant in cardiac development. Postnatally, the phenotypes

differ: more severe defects are found in mice lacking the WT1(–KTS) variant. These animals display hypoplastic kidneys and streak gonads with an increased number of apoptotic cells in the gonads during embryonic development, indicating that the WT1(–KTS) variant is required for the survival of embryonic kidneys and gonads. Homozygous animals lacking the WT1(+KTS) variant also die soon after birth, owing to renal failure, but the phenotype seems to be caused by a lack of podocyte differentiation. In addition, gonads develop as ovaries in both XX and XY animals owing to reduced expression levels of *Sry*, a gene essential for the initiation of male development (see below). Heterozygous animals develop normally but, interestingly, mice that have reduced levels of WT1(+KTS) variants die after several months owing to renal insufficiency caused by focal segmental glomerular sclerosis combined with diffuse mesangial sclerosis. Hammes et al. have thus proposed that the WT1(+KTS) isoforms are more important for maintenance of podocyte function (Hammes et al., 2001). The mice lacking the WT1(+KTS) variants represent a good model for the human Frasier syndrome, which is characterised by reduced levels of the WT1(+KTS) variants due to mutations in the splice donor site of *WT1* exon 9, the development of renal insufficiency with focal and segmental glomerulosclerosis and male-to-female sex reversal (Barbaux et al., 1997; Klamt et al., 1998). Further analysis of this mouse model may help us to understand the molecular processes leading to the development of the human syndrome.

WT1 and sex determination: at the heart of a complex network

We have seen that WT1 has important functions in the development and maintenance of many organs. But, at least in some organs, WT1 seems to be involved in multiple developmental steps. In the final part of this *Commentary*, we focus on WT1 function during gonad formation and sex determination, which can serve as a paradigm for how and at what point different isoforms of WT1 feed into a complex molecular network during development.

In the mouse, gonads form at E9.5 through proliferation of the coelomic epithelium overlying the mesonephros. At E10.5, this primordium is bi-potential and can differentiate along the male or the female pathway, depending on the presence or absence of the sex-determining gene *Sry*. Expression of *Sry* leads to the activation of a molecular cascade involving the action of genes such as *Sox9* and *MIS*, and the differentiation of cells into the components of a testis, including Sertoli, Leydig and myotubular cells. In contrast, absence of *Sry* expression results in the differentiation of the gonads into ovaries.

WT1 is already expressed within the undifferentiated gonad, and its importance there has been demonstrated in *Wt1*$^{-/-}$ mice, in which the gonads undergo apoptosis. Since the gonads and adrenal cortex share a common primordium (Hatano et al., 1996) it is perhaps unsurprising that adrenal glands are also affected in these mice (Moore et al., 1999). This phenotype is reminiscent of that found in homozygous *Sf1*-knockout animals, which also lack gonads and adrenals owing to massive apoptosis in the adreno-genital primordium (Luo et al., 1994). Moreover, expression of Sf1 and expression of WT1 in the gonad begin at roughly the same time and might be dependent. Indeed, Wilhelm and Englert recently demonstrated that WT1, and more specifically its DNA binding form WT1(–KTS), can activate the *Sf1* promoter in vitro and in vivo (Wilhelm and Englert, 2002). The apoptotic phenotype in *Wt1*-knockout mice might therefore be caused by a lack of Sf1 expression. Alternatively, WT1 might activate an Sf1-independent pathway required for adrenogenital survival. This could include the anti-apoptotic factor Bcl-2, which might be directly activated by WT1 (Mayo et al., 1999).

A second role for WT1 during gonad formation occurs at the level of sex determination. We have seen that expression of Sry is important for the activation of the male differentiation pathway. In vitro experiments suggested that WT1 is responsible for transcriptional activation of *Sry*. As expected, only the DNA binding variant of WT1(–KTS) can transactivate this gene; WT1(+KTS) isoforms showed no effect in co-transfection assays (Hossain and Saunders, 2001). Interestingly, our own analysis in vivo paints a somewhat different picture. Mouse mutants lacking WT1(–KTS) products still show some expression of the downstream gene *Sox9* at E12.5, albeit the expression is restricted to a relatively small number of cells (Hammes et al., 2001). Hence, the male sex determination pathway in this strain seems to be active. In contrast, gonads in mice lacking WT1(+KTS) variants completely lack Sox9 expression, as well as that of its putative downstream target the Müllerian-inhibiting substance (MIS), also known as AMH. The lack of activation of male-specific genes could be traced back to a significant reduction of Sry expression to ~25% of wild-type levels, which is known to be insufficient to induce testis formation in mice. Consequently, gonads in mice lacking WT1(+KTS) products develop along the female pathway. At the moment it is not clear how WT1(+KTS) proteins act on *Sry*. Further analysis should shed light on this important question.

Other potential targets during gonad formation include *Wnt-4* (Sim et al., 2002), *Dax1* (Kim et al., 1999) and *MIS*, all of which seem to be activated by WT1(–KTS) variants. The best studied is certainly the regulation of *MIS*, and in vitro experiments suggested that WT1(–KTS) transactivates its promoter through direct interaction with Sf1 protein (Nachtigal et al., 1998). This transactivation of the *MIS* promoter, but also of the *Dax1* promoter, seems to be further increased through interaction of WT1 with the LIM-only co-activator FHL2 (Du et al., 2002). *Dax1*, a gene that can interfere with male sexual development in mouse and man was proposed to be a repressor of the *MIS* promoter that could displace WT1 from its complex with Sf1. By using site-specific mutagenesis in ES cells, Arango et al. demonstrated that the Sox-binding site in the *MIS* promoter region is absolutely required for activation of this gene, whereas the Sf1/WT1 binding site acts only as a quantitative regulator (Arango et al., 1999). MIS expression is ~30% of wild-type levels when it is detected. A schematic representation of these interactions is shown in Fig. 2.

Finally, the continuous expression of WT1 in Sertoli cells in testes and granulosa cells in ovaries throughout life might mean that it has an additional role in the maintenance of cellular functions in these cell types. Analysis of this role will have to wait for the generation of conditional WT1-knockout mice.

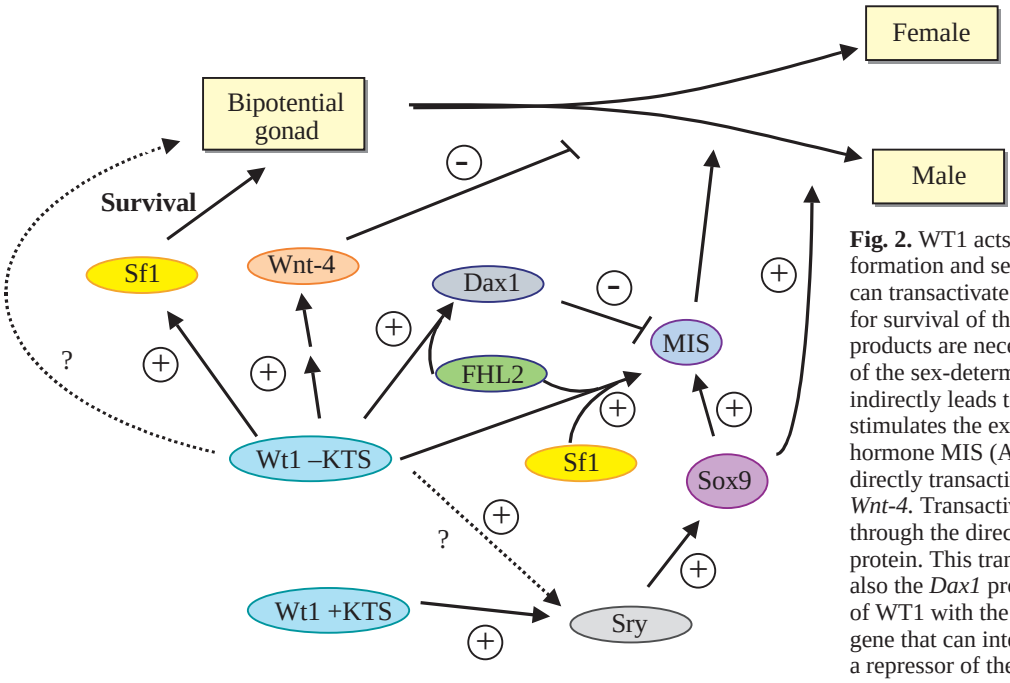

Fig. 2. WT1 acts at multiple steps during gonad formation and sex determination. WT1(–KTS) variants can transactivate Sf1. Both WT1 and Sf1 are required for survival of the bi-potential gonads. WT1(+KTS) products are necessary for the expression of high levels of the sex-determining gene *Sry*. Sry directly or indirectly leads to the activation of *Sox9*, which in turn stimulates the expression of the anti-Mullerian hormone MIS (AMH). Furthermore, WT1(–KTS) can directly transactivate *MIS*, *Dax1* and at least indirectly *Wnt-4*. Transactivation of the MIS promoter is achieved through the direct interaction of WT1(–KTS) with SF1 protein. This transactivation of the *MIS* promoter, but also the *Dax1* promoter is increased through interaction of WT1 with the LIM-only co-activator FHL2. *Dax1*, a gene that can interfere with male sexual development is a repressor of the *MIS* promoter.

Conclusions

From the above it is clear that the past few years have significantly improved our understanding of WT1 function. However, similarly evident are the open questions that need to be addressed. Most crucially, we must elucidate the molecular function of WT1(+KTS) products. This might prove difficult, however, and may require a combination of genetic, biochemical and molecular approaches. Identification of WT1(+KTS) function would allow us to interpret the various clues we have received from the analysis of cells, mouse models and human diseases.

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