A CTCF-binding silencer regulates the imprinted genes AWT1 and WT1-AS and exhibits sequential epigenetic defects during Wilms’ tumourigenesis

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We have shown previously that AWT1 and WT1-AS are functionally imprinted in human kidney. In the adult kidney, expression of both transcripts is restricted to the paternal allele, with the silent maternal allele retaining methylation at the WT1 antisense regulatory region (WT1 ARR). Here, we report characterization of the WT1 ARR differentially methylated region and show that it contains a transcriptional silencer element acting on both the AWT1 and WT1-AS promoters. DNA methylation of the silencer results in increased transcriptional repression, and the silencer is also shown to be an in vitro and in vivo target site for the imprinting regulator protein CTCF. Binding of CTCF is methylation-sensitive and limited to the unmethylated silencer. Potentiation of the silencer activity is demonstrated after CTCF protein is knocked down, suggesting a novel silencer-blocking activity for CTCF. We also report assessment of WT1 ARR methylation in developmental and tumour tissues, including the first analysis of Wilms’ tumour precursor lesions, nephrogenic rests. Nephrogenic rests show increases in methylation levels relative to foetal kidney and reductions relative to the adult kidney, together with biallelic expression of AWT1 and WT1-AS. Notably, the methylation status of CpG residues within the CTCF target site appears to distinguish monoallelic and biallelic expression states. Our data suggest that failure of methylation spreading at the WT1 ARR early in renal development, followed by imprint erasure, occurs during Wilms’ tumourigenesis. We propose a model wherein imprinting defects at chromosome 11p13 may contribute to Wilms’ tumourigenesis.

INTRODUCTION

The Wilms’ tumour (WT)-suppressor gene (WT1) is a highly conserved, developmentally regulated gene with transcriptional and post-transcriptional regulatory functions in a variety of tissues. It was originally identified as a tumour-suppressor gene on human chromosome 11p13, a locus implicated in WT, a paediatric kidney tumour derived from embryonal blastemal stem cells (1). Subsequently, studies on wt1-knockout mice have shown that wt1 protein is essential for the normal development of the kidney and urogenital system, mesothelium, heart, and lungs, with homozygous wt1 deletion being embryonically lethal as a result of developmental failure (2). It has further been shown that WT1 is involved in the development of the spleen and retina and in sex-determination (3). A tumour-suppressor function for WT1 in carcinogenesis is indicated by mutations of the gene detected in ~10% of WTs (4,5) and acute myeloid leukaemias (AMLs) (6). Mutations of the WT1 gene

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in WTs are mostly nonsense truncating mutations that eliminate gene activity, but missense mutations are also observed. These are especially common as germline defects in Denys Drash syndrome, a congenital disease characterized by nephrotic syndrome ultimately resulting in renal failure, XY pseudohermaphroditism and WT. Paradoxically, WTs and AMLs without WT1 mutations often express high levels of WT1 proteins, as do some adult cancers, and WT1 has varying effects on cellular differentiation (1,4,7).

In the developing kidney, WT1 expression is stringently regulated and is associated with the transition of mesodermal components to epithelium. Expression is low in the condensing mesenchyme but rises as cells progress towards an immature epithelial cell phenotype, attenuating as epithelial cells mature. High level expression of WT1 is maintained postnatally specifically in the podocytes lining the glomerular filtration units. Strict temporal and spatial control of WT1 expression is maintained, although the mechanisms by which control is exerted remain unclear. Regulation of WT1 cellular function involves at least 32 protein isoforms of WT1, which overlap the 5′-end of the WT1 sense transcript. These analyses also indicated that the allelic methylation characteristic of an imprinting mark does not occur at the WT1-AS or AWT1 promoters, or at other CpG islands at the 5′-end of the WT1 gene. Thus, the imprinting and expression status of AWT1 and WT1-AS is closely associated with differential methylation of the WT1 gene.

Our previous work has identified a new human-imprinted locus on chromosome 11p13. We have demonstrated that the WT1 antisense regulatory region (WT1-ARR) is differentially methylated in human kidney, accompanied by parent-of-origin-dependent allele-specific WT1-AS expression. WT1-AS expression is biallelic in the FK, but restricted to the paternal allele. This is in contrast to WT1, which is not monoallelically expressed in the kidney, with mosaic and polymorphic imprinting confined to placenta and brain (15). Importantly, WTs display a high frequency of hypomethylation at the WT1 ARR, accompanied by biallelic expression of WT1-AS and AWT1. These analyses also indicated that the allelic methylation characteristic of an imprinting mark does not occur at the WT1-AS or AWT1 promoters, or at other CpG islands at the 5′-end of the WT1 gene. Thus, the imprinting and expression status of AWT1 and WT1-AS is closely associated with differential methylation of the WT1 gene.

This study reports the first analysis of epigenetic mechanisms operating at human chromosome 11p13 and describes the genetic function of the WT1 ARR. We demonstrate that the ARR contains a cis-acting element able to modulate both the WT1-AS and AWT1 promoters and has attributes necessary for an imprinting regulatory element. Focusing on this region, we also assess methylation changes occurring during kidney development and tumorigenesis, including the first high-resolution methylation analysis of WT precursor lesions, known as nephrogenic rests (NRs) (16). On the basis of our data, we propose a model for Wilms’ tumorigenesis involving epigenetic lesions at the AWT1/WT1-AS regulatory region.

**RESULTS**

A methylating-sensitive silencer element maps to the WT1 ARR DMR

Our previous characterization of the WT1-AS promoter indicated the presence of negative regulatory sequences adjacent
to the promoter (10), and our subsequent epigenetic analyses demonstrated methylation patterns characteristic of imprinted genes also occurring within the region, the WT1 ARR (8,14).

Figure 1 illustrates the relative positions of the WT1-AS and AWT1 promoters, together with the differentially methylated region (DMR). In order to define the negative regulatory element, deletions of the WT1 intron 1 region were made and used in transient transfections of 293 cells (Fig. 2A). In

**Figure 2.** Delineation of the silencer element in the WT1 ARR. (A) A schematic restriction map of the WT1 ARR, numbered relative to the WT1-AS start, with the core silencer (solid box), promoter (striped box) and putative Sp1 (solid circles) and WT1 (open circles) binding sites shown. Promoter constructs bearing WT1 ARR deletions (stippled bars) were transiently transfected into 293 cells. Luciferase reporter activities relative to pINE 16 are shown in percentage terms (open bars). (B) Silencer promoter constructs transiently transfected into 293 cells are shown. Striped arrows, the basic silencer fragment, SIL (nucleotides −726 to −592). Genomic sequence 5′ to SIL (DMR extension), black box. Forward orientation of the arrow represents the orientation of the silencer relative to the promoter in its endogenous location. Promoters are WT1-AS (open box), WT1 sense (vertically striped box) and SV40 (horizontally striped box). Zig-zag lines represent 2.8 kb intervening plasmid backbone. Luciferase reporter activities relative to each appropriate promoter-only construct are shown in percentage terms (dark bars). (C) Silencer-AWT1 promoter constructs. Striped arrow indicates the silencer, and the open circle represents the SV40 enhancer in pGL3E-based plasmids, and constructs labelled. NAT labelling of plasmids indicates natural (genomic context) orientation of the silencer relative to the AWT1 promoter.
comparison with pNE16, which contains the WT1-AS promoter only, constructs which included upstream flanking sequences display a graded decrease of luciferase activity. The greatest decrease in luciferase activity is seen between ΔSL8 (78% of pNE16) and ΔSL7 (26% of pNE16). This result maps a core silencer region to between –627 and –726 relative to the WT1-AS transcriptional start site, adjacent to the WT1 ARR DMR. To assess regulatory capacity, a 134 bp silencer fragment (SIL, Fig. 1) was cloned into the WT1-AS reporter plasmid αSP (Fig. 2B). In transient transfections, a single copy of the silencer fragment confers a reduction in luciferase activity down to 59% of that with the promoter alone confirming that the fragment is a negative regulator of the WT1-AS promoter. The silencing activity is reduced by reversing its orientation (REV-αSP) and is shown to be position and orientation independent when located 2.8 kb upstream of the promoter. Placement of the silencer downstream of the promoter shows comparable repression. Single and tandem copies of the silencer were also positioned immediately upstream of two heterologous promoters. The WT1 sense promoter is weakly repressed and the SV40 early promoter is weakly induced by a single silencer, but tandem copies of the element silenced both WT1 and SV40 promoters, reducing luciferase activity to 50 and 67%, respectively.

Owing to the proximity of the basic silencer fragment to the DMR, we sought to determine whether silencer activity extended upstream into the DMR. We constructed DMR-SIL-αSP, containing upstream sequences –838 to –592 (Fig. 1). As shown in Figure 2B, extending the silencer fragment upstream of –726 gives a stronger silencing effect on the WT1-AS promoter than on SIL-αSP, reducing luciferase activity down to 35% that of αSP. DMR-SIL in the reverse orientation was not clonable. As the silencer element/DMR resides between the WT1-AS promoter and the AWT1 promoter, we also examined the effects of the silencer element on the AWT1 promoter. As shown in Figure 2C, the silencer repressed luciferase activity driven by the AWT1 promoter in constructs including or excluding the SV40 enhancer in an orientation-independent manner. The silencer is therefore predicted to be bidirectional in its native (genomic) context, co-ordinately regulating the promoters of both imprinted transcripts. Silencing in the absence of the SV40 enhancer suggests a mechanism other than enhancer blocking. Interestingly, the silencer-only construct, p3BSILNat, showed a markedly reduced luciferase activity in comparison with the basal luciferase vector alone, presumably due to the repression of cryptic promoter activity inherent in pGL3-B.

As the silent maternal allele is hypermethylated in the NK, we hypothesized that in vitro methylation of the ARR would strengthen its silencing activity. We therefore employed a patch methylation assay to assess the silencer effect on the promoters of both imprinted transcripts. As shown in Figure 3, the promoter activity of the patch-methylated silencer constructs is reduced by about half the level of the mock-methylated control. These results show that increased methylation can augment silencer activity, although the silencer has intrinsic repressive capacity. Thus, the silencer is a cis-acting element controlling expression of the WT1-AS and AWT1 promoters, and a candidate methylation-sensitive imprinting regulatory element.

Figure 3. Patch methylation of the ARR silencer region. A silencer fragment was methylated with SsoI methylase (meth) or mock-methylated (mock), ligated into WT1-AS and AWT1 promoter plasmids, transfected and luciferase activity assayed (top panel). An aliquot of methylation-modified DNA was digested with BsoI(1236I) and then electrophoresed together with undigested DNA (Und) in order to check the efficacy of methylation. Methylated DNA protected from digestion is 712 bp. BsoI(1236I) digestion gives a 563 bp band (bottom panel).

The WT1 ARR silencer region binds the imprinting regulator CTCF

We assayed the WT1 ARR for DNaseI hypersensitivity and found that the unmethylated paternal allele showed greater DNaseI digestion, suggesting an open conformation available for protein–DNA interactions (data not shown). The multivalent zinc-finger protein CTCF has previously been shown to interact with the chicken lysozyme silencer (17) and the imprinting control regions of several imprinted genes, including IGF2-H19, Kcnq1 and TSIX (18). Furthermore, loss of heterozygosity (LOH) of chromosome 16q, resulting in haploinsufficiency of CTCF, has been suggested to contribute to epigenetic perturbations in WTs (19). Having identified the WT1 ARR as a region involved in the regulation of WT1-AS and AWT1 expressions, we were interested to examine whether the region might also be a target for CTCF binding. Fragments spanning the ARR were assessed for their ability to bind CTCF in electrophoretic mobility shift assays (EMSAs), as shown in Figure 4. CTCF binding was apparent with probes F2 and F3, but not with F1, F4 or F5 (Fig. 4A and B). Binding to probes F2 and F3 was shown to be methylation-sensitive, as methylated probes were not bound at all by CTCF (Fig. 4D). Remarkably, the CTCF-binding region overlaps precisely with the silencer element identified by transfections with SIL and DMR-SIL constructs in Figure 2. This CTCF target site (CTS) also spans the BstUII site (BstUII[−734], Fig. 4E), which displays consistent methylation changes in WTs relative to the NK (8,14),
suggesting that the methylation status of CpGs 13 and 14 is critical for the regulation mediated by CTCF (Fig. 4E). To verify that CTCF physically interacts with the WT1 ARR in vivo, we performed chromatin immunoprecipitation (ChIP) analyses with the NK and WT samples (Fig. 4F). CTCF antibody, not the normal serum control, pulled down ARR sequences spanning the differentially methylated silencer region in both the NK and WT samples. The band amplified in Figure 4F corresponds to nucleotide positions \(2554\) to \(2919\) relative to Figures 1A, 2A and 4A. The methylation-sensitive binding of CTCF at the ARR silencer supports the possibility that this region is involved in imprinting with one possible role for CTCF binding being to maintain differential methylation as previously demonstrated for \(Igf2/H19\) (20).

Given the overlap of the CTS with the silencer element, we were also interested in how CTCF may influence silencer activity. To investigate possible silencer modulation by
CTCF in a minimal system, we exploited the silencer effect on the basal luciferase shown in Figure 2C. Plasmid pGL3-B constructs with and without the silencer (p3BSIL and p3B, respectively) were transfected into 293 cells, which were then treated with a negative control siRNA or CTCF siRNA (Fig. 4G, top). As expected, the silencer reduced the basal luciferase activity of pGL3-B in the presence of the negative control siRNA by ~19%. When CTCF was knocked down, silencer-mediated repression was increased more than 2-fold to 42%. In contrast, vector-only luciferase activity was marginally higher after CTCF depletion, indicating a minimal and opposite effect of CTCF knockdown on cryptic promoter activity, emphasizing the specificity of silencer-associated changes. In all assays, cell viability and house-keeping gene expression remained comparable (data not shown). This experiment suggests a secondary role for CTCF interaction with the unmethylated WT1 ARR silencer, which is to block its methylation-independent repressive properties and maintain expression from the paternal allele.

Methylation analysis of the WT1 ARR in developmental and tumour samples

Our demonstration of CTCF involvement in AWT1/WT1-AS regulation, allied with the documented genetic lesions of chromosome 16 during Wilms’ tumourigenesis, suggested the possibility of epigenetic deregulation at 11p13 arising due to 16q LOH. Indeed our group has previously demonstrated that 16q LOH is a late event in Wilms’ tumourigenesis (21). Thus, having verified that the WT1 ARR DMR contains a methylation-sensitive CTCF-binding silencer involved in the regulation of gene expression, and probably imprinting, we sought to assess by methylation analyses the timing of epigenetic changes occurring during development and tumourigenesis. As previously demonstrated, Southern blot analysis of the WT1 ARR in the NK shows two major bands representative of a methylated maternal allele (731 bp band) and an unmethylated paternal allele (542 bp band). Analysis of four FK DNAs consistently showed that the WT1 ARR BstUI site distinguishing the 731 bp band and the 542 bp band (BstUI[−734]) is predominantly hypomethylated (Fig. 5A). To observe methylation variations during Wilms’ tumourigenesis, we analysed two non-LOH WT samples together with their matched NK and NRs. Consistent with imprinting defects observed previously (8), both left and right tumours (T65L and T65R) from patient 65 and a WT from a second bilateral patient (T62) show lack of methylation of BstUI[−734] on both alleles (Fig. 5A). NRs display predominantly BstUI[−734] hypomethylation similar to FK samples. Some methylation of BstUI[−734] and sites towards the antisense promoter is indicated by the fainter 850 bp band in NRs, similar to NKS.

As Southern blot analysis is limited to assessing CpG residues in restriction enzyme sites, we conducted a high-resolution methylation analysis of the WT1 ARR, using bisulphite-modified DNA spanning the WT1 ARR DMR (Fig. 5B). Combined bisulphite sequencing data from this study and a previous study (one matched pair of NK and WT) (14) are summarized graphically in Figure 5C. In general, bisulphite sequencing reflected the Southern blotting with the methylation status of BstUI[−734] being (i) predominant hypomethylation in the FK and rests, (ii) hemimethylation in the adult kidney and (iii) hypomethylation in WTs. However, bisulphite analysis also shows ARR methylation in early stages of renal development (represented by the FK and NRs). Additionally, the ratio of methylated and unmethylated alleles, irrespective of the density of methylation, is comparable. This suggests that methylation at the ARR may represent the primary imprint for AWT1/WT1-AS expression, i.e. the signal that distinguishes inherited alleles maternally and paternally. In normal paediatric kidney, equivalence of allelic methylation extends to CpG 13, with BstUI[−734], as is expected from Southern blotting data. Interestingly, methylation density was lower in NK62 relative to the other kidneys. This patient had anaplastic WT, suggesting a possible association between lower DMR methylation density and poor prognosis subsequent to WT development. Finally, WTs display almost total absence of methylation throughout the ARR.

The pattern and levels of methylation in NRs relative to the FKs and WTs were surprising considering that rests are precursors for WTs, and that WTs are considered to represent an expansion of foetal blastemal cells. If this was the case, it might be expected that increased hypomethylation would be observed in rests relative to FK, with NR methylation being more comparable with WTs. However, whereas FKs showed an average methylation density of 13%, rests showed 22% methylation, suggesting that methylation in NR cells is increasing towards the epigenotype observed in the fully developed kidney, rather than decreasing towards the hypomethylated state seen in WTs (Fig. 5D). This is consistent with the tumour epigenotype resulting from a loss of methylation (imprinting) rather than expansion of a FK cell with a hypomethylated epigenotype.

Notably, methylation of BstUI[−734] in NRs and the FKs remains comparable, and low relative to the adult kidney. We therefore examined whether the differences in methylation states correlated with allelic expression patterns of AWT1/WT1-AS. As shown in Figure 6, low level, monoallelic expression of WT1-AS and AWT1 is observed in NK62 and NK65, correlating with differential methylation of the WT1 ARR DMR. Both corresponding NR samples display biallelic expression of WT1-AS (Fig. 6A and B), and biallelic AWT1 expression is also apparent in NR62 (Fig. 6C). Patient 65 samples were not informative for allelic expression analysis of AWT1. Thus, the increased methylation observed in NRs relative to the FK is not able to impose monoallelic expression of the imprinted transcripts. High level, biallelic expression of AWT1/WT1-AS is associated with hypomethylation of both alleles in WT, confirming and extending our previous findings (8,14). This suggests that spreading of methylation to include BstUI[−734] may be necessary for switching to monoallelic expression of AWT1/WT1-AS. However, this does not occur in NRs despite an overall increase in methylation. Taken together, our methylation and expression analyses suggest that sequential epigenetic errors are required to attain the WT epigenotype, beginning with aberrant lowering of the methylation necessary for normal transcriptional repression of the maternal allele, followed by demethylation and erasure of the imprint.
DISCUSSION

In order to investigate inappropriate expression and imprinting of AWT1 and WT1-AS, we have characterized a candidate epigenetic regulatory element in the WT1 ARR DMR. This element acts as a transcriptional silencer of both AWT1 and WT1-AS promoters in reporter assays and is also capable of silencing heterologous promoters. The coincidence of high level, biallelic AWT1 and WT1-AS expression and hypomethylation of the WT1 ARR DMR in WTs (8, 14) suggested that methylation of the silencer would enhance its activity, and this is confirmed in this study, although the silencer also has intrinsic repressive activity in the absence of methylation. The silencer is also a CTS, with CTCF binding restricted to the unmethylated silencer and be able to partially alleviate silencing in vitro. Assessment of silencer region methylation demonstrates epigenetic changes occurring during development and tumourigenesis. Together, our data suggest that (i) the silencer is a candidate imprinting region, (ii) spreading of methylation is necessary for monoallelic expression, (iii) CTCF is involved in the expression and imprinting of this locus and (iv) erasure of methylation and imprinting are necessary for the WT epigenotype.

Our studies indicate a dual regulatory role for the ARR silencer, as it acts as an epiregulatory element as well as a cis-acting repressor of transcription. Deletion analysis and EMSA scanning of the region upstream of the WT1-AS promoter revealed a striking overlap of CTCF binding with silencer activity, and ChIP confirmed CTCF binding to the silencer region in vivo. Using coupled transient transfection, siRNA knockdown in a minimal reporter assay, we found that decreased CTCF potentiated silencing in 293 human embryonic kidney cells. Extrapolating our data to a cellular context, hypermethylation of the silencer element (exemplified by the maternal allele in the NK) leads to complete inhibition of AWT1 and WT1-AS expression, whereas CTCF binding on the unmethylated paternal allele permits expression. CTCF may therefore regulate the maintenance of differential

Figure 5. Methylation analysis of NRs. (A) Southern blot showing differential methylation of the WT1 ARR DMR. DNAs are from matched sets (62 and 65) of NK, NR and T (Wilms’ tumor), where L (left) and R (right) refer to bilateral tumours from the same patient, and from four independent FK samples. The 542 bp band is indicative of lack of methylation at BstUI−734 (Fig. 1) (14), whereas methylation of BstUI sites gives rise to higher bands. (B) CpG dinucleotide distribution in the WT1 ARR DMR. (C) Bisulfite analysis of developmental and tumour DNAs. Rows in the result table represent individual clones, and columns the CpG dinucleotides as depicted in (B), with CpG14 on the left, and CpG1 on the right. Filled boxes represent methylation and open boxes represent no methylation. CpGs not assessed due to primers are indicated by ‘−’. (D) Percentage methylation calculated from the total number of methylated cytosines in 10 bisulphite sequence clones from each tissue sample, covering CpGs 1–14 (140 CpG dinucleotides in total for each sample). Values plotted are the mean for each tissue type (FK, NK, NR, WT). Error bars show standard deviations.
methylation necessary for imprinted expression as demonstrated for the *Igf2-H19* locus (20), but also have a secondary function as a transcriptional activator via partially blocking the silencer. We note that mutation of CTCF binding sites within the *Igf2/H19* ICR led to a decrease of *H19* expression from the maternal allele, implying a similar positive role for CTCF in *H19* transcription (20). CTCF-binding silencers have been demonstrated at other imprinted genes, such as *Igf2* (22) and *KCNN1* (23). Although their precise mechanisms remain obscure, silencers are thought to function by recruiting protein complexes to chromatin and forming repressive domains by DNA bending or looping (24,25). In the case of *Igf2*, the unmethylated maternal allele DMR1 silencer binds CTCF, which facilitates formation of a repressive chromatin loop (22). *Igf2* is expressed from the paternal allele where the silencer is methylated and therefore acts oppositely to the ARR silencer. Examples of CTCF-binding silencers at non-imprinted genes include the chicken lysozyme silencer (17) and the chicken α-globin cluster (26). Again, both act oppositely to the ARR silencer in that CTCF binding is necessary for silencer activity. Together with our study, this suggests that as well as the documented role for CTCF in insulator control (27), CTCF may also have a genome-wide role in gene regulation via silencer modulation, a point highlighted by a recent analysis of human promoters, which suggested that more than half may have upstream negative regulatory elements (28).

As well as being involved in genomic imprinting, the ARR silencer may function in tissue or cell-type-specific expression, given its intrinsic repression activity. It is well documented that WT1 proteins are only detectable in podocytes in the adult kidney, although ARR differential methylation in the adult kidney might suggest uniform expression in all cell types. The intrinsic repressor activity of the silencer may serve to shut down *AWT1/WT1-AS* expression by recruitment of cell-type-specific repressors unavailable in podocytes. A similar silencer activity has been invoked to explain tissue-specific regulation by the 1A DMR-imprinting control region of *G_{2}alpha* (29). Our *in vitro* studies of silencer functions outside its native chromatin context highlight the importance of further functional analyses *in vivo*.

Assessment of DNA methylation at CpG islands located across the *WT1* locus showed allelic differential methylation clearly apparent at the *WT1* ARR, together with an absence of allelic methylation changes at the *WT1-AS* and *AWT1* promoters, and CpG islands at the *WT1* locus (8,14). Thus, the region contains a genuine DMR at which hypermethylation and hypomethylation are strictly confined to parent-of-origin-specified alleles and tightly coupled to the control of imprinted gene expression, suggesting that the *WT1* ARR DMR is central to imprinting control. Although definitive evidence requires methylation analysis in the germline, the methylation-sensitive regulatory capacity and CTCF binding of the silencer, together with allelic methylation suggested by bisulphite analysis of the developmental kidney DNAs, support the silencer as a candidate primary imprinting control region. Such an element would bear the signal that distinguishes maternally and paternally inherited alleles post-fertilization and maintain that signal throughout development. This has important implications for the interpretation of our results, as it permits discriminating between the WT epigenotype arising from the expansion of foetal progenitors with both alleles hypomethylated (possible if the ARR methylation is not a primary imprint), or an active loss of a primary imprint, which in turn would imply a causative role of deregulated genes/proteins. Our finding that NRs display methylation patterns and densities between the FK and adult kidney, rather than between FKs and WTs, supports the latter alternative. As our previous work with LOH in NRs has confirmed the efficacy of microdissection (21), we do not consider it likely that NR DNAs would be contaminated with the adult kidney DNA that might give a false impression of methylation patterns in NRs.

A variety of other imprinted genes are epigenetically deregulated in WT, including *IGF2*, *IGF2-AS*, *HI9*, *CDK2J/ICp57KIP2*, *BWR1A* and *BWR1C* (30). Some of these events have been shown to occur early in tumourigenesis. For instance, *HI9* and *IGF2* imprinting changes can be detected not only in WT, but also in the surrounding kidney cells (31). Our analyses of NRs provide further evidence for a multi-step pathway leading to WT. Importantly, this analysis suggests the need to discriminate between limitations in the spreading of methylation necessary for imposing monoallelic expression in hyperplastic NRs, and the subsequent loss of imprinting that occurs as a subpopulation of NR cells develops the tumour epigenotype. Thus, although biallelic expression indicative of relaxation or loss of imprinting (ROI/LOI) is apparent early in tumourigenesis, this appears to be attributable to undermethylation as opposed to a lack of the imprint *per se*. However, for tumours to arise from

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**Figure 6.** RT–PCR analysis of *WT1-AS* and *AWT1* allelic expression. (A) *WT1-AS* expression in patient 62 samples showing monoallelic expression in the NK, and biallelic expression in the NR and WT. Individual alleles are arrowed (A1, A2), with sizes on the right. (B) *WT1-AS* expression in patient 65 samples showing similar expression patterns to patient 62. (C) *AWT1* analysis of patient 62, demonstrating monoallelic expression in the adult kidney and biallelic expression in rest and tumour. The very faint band of ~56 bp in NK62+ represents a PCR stutter band. (D) DNA: ′−′, without reverse transcriptase control; ′+′, with reverse transcriptase.
cells with an NR epigenotype, complete erasure of the imprint is necessary, as ARR methylation in tumour DNAs is virtually absent. It is therefore reasonable to propose that the early curtailed methylation is an early initiation step, necessary but insufficient in itself for tumour development, whereas complete loss of imprinting is a later progression step.

It is intriguing that the imprinting regulator CTCF is an upstream determinant of AWT1/WT1-AS expression, as LOH of chromosome 16q and CTCF missense mutations have been reported in WTs (32,33). CTCF haploinsufficiency resulting from 16q LOH has been linked with LOI of IGF2-H19 (19,34), but another report showed that CTCF transcripts were unaltered in samples with 16q LOH and argued against a link between genetic lesions at 16q and LOI (32). Although there is conflicting data regarding the possible role of CTCF as a tumour-suppressor gene involved in Wilms’ tumourigenesis, our finding of CTCF binding at the silencer raises several possibilities regarding deregulated CTCF-mediated control and its role in cancer. CTCF mutations occur in the zinc-finger region and diminish DNA binding greatly at promoters of growth regulatory genes, whereas binding to promoters of proliferation neutral genes is unaffected (18). However, CTCF mutations are infrequent (35) and an association between changes in CTCF and expression of IGF2-H19 is equivocal (19,32,34). At present, no studies have evaluated alternative modes of CTCF deregulation in tumours, such as the post-transcriptional modifications of CTCF shown to affect imprinting regulation (36) or DNA-binding site competition by the CTCF paralogue, BORIS (37). Poly(ADP-ribosyl)ation has been shown to be essential for CTCF-dependent chromatin insulation (36), and BORIS, which is frequently overexpressed in cancer, can modulate the epigenetic status of promoters targeted by CTCF (38,39). Our preliminary studies suggest that there are CTSs, in addition to those reported here, across the WT1 locus, emphasizing the need for in-depth analysis of CTCF/BORIS-mediated regulation of transcription from the WT1 locus.

We propose a model (Fig. 7) wherein the failure to establish monoallelic expression of AWT1/WT1-AS during early development permits unscheduled expansion of preneoplastic NR cell subpopulations owing to the growth-promoting and anti-apoptotic activities of deregulated AWT1 and WT1 (via WT1-AS deregulation). AWT1 has been shown to have potential oncogenic properties in activating the cyclin E promoter and cooperating with Ras in transforming primary fibroblasts in vitro (9). WT1 has been shown to transactivate expression of the anti-apoptotic genes BCL2 (40) and BFL1 (41), and certain isoforms of WT1 can increase tumour growth rate when ectopically expressed (42). The possible involvement of deregulated AWT1/WT1 expression in tumourigenesis is
also supported by the observation that Beckwith–Wiedemann syndrome patients with paternal uniparental disomy extending to 11p13 appear likelier to develop WT (43). Also, LOH at 11p13 unaccompanied by WT1 mutation is often seen in WTs (44) and is unusual relative to other loci in that it always involves mitotic recombination, which reduplicates the remaining paternal allele (45). Thus, genetic and epigenetic events at the WT1 locus manifest similar effects in terms of gene dosage, which is an increase of paternally expressed genes, alluding to this being a requirement for tumourigenesis in a subset of WTs.

The deficit in methylation at the first stage of WT development is unlikely to result from a genetic defect at the CTCF locus, which is a later event, but may be attributable to protection from methylation by modified CTCF, altered CTCF/BORIS ratios or other transcription factors. Thereafter, NRs can be eliminated or regress, but a second hit, which may be genetic or epigenetic, leads to complete erasure of the imprint and neoplastic progression. It is interesting to note that both tumours 62 and 65 studied here have 16q LOH, which is not apparent in the NRs (21; unpublished data), suggesting that lowered CTCF and/or BORIS activity may drive the erasure stage. Although the involvement of BORIS is at present speculative, it is noteworthy that it binds CTSs and promotes tumourigenesis in a subset of WTs.

In summary, this first analysis of epigenetic mechanisms operating at the WT1 locus underlines the complexity of expression control necessary for normal development orchestrated by WT1-related proteins and transcripts. Deregeneration of CTCF/BORIS may be pivotal in generating epigenetic lesions, and our studies suggest that the absence of imprinting observed in WTs is not simply a manifestation of onco-foetal epigenotype. This in turn implicates AWT1, WT1 and WT1-AS overexpression in the aetiology of WT.

MATERIALS AND METHODS

Clinical materials
All tissues used were collected with appropriate ethical approval and processed as described previously (14,21). Both patient 62 and 65 had sporadic bilateral tumours with triphasic histology. Patient 65 was responsive to therapy and survived, whereas patient 62 had anaplastic WT and did not. FK samples ranged 19–22 weeks of gestation.

Cell culture, transient transfections and siRNA knockdown
All cells were maintained as previously described (46). For reporter assays, 100 ng of each promoter construct was transfected using 1 μL Lipofectamine and 2 μL Plus reagent (Gibco BRL) into 2 × 10⁵ 293 cells. Cells were incubated at 37°C for 40 h and subsequently lysed and analysed for luciferase activity according to the manufacturer’s protocol (Promega). For patch methylation experiments, ~500 ng of each ligated construct was transfected using 3 μL Lipofectamine and 1 μL Plus reagent and harvested 48 h post-transfection. Plasmid pGL2E, pGL2B, pGL3B or pGL3E was used as the negative control as appropriate and all transfection assays were performed at least three times in triplicate. Co-transfection with internal control reporter plasmids was precluded by transfections exerted by silencer fragments. For transient transfection/siRNA knockdowns, 2 × 10⁵ 293 cells/well were seeded and transfected the following day with 100 ng of the relevant plasmid, using Fugene 6 (Roche). After 24 h, the cells were treated with 100 nm of CTCF or non-targeting control SMARTpool siRNAs together with transfection reagent, DharmaFECT 1 (Dharmacon, Inc.), following the manufacturer’s protocol. Luciferase lysates and protein were harvested after a further 48 h. Western blotting was carried out as previously described (8).

Transfection constructs
Silencer mapping plasmids were constructed by subcloning WT1 intron 1 as a series of deletion mutants in pGL2E (Promega). Silencer characterization plasmids were constructed in pGL2B (Promega) by subcloning the 134 bp Sau3AI fragment, except plasmid DMR-SIL-αSP, which was made by subcloning a Kpn1-restricted PCR product amplified using the primers HMF (5’-GGGGTTACGACCCAATCTCCAGGTTCG-3’) and M.Kpn1 (5’-CCGTTACCAGATCTGTCCTGGAGG-3’). Fragments were cloned upstream of the relevant promoter constructs. Patch methylation assay plasmids and AWT1 promoter constructs were in pGL3E and pGL3B (Promega) and used the silencer fragment between the Kpn1 site and SpeI site shown in Figure 1.

Patch methylation analysis
Plasmid pGL3E-550 was constructed by ligating an Sau3AI/PvuII WT1-AS promoter fragment (~592 to ~43 relative to Fig. 1) into pGL3E. To generate promoter plasmid backbone, 10 μg pGL3E-550 was digested with SacI and Nhel. For preparation of methylated and unmethylated silencer fragments, plasmid pXS SIL was used. This plasmid contains the ARR region-spanning nucleotides ~592 to ~1276, with XbaI and SacI terminus sites generated by PCR primers. This insert was cloned into pUC18, and the resultant pXS SIL was propagated in bacterial strain SCS110 to limit methylation. For patch methylation studies, the insert was excised as an XbaI/SacI fragment and methylated using SssI methylase (NE Biolabs) with 160 μM S-adenosyl methionine under the recommended conditions for 2 h. The DNA was mock-methylated under the same conditions minus enzyme. Aliquots were removed and digested with Bsh1236I to ensure methylation. For ligation of XS SIL insert into pGL3E-550 promoter backbone, DNA was purified by phenol extraction and ethanol precipitation and then ligated to plasmid backbone at a ratio of ~2 μg plasmid backbone:1 μg insert (~3-fold molar excess of insert) using 800 cohesive end units T4 DNA ligase (NE Biolabs) under the recommended conditions. Ligation products were re-purified as described previously, resuspended in TE and analysed by gel electrophoresis prior to transfection. A similar procedure was used for WT1 promoter constructs using plasmid 400F (8) as the core WT1 promoter.

Electrophoretic mobility shift assay
The 11-ZF DNA-binding domain and full-length human CTCF proteins were synthesized from pET-12Zf and the
pET-7.1 constructs, respectively (47,48), with the TnT reticulocyte lysate-coupled in vitro transcription–translation system (Promega). An 850 bp sequence spanning the WT1 ARR was amplified by PCR and TA cloned, and used for subsequent PCR of five fragments ranging from 192 to 254 bp (F1–F5). Primers for F2 were ARR2F (5'-GAGAATCGAAGCCCGTCTTCTCT-3') and ARR2R (5'-CAAGGTTGCGCCGCATCGATCAAAGAC-3'), and for F3, ARR3F (5'-CTCAAGGGGGTTTGGGACACAATCGGTTGTT-3') and ARR3R (5'-GATGGGTGCGAGTTCCATATTTAAAAG-3'). Gel-extracted PCR products were γ-32P-labelled, Microspin G-25 column (Amersham Biosciences)-purified and used as DNA probes for EMSA with the in vitro-translated proteins, as described (47,48). Binding reactions were carried out in the buffer containing standard 50 mM Tris (pH7.5) with 5 mM MgCl2, 0.1 mM ZnSO4, 0.1% NP40 and 10% glycerol in the locyte lysate-coupled in vitro pET-7.1 constructs, respectively (47,48), with the TnT reticulocyte lysate-coupled in vitro translation system (Promega). An 850 bp sequence spanning the WT1 ARR was amplified by PCR and TA cloned, and used for subsequent PCR of five fragments ranging from 192 to 254 bp (F1–F5). Primers for F2 were ARR2F (5'-GAGAATCGAAGCCCGTCTTCTCT-3') and ARR2R (5'-CAAGGTTGCGCCGCATCGATCAAAGAC-3'), and for F3, ARR3F (5'-CTCAAGGGGGTTTGGGACACAATCGGTTGTT-3') and ARR3R (5'-GATGGGTGCGAGTTCCATATTTAAAAG-3'). Gel-extracted PCR products were γ-32P-labelled, Microspin G-25 column (Amersham Biosciences)-purified and used as DNA probes for EMSA with the in vitro-translated proteins, as described (47,48). Binding reactions were carried out in the buffer containing standard 50 mM Tris (pH7.5) with 5 mM MgCl2, 0.1 mM ZnSO4, 0.1% NP40 and 10% glycerol in the presence of poly(deoxyinosinic-deoxycytidylic acid). Final reaction mixtures of 20 μl were incubated for 30 min at room temperature and then analysed on 5% non-denaturing PAGE run in 0.5 × Tris-borate–EDTA buffer.

**ChIP analysis**

Formaldehyde-crosslinked nuclei from WT and the NK tissue were isolated and chromatin immunopurified using a CTCF antibody, as described previously (49). The CTFC-binding ARR fragment was PCR-amplified for 25 cycles (94°C for 30 s, 64°C for 30 s and 72°C for 30 s), using the following primer set: ARR forward (5'-GAGAATCGAAGCCCGTCTTCTCT-3') and ARR reverse (5'-GATGGGTGCGAGTTCCATATTTAAAAG-3'). ChIP material from WT, normal tissue and serum control corresponding to 5 μg input genomic DNA was used as template. The PCR products were analysed on a non-denaturing 5% polyacrylamide gel stained with SYBR-green (Molecular Probes) and visualized using an FLA-3000 phosphorimager (Fuji).

**Southern blot analysis and bisulphite DNA sequencing**

Methylation-sensitive Southern blotting and bisulphite DNA sequencing of the WT1 ARR were performed as previously described (14).

**RT–PCR for allelic expression**

Allelic expression of WT1-AS by RT–PCR was determined using transcribed polymorphisms in the region of WT1-AS upstream of WT1, detected by MnlI digestion, as described previously (14), or by DdeI digestion (50) of nested PCR products [first-round primers: R1 (CATGTGGATCCGTTGGGGTC) and F3A (GTGCGCGGATCTTATGGGCA); second-round primers: 18 (CTTAGACCTTCTCTTGGC) and F3B (GGCGCGGATCTTATGGGCA)]. Similarly, allelic expression of AWT1 using a polymorphism in the 5'-UTR of exon 1A (SNP database ref. rs12575247) was determined by MnlI digestion of semi-nested PCR products [first round primers: SN1 (GCCACTCGATCTCTCTCCT) and Ex2AS (TGGCCCAGGATGTCTTCTG); second-round primers: SN1 and ASN2 (CAGCGCTTGGCGTCGCGCG)]. For nested and semi-nested PCRs, both rounds of amplification were for 30 cycles at an annealing temperature of 60°C. The template for the second round was 1 μl of the 25 μl first-round reaction.

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**Conflict of Interest statement.** There are no conflicts of interest.

**REFERENCES**


