Development of an siRNA-based method for repressing specific genes in renal organ culture and its use to show that the Wt1 tumour suppressor is required for nephron differentiation

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Wt1 is a tumour suppressor gene, mutation of which is a cause of Wilms’ tumour, a childhood renal nephroblastoma. Wt1 is expressed in a rich pattern during renal development suggesting that it acts at three stages: determination of the kidney area, the differentiation of nephrons and maturation of glomeruli. Wt1−/− mice confirm that Wt1 is essential for the inception of kidney development; cells that ought to form kidneys die by apoptosis instead. Specific human WT1 mutations cause defects of glomerular maturation (Denys–Drash and Frasier syndromes), providing circumstantial evidence for action of Wt1 during glomerular maturation. There is, however, no genetic evidence for a function during nephron differentiation because this stage is never reached in Wt1−/− mice. We have therefore developed a novel technique, based on small interfering RNA (siRNA), to repress the expression of Wt1 and other specific genes at different stages of kidney development in culture. We find that early repression of Wt1 phenocopies the Wt1−/− mouse, but later repression prevents cells differentiating into nephrons and causes them instead to proliferate abnormally, possibly mimicking aspects of Wilms’ tumour. In line with established hypotheses about genetic pathways that control kidney development, we find that repressing Pax2 using siRNAs represses Wt1 expression and blocks both bud growth and nephron differentiation, but that repressing Wnt4 blocks nephron differentiation without affecting Wt1 expression. As well as illuminating previously inaccessible aspects of Wt1 biology, our results suggest that siRNA in organ culture will be a powerful method for analyzing other developmental pathways and testing the effects of stage-specific loss of tumour suppressor genes.

INTRODUCTION

Production of knockout mice using ES cells (1) has become one of the most powerful techniques for studying the molecular genetics of mammalian development. For many projects, the technology is ideal, except for its cost (2), but standard knockouts are not suitable for studying genes that are required at multiple stages of development; embryos die or become abnormal at the first stage that the gene is needed, making later events inaccessible to observation. Advanced transgenic techniques, such as cre-lox (3,4) can ameliorate these difficulties but they depend on having tissue-specific promoters, which are not always available especially in early development before cells differentiate in an organ-specific manner. For this reason, the recently-developed technique of RNA interference (RNAi) mediated by small interfering RNAs (siRNAs) is interesting because it has the potential to inhibit gene expression of any gene at a time of the experimenter’s choosing (5–7). So far, most applications of siRNA have been to cells growing in monolayer culture (6–9). We have adapted the
but outgrowth of the ureteric bud does not occur and the yme, including expression of Pax2, Six2 and GDNF, is normal. rudiments to investigate previously inaccessible aspects of one of Denys–Drash and Frasier syndromes (19–26). The mutants the kidneys with specific glomerular defects including those of beginning of renal development. Second, low gene dosage of be difficult to explain if Wt1 functioned only once, at the very futher in glomerular podocytes (14,18). This expression would of metanephric development, then being upregulated in the appearing as general low-level expression right at the beginning of main stages in the developing kidneys of mice and humans, expressed in a complex spatiotemporal pattern which has three of human Wilms’ tumour, a nephroblastoma affecting 1 : 10 000 children that is thought to result from maldevelopment of the embryonic kidney. It encodes a zinc finger containing protein that exists in several isoforms, produced by alternative splicing, which is associated with transcription repression, transcription activation and are also implicated in mRNA splicing (10). Wt1 is a tumour suppressor gene, and its mutation accounts for some 10% of cases of Wilms’ tumours, as well as for several additional developmental syndromes characterized by a combination of Wilms’ tumours and other urogenital abnormalities (11).

Mice lacking both copies of the Wt1 gene die during foetal development, probably as a result of having a badly malformed heart (12,13). In the urogenital system, the Wt1 phenotype is dramatic: no metanephric kidneys form. Lack of Wt1 appears to disrupt renal development at its earliest stages. In normal embryos, kidney development begins when a group of cells at the caudal end of the intermediate mesoderm expresses transcription factors such as Wt1, Six2 and Pax2, and produces diffusible signalling molecules such as GDNF (14,15). These induce the Wolffian duct that runs along the edge of the intermediate mesoderm to produce a side-branch, the ureteric bud. The bud invades the metanephric mesenchyme and, once inside, it is induced to branch to form the collecting duct tree. As it does, the bud induces groups of mesenchymal cells to undergo a mesenchyme-epithelial transition and form excretory nephrons which eventually contain the specialized segments of a mature nephron and connect to the collecting ducts (16,17). In Wt1−/− embryos, the initial differentiation of the mesenchyme, including expression of Pax2, Six2 and GDNF, is normal (15) but outgrowth of the ureteric bud does not occur and the mesenchyme dies by apoptosis (12).

The dramatic phenotype of Wt1−/− mice demonstrates that Wt1 is essential for renal development, but a paradox emerges when the knockout phenotype is considered along with the tumour data; loss of heterozygosity for functional Wt1 during human renal development results in formation of a tumour, yet complete absence of Wt1 in developing mouse kidneys results not in neoplasia but rather in complete loss of cells by apoptosis. There are two possible explanations for this; there may be an intrinsic difference between the two species (though this seems unlikely given the fundamental biology involved) or Wt1 may function at more than one stage of renal development with only some of these being able to generate the tumour phenotype on loss of Wt1.

There are several reasons for supposing that Wt1 acts at multiple stages of metanephric development. First, Wt1 is expressed in a complex spatiotemporal pattern which has three main stages in the developing kidneys of mice and humans, appearing as general low-level expression right at the beginning of metanephric development, then being upregulated in the mesenchyme as it differentiates into nephrons and later increasing further in glomerular podocytes (14,18). This expression would be difficult to explain if Wt1 functioned only once, at the very beginning of renal development. Second, low gene dosage of Wt1, or specific Wt1 mutations in humans and in mice, result in the kidneys with specific glomerular defects including those of Denys–Drash and Frasier syndromes (19–26). The mutants therefore provide sufficient function for kidney development to begin, but not for every aspect of kidney maturation to take place normally. Finally, while mice lacking their own Wt1 genes fail to form kidney rudiments, similar mice provided with a YAC containing the human Wt1 locus can develop kidney rudiments, but these fail to form glomeruli presumably because the mice have an abnormal gene dosage (13).

There is, therefore, good evidence that Wt1 acts at the first stage described above (onset of renal development) and at the third stage (glomerular maturation) but there is only circumstantial evidence that it acts at the middle stage, that of nephron differentiation.

Persuasive as the argument summarized above may be, a requirement for Wt1 in nephrogenesis can be proved only by allowing the early stages of organogenesis to proceed with normal expression of Wt1, then removing Wt1 and determining whether development can still proceed. Until the advent of siRNA, the only method that has been used for gene knockdown in kidneys has been the application of phosphorothioate antisense oligonucleotides, whose use has proved controversial. They first showed promise in a study on the role of the neurotrophin receptor p75NGFR; 5 μM antisense p75NGFR inhibited the development of ureteric bud and nephrons but sense did not (27). Shortly afterwards, though, a research group attempting to repeat this observation using the same sequences from the same source found no specific effects and concluded that ‘phosphorothioate oligonucleotides cause non-specific toxicity in organ cultures of mouse and rat kidneys’ (28). Further examination of this issue suggested that, in kidneys at least, the range of concentrations at which phosphorothioate reagents are effective but not toxic is very narrow and that, because of inevitable small variations in the way that individuals handle cultures, reproducibility between laboratories can be difficult (29). There have been promising applications of phosphorothioate antisense reagents to kidneys, notably the very closely controlled study on Pax 2 (30). In general, though, the issues described above, and later discoveries about unexpected side-effects of phosphorothioates such as displacement of heparin-binding growth factors from their low-affinity receptors (31), dissuaded us from choosing phosphorothioate oligonucleotides to inhibit the expression of Wt1.

So far, it seems that siRNA is relatively free from non-sequence-specific effects, possibly because of its different structure or possible because it is usually applied at a much lower concentration than the μM range that is required for phosphorothioates. We have therefore developed a technique for applying siRNA to kidney rudiments developing in organ culture. Using siRNA to repress Wt1 expression at different stages of renal development, we find that Wt1 is required not just at the beginning but also later for nephron differentiation.

RESULTS

Wt1 siRNA represses Wt1 expression in murine M15 cells

It has been documented that, while siRNA is generally highly effective in repressing gene expression in mammalian cells, the
effectiveness of any particular siRNA is difficult to predict (32,33). It was therefore important for us to develop a system in which we could test the effectiveness of candidate siRNAs, designed to repress expression of Wt1, without the additional complications and variables expected to arise from their use in three-dimensional organ culture.

The M15 cell line, derived from the mesonephric region of an embryonic mouse, grows well in conventional two-dimensional cell culture and expresses Wt1 protein at high levels (34). M15 cells treated with irrelevant siRNA continued to stain strongly with anti-Wt1 antibody (Fig. 1A), demonstrating that the presence of siRNA in general, or of the Oligofectamine vector, does not repress Wt1. In contrast, an siRNA (Wt1_913) designed to target Wt1 mRNA caused a profound repression of Wt1 expression in these cells (Fig. 1A). This repression of Wt1, and also the continued normal expression of other messages and proteins (β-actin and p116), was confirmed by both northern and western blotting (Fig. 1B and C). Scanning and quantifying the northern and western blots revealed the degree of protein repression to be ~80%.

**Wt1 siRNA represses Wt1 expression in cultured embryonic kidney rudiments and blocks nephron development at the pre-epithelial stage**

Application of siRNA to kidney rudiments growing in culture required the development of a modified culture technique. Our initial attempts to economize on siRNA by growing kidneys in hanging drops, as salivary glands are often grown, met with failure because kidneys always grew very poorly in hanging drops even in serum-enriched medium. It seemed unlikely that this was due to exhaustion of nutrients or build-up of toxins, because it was true even if the medium was changed frequently; it may reflect a requirement for cell traction which can only take place against other tissues or a solid substrate (35,36). A miniaturized standard kidney culture system, in which kidneys were grown on polycarbonate filters supported at the surface of serum-containing medium, resulted in vigorous growth but siRNA was ineffective, neither reducing expression of Wt1 nor having any discernable effect on renal development. Use of transferrin-supplemented serum-free medium (37) for the full 72 h of culture allowed siRNA to work well but resulted in fewer nephrons (mean 2.6, σ = 1.6), even in untransfected controls, than are produced in serum-containing medium (mean 4.8, σ = 2.5, from the same batch of embryos). Combining these techniques, by culturing kidneys in transferrin-supplemented serum-free medium with siRNA for the first 24 h followed by the addition of 10% serum for the remaining 48 h, produced vigorous development in control kidneys while still allowing siRNA to inhibit gene expression: this method was therefore adopted for all subsequent siRNA studies.

In control kidney cultures isolated at E11 and cultured for 72 h, and also in those treated with luciferase siRNA (Fig. 2A), there was no detectable expression of Wt1 protein in the ureteric bud but there was low level expression in the mesenchyme and a high level of expression in nephrons. This expression accords well with the observations of others who have studied Wt1 expression *in vivo* and in culture (14,38). Culture of kidney rudiments in the presence of Wt1_913 siRNA resulted in a dramatic reduction of Wt1 protein expression (Fig. 2B), although a few cells seemed to escape inhibition as we had previously observed with the M15 cells (Fig. 1A).

As well as inhibiting the expression of Wt1, Wt1_913 siRNA affected the morphogenesis of the kidney rudiments. There are two simple ways by which such morphogenesis can be quantified; by the number of epithelial nephrons produced in the mesenchyme and by the number of branches made by the ureteric bud. For the purposes of this report, the status ‘epithelial nephron’ was determined by possession of a basement membrane containing laminin A, which is acquired at the same time as other epithelial markers such as E-cadherin and desmocollins (39,40). Control kidneys grown in this culture system but without siRNA produced a mean of 4.8 (SEM = 1.25) epithelial nephrons per kidney after 72 h. Kidneys grown in the presence of luciferase siRNA produced a mean of 4.5 (SEM = 0.77) epithelial nephrons, a number not significantly different from untreated controls (Figs. 2B, D and E). In contrast, those cultured in the presence of Wt1_913 siRNA produced a mean of only 0.9 (SEM = 0.35) epithelial nephrons per kidney, a number significantly lower than controls (Fig. 2B, D and E). Where they did form, these nephrons expressed some Wt1, so presumably arose from cells that had not been transfected efficiently with siRNA. Development of the ureteric bud tree was not affected significantly by siRNA-mediated Wt1 repression over the time-course of this experiment (Fig. 2F). The shape of the bud was often rather

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**Figure 1.** siRNA-mediated repression of Wt1 in M15 cells. (A) Immunostaining of M15 cells (nuclear DAPI staining shown in blue, Wt1 expression in red), shows that Wt1 expression remains in cells treated for 48 h with luciferase (‘Luc’) siRNA but is repressed in most cells treated with Wt1 siRNA. This repression is confirmed by northern blotting (B) and western blotting (C).
disorganized (see, for example, Fig. 2D) but it was not clear whether this reflected altered morphogenesis of the bud itself, or was secondary to disorganization of the mesenchyme caused by the physical absence of nephrons. The other siRNA we designed against Wt1, Wt1_1180, also significantly suppressed nephron differentiation without blocking ureteric bud branching. Its effect was not as marked as that of Wt1_913, though, which is why the rest of our experiments concentrated on the latter siRNA.

**Application of Wt1 siRNA to E9 intermediate mesoderms phenocopies the Wt1^{+/−} mouse**

The absence of a clear ureteric bud phenotype in E11 kidney rudiments described above seemed surprising because ureteric bud development fails completely in Wt1^{-/-} mice (12). There are several possible reasons that loss of mesenchymal Wt1 function once the kidney has already reached the T-bud stage may have a different effect on ureteric bud development than would absence of Wt1 from the very beginning of renal development (see Discussion), but it was important to exclude the possibility that the difference reflected some inherent weakness of the siRNA approach. We therefore separated the issues of timing and method of gene repression by applying siRNA before metanephric Wt1 expression normally begins, to mimic the knockout as closely as possible. The caudal region of the intermediate mesenchyme was isolated from E9 embryos and maintained in culture; at the time of its isolation, no signs of metanephric development were visible in the mesenchyme, there was no ureteric bud and the Wolffian duct had not yet reached the cloaca. Over 48–72 h in culture, control explants and those incubated in luciferase siRNA extended their Wolffian ducts and produced ureteric buds which invaded the nascent metanephric mesenchyme and began to branch (Fig. 3A). Explants incubated in Wt1_913 siRNA also showed extension of the Wolffian duct, but ureteric buds failed to form (Fig. 3B). This result suggests that, when applied sufficiently early, Wt1 siRNA causes defects in ureteric

![Figure 2](https://example.com/figure2.png)

*Figure 2.* Effect of Wt1 siRNA on cultured kidneys stained for laminin (red) and Wt1 (green). (A) A control kidney, with luciferase siRNA, shows a typical pattern of Wt1 expression; Wt1 is absent from the ureteric bud (‘ub’), present at a low level in condensing mesenchyme (‘cm’) and upregulated strongly in the presumptive glomeruli (‘g’). (B) In kidneys treated with Wt1 siRNA, some low level expression of Wt1 remains in the mesenchyme but epithelial nephrons are absent and only a few scattered cells express high levels of Wt1. Inhibition of nephrogenesis can be seen clearly by comparing (C), a low-power view of a kidney cultured in luciferase siRNA, in which there are many nephrons (arrows show examples) and (D) a kidney cultured in Wt1 siRNA in which no nephrons are present. (E) Counting of epithelial nephrons detectable by 72 h by expression of laminin A-rich basement membranes shows no significant difference between no-siRNA and luciferase siRNA samples, but a highly significant reduction in nephron number in kidneys treated with Wt1 siRNA. (F) There is no significant difference in the extent of ureteric bud branching, assessed by counting tips, by 72 h. Error bars represent standard errors of the mean.
bud development similar to those seen in Wt1−/− mice, so that the differences seen when Wt1 siRNA is applied only later in development are a result of the different timing rather than any inherent problem with the siRNA method of gene repression.

**Loss of Wt1 function during nephrogenesis causes cells to proliferate abnormally rather than differentiate**

The Wt1 gene was first identified because of the association between the Wt1 mutation and the development of Wilms' tumour which, like all tumours, is associated with aberrant differentiation, proliferation and apoptosis (41,42). The results presented in Figure 2 and described above show Wt1 to be required for normal differentiation of mesenchyme cells into nephrons; they also raise the question of whether failure of differentiation is accompanied by unusual proliferation or apoptosis.

We assessed proliferation in cultured kidney rudiments by the BrdU incorporation method (43). Untreated kidneys, and also those treated with luciferase siRNA, showed a typical pattern of cell proliferation (Fig. 4A). The ureteric bud was undergoing some proliferation, which was confined mainly to the tips as previously reported (43). There was also a zone of intense proliferation in the mesenchyme condensing around those tips. In the regions left behind by the advancing ureteric bud tips, where nephron differentiation was taking place, proliferation was much lower than in the mesenchymal halo around those tips. In kidneys treated with Wt1_913 siRNA (Fig. 4B), proliferation was similar, both in appearance and quantitatively (Fig. 4C), in the ureteric bud itself and in the mesenchyme around its tips. It was, however, abnormally high in the regions left behind by the tips, where nephrons ought to have been differentiating but were not; quantitatively, the difference in proliferation between luciferase siRNA and Wt1 siRNA treated kidneys was statistically significant in this zone (Fig. 4C).

We assessed apoptosis in these rudiments by nuclear morphology (DAPI staining, data not shown) and by TUNEL (Fig. 5A). The thickness of the tissue, coupled with the fact that DAPI stains even normal nuclei, meant that images obtained by this method were not of sufficient quality to permit accurate counting and only TUNEL yielded data clear enough to quantify accurately. In control kidneys, most apoptosis took place in a circumferential belt of cells around the edge of the culture, where the rudiment was at its thinnest and cells were furthest from developing nephrons and the ureteric bud. Cells at the top surface of the centre of the bud, which were as accessible to medium (and later to TUNEL reagents) as the circumferential cells, but which were closer to the bud and developing nephrons, showed little apoptosis. The general pattern of apoptosis was similar in cultures grown in Wt1 siRNA, except that the zone of circumferential apoptosis was wider and levels of apoptosis there were significantly elevated (Fig. 5B and C).

**Wnt4 siRNA expression blocks nephrogenesis without blocking mesenchymal WTI expression**

Wnt4 is a signalling protein expressed during nephron differentiation which functions in an autocrine manner to stimulate its own synthesis (44); it has been proposed that the local build-up of Wnt4 may be a mechanism that allows aggregating mesenchymal cells to sense when they are of sufficient number to undergo epithelial differentiation (45). Wnt4 is first expressed many hours later than Wt1 but, unlike Wt1, it is expressed only in differentiating cells. Wnt4 has been identified as a potential target of Wt1, at least in cell lines (46), but given the difference in expression patterns of these molecules the connection between them must be complex and conditional. The effects of Wnt4 loss have been studied using knockout mice, whose kidneys fail to form nephrons (44). We have developed a Wnt4 siRNA to try to phenocopy the wnt4−/− kidneys and to determine whether siRNA targeting Wnt4 has any effect on expression of Wt1.

We tested four potential Wnt4 siRNAs in M15 cells (Table 1), assessing efficiency by western blotting. Two of them showed no detectable effects (data not shown), one showed only a small effect but one was a powerful suppressor of Wnt4 expression in M15 cells (Fig. 6A) and the same siRNA was also the most effective in cultured kidneys. Application of this siRNA to cultured kidneys caused a significant reduction in the numbers of epithelial nephrons compared with controls treated with luciferase siRNA (Fig. 6B). Expression of Wt1 in the mesenchyme of Wnt4 siRNA-treated kidneys remained, however, and was particularly strong in the mesenchyme near
We tested three potential siRNAs targeting Pax2 (Table 1). Two produced mediocre repression and one produced powerful repression; we used only that siRNA in subsequent experiments. Kidney rudiments cultured in the presence of luciferase siRNA expressed Pax2 strongly in their ureteric buds and in developing nephrons and expressed it more weakly in the bulk of the mesenchyme (Fig. 7A); this is the normal pattern of expression that has been described before (30). Kidneys cultured with Pax2 siRNA showed a profound depression of Pax2 expression in most of the mesenchyme, although some cultures still had scattered areas that retained some expression (Fig. 7B). Expression in nephrons could not be assessed because there were none. Expression in the ureteric bud was only mildly repressed, however, and remained at levels at least 50% of those in controls as judged by brightness in micrographs taken at equal exposure such as Figure 7A and B. Therefore it is clear that siRNAs delivered by our method share with phosphorothioate oligonucleotides the property of not being efficient at repressing expression of genes in the ureteric bud.

Despite the poor knockdown of Pax2 in the ureteric bud, Pax2 siRNA had a profound effect on morphogenesis of both the nephrons and the ureteric bud. In the series of kidneys used to obtain the quantitative data shown in Figure 7C and D, no epithelial nephrons (defined as before) formed. Unlike Wt1 siRNA, Pax2 siRNA had a dramatic effect on ureteric bud branching, reducing it by over 50%. It is not clear whether this was due to the mild repression of the Pax2 that was expressed in the bud itself or to an indirect effect of Pax2 loss in the mesenchyme. Pax 2 is widely regarded as acting ‘upstream’ of Wt1 in controlling nephrogenesis (48); it was therefore interesting to determine what effect Pax2 siRNA had on the expression of Wt1. Wt1 expression in kidneys grown in luciferase siRNA followed the same pattern as before, being absent in the ureteric bud, present at low levels in the mesenchyme and at high levels in the developing nephrons (Fig. 7E). In kidneys treated with Pax2 siRNA, low level expression of Wt1 remained but there were only occasional areas of elevated expression, which did not reach the levels seen in developing nephrons. Full elevation of Wt1 expression in potentially nephrogenic areas is not therefore possible in the absence of Pax2.

### DISCUSSION

The research reported here had two main aims; to develop an siRNA-based method for repression of specific gene expression...
in kidneys developing in culture, and to use this method to investigate the aspects of gene function that could not be investigated adequately using a knockout mouse.

**siRNA as a research tool in mammalian organogenesis**

Our results have shown siRNA to be capable of mediating specific gene repression in mammalian kidney rudiments developing in culture. Where the target genes are expressed only by mesenchymal cells, or by cells that are mesenchymal at the time of treatment, siRNAs produce a profound reduction in synthesis of their target proteins. The pooled data from our experiments suggest that siRNA is a well-behaved reagent for gene repression:

- siRNAs affect their target gene without affecting irrelevant genes (e.g. Wt1 siRNA repressed Wt1 without affecting actin and p116 in M15 cells; Wnt4 siRNA repressed Wnt4 expression without affecting basal Wt1 expression in kidneys).

- There is no evidence of non-specific toxicity (e.g. phenotypes of Pax2 and Wt1 siRNA differ, which would not be expected if they arose from non-sequence-specific side effects; there is no widespread cell death as described (28) for phosphorothioates; cell proliferation in many compartments of kidneys treated with Wt1 siRNA is unaffected, while it increases in one specific compartment—increased proliferation is unlikely to occur in cells that have simply been poisoned).

- When applied at a time appropriate to phenocopy a knockout, Wt1 and Wnt4 siRNAs have the effect predictable from knockout mice.

The general pattern of inhibition is similar in both cell lines and organ rudiments, in that most target cells are inhibited strongly but a small minority seem completely unaffected by their treatment. Future developments in transfection agents may result in all cells being affected but, in principle, the ability of some cells to escape inhibition could be exploited to test whether the effects of particular genes are cell-autonomous.

When applied early enough to block the onset of gene expression, an effective technique for gene repression should produce a phenotype similar to that of a conventional knockout mouse. We tested this in two contexts: repression of Wt1 in E9 intermediate mesenchymes and repression of Wnt4 in E11 metanephroi. The morphological phenotype resulting from early repression of Wt1 was the same as that seen in the knockouts (no ureteric bud). The phenotype resulting from Wnt4 (nephrogenesis inhibited) was similar to that seen in knockout mice, but was not as absolute; a few nephrons still formed in the siRNA whereas none do in the knockout mouse (44). In this respect siRNA behaves rather like a hypomorphic allele and the difference between knockdown and knockout becomes important. It is perhaps significant that Wnt4 is a signalling molecule so that its production by a few uninhibited cells may be able to assist neighbouring cells that cannot produce it for themselves.
The most serious weakness of our siRNA method is that it is not an efficient method for repressing gene expression in the ureteric bud. A similar problem affects phosphorothioate antisense oligonucleotides which can repress gene expression incompletely in renal mesenchyme but not at all in the ureteric bud (28,30). In an epithelial tubule, the apical domains of cells—the domains most exposed to siRNA in simple two-dimensional culture—are orientated towards the centre of the tubule and are least accessible to siRNAs diffusing in the surrounding tissue while the basal domains are separated from the surrounding tissue by a basement membrane. The unique biology of developing kidneys, in which nephric epithelia arise from initially-mesenchymal cells, still allows experimenters to test the effects of gene loss during the formation of epithelia (as we have done here), but in general our method will not be suitable for studying the functions of epithelial proteins in the ureteric bud until an improved method of delivery can be devised. We note that, in the only other report of application of siRNA to organ culture of which we are aware, siRNA bound to oligofectamine was readily taken up by and was functional in the branching epithelium of salivary glands (49). It may be that siRNAs have better access to the salivary gland epithelium.

Figure 7. Effect of Pax2 siRNA on renal development and on Wt1 expression. (A) In a control kidney grown in the presence of luciferase siRNA, Pax 2 is expressed strongly in the ureteric bud (‘ub’) and the developing nephrons (‘n’) and rather less strongly in the mesenchyme surrounding the ureteric bud (‘mm’). (B) In kidneys grown in the presence of Pax2 siRNA, expression is greatly reduced in most of the mesenchyme, although some patches of expression remain (‘mm’) and there are no nephrons. Expression in the ureteric bud (‘ub’), though somewhat reduced, is affected much less than elsewhere in the kidney (see main text). (C, D) Quantitative analysis of renal development shows that Pax 2 siRNA causes a significant reduction in both nephrons count and ureteric bud branching; in (C) the Pax 2 data point and error bars are hidden by the axis: there were no nephrons found in any kidneys in this series so both data point and error bar are zero. (E) In control kidneys, Wt1 is expressed at a low level in the mesenchyme (‘mm’) and strongly in the developing nephrons (‘n’). (F) In kidneys treated with Pax2 siRNA, some weak mesenchymal expression remains especially in small isolated groups of cells but no clumps of strongly-expressing cells, characteristic of developing nephrons, can be found.
TIMP-2, FGF-2 and TGF are both sources of survival factors; the ureteric bud produces the most survival factors. However, when epithelial cells in the ureteric bud are removed, the ureteric bud still produces furthest from the ureteric bud and developing nephrons, which are essential for normal kidney development. The ureteric bud produces BMP7 (60–62). The most obvious explanation for the increased circumferential apoptosis seen in Wt1 knockdowns is that it loses its tissue integrity during early branching morphogenesis (50).

The application of siRNA to developing mammalian kidneys may at last allow their molecular genetics to be studied by high-throughput siRNA-based screening techniques that have been used to great effect on lower animals such as C. elegans (51,52). Coupling of siRNA-based high-throughput screens with methods for microarray analysis (53,54) and bioinformatics resources developed to support research into renal organogenesis (55) promises repaid progress in analysis of key developmental pathways. Another powerful advantage of siRNA being applied to organ development, aside from the substantial economic and space savings of not requiring colonies of transgenic mice, is that genes can be repressed at a time of the experimenter’s choosing. This is very important for a gene that plays multiple roles at different stages of development, because a researcher can allow early events to proceed normally and then to test the consequences of gene loss. In a similar vein, it allows candidate tumour suppressor genes to be repressed at different stages of development to allow the ‘window of opportunity’ for neoplastic transformation to be characterised and better understood. Both applications are important to Wt1.

New insights into the function of Wt1

Wt1 has been studied intensively for over a decade, yet a number of fundamental questions remain unresolved. Wt1 is expressed at the earliest stages of kidney formation and in its absence, no kidneys form (12); it therefore functions right at the start of renal development. During kidney development, its expression becomes more complex and it increases in level at least twice, once locally in cells condensing around the ureteric bud as they begin to form nephrons, and again when the podocytes of the glomerulus differentiate (14). In vitro studies have identified several potential target genes, many of which play a role in nephron development, for regulation by Wt1. We have shown here that knock-down of Wt1 only at a stage beyond the point at which kidney development fails in the Wt1−/− mouse still has a dramatic effect on organogenesis, and an effect that is quite distinct from that of the knockout. Loss of Wt1 at the onset of kidney development, whether by transgenic knockout or by siRNA, blocks development of the ureteric bud but loss of Wt1 only from E11 allows bud branching to continue. It may be that by the time that Wt1 siRNA takes full effect, the mesenchyme has made sufficient branch-promoting growth factors to support bud branching for a few days.

The most dramatic effect of knockdown of Wt1 at E11 is inhibition of nephron formation and its replacement with abnormal cell proliferation. There is also a small rise in apoptosis at the circumference of the cultures, where even controls showed most of their apoptosis. This is the zone at which cells are at their furthest from the ureteric bud and developing nephrons, which are both sources of survival factors; the ureteric bud produces TIMP-2, FGF-2 and TGFβ (56–59) and differentiating mesenchyme produces BMP7 (60–62). The most obvious explanation for the increased circumferential apoptosis seen in Wt1 knockdowns would be that, without mesenchymal differentiation taking place, levels of survival factors such as BMP7 are lower than normal at the centre of the culture and their concentration in the circumferential zone is correspondingly lower, resulting in more apoptosis.

Knockdown of Wt1, Wnt4 and Pax2 all resulted in inhibition of nephrogenesis, though with some differences. Knockdown of Pax2 partially repressed the expression of Wt1 in the mesenchyme and prevented it from increasing in cells that should have been destined for nephrogenesis, but Wnt4 did not repress mesenchymal expression of Wt1. These observations are compatible with in vitro and cell line observations made on the interactions between these genes, but should nevertheless be interpreted with caution. Pax2 is known to bind and transactivate the Wt1 promoter in vitro (63,64) and this fact, coupled with the manner in which Pax2 expression precedes the rise in Wt1 expression associated with nephrogenesis, has led several authors to propose that Pax2 is likely to be a necessary positive regulator of Wt1 in kidney development (48). In the light of this hypothesis, it is not surprising that Wt1 fails to reach its normal levels of expression in kidneys treated with Pax2 siRNA, but it remains possible that in our system the connection between the two genes was indirect and due to the failure of bud development in the Pax2 siRNA-treated organs. Studies in M15 and HEK cells have shown that Wt1 positively regulates the expression of Wnt4 (65), and have given rise to the idea that Wt1 lies downstream of Pax2 but upstream of Wnt4 in the chain of events leading to nephron differentiation (66). Our observations that Wnt4 blocks nephrogenesis without blocking mesenchymal expression of Wt1 bear this out, although the absence of high levels of Wt1 associated with glomerular development shows that later aspects of Wt1 expression do depend, however indirectly, on Wnt4 function. Again, our siRNA-based system has produced a good phenocopy of the Wnt4−/− knockout mouse, in which the basic mesenchymal expression of Wt1 was unchanged (44). Before the current study, there was genetic evidence for Wt1 being required at the first and the last of the three stages at which expression data suggests that it acts (see Introduction); our data now provides evidence for action in the middle one, nephrogenesis, as summarized in Figure 8.

The cell biology of mesenchymal cells in Wt1 siRNA-treated kidneys is especially interesting from the point of view of cancer biology. We have identified a developmental period in which Wt1 knockdown results not in massive apoptosis but rather in a failure of cell differentiation and its replacement by abnormal proliferation; this combination is one of the hallmarks of neoplastic change (41,42,67). While it would most certainly be premature to conclude that these cells are representative of those that give rise to Wilms’ tumours in humans, the potential connection is an intriguing one that deserves further investigation.

MATERIALS AND METHODS

Cell and organ culture

Mouse mesonephric M15 cells (68) were cultured in Dulbecco’s modified Eagle medium (DMEM, Gibco BRL)
with 10% FCS (foetal calf serum). Mouse metanephric rudiments isolated at embryonic day 11 (T-bud stage), or intermediate mesoderms isolated at E9, were cultured on 5 μm pore-size Isopore polycarbonate filters supported at surface of the medium. For the first 24 h, the medium was Richter’s Modified Improved MEM (Gibco 10373-017) supplemented with 10 μg/ml iron-loaded human transferrin, an essential factor for kidney development (37) and in some cases with siRNA as described below. After 24 h, the medium was changed for conventional MEM (Sigma M5650) with 10% newborn calf serum, penicillin and streptomycin. 100 μM BrdU (bromodeoxyuridine) was added to some cultures for their last 24 h. Cells and organs were cultured at 37°C with 5% CO₂.

Transfection of siRNA

M15 cells were seeded onto 90mm plates including coverslips. siRNAs were either chemically synthesized (Wt1 and luciferase siRNAs, Dharmacon or Qiagen) or made by us using Ambion’s Silencer kit (Wt1, Wnt4, Pax2 and luciferase) according to the supplied protocol. Where both methods were used for the same target sequences (Wt1-913 and luciferase) no differences in knock down efficiency were apparent. Target selection was based on the standard Tuschl rules (Wt1 targets) or using the on-line design tool at the Ambion website (http://www.ambion.com/techlib/misc/siRNA_finder.html) for the Wnt4 and Pax2 targets. For target sequences not published before see Table 1. Sequence GL2 against luciferase has been described before (7). Transfections were as detailed in the oligofectamine (Invitrogen) protocol, using 60 pmol of siRNA and 3 μl oligofectamine for every 100 μl of transfection mix for M15 cells. For organ cultures, the transfection mix was made the same way but with Richter’s Modified Improved MEM (Gibco 10373-017) in place of the ‘Optimem’ of the Invitrogen protocol. The transfection mix was diluted 1:2 in Richter’s Modified Improved MEM with transferrin and applied directly to organ cultures for 24 h, after which time the medium of the cultures was changed as described above.

Immunofluorescence

Cells were fixed for 10 min in 1:1 acetone : methanol and blocked in 2% BSA in PBS (phosphate buffered saline); 7% (v/v) glycerol; 0.02% (v/v) sodium azide. For immunofluorescence for laminin, Wt1 and Calbindin-D-28K, organ rudiments were fixed for 10 min in cold methanol and blocked in 2% BSA in PBS, while for immunofluorescence for Pax2 they were fixed in 2% paraformaldehyde in PBS for 1 h and permeabilized using 1% Triton X-100 in PBS for 10 min at room temperature. Primary antibody dilutions used were: anti-WT1 rabbit polyclonal C19 (Santa Cruz Biotech) 1:100; anti laminin (Sigma) 1:400; anti BrdU (Sigma) 1:100; anti-Calbindin-D-28K (Chemicon) 1:100 and anti Pax2 (Santa Cruz) 1:100. Secondary dilutions used were: Alexa 594-conjugated goat anti-rabbit (Molecular Probes) 1:100; FITC-conjugated anti mouse (Sigma) 1:100; Texas Red-conjugated anti rabbit (molecular probes) 1:200 and FITC-conjugated...
anti-goat (Sigma) 1:200. BrdU was detected using the method of Fisher et al. (43). Immunofluorescence of cells was observed and recorded using a Zeiss Axioplan microscope, 63× objective, with a Micro Imager 1400, and immunofluorescence of organs was observed using a Leica epifluorescence microscope and a Leica confocal microscope. TUNEL was performed using the Roche kit (Cat 1-684-795) according to the manufacturer’s directions.

Nuclear extracts and western blots

Nuclear extract preparation and western blots for Wt1 were as previously described using 10 μg nuclear extracts (69). The antibodies used were: anti-WT1, rabbit polyclonal C19 (Santa Cruz Biotech.); M70 anti Wnt4 (Santa Cruz) and ‘Stan’, a rabbit polyclonal raised against the splice factor p116, gift of P. Fabrizio and R. Lührmann. For western blots for Wnt4, M15 cells were lysed on the plate directly in 1× laemlli loading sample buffer followed by 5 min boiling. Samples were separated on 10% SDS-PAGE and transferred to Hybond-P membrane (Amersham Biosciences). Membranes were hybridized for 45 min in TBST with 5% non-fat dry milk with M70 rabbit polyclonal antibody diluted 1:500, washed 2× 10 min in TBST, 45 min incubated with anti-rabbit-HRP conjugate, washed 6× 10 min in TBST and visualized using ECL² (Amersham Biosciences).

RNA extraction and northern blots

Cells were washed twice in cold PBS, spun down, and raised in 3–5 ml of lysis buffer per 90 mm plate. Genomic DNA was sheared by passing through a 21G needle three times. 7.5 ml of cell lysate was layered onto a 3 ml Caesium cushion [5.7 M CsCl; 25 mM NaOAc, pH 5.5; in DEPC (diethylpyrocarbonate)-treated dH2O] and centrifuged on a SW41 Swing Out rotor (Sorvall) in an 11 ml tube at 30 000 rpm for 20 h at 20°C. Following centrifugation the supernatant was removed using a 10 ml pipette and the tube inverted quickly to remove CsCl by draining onto tissue. The walls of each tube were dried with fresh tissue taking care not to touch the pellet. The pellet was rinsed with 0.5 ml 70% ethanol and air dried for 5–15 min, then resuspended in a suitable volume of TES (10 mM Tris–HCl, pH 7.5; 1 mM EDTA; 0.2% w/v SDS). RNA was precipitated with 1/10 volume 2 M sodium acetate pH 5.5 and 2 volumes ethanol after being kept at −20°C for at least 1 h. Samples were spun at 14 000 rpm on a microfuge for 15 min at 4°C, dried and resuspended in DEPC-dH2O. RNA was quantified and 10 μg was run on northern blots using standard methods. The probes used for the northern blots were: mouse WT1, a cDNA encompassing the entire coding region; and for β-actin, a cDNA corresponding to bases 251–710. Examples of northern and western blots were analysed quantitatively using the histogram function of Adobe Photoshop, normalized to the control (actin/p116).

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