Angioblast-mesenchyme induction of early kidney development is mediated by Wt1 and Vegfa

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Summary

Most studies on kidney development have considered the interaction of the metanephric mesenchyme and the ureteric bud to be the major inductive event that maintains tubular differentiation and branching morphogenesis. The mesenchyme produces Gdnf, which stimulates branching, and the ureteric bud stimulates continued growth of the mesenchyme and differentiation of nephrons from the induced mesenchyme. Null mutation of the *Wt1* gene eliminates outgrowth of the ureteric bud, but Gdnf has been identified as a target of Pax2, but not of Wt1. Using a novel system for microinjecting and electroporating plasmid expression constructs into murine organ cultures, it has been demonstrated that Vegfa expression in the mesenchyme is regulated by Wt1. Previous studies had identified a population of Flk1-expressing cells in the periphery of the induced mesenchyme, and adjacent to the stalk of the ureteric bud, and that Vegfa was able to stimulate growth of kidneys in organ culture. Here it is demonstrated that signaling through Flk1 is required to maintain expression of Pax2 in the mesenchyme of the early kidney, and for Pax2 to stimulate expression of Gdnf. However, once Gdnf stimulates branching of the ureteric bud, the Flk1-dependent angioblast signal is no longer required to maintain branching morphogenesis and induction of nephrons. Thus, this work demonstrates the presence of a second set of inductive events, involving the mesenchymal and angioblast populations, whereby Wt1-stimulated expression of Vegfa elicits an as-yet-unidentified signal from the angioblasts, which is required to stimulate the expression of Pax2 and Gdnf, which in turn elicits an inductive signal from the ureteric bud.

Key words: Mouse, Vegf, Wt1, Kidney, Angioblast, Flk-1/Flk1

Introduction

Epithelial-mesenchymal inductive interactions have long been the hallmark of organ development, especially of those organs that develop specialized epithelial and/or undergo branching morphogenesis, such as the kidney, lung, liver, pancreas and other secretory glands. In the case of the kidney, interaction of the epithelial ureteric bud with the metanephric mesenchyme, a histologically distinct patch of cells in the urogenital ridge, leads to condensation of the mesenchyme around the branching ureteric bud, and the subsequent induction of a mesenchymal-to-epithelial transformation of the condensed mesenchyme into tubular elements called nephrons (Saxen, 1987). This process is reiterated by continual branching of the ureteric bud and expansion of the mesenchyme until, in the human kidney, approximately one million nephrons have been induced. Many genes have been identified with a function required for these initial interactions. For example, the transcription factors *Wt1*, *Pax2*, *Six1* and *Eya1* are expressed in the metanephric mesenchyme, and in the absence of any one of these factors, the ureteric bud either fails to emerge from the Wolffian duct or fails to invade the metanephric mesenchyme, thus blocking kidney development at its earliest stages (Kreidberg et al., 1993; Torres et al., 1995; Xu et al., 1999; Xu et al., 2003).

Many recent studies have significantly advanced our understanding of the molecular basis of ureteric bud-mesenchymal interactions that initiate kidney development. For example, *Pax2* has been shown to regulate the expression of glial cell line derived neurotrophic factor (*Gdnf*) (Brophy et al., 2001), a member of the neurotrophin family expressed by the metanephric and condensed mesenchyme, which binds the c-Ret receptor expressed at the tip of the ureteric bud to stimulate growth and branching of the bud (Durbec et al., 1996; Sanicola et al., 1997; Trupp et al., 1996). In *Gdnf*-deficient embryos, there is no outgrowth of the ureteric bud (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). Compared with that of *Pax2*, our understanding of how the *Wt1* gene regulates the interactions that initiate kidney development is less well understood. *Wt1* encodes a zinc finger transcription factor; this gene was first identified as a tumor suppressor gene for Wilms' tumor, a neoplasm of the kidney that occurs in young children (Call et al., 1990; Gessler et al., 1990; Glaser et al., 1989). *Wt1* is expressed in the metanephric mesenchyme and increases in expression as the mesenchyme condenses around the ureteric bud, finally being restricted to the podocyte

population of the glomerulus, where it continues to be expressed in the mature kidney. Many genes have been identified as potential targets for *Wt1* (Scharnhorst et al., 2001), but it has been difficult to reconcile these findings with the renal agenesis phenotype of *Wt1*-deficient embryos (Kreidberg et al., 1993). For example, amphiregulin has been identified as a target for *Wt1* (Lee et al., 1999) and stimulates branching morphogenesis in kidney organ cultures, but amphiregulin mutant mice demonstrate normal kidney development (Luetteke et al., 1999). Whether this can be accounted for by redundancy among EGF family members is not known.

Recently, we reported the phenotype of transgenic mice in which a truncated form of *Wt1*, expected to act in a dominant-negative fashion, was specifically expressed in glomerular podocytes, which are *Wt1*-expressing cells that form part of the filtration unit of the kidney (Natoli et al., 2002a). Surprisingly, instead of yielding defects in podocyte differentiation, expression of the mutant form of *Wt1* resulted in abnormal development of the glomerular capillaries that form in intimate contact with the podocytes. This observation has led us to hypothesize that one function of *Wt1* is to regulate the expression of growth factors that are involved in vascular development. A role for growth factors that stimulate vascular development in kidney development was first suggested by Tufro, who demonstrated that vascular endothelial growth factor A (Vegfa), or hypoxic culture conditions, could stimulate proliferation and nephrogenesis in metanephric kidney organ cultures (Tufro, 2000; Tufro et al., 1999; Tufro-McReddie et al., 1997). Here we present results demonstrating that *Wt1* does indeed regulate the expression of Vegfa. Furthermore, it is shown that Vegfa produced by the mesenchyme stimulates branching morphogenesis and nephrogenesis in the early kidney through reciprocal inductive events that involve a population of Flk1 (Vegfr2; Kdr – Mouse Genome Informatics)-expressing angioblasts. Thus, similarly to recent studies identifying a role for angiogenic cells in hepatogenesis and pancreatic development (Lammert et al., 2001; Matsumoto et al., 2001), this work identifies an interaction between angioblasts and the condensed mesenchyme as an additional crucial interaction involved in early kidney development, in addition to the classically recognized inductive interaction between the mesenchyme and the ureteric bud (Grobstein, 1953).

Materials and methods

Antibodies and other materials

Recombinant mouse Vegfa (#493-MV), recombinant rat Gdnf (#512-GF) and goat polyclonal anti-Vegfa antibody (#AF-493-NA) were purchased from R&D Systems (Minneapolis, MN); rat polyclonal anti-Flk1 antibody (#550549) for immunostaining was purchased from BD Biosciences (San Diego, CA); rat polyclonal anti-Flk1 antibody (clone DC101) was obtained from ImClone Inc. (New York, NY); mouse polyclonal anti-pan-cytokeratin antibody was purchased from Sigma (St Louis, MO); rabbit polyclonal anti-Pax2 antibody (#71-600) was purchased from Zymed (South San Francisco, CA) or obtained from Dr Greg Dressler, (University of Michigan); rabbit polyclonal anti-Pecam antibody (H-300)), goat polyclonal anti-Pecam antibody (M-20) and rat-IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit polyclonal anti-Brush Border antibody (Miettinen and Linder, 1976) was obtained from Hannu Sariola (University of Helsinki). All secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). Tyrphostin SU1498 (#T-2710) was purchased from Calbiochem (La Jolla, CA); DMSO was purchased from Fisher Scientific (Pittsburgh, PA). A plasmid encoding a truncated version of Flk1 (Tsou et al., 2002) was obtained from Dr F. Frank Isik, (University of Washington, Seattle). Lotus lectin (FL-1321) was purchased from Vector laboratories, Inc. (Burlingame, CA).

Microinjection and electroporation

The electroporation system modified from that previously used to microinject chick neural tubes (Itasaki et al., 1999; Nakamura et al., 2000) is depicted in Fig. 1A. Metanephric kidney organ cultures were placed in a dish under a drop of buffer, and plasmid vectors were injected into the culture using 15-20 μm diameter glass needles prepared using a Sutter Instrument micropipette puller model P-87 (Sutter Instruments, Novato, CA). Settings on the pipette puller were: Heat 400; Pull 150; Velocity 100; Time 100. The glass tubes for making needles were obtained from Sutter, (#BF100-50-10), outer diameter 1.0 mm, inner diameter 0.5 mm, 10 cm length. Needles were connected by thin tubing to a FemtoJet microinjection device (Eppendorf, Hamburg, Germany) using the following settings: injection Pressure 6.00 psi; compensation pressure 1.00 psi; injection time 0.4 seconds, two injections per injection site. A square wave electroporator (BTX ECM830, Genetronics Inc., San Diego, CA) was used to pulse the culture immediately after the injection, using rectangular electrodes (gold plated, Model 516, Genetronics Inc.) placed in parallel on either side of the organ culture before the injection. The parameters used here were voltage: 36 V; number of pulse: 5; pulse length: 50 ms; internal time: 100 ms. After growing for 6-48 hours, cultures were analyzed to examine gene expression, culture growth and differentiation.

In-vitro kidney cultures and immunocytochemistry

Embryonic day (E) 11.5 metanephric rudiments were isolated from embryos of FVB mice, placed on nitrocellulose filters (0.1 μm pore size, Nuclepore Track-Etch Membrane, Whatman 0930059), suspended over DMEM/10% fetal calf serum (FCS) and cultured at the air/medium interface for 24, 36, 48 or 72 hours at 37°C with 5% CO_2. Blocking antibodies or pharmacological agents were added to the growth medium as mentioned for each experiment. Kidney cultures were fixed in 4% paraformaldehyde (PFA) for in situ hybridization with RNA probes, or fixed in methanol and stained with antibodies. Branch tips were quantified by manual counting after anti-cytokeratin staining, nephron proximal tubule units were identified by staining with Lotus lectin or Brush Border antibody. Results were statistically analyzed by Student's *t*-test. Antibody staining: kidney cultures were fixed in ice-cold methanol for 10 minutes, washed in PBS containing 1% bovine serum albumin (BSA) at room temperature and incubated overnight in primary antibodies at 4°C. The samples were then washed three times for 2 hours each in PBS at room temperature and incubated overnight in secondary antibodies at 4°C. Finally, after washing three times for 2 hours each in PBS at room temperature, organ cultures were analyzed using an inverted fluorescence microscope (Nikon Eclipse TE 300), and imaged using a Spot 1.4.0 digital camera (Diagnostic Instruments, Sterling Heights, MI). Images were processed on Macintosh computers using Photoshop 6.0 (Adobe Systems, Inc., San Jose, CA). Fluorescent alkaline phosphatase (AP) detection used for Flk1 staining was essentially as published (Natoli et al., 2004).

In situ hybridization

Riboprobes were obtained or generated from the coding region of mouse *Gdnf* (Srinivas et al., 1999) (obtained from F. Costantini, Columbia University), *Wt1* (Pelletier et al., 1991), *Vegf164* (bases 75-669 in the murine cDNA, amplified by PCR), *Pax2* [(Dressler et al., 1990), obtained from P. Grus], *Nanog* [generated in our laboratory

cDNA 558-1140 (Chambers et al., 2003; Mitsui et al., 2003)], *Osr1* [(So and Danielian, 1999), obtained from P. Danielian, Chester Beatty Laboratories, London] and *Wnt4* [(Stark et al., 1994), obtained from S. Vainio, University of Oulu, Finland], and subcloned in pCDNA3 or pCRII-TOPO (Invitrogen). Sense and antisense probes were synthesized and labeled with digoxigenin-UTP (Roche). The protocol used for whole-mount in situ hybridization was as published (Wilkinson and Nieto, 1993). For section in situs, material was fixed in 4% paraformaldehyde and embedded in paraffin. Sections were cut at 8uM and deparaffinized through xylene and a standard ethanol series.

Real-time PCR analysis

As specified in the Results section, RNA was prepared either from whole organ cultures or from sections co-injected with a GFP expression plasmid, in which case GFP-expressing sections were micro-dissected apart from the rest of the organ culture. Total RNA was isolated from kidney organ cultures, digested with DNase 1 (Qiagen) and reverse-transcribed with SuperScript First-Strand Synthesis System kit (Invitrogen) to get cDNA template. For Smart Cycler Real-Time PCR reaction, a mastermix of the following reaction components was prepared to the indicated end-concentration: Water (RNase free), forward primer (0.3 μmol/l), reverse primer (0.3 μmol/l), TaqMan probe (0.2 μmol/l, Cepheid), Q solution (1×, Qiagen), dNTPs (0.2 mmol/l, Biolabs), PCR buffer (1×, containing 15 mmol/l MgCl2, Qiagen), Taq DNA polymerase (0.1 unit/μl, Biolabs), cDNA template (5 ng/μl). Smart Cycler reaction mastermix was filled in the Smart Cycler reaction tubes [Cepheid (Sunnyvale, CA)], which were closed, centrifuged and placed into the Smart Cycler processing block (Cepheid). The following protocols were used: for *Gapdh* and *Vegfa*, 94°C for 120 seconds; 94°C for 60 seconds, 58°C for 30 seconds, 72°C for 30 seconds, 40 cycles; cool to 4°C. For *Pax2*, 94°C for 120 seconds; 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 45 seconds, 40 cycles; cool to 4°C. For *Gdnf*, 94°C for 120 seconds; 94°C for 30 seconds, 60°C for 45 seconds, 72°C for 45 seconds, 40 cycles; cool to 4°C. Primers used were: *gapdh* (Forward: TCC ACC CAT GGC AAA TTC A; Reverse: TCG CTC CTG GAA GAT GGT), *Vegfa* (Forward: AGC AAC ATC ACC ATG CAG AT; Reverse: TCA CAG TGA TTT TCT GGC TTT G), *Pax2* (Forward: TGG CTG TGT CAG CAA AAT CCT; Reverse: ATTCGGCAATCTTGTCCACCA), and *Gdnf* (Forward: TTC GCG CTG ACC AGT GA; Reverse: CTC TCT TCG AGG AAG CGC T). Standard curves of *Gapdh*, *Vegfa*, *Pax2* and *Gdnf* were generated using cDNA dilutions from 10^{-3} μg/μl to 10^{-7} μg/μl (data not shown); each dilution was run in triplicate, and Ct values all varied by less than ±0.4 unit cycles. *Vegfa*, *Pax2* and *Gdnf* Ct values were normalized using *Gapdh* Ct values using the following equation: Vegfa corrected Ct (sample 1) = Vegfa obtained Ct (sample 1) × Gapdh Ct (sample 2) / Gapdh Ct (sample 1). Ct values were converted to relative mRNA values using the *Vegfa*, *Pax2* or *Gdnf* standard curves.

Results

Organ culture microinjection and electroporation

Using *Wt1*$^{-/-}$ embryos to evaluate potential downstream targets of the *Wt1* gene has been problematic, as the metanephric mesenchyme in these mutant embryos begins to undergo apoptosis almost immediately upon its formation as a histologically distinct structure (Kreidberg et al., 1993). Therefore, whether a gene is expressed or not may be due to the apoptotic program rather than whether it is a valid target of *Wt1*. To circumvent this issue, we adapted a microinjection/electroporation system previously used in chick neural tubes (Itasaki et al., 1999; Nakamura et al., 2000) to the study of *Wt1* function in murine organ culture (Fig. 1A) because it allows higher-throughput experimentation than the derivation of a novel transgenic mouse line for each individual transgene. Additionally, this approach allows gene expression in locations for which no appropriate promoter is available, such as the metanephric mesenchyme. Thus, rather than studying loss-of-function phenotypes in *Wt1* mutant embryos, this system allows the observation of gain-of-function phenotypes in organ cultures in which *Wt1* or other genes have been overexpressed. This system also allows the evaluation of *Wt1* target genes in non-immortalized cells, avoiding the

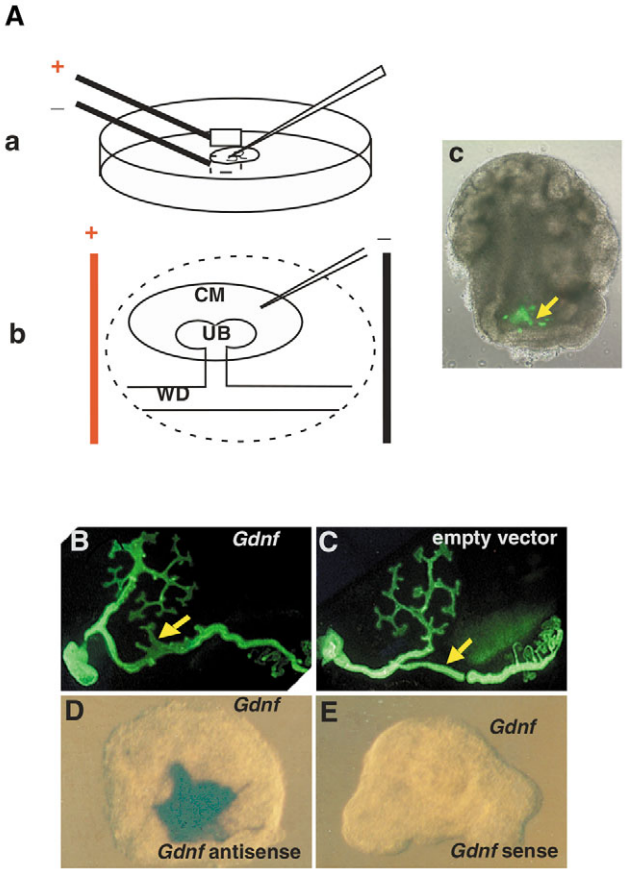


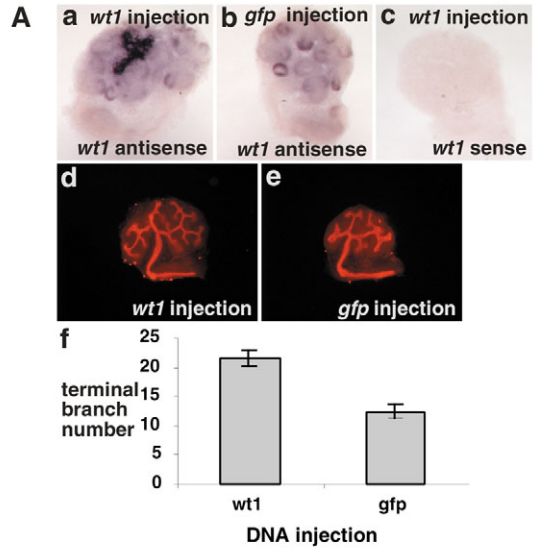
Fig. 1. Microinjection and electroporation system for kidney organ cultures. (A) A diagram of the microinjection and electroporation system. (a) A three-dimensional depiction, showing a pair of electrodes on the left, and the injection needle on the right. (b) A view from above the organ culture; showing the injection needle placed in the condensed mesenchyme. The dotted line shows the boundary of the whole organ culture. Injections can also be placed outside the condensed mesenchyme, within the looser mesenchyme of the organ culture. (c) Whole-mount double exposure of brightfield image of organ culture and GFP fluorescence 24 hours after injection. (B-E) *Gdnf* injection/electroporation. (B,D,E) A *Gdnf* expression vector was injected outside the condensed mesenchyme, adjacent to the Wolffian duct, as shown by the arrow in B. (C) An empty vector was injected as a negative control. (B,C) Cytokeratin stains done 36 hours post-injection to demonstrate the presence of ectopic ureteric buds emerging from the Wolffian duct. Several are present in B, none in C, next to the yellow arrows. (D,E) In situ hybridization for *Gdnf*, using antisense (D) and sense (E) probes. The endogenous *Gdnf* mRNA signal is too weak to be detected under these hybridization conditions. CM, condensed mesenchyme; UB, ureteric bud.

possibility that *Wt1* function is modified when co-expressed with immortalizing proteins. As described in the Materials and methods section, an organ culture was placed between two electrodes, DNA plasmid expression constructs were microinjected into the mesenchymal component of the organ rudiment, and the culture was electroporated immediately after the injection. After an additional 6-48 hours in culture, these cultures were analyzed to examine gene expression, culture growth and differentiation.

As a first test of this system, a green fluorescent protein (GFP) expression construct (pCS2-EGFP) was microinjected into the E11 kidney organ cultures (Fig. 1A, part c). GFP was highly expressed in multiple cell layers at the site of injection (see Fig. S1 in the supplementary material; additionally, Fig. S2 shows a slight decrease in branching number as a consequence of electroporation, but the pattern of Pax2 and Wt1 expression is preserved). To further examine the ability of this system to manipulate biologically relevant aspects of kidney development, a vector encoding *Gdnf*, the major growth factor responsible for stimulating growth of the ureteric bud (Sainio et al., 1997; Sanicola et al., 1997), was introduced into the organ culture by microinjection/electroporation. Localized expression of *Gdnf* adjacent to the Wolffian duct led to outgrowth of ectopic ureteric buds from the Wolffian duct, adjacent to the site of injection (Fig. 1B, compare with Fig. 1C). This was observed in 100% of injections (the success rate for all experiments is presented in Table 1). Injection of an empty vector that did not contain the *Gdnf* cDNA never resulted in ectopic bud outgrowth from the Wolffian duct (Fig. 1C). In situ hybridization with a *Gdnf* RNA probe showed strong expression of *Gdnf* at the site of injection (Fig. 1D, compare with sense probe, Fig. 1E).

Overexpression of *Wt1* stimulates branching of the ureteric bud

Having established the efficacy of the microinjection/electroporation system for use in metanephric kidney organ culture, *Wt1* was overexpressed in an attempt to identify possible Wt1 target genes. In situ hybridization with a *Wt1* antisense probe showed increased signal at the site of the *Wt1* plasmid injection (Fig. 2A, part a, compare with sense probe Fig. 2A, part c), whereas no increased signal was found after injection of a control GFP expression vector (Fig. 2A, part b). Overexpression of *Wt1* in the condensed mesenchyme (after injection at multiple sites) led to increased branching of the ureteric bud (Fig. 2A, parts d,f, compare with Fig. 2A, part e). The condensed mesenchyme refers to the portion of the metanephric mesenchyme that condenses around the ureteric bud upon its invasion of the metanephric mesenchyme, and in which expression of genes such as *Pax2* and *Wt1* increases as a consequence of induction by the bud (Schedl and Hastie, 2000). These results indicate that this system might be suitable for the evaluation of potential *Wt1* target genes involved in early kidney development.



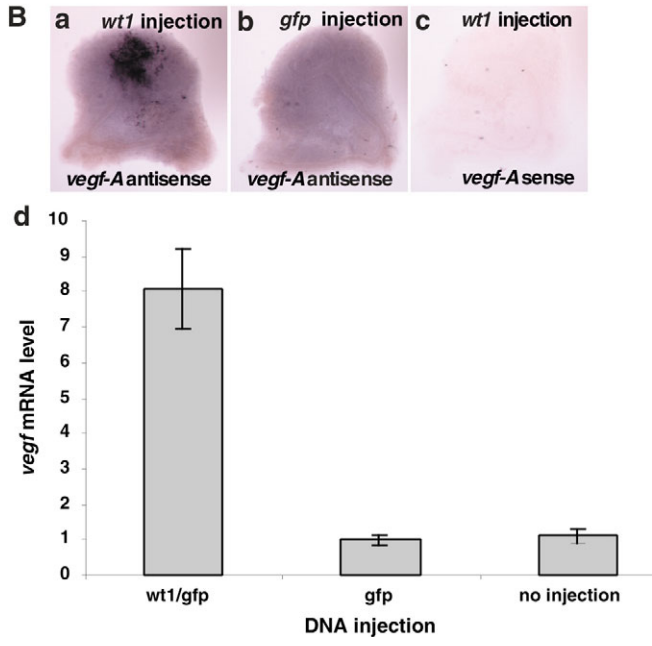


Fig. 2. Injection of the *Wt1* expression vector. (A) Effect on branching morphogenesis. (a,c,d) Injection of Wt1 cDNA. (b,e) Injection of GFP vector. In situ hybridization with *Wt1* antisense probe (a,b), or sense probe (c). The strong area of hybridization is at the single injection site (a), and the endogenous Wt1 signal is observed at several induced nephrons (a,b). (d,e) Cytokeratin staining demonstrating effect of Wt1 on branching. In experiments designed to observe an effect on branching, the vector is injected at two sites, one on either side of the ureteric bud. The branching comparisons shown in each figure are between organ cultures obtained from the same embryo. (f) Overall quantitation of 12 pairs of experiments, represented by d,e. Significant differences in branching were detected after *Wt1* injection ($P<0.001$). (B) *Wt1* stimulation of *Vegfa* expression. Injection of *Wt1* (a,c) and *Gfp* (b) expression vectors. In situ hybridization with *Vegfa* antisense (a,b) and sense (c) probes. (d) Real-time PCR with *Vegfa* primers in response to microinjections. As described in the text, a *Gfp* expression vector was co-injected, and areas of *Gfp* expression were separated from the remainder of the organ culture. The expression vectors used are shown at the bottom of each column. Increased *Vegfa* mRNA is observed in response to injection of a *Wt1* expression vector. All *Vegfa* Ct values were normalized to the Gapdh Ct value from real-time PCR for the injected tissue.

Table 1. Summary

Figure	Injection	Treatment	Observation	Total number	Positive
Fig. 1B,C	Empty vector	None	Ectopic ureteric bud	8	0
Fig. 1B,C	*Gfnf* cDNA	None	Ectopic ureteric bud	8	8
Fig. 2A, parts d,e	*Gfp* vector	None	Increased branching	12	0
Fig. 2A, parts d,e	*Wt1*	None	Increased branching	12	11
Fig. 2B, parts a,b	*Gfp* vector	None	Increased *Vegfa* expression	12	0
Fig. 2B, parts a,b	$Wt1^{-/-}$	None	Increased *Vegfa* expression	20	19
Not shown*	$Wt1^{-/+}$	None	Increased *Vegfa* expression	6	6
Not shown*	$Wt1^{+/-}$	None	Increased *Vegfa* expression	3	3
Not shown*	$Wt1^{+/+}$	None	Increased *Vegfa* expression	3	3
Fig. 3B	None	PBS buffer	Increased branching and nephrons	4	0
Fig. 3B	None	Vegfa protein	Increased branching and nephrons	4	4
Not shown	*Gfp* vector	None	Increased branching	10	0
Not shown	*Vegfa* cDNA	None	Increased branching	10	10
Fig. 3C	None	Goat IgG	Decreased branching and nephrons	4	0
Fig. 3C	None	Vegfa Ab	Decreased branching and nephrons	4	4
Fig. 4B	None	Rat-IgG	Decreased branching and nephrons	8	0
Fig. 4B	None	Flk1 Ab	Decreased branching and nephrons	8	8
Not shown*	*Gfp* vector	None	Decreased branching	5	0
Not shown*	dn*Flk1* cDNA†	None	Decreased branching	5	5
Fig. 5A	None	Rat-IgG	Decreased *Pax2* expression	6	0
B	None	Flk1 Ab	Decreased *Pax2* expression	6	5
C	None	Rat-IgG	Decreased *Osr1* expression	3	0
D	None	Flk1 Ab	Decreased *Osr1* expression	3	0
E	None	Rat-IgG	Decreased *Nanog* expression	3	0
F	None	Flk1 Ab	Decreased *Nanog* expression	3	3
G	None	Rat-IgG	Decreased *Wnt4* expression	3	0
H	None	Flk1 Ab	Decreased *Wnt4* expression	3	3
I	None	Rat-IgG	Decreased *Wt1* expression	3	0
J	None	Flk1 Ab	Decreased *Wt1* expression	3	0
Fig. 6A, part a	*Gfp*	None	Increased *Pax2* expression	8	0
A, part b	*Vegfa*	None	Increased *Pax2* expression	11	10
Fig. 6A, parts d,e	*Gfp* vector	Rat-IgG (as control)	Decreased *Pax2* expression	6	0
Fig. 6A, parts d,e	*Vegfa* cDNA	Rat-IgG (as control)	Decreased *Pax2* expression	6	0
Fig. 6A, parts d,e	*Gfp* vector	Flk1 Ab	Decreased *Pax2* expression	6	6
Fig. 6A, parts d,e	*Vegfa* cDNA	Flk1 Ab	Decreased *Pax2* expression	6	6
Not shown	*Gfp* vector	DMSO (as control)	Decreased *Pax2* expression	6	0
Not shown	*Vegfa* cDNA	DMSO (as control)	Decreased *Pax2* expression	6	0
Not shown	*Gfp* vector	SU1498	Decreased *Pax2* expression	6	6
Not shown	*Vegfa* cDNA	SU1498	Decreased *Pax2* expression	6	6
Fig. 6B	Empty vector	Rat-IgG (as control)	*Pax2* stimulation of Gdnf expression	3	0
Fig. 6B	*Pax2* cDNA	Rat-IgG (as control)	*Pax2* stimulation of Gdnf expression	3	3
Fig. 6B	Empty vector	Flk1 Ab	*Pax2* stimulation of Gdnf expression	3	0
Fig. 6B	*Pax2* cDNA	Flk1 Ab	*Pax2* stimulation of Gdnf expression	3	0
Fig. 6B	Empty vector	DMSO (as control)	*Pax2* stimulation of Gdnf expression	3	0
Fig. 6B	*Pax2* cDNA	DMSO (as control)	*Pax2* stimulation of Gdnf expression	3	3
Fig. 6B	Empty vector	SU1498	*Pax2* stimulation of Gdnf expression	3	0
Fig. 6B	*Pax2* cDNA	SU1498	*Pax2* stimulation of Gdnf expression	3	0
Fig. 6D, parts a-d	*Pax2* cDNA	Rat-IgG	Increased *Pax2* mRNA expression	3	3
Fig. 6D, parts a-d	*Pax2* cDNA	Flk1 Ab	Increased *Pax2* mRNA expression	3	0
Fig. 6D, parts a-d	*Pax2* cDNA	DMSO	Increased *Pax2* mRNA expression	3	3
Fig. 6D, parts a-d	*Pax2* cDNA	SU1498	Increased *Pax2* mRNA expression	3	0
Fig. 6D, parts e-h	*Pax2* cDNA	Rat-IgG	Increased Pax2 protein expression	3	3
Fig. 6D, parts e-h	*Pax2* cDNA	Flk1 Ab	Increased Pax2 protein expression	3	0
Fig. 6D, parts e-h	*Pax2* cDNA	DMSO	Increased Pax2 protein expression	3	3
Fig. 6D, parts e-h	*Pax2* cDNA	SU1498	Increased Pax2 protein expression	3	0
Fig. 6D, parts i,j	*Gfp* vector	Rat-IgG	GFP fluorescence	3	3
Fig. 6D, parts i,j	*Gfp* vector	Flk1 Ab	GFP fluorescence	3	3
Fig. 6D, parts i,j	*Gfp* vector	DMSO	GFP fluorescence	3	3
Fig. 6D, parts i,j	*Gfp* vector	SU1498	GFP fluorescence	3	3
Fig. 7A	None	PBS/Flk1 Ab	Increased branching and nephrons	4	0
Fig. 7A	None	Gdnf/Flk1Ab	Increased branching and nephrons	4	4
Fig. 7B	None	PBS/Flk1 Ab	Increased *Pax2* mRNA expression	4	0
Fig. 7B	None	Gdnf/Flk1Ab	Increased *Pax2* mRNA expression	4	4
Fig. 7C	None	Rat-IgG(48/48)	Decreased branching and nephrons	4	0
Fig. 7C	None	Flk1 Ab(48/48)	Decreased branching and nephrons	4	0

The number of times each experiment was performed and the outcomes discussed in the text and shown in each figure.
*Data not shown in figures but referred to in the text.
†Dominant negative *Flk1* cDNA referred to in the text.

Stimulation of Vegfa expression in kidney organ culture by injection of Wt1

Our previous work suggested that *Wt1* may regulate the expression of factors that regulate vascular development (Natoli et al., 2002a). One such factor is *Vegfa* (Neufeld et al., 1999). *Wt1* and *Vegfa* are both highly expressed by podocytes in the kidney glomerulus (Armstrong et al., 1993; Eremina and Quaggin, 2004), leading us to hypothesize that *Vegfa* may be a regulatory target of *Wt1*. Injection of a *Wt1* expression plasmid containing a *Wt1* cDNA, without alternatively spliced exon 5 or the KTS sequence present at the end of exon 9, led to high-level expression of *Vegfa* at the site of injection [Fig. 2B, part a, compare with sense probe (part c) and with control injection of a vector containing the *Gfp* cDNA (part b)]. Injection of a vector encoding *Pax2* (Dressler et al., 1990) also failed to induce expression of *Vegfa* (data not shown). Real time-PCR for *Vegfa* also demonstrated increased expression of *Vegfa* RNA at the Wt1 injection site (Fig. 2B, part d). Previous studies have ascribed distinct functions to the different splice forms of *Wt1*. However, the ability to induce *Vegfa* expression was not restricted to a single splice form of *Wt1*. Rather, all four major splice forms were able to induce expression of *Vegfa* (Table 1, data not shown). This finding is, perhaps, not surprising in view of recent results in which we had shown that a targeted deletion of alternatively spliced exon 5 in mice had no apparent phenotype (Natoli et al., 2002b), and Hammes et al. (Hammes et al., 2001) had shown that embryos singly expressing either the –KTS or +KTS versions of *Wt1* experience normal early kidney development (see Discussion for additional detail). Because we found no apparent difference in the ability of *Wt1* alternatively spliced isoforms to induce expression of *Vegfa*, the remainder of the studies in this report used the version of the *Wt1* cDNA that did not include either exon 5 or the KTS sequence.

Vegfa is expressed in the metanephric kidney

The expression pattern of Vegfa in the early kidney is shown in Fig. 3A. Vegfa expression was present in the condensed mesenchyme and highest in pretubular aggregates (Fig. 3A). Lower levels of Vegfa were also present in the ureteric bud. In older kidneys, expression was highest in podocytes of maturing glomeruli. The mesenchymal expression of Vegfa overlapped with that of Wt1, which was also in condensed mesenchyme and in pretubular aggregates, and highest in podocytes of older kidneys (data not shown) (see Armstrong et al., 1993; Eremina et al., 2003).

Overexpression of *Vegfa* stimulates branching of the ureteric bud

Vegf164 is a predominant isoform of *Vegfa* (Neufeld et al., 1999). The findings of Tufro, that Vegfa could stimulate branching morphogenesis and induction of nephrons were confirmed both by adding Vegfa to the organ culture (Fig. 3B), and by microinjection/electroporation of a Vegfa expression vector (not shown). Addition of Vegfa neutralizing antibodies to the organ culture also resulted in decreased branching and nephron induction (Fig. 3C).

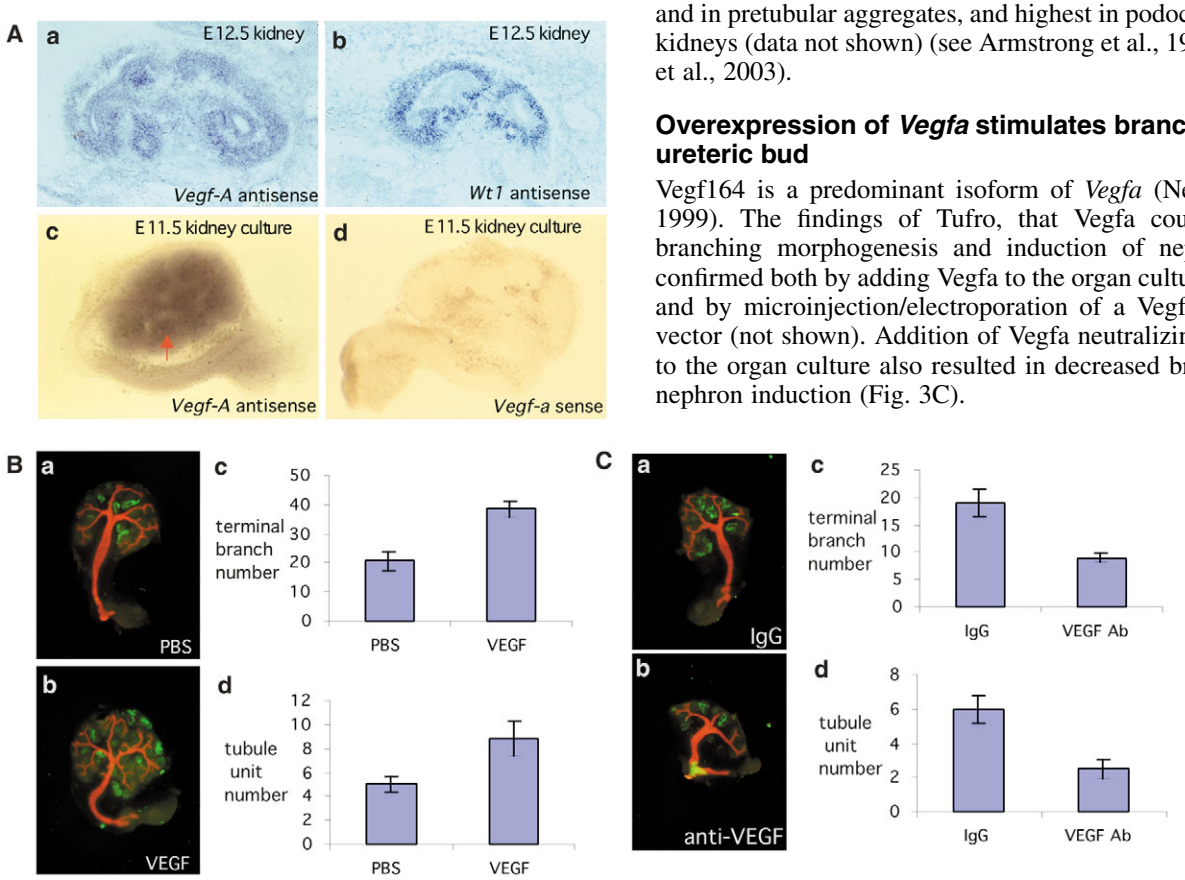


Fig. 3. (A) *Vegfa* expression in early kidney development compared to *Wt1*. (a,b) E12.5 kidneys, fixed immediately after dissection and sectioned. (c,d) E11.5 metanephric kidney rudiments placed in culture for 48 hours. (a,c) *Vegfa* antisense probe, (b) *Wt1* antisense probe and (d) *Vegfa* sense probe. Clear *Vegfa* expression is present in the condensed mesenchyme of the E12.5 kidney (a) overlapping significantly with *Wt1* expression at the same timepoint (b). (B) Vegfa stimulation of branching and tubulogenesis. (a) Control PBS addition, (b) Vegfa addition, Cytokeratin stain red, proximal tubule-binding lectin green. (c) Terminal branch tip ($P<0.001$) and (d) proximal tubule ($P<0.001$) quantitation showing results of four pairs of organ cultures represented by (a) and (b), which are from the same embryo. (C) Antibody inhibition of branching. (a) Control organ culture treated with rabbit IgG. (b) Organ culture from the same embryo as (a) treated with anti-Vegfa neutralizing antibody. (c,d) Quantitation of four pairs of organ cultures treated as in (a) and (b); (c) terminal branch tips ($P<0.001$); (d) tubules as identified by lectin staining for proximal tubules ($P<0.001$).

Expression of the Vegfa receptor Flk1 in the early kidney

Understanding the role of *Vegfa* in early kidney development requires identification and localization of the Vegfa receptors expressed at the initiation of kidney development. The two major receptors for Vegfa are Flt1 (Vegfr1) and Flk1 (Vegfr2) (Neufeld et al., 1999); Flk1 appears to be essential for endothelial differentiation, whereas Flt1 appears to have a more crucial role in vascular assembly (Fong et al., 1995; Fong et al., 1999; Shalaby et al., 1997). Previous reports using *Flk1-lacZ* knock-in mice and immunostaining for Flk1 have identified a population of Flk1-expressing cells at the periphery of the condensed mesenchyme and adjacent to the stalk of the ureteric bud (Loughna et al., 1998; Robert et al., 1998; Tufro, 2000; Tufro et al., 1999; Tufro-McReddie et al., 1997). Immunostaining of E13 kidneys and organ cultures with an Flk1 antibody confirmed this expression pattern (Fig. 4A, part a), and co-staining with anti-Pax2 (Fig. 4A, part b), a marker of the condensed mesenchyme and the ureteric bud, demonstrated their non-overlapping expression patterns (Fig. 4A, part c), thus demonstrating that the Flk1- and Pax2-expressing cells represent distinct compartments in the early kidney. Furthermore, staining for Pecam, a marker of mature endothelial cells (Newman, 1997), identified a small subset of the Flk1-positive cells (Fig. 4A, parts g,h,i), indicating that the majority of Flk1-expressing cells in the early kidney are angioblasts or immature endothelial cells.

Flk1 signaling is required to maintain differentiation of the condensed mesenchyme

Next it was determined whether the stimulation of kidney development by Vegfa required signaling through Flk1. Signaling by Flk1 was blocked by three independent methods, all of which yielded similar results. Addition of an Flk1-blocking antibody (DC101 from ImClone Systems) (Fig. 4B), addition of a chemical inhibitor of the kinase activity of Flk1 (tyrphostin SU1498) (data not shown) or microinjection and electroporation of a dominant-negative truncation mutant form of Flk1 (Tsou et al., 2002) (data not shown), all led to reduced branching of the ureteric bud and reduced numbers of induced nephrons (Fig. 4B).

To further examine the effect of angioblast-derived signals on the differentiation of the mesenchymal component of the early kidney, it was determined whether signaling through Flk1 is involved in maintaining expression of several genes characteristically expressed by the condensed mesenchyme. The expression of several known, and one novel, marker of the condensed mesenchyme was examined by whole-mount in situ hybridization in the presence or absence of the Flk1-blocking antibody. *Pax2* expression in the mesenchyme was decreased in the presence of the Flk1-blocking antibody, as shown both by in situ hybridization and real-time PCR, the latter indicating an approximately fourfold decrease in expression level (Fig. 5A,B,K). Whether the expression of *Pax2* in the ureteric bud was affected is difficult to determine using whole-mount in situ hybridization.

Pax2 has previously been shown to regulate the expression of *Gdnf* (Brophy et al., 2001); and, as would be predicted in the presence of decreased Pax2, *Gdnf* RNA levels measured by real-time PCR were also decreased in the presence of the Flk1-blocking antibody (Fig. 5L).

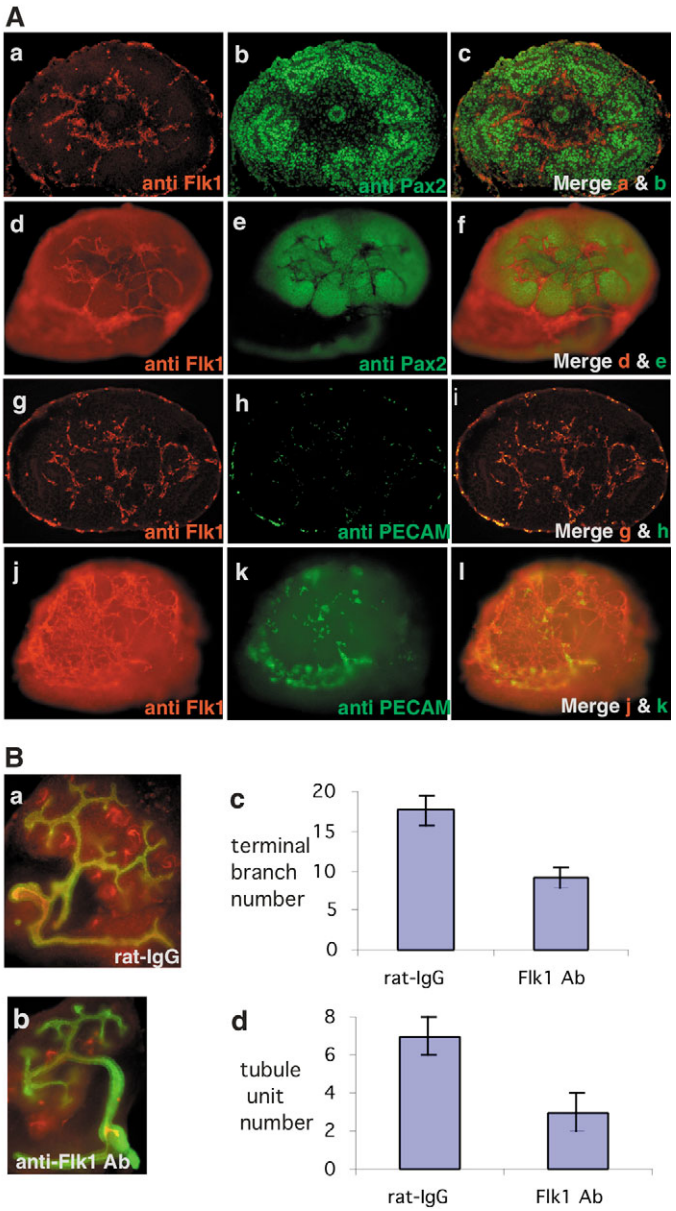


Fig. 4. (A) Antibody staining of Flk1, Pax2 and Pecam in E12.5 kidney frozen sections (a-c, g-i) and whole mounts of E11.5 kidney organ cultures placed in culture for 48 hours (d-f, j-l). (a,d) Flk1; (b,e) Pax2; (c,f) merge of preceding panels showing non-overlapping expression of Flk1 and Pax2. (g,j) Flk1; (h,k) Pecam; (i,j) merge of preceding two panels showing Pecam-expressing cells are a subset of Flk1-expressing cells. (B) Flk1-blocking antibody effect on branching and nephrogenesis. Cytokeratin green; proximal tubule brush border antigen red. (a,b) From the same embryo. (a) Treatment with rat IgG; (b) treatment with anti-Flk1; (c,d) quantitation of four pairs of organ cultures represented by (a) and (b), (P<0.001 for both terminal branch number and tubules).

Osr1 (odd-skipped related 1) is a marker of the intermediate mesoderm that continues to be expressed in the condensed mesenchyme (So and Danielian, 1999). By contrast to those of *Pax2*, *Osr1* expression levels appeared unchanged in the presence of the Flk1-blocking antibody (Fig. 5C,D), indicating

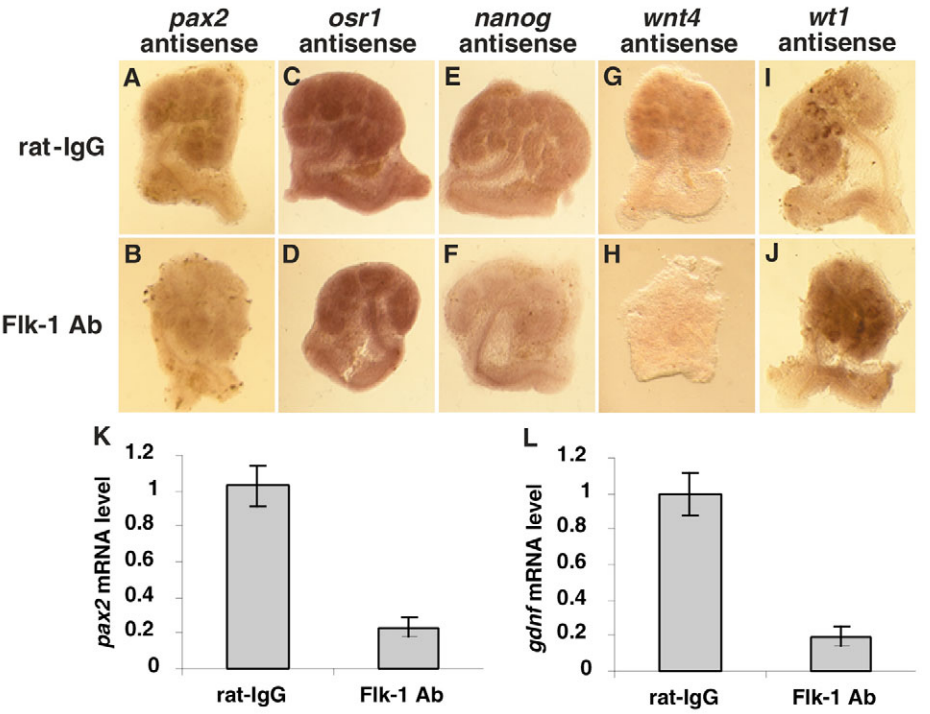


Fig. 5. Flk1 dependency of gene expression. (A,C,E,G,I) treatment with Rat IgG; (B,D,F,H,J) treatment with Flk1 blocking antibody. The probe used for in situ hybridization is shown to the left of each pair of panels. Each pair of panels is representative of the number of experiments noted in Table 1. Similar results were obtained with SU1498, (not shown). (K,L) Real-time PCR for *Pax2* (K) and *Gdnf* (L) demonstrated decreased *Pax2* and *Gdnf* mRNA ($P<0.001$) after treatment of organ cultures with the Flk1 blocking antibody. All *Pax2* and *Gdnf* Ct values were normalized to Gapdh Ct values and then converted to relative mRNA levels using standard curves, as described in the Materials and methods.

that Flk1 blockade did not non-specifically affect expression of all genes in the organ culture.

Nanog, a homeobox-containing gene recently shown to be required to preserve the multipotent state of embryonic stem cells (Chambers et al., 2003; Mitsui et al., 2003) was also identified as a novel marker of the condensed mesenchyme, with an expression that decreased after treatment with Flk1-blocking antibody (Fig. 5E,F), suggesting that it may be not a marker of the most undifferentiated mesenchyme. *Wnt4* is a signaling molecule required for the mesenchymal-to-epithelial transformation by which aggregates of condensed mesenchyme begin to form tubules (Stark et al., 1994). As would be predicted for a later marker involved in the differentiation of nephrons, *Wnt4* expression also decreased in the presence of the Flk1-blocking antibody (Fig. 5G,H). By contrast to *Pax2* expression, which overlaps *Wt1* in the condensed mesenchyme, *Wt1* expression itself did not decrease in the presence of Flk1 blockade. However, instead of the usual pattern of *Wt1* expression, which is highest in the discrete mesenchymal condensates around each derivative of the ureteric bud (Fig. 5I) a more diffuse *Wt1* expression pattern was apparent (Fig. 5J), indicative of the decreased branching and failure to form the usual discrete mesenchymal condensates around each tip of the ureteric bud derivatives. Similar results for each gene were obtained using SU1498, the pharmacological inhibitor of signaling through Flk1 (data not shown).

The Flk1-dependent signal acts on the condensed mesenchyme

The reduced branching of the ureteric bud observed after blockade of signaling though Flk1 could be due to a signal from the angioblasts that acts directly on the ureteric bud, or that acts on the mesenchyme, to indirectly affect branching. To distinguish these possibilities, Vegfa was expressed by microinjection/electroporation at the periphery of the condensed mesenchyme. This resulted in a localized increase in *Pax2* expression adjacent to the injection site (Fig. 6A, parts a,b), that was apparent within 6 hours of the injection, and that could be inhibited by either the Flk1-blocking antibody (Fig. 6A, parts d,e) or SU1498 (data not shown). This observation is most consistent with the possibility that the Flk1-dependent signal is acting directly on the mesenchyme. By contrast, if the Flk1-dependent signal were acting directly on the ureteric bud, it would be expected that the ureteric bud would then induce higher levels of *Pax2* in its usual pattern within the condensed mesenchyme around the ureteric bud.

The Flk1-dependent signal regulates stability of *Pax2* mRNA

As signaling through Flk1 is apparently required to maintain high levels of *Pax2* RNA in the condensed mesenchyme, it can be hypothesized that reduced branching of the ureteric bud upon blockade of Flk1 signaling would be due to reduced expression of *Pax2* and consequent decreased stimulation of *Gdnf* expression. To further examine the role of the Flk1-dependent signal, *Gdnf* expression in response to *Pax2* microinjection/electroporation in the condensed mesenchyme was examined, in the presence or absence of Flk1 blockade. *Gdnf* was abundantly expressed in response to *Pax2*, but Flk1 blockade eliminated *Gdnf* induction by *Pax2* (Fig. 6B).

To further examine the mechanism by which the Flk1-dependent signal may regulate *Pax2*, the levels of *Pax2* RNA expression were measured in response to either a generalized blockade of transcription by actinomycin-D (act-D) or a specific blockade of Flk1 signaling by a pharmacological agent, SU1498. As measured by real-time PCR, levels of *Pax2* decreased 1000-fold over an 8-hour treatment period with act-

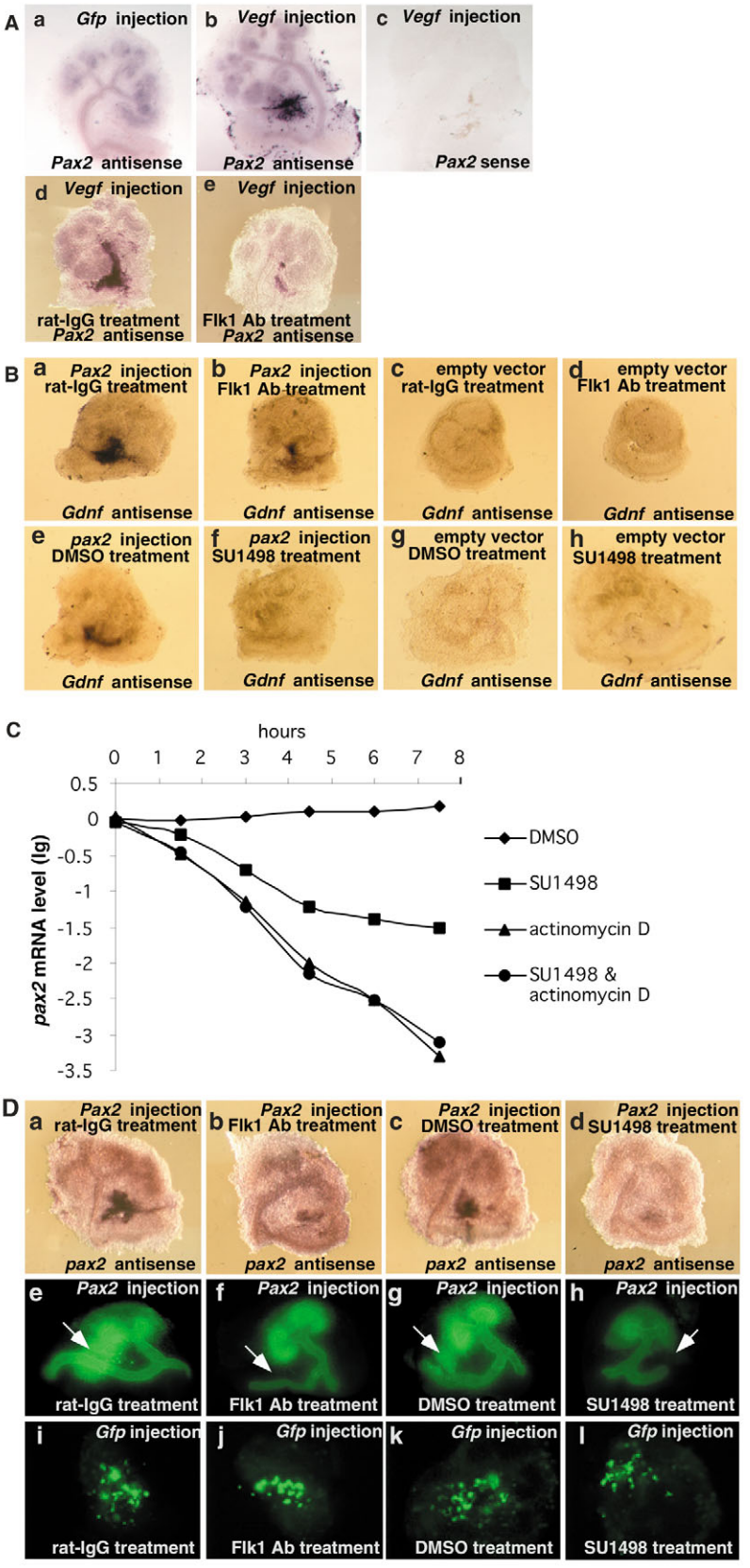


Fig. 6. (A) Flk1-dependent *Vegfa* induction of *Pax2*. The injection is noted at the top of each panel, and the antibody treatment and hybridization probes are noted at the bottom of each panel. *Gfp* (a) or *Vegfa* (b-e) expression vectors were injected at the edge of the condensed mesenchyme. Rat IgG (d) or Flk1-blocking antibody (e) were added at the time of injection. Six hours after injection, cultures were analyzed by in situ hybridization with *Pax2* antisense (a,b,d,e) or sense (c) probe. (B) Flk1-dependent *Pax2* induction of *Gdnf* expression. Panels are labeled as in A. *Pax2* expression (a,b.d,f) or control (c,d,g,h) vectors were injected and *Gdnf* antisense probe was used in all panels. (C) Real-time PCR analysis of *Pax2* mRNA expression level in organ cultures after Flk1 blockade or treatment with actinomycin-D. Treatments are shown in the figure box. SU1498, actinomycin-D, SU1498 with actinomycin-D or DMSO, were added to the culture medium at the beginning of the culture period, (time 0). At 0, 1.5, 3, 4.5, 6, or 7.5 hours after beginning cultures, real-time PCR was performed to measure levels of *Pax2* mRNA. All *Pax2* Ct values were normalized to the Gapdh Ct values and converted to relative mRNA levels as described in the methods. Relative RNA levels are shown on a log scale. (D) Flk1 dependency of *Pax2* expression. Panels a-h were injected with the *Pax2* expression vector. The hybridization probes are noted at the bottom of each panel (a-d), and the antibody treatment is noted at the top (a-d) or bottom (e-h) of each panel. (a,e) Rat IgG; (b,f) Flk1-blocking antibody; (c,g) DMSO 1:1000; (d,h) SU1498 treatment. (a-d) In situ hybridization with *Pax2* antisense probe; (e-h) staining with anti-Pax2 antibody. *Pax2* mRNA or protein expression in response to injection of the *Pax2* expression vector could be blocked by antibody or pharmacological inhibition of signaling through Flk1. (i-l) Injections of the GFP expression vector, detecting direct GFP fluorescence.

D (Fig. 6C). By contrast, on treatment with SU1498, *Pax2* mRNA levels initially decreased but reached a steady-state level significantly higher than that resulting from treatment with act-D. No additive effects were observed when act-D and SU1498 were combined. At least three possible explanations can be offered to account for these observations: (1) the Flk1-dependent signal acts to stabilize *Pax2* RNA in the mesenchyme, and the lower level observed after Flk1 blockade reflects a high-turnover state of non-stabilized *Pax2* RNA; (2) the difference in *Pax2* RNA levels between act-D treatment and Flk1 blockade is due to retention of ureteric bud expression of *Pax2* in the latter treatment, compared with complete loss of *Pax2* RNA in the former treatment; (3) a third possibility, that the angioblast signal stimulates supra-basal transcription of *Pax2*, is also possible, although the failure of ectopically expressed *Pax2* to induce expression of Gdnf under conditions of Flk1 blockade strongly suggests some degree of post-transcriptional regulation.

Organ culture experiments do not allow the use of nuclear run-on experiments to directly measure transcription rates. However, the hypothesis that Flk1-dependent signals regulate post-transcriptional regulation of *Pax2* expression was tested

further by examining *Pax2* expression itself in response to injection and electroporation of the *Pax2* expression construct, in the presence or absence of inhibitors of Flk1. It was observed that when Flk1 inhibitors were present, much less *Pax2* RNA or protein could be detected in response to *Pax2* injection (compare Fig. 6D, parts a,c and b,d; and Fig. 6D, parts e,g and f,h). By contrast, Flk1 blockade had no effect on expression of a *Gfp* construct expressed from the same CMV promoter-enhancer used to express *Pax2* (Fig. 6D, parts i-l), demonstrating that Flk1 blockade is not simply inhibiting transcription from the CMV promoter used in the *Pax2* (or *Gfp*) expression vector(s). Thus, the results of this last experiment are also consistent with post-transcriptional regulation of *Pax2* by an Flk1-dependent signal.

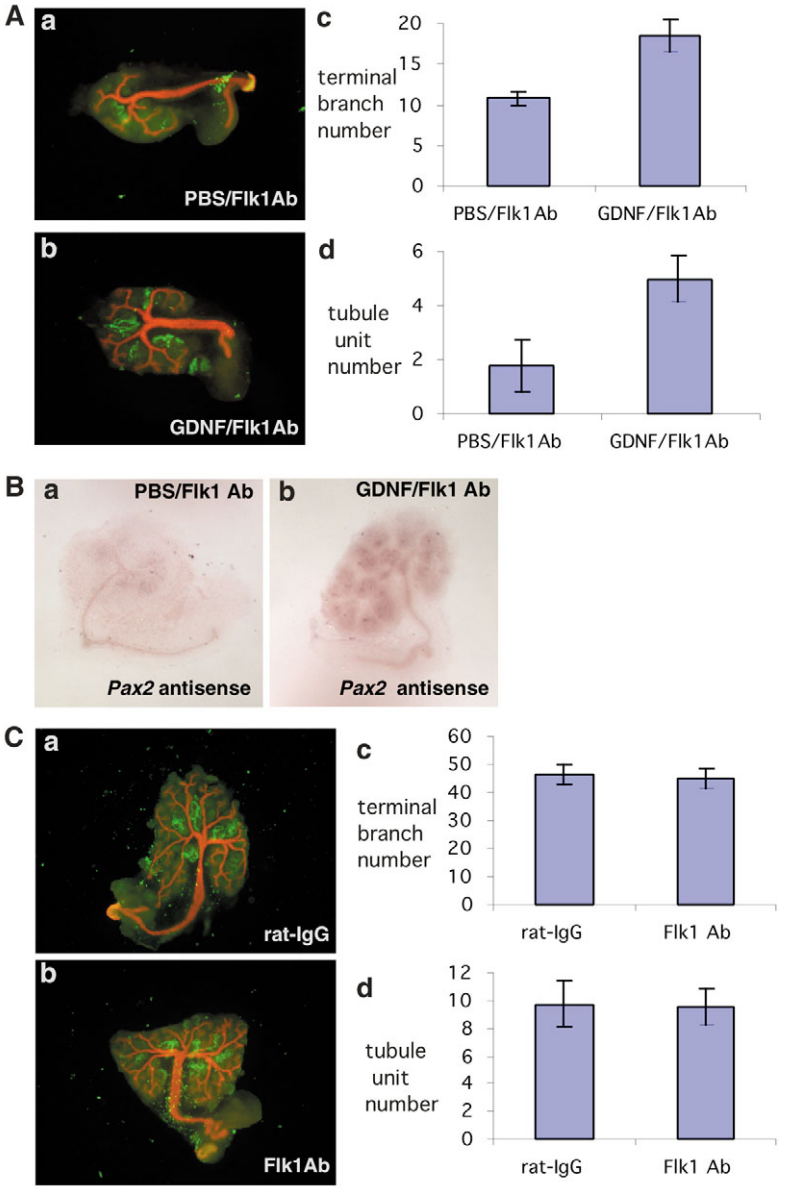


Fig. 7. (A) Gdnf rescue of Flk1 blockade. (a) Flk1 block and (b) Flk1 block with addition of Gdnf are stained for cytokeratin (red) and proximal tubule-binding lectin (green). (a,b) From the same embryo. (c) Terminal branch number (P<0.001) and (d) tubule number (P<0.001) quantification from four pairs of cultures represented by (a) and (b). (B) *Pax2* in situ hybridization of organ cultures from E11.5 embryos cultured for 48 hours in the presence of (a) Flk1 block and (b) Flk1 block with addition of Gdnf. (C) Late addition of Flk1 blockade. (a,b) Organ cultures from the same embryo, obtained from E11.5 embryos, cultured for 48 hours before addition of Flk1-blocking antibody (b) or control IgG (a), and cultured for an additional 48 hours. (c,d) Quantitation of four pairs of organ cultures represented by (a) and (b). Differences are not significant.

The Flk1-dependent signal is dispensable after initiation of kidney development

To further test the hypothesis that the Flk1-dependent signal acts primarily on the mesenchyme, Gdnf was added to the organ culture medium in the presence of the Flk1 blockade. As Gdnf acts directly on the ureteric bud, this should be able to overcome any deficiency in the condensed mesenchyme due to the absence of the Flk1-dependent signal from the angioblasts. Addition of Gdnf in the presence of the Flk1 blockade stimulated branching of the ureteric bud (Fig. 7A). Surprisingly, addition of Gdnf also rescued induction of nephrons (Fig. 7A); this was not predicted because expression of *Pax2* and *Wnt4* were greatly decreased upon blockade of the Flk1-dependent signal. However, it was also observed that expression of *Pax2* in the condensed mesenchyme was also rescued by addition of Gdnf (Fig. 7B). Together these results suggested that the Flk1-dependent signal was required to initiate kidney development, but that once *Pax2* was able to stimulate expression of Gdnf, and Gdnf was able to elicit an inductive signal from the ureteric bud, the Flk1-dependent signal might no longer be required. As further support for this possibility, it was observed that addition of the Flk1-blocking antibody after organ cultures had already been established for 48 hours, had little effect on the extent of branching and nephron induction (Fig. 7C).

Discussion

Previous studies on the induction of the metanephric kidney have considered the interaction of two cell lineages, the metanephric mesenchyme and the epithelial cells of the ureteric bud (Schedl and Hastie, 2000). Our results provide support for the requirement for a third cell type, the angioblast, which appears to be involved in maintaining the condensed mesenchyme in a differentiated state capable of responding to the inductive influence of the ureteric bud. We have shown that the *Wt1* tumor suppressor gene, previously shown to be required for the initiation of kidney development, stimulates the expression of *Vegfa* within the mesenchyme. The target cells of Vegfa appear to be a population of Flk1-expressing angioblasts found at the periphery of the mesenchymal condensations. There appears to be a reciprocal set of interactions between the angioblasts and the mesenchyme that are required to maintain the mesenchyme in

differentiated state characterized by high expression of *Pax2* and *Gdnf*. Vegfa appears to be the major component of the mesenchyme-derived component of this reciprocal set of inductive signals, and the angioblast signal remains to be identified. However, once Gdnf expression has stimulated the initial branching of the ureteric bud and the induction of nephrons has begun, the Flk1-dependent signal appears not to be required, and kidney development is maintained by the interaction of the ureteric bud and the mesenchyme.

In embryos carrying a homozygous targeted mutation in the *Wt1* gene, the ureteric bud fails to grow out from the Wolffian duct, and there is consequent apoptosis of the metanephric mesenchyme (Kreidberg et al., 1993). It has been difficult to identify potential targets of *Wt1* for which a deficiency could account for the renal agenesis phenotype, even though several studies have identified potential *Wt1* target genes in the gonad, including *Sry* (Hammes et al., 2001; Hossain and Saunders, 2001) and *Sf1* (*Zfp162* – Mouse Genome Informatics) (Wilhelm and Englert, 2002). Low levels of *Gdnf* were detected in the metanephric mesenchyme of *Wt1*-deficient embryos (Donovan et al., 1999), suggesting that *Gdnf* may not be a target of *Wt1*, although it remains possible that *Wt1* is involved in boosting levels of *Gdnf* to achieve bud outgrowth. More recently, siRNA to *Wt1* was used in an organ culture system to confirm the requirement for *Wt1* in early kidney development, but this approach was less amenable for identifying novel target genes, because of the requirement for *Wt1* to maintain cell viability (Davies et al., 2004). The present study suggests *Vegfa* as a novel candidate target gene for Wt1. The angioblast-mesenchyme interaction mediated by Vegfa appears to be crucial in maintaining the initial differentiated state of the mesenchyme, and in the absence of this interaction, it is likely that insufficient levels of Pax2 and Gdnf are present to elicit outgrowth of the ureteric bud.

The conclusion that Flk1-expressing angioblasts express a signal that is required to initiate kidney development depends on their identification as the major, if not sole, cell type expressing Flk1 in the early metanephric kidney. Immunostaining for Flk1 and the use of *Flk1-LacZ* knock-in mice has failed to detect expression in other cell types, and particularly not in the ureteric bud. Furthermore, the observations that (1) localized *Vegfa* injection/electroporation results in adjacent Pax2 expression, as opposed to enhanced Pax2 expression around the ureteric buds, and (2) addition of Gdnf can overcome the Flk1 blockade, are both more consistent with a model that invokes the requirement for angioblasts, rather than direct stimulation of the ureteric bud by Vegfa. However, the possibility that very low levels of Flk1, not detectable by immunostaining or the use of knock-in mice, are expressed by the ureteric bud and involved in Vegfa stimulation of branching, remains a possibility that might be examined by genetic ablation of the *Flk1* gene in the ureteric bud.

There are four major splice forms of the *Wt1* gene, due to alternative splicing of exon 5, and alternative insertion of a three amino acid sequence, lysine-threonine-serine (KTS), at the end of exon 9 (Haber et al., 1991). We recently showed that elimination of exon 5 had no effect on kidney development and function, and the role of this alternatively spliced exon remains unknown (Natoli et al., 2002b). Hammes et al. (Hammes et al., 2001) have published the results of gene targeting experiments that allowed expression of either the +KTS or –KTS forms of *Wt1*. In each case, homozygous mutant embryos showed defective kidney development, with poorly developed glomeruli, a phenotype more pronounced in the +KTS-only kidneys. The difference in these phenotypes raised the question of whether this indicated that the two splice forms of *Wt1* have entirely distinct functions, or whether the two phenotypes are due to differences in severity, but due to the same function of the different splice forms. Supporting the former possibility are observations that the +KTS form of Wt1 associates with spliceosomes (Larsson et al., 1995), and has a speckled nuclear staining pattern, whereas the –KTS form has a more diffuse staining pattern in the nucleus. In our microinjection and electroporation system it has been observed that both the +KTS and –KTS forms of *Wt1* are capable of inducing expression of Vegfa. However, this does not necessarily conflict with the likely possibility that the +KTS and –KTS forms of *Wt1* have common functions early in kidney development but acquire distinct functions during podocyte differentiation. Indeed, both +KTS- and –KTS-only embryos apparently undergo normal early kidney development (Hammes et al., 2001), implying that they are interchangeable with regard to early functions of *Wt1*.

Previous studies examining liver and pancreas development have identified roles for the vascular system, and in particular Flk1-expressing cells in organogenesis (Lammert et al., 2001; Matsumoto et al., 2001). Thus it appears to be an emerging paradigm that organs require signals from angioblast-type cells to stimulate or maintain organ-specific patterns of differentiation. It is not known whether liver- and pancreas-associated angioblastic cells are responding to Vegfa produced by the mesenchymal components of these organs. It will also be of great interest to eventually determine whether these signal elaborated by pancreas-, liver- and kidney-associated angioblasts are identical or differ between these organs.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/24/5437/DC1

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