

Wilms' tumour protein *Wt1* stimulates transcription of the gene encoding vascular endothelial cadherin

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Abstract The Wilms' tumour gene, *Wt1*, encodes a zinc finger protein, which is mutated in a subset of paediatric renal carcinomas known as Wilms' tumours (nephroblastomas). Recent findings indicate that *Wt1*, beside its role in genitourinary development, is also necessary for normal vascularisation of the embryonic heart, and may even be involved in tumour angiogenesis. The original purpose of this study was to decipher potential downstream signalling pathways of *Wt1* for blood vessel formation. We found that the *Wt1*(-KTS) protein, which functions as a transcription factor, stimulated the expression of *cadherin 5* (*CDH5*, *vascular endothelial (VE) cadherin*) and other vascular genes, i.e. those encoding vascular endothelial growth factor receptors 1 and 2, and angiopoietin-2. Furthermore, an enhancer element was identified in the first intron of the *CDH5* gene, which bound to the *Wt1*(-KTS) protein and was necessary for reporter gene activation by *Wt1*(-KTS) in transiently transfected cell lines. *Wt1* and VE-cadherin proteins could be co-localised by double immunofluorescence staining in maturing glomeruli of embryonic murine kidneys. *VE-cadherin* transcripts were reduced in some but not all tissues of *Wt1*-deficient mouse embryos. These results indicate that *Wt1* can stimulate vascular gene transcription. By demonstrating that *Wt1*(-KTS) protein *trans*-activates an enhancer element in the first intron we identified *CDH5* as a novel target gene of *Wt1*. It is suggested that transcriptional activation of *CDH5* by *Wt1* fulfils regulatory functions during vascular development and kidney formation.

Keywords Transcription factor · Angiogenesis · Kidney development · Cadherin · Vascular endothelial growth factors · Enhancer element

Introduction

Wt1 was originally identified as a tumour suppressor gene by reason of loss-of-function mutations in a childhood malignancy of the kidneys known as nephroblastoma or Wilms' tumour [32]. Wilms' tumours can arise when pluripotent progenitor cells in the developing kidney continue to proliferate instead of differentiating to glomeruli and tubules [28, 32]. It is assumed that the *Wt1* gene product enables cells to switch between an epithelial and mesenchymal state [2, 25, 26]. Failure of epithelial cell differentiation in the kidneys due to *Wt1* inactivation is responsible for approximately 10% of Wilms' tumours. The *Wt1* gene encodes a zinc finger protein that can function as transcription factor [33]. Alternative RNA splicing gives rise to the insertion of three additional amino acids (lysine, threonine, serine (KTS)) in the C-terminal zinc finger domain [13]. Molecules with the KTS tripeptide exhibit increased RNA-binding affinity and are presumably involved in post-transcriptional processes [21, 27].

Besides its role in tumourigenesis, *Wt1* is also necessary for the development of certain organs. Mice with homozygous disruption of *Wt1* (*Wt1*^{-/-}) are embryonic lethal due to heart failure resulting from myocardial growth arrest [20, 26]. Other abnormalities of *Wt1*-null mice include defects of the kidneys and gonads, mesothelial tissues, spleen, adrenal glands, retina and olfactory epithelium [14, 20, 26, 39, 41]. We have previously found that *Wt1* is also required for blood vessel formation in the developing heart [40]. Coronary vascular cells are derived from the epicardium, which forms

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an epithelial sheet on the outer heart surface [31]. During cardiogenesis Wt1-positive epicardial cells undergo epithelial-to-mesenchymal transition (EMT), which permits them to become cardiovascular progenitor cells [11]. It has been shown recently that EMT does not occur in embryoid bodies of *Wt1*-knockout mice [25]. Furthermore, *Wt1*-deficient embryonic bodies expressed endothelial genes, i.e. *Flk1/Kdr* and *cadherin 5 (Cdh5, vascular endothelial (VE)-cadherin)*, at a rather low level [25]. These findings raise the interesting possibility that Wt1 facilitates the differentiation of progenitor cells along the endothelial lineage through up-regulation of vascular growth factor receptors and endothelial cell adhesion molecules. Consistently, Wt1 was expressed de novo in resident vascular smooth muscle and endothelial cells after myocardial infarction in rats suggesting a role in neovascularisation of the ischemic heart [38]. Wt1 was recently also detected in the vascular endothelium of various tumours [34, 37]. While these observations provided first evidence for a link between Wt1 and tumour angiogenesis, little is known about the function of Wt1 in the vasculature of normal and neoplastic tissues. Important hints hereunto can be obtained from the knowledge of candidate target genes that are regulated by Wt1. As a step towards this issue, we explored vascular gene regulation by Wt1 in cells derived from osteosarcoma, a highly vascularised bone tumour with strong metastatic potential and the capacity of vascular mimicry [3, 7]. We report here that Wt1 stimulates indeed the expression of several vascular genes including *CDH5*, VEGF receptor 1 and 2 (*Flt1* and *Flk1*), and angiopoietin-2 (*angpt2*). Furthermore, we identified an enhancer element in the first intron of the *CDH5* gene, which is *trans*-activated by the Wt1(-KTS). It is proposed that activation of *CDH5* and other vascular genes by Wt1 supports blood vessel assembly in the heart and may also promote tumour angiogenesis.

Materials and methods

Cell culture

The UB27 and UD28 lines, which express the Wt1(-KTS) and Wt1(+KTS) proteins under control of a tetracycline-regulated promoter, were the gift of Dr. Christoph Englert [8]. These cells are derived from the parental U-2OS osteosarcoma cell line (ATCC no. HTB-96) and were grown in DMEM nutrient (PAA Laboratories, Pasching, Austria) supplemented with 10% FCS (Biochrom KG, Berlin, Germany), 100 IU/ml penicillin (Invitrogen GmbH, Karlsruhe, Germany), 100 µg/ml streptomycin (Invitrogen), 1 µg/ml tetracycline, and 1 µg/ml puromycin. Stimulation of Wt1 expression was achieved by incubation of UB27 and UD28 cells in tetracycline-free DMEM as described [8, 40]. Human

embryonic kidney (HEK) 293 cells (catalogue no. ACC 305) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and kept in supplemented DMEM.

Cell transfections and reporter assays

UB27 and UD28 cells were expanded to approximately 50% confluence in 24-well tissue culture plates. Tetracycline was removed from the medium 24 h before the transfections in the moiety of cells to stimulate Wt1(-KTS) and Wt1(+KTS) expression, respectively [8]. Nine hundred nanograms of firefly luciferase constructs harbouring regulatory sequences of the *VE-cadherin* gene and 100 ng of a renilla luciferase plasmid were transiently co-transfected with the Fugene6® reagent (3 µl per well) according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). Transfection of the empty pGL3-basic reporter vector served as negative control. The transfected cells were incubated for 48 h with and without tetracycline (1 µg/ml) prior to lysis in Reporter Lysis Buffer (Promega, Mannheim, Germany). Luciferase activities were measured in a luminometer (Micro-lite TLX1, MGM Instruments, Hamden, CT) as described [5, 17]. Data are presented as relative light units normalised to renilla luciferase activities. Transfection of HEK 293 cells was performed as described [17].

Reverse transcription real-time PCR

Total RNA was isolated from cultured cells and tissues with the Trizol reagent (Invitrogen) according to the manufacturer's protocol. First-strand cDNA synthesis was performed with 2 µg of total RNA using oligo(dT) primers and superscript II reverse transcriptase (Invitrogen). Forty nanograms of cDNA were taken for real-time PCR amplification with SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, USA). The PCR reactions were carried out on a StepOnePlus thermocycler (Applied Biosystems, Darmstadt, Germany) as follows (45 cycles): DNA denaturation (15 s) at 94°C, primer annealing (15 s) and extension (60 s) of double-stranded DNA at 60°C, detection of SYBR® Green fluorescence at 77°C (30 s). The PCR primers used for the amplification reactions are listed in Table 1. The C_t values for the genes of interest were subtracted by the C_t values for β -actin to obtain delta C_t values. Differences in transcript levels were calculated according to the equation $2^{\text{deltadelta}C_t}$.

Plasmids

A DNA sequence extending from -2,790 to +99 base pairs (bp) relative to the transcription start site in the human *cadherin 5 (CDH5, VE-cadherin)* gene (National Center for Biotechnology Information (NCBI) accession no. NT_010498) was amplified

Table 1 Oligonucleotides used for quantification by PCR of the indicated mRNAs

Primer name	Gene name (mRNA)	NCBI reference sequence	Primer sequence
hVE-cadherin-F	<i>Human VE-cadherin, cadherin 5, CDH5</i>	NM_001795	5'-CCTACCAGCCCAAAGTGTGT-3'
hVE-cadherin-R			5'-GACTTGGCATCCCATTGTCT-3'
hVEGFR1-F	<i>Human vascular endothelial growth factor receptor 1, VEGFR1, FLT1</i>	AF063657	5'-ACCCAGATGAAGTTCTTTGGA-3'
hVEGFR1-R			5'-TTAATGCCAAATGCTGATGCTT-3'
hVEGFR2-F	<i>Human vascular endothelial growth factor receptor 2, VEGFR2, FLK1, KDR</i>	AF035121	5'-TGGCATCGCGAAAAGTGTATC-3'
hVEGFR2-R			5'-AAAGGGAGGCGAGCATCTC-3'
hAngpt1-F	<i>Human angiopoietin-1, ANGPT1</i>	NM_001146	5'-TATGCCAGAACCCAAAAAGG-3'
hAngpt1-R		NM_139290	5'-TTCACCGGAGGGATTCCA-3'
hAngpt2-F	<i>Human angiopoietin-2, ANGPT2</i>	NM_001147	5'-CGTTGATTTTCAGAGGACTTGGA-3'
hAngpt2-R			5'-CTCATTCCCTCCAGTCTTTAAG-3'
hTie2-F	<i>Human endothelial TEK tyrosine kinase, TEK, TIE2</i>	L06139	5'-TTGTGAACTGCACACGTTTGG-3'
hTie2-R			5'-CCTCGGTATGCCTTCATTGC-3'
hGAPDH-F	<i>Human glyceraldehyde-3-phosphate dehydrogenase, GAPDH</i>	NM_002046	5'-ACAGTCAGCCGCATCTTCTT-3'
hGAPDH-F			5'-GACAAGCTTCCCGTTCTCAG-3'
mVE-cadherin-F	<i>Murine VE-cadherin, cadherin 5, cdh5</i>	NM_009868	5'-CACTGCTGTACACGGATGAC-3'
mVE-cadherin-R			5'-GGCAAGATCTTCGGGGTCGAT-3'
mGAPDH-F	<i>Murine glyceraldehyde-3-phosphate dehydrogenase, Gapdh</i>	NM_008084	5'-ACGACCCCTCATTGACCTCA-3'
NM_008084			5'-TTGGCTCCACCCTTCAAGTG-3'

by PCR using a bacterial artificial chromosome (imaGenes, Berlin, Germany, clone RP11-93H5) as template. The PCR product was ligated into the *KpnI* restriction site of the pGL3-basic reporter plasmid (Promega). This construct was designated *pGL3bVEcadprom*. Likewise, various regions of the first intron of the *CDH5* gene were cloned and ligated downstream of the promoter into the *SacI/HindIII* sites of *pGL3bVEcadprom*. Site-directed base-pair mutations were introduced in the first intron of the *VE-cadherin* gene by PCR as described elsewhere [5, 17]. All constructs were analysed by automated DNA sequencing.

Immunoprecipitation and SDS-PAGE

UB27 osteosarcoma cells were kept for 72 h either in the absence or presence of tetracycline (1 µg/ml) to stimulate or inhibit Wt1 expression. Cell lysates were prepared in RIPA buffer (1× PBS, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mg/ml PMSF, 0.1 IU/ml aprotinin, 1 mM sodium orthovanadate) as described elsewhere [5, 17]. The lysates (250 µg protein each) were pre-cleared for 1 h at 4°C with Protein A Sepharose™ CL-4B (Amersham Bioscience, Uppsala, Sweden), and the beads were pelleted by centrifugation at 1,000×g (5 min, 4°C). Prior to immunoprecipitation, aliquots of the supernatants were removed for immunoblotting with anti-actin antibody (see below). One microgram of mouse monoclonal anti-VE-cadherin antibody (C-19, catalogue no. sc-9989, Santa Cruz Biotechnology) was added to the supernatants (1 ml), which

were then incubated for 2 h at 4°C. Control experiments were performed with mouse IgG instead of primary antibody. After addition of pre-washed Protein A Sepharose beads (50 µl each) the tubes were shaken overnight at 4°C on a rocker platform. The next morning, the beads were centrifuged at 1,000×g for 5 min (4°C), washed four times with RIPA buffer, and after the final wash resuspended in Laemmli buffer and heated to 95°C for 3 min. Following centrifugation (1,000×g for 5 min) the supernatants were loaded and separated on a 7.5% polyacrylamide gel. The proteins were transferred onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Freiburg, Germany) with the use of a semidry blotting apparatus (BioRad, München, Germany). Non-specific binding activity was reduced by incubating the membranes for 60 min at room temperature in PBS, 5% non-fat milk (Roth, Karlsruhe, Germany), 0.05% Tween-20 (Serva, Heidelberg, Germany). Incubation with anti-VE-cadherin antibody from goat (catalogue no. sc-6458, diluted 1:200 in PBS, 2.5% non-fat milk, 0.05% Tween-20) was performed overnight at 4°C. The primary antibody was detected with a peroxidase-coupled donkey anti-goat-IgG (catalogue no. sc-2020, Santa Cruz Biotechnology, 1:1,000 dilution), and the reaction products were visualised with the enhanced chemiluminescence system (Amersham Pharmacia Biotech.). A goat polyclonal anti-β-actin antibody (catalogue no. sc-1615, Santa Cruz Biotechnology, diluted 1:500) was applied for immunoblotting of aliquots that had been collected before immunoprecipitation to assess equal protein content of the samples.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed as described in detail elsewhere [24]. In brief, UB27 cells were grown for 48 h either in the presence or absence of tetracycline (1 µg/ml) to inhibit or stimulate expression of Wt1(-KTS). Cross-linking of DNA-bound protein was accomplished by incubating the cells for 10 min in a 1% formaldehyde solution. The cell lysates were ultrasonicated to obtain an average DNA fragment size of 200–1,000 bp. Immunoprecipitations were performed with the following antibodies (1 µg each) overnight at 4°C: rabbit polyclonal anti-Wt1 antibody (sc-192, Santa Cruz Biotechnology), rabbit polyclonal anti-acetylated histone 3 antibody (cat. no. 06–599, Upstate-Millipore, Darmstadt, Germany). Incubation with normal rabbit IgG (Cell Signaling, Boston, USA) served as a negative control. The antibody-bound proteins were precipitated for 1 h at 4°C with DNA-blocked protein G-agarose (Millipore, Darmstadt, Germany). After several washes in low- and high-salt buffer, the DNA was eluted from the agarose beads, extracted by phenol:chloroform treatment, and precipitated in 100% ethanol. The DNA pellet was resuspended in 30 µl of ddH₂O and 2 µl of a 1:40 dilution was taken for quantitative PCR amplification of the first intron of the human *CDH5* gene using the following primers: 5'-GAGCTGAGCCGATCTCAC-3' (forward), 5'-GCCGGTCATGTGGTGAAA-3' (reverse) on a StepOnePlus thermocycler (Applied Biosystems). Amplification of genomic *β-actin* DNA with the following primers 5'-GTGAGTGGCCCGCTACCT-3' (forward) and 5'-CCTTGTCACACGAGCCAG-3' (reverse) served as a control.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSA) were performed with GST-purified recombinant Wt1 protein as described in detail elsewhere [5, 17, 40]. In brief, a double-stranded oligonucleotide (5'-GTCTGTATGCTCCCA CAGCCTCCTCGAT-3') was selected on the basis of a predicted Wt1 binding site in the first intron of the *CDH5* gene and end-labelled with [γ -³²P]ATP (7,000 Ci/mmol, catalogue no. 35020, ICN Biochemicals, Eschwege, Germany). Likewise, a second double-stranded oligonucleotide with mutation of the core Wt1 binding motif (underlined) was used for the binding reactions: 5'-GTCTGTATGC GAATTCAGCCTCCTCGAT-3'. Competition experiments were performed with an unlabelled oligonucleotide carrying the previously identified Wt1 binding site [40] from the human *NTRK2* gene (NCBI accession no. AL390777): 5'-TGTGAACCTCCACATGCTGCTG-3'. The binding reactions were separated on a non-denaturing 4% polyacrylamide gel. The dried gels (gel dryer, model 583, BioRad) were exposed on X-ray film for 6 h and developed.

Immunohistochemistry

Morphological studies were performed as described previously [5, 17, 19, 38, 40]. Staged embryos were fixed overnight in paraformaldehyde (3% in PBS) at 4°C and frozen in tissue-Tek O.C.T. compound (Sakura Finetek, Zoeterwoude, Netherlands). Ten-micrometre cryostat sections were permeabilised with 0.1% Triton X-100 in PBS and blocked for 5 min at room temperature in serum-free DakoCytomation protein block (catalogue no. X0909, Dako, Hamburg, Germany). The following primary antibodies were diluted 1:50 in ready-to-use antibody diluent (Zymed Laboratories Inc., Berlin, Germany): rabbit polyclonal anti-Wt1 antibody (C-19, catalogue no. sc-846, Santa Cruz Biotechnology) and goat polyclonal anti-VE-cadherin antibody (C-19, catalogue no. sc-6458, Santa Cruz Biotechnology). The reaction products were visualised by incubation (1.5 h at room temperature) with Cy3 (Wt1) and Cy2 (VE-cadherin) conjugates. Nuclei were sometimes counterstained with 4',6-diamidino-2-phenylindole [5, 17]. Tissue sections were viewed under an epifluorescence microscope (Axiovert S100, Zeiss, Jena, Germany), which was connected to a digital camera (Spot RT Slider, Diagnostic Instruments, Sterling Heights, USA) equipped with the Metamorph V4.1.2 software (Molecular Devices Inc. Downington, USA).

Chemicals

If not otherwise indicated, all chemicals were obtained from Sigma-Aldrich, Hamburg, Germany.

Statistics

Values are presented as means ± SD. ANOVA with Bonferroni test as post hoc calculation and Student's *t* test were performed as indicated to reveal statistical significances. Values of *P* < 0.05 were considered statistically significant.

Results

Wt1 activates expression of vascular genes in osteosarcoma cells

Genetically modified cells are valuable tools to identify transcription factor target genes. We took advantage of a previously engineered osteosarcoma cell line (U-2OS) harbouring a *Wt1* expression cassette under control of the tetracycline repressor [8] to identify putative downstream effectors of the Wt1 protein. Osteosarcoma is a highly vascularised bone tumour whose cells are capable of vascular mimicry, e.g. forming blood vessel-like tubular networks with concomitant expression of vascular cell markers [3, 7]. Thus,

one can assume that osteosarcoma-derived cell lines are endowed with a pro-angiogenic programme, which makes them suitable for studying vascular gene regulation. The two clonal osteosarcoma cell lines that we used differ in their expression either of the Wt1(-KTS) (UB27 cells) or the Wt1(+KTS) (UD28 cells) protein [8]. Wt1 isoforms without the lysine–threonine–serine (KTS) splice insertion in the zinc finger domain can function as transcription factors, whereas Wt1(+KTS) molecules have a presumed role in mRNA processing [21, 27]. Total RNA was obtained from four independent experiments, and a quantitative reverse transcription (RT)-PCR technique was applied to determine relative transcript levels of genes involved in blood vessel formation. Remarkably, the mRNAs of *CDH5*, *VEGFR1* (*Flt1*), *VEGFR2* (*Flk1*) and *angiopoietin-2* (*angpt2*) were enhanced significantly upon induction of the Wt1(-KTS) protein in UB27 cells (Fig. 1). In contrast, no significant differences in *angiopoietin-1* (*angpt1*) and *Tie2* mRNAs were observed between cells with high and low Wt1(-KTS) expression (Fig. 1). No changes in vascular gene transcripts were detected upon induction of Wt1(+KTS) protein in UD28 cells (Fig. 1).

Expression of Wt1(-KTS) and VE-cadherin is correlated in osteosarcoma cells

Since Wt1 exerted by far the strongest effect on *VE-cadherin* mRNA, we studied the interaction of these molecules in

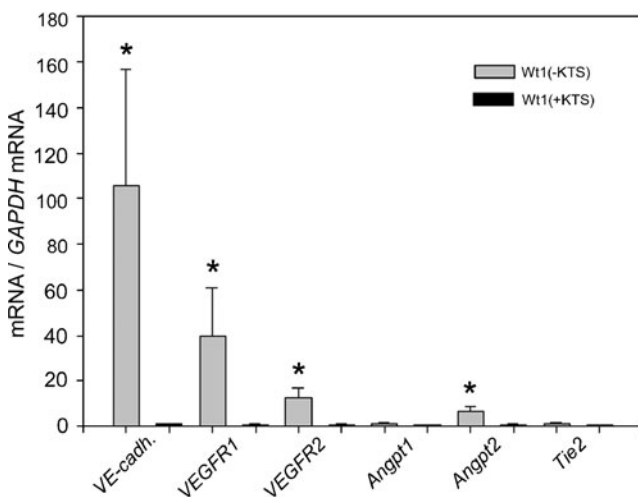


Fig. 1 Messenger RNA levels of vascular genes in osteosarcoma cells. Transcripts of *VE-cadherin* (*VE-cadh.*), *vascular endothelial growth factor receptors* (*VEGFR*) 1 and 2, *angiopoietins* (*Angpt*) 1 and 2, and *Tie2* were measured by real-time RT-PCR in stable osteosarcoma cell lines with inducible expression of Wt1(-KTS; clone UB27) and Wt1(+KTS) proteins (clone UD28), respectively [8]. Relative mRNA levels were determined on the basis of deltaC_t values. Shown are the fold differences of gene-specific mRNAs normalised to *Gapdh* mRNA in cells with stimulated vs. repressed Wt1(-KTS) (grey bars) and Wt1(+KTS) (black bars), respectively. Values are means ± S.D. of four independent experiments, each. Statistical significances are indicated (asterisk $P < 0.001$, Student's *t* test)

more detail. Omission of tetracycline from the culture medium of UB27 cells for 24, 48 and 72 h caused an increase of Wt1(-KTS) protein that was paralleled by a significant rise in *VE-cadherin* mRNA ($n=4$, Fig. 2a). For comparison, a similar Wt1(+KTS) protein content in UD28 cells had no significant effect on *VE-cadherin* transcripts (Fig. 2a). Immunoprecipitation followed by SDS-PAGE was performed to verify the ~130 kDa VE-cadherin protein in UB27 cells with high Wt1(-KTS) expression (Fig. 2b). Appropriate negative controls were performed with normal mouse serum instead of anti-VE-cadherin antibody (Fig. 2b). VE-cadherin could not be immunoprecipitated in uninduced UB27 cells with low Wt1(-KTS) protein (Fig. 2b). These findings indicate that VE-cadherin gene expression in osteosarcoma cells is positively correlated with Wt1(-KTS), but not with Wt1(+KTS) protein. Considering the previously recognised functions of the different Wt1 proteins [21, 27] our observations suggest a transcriptional mechanism of *CDH5* gene regulation by Wt1.

Wt1(-KTS) activates an enhancer element in the first intron of the *CDH5* gene

To validate the relationship between Wt1 and *VE-cadherin* and to analyse the underlying regulatory mechanism, we investigated whether Wt1 can activate the promoter of the *CDH5* gene directly. For this purpose, a firefly luciferase construct carrying a region between -2,790 and +99 bp relative to the transcription start site in the human *CDH5* gene was transiently co-transfected into UB27 and UD28 cells along with a renilla luciferase plasmid for normalisation of transfection efficiencies. After transfection, the cells were grown for 48 h either in the presence or absence of tetracycline to adjust low and high Wt1 protein levels, respectively. Stimulation of Wt1(-KTS) in UB27 cells nearly doubled the activity of the *CDH5* promoter, whereas Wt1(+KTS) in UD28 cells had no significant effect ($n=5$, Fig. 3b). The relatively weak stimulatory effect of Wt1(-KTS) on reporter gene activity compared to its strong impact on *VE-cadherin* mRNA levels in UB27 cells (Fig. 1b) let us assume that additional, Wt1-sensitive *cis*-regulatory elements may exist outside of the promoter in the *CDH5* gene. The *CDH5* gene is organised such that the first intron, which consists of approximately 12 kb, is contained in the 5' untranslated region (UTR), and the protein coding sequence starts in exon 2 [23] (Fig. 3a). Notably, the first intron has previously been found to be important for high-level expression of VE-cadherin [15]. To test for the presence of Wt1 inducible enhancer elements in the first intron of the *CDH5* gene, reporter constructs were generated in which we combined the 2,790 bp 5'-upstream region with different parts of the first intron each comprising approximately 4,000 bp. While two of these constructs had no effect

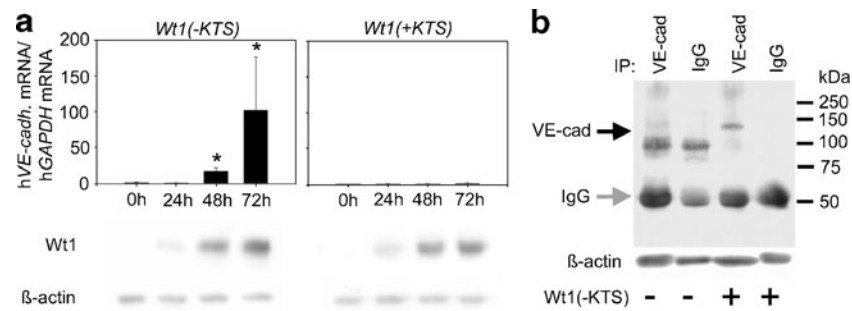


Fig. 2 VE-cadherin in osteosarcoma cells with inducible expression of different Wt1 proteins. *Panel a* shows the time course of *VE-cadherin* transcripts upon induction of the Wt1(-KTS) and Wt1(+KTS) splice variants in two clonal cell lines, UB27 and UD28, respectively. Representative immunoblots of Wt1 and β -actin proteins are depicted below. Note, that *VE-cadherin* transcripts were increased only in Wt1(-KTS) but not in Wt1(+KTS) expressing cells. Values are means \pm S.D. of $n=4$ experiments. Statistical significance vs. control (0 h) is marked by asterisks (asterisk $P<0.001$, ANOVA with Bonferroni as post hoc test). The ≈ 130 kDa *VE-cadherin* protein

(*VE-cad*) was detected in stimulated UB27 cells by immunoprecipitation (IP) and subsequent immunoblot analysis (*lane 3* in *panel b*). Appropriate control experiments were performed with the use of mouse IgG instead of anti-*VE-cadherin* antibody for immunoprecipitation (*lanes 2* and *4* in *panel b*). *VE-cadherin* could not be clearly verified in UB27 cells with low Wt1(-KTS) protein (*lane 1* in *panel b*). Anti- β -actin antibody was applied to assess equal protein content in aliquots that had been removed before immunoprecipitation (*panel b*, lower part)

on the *CDH5* promoter in Wt1(-KTS) expressing UB27 cells, a sequence extending from +4,530 to +8,811 bp significantly enhanced *CDH5* promoter activity ($n=5$, Fig. 3b). The same reporter plasmid did not respond to changes of Wt1(+KTS) protein in UD28 cells (Fig. 3b), but was stimulated approximately sixfold in HEK 293 cells upon co-transfection of a Wt1(-KTS) expression construct ($n=5$, Fig. 3c). A reporter plasmid carrying the *CDH5* promoter

(from -2,790 to +99 bp) alone, e.g. without additional intron 1 sequence, was insensitive to Wt1(-KTS) in transiently transfected HEK 293 cells (Fig. 3c). We also tested whether the identified intron sequence in the *CDH5* gene conferred Wt1 inducibility to a heterologous promoter. For this purpose, the respective region was ligated downstream of the SV40 promoter in the pGL3promoter plasmid. While Wt1(-KTS) exerted no effect on the activity of the empty

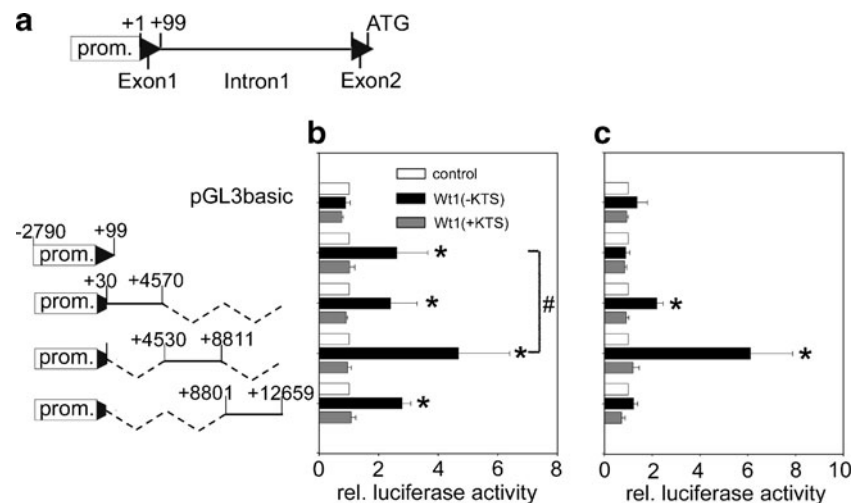


Fig. 3 Regulation of different *VE-cadherin* reporter constructs by Wt1. **a** Scheme of the 5'-flanking region of the human *VE-cadherin* gene. The transcription start site is marked by +1 and the start codon (ATG) in exon 2 is indicated. The position of nucleotides is given relative to the transcription start according to the reference sequence (NCBI AC012325). **b** Effect of Wt1(-KTS) and Wt1(+KTS) proteins on transcriptional activity of the *VE-cadherin* promoter alone and in combination with different parts of the first intron. Reporter constructs in pGL3-basic containing the human *VE-cadherin* promoter (from -2,790 to +99 bp) with and without intron 1 sequence were transiently transfected in UB27 and UD28 cells. The cells were grown either in

the presence or absence of tetracycline to suppress (*open bars*) or stimulate (*black and grey bars*) Wt1 expression, respectively. **c** Reporter gene activity in HEK 293 cells co-transfected with empty vector (*open bars*) or expression constructs for Wt1(-KTS) and Wt1(+KTS) proteins (*black and grey bars*). A renilla luciferase vector was used for the normalisation of transfection efficiencies. All values are means \pm SD of five independent experiments, each performed in triplicate. Significant differences vs. empty pGL3 reporter vector (asterisk $P<0.01$, ANOVA with Bonferroni as post hoc test) and between two reporter constructs are indicated (*number sign* $P<0.01$, Student's *t* test)

pGL3promoter vector in UB27 cells, a more than fourfold activation was elicited upon inclusion of the intron one sequence (data not shown). To identify the relevant *cis*-element in the first intron of the *CDH5* gene, the ≈ 4.3 -kb sequence was curtailed down to a 569-bp fragment (+4,530 to +5,098 bp), which was as efficient as the ≈ 4.3 -kb piece in terms of being stimulated by Wt1(-KTS) protein (Fig. 4a). For comparison, an adjacent 522-bp sequence (+5,099 to +5,620 bp) did not significantly ($P>0.05$) enhance *CDH5* promoter activity in the presence of Wt1(-KTS) (Fig. 4a). Mutation of a predicted Wt1(-KTS) binding site in the 569-bp intron fragment (+4,530 to +5,098 bp) abolished the stimulatory effect of Wt1(-KTS) protein (Fig. 4a). EMSAs were performed to investigate whether the identified element could interact with Wt1 proteins (Fig. 4b). Specific retardation bands indicating the binding of recombinant Wt1(-KTS) protein were obtained with a 28-bp oligonucleotide carrying a predicted Wt1 consensus element. Interaction of Wt1(-KTS) with this sequence could be competed

with unlabeled DNA including the previously identified Wt1 (-KTS) binding site in the promoter of the *NTRK2* gene [40]. Introducing a mutation in the oligonucleotide abolished its binding affinity for the Wt1(-KTS) protein (Fig. 4b). Notably, the Wt1(+KTS) molecule did not interact with the enhancer element in the first intron of the *CDH5* gene (Fig. 4b).

ChIP was applied to explore whether Wt1 interacts with the *CDH5* enhancer also in a natural chromosomal configuration. Repeated analyses were carried out with chromatin prepared from UB27 cells with high and low Wt1(-KTS) protein content, respectively. An approximately fivefold enrichment of genomic DNA from the first intron of the *CDH5* gene was obtained with anti-Wt1 antibody vs. normal rabbit serum (Fig. 5a). *CDH5* intron one sequence could not be enriched from UB27 cells with low Wt1 protein content (Fig. 5a). Likewise, anti-Wt1 antibody did not immunoprecipitate genomic DNA sequence from the human β -actin gene (data not shown).

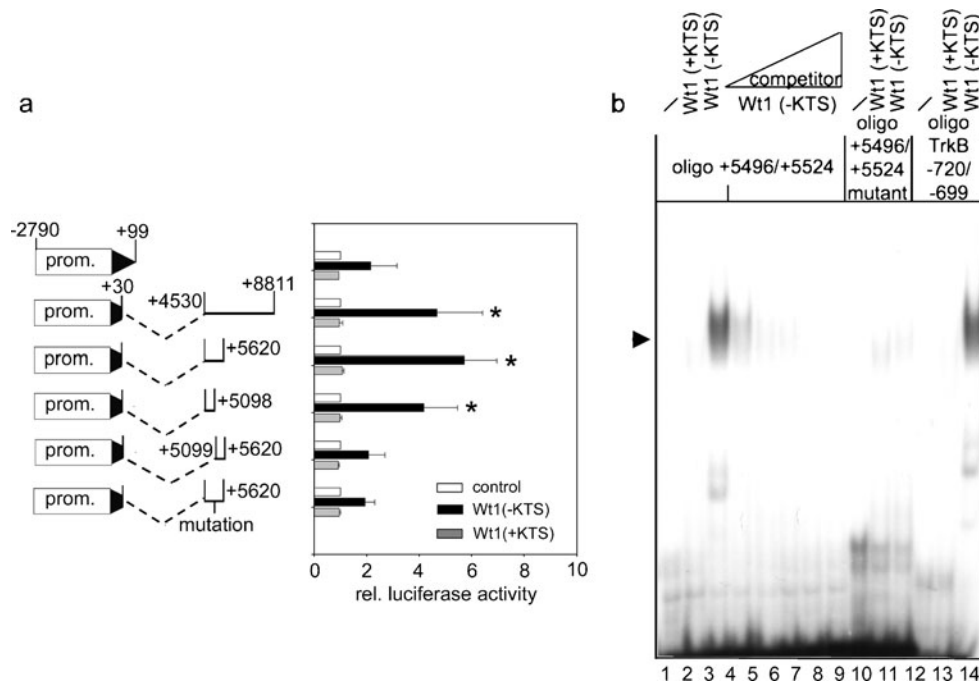
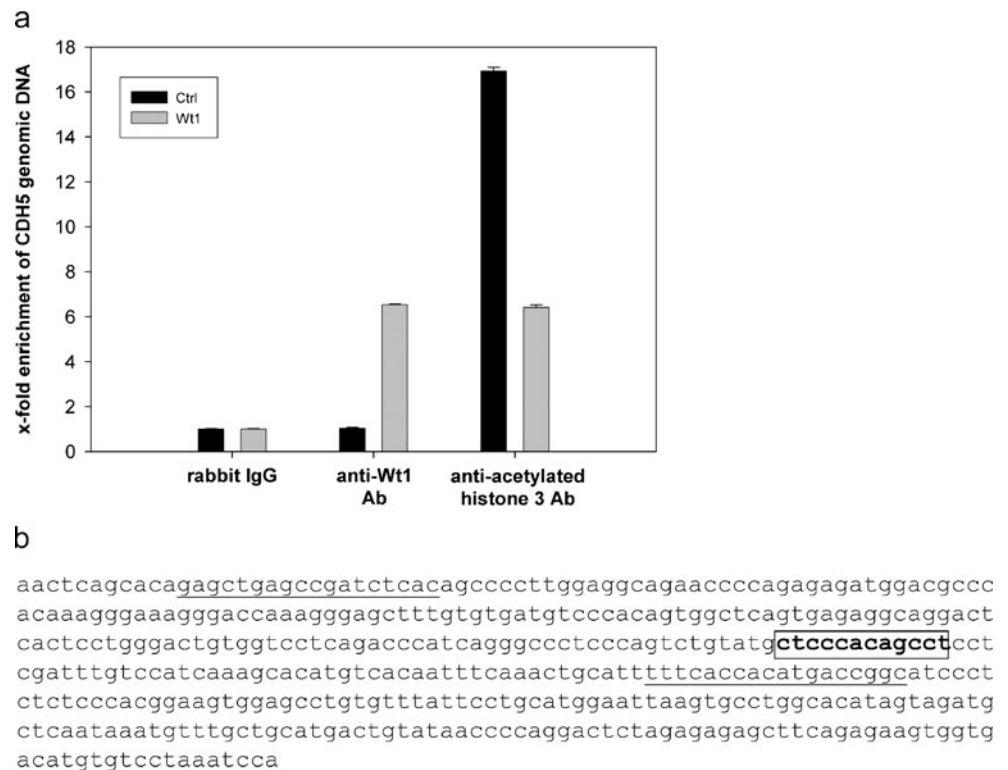


Fig. 4 Identification of a Wt1(-KTS) activated *cis*-element in the first intron of the *VE-cadherin* gene. **a** Normalised luciferase activities of the indicated reporter constructs in UB27 (black bars) and UD28 cells (grey bars) expressing Wt1(-KTS) and Wt1(+KTS) proteins, respectively. Cells were grown in the presence of tetracycline to keep Wt1 expression suppressed (open bars). The Wt1(-KTS) sensitive region could be mapped to a 569 bp fragment extending from +4530 bp to +5098 bp relative to the transcription start site in the *VE-cadherin* gene. Mutation of a predicted Wt1 binding motif resulted in loss of stimulation by the Wt1(-KTS) molecule. Values represent means \pm SD of five independent experiments, each performed in triplicate. Asterisks indicate significant differences vs. the effect of Wt1(-KTS) on a reporter construct containing the *CDH5* promoter alone. Asterisk $P<0.01$, ANOVA with Bonferroni as post hoc test. **b** Electrophoretic mobility shift assay demonstrating interaction of recombinant Wt1

(-KTS) protein with a 28 bp oligonucleotide (oligo +5,496 to +5,524 bp) in the first intron of the human *VE-cadherin* gene (lane 3). The arrowhead points to the retardation band produced by interaction of Wt1(-KTS) protein with the oligonucleotide. Wt1 (+KTS) protein, which fulfils a presumed role in RNA processing [21], exhibited much lower affinity for the oligonucleotide (lane 2). Interaction could be competed with unlabelled oligonucleotide containing the previously identified Wt1(-KTS) binding motif from the promoter of the human *NTRK2/TrkB* gene [40] (lanes 4–8). A mutation in the *VE-cadherin* oligonucleotide diminished interaction with Wt1(-KTS) protein (lanes 9–11). Note, that the same mutation prevented reporter gene activation by Wt1(-KTS) in transient transfection experiments (panel a). An oligonucleotide with the Wt1(-KTS) binding motif in the *NTRK2/TrkB* gene promoter was used as positive control (lanes 12–14)

Fig. 5 Representative chromatin immunoprecipitation (ChIP) assay with anti-Wt1 antibody demonstrating an approx. Fivefold enrichment of DNA in the first intron of the *CDH5* gene from UB27 cells with high Wt1 protein (*panel a*). *VE-cadherin* DNA could not be enriched from UB27 cells with low Wt1 expression. Normal rabbit serum and anti-acetylated histone 3 antibody were used as negative and positive controls, respectively. *Panel b* shows enhancer sequence from the first intron of the *CDH5* gene. The Wt1-responsive element is marked by the *box*, the oligonucleotides used for PCR amplification are *underlined*



Wt1 and VE-cadherin are co-expressed in glomeruli of the developing kidney

Wt1 expression is particularly high in the genitourinary system of embryos [1, 30], and inactivation of *Wt1* in mice caused a failure of normal formation of the kidneys and gonads [20]. We used a double immunofluorescent labelling technique to analyse the distribution of Wt1 and VE-cadherin expressing cells in mouse embryonic kidneys. After treatment with primary antibodies the frozen tissue sections were incubated with Cy3-coupled secondary antibody to visualise Wt1 protein (red fluorescence) and with a Cy2-conjugate to detect VE-cadherin-expressing cells (green fluorescence). In agreement with previous studies [1, 30] we localised Wt1 protein to the condensing mesenchyme and immature glomeruli of E12.5 foetal kidneys (Fig. 6a). No VE-cadherin expressing renal cells could be identified in this developmental stage (Fig. 6b). However, VE-cadherin was clearly visible in developing glomeruli of E19.5 mice (Fig. 6e and h). Notably, VE-cadherin positive glomeruli were distributed mainly in the deeper zones of embryonic kidneys reflecting a more advanced developmental state than superficial glomeruli (Fig. 6d–f). High power magnification revealed co-expression of Wt1 and VE-cadherin proteins in the developing podocytes. Expectedly, Wt1 was located in the cell nuclei whereas VE-cadherin was associated with the plasma membranes (Fig. 6g–i). In addition to the developing kidneys, an overlapping pattern of Wt1 and VE-cadherin was also seen in the epicardium

of embryonic mice (data not shown). While VE-cadherin was present in the developing vasculature, we observed no general vascular expression of Wt1 protein in murine foetal tissues (data not shown).

VE-cadherin mRNA is reduced in the livers and hearts of Wt1-deficient mouse embryos

To examine whether Wt1 is necessary for normal expression of VE-cadherin in vivo, transcript levels were measured by real-time RT-PCR in hearts, livers and urogenital ridges [18] of wild-type (*Wt1*^{+/+}), heterozygous (*Wt1*^{+/-}) and *Wt1*-deficient (*Wt1*^{-/-}) mouse embryos at E12.5 (Fig. 7). Livers, in addition to urogenital ridges and hearts were chosen, because we have previously found that Wt1 is contained in CD117⁺ haematopoietic cells from mouse foetal liver [5, 17]. Furthermore, VE-cadherin is transiently expressed by foetal liver haematopoietic stem cells [16]. Messenger RNA measurements were not performed at later stages because most *Wt1*^{-/-} embryos fail to develop beyond 13 days post conception [20]. *VE-cadherin* mRNA levels were reduced by approximately 42% and 40% in the livers and hearts, respectively, of *Wt1*-deficient embryos compared to their wild-type (*Wt1*^{+/+}) littermates (Fig. 7). No differences in *VE-cadherin* transcripts were observed in the urogenital ridges of wild-type and *Wt1*-deficient embryos (Fig. 7). *VE-cadherin* transcripts were not significantly different in all tissues of wild-type and heterozygous (*Wt1*^{+/-}) embryos (Fig. 7).

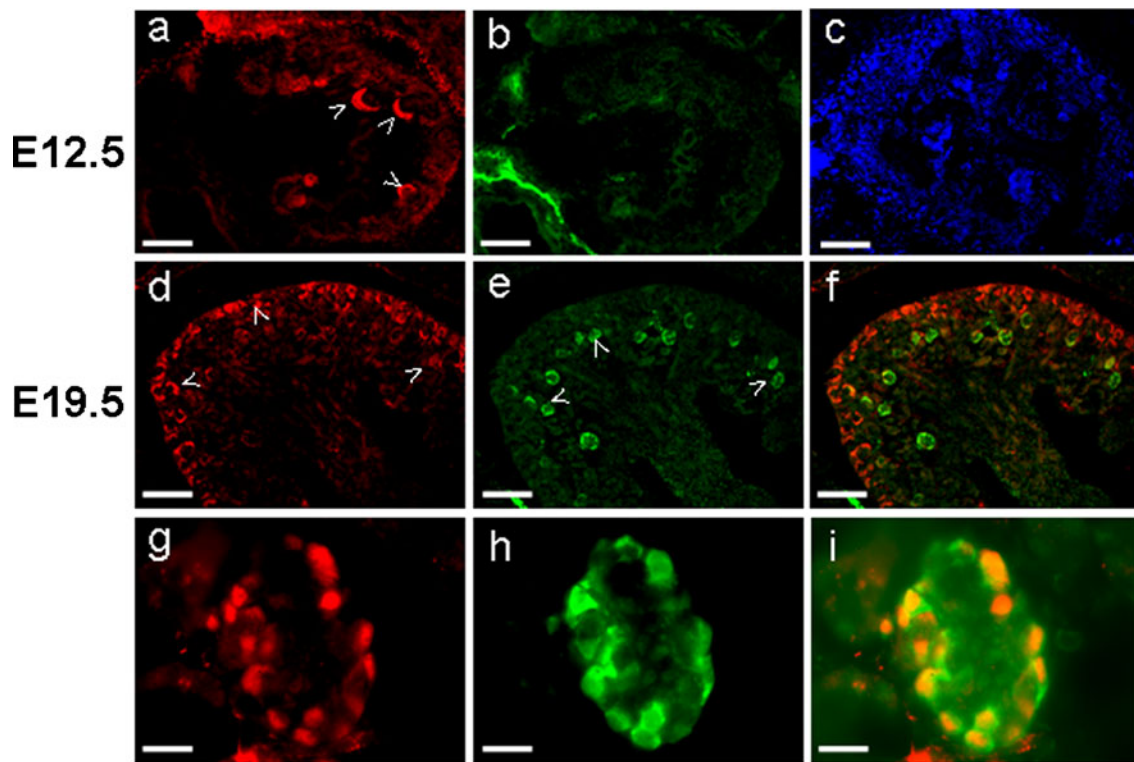


Fig. 6 Representative VE-cadherin expression analysis in mouse embryonic kidney. Double-immunofluorescence labelling of Wt1 (red) and VE-cadherin (green) proteins in frozen sections from murine kidneys at embryonic days E12.5 and E19.5. Cells with co-expression of nuclear Wt1 and membrane-bound VE-cadherin can be clearly identified in the high power images (g–i). Note, that Wt1 is strongly expressed in comma-shaped bodies at E12.5 (arrowheads in a),

whereas VE-cadherin is absent from embryonic kidneys at this early stage (b). Co-localization of Wt1 and VE-cadherin in developing glomeruli in deeper zones of embryonic kidneys at E19.5 is clearly visible (d–i). Scale bars indicate 200 μm (a–f) and 10 μm (g–i), respectively. No specific fluorescence signal was obtained with the use of normal serum instead of primary antibody (c). Nuclei in c were stained with Dapi

Discussion

The Wilms' tumour gene, *Wt1*, encodes zinc finger proteins that can function as tumour suppressors and also play important roles in the development of the genitourinary system and several other tissues [32]. The purpose of this study was to identify novel putative downstream target genes of Wt1, particularly with regard to its proposed contribution to blood vessel formation [34, 37, 38, 40]. We found that Wt1 indeed stimulates the expression of vascular genes in cells derived from osteosarcoma, an aggressive bone tumour, which is capable of vasculogenic mimicry by forming a tubular network reminiscent of blood vessels [7, 9]. The observed effects were caused by the Wt1(–KTS) molecule only, but not by the Wt1(+KTS) protein suggesting that activation of a vascular gene programme was related to the transcription regulatory function of Wt1 (Figs. 1 and 2).

Furthermore, the activity of a *CDH5* promoter reporter construct was markedly enhanced by the Wt1(–KTS) isoform in osteosarcoma cells (Fig. 3). VE-cadherin (CD144) is a member of the cadherin family of adhesion proteins that associates with intracellular catenins and allows contact formation among vascular endothelial cells [35]. VE-

cadherin is necessary for blood vessel assembly [12, 36] and plays a pivotal role in the control of vascular permeability [10]. Previous findings indicated that the promoter of the *cadherin 5* gene (*CDH5*, *VE-cadherin*) confers tissue-specific

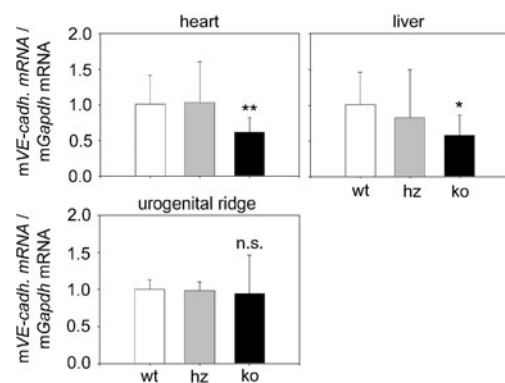


Fig. 7 *VE-cadherin* mRNA in different tissues of wild-type (wt, open bars), heterozygous (hz, grey bars) and homozygous *Wt1*-deficient (ko, black bars) embryos at E12.5. *VE-cadherin* transcripts were measured by real-time RT-PCR and normalised to *Gapdh* transcripts. Values are means \pm SD, $n=5$ each. Asterisks indicate significant differences vs. wild-type embryos. One asterisk, $P<0.05$; two asterisks, $P<0.01$, ANOVA with Bonferroni as post hoc test; n.s., not significant

(*trans*)gene expression to the vasculature [29], but robust expression of *VE-cadherin* required interaction of the 5'-flanking region with intron one sequence [15]. In this study, we identified an enhancer element in the first intron of the *CDH5* gene, which binds to the Wt1(-KTS) molecule and is required for *trans*-activation by Wt1(-KTS) (Figs. 3, 4, 5). Remarkably, the core binding motif (-CTCCACA-) of the identified sequence is identical to the previously discovered Wt1-sensitive *cis*-regulatory element in the promoter of the *NTRK2* gene, which encodes the TrkB neurotrophin receptor [40]. Activation of the TrkB receptor by its ligand, brain-derived neurotrophic factor, is a critical mechanism during blood vessel formation in the developing heart [39]. Thus, one can assume that the Wt1(-KTS) protein interacts with *cis*-elements that are conserved in the regulatory regions of genes involved in blood vessel configuration.

It is important to note that Wt1 is expressed in a rather restricted fashion in the developing organism including the kidneys and blood vessels of embryonic hearts [1, 40]. However, Wt1 is absent from the vasculature of most organs, and *Wt1*-depleted embryos exhibit no obvious vascular phenotype [20] except for a reduced blood vessel density in their hearts [40]. These observations indicate that the contribution of Wt1 to vascular organisation is confined to distinct tissues, i.e. the myocardium and possibly certain tumours [34, 37, 38, 40]. Blood vessel formation in the heart relies on a special process of EMT, which enables epicardial cells to invade the myocardium and eventually become vascular smooth muscle and endothelial cells [11]. As shown recently, Wt1 mediates EMT in epicardial cells and during embryonic stem cell differentiation, and a failure of epicardial cells to undergo this transition likely contributes to the vascular defects in *Wt1*-deficient hearts [25, 40]. Remarkably, the transcription factor Snail (*Snail*), which is a molecular downstream target of Wt1 in EMT [25], has previously been identified as direct regulator of the *CDH5* gene [22]. Furthermore, *VE-cadherin* transcripts were significantly reduced in *Wt1*-knockout compared to wild-type embryoid bodies indicating that Wt1 is necessary for normal expression of *VE-cadherin* during early embryogenesis [25]. The importance of Wt1 for *CDH5* gene regulation *in vivo* is underscored by the lower *VE-cadherin* mRNA levels in the hearts and livers of *Wt1*-deficient murine embryos compared to their wild-type and heterozygous (*Wt1*^{+/-}) littermates (Fig. 7). The latter observation may reflect a reduction of *VE-cadherin* transcripts in haematopoietic progenitor cells [16], which normally also express Wt1 [17].

Another novel observation of this study is the robust expression of *VE-cadherin* in embryonic kidneys of mice (Fig. 6). Development of the mammalian kidney depends on the interaction of the ureteric bud, an outgrowth of the primary nephrogenic duct, with the surrounding metanephric mesenchyme [6]. Mesenchyme-to-epithelial conversion in

vicinity of the newly formed ureteric bud tips is among the early events in nephrogenesis. Absence from embryonic mouse kidneys at E12.5 indicates that *VE-cadherin* is likely dispensable for the initial induction of the metanephric mesenchyme. Accordingly, no differences in *VE-cadherin* mRNA were found in the urogenital ridges of wild-type and *Wt1*-deficient embryos at E12.5 (Fig. 7). However, cellular co-expression with Wt1 suggests a possible role for *VE-cadherin* in the formation of the glomerular podocytes (Fig. 6). Podocytes are epithelial cells that cover the fenestrated capillaries of renal glomeruli and contribute to the kidney filtration barrier formed by the so-called “slit diaphragm” between their foot processes. Interestingly, a comparative promoter analysis identified *VE-cadherin* as novel slit diaphragm molecule that was co-regulated with nephrin and ZO-1, two other major components of the renal filtration barrier, in human glomerular disease [4]. Thus our findings raise the possibility that transcriptional activation of the *CDH5* gene by Wt1 contributes to intercellular contact formation between glomerular podocytes.

In summary, the results of this study indicate that Wt1 is capable of activating vascular gene transcription. Furthermore, we identified the gene encoding *VE-cadherin* (*cadherin 5*, *CDH5*) as a novel candidate target of Wt1. Absence from vascular endothelial cells in most tissues excludes Wt1 as a general regulator of *VE-cadherin* in the vasculature. Instead, stimulation of *VE-cadherin* expression by Wt1 may rather become relevant during blood vessel assembly in the embryonic heart and possibly play a role in podocyte maturation in the developing kidney.

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We declare that the experiments comply with the current laws of Germany. We also indicate that we have no conflict of interest with regard to the financial support to this study.

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