THE WILMS’ TUMOR SUPPRESSOR WT1 PROMOTES CELL ADHESION THROUGH TRANSCRIPTIONAL ACTIVATION OF THE \( \alpha_4 \) INTEGRIN GENE

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Running title: Wt1 activates transcription of the \( \alpha_4 \) integrin gene

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Cell-matrix interaction through specific adhesion molecules is a critical step during organ development. In addition, down-regulation of cell adhesion receptors may promote tumor invasion and metastasis. We show here that the Wilms’ tumor suppressor Wt1, which is necessary for normal development of the epicardium, coronary vessels, genitourinary system, and other tissues, activates transcription of the α4 integrin gene. Binding of the Wt1(-KTS) form, which is transcriptionally active, to the proximal α4 integrin promoter was demonstrated by electrophoretic mobility shift assay and chromatin immunoprecipitation. A reporter construct harboring approximately 1.9 kilobases of the human α4 integrin gene promoter was activated significantly by transient co-transfection of a Wt1(-KTS) expression plasmid. Introducing mutations in two identified Wt1(-KTS) binding motifs in the proximal promoter of the α4 integrin gene abrogated this stimulatory effect. Endogenous α4 integrin transcripts were increased more than 3-fold in human embryonic kidney (HEK) 293 cells with stable expression of the Wt1(-KTS) protein. Wt1 overexpressing cells showed augmented adhesion to the α4 integrin ligand VCAM-1, which was abolished upon incubation with an inhibitory α4 integrin antibody. Double-immunofluorescent staining revealed co-localization of Wt1 and α4 integrin in the developing epicardium of mouse embryos. Cardiac expression of α4 integrin was reduced significantly in embryos with a homozygous Wt1 defect (Wt1-/-). These findings demonstrate that Wt1 can support cell adhesion through enhanced expression of α4 integrin. This transcriptional activation of the α4 integrin gene by Wt1(-KTS) might contribute to normal formation of the epicardium and other tissues in the developing embryo.

The Wilms’ tumor gene, Wt1, encodes a zinc finger protein, which exerts a dual role as a tumor suppressor and a critical regulator of embryonic development (1-3). Inactivating mutations in the Wt1 gene on human chromosome 11p13 occur in approx. 10% of sporadic Wilms’ tumors (4), a common childhood malignancy that arises from a failure of pluripotent cells in the developing kidney to differentiate into glomeruli and tubules (5,6).

Targeted gene inactivation revealed a requirement of Wt1 for cardiac development and the formation of the genitourinary system and other tissues. Wt1-deficient mouse embryos (Wt1-/-) are lethal with a failure of normal development of the heart, kidneys, gonads (3), spleen (7), adrenal glands (8) and mesothelial structures (3,8). We have recently found that Wt1 is also necessary for the formation of neural tissues, i.e. the retina (9) and olfactory epithelium (10).

How the Wt1 suppressor fulfills its diverse roles on a molecular basis is still unclear. More than two dozens Wt1 proteins are generated by alternative mRNA splicing (11), the use of variable translation start sites (12,13), and RNA editing (14). Among the Wt1 isoforms alternatively spliced exon 5 encodes 17 amino acids, and the use of two alternative splice donor sites at the end of exon 9 results in the insertion/omission of a tripeptide (lysine-threonine-serine, KTS) in the zinc finger domain of the Wt1 protein (11). The Wt1(+KTS) forms with the tripeptide insertion have a presumed role in mRNA processing (15-18), whereas Wt1(-KTS) proteins, which lack the KTS peptide, function as transcription factors (reviewed in 19,20). A variety of Wt1 candidate target genes were identified, mainly by assessing the effect of transiently transfected Wt1 on their promoter activities. Putative downstream mediators of Wt1 include genes encoding growth factors and their receptors (21-23), transcription factor genes (24-26), genes for extracellular proteins (27,28), cell cycle regulators (29), and others (reviewed in 19). However, only very few of the proposed targets could be verified as bona fide downstream effectors of Wt1.

Another group of genes that are considered as potential Wt1 targets encode for cell adhesion molecules. Cell-cell contact formation and cell-matrix interaction through specific membrane proteins is a critical step during organ development (reviewed in 30). Moreover, down-regulation of adhesion
receptors may enable malignant cells to migrate from a primary neoplasm into the adjacent tissue and the circulatory system, thereby promoting tumor invasion and metastasis (reviewed in 31). Among the cell adhesion receptors, integrins comprise a family of \( \alpha\beta \) heterodimeric glycoproteins, which have been implicated in development and disease (reviewed in 32). Integrins that contain the \( \alpha 4 \) subunit associate with \( \beta 1 \) or \( \beta 7 \), and bind to fibronectin, a major constituent of the extracellular matrix (33,34). Vascular cell adhesion molecule-1 (VCAM-1) is another binding partner of \( \alpha 4\beta 1 \) integrin (35,36).

The requirement for \( \alpha 4 \) integrin during murine embryogenesis is indicated by the early lethality of embryos with homozygous disruption of the \( \alpha 4 \) integrin gene (37). Remarkably, defects seen in the \( \alpha 4 \) integrin-null mutants are reminiscent of the phenotype of mice with \( Wt1 \) deficiency, i.e. \( \alpha 4 \) integrin-deficient embryos – like \( Wt1 \) knockouts (3,8,38) – have an abnormal formation of the epicardium and coronary vessel system (37). Furthermore, \( \alpha 4 \) integrin and \( Wt1 \), by regulating the proliferation of progenitor cells, have both been implicated in normal hematopoiesis of murine embryos (39-41). These findings suggest that \( Wt1 \) and \( \alpha 4 \) integrin may act synergistically during critical steps of development. Our present results support a coordinated action of both molecules in epicardial formation by demonstrating that \( Wt1 \) activates transcription of the \( \alpha 4 \) integrin gene, which promotes cell adhesion.

**EXPERIMENTAL PROCEDURES**

**Animals** - A mouse breeding pair [C57BL/6 strain] with heterozygosity for the \( Wt1 \) allele (\( Wt1^{+/-} \)), was obtained from the Jackson Laboratory (Bar Harbor, ME) and genotyped by PCR (9).

**Cell culture** - Human embryonic kidney (HEK) 293 cells (accession no. ACC 305) and K562 leukemia cells (accession no. ACC 10) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The HEK293 cells were grown in Dulbecco’s modified Eagle’s medium and the K562 cells were kept in RPMI 1640 nutrient (PAA Laboratories, Pasching, Austria) each supplemented with 10% FCS (Biochrom KG, Berlin, Germany), 100 IU/ml penicillin (Invitrogen, Karlsruhe, Germany), and 100 µg/ml streptomycin (Invitrogen). Stable transfections with murine \( Wt1 \) expression constructs (\( Wt1 \) cDNA in pCB6+) and with empty vector were performed as described (42,43). Clones were selected with 300 µg/ml G418 (Invitrogen) and expanded as single colonies.

**Cell adhesion assay** - Adhesion of pCB6+- and \( Wt1 \)-transfected HEK293 cells was assayed according to our following protocol: Ninety-six well tissue culture plates were coated with 10 µg/ml of recombinant human VCAM-1 (R&D Systems, Bad Nauheim, Germany) in Dulbecco’s PBS (DPBS with 1.8 mmo/l \( Ca^{2+} \) and 1.5 mmo/l \( Mg^{2+} \)) for 1h followed by blocking with DPBS, 1% BSA (Serva, Heidelberg, Germany) for 30 min. Control plates were prepared by incubation with DPBS in the absence of VCAM-1. HEK293 cells at 60% confluence were used for the experiments. The cells were gently detached from the bottom of the tissue culture flasks with accutase™ (PAA Laboratories), washed three times with DPBS, 1% BSA, and incubated in DPBS, 1% BSA, 2 µM CellTracer (Invitrogen, Molecular Probes) for 1h. In some experiments, the cells were additionally treated with an inhibitory monoclonal anti-\( \alpha 4 \) integrin antibody (10 µg/ml, cat.# BBA37, R&D Systems, 44). After incubation, the cells were washed and seeded into 96-well plates at a density of 5 x 10⁴ cells/well in DPBS, 1% BSA. After 30 min at room temperature, the wells were washed 4-times with PBS, 0.1% BSA. Images were taken with an epifluorescence microscope (Axiovert100, Carl Zeiss, Berlin, Germany) connected to a digital camera (Spot RT Slider, Diagnostic Instruments, Sterling Heights, MI) with the Spot software (Universal Imaging Corp., Marlow Buckinghamshire, UK). At least 500 cells in 12 optical fields were randomly counted.

**Cell transfection experiments and reporter gene assays** - HEK293 cells were grown to approximately 60% confluence in 24-well tissue culture plates. One hundred ng of the reporter constructs together with 25 ng of a cytomegalovirus (CMV)-driven \( \beta \)-galactosidase plasmid, and 125 ng of expression constructs encoding different \( Wt1 \) forms were transiently co-transfected with the use of the Fugene®
reagent (1 µl per well) according to the supplier’s protocol (Roche Diagnostics). The pG2basic vector (Promega, Mannheim, Germany) and the pCB6+ plasmid were transfected for control purpose. Luciferase and β-galactosidase activities were measured in the cell lysates after 48h as described previously (9,38,45). Values are presented as relative light units normalized to β-galactosidase activities for internal control of transfection efficiencies. The results shown are averages of 5 transfection experiments each performed in duplicate.

**Alpha4 integrin promoter constructs** - A bacterial artificial chromosome (BAC) carrying the human gene for α4 integrin was obtained from the German Resource Center for Genome Research (clone ID RZPDB737G04151D, Berlin, Germany). Different sized fragments being located between -1864 bp and +1 bp relative to the transcription start site (NCBI accession no. L26059, 46,47) were amplified with the use of the Expand Long Template PCR System (Roche Diagnostics). Sequence information of the primers that were used for PCR amplification is given in Table 1. The PCR products were ligated into the SacI and HindIII sites of the pGL2basic reporter plasmid (Promega). A PCR-based strategy was applied to introduce site-directed mutations into two identified Wt1 binding elements (oligo A and oligo B) of the α4 integrin promoter (see below). The correct identity of all constructs was verified by automated dideoxy sequencing.

**Reverse transcription (RT) real-time PCR** - Total RNA was isolated from cultured cells and embryonic mouse tissues (E12.5) using the Trizol reagent (Invitrogen). The RNA pellets were dissolved in diethyl pyrocarbonate-treated H2O. First-strand cDNA synthesis was performed with 2 µg of total RNA using oligo(dT) primers and superscript II reverse transcriptase (Invitrogen). One percent of the volume of the reaction product was taken for quantitative real-time PCR amplification with Sybr® Green PCR Master Mix (Roche, Applied Biosystems) according to the following protocol: DNA denaturation at 94°C, primer annealing (10 sec) and extension of double-stranded DNA at 60°C (60 sec), measurement of Sybr® Green fluorescence at 77°C (30 sec) for 45 cycles (GeneAmp5700, Applied Biosciences, Foster City, CA). The primers that were used for real-time RT-PCR are listed in Table 1. Transcript levels were determined on the basis of differences in the C_t values as described previously (48).

**Electrophoretic mobility shift assays** - Electrophoretic mobility shift assays (EMSA) were performed with purified recombinant GST-Wt1 protein as described in detail elsewhere (45). The DNA binding reactions were carried out on ice for 15 min in 20 µl of a 1x reaction buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 50 mM NaCl, 1 mM MgCl2, 1 mM EDTA, 5 mM DTT, 5% glycerol, 0.05 mg/ml herring sperm DNA). Double-stranded oligonucleotides, which are contained within the Wt1-inducible α4 integrin promoter fragment, were selected by their sequence homology with known Wt1 consensus sites: oligoA (-263 to -238 bp): 5’-TGGCAACCTGATGGTGCGTGAAAAG-3’; oligoB (-70 to -47 bp): 5’-AGAGGAAGTGTGGGGAGGAAGAAGGAGAA-3’). Single nucleotide mutations were designed to test for the specificity of Wt1 binding: mutant oligoA: 5’-TGTCACCTGATTGTTCTTGTGAGAAAGG-3’; and mutant oligoB: 5’-GAGGAAGTGTGGGGAGGAAGTAA-3’. Competition experiments were performed with excess amounts of a non-radioactive 21-bp oligonucleotide containing the previously identified Wt1(-KTS) binding site in the vitamin D receptor gene promoter (5’-TGAACTTAGTGGGCGTGGTTG-3’) (42).

The binding reactions were run for 3h on non-denaturing 8% polyacrylamide gels at 4°C and autoradiographed.

**Chromatin immunoprecipitation (ChIP) assay** - Chromatin immunoprecipitation assay (ChIP) was done with K562 cells, which express the α4 integrin gene (49), as described (50). Antibodies (2 µg each) against acetylated histone 3 (rabbit polyclonal antibody, #06-599, Upstate, Lake Placid, NY), and Wt1 (rabbit polyclonal antibody, C-19, Santa Cruz Biotechnology) were used. Normal rabbit serum served as negative control, a 1:10 dilution of the ‘input’ sample as positive control. Following immunoprecipitation, the purified DNA was eluted in 50 µl UltraPure DNAse, RNAsel free ddH2O (Gibco/Invitrogen, #10977-015). For amplification of purified DNA fragments by PCR, 0.5 µl of the input DNA (diluted in a total
volume of 5 µl ddH\(_2\)O, 5 µl of the “normal serum” sample, and 5 µl of immunoprecipitated DNA were mixed each with primers, PCR buffer, dNTPs, and AmpliTaq DNA Polymerase (Applied Biosystems, Roche). The following primer pairs were used: human \(\alpha_4\)integrin promoter (NCBI accession no. L26059): 5'-CGTGAGCTCTAAAAGCAGGTGTCCCGT-3' (forward primer), 5'-CGCAAGCTTTGGCTATTCTCTTCAAGACC-3' (reverse primer), human \(\alpha_4\)integrin 3'-region (NCBI accession no. BC080190): 5'-TAAGAGAGCTGTGGCCGAAT-3' (forward primer), 5'-TCTGGGTTCAGGAGTTTTC-3' (reverse primer). All samples were processed in the same PCR reaction (32 cycles) at an annealing temperature of 55°C. The PCR products were electrophoresed on a 1.2% agarose gel yielding DNA fragments of 283 bp (\(\alpha_4\)integrin promoter) and 235 bp (\(\alpha_4\)integrin 3'-region), respectively.

**SDS-PAGE** -Membrane fractions were prepared from subconfluent cultures of HEK293 cells as described (51) and separated on a 10% polyacrylamide gel. The proteins (20 µg per lane) were transferred onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Freiburg, Germany) with the use of a semidry blotting apparatus (BioRad, München, Germany). After blocking at room temperature for 60 min in PBS, 5% non-fat milk (Roth, Karlsruhe, Germany), 0.05% Tween-20 (Serva, Heidelberg, Germany), incubation with one the following primary antibodies was performed in PBS, 2.5% non-fat milk, 0.05% Tween-20: polyclonal anti-\(\alpha_4\)integrin antibody from goat (C-20, cat.# sc-6589, Santa Cruz Biotechnology, 1:1,000 dilution), polyclonal anti-Wt1 antibody from rabbit (C-19, cat.# sc-846, Santa Cruz Biotechnology, 1:1,000 dilution), mouse monoclonal antibody against \(\beta\)-actin (cat.# MAB1501R, Chemicon, 1:1,000 dilution). Incubation with peroxidase-coupled secondary antibodies and visualization with the enhanced chemiluminescence system (Amersham) was performed as reported earlier (38,42,45).

**Histology and immunohistochemistry** - Morphological studies were performed as described in detail elsewhere (9,38,43). Staged embryos were fixed overnight at 4°C in paraformaldehyde (3% in PBS) and frozen in tissue-Tek O.C.T. compound (Sakura Finetek, Zoeterwoude, Netherlands). Ten µm cryostat sections were permeabilized with 0.1% Triton X-100 in PBS and blocked for 5 min at room temperature in serum-free DakoCytomation protein block (cat.# X0909, Dako, Hamburg, Germany). An indirect immunofluorescent double-labeling technique was used to mark the Wt1 and \(\alpha_4\)integrin expressing cells with the following primary antibodies (1:50 dilution in ready-to-use antibody diluent, cat.# 00-3218, Zymed, Berlin, Germany): rabbit polyclonal anti-Wt1 antibody (C-19, cat.# sc-846, Santa Cruz Biotechnology), goat polyclonal anti-\(\alpha_4\)integrin antibody (C-20, cat.# sc-6589, Santa Cruz Biotechnology). The reaction products were visualized by incubation (1.5 h at room temperature) with Cy3- and Cy2-conjugates as described previously (9,38,43). The nuclei were counterstained with 4'-6-Diamidino-2-phenylindole (Dapi). Alternatively, three µm paraffin sections from wild-type embryos (E11.5) and their Wt1-deficient littermates were stained with an immunoperoxidase technique (38). For this purpose, the slides were incubated with goat polyclonal anti-\(\alpha_4\)integrin antibody (1:50 dilutions in PBS, 0.1% Triton X-100, 3% BSA, C-20, cat.# sc-6589, Santa Cruz Biotechnology) or with goat polyclonal anti-vimentin antibody (1:50 dilutions in PBS, 0.1% Triton X-100, 3% BSA, cat.# sc-7557, Santa Cruz Biotechnology). The antigen detection was performed with a biotinylated anti-goat antibody (Vector Laboratories) followed by incubation with peroxidase-coupled Streptavidin (Sigma). The reaction was visualized with DAB substrate (Vector Laboratories, catalog no. SK-4100).

**Statistics** - ANOVA with Bonferroni-test as post-hoc calculation and students’ t-test were performed as indicated to reveal statistical significances. A \(P\)-value of less than 0.05 was considered statistically significant.

**RESULTS**

To explore whether Wt1 can activate the \(\alpha_4\)integrin gene, we took advantage of our previously established human embryonic kidney (HEK) 293 cells with stable Wt1 expression (42,43). Gene expression was measured by real-time RT-PCR in HEK293 cells with stable integration either of a Wt1(-KTS) expression plasmid or empty pCB6\(^+\) vector. Alpha4integrin
mRNA, which was normalized against GAPDH transcripts, was increased more than 3-fold in Wt1(-KTS)-expressing compared to pCB6'-transfected cells (Fig. 1A). The increase of α4integrin in the Wt1(-KTS)-expressing HEK293 cells was confirmed at the protein level using a polyclonal antibody from goat for immunoblotting (Fig. 1B). A lesser, though significant increase of β3integrin transcripts was also seen in HEK293 cells with forced expression of Wt1(-KTS) (Fig. 1A). In contrast, VCAM-1 mRNA levels were not significantly different between pCB6'- and Wt1(-KTS)-transfected cells (Fig. 1A). These findings demonstrate that forced expression of the Wt1(-KTS) protein stimulates α4integrin expression in kidney-derived HEK293 cells.

To validate the relationship between Wt1 and α4integrin and to analyze the underlying regulatory mechanism(s), we investigated whether Wt1 can activate the promoter of the α4integrin gene directly. For this purpose, a luciferase reporter construct containing the nucleotides between -1864 and +1 bp relative to the transcription start site of the human α4integrin gene was transiently co-transfected into HEK293 cells along with expression constructs encoding for two different Wt1 protein forms. These two Wt1 variants differ by the presence/absence of three amino acids (lysine-threonine-serine, KTS) in the zinc finger domain of the molecule. Compared with co-transfection of the empty pCB6' vector, reporter activity was stimulated over 3-fold by the Wt1(-KTS) expression construct, which lacks the tripeptide insertion (Fig. 2A). In contrast, the Wt1(+KTS) molecule, which has a role in mRNA processing rather than transcriptional regulation (15-18), did not activate the promoter of the α4integrin gene (Fig. 2A). To delineate the cis-element(s) necessary for activation of the α4integrin promoter by Wt1(-KTS), we generated different constructs with variable truncations and/or deletions of the original sequence (Fig. 2A). Remarkably, a promoter sequence from -283 to +1 bp relative to the transcription initiation site was stimulated more than 10-fold by the Wt1(-KTS) protein, but not by the Wt1(+KTS) variant (Fig. 2A). Luciferase activity under control of an α4integrin promoter fragment, which extended from -1864 to -263 bp, was not changed significantly by co-transfection of Wt1(-KTS) (Fig. 2A). These results suggest that the regulatory binding motif(s) for Wt1 are located between -283 and +1 bp relative to the transcription start site in the α4integrin gene. For further analysis, the GC-rich proximal α4integrin promoter region was subdivided into two shorter fragments between -283 and -171 bp, and between -104 and +1 bp, respectively. Remarkably, co-transfection of Wt1(-KTS) with either one of these constructs increased the reporter activity more than 10-fold (Fig. 2A) indicating that at least two separate elements in the α4integrin promoter can mediate the stimulatory effect of Wt1(-KTS).

A survey of the Wt1(-KTS)-responsive α4integrin promoter sequence revealed two predicted Wt1 consensus elements that were located between -263 and -238 bp (oligo A) and between -70 and -47 bp (oligo B) relative to the transcription start site (Fig. 2B). Electrophoretic mobility shift assays were performed to investigate whether these two oligonucleotides can interact with the Wt1 molecules. Specific retardation bands reflecting binding of recombinant Wt1(-KTS) protein were obtained with oligos A and B (Fig. 3). For comparison, the Wt1(+KTS) molecule had a lower affinity for oligo B and did not interact with oligo A (Fig. 3). Binding of the Wt1(-KTS) protein to oligos A and B could be competed with an unlabeled DNA, which contained the previously identified Wt1(-KTS) consensus site of the vitamin D receptor gene promoter (42). Introducing point mutations into each one of the two oligonucleotides abolished binding of the Wt1(-KTS) protein (Fig. 3). Consistent with these findings, activation of the α4integrin promoter by the Wt1(-KTS) form was abrogated with a 1.9 kb reporter construct, which harbored both mutations (mutation A and mutation B) in combination (Fig. 4A). Introducing either mutation A or mutation B alone did not abolish activation of the α4integrin promoter by Wt1(-KTS) (data not shown). These findings indicate that two cis-regulatory elements mediate the activation of the α4integrin promoter by the Wt1(-KTS) protein.

Binding of Wt1 to the α4integrin promoter was confirmed by ChIP assay in K562 leukemia cells (Fig. 4B), which are widely used to study Wt1 regulation (52, 53) and also express
the α4integrin gene (49). The specificity of Wt1 interaction with the α4integrin promoter is indicated by the lack of a PCR product when the same samples were amplified with primers for the 3′-region of the gene (Fig. 4B).

Next we explored whether enhanced α4integrin expression would increase the adhesion of Wt1-transfected HEK293 cells to VCAM-1, one natural ligand of α4integrin (35,36). For this purpose, HEK293 cells with stable expression of the Wt1(-KTS) protein were seeded on tissue culture plates that had been coated with VCAM-1. HEK293 cells with stable integration of the empty pCB6+ vector were used as a negative control. Remarkably, 87±6% of the Wt1(-KTS)-transfected cells became adherent within 30 min after seeding on the VCAM-1-coated plates (Fig. 5). For comparison, only 12±7% of the pCB6+-transfected HEK293 cells were attached after 30 min (Fig. 5). To test whether cell adhesion was mediated by interaction of α4integrin with VCAM-1, the cells were incubated with an inhibitory α4integrin antibody for 1h before seeding (44). Preincubation with this antibody reduced the fraction of adherent Wt1-transfected HEK293 cells from 87±6% to 12±7% (Fig. 5). Less than 3% of all cells, no matter whether they had been transfected with the pCB6+ plasmid or with the Wt1(-KTS) expression construct, became adherent to uncoated (without VCAM-1) tissue culture plates within the 30 min period (Fig. 5). Cell viability, which was tested with the use of the Celltracer reagent (Invitrogen, Molecular Probes), was above 95% in suspensions of both pCB6+ and Wt1(-KTS)-transfected cells. Consequently, almost 100% of the pCB6+-transfected HEK293 cells became adherent to the bottom of the tissue culture plates within 4h after seeding indicating that their general capacity to grow adherent was maintained. These findings show that forced expression of Wt1 stimulates adhesion of HEK293 cells through the binding of α4integrin to its receptor VCAM-1.

Finally we explored whether Wilms’ tumor proteins can possibly regulate α4integrin gene expression also in vivo. A double immunofluorescent technique for labeling of the Wt1 (red fluorescence, Cy3) and α4integrin expressing cells (green fluorescence, Cy2) was applied to analyze the distribution of both molecules in the developing heart of murine embryos. As reported earlier (8,54,55), Wt1 was present in the epicardium of mouse embryos at 11.5 d.p.c. (Fig. 6). High power magnification and counterstaining with Dapi revealed that Wt1 expression in epicardial cells was nuclear, whereas α4integrin could be detected in the plasma membrane (Fig. 6). In contrast, myocardial cells did not stain for Wt1 or α4integrin (Fig. 6).

To examine whether Wt1 is necessary for normal expression of α4integrin in vivo, we measured transcript levels by real-time RT-PCR in the hearts of wild-type and Wt1-deficient mouse embryos (Wt1−/−) at 12.5 d.p.c. Hearts were chosen, because α4integrin and Wt1 are co-localized in the epicardial cells (Fig. 6), and we could show recently that Wt1 is required for normal cardiac development (38). Compared to wild-type mice, α4integrin transcripts were reduced by 57% in the hearts of Wt1−/− embryos (Fig. 7A). In contrast, no significant differences in α4integrin mRNA were seen between the livers of normal and Wt1−/− embryos (Fig. 7B). Unlike the α4integrin mRNA, the transcripts for β1integrin and VCAM-1 were not significantly different between the hearts of normal and Wt1−/− mice (Fig. 7C,D) indicating that gene expression was not generally down-regulated in Wt1-deficient tissues. Similarly, endo B cytokeratin (K18), which is expressed in the epicardium but not in cardiac myocytes (56), was insignificantly reduced in Wt1-deficient vs. wild-type embryos (Fig. 7E).

Murine embryos with inactivated Wt1 gene have previously been reported to lack part of their epicardium (8). To explore whether Wt1 would be necessary for the expression of α4integrin in the remaining epicardial cells in Wt1-deficient embryos, we applied an immunoperoxidase-based staining technique. Figure 8 shows that α4integrin could be readily detected in the epicardium of wild-type embryos, but was barely seen in Wt1−/− littermates at E11.5. For comparison, immunoactivity of vimentin, which is expressed in mesenchymal cells including the immature epicardium (57), was maintained in Wt1−/− embryos (Fig. 8). These results suggest that Wt1 is a critical factor for the
normal expression of α4integrin in the developing mouse heart.

DISCUSSION

We have recently demonstrated that the Wilms' tumor suppressor Wt1, in extension to its previously recognized functions during organ development, is also required for blood vessel formation in the embryonic heart (38). In the same study, Ntrk2, the gene encoding the TrkB neurotrophin receptor, was identified as a first candidate target for the vasculogenic action of Wt1 in the myocardium (38). Enhanced transcription of Ntrk2 by Wt1 may prevent the newly formed blood vessels in the developing heart from becoming apoptotic (58). Whilst these findings for the first time established a role for Wt1 during later stages of blood vessel formation in the heart, little is known about the molecular signaling pathways of Wt1 during early steps of cardiac development. Our present findings provide circumstantial evidence that transcriptional activation of the α4integrin gene, which encodes a transmembrane cell adhesion molecule, could be a regulatory mechanism for the formation of the epicardium.

Notably, the epicardium is of critical importance for the configuration of the intramyocardial vasculature as it supplies the progenitors for vascular smooth muscle and endothelial cells as well as perivascular fibroblasts (59,60). Unraveling the transcriptional program in epicardial cells may therefore permit significant novel insights into the early steps of blood vessel formation in the heart. The epicardium is a single-layered mesothelial tissue on the outer surface of the myocardium, which originates from an extracardiac primordium, the so-called proepicardial serosa (reviewed in 61,62). Remarkably, embryos with inactivation of the α4integrin gene (α4integrin-/-), like Wt1-null mutants (3,8,38), exhibit a failure of normal formation of the epicardium and the coronary vasculature (37). An essential step in the development of the epicardium is the attachment of the progenitor cells to the outer surface of the heart. The cardiac phenotype of the α4integrin knockout embryos suggests that α4integrin is necessary for both, the initial migration of the epicardial progenitor cells and their contact formation with the myocardium (37,62). In support of this, similar epicardial defects were observed in mice lacking VCAM-1 (63), a natural ligand of α4integrin (35,36). Extending previous reports by other groups (8,64,65), our observations demonstrate that α4integrin and Wt1 are co-expressed in the developing epicardium. Unfortunately, epicardial cell lines are not available to study the relationship between Wt1 and α4integrin in a more physiological cellular context. However, since Wt1(-KTS) promotes the adhesion of cultured embryonic kidney cells by upregulating the α4integrin gene, it is intriguing that Wt1 may facilitate the attachment of proepicardial cells to the surface of the heart through a similar mechanism. Remarkably, α4integrin mRNA was reduced significantly in the hearts of Wt1-deficient vs. wild-type embryos. Furthermore, α4integrin immunoreactivity was lost in the remaining epicardial cells of the Wt1-/- mutants. While a partial defect of the epicardium (8) may contribute to the lower α4integrin content in the Wt1-/- hearts, our findings suggest that Wt1 is indeed necessary for normal expression of α4integrin in the developing epicardial cells.

The two cis-elements in the α4integrin promoter that mediate the transactivation by Wt1(-KTS) are highly homologous with previously recognized binding motifs in other Wt1 target genes (38,45,66,67). Each one of the identified regulatory elements alone was sufficient to confer the full Wt1(-KTS)-responsiveness to a heterologous α4integrin promoter-reporter construct. Accordingly, the stimulatory effect of Wt1(-KTS) on the α4integrin promoter was not significantly attenuated unless both bindings sequences (oligo A and oligo B) were mutated. These observations suggest that Wt1(-KTS) activates the α4integrin promoter with some functional redundancy, thus underlining its importance for α4integrin regulation. In general, the shorter proximal promoter pieces were more susceptible to stimulation by Wt1(-KTS) than the larger ≈1.9 kb construct. We therefore assume that additional, inhibitory elements are present in the α4integrin promoter, which are located 5' to the Wt1-responsive sites and counteract the effect of Wt1(-KTS). This structural organization permits a balanced regulation of promoter activity.
through the recruitment of trans-acting factors to their available cis-regulatory binding sites. Notably, the proximal Wt1(-KTS)-responsive sequence (oligo B) overlapped with three nearby consensus elements (GGAA/T) for Ets transcription factors. In addition to the Pax-6 gene product, which acts through another cis-regulatory site (68), Ets proteins have previously been reported to stimulate the \( \alpha_4 \) integrin promoter through binding to these elements (46). Like Pax-6 and Wt1, the Ets transcription factors are expressed in a developmental pattern and were detected in lymphatic and hematopoietic tissues in addition to vascular endothelial and smooth muscle cells (reviewed in 69). In the light of our recent findings demonstrating Wt1 in vascular endothelial and smooth muscle cells of the coronary system (38,70), it is conceivable that Wt1(-KTS) and Ets factors may act synergistically to stimulate \( \alpha_4 \) integrin expression. Moreover, transcriptional control of the \( \alpha_4 \) integrin promoter seems to fulfill a major purpose by restricting gene expression in a tissue- and developmental-specific mode.

In summary, we identified an alternative splice variant of the Wilms’ tumor suppressor Wt1 as a novel transcriptional activator of the \( \alpha_4 \) integrin gene. It is suggested that stimulation of \( \alpha_4 \) integrin expression by Wt1 may promote cell adhesion in the epicardium and possibly also in other tissues during embryonic development. Considering the proposed role of cell adhesion receptors in tumor invasion and metastasis (reviewed in 31), one can speculate whether down-regulation of \( \alpha_4 \) integrin in nephroblastoma would favor progression of the disease. More elaborate studies will be necessary to further explore the link between Wt1 and \( \alpha_4 \) integrin and its potential role in tumorigenesis.
REFERENCES


FOOTNOTES
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FIGURE LEGENDS

Figure 1
Alpha4-, β1integrin, and VCAM-1 in human embryonic kidney (HEK) 293 cells with stable expression of the Wt1(-KTS) protein relative to “empty” pCB6’ vector-transfected cells. The mRNAs were quantified by reverse transcription real-time PCR and normalized to GAPDH transcripts (A). Asterisks indicate significant differences between Wt1- and pCB6’-transfected cells (students’ t-test with P<0.001, n=7). Increased expression of α4integrin in the Wt1-transfected cells was confirmed by immunoblotting with specific antibodies (B).

Figure 2
(A) Effect of Wt1(+/-KTS) proteins on the transcriptional activity of α4integrin promoter reporter constructs. The different luciferase reporter vectors were transiently co-transfected into HEK293 cells along with expression plasmids for the Wt1(-KTS) and Wt1(+KTS) proteins. The luciferase activities were normalized to β-galactosidase in each sample. Values are means ± S.E.M. of 5 transfection experiments each performed in duplicate. Asterisks indicate significant differences vs. the respective pCB6’ vector controls (students’ t-test, P<0.001). The Wt1(-KTS)-responsive element(s) could be mapped to two distinct regions between -283 and -171 bp, and between -104 and +1 bp relative to the transcription start site in the α4integrin promoter.
(B) DNA sequence of the 5’-flanking region of the human α4integrin gene (NCBI no. L26059). The two Wt1 binding motifs (Wt1(oligo A) and Wt1 (oligo B)) are indicated in bold. The major transcriptional start site is designated “+1” (according to ref. 46).

Figure 3
(A) Electrophoretic mobility shift assay (EMSA) demonstrating binding of Wt1(+/-KTS) proteins to two predicted oligonucleotides (Oligo A and Oligo B) in the proximal α4integrin promoter. Introducing single base-pair mutations into each one of the two oligonucleotides abrogated physical interaction with the Wt1 proteins. Binding of the Wt1(-KTS) molecule to the α4integrin promoter sequence could be competed by incubation with the previously identified Wt1-consensus motif from the vitamin D receptor promoter (42), which was used at 1-, 10- and 100-fold molar concentrations.
(B) Sequences of oligonucleotides that were used for the gel shift experiments.

Figure 4
(A) Luciferase reporter assay demonstrating that the two identified Wt1-binding sites can mediate activation of the α4integrin promoter by the Wt1(-KTS) protein. The indicated ≈1.9 kb reporter constructs were transiently co-transfected into HEK293 cells along with Wt1(-KTS) and Wt1(+KTS) expression plasmids. Shown are luciferase activities that were normalized to β-galactosidase in each sample. Note, that introducing single base-pair mutations in oligos A and B (see Fig. 3B) abrogated stimulation of the α4integrin promoter by Wt1(-KTS). Values are means ± S.E.M. of 5 experiments each performed in duplicate. Asterisks indicate significant differences vs. the respective pCB6’ vector controls (students’ t-test, P<0.001).
(B) Chromatin-immunoprecipitation (ChIP) assay demonstrating binding of Wt1 protein to the α4integrin promoter in K562 leukemia cells. PCR-amplified products of the immunoprecipitates were electrophoresed in a 1.5% agarose gel and stained with ethidium bromide; for better visualization the gel photograph is presented as negative of the original. Apparently, Wt1 binds to the 5’-promoter, but not to the 3’-region of the α4integrin gene. Input DNA (1:10 dilution) and immunoprecipitates obtained with acetylated histone 3 antibody (Acetyl. H3) served as positive controls. Additional negative control experiments were performed with normal rabbit serum instead of specific antibodies.
Figure 5
Adhesion of human embryonic kidney (HEK) 293 cells with forced expression of the Wt1(-KTS) protein and with stable integration of the pCB6+ plasmid. Suspensions of single HEK293 cells were seeded at a density of 5 x 10^4 cells/well either in untreated 96-well plates or in dishes that had been coated with the α4integrin ligand VCAM-1. Thirty minutes after seeding the adherent cells were identified by their characteristic flat shapes (arrows in the micrographs). More than 500 cells in 12 optical fields were counted under the microscope. Note, that stable transfection of Wt1(-KTS) enhanced cell adhesion to VCAM-1-coated plates, and this effect was reversed upon pre-incubation with an inhibitory anti-α4integrin antibody (VCAM-1 + α4integrin Ab). Marks indicate significant differences (P<0.001, ANOVA with Bonferroni test as post-hoc calculation) in cell adhesion between the Wt1(-KTS)- and pCB6+-transfected cells (*), and between cells that had been treated or not with an inhibitory anti-α4integrin antibody (#). Values are means±S.E.M. of 3 experiments, each performed in duplicate.

Figure 6
Double-immunofluorescent labeling of α4integrin (green, Cy2-conjugate) and Wt1 (red, Cy3-conjugate) in the heart of a mouse embryo at 11.5 d.p.c.. The images are representative for the more than 20 tissue sections that were analyzed from 4 different animals. Co-localization of both proteins is evident in the developing epicardium, but not in myocardial cells, which contain neither Wt1 nor α4integrin at a detectable level. The nuclei were counterstained in blue with Dapi in all panels except one.

Figure 7
Expression analysis of α4-, β1integrin, endo B cytokeratin (K18), and VCAM-1 in the hearts and livers of wild-type (Wt1+/+) and Wt1-deficient murine embryos (Wt1−) at E12.5. The transcripts were measured by real-time RT-PCR and normalized to GAPDH mRNA in each sample. The number of embryos that were analyzed is indicated at the bottom of each bar. Asterisks indicate significant differences in Wt1− vs. normal (Wt1+/+) embryos. A P-value of less than 0.05 (P<0.05) was considered statistically significant (students’ t-test).

Figure 8
Representative immunoperoxidase staining of α4integrin and vimentin in the heart of a wild-type (Wt1+/+) and a Wt1-deficient (Wt1−) murine embryo at E11.5. Alpha4 integrin can be readily seen in epicardial cells of the normal embryo (a, c), but is hardly detectable in the Wt1− mutant (e, g). Note, that the mesenchymal protein, vimentin, which is expressed in the epicardium of the normal embryo (b, d), was also present in the remaining epicardial cells of the Wt1− mutant (f, arrows in h). No staining was obtained with the use of normal goat serum instead of specific antibodies (not shown). Scale bars indicate 50 μm.
Figure 1
Figure 2

A

\[ \alpha_4 \text{Integrin promoter} \]

\[
\begin{align*}
-1864 & \quad \text{luc} \quad +1 \\
-1864 & \quad \text{ } \\
-263 & \quad \text{ } \\
-283 & \quad +1 \\
-283-171 & \quad \text{ } \\
-104 & \quad +1
\end{align*}
\]

\[ \text{fold increase of luciferase activity} \]

\[ \square \text{control} \quad \blacksquare \text{Wt1 (-KTS)} \quad \textsf{Wt1 (+KTS)} \]

\[ \star \]

B

\[
\begin{align*}
-290 & \quad \text{CCACACTGGGTGTTACACCCCTAAAGCAGGTGTGCCCCGG} \\
-260 & \quad \text{TGGCAACTGAGGTTGCGTAAAGG} \\
-180 & \quad \text{GAATTCATCAAATACAGCTGGG} \\
-100 & \quad \text{CAAT-Box} \\
-80 & \quad \text{CCAACCTCTCTTGTGGGTCAAGCCACAGCCCTAAACCCGCTGCGTTCAAGCTGAGGGCTCTTAGC} \\
+1 & \quad \text{Wt1 (oligo A)} \\
\end{align*}
\]

\[
\begin{align*}
-290 & \quad \text{CTGTCACGCTGACCGCCGCTGCAAAGGAGGATTGGGTGCT} \\
-260 & \quad \text{TATA-Box} \\
-180 & \quad \text{GAGATGTGGTCTTGAAGGAATAGCCGTTAACGTCT} \\
+1 & \quad \text{Wt1 (oligo B)}
\end{align*}
\]
A

Oligonucleotides used for the EMSA experiments:

- oligoA: 5’-TGGCAACTGAGTGCGTGAAAAG-3’ (-263 to -238 bp)
- mutant oligoA: 5’-TGTCAACTGAGTGCGTGAAAAG-3’
- oligoB: 5’-AGAGGAAGTGTGGGGAGGAAGGAA-3’ (-70 to -47 bp)
- mutant oligoB: 5’-AGAGGAAGTGTGGGGAGGAAGGAA-3’
- competitor: 5’-TGAACTTAGTGCGTGTTG-3’

B

Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Table 1: Single-stranded DNA oligonucleotides used for PCR amplification of the human \(\alpha_4\)-integrin gene promoter (NCBI accession no. L26059).

<table>
<thead>
<tr>
<th>promoter region</th>
<th>oligonucleotide sequence</th>
</tr>
</thead>
</table>
| -1864 to +1 bp  | forward: 5'-CGTGAGCTCGAGGTAATGTAGGGTGGCTAT-3'  
                  reverse: 5'-CGCAAGCTTACGGGACACACCTGCTTTTA-3' |
| -1864 to -263 bp| forward: 5'-CGTGAGCTCAGAATCAAGTCACAGTGCTTCC-3'  
                  reverse: 5'-CGCAAGCTTACGGGACACACCTGCTTTTA-3' |
| -283 to +1 bp   | forward: 5'-CGTGAGCTCTAAAAGCAGGTGTGTCCCGT-3'  
                  reverse: 5'-CGCAAGCTTACGGGACACACCTGCTTTTA-3' |
| -283 to -171 bp | forward: 5'-CGTGAGCTCTAAAAGCAGGTGTGTCCCGT-3'  
                  reverse: 5'-GGCAAGCTTGGAAGCACTGTGACTTGATTC-3' |
| -104 to +1 bp   | forward: 5'-TACGAGCTCTGCTAGCTAGCTCACGCACAT-3'  
                  reverse: 5'-CGCAAGCTTACGGGACACACCTGCTTTTA-3' |

Single-stranded DNA oligonucleotides used for RT-PCR amplification of gene transcripts

<table>
<thead>
<tr>
<th>gene</th>
<th>NCBI accession no.</th>
<th>oligonucleotide sequence</th>
</tr>
</thead>
</table>
| mouse \(\alpha_4\)-integrin | BC068313          | forward: 5'-GGATCATCGTACTGGACTGGC-3'  
                                reverse: 5'-GAGCTCCAACAAAGGAGATCTG-3' |
| mouse \(\beta_1\)-integrin | NM_010578         | forward: 5'-GGTGTCGTGTTTGTGAATGC-3'  
                                reverse: 5'-CTCCTGTGCACACGTCTT-3' |
| mouse VCAM-1          | NM_011693          | forward: 5'-TGACCTGTCTGCAAAGGAGACAC-3'  
                                reverse: 5'-TCAATGGTGGGGATGAAGGTC-3' |
| mouse endo B cytokeratin (K18) | M11686            | forward: 5'-AGAACAGGAGAGACCTGAGAGCAC-3'  
                                reverse: 5'-TGCTTTGTGGGGATGAAGGTC-3' |
| mouse GAPDH          | BC083149          | forward: 5'-ACGACCCTTTCATTTGCAGCT-3'  
                                reverse: 5'-TTTGGCTCCACCCCTCAAGTG-3' |
| human \(\alpha_4\)-integrin | X16983            | forward: 5'-CTCGCCAACGTTCAGTGACT-3'  
                                reverse: 5'-TCGTAAATCGAGGGGACTCC-3' |
| human \(\beta_1\)-integrin | NM_002211         | forward: 5'-ATGAAGGGCAGTGGTTGTAAG-3'  
                                reverse: 5'-CGTTGTGGGCTTCCAGAATT-3' |
| human VCAM-1         | NM_080682         | forward: 5'-CCATTTGACACGGGACTGAGAT-3'  
                                reverse: 5'-TTCCTTGGCAGCTTTGGAAGTG-3' |
| human \(\beta\)-actin | NM_001101         | forward: 5'-AGAAAATCTGACACCCACACC-3'  
                                reverse: 5'-GCCATCTCTAGCTCGAAGGT-3' |
The Wilms' tumor suppressor WT1 promotes cell adhesion through transcriptional activation of the α4 integrin gene

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