Versican Mediates Mesenchymal–Epithelial Transition[□]

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Versican is a large extracellular chondroitin sulfate proteoglycan that belongs to the family of lecticans. Alternative splicing of versican generates at least four isoforms named V0, V1, V2, and V3. We show here that ectopic expression of versican V1 isoform induced mesenchymal–epithelial transition (MET) in NIH3T3 fibroblasts, and inhibition of endogenous versican expression abolished the MET in metanephric mesenchyme. MET in NIH3T3 cells was demonstrated by morphological changes and dramatic alterations in both membrane and cytoskeleton architecture. Molecular analysis showed that V1 promoted a “switch” in cadherin expression from N- to E-cadherin, resulting in epithelial specific adhesion junctions. V1 expression reduced vimentin levels and induced expression of occludin, an epithelial-specific marker, resulting in polarization of V1-transfected cells. Furthermore, an MSP (methylation-specific PCR) assay showed that N-cadherin expression was suppressed through methylation of its DNA promoter. Exogenous expression of N-cadherin in V1-transfected cells reversed V1's effect on cell aggregation. Reduction of E-cadherin expression by Snail transfection and siRNA targeting E-cadherin abolished V1-induced morphological alteration. Transfection of an siRNA construct targeting versican also reversed the changed morphology induced by V1 expression. Silencing of endogenous versican prevented MET of metanephric mesenchyme. Taken together, our results demonstrate the involvement of versican in MET: expression of versican is sufficient to induce MET in NIH3T3 fibroblasts and reduction of versican expression decreased MET in metanephric mesenchyme.

INTRODUCTION

Versican is a large chondroitin sulfate proteoglycan and belongs to the family of lecticans (Aspberg *et al.*, 1997; Yamaguchi, 2000). It was initially identified in cultured fibroblasts (Zimmermann and Ruoslahti, 1989) and its chicken homologue (called PG-M) was isolated from developing limb buds (Shinomura *et al.*, 1993). Versican possesses two globular domains, the N-terminal (G1 domain) and the C-terminal (G3 domain), separated by a glycosaminoglycan (GAG) attachment region. The G3 domain is composed of two epidermal growth factor (EGF)-like repeats, a lectin-like motif, and a complement binding protein (CBP)-like motif. The chondroitin sulfate (CS)-attachment domains present in the middle region are divided into two alternatively spliced domains named CS α and CS β . Alternative splicing of versican generates at least four versican isoforms: V0, V1, V2, and V3 (Ito *et al.*, 1995; Zako *et al.*, 1997; Lemire *et al.*, 1999). Different numbers of CS chains are observed in the V0, V1,

and V2 versican isoforms. CS chains are absent in the versican V3 isoform. The G1 domain of versican binds hyaluronan (LeBaron *et al.*, 1992), whereas the G3 domain not only interacts with extracellular matrix (ECM) proteins (Zheng *et al.*, 2004b; Wu *et al.*, 2005b), but also interacts with sulfated glycolipids (Miura *et al.*, 1999). Interaction of versican with ECM and cell surface proteins is believed to provide structural integrity to tissues and regulate cell proliferation and differentiation.

Tissue distribution of versican has been studied, but physiological and pathological roles of versican remain unclear. Two expression constructs for chicken versican isoforms, V1 and V2, were recently generated in our laboratory and adopted to study their biological effects on mediating cell activities. In our previous studies, we have shown that the V1 isoform displayed an ability to enhance cell growth, whereas the V2 isoform exerted an inhibitory effect (Sheng *et al.*, 2005). Molecular analyses indicated that V1 up-regulates EGF receptor (EGFR) expression and activates its downstream signaling pathway, but V2 has an opposing effect. This suggests that EGFR and its downstream signaling pathway are involved in V1- and V2-mediated cell proliferation. We have also demonstrated that V1 and V2 are able to regulate the expression of EGFR in PC12 cells (Wu *et al.*, 2004b). Our results have provided evidence that the CS β domain and the CS α domain exert different effects on the EGFR signaling pathway. The opposite functions of V1 and V2 on cell proliferation reveal that a dynamically balanced expression pattern of these two isoforms may provide a suitable extracellular environment for normal proliferation and survival of cells.

Earlier studies have shown that versican V1/V0 and V2 have complementary expression patterns (Bandtlow and

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Abbreviations used: MET, mesenchymal–epithelial transition; G3, selectinlike domain; CS, chondroitin sulfate; GAG, glycosaminoglycan; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FGF, fibroblast growth factor; LIF, leukemia inhibitory factor; TGF, transforming growth factor; CBP, complement binding protein; siRNA, small interfering RNA.

Zimmermann, 2000). While versican V1/V0 is mainly expressed in the late stages of embryonic development (Landolt *et al.*, 1995), versican V2 becomes a major chondroitin sulfate proteoglycan in the mature brain (Schmalfeldt *et al.*, 1998). These observations suggest that different versican isoforms may play distinct functions in morphogenesis and tissue development. We demonstrate here that versican V1 induces mesenchymal–epithelial conversion in NIH3T3 cells through regulation of the expression of cadherin family proteins, resulting in a “switch” in expression from N- to E-cadherin. Furthermore, we demonstrate that versican is essential in MET of metanephric mesenchyme.

MATERIALS AND METHODS

Materials and Cell Culture

DMEM, fetal bovine serum (FBS), Hanks' balanced salt solution (HBSS), trypsin/EDTA, Lipofectamine, and geneticin (G418) were purchased from Invitrogen (Burlington, Ontario, Canada). Tissue culture plates were purchased from Sarstedt (Montreal, Quebec, Canada). ECL Western blot detection kit was from Amersham Life Science (Baie d'Urfé, Quebec, Canada); horseradish peroxidase–conjugated goat anti-mouse IgG secondary antibody, oligonucleotides, and all chemicals were purchased from Sigma (Oakville, Ontario, Canada). Antibodies used are as follows: anti-E-cadherin, anti-N-cadherin, anti-p120 (all from Transduction Laboratories, Oakville, Ontario, Canada), anti- β catenin (c-18; Santa Cruz Biotechnology, Santa Cruz, CA), anti-occludin (Zymed Laboratories, Toronto, Ontario, Canada), anti- β actin (Sigma) and anti-vimentin (BD PharMingen, Oakville, Ontario, Canada), Texas red dye–conjugated donkey anti-goat IgG antibody (Jackson ImmunoResearch Laboratories, Etobicoke, Ontario, Canada), and FITC-conjugated anti-mouse IgG antibody (Sigma). Chicken N-cadherin expression plasmid (pMiwcN) was kindly provided by Dr. M. Takeichi (Kyoto University, Japan). Mouse E-cadherin expression plasmid (pBATEM2) was kindly provided by Dr. S. Dufour (Institute Curie, France). Mouse snail expression plasmid (pcDNA3-Snail) was kindly provided by Dr. A. Cano (Instituto de Investigaciones Biomédicas, Spain). NIH3T3 fibroblasts were from the American Type Culture Collection (Rockville, MD). RIMM-18 and RUB1 cell lines were kindly provided by Dr. A. Perantoni (National Institutes of Health, Maryland). RIMM-18 cells were grown in Ham's F12/DMEM 1:1 medium (Invitrogen, Mississauga, Ontario, Canada) with 5% FBS and 10 ng/ml FGF2 and 100 nmol/l estradiol. RUB1 cells were grown in Ham's F12/DMEM 1:1 medium with 5% FBS. Epithelial conversion of RIMM-18 cells was carried out by removing estradiol and serum from the culture medium and by addition of cytokines and growth factors known to be inductive for tubule formation in primary mesenchymal explants, including 50–100 ng/ml human fibroblast growth factor 2 (FGF2; R&D Systems, Hornby, Ontario, Canada), 30–50 ng/ml mouse leukemia inhibitory factor (LIF; Sigma), 20 ng/ml human transforming growth factor (TGF; Sigma), and 0.1–6 ng/ml human TGF- β 2 (Sigma). Conditioned medium was prepared from confluent cultures of RUB1 cells as described previously (Plisov *et al.*, 2001).

Construct Generation and Expression

To study the effect of versican on mediating cell activities, we used two constructs: versican V1 and versican V2, the structures of which have been previously described (Wu *et al.*, 2004b; Sheng *et al.*, 2005).

To generate three constructs expressing small interfering RNA (siRNA) targeting mouse E-cadherin, three target sequences (nucleotides 1060–1079, ggcgaagcttgagcaca; nucleotides 1236–1255, agctgtgtacaccgtatg; and nucleotides 2010–1029, gctcgcgataaccagaac) were selected and inserted into the pSuper plasmid according to the manufacturer's instructions. After DNA sequencing, three constructs containing correct inserts, named Ecad-siRNA-1, Ecad-siRNA-2, and Ecad-siRNA-3, were obtained. To examine the efficiency of these siRNA constructs, COS-7 cells were cotransfected with the recombinant E-cadherin expression construct and one of the siRNA construct or the control vector pSuper. Cell lysate was prepared and analyzed on Western blot probed with anti-E-cadherin antibody to estimate the silencing efficiency. The same membrane was reprobed with anti-mouse β -actin antibody to ensure equal loading. After exposure, the films were overlapped for picture taking. One of them, the Ecad-siRNA-1 construct, was used for silencing experiments by cotransfection with the pcDNA3.1/Hygro plasmid in V1-transfected NIH3T3 fibroblasts.

To generate siRNA constructs targeting the V1 construct expressed in NIH3T3 fibroblasts, several target sequences (including nucleotides 5337–5355, gctgacatgactgtctt; nucleotides 9151–9170, gaggttagttctgatgtg; and nucleotides 10789–10808, cactacacgtcggtgatca) of chicken versican was inserted into the pSuper plasmid according to the manufacturer's instructions. After DNA sequencing, the silencing effects of these siRNA constructs were analyzed. These three constructs were shown to greatly reduce versican expression. One of them, containing the target sequence, nucleotides 5337–5355

(V1-siRNA), was used for stable expression in the V1-transfected NIH3T3 fibroblasts.

Similarly, three constructs were generated to silence endogenous rat versican expression, using the sequences 5'-cagcaaatgtcagtagac (Ratver3263), 5'-gcagtc-aaggagacagcat (Ratver4369), and 5'-gtgctgtgctaattgaag (Ratver5616). The construct (Ratver5616) producing the best silencing effect was used to generate stable cell lines in RIMM-18 cells.

NIH3T3 fibroblasts were stably transfected with versican V1 and V2 expression constructs, the E-cadherin expression construct, or a control vector pcDNA3. The transfected cells and parental NIH3T3 were grown in DMEM with or without G418 in the presence of 10% FBS, 100 IU/ml penicillin, and 100 μ g/ml streptomycin at 37°C in a humidified CO₂ atmosphere. The V1-expressing cells were also stably transfected with versican V1 siRNA (V1-siRNA), snail expression construct, or N-cadherin expression construct, and RIMM-18 cells were transfected with siRNA construct Ratver5616. For these studies, cotransfection was carried out with vector pcDNA3.1/Hygro using Lipofectamine Plus, according to the manufacturer's instructions. Stable transfectants were selected with hygromycin B at 0.3 mg/ml. Cotransfected cell lines were cultured in media with both neomycin and hygromycin for functional studies.

Migration Assay

In wound healing experiments, cells were seeded at 70% confluence on tissue culture plates in DMEM supplemented with 10% FBS. Twenty-four hours after cell inoculation, confluent monolayers were wounded linearly by scraping with p1000 pipette tips, washed to remove cell debris, and refilled with fresh media. Cells were fixed in 3.7% paraformaldehyde at the indicated time intervals and photographed with a phase-contrast microscope.

Immunofluorescence and Western Blot

Cells cultured on chambered slides were fixed in 3.7% paraformaldehyde at 37°C for 30 min, permeabilized by incubation for 15 min with 0.1% Triton X-100, and blocked with 1% BSA in phosphate-buffered saline (PBS). They were then immunostained with primary antibody diluted in blocking solution at room temperature for 1 h. Cells were rinsed three times with PBS and incubated with secondary antibody coupled with Texas red and FITC for 45 min. Slides were then rinsed three times with PBS before mounting. Actin fibers were stained by incubation with TRITC-labeled phalloidin. Preparations were visualized using a Carl Zeiss confocal microscope (Göttingen, Germany). Western blots were carried out as previously described (Wu *et al.*, 2004a; Wu *et al.*, 2005a).

RT-PCR and PCR

Cells (2.5×10^6) were harvested, and total RNA was extracted with Qiagen's RNeasy Mini Kit (Santa Clarita, CA) according to the manufacturer's instructions. RT-PCR assays were performed as previously described (Yang *et al.*, 2003; Zheng *et al.*, 2004a). Briefly, 2 μ g of total RNA was used to synthesize cDNA by reverse transcription, a portion of which (equal to 0.2 μ g of RNA) was used in a PCR with two appropriate primers. PCR products were visualized through agarose gel electrophoresis using ethidium bromide staining. The primers used are: mo-E-cadherin2401, 5' tggatgccgacggagtgact and mo-E-cadherinC, 5'-ctagctgtctctgcaccgccta for E-cadherin; mo-N-cadherin2761F, 5'-acgagagcctatc-catgctgac and mo-N-cadherinC, 5'-tcagtctgaccaccgcctacat for N-cadherin; mo-Occludin1441F, 5'-tctctcgaggagctggaggagac and mo-OccludinC, 5'-ctaagg-ttccgtctgtcatatgc for occludin; mSnailNBamHI, 5'-cccgatccccgcctctctcgtg-caggag and mSnailCXbaI, 5'-ccctctagagcgggctcctggagcagcaga for snail; mo-actin121F, 5'-ccggcatgtgcaagccggtctgc and mo-actin-360R, 5'-gctcattgtagaag-gtctgtgtgc for β -actin (nucleotides 121–360); 5'-gagcaagacacagagact and 5'-tgtctctcttcttcaggt for the CRD motif of versican. To detect rat versican isoforms expressed in RIMM-18 cells, the following primers were used: ratverCSaF (5'-catcttatcagggtgtgtgcaatgacac) and ratverCS β R (5'-ctctctttagattctgaatctatgg) for rat versican V0 isoform; ratverG1F (5'-gctctcgatgcagcgtgcggcacc) and ratverCS β R for V1 isoform; ratverCSaF and ratverG3R (5'-ggctcattccga-caagggttagatgtg) for V2 isoform; and ratverG1F and ratverG3R for V3 isoform. Genomic DNA was extracted from cells by phenol-chloroform treatment and ethanol precipitation, followed by PCR amplification of the integrated plasmid DNA using two primers (5'-caggaaatcgaacgtgacgt and 5'-gagggtatcgataagcttttcaaaaa). To analyze silencing of versican, RT-PCR was performed using two primers ratverG3N (5'-ggacctgtatctctgcaaaaacaaacca) and ratverG3C (5'-gcgc-ctgttctctgcacctccggt).

Methylation-Specific PCR (MSP) Assay

Total DNA from the V1-transfected cells was extracted by phenol-chloroform before ethanol precipitation. DNA (1 μ g) was denatured using 0.2 M NaOH and modified with 3 M sodium bisulfate and 10 mM hydroquinone at 50°C for 16 h according to Herman *et al.* (1996). Modified DNA was purified using the Wizard DNA purification resin and eluted into 50 μ l of water. Bisulfate-treated DNA was used as a PCR template and amplified with specific primer pairs for methylated DNA (sense: 5'-tgccaggttcggcgatttcg and anti-sense: 5'-aacgcgaaatcgactctga), and for unmethylated DNA (sense: 5'-tgtgagttgtgtgatttcg and anti-sense: 5'-aacacaaaatcaaataccaa). Untreated DNA was ampli-

Figure 1. Exogenous expression of the versican V1 isoform induces cell aggregation, morphological changes, and a cadherin “switch.” (A) A leading peptide (LP) was tagged at the N-terminal of both V1 and V2 constructs. The V1 isoform contains the CSβ domain and two globular domains, G1 and G3. Replacing the CSβ domain with the CSα domain produced the V2 construct. (B) Expression of V1 and V2 was illustrated by immunofluorescence using the mAb 4B6, which recognizes an epitope in the leading peptide. Vector-transfected cells were used as a negative control. Images were captured using a confocal microscope (scale bar, 25 μm). (C) Vector-, V1-, and V2-transfected cells were examined under a light microscope. Morphological changes were observed in the V1-transfected cell lines (scale bar, 150 μm). (D) Total cell lysate (40 μg total protein) from each cell line was analyzed on Western blot probed with monoclonal antibodies against N- and E-cadherin. β-actin was used as a loading control. Transcriptional expression of N- and E-cadherin was detected by RT-PCR. Total RNA was extracted from each of the transfectants. After treatment with DNase 1, cDNA was synthesized from RNA and amplified by PCR using the primers described in *Materials and Methods*. PCR products were analyzed on agarose gel electrophoresis. Up-regulation of E-cadherin and down-regulation of N-cadherin were detected in the V1-transfected cells. (E) The vector-, V1-, and V2-transfected cells were immunostained for N- and E-cadherin. Expression and distribution of N- and E-cadherin were analyzed with a fluorescence confocal microscope (scale bar, 25 μm).

fied by PCR using primers (5'-tccgcccgcagctccgagcccg and 5'-cagcgcggagtcgctccgg). The PCR mixture contained 1× buffer with 1.5 mM MgCl₂, 40 pM of each primer, 0.2 mM dNTPs, and 2.5 U HotStarTaq DNA polymerase. The PCR conditions were as follows: denaturing cycle at 95°C for 10 min; amplification cycles (35) at 94°C for 30 s, 55°C for 45 s, and 72°C for 1 min; and extension cycle at 72°C for 10 min. PCR products were separated in 1.5% agarose gel electrophoresis and visualized with ethidium bromide staining.

RESULTS

Versican V1 Induces an Epithelial-like Morphological Conversion and a “Switch” of Cadherin Expression

The expression constructs of the versican V1 and V2 isoforms, which contain an N-terminal leading peptide (LP) recognized by the monoclonal antibody (mAb) 4B6, were generated as described previously (Figure 1A; Sheng *et al.*, 2005). The constructs and a control vector were stably expressed in NIH3T3 fibroblasts. Expression and secretion of V1 and V2 has been previously demonstrated using Western blotting and ELISA (Sheng *et al.*, 2005). In the present study, expression of V1 and V2 in NIH3T3 fibroblasts was analyzed by immunostaining (Figure 1B). After cell line selection, an obvious morphological change was observed in all V1-trans-

fected NIH3T3 cells, whereas the vector- and V2-transfected cells maintained the mesenchymal morphology of the parental cells (Figure 1C). The V1-transfected cells aggregated and formed epithelial islands with an increased capacity for adhesion to the tissue culture plates. Twelve V1-transfected cell lines collected from transfection of two individual V1 expression constructs after G418 selection showed identical morphological changes (unpublished data).

The morphological changes in the V1-transfected cells implied an alteration in intracellular adhesion. To investigate the mechanism by which V1-mediated cell adhesion and morphological change, we analyzed the expression of two cell adhesion-related molecules, N- and E-cadherin, with both Western blotting and RT-PCR. Our data showed that N-cadherin expression was detected in parental NIH3T3 cells as well as in the vector-transfected cells and three individual V2-transfected cell lines. Surprisingly, N-cadherin expression was suppressed and E-cadherin expression was transactivated in the V1-transfected cells. Three V1-transfected clones showed identical E-cadherin transactivation by either Western blotting or RT-PCR (Figure 1D).

Expression and subcellular distribution of N- and E-cadherin were examined by immunostaining. Strong staining of N-cadherin was detected at cell–cell contact sites in the vector- and V2-transfected cells. By contrast, E-cadherin was identified only in the V1-transfected cells and showed a prominent distribution on the cell membrane (Figure 1E). The results of RT-PCR, Western blotting, and immunofluorescence staining were consistent, and they suggest that V1 is able to induce a switch of cadherin expression, from N- to E-cadherin.

Versican Induces Epithelial-Type Adhesive and Tight Junctions and Cytoskeletal Rearrangement

N- and E-cadherin are both classical cadherins (Yagi and Takeichi, 2000). N-cadherin is preferentially expressed in mesenchymal-type cells, such as fibroblasts, whereas E-cadherin is predominant in epithelial cells, and is involved in the formation and maintenance of epithelial structures. Classical cadherins are single-span transmembrane domain glycoproteins, which mediate Ca^{2+} -dependent homophilic interactions through their extracellular domains. The intracellular domains of classical cadherins interact with β -catenin and p120^{cas} (hereafter p120) to form cytoplasmic cell adhesion complexes, which are linked to the actin cytoskeleton through α -catenin. The linkage between cadherins and the actin cytoskeleton is critical for cadherin-mediated cell–cell adhesion.

After confirming that versican V1 induced a conversion from N- to E-cadherin expression in NIH3T3 cells, we further characterized the formation of the cadherin-catenin adhesive complex in transfectants by examining two cadherin-binding proteins, β -catenin and p120. The expression of both β -catenin and p120 was detected in parental NIH3T3 fibroblasts and in the V1-, V2-, and vector-transfected cells by Western blotting (Figure 2A).

Subcellular distribution of β -catenin and p120 was further examined by immunofluorescence staining. The staining pattern showed that the β -catenin signal not only localized in the cytoplasm but was also found at the sites of cell–cell contacts in vector- and V2-transfected cells (Figure 2B), in which N-cadherin is present. By contrast, β -catenin was displaced and localized extensively at the boundary of cell–cell contacts in V1-transfected cells, in which E-cadherin is expressed. p120 signals are apparently localized to the cytoplasm in the vector- and V2-transfected cells, and no extra signal was detected at sites of cell–cell contact. By contrast, p120 was prominently recruited from the cytoplasm to cell–cell junction sites, forming a boundary between cells in the V1-transfected cells (Figure 2B). These results suggest that versican V1, but not V2, regulates subcellular distribution of β -catenin and p120. Similarly, F-actin was localized to cytoplasmic stress fibers in the vector- and V2-transfected cells, but it was redistributed to the cell membrane in the V1-transfected cells. The colocalization of E-cadherin, β -catenin, p120, and F-actin on the cell membrane and at the boundary of cell contacts indicates that V1-transfected cells formed functional E-cadherin-based epithelial-type adhesive junctions.

Morphological changes and establishment of epithelial-type junctions in V1-transfected cells led us to examine whether these cells were losing mesenchymal properties. We tested the expression of vimentin, a mesenchymal protein that is normally expressed by NIH3T3 cells. Western blot analysis showed that the expression of vimentin was reduced in the V1-transfected cells compared with that of the parental NIH3T3 cells as well as in the vector- and V2-transfected cells. Three distinct V1-transfected clones showed identical reduction in vimentin expression (Figure

Figure 2. Versican V1 induces epithelial-type adhesion junctions. (A) Cell lysate was analyzed on Western blot probed with antibodies against β -catenin, p120, and β -actin. Little difference was observed between the cell lines tested. (B) Subcellular distribution of β -catenin and p120 was illustrated by immunofluorescence. F-actin localization was assessed by rhodamine-phalloidin staining. Images were captured by confocal microscope. Relocalization of β -catenin and p120 was detected in the V1-transfected cells (scale bar, 25 μm).

3A). The presence of vimentin in cells was also examined using immunofluorescence staining. In agreement with the Western blot, stronger vimentin signals were observed in the cytoplasm in the vector- and V2-transfected cells, and weaker signals were detected in the V1-transfected cells (Figure 3C).

One hallmark of epithelial cells is their apical-basolateral polarity. We further determined whether epithelial phenotypic V1-transfected cells developed apical-basolateral polarity by characterizing the distribution of the tight junction-related protein, occludin, which is known to be a reliable epithelial marker (Tsukita and Furuse, 1999). Western blot and RT-PCR detected occludin expression in the V1-transfected cells (Figure 3B). Two bands were detected in the V1-transfected cells on Western blot, corresponding to high- and low-molecular-weight occludin, as reported previously (Wong, 1997). In contrast, this protein was not found in parental NIH3T3 cells or in the vector- or V2-transfected cells. The expression of occludin in the V1-transfected cells was further confirmed by immunofluorescence staining. Occludin staining signals were specifically detected in the cytoplasm and at sites of cell–cell contact in the V1-transfected

Figure 3. V1 regulates expression and distribution of vimentin and occludin. (A) The expression of vimentin was assessed by Western blotting. β -actin expression was used for the loading control. (B) The expression of occludin in the V1-transfected cells was visualized by Western blot and RT-PCR. The NIH3T3 cell line served as the negative control. Two signal bands were detected in the V1-transfected cells on Western blot using mAb against occludin, corresponding to the high and low molecular weights of occludin. Positive responses for occludin expression were only observed in V1-transfected cells by RT-PCR. (C) Subconfluent monolayer cultures of transfectants were immunostained for vimentin and occludin (scale bar, 25 μ m).

cells, while no staining was observed in the vector- or V2-transfected cells (Figure 3C), consistent with the results obtained by Western blot and RT-PCR. This data confirmed that ectopic expression of versican V1 in NIH3T3 cells resulted in the formation of tight junctions and the acquisition of apical-basolateral cell polarity. Epithelial-like morphological changes and establishment of epithelial-type adhesion and tight junctions in the V1-transfected cells suggest that the versican V1 isoform induces mesenchymal-epithelial transition (MET) in NIH3T3 fibroblasts.

Suppression of N-Cadherin Is Essential in V1-mediated Morphological Changes and Cell Transition

We next attempted to determine the mechanism of V1 action. Because the expression of N-cadherin was completely suppressed by versican V1, we examined the methylation of the N-cadherin promoter by PCR as described (Herman *et*

Figure 4. Versican V1 induces methylation of the N-cadherin promoter. Purified DNA was first modified by sodium bisulfate treatment. Both bisulfate-treated and untreated DNA were then subjected to PCR amplification with specific primer pairs for methylation detection. The methylated state was detected in V1-transfected cells, whereas the DNA samples from the V2- and vector-transfected cells as well as the parental NIH3T3 fibroblasts exhibited an unmethylated N-cadherin promoter. Normal DNA was amplified by PCR for the DNA extraction control.

al., 1996). Total DNA extracted from parental NIH3T3 cells and all transfectants was modified by sodium bisulfate. Treated DNA was used as the PCR template and amplified using primers specifically designed for methylated and unmethylated DNA amplification. The experiments indicated that the N-cadherin promoter was methylated in the V1-transfected cells, but the methylation was not detected in the parental, vector-, or V2-transfected NIH3T3 cells (Figure 4). This suggests that versican V1 suppressed the expression of N-cadherin by inducing methylation of its DNA promoter.

To corroborate this finding, we reexpressed N-cadherin exogenously in the V1-transfected cells. An N-cadherin expression construct was cotransfected with the pcDNA3.1/hygro plasmid, or pcDNA3.1/hygro alone as control, into V1-expressing cells. Stable cell lines were selected by hygromycin at 0.3 mg/ml. The expression of N-cadherin in transfected cells was confirmed by Western blot (Figure 5A, top). However, complementary expression of N-cadherin in the V1-transfected cells had no effect on E-cadherin expression (Figure 5A, bottom). Examination of cell morphology indicated that pcDNA3.1/hygro transfection had no effect on cell morphology. However, exogenous N-cadherin expression induced cell dissociation, as the cells no longer formed cell islands (Figure 5B). These results suggest that exogenous expression of N-cadherin prevented V1-mediated cell-cell adhesion and aggregation. Moreover, monolayer cultures showed phenotypic epithelial-to-mesenchymal reversion of N-cadherin-expressing cells. Reversion to the mesenchymal phenotype was revealed by cell morphological changes from a cuboidal form to a more elongated stellate morphology. Suppression of N-cadherin expression by versican V1 was therefore necessary for V1-mediated morphological changes and cell transition.

The expression of N-cadherin and the distribution of β -catenin, p120, and E-cadherin in the V1-transfected cells were examined by immunofluorescence. As expected, the expression of exogenous N-cadherin was visualized at sites of cell-cell contact (Figure 5C). The subcellular localization of β -catenin was not affected by transfection of the control vector in the V1-transfected cells, in which β -catenin was primarily localized at the cell membrane at cell contact sites. By contrast, ectopic expression of N-cadherin in the V1-

Figure 5. Exogenous expression of N-cadherin destroyed V1-mediated cell aggregation and induced redistribution of β -catenin and p120. (A) The V1-transfected cells were stably transfected with mouse N-cadherin or a control vector. After selection of stably expressing cell lines, the expression of exogenous N-cadherin in V1 cells was analyzed by Western blotting. Parental NIH3T3 cells and V1 cells served as positive and negative controls, respectively. E-cadherin expression was analyzed in the N-cadherin-transfected cells by Western blot. The V1-transfected cells and NIH3T3 cells served as positive and negative controls, respectively. Little change in E-cadherin expression was detected (bottom). (B) Exogenous expression of N-cadherin induced dissociation of V1-transfected cells, but vector transfection had no effect on cell morphology (scale bar, 150 μ m). (C) The expression of exogenous N-cadherin in transfected cells was visualized by immunofluorescence. The vector-transfected V1 cells were adopted as negative controls. Immunofluorescence images were captured by confocal microscope. Exogenous expression of N-cadherin induced redistribution of β -catenin, p120, and E-cadherin (scale bar, 25 μ m).

transfected cells triggered a relocation of β -catenin. Staining signals for β -catenin were identified not only at sites of cell contact, but also in cell nuclei (Figure 5C). A dramatic movement of p120 from the cell membrane at cell contact sites to the cytoplasm was also observed, whereas E-cadherin was predominantly visualized on the cell membranes (Figure 5C). Relocation of β -catenin and p120 from cell contact sites suggests that N-cadherin obliterated E-cadherin-mediated cell adhesion junctions in the V1-transfected cells.

Cadherin family proteins modulate cell motility and migration. Because versican V1 induced a conversion of expression from N- to E-cadherin in NIH3T3 cells, we decided to examine the mobility of the versican-transfected cells. As shown in Figure 6, within 24 h, the vector- and V2-trans-

fected cells were able to migrate into empty surface areas at a rate greater than that caused by proliferation, whereas the V1-transfected cells migrated into the wound areas by proliferation-directed forward movement (Figure 6). These observations suggest that V1 decreased cell motility and migration, but that V2 had no effect. To determine whether motility changes were linked with cadherin conversion, the N-cadherin-transfected V1 cells were also assessed by migration assays. These experiments showed that N-cadherin expression promoted cell motility and migration (Figure 6), suggesting that cadherin conversion was involved in the V1-mediated motility decrease. It is plausible that V1 reduced the motility rate by inhibiting N-cadherin expression. Taken together, ectopic expression of N-cadherin induced

Figure 6. N-cadherin is involved in the V1-mediated reduction of cell motility and migration. Cells were seeded at 70% confluence in DMEM supplemented with 10% serum and grown to confluence. Confluent monolayers were wounded by scraping with micropipette tips, washed to remove cell debris, and refilled with fresh media. Cells were fixed with 3.7% paraformaldehyde at the indicated time intervals and photographed with a phase-contrast microscope.

Figure 7. Exogenous expression of E-cadherin in V1-transfected cells failed to induce an epithelial-like morphological conversion. (A) A mouse E-cadherin construct was stably expressed in NIH3T3 cells. After cell line selection, protein lysate was analyzed on Western blot for E-cadherin expression. Parental NIH3T3 cells and vector-transfected cells were used as negative controls (top). N-cadherin expression was analyzed in the E-cadherin-transfected NIH3T3 cells by Western blot. NIH3T3 cells and the V1-transfected cells were used as positive and negative controls, respectively. No change in N-cadherin levels was observed (bottom). (B) E-cadherin- and vector-transfected NIH3T3 cells were immunostained to determine E-cadherin localization. Staining patterns were visualized with a confocal microscope. As expected, staining signals of E-cadherin were stronger at sites of cell contact. Little signal was found in vector-transfected cells (scale bar, 25 μ m). (C) The monolayer culture of E-cadherin- and V1-transfected cells was photographed with a phase-contrast microscope. Although V1-transfected cells displayed an epithelial-like morphology, E-cadherin-transfected cells exhibited a fibroblastlike morphology (scale bar, 150 μ m).

cell dissociation in the V1-transfected cells probably by promoting cell motility and migration.

Activation of E-Cadherin Is Indispensable to V1-mediated Cell Changes and Cell Transition

Transactivation of E-cadherin was found in the V1-transfected cells, in which functional E-cadherin-based epithelial-type adhesion junctions were also identified. To determine whether V1 induced cell morphological conversion through activation of E-cadherin, we expressed E-cadherin in NIH3T3 cells. The cells were cotransfected with pcDNA3 and a mouse E-cadherin expression construct or with pcDNA3 alone as control, followed by neomycin selection. After confirming the expression of E-cadherin (Figure 7A, top), we detected no inhibitory effect of E-cadherin transfection on N-cadherin expression (Figure 7A, bottom). Subcellular distribution was examined by immu-

nofluorescence staining. As expected, E-cadherin signals were detected prominently at sites of cell contact (Figure 7B). However, extensive expression of E-cadherin was insufficient to drive NIH3T3 cells to the V1-transfected cell morphology (Figure 7C). These experiments suggest that versican V1 does not induce morphological conversion simply through transactivation of E-cadherin expression.

We then examined whether E-cadherin activation is linked with V1-mediated cell property changes. It is known that Snail is able to down-regulate E-cadherin expression (Cano *et al.*, 2000). The E-cadherin-expressing V1-transfected cells were cotransfected with the pcDNA3.1/hygro plasmid and a Snail expression construct, or with pcDNA3.1/hygro plasmid alone. Snail expression was confirmed by RT-PCR (Figure 8A). Three different Snail-positive clones were analyzed for E-cadherin expression by RT-PCR (Figure 8A) and Western blot (Figure 8B), and the reduction in E-cadherin expression was semiquantified using densitometry (Figure 8C). Interestingly, inhibition of E-cadherin expression by Snail transfection facilitated rescue of mesenchymal morphology in the V1-transfected cells (Figure 8D). These results suggest the V1-induced cell aggregation and adhesion occurred through an E-cadherin-mediated pathway, as down-regulation of E-cadherin expression abolished V1-mediated changes.

To corroborate the role of E-cadherin in mediating V1 activity, we further specifically silenced E-cadherin by using siRNA. Three siRNA constructs targeting different sequences of mouse E-cadherin were generated. All siRNA constructs greatly reduced mouse E-cadherin expression (Supplementary Data 1A). One of the siRNA constructs, Ecad-siRNA-1, was stably introduced into the V1-transfected cells to silence endogenous E-cadherin expression. After cell line selection, inhibition of E-cadherin expression was analyzed by RT-PCR (Supplementary Data 1B) and Western blot (Supplementary Data 1C). E-cadherin silencing resulted in translocation of β -catenin and p120 from the cell membrane at cell-cell contact sites to the cytoplasm (Supplementary Data 2A). The typical V1-mediated islandlike growth formation was prevented after E-cadherin silencing. Instead, siRNA-transfected cells were loosely associated and had an elongated fibroblastlike appearance (Supplementary Data 2B). These results indicate that E-cadherin was essential for the maintenance of V1-mediated changes in cell morphology.

Silencing of V1 Expression Reverses Its Effects on Cell Morphological Change and Cell Transition

To further elucidate the direct involvement of the versican V1 isoform in cell morphological change and cell transition, we used siRNA to reduce V1 expression in V1-transfected NIH3T3 cells. One construct (V1-siRNA), containing a target sequence against the CS β domain, was stably expressed in the V1-transfected cells. After cell line selection, silencing of V1 was analyzed by RT-PCR. Two typical cell lines (V1-siRNA-1 and V1-siRNA-2) revealed significant V1 mRNA down-regulation through semiquantitative RT-PCR analysis (Figure 9A). We then examined the expression of E- and N-cadherin. Immunoblot analysis demonstrated that the expression of E-cadherin was down-regulated in V1-siRNA-transfected cells compared with vector pSuper-transfected cells (Figure 9B). Although V1 silencing induced a dramatically decreased E-cadherin expression, the expression of N-cadherin was detected only in the vector-transfected NIH3T3 cells. These data suggest that the expression of E-cadherin in our experiment is V1-dependent.

Figure 8. Expression of Snail in the V1-transfected cells inhibits E-cadherin expression and induces cell dissociation. (A) The expression construct of Snail was cotransfected with pcDNA3.1/hygro in the V1-expressing cells. Stably transfected cell lines were selected with hygromycin at 0.3 mg/ml. After confirming Snail expression by RT-PCR, three clones were examined for E-cadherin expression. Vector-transfected cells served as a negative control. Inhibition of E-cadherin expression was seen in the Snail-transfected V1-expressing cells. Actin amplification was used as a control for the assay system. (B) The expression of E-cadherin in Snail-transfected V1-expressing cells was compared with that in vector-transfected V1-expressing cells on Western blot. Reduced expression of E-cadherin was found in all of the three Snail-transfected cell lines, but not in vector-transfected cells. (C) The intensities of RT-PCR and Western blot bands were semiquantified using a densitometer. (D) Cell morphology was examined under a light microscope. Snail expression induced dissociation of V1-transfected cells (scale bar, 150 μ m).

Immunofluorescence staining confirmed the disappearance of E-cadherin in the V1-siRNA-transfected cells, which further led to the translocation of β -catenin and p120 to the cytoplasm (Figure 9C). Further, V1 silencing resulted in cell dissociation and morphological reversion; phenotypic mesenchymal reversion was revealed by shape changes, which reverted to elongated fibroblast morphology (Figure 9D). Our results confirm a key role for E-cadherin in the V1-mediated transition from mesenchymal- to epithelial-type cells.

Inhibition of Endogenous Versican Expression by siRNA Prevents Conversion and Survival of Metanephric Mesenchyme

MET takes place during kidney development. Metanephron formation begins with the outgrowth and invasion of the ureteric bud into mesenchymal metanephric blastema. The ureteric bud induces the metanephric mesenchyme to condense and convert into nephron epithelial cells. Our exper-

Figure 9. Reduction in V1 function using siRNA. (A) V1-expressing cells were stably cotransfected with pcDNA3.1/hygro, and V1-siRNA or pSuper (V1-pSuper). Hygromycin-resistant cell lines were selected. Two cell lines (V1-siRNA-1 and V1-siRNA-2) were used to analyze V1 expression by RT-PCR. Vector-transfected NIH3T3 fibroblasts (vector) served as a negative control. β -actin served as an experimental control. (B) The expression of N- and E-cadherin in the above cell lines was analyzed on Western blot. The expression of β -actin served as a loading control. (C) The expression and distribution of E-cadherin, β -catenin, and p120 were examined by immunostaining in the pSuper- and siRNA-transfected V1-expressing cells (scale bar, 25 μ m). (D) Cell morphology was examined. Transfection of V1-siRNA facilitated dissociation of the grouped cells compared with pSuper-transfection (scale bar, 150 μ m).

Figure 10. Reduction of endogenous versican expression by siRNA prevents conversion and survival of metanephric mesenchyme. (A) Expression of versican in RIMM-18 and RUB1 cells was assayed by RT-PCR (RT). Purified mRNA (mRNA) was also used as a template serving as a negative control. Versican was not detected in RUB1 cells, whereas RIMM-18 cells express versican isoforms V0, V1, and V3 (left panel). Versican silencing was carried out by stable expression of three siRNA constructs (Ratversi3263, Ratversi4369, and Ratversi5616). Three cell lines showed significant down-regulation of versican expression (right panel). (B) RIMM-18 cells, vector-transfected (RIMM-V), and Ratver5616-transfected (Ratver5616) cells were analyzed for cell differentiation after inductive stimulation. Cell condensation appeared in parental and vector-transfected RIMM-18 cells, but not in the Ratver5616-transfected cells (left). The number of cells was much lower in the Ratver5616-transfected RIMM-18 cells (siRNA, right). White bar, inoculated cell number; gray bar, cell number at the end point. (C) Seeded at lower density, RIMM-18 cells could undergo condensational conversion, but Ratver5616-transfected cells failed to do so (left). Under inductive conditions, the numbers of RIMM-18 cells increased slowly, but that of Ratver5616-transfected RIMM-18 cells (siRNA) apparently diminished (right). White bar, inoculated cell number; gray bar, cell number at the end point. Immunofluorescent staining displayed E-cadherin-positive response in the condensed areas at high (D) and low (E) cell densities.

iments demonstrate that exogenous expression of the versican V1 isoform in mesenchymal cells induces MET. We further investigated whether versican plays roles in the process of MET in metanephric mesenchyme. We initially examined the expression of versican in RIMM-18 and RUB1 cells by RT-PCR. Versican was not detected in RUB1 cells, whereas RIMM-18 cells express versican isoforms V0, V1, and V3 (Figure 10A, left panel). It has been shown that RIMM-18 cells can be converted into epithelium by induction with several cytokines in combination with conditioned medium from RUB1 cultures (Levashova *et al.*, 2003).

To investigate the effect of versican in epithelial conversion of metanephric mesenchyme, we silenced the expression of versican with Ratver5616 before induction. After cell line selection, silencing of versican was confirmed by semiquantitative RT-PCR analysis. Three cell lines (Ratver5616-1, -2, and -3) showed significant down-regulation of versican expression (Figure 10A, right panel). RIMM-18, vector- and Ratver5616-

transfected cells were further analyzed for cell conversion after inductive stimulation by cytokines and growth factors in combination with conditioned medium from RUB1 cells. Cell condensation (positive for γ -glutamyl transpeptidase, a marker for proximal tubular epithelia) appeared in parental and vector-transfected RIMM-18 cells, but not in the Ratver5616-transfected cells (Figure 10B, left panel). A dramatic diminution in cell number was observed in Ratver5616-transfected RIMM-18 cells (Figure 10B). We therefore questioned whether failure to form condensed cell heaps in Ratver5616-transfected RIMM-18 cells resulted from its low cell density. To address this question, RIMM-18- and Ratver5616-transfected cells were plated at low cell density for inductive treatment. Although parental RIMM-18 cells were able to form condensed cell heaps at low cell density, no condensed heaps were observed in Ratver5616-transfected cells (Figure 10C, left panel). The numbers of RIMM-18 cells increased slowly in inductive medium, whereas the numbers of Ratver5616-transfected RIMM-18 cells de-

creased (Figure 10C, right panel). Immunofluorescent staining displayed E-cadherin-positive response in the condensed areas, which were seen only in parental and vector-transfected RIMM-18 cells at high (Figure 10D) and low (Figure 10E) cell densities. These results revealed that versican expression is closely linked to the condensation of metanephric mesenchyme: silencing of versican expression prevented conversion and survival of the cells.

DISCUSSION

Our studies have demonstrated that ectopic expression of the versican isoform V1 in NIH3T3 fibroblasts induces cell aggregation and remarkable cell morphological changes. We further showed that V1 was able to reduce cell migration and mediate mesenchymal-epithelial conversion. In addition, silencing of versican expression using siRNA prevented the condensation, conversion, and survival of metanephric mesenchyme. In our transfection experiments, molecular analyses showed that the V1-induced changes in cell behavior were linked with changes in the expression of adhesion molecules. Our experiments demonstrate that N-cadherin expression was completely suppressed in the V1-transfected cells, whereas E-cadherin expression was transactivated. Thus, V1 was able to induce a cadherin switch from N- to E-cadherin in NIH3T3 cells. Further data confirmed that the inactivation of N-cadherin and transactivation of E-cadherin were both involved in V1's effects on NIH3T3 cells. The involvement of N-cadherin in V1's effects was shown by the complementary expression of exogenous N-cadherin in the V1-transfected cells, which destroyed V1-mediated changes in cell behavior. Immunofluorescence showed that N-cadherin expression triggered redistribution of β -catenin and p120 and increased cell migration, resulting in disruption of cell aggregation and dissociation of the V1-transfected cells. To corroborate the role of E-cadherin in mediating V1 actions, specific silencing of E-cadherin expression by transfecting either Snail or siRNA targeting E-cadherin prevented the formation of adhesion junctions, resulting in disruption of the aggregation and adhesion of the V1-transfected cells. Transactivation of E-cadherin was therefore essential for the maintenance of the V1-mediated cell aggregation and morphological features.

A prominent epithelioid phenotype was observed in the V1-transfected cells. Further examination of these cells by Western blot and immunofluorescence staining demonstrated that the V1-transfected cells formed a functional adhesion junction mediated by E-cadherin, which is characterized by the arrangement of the cytoskeleton, and by colocalization of E-cadherin with β -catenin and p120 at sites of cell contact (Wheelock and Johnson, 2003). E-cadherin is an epithelial-specific cadherin, whereas N-cadherin is the major mesenchymal cadherin in NIH3T3 fibroblasts. The conversion from N- to E-cadherin revealed that the V1-transfected cells developed intercellular adhesion junctions that are highly characteristic of epithelial cell types. This induction of markers of epithelial differentiation in the V1-transfected cells was consistent with the results of the morphological alterations, indicating that differentiation toward an epithelial cell type was modulated by alterations at the molecular level. Intracellular cytoskeletal proteins and tight junction-related proteins serve as good markers for cell transition, because mesenchymal and epithelial cells express different patterns of these proteins. The expression of vimentin, a mesenchymal intracellular intermediate filament protein, decreased in the V1-transfected cells, which correlates with the transition that was found.

In addition to E-cadherin, the expression of occludin was also induced in the V1-transfected cells. Occludin is an integral membrane protein of tight junctions and can be detected in tight junction strands by immunolabeling freeze-fracture replicas (Fujimoto, 1995). Of all tight junction proteins, occludin is the most ubiquitously expressed at apical-basolateral membranes, and is considered the most reliable immunohistochemical marker for tight junctions (Tsukita and Furuse, 1999). Both lower- and higher-molecular-weight forms of occludin were detected by Western blot in the V1-transfected cells. The higher-molecular-weight band is a hyperphosphorylated form of occludin (Wong, 1997). Phosphorylation of occludin allows it to be recruited from the plasma membrane and intracellular vesicles, and stabilizes occludin assembly at tight junctions (Wong, 1997). The phosphorylation and distribution of occludin on the cell membrane implies the formation of tight junctions in the V1-transfected cells. Tight junctions play a fundamental role during the development of cell surface polarity, which is a hallmark of epithelial cells (Rodriguez-Boulant and Powell, 1992). Thus, the presence of E-cadherin and occludin provides evidence for a mesenchymal-epithelial transition in the V1-transfected NIH3T3 cells.

Versican is a large chondroitin sulfate proteoglycan identified as one of the major extracellular molecules in the prechondrogenic mesenchymal condensation area (Kimata *et al.*, 1986; Shinomura *et al.*, 1990). Four different isoforms of versican have been found, which are determined by alternate splicing. Although all the isoforms share identical N- and C-terminal domains, different splicing of the CS domains with differing numbers of GAG chains, and different tissue distributions of these isoforms indicate that they may have distinct biological functions. Indeed, in our previous studies, ectopic expression of chicken V1 and V2 in NIH3T3 fibroblasts and neuron cells demonstrated that these two versican isoforms have different effects on cell proliferation, apoptosis, and differentiation (Wu *et al.*, 2004b; Sheng *et al.*, 2005). It is expected that the background of V1 isoform expression in NIH3T3 fibroblasts is low, as it has been reported that skin expresses very low levels of V1 (Cattaruzza *et al.*, 2002). This has allowed us to demonstrate that ectopic expression of V1 in NIH3T3 cells induces cell aggregation and a mesenchymal-epithelial transition, whereas V2 had little effect on cell morphology and property changes. Mesenchymal condensation has been found at the early steps of chondrogenesis and osteogenesis and in organ formation such as kidney development (Thesleff *et al.*, 1995). During embryo nephrogenesis and somitogenesis, mesenchymal condensation also serves as an initial step in mesenchymal-epithelial transition, which is critical for vertebrate organogenesis. In mammalian kidney development, the metanephric mesenchyme condenses at the tips of ureteric buds and undergoes a mesenchymal-epithelial transition (Horster *et al.*, 1999). Our experiments show that versican is expressed in metanephric mesenchyme, but not in ureteric bud cells. Embryonic metanephric mesenchyme could be converted into nephron epithelium. Condensed areas are positive for E-cadherin and γ -glutamyl transpeptidase, markers for proximal tubular epithelia. This model reflects the real conversion process during kidney development, which is divided into two stages: the inductive step, when metanephric mesenchyme condenses at the tips of ureteric buds in response to inducing signals, and the morphogenetic step, when epithelial conversion takes place. Our observation that exogenous expression of the versican V1 isoform in NIH3T3 cells induced cell condensation and MET led us to investigate whether versican was involved in the MET of metanephric mesenchymes. Metaneph-

ric mesenchymes failed to condense and no significant E-cadherin activation was visualized in these cells, when expression of endogenous versican was inhibited by siRNA before cell induction. Our results indicate that versican is essential in the condensation and conversion of metanephric mesenchyme.

Several genes have been shown to induce epithelialization of NIH3T3 cells. The expression of WT-1 in NIH3T3 cells induces a cell type that manifests several epithelial features. Desmosome-like structures were identified in WT-1-transfected cells, in which several epithelial marker genes are up-regulated, such as collagen IV, cytokeratin, and uromodulin, and various mesenchymal marker genes are down-regulated, such as vimentin and integrin $\alpha 8$ (Hosono *et al.*, 1999). As tight junctions were not detected in WT-1-transfected NIH3T3 cells, epithelialization induced by WT-1 was considered to be only partial (Hosono *et al.*, 1999). In contrast, the formation of tight junctions was found in the V1-transfected NIH3T3 cells, suggesting that these transfected cells are polarized, and epithelialization of NIH3T3 cells induced by V1 can therefore be considered complete. The expression of versican and WT-1 were both detected during renal development (Pritchard-Jones *et al.*, 1990; Hosono *et al.*, 1999; Erickson and Couchman, 2001; Steer *et al.*, 2004).

Versican displayed an effect on MET in two different kinds of mesenchymes, and this effect appears to be isoform specific. The expression of versican isoforms V1 and V2 in NIH3T3 fibroblasts revealed different effects on cell aggregation, motility, morphology, and epithelialization. Our results demonstrated that, in contrast to V1, V2 had no significant association with a mesenchymal-epithelial transition. In fact, predominant expression of V2 was detected recently in the chicken embryo aorta at sites where endothelial cells transformed into mesenchymal cells (Arciniegas *et al.*, 2004). The question remaining is whether versican V2 is involved in endothelial-mesenchymal transition (EMT) in the development of the embryo aorta.

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