Abstract. The Wnt-β-catenin pathway plays key roles in embryogenesis. Wnt-4 is known to be expressed in the mesonephric duct in embryonic development. It is tempting to speculate that the Wnt-4-β-catenin pathway contributes to the recovery from acute renal failure (ARF). This study used an in vivo model of ARF rats to clarify the significance of the Wnt-4-β-catenin pathway in ARF. ARF was induced by clamping the rat left renal artery for 1 h. At 3, 6, 12, 24, 48, and 72 h after reperfusion, whole kidney homogenate and total RNA were extracted for examination by Western blot analysis and real-time RT-PCR. Wnt-4 mRNA and protein expression were strongly increased at 3 to 12 h and 6 to 24 h after ischemia, respectively. In immunohistologic examination, Wnt-4 was expressed in the proximal tubules and co-expressed with aquaporin-1, GM130, and PCNA. Cyclin D1 and cyclin A were expressed at 24 to 48 h after reperfusion. In addition, the overexpression of Wnt-4 and β-catenin promoted the cell cycle and increased the promoter activity and protein expression of cyclin D1 in LLC-PK1 cells. Taken together, these data suggest that the Wnt-4-β-catenin pathway plays a key role in the cell cycle progression of renal tubules in ARF. The Wnt-4-β-catenin pathway may regulate the transcription of cyclin D1 and control the regeneration of renal tubules in ARF.

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Expression and Function of the Developmental Gene Wnt-4 during Experimental Acute Renal Failure in Rats

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Ischemic acute renal failure (ARF) is the most common form of ARF in the adult population. The molecular mechanisms of tubular regeneration after ischemic renal injury remain largely unknown (1–3). An understanding of the mechanisms that lead to renal cell proliferation and regeneration will be necessary for the exploration of novel therapeutic strategies for the treatment of ischemic ARF. Some reports have proposed that regeneration processes may recapitulate developmental processes to restore organ or tissue function (4,5). The adult tubular epithelial cells have a potent ability to regenerate after cellular damage. Under a condition of ischemic renal damage, normally quiescent cells undergo de-differentiation and acquire the ability to proliferate after their DNA synthesis is enhanced (6,7). The regulation of cyclin and cyclin-dependent kinase (CDK) inhibitor has been reported in ARF (8,9). The restriction point of the G1-to-S phase is determined by the activities of cyclin D1, cyclin A, cyclin E, and CDK1 (10,11). Cyclin D1 and cyclin A play key roles in G1-S regulation of renal tubular epithelial cells (12).

Members of the Wnt family of signaling molecules have been shown to have dramatic effects on the induction of metanephric mesenchyme. Wnt are cell-surface molecules that bind to and receptors of the “frizzled” class and activate them (13,14). In early organ culture experiments, Wnt-frizzled interactions appeared to form the base requirement for cell-cell contact between metanephric mesenchyme and inducer tissue (15). Among the Wnt family, Wnt-4 is expressed in developing kidney and may initiate differentiation of the metanephric mesenchyme (15). Mutant mice lacking Wnt-4 show a complete lack of tubular development despite initially normal ureteric bud branching and aggregation of cells at ureteric bud tips, indicating that Wnt-4 is necessary for induction (15). The mutant Wnt4−/− metanephric mesenchyme can be induced with exogenous Wnt-4 into a substantial portion of the epithelialization pathway, suggesting that Wnt-4 is sufficient for induction (15). In the proposed pathway for Wnt signaling, the inhibition of glycogen synthase kinase (GSK)-3β leads to posttranscriptional stabilization of soluble β-catenin, which in turn leads to the accumulation of β-catenin in the cytoplasm and nucleus (13,14,16). Within the nucleus, β-catenin interacts with members of the TCF (T cell factor)/LEF family of transcription factors to regulate gene expression (17,18). Tetsu and McCormick (19) recently reported that β-catenin activates transcription from the cyclin D1 promoter and that sequences within the promoter related to consensus TCF/LEF-binding sites are required for activation in colon carcinoma cells.

In this study, we hypothesized that the developmental gene, Wnt-4, is re-expressed during regeneration after acute renal failure and plays a key role in transcriptional regulation of cyclin D1 and cell cycle progression in renal tubular cells. To test this hypothesis, we examined the expression pattern of Wnt-4 expression during the recovery phase of ischemia/reperfusion kidney. Our data demonstrate that Wnt-4 is upregulated in the early phase of ischemia/reperfusion kidney, and that the
Wnt-4-β-catenin pathway regulates the transcription of cyclin D1 and cell cycle progression in renal tubules in ARF.

Materials and Methods

Induction of ARF

Male Sprague-Dawley (SD) rats (Saitama Experimental Animal Supply, Saitama, Japan) weighing 150 to 200 g were anesthetized intraperitoneally with sodium pentobarbital (30 mg/kg), and the left renal arteries were occluded with Sugita aneurysm clips (Mizuhiko Ikakogyo, Tokyo, Japan) for 60 min. The clamps were removed, the incisions were closed, and the rats were sacrificed at 0, 3, 6, 12, 24, 48, 72, and 96 h (n = 5). The left kidneys were quickly removed and processed for histologic evaluation, protein extraction, or RNA extraction. Age-matched and weight-matched SD rats that received sham operations without clamping of the renal arteries served as normal controls (n = 5). All animal experiments described here were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Tokyo Medical and Dental University Review Boards (#0010277).

Isolation of Kidney Tissue and Histologic Examination

Rats were anesthetized with pentobarbital at indicated times after the ischemic event. After perfusing their kidneys with sterile phosphate-buffered saline (PBS), the left kidneys were quickly excised, frozen in liquid nitrogen, and homogenized in the SDS sample buffer described later. For immunohistochemical studies, kidneys were removed after in vitro perfusion with 4% paraformaldehyde in a phosphate buffer and immersed overnight in the same fixative at 4°C. The fixed tissue was cryoprotected by immersion in 20% sucrose in PBS at 4°C and then shock-frozen in liquid nitrogen. Frozen 10-μm sections were cut with cryostat, thaw-settled on APS-coated slides, mounted with an aqueous mounting medium (Mount-Quick Aqueous; Daido Sangyo, Tokyo, Japan), and examined under a confocal laser microscope (Carl Zeiss Japan, Tokyo, Japan).

Frozen sections prepared in the manner described above were used for immunohistochemistry. The primary antibodies included anti-Wnt-4 antibody, anti-PCNA-specific antibody (Santa Cruz Biochemical Inc. Santa Cruz, CA), anti-GM130-specific antibody (Transduction Laboratories, San Jose, CA) as a marker for golgi (20), and an aquaporin-1-specific antibody (Santa Cruz Biochemical Inc.) as a marker for proximal tubules (21–23). The blocking peptide for the Wnt-4 antibody was purchased from Santa Cruz Biochemical Inc. The anti-Wnt-4 antibody was generated in goat. The anti-PCNA antibody and the anti-GM130 antibody were mouse monoclonal antibodies. The anti-aquaporin-1 antibody was generated in rabbits. The secondary antibody for the anti-Wnt 4 antibody was an anti-goat IgG FITC-conjugated antibody (Sigma, St. Louis, MO). The secondary antibody for the anti-PCNA antibody and the anti-GM130 antibody was an anti-mouse IgG Cy3-conjugated antibody (Sigma). The anti-aquaporin-1 antibody was an anti-rabbit IgG Cy3-conjugated antibody (Sigma).

After blocking, frozen sections were incubated at room temperature for 1 h with the anti-Wnt 4, anti-PCNA, anti-GM130, and anti-aquaporin-1 antibodies at dilutions of 1:100. The sections were then washed several times and incubated at room temperature for 1 h with the secondary antibodies at dilutions of 1:200. After more washing, the slides were mounted and examined under a confocal laser microscope.

Western Blot Analysis

Homogenized total renal tissue or LLC-PK1 cells were lysed in SDS sample buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM sodium orthovanadate) at 4°C (24). Protein was transferred to nitrocellulose membrane and probed with polyclonal antibodies against Wnt-4, cyclin D1, cyclin A, and actin (Santa Cruz Biotechnology). The primary antibodies were detected using horseradish peroxidase (HRP)–conjugated rabbit anti-mouse IgG and visualized by the Amersham ECL system (Amersham Corp., Arlington Heights, IL).

Real-Time Quantitative PCR

Reverse transcription-PCR (RT-PCR) reaction was used to investigate Wnt-4 from RNA extracted from the renal cortices of ischemia/reperfusion kidneys. The RNA was extracted from the renal cortex using TRI-REAGENT (Life Technologies, Gaithersburg, MD) (25). One microgram of total RNA was used for RT-PCR as follows. The real-time quantitative PCR method was used to accurately detect the changes of Wnt-4 gene copies. The cycle at which the amplification plot crosses the threshold (CT) is known to accurately reflect relative mRNA values (26,27). Total RNA was harvested from renal tissue. Rat Wnt-4 and GAPDH mRNAs were amplified. Wnt-4 primer 1 (antisense) encompassed bases 343 to 362, and primer 2 (sense) encompassed bases −4 to 16 (Genebank accession No. AF188698). The sequence of primer 1 was 5'-ATGGAGCCGGATCCGGTCCAG-3', and the sequence of primer 2 was 5'-CACCATGCACCTCCTCCAGC-3'. The predominant cDNA amplification product was predicted to be 366 bp in length (the distance between primers plus the primer length). RT and PCR of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) served as positive controls. The primers were defined by the following cDNA base sequences (28): primer 1 (antisense), bases 795 to 814, sequence, 5'-AGATCCCAACGG-ATACATT-3'; primer 2 (sense), bases 506 to 525, sequence, 5'-TCCCTCAAGATGGTGCAGCA-3'. The predominant cDNA amplification product was predicted to be 309 bp in length. PCR products were detected and quantitated in real time using the LightCycler Real time PCR (Roche Molecular Biochemicals, Tokyo, Japan) as described previously (26,27). The amplification mixture contained 5 nM of template DNA and 50 μM of primer DNA in 50 nM salt and 1 mM Mg (2) A three-step PCR consisting of denaturation at 94°C for 20 s, annealing at 55°C for 20 s, and extension at 72°C for 30 s was performed for 35 cycles. The reaction produced a 366-bp PCR product for Wnt-4 and a 309-bp product for GAPDH. The PCR cycle at which the amplification plot crossed a threshold of 10 SD above the baseline was defined as the CT value. While the normalized reporter signal remained unchanged in control sample without RNA, the PCR amplification climbed to the threshold value in the sample treated with RNA from renal tissue. In calculating the relative mRNA level, the CT values were assumed to increase by approximately 1 for each twofold dilution. To examine the precision of the assay, mRNA from renal tissue was reverse-transcribed and amplified on three separate days. The mean CT values ranged from 22.28 to 22.65. The PCR products of Wnt-4 and GAPDH were subcloned to TA cloning vector TM (Promega, Biotec, Madison, WI) as described previously (25). The plasmids containing Wnt4 cDNA and GAPDH cDNA were used to make standard curves of quantitative PCR.

Reporter Constructs

The cyclin D1 reporter construct used for the luciferase assays contained human cyclin D1 promoter from residues −944 to +139 cloned upstream of the luciferase gene (generous gift of Dr. M. Eilers) (29). Wnt-4 and β-catenin-dN (deletion of the amino-terminal region,
Cell Cycle Analysis by [H]-Thymidine Incorporation and Flow Cytometry

After transfection, the LLC-PK1 cells were plated in 24-well plates and incubated in a medium without FCS for 20 h. For the last 4 h, the cells were pulsed with 1 μCi [H]-thymidine (Amersham). After the incubation, the cells were washed three times in ice-cold PBS, precipitated two times with 10% TCA, redissolved in 0.5 M NaOH, and counted in Aquasol-2 scintillation cocktail (NEN Research Products, Boston, MA).

LLC-PK1 cells were cultured in a 75-cm² flask. After transfection, the cells were incubated in a medium plus 10% FCS for 24 h, washed twice with PBS, and then resuspended in 70% ethanol for at least 12 h at 4°C. Fixed and permeabilized cells were collected by centrifugation and washed with PBS. The cells were stained with propidium iodide and analyzed by flow cytometry using a Coulter EPICS 753 flow cytometer to determine the percentages of the cells in the G1, S, and G2/M phases (31). All experiments were repeated at least four times.

Statistical Analyses

Results are given as means ± SD when comparing groups with equal sample numbers. Results are given as means ± SEM when comparing groups with different sample numbers. The differences were tested using two-way ANOVA followed by the Scheffe test for multiple comparisons. Two groups were compared by the unpaired t test. P < 0.05 was considered significant.

Results

Western Blot Analysis of the Protein Expressions of Wnt-4, Cyclin D1, and Cyclin A after Ischemic Renal Failure

The left renal artery was clamped for 60 min, and the left kidney was excised at 6, 12, 24, 48, and 72 h after reperfusion. Western blot analysis was used to detect the protein levels of Wnt-4, cyclin D1, and cyclin A. In an earlier study, Wnt-4 was reported to be expressed in the renal tubular cells of both embryonic and neonatal kidneys (32). For a positive control in our study, we used the neonatal rat kidney (Figure 1A), an organ that exhibits a clear positive signal. Weak expression of Wnt-4 was detected in the control adult kidney at 0 h (Figure 1A, A and C). Wnt-4 dramatically increased at 6 to 12 h after ischemia/reperfusion (Figure 1C). The upregulation of Wnt-4 protein expression was temporary. The intensity of the Wnt-4 band declined at 24, 48, and 72 h after ischemia/reperfusion. We also examined the protein expressions of cyclin D1 and cyclin A. The band intensity of cyclin D1 was detectable in control (0 h) and at 6 and 12 h after ischemia/reperfusion and increased at 24 h after ischemia/reperfusion (Figure 1E). The band intensity of cyclin A was very weak in control (0 h), as well as in the ischemia/reperfusion kidneys at 6 and 12 h. At 24 h after ischemia/reperfusion, the expression of cyclin A increased (Figure 1G).

Immunohistochemical Examination of Wnt-4 in ARF

We next performed immunohistologic studies on Wnt-4 using confocal microscopy (Figures 2 and 3). In the lower-power view, Wnt-4 expression was observed in cortical renal tubules at 12 h after ischemia/reperfusion. (Figure 2A). Anti-aquaporin-1 antibody was used as a marker of proximal tubules (21–23). The expression of Wnt-4 was apparently co-localized with aquaporin-1 in the lower-power view (Figure 2, B and C) but undetectable in the renal cortices from the control rats (Figure 2G). The expression of aquaporin-1 was clearly observed in the renal cortices from the control rats (Figure 2H). Taken together, these results demonstrate a prevalence of Wnt-4 expression in the proximal tubules of the renal cortex 12 h after ischemia/reperfusion (Figure 2, A through C). Wnt-4 was undetectable in the renal medullae of both the ischemia/reperfusion kidneys and control kidneys (Figure 2, D and J). Only minimal staining of aquaporin-1 was observed in the medullae of the ischemia/reperfusion and control kidneys (Figure 2, E and K). In the higher-power view, Wnt-4 staining was observed in intracellular location of proximal tubular cells (Figure 3A). In double-staining, these cells were stained positively by aquaporin-1 antibody (Figure 3, B and C). When blocking peptide was added to the anti-Wnt-4 antibody solution, no positive signal was observed (Figure 3D).

To examine the intracellular localization of Wnt-4 in renal tubules, we performed immunohistochromic studies on the colocalization using anti-Wnt-4 and anti-GM130 antibodies. The anti-GM130 antibody was used as a Golgi apparatus marker (20). Wnt-4 staining was observed intracellularly at locations (Figure 4, A and D) overlapping the areas stained by GM130 (Figure 4, B and E). In the double-staining of Wnt-4 and GM130, the Wnt-4 staining appeared in the Golgi apparatus (yellow staining in Figure 4, C and F).

To colocalize Wnt-4 with dividing cells, a lower-power view of Wnt-4 staining and PCNA staining was examined at 24 h after ischemia/reperfusion (Figure 5). Wnt-4 staining and PCNA staining were both observed at the proximal tubules of the cortex (Figures 5, A, B, and C). In the higher-power view of Wnt-4 staining, Wnt-4 and PCNA were co-localized in the same tubular cells at 24 h after ischemia/reperfusion (Figure 5, D, E, and F).

Real-Time PCR

Quantitation of Wnt-4 mRNA transcript using the real-time quantitative PCR method revealed 6.5-fold (3 h), 15.2-fold (6 h), 8.1-fold (12 h), and 3.7-fold (24 h) increases in Wnt-4 mRNA levels, compared with the 0 h value (Figure 6A). With
Figure 1. Protein expressions of Wnt-4, cyclin D1, and cyclin A in the kidneys of rats subjected to 60 min of renal ischemia. Bilateral renal arteries were clamped for 60 min, and kidneys were excised at 6, 12, 24, 48, and 72 h after reperfusion. Rats that received sham operations at 0 h served as controls. In the case of Wnt-4, neonatal rat renal protein (20 μg) was loaded as a positive control (right lane) (A). Extracted protein (20 μg) from renal tissue was separated by SDS-PAGE gels. Wnt-4, cyclin D1, and cyclin A protein levels were detected by Western blot analysis (C, F, G). Western blots of actin as loading controls are shown (B, D, F, H). Three membranes were used for panels C through H: the first for panels C (Wnt-4) and D (actin), the second for panels E (cyclin D1) and F (actin), and the third for panels G (cyclin A) and H (actin). These three membranes were equally loaded of the renal protein.
Figure 2. Immunohistologic examination of Wnt-4 in ischemic-reperfusion kidneys and control kidneys. Immunohistochemical analyses of lower-power views (×100) of the renal cortex were performed with antibody against Wnt-4 (A), antibody against aquaporin-1 (B), and in a merged condition (C) at 12 h after ischemic injury. Immunohistochemical analyses of lower-power views (×100) of the renal medulla were performed with antibody against Wnt-4 (D), antibody against aquaporin-1 (E), and in a merged condition (F) at 12 h after ischemic injury. Immunohistochemical analyses of the lower-power view (×100) of the renal cortex were performed with antibody against Wnt-4 (G), antibody against aquaporin-1 (H), and in a merged condition (I) at the control kidney. Immunohistochemical analyses of lower-power views (×100) of the renal medulla were performed with antibody against Wnt-4 (J), antibody against aquaporin-1 (K), and in a merged condition (L) at the control kidney.
the use of the Wnt-4 and GAPDH cDNA plasmids, the linear curve between the PCR product and quantity of cDNA (10 pg/μl to 100 ng/μl) was observed in the utilized range. Wnt-4 mRNA expression was also significantly upregulated in neonatal kidney (Figure 6B). GAPDH signal was not significantly changed by ischemia/reperfusion (Figure 6C).

**Cell Cycle Progression by Overexpression of Wnt-4 in LLC-PK1 Cells**

Initially, we examined how the Wnt-4-β-catenin pathway affected the proliferation of LLC-PK1 cells by [3H]-thymidine uptake. Figure 7A shows the effects of the Wnt-4-β-catenin pathway on [3H]-thymidine uptake. Transfection of Wnt-4, β-catenin dN, and co-transfection of Wnt-4 and β-catenin dN stimulated the [3H]-thymidine uptake to 186, 137, and 225%, respectively. We next used flow cytometry to examine the effects of the Wnt-4-β-catenin pathway on cell cycle progression. When LLC-PK1 cells expressing Wnt-4 and β-catenin were incubated without FCS for 24 h, the percentages of S and G2/M phases were increased from 15.5% to 23.5% and from 30.5% to 47.3%, respectively, compared with the levels in the cells transfected with empty vector (Figure 7, B and C). These results demonstrated the stimulatory effects of the Wnt-4 and β-catenin pathways on cell cycle progression.

**Stimulation of Cyclin D1 Promoter Activities by Overexpression of Wnt-4 and β-Catenin**

We next examined the role of the Wnt-4-β-catenin signaling pathway in the regulation of cyclin D1 promoter activity and protein expression. We performed a transient transfection with
either an empty vector or vector containing Wnt-4 and β-catenin dN, together with the cyclin D1-luciferase reporter gene and β-galactosidase expression vector. When Wnt-4, β-catenin dN, and Wnt-4-β-catenin dN were expressed in LLC-PK1 cells cultured in a DMEM medium without FCS, cyclin D1 promoter activity increased significantly to 2.5-, 2.8-, and 4.8-fold their control levels, respectively (Figure 8A). We further examined the regulation of cyclin D1 protein expression when Wnt-4 and β-catenin dN plasmids were co-transfected. When Wnt-4 and β-catenin dN plasmids were transfected, the cells expressed higher levels of cyclin D1 than the cells transfected with the empty vector (Figure 8B). This result suggested that the Wnt-4-β-catenin pathway positively regulates cyclin D1 promoter activity and protein expression in renal tubular cells.

**Discussion**

In the present study, we demonstrate (1) that Wnt-4 is upregulated in the proximal tubules during recovery from ARF and (2) that Wnt-4 expression is localized in the Golgi apparatus and co-localized with PCNA in the renal tubules during ARF. Our findings additionally suggest that the Wnt-4-β-catenin pathway may regulate the transcription of cyclin D1 and cell cycle progression in the renal tubules during ARF.

Recovery from ARF requires the replacement of damaged cells with new cells that restore tubule epithelial integrity. Regeneration processes are characterized by proliferation of dedifferentiated cells and subsequent redifferentiation of the daughter cells into the required cell phenotypes. Noting the similarity between this process and the cellular phenomena observed during embryogenesis, investigators have postulated
that regeneration processes to reestablish proper tissue function after damage may repeat parts of the genetic program that serve during organogenesis (5,33). To test this hypothesis, we examined the patterns of Wnt-4 expression in a model of acute renal ischemia.

Our study is the first to demonstrate that Wnt-4 expression is upregulated in the early phase of ischemic acute renal failure. Wnt-4 expression in the proximal tubule was localized exclusively to the site of tubule regeneration, where PCNA is also expressed. Accordingly, we speculated that Wnt-4 protein may induce the transformation of regenerative renal tubular cells. In the developmental stage, Wnt-4 is expressed in pretubular aggregates of the condensing mesenchyme during development to the comma- and S-shaped bodies of metanephric kidneys (15). Thus, our data suggested that the cells that express Wnt-4 after ischemic injury have characteristics of embryonic renal cells such as mesenchymal-to-epithelial progression and proliferation. The origin of the Wnt-4-positive cells is an interesting issue. At least two possible origins can be considered: the primitive cells relining the outer medullary portion of the proximal tubule, and the differentiated surviving cells of the superficial cortex. We are inclined to believe the latter possibility, given the presentation of the Wnt-4–positive cells mainly in the cortex, as shown in Figure 2A. These findings suggest that the differentiated surviving cells of the cortical tubules may express Wnt-4 after ischemic injury.

In a study on the expression of the Wnt gene family (Wnt-4, -7b, and -11) during late nephrogenesis and complete ureteral obstruction, Nguyen et al. (32) found that Wnt-4 and Wnt-11 were important mediators of the transformation of mesenchyme to epithelium in the kidney. It thus seems that other members of the Wnt family may work together with Wnt-4 in regenerating the renal tubules after ischemic ARF. Further studies may be necessary to clarify these issues.

Surendran et al. (34) recently observed low levels of Wnt-4 mRNA in the normal adult mouse kidney and that high levels

Figure 5. Immunohistologic colocalization of Wnt-4 and PCNA in proximal tubules of ischemic-reperfusion kidneys. Immunohistochemical analyses of lower-power views (×100) of the renal cortex were performed with antibody against Wnt-4 (A), antibody against PCNA (B), and in a merged condition (C) at 24 h after ischemic injury. Immunohistochemical analyses of higher-power views (×400) of the renal cortex were performed with antibody against Wnt-4 (D), antibody against PCNA (E), and in a merged condition (F) at 24 h after ischemic injury. The arrows indicate Wnt-4–positive and PCNA–positive cells.
of Wnt-4 expression induced in the collecting ducts in murine models of renal injury that produced tubulointerstitial fibrosis. In the ischemia/reperfusion model in our study, we did not observe a fibrotic area in the proximal tubules. It will be of interest to examine whether Wnt-4 plays a role in fibrogenesis in renal tubular cells.

Our data are also the first to demonstrate the contribution of Wnt-4/β-catenin signaling to the activation of cyclin D1 and promoter and protein expression in renal tubular cells. The Wnt-β-catenin pathway plays a key role in normal embryonic development and in malignant transformation of many types of human cells (13). Recent reports indicate that β-catenin activates transcription from the cyclin D1 promoter and also that sequences within the promoter related to consensus TCF/LEF-binding sites are necessary for activation in colon carcinoma cells (19). However, the functional role of the Wnt-4/β-catenin signaling pathway in non-carcinoma cells is not known well. Our data demonstrate that overexpression of Wnt-4 increases cyclin D1 promoter activity and protein expression in non-carcinoma, renal epithelial cells. Moreover, the co-expression of β-catenin further increased the promoter activity and protein expression of cyclin D1. These data suggest that the Wnt-4 protein might activate a cascade of signaling events that leads to an increase in the intracellular availability of β-catenin in renal tubular cells. Within the nucleus, β-catenin interacts with the TCF family and regulates gene expression via the TCF binding site (13,16). Our data suggest that the overexpression of Wnt-4 and β-catenin induce cell cycle progression in renal tubular cells. If this is the case, then the expression of Wnt-4 in the recovery phase of ARF could be expected to promote cell cycle progression after tubular injury. While we lack clear evidence that Wnt-4 is a proliferative signal during tubulogenesis, our data demonstrate that in vitro Wnt-4 and β-catenin proliferate renal tubular cells. Wnt-4 has been proven to be required for metanephric condensation during development, and there is no evidence suggesting that Wnt-4 is a proliferative signal in metanephric formation. In our ischemia/reperfusion model, Wnt-4 was co-localized with PCNA, suggesting that Wnt-4 might be a proliferative signal in regenerating renal tubules. Dysregulated activation of Wnt-4 and β-catenin pathways has been reported in malignancies such as colon carcinoma cells and melanoma. The transient but less persistent expression may prevent renal tubular cells from becoming hyperplastic or malignant.
Figure 8. Effects of Wnt-4 and β-catenin on the transcriptional activity and protein expression of cyclin D1. (A) After co-transfecting the cells with cyclin D1 promoter-luciferase construct together with β-galactosidase reporter construct and expression vectors containing Wnt-4, β-catenin dN, or pCDNA3 (vector), they were incubated with DMEM medium without FCS. Cyclin D1 promoter luciferase activities were measured as described in the Materials and Methods section. Results are means ± SEM of four or five independent experiments. * P < 0.05; # P < 0.01. (B) LLC-PK1 cells were infected with Wnt-4, β-catenin dN plasmids, or pCDNA3 (vector) and incubated for 48 h. Whole cell lysates were separated by SDS-PAGE gels. Western blot analysis was used to detect the cyclin D1 protein levels.

Further studies will be necessary to gain a more precise understanding of the molecular mechanisms of renal recovery after ischemia/reperfusion injury.

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