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Tubule-specific ablation of endogenous β-catenin aggravates acute kidney injury in mice

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Abstract

β-catenin is a unique intracellular protein functioning as an integral component of the cell-cell adherens complex and a principal signaling protein mediating canonical Wnt signaling. Little is known about its function in adult kidneys in the normal physiologic state or after acute kidney injury (AKI). To study this, we generated conditional knockout mice in which the β-catenin gene was specifically disrupted in renal tubules (Ksp-β-cat−/−). These mice were phenotypically normal with no appreciable defects in kidney morphology and function. In the absence of β-catenin, γ-catenin functionally substituted it for E-cadherin binding, thereby sustaining the integrity of epithelial adherens junctions in the kidneys. In AKI induced by ischemia reperfusion or folic acid, the loss of tubular β-catenin substantially aggravated renal lesions. Compared with controls, Ksp-β-cat−/− mice displayed higher mortality, elevated serum creatinine and more severe morphologic injury. Consistently, apoptosis was more prevalent in kidneys of the knockout mice, which was accompanied by increased expression of p53 and Bax, and decreased phosphorylated Akt and survivin. *In vitro*, activation of β-catenin by Wnt1 or stabilization of β-catenin protected tubular epithelial cells from apoptosis, activated Akt, induced survivin, and repressed p53 and Bax expression. Hence, endogenous β-catenin is pivotal for renal tubular protection after AKI by promoting cell survival through multiple mechanisms.

Keywords

Wnt; β-catenin; acute kidney injury; apoptosis

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Disclosures

None.

Introduction

Acute kidney injury (AKI), often resulting from ischemic, toxic and septic insults, is a common kidney disorder associated with high rate of morbidity and mortality.[1, 2] Although a number of cellular events may contribute to the pathogenesis of AKI, excessive apoptosis of renal tubular epithelial cells is increasingly recognized as a major mechanism leading to tubular collapse and an abrupt decline in kidney function.[3] Tubular cell apoptosis is tightly controlled by the delicate balance between pro- and anti-apoptotic forces, in which Akt kinase, p53 transcription factor, and pro-apoptotic Bax are key players.[2, 3] In this context, elucidation of their regulation *in vivo* is essential for understanding of the pathogenesis of AKI, as well as for designing rational intervention strategies.

β-catenin is unique intracellular protein that possesses distinctive, dual functions. In addition to playing a role in establishing cell-cell adhesion as an integral component of the adherens junction complex, β-catenin is also the principal signaling protein that mediates the canonical Wnt signaling.[4, 5] As a structural protein in the cell adherens complex, β-catenin physically binds to and bridges E-cadherin to actin cytoskeleton, thereby stabilizing cell adherens junction. As a signaling protein, β-catenin is controlled in the cytoplasm under normal resting conditions, and constitutively undergoes phosphorylation and subsequent ubiquitin-mediated degradation. However, upon activation by its upstream signaling, β-catenin is stabilized and translocates into the nucleus, where it binds to members of the T-cell factor (TCF)/lymphoid enhancer-binding factor (LEF) family of transcription factors, and drive the expression of its target genes. Aside from Wnt signaling, β-catenin activation is also regulated by other signal pathways as well, such as integrin-linked kinase (ILK) and TGF-β1.[6–9] In that regard, β-catenin could serves as a converging downstream effector that mediates the actions of multiple key intracellular signaling. Not surprisingly, extensive studies have demonstrated that β-catenin is essential in regulating diverse arrays of biologic processes such as organ development, tissue homeostasis and injury repair.[5, 10]

Activation of β-catenin signaling in a temporally and spatially controlled fashion is indispensible for nephron formation and kidney development.[11, 12] Inappropriate activation of β-catenin, however, has been shown to implicate in the pathogenesis of various chronic kidney diseases such as obstructive nephropathy, diabetic nephropathy, adriamycin nephropathy, polycystic kidney disease and chronic allograft nephropathy.[13–17] These results suggest that a properly controlled β-catenin signaling is necessary and essential for the maintenance of kidney tissue integrity and homeostasis.[18–20] β-catenin is ubiquitously expressed, at low level, in normal kidneys.[14] However, little is known about its function in adult kidneys in normal physiologic setting. Furthermore, whether it plays any role in regulating tissue damage or protection after AKI is completely unknown.

In this study, we studied β-catenin expression in mouse models of AKI, and investigated its function in regulating tubular cell injury/survival in conditional knockout mice in which β-catenin is specifically ablated in renal tubules. Our results suggest that endogenous β-catenin is crucial for renal tubular protection after AKI, which is primarily mediated by promoting cell survival through multiple mechanisms.

Results

Induction of β-catenin in renal tubules after acute kidney injury

We first examined the expression of β-catenin in AKI induced by folic acid, a model characterized by renal tubule injury, cell apoptosis and acute renal failure.[21–23] Immunohistochemical staining demonstrated an increased β-catenin protein in renal tubules at 2 days after folic acid injection (Figure 1b), compared with the controls (Figure 1a). Cytoplasmic and nuclear localization of β-catenin was clearly evident in renal tubules (Figure 1b, arrowheads in the boxed area), indicating its activation after AKI. To confirm this finding, we quantitatively assessed renal β-catenin abundance by utilizing Western blot analysis of whole kidney lysates. As shown in Figure 1, c and d, more than 6-fold induction of β-catenin abundance was observed in the injured kidneys after folic acid injection, compared to the controls (Figure 1d). Similar induction of β-catenin protein was also observed in the AKI induced by renal ischemia/reperfusion injury (IRI) (data not shown).

To determine tubular segment-specificity of β-catenin induction in AKI, we employed double immunostaining for β-catenin (green) and various tubular markers (red) in the kidneys after folic acid injection. As illustrated in Figure 1e, β-catenin protein was apparently detectable in all tubular segments, including proximal tubule, cortical thick ascending limb, distal tubule and collecting duct epithelia in AKI after folic acid injection.

Mice with tubule-specific ablation of β-catenin are phenotypically normal

We sought to determine the potential role of endogenous β-catenin in normal renal physiology and in regulating tissue injury in AKI. To this end, we generated conditional knockout mice in which β-catenin gene is specifically disrupted in renal tubules by utilizing the Cre-LoxP system. Homozygous β-catenin floxed mice were mated with Ksp-Cre transgenic mice expressing Cre recombinase under the control of Ksp-cadherin promoter. As shown in Figure 2, a and b, conditional knockout mice with tubule-specific ablation of endogenous β-catenin (designated as Ksp-β-cat−/−) were generated (genotype: β-cat$^{fl/fl}$, Cre) (Figure 2b, lane 2). Age- and sex-matched β-catenin floxed mice (genotype: β-cat$^{fl/fl}$) from the same litters were used as controls (Figure 2b, lane 1). To confirm conditional ablation of β-catenin, we examined its expression in the kidneys after folic acid injection, since renal β-catenin was induced in that setting (Figure 1). As shown in Figure 2c, Western blot analyses of whole kidney lysates revealed a marked reduction of renal β-catenin protein in Ksp-β-cat −/− mice, comparing with the controls. Immunohistochemical staining for β-catenin also showed a tubule-specific reduction of β-catenin protein in the kidneys of Ksp-β-cat−/− mice (Figure 2, d and e).

Mice with tubule-specific deletion of β-catenin (Ksp-β-cat−/−) were phenotypically normal. There was no appreciable abnormality in kidney morphology of Ksp-β-cat−/− mice (Figure 3a). Ksp-β-cat−/− mice exhibited similar body weights as the controls (Figure 3b). Kidney function, as reflected by serum creatinine and urinary albumin, was indistinguishable between Ksp-β-cat−/− mice and the controls (Figure 3, c and d).

As β-catenin is known to bind to E-cadherin and mediates epithelial adherent interaction, this prompted us to investigate the reason why ablation of this protein has not resulted in a

defect at epithelial adherens junctions. As shown in Figure 3e, co-immunoprecipitation demonstrates that β-catenin physically interacted with E-cadherin in the control kidneys. As expected, this interaction of β-catenin/E-cadherin was reduced in the kidneys of Ksp-β-cat−/− mice (Figure 3e). Interestingly, the interaction between E-cadherin and γ-catenin, another member of the catenin family, was markedly induced in Ksp-β-cat−/− kidneys, compared to the controls (Figure 3e). The protein levels of γ-catenin and E-cadherin, however, were not changed in the Ksp-β-cat−/− kidneys (Figure 3f), indicating that the increased association of E-cadherin/γ-catenin is not due to their upregulation. These results suggest that in the absence of β-catenin, γ-catenin functionally substitutes for its role in maintaining the integrity of epithelial cell-cell adherens junctions.

Tubule-specific ablation of β-catenin aggravates acute kidney injury

We next examined the effects of β-catenin ablation on acute tubular injury after folic acid injection. Of interest, nine out of eighteen Ksp-β-cat−/− mice died (50% mortality rate) within 2 days after folic acid administration, whereas only four out of seventeen control mice deceased (23.5% mortality rate) in the same period under the identical conditions. This suggests that loss of endogenous β-catenin increases mortality rate in mice after AKI. In the surviving mice, serum creatinine levels at 2 days after folic acid were significantly higher in Ksp-β-cat−/− mice than that in the controls (Figure 4a). Accordingly, Ksp-β-cat−/− kidneys exhibited more severe morphological injury, particularly in the outer stripe of out medulla region, characterized by loss of brush border, tubular cell depletion and cast formation in the lumen (Figure 4b, yellow asterisks). Quantitative assessment of kidney morphological injury between control and Ksp-β-cat−/− groups at 2 days after folic acid injection is presented in Figure 4c. Together, it is clear that loss of endogenous β-catenin aggravates tubular lesions and acute kidney failure induced by folic acid.

Ablation of β-catenin promotes tubular cell apoptosis and Bax expression

To explore the mechanism underlying the cytoprotective role of endogenous β-catenin in AKI, we further examined apoptotic cell death in the kidneys of control and Ksp-β-cat−/− mice after folic acid injection. As shown in Figure 5a, TUNEL staining revealed considerable apoptosis in both cortical and medullar regions of the kidneys in control mice at 2 days after folic acid administration. However, the frequency of apoptosis in the Ksp-β-cat−/− kidneys was significantly higher than that in the controls under same conditions (Figure 5a, arrows). Quantitative data on apoptotic cells in both cortical and medullar regions of control and Ksp-β-cat−/− mice are presented in Figure 5b. These results suggest that tubule-specific loss of β-catenin exacerbates kidney injury by promoting apoptosis.

We further examined renal expression and distribution of Bax, a pro-apoptotic member of Bcl-2 family, in control and Ksp-β-cat−/− mice, since it is a central player in mediating mitochondrial dysfunction and cell apoptosis.[24, 25] As shown in Figure 5, c and d, Bax protein was markedly increased in the kidneys of Ksp-β-cat−/− mice at 2 days after folic acid injection, when compared to the controls. Immunohistochemical staining also revealed a substantial increase of Bax protein in renal tubules in the kidneys of Ksp-β-cat−/− mice (Figure 5e, arrowheads).

Ablation of endogenous β-catenin activates multiple pro-apoptotic pathways

To elucidate the upstream signaling that is responsible for Bax induction in Ksp-β-cat−/− mice, we further examined renal expression of p53, a tumor suppressor protein that promotes apoptosis by regulating Bax expression.[26] As shown in Figure 6, a and b, p53 protein was significantly upregulated in the kidneys of Ksp-β-cat−/− mice at 2 days after folic acid injection, comparing with the controls. These data suggest that p53 upregulation could be a potential upstream signaling that leads to renal Bax induction in Ksp-β-cat−/− mice after injury.

Bax protein is also subjected to regulation by Akt-mediated phosphorylation.[27] Therefore, we also examined the phosphorylation status of renal Akt *in vivo*. As shown in Figure 6c, tubule-specific loss of β-catenin substantially inhibited Akt phosphorylation at Serine 473 in the Ksp-β-cat−/− mice, although total Akt abundance was unaltered (Figure 6, c and d).

We reasoned that in the setting of AKI, activated β-catenin might also directly control the transcription of pro-survival genes. In that regards, previous studies indicate that survivin, a member of the inhibitors of apoptosis proteins (IAPs) family that promotes cell survival by preventing apoptosis,[28, 29] is a direct downstream target gene of β-catenin.[30, 31] Hence, we examined survivin mRNA expression in the kidneys by quantitative, real-time RT-PCR (qRT-PCR). As shown in Figure 6e, the steady-state level of survivin mRNA in Ksp-β-cat−/− mice at 2 days after folic acid injection was significantly lower than that in the controls. All together, as illustrated in Figure 6f, it becomes clear that loss of β-catenin stimulates multiple signaling pathways leading to tubular cell apoptosis after AKI.

Loss of tubular β-catenin also aggravates AKI induced by ischemia/reperfusion injury

We also investigated the cytoprotective role of endogenous β-catenin by utilizing another model of AKI, renal ischemia/reperfusion injury (IRI). At 1 day after IRI, all control mice (n = 4) survived, while one out of four Ksp-β-cat−/− mice (n = 4) died. As shown in Figure 7a, serum creatinine levels at 1 day after IRI were significantly higher in Ksp-β-cat−/− mice than that in the controls. Ksp-β-cat−/− kidneys also showed more severe morphological injury, characterized by loss of brush border and tubular cell loss (Figure 7, b and c). Similarly, TUNEL staining also exhibited more apoptosis in the kidneys after IRI in Ksp-β-cat−/− mice than that in the controls (Figure 7, d and e). Renal expression of Bax protein was markedly increased in the kidneys of Ksp-β-cat−/− mice at 1 day after IRI, compared to the controls (Figure 7, f and g). In short, these results indicate that loss of endogenous β-catenin exacerbates ischemic AKI as well.

Activation of β-catenin protects tubular cells against apoptosis in vitro

To provide direct evidence that links the loss of β-catenin to tubular cell apoptosis, we finally investigated the potential role of β-catenin activation in regulating tubular cell survival after injury by using *in vitro* system. For activating endogenous β-catenin, human proximal tubular epithelial cells (HKC-8) were transfected with the expression vector encoding Wnt1, the prototype member of Wnt family that activates β-catenin via canonical pathway. Previous studies have shown that ectopic expression of Wnt1 causes endogenous β-catenin activation in HKC-8 cells.[32] As shown in Figure 8, a and b, significant apoptosis

was observed in HKC-8 cells after treatment with staurosporine (STS), a potent apoptosis inducer,[33, 34] for a short period of incubation, as illustrated by TUNEL staining. However, transfection of Wnt1 expression vector (pHA-Wnt1) completely protected HKC-8 cells from STS-induced apoptosis under same conditions (Figure 8, a and b).

Wnt1 also induced survivin mRNA expression in tubular epithelial cells, as demonstrated by qRT-PCR (Figure 8c). As shown in Figure 8d, tubular cell apoptosis induced by STS was associated with Bax induction in HKC-8 cells. However, ectopic expression of Wnt1 substantially abolished Bax induction in HKC-8 cells (Figure 8d). Consistent with the *in vivo* data, activation of endogenous β-catenin by Wnt1 also promoted Akt phosphorylation and inhibited p53 expression in tubular cells after injury (Figure 8e). Similarly, ectopic expression of exogenous β-catenin by transfecting of HKC-8 cells with N-terminally truncated, stabilized β-catenin expression vector (pDel-β-cat) also prevented STS-induced apoptosis (Figure 8f), induced survivin mRNA expression (Figure 8g) and abolished Bax induction (Figure 8h). These data suggest that activation of β-catenin *in vitro* is sufficient for protecting tubular epithelial cells from apoptosis by multiple mechanisms.

Discussion

By generating conditional knockout mouse model in which β-catenin is selectively ablated in renal tubules, the present study represents the first attempt to elucidate the function of endogenous β-catenin in normal renal physiology and in regulating tubular cell damage/survival after acute injury. Our data indicate that activation of β-catenin in the setting of AKI is advantageous and renal protective, as tubule-specific loss of endogenous β-catenin results in higher mortality rate, elevated serum creatinine and worsened morphologic lesions after folic acid and ishemia/reperfusion injury (Figures 4 and 7). Furthermore, loss of β-catenin also leads to an increased tubular cell apoptosis after AKI, which is associated with an increased renal expression of p53 and Bax, and decreased Akt phosphorylation and survivin expression (Figures 5 and 6). It should be pointed out that the difference in renal injury and kidney dysfunction between control and Ksp-β-cat−/− groups is mostly likely underestimated, because more mice with the most severe AKI died selectively in the Ksp-β-cat−/− group, compared to the controls. These results clearly indicate that activation of β-catenin in renal tubules after AKI is a defensive response of the kidneys in an attempt to protect against the catastrophic damage to tubule cells.

Kidney tubular cells are susceptible to various toxic and metabolic injuries to undergo apoptotic cell death, mainly by a mitochondria-dependent pathway. In AKI induced by folic acid or IRI, tubular cell apoptosis is a major pathogenic mechanism leading to acute renal failure.[3, 22] Because β-catenin is a survival factor *in vitro* for tubular epithelial cells (Figure 8),[35] loss of β-catenin would eradicate an endogenous survival mechanism that normally safeguards renal tubules, leading to an exaggerated apoptosis after injury. This notion is substantiated experimentally in two models of AKI induced by folic acid (Figure 5) and IRI (Figure 7). However, we cannot exclude the possibility that endogenous β-catenin may also affect other forms of tubular cell death such as necrosis and necroptosis after injury.[3, 36]

Comparing to renal medulla, only a fraction of proximal tubules in the cortical region in Ksp-β-cat−/− kidneys exhibited an increased TUNEL staining and Bax induction (Figure 5). This is probably related to the unique expression pattern of Cre recombinase in Ksp-Cre mice,[37, 38] which closely imitates endogenous Ksp-cadherin, a tissue-specific member of the cadherin family of cell adhesion molecules.[37, 39] Although Cre is expressed in all segments of the nephron and collecting ducts in Ksp-Cre mice, not all proximal tubules express it.[37, 38] In fact, earlier studies using reporter gene indicate that only 21% of proximal tubular cells, but more than 92% of distal tubular and collecting duct cells, actually express sufficient Cre to mediate the deletion of a floxed DNA segment.[38] This could result in an incomplete ablation of β-catenin in selective proximal tubules, thereby contributing to the discriminatory apoptosis and Bax induction in the cortical region of the Ksp-β-cat−/− kidneys.

The present study indicates that activation of β-catenin may promote tubular epithelial cell survival by a multitude of mechanisms *in vivo*. Loss of β-catenin results in Bax induction in renal tubules after injury, suggesting that Bax, a pro-apoptotic member of Bcl-2 family proteins, may play a critical role in mediating pro-apoptotic effect of β-catenin ablation *in vivo*. This notion is in line with previous *in vitro* studies,[35] and is substantiated by the observations that either activation of endogenous β-catenin by expressing Wnt1 or ectopic expression of exogenous β-catenin effectively prevents Bax induction and tubular epithelial cell apoptosis after incubation with staurosporine, an apoptosis inducer (Figure 8). It is conceivable that p53, a tumor suppressor with pro-apoptotic activity, could be an upstream regulator that is responsible for Bax induction, as it is induced in Ksp-β-cat−/− kidneys as well after folic acid injury (Figure 6). It has been shown that p53, as a transcription factor, regulates Bax by controlling its transcription.[40] Furthermore, p53 also exerts its pro-apoptotic activity in a transcription-independent fashion by interacting with Bax, which results in promotion of Bax activation as well as its insertion into the mitochondrial membrane.[40, 41]

Loss of β-catenin also leads to reduction of Akt phosphorylation and survivin expression, two pro-survival signals. Akt, a protein kinase activated by phosphatidylinositol 3-kinase (PI3K), is an essential regulator of cell survival/apoptosis.[42] Although β-catenin might activate Akt through stimulating its upstream PI3K and/or inducing its expression,[35] our results indicate that β-catenin ablation inhibits Akt phosphorylation, but does not affect Akt abundance *in vivo* (Figure 6). As Akt can phosphorylate Bax leading to its inactivation, a reduced Akt phosphorylation would enhance the pro-apoptotic activity of Bax. Similarly, survivin, a member of the IAPs family proteins that promotes cell survival by inhibiting caspase activity, is a direct transcription target of β-catenin.[31, 43] Therefore, loss of β-catenin inevitably reduces the expression of this survival gene. Taken together, as summarized in Figure 6f, deletion of β-catenin in a tubule-specific fashion leads to an increased apoptosis after AKI by multiple mechanisms. On one hand, β-catenin ablation causes p53 induction and Akt inhibition, resulting in Bax induction and activation, respectively, which lead to subsequent activation of caspases (Figure 6f). On the other hand, loss of β-catenin reduces survivin expression, thereby effectively eliminating the negative inhibitor of caspases (Figure 6f). Undoubtedly, these effects resulted from loss of β-catenin would make tubular

cells extremely vulnerable to injury, leading to an enhanced tubular cell apoptosis. In addition, as Akt is also involved in promoting tubular cell proliferation,[44] reduced Akt activation in β-catenin-deficient tubules may contribute to AKI via an impaired renal regeneration.

It is interesting to point out that β-catenin is also important in mediating cell-cell adhesion, and is a constituent of adherens junctions where it links E-cadherin to the actin cytoskeleton. However, tubule-specific ablation of β-catenin seems not to cause any phenotypic abnormality, suggesting that β-catenin is dispensable for maintaining the tubular integrity and homeostasis in adult kidneys. This observation is also in line with recent reports demonstrating that genetic deletion of β-catenin in various tissues including glomerular podocytes, hepatocytes and cardiomyocytes does not cause significant pathologic lesions.[13, 45–47] Although β-catenin is a component of cell adherens junction complex, our studies indicate that its function as a structural protein can be substituted by γ-catenin, also known as plakoglobin, a structurally related protein that also binds to E-cadherin (Figure 3e).[46, 47]

In summary, we report herein that β-catenin is induced in mouse models of AKI induced by folic acid or IRI, and loss of endogenous β-catenin in a tubule-specific fashion aggravates kidney injury by promoting apoptosis via multiple mechanisms. These findings suggest that renal activation of β-catenin after AKI is a protective response in attempt to minimize cell damage. This seems in sharp contrast to the setting of chronic kidney diseases, in which sustained activation of β-catenin after chronic injury is shown to lead to tubular epithelial-mesenchymal transition (EMT) and renal fibrogenesis.[17, 19, 20] Therefore, a better understanding of the role and mechanism of endogenous β-catenin signaling in different settings would be essential for designing rational strategies for therapeutic interventions. Clearly, more studies are needed in this area.

Materials and methods

Mice and Genotyping

Homozygous β-catenin floxed mice (C57BL/6J background) were obtained from the Jackson Laboratories (Bar Harbor, ME), as described previously.[13] Transgenic mice that expressed Cre recombinase under the control of kidney-specific Ksp-cadherin promoter (ksp-Cre) was reported elsewhere.[37] By mating β-catenin floxed mice with Ksp-Cre transgenic mice, conditional knockout mice (Ksp-β-cat−/−) in which β-catenin gene was specifically disrupted in renal tubular epithelial cells (genotype β-cat$^{fl/fl}$, Cre+/−) were created. These mice were cross-bred with homozygous β-catenin floxed mice (genotype β-cat$^{fl/fl}$) to generate offspring with 50% Ksp-β-cat−/− mice and 50% control mice (β-catenin floxed mice) within the same litters. A routine PCR protocol was used for genotyping of tail DNA samples with the following primer pairs: Cre transgene, 5′-AGG-TGT-AGA-GAA-GGC-ACT-TAGC-3′ and 5′-CTA-ATC-GCC-ATC-TTC-CAG-CAG-G-3′, which generated a 411-bp fragment; and β-catenin genotyping, 5′-AAGGTA-GAG-TGA-TGA-AAG-TTG-TT-3′ and 5′-CAC-CAT-GTCCTC-TGT-CTA-TTC-3′, which yielded 324-bp band for the floxed alleles. All animals were born normally at the expected Mendelian frequency; and they were normal in size and did not display any gross physical or behavioral abnormalities.

Animal experiments were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

Mouse models of Acute Kidney Injury

Acute kidney injury in mice was induced by a single intraperitoneal injection of folic acid (Sigma, St. Louis, MO) dissolved in 150 mM sodium bicarbonate (vehicle) at 250 mg/kg body weight, as described previously in rats.[21] At 48 hours after injection, mice were sacrificed, and serum and kidney samples were collected for various analyses. We also utilized another AKI model by inducing renal ischemia/reperfusion injury (IRI).[36] Briefly, after mice were anesthetized, a midline abdominal incision was made and bilateral renal pedicles were clipped for 35 minutes using microaneurysm clamps. After removal of the clamps, reperfusion of the kidneys was visually confirmed. The incision was then closed and the animal was allowed to recover. During the ischemic period, body temperature was maintained between 35~37.5°C using a temperature-controlled heating system. Blood and tissue samples were obtained at 24 hours post-IRI.

Determination of Serum Creatinine

Serum was collected from mice at 48 hours after folic acid injection. Serum creatinine level was determined by use of a QuantiChrom creatinine assay kit, according to the protocols specified by the manufacturer (BioAssay Systems, Hayward, CA). The level of serum creatinine was expressed as milligrams per 100 ml (dl).

Histology and Immunohistochemical Staining

Paraffin-embedded mouse kidney sections (3-μm thickness) were prepared by a routine procedure. The sections were stained with hematoxylin-eosin (HE), periodic acid-Schiff (PAS) reagent by standard protocol. Immunohistochemical staining was performed according to the established protocol as described previously.[19] The antibodies used were as follows: rabbit polyclonal to β-catenin (ab15180; Abcam, Cambridge, MA) and rabbit polyclonal to Bax (sc-493; Santa Cruz Biotechnology, Santa Cruz, CA).

Immunofluorescence Staining and Confocal Microscopy

Kidney cryosections were fixed with 3.7% paraformalin for 15 min at room temperature and immersed in 0.2% Triton X-100 for 10 min. After blocking with 10% donkey serum in PBS for 1 hour, slides were double immunostained with anti-β-catenin and one of the following antibodies: anti-aquaporin 1 (AQP1; sc-9878, Santa Cruz Biotechnology, Santa Cruz, CA), anti-aquaporin 3 (AQP3; AB2219, Millipore, Billerica, MA), anti-Tamm-Horsfall protein(THP; sc-19554, Santa Cruz Biotechnology), or anti-Thiazide-sensitive Nacl Cotransporter(NCC; AB3553, Millipore) to determine β-catenin expression in different tubule segments, as described previously.[48] To visualize the primary antibodies, slides were stained with cyanine Cy2- or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Stained slides were viewed under a Leica TCS-SL confocal microscope equipped with a digital camera (Buffalo Grove, IL).

Detection of Apoptotic Cells

Apoptotic cell death was determined by using terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) staining with DeadEnd Colorimetric or Fluorometric Apoptosis Detection System (Promega, Madison, WI), as described previously.[49]

Real-Time RT-PCR

Total RNA isolation and real-time RT-PCR were carried out by the procedures described previously.[20] Briefly, the first strand cDNA synthesis was carried out by using a reverse transcription system kit according to the instructions of the manufacturer (Promega). Quantitative, real-time RT-PCR (qRT-PCR) was performed on ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) as described previously.[20] The sequences of the primer pairs were as follows: mouse survivin, 5-GTT TGA GTC GTC TTG GCG-3 (sense) and 5-TCA GGC TCG TTC TCG GTA-3 (anti-sense); human survivin, 5-GCA CCA CTT CCA GGG TTT ATT C-3 (sense) and 5-TCT CCT TTC CTA AGA CAT TGC TAA GG-3. PCR was run by using standard conditions. The mRNA levels of various genes were calculated after normalizing with β-actin.

Western Blot Analysis

Kidney tissues were lysed with radioimmune precipitation assay (RIPA) buffer containing 1% NP-40, 0.1% SDS, 100 μg/ml PMSF, 1% protease inhibitor cocktail, and 1% phosphatase I and II inhibitor cocktail (Sigma) in PBS on ice. The supernatants were collected after centrifugation at 13,000×*g* at 4°C for 15 min. Protein expression was analyzed by Western blot analysis as described previously.[33] The primary antibodies used were as follows: anti-β-catenin (#610154; BD Transduction Laboratories, San Jose, CA), anti-cleaved caspase-3 (#9661), anti-Akt (#4685), anti-phospho-Akt (Ser473) (#4060), anti-E-cadherin ((#3195), anti-γ-catenin (#2309) (Cell Signaling Technology, Danvers, MA), anti-Bax (sc-493), anti-p53 (sc-6243), anti-actin (sc-1616) (Santa Cruz Biotechnology), and anti-GAPDH (AM4300; Ambion, Austin, TX).

Co-immunoprecipitation

Co-immunoprecipitation was carried out by using an established method.[50] Briefly, kidneys from control and Ksp-β-cat−/− mice were lysed on ice in 1 ml of non-denaturing lysis buffer that contained 1% Triton X-100, 0.01 M Tris-HCl (pH 8.0), 0.14 M NaCl, 0.025%NaN3, 1% protease inhibitors cocktail, and 1% phosphatase inhibitors cocktail I and II (Sigma). After preclearing with normal IgG, kidney lysates (0.5 mg of protein) were incubated overnight at 4°C with 2 μg of anti-E-cadherin (#610182; BD Transduction Laboratories, San Jose, CA), followed by precipitation with 100 μl of protein A/G Plus-agarose for 3 hours at 4°C. The precipitated complexes were separated on SDS-PAGE and immunoblotted with specific antibodies against E-cadherin (#3195; Cell Signaling Technology), β-catenin (#610154; BD Transduction Laboratories) and γ-catenin (#2309, Cell Signaling Technology), respectively.

Cell Culture and Transfection

Human proximal tubular epithelial cell line (HKC, clone 8) was provided by Dr. L. Racusen of the Johns Hopkins University (Baltimore, MD). Cells were maintained as described previously.[9] Serum-starved HKC-8 cells were transfected with Wnt1 expression vector (pHA-Wnt1), N-terminally truncated, constitutively active β-catenin expression vector (pDel-β-cat), or empty plasmid pcDNA3, respectively. For some experiments, the transfected cells were collected at 24 and 48 hours after transfection, and total RNA was prepared for qRT-PCR analysis. The transfected cells were also treated at 48 hours after transfection with 1 μM staurosporine (S4400; Sigma, St. Louis, MO) for various periods of time as indicated to induce apoptosis, and then subjected to TUNEL staining and Western blot analyses, respectively.

Statistical Analyses

All data were expressed as mean ± SEM. Statistical analysis of the data was performed using SigmaStat software (Jandel Scientific Software, San Rafael, CA). Comparison between groups was made using one-way ANOVA, followed by the Student-Newman-Keuls test. $P < 0.05$ was considered significant.

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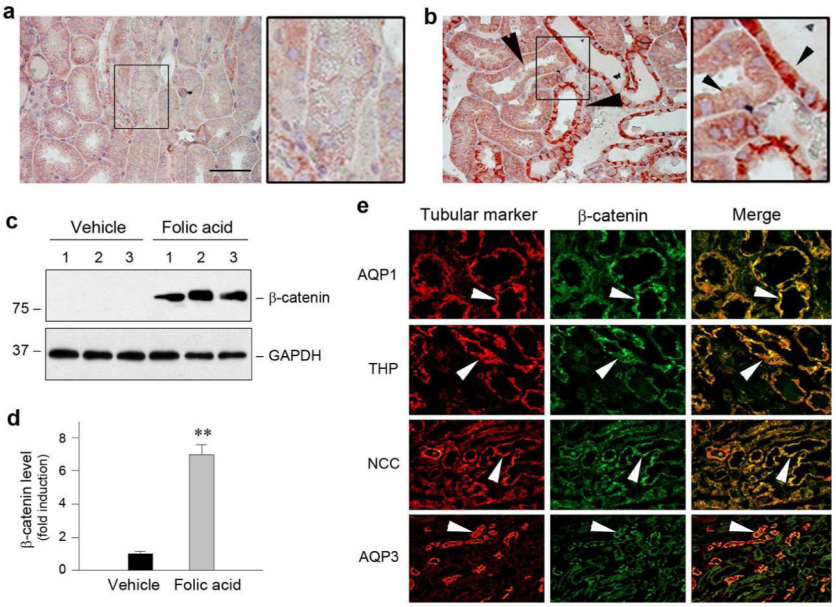

Figure 1. Up-regulation of renal β-catenin in mouse model of acute kidney injury induced by folic acid

(**a, b**) Immunohistochemical staining shows the expression and localization of β-catenin in the kidneys at 2 days after folic acid injection. Arrowheads indicate positive staining. Boxed area was enlarged. Scale bar, 50 μm. (**c, d**) Western blots demonstrate renal β-catenin protein levels at 2 days after folic acid injection. Representative Western blot (**c**) and quantitative data (**d**) are presented. Numbers (1, 2 and 3) indicate each individual animal in a given group. $^{**}P < 0.01$ versus vehicle controls (n = 4). (**e**) Co-staining for β-catenin and tubular segment-specific markers in the injured kidneys after folic acid injection. Immunofluorescence staining demonstrated the co-staining of β-catenin (green) and various tubular markers (red) in the kidneys at 2 days after folic acid injection. Segment-specific tubular markers used are as follows: proximal tubule, aquaporin-1 (AQP1); cortical thick ascending limb, Tamm-Horsfall glycoprotein (THP); distal tubule, thiazide-sensitive NaCl cotransporter (TSC)/NCC; and collecting duct, aquaporin-3 (AQP3). Arrowheads indicate β-catenin-positive tubules.

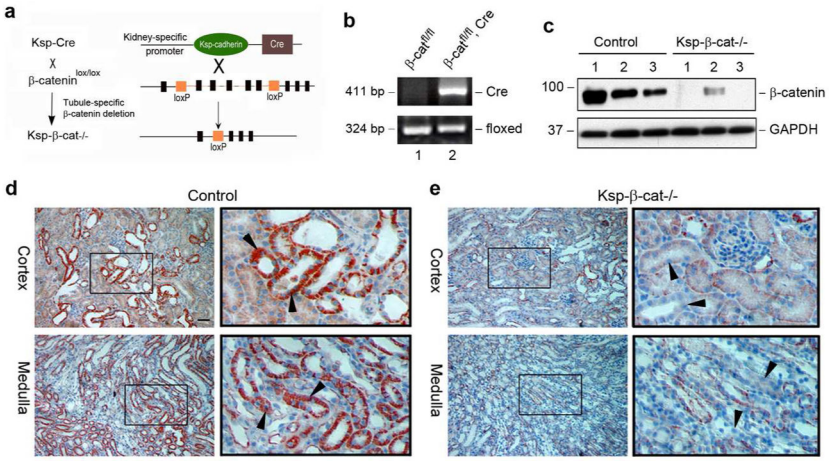

Figure 2. Generation of conditional knockout mice with tubule-specific ablation of endogenous β-catenin

(**a**) Experimental design shows the strategy of cross-breeding of the β-catenin floxed mice (β-cat$^{fl/fl}$) with Cre transgenic mice under the control of Ksp-cadherin promoter (Ksp-Cre). Black boxes indicate the exons of β-catenin gene. Orange boxes denote LoxP site. (**b**) Genotyping of the mice by PCR analysis of genomic DNA. Lane 1 shows the genotyping of the control mice used in this study (genotype: β-cat$^{fl/fl}$), whereas lane 2 denotes the genotyping of the tubule-specific β-catenin knockout mice (genotype: β-catenin$^{fl/fl}$,Cre), designated as Ksp-β-cat−/−. (**c**) Western blot analyses demonstrated a substantial reduction of renal β-catenin protein in Ksp-β-cat−/− mice. Kidney lysates were made from control and Ksp-β-cat−/− mice at 2 days after folic acid injection, and immunoblotted with specific antibodies against β-catenin and GAPDH, respectively. Numbers (1, 2 and 3) indicate each individual animal in a given group. (**d, e**) Representative micrographs show renal β-catenin staining in the control (**d**) and Ksp-β-cat−/− (**e**) mice at 2 days after folic acid injection. Boxed areas in cortex and medulla regions are enlarged. Arrowheads indicate renal tubules. Scale bar, 50 μm.

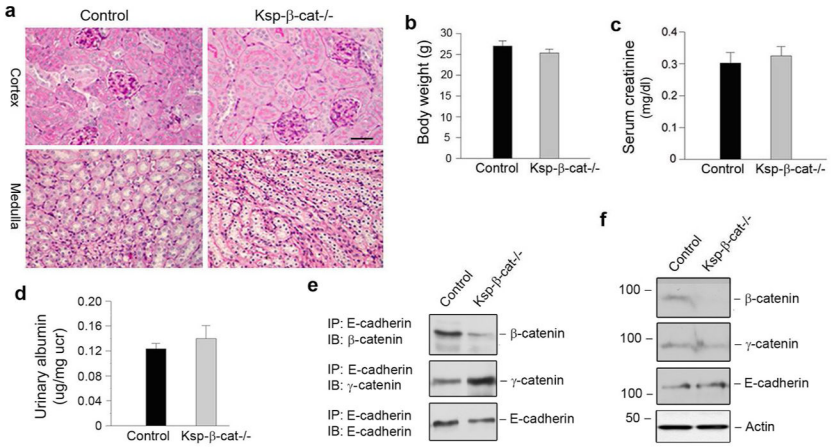

Figure 3. Mice with tubule-specific ablation of endogenous β-catenin are phenotypically normal

(**a**) Representative micrographs show the morphology of control and Ksp-β-cat−/− kidneys. Cortical and medullar regions were shown. Scale bar, 50 μm. (**b–d**) There was no difference in body weight (b), serum creatinine (**c**) and urinary albumin level (**d**) between control and Ksp-β-cat−/− mice (n = 3). (**e**) γ-catenin functionally compensates for the lost β-catenin at adherens junctions by augmenting interaction with E-cadherin in the kidneys of Ksp-β-cat−/− mice. Kidney lysates from control and Ksp-β-cat−/− mice were immunoprecipitated with anti-E-cadherin antibody, followed by immunoblotting with antibodies against β-catenin, γ-catenin and E-cadherin, respectively. (**f**) Loss of tubular β-catenin did not affect the expression of γ-catenin and E-cadherin in the kidneys. Kidney lysates were immunoblotted with antibodies against β-catenin, γ-catenin, E-cadherin and actin, respectively.

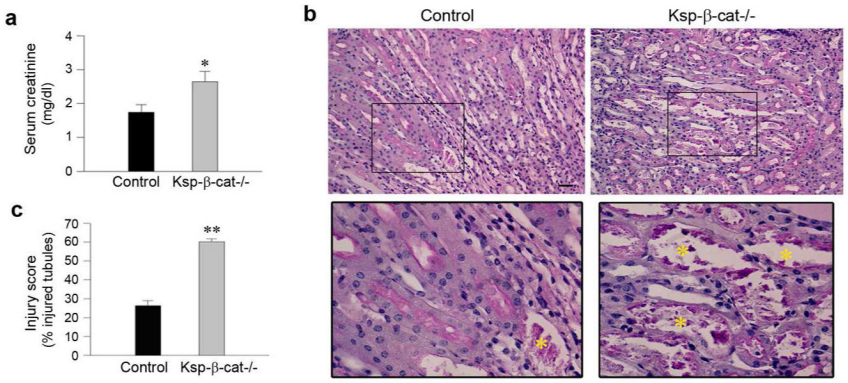

Figure 4. Tubule-specific ablation of β-catenin aggravates acute kidney injury induced by folic acid in mice

(**a**) Serum creatinine level in control and Ksp-β-cat−/− mice at 2 days after folic acid injection. **P* < 0.05 (n = 9–10). (**b, c**) Morphological injury assessed in the PAS-stained kidney sections in control and Ksp-β-cat−/− mice. Representative micrographs of the kidneys at 2 days after folic acid injection (**b**) and quantitative assessment of injury (**c**) are presented. Yellow asterisks in the enlarged boxed areas indicate injured tubules. ***P* < 0.01 (n = 4).

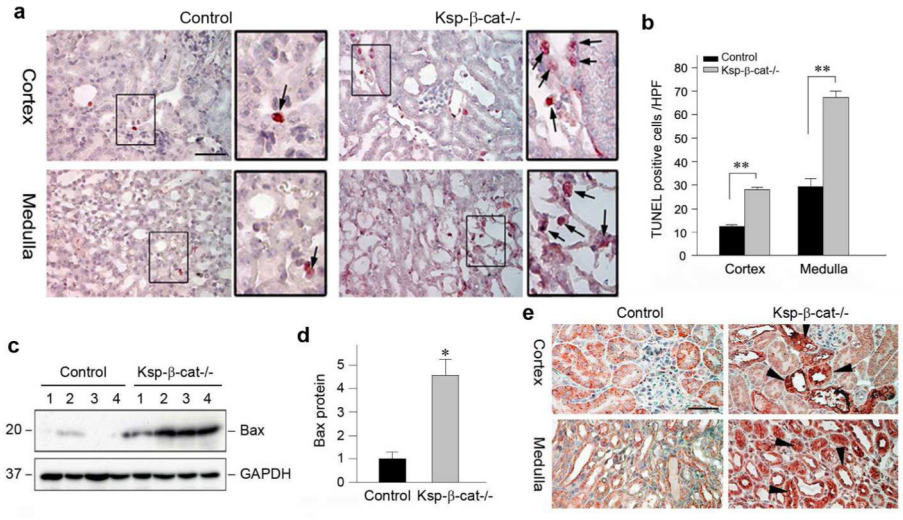

Figure 5. Tubule-specific ablation of β-catenin promotes apoptosis and Bax expression after acute kidney injury

(**a, b**) Tubule-specific loss of β-catenin aggravates apoptosis in acute kidney injury. (**a**) Representative micrographs show apoptotic cell death detected by TUNEL staining. Arrows in the enlarged boxed areas indicate apoptotic cells. Scale bar, 50 μm. (**e**) Quantitative determination of apoptotic cells in renal cortex and medulla regions at 2 days after folic acid injection. Data are presented as numbers of apoptotic cells per high power field (HPF). ***P* < 0.01 (n = 4). (**c, d**) Loss of β-catenin promoted renal Bax protein expression. Representative Western blot data (**c**) and quantitative analysis (**d**) are presented. Numbers (1, 2, 3 and 4) indicate each individual animal in a given group. **P* < 0.05 (n = 4). (**e**) Representative micrographs showed immunohistochemical staining for Bax in the kidneys at 2 days after folic acid injection. Arrows indicate Bax-positive tubules in renal cortex and medulla. Scale bar, 50 μm.

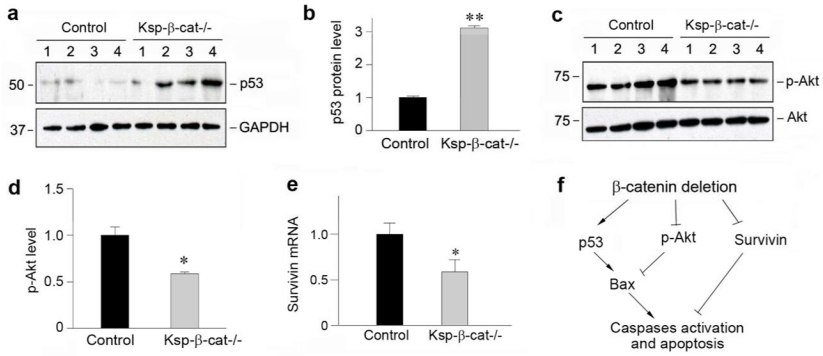

Figure 6. Tubule-specific ablation of β-catenin induced p53 and inhibited Akt phosphorylation and surviving expression *in vivo*

(**a, b**) Western blot analyses demonstrated that tubule-specific ablation of endogenous β-catenin induced renal expression of p53 protein. Numbers (1, 2, 3 and 4) indicate each individual animal in a given group. Quantitative data are presented in Panel (**b**). $^{**}P < 0.01$ (n = 4). (**c, d**) Tubule-specific ablation of endogenous β-catenin suppressed renal Akt phosphorylation. Kidney lysates at 2 days after folic acid injection were immunoblotted with specific antibodies against phosphorylated Akt (Ser473) and total Akt, respectively. Numbers (1, 2, 3 and 4) indicate each individual animal in a given group. Quantitative data are presented in Panel (**d**). $^{*}P < 0.05$ (n = 4). (**e**) qRT-PCR demonstrated a decreased expression of survivin mRNA in Ksp-β-cat−/− kidneys. $^{*}P < 0.05$ (n = 5–6). (**f**) Diagram shows the potential pathways leading to apoptosis induced by the tubule-specific ablation of endogenous β-catenin.

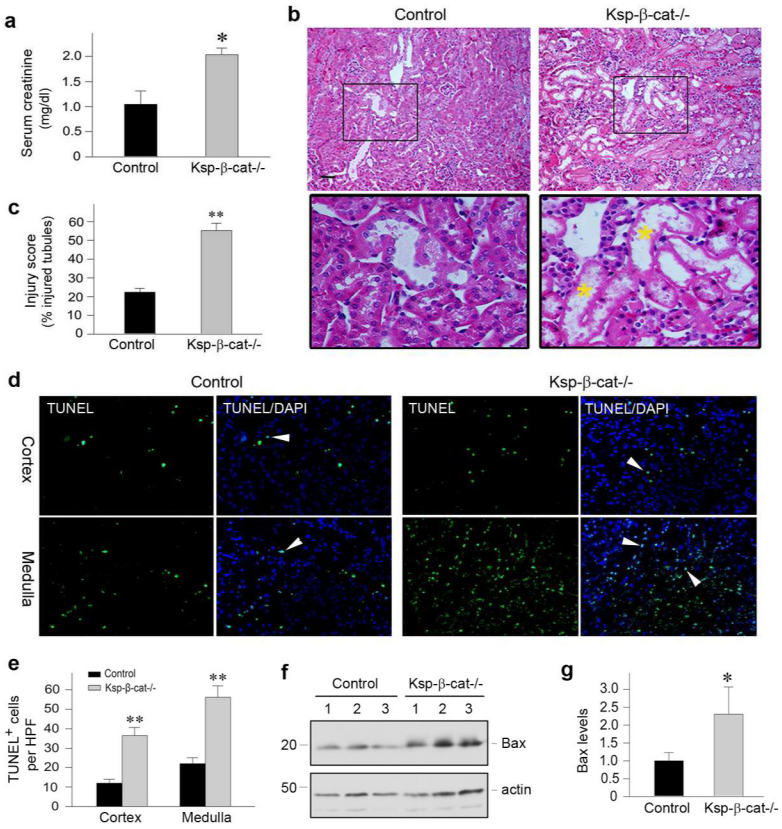

Figure 7. Loss of tubular β-catenin aggravates AKI in renal ischemia/reperfusion injury model

(**a**) Serum creatinine level in control and Ksp-β-cat−/− mice at 1 day after renal ischemia/reperfusion injury. **P* < 0.05 (n=3–4). (**b**) Representative micrographs of the kidneys in control and Ksp-β-cat−/− mice at 1 day after renal ischemia/reperfusion injury. Yellow asterisks in the enlarged boxed areas indicate injured tubules. Scale bar, 50 μm. (**c**) Quantitative assessment of renal injury. Injury score (% of injured tubules) are presented. ***P* < 0.01 (n=3). (**d, e**) Tubule-specific ablation of β-catenin aggravates apoptosis after renal ischemia/reperfusion injury. (**d**) Representative micrographs show apoptotic cell death detected by TUNEL staining. White arrows indicate apoptotic cells. (**e**) Quantitative determination of apoptotic cells in renal cortical and medullar regions at 1 day after renal ischemia/reperfusion injury. Data are presented as numbers of apoptotic cells per high power field (HPF). ***P* < 0.01 (n = 3). (**f, g**) Loss of β-catenin promoted renal Bax protein expression in ischemic AKI. Western blot (**f**) and quantitative analysis (**g**) are presented. Numbers (1, 2 and 3) indicate each individual animal in a given group. **P* < 0.05 (n= 3).

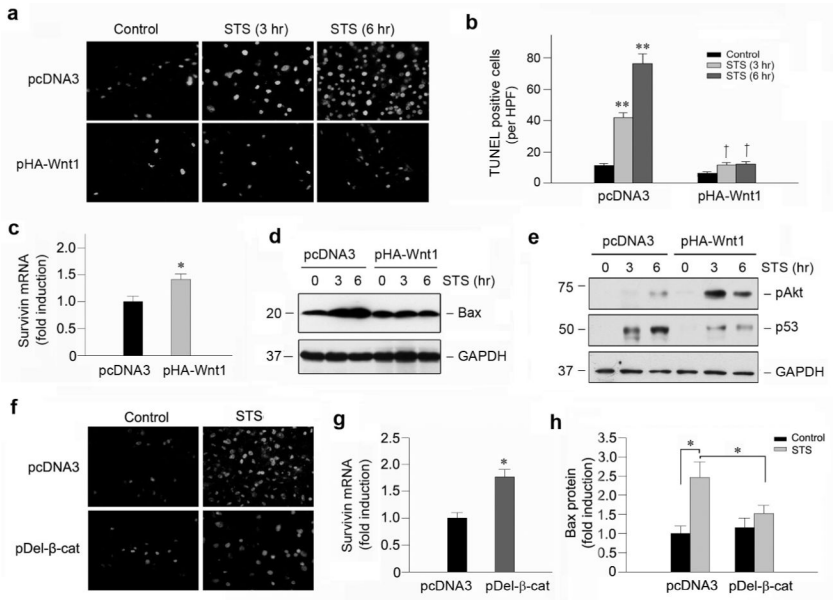

Figure 8. Activation of endogenous β-catenin promotes kidney tubular cell survival *in vitro*.

(**a, b**) Ectopic expression of exogenous Wnt1 protects tubular cells against apoptosis induced by staurosporine (STS). HKC-8 cells were transfected with Wnt1 expression vector (pHA-Wnt1) or empty vector (pcDNA3), followed by treatment with STS (1 μM) for various periods of time as indicated. Apoptosis was assessed by TUNEL staining. Representative micrographs of TUNEL staining (**a**) and quantitative data of apoptotic cells per high power field (HPF) (**b**) are presented. $^{**}P < 0.01$ versus vehicle control (n = 3); †$P < 0.05$ versus pcDNA3 control (n = 3). (c) qRT-PCR showed that activation of endogenous β-catenin by Wnt1 induced survivin mRNA expression in tubular epithelial cells. $^{*}P < 0.05$ (n = 3). (**d, e**) Ectopic expression of Wnt1 prevented Bax (**d**) and p53 (**e**) expression induced by STS in tubular epithelial cells. Wnt1 also promoted Akt phosphorylation after STS incubation (**e**). (**f–h**) Ectopic expression of stabilized β-catenin also prevented tubular cell apoptosis (**f**), induced survivin expression (**g**) and blocked STS-induced Bax expression (**h**). HKC-8 cells were transfected with N-terminally truncated, stabilized β-catenin (pDel-cat) or empty vector (pcDNA3), respectively. Apoptosis was assessed by TUNEL staining after incubation with STS (1 μM) for 6 hours (**f**). Survivin mRNA expression was detected by qRT-PCR (**g**). Bax protein expression was assessed by Western blot and quantified (**h**). $^{*}P < 0.05$ (n = 3).