Impaired Wound Healing in Hypoxic Renal Tubular Cells: Roles of Hypoxia-Inducible Factor-1 and Glycogen Synthase Kinase 3 β / β -Catenin Signaling

Jianping Peng, Ganesan Ramesh, Lin Sun, and Zheng Dong

Department of Cellular Biology and Anatomy (J.P., Z.D.), Vascular Biology Center (G.R.), Georgia Health Sciences University, Augusta, Georgia; Department of Nephrology, Second Xiangya Hospital, Central South University, Changsha, Hunan, China (L.S.); Department of Urology, Renmin Hospital, Wuhan University, Wuhan, China (J.P.); and Renal Research Laboratory, Charlie Norwood VA Medical Center, Augusta, Georgia (Z.D.)

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ABSTRACT

Wound and subsequent healing are frequently associated with hypoxia. Although hypoxia induces angiogenesis for tissue remodeling during wound healing, it may also affect the healing response of parenchymal cells. Whether and how wound healing is affected by hypoxia in kidney cells and tissues is currently unknown. Here, we used scratch-wound healing and transwell migration models to examine the effect of hypoxia in cultured renal proximal tubular cells (RPTC). Wound healing and migration were significantly slower in hypoxic (1% oxygen) RPTC than normoxic (21% oxygen) cells. Hypoxia-inducible factor-1 α (HIF-1 α) was induced during scratch-wound healing in normoxia, and the induction was more evident in hypoxia. Nevertheless, HIF-1 α -null and wild-type cells healed similarly after scratch wounding. Moreover, activation of HIF-1 α with di-

methyloxalylglycine in normoxic cells did not suppress wound healing, negating a major role of HIF-1 α in wound healing in this model. Scratch-wound healing was also associated with glycogen synthase kinase 3 β (GSK3 β)/ β -catenin signaling, which was further enhanced by hypoxia. Pharmacological inhibition of GSK3 β resulted in β -catenin expression, accompanied by the suppression of wound healing and transwell cell migration. Ectopic expression of β -catenin in normoxic cells could also suppress wound healing, mimicking the effect of hypoxia. Conversely, inhibition of β -catenin via dominant negative mutants or short hairpin RNA improved wound healing and transwell migration in hypoxic cells. The results suggest that GSK3 β / β -catenin signaling may contribute to defective wound healing in hypoxic renal cells and tissues.

Introduction

Wound healing in tissues and organs is characterized by hypoxia, a condition of decreased availability of oxygen. Hypoxia in wounded tissues is caused in part by the vascular damage and decreased blood supply, but also, in a large part, by the O₂ consumption of the cells in the wound that are metabolically activated for migration, proliferation, and wound healing (Tandara and Mustoe, 2004). Under this con-

dition, hypoxia contributes to the stimulation of the angiogenesis and tissue remodeling by activating a myriad of signaling pathways and inducing key transcription factors such as hypoxia-inducible factors (HIFs) (Tandara and Mustoe, 2004). In wounded skin, hypoxia has also been shown to promote the migration of keratinocytes for restoration of the epithelium (Benizri et al., 2008). However, whether and how hypoxia affects wound healing in parenchymal cells in injured organs, such as the kidneys, is unknown.

HIF is a family of transcription factors that are induced by hypoxia to regulate gene expression (Semenza, 2007). Recent studies have further revealed HIF activation by nonhypoxic conditions or stimuli. HIF consists of α and β subunits. HIF β is constitutively expressed, whereas HIF α is hydroxylated at several proline and asparagine sites in the presence of oxygen, targeting it for von Hippel-Lindau-mediated ubiquitination and proteosomal degradation. In hypoxia, the lack of oxygen prevents HIF α hydroxylation and degradation, lead-

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ABBREVIATIONS: HIF, hypoxia-inducible factor; DMOG, dimethyloxalylglycine; GSK3 β , glycogen synthase kinase 3 β ; MEF, mouse embryonic fibroblast; RPTC, renal proximal tubular cells; LiCl, lithium chloride; shRNA, short hairpin RNA; HEK, human embryonic kidney; PI, propidium iodide; SB216763, 3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione.

ing to HIF α accumulation and dimerization with HIF β to form a functional transcription factor (Semenza, 2007; Kaelin and Ratcliffe, 2008). Pharmacological inhibitors of HIF α prolyl hydroxylase, such as dimethylxaloylglycine (DMOG), can suppress HIF α hydroxylation and induce HIF α under normoxia. During ischemia-reperfusion, an *in vivo* condition of hypoxia, HIF family members are induced to regulate tissue repair and remodeling. In kidneys, HIF-1 is induced in renal tubules, whereas HIF-2 is expressed by interstitial cells (Rosenberger et al., 2002; Sutton et al., 2008). Although HIF induction has been shown to protect kidney tissues against ischemic and nephrotoxic injury (Bernhardt et al., 2006; Weidemann et al., 2008; Ma et al., 2009), it remains unclear whether it does so by directly protecting renal tubular cells, promoting wound healing in these cells, or enhancing angiogenesis and tissue remodeling (Haase, 2006; Hill et al., 2008; Gunaratnam and Bonventre, 2009).

Another important signaling pathway that may contribute to the regulation of wound healing involves glycogen synthase kinase 3 β (GSK3 β) and β -catenin. In unstimulated cells, β -catenin exists in a multiprotein complex with GSK3 β , axin, and adenomatous polyposis coli. Phosphorylation of β -catenin by GSK3 β in the complex results in the degradation of β -catenin. Activation of Wnt signaling leads to GSK3 β phosphorylation and inactivation, resulting in the accumulation of β -catenin, which may translocate into the nucleus to induce gene expression. GSK3 β / β -catenin signaling plays a critical role in nephron formation in the early stage of kidney development (Carroll et al., 2005; Iglesias et al., 2007; Lyons et al., 2009). In adult kidneys, changes of GSK3 β / β -catenin signaling in renal tubules, podocytes, and interstitial cells are associated with numerous kidney diseases, including acute kidney injury, diabetic nephropathy, renal cancers, cystic kidney diseases, albuminuria, and renal fibrosis (Pulkkinen et al., 2008; Dai et al., 2009; He et al., 2009; Wang et al., 2009b, 2010). In kidney interstitial cells, β -catenin promotes fibrosis (He et al., 2009). In podocytes, β -catenin promotes podocyte dysfunction, inducing albuminuria, and blockade of Wnt/ β -catenin signaling by paricalcitol ameliorates adriamycin-induced proteinuria and kidney injury (Dai et al., 2009; He et al., 2011). In renal tubular cells, β -catenin promotes cell survival by inhibiting Bax, a well documented proapoptotic protein, whereas GSK3 β promotes tubular apoptosis after acute ischemic kidney injury (Wang et al., 2009b, 2010). Although these studies have suggested a role of GSK3 β / β -catenin signaling in acute kidney cell injury, whether and how this signaling pathway contributes to kidney repair or wound healing after initial injury remains poorly understood.

In this study, we first demonstrated the inhibitory effect of hypoxia on wound healing in renal tubular cells. We then examined the roles played by HIF-1 and GSK3 β / β -catenin signaling in the wound-healing defect of hypoxic cells. The results suggest that GSK3 β / β -catenin signaling, but not HIF, contributes to the wound-healing defect of renal proximal tubular cells during hypoxia.

Materials and Methods

Antibodies and Special Reagents. Antibodies were purchased from the following sources: polyclonal anti- β -catenin, antiphospho-GSK3 β -ser9, and anti-total-GSK3 β from Cell Signaling Technology

(Danvers, MA), monoclonal anti-HIF-1 α from BD Biosciences (San Jose, CA), the secondary antibodies for immunoblot analysis from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA), and the secondary antibodies for immunofluorescence from Millipore (Billerica, MA). Lithium chloride (LiCl), 3-(2,4-dichlorophenyl)-4-(1-methyl-1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione (SB216763), and DMOG were purchased from Acros Organics (Fairlawn, NJ), Tocris Bioscience (Ellisville, MO), and Sigma (St. Louis, MO), respectively.

Cell Culture. Immortalized rat kidney proximal tubular cells (RPTC) were originally obtained from Dr. Ulrich Hopfer (Case Western Reserve University, Cleveland, OH). Human embryonic kidney (HEK) 293 cells were purchased from the American Type Culture Collection (Manassas, VA). Wild-type and HIF-1 α -null mouse embryonic fibroblasts (MEFs) were obtained from Dr. Gregg Semenza (The Johns Hopkins University, Baltimore, MD) (Zhang et al., 2008).

Hypoxic Incubation. RPTC were plated at a density of 1×10^6 cells/35-mm dish or 2.5×10^6 cells/60-mm dish to grow overnight for experiment. Hypoxia treatment was conducted in a hypoxia chamber as described previously (Wang et al., 2006). In brief, dishes with cells were transferred into a hypoxia chamber (COY Laboratory Products, Ann Arbor, MI) containing 1% oxygen. The oxygen tension in the chamber was monitored and maintained by a computerized sensor probe. Humidity was maintained with a humidifier.

Scratch-Wound Healing Assay. For morphological examination, a scratch-wound healing model was modified from Zhuang et al. (2011). In brief, a monolayer of confluent RPTC grown in a 35-mm dish was linearly scratched with a sterile 1000- μ l pipette tip. Phase-contrast images were recorded at 0 and 18 h after scratching. The width of the wound was measured at various time points to determine the healed distance. For biochemical analysis, multiple uniformed wounds were created in a RPTC monolayer in 60-mm dishes with a wounding device that makes concentric circular 130- μ m-wide cell bands separated by 750- μ m-wide wounds (Lan et al., 2010). Whole-cell lysate was collected at different time points after wounding for immunoblot analysis.

Transwell Cell Migration Assay. Transwell cell migration was measured as described previously (Wang et al., 2009a). In brief, the undersurfaces of transwells (Costar; Corning Life Sciences, Lowell, MA) were coated with 10 μ g/ml collagen I (Millipore) overnight at 4°C. Coated wells were then placed into a 24-well plate containing 600 μ l of culture medium. RPTC were detached and suspended at 1.5×10^6 cells/ml in culture medium. The cells were then added into transwells (200 μ l, 3×10^5 cells/well) and allowed to migrate for 6 h at 37°C. Cotton swabs were used to remove cells in the upper surface of the transwells, and migratory cells attached on the undersurface were stained with propidium iodide (PI). The numbers of migrated cells were counted by using an inverted microscope. To determine the effect of GSK3 inhibitors on cell migration, the inhibitors were added in the bottom-chamber medium. To determine the effect of β -catenin knockdown on cell migration, cells were infected with lentivirus containing scramble or β -catenin-short hairpin RNA (shRNA) sequence for 3 days before adding them to the transwell for cell migration assay.

Lentivirus-Mediated β -Catenin Overexpression. A mouse β -catenin cDNA plasmid (gift from Dr. Lin Mei, Georgia Health Sciences University, Augusta, GA) was used as the template for polymerase chain reaction-based deletion to generate active β -catenin (β -catenin_{152–781}) and dominant negative β -catenin (β -catenin_{152–694}), which were subcloned into the lentiviral vector pCDH-CMV-MCS-EF1-copGFP (System Biosciences, Mountain View, CA) at XbaI and NotI sites. The β -catenin plasmids were cotransfected into 293FT cells (Invitrogen, Carlsbad, CA) with three packaging plasmids (pLP1, pLP2, and pLP/VSV-G) to collect the culture medium at 48 h. The culture medium with packaged lentivirus was added to RPTC for 1 day of infection. The medium was then replaced with fresh medium for an additional 2 days of culture before the wound-healing test.

Lentiviral shRNA-Mediated β -Catenin Knockdown. Lentiviral shRNA plasmid targeting β -catenin and a scrambled nontargeting control plasmid were made by using the pLV-mU6-EF1a-GFP vector (Biosettia, San Diego, CA). The target sequence of the β -catenin shRNA was GCTGACCAAACTGCTAAATGA. The lentivirus package and transduction methods were the same as those described above for lentivirus-mediated β -catenin overexpression.

Immunofluorescence. RPTC grown on glass coverslips were scratch-wounded with a sterile 1000- μ l pipette tip and incubated in hypoxia or normoxia for 6 h. Then the cells were fixed with 4% paraformaldehyde and permeabilized with 0.4% Triton X-100 in a blocking buffer. The cells were then exposed to β -catenin antibody, followed by incubation with Cy3-labeled goat anti-rabbit secondary antibody. After washes, the coverslips were mounted on slides with Antifade for examination by fluorescence microscopy using the Cy3 channel.

Immunoblot Analysis. Protein concentration was determined by using the BCA reagent (Thermo Fisher Scientific, Waltham, MA). Equal amounts of protein were loaded in each well for electrophoresis by using the NuPAGE Gel System (Invitrogen), followed by transferring them onto polyvinylidene fluoride membranes. The blots were then incubated in blocking buffer for 1 h, then in primary antibodies overnight at 4°C. After washes, the blots were incubated with horseradish peroxidase-conjugated secondary antibody, and the antigens on the blots were revealed by using the enhanced chemiluminescence kit from Thermo Fisher Scientific.

Statistical Analysis. Quantitative data were expressed as means $\pm$ S.D. ($n \geq 4$). Statistical differences between two groups were determined by Student's *t* test. $p < 0.05$ was considered significantly different. Qualitative results, including immunoblots, were representatives of at least three separate experiments.

Results

Hypoxia Inhibits Scratch-Wound Healing and Migration in RPTC. Ischemia or hypoxia is a common feature of injured tissues, including kidneys (Haase, 2006; Gunaratnam and Bonventre, 2009). Whether and how wound healing is affected by hypoxia is largely unknown. We examined the effect of hypoxia in a scratch-wound healing model of cultured RPTC. In this model, RPTC were scratch-wounded with a sterile pipette tip and then incubated in hypoxia (1% O_2) or normoxia (21% O_2) for recovery or healing. Wounds were recorded and measured immediately after scratching (0

h) and at different time points postscratching. Representative wounds are shown in Fig. 1A. Under normoxia, most of the wound healed within 18 h. In contrast, a significant wound remained in the cells recovered under hypoxia. Measurement of the healed distance showed that wound healing was suppressed by hypoxia in a time-dependent manner (Fig. 1B).

In the scratch model, wound healing involves a rapid mobilization of the wound edge cells to migrate into the wound. We hypothesized that hypoxia might suppress wound healing in part by blocking cell migration. To test this possibility, we determined the effect of hypoxia on cell migration by using the transwell cell migration assay (Zhuang et al., 2011). RPTC were seeded in transwell inserts, which were then placed in the wells of 24-well plates containing culture medium under normoxia or hypoxia. The cells that migrated to the undersurface of the transwell inserts were recorded after PI staining and counted. As shown in Fig. 1C, apparently fewer cells migrated under hypoxia than under normoxia. Cell counting showed that in 6 h $\sim$ 720 cells migrated under normoxia, whereas $\sim$ 520 did so under hypoxia (Fig. 1D).

HIF-1 α Induction during Wound Healing. To understand the mechanism of wound-healing defects in hypoxic cells, we initially focused on HIFs, which mediate a variety of cellular responses to hypoxia (Semenza, 2007). Multiple circular wounds were created in RPTC with a “stamp wounding” device (Lan et al., 2010), which generated multiple uniformed wounds in the cell layer (Fig. 2A). After wounding, the cells were incubated under normoxia or hypoxia for various durations of healing. As shown in Fig. 2B, left, HIF-1 α was induced during wound healing in normoxic RPTC. The induction was detectable at 1 h postwounding, further increased at 3 and 6 h, and then decreased. In hypoxia, HIF-1 α induction during wound healing was stronger and lasted longer (Fig. 2B, right). A marked HIF-1 α induction was detected at 1 h postwounding in hypoxic cells, and the induction reached the highest level at 3 h. Thereafter HIF-1 α decreased but it remained higher than the basal level at 24 h (Fig. 2B, right). The time-dependent changes in HIF-1 α expression during scratch-wound healing were further confirmed by quantification via densitometry (Fig. 2C).

Fig. 1. Hypoxia inhibits scratch-wound healing and transwell migration in RPTC. A and B, scratch-wound healing. RPTC were scratch-wounded with a sterile pipette tip and then incubated under hypoxia (1% O_2) or normoxia (21% O_2). A, representative wounds immediately after scratch wounding and after 18 h of healing were recorded with a phase-contrast microscope. B, the wound width was measured at 6, 12, and 18 h after scratching to determine the healed distance. C and D, transwell migration. A total of 3×10^5 cells were added to each transwell insert, which was put in a 24-well plate with 600 μ l of culture medium in normoxia or hypoxia for 6 h. C, representative PI staining of migratory cells was recorded with a fluorescence microscope. D, migratory cells attached on the undersurface were counted after PI staining. In B and D, data are expressed as mean $\pm$ S.D. ($n = 6$). *, $p < 0.05$ versus normoxia.

Fig. 2. HIF-1 α induction during wound healing in normoxia and hypoxia. A, multiple uniform wounds made with a wounding device. RPTC grown in 60-mm dishes were pressed with a multiple-wounding device to induce concentric circular cell bands of 130- μ m width separated by wounds of 750- μ m width. B, time-dependent HIF-1 α induction during wound healing in normoxia and hypoxia. RPTC in 60-mm dishes were wounded by the multiple-wounding device and then incubated in normoxia or hypoxia to collect whole-cell lysates at different time points for immunoblot analysis of HIF-1 α and β -actin. C, densitometric analysis of HIF-1 α expression during scratch-wound healing. Immunoblots from three separate experiments were analyzed by densitometry, and the signal of HIF-1 α was normalized with control (0 h) that was arbitrarily set as 1. Data are expressed as mean $\pm$ S.D. ($n = 3$). *, $p < 0.05$ versus normoxia.

Pharmacological Induction of HIF Does Not Block Wound Healing. Wound healing was defective in hypoxic cells (Fig. 1) that had a notable HIF-1 α induction (Fig. 2B), suggesting that HIF-1 may contribute to the wound-healing defects under hypoxia. To test this possibility, we first determined the effects of DMOG, a pharmacological inducer of HIF that prevents HIF-1 α degradation under normoxia by inhibiting prolyl hydroxylases (Fraisl et al., 2009). It was reasoned that, if hypoxia suppresses wound healing via HIF induction, then HIF induction by DMOG may block wound healing in normoxic cells. Our pilot experiments titrated the condition of DMOG treatment. As shown in Fig. 3A, 6 h of wound healing led to HIF-1 α induction in both RPTC and HEK cells under normoxia, which was dramatically enhanced by DMOG. Despite marked HIF-1 induction, DMOG did not attenuate wound healing in either RPTC or HEK cells; instead it slightly improved wound healing in these cells (Fig. 3, B and C). The results therefore do not support the hypothesis that HIF induction accounts for the wound-healing defects in hypoxic cells.

Wound Healing Is Not Affected by HIF-1 Deficiency in MEF Cells. To further delineate the role of HIF in wound healing, we compared wild-type [HIF-1 α (+/+)] and HIF-1 α -null [HIF-1 α (-/-)] MEFs. As expected, HIF-1 α was induced by hypoxia during wound healing in HIF-1 α (+/+) MEF cells,

but not in HIF-1 α (-/-) cells (Fig. 4A). When these two genotype cells were subjected to scratch wounding, their healing was very similar in both normoxia and hypoxia (Fig. 4B). The results suggest that HIF-1 does not play a critical role in wound healing in this cellular model.

Activation of GSK3 β / β -Catenin Signaling during Wound Healing. In the canonical pathway of GSK3 β / β -catenin signaling, GSK3 β phosphorylates β -catenin, leading to its degradation. GSK3 β / β -catenin signaling plays an important role in wound healing (Cheon et al., 2002). In normoxic RPTC, wound healing induced GSK3 β phosphorylation at serine-9 (indicative of GSK3 β inactivation), which was accompanied by increased expression of β -catenin (Fig. 5A, left). GSK3 β phosphorylation and β -catenin expression were also induced during wound healing in hypoxic cells (Fig. 5A, right). It is noteworthy that compared with normoxic cells hypoxic cells showed higher GSK3 β phosphorylation at 3 h postwounding and higher β -catenin expression at all time points examined (Fig. 5, A–C). We further examined β -catenin expression by immunofluorescence (Fig. 5D). Under normoxia, some of the cells at the wound edge showed an increase in β -catenin staining. Under hypoxia, β -catenin staining was detected in more cells and seemed to be more intense. It was noted that in some of the hypoxic cells β -catenin staining appeared in nuclei, in addition to plasma

Fig. 3. DMOG induces HIF-1 α without affecting wound healing. A, RPTC and HEK cells were wounded with the multiple-wounding device and incubated in full medium without (-) or with (+) 100 mM DMOG in normoxia for 6 h to collect whole-cell lysates for immunoblot analysis of HIF-1 α . B and C, scratch-wound healing. RPTC and HEK cells were scratch-wounded and then incubated in normoxia without (-) or with (+) 100 mM DMOG for 18 h to measure the healed distance. Data are expressed as mean $\pm$ S.D. ($n = 6$).

Fig. 4. Wound healing in HIF-1 α -null cells. A, HIF-1 α induction during wound healing in hypoxia in HIF-1 α -null [HIF-1 α (-/-)] and wild-type [HIF-1 α (+/+)] MEF cells. MEF cells were wounded by the multiple-wound device and incubated in hypoxia to collect whole-cell lysates at different time points for immunoblot analysis of HIF-1 α and β -actin. B, scratch-wound healing. Wild-type and HIF-1 α -null MEF cells were scratch-wounded and then incubated in hypoxia for 18 h to measure the healed distance. Data are expressed as mean $\pm$ S.D. ($n = 6$).

membrane and cytoplasm (Fig. 5D, right). Together, these data demonstrated an enhanced GSK3 β / β -catenin signaling in hypoxic cells.

GSK3 β Inhibitors Suppress Wound Healing. Based on the above observations (Fig. 5), we hypothesized that the enhanced GSK3 β / β -catenin signaling may contribute to wound-healing defects in hypoxic cells. To test this possibility, we determined the effects of GSK3 β inhibitors on wound healing. The rationale is that if hypoxia suppresses wound healing by blocking GSK3 β , resulting in β -catenin activation, then similar effects would be induced by inhibitors of GSK3 β . We first titrated the concentrations of two GSK3 β inhibitors

and found that 10 mM LiCl and 10 μ M SB216763 could effectively induce β -catenin expression in both normoxia and hypoxia (Fig. 6, A and B). By immunofluorescence, we further confirmed the induction of β -catenin by these GSK3 β inhibitors (shown in Fig. 6C for LiCl effect). It is noteworthy that both LiCl and SB216763 suppressed scratch-wound healing and transwell cell migration in normoxia (Fig. 6, D and E). We further examined the effects of these inhibitors on wound healing in hypoxia. As shown in Fig. 6F, whereas hypoxia per se suppressed wound healing markedly, the GSK3 β inhibitors exerted marginal additive effects, suggesting that GSK3 β / β -catenin signaling is a major, but not the only, pathway that contributes to the wound-healing defects of hypoxic cells.

Active β -Catenin Suppresses and Dominant Negative β -Catenin Enhances Wound Healing. The above results (Fig. 6) suggest that GSK3 β inhibitors may induce β -catenin to suppress wound healing. To directly examine the role of β -catenin on wound healing, we determined the effects of stable active- β -catenin (β -catenin₁₅₂₋₇₈₁) or dominant negative β -catenin (β -catenin₁₅₂₋₆₉₄). β -Catenin undergoes degradation after GSK3 β -mediated phosphorylation at the N terminus. β -Catenin₁₅₂₋₇₈₁ is resistant to GSK3 β -mediated degradation due to the deletion of the N-terminal 151 amino acid residues, including the GSK3 β phosphorylation sites, and as a result, it functions as active β -catenin when expressed in cells. In contrast, β -catenin₁₅₂₋₆₉₄ has an additional deletion at the C terminus that is critical to the gene transcription activity of β -catenin and therefore functions as a dominant-negative mutant in cells (Fig. 7A). These constructs have been used to determine the role of β -catenin in renal tubular cell death or survival (Wang et al., 2009b). After transfection into HEK cells, the expression of β -catenin₁₅₂₋₇₈₁ was detected by the β -catenin antibody that recognized C terminus of this protein, where β -catenin₁₅₂₋₆₉₄ was not detected by this antibody because of its C-terminal deletion (Fig. 7B). Consistent with previous results (Fig. 1),

Fig. 5. GSK3 β and β -catenin expression during wound healing in RPTC. A, GSK3 β phosphorylation and β -catenin induction during wound healing in RPTC under normoxia and hypoxia. RPTC were wounded by the multiple-wound device and incubated in normoxia or hypoxia to collect whole-cell lysates at different time points for immunoblot analysis of serine-15-phosphorylated and total GSK3 β , β -catenin, and β -actin. B and C, densitometric analysis of p-GSK3 β and β -catenin induction during 3 h of wound healing. The signals of p-GSK3 β and β -catenin in the immunoblots from three separate experiments were analyzed by densitometry and normalized with control (0 h). Data are expressed as mean $\pm$ S.D. ($n = 3$). #, $p < 0.05$ versus control; *, $p < 0.05$ versus 3 h after scratch wounding in normoxia. D, immunofluorescence analysis of β -catenin expression. RPTC cells were subjected to 6 h of scratch-wound healing under normoxia or hypoxia. The cells were fixed for immunofluorescent staining of β -catenin (red) and nuclear staining with Hoechst33342 (blue).

Fig. 6. GSK3 β inhibitors suppress wound healing. A and B, β -catenin up-regulation by GSK3 β inhibitors. RPTC were scratch-wounded and then incubated for 6 h in normoxia or hypoxia with or without 10 mM LiCl or 10 μ M SB216763. A, whole-cell lysate was collected for immunoblot analysis of serine-9-phosphorylated GSK3 β , total GSK3 β , β -catenin, and β -actin. B, densitometric analysis of β -catenin expression. The signal of β -catenin in the immunoblots from three separate experiments was analyzed by densitometry and normalized with control (0 h). Data are expressed as mean $\pm$ S.D. ($n = 3$). #, $p < 0.05$ versus control; *, $p < 0.05$ versus 6 h of scratch-wound healing without inhibitors. C, another group of cells was fixed for immunofluorescent staining of β -catenin (red) and nuclear staining with Hoechst33342 (blue). D, suppression of wound healing in normoxia by GSK3 β inhibitors. RPTC were scratch-wounded and incubated in normoxia with or without 10 mM LiCl and 10 μ M SB216763 for 18 h to measure the healed distance. E, suppression of cell migration in normoxia by GSK3 β inhibitors. A total of 3×10^5 RPTC were seeded in a transwell insert, which was put in a 24-well plate containing 600 μ l of medium with or without 10 mM LiCl and 10 μ M SB216763 in normoxia for 6 h. The cells that migrated to the undersurface of the insert were stained with PI and counted. F, effect of GSK3 β inhibitors on wound healing in hypoxia. RPTC were scratch-wounded and incubated in hypoxia with or without 10 mM LiCl and 10 μ M SB216763 for 18 h to measure the healed distance. In D to F, data are expressed as mean $\pm$ S.D. ($n = 6$). *, $p < 0.05$ versus control; #, $p < 0.05$ versus hypoxia-only group.

hypoxia suppressed scratch-wound healing in both RPTC and HEK cells. This suppressive effect of hypoxia was blocked by dominant-negative β -catenin_{152–694}. In addition, active β -catenin_{152–781} could suppress wound healing in normoxic cells (Fig. 7, C and D). These results suggest that β -catenin is inhibitory to wound healing, and its induction by hypoxia may partially account for the wound-healing defects in hypoxic cells.

β -Catenin Knockdown Abrogates Wound-Healing Defects in Hypoxic Cells. To further establish the role of β -catenin in wound-healing defects in hypoxia, we tested the effect of β -catenin knockdown with shRNA. RPTC and HEK cells were infected with lentivirus containing β -catenin-shRNA or scrambled control sequence, followed by scratch wounding or transwell migration assay. By immunoblot analysis, we confirmed that β -catenin-shRNA, but not the scrambled sequence, reduced β -catenin expression in both RPTC and HEK cells (Fig. 8A). It is noteworthy that although hypoxia suppressed wound healing in scrambled sequence-transfected cells, the suppressive effect was reversed by shRNA-mediated β -catenin knockdown (Fig. 8, B and C). In

addition, migration defect of hypoxic cells was prevented by β -catenin shRNA (Fig. 8D). The restoration of wound healing and migration in hypoxic cells by knocking down β -catenin supports a role for β -catenin in wound-healing defects in hypoxia.

Discussion

Wound healing in tissues and organs is characterized by hypoxia. However, the effect of hypoxia on wound healing is not well understood. On one hand, hypoxia stimulates angiogenesis and tissue remodeling for healing; on the other hand, lack of oxygen may adversely affect parenchymal cells to prevent the repair of the wound. In this study, we have used cell culture models to examine the effect of hypoxia on wound healing in renal tubular cells. The results demonstrate that wound healing is impaired in hypoxic cells. Mechanistically, GSK3 β / β -catenin signaling, but not HIF-1, seems to contribute to the wound-healing defect.

A renoprotective role of HIF has been suggested during acute kidney injury. Hill et al. (2008) showed that pharmacological

Fig. 7. Active β -catenin suppresses and dominant negative β -catenin enhances wound healing. RPTC and HEK cells were infected with empty virus (EV), active- β -catenin virus (β -catenin¹⁵²⁻⁷⁸¹), or dominant negative β -catenin virus (β -catenin¹⁵²⁻⁶⁹⁴). A, diagram of wild type (WT) and deletion β -catenin mutants. B, β -catenin expression after lentivirus-mediated infection in HEK cells. Whole-cell lysates were collected for immunoblot analysis using an antibody recognizing the C terminus of β -catenin. C and D, scratch-wound healing. RPTC and HEK cells after lentiviral infection were scratch-wounded and incubated in normoxia or hypoxia for 18 h to measure the healed distance. Data are expressed as mean $\pm$ S.D. ($n = 6$). *, $p < 0.05$ versus normoxia with empty virus group; #, $p < 0.05$ versus hypoxia with empty virus group.

Fig. 8. Knockdown of β -catenin restores wound healing in hypoxic cells. RPTC and HEK cells were infected with lentivirus containing scramble control sequence (Scr) or β -catenin shRNA sequence (shRNA). A, knockdown effect of β -catenin shRNA. After infection, whole-cell lysates were collected for immunoblot analysis of β -catenin. B and C, scratch-wound healing. After lentivirus infection, RPTC and HEK cells were scratch-wounded and incubated in normoxia or hypoxia for 18 h to measure the healed distance. D, transwell cell migration. After lentivirus infection, 3×10^5 RPTC were seeded in a transwell insert, which was then put in a 24-well plate containing culture medium in normoxia for 6 h. The cells that migrated to the undersurface were stained with PI and counted. In B to D, data are expressed as mean $\pm$ S.D. ($n = 6$). *, $p < 0.05$ versus normoxia; #, $p < 0.05$ versus hypoxia without β -catenin shRNA (with scrambled sequence).

activation of HIF by DMOG protected against renal ischemia-reperfusion injury in mice. Weidemann et al. (2008) further showed that hypoxic preconditioning protected renal tubular cells against cisplatin injury by inducing HIF. The preconditioning effect of HIF was also reported to ameliorate ischemic kidney injury and renal failure (Bernhardt et al., 2006; Ma et al., 2009; Yang et al., 2009). Despite these reports, the mechanism underlying the renoprotective effect of HIF remains unclear. In cultured renal tubular cells, the cytoprotective effect of hypoxia

was shown to be HIF-independent, suggesting that HIF may not directly protect renal parenchymal cells (Wang et al., 2006). The results of the present study further indicate that HIF may not play a major regulatory role in the wound-healing response of renal tubular cells. This conclusion is supported by the observation that scratch-wound healing was not significantly affected by either pharmacological activation of HIF or genetic deletion of HIF-1 (Figs. 3 and 4). It is noteworthy that these are in vitro experiments using cultured cells, and it is important to

determine whether HIF regulates parenchymal cell recovery and regeneration during kidney repair *in vivo*. In addition to parenchymal cell regulation, HIF may participate in kidney repair by inducing angiogenesis and the development of renal fibrosis (Higgins et al., 2007).

The role of GSK3 β / β -catenin in wound healing is very complex and varies between cell and tissue types. β -catenin inhibited keratinocyte migration and activated fibroblast proliferation, causing aggressive fibromatosis and hyperplasia in cutaneous wounds (Cheon et al., 2002). In chronic ulcers, β -catenin was shown to be induced in the nonhealing epidermal edge to inhibit keratinocyte migration and dedifferentiation, resulting in impaired healing. Consistently, β -catenin inhibited epithelial cell migration in wounds in a human skin organ culture model (Stojadinovic et al., 2005). In contrast, other studies have suggested that β -catenin promotes proliferation and repair. For example, during the repair of bone fracture β -catenin positively regulated osteoblasts, and activation of β -catenin by lithium treatment improved fracture healing (Chen et al., 2007). In kidney injury models, GSK3 β / β -catenin signaling has been studied mainly in the injury phase. Price et al. (2002) reported that β -catenin and T-cell factor proteins transiently accumulated in the nucleus in response to sublethal renal ischemic injury. Wang et al. (2010) demonstrated that inhibition of GSK3 β protected kidney tubular cells from ischemic injury by inhibiting tubular cell apoptosis. Moreover, β -catenin overexpression promoted survival of renal tubular cells by inhibiting Bax (Wang et al., 2009b). In podocytes, however, β -catenin was shown to promote cell injury and dysfunction during adriamycin-induced kidney injury (Dai et al., 2009; He et al., 2011). In these studies, whether GSK3 β / β -catenin signaling regulates the wound-healing response of parenchymal kidney cells was not examined. Our current results demonstrate GSK3 β inactivation with concurrent β -catenin induction during wound healing in renal tubular cells, indicative of the activation of GSK3 β / β -catenin signaling. It is noteworthy that inhibition of GSK3 β and β -catenin activation or overexpression can suppress wound healing and cell migration, suggesting that GSK3 β / β -catenin signaling antagonizes wound healing in renal tubular cells. Considering the published work reporting the protective role of β -catenin (Wang et al., 2009b), it is suggested that β -catenin may play a dual role in kidney injury: protecting against tubular cell injury but suppressing kidney repair during recovery. If proven true *in vivo*, inhibition of GSK3 β and consequent activation of β -catenin may offer a useful therapeutic strategy when administered before and during kidney injury; however, it would be important to suppress β -catenin during subsequent kidney repair. Obviously, it is important to investigate the distinct roles played by GSK3 β / β -catenin signaling during kidney injury and repair phases.

We demonstrated β -catenin expression in the cells at the wound edge (Fig. 5D). Our immunoblot analysis further showed that β -catenin expression was accompanied by GSK3 β phosphorylation or inactivation, suggesting the involvement of the canonical Wnt/GSK3 β pathway in β -catenin activation during wound healing. Consistently, in cutaneous wounds β -catenin expression is not associated with a change in its mRNA level, but correlates with GSK3 β phosphorylation or inactivation (Cheon et al., 2005). Upon activation, β -catenin inhibited wound healing in renal tubular cells (Figs. 7 and 8); however, the underlying mechanism is currently unclear. β -Catenin activates multiple target genes that potentially promote epithelial

cell survival, including insulin-like growth factor II, Akt, inhibitor of apoptosis proteins, proliferin, and Wnt-1- β -catenin-secreted proteins 1 and 2 (Longo et al., 2002; Dihlmann et al., 2005). It is noteworthy that, in addition to the cell survival effects, β -catenin is an important integrator of cell proliferation and differentiation (Brembeck et al., 2006), and, as a result, inappropriate activation of β -catenin and downstream proteins may lead to deregulation of proliferation and differentiation, resulting in impaired wound healing. In line with this possibility, Stojadinovic et al. (2005) reported that β -catenin accumulated in the nucleus of keratinocytes at the nonhealing wound edge of chronic ulcers, leading to c-myc expression and resulting in cellular hyperproliferation and inhibition of migration and wound healing. In our study, cell migration was also suppressed when β -catenin expression was elevated with GSK3 β inhibitors (Fig. 6E). Several mechanisms may account for the inhibitory effect of β -catenin on cell migration. It was reported that β -catenin was elevated in mesenchymal cells during the proliferative phase. In hyperplastic wounds there was a prolonged duration of β -catenin elevation, maintaining the mesenchymal cells in a prolonged proliferative state that may prevent the cells from entering into the migration state (Cheon et al., 2005). In addition, β -catenin is known to be a key component of adherens junctions, which are required to disassemble to allow cell migration (Nelson and Nusse, 2004). β -Catenin activation may enhance the adherens junctions to suppress migration. Furthermore, β -catenin may inhibit cell migration by regulating gene expression. In this regard, Stojadinovic et al. (2005) reported that β -catenin can suppress the expression of cytoskeletal components K6/K16 keratins that are important for keratinocyte migration in chronic wounds.

The observations the β -catenin was inhibitory in wound healing of renal tubular cells (Figs. 7 and 8) and hypoxia further activated β -catenin (Figs. 5 and 6) suggest that GSK3 β / β -catenin signaling may contribute to impaired wound healing in hypoxic tubular cells. Indeed, blockade of β -catenin via dominant negative mutants and shRNAs could restore wound healing and cell migration during hypoxia (Figs. 7 and 8). It remains unclear how β -catenin is induced by hypoxia in RPTC during wound healing. Mazumdar et al. (2010) showed that hypoxia could not induce β -catenin in HIF-1 α -null embryonic stem cells, suggesting a role of HIF-1 in β -catenin induction during hypoxia. However, in our study wound healing was not affected by HIF-1 α deficiency, negating the involvement of HIF in wound-healing signaling, including β -catenin. Alternatively, hypoxic induction of β -catenin may be mediated by GSK3 β inactivation. This possibility is supported by our observation that β -catenin induction during hypoxic wound healing was accompanied by GSK3 β inactivation (indicated by phosphorylation at serine-9) (Fig. 6). In this regard, GSK3 β is known to be modulated by Akt (Patel and Woodgett, 2008), a key signaling kinase that has been reported to be activated by hypoxia in renal tubular cells (Zeng et al., 2008). Consistently, we demonstrated significantly higher Akt activation during wound healing in hypoxic tubular cells than normoxic cells (data not shown). Thus we speculate that hyperactivation of Akt by wound healing in hypoxic cells may inactivate GSK3 β , inducing β -catenin to prevent wound healing in renal tubular cells under hypoxia.

Our results showed that β -catenin was expressed in nucleus in some of the wound edge cells, suggesting the translocation and transcriptional activation of β -catenin. β -Catenin in nucleus can bind to lymphoid enhancer factor/T-cell factor, leading

to increase in target gene expression. Consistently, β -catenin was shown to be transcriptionally active in mesenchymal cells during the proliferative phase of wound healing, as demonstrated by activation of a reporter construct in primary cell cultures and up-regulation of the β -catenin target genes: matrix metalloproteinase 7 and fibronectin (Cheon et al., 2005). Moreover, β -catenin and downstream target gene c-myc expression in keratinocytes inhibited epithelialization during wound healing in chronic wounds (Stojadinovic et al., 2005). It would be interesting to investigate the downstream transcriptional targets of β -catenin in renal tubular cells that affect wound healing in kidney tissues.

In conclusion, this study has determined the roles played by HIF-1 α and GSK3 β / β -catenin signaling during wound healing in renal tubular cells. Both HIF-1 α and β -catenin are induced during wound healing in normoxic cells. Hypoxia leads to further activation of HIF-1 α and β -catenin, which is accompanied by impaired wound healing. GSK3 β / β -catenin signaling, but not HIF-1 α , seems to contribute to the wound-healing defect in hypoxic cells.

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Authorship Contributions

Participated in research design: Peng and Dong.

Conducted experiments: Peng and Dong.

Contributed new reagents or analytic tools: Ramesh and Sun.

Performed data analysis: Peng and Dong.

Wrote or contributed to the writing of the manuscript: Peng, Ramesh, Sun, and Dong.

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Address correspondence to: Dr. Zheng Dong, Department of Cellular Biology and Anatomy, Georgia Health Sciences University, 1459 Laney Walker Blvd., Augusta, GA 30912. E-mail: zdong@georgiahealth.edu