JPET Fast Forward. Published on October 18, 2011 as DOI: 10.1124/jpet.111.187427
JPET #187427

Impaired Wound Healing in Hypoxic Renal Tubular Cells: roles of HIF-1 and GSK3β/β-catenin Signaling

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JPET #187427

Running Title: Impaired wound healing in renal hypoxia

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Manuscript Information:

Text Pages: 32
Figures: 8
Tables: 0
References: 40
Abstract: 230
Introduction: 645
Discussion: 1283

Non-standard Abbreviations:

dimethyloxalylglycine (DMOG)
glycogen synthase kinase 3β (GSK3β)
hypoxia-inducible factor (HIF)
mouse embryonic fibroblasts (MEF)
renal proximal tubular cells (RPTC)
short hairpin RNA (shRNA)

Section Assignment: Gastrointestinal, Hepatic, Pulmonary, and Renal

ABSTRACT

Wound and subsequent healing are frequently associated with hypoxia. While 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-1α (HIF-1α) was induced during scratch wound healing in normoxia and the induction was more evident in hypoxia. Nevertheless, HIF-1α-null and wild-type cells healed similarly after scratch wounding. Moreover, activation of HIF-1α with dimethyloxalylglycine (DMOG) in normoxic cells did not suppress wound healing, negating a major role of HIF-1α in wound-healing in this model. Scratch wound healing was also associated with GSK3β/β-catenin signaling, which was further enhanced by hypoxia. Pharmacological inhibition of GSK3β 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 shRNA improved wound healing and transwell migration in

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hypoxic cells. The results suggest that GSK3β/β-catenin signaling may contribute to the 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 due in part to the vascular damage and decreased blood supply, but also, in a large part, to the O_2 consumption of the cells in the wound that are metabolically activated for migration, proliferation and wound healing (Tandara and Mustoe, 2004). Under this condition, 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, HIF (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 non-hypoxic conditions or stimuli. HIF consists of α and β subunits. HIF β is constitutively expressed, whereas HIFα is hydroxylated at several proline and asparagine sites in the presence of oxygen, targeting it for VHL-mediated ubiquitination and proteosomal degradation. In hypoxia, the lack of oxygen prevents HIFα hydroxylation and degradation, leading to HIFα accumulation and dimerization with HIF β to form a functional transcription factor (Semenza, 2007; Kaelin and

Ratcliffe, 2008). Pharmacological inhibitors of HIFα prolyl hydroxylase, such as DMOG, can suppress HIFα hydroxylation and induce HIFα 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). While 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 if 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 3β (GSK3β) and β-catenin. In unstimulated cells, β-catenin exists in a multi-protein complex with GSK3 β, Axin and adenomatous polyposis coli (APC). Phosphorylation of β-catenin by GSK3β in the complex results in the degradation of β-catenin. Activation of Wnt signaling leads to GSK3β phosphorylation and inactivation, resulting in the accumulation of β-catenin, which may translocate into the nucleus to induce gene expression. GSK3β/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 GSK3β/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

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diseases, albuminuria and renal fibrosis (Pulkkinen et al., 2008; Dai et al., 2009; He et al., 2009; Wang et al., 2009b; Wang et al., 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 pro-apoptotic protein, while GSK3β promotes tubular apoptosis after acute ischemic kidney injury (Wang et al., 2009b; Wang et al., 2010). While these studies have suggested a role of GSK3β/β-catenin signaling in acute kidney cell injury, whether and how this signaling pathway contributes to kidney repair or wound healing following 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 GSK3β/β-catenin signaling in the wound-healing defect of hypoxic cells. The results suggest that GSK3β/β-catenin signaling, but not HIF, contributes to the wound-healing defect of renal proximal tubular cells during hypoxia.

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METHODS

Antibodies and Special Reagents: Antibodies were purchased from the following sources: polyclonal anti-β-catenin, anti-phospho-GSK3β-ser9 and anti-total-GSK3β from Cell Signaling Technology (Beverly, MA), monoclonal anti-HIF-1α from BD Biosciences (Rockville, MD), the secondary antibodies for immunoblot analysis from Jackson ImmunoResearch (West Grove, PA), and the secondary antibodies for immunofluorescence from Chemicon (Temecula, CA). Lithium chloride (LiCl), 3-(2,4-Dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-py rrole-2,5-dione (SB216763), and DMOG were purchased respectively from Acros Organics (Morris Plains, NJ), Tocris Bioscience (Ellisville, Missouri), and Sigma (St. Louis, MO).

Cell Culture: Immortalized rat kidney proximal tubular cell line (RPTC) was originally obtained from Dr. Ulrich Hopfer (Case Western Reserve University, Cleveland, OH). Human embryonic kidney (HEK293) cells were purchased from American Type Culture Collection (Manassas, VA). Wild-type and HIF-1α-null mouse embryonic fibroblasts (MEF) were obtained from Dr. Semenza at John Hopkins University (Zhang et al., 2008).

Hypoxic Incubation: RPTC cells 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 before (Wang et al., 2006).

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Briefly, 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 and colleagues (Zhuang et al.). Briefly, a monolayer of confluent RPTC cells grown in a 35 mm dish was linearly scratched with a sterile 1000-μl pipette tip. Phase-contrast images were recorded at 0 and 18 hours following scratching. The width of the wound was measured at various time points to determine that healed distance. For biochemical analysis, multiple uniformed wounds were created in a RPTC cell monolayer in 60-mm dishes with a wounding device that makes concentric circular 130 μm wide cell bands separated by 750 μm 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). Briefly, the undersurfaces of Transwells (Costar, Corning, NY) were coated with 10 μg/ml of collagen I (Upstate Biotechnology, Lake Placid, NY) overnight at 4 °C. Coated wells were then placed into a 24-well plate containing 600 μl of culture medium. RPTC cells were detached and suspended at 1.5×10^6 cells/ml in culture medium. The cells were then added into

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transwells (200μl, 3×10^5 cells/well) and allowed to migrate for 6 hours 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 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-shRNA sequence for 3 days before adding to the transwell for cell migration assay.

Lentivirus-Mediated β-Catenin Overexpression: A mouse β-catenin cDNA plasmid (gift from Dr. Lin Mei at Georgia Health Sciences University, Augusta, GA) was used as the template for PCR-based deletion to generate active β-catenin (β-catenin$_{152\text{-}781}$) and dominant negative β-catenin (β-catenin$_{152\text{-}694}$), which were subcloned into the lentiviral vector pCDH-CMV-MCS-EF1-copGFP (System Biosciences, Mountain View, CA) at Xbal and Not1 sites. The β-catenin plasmids were co-transfected into 293FT cells (Invitrogen) with three packaging plasmids (pLP1, pLP2, pLP/VSV-G) to collect the culture medium at 48 hours. The culture medium with packaged lentivirus was added to RPTC cells for one day of infection. The medium was then replaced with fresh medium for additional two days of culture prior to wound healing test.

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Lentiviral shRNA-Mediated β-catenin Knockdown: Lentiviral shRNA plasmid targeting β-catenin and a scrambled non-targeting control plasmid were made 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 cells grown on glass coverslips were scratch-wounded with a sterile 1000-μl pipette tip, and incubated in hypoxia or normoxia for 6 hours. 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 Cy3 channel.

Immunoblot Analysis: Protein concentration was determined using the BCA reagent (Pierce Chemical Co., Rockford, IL). Equal amounts of protein were loaded in each well for electrophoresis using the NuPAGE Gel System, followed by transferring onto polyvinylidene fluoride membranes. The blots were then incubated in blocking buffer for 1 hour, then in primary antibodies overnight at 4°C. After washes, the blots were incubated with the horseradish peroxidase-conjugated

secondary antibody, and the antigens on the blots were revealed using the enhanced chemiluminescence (ECL) kit from Pierce.

Statistical Analysis: Quantitative data were expressed as Means±SD ($n \geq 4$). 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 cells.

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 are largely unknown. We examined the effect of hypoxia in a scratch wound healing model of cultured rat renal proximal tubular cells (RPTC). In this model, RPTC cells 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 hour) and at different time points post-scratching. Representative wounds were shown in Figure 1A. Under normoxia, most of the wound healed within 18 hours. 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 (Figure 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 using the transwell cell migration assay (Zhuang et al.). RPTC cells 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

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recorded after PI staining and counted. As shown in Figure 1C, apparently fewer cells migrated under hypoxia than normoxia. Cell counting showed that in six hours, ~720 cells migrated under normoxia whereas ~520 did so under hypoxia (Figure 1D).

HIF-1α induction during wound healing.

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

Pharmacological induction of HIF does not block wound healing.

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Wound healing was defective in hypoxic cells (Figure 1) that had a notable HIF-1α induction (Figure 2B), suggesting that HIF-1 may contribute to the wound healing defects under hypoxia. To test this possibility, we first determined the effects of dimethyloxaloylglycine (DMOG), a pharmacological inducer of HIF that prevents HIF-1α 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 Figure 3A, 6 hours of wound healing led to HIF-1α 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 (Figure 3B, 3C). 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-1α+/+) and HIF-1α-null (HIF-1α-/-) mouse embryonic fibroblasts (MEF). As expected, HIF-1α was induced by hypoxia during wound healing in HIF-1α+/+ MEF cells, but not in HIF-1α-/- cells (Figure 4A). When these two genotype cells were subjected to scratch-wounding, their healing was very similar in both normoxia and

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hypoxia (Figure 4B). The results suggest that HIF-1 does not play a critical role in wound healing in this cellular model.

Activation of GSK3β/β-catenin signaling during wound healing.

In the canonical pathway of GSK3β/β-catenin signaling, GSK3β phosphorylates β-catenin leading to its degradation. GSK3β/β-catenin signaling plays an important role in wound healing (Cheon et al., 2002). In normoxic RPTC cells, wound healing induced GSK3β phosphorylation at serine-9 (indicative of GSK3β inactivation), which was accompanied by increased expression of β-catenin (Figure 5A: left panels). GSK3β phosphorylation and β-catenin expression were also induced during wound healing in hypoxic cells (Figure 5A: right panels). It is noteworthy that compared with normoxic cells, hypoxic cells showed higher GSK3β phosphorylation at 3 hours post-wounding and higher β-catenin expression at all time points examined (Figure 5A, 5B, 5C). We further examined β-catenin expression by immunofluorescence (Figure 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 membrane and cytoplasm (Figure 5D: right panel). Together, these data demonstrated an enhanced GSK3β/β-catenin signaling in hypoxic cells.

GSK3β inhibitors suppress wound healing

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Based on above observation (Figure 5), we hypothesized that the enhanced GSK3β/β-catenin signaling may contribute to wound healing defects in hypoxic cells. To test this possibility, we determined the effects of GSK3β inhibitors on wound healing. The rationale is that if hypoxia suppresses wound healing by blocking GSK3β resulting in β-catenin activation, then similar effects would be induced by inhibitors of GSK3β. We first titrated the concentrations of two GSK3β inhibitors and found that 10 mM LiCl and 10 μM SB216763 could effectively induce β-catenin expression in both normoxia and hypoxia (Figure 6A, 6B). By immunofluorescence, we further confirmed the induction of β-catenin by these GSK3β inhibitors (shown in Figure 6C for LiCl effect). Importantly, both LiCl and SB216763 suppressed scratch wound healing and transwell cell migration in normoxia (Figure 6D, 6E). We further examined the effects of these inhibitors on wound healing in hypoxia. As shown in Figure 6F, while hypoxia per se suppressed wound healing markedly, the GSK3β inhibitors exerted marginal additive effects, suggesting that GSK3β/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 (Figure 6) suggest that GSK3β 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-catenin$_{152\text{-}781}$) or

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dominant negative β-catenin (β-catenin$_{152-694}$). β-catenin undergoes degradation after GSK3β-mediated phosphorylation at the N-terminus. β-catenin$_{152-781}$ is resistant to GSK3β-mediated degradation due to the deletion of the N-terminal 151 amino acid residues including the GSK3β phosphorylation sites and as a result, it functions as active β-catenin when expressed in cells. In contrast, β-catenin$_{152-694}$ 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 (Figure 7A). These constructs have been used recently 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-catenin$_{152-781}$ was detected by the β-catenin antibody that recognized C-terminus of this protein, where β-catenin$_{152-694}$ was not detected by this antibody due to its C-terminal deletion (Figure 7B). Consistent with previous results (Figure 1), 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 (Figure 7C, 7D). 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 short hairpin RNA (shRNA). RPTC

and HEK cells were infected with lentivirus containing β-catenin-shRNA (shRNA) or scrambled control sequence (Scr), 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 (Figure 8A). Importantly, while hypoxia suppressed wound healing in scrambled sequence-transfected cells, the suppressive effect was reversed by shRNA-mediated β-catenin knockdown (Figure 8B, 8C). In addition, migration defect of hypoxic cells was also prevented by β-catenin shRNA (Figure 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, GSK3β/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. showed that pharmacological activation of HIF by DMOG protected against renal ischemia-reperfusion injury in mice (Hill et al., 2008). Weidemann et al. further showed that hypoxic preconditioning protected renal tubular cells against cisplatin injury by inducing HIF (Weidemann et al., 2008). 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

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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 (Figures 3 and 4). Of note, 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 GSK3β/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, GSK3β/β-catenin signaling has been studied mainly in the injury phase. Price et al. reported that β-catenin and T-cell factor (TCF) proteins transiently accumulated in the nucleus in response to sub-lethal renal ischemic injury (Price et al., 2002). Wang et al. demonstrated that inhibition of GSK3β protected kidney tubular cells from

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ischemic injury by inhibiting tubular cell apoptosis (Wang et al., 2010). 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 GSK3β/β-catenin signaling regulates the wound healing response of parenchymal kidney cells was not examined. Our current results demonstrate GSK3β inactivation with concurrent β-catenin induction during wound healing in renal tubular cells, indicative of the activation of GSK3β/β-catenin signaling. Importantly, inhibition of GSK3β and β-catenin activation or overexpression can suppress wound healing and cell migration, suggesting that GSK3β/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 GSK3β 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 GSK3β/β-catenin signaling during kidney injury and repair phases.

We demonstrated β-catenin expression in the cells at the wound edge (Figure 5D). Our immunoblot analysis further showed that β-catenin expression was accompanied by GSK3β phosphorylation or inactivation, suggesting the involvement

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of the canonical Wnt/GSK3β 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 GSK3β phosphorylation or inactivation (Cheon et al., 2005). Upon activation, β-catenin inhibited wound healing in renal tubular cells (Figures 7 and 8); however the underlying mechanism is currently unclear. β-catenin activates multiple target genes that potentially promote epithelial cell survival, including IGF 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). Notably, 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. reported that β-catenin accumulated in the nucleus of keratinocytes at the nonhealing wound edge of chronic ulcers leading to c-myc expression, resulting in cellular hyperproliferation and inhibition of migration and wound healing (Stojadinovic et al., 2005). In our study, cell migration was also suppressed when β-catenin expression was elevated with GSK3β inhibitors (Figure 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 migration state (Cheon et al., 2005).

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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. reported that β-catenin can suppress the expression of cytoskeletal components K6/K16 keratins that are important for keratinocyte migration in chronic wounds (Stojadinovic et al., 2005).

The observations the β-catenin was inhibitory in wound healing of renal tubular cells (Figures 7, 8) and that hypoxia further activated β-catenin (Figures 5, 6) suggest that GSK3β/β-catenin signaling may contribute to the 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 (Figures 7, 8). It remains unclear how β-catenin is induced by hypoxia in RPTC cells during wound healing. Mazumdar et al. showed that hypoxia could not induce β-catenin in HIF-1α-null embryonic stem cells, suggesting a role of HIF-1 in β-catenin induction during hypoxia (Mazumdar et al., 2010). However, in our study wound healing was not affected by HIF-1α-deficiency, negating the involvement of HIF in wound healing signaling including β-catenin. Alternatively, hypoxic induction of β-catenin may be mediated by GSK3β inactivation. This possibility is supported by our observation that β-catenin induction during hypoxic wound healing was accompanied by GSK3β inactivation (indicated by phosphorylation at serine-9) (Figure 6). In this regard, GSK3β is known to be modulated by Akt (Patel and Woodgett, 2008), a key

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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 hyper-activation of Akt by wound healing in hypoxic cells may inactivate GSK3β, 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 LEF/TCF, 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 by upregulation of the β-catenin target genes: matrix metalloproteinase seven (MMP-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-1α and GSK3β/β-catenin signaling during wound healing in renal tubular cells. Both HIF-1α and β-catenin are induced during wound healing in normoxic cells. Hypoxia leads to

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further activation of HIF-1α and β-catenin, which is accompanied by impaired wound healing. GSK3β/β-catenin signaling, but not HIF-1α, appears to contribute to the wound healing defect in hypoxic cells.

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ACKNOWLEDGMENTS

We thank Dr. Steven Borkan at Boston University and Dr. Lin Mei at Georgia Health Sciences University for β-catenin plasmids. We also thank Dr. Gregg Semenza at John Hopkins University for providing HIF-1α-null and wild-type MEF cell lines. Zheng Dong is a Research Career Scientist of Department of Veterans Affairs (VA).

Authorship Contributions

Participated in research design: Peng, Dong

Conducted experiments: Peng, Dong

Contributed new reagents or analytic tools: Ramesh, Sun

Performed data analysis: Peng, Dong

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

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Footnotes

The study was supported in part by the National Institutes of Health [DK058831, DK087843] and VA Merit grant.

JP is an exchange graduate student conducting thesis research at Georgia Health Sciences University, as part of the International Cooperative Agreement between Georgia Health Sciences University and Wuhan University in China.

FIGURE LEGENDS

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

Figure 2. HIF-1α induction during wound healing in normoxia and hypoxia. (**A**) Multiple uniform wounds made with a wounding device. RPTC cells grown in 60-mm dishes were pressed with a multiple wounding device to induce concentric circular cell bands of 130 µm width separated by wounds of 750 µm width. (**B**) Time dependent HIF-1α induction during wound healing in normoxia and hypoxia. RPTC cells 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-1α and β-actin. (**C**) Densitometric analysis of

HIF-1α expression during scratch-wound healing. Immunoblots from three separate experiments were analyzed by densitometry and the signal of HIF-1α was normalized with Control (0 hour) that was arbitrarily set as 1. Data are expressed as mean ± SD (n=3). * $p<0.05$ versus normoxia.

Figure 3. DMOG induces HIF-1α 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 hours to collect whole cell lysates for immunoblot analysis of HIF-1α. (**B**, **C**) Scratch wound healing. RPTC and HEK cells were scratch wounded and then incubated in normoxia without (-) or with (+) 100 mM DMOG for 18 hours to measure the healed distance. Data are expressed as mean ± SD (n=6).

Figure 4. Wound healing in HIF-1α-null cells. (**A**) HIF-1α induction during wound healing in hypoxia in HIF-1α-null (HIF-1α -/-) and wild-type (HIF-1α+/+) 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-1α and β-actin. (**B**) Scratch wound healing. Wild-type and HIF-1α-null MEF cells were scratch wounded and then incubated in hypoxia for 18 hours to measure the healed distance. Data are expressed as mean ± SD (n=6).

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Figure 5. GSK3β and β-catenin expression during wound healing in RPTC cells. (**A**) GSK3β phosphorylation and β-catenin induction during wound healing in RPTC cells under normoxia and hypoxia. RPTC cells 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 GSK3β, β-catenin, and β-actin. (**B**)(**C**) Densitometric analysis of p-GSK3β and β-catenin induction during 3 hours of wound-healing. The signals of p-GSK3β and β-catenin in the immunoblots from three separate experiments were analyzed by densitometry and normalized with Control (0 hour). Data are expressed as mean ± SD (n=3). #, $p<0.05$ versus Control; * $p<0.05$ versus 3 hours after scratch-wounding in normoxia. (**D**) Immunofluorescence analysis of β-catenin expression. PRTC cells were subjected to 6 hours of scratch wound healing under normoxia or hypoxia. The cells were fixed for immunofluorescence staining of β-catenin (red) and nuclear staining with Hoechst33342 (blue).

Figure 6. GSK3β inhibitors suppress wound healing. (**A**, **B**) β-catenin up-regulation by GSK3β inhibitors. RPTC cells were scratch-wounded and then incubated for 6 hours in normoxia or hypoxia with or without 10 mM LiCl or 10 μM SB216763. (**A**) Whole cell lysate was collected for immunoblot analysis of serine-9 phosphorylated GSK3β, total GSK3β, 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

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Control (0 hour). Data are expressed as mean ± SD (n=3). #, $p<0.05$ versus Control; * $p<0.05$ versus 6 hours of scratch-wound healing without inhibitors. (**C**) Another group of cells was fixed for immunofluorence staining of β-catenin (red) and nuclear staining with Hoechst33342 (blue). (**D**) Suppression of wound healing in normoxia by GSK3β inhibitors. RPTC cells were scratch wounded and incubated in normoxia with or without 10 mM LiCl and 10 μM SB216763 for 18 hours to measure the healed distance. (**E**) Suppression of cell migration in normoxia by GSK3β inhibitors. 3×10^5 RPTC cells were seeded in a transwell insert, which was put in a 24-well plate containing 600 μl medium with or without 10 mM LiCl and 10 μM SB216763 in normoxia for 6 hours. The cells that migrated to the undersurface of the insert were stained with PI and counted. (**F**) Effect of GSK3β inhibitors on wound healing in hypoxia. RPTC cells were scratch wounded and incubated in hypoxia with or without 10 mM LiCl and 10 μM SB216763 for 18 hours to measure the healed distance. In (**D**, **E, F**), data are expressed as mean ± SD (n=6), * $p<0.05$ versus control; # $p<0.05$ versus Hypoxia-only group.

Figure 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 ($\beta\text{-catenin}_{152\text{-}781}$), or dominant negative β-catenin virus ($\beta\text{-catenin}_{152\text{-}694}$). (**A**) Diagram of wild type 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

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C-terminus of β-catenin. (**C**, **D**) Scratch wound healing. RPTC and HEK cells after lentiviral infection were scratch-wounded and incubated in normoxia or hypoxia for 18 hours to measure the healed distance. Data are expressed as mean ± SD (n=6), * $p<0.05$ versus normoxia with empty virus group; # $p<0.05$ versus hypoxia with empty virus group.

Figure 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**, **C**) Scratch wound healing. After lentivirus infection, RPTC and HEK cells were scratch-wounded and incubated in normoxia or hypoxia for 18 hours to measure the healed distance. (**D**) Transwell cell migration. After lentivirus infection, 3×10^5 RPTC cells were seeded in a transwell insert, which was then put in a 24-well plate containing culture medium in normoxia for 6 hours. The cells that migrated to the undersurface were stained with PI and counted. In (**B**, **C**, **D**), data are expressed as mean ± SD (n=6), * $p<0.05$ versus normoxia; # $p<0.05$ versus hypoxia without β-catenin shRNA (with scrambled sequence).

Figure 1

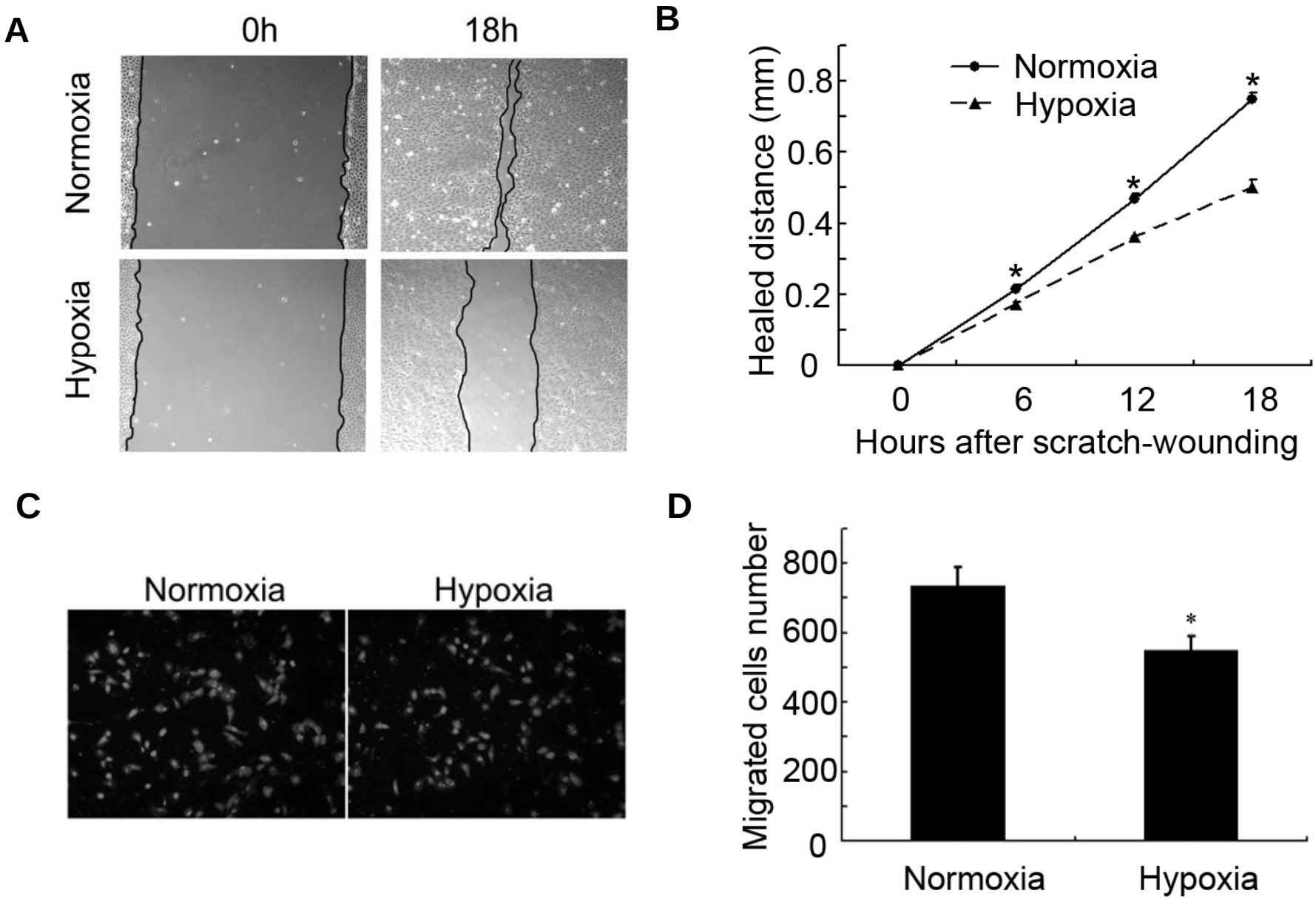
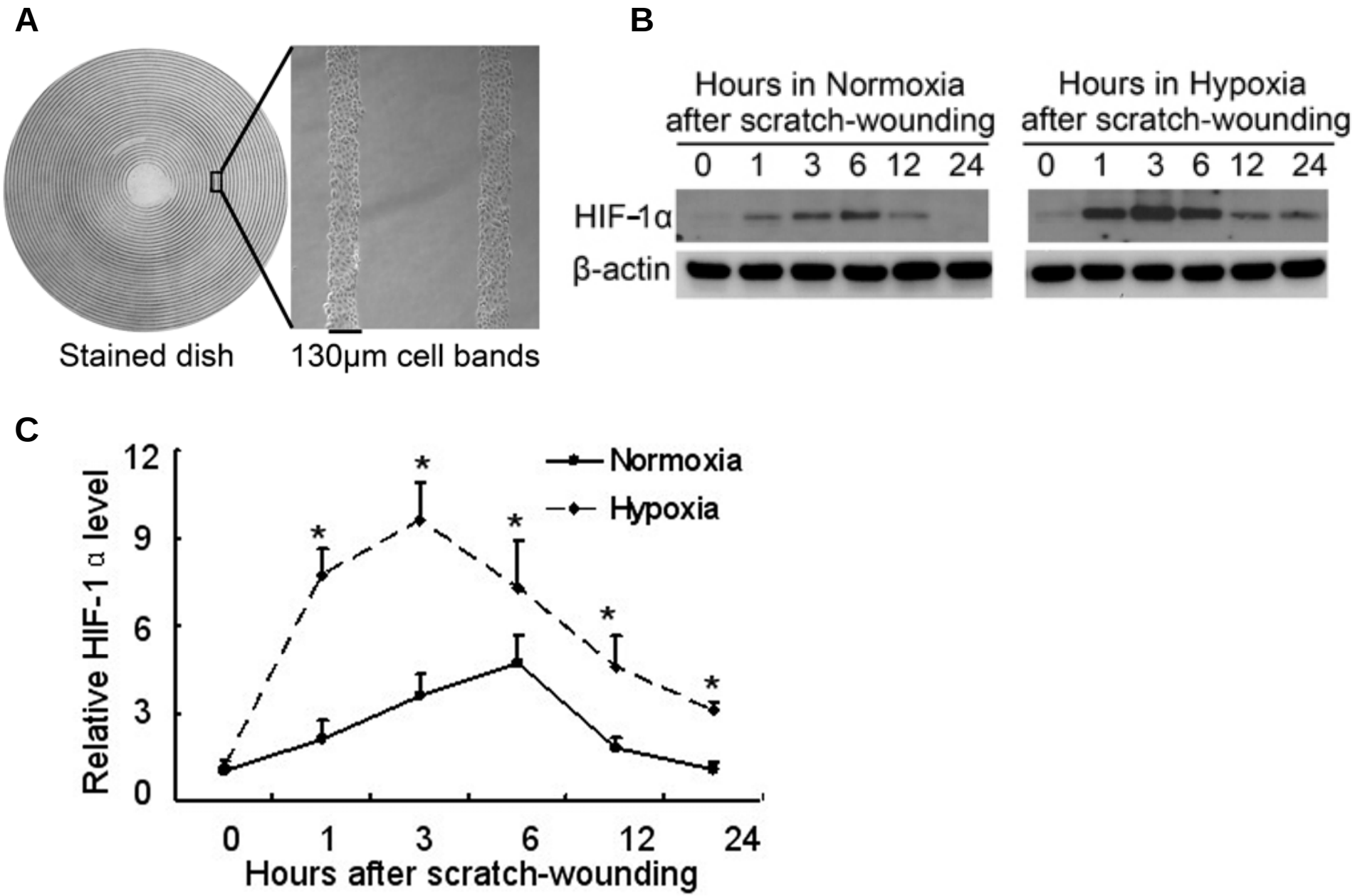


Figure 2



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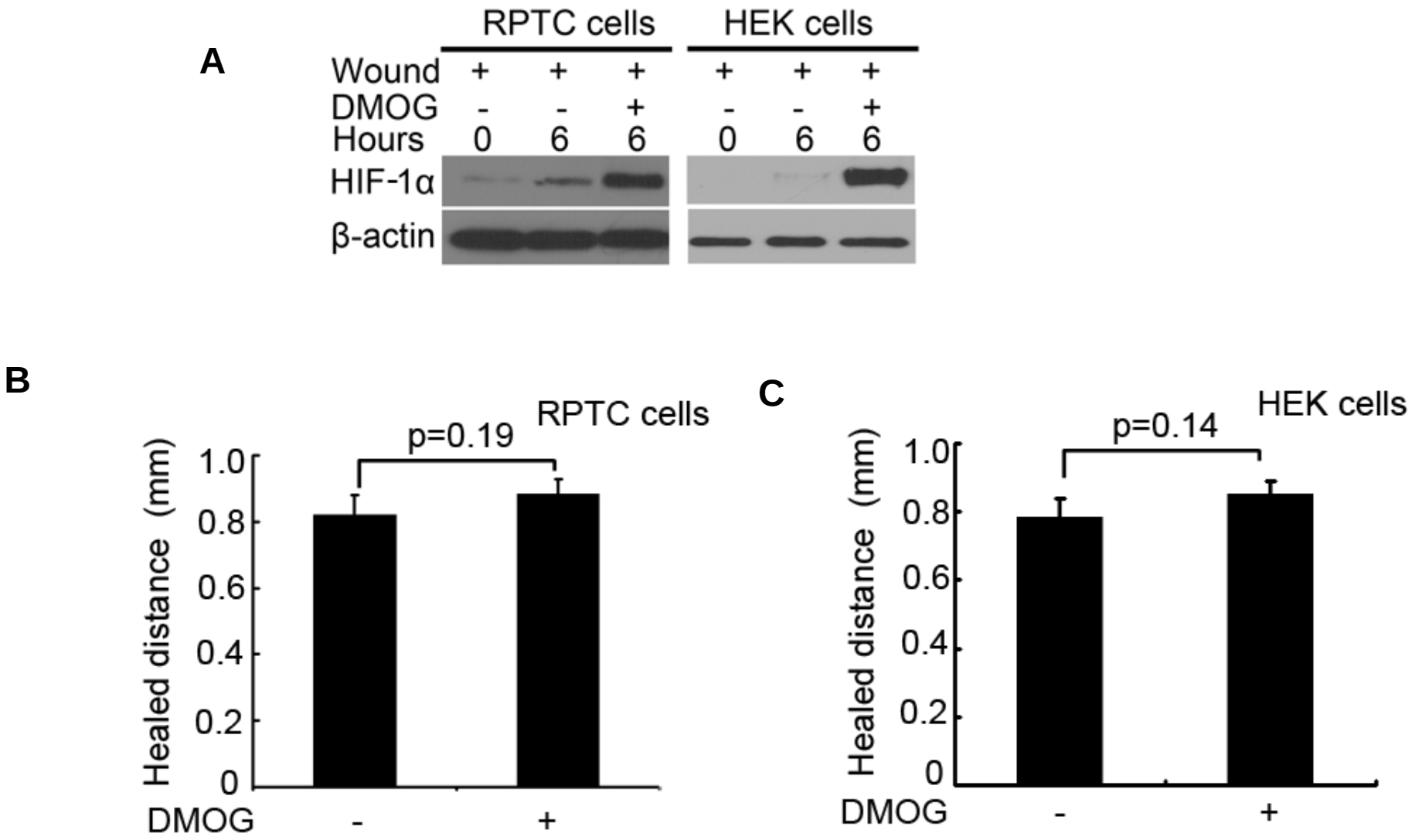

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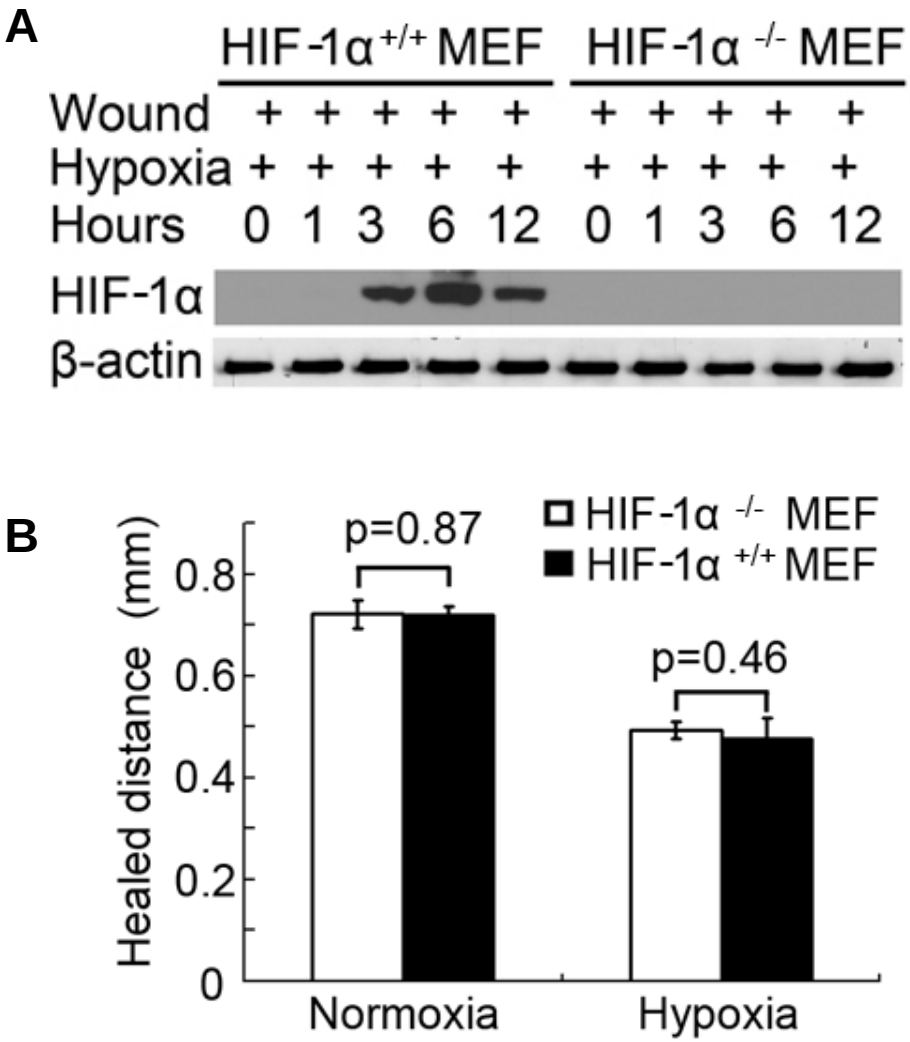


Figure 5

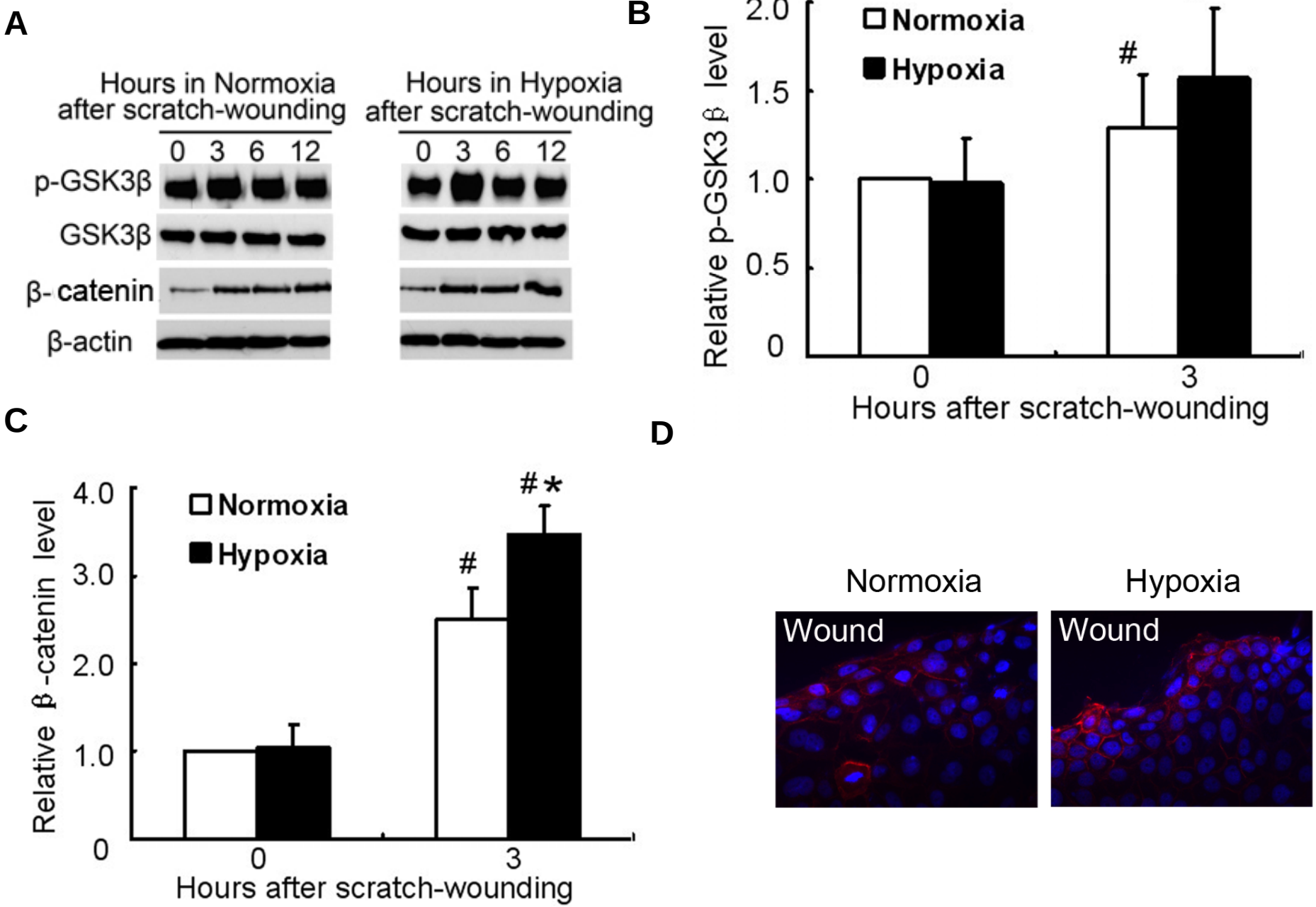
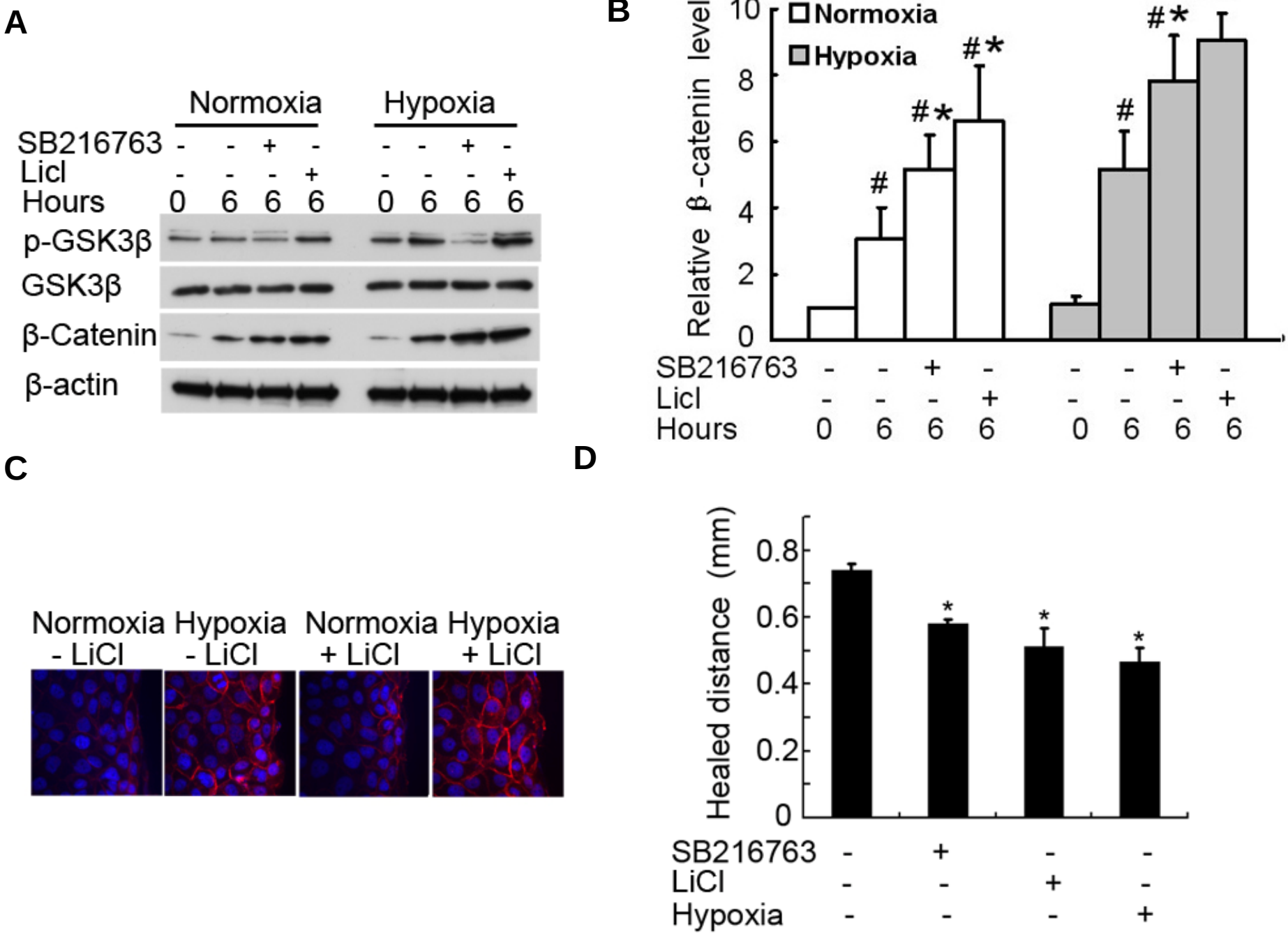


Figure 6



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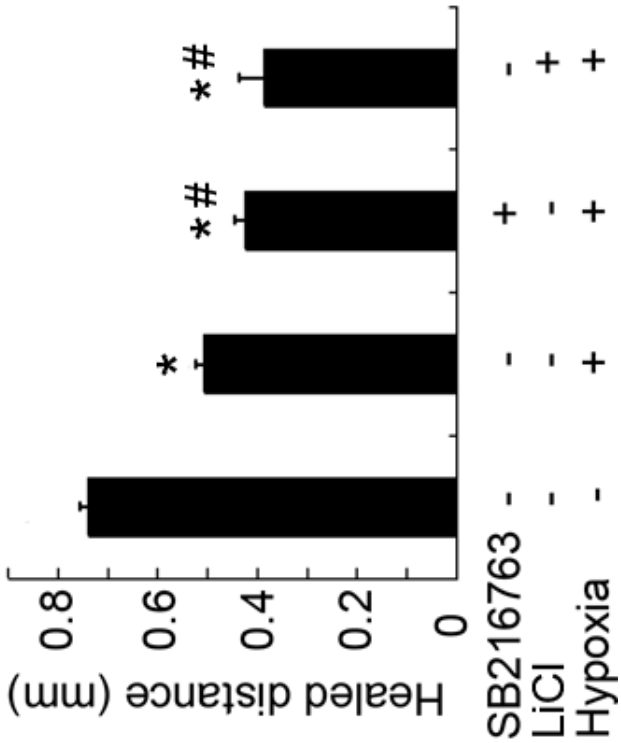

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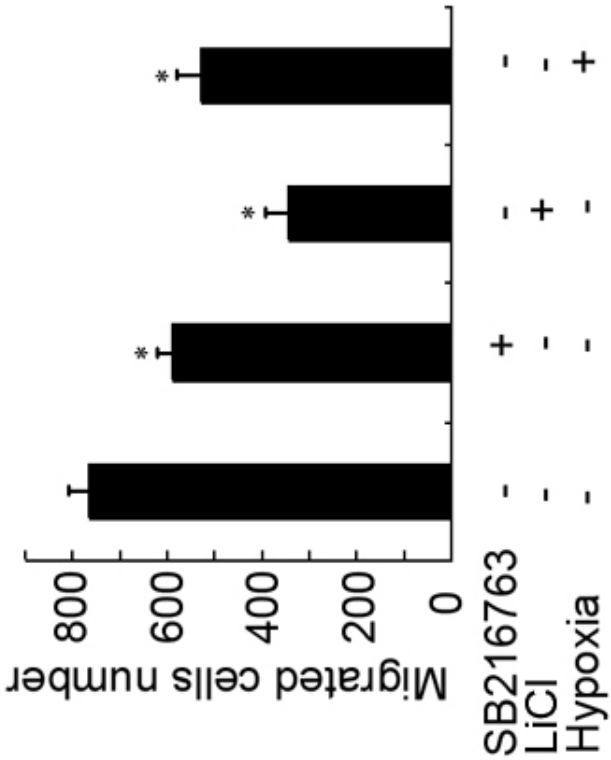

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Figure 7

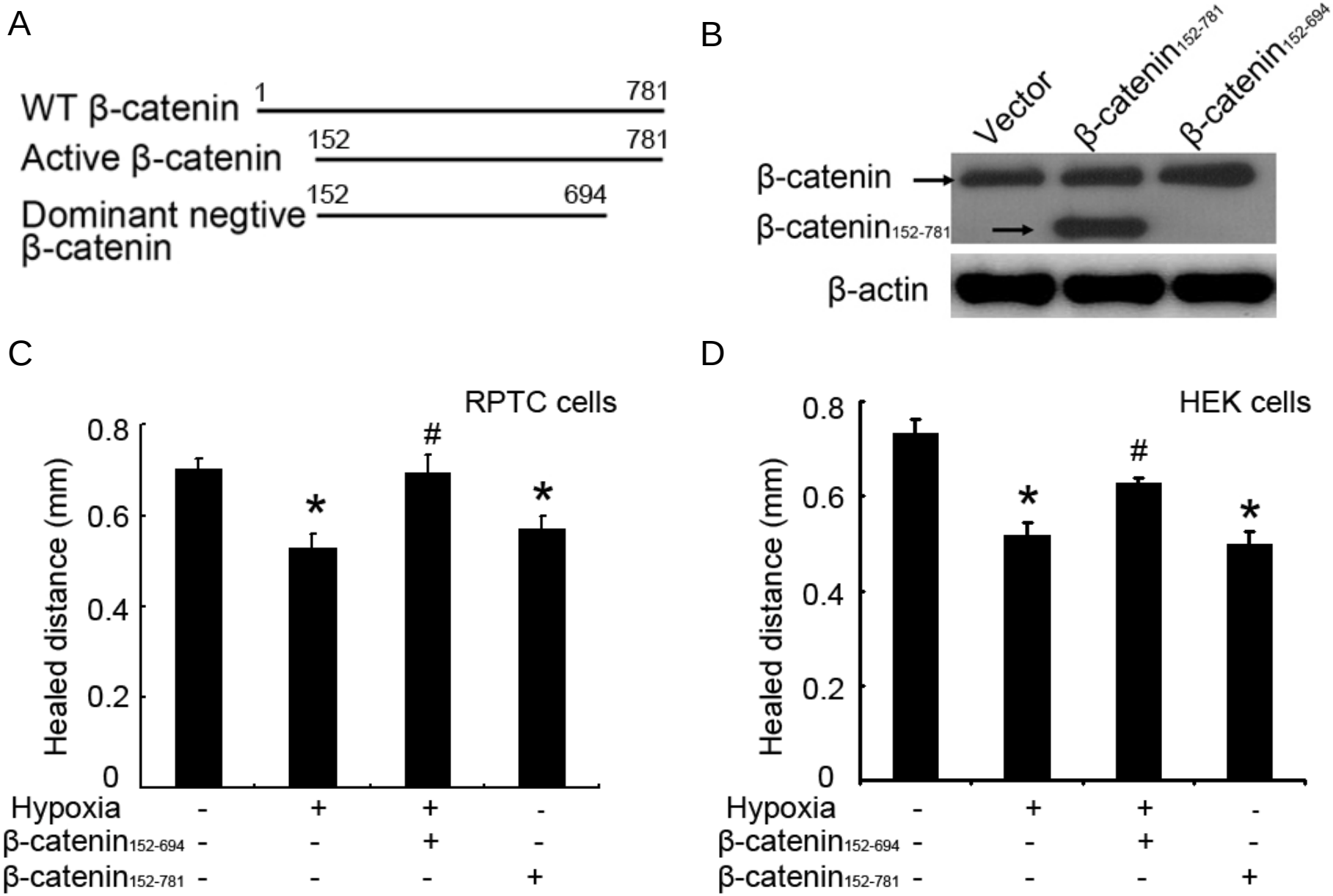

Figure 8