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Secreted frizzled related protein 2 protects cells from apoptosis by blocking the effect of canonical Wnt3a

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Abstract

We have demonstrated that mesenchymal stem cells overexpressing the survival gene Akt can confer paracrine protection to ischemic myocytes both in vivo and in vitro through the release of secreted frizzled related protein 2 (Sfrp2). However, the mechanisms mediating these effects of Sfrp2 have not been fully elucidated. In this study, we studied rat cardiomyoblasts subjected to hypoxia reoxygenation (HR) injury to test the hypothesis that Sfrp2 exerts anti-apoptotic effect by antagonizing pro-apoptotic properties of specific Wnt ligands. We examined the effect of Wnt3a and Sfrp2 on HR-induced apoptosis. Wnt3a significantly increased cellular caspase activities and TUNEL staining in response to HR. Sfrp2 attenuated significantly Wnt3a-induced caspase activities in a concentration dependent fashion. Using a solid phase binding assay, our data demonstrates that Sfrp2 physically binds to Wnt3a. In addition, we observed that Sfrp2 dramatically inhibits the beta-catenin/TCF transcriptional activities induced by Wnt3a. Impressively, Dickkopf-1, a protein that binds to the Wnt coreceptor LRP, significantly inhibited the Wnt3a-activated caspase and transcriptional activities. Similarly, siRNA against beta-catenin markedly inhibited the Wnt3a-activated caspase activities. Consistent with this, significantly fewer TUNEL positive cells were observed in siRNA transfected cells than in control cells. Together, our data provide strong evidence to support the notion that Wnt3a is a canonical Wnt with pro-apoptotic action whose cellular activity is prevented by Sfrp2 through, at least in part, the direct binding of these molecules. These results can explain the in vivo protective effect of Sfrp2 and highlight its therapeutic potential for the ischemic heart.

Keywords

Sfrp2; Wnt3a; apoptosis; hypoxia reoxygenation; beta-catenin

1. Introduction

It has been shown that intramyocardial transplantation of mesenchymal stem cells (MSCs) over-expressing the survival gene Akt result in significant increase in cell viability and engraftment, reduction of infarct size and improvement of cardiac function [1,2]. Recently, our

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laboratory has demonstrated that Akt MSCs release Sfrp2 that mediate the pro-survival effects of these cells on ischemic cardiomyocytes. Indeed the cytoprotective effects of these cells were markedly attenuated upon knockdown of Sfrp2 with siRNA [3]. Taken together, these data support a key role played by Sfrp2 in mediating the paracrine effects of Akt-MSC on myocardial protection.

The mechanisms mediating Sfrp2's cellular survival effect has not been precisely elucidated. It is currently thought that Sfrps compete with the Frizzled receptor for Wnt ligands thereby preventing the activation of Wnt signaling [4]. Through this interaction, Sfrps could potentially influence cell fate and survival. Accordingly, we hypothesize that Sfrp2 exerts anti-apoptotic effect by antagonizing Wnts with pro-apoptotic properties. To further explore this possibility, we studied rat cardiomyoblasts subjected to hypoxia reoxygenation (HR). We examined the effect of Wnt3a on HR-induced apoptosis and then studied the nature of the interaction of Sfrp2 with Wnt3a. Wnt3a was selected since its expression is up-regulated in response to hypoxia [3] and it has been shown to induce apoptosis.

We examined the effect of Sfrp2 on canonical pathway activation induced by Wnt3a. Wnt ligands bind to the frizzled receptors and co-receptors LRP5/6, leading to phosphorylation of the disheveled protein, which, through its association with Axin and the adenomatous polyosis coli (APC) tumor suppressor, prevents glycogen synthase kinase 3beta from phosphorylating beta-catenin. Unphosphorylated beta-catenin is stabilized by escaping recognition by beta-TrCP, a component of an E3 ubiquitin ligase. Free beta-catenin translocates to the nucleus, where it engages transcription factors T cell factor/lymphoid enhancer factor (TCF/LEF) to activate downstream genes [5,6]. This signaling pathway can be modulated by Sfrp [4,7–11]. In this article, we demonstrated that Wnt3a induces apoptosis via the canonical pathway and that Sfrp2 protects hypoxic and reoxygenated cells by inhibiting the pro-apoptotic effect of canonical Wnt3a. Furthermore, our data showed that Sfrp2 physically binds to Wnt3a. This may explain, at least in part, how Sfrp2 antagonizes the Wnt3a cellular effect. In addition, we observed that Sfrp2 inhibits the beta-catenin/TCF transcriptional activities induced by Wnt3a. Impressively, Dickkopf-1 (DKK1) and siRNA against beta-catenin significantly inhibited the Wnt3a-activated apoptosis and transcriptional activities. Together, our data provide strong evidence to support the notion that Wnt3a is a canonical Wnt with pro-apoptotic action whose cellular activity is prevented by Sfrp2 through, at least in part, direct binding of these molecules.

2. Materials and Methods

2.1. Cell cultures and hypoxia/reoxygenation treatment

H9C2, a clonal line of rat embryonic heart-derived myoblasts, was obtained from American Type Culture Collection (CRL-1446, Manassas, VA USA). The cells were maintained in culture medium consisting of Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Grand Island, N.Y. USA), 10% fetal bovine serum (Hyclone, Logan, UT USA), penicillin (100U/ml), streptomycin (100U/ml), and glutamine (4mM). To prevent loss of differentiation potential, cells were not allowed to become confluent. Passages 12–25 were used for all experiments described in this article. Cell density and viability were determined by Trypan blue dye exclusion test. After changing to hypoxic serum-free medium, cells were incubated under hypoxia and then putinto normoxia for indicated hours.

2.2. Western Blotting

Nuclear fractions of protein were isolated according to the protocol of FractionPREP Cell Fractionation System (Biovision, Mountain View, CA USA). Proteins from H9C2 cell lysates were separated by SDS page gel (Invitrogen, Carlsbad, California USA) and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA USA). The blots were incubated with beta-

catenin primary antibody (Cell Signaling, Danvers, MA USA) and then with appropriate second antibody conjugated with horseradish peroxidase (Amersham Biosciences, Piscataway, NJ USA). Complexes were detected by chemiluminescence (LumiGLO, Cell Signaling, Danvers, MA USA).

2.3. In situ cell death (TUNEL) analysis

The TUNEL assay (terminal deoxynucleotidyl transferase [TdT]-mediated 3′ ends of cleaved DNA labeling) was carried out according to the protocols of DeadEnd Colorimetric TUNEL System (Promega, Madison, WI USA). H9C2 cells, transfected with siRNAs against rat beta-catenin or scrambled control siRNA, were seeded on glass slide chambers and then treated with hypoxia/reoxygenation. Cells were fixed with 4% formaldehyde, permeabilized with proteinase K 20ug/ml following by the incubation with rTdT enzyme and biotinylated nucleotides. Thenstrepavidin-horseradish peroxidasewas developed. TUNEL staining is quantified by calculating the percentage of TUNEL positive nuclei in total nucleiin each low magnification field.

2.4. Quantification of caspases

Apoptosis was determined by measuring the activity of cleaved-caspase 3/7 using a caspase-specific fluorogenic substrateaccording to the protocol for Caspas 3/7assay kit (Sigma, St. Lousi, MOUSA). H9C2 cellswere lysed after treatment with Wnt3a and/or Sfrp2 for 24 hours under hypoxia/reoxygenation. 5ul of cell extract was incubatedin reaction buffer at room temperature for 1 hour. The enzyme-catalyzed release of 7-amino-4-methyl coumarin (AMC) was measured by a fluorescence microplate reader. Fluorescent units were converted to pmole AMC/hr/ug protein, using a standard curve of AMC.

2.5. Luciferase reporter assay

Cells were plated to for 60–80% confluent cultures in 12-well dishes. All transfection experiments were performed in triplicate with Lipofectamine kit (Invitrogen, Carlsbad, California USA), in accordance with the manufacture's instructions. TopFlash and the negative control counterpart FopFlash contain TCF binding sites (Top) and inactive TCF binding sites (Fop) (catalog #17-285, Upstate, Billerica, MA USA), were used to report TCF/beta-catenin signaling in the cells. The pRL-CMV renilla luciferase (Promega, Madison, WI USA) was co-transfected to normalize for transfection efficiency and total amount of transfected plasmid was made equal by addition of pUC18 vector (Stratagene, La Jolla, CA USA). Luciferase activity was assayed 48 hours after transfection, using a dual-luciferase reporter assay system (Promega, Madison, WI USA).

2.6. Sfrp2/Wnt3a ELISA binding assays

Different doses of Sfrp2 (R&D System, Minneapolis, MN USA) diluted in 0.1% bovine serum albumin(BSA)/PBS was incubated in 96-well Falcon ELISA (100ul/well) for over night at room temperature (RT). After decanting, all wells were filled with 5%BSA/PBS (300ul/well) and incubated for an additional 3 h at 37°C to block the plate. Following three washes with wash buffer (0.05% Tween 20 in PBS, pH 7.4), 100ul aliquots of 3 or 15nMofwnt3a (R&D Systems, Minneapolis, MN USA) diluted in 0.1% BSA/PBS were added and incubated for 2 h at RT. After three washes, 100ul/well of Wnt3a antibody (R&D Systems, Minneapolis, MN USA) diluted in 01% BSA/PBS to a final concentration of 1ug/ml was incubated for1 h at RT. Another three washes were followed by a 1-h treatment at RT with 1: 10000 dilution of conjugated HRP goat anti-rat IgG (abcam, Cambridge, MA USA). After a final set of three washes, 100ul/well of Color reagent (R&D Systems, Minneapolis, MN USA) was added and incubated for 20mins at RT following by 50ul/well of Stop Solution (R&D Systems,

Minneapolis, MN USA). Absorbance at 450nm was measured with an ELISA plate reader (BMG LABTECH, Durham, NC USA).

2.7. Sfrp2/Wnt3a immuno-precipitation

15nM of Sfrp2 and/or Wnt3a were incubated in binding buffer (0.1% BSA/PBS) at RT for overnight following by pre-cleaning with 50ul of Protein G beads (Pierce, Rockford, IL). At 4°C, the samples were reacted for overnight with Wnt3a antibody (R&D Systems, Minneapolis, MN USA) dilutedto a final concentration of 1ug/ml.50ul of Protein G beads was added in the samples and incubated with mixing at RT for 1 h. Collected beads were denatured for SDS page and blotted by Sfrp2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Recombinant Sfrp2 (R&D System, Minneapolis, MN USA) was loaded as a size control.

2.7. Electroporation

Pelleted cells were resuspended in fresh medium and counted, and sufficient cells for all transfections (1×10^6cells per transfection) were transferred to a fresh tube and centrifuged at 80×g for 10 minutes. After carefully removing the supernatant, the cell pellet was resuspended in 100ul of cellline nucleofector solution L (Amaxa biosystems, Gaithersburg, MD USA) per transfection, then each transfection was added with 2nmole of siRNA against rat beta-catenin or scrambled control siRNA. The solution was putto Amaxa electrode cuvettes and electroporated in an Amaxa Nucleofector using program C-20 (Amaxa biosystems, Gaithersburg, MD USA). Immediately afterward, cells were diluted in 0.5mL DMEM (supplemented with 10% FCS, prewarmed at 37 °C) and incubated the cells at 37 °C for 10 minutes. Cells were then seeded into dishes or slide chambers and incubated for the indicated hours.

2.8. Suppression of rat beta-catenin by siRNA

H9C2 cells with 2nmole of siRNAs against rat endogenous beta-catenin (siRNA1, sense (5′→3′): CCUCCCAAGUCCUUUAUGAtt, antisense (5′→3′): UCAUAAAGGACUUGGGAGGtg; siRNA2, sense (5′→3′): GCUGACCAAACUGCUAAAUtt, antisense (5′→3′): AUUUAGCAGUUUGGUCAGCtc, Ambion, Austin, TX USA) or scramble control siRNA (sense (5′→3′): CCUCCCAAGUCCUUUAUGAtt, antisense (5′→3′): UCAUAAAGGACUUGGGAGGtg, Ambion, Austin, TX USA) were electroporated. After 18 hours incubation, the cells were exposed to hypoxia and reoxygenation.

2.9. Myocardial infarction models and Sfrp2 expression in these models

Ligation of the left anterior descending (LAD) coronary artery was performed on 170- to 200-g female Sprague-Dawley rats (Harlan World Headquarters, Indianapolis, IN). Briefly, a left thoracotomy was performed under anesthesia, and the vessel was ligated with a silk suture at midway between the left atrium and the apex of the heart. The infarction then was assessed by the change of color and kinesis of the apex and the anterior-lateral wall. Then, the wound was sutured immediately. In permanent ligation model, the LAD coronary artery was occluded permanently. In reperfusion model, 30mins after ligation, the ligature was loosed. Sham-operated mice underwent the same procedures without ligation and reperfusion. All the procedures were approved by the Harvard Medical Area Standing Committee on Animal Care and Use.

Protein and RNA were isolated from sham, permanent ligation and reperfusion injured hearts. Sfrp2 protein levels were detected by western blot with Sfrp2 primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and then with anti-goat secondary antibody conjugated with horseradish peroxidase (Amersham Bioscienes, Piscatoway, NJ USA). RNA from these

samples were reverse transcripted to DNA and detected by primers (forward: CACGAGACCATGAAGGAGGT; reward: TGAGCCACAGCACTGATTTC) to measure mRNA levels of Sfrp2. GAPDH mRNA levels were used as a loading control (forward primer: CCCCTGGCCAAGGTCATCCATGACAACTTT; reward primer: GGCCATGAGGTCCACCACCCTGTTGCTGTA).

3. Results

3.1. Cardiac expression of Sfrp2

mRNA and protein levels were detected in rat hearts subjected to sham, permanent ligation and reperfusion injuries (Fig. 1A). Sfrp2 protein levels were significantly decreased in respond to permanent ligation and reperfusion. Interestingly, Sfrp2 mRNA levels were up-regulated in permanent ligation and reperfusion injuries, indicating that in vivo heart needs Sfrp2 during reperfusion.

3.2. Wnt3a increases apoptosis during hypoxia reoxygenation in H9C2 cells

H9C2 cells were incubated with Wnt3a recombinant protein under hypoxia followed by reoxygenation and the effects on caspase 3/7 activities were studied. As shown in Fig. 1B and C, 24 hours hypoxia followed by 24 hours reoxygenation resulted in marked cell apoptotic death in H9C2 cells. The addition of 3nM Wnt3a in serum free medium significantly increased cellular caspase activities by 35% in response to hypoxia and reoxygenation. To further characterize the effect of Wnt3a, we performed TUNEL staining. Consistent with the caspase results, the number of TUNEL positive cells was augmented in Wnt3a treated group compared with control (Fig. 1C).

3.3. Sfrp2 blocks the apoptotic effect induced by Wnt3a

To assess the effect of Sfrp2 on Wnt3a-induced caspase activity, H9C2 cells in serum free medium were treated with Wnt3a alone or Wnt3a in combination with increasing concentrations of recombinant Sfrp2 protein (3, 30 and 300 nM) during hypoxia and reoxygenation. Sfrp2 attenuated significantly Wnt3a-induced caspase activity in a concentration dependent fashion, achieving 36% inhibition at a concentration of 300nM (Fig. 1B). The similar results were shown in TUNEL staining and its quantification (Fig. 1C).

3.4. Sfrp2 inhibits Wnt3a effect on nuclear beta-catenin accumulation and activity

To examine the mechanism for Sfrp2 inhibitory effect on Wnt3a induced apoptosis, nuclear beta-catenin levels were determined with Western blot analysis in hypoxic and reoxygenated H9C2 cells incubated with Wnt3a alone or Wnt3a in combination with different concentrations of Sfrp2. As shown in Fig. 2A, the addition of Wnt3a significantly increased nuclear beta-catenin levels, indicating that Wnt3a activated canonical signaling pathway. Sfrp2 reduced significantly beta-catenin levels induced by Wnt3a in isolated nuclear fractions as compared to Wnt3a alone. This effect is particularly remarkable at Sfrp2 concentrations of 30nM and 300nM. Thus the apoptosis-inhibitory effect of Sfrp2 is associated with the suppression of canonical beta-catenin signaling activated by Wnt3a.

To further confirm the inhibitory effect of Sfrp2 on beta-catenin signaling, Top/Fop Flash assay was performed. It has been established that association of beta-catenin with the TCF/LEF family of transcription factors promotes the expression of many genes [12]. Accordingly the beta-catenin-dependent TCF transcriptional activity was measured in H9C2 cells during hypoxia reoxygenation with Wnt3a alone or Wnt3a in combination of increasing concentrations of Sfrp2. As shown in Fig. 2B, Wnt3a activated TCF transcriptional activity during hypoxia reoxygenation. Treatment with 300nM of Sfrp2 significantly suppressed beta-

catenin/TCF transcriptional activity induced by Wnt3a by 47%. This observation provides further evidence for the apoptosis-inhibitory effect of Sfrp2 by interfering with Wnt3a canonical activity.

3.5. Direct binding of Sfrp2 with Wnt3a

Next, we tested for a direct interaction between Sfrp2 and Wnt3a, we used the ELISA method to study potential binding of these two proteins. This method has been used by Uren A, et al. to observe the binding of Sfrp1 with Wnts [13]. As shown in Fig. 3A, Wnt3a bound avidly to Sfrp2 in a concentration-dependent manner. Using 15nM of Wnt3a, we noticed that the binding was saturated when Sfrp2 is 15nM (Fig. 3A). While using 3nM of Wnt3a, we observed that the binding was saturated when the concentration of Sfrp2 is 3nM (Fig. 3B). We found no binding of Wnt3a to Sfrp3 indicating the specificity of binding between Wnt3a and Sfrp2 (Fig. 3C). Immuno-precipitation results confirmed that Sfrp2 interacted with Wnt3a (Fig. 3D).

3.6. DKK1 inhibits apoptosis induced by Wnt3a

To further explore the mechanism for the blocking effect of Sfrp2 on apoptosis, caspase activities were measured in hypoxic and re-oxygenated H9C2 cells incubated with Wnt3a or Dickkopf1 (DKK1) alone or Wnt3a in combination of increasing concentration of DKK1. DKK1 is a secreted protein that binds to LRP5/6 thereby preventing the formation of Wnt-Frizzled-LRP5/6 receptor complexes and consequently blocking canonical Wnt signaling [14]. Consistent with earlier data (Fig. 1), Wnt3a gave a 44% increase of caspase activities in hypoxic and re-oxygenated cells (Fig. 4A). DKK1 had no effect on the caspase activity in these hypoxic and reoxygenated H9C2 cells, but markedly inhibited Wnt3a-induced caspase activity in a concentration dependent manner (Fig. 4A). The caspase activities were decreased 28% with 30nM and 31% with 300nM of DKK1, down to the levels of hypoxic and re-oxygenated control. Taken together, our data demonstrated that Wnt3a induced apoptosis via the canonical beta-catenin signaling pathway.

3.7. DKK1 blocks Wnt3a-induced beta-catenin/TCF transcriptional activities

In order to confirm the blocking effect of DKK1 on Wnt3a related canonical activity, beta-catenin/TCF transcriptional activities were determined using Top/Fop Flash in hypoxic and reoxygenated H9C2 cells incubated with Wnt3a or DKK1 alone or Wnt3a in combination of different concentrations of DKK1. As shown in Fig. 4B, and consistent with the results in Fig. 2, Wnt3a dramatically enhanced TCF activities in hypoxic and re-oxygenated cells. While DKK1 alone slightly reduced intrinsic beta-catenin/TCF activities, DKK1 significantly inhibited Wnt3a-induced beta-catenin/TCF transcriptional activities in a concentration-dependent manner during hypoxia and reoxygenation. Treatment with DKK1 (from 3nM to 300nM) decreased beta-catenin/TCF activities to, 35% at 3nM, 62% at 30nM and 77% at 300nM, of those for Wnt3a alone. Our data demonstrated that DKK1 blocked Wnt3a induced apoptosis through the inhibition of beta-catenin/TCF transcriptional activities.

3.8. siRNA against beta-catenin blocked the pro-apoptotic effect of Wnt3a in hypoxic and reoxygenated cells

Finally, to establish the direct relationship of Wnt3a-induced beta-catenin with apoptosis, siRNAs against rat beta-catenin or their scrambled control siRNA were transfected into hypoxic and re-oxygenated H9C2 cells with nucleofector technology. As shown in Fig. 5A, siRNAs directed toward endogenous beta-catenin dramatically inhibited nuclear beta-catenin protein levels as compared to scrambled control siRNA. Wnt3a markedly increased nuclear beta-catenin in the cells transfected with scrambled control siRNA. To observe the inhibitory effect of siRNAs, caspases activities were measured in these samples. As expected, Wnt3a induced 22% increase of caspase activities in the cells transfected with scrambled control

siRNA. Impressively, in the cells transfected with siRNAs against beta-catenin, Wnt3a lost its pro-apoptotic effect by 59%, suggesting that beta-catenin contributes to the apoptosis induced by Wnt3a in the cells subject to hypoxia and re-oxygenation.

To further confirm the effect of endogenous beta-catenin on apoptosis of H9C2 cells, TUNEL staining was performed on siRNAs transfected, hypoxic and re-oxygenated H9C2 cells. As shown in Fig. 5B, there were significantly fewer TUNEL positive cells in siRNA transfected cells than scramble control cells. The quantification of TUNEL staining gave us similar results. Interestingly, we observed changes in H9C2 cell morphology after transfection of siRNA there are more well spread cells indicating increased viability in beta-catenin knock down group than in scrambled control siRNA group. A significant increase in H9C2 cell number was observed after beta-catenin siRNA transfection compared with the control. These results confirmed that beta-catenin plays an important role in Wnt3a-induced apoptosis in the cells exposed to hypoxia and reoxygenation.

4. Discussion

The Wnt family comprises 19 different proteins and has been implicated in diverse cellular processes including apoptosis and cell survival [15]. However, the precise cellular effect depends on the dose and type of Wnt, tissue, cells as well as environmental cues. For example, Wnt1 inhibits TNF mediated apoptosis of certain cancer cells [16], while Wnt7b has been recently identified to induce apoptosis in human vascular endothelial cells [17]. Wnt3a induced dramatic growth retardation in untransformed cell lines of mouse embryonic fibroblasts, by augmenting beta-catenin/TCF activity [18]. Interestingly, Wnt3a also inhibits the proliferation and induces beta-catenin accumulation, nuclear translocation and TCF/LEF-1 transcriptions in several leukaemia cell lines of Nalm-6, BV173 and Reh cells [19]. A study recently published by our group indicates that Sfrp2 regulates differentiation of mouse embryonic carcinoma stem cells by inhibition Wnt3a gene transcription [20].

For the relationship of Wnt/beta-catenin and cellular apoptosis, it has been demonstrated that over expression of beta-catenin, a downstream mediator of Wnt signaling, promotes apoptosis in several mammalian cell lines [21,22]. Moreover, transfection with beta-catenin small interfering RNA or expression of dominant negative TCF inhibited apoptosis, whereas expression of dominant stable beta-catenin caused significant apoptosis in human ovarian surface epithelial cells [23]. Similar results were also obtained in human cell line of T/C28a [24], indicating a pro-apoptotic role of beta-catenin/TCF. Interestingly, in our study, the siRNA inhibition of beta-catenin in presence of added Wnt3a reduces the caspase activity not only well below the control (no inhibition but added Wnt3a), but also markedly below the control (no inhibition and no Wnt3a), indicating that beta-catenin siRNA could suppress apoptosis induced by Wnt3a plus hypoxia reoxygenation. It is interesting that a paper in “Acta Biochim Biophys Sin” [25] shows that Wnt3a in H9C2 cells promoted cell cycling. For the discrepancies between our paper and theirs, there are two major reasons: First, they transfected plasmids with Wnt3a full-length into H9C2 cells, while we added purified Wnt3a into the medium of H9C2; Second, they just cultured the H9C2 cells at 37 °C with 5% CO_2 in a humidified atmosphere, while our H9C2 cells were in hypoxic reoxygenation.

Further evidence implicating the roles of Wnt signaling in apoptosis is supported by observations demonstrating embryonic lethality and widespread apoptosis in various tissues in GSK3beta knockout mice [26], GSK3beta being a negative regulator of Wnt signaling. In addition, functional loss of the APC gene, which is a negative regulator of Wnt signaling through downregulation of beta-catenin, causes accumulation of beta-catenin and leads to severe apoptosis in neural crest cells [27]. These observations clearly support a role of canonical Wnt/beta-catenin signaling in mediating apoptosis in specific cells.

Previous studies have indicated an anti-apoptotic role of Sfrp2 in mediating cellular resistance to UV and TNF induced apoptosis in mammalian cell lines [10,28,29]. Furthermore, over-expression of Sfrp1 reduced infarct size and improved ventricular function in mice of myocardial infarction and was associated with the decrease of beta-catenin and attenuation of Wnt signaling [30]. Additional strong circumstantial evidence exists supporting the role of Sfrps in regulation of apoptosis. For example, Sfrp1 and Sfrp2 are produced by long-term and ex vivo malignant glioma cells and are thought to act as survival and proliferation promoting factors [31]. These observations demonstrate a role of Sfrp in the regulation of apoptosis.

Sfrps possess a cysteine rich domain (CRD) that is similar to a homologous region on the frizzled receptor that binds Wnts [32–34], and are thought to bind and sequester Wnts away from active receptor complexes. Thus, the shared sequence homology between the Frizzled and Sfrp CRDs suggests that the binding of Wnt to the Sfrp CRD is responsible for the inhibition of Wnt activity by Sfrps. Consistent with this notion, the CRD of Sfrp has also been shown to be sufficient and necessary for interaction with Wnt proteins [35–37]. In the neural tube, Sfrp1,2 and 3 are expressed in or adjacent to the Wnt1/Wnt3a expression domain in the dorsal neural tube, suggesting that they might serve as regulators of Wnt1 and/or Wnt3a activity [38–43]. In addition, Sfrp1 and 2 are able to attenuate Wnt3a-induced accumulation of beta-catenin in L cells, while Sfrp3 can not [44]. Their results also show that the inhibition of Wnt3a activity occurs at the cell surface. Interestingly, Sfrp2 and Wnt7b, which are normally expressed in overlapping regions in the ventral and intermediate zones of the spinal cord, are also missing in this region in Pax-6 mutants [45], indicating a role of Sfrp2 in regulation of Wnt7b. Interestingly, Wnt7b has been identified to induce apoptosis in human vascular endothelial cells [17]. Wawrzak et al. [46], using Plasmon Resonance technique to measure the affinity of Wnt3a binding with Sfrp, supported our ELISA and immuno-precipitation data. Consistent with the above results of Sfrp and Wnt interactions, our studies show that Sfrp2 inhibits Wnt3a induced apoptosis of cardiomyoblasts by direct specific binding to Wnt3a.

Our studies elucidate the molecular mechanisms of interruption of Wnt signaling by Sfrp2, the key Akt-MSC released paracrine factor. H9C2, a rat heart cell line, was exposed to hypoxic reoxygenation to mimic reperfusion myocardial infarction model in vitro. It is difficult to get physiological concentrations of Wnt3a and Sfrp2 [47]. Our results show that Sfrp2 could be clearly detected in 10ug heart tissue protein, suggesting that there is more than 1ng Sfrp2 in 1ug heart protein. These findings suggest that Sfrp2 induces cytoprotection of H9C2 cells through inhibition of canonical Wnt pathway in the cells, supporting its in vivo protective effect and the therapeutic potential for the ischemic heart. Thus conceivably, in hypoxia reoxygenation induced injury, Wnt signaling plays a pathophysiological role, and its antagonism may be important for cell protection. Furthermore, since the wnt signaling pathways play important roles in embryogenesis and oncogenesis, our data should provide insight into the mechanism of sfrps in the modulation of these processes.

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Abbreviations

Sfrp2
: secreted frizzled related protein 2

HR
: hypoxia reoxygenation

MSCs
: mesenchymal stem cells

APC
: adenomatous polyosis coli

TCF/LEF
: T cell factor/lymphoid enhancer factor

DKK1
: Dickkopf-1

TdT
: terminal deoxynucleotidyl transferase

AMC
: 7-amino-4-methyl coumarin

BSA
: bovine serum albumin

RT
: room temperature

CRD
: cysteine rich domain

References

1. Mangi AA, Noiseux N, Kong D, He H, Rezvani M, Ingwall JS, et al. Mesenchymal stem cells modified with Akt Prevent remodeling and restore performance of infarcted hearts. Nat Med Sep;2003 9(9): 1195–1201. [PubMed: 12910262]
2. Gnecchi M, He H, Liang OD, Melo LG, Morello F, Mu H, et al. Paracrine action acconts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat Med Apr;2005 11(4):367–368. [PubMed: 15812508]
3. Mirotsou M, Zhang Z, Deb A, Zhang L, Gnecchi M, Noiseux N, et al. Secreted frizzled related protein 2 (Sfrp2) is the key Akt-mesenchymal stem cell-released paracrine factor mediating myocardial survival and repair. Proc Natl Acad Sci U S A Jan;2007 104(5):1643–1648. [PubMed: 17251350]
4. Dann CE, Hsieh JC, Rattner A, Sharma D, Nathans J, Leahy DJ. Insights into Wnt binding and signaling from the structures of two Frizzled cysteine-rich domains. Nature Jul 5;2001 412(6842):86–90. [PubMed: 11452312]
5. Nelson WJ, Nusse R. Convergence of Wnt, beta-catenin, and cadherin pathways. Science Mar 5;2004 303(5663):1483–1487. [PubMed: 15001769]
6. Gordon MD, Nusse R. Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors. J Biol Chem Aug 11;2006 281(32):22429–22433. [PubMed: 16793760]
7. Wang S, Krinks M, Lin K, Luyten FP, Moos M Jr. Frzb, a secreted protein expressed in the Spemann organizer, binds and inhibits Wnt-8. Cell Mar 21;1997 88(6):757–766. [PubMed: 9118219]
8. Leyns L, Bouwmeester T, Kim SH, Piccolo S, De Robertis EM. Frzb-1 is a secreted antagonist of Wnt signaling expressed in the Spemann organizer. Cell Mar 21;1997 88(6):747–756. [PubMed: 9118218]
9. Finch PW, He X, Kelley MJ, Uren A, Schaudies RP, Popescu NC, et al. Purification and molecular cloning of a secreted, Frizzled-related antagonist of Wnt action. Proc Natl Acad Sci U S A Jun 24;1997 94(13):6770–6775. [PubMed: 9192640]
10. Melkonyan HS, Chang WC, Shapiro JP, Mahadevappa M, Fitzpatrick PA, Kiefer MC, et al. SARPs: a family of secreted apoptosis-related proteins. Proc Natl Acad Sci U S A Dec 9;1997 94(25):13636–13641. [PubMed: 9391078]

11. Bafico A, Gazit A, Pramila T, Finch PW, Yaniv A, Aaronson SA. Interaction of frizzled related protein (FRP) with Wnt ligands and the frizzled receptor suggests alternative mechanisms for FRP inhibition of Wnt signaling. J Biol Chem Jun 4;1999 274(23):16180–16187. [PubMed: 10347172]
12. Daniels DL, Weis WI. Beta-catenin directly displaces Groucho/TLE repressors form Tcf/Lef in Wnt-mediated transcription activation. Nat Struct Mol Biol Apr;2005 12(4):364–371. [PubMed: 15768032]
13. Uren A, Reichsman F, Anest V, Taylor WG, Muraiso K, Bottaro DP, et al. Secreted frizzled-related protein-1 binds directly to Wingless and is a biphasic modulator of Wnt signaling. J Biol Chem Feb 11;2000 275(6):4374–4382. [PubMed: 10660608]
14. Bafico A, Liu G, Yaniv A, Gazit A, Aaronson SA. Novel mechanism of Wnt signaling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow. Nat Cell Biol Jul;2001 3(7):683–686. [PubMed: 11433302]
15. Moon RT, Brown JD, Yang-Snyder JA, Miller JR. Structurally related receptors and antagonists complete for secreted Wnt ligands. Cell Mar 21;1997 88(6):725–728. [PubMed: 9118212]
16. Yang F, Zeng Q, Yu G, Li S, Wang CY. Wnt/beta-catenin signaling inhibits death receptor-mediated apoptosis and promotes invasive growth of HNSCC. Cell Signal May;2006 18(5):679–687. [PubMed: 16084063]
17. Lobov IB, Rao S, Carroll TJ, Vallance JE, Ito M, Ondr JK, et al. WNT7b mediates macrophage-induced programmed cell death in patterning of the vasculature. Nature Sep 15;2005 437(7057):417–421. [PubMed: 16163358]
18. Nakagiri S, Murakami A, Takada S, Akiyama T, Yonehara S. Viral FLIP enhances Wnt signaling downstream of stabilized beta-catenin, leading to control of cell growth. Mol Cell Biol Nov;2005 25 (21):9249–9258. [PubMed: 16227577]
19. Nygren MK, Dosen G, Hystad ME, Stubberud H, Funderud S, Rian E. Wnt3A activates canonical Wnt signaling in acute lymphoblastic leukaemia (ALL) cells and inhibits the proliferation of B-ALL cell lines. Br J Haematol Feb;2007 136(3):400–413. [PubMed: 17156404]
20. Deb A, Davis BH, Guo J, Ni A, Huang J, Zhang Z, Mu H, Dzau VJ. SFRP2 regulates cardiomyogenic differentiation by inhibiting a positive transcriptional autofeedback loop of Wnt3a. Stem Cells Jan; 2008 26(1):35–44. [PubMed: 17916803]
21. Kim K, Pang KM, Evans M, Hay ED. Overexpression of beta-catenin induces apoptosis independent of its transactivation function with LEF-1 or the involvement of major G1 cell cycle regulators. Mol Biol Cell Oct;2000 11(10):3509–3523. [PubMed: 11029052]
22. Olmeda D, Castel S, Vilaro S, Cano A. Beta-catenin regulation during the cell cycle: implications in G2/M and apoptosis. Mol Biol Cell Jul;2003 14(7):2844–2860. [PubMed: 12857869]
23. Pon YL, Wong AS. Gonadotropin-induced apoptosis in human ovarian surface epithelial cells is associated with cyclooxygenase-2 up-regulation via the beta-catenin/T-cell factor signaling pathway. Mol Endocrinol Dec;2006 20(12):3336–3350. [PubMed: 16945989]
24. Jullig M, Zhang WV, Ferreira A, Stott NS. MG132 induced apoptosis is associated with p53-independent induction of pro-apoptotic Noxa and transcriptional activity of beta-catenin. Apoptosis Apr;2006 11(4):627–641. [PubMed: 16673057]
25. Liu X, Liu W, Yang L, Xia B, Li J, Zuo J, Li X. Increased connexin 43 expression improves the migratory and proliferative ability of H9C2 cells by Wnt3a overexpression. Acta Biochim Biophys Sin (Shanghai) Jun;2007 39(6):391–8. [PubMed: 17558443]
26. Hoeflich KP, Luo J, Rubie EA, Tsao MS, Jin O, Woodgett JR. Requirement for glycogen synthase kinase-3beta in cell survival and NF-kappaB activation. Nature Jul 6;2000 406(6791):86–90. [PubMed: 10894547]
27. Satoh J, Kuroda Y. Beta-catenin expression in human neural cell lines following exposure to cytokines and growth factors. Neuropathology Jun;2000 20(2):113–123. [PubMed: 10935448]
28. Lee JL, Lin CT, Chueh LL, Chang CJ. Autocrine/paracrine secreted Frizzled-related protein 2 induces cellular resistance to apoptosis: a possible mechanism of mammary tumorigenesis. J Biol Chem Apr 9;2004 279(15):14602–14609. [PubMed: 14709558]
29. Lee JL, Chang CJ, Chueh LL, Lin CT. Secreted frizzled related protein 2 (sFRP2) decreases susceptibility to UV-induced apoptosis in primary culture of canine mammary gland tumors by NF-

kappaB activation or JNK suppression. Breast Cancer Res Treat Nov;2006 100(1):49–58. [PubMed: 16791480]

30. Barandon L, Couffinhal T, Ezan J, Dufourcq P, Costet P, Alzieu P, et al. Reduction of infarct size and prevention of cardiac rupture in transgenic mice overexpressing FrzA. Circulation Nov 4;2003 108(18):2282–2289. [PubMed: 14581414]
31. Roth W, Wild-Bode C, Platten M, Grimmel C, Melkonyan HS, Dichgans J, et al. Secreted frizzled-related proteins inhibit motility and promote growth of human malignant glioma cells. Oncogene Aug 31;2000 19(37):4210–4220. [PubMed: 10980594]
32. Jones SE, Jomary C. Secreted frizzled-related proteins: searching for relationships and patterns. Bioessays Sep;2002 24(9):811–820. [PubMed: 12210517]
33. He X, Saint-Jeannet JP, Wang Y, Nathans J, Dawid I, Varmus H. A member of the Frizzled protein family mediating axis induction by Wnt-5A. Science Mar 14;1997 275(5306):1652–1654. [PubMed: 9054360]
34. Wu CH, Nusse R. Ligand receptor interactions in the Wnt signaling pathway in Drosophila. J Biol Chem Nov 1;2002 277(44):41762–41769. [PubMed: 12205098]
35. Lin K, Wang S, Julius MA, Kitajewski J, Moos M Jr, Luyten FP. The cysteine-rich frizzled domain of Frzb-1 is required and sufficient for modulation of Wnt signaling. Proc Natl Acad Sci U S A Oct 14;1997 94(21):11196–11200. [PubMed: 9326585]
36. Bafico A, Gazit A, Wu-Morgan SS, Yaniv A, Aaronson SA. Characterization of Wnt-1 and Wnt-2 induced growth alterations and signaling pathways in NIH3T3 fibroblasts. Oncogene May 28;1998 16(21):2819–2825. [PubMed: 9652750]
37. Lescher B, Haenig B, Kispert A. sFRP-2 is a target of the Wnt-4 signaling pathway in the developing metanephric kidney. Dev Dyn Dec;1998 213(4):440–451. [PubMed: 9853965]
38. Duprez D, Leyns L, Bonnin MA, Lapointe F, Etchevers H, De Robertis EM, et al. Expression of Frzb-1 during chick development. Mech Dev Dec;1999 89(1–2):179–183. [PubMed: 10559495]
39. Baranski M, Berdougo E, Sandler JS, Darnell DK, Burrus LW. The dynamic expression pattern of frzb-1 suggests multiple roles in chick development. Dev Biol Jan 1;2000 217(1):25–41. [PubMed: 10625533]
40. Esteve P, Morcillo J, Bovolenta P. Early and dynamic expression of cSfrp1 during chick embryo development. Mech Dev Oct;2000 97(1–2):217–221. [PubMed: 11025229]
41. Ladher RK, Church VL, Allen S, Robson L, Abdelfattah A, Brown NA, et al. Cloning and expression of the Wnt antagonists Sfrp-2 and Frzb during chick development. Dev Biol Feb 15;2000 218(2): 183–198. [PubMed: 10656762]
42. Terry K, Magan H, Baranski M, Burrus LW. Sfrp-1 and sfrp-2 are expressed in overlapping and distinct domains during chick development. Mech Dev Oct;2000 97(1–2):177–182. [PubMed: 11025221]
43. Jin EJ, Erickson CA, Takada S, Burrus LW. Wnt and BMP signaling govern lineage segregation of melanocytes in the avian embryo. Dev Biol May 1;2001 233(1):22–37. [PubMed: 11319855]
44. Galli LM, Barnes T, Cheng T, Acosta L, Anglade A, Willert K, et al. Differential inhibition of Wnt-3a by Sfrp-1, Sfrp-2, and Sfrp-3. Dev Dyn Mar;2006 235(3):681–90. [PubMed: 16425220]
45. Kim AS, Anderson SA, Rubenstein JL, Lowenstein DH, Pleasure SJ. Pax-6 regulates expression of SFRP-2 and Wnt-7b in the developing CNS. J Neurosci Mar 1;2001 21(5):RC132. [PubMed: 11222670]
46. Wawrzak D, Métioui M, Willems E, Hendrickx M, de Genst E, Leyns L. Wnt3a binds to several Sfrps in the nanomolar range. Biochem Biophys Res Commun Jun 15;2007 357(4):1119–23. [PubMed: 17462603]
47. Sun X, Zhang R, Lin X, Xu X. Wnt3a Regulates the Development of Cardiac Neural Crest Cells by Modulating Expression of Cysteine-Rich Intestinal Protein 2 in Rhombomere 6. Circ Res Apr 11;2008 102(7):831–9. [PubMed: 18292601]

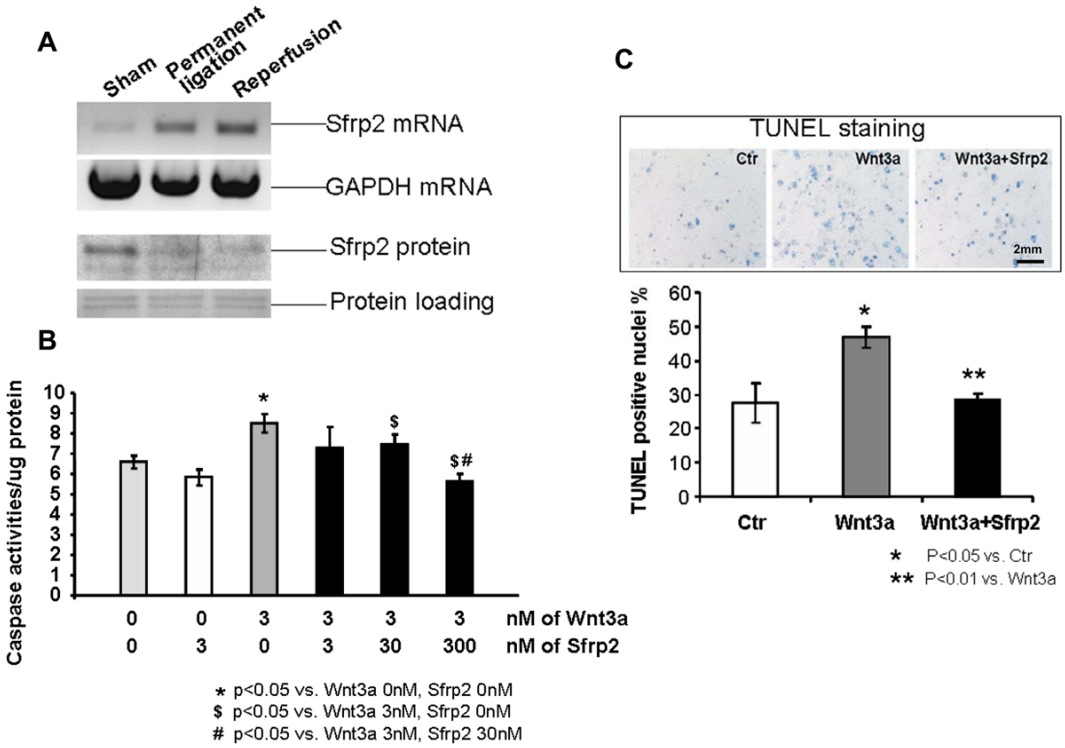

Figure 1.

The expression levels of Sfrp2 and the effects of Wnt3a/Sfrp2 on apoptosis. (A) The mRNA and protein expression levels of Sfrp2 in sham, permanent ligation and reperfusion rat hearts. GAPDH was used as RT-PCR internal control; while CBB staining to show the protein loading. (B) and (C), H9C2 cells were exposed to hypoxia (24hrs) and reoxygenation (24 hrs) with Wnt3a or Sfrp2 or Wnt3a with the addition of different doses of Sfrp2. Then the cells were assayed for caspases activities or TUNEL staining. The data of caspase are expressed as mean ± SD (B). In Fig C, hypoxic (24hrs) and reoxygenated (24hrs) cells, which were cultured in 4-well slide-chambers with 3nM Wnt3a or Wnt3a(3nM)/Sfrp2(300nM), were stained with DeadEnd Colorimetric TUNEL System. The blue color indicated the nuclei of TUNEL positive cells. Quantification of TUNEL positive cells were presented as the percentage of TUNEL positive nuclei in total nuclei.

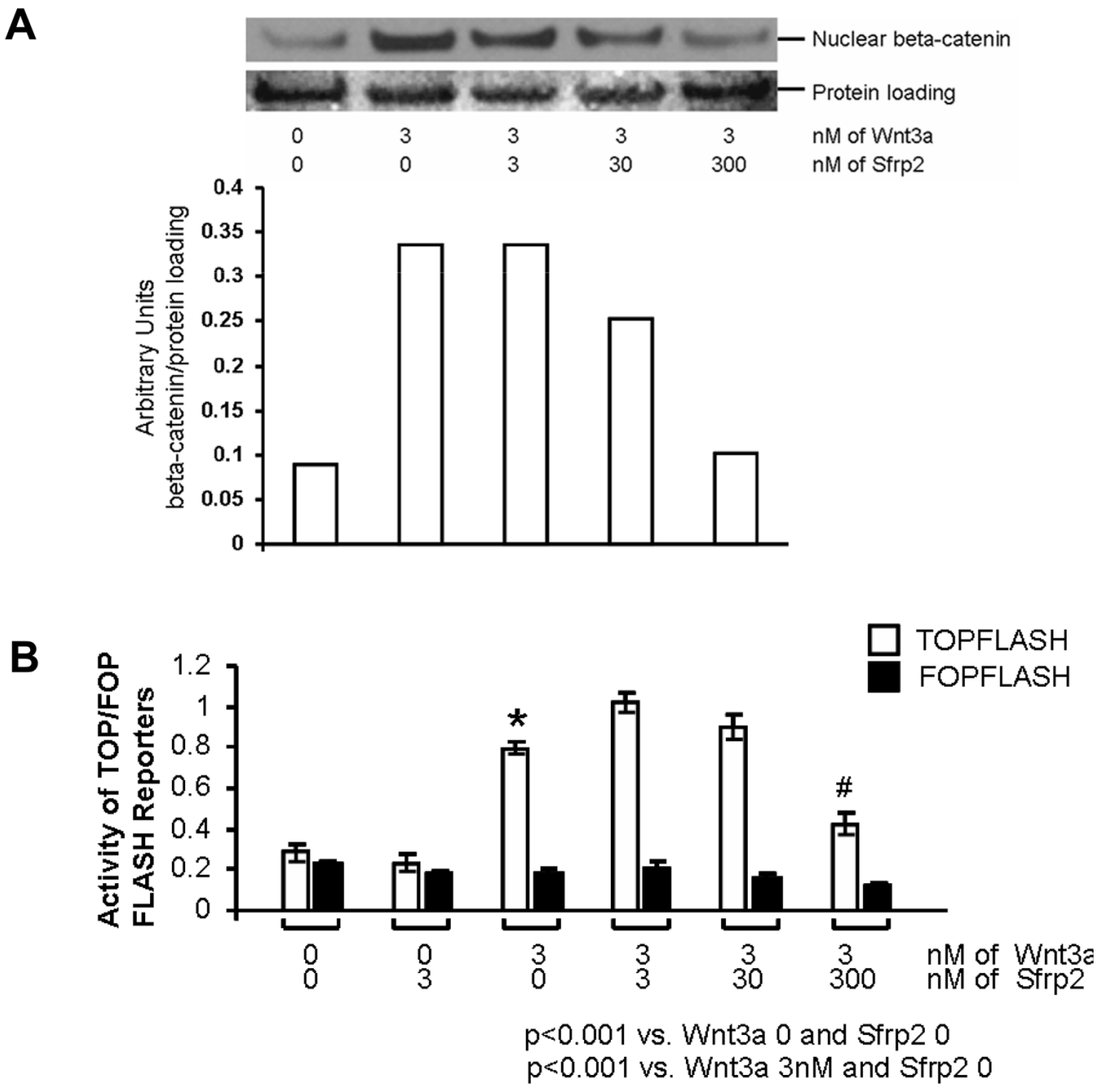

Figure 2.

The effect of Wnt3a and Sfrp2 on beta-catenin/TCF transcriptional activities. (A) Nuclear beta-catenin levels. H9C2 cells were treated with Wnt3a alone or Wnt3a in combination of increasing dose of Sfrp2 during 24-hour hypoxia and 24-hour reoxygenation. Then nuclear beta-catenin was isolated with FractionPREP Cell Fractionation System, and the protein samples were separated by SDS-PAGE. Protein loading was shown by CBB staining. (B) Beta-catenin/TCF transcriptional activities. The cells were transfected by Top or Fop Flash with pRL-CMV renilla. 24 hours later, transfected cells were treated by Wnt3a with addition of Sfrp2 or not. They were then exposed to 12-hour hypoxia and 12-hour reoxygenation, following by dual-luciferase reporter assay. The data are expressed as mean ± SD.

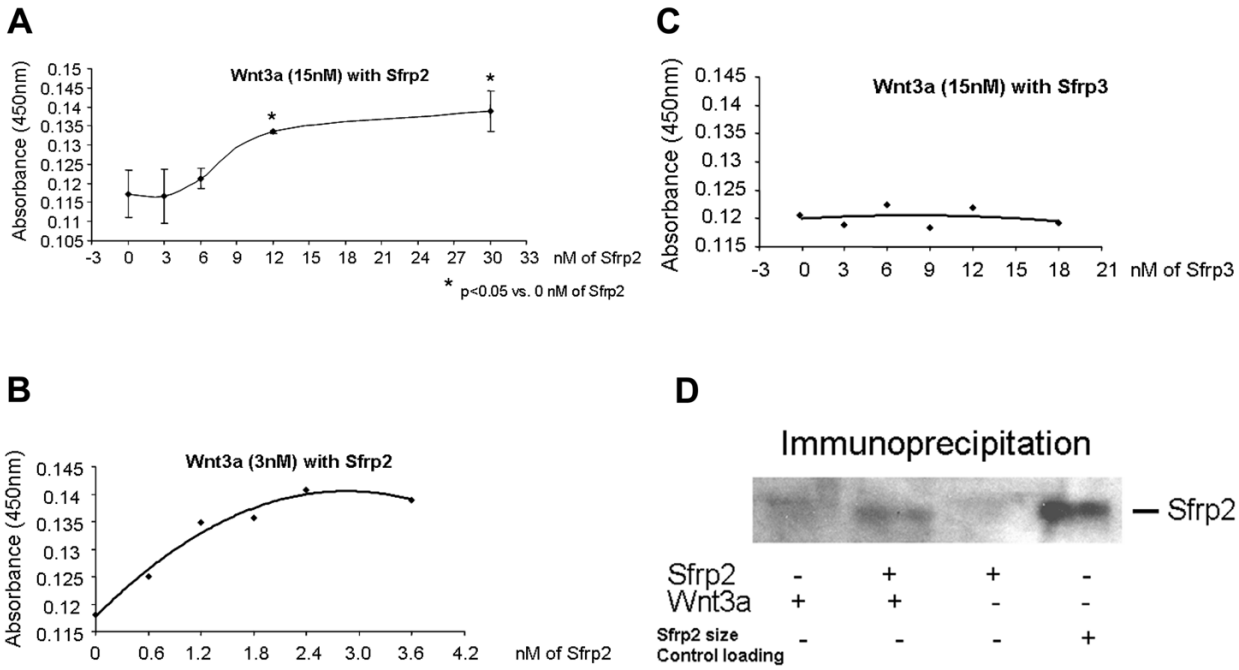

Figure 3.

The ELISA binding of Sfrp2 with Wnt3a. (A) ELISA wells were coated with different doses (from 3nM to 30nM) of Sfrp2 or BSA alone and incubated with 15nM of Wnt3a. The bound Wnt3a protein was detected with anti-Wnt3a and secondary immune reagents as described in Materials and Methods. (B) Sfrp2 (from 0.6nM to 3.6nM) were coated on 96-well Falcon ELISA plates and incubated with 3nM of Wnt3a. Absorbance at 450nm was measured with an ELISA plate reader. (C) ELISA plates were pre-incubated with 3~18nM of Sfrp3, a member of Sfrps, and then 15nM of Wnt3a was added on Sfrp3. In contrast, no increase in absorbance was observed with any concentration and the readings are comparable to basal BSA levels. Each panel is the representative of three experiments. (D) In the immunoprecipitation, the binding proteins were precipitated by Wnt3a antibody and were detected by Sfrp2 antibody in SDS page.

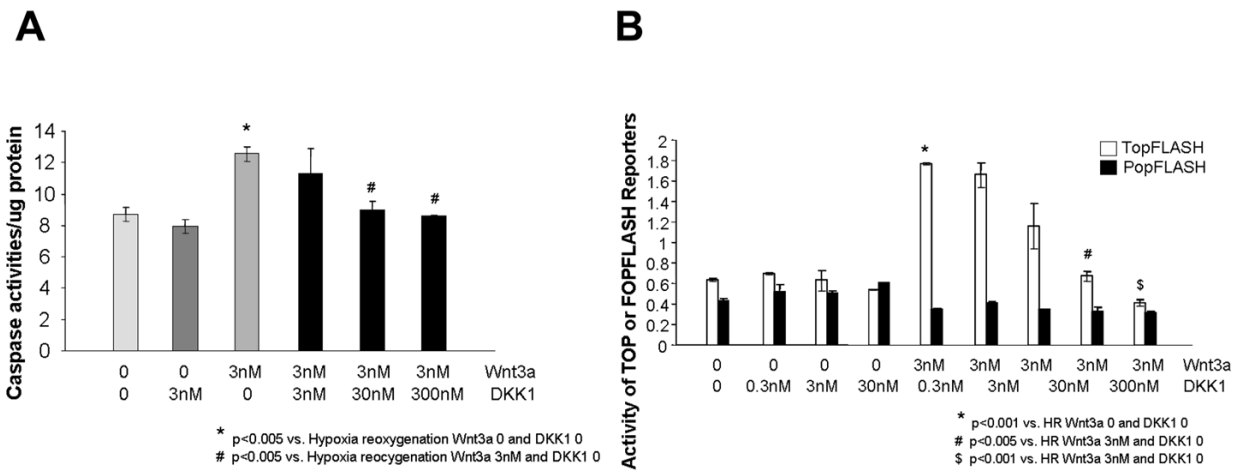

Figure 4.

The effect of DKK1 on caspases and transcriptional activities. (A) caspase activities. Wnt3a or DKK1 or Wnt3a with increasing concentration of DKK1 was added to H9C2 cells, which then exposed to 24-hour hypoxia and 24-hour reoxygenation. Dose-response inhibitory effect of DKK1 was observed. All data are represented as means ± SD. (B) transcriptional activities. Co-transfection of Top or Fop Flash with pRL-CMV renilla was performed in H9C2 cells, following by 12-hour hypoxia and 12-hour reoxygenation with Wnt3a or DKK1 or Wnt3a plus indicated amounts of DKK1. Dkk1-mediated repression of TCF activity was shown dose dependently and dramatically. The data are expressed as mean ± SD.

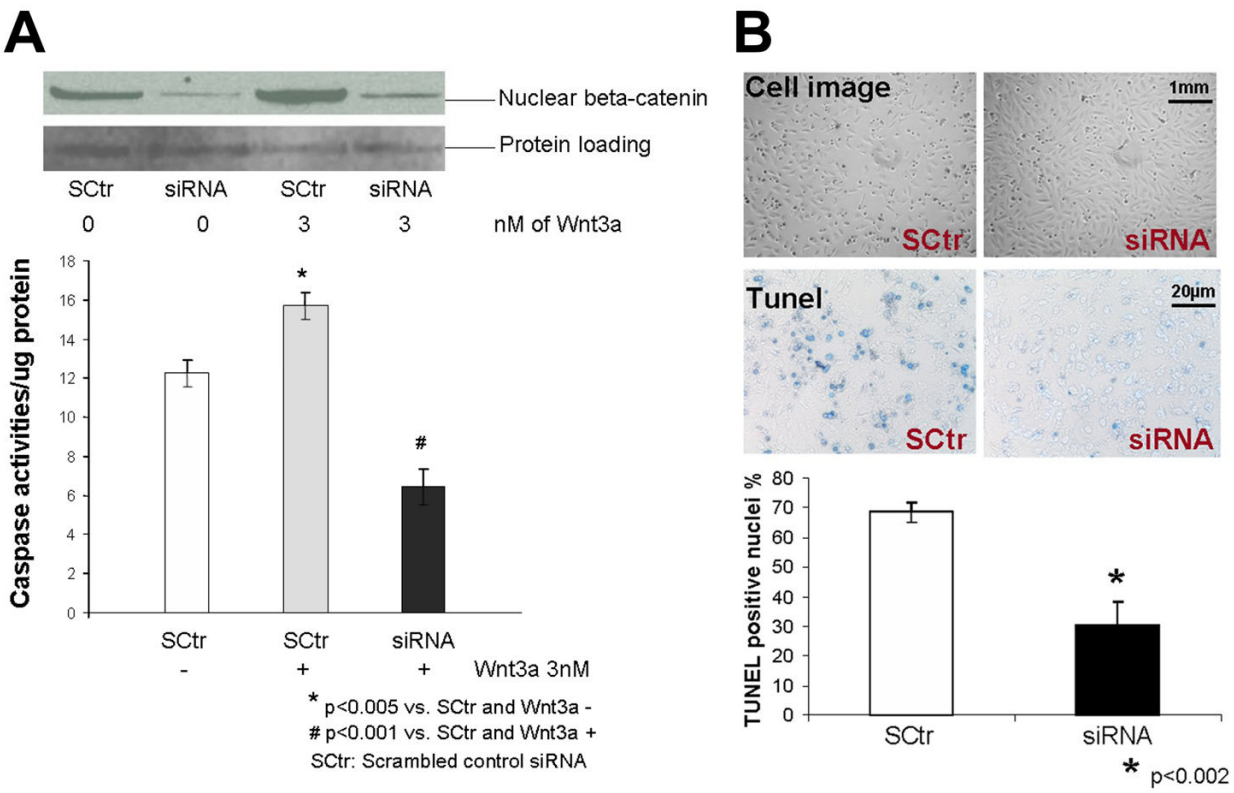

Figure 5.

The effect of Wnt3a on apoptosis in beta-catenin knock down cells. (A) caspase activities and beta-catenin. H9C2 cells were electroporated by beta-catenin siRNA and incubated for 24 hours following by 24-hour hypoxia and 24-hour reoxygenation with or without Wnt3a. Cells were harvested for caspase and Western assays. Protein loading was shown by CBB staining. The results are expressed as mean ± SD. (B) TUNEL staining and cell images. Beta-catenin siRNA was transfected into H9C2 cells with nucleofector technology following by hypoxia (24 hours) and reoxygenation (24 hours). In TUNEL staining, the blue color indicated the nuclei of TUNEL positive cells. Quantification of TUNEL positive cells were presented as the percentage of TUNEL positive nuclei in total nuclei. In the pictures of cell image, the survival of cells is indicated by increasing of cell number and spread morphology.