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Dickkopf-1 enhances migration of HEK293 cell by beta-catenin/E-cadherin degradation

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1. ABSTRACT

Migration is an important process during cellular activity and embryo development. We recently showed that Dickkopf-1(Dkk-1), an antagonist of Wnt/ beta-catenin signaling pathway, could promote trophoblast cell invasion during murine placentation. However, mechanism of Dkk-1 action on cell migration was not clear. The objective of this study was to further evaluate the effect of Dkk-1 on cell migration and to identify the underlining mechanisms. Functional assays with stable Dkk-1 transfected HEK293 cells revealed that Dkk-1 expression increased cell migration by decreasing cell-cell adhesion, not cell-matrix adhesion. Treatment with LiCl and Genistein (widely used inhibitor of glycogen synthase kinase-3 and tyrosine protein kinase, respectively.) could inhibit the migration effect of Dkk-1, and significantly increased the membrane localization of beta-catenin and E-cadherin in HEK293 cells transfected with Dkk-1. Further data showed that HEK293 cells transfected with Dkk-1 have significantly decreased accumulation of both beta-catenin and E-cadherin at the cell membrane. Together, our data suggest that Dkk-1 stimulates the release of beta-catenin from cell membrane and facilitates cell migration which accompanies degradation of beta-catenin/E-cadherin.

2. INTRODUCTION

Cell migration plays an important role in a wide variety of biological phenomena (1). Among numerous factors affecting this process, the membrane complex formed by E-cadherin and beta-catenin guides the cell-cell adhesion and regulates the migration of cells (2). Cadherin is highly conserved transmembrane protein (3). The extra-cellular domain of cadherin maintains the cell-cell adhesion and the cytoplasmic domain of it interacts with beta-catenin, α-catenin and p120-catenin (4), where beta-catenin bridges the cadherin complex to the actin cytoskeleton via α-catenin (5). The assembly-disassembly of the cadherin–catenin complex is in a dynamic state (6), and the association-disassociation of beta-catenin from membrane complex is supposed to be an important mechanism to regulate cell-cell adhesion and signal transmission (7). Disassociation of beta-catenin from cadherin–catenin complex could reduce the stability of cell-cell adhesion and facilitate migration (8). Beta-catenin, a pivotal molecule in Wnt signaling (9), has three distinct cellular locations (cell membrane, cytosol and nucleus) (10). Cadherin–catenin complex is an important reservoir of beta-catenin in membrane, where disassociation of beta-catenin from cadherin–catenin complex may lead to the accumulation of

beta-catenin in cytosol and even fluctuation of Wnt signaling.

Dickkopf-1(Dkk-1) is a well known canonical Wnt signaling antagonist (11-12). The expression of Dkk-1 is temporally and spatially regulated during embryogenesis and in adults (13). In Wnt pathway, Wnt-induced inhibition of glycogen synthase kinase-3 (GSK-3) leads to stabilization and accumulation of beta-catenin in the cytosol pool (14). After accumulation of beta-catenin in cytoplasm, it could likely be transported into nucleus and interact with lymphoid enhancer binding factor /T cell-specific factor (LEF/TCF) to start the transcription of downstream genes (15). In this process, Dkk-1 has been shown to inhibit Wnt/beta-catenin signaling by antagonizing LRP5/6 and binding to trans-membrane proteins Kremen1 or Kremen2 (16).

Expression levels of Dkk-1 in various cancers and normal tissues are heterogeneous. Most studies showed that Dkk-1 was downregulated in cancer compared with healthy tissue and had anti-tumor effect (12, 17). However, some reports demonstrated that Dkk-1 is preferentially expressed in hormone-resistant breast tumors and other common tumors, and Dkk-1 appears in tumors with metastasis and poor outcome (18-20). So the exact roles of Dkk-1 in tumor progression and metastasis have not yet been certain and under investigation so far (18, 21). We recently showed that Dkk-1 secreted by decidual cells could promote trophoblast cell invasion during murine placentation which unveils a new aspect of Dkk-1 (22-23). Since there are striking similarities between trophoblast cells and cancer cells (24), here we are aimed to examine the function and potential mechanism of Dkk-1 in cell migration. In this study, HEK293 cells transfected with Dkk-1 were chosen as a model to investigate cell migration (25). Through migration assay, we found Dkk-1 could prominently enhance the migration ability of HEK293 cells, accompanied disruption of cell-cell adhesion and degradation of beta-catenin/E-cadherin.

3. MATERIALS AND METHODS

3.1. Migration assay and adhesion assay

In migration assay, cells were cultured in plates precoated with fibronectin (Sigma, St. Louis MO, USA) (20μg/ml) until subconfluence, and starved for 24 h, then treated with 10 μg/ml mitomycin C for 2 h to avoid proliferative effect on cells and finally subjected to scratch wound assays. Cell-free area was introduced by scraping the monolayer cell with a 200μl pipette tip. Cell migration to the cell-free area for another 24 h or 48h was evaluated in the absence of exogenous ligands. Photographs were taken using a phase-contrast microscope. The number of migrating cells was counted after taking photographs of more than three non-overlapping fields and migrating cells in the 150μm wide cell-free areas were counted. Values represent the mean of migrating cells beyond the frontiers of the in vitro wound edge. Migration assay was also examined using transwell assays, which were performed using a Neuro Probe (Cabin John, MD) 24-well chemotaxis Boyden chamber. Cells were trypsinized and seeded in the upper chamber at 1×10^4 cells/well in serum-free Dulbecco's modified Eagle's medium (DMEM) (Gibco Invitrogen, San Diego, CA). DMEM supplemented with 10% FCS (used as a chemoattractant) was placed in the bottom well, and the cells were allowed to migrate for 4 h in CO_2 incubator. At the end of experiment, Nonmigratory cells in the upper chamber then were removed with a cotton-tip applicator. Migrated cells on the lower surface were fixed with methanol and stained with hematoxylin. The number of migrating cells was determined by counting five high-powered fields (200×) on each membrane.

In adhesion assay, a 96-well-plate was coated with fibronectin (20μg/ml). Some wells uncoated with fibronectin were negative control. Nonspecific binding sites were blocked with blocking buffer (0.5% BSA in medium DMEM) at 37Cfor 45-60 minutes. 2×10^4 cells were plated in each well and incubated in CO_2 incubator at 37C for 30 minutes. Adherent cells were fixed with 4% paraformaldehyde and stained with Crystal Violet for 10 minutes. A595 was measured from the extracted dye using an enzyme-linked immunosorbent assay reader. For experiments investigating cell-cell adhesion, 96-well-plate was precoated with confluence HEK293 cells. While investigating cell-extracellular matrix adhesion, 96-well-plate was precoated with fibronectin (20μg/ml).

3.2. Stable transfection

The cDNA of Dkk-1 was acquired from plasmid pcDNA3.1-Dkk-1 (gift from Dr Zhijie Chang, Tsinghua University, China). The plasmid pcDNA3.1-beta-cateninS37A was a kind gift from Dr Chaojun Li (Nanjing normal University, China). The cDNA of beta-cateninS37A was utilized under the permission of Dr Frank McCormick (UCSF Comprehensive Cancer Center, USA), and beta-cateninS37A is a mutant form of beta-catenin whose serine residue (S37) is mutated into alanine residue, thus the phosphorylation by GSK-3 is inhibited and ubiquitination is blocked, leading to long term accumulation of beta-catenin. For transfection experiments, 2×10^5 cells/well were plated in six-well plates 24h before transfection. Lipofectamine 2000 (Invitrogen, Carlsbad CA, USA) was used to mediate transfection, and transfection and G418 selection were performed according to the manufacture's protocol.

3.3. Immunofluorescence

Cells were fixed in 4% paraformaldehyde solution and blocked with 5% bovine serum albumin (BSA) before incubation at 4C overnight with goat anti-Dkk1 or mouse anti-beta-catenin (Santa Cruz ,CA, USA) or rat anti- E-cadherin(Sigma, St. Louis MO, USA). Thereafter, the cells were incubated in FITC-conjugated secondary antibody (ZSB, Beijing, China) at a dilution of 1:100 in PBS for 1 h at 37C. Nuclei were stained with 0.01mg/ml propidium iodide (PI, 10μg/ml) for 10 min and viewed under a laser scanning confocal microscope. For negative controls, parallel experiments were performed with cells using pre-immune goat or mouse or rat serum.

3.4. Western blot analysis

Proteins were extracted from cells of each sample. About 20μg proteins were loaded onto 15% SDS–PAGE

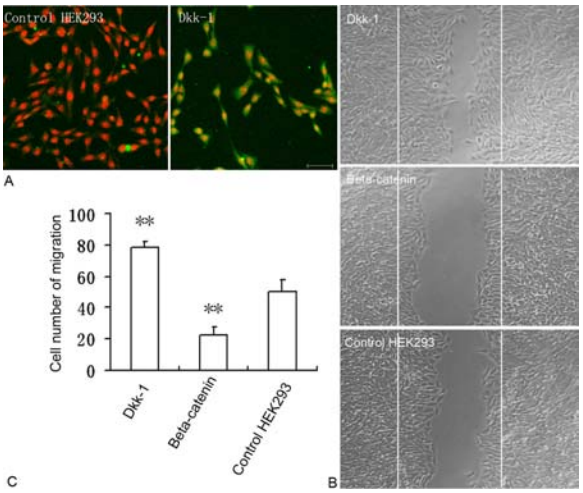

Figure 1. Dkk-1 prominently enhances migration of HEK293 cells. (A) Dkk-1 staining in HEK293 cells transfected with an empty vector (Control HEK293) or Dkk-1 expression vector (Dkk-1). Scale bar: 50μm. (B) Scratch wound assays of HEK293 cells over expressing Dkk-1, beta-catenin and control HEK293 cells. The photographs were taken 24 h after the wounds were made. Original magnification: ×100. (C) Cell numbers calculation in migration assay. **P< 0.01 vs. control HEK293 cells.

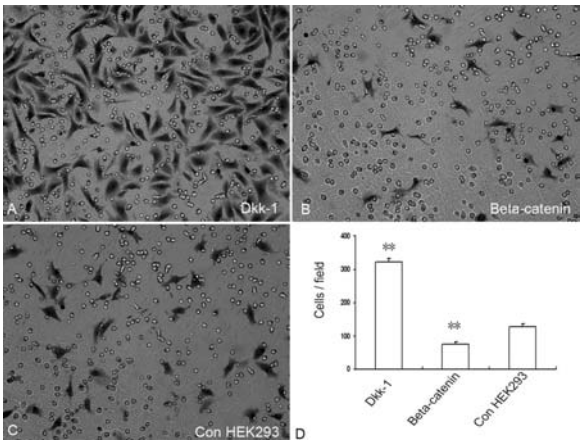

Figure 2. Migration assays of HEK293 cells over expressing Dkk-1, beta-catenin and control HEK293 cells. Migration assays were performed using a 24-well chemotaxis Boyden chamber. Migrated cells on the lower surface were stained with hematoxylin and photographed. (A, B, C) Microscopic image of migrated HEK293 cells over expressing Dkk-1, beta-catenin and control HEK293 cells, respectively. Original magnification: ×200. (D) Cell numbers calculation in migration assay. The results of transwell assay also showed that the migration ability of HEK293 cells over expressing Dkk-1 was significantly enhanced compared with the cells stably expressing beta-catenin and control HEK293 cells. ** P< 0.01 vs. Control HEK293 cells.

and transferred onto a PVDF membrane (Bio-Rad, Hercules, CA). Primary antibodys are beta-actin antibody (LAB VISION, USA), E-cadherin (Sigma, St. Louis MO, USA), beta-catenin (Santa Cruz, CA, USA), and second antibodys are HRP–conjugated rabbit anti-goat IgG or mouse anti-rat IgG and goat anti-mouse IgG (ZBS, Beijing, China), the blot was detected using the western blotting luminol reagent (Santa Cruz, CA, USA) according to the instructions of the manufacturer.

3.5. Apoptosis analysis and cell cycle analysis

Sample cells were harvested by trypsinization and stained using an Annexin V FITC Apoptosis Detection kit (Biosea Biotechnology Co. LTD, Beijing, China), according to the manufacturer's protocol. Briefly, cells were washed in cold PBS three times, followed by resuspension in cold binding buffer and incubated with anti-AnnexinV FITC-conjugated antibody for 30 min on ice. PI was added 10 min before analysis using Flow Cytometer (FACSCalibur□BioRAD, USA).

In cell cycle analysis, cells were harvested by trypsinization and fixed in 70% ethanol over night at 4C, washed with PBS and incubated with 50μg/ml RNase, 40μg/ml PI and 0.1%Triton X-100 for 30 minute at 37C. DNA content was measured using an excitation wavelength of 488 nm and an emission wavelength of 635 nm, and analyzed using the ModFit software program.

3.6. Statistical analysis

Each value represents the mean ± SEM. The data were analyzed using one-way ANOVA followed by LSD's post-hoc test. The P values less than 0.05 were considered statistically significant. All statistical analyses were performed using SPSS 10.

4. RESULTS

4.1. Dkk-1 prominently enhances migration of HEK293 cells

HEK293 cells over expressing Dkk-1 (Figure 1A) were chosen as model to investigate cell migration. In migration assay, we found the migration ability of HEK293 cells over expressing Dkk-1 was significantly enhanced compared with the cells stably expressing beta-cateninS37A (defined as beta-catenin in this paper) and HEK293 cells transfected with empty vector (defined as control HEK293) (Figure 1B). By calculating the number of migrate cells (see details in MATERIALS AND METHODS), we found Dkk-1 enhances the migration ability of HEK293 cells over 3 and 1.3-fold compared with HEK293 cells over expressing beta-cateninS37A and control HEK293 cells respectively (Figure 1C). Furthermore, Migration assays were also performed using transwell assays. The results also demonstrated that the migration ability of HEK293 cells over expressing Dkk-1 was significantly enhanced compared with the cells stably expressing beta-cateninS37A and control HEK293 cells (Figure 2A, B, C, D). To explain the reason for the mobilization effect of Dkk-1, we utilized adhesion assay (see details in MATERIALS AND METHODS) to investigate the cell-cell and cell-extra cellular matrix adhesion. We found Dkk-1 evidently decreased the cell-cell adhesion (P<0.01), whereas beta-cateninS37A increased the cell-cell adhesion (p<0.01), compared to control

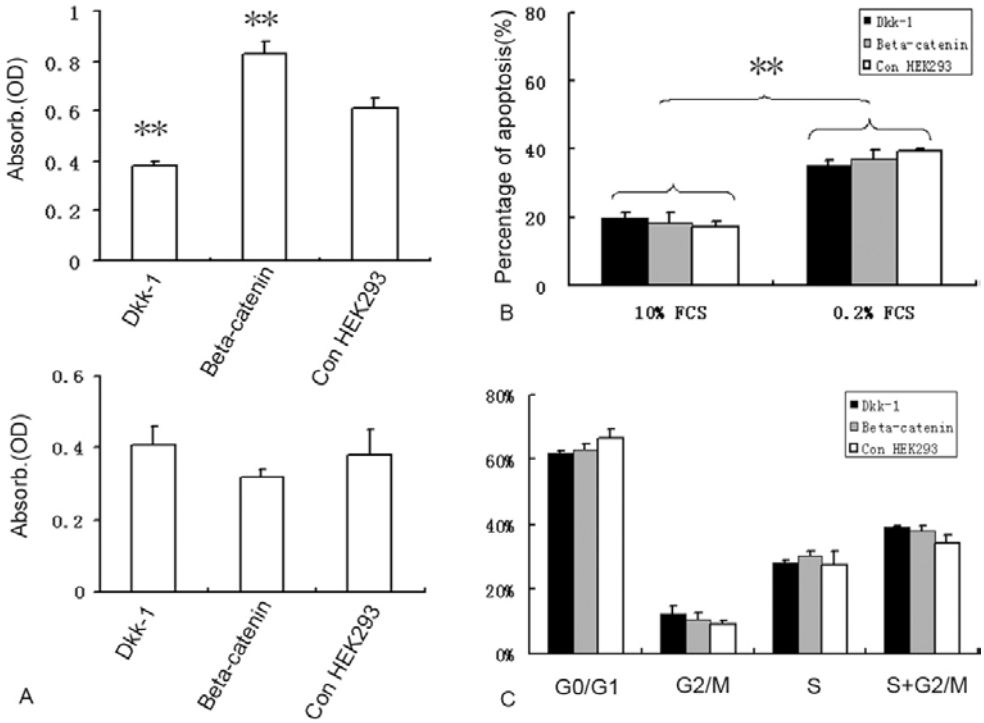

Figure 3. Effects of Dkk-1 on the cell adhesion, cell apoptosis and cell cycle of HEK293 cell. (A) Adhesion assays between cell-cell (Top) and cell-extracellular matrix (Bottom). 96-well-plate was precoated with HEK293 cells or fibronectin (20 μg/ml), and 2×10^4 cells expressing Dkk-1, beta-catenin and control HEK293 cells were plated in each well. Adherent cells were detected by Crystal Violet for 10 minutes. **P< 0.01 vs. control HEK293 cells. (B, C) Cell apoptosis and cell cycle analysis in HEK293 cells over expressing Dkk-1, beta-catenin and control HEK293 cells. Serum starvation (0.2% FCS) induced prominent cell apoptosis compared with normal culture (10% FCS) (P< 0.01), but no statistic significant differences were observed under the same situations (with serum or serum starvation) among these groups.

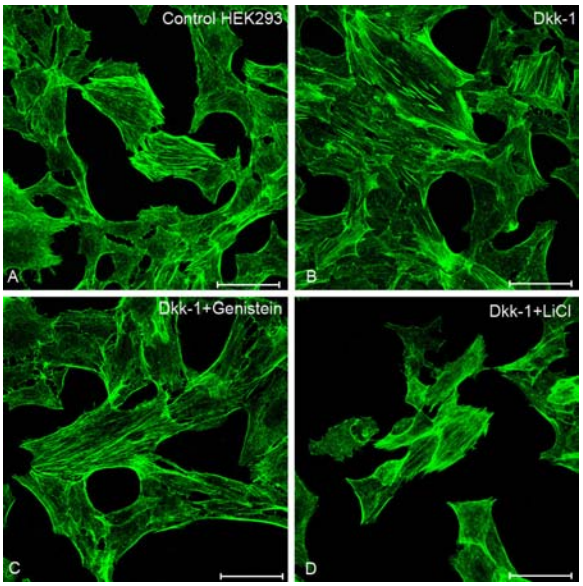

Figure 4. Effect of Dkk-1 on the structure of actin filament. Actin filaments in control HEK293 cells (A), HEK293 cells over expressing Dkk-1(B), HEK293 cells over expressing Dkk-1 with Genistein stimulation (C) and LiCl stimulation (D) were stained with FITC-phalloidin. No significant changes were observed in regards to actin structure. Scale bar: 50μm.

HEK293 cells (Figure 3A). On the other hand, the cell-extracellular matrix adhesions of these cells did not have significant difference (P>0.05) (Figure 3A), and we investigated whether Dkk-1 or beta-cateninS37A could influence the growth of HEK 293 cells. Through BrdU incorporation analysis (data not shown), apoptosis analysis (Figure 3B) and cell cycle analysis (Figure 3C), we found no significant differences existed among HEK 293 cells over expressing Dkk-1, beta-cateninS37A and control HEK293 cells.

4.2. LiCl and Genistein stimulus reverse the mobilization effect of Dkk-1

Previous researches suggested there are close relations between migration and cytoskeleton. To investigate the possible mechanism involved in the mobilization effect of Dkk-1, we examined the structure of actin filament in control HEK293 cells and HEK293 cells over expressing Dkk-1. However, no prominent alteration was observed (Figure 4A, B). So, we speculated the mobilization effect of Dkk-1 is more likely relative to beta-catenin and Wnt components. As the Wnt induced stabilization of cytoplasmic beta-catenin can be simulated by LiCl, we used the method of LiCl stimulus, and found LiCl stimulus greatly reverses the mobilization effect of Dkk-1 in a dose-dependent manner compared to control HEK293 cells (Figure 5A, B, C, D and E). Also the cell-cell adhesion was increased after LiCl stimulus (data not

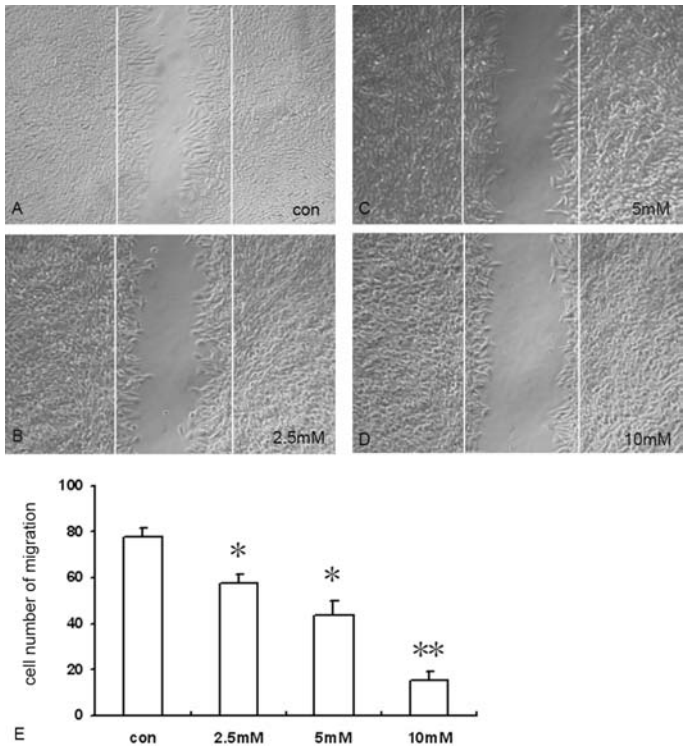

Figure 5. LiCl prominently suppresses the migration of HEK293 cells over expressing Dkk-1 in a dose-dependent manner. (A-D) Dosedependent effect of LiCl on cell migration. HEK293 cells over expressing Dkk-1 were treated with varying doses of LiCl (0 mM , 2.5 mM, 5 mM, 10 mM respectively).The photographs were taken 24 h after the wounds were made. Original magnification: ×100. (E) Cell numbers calculation in migration assay, the method to calculate the number of migration cell is described in materials and methods in detail. *P< 0.05, **P< 0.01 vs. control.

shown). Furthermore, we used Genistein, a widely used inhibitor of tyrosine protein kinase, to prevent beta-catenin dissociation in our experiments. Genistein could inhibit the migration of HEK293 cells over expressing Dkk-1 and HEK293 cells over expressing beta-cateninS37A at 48 h (Figure 6A, B, C, and D). These results indicated accumulation of beta-catenin in cytosol might stabilize the cadherin–catenin complex and inhibit the migration ability of cells.

4.3. Mobilization effect of Dkk-1 is accompanied with beta-catenin dissociation from cadherin–catenin complex and E-cadherin degradation

To further demonstrate our hypothesis, we used immunofluorescence to see whether cadherin–catenin complex is disrupted and beta-catenin is dissociated from membrane complex of HEK293 cells over expressing Dkk-1. In this experiment, we found Dkk-1 decreases the accumulation of beta-catenin in membrane compared with control HEK293 cells (Figure 7A, B). Moreover, both LiCl and Genistein stimulus increase the accumulation of beta-catenin in membrane (Figure 7C, D) and the protein level of beta-catenin is elevated compared with HEK293 cells over expressing Dkk-1 by western blotting analysis (Figure 7E, F).

Next, we tried to see whether the dissociation of beta-catenin from cadherin–catenin complex could influence the status of E-cadherin. We found E-cadherin of HEK293 cells over expressing Dkk-1 was greatly reduced. In contrast, it was elevated in HEK293 cells over expressing beta-cateninS37A (Figure 8A, B, C), and both LiCl and Genistein stimulation significantly enhance E-cadherin expression in membrane of HEK293 cells over expressing Dkk-1 (Figure 8D, E). These results were confirmed at protein level by western blotting analysis (Figure 8G).

5. DISCUSSION

In the present study, we demonstrated Dkk-1 could significantly enhance the migration of HEK293 cells through wound scratch assay and transwell assay, which is consistent with our observation during trophoblast invasion (22-23). To elucidate underling mechanisms to the mobilization effect of Dkk-1, we utilized adhesion assay to examine the cell-cell and cell-extra cellular matrix adhesion. We found Dkk-1 evidently decreased the cell-cell adhesion, supposing the mobilization effect of Dkk-1 is correlated with cell-cell adhesion disruption. In the case of the widely distributed adherens junctions, classical cadherins self-associate extracellularly, and intracellularly interact with β-catenin, which interacts with the actin cytoskeleton via α-catenin (26-27). So we tried to see whether the mobilization effect of Dkk-1 is related to beta-catenin and E-cadherin in our experiment. We used the method of LiCl and Genistein stimulus, and found that both LiCl and Genistein could reverse the mobilization effect of

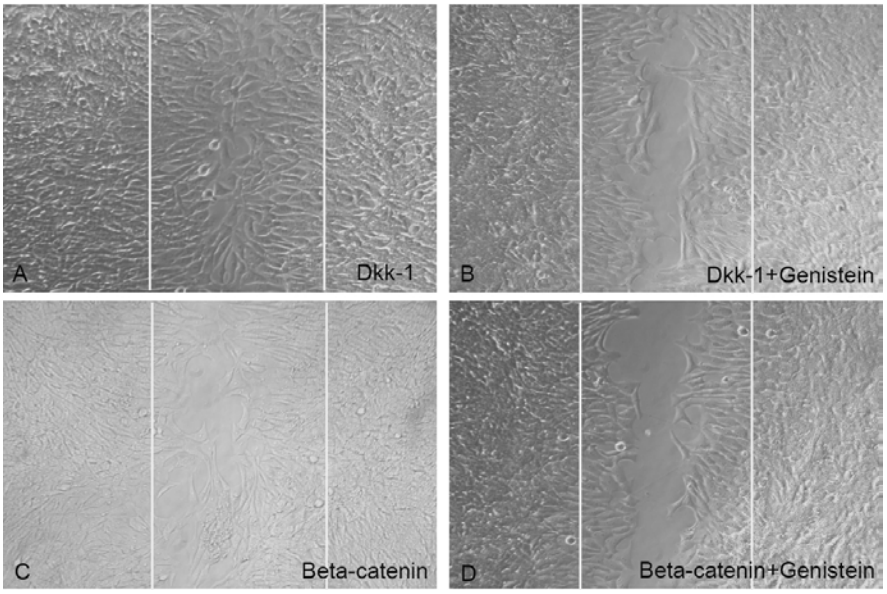

Figure 6. Genistein greatly inhibits the migration ability of HEK293 cells over expressing Dkk-1 and beta-catenin. (A, C) Scratch wound assays of HEK293 cells over expressing Dkk-1 and beta-catenin. (B, D) Scratch wound assays of HEK293 cells over expressing Dkk-1 and beta-catenin with Genistein treatment. The photographs were taken 48 h after the wounds were made. Original magnification: ×100.

Dkk-1. Immunostaing showed that LiCl and Genistein significantly increased the membrane localization of beta-catenin and E-cadherin in HEK293 cells transfected with Dkk-1, suggesting the Dkk-1 stimulated cell migration was closely related to membrane bound beta-catenin and E-cadherin, and dissociation of beta-catenin from the cadherin-catenin complex localized in the membrane was induced by Dkk-1 over- expression. In addition, to investigate whether adhesion disruption was relative to actin filaments alteration, cells were stained with FITC-phalloidin. However, no significant changes were observed in regards to actin structure between control HEK293 cells and HEK293 cells over expressing Dkk-1. This unexpected result suggested a non-actin-based classical cadherin involved in the process.

Beta-catenin localized in the membrane and cytosol is in equilibrium and one side declination might promote beta-catenin to stream toward the other side. Since Dkk-1 is a well-known Wnt signaling antagonist (11, 28), It might enhance the degradation function of GSK-3 against beta-catenin (11, 16), which leads to the decrease of beta-catenin in cytosol, and the present study demonstrated that transfection with Dkk-1 decreased cellular level of beta-catenin. Then the balance between membrane and cytosol pool of beta-catenin was broken, causing release of membrane associated beta-catenin, and accompanying destabilization of cadherin-catenin complex and degradation of E-cadherin. There might be some unknown mechanism to regulate the balance of beta-catenin between membrane and cytosol. Previous researches indicated the dissociation of beta-catenin from cadherin-catenin complex was accompanied with specific trysoine residue phosphorylation. In this process some trysoine kinase might play an important role (29-30).

Previous researches suggest E-cadherin might be a direct target gene of Wnt signaling, which is regulated at transcriptional level (31). However, here in our work we did not detect significant change of E-cadherin at transcription level after Dkk-1 transfection(data not shown), indicating in this situation E-cadherin was not regulated at transcription level but rather at protein level, and more likely the stability of E-cadherin or the degradation mechanism could regulate cellular level of E-cadherin.

Because of the antagonist effect of Dkk-1 in Wnt signaling, most researchers believe primary function of Dkk-1 inhibits cell proliferation and survival, and the loss of Dkk-1 expression in tissues may open the door to cancer by removing the inhibitory effect on the Wnt/beta-catenin pathway (12). Therefore, Dkk-1 is suggested as an anti-tumor gene (12, 32). However, the expression pattern of Dkk-1 in different cancers is various, and some research found its elevated expression in numerous cancer cells, and Dkk-1 appears in some tumors with a poor outcome (18-19). In our research, we found Dkk-1 enhanced the migration of HEK293 cells through beta-catenin/E-cadherin degradation. Recently, our lab has found Dkk-1 expressed dynamically in both mouse embryos and uterine decidual tissue during embryonic implantation, Dkk1 secreted by decidual cells may induce trophoblast cell invasion and beta-catenin may be involved in such function of Dkk1. The results may partly explain why Dkk-1 appears in some tumors with a poor outcome, and increase our understanding of its function in cancer cells and non-cancer cells.

Cadherin-catenin membrane complex not only regulate the cell-cell adhesion but also are very important

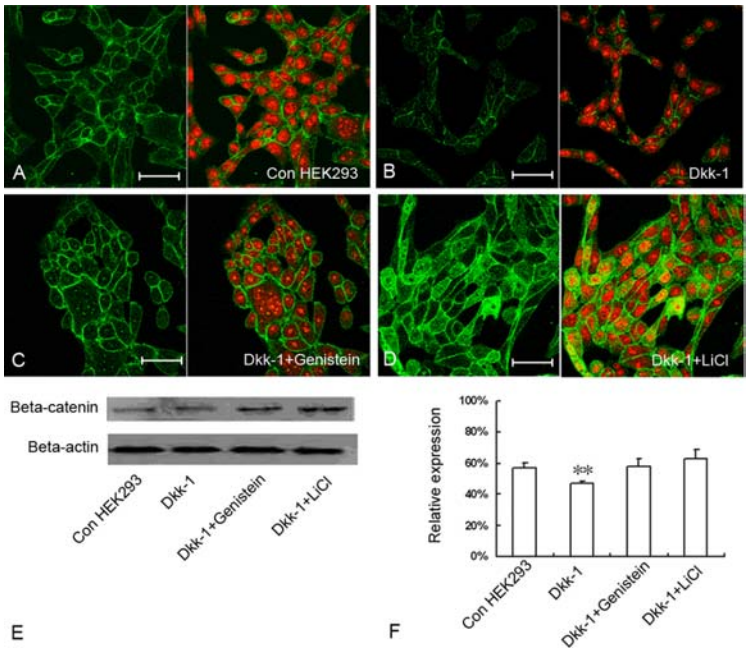

Figure 7. Dkk-1 decreases the accumulation of beta-catenin in membrane, whereas Genistein or LiCl stimulation significantly reverses the effect of Dkk-1. Green coloration represents beta-catenin staining and red coloration indicates nuclear staining. Beta-catenin staining in HEK293 cells(A), HEK293 cells over expressing Dkk-1(B), HEK293 cells over expressing Dkk-1 with Genistein (50μM) treatment(C), and HEK293 cells over expressing Dkk-1 with LiCl (10mM) treatment(D). Scale bar: 50μm. (E, F) Western blot and intensity histogram determine relative protein level of beta-catenin. Beta-actin is probed as a loading control. ** P< 0.01 vs. control HEK293 cells.

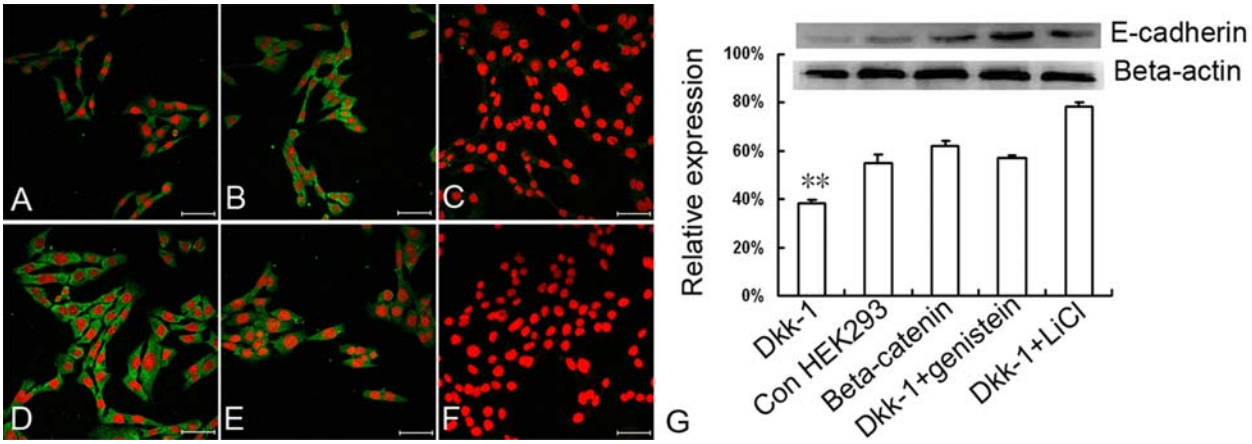

Figure 8. Dkk-1 decreases the protein level of E-cadherin, whereas Genistein or LiCl treatment prominently reverses the effect of Dkk-1. Green coloration represents E-cadherin staining and red coloration indicates nuclear staining. E-cadherin staining in control HEK293 cells(A), HEK293 cells over expressing beta-catenin(B), HEK293 cells over expressing Dkk-1(C), HEK293 cells over expressing Dkk-1 with Genistein(50μM) and LiCl (10mM) stimulation(D, E). (F)Negative control: primary antibodies replaced by rat IgG. Scale bar: 50μm. (G) Western blot and intensity histogram determine relative protein level of E-cadherin. Beta-actin is probed as a loading control. ** P<0.01 vs. con HEK293.

for signaling transduction from external cellular environment (7, 33). Previous researches indicate the abnormalities caused by cadherin-catenin mutant result in signaling defects (33), suggesting cadherin-catenin is an important signaling center underestimated before. For example, p120, a component of cadherin-catenin complex linking to other signaling pathways directly, which contains binding motifs that recruit a variety of cytoplasmic PDZ motif-containing proteins (34-35), could activate Rac and inhibit Rho (36), recruit the cytoplasmic tyrosine kinases for molecular medicine Fer and Fyn (37).

In summary, we found Dkk-1 enhances the migration ability of HEK293 cells through disruption of

cell-cell adhesion, whereas LiCl and Genistein stimulation prominently reverse the mobilization effect of Dkk-1, indicating Dkk-1 stimulated cell migration is closely correlated with beta-catenin. We suppose Dkk-1 stimulates the release of beta-catenin from membrane and facilitates cell migration that accompanies membrane adhesion disruption and degradation of beta-catenin/E-cadherin.

6. ACKNOWLEDGEMENT

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Abbreviations: Dkk-1: Dickkopf-1, GSK-3: glycogen synthase kinase-3, LEF/TCF: lymphoid enhancer binding factor /T cell-specific factor

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