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## **Cdc42 is essential for the polarized movement and adhesion of human dental pulp stem cells**

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## **Abstract**

**Objective:** Stem cell-based tissue repair and regeneration require the regulation of cell migration and adhesion. As a regulator of cell polarization, Cdc42 (cell division control protein 42) plays a basic role at the initial stage of cell migration and adhesion. This study explores the effect of Cdc42 on the polarized migration and adhesion of hDPSCs (human dental pulp stem cells).

**Design:** HDPSCs were isolated from extracted third molars and transfected with siRNA targeted against Cdc42. Scratch wound assays and transwell assays were performed to detect the migration of human dental pulp stem cells. Polarization assays were applied to explore the polarized movement of Golgi bodies and nuclei.

Western blot was used to examine the expression of related proteins.

**Results:** The expression of Cdc42 was knocked down by siRNA transfection, which inhibited the migration of hDPSCs in both the scratch wound assays and transwell assays. Meanwhile, the proportion of polarized hDPSCs during migration was also decreased, and the adhesion ability of hDPSCs was downregulated. Western blot demonstrated that these effects were dependent on FAK (focal adhesion kinase),  $\beta$ -catenin and GSK3 $\beta$  (Glycogen synthase kinase-3 $\beta$ ).

**Conclusion:** Our study demonstrates that Cdc42 plays an essential role during the polarized movement and adhesion of hDPSCs.

**Keywords:** cell division control protein 42; human dental pulp stem cells; migration; polarization; adhesion

## **Introduction**

When exposed to the external environment or infected by bacterium, dental pulp may be damaged. Wound healing involves a series of biological and molecular processes, including cell migration, adhesion, proliferation, and extracellular matrix (ECM) deposition (Rodero & Khosrotehrani, 2010; Rorth, 2009). Meanwhile, more and more evidence has shown that stem cells hold therapeutic potential for tissue repair (Lee, Jung, Oh, Yun & Han, 2014). Human dental pulp stem cells (hDPSCs), as one type of mesenchymal stem cell (MSC) present in the cell-rich zone and core of the dental pulp, are thought to be effective stem cells for dental pulp wound repair. Under the effects of the molecular cues, such as the chemokines expressed by the odontoblasts, dental pulp cells or immunocytes, hDPSCs migrate to the wound area and differentiate into relevant cells to repair the damaged tissue.

During this process, directional cell movement is necessary for hDPSCs to migrate into the injured area. Cell migration is an organic behaviour that requires cytoskeletal rearrangement, remodelling of cell adhesion, translocation of cellular organelles and movement of the cell body (Etienne-Manneville, 2006). Rho GTPases link outside signals to the cytoskeleton, act as the upstream regulators of actin polymerization and control cell functions, including protrusion formation and cell adhesion, which are the basis of cell migration (Donnelly, Bravo-Cordero & Hodgson, 2014; Ivetic & Ridley, 2004). Cell movement during wound healing is a polarized process for fibroblasts and keratocytes. Cells are polarized as the microtubule organizing centre (MTOC) and the Golgi body move towards the wound. Front-back polarity emerges when cells move in a certain direction towards molecular cues or along physical forces and are formatted by the asymmetric distribution of the cell contents, including cell organs and proteins on the cell membrane (Woodham & Machesky, 2014). This polarity is reinforced by the Golgi

reorientation towards the leading edge to facilitate directed vesicular transport (Woodham & Machesky, 2014).

As a Rho GTPase, Cdc42 (cell division control protein 42) regulates cytoskeletal reorganization by switching between an active GTP-binding state and an inactive GDP-binding state (Tcherkezian & Lamarche-Vane, 2007). Cytoskeletal remodelling plays a basic role during the formation of cellular protrusions, which is the first step in cell adhesion and migration (Zegers & Friedl, 2014). Two kinds of cellular protrusions, filopodia and lamellipodia, have been proven to be necessary for polarized migration in fibroblasts, keratocytes and mesenchymal cells (Wehrle-Haller & Imhof, 2003; Yang, Wang & Zheng, 2006). Filopodia are thin membrane protrusions, while lamellipodia are large actin-based sheet-like protrusions (Zegers & Friedl, 2014). Previous studies have shown that Rho GTPases promote the formation of filopodia and lamellipodia in response to environmental stimulation (Donnelly et al., 2014). As a polarity protein, Cdc42 affects many aspects of cell behaviour, including spindle orientation in the mitosis, the Golgi body and nucleus reorientation (Mitsushima, Toyoshima & Nishida, 2009). In migration and polarization of a single cell and loosely connected astrocytes, Cdc42-dependent cell leading edges are also well established (Mitsushima et al., 2009; Osmani, Vitale, Borg & Etienne-Manneville, 2006).

Despite the importance of Cdc42 in cell polarization during wound healing, little information is currently available on the influence of Cdc42 on human dental pulp stem cells after dental pulp injury. We hypothesized that Cdc42 is necessary for the normal movement of hDPSCs in wound repair. Therefore, we set out to investigate the effect of Cdc42 on the adhesion and polarized migration of hDPSCs.

# **Materials and Methods**

## **Cell culture**

Third molars obtained from patients undergoing routine extraction were used for the isolation and culture of primary hDPSCs. All the procedures were performed within the approval of the Ethics Committee of the Fourth Military Medical University (FMMU). We ensured that all the methods were performed in accordance with the relevant guidelines, and informed consent was collected from all the subjects. Briefly, freshly extracted teeth were collected from patients between the ages of 18 and 23 who underwent the cell isolation process within 1 hour. After dissection, dental pulp was cut into pieces and digested in PBS with 4 mg/mL dispase and 3 mg/mL collagenase type I (Sigma, USA) at 37°C for 45 min. Subsequently, the tissue mixtures were filtered using a 70- $\mu$ m cell strainer to obtain single-cell suspensions. The cells isolated from one tooth were seeded into a single well of 6-well plate. The suspensions were cultured in  $\alpha$ -MEM with 20% foetal bovine serum (FBS, HyClone, USA), 100 U/mL penicillin-G and 100 mg/mL streptomycin (Roche) in 5% CO<sub>2</sub> at 37°C. The medium was changed every 2 days. After the cells were grown to 60% confluence, they were trypsinized and reseeded to obtain single-cell clones by limiting dilution. The single-cell clones were passaged and were used for this study after they were passaged 3-4 times.

## **Flow cytometric analysis of cell surface markers**

The hDPSCs of passage 3 were harvested and resuspended in PBS with 3% FBS for flow cytometric analysis. Subsequently, anti-human monoclonal antibodies (CD29-PE, CD34-PE, CD45-PE, CD90-PE, CD105-FITC, CD146-PE) were used for cell staining for 1 hour in the dark at room temperature. After the cells were stained and washed twice with ice-cold PBS, they were resuspended in PBS with 3% FBS

and analysed with a FACSCalibur Flow Cytometer (BD, USA).

### **Multiple lineage differentiation**

Cells were cultured in 6-well plates to reach 80% confluence and then incubated in odontogenic/osteogenic medium ( $\alpha$ -MEM with 50 mg/mL ascorbic acid, 10 nM dexamethasone, 10 mM  $\beta$ -glycerophosphate and 10% FBS) for 4 weeks for odontogenic/osteogenic induction. After fixation in 4% paraformaldehyde in PBS for 15 min, the cells were washed with ddH<sub>2</sub>O and stained with 40 mM Alizarin Red S (pH 4.2). For adipogenic induction, the cells were cultured until they were subconfluent and incubated in adipogenic medium ( $\alpha$ -MEM with 0.5 mM isobutyl-methylxanthine, 1  $\mu$ M dexamethasone, 0.2 mM indomethacin, 0.01 mg/ml insulin and 10% FBS) for 5 weeks. After the cells were fixed in 4% paraformaldehyde in PBS for 15 min and washed with ddH<sub>2</sub>O, they were stained with 0.3% Oil Red O.

### **siRNA treatment of cells**

Two pairs of siRNAs targeting Cdc42 and one pair of scrambled sequences were custom designed and purchased from RiboBio (Guangzhou, China). The sequences of the Cdc42 siRNAs were 5'-GACUCAAUUGAUCUCAGAdTdT-3'/ 5'-UCUGAGAUCAAUUUGAGGUCdTdT-3' and 5'-GCAAGAGGAUUUGCAGAdTdT-3'/ 5'-UCUGUCAUAAUCCUCUUGCdTdT-3'. The transfections were performed according to the manufacturer's instructions with a final siRNA concentration of 50 nM. Briefly,  $1 \times 10^5$  cells were seeded into each well of a six-well plate with antibiotic-free medium, grown to 60%-70% confluence and then incubated with siRNA diluted with transfection reagent and medium for the indicated time. The scrambled siRNA was transfected as a negative control.

### **RT-PCR**

After transfection with a negative control or Cdc42 siRNA for 24 h, the total RNA was extracted from hDPSCs using the Trizol reagent (Takara, Dalian, China) and then converted into cDNA according to the manufacturer's protocol. Afterwards, gene expression analyses were performed on an ABI Prism 7500 Real-Time PCR System (Applied Biosystems) using the following primers for Cdc42: forward 5'-GGTGGAGAAGCTGAGGTCAT-3', reverse 5'-TTTACCAACAGCACCATCGC-3'.

### **Western blot**

hDPSCs were washed with ice-cold PBS after transfection for 48 hours. Then, ice-cold radio-immunoprecipitation (RIPA) lysis buffer (Santa Cruz Biotechnology) containing a protease inhibitor cocktail (Sigma) was added into the wells for 10 min on ice. The proteins were resolved by SDS-PAGE and transferred to a PVDF membrane. After blocking with 5% BSA in Tris-buffered saline with Tween (TBST) for 1 h, the membranes were incubated with primary antibodies (anti-Cdc42 from Abcam; anti-FAK, p-FAK,  $\beta$ -catenin, p- $\beta$ -catenin, GSK3 $\beta$ , p-GSK3 $\beta$  from Cell Signaling) overnight at 4°C. Following a 5-min wash in TBST, the membranes were incubated with IRDye TM800CW-conjugated secondary antibody for 1 hour at room temperature. After washing, the membranes were scanned using an Odyssey infrared imaging system (LI-COR). The signal intensities of the bands were analysed by ImageJ (NIH, USA). B-actin was also detected in this experiment as an internal control.

### **Scratch wound assay**

Dominant negative siRNA- or siCdc42-transfected hDPSCs and hDPSCs without transfection were seeded into 6-well plates at a density of  $1 \times 10^5$  cells per well, cultured to obtain a completely confluent monolayer and then subjected to wound healing assays. Briefly, the hDPSC monolayers were incubated



for 24 hours in serum-free medium and then physically wounded with a sterile 1000- $\mu$ l pipette tip. Detached cells were washed away with PBS. Fresh  $\alpha$ -MEM medium with 5% FBS was added into the transfection wells. Fresh medium with DMSO or 10  $\mu$ M ML141 (an inhibitor of Cdc42) was added into the rest of the wells. Microphotographs of the scratch were acquired at the defined positions after 0 and 36 hours of migration using an Olympus light microscope. The scratch wound closure was analysed with ImageJ and was expressed as a percentage of the scratch surface area covered by hDPSCs. The assays were carried out in three independent experiments.

### **Transwell assay**

Using a two-chamber transwell system with a pore size of 8  $\mu$ m and a diameter of 6.5 mm, a cell migration assay was performed as previously described. Briefly, dominant negative siRNA- or siCdc42-transfected hDPSCs ( $5 \times 10^4$ ) were seeded into the top chamber of the insert and placed in a 24-well plate filled with 600  $\mu$ l serum-free medium. After cell adhesion, the culture medium in the upper chamber was replaced with medium containing 2% FBS, while the lower chamber was filled with fresh medium containing 15% FBS. After the 24-hour incubation in 5% CO<sub>2</sub> at 37°C, the inserts were removed, fixed with 100% methanol for 10 min at -20°C, and stained with 0.1% crystal violet. The cells remaining on the upside of the inserts were scratched with a cotton swab. At least three random microscopic fields were chosen for cell counts in each group.

### **Polarization assay**

hDPSCs transfected with negative control or siCdc42 were seeded on glass coverslips to reach complete confluence. After starvation in serum-free medium for 24 hours, the cells were wounded with a 200- $\mu$ l pipette tip across the confluent monolayer. Subsequently, cells were incubated in medium with 5% FBS

for 12 hours, fixed in 4% paraformaldehyde for 15 min, permeated for 5 min in 0.2% Triton X-100 and blocked for 30 min with 5% BSA in PBS, followed by immunofluorescence staining for Golgi bodies using an antibody against GM130 (#12480, Cell Signaling). Afterwards, the cells were incubated with Tetramethylrhodamine isothiocyanate (TRITC)-conjugated secondary antibodies to detect GM130 and phalloidin-FITC to detect actin for 1 h at room temperature. Nuclei were stained with DAPI. The immunostained cells were then visualized with an Olympus FV1000 confocal microscope, and the images were acquired using FV10-ASW3.1 Viewer software. Golgi apparatus polarization and nucleus reorientation were measured as previously reported (Nobes & Hall, 1999). Briefly, for Golgi apparatus polarization, the cells located at the wound edge were divided into three sectors. These sectors were centred on the cell nucleus, and each of them contained 120°. One of them faced the wound (Fig. 4). Only the cells with Golgi apparatuses located in the sector facing the wound were considered positive. At least 5 random fields and 100 cells were taken into consideration for each group. For nucleus reorientation, the positive cells were determined from the description above. Nucleus reorientation was measured by the angle between the long axis of the cell nucleus and the edge of the wound. Angles no more than 90° were used for the analyses.

### **Cell adhesion**

To test cell adhesion, 48-well plates were coated with 40 mg/L of collagen type I in PBS overnight at 4°C and then blocked with 1% (w/v) BSA in PBS for 1 h at 37°C. Dominant negative- or siCdc42-transfected hDPSC suspensions with a density of  $1 \times 10^5$  cells/ml in serum-free media were prepared. After removing the blocking buffer, 100 µl of cell suspension was added to each well and incubated for 60 min at 37°C. Subsequently, non-adherent cells were washed away with PBS. Adherent cells were fixed in 100%

methanol for 10 min at -20°C and stained with 0.5% crystal violet for 15 min.

### **Statistical Analysis**

Data shown in this experiment are expressed as the mean  $\pm$  standard deviation (SD). Significant differences between two groups were analysed by Student's t test, and the statistically significant differences were identified by P-values less than 0.05.

## **Results**

### **Characterization of human dental pulp stem cells**

Primary cells formed cell clones after being continuously cultured for 2 weeks (Fig. 1B). Flow cytometric analyses showed that these cells were stained and had positive expression of CD29, CD90, CD105 and CD146, while they were negative for the expression of CD34 and CD45 (Fig. 1A). The multiple lineage differentiation tests demonstrated that these cells formed mineralized nodules and stained positive with Alizarin Red S after 4 weeks of culture with odontogenic/osteogenic medium (Fig. 1C). They also stained positive for lipid droplets with Oil Red O after 5 weeks of induction in adipogenic medium (Fig. 1D).

### **Cdc42 is knocked down by siRNA**

Cdc42 plays an important role in cytoskeletal rearrangements and cell migration. We knocked down the expression of Cdc42 in human dental pulp stem cells by transfecting siRNA targeting Cdc42 into the cells. The effect of the knockdown was confirmed by real time-PCR and Western blot (Fig. 2). Compared with that of the control group, the mRNA expression of Cdc42 was significantly downregulated by approximately 90% after transfection with the siRNA targeting Cdc42 (Fig. 2A). The siRNA directed to

Cdc42 successfully reduced the protein levels after 48 h of transfection, with an extremely efficient knockdown of more than 90% (Fig. 2B and C).

### **Cdc42 siRNA inhibits the migration ability of hDPSCs**

To study the role of Cdc42 during the migration of hDPSCs, the cells were cultured to reach complete confluence, and the scratch wound assays were performed using a sterile pipette tip to cut through the monolayer. Compared with that observed in the control siRNA-transfected cells, wound closure was delayed in the cells transfected with siRNA targeting Cdc42 after 36 h (Fig. 3A and B). To confirm this phenomenon, transwell migration assays were performed as mentioned above. As shown in the figure, siRNA targeting Cdc42 significantly reduced the number of migrated cells when compared with that in the control group after 24 h (Fig. 3C and D). Treatment with 10  $\mu$ M ML141 also markedly decreased the migration of hDPSCs in both the scratch wound assay and transwell assay (Fig. 3A-D).

### **Cdc42 knockdown affects the polarization of the Golgi apparatus (GA) and nucleus reorientation in migrating hDPSCs**

After hDPSCs have been migrating for 12 h in the scratch wound assays, the GA was detected with an antibody against GM130, and the nucleus was stained with DAPI (Fig. 4A-I). Cells reoriented their Golgi bodies and nuclei in all the groups in response to the wound. As shown in Figure 4, cells transfected with siRNA targeting Cdc42 showed a significant reduction in the proportion of cells exhibiting GA polarization (Fig. 4J) and nucleus reorientation (Fig. 4K). Only those cells migrating into the scratched area were counted.

### **Effects of Cdc42 knockdown on the adhesion of hDPSCs**

To detect the effects of Cdc42 on the adhesion of hDPSCs, control siRNA- or Cdc42-siRNA,

transfected cells were allowed to spread on the plates coated with collagen I. As shown in figure 5, the adhesion ability of hDPSCs transfected with Cdc42 siRNA was markedly inhibited when compared with that of the cells transfected with the control siRNA (Fig. 5A-C). Statistical analysis of the number of adherent cells in the Cdc42 siRNA group revealed a significant change showing only approximately 60% of the adherent cells in the control group (Fig. 5G). A remarkable difference in the morphology of the hDPSCs was also observed with a reduction in the leading edge after Cdc42 siRNA transfection (Fig. 5D-F). The projected cell area was significantly smaller than that of the control group.

### **Cdc42 affects the migration and adhesion of hDPSCs by regulating the phosphorylation of $\beta$ -catenin, FAK and GSK3 $\beta$**

To investigate the participation of Cdc42 in the migration and adhesion of hDPSCs in more detail, we measured the protein levels of  $\beta$ -catenin, FAK and GSK3 $\beta$ , which have been reported to play roles in these cell behaviours. hDPSCs without siRNA transfection were used as a blank control. The knockdown of Cdc42 reduced the expression level of  $\beta$ -catenin but not FAK or GSK3 $\beta$  (Fig. 6A-C). Considering that the function of these proteins mainly depends on their phosphorylation, we measured the protein levels of p- $\beta$ -catenin, p-FAK and p-GSK3 $\beta$  after transfection (Fig. 6A-C). The levels of p- $\beta$ -catenin, p-FAK and p-GSK3 $\beta$  showed significant alterations after Cdc42 knockdown when normalized to  $\beta$ -catenin, FAK and GSK3 $\beta$ , respectively (Fig. 6D).

## **Discussion**

After tissue injury, soluble molecules are rapidly released into the microenvironment around the damaged tissue and then attract immunocytes for the inflammatory response or stem cells for tissue

repair (Biro, Munoz & Weninger, 2014). The important role of stem cells in tissue repair has been highlighted in many types of tissue injury. The motility and recruitment of stem cells are essential for the repair of damaged tissue. HDPSCs remain at the cell-rich zone, where they begin the repair process. The abovementioned situation implies that stem cells need to move effectively towards the injury and adhere successfully.

HDPSCs are the source of many kinds of dental pulp cells and contain two critical features, self-renewal and multi-lineage differentiation potential (Nayak, Chang, Vaitinadin & Cancelas, 2013). Understanding the signals that regulate stem cell motility is necessary for their manipulation and the application of stem cell therapies in the areas of dental pulp repair and regeneration. In response to environmental cues, hDPSCs activate regulators of cell morphology and migration. Single cell migration usually contains four steps: polarization of the cell body, extension of a protrusion, attachment near the leading edge and retraction at the cell rear (Kumar, Ho & Co, 2015). Cdc42 plays an important role during this process by regulating the cytoskeletal rearrangements and the formation of focal adhesions (Ridley, 2006; Spiering & Hodgson, 2011). Studies in corneal epithelial cells, mouse embryonic fibroblasts and cancerous cell lines found that downregulation or excision of Cdc42 results in reduced cell migration (Gadea, Lapasset, Gauthier-Rouviere & Roux, 2002; Hou, Toh, Gan, Lee, Manser & Tong, 2013). Previous studies have shown that defects of Cdc42 are related with Fanconi anaemia, whose characteristics include defects in homing and adhesion of haematopoietic progenitors (Stengel & Zheng, 2011; Zhang et al., 2008). These findings remind us that the normal function of Cdc42 is necessary for stem cell migration and adhesion.

However, there is still not enough evidence regarding the role of Cdc42 in the migration and

adhesion of hDPSCs after dental pulp is damaged. In the present study, siRNAs targeting Cdc42 were transfected into hDPSCs to obtain stem cells with low-expression of Cdc42. According to the quantitative real-time PCR analysis and Western blot, the expression of Cdc42 in the transfection group was significantly lower than that of the control group. Studies in other cells found that downregulation of Cdc42 could obviously inhibit cell motility, which is consistent with the phenomenon we observed in this study. In the wound healing assay, hDPSCs showed a significant decrease in migration when activity of Cdc42 was inhibited by ML141 or when expression of Cdc42 was reduced by siRNA. This result was also detected in the transwell assay in which hDPSCs were induced by a high proportion of FBS to pass through the membrane. The abovementioned results indicate that the migration of hDPSCs will be delayed when the normal function of Cdc42 is disturbed.

Cell polarization has been well studied in the migration of several cell lines and shows specific characteristics in different cells. Cells first relocate the Golgi apparatus and microtubule organizing centre towards the front of the nucleus and towards the direction of the motion, following the extraction of an actin-rich protrusion that adheres to the extracellular matrices (ECM) that lead cell migration (Vicente-Manzanares, Webb & Horwitz, 2005). Golgi body positioning involves many proteins and could be affected by cell shape and confluence (Hoon, Li & Koh, 2014). In the current study, we focused on Golgi reorientation in a confluent stem cell monolayer. Previous studies have shown that the position of the Golgi is closely related to the function of Cdc42, which determines the position through cytoskeletal rearrangements, and Cdc42 is usually thought to be necessary for the establishment of the centre of cell polarity (Etienne-Manneville & Hall, 2002; Ridley et al., 2003). A study in *Drosophila* wound healing demonstrated that Cdc42 is required for polarization in cells next to the wound's leading edge (Baek,

Kwon, Lee & Choe, 2010). In this study, expression of Cdc42 in hDPSCs was also tightly connected with GA reorientation. After inhibiting the expression of Cdc42 by siRNA, the proportion of polarized GAs in the migrating hDPSCs next to the wound was obviously lower than that of the control group. Consistently, the diversification of nucleus reorientation paralleled the decrease in GA polarization.

Cell migration relies on an organic and spatiotemporal regulation of cytoskeletal dynamics, in which Cdc42 is a fundamental regulator (Renkawitz & Sixt, 2010). The generation of lamellipodia and filopodia at the leading edge of cells is necessary for mesenchymal cell migration (Boer, Howell, Schilling, Jette & Stewart, 2015). The leading edges are stable and thin sheets that extend from the plasma membrane by actin polymerization (Biro et al., 2014). Cdc42 affects the filopodia formation of migrating cells, and filopodia are rapidly induced in fibroblasts when Cdc42 is activated before developing into lamellipodia (Burrige & Wennerberg, 2004). According to the previous studies, activated Cdc42 is localized in large dynamic protrusions in a short time after cell adhesion, which suggests that Cdc42 is critical at the initial stage of protrusion formation (Donnelly et al., 2014). To assess whether Cdc42 is involved in the protrusion formation of hDPSCs, cells transfected with siCdc42 or control siRNA were fixed after adhering to type I collagen-coated culture plates for 1 hour. Spreading cells were identified under phase contrast microscopy, and the spreading of hDPSCs was inhibited by the siRNA targeting Cdc42, with cells transfected with siCdc42 covering a smaller surface area. The results demonstrated that the formation of both filopodia and lamellipodia in hDPSCs were downregulated during this period.

In migrating cells, cytoskeletal rearrangements regulated by Cdc42 at the cell leading edge participate in the construction of cell adhesion to the extracellular matrices (Donnelly et al., 2014; Zegers & Friedl, 2014). Directional cell movement requires cytoskeletal rearrangements to generate force and pull the cell



body forward, while force generation relies on the focal adhesions (FAs) that connect the cell membrane to the extracellular matrices (Stehbens & Wittmann, 2012). FAK is an integrin-binding non-receptor tyrosine kinase. As a major modulator of FA dynamics, FAK is necessary for microtubule-mediated FA disassembly and is recruited to the sites where cell adhesion molecules bind with the ECM (Ezratty, Partridge & Gundersen, 2005; Schober et al., 2007). The increased expression of activated FAK at the leading edge of migrating cells promotes the establishment of polarity and improves cell motility (Kiwanuka et al., 2016). Dephosphorylation of FAK is related to the disassembly of focal adhesions, while hyperphosphorylation of FAK promotes the spreading of protrusions and affects cell migration. Although the expression level of FAK was not affected by Cdc42 knockdown in this study, its phosphorylation was obviously downregulated, which indicated that Cdc42 may control the migration of hDPSCs by regulating the phosphorylation of FAK.  $\beta$ -catenin, which can be found in centrosomes, is tightly connected with cell polarity in the developing midbrain. As an important component of cell adhesion,  $\beta$ -catenin forms a dynamic link between cell adhesion molecules and the cytoskeleton, which makes it a connector between cell adhesion and migration (Nelson, 2008). Phosphorylation of  $\beta$ -catenin leads to its localization to the cell adherens junction and protrusion in MDCK cells (Faux, Coates, Kershaw, Layton & Burgess, 2010). Studies show that it could be phosphorylated by GSK3 $\beta$  at Ser45, Ser33/37 and Thr41. GSK3 $\beta$  is a multifunctional serine/threonine kinase and affects numerous cellular processes, including cell migration (Doble & Woodgett, 2003; Frame & Cohen, 2001). It also regulates the turnover and subcellular localization of  $\beta$ -catenin (Phukan, Babu, Kannoji, Hariharan & Balaji, 2010). At the same time, GSK3 $\beta$  could regulate the disassembly of focal adhesions to promote cell migration (Kobayashi et al., 2006). The activation of Cdc42 induced by a wound could also phosphorylate GSK3 $\beta$

to promote microtubule anchoring and cell polarization (Manneville, Jehanno & Etienne-Manneville, 2010). Our studies showed that the phosphorylation of GSK3 $\beta$  and  $\beta$ -catenin are both inhibited after Cdc42 knockdown. Although the relationship between GSK3 $\beta$  and  $\beta$ -catenin were not clearly investigated in the present study, both could still play roles in the adhesion and migration of hDPSCs.

## **Conclusion**

Our study demonstrates that Cdc42 is necessary for the migration of hDPSCs and that GA polarization and nucleus reorientation require the normal function of Cdc42, which participates in these processes from the initial stage of cell adhesion and migration. Although the crosstalk among the proteins examined in this study is limited, downregulation of Cdc42 was still found to decrease hDPSC motility by altering the phosphorylation levels of FAK, GSK3 $\beta$  and  $\beta$ -catenin. In the following studies, the precise relationships among these proteins in the migration and adhesion of hDPSCs need to be confirmed through detailed experiments, which may provide the foundations for a therapy for dental pulp injury.

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## Figure legends

**Figure 1.** Characterization of human dental pulp stem cells (hDPSCs). **(A)** Flow cytometric analysis of molecular surface antigen markers in hDPSCs (B refers to the positive percentage for the antigen). **(B)** The primary cells formed clones after being cultured for 2 weeks. **(C)** Positive staining of mineralized nodules with Alizarin Red S. **(D)** Positive staining of lipid droplets with Oil Red O.

**Figure 2.** Knockdown of Cdc42 by small interfering RNAs (siRNAs). **(A)** Human dental pulp stem cells (hDPSCs) transfected with control or Cdc42 siRNA were cultured in normal medium for 24 h to assess the effects of siRNA regulation on the mRNA transcription levels of Cdc42. **(B, C)** Cells were cultured for 48 h and subjected to Western blot using the indicated antibodies. The quantitative results **(A and C)** are the means  $\pm$  SD of three independent experiments. NS means not significant, \*\*P<0.01.

**Figure 3.** Regulating the migration of hDPSCs by knockdown or inhibition of Cdc42. **(A, B) Confluent monolayer of** human dental pulp stem cells transfected with control or Cdc42 siRNA were scratched using 1000- $\mu$ l pipette tips. Representative images **(A)** were taken at 0 h and 36 h after wounding. **(C, D)**

Cells transfected with control or Cdc42 siRNA were subjected to the transwell assays. Cells that had migrated onto the down side of the transwell were fixed and stained after 24 h of migration, and representative images (**D**) are shown. The quantitative results (**B** and **C**) are the means  $\pm$  SD of three independent experiments. In **A** scale bars = 500  $\mu$ m; in **D** scale bars = 200  $\mu$ m; \*\*P<0.01.

**Figure 4.** Inhibition of the reorientation of the Golgi and nucleus by knockdown of Cdc42. Human dental pulp stem cells transfected with control or Cdc42 siRNA were subjected to a scratch wound assay and stained with an anti-GM130 antibody, FITC-conjugated phalloidin and DAPI. Representative images (**A-I**) and quantitative results (**J, K**) are shown. (**J**) The quantitative results shown are the means  $\pm$  SD of three independent experiments in which at least five randomly chosen fields were analysed in each experiment. (**K**) The box represents the middle 50% and is derived from the upper and lower quartile values. The line within the box indicates the median value. The minimum and maximum values are displayed by the whiskers connecting to the box. In **A, B, D, E, G** and **H**, scale bars = 100  $\mu$ m; in **C, F** and **I**, scale bars = 25  $\mu$ m; \*\* P<0.01.

**Figure 5.** The effect of Cdc42 knockdown on the adhesion of hDPSCs. Cells transfected with control or Cdc42 siRNA were subjected to an adhesion assay. Adherent cells were fixed and stained after 1 hour. Representative images (**A-F**) and quantitative results (**G**) are shown. The quantitative results shown are the means  $\pm$  SD of three independent experiments in which at least three randomly chosen fields were analysed in each experiment. In **A-C**, scale bars = 200  $\mu$ m; in **D-F**, the white arrows indicate filopodia, while the black arrows indicate lamellipodia, scale bars = 20  $\mu$ m; \*\*P<0.01.

**Figure 6.** The effect of Cdc42 knockdown on the expression of FAK,  $\beta$ -catenin and GSK3 $\beta$ . Lysates of human dental pulp stem cells transfected with control or Cdc42 siRNA were subjected to Western blot.



The expression and phosphorylation of FAK (A),  $\beta$ -catenin (B) and GSK3 $\beta$  (C) were detected by the indicated antibodies. Representative images and quantitative results are shown. The quantitative results (D) are the means  $\pm$  SD of three independent experiments. NS means not significant, \*P<0.05.

Fig.1

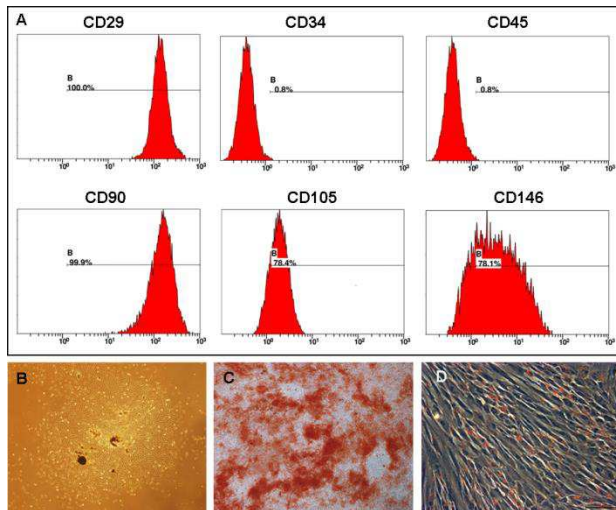


Fig.2

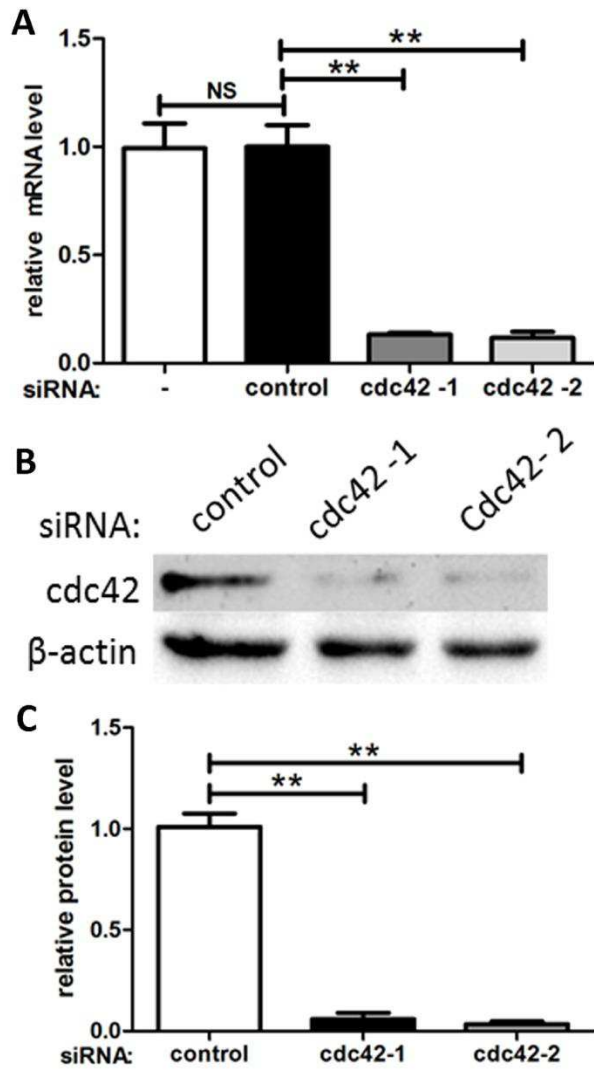


Fig.3

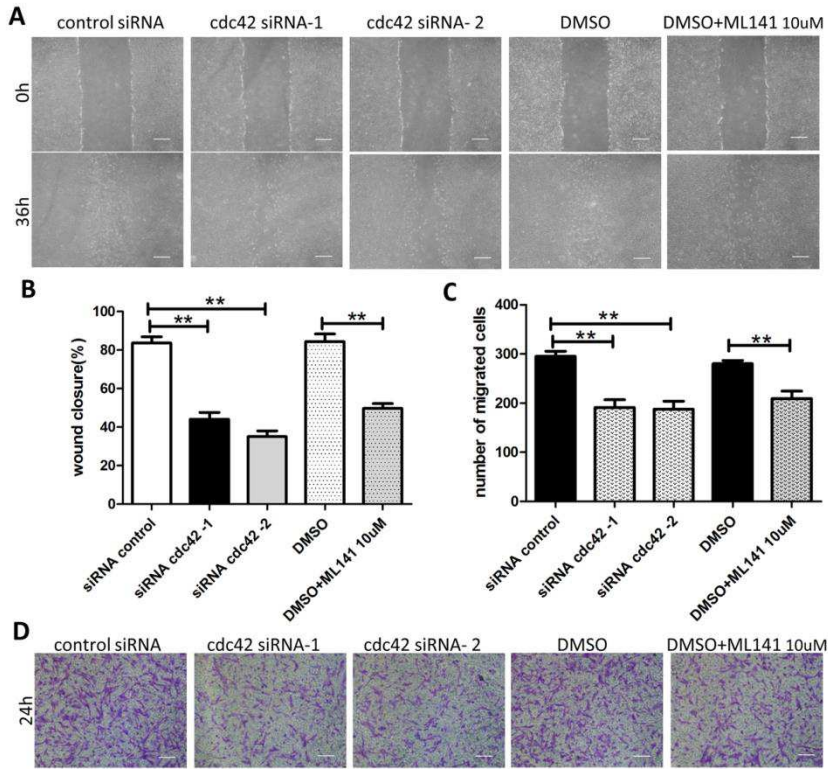


Fig.4

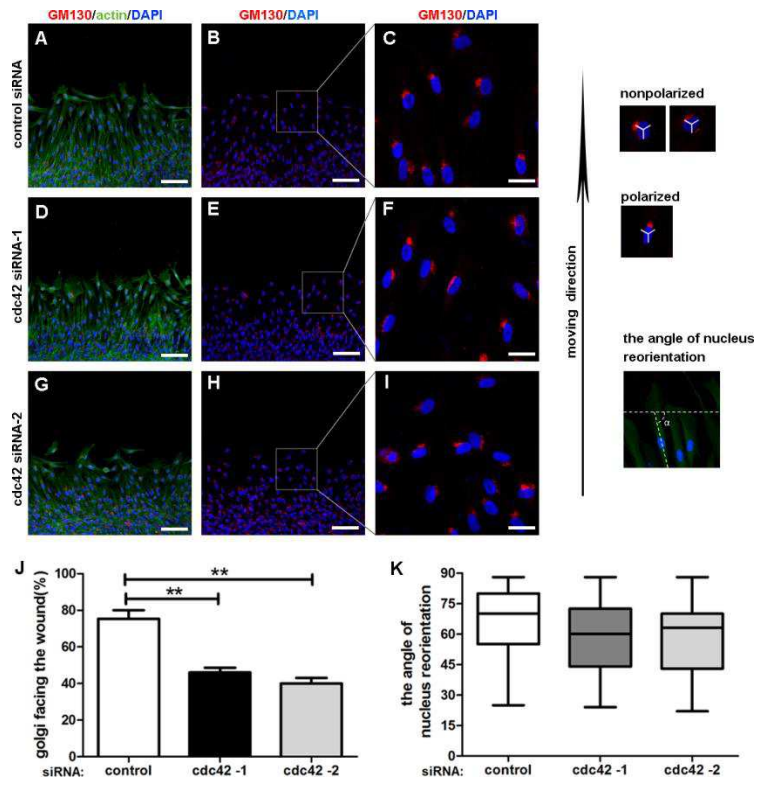


Fig.5

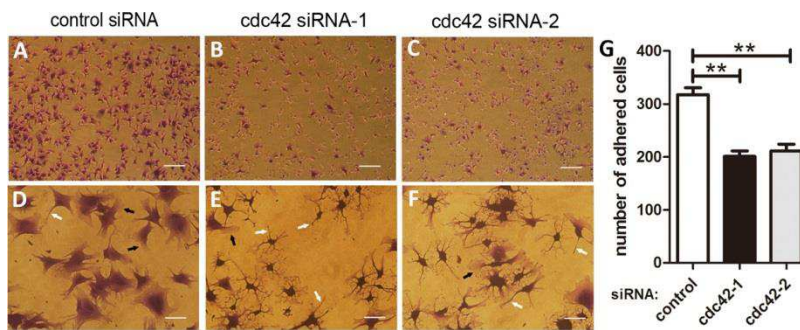


Fig.6

