Int J Clin Exp Pathol 2015;8(11):14425-14432
www.ijcep.com /ISSN:1936-2625/IJCEP0017028

Original Article

Effect of ITGA5 down-regulation on the migration capacity of human dental pulp stem cells

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Received September 28, 2015; Accepted October 28, 2015; Epub November 1, 2015; Published November 15, 2015

Abstract: Background: The purpose of this study was to evaluate the role of integrin-α5 (ITGA5) in regulating the migration capacity of human dental pulp stem cells (hDPSCs), which might provide new evidence for understanding the repair and regeneration mechanisms of dental pulp tissues. Materials and methods: The enzyme digestion method was employed to isolate the hDPSCs from dental pulp tissues. The cell surface markers of hDPSCs were detected using flow cytometry analysis. Then the colony forming and multi-differentiation capacity of hDPSCs were evaluated. The lentivirus vector that carried the ITGA5 shRNA was constructed and real-time PCR was used to examine the effectiveness of ITGA5 shRNA lentivirus. Then transwell assay was performed to evaluate the impact of ITGA5 inhibition on the migration capability of hDPSCs. Results: Our results showed that the cells we isolated from the dental pulps were positive for mesenchymal stem cells biomarkers. In addition, the cells possessed both colony forming capacity and multi-differentiation potential. ITGA5 shRNA lentivirus could not only infect hDPSCs with high efficiency, but also down-regulate the expression level of ITGA5 mRNA significantly ($P<0.01$). The transwell assay revealed the number of cells that migrated to the lower chamber was significantly less in the ITGA5 shRNA group compared with that in the scrambled shRNA group ($P=0.016$). Conclusion: ITGA5 plays an important role in maintaining and regulating the normal migration capacity of hDPSCs.

Keywords: hDPSCs, ITGA5, migration, lentivirus

Introduction

Human dental pulp stem cells (hDPSCs) were first isolated from dental pulp tissues and named by Gronthos and his colleagues in 2001 [1]. The clonogenic cells were demonstrated to possess both self-renewal and multi-lineage differentiation capability [2, 3]. In addition, hDPSCs could form dentin-like structures under both ex vivo and in vivo conditions [1, 3, 4]. Thus these cells have great potential for regenerative dentistry [5, 6].

Integrin-α5 (ITGA5), which is a member of intergrins, has been demonstrated to regulate various vital cellular processes such as proliferation, migration, differentiation and development. Chen et al showed that ITGA5 was a mediator for epidermal growth factor-induced retinal pigment epithelial cell proliferation and migration [7]. The cytoplasmic domain of ITGA5 is essential for cell migration and the radial migration capacity was perturbed in ITGA5 knockdown neural precursor cells [8-10]. ITGA5 also plays an important role in the regulation of differentiation of various cell types such as myoblast, adipocyte and mesenchymal stem cell [11-13]. Moreover, ITGA5 involves in regulating a number of early developmental processes, including somite boundary maintenance, morphorgenesis and organ development [14-16].

We have demonstrated that ITGA5 is important for maintaining hDPSCs in a proliferative state. Inhibition of ITGA5 signaling promotes the odontogenic differentiation of hDPSCs [17]. However, whether ITGA5 involves in regulating the migration capacity of hDPSCs remains unknown. The migration capacity of hDPSCs is essential for dental pulp repair and regeneration. Therefore, the aim of the present study was to elucidate the role of ITGA5 in regulation of hDPSC migration.

Materials and methods

hDPSC isolation and culture

Human dental pulps were isolated from complete wisdom teeth of healthy subjects at the Department of Stomatology, Nanfang Hospital and written informed consent was obtained from the donors. The enzyme digestion method was used to separate the hDPSCs from dental pulp tissues, which has been previously described [17]. The cells were cultured in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 mg/mL streptomycin (Gibco-BRL, Grand Island, NY, USA), seeded in 25 cm^2 culture flasks (Corning Inc, Corning, NY, USA) and incubated in the humidified incubator at 37°C with 5% CO_2.

Flow cytometry analysis

At least 1×10^6 cells were prepared in cold PBS for each test. Cell phenotype analysis was performed by flow cytometric detection of CD29/PE, CD34/PE CD90/PE (PharMingen-BD Biosciences, San Diego, CA, USA), CD44/FITC (Beckman Coulter, Fullerton, CA, USA), CD45/PE (Invitrogen, Carlsbad, CA, USA) and CD105/FITC (Biolegend, San Diego, CA, USA) according to manufacturers' instructions.

In vitro analysis of hDPSCs multi-lineage differentiation potential

For odontogenic differentiation, hDPSCs were seeded in 2 mL complete culture medium at 3×10^4/35 mm plate and cultured to 70% confluence. Differentiation was induced by culturing cells in complete medium supplemented with 10 mM β-glycerol phosphate, 50 μg/mL ascorbic acid, and 10^{-7} M dexamethasone for 3 weeks. The induced cells were fixed in 70% ice-cold ethanol for 20 min at room temperature (RT) and then stained with 2% Alizarin Red. For adipogenic differentiation, hDPSCs were seeded into 24-well plates at a density of 1×10^4/well and cultured to 70% confluence. Differentiation was induced by culturing cells in complete medium supplemented with 0.5 mM methylisobutylxanthine, 0.5 mM hydrocortisone, and 60 mM indomethacin for 3 weeks. The cells were fixed in 4% paraformaldehyde (PFA) for 20 min at RT and then stained with Oil Red O. For chondrogenic differentiation, hDPSCs were prepared as described for adipogenic differentiation. However, the cells were incubated with chondrogenic differentiation medium supplemented with 10 ng/mL transforming growth factor-β_3 for 3 weeks. Finally, the cells were then fixed in 4% PFA for 20 min at RT and then stained with Alcian Blue.

Lentivirus construction and infection

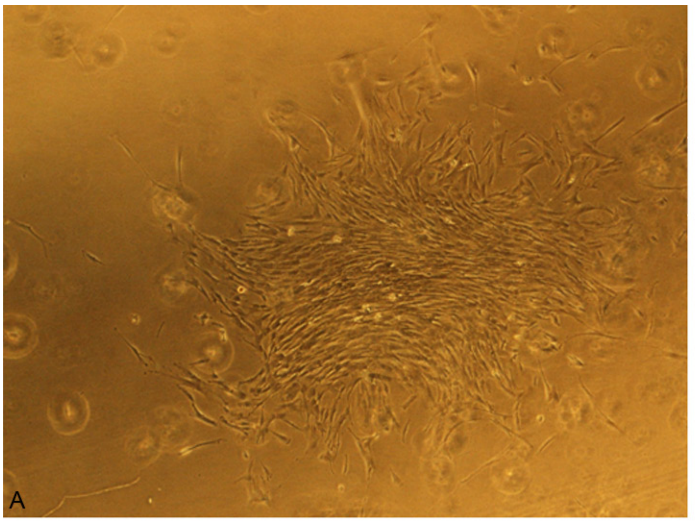
The lentiviral vector GV118-GFP (GeneChem, Shanghai, China) was used for inserting the oligonucleotides (ITGA5 shRNA and scrambled shRNA). Then the recombinant lentiviral vectors and packaging vectors were transfected into 293T cells. The supernatant was harvested 72 h after transfection. The lentiviruses were then purified by ultracentrifugation, and the titer of lentiviruses was determined. hDPSCs were infected with the ITGA5 shRNA lentivirus at multiplicity of infection (MOI) of 100, and scrambled shRNA hDPSCs were used as negative controls.

Real-time PCR

Total RNA was isolated from hDPSCs using TRIzol (Takara Bio, Kyoto, Japan) according to the manufacturer's protocol. PrimeScript® RT reagent Kit (TaKaRa) was employed to synthesize the first-strand cDNA. Then the SYBR® Premix DimerEraser™ kit (TaKaRa) was used to perform the real-time PCR on a Stratagene MX3000P real-time PCR system (Agilent Technologies, La Jolla, CA). Gene expression was normalized to GAPDH. Triplicate reactions were carried out in three separate experiments. The primers were as follows: ITGA5 Forward: 5'-GTCGGGGGCTTCAACTTAGAC-3'; ITGA5 Reverse: 5'-CCTGGCTGGCTGGTATTAGC-3'; GAPDH Forward: 5'-TGTTCGTCATGGGTGTGAAC-3'; GAPDH Reverse: 5'-ATGGCATGGACTGTGGTCAT-3'.

Transwell migration assay

The migration capacity of ITGA5 shRNA hDPSCs and scrambled shRNA hDPSCs were evaluated using transwell chambers (8 μm pore size, Corning, NY, USA). Following overnight serum starvation, cells were harvested and re-suspended in DMEM containing 0.1% FBS, then added to the upper transwell chamber. The migration ability of ITGA5 shRNA hDPSCs and scrambled shRNA hDPSCs were stimulated by the addition of DMEM+10% FBS to the lower chamber. Following 48 h culture, the cells that transversed through the membrane were fixed



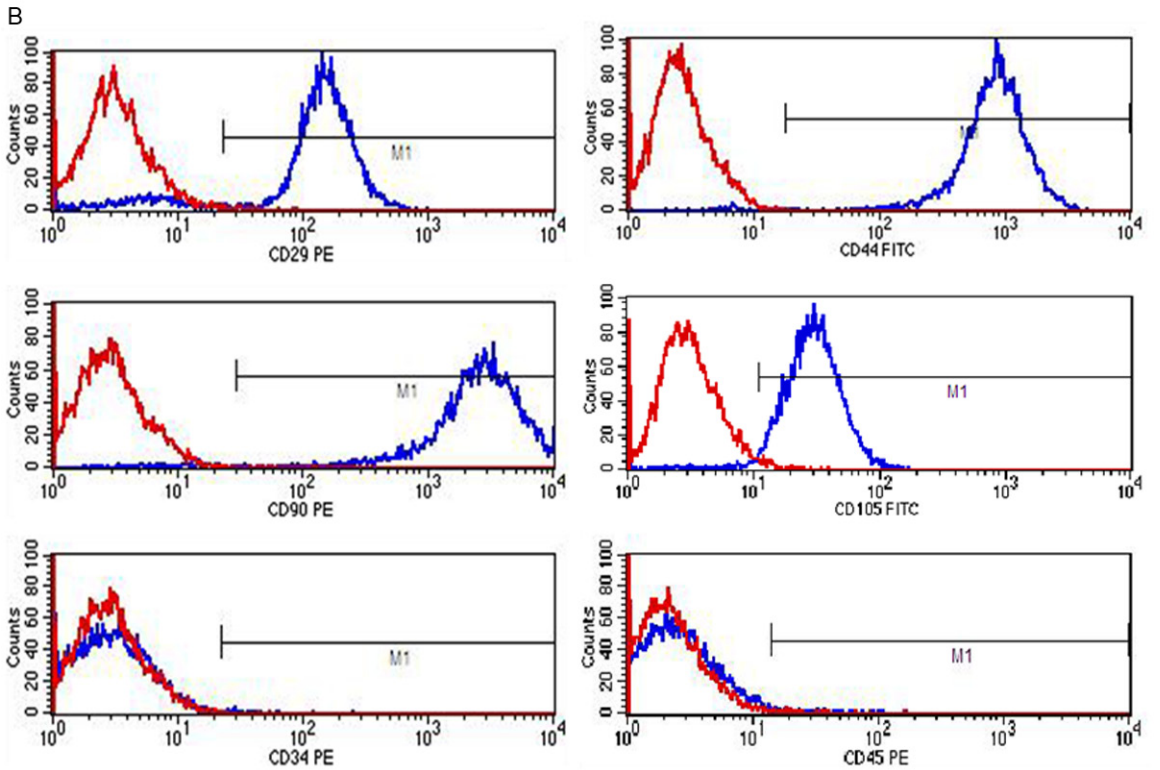


Figure 1. Colony forming morphology and phenotypic characteristics of hDPSCs. A. The hDPSCs isolated from the dental pulp were spindle-shaped and fibroblast-like. B. Cell surface markers expression examined by flow cytometric analysis. Positive expression of CD29, CD44, CD90, CD105 (MSC surface markers) was 90.82%, 99.45%, 98.79%, 97.92% respectively, and that of CD34 and CD45 (hematopoietic surface protein) was 0.23% and 0.75%, respectively.

in methanol and stained with 0.1% crystal violet. Cells were counted in four random fields visualized under a light microscope and expressed as the average number of cells per field.

Statistical analysis

Data were expressed as mean ± standard deviation and all statistical analysis was performed using the SPSS version 21.0 (SPSS, Inc, Chicago, IL). Differences were considered statistically significant when *P* value was less than 0.05.

Results

Isolation and surface antigen phenotypes of hDPSCs

We successfully expanded hDPSCs from human dental pulp. The hDPSCs formed single colonies in culture. A typical colony of hDPSCs growing in 25 cm^2 culture flask for two weeks was

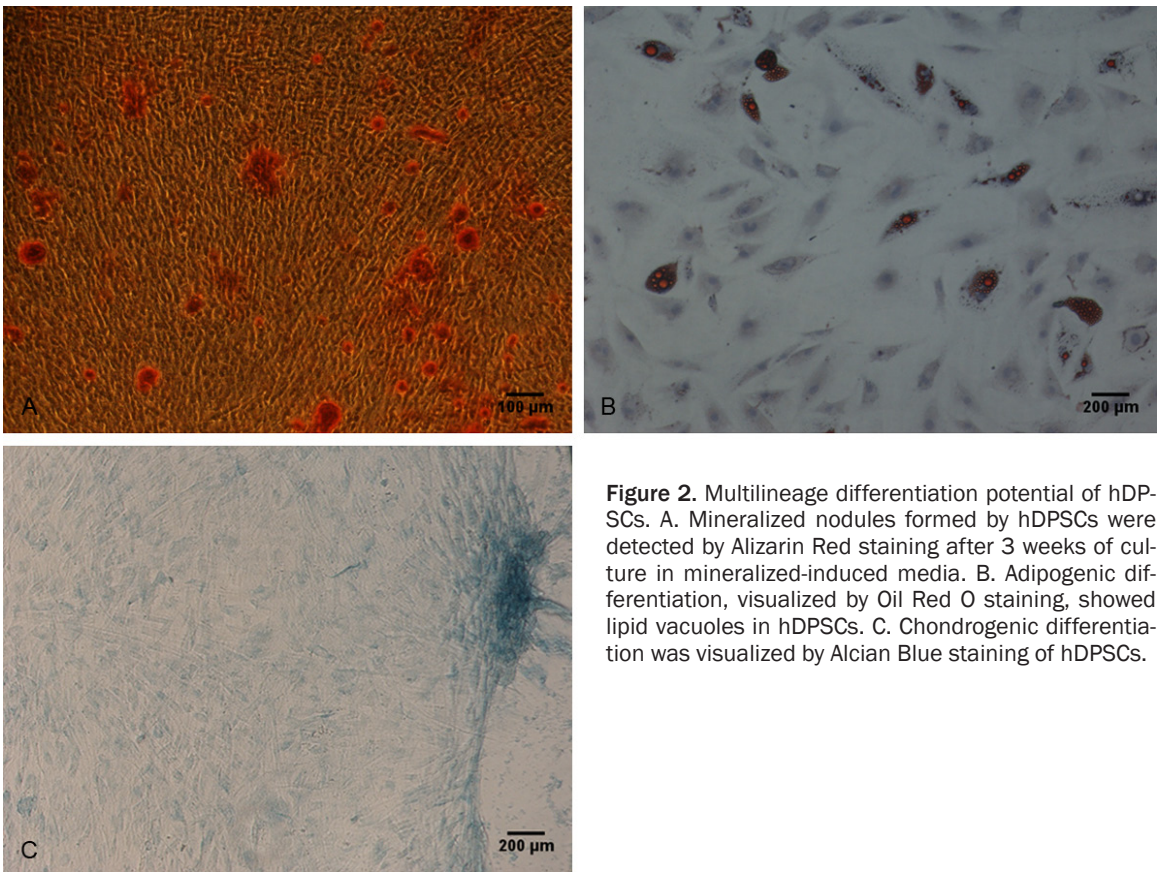


Figure 2. Multilineage differentiation potential of hDPSCs. A. Mineralized nodules formed by hDPSCs were detected by Alizarin Red staining after 3 weeks of culture in mineralized-induced media. B. Adipogenic differentiation, visualized by Oil Red O staining, showed lipid vacuoles in hDPSCs. C. Chondrogenic differentiation was visualized by Alcian Blue staining of hDPSCs.

identified after seeding as a single cell suspension. The cells were spindle-shaped and fibroblast-like (**Figure 1A**). Surface antigen expression of hDPSCs was analyzed by flow cytometry. The positive expression rate of CD34 and CD45 (hematopoietic progenitor cell surface molecules) was 0.23% and 0.75%, respectively However, for mesenchymal stem cell surface markers (CD29, CD44, CD90 and CD105), the positive expression rate was 90.82%, 99.45%, 98.79%, 97.92% respectively (**Figure 1B**).

Multi-potent differentiation capacity of hDPSCs

To evaluate the multi-potent differentiation capacity, hDPSCs were treated with various differentiation-inducing media. Odontoblastic differentiation was indicated by the detection of mineralized nodules after 3 weeks of culture in mineralization medium (**Figure 2A**). Following induction of adipogenic differentiation, the accumulation of lipid-rich vacuoles was visualized within hDPSCs by Oil Red O staining (**Figure 2B**). The induction of chondrogenic differentiation was demonstrated by the accretion of sulfated matrix stained with Alcian Blue (**Figure 2C**). Thus, these data demonstrate the multilineage differentiation potential of the hDPSCs isolated from human dental pulp.

Lentivirus infection of hDPSCs

Green fluorescent protein (GFP) was used to monitor the infection efficiency. hDPSCs were successfully infected with lentiviruses expressing either ITGA5 shRNA or scrambled shRNA (**Figure 3A**). Real-time PCR showed that the expression level of ITGA5 mRNA was significantly lower in the ITGA-5 knockdown cells compared to the controls (***P*<0.01) (**Figure 3B**).

The role of ITGA5 on cell migration in hDPSCs

The role of ITGA5 in the migration of hDPSCs was investigated by RNA interference analysis in a transwell chamber system. Compared with scrambled shRNA hDPSCs, ITGA5 shRNA hDPSCs showed about a 50% reduction in the number of cells that crossed the membrane (193±24 vs. 97±23, ***P*=0.016) (**Figure 4A**, **4B**).

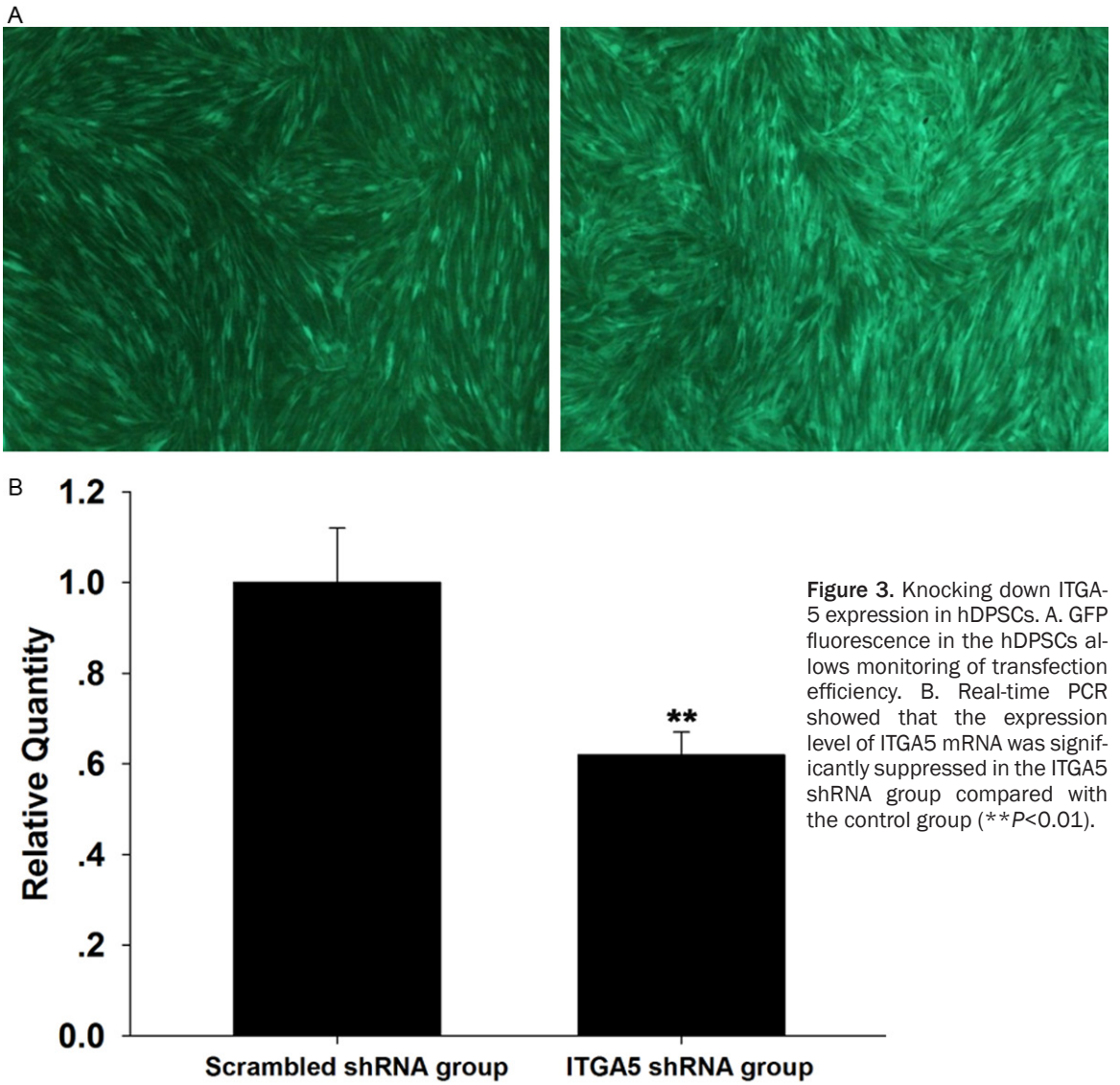


Figure 3. Knocking down ITGA-5 expression in hDPSCs. A. GFP fluorescence in the hDPSCs allows monitoring of transfection efficiency. B. Real-time PCR showed that the expression level of ITGA5 mRNA was significantly suppressed in the ITGA5 shRNA group compared with the control group (**P<0.01).

Discussion

The success of tissue engineering relies on three key ingredients: seeded cells, scaffold and morphogens. hDPSCs not only are suitable for use as seed cells in regenerative dentistry, but also show great value in treating various types of diseases such as diabetes, nerve injury, and neurodegenerative diseases [18-20]. Therefore, understanding the molecular mechanisms that regulation the biological functions of hDPSCs is important and imperative.

In this study, we demonstrated that the cells that isolated from the dental pulp tissues possessed the properties of stem cells such as colony forming ability, expression of positive MSC surface markers and multi-lineage differentiation potential. Colony formation capacity is an important character of stem cells, expanding a large number of stem cells is crucial for tissue regeneration and therapy purpose. The cell surface marker expression analysis showed that these isolated cell clones were positive for known MSC markers, such as CD29, CD44, CD90, and CD105, and were negative for the hematopoietic markers, CD34 and CD45. However, currently no specific biomarker is available for hDPSCs identification; further investigations of biomarker screening are needed to perform to address this problem. These cells were also shown to be capable of differentiating into various cell types including odontoblasts, adipocytes and chondrocyte. hDPSCs has been demonstrated to have the

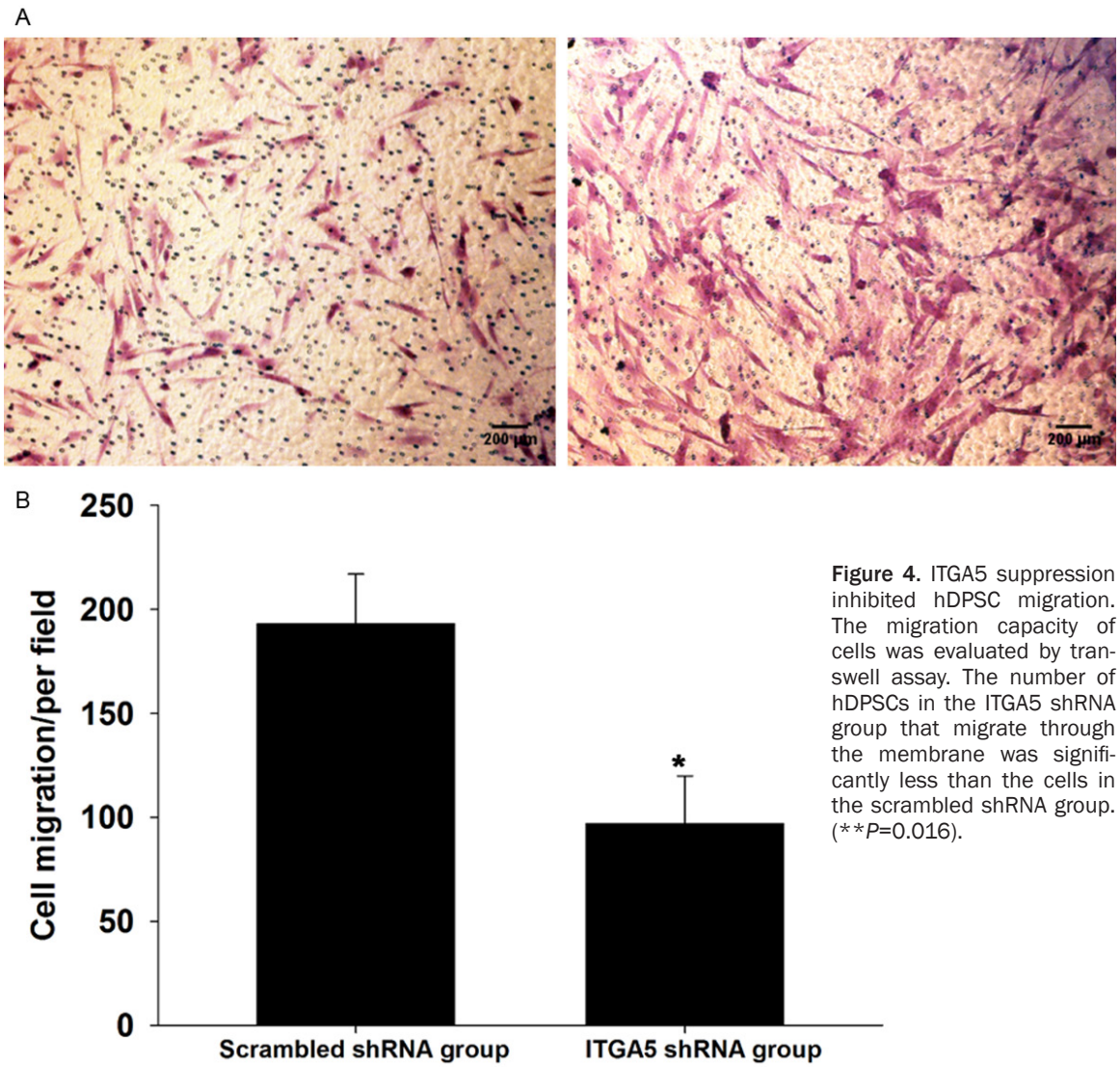


Figure 4. ITGA5 suppression inhibited hDPSC migration. The migration capacity of cells was evaluated by transwell assay. The number of hDPSCs in the ITGA5 shRNA group that migrate through the membrane was significantly less than the cells in the scrambled shRNA group. (***P*=0.016).

potential to differentiate into various cell types such as neurons, insulin producing cells and cornea epithelial cells [18, 21, 22].

The lentivirus we constructed could infect the hDPSCs with high efficiency. In addition, the expression level of ITGA5 mRNA was significantly reduced after ITGA5 shRNA lentivirus infection, indicating the effectiveness of the ITGA5 shRNA sequence. The reasons that we used lentiviral vectors rather than siRNA for ITGA5 suppression are as follows. Firstly, hDPSCs are primary cells which are difficult for transducing the chemical synthesized siRNA into nucleus to inhibit expression of target gene. Moreover, siRNA is unstable and might lose its function in a relative short time.

Then our findings demonstrated that ITGA5 suppression could significantly inhibit the migration capacity of hDPSCs, indicating ITGA5 is important for hDPSC migration. In agreement with our data, ITGA5 was reported to play an important role in regulation of migration in other cell types as well as cancer cells. The capability of neural precursors to migrate towards cerebral cortex is impaired in ITGA5-null animal models, suggesting ITGA5 might be crucial for cortical development [10]. Qiao et al showed that depletion of Snai1b function in the zebrafish embryos could significantly suppress the migration of cardiac precursors via down-regulating the expression of ITGA5, which resulted in defects in cardiac morphology and function in zebrafish embryos. In addition,

injection of α5β1 protein could rescue the myocardia precursor migration in Snai1b knockdown embryos, indicating the essential role of ITGA5 for normal migration capacity of cardiac precursors [23]. Larzabal et al revealed that ITGA5 suppression in lung cancer cells could not only completely abrogate cell migration, but also decrease adherence capacity to fibronectin as well as reduce tumor growth in vivo. Moreover, ITGA5 was identified as a direct target of miR-205 [24]. However, enforced expression ITGA5 in breast cancer cells could inhibit cell proliferation, migration and invasion capacity, indicating ITGA5 may be a metastasis suppressor in breast cancer [25]. Therefore, it is probably that the migration function of ITGA5 is cell type dependent and closely associated with the microenvironment the cells reside in.

Conclusion

Our data indicated that ITGA5 played an important role in regulating the migration capacity of hDPSCs. Further studies are needed to elucidate the concrete molecular mechanisms.

Disclosure of conflict of interest

None.

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