Indian Journal of Animal Sciences **84** (10): 1055–1060, October 2014/Article

Knockdown Fascin impairs bone marrow mesenchymal stem cells migration capacity of Wuzhishan Miniature pig

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Received: 26 April 2014; Accepted: 2 June 2014

ABSTRACT

The bone marrow mesenchymal stem cells (BM-MSCs) from Wuzhishan pig (WZSP) with knockdown expression of Fascin were used to make out the role Fascin gene in migratory potential of BM-MSCs. The role of Fascin gene is well established in cellular migration, but it is unclear for its activity in porcine BM-MSCs. At present study, BM-MSCs were isolated from femur and tibia of the WZSP with about 42 day-old, and had been evaluated by previous study. At first, 4 vectors encoding different short hairpin RNA (shRNA) for Fascin were designed to knock down Fascin, and qRT-PCR was employed to detect the expression of Fascin mRNA by BM-MSCs after transfection, and the vector with best interference effect was selected to be used in the following experiments. And then, the effect of knock down Fascin was further determined through Western blot using Fascin antibody. Finally, the migration capacity of the BM-MSCs was evaluated through scratch assay and transwell migration assay. The results showed that the specific shRNA for knocking down Fascin efficiently was found for the BM-MSCs from WZSP, and Fascin was involved in regulating the migration capacity of the BM-MSCs *in vitro*, which may be useful for the BM-MSCs from WZSP to be utilized in regenerative therapy for human.

Key words: Bone marrow mesenchymal stem cells, Fascin, Migration capacity, Specific short hairpin RNA, Wuzhishan pig

Mesenchymal stem cells (MSCs) are multipotent adult stem cells that can differentiate into various mesodermal lineages, including osteoblasts, adipocytes, and chondrocytes as well as myocytes and neuron-like cells (Pontikoglou *et al.* 2011). MSCs are derived from marrow tissues, umbilical cord blood, adipose tissue, adult muscle, corneal stroma, and can easily be isolated from bone marrow and subsequently expand *in vitro*. Bone marrow MSCs (BM-MSCs) are the most widely used MSCs in regenerative medicine and tissue engineering. MSCs exhibit potent mechanisms to avoid allogeneic rejection (Ryan *et al.* 2005), and BMMSC transplantation has therapeutic benefits to many kinds of diseases such as Alzheimer's disease, heart infarction, and rheumatoid arthritis (Lee *et al.* 2010, Loffredo *et al.* 2011, Papadopoulou *et al.* 2012).

Fascin, encoded by the *FSCN1* gene in human, is an actin cross-linking protein that shares no homology with other actin-bundling proteins, which contributes to cell migration by providing rigidity to filopodia and microspikes (Adams 2004). Fascin is a structural protein which is found in mesenchyme, nervous, and retinal tissue, which plays a central role in the regulation of cell adhesion, migration and invasion through bundling to actin molecules (Jayo and Parsons 2010). Fascin mainly localizes to actin-rich protrusions at the cell surface, and also localizes to invadopodia, membrane protrusions at the adherent cell surface, which facilitates cell migration.

As a special miniature pig in China, the adult Wuzhishan pig (WZSP) has a small body size, with physiological and general biochemical indices very similar to that of human being. The heart, small intestine and guts of WZSP are similar to that of human body. Therefore, WZSP can be used as a research model for human disease and organ

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transplantation, and its skin can also be used for skin grafting. In addition, WZSP is considered useful for medical and veterinary research due to its small size (Wang *et al.* 2006). Treatment with autologous BM-MSCs can increase cardiac function and promote myocardial viability by intracoronary transplantation on myocardial infarction in swine (Peng *et al.* 2013). Comparing to adipose-derived stem cells (ASC), BM-MSC had a greater cell growth and proliferation, while there was an overall greater lipid metabolism in ASC from pig (Monaco *et al.* 2012). FGF-2 can significantly enhance porcine BM-MSC osteogenic differentiation and proliferation, and porcine BM-MSCs can integrate readily with Gelfoam scaffolds and maintain viability and proliferative ability (Sun *et al.* 2013).

However, it is not clear whether knockdown Fascin have any effect on the migration capacity of BM-MSCs from WZSP. In this study, the specific short hairpin RNA (shRNA) for Fascin was designed to explore the influence on expression of Fascin in BM-MSCs. In addition, the effect of knockdown Fascin on the migration capacity of BM-MSCs from WZSP was evaluated. Although role of Fascin gene is well established in cellular migration, the novelty of this study aims to make out the role Fascin gene in migratory potential of BM-MSCs from WZSP, which may be helpful for the BM-MSCs from WZSP to be utilized in regenerative therapy for human.

MATERIALS AND METHODS

Culture of porcine BM-MSCs: The BM-MSCs were isolated from femur and tibia of the WZSP with about 42 day-old, and had been evaluated by flow cytometric analysis, adipogenic and osteogenic differentiation as described previously (He 2013, He *et al.* 2013). The BM-MSCs were cultured in Dulbecco's modified Eagle's medium with F12 salts containing penicillin/streptomycin (50 IU/mL, 50 µg/mL) with 10% fetal bovine serum, and incubated at an atmosphere of 5% CO_2 in air at 37 °C in 100% humidified air. Media were changed every other day.

Design and assessment of specific short hairpin RNA for Fascin: Specific short hairpin RNA (shRNA) sequences for Fascin were designed based on Fascin gene order from NCBI Gene database (Gene ID 100286741) and design principle for shRNA (Moore *et al.* 2010), and synthesized by commercial firms (Table 1). In addition, the negative control was designed and synthesized, which had the same composition of nucleic acids to the specific shRNA sequences with no homology to the gene sequence. The vectors encoding different shRNA included Fascin-sus-241, Fascin-sus-1115, Fascin-sus-1145 and Fascin-sus-1180. The BM-MSCs with about fourth to fifth generation were electroporated, and then were transfected by above 4 vectors and non-specific vector of shRNA (shRNA-NC, Control) using X-tremeGENE HP DNA transfection reagent following the manufacturer's protocol. The qRT-PCR was employed to detect the expression of Fascin mRNA after transfection for 24 h, and the vector with best interference effect was used in the following experiments. The best vector (Fascin-sus-1180) was recovered after digested with the restriction endonuclease ApaLI.

The linearized shRNA vector with best interference effect on BM-MSCs was named as shRNA-1180, and the linearized shRNA vector with no interference effect on BM-MSCs was shRNA-NC. The BMMSC treated with shRNA-1180 was experimental group, and the BMMSC treated with shRNA-NC was the control group. The culture medium supplemented with G418 (200 µg/mL) was used after the BM-MSCs had been transfected with the vectors of shRNA-NC and shRNA-1180 for 36 h, and the culture medium was changed once every 2 days. The stable cell line transfected with the vectors of shRNA-NC and shRNA-1180 were

Table 1. The sequence of specific short hairpin RNA for Fascin

Fascin-sus-241	S	5'-CACCGCCTGAAGAAGAAGCAGATCTTTCAAGAGAAGATCTGCTTCTT CTTCAGGCTTTTTTG-3'
	A	5'-GATCCAAAAAAGCCTGAAGAAGAAGCAGATCTTCTCTTGAAAGATCTGCTT CTTCTTCAGGC-3'
	Transcript	GCCTGAAGAAGAAGCAGATCTTTCAAGAGAAGATCTGCTTCTTCTTCAGGCTT
Fascin-sus-1115	S	5- CACCGGATGCCAGCTGCTACTTTGATTCAAGAGATCAAAGTAGCAGCTGGCATC CTTTTTTG -3'
	A	5'-GATCCAAAAAAGGATGCCAGCTGCTACTTTGATCTCTTGAATCAAAGTA GCAGCTGGCATCC-3'
	Transcript	GGATGCCAGCTGCTACTTTGATTCAAGAGATCAAAGTAGCAGCTGGCATCCTT
Fascin-sus-1145	S	5'-CACCGCGTGATCGGAGGATCATACTTTCAAGAGAAGTATGATCCTCCGATCACG CTTTTTTG-3'
	A	5'-GATCCAAAAAAGCGTGATCGGAGGATCATACTTCTCTTGAAAGTATGAT CCTCCGATCACGC-3'
	Transcript	GCGTGATCGGAGGATCATACTTTCAAGAGAAGTATGATCCTCCGATCACGCTT
Fascin-sus-1180	S	5'-CACCGCAAGTTTGTGACGGCCAAGATTCAAGAGATCTTGGCCGTCACAAACTTG CTTTTTTG-3'
	A	5'-GATCCAAAAAAGCAAGTTTGTGACGGCCAAGATCTCTTGAATCTTGGCCG TCACAAACTTGC-3'
	Transcript	GCAAGTTTGTGACGGCCAAGATTCAAGAGATCTTGGCCGTCACAAACTTGCTT

obtained through elimination of non-transfected cells after selection for 14 days.

RNA extraction and qRT-PCR assay: Total RNA was extracted using the total RNA kit. The cDNA was synthesized using RevertAid H Minus M-MuLV Reverse transcriptase. The qRT-PCR assay was performed using TaKaRa RNA PCR kit. Primer sequences of Fascin (forward: 52 -TTTTGTAGAGCGCTGCTGAG-32, reverse: 52 -ACCGTTTTCTCTGGGTTTCC-32) and GAPDH (forward: 52 -GTGAAGGTCGGAGTGAACG-32, reverse: 52 -CTCGCTCCTGGAAGATGGTG-32) were got designed and synthesized commercially. GAPDH was used as an internal control gene to calculate relative gene expression of the mRNA levels.

Western blot: The BM-MSCs were washed and lysed, and protein concentration was measured by BCA protein assay kit. Total protein of 10 μg was separated using 12% SDS-PAGE and transferred to PVDF membranes. The PVDF membranes were blocked in 5% non-fat milk. Fascin primary antibody was used to probe the membrane. The immunoreactive bands were detected by chemiluminescent substrate and recorded on x-ray films. A GAPDH antibody was used to monitor variation in loading of samples.

BM-MSCs migration analysis by scratch assay and transwell migration assay: The scratch assay and transwell migration assay were used to assess migration capacity of the BM-MSCs. The BM-MSCs were cultured in 60 mm culture plate to confluence, and then incubated with 10 μg/mL mitomycin-C for 2 h. The growth arrested BM-MSCs were transferred into 6 well-plate in a density of 3×10^6 per well, and the 'scratches' were made using a 10 μL pipette tip along the bottom of the plate after the BM-MSCs were cultured for 6 h, and then the BM-MSCs were cultured for another 48 h. The images of the BM-MSCs were acquired from the culture dishes using phase contrast microscope at the selected time points (0, 6, 12, 24, 36 and 48 h).

The BM-MSCs with 100 μL serum-free medium were placed in the apical well of the transwell assembly (6.5 mm diameter inserts, 8.0 μm pore size) at a density of 4×10^6 cells/mL, and 800 μL culture medium containing 10% FBS was filled into lower compartments as a source of chemoattractants. The nucleus was stained with Hochest 33342 after incubated at 37°C for 12 h, and the number of migrating cells was determined through counting nine fields per-well at random under the fluorescence microscope at 40× magnification.

Statistical analysis: The experiment was repeated at least 3 times, and the results were expressed as the mean ± SD. Statistical analyses were performed by Student *t* test. $P < 0.05$ was considered statistical significance.

RESULTS AND DISCUSSION

Assessment of specific shRNA for Fascin: RNA interference is to target complementary RNAs for destruction using double-stranded RNAs. In mammalian systems, shRNAs can be expressed continuously to establish stable gene silencing (Paddison *et al.* 2004). Several microRNAs can regulate expression of Fascin gene. In this study, the specific shRNAs for Fascin was used to knock down Fascin expression in the BM-MSCs from WZSP. The expression of Fascin by the BM-MSCs was evaluated through qRT-PCR assay after the BM-MSCs were transfected with the vectors of shRNA-NC (control), Fascin-sus-241, Fascin-sus-1115, Fascin-sus-1145 and Fascin-sus-1180. The result showed that there was a decreased expression of Fascin in the BM-MSCs transfected with the vectors of Fascin-sus-241, Fascin-sus-1115, Fascin-sus-1145 and Fascin-sus-1180 comparing with that transfected with the vector of shRNA-NC (control) ($P < 0.05$), and the shRNA interference for Fascin induced efficiently knockdown of Fascin in the BM-MSCs (Fig. 1). The expression level of Fascin transfected with the vector of Fascin-sus-1180 was the lowest one among the 4 experimental groups.

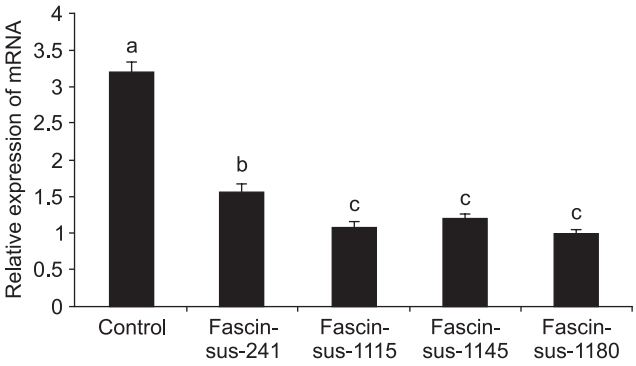


Fig. 1. The expression level of Fascin in the BM-MSCs through qRT-PCR analysis. The groups of control, Fascin-sus-241, Fascin-sus-1115, Fascin-sus-1145 and Fascin-sus-1180 were the BM-MSCs which were transfected with the vectors of shRNA-NC, Fascin-sus-241, Fascin-sus-1115, Fascin-sus-1145 and Fascin-sus-1180. Significant differences ($P < 0.05$) are indicated by different letters within different columns.

As illustrated in Fig. 2A that the linearized shRNA vectors for shRNA-NC (control) and shRNA-1180 were reclaimed efficiently. It was showed in Fig. 2B that all BM-MSCs were observed with fluorescence, and the stable cell lines transfected with the vectors of shRNA-NC (control) and shRNA-1180 were obtained through selection for 14 days and non-transfected cells were removed. There was a significant difference (Fig. 2C) in expression of Fascin mRNA between the group of shRNA-NC (control) and shRNA-1180 ($P < 0.05$) through qRT-PCR assay, and the Fascin gene was knocked down effectually in the BM-MSCs which were transfected with the vector of shRNA-1180. The GAPDH protein was expressed equally in both the groups of shRNA-NC (control) and shRNA-1180, but there was an obviously decreasing expression of Fascin protein in the group of shRNA-1180 comparing with that in the control group by Western blot analysis (Fig. 2D).

The results indicated that specific shRNAs for Fascin

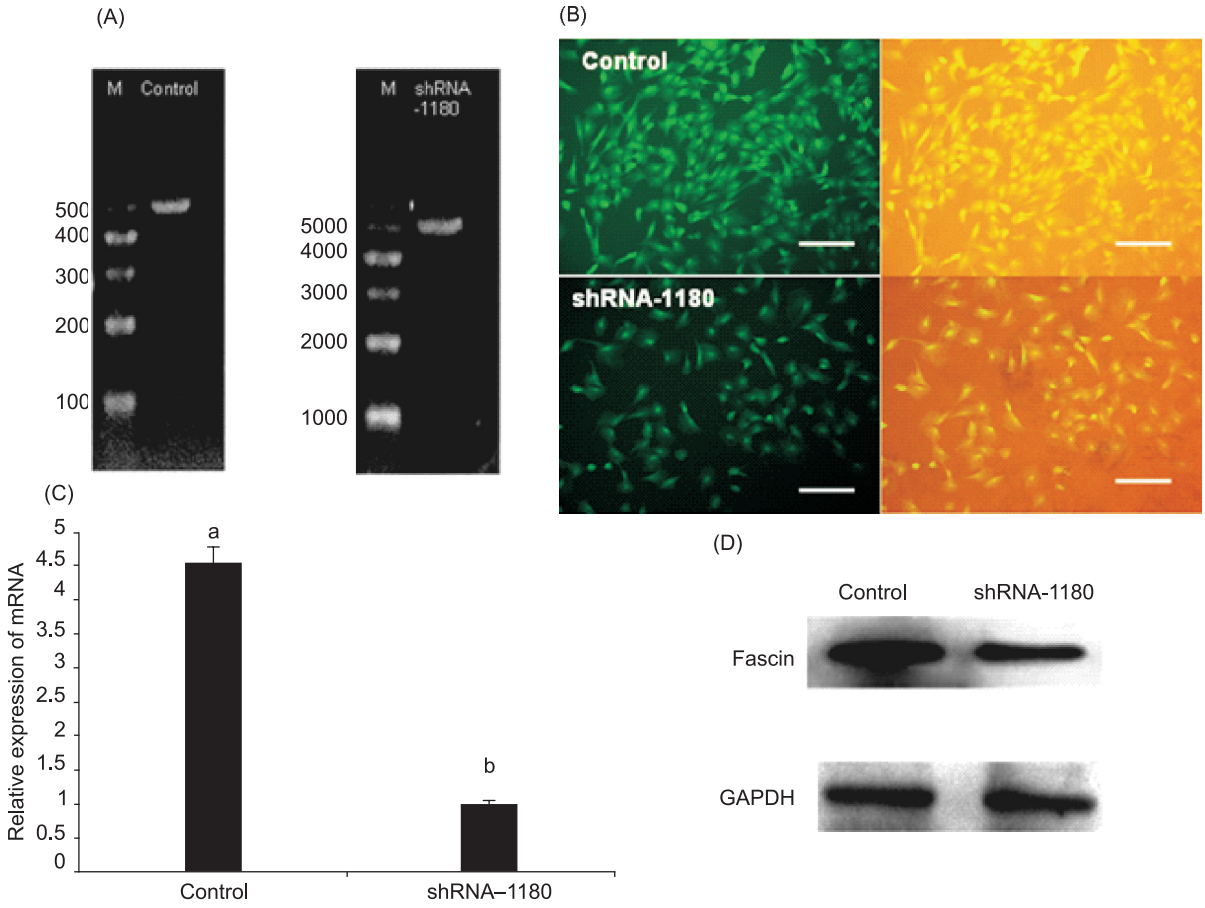


Fig. 2. Effect of the specific shRNA for Fascin on knockdown Fascin. **A**: The linearized shRNA vectors for shRNA-NC (control) and shRNA-1180 were reclaimed. M: maker. **B**: All BM-MSCs were observed with fluorescence, and the stable cell line transfected with the vectors of shRNA-NC (control) and shRNA-1180 were obtained. Bar: 100 μm. **C**: The expression level of Fascin mRNA in the BM-MSCs through qRT-PCR analysis. Significant differences ($P < 0.05$) are indicated by different letters within different column. **D**: The expression of Fascin protein in the BM-MSCs through Western blot analysis. GAPDH antibody was used to monitor variation in loading of samples.

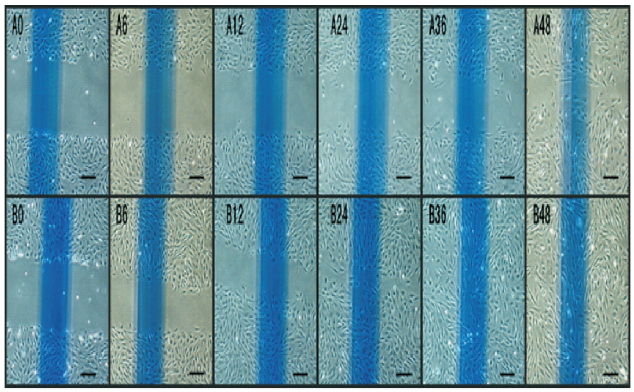


Fig. 3. BM-MSCs migration capacity analyzed by scratch assay. The blue zone was marked outside of plate bottom by a pen with blue ink. A0, A6, A12, A24, A36 and A48 were images of the BM-MSCs transfected with the vector of shRNA-1180, which were acquired at the selected time points (0 h, 6 h, 12 h, 24 h, 36 h and 48 h) after scratched. B0, B6, B12, B24, B36 and B48 were images of the BM-MSCs transfected with the vector of shRNA-NC (control), which were acquired at the selected time points (0 h, 6 h, 12 h, 24 h, 36 h and 48 h) after scratched. Bar: 100 μm.

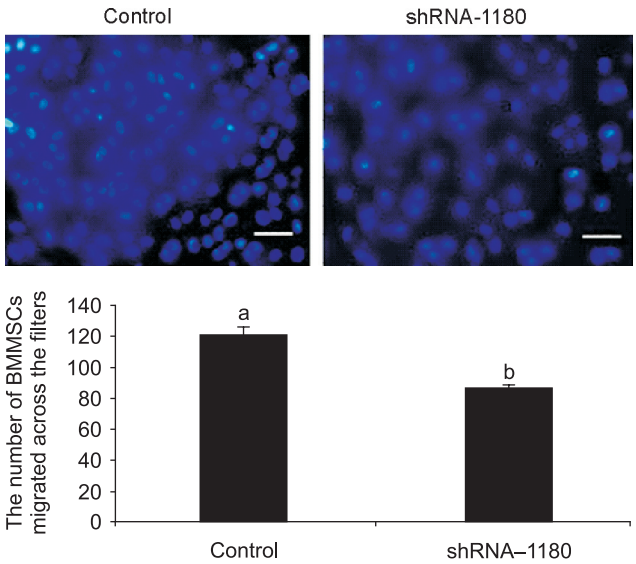


Fig. 4. The transwell results of the BM-MSCs. Control (*top left*) was the BM-MSCs transfected with the vector of shRNA-NC (*top right*). The shRNA-1180 was the BM-MSCs transfected with the vector of shRNA-1180. Bar: 100 μm. Significant (*bottom*) differences ($P < 0.05$) are indicated by different letters within different column.

can decrease the expression of Fascin effectively through the qRT-PCR detection and Western blot analysis (Figs 1, 2). Therefore the design of specific shRNAs for Fascin was suitable for knockdown Fascin in the BM-MSCs, and a BM-MSCs line with stable Fascin gene silencing was established, and the specific shRNA that knocked down Fascin efficiently was found. However, the Fascin was not knocked down completely, which may be due to the liposomal transfection method.

Role of Fascin on BM-MSCs migration capacity: Fascin gene encodes a member of actin-binding proteins which organize F-actin into parallel bundles, and are required for the formation of actin-based cellular protrusions. Fascin protein plays a key role in the organization of actin filament bundles and the formation of microspikes, membrane ruffles and stress fibers (Jayo and Parsons 2010). Our results showed that the scratched BM-MSCs transfected with the vector of shRNA-1180 (Fig. 3, A0 to A48) confluenced after cultured for about 48 h, but the scratched BM-MSCs transfected with the vector of shRNA-NC (B0 to B48, control) confluenced after about 24 h (Fig. 3).

The results of the transwell migration assay manifested that the number of the BM-MSCs that migrated across the filters with Fascin gene knockdown was 85.50 ± 2.56 (shRNA-1180), but the number of the BM-MSCs that migrated across the filters with normal Fascin expression was 122.06 ± 3.32 (control). The scratched BM-MSCs transfected with the vector of shRNA-1180 migrated more slowly than the scratched BM-MSCs transfected with the vector of shRNA-NC (control) (Fig. 4).

The scratch assay and transwell migration assay indicated that the knockdown of Fascin gene cut down the migration capacity of BM-MSCs significantly (Figs 3, 4), and it was important for the BM-MSCs to maintain expression of Fascin at a high level to keep their migration capacity. Fascin plays an important role in the formation of actin-based structures in the migratory behavior of cells (Hwang *et al.* 2008). Therefore, Fascin was a key gene for regulating the migration capacity of the BM-MSCs from WZSP. It was reported that cell motility of human adamantinomatous craniopharyngiomas tumor cells was significantly reduced in Boyden chamber and wound-healing experiments *in vitro* when transfected with Fascin small interfering RNA (Hölsken *et al.* 2010), which was in coincidence with our result in the BM-MSCs from WZSP. It is known that Fascin is expressed at a high level in the bone marrow, so it is not necessary to overexpress this gene to explore the effect of overexpression of Fascin on the migration capacity of the BM-MSCs.

Migration capacity of BM-MSCs from WZSP: BM-MSCs are the most important cell source for stem cell transplant therapy, and the migration capacity of BM-MSCs is the most essential determinant for the efficiency of their transplant therapy (Zhang *et al.* 2013). As an ideal stem cell candidate for tissue engineering and regenerative medicine, BM-MSCs have properties of multipotency, paracrine effects, and immune-modulation (Arthur *et al.* 2009). BM-MSCs can operate cell renewal and migrate to the damaged tissues to regenerate the injury *in vivo*, when trauma happens. BM-MSCs also can proliferate and differentiate to a variety of cell lineages *in vitro* (Mohammadian *et al.* 2013). It was demonstrated that human BM-MSCs were homogeneous and differentiated with high fidelity to osteogenic, adipogenic, neurogenic or chondrogenic lineages (Zheng *et al.* 2013). BM-MSCs play a key role in cell-based regenerative therapy.

In this study, the BM-MSCs from WZSP were used to make out the role Fascin gene in migratory potential of BM-MSCs. WZSP is considered useful for medical and veterinary research due to its small size, with physiological and general biochemical indices similar to that of human being. It was reported that there was a limited number of active porcine endogenous retroviruses (PERVs) and a natural lack of PERV-C in the WZSP genome, which indicated that the risk of pig-to-human infection was very low during xenotransplantation, and these pigs may be used as potential organ donors for human (Fang *et al.* 2012). Therefore our results may be useful for the BM-MSCs from WZSP to be utilized in regenerative therapy.

In conclusion, Although role of Fascin gene is well established in cellular migration, it is unclear for its activity in porcine BM-MSCs. Our results showed that the specific shRNA for knocking down Fascin efficiently was found for the BM-MSCs from WZSP, and Fascin was involved in regulating the migration capacity of the BM-MSCs, which may be useful for the BM-MSCs from WZSP to be utilized in regenerative therapy for human.

ACKNOWLEDGMENTS

This work was supported by National Natural Science Foundation of China (31272404), National High Technology Research and Development program of China (2012AA020603).

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