Research Article **Cell Biology International**
10.1002/cbin.10757

SFRP2 enhanced the adipogenic and neuronal differentiation potentials of stem cells from apical papilla†

Xiao Lin[1, 2], Rui Dong[1],ShuDiao[1, 3],Guoxia Yu[1, 4, 5], Liping Wang[1], Jun Li[2, *], Zhipeng Fan[1, *]

[1]Laboratory of Molecular Signaling and Stem Cells Therapyand [2]Department of Implant Dentistry, [3]Department of Pediatric dentistry and [4]Molecular Laboratory for Gene Therapy and Tooth Regeneration, Beijing Key Laboratory of Tooth Regeneration and Function Reconstruction, Capital Medical University School of Stomatology, Beijing, China; And [5]Department of Stomatology, Beijing Children's Hospital,Capital Medical University, Beijing,China

Running title: SFRP2 regulated MSC differentiation

correspondence: Dr. Zhipeng Fan, Laboratory of Molecular Signaling and Stem Cells Therapy, Beijing Key Laboratory of Tooth Regeneration and Function Reconstruction, Capital Medical University School of Stomatology, Tian Tan Xi Li No.4, Beijing 100050, P.R. China.Tel: +86 10 6706 2012; Fax: +86 10 6706 2012; E-mail: zpfan@ccmu.edu.cn; Dr. Jun Li, Department of Implant Dentistry, Capital Medical University School of Stomatology, Tian Tan Xi Li No.4, Beijing 100050, P.R. China. Tel: +86 10 5709 9171; Fax: +86 10 5709 9171; E-mail: lijun3021@aliyun.com

†This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/cbin.10757]

Received: 6 August 2016; Revised: 27 January 2017; Accepted: 24 February 2017

Abbreviations:

BMMSCs, bone marrow mesenchymal stem cells; CEBPA/B, transcription factors CCAAT/enhancer binding protein alpha/beta; COL2/5, collagen type II/V; DFSCs, dental follicle stem cells; DPSCs, dental pulp stem cells; EGE, epidermal growth factor; ESCs, embryonic stem cells; FGF, fibroblast growth factor; iPSCs, induced pluripotent stem cells; Klf 4, kruppel like factor; LPL, lipoprotein lipase; MSCs, mesenchymal stem cells; NCAM, neural cell adhesion molecule; NEF/S100B, S100 calcium binding protein B; OCT4/POU5F1, POU class 5 homeobox 1; PDLSCs, periodontal ligament stem cells; SCAPs, stem cells from apical papilla; SFRPs, the secreted Frizzled-related proteins; SHEDs, stem cells from human exfoliated deciduous teeth; shRNAs, short-hairpin RNAs; SOX2/9, SRY-box 2/9; TH, tyrosine hydroxylase

Abstract: Dental tissue-derived mesenchymal stem cells (MSCs) are easily obtained and considered as a favorable cell source for tissue engineering, but the regulation of direct differentiation is unknown which restricts their application. The present study investigated the effect of SFRP2, a WNT signaling modulator, on MSC differentiation

using stem cells from apical papilla (SCAPs). The cells were cultured in specific inducing medium for adipogenic, neurogenic, or chondrogenic differentiation. Over-expression of *SFRP2* via lentiviral infection enhanced the adipogenic and neurogenic differentiation of SCAPs. While inhibit of WNT pathway by IWR1-endo could enhance the neurogenic differentiation potentials of SCAPs, similar with the function of SFRP2. In addition, over-expression of SFRP2 up-regulated the expression of stemness-related genes *SOX2* and *OCT4*. Furthermore, *SOX2* and *OCT4* expression was significantly inhibited after lentiviral silencing of *SFRP2* in SCAPs. Therefore, our results suggest that SFRP2 enhances the adipogenic and neurogenic differentiation potentials of SCAPs by up-regulating *SOX2* and *OCT4*. Moreover, the effect of SFRP2 in neurogenic differentiation of SCAPs maybe also associated with WNT inhibition. Our results provided useful information about the molecular mechanism underlying directed differentiation in dental tissue-derived MSCs.

Keywords: adipogenic differentiation, neurogenic differentiation, SFRP2, stem cells from apical papilla (SCAPs), stemness

1. Introduction

Mesenchymal stem cells (MSCs) were first identified from bone marrow tissues. MSCs can differentiate into multiple cell-restricted lineages, including osteoblasts, odontoblasts, adipocytes, neurocytes, and chondrocytes. Recent research confirmed that MSCs are found in a variety of dental tissues, as well as craniofacial tissues, and can be divided into several cell types based on the tissue of origin, including but not limited to dental pulp stem cells (DPSCs)(Gronthos et al. , 2000), stem cells from human exfoliated deciduous teeth (SHEDs)(Miura et al. , 2003), periodontal ligament stem cells (PDLSCs)(Seo et al. , 2004), dental follicle stem cells (DFSCs)(Morsczeck et al. , 2005), and stem cells from apical papilla (SCAPs)(Sonoyama et al. , 2008). All of these dental tissue-derived MSCs exhibit higher proliferation potential than bone marrow mesenchymal stem cells (BMMSCs), which is considered an indicator of MSC (Huang et al. , 2010). In addition, dental tissue-derived MSCs possess immunomodulatory properties, though the molecular mechanisms are still unclear (Liu et al. , 2015). Due to their accessibility

and superior properties, studies of the special populations of MSCs have gradually increased and they are considered a favorable cell source for tissue engineering. However, the direct differentiation of MSCs is still unclear, which restricts the application of MSCs in tissue regeneration. Therefore, finding a way to ensure the direct differentiation of MSCs *in vivo* would be an important development.

The WNT signaling pathway has been implicated in the modulation of self-renew and cell fate in diverse stem cells (Anakwe et al. , 2003, Aubert et al. , 2002, Bi et al. , 2009, Cain and Manilay, 2013, Huang et al. , 2015, Lee et al. , 2016, Leiros et al. , 2017, Selvaraj et al. , 2016). The secreted Frizzled-related proteins (SFRPs) are considered to be the endogenous antagonists of the Frizzled receptors of Wnt proteins, which contain a homologous region to Frizzled but lack a transmembrane domain to transmit signals (Lee et al. , 2000). One member of the SFRP family, SFRP2, participates in various cellular activities, including proliferation, apoptosis, migration, and cell differentiation (Boland et al. , 2004, Crowley et al. , 2016, Lin et al. , 2016, Majchrzak-Celinska et al. , 2016, Schmeckpeper et al. , 2015, Skah et al. , 2015). Some investigations have reported that SFRP2 plays a role in the biphasic

regulation of Wnt signaling(Kele et al. , 2012, Lin, Angeli, 2016, Majchrzak-Celinska, Slocinska, 2016, Skah, Nadjar, 2015). Moreover, the role of SFRP2 in stem cell differentiationwas still debated based on the limited investigations. Some studies demonstrated that SFRP2 is up-regulated during MSC osteogenesis (Boland, Perkins, 2004, Yu et al. , 2016), whereas some studies reported that SFRP2 decreases the osteogenic and chondrogenic differentiation potentials of murine MSCs (Alfaro et al. , 2010, Zhang et al. , 2014). In Bone marrow stem cell (BMSC) precursor, the higher level of SFRP2 enhanced the adipogenic differentiation and the decrease of SFRP2 suppressed adipogenesis in the MSCs of mice (Cianferotti and Demay, 2007, Zhou et al. , 2016). Furthermore, SFRP2 prompts the dopamine neuron differentiation of embryonic stem cells (ESCs) through the activation of WNT signaling (Kele, Andersson, 2012).

Despite increasing knowledge of the effects of SFRP2 on diverse stem cells, the function of SFRP2 in regulating the differentiation of dental tissue-derived MSCs remains to be identified. Here, we studied the effects of SFRP2 on the adipogenic, neurogenic, and chondrogenic differentiation potentials of SCAPs.

2. Materials and methods

2.1 MSC isolation and culture

All research involving human stem cells complied with the 'Guidelines for the Conduct of Human Embryonic Stem Cell Research' of the International Society for Stem Cell Research. Human impacted third molars with immature roots were collected from healthy patients following the approved guidelines set by the Beijing Stomatological Hospital, Capital Medical University, with informed patient consent. Molars were removed, disinfected with 75% ethanol, and then washed with PBS. SCAPs were isolated, cultured, and identified as described previously (Sonoyama, Liu, 2008, Yu, Wang, 2016). Five human impacted third molars with immature roots (wisdom teeth) were collected from five healthy individuals (16–20 years old) (Yu, Wang, 2016). Briefly, the apical papilla was gently separated from the root and then digested in a solution of 3 mg/mL collagenase type I (Worthington Biochem, USA) and 4 mg/mL dispase (Roche, Germany) for 1 h at 37°C. Single-cell suspensions were obtained by passing the cells through a 70-μm strainer (Falcon, BD Labware, USA). MSCs were grown in a humidified 5% CO_2 incubator at 37°C in α-MEM (Gibco, USA) supplemented with 15% fetal

bovine serum (Gibco, USA), 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, USA). The culture medium was changed every 3 days. Characterization of these MSCs has been shown in our previous study (Sonoyama, Liu, 2008, Yu, Wang, 2016). MSCs at passages 3-5 were used in subsequent experiments.

2.2 Plasmid construction and viral infection

Plasmids were constructed following standard methods and verified by appropriate restriction digestion and/or sequencing. Human full-length *SFRP2* cDNA was produced using a standard gene synthesis method and subcloned into the LV5 lentiviral vector (GenePharma Company, Suzhou, China). Short-hairpin RNAs (shRNAs) with the complementary sequences to*SFRP2* were subcloned into the pLKO.1lentiviral vector (Addgene, Cambridge, MA, USA). For viral infection, MSCs were plated over night and infected with lentivirus in the presence of polybrene (6 μg/mL, Sigma-Aldrich, St. Louis, MO, USA) for 12 h. After 48 h, infected cells were selected with 2μg/mL puromycin for 7 days. A Scramble shRNA control (Scramsh) was purchased from Addgene (USA). The target sequence for the shRNAs was 5’-ttgatgtaggttatctccttc-3’ (SFRP2sh).

2.3 Adipogenic differentiation and Oil Red Ostaining

Adipogenic differentiation was induced using the StemProadipogenesis differentiation kit (Invitrogen, USA). MSCs were grown in adipose-inducing medium for 3 weeks. To confirm the effect of Wnt pathway in MSCs adipogenic differentiation, Wnt pathway antagonist, IWR1-endo (10uM, MedChemExpress, New Jersey, USA) and Wnt pathway agonist, AZD2858 (1uM, Selleck, Texas, USA) were used as the positive and negative control. For Oil Red O staining, cells were fixed with 10% formalin for at least 1 h at room temperature. Next, cells were stained with the 60% Oil Red O in isopropanol for 10 min. The proportion of Oil Red O-positive cells was determined by counting stained cells under a light microscope. The Oil Red O dye was eluted with 100% isopropanol for 10 min and the OD measured at 500 nm using 100% isopropanol as the blank. The final OD value in each group was normalized to the total protein concentrations determined on a duplicate plate.

2.4 Neurogenic differentiation and Immunofluorescence Staining

Neurogenic differentiation was induced by Neurobasal A (Gibco, USA) supplemented with B27 (Gibco, USA), 40ng/mLFGF, 20 ng/ml EGF, 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco,

USA). Cells were resuspended in the neuron-inducing medium and then transferred to an ultra-low attachment culture dish (Corning, USA) at a density of 10^6cells/mL and cultured for 9 days. The culture medium was changed every 3 days. To confirm the effect of Wnt pathway in MSCs neurogenic differentiation, 10uM IWR1-endo (MedChemExrpess) and 1uM AZD2858 (Selleck) were used as the positive and negative control. After 9 days, we observed the changes in cell morphology under a microscope. For immunofluorescence staining, the collected neuron-like cells were fixed with 4% paraformaldehyde for 10min at room temperature. Next, the fixed cells were suspended in 1%Triton X-100 and blocked with 5% BSA for 30 min. And then the cells were incubated with primary antibodies associated with neural markers, including mouse monoclonal anti-Nestin antibody (1:500; Clone No.3k1, Abcam, Cambridge, US) and rabbit monoclonal anti-βIII-tubulinantibody (1:500; Clone No.EPR1568Y, Abcam) overnight. The secondary antibody (1:500 goat anti-mouse Alexa 488, and 1:500 goat anti-rabbit Alexa 488; Invitrogen, California, USA) was used followed by TBST washing. After nuclear staining using 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Invitrogen, USA), the cells were washed and mounted on poly-L-

lysine-coated slides, then examined by fluorescence microscopy. The images were captured and the cells with positive staining were counted.

2.5 Chondrogenic differentiation and Alcianblue staining

Chondrogenic differentiation was induced using the StemProchondrogenesis differentiation kit (Invitrogen, USA). MSCs were grown in the chondrogenic medium for 2 weeks. For Alcian blue staining, cells were rinsed once with PBS and fixed with 4% formaldehyde solution for 30 min. After fixation, the wells were rinsed with PBS and the cells stained for 30 min with 1% Alcian blue solution prepared in 0.1 N HCl. Next, the wells were rinsed three times with 0.1 N HCl and distilled water added to neutralize the acidity. Cells were visualized under a light microscope and images captured for analysis. Blue staining indicated the synthesis of proteoglycans by chondrocytes. To quantify proteoglycan synthesis, Alcian blue was extracted by treatment with 4 M guanidine-HCl overnight at 4°C. Absorbance values were read at 600 nm after equilibrating the temperature. The final OD value in each group was normalized to the total protein concentrations determined on a duplicate plate.

2.6 Real-time RT-PCR

Total RNA was isolated from MSCs using TRIzol reagent (Invitrogen, USA). For real-time RT-PCR, 2 μg aliquots of RNA were synthesized using random hexamers or oligo (dT) and reverse transcribed according to the manufacturer's protocol (Invitrogen, USA). Real-time PCR reactions were performed using the SYBR Green PCR kit (Qiagen, Germany) and the IcycleriQ Multi-color real-time PCR detection system. The primers were designed using the online D-LUX Designer™ program (Invitrogen, USA) and listed in Supplementary Table 1.

2.7 Statistical analysis

All statistical analyses were performed using SPSS17.0 software (SPSS Inc., Chicago, IL). Significance was determined by the Student's *t* test. $P \leq 0.05$ was considered significant.

3. Results

3.1 SFRP*2 Over-expression enhanced adipogenic differentiation potentials in SCAPs*

To study the function of SFRP2 during SCAP differentiation, we over-expressed *SFRP2*vialentivirus infection. Strong ectopic *SFRP2* expression

was confirmed by real-time RT-PCR (**Figure 1A**). The SCAPs infected with *SFRP2* or empty vector were cultured with adipose-inducing medium for 3 weeks and stained with Oil Red O solution, which allows visualization of lipid deposits. The Oil Red O staining revealed increased lipid deposits in cells over-expressing *SFRP2* compared to controls (**Figure 1B**). After normalization to total protein content, the quantitative analysis results implied that the adipogenic differentiation potential was stronger in the cells over-expressing *SFRP2* (**Figure 1C**). To further determine the relationship between SFRP2 and adipogenesis, we evaluated the expression of some adipogenesis-associated genes using real-time RT-PCR, including lipoprotein lipase (*LPL*) andtranscription factors CCAAT/enhancer binding protein alpha (*CEBPA*) and CCAAT/enhancer binding protein beta (*CEBPB*). The *LPL*mRNA level was increased more significantlyin SCAPs over-expressing *SFRP2*at 0, 1, 2, and 3 weeks after induction compared to the control group (**Figure 1D**), whereas the *CEBPA*mRNA level was increased more significantly in SCAPs over-expressing *SFRP2* at 3 weeks after induction, and *CEBPB* mRNA level was increased at 1 week and 3 weeks after induction compared to the control group (**Figure 1E**, **1F**). Taking together, the results indicated that

SFRP2 over-expression enhanced the adipogenic differentiation potential of SCAPs.

3.2 SFRP2 *over-expression enhanced the neurogenic differentiation potential in SCAPs*

To understand the effect of SFRP2 on neurogenic differentiation, we used modified neural differentiation medium to culture SCAPs. During culture, the morphology of the control cells, the cells over-expressing SFRP2 and the cells treated with IWR1-endo became shorter, fatter, and more ball-shaped, eventually changing into neuron-like cells. After 9 days inducing, more neuron-like cells formed amongSCAPs over-expressing *SFRP2*than among control SCAPs (**Figure 2A**). The immunofluroscence staining results showed that the SCAPs-derived spheres were Nestin-positive and βIII-tubulin positive (**Figure 2B-C**). That indicated the SCAPs-derived spheres were similar to neuron with regard to neural markers. Then, the positive stained cells were counted, showing that SFRP2 over-expressed group revealed more Nestin and βIII-tubulin positive cell clusters compared to control group (**Figure 2D**).

In addition, real-time RT-PCR showed that the neuron-associated genes neural cell adhesion molecule (*NCAM*; **Figure 2E**), *NeuroD* (**Figure 2F**),

S100 calcium binding protein B (S100B, also known as *NEF*; **Figure 2G**), and tyrosine hydroxylase (*TH1*; **Figure 2H**) increased 9 days after induction compared to the control group. These results indicated that the over-expression of *SFRP2* enhanced the neurogenic differentiation potential of SCAPs.

3.3 SFRP2 *over-expression did not distinctly affect the chondrogenic differentiation potential in SCAPs*

Alcian Blue Staining was used to investigate the chondrogenic differentiation of SCAPs in the presence or absence of *SFRP2* over-expression. After 2 weeks of culture in chondrogenic inducing medium, Alcian Blue staining revealed similar proteoglycan production in SCAPs over-expressing *SFRP2* and the control group (**Supplementary Figure 1A**). After normalizing the data to the total protein content, we found no significant difference (**Supplementary Figure 1B**). We also examined chondrogenic differentiation markers SRY-box 9 (*SOX9*), collagen type II (*COL2*), and collagen type V (*COL5*) by real-time RT-PCR and found no significant difference in the gene expression in SCAPs over-expressing *SFRP2* compared to the control group (**Supplementary Figure 1C-E**).

Thus, *SFRP2* over-expression did not distinctly affect the chondrogenic differentiation potential in SCAPs.

3.4 SFRP2 *expression positively regulated* SOX2 *and* OCT4 *mRNA expression in SCAPs*

OCT4, also known as POU class 5 homeobox 1 (POU5F1), is one of the transcription factors that play a vital role in embryonic development and stem cell fate determination, as well as SRY-box 2 (SOX2). We detected the expression of *SOX2* and *OCT4* mRNA to determine whether SFRP2 affects SCAP function in vitro via the regulation of these two factors. Real-time RT-PCR demonstrated that over-expression of *SFRP2* increased both *SOX2* (**Figure 3A**) and *OCT4* (**Figure 3B**) expression in SCAPs. To further evaluate whether SFRP2 regulates the expression of *SOX2* and *OCT4*, we suppressed endogenous SFRP2 in SCAPs via lentiviral infection. After selection, the knock-down efficiency (90%) was verified by real-time RT-PCR using Scramsh for comparison (**Figure 4A**). Depletion of SFRP2 abrogated the expression of *SOX2* (**Figure 4B**) and *OCT4* (**Figure 4C**). Thus, SFRP2 positively regulated the expression of *SOX2* and *OCT4* in SCAPs.

3.5 *The enhanced function of SFRP2 for neurogenic differentiation was similar with WNT pathway inhibitor, IWR1-endo in SCAPs*

We used IWR1-endo (antagonist of WNT pathway) and AZD2858 (agonist of WNT pathway) as the positive and negative control in adipogenic differentiation of SCAPs. Unfortunately, after adipogenic induction, the SCAPs treated with 1 uM AZD2858 were dead. The Oil Red O staining revealed increased lipid deposits in cells over-expressing *SFRP2* compared to controls and the cells treated with 10 uM IWR1-endo (**Figure 5A**). The quantitative analysis results showed that the adipogenic differentiation potential was stronger in the cells over-expressing *SFRP2* compared with control group and the cells treated with IWR1-endo. However, there was no statistical difference between control group and the cells treated with IWR1-endo (**Figure 5B**).

Then we used IWR1-endo and AZD2858 as the positive and negative control in neurogenic differentiation of SCAPs. While, after neurogenic induction, the SCAPs treated with 1 uM AZD2858 were also dead without sphere-like clusters generating. And during neurogenic inducing culture, the morphology of the control cells, the cells over-expressing SFRP2 and the cells treated with 10 uM IWR1-endo became shorter, fatter, and more ball-shaped, eventually changing into neuron-like cells.

But the cells treated with IWR1-endo changed rapidly into neuron-like spheres in 3 days, and then gradually went dead. So this IWR1-endo treated group was failed for quantitative analysis. After 9 days inducing, the immunofluroscence staining results showed the SCAPs-derived spheres in control cells, the cells over-expressing SFRP2 and the cells treated with 10 uM IWR1-endo were Nestin-positive and βIII-tubulinpositive (**Figure 5C-D**). These indicated the SCAPs-derived spheres were similar to neuron with regard to neural markers.

4. Discussion

Due to the special characteristics of stem cells, stem cell therapies, including but not limited to tissue regeneration and immune regulation, have come to the forefront of modern medical science. Nonetheless, several difficulties need to be resolved, such as the modulation of cell plasticity and proliferation, the prospective harmful side effects, and the elimination of transplanted cells (Somoza and Rubio, 2012). Numerous factors are being investigated in the regulation of cell plasticity and to enhance the success rate of tissue regeneration.

The WNT signaling pathway plays an important role in stem cell commitment, maintenance, and function, and SFRP2 protein is a modulator of WNT (Ehrlund et al. , 2013, Yuan et al. , 2016). SFRP2 is also known to be a secreted apoptosis-related protein that contributes to apoptotic resistance (Kele, Andersson, 2012). Promoting cell growth, proliferation, and viability could increase the tissue regeneration potential. In our present study, we investigated the effect of *SFRP2* expression on the adipogenic, neurogenic, and chondrogenic differentiation potentials using dental tissue-derived MSCs. Some SCAPs eventually develop into dental pulp, which consists of blood vessels and nerves. Due to their origin, SCAPs express neurogenic markers without neurogenic stimulation. In addition, they express higher levels of neurogenic markers than DPSCs and PDLSCs after neurogenic induction (Huang et al. , 2009, Lee et al. , 2014). Thus, SCAPs could be a favorable cell source for neuron regeneration. We demonstrated enhanced neurogenic differentiation potential in SCAPs after *SFRP2*over-expression, indicating that SFRP2 maybe an accelerative factor for SCAP differentiation into neurons. Similarly, SFRP2 has been reported to promote the dopamine neuron development of ESCs*in vivo* (Kele, Andersson, 2012). Therefore, SFRP2 could be a candidate-secreted

factor for enhanced neurogenic tissue regeneration. Since SFRP2 is a well-known WNT pathway modulator, we used antagonist, IWR1-endo and agonist, AZD2858 of WNT pathway as the positive and negative control in adipogenic and neurogenic differentiation procession to investigate the relationship between SFRP2 and WNT pathway. Unfortunately, after treated with AZD2858, the SCAPs were died after whatever adipogenic or neurogenic induction. While inhibit of WNT pathway by IWR1-endo could enhance the neurogenic differentiation potentials of SCAPs, similar with the function of SFRP2. However, IWR1-endo didn't affect the adipogenic differentiation potential of SCAPs. These results indicated that SFRP2 enhanced neurogenic differentiation might via WNT inhibition in SCAPs.

Our results also implied that SFRP2 promoted the adipogenic differentiation of SCAPs. Although over-expression of *SFRP2* did not affect the chondrogenic differentiation of SCAPs, we verified that SFRP2 enhanced the osteo/dentinogenic differentiation potentials of SCAPs (data not shown). Taken together, these findings indicated that SCAPs over-expressing *SFRP2* have more powerful multi-differentiation potential and may possess greater "stemness". Therefore, we evaluated

the expression of stemness-related genes *SOX2* and *OCT4*. Over-expression or knockdown of *SFRP2* resulted in positive regulation of *SOX2* and *OCT4* expression. These findings suggested that SFRP2 enhanced MSCs with stemness features that may be more effective in tissue regeneration.

ESCs are limited to use incell-based therapies for humans mainly because of the ethics problem. Induced pluripotent stem cells (iPSCs) are considered to be the favorable alternative to ESCs. iPSCs are generated by introducing four factors into somatic cells to reprogram them: c-Myc, Klf4, Sox2, and Oct4, or Lin28, Nanog, Sox2, and Oct4 (Yan et al. , 2010). We could probably substitute *SFRP2* for *SOX2* and *OCT4*. Some studies have found that a few cells, such as neural stem cells derived from mice, are easily reprogrammed with fewer than four factors (Kim et al. , 2009, Shi et al. , 2008). Thus, maybe iPSCs can be induced by only over-expressing *SFRP2*.

The other problem during the induction of iPSCs is vector retention. Much time and effort has been spentin the attempt to remove vectors for long-term safety (Zou et al. , 2012). However, if we approach it

another way and abandon the vector system, this will not be a problem. SFRP2 protein has been commercialized andit isconvenient to use exogenous SFRP2 protein in cell culture. However, further studies are needed to determine whether such an approach is feasible.

5. Conclusion

In summary, the present study demonstrated that over-expression of SFRP2 enhanced the adipogenic and neurogenic differentiation potentials in SCAPs. The function of SFRP2 in SCAP differentiation maybe exerted by positive regulation of the expression of stemness transcription factors SOX2 and OCT4. Moreover, the effect of SFRP2 in neurogenic differentiation of SCAPs maybe also associated with WNT inhibition.

6. Acknowledgements

None.

7. Funding

This work was supported by grants from the National Natural Science Foundation of China (81625005 to Z.P.F., 81271100 and 81570936 to R.D., 81371115 to J.L.)

Disclosure of conflict of interest

None.

References

Alfaro MP, Vincent A, Saraswati S, Thorne CA, Hong CC, Lee E, et al. sFRP2 suppression of bone morphogenic protein (BMP) and Wnt signaling mediates mesenchymal stem cell (MSC) self-renewal promoting engraftment and myocardial repair. J Biol Chem. 2010;285:35645-53.

Anakwe K, Robson L, Hadley J, Buxton P, Church V, Allen S, et al. Wnt signalling regulates myogenic differentiation in the developing avian wing. Development. 2003;130:3503-14.

Aubert J, Dunstan H, Chambers I, Smith A. Functional gene screening in embryonic stem cells implicates Wnt antagonism in neural differentiation. Nat Biotechnol. 2002;20:1240-5.

Bi Y, Huang J, He Y, Zhu GH, Su Y, He BC, et al. Wnt antagonist SFRP3 inhibits the differentiation of mouse hepatic progenitor cells. J Cell Biochem. 2009;108:295-303.

Boland GM, Perkins G, Hall DJ, Tuan RS. Wnt 3a promotes proliferation and suppresses osteogenic differentiation of adult human mesenchymal stem cells. J Cell Biochem. 2004;93:1210-30.

Cain CJ, Manilay JO. Hematopoietic stem cell fate decisions are regulated by Wnt antagonists: comparisons and current controversies. Exp Hematol. 2013;41:3-16.

Cianferotti L, Demay MB. VDR-mediated inhibition of DKK1 and SFRP2 suppresses adipogenic differentiation of murine bone marrow stromal cells. J Cell Biochem. 2007;101:80-8.

Crowley RK, O'Reilly MW, Bujalska IJ, Hassan-Smith ZK, Hazlehurst JM, Foucault DR, et al. SFRP2 Is Associated with Increased Adiposity and VEGF Expression. PloS One. 2016;11:e0163777.

Ehrlund A, Mejhert N, Lorente-Cebrian S, Astrom G, Dahlman I, Laurencikiene J, et al. Characterization of the Wnt inhibitors secreted frizzled-related proteins (SFRPs) in human adipose tissue. J Clin Endocrinol Metab. 2013;98:E503-8.

Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci U S A. 2000;97:13625-30.

Huang GT, Gronthos S, Shi S. Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. J Dent Res. 2009;88:792-806.

Huang GT, Yamaza T, Shea LD, Djouad F, Kuhn NZ, Tuan RS, et al. Stem/progenitor cell-mediated de novo regeneration of dental pulp with newly deposited continuous layer of dentin in an in vivo model. Tissue Eng Part A. 2010;16:605-15.

Huang TS, Li L, Moalim-Nour L, Jia D, Bai J, Yao Z, et al. A Regulatory Network Involving beta-Catenin, e-Cadherin, PI3k/Akt, and Slug Balances Self-Renewal and Differentiation of Human Pluripotent Stem Cells In Response to Wnt Signaling. Stem Cells. 2015;33:1419-33.

Kele J, Andersson ER, Villaescusa JC, Cajanek L, Parish CL, Bonilla S, et al. SFRP1 and SFRP2 dose-dependently regulate midbrain dopamine neuron development in vivo and in embryonic stem cells. Stem cells. 2012;30:865-75.

Kim JB, Sebastiano V, Wu G, Arauzo-Bravo MJ, Sasse P, Gentile L, et al. Oct4-induced pluripotency in adult neural stem cells. Cell. 2009;136:411-9.

Lee CS, Buttitta LA, May NR, Kispert A, Fan CM. SHH-N upregulates Sfrp2 to mediate its competitive interaction with WNT1 and WNT4 in the somitic mesoderm. Development. 2000;127:109-18.

Lee H, Haller C, Manneville C, Doll T, Fruh I, Keller C, et al. Identification of Small Molecules Which Induce Skeletal Muscle Differentiation in Embryonic Stem Cells via Activation of the Wnt and Inhibition of Smad2/3 and Sonic Hedgehog Pathways. Stem Cells. 2016;34:299-310.

Lee JH, Um S, Song IS, Kim HY, Seo BM. Neurogenic differentiation of human dental stem cells in vitro. J Korean Assoc Oral Maxillofac Surg. 2014;40:173-80.

Leiros GJ, Ceruti JM, Castellanos ML, Kusinsky AG, Balana ME. Androgens modify Wnt agonists/antagonists expression balance in dermal papilla cells preventing hair follicle stem cell differentiation in androgenetic alopecia. Mol Cell Endocrinol. 2017;439:26-34.

Lin H, Angeli M, Chung KJ, Ejimadu C, Rosa AR, Lee T. sFRP2 activates Wnt/beta-catenin signaling in cardiac fibroblasts: differential roles in cell growth, energy metabolism, and extracellular matrix remodeling. Am J Physiol Cell Physiol. 2016;311:C710-C9.

Liu J, Yu F, Sun Y, Jiang B, Zhang W, Yang J, et al. Concise reviews: Characteristics and potential applications of human dental tissue-derived mesenchymal stem cells. Stem Cells. 2015;33:627-38.

Majchrzak-Celinska A, Slocinska M, Barciszewska AM, Nowak S, Baer-Dubowska W. Wnt pathway antagonists, SFRP1, SFRP2, SOX17, and PPP2R2B, are methylated in gliomas and SFRP1 methylation predicts shorter survival. J Appl Genet. 2016;57:189-97.

Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, et al. SHED: stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci U S A. 2003;100:5807-12.

Morsczeck C, Gotz W, Schierholz J, Zeilhofer F, Kuhn U, Mohl C, et al. Isolation of precursor cells (PCs) from human dental follicle of wisdom teeth. Matrix Biol. 2005;24:155-65.

Schmeckpeper J, Verma A, Yin L, Beigi F, Zhang L, Payne A, et al. Inhibition of Wnt6 by Sfrp2 regulates adult cardiac progenitor cell differentiation by differential modulation of Wnt pathways. J Mol Cell Cardiol. 2015;85:215-25.

Selvaraj P, Xiao L, Lee C, Murthy SR, Cawley NX, Lane M, et al. Neurotrophic Factor-alpha1: A Key Wnt-beta-Catenin Dependent anti-Proliferation Factor and ERK-Sox9 Activated Inducer of Embryonic Neural Stem Cell Differentiation to Astrocytes in Neurodevelopment. Stem Cells. 2016.

Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J, et al. Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet. 2004;364:149-55.

Shi Y, Desponts C, Do JT, Hahm HS, Scholer HR, Ding S. Induction of pluripotent stem cells from mouse embryonic fibroblasts by Oct4 and Klf4 with small-molecule compounds. Cell Stem Cell. 2008;3:568-74.

Skah S, Nadjar J, Sirakov M, Plateroti M. The secreted Frizzled-Related Protein 2 modulates cell fate and the Wnt pathway in the murine intestinal epithelium. Exp Cell Res. 2015;330:56-65.

Somoza RA, Rubio FJ. Cell therapy using induced pluripotent stem cells or somatic stem cells: this is the question. Curr Stem Cell Res Ther. 2012;7:191-6.

Sonoyama W, Liu Y, Yamaza T, Tuan RS, Wang S, Shi S, et al. Characterization of the apical papilla and its residing stem cells from human immature permanent teeth: a pilot study. J Endod. 2008;34:166-71.

Yan X, Qin H, Qu C, Tuan RS, Shi S, Huang GT. iPS cells reprogrammed from human mesenchymal-like stem/progenitor cells of dental tissue origin. Stem Cells Dev. 2010;19:469-80.

Yu G, Wang J, Lin X, Diao S, Cao Y, Dong R, et al. Demethylation of SFRP2 by histone demethylase KDM2A regulated osteo-/dentinogenic differentiation of stem cells of the apical papilla. Cell Prolif. 2016;49:330-40.

Yuan Z, Li Q, Luo S, Liu Z, Luo D, Zhang B, et al. PPARgamma and Wnt Signaling in Adipogenic and Osteogenic Differentiation of Mesenchymal Stem Cells. Curr Stem Cell Res Ther. 2016;11:216-25.

Zhang WB, Zhong WJ, Wang L. A signal-amplification circuit between miR-218 and Wnt/β-catenin signal promotes human adipose tissue-derived stem cells osteogenic differentiation. Bone. 2014;58:59-66.

Zhou Y, Song T, Peng J, Zhou Z, Wei H, Zhou R, et al. SIRT1 suppresses adipogenesis by activating Wnt/beta-catenin signaling in vivo and in vitro. Oncotarget. 2016;7:77707-20.

Zou XY, Yang HY, Yu Z, Tan XB, Yan X, Huang GT. Establishment of transgene-free induced pluripotent stem cells reprogrammed from human stem cells of apical papilla for neural differentiation. Stem Cell Res Ther. 2012;3:43.

Figure Legends

Figure 1. *SFRP2* over-expression enhanced adipogenic differentiation potentials in SCAPs. A, Real-time RT-PCR results showed SCAPs over-expressed *SFRP2* with lentivirus infecting. B and C, Lipid deposit amounts were relatively increased in SCAPs over-expressed SFRP2. SCAPs were cultured with adipogenesis differentiation medium for three weeks. Cells were stained with Oil Red O solution. The lipid deposits were increased in SFRP2 over-expressed group compared with control group. Scale bar: 50um (B). After data was normalized with the total protein, the relative lipid deposits were stronger in SFRP2 over-expressed group than in

control group after adipogenic induction (C). D-F, Real-time RT-PCR results showed that the *LPL* (D), *CEBPA* (E), and *CEBPB* (F) expressions were higher in SFRP2 over-expressed group than control group after adipogenic induction. *GAPDH* was used as an internal control. Statistical significance was determined by Student's t test. All error bars represent SD (n = 3). **P*< 0.05. ***P*< 0.01.

Figure 2. *SFRP2* over-expression enhanced the neuronal differentiation potentials in SCAPs. SCAPs were cultured with modified neural differentiation medium for the indicated time periods. A, Neuron-like cells were observed by using microscope. Scale bar: 100um. B and C, The neuron-like cells in control group, SFRP2 over-expressed group were positive for Nestin (green, B) and βIII-tubulin (red, C) expression by using immunofluorescence staining. The nuclie were counterstained with DAPI (blue). Scale bar: 100um. D, The Nestin or βIII-tubulin positive staining cells were increased in SFRP2 over-expressed group than in control group. E-H, Real-time RT-PCR results showed that the expressions of *NCAM* (E), *NeuroD* (F), *NEF* (G) and *TH* (H) were increased in SFRP2 over-expressed group than control group at time points 9d after neuronal induction. *GAPDH* was used as an internal control. Statistical significance

was determined by Student's t test. All error bars represent SD (n = 3). *P< 0.05. **P< 0.01.

Figure 3. *SFRP2* over-expression increased the mRNA expressions of *SOX2* and *OCT4* in SCAPs. A, The *SOX2* mRNA was higher in SFRP2 over-expressed group than control group. B, *OCT4* expression was increased in SCAPs after over-expression of SFRP2. Gene expressions were determined by real-time RT-PCR. *GAPDH* was used as an internal control. Statistical significance was determined by Student's t test. All error bars represent SD (n = 3). *P< 0.05. **P< 0.01.

Figure 4. Depletion of SFRP2 decreased the mRNA expressions of *SOX2* and *OCT4* in SCAPs. A, The knock-down of SFRP2 in SCAPs. SCAPs were infected with lentiviruses expressing SFRP2 shRNA (SFRP2sh) or Scramsh. After selection with 2μg/mL puromycin for 7 days, *SFRP2* expression was determined by real-time RT-PCR. The results showed *SFRP2*was 70% knocked down by SFRP2sh compared with Scramsh in SCAPs. B, The *SOX2* mRNA was decreased in SFRP2 knock-down group than control group. C, The *OCT4* expression was decreased after depletion of SFRP2. Gene expressions were determined by real-time RT-PCR. *GAPDH* was

used as an internal control. Statistical significance was determined by Student T-test. All error bars represent SD (n = 3). **P< 0.01.

Figure 5. IWR1-endo enhanced neurogenic, but not adipogenic differentiation potentials in SCAPs. A and B, SCAPs were cultured with adipogenesis differentiation medium for three weeks. Cells were stained with Oil Red O solution. The lipid deposits were increased in SFRP2 over-expressed group compared with 10 uM IWR1-endo treated group and control group. Scale bar: 100um (A). After data normalized with the total protein, the relative lipid deposits were stronger in SFRP2 over-expressed group than that in control group after adipogenic induction. But there was no statistical difference between 10 uM IWR1-endo treated group and control group (B). C and D, SCAPs were cultured with modified neural differentiation medium for the indicated time periods. The neuron-like cells in control group, SFRP2 over-expressed group and 10uM IWR1-endo treated group were positive for Nestin (green, C) and βIII-tubulin (red, D) expression by using immunofluorescence staining. The nuclie were counterstained with DAPI (blue). Scale bar: 100um. Statistical significance was determined by Student's t test. All error bars represent SD (n = 3). *P< 0.05. **P< 0.01.

Supplementary Figure 1. *SFRP2* over-expression did not distinctly affect the chondrogenic differentiation potentials in SCAPs. A and B, Proteoglycan amounts were similar after over-expression of SFRP2. SCAPs were cultured with chondrogenic differentiation medium for two weeks. Cells were stained with Alcian Blue solution. The Proteoglycans were similar in SFRP2 over-expressed group with control group (A). After data was normalized with the total protein, the relative proteoglycans had no statistical difference between SFRP2 over-expressed group and control group after chondrogenic induction (B). C-E, Real-time RT-PCR results showed that the *SOX9* (C), *COL2* (D), and *COL5* (E) expressions showed no statistical difference in SFRP2 over-expressed group and control group after chondrogenic induction. *GAPDH* was used as an internal control. Statistical significance was determined by Student's t test. All error bars represent SD (n = 3). **P*< 0.05. ***P*< 0.01.

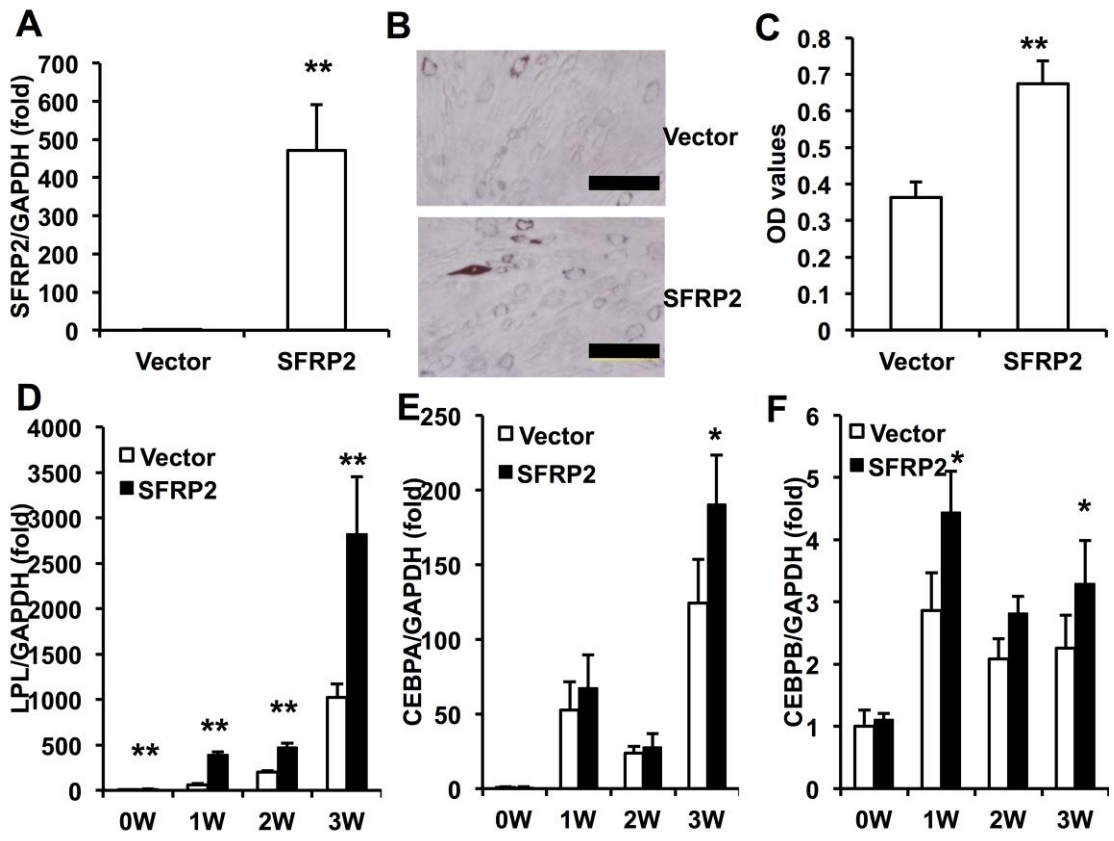

Figure 1

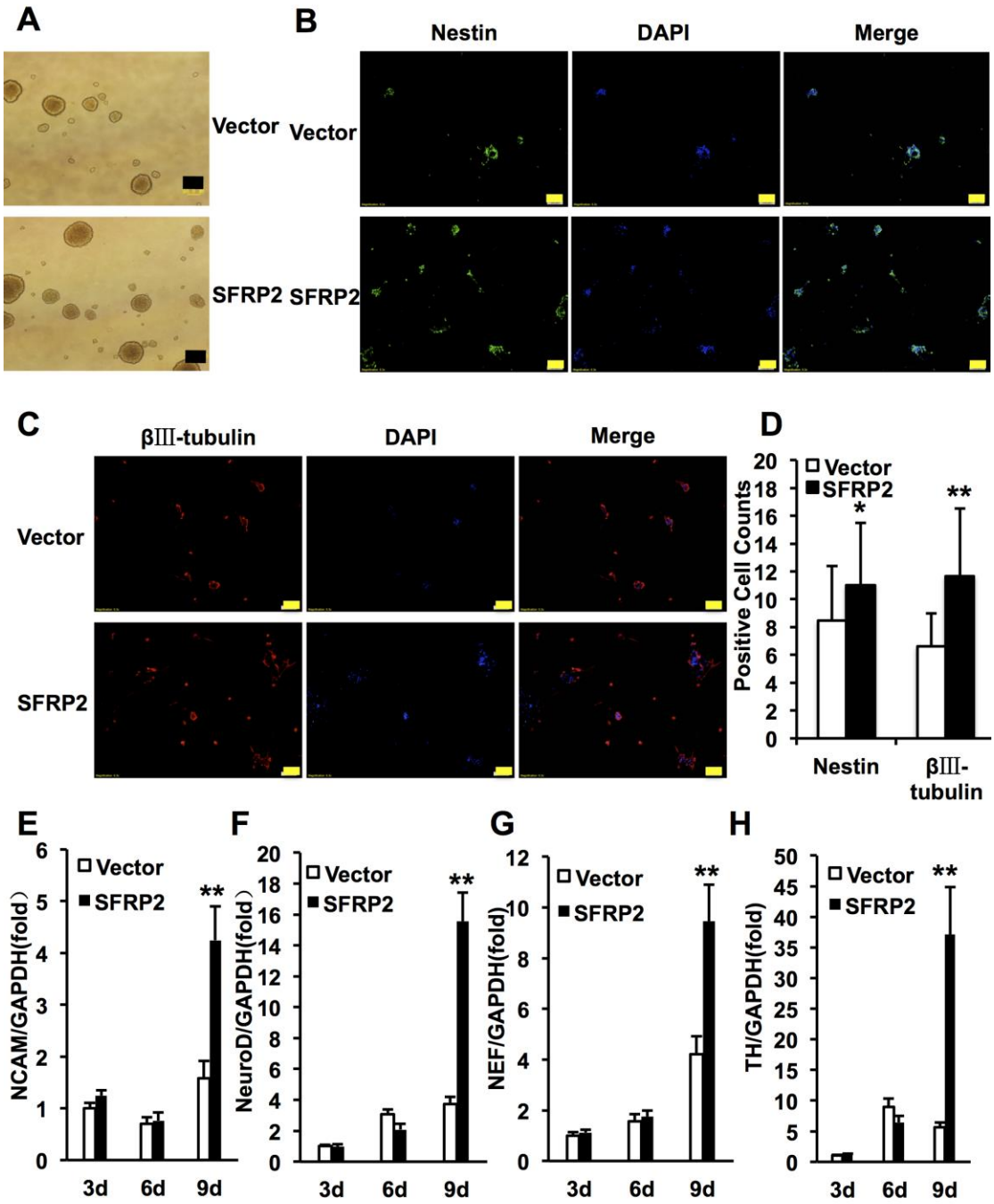

Figure 2

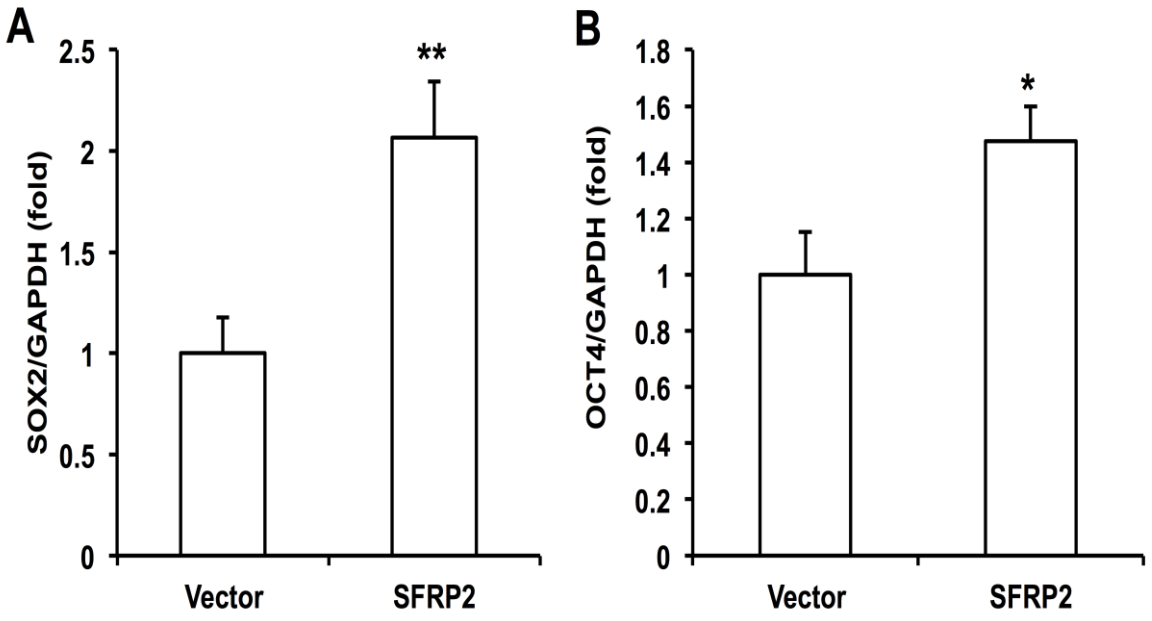

Figure 3

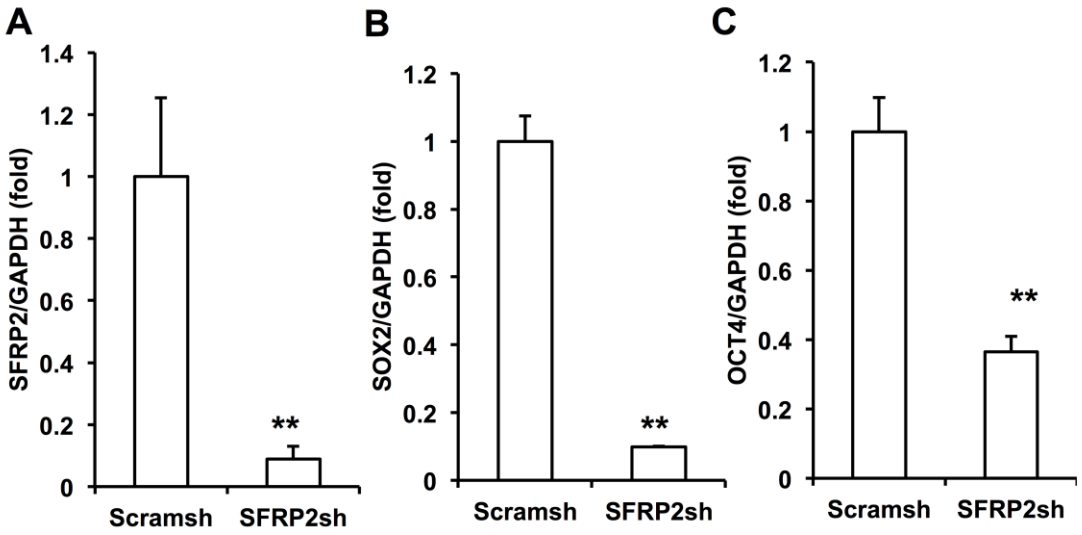

Figure 4

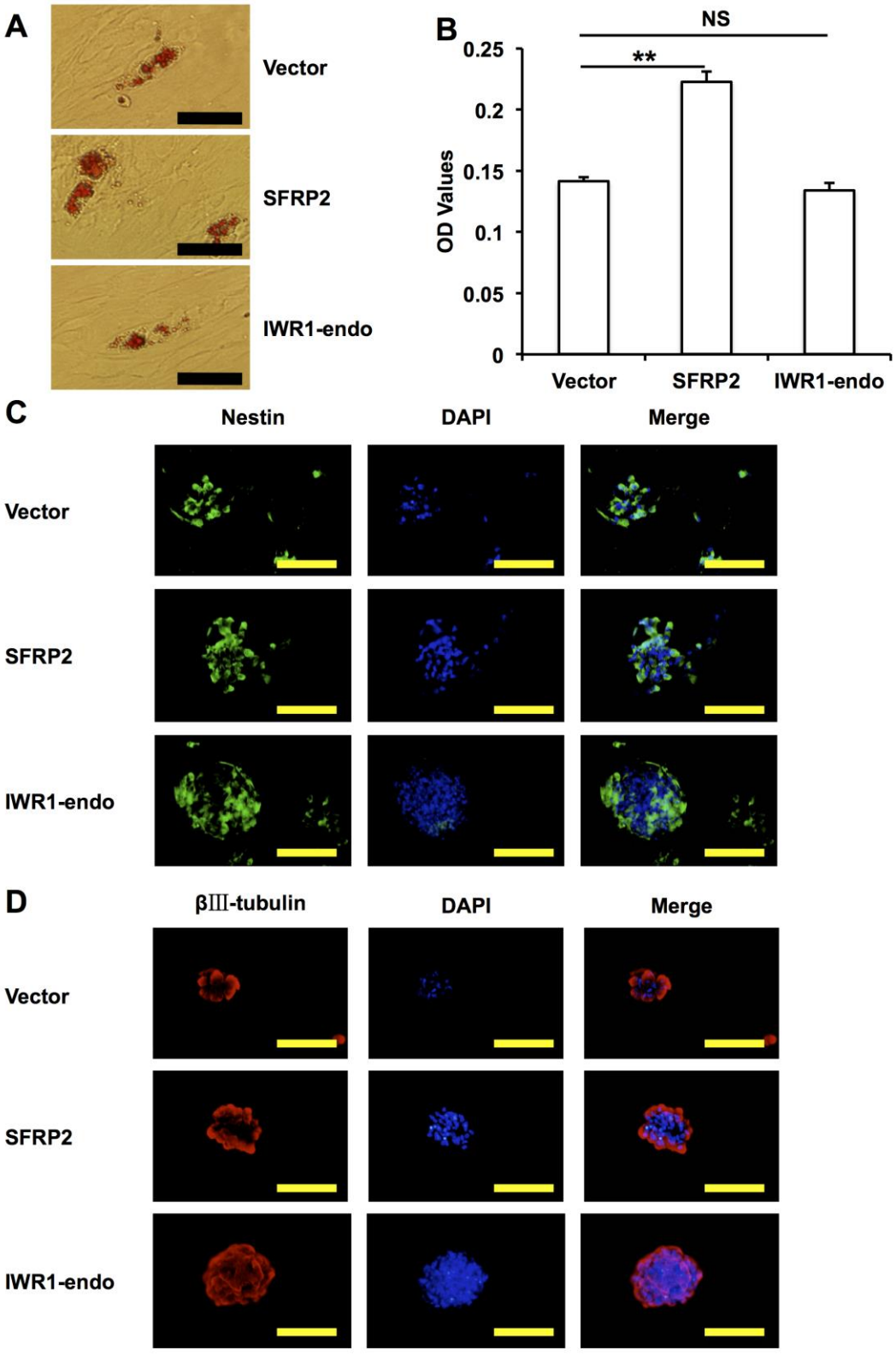

Figure 5