Am J Transl Res 2018;10(8):2619-2627
www.ajtr.org /ISSN:1943-8141/AJTR0072641

*Original Article*

# Identification and differentiation therapy strategy of pterygium in vitro

Xinyuan Hu*, Nitin Tandra*, Zhijian Zhang, Aihua Gong, Jingyan Chen, Yang Li, Qian Chen, Wenrong Xu, Hui Qian

*Zhenjiang Key Laboratory of High Technology Research on Exosomes Foundation and Transformation Application, Key Laboratory of Laboratory Medicine of Jiangsu Province, School of Medicine, Jiangsu University, Zhenjiang, People's Republic of China. *Equal contributors.*

Received January 12, 2018; Accepted July 5, 2018; Epub August 15, 2018; Published August 30, 2018

**Abstract:** Pterygium is an invasive hyperplasia, resulting from the hyper-proliferation of epithelial cells in cornea. The aim of our present study is to identify stem cells derived from pterygiums and detect differentiation strategy of the pterygium stem cells in vitro. Fundamental properties of the cultured pterygium stem cells were mainly studied using immunofluorescence staining. While clone formation and MTT assay were utilized to evaluate the cell viability. Pterygium stem cells could be cultivated easily, expanded efficiently, but expressed multilineage stem cell markers, which could be differentiated into neuron, osteocytes and adipocytes in vitro. Pterygium-derived spheres expressed stem cell markers and the epithelial-mesenchymal transition markers after treating with the inducing assays. Proliferation and viability of pterygium stem cells could be inhibited after inducing differentiation indicating that differentiation strategy will be a promising strategy in future therapies of pterygium. Cells derived from pterygiums express multilineage stem cell markers and could be induced differentiation. Differentiation therapy strategy could inhibit pterygium stem cell in vitro.

**Keywords:** Pterygium stem cell, sphere formation, neurogenic differentiation, differentiation therapy

## Introduction

Pterygium is the hyperplasia of epithelial cells alongside triangular-shaped growth of fibrovascular tissue from the bulbar conjunctiva onto the cornea [1]. It has been one of the most commonly occurring eye diseases, which could lead to astigmatism and vision loss, it is characterized by fibro-vascularization, conjunctiva invasion and degeneration of collagen [2]. Epidemiological studies around the world have shown that pterygium prevalence rates range from 0.3% to 37.46% [3, 4] and vary according to age, gender and genetic predisposition [5]. Surgery has been considered as the most appropriate treatment for pterygium [1, 6]. However the recurrence of pterygium is still a challenge for its treatment. Recently, Mitomycin-C [7, 8], subconjunctival membrane graft [9, 10] and 5-Fluorouracil [11, 12] have been employed to lower the recurrence of pterygium after the surgery.

Researches focus on pterygium increased, but the detail mechanism of pterygium is still unclear [13-15]. Previous studies have proposed that formation and recurrence of pterygium are related to the stem cells in situ. Conjunctival epithelium has proved is a stem cell-rich zone [16, 17]. NESTIN-positive cells were present at the superficial layer of the epithelium and NESTIN is also an important molecular marker of various other types of stem/progenitor cells [2, 18, 19]. Alteration of epithelial stem cells is the fundamental cause of pterygium formation and microenvironment in cornea is of importance for the tissue maintenance. Microenvironment aberration is associated with the epithelial stem cells fate in the pterygium [20].

The aim of our study was to identify stem cells distributed in the superficial layer both in the primary and the recurrent pterygiums and detect inducing differentiation strategy of the pterygium stem cells in vitro. These stem cells were strongly positive for the multiple stem cell markers and could be differentially induced into neuron, osteocytes and adipocytes. Meanwhile, inducing differentiation perhaps be a novel therapy strategy of the pterygium in vitro and

further research in the physiology of the pterygium is required for the development of new effective and curative strategy for it.

## Materials and methods

### *H&E and immunohistochemistry staining of pterygium tissues*

Primary and recurrence pterygium tissues were fixed in 10% formalin and embedded in paraffin before sectioning and staining. Tissue sections (3 μm) were dewaxed in xylene and rehydrated in an ethanol series. Hematoxylin and eosin (H&E) staining was performed according to standard protocol. For immunohistochemistry staining of stem cells located in pterygium tissues, the sections were incubated with primary antibodies anti-SOX2 (rabbit monoclonal: dilution 1:300: abcam), anti-NESTIN (rabbit polyclonal; dilution 1:100: boster), anti-VIMENTIN (mouse polyclonal; dilution 1:100: boster), and anti-CD44 (rabbit polyclonal; dilution 1:100: boster) at 4°C overnight, followed by exposure to 3% $H_2O_2$ and blocked by 5% BSA. Following a rinse in PBS, the sections were incubated with biotinylated polyclonal secondary antibodies. Washed and incubated with horseradish peroxidase-label streptavidin, then the sections were developed with diaminobenzidine and counterstain with hematoxylin. Representative images were acquired using a digital microscope (Nikon, Tokyo, Japan).

### *Isolation of pterygium stem cells*

Primary pterygium stem cells were cultured as monolayers [21]. Briefly, the pterygium tissues were kept in L-DMEM medium and transportation, then were minced into 1×1 $mm^3$ pieces and explanted onto the bottoms of plastic flasks and incubated with fresh medium (L-DMEM, 10% FBS 100 ng/ml penicillin and streptomycin) at 37°C, humidified atmosphere of 95% air and 5% $CO_2$. The cells were resuspended when the plastic flasks were 90% confluence, typically after 3 days depending on the initial pterygium population size and the cell proliferation rate. Adherent cells and suspended spheres derived from pterygium were collected for subsequent experiments.

### *Sphere formation of pterygium stem cells*

Pterygium stem cells were seeded into plastic flasks and cultured in serum-free L-DMEM medium supplemented with 2% B27, 20 ng/ml EGF and 20 ng/ml bFGF. The cells were cultured for 4 days and added EGF and bFGF daily. For secondary sphere culture, the primary spheres were dissociated into single cell and seeded into a new plastic flask. The primary and secondary spheres were identified by immunofluorescence staining.

### *Immunophenotype*

Adherent cells and suspended spheres derived from pterygium were fixed with 4% PFA solution at 4°C overnight. These cells and spheres were rinsed three times with cold PBS, and then incubated with 5% BSA (bovine serum album) solution and 0.3% triton solution at 37°C for half an hour. These cells and spheres were incubated with primary antibodies anti-SOX2, anti-NESTIN, anti-VIMENTIN, anti-CD44, anti-Ki67 (rabbit polyclonal; dilution 1:100: boster), anti-CD133 (rabbit polyclona; dilution 1:100: boster), anti-CD90 (rabbit polyclona; dilution 1:100: boster), anti-CD105 (rabbit polyclonal; dilution 1:100: boster), anti-YAP1 (rabbit polyclonal; dilution 1:100: boster), anti-P63 (rabbit polyclona; dilution 1:100: boster), anti-E-Cadherin (rabbit polyclonal; dilution 1:100: boster), anti-N-cadherin (rabbit polyclona; dilution 1: 100: boster), anti-stat3 (rabbit monoclonal; dilution 1:300: abcam), anti-snail (rabbit monoclonal; dilution 1:300: abcam), anti-SOX9 (mouse polyclonal; dilution 1:100: boster), anti-SOX10 (mouse polyclonal; dilution 1:100: boster), at 4°C overnight and incubated with cy3-labeled secondary antibody (dilution 1:300, Invitrogen) at 37°C for 60 mins. These cells and spheres were washed three times with PBS, subsequently the nuclei were counterstained with Hoechst 33342 (dilution 1:500, Sigma) at room temperature for 5 mins.

### *Differentiation capacity*

The pterygium stem cells were cultured in neurogenic-inducing medium (neurobasal medium supplemented with 10% FBS, 2% B27, 3.3 μM ARTA, 0.5 μM TSA, 20 ng/ml NGF and 2 mM L-glutamine) for 3 week, the medium was being replaced every 3 days. The induced neurons were fixed with 4% paraformaldehyde prior to incubation with antibodies against the neuron-specific markers anti-NF-200 (rabbit polyclonal; dilution 1:100: boster), anti-PERIPHERIN (Rabbit polyclonal dilution 1: 300: abcam) and anti-

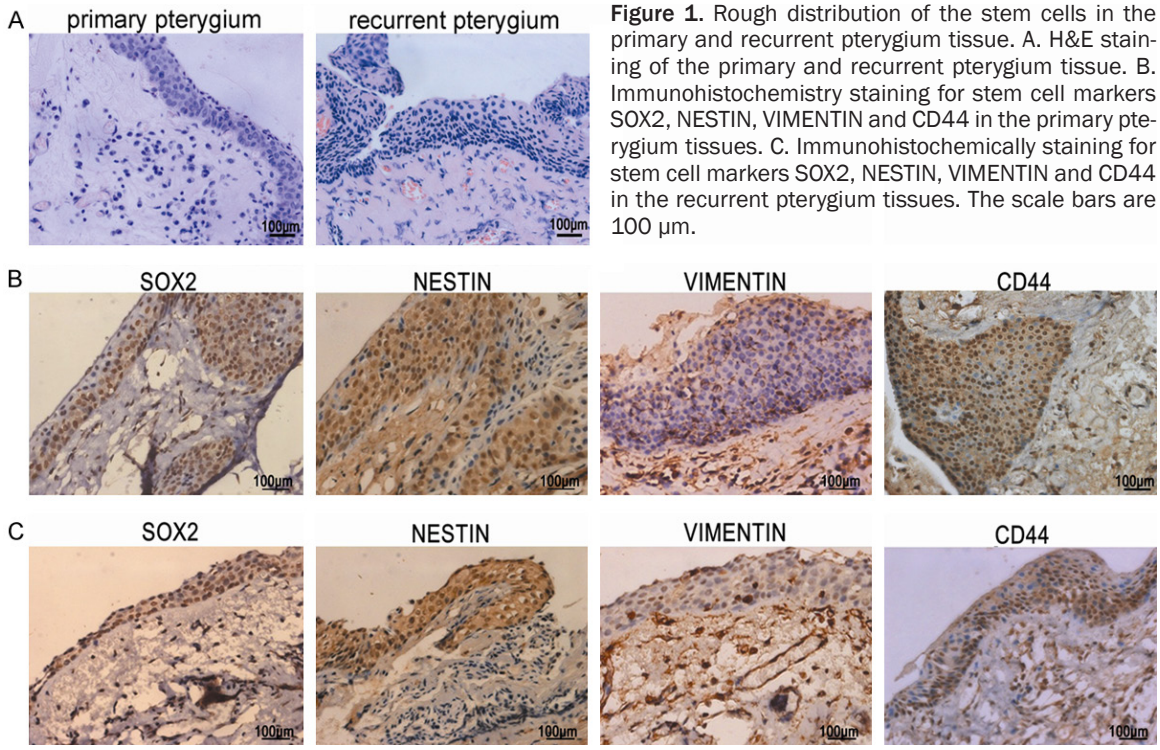


**Figure 1.** Rough distribution of the stem cells in the primary and recurrent pterygium tissue. A. H&E staining of the primary and recurrent pterygium tissue. B. Immunohistochemistry staining for stem cell markers SOX2, NESTIN, VIMENTIN and CD44 in the primary pterygium tissues. C. Immunohistochemically staining for stem cell markers SOX2, NESTIN, VIMENTIN and CD44 in the recurrent pterygium tissues. The scale bars are 100 μm.

SYNAPTOTAGMIN (mouse monoclonal: dilution 1:300: abcam). The pterygium stem cells were cultured in osteogenic-inducing medium (L-DMEM supplement with 10% FBS, 0.1 μM dexamethasone, 10 mM β-glycerophosphate disodium and 0.2 mM L-ascorbic acid -2-phosphate) for more than 2 weeks. The osteogenic activity of induced pterygium stem cells was tested by alkaline phosphatase activity with NAT staining. The ossification potential of pterygium stem cells was analyzed via detection of bone nodules on the surface of induced osteoblasts with Alizarin Red S staining. The pterygium stem cells were cultured in adipogenic inducing medium (L-DMEM supplement with 10% FBS, 2 μM insulin, 500 μM IBMX, 1 μM dexamethasone and 200 μM indomethacin) for 3 days. Then the cells were cultured in the maintained medium containing 2 μM insulin for 1 day. After three inducing cycles, induced pterygium stem cells were observed by phase contrast microscope and stained with Oil red O staining to reveal the lipid droplets in these cells.

*Inducing therapy strategy of pterygium stem cells in vitro*

Cell viability of pterygium stem cells were detected by MTT assay and clone formation assay. Pterygium stem cells ($5\times10^3$/well) were seeded into the 96-well plates in 100 μL of medium for 24 h, Subsequently, the differentiation assays were changed and the cells were cultured for 4 more days at 37°C. 20 μL (10 μL/mL) MTT (Sigma, St. Louis, MO, USA) was added to each well, and these cells were incubated for 4h at 37°C. The supernatant was removed and 100 μL DMSO was added. The proliferation was tested by measuring absorbance at 490 nm (OD value). Pterygium stem cells were seeded for colony assay in 6-well plates at 5000 cells per well. Neurogenic and osteogenic inducing media were replaced immediately after the cells attached to the bottom of the plates and the medium were replaced every 3 days. The colony were counted only when if it contained more than 50 cells. Every treatment was carried out triplicated.

*Statistical analysis*

All data were presented as means 6 standard deviation (SD). The statistically significant differences between groups were assessed by analysis of variance (ANOVA) or t-test with GraphPad Prism software (GraphPad, San Diego, U.S.). *P* value <0.05 was considered significant.

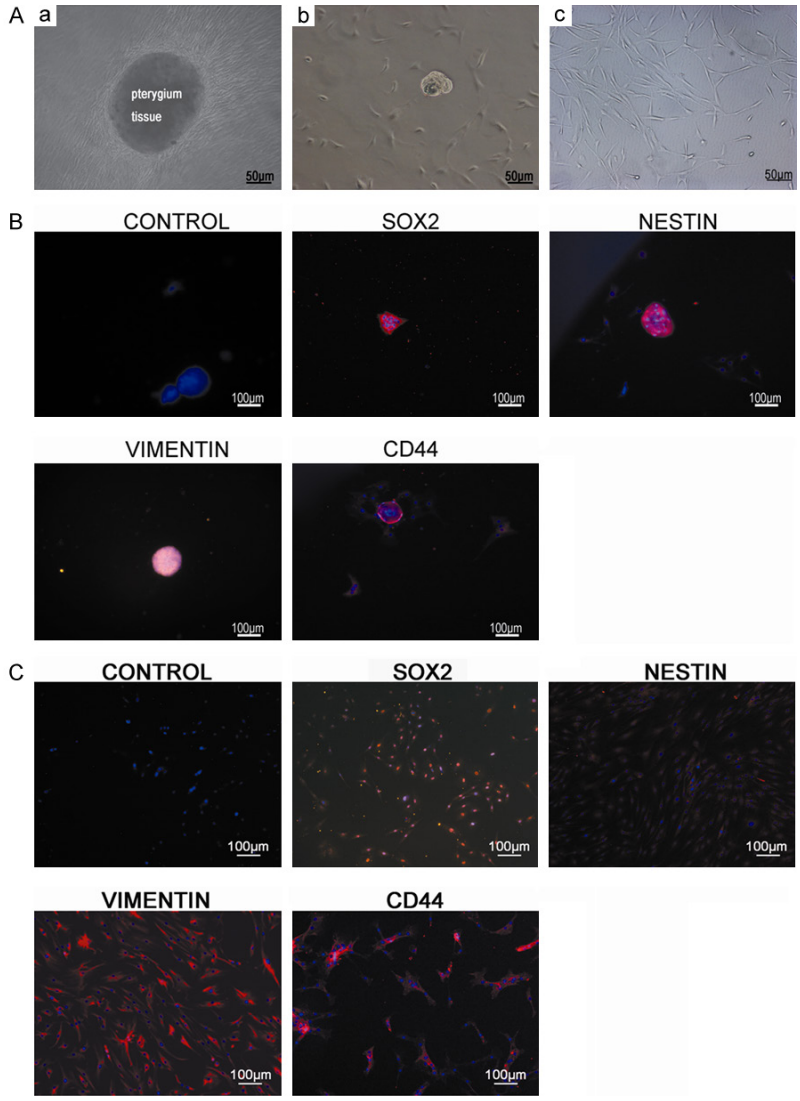


**Figure 2.** Pterygium stem cells express mutlineage stem cell markers in vitro. (A) The pterygium stem cells migrating out of the pterygium tissue (a); a microscopic image of the pterygium stem cells (b); a microscopic image of the spheres aroused spontaneously during the primary culture of pterygium (c). (B) Spontaneously formed spheres express mutlineage stem cell markers in vivo. (C) Cultured pterygium stem cells express mutlineage stem cell markers. IgG-Cy3 (red) was used as the secondary antibody. The nuclei were counterstained with Hoechst 33342 (blue). The scale bars are 50 μm (A) and the scale bars are 100 μm (B, C).

## Results

*Stem cells mainly located in the superficial layer of pterygium tissues*

Polarities and shapes of the cells in the superficial layer of pterygium were irregular. Abundant of blood capillaries, fibrocytes and inflammatory cells were presented in the loose connective tissue sub-epithelial. More cell layers and irregular arrangement had been seen in the superficial layer of recurrent pterygium (**Figure 1A**). After immunohistochemistry staining, the pterygium stem cell could be observed. As shown in **Figure 1** the stem cells express pluripotency stem cell marker SOX2, neural crest stem cell marker NESTIN and mesenchymal stem cell markers VIMENTIN and CD44, distributed in the superficial layer both in the primary and recurrent pterygium tissues. Respectively, the cells exhibiting SOX2, NESTIN and CD44 were mainly distributed in the superficial layer of both the primary and recurrent pterygiums. VIMENTIN is a protein that expressed in mesenchymal stem cells and mesenchymal cells, so it could be seen both in the epithelial and sub-epithelial of the primary and recurrent pterygiums (**Figure 1B**, **1C**). These results demonstrated that stem cells are mainly located in the superficial layer of the primary and recurrent pterygiums.

*Pterygium stem cells express multilineage stem cell markers*

When culture on polylysine-coated flask, the spindle pterygium stem cells could migrate from the pterygium tissues, and attach well and expand easily in adherent monolayer (**Figure 2Aa**). During the primary culture of the pterygium stem cells, a large amount of neuron sphere-like cell aggregates could be observed floating in the medium (**Figure 2Ab**). Immunofluorescence staining showed that these spheres express stem cell markers, including SOX2, NESTIN, VIMENTIN and CD44 (**Figure 2B**). Adherent pterygium cells could maintain stable spindle morphologi-

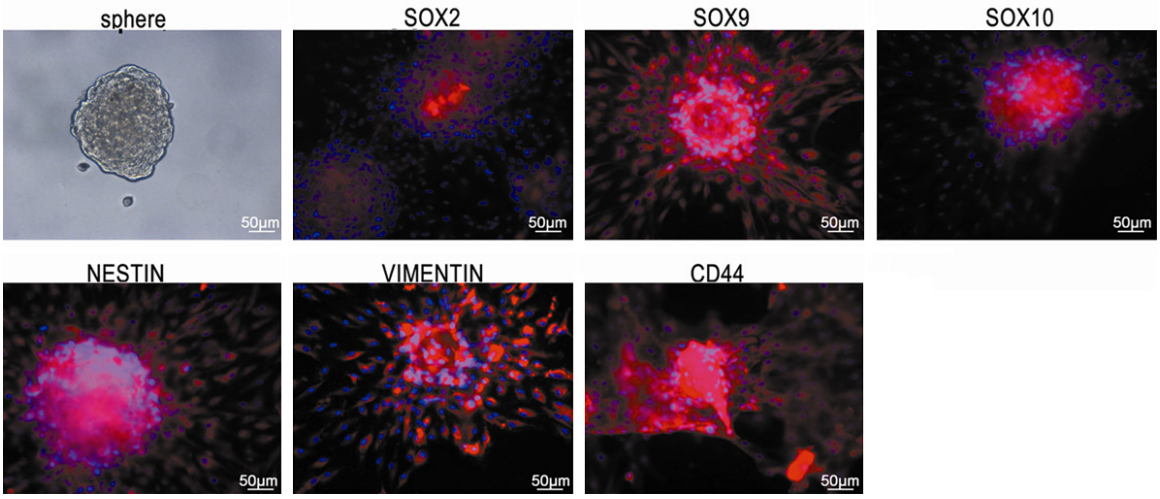


**Figure 3.** Pterygium stem cells induced spheres retain characteristic of stem cells. The spheres expressed multilineage stem cells markers including SOX2, SOX9, SOX10, NESTIN, VIMENTIN and CD44. IgG-Cy3 (red) was used as the secondary antibody. The nuclei were counterstained with Hoechst 33342 (blue). The scale bars are 50 μm.

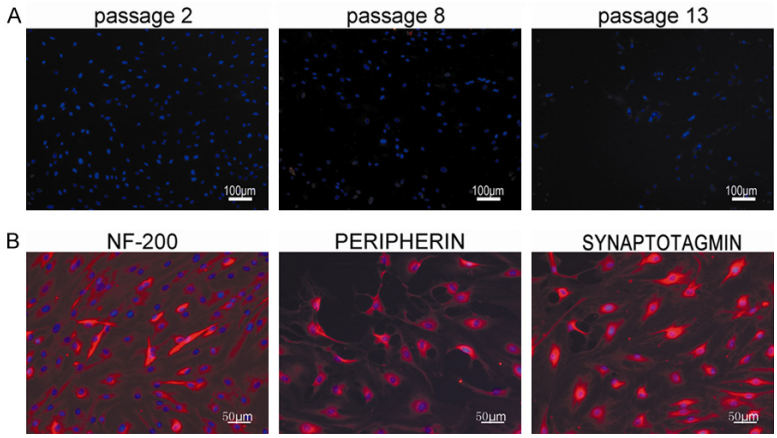


**Figure 4.** Neural differentiation of the pterygium stem cells. (A) Pterygium stem cells show no signs of neural differentiation after continuous treating with neurogenic differentiation assay. (B) Pterygium stem cells express strongly positive for neural markers NF-200, PERIPHERIN and SYNAPTOTAGMIN after treating with the neurogenic differentiation assay. IgG-Cy3 (red) was used as the secondary antibody. The nuclei were counterstained with Hoechst 33342 (blue). The scale bars are 100 μm (A) and the scale bars are 50 μm (B).

cal phenotype (**Figure 2Ac**) and were positive of multilineage stem cell markers, including SOX2, NESTIN, VIMENTIN and CD44 (**Figure 2C**). Meanwhile, cultured pterygium stem cells were strongly positive for the proliferation marker Ki67 and stem cell markers CD133 and CD90 (Supplementary Figure 1). Our findings supported that these stem cells derived from pterygium tissues have significant stem cell potential and were able to culture and expand quickly in vitro.

*Pterygium stem cells sphere formation*

Neural-sphere formation is a classic method to collect stem cells due to the low adhesion ability of stem cells. A significant amount of the spheres were found floating in the medium, during the different stages of subculture of the pterygium stem cells in the neural basal medium. Immunofluorescence staining showed that induced spheres express positive for pluripotency stem cell markers SOX2, SOX9, SOX10, YAP1, neural crest stem cell marker NESTIN, mesenchymal stem cell markers VIMENTIN, CD44 and CD105, epithelial stem cell marker p63 and proliferation marker Ki67 (**Figure 3**; Supplementary Figure 2A). Concurrently, induced spheres were also positive for two key molecular markers of epithelial-mesenchymal transition (EMT), E-Cadherin and N-Cadherin (Supplementary Figure 2B), Two transcription factors that may be involved in EMT, stat3 and snail, could be located in the nucleus (Supplementary Figure 2C), indicated that the pterygium stem cells were in the process of EMT and transformed into mesenchymal cell type.

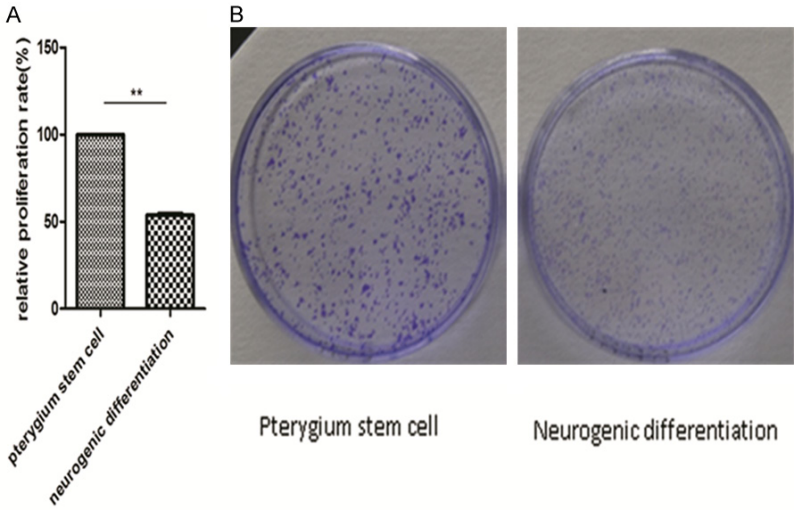


**Figure 5.** Neurogenic differentiation reduces viability of pterygium stem cells in vitro. Cell viability was detected by MTT and clone formation assay. The proliferation of the pterygium stem cells decreased after treating with the neurogenic differentiation assay (A) (n=3, **, P<0.05) and the colonies were smaller (B).

*Inducing pterygium stem cell differentiation into neuron, osteocytes and adipocytes*

Pterygium stem cells were consistent with negative expression of neuron marker NF-200 after continuous culture in vitro at passage 2, passage 8 and passage 13 (**Figure 4A**). When treated with the neurogenic differentiation assay, the pterygium stem cells were changed into neuron-like appearance and strongly positive for neuron markers NF-200, PERIPHERIN and SYNAPTOTAGMIN (**Figure 4B**). It suggested that the pterygium stem cells possess the differentiation potential and could be induced into neuron. To evaluate the osteogenic ability of the pterygium stem cells, the pterygium stem cells were cultured in osteogenic induction medium. The Alizarin Red S staining demonstrated that calcium deposition and mineralization of the differentiated pterygium stem cells increased gradually in 3 weeks of induction (Supplementary Figure 3A). To detect whether pterygium stem cells could differentiate into adipocytes, the pterygium stem cells were cultured in the adipogenic induction medium/maintenance medium. The results shown that lipid droplets were produced and accumulated in the adipogenic differentiated pterygium stem cells (Supplementary Figure 3B).

*Differentiation into neuron and osteocytes reduce the viability of pterygium stem cells*

Differentiation therapy is a novel strategy of curing disease resulted from stem cells, it aims to induce stem cells to differentiate and inhibit their proliferation and migration. After incubation in the neurogenic induction assay, MTT assay found that the cell viability of the pterygium stem cells decreased (**Figure 5A**). Further, the colony-forming unit assay found that the self-renewing capacity of the pterygium stem cells favorably decreased (**Figure 5B**).

As well, colony-forming assay showed that osteogenic differentiation assay could inhibit the self-renew capacity of pterygium stem cells in vitro, however, MTT assay showed no statistical difference between pterygium stem cells and osteogenic differentiation pterygium stem cells (Supplementary Figure 4).

## Discussion

Pterygium is one of the most commonly occurring eye diseases leading to astigmatism and vision loss induced by a variety of factors [1, 22]. Better understanding of the biological characteristics of pterygium is very important for improving the clinical therapy for curing it. In this study, we try to clarify the role of stem cells in the development and progression of primary and recurrent pterygiums.

The exact pathogenesis of pterygium has not been completely elucidated, even though there existed many researches. Najafi had reported that two common tumor suppressor genes, LATS1 and LATS2 were induced through methylation by the UV light exposure, resulting in the pterygium initial development [23]. Mutation of stem cells resident in the conjunctiva perhaps is one of dominating causes of the pterygium. Harun reported that stem cell behavior is dependent on the specific microenvironment on which they inhabit and aberration or alteration within signaling pathway between stem cells and micro-environmental networking, which provokes disease development [16]. These variations, mutation and alteration of the microenvironment, are all considered to play key roles in the formation and development of the pterygium.

Stem cells are a subtype of cells that possess the capability of self-renewal and multi-potential differentiation, offer diverse options for treatment of degenerative diseases [24, 25]. In our present study, we examine the presence and distribution of stem cells in the pterygium tissues. Pluripotency stem cell marker SOX2, neural stem cell marker NESTIN, mesenchymal stem cell markers VIMNENTIN and CD44 were selected to detect stem cells in pterygium tissues, VIMNENTIN is a type III intermediate filament (IF) protein that is expressed in mesenchymal cells and mesenchymal stem cells. Immunochemical staining results showed that stem cells positive of these markers and were mainly distributed in the head of primary alongside recurrence pterygium tissues. Stem cell markers including SOX2, NESTIN and CD44 were primarily found in the superficial layer of both the primary and recurrent pterygium. However the cell marker VIMENTIN was mainly positive of the mesenchymal stem cells and mesenchymal cells located in the superficial layer as well as sub-epithelial layer of both the primary and recurrent pterygium. Stem cells presented in the head of primary and recurrence pterygium may be associated with formation and recurrence of pterygium.

We obtained the pterygium stem cell using the method of primary culture of the pterygium tissues and found that the pterygium stem cells could migrate from the tissues. The pterygium stem cells were cultivated, expanded easily and able to keep stable phenotype, suggesting that it is an ideal cellular modal for exploring the characteristics of the pterygium in vitro. To evaluate the stemness of the cultured pterygium stem cells, these cells were fixed and incubated with the antibody against SOX2, NESTIN, VIMENTIN and CD44. Immunofluorescence staining show that the pluripotent stem cell marker SOX2 was mainly located in nuclei and the three other stem cell markers were mainly localized in cytoplasm or on the membrane.

Many neural sphere-like cell aggregates floated in the media during the primary culture of pterygium. Sphere culture technique is a convenient method to isolate the stem cells especially for the isolation of the neural stem cells and neural crest cells [26, 27]. These spheres were thought to be derived from stem cells in the pterygium tissues and the sub-cultured pterygium stem cells. Immunofluorescence staining showed that all these spontaneous formed spheres strongly positive for diverse stem cell markers, including the pluripotency stem cell marker SOX2, neural stem cell marker NESTIN, mesenchymal stem cell markers VIMNENTIN and CD44.

After inducing the sphere formation assay for 4 days, almost all the pterygium stem cells attached to the bottom of the flask formed sphere-like cell aggregates. These induced-formed spheres consists of more cells than the spontaneous formed spheres and positive expression of the SOX2, SOX9, SOX10, YAP1, neural crest stem cell marker NESTIN, mesenchymal stem cell markers VIMENTIN, CD44 and CD105, proliferation marker Ki67 and epithelial stem cell marker P63. Expression of these stem cell markers indicated that the pterygium stem cells we obtained from the pterygium possess stemness. Meanwhile, positive expression of E-Cadherin and N-Cadherin, combined with localization of stat3 and snail in the nucleus, indicated that the pterygium stem cells were in the process of EMT and transforming to mesenchymal phenotype. Expression and localization of YAP1 in the nucleus indicate that the proliferation of cells is not limited by hippo pathway [28, 29]. Positive expression of SOX2, SOX9, SOX10, NESTIN and formation of the spheres reflect that pterygium cells were subsets of neural stem cells or the neural crest cells [30, 31].

Multilineage differentiation is a major characteristic of stem cells. We detected the multi-differentiation ability of the pterygium stem cells and the efficacy of the inducing differentiation therapy strategy. These cells were differentially induced into multi-lineage cell types with various inducing assays. Results showed that there were no signs of neural differentiation after continuous culture of the pterygium stem cells in vitro. After treating with neurogenic induction assays, the pterygium stem cells expressed neuron and neurogliocyte markers, including NF-200, PERIPHERIN and SYNAPTOTAGMIN. After osteogenic induction, calcium depositions were observed on the surface of the differentiated pterygium stem cells. It was also observed that the calcium deposition increase in the process of inducing differentiated into osteocytes. For detecting the adipogenic differentiation ability of the pterygium stem cells, the cells were cultured in the in-

duction medium for 3 days and then cultured in the maintenance medium for 1 day. After 3 cycles, large amount of lipid droplets could be observed on the surface or in the induced pterygium stem cells and the Oil Red O staining showed that induced pterygium stem cells produced a lot of lipid droplets which occupied the space in the cytoplasm. These results strongly indicated that the pterygium stem cells possessed differentiation ability and can be induced into neuron, osteocytes and adipocytes.

Our results have shown that abundant stem cells existed in the head of the primary and recurrence pterygium. Growth and infiltration of pterygium tissues may be closely related to the presence of stem cells in situ and inhibition of the activity of these stem cells is an effective strategy for alleviation and treatment of pterygium. We demonstrated that the cell viability and self-renewing ability of pterygium stem cells were inhibited after incubated with differentiation assays in vitro and inducing differentiation therapy strategy will be a potential treatment strategy in the future.

Here we have investigated that the pterygium stem cells obtained from the pterygium tissues were stem cells and possessed multilineage differentiation ability. These findings supporting that pterygiums were a disease of stem cell origin. In addition, these results showed that the proliferation rate and the colony forming ability of the pterygium stem cells decreased after differentiation, suggesting that inducing differentiation therapy may be a novel strategy for treating the pterygium and reduce the risk of recurrence after the operation.

## Acknowledgements

The study was supported by the National Natural Science Foundation of China (Grant No 81571830, 81501077), the Innovation Project for Graduate Student Research of Jiangsu Province (Grant No KYLX16_0919).

## Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Hui Qian, Zhenjiang Key Laboratory of High Technology Research on Exosomes Foundation and Transformation Application, Key Laboratory of Laboratory Medicine of Jiangsu Province, Medical School of Jiangsu University, 301 Xuefu Road, Zhenjiang 212013, Jiangsu, People's Republic of China. Tel: 86-511-86102007; Fax: 86-511-86102010; E-mail: lstmmmlst@163.com

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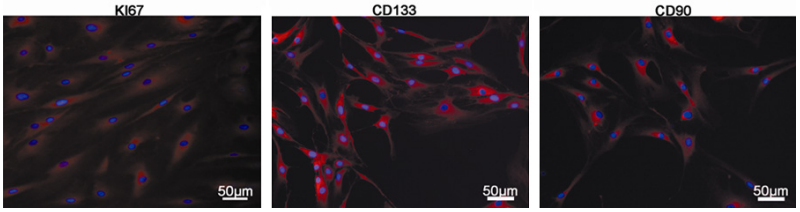
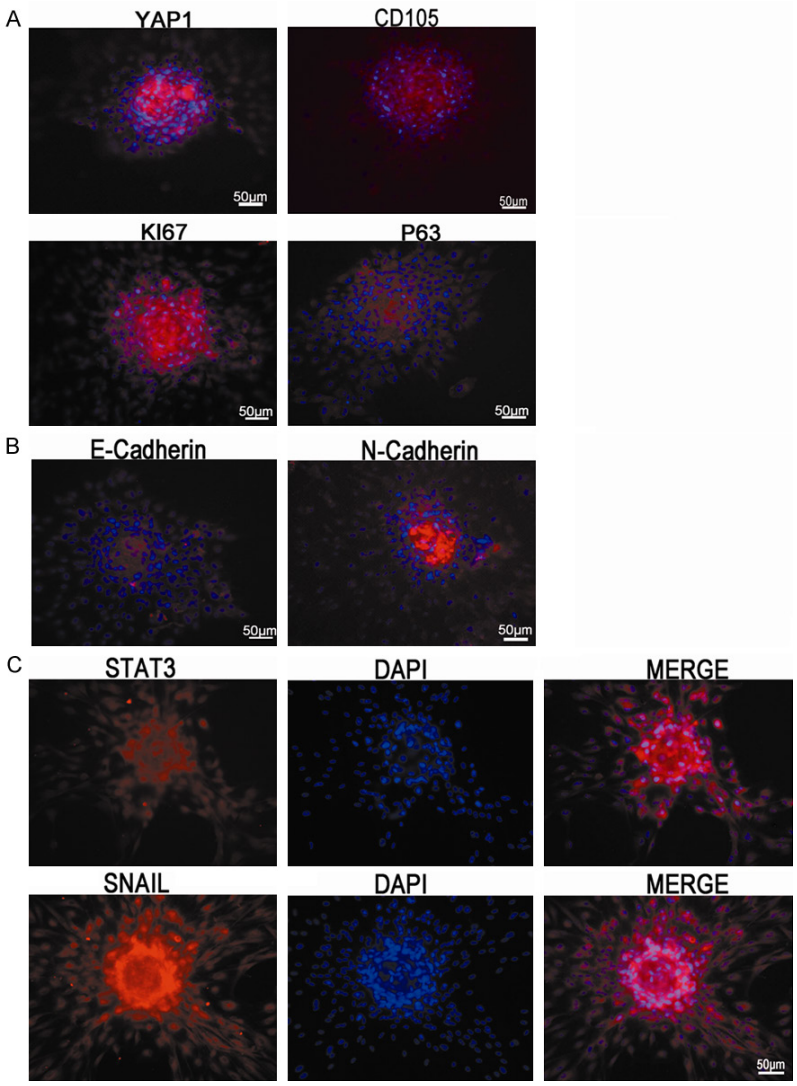
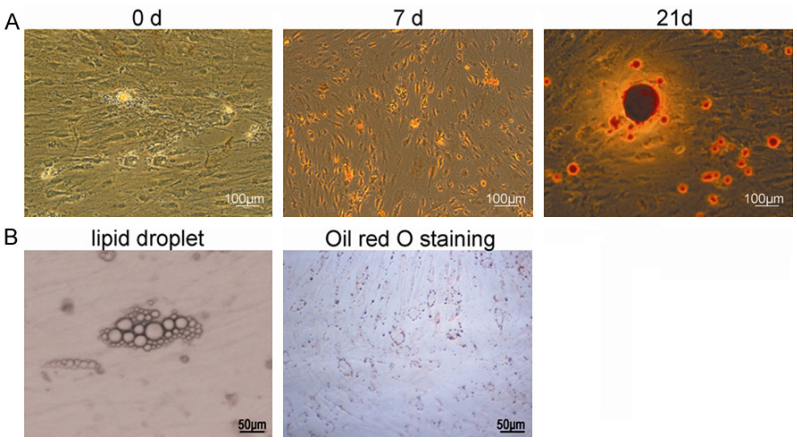


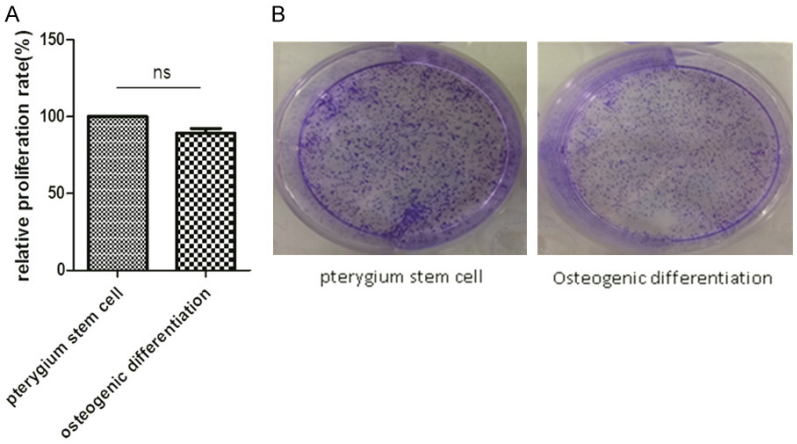

**Supplementary Figure 1.** Pterygium stem cells strongly positive for the proliferation and mesenchymal stem cells markers. Immunofluorescence staining showed that pterygium stem cells were strongly positive for the proliferation marker Ki67 and mesenchymal stem cells markers CD133 and CD90. IgG-Cy3 (red) was used as the secondary antibody. The nuclei were counterstained with Hoechst 33342 (blue). The scale bars are 50 μm.




**Supplementary Figure 2.** Characterization of the pterygium induced spheres. A. Immunofluorescence staining shows that pterygium-induced spheres expressed stem cells marker YAP1, mesenchymal stem cell marker CD105, proliferation marker Ki67 and epithelial stem cells marker P63. B. Pterygium-induced spheres were positive for both E-Cadherin and N-Cadherin, indicated that these cells were in the process of EMT. C. Transcription factors participated in the process of EMT, stat3 and snail, located in the Nuclei. IgG-Cy3 (red) was used as the secondary antibody. The nuclei were counterstained with Hoechst 33342 (blue). The scale bars are 50 μm.




**Supplementary Figure 3.** Osteogenic and adipogenic differentiation of pterygium stem cells. (A) After treating with the osteogenic differentiation assay, Alizarin Red S staining reflected that the number of the calcium deposition and the level of mineralization in pterygium stem cells increased. (B) Culturing in the adipogenic differentiation assay for 2 weeks, lipid droplets could be observed in induced pterygium stem cells. The scale bars are 100 μm (A) and the scale bars are 50 μm (B).




**Supplementary Figure 4.** Osteogenic differentiation strategy reduces viability of pterygium stem cells in vitro. The proliferation of the pterygium stem cells decreased after culturing in osteogenic differentiation medium.