Culture and Identification of Multipotent Stem Cells in Guinea Pig Sclera

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Research Article

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Abstract

Background: to investigate whether the sclera of guinea pig contains stem cells with multiple differentiation potentials.

Methods: Scleral tissue from guinea pig was separated from the retina and choroid and digested to release single cells. The cells cultured was identified as stem cells by flow cytometric analysis, semiquantitative RT-PCR. Abilities for multipotent differentiation were analyzed by histochemical staining technique (oil-red-O staining, alcian blue staining and alizarin red staining).

Results: The cultured scleral stem cells were positive for CD44 and CD105 (mesenchymal stem cell surface markers) by flow cytometry. The cells cultured expressed stem cell markers ABCG2, Notch1, Six2 and Pax6, and the most important component of sclera type I collagen. The positive staining informed that the cells cultured were able to differentiate to adipogenic, chondrogenic, and osteogenic lineages.

Conclusion: The guinea pig sclera contained stem cells with multiple differentiation potentials. The cells were also related to scleral collagen and cartilage related proteins. The finding may provide a new tool to help clarify mechanisms of sclera related disease in further studies.

Background

Stem cells are a special cell population able to self-renew, proliferate and differentiate multi-directionally. Under certain conditions, they can differentiate into one or more kinds of functional cells and this has been a hot topic in various research fields. At present, in the field of ophthalmology, stem cells are mainly used in the research of retinal degenerative diseases,[1] ocular surface diseases [2], and glaucoma. [3] However, few researches have reported that stem cells have been applied in myopia research.

Previous studies have found that sclera is constantly modified in the process of eyeball growth. Axial myopia is often accompanied by sclera thinning, especially in the posterior pole. Therefore, sclera, as the final target organ of intraocular regulation mechanism in the occurrence and development of myopia[4], plays a very important role in regulating the development of myopia.[5] Scleral extracellular matrix is derived from scleral fibroblasts [6], which mainly consists of a large amount of type I collagen.[7] Sclera is one of the ocular tissues closely related to the occurrence and development of myopia.[8] “Scleral active remodeling mechanism” was considered to be an important breakthrough in the etiology of experimental myopia. It is a very strict and fine regulatory process that depends on the biochemical and biomechanical changes of extracellular matrix. However, its regulatory mechanism still needs further study. Sclera cells may play an important role in the process of sclera remodeling. Some scholars have successfully isolated and cultured mouse scleral stem cells with multiple differentiation potential[9], which provided a new idea for myopia research. At present, guinea pig is widely accepted as a good model for myopia research because its sclera structure is similar to that of human sclera. However, it is still unknown whether there are stem cells contained in guinea pig sclera. The purpose of our study was

to culture guinea pig sclera stem cells and examine their multiple differentiation potentials, which can provide a new research model and research ideas for myopia research.

Materials And Methods

Animal

Four-week-old pigmented guinea pigs were obtained from the Shanghai Laboratory Animal Center (Shanghai, China). All animals were clinically examined to confirm the corneal transparency of each eye and lack of injuries or infections in the eyes. All animals in our study were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Primary Culture of multipotent Stem Cells in guinea pig sclera

Eyeballs were removed from each guinea pig under sterile conditions and rinsed three times with saline that contained gentamicin and phosphate-buffered saline (PBS) containing 10% penicillin and streptomycin. The sclera was carefully dissected away from the corneal limbus and optic disc under micro-scissors. Then the scleral tissue was cut into small pieces and digested with 1.5 mg/mL collagenase type I (Worthington, Lakewood, NJ) and 2 mg/mL Dispase (Roche, Indianapolis, IN) for one and half hours at 37°C to separate the individual cells. The cells were cultured in low glucose DMEM supplemented with 20% lot-selected FBS (Equitech-Bio, Kerrville, TX), glutamine, penicillin/streptomycin, and 50 mmol mercaptoethanol for 10 to 14 days at 37°C in 5% CO2. Cells of 80% confluence were passaged by 0.25% trypsin-EDTA (Gibco, USA).

Flow Cytometric Analysis of multipotent Stem Cells in guinea pig sclera

The cultured scleral stem cells were treated with 0.25% EDTA trypsin. The cells were resuspended with PBS buffer containing 1% BSA, and the cell concentration was adjusted to 1 × 106. The cells were incubated with 1 μl of CD105, CD44 and CD105 flow cytometry antibodies on ice for half an hour. The cells were then washed two to three times with PBS buffer, and finally resuspended with 500 μl PBS. The expression of cell surface markers was detected by flow cytometry (Dxflex, Backman).

Semiquantitative RT-PCR

Total RNA was isolated from the cultures (TRIzol Reagent; Takara), according to the manufacturer's protocols. The cDNA was synthesized from 1000 ng of total RNA according to the manufacturer's protocols. Primers were designed with Primer-BLAST software. The primers used are shown in Table 1. The amplified PCR products were put in IQ5 PCR instrument and were analyzed with SYBR Green method.

Adipogenic Differentiation

For adipogenic induction in vitro, the cells were cultured in an adipogenic differentiation medium kit (cyagen) with adipogenic medium for two weeks. The cells were stained with 0.3% oil-red-O (Sigma-Aldrich, St. Louis, MO) to detect lipid droplets.

Chondrogenic Differentiation

The digested cells were cultured into a cartilage culture medium to make cell suspension droplets on the inside of the upper cover of the culture dish. The resulting suspension was aggregated into the pelleted cells which were blown into chondrogenic medium in order to be harvested at 14 or 21 days. After embedding, the pelleted cells were frozen into sections and stained with alcian blue.

Osteogenic Differentiation

The digested cells were added to the complete osteogenic differentiation medium of stem cells. The medium was changed every three days. After two to four weeks, the induced cells on the dishes were detected by staining with 2% alizarin red (pH 4.2; Sigma-Aldrich).

Results

1. Primary culture of guinea pig scleral stem cells

On the third day of primary culture, a small number of cells appeared. On the fifth day, the cell density was about 30%. The cells were spindle-shaped with high nucleocytoplasmic ratio. On the sixth day, the density of cells reached 50% and the cells were arranged in clusters or whirlpools. (Fig 1)

2. Identification of guinea pig scleral stem cells

Using flow cytometry, the cultured scleral stem cells tested positive for mesenchymal stem cell surface markers CD44 and CD105 and negative for leukocyte markers CD45. Fig 2.A, Fig 2.B, Fig 2.C

The RNA extracted from cultured scleral stem cells was detected by PCR. The results showed that the scleral stem cells of guinea pig expressed stem cell markers ABCG2, Notch1, Six2 and Pax6, and the scleral stem cells also expressed type I collagen, the most important component of sclera. Fig. 3

3. Multipotent Differentiation of guinea pig scleral stem cells

In guinea pig scleral stem cells that were induced in adipogenic medium, lipid droplets were detected in the differentiated cells by oil red staining.Fig.4 A

Positive Alizarin red staining was observed in cultured guinea pig scleral stem cells induced in osteogenic medium. (Fig.4 B

The pelleted cells induced in chondrogenic medium were positive with toluidine blue staining. (Fig.4 C

Discussion

Stem cells are a special cell population able to self-renew, proliferate and differentiate multi-directionally. Under certain conditions, they can differentiate into one or more kinds of functional cells and this has been a hot topic in various research fields. The structure of guinea pig sclera is similar to that of human sclera, therefore, guinea pig is often used as an animal model to study sclera-related diseases. In the study of myopia, sclera is the final target organ of myopia development, and guinea pigs are often used to build experimental myopia models.[10, 11] In this study, we isolated stem cells from guinea pig sclera, which are able to differentiate multi-directionally and self-renew. The cultured guinea pig scleral stem cells can provide a new perspective and intervention target for the treatment of scleral tissue-related diseases.

In this study, the CD44 and CD105 surface markers of mesenchymal stem cells from cultured guinea pig scleral stem cells tested positive, which proved that the cells belonged to mesenchymal stem cells. Some studies showed that stem cells, which expressing high levels of CD44 and CD105 markers, may play an important role in cartilage formation. [12] The surface marker CD105 is the main glycoprotein of human vascular endothelium. It is a complete type I membrane protein with a large extracellular region, a hydrophobic transmembrane region and a short cytoplasmic tail. Alsalameh et al.[13] , who found a subset of positive CD105 cells that can be differentiated into chondrocytes in human articular cartilage, suggested that cells which expressed CD105 may be good markers of cartilage development. Therefore, we believe that the cultured guinea pig scleral stem cells may become a new carrier for the study of cartilage development related diseases.

Secondly, we detected the gene expression of scleral stem cell markers ABCG2, Notch1, Six2, Pax6 and type I collagen by PCR. ABCG2 is a member of ATP binding cassette (ABC) transporter family. It is a cell surface protein, mainly located in plasma membrane. Based on the principle of hoechst33342 dye efflux, ABCG2 + cells can be separated by flow cytometry. [14] The expression of ABCG2 could increase colony formation efficiency and growth ability.[15] It is suggested that ABCG2 is a positive marker of scleral stem cells. Notch-1 is a transmembrane receptor, which is involved in maintaining the undifferentiated state of cells. Notch-1 existed in the cell sub-populations expressing ABCG2, and can also be highly expressed in quiescent cells. [16] Thomas et al. [17] found that all Notch-1 positive cells in the basal layer of limbal epithelium could simultaneously express ABCG2. Although all Notch-1 cells were ABCG2 positive, not all ABCG2 positive cells expressed Notch-1. Therefore, Notch-1 has a good application prospect as a marker of scleral stem cells. It is reported that Pax6 haplotype is related to the high susceptibility to myopia that Chinese people exhibit. [18] Pax6 gene is a major gene in the development of the central nervous system and eyeballs, especially in the differentiation of lens and retina. Six2 is a transcription factor containing homologous domains[19]. Sox2 is expressed in many stages of embryonic development and cell differentiation, which is crucial to the regeneration ability of undifferentiated embryonic stem cells, nerves, stem cells, etc., and the pluripotency of stem cells. Sox2 can play a role in the earliest stage of embryonic development.[20, 21] Importantly, Sox2 is found to be a key downstream mediator of Six2, which promotes stem cell phenotype in vitro and in vivo. Previous study [22] demonstrated for the first

time that the expression of Six2 and Sox2 was positively correlated in human breast cancer, and Six2 mediated gene markers were significantly correlated in distant metastasis free survival, recurrence free survival and recurrence free survival. These data suggest that Six2 may regulate stem cell-like properties in multiple tissue types, in both normal and disease states.

The multi-directional differentiation ability of bone marrow mesenchymal stem cells, which can differentiate into cartilage, osteoblast, adipose tissue and nerve cells, has been confirmed.[23] We found that scleral stem cells could be induced to differentiate into chondrocytes, osteocytes and adipocytes in vitro. The chondrogenic differentiation of scleral stem cells was round and enlarged, and toluidine blue staining was positive. These results indicate that the culture of guinea pig scleral stem cells in cartilage differentiation medium for 14 days is enough to obtain chondrocyte-like cells. The osteogenic differentiation was spindle-shaped to cube-shaped, and alizarin red staining was positive. The results showed that osteoblast-like cells could be obtained from guinea pig scleral stem cells cultured in osteogenic differentiation medium for 14 days. The differentiated scleral stem cells showed adipocyte-like morphology and formed lipid droplets and oil bubbles. Oil red staining could detect the presence of colored lipid droplets in the differentiated cells, which confirmed the adipogenic differentiation of scleral stem cells. These results indicated that adipose-like cells can be obtained from guinea pig scleral stem cells cultured in an adipogenic differentiation medium for 14 days. Our data show that: (1) guinea pig scleral stem cells have the characteristics of bone marrow mesenchymal stem cells; (2) guinea pig scleral stem cells can express stem cell surface markers; (3) guinea pig scleral stem cells have the potential to differentiate into chondrocytes, osteocytes, adipocytes and so on. Therefore, guinea pig scleral stem cells can be used as a new source of cells for the study of scleral-related diseases.

Sclera, as the ultimate target organ of myopia, is an important focus of myopia research. The sclera of guinea pig is similar to human sclera, so guinea pig is a classical animal model of experimental myopia, and its sclera is also an important carrier of research. About 90% of mammalian sclera is composed of type I collagen.[7] Research found that in the development of myopia, the content of type I collagen decreased significantly and sclera became thinner, which participated in the regulation of myopia related sclera remodeling.[24] During the development of myopia, the expression of α - smooth muscle actin (α - SMA) increased.[25] Artilage-associated protein in extracellular matrix of sclera was found to be increased in the bird myopia model, which was considered to be related to the change of extracellular matrix of sclera. [26] Although the extracellular matrix of mammalian sclera is mainly composed of type I collagen, the cells isolated and cultured from human sclera can detect the early marker of cartilage formation, type II collagen, and persistent expression.[27] The expression of cartilage related proteins can also be detected in human sclera [28] , indicating that the cartilage components retained in mammalian sclera may affect the biochemical and biomechanical properties of sclera. However, the pathogenesis of myopia still needs to be further studied. The mechanism of type I collagen and cartilage related protein in myopia-related scleral remodeling is not clear. In this study, the mRNA expression of type I collagen could be detected in cultured guinea pig scleral stem cells, and CD105 (endoglin) is the main glycoprotein of human vascular endothelium, which is a complete type I membrane protein, with a large extracellular region, a hydrophobic transmembrane region and a short cytoplasmic tail. Alsalameh et al.[13] found a

subset of CD105 + cells that can differentiate into chondrocytes in human articular cartilage, suggesting that CD105 cells may be a good marker of cartilage development. At the same time, guinea pig scleral stem cells can differentiate into chondrocytes by directional differentiation, which also suggests that guinea pig scleral stem cells may provide a new intervention target for elucidating the cellular and molecular mechanism of myopia-related scleral remodeling.

In conclusion, we found that there are stem cells with multiple differentiation potentials in guinea pig sclera, and also found that the cells are related to scleral collagen and cartilage related proteins. The finding may provide a tool to help clarify mechanisms of sclera-related ocular development and disease, and will allow us to investigate the possible role of this group of cells in further studies.

Declarations

Conflict of Interest statement:

There were not any competing financial interests in relation to the work described. The authors disclosed there were not the sources of any support for the work, received in the form of grants and equipment and drugs.

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Data availability statement:

The data used to support the findings of this study are available from the corresponding author upon request.

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Tables

Table 1. Primers for Reverse Transcription-Polymerase Chain Reaction

Gene	Primer sequence(5'-->3')
ABCG2-F	TGTTGACCTGCGCTGAACTT
ABCG2-R	CCCGGTCTCATGATTCCATTAAC
Notch1-F	ATGACTGCCCGGGAAATAGC
Notch1-R	TGCAGAACTGACCTGTCCAC
Six2-F	AAGGAAAGGTACGAGGAGAACA
Six2-R	TCCTCAGAGCTGCCTAGCAC
Pax6-F	CCGAATTCTGCAGGTGTCCAA
Pax6-R	CTTTTCGCTAGCCAGGTTGC
collagen I-F	GGCCAAGATATCAGGCAACCA
collagen I-R	GGCAGCAAAGTTTCCACCAA

F, forward; R, reverse.

Figures

Fig.1

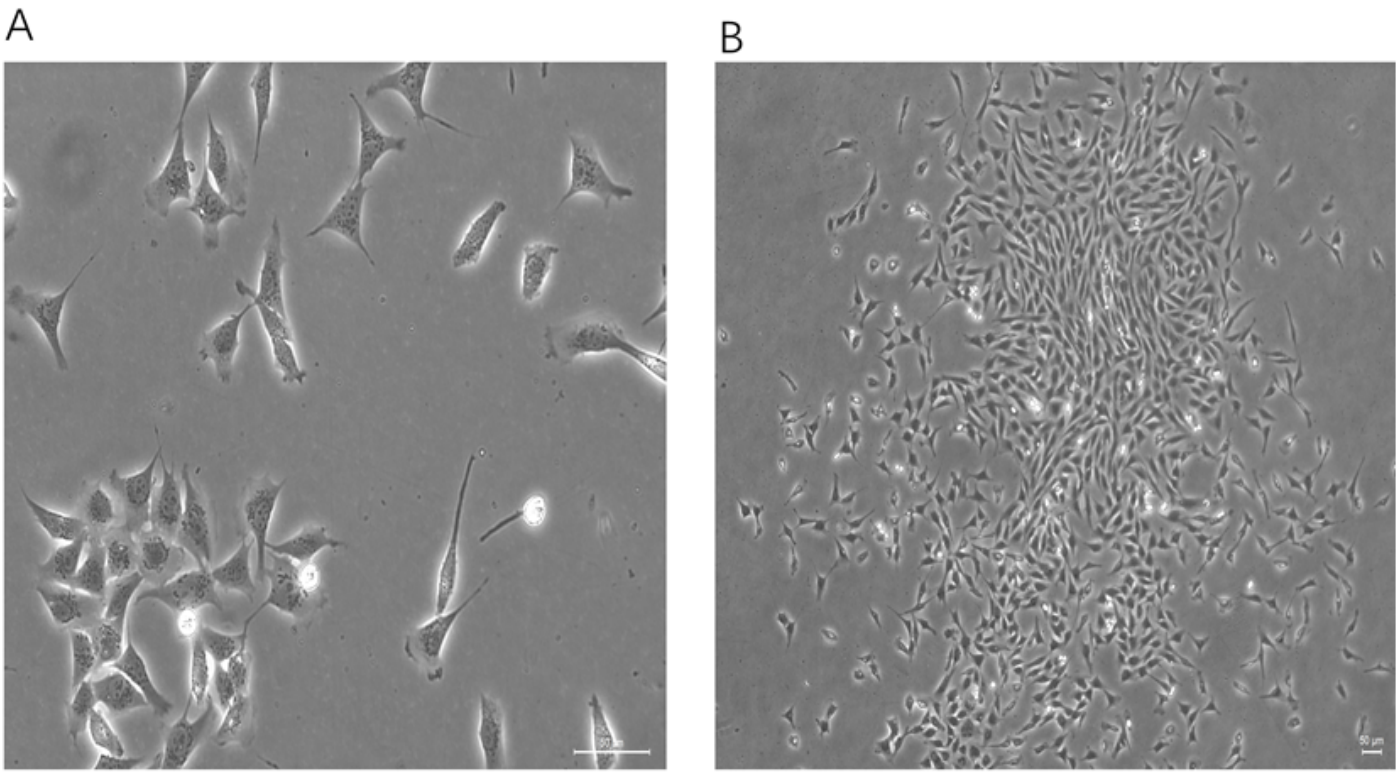

Figure 1

Primary culture of guinea pig scleral stem cells. On the sixth day, the density of cells reached 50% and the cells were arranged in clusters or whirlpools.

Fig.2

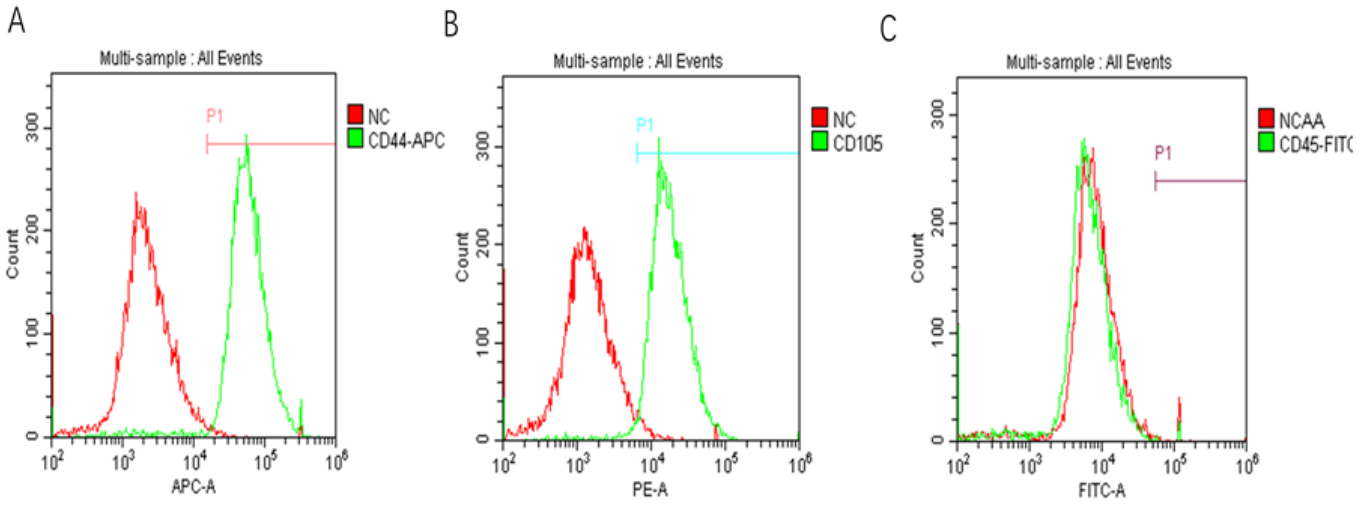

Figure 2

Expression of surface markers in guinea pig scleral stem cells by flow cytometry. The mesenchymal stem cell surface markers CD44 and CD105 were positive and leukocyte markers CD45 was negative.

Fig.3

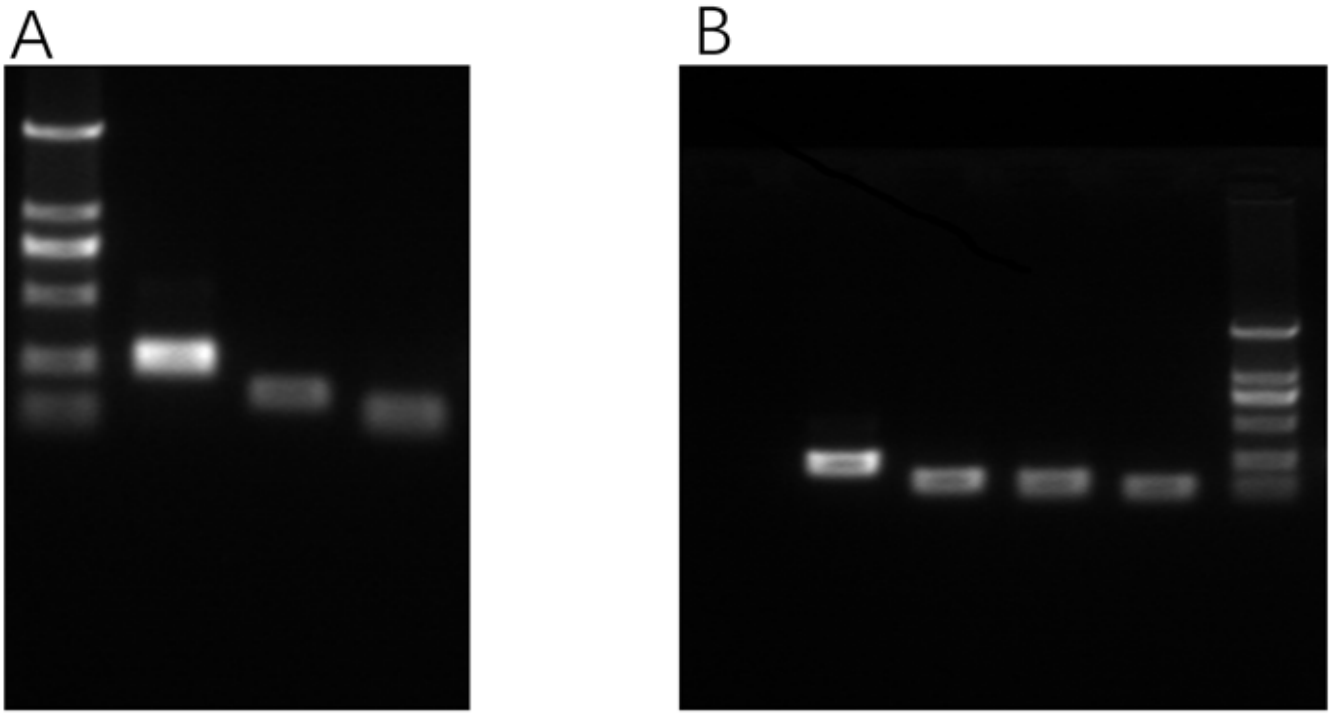

Figure 3

Expression of stem cell markers and type I collagen in guinea pig scleral stem cells by RT-PCR. The cells expressed stem cell markers ABCG2, Notch1, Six2 and Pax6, and also expressed sclera marker type I collagen.

Fig.4

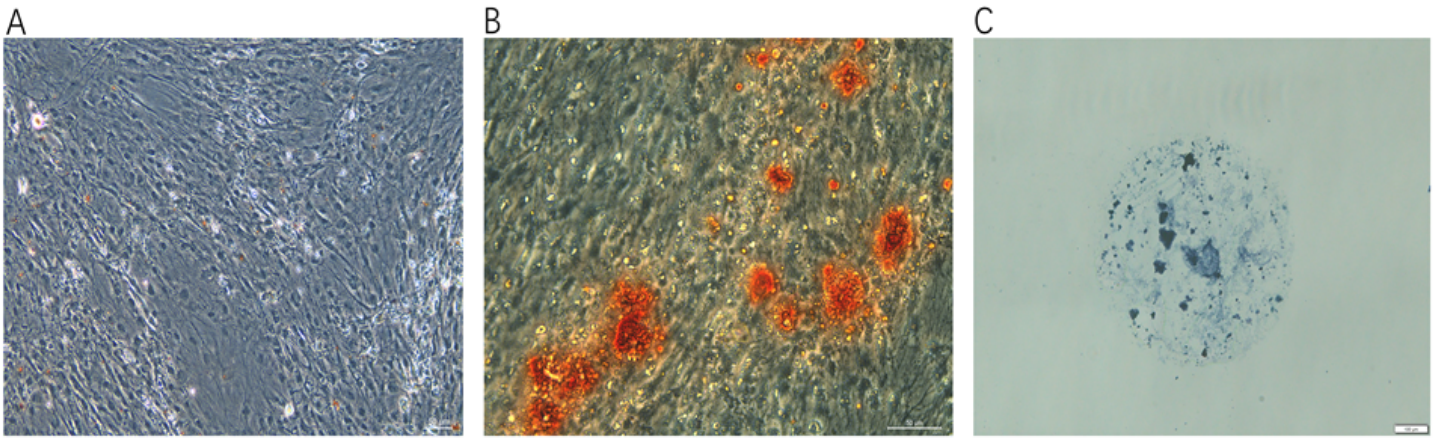

Figure 4

Multipotent Differentiation of guinea pig scleral stem cells. A. Detection of guinea pig scleral stem cells induced by adipogenic medium by oil red staining. lipid droplets were detected in the differentiated cells. B. Detection of guinea pig scleral stem cells induced by osteoblast medium by alizarin red staining. Positive alizarin red staining was observed in cell cultured . C. The pelleted cells induced in chondrogenic medium detected by toluidine blue staining.