In Vitro and *In Vivo* Osteogenesis of Human Orbicularis Oculi Muscle-Derived Stem Cells

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Abstract

BACKGROUND: Cell-based therapies for treating bone defects require a source of stem cells with osteogenic potential. There is evidence from pathologic ossification within muscles that human skeletal muscles contain osteogenic progenitor cells. However, muscle samples are usually acquired through a traumatic biopsy procedure which causes pain and morbidity to the donor. Herein, we identified a new alternative source of skeletal muscle stem cells (SMSCs) without conferring morbidity to donors.

METHODS: Adherent cells isolated from human orbicularis oculi muscle (OOM) fragments, which are currently discarded during ophthalmic cosmetic surgeries, were obtained using a two-step plating method. The cell growth kinetics, immunophenotype and capabilities of *in vitro* multilineage differentiation were evaluated respectively. Moreover, the osteogenically-induced cells were transduced with GFP gene, loaded onto the porous β -tricalcium phosphate (β -TCP) bioceramics, and transplanted into the subcutaneous site of athymic mice. Ectopic bone formation was assessed and the cell fate *in vivo* was detected.

RESULTS: OOM-derived cells were fibroblastic in shape, clonogenic in growth, and displayed phenotypic and behavioral characteristics similar to SMSCs. In particular, these cells could be induced into osteoblasts *in vitro* evidenced by the extracellular matrix calcification and enhanced alkaline phosphatase (ALP) activity and osteocalcin (OCN) production. New bone formation was found in the cell-loaded bioceramics 6 weeks after implantation. By using the GFP-labeling technique, these muscle cells were detected to participate in the process of ectopic osteogenesis *in vivo*.

CONCLUSION: Our data suggest that human OOM tissue is a valuable and noninvasive resource for osteoprogenitor cells to be used in bone repair and regeneration.

Keywords Skeletal muscle stem cells · Orbicularis oculi muscle · Osteogenic differentiation

1 Introduction

Repair of large bone defects caused by trauma, infection, cancer or congenital malformation still remains a major surgical challenge throughout the world. Stem cell-based therapies hold great promise for the repair and regeneration of various tissues and organs, including bone tissue, because of their capabilities to undergo self-renewal and multilineage differentiation [1]. Skeletal muscle tissue has been extensively investigated as a potential source for isolating skeletal muscle stem cells (SMSCs) recently [2].

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SMSCs exhibit capacities of undergoing myogenic, osteogenic, chondrogenic and adipogenic differentiation *in vitro* and forming bone and cartilage *in vivo*, receiving widespread interest in the research field of repairing bone defects [3–5]. However, the skeletal muscle is usually acquired through an invasive biopsy procedure. Its related complications, such as infection, bleeding, injury, morbidity and chronic pain to the donor muscles, limit the availability of muscle tissue which can be harvested and the amount of SMSCs which can be isolated. In some studies, SMSCs used for investigation had to be obtained from human traumatized muscles [6, 7].

The blepharoplasty is one of the most performed ophthalmic and cosmetic surgeries, during which part of the orbicularis oculi muscle (OOM) is routinely removed and discarded as medical waste. OOM is the sphincter muscles of the eyelids, a distinctive group of highly specialized and vascularized skeletal muscles, whose main function is to narrow the palpebral fissure and modulate facial expression [8]. We speculate that OOM is an excellent tissue source for muscle stem cells due to the easy accessibility and rich vascularization. To date, however, little information is available about whether SMSCs are present in OOM tissue.

In the current study we identified an adherent cell population isolated from human OOM tissue using collagenase digestion combined with a two-step plating method, which was capable of undergoing self-renewal, displaying a phenotype profile similar to muscle stem cells and differentiating towards myogenic, adipogenic, chondrogenic and osteogenic cell fates *in vitro*. Furthermore, these cells were proven to be able to initiate ectopic bone formation *in vivo* when implanted into nude mice in combination with biodegradable scaffolds. Collectively, our findings suggest that OOM is a less invasive and more accessible source of SMSCs that might have the potential application for cellular therapies of bone reconstruction and regeneration.

2 Materials and methods

2.1 Cell isolation and expansion

Experimental protocols for this study were approved by the Research Ethics Committee of the Sun Yat-Sen Memorial Hospital of Sun Yat-Sen University (No. 2017-0081) and conformed to the principles outlined in the Declaration of Helsinki. Muscle samples were surgically harvested from the tarsal portions of OOM during the upper eyelid blepharoplasty from 6 female healthy patients (mean age 23.5 ± 4.2 years old) with written informed consent. After histological (HE staining) and histochemical (Myogenin and Pax7) analyses, muscle fragments were washed with phosphate-buffered saline (PBS, Hyclone, UT, USA),

minced with sterile scissors and digested in 0.1% collagenase type I solution (Worthington, NJ, USA) at 37 °C for 4 h under constant shaking. Collagenase was neutralized with low-glucose Dulbecco modified Eagle's medium (LG-DMEM, Gibco, NY, USA) containing 10% fetal bovine serum (FBS, Hyclone). Digested tissues were filtered through a 40 µm cell strainer, centrifuged at 250g for 5 min, and the resulting cell pellets were resuspended in the growth medium [LG-DMEM, 10% FBS, 2 mM L-glutamine (Sigma, MO, USA), 100 units/mL penicillin (Sigma), and 100 mg/mL streptomycin (Sigma)]. The cells were transferred into a 35-mm tissue culture dish (Falcon, NJ, USA) and incubated at 37 °C and 5% CO₂ for 6 h. Then the supernatant was withdrawn and replated onto a new culture dish. After 18 h, the medium was replaced for the first time to remove the non-adherent tissue fragments. Cells that adhered to the tissue culture plastic were maintained in the growth medium with changes twice a week. Immunofluorescence analysis of satellite cell markers, Pax7 and Myf5 (antibodies from BD Sciences, CA, USA), was performed after 6 days in culture [1]. SMSCs were digested by 0.25% trypsin–EDTA solution (Sigma) and subcultured into new dishes when reaching approximately 85% confluent and designated as passage 1 (P1). Cells of P3 or P4 were used for the following experiments.

2.2 Characterization and multilineage differentiation

To characterize the cell population *in vitro*, flow cytometric analysis of cell surface antigens (CD markers) was performed as previously reported [8]. A total of 10^5 cells were collected, washed with PBS and incubated at 4 °C for 30 min with the following fluorescein isothiocyanate (FITC)-conjugated or phycoerythrin (PE)-conjugated monoclonal anti-human antibodies: CD29-FITC, CD31-FITC, CD34-FITC, CD45-PE, CD56-FITC, CD73-PE, CD90-FITC and CD105-FITC. Labeled cells were analyzed by flow cytometry (FACSCalibur, Becton Dickson, CA, USA), and cells stained with FITC- or PE-conjugated non-specific IgG were used as isotype controls (all antibodies were purchased from Abcam, MA, USA). *In vitro* multilineage differentiation capabilities of SMSCs were assessed as followed with methods described before [1, 2, 8, 9]. To initiate myogenic differentiation, cells were replated at 2×10^4 cells/cm² in 35-mm dishes containing glass coverslips, which had been coated with 1:200 PBS-diluted (50 µL/cm²) Matrigel™ (BD Biosciences, NJ, USA) at 37 °C overnight prior to cell plating. After maintained in the growth medium for 12 h to allow cell adherence, fresh myogenic medium (containing the growth medium supplemented 5% horse serum, 0.1 µM dexamethasone, 50 µM hydrocortisone (all from Sigma)) was

replenished. Medium was changed twice weekly for 3 weeks and myogenic differentiation was assessed by fluorescence immunocyto staining with antibody of mouse anti-human Desmin (Santa Cruz, CA, USA), a late differentiation marker of skeletal muscle cells. For chondrogenic differentiation, cell pellets were prepared (2.5×10^6 per pellet) in 15-mL polypropylene tubes (Falcon) and induced in the chondrogenic medium (containing high-glucose DMEM supplemented with 10% FBS, 10 ng/mL transforming growth factor- β 1 (TGF- β 1), 100 ng/mL insulin-like growth factor (IGF), 40 ng/mL dexamethasone and 6.25 mg/mL transferrin (all from Sigma except for FBS from Hyclone)). Half media were changed every 3 days for 3 weeks. Afterwards, the pellets were fixed with methanol, embedded in paraffin and processed for immunohistochemical staining of collagen type II (mouse anti-human Collagen II antibody, from Abcam). To induce adipogenic differentiation, cells were replated at 2×10^4 cells/cm² in 35-mm dishes and cultured for 2 weeks in the adipogenic medium (consisting of the basic culture medium plus 1 μ M dexamethasone, 0.5 mM isobutyl-methylxanthine (IBMX), 10 μ g/mL insulin and 100 μ M indomethacin (all from Sigma)). Medium was changed twice weekly and intracellular lipid accumulation was detected by Oil red O staining (Sigma) after 14 days.

2.3 Osteogenic differentiation *in vitro*

OOM-derived cells were replated at a density of 2×10^4 cells/cm² in 35-mm dishes and maintained for 2 weeks in the osteogenic medium [consisting of the growth medium plus 0.1 μ M dexamethasone, 10 mM β -phosphoglycerol and 50 μ M ascorbic acid (all from Sigma)]. The medium was renewed every 3 days and the mineralization of extracellular matrix was characteristically evaluated by Alizarin red S staining (Sigma). Calcium deposition within the cells was semi-quantitatively measured using a colorimetric assay of the solubilized Alizarin red stain at days of 3, 6, 9, 12 and 15 in culture [10]. Alkaline phosphatase (ALP) activity and osteocalcin (OCN) synthesis, the most specific markers for osteoblasts in their early and late stages of differentiation, were also analyzed quantitatively at these time points respectively [11]. Briefly, at each time point cells were homogenized and digested in proteinase K (Sigma) at 56 °C for 16 h. DNA content in the cell lysate was quantified spectrofluorometrically using Hoechst 33,258 dye (Sigma) and the cell number was calculated by correlating with a standard curve, which was generated by lysing serial dilutions of a known concentration of cells. ALP activity was measured using an ALP assay (Sigma) and OCN production in the medium was determined using an ELISA assay (Invitrogen, CA, USA). The amounts of OCN and ALP were divided by the total cell number

obtained from DNA measurement, with the non-induced cells served as the controls.

2.4 Ectopic bone formation *in vivo*

SMSCs were transduced with the green fluorescent protein (GFP) gene as a method of monitoring their *in vivo* fate [12], and the gene-transduction efficiency was observed using a fluorescence microscope (Zeiss Axiophot, Heidenheim, Germany). Porous β -tricalcium phosphate (β -TCP, Bio-lu Biomaterials, Shanghai, China) ceramic cylinders (6 mm in length and 3 mm in diameter), which had a porosity of 70% with a mean pore size of 400 μ m and interconnecting pores of 100 μ m, were loaded with the GFP-transduced SMSCs (10^7 cells per implant) according to cell-seeding methods previously described [13]. The cell-TCP composites were further incubated in the osteogenic medium for 7 days, and scanning electron microscopy (SEM, Jeol, Tokyo, Japan) examination was performed to evaluate cell adhesion and growth within the implants. Then the composites were surgically implanted into subcutaneous sites of BALB/c athymic nude mice (6 weeks old, female). Each animal received one scaffold loaded with cells and one scaffold without cells under general anesthesia. All the animals enrolled in this study were processed in accordance with protocols approved by the Institutional Animal Care and Use Committee of Tongji University. After 6 weeks, the animals were sacrificed and radiographed. The specimens were harvested, decalcified in HCl, embedded in paraffin, sectioned and prepared for the standard histological evaluation (HE staining) to assess ectopic bone formation. To trace the *in vivo* fate of transplanted human SMSCs and determine the origin of newly formed tissue, sections were further observed with the fluorescence microscopy to detect the presence of GFP-positive cells.

2.5 Statistical analysis

All data collected were presented as mean $\pm$ standard deviation (SD) and Student-Newman-Kewels paired *t* test was used to determine possible significant differences ($p < 0.05$) between groups.

3 Results

3.1 Cell isolation and culture

Human OOM can be subdivided into tarsal, septal and orbital portions. Muscle samples used in this study were harvested from the tarsal portion (Fig. 1A), with an average weight of (0.456 ± 0.215) g ($n = 6$). Histological evaluation showed

Fig. 1 Isolation of SMSCs from OOM myofibers. **A** Schematic illustration of human OOM anatomic structure. T: the tarsal portion of OOM; S: the septal portion; O: the orbital portion. The rectangular region indicates the harvest site of OMM samples for this study. **B** By HE staining evaluation, OOM samples comprised striated muscles (pink) with no fat tissue. **C** By immunohistochemical analysis, OOM positively expressed the myofiber marker of myogenin (brown, cross

section of the muscle). **D** Satellite cells of the OOM myofibers were shown by immunoreaction with an anti-Pax7 antibody (green, arrows, cross section of the muscle). **E** The isolated adherent cells showed positive fluorescent immunostaining of Myf5 (red) and Pax7 (green) when cultured *in vitro* for 6 days, indicating their myogenic origin. Cell nuclei were visualized by DAPI. Scale bars for **B–E**: 100 μ m. (Color figure online)

the samples were mainly composed of skeletal muscle and some fibrous tissue (Fig. 1B, C). The OOM satellite cells, which are located beneath the myofiber membrane and normally proposed as SMSCs, could be detected by immunostaining of anti-Pax7 antibody (Fig. 1D). Cells obtained from OOM myofibers on the basis of collagenase digestion and adhesion characteristics positively expressed satellite cell markers of Myf5 and Pax7 when cultured *in vitro* for 6 days (Fig. 1E). The mean yield of OOM-derived cells in primary culture was $(2.79 \pm 0.82) \times 10^5$ cells per gram of muscle tissue ($n = 6$). These cells underwent extensive proliferation *in vitro*. They could be maintained up to passage 10 without obvious changes in cell phenotype and their average population doubling time was approximately 60–70 h (data not shown).

3.2 Cell characterization and differentiation

Adherent cells isolated from digested muscle tissue could form colonies and grow rapidly in culture (Fig. 2A, B). After passaging, the cells appeared homogenous fibroblast-like morphology (Fig. 2C). Flow cytometry analysis showed that these cells were positive for mesenchymal stem cell (MSC) antigen markers (CD29, CD73, CD90 and CD105), and negative for CD31 (endothelial marker) and

CD45 (monocyte-macrophage antigen). They also expressed CD34 and CD56, characteristic markers of SMSCs [14] (data now shown). The multilineage differentiation potentials were confirmed by positive results of Desmin expression (myogenic marker, Fig. 2D), collagen type II expression (chondrogenic marker, Fig. 2E) and Oil red O staining (adipogenic marker, Fig. 2F), respectively. No positive staining results were found in the non-induced control cells (data not shown).

3.3 Osteogenesis *in vitro*

In contrast to the non-induced cells, OOM-derived SMSCs cultured in the osteogenic differentiation medium for 15 days could form densely calcified nodules, which were stained specifically by Alizarin red S (Fig. 3A, B). When quantitatively measured, the calcium deposition in cells showed an increasing trend in the osteogenic-induced group, and became significantly higher than that in the control cells from day 9 (Fig. 3C). The cell growth, ALP activity and OCN release were determined at days 3, 6, 9, 12 and 15 of culture. Based on DNA assay, cells proliferated steadily in both groups, showing no significant difference in terms of cell growth rate (Fig. 3D). But from day 12, both ALP activity and OCN content became

Fig. 2 *In vitro* proliferation and multilineage differentiation of SMSCs. **A** Freshly isolated cells formed colonies within 48 h in primary culture. **B** They proliferated to approximately 85% confluence after 14 days. **C** The subcultured cells appeared homogenous fibroblast-like morphology after passaging. **D** After myogenic differentiation for 2 weeks, OOM-derived cells were positive for desmin, a late differentiation marker of the muscle cells, and fused into

elongated, multinucleated myotube-like structure. **E** The chondrogenic differentiation in cell pellet culture was identified by the positive collagen type II staining. **F** The adipogenic differentiation was confirmed by positive Oil red O staining after adipogenic induction. Cell nuclei were visualized by DAPI. Scale bars: 100 μ m. (Color figure online)

approximately 5–10 times higher in the osteo-induced cells than those in the non-induced cells (Fig. 3E, F).

3.4 Osteogenesis *in vivo*

Greater than 95% of the GFP-transfected cells expressed the green fluorescence (Fig. 4A), and could homogeneously adhere to the surface of β -TCP ceramics after loading (Fig. 4B). Then the implants were co-cultivated *ex vivo* for 7 days, and SEM observation showed that SMSCs were present within the TCP pores, secreted and formed an extracellular matrix network through the interconnected pores (Fig. 4C, D). 6 weeks after *in vivo* implantation, the cell-TCP composites displayed evidently higher radiographic density than the scaffolds alone (Fig. 4E, F). Histological evaluation revealed new bone matrix formation in the cell-loaded implants. Active osteoblasts aggregated on the edge of newly formed osteoid and osteocytes became embedded in the bone matrix lacunae (Fig. 4G). The presence of green luminescence indicated that a certain proportion of GFP-labeled human cells differentiated into osteoblasts and participated in the *de novo* bone formation (Fig. 4H). In contrast, no evidence of ectopic osteogenesis or green fluorescence was observed in the control TCP scaffolds without loading cells after implantation (data not shown).

4 Discussion

Heterotopic ossification within skeletal muscles is very common in many clinical conditions, such as fracture healing, trauma and immobilization, suggesting that skeletal muscle tissue may contain a population of osteoprogenitor cells [4, 15]. The interest in this phenomenon and the need to find osteogenic precursors for bone repair and regeneration have promoted investigation on isolating multipotent stem cells from skeletal muscle tissues [2]. Several research groups have identified the existence of SMSCs not only in the limb muscles but also in the craniofacial muscles, such as the masseter muscle and the orbicular oris muscle [2, 9, 16]. In the present study, we reported for the first time that human OOM contains SMSCs that are capable of differentiating into osteogenic progenitor cells and initiating *de novo* osteoid formation by using *in vitro* and *in vivo* assays.

Cells obtained from the collagenase-digested OOM tissue were selected according to their high adhesion characteristics to the tissue culture plastic. The positive expression of Pax7 and Myf5, two specific markers of satellite cells and myoblasts, displayed their myogenic origin [17]. When cultured *in vitro*, these cells exhibited self-renewal and multipotent differentiating capabilities.

Fig. 3 *In vitro* osteogenic differentiation of SMSCs. **A** After induced for 15 days, osteogenic differentiation was verified by the positive Alizarin red staining. Inset photograph is the gross view of staining. **B** The non-induced cells revealed negative staining. **C** Calcium content within the cells was measured using a colorimetric assay of the solubilized Alizarin red stain. At days of 9, 12 and 15 in culture, more Ca^{2+} was deposited in the osteogenically induced cells compared to the control cells. **D** Growth rates of osteo-induced and

non-induced SMSCs were determined by DNA assay. No significant difference in cell numbers was found between two groups. **E** Greater ALP activity was expressed when cells were osteo-induced compared to the non-induced cells from day 12. **F** More OCN was produced in the osteo-induced cells than in the control cells from day 12. Error bars represent SD with $n = 6$ (* $p < 0.05$; # $p < 0.01$). (Color figure online)

Fig. 4 *In vivo* osteogenic differentiation of SMSCs. **A** After GFP-transduction, the majority of the cells expressed green fluorescence. **B** The GFP-labeled SMSCs distributed evenly on the β -TCP ceramics after loading. **C** SEM observation showed the porous structure of the ceramic scaffold. **D** 7 days after cell seeding, SMSCs deposited large amounts of extracellular matrix covering the ceramic pores and formed a matrix network through the interconnected pores. **E** X-ray photograph showed higher density in the cell-TCP composite (top) than in the cell-free scaffold (bottom) at 6 weeks of transplanting

in vivo. **F** Quantitative analysis of X-ray grey values demonstrated significant difference between two groups ($n = 6$). **G** HE staining revealed typical woven bone formation (red) after implantation of the GFP-labeled osteo-induced cells. **H** When observed with a fluorescent microscope, these active osteoblasts lining on the bone surface in the adjacent section of the HE-stained specimen showed the green fluorescence. Scale bars: 100 μm for **A–B**, 200 μm for **C–D** and 25 μm for **G–H**. (Color figure online)

Their osteogenic phenotype was proven by the sign of extracellular matrix mineralization and increases of ALP activity and OCN secretion in the cells exposed to the osteogenic induction medium. Furthermore, the muscle-derived cells could undergo osteogenic differentiation *in vivo* and were themselves responsible for the osteoid formation when introduced into a mouse model of ectopic osteogenesis, suggesting their potential applications in bone engineering and reconstruction.

SMSCs are believed heterogeneous in nature and pre-plate techniques are suggested to purify the stem cells in some reports [3, 18]. In this current study we adopted a modified two-step plating method. Cells that adhered rapidly were mostly non-myogenic fibroblasts and the slow-adhering cells were mainly composed of myogenic progenitor cells with stem cell properties [3, 4, 18, 19]. Therefore, in our investigation we selected the cells that adhered to the plastic substrate 6–24 h after initial plating. The adherent cells showed clonogenic proliferative behavior and fibroblast-like morphology, indicating that they were relatively homogenous. After passaging, the subcultured cells displayed a similar expression profile of MSCs markers (CD29, CD73, CD90 and CD105) and were positive for the characteristic antigens of SMSCs (CD34 and CD56). Contamination of endothelial cells or leukocytes could be excluded by evidence of being negative for CD31 and CD45. Taken together, this two-step plating method is proven to be an easy technique to isolate and purify SMSCs from OOM tissues.

The blepharoplasty or eyelid cosmetic procedure is a widely performed plastic surgery, during which some OOM tissue is currently removed and discarded as medical waste. Compared to muscles in other sites, OOM is easily accessible under local anesthesia with little patient discomfort, and its removal causes negligible function loss and morbidity to donors. OOM is composed of three different anatomic regions (i.e. the tarsal part, septal part and orbital part), and each portion varies in the histological microstructure suggesting divergent physiological functions [20]. The OOM tissue used in this study was obtained from the tarsal portion, which contains the highest ratio (83.5%) of muscle tissue and lowest percentage of fatty tissue (0%) [20] (Fig. 1A–C). Approximately $(2.79 \pm 0.82) \times 10^5$ adherent cells could be obtained from 1 g of OOM tissue, higher than the yield of 10^5 SMSCs from 1 g of human skeletal muscle [21], indicating that OOM is an abundant source of muscle stem cells. OOM-derived SMSCs could be expanded to $30\text{--}40 \times 10^6$ after 4 passages in *in vitro* culture (data not shown), a sufficient number required for the cellular therapies of bone loss or defect [5].

In summary, the results of this study showed that SMSCs can be conveniently obtained from human OOM

when this tissue is exposed for surgical procedure. These cells are capable of differentiating into osteogenic lineage under simple inductive stimulus and forming osteoid tissue *in vivo* when implanted with β -TCP biomaterials. Our findings support that OOM tissue may be a valuable and less invasive resource of osteoprogenitor cells to be used in clinical setting to improve bone healing.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval The protocol for this study was approved by the Research Ethics Committee of the Memorial Hospital of Sun Yat-Sen University (No. 2017-0081) and conformed to the principles outlined in the Declaration of Helsinki.

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