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Isolation and *in vitro* propagation of human skeletal muscle progenitor cells from fetal muscle

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Abstract

Skeletal muscle progenitor cells (SMPCs) are considered one of the most valuable cells for cell-based therapy targeting skeletal muscle. However, an efficient protocol for isolating and maintaining human myogenic progenitors *in vitro* has not been fully established. In this study, we demonstrate that human myogenic progenitors can be expanded and proliferated from human fetal muscles. Human SMPCs were prepared from fetal hind limb muscles and induced to proliferate as free-floating spheres termed myospheres in the medium containing basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). Both myogenic progenitors and myoblast populations from human fetal muscles were effectively propagated in myospheres and passed by a mechanical chopping. After expanding these spheres in culture, we tested whether myogenic progenitor cells can differentiate into multinucleated myotubes. The myospheres were dissociated, plated down on coverslips, and cultured in the medium for terminal differentiation. We could confirm that the plated cells formed well-developed, multinucleated myotubes. This culture method using myospheres is an effective protocol to isolate and maintain SMPCs from human fetal skeletal muscles in culture.

Keywords

skeletal muscle progenitor cells; human fetal muscle; myosphere; basic fibroblast growth factor; epidermal growth factor

Introduction

Cell-based therapy is a promising therapeutic approach for neuromuscular diseases. Skeletal muscle progenitor cells (SMPCs) are one of the most valuable cell types for this approach (Montarras et al., 2005; Partridge et al., 1989; Tedesco et al., 2010). SMPCs can be isolated from various sources including adult tissues and pluripotent stem cells (Chang et al., 2009; Darabi et al., 2008; Ryan et al., 2011). Satellite cells are a type of SMPCs in postnatal and adult skeletal muscles (Bischoff, 1986).

Fetal tissue-derived stem/progenitor cells also have many potential advantages because of their immature immune status and greater capacity for proliferation (Gotherstrom et al., 2004; Rollini et al., 2004; Van and Liang, 2003). This was confirmed by showing that SMPCs exist in human fetal skeletal muscles *in vivo* and contribute to muscle development and growth (Frank et al., 2006). However, a culture method for propagating human fetal-

derived SMPCs has not been fully established, although there is one report of isolating and culturing myoblasts from fetal muscles (Hirt-Burri et al., 2008).

We show the possibility of fetal skeletal muscle as a source of SMPCs using free-floating myosphere culture, which have been established for adult muscle-derived SMPCs (Arsic et al., 2008; Sarig et al., 2006; Wei et al., 2011; Westerman et al., 2010).

Materials and Methods

Preparation of human fetal skeletal muscle

Human fetal tissue (10 weeks post-conception) was provided by University Hospital Freiburg, Freiburg, Germany. The method of collection conformed to the guidelines recommended by National Institute of Health for the collection of tissue and set out by the University of Wisconsin, Madison. Institutional Review Board approval was obtained for all studies.

Culture of human fetal myospheres

A schematic illustration of the culture is given in Fig. 1A. Human skeletal muscle progenitor/stem cells were prepared from fetal hind limb muscles (quadriceps) and induced to proliferate as myospheres. We modified our previous protocols for the long-term growth of human neural progenitor cells from fetal brain tissue (Svendsen et al., 1998) and human pluripotent stem cells (Ebert et al., 2009). Freshly isolated tissue was dissociated in 0.1% collagenase (Sigma-Aldrich) and seeded into a T25 flask at 200,000 cells per ml maintenance medium [Stemline medium (S-3194, Sigma-Aldrich) supplemented with penicillin/streptomycin/amphotericin B (PSA, 1% v/v), 100 ng/ml human basic fibroblast growth factor (bFGF, WCell Research Institute), 100 ng/ml human epidermal growth factor (EGF, Millipore), and 5 ng/ml Heparin (Sigma-Aldrich)]. The culture flask was pre-coated with poly-HEMA (poly 2-hydroxyethyl methacrylate; Sigma-Aldrich) to prevent the attachment of cells on the surface. After 1 week, the cells formed myospheres (hfMyosphere; passage 0). All cultures were maintained in a humidified incubator at 37°C, and half the growth medium was replenished every 2–3 days. The spheres were passaged by mechanically chopping them into 200 µm cubes (passage 1) using a McIlwain tissue chopper (Mickle Laboratory Engineering). Bright field images of cultured myospheres were obtained using an inverted microscope (TS100, Nikon) with a charge-coupled device (CCD) camera (QICAM Fast 1394, QImaging) and imaging software (Q capture pro, QImaging).

Differentiation of myosphere-derived cells

To test whether sphere-cultured cells maintain features of muscle cells such as terminal differentiation, we used an adherent culture of myosphere-derived cells using a standard culture protocol for muscle cells (Yaffe and Saxel, 1977). hfMyospheres (passage 1) were dissociated with TrypLE (Life Technologies Corp.) for 10 min. The dissociated cells were then on a 24-well culture dish coated with laminin (Sigma-Aldrich) and cultured in the proliferation medium [Dulbecco's modified Eagle medium (DMEM, Sigma-Aldrich) containing 10% fetal bovine serum (Life Technologies)] for 7 days. The cell density was sufficient at 7 days for cell-cell fusion to differentiate into multinucleated myotubes. The medium was switched to DMEM supplemented 2% horse serum (Life Technologies) for an additional 3 days to bring the cell out of cycle.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA isolation from myospheres and RT-PCR were done as previously described (Suzuki et al., 2008). Total RNA was extracted from whole myospheres grown using RNeasy purification systems (Qiagen). RT-PCR was run for a maximum of 30 cycles on a

thermal cycler (Eppendorf). Primers were obtained from Integrated DNA Technologies and were combined with PCR Master Mix (Promega Corp.). All primers were prepared from human cDNA sequences for Pax3, Pax7, Myf5, MyoD, Myogenin, and β -Actin, which were obtained from GenBank database (Table.1). The sequences were designed to cross an intron-exon boundary to prevent a false-positive signal due to genomic DNA.

Immunocytochemistry

Immunocytochemical detection of skeletal muscle markers was done as before (Suzuki et al., 2008). Briefly, plated cells were fixed in 4% paraformaldehyde [PFA, in phosphate-buffered saline (PBS)] at room temperature for 20 min and washed with PBS 3 times. After blocking in 5% normal goat serum, the cells were incubated with primary antibodies against PAX7 [1:50; Developmental Studies Hybridoma Bank (DSHB)], MYOD (5.8A, 1:50; Vector laboratories), MYOGENIN (F5D, 1:50; DSHB), or Myosin heavy chain (MHC) (MF20, 1:20, DSHB). After incubation with the primary antibodies, the cultures were rinsed in PBS, and incubated for 30 min with secondary antibody conjugated to Cy3 (goat anti-mouse IgG, 1:1000, Jackson ImmunoResearch Laboratories). Hoechst 33258 (0.5 μ g/ml in PBS, Sigma-Aldrich) was added for 10 min after completion of the secondary antibody incubation as a nuclear stain. Immunocytochemical images were acquired using a Nikon Eclipse 80i fluorescence microscope and a DS-QiIMC CCD camera (Nikon). Cell counts were performed with NIS-Elements Imaging software (Nikon). The percentage of positive cells for each antibody was determined by averaging counts from 10 different, randomly chosen microscopic fields.

Results

Myosphere culture of human fetal-derived skeletal muscle cells

Human fetal hind limb muscles were dissected and collected muscle cells were cultured in the maintenance medium (Fig. 1A). After 1 week of culture, the cells formed spherical aggregates (hfMyospheres) (Fig. 1B). These spheres were passaged by mechanically chopping them using a tissue chopper and further propagation in the maintenance medium.

Expression of marker genes for both myogenic progenitor cells and myoblasts

We determined whether hfMyospheres could maintain SMPCs. RT-PCR results showed that hfMyospheres at passage 1 expressed specific markers for muscle progenitor cells (Pax3 and Pax7) and myogenic cells (Myf5, MyoD, and Myogenin) (Fig. 2A). These results indicate that hfMyospheres can maintain both SMPC and myoblast populations.

Muscle differentiation of SMPCs in myospheres

To see whether SMPCs in hfMyospheres can differentiate into multinucleated myotubes, hfMyosphere-derived cells (passage 1) were cultured in the proliferation medium for 7 days to produce enough cells for fusion. At day 1, the cells expressed both PAX7-positive myogenic progenitors and MYOD- or MYOGENIN-positive myoblast markers. Similar patterns of gene expression were observed at day 4 (Fig. 2B), indicating that gene expression profiles were sustained. Immunocytochemistry showed that approximately 16, 28, and 30% of the cells expressed PAX7, MYOD and MYOGENIN at day 1, respectively (Fig. 3). The number of myoblasts in early stage (MYOD positive: 22%) was similar at day 4 compared to the level at day 1, while myoblasts in late stage (MYOGENIN positive) and the progenitor cell population (PAX7 positive) decreased to 17% and 5%, respectively (Fig. 3). Finally, the cells were placed in the differentiation medium at day 7 and completed terminal differentiation for 3 days. Well-developed multinucleated myotubes (MHC⁺: 16%

of fusion index) were observed at day 10, indicating that hfMyosphere-derived cells terminally differentiate into myotubes (Fig. 4).

Discussion

Free-floating spherical culture has been applied to a variety of cell types, such as neural and cardiac progenitor cells, and adult SMPCs (Messina et al., 2004; Sarig et al., 2006; Svendsen et al., 1998). We have demonstrated that this method can also be applied to human fetal-derived SMPCs to expand in culture. A new technique to passage myospheres has been developed using mechanical chopping, which can reduce the damage done to cell surface proteins and help maintain cell-cell contact (Collins et al., 2005).

Fetal tissue-derived myospheres maintained both SMPCs and myoblasts. In contrast, adult muscle-derived myospheres predominantly consist of satellite cells (Sarig et al., 2006; Westerman et al., 2010). Our results indicate that prenatal muscle tissue could maintain more immature progenitor cells compared to postnatal tissues. Spherical culture can expand progenitor cells to a large scale, but it produces heterogeneous cellular populations. This characteristic might be helpful for propagation of myoblasts as well as SMPCs, although more work is needed to see whether hfMyosphere can be cultured long-term without losing myogenic potential.

Formation of multinucleated myotubes indicates that hfMyosphere-derived cells maintain myogenic character, and suggests that spherical culture does not affect the myogenic characteristics of fetal-derived SMPCs and myoblasts. Interestingly, the number of Pax7-positive cells gradually decreased during adherent culture. Myospheres derived from human adult skeletal muscle maintain SMPCs in an undifferentiated state (Wei et al., 2011). This indicates that spherical aggregation might be effective for maintaining fetal-derived progenitor populations *in vitro*. Examining the ability of intramuscular integration and migration following transplantation is necessary. MHC staining at day 1 for the plated cells did not show any MHC-positive myotubes (data not shown). However, our present results fall short of deciding whether the differentiated myotubes originated from SMPC or myoblasts in hfMyosphere-derived cells. To answer this question, further studies are necessary to characterize and isolate SMPC in hMyospheres by using the other protocols such as flow cytometry.

In summary, we demonstrate that fetal-derived SMPCs can be maintained and propagated using myosphere culture. Cell-based therapy is a promising therapeutic approach for neuromuscular diseases. Amongst transplantable cells into skeletal muscle, SMPCs are considered one of promising cell types for neuromuscular diseases. Thus fetal-tissue derived SMPCs provide an alternative choice of cell type for cell-based therapy targeting muscle.

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Abbreviations

SMPCs	skeletal muscle progenitor cells
hfMyosphere	human fetal-derived myosphere
bFGF	basic fibroblast growth factor
EGF	epidermal growth factor
poly-HEMA	poly 2-hydroxyethyl methacrylate
DMEM	Dulbecco's modified eagle medium
RT-PCR	reverse transcription-polymerase chain reaction
MHC	myosin heavy chain

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Figure 1. Sphere-based culture of human fetal-derived skeletal muscle cells

(A) Schematic describing myosphere culture of human fetal-derived SMPCs. SMPCs are isolated from hind limb muscles of human fetus and cultured in the sphere maintenance medium for 1 week. Human fetal-derived myospheres (hfMyospheres) are mechanically chopped to passage them. Small pieces of hfMyospheres were cultured for an additional 1 week to propagate SMPCs. (B) A representative image of hfMyospheres developed from human fetal skeletal muscle-derived cells. Original magnification, $\times 100$.

Figure 2. Expression of myogenic marker genes in human fetal-derived myospheres
(A) hfMyospheres express marker genes for both progenitor cells (Pax3 and Pax7) and myoblasts (Myf5, MyoD, and Myogenin). p1: passage 1. **(B)** hfMyospheres-derived cells express myogenic marker genes in adherent culture. Gene expression was analyzed at days 1 and 4.

Figure 3. Expression of myogenic marker proteins in hfMyosphere-derived cells
The plated cells derived from hfMyospheres expressed marker proteins for myogenic progenitor cells (PAX7) and myoblasts (MYOD and MYOGENIN) at day 1 and 4. Scale bar: 100 μ m.

Figure 4. Terminal differentiation of myosphere-derived cells into multinucleated myotubes
Dissociated cells were plated in the proliferation medium for 7 days and switched to DMEM supplemented 2% horse serum for an additional 3 days to withdraw cells from cell cycle. After 10 days of differentiation, hfMyospheres-derived cells formed multinucleated myotubes. MHC: Myosin heavy chain. Scale bar: 100 μ m.

Table 1

Sequences of primers used for RT-PCR.

	Primer	Oligonucleotide sequence	Size (bp)	GenBank Accession No.
Pax3	Sense	5'-AAA GAG GAA CAG CGC AGA A-3'	269	NM_181461.3
	Antisense	5'-GAG GTC TCC GAC AGC TGG TA-3'		
Pax7	Sense	5'-GGG AAG AAA GAG GAG GAG GA-3'	253	NM_002584.2
	Antisense	5'-CCT CGC GGG TGT ATA TGT CT-3'		
Myf5	Sense	5'-CCA CCT CCA ACT GCT CTG AT-3'	152	NM_005593.2
	Antisense	5'-AGG TGA TCC GGT CCA CTA TG-3'		
MyoD	Sense	5'-GAC GGC ATG ATG GAC TAC AG-3'	134	NM_002478.4
	Antisense	5'-AGG CAG TCT AGG CTC GAC AC-3'		
Myogenin	Sense	5'-GCC ACA GAT GCC ACT ACT TC-3'	569	NM_002479.4
	Antisense	5'-CAA CTT CAG CAC AGG AGA CC-3'		
β -Actin	Sense	5'-CCG ACA GGA TGC AGA AGG AG-3'	663	NM_001101.3
	Antisense	5'-CCG ACA GGA TGC AGA AGG AG-3'		