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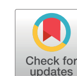
Establishment of primary myoblast cell cultures from cryopreserved skeletal muscle biopsies to serve as a tool in related research & development studies



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ABSTRACT

Background: Primary myoblast cell cultures display the phenotypic characteristics and genetic defects of the donor tissue and represent an *in vitro* model system reflecting the disease pathology. They have been generated only from freshly harvested tissue biopsies. Here, we describe a novel technique to establish myoblast cell cultures from cryopreserved skeletal muscle biopsy tissues that are useful for diagnostic and research purposes.
Methods and results: This protocol was performed on seven gradually frozen muscle biopsy specimens from various neuromuscular disorders that were stored in dimethylsulfoxide (DMSO)-supplemented freezing media at −80 °C for up to one year. After storage for varying periods of time, primary myoblast cultures were successfully established from all cryopreserved biopsy tissues without any chromosomal abnormality. Desmin immunoreactivity confirmed that the cell cultures contained > 90% pure myoblasts. The myoblasts differentiated into multinucleated myotubes successfully. Furthermore, there were no statistically significant differences in cell viability, metabolic activity, population doubling time, and myocyte enhancer factor 2 (*MEF2C*) expression between cell cultures established from freshly harvested and one year-stored frozen tissue specimens.
Conclusions: This protocol opens up new horizons for basic research and the pre-clinical studies of novel therapies by using cryopreserved skeletal muscle biopsies stored under suitable conditions in tissue banks.

1. Introduction

A number of rodent myoblast cell lines, especially the mouse cell line, C2C12, [1,2] and the rat cell line, L6, [3] have been extensively used to investigate the molecular mechanisms of myoblast proliferation and differentiation. In addition, they serve as important experimental models for identifying the pathogenic and pathophysiological mechanisms of various neuromuscular disorders and are also used in therapeutic drug discovery approaches. However, immortalized cell lines do not behave identically with primary cells. They are often genetically and phenotypically unstable and are maintained under artificial conditions in culture over an extended period, which can cause extensive alterations in gene expression profile, native cell behavior, and their responses to a number of stimuli [4–6]. Moreover, there exist significant interspecies differences between humans and rodents at a molecular level [7], so rodent cell lines may not be representative genomic models for every human disease. Therefore, primary myoblast cell cultures established from skeletal muscle biopsies are believed to be more relevant biological tools than cell lines for multiple research applications including skeletal muscle function, tissue regeneration or renewal for tissue engineering purposes, gene therapy, and drug screening [8,9]. They are the most suitable experimental models for evaluating a variety of mutations in their natural environment as they reflect the genetic background of the primary tissues from which they are derived and mimic the disease condition *in vitro* very well [8]. When skeletal muscle biopsies are cultured under specific artificial conditions, quiescent satellite cells underneath the basal lamina are activated to enter the cell cycle and give rise to proliferating myoblasts before being terminally differentiated into multinuclear myotubes [10,11]. In addition, the immortalization of proliferating myoblasts offers the possibility of establishing cell lines that have an unlimited life span and a specific genetic background [12,13].

To date, primary myoblast cell cultures have been generated only from freshly harvested tissue biopsies. Although skeletal muscle stem

Abbreviations: DMSO, Dimethylsulfoxide; MEF2C, Myocyte enhancer factor 2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PDT, Population doubling time

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cells have the ability to survive for extended periods *post mortem* [14,15], it is recommended to establish the primary myoblast cell culture in a few hours after the muscle biopsy specimen is dissected. However, establishing a myoblast cell culture from each and every biopsy material is an expensive and time-consuming procedure. Furthermore, in terms of time, labor and laboratory conditions, it is not always possible to initiate a cell culture from every biopsy material taken for diagnostic purposes. A simple method for storing cryopreserved tissue samples for subsequent culture will be useful in these circumstances and also have other practical uses in retrospective studies. In this study we report a simple method for storing skeletal muscle biopsies (~2 mm^3) at −80 °C in dimethylsulfoxide (DMSO)-supplemented freezing media for up to one year without loss of myoblastic cell growth and differentiation capacity.

2. Materials and methods

2.1. Patients and muscle biopsies

The study protocol was approved by the Hacettepe University Faculty of Medicine Ethical Review Board (GO 17/656–19). Written informed consent was obtained from all the patients at the time of the diagnostic muscle biopsy. Between January 2016 and May 2017, eight skeletal muscle biopsies from patients with a clinicopathological diagnosis of various forms of muscular dystrophy were included in this study. The biopsies were taken from the left biceps brachii muscle in all patients. The muscle biopsy specimens (approximately 0.3 cm^3) were transferred into 2 ml cryovials containing DMSO-supplemented freezing media (10% DMSO, 45% FBS, 45% DMEM supplemented with 2 mM L-glutamine, and 3% Pen/Strep), were then gradually frozen in a freezing container (Nalgene, Rochester, USA), and maintained at −80 °C for up to one year.

2.2. Establishing primary myoblast cell cultures

After storage for varying periods of time (1 and 6 weeks; 2, 3, 5, and 7 months; 1 year) at −80 °C, cryopreserved biopsy samples were thawed completely for 1 min in a 37 °C water bath, washed twice with PBS and then cut into smaller pieces in a biopsy culture media (Skeletal Muscle Cell Growth Medium, Promocell supplemented with 2 mM L-glutamine and 1% Pen/Strep). Two drops of autoclaved silicone grease were placed in a 100-mm plastic Petri dish, and minced pieces of the biopsy sample were placed around each drop. Coverslips were placed on top of each silicone grease/tissue sample, and biopsy culture media was added to the dish (Fig. 1). Tissue samples were left undisturbed for 7–10 days. The medium was then replaced every 2 days, and culture dishes were monitored for the appearance of myoblast outgrowth by inverted microscopy (Leica DMIL). Outgrowing cells were detached with trypsin, transferred to T25 cell culture flasks and subcultured until sufficient numbers were generated for cryogenic preservation.

2.3. Phenotypic characterization of myogenic cells and karyotyping

After the myogenic precursors became 70–80% confluent, they were differentiated into myotubes by replacing the biopsy culture medium with a differentiation medium (DMEM supplemented with 2% horse serum, 2 mM L-glutamine, and 1% Pen/Strep). Images of the myogenic cells were captured at different time points using a Leica DMIL microscope equipped with a Leica DFC320 camera (Leica, Switzerland) for morphological analysis. Desmin expression in confluent myoblasts at passage 2 was measured using indirect fluorescent immunolabeling to assess the myogenic purity of the primary skeletal muscle cell cultures. Cells were fixed in 4% paraformaldehyde for 10 min, blocked with phosphate buffered saline (PBS) containing 10% normal goat serum, 10% bovine serum albumin, and 0.1% Tween-20 at 20–25 °C for 1 h. To detect desmin-positive myoblasts, fixed cells were incubated with mouse anti-desmin antibody clone DEU-10 (1:10; Sigma) overnight at 4 °C and then labeled with Alexa Fluor 488 goat anti-mouse IgG antibody (Molecular Probes) at 1:1000 for 45 min at room temperature. Immunostained cells were observed under an inverted-fluorescent microscope (Leica DMIL). Additionally, the karyotype of the myogenic cells was examined by G-banding, and at least 20 metaphases were checked for each sample.

2.4. Real-time RT-PCR

RNA was isolated from the primary myoblast cells (passage 2) established from a frozen tissue sample stored for one year at −80 °C (Aurum Total RNA Mini kit, BioRad). After checking for purity and quantity, 800 ng of RNA was used as a template for cDNA synthesis (QuantiTect Reverse Transcription kit, Qiagen). Quantitative PCR reactions were carried out using the iQ5 Real-Time PCR Detection System (Bio-Rad Lab, Hercules, CA, USA). The reactions were run in triplicate using the primers: *MEF2C*/F: 5′-GGTCTCACCTGGTAACTTGAACA-3′ and *MEF2C*/R: 5′-GCCGACTGGGAGTTATTTATC-3′; *GAPDH*/F: 5′-CACCAGGGCTGCTTTTAACTCTGGTA-3′ and *GAPDH*/R: 5′-CCTTGACGGTGCCATATTTGC-3′; *ACTB*/F: 5′-CGCAAAGACCTGTACGCCAAC-3′ and *ACTB*/R: 5′-GAGCCGCCGATCCACACG-3′. Each 25 μl of qPCR reaction

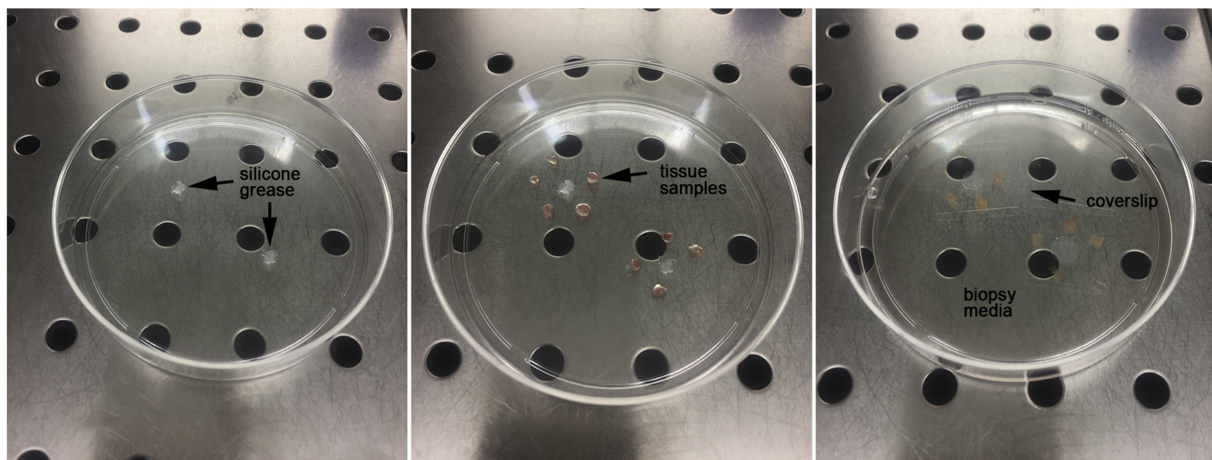

Fig. 1. Experimental protocol for plating cryopreserved skeletal muscle biopsy samples on a culture dish. (Left) Transfer two drops of sterile silicone grease to an empty 100 mm Petri dish; (Middle) Mince the biopsy tissue into small pieces, place them around each silicone grease, and top them with a coverslip; (Right) Gently add the biopsy culture media into the dish.

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