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Functional validation and expression analysis of myotubes converted from skin fibroblasts using a simple direct reprogramming strategy

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ABSTRACT

Previously, we reported that MyoD, a master gene for myogenic cells, could efficiently convert primary skin fibroblasts into myoblasts and myotubes, thereby effecting direct reprogramming. In this study, we further demonstrated that MyoD-expressing primary fibroblasts displayed rapid movement in culture, with a movement velocity that was significantly faster, almost four times, than mouse primary myoblasts. MyoD-transduced cells obtained the characteristics of Ca^{2+} release and electrically-stimulated contraction, which was comparable to C2C12 myotubes, suggesting that the essential features of muscle were observed in the transduced cells. Furthermore, the ability to fuse to the host myoblasts means that gene transfer from MyoD-transduced cells to host muscle cells could be obtained by cell fusion. In comparison with the iPS method (indirect reprogramming), our transduction method has a low risk for tumorigenesis and carcinogenesis because the starting cells are fibroblasts and the transduced cells are myoblasts, both normal and mortal cells. Accordingly, MyoD transduction of human skin fibroblasts using the adenoviral vector is a simple, inexpensive and promising candidate as a new cell transplantation therapy for patients with muscular disorders.

1. Introduction

Duchenne muscular dystrophies (DMD) is X-linked recessive disorder caused by loss of function with a mutation in the dystrophin gene. Although one of fundamental treatments for DMD is muscle transplantation, a clinically significant transplantation requires plenty of muscle cells from the donor, which do not have high proliferation potency in culture. As a solution, we chose skin fibroblast, which is known to be easily obtained and have a high proliferation potency especially in young child. In addition, direct reprogramming from skin fibroblasts to muscle cells is previously reported [1].

For many hereditary and refractory diseases, treatment strategies using iPS cell reprogramming have been explored worldwide. iPS cells have the potential to grow indefinitely, which is beneficial for obtaining sufficient amounts of cells for use in clinical treatment. However, the drawback of immortalization is the possibility of inducing carcinogenesis in culture and in the body. In 1987, cellular reprogramming was utilized in an attempt to generate myoblast cells from fibroblasts using the MyoD gene [2]; a strategy now called "direct reprogramming" as compared to "indirect reprogramming" through the use of iPS cells. The advantages (pluripotency and permanent cell growth) and disadvantages (possibility of carcinogenicity) of the iPS reprogramming method are two sides of the same coin.

Similarly, gene transfer using viral vectors can be broadly classified into two methods, transient and stable expression, each of which has advantages and disadvantages. Adenovirus vectors have the advantage of achieving high titers and consequently a high percentage of transduction and reprogramming. In our previous report, transient expression of the MyoD gene enabled myogenesis from skin fibroblasts. We found that transferring the MyoD gene into skin fibroblasts using an adenoviral vector (*Ad.CAGMyoD*) generated myoblast morphology and gene expression, and the development of myotubes in culture [1].

In this study, we examined whether *Ad.CAGMyoD*-induced myogenesis produced functional characteristics of muscle cells (contraction and intracellular Ca^{2+} release) *in vitro*. Furthermore, the directly reprogrammed cells were tested for motility and the capability to fuse with host myoblasts, which are vital factors in transplantation as gene transfer to host muscle cells from donor cells is desirable.

2. Materials and methods

2.1. Cell culture

Human primary fibroblast cells were obtained from Cell Systems Corporation (Kirkland, WA, USA). Primary mouse myoblasts were

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isolated from the hind limbs of 6-week-old C57BL/10J mice. Animal experiments were carried out according to the guidelines of the Laboratory Protocol for Animal Handling, Sojo University Faculty of Pharmaceutical Sciences. The C2C12 mouse myoblast cell line was obtained from ATCC (Manassas, VA, USA). All cell types were grown in high glucose Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin-streptomycin, and 1% GlutaMax supplement (Invitrogen, Tokyo, Japan) at 37 °C under humidified 5% (v/v) CO_2.

2.2. Adenoviral vectors

We constructed five adenoviral vectors. Among these, the *Ad.CAGMyoD* and control *Ad.CAGEGFP* vectors were previously described [1,3]. The *Ad.CAG-MyoD-ires-EGFP* (*Ad.CAGMiG*) vector was generated from *Ad.CAGMyoD* by adding an internal ribosome entry site (IRES) linked to the enhanced green fluorescence protein (EGFP) reporter gene. The *Ad.CAG-MyoD-ires-Cherry* (*Ad.CAGMiC*) and *Ad.CAGCherry* vectors were generated from *Ad.CAGMiG* and *Ad.CAGEGFP* by replacing EGFP with the red fluorescent protein Cherry. The organization of the adenovirus vectors is depicted in Fig. S1. The expression units were inserted into the E1 region of E1-E3-deleted human adenovirus type 5. Each vector was amplified in HEK-293 cells (ATCC) and was purified using double CsCl gradient ultra-centrifugation [4]. The biological titer for each adenoviral vector is shown in Table 1A.

2.3. Adenoviral transduction

Human fibroblasts were seeded at a density of 7×10^5 cells/well in 4-well plates or 2×10^5 cells/well in 12-well plates coated with matrigel before adenoviral transduction. Cells were transduced with adenoviral vectors at multiplicities of infection (MOI) of 10–30 for 1 h in 2% FBS-DMEM (Dulbecco's Modified Eagle's Medium supplemented with 2% fetal bovine serum and 1% penicillin-streptomycin). The medium containing the virus vector was removed and the cells were washed twice with fresh 10% FBS-DMEM. Subsequently, the cells were cultured for 2 days in 10% FBS-DMEM. To induce differentiation, the culture medium was replaced with differentiation medium (2% FBS-DMEM) (day0 in this experiment).

2.4. RNA isolation and reverse transcription

Total RNA was isolated using Nucleo Spin RNA® (Takara, Tokyo, Japan) according to the manufacturer's instructions at day-2 (no virus vector), 0, 3, 5, 8, 12 after replacing the differentiation medium. Reverse transcription was performed using a PrimeScript® RT reagent Kit (Perfect Real Time) (Takara). PCR was performed with Takara Ex Taq® (Takara). RT-PCR conditions and primers for each gene are shown in Table 1B.

2.5. Ca^{2+} measurement

Calcium imaging was carried out using Fluo4-AM special packaging® (Dojindo, Kumamoto, Japan) according to the manufacturer's protocol. To measure intracellular Ca^{2+}, cells were loaded with Fluo4-AM at 37 °C for 1 h, washed, and changed to recording medium (Fluo4-AM special packaging® kit) for cellular analysis. For each culture, one of the following reagents was added 10 s after the start of observation: adenosine triphosphate (ATP) (MP Biomedicals, Tokyo, Japan), or the chlorophenol derivatives 4-chloro-3-ethylphenol (4-CEP) (Sigma-Aldrich, Tokyo, Japan) or 4-chloro-m-cresol (4-CmC) (Sigma-Aldrich). Fluorescence intensity was calculated using a BZ-X Analyzer (Keyence, Osaka, Japan). A total of 15 individual cells from the same dish were studied and maximal peak amplitudes were measured. Ca^{2+} response data were expressed as mean ± S.E.M. The student's *t*-test was used to determine significant differences between groups. The significance level was set at $P < 0.05$.

Table 1A
Table of Adenoviral vectors.

Adenovirus vector	Titer
Ad.CAG-MyoD	1.0×10^9 PFU/ml
Ad.CAG-MyoD-ires-EGFP	1.0×10^9 PFU/ml
Ad.CAG-EGFP	1.0×10^9 PFU/ml
Ad.CAG-MyoD-ires-Cherry	2.5×10^9 PFU/ml
Ad.CAG-Cherry	1.0×10^9 PFU/ml

Table 1B
Sequences of primers used for mMyoD, hMyoD, hCK-M, hMyogenin, hMHC, hDystrophin, hMyomaker.

Gene name	Number cycle	Ann. temp.	Sequence
hβactin	30	55	CTCTTCCAGCCTTCCTTCCT
			CACCTTCACCGTTCCAGTTT
mMyoD	30	55	CTTCTATGACCCGTGTTTCGAC
			CTGGGTTCCCTGTTCTGTGT
hMyoD	30	61	CACTCCGGTCCCAAATGTAG
			TTCCCTGTAGCACCACACAC
hCK-M	30	55	ACATGGCCAAGGTACTGACC
			TGATGGGGTCAAAGAGTTCC
hMyogenin	30	64	TAAGGTGTGTAAGGGAAGTCG
			CCACAGACACATCTTCCACTGT
hMHC	30	61	CTGCTGAAGGAGAGGGAGCT
			TGATTAGCTGGTCACACCTT
hDystrophin	30	61	GATGCACGAATGGATGACAC
			TGTGCTACAGGTGGAGCTTG
hMyomaker	30	61	GAAGGAGAAGAAGGGCCTGT
			CCTTCTTGTTGACCTTGGGC

2.6. Electrical stimulation

The MyoD-transduced fibroblasts at day 14 after the myogenic-induction and C2C12 myotubes were electrically stimulated as previously described [5,6]. The medium was replaced with 3 ml of fresh medium before electrical stimulation using the C-dish system (ION Optix, MA, USA) with an electric stimulator (Uchida Denshi, Hachioji, Japan). The electric pulse used was 150 V for 3 ms with a 997 ms interval. Cellular contraction was evaluated as the change in distance between two points on individual cells using a motion analyzer (BZ-X Analyzer).

2.7. Time-lapse imaging

Primary mouse myoblasts or adenoviral transduced human skin fibroblast cells were seeded at 5×10^5 cells/well of 35-mm collagen 1-coated Petri dishes and cultivated in DMEM containing 2% FBS. The culture dish was secured on a dish holder on the stage and covered with a heated quartz glass lid that allowed for long-term imaging without condensation. Humidified air with 5% CO_2 was supplied to the chamber through a flexible pipe. Morphological changes in cells were recorded using a fluorescent Keyence BZ-8000 microscope time-lapse photo system. Individual Z-stacks (1 μm thick) were captured every 10 min over 2–5 days. Cellular velocity was calculated using BZ-H1M software (Keyence) by tracking the paths of individual cells. For each isolate, 15 individual cells chosen at random from the same dish were analyzed. The student's *t*-test was used to determine significant differences between groups. The significance level was set at $P < 0.05$. Motion pictures were created from time-lapse images at 10 frames per second.

2.8. Cell fusion assay

Ad.CAGEGFP-transduced mouse primary myoblasts and *Ad.CAGMyoD*-transduced human primary fibroblasts were co-seeded at 1×10^5 for each cell type in 24-well tissue culture plates containing