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Induction of motor neuron differentiation by transduction of Olig2 protein

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ABSTRACT

Olig2 protein, a member of the basic helix-loop-helix transcription factor family, was introduced into the mouse embryonic carcinoma cell line P19 for induction of motor neuron differentiation. We show that Olig2 protein has the ability to permeate the cell membrane without the addition of a protein transduction domain (PTD), similar to other basic helix-loop-helix transcription factors such as MyoD and NeuroD2. Motor neuron differentiation was evaluated for the elongation of neurites and the expression of choline acetyltransferase (*ChAT*) mRNA, a differentiation marker of motor neurons. By addition of Olig2 protein, motor neuron differentiation was induced in P19 cells.

1. Introduction

Motor neurons (MNs) are located in the central nervous system and control muscles. Degeneration of MNs caused by disease or injury leads to serious health problems. However, there are no effective treatments for MN diseases. Therefore, regulation of MN differentiation is receiving considerable attention in the field of regenerative medicine.

MN differentiation from embryonic stem (ES) cells [1–3] or induced pluripotent stem (iPS) cells has been well studied [4–6]. Generally, for induction of MN differentiation, ES and iPS cells have been stimulated with retinoic acids and sonic hedgehog after formation of embryoid body (EB). Under those culture conditions, cells sequentially expressed MN-specific transcription factors and MN phenotypes.

During animal development, transcription factors play important roles in the regulation of cellular differentiation. Olig2 is a member of the basic helix-loop-helix (bHLH) family of transcription factors. Olig2 knockout results in the absence of MNs and oligodendrocytes (OLs) [7–9], suggesting that Olig2 is required for MN and OL differentiation. Moreover, forced Olig2 expression induces MN and/or OL differentiation [9,10].

In our previous experiments, instead of gene transfection, tissue-specific bHLH transcription factor proteins were introduced into cells [11,12]. Neural- and muscle-specific transcription factor proteins, NeuroD2 and MyoD, were introduced into neuroblastoma and myoblast cells, treatments that induced cell differentiation. Other groups also succeeded in inducing differentiation by transduction of tissue-specific transcription factors [13–15]. Thus, it was demonstrated that cell differentiation could be regulated by introduction of tissue-specific transcription factor proteins.

Generally, to introduce proteins into cells, proteins of interest are fused with a short peptide called the protein transduction domain (PTD). However, we have shown that bHLH type transcription factors have PTDs within their primary sequences. In this experiment, the oligodendrocyte- and motor neuron-specific transcription factor Olig2 protein was transduced into mouse embryonic carcinoma P19 cells to induce motor neuron differentiation. In the present report, the transduction ability of Olig2 and the induction of motor neuron differentiation were shown.

2. Materials and methods

2.1. Construction of protein expression vectors

The mouse *Olig2* gene (GenBank NM_016967) was cloned from Mouse Fetal Normal Tissue 17-day embryo Total RNA (BioChain) by reverse transcription-PCR using primer sets containing restriction enzyme sites and the His-tag sequences (GGAATTCATATGCACCATCATCATCATCATCATGCTAGCATGGACTCGG and CCCAAGCTTTCACTTGGCGTCGGAGGTGAG). The amplified *His-Olig2* gene was digested with *Eco*R I and *Hind* III and cloned into pBluescript II (stratagene) digested with same restriction enzymes (pBS-His6-Olig2). Plasmid, pBS-His6-Olig2, was digested with *Nde* I and *Hind* III. The digested fragment was inserted into pET32c digested with same restriction enzymes. The resulting plasmid for protein expression in *Escherichia coli* was termed pET-His6-Olig2.

For construction of the variants, $Olig2_{1-120}$ lacking the sequences after helix-loop-helix (HLH) domain and $Olig2_{1-105}$ lacking the sequences after basic domain, the gene fragments encoding these variants were amplified by PCR from

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pET-His6-Olig2 as a template using primer sets (GGAATTCATATG-CACCATCATCATCATCATGCTAGCATGGACTCGG and TGCAAGCTTCT CAGGCGATGTTGAGGTCGTGCAT for $Olig2_{1-120}$ or ATTTAAGCTTT CACTGCTGCAGCTCGGGCTCAGT for $Olig2_{1-105}$). The amplified fragments were digested with *Nde* I and *Hind* III and inserted to pET32c digested with same restriction enzymes. The resulting plasmids were termed pET-His6-$Olig2_{1-120}$ and pET-His6-$Olig2_{1-105}$. For construction of other variant, $Olig2_{HLH}$ lacking nuclear localized signal like domain (NLS-L) and basic domain, pET-His6-Olig2 was digested with *Nco* I and ligated (pET-His6-$Olig2_{HLH}$).

2.2. Expression and purification of Olig2 proteins

For expressions of Olig2 and $Olig2_{HLH}$ proteins, *E. coli* BL21(DE3) were transformed with pET-His6-Olig2 and pET-His6-$Olig2_{HLH}$ respectively. Transformed cells were cultured in LB media with 50 μg/ml ampicillin at 37 °C until $O.D._{660}$ reached around 0.6. Protein expressions were induced by addition of isopropyl-β-D(-)-thiogalactopyranoside (IPTG: 0.5 mM) followed by culture for 4 h at 37 °C (Olig2) or for 14 h at 30 °C ($Olig2_{HLH}$). For expressions of $Olig2_{1-120}$ and $Olig2_{1-105}$, *E. coli* KRX were transformed with pET-His6-$Olig2_{1-120}$ and pET-His6-$Olig2_{1-105}$. Transformed cells were cultured in LB media with 50 μg/ml ampicillin at 37 °C until $O.D._{660}$ reached around 0.6. Protein expressions were induced by addition of rhamnose (0.1%) and IPTG (0.5 mM) followed by culture for 4 h at 37 °C. After protein induction, cells were harvested by centrifugation and the cell pellets were resuspended in phosphate buffered saline (PBS: 150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.4). Then, resuspended cells were broken by sonication (Biorupter). The cell lysates were separated into soluble and insoluble fractions by centrifugation. Except $Olig2_{HLH}$, the supernatants were added to His-select Nickel affinity Resin (SIGMA). After rotation for 45 min at 4 °C, samples were washed with Ni–NTA buffer (0.5 M NaCl, 20 mM phosphate buffer) including 10 mM imidazole. Then proteins were eluted with Ni–NTA buffer with 500 mM imidazole. Those solutions were dialyzed against PBS using Slide-A-lyzer dialysis cassettes (Pierce: MW 10,000, 0.5–3 ml).

$Olig2_{HLH}$ was obtained from insoluble fraction. The soluble fraction was dissolved with 8 M urea and added to His-select Nickel affinity Resin. After rotation for 45 min at 4 °C, samples were washed with Ni–NTA buffer (0.5 M NaCl, 20 mM phosphate buffer) including 20 mM imidazole and 4 M urea. Then proteins were eluted with Ni–NTA buffer with 500 mM Imidazole and 4 M urea. The eluted sample was dialyzed against PBS with urea using Slide-A-lyzer dialysis cassette. The urea concentration was reduced gradually by carrying out dialysis at 4, 2, 0.5 M and finally 0 M urea.

Purified proteins were analyzed by SDS–PAGE (12% acrylamide gel) and their concentrations were determined using BCA assay kit (Pierce).

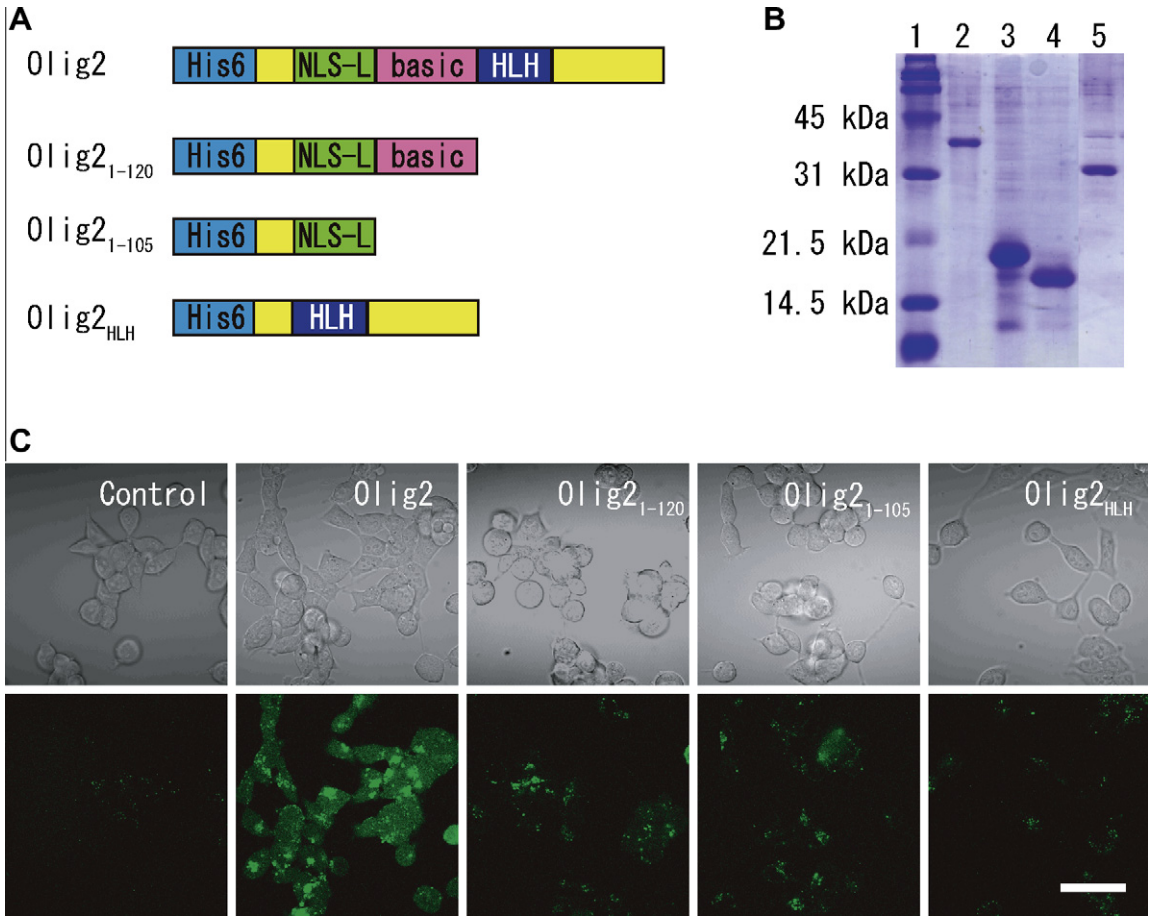

Fig. 1. Transduction ability of Olig2 and its variant proteins. (A) Schematic drawings of constructed proteins. NLS-L represents the NLS-like sequence located from position 95 to 105. Basic represents the basic domain of the basic helix-loop-helix sequence. Olig2 is a full Olig2 sequence with a His-tag (33 kDa). $Olig2_{1-120}$ is Olig2 lacking the HLH domain and the following sequence with a His-tag (14.2 kDa). $Olig2_{1-105}$ is Olig2 lacking the basic-HLH domain and the following sequence with a His-tag (11.8 kDa). $Olig2_{HLH}$ is Olig2 lacking the NSL-L and the basic domain with the His-tag (26.2 kDa). (B) Confirmation of purified Olig2 and its variant proteins by SDS–PAGE. Lane 1: marker; lane 2: Olig2; lane 3: $Olig2_{1-120}$; lane 4: $Olig2_{1-105}$; lane 5: $Olig2_{HLH}$. (C) Transduction of Olig2 and variant proteins. Olig2 and variant proteins were modified with Oregon Green. Cells were observed using laser scanning confocal microscopy. Scale bar represents 50 μm.

2.3. Cell culture

Mouse embryonic carcinoma cell line P19 (Riken cell bank) was cultured in αMEM including 10% (v/v) fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (0.1 mg/ml). During induction of differentiation, cells were cultured in DMEM/Ham's F-12 with 2% (v/v) B-27™ Supplement minus Vitamin A (Gibco). Cells were incubated in a 37 °C, humidified chamber (95% (v/v) air and 5% (v/v) CO_2).

2.4. Evaluation of cellular membrane transduction ability

For evaluation of Olig2 and its variants protein transduction abilities, purified proteins were modified with Oregon Green 488 carboxylic acid, succinimidyl ester (Molecular Probes) as described by the manufacturer. P19 cells, seeded at 7×10^4 cells/35 mm tissue culture dish (Falcon), were incubated for 12 h with fluorescently labeled protein at a final concentration of 0.2 μM. Then cells were trypsinized and inoculated on 35 mm glass base dishes (Iwaki). After 2–3 h, cells were observed using laser scanning confocal microscopy (Olympus: FV300 system).

2.5. Induction of neural differentiation by addition of Olig2 protein

P19 cells were plated on a 6-well tissue culture plate (Falcon) at 6×10^4 cells/well. After overnight incubation, medium was changed to DMEM/Ham's F-12 with 2% (v/v) B-27™ Supplement minus Vitamin A and then Olig2 protein was added to cells. The final concentration of Olig2 protein was 0.1 μM. Same procedure was performed every 24 h.

2.6. Immunostaining of β-III tubulin

After five days of culture with Olig2 protein, cells were washed with TBS (10 mM Tris–HCl. 150 mM NaCl, pH 7.4) and fixed with 4% (w/v) paraformaldehyde for 30 min at room temperature. After washing with TBS, fixed cells were treated with TBS containing 0.2% Tween 20 for 40 min. After blocking with 3% (w/v) bovine

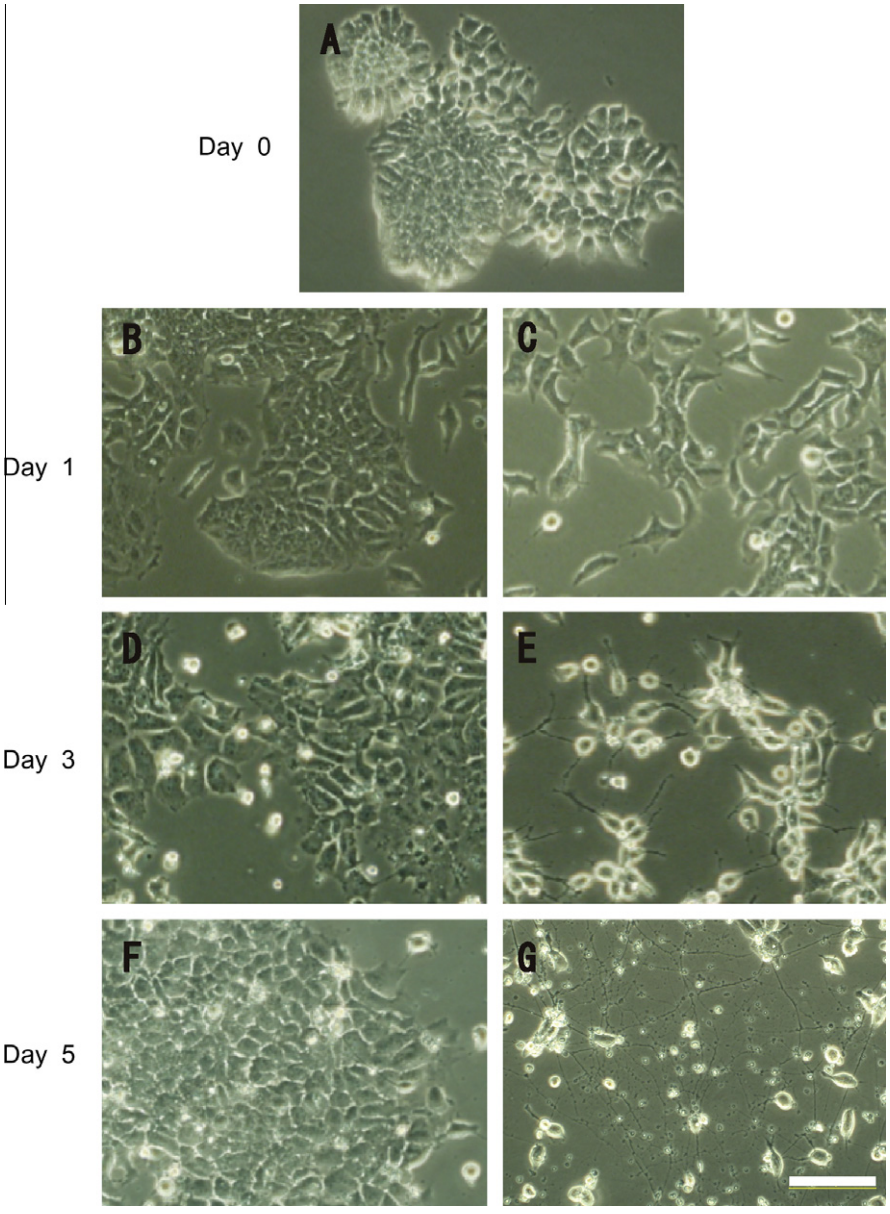

Fig. 2. P19 cells were cultured with Olig2 proteins. P19 cells were cultured with or without Olig2 protein in the presence of B-27 supplement minus vitamin A. The Olig2 concentration was 0.2 μM. P19 cells before addition of Olig2 (A). Culture without Olig2 (B, D, F) and with Olig2 (C, E, G). Scale bar represents 100 μm.

serum albumin (BSA) in TBS for 60 min, mouse anti β-III tubulin antibody (R&D Systems: MAB1195) was added (1:200 (v/v) dilution). After overnight incubation at 4 °C, fixed cells were washed with TBS containing 0.05% Tween 20 followed by addition of anti-mouse Alexa 488 conjugated antibody (Molecular Probes) diluted in TBS containing 1% (w/v) BSA and 0.1% Tween 20 at a 1:500 (v/v) dilution. After 1 h incubation, fixed cells were washed with TBS and incubated with Hoechst 33258 (1 μg/ml). Finally, cells were washed with TBS containing 0.05% Tween 20 and observed using fluorescence microscopy.

2.7. Evaluation of choline acetyltransferase mRNA expression by semi-quantitative RT-PCR

After five days of culture with or without Olig2 protein, total RNA was extracted from P19 cells using TRIzol reagent (Invitrogen) following the manufacturer's protocol. cDNA was constructed with SuperScript III Reverse Transcriptase and Oligo dT primer (Invitrogen). The cDNA was amplified by polymerase chain reaction using KOD plus polymerase (TOYOBO) with denaturing at 98 °C for 15 s, annealing at 60 °C for 25 s and extension at 72 °C for 45 s. The reaction cycles were 30 cycles for choline acetyltransferase (*ChAT*: GenBank BC119322.1) and 19 cycles for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*: GenBank NM_008084). Primer sets for each PCR were as follows: *ChAT* (GCCTGGTATGCCTGGATGGTC and TGGAGGGCCACCTGGATGAAG) and *GAPDH* (CCATCACCATC TTCCAGGAG and GCATGGACTGTGGTCATGAG). After PCR, samples were separated by agarose gel electrophoresis and stained with Ethidium Bromide. Fluorescent image was analyzed using Image J software.

3. Results and discussion

3.1. Transduction of Olig2 and its variant proteins

In general, to achieve protein transduction, it is necessary to add a protein transduction domain (PTD), such as HIV-1 TAT PTD and arginine repeats sequences [16–18]. However, several proteins such as bHLH transcription factors possess their own PTDs [11,12,15]. In our previous experiments, NeuroD2 and MyoD proteins were shown to have transduction abilities and the essential sequences for protein transduction were clarified. Because Olig2 is a bHLH transcription factor, it was expected that it also possessed transduction ability. To clarify its transduction ability, Olig2 and its variant proteins were expressed and purified. Studies have shown that the protein transduction abilities of other bHLH transcription factors are due to their nuclear localization sequences (NLS) and/or basic domains [11,12,15]. In Olig2, an NLS was not identified, but an NLS-like (NLS-L) sequence was predicted. Based on such information, variants which lacked the NLS-L and/or basic domain were constructed. Constructed proteins are shown in Fig. 1A. All proteins were expressed in *E.coli* and purified using His-tags. After purification, proteins were confirmed by SDS–PAGE (Fig. 1B). All proteins appeared larger than expected, due to the Olig2 sequence. After evaluation of protein concentrations, purified proteins were modified with fluorescent molecules and added to P19 cells. Fig. 1C shows cells observed using laser scanning confocal microscopy. Control cells (treated only with fluorescent dye), did not show fluorescence. However, cells with Olg2 protein showed strong fluorescence. These results showed that Olig2 itself had a transduction ability similar to other bHLH transcription factors. Variants lacking an NLS-L and a basic domain did not show a fluorescent signal. Other variants that included a basic domain and/or an NLS-L showed a fluorescent signal but they were much weaker than Olig2. Chen et al. reported that an HLH sequence was also important for protein transduction [19]. However, variants with an HLH sequence did not show fluorescence. Therefore, its requirement for transduction is unclear. However, from these results, it was shown that Olig2 is capable of transduction.

3.2. Induction of differentiation by addition of Olig2 protein to P19

Olig2 plays important roles in development of the central nervous system. It was reported that Olig2 was essential for MN and

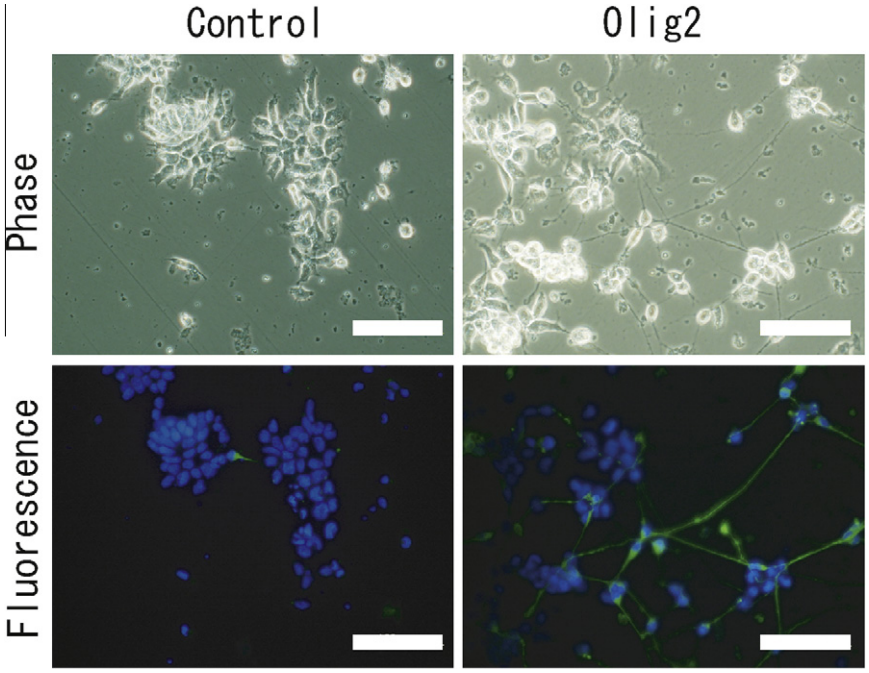

Fig. 3. Immunostaining of the neural differentiation marker, β-III tubulin. Upper columns were observed using phase contrast microscopy. Lower columns were observed using fluorescence microscopy. β-III tubulin (Green) and Hoechst 33258 stained nuclei (Blue). Scale bars represent 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

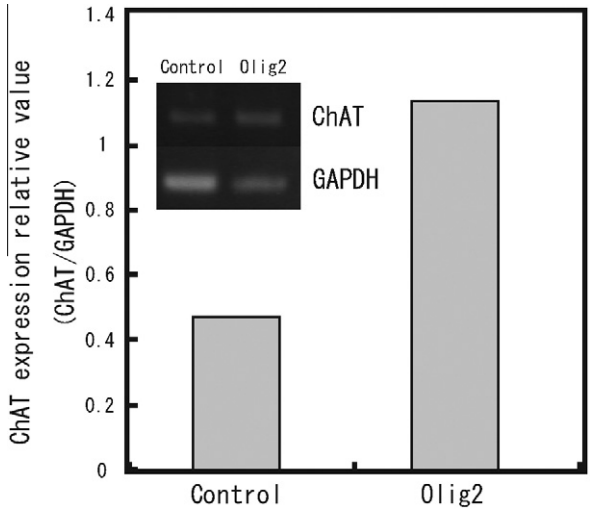

Fig. 4. Evaluation of motor neuron differentiation by RT-PCR. Expression of choline acetyltransferase mRNA was evaluated. The fluorescent signals were analyzed with Image J software and normalized to *GAPDH* mRNA expression signals.

OL generation. Here, P19 cells were treated with Olig2 protein. Before addition of Olig2, cells were attached to one another (Fig. 2), due to E-cadherin expressed by undifferentiated P19 [20]. However, following addition of Olig2 protein, cells were dispersed within 24 h. The data suggested that E-cadherin expression was decreased by addition of Olig2 protein. After three days, P19 cells cultured without Olig2 were still attached to one another, while cells cultured with Olig2 showed elongation characteristic of neurites. After five days of culture with Olig2 protein, cells were elongated compared to control cells. Differentiation was confirmed by immunostaining for β-III tubulin (Fig. 3). This protein is well expressed in neurons and is used as a neural marker. In P19 cells, β-III tubulin expression was increased by addition of retinoic acids that induced neural differentiation [21]. Cells cultured without Olig2 protein were not stained by anti-β-III tubulin antibody. On the other hand, cells cultured with Olig2 protein showed a strong fluorescent signal. These results showed that addition of Olig2 protein induced neural differentiation in P19 cells.

3.3. Evaluation of motor neuron differentiation by ChAT mRNA expression

Neural differentiation of P19 was confirmed by immunostaining. As described before, Olig2 has the ability to induce MN and OL differentiation. Thus, MN differentiation was evaluated by assessing *ChAT* mRNA expression (Fig. 4). Choline acetyltransferase is an enzyme that converts Acetyl-CoA to choline and is well known as an MN-specific marker. The expression of *ChAT* mRNA in P19 cells cultured with or without Olig2 protein was evaluated by semi-quantitative RT-PCR. The PCR cycles were stopped before saturation of amplification and normalized to *GAPDH* mRNA expression. *ChAT* mRNA expression of P19 cell cultured with Olig2 was not strong, but about twice that of cells cultured without Olig2. We also evaluated the expression of myelin basic protein (MBP), a marker of OL. However, the expression of *MBP* mRNA in P19 cells culture with Olig2 was lower than in cells cultured without Olig2 (data not shown). From these results, it was shown that Olig2 protein could induce P19 cells to differentiate toward MN cells.

The mechanism by which Olig2 induces MN differentiation is still unclear. Novitch et al. reported that Olig2 promotes the expression of MNR2 and/or Lim3 homeodomain proteins, which are motor neuron determinants, by repression of Irx3 homeodomain protein expression [22]. Recently, Li et al. reported that phosphorylation of Olig2 regulates MN and OL differentiation [23]. In their experiments, OL differentiation was induced by introduction of an Olig2 mutant with an altered phosphorylation site. They suggested that phosphorylated Olig2 proteins form stable homodimers, whereas unphosphorylated Olig2 prefers to form heterodimers with other bHLH proteins such as Neurogenin 2. Therefore, by addition of phosphorylation signals, Olig2 protein transduction could effectively induce MN differentiation. Moreover, by introduction of an unphosphorylated Olig2 mutant and a wild-type Olig2 protein, it might be possible to regulate MN and OL differentiation.

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