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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).

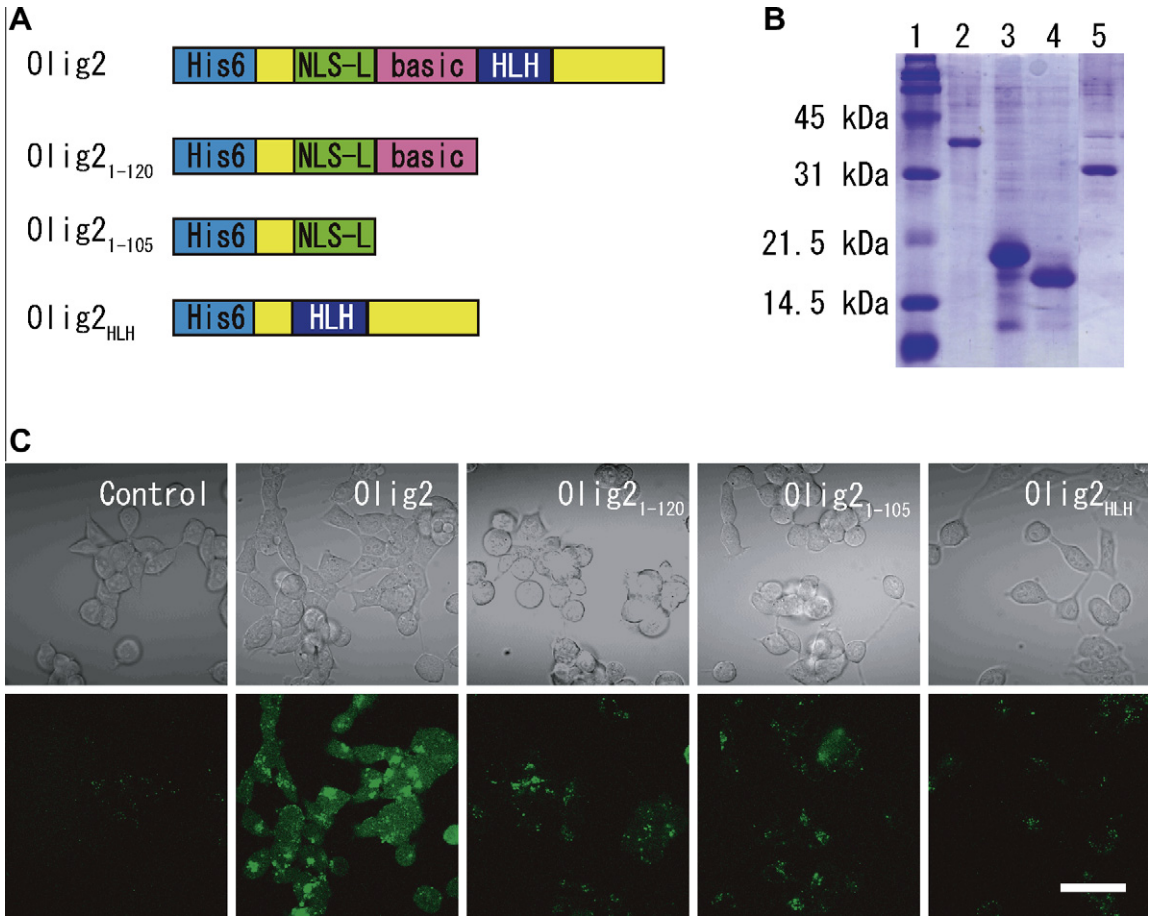

Fig. 1. Transduction ability of Olig2 and its variant proteins. (A) Schematic drawings of constructed proteins. NLS-L represents the NLS-like sequence located from position 95 to 105. Basic represents the basic domain of the basic helix-loop-helix sequence. Olig2 is a full Olig2 sequence with a His-tag (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.