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Myt/NZF family transcription factors regulate neuronal differentiation of P19 cells

Toshiki Kameyama[a,b,*], Fumio Matsushita[b,c], Yuzo Kadokawa[b,d], Tohru Marunouchi[b]

[a] Division of Gene Expression Mechanisms, Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Aichi 470-1192, Japan
[b] Division of Cell Biology, Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Aichi 470-1192, Japan
[c] Department of Biology, School of Health Science, Fujita Health University, Toyoake, Aichi 470-1192, Japan
[d] Department of Biology, School of Medicine, Fujita Health University, Toyoake, Aichi 470-1192, Japan

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ABSTRACT

During mammalian central nervous system development, neural stem cells differentiate and then mature into various types of neurons. Myelin transcription factor (Myt)/neural zinc finger (NZF) family proteins were first identified as myelin proteolipid protein promoter binding factors and were shown to be involved in oligodendrocyte development. In this study, we found that Myt/NZF family molecules were expressed during neuronal differentiation *in vivo* and *in vitro*. Transient over-expression of Myt/NZF family genes could convert undifferentiated P19 cells into neurons without induction by retinoic acid (RA), and the ability of these genes to induce neuronal differentiation was comparable to that of Neurog1 and Neurod1. Additionally, we found that St18 (or NZF-3) was induced by several bHLH transcription factors. When NZF-3 and Neurog1 were co-expressed in P19 cells, the rate of neuronal differentiation was significantly increased. These data suggest not only that NZF-3 works downstream of Neurog1 but also that it plays a crucial role together with Neurog1 in neuronal differentiation.

Myelin transcription factor 1 (Myt1)/neural zinc finger 2 (NZF-2), a protein with six clustered Cys-X_5-Cys-X_{12}-His-X_4-Cys (C2HC)-type zinc finger motifs, was first identified as a myelin proteolipid protein (PLP) promoter binding factor [1] and was shown to be involved in oligodendrocyte development [14,19]. In *Xenopus* nervous system development, Myt1 (X-MyT1) has been shown to be expressed during primary and secondary neurogenesis and to act downstream of Neurog2 (X-NGNR-1), in part by allowing cells to escape lateral inhibition and to differentiate during later phases of neuronal specification [4]. Other similar molecules, including myelin transcription factor 1-like (Myt1l or NZF-1) and suppression of tumorigenicity 18 (St18 or NZF-3), have been identified in rat, mouse, *Xenopus* and human nervous tissue [4,9,10,17,24,25]. However, little is known about the function of the Myt/NZF family gene products in mammalian neuronal differentiation.

We investigated the ability of the Myt/NZF family to direct neuronal differentiation using pluripotent mouse P19 embryonal carcinoma cells. P19 cells are derived from mouse embryos and have been used as a model system for *in vitro* differentiation [2]. After treatment with a high dose of retinoic acid (RA) and cellular aggregation, P19 cells differentiate into neurons and glia; however, under other conditions, these cells can differentiate into skeletal or cardiac muscle, endoderm or other cell types [11,18]. Numerous genes are known to be activated in P19 cells in response to RA treatment and are involved in the differentiation of the cells themselves. Transient transfection of some of the neuronal basic helix-loop-helix (bHLH) proteins can differentiate P19 cells into neurons without RA treatment [6]. In this report, we demonstrate that Myt/NZF family transcription factors are expressed during neural induction and differentiation of P19 cells and can cause differentiation into neurons without RA treatment, similar to bHLH proteins.

Mouse embryos were fixed at 11.5 days postcoitum (dpc) with 4% paraformaldehyde in PBS at 4 °C overnight. Fixed embryos were washed twice with PBS and dehydrated stepwise with ethanol. The fixed and dehydrated embryos were transferred into methyl benzoate and benzene as mediators and then embedded in paraffin. Serial paraffin sections (10 μm) were processed for immunohistochemistry and *in situ* hybridization. *In situ* hybridization experiments using digoxigenin-labeled mouse NZF-1, NZF-2b and NZF-3 probes were performed as described previously [17]. Rehydrated paraffin sections were treated at room temperature with 0.3% hydrogen peroxide in methanol for 30 min, with blocking solution [2% skim milk (Nacalai Tesque, Japan)/0.3% Triton X-100 (Sigma–Aldrich, Japan) in PBS] for 1 h, and then incubated with anti-neural class III β-tubulin (TuJ1, 1:500, COVANCE) overnight at 4 °C. After several washes, the sections were incubated with biotin-labeled affinity-purified anti-mouse IgG secondary antibody (Kirkegaard & Perry Laboratories, MD, USA) in blocking solution for

* Corresponding author at: Division of Gene Expression Mechanisms, Institute for Comprehensive Medical Science, Fujita Health University, 1-98 Dengakugakubo, Kutsukakecho, Toyoake, Aichi 470-1192, Japan. Tel.: +81 562 93 9378; fax: +81 562 93 8834.
E-mail address: tkame@fujita-hu.ac.jp (T. Kameyama).

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1 h at room temperature. Immunoreactive signals were detected with the Vectastain Elite ABC kit (Vector) using diaminobenzidine and hydrogen peroxide as substrates for horseradish peroxidase.

P19 cells were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer at Tohoku University. P19 cells were cultivated in MEM-alpha medium (Sigma) with 10% fetal bovine serum (JRH Biosciences) and maintained as subconfluent cultures prior to experiments. Neuronal differentiation was performed as previously described [21].

Total RNA of undifferentiated, RA-inducing, differentiated and transfected P19 cells was isolated using an acid guanidium thiocyanate-phenol-chloroform extraction method [5]. Five micrograms of total RNA was reverse-transcribed using an oligo (dT) primer and SuperScriptII RTase (Invitrogen) according to the manufacturer's instructions. A total of 0.5 μl of RT product was amplified using ExTaq DNA polymerase for 25 cycles.

The sequences of the DNA primers were as follows: 1F (5′-AAGCGGTACTGCAAGAATGC-3′) and 1R (5′-TGTGGACAGGTTGAGAATGG-3′) for the detection of NZF-1, 2F (5′-AAGGCTGTGGACATTGAGG-3′) and 2R (5′-TGAGGTCATAGAGCTGCTGG-3′) for the detection of NZF-2b, and 3F (5′-GCAATGAGAGTCTGCTGAAGG-3′) and 3R (5′-TCGTTCTACACATGGATGCC-3′) for the detection of NZF-3.

We isolated an NZF-1 cDNA clone (clone 5-2-1; GenBank accession number AB212898) encoding the entire open reading frame of mouse NZF-1 from an embryonic mouse brain cDNA library [12] using an RT-PCR product as a probe (GenBank accession number U86338; nucleotides 211–4305). To identify the mouse homologue of NZF-3, we used degenerate RT-PCR to amplify the product from the total RNA of an E12.5 mouse brain (forward primer, 5′-GCCATGCATGARAAYGTWCTSAAG-3′; reverse primer, 5′-GTTGGCAGCCATGAGAGACTTCAG-3′). Using this product as a probe, we isolated a cDNA clone (GenBank accession number AB097467).

Open reading frames of NZF-1, NZF-2b and NZF-3 were cloned into the EcoRI site of a pCAGGS expression vector (a gift from Dr. Miyazaki, Osaka University) [20]. Full-length Neurogenin1 (Neurog1), Neurogenin2 (Neurog2), Neurogenic differentiation 4 (Neurod4) and Neurogenic differentiation 1 (Neurod1) were obtained using RT-PCR and subcloned into the EcoRI site of pCAGGS. To construct selection vectors, pCXP, a puromycine-resistant gene expression unit (PGK-puro) was introduced into the SalI site of pCAGGS. Then, to construct pCXP-EGFP and pCXP-nEGFP plasmids, EGFP or nEGFP, which is EGFP fused to a nuclear localization signal, was subcloned into the EcoRI site of pCXP.

A total of 1×10^5 cells were plated in a 12-well multiwell plate (Falcon 353043) prior to a 12-h transfection. Transfection was performed using the FuGENE6 transfection reagent (Roche) and the BoosterExpress reagent kit (Gene Therapy Systems) according to the manufacturer's instructions. Briefly, a total of 1 μg of plasmid (a mixture of 0.8 μg pCAGGS-NZFs and 0.2 μg pCXP-EGFP) and 3 μl of FuGENE6 was added to P19 cells. Four hours after transfection, Booster #2 reagent was added to the medium to a final concentration of 1×. Then 12 h after transfection was initiated, transfection reagent was removed using a medium change. Cells were dissociated by trypsinization and re-plated on poly-D-lysine- (20 μg/ml, Sigma) and fibronectin-coated (1 μg/ml, Sigma) cover slips 24 h after transfection. Transfected cells were partially selected using 0.5 μg/ml puromycin 24–48 h post-transfection. Eighty-four hours after transfection, transfected cells were subjected to RT-PCR or immunocytochemistry.

Transfected cells were labeled with 10 μM bromodeoxyuridine (BrdU) for 12 h before fixation. To visualize BrdU labeling, fixed cells were treated with 2N HCl for 15 min at room temperature and then neutralized using 0.15 M borate buffer (pH 8.5) for 15 min. Cells were then blocked for 1 h with 4% skim milk and 0.1% Triton X-100 in Tris-buffered saline (TBST). Anti-BrdU antibody (1:100, MBL) diluted in 4% skim milk in TBST was added to cells and allowed to incubate overnight at 4 °C. Alexa Fluor 594 goat anti-mouse (1:1000, Molecular Probes) was used as a secondary antibody.

Transfected cells were fixed for 1 h using 4% paraformaldehyde in PBS and then blocked overnight with 4% skim milk in TBST. Primary antibodies, TuJ1 (1:500) or GFAP (1:50, IMMUNON) diluted in 4% skim milk in TBST, were added to cells that were incubated overnight at 4 °C. Alexa Fluor 594 goat anti-mouse or anti-rabbit IgG (H+L) was used as a secondary antibody. Cells were mounted in Vectashield Mounting medium with DAPI (Vector Laboratories). Immunofluorescence was examined with a Zeiss Axioplan microscope and photographed with a Zeiss AxioCam-HRc.

Statistical analysis was performed using a Student's *t*-test to compare the mean of each group to that of a control pCAGGS transfectant group. A value of $p < 0.05$ was considered statistically significant.

First, we compared the expression patterns of Myt/NZF family genes during the development of nervous tissues. Transcripts of Myt/NZF family genes were detected in the subventricular zone (SVZ) of almost all regions of the central nervous system at 11.5 dpc (Fig. 1A–C and F.M. unpublished observation). During the development of the mouse cerebral cortex, many neocortical neurons are produced by neural precursor cells within the ventricular zone (VZ) surrounding the lateral ventricles of the embryonic cerebral wall [3]. Postmitotic young neurons, which express neuron-specific β-III tubulin, migrate radially toward the pial surface (Fig. 1D). NZF-3 displayed restricted expression to the SVZ where newly generated neurons are located (Fig. 1C). NZF-3 expression could only be detected in a very small number of VZ cells and could not be detected in the differentiated neurons located in the marginal zone (MZ). NZF-2b had a slightly broader expression pattern than NZF-3. NZF-2b was also strongly expressed in the SVZ. In addition, NZF-2b showed a dotted pattern of expression in the VZ and was expressed in the MZ (Fig. 1B and described previously) [13,17]. NZF-1 was expressed latest among the three members of this family during neuronal differentiation. NZF-1 transcripts were only detected in the MZ and overlapped with TuJ1 immunostaining (Fig. 1A and D and described previously) [10,24].

To investigate the function of Myt/NZF family genes in mammalian neurogenesis, we used P19 cells. First, we tested the expression profiles of Myt/NZF family genes in P19 cells during neuronal induction and differentiation using semi-quantitative RT-PCR. No NZF genes were expressed in undifferentiated P19 cells (Fig. 1E), but they were expressed after neuronal induction and differentiation. NZF-1 was very weakly induced by RA treatment and was strongly upregulated after neuronal differentiation. NZF-2 and NZF-3 expression was also induced by RA treatment, and both genes were strongly expressed after neuronal differentiation. These data suggest that NZF-2 and NZF-3 are expressed in differentiating and differentiated neurons, while NZF-1 is expressed mainly in differentiated neurons. These findings closely resemble the expression patterns of the NZF family genes *in vivo*.

In situ hybridization and RT-PCR analysis revealed the pattern of Myt/NZF family gene expression during neuronal differentiation. To investigate whether Myt/NZF family molecules are involved in neuronal differentiation, P19 cells were transiently cotransfected with plasmid expression vectors for Myt/NZF family molecules, neural bHLH family molecules and green fluorescent protein (GFP). Cells were cultured in a monolayer in media without RA. Transfected cells could be identified by GFP fluorescence. First, we demonstrated the importance of NZF-3, which has the most restricted