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Overexpression of MCT8 enhances the differentiation of ES cells into neural progenitors

Mika Sugiura [a], Masato Nagaoka [a], Hikaru Yabuuchi [b], Toshihiro Akaike [a,*]

[a] *Department of Biomolecular Engineering, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259-B-57 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan*
[b] *GenoMembrane Inc., 501 Leading Venture Plaza 75-1, Ono-cho, Tsurumi-ku, Yokohama 230-0040, Japan*

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Abstract

Embryonic stem (ES) cell differentiation is regulated by cytokines and growth factors, as well as small-compound chemicals incorporated into cells by transporter proteins. Little is known regarding the effect of transporters on ES cell differentiation. This study focused on the effect of transporters during the neural-lineage differentiation of ES cells. Among the 27 types of SLC family transporters, MCT8 expression was coincident with that of neural stem cell markers, and the overexpression of MCT8 accelerated the differentiation into neural cells. These results suggested that the transporters and their substrates also play a crucial role in the regulation of ES cell differentiation.

Keywords: ES cells; Transporter; Differentiation; Slc family; MCT8 (slc16a2); Neural cells

Embryonic stem (ES) cells are derived from the inner cell mass of a blastocyst [1,2]. ES cells by definition have the potential to differentiate into any cell type *in vivo* and *in vitro* [3]. These cells are therefore valuable tools for identifying key molecules in development and for differentiation into specific cell lineages. Much research has focused on supplementing cells with soluble proteins for targeted differentiation, however few studies have investigated the small compounds important in differentiation that are incorporated into cells by transporter family proteins.

The solute carrier (SLC) family of transporters is critical for a variety of cellular physiological processes including the import and export of neurotransmitters, nutrients, and metabolites. More than 300 SLC genes have been cloned and each SLC family transports specific substrates, such as amino acids, oligopeptides, sugars, monocarboxylic acid, organic cations, anions, phosphates, nucleosides, metals, or water-soluble vitamins.

This study investigated the role of SLC family transporter proteins during neural differentiation from mouse ES cells. We characterized the expression pattern of SLC family proteins during this differentiation process and analyzed the direct effect of forced SLC transporter expression on ES cell differentiation.

Materials and methods

Cell culture. Feeder-free murine ES cells, EB3 [4] and MG1.19 [5], were maintained on 0.1% gelatin-coated surfaces in KNOCKOUT-DMEM (Invitrogen) or GMEM (Sigma Chemical) supplemented with 1 mM sodium pyruvate, respectively. Each culture media contained 1 mM L-glutamine, 1% nonessential amino acids

Abbreviations: afp, α-fetoprotein; CNS, central nervous system; EB, embryoid body; ES cells, embryonic stem cells; *gfap*, glial fibrillary acidic protein; ITS, insulin–transferrin–selenium; LIF, leukemia inhibitory factor; *map2*, microtubule-associated protein 2; MCT, monocarbonate transporter; slc, solute carrier; *ttr*, transthyretin; *vtn*, vitronectin.

* Corresponding author. Fax: +81 45 924 5815.
E-mail address: takaike@bio.titech.ac.jp (T. Akaike).

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(Invitrogen), 0.1 mM β-mercaptoethanol (Sigma Chemical), 10% fetal bovine serum (FBS) and 1000 U/ml ESGRO® (Chemicon). HEK293 cells were cultured in DMEM (Sigma) supplemented with 10% FBS.

RT-PCR analysis. Total RNA was isolated with Trizol reagent (Invitrogen). First strand cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen), and PCR was carried out with rTaq polymerase (TOYOBO). Primers used in this study are listed in Supplementary Table 1, which is available online. PCR products were analyzed by 1% agarose gel electrophoresis.

Cloning of mouse MCT8 and construction of the expression vector. The cDNA encoding mouse MCT8 was amplified with KOD plus polymerase (TOYOBO) from mouse liver cDNA using the specific primer pair for mouse MCT8: 5′-CTCGA GCCCC TAGCC ACGAT GGCGC T-3′ and 5′-CTCGA GAGGA GCACA CAATG GCAAG A-3′; underlining represents the XhoI recognition site. pGEM-T easy vector containing mouse MCT8 cDNA was digested by Xho1, and subcloned into pHPCAG vector [6] to produce pHPCAG/MCT8. pHPCAG is a polyoma-based episomal expression vector reported to be stable in ES cells. Sequence was confirmed by Hitachi bioscience systems.

Transport assay. Since rat MCT8 was reported to transport thyroxine [7], transport activity of cloned MCT8 was measured as [^{125}I] thyroxine uptake. HEK293 cells were transfected with pHPCAG/MCT8 vector (MCT8/HEK293 cells). At 48 h after transfection, cells were harvested and suspended in the transport buffer (125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 25 mM Hepes pH 7.4). The cell suspension and a solution containing 0.1 nM [^{125}I] thyroxine in transport buffer were separately incubated at 37 °C for 10 min and then mixed to initiate transport. At appropriate incubation times, 100-μL aliquots of the mixture were withdrawn, and the cells were separated by centrifugation through a layer of silicon oil mixed with liquid paraffin to a density of 1.03 g/ml. The cell pellets were solubilized in 50 μL 3N KOH, and then neutralized using 150 μL 0.1 M HCl. The associated radioactivity was quantitated in a liquid scintillation counter (Beckman). For normalization, the protein concentration of each cell lysate was determined using the *DC* Protein Assay kit (Bio-Rad Laboratories).

Western blotting. Total protein was extracted with RIPA buffer (10 mM Tris–HCl pH 7.8, 150 mM NaCl, 1% Nonidet P40, 0.1% sodium deoxyglycolate, 0.1% SDS, 1 mM EDTA, 8 M urea, 10 μg/ml aprotinin, and 1 mM PMSF). Samples were separated by 12% polyacrylamide gel electrophoresis and electrophoretically transferred to PVDF membrane (Immobilon-P; Millipore). Blots were probed with anti-MCT8 polyclonal antibody (Chemicon) followed by horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (Jackson Immunoresearch Laboratories), and developed by ECL reagent (GE Healthcare Biosciences).

Induction of neuronal differentiation in ES cells. The hanging drop method was used to induce multilineage differentiation of ES cells and neuronal differentiation was carried out as previously described [8]. Briefly, undifferentiated ES cells were suspended without leukemia inhibitory factor (LIF) and spotted onto a petri dish to form EBs (day 0). Two days later, cells were transferred onto a 0.5% agarose gel-coated dish (day 2). EBs were then adhered onto a 0.1% gelatin-coated culture dish (day 4). After 24 h, the medium was changed to DMEM/F12 (SIGMA) containing ITS-G (SIGMA) and 5 μg/ml fibronectin (KOKEN), and cultured for 1 week. Cells were trypsinized and plated onto dishes coated with 15 μg/ml poly-L-ornithine (SIGMA) and 1 μg/ml laminin I (KOKEN) (day 12). Cells were cultured for an extra 6 days in DMEM/F12 containing ITS-G, 20 nM progesterone (SIGMA), 100 μM putrescine (SIGMA), 1 μg/ml laminin I, 10 ng/ml bFGF, and 20 ng/ml EGF. Cells were harvested on days 0, 4, 12, and 18.

To clarify the effect of MCT8 on neuronal differentiation, MG1.19 cells were transfected with pHPCAG/MCT8 using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions (MCT8/ES cells). Transfected cells were selected for 1 week by culturing in the presence of 80 μg/ml hygromycin B (nacalai tesque) and were differentiated into neural precursor cells as described above.

Results and discussion

Expression pattern of SLC family transporters during neural differentiation of ES cells

First, we studied the expression pattern of marker genes for three germ layer derivatives (Fig. 1A). Although early neural markers (*neurod3*/*ngn1* and *sox-1*) were observed from day 12, neither the neuronal marker (*map2*) nor astrocyte marker (*gfap*) could be detected throughout differentiation. The expression of *nestin*, a marker of neural precursor cells, appeared from day 12 and progressively increased until day 18. Mesodermal markers (*flk-1* and *hbb*) and endodermal markers (α-*fetoprotein*, *transthyretin*, and *vitronectin*) were expressed strongly on day 12, but had disappeared by day 18. These results indicated that three germ layer-derived cells coexisted at day 12, and that neural cell selection was achieved by day 18. Next, we investigated the expression of SLC family transporters during neural differentiation. Among the 27 transporters tested, the expression of MCT8 coincided with that of neural stem cell markers (Fig. 1B). MCT8 was not expressed in undifferentiated ES cells, but gradually increased following differentiation. The expression pattern of MCT8 was similar to that of nestin, indicating MCT8 induction parallels neural differentiation. MCT8 (slc16a2) is a member of the monocarboxylate transporter (MCT) family, expressed in brain, liver, kidney, and heart [9]. Thyroid hormone transport activity was demonstrated recently in the rat homologue of MCT8 by *in vitro* expression of this protein in *Xenopus* oocytes causing a 10-fold increase in the uptake of iodothyronines [7]. These thyroid hormones are important in the neural function of mammalian CNS, particularly during critical developmental stages [10]. Iodothyronine deficiency induces severe brain dysfunction, causing irreversible cretinism and hypothyroidism that may seriously damage neural cells [11,12]. These findings suggested that expression of MCT8 proteins is essential for normal neural development; further experiments in this study therefore focused on this molecule.

Cloning of mouse MCT8

The cloned *MCT8* mouse gene had no double repeated PEST sequence and showed high homology to human MCT8 (93.6%) and rat MCT8 (98.5%); this was slightly different from the gene reported previously [13] (AF045692; NM_009197) (Fig. 2). To determine whether our cloned gene was the correct MCT8 cDNA, we designed primers on both sides of PEST domain and performed PCR using cDNA prepared from the liver, heart, and brain of fetal or adult mice, and cDNA obtained from differentiated mouse ES cells. As a result, only the gene containing a single PEST sequence was detected (data not shown), as with the MGC sequence [14] (BC080678). The obtained gene (AB265788) was confirmed to encode mouse MCT8 protein, and we used this gene for further experiments.