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Research paper

Analysis of ZIP (Zrt-, Irt-related protein) transporter gene expression in murine neural stem/progenitor cells

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ABSTRACT

Zinc plays important roles for brain development. Zrt-, Irt-related protein (ZIP) is a major transporter family to regulate the intracellular zinc levels. Neural stem/progenitor cells (NSPCs) are more sensitive than their differentiated progeny (neural/glial cells) to zinc *in vitro* (Nishikawa et al., 2015). We analyzed relative gene expression of 14 different ZIPs in murine NSPCs and differentiated cells by real-time polymerase chain reaction technique. Expression of *Zip4* and that of *Zip12* drastically increased, while that of *Zip8* clearly decreased after differentiation of NSPCs. Downregulation of NSPC's marker (*Nes*) and upregulation of differentiated cell markers (*Tubb3*; neuron, *Gfap*; astrocyte) occurred simultaneously. ZIP8 protein was immunochemically detected both in cultured neurospheres consisting of NSPCs *in vitro* and in subventricular zone of embryonic mouse brain *in vivo*, like a novel surface marker of NSPCs. We considered that required types of ZIP changed during the differetiation of NSPCs.

1. Introduction

Zinc is the second most abundant essential trace element and performs various physiological roles as structural components of proteins, cofactor of enzymes and mediator in signal transduction (Vallee and Falchuk, 1993; Maret and Li, 2009; Kambe et al., 2015). During the development of central nervous system (CNS), zinc is essential for the proper functioning of zinc binding protein such as Klf4, a transcription factor keeping the undifferentiated state of NSPCs and matrix metalloproteinase which regulates migration of cells (Small and Crawford, 2016). It was also reported that zinc deficiency in pregnancy caused abnormality in development of mouse fetal CNS (Tian et al., 2014).

Neural stem/progenitor cells (NSPCs) are capable to self-renew in immature state and are also multipotent to differentiate into both neurons and glial cells of CNS (Reynolds et al., 1992). NSPC is a promising tool to supply neuronal cells for cell-transplantation therapy of intractable CNS diseases and those for pharmacological assay in drug screening since they can be proliferated *in vitro* and differentiated into neuronal and glial cell (Iwanami et al., 2005; Mori and Hara, 2013).

Mammalian CNS development is a very complex multi-step process (Silbereis et al., 2016). Simply speaking, it proceeds by proliferation of immature neural stem cells, differentiation of the cells into neurons, glial cells (astrocytes and oligodendrocytes) with different timing and migration of those cells into proper location to form layer structure of brain. Neurons are mostly produced in prenatal stage and early neonatal stage of brain development. Astrocytes are produced much in neonatal stages while oligodendrocytes, myelin-forming cells, are produced in later stages. Zinc is essential for normal development of central nervous system as previously reported (Di et al., 2001). Zinc deficiency in food reduce stem cell proliferation during brain development of mouse and also affect neurogenesis in adult brain resulting the change in stem cell proliferation, neuronal precursor survival and neuronal differentiation (Levenson and Morris, 2011). We hope to answer the question why zinc is necessary for normal development of central nervous systems and then intend to clarify the role of zinc ion on neurogenesis accompanied with change in expression of zinc transporters during the neurodevelopmental stages.

We previously demonstrated that zinc tolerance was strengthened during the differentiation of NSPCs into neuronal/glial cells. Those change in tolerance was accompanied with the upregulation of metallothioneins (MTs), metal-binding proteins (Nishikawa et al., 2015). MTs store cytosolic zinc and also attenuate the heavy metal-induced cytotoxicity by chelating these metals. Cellular zinc homeostasis is principally controlled by three major factors, MTs and two different families of zinc transporter proteins (importers and exporters), as proved by various studies on small intestinal epithelial cells and other cells (Szewczyk, 2013; Kimura and Kambe, 2016). Transporter proteins have been classified into two families, ZIP (Zrt-, Irt-related protein, Slc39a) of 14 different types and 9 types of ZnT (Zn

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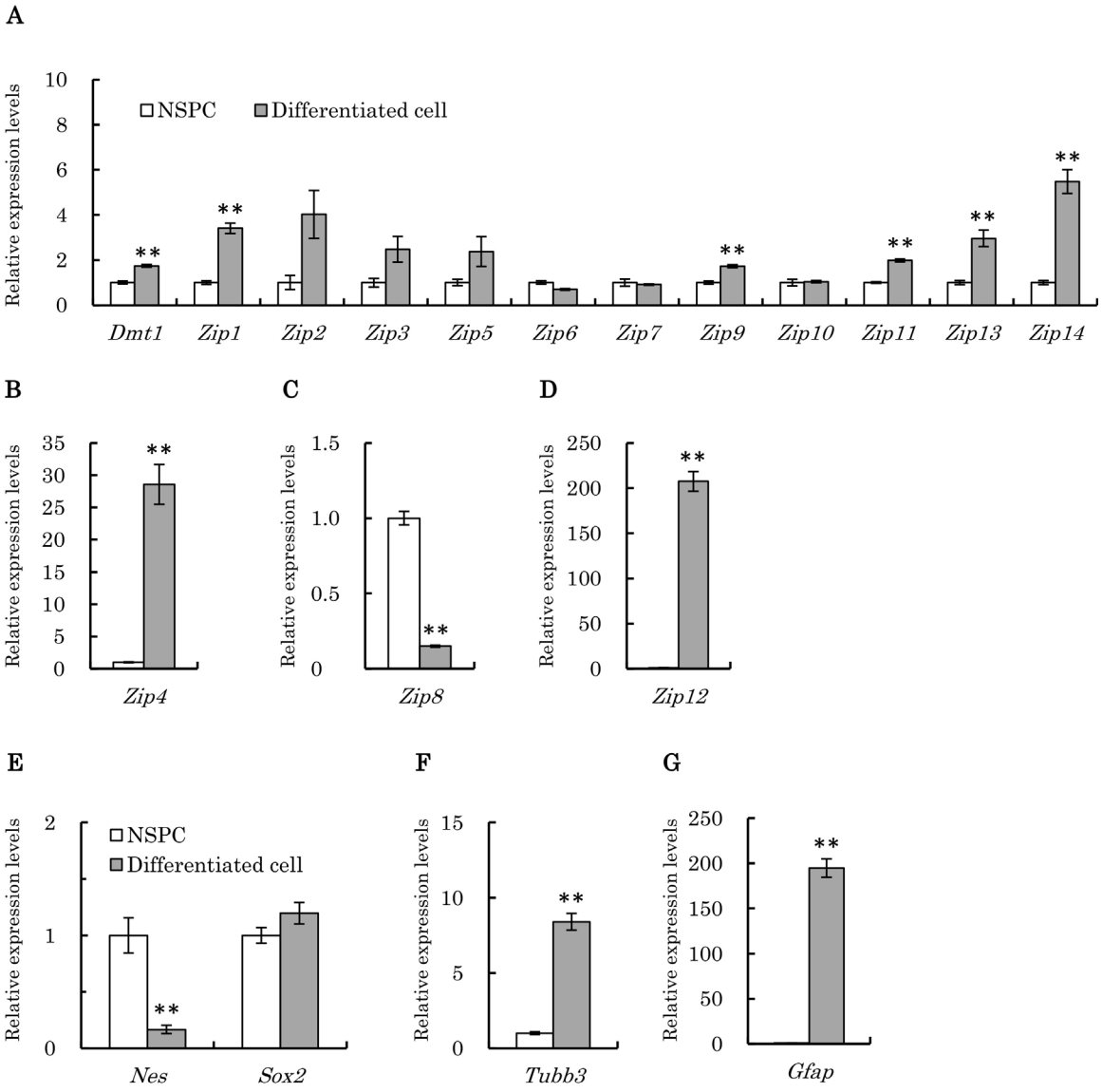

Fig. 1. Expression levels of ZIP family genes and that of cell differentiation marker genes in NSPCs (open bar) and the differentiated cells (gray bar) were determined by qRT-PCR. Dmt1, *Zip1, Zip2, Zip3, Zip5, Zip6, Zip7, Zip9, Zip10, Zip11, Zip13* and *Zip14* are shown in (A). *Zip4, Zip8* and *Zip12* are shown in (B), (C) and (D), respectively. NSPC marker *Nes* and *Sox2* are in (E). Neuronal marker *Tubb3* and astrocyte matker *Gfap* are (F) and (G), respectively. Expression levels are normalizing the amount of mRNA in NSPCs. Each bar represents the mean ± SD (n = 4, **: $p < 0.05$ via Student's *t*-test).

transporter, Slc30a) of 9 different types. ZIPs transport zinc from the extracellular space into cells or otherwise transport zinc out of organelles to increase the cytoplasmic concentration of free zinc. ZnTs transport zinc in the opposite direction to reduce cytoplasmic concentration of free zinc (Fukada and Kambe, 2011).

Little has been known about sensitivity of zinc and its regulatory mechanism in NSPCs until now although zinc is necessary for normal CNS development. Therefore, we focused on ZIP transporters for uptake of zinc into the cytoplasm, and examined their gene expression in mouse NSPCs and their differentiated cells to understand the regulatory mechanism of zinc in this study.

2. Materials and methods

2.1. Materials

Coverslips and glass slides were purchased from Matsunami Glass Ind., Ltd. (Kishiwada, Japan), PermaFluor mounting medium and cell culture plates from Thermo Fisher Scientific (Fremont, CA, USA), and poly-*L*-ornithine hydrobromide (PLO) and fetal bovine serum (FBS) from Sigma Aldrich (St. Louis, MO, USA). B27 Supplement and Alexa Fluor 568-conjugated goat anti-mouse IgG were purchased from Life Technologies (Carlsbad, CA, USA). FITC-conjugated goat anti-rabbit IgG was from Invitrogen (Carlsbad, CA, USA). Recombinant human epidermal growth factor (EGF) and recombinant human basic fibroblast growth factor (b-FGF) were obtained from PeproTech Inc. (Rocky Hill, NJ, USA). O.C.T. compound was from Sakura Finetek Japan (Tokyo, Japan). Normal goat serum was purchased from Vector Laboratories (Burlingame, CA, USA). Rabbit polyclonal anti-SLC39A8(ZIP8) antibody and mouse monoclonal anti-Nestin antibody (MAB353) were from ProteinTech Group, Inc. (Chicago, IL, USA) and Merck Millipore (Darmstadt, Germany), respectively. Solution of 4′,6-diamidino-2-phenylindole (DAPI) was from Dojindo Laboratories (Mashikimachi, Japan). Zinc chloride ($ZnCl_2$) was from Nacalai Tesuque (36920-24, guaranteed reagent grade; 98% purity; solubility in water 4.32 g/ml). All other reagents were from Nacalai Tesque (Kyoto, Japan).