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Ect2, an ortholog of *Drosophila Pebble*, regulates formation of growth cones in primary cortical neurons

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ABSTRACT

In collaboration with Marshall Nirenberg, we performed *in vivo* RNA interference (RNAi) genome-wide screening in *Drosophila* embryos. *Pebble* has been shown to be involved in *Drosophila* neuronal development. We have also reported that depletion of Ect2, a mammalian ortholog of *Pebble*, induces differentiation in NG108-15 neuronal cells. However, the precise role of Ect2 in neuronal development has yet to be studied. Here, we confirmed in PC12 pheochromocytoma cells that inhibition of Ect2 expression by RNAi stimulated neurite outgrowth, and in the mouse embryonic cortex that Ect2 was accumulated throughout the ventricular and subventricular zones with neuronal progenitor cells. Next, the effects of Ect2 depletion were studied in primary cultures of mouse embryonic cortical neurons: Loss of Ect2 did not affect the differentiation stages of neuritogenesis, the number of neurites, or axon length, while the numbers of growth cones and growth cone-like structures were increased. Taken together, our results suggest that Ect2 contributes to neuronal morphological differentiation through regulation of growth cone dynamics.

1. Introduction

RNA interference (RNAi) has been used successfully to screen large numbers of genes in *Caenorhabditis elegans* (Gonczy et al., 2000), *Drosophila* embryos (Kim et al., 2004), and *Drosophila* cell cultures (Boutros et al., 2004; Kiger et al., 2003) to identify genes that are involved in biological phenomena. In collaboration with Marshall Nirenberg, we previously performed *in vivo* RNAi to screen for *Drosophila* genes required in the development of the embryonic nervous system (Ivanov et al., 2004; Koizumi et al., 2007). Two genetic screening studies, including our RNAi screens, indicated that *pebble* is a gene affecting development of the *Drosophila* nervous system (Kraut et al., 2001; Prokopenko et al., 2000; Ivanov et al., 2004; Koizumi et al., 2007). However, the functional role of *Ect2*, a mammalian ortholog of *Pebble*, has not been examined in the development of the mammalian nervous system, except in pituitary development (Islam et al., 2010).

Ect2 and Pebble are guanine nucleotide exchange factors (GEF) that function during cytokinesis. They both activate RhoA (Schmidt and Hall, 2002), which regulates contractile ring formation through actomyosin contraction (Kishi et al., 1993; Mabuchi et al., 1993). Prokopenko et al. reported that Pebble mutants failed to form the contractile ring and showed defects in cytokinesis (Prokopenko et al., 1999). Tatsumoto et al. reported that downregulation of Ect2 function, either by dominant-negative Ect2 expression or by microinjection of anti-Ect2 antibody, blocked cell division and generated multinucleate cells (Tatsumoto et al., 1999).

We previously showed the induction of binucleate cells by Ect2 RNAi in mouse neuroblastoma × rat glioma hybrid NG108-15 cells, a useful neuronal model produced in Nirenberg's laboratory (Nirenberg et al., 1983; Puro and Nirenberg, 1976; McGee et al., 1978; Tsuji et al., 2011). Although there have been many analyses of Ect2 in cytokinesis, a recent study revealed possible functions of Ect2 in cellular differentiation and neuronal regulation; Smallhorn et al. reported that Pebble was involved in epithelial-mesenchymal transition and mesoderm migration in *Drosophila* (Smallhorn et al., 2004). Pebble was identified as a candidate substrate of UBE3A ligase, a gene responsible for Angelman syndrome that causes severe developmental delay and mental retardation, associated with autism in a certain ratio (Steffenburg et al., 1996), by two-dimensional gel and MALDI-TOF analyses (Reiter et al., 2006).

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Moreover, the expression level and pattern of Ect2 were remarkably altered in the hippocampus and cerebellum of UBE3A null mice (Reiter et al., 2006). However, no previous reports have revealed the precise roles of Ect2 in mammalian neuronal development.

In the present study, we showed that inhibition of Ect2 by RNAi also stimulated neurite outgrowth in PC12 cells, a nerve growth factor (NGF)-regulated adrenergic clone derived from pheochromocytoma cells (Greene and Tischler, 1976). Next, we examined Ect2 expression in the mouse embryonic cortex and found its accumulation throughout the ventricular and subventricular zone (VZ, SVZ). Furthermore, to assess the role of Ect2, RNAi was performed in primary cultures of mouse embryonic cortical neurons. We demonstrated that Ect2 depletion did not affect the defined stages of neuritogenesis (de Lima et al., 1997) of cultured cortical neurons. While neither the number of neurites nor axon length showed differences related to the loss of Ect2, the numbers of growth cones and growth cone-like structures were increased by Ect2 depletion.

2. Materials and methods

2.1. RNA interference in PC12 cells

PC12 cells were maintained in DMEM supplemented with 5% bovine serum and 10% horse serum (Torocsik et al., 2002). For Ect2 knockdown, siRNA Ect2 #1 (Ect2-RSS360274; Invitrogen, Carlsbad CA, USA) and #2 (Ect2-RSS360275; Invitrogen) were used. Stealth RNAi Negative Control Duplex (Invitrogen) was used for control RNAi. The siRNA (0.6 μl of 20 μM siRNA duplex) and 2 μl of Lipofectamine RNAiMAX (Invitrogen) were mixed in 200 μl DMEM and added to 1 ml of culture medium according to the manufacturer's protocol. After 48 h, NGF (50 ng/ml) was applied to the replaced culture medium (DMEM supplemented with 0.05% bovine serum and 0.1% horse serum). As the medium was replaced, the cells were again transfected with each siRNA. For morphological analyses, cells were seeded onto glass coverslips coated with poly-D-lysine (0.1 mg/ml; Sigma, St. Louis, MO, USA). Cells were fixed with 3.7% paraformaldehyde (PFA) and stained with DAPI. To determine the knockdown efficiency, the whole cell lysates of PC12 cells were subjected to SDS–PAGE, followed by immunoblotting as described (Islam et al., 2010).

2.2. Immunohistochemistry

All animal experiments were performed in Slc:ICR mice purchased from Japan SLC, Inc. (Hamamatsu, Japan). The experiments were carried out in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology of Japan. For immunohistochemistry, embryonic day (E) 14 mice were immersion fixed with 4% PFA in PBS for 16 h. Procedures for immunohistochemistry using cryosections were conducted essentially as reported (Islam et al., 2010).

2.3. Primary cultures of embryonic cortical neurons

Cultures of primary cortical neurons were prepared from E14 mice essentially as described with minor modifications (de Lima et al., 1997). Briefly, dissected cortical tissue was minced and dissociated with a solution of trypsin–EDTA in PBS for 5 min at 37 °C. Dissociation was completed by repeatedly passing the suspension through a Pasteur pipette. Dissociated cells (1×10^6 cells) were resuspended in 8 μl of Resuspension buffer (Invitrogen) with 60 pmol of siRNA Ect2 (Ect2-MSS203768; Invitrogen) or Stealth RNAi Negative Control Duplex. Neurons were transfected by electroporation (MicroPorator MP-100; NanoEnTek, Seoul, Korea) according to the optimized protocol for primary mouse neurons. Neurons were plated onto glass coverslips coated with poly-D-lysine (0.1 mg/ml; Sigma) and cultured in Neurobasal culture medium supplemented with 2% B27, 0.5 mM L-glutamine, and 1% FBS. After 24 h, medium was replaced with serum-free culture medium. Neurons were fixed 48 h after plating with 3.7% PFA and stained with Texas Red-X phalloidin (Invitrogen). The efficiency of knockdown was examined by immunoblotting and RT-PCR analysis as described previously (Islam et al., 2010; Tsuji et al., 2011).

2.4. Analysis of cell morphology

Images were taken using an Olympus IX71 inverted microscope equipped with a CoolSNAP HQ2 camera (Molecular Devices, Sunnyvale, CA, USA) to determine the morphology of PC12 cells and cortical primary neurons. Using the MetaMorph software (Molecular Devices, Sunnyvale, CA, USA), images were analyzed manually by the experimenter blind to the experimental groups. PC12 cells possessing at least one neurite with a length longer than one cell diameter ($\geqslant$22 μm) were defined as neurite-bearing cells. In the cortical primary neurons, growth cones or growth cone-like struc-

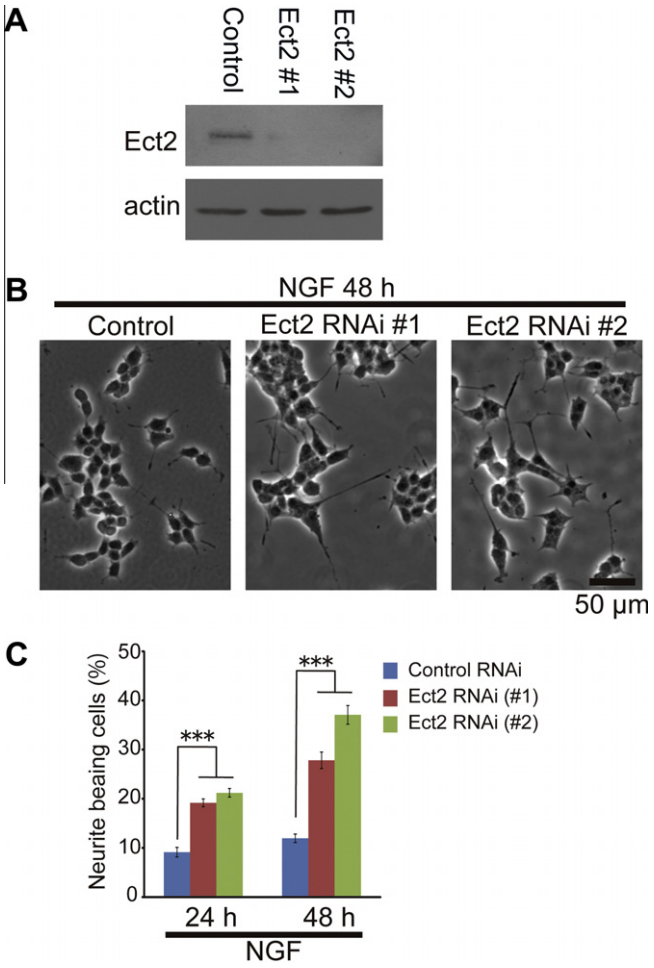

Fig. 1. Inhibition of Ect2 expression induces neurite formation by NGF-stimulated PC12 cells. (A) Depletion of Ect2 protein by siRNA treatment. PC12 cells were transfected with control siRNA or two Ect2 siRNAs, #1 and #2. After 48 h, the cells were harvested and subjected to immunoblotting with anti-Ect2 or anti-actin antibody. (B) Representative images of PC12 cells stimulated with NGF for 48 h. siRNAs were transfected 48 h prior to the application of NGF. (C) Frequencies of neurite-bearing PC12 cells (means ± SEM; $n = 4$ independent experiments, in each of which >368 cells were examined). $^{***}P < 0.001$ compared with controls.