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Induction by NeuroD of the components required for regulated exocytosis

Nobuko Ishizuka [a,b], Kohtaro Minami [a], Akinori Okumachi [a,b], Masaaki Okuno [a], Susumu Seino [a,c,*]

[a] Department of Experimental Therapeutics, Translational Research Center, Kyoto University Hospital, Kyoto, Japan
[b] Department of Diabetes and Clinical Nutrition, Graduate School of Medicine, Kyoto University, Kyoto, Japan
[c] Division of Cellular and Molecular Medicine, Kobe University Graduate School of Medicine, 7-5-1, Kusunokicho, Chuo-ku, Kobe 650-0017, Japan

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Abstract

NeuroD is a transcriptional factor critical in differentiation of neuronal cells, enteroendocrine cells, and pancreatic endocrine cells. However, little is known of its roles in cellular functions. We show here that introduction of NeuroD into human fetal epithelial cell line Intestine 407 cells induces neuron-like morphology. In addition, multiple genes associated with vesicular trafficking and exocytotic machinery, including Sec24D, carboxypeptidase E, myosin Va, SNAP25, syntaxin 1A, Rab, Rims, Munc18-1, and adenylyl cyclase, were up-regulated by NeuroD gene transfer. Moreover, low osmotic pressure-induced exocytosis monitored by FM1-43 was enhanced by overexpression of NeuroD. These results suggest that NeuroD plays an important role in regulated exocytosis by inducing expressions of various components required in the process.

Keywords: NeuroD; SNAREs; Rabs; Exocytosis

NeuroD (also known as BETA2) is a basic helix–loop–helix (bHLH) transcription factor expressed in pancreatic endocrine, enteroendocrine, and neuronal cells [1,2]. Lack of NeuroD expression in mice results in a reduction of mature pancreatic islets and decreased numbers of secretin- and cholecystokinin-producing enteroendocrine cells, leading to perinatal death [3]. When NeuroD expression is rescued by a transgene under the insulin promoter, the mice survive but exhibit severe loss of inner sensory neurons and granule cells in cerebellum and hippocampus [4,5]. In addition, NeuroD has been implicated in cell fate determination, differentiation, and cell survival in neurons [6,7]. These findings indicate that NeuroD is essential in normal development of pancreatic endocrine, enteroendocrine, and neuronal cells.

NeuroD is known to directly regulate the expression of genes in differentiated cells. NeuroD binds and transactivates the insulin gene upon dimerization with ubiquitous bHLH transcription factor E47 [8]. Sulfonylurea receptor 1 (SUR1), the regulatory subunit of the ATP-sensitive K^+ channel in pancreatic β-cells, is up-regulated by NeuroD [9]. Expression of glucokinase, the rate-limiting enzyme of glucose metabolism in pancreatic β-cells, also is activated by NeuroD [10]. In addition, NeuroD activates promoters of the islet-specific glucose-6-phosphatase catalytic subunit-related protein, secretin, and Pax6 [11–13]. However, the roles of NeuroD in the regulation of cellular functions are poorly understood.

The human fetal epithelial cell line Intestine 407 possesses undifferentiated enteric cell properties [14] without expression of NeuroD. IEC6, a cell line derived from rat immature intestinal epithelium, has been shown to differentiate into insulin-secreting cells by introduction of transcription factors important in pancreatic β-cell development [15,16]. These findings indicate that undifferentiated

Abbreviations: SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor; SNAP25, synaptosome-associated protein of 25 kDa; cAMP, cyclic AMP.

* Corresponding author. Fax: +81 78 304 6064.
E-mail address: seino@med.kobe-u.ac.jp (S. Seino).

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intestinal cells possess potency to differentiate into cells with endocrine phenotype. In the present study, we have used Intestine 407 cells to clarify the roles of NeuroD in the regulation of gene expression and exocytosis including vesicle trafficking and membrane fusion.

Materials and methods

Cell culture. Intestine 407 (Dainippon Sumitomo Pharma, Osaka, Japan) human fetal intestinal epithelial cells were cultured in DMEM (Sigma, St. Louis, MO) containing 10% fetal calf serum under humidified condition of 5% CO_2/95% air at 37 °C.

Adenoviruses. Human NeuroD recombinant adenovirus (Ad-NeuroD) was constructed under cytomegalovirus (CMV) promoter, using an adenovirus expression vector kit (Takara, Ohtsu, Japan) according to the manufacturer's instruction. Adenovirus expressing β-galactosidase (Ad-LacZ) was used as a negative control.

Immunoblotting of NeuroD. Cells were washed with PBS and lysed in a buffer containing 150 mM NaCl, 20 mM Tris (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM PMSF, and protein inhibitor cocktail (Nacalai Tesque, Kyoto Japan). The total cell lysates (20 μg of protein) were subjected to SDS–PAGE and separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA). The membranes were probed with anti-NeuroD antibody (N-19) (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-actin antibody (I-19) (Santa Cruz Biotechnology). The secondary antibody used was horseradish peroxidase-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology). Signals were visualized using ECL plus Western Blotting Detection System (GE Healthcare Bio-Sciences, Piscataway, NJ). Blocking peptide (N-19P) (Santa Cruz Biotechnology) for anti-NeuroD antibody was used to confirm specificity of the reaction.

Reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was isolated using the RNeasy Mini kit (Qiagen, Venlo, The Netherlands). After treatment with DNaseI (Qiagen), 1 μg of the RNA was reverse-transcribed by RevaTraAce (Toyobo, Osaka, Japan) with random priming. PCR primers were designed such that the amplified regions spanned introns in the gene. β-Actin was amplified to confirm equivalence between samples. The PCR conditions were as follows: denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 45 s. The cycle number was 35, 33, and 23 for glucokinase, SUR1, and β-actin, respectively.

For real-time RT-PCR analysis, TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) were used. All reactions and analyses were performed using ABI PRISM 7300 Sequence Detection System (Applied Biosystems). Gene expression levels were calculated using comparative threshold cycle methods according to ABI PRISM 7700 Sequence Detection System User Bulletin #2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The GAPDH expression level was unchanged even when NeuroD was overexpressed. Experiments were repeated four to seven times, each of which included duplicate measurements.

GeneChip analysis. Intestine 407 cells were infected with Ad-LacZ or Ad-NeuroD at 25 multiplicity of infection (moi). Two days after infection, total RNA isolated as described was subjected to GeneChip analysis. Labeled cRNA was synthesized and hybridized by Human Genome Focus Array (Affymetrix, Santa Clara, CA), which represents over 8500 human genes, according to the manufacturer's instruction. The probe arrays were washed and stained. The signals were scanned using GeneChip Scanner 3000 (Affymetrix). The scanning data were analyzed using Microarray Suite 5.1 (Affymetrix).

Measurement of exocytosis with FM1-43. Intestine 407 cells were seeded on a 96-well plate the day before infection. Cells were infected with Ad-LacZ or Ad-NeuroD (100 moi) and cultured for 72 h. After washing with Opti-MEM (Invitrogen), 2 μM FM1-43 (Molecular Probes, Eugene,

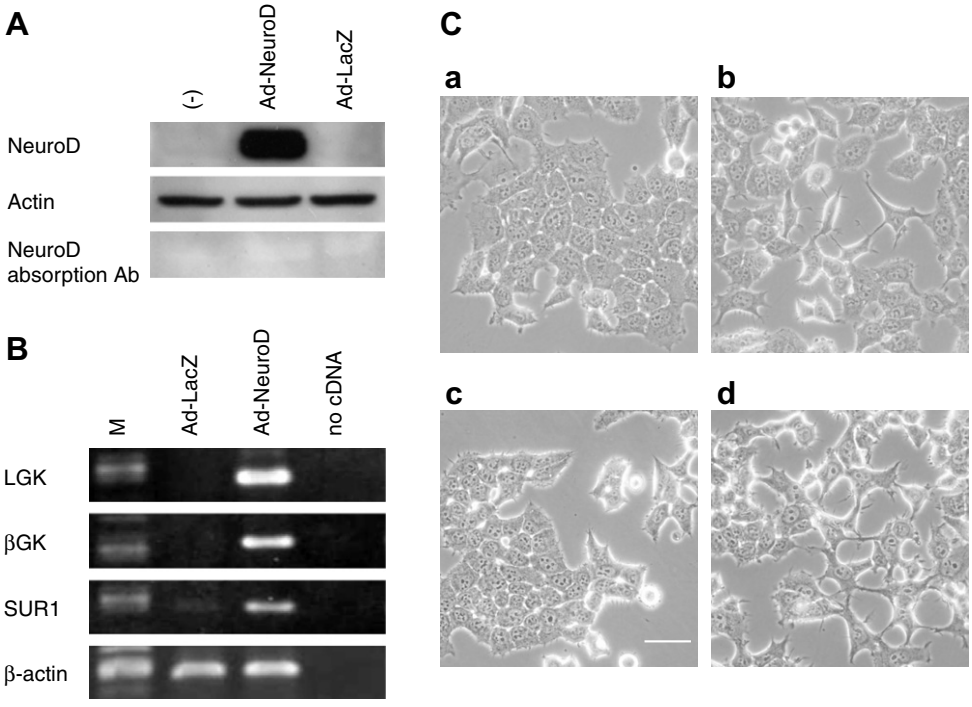

Fig. 1. Validation of adenovirus and morphological changes of the cells. (A) NeuroD expression by Ad-NeuroD infection. Intestine 407 cells were infected with Ad-NeuroD or Ad-LacZ (25 moi). NeuroD protein was detected by SDS–PAGE and immunoblotting. (−), without infection. (B) Induction of glucokinase and SUR1 by NeuroD overexpression. Total RNAs were isolated 48 h after infection. Gene expressions of glucokinase and SUR1 were assessed by RT-PCR. Lane M, molecular weight marker (Hinc II-digested ϕX174 DNA fragments); LGK, liver-type glucokinase; βGK, β-cell-type glucokinase. (C) Phase-contrast photomicrographs of NeuroD-infected cells 48 h after infection. Cells grown on a tissue culture dish were infected with Ad-LacZ (25 moi (a) and 100 moi (c)) or Ad-NeuroD (25 moi (b) and 100 moi (d)). Cells were photographed through a microscope. Bar, 50 μm.

OR) diluted with 50 μl of Opti-MEM was added to the cells. Fluorescence intensity was monitored by Fluoroskan Ascent CF fluorometer (Thermo Labsystems, Vantaa, Finland) [17], with excitation at 485 nm and emission at 590 nm. Baseline fluorescence was obtained after 5-min incubation at 37 °C. Subsequently, distilled water containing 2 μM FM1-43 was added to the cells for hypotonic stimulation [18,19].

Statistical analysis. Groups were compared using Mann–Whitney *U* test.

Results and discussion

Morphological changes induced by NeuroD gene transfer

To validate adenovirus vector expressing human NeuroD under control of CMV promoter (Ad-NeuroD) and to confirm usefulness of the Intestine 407 cells, we evaluated changes in the expression of genes known to be regulated directly by NeuroD. NeuroD protein expression was not detected in uninfected or negative control adenovirus (Ad-LacZ)-infected Intestine 407 cells, but was clearly detected in Ad-NeuroD-infected cells (25 moi) (Fig. 1A). As reported previously [9,10], expressions of β-cell-type glucokinase and SUR1 were induced by NeuroD overexpression. Liver-type glucokinase, the first exon of which is specific to the liver transcripts, was also induced (Fig. 1B).

NeuroD overexpression remarkably changed the cell morphology. While the LacZ-infected (control) cells were flat and cobblestone-like (Fig. 1C-a and -c), NeuroD-infected cells were changed to polygonal and spindle-like morphology in a moi-dependent manner. In addition, neuron-like processes were observed in NeuroD-infected cells (Fig. 1C-b and -d).

Comprehensive gene expression analysis of Intestine 407 cells with or without NeuroD gene transfer

We then analyzed global gene expression profiles of Intestine 407 cells with or without NeuroD gene transfer. Intestine 407 cells were infected with either Ad-NeuroD or Ad-LacZ (25 moi), and total RNA isolated 48 h after the infection was subjected to GeneChip analysis. The total number of up-regulated (>2.0-fold) and down-regulated (<0.5 fold) genes was 119 and 39, respectively (Supplementary Tables 1 and 2).

The expressions of Keratin 7 and Keratin 17, intermediate filaments in cytoplasm of epithelial cells [20], were decreased, and the expression of brain-specific α-tubulin, a major component of microtubules, was increased by NeuroD overexpression (Table 1). As brain-specific α-tubulin is especially enriched in regions actively undergoing neurite extension [21], the increased expression of brain-specific α-tubulin might contribute to the neuron-like cell shape in Ad-NeuroD-infected cells. These results suggest that NeuroD changes the composition of cytoskeleton from epithelial to neuronal.

While endocrine hormones were not detected by GeneChip analysis, a set of genes associated with exocytosis was found to be up-regulated by NeuroD (Table 1 and Supplementary Table 1). Since NeuroD is expressed in pancreatic endocrine, enteroendocrine, and neuronal cells, all of which manifest regulated exocytosis [22], we analyzed the effect of NeuroD expression on common required components in the exocytotic machinery.

Table 1
Partial list of genes up-regulated (>2-fold) or down-regulated (<0.5 fold) by NeuroD overexpression (for complete lists of up- and down-regulated genes, see Supplementary Tables)

Gene name	Fold change
Up-regulated genes	
Cell structure	
Tubulin, α, brain specific (TUBA3)	6.1
Ankyrin 3	11.3
Transcriptional regulation	
ID3	2.3
ID1	2.1
Neuronal differentiation	
Wolframin	2.1
Endocytosis	
Rab31	4.3
Myosin IV	3.2
Transporters	
ABCC4	2.8
ABCG1	14.9
SLC22A4	2.1
SLC7A8	2.3
SLC12A2	3.2
Cell cycle and apoptosis	
CDK inhibitor 1A	3.2
Channel	
KCNK3	2.8
CLCN5	2.5
Synaptic transmission	
Cholinergic receptor (nicotinic)	3.0
Exocytosis	
Sec24D	2.1
Carboxypeptidase E	3.2
Myosin Va	2.3
SNAP25	2.6
Rab27A	2.3
Rab26	4.9
Adenylate cyclase 7	4.0
Down-regulated genes	
Cell structure	
Keratin 17	0.31
Keratin 7	0.47
Enzyme	
Monoamine oxidase B	0.44
Transporters	
SLC16A6	0.38

ID, inhibitor of DNA binding; ABCC4, ATP-binding cassette, subfamily C (CFTR/MRP), member 4.
ABCG1, ATP-binding cassette, sub-family G (WHITE), member 1; SLC, solute carrier family; CDK, cyclin-dependent kinase; SNAP25, synaptosomal-associated protein, 25 kDa; CLCN5, chloride channel 5; KCNK3, potassium channel subfamily K, member 3.

Expression of genes involved in vesicle trafficking and exocytosis

A series of biological events is required for completion of exocytosis. Vesicles containing secretory products are

formed from endoplasmic reticulum (ER) membrane, which is coated by coat protein complex, and sorted at the *trans*-Golgi network [23,24]. The secretory vesicles are transported to the plasma membrane by several motor proteins moving along with microtubules or actin filaments [25]. The vesicles are then docked and fused with the plasma membrane, a process in which SNARE proteins are critically involved, to release secretory products. The

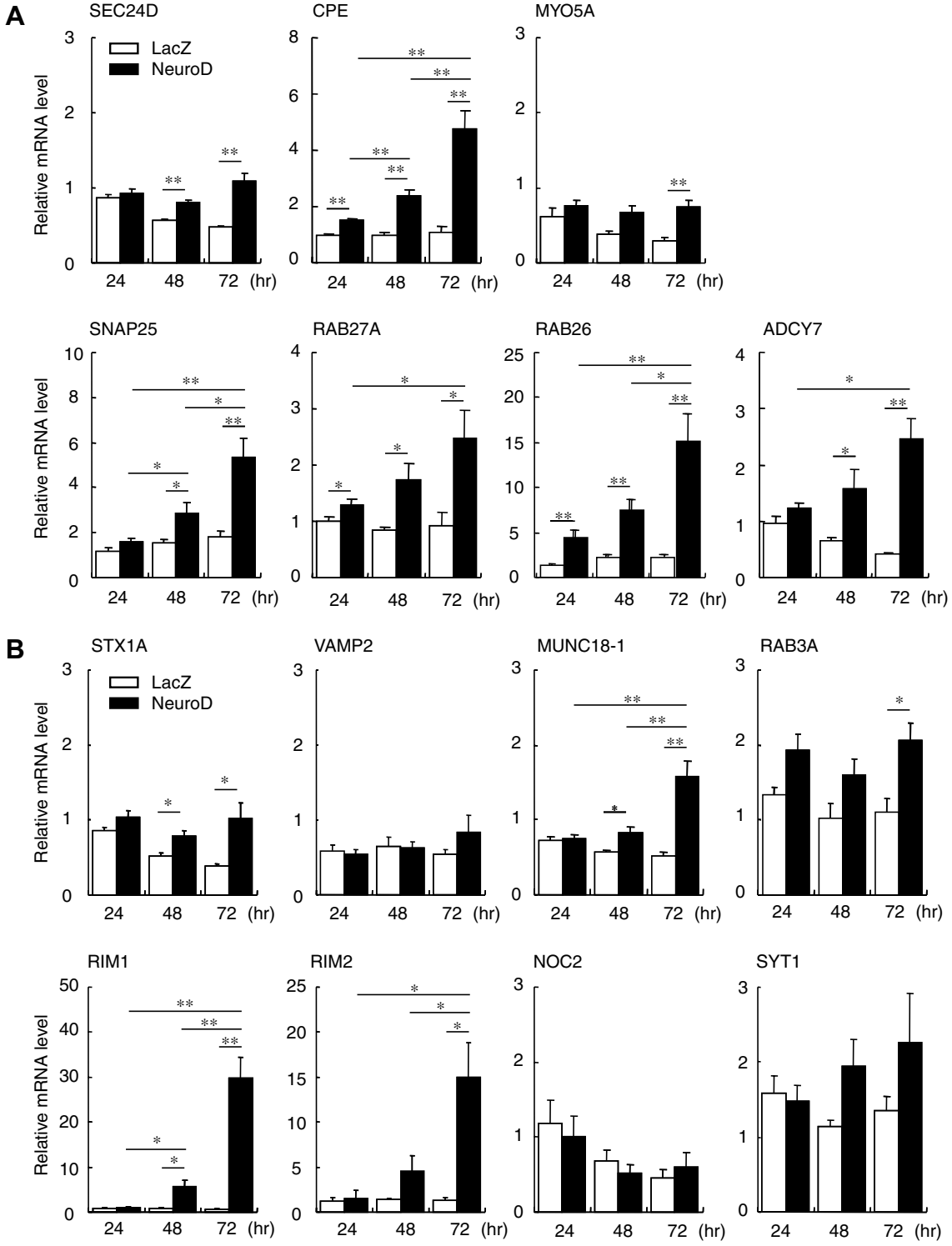

Fig. 2. Changes in expression of genes related to exocytosis in Intestine 407 cells after transfer of NeuroD gene by adenovirus system (25 moi). (A,B) Time-course analysis of changes in gene expression. Gene expression was assessed by real-time RT-PCR using comparison C_t method. PCRs were performed in duplicate. Experiments were repeated independently four to seven times. Data are expressed as average ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$. CPE, carboxypeptidase E; MYO5A, myosin Va; ADCY7, adenylyl cyclase 7; STX1A, syntaxin 1A; SYT1, synaptotagmin 1.

conserved components of the exocytotic machinery include SNAREs, SM proteins (Sec1/Munc18-like proteins), and Rab proteins [26–29]. Regulated exocytosis occurs in response to physiological stimulation, which causes a transient rise of intracellular second messengers such as Ca^{2+} [30]. While Ca^{2+} triggers exocytosis in many cell types, cAMP potentiates the process [22,31].

Sec24D, a component of the coat protein complex [23,32], was up-regulated by NeuroD expression (Table 1). Quantitative real-time RT-PCR analysis showed that the gene was up-regulated compared to LacZ control, but there was no time-dependent increase in the expression (Fig. 2A). The expression of carboxypeptidase E (CPE) was enhanced by NeuroD (Table 1). CPE acts as a sorting receptor both at the *trans*-Golgi network and secretory granules [24,33,34]. Up-regulation of the CPE gene was confirmed by real-time RT-PCR (Fig. 2A). Myosin Va, a motor protein participating in vesicle docking to the plasma membrane [25,35,36], was also up-regulated by NeuroD compared to LacZ control (Table 1 and Fig. 2A). A synaptosome-associated protein of 25 kDa (SNAP25) is one of the basic components of SNAREs, and Rab proteins are known to be involved in regulation of exocytosis [26,27,37–39]. The expressions of SNAP25, Rab27a, and Rab26 in NeuroD-infected cells were significantly elevated compared with those in LacZ-infected cells (Table 1 and Fig. 2A), and mRNAs of SNAP25, Rab27a, and Rab26 were increased in a time-dependent manner (Fig. 2A). We also found that adenylyl cyclase 7 (ADCY7), a widely expressed enzyme that synthesizes cyclic AMP from ATP [31], was up-regulated by NeuroD, and that mRNA of ADCY7 was increased in a time-dependent manner (Table 1 and Fig. 2A). Accordingly, NeuroD could well affect the expression of other genes that participate in the regulation of exocytosis but are overlooked due to detection sensitivity limitations of GeneChip analysis.

By use of quantitative real-time RT-PCR, changes in the expression of genes having roles in exocytosis, including SNARE proteins (syntaxin 1A and VAMP2), a SM protein (Munc18-1), Rab3A and its effectors (Noc2, Rim1, and Rim2), and a calcium-sensing protein synaptotagmin 1 [40–47] were evaluated. Among these, the expressions of syntaxin 1A, Munc18-1, and Rab3A were up-regulated by NeuroD, and mRNA of Munc18-1 was increased in a time-dependent manner (Fig. 2B). In addition, introduction of NeuroD remarkably increased expressions of both Rim1 and Rim2 (Fig. 2B). Since Rim1 is expressed predominantly in brain and Rim2 is found predominantly in neuroendocrine and endocrine cells [46], factors other than NeuroD may be responsible for the tissue-specific expression of Rim proteins while NeuroD is essential for their expressions. These results demonstrate that NeuroD regulates expression of a variety of genes associated with vesicle trafficking and exocytosis.

Vesicular exocytosis monitoring using FM1-43

We then investigated the effects of NeuroD expression on regulated exocytosis in Intestine 407 cells at the functional level. Regulated exocytosis includes both secretory exocytosis and non-secretory exocytosis [48]. It is well known that non-secretory exocytosis leads to rapid membrane expansion in hypotonic solution to correct cell surface tension [18,19,48,49]. Because Intestine 407 cells are not secretory cells and secretory hormones were not induced by NeuroD overexpression, we examined hypotonicity-induced exocytosis using FM1-43. FM1-43, a lipophilic fluorescence dye, stains only the external leaflet of plasma membrane, and additional fusion of vesicle to the plasma membrane raises FM1-43 fluorescence, permitting monitoring of vesicle exocytosis [50,51]. *N*-Ethylmaleimide or *Clostridium botulinum* toxin F abolishes the increase in FM1-43 fluorescence induced by hypotonic conditions in Intestine 407 cells [18], indicating that hypotonicity-induced exocytosis requires the SNARE complex. As reported [18], hypotonic conditions induced a rise in fluorescence intensity of FM1-43-loaded Intestine 407 cells (Fig. 3A),

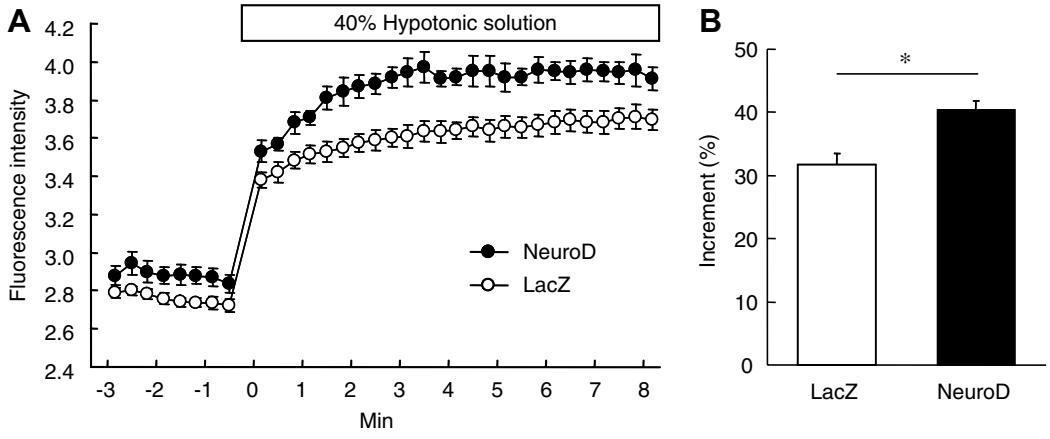

Fig. 3. Facilitation of vesicular exocytosis by NeuroD overexpression. Intestine 407 cells were seeded on a 96-well plate and infected with Ad-NeuroD or Ad-LacZ (100 moi). (A) Fluorescence changes of FM1-43-loaded cells. Vesicle exocytosis was stimulated by 40% hypotonic solution 72 h after infection ($n = 6$). Representative results of three independent experiments are shown. (B) Quantification of increment of fluorescence. Data represent average increments in fluorescence at 4 min after 40% hypotonic stimulation of three independent experiments ± SE. $^*P < 0.05$.

indicating that vesicle exocytosis occurred. When the cells were infected with Ad-NeuroD, the hypotonicity-induced rise in fluorescence was enhanced in comparison with cells infected with Ad-LacZ at 72 h after infection (Fig. 3A). The increment in fluorescence intensity over the unstimulated condition was significantly higher in Ad-NeuroD-infected cells than in Ad-LacZ-infected cells ($40.3 \pm 1.45\%$ vs. $31.7 \pm 1.86\%$ at 4 min after hypotonic stimulus; $n = 3$, $P < 0.05$) (Fig. 3B). These results demonstrate that NeuroD overexpression facilitates vesicular exocytosis in Intestine 407 cells. The neuron-like morphology and elevated expression of brain-specific α-tubulin in Ad-NeuroD-infected cells indicate the development of microtubules. Because the vesicles and organelles move along with the microtubules, the microtubule development induced by NeuroD, as well as the elevated expressions of SNAREs and their associated proteins, could be responsible for the acceleration of vesicle trafficking in hypotonicity-induced exocytosis.

Although NeuroD was known to be expressed in cells in which regulated exocytosis occurs, including neuronal, enteroendocrine, and pancreatic endocrine cells [22], its role in exocytosis was unknown. The present study demonstrates that NeuroD induces expressions of fundamental components of the exocytotic machinery. Thus, in addition to its critical role in differentiation, NeuroD may also play an important role in maintaining normal function of regulated exocytosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.12.197.

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