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Overexpression of neurogenin1 induces neurite outgrowth in F11 neuroblastoma cells

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Abbreviations: bHLH, basic helix-loop-helix; Ngn1, neurogenin1; RIPE3, rat insulin promoter enhancer 3

Abstract

Neurogenin1 (Ngn1) is a basic helix-loop-helix (bHLH) transcription factor expressed in neuronal precursors in the developing nervous system. The function of Ngn1 in neurogenesis has been shown in various aspects. In this study, we investigated the neurogenic potential of Ngn1 using neuroblastoma cell line, F11, which could be induced to differentiate into neurons in the presence of cAMP. To investigate the expression of Ngn1, expression vectors for the full-length and the C-terminal deletion mutant of Ngn1 were constructed and their transactivation potential was verified using reporter gene containing the E-box sequence. Overexpression of the full-length Ngn1 induced neurite outgrowth in F11 cells in the absence of cAMP. A C-terminal deletion mutant, Ngn1(1-197), inhibited neurite outgrowth induced by cAMP in F11 cells. These results indicate that the Ngn1 plays an important role in differentiation of neuroblastoma cells and the C terminus of Ngn1 is essential for the efficient differentiation.

Keywords: cell differentiation; genes; mutation; neuroblastoma; neurons; reporter; transcription factors

Introduction

Several basic helix-loop-helix(bHLH) proteins are sequentially expressed in developmental stages in neural lineages (Jan and Jan, 1993). Tissue specific bHLH proteins of the class B interact with ubiquitous bHLH proteins of the class A, E proteins, to form heterodimers (Lassar *et al.*, 1991). The heterodimer binds to the E-box (CANNTG) of target genes (Murre *et al.*, 1989) and regulates various transcriptional events, such as differentiation procedures. Tissue specific class B proteins include the neurogenic proteins such as neurogenins (Ngns) (Gradwohl *et al.*, 1996; Ma *et al.*, 1996), Mash1 (Johnson *et al.*, 1990), Math3 and NeuroD/BETA2 (Lee *et al.*, 1995). Ngns and Mash1 are expressed in dividing cells in the complementary regions of the developing central nervous system and in distinct sublineages of the peripheral nervous system and specify cell fates (Ma *et al.*, 1997). Especially Ngns are *Drosophila* atonal-related bHLH factors that are expressed in precursors of both placode- and neural crest-derived sensory neurons but not in those of autonomic neurons (Gradwohl *et al.*, 1996; Ma *et al.*, 1996; Ma *et al.*, 1997). Expression of Ngn1 and Ngn2 is overlapped in the central nervous system although they control two distinctive waves of neurogenesis in developing dorsal root ganglia (Ma *et al.*, 1999). Ngn1 and Ngn2 knock out mice exhibit defects in cranial ganglia and fail to activate downstream genes including Math3, NeuroD/ BETA2 and the notch ligand Delta 1 (Fode *et al.*, 1998; Ma *et al.*, 1998). In mammals, Ngn1/MATH4C (Cau *et al.*, 1997; Ma *et al.*, 1999) performs a determination function similar to that of the *Drosophila* proneural gene in neural development. Ngn1 knock out mice revealed defects in the midbrain, the dorsal root ganglia, and a subset of cranial sensory ganglia (Ma *et al.*, 1998).

Neuronal differentiation has been extensively studied in various cell types including PC12 cells. PC12 cells are pheochromocytoma cells characterized by electrical excitability and induction of neuron specific genes including tyrosine hydroxylase (Czyzyk-Krzeska *et al.*, 1994) when differentiated into sympathetic-like neurons by nerve growth factor (NGF). F11 neuroblastoma cells are hybrid cells of rat dorsal root ganglion and mouse neuroblastoma N18TG2 (Platika *et al.*, 1985). F11 cells can differentiate into neurons in the presence of cAMP or prostaglandins and express bradykinin and prostaglandin receptors, which are involved in the transduction of pain (Francel *et al.*, 1987). In this report, we investigated E-box mediated gene transcription of full-length Ngn1 and a

C-terminal truncated form, Ngn1(1-197). Full-length Ngn1 was able to induce neurite outgrowth in F11 cells but C-terminal deletion mutant was not.

Materials and Methods

Materials

F11 cells were kindly provided by Dr. M. Fishman (Harvard University, Cambridge, MA). Dulbecco's modified Eagle's medium (DMEM), 0.25% trypsin-EDTA, fetal bovine serum (FBS) and LipofectAMINE Plus Reagent were purchased from Gibco-BRL (Grand Island, NY); poly-D-lysine and N^6, 2-*O*-dibutytyladenosine 3',5'-cyclic monophosphate (db-cAMP) from Sigma (St. Louis, MO); pCR2.1-TOPO, pcDNA3.1 His from Invitrogen (Carlsbad, CA); anti-Ngn1 antibody from Santa Cruz Biotechnology (Santa Cruz, CA); pEGFP from Clontech (Palo Alto, CA); pGL3-promoter vector and luciferase assay system from Promega (Madison, WI); Super Signal West Pico chemiluminescent substrate from Pierce (Rochford, IL).

Plasmids and oligonucleotides

The full-length mouse Ngn1 cDNA (GenBank U63841) was obtained by PCR using oligonucleotides TGC AAG ATG CCT GCC CCT TT (forward) and GCC ATA GGT GAA GTC TTC TGA AGC CGA GGG ACT ACT G (reverse) as primers and genomic DNA of Balb/c mouse as a template, subcloned into the pCR2.1-TOPO plasmid and sequenced. The cDNA was isolated from pCR2.1-TOPO and inserted into the *Eco*RI site of the expression vector, pcDNA3.1His, to yield a full-length expression vector, pcDNA3.1His/Ngn1. To construct an expression vector for a truncated Ngn1, pcDNA3.1His/Ngn1(1-197), the *Bam*HI and *Sma*I fragment of pcDNA3.1His/Ngn1 was inserted into pcDNA3.1His plasmid restricted with *Bam*HI and *Eco*RV. The pRIPE3(3+)-luc luciferase reporter construct was generated by inserting the three copies of rat insulin promoter enhancer 3 (RIPE3), CGG ATC AGC AGA TGG CCA GAG GGG CTG AAG CTG CAG TTT CCA GAT C, as suggested by Kim *et al.* (2002), into pGL3-promoter luciferase vector.

Cell culture and transfection assays

293T, F11, HeLa and HIT cells were maintained in growth medium, DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 mg/ml streptomycin, at 37°C in a humidified 5% CO_2 atmosphere. 293T cells were transfected by the calcium phosphate precipitation method as previously described (Graham and Eb, 1973). Transient transfection of HeLa and HIT cells was carried out with 0.3 μg of reporter gene and indicated amounts of expression vectors using LipofectAMINE Plus Reagent as suggested by the manufacturer. For each transfection, pCMV-βgal (0.1 μg/dish) was included as an internal control and the total DNA amount was kept constant with the addition of pcDNA3.1His. The cells were harvested 40 h after transfection and 10-20 μg proteins were used for luciferase assay. Luciferase activity was normalized for the transfection efficiency with β-galactosidase activity. For neurite outgrowth assay, F11 cells were plated onto collagen and poly-D-lysine coated culture plates (1.2×10^5 cells/35 mm dish) and cotransfected with pEGFP as a transfection marker using LipofectAMINE Plus Reagent as suggested by the manufacturer. To ensure that all GFP positive cells also contained both Ngn1 and E47, cotransfection was performed with 1 μg of each expression vector for Ngn1 and E47, and 0.3 μg of pEGFP using LipofectAMINE Plus. To induce differentiation of F11 cells, cells were incubated in DMEM supplemented with 0.5 % FBS and 0.5 mM db-cAMP for 4 days after transfection (Ghil *et al.*, 2000). Fluorescent images of GFP positive cells were acquired and analyzed using image analysis software, Image-Pro Plus (Media Cybernetics). The processes greater than diameter of cell body were counted as neurites.

Western blot analysis

293T and F11 cells were harvested 40 h after transfection and lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) with 0.01% phenylmethylsulfonyl fluoride for 10 min on ice and centrifuged at 14,000 rpm for 10 min. The supernatants were harvested and 20-50 μg proteins were subjected to SDS-PAGE. The proteins on the gel were transferred electrophoretically onto Immobilon-P membranes and Western blot analysis was performed with an antibody against Ngn1 (1:500 dilution). Finally, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse IgG and proteins were visualized using a Super Signal West Pico chemiluminescent substrate following the manufacturer's recommendation.

Results

Cloning of Ngn1 cDNA

To search for roles of Ngn1 in neuronal differentiation, we generated expression vectors for the full-length and truncated mutant form of Ngn1(1-197) (Figure 1A). We premised that the C-terminus might contain the activation domain and the truncated mutant form, Ngn1(1-197) might function dominant negatively

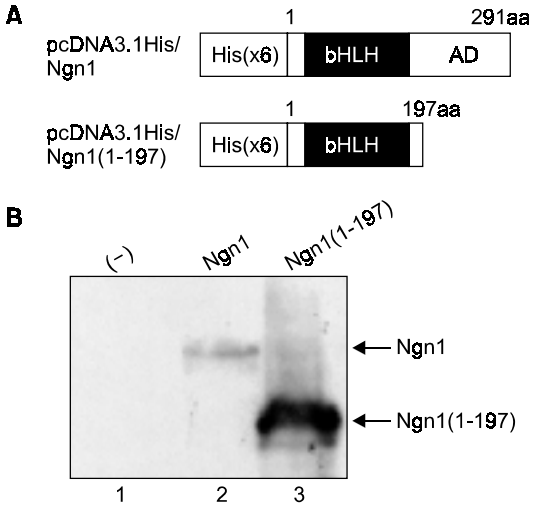

Figure 1. Expression vectors for full length and truncated mutant of Ngn1. (A) A schematic diagram of full-length Ngn1 and truncated form Ngn1(1-197) peptide which were epitope tagged with 6xHis at the N-terminus. bHLH, basic helix-loop-helix domain; AD, activation domain. (B) Western blot analysis of 293T cells transiently transfected with pcDNA3.1 (a negative control, lane1), pcDNA3.1His/Ngn1 (lane2) and pcDNA3.1His/Ngn1(1-197)(lane3).

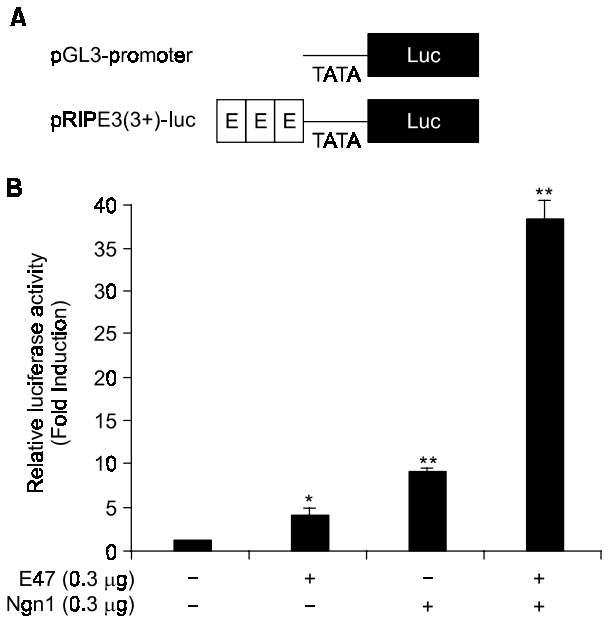

Figure 2. Analysis of transactivation potential of full-length Ngn1. (A) Reporter gene, pRIPE3(3+)-luc, was constructed by ligating three copies of the rat insulin E-box to pGL3 promoter. (B) HeLa cells were transiently transfected with pcDNA3.1His/Ngn1, pSVE2-5, and pRIPE3 (3+)-luc as a reporter gene. Ngn1 enhanced the E-box mediated gene expression. Data are shown as averages ± S.E. from three independent experiments that were normalized to β-galactosidase assay (*$P <$ 0.005 and **$P < 0.0001$, both P values were estimated from t-test compared with the values of reporter gene alone).

against the full-length. To verify proper expression of these recombinant Ngn1 proteins, the expression vectors were transfected into 293T cells and whole cell extracts were used for Western blot analysis using anti-Ngn1 antibody (Figure 1B). The full-length and the truncated mutant form of Ngn1 proteins were detected as 35 kDa and 24 kDa proteins, respectively.

Transactivation potential of recombinant Ngn1 proteins was determined using a luciferase reporter gene, pRIPE3(3+)-luc containing three copies of E-box (CANNTG) of the rat insulin promoter enhancer3 (RIPE3) (Figure 2A). We have previously shown that NeuroD/BETA2 binds to the RIPE and activates luciferase activity of pRIPE3(3+)-luc (Kim *et al.*, 2002). HeLa cells were transfected with pRIPE3(3+)-luc, pcDNA3.1His/Ngn1, and pSVE2-5, an expression vector for E47. The expression of Ngn1 or E47 alone enhanced the promoter activity of the reporter gene by 6- and 9-fold, respectively. Importantly, coexpression of both Ngn1 and E47 increased luciferase activity about 40-fold compared to that of the reporter gene alone. The result suggests that the Ngn1 interacts with E47 and synergistically increases the E-box mediated gene expression (Figure 2B).

To determine whether the C-terminus might contain transactivation domain of Ngn1, Ngn1(1-197) was transfected into fibroblastic HeLa cells and hamster insulinoma HIT cells. In HeLa cells, reporter gene activity was examined by transfecting pcDNA3. 1His/Ngn1, and pSVE2-5 (Figure 3A). During transfection, increasing amount of Ngn1(1-197) were added. Expression of Ngn1(1-197) decreased the E-box mediated gene expression in a partially dose dependent manner. Interestingly, the repressive effect of Ngn1 (1-197) was less prominent than that of Id1, a negative regulator for bHLH transcription factors (Kadesch, 1993; Littlewood and Evan, 1995), and NeuroD(1-233), a dominant negative form of NeuroD/ BETA2 (Cho *et al.*, 2001) (compare the luciferase activity with 0.1-0.3 μg each of expression vectors).

In HIT cells, due to the presence of endogenous E47 and other bHLH transcription factors, such as NeuroD/BETA2, the luciferase activity was exhibited reasonably high (Cho *et al.*, 2001), which allowed us to detect repressive effect of Ngn1(1-197) (Figure 3B). Addition of Ngn1(1-197) during transfection decreased the insulin promoter activity in a dose dependent manner. More than 0.3 μg of pcDNA3.1His/Ngn1 (1-197) decreased the promoter activity although 0.1 μg of pcDNA3.1His/Ngn1(1-197) yielded higher luciferase activity (see the Discussion). These results suggest that the truncated mutant of Ngn1 may compete with the endogenous bHLH proteins for interacting endogenous E47 and binding to the E-box sequence.

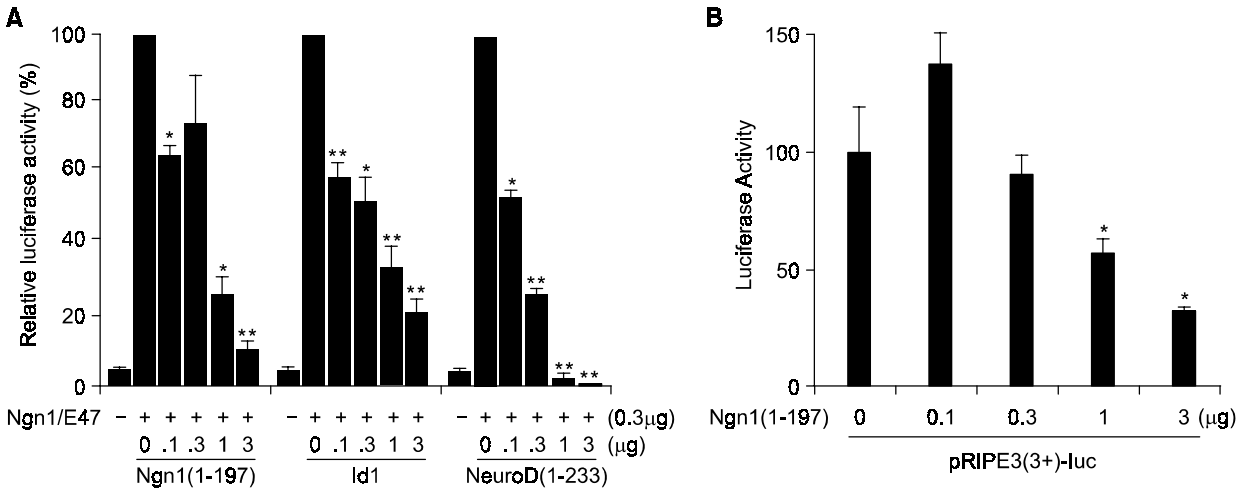

Figure 3. Repression of E-box mediated promoter activation by Ngn1(1-197). (A) Reporter gene was activated by transient transfection of pSVE2-5 and pcDNA3.1/His-Ngn1 in HeLa cells. But additional expression of Ngn1(1-197), Id1 and NeuroD(1-233) during transfection decreased the insulin promoter activity (*$P<0.005$ and **$P<0.0005$, all P values were estimated from t-test compared with the values of luciferase activity of pRIPE3 (3+)-luc, Ngn1, and E47). (B) HIT cells were transiently transfected with pcDNA3.1His/Ngn1(1-197) and pRIPE3(3+)-luc as a reporter gene. Ngn1 (1-197) decreased the insulin promoter activity in a partially dose dependent manner (*$P<0.05$, both P values were estimated from t-test compared with the values of pRIPE3(3+)-luc alone). Above values (A and B) represent averages ±S.E. from three independent experiments that were normalized to β-galactosidase assay.

Regulation of neurite outgrowth by Ngn1

To determine the role of Ngn1 in neuronal differentiation, we overexpressed the full-length Ngn1 and the truncated mutant, Ngn1(1-197), in F11 cells. F11 cells are hybrid cells between dorsal root ganglion and neuroblastoma. Several studies have shown that F11 cells exhibited characteristics of dorsal root ganglionic neurons (Platika *et al.*, 1985; Francel *et al.*, 1987). F11 was earlier found to provide an efficient *in vitro* system where the neurogenic effect of bHLH transcription factors including NeuroD/BETA2 can be evaluated (Cho *et al.*, 2001). F11 cells exhibited round shapes in the proliferating condition (Figure 4A*a*). Treatment of db-cAMP, a permeable analogue of cAMP, caused neurite outgrowth in F11 cells (Figure 4Ab) and thus 39.1% cells bore neurites (Figure 4B). Expression of the full-length Ngn1 induced neurite outgrowth in non-differentiating condition (in the absence of db-cAMP) (Figure 4Ac). With the full- length Ngn1, the number of neurite bearing cells was 2.99-fold higher than the control in the absence of db-cAMP (Figure 4B, *$P<0.01$). The additional effect of db-cAMP on neurite outgrowth was minimal in the presence of Ngn1 (Figure 4B). Importantly, expression of Ngn1(1-197) inhibited neurite outgrowth in the differentiating condition (Figure 4Af). In the presence of db-cAMP and Ngn1(1-197), percentage of neurite bearing cells was 17.2%. Thus, in the presence of db-cAMP and Ngn1(1-197), the number of neurite bearing cells was 2.27-fold lower than that of the control in the presence of db-cAMP (Figure 4B, **$P<0.05$). These results indicate that sustained expression of the full-length Ngn1 can substitute cAMP to induce neuronal differentiation and the Ngn1(1-197) functions as a negative regulator to decrease the neurite outgrowth. To determine whether F11 cells endogenously express Ngn1, Western blot analysis was carried out. Despite of the extensive search, we failed to detect Ngn1 in presence or absence of db-cAMP in F11 cells (Figure 4C).

Discussion

The role of Ngn1 during neurogenesis has been suggested by experiments with *Xenopus* homologues of ngn (X-Ngnr) in *Xenopus* embryos. For example, forced expression of X-Ngnr-1 leads to unidirectional ectopic expression of NeuroD/BETA2 and other neuronal marker showing that X-Ngnr-1 proteins can impose a neuronal fate on some cells (Ma *et al.*, 1996; Olson *et al.*, 1998). However, X-Ngnr-1 shows 78% identity with the mouse Ngn1 over the 65 amino acid stretch in the bHLH domain, which may cause diverse effects from the mammalian system. Thus, it is necessary to determine the role of mammalian Ngns by gain-of-function study in mammalian cells. In this study, using *in vitro* cultured neuroblastoma cells we showed that the full-length Ngn1 is sufficient to induce neurite outgrowth and the C-terminal deletion

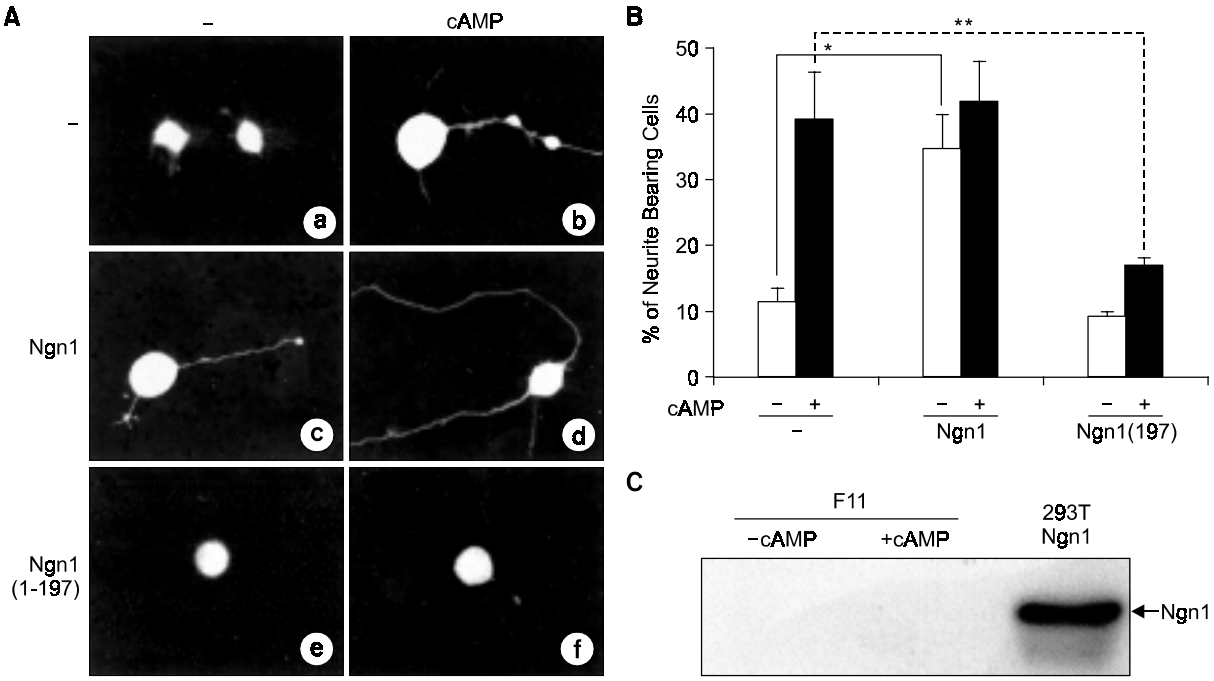

Figure 4. Induction of neurite outgrowth by Ngn1 in F11 cells. (A) F11 cells were transfected (a and b) with pcDNA3.1; (c and d) with pcDNA3.1His/Ngn1; (e and f) with pcDNA3.1His/Ngn1(1-197). Transfection was carried out with limited amount of pEGFP as a transfection marker. Differentiation was induced in the presence of 0.5% FBS and 0.5 mM db-cAMP (b, d, f) for 4 days after transfection. Scale bar = 30 μm. (B) Measurement of neurite bearing cells. Data are shown as the percentage of neurite bearing cells among GFP-positive cells. In the absence of cAMP, Ngn1 increased neurite bearing cells (*$P < 0.01$). In the presence of cAMP, Ngn1 (1-197) decreased neurite bearing cells (**$P < 0.05$). (C) F11 cells treated with or without db-cAMP were harvested and subjected to Western analysis.

mutant blocks neurite outgrowth induced by db-cAMP.

Generally the transactivation potential of Ngn1 is proportional to the neurogenic potential in F11 cells. In F11 cells, the full-length Ngn1 that can transactivate the insulin E-box is also able to induce neurite outgrowth in proliferating condition (in the absence of db-cAMP). Importantly, the C-terminal deletion mutant, Ngn1(1-197) that completely represses the insulin E-box in HeLa is able to inhibit neurite outgrowth induced by db-cAMP. This result suggests that induction of neuronal differentiation requires the activation domain located in the C-terminal region. It has been shown that bHLH transcription factors such as NeuroD/BETA2 (Sharma *et al.*, 1999) and NDRF/neuroD2 (Konishi *et al.*, 2000) interact with transcription machinery including CBP/p300 *via* the activation domain located in the C-terminus. We have shown that the C-terminal deleted mutant of NeuroD/BETA2 competes with the full- length for binding to E-box and functions as a dominant negative mutant (Cho *et al.*, 2001). We have also shown that Id2, a negative regulator of bHLH factors, interacts with bHLH protein to inhibit E-box mediated gene expression (Ghil *et al.*, 2002). Compared NeuroD(1-233) and Id1, the repressive effect of Ngn1(1-197) is less prominent, especially when low amount of Ngn1(1-197) is added during transfection. In HeLa cells (Figure 3A), the N terminal part of 197 amino acid of Ngn1(1-197) can partially activate the E-box mediated gene expression in HIT cells. These results support the idea that an additional activation domain may reside at the N-terminal part in Ngn1.

F11 is a hybrid cell line between neuroblastoma N18TG2 and dorsal root ganglionic neurons (Platika *et al.*, 1985). F11 cells differentiate into neurons in the presence of cAMP or prostaglandins and express neuronal markers including receptor for bradykinin, prostaglandin, opioids, and N- and L-type voltage dependent Ca^{2+} channel (Francel *et al.*, 1987; Boland and Dingledine, 1990; Fan *et al.*, 1992; Cruciani *et al.*, 1993), suggesting that F11 cells are similar to dorsal root ganglionic neurons. Signaling pathways for neurite outgrowth of F11 cells are involve with protein kinase A (PKA) and cAMP responsive element binding protein (CREB). Interference of this pathway inhibits neurite outgrowth as well as CREB activation (Ghil *et al.*, 2000). It will be interesting to know whether full-length Ngn1 induces neurite outgrowth by modulating this pathway. The expression of Ngn1 as well as Ngn2 is high in developing dorsal root ganglia

during early embryonic period but gradually decreases as development proceeds (Ma *et al.*, 1999). However, we could not detect expression of endogenous Ngn1 in F11 cells under our experimental condition (Figure 4C). Ngn1(1-197) exhibited negative effect in the absence of endogenous Ngn1 in F11 cells. From these data, we can draw the following possibilities. First, F11 cells may not represent the subset of Ngn1 expressing neurons in dorsal root ganglion. There are several types of sensory neurons in dorsal root ganglion, including large diameter neurons expressing trkB and trkC, small diameter neurons expressing NGF receptor, trkA, and purinoreceptor, P2X3-expressing neurons (Averill *et al.*, 1995). Ngn2 is required primarily for the generation of $trkC^+$ and $trkB^+$ neurons, whereas Ngn1 is for most of all $trkA^+$ neurons requires Ngn1. Second, Ngn1-like bHLH factors may be involved in neuronal differentiation of F11 cells. Several bHLH factors such as Mash1 or Ngn2 sharing functional similarity with Ngn1 have been isolated and studied in neuronal differentiation (Gowan *et al.*, 2001; Parras *et al.*, 2002). The neurogenesis in the Ngn2 $-/-$ chick embryos is only transiently defective but later compensated by Ngn1-dependent precursors, suggesting that feedback or competitive interactions between these bHLH factors may control the different neuronal subtypes (reviewed by Anderson, 1999). Third, as mentioned earlier, Ngn1(1-197) may interact with a transcriptional machinery and quench transactivation potential of yet unidentified transcription factor(s) whose expression is induced by cAMP.

Our data suggest that Ngn1 play an important role in differentiation of neuroblastoma cells and the C terminus of Ngn1 is essential for the efficient differentiation. Finally, F11 cells may provide a useful system to examine the role of Ngn1-like factors in neuronal differentiation *in vitro*.

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