Mash1 and Neurogenin 2 Enhance Survival and Differentiation of Neural Precursor Cells After Transplantation to Rat Brains via Distinct Modes of Action

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Neural precursor cells (NPCs) are regarded as a promising source of donor cells in transplantation-based therapies for neurodegenerative disorders. However, poor survival and limited neuronal differentiation of the transplanted NPCs remain critical limitations for developing therapeutic strategies. In this study, we investigated the effects of the proneural basic helix–loop–helix (bHLH) transcription factors Mash1 and Neurogenin 2 (Ngn2) in neuronal differentiation and survival of NPCs. Induction of Mash1 or Ngn2 expression strikingly enhanced neuronal differentiation of cultured NPCs *in vitro*. Ngn2-transduced NPCs underwent a rapid cell cycle arrest, which often accompanies differentiation. In contrast, cells continuously expanded upon Mash1 expression during NPC differentiation. Notably, sonic hedgehog (SHH) was upregulated by Mash1 and mediated the proliferative and survival effects of Mash1 on NPCs. Upon transplantation into adult rat brains, Mash1-expressing NPCs yielded large grafts enriched with neurons compared to control LacZ-transduced NPCs. Interestingly, enhancements in neuronal yield, as well as in donor cell survival, were also achieved by transplanting Ngn2-transduced NPCs. We show that a differentiation stage– and cell density–dependent survival effect of Ngn2 involves neurotrophin3 (NT3)/TrkC-mediated signaling. Together, these findings suggest potential benefits of *bHLH* gene manipulation to develop successful transplantation strategies for brain disorders.

INTRODUCTION

The adult mammalian brain has long been regarded as an organ that is devoid of intrinsic repair capacity after injury. Such a view, however, is now abandoned as studies have clearly demonstrated that *de novo* generation of neurons in adult central nervous system does occur,[1,2] and that self-repair via endogenous neurogenesis in response to CNS injury and disease is induced.[3,4] Stimulation of self-repair processes by manipulating endogenous precursor cell behaviors may be an ideal therapeutic approach for the functional recovery of damaged brains. However, at present, this is impossible and will not likely be achievable until the critical self-repair mechanisms are clearly understood. Given over a decade of clinical experiences with human fetal midbrain transplantation for patients with Parkinson's disease,[5] a cell transplantation–based approach seems to be a viable alternative. Stem or precursor cells with self-renewal and multilineage developmental capacities are a promising cell source for the replacement strategy because *in vitro* manipulation for the proliferation and guided differentiation of these cells can potentially yield the optimal number and types of transplantable cells. Neural precursor cells (NPCs) can be isolated from both developing and adult brains, and propagated *in vitro*.[6,7] Upon being reimplanted into the lesioned brain, the NPCs can integrate into the host parenchyme, differentiate into a range of cell types including the "replacement" cells, and ultimately result in functional recovery of the neurologic deficits of the grafted host.[8] At the same time, several studies have also raised major concerns with regard to donor cell survival and neuronal yield *in vivo*. In fact, only minor proportions of donor NPCs appear to be viable in the host brains, and the long-term efficacy of cell transplantation accompanied by long-term cell survival has yet to be reported.[9] Furthermore, after engraftment into the injured or diseased brain, NPCs differentiate mainly into glia[10] or often remain in an undifferentiated precursor state.[11] These limitations in turn suggest that *in vitro* manipulations that enhance the survival and neuronal differentiation of NPCs may be valuable,[12] if not indispensable before transplantation.

Proneural basic helix–loop–helix (bHLH) proteins are transcription factors expressed in both the central and the peripheral nervous systems during development that can promote neuronal

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determination and differentiation.[13,14] The first bHLH protein to be discovered in the mammalian system was Mash1, which appears to be the ortholog of the *Drosophila* achaete scute, whereas subsequently identified neurogenins (Ngns) belong to the atonal subfamily.[15] Because of their neurogenic effects, these bHLH factors constitute prime candidate molecules whose expression in donor cells could yield neuron-enriched grafts. In this study, we show that transduction of Mash1 or Ngn2 into donor NPCs before transplantation dramatically enhances neuronal yield and donor cell survival, both *in vitro* and *in vivo*. As a consequence, larger grafts enriched with neuronal cells were generated upon transplantation. We describe further *in vitro* mechanistic analyses of the role of Mash1 and Ngn2 in neuron formation, cell survival, and proliferation. Interestingly, Mash1 and Ngn2 enhance donor cell survival through distinct signal pathways involving sonic hedgehog (SHH) and neurotrophin3 (NT3)/TrkC, respectively. Our results have significant implications for using extracellular signals and transcription factors to modify donor cells for maximal restoration of injured or diseased brains.

RESULTS

Distinct modes of neuronal differentiation induced by Ngn2 and Mash1

NPCs isolated from the cortices of rat fetal brains at E14 were transduced with retroviruses expressing Ngn2, Mash1, LacZ, or empty vector (control), and then induced to differentiate 24 hours afterward. Consistent with the established neurogenic roles of these proneural bHLH factors, robust increases of neuronal cell populations, estimated by the numbers of cells positive for the neuron-specific markers β-tubulin III (TuJ1) and microtubule-associated protein 2, were observed 3 days after transduction with Mash1 or Ngn2, but not with LacZ (**Figure 1a–d**). Although the total number of neurons induced by Ngn2 and Mash1 were indistinguishable, Ngn2-induced neurons, especially in cultures at low cell densities, displayed a more mature neuronal morphology with extensive neurite outgrowth compared to Mash1-transduced neuronal cells. The total TuJ1+ fiber lengths per cell were $156.43 \pm 8.6\,\mu m$ in Ngn2-, $75.64 \pm 5.87\,\mu m$ in Mash1-, and $52.22 \pm 2.3\,\mu m$ in LacZ-transduced cultures ($n = 130$, $P < 0.001$, **Supplementary Figure S1**).
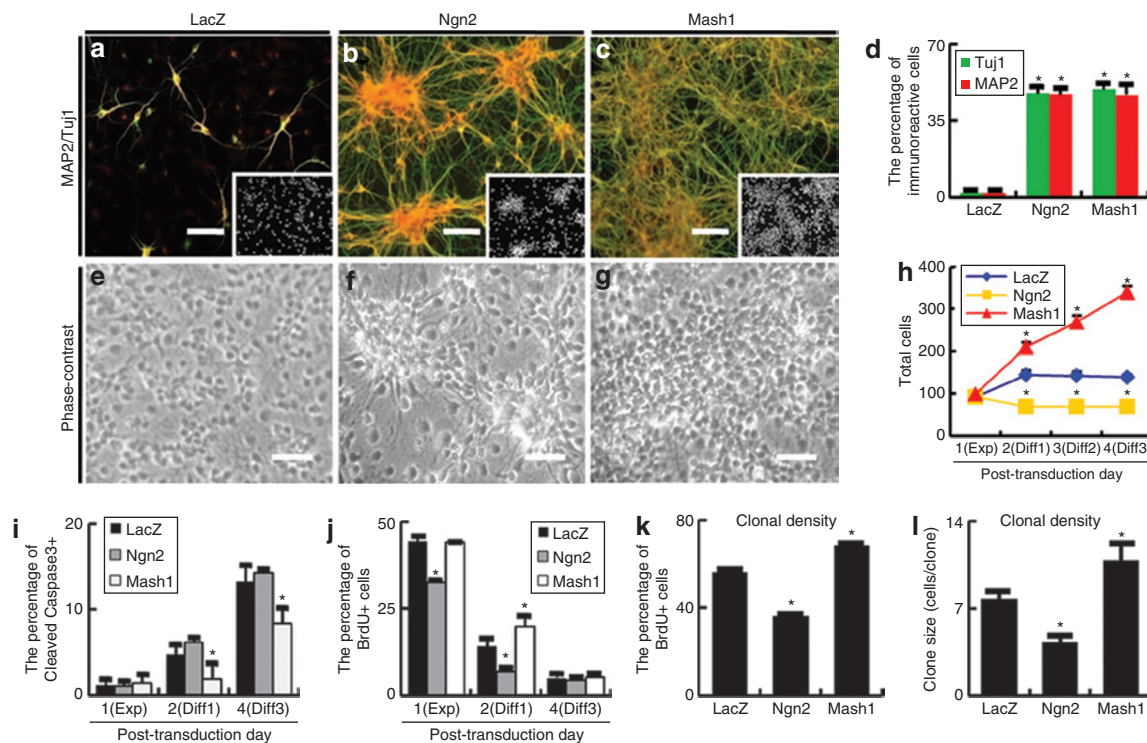

Figure 1 Effects of exogenous expression of the proneuronal basic helix–loop–helix (bHLH) factors neurogenin 2 (Ngn2) and Mash1 on neural precursor cell (NPC) differentiation, growth, and survival *in vitro*. NPCs from rat embryonic cortices at E14 were cultured in the presence of the mitogen basic fibroblast growth factor (bFGF) and transduced with retroviruses expressing Ngn2, Mash1, or LacZ (control). Differentiation of the transduced NPCs was induced for 3 days by withdrawing bFGF 1 day after viral transduction. (**a–d**) Neuronal differentiation *in vitro*. Shown in **a–c** are representative images of Tuj1/MAP2-immunoreative cells in cultures transduced with (**a**) LacZ-control, (**b**) Ngn2, and (**c**) Mash1. Insets, 4′,6′-diamidino-2-phenylindole (DAPI) nuclear staining of the same field. Scale bars = 40 μm. Graph (**d**) represents the percentage of TuJ1+ or MAP2+ neurons out of total DAPI+ cells at differentiation day 3. Data are expressed as the mean and SEM. (**e–h**) Cell growth pattern. Phase-contrast microscopic views of cell clusters of (**e**) LacZ, (**f**) Ngn2-, and (**g**) Mash1-transduced cultures. (**h**) Cell growth profiles of the control, Ngn2-, and Mash1-transduced cultures were determined by counting total viable cells at post-transduction day 1 (expansion day; Exp), 2 (differentiation day 1; Diff1), 3 (Diff2), and 4 (Diff3). The cell counting was carried out for phase-contrast microscopic fields that were randomly chosen across the culture area, using an eyepiece grid at the final magnification of ×200 ($n = 40$–60 microscopic fields from three culture wells of a 24-well plate). Apoptosis and proliferation were estimated by the fraction of cells positive for (**i**) activated caspase 3 and (**j**) percentage of BrdU-incorporated cells, respectively, over the indicated time periods. Proliferating NPCs were treated with BrdU (10 μmol/l) for 30 minutes and visualized by immunocytochemical detection. Effects of Ngn2 and Mash1 expression on cell survival and proliferation during NPC differentiation were examined in the cultures plated at a clonal density (2,000 cells/6-cm dish) based on percentage of cells viable (**k**) 1 day after cell plating and (**l**) clone size 4 days after plating (day 3 of differentiation), respectively. *Significantly different from the respective LacZ-transduced controls at $P < 0.001$.

In addition to the difference in neuronal maturation, Ngn2- and Mash1-transduced cells displayed distinct shapes of cell clusters and distinct patterns of proliferation and apoptosis. Ngn2-transduced precursors migrated and assembled into small compact clusters (**Figure 1f**), which is an authentic feature of *in vitro* neuronal differentiation.[16] Such a characteristic cluster shape was not clear in the control and Mash1-transduced cultures (**Figure 1e** and **g**). The total cell number in Ngn2-cultures was significantly less than that in LacZ-transduced controls throughout the 3 days of *in vitro* differentiation (**Figure 1h**). In contrast, cells in Mash1-transduced cultures continuously proliferated during the differentiation period. The common neurogenic but distinct effects of Ngn2 and Mash1 in cell cluster shape and growth pattern were similarly observed regardless of the brain regions and embryonic ages from which NPCs were isolated (**Supplementary Figure S2** and data not shown).

Cell differentiation is generally considered to be initiated upon cell cycle arrest of proliferating precursor cells. At the same time, subpopulations of differentiating cells undergo apoptotic cell death, which is one of the principal processes controlling the number of differentiated cells for a given tissue or organ.[17] A decrease in the number of 5-bromo-2-deoxyuridine (BrdU)+ proliferating cells was evident the day after Ngn2 transduction (**Figure 1j**), indicating a rapid promotion of cell cycle exit by Ngn2. No significant difference in the number of cells positive for activated (cleaved) caspase-3 was observed between Ngn2-transduced and control cultures ($6.18 \pm 0.51\%$ (Ngn2), $4.83 \pm 1.3\%$ (control) at *in vitro* differentiation day 1, and $n = 24$, $P = 0.56$, $14.28 \pm 2.22\%$ (Ngn2), $13.17 \pm 2.37\%$ (control) at differentiation day 3, $n = 24$, $P = 0.61$, **Figure 1i**). In contrast to the retarded cell growth in Ngn2-transduced cultures, the total cell number increased continuously in Mash1-transduced cultures during the 3 days of cell differentiation (**Figure 1h**). The proportion of BrdU+ cells in Mash1-transduced cultures was significantly greater than that in the control or Ngn2-transduced cultures; *e.g.*, BrdU+ cells at day 1 accounted for $20.21 \pm 2.88\%$ (Mash1), $7.25 \pm 1.06\%$ (Ngn2), or $14.36 \pm 2.03\%$ (control) ($n = 42$, $P < 0.001$) (**Figure 1j**). Reduced indices of cell apoptosis in Mash1-cultures compared to those in the controls and Ngn2 cultures were also noted; the percentage of activated caspase-3+ cells in Mash1-transduced cultures were $1.83 \pm 1.77\%$ and $8.31 \pm 1.52\%$ at differentiation days 1 and 3, respectively ($n = 24$, which were different from the values of LacZ controls and Ngn2 shown above at $P < 0.001$) (**Figure 1i**).

We next evaluated cell survival and proliferation effects of the proneural bHLH factors by determining the percentage of viable cells and clone size in the cultures plated at a clonal density (see Materials and Methods). As cell survival and proliferation can be influenced by close cell-to-cell interactions, clonal density cultures allow a clearer characterization of the activity of transduced bHLHs. In addition, these indices of the clonal density cultures represent intrinsic cell-autonomous activities. The percentage of viable cells and clone size were significantly less in the cultures transduced with Ngn2 than those with the LacZ controls (**Figure 1k** and **l**), confirming Ngn2-mediated inhibition of cell survival and proliferation. Conversely, significant increases in both cell survival and proliferation indices were observed by Mash1 transduction. The percentage of viable cells at the day following

cell plating was $36.75 \pm 2.39\%$ in Ngn2-, $68.87 \pm 1.45\%$ in Mash1-, and $56.57 \pm 4.18\%$ in LacZ-transduced cultures ($n = 16$ of 6-cm dishes for each group), whereas clone sizes 4 days after plating were 4.39 ± 0.42 cells/clone in Ngn2-, 10.9 ± 1.33 cells/clone in Mash1-, and 7.79 ± 0.69 cells/clone in LacZ-transduced cultures ($n = 70–117$ clones for each group). These findings suggest that
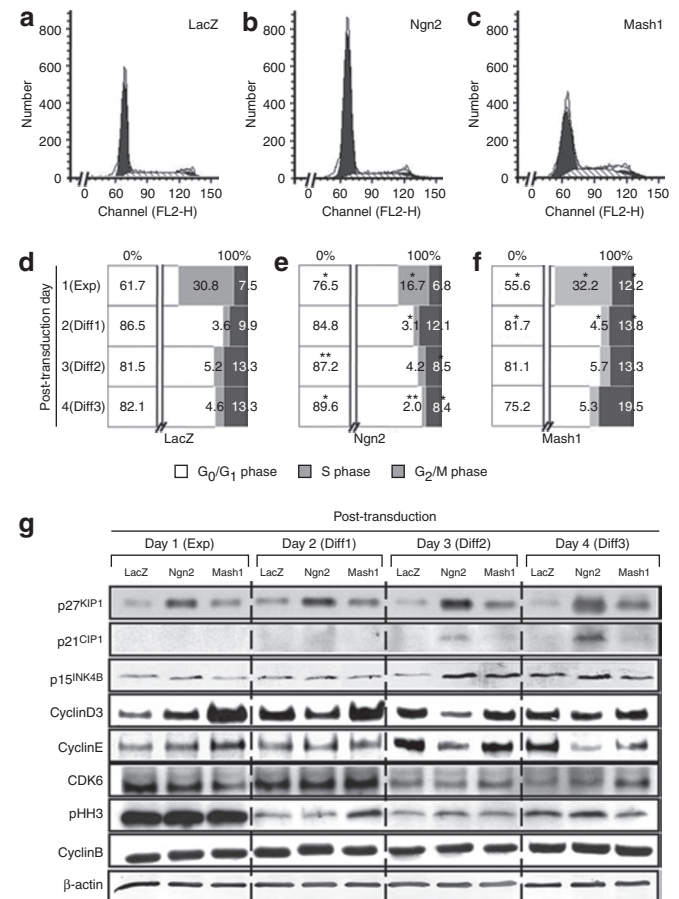

Figure 2 Distinct activities of neurogenin 2 (Ngn2) and Mash1 in cell cycle regulation during neuronal differentiation of neural precursor cells (NPCs). (**a–f**) Flow cytometric analysis for determining cell cycle status of (**b**, **e**) Ngn2-, (**c**, **f**) Mash1-, and (**a**, **d**) LacZ-transduced NPCs. Cortical NPCs were cultured and transduced as indicated in the legend of **Figure 1**, and subjected to flow cytometric analyses. Propidium iodide staining was used to quantify DNA content of individual cells. Graphs **a–d** are the representative flow cytometry profiles of (**a**) LacZ-, (**b**) Ngn2-, and (**c**) Mash1-transduced cells at post-transduction day 1. Shown in **d–f** are the fraction of cells distributed at G_0/G_1, S, and G_2/M phases in the cultures transduced with (**d**) LacZ, (**e**) Ngn2, and (**f**) Mash1 at the indicated post-transduction days. It is noted that a higher proportion of Ngn2-transduced cells, compared to those of the control, are distributed in the G_0/G_1 phases, whereas Mash1-transduced cells are accumulated in the S and G_2/M phases at the expense of those in the G_0/G_1 phases. Significantly different from the respective LacZ-transduced controls at *$P < 0.001$ or at **$P < 0.01$. (**g**) Time course–dependent activation of proteins specific to cell cycle promotion and arrest. Western blot analyses were carried out to determine the level of protein activated by Ngn2 and Mash1 transduction. Ngn2-specific activation was noted in the cell cycle arrest proteins p27[KIP1] and p21[CIP1]. In contrast, cyclin D3 and E protein levels, compared to those of the control and Ngn2-transduced cultures, were higher at post-transduction day 1 in Mash1-transduced cells. Mash1-specific induction of the M phase–specific protein phosphohistone H3 (pHH3) followed subsequently at post-transduction day 2.

although Ngn2 and Mash1 have a common role in inducing neuronal differentiation, they possess distinct regulatory roles in cell proliferation and survival.

Induction of distinct subsets of cell cycle regulatory proteins by Mash 1 and Ngn2

We further examined cell cycle regulation using flow cytometry. As expected, Ngn2-transduced cells accumulated in the G_0/G_1 phases (**Figure 2a–f**). In contrast, a significantly larger portion of Mash1-transduced cells was distributed in the S and G2/M phases with a concomitant decrease in the proportion of those in the G_0/G_1 phase ($n = 6$ from two different experiments). These results confirm contrasting roles of Ngn2 and Mash1 in cell cycle progression. To gain further insight into the differential effects of Mash1 and Ngn2 in the proliferation of cortical precursor cells, we explored the time-course patterns of proteins activated during cell cycle progression (cyclin B, D3, E, cyclin-dependent kinase 4, 6, and phospho-histone H3) and cell cycle arrest (cyclin-dependent kinase inhibitors: p21[CIP1], p27[KIP1], and p15[INK4B]). Notably, among the proteins tested, an increase in cyclin D3 and cyclin E protein levels were noted in Mash1-transduced cultures at day 1 after transduction, compared to those in the controls and Ngn2-transduced cells (**Figure 2g**). In contrast, Ngn2 caused a clear increase in protein levels of the cell cycle inhibitors p27[KIP1] and p21[CIP1] compared to those in the control LacZ- and Mash1-transduced cells. Specifically, p27[KIP1] showed an immediate and lasting upregulation, whereas p21[CIP1] upregulation was observed 3 days post-transduction. This sequential pattern of p27[KIP1] and p21[CIP1] activation has been similarly demonstrated in cortical brain development *in vivo*.[18] Changes in cyclin-dependent kinase levels by Mash1 and Ngn2 were not observed.

NeuroD1 is a downstream molecule mediating Ngn2 effect on NPC growth inhibition

The expression of Ngn1/2 spatially overlaps and often temporally precedes that of NeuroD in the developing brain, suggesting that Ngn1/2 and NeuroD function in a cascade.[19] Indeed, exogenous Ngn2 expression strongly induced the expressions of NeuroD1 in cultured NPCs at both the mRNA and protein levels[19] (**Figure 3a** and **b**). In contrast, no detectable change in NeuroD1 expression was induced by exogenous Mash1 transduction in NPCs. These findings suggest the possibility that the different activities of Mash1 and Ngn2 can be attributed to whether NeuroD1 expression is induced or not. Exogenous NeuroD1 expression in the NPC cultures exactly phenocopied the effects of Ngn2 on neuronal differentiation, cell cluster shape, and cell growth (data not shown). Furthermore, knockdown of NeuroD using micro-NeuroD not only resulted in a significant reduction of Ngn2 activities in cell growth (**Figure 3f**), but also abolished the characteristic feature of cell clusters observed in Ngn2-tranduced cells (**Figure 3c–e**). However, the TuJ1+ neuron yield was not significantly altered by NeuroD knockdown in Ngn2-transduced cultures (**Figure 3c–e** and **g**). These findings collectively indicate that NeuroD1 mediates, at least in part, the effect of Ngn2 in cell cycle arrest and that the different regulatory actions of Ngn2 and Mash1 in NPC proliferation are due to differences in NeuroD1 induction.
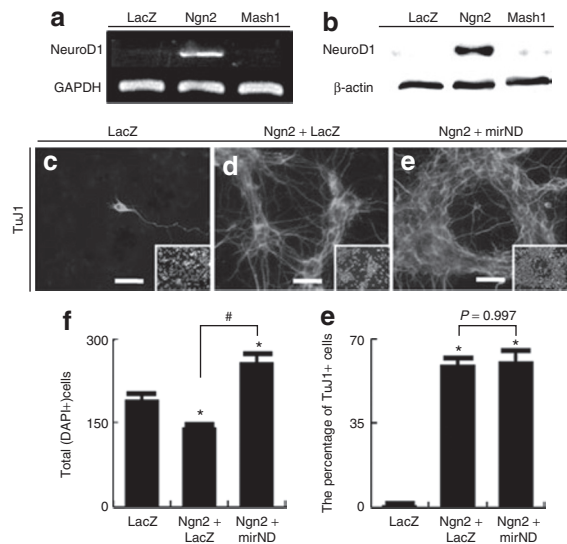

Figure 3 NeuroD1 as a mediator of neurogenin 2 (Ngn2)-induced retardation of cell growth during neural precursor cell (NPC) differentiation. (**a**, **b**) Ngn2 induced an upregulation of NeuroD1 expression. NeuroD1 mRNA and protein levels were determined at differentiation day 3 using (**a**) semiquantitative reverse transcriptase PCR and (**b**) western blot analyses. (**c–g**) Loss-of-function experiments of NeuroD1 in Ngn2-expressing NPCs. Precursors from E14 cortices were transduced with the retroviruses expressing LacZ, Ngn2+LacZ, and Ngn2+NeuroD microRNA (mirND), and then numbers of total 4′,6′-diamidino-2-phenylindole (DAPI)+ cells and TuJ1+ cells were determined 3 days after differentiation. For the co-transductions, mixtures (1:1, vol:vol) of viral soups of Ngn2 and LacZ (Ngn2+LacZ) or Ngn2 and mirND (Ngn2+mirND) were applied to the cells. (**c–e**) Representative TuJ1-immunofluorescent views of the cultures transduced with (**c**) LacZ, (**d**) Ngn2+LacZ, and (**e**) Ngn2+mirND. Insets, DAPI nuclear staining of the same fields. Graphs **f** and **g** show total cell numbers and percentage of TuJ1+ cells, respectively. Scale bar = 40 µm. Significantly, different from the controls (*) at $P < 0.001$ and from Ngn2+LacZ-transduced cultures (#) at $P < 0.001$. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Activated SHH signaling in Mash1-induced cell survival/proliferation

To understand how Mash1 induces NPC proliferation, we examined the expression of cytokines and receptors of various signaling pathways known to be involved in NPC proliferation. These included components of fibroblast growth factor (FGF), epidermal growth factor, leukemia inhibitory factor/ciliary neurotrophic factor/gp130, Wnt, Notch, and SHH-signaling pathways. Interestingly, a robust induction of SHH mRNA expression was noted in Mash1-transduced cultures (**Supplementary Figure S3** and **Figure 4a**). SHH is a secreted protein, which promotes the survival and proliferation of NPCs.[20,21] The enhanced synthesis and secretion of SHH proteins from Mash1-transduced NPCs were further confirmed by western blot analysis and enzyme-linked immunosorbent assay (**Figure 4b** and **c**). Enzyme-linked immunosorbent assay analysis showed that the SHH protein level in media conditioned in Mash1-transduced cultures was 72.57 ng/ml, which was significantly different from the values of Ngn2- (27.58 ng/ml) and LacZ-transduced (25.91 ng/ml) cultures at $P < 0.05$. Among the genes downstream of SHH are the Gli-family transcription factors (Gli1-3).[22] Gli1 is the principal mediator of SHH signaling in NPCs, and the expression of Gli1 has been shown to accompany that of SHH in the
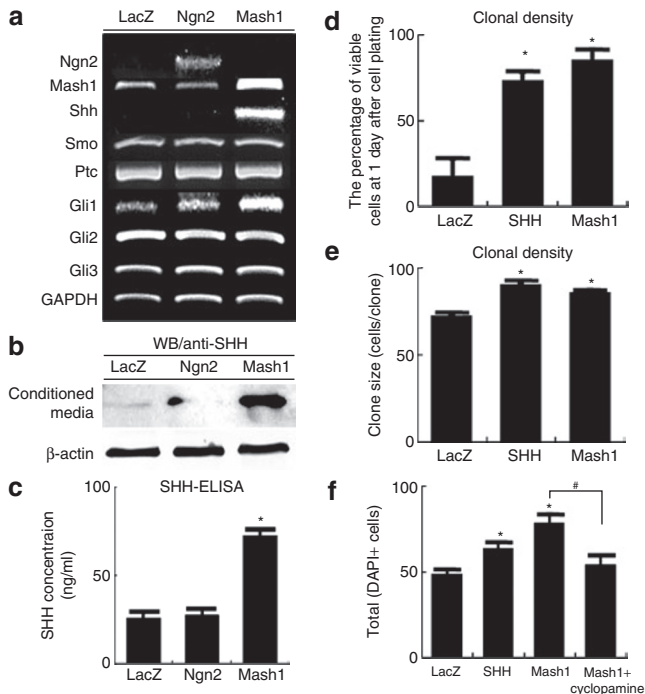
Figure 4 Involvement of sonic hedgehog (SHH) signaling in Mash1-induced cell survival and proliferation of neural precursor cells (NPCs). (**a**–**c**) Increased expression of SHH in Mash1-transduced NPCs. All SHH expression analyses were performed 3 days after differentiation. (**a**) Reverse transcriptase PCR analyses for SHH, SHH receptor components (Smo, Ptc), SHH downstream transcription factors of the Gli-family (Gli1,2,3). Mash1-induced increases were observed for SHH and Gli1 mRNAs. (**b**) Western blot (WB) analysis for SHH proteins in the cell lysates. (**c**) Enzyme-linked immunosorbent assay (ELISA) determination of SHH protein levels in the media conditioned via cultures transduced with LacZ (control), neurogenin 2 (Ngn2), and Mash1. For ELISA detection, media conditioned by the cultures indicated were collected for 3 days of differentiation. (**d**–**f**) Role of SHH in NPC survival and proliferation activated by Mash1. Activities of SHH in cell survival and proliferation were determined by the percentage of viable cells (**d**) 1 day after cell plating and (**e**) clone size 4 days after cell plating in cultures plated at a clonal density of 2,000 cells/6-cm dish. (**f**) The Mash1-induced increase of cell growth (total cells) was abolished by cyclopamine treatment (1 µg/ml), a specific inhibitor of SHH-mediated signaling. Significances in comparison to the respective LacZ-transduced controls were *$P < 0.001$ and to the Mash1-transduced cultures were #$P < 0.005$. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

proliferative zone of the neocortex.[23,24] Consistent with this, we noticed that the expression of Gli1 was specifically upregulated in Mash1-transduced cultures, but not in control or Ngn2-transduced cultures (**Figure 4a**). We further showed that SHH treatment can also phenocopy Mash1 in NPC survival (**Figure 4d**) and proliferation (**Figure 4e**). In addition, cell growth was retarded by cyclopamine, an inhibitor of SHH signaling (**Figure 4f**). Collectively, the effects of Mash1 in cell survival and proliferation is, at least in part, mediated by SHH protein synthesis and secretion.

Mash1 and Ngn2 transductions in NPCs enhance cell survival *in vivo* after transplantation

Limited neuronal differentiation and poor survival of donor cells after transplantation have been reported in cell transplantation trials using NPCs.[25,26] To examine whether expression of the neurogenic

bHLH genes in donor cells could lead to a more robust neuronal engraftment in transplanted brains, NPCs transduced with Mash1 or Ngn2 were implanted into the striatum (nonneurogenic) and hippocampus (neurogenic region) of adult rat brains. To label donor cells, NPCs were transduced with viruses carrying bicistronic vectors *pMash1-IRES-eGFP*, *pNgn2-IRES-eGFP*, and *pLacZ-IRES-eGFP*, which are designed to express green fluorescent protein (GFP) concomitantly. However, consistent with previous studies,[27] the GFP labeling was inefficient, with <30% of the transduced cells expressing detectable levels of GFP 2 weeks after transplantation. In contrast, when a labeling method using BrdU incorporation (see Materials and Methods) was applied, >90% cells in grafts were positive for BrdU 8 weeks afterward. We thus performed most of the *in vivo* studies with donor cells labeled with BrdU.

Consistent with the *in vitro* findings, Mash1-transduced NPCs generated considerably larger grafts than those in the brain of the animals implanted with LacZ-transduced control cells, regardless of the target brain regions of the striatum (**Figure 5**) and hippocampus (**Supplementary Figure S4**). For example, the graft volumes in the striatum at 8 weeks after transplantation were $96.46 \pm 3.47\,\mu m^3$ (Mash1) versus $14.86 \pm 3.2\,\mu m^3$ (LacZ). Furthermore, strikingly higher proportions of cells were positive for the mature neuronal marker NeuN in the grafts generated by Mash1-NPCs (**Figure 5** and **Supplementary Figure S4**); the percentage of NeuN+ cells in the striatal graft at 8 weeks were $21.08 \pm 2.87\%$ (Mash1) versus $4.88 \pm 1.09\%$ (LacZ).

Exogenous Ngn2 was even more efficient at generating neurons *in vivo*. Up to $56.79 \pm 3.64\%$ of the transplanted cells were NeuN+ neurons when examined 8 weeks post-transplantation (**Figure 5**). In contrast to the *in vitro* findings of Ngn2-mediated decreases in NPC proliferation and survival (**Figures 1** and **2**), transplantation with Ngn2-transduced NPCs generated far better grafts in terms of volume and total cell number than the LacZ-transduced controls and Mash1-transduced cells (**Figure 5**). In sum, introduction of Ngn2 and Mash1 into donor NPCs yielded a massive improvement of NeuN+ neuron engraftments by 842 and 192-fold, respectively, when examined after 8 weeks. These effects were due to a combination of improved cell differentiation and survival.

Cell density– and differentiation period–dependent cell survival effect of Ngn2 via NT3/TrkC-mediated signaling

The enhancement of cell survival/proliferation indices by exogenous Ngn2 *in vivo* (**Figure 5**) was unexpected and inconsistent with the results from the *in vitro* experiments shown in **Figure 1**. The difference may result from the more compact cellular environment and the longer differentiation duration of the grafted NPCs *in vivo*. We thus wondered whether Ngn2 could have different effects on cell survival depending on the cell density and cell differentiation stage/period. The methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay in **Figure 6a** shows invariably higher viable cell numbers in Mash1-transduced cultures compared to the control cultures regardless of the experimental conditions. In contrast, MTT values were lower in Ngn2-tranduced cultures than those of the controls at low cell densities and during early differentiation periods as shown in **Figure 1**. Interestingly, the trend reversed at higher cell densities after longer differentiation
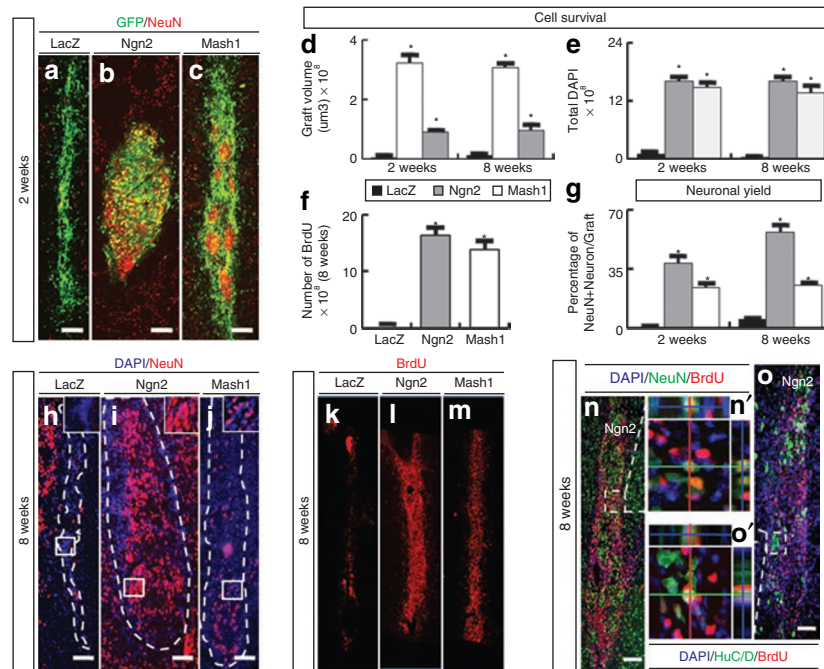
Figure 5 Effect of Mash1- and neurogenin 2 (Ngn2) transduction on donor cell survival and neuron formation in vivo after transplantation. Neural precursor cells (NPCs) from E14 cortices were transduced and transplanted into the striatum of adult rat brains as indicated. Donor cells were labeled with green fluorescent protein (GFP) by transduction with viruses carrying the bicistronic GFP-expressing vectors *pLacZ-IRES-GFP*, *pMash1-IRES-GFP*, or *pNgn2-IRES-GFP*. Alternatively, donor NPCs incorporated BrdU for 24 hours during the cell-proliferation period. Two and eight weeks after transplantation, donor cell survival and neuronal yield were determined. Representative confocal image stacks (35 µm in the *z* direction) of the NeuN+/GFP+ striatal grafts at 2 weeks (**a–c**) and 8 weeks (**h–j**) for NeuN+ cells; (**k–m**) for BrdU+ cells after transplantation. The borders of the grafts are shown in **h–j** and are indicated by dashed lines. Insets of **h–j** are high-magnification images of the respective boxed areas. (**n,n',o,o'**) Colocalizations of the neuronal markers NeuN (**n**) and HuC/D (**o**) in donor (BrdU+/DAPI+) cells within the grafts generated by Ngn2-NPC transplantation. The boxed areas of **n** and **o** are magnified in **n'** and **o'**. *In vivo* donor cell survival was determined by cell graft volume (**d**), total number of cells (**e**) at 2 and 8 weeks and BrdU+ cells (**f**) in grafts at 8 weeks. (**g**) Percentage of NeuN+ donor cells in graft are shown in the increase of neuronal yield compared to LacZ control. (Asterisks) Significantly different from the LacZ-controls at $P < 0.001$, $n = 5$ for 2 weeks and $n = 3$ for 8 weeks. Scale bars = 80 µm. DAPI, 4',6'-diamidino-2-phenylindole.

periods *in vitro*, and Ngn2-transduced cultures produced higher MTT values than control cultures. In fact, the cell viability value of Ngn2-transduced cells plated at the highest cell density tested (3,200 cells/mm²) exceeded even the Mash1 value at *in vitro* differentiation day 12. Similar patterns were observed with regard to the percentage of apoptotic cells (**Figure 6c**). In addition, to exclude cell-proliferation activity,[28] MTT assays were carried out in the presence of aphidicolin, an inhibitor of DNA polymerase; a higher MTT value was observed in the Ngn2-transduced cultures even soon post-transduction if cells were plated at a high cell density (**Figure 6b**). These findings suggest that extrinsic cell–cell contacts and/or diffusible factor-mediated signals produced by Ngn2-transduced cells at high cell densities can override intrinsic cell apoptosis shown in cultures at clonal density (**Figure 1k**).

Survival of neurons requires trophic support mediated by neurotrophic factors.[29,30] As the findings above suggest a possibility of paracrine involvement in Ngn2-mediated cell survival, we examined the expression of neurotrophic factors and their receptor Trk family. Compared to the controls, NT4/5, nerve growth factor, glial cell–derived neurotrophic factor, and glial cell–derived neurotrophic factor receptor c-ret mRNAs were expressed at higher levels in cultures containing Ngn2- and Mash1-NPCs. In addition, a striking and specific induction of mRNA expression of NT3 and its receptor TrkC was evident in Ngn2-transduced

cultures (**Figure 7a**). Increased synthesis and secretion of NT3 proteins by Ngn2 cells were confirmed further by western blot analysis (**Figure 7b** and **c**). In addition, Ngn2-induced NT3 protein expression gradually increased during the differentiation period (**Figure 7d** and **e**), suggesting that the differentiation period–dependent cell survival effect of Ngn2 is specifically conferred by NT3/TrkC signaling. Blockage of the neurotrophic factor–mediated signals with a specific inhibitor K-252a completely abolished Ngn2-mediated cell survival. Consistent with this, treatment of cells with NT3 cytokine (20 ng/ml) during the differentiation period resulted in a cell survival effect comparable to that of Ngn2 (**Figure 7f** and **g**). Finally, *in situ* hybridization clearly demonstrated robust NT3 mRNA expression in the grafts generated by Ngn2-transduced cells (**Figure 7h**), but not in Mash1- and LacZ-transduced cell grafts (data not shown). These findings collectively indicate that NT3 is responsible for the cell density– and differentiation period–dependent cell survival effects of Ngn2.

DISCUSSION

We demonstrated that although both Mash1 and Ngn2 show neurogenic consistent with their role as proneural genes, there are significant differences in mechanistic details. Notably, Mash1-transduced precursor NPCs kept growing during *in vitro*
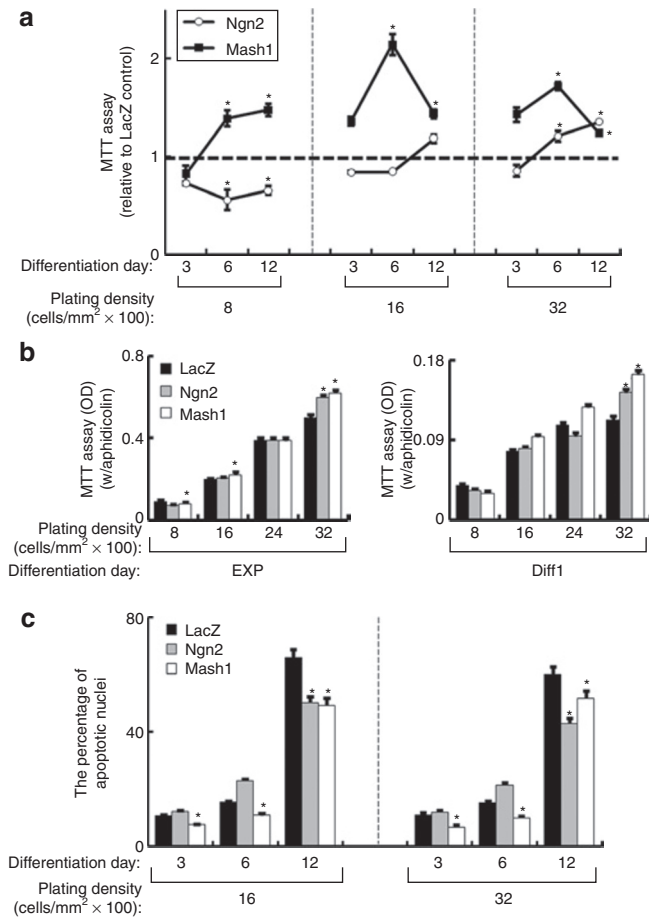
Figure 6 Cell density– and differentiation period/stage–dependent effects of neurogenin 2 (Ngn2) on cell survival. (a) Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. LacZ-, Ngn2-, and Mash1-transduced neural precursor cells (NPCs) were plated on 96-well plates at different cell densities and allowed to differentiate for 12 days. The cultures at each time point were subjected to MTT assays to quantify cell viability. It is noted that MTT values in Ngn2-transduced cultures were lower at the lowest plating cell density (3 cells/mm² × 100), but higher than those of the controls at higher plating cell densities (16 and 32 cells/mm² × 100) after extended differentiation (>6 days). **(b)** MTT assay in the presence of aphidicolin. To eliminate cell proliferation during the early differentiation period, MTT assays were carried out in the presence of aphidicolin, an inhibitor of DNA polymerase. *n* = 8 wells of 96-well plates for each value. **(c)** Percentage apoptotic cells. *n* = 20–40 microscopic fields for each value. *Significantly different from the LacZ-controls at *P* < 0.001. OD, optical density.

differentiation, whereas Ngn2 induced NPCs to exit the cell cycle rapidly (**Figure 2a–f**). We further showed corresponding changes in the levels of protein involved in cell cycle regulation by Mash1 and Ngn2 (**Figure 2g**). The cell cycle promotion activity of the proneural factor Mash1 appears to be in conflict with the notion that differentiation-inducing factors reduce proliferative capacity and result in cell cycle exit. It has, however, been reported that Mash1 deletion in mice leads to a severe decrease in the incorporation of BrdU in the neuroepithelium and to enlargement of the ventricles, indicating a role for Mash1 in cell proliferation.[31] The reduced proliferation that results from Mash1 deletion is more dramatic during the early neurogenic period in the subventricular zone, which harbors more neuron-restricted progenitors than in
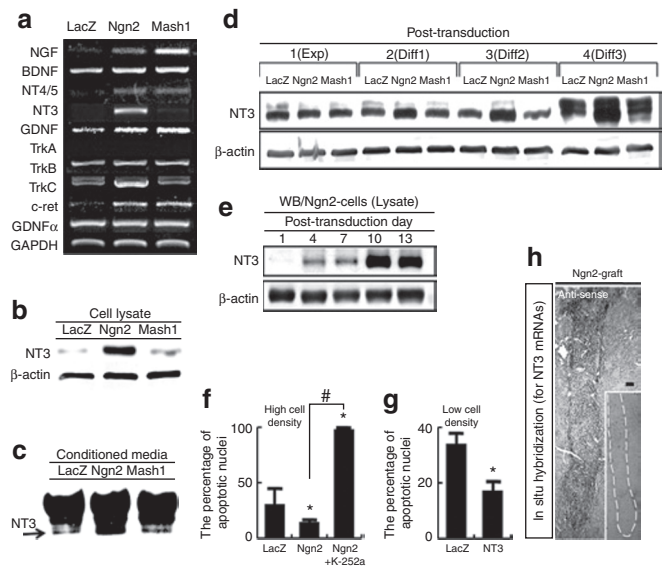

Figure 7 Neurotrophin3 (NT3)-signal activation by Ngn2. (a) Reverse transcriptase PCR analysis for the neurotrophic factors (NGF, BDNF, NT4/5, NT3, and GDNF) and their receptors (TrkA, B, C, GDNFRα, and c-ret). **(b–e)** Western blot (WB) analyses for NT3 protein expression. NT3 protein levels were determined in (**b, d, e**) cell lysates and (**c**) media conditioned in the cultures indicated. **(f)** Ngn2-induced cell survival involves NT3-mediated signaling. Neural precursor cells (NPCs) transduced with Ngn2 or LacZ (control) were plated at 3,200 cells/mm² (high cell density, **f**) or 300 cells/mm² (low cell density, **g**) and differentiated in the presence or absence of (**g**) NT3 cytokine or (**f**) K-252a, an inhibitor of protein kinases activated by Trk-mediated signals. The percentage of cells with apoptotic nuclei out of total cells was determined 12 days after differentiation. Significantly different from the LacZ-transduced controls (*) and from Ngn2-transduced cultures (#) at *P* < 0.001, *n* = 30–45 microscopic fields for each value. **(h)** *In situ* hybridization for NT3 mRNA expression. A representative image of digoxigenin-labeled NT3 anti-sense mRNA in the graft of Ngn2-transduced NPCs 2 weeks after cell transplantation is shown. Inset, negative control with NT3 sense probe. Scale bars = 200 µm.

the ventricular zone where uncommitted neural stem cells reside. These findings indicate that Mash1-mediated cell proliferation is specific to committed neuronal progenitor cells. Compared to Ngn2-generated neurons, which were morphologically mature, Mash1-induced neurons were relatively less differentiated in terms of neuronal morphology (**Supplementary Figure S1**). These findings collectively indicate that in addition to acting as a neuronal determination factor, Mash1 may regulate the proliferation of neuronal precursors early in neurogenesis, whereas Ngn2 acts on the overt terminal differentiation and maturation of postmitotic neurons at a late developmental stage. In support of this idea, sequential roles of Mash1 and Ngn2 have been proposed in the generation of neurons in the dorsal spinal cord[32] and in the olfactory epithelium.[33] We further demonstrate that induction of NeuroD by Ngn2, but not by Mash1, underlies the key differences in function between Mash1 and Ngn2 (**Figure 3**).

SHH, a secreted signaling factor, plays a pivotal role in vertebrate development. It is a well-known morphogen, which emanates from the floor plate and the notochord, that has a ventralizing effect on the developing brain and spinal cord.[34,35] SHH is also required for the generation of specific neuron types, such as the medial and lateral ganglionic eminences in the ventral forebrain

and dopaminergic and serotonergic neurons in the midbrain and hindbrain.[36] In addition, SHH promotes proliferation and cell survival of NPCs.[20,21] Interestingly, this study demonstrates that SHH expression is upregulated in Mash1-transduced NPCs, and that elevated SHH expression was responsible for the effects of Mash1 on cell survival and proliferation (**Figure 4**). Similar to our findings, a recent study has shown that Notch ligand treatment promotes NPC survival *in vitro* and *in vivo*, and that SHH is the molecule that mediates the Notch-signaling effect.[37] Because Mash1 can activate Notch signaling in the "lateral inhibition" process, which is required for the development of appropriate number of neurons via neuron-to-glia fate transition during brain development,[38] Notch activation can be assigned to a delineable signal transduction pathway for Mash1-induced SHH expression. We have initiated an investigation to determine the molecular mechanisms underlying Mash1-induced SHH expression.

The result of greatest interest from our study is the different activity of Ngn2 in cell survival depending on the cell density and differentiation period *in vitro* (**Figure 6**). We show that NT3 ligand and TrkC receptor are strikingly upregulated by Ngn2 (**Figure 7**), raising the possibility that the cell density– and differentiation period–dependent survival effect of Ngn2 is achieved through NT3/TrkC activation. To our knowledge, this is the first report of simultaneous Ngn2-mediated cell survival activity and control of NT3 ligand and receptor expression by Ngn2. Nevertheless, this is not an unimaginable result given that a close association between proneural bHLH factors and NT/Trk signal pathways has been frequently suggested.[39–41] NPCs have been reported to differentiate principally into astrocytes after transplantation into nonneurogenic regions of uninjured adult CNS[42] and injured spinal cord.[43] Attempts have been made to drive the implanted cells into a neuronal fate via proneural genes more efficiently. However, the effects of proneural bHLHs on neuronal differentiation *in vivo* have been controversial, and have yet to be clarified. Facilitated neuronal engraftments have been reported in the inner ear grafted with Ngn2-transfected NPCs[44] and spinal cord engrafted with neuronal progenitors derived from Mash1-infected embryonic stem cells.[45] In contrast, transduction of NPCs with Ngn2 largely enhanced oligodendroglial differentiation in injured spinal cord with an unexpectedly limited extent of neuronal differentiation,[27] suggesting that the commitment activity of the proneural gene can be overridden by environmental cues.[46] This study shows, through systemic and comparative analyses, that transduction of Ngn2 or Mash1 into NPCs yields enriched engraftments of mature neuronal populations in the adult CNS regardless of whether the brain region is neurogenic (hippocampal dentate gyrus) or nonneurogenic (striatum). Furthermore, consistent with the *in vitro* findings of SHH- and NT3/TrkC-mediated cell survival activity of Mash1 and Ngn2, respectively, transduction of Mash1 and Ngn2 in NPCs strongly enhanced donor cell survival in grafted brains.

Tumor formation in the grafted brain could be another concern as Mash1 stimulates proliferation. The following considerations, however, strongly argue against such a possibility. First, the increase in the cell growth observed *in vitro* during the early differentiation period of Mash1-NPCs leveled off, and no further increase in cell number was observed after *in vitro* differentiation for 6 days (**Supplementary Figure S5a**). Consistent with this, no

significant change in the cell number or graft volume was observed between the 2 week and 8 week time points (**Figure 5d** and **e**). Second, very few cells in the grafts (0–20 cells/graft) were positive for the M phase–specific marker phospho-histone H3 at 2 weeks post-transplantation (**Supplementary Figure S5b–d**). Finally, histological examinations showed that cells were uniformly arranged in the Mash1-NPC-derived grafts without forming clusters or any tumor-like structures (data not shown).

In sum, the findings presented in this study suggest that proneural *bHLH* gene manipulation before transplantation could lead to more efficient and long-lasting engraftment of NPCs in the CNS without tumor formation, and should contribute to the development of efficient NPC-based neural replacement strategies for CNS disorders.

MATERIALS AND METHODS

Primary CNS precursor culture **in vitro**. Embryonic brain tissues were dissected from the cortices, ventral parts of the midbrains, hindbrains, and spinal cords of E13–15 rat embryos (Sprague Dawley, Seoul, Korea). After mechanical trituration, cells were plated at 15,000 cells/cm^2 on 10-cm culture dishes precoated with poly-L-ornithine/fibronectin (15 µg/ml; Sigma, St. Louis, MO) at 37 °C overnight followed by fibronectin (1 µg/ml, Sigma) for at least 2 hours in N2 medium (Dulbecco's modified Eagle's medium/F12, 4.4 µmol/l insulin, 100 mg/l transferrin, 30 nmol/l selenite, 0.6 mol/l putrescine, 20 nmol/l progesterone, 0.2 mmol/l ascorbic acid, 200 mmol/l L-glutamine, 8.6 mmol/l D(+) glucose, and 20 mmol/l NaHCO$_3$) supplemented with basic fibroblast growth factor (bFGF, 20 ng/ml; R&D system, Minneapolis, MN). After 4–5 days of culture, cell clusters generated by cell proliferation were dissociated and plated at 10,000–90,000 cells/cm^2 on freshly coated culture surfaces to obtain a uniform population of NPCs. Upon reaching 50–60% cell confluency, cells were subjected to retroviral transductions as described below. On the day following transduction, cell differentiation was induced by withdrawing the mitogen bFGF from the media. In certain experiments, cells were treated with SHH (100–750 ng/ml, R&D systems), cyclopamine (1 µg/ml, Sigma), NT3 (20 ng/ml; Prospec-Tany TechnoGene, Rehovot, Israel), or K-252a (100 nmol/l; Calbiochem, Darmstadt, Germany). Cultures were maintained at 37 °C in a 5% CO$_2$ incubator, and the media were changed every other day with or without daily supplementation of bFGF.

Retroviral infection. Retroviral vectors expressing Ngn2, Mash1, or LacZ (control) were constructed by inserting the respective complementary DNAs into monocistronic (*pCL*) or bicistronic (*pIRES-eGFP*) retroviral vectors as described previously.[47] The retroviral vector expressing NeuroD microRNA (*shRNAmir-NeuroD-pSM2C*) was purchased from Open Biosystems (Huntsville, AL). For the production of retroviruses, the plasmids were introduced into the retrovirus packaging cell line 293gpg by transient transfection with Lipofectamine (Invitrogen, Carlsbad, CA). After 72 hours, supernatant fractions were harvested and maintained at −70 °C until use. For viral transduction, NPCs cultured *in vitro* were incubated with the viral supernatant (3–5 × 10^6 particles/ml) containing polybrene (hexadimethrine bromide: 1 µg/ml, Sigma) for 2 hours, followed by medium change with bFGF-supplemented N2. For coexpression, each of the viral soups was mixed 1:1 (vol:vol) and applied to cells for infection.

Immunostaining cultured cells and brain slices. Cultured cells or cryosectioned brain slices were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS). In the case of BrdU staining, the postfixation procedure was applied by sequentially incubating samples in 1 N HCl, and then in 0.1 mol/l sodium borate solution (pH 8.0). The following primary antibodies were used at the specified concentrations: anti-rabbit TuJ1 (Covance, Richmond, CA; 1:2000), HuC/D (Invitrogen, 1:100), cleaved (activated)

caspase-3 (Cell Signaling Technology, Beverly, MA; 1:500), antimouse microtubule-associated protein 2 (Sigma; 1:1,000), NeuN (Chemicon, Temecular, CA; 1:100), GFP (Invitrogen; 1:400), and anti-rat BrdU (AbD serotec, Oxford, UK; 1:400). Appropriate fluorescence-tagged secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) were used for visualization. Stained samples were mounted in Vectashield with 4′,6′-diamidino-2-phenylindole-mounting medium (Vector Laboratories, West Grove, CA), and photographed using epifluorescence (Nikon, Tokyo, Japan) and confocal microscopes (Olympus, Tokyo, Japan).

Cell viability and proliferation assays. The total number of viable cells was monitored over the entire experimental period via phase-contrast microscopy. Cell viability was further determined by the MTT (Sigma) assay as previously described[48] in the absence or presence of aphidicolin (Sigma). Cells undergoing apoptosis were visualized by their characteristic fragmented and condensed apoptotic nuclei under 4′,6′-diamidino-2-phenylidole staining, as well as by immunocytochemistry against activated (cleaved) caspase-3. Cell proliferation was determined using a BrdU-incorporation assay. Cells were pulsed with 10 µmol/l BrdU (Roche, Indianapolis, IN) for 30 minutes, and BrdU-labeled proliferating cells were detected by immunocytochemistry using an anti-BrdU antibody as described above. Cell survival and proliferation were further determined in the cultures plated at a clonal density, as previously described.[49] Briefly, retrovirally transduced cells were dissociated into single cells and plated at 2,000 cells/6-cm dish. After 6 hours of settling, well-isolated cells were marked with a 3-mm circle marker (Nikon) on the bottom of the plate. Only cells within the marked circles were analyzed. The percentage of viable cells out of the marked cells 1 day after plating and the number of cells per clone 4 days after plating were determined as the indices for cell survival and proliferation, respectively.

Cell cycle analysis by flow cytometry. Cells were harvested by trypsinization, fixed in cold methanol, and labeled with 50 µg/ml propidium iodide (Invitrogen) containing 125 U/ml protease-free RNase (Roche). Harvested cells were filtered through a 95-µm pore size nylon mesh into polystyrene round-bottom tubes with cell-strainer cap, and a total of 10,000 stained nuclei were analyzed in a fluorescence-activated cell sorting/Calibur Flow Cytometer (BD Biosciences, Franklin Lakes, NJ).

Transplantation and histological procedures. To prepare cells for transplantation, proliferating NPCs were labeled by treating them with BrdU (10 µmol/l) for 24 hours. In other cases, donor cells were transduced with the viruses carrying GFP-expressing bicistronic vectors (see "retroviral infection") and detected by immunofluorescent staining for GFP. Single-cell dissociates of the labeled NPCs (3 µl, 1.5 × 10⁵ cells/µl in PBS) were injected over a 3-minute period into the striatum (0 mm posterior to bregma; −0.3 mm lateral to midline; −0.5 mm ventral to the dura) and hippocampal dentate gyrus (−0.35 mm; −0.2 mm; −0.3 mm of adult rats (Sprague Dawley, 200–250 g) under anesthesia induced by Ketamine (4.5 mg/kg) mixed with Rompun (93.28 µg/kg)). The incisor bar was set at 3.5 mm below zero. The needle (22 gauge) was left in place for 3–5 minutes after completion of each injection. The rats received daily injections of cyclosporin A (10 mg/kg, intraperitoneally) starting 24 hours before the grafting and for 3 weeks thereafter. Two or eight weeks after transplantation, animals were anesthetized (50 mg/kg pentobarbital) and perfused intracardially with 4% paraformaldehyde in PBS. Brains were removed and immersed in 30% sucrose in PBS overnight, frozen in Tissue-Tek (Sakura Finetek USA, Torrance, CA), and then sliced on a freezing microtome (CM 1850; Leica, Wetzlar, Germany). Free-floating brain sections (35-µm thick) were subjected to immunohistochemistry as described above. The total numbers of cells positive for NeuN, BrdU, GFP, and 4′,6′-diamidino-2-phenylindole in the graft were estimated as previously described with the Abercrombie correction factor.[50]

NT3 in situ hybridization. A digoxigenin-labeled single-stranded RNA probe was prepared from a linearized 774-base pair full-length NT3 complementary DNA by T7 RNA polymerase using a Digoxigenin RNA Labeling kit (Roche Diagnostics, Mannheim, Germany), and was purified by a nucleotide removal kit (28304; Qiagen, Hilden, Germany). The riboprobe was treated with 10 µg/ml proteinase K (Sigma), acetylated with 0.25% acetic anhydride (Sigma), and denatured at 80 °C immediately before hybridization reactions. Cryosectioned brain slices of the grafts (30 µm) were dried for 1 hour at 50 °C, fixed in 4% paraformaldehyde in PBS (diethylpyrocarbonate-treated), and hybridized at 60 °C overnight in a hybridization solution (50% deionized formamide (Fluka, Buchs, Switzerland), 10% dextran sulfate (Sigma), 1× Denhart's solution (Sigma), 50 mmol/l dithiothreitol (Sigma), 250 µg/ml yeast tRNA purified from brewer's yeast (Ambion, Austin, TX), and 500 µg/ml fish sperm DNA (Roche)). After washing twice (2 hours each) in 0.5× SSC (Fluka) at 68 °C for 2 hours, cells hybridized with the digoxigenin probe were visualized using an alkaline phosphatase–based colorimetric reaction with NBT/BCIP substrate (Roche). The tissues were dehydrated through a graded series of ethanol solutions and mounted for analysis.

Reverse transcriptase PCR, western blot analyses, and enzyme-linked immunosorbent assay for secreted SHH protein quantification. For details of these procedures, see **Supplementary Materials and Methods**.

SUPPLEMENTARY MATERIAL
Figure S1. Morphologic maturation of the neurons differentiated from NPCs expressing the proneural bHLH transcription factors Ngn2 and Mash1.
Figure S2. Ngn2 and Mash1 effects on neuronal yield and cell growth of NPCs isolated from various regions and days of rat embryonic brains.
Figure S3. Semi-quantitative RT-PCR analyses for the ligands and receptors involved with signaling pathways specific to NPC proliferation including FGF-, EGF-, LIF-, Wnt-, Notch-, and SHH-signaling pathways.
Figure S4. *In vivo* survival and neuronal differentiation of Ngn2 or Mash1-transduced NPCs grafted in adult rat hippocampus.
Figure S5. Absence of prolonged cell proliferation in Mash1-transduced precursors.
Materials and Methods.

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