Foxn4 acts synergistically with Mash1 to specify subtype identity of V2 interneurons in the spinal cord

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Neuronal subtype diversification is essential for the establishment of functional neural circuits, and yet the molecular events underlying neuronal diversity remain largely to be defined. During spinal neurogenesis, the p2 progenitor domain, unlike others in the ventral spinal cord, gives rise to two intermingled but molecularly distinct subtypes of interneurons, termed V2a and V2b. We show here that the Foxn4 winged helix/forkhead transcription factor is coexpressed with the bHLH factor Mash1 in a subset of p2 progenitors. Loss of Foxn4 function eliminates Mash1 expression and V2b neurons and causes a fate switch to V2a neurons, whereas the absence of Mash1 displays a similar but less severe phenotype. Overexpression of Foxn4 alone in spinal neural progenitors promotes the V2a fate at the expense of the V2b fate, whereas Mash1 suppresses both the V2a and V2b fates. However, coexpression of both Foxn4 and Mash1 promotes the V2b fate while inhibiting the V2a fate, indicating that Foxn4 cooperates with Mash1 to specify the identity of V2b neurons from bipotential p2 progenitors.

Materials and Methods

Animals. The Foxn4 and Mash1 knockout mice were generated previously (28, 30) and maintained in our laboratories. The stage of mouse embryos was determined by taking the morning when the copulation plug was seen as embryonic day 0.5 (E0.5). All genotypes described were confirmed by PCR.

Immunofluorescence and In Situ Hybridization. Staged mouse embryos were fixed in 4% paraformaldehyde/PBS at 4°C for 20–30
min, infiltrated with 30% sucrose/PBS, and embedded in the OCT compound for cryosection preparation. Immunofluorescent staining of cryosections was then performed as described (28). Antibodies used were: mouse anti-Mash1 (BD Biosciences) at 1:100; rabbit anti-β-gal (Cappel, ICN Pharmaceuticals) at 1:2,000; mouse anti-β-gal (Promega) at 1:300 (with tyramide amplification, Molecular Probes); rabbit anti-Foxn4 at 1:50 (28); rabbit anti-Gata2 (Santa Cruz Biotechnology) at 1:200; mouse anti-Gata3 (Santa Cruz Biotechnology) at 1:50; mouse anti-BrdUrd (BD Biosciences) at 1:100; sheep anti-Chx10 (Exalpha Biologicals) at 1:1,600; mouse anti-Lhx3 (Developmental Studies Hybridoma Bank, DSHB) at 1:100; mouse anti-Mnr2βHb9 (DSHB) at 1:100; mouse anti-Nkx2.2 (DSHB) at 1:50; mouse anti-En1 (DSHB) at 1:25; mouse anti-Isl1 (DSHB) at 1:50; mouse anti-Pax6 (DSHB) at 1:16000; and anti-rabbit phosphorylated caspase-3 (IDUN Pharmaceuticals) at 1:250.

RNA in situ hybridization was carried out as described by using digoxigenin-labeled anti-sense riboprobes (31). The probes used were a mouse Gata2 (21) and chicken Foxn4 cDNA. To generate the chicken Foxn4 probe, primers were designed from an EST containing chicken Foxn4 (ChEST852118) and were used for PCR on chick genomic DNA. The following primer pair was used to amplify a 389-bp fragment of the coding region of chicken Foxn4 gene corresponding to 915–1,309 nt of the mouse Foxn4 gene: forward, 5′-AGGAGCTGGACAAGCTGATTACTG-3′ and reverse, 5′-TACCTTGCAATGCAAATCCATAAT-3′.

**BrdUrd Pulse-labeling and X-Gal Staining.** Staged pregnant mice were injected i.p. with BrdUrd at 100 μg per g of body weight. Two hours later, the injected female was killed, and the embryos were collected and processed for detection of BrdUrd labeling as described (28, 32). X-Gal staining was also performed as described (32).

**Quantitation of V2 Neurons.** To quantify the number of V2 neurons, serial cross-sections of E10.5–10.75 spinal cords were immunostained with anti-Chx10, anti-Gata3, or anti-Nkx2.2 antibodies. Three slides of sections spanning the thoracic to lumbar region were selected and scored under a fluorescent microscope. Three to six samples were collected for each genotype. All data were tested for significance by using two sample Student’s t test.

**Transfection Constructs and in Ovo Electroporation of Chick Embryos.** Fertilized White Leghorn chicken eggs (SPAFAS, Preston, CT) were incubated at 39°C and 50–60% humidity. Electroporations were performed at stages 12–14 by using a BTX square-wave electroporator as described (33). Transfected embryos were incubated for 24 or 48 h and then processed for immunohistochemistry as described (33). Mouse full-length Foxn4 (28) and Mash1 cDNAs were subcloned into the bicistronic pCIG vector also encoding eGFP (6). Only embryos showing strong GFP expression were included in the analysis, which was based on at least three embryos for each experiment.

**Results and Discussion**

**Foxn4 and Mash1 Are Expressed in a Subpopulation of p2 Progenitor Cells.** As a first step to understand the role of Foxn4 during mouse spinal cord development, we characterized the types of cells that express Foxn4 by immunostaining. Starting from E9.5 and at E10.5–11.5, Foxn4 is prominently expressed in a
small cluster of cells located primarily within the ventral ventricular zone (Fig. 1). These cells coexpress Pax6, Mash1, and Lhx3, but not Gata3 or Chx10, even though they are positioned at the level of Gata3" or Chx10" cells (Fig. 1 B–F), indicating that Foxn4 is expressed in a subset of cells within the p2 progenitor domain. Consistent with this, Foxn4-expressing cells are located ventral to En1" V1 interneurons but dorsal to Nkx2.2" V3 interneurons and Hb9" and Isl1/2" motor neurons (Fig. 1 G–J). In addition, most Foxn4" cells are mitotically active as they can be labeled by short pulses of BrdUrd; only a small number do not incorporate BrdUrd in these experiments, and thus may be postmitotic (Fig. 1K). A small number of Lhx3" or Mash1" cells are found to express Gata2 (Fig. 1 L and M). Similarly, Gata2 and Chx10 proteins are detected in some Foxn4" cells and/or their progeny marked by β-gal expressed from the knock-in lacZ reporter in Foxn4lacZ/+ mice (Fig. 1 N and O and data not shown) (28), indicating that both Foxn4 and Mash1 may be expressed in a subset of p2 progenitors that can give rise to either V2a or V2b subtypes. This result is consistent with a Cre-loxP lineage mapping study which shows that Mash1-expressing cells can generate both V2a and V2b neurons (J. Johnson, personal communication).

**Loss of Foxn4 or Mash1 Function Causes a Switch in Cell Fate of p2 Progenitors.** To investigate the requirement of Foxn4 for neurogenesis in the ventral spinal cord, we followed the fate of cells that would normally express Foxn4 in Foxn4lacZ/lacZ mutants (Fig. 2). In heterozygotes, X-Gal staining showed β-gal activity in the mesencephalon and rhombencephalon and throughout the spinal cord at E10.5–11.5 (Fig. 2 A and C), in a pattern closely resembling that revealed by RNA in situ hybridization (29). In the spinal cord, β-gal expression, which is largely confined to the ventricular zone with some weak activity in the ventrolateral funiculus (Fig. 2G), begins to be down-regulated at E12.5 and...
disappears by E13.5 (Fig. 2E). Foxn4 null mutants exhibited a similar pattern of lacZ expression in the developing CNS at E10.5–11.5 (Fig. 2A–D). In the spinal cord, however, lacZ is expressed by a much broader, more lateral group of cells that project their axons in the ventrolateral funiculus (Fig. 2H). Moreover, lacZ expression is not completely down-regulated until E14.5 (Fig. 2E and F), similar to a delay in the down-regulation of lacZ expression in Foxn4lacZ/lacZ retinas (28).

We next analyzed the identity of lacZ+ cells in E10.5–11.5 Foxn4lacZ/lacZ spinal cords using antibodies against a series of cell type-specific markers. There was a complete absence of cells expressing Mash1, Gata3, or Gata2 in the mutant (Figs. 3A–F and 4F), indicating a total failure in the genesis of V2b interneurons. This notion was further supported by the failure to detect Gata2 RNA in situ signals in the Foxn4lacZ/lacZ spinal cord (data not shown). In contrast, there was on average a 3-fold increase in the number of Chx10+/Lhx3+ neurons that simultaneously expressed β-gal (Figs. 3G–L and 4F), indicating that p2 progenitors that would normally express Foxn4 now generate only V2a but not V2b neurons in the mutant spinal cord. Loss of Foxn4 function has no effect on the generation of V1, V3, or motor neurons as no changes were detected in the number of neurons expressing En1, Nkx2.2, Hb9, or Isl1/2 in the null mutant (Figs. 3M–T and 4F). Similarly, it does not affect the expression of prepattern homeodomain factors Pax6 or Irx3 that are involved in the partitioning of p2 progenitor domain (data not shown).

Given the coexpression of Foxn4 and Mash1 and the dependence of Mash1 expression on Foxn4 in p2 progenitors (Figs. 1 and 3), we examined the role of Mash1 in the specification of V2 neurons. To this end, we compared the number of Mash1, Gata2, or Gata3 immunoreactive cells in Foxn4lacZ/lacZ and Foxn4+/−/(H9252) mutant embryos (Figs. 3 and 4). In the spinal cord, however, we observed a significant decrease of neurons immunoreactive for Gata3 or Gata2, a significant increase of neurons immunoreactive for both Chx10 and Lhx3, but no change of Foxn4+ cells. (E and F) Quantitation of neurons that are immunoreactive for Chx10, Gata3, or Nkx2.2 in E10.5 Mash1+/− and Mash1−/− (E) or Foxn4+/− and Foxn4+/+ (F) spinal cords. Each histogram represents the mean ± SD for three to six spinal cords. (G) Summary of changes in V2 cell fate in Foxn4 and Mash1 mutant spinal cords. (Bar, 50 μm in A and B and 20 μm in C and D.)
**Fxn4 and Mash1 Cooperate to Specify V2b Interneurons.** To further clarify the relationship between Fxn4 and other factors that are involved in V2 neuron specification, we performed gain-of-function analyses in the chick spinal cord by using in ovo electroporation. Like in the mouse, chicken Fxn4 expression is confined to progenitors within the V2 domain (Fig. 7 A and B, which is published as supporting information on the PNAS web site). Although Fxn4 is required in mouse for the V2b but not V2a lineage, misexpression of a full-length mouse Fxn4 cDNA in progenitors induced up-regulation of ectopic Chx10 (V2a) expression at E3 and to a lesser degree E4, but suppressed both Gata2+ and Gata3+ V2b cells (Fig. 5 A–C and E–G and Fig. 8, which is published as supporting information on the PNAS web site). Furthermore, expression of endogenous Cash1 was suppressed dorsally but not ventrally, and apoptosis was induced in transfected cells (Figs. 5D and 7C–F and data not shown). In comparison, even though Mash1 is also required for the V2b but not V2a lineage, electroporation of full-length mouse Mash1 alone suppressed Chx10+ and Gata2/3+ cells and induced exclusively dorsal interneuron fates as previously shown (Fig. 5 H–J and data not shown) (34). In contrast to these individual activities, cotransfection of both Fxn4 and Mash1 together strongly induced ectopic Gata2+ and, to a limited degree, Gata3+ V2b cells (Fig. 5 K–M). Notably, Chx10 expression was not up-regulated in these experiments (Fig. 5K). These data indicate that Fxn4 and Mash1 together can cooperate to specify the V2b lineage but have distinct activities on their own, and provide a framework for understanding their individual roles in specifying V2 subtypes.

**Transcriptional Network That Controls Subtype Specification of V2 Interneurons.** The complete absence of Gata3+ cells and dramatic increase of Chx10+ cells in Fxn4 null mutant spinal cords suggest that Fxn4 is a determinant of the V2b lineage and that loss of Fxn4 function results in a p2b-to-p2a fate change in p2 progenitors (Figs. 4G and 6). Fxn4 is required for the expression of Mash1 and Gata2 because Mash1+ or Gata2+ cells disappear in the ventral spinal cord of the Fxn4 mutant. Indeed, Fxn4 function appears to be partly mediated by Mash1 and Gata2 because the absence of Mash1 results in a phenotype similar to but milder than the Fxn4 mutant phenotype, and Gata2 has been shown to play a key role in promoting the differentiation of V2b neurons (19, 21). However, although it is necessary, Fxn4 does not appear to be sufficient on its own for the expression of either Mash1 or Gata2 (Fig. 5 and data not shown). To the contrary, misexpression of Fxn4 alone suppresses the expression of Gata2 while activating that of Chx10, whereas misexpression of Mash1 alone inhibits Chx10 and Gata2 expression. Despite these individual activities, we have found that Fxn4 and Mash1 together exhibit a completely distinct function: they activate the expression of Gata2 and Gata3 while inhibiting that of Chx10, strongly suggesting that Fxn4 and Mash1 act synergistically to promote the V2b lineage (Fig. 6). Such transcriptional cooperation may represent a general mech-

![Fig. 5. Transfection of Fxn4 and/or Mash1 induces V2 neuron subtypes. (A–G) Electroporation (EP) of mouse Fxn4 alone induces ectopic Chx10 expression in the dorsal spinal cord at E3 (stages 18) and E4 (stage 22) (arrowheads), but suppresses Gata2, Gata3, and Cash1 expression (yellow asterisks). (H–J) EP of Mash1 blocks Chx10, Gata2, and Gata3 expression (yellow asterisks). (K–M) Co-EP of Fxn4 and Mash1 blocks Chx10 expression (yellow asterisks) but up-regulates Gata2/Gata3 expression (arrowheads). Images in E–M are from embryos taken 48 h after transfection. Right side was transfected in all images; inset in each panel includes GFP channel to show extent of transfection. (Bar, 50 μm.)](image)

![Fig. 6. Proposed role of Fxn4 and Mash1 in the specification of V2 subtype identity. In the p2 progenitors, Fxn4 is required for the expression of Mash1 and Gata2. Although Fxn4 and Mash1 have distinct individual activities, they function synergistically to specify the V2b lineage. See text for more details. The thin arrows indicate necessity but not sufficiency for expression, and thick arrows indicate sufficiency.](image)
anism for the specification of spinal neuron subtypes. For instance, the specification of motor neurons has been shown to involve the synergistic activities of bHLH and homeodomain transcription factors (35).

Our overexpression results indicate that additional factors likely play a role in enacting the complete differentiation program of V2 subtypes. Induction of Chx10 by Foxn4 occurs in progenitor cells and is accompanied by widespread apoptosis, and even though Mash1 cotransfected with Foxn4 results in Gata2/3 and not Chx10 induction, cell death was still induced (Fig. 7F). In contrast, misexpression of Lhx3 induces Chx10, but not Gata2/3 cells (data not shown), and does so in the context of normal neurogenesis (22, 23). Therefore, it seems likely that other neurogenic factors expressed in the V2 domain that are related to Mash1, such as Ngn1 and Ngn2, and which have also been shown to be required for ventral neuronal development (36), may play additional roles in regulating V2 subtype specification.

In conclusion, our studies define a critical role for Foxn4, functioning in cooperation with Mash1, in establishing the identity of Gata2+/Gata3+ V2b cells from common p2 progenitors via a mechanism that involves the simultaneous activation of Gata2 and suppression of Chx10 lineages in a subset of p2 progenitors (Fig. 6). Because both Foxn4 and Mash1 have distinct activities on their own, these results clearly illustrate that distinct and mutually exclusive differentiation programs can be initiated in closely developing (intermingled) cell populations via intrinsic genetic mechanisms that depend on the synergistic function of two separate transcription factors acting in progenitors. Furthermore, our results suggest that unique signaling mechanisms, distinct from those such as Shh and BMPs, which have been shown to broadly specify unitary progenitor domains, likely function locally and in a cell-specific manner to segregate the two different but closely apposed p2 progenitor populations during neurogenesis.

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