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A Cre Transgenic Line for Studying V2 Neuronal Lineages and Functions in the Spinal Cord

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Abstract

During spinal neurogenesis, the p2 progenitor domain generates at least two subclasses of interneurons named V2a and V2b which are components of the locomotor central pattern generator. The winged-helix/forkhead transcription factor Foxn4 is expressed in a subset of p2 progenitors and required for specifying V2b interneurons. Here we report the generation of a Foxn4-Cre BAC transgenic mouse line that drives Cre recombinase expression mimicking endogenous *Foxn4* expression pattern in the developing spinal cord. We used this transgenic line to map neuronal lineages derived from Foxn4-expressing progenitors and found that they gave rise to all neurons of the V2a, V2b as well as the newly identified V2c lineages. These data suggest that Foxn4 may be transiently expressed by all p2 progenitors and that the Foxn4-Cre line may serve as a useful genetic tool not only for lineage analysis but also for functional studies of genes and neurons involved in locomotion.

Keywords

Foxn4; transcription factor; V2 interneuron; CPG

INTRODUCTION

Spinal cord development begins with the formation of the neural tube, followed by the generation of distinct neuronal and glial cell types in the tube, and concludes with the establishment of functional sensory and motor circuits in the mature cord. The neuronal fates along the dorsoventral axis of the neural tube are controlled in part by signaling molecules secreted from the dorsal and ventral midlines in a concentration-dependent manner (Tanabe and Jessell, 1996; Ericson et al., 1997a; Ericson et al., 1997b; Briscoe et al., 1999; Briscoe et al., 2000; Jessell, 2000). For instance, the opposing activity gradients of Shh and BMPs/Wnts are shown to activate the expression of transcription factors (Liem et al., 1997; Lee et al., 1998; Lee et al., 2000; Liem et al., 2000; Mekki-Dauriac et al., 2002; Patten and Placzek, 2002), which in turn define different progenitor domains from which distinct neuronal cell types arise (Lee and Jessell, 1999; Briscoe et al., 2000; Jessell, 2000; Shirasaki and Pfaff, 2002; Caspary and Anderson, 2003).

In the ventral neural tube, the ventricular zone is organized into one motor neuron progenitor domain (pMN) and four interneuron progenitor domains (p0-p3) that give rise to motor neurons (MN) and several classes of interneurons (V0-V3), respectively (Burrill et al., 1997; Ericson et al., 1997a; Matise and Joyner, 1997; Pierani et al., 1999; Briscoe et al., 2000).

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Each of the progenitor domains is defined by the combinatorial activities of a set of transcription factors, in particular, the homeoproteins and bHLH factors (Pierani et al., 1999; Briscoe et al., 2000; Jessell, 2000; Zhou et al., 2000; Mizuguchi et al., 2001; Novitsch et al., 2001; Zhou et al., 2001; Shirasaki and Pfaff, 2002; Muroyama et al., 2005). The p2 domain generates at least two molecularly distinct interneuron subclasses named V2a and V2b (Briscoe et al., 2000; Karunaratne et al., 2002; Li et al., 2005; Muroyama et al., 2005). V2a cells are mostly glutamatergic excitatory neurons defined by their expression of Chx10; whereas V2b cells are mostly inhibitory GABAergic/glycinergic neurons characterized by their expression of Gata2 and Gata3 (Briscoe et al., 2000; Karunaratne et al., 2002; Al-Mosawie et al., 2007; Lundfald et al., 2007). The binary V2a and V2b fates are selected by Dll-Notch signaling and consolidated by the activity of V2a- and V2b-specific LIM protein complexes involving Lhx3, LMO4, NLI, Scl, and Gata2 (Del Barrio et al., 2007; Peng et al., 2007; Joshi et al., 2009). Although the V2b function is currently unknown, V2a neurons have been shown recently to control speed-dependent left-right locomotor coordination (Crone et al., 2008; Crone et al., 2009).

Foxn4 is a winged-helix/forkhead transcription factor transiently expressed in a subset of progenitors during spinal neurogenesis and retinogenesis. It has been shown by gene targeting and gain-of-function analyses to play a crucial role in the generation of amacrine and horizontal cells during retinal development (Li et al., 2004). In the spinal cord, Foxn4 is expressed by a subset of cells in the p2 progenitor domain (Li et al., 2005). *Foxn4* inactivation causes a complete loss of V2b neurons with a concomitant fate-switch to V2a neurons whereas its misexpression promotes the V2b fate (Li et al., 2005; Del Barrio et al., 2007), thus demonstrating both the necessity and sufficiency for Foxn4 to promote V2b neuron differentiation. Foxn4 acts genetically upstream of the bHLH factors Mash1 and Scl, both of which are required for specifying the V2b fate (Li et al., 2005; Muroyama et al., 2005), as well as controls the expression of Dll4, a ligand for Notch signaling (Del Barrio et al., 2007).

Delineation of neuronal lineages is important for providing a framework to understand the molecular basis of neurogenesis. Our previous immunostaining and knockin analyses have localized Foxn4 to a subset of p2 progenitors and identified a subset of V2a and V2b cells as the progeny of Foxn4-expressing progenitors (Li et al., 2005; Del Barrio et al., 2007), implicating the expression of Foxn4 only by a subset of p2 progenitors. However, due to the transient nature of Foxn4 expression, a possibility exists that Foxn4 may be transiently expressed by all p2 progenitors which give rise to all V2a and V2b cells. Thus, the neural lineages of Foxn4-expressing progenitors have yet to be clearly described during spinal cord development. In this work, we generated a Foxn4-Cre BAC transgenic mouse line and employed the Cre-loxP fate-mapping strategy to determine the cell lineages derived from Foxn4-expressing progenitors in the ventral spinal cord. Our results indicate that the Foxn4-Cre transgene can drive Cre recombinase expression mimicking that of the endogenous *Foxn4* in the developing spinal cord and therefore should be suitable for conditional deletion/ablation of genes and neurons in the V2 domain.

RESULTS AND DISCUSSION

To generate the Foxn4-Cre mice, a BAC containing the *Foxn4* locus was modified by recombineering in *E. coli* to insert the *Cre* coding region at the Foxn4 translation initiation site (Fig. 1a) (Copeland et al., 2001; Gong et al., 2002; Warming et al., 2005; Yang et al., 2006). This BAC transgenic construct contains ~125 kb 5' flanking sequence, all exons and introns, and ~90 kb 3' flanking sequence (Fig. 1a), thereby increasing the likelihood of sufficient regulatory information to recapitulate the endogenous *Foxn4* expression pattern as demonstrated for many other genes (Yang et al., 1997; Heintz, 2001; Battiste et al., 2007).

We obtained three founder lines for this transgenic construct and all of them displayed a similar Cre expression pattern in the developing ventral spinal cord (Fig. 1c-g).

To ask whether Foxn4-Cre can mimic the spatial expression pattern of the endogenous *Foxn4* gene (Fig. 1b), we carried out double-immunolabeling between Cre and markers for p2/V2 and adjacent domains. In the ventral spinal cord of E10.5 Foxn4-Cre transgenic embryos, Cre was seen in clusters of cells at the p2/V2 position, many of which co-expressed Foxn4, Chx10 or Gata2 (Fig. 1c-e). The presence of Cre in Chx10⁺ and Gata2⁺ cells is likely due to the perdurability of Cre since Foxn4 is normally expressed only in progenitors but not in V2a and V2b interneurons (Li et al., 2005). This perdurability may also explain why Cre is present in more cells than Foxn4 (Fig. 1c). There was no expression of Cre in En1⁺ V1 cells or in Isl1/2⁺ motor neurons (Fig. 1f,g). Thus, the Foxn4-Cre transgene appears to be able to largely recapitulate the expression pattern of the endogenous *Foxn4* gene in the developing spinal cord. However, given the presence of Cre in more numerous cells than Foxn4, there is still a possibility that the BAC used may not contain all the regulatory elements necessary to completely mimic endogenous *Foxn4* expression.

To determine the neuronal offspring of Foxn4-expressing progenitors, we crossed the Foxn4-Cre line with the conditional reporter R26R-YFP mice, in which all cells would constitutively express YFP following a Cre-mediated recombination event to remove a transcription stop cassette (Fig. 1a) (Srinivas et al., 2001). When spinal cord sections from E14.5 *Foxn4-Cre;R26R-YFP* embryos were double-labeled with an anti-GFP antibody and the nuclear marker DAPI, we found that Foxn4-expressing progenitors gave rise to many YFP⁺ cells scattered within the ventral gray matter with their axons projected into the ventrolateral funiculus (Fig. 2a,e). The ventral commissure appeared to be largely devoid of YFP⁺ processes (Fig. 2a,e), consistent with the finding that V2 interneurons are mostly non-commissural neurons that project ipsilaterally (Al-Mosawie et al., 2007; Lundfald et al., 2007).

Double-immunostaining of E14.5 spinal cord sections with antibodies against V2 markers revealed the expression of YFP in all Chx10⁺ and Gata2⁺ cells (Figs. 2a-h, 3a), indicating that all V2a and V2b interneurons arise from Foxn4-expressing progenitors. However, only about 37.2% and 25.5% of YFP⁺ cells co-expressed Chx10 and Gata2, respectively (Figs. 2a-h, 3b), suggesting that many V2 interneurons may completely downregulate the expression of V2 markers by E14.5 and/or Foxn4 may be expressed in additional unidentified V2 interneurons or glial cells. In spinal cord sections of E12.5 *Foxn4-Cre;R26R-YFP* embryos, we investigated whether YFP⁺ cells co-express Sox1, which is found in a small subset of interneurons that are derived from the p2 domain and designated as V2c (Panayi et al., 2010). As shown in Fig. 2I-L and by quantification (Fig. 3a), all ventrolaterally located Sox1⁺ cells were immunoreactive for GFP, demonstrating that Foxn4-expressing progenitors also give rise to all Sox1⁺ V2c interneurons. Approximately 4% of all YFP⁺ cells co-expressed Sox1 in the ventrolateral area (Fig. 3b), consistent with V2c cells forming a very small subclass of V2 interneurons (Panayi et al., 2010). By contrast, motor neurons are not generated from the p2 progenitor domain and correspondingly we did not observe YFP⁺ cells that co-expressed the motor neuron marker Isl1/2 (Fig. 2m-p).

Foxn4 is seen only in a limited number of p2 progenitors at a given stage during spinal cord development (Fig. 1b,c) (Li et al., 2005). Moreover, the progeny marked by β -gal expressed from the knock-in *lacZ* reporter in *Foxn4^{lacZ/+}* spinal cords encompasses only a proportion of V2a and V2b interneurons (Li et al., 2005; Del Barrio et al., 2007). These observations raise questions about whether all p2 progenitors transiently express Foxn4 and whether Foxn4-expressing progenitors give rise to all V2 interneurons or only a subset. Our lineage

analyses suggest that all V2a, V2b and V2c interneurons arise from p2 progenitors that express *Foxn4* at some point in life. Consistent with this, there is a complete absence of V2b and V2c neurons in the spinal cord of *Foxn4* null mutant mice (Li et al., 2005; Panayi et al., 2010). Thus, despite the apparent expression of *Foxn4* only in a subset of p2 progenitors (Li et al., 2005), a broader than anticipated complement of V2 interneurons arise from *Foxn4*-expressing progenitors. Similarly, a recent fate-mapping study has revealed that *Ascl1*-expressing progenitors in the spinal cord generate oligodendrocytes more extensively than previously appreciated (Battiste et al., 2007).

V2 neurons constitute part of the locomotor central pattern generator (CPG) network in the spinal cord, which includes also V0, V1 and V3 interneurons and motor neurons (Goulding, 2009). The CPG is an intrinsic neural network capable of generating rhythmic patterns of motor activity without sensory inputs. At present, the developmental, cellular and physiological events leading to locomotor rhythm still remain poorly defined. Gene targeting experiments have shown that V0 neurons are essential for proper coordination of left-right locomotor activity (Lanuza et al., 2004). Genetic ablation of V1 cells demonstrates a key role for these interneurons in the generation of fast locomotor outputs (Gosgnach et al., 2006). V2a cells are mostly glutamatergic excitatory interneurons (Al-Mosawie et al., 2007; Lundfald et al., 2007). Selective ablation of V2a interneurons by diphtheria toxin results in disrupted left-right coordination at high speeds and increased variability in frequency and amplitude of locomotor rhythm (Crone et al., 2008; Crone et al., 2009). This subclass of interneurons is therefore required for maintaining left-right alternation at high speeds of locomotion as well as for stabilizing the locomotor rhythm. V2b cells on the other hand are mostly GABAergic/glycinergic inhibitory interneurons (Al-Mosawie et al., 2007; Lundfald et al., 2007). It is currently unknown how the V2b and V2c subclasses of V2 interneurons participate in the CPG network. In this regard, our *Foxn4*-Cre transgenic line should provide a useful genetic tool not only for lineage analysis but also for domain- and neuron-specific gene manipulation to study functions of genes and neurons in the V2 domain.

MATERIALS AND METHODS

Generation of Transgenic Mice by BAC Recombineering

A *Foxn4* BAC (Clone-ID: RP23-154G3) was purchased from Invitrogen. The *Foxn4*-Cre BAC transgenic construct was prepared primarily following the online BAC recombineering protocols (<http://web.ncifcrf.gov/research/brb/recombineeringInformation.aspx>) (Copeland et al., 2001). Two adapters located in the exon with the translation start site were used for homologous recombination, which resulted in the immediate control of *Cre* gene under the endogenous *Foxn4* promoter without any extra sequence. The two adapters are from two pairs of oligos: 5'end: 5' CCGCTCGAGATTGTCCTGCAATTGTGACCCTTTTGG 3' and 5' CGCAAGCTTATTTTCCTTAGGGATGAAAAAAAAAAG 3' and 3' end: 5' CGGACTAGTATAGAAAGTGGCATTGTTCCAGAATG 3' and 5' TCCCCGCGGGTATTCCTGTGGAGAGCAGTGGTGG 3'. Three *Foxn4*-Cre transgenic founder lines were produced at the transgenic/knockout core facility of the Cancer Institute of New Jersey using F1 (C57BL/6×CBA) with the finished construct. All three lines exhibited a similar reporter expression pattern in the spinal cord and the final analysis was mainly based on one line. The conditional reporter R26R-YFP mice were purchased from the Jackson Laboratory (Srinivas et al., 2001).

Immunofluorescence

Staged mouse embryos were collected and fixed with 4% PFA/PBS and processed for cryosections. Immunofluorescent staining of cryosections was then carried out as previously

described (Li et al., 2004). The antibodies used in the immunostaining analysis are: anti-GFP (for YFP) (goat, 1:1000, Abcam), anti-GFP (for YFP) (rabbit, 1:400, MBL International), anti-Foxn4 (rabbit, 1:50) (Li et al., 2004), anti-Cre (mouse, 1:1000, Covance), anti-Cre (rabbit, 1:10000, EMD Chemicals), anti-Chx10 (sheep, 1:1600, Exalpha Biologicals), anti-Gata2 (rabbit, 1:200, Santa Cruz Biotechnology), anti-Gata2 (guinea pig, 1:2000) (Peng et al., 2007), anti-En1 [mouse, 1:25, Developmental Studies Hybridoma Bank (DSHB)], anti-Nkx2.2 (mouse, 1:50, DSHB), anti-Isl1 (mouse, 1:50, DSHB), and anti-Sox1 (guinea pig, 1:500) (Panayi et al., 2010).

Quantification

To quantify YFP⁺ cells and YFP⁺ cells colocalized with cell type-specific markers in the spinal cord, three comparable samples of each type were counted for positively labeled cells on serial semi-sections located at the thoracic region. The results were analyzed statistically.

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FIG. 1.

Generation of Foxn4-Cre transgenic mice that express Cre recombinase in the p2/V2 domain of the spinal cord. **(a)** The Cre-loxP system for conditional activation of reporter YFP expression using Foxn4-Cre and R26R-YFP mice. To generate the Foxn4-Cre BAC, a BAC containing the *Foxn4* locus was modified by recombineering to insert the *Cre* coding region (Cre-pA) at the Foxn4 translation initiation site (ATG) to generate the Foxn4-Cre BAC. The exons are indicated by vertical black bars and the estimated lengths of 5' and 3' flanking sequences are also indicated. The transgenic line was crossed with R26R-YFP mice to activate YFP expression in the spinal cord. **(b-g)** Spinal cord sections from E10.5 wild-type (b) and Foxn4-Cre (c-g) embryos were labeled by double-immunofluorescence using the indicated antibodies and weakly counterstained with nuclear DAPI. There is colocalization between Cre and Foxn4, Chx10 and Gata2 in cells of the p2/V2 domain but no colocalization between Cre and En1 or Isl1/2. Insets in (c-e) show corresponding outlined regions at a higher magnification. Scale bar equals 25 μ m (b-g).

FIG. 2.

Foxn4-expressing cells give rise to all interneurons of the three known V2 lineages. **(a-p)** Spinal cord sections from E14.5 (a-h) and E12.5 (i-p) *Foxn4-Cre;R26R-YFP* embryos were stained by double-immunofluorescence using the indicated antibodies. Sections in (a,e,i,m) were also weakly counterstained with DAPI. Note the colocalization between YFP and Chx10 in V2a neurons (a-d), between YFP and Gata2 in V2b neurons (e-h), and between YFP and Sox1 in V2c neurons (indicated by arrows) (i-l). YFP and Isl1/2 are not colocalized in motor neurons (m-p). The dashed ovals in (i) outline the regions where Sox1-immunoreactive V2c cells are located. CC, central canal; VLF, ventrolateral funiculus; VZ, ventricular zone. Scale bar equals 50 μ m (a,e,i,m), 12.5 μ m (b-d,f-h,j-l), and 12 μ m (n-p).

FIG. 3.

Percentages of V2 marker-positive cells that are immunoreactive for GFP (**a**), and GFP-positive cells that are immunoreactive for V2 cell markers (**b**) in *Foxn4-Cre;R26R-YFP* spinal cords. Each histogram in (b) represents the mean $\pm$ SD for 3 different spinal cords. Note that Chx10⁺ and Gata2⁺ cells were counted at E14.5 but counts of Sox1⁺ cells were obtained at E12.5 when these cells could be optimally labeled.