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A Requirement for Retinoic Acid-Mediated Transcriptional Activation in Ventral Neural Patterning and Motor Neuron Specification

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Summary

The specification of neuronal fates in the ventral spinal cord depends on the regulation of homeodomain (HD) and basic-helix-loop-helix (bHLH) proteins by Sonic hedgehog (Shh). Most of these transcription factors function as repressors, leaving unresolved the link between inductive signaling pathways and transcriptional activators involved in ventral neuronal specification. We show here that retinoid signaling and the activator functions of retinoid receptors are required to pattern the expression of HD and bHLH proteins and to specify motor neuron identity. We also show that fibroblast growth factors (FGFs) repress progenitor HD protein expression, implying that evasion of FGF signaling and exposure to retinoid and Shh signals are obligate steps in the emergence of ventral neural pattern. Moreover, joint exposure of neural progenitors to retinoids and FGFs suffices to induce motor neuron differentiation in a Shh-independent manner.

Introduction

Deciphering the mechanisms by which cells acquire distinct identities is key to understanding the logic of embryonic patterning and tissue morphogenesis. In many developing tissues, secreted inductive factors initiate cell fate decisions by regulating the expression of transcription factors that impose developmental restrictions on progenitor cells. Typically, these transcription factors operate in a hierarchical manner, such that proteins expressed early in a temporal program of cell differentiation direct the expression of downstream factors (Busslinger et al., 2000; Isshiki et al., 2001; Novitch et al., 2001; Bertrand et al., 2002). Somewhat counterintuitively, studies of transcriptional networks in a variety of developing tissues have revealed that many transcription factors that serve pivotal roles in directing cell fate act as repressors, promoting the differentiation of individual cell types through the repression of other repressors (Mannervik et al., 1999; Busslinger et al., 2000; Barolo and Posakony, 2002). The emergence of an individual cell type is therefore achieved by repressing alternative cell fates—a derepression strategy. Embryonic cell fate decisions in tissues as diverse as sea urchin endoderm and vertebrate hematopoietic and nervous systems depend on such strategies (Busslinger et al., 2000; Muhr et al., 2001; Davidson et al., 2002).

One aspect of cell fate determination by derepression that has yet to be resolved is the pathway responsible for activating the expression of target transcription factors in appropriately derepressed cellular contexts. For example, in the hierarchical transcriptional cascades that operate in many pathways of cell fate determination, it is unclear how downstream transcription factors are activated if the key upstream transcription factors work as repressors. At one extreme, target gene activation could be achieved through uniformly expressed transcriptional activators whose constitutive and subordinate functions become evident only in appropriately derepressed cellular contexts. Alternatively, the relevant transcriptional activators could themselves be regulated by extrinsic signaling pathways, and different activators may be dedicated to the generation of specific cell types. Identification of the activator pathways implicit in these derepression strategies is therefore an important step in clarifying the general logic of cell fate determination.

The vertebrate central nervous system is one tissue in which cell fate determination depends on inductive signals that act through a transcriptional derepression strategy (Muhr et al., 2001). Here, the identity of repressor cascades has been defined in most detail for pathways of motor neuron (MN) and interneuron generation in the ventral spinal cord (Briscoe and Ericson, 2001; Muhr et al., 2001). MNs and ventral interneurons are generated through a pathway that involves the secretion of Sonic hedgehog (Shh) from the notochord and floor plate (Patten and Placzek, 2000). Shh signaling is critical for ventral neural differentiation, since embryos lacking *Shh* function fail to generate MNs and most ventral interneurons (Chiang et al., 1996; Pierani et al., 1999; Litingtung and Chiang, 2000). Moreover, Shh can induce MNs and ventral interneurons in neural plate explants (Roelink et al., 1994, 1995; Marti et al., 1995; Ericson et al., 1996), suggesting that Shh signaling is sufficient to specify ventral neuronal fates.

The pathway by which Shh directs MN and ventral interneuron fates involves a transcriptional hierarchy that appears to operate in three sequential phases (Figure 1B). In a first phase, as the neural plate folds to form the neural tube, distinct ventral progenitor domains are defined by the profile of expression of homeodomain (HD) transcription factors (Briscoe and Ericson, 2001; Lee and Pfaff, 2001). One set of HD proteins, termed class II proteins (the Nkx6 and Nkx2 proteins), requires Shh signaling for expression (Briscoe and Ericson, 2001; Lee and Pfaff, 2001). But a second set, the class I proteins (Pax7, Pax6, Dbx1/2, and Irx3), is expressed in the absence of Shh signaling, and is instead repressed by Shh (Briscoe and Ericson, 2001). Subsequently, cross-

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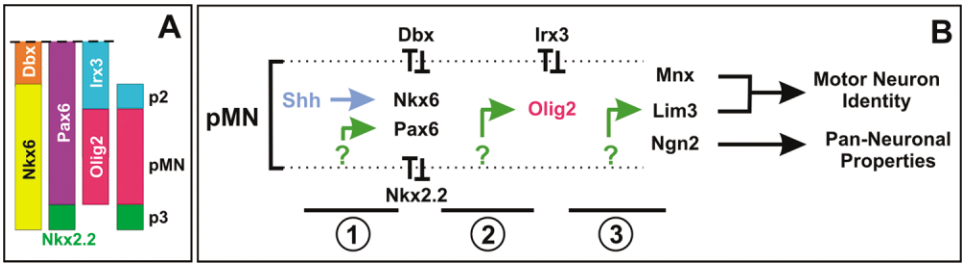

Figure 1. Pathway of Ventral Patterning and Motor Neuron Differentiation

(A) Expression domains of HD and bHLH transcription factors in the ventral spinal cord. p2, V2 neuron progenitors; pMN, MN progenitors; p3, V3 neuron progenitors (for more details, see Briscoe et al., 2000; Novitch et al., 2001).

(B) The cascade of transcriptional repressors that direct MN generation within the pMN domain. Step 1: Shh participates in MN generation through activation of Nkx6 expression. The repressor activity of Nkx6, in conjunction with that of Pax6, excludes the Dbx1/2 and Nkx2 HD repressor proteins. Step 2: the exclusion of Dbx and Nkx2 proteins permits the expression of Olig2, a repressor that helps to prevent the expression of Irx3, another HD repressor of MN differentiation. Step 3: Olig2 repressor function permits the expression of downstream HD and bHLH transcription factors. This repressor pathway presumably requires transcriptional activators (green arrows) to initiate expression of Pax6, Olig2, and downstream transcription factors.

repressive interactions between class I and II HD proteins sharpen and stabilize the boundaries between these ventral progenitor domains (Figure 1A; Briscoe and Ericson, 2001).

In a second phase, after neural tube closure, the profile of class I and II HD proteins within a progenitor domain leads to the expression of domain-restricted determinants of neuronal subtype identity (Figure 1B; Briscoe and Ericson, 2001; Muhr et al., 2001). This transitional phase has been best characterized in the context of MN differentiation (Novitch et al., 2001; Shirasaki and Pfaff, 2002). Prospective MN progenitors are distinguished by their class I (Pax6 and not Irx3) and class II (Nkx6 and not Nkx2) HD protein profile (Briscoe and Ericson, 2001). Yet Pax6 and the Nkx6 proteins are also expressed by other ventral progenitor domains (Briscoe et al., 2000), implying that the emergence of a definitive MN progenitor state occurs downstream of their expression. In support of this, MN progenitors are marked, selectively, by the expression of Olig2, a basic-helix-loop-helix (bHLH) protein that is a determinant of MN fate (Mizuguchi et al., 2001; Novitch et al., 2001; Lu et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002). Moreover, although the Pax6 and Nkx6 proteins are clearly involved in Olig2 expression and MN generation (Ericson et al., 1997; Briscoe et al., 2000; Sander et al., 2000; Novitch et al., 2001; Vallstedt et al., 2001), their primary function within MN progenitors is to prevent the expression of transcription factors capable of repressing Olig2 expression (Novitch et al., 2001). Indeed, a few MNs are generated in *Nkx6* and *Pax6* mutant mice (Ericson et al., 1997; Sander et al., 2000; Vallstedt et al., 2001), indicating that these HD proteins are not required, in an absolute sense, for the activation of Olig2 expression or for MN specification.

In a third phase of this transcriptional hierarchy (Figure 1B), just prior to the emergence of postmitotic neurons, neuronal subtype determinants appear to coordinate panneuronal with subtype-specific programs of differentiation (Muhr et al., 2001). Olig2 functions as such a determinant, promoting the expression of Neurogenin2 (Ngn2) and other bHLH proteins that drive progenitors out of the cell cycle, inducing panneuronal markers and directing the expression of downstream HD regulators of MN identity, notably the Mnx (Mnr2 and Hb9), Lim3, and Isl1/2 proteins (Figure 1B; Mizuguchi et al., 2001; Novitch et al., 2001; Scardigli et al., 2001; William et al., 2003). Moreover, Olig2, like the Nkx6 and Pax6 proteins, functions as a transcriptional repressor (Novitch et al., 2001), revealing that an analogous derepression strategy is at work during this late phase of MN specification.

Despite advances in defining the role of Shh signaling and transcriptional cascades, many critical steps in ventral neuronal specification have yet to be resolved. First, despite the focus on Shh as a regulator of progenitor HD protein expression, the initial activation of class I HD protein expression does not involve Shh, although the relevant signals have not been defined. Ventral neural progenitors are located near sources of other signaling factors, notably FGFs in the node and presomitic mesoderm (Niswander and Martin, 1992; Crossley and Martin, 1995; Riese et al., 1995; Bueno et al., 1996; Diez del Corral et al., 2002) and retinoids in the paraxial mesoderm (Niederreither et al., 1997; Swindell et al., 1999). FGFs and retinoids have been reported to regulate the expression of certain class I HD proteins (Pierani et al., 1999; Bertrand et al., 2000; Diez del Corral et al., 2002), but their general role in the temporal and spatial sequence of progenitor HD protein expression and neural patterning remains unclear.

A second unresolved issue, posed by the finding that most transcription factors involved in MN specification function as repressors, is the identity of the transcriptional activators that drive sequential phases of ventral neuronal specification (Figure 1B). Is the expression of the factors involved in this transcriptional hierarchy achieved through unregulated and constitutive transcriptional activators? Or might inductive signals regulate neuronal subtype selective activators as well as repressors? Defining the link between inductive signaling and transcriptional activator function is a necessary step in resolving the pathway of MN differentiation and may provide more general insights into neuronal specification and the workings of derepression programs used to determine cell fate in other tissues. In this study, we show that retinoid signaling, mediated by the transcrip-

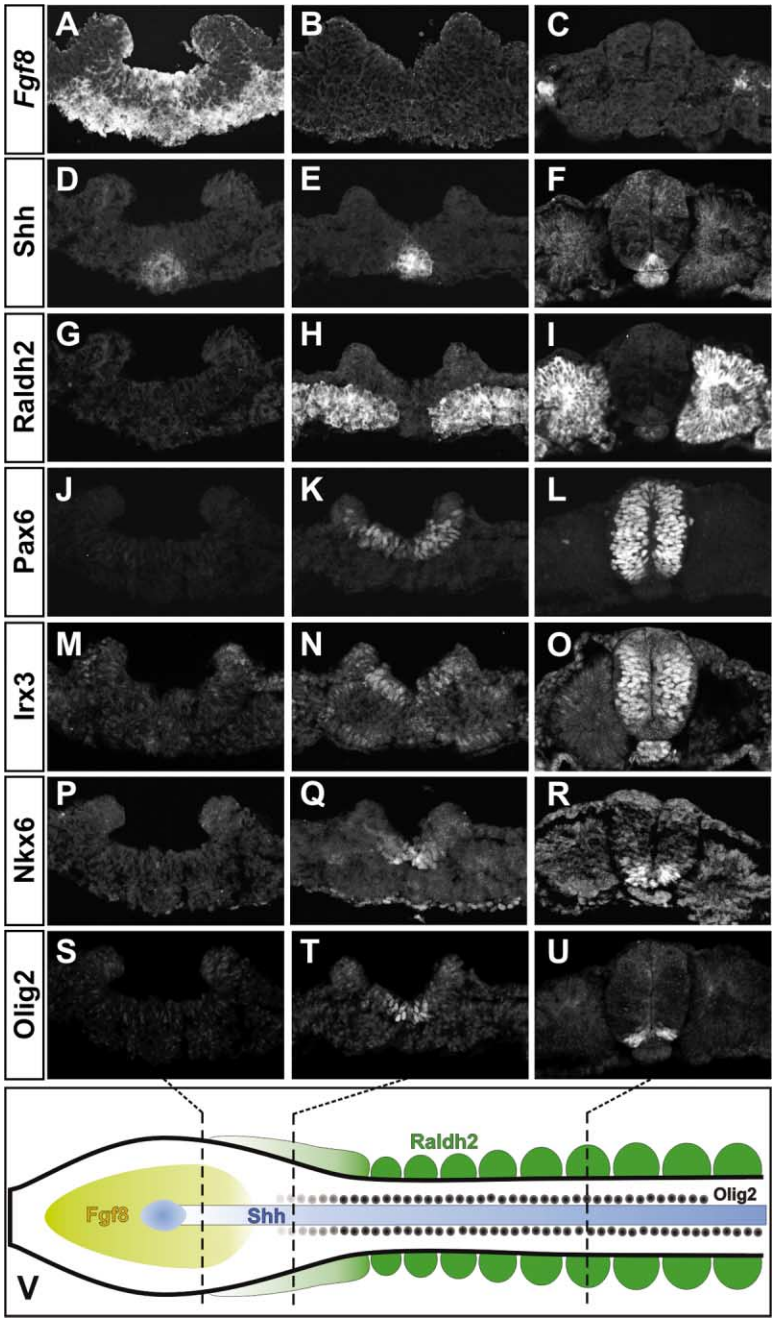

Figure 2. Developmental Changes in Expression of Signaling Factors and HD and bHLH Proteins

(A–U) Analysis of *Fgf8* mRNA and Shh, Raldh2, Olig2, Pax6, Irx3, and Nkx6 protein expression in the neural plate and neural tube of stage 10 chick embryos. Left column, caudal (immature) neural plate; center column, rostral (mature) neural plate; right column, closed neural tube.

(V) Caudal end of a stage 10 chick embryo (top-down view) showing expression of *Fgf8* (yellow) in the node and presomitic mesoderm; the rostral expression of Shh (blue) in the node and notochord; and expression of Raldh2 (green) in the presomitic mesoderm and somites. The onset of Olig2 expression in ventral neural progenitors is shown (gray/black circles).

tional activator function of retinoid receptors, plays a crucial role in ventral progenitor patterning and MN specification.

Results

Olig2 Expression Coincides with the Extinction of *Fgf8* and the Onset of Shh and Raldh2 Expression

To provide information on the nature of the transcriptional activators involved in ventral neuronal specification, we first considered whether other secreted signaling factors might participate with Shh in patterning the ventral neural tube. We therefore analyzed signaling factors known to be expressed in or near the neural tube. In caudal regions of stage 10 chick embryos, soon after differentiation of the neural plate, *Fgf8* is expressed by the node, by the mesoderm that underlies the neural plate, and by cells within the neural plate itself (Figure 2A). Later, during neural tube formation, *Fgf8* expression is extinguished from both neural and adjacent mesodermal cells (Figures 2B and 2C). This temporal decline in *Fgf8* expression is complemented by a progressive increase in the level of Shh expression by the notochord and floor plate (Figures 2D–2F) and by an elevation in the level of expression of Raldh2, a key enzyme in retinoic acid (RA) synthesis, by the paraxial mesoderm (Figures 2G–2I). Thus, as neural progenitor cells mature,

their signaling environment appears to change markedly: from high FGF-low Shh/RA signaling to low FGF-high Shh/RA signaling (Figure 2V).

This temporal switch in signaling factors in the vicinity of neural cells is accompanied by the onset of expression of class I and II HD proteins. In the newly formed neural plate, cells lack expression of the class I HD proteins Pax6, Irx3, and Dbx1/2 and the class II HD proteins Nkx6 and Nkx2.2 (Figures 2J, 2M, and 2P; data not shown). But as the neural tube closes, these class I and class II HD proteins begin to be expressed, and in the specific context of MN generation, the expression of Pax6, Nkx6, and Olig2 is first detected (Figures 2K, 2Q, and 2T; data not shown). The onset of expression of class I and II HD proteins and Olig2 by ventral neural progenitors therefore coincides with the transition from *Fgf8* to Shh and Raldh2 expression (Figure 2V).

FGF Signaling Suppresses the Expression of Class I and II HD Proteins

The coincidence in onset of class I and II HD protein expression and the extinction of *Fgf8* expression, together with evidence that ectopic FGF signaling prevents the onset of Pax6 expression (Bertrand et al., 2000; Diez del Corral et al., 2002), suggested that the evasion of FGF signals by neural precursors might contribute to the timing of onset of class I and II HD protein expression. If this is the case, maintained exposure of neural plate cells or re-exposure of neural tube cells to FGF signals might be expected to suppress expression of class I and II HD proteins.

To test this possibility, we isolated intermediate neural plate ([i]) explants from stage 10 chick embryos (Yamada et al., 1993), cultured them for 18–24 hr alone or with FGF2 (10 ng/ml), and monitored the expression of class I HD proteins. When grown alone, cells in [i] explants expressed low levels of the class I HD proteins Pax6, Irx3, Dbx1, and Dbx2 and did not express the class II HD proteins Nkx6 and Nkx2.2 (Figures 3A–3D, see Supplemental Figure S1 at http://www.neuron.org/cgi/content/full/40/1/81/DC1). In the presence of FGF2, the expression of Pax6, Irx3, Dbx1, and Dbx2 was suppressed (Figures 3E and 3F, Supplemental Figure S1), but these cells still expressed Sox1 and Sox3 (data not shown), indicating that they retain a neural progenitor character. Thus, FGF signaling exerts a general repressive action on class I HD protein expression in neural cells in vitro, mimicking the actions of Shh signaling.

To test whether FGF also influences the expression of class II HD proteins in vitro, we examined [i] explants cultured in the presence of FGF2 (10 ng/ml). No expression of Nkx6 or Nkx2.2 was detected (Figures 3G and 3H), providing evidence that FGF signaling does not mimic the actions of Shh on class II HD protein expression and suggesting that the extinction of class I HD protein expression is not a sufficient condition for the onset of class II HD protein expression. To explore this issue in more detail, we examined whether FGF signaling influences the expression of class II HD protein expression elicited by Shh signaling. We exposed [i] explants to the Hedgehog agonist HhAg1.3 (500 nM; Frank-Kamenetsky et al., 2002; Wichterle et al., 2002) alone and with differing concentrations of FGF2 (1–10 ng/ml). Exposure of cells to HhAg1.3 alone induced Nkx6 expression in >90% of cells and Nkx2.2 expression in ~25% of cells (Figures 3O and 3P). Exposure of [i] explants to 1 ng/ml FGF2 in combination with HhAg1.3 (500 nM) did not change the expression of Nkx6 but resulted in a marked increase in Nkx2.2 expression (Supplemental Figure S2). This enhancement of Nkx2.2 expression is likely to result from the virtually complete repression of Pax6 expression achieved by exposure of cells to low FGF and Shh signaling (Supplemental Figure S2). However, joint exposure of cells to HhAg1.3 and 10 ng/ml FGF2 markedly reduced Nkx6 expression and, to a lesser extent, Nkx2.2 expression (Figures 3S and 3T). Thus, high-level FGF signaling in vitro exerts a repressive effect on class I and II HD protein expression.

To test whether FGF signaling similarly regulates class I and II HD protein expression in vivo, we electroporated the neural tube of chick embryos with expression constructs encoding either a constitutively activated derivative of FGF receptor 1 (FGFR1*) or Fgf8 (Liu et al., 2001).

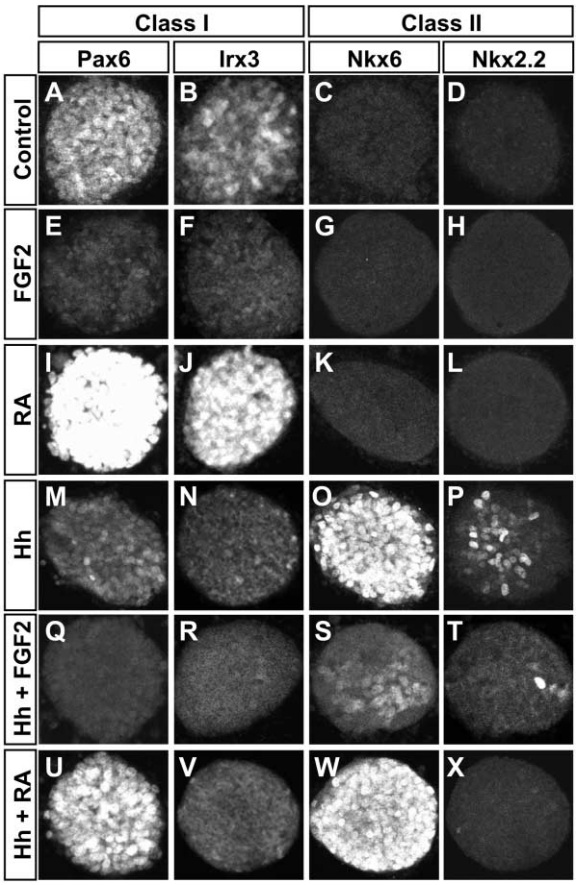

Figure 3. Modulation of Class I and II HD Protein Expression in Neural Plate Explants by FGFs, Retinoids, and Shh

HD protein expression in stage 10 [i] explants grown for 18–24 hr in the absence or presence of different combinations of growth factors (10 ng/ml FGF2, 100 nM retinoic acid [RA], or 500 nM Hh agonist Hg-Ag1.3 [Hh]).

Images are representative of >5 explants cultured under indicated conditions.

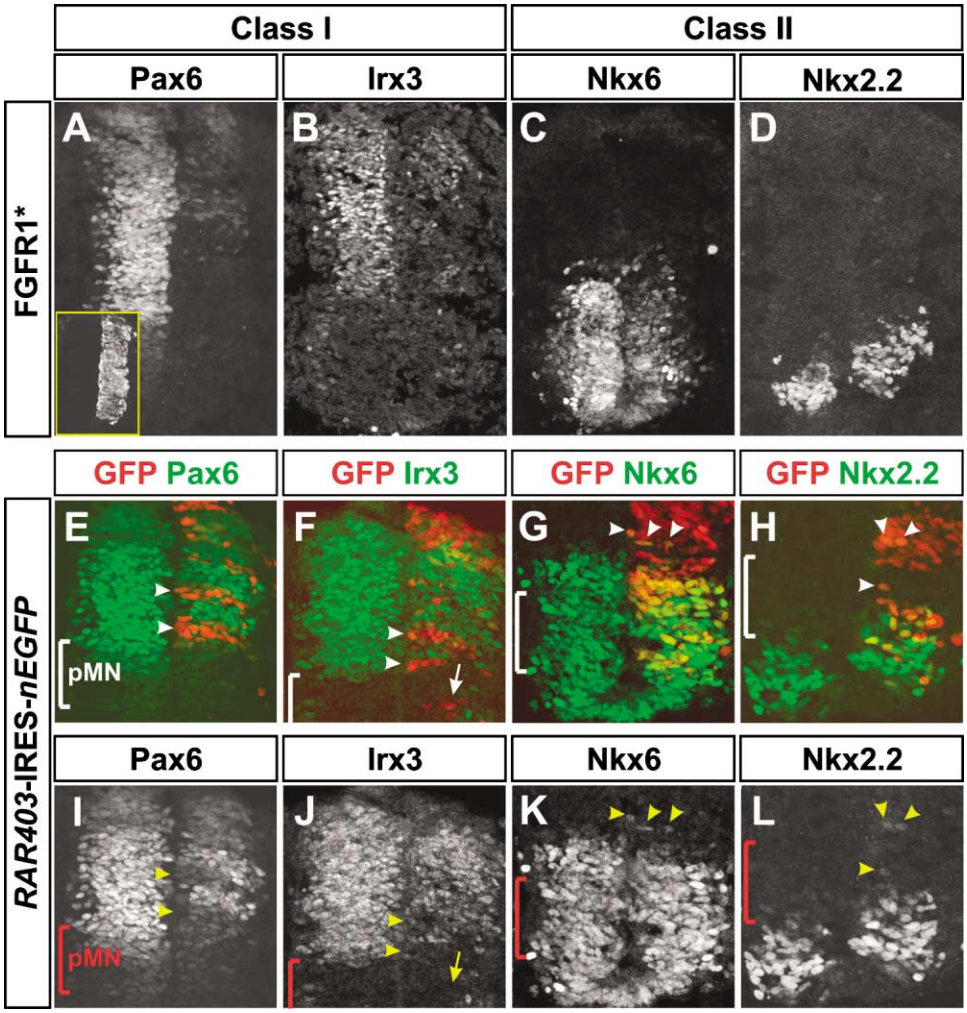

Figure 4. Modulation of Class I and II HD Protein Expression In Vivo by Activation of FGF Signaling or by Blockade of Retinoid Receptor Activation

(A–D) Effects of misexpression of a constitutively activated form of fibroblast growth factor receptor 1 (FGFR1*) on class I and II HD protein expression in the chick spinal cord. Serial sections of a representative transfected embryo are displayed. Transfection of the right half of the neural tube is confirmed by viral envelope protein expression (inset in A).

(E–H) Reduction in class I but not class II HD proteins by misexpression of a dominant-negative retinoic acid receptor (RAR403) construct containing an IRES-nEGFP cassette. Transfection of cells is revealed by GFP staining.

(I–L) Single-channel display of green images from (E) to (H). Note that low levels of Pax6 persist in cells expressing RAR-403-IRES-nEGFP.

The position of the pMN is indicated by brackets. Arrowheads indicate transfected cells. The arrows in (F) and (J) indicate transfected cells in the pMN.

Images are representative of >5 embryos for each experiment.

Misexpression of FGFR1* or Fgf8 in neural cells reduced the level of Pax7 and resulted in a marked suppression of Pax6, Irx3, Dbx1, and Dbx2 expression (Figures 4A and 4B; data not shown), whereas Sox2 and Sox3 expression was retained (data not shown). In addition, the level of Nkx6 expression within its normal domain was reduced (Figure 4C). The expression of Nkx2.2 within its normal domain was not obviously altered, but we observed that the dorsal extent of Nkx2.2 expression was slightly expanded (Figure 4D), most likely a consequence of Pax6 repression. Thus, elevated FGF signaling represses class I HD protein expression in vivo, as in vitro, but has only a limited effect on class II HD protein expression. Together, these findings support the idea that the evasion of FGF signaling that accompanies the maturation of neural cells helps to define the time of onset of class I HD protein expression in vivo.

Retinoid Signaling Selectively Enhances Class I HD Protein Expression in Neural Progenitor Cells

Both FGF and Shh repress class I HD protein expression, raising the issue of whether the high level of expression of these proteins in the absence of these two signals is constitutive or if it requires exposure to an inductive signal. The temporal link between the elevation of Raldh2 expression and the onset of expression of class I HD proteins (Figure 2) is supported by evidence that cells

in the neural tube are exposed to retinoids (Mendelsohn et al., 1991; Balkan et al., 1992; Solomin et al., 1998). Moreover, retinoids activate Dbx expression in neural cells in vitro (Pierani et al., 1999). We therefore examined whether retinoids are involved more generally in the activation of expression of class I HD proteins.

To test this possibility, we compared the profile of class I HD protein expression in [i] explants grown alone or in the presence of retinoic acid (RA; 100 nM). In [i] explants exposed to RA, the level of Pax6, Irx3, Dbx1, and Dbx2 expression by neural progenitor cells was markedly enhanced (Figures 3I and 3J; Supplemental Figure S1 at http://www.neuron.org/cgi/content/full/40/1/81/DC1). Thus, retinoids and FGFs exert opponent influences on the expression of class I HD proteins in vitro. Moreover, joint exposure of [i] explants to retinoids and HhAg1.3 resulted in the persistence of Pax6 but extinction of Irx3 expression (Figures 3U and 3V), a class I HD protein profile characteristic of prospective MN progenitors. Exposure of cells in [i] explants to RA alone did not induce class II HD protein expression (Figures 3K and 3L). Joint exposure of neural cells to retinoids and HhAg1.3 did not significantly alter the expression of Nkx6 protein induced by HhAg1.3 alone (Figure 3W) but suppressed the expression of Nkx2.2 (Figure 3X), a finding that likely reflects the retinoid-mediated upregulation of Pax6 (Figure 3U; Briscoe et al., 2000; Muhr et al., 2001). Thus, retinoids are selective activators of class I HD protein expression in neural cells in vitro.

To determine whether retinoid signaling is needed for high-level expression of class I or II HD proteins in vivo, we assayed the consequences of blocking retinoid receptor function through the misexpression of a dominant repressor derivative of the human retinoic acid receptor α (RAR403) (Damm et al., 1993; Gould et al., 1998). Misexpression of RAR403 in neural progenitors resulted in a marked and cell-autonomous decrease in the level of expression of the class I HD proteins Pax6, Irx3, Dbx1, and Dbx2 (Figures 4E, 4F, 4I, and 4J; data not shown), complementing the retinoid-mediated elevation of expression of class I HD proteins detected in vitro (Figures 3I and 3J).

In contrast, misexpression of RAR403 in ventral progenitors did not inhibit expression of the class II HD proteins Nkx6 and Nkx2.2 within their normal domains (Figures 4G and 4H). Indeed, we occasionally observed RAR403-transfected cells that expressed Nkx6 and Nkx2.2 in ectopic dorsal positions (Figures 4G, 4H, 4K, and 4L, arrowheads). The dorsal expansion of Nkx6 and Nkx2.2 appeared to be limited by class I HD protein expression, which (although markedly reduced by RAR403) was not completely eliminated (Figures 4E and 4I; data not shown). Thus, retinoid signaling appears to be required for high-level class I HD protein expression in vivo, complementing the activation of class I HD protein expression by retinoids in vitro. Together, these findings support the idea that the onset of high-level class I HD protein expression in the neural tube depends on the exposure of cells to retinoid signals, whereas the onset of class II HD protein expression is governed primarily by the onset of Shh signaling.

Blockade of Retinoid Signaling Prevents the Specification of Olig2$^+$ Motor Neuron Progenitors

At the time of neural tube closure, neural progenitor cells have escaped FGF signaling and appear to be exposed to retinoid signals. We therefore examined whether retinoids might have additional roles in promoting the differentiation of ventral neuronal subtypes, independent of their early function in activating class I HD protein expression. To test this possibility, we focused on the specification of MN identity, since the transcriptional hierarchy for MN generation is understood in greater detail than for other classes of spinal neurons (Lee and Pfaff, 2001; Novitch et al., 2001).

MN progenitors can be defined by the onset of expression of Olig2, a critical determinant of MN differentiation (Mizuguchi et al., 2001; Novitch et al., 2001). We therefore examined the influence of retinoid signaling on the progression of Pax6$^+$, Nkx6$^+$ ventral progenitors to an Olig2$^+$ MN progenitor state. We first examined the influence of retinoids on the generation of Olig2$^+$ cells in vitro, comparing Olig2 expression in [i] explants grown alone or exposed to RA (100 nM) or HhAg1.3 (500 nM) or to a combination of RA and HhAg1.3. In [i] explants grown alone or in the presence of RA, no Olig2$^+$ cells were detected (Figures 5A and 5B). In [i] explants grown in the presence of HhAg1.3 alone, Olig2 expression was detected in $\sim$60% of cells (Figures 5C and 5E; Table 1). But when cells in [i] explants were exposed to both RA and HhAg1.3, Olig2 expression was now detected in $\sim$85% of cells and the level of Olig2 expression in each cell was greatly increased compared to the level obtained after exposure to HhAg1.3 alone (Figures 5C–5E; Table 1). Thus, joint exposure of neural cells to retinoid and Hh signaling in vitro enhances the formation of Olig2$^+$ MN progenitors.

The enhancement of Olig2 expression by joint exposure of cells in [i] explants to retinoid and Shh signaling raised the converse issue of whether retinoid signaling is required for the Shh-mediated expression of Olig2 and MN generation in [i] explants grown in vitro (Figure 5C; Marti et al., 1995; Roelink et al., 1995; Ericson et al., 1996; Lu et al., 2000). To test this, we electroporated neural plate cells with RAR403 in ovo and grew [i] explants isolated from electroporated neural tissue for 18–24 hr in the presence of HhAg1.3. Cells in [i] explants that expressed RAR403 exhibited high levels of Nkx6 expression, but few cells transfected with RAR403 expressed Olig2 (Figures 5F and 5G). These results provide evidence that retinoid receptor activation is required for Hh induction of Olig2 expression in vitro. The ability of Shh to induce Olig2 expression and MN differentiation in [i] explants grown in the absence of added retinoids is likely therefore to reflect the prior exposure of neural cells to retinoids before their isolation and culture.

To determine whether retinoid signaling is required for the generation of MN progenitors in vivo, we expressed RAR403 in ventral progenitor cells and monitored the expression of Olig2. The expression of RAR403 did not affect the level of Nkx6 expression in ventral progenitor cells (Figures 4G and 5I) and did not completely repress Pax6 expression (Figures 4E and 4I), but produced a virtually complete, and cell-autonomous, inhibition of

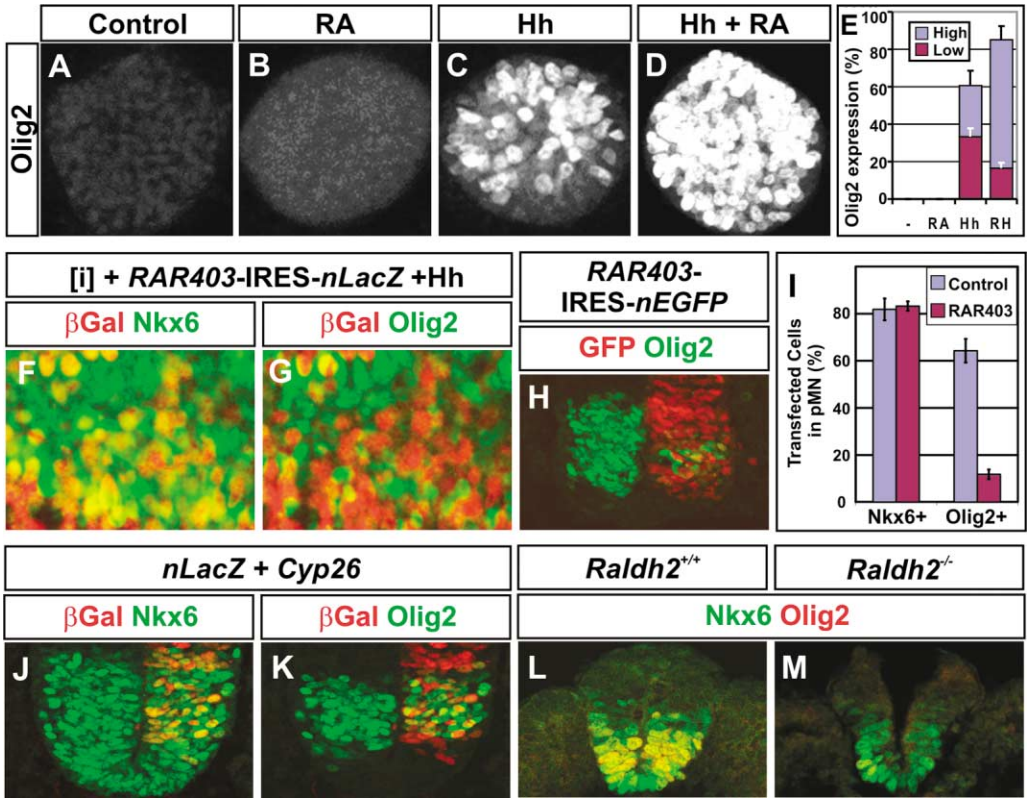

Figure 5. A Requirement for Retinoid Signaling in the Formation of Olig2$^+$ Motor Neuron Progenitors

(A–D) Stage 10 [i] explants grown for 18–24 hr in the absence of growth factors (control), 100 nM retinoic acid (RA), 500 nM Hg-Ag1.3 (Hh), or a combination of 500 nM Hg-Ag1.3 and 100 nM retinoic acid (Hh + RA).

(E) Quantitation of Olig2 expression in [i] explants displayed as % total cells within each explant. High, cells expressing high levels of Olig2 (blue bars); Low, cells expressing low levels of Olig2 (purple bars). Data points taken from >5 different explants (mean ± SEM).

(F and G) Hh-mediated induction of Olig2 within [i] explants requires retinoid receptor signaling.

(H) Electroporation of a RAR403-IRES-nEGFP construct blocks Olig2 expression in the ventral neural tube.

(I) Quantitation of results obtained from chick embryos electroporated with control GFP expression constructs or those encoding RAR403. The % of cells within the presumptive MN progenitor domain is displayed. Data points taken from >5 sections from three different embryos (mean ± SEM).

(J and K) Reduction in Olig2 expression in embryos coelectroporated with *nLacZ* and *Cyp26*.

(L and M) Analysis of Nkx6 and Olig2 expression in control and *Raldh2* mutant mice. Note that Raldh2 mutant mice at e9.5 are delayed in their overall development (Niederreither et al., 1999). For a comparative stage of neural development, a somite-matched e8.5 control embryo is displayed.

Olig2 expression in prospective MN progenitors (Figures 5H and 5I). Thus, retinoid receptor activation appears to be required in vivo for the progression of Pax6$^+$, Nkx6$^+$ ventral progenitor cells to an Olig2$^+$ MN progenitor state.

We also examined the consequences of reducing retinoid signaling in the neural tube through two other measures. First, we expressed Cyp26, a cytochrome P450-related enzyme that degrades RA into oxidized forms that are less effective in stimulating RAR activity (Niederreither et al., 2002a), in ventral progenitors. After expression of Cyp26, no change in Nkx6 expression was detected, but

Table 1. Quantification of Transcription Factor Expression in Neural Plate Explants

	Control	RA	Hh	RA + Hh
Class I				
Pax6	82% ± 7%/0% ± 0%	7% ± 1%/77% ± 7%	78% ± 11%/1% ± 0%	17% ± 1%/68% ± 3%
Irx3	63% ± 6%/1% ± 0%	22% ± 1%/56% ± 3%	6% ± 1%/1% ± 1%	12% ± 3%/4% ± 2%
Class II				
Nkx2.2	0% ± 0%/0% ± 0%	0% ± 0%/0% ± 0%	17% ± 4%/9% ± 6%	0% ± 0%/0% ± 0%
Nkx6	0% ± 0%/0% ± 0%	0% ± 0%/0% ± 0%	20% ± 2%/55% ± 4%	34% ± 5%/49% ± 6%
Olig2	0% ± 0%/0% ± 0%	0% ± 0%/0% ± 0%	33% ± 4%/27% ± 8%	16% ± 3%/69% ± 7%

Data is presented in the following format: low-expressing cells ± SEM/high-expressing cells ± SEM

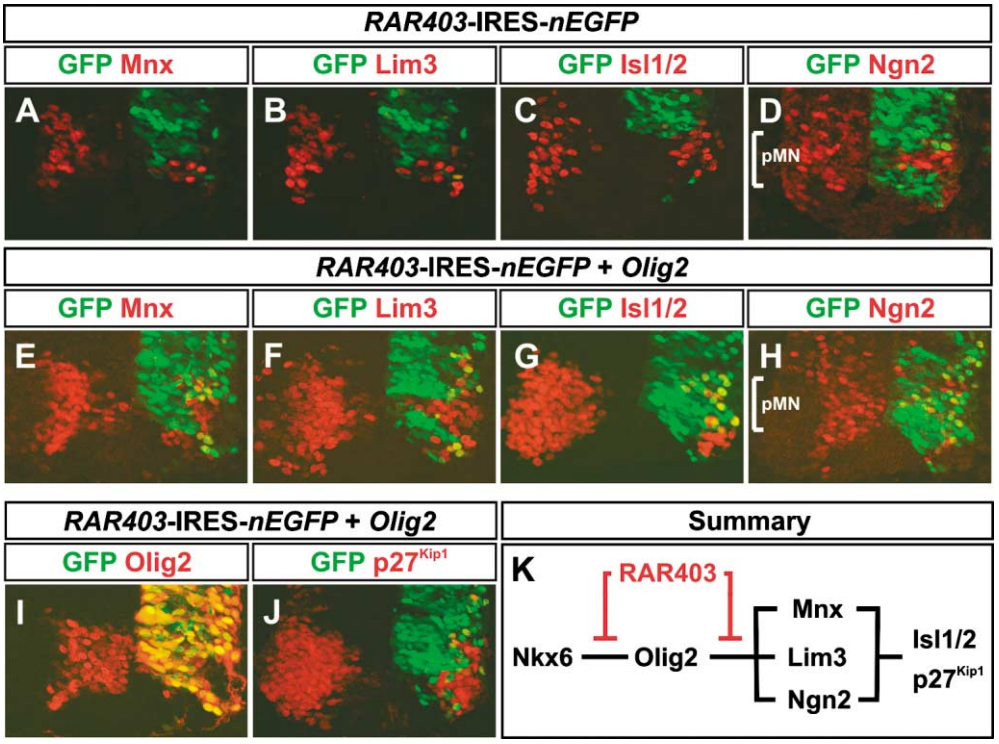

Figure 6. Retinoid Signaling Is Required for Motor Neuron Differentiation

(A–D) Analysis of MN-specific and panneuronal differentiation in the ventral neural tube of embryos electroporated with *RAR403*-IRES-*nEGFP*.

(E–J) Analysis of MN-specific and generic differentiation markers in the ventral neural tube of embryos electroporated with *RAR403*-IRES-*nEGFP* and *Olig2*.

Images representative of >5 embryos for each experiment.

(K) Summary indicating blockade of MN differentiation by RAR403 at steps upstream and downstream of Olig2.

the number of Olig2$^+$ cells was reduced ~4-fold (Figures 5J and 5K). This finding suggests that the degradation of active retinoid ligands recapitulates the effects of blocking retinoid receptor activation. Second, we examined mutant mouse embryos that lack *Raldh2* function, and consequently have markedly reduced levels of RA production (S.S. and T.M.J., unpublished; Niederreither et al., 1999). The caudal neural tube of *Raldh2*$^{-/-}$ embryos was patterned along the dorsoventral axis, as revealed by the ventrally restricted expression of Nkx6 (Figure 5M) and dorsal expression of Pax7 (data not shown). Nevertheless, *Raldh2* mutant embryos exhibited a ~7-fold decrease in the proportion of Nkx6$^+$ progenitor cells that expressed Olig2 (Figures 5L and 5M; data not shown). Together, these findings indicate that a reduction in the availability of retinoid ligands impairs the progression of Pax6$^+$, Nkx6$^+$ ventral progenitor cells to an Olig2$^+$ MN progenitor state, consistent with the influence of retinoid receptor blockade on MN progenitor specification.

Retinoid Signaling Is Required for Motor Neuron Generation In Vivo

Olig2 function is required for expression of Mnx, Lim3, and Isl1/2, HD proteins that define successive steps in MN differentiation (Mizuguchi et al., 2001; Novitch et al., 2001; Lu et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002; William et al., 2003). We therefore examined whether the loss of Olig2 after RAR403 expression affects later steps in the differentiation of Pax6$^+$, Nkx6$^+$ ventral progenitor cells into MNs. Expression of RAR403 in Pax6$^+$, Nkx6$^+$ progenitors blocked the expression of Mnx, Lim3, and Isl1/2 proteins (Figures 6A–6C), providing evidence that retinoid receptor activation is also required for expression of the HD proteins that function downstream of Olig2 in the hierarchy of MN specification.

Olig2, like the Nkx6 proteins, promotes MN differentiation through its function as a transcriptional repressor (Novitch et al., 2001), raising the issue of how activation of the HD transcription factors that function downstream of Olig2, notably Mnx, Lim3, and Isl1/2, is achieved. We considered whether retinoid receptor activation might also be required downstream of Olig2 for the expression of these HD transcription factors. To test this, we examined the consequences of coexpression of Olig2 and RAR403 in prospective MN progenitor cells. After coexpression of RAR403 and Olig2, high levels of expression of Olig2 were detected in Nkx6$^+$ progenitor cells (Figure 6I). Despite this, very few RAR403$^+$, Olig2$^+$ cells expressed Mnx, Lim3, or Isl1/2 (Figures 6E–6G). These findings support the idea that retinoid receptor activation is also required downstream or in parallel with Olig2 to activate the expression of transcription factors that direct later steps in MN differentiation (Figure 6K).

We next examined whether Nkx6$^+$ progenitors that fail

to express Olig2 under conditions of RAR403 expression exhibit defects in panneuronal differentiation, assessed by expression of Ngn2, a bHLH protein that directs neural progenitor exit from the cell cycle (Novitch et al., 2001), and of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor that marks postmitotic neurons (Farah et al., 2000; Novitch et al., 2001). $Pax6^+$, $Nkx6^+$ ventral progenitor cells that expressed RAR403 rarely differentiated into postmitotic neurons, as assessed by the absence of Ngn2 and $p27^{Kip1}$ expression, even when Olig2 was expressed (Figures 6D, 6H, and 6J; data not shown). Thus, blockade of retinoid signaling causes $Pax6^+$, $Nkx6^+$ cells to maintain a progenitor state and blocks the acquisition of panneuronal as well as MN subtype properties.

Ngn2 is widely expressed in the ventral neural tube and has been implicated in the specification of ventral interneurons as well as MNs (Scardigli et al., 2001), prompting us to examine whether blockade of retinoid receptor signaling in other ventral progenitor domains has an influence on Ngn2 expression and, consequently, on interneuron generation. We found that expression of RAR403 within the p0, p1, and p2 progenitor domains (Briscoe and Ericson, 2001; Lee and Pfaff, 2001) blocked Ngn2 expression in a manner similar to that observed in the MN progenitor domain (Figure 6D; data not shown). In addition, the generation of V0, V1, and V2 neurons, defined by expression of Evx1/2, En1, and Chx10, respectively (Briscoe et al., 2000), and $p27^{Kip1}$ was blocked in a cell-autonomous manner by expression of RAR403 (data not shown). Thus, in addition to its roles in activating Olig2 expression and driving the progression of MN differentiation, retinoid receptor signaling appears to have a more general involvement in ventral interneuron generation, mediated at least in part through the regulation of Ngn2 expression.

Retinoids Induce Olig2 Expression and Motor Neuron Generation in a Derepressed Cell Context Achieved by FGF Exposure

We next addressed the issue of whether exposure of neural progenitor cells to retinoids is sufficient to activate Olig2 expression and to promote MN differentiation in a derepressed cell context. To explore this possibility, we needed to find a condition in which the expression of class I HD repressors of MN fate is eliminated, without the exposure of neural cells to Hh signals and consequent Nkx6 expression. The ability of FGF signaling to block expression of class I HD proteins suggested a means of examining whether retinoid signaling is sufficient to induce the formation of $Olig2^+$ MN progenitors in a broadly derepressed cellular context. To test this idea, we exposed cells in [i] explants to FGF (10 ng/ml) and RA (100 nM). Exposure of cells in [i] explants to FGF2 and RA resulted in the expression of moderate levels of Pax6 (Figure 7A), but no expression of Irx3 and Dbx2 was detected (Figure 7B; Supplemental Figure S1 at http://www.neuron.org/cgi/content/full/40/1/81/DC1). Moreover, cells in [i] explants cultured in the presence of both RA and FGF lacked significant Nkx6 or Nkx2.2 expression (Figure 7C; data not shown). Thus, a class I HD derepressed state of neural progenitors conducive to MN specification is achieved under conditions of joint FGF and retinoid exposure in the absence of expression of class II HD proteins.

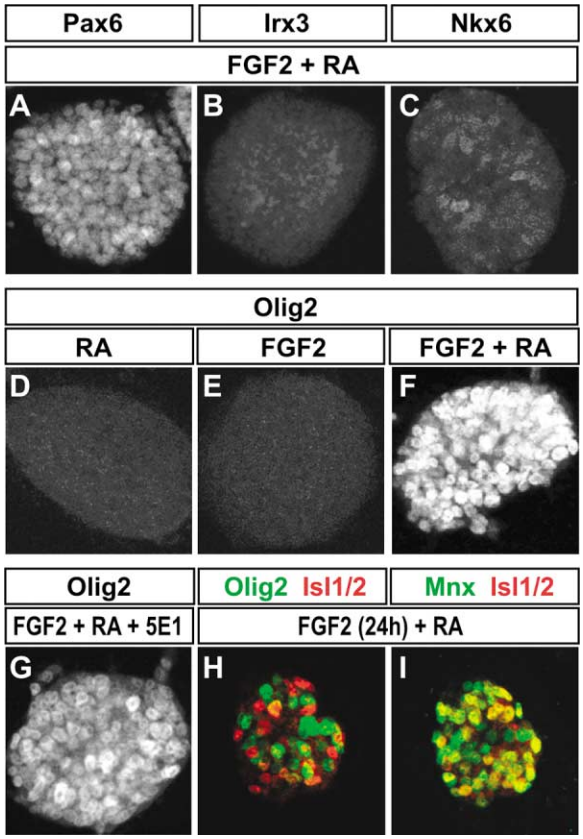

Figure 7. Hh-Independent Induction of Olig2 Expression and Motor Neuron Differentiation In Vitro by Joint Exposure to RA and FGF

(A–C) Induction of Pax6 but not Irx3 or Nkx6 in stage 10 [i] explants cultured in the presence of 10 ng/ml FGF2 and 100 nM RA.

(D–F) Induction of Olig2 expression in [i] explants by 10 ng/ml FGF2 and 100 nM RA.

(G) Induction of Olig2 by FGF and RA is not blocked by mAb 5E1.

(H and I) $Olig2^+$ cells induced by the combination of FGF and RA can differentiate into MNs. $Olig2^+$ progenitors were induced in [i] explants by exposure to FGF2 and RA for 18–24 hr. Explants were washed with PBS and cultured for an additional 24 hr in the presence of RA alone.

Images are representative of >5 explants cultured under indicated conditions.

We next examined the consequences of combined RA and FGF application on Olig2 expression. Neither FGF nor RA signaling alone was sufficient to induce Olig2 expression in neural cells (Figures 7D and 7E), but joint RA and FGF exposure induced high levels of Olig2 expression in many cells (Figure 7F). Thus, combined exposure of neural progenitor cells to FGF and RA promotes the formation of $Pax6^+$, $Olig2^+$ progenitors in the absence of Nkx6 expression. To confirm that Olig2 expression under these conditions was achieved independently of Hh signaling, we grew [i] explants in the presence of RA and FGF, together with the function blocking Hh antibody 5E1 (30 μg/ml) (Ericson et al., 1996). Even in the presence of this Hh inhibitor, many $Olig2^+$ cells were induced by FGF and RA exposure

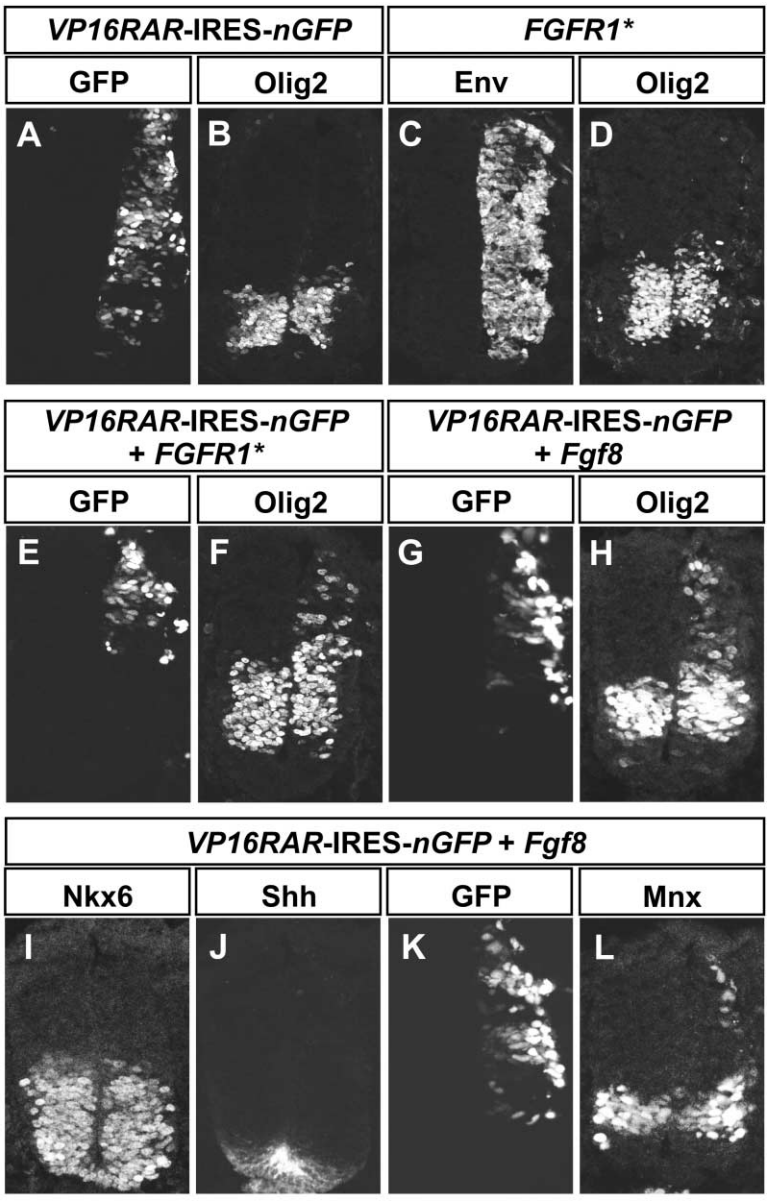

Figure 8. Activation of Both Retinoid and FGF Signaling Pathways Suffices to Induce Olig2 Expression in Vivo

(A–D) Misexpression of a constitutively activated retinoic acid receptor α derivative (VP16RAR) or a constitutively activated FGFR1 (FGFR1*) alone.

(E–H) Combined misexpression of VP16RAR and FGFR1* or Fgf8 activates expression of Olig2. Typically, Olig2 expression was detected in cells that expressed low or intermediate levels of GFP.

(I and J) Combined misexpression of VP16RAR and Fgf8 does not induce ectopic expression of either Nkx6 or Shh.

(K and L) Combined misexpression of VP16RAR and Fgf8 leads to the appearance of ectopic Mnx^+ neurons.

Images are representative of >5 embryos for each experiment.

(Figure 7G), indicating that retinoid signaling can induce Olig2 expression in a class I HD derepressed cell context, independent of Hh signaling.

We next examined whether $Pax6^+$, $Olig2^+$ progenitor cells generated in [i] explants in response to FGF and RA are able to differentiate into postmitotic MNs. To test this, we grew [i] explants in the presence of FGF and RA for 24 hr to initiate Pax6 and Olig2 expression and cultured explants for a further 24 hr in the presence of RA, but without FGF. Under these conditions, many Mnx^+, $Isl1/2^+$ MNs were detected, even in the presence of the Hh blocking antibody 5E1 (Figures 7H and 7I; data not shown). Thus, $Pax6^+$, $Olig2^+$ progenitors generated by exposure to FGF and RA can differentiate into MNs. Together, these findings reveal that retinoid signaling can induce MN differentiation under in vitro conditions in which the expression of class I HD repressors is eliminated, even though cells have not been exposed to Hh signals and do not express Nkx6 proteins.

An Activator Form of Retinoid Receptor α Induces Olig2 Expression and Motor Neuron Generation In Vivo

To assess more directly whether retinoid receptors function as transcriptional activators during the process of MN generation in vivo, we examined the effects of neural expression of a VP16 derivative of retinoic acid receptor α (VP16-RAR), which activates retinoic acid target genes in a ligand-independent manner (Castro et al., 1999). Expression of VP16-RAR alone induced a few ectopic $Olig2^+$ cells in p1 and p2 progenitors, but no ectopic $Olig2^+$ cells were detected in the dorsal spinal cord (Figures 8A and 8B). Since this restriction is likely to reflect the persistent expression of class I HD repressor factors in dorsal neural cells, we coexpressed VP16-RAR with FGFR1* or Fgf8. Expression of FGFR1* or Fgf8 alone was not sufficient to induce ectopic Olig2 expression in the dorsal spinal cord (Figures 8C and 8D; data not shown), but the activation of both retinoid and

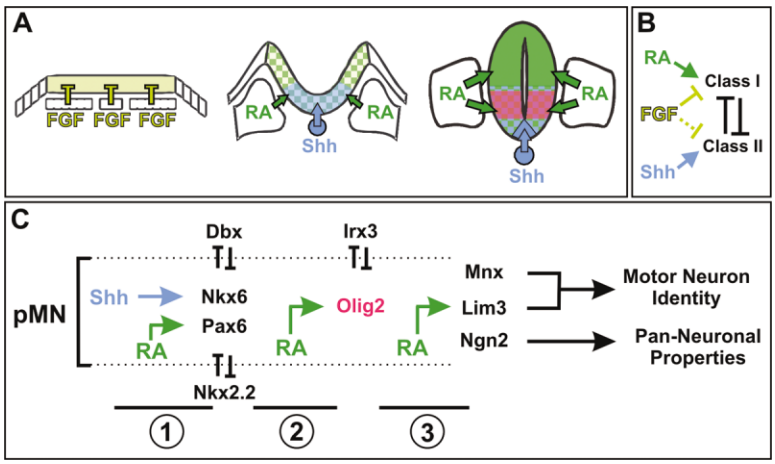

Figure 9. Inductive Signals and the Transcriptional Network That Controls Ventral Patterning and Motor Neuron Differentiation

(A) Schematic displaying the sources of FGFs, RA, and Shh and their influence on neural progenitors. Left column, nascent neural plate; center column, mature neural plate; right column, neural tube.

(B) Influence of signaling factors on class I and II HD protein expression. FGF signals exert a potent suppressive effect on the expression of class I HD proteins and a weaker repression of class II HD proteins. In the absence of FGF signaling, expression of class I HD proteins is enhanced by retinoid signaling, whereas expression of class II HD proteins is initiated by Shh signaling. Once expressed, complementary pairs of class I and II HD proteins exhibit cross-repressive interactions that partition the ventral neural tube into discrete progenitor domains (Briscoe and Ericson, 2001; Lee and Pfaff, 2001; Muhr et al., 2001).

(C) Involvement of retinoid-mediated transcriptional activation in three sequential steps in MN specification. Step 1: RA bound receptors activate the expression of Pax6, providing a derepressed context in which Olig2 expression is permitted. Step 2: RA bound receptors activate expression of Olig2 in derepressed ventral progenitors. Step 3: RA bound receptors act downstream of Olig2 expression to activate the expression of HD that confer MN identity, and bHLH proteins that promote panneuronal differentiation. Our results do not exclude that retinoids may act in parallel with Olig2 for activation of expression of downstream MN HD and bHLH proteins. For details, see text.

FGF signaling pathways in dorsal neural tube cells induced many ectopic Olig2$^+$ cells (Figures 8E–8H). Moreover, these dorsal Olig2$^+$ cells appeared to be generated in the absence of Shh signaling, as assessed by the lack of ectopic Shh or Nkx6 expression in dorsal progenitors (Figures 8I and 8J).

Finally, we determined whether these ectopic dorsal Olig2$^+$ cells acquire later features of MN identity. In the dorsal spinal cord of embryos coexpressing VP16-RAR and FGFR1* or Fgf8, many laterally positioned cells expressed the Mnx proteins, definitive markers of spinal MNs (Figures 8K and 8L; William et al., 2003). Together, these findings provide evidence that the ligand-gated transcriptional activator function of retinoid receptors can induce Olig2 expression and MN generation in a class I HD derepressed context, independently of Shh signaling.

Discussion

The patterning of cells in the ventral spinal cord requires the expression of HD and bHLH transcription factors that function as repressors in a regulatory cascade of neuronal fate specification (Lee and Pfaff, 2001; Muhr et al., 2001). But the identity of the complementary activators implicit in this transcriptional network has remained unclear, as has the contribution of other inductive signals to ventral patterning. In this study we establish a link between Shh-independent inductive signals and the elusive transcriptional activators, showing that retinoid receptor activation directs progressive steps in the differentiation of neural progenitor cells to specific ventral neuronal subtypes. Our findings clarify how transcriptional repressors and activators interact in one system in which derepression drives cell fate decisions, and they may have implications for mechanisms of neuronal specification in other regions of the CNS.

Retinoid Signals and the Activation of Progenitor Homeodomain Protein Expression

The onset of expression of class I and II HD proteins by neural progenitor cells is a critical early step in ventral patterning (Briscoe and Ericson, 2001). Previous studies have emphasized the role of Shh signaling in establishing the pattern of progenitor HD protein expression (Briscoe and Ericson, 2001), yet our findings indicate that Shh is but one of three classes of signaling factors that control the temporal and spatial pattern of class I and II HD protein expression in ventral progenitor cells, with retinoid and FGF signals having crucial accessory roles.

These three signaling factors appear to have different temporal contributions to ventral patterning. During the early stages of neural differentiation, progenitor cells are located close to sources of *Fgf8* expression in the node, the presomitic mesoderm, and the neural plate itself, whereas levels of retinoid synthesis and Shh are low (Figure 9A). Since FGFs are repressors of class I and II HD protein expression, the early exposure of neural progenitors to high FGF-low retinoid/Shh signaling is likely to underlie the initial absence of class I and II HD protein expression, as well as the proliferative capacity of early neural cells (Mathis et al., 2001). As the neural tube forms, however, *Fgf8* expression in the vicinity of neural cells is extinguished, coincident with the onset of high-level retinoid signaling from the paraxial mesoderm and of Shh signaling from the notochord and floor plate (Figure 9A). This switch is accompanied by the onset of neural expression of class I and II HD proteins. Our data indicate that a paraxial mesodermal source of retinoid signaling promotes generic high-level expression of class I HD proteins, whereas a focal ventral source of Shh repressive signals counteracts the action of retinoids and patterns class I HD protein expression, and in addition induces class II HD protein expression.

Details of the circuitry through which Shh coordinately regulates class I and II HD protein expression have re-

mained unclear, however, mainly because class I and II HD proteins exhibit mutual cross-repressive interactions (Briscoe and Ericson, 2001; Lee and Pfaff, 2001; Muhr et al., 2001). Thus, the role of Shh in promoting class II HD protein expression could be achieved through a direct pathway, likely involving Gli proteins (Matise and Joyner, 1999; Persson et al., 2002; Wijgerde et al., 2002; Meyer and Roelink, 2003), or indirectly by repression of class I HD proteins. Conversely, the repression of class I HD proteins by Shh could be direct or could be mediated through activation of class II HD proteins. We find that exposure of neural cells to low-level FGF signaling represses class I without eliciting class II HD protein expression. This finding suggests that Shh signaling is needed for class II HD protein expression, even under conditions of class I HD protein repression (Figure 9B). Moreover, in *Nkx6* mutant mice, the domain of expression of the Dbx class I HD proteins expands ventrally in the face of high-level Shh signaling (Sander et al., 2000; Vallstedt et al., 2001), consistent with the idea that Shh-induced expression of class II HD proteins is needed for ventral exclusion of class I HD proteins.

Retinoid Activation of Olig2 Expression and Motor Neuron Progenitor Identity

Class I and II HD proteins define individual progenitor domains only in combinatorial fashion, and thus their expression, per se, does not mark the emergence of neuronal subtype-restricted progenitors. This step appears to be associated with the onset of expression of neuronal subtype determinants within an individual progenitor domain (Briscoe and Ericson, 2001; Muhr et al., 2001). Within the MN lineage, the transition from a class I and II HD protein-defined progenitor domain to MN progenitor status is marked by onset of expression of Olig2 (Mizuguchi et al., 2001; Novitch et al., 2001). The requirement for retinoid-mediated transcriptional activation in Olig2 expression clarifies a crucial step in the derepression strategy of MN specification. The class I and II HD proteins that are expressed in MN progenitors, Pax6 and Nkx6 proteins, are important for effective Olig2 expression (Mizuguchi et al., 2001; Novitch et al., 2001), but only through their ability to exclude repressors of Olig2 from the MN progenitor domain (Ericson et al., 1997; Novitch et al., 2001; Supplemental Figure S3 at http://www.neuron.org/cgi/content/full/81/DC1). Our studies indicate that retinoid signaling through retinoid receptors underlies this missing activator step, promoting the transition of $Pax6^+$, $Nkx6^+$ ventral progenitors to $Olig2^+$ MN progenitors (Figure 9C).

Retinoids and Shh therefore appear to have crucial and complementary roles in directing the transcriptional cascade that specifies MN progenitor identity. Shh signals direct expression of the Nkx6 class II HD proteins, whereas retinoid signaling initially ensures expression of the class I HD protein Pax6 and later, once a progenitor domain expressing the Pax6 and Nkx6 repressors has been established, exerts an additional function in activating Olig2 expression (Figure 9C). Strikingly, the coincident exposure of neural progenitor cells to FGF and retinoid signals induces Olig2 expression and MN generation through a pathway that bypasses the requirement for Shh signaling and class II HD protein expression. These findings therefore suggest a basis for the persistence of Olig2 expression observed in the hindbrain of *Nkx6* mutant mice (Pattyn et al., 2003).

Coordinated Shh and retinoid signaling influences cell patterning in other tissues, for example, during limb and cranio-facial morphogenesis (Helms et al., 1994, 1997). But here, retinoid signaling influences the expression of *Shh* (Helms et al., 1997; Schneider et al., 2001; Niedereither et al., 2002b), rather than converging on the Shh signal transduction pathway at the level of target gene regulation. Studies of the establishment of left-right asymmetry in early vertebrate embryos, however, have revealed that the expression of *Lefty-1*, an asymmetry gene, is regulated in parallel by Shh and retinoids (Tsukui et al., 1999). Thus, the convergence of Shh and retinoid signaling may have a more widespread influence on target gene regulation during cell fate specification.

Retinoid-Dependent Activation and the Coordination of Neuronal Differentiation

Olig2 functions as a determinant of MN identity, directing MN progenitors out of the cell cycle and imposing panneuronal properties, as well as promoting expression of downstream HD transcription factors (Mizuguchi et al., 2001; Novitch et al., 2001). Yet Olig2 also functions as a repressor, implying that an analogous derepression strategy is used during this late phase of MN specification. Our data provide evidence that retinoid receptor activation is required downstream of Olig2 expression, for induction of Ngn2 and panneuronal differentiation, as well as for the expression of downstream HD protein effectors of MN differentiation. Thus, retinoid receptor signaling appears to supply missing activator functions that operate at sequential phases in the transcriptional specification of MN identity (Figure 9C).

The derepression strategy of neuronal fate specification is not restricted to MN generation and appears to control the specification of many ventral interneuron classes (Muhr et al., 2001). Retinoid signaling may also be involved in the differentiation of many of these ventral interneuron populations, since the expression of class I HD proteins that define each ventral progenitor domain and interneuron class (Briscoe et al., 2000) appears to be enhanced by retinoid signaling. Indeed, previous studies have provided evidence that retinoid signaling is involved in the generation of V0 and V1 neurons (Pierani et al., 1999). Retinoid receptor activation, however, appears not to be involved in the specification of all ventral interneuron subtypes. Progenitors in the p3 domain do not express a class I HD protein, raising the possibility that this domain requires the evasion of retinoid signals. Consistent with this idea, in vitro expression of the class II HD repressor protein Nkx2.2, which specifies the p3 progenitor domain (Briscoe et al., 1999; Briscoe and Ericson, 2001), can be suppressed, albeit indirectly, by exposure to retinoids. Our findings therefore suggest a widespread, but not universal, role for retinoid-mediated transcriptional activation in the specification of ventral neuronal subtypes.

Retinoid-mediated activation also appears to operate during the core program of neurogenesis that is controlled, in part, by neuronal subtype determinants such

as Olig2. The dependence of Ngn2 expression on retinoid receptor activation is evident not only within the domain of MN progenitors, but also within other regions of the ventral neural tube. Studies in *Xenopus* embryos have implicated retinoid receptor signaling in neurogenesis (Sharpe and Goldstone, 1997) and, together with our results, suggest that this action involves the retinoid-mediated activation of neurogenic bHLH protein expression, independent and downstream of the earlier roles of retinoids in progenitor cell specification.

Coordinated Activities of Retinoid and FGF Signals

The influence of retinoids and FGFs on the expression of class I and II HD proteins provides an indication of how ventral progenitor cell pattern can be regulated independently of Shh signaling. These observations may be relevant to the discovery of a surprisingly normal degree of ventral patterning in the spinal cord of *Shh* null mutant embryos, when negative intracellular regulators of Hh signal transduction, Gli3 or Rab23, are also eliminated (Litingtung and Chiang, 2000; Eggenschwiler et al., 2001; Persson et al., 2002; Wijgerde et al., 2002). One explanation proposed for the restoration of ventral neuronal pattern is the existence of a Shh-independent signaling pathway, and our studies raise the possibility that this subordinate signal involves the conjoint exposure of ventral neural progenitor cells to retinoids and FGFs.

Although the molecular basis of the opponent actions of retinoids and FGFs on class I HD protein expression has not been resolved, this study and work by others has begun to indicate that coordinate retinoid and FGF signaling exerts a pervasive influence on neural cell patterning. Coordinate retinoid and FGF signaling has, for example, been implicated in the regional specification of the neural plate along its anteroposterior axis (Muhr et al., 1999; Mercader et al., 2000; Liu et al., 2001; Bel-Vialar et al., 2002; Kudoh et al., 2002), and the later opponent activities of FGFs and retinoids establish a spatial profile of Hox protein expression along the rostrocaudal axis of the hindbrain and spinal cord (Liu et al., 2001; Bel-Vialar et al., 2002; Kudoh et al., 2002). Similarly, opponent roles for retinoids and FGF have been suggested to control cell differentiation along the proximodistal axis of the limb (Mercader et al., 2000) and may influence the rostrocaudal program of paraxial mesodermal differentiation (Deschamps et al., 1999; Dubrulle et al., 2001).

Finally, our findings on neuronal pattering in the ventral spinal cord may provide more general insights into the nature of the interplay between transcriptional activators and repressors involved in cell fate specification. In the ventral spinal cord, the function of one set of activators used in neuronal specification is not constitutive but is gated by extrinsic signaling factors. Moreover, during MN differentiation, the same activator mechanism appears to be used at several sequential steps in a transcriptional cascade of neuronal specification, at each step complementing the functions of a distinct set of repressors. Since cell fate specification in other regions of the CNS (Schuurmans and Guillemot, 2002) and in many nonneural tissues (Busslinger et al., 2000; Davidson et al., 2002; Han et al., 2002) relies on similar derepression strategies, it will be informative to determine whether the transcriptional logic of neuronal patterning revealed in the ventral spinal cord is predictive of that used to generate cell diversity in other developing systems.

Experimental Procedures

Animal Preparation

Fertilized chick eggs (Spafas) were incubated at 38°C and staged (Hamburger and Hamilton, 1951). *Raldh2* null mice were generated by replacement of exon 4 of the *Raldh2* locus with an IRES-tau-myc tk-neomycin/hygromycin cassette. Details are available upon request.

In Situ Hybridization Histochemistry and Immunohistochemistry

Fgf8 localization was performed as described (Liu et al., 2001). Images were collected on a Zeiss Axioskop microscope. Antibody staining was performed as described (Sockanathan and Jessell, 1998; Briscoe et al., 2000; Novitch et al., 2001), and immunofluorescence images were collected on a BioRad MRC1024 confocal microscope. Note that the anti-Nkx6 antibody used recognizes both Nkx6.1 and Nkx6.2.

Neural Plate Explant Culture

Intermediate [i] neural plate tissue was isolated from stage 10 chick embryos as previously described (Yamada et al., 1993; Ericson et al., 1996). Explants were grown in collagen matrices in Ham's F12 media (Specialty Media) supplemented with 3% glucose (Sigma), 1× N2 supplement, Penicillin-Streptomycin, 2 mM L-glutamine (all from Invitrogen), and 0.5 U/ml heparin (Sigma). 200–1000 nM of Hedgehog agonist Hh-Ag1.3 (Curis; Frank-Kamenetsky et al., 2002; Wichterle et al., 2002), 100 nM all-trans-retinoic acid (Sigma), or 1–10 ng/ml of FGF2 (R&D Systems) were added to selected culture media. To test the Hh independence of MN production, explants were cultured in the presence of 30 μg/ml Hh blocking antibody 5E1 (Yamada et al., 1993; Ericson et al., 1996).

Expression Constructs and In Ovo Electroporation

cDNAs encoding human retinoic acid receptor α truncation mutant 403 (RAR403) (Damm et al., 1993), a VP16-retinoic acid receptor α fusion (Castro et al., 1999), and Cyp26 (Swindell et al., 1999) were cloned into the pCAGGS expression vector (Niwa et al., 1991) or IRES-nLacZ or IRES-nEGFP derivatives. The activated FGFR1 vector was as described (Liu et al., 2001). Gene misexpression was achieved by in ovo electroporation (Briscoe et al., 2000; Novitch et al., 2001) of constructs alone or coexpressed with a nLacZ plasmid to distinguish transfected cells. Embryos were typically electroporated at stages 11–13 and analyzed at stages 17–22.

Explant Culture of Electroporated Neural Tissue

Stage 9 chick embryos were electroporated with nLacZ or RAR403-IRES-nLacZ expression plasmids and were incubated in ovo for ~2 hr. Neural explants were isolated from [i] regions of the transfected neural tube. Explants were cultured for 18–24 hr with or without 1 μM HhAg1.3.

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