Distinct activities of Msx1 and Msx3 in dorsal neural tube development

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Summary

Patterning of the dorsal neural tube involves Bmp signaling, which results in activation of multiple pathways leading to the formation of neural crest, roof plate and dorsal interneuron cell types. We show that constitutive activation of Bmp signaling at early stages (HH10-12) of chick neural tube development induces roof-plate cell fate, accompanied by an increase of programmed cell death and a repression of neuronal differentiation. These activities are mimicked by the overexpression of the homeodomain transcription factor Msx1, a factor known to be induced by Bmp signaling. By contrast, the closely related factor, Msx3, does not have these activities. At later stages of neural tube development (HH14-16), dorsal progenitor cells lose their competence to generate roof-plate cells in response to Bmp signaling and instead generate dorsal interneurons. This aspect of Bmp signaling is phenocopied by the overexpression of *Msx3* but not *Msx1*. Taken together, these results suggest that these two different Msx family members can mediate distinct aspects of Bmp signaling during neural tube development.

Key words: Spinal cord development, Roof plate, Homeodomain factors, Basic-helix-loop-helix transcription factors, Chick in ovo electroporation, Math1/Cath1, Mash1/Cash1, Bmp signaling, Dorsal-ventral patterning

Introduction

During the development of the vertebrate nervous system, multiple signaling pathways play instructive roles in patterning the dorsoventral axis of the neural tube. Sonic hedgehog secreted from the notochord and floor plate induces the differentiation of motoneurons and ventral interneurons in a concentration-dependent manner in the ventral neural tube (reviewed by Briscoe and Ericson, 2001). Similarly, signaling molecules secreted from the epidermal ectoderm and roof plate are involved in patterning the dorsal neural tube. Both Wnt and Bmp signaling pathways have been implicated to be important for dorsal neural tube patterning (Garcia-Castro et al., 2002; Liem et al., 1995; Nguyen et al., 2000). The downstream effectors that mediate these activities of Wnt and Bmp signaling in regulating dorsal neural tube development have been poorly defined. We address the roles of the Msx family of transcription factors in mediating downstream effects of Bmp signaling in chick neural tube development.

Three populations of cells develop from the dorsal neural tube/lateral neural plate. Neural crest cells that give rise to the peripheral nervous system are generated from the border region between the neural plate and adjacent ectoderm and migrate out of the neural tube before or during neural tube closure (Le Douarin, 1982). Roof-plate cells develop at the dorsal midline of the neural tube and are a group of specialized glial cells. Both neural crest and roof-plate cells are induced by Bmp signals from the epidermal ectoderm (Liem et al., 1995). The roof-plate cells themselves subsequently become a source of several Bmps, which can promote the differentiation of a third population of cells, the dorsal spinal cord interneurons (Lee et al., 2000; Lee et al., 1998; Liem et al., 1997). Genetic studies of zebrafish mutants with different components of Bmp signaling pathways disrupted (*swirl/bmp2b*, *snailhouse/bmp7* and *somitabun/smad5*) provide direct evidence for the involvement of Bmps in the specification of neural crest and dorsal neurons (Barth et al., 1999; Nguyen et al., 2000).

It is not clear how Bmp signaling can mediate such a diverse array of activities in the dorsal neural tube. It has been suggested that the competence of neural progenitor cells in the lateral neural plate/dorsal neural tube changes so that early progenitor cells generate neural crest and roof-plate cells in response to Bmp signaling whereas late progenitor cells generate dorsal interneurons, and in vitro explant studies provide evidence consistent with this hypothesis (Liem et al., 1997). Nevertheless, the question remains of what downstream effectors mediate these activities of Bmps, and whether different downstream mediators display a different subset of Bmp initiated activities. One group of candidate effectors for Bmp signaling is the Msx family of transcription factors.

Msx genes encode homeodomain transcription factors related to the *Drosophila msh* gene (for a review, see Cornell and Ohlen, 2000). These genes have been implicated as downstream targets of the Bmps because members of the *Msx* family are induced in regions where Bmp signaling is active, such as the hindbrain, spinal cord, telencephalon, tooth, facial primordium and limb (Barlow and Francis-West, 1997; Bei and

Maas, 1998; Furuta et al., 1997; Ganan et al., 1996; Graham et al., 1994; Liem et al., 1995; Shimeld et al., 1996; Timmer et al., 2002; Vainio et al., 1993). The mouse Msx family has three genes, *Msx1*, *Msx2* and *Msx3* (reviewed by Davidson, 1995). *Msx1* and *Msx2* have largely overlapping expression patterns in a variety of tissues including the roof-plate and adjacent cells in the dorsal neural tube and neural crest (Wang et al., 1996). *Msx3*, however, is expressed exclusively in the dorsal neural tube (Shimeld et al., 1996; Wang et al., 1996).

The functions of *Msx1* and *Msx2* have been studied genetically and biochemically. Targeted inactivation of *Msx1* in mouse reveals a role in the development of the molar tooth and palate (Chen et al., 1996; Satokata and Maas, 1994), and a role in the development of the midline structure in the forebrain (Bach et al., 2003). Inactivation of *Msx2* causes defects in calvarial bones, skin and mammary glands (Satokata et al., 2000). Genetic mutation of *Msx3* in mouse has not been reported. Gain-of-function analysis of Msx1 function in cell culture and in vivo model systems suggest that Msx1 acts as a negative regulator of differentiation (Bendall et al., 1999; Hu et al., 2001; Song et al., 1992; Woloshin et al., 1995). This is achieved through its ability to repress the transcription of differentiation genes such as *MyoD* (Bendall et al., 1999) and to regulate the expression and activity of cell cycle molecules such as cyclin D1 and CDK4 (Hu et al., 2001). Biochemical studies show that Msx1 and Msx2 are potent transcriptional repressors (Catron et al., 1996; Catron et al., 1995; Newberry et al., 1997; Zhang et al., 1996). Although optimal DNA-binding sites for Msx proteins have been identified, in certain contexts, the repressor activity does not require DNA-binding of these factors. Even though less is known about Msx3, biochemical studies suggest that it is also a transcriptional repressor (Mehra-Chaudhary et al., 2001).

In the developing mouse spinal cord, all three members of the Msx family are expressed in the dorsal neural tube from E9.0 or earlier (Hill et al., 1989; Robert et al., 1989; Shimeld et al., 1996; Wang et al., 1996). As development proceeds, *Msx1* and *Msx2* expression becomes restricted to the dorsal midline roof-plate cells while the expression of *Msx3* is in the ventricular zone of the dorsal one-third of the neural tube but excluded from the roof plate. Loss-of-function studies have not been informative on the role of the Msx factors in spinal cord development possibly owing to redundant activity and the overlapping expression of Msx1, Msx2, and Msx3 in this tissue.

To begin to address the roles of Msx factors in the developing spinal cord, we have analyzed the fate of neural progenitor cells in chick neural tube upon overexpression of the mouse Msx genes by in ovo electroporation. For comparison, we also examined the phenotypes of chick embryos with the Bmp signaling pathway activated at different stages by overexpressing the constitutively active forms of Bmp receptors. We show that Bmp signaling and Msx factors have stage-dependent activities in determining dorsal cell fates. Activation of Bmp signaling in HH10-12 embryos resulted in an increase of roof-plate cells with a concurrent increase of apoptosis and repression of neuronal differentiation. This set of phenotypes was mimicked by overexpression of Msx1, but not Msx3. By contrast, when activated Bmp receptors were introduced into later stage neural tubes (HH14-16), dorsal interneuron cell fates were induced rather than roof plate. In this case, Msx3 but not Msx1 induced the same phenotypes. Together, these results demonstrate that Msx1 and Msx3 have differential functions in spinal cord development and each may mediate a subset of Bmp activities in patterning the dorsal neural tube.

Materials and methods

Plasmid construction

Plasmids expressing mouse *Msx1* and *Msx3* were constructed by subcloning the full-length coding sequences into the chick expression vector pMiWIII (Muramatsu et al., 1997). Msx1a contains substitutions in the N-terminal arm of the homeodomain replacing K182, R184 and F187 with Alanines (Zhang et al., 1996). Similarly, Msx3a contains Alanine substitutions of K89, R91 and F94 in its homeodomain. The expression plasmids for Msx1a and Msx3a were constructed by site-directed mutagenesis (Stratagene). Constitutively active forms of the chick Bmp receptors 1a (in pCAGGS) and 1b (in pMiWIII) were kindly provided by J. Timmer (Timmer et al., 2002).

Chick in ovo electroporation

Fertilized White Leghorn eggs were incubated at 39°C and embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1992). Stage HH10-12 chick embryos used for electroporation were analyzed after 24 hours (HH15-18) or 48 hours (HH20-22). Stage HH14-16 embryos used for electroporation were analyzed after 24 hours (HH20-22). Plasmid DNA was injected into the lumen of the chick neural tubes at a concentration of 2-3.5 mg/ml (except for ca-Bmpr1b, which was injected at a concentration of 0.3-1.2 mg/ml). A CMV-EGFP expression plasmid (Clontech) was co-injected at a concentration of 2-3 mg/ml for visualization of the electroporated cells. Electric square pulses were applied five times at 25 volts for 50 mseconds with an electro-square porator T830 (BTX, Genetronics). Only embryos with high levels of GFP fluorescence in the neural tube were processed for each experiment. Each embryo was fixed in 4% paraformaldehyde/PBS at 4°C for 1 hour, washed extensively in PBS and cryoprotected in 30% sucrose/PBS at 4°C overnight. Embryos were embedded in OCT and cryosectioned at 20-30μm. Sections were taken in between forelimb and hindlimb levels and only sections with high levels of GFP were used for analysis. All results were repeated in at least four embryos.

In situ hybridization and immunofluorescence

In situ hybridization was performed as previously described (Birren et al., 1993). Chick *Bmp4*, *Wnt1*, *Cash1*, *Ngn1* and *Ngn2* antisense probes were labeled with digoxigenin and hybridized to frozen sections of the embryos in a concentration of 1-2 μg/ml. *Bmp4* plasmid was obtained from University of Delaware chick EST database (clone pgf2n.pk004.m4). Immunofluorescence was performed as described (Gowan et al., 2001) with the following antibodies: rabbit anti-Math1 (Helms and Johnson, 1998), rabbit anti-Lhx2/9 (L1) (Liem et al., 1995), rabbit anti-Pax2 (Zymed Laboratories), rabbit anti-Dlx (Panganiban et al., 1995), mouse monoclonal antibody Tuj1 (Lee et al., 1990) and monoclonal antibodies obtained from Developmental Studies Hybridoma Bank (DSHB), including anti-Msx (4G2) (Liem et al., 1995), anti-Pax7 (Ericson et al., 1996), anti-Lhx1/5 (4F2) (Tsuchida et al., 1994), anti-Isl1/2 (39.4D5) (Ericson et al., 1992), anti-Lmx1 (50.5A5) (Riddle et al., 1995) and anti-Mnr2 (81.5C10) (Tanabe et al., 1998). Immunofluorescence images were taken on a BioRad MRC 1024 confocal microscope.

BrdU labeling and TUNEL assay

BrdU (100-150 μl of 5 mg/ml in PBS) was injected to the vicinity of the heart of the embryo 1 hour before harvesting. Incorporation of BrdU was detected with mouse anti-BrdU (Beckton-Dickinson).

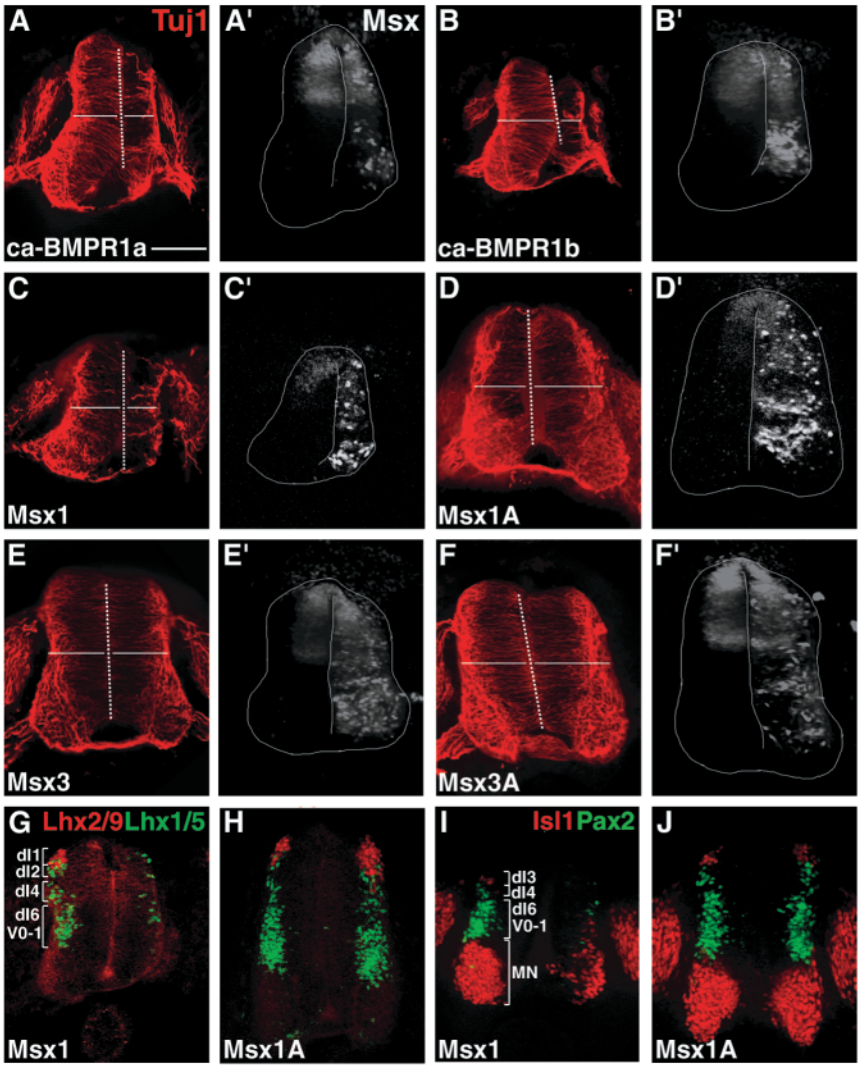
TUNEL assays were performed using the In Situ Cell Death Detection Kit (Roche) according to the manufacturer's instructions.

Results

Msx1, but not Msx3, when overexpressed at early stages of neural tube development, represses neuronal differentiation

Expression of the Msx family of transcription factors is induced by Bmp signaling in multiple systems, including the neural tube (Furuta et al., 1997; Liem et al., 1995; Timmer et al., 2002) (Fig. 1A′,B′). To examine the roles of Msx1 and Msx3 in spinal neural tube development, and to determine if these roles correlate with the function of Bmp signaling, we used electroporation to overexpress *Msx1*, *Msx3* and constitutively active forms of Bmp receptors (*ca-Bmpr1a* and *ca-Bmpr1b*) in chick neural tubes. Electroporation was performed at stage HH10-12 or HH14-16 and embryos were analyzed 24 or 48 hours later as specified in each experiment. Although endogenous Msx expression is restricted to the dorsal half of the neural tube, expression of the constructs occurs along the length of the dorsoventral axis when electroporated into one side of the neural tube (Fig. 1A′-F′, compare left and right sides). In all experiments, the electroporated side is shown on the right and should be compared with the control side on the left.

The most obvious phenotypic change that occurred with overexpression of *ca-Bmpr1a/b*, or *Msx1* into HH10-12 embryos was the decrease in the size of the neural tube on the electroporated side relative to the control side when we analyzed the embryos 48 hours post electroporation (Fig. 1A-C). This phenotype is illustrated here using a marker for neuronal differentiation Tuj1 (Fig. 1). The decrease in size of the neural tube reflects a subtle decrease in the size of the ventricular zone (see Fig. 3F-J), and a more dramatic reduction in the size of the mantle layer where differentiating Tuj1-expressing neurons reside. The decrease in size of the neural tube was specific to Msx1, as the related Msx3 did not have this phenotype (Fig. 1E). Msx1a, a previously described mutant form of Msx1, contains three amino acid substitutions in the N-terminal arm of the homeodomain and cannot bind DNA (Bendall et al., 1999; Hu et al., 2001; Zhang et al., 1996; Zhang et al., 1997). Ectopic expression of Msx1a did not cause a small neural tube phenotype or a decrease of neuronal differentiation (Fig. 1D), suggesting that the N-terminal arm of the Msx1 homeodomain is critical for its activity. A similar



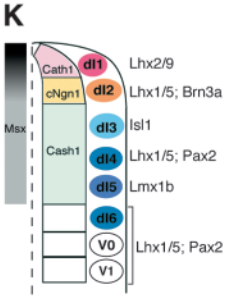


Fig. 1. Early expression of Bmp receptors and Msx1 but not Msx3 repress neuronal differentiation. (A-F) Immunofluorescence labeling with the antibody Tuj1 in embryos electroporated at HH10-12 and assayed 48 hours post electroporation at HH20-22. Electroporated constructs are indicated. Broken lines indicate the midline of the neural tube. Horizontal lines indicate the width of the control (left) versus the electroporated (right) side of the neural tube. (A′-F′) Immunofluorescence labeling with the Msx antibody showing the endogenous Msx expression on the control side and the ectopic expression of Msx on the electroporated side by Bmp signaling induction (A′ and B′) or overexpression of *Msx* constructs (C′-F′). (G-J) Immunofluorescence labeling of neuronal differentiation markers Lhx2/9 (red) and Lhx1/5 (green) (G,H), and Isl1 (red) and Pax2 (green) (I,J) in neural tubes electroporated with *Msx1* or *Msx1a*. (K) Schematic showing the dorsoventral boundaries of the ventricular zone markers Msx, Cath1, chick Ngn1, Cash1, and the differentiation markers Lhx2/9, Lhx1/5, Brn3a, Isl1, Pax2 and Lmx1b. dI1-6, dorsal interneuron populations 1-6; MN, motor neuron; V0-1, ventral interneuron populations 0 and 1. In all panels left is the control side and right is the electroporated side. Scale bar: 150 μm.

mutant for Msx3, Msx3a, did not change the differentiation status or size of the neural tube (Fig. 1F). Thus, Bmp signaling and Msx1 overexpression result in a smaller neural tube with a specific reduction in neuronal differentiation.

To determine if the repression of neuronal differentiation was general or restricted to defined neuronal populations, expression of the LIM-homeodomain proteins Lhx2/9, Lhx1/5 and Isl1, as well as Paired-homeodomain protein Pax2, was examined. These transcription factors mark different populations of dorsal and ventral interneurons as well as motoneurons in the developing spinal cord (Fig. 1K). Currently, six different dorsal interneuron populations (dI1-dI6) and four ventral interneuron populations (V0-V3) have been identified based on the expression of different markers (for reviews, see Briscoe and Ericson, 2001; Helms and Johnson, 2003). As illustrated in Fig. 1K, Lhx2/9 marks the dI1 population; Lhx1/5 marks the dI2, 4 and 6, as well as V0 and V1 populations; Isl1 marks the dI3 and the motoneuron populations; and Pax2 marks the dI4, dI6, V0 and V1 neuronal populations. Expression of all these differentiation markers was repressed by Msx1 (Fig. 1G,I), resulting in a significant reduction of cells expressing these markers on the electroporated side in comparison with the control side of the neural tube (Table 1). Overexpression of the mutant Msx1a, however, caused a subtle increase in each neuronal population, suggesting it may be acting as a dominant negative (Fig. 1H,J, Table 1). Repression of all differentiation markers was also observed in embryos overexpressing *ca-Bmpr1a/b* at stage HH10-12 (Table 1). Therefore, at early stages of neural tube formation, overexpression of Msx1 and Bmp signaling inhibit differentiation of neurons throughout the dorsoventral axis of the neural tube.

Repression of neuronal differentiation gene expression by *Msx1*

Msx1 is a potent transcriptional repressor; therefore, the repression of neuronal differentiation by Msx1 could be achieved through its ability to repress the transcription of neuronal differentiation genes. We examined the expression of early differentiation genes expressed in the ventricular zone in *Msx1*-electroporated neural tube. The basic helix-loop-helix (bHLH) transcription factors Math1, Mash1, neurogenin1 and neurogenin2 (Ngn1/2) are involved in neurogenesis and specification of dorsoventral neuronal cell types in the developing spinal cord (Gowan et al., 2001). Overexpression of *Msx1* but not the mutant *Msx1a* resulted in loss or dramatic reduction in expression of each of these regulatory genes (Fig. 2). Cath1 (chick ortholog of Math1) expression was dramatically repressed by Msx1 (Fig. 2A). Significant repression was also observed for the chick orthologs of *Mash1* (*Cash*), *Ngn1* and *Ngn2* (Fig. 2C,E,G), although in these cases, expression was not completely lost but was rather decreased with a disruption in the pattern. An additional marker of progenitor cells in the dorsal neural tube was examined. Pax7, a paired homeodomain factor, is expressed in the dorsal half of the neural tube (Mansouri and Gruss, 1998), and its expression was also dramatically downregulated by Msx1 (Fig. 2I). Overexpression of the mutant *Msx1a* did not repress expression of the differentiation genes (Fig. 2B,D,F,H,J). Expression of *Msx1a* was confirmed by immunostaining with the Msx antibody (data not shown). Thus, the repression activity of Msx1 depends on an intact N-terminal arm of its homeodomain. Taken together, Msx1 inhibits neuronal differentiation at least in part through repression of regulatory genes such as the bHLH and paired homeodomain transcription factors.

Msx1, but not Msx3, when overexpressed at early stages of neural tube development, induces apoptosis

In addition to the decrease in neuronal differentiation, we also examined whether the small neural tube size could be due to an increase in apoptosis and/or a decrease in proliferation. A clear increase in the number of apoptotic cells was observed in embryos overexpressing *ca-Bmpr1* or *Msx1*, as revealed by TUNEL labeling (Fig. 3A-E). There was a two- to fourfold increase in $TUNEL^+$ cells on the electroporated side compared to the control side in *ca-Bmpr1*- and *Msx1*-expressing embryos 24 hours post electroporation (Fig. 3A,B,E), and a three- to fivefold increase 48 hours post electroporation (Fig. 3C,D,E). These data suggest that increased apoptosis is one factor contributing to the smaller neural tube phenotype observed in *ca-Bmpr1*- and *Msx1*-electroporated embryos.

We measured neural progenitor cell proliferation by examining incorporation of the thymidine analog BrdU. In contrast to the dramatic reduction of the overall size of the neural tube, the size of the ventricular zone of *ca-Bmpr1*- or *Msx1*-expressing neural tube was only slightly reduced (Fig.

Table 1. Comparison of number of cells expressing various differentiation markers in electroporated embryos

	Lhx2/9	Lhx1/5	Isl1 (total)	Isl1 (dI3)	Isl1 (MN)	Mnr2	Pax2
Msx1	10.0±1.13	38.4±2.71	92.3±8.82			ND	36.4±2.03
	1.2±0.53***	10.5±1.69***	46.6±8.48***			ND	6.9±1.20***
Msx1a	15.1±0.55	58.0±6.81		8.2±2.94	120.0±12.49	ND	35.8±11.04
	25.3±1.98***	76.3±6.48 (ns)		13.0±1.30 (ns)	128.2±6.88 (ns)	ND	53.4±6.05 (ns)
ca-Bmpr1 (early)	10.7±1.11	57.4±1.49	95.0±4.03			ND	38.5±2.99
	1.6±0.69***	35.7±4.26***	46.6±4.26***			ND	9.3±1.05***
Msx3	10.1±1.39	39.4±3.38		12.6±0.98	120.1±3.25	80.0±0.20	59.1±2.06
	25.7±3.15***	23.6±2.48**		22.5±1.36***	104.5±5.45*	72.8±3.66 (ns)	38.7±3.06***
Msx3a	18.5±1.23	79.8±4.29		12.0±0.89	117.1±3.33	87.5±4.55	53.3±2.69
	13.7±1.43*	50.8±3.24***		9.0±1.38 (ns)	99.1±2.45**	86.5±3.43 (ns)	43.1±5.62 (ns)
ca-Bmpr1 (late)	10.0±1.73	60.3±1.03		7.1±0.74	95.5±3.69	97.7±3.09	47.9±4.07
	18.2±2.64*	57.8±2.75 (ns)		15.8±1.26***	85.1±3.50 (ns)	97.1±3.29 (ns)	37.6±4.89 (ns)

Results are mean±s.e.m. (n>10; *** P<0.001; **P<0.005; *P<0.05).
ND, not determined; ns, not significant.
For each set of data, top is the number of cells on the control side, and bottom is the number of cells on the electroporated side.

3F-J). We observed a 20-30% reduction of mitotic cells on the electroporated side compared with the control side both 24 hours and 48 hours post electroporation (Fig. 3J). However, most of the reduction can be accounted for by the increase of apoptosis in the neural tube, therefore it is not likely that ca-Bmpr1 or Msx1 has a direct effect on proliferation in this assay.

Altogether, these results demonstrate that increased cell death and decreased neuronal differentiation contribute to the small neural tube phenotype caused by *ca-Bmpr1* or *Msx1* overexpression. The fact that expression of *ca-Bmpr1a/b* cause ectopic Msx expression (Fig. 1A′,B′) suggests that Msx1 is an effector in the Bmp signaling pathway for these specific phenotypes in the developing neural tube.

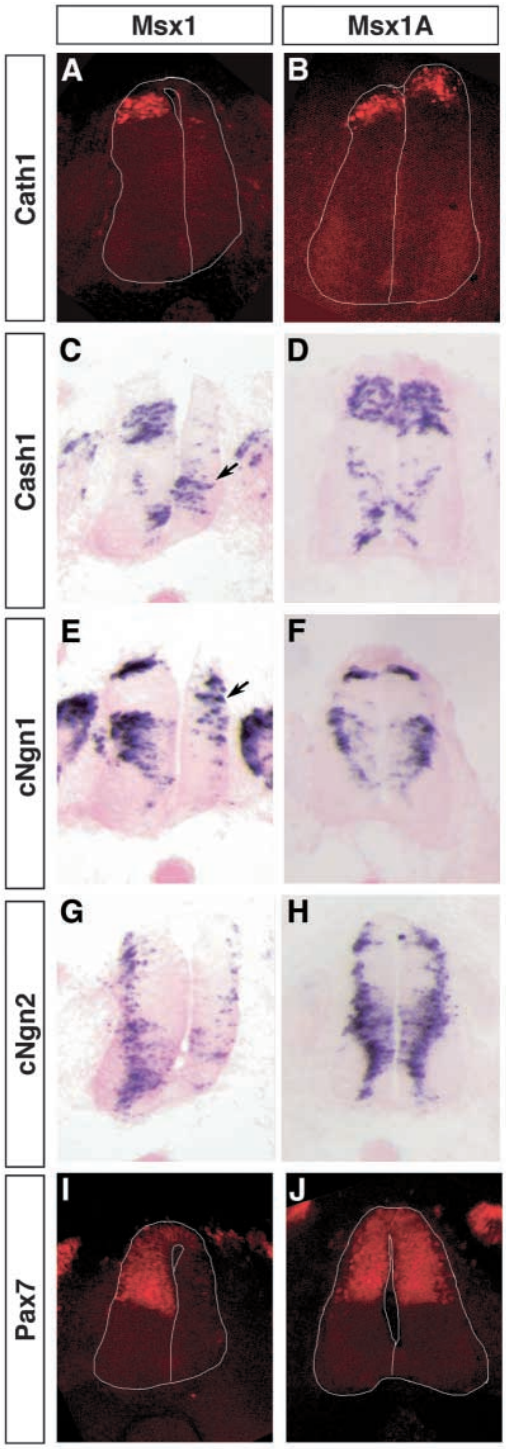


Fig. 2. Msx1 represses the expression of neural progenitor genes. Neural progenitor markers Cath1 (A,B) and Pax7 (I,J), which are detected by immunofluorescence labeling; as well as *Cash1* (C,D), chick *Ngn1* (E,F), chick *Ngn2* (G,H), which are detected by in situ hybridization, are repressed by Msx1 (A,C,E,G,I) but not Msx1a (B,D,F,H,J). Arrows in C and E indicate the disruption of dorsoventral patterns of Cash1 and chick Ngn1 expression. In all panels, left is the control side and right is the electroporated side.

Overexpression of *Msx1* but not *Msx3* promotes roof-plate cell fate in a stage-dependent manner

Bmp signaling is involved in the generation of dorsal cell types including neural crest and roof plate, and dorsal interneurons (reviewed by Helms and Johnson, 2003; Lee and Jessell, 1999). To determine whether Msx1, Msx3, or both, are involved in the generation of any of these cell types, we examined markers for each of these cell types in the electroporated neural tubes. Expression of the *ca-Bmpr1* constructs resulted in an increase in the number of cells migrating from the dorsal aspect of the neural tube expressing Dlx, a marker for neural crest (Fig. 4A,D). The ca-Bmpr1 constructs also induced the expression of the neural crest marker *Slug* on the electroporated side of the neural tube (Fig. 4E), consistent with the ability of Bmps to induce *Slug* expression in chick neural plate explant culture (Liem et al., 1995). Electroporation of both mouse *Msx1* and *Msx3* resulted in an increase in the number of Dlx-expressing cells migrating from the neural tube (Fig. 4B-D). However, in contrast to the activity of ca-Bmpr1, there was no induction of *Slug* in the neural tube of *Msx1*- or *Msx3*-expressing embryos (Fig. 4F,G). Thus, Msx1 and Msx3 can phenocopy some aspects of activating Bmp signaling, but they cannot fully mimic these activities in induction of neural crest in this assay.

To determine whether the mouse Msx genes could influence roof-plate differentiation in chick neural tube, we examined three roof-plate markers: Bmp4 (Liem et al., 1995), Wnt1 (Parr et al., 1993) and Lmx1 (Millonig et al., 2000; Yuan and Schoenwolf, 1999) in electroporated embryos. Activation of Bmp signaling by ca-Bmpr1 induced the expression of *Bmp4*, *Wnt1* and *Lmx1* in the dorsal neural tube after 24 hours (Fig. 5A,F,K,P). The Lmx1 family members (Lmx1a and Lmx1b) are expressed in the roof plate, floor plate and dI5 population of dorsal interneurons (Gross et al., 2002; Millonig et al., 2000; Muller et al., 2002; Pierani et al., 2001). At 24 hours post electroporation, the induction of Lmx1 expression most probably reflects an induction of roof-plate cell fate because differentiation of dorsal interneurons has not occurred at this stage (Fig. 5K, see control side). Overexpression of *Msx1*, but not *Msx3*, also induced all three roof-plate markers after 24 hours, resulting in a ventral expansion of the roof-plate marker expression (Fig. 5, compare B,G,L with C,H,M). However, Bmp signaling is more potent in its ability to induce roof-plate development than Msx1, with more cells ectopically expressing the roof-plate markers and the ectopic expression extended more ventrally, even though the electroporation efficiency in all experiments is comparable, as indicated by the control GFP expression (Fig. 5A-E, insets).

A dramatic induction of *Bmp4*, *Wnt1* and *Lmx1* was seen in *ca-Bmpr1*-electroporated embryos 48 hours after

electroporation (Fig. 5D,I,N,P). By contrast, little or no induction of the roof-plate markers was detected in *Msx1*-expressing embryos at this stage (Fig. 5E,J,O,P), suggesting that Msx1 is sufficient to initiate the expression of roof-plate markers but not sufficient to maintain their expression. Induction of these three markers was not observed in embryos overexpressing Msx3 48 hours post electroporation (data not shown). Taken together, these results indicate that Msx1, but not Msx3, can mediate a subset of Bmp activities to induce roof-plate development. We found that both Bmp signaling and Msx1 are capable of inducing roof-plate markers only when they are overexpressed at stage HH10-12, but not at HH14-16

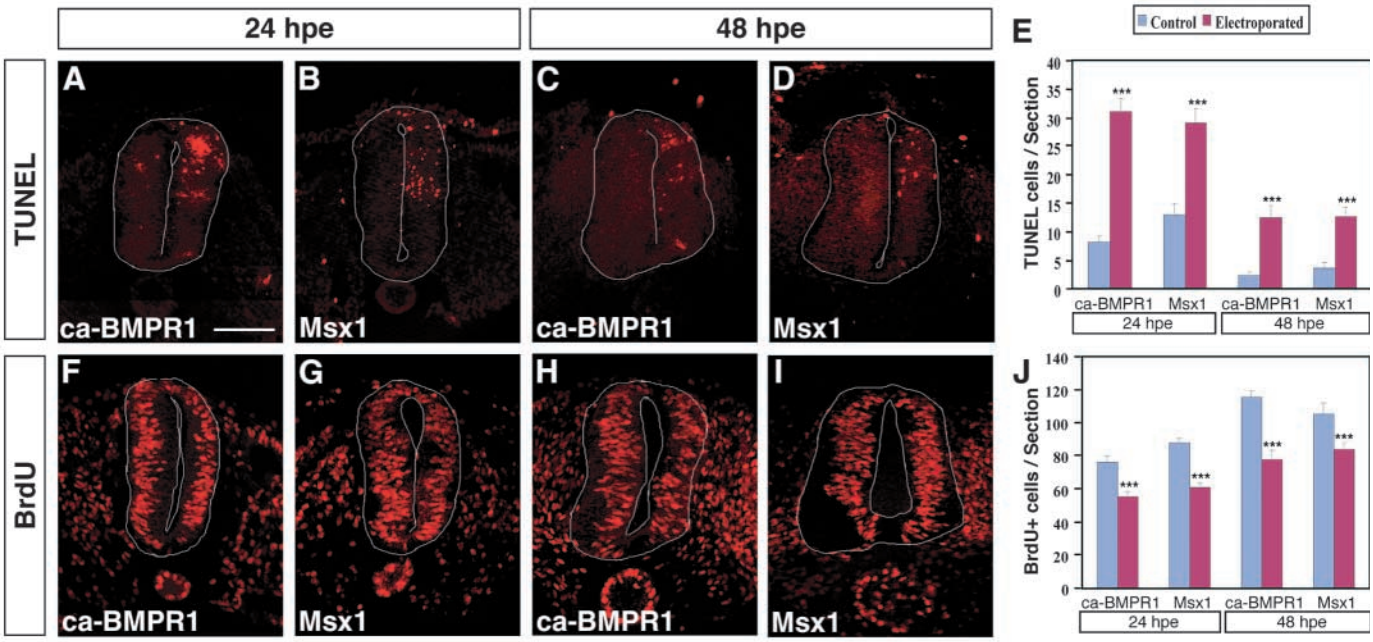


Fig. 3. Early expression of Bmp receptors and Msx1 induce apoptosis and decrease proliferation. TUNEL labeling (A-D) and BrdU incorporation (F-I) in embryos electroporated at HH10-12 with *ca-Bmpr1a/b* or *Msx1* as indicated and viewed at 24 (A,B,F,G) or 48 (C,D,H,I) hpe. (E,J) Graphs showing number of TUNEL$^+$ (E) or BrdU$^+$ (J) cells/section (mean±s.e.m., n=16 sections from at least four embryos; ***P<0.001). hpe, hour post-electroporation. In all panels, left is the control side and right is the electroporated side. Scale bar: 150 μm.

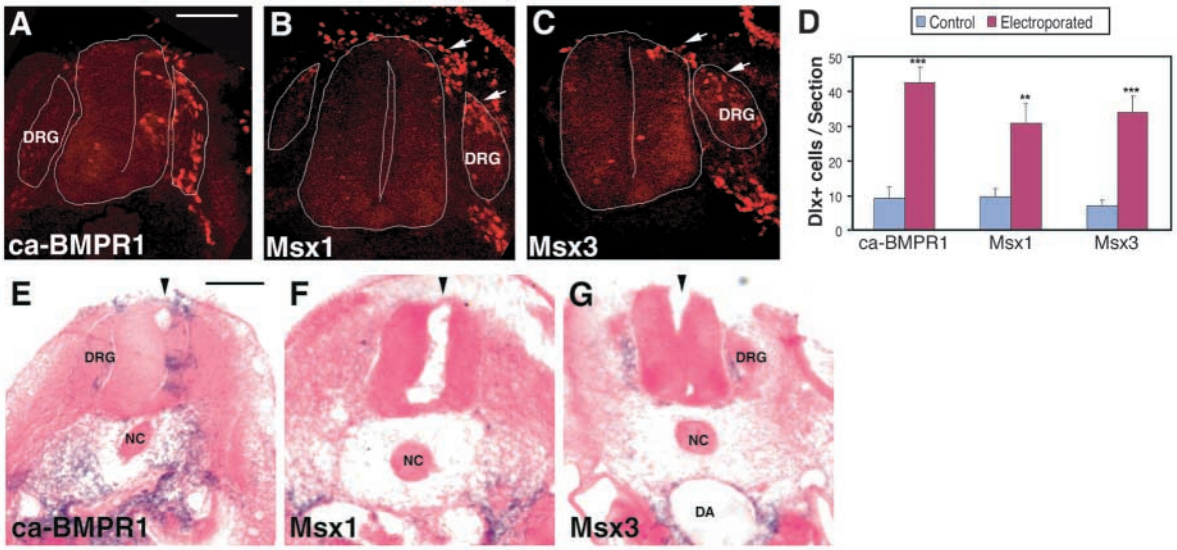


Fig. 4. Neural crest marker Dlx is induced by Bmp signaling, Msx1 and Msx3. Immunofluorescence labeling showing an increase in number of migrating Dlx$^+$ cells induced by overexpression of *ca-Bmpr1* (A), *Msx1* (B) and *Msx3* (C). Arrows indicate migrating Dlx$^+$ cells. ca-Bmpr1 also induces ectopic Dlx expression within the neural tube (A). (D) Quantification of Dlx$^+$ cells/section on the control side and the electroporated side of the neural tubes shown in A-C. Data shown as mean±s.e.m., n=16 sections from at least four embryos; ***P<0.001; **P<0.005. (E-G) The expression of another neural crest marker, *Slug*, detected by in situ hybridization, is induced within the neural tube by ca-Bmpr1 (E), but not Msx1 (F) or Msx3 (G). DA, dorsal aorta; DRG, dorsal root ganglia; NC, notochord. In all panels, left is the control side and right is the electroporated side. Scale bars: 150 μm.

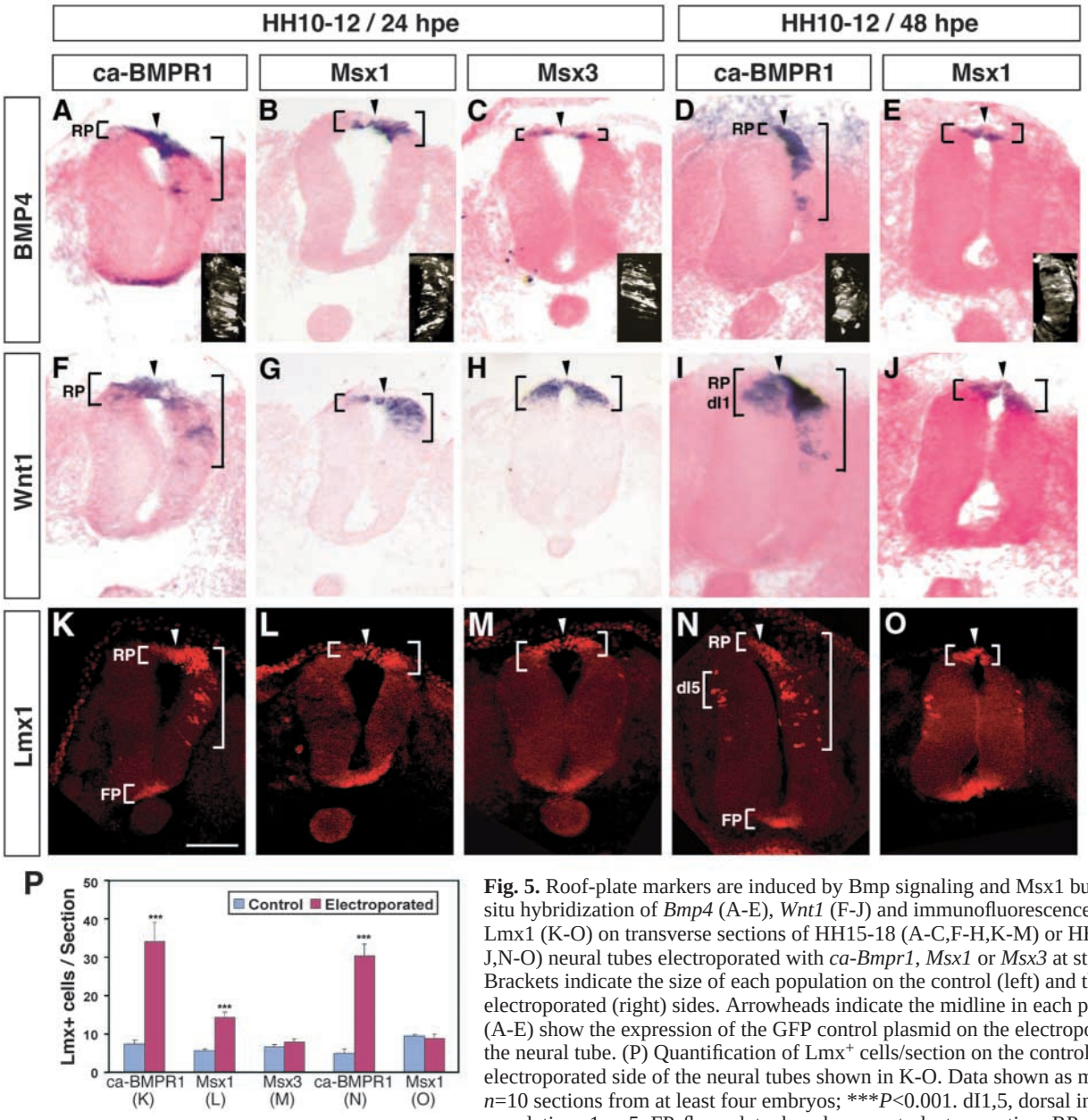


Fig. 5. Roof-plate markers are induced by Bmp signaling and Msx1 but not Msx3. In situ hybridization of *Bmp4* (A-E), *Wnt1* (F-J) and immunofluorescence labeling of Lmx1 (K-O) on transverse sections of HH15-18 (A-C,F-H,K-M) or HH20-22 (D-E,I-J,N-O) neural tubes electroporated with *ca-Bmpr1*, *Msx1* or *Msx3* at stages indicated. Brackets indicate the size of each population on the control (left) and the electroporated (right) sides. Arrowheads indicate the midline in each panel. Insets in (A-E) show the expression of the GFP control plasmid on the electroporated side of the neural tube. (P) Quantification of Lmx$^+$ cells/section on the control side and the electroporated side of the neural tubes shown in K-O. Data shown as mean±s.e.m., n=10 sections from at least four embryos; ***P<0.001. dI1,5, dorsal interneuron populations 1 or 5; FP, floor plate; hpe, hour post-electroporation; RP, roof plate. In all panels left is the control side and right is the electroporated side. Scale bar: 150 μm.

(data not shown), suggesting a switch in the competence of progenitor cells in the neural tube between these stages.

Msx3 and late activation of Bmp signaling induce dorsal interneuron cell fates

Activation of Bmp signaling in late chick embryos (HH14-16) induces neural progenitor cells to adopt a dorsal neuronal cell fate marked by Math1/Cath1 (Lee et al., 1998; Timmer et al., 2002). Therefore, we examined Cath1 expression when Msx1 and Msx3 were overexpressed at this later stage. No significant change in the number of Cath1$^+$ cells between the control and electroporated sides was detected when Msx1 was electroporated into HH14-16 embryos and analyzed 24 hours later at HH20-22 (data not shown). By contrast, a twofold increase of Cath1$^+$ cells was detected in embryos overexpressing Msx3 at HH14-16 (Fig. 6D,E,G). In fact, a small increase of Cath1$^+$ cells was also detected when Msx3 was electroporated into HH10-12 embryos and analyzed 48 hours later (Fig. 6A,B,G). Using double label immunofluorescence we found many Cath1 and Msx double positive cells (Fig. 6B,E, arrows), suggesting a cell-autonomous mechanism. These results indicate that even though Msx3 exhibits similar DNA-binding and transcriptional repression activity in vitro as Msx1 (Mehra-Chaudhary et al., 2001), the two proteins have very different in vivo activities in terms of regulating *Cath1* expression. Induction of *Cath1* expression is dependent on the N-terminal arm of Msx3 homeodomain because Msx3a did not significantly increase the number of Cath1$^+$ cells (Fig. 6C,F,G).

Consistent with the increase of Cath1-expressing dorsal

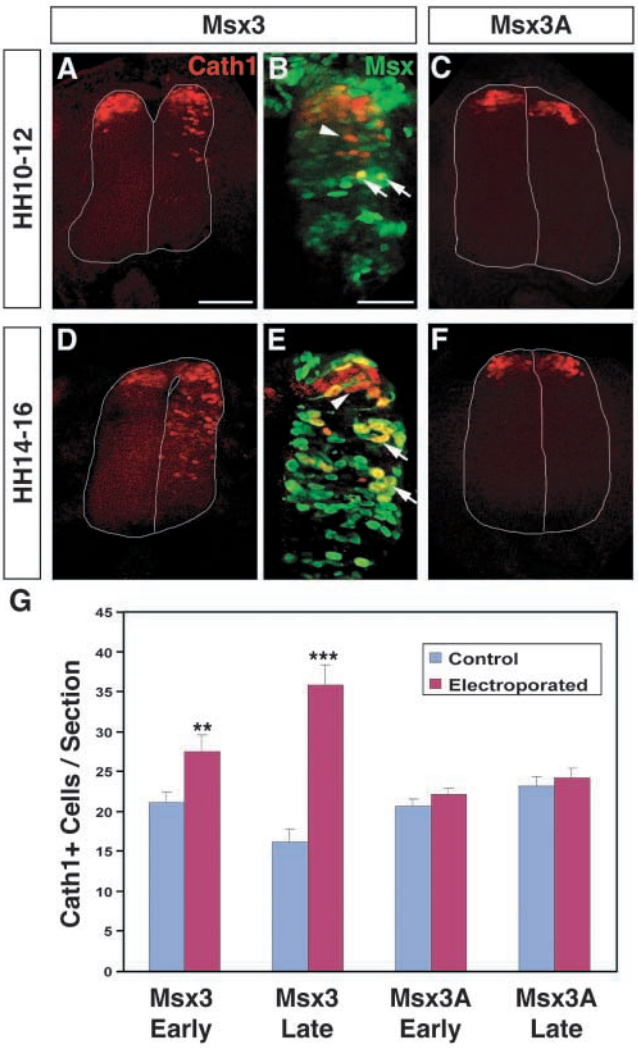


Fig. 6. Msx3 induces neural progenitor cells to adopt a dorsal cell fate. (A-F) Immunofluorescence labeling of Cath1 in HH20-22 neural tubes. Electroporation of *Msx3* in HH10-12 (A,B) or HH14-16 (D,E) embryos induces ventral ectopic expression of Cath1 at HH20-22. (B,E) Higher magnification images of the electroporated (right) side of the neural tubes in A and D showing Cath1 (red) and Msx (green) double positive cells (yellow, arrows) and Cath1$^+$Msx$^-$ cells (red, arrowheads). Overexpression of *Msx3a* does not induce ectopic Cath1 expression (C,F). (G) Bar graph indicating number of Cath1$^+$ cells/section in *Msx3*- versus *Msx3a*-electroporated embryos (late indicates electroporation at HH14-16; early indicates electroporation at HH10-12) (mean±s.e.m., n=16 sections from at least four embryos; ***P<0.001, **P<0.005). In all panels, left is the control side and right is the electroporated side. Scale bars: 150 μm in A,C,D,F; 75 μm in B,E.

progenitor cells in *Msx3*-electroporated neural tube, the interneurons derived from these progenitors, dI1 neurons marked by Lhx2/9, were also expanded ventrally (Fig. 7A,B). The expansion of Lhx2/9 interneurons was at the expense of cells that have more ventral cell fates, illustrated by the reduction in the marker Lhx1/5 that marks dI2, dI4, dI6 and V0-V1 populations (Fig. 7A,B, Table 1). The ventral expansion of Lhx2/9 in these embryos is similar to that seen with activation of Bmp signaling at late stages (Fig. 7D) (Timmer et al., 2002). Ectopic expression of another differentiation marker Isl1 was also observed in both *Msx3*- and *ca-Bmpr1*-electroporated embryos (Fig. 7E,F,H, Table 1). Because Isl1 marks both the dI3 interneuron and the motoneuron populations, ectopic expression of *Isl1* could be the result of a ventral expansion of dI3 neurons or a dorsal expansion of motoneurons. To distinguish between these two possibilities, we examined the expression of motoneuron marker Mnr2 (Tanabe et al., 1998). Staining of Mnr2 showed that although the shape of the motoneuron pool seemed to be elongated in *Msx3*-overexpressing neural tubes (Fig. 7I,J), no ectopic Mnr2$^+$ cells were detected outside of the MN domain and there was not a significant increase of the number of Mnr2$^+$ cells on the electroporated side (Table 1). This suggests that the ectopic expression of *Isl1* reflects the ventral expansion of the dI3 interneurons. This expansion was accompanied by a reduction of the more ventral Pax2$^+$ neuronal populations (Fig. 7E,F,H,I, Table 1). Electroporation of the mutant *Msx3a* did not cause the ventral expansion of dI1 or dI3 interneurons (Fig. 7C,G,K, Table 1).

Taken together, these results indicate that Msx3 and late activation of Bmp signaling promote the formation of dorsal interneurons at the expense of ventral neurons. The induction of dorsal interneuron cell fate is an activity specific to Msx3 because we did not see expansion of dorsal neuron populations in *Msx1*-expressing embryos electroporated at early or late stages (Fig. 1H,J, data not shown).

Discussion

Msx family members mediate stage-dependent activities of Bmps in patterning the dorsal neural tube

The instructive roles that Bmp signaling plays in patterning the dorsal neural tube are well established (for reviews, see Helms and Johnson, 2003; Lee and Jessell, 1999). Loss-of-function studies in mouse and zebrafish in combination with gain-of-function studies in mouse and chick have provided evidence for the involvement of Bmps in specifying dorsal cell fates (Barth et al., 1999; Hebert et al., 2002; Lee et al., 2000; Liem et al., 1997; Liem et al., 1995; Nguyen et al., 2000; Panchision et al., 2001; Timmer et al., 2002). Furthermore, a temporal switch in the competence of neural progenitor cells to respond to Bmp signaling has been demonstrated (Liem et al., 1997). For example, stage HH10 chick neural plate explant generated neural crest cells when cultured with Bmp4 for 24 hours. By contrast, explants from HH15 embryos failed to generate neural crest cells, but rather generated dI1 and dI3 dorsal interneurons in response to 24 hours of Bmp treatment. Such a change in the competence of the neural progenitor cells ensures that the proper cell types in proper numbers are produced in the developing spinal cord.

The chick in ovo electroporation system provides a unique advantage to study stage-dependent activities of signaling pathways and transcription factors. Consistent with the explant studies, our results provide in vivo evidence for a temporal switch in the competence of neural progenitor cells in response to Bmp signaling. Overexpression of *ca-Bmpr-1a* and *ca-Bmpr-1b* in chick neural tube before or during neural tube closure (HH10-12) increased the number of cells expressing neural crest and roof-plate markers, while repressing neuronal

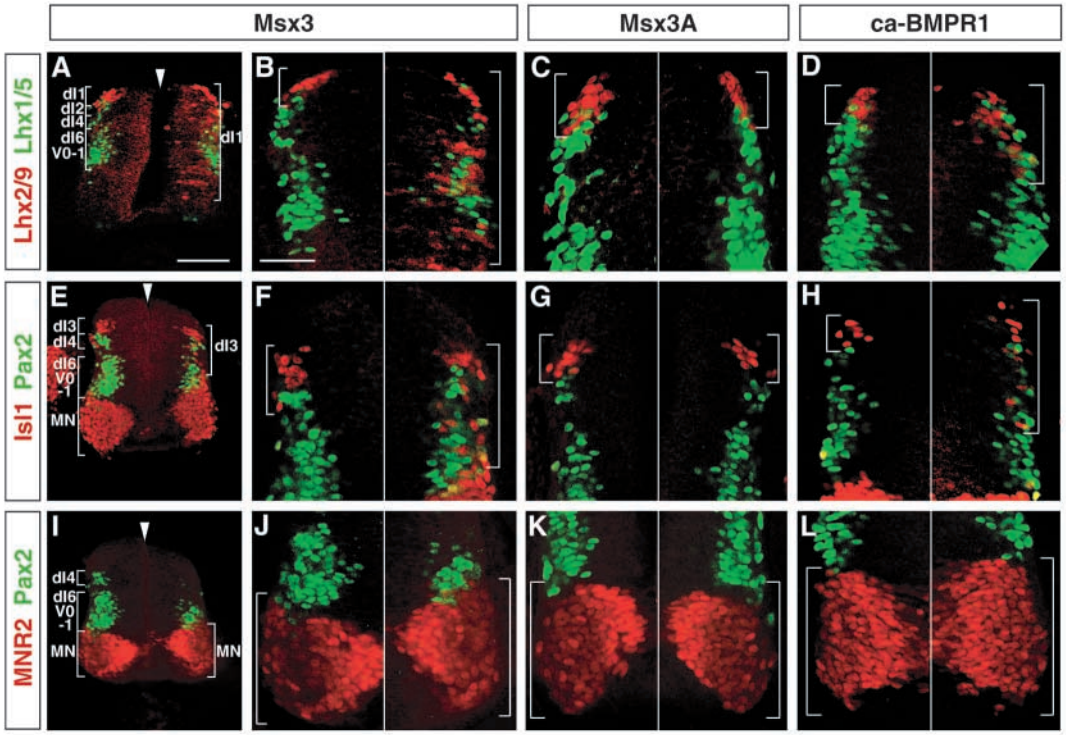


Fig. 7. Msx3 and late activation of Bmp signaling induce dorsal interneuron cell fates at the expense of ventral cell fates. (A-D) Lhx2/9 (red) and Lhx1/5 (green) immunofluorescence labeling shows a ventral expansion of Lhx2/9$^+$ dI1 population in embryos overexpressing *Msx3* (A,B), *ca-Bmpr1a/b* (D) but not *Msx3a* (C). (E-H) Isl1 (red) and Pax2 (green) labeling shows a ventral expansion of Isl1$^+$ dI3 population in embryos electroporated with *Msx3* (E,F), *ca-Bmpr1a/b* (H) but not *Msx3a* (G). (I-L) Mnr2 (red) and Pax2 (green) labeling shows no significant dorsal expansion of Mnr2$^+$ motoneurons. dI1-6, dorsal interneuron populations 1-6; MN, motoneuron; V0-1, ventral interneuron populations 0 and 1. Brackets outline the size of each neuronal population indicated. Arrowheads in A,E,I indicate the dorsal midline. In all panels, left is the control side and right is the electroporated side. Scale bars: 150 μm in A,E,I; 75 μm in B-D,F-H,J-L.

differentiation and inducing apoptosis. By contrast, activation of Bmp signaling after neural tube closure (HH14-16) promoted the dorsal progenitor cells to adopt dI1 and dI3 interneuron cell fates rather than neural crest or roof-plate cell fate.

In this study, we found that mouse Msx transcription factors displayed a similar set of activities in a stage-dependent fashion. Overexpression of *Msx1* in HH10-12, but not HH14-16 embryos, induced the expression of roof-plate markers, caused ectopic apoptosis and repressed neuronal differentiation. On the contrary, dorsal interneurons were induced by both early and late overexpression of *Msx3*. Although the functions of Bmp signaling in the development of dorsal midline have been established by both loss-of-function and gain-of-function studies (Hebert et al., 2002; Liem et al., 1995; Panchision et al., 2001), a role for *Msx1* in dorsal midline development has only recently been demonstrated in mice (Bach et al., 2003). *Msx1*$^{-/-}$ mutant embryos lack a functional dorsal midline in prosomere 1 of the developing diencephalon. Roof-plate defects were not detected in the hindbrain and spinal cord of *Msx1*$^{-/-}$ mutant or *Msx1*$^{-/-}$/*Msx2*$^{-/-}$ compound mutant embryos, possibly owing to compensatory activity from *Msx3* (Bach et al., 2003). However, in our chick electroporation assay, Msx3 displayed minimal activity in inducing the roof-plate markers. Because the competence of dorsal progenitor cells to respond to signals changes rapidly from stage to stage, it is possible that Msx3 expressed at a different stage could induce midline gene expression, and, thus, compensate for the absence of Msx1 and Msx2. How molecular mechanisms control the temporal switch of neural progenitor cells to respond to activating Bmp signaling pathway, and to activating Msx transcription factors, remains an important question in dorsal neural tube development.

The role of Msx factors as downstream effectors of Bmp signaling is supported by experiments in multiple systems. The activation of the Bmp signaling pathway is sufficient to induce the expression of *Msx* genes at different sites (reviewed by Davidson, 1995), and genetic ablation of the roof plate in mouse resulted in the loss of expression of Msx1 and Msx3 (Lee et al., 2000) (K. Lee, personal communication). The *Xenopus Msx1* gene acts downstream of Bmp signaling in epidermal induction and inhibition of neural differentiation in early *Xenopus* embryos (Suzuki et al., 1997). Our data support a model in which Msx factors mediate the stage-dependent activities of Bmp signaling in patterning the dorsal neural tube. However, in some instances, overexpression of Msx genes does not fully recapitulate the activities of Bmps. Thus, although Msx transcription factors appear to mediate multiple aspects of Bmp signaling, there are likely other parallel pathways required to account for the whole program induced by Bmp signaling.

Distinct functions of Msx genes

The biochemical properties of two Msx family members, Msx1 and Msx2, have been compared (Catron et al., 1996). These two proteins bind a common consensus DNA site and exhibit similar DNA-binding site preferences. Both factors function as

transcriptional repressors independent of DNA-binding in transfection assays. There are subtle functional differences between the two transcription factors, including a higher DNA-binding affinity of Msx2 and a greater potency of repression by Msx1. Msx3 has also been shown to be a transcriptional repressor in transfected cells (Mehra-Chaudhary et al., 2001). It has not been previously shown whether different Msx factors have similar or distinct biological functions in any given developmental system in vivo.

Our analysis of the functions of *Msx1* and *Msx3* in neural tube development allowed us to uncover the distinct functions of these two genes. We found three activities of Msx1 that are not shared by Msx3. First, early overexpression of *Msx1* but not *Msx3* promotes roof-plate cell fate. This is consistent with the observation that in E10.5 or older mouse embryos the expression of *Msx1* in the neural tube is restricted to the roof plate, whereas that of *Msx3* is in the dorsal ventricular zone but devoid from the roof plate (Wang et al., 1996). Second, Msx1 represses neuronal differentiation along the entire dorsoventral axis of the neural tube, whereas Msx3 induces the differentiation of dorsal interneurons at the expense of ventral neurons. Inhibition of terminal differentiation by Msx1 has been observed in multiple developmental systems and two mechanisms have been proposed to account for this activity. Forced expression of *Msx1* efficiently blocks terminal differentiation of multiple mesenchymal and epithelial progenitor cell types in culture and differentiation of the mammary epithelium in transgenic mice, and this activity is associated with the upregulation of cyclin D1 expression and an increase of Cdk4 activity (Hu et al., 2001). Msx1 has also been shown to inhibit muscle differentiation by repressing the expression of myogenic bHLH gene *MyoD* (Bendall et al., 1999). We observed a similar repression of early differentiation genes by Msx1 in nervous system development including expression of chick neural bHLH genes *Cath1*, *Cash1*, *Ngn1* and *Ngn2*, and the paired-homeobox gene *Pax7*. Taken together, the data in the different systems suggest that Msx1 blocks terminal differentiation by repressing the expression of differentiation genes and by modulating cell cycle exit.

The third activity specific to Msx1 and not shared by Msx3 is that Msx1 induces apoptosis in developing neural tube. For proliferating cells, death represents an alternative pathway to differentiation. Therefore, the increased apoptosis could result from the inability of the progenitor cells overexpressing *Msx1* to properly leave the cell cycle and undergo differentiation. It is interesting that increased apoptosis, decreased proliferation and repression of neuronal differentiation have all been described as properties of the dorsal midline of the developing telencephalon (Furuta et al., 1997; Hebert et al., 2002; Monuki et al., 2001). Therefore, it is possible that these activities we observed when *ca-Bmpr1* or *Msx1* were electroporated into early neural tubes may be connected to their ability to promote roof-plate development.

In contrast to the activities of Msx1 detailed above, the primary activity of Msx3 in these assays seems to be the specification of dorsal interneurons. In mouse, *Msx3* is expressed specifically in the dorsal region of the neural tube in early embryos prior to the onset of dorsal neuronal differentiation (Wang et al., 1996). We show that overexpression of *Msx3* in the developing neural tube induces Cath1-expressing progenitor cells and the ventral expansion of dI1 and dI3 neurons. This activity is distinct from that of Msx1 as induction of dorsal neuron differentiation was never observed in *Msx1*-electroporated embryos.

It is important to note that in these studies we are overexpressing mouse *Msx1* and *Msx3* in chick neural tubes. Although two chick Msx genes have been cloned (*Msx1/GHox7* and *Msx2/GHox8*), which show high sequence homology and similar tissue distribution to the murine *Msx1* and *Msx2* genes (Coelho et al., 1991; Yokouchi et al., 1991), the chick *Msx3* gene has not been identified. Blast searches of two chick EST databases (http://www.chickest.udel.edu/ and http://chick.umist.ac.uk/) using the mouse *Msx3* sequence failed to reveal a Msx3 ortholog. As the complete sequence of the chick genome is not available, it is not yet clear if a chick *Msx3* gene exists. In the chick electroporation assay, both chick *Msx1* and *Msx2* behaved like the mouse *Msx1*, causing a reduction in the size of the neural tube and repressing neuronal differentiation (Y.L. and J.E.J., unpublished). It is possible that the differences seen in the activities of Msx1 and Msx3 in the chick neural tube are due to evolutionary differences in the factors that they interact with in chick, and thus, caution should be taken in assigning specific functions for the mouse genes. Assignment of specific functions will require additional loss-of-function analysis. Nevertheless, previous work in both mouse and chick suggest that Msx3 and Msx1/2 may have opposing functions. Whereas Msx2 acts to mediate an apoptotic response induced by Bmp signaling in rhombomeres 3 and 5 of the hindbrain (Graham et al., 1994), the expression of *Msx3* in these rhombomeres is selectively repressed (Shimeld et al., 1996). Furthermore, targeted disruption of Smad4, a downstream mediator of TGFβ signaling pathway, results in a reduction of *Msx2* expression and an activation of *Msx3* expression in fibroblasts and differentiating ES cells (Sirard et al., 2000). The difference in activities revealed in chick neural tube in our study demonstrates that Msx1 and Msx3 have distinct activities and likely interact with different co-factors.

Transcriptional regulation of neural differentiation genes by Msx

Two lines of evidence suggest that the neural bHLH genes that are crucial for neuronal differentiation might be direct transcriptional targets of Msx1. First, in our pursuit of factors that regulate the expression of the neural bHLH genes by yeast one-hybrid screening, Msx1 was identified to be potential regulator for both *Math1* and *Mash1* and several consensus sites for Msx1 binding are present in the enhancer regions of *Math1/Cath1* and *Mash1/Cash1* (S. Verma-Kurvari, P. J. Ebert, and J.E.J., unpublished). Furthermore, both Msx1 and Msx3 can bind to these consensus sites in vitro (Y.L. and J.E.J., unpublished). However, because in vivo Msx1 represses the bHLH factor expression and Msx3 induces Cath1 expression, additional in vivo co-factors or chromatin properties that modulate these activities must be invoked.

The analysis of the mutants Msx1a and Msx3a underscores the importance of the homeodomains of Msx proteins for the activities seen in the overexpression assays. Both mutants have three amino acids in the N-terminal arm of the homeodomain replaced by arginine. Msx1a has previously been shown to be unable to bind DNA (Zhang et al., 1996; Zhang et al., 1997). Unfortunately, the N-terminal arm is not only responsible for

DNA-binding, it is also a major contact point for protein-protein interactions between Msx1 and basal transcription factors such as TBP or other homeodomain proteins such as Dlx (Zhang et al., 1996; Zhang et al., 1997). Therefore, these data alone are not sufficient to distinguish whether the regulation of neural differentiation genes by Msx1 depends upon its ability to bind DNA. However, Msx1a was not inert when electroporated into the neural tube but rather appeared to function as a dominant negative, suggesting that the mutant protein could form a non-functional complex with co-factor(s) important for Msx1 activity. The fact that Msx1a and Msx3a did not show similar dominant-negative activity (Table 1) implies that these two related factors use different mechanisms for activity, and suggest that they do not share protein interaction surfaces. Consistent with this notion, sequences outside the homeodomains in Msx1 and Msx3 are not related.

Concluding statements

The data presented here clearly demonstrate distinct activities for the related transcription factors Msx1 and Msx3. The activities shown for Msx1 and Msx3 are not necessarily indicative of their in vivo functions under normal expression conditions, as this would require additional loss-of-function experiments. However, in the dorsal neural tube, Msx genes are likely to be effectors of the Bmp pathway because they are induced upon activation of the pathway, and we show that the two Msx factors tested can mimic multiple distinct phenotypes seen with Bmp signaling activation. It is important to note that the activities of the Msx factors alone do not account for all the phenotypes seen with activation of the Bmp pathway. Important directions for the future will be to determine other effectors of this signaling pathway, how they interact with the Msx factors and the mechanisms controlling the temporal competence of the developing neural tube to respond to these signals.

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