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The TGFβ intracellular effector Smad3 regulates neuronal differentiation and cell fate specification in the developing spinal cord

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Here we show that Smad3, a transforming growth factor β (TGFβ)/activin signaling effector, is expressed in discrete progenitor domains along the dorsoventral axis of the developing chick spinal cord. Restriction of Smad3 expression to the dP6-p2 and p3 domains together with exclusion from the motoneuron progenitor domain, are the result of the activity of key transcription factors responsible for patterning the neural tube. Smad3-mediated TGFβ activity promotes cell-cycle exit and neurogenesis by inhibiting the expression of Id proteins, and activating the expression of neurogenic factors and the cyclin-dependent-kinase-inhibitor $p27^{kip1}$. Furthermore, Smad3 activity induces differentiation of selected neuronal subtypes at the expense of other subtypes. Within the intermediate and ventral domains, Smad3 promotes differentiation of ventral interneurons at the expense of motoneuron generation. Consequently, the absence of Smad3 expression from the motoneuron progenitor domain during pattern formation of the neural tube is a prerequisite for the correct generation of spinal motoneurons.

KEY WORDS: Spinal cord, Pattern formation, Neurogenesis, Basic helix-loop-helix (bHLH) proteins, Motoneurons, Interneurons, Transforming growth factor β (TGFβ), Smad3, Sonic hedgehog, Chick

INTRODUCTION

The embryonic rudiment of the central nervous system (CNS), the neural tube, is composed of a pseudo-stratified neuroepithelium named the ventricular zone (VZ). Shortly after neural tube closure, scattered neuroepithelial cells exit the cell cycle, migrate laterally from the VZ and begin to exhibit specific neuronal identities at the lateral edge of the neural tube, now forming the mantle zone (Ramón and Cajal, 1911). There is complex interplay between intrinsic and extrinsic factors that mediate the acquisition of progenitor cell identity, the precise timing of cell-cycle exit and the initiation of neuronal differentiation, and the spinal cord provides a model to study these factors.

Selected members of the basic helix-loop-helix (bHLH) family of transcription factors appear to play multiple key roles in driving progenitor cells from a proliferative to a terminally differentiated state at the proper times and locations. To supply the spinal cord with the appropriate number of cells, neural progenitors must proliferate sufficiently before differentiating. Id proteins (inhibitory bHLH that lack the basic DNA-binding region) promote cell-cycle progression by interacting with components of the cell-cycle machinery, and inhibit neurogenic bHLH activity by sequestering E proteins from bHLH factors (Ruzinova and Benezra, 2003). Subsequently, the transition from proliferation to neurogenesis involves a coordinated increase in proneural bHLH activity and a decrease in Id activity. Proneural bHLH factors upregulate the expression of cell-cycle inhibitors, such as $p27^{kip1}$, and promote cell-cycle withdrawal (Bertrand et al., 2002). Because these classes of transcriptional regulators functionally antagonize one another, reciprocal changes in their activities initiate an irreversible cascade, which culminates in neuronal differentiation. Finally, neuro-specific differentiation bHLH factors mediate terminal differentiation.

Furthermore, positional information in the developing spinal cord is acquired by a gradient of extracellular signals that set up a combinatorial code of homeodomain (HD) and bHLH transcription factors. Members of the transforming growth factor β (TGFβ) family, including several bone morphogenetic proteins (BMPs), secreted from the ectoderm overlaying the neural tube and from the roof plate, together with the morphogen sonic hedgehog (Shh) secreted from the notochord and from the floor plate, are the major extracellular signaling pathways that lead to the generation of distinct classes of neural progenitors at specific dorsoventral (DV) locations within the spinal cord. These signals regulate the spatial expression of transcription factors, which in turn act as cell fate determinants (Briscoe and Ericson, 2001; Helms and Johnson, 2003; Jessell, 2000; Martí et al., 2005). The subdivision of progenitors within the VZ is the initial requirement for the generation of distinct neuronal subtypes. Subsequently, the profile of progenitor proteins expressed by precursor cells, acting together with neurogenic factors, specify cell identity and initiate the differentiation of neurons from each progenitor domain (Bertrand et al., 2002).

To gain further insight into the role of TGFβ signaling in the developing spinal cord, we analyzed the expression of several components of the pathway during neural tube development. We found TGFβ2 to be highly expressed in the notochord and the floor plate suggesting a role in ventral neural tube development. Smad proteins are the main TGFβ/BMP-receptor substrates that transduce signals (Massagué et al., 2005). R-Smads fall into two groups, each serving one branch of the TGFβ superfamily of ligands; Smad2/3 transduce TGFβ/activin responses, and Smad1/5/8 lie on the BMP pathway. Receptor-mediated phosphorylation of R-Smads increases their affinity for a common Smad (Smad4), an essential component for the assembly of transcriptional complexes and for the generation of specific Smad responses (Massagué et al., 2005). We found

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Smad3 mRNA expression in discrete progenitor domains of the developing neural tube whereas differentiated neurons showed no expression. We demonstrate that Smad3-mediated TGFβ activity hinders progenitor features and induces the expression of neurogenic genes, such as NeuroM, and the expression of cell-cycle inhibitors, such as $p27^{kip1}$, thereby resulting in cell-cycle exit and neurogenesis. However, TGFβ/Smad3 activity does not induce an overall differentiation of neural tube progenitor cells, but instead the preferential differentiation of certain neuronal subtypes at the expense of others. In vivo overexpression and loss-of-function experiments indicate that Smad3 activity promotes differentiation of ventral interneurons (INs), at the expense of motoneuron (MN) generation. Taken together, these findings indicate that Smad3 plays a key role in the regulation of neuronal differentiation and cell fate specification in the ventral spinal cord.

MATERIAL AND METHODS

DNA constructs

Human N-terminal Flag-tagged Smad3 was cloned into a *pCIG* vector (Megason and McMahon, 2002), upstream of an internal ribosomal entry site (IRES) and three nuclear localization sequence-tagged enhanced GFP in pCAGGS. Site-directed mutagenesis performed by PCR was used to generate two Smad3 mutant versions; Smad3-3S/D (Liu et al., 1997), in which three serines in the C-terminal were mutated to aspartic acid residues, and Smad3-3S/A (Lagna et al., 1996), in which the three serines were mutated to alanine, and further cloned into pCIG. To monitor endogenous Smad activity, we used the constitutively active form of the human TGFβ type I receptor, TβR-I (T204D), in which Thr204 was replaced by aspartic acid (Wieser et al., 1995). *pcDNA-3.1*-mouse Olig2 (Novitch et al., 2001), *pCAAGS*-chick Nkx2.2, -rat Nkx6.1 and -mouse Irx3 (Briscoe et al., 2000) were used for in ovo electroporation.

Non-overlapping sequences of chicken Smad3 (bp 550-569 and bp 711-730) were targeted based on published protocol, and further cloned into pSUPER vector (Brummelkamp et al., 2002) to deliver short hairpin RNA (shRNA) against chicken Smad3, into the neural tube. Controls were performed by electroporation of the empty pSUPER vector and by the use of mismatched sequences that gave no phenotypes.

Chick in ovo electroporation

Eggs from White-Leghorn chickens were incubated at 38.5°C in an atmosphere of 70% humidity. Embryos were staged following Hamburger and Hamilton (HH) (Hamburger and Hamilton, 1951).

In vivo experiments were performed by in ovo electroporation. Chick embryos were electroporated with Clontech purified plasmid DNA at 1-5 μg/μl in H_2O with 50 ng/ml of Fast Green. Briefly, plasmid DNA was injected into the lumen of HH stage 14-16 neural tubes, electrodes were placed at both sides of the neural tube and electroporation was performed using an Intracel Dual Pulse (TSS-100) electroporator delivering five 50 ms square pulses of 40 V. Transfected embryos were allowed to develop for 10-48 hours, then dissected out, fixed and processed for immunohistochemistry or in situ hybridization.

Immunohistochemistry

Embryos were fixed for 2-3 hours at 4°C in 4% paraformaldehyde in PBS, rinsed, sunk in 30% sucrose solution, embedded in OCT and sectioned in a Leica cryostate (CM 1900). Immunostainings were performed following standard procedures.

Foxa2 (4C7), Nkx2.2 (74.5A5), MNR2 (81.5C10), Isl1 (40.2D6), Pax7, Lhx3 (67.4E12), Lhx1/5 (4F2) and BrdU (G3G4) were detected with monoclonal antibodies obtained from the Developmental Studies Hybridoma Bank (The University of Iowa). Additional antibodies used were: rabbit anti- Pax2 (Zymed), rabbit anti-Green Fluorescence Protein (GFP, Molecular Probes), rabbit anti-activated Caspase-3 (BD Transduction Laboratories), rabbit anti-Pax6 (CRP Inc.), mouse anti-$p27^{kip1}$ (BD Transduction Laboratories) and mouse anti-neural β-Tubulin III (Tuj1, BabCO). Rabbit polyclonal antisera were used to detect Olig2 (Sun et al., 2001) and Nkx6 (Sander et al., 2000).

Single- and double-label analyses were performed using Alexa488-, Alexa555- (Molecular Probes) and Cy5-conjugated (Jackson Immuno Research Inc.) secondary antibodies. Images were collected by confocal microscopy. Alternatively, embryos were sectioned in a Leica vibratome (VT 1000S), and protein expression was localized by indirect peroxidase immunochemistry and stained with DAB.

BrdU incorporation

A total of 5 μg/ml BrdU was injected into the lumen of the neural tube of chick embryos 30 minutes before harvesting. BrdU detection was performed on sections by treatment with 2N HCl for 30 minutes, 0.1 M NaBorate (pH 8.5) and incubation with anti-BrdU antibody.

In situ hybridization

Embryos were fixed overnight at 4°C in 4% paraformaldehyde in PBS, rinsed and processed for whole-mount RNA in situ hybridization following standard procedures, using probes for chick ActRII-b, TβRII, TGFβ2, Smad2, Smad3, Sim1, Nkx6.1, Irx3, Dbx1, Olig2, Chx10, En1, Evx1, and NeuroM. After hybridization, embryos were post-fixed in 4% paraformaldehyde and sectioned in a vibratome.

Luciferase-reporter assay

A transcriptional activity assay of distinct Smad3 versions was performed in vivo. Chick embryos were electroporated at HH stage 14-16 with pCIG-Smad3, pCIG-Smad3-3S/D or empty pCIG vector as control, together with a firefly-luciferase reporter construct containing the plasminogen activator inhibitor-1 (PAI-1) promoter, p3TP-Lux (Wrana et al., 1992), and a renilla-luciferase reporter construct carrying the CMV immediate early enhancer promoter (Promega) for normalization. Similarly, the pCIG-Smad3-3S/A mutant version was assayed in the presence of constitutively active pCIG-TβR-I construct. Embryos were harvested after 24 hours incubation in ovo, and GFP-positive neural tubes were dissected and homogenized with a douncer in Passive Lysis Buffer on ice. Firefly- and renilla-luciferase activities were measured by the Dual Luciferase Reporter Assay System (Promega).

Statistical analysis

Quantitative data were expressed as mean ± s.d. or mean ± s.e.m. Significant differences between groups were tested by Student's *t*-test.

RESULTS

Smad3 is expressed in discrete domains of the neural tube

TGFβ proteins signal by binding to pairs of membrane receptor serine/threonine kinases (receptors type I and II) responsible for phosphorylation of R-Smads (Massagué et al., 2005). Whereas type II receptors (TβR-II and ActR-II) were expressed throughout the DV axis of the early developing neural tube (Fig. 1A, and data not shown), TGFβ2 was highly expressed in the notochord and the surrounding ventral mesoderm (Fig. 1B). High expression of TGFβ2 was observed in the floor plate at HH18 (Fig. 10), coincident with high floor plate-signaling activity and with high Shh-expression (Martí et al., 1995).

To begin to examine the role of TGFβ in early spinal cord development, we studied the pattern of Smad2 and Smad3 expression in the chick neural tube of HH stages 6 to 25 (Hamburger and Hamilton, 1951). By in situ hybridization, Smad2 mRNA was not detected at any stage analyzed (data not shown), whereas Smad3 was first detected before neural tube closure (HH stage 6+) at the intermediate neural plate (Fig. 1D). From HH stage 13, Smad3 was found in a broad domain along the DV axis of the neural tube, and was absent from dorsal and ventral midline cells (Fig. 1E). High expression was also detected at tail-bud regions (Fig. 1E). At HH stage 18, expression was further restricted to sharp boundary domains, including a dorsal domain which showed high levels ($Smad3^{high}$) corresponding to dP1-2 progenitors, a broad

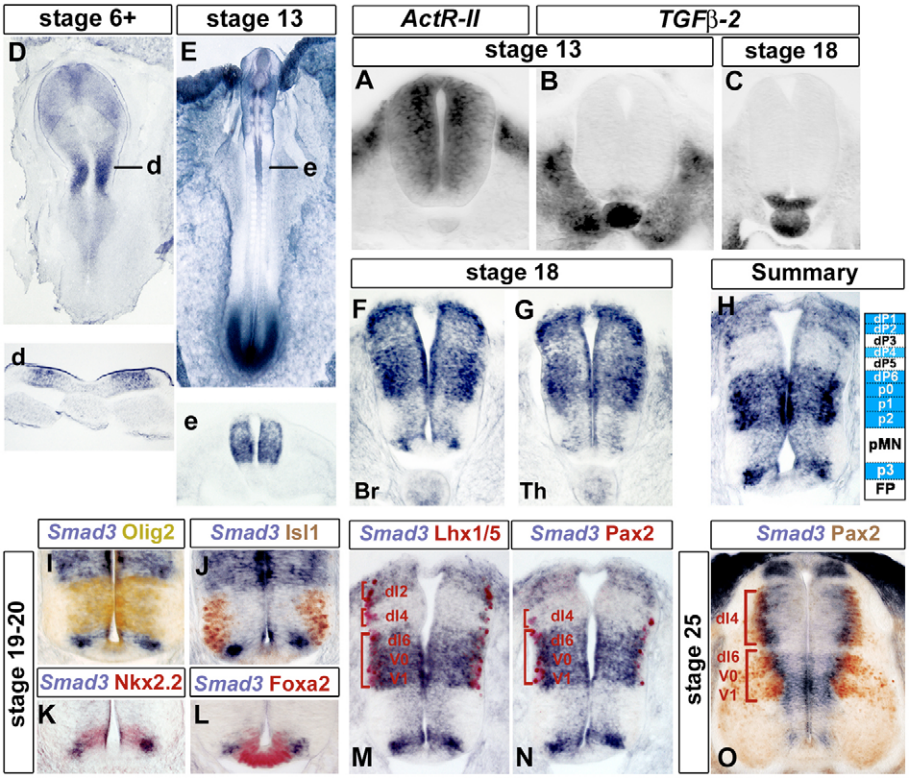

Fig. 1. Expression of TGFβ2/Smad3 in the developing chick spinal cord. (**A**) At stage HH13, ActR-II is expressed throughout the DV axis. (**B**) TGFβ2 is highly expressed in the notochord and ventral mesoderm at HH13, and weakly in the floor plate. (**C**) At stage HH18, TGFβ2 is highly expressed in the notochord and the floor plate. (**D**) Expression of Smad3 in the opened neural plate of a stage 6 chick embryo. (**E**) Smad3 is expressed at rostral levels of the neural tube by stage 13, along the DV axis, excluding dorsal and ventral midline cells. (**F**,**G**) Smad3 expression at stage 18 predominates in two stripes encompassing dorsal and intermediate domains of the VZ. (F) A third ventral stripe is detected at brachial levels. (**H**) Summary of the pattern of Smad3 expression along the DV axis of the stage 18 chick neural tube. Panels on the right indicate progenitor domains. (**I**,**J**) From stage HH18-19, Smad3 is absent from Olig2-expressing pMN progenitors (I) and from postmitotic Isl1+ MNs (J). (**K**) Smad3 is co-expressed with Nkx2.2 within p3 progenitor domain. (**L**) Smad3 is absent from Foxa2-expressing floor plate cells. (**M**) Double-labelling Smad3 with anti-Lhx1/5 assigns Smad3-expressing domains to V1-dI6, dI4 and dI1-2. (**N**) Double-labelling Smad3 with anti-Pax2 assigns Smad3 expressing domains to V1-dI6 and dI4. (**O**) At stage 25, Smad3 expression is high at the lateral edge of the VZ at most levels of the DV axis except in the dorsal domain. Virtually all Smad3+ cells at the edge of the VZ co-express Pax2.

intermediate domain of Smad3^{high} including dP6-p2 progenitors, and a discrete group of ventral cells flanking the floor plate corresponding to p3 domain progenitors (Fig. 1F,G). This ventral-most (p3) domain of Smad3^{high} became more evident at ~HH stage 20, and expression of Smad3 was detected at both brachial and thoracic levels (Fig. 1G). The ventral-most domain of Smad3^{high} was defined as p3 by the co-expression of the HD factor Nkx2.2 (Fig. 1K) and by the exclusion from Foxa2+ floor plate cells (Fig. 1L). Furthermore, at ~HH stage 20, Smad3^{high} was absent from Olig2-expressing motoneuron progenitors (pMN) (Fig. 1I) and from postmitotic MNs expressing Isl1 (Fig. 1J). The intermediate and dorsal domains of Smad3^{high} were defined as V1-dI6 and dI4, respectively, by the co-expression of Lhx1/5 (Fig. 1M) and Pax2 (Fig. 1N,O), whereas the dorsal dI1-2 domains was defined by the expression of Lhx1/5 in the absence of Pax2 (Fig. 1M,N).

Moreover, at early developmental stages, Smad3 was expressed by progenitor cells encompassing the entire mediolateral axis of the VZ, whereas at later stages (HH stage 20-25), mRNA was clearly enriched in laterally positioned progenitors, where neurogenesis occurs (Fig. 1O). Furthermore, differentiated neurons showed no expression of Smad3.

Taken together, these findings indicate that the regionalized expression of Smad3 might be regulated to discrete domains during development of the neural tube. Therefore, we next analyzed possible interactions between Smad3 and proteins known to be involved in pattern formation.

Regionalized expression of Smad3 depends on progenitor proteins

In the developing neural tube, Gli-mediated Shh signaling induces the expression of class II progenitor proteins following the temporal sequence of Nkx6, Olig2, Nkx2, to determine ventral progenitor identities (Jeong and McMahon, 2005; Stamataki et al., 2005). To study whether regionalized expression of Smad3 is dependent on the activity of class II proteins, we misexpressed Nkx6.1, Olig2 and Nkx2.2 by chick in ovo electroporation, and examined the resulting pattern of Smad3 expression.

The onset of Smad3 expression occurred before neural tube closure and was already absent from the medial neural plate cells, where the HD factor Nkx6.1 was expressed, suggesting an early role for Nkx6 in restricting Smad3 expression (Fig. 2A-C). Ectopic expression of Nkx6.1 (Fig. 2D) resulted in decreased Smad3^{high} in

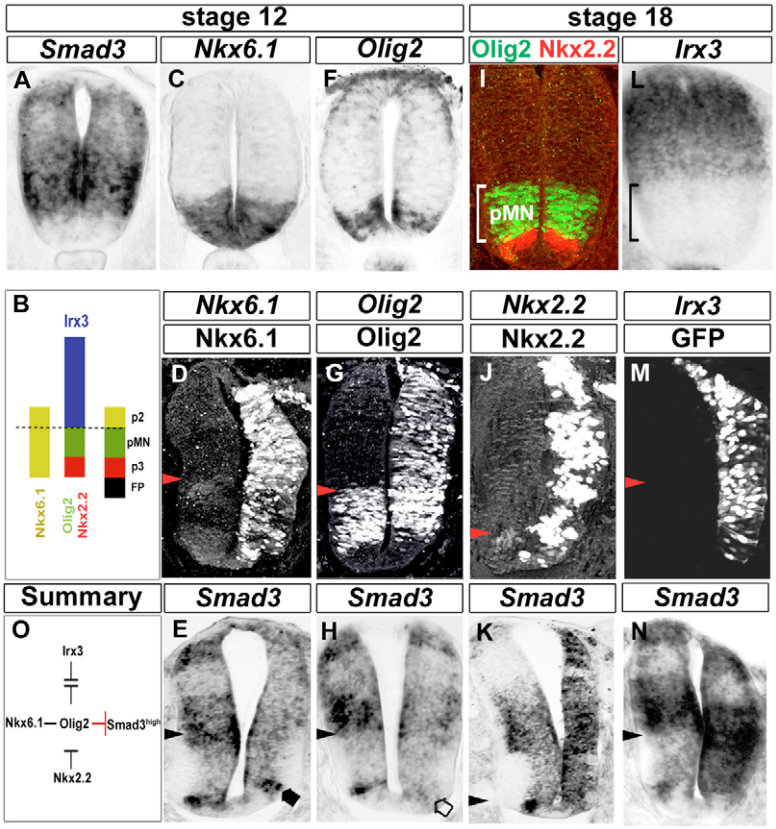

Fig. 2. Regionalized expression of Smad3 depends on patterning genes. (**A**,**C**,**F**,**I**,**L**) Comparative expression analysis of Smad3 and progenitor class I/II genes at rostral levels of the chick neural tube at stages 12-18. (**B**) Diagram showing the expression pattern of selected class I/II genes in ventral progenitor domains. (**D**,**E**) Misexpression of Nkx6.1 (D) leads to a decrease in Smad3 in a position-dependent manner (E). Smad3 is repressed from intermediate and dorsal domains, but not from the p3 domain, filled arrow in (E). (**G**,**H**) Misexpression of Olig2 (G) leads to a decrease in Smad3 (H), including the p3 domain, open arrow in (H). (**J**,**K**) Misexpression of Nkx2.2 (J) induces ectopic expression of Smad3 along the DV axis (K). (**M**,**N**) Misexpression of Irx3 (M) induces Smad3 expression within the pMN domain (N). (**O**) Summary of regulatory interactions between class I/II proteins that prevent the maintenance of Smad3 expression in the pMN domain.

the intermediate (p2-dP6) domain (Fig. 2E), indicating that Nkx6.1 might normally limit the ventral boundary of Smad3^{high} outside the MN progenitor domain. However, the late onset of Smad3^{high} expression at the p3 domain, where Nkx6 and Smad3 were normally co-expressed, was not altered by overexpression of Nkx6.1 (Fig. 2E, filled arrow).

The onset of Olig2 expression followed that of Nkx6.1 in a small group of cells that initially encompassed the ventral midline of the neural plate. During neural tube development, Olig2 was gradually repressed from midline cells and from the ventral-most p3 progenitor domain, to unequivocally define the pMN domain (Jeong and McMahon, 2005; Novitch et al., 2001; Stamataki et al., 2005) (Fig. 2F,I). Ectopic expression of Olig2 in the neural tube (Fig. 2G) resulted in the repression of Smad3^{high} from the intermediate (p2-dP6) domain (Fig. 2H). Furthermore, Smad3^{high} in the p3 domain, where Olig2 is normally absent, was efficiently repressed by Olig2 activity (Fig. 2H, open arrow). These results suggest that Olig2 represses Smad3^{high} to refine the p2/pMN domain boundary whereas at early time points, before the onset of Olig2 expression, the main dorsal repression might have already been accomplished by Nkx6 activity.

Nkx2 class genes are selectively expressed by ventral p3 progenitors (Briscoe et al., 1999). The onset of Nkx2.2 expression follows that of Nkx6 and Olig2 in ventral midline cells. During neural tube development, however, Nkx2.2 is absent from midline cells and confined to the p3 domain (Jeong and McMahon, 2005; Stamataki et al., 2005) (Fig. 2I). The late onset of Smad3 expression in the p3 domain, only after Nkx2.2 is confined to p3 domain progenitors and excluded from floor plate cells, suggests that Nkx2.2 also regulates Smad3 expression. Indeed, ectopic expression of Nkx2.2 throughout the DV axis (Fig. 2J) fully induced Smad3^{high} dorsally (Fig. 2K).

These results indicate that progenitor class II proteins restrict Smad3 expression during neural tube development to discrete ventral spinal cord progenitor domains. Additionally, class I proteins are broadly expressed in early unpatterned neural tube, with their expression being dorsally restricted by Gli-mediated Shh activity. To study whether this class of proteins is involved in the regionalized expression of Smad3, we misexpressed Irx3 and examined the resulting Smad3 expression. Irx3 and Smad3 were partially co-expressed within the early unpatterned neural tube, and neither RNA was present in pMN progenitors (Fig. 1I and Fig. 2L). Ectopic expression of Irx3 (Fig. 2M) was sufficient to ventrally expand the intermediate domain of Smad3^{high}, without changing the dorsal pattern (Fig. 2N), thus suggesting that Irx3 participates in maintaining Smad3^{high} expression within p2-dP6 progenitor domains.

All together, these findings indicate that the activity of class I and class II progenitor HD and bHLH proteins contribute to regulate the expression of Smad3 to discrete domain boundaries during pattern formation of the neural tube (Fig. 2O). We therefore proceeded to analyze the contributions of Smad3 activity to spinal cord development. Converse experiments revealed that overexpression of this protein substantially hindered DV progenitor features, without increasing progenitor cell death (see Fig. S1 in the supplementary material). This observation indicates that loss of progenitor proteins might be a consequence of cells progressing into a differentiated state.

Smad3 activity promotes neurogenesis

The finding that Smad3 overexpression repressed progenitor features prompted us to examine, by chick embryo electroporation, whether the activity of this protein influences neurogenesis. We misexpressed various Smad3 versions that show differential transcriptional activity (Lagna et al., 1996; Liu et al., 1997; Weiser et al., 1995) (see Fig. S2

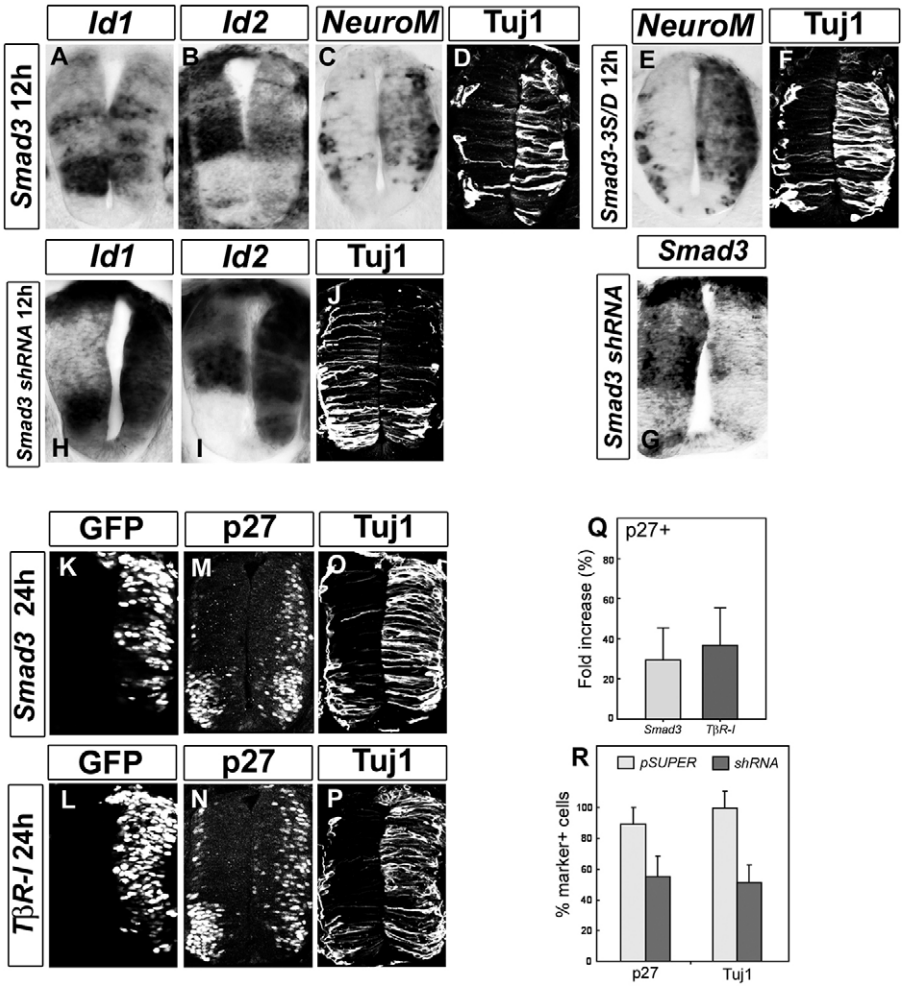

Fig. 3. Smad3 activity reduces expression of progenitor proteins and promotes neuronal differentiation. (**A**,**B**) Forced expression of Smad3 reduces expression of progenitor markers Id1 and Id2. (**C**,**D**) Forced expression of Smad3 induces neural differentiation markers, 12 hours after electroporation, transfected cells upregulate the expression of NeuroM (C) and Tuj1 (D). (**E**,**F**) Smad3-3S/D mutant version mimics the induction of neural differentiation markers. (**G**) Electroporation of Smad3 shRNA efficiently reduces Smad3 endogenous expression. (**H-J**) Endogenous Smad3 activity is required for neuronal differentiation. 12 hours after electroporation of Smad3 shRNA, expression of Id1 (H) and Id2 (I) are ectopically activated, and Tuj1 expression is reduced (J). (**K-P**) 24 hours after transfection of either Smad3 (K) or TβR-I (L) most cells have upregulated the expression of the cyclin-dependent kinase inhibitor $p27^{kip1}$ (M,N), and the pan-neuronal marker Tuj-1 (O,P). (**Q**) Quantitative analysis shows an increase in expression of $p27^{kip1}$ after Smad3 or TβR-I transfection compared with the non-electroporated control side. Histograms show data points as mean values ± s.d. (n>3 embryos, six sections). (**R**) 36 hours after transfection of Smad3 shRNA reduces the expression of neurogenic markers $p27^{kip1}$ and Tuj-1, compared with control embryo transfected with the empty pSUPER vector. Histograms show data points as mean values ± s.d. (n>3 embryos).

in the supplementary material), and analyzed the resulting neural tubes for the expression of markers defining progenitor cells or differentiated neurons.

Id genes (Id1-4) encode helix-loop-helix (HLH) proteins that inhibit neural differentiation and maintain neuroepithelial cells in a progenitor state. These genes are therefore widely expressed in the VZ and absent from differentiated neurons (Kee and Bronner-Fraser, 2001). In the developing spinal cord, the four Id genes displayed nonoverlapping patterns of expression, Id1 and Id2 being highly complementary along the DV axis (Fig. 3A,B, left side). Overexpression of Smad3 caused a rapid decrease in progenitor markers and induction of differentiation markers. Indeed, an efficient reduction of Id1 and Id2 expression was detected 12 hours after electroporation of Smad3 or Smad3-3S/D (Fig. 3A,B and data not shown). In addition, expression of NeuroM, a neural-specific bHLH factor sufficient for triggering neuronal differentiation (Roztocil et al., 1997; Lee and Pfaff, 2003), was induced 12 hours after electroporation (Fig. 3C,E), together with the pan-neuronal marker Tuj-1 (Fig. 3D,F).

As all P-Smad proteins interact with the common co-factor Smad4 for their activity (Massagué et al., 2005), overexpression of Smad3 might be sequestering Smad4 therefore resulting in the blockade of other Smad function. To test this possibility we co-transfected Smad4 with Smad3, which resulted in a comparable induction of Tuj-1 expression (see Fig. S3 in the supplementary material). These indicated that Smad4 was not a limiting factor for Smad3 activity and that the neurogenic activity of Smad3 was not a consequence of Smad4 being sequestered from other Smads.

Given the finding that Smad3 activity was sufficient to promote neural differentiation, we next asked whether endogenous Smad3 activity was required for neurogenesis. Electroporation of Smad3 short RNA hairpin (shRNA) resulted in an effective reduction of endogenous Smad3 expression, as assessed by in situ hybridization (Fig. 3G). Interestingly, loss of endogenous Smad3 resulted in a dramatic induction of Id1 (Fig. 3H) and Id2 (Fig. 3I) overexpression along the entire DV axis, thus suggesting that neuroepithelial cells are forced to be maintained in a progenitor state, and therefore resulting in the loss of neural markers such as Tuj-1 (Fig. 3J).

At 24 hours after overexpression of Smad3 (Fig. 3K), or activation of endogenous Smad3 proteins by the electroporation of a constitutively active form of the type I TGFβ receptor (TβR-I) (Weiser et al., 1995) (Fig. 3L), the expression of the cyclin-dependent kinase inhibitor $p27^{kip1}$ was significantly increased throughout the developing spinal cord ($p27^{kip1+}$ cells in Smad3 control side: 88.21±28.37%; electroporated side: 111.38±34.40%; TβR-I control side: 85.99±15.49%; electroporated side 117.91±32.23%) (Fig. 3M,N,Q). Expression of Tuj-1 (Fig. 3O,P) and NeuroM (not shown) were further increased in the electroporated side of the neural tube 24 hours after misexpression of Smad3 or TβR-I.

Converse experiments were analyzed 36 hours post-electroporation of Smad3 shRNA. Loss of endogenous Smad3 activity led to a significant reduction of postmitotic markers compared with control pSUPER transfected embryos, without inducing apoptosis (not shown): $p27^{kip1+}$ cells, control

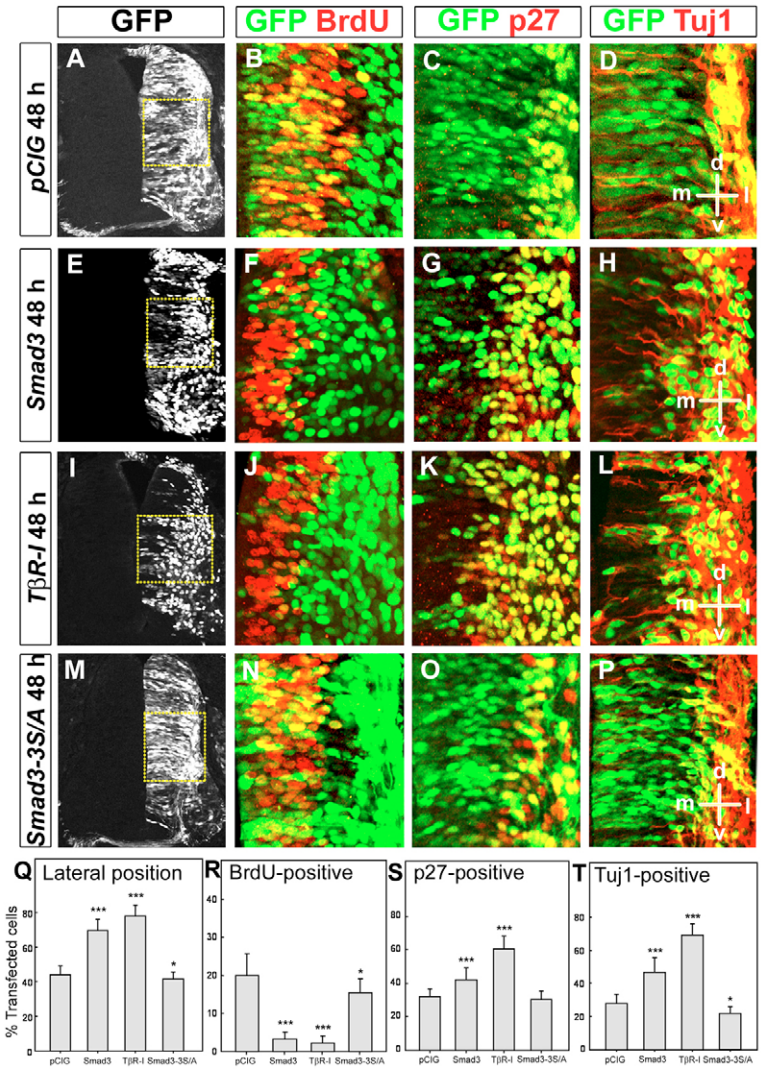

Fig. 4. TGFβ signaling through Smad3 promotes neurogenesis in the spinal cord. HH stage 14-16 embryos electroporated with pCIG-based vectors driving the expression of nuclear GFP as reported, were harvested 48 hours later. (**A-D**) Control embryos electroporated with the empty pCIG vector. (**E-H**) Electroporation of Smad3 causes mediolateral displacement of GFP-expressing cells by >55% (E), decreases BrdU incorporation by >80% (F), cell autonomously increases $p27^{kip1}$ expression by >30% (G) and cell-autonomously induces Tuj1 by >65% (H) compared with the empty pCIG vector transfection. (**I-L**) Electroporation of TβR-I causes similar phenotype changes to Smad3 electroporation. (**M-P**) Electroporation of Smad3-3S/A reverts all aspects of the Smad3 phenotype; lateral GFP+ cells (M) are reduced by ~40%, transfected cells incorporating BrdU (N) are increased four- to five-fold and transfected cells co-expressing $p27^{kip1}$ (O) or Tuj1 (P) are reduced by more than 27% and 53% respectively. (**Q-T**) Percentages of transfected cells at lateral positions (Q), double labeled GFP/BrdU (R), co-expressing GFP/$p27^{kip1}$ (S) and co-expressing GFP/Tuj1 (T). The medial (m) or lateral (l) position of transfected cells was defined by cell location in relation to $p27^{kip1}$+ cells. Histograms show data points as mean values ± s.d. (n=6 embryos, >6 sections were assessed in each group of experiments) *P<0.05; **P<0.01; ***P<0.001.

89.23±10.9%, shRNA 55.33±13.1%, P=0.000031; and Tuj-1, control 99.73±10.8%, shRNA 51.40±11.6%, P=0.000043 (Fig. 3R). Taken together, these data indicate multiple neurogenic roles for Smad3 in spinal cord development by: (a) decreasing the expression of the inhibitor Ids; (b) inducing expression of the cell-cycle inhibitor $p27^{kip1}$; and, (c) inducing expression of the required neurogenic bHLH factor NeuroM.

TGFβ signaling through Smad3 activity promotes cell-cycle exit, lateral migration and neuronal differentiation

In the developing neural tube, dividing progenitor cells reside in the VZ. As these cells differentiate, they move laterally out of the VZ, exit the cell cycle and begin to express neuronal specific markers. Using these three criteria for neuronal differentiation, we found that TGFβ signaling through activation of Smad3 directs neuroepithelial progenitor cells to exit the cell cycle and undergo neuronal differentiation.

Electroporation of the control expression vector pCIG at HH stage 14-16, and analysis 48 hours later, revealed transfected cells encompassing the entire mediolateral axis of the neural tube (Fig. 4A-D). By contrast, electroporation of Smad3 resulted in transfected cells preferentially located lateral to the VZ within the mantle layer where differentiated neurons reside (percentage of GFP+ cells: pCIG 43.77±4.87%, Smad3 69.44±6.64%; P<0.001; Fig. 4E,Q). Furthermore, cells overexpressing Smad3 had a reduced capacity to incorporate BrdU (percentage of BrdU+/GFP+ cells: pCIG 20.02±5.67%, Smad3 3.36±1.75%; P<0.001; Fig. 4F,R), and increased expression of $p27^{kip1}$ (percentage of $p27^{kip1}$+/GFP+ cells: pCIG 31.77±4.85%, Smad3 41.85±7.37%; P<0.001; Fig. 4G,S), and of Tuj-1 (percentage of Tuj1+/GFP+ cells: pCIG 27.82±5.40%, Smad3 46.76±8.66%; P<0.001; Fig. 4H,T), compared with cells transfected with the control empty vector (Fig. 4A-D). These data indicate that Smad3 overexpression caused progenitor cells to exit the cell cycle and differentiate into neurons. Similar changes were observed when endogenous Smad proteins were activated by electroporation of TβR-I (77.96±6.19% of transfected cells laterally located, 2.23±1.95% of transfected cells expressing BrdU, 60.31±7.84% of transfected cells expressing $p27^{kip1}$, and 69.21±6.73% cells expressing Tuj-1; P<0.001; Fig. 4I-L,Q-T), suggesting that the neurogenic activity of Smad3 mediates a TGFβ-like activity.

Furthermore, electroporation of the Smad3-3S/A mutant version (see Fig. S2 in the supplementary material), which lacks the capacity to be activated by receptor phosphorylation and therefore fails to transduce TGFβ signal, reverted the Smad3 phenotype. Cells expressing Smad3-3S/A were equally distributed within the VZ and the mantle zone (41.55±3.91% of transfected cells at lateral position,

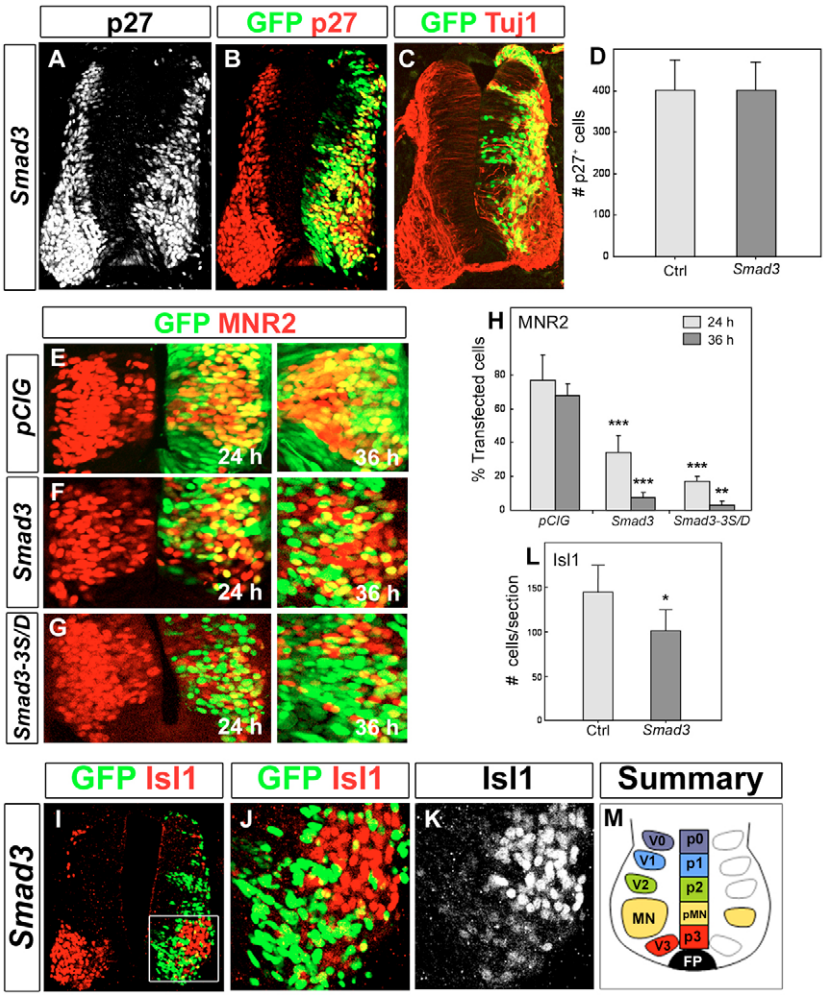

Fig. 5. Smad3 activity inhibits MN differentiation. HH14-16 embryos transfected with Smad3 were analyzed 48 hours after electroporation for the expression of $p27^{kip1}$, Tuj1 and MN markers. (**A-D**) At this stage, the total number of cells that exited the cell cycle in the non-electroporated control side and the electroporated side does not differ significantly (~400 cells, *n*=6 embryos) (D). Immunohistochemical analysis of MN differentiation (MNR2+ cells) in embryos electroporated with pCIG empty vector (**E**), Smad3 (**F**) or Smad3-3S/D (**G**), and analyzed 24 hours (left panel) or 36 hours (right panel) after transfection. Overexpression of Smad3 decreases the number of MNR2+ cells from ~56% at 24 h (F, left panel) to ~90% at 36 h after transfection (F, right panel) compared with the pCIG empty vector. Forced expression of the Smad3-3S/D mutant version further reduces GFP+/MNR2+ cell numbers, ~90% reduction at 24 h (G, left panel) to ~96% at 36 h (G, right panel). (**H**) Quantitative data of transfected cells that co-express GFP/MNR2 within the domain of MN generation (*n*=6 embryos were assessed in each experiment). Histograms show data points as mean values ± s.d. ***P*<0.01; ****P*<0.001. (**I-L**) 48 hours after electroporation of Smad3, transfected cells (GFP+ cells) do not express Isl1 in a cell-autonomous way. (L) Quantitative data on Isl1-expressing cells, 48 hours after Smad3 electroporation. Isl1+ cells are reduced by ~30% compared with the non-electroporated control side (*n*>6 embryos, at least four sections/embryo). Histograms show data points as mean values ± s.d. **P*<0.05. (**M**) Diagrammatic representation of ventral progenitor domains and ventral neuronal subtypes, generated in a normal spinal cord and after Smad3 misexpression.

P<0.05; Fig. 4M,Q), incorporated BrdU at lower levels than control transfected cells (15.38±3.86% of transfected cells; *P*<0.05; Fig. 4N,R), expressed $p27^{kip1}$ at comparable levels to pCIG-transfected cells (30.18±5.28 % of transfected cells; Fig. 4O,S), and expressed Tuj-1 at lower levels than control transfected cells (21.79±4.11% of transfected cells; *P*<0.05; Fig. 4P,T). All these observations indicate that phosphorylation is a requisite for Smad3-induced neuronal differentiation in the developing spinal cord, and that Smad3-3S/A acts as a weak dominant negative protein.

Motoneuron differentiation is impaired by Smad3 activity

Although overexpression of Smad3 increased neuronal differentiation, the final size of the spinal cord was similar on the electroporated and the non-electroporated control side of the spinal cord, indicating that Smad3 activity did not cause a significant change in the final postmitotic cell numbers. Indeed, total numbers of $p27^{kip1+}$ cells remained constant between electroporated and non-electroporated sides (control side 400.42±74.9 cells; Smad3 electroporated-side 400.37±68.8; Fig. 5A-D). This finding suggests that TGFβ/Smad3 activity does not induce an overall differentiation of neural tube progenitor cells but instead the preferential differentiation of certain neuronal subtypes at the expense of others. Supporting this possibility we observed a morphological disruption of the ventral horn that might reflect a reduction of the MN pool (Fig. 5A-C).

To test this possibility, we first analyzed changes in the expression of selective MN markers after electroporation of Smad3 or Smad3-3S/D. MNR2 is a HD protein that acts as a determinant of MN identity. MNR2 is selectively expressed by late MN progenitors and transiently by postmitotic somatic MNs (Tanabe et al., 1998). Misexpression of Smad3 within the pMN domain resulted in the suppression of MNR2, without inducing cell death (data not shown). Loss of MNR2 expression was both time- and dose-dependent on Smad3 activity (Fig. 5E-H). As such, 24 hours after misexpression of Smad3 within the pMN domain, the number of MNR2+ cells was reduced by ~55.84% compared with pCIG control transfection (percentage of MNR2+/GFP+ cells: pCIG 77±15%, Smad3 34±10%; *P*<0.001; Fig. 5E,F [left panel], H), whereas 36 hours after, Smad3 electroporation caused a ~89.71% reduction in MNR2+ cells (percentage of MNR2+/GFP+ cells: pCIG 68±7%, Smad3 7±3%; *P*<0.001; Fig. 5E,F [right panel], H). Furthermore, higher transcriptional activity, accomplished by misexpression of the Smad3-3S/D mutant version, caused a more dramatic reduction in the number of MNR2+ cells; a further ~50% and ~57.14% by 24 hours (17±3% of MNR2+/GFP+ cells; *P*<0.001; Fig. 5E,G [left panel], H), and 36 hours after electroporation (3±2% of MNR2+/GFP+ cells; *P*<0.01; Fig. 5E,G [right panel], H) respectively, compared with the wild-type Smad3. Thus, MNR2 expression was efficiently suppressed by increasing levels of Smad3 transcriptional activity, which indicates a dose response of Smad3 activity in repressing MN generation.

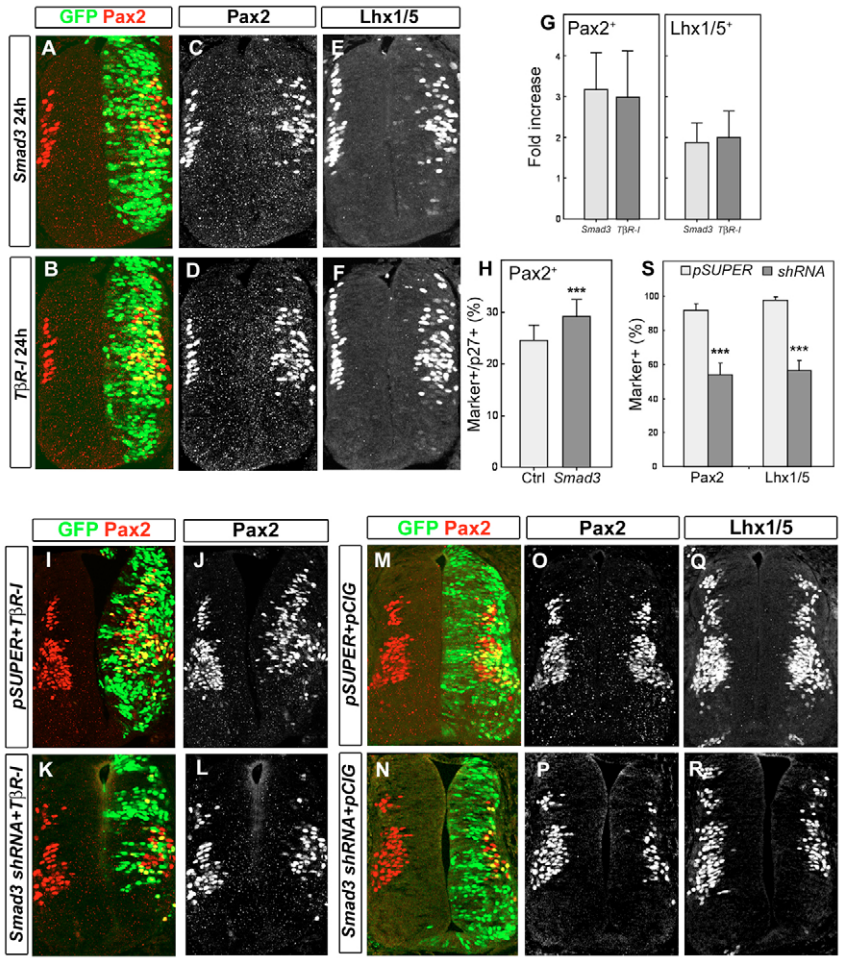

Fig. 6. Smad3 activity is both required and sufficient for the generation of ventral interneurons. (**A-F**) HH stage 14 embryos electroporated with Smad3 or TβR-I were analyzed 24 hours later for the expression of selective IN markers. Pax2+ (A-D) and Lhx1/5+ (E,F) were cell-autonomously induced in the electroporated side. (**G**) Quantitative analysis shows an increase in expression of Pax2+ and Lhx1/5+ cells after Smad3 or TβR-I transfection compared with the non-electroporated control side. (**H**) Quantitative analysis of $p27^{kip1}$/Pax2 double labeled cells after electroporation of Smad3 shows a increased proportion of Pax2+cells. Histograms show data points as mean values ± s.d. (*n*=embryos, 6 sections). (**I**,**J**) 48 hours co-electroporation of pSUPER with TβR-I resulted in a dramatic increase in Pax2+ cells. (**K**,**L**) Co-electroporation of pSUPER-Smad3 shRNA with TβR-I resulted in the rescue of Pax2 ectopic expression. (**M-R**) HH stage 14-16 embryos electroporated with pSUPER-Smad3 shRNA were analyzed 48 hours later for the expression of selective LIM-HD factors. shRNA Smad3 and empty pSUPER vector were co-electroporated with pCIG (5:1) to use GFP as a reporter protein. 48 hours after electroporation (M,N) the expression of interneuron markers such as Pax2 (O,P) and Lhx1/5 (Q,R) is reduced. (**S**) Percentage of cells that express Pax2, and Lhx1/5 in control vs. electroporated sides. Pax2+ cells are reduced by 41.29%, Lhx1/5+ cells by 42.31% (*n*=5 embryos were assessed in each experiment). Histograms show data points as mean values ± s.e.m. Lhx1/5, *P*=0.00022; Pax2, *P*=0.000025; using the two-tailed Student's *t*-test.

We next analyzed the expression of the LIM-HD protein Isl1, which is restricted to terminally differentiated MNs (Pfaff et al., 1996). Misexpression of Smad3 within the pMN domain resulted in a ~30.17% reduction of Isl1+ MNs (number of Isl1+ cells: control side 144.01±30.39, electroporated side 100.56±23.83; $P<0.05$; Fig. 5I-L). High levels of Smad3 resulted in a cell-autonomous inhibition of Isl1 expression, whereas lower levels did not completely repress Isl1 (Fig. 5K). Interestingly, many Smad3-transfected cells within the MN domain, cell-autonomously expressed $p27^{kip1}$ (Fig. 5B), indicating that these cells have been driven into terminal differentiation, although they are likely being mis-specified to assume different identities.

In the ventral spinal cord, together with MNs, four neuronal subtypes (V3, V2, V1 and V0 INs) generate from individual progenitor domains (summarized in Fig. 5M). Ventral interneuron identities are defined by expression of a set of HD proteins and DV position within the ventral spinal cord (Briscoe and Ericson, 2001; Jessell, 2000; Martí et al., 2005). We next asked whether Smad3 activity was promoting ventral IN differentiation at the expense of MN generation.

Smad3 activity promotes differentiation of ventral interneurons

Our expression analysis showed Smad3 in an intermediate progenitor domain encompassing p1-dP6 (Fig. 1). INs generated from these progenitor domains can be identified by the expression of the postmitotic markers Pax2 (Burrill et al., 1997), and the LIM-HD factor Lhx1/5 (Helms and Johnson, 2003). At 24 hours after electroporation of Smad3 (Fig. 6A) or TβR-I (Fig. 6B), cell-autonomous induction of Pax2+ cells (fold increase: Smad3 3.17±0.90, TβR-I 2.98±1.15; Fig. 6C,D,G) and Lhx1/5+ cells was seen (fold increase: Smad3 1.87±0.49, TβR-I 1.99±0.65; Fig. 6E,F,G). Additionally, the proportion of Pax2+ cells among differentiated $p27^{kip1+}$ cells had significantly increased 48 hours after electroporation of Smad3 (19% increase in $p27^{kip1}$/Pax2 double labeled cells, Fig. 6H), indicating that a subset of differentiated cells are being misspecified to assume a different phenotype.

At 48 hours after activation of endogenous Smad proteins by the electroporation of TβR-I, a further expansion of Pax2+ cells was evident (Fig. 6I,J). To test whether this activity was dependent on endogenous Smad3 proteins, we co-electroporated this activated receptor with Smad3 shRNA. Inhibition of endogenous Smad3 was sufficient to revert expansion of Pax2 (Fig. 6K,L), indicating that Smad3 activity was indeed required for the expansion of Pax2+ cells.

The observation that a TGFβ mediated Smad3 activity was sufficient for the cell-autonomous induction of IN markers such as Pax2 and Lhx1/5 prompted us to examine whether endogenous Smad3 activity was required for the generation of ventral INs. Inhibition of endogenous Smad3 by electroporation of shRNA resulted in a significant reduction in Pax2+ cells within V1-dI6/dI4 compared with control pSUPER transfected embryos (percentage of Pax2+ cells: control 91.7±3.9%, shRNA 58.71±7.2%; $P=0.000025$; Fig. 6M-P,S). Furthermore, inhibition of Smad3 resulted in a

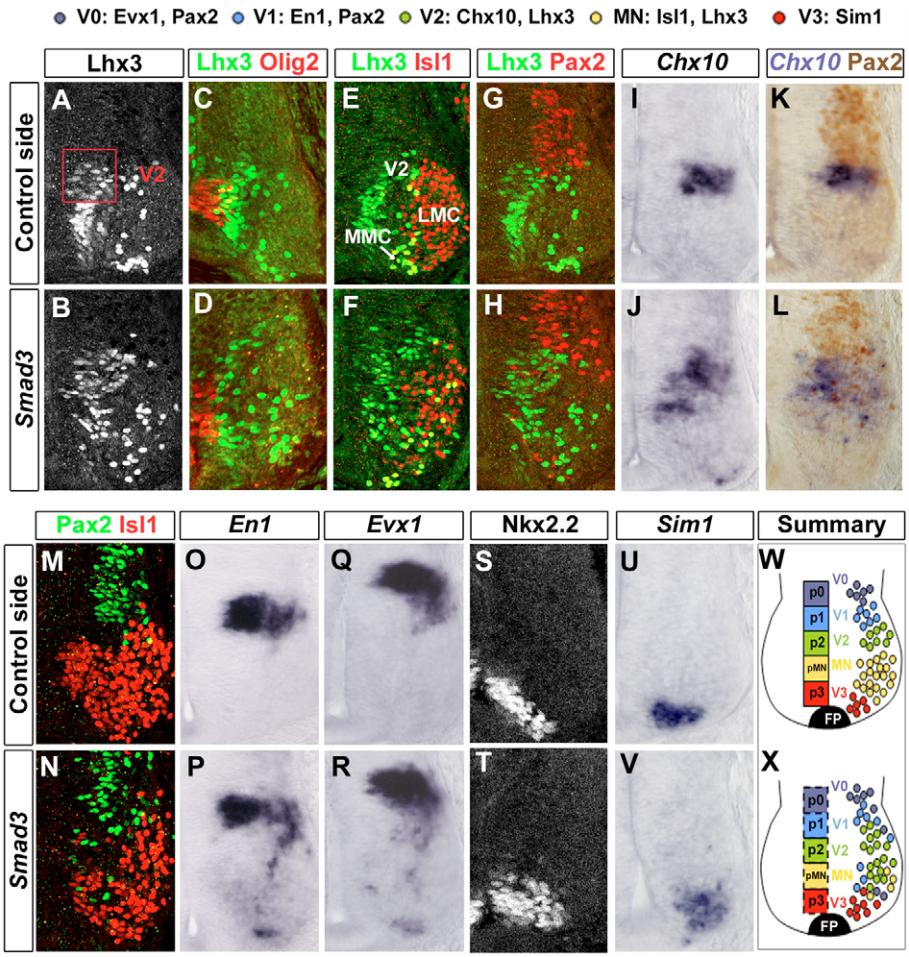

Fig. 7. Smad3 activity promotes differentiation of V0-V2 and V3 interneurons at the expenses of MNs. HH stage 14-16 embryos electroporated with Smad3 were analyzed 48 hours later for the expression of selective LIM-HD factors. (**A**,**C**,**E**,**G**,**I**,**K**) Control embryos showing markers for specific neuronal subpopulations. Lhx3 expression identifies postmitotic pre-migratory MNs and V2 INs. (**B**,**D**,**F**,**H**,**J**,**L**) Forced expression of Smad3 results in the ectopic induction of V2 INs. Lhx3+; Isl1- V2 INs are dorsally and ventrally expanded (F). LMC, lateral MN column; MMC, medial MN column (E). Chx10-expressing V2 INs (I-L) are also dorsally and ventrally expanded. (**M**,**O**,**Q**,**S**,**U**) Control embryos showing markers for specific ventral IN populations. (**N**,**P**,**R**,**T**,**V**) Misexpression of Smad3 induces Pax2+, En1+ V1 and Evx1+ V0 INs. Ectopic expression of Pax2 (N), En1 (P) and Evx1 (R) is detected ventral to the p2/p1 domain boundary. (S-V) Misexpression of Smad3 induces few ectopic Nkx2.2+ cells (T) and a dorsal expansion of Sim1+ V3 INs (V). (**W**,**X**) Diagrammatic representation of ventral progenitor domains and ventral neuronal subtypes, generated in a normal spinal cord and after Smad3 misexpression.

significant reduction in Lhx1/5+ cells (percentage of Lhx1/5+ cells: control 108.00±4.0%, shRNA 57.64±5.7%; P=0.00022; Fig. 6Q-S). Interestingly, loss of endogenous Smad3 did not affect the final number of differentiated MNs (percentage of islet1+ cells: in control condition [pSUPER empty vector] 104.87±7.76%, Smad3 shRNA 99.25±9.24%; i.e. less than 5% reduction of Isl1+ MNs compared with ~36% and ~47% reduction of Pax2+ and Lhx1/5+ interneurons, respectively).

All together our data showed that ectopic expression of Smad3 within the MN progenitor domain was sufficient for the loss of MN generation, whereas endogenous Smad3 activity was required for the generation of certain subtypes of spinal cord INs. However, our expression analysis showed that Smad3^{high} was expressed in additional progenitor populations flanking the pMN domain. These observations prompted us to analyze whether Smad3 activity was sufficient for the generation of additional ventral IN subtypes.

Dorsal to MNs, Lhx3 is expressed by the small population of V2 INs, as well as by MNs as they exit the cell cycle (Thaler et al., 2002; Tsuchida et al., 1994) (Fig. 7A). The small population of V2 INs, marked by the expression of Lhx3 in the absence of Isl1, expanded a few cell diameters dorsally and ventrally to occupy the MN domain (Fig. 7A,B,E,F). Lhx3+ cells within the MN domain did not express Olig2, indicating that these cells terminally differentiated (Fig. 7C,D). Neither did these cells express Isl1 (Fig. 7E,F) or Pax2 (Fig. 7G,H), indicating that they maintained their V2 identity in spite of ectopic locations. Furthermore, expression of the V2 specific marker Chx10 was also expanded to partially occupy the MN domain (Fig. 7I-L).

Differential cell identities among the Pax2+ INs can be further delineated by the expression of En1 (V1 INs) and Evx1 (V0 INs) (Ericson et al., 1997; Pierani et al., 2001). Misexpression of Smad3 expanded Pax2+ cells ventrally to partially occupy the MN domain (Fig. 7G,H,K-N). Ectopic expression of Smad3 resulted in the cell-autonomous induction of Pax2+ cells within the pMN domain (Fig. 7G,H), as well as in the corresponding ventral expansion of En1-expressing V1 INs (Fig. 7O,P) and Evx1-expressing V0 INs (Fig. 7Q,R). Interestingly, ectopic Pax2+ cells showed no Isl1 expression either (Fig. 7M,N), further indicating that these cells maintained their V1/V0 IN identity.

Following Nkx2.2 restriction to the ventral-most p3 domain progenitors, and depending on Nkx2.2 activity, a further discrete domain of Smad3 expression was observed in the ventral neural tube (Fig. 1K,L). Misexpression of Smad3 along the DV axis of the developing neural tube caused a moderate (few cell diameters) dorsal expansion of Nkx2.2-expressing cells (Fig. 7S,T), at the expense of pMN markers (see Fig. 1C in the supplementary material), which resulted in a moderate dorsal expansion of Sim1+ V3 INs (Fig. 7U,V).

Taken together, our results indicate that ectopic Smad3 activity within the pMN domain resulted in a dramatic dose- and time-dependent suppression of MN generation. Instead, an array of ventral IN subtypes, including V3, V2, V1 and V0 cell identities, were generated to occupy the MN domain (Fig. 7W,X). This observation supports a model in which absence of Smad3 expression in the pMN domain during early stages of neural tube development is a pre-requisite for spinal MN specification and terminal differentiation, whereas Smad3 activity promotes the generation of ventral spinal cord INs.

DISCUSSION

The TGFβ superfamily of growth factors exerts a wide range of biological responses on a variety of cells belonging to different organs. These responses include cell fate decisions, tissue patterning, cell-cycle arrest, cell death and differentiation. Members of the TGFβ family, including BMPs, control key steps in the formation and differentiation of the vertebrate nervous system (Liu and Niswander, 2005). Indications of a role of the TGFβ/activin branch of the family in spinal cord development came from the description of several components of the pathway including ligands such as activin, dorsalin, or Vg1 (Liem et al., 1997; Shah et al., 1997), membrane receptors (our results) (Stern et al., 1995; Verschueren et al., 1995), and the common intracellular effector Smad4 (Chesnutt et al., 2004), being expressed throughout the DV axis of the developing neural tube.

TGFβ/activin-receptors signal through the activation of both Smad2 and Smad3 intracellular effector proteins (Massagué et al., 2005). To begin to understand the role of TGFβ/activin signaling in spinal cord development, we have set forth the expression and functional analysis of Smad proteins. By in situ hybridization we found Smad3, but not Smad2, to be expressed in the developing chick spinal cord, although our experimental approach cannot unequivocally rule out low levels of Smad2 being expressed below threshold detection. Whereas Smad2 null mice show an early embryonic lethality (Waldrip et al., 1998; Nomura and Li, 1998; Weinstein et al., 1998), Smad3 null mice appeared fertile and viable (Datto et al., 1999; Zhu et al., 1998). However, a detailed analysis of the Smad3–/– mouse CNS, has not been reported and should be performed to better understand the role of Smad3 in mouse spinal cord development.

Here we found Smad3 to be restricted to discrete progenitor domains of the neural tube. The onset of Smad3 expression occurred before neural tube closure. Concomitant to DV patterning of the neural tube, this protein became restricted to discrete progenitor domains, including a dorsal domain of $Smad3^{high}$ corresponding to dP1-2 progenitors, a broad intermediate domain of $Smad3^{high}$ including dP6-p2 progenitors, and a discrete group of ventral cells flanking the floor plate corresponding to the p3 progenitor domain. Our results show that the pattern of Smad3 expression is refined along neural tube development by the activity of patterning genes.

Soon after neural tube closure, ventral $Smad3^{high}$ was restricted, initially by the activity of Nkx6.1 HD factor, and subsequently by the activity of Olig2 bHLH factor. Thus, during ventral patterning of the spinal cord, $Smad3^{high}$ was efficiently excluded from the pMN domain. Our results show that this was a pre-requisite for subsequent MN generation. Within the intermediate dP6-p2 domain, $Smad3^{high}$ expression might be achieved by the activity of the HD factor Irx3, as both factors are partially co-expressed along neural tube development, and misexpression of Irx3 within pMN domain was sufficient for the ectopic ventral induction of $Smad3^{high}$. Interestingly, dorsal domains of Smad3 were not regulated by Irx3, further suggesting unresolved genetic interactions.

During spinal cord development, the expression of Smad3 was restricted to progenitor cells within the VZ, and excluded from differentiated neurons in the mantle layer. Interestingly, within specific progenitor domains, high levels of Smad3 were observed at the lateral edge of the VZ, the transition zone, where progenitor cells exit the cell cycle and enter the differentiation pathway. This observation suggests that Smad3 participates in neurogenesis. Indeed, overexpression of Smad3 or activation of endogenous Smad3 proteins with the constitutively active TGFβ receptor, resulted in the regulation of multiple key steps that culminate in neuronal differentiation.

Id proteins inhibit neurogenic bHLH activity by sequestering E proteins from bHLH factors (Ruzinova et al., 2003). In the developing spinal cord, we found Id1 and Id2 expression to be highly complementary, and overexpression of Smad3 repressed Id1 and Id2 expression. Furthermore, loss of endogenous Smad3 resulted in the ectopic activation of Id1 and Id2. These findings point to a model in which high Smad3 activity at the lateral edge of the VZ, is required for the repression of Id proteins, this allows E proteins to bind neurogenic bHLH factors and to initiate neurogenesis. Furthermore, overexpression of Smad3 resulted in rapid induction of NeuroM expression. NeuroM, is a neural-specific bHLH factor, transiently expressed at the lateral edge of the VZ, whose activity is essential for triggering neuronal differentiation (Roztocil et al., 1997; Lee and Pfaff, 2003). Thus our results indicate a dual role for Smad3 in neurogenesis by repressing bHLH inhibitors and by inducing neurogenic bHLH.

Furthermore, cell-cycle arrest is yet another pre-requisite for neurogenesis. One of the most common cellular responses to TGFβ signaling, cell-cycle arrest is achieved by multiple mechanisms, including transcriptional activation of cell-cycle inhibitors (Massagué et al., 2005). In the developing spinal cord, our results show that Smad3 overexpression upregulated the cyclin-dependent kinase inhibitor $p27^{kip1}$, resulting in cell-cycle exit and further contributing to neuronal differentiation. Induction of neuronal differentiation by activin signaling has also been reported in the developing spinal cord (Timmer et al., 2005), and our results suggest that this activity is mediated by Smad3. In addition, loss of endogenous Smad3 by in ovo RNA interference resulted in reduced expression of $p27^{kip1}$, thereby supporting a model in which Smad3 activity regulates $p27^{kip1}$ expression in the developing spinal cord and contributes to neurogenesis.

Interestingly, overexpression of Smad3 did not cause an overall change in the final number of postmitotic cells, because total numbers of $p27^{kip1+}$ cells remained constant between electroporated and non-electroporated sides of the spinal cord. This observation suggests that TGFβ/Smad3 activity does not induce an overall differentiation of neural tube progenitor cells but instead the preferential differentiation of certain neuronal subtypes at the expense of others.

In the ventral neural tube, Smad3 expression was excluded from pMN domain by cross-regulatory interactions between Smad3 and Shh-induced class II genes expressed in the pMN domain (Briscoe and Ericson, 2001). Our results indicate that exclusion of Smad3 from pMN was a pre-requisite for MN generation, because ectopic expression of Smad3 in the pMN domain led to a cell-autonomous dose-dependent suppression of the MN markers MNR2 and Isl1. The response of ventral progenitors to specific levels of Shh signaling is dependent on ambient TGFβ and BMPs (Liem et al., 2000). Our results further contribute to the molecular explanation for this antagonism.

In the ventral spinal cord, together with MNs, four neuronal subtypes (V3, V2, V1 and V0) of INs generate from individual progenitor domains. Our expression analysis showed high levels of Smad3 in all ventral progenitor domains; p3, p2-p0, from where ventral INs generate. Overexpression and loss-of-function experiments indicate that Smad3 activity is both necessary and sufficient for differentiation of ventral and various dorsal IN subtypes, at the expense of MN generation. These observations support a model in which Smad3 activity promotes differentiation of specific progenitor populations, particularly those in which Smad3 is highly expressed. As the Shh/Gli canonical pathway has recently been shown to contribute to the maintenance of spinal cord progenitors in a proliferative state (Cayuso et al., 2006), our results point to an additional level of Shh/TGFβ functional antagonism in spinal cord development.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/1/65/DC1

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