Direct action of the Nodal-related signal Cyclops in induction of sonic hedgehog in the ventral midline of the CNS

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SUMMARY

The secreted molecule Sonic hedgehog (Shh) is crucial for floor plate and ventral brain development in amniote embryos. In zebrafish, mutations in cyclops (cyc), a gene that encodes a distinct signal related to the TGFβ family member Nodal, result in neural tube defects similar to those of shh null mice. cyc mutant embryos display cyclopia and lack floor plate and ventral brain regions, suggesting a role for Cyc in specification of these structures. cyc mutants express shh in the notochord but lack expression of shh in the ventral brain. Here we show that Cyc signalling can act directly on shh expression in neural tissue. Modulation of the Cyc signalling pathway by constitutive activation or inhibition of Smad2 leads to altered shh expression in zebrafish embryos. Ectopic activation of the shh promoter occurs in response to expression of Cyc signal transducers in the chick neural tube. Furthermore, an enhancer of the shh gene, which controls ventral neural tube expression, is responsive to Cyc signal transducers. Our data imply that the Nodal related signal Cyc induces shh expression in the ventral neural tube. Based on the differential responsiveness of shh and other neural tube specific genes to Hedgehog and Cyc signalling, a two-step model for the establishment of the ventral midline of the CNS is proposed.

Key words: TGFβ, Smad2, FAST-1, FoxH1, Nodal, HNF3β, Floor plate, Chick electroporation, shh promoter

INTRODUCTION

The secreted signalling molecule sonic hedgehog (Shh) plays a crucial role in the specification of the floor plate and ventral brain identity. Shh is expressed in the organizer and subsequently in the axial mesoderm and ventral neural tube, regions shown by tissue recombination experiments in amniote embryos to secrete ventral neural tube inducing signals (Placzek et al., 1990; Yamada et al., 1991). Mouse embryos that carry null alleles of shh fail to form ventral neural tube structures and display cyclopia (Chiang et al., 1996). Similarly, floor plate differentiation is blocked in mice deficient in Gli2, a mediator of Shh signalling (Ding et al., 1998; Matise et al., 1998). Furthermore, recombinant Shh induces floor plate and ventral brain gene expression when administered to neural plate explants of amniote embryos in vitro (Roelink et al., 1994; Marti et al., 1995). Taken together, this body of evidence strongly suggests that shh is both necessary and sufficient for specification of the ventral neural tube. It has been suggested that Shh may control its own expression in the ventral neural tube. This autoregulatory loop was proposed to involve the winged helix transcription factor HNF3β, which is both an immediate target of Shh signalling (Ruiz i Altaba et al., 1995a; Sasaki et al., 1997) and a regulator of the shh gene (Ruiz i Altaba et al., 1995b; Chang et al., 1997). There is, however, little direct evidence supporting a role for Shh in regulating its own expression. Moreover, based on the analysis of enhancers of the mouse and zebrafish shh gene, it is apparent that HNF3β-independent mechanisms also contribute to controlling shh expression in the ventral neural tube (Epstein et al., 1999; Müller et al., 1999a).

Unlike shh-/- mouse embryos, zebrafish embryos lacking shh activity (sonic-you mutants), show only moderate defects in the ventral neural tube. sonic-you mutants lack lateral floor plate cells but form the ventralmost part of the neural tube, which is known as the floor plate proper or medial floor plate (Odenthal et al., 2000; Schauerte et al., 1998). Thus, shh appears to be less critically required for floor plate in zebrafish, although the activity of the related genes, tiggy-winkle hedgehog (Ekker et al., 1995) and echidna hedgehog (Currie and Ingham, 1996) are also expressed in the developing body axis and may partially compensate for the lack of shh activity in the mutant.

In contrast to mutations in shh, mutations in cyclops (cyc) and squint (sqt) result in a loss of floor plate, defects in the ventral forebrain and cyclopia (Hatta et al., 1991; Feldman et al., 1998). Very similar to the phenotype of shh-/- mouse embryos (Chiang et al., 1996). The cyc and sqt genes encode TGFβ-like molecules, which are very closely related to mouse Nodal (Feldman et al., 1998; Rebagliati et al., 1998a; Sampath et al., 1998). Although cyc-/- mutant embryos form a notochord that expresses shh mRNA, mutant embryos fail to
form a correctly differentiated ventral neural tube and 1-day-old embryos lack expression of genes normally expressed in the medial floor plate, including shh, twhh, netrin1 and axial/HNF3\(\beta\) (Krauss et al., 1993; Strähle et al., 1993, 1996, 1997a; Ekker et al., 1995). In addition, cyc/sqt double mutants fail to form mesendoderm (Feldman et al., 1998), a phenotype also observed in Nodal-deficient mouse embryos (Zhou et al., 1993; Conlon et al., 1994).

Although it has been demonstrated that Cyc activity is required for development of the ventral neural tube, it remains unclear whether Cyc regulates this process by acting directly on neuroectodermal cells or whether it controls the expression and/or processing of ventral neural tube inducing signals in the midline mesoderm. In particular, it is unknown how Cyc signalling interacts with or impinges upon the known function of Hedgehog proteins in specifying ventral neural tube identity. We therefore investigated the role of Cyc in neural tube patterning in the zebrafish by employing expression of dominant negative and constitutively active signal transducers of Cyc signalling in wild-type and mutant zebrafish embryos. We show that a constitutively active form of the Cyc signal transducer Smad2 can activate shh expression in the neural tube in a cell-autonomous fashion. In addition, an enhancer that was previously shown to mediate ventral neural tube expression of shh in zebrafish and mouse embryos was demonstrated to be responsive to Cyc signalling by electroporation in the chick neural tube. In contrast, the shh gene has been found to be much less responsive to Hh signals than other floor plate markers such as netrin1 or axial/HFN3\(\beta\), suggesting that autoinducing mechanisms play a less important role in the establishment of shh expression in the zebrafish neural tube. Taken together our experiments provide evidence for a crucial role for Cyc signalling in control of shh expression in the ventral neural tube.

**RESULTS**

Smad2 and FAST-1 act in the Cyc signalling pathway

To investigate the possible role of Cyc in patterning of ventral neural tube, we first tested whether putative downstream mediators of Cyc/Nodal signalling could be used as tools to mimic or interfere with Cyc signalling. TGF\(\beta\) signals are transduced intracellularly by Smad proteins (reviewed in Attisano and Wrana, 1998; Kretzschmar and Massague, 1998; Whitman, 1998). Indirect evidence in mouse and zebrafish indicated that Smad2 is a downstream, intracellular transducer of the Nodal subclass of TGF\(\beta\) signals (Gritsman et al., 1999; Nomura and Li, 1998). Consistent with this idea, injection of synthetic mRNA for an amino-terminal truncated, constitutively active form of smad2 (smad2\(^{CA}\)) into early cleavage-stage zebrafish embryos resulted in malformations in 1-day-old embryos (94.0%, n=310) in a manner reminiscent of those injected with cyc mRNA (Fig. 1A-C; Erter et al., 1998; Rebagliati et al., 1999b; Müller, 1999b). Most strikingly, smad2\(^{CA}\)-injected embryos lacked posterior structures. Furthermore, those embryos expressed shh ectopically (90.5%, n=74, Fig. 1E,F) similarly to Cyc-injected embryos (Kiecker et al., 2000).

Biochemical evidence suggests that Smad2-dependent gene regulation involves the DNA binding partner FAST-1, a forkhead/winged helix domain protein (Chen et al., 1997). Deletion of the DNA binding domain of FAST-1 generates a dominant inhibitor of Smad2 activity, (FAST-1\(^{SD}\), Chen et al., 1997; Watanabe and Whitman, 1999). As predicted, the phenotype of FAST-1\(^{SD}\) mRNA-injected embryos was very similar to that of compound mutants for cyc and sqt (85.0%, n=56; Fig. 1D), displaying deficiencies in mesendoderm formation, ventral neural tube patterning and profound cyclopia (Figs 1D, 2C). FAST-1\(^{SD}\) mRNA-injected embryos lacked shh expression (94.7%, n=38; Fig. 1G). Moreover, FAST-1\(^{SD}\) efficiently blocked the activity of coinjected Smad2\(^{CA}\) (Fig. 1H; 96.2%, n=56) or Cyc (88.0%, n=75; data not shown). Together, these data show that Smad2\(^{CA}\) and FAST-1\(^{SD}\) are efficient tools to manipulate the Cyc/Sqt signalling pathway, consistent with important roles for Smad2 and FAST-1 downstream of Cyc/Sqt signals in the zebrafish embryo.

**Modulation of the Cyc signal transduction pathway affects shh expression in the neural tube**

In addition to posterior truncations, Smad2\(^{CA}\)-injected embryos displayed malformations of the eyes characterised by an expansion of the pax-2.1 expression domain into the distal
Smad2CA and FAST-1SID mimic gain- and loss-of-function phenotypes of the Cyc/Sqt signals, respectively. (A) Uninjected control embryo. B,C: expression of constitutively active Smad2CA (B) causes malformations similar to the effects of cyc misexpression (C). Injected embryos lack posterior trunk and tail (arrows in B,C). (D) Injection of dominant negative FAST-1SID mimics the cyc/sqt double mutant phenotype (Feldman et al., 1998), characterised by severely disrupted dorsoventral patterning of the neural tube, lack of mesendoderm and a cyclopic eye (arrow). Arrowhead indicates the position of the otic vesicle. (A–D) Lateral views anterior to the left. (E–H) FAST-1SID blocks the expression in the midline. (F) Arrowhead points at an embryo with complete loss of shh expression. (G) Embryos injected with FAST-1SID. Arrowhead points at an embryo with no expression of shh in the midline. (H) Embryos coinjected with Smad2CA and FAST-1SID. Arrowhead points at an embryo with complete loss of shh expression.

(dorsal) parts of the eye (53.0%, n=45; Fig. 2A,B). This expansion of pax2.1 expression is reminiscent of the effects of misexpression of shh (Ekker et al., 1995; MacDonald et al., 1995). Moreover, a loss of the brain ventricles was also observed, again similar to the effects caused by ectopic expression of shh mRNA, suggesting that Smad2CA may affect eye development indirectly through activation of shh expression. Consistent with this interpretation, shh expression in the brain was increased in Smad2CA-injected embryos (Fig. 1F; compare Fig. 2A with B and F with G). In contrast, injection of mRNA encoding the structurally related but functionally distinct smad1 (Müller et al., 1999b) affected neither shh nor pax2.1 expression (data not shown and Kiecker et al., 2000). Finally, when Smad2 activity was blocked by expression of Fast-1SID, shh and pax2.1 expression were abolished in the ventral brain and eye, respectively, (Fig. 2C; 70.0%, n=30), reminiscent of the cyc mutant phenotype (Fig. 2D).

We next tested whether misexpression of smad2CA can rescue the cyc− phenotype in cycb16 embryos. Injection of smad2CA mRNA at the 16- to 32-cell stage resulted in a mosaic distribution of the expressed protein, and hence allowed the unambiguous identification of mutant embryos due to mosaic rescue. Smad2CA partially rescued both shh and pax2.1 expression in cycb16 mutant embryos (53.8%, n=26 embryos with a cyc mutant phenotype; Fig. 2E,H). Moreover, injection of Smad2CA into early embryos resulted in rescue of the mutant eye phenotype (only 14.4% injected embryos showed cyclopia (n=188), versus 25.9% in the uninjected control batch (n=266).

To address whether expression of Smad2CA affects shh in the neurectoderm directly, we investigated whether Smad2CA acts on shh expression in a cell-autonomous manner. For this purpose, a Myc-epitope tagged version of smad2CA was expressed from a plasmid (CMV::smad2CA), resulting in a highly mosaic distribution of expression in order to allow analysis at a single cell resolution (Müller et al., 1999a). In the neurectoderm of early somite-stage cyc mutant and wild-type embryos injected with CMV::smad2CA, ectopic expression of shh was only detected in neural cells, which also expressed the myc epitope (Fig. 2I, 79.5% ectopic shh+ cells were also myc+; n=39). A similar result was obtained in the neural tube of 24 hour wild-type injected embryos (Fig. 2J). Such a high frequency of colocalisation suggests that shh can be activated cell-autonomously by Smad2CA, and is inconsistent with an indirect action of Smad2CA via induction of a second signal. Taken together, these results imply that the Cyc pathway can regulate expression of shh in neural epithelial cells.

We have also addressed whether smad2CA is able to induce other ventral neural marker genes. Both axial/HNF3β (82.2%, n=37) and netrin1 (78.9%, n=38) are expressed ectopically in the neural tube of smad2CA-injected embryos (Fig. 2; compare K with L and M with N, respectively). Expression of either netrin1 (64.8%, n=37; Fig. 6B) and axial/HNF3β (data not shown) was abolished or significantly reduced in the neural tube of FAST-1SID expressing embryos, similar to Shh expression.

The zebrafish shh promoter is activated by Smad2CA in the chick neural tube

We next asked whether previously identified shh promoter/enhancer regions, which drive expression in the ventral neural tube, could be activated by Smad2 and FAST-1. We utilised the 11+12→−2.2shh::lacZ construct that harbours the intronic enhancer region in front of the −2.2 kb shh promoter. This construct drives lacZ expression in the midline of fish and mouse embryos (Müller et al., 1999a), suggesting conservation of the underlying regulatory mechanisms. We employed electroporation into the chick neural tube as this technique
directly targets expression into neuroepithelial cells in situ (reviewed in Itasaki et al., 1999) and leaves other tissues such as notochord unaffected, as demonstrated by the pattern of expression of Green Fluorescent Protein (GFP) from coelectroporated CMV::GFP in stage-19 embryos (Fig. 3A,B).

The I1+I2/-2.2shh::lacZ construct was electroporated into the folding neural plate in stage 10-12 chicken embryos, either alone or together with plasmids encoding regulators or empty plasmid vector as controls. Floor plate-restricted expression of lacZ was observed in the chick embryos electroporated with I1+I2/-2.2shh::lacZ (10/10 embryos, Fig. 3C,D). Coelectroporation of CMV::cyc and I1+I2/-2.2shh::lacZ resulted in the ectopic activation of lacZ in cells of the lateral neural tube (8/10 embryos; Fig. 3E). Similarly, ectopic activation of lacZ was noted when CMV::smad2CA and I1+I2/-2.2shh::lacZ were coelectroporated (8/10 embryos, Fig. 3F). Ectopic activation of endogenous shh expression was also detected by in situ hybridisation of Smad2CA expressing embryos, albeit at a lower frequency (3/15 embryos, data not shown). As a control, no ectopic lacZ expressing cells were detected when CMV::smad1 was electroporated together with the I1+I2/-2.2shh::lacZ reporter construct, although floor plate-specific expression was maintained (n=7 embryos; data not shown).

Dorsal expansion and an increase in the number of lacZ expressing cells were observed in the lateral neural tube when embryos were electroporated with I1+I2/-2.2shh::lacZ, CMV::smad2CA and CMV::FAST-1 (8/13 embryos, Fig. 3G,H).

Taken together these results suggest that Cyc, and its downstream transducers Smad2 and FAST-1, are regulators of shh expression in neuroepithelial cells. The shh gene harbours at least one Cyc responsive enhancer

The regulatory architecture of the shh gene is complex. As previously shown, multiple enhancer regions contribute to the
Cyc signalling induces shh in the neural tube

control of shh expression in the body axis. To locate the region responsible for activation by FAST-1/Smad2CA, deletion constructs were tested by electroporation into the chick neural tube. The −2.2shh::lacZ reporter, which contains 2430 bp upstream of the transcription start site but lacks the intronic enhancer regions, was shown to drive expression in the ventral neural tube of zebrafish embryos (Müller et al., 1999a). When the −2.2shh::lacZ construct was electroporated alone into the chick neural tube (Fig. 4C) lacZ expression was confined to the floor plate (5/6 embryos), as in the case of the full-length I1+I2/−2.2shh::lacZ construct (Fig. 4A). Also, expression from the −2.2shh::lacZ construct could be activated ectopically by coexpression of FAST-1/Smad2CA (6/6 embryos, Fig. 4D). In contrast, expression from construct −563shh::lacZ, which contains 563 bp upstream of the transcription start site of the shh promoter (Chang et al., 1997), failed to drive expression.

Fig. 3. Regulatory regions in the I1+I2/−2.2shh::lacZ reporter construct drive expression in the floor plate and respond to Cyc signals upon electroporation into the chicken neural tube. (A,B) Bright field (A) and fluorescent image (B) of a cross section of a chick neural tube anterior to the hindlimb bud at stage 19. The neural tube was transfected by injection with a CMV::GFP construct into the groove of the folding posterior neural plate at stage 10-12 and electroporation in situ. GFP expression is evident throughout the right half of the neural tube, but never detected in the notochord (depicted with red dots). (C,D) β-gal activity is restricted to the floor plate (arrows) in neural tubes coelectroporated with I1+I2/−2.2shh::lacZ and CMV::GFP. (E-H) Electroporation of CMV::cyc (E), CMV::smad2CA (F), or CMV::smad2CA with CMV::FAST-1 (G,H) causes ectopic activation of I1+I2/−2.2shh::lacZ (arrowheads in E-H). Arrows in E,F and H indicate lacZ expression in the floor plate. A-C and E-G are cross sections, dorsal up. (D,H) Lateral views of the spinal cord between the limb buds. (C-H) Expression vectors coelectroporated with I1+I2/−2.2shh::lacZ are indicated in the top right corner of panels. fp, floor plate; n, notochord.

Fig. 4. The upstream enhancer region of the shh gene is responsive to Cyc signalling. (A,B) Neural tubes electroporated with I1+I2/−2.2shh::lacZ alone (A) or together with CMV::smad2CA and CMV::FAST-1 (B). (C,D) Neural tubes electroporated with −2.2shh::lacZ alone (C) or together with CMV::smad2CA and CMV::FAST-1 (D). The 2430 bp shh upstream sequence in −2.2shh::lacZ drives floor plate-specific expression (C, arrow) and can be activated ectopically by coexpression of CMV::smad2CA and CMV::FAST-1 (D, arrowhead) similarly to I1+I2/−2.2shh::lacZ (B, arrowhead). (E,F) The −563shh::lacZ construct containing the 563 bp proximal promoter region does not mediate floor plate specific expression (E), and can not be activated by coexpressed CMV::smad2CA and CMV::FAST-1. Weak expression of lacZ is seen in few scattered cells in the electroporated neural tube (arrowheads in E,F). DNA constructs containing different genomic fragments of the shh locus are indicated schematically on the left. (A-H) Lateral views onto the trunk between the limb buds of stage 18-19 chick embryos. Anterior to the left. White dots indicate the dorsal border of the floor plate.
of lacZ in the floor plate (Fig. 4E). Instead, weak expression was detected in very few scattered cells throughout the neural tube (7/6 embryos), consistent with previous findings that the −563shh promoter region is not sufficient to drive floor plate specific expression (Müller et al., 1999a). Moreover, the −563shh::lacZ construct was not responsive to coexpressed FAST-1/Smad2CA (7/8 embryos, compare Fig. 4F with E). It was upregulated in response to coexpressed axial/HNF3β (data not shown), consistent with the presence of multiple HNF3β binding sites in the promoter (Chang et al., 1997). The finding that −2.2shh::lacZ but not −563shh::lacZ was responsive to Smad2/FAST-1 suggests that the upstream region between −2430 and −563 contains elements that are required for activation by FAST-1/Smad2.

**shh expression cannot be activated by Hh signalling as efficiently as other floor plate marker genes**

The direct effect of Cyc signalling on shh expression in the neural tube prompted us to re-examine the role of Hedgehogs in this process. We utilised expression of a dominant negative regulatory subunit of protein kinase A (PKI), which mimics Hh signalling (Conordet et al., 1996; Hammerschmidt et al., 1996) and compared the responsiveness of shh expression with that of other floor plate markers such as netrin1 and axial/HNF3β. To minimise variation, PKI mRNA was injected into a large batch of zebrafish embryos, which was then split for staining with the different probes. Ectopic activation of shh by PKI was restricted to an expansion of shh expression in the zona limitans of the diencephalon (56.3%, n=126; Fig. 5A,B). Similarly, a restricted activation was observed for the shh homolog twhh in the diencephalon and the posterior mesencephalon (67.9%, n=112; Fig. 5C,D). In contrast, expression of PKI elicited strong ectopic activation of the floor plate marker genes netrin1 (88.7%, n=71) and axial/HNF3β (83.9%, n=93; Fig. 5E,F and G,H, respectively), beginning from the diencephalon and extending into the rest of the brain overlying the floor plate. Thus, shh and twhh appear to be less responsive to Hedgehog signalling than netrin1 and axial/HNF3β. This suggests that the regulatory mechanisms underlying the expression of shh/twhh in the floor plate differ from those controlling netrin1 and axial/HNF3β.

cyc mutants may lack expression of ventral neural tube markers such as netrin1 because they fail to establish shh (and twhh) expression in the neurectoderm. If this epistatic relationship were true, one would predict that netrin1 or the ventral eye marker pax2.1 can be induced by forced activation of Hh signalling when Cyc/Sqt signalling is blocked. To this end Cyc/Sqt signalling was inhibited by expression of FAST-1SD, while the Hh pathway was simultaneously activated by coexpression of PKI. In the resulting double-injected zebrafish embryos, netrin1 (Fig. 6A-C) was broadly activated ectopically (79.6% n=37) while no netrin1 expression or only traces in the midbrain were observed in FAST-1SD-injected embryos (80.6% n=31). axial/HNF3β was activated by PKI in a similar manner in embryos with blocked Cyc/Sqt signalling (data not shown). pax2.1 expression in the eye was strongly induced (85.7%, n=49; Fig. 6D-F) while shh expression was only rescued in the area of the zona limitans of FAST-1SD-expressing embryos (77.1% of embryos showed complete lack of shh expression or were restricted to the zona limitans, n=35). These results show that netrin1, axial/HNF3β and pax2.1 can be activated by Hh signalling in the absence of Cyc signalling. These results are, furthermore, consistent with the notion that cyc mutants lack expression of ventral neural tube marker genes because they failed to establish shh (twhh) expression in the CNS.

**DISCUSSION**

We provide evidence that Smad2 and FAST-1 are regulators of the shh gene. Our data furthermore suggest that Smad2 and FAST-1 or closely related molecules are transducers of the Nodal-like signals Cyc and Sqt, consistent with previous findings in both mouse and zebrafish embryos (Gritsan et al., 1999; Nomura and Li, 1998). It has been shown that cyc mutants lack shh expression in the neural tube but express shh mRNA in the notochord (Krauss et al., 1993). Our results support a direct role for Cyc signalling in the regulation of shh expression in the neural tube. In particular, we have shown that Smad2CA activates shh in a cell-autonomous fashion in the zebrafish embryo. In addition, the upstream shh promoter/
Fig. 6. PKI can activate netrin1 and pax2.1 in the absence of Cyc signalling. (A-D) Control uninjected embryos. (B,E) Embryos injected with FAST-1 SID mRNA. (C,F) Embryos double-injected with FAST-1 SID and PKI mRNA. Embryos were stained with either netrin1 probe (A-C) or with a combination of shh (blue/black) and pax2.1 (red) probes (D-F). Expression of netrin1 and shh in the ventral neural tube and pax2.1 in the eye is lost in embryos injected with FAST-1 SID (B,E). Traces of netrin1 expression and unaffected pax2.1 activity are detected in the more dorsal domain in the midbrain (arrowhead in D) and in the midbrain-hindbrain boundary (mhb) respectively. (C,F) Activation of netrin1 is evident throughout the brain of embryos coinjected with FAST-1 SID and PKI (arrowheads in C) while shh expression was restricted to the zona limitans region (arrowhead in F). pax2.1 expression in the anterior brain is strongly activated (arrow in F). All panels are lateral views of the head of 24 hour embryos. fp, floor plate; mhb, midbrain/hindbrain boundary; zl, zona limitans.

Fig. 7. A model of the function of Nodal and Shh signalling for activation of ventral neurectoderm markers during floor plate induction. Note that the model depicts developmental pathways; arrows may therefore not reflect single steps or direct molecular interactions.
–563shh promoter, which contains functional HNF3β sites. Furthermore, the –563shh promoter is not able to drive floor plate expression (Müller et al., 1999a; this study), suggesting that the HNF3β binding sites in the promoter region are not sufficient.

Bone morphogenetic proteins (BMP) inhibit development of ventral neural character in explant cultures (Liem et al., 1995). Furthermore, mouse embryos that lack the BMP inhibitor Noggin fail to develop a floor plate, suggesting that the activity of BMPs has to be blocked for floor plate, differentiation to occur (McMahon et al., 1998). It was previously noted that Smad2 and the BMP transducer Smad1 can compete for the common partner Smad4 in Xenopus embryos (Candia et al., 1997). Although we regard this competition as unlikely to be the main mechanism, Cyc signalling could nevertheless contribute to inhibition of BMP signalling via activation of Smad2 and subsequent sequestering of Smad4.

Transplantation and ablation experiments showed that the notochord has floor plate inducing activity, leading to the proposal that signals from the notochord induce floor plate (Placzek et al., 1990). Results of cell labelling experiments carried out in chick embryos, together with the expression patterns of floor plate and notochord-specific genes, suggested, however, that floor plate differentiation may be well under way before cells have taken up their final position in notochord and floor plate (Catala et al., 1996). Arguments based on marker gene expression in the zebrafish led to similar conclusions (Le Douarin and Halpern, 2000). Our results, which support a direct role for Cyc signalling in inducing expression of shh in the CNS and the early expression of cyc in the zebrafish shield and tail bud (Rebagliati et al., 1998a,b), argue in favour of an early specification of floor plate identity before the notochord has formed.

Shh expressed from the mature notochord is apparently not sufficient to induce its own expression in the zebrafish embryo as cyc mutants strongly express shh in the notochord (Krauss et al., 1993; Strähle et al., 1997b; Odenthal et al., 2000). This raises the question of the function of Hh signalling in this process in the zebrafish. When we overexpressed the Hh signal transducer PKI in the zebrafish embryo, we observed an expansion of shh expression, although this was limited to the zona limitans. This suggests that shh can induce its own expression, to some extent. Other ventral genes such as netrin1 or pax2.1 were, however, much more strongly activated by the same concentration of the mimic of Hh activity, PKI. One possibility is that only very high concentrations of Shh signalling may be able to efficiently auto-induce shh expression, a situation that we may not be able to achieve by injection of PKI mRNA. The presence of multiple hh related genes has so far precluded an analysis of Hh function in medial floor plate induction in the zebrafish (Currie and Ingham, 1996; Ekker et al., 1995; Krauss et al., 1993; Schauerte et al., 1998).

Irrespective of the role of Hh signals, the cyc mutant phenotype, together with our results, points to a prominent role for the Nodal related signal Cyc in the induction of shh expression in the zebrafish neural tube.

The mouse knock-out of shh is, due to its design, not informative as to whether shh is required to induce its own expression (Chiang et al., 1996). This raises the question whether Nodal or Nodal-related factors may also play a role in regulating shh expression in amniotes. Mouse embryos lacking Nodal activity are so severely malformed (Zhou et al., 1993; Conlon et al., 1994) that they do not allow conclusions to be made regarding whether Nodal has a function in the ventral neural tube. Several lines of indirect evidence argue, however, in favour of an involvement of Nodal signalling. Firstly, transgenes harbouring the control regions of the zebrafish shh gene drive reporter gene expression in the ventral neural tube of mouse embryos (Müller et al., 1999a). Secondly, mutations that affect the Nodal signal pathway in the mouse also cause defects in the ventral CNS. smad2+/−, nodal+/− trans-heterozygote mouse embryos show defects similar to shh mutant mice (Nomura and Li, 1998). Moreover, loss of shh expression occurs in the anterior brain in mouse embryos lacking smad2 gene function, suggesting that a similar regulatory relationship exists between Smad2 and shh in the mouse (Heyer et al., 1999). We thus propose that the induction of shh expression by Nodal signals is fundamental to ventral neural tube development in higher vertebrates.

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