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# Lack of Motor Neuron Differentiation is an Intrinsic Property of the Mouse Secondary Neural Tube

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## Abstract

The cranial part of the amniote neural tube is formed by folding and fusion of the ectoderm-derived neural plate (primary neurulation). After posterior neuropore closure, however, the caudal neural tube is formed by cavitation of tail bud mesenchyme (secondary neurulation). In mouse embryos, the secondary neural tube expresses several genes important in early patterning and induction, in restricted domains similar to the primary neural tube, yet it does not undergo neuronal differentiation, but subsequently degenerates. Although the secondary neural tube, isolated from surrounding tissues, is responsive to exogenous Sonic Hedgehog proteins in vitro, motor neuron differentiation is never observed. This cannot be attributed to the properties of the secondary notochord, since it is able to induce motor neuron differentiation in naïve chick neural plate explants. Taken together, these results support that the lack of motor neuron differentiation is an intrinsic property of the mouse secondary neural tube.

### Keywords

Shh; tail bud; secondary neurulation; neuronal differentiation; neural tube patterning; mouse embryo

## Introduction

The development of the body of vertebrate embryos occurs in two distinct and separate phases. The initial phase is called “primary body development” (Holmdahl, 1925), and is characterized by gastrulation. During gastrulation, cells of the epiblast ingress through the primitive streak to establish the three definitive germ layers: ectoderm, mesoderm and endoderm, from which all tissues in the anterior and greater portion of the body are derived (Lawson et al., 1991). This is followed by the next phase called “secondary body development” (Holmdahl, 1925), in which the remnants of the node and the primitive streak consolidate into a spherical mass of mesenchymal cells named the tail bud. This structure

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subsequently lays down the secondary neural tube, notochord, and gut endoderm, surrounded by mesenchymal cells. The tail bud is a single tissue from which all three germ layers are derived (Griffith et al., 1992). In mammals, tails are the sole adult structures derived from the tail bud. It is intriguing why the formation of the tail occurs via such apparently distinct mechanisms, and raises the questions how tissues derived from the tail bud form and whether they serve the same function as those derived from the three germ layers. In this study, we have focused on secondary neurulation in the mouse embryo and examine the potency of the secondary notochord to induce patterning and the competency of the secondary neural tube to form the cell types normally present in the primary neural tube.

In mice, primary neurulation begins with the induction of the neural plate from the ectoderm at the dorsal aspect of the embryo. The margins of the neural plate then elevate until they appose in the midline and fuse together to form the neural tube (reviewed by Copp et al., 1990; Schoenwolf and Smith, 1990). Primary neurulation finishes with the closure of the posterior neuropore, just posterior to the future hindlimbs. Secondary neurulation begins with a group of cells clustering at the dorsal midline forming the tail bud. These cells undergo mesenchymal to epithelial transformation and orientate radially around a small lumen. The lumen gradually enlarges as more cells are recruited to form the wall of the secondary neural tube (Schoenwolf, 1984). The lumina of the primary and secondary neural tube join end-to-end (Shum and Copp, 1996).

Although development of the nervous system is a major area of research, so far, studies have been mainly focused on the primary neural tube. In contrast, the molecular control and subsequent development of the mouse secondary neural tube remains largely unknown. Here we show that, while the secondary neural tube expresses several genes known to have critical functions in neural tube development in restricted domains similar to those in the primary neural tube, it fails to develop a functional floor plate and lacks any neuronal differentiation. This is unlikely to be the result of absence of signaling from the surface ectoderm and the secondary notochord because both *Bone Morphogenetic Protein 4* (*Bmp4*) *a*nd *Sonic Hedgehog* (*Shh*) are expressed appropriately, and the secondary notochord is competent to induce motor neurons in naïve neural plate explants in vitro. Furthermore, isolating the secondary neural tube from surrounding tissues and culturing it in the presence of exogenous Shh is nevertheless insufficient to induce motor neuron differentiation. Taken together, our findings support the notion that the lack of motor neuron differentiation is an intrinsic property of the mouse secondary neural tube. It is concluded that the neural tube formed by the tail bud does not follow the same developmental pathway as the primary neural tube.

## Results

### Expression of Inducers of Neural Tube Patterning is Retained in and around the Secondary Neural Tube

In the mouse embryo, complete closure of the posterior neuropore occurs at the 29-30 somite-stage, and this event marks the beginning of secondary neurulation (Shum and Copp, 1996). Taking into account that another 6-7 somites will form from the unsegmented paraxial mesoderm (Tam, 1986), the level of transition from primary to secondary

neurulation occurs around somite-level 36. In the following analysis we compared the expression of genes both anterior (primary neural tube) and posterior (secondary neural tube) to somite 36.

Dorsoventral patterning of the primary neural tube is mediated by a limited number of inducers (Ulloa and Briscoe, 2007). BMPs and Wnts are the principal molecules inducing dorsal cell types, while Shh is involved in the induction of ventral cell types. To assess if the secondary neural tube is exposed to a similar environment of inducers as the primary neural tube, we determined the expression pattern of *Bmp4, Wnt1, Wnt3a* and *Shh*. Several roles in early neural tube development have been attributed to activities mediated by *Wnt1* and *Wnt3a*. For instance, in *Wnt1/Wnt3a* double knock out mice, there is a significant reduction in neural crest derivatives (Ikeya et al., 1997). Wnts have further been implicated in the growth of the neural tube (Ikeya and Takada, 1998). In the primary neural tube, *Wnt1* and *Wnt3a* expression patterns are largely overlapping in the roof plate (Fig. 1A,C). Although histologically, the dorsal midline of the secondary neural tube does not resemble a roof plate, *Wnt1* and *Wnt3a* are expressed at this site (Fig. 1B,D).

Many of the dorsal neurons in the primary neural tube are induced by BMP-mediated signals derived from the dorsal ectoderm and the roof plate (Fig. 1E) (Liem et al., 1995). In the secondary neural tube, *Bmp4* is expressed at a low level in the dorsal midline region. In contrast, strong *Bmp4* expression is detected in the dorsal ectoderm and the mesenchyme underlying it and surrounding the secondary neural tube (Fig. 1F).

Differentiation of ventral cell types in the primary neural tube is regulated by *Shh*, which is expressed in the notochord and floor plate (Fig. 1G). Shh-mediated signaling is sufficient and required (Roelink et al., 1995; Chiang et al., 1996) for the induction of ventral cell types, including the floor plate and motor neurons. *Shh* is strongly expressed in the secondary notochord, but is not expressed in the ventral midline of the secondary neural tube (Fig. 1H). Since in the primary neural tube the expression of *Shh* in the floor plate is induced by Shh released from the notochord (Roelink et al., 1994), the absence of *Shh* expression in the secondary neural tube might indicate that Shh is unable to induce floor plate tissue. In tissues, Shh has a very limited diffusion ability (Lewis et al., 2001; Etheridge et al., 2010) and its inductive effects are concentration-dependent (Roelink et al., 1995). A possible explanation for the lack of *Shh* expression in the secondary neural tube might be the relatively large distance between the secondary neural tube and the secondary notochord resulting in failure of floor plate differentiation (Fig. 1H). To further investigate this possibility, we examined the expression of *FoxA2*, which is both expressed in the floor plate and required for floor plate development (Sasaki and Hogan, 1994; Weinstein et al., 1994).

In the secondary neural tube, *FoxA2* is expressed in the ventral midline as in the primary neural tube (Fig. 1I,J). Since the induction of *FoxA2* in the primary neural tube requires Shh activity (Chiang et al., 1996), it would appear that the secondary neural tube can respond to Shh, and Shh levels are sufficient to induce this gene. However, in the primary neural tube, Shh expression in the notochord precedes *FoxA2* expression in the floor plate, consistent with its induction by notochord-derived Shh. In contrast, *FoxA2* is expressed in the secondary neural tube as soon as it is formed. The simultaneous initiation of *Shh* expression

in the notochord and *FoxA2* in the secondary neural tube indicates that *FoxA2* expression is not induced by Shh, although we demonstrate below that the secondary neural tube is Shh responsive.

Further indication that cells in the ventral midline of the secondary neural tube do not differentiate into a functional floor plate is demonstrated by the absence of *Netrin1*, which is normally expressed in the floor plate of the primary neural tube (Serafini et al., 1994; Serafini et al., 1996). Nevertheless, the lower level of expression in the lateral region of the neural tube is present in both (Fig. 1K,L).

We conclude that the overall inductive environment of the secondary neural tube is not fundamentally different from the primary neural tube. However, the absence of a functional floor plate indicates that the secondary neural tube has differentiated along pathways distinct from the primary neural tube.

## Initial Neural Dorsoventral Differentiation is Conserved in the Secondary Neural Tube, but It Lacks Subsequent Neuronal Differentiation

We have assessed the expression of *Pax3* and *Pax6* because these genes are expressed in restricted domains during early patterning of the primary neural tube. *Pax3* is expressed in the dorsal and *Pax6* in the lateral neural tube. The localized expression of these genes is important in the subsequent steps in neural differentiation (Goulding et al., 1991; Walther and Gruss, 1991; Ericson et al., 1997; Mansouri and Gruss, 1998).

The expression domains of *Pax3* (Fig. 2A) and *Pax6* (not shown) change abruptly at somite-level 36, coincident with the border between the primary and secondary neural tube. Anterior to this level, the expression domain is broad; while caudal to this level, the expression domain is significantly smaller. The border between the primary and secondary neural tube is easily recognized as an apparent bulge (Fig. 2B), which becomes more prominent as development proceeds (Fig. 2C). The increasing size difference shows that the secondary neural tube fails to undergo growth proportional to the primary neural tube. Nevertheless, despite this difference in size, the dorsoventral expression patterns of *Pax3* and *Pax6* are conserved between the primary (Fig. 2D,F) and the secondary neural tube (Fig. 2E,G). *Pax3* is restricted to the dorsal half of the neural tube (Fig. 2D,E), while *Pax6* is expressed in mid-lateral regions (Fig. 2F,G). In the primary neural tube, the expression of Pax3 and Pax6 is inhibited by Shh (Ericson et al., 1997), suggesting that Shh plays a similar role in the initial patterning events of the secondary neural tube.

To follow the subsequent development of the secondary neural tube, we carried out histological examination of sections taken from the same axial level of embryos from E10.5 to E18.5. Immediately after its formation, the secondary neural tube is similar to the primary neural tube (Fig. 3A,B). It is composed of an actively proliferating neuroepithelium, and dividing cells are indicated by arrowheads in Fig. 3B. However, while the primary neural tube continues to develop and grow, the secondary neural tube stops growing within one day of its formation, and cells begin to die, as indicated by the presence of pyknotic nuclei (Fig. 3C green arrowheads). In subsequent days, the secondary neural tube regresses further, losing its epithelial character as indicated by the layering of the cell nuclei (Fig. 3E, green

arrow). In addition, a distinct bundle-like structure is present at the ventral side of the secondary neural tube at this stage (Fig. 3D, yellow arrow). A similar structure is not present ventral to the primary neural tube, and we will refer to this structure as the ventral bundle. By E18.5, the secondary neural tube is reduced greatly in size and is surrounded by mesenchymal-like cells (Fig. 3E). The ventral bundle appears to be a transient structure and is no longer detected at this stage of development.

Early neuronal differentiation in the primary neural tube includes the formation of motor neurons, which can be identified using an antibody against Isl1/2 (Fig. 3F). Isl1/2 is also expressed in sensory neurons in the dorsal root ganglia (Fig. 3F). In contrast, the secondary neural tube and surrounding cells are devoid of Isl1/2 expressing cells (Fig. 3G), nor could we detect *Isl1* mRNA. Expression of *Isl1* in the dorsal root ganglia terminates at the transition between primary and secondary neural tube (Fig. 3H), indicating that there are no dorsal root ganglia caudal to this level. Hence, the secondary neural tube neither differentiates into motor neurons, nor does the neural crest form dorsal root ganglia. In addition, we found that the secondary neural tube lacks *En1* transcripts, indicating the absence of circumferential ascending (CiA) interneuron differentiation (Fig. 3I,J) (Ridgeway et al., 2000). In further in situ hybridization studies, we sought expression of several other markers of neuronal differentiation. While we could detect *Nkx6.1* transcripts in the secondary neural tube, we did not observe expression of *Nkx2.2*, *HB9*, *Ngn1*, *Ngn2*, *LH2A*, *Bmp2*, *Math1*, *Dbx1*, *Olig1* and *Olig2* (data not shown). This further indicates that neurons do not form in the secondary neural tube.

To further assess if any neuronal differentiation could be detected in the secondary neural tube, we examined the expression of neurofilament. While staining was detected in the cell bodies and nerve fibers of neurons in the primary neural tube (Fig. 3K), staining was absent in all cells located in the secondary neural tube (Fig. 3L). Importantly, at later developmental stages, the bundle ventral to the secondary neural tube contains neurofilament (Fig. 3M,N), showing that the ventral bundle consists of nerve fibers. In addition, two paired nerve bundles running parallel to the notochord could be detected (Fig. 3M). However neither of these nerve bundles were associated with cells located inside the secondary neural tube.

In the complete absence of neuronal differentiation in the secondary neural tube, the question arose where the nerve fibers in the ventral bundle originate. To determine if these fibers extended from neurons located in the primary neural tube, a DiI crystal was inserted into the spinal cord at the hindlimb level (Fig. 4 A,B). After three months incubation, the dye had diffused along the nerve fibers (Fig. 4C). Examination of serial transverse sections demonstrated significant changes in labeling pattern along the body axis. Just caudal to the site of DiI insertion (L1), the developing spinal cord was heavily labeled (Fig. 4D). Further caudally (L2), the longitudinal fibers in the dorsal region of the marginal layer of the spinal cord were heavily labeled, while labeling of fibers in the ventral region of the marginal layer was weaker (Fig. 4E). Outside the spinal cord, the dorsal root ganglia, and dorsal and ventral spinal nerves were labeled. In the secondary body (L3), the ventral bundle was labeled, while staining was absent from the secondary neural tube (Fig. 4F). This demonstrates that the nerve fibers running in the ventral bundle of the secondary neural tube originate from cell bodies located in the primary neural tube. These results further demonstrate that the

secondary neural tube not only does not contain neurons, but it also does not appear to serve as an axonal conduit. However, given the close association of the ventral bundle with the secondary neural tube, it remains a possibility that guidance factors are released from the secondary neural tube.

## Secondary Notochord is Able to Induce Neuronal Differentiation in Primary Neural Plate Explants

The complete absence of neuronal differentiation in the secondary neural tube raises the question if this is caused by a lack of competence of this structure to form neurons, or if the environment is unsuitable for neuronal induction. Signals released from the primary notochord are able to induce motor neurons in neural plate explants (Yamada et al., 1993). To determine whether the secondary notochord has the same inductive ability, we cultured naïve chick neural plate explants in contact with secondary notochord tissue. Because of the small size and fragility of the mouse secondary notochord, rat embryonic tissue was used to isolate this structure. We were unable to successfully culture the rat secondary notochord in isolation, but leaving the secondary neural tube attached allowed survival of the secondary notochord. Explants were cultured for 36 hours and examined for the expression of Isl1/2. The rat secondary neural tube attached to the notochord contained no Isl1/2 positive cells (not shown). However, chick primary neural plate explants cultured in association with secondary notochord/neural tube tissue contained many Isl1/2 positive cells (Fig. 5A), but not the co-cultured rat secondary neural tube. Chick neural plate explants co-cultured with the secondary neural tube alone contained no Isl1/2 positive cells (Fig. 5B). This demonstrates that signals released by the secondary notochord are sufficient to induce motor neurons in primary neural plate tissue.

## Cells of the Secondary Neural Tube are Responsive to Shh

In the primary neural tube, Shh can repress the expression of dorsal genes, such as *Pax7* and *Msx1/2*, antagonizing the effect of BMP-mediated signals from the epidermal ectoderm (Liem et al., 1995; Ericson et al., 1996; Liem et al., 2000). To determine whether the secondary neural tube can respond to Shh, in the absence of possible antagonistic effects from surrounding tissues, we cultured isolated secondary neural tubes in the presence of a soluble form of Shh (ShhN) (Roelink et al., 1995). In the absence of ShhN, FoxA2 was expressed in the isolated secondary neural tube in a stripe of cells running the length of the explant, most likely representing the ventral midline (Fig. 5C). In the presence of ShhN, the number of FoxA2 positive cells increased dramatically so that, in many cases, the majority of cells in the explant were FoxA2 positive (Fig. 5D). Conversely, the expression of Pax7 (Fig. 5E) and Msx1/2 (Fig. 5G) was repressed when the secondary neural tube explants were cultured in the presence of ShhN (Fig. 5F,H). Thus, cells in the secondary neural tube exhibit some responses to ShhN similar to those in the primary neural tube. Nevertheless, even though the ShhN concentration in the cultures was sufficient to induce FoxA2 expression, and should therefore be in excess of the concentration needed to induce motor neurons (Roelink et al., 1995), we never observed any Isl1/2 positive cells in the secondary neural tube explants (Fig. 5I,J). These findings support the idea that the inability to form motor neurons is an autonomous characteristic of the mouse secondary neural tube, rather than an effect of extra-neural inhibitory signals.

### The Secondary Neural Tube and Notochord as Signaling Centers for Surrounding Mesoderm

The primary neural tube and notochord are important sources of signals required for normal development of the surrounding mesoderm (Pourquié et al., 1993). In the secondary body, *Pax3*, a marker for dermomyotome in the primary body (Fig. 6A) (Goulding et al., 1994), is expressed in the secondary mesoderm immediately beneath the entire surface ectoderm, except at the dorsal and ventral midline regions (Fig. 6B). *Pax1*, which is expressed in the sclerotome in the primary body (Fig. 6C) (Wallin et al., 1994), and induced by Shh (Marcelle et al., 1999; Rodrigo et al., 2003) is expressed in two large domains on either side of the secondary notochord, and is not expressed in a domain lateral to the secondary neural tube (Fig. 6D). This pattern is highly suggestive that also in the forming tail, *Pax1* expression is induced by notochord-derived Shh. As development proceeds in the primary body, sclerotomal cells form the vertebral body surrounding the notochord, and the neural arches and spinous process that completely encase the spinal cord (Fig. 6E). In contrast, the secondary neural tube is surrounded by bundles of muscle and tendons. Neural arches and spinous processes are absent although there is a large vertebral body organized around the secondary notochord (Fig. 6F). Together these expression patterns indicate that several aspects of primary notochord signaling are conserved in the secondary body, in particular the osteogenesis around the notochord.

## Discussion

### Lack of Floor Plate and Neuronal Differentiation in the Secondary Neural Tube

In this study, we find that the secondary neural tube fails to undergo neuronal and floor plate differentiation, despite appropriate expression of *Pax3* and *Pax6*, two early dorsoventrally restricted genes that are essential for regulating the differentiation of various types of neurons (Mansouri et al., 1994; Osumi et al., 1997), and *FoxA2*, which regulates early floor plate development (Sasaki and Hogan, 1994; Ruiz i Altaba et al., 1995).

Shh is one of the critical factors for patterning and differentiation of the primary neural tube (Echelard et al., 1993; Roelink et al., 1994). The effects of Shh on neural plate cells have both early and later components (Ericson et al., 1996). The early response consists of ventralization by *Pax3* and *Pax7* repression and *FoxA2* induction in the ventral midline (Goulding et al., 1993; Ericson et al., 1996), whereas the later response includes the induction of neuron-specific gene expression like *Isl1* (Ericson et al., 1996), and floor plate-specific gene expression like *Shh* itself and *Netrin1* (Echelard et al., 1993; Kennedy et al., 1994; Roelink et al., 1994; Hynes et al., 2000). Interestingly, we find that such early responses to Shh are retained in the secondary neural tube whereas the late responses are not. These observations in conjunction with our findings that secondary neural tube explants respond to Shh, by induction of FoxA2 and repression of Pax7 and Msx1/2, demonstrate that the Shh response pathway is functional in the secondary neural tube, and is thus unlikely to be responsible for its failure to undergo neuronal differentiation.

### Potential of the Secondary Neural Tube to Undergo Neuronal Differentiation

While the mouse secondary neural tube is clearly responsive to Shh in explant culture, we did not observe induction of Is11/2-positive neuronal differentiation. Interestingly, transplantation of the chick secondary neural tube to the primary body can induce motor neuron formation (Afonso and Catala, 2005) indicating that the absence of neurons in the chick secondary neural tube may result from the lack of an inducing signal, or presence of an inhibitor, in the local environment. Transplantation of somites from the secondary body adjacent to the primary neural tube did not abrogate motor neuron formation, while local application of retinoic acid was unable to rescue motor neuron formation in the secondary neural tube (Afonso and Catala, 2005). It appears, therefore, that an unknown factor present in the primary body is necessary to induce motor neuron formation in the chick neural tube. If the mechanisms regulating neuronal differentiation are conserved between mouse and chick, then the combined results of our study and of Afonso and Catala (2005) would suggest that the absence of neuronal differentiation in the secondary neural tube, which is observed in both species, is determined by a combination of cell autonomous factors and permissive signals present in the primary but not secondary body. It remains to be determined whether the molecular mechanisms underlying the suppression of neuronal differentiation might be similar or different in birds and mammals.

Our finding that *Pax3, 6, and 7* and *FoxA2* are expressed suggests that at least some neuronal precursors are present in the secondary neural tube. These precursors might be lost as a result of the significant cell death we observed in the secondary neural tube starting around E12.5, consistent with the idea of the absence of a survival factor. In chick, a survival factor (likely Shh) released from Hensen's node-derived structures prevents massive apoptosis in the primary neural tube (Charrier et al., 1999; 2001). It remains a possibility that the significant cell death observed in the secondary neural tube of mice could also be caused by insufficient levels of this survival factor, due to the separation of the notochord and the secondary neural tube, and the absence of a neural source of Shh.

### Lack of Roof Plate, Dorsal Interneurons and Neural-Crest Derived Sensory Neurons in the Secondary Neural Tube

Specification of dorsal identity in the neural tube is initiated by BMP-mediated signals derived from the epidermal ectoderm that overlies the neural plate. Subsequently, roof plate cells become a secondary source of BMP4 and BMP7, to control the generation of distinct classes of dorsal interneurons (Liem et al., 1997; Lee et al., 1998; McMahon et al., 1998). In the secondary body, we found *Bmp4* is expressed at very high levels in the dorsal ectoderm, in the underlying mesenchyme and also in the mesenchyme surrounding the secondary neural tube. In contrast, *Bmp4* expression in the dorsal region of the secondary neural tube is much weaker than in the primary neural tube, therefore likely affecting patterning and differentiation of the secondary neural tube. Indeed, histologically, the dorsal midline does not resemble a secondary roof plate although it does express *Wnt1* and *Wnt3a*, like the primary roof plate. Moreover, the absence of Isl1/2, which is expressed in a subset of dorsal interneurons in the primary neural tube (Liem et al., 1997), together with the lack of neurofilament staining in the secondary neural tube, argues against any dorsal neuronal differentiation.

BMPs mediate the induction of neural crest cells in the primary neural tissue (Liem et al., 1995), while *Wnt1* and *Wnt3a* play an important role in controlling the expansion of the neural crest (Ikeya et al., 1997). However, despite the high levels of *Bmp4*, *Wnt1* and *Wnt3a* expression in the mouse secondary body, the complete absence of dorsal root ganglia indicates that sensory neurons are not formed. Similarly, there is a lack of dorsal root ganglion formation in the chick secondary body (Osorio et al., 2009a). Interestingly, genes associated with neural crest formation appear to be expressed normally in the chick secondary body (Osorio et al., 2009b). Unlike the mouse, the chick exhibits *Bmp4* expression only in the dorsal midline region, and not in the mesenchyme surrounding the secondary neural tube. However, Bmp4-Wnt1 signaling is impaired in chick, owing to enhanced expression of the BMP antagonist Noggin, which leads to absence of neuronal derivatives from the neural crest (Osorio et al., 2009a).

The absence of sensory neuron formation in the mouse secondary body raises the question of how tail sensation is mediated postnatally. We noted a ventral bundle associated with the secondary neural tube and, by DiI tracing, demonstrate that this comprises nerve fibers originating from the primary neural tube and travelling down the body axis into the tail. Also present are paired dorsal and ventral nerve bundles, outside the secondary neural tube, which also likely originate in the primary body. We conclude that sensation and muscle innervation in the mouse tail are most likely mediated via sensory and motor neurons located in the primary body.

Besides forming sensory neurons, neural crest cells also form non-neural cell types. It has been demonstrated that the chick secondary neural tube can form neural crest, but the developmental potential is restricted to melanocytes and glial cells (Catala et al., 2000; Osorio et al., 2009a). It remains to be determined whether the mammalian secondary neural tube also gives rise to neural crest cells, and if it does, whether the neural crest cells have similar developmental potentials as those in the chick.

## Effect of Secondary Neural Tube on Somite Patterning

Other than serving sensory and motor functions, the primary neural tube is an important source of inductive signals involved in the patterning and differentiation of adjacent somites (Pourquié et al., 1993). The ventromedial aspect of the somite differentiates into sclerotome in response to Shh derived from the notochord and floor plate (Fan and Tessier-Lavigne, 1994; Johnson et al., 1994; Borycki et al., 1998). On the other hand, Wnt signals derived from the dorsal neural tube are involved in the differentiation of dermatome and myotome (Fan et al., 1997; Capdevila et al., 1998; Ridgeway et al., 2000).

Vertebrae in the primary body are formed by sclerotomal cells, which migrate ventrally to surround the notochord, forming the vertebral body, and dorsally to form the neural arches and spinous process (Johnson et al., 1994; Watanabe and Le Douarin, 1996). Only some of these processes appear to be conserved in the secondary body. The vertebral body is organized around the notochord, implicating the sclerotome inducing ability of Shh derived from the secondary notochord. However, the neural arches and spinous process fail to form. In this study, we find that *Bmp4* is expressed strongly in the mesenchyme located between the dorsal ectoderm and the secondary neural tube, with the expression domain extending

into the mesenchyme surrounding the neural tube. However, *Bmp4* is not expressed at these sites in the primary body. *Bmp4* has been shown to exert a negative effect on the expression of *Pax1* in the somitic mesenchyme, inhibiting sclerotome cell growth and differentiation into cartilage (Monsoro-Burq et al., 1996). Consistent with this observation, the mRNA level of *Pax1* surrounding the secondary neural tube is very much weaker in comparison to the same region in the primary body level. It is possible, therefore, that the lack of neural arches and spinous process formation around the secondary neural tube may be attributed by the inhibitory effect of BMP4 on osteogenesis.

The primary neural tube also regulates myogenesis. *Wnt1*, *Wnt3a* and *Wnt4*, which are expressed in the dorsal region of the neural tube, in conjunction with floor plate and notochord-derived Shh (Munsterberg et al., 1995; Ridgeway et al., 2000), regulate myogenesis in the medial compartment of the developing somite that forms the epaxial muscles associated with the vertebral column. Although *Shh* expression is absent in the secondary neural tube, the notochord expresses high levels of *Shh*. Moreover, the localization of *Wnt1* and *Wnt3a* is essentially conserved between the primary and secondary neural tube. It is thus likely that the secondary neural tube and notochord contribute to the induction of epaxial muscles and tendons surrounding the secondary neural tube.

## Fate of the Secondary Neural Tube

While the primary neural tube develops into the brain and the spinal cord, the contribution of the secondary neural tube to the central nervous system is still controversial. Some studies suggest that the whole lumbosacral segment of the spinal cord in humans is the result of secondary neurulation (Lemire et al., 1975), while others suggest that secondary neurulation leads to the formation of only the lower sacral and all coccygeal segments of the spinal cord (Copp and Brook, 1989; Muller and O'Rahilly, 1989). Others suggest that all spinal cord segments and spinal ganglia are formed by primary neurulation (Nievelstein et al., 1993). In the present study, we found that the mouse secondary neural tube ultimately regresses to a small structure with a tiny lumen in which the cells have lost epithelial characteristics, histologically similar to the human filum terminale that bears the ependymal epithelium (Nievelstein et al., 1993; Marin-Garcia et al., 1995). Moreover, mesenchymal-like cells are found to surround the regressing secondary neural tube at advanced stages, which may differentiate to form the primitive pia mater. These findings are consistent with the idea that the fate of the secondary neural tube is to become the filum terminale.

In conclusion, the secondary neural tube does not undergo neuronal differentiation, a feature that appears to result from intrinsic, neural tube-specific, factors as well as extrinsic influences. Understanding the molecular basis of the failure in neuron formation in the secondary neural tube might help to define the autonomous and non-autonomous factors that allow neuronal differentiation in the primary neural tube.

## Experimental Procedures

### Mouse and Rat Strains

ICR mice and Sprague Dawley rats were kept on a 12:12 hour light-dark cycle with the mid-point of the dark period at midnight. Females were housed with stud males overnight and checked for copulation plugs the following morning. Noon on the day of finding a plug was considered embryonic day (E) 0.5.

### In Situ Hybridization

Expression patterns for *Pax1* (Ebensperger et al., 1995), *Pax3* (Goulding et al., 1991), *Pax6* (Walther and Gruss, 1991), *Wnt1* (Wilkinson et al., 1987), *Wnt3a* (Roelink and Nusse, 1991), *En1* (Davis and Joyner, 1988), *Netrin1* (Kennedy et al., 1994), *Shh* (Echelard et al., 1993), *FoxA2* (Sasaki and Hogan, 1993), *Islet1* (Ericson et al., 1992) and *Bmp4* (Winnier et al., 1995) were studied in embryos of E9.5 to E14.5. For detailed analysis of the spatial expression patterns, the portions of the body at somite-level 12-17 from embryos at E9.5 and somite-level 48-53 from embryos at E12.5 were cut out and prepared as 50 μm transverse vibratome sections. Sections collected from these two axial levels represented the newly formed tissues at the primary body and the secondary body respectively. At least 8 embryos of each stage for each gene were studied.

### Histological Examination of Neural Tube Development

Two portions of the body, corresponding to somite-levels 28-32 and 38-42, were collected from embryos at E10.5 to E18.5 in order to follow the development of the neural tube at a particular axial level. The neural tube in these two levels is derived from primary and secondary neurulation respectively. The tissue was fixed in Bouin's fixative and prepared as 7 μm transverse paraffin sections stained with haematoxylin and eosin. About 3-10 embryos of each stage were studied.

### Immunohistochemical Detection of Neurofilaments and Isl1/2

Two portions of the body, corresponding to somite-levels 28-32 and 38-42, were collected from embryos at E11.5 to E16.5 and fixed in Carnoy's solution at 4°C overnight. The tissue was prepared as 10 μm transverse paraffin sections. Neurofilaments and Isl1/2 were stained by standard immunohistochemical techniques, using the 2H3 (mouse IgG) and 39.4D5 (mouse IgG) supernatants respectively (1:5, Developmental Studies Hybridoma Bank). The secondary antibodies employed were biotinylated anti-mouse IgG, which were detected by using the Vectastain ABC peroxidase kit (Vector). About 3-14 embryos of each stage for each primary antibody were studied.

### Tracing of Nerve Fibers with DiI

Mouse embryos at E14.5 (n=20) were fixed in 4% paraformaldehyde in phosphate buffered saline for 24 hours at 4°C. A DiI crystal (Molecular Probes) was inserted into the spinal cord at the axial level of the developing hindlimb. The embryo was incubated in 2% formalin in 0.1 M phosphate buffer in the dark at room temperature. The extent of labeling was periodically checked by fluorescence microscopy under a rhodamine filter. After about 6

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months, labeling along the nerve fibers had extended to the caudal end. The embryo was photographed and then cut into 75 μm transverse or longitudinal vibratome sections. The sections were analyzed by confocal microscopy.

### Secondary Notochord and Neural Plate Co-culture Assay

The secondary notochord with neural tube attached, or the secondary neural tube alone, was isolated from the caudal end up to the last 5 somites of E13.5 rat embryos after dispase digestion. Chick intermediate neural plate tissue was isolated from Hamburger-Hamilton stage 10 chick embryos. The rat secondary notochord with neural tube attached (n=10) or the secondary neural tube alone (n=4) was placed in contact with the chick neural plate explant and the tissues were embedded within three-dimensional collagen gels and cultured for about 36 hours as described (Yamada et al., 1993). Explants were fixed in 4% paraformaldehyde for 1 hour and then processed for immunocytochemistry.

### Secondary Neural Tube Explant Culture

The secondary neural tube, from the caudal end up to the last 10 somites, was isolated from E13.5 rat embryos. Explants were cultured within three-dimensional collagen gels in the presence of conditioned medium containing ShhN (n=29), derived from 293T cells transfected with ShhN plasmid DNA (Roelink et al., 1995) using Lipofectamine (Life Technologies), or conditioned medium from untransfected 293T cells (n=25). Culture was terminated after around 36 hours. Explants were fixed in 4% paraformaldehyde for 1 hour and then processed for immunocytochemistry.

### Whole Mount Immunocytochemistry

Explants were subject to whole mount immunocytochemical detection of Isl1/2 proteins with 39.4D5 (mouse IgG), FoxA2 with 4C7 (mouse IgG), Pax7 with Pax7 (mouse IgG1) and Msx1/2 with 4G1 (mouse IgG) supernatants (1:5, Developmental Studies Hybridoma Bank), followed by detection using fluorescein or texas red-conjugated hamster anti-mouse IgG antibody (1:100, Jackson ImmunoResearch). Explants were examined by confocal microscopy.

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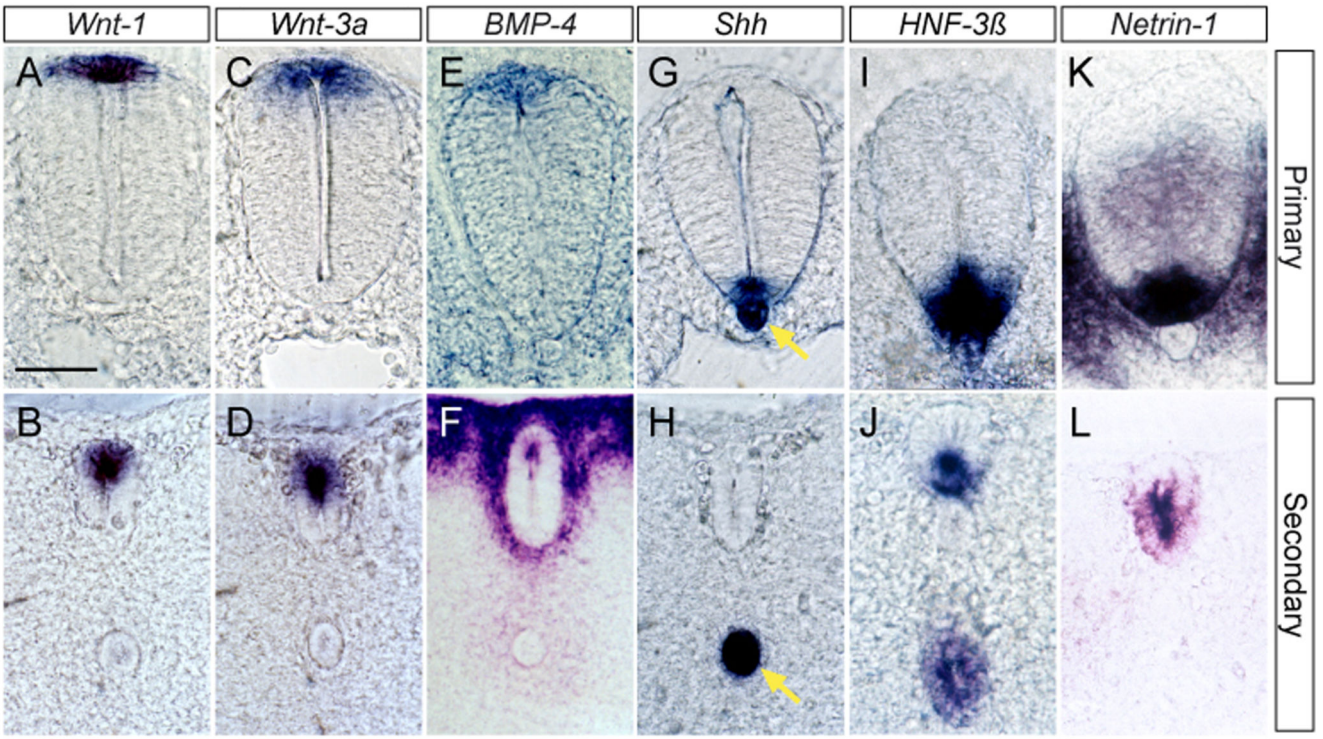


**Fig. 1.**

A comparison of the mRNA expression patterns of several genes important in neural tube patterning in the primary and secondary neural tube. (A-L) Vibratome sections taken from the last 5 somites at E9.5 (A,C,E,G,I,K) and E12.5 (B,D,F,H,L) or E11.5 (J) embryos to show the primary and secondary neural tube at similar developmental stages. The dorsally restricted expression domain of *Wnt1* and *Wnt3a* in the primary neural tube (A,C) is retained in the secondary neural tube (B,D). While *Bmp4* is expressed in the roof plate of the primary neural tube and weakly in the overlying dorsal ectoderm (E), very high levels of *Bmp4* are detected in the dorsal ectoderm and the underlying mesenchyme surrounding the secondary neural tube (F). The expression of *Shh* in the ventral midline (floor plate) of the primary neural tube (G) is missing from the secondary neural tube, although strong expression is detected in the notochord (arrow; H). The ventral midline of the secondary neural tube expresses *FoxA2* (J) as in the primary neural tube (I). However, the expression of *Netrin1* in the secondary neural tube is retained only in the lateral neural tube (L), but not in the ventral midline region as in the primary neural tube (K). Scale bar: 50 μm (A-L).

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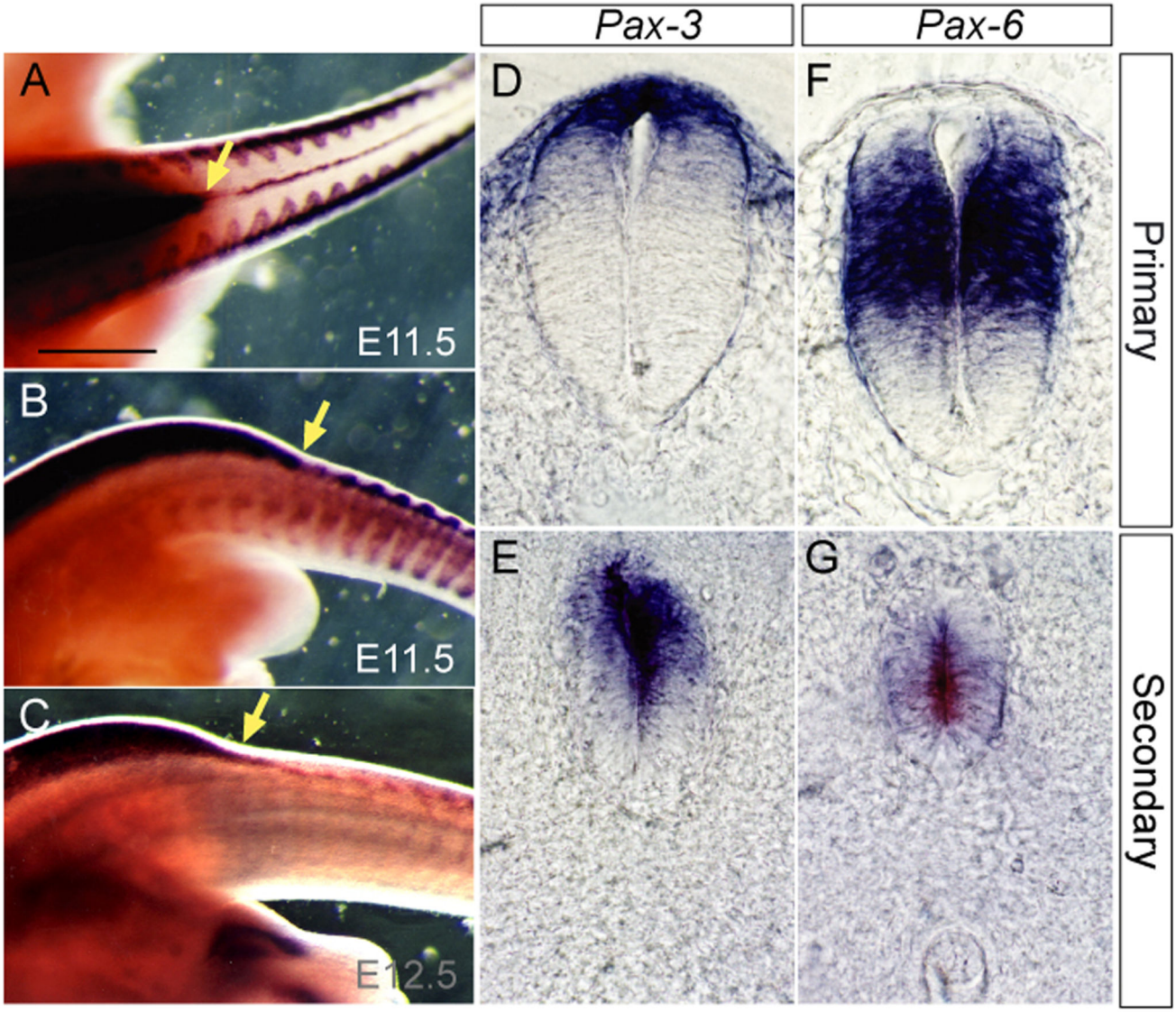


**Fig. 2.**

Early neural genes *Pax3* and *Pax6* are expressed in similar dorsoventrally restricted domains in the primary and secondary neural tube. (A-C) The mRNA expression pattern of *Pax3* at E11.5 (A,B) and E12.5 (C). A sharp transition of expression from a broad to a narrow domain occurs at somite-level 36 (arrow). (D,E) *Pax3* is expressed in the dorsal region in both the primary (D) and secondary neural tube (E). (F,G) Expression of *Pax6* in the lateral region of the primary neural tube (F) is retained in the secondary neural tube (G). Scale bar: 150 μm (A-C); 50 μm (D-G).

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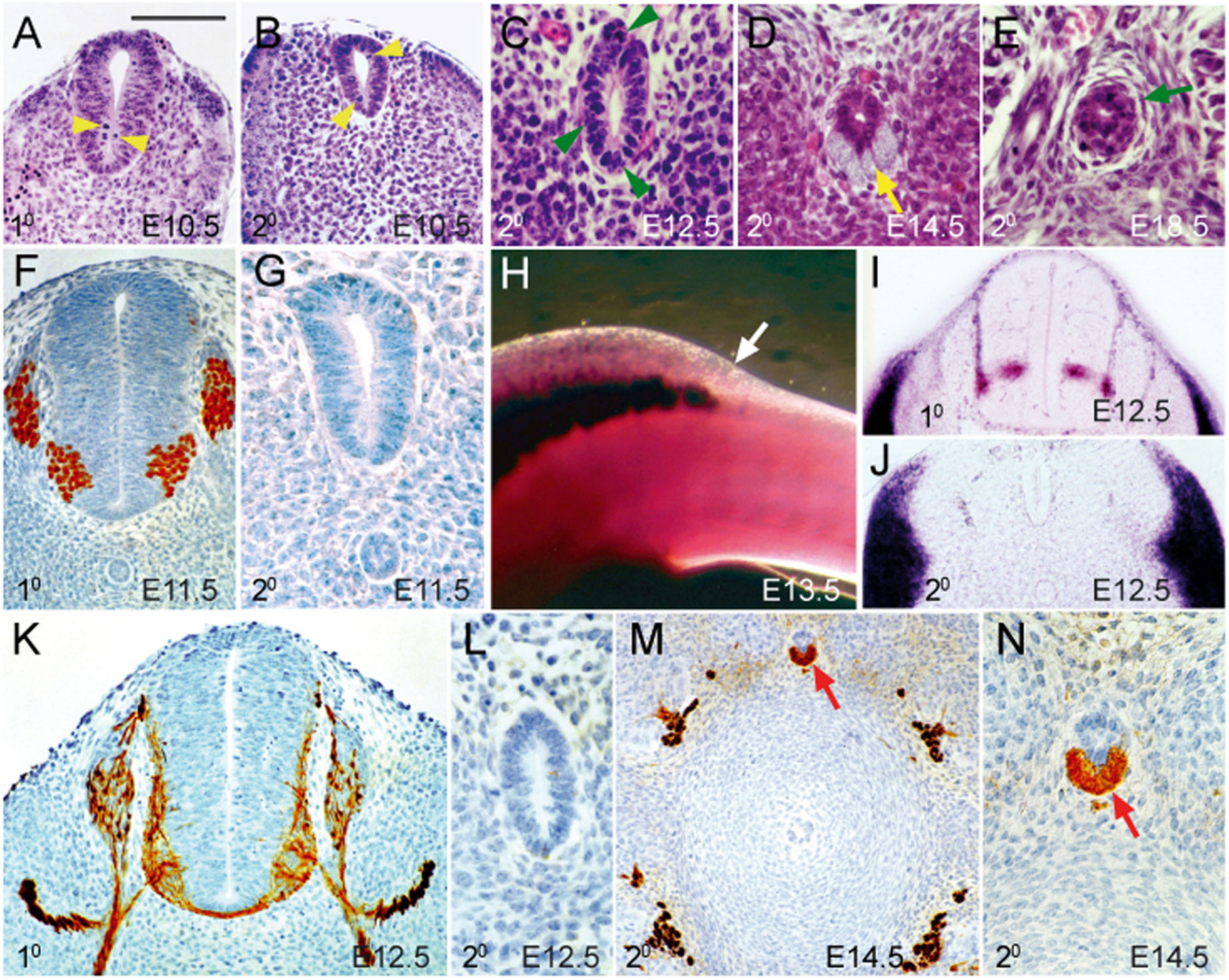


**Fig. 3.**

Lack of neuronal differentiation and dorsal root ganglion formation in the secondary neural tube. (A-E) Haematoxylin and eosin stained paraffin sections of the primary ($1^0$; A) and secondary neural tube ($2^0$; B-E). A newly formed secondary neural tube (B) is histologically similar to a newly formed primary neural tube (A). Both are actively proliferating as shown by the presence of mitotic figures (yellow arrowheads) located at the luminal side. Two days after formation, there are many pyknotic nuclei (green arrowheads) in the secondary neural tube (C). A bundle of tissue (yellow arrow) transiently appears in the ventral part of the secondary neural tube at E14.5 (D). Subsequently, the secondary neural tube regresses to a tiny structure surrounded by mesenchymal-like cells (green arrow; E). (F-H) Detection of Isl-1/2 proteins by immunohistochemistry (F,G) and *Isl1* mRNA by in situ hybridization (H). Isl1/2 is expressed in sensory neurons in the dorsal root ganglia and in motor neurons in the ventrolateral region of the primary neural tube (F). No Isl1/2 immunoreactivity could be detected in and around the secondary neural tube (G). The expression of *Isl1* mRNA in the dorsal root ganglia abruptly terminates at somite-level 36 (white arrow; H). (I,J) *En1* mRNA detected by in situ hybridization. *En1* is expressed in a subset of interneurons in the primary

neural tube (I), but no *En1* expression could be detected in the secondary neural tube (J). Expression of *En1* in lateral mesoderm at both levels of the body axis provides a positive expression control. (K-N) Immunohistochemical detection of neurofilaments which are heavily labeled in the cell bodies and nerve fibers of the primary neural tube, in both dorsal root ganglia and spinal nerves (K). In contrast, no neurofilament staining is detected in the newly formed secondary neural tube (L). However, at E14.5, neurofilaments can be detected in the ventral bundle (red arrow) of the secondary neural tube (M,N). In addition, two pairs of nerves, located dorsolateral and ventrolateral to the notochord of the tail are labeled (M). Scale bar: 100 μm (A,B,F,J,K,M); 50 μm (C,D,E,G,L,N); 500 μm (H); 200 μm (I).



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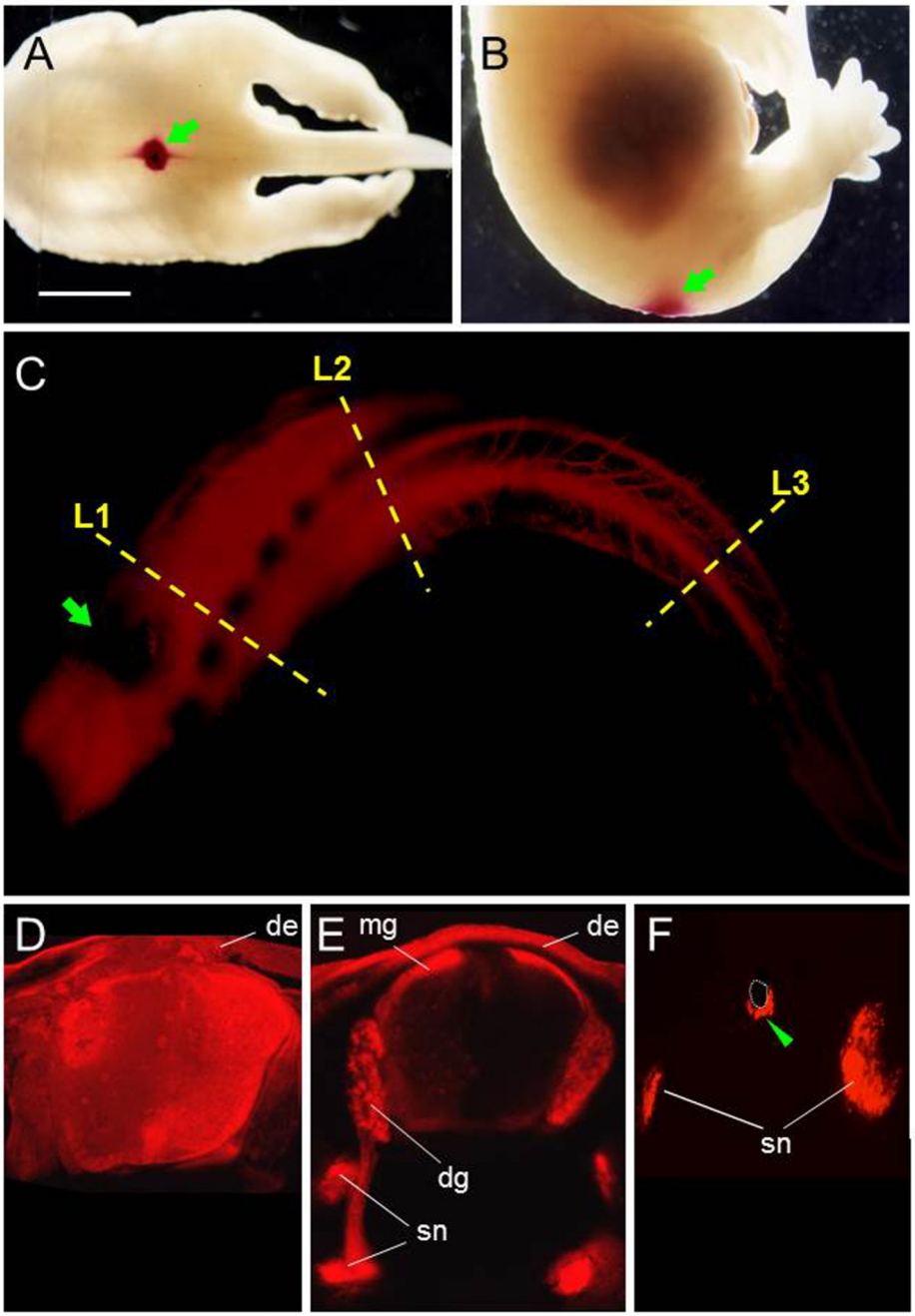


**Fig. 4.**

The ventral bundle of nerve fibers in the secondary neural tube is derived from neuronal cell bodies at the primary body level. (A,B) Dorsal (A) and lateral (B) views of a E14.5 embryo with a DiI crystal (arrow) inserted into the spinal cord at the hindlimb level. (C-F) Fluorescence examination of the pattern of DiI labeling after several months incubation. Areas around the site of insertion (arrow) are heavily labeled. DiI has diffused caudally into the secondary body region along the cell membrane of the nerve fibers. The levels of transverse sections shown in D, E, F are marked as L1, L2 and L3 respectively (C). At L1,

near the site of insertion, the entire spinal cord and the dorsal ectoderm (de) are heavily labeled (D). At L2, heavy labeling is detectable in the nerve fibers of the dorsal marginal layer (mg) of the primary neural tube, in the dorsal root ganglia (dg) and in the spinal nerves (sn) (E). At L3, the ventral bundle (arrowhead) is labeled although the secondary neural tube (circled) itself is free of DiI (F). Spinal nerves are also labeled at this level. Scale bar: 1600 μm (A,B); 400 μm (C); 200 μm (D,E,F).



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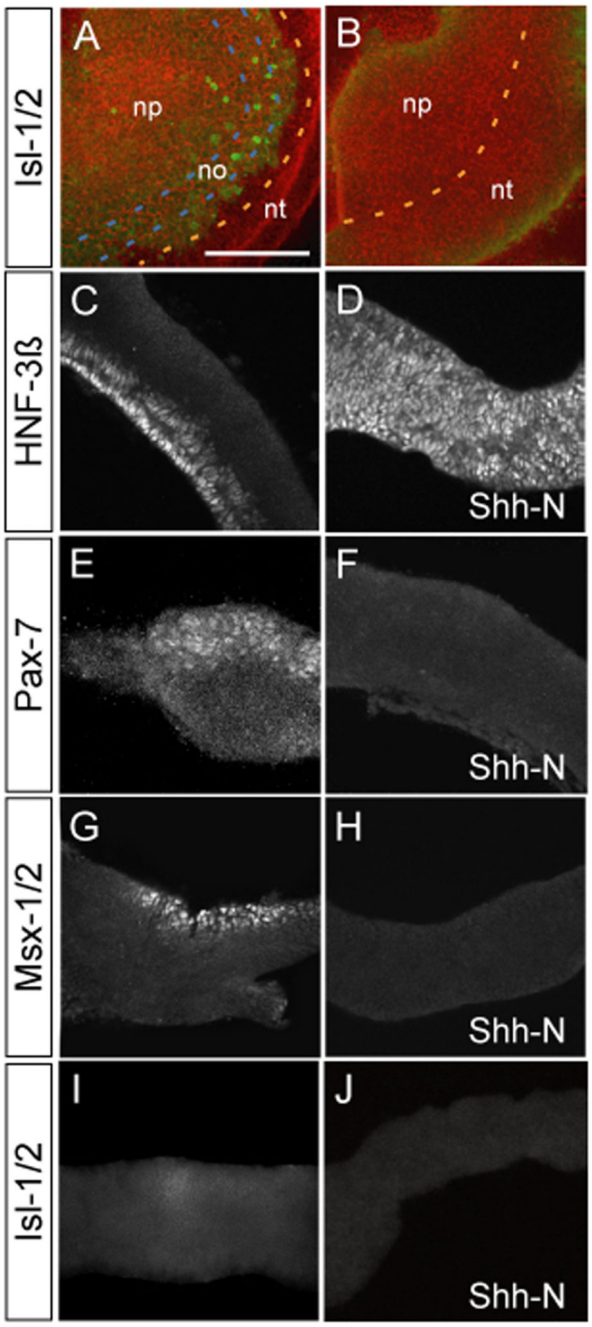


**Fig. 5.**

The secondary neural tube is unable to undergo motor neuron differentiation although it can respond to Shh. (A,B) Co-culture of the chick neural plate (np, border is indicated with a yellow dotted line) with the rat secondary neural tube (nt), with or without the secondary notochord (no, outlined by blue dotted lines in A). The secondary notochord is below the focal plane shown in A. Many Isl1/2 positive cells (green) were induced in the chick neural plate when co-cultured with the secondary notochord/neural tube explant (A), but not with the secondary neural tube alone (B). (C-J) The rat secondary neural tube explant cultured in

the absence (C,E,G,I) or presence (D,F,H,J) of ShhN. The secondary neural tube can respond to ShhN by ectopic expression of FoxA2 (D) and repression of Pax7 (F) and Msx1/2 (H). However, Isl1/2 positive cells are not induced by ShhN (J). Scale bar: 100 μm (A-J).



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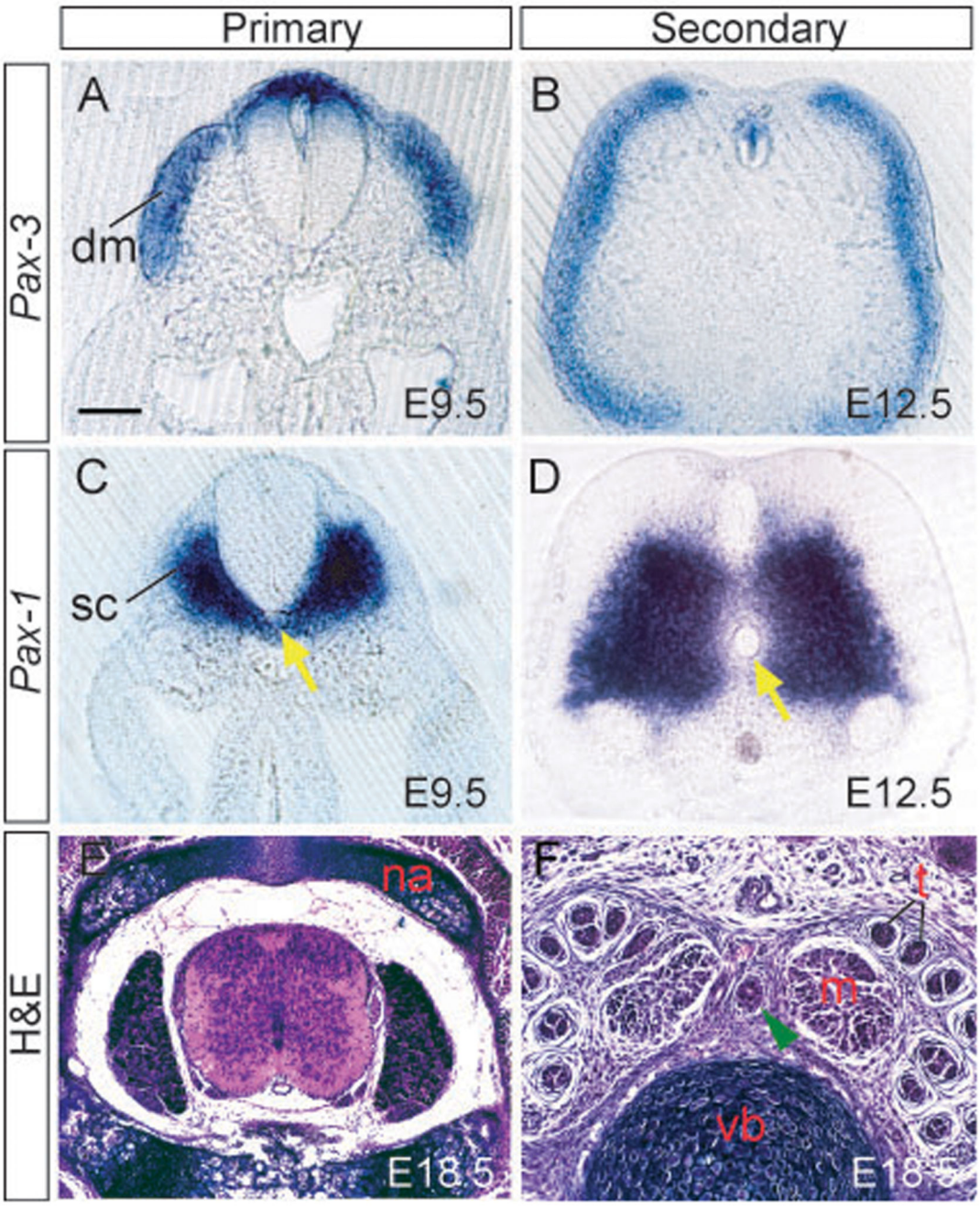


**Fig. 6.**

A comparison of somite patterning and development in the primary and secondary body. (A-D) Vibratome sections taken from the primary (A,C) and secondary (B,D) body level of embryos hybridized with *Pax3* (A,B), a molecular marker for dermomyotome (dm) and *Pax1* (C,D), a marker for sclerotome (sc) that develops around the notochord (arrow). Both *Pax3* and *Pax1* are expressed in expanded domains in the secondary body (B,D) compared with the primary body (A,C). (E,F) Haematoxylin and eosin stained paraffin sections taken from the primary (E) and secondary (F) body levels at E18.5. Unlike the spinal cord in the

primary body, which is encased by the neural arches (na) (E), the secondary neural tube (arrowhead) is flanked by bundles of tendon (t) and muscle (m) with no vertebral segments surrounding it, apart from a large vertebral body (vb) located ventrally. Scale bar: 50 μm (A-D); 150 μm (E); 75 μm (F).



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