Ectopic expression of Gcm1 induces congenital spinal cord abnormalities

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INTRODUCTION

The neuroectodermal cells of the primitive neural tube are the common progenitor cells of the neurons and glia of the CNS. In Drosophila, the transcription factor Glial cells missing (Gcm) is believed to play a central role in the cell-fate specification of neurons and glia (Hosoya et al., 1995; Jones et al., 1995). The observations that Drosophila embryos with an impaired gcm gene are impoverished of glia, while embryos ectopically expressing gcm have an excess of glia, usually at the expense of neurons, have led to the proposition that gcm is a binary switch controlling the flow of progenitor cells into glial or neuronal cell lineages. We and other have identified two vertebrate homologues of gcm, named Gcm1 and Gcm2 (also called Gcma and Gcmb, respectively) (Akiyama et al., 1996; Alshuller et al., 1996; Basyuk et al., 1999; Kim et al., 1999). Mammalian Gcm1 and Gcm2 share a highly conserved DNA binding domain with the Drosophila gcm which does not resemble known DNA binding domains. Consistent with their nuclear localization and specific DNA-binding to the motif 5'-(G/A)CGGGT-3', Gcm proteins were proposed to be a novel class of transcription factors (Schreiber et al., 1997; Schreiber et al., 1998). Surprisingly, the mammalian Gcm1 and 2 are more highly expressed in non-neuronal tissue than in brain. However, both Gcm1 and 2 are expressed in the mammalian developing CNS. Furthermore, the mouse Gcm1 can fulfill many of the functions of the Drosophila gcm gene when introduced into the fly (Kim et al., 1998; Reifegerste et al., 1999). Thus, ectopic expression of mouse Gcm1 in Drosophila gcm null mutant embryos induces gliogenesis. A direct assessment of the role of the mouse Gcm1 gene is complicated by the fact that Gcm1 null mutant conceptuses fail to develop a competent placenta (Anson-Cartwright et al., 2000; Schreiber et al., 2000) and die at E10, a time well before gliogenesis occurs.

We have assessed the potential role of Gcm1 in murine CNS development using transgenic mice that express Gcm1 under the control of the mouse Hoxa7 enhancer. We show that ectopic expression of Gcm1 during early embryogenesis leads to two severe neural tube defects that have counterparts in human disease: failure of the neural tube to close (spina bifida or more precisely, myelocele) and multiple neural tubes (diastematomyelia). The dysraphisms develop during the period of transgene expression and within the zone of expression. After transgene expression ceases, the dysraphisms are progressively resolved and the open neural tube closes. Neonatal animals, while showing signs of scarring and tissue resorption, have a closed vertebral column. The multiple spinal cords remain but are enclosed in a single spinal column as in the human diastematomyelia. The animals live a normal life time, are fertile and do not exhibit any obvious weakness or motor disabilities.

Key words: glial cells missing, Secondary neurulation, Cell fate specification, Neural tube defects, Tail bud, Mouse

SUMMARY

Brief ectopic expression of Gcm1 in mouse embryonic tail bud profoundly affects the development of the nervous system. All mice from 5 independently derived transgenic lines exhibited either one or both of two types of congenital spinal cord pathologies: failure of the neural tube to close (spina bifida) and multiple neural tubes (diastematomyelia). Because the transgene is expressed only in a restricted caudal region and only for a brief interval (E8.5 to E13.5), there was no evidence of embryonic lethality. The dysraphisms develop during the period and within the zone of transgene expression. We present evidence that these dysraphisms result from an inhibition of neuropore closure and a stimulation of secondary neurulation. After transgene expression ceases, the spinal dysraphisms are progressively resolved and the neonatal animals, while showing signs of scarring and tissue resorption, have a closed vertebral column. The multiple spinal cords remain but are enclosed in a single spinal column as in the human diastematomyelia. The animals live a normal life time, are fertile and do not exhibit any obvious weakness or motor disabilities.
MATERIALS AND METHODS

Hoxa7-Gcm1 transgene construct
An AccI-EcoRI cDNA fragment containing the entire coding sequence of Gcm1 was blunt-ended and cloned into Smal site of Bluescript SKII, generating pGcm1. A 190-bp Spel fragment containing the SV40 polyadenylation signal was directionally cloned at the 3’ end of the Gcm1 coding region creating the pGcm1/SV40 plasmid. The Hoxa7-Gcm1 transgene was inserted by using the Hoxa7 enhancer and the TK minimal promoter from the pAX470 plasmid (gift from P. Gruss) (Fig. 1A).

Generation of transgenic mice
The entire transgene was released from the Hoxa7-Gcm1 plasmid by digestion with ClaI and SacI. A transgene containing lacZ instead of Gcm1 was obtained from pAX470 plasmid by digestion with HindIII and Snabl. These fragments were gel purified, dialyzed with injection buffer (10 mM Tris, pH 7.4, 0.2 mM EDTA) and filtered through a 0.22 μm filter (Millex-GV4, Millipore). They were diluted to 2-5 μg/ml in injection buffer and co-injected into pronuclei twice through a 0.22 μm filter. These fragments were gel purified, dialyzed with dIII and used as an injection. The entire transgene was released from the Gcm1 plasmid by digestion with HindIII and SnaBI. The transgene containing lacZ instead of Gcm1 was obtained from pAX470 plasmid by digestion with HindIII and Snabl. These fragments were gel purified, dialyzed with injection buffer (10 mM Tris, pH 7.4, 0.2 mM EDTA) and filtered through a 0.22 μm filter (Millex-GV4, Millipore). They were diluted to 2-5 μg/ml in injection buffer and co-injected into pronuclei twice through a 0.22 μm filter. These fragments were gel purified, dialyzed with dIII and used as an injection.

Northern blot analysis
Total RNA was extracted from E9.5 transgenic and wild-type embryos using guanidine thiocyanate-cesium chloride method (Sambrook et al., 1989). Poly(A) selected RNA were purified on oligo(dT) resin using mRNA isolation kit (Ambion). RNA were then separated on 1% agarose gel in 6.3% formaldehyde containing buffers, transferred to nylon membrane (Nytran) and hybridized with a 810-bp Gcm1-specific cDNA probe-labeled by random priming (NEB blot). After hybridization with the Gcm1 probe, the blot was stripped and probed with a GAPDH-specific probe used as standard control of RNA loading.

In situ hybridization
In situ hybridization was carried out on 10 μm paraffin sections of embryos as described by Wilkinson (Wilkinson et al., 1987). The following probes were used to generate sense and antisense riboprobes: Shh probe corresponding to a 642-bp EcoRI fragment (a gift from Dr A. McMahon, Harvard University), Pax3 probe [530-bp PstI/HindIII fragment from the 3’-end of the gene (Goulding et al., 1991)], Gcm1 probe [800-bp fragment, bases 710-1510 in the sequence in Altshuller et al. (Altshuller et al., 1996)], Tbx6 probe was generated from an EST clone (IMAGE consortium clone ID 1446422), Fgfr1 probe, corresponding to a 400-bp fragment covering the Ig.II and 5’ half of the Ig.III domains, was generated by PCR using Fgfr1 cDNA as a template (a gift from Dr M. Goldfarb, Mount Sinai School of Medicine, New York), Notc1 probe corresponds to a 422-bp fragment (a gift from Dr J. Kitajewski, Columbia University, New York, NY). Sense and anti-sense 35S-UTP-labelled riboprobes were generated with T3 or T7 RNA polymerases using a standard in vitro transcription protocol.

Immunohistochemistry
Embryos were dissected in cold PBS and fixed in 4% paraformaldehyde overnight at 4°C and embedded in paraaffin according to standard protocols. Ten μm sections were deparaffinized in xylene and hydrated through a graded ethanol series, then treated with 1% H2O2/10% methanol in 0.1 M PBS for 20 minutes, washed extensively in PBS and blocked for 1 hour in 10% normal goat serum, 1% gelatin, 5% BSA, 0.05% sodium azide in 0.1 M PBS. They were incubated overnight with the primary antibody diluted in 10% normal goat serum/0.1 M PBS in a humid chamber. Sections were then washed in 0.1 M PBS/0.1% Triton X-100 (Sigma) 3 time for 20 minutes and then incubated with the appropriate peroxidase-conjugated secondary antibody for 1 hour at room temperature. Sections were then washed in 0.1 M PBS/0.1% Triton X-100 and transferred in 0.1 M Tris-HCl pH 7.6. Peroxidase histochemistry was performed in 0.1 M Tris-HCl pH 7.6 containing 0.03% diaminobenzidine (DAKO, Carpinteria, CA), 0.1% NiCl2 and 0.003% H2O2 and stopped in water. Sections were dehydrated, cleared in xylene and mounted in cytoseal (Stephens Scientific). The following primary antibody were used: mouse monoclonal anti-Islet-1 (1/20 dilution, mouse IgG2b, 39.4DS; DSHB, University of Iowa), a mouse monoclonal anti-tubulin β3 (mouse IgG2b, clone SDL.3D10, 1/100 dilution; Sigma), a mouse monoclonal anti-MAP2 (mouse IgG1, 1/100 dilution; Sigma), and a mouse monoclonal anti-neurofilament 160 (mouse IgG1, 1/200 dilution; Sigma).

Magnetic resonance imaging
Each E16.5 embryo was positioned within a 1.5 cm diameter polyethylene tube filled with Fomblin (perfluoropolyether; Ausimont, Thorofare, New Jersey) used as a wetting and embedding agent to prevent dehydration and to reduce artifacts at tissue margins. Where present, air bubbles were aspirated from the interstices of the embryo with a very fine needle and syringe. The sample was wedged in place between two styrofoam plugs to reduce tissue vibration and the tube was sealed to prevent evaporation and re-entry of air bubbles.

The magnetic resonance images in the axial, coronal and sagittal planes were obtained on a 9.4 T superconducting magnet with a vertical 89 mm bore using a 25 mm birdcage coil. An automated water cooling system maintained the temperature within the bore at less than 30°C (Bruker Avance System with microimaging; Bruker Analytik, Rheinstetten, Germany). Pilot studies of diverse T1, T2 and intermediate-weighted (Int-w) sequences led us to select the Int-w sequence for spinal cord analysis, specifically; TR=2000 mseconds, TE=40 mseconds, slice thickness 0.5 mm, field of view 15×15 mm, data matrix 512×512, and number of excitations=50. This corresponds to an in-plane resolution of 29×29 μm and a slice thickness of 500 μm. Each sample was run overnight for a total acquisition time of 14 hours, 17 minutes.
RESULTS

An experimental paradigm for transient, ectopic expression of Gcm1

In order to assess the effects of Gcm1 during CNS development, we have generated transgenic mice expressing Gcm1 and lacZ under the control of the mouse Hoxa7 enhancer. The 470-bp Hoxa7 enhancer was originally identified by Knittel et al. (Knittel et al., 1995) as a minimal control element specifying the anterior boundary of Hoxa7 expression. This element directs expression in the developing tail bud during the period E9 to E13. The anterior boundary of Hoxa7 expression starts at the hind limb bud and recedes rostrally with time, so that by E12.5 only the tip of the tail bud shows transgene expression. This element directs expression in most neuroepithelial and mesodermal derivatives in the caudal region. By limiting the transgene expression to the caudal region and to only a brief period, we hoped to diminish the likelihood of embryonic lethality due to transgene expression.

Five independent Gcm1 founder transgenic mice were generated with these constructs (see Materials and Methods for details). In all animals, ectopic expression of Gcm1 leads to congenital CNS abnormalities. Most analyses described here were carried out on two permanent transgenic lines that were established. To illustrate the domain of transgene expression, embryos (E9.5 to E16.5) bearing the Hoxa7-lacZ transgene were stained with X-gal as whole mounts. As shown in Fig. 1D,E, the expression domain of the transgene is restricted to the caudal part of the embryo with an anterior boundary at the level of somite 18-20. At E9.5, note the enlargement of the posterior neuropore in the transgenic embryo as compared to the small oval shape of the posterior neuropore in wild type (arrow in C,D).

Gcm1 expression induces severe neural tube malformations

Gross morphologic examination of transgenic embryos revealed two types of severe neural tube defects. All transgenic mice derived from the 5 founders showed either one or both of the two pathologic phenotypes: failure of neural tube closure (spina bifida) and the presence of ectopic neural tubes (diastematomyelia). A summary of the examination of 62 transgenic embryos, shown in Table 1, reveals that 100% of the embryos had multiple neural tubes and 26% had open neural tube. No signs of embryonic lethality were observed. Most remarkably the neonatal animals exhibit a progressive resolution of their spina bifida and by 2 months of age show no outward signs of the embryonic neural defect.

Neural tube closure defect

As early as E9.5, transgenic embryos showed an enlargement of the posterior neuropore as compared with the small oval

Table 1. Frequency of ectopic neural tubes and spina bifida in transgenic embryos

<table>
<thead>
<tr>
<th>Stage</th>
<th>No. of transgenics</th>
<th>Spina bifida</th>
<th>Ectopic neural tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td>E9.5</td>
<td>7</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>E10.5</td>
<td>17</td>
<td>2</td>
<td>17</td>
</tr>
<tr>
<td>E12.5</td>
<td>20</td>
<td>7</td>
<td>20</td>
</tr>
<tr>
<td>E14.5</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>E16.5</td>
<td>15</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td>62</td>
<td>16 (25.8%)</td>
<td>62 (100%)</td>
</tr>
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</table>
shape of the posterior neuropore in wild-type embryos (Fig. 1C,D). This difference suggests an inhibition of the neural tube closure in the transgenic mice. Neural tube defects in the lumbo-sacral region were more obvious when E12.5-E16.5 embryos were examined (Fig. 2). The spina bifida extended from the level of the hind limb bud to the sacral region (Fig. 2B,C). The neural tube in this region remains flattened on the top of the ectoderm forming a neural placode, characteristic of the spina bifida aperta (Fig. 2D and Fig. 3A). At postnatal day 7, transgenic mice with this pathology showed a subcutaneous plate-like structure in the lumbo-sacral region with signs of necrosis (Fig. 2E). After the second postnatal week, this subcutaneous neural plate-like structure was totally resorbed and signs of scarring were no longer visible. Furthermore, all transgenic mice (100% penetrance) developed a kinky tail (Fig. 2E) unlike their wild-type littermates (Fig. 2F).

Multiple neural tubes

The second type of neural tube defects is revealed in histological examination of lumbo-sacral sections of transgenic embryos. Sections through this region showed the presence of ectopic neural tube structures (Fig. 3B), similar to human diastematomyelia. Two ectopic neural tubes were most frequently seen in the transgenic animals, but some had three or more. Serial sections revealed that the ectopic neural tubes were attached at their rostral end to the primary neural tube and formed a bifid spinal cord (data not shown). The presence of dorsal root ganglia associated with the primary neural tube at this level demonstrates that the ectopic tubes extended up to the region generated during primary neurulation. The ectopic neural tubes are confined to the region of the hind limb bud and tail bud. Frequently there is also a lipoma at the tip of the spinal cord (Fig. 3D,E), a characteristic also shared with the human spinal dysraphias. The physical relationship between the ectopic neural tubes and the neural tube closure defect can best be appreciated in the magnetic resonance images of an E16.5 transgenic embryo presented in Fig. 4. The ectopic neural tube can be seen emerging from the primary neural tube in the vicinity of the posterior neuropore where the neural tissue is hyperplastic and convoluted (Fig. 4D). The ectopic neural tube extends rostrally with progressively reduced caliber to a level above the kidneys (Fig. 4A-C). The MR images also reveal evidence of a neural tube closure defect and the discontinuity of the surface ectoderm (arrowheads, Fig. 4D).

A second type of ectopic tubular structure is observed at the tip of the tail bud. Examination of serial histological sections through the tail bud of E12.5 transgenic embryos revealed the presence of ectopic tubular structures with a neuroepithelial-like feature (Fig. 5B) that were free floating and were not connected to the primary neural tube. The neuroepithelial identity of these ectopic tubular structures was confirmed by immunostaining for tubulin β 3 (Fig. 5C). The presence of ectopic neural tubes in this region suggests that ectopic expression of Gcm1 in mesenchymal cells may promote the formation of secondary neural tubes by a process similar to secondary neurulation.

![Fig. 2. The gross morphology of Hoxa7-Gcm1 transgenic mice. (A,B) Lateral views of E12.5 wild-type (A) and transgenic (B) embryos. The arrow in B indicates spina bifida in the transgenic embryo. (C,D) Lateral and posterior aspect of a transgenic E16.5 embryo revealing a subcutaneous neural plate-like structure (arrow). (E,F) Posterior aspects of 1-week-old wild-type and transgenic littermates. Scarring and necrosis is evident in the transgenic pup (arrow).](image)

![Fig. 3. Histologic analyses of the spina bifida and ectopic neural tubes in transgenic mice. (A,B) Transverse sections through the lumbo-sacral level of the spinal cord showing the histological features of the spina bifida (A) and an ectopic neural tube (B) in E12.5 transgenic embryos. Dorsal root ganglia are indicated by the arrows. (C,D) View of the filum terminale (ft) in 1-month-old wild-type (C) and transgenic (D) spinal cord. Note the presence of a lipoma (lp) in the transgenic spinal cord. (E) Transverse section of the adult transgenic spinal cord illustrating the attached lipoma and the split cord. cc, central canal; A,B,E, Hematoxylin and Eosin staining. Bars: 100 μm.](image)
Ectopic expression of Gcm1 induces neural tube defects

Patterning and cellular differentiation within the ectopic neural tubes

We assessed the neural identity, the cyto-architecture and the degree of differentiation within the ectopic neural tubes by in situ hybridization and immunomicroscopy. In situ hybridization was then performed to examine the dorsoventral properties of the endogenous and ectopic neural tubes of the transgenic embryos and compare it to that found in non-transgenic littermates. Sonic hedgehog, Shh, is a notocord and floor plate marker of the neural tube. In situ hybridization with Shh probes showed the presence of a single notocord in all wild-type embryos, and even transgenic embryos with three neural tubes, showed expression in all the neural tubes (Fig. 6A,C). Probes for the pair rule gene Pax3 (a marker for dorsal neural tube and dermatomyotome) revealed expression in the expected domain of the primary neural tube of both the transgenic and non-transgenic embryos (Fig. 6A,C). Probes for the pair rule gene Pax3 (a marker for dorsal neural tube and dermatomyotome) revealed expression in the expected domain of the primary neural tube of both the transgenic and non-transgenic embryos (Fig. 6B,D). However, expression of this marker in ectopic neural tubes was variable and appeared to depend upon the position of the tubes in the embryo. When the tubes lay close to the surface ectoderm of the embryo, they expressed Pax3. When they lay at a distance from the surface ectoderm, as in Fig. 6D, they did not express the marker, perhaps because the Pax3 inducing BMP signal is not sufficiently strong at this remote location. Nonetheless, expression domains of Shh and Pax3 appear normal in transgenic animals.

Immunomicroscopic analyses revealed that both the primary and ectopic neural tubes were differentiating and adopting the neural tube cyto-architecture apace with neural tubes in age-matched non-transgenic littermates. Tubulin β3- and MAP2-positive cells, which are presumptive differentiating neurons, formed the outer layer of the neural tube, while the cell layer adjacent to the central canal remained undifferentiated (Fig. 7A,B). In transverse sections of non-transgenic E12.5 embryos, Islet-1 immunoreactivity is detected in the motoneuron precursors of the forming ventral horn of the spinal cord (Fig. 7C). In E12.5 transgenic embryo, Islet-1 staining was detected in both its expected domain in the primary neural tube and also in two ventrally located ectopic neural tubes (Fig. 7D), indicating that motoneurons were developing in all of these structures.

Gcm1 restricts expression of mesodermal differentiation factors

We investigated the consequences of Gcm1 expression in the tail bud of E9.5 wild-type and transgenic embryos by in situ hybridization. We were unable to detect any Gcm1 mRNA in non-transgenic embryos (Fig. 8A). The absence of Gcm1 mRNA in these tissues suggests that it does not play an important role in secondary neurulation. However, in the transgenic tail bud, transcripts of the Gcm1 transgene were...
easily detected in small clusters of cells condensing to form tubular structures inside the mesenchyme surrounding the hindgut (Fig. 8B). The Gcm1 transgene is not expressed uniformly in the seemingly homogeneous mesenchyme but rather in discrete islands. Furthermore, the cells expressing Gcm1 are driven toward a tubular organization. To further investigate this cellular diversity, we assessed the level and uniformity of Fgfr1, Notch1 and Tbx6 expression by in situ hybridization in both transgenic and non-transgenic E9.5 tail buds. These genes are essential for the specification of a mesodermal cell fate as previously reported (Beck and Slack, 1999; Chapman et al., 1996; Ciruna and Rossant, 2001). The expression of Notch1 (Fig. 8E,F) and Tbx6 (Fig. 8G,H) appears Gcm1 restricts expression of genes encoding mesodermal differentiation factors. Transverse sections through the tail bud of E9.5 wild-type (A,C,E,G) and transgenic (B,D,F,H) embryos were hybridized with Gcm1 (A,B), Fgfr1 (C,D), Notch1 (E,F) and Tbx6 (G,H) 35S-labeled riboprobes, and then counterstained with Hematoxylin. In the transgenic tail bud, Gcm1-expressing cells form secondary neural tube-like structures (arrowheads, B). In contrast, no expression of Gcm1 is detected in the wild-type tail bud (A). Expression levels of Fgfr1, Notch1 and Tbx6 are reduced in the secondary neural tubes of the transgenic tail buds (D,F,H). (H) The small rosettes of cells with no Tbx6 expression are those that tend to form secondary neural tubes (arrowheads). Bar: 50 μm.
to be specifically excluded from the cells condensing into tubular structures.

The formation of the paraxial mesoderm depends on a transcriptional cascade that involves the FGFRI, Notch1 and Tbx6 (Beck and Slack, 1999; Chapman et al., 1996; Ciruna and Rossant, 2001). The preferential exclusion of Notch1 and Tbx6 mRNAs from condensing mesenchymal cells and the expression of Gcm1 in these same cells, suggests a reciprocal expression pattern in which ectopic expression of Gcm1 overrides and suppresses a mesodermal cell fate, channeling the cells towards the default neural pathway.

**DISCUSSION**

The glial cell missing gene of *Drosophila* has been described as a master regulator of glial cell fate specification (Anderson, 1995). The mammalian homologues of *gcm* are expressed in both neural and non-neural tissues and they appear to participate in the specification of several diverse cell fates. In the present study, we show that transient expression of Gcm1 in the developing tail bud consistently leads to two severe neural tube defects: a neural tube closure defect (spina bifida) and the induction of ectopic neural tubes (diastematomyelia).

The transgene that we employed initiates expression of Gcm1 at a time and place that are of critical importance to neurulation: E9.5 and the tail bud, the time and place of the posterior neuropore closure and secondary neurulation (Nievelstein et al., 1993; Schoenwolf, 1984). At that time the caudal tip of the neural tube lies adjacent to the tail bud (Griffith et al., 1992). The embryonic tail bud comprises a seemingly homogeneous mass of cells and represents the remains of Hensen’s node and the primitive streak. This cell mass has remarkable developmental potential and gives rise, during subsequent development, to a variety of structures including the secondary neural tube, the caudal notochord, hind gut, part of the vertebral column and musculature; all without the previous formation of the three germ layers (Griffith et al., 1992; Tam and Trainor, 1994). This seemingly direct generation of ecto-, endo- and mesodermal cell types is in stark contrast to the generation of similar cell types in the rostral regions, and the details of this process are incompletely understood.

After closure of the posterior neuropore (E9.5-10) the primary neural tube is extended caudally through a process of secondary neurulation (Naidich et al., 1996; Nievelstein et al., 1993; Schoenwolf, 1984). At that time the closed primary neural tubes extends to the level of somite 32-34, the future level of the third sacral vertebra and thus primary neurulation forms the parts of the wild-type spinal cord that have dorsal and ventral roots. The secondary neurulation produces the caudal spinal cord, including the portions that are the primordia of the filum terminale, the ventriculus terminalis and part of the conus medullaris. In mice, the observed earliest event in secondary neurulation is the continuous accretion of aggregates of the tail bud mesenchymal cells in the form of medullary rosettes at the caudal end of the primary neural tube ( Muller and O’Rahilly, 1987; Muller and O’Rahilly, 1988; Nievelstein et al., 1993; Schoenwolf, 1984). These cells take on a columnar epithelial appearance and form a neurocoele by cavitation, the lumen being always in contact with the lumen of the primary neural tube.

Several lines of evidence suggest that the complex phenotype of the transgenic mice described here (spina bifida and diastematomyelia) is a direct result of two distinct phenomena: the inhibition of posterior neuropore closure and the indirect stimulation of secondary neurulation. That the spina bifida observed in the transgenic mice results from an inhibition of neuropore closure by Gcm1 is supported by our estimates of the anatomical level of the defect. The position of the closure defect in affected transgenic mice, whether visualized in histologic sections or in whole mounts by MR imaging, is same in different animals and corresponds to the position of the posterior neuropore. The value of 26% reported in Table 1 for the proportion of transgenic embryos with recognizable spina bifida may be strongly affected by two competing secondary processes: progressive resolution of the defect and the neuroepithelial hyperplasia, which exaggerates the defect.

The possibility that Gcm1 directly stimulates secondary neurulation is difficult to reconcile with our inability to detect any Gcm1 mRNA in the wild-type tail bud during active secondary neurulation (Fig. 8). Rather, we believe that the transgene-derived Gcm1 indirectly stimulates secondary neurulation that results in the generation of ectopic neural tubes. This hypothesis is supported by two lines of evidence. The time and place of transgene expression indicate that the ectopic neural tubes are not derived from primary neurulated tissue. Further, the appearance of numerous, small “free-floating” neural tube-like structures at the very tip of the tail bud suggests that the transgene-derived Gcm1 stimulates the neuroepithelial differentiation of tail bud mesenchyme, a process already underway at that time and place. There is mounting evidence to suggest that when tail bud mesenchymal cells are prevented from differentiating into paraxial mesoderm they follow a pathway leading to the formation of secondary neural tubes. Much of the evidence comes from studies of the effects of mutations in the transcriptional or signaling factors known to be active in the mesenchyme to paraxial mesoderm transformation, an early stage in the differentiation of somites. Transcriptional cascades initiated by FGFRI (Ciruna and Rossant, 2001) or Wnt3a (Yamaguchi et al., 1999a; Yamaguchi et al., 1999b; Yoshikawa et al., 1997) have been identified in the somite progenitor cells. Interruption of these cascades by null mutations in the genes encoding them leads not only to a deficit in paraxial mesoderm but also to the formation of ectopic neural tubes. Thus mutations in either Fgfr1 (Ciruna et al., 1997; Deng et al., 1997), Wnt3a (Yoshikawa et al., 1997), Tbx6 (Chapman and Papaioannou, 1998) or mutations in both Lef1 and Tcf1 (Galceran et al., 1999) lead to the formation of ectopic neural tubes. The transformation of paraxial mesoderm progenitors to neural progenitors that is apparent in these null mutants has been attributed to diversion of the progenitor cells to a default neural pathway when mesodermal differentiation is inhibited. Our results are compatible with this interpretation if ectopic expression of Gcm1 in tail bud mesenchymal cells specifically interferes with their differentiation to paraxial mesoderm. Gcm-induced interference with both epidermal and mesodermal differentiation has been observed in *Drosophila* (Akiyama-Oda et al., 1998; Bernardoni et al., 1998; Reifegerste et al., 1999). In our study, we showed that ectopic expression of Gcm1 in multipotential mesenchymal cells induces the down regulation of two factors normally required
for mesodermal differentiation, Notch1 and Tbx6, in cells assuming neuroepithelial cell type. We believe that the ectopic neural tubes in our transgenic mice result from the incomplete suppression of the differentiation of mesenchyme to paraxial mesoderm and the diversion of precursors to the default neural pathway. However the possibility that Gcm1 may directly induce neural-specifying genes can not be ruled out.

A striking difference between our transgenic mice and those bearing the aforementioned null mutations is that our transgenic animals are viable, fertile and live a normal life span. They show no obvious signs of posterior limb weakness. All mesoderm-derived organs and tissues are present in normal amount. Thus, the cells fated to the ectopic neural tubes seem to be in addition to, not instead of, paraxial mesoderm tissues. Thus, we further speculate that this interference with mesenchymal cell differentiation occasions a limited hyperplasia that brings the paraxial mesoderm generation to the normal range and accounts for the increase in neuroepithelial cells that we see in the transgenic embryos. These results are corroborated by the observation that the tail buds of E9.5 transgenic mice are consistently larger than those of their wild-type littersmates. This hyperplasia seems restricted to the neuroepithelium and is evident in the MRI images of Fig. 4.

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REFERENCES


