XMeis3 protein activity is required for proper hindbrain patterning in *Xenopus laevis* embryos

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**SUMMARY**

Meis-family homeobox proteins have been shown to regulate cell fate specification in vertebrate and invertebrate embryos. Ectopic expression of RNA encoding the *Xenopus* Meis3 (XMeis3) protein caused anterior neural truncations with a concomitant expansion of hindbrain and spinal cord markers in *Xenopus* embryos. In naïve animal cap explants, XMeis3 activated expression of posterior neural markers in the absence of pan-neural markers. Supporting its role as a neural caudalizer, XMeis3 is expressed in the hindbrain and spinal cord. We show that XMeis3 acts like a transcriptional activator, and its caudalizing effects can be mimicked by injecting RNA encoding a VP16-XMeis3 fusion protein. To address the role of endogenous XMeis3 protein in neural patterning, XMeis3 activity was antagonized by injecting RNA encoding an Engrailed-XMeis3 antimorph fusion protein or XMeis3 antisense morpholino oligonucleotides. In these embryos, anterior neural structures were expanded and posterior neural tissues from the midbrain-hindbrain junction through the hindbrain were perturbed. In neuralized animal cap explants, XMeis3-antimorph protein modified caudalization by basic fibroblast growth factor and Wnt3a. XMeis3-antimorph protein did not inhibit caudalization per se, but re-directed posterior neural marker expression to more anterior levels; it reduced expression of spinal cord and hindbrain markers, yet increased expression of the more rostral En2 marker. These results provide evidence that XMeis3 protein in the hindbrain is required to modify anterior neural-inducing activity, thus, enabling the transformation of these cells to posterior fates.

Key words: *Xenopus laevis*, XMeis3, Antimorph, Antisense morpholino oligonucleotides, Caudalization, Hindbrain

**INTRODUCTION**

Formation of the central nervous system (CNS) in vertebrates such as *Xenopus* is initiated during gastrulation and largely depends on the inductive interaction between the ectoderm and adjacent dorsal mesoderm (Spemann organizer). The CNS is characterized by distinct anteroposterior (AP) and dorsoventral patterning (review by Hamburger, 1988; Doniach, 1993). The predominant concept of how AP patterning is established was suggested by Nieuwkoop (Nieuwkoop, 1952). In this two-step model, the initial neural inducing signal is thought to specify anterior neuroectodermal structures, such as cement gland and forebrain; this first step is referred to as ‘activation’. The second caudalizing step is called ‘transformation’. During this second step, anterior neural tissue is respecified to more posterior fates, such as midbrain, hindbrain and spinal cord.

Several molecules have been identified which participate in the ‘activation’ and ‘transformation’ processes. Non-neural ectoderm is induced to anterior-neural tissue by inhibition of bone morphogenetic protein (BMP) activity (reviewed by Harland and Gerhart, 1997). Secreted BMP antagonist molecules bind the BMP molecule and inhibit its receptor binding activity (Zimmerman et al., 1996; Piccolo et al., 1996; Fainsod et al., 1997; Hsu et al., 1998). BMP antagonists are expressed in Spemann’s organizer during gastrulation and induce anterior neural tissue in adjacent ectoderm (Lamb et al., 1994; Hemmati-Brivanlou and Melton, 1994; Sasai et al., 1994).

Three secreted ‘transformation’ factors have been shown to caudalize neural tissue in whole embryos or explants: retinoic acid (Durston et al., 1989; Sive et al., 1990; Ruiz i Altabaa and Jessell, 1991; Sharpe, 1991; Kolm and Sive, 1995; Papalopulu, and Kintner, 1996; Godsave et al., 1998), basic fibroblast growth factor (bFGF; Kenkgaku and Okamato, 1995; Lamb and Harland, 1995; Cox and Hemmati-Brivanlou, 1995) and Xwnt3a (McGrew et al., 1995; McGrew et al., 1997). These factors and/or their receptors are expressed in the neural plate in a temporal and regional manner, supporting their roles as caudalizers of the nervous system. All three of these molecules caudalize in non-equivalent manners and it is still not clear how they interact to specify proper AP pattern in the CNS (Kolm et al., 1997; rev. in Gamse and Sive, 2000).

In *Xenopus* embryos and explants, Meis homeobox proteins have been shown to caudalize and dorsalize the CNS (Salzberg et al., 1999; Maeda et al., 2001). The caudalizing *Xenopus Meis3* gene (Salzberg et al., 1999) was originally identified as a *Drosophila homothorax* (*hth*) gene homolog (Rieckhof et al., 1997; Kurant et al., 1998). In neurula embryos, XMeis3 is
expressed in the hindbrain from rhombomere 2 (r2) to rhombomere 4 (r4), and in the anterior spinal cord (Salzberg et al., 1999). Ectopic XMeis3 expression in embryos causes anterior truncations, with a loss of anterior neural tissues from the cement gland/forebrain until the midbrain-hindbrain junction. In parallel, hindbrain and spinal cord cell types are expanded in embryos that overexpress XMeis3. Expression of pan-neural markers is unaltered by ectopic XMeis3 expression.

In neutralized animal cap explants, ectopic XMeis3 expression inhibits anterior neural induction by BMP antagonists such as noggin or the BMP2/4 dominant-negative (DN) receptor; however, XMeis3 does not inhibit the ability of these BMP antagonists to induce pan-neural markers (Salzberg et al., 1999). Strikingly, in naïve animal cap ectoderm, ectopic XMeis3 expression induces transcriptional activation of hindbrain and spinal cord neural markers, albeit in the absence of pan-neural marker expression (Salzberg et al., 1999). This effect is ectoderm-specific, as XMeis3 does not activate transcription of mesodermal markers in injected animal cap explants (Salzberg et al., 1999). Thus, the XMeis3 protein 'uncouples' neural caudalization from neural induction.

To further examine the role of XMeis3 protein in Xenopus neural development, two distinct strategies have been used to inhibit endogenous XMeis3 protein activity. In the first strategy, fusions of a XMeis3 open reading frame to either the Engrailed transcriptional repressor domain or the VP16 transcriptional activation domain were compared in embryos and explants. We found that the Eng-XMeis3 fusion protein acted as an antimorph, blocking the effects of wild-type XMeis3-encoding RNA in Xenopus embryos and explants, while the VP16-XMeis3 fusion protein acted as a transcriptional activator to caudalize embryos and explants. In embryos, ectopic XMeis3-antimorph (XMeis3-AM) protein expression caused a loss of hindbrain marker expression, with a concomitant posterior expansion of anterior neural markers into the hindbrain region. Spinal cord and pan-neural marker expression was unaltered by the XMeis3-AM protein. In a second experimental approach, inhibition of XMeis3 mRNA translation by injection of XMeis3 antisense morpholino oligonucleotides (AMOs) also disrupted Xenopus hindbrain formation.

In animal cap explants caudalized by bFGF or Wnt3a, antagonism of XMeis3 protein activity did not specifically inhibit caudalizer activity, but it did rostralize the AP extent of posterior neural marker expression. XMeis3 activity is probably required for cells to overcome anterior neural signaling, thus enabling proper hindbrain cell fate identity in the developing Xenopus CNS.

MATERIALS AND METHODS

Construction of XMeis3-antimorph constructs

Two sets of Eng-XMeis3 and VP-XMeis3 fusion proteins were constructed. In the first set, PFU DNA polymerase (Promega) generated full-length fragments (amino acids 1-385) that were cloned in frame 3' to the VP16 or Engrailed domains in the pCS2 vector (Kessler, 1995). In the second set of constructs, the region spanning amino acids 1-333 was cloned in the same manner. These latter two constructs contain the Meis homology-box and homeodomain regions, but lack all of the region C-terminal to the homeodomain. All four of these constructs were subcloned into the pSP64TI+globin vector. Both sets of Eng-XMeis3 constructs acted as antimorphs; the shorter version was more effective and was used in the shown experiments. Both sets of VP-XMeis3 constructs acted as caudalizers; the longer version was more effective and was used in the shown experiments.

Xenopus embryos, explants and inducing factors

Ovulation, in vitro fertilization, embryo culture and dissections were carried out as described by Re’em-Kalma et al. (Re’em-Kalma et al., 1995). Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Xenopus bFGF (XbFGF) treated (50 ng/ml) animal cap explants were cultured as described by Lamb and Harland (Lamb and Harland, 1995).

RNA injections

Different concentrations of capped sense in vitro transcribed full-length XMeis3 (Salzberg et al., 1999), Eng-XMeis3 and VP16-XMeis3 (0.1-1.8 ng in a volume of 5-10 nl) were injected into the animal hemisphere of embryos at the one or two-cell stages. Capped in vitro transcribed Xenopus noggin RNA (200 pg) and mouse Wnt3a RNA (100 pg) were injected into the animal hemisphere of embryos at the one-cell stage (Smith and Harland, 1992; Baker et al., 1999).

Injection of antisense morpholino oligonucleotides (AMOs)

Antisense morpholino oligonucleotides (AMOs) complementing the 5' region of the XMeis3 mRNA were designed by and purchased from Gene Tools, LLC; Corvallis, OR (www@gene-tools.com; Heasman et al., 2000; Nasevicius and Ekker, 2000). The XMeis3 AMO sequence is 5'-ATACCTTTGTGCCATTCCGAGTTGG-3'. A standard control morpholino oligonucleotide (CMO) was also used in each experiment (Gene Tools). AMOs and CMOs were dissolved at 2 mg/ml in sterile water. One-cell embryos were routinely injected in the 10-20 ng range in 5-10 nl volumes. In two-cell stage embryos, one blastomere was injected with 7.5 ng in a 5 nl volume. The AMO was toxic at levels above 30 ng/embryo and experiments were performed at significantly lower concentrations.

In situ hybridization

Whole-mount in situ hybridization was carried out with digoxigenin-labeled probes, as described previously (Hemmati-Brivanlou et al., 1990; Harland, 1991; Knecht et al., 1995). Double in situ hybridization experiments were performed with probes generated from fluorescein and digoxigenin RNA-labeling mixes (Roche). Embryos were stained with BM purple and Fast Red substrates (Roche; Hollemann et al., 1998). In some cases, both probes were stained with BM purple. Two-cell stage albino embryos were injected unilaterally into the animal hemisphere of one-cell with 50-100 pg of RNA encoding the XMeis3-AM protein or 6-7.5 ng of the AMO. Embryos were cultured until late neurula stages and subsequently fixed for in situ hybridization. The uninjected side served as an internal control in all experiments. For lineage tracing analysis, 50 pg of RNA encoding the β-galactosidase protein (β-gal; Smith and Harland, 1991) and RNA encoding the XMeis3-AM protein were co-injected unilaterally at the two-cell stage. Embryos were stained in red for β-gal activity and fixed for whole-mount in situ hybridization as described previously (Bonstein et al., 1998). The perturbations seen in the embryos were always seen on the red stained hemisphere of embryos at the one or two-cell stages. Capped in vitro transcribed RNA (200 pg) and mouse Wnt3a RNA (100 pg) were injected into the animal hemisphere of embryos at the one-cell stage (Smith and Harland, 1992; Baker et al., 1999).

RT-PCR analysis

RT-PCR was performed as described previously (Wilson and Melton, 1994), except that random hexamers (100 ng/reaction) were used for reverse transcription. Primers for EF1α, En2, Krox20 and HoxB9 have been described elsewhere (Hemmati-Brivanlou and Melton, 1994). The primers for HoxD1 and RARα2 have been described by Kolm et al. (Kolm et al. 1997). The otx2 and XAG1 primers are described...
RESULTS

ENG-XMeis3 protein antagonizes XMeis3 caudalizing activity in contrast to VP16-XMeis3 protein

To inhibit XMeis3 protein activity during early development, we constructed an XMeis3 antimorph protein by fusing two different XMeis3 open reading frames (see Materials and Methods) to either the Engrailed (ENG) transcriptional repressor domain or the VP16 transcriptional activator domain (Fig. 1). In a relatively simple animal cap explant assay, antimorph candidate RNA molecules were screened by co-injection with wild-type XMeis3-encoding RNA (Fig. 2A-B). XMeis3 RNA activates expression of a panel of posterior neural markers in animal cap ectoderm (Salzberg et al., 1999); thus, any bona fide antimorph-encoding RNA should antagonize wild-type XMeis3 protein caudalizing activity in co-injected animal cap explants.

Co-injection of RNA encoding the ENG-XMeis3 chimera protein inhibited wild type XMeis3 caudalization activity in animal caps (Fig. 2A). As expected, XMeis3 activated Krox20, HoxB1 and HoxB9 expression in animal caps (Fig. 2A, lane 4), whereas injection of XMeis3-AM alone did not transcriptionally activate these markers (Fig. 2A, lane 5). However, in the XMeis3/XMeis3-AM co-injected group, posterior neural marker expression was eliminated (Fig. 2A, compare lane 4 with lane 6). By contrast, injection of RNA encoding the VP16-XMeis3 chimera protein induced posterior neural marker transcription in animal cap explants (Fig. 2B, compare lane 4 with lane 3). Ectopic VP16-XMeis3 expression caused anterior truncations in whole embryos (Fig. 2C); this is the same phenotype observed in embryos ectopically expressing wild-type XMeis3 RNA (Salzberg et al., 1999). These results suggest that XMeis3 protein may act as a transcriptional activator in the embryo to induce posterior neural gene expression.

Ectopic XMeis3-antimorph (XMeis3-AM) expression eliminates the hindbrain region and expands anterior neural tissues

To address the role of endogenous XMeis3 protein in early development, we injected 400-800 pg of in vitro synthesized XMeis3 antimorph (XMeis3-AM) -encoding RNA into the animal hemisphere of one-cell Xenopus embryos. Embryos were scored at tailbud to tadpole stages for phenotypes (Fig. 2D). Overexpression of XMeis3-AM RNA caused anterior expansions in over 80% (n=40/48) of the injected embryos in the shown experiment. In comparison with control embryos, these embryos had enlarged cement glands and a shortened body axis (Fig. 2D).

To further examine the effects of ectopic XMeis3-AM expression on spatial expression of neural markers, whole-mount in situ hybridization was performed on XMeis3-AM-injected late neurula stage embryos. Embryos were unilaterally injected (50-100 pg of XMeis3-AM RNA) into the animal hemisphere of one-cell Xenopus embryos. Embryos were scored at tailbud to tadpole stages for phenotypes (Fig. 2D). Overexpression of XMeis3-AM RNA caused anterior expansions in over 80% (n=40/48) of the injected embryos in the shown experiment. In comparison with control embryos, these embryos had enlarged cement glands and a shortened body axis (Fig. 2D).

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Table 1. Ectopic XMeis3-antimorph action on neural marker expression

<table>
<thead>
<tr>
<th>Gene analyzed</th>
<th>Number of embryos with modified gene expression on injected side</th>
<th>Number of embryos with unchanged gene expression on injected side</th>
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<tbody>
<tr>
<td>En2</td>
<td>48/67 (76%)</td>
<td>19/67 (24%)</td>
</tr>
<tr>
<td>Krox20</td>
<td>38/50 (76%)</td>
<td>12/50 (24%)</td>
</tr>
<tr>
<td>XE10</td>
<td>22/22 (100%)</td>
<td>0</td>
</tr>
<tr>
<td>HoxB1</td>
<td>7/12 (58%)</td>
<td>5/12 (42%)</td>
</tr>
<tr>
<td>HoxB3</td>
<td>28/38 (74%)</td>
<td>10/38 (26%)</td>
</tr>
<tr>
<td>HoxB9</td>
<td>1/22 (5%)</td>
<td>21/22 (95%)</td>
</tr>
<tr>
<td>n-tubulin</td>
<td>13/13 (100%)</td>
<td>0</td>
</tr>
<tr>
<td>Nrp1</td>
<td>0</td>
<td>13/13 (100%)</td>
</tr>
</tbody>
</table>
**Fig. 2.** Eng-XMeis3 RNA encodes an antimorph protein; VP16-XMeis3 RNA encodes a caudalizing protein. (A) One-cell stage embryos were injected in the animal hemisphere with 1.0 ng of XMeis3 RNA (lane 4), 1.6 ng of Eng-XMeis3 RNA (XMeis3-AM; lane 5) or both (lane 6). Eighteen animal cap explants were removed from uninjected (lane 3) and injected groups (lanes 4-6) of blastula embryos (stage 8-9). Explants from each group were grown to stage 20 and total RNA was isolated. RT-PCR analysis was performed with the markers: Krox20, HoxD1 and HoxB9. EF1α served as a control for quantifying RNA levels in the different samples. For controls, RT-PCR (lane 2) and –RT-PCR (lane 1) were performed on total RNA isolated from normal embryos. (B) One-cell stage embryos were injected in the animal hemisphere with 1.6 ng of VP16-XMeis3 RNA (lane 4). Eighteen animal cap explants were removed from uninjected (lane 3) and injected groups (lane 4) of blastula embryos (stage 8-9). Explants from each group were grown to stage 20 and total RNA was isolated. RT-PCR analysis was performed with the markers: Krox20, HoxB3, HoxD1 and RARα2.2. EF1α served as a control for quantifying RNA levels in the different samples. For controls, RT-PCR (lane 2) and –RT-PCR (lane 1) were performed on total RNA isolated from normal embryos. (C) Embryos at the one-cell stage were injected with 1.3 ng of in vitro transcribed VP16-XMeis3. The upper embryo serves as an uninjected control. Injected embryos had anterior truncations and highly reduced cement gland formation (lower panel). The dorsal anterior index (DAI, Kao and Elinson, 1988) was 2.5 (n=39); over 20% of the embryos completely lacked cement glands and another 80% had extreme posterior truncations, with partial cement gland formation (lower panel). Embryos are oriented posterior to anterior: left to right. Embryos were fixed for photography at stages 35/36. (D) Embryos at the one-cell stage were injected with 1.0 ng of XMeis3-AM antimorph RNA. The top embryo serves as an uninjected control. In this representative experiment, over 80% of the XMeis3-AM injected displayed phenotypes. Embryos are oriented posterior to anterior: left to right. Embryos were fixed for photography at stages 30/31.

posteriorly into the r4/r5 boundary (Fig. 3B). cpl-1 expression was also shifted into the hindbrain region, as shown by the double in situ with En2 (Fig. 3D). In the cement gland, XAG1 and XA1 (not shown) expression (Sive et al., 1989) spread posteriorly in over 60% of the embryos (n=25/40). XAG1 expression appeared to extend into both spinal cord and lateral epidermal regions (Fig. 3E).

Hindbrain marker expression was severely inhibited in XMeis3-AM-injected embryos. Krox20 (Bradley et al., 1992) expression in r3/r5 was reduced in over 75% of the injected embryos (Fig. 3G,B,K-M; Table 1). In the mildest of phenotypes, r3/r5 expression is pushed posteriorly into r5/r7 (Fig. 3K). Rhombomere 4–specific markers, such as XE10 (Weinstein et al., 1996) and HoxB1 (Godsave et al., 1998) were also highly reduced in XMeis3-AM-injected embryos (Fig. 3H-I; Table 1). Interestingly, HoxB3 (Godsave et al., 1998) expression in r5/r6 was also eliminated, yet XMeis3 is not expressed at high levels in these rhombomeres (Fig. 3J; Table 1).

*En2* (Hemmatti-Brivanlou and Harland, 1989) expression was altered in the mid-hindbrain junction in over 75% of the XMeis3-AM-injected embryos (Table 1). However, the perturbed expression of *En2* was of a more subtle nature in comparison with hindbrain markers. In embryos displaying the most moderate XMeis3-AM phenotypes, we did not observe major reductions in *En2* expression, but expression expanded as far as r3/r4 (Fig. 3C,K). In intermediate phenotypes, we observed a posterior spreading of *En2* to r2/r3 with a concomitant loss of *Krox20* expression (Fig. 3L). In more extreme phenotypes, *En2* expression was lost, together with *Krox20* (Fig. 3M,D).

Expression of the pan-neural *nrp1* marker (Fig. 3F; Table 1; Richter et al., 1988) and the spinal cord-specific *HoxB9* marker (Fig. 3G; Table 1; Wright et al., 1990) was unaltered by ectopic *XMeis3-AM* expression, despite overlapping *XMeis3* mRNA expression in the hindbrain and anterior spinal cord (Salzberg et al., 1999). Interestingly, expression of the neuron specific n-tubulin marker (Hollemann et al., 1998) was highly inhibited (Fig. 3N; Table 1) by ectopic XMeis3-AM activity. In strong phenotypes, both the r2-derived trigeminal neuron as well as the more posterior neural expression was eliminated. In more moderate phenotypes, the trigeminal was still missing but posterior expression was less inhibited (not shown). Thus, XMeis3 may have a role in early neuron specification.

These results demonstrate that ectopic XMeis3-AM expression can cause an anterior transformation of the hindbrain by inhibiting caudalization. Thus, functional XMeis3
protein appears to be required for correct specification of the hindbrain in early Xenopus development.

**Antisense morpholino oligonucleotides also inhibit XMeis3 caudalizing activity and hindbrain pattern**

An additional molecular tool for antagonism of in vivo XMeis3 protein activity is antisense morpholino oligonucleotides (AMOs). AMOs have recently been shown to inhibit mRNA translation in Xenopus and zebrafish embryos (Hasman et al., 2000; Nasevicius and Ekker, 2000). AMOs, complementary to the 5’ UTR and spanning the initial translated codons of the XMeis3 mRNA (see Materials and Methods) were injected at the one-cell stage into the animal hemisphere of embryos. In every experiment, a control morpholino oligonucleotide (CMO) was injected at an identical concentration to the AMO (see Materials and Methods). As described previously for XMeis3-AM, the specific inhibitory effect of AMOs on XMeis3 activity was screened by co-injection with wild-type XMeis3 RNA. Injection of XMeis3 AMOs together with XMeis3 wild-type RNA inhibited caudalizing activity in both animal cap explants and whole embryos (Fig. 4A). In animal cap explants, XMeis3 ectopically activated expression of the Krox20, HoxB9, and HoxB3 genes (Fig. 4A, lane 7), but in explants co-expressing XMeis3 and the AMO, posterior neural marker expression was eliminated (Fig. 4A, lane 9). In whole embryos, ectopic XMeis3 significantly increased Krox20 and HoxB3 gene expression (Fig. 4A, lane 2); this increase was inhibited by co-expression with the AMO (Fig. 4A, lane 5). Strengthening this observation, expression of the AMO in embryos significantly reduced normal Krox20 and HoxB3 expression levels, in comparison with CMO-injected control embryos (Fig. 4A, compare lanes 2 and 4). HoxB9 expression

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**Fig. 3.** Expression pattern of neural markers in embryos injected with XMeis3-AM RNA. Two-cell albino embryos were injected unilaterally into the animal hemisphere of one blastomere with 50-100 pg of XMeis3-AM RNA. The red arrow delineates the dorsal midline. In all embryos, XMeis3-AM injection is on the left side. In all cases (except Fig. 3M), embryos are viewed dorsally; embryos are oriented anterior (top) to posterior (bottom). (A) In situ hybridization with otx2. Expression is expanded posteriorly on the XMeis3-AM injected side. The red lines delineate the AP extent of otx2 expression on the uninjected versus injected side. (B) In situ hybridization with otx2 and Krox20. otx2 expression is expanded posteriorly and Krox20 expression (blue arrows) is lost on the XMeis3-AM-injected side. The red lines delineate the AP extent of otx2 expression on the uninjected versus injected side. (C) In situ hybridization with otx2 and En2 (red). Expression of otx2 and En2 (blue arrows) is expanded posteriorly. (D) In situ hybridization with cpl-1 and En2 (red). cpl-1 expression is expanded posteriorly and En2 expression (blue arrow/uninjected side) is lost on the XMeis3-AM-injected side. The red lines delineate the AP extent of cpl-1 expression on the uninjected versus injected side. (E) In situ hybridization with XAG1; expression is expanded posteriorly and laterally on the XMeis3-AM injected side. (F) In situ hybridization with arpl1; expression is unchanged on the XMeis3-AM injected side. (G) In situ hybridization with Krox20 and HoxB9; Krox20 expression (blue arrows/uninjected side) is eliminated on the XMeis3-AM-injected side. HoxB9 expression is unchanged on the XMeis3-AM injected side. (H) In situ hybridization with XE10. XE10 expression is eliminated on the XMeis3-AM-injected side. (I) In situ hybridization with HoxB1. HoxB1 expression is eliminated on the XMeis3-AM injected side. (J) In situ hybridization with HoxB3. HoxB3 expression is eliminated on the XMeis3-AM-injected side. (K) In situ hybridization with En2 (red) and Krox20. En2 expression is pushed posteriorly to the r3/r4 boarder and Krox20 expression is pushed posteriorly to the r5/r7 boarder on the XMeis3-AM-injected side. (L) In situ hybridization with En2 and Krox20. En2 expression is pushed posteriorly to r3 on the XMeis3-AM-injected side. The blue arrow delineates the reduced Krox20 expression (fused stripes) on the injected side. (M) In situ hybridization with En2 (red) and Krox20. Expression of En2 and Krox20 (blue arrows/uninjected side) is eliminated on the XMeis3-AM-injected side. An anterior view of the embryo: dorsal (top) to ventral (bottom). (N) In situ hybridization with n-tubulin. n-tubulin expression is eliminated on the XMeis3-AM-injected side. The trigeminal neuron is marked by blue arrows on both sides.
was not disrupted in AMO-injected embryos (Fig. 4A, lanes 2-5).

To demonstrate that the AMO was indeed inhibiting translation of XMeis3 RNA, the AMO was co-injected with RNA encoding a XMeis3-Myc-tagged protein. As determined by Western blot analysis, co-injection of AMO prevented translation of this XMeis3-Myc tagged protein in comparison with embryos co-injected with the CMO (Fig. 4B, compare lanes 2 and 3). The effects on XMeis3-Myc translational inhibition were specific, as AMO and CMO injections did not inhibit endogenous Xenopus proteins; Erk levels were identical in all groups (Fig. 4B, compare lanes 1-3). In contrast to protein levels, the AMO did not alter XMeis3-Myc RNA levels, as RNA levels were identical in the AMO- and CMO-injected groups (Fig. 4C, compare lanes 1 and 2).

In XMeis3-Myc/CMO-injected embryos, XMeis3-Myc protein acts as a caudalizer, increasing Krox20 expression in these embryos (Fig. 4C, compare lanes 2 and 3). However, when XMeis3-Myc/AMO was co-injected, levels of Krox20 RNA were highly reduced, significantly below levels in control embryos (Fig. 4C, lanes 2-4).

To further determine the role of endogenous XMeis3 protein in the embryo, we used the AMO to inhibit endogenous XMeis3 mRNA translation during early development. We injected 12.5-20 ng of the AMO into the animal hemisphere of one-cell stage embryos; these embryos were scored at tailbud to tadpole stages for phenotypes (Fig. 5). Like ectopic XMeis3-AM expression, the AMO (17.5-20 ng) caused anterior expansions and cement gland enlargement in over 80% (n=41/49) of the injected embryos (Fig. 5A, lower panel), in comparison with control CMO-injected embryos (Fig. 5A, upper panel). As in the case of the XMeis3-AM phenotypes, these embryos also had a much shorter body axis; body length was reduced by approximately 25-33% in AMO phenotypic embryos. At lower AMO concentrations (12.5-15 ng), body length was still altered in over 75% of the embryos (n=37/54), and anterior expansion phenotypes were weaker (Fig. 5A, middle panel). Thus, like the XMeis3-AM protein, injection of the AMO caused a prominent dose-dependent anteriorized phenotype in embryos.

By RT-PCR analysis, neural marker expression was examined over the 12.5-20 ng AMO concentration range (Fig. 5B). Krox20 expression is the most sensitive to loss of XMeis3
Xmeis is required for hindbrain patterning activity, being highly reduced at all concentrations examined (Fig. 5B). HoxB3 expression was also inhibited (not shown). En2 expression is lost, but in a graded manner and only at higher AMO concentrations (Fig. 5B). HoxB9 expression was not reduced, being slightly increased in AMO-injected embryos (Fig. 5B). Expression levels of the ectodermal and mesodermal markers, epidermal cytokeratin and muscle actin were not significantly altered by the AMOs (not shown).

To demonstrate AMO specificity, XMeis3 and Drosophila hth-encoding RNAs were separately co-injected into embryos together with the AMO. We have previously shown that ectopic hth expression can caudalize Xenopus embryos and animal cap explants, in the same way as XMeis3 (Salzberg et al., 1999). As the hth gene lacks the XMeis3 5' region encoded by the AMO, we expect that its caudalizing activity should not be affected by the AMO. Indeed, both RNAs caudalized embryos.

**Fig. 5.** XMeis3 antisense morpholino oligonucleotide causes posterior truncations and anterior expansions in embryos. (A, top) Embryos at the one-cell stage were injected with 20 ng of the CMO. These embryos resembled uninjected controls. All embryos were fixed for photography at stage 38. (A, middle) Embryos at the one-cell stage were injected with 12.5 – 15 ng of the AMO. (A, bottom) Embryos at the one-cell stage were injected with 17.5-20 ng of the AMO. (B) Embryos from the experiment in A were grown to late neurula stages. Total RNA was also isolated from pools of seven embryos from each of the injected groups (lanes 1-6). RT-PCR analysis was performed with the markers: En2, Krox20 and HoxB9. EF1α served as a control for quantifying RNA levels in the different samples. For controls, RT-PCR (lane 2) and –RT-PCR (lane 1) were performed on total RNA isolated from control CMO injected embryos (lane 2). (C) Embryos at the one-cell stage were injected with 15 ng of the CMO (top) or the AMO (bottom). CMO- and AMO-injected embryos were co-injected with either 1.6 ng of XMeis3 RNA (middle) or 1.6 ng of hth RNA (right). White arrows mark the cement glands. All embryos were fixed for photography at stage 27/28.
in a similar manner: 85% of the XMeis3/CMO injected embryos (n=8) and 75% of the hth/CMO (n=16) -injected embryos had small cement glands (Fig. 5C, upper panel). XMeis3/AMO-injected embryos had rescued caudalized phenotypes: nearly 80% of the embryos (n=18) had normal or expanded cement glands (Fig. 5C, lower panel). In sharp contrast, 70% (n=20) of the hth/AMO-injected embryos had small cement glands (Fig. 5C, lower panel), like the XMeis3/CMO-injected group (Fig. 5C, upper panel). In the AMO-injected control group, 75% (n=16) of the embryos had expanded cement glands (lower left panel), in comparison with the CMO-injected (upper left panel) group (n=10). Similar results were also seen in animal cap explants; expression of Krox20 was reduced in XMeis3/AMO versus XMeis3/CMO-injected explants, but levels of Krox20 expression were identical in hth/AMO- and hth/CMO-expressing explants (not shown). These results show that the AMO cannot inhibit hth caudalizing activity, thus, the AMO is indeed specific to the XMeis3 gene.

To further examine the role of the AMO in neural patterning, whole embryos at the two-cell stage were injected unilaterally into one blastomere with 6-7.5 ng of AMO, and whole-mount in situ hybridization was performed. We saw a dramatic reduction in hindbrain marker expression on the injected side: Krox20 (Fig. 6A), XE10 (Fig. 6B) and HoxB3 (Fig. 6C). HoxB9 expression in the spinal cord was not decreased in AMO-injected explants (Fig. 6A,C). In AMO-injected embryos, XE10 expression was exclusively inhibited in the hindbrain (where XMeis3 expression overlaps), but not in ectoderm regions found lateral to the neural tube, where XMeis3 was not expressed (Fig. 6B). We also examined how AMO injection altered endogenous XMeis3 expression in r2-r4 and the anterior spinal cord. In moderate phenotypes (Fig. 6D), we detected a shift of the XMeis3 rhombomeric expression from r2-r4 to r5-r7 with a fusion of the expression domain to the spinal cord. In the same embryo, En2 expression is pushed to the approximate r2/r3 boarder (Fig. 6D). In more extreme phenotypes (Fig. 6E), the XMeis3 expression pattern is again shifted posteriorly, but XMeis3 mRNA levels are also highly reduced. These data strongly corroborate the results obtained with the XMeis3-AM protein (Fig. 3), providing substantial proof that XMeis3 protein activity is obligatory for proper cell fate determination in the hindbrain.

Caudalized animal caps: XMeis3-AM protein rostralizes AP coordinates

To elucidate specific XMeis3 interactions with caudalizing pathways, XMeis3-AM was ectopically expressed in animal caps caudalized by either Xenopus bFGF (XbFGF) or mouse Wnt3a proteins. At the one-cell stage, XMeis3-AM, noggin and/or mouse Wnt3a RNAs were injected in the animal hemisphere; at blastula stages animal cap explants were removed. In the experiments with XbFGF, animal caps were aged until stage 10.25, when XbFGF treatment was initiated (Lamb and Harland, 1995). Total RNA was isolated for RT-PCR analysis at late neurula stages.

In noggin-neutralized animal cap explants, XMeis3-AM protein modified caudalization by XbFGF and mouse Wnt3a. In these caps, the perturbation of XMeis3 activity did not inhibit caudalization per se, but did bias neural marker expression in a more anterior manner. In both noggin/XbFGF- and XbFGF-treated animal caps explants, XMeis3-AM protein decreased expression of spinal cord-specific HoxB9 marker, yet increased expression of the more anterior En2 marker (Fig. 7A). This effect was dependent on the initial AP coordinates of the explant. When caps are treated with XbFGF, only HoxB9 is induced (Fig. 7A, lane 3), yet in the presence of the XMeis3-AM protein, both HoxB9 (reduced levels) and En2 are expressed (Fig. 7A, lane 4). In XbFGF/noggin-treated caps, both En2 and HoxB9 are expressed (Fig. 7A, lane 5); however, in the presence of XMeis3-AM protein, En2 is exclusively expressed and at increased levels (Fig. 7A, lane 6). Thus, the final extent of anteriorization in the XMeis3-AM injected explants appears determined by the initial AP patterning coordinates in the explant that were pre-determined by FGF+noggin. The presence of the XMeis3-AM protein shifted neural marker expression in favor of the more anterior mid-hindbrain.
We have used two experimental approaches to inhibit endogenous XMeis3 protein activity in developing Xenopus embryos. In the first approach, we constructed an antimorph protein, by fusing XMeis3 open reading frames to the engrailed repressor or VP16 activator transcriptional domains. In the second approach, we injected an antisense morpholino oligonucleotide (AMO) homologous to the 5′ end of the XMeis3 mRNA. In a relatively simple bioassay in animal cap explants, we determined that the Eng-XMeis3 fusion protein inhibited caudalizing activity when co-injected with wild-type XMeis3 RNA. In the same animal cap assay, the VP16-XMeis3 fusion protein strongly activated expression of posterior neural markers. We thus concluded that the Eng-XMeis3 fusion protein acted as a bona fide antimorph protein.

Further supporting this observation, studies in transgenic flies that express either Xenopus Eng-XMeis3 or Drosophila Eng-HTH chimera proteins demonstrated lth loss-of-function like phenotypes (Inbal et al., 2001). In a complementary manner, transgenic flies expressing VP16-HTH chimera protein displayed lth gain-of-function like phenotypes; VP16-HTH also rescued phenotypes in lth mutant embryos (Inbal et al., 2001). Our previous studies demonstrated that ectopic expression of either wild-type HTH or XMeis3 proteins caudalized Xenopus embryos and animal cap explants (Salzberg et al., 1999). These results suggest that the Meis family transcriptional activator function has been conserved for nervous system development in such diverse organisms as flies and frogs.

Like the XMeis3-AM protein, injection of AMOs also inhibited wild-type XMeis3 caudalizing activity in embryos and animal cap explants. XMeis3-Myc protein levels were eliminated by the AMO. These results show that the AMO also acts as a potent inhibitor of XMeis3 activity, by preventing mRNA translation.

To address the role of XMeis3 during Xenopus CNS development, one-cell embryos were injected with the ENG-XMeis3 (XMeis3-AM) RNA, VP16-XMeis3 RNA or AMOs. Ectopic expression of VP16-XMeis3 RNA caudalized Xenopus embryos in a manner similar to the wild-type XMeis3-encoding RNA. By contrast, both XMeis3-AM and AMOs had distinct posterior-truncation/anterior-expansion phenotypes. In these embryos, the cement gland was expanded and the body axis was shortened.

To further address this point, albino embryos were unilaterally injected with XMeis3-AM or AMOs into one blastomere at the two-cell stage. At neurula stages, a wide array of neural markers were examined by whole-mount in situ hybridization. Confirming the phenotypic observations, in XMeis3-AM-injected embryos, we saw an expansion of expression of anterior markers such as XAG1, cpl-1, otx2 and En2 into more posterior regions of the brain. In the most extreme cases, we saw otx2 expression shifted as far back as r4/r5 and En2 expression was eliminated. In more moderate phenotypes, otx2 expression was shifted to r1/r2 and En2 expression was shifted to r3/r4. The posterior spread and loss of En2 and endogenous XMeis3 expression, and the concomitant loss of the r2-derived trigeminal neuron demonstrate that patterning in the most anterior hindbrain r1/r2 regions is greatly disrupted by the loss of endogenous XMeis3.
activity. Rhombomeric expression of the Krox20, XE10, HoxB1 and HoxB3 markers was severely reduced by the XMeis3-AM protein or the AMO. In the most moderate hindbrain phenotypes, we could detect a two rhombomeric-shift of Krox20 expression from r3/r5 to r5/h7. By contrast, the spinal cord and pan-neural markers, HoxB9 and nrl were unaffected by XMeis3-AM or AMO activity.

It also appears that neurogenesis may be affected by the loss of XMeis3 activity. In XMeis3-AM injected embryos, n-tubulin expression is always lost in the r2-derived trigeminal neuron, and this could be a reflection of rhombomeric identity loss in this region. In most cases, injection of the XMeis3-AM also inhibited posterior n-tubulin expression. Neither XMeis3 nor noggin strongly induces n-tubulin expression in animal cap explants; however, in the presence of both molecules, we detected high levels of n-tubulin expression in animal caps (S. E. and D. F., unpublished). Thus, further experiments need to be performed to determine the exact role for XMeis3 protein in specifying neuron cell fates along the AP axis.

These results show that functional XMeis3 protein maintains a proper AP balance required for hindbrain formation. The spread of expression of anterior neural markers posteriorly into the hindbrain suggests that XMeis3 is essential for actively maintaining a caudalized state in the hindbrain. While XMeis3 does not seem required for neural induction, it seems to fine tune the AP pattern in the forebrain-hindbrain region. The conversion of hindbrain regions to more anterior fates, with concomitant posterior expansion of XAG1, cpl-1, otx2 and En2 expression emphasizes the role of XMeis3 in this AP fine-tuning process. XMeis3 is expressed in the anterior spinal cord; however, it may not be required for proper spinal cord formation.

Animal cap assays shed an interesting light on the interactive role of XMeis3 with caudalizing signaling molecules such as XbFGF and Wnt3a, confirming a role for XMeis3 as a neural patterning gene. Ectopic XMeis3-AM expression did not specifically inhibit caudalizing activity by these signaling molecules. However, the lack of XMeis3 activity did lead to a rostral shift in the AP levels of these explants that was dependent on the initial AP coordinates in the explants. In the case of animal cap explants treated solely with XbFGF, these caps expressed HoxB9 but not En2; however, these explants expressed both HoxB9 (reduced) and En2, when XMeis3 activity was inhibited. XbFGF/noggin-treated animal caps expressed both HoxB9 and En2, yet in the presence of the XMeis3-AM, these caps ceased to express HoxB9 and had increased levels of En2. Thus, in XMeis3-AM-expressing animal caps, the final AP output was determined by the initial AP status of the explant. XMeis3-AM protein shifted posterior neural marker expression to the anterior mid-hindbrain junction, while inhibiting expression of spinal cord and hindbrain markers. Our previous studies have shown that XMeis3 caudalization activity requires functional FGF/mitogen-activated protein kinase signaling (Ribisi et al., 2000); however, this relationship is not reciprocal, as bFGF caudalizing activity per se is not dependent on XMeis3 activity. This result strongly supports a role for XMeis3 as a cell patterning protein that interprets and maintains a given AP status in the CNS.

The interpretation of experiments in which animal cap explants are caudalized by mouse Wnt3a is more complicated, but supportive of the results with bFGF. In noggin/Wnt3a-expressing caps, a similar rostralization was observed in the presence of XMeis3-AM, a gain of En2 expression, with a concomitant loss of HoxB9, HoxB3 and Krox20 expression. However, a somewhat contrasting result was seen with the coinjection of mouse Wnt3a and XMeis3-AM in the absence of noggin. In these experiments, there was indeed an increase in En2 expression and a decrease in hindbrain marker expression, but we did not observe a reduction in HoxB9 expression. In some experiments, we even saw an increase in HoxB9 expression (not shown). It appears that in the absence of a strong neural inducer, mouse Wnt3a is a relatively weak inducer of hindbrain in comparison with XMeis3; however, mouse Wnt3a may actually induce spinal cord better than XMeis3. We have found that when XMeis3 induces maximal levels of hindbrain markers, HoxB9 expression is reduced in animal cap explants (not shown). Apparently, when mouse Wnt3a caudalizes alone, its induction of the HoxB9 spinal cord marker is optimal when some XMeis3 target genes are inhibited by the XMeis3-AM. Perhaps, antagonism of specific XMeis3 target genes by the antimorph protein may enable mouse Wnt3a to more efficiently activate spinal cord markers instead of hindbrain markers in the absence of neural induction. However, in the presence of noggin, XMeis3 target genes appear to be required for high HoxB9 and hindbrain marker expression by mouse Wnt3a. In whole embryos, HoxB9 expression is unchanged or even slightly increased in the presence of XMeis3-AM or the AMO. Inhibition of Xwnt-3a activity reduces HoxB9 expression in embryos and explants (McGrew et al., 1997), so a delicate balance between Wnt and XMeis3 activities may maintain optimal HoxB9 expression levels in the spinal cord. The implications of these wnt/XMeis3 interactions are still unclear, and further experiments are being carried out to understand how Wnt and XMeis3 pattern posterior neural tissue.

Using two distinct molecular approaches, we have inhibited XMeis3 protein activity in Xenopus embryos. In these embryos, a clear perturbation of the posterior CNS is observed, most specifically in the hindbrain region. XMeis3 appears to give distinct spatial identity to hindbrain cells. Without proper XMeis3 activity, anterior neural tissue spreads posteriorly and hindbrain identity is lost. The hindbrain is trapped in a more rostral cell fate. XMeis3 caudalizes the CNS to hindbrain, without inducing neural tissue. When viewing the ‘activation’ and ‘transformation’ model of neural induction, functional XMeis3 activity may be prerequisite for the ‘transformation’ step. XMeis3 probably interprets spatial information along AP axis in hindbrain cells, thus enabling them to differentiate in a proper manner. Future studies will focus on how XMeis3 functions as a transcriptional activator to caudalize the brain. By identifying genes directly targeted by XMeis3, we intend to determine the genetic hierarchy regulating hindbrain formation in the developing CNS.

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