Knockdown of the complete Hox paralogous group 1 leads to dramatic hindbrain and neural crest defects

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Summary

The Hox paralogous group 1 (PG1) genes are the first and initially most anterior Hox genes expressed in the embryo. In *Xenopus*, the three PG1 genes, *Hoxa1*, *Hoxb1* and *Hoxd1*, are expressed in a widely overlapping domain, which includes the region of the future hindbrain and its associated neural crest. We used morpholinos to achieve a complete knockdown of PG1 function. When *Hoxa1*, *Hoxb1* and *Hoxd1* are knocked down in combination, the hindbrain patterning phenotype is more severe than in the single or double knockdowns, indicating a degree of redundancy for these genes. In the triple PG1 knockdown embryos the hindbrain is reduced and lacks segmentation. The patterning of rhombomeres 2 to 7 is lost, with a concurrent posterior expansion of the rhombomere 1 marker, *Gbx2*. This effect could be via the downregulation of other Hox genes, as we show that PG1 function is necessary for the hindbrain expression of Hox genes from paralogous groups 2 to 4. Furthermore, in the absence of PG1 function, the cranial neural crest is correctly specified but does not migrate into the pharyngeal arches. Embryos with no active PG1 genes have defects in derivatives of the pharyngeal arches and, most strikingly, the gill cartilages are completely missing. These results show that the complete abrogation of PG1 function in *Xenopus* has a much wider scope of effect than would be predicted from the single and double PG1 knockouts in other organisms.

Key words: Hox, PG1, *Xenopus*, Hindbrain, Neural crest

Introduction

A vital aspect of vertebrate development is the organisation and patterning of different tissues along the various axes of the embryo. Along the anteroposterior (AP) axis this occurs partly under the control of the Hox family of transcription factors. These homeobox-containing genes have been conserved throughout evolution and are responsible for the patterning of various tissues at specific axial levels (McGinnis and Krumlauf, 1992). In vertebrates, the Hox genes are organised in multiple clusters, which contain 13 paralogous groups, identified on the basis of DNA sequence and the position on the chromosome. These clusters are believed to be derived from the successive duplications of an ancestral Hox cluster. The subsequent loss of certain genes, and divergence and function shuffling in the remaining genes resulted in the clusters as they are now (Prince and Pickett, 2002). One interesting aspect of the Hox genes is that they are expressed in a colinear fashion. This means that genes which are located more 3′ in the cluster are generally expressed at an earlier developmental time point [temporal colinearity (reviewed by Deschamps et al., 1999)] and also at a more anterior position in the embryo [spatial colinearity (reviewed by Duboule and Dolle, 1989; Graham et al., 1989)]. The mechanism behind this colinearity is not yet delineated but the final effect is to produce a complex pattern of Hox gene expression, known as the 'Hox code', which defines particular AP axial levels (Deschamps et al., 1999; Maconochie et al., 1996).

Often however, the removal of the function of one particular Hox gene, or even one complete cluster, does not have dramatic consequences for the embryo (Spitz et al., 2001; Suemori and Noguchi, 2000). This, together with the observation that paralogous genes often have similar functions as well as similar expression domains, points to the possibility of functional redundancy between genes from the same paralogous group (PG). This indeed appears to be the case. The loss of function of complete paralogous groups have been shown to be more severe than knockouts of single Hox genes in both zebrafish [PG2 (Hunter and Prince, 2002)] and mouse [PG8 (van den Akker et al., 2001)]. Thus the knockdown of a single Hox gene may not reveal its complete function, and entire paralogous groups may need to be abrogated before their shared role can be illuminated.

Here we investigate the function of the PG1 genes, which are the homologues of the *Drosophila labial* gene. These are the earliest of the Hox genes, with expression starting during gastrulation, and they eventually have their anterior boundary in the hindbrain at the level of rhombomeres (r) 3/4 in most vertebrates (Frohman et al., 1990; Frohman and Martin, 1992; Godsave et al., 1994; Kolm and Sive, 1995; Murphy and Hill, 1991; Sundin et al., 1990; Wilkinson et al., 1989). *Hoxa1* is also expressed at a later stage in the fore/midbrain (McClintock et al., 2002; Shih et al., 2001).

Knockout studies in mouse have concentrated on the *Hoxa1* and *-b1* genes, and have implicated these genes in hindbrain and craniofacial development. In the *Hoxa1* null mutant, r5 is either absent or severely reduced, r4 is reduced and there are

defects in the hindbrain and associated nerves in the region between r3 and r8 (Carpenter et al., 1993; Chisaka et al., 1992; Dolle et al., 1993; Lufkin et al., 1991; Mark et al., 1993). In the *Hoxb1* knockout the identity of r4 is altered, but segmentation is not affected (Goddard et al., 1996; Studer et al., 1996). When both *Hoxa1* and *Hoxb1* are deleted, a more severe phenotype is observed than in either of the single knockouts, with both r4 and r5 being mis-specified (Gavalas et al., 1998; Studer et al., 1998) and eventually deleted (Rossel and Capecchi, 1999) as well as the 2nd pharyngeal arch and its derived tissues being lost. In zebrafish, knockdown of the *Hoxb1b* gene (thought to be the functional equivalent of the mouse *Hoxa1* gene) leads to disruption of r4 and *Hoxb1a*, like mouse *Hoxb1*, is involved in the specification of nerves originating in r4. The double knockdown of *Hoxb1b* and *Hoxb1a* also implies some degree of functional redundancy between these genes. However, the phenotype of the double knockdown is not as severe as that observed in mouse, with r4 and r5 always present, albeit reduced (McClintock et al., 2002).

Despite the intense interest in these anterior Hox genes, a complete knockdown of all PG1 genes has not yet been performed. Therefore any function that is shared between all of the genes may still be hidden. To address this question we knocked down all the *Xenopus laevis* Hox PG1 genes. In *Xenopus*, the early expression of the three PG1 genes (*Hoxa1*, *Hoxb1* and *Hoxd1*) is highly overlapping and they are all expressed in the presumptive hindbrain region. Here we use the morpholino (MO) knockdown technique to demonstrate that the complete loss of PG1 gene function has deeper implications for the development of the embryo than the loss of the individual genes.

Materials and methods

Embryos and explants

Xenopus laevis embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1956). Culture of embryos and buffers was as described (Winklbauer, 1990).

Cloning of the *Xenopus Hoxd1* morpholino insensitive construct

The complete open reading frame of *Xenopus Hoxd1* (Sive and Cheng, 1991) was amplified using primers incorporating *Bam*HI and *Xho*I restriction sites (for: 5′CCGGGATCCGCCGCCACCATGAATTCCTACCTAGAATACACTTCTTGCGGG; rev: 5′TGCACTCGAGCTAGGGTGAAGCGTCCTTGGATGGCG). After ligation of the PCR product into the pGEM-T Easy vector (Promega), *Hoxd1* was excised by *Bam*HI/*Xho*I digestion, ligated into the CS2+ vector (Turner and Weintraub, 1994), and checked by sequencing.

Injection of morpholinos and mRNA

Two morpholinos were designed for each Hox PG1 gene (Gene-Tools Inc.) The pseudo-tetraploidy of *Xenopus laevis* was taken into consideration and the morpholinos were all designed to be effective against both alleles present – determined by 5′ RACE analysis for *Hoxd1* and comparison with available EST data for *Hoxa1* and *Hoxb1*. Sequence of morpholinos is as follows: *Hoxa1*, MO1-5′CTCATCCTCCTCGCATAGTCCATCT, MO2-5′CTCGCATAGTCCATCTATCACTAGG; *Hoxb1*, MO1-5′AGGAACTCATTCTGCTATTGTCCAT, MO2-5′ATCTGCCAGTGATGGAGGAGGGTCA; *Hoxd1*, MO1-5′AGGAACCTGCTGATCCCTCAATCTT, MO2-5′TAGGTAGGAATTCATCTCTAGGGAG. Morpholinos and mRNAs were diluted in Gurdon's buffer (15 mM Tris pH 7.5, 88 mM NaCl, 1 mM KCl) and injected at the four-cell stage into both left blastomeres with *GFP* mRNA co-injected as a lineage tracer. Before fixation the GFP was checked to ensure that the injections were on the correct side. Amounts injected ranged from 5 ng to 30 ng of PG1 morpholinos and 7.5 ng to 60 ng of control morpholino. Whenever a comparison was made between single, double or triple MO injections the total amount of MO was equalised by adding control morpholino. Several combinations of the 1st and 2nd MOs were analysed with the *Krox20* and *Engrailed-2* probes and gave the same result. In all experiments control morpholino (standard control, Gene-Tools Inc.) was also injected and the embryos included in subsequent analyses, but this never gave different results from the non-injected controls. CS2+GFP (25 pg) and CS2+xHoxd1 (100 pg) were linearised with *Not*I and transcribed with Sp6 polymerase.

Detection of gene expression by in situ hybridisation

The whole mount in situ hybridisation protocol used was described previously (Wacker et al., 2004b), as modified from a previous protocol (Harland, 1991). Antisense, digoxigenin-labelled probes were: *Hoxd1* (Sive and Cheng, 1991); *Hoxa1*, *Hoxb1*, *Hoxc6*, *Hoxb9* (Wacker et al., 2004a); *Krox20* (Bradley et al., 1993); *Engrailed-2* (Hemmati-Brivanlou et al., 1991); *Nrp1* (Richter et al., 1990); *Gbx2* (von Bubnoff et al., 1996); *Xslug* (Mayor et al., 1995); *Xsnail* (Mayor et al., 1993); *dll4* (Papalopulu and Kintner, 1993); *Otx2* (Pannese et al., 1995); *Hoxa2* (Pasqualetti et al., 2000); *Myod* (Hopwood et al., 1989); EST clones from the I.M.A.G.E. Consortium [LLNL] cDNA library (Lennon et al., 1996), *Hoxa3* (IMAGE4405749), or the NIBB library, *Hoxd3* (XL012i13); *Hoxd4* (XL094l20); *Hoxa5* (XL045g13).

Neural antibody analysis and cartilage staining

After bleaching (80% methanol, 6% H_2O_2, 15 mM NaOH), stage 46 embryos were washed (4×30 minutes PBS+0.2% Tween), blocked (30 minutes PBS+ 0.2% Tween, 3% BSA) and incubated overnight at 4°C with the neural antibody 2G9 (Jones and Woodland, 1989). The embryos were then washed and incubated overnight at 4°C with a secondary antibody conjugated to the Cy5 fluorophore. After washing, the embryos were dehydrated and fixed step-wise in methanol (25%, 50%, 75%, 100%). Before analysis embryos were cleared in Murrays and the hindbrain was visualised using scanning confocal microscopy (Leica TCS-NT). To visualize the cartilage, stage 49 embryos were fixed and stained with Alcian Blue (Pasqualetti et al., 2000).

Results

Hox PG1 genes are expressed in overlapping domains throughout development

To gain as complete a picture as possible of the expression of the PG1 genes, and to determine the possibilities for overlap of function, we performed an extensive whole mount in situ hybridization study of Hox PG1 gene expression (Fig. 1). This is the first comparative study of all three genes, although there is data on aspects of the individual genes (Godsave et al., 1994; Kolm and Sive, 1995; Wacker et al., 2004a). All three PG1 Hox genes begin to be expressed during gastrulation, where they are seen in a ring around the blastopore, with a gap on the dorsal side. When this expression is compared with the expression of the forebrain/midbrain marker, *Otx2*, it can be seen that whilst none of the Hox genes' anterior expression reaches the midbrain, *Hoxb1* appears to be expressed more anteriorly than *Hoxa1* and *Hoxd1*, almost fusing with the *Otx2* domain (Fig. 1A,I,Q). As elongation procedes, differences in the gene expression patterns begin to arise. As early as stage 14, a stripe of *Hoxb1* expression in the future hindbrain is observed and this becomes further defined by stage 18 (Fig. 1K-N). *Hoxa1*

and *Hoxd1* on the other hand do not exhibit this early stripe of expression, although they are both expressed in the hindbrain region (Fig. 1E,U). Expression of *Hoxd1* thereafter decreases and from stage 26 there only remains faint expression in the pharyngeal arches and the tailbud (Fig. 1W,X). The hindbrain stripe of *Hoxb1* expression persists until at least stage 30, accompanied by faint expression in the pharyngeal arches (Fig. 1O,P). *Hoxa1* remains expressed in a comparatively large domain along the axis, including the pharyngeal arches. In addition, as has been previously reported (McClintock et al., 2002), we observed midbrain expression of *Hoxa1* at stage 26 and 30 (Fig. 1G,H). Thus, the Hox PG1 genes are expressed in expansive and overlapping domains throughout early development, and all three of them are expressed in the hindbrain while it is being specified and patterned (Melton et al., 2004), and at a later stage in the pharyngeal arches.

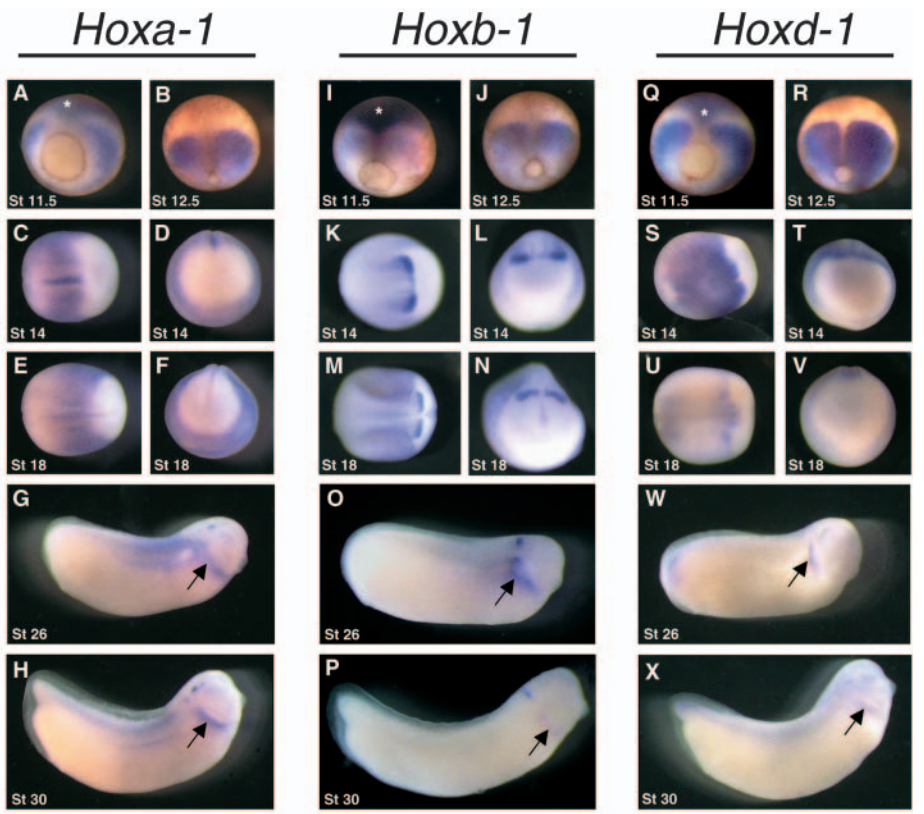

Fig. 1. Hox PG1 genes have overlapping expression domains during *Xenopus laevis* development. *Hoxa1* (A-H), *Hoxb1* (I-P) and *Hoxd1* (Q-X) have overlapping expression patterns. Gastrula stage embryos (A,B,I,J,Q,R) are shown from the vegetal side, dorsal up. When compared with Otx-2 expression (* in A,I,Q), it can be seen that the Hox expression does not reach the presumptive midbrain region. Neurula embryos are shown from the dorsal side, anterior to the right (C,E,K,M,S,U) or from the anterior side, dorsal up (D,F,L,N,T,V). Later embryos are shown from the lateral side, anterior to the right (G,H,O,P,W,X). Note pharyngeal arch expression of all three PG1 genes (arrows in G,H,O,P,W,X).

Knockdown of individual Hox PG1 genes leads to defects in rhombomere 4 formation

To analyse the role of the Hox PG1 genes we used the morpholino knockdown approach. Two morpholinos were designed for each PG1 gene and the effect on hindbrain patterning was investigated. To have an internal control, injections were performed at the four-cell stage into the left-hand side (lhs) of the embryo only (Fig. 2). For all three genes, both morpholinos gave the same phenotype, albeit at different concentrations (within a range of 10-30 ng), confirming the specificity of these morpholinos. The knockdown of each of the PG1 genes led to a defect in hindbrain patterning. For all three of them, r3 and r5 (shown by *Krox20* expression) were closer together on the injected side, indicating the reduction of r4, with the greatest reduction seen with the *Hoxa1* morpholinos (Fig. 2B,C). The expression domain of *Engrailed-2* however, a marker of the midbrain-hindbrain boundary (Hemmati-Brivanlou et al., 1991), was generally unaffected, although the width of this stripe often appeared to be smaller on the injected side.

For *Hoxa1* and *Hoxb1* this reflects the phenotypes observed in zebrafish and mice (Carpenter et al., 1993; Goddard et al., 1996; McClintock et al., 2002), but there is less information on the *Hoxd1* loss function phenotype. Therefore we decided to further check the specificity of the *Hoxd1* morpholino. To this end we tried to rescue the hindbrain phenotype with a *Xenopus Hoxd1* mRNA construct which lacks the 5′ UTR and therefore is not recognised by any of the morpholinos. Co-injection (in the lhs) of *Hoxd1* mRNA rescued the *Hoxd1* morpholino phenotype, as shown by Krox20 expression (Fig. 2K,L). Rhombomeres 3 and 4 were restored and in some cases were even larger than on the control side, as also seen in the simple *Hoxd1* overexpression phenotype (Fig. 2J). This demonstrates that the *Hoxd1* morpholino is specific and that in *Xenopus laevis*, *Hoxd1* has a role in hindbrain patterning.

Simultaneous knockdown of all three Hox PG-1 genes leads to severe hindbrain defects

Having established the specificity of the morpholinos we wanted to examine the effect of knocking down all three PG1 genes simultaneously. The morpholinos were injected (on the lhs) either in double combinations, or in the triple combination to remove all Hox PG1 function (Fig. 3). In all the double morpholino combinations the *Krox20* stripes are still present, although somewhat reduced and closer together (Fig. 3D,E,F). However, with the triple MO combination, *Krox20* expression in the hindbrain is either severely reduced or completely absent, with only a vestige of expression in the neural crest region (Fig. 3C). This indicates that an area encompassing at least r3-r5 is affected when all three PG1 genes are knocked down. The same effect was noted when the morpholinos were injected into the whole embryo (data not shown).

We attempted to rescue this phenotype with the morpholino insensitive *xHoxd1* expression construct (Fig. 4). When this

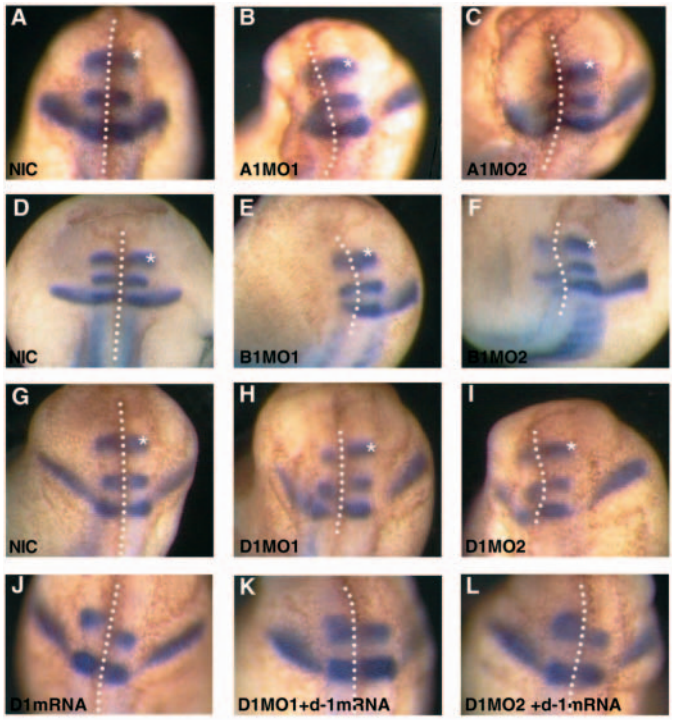

Fig. 2. Specificity of PG1 morpholinos. Two morpholinos for each Hox PG1 gene were injected into the left-hand side of the embryo and *Krox20* (r3 and r5) and *Engrailed-2* (midbrain/hindbrain boundary: * in A-I) expression analysed. The hindbrain region of early tailbud stage embryos (anterior to the top) is shown for non injected controls (NIC; A,D,G), *Hoxa1* 1st (B: 80%, *n*=15) and 2nd (C: 70%, *n*=10) morpholinos, *Hoxb1* 1st (E: 85%, *n*=13) and 2nd (F: 70%, *n*=20) morpholinos and *Hoxd1* 1st (H: 90%, *n*=11) and 2nd (I: 80%, *n*=15) morpholinos. Overexpression of *Hoxd1* morpholino insensitive RNA (J) and rescue of *Hoxd1* 1st and 2nd morpholinos with *Hoxd1* RNA is also shown (K,L). Dotted line indicates the midline.

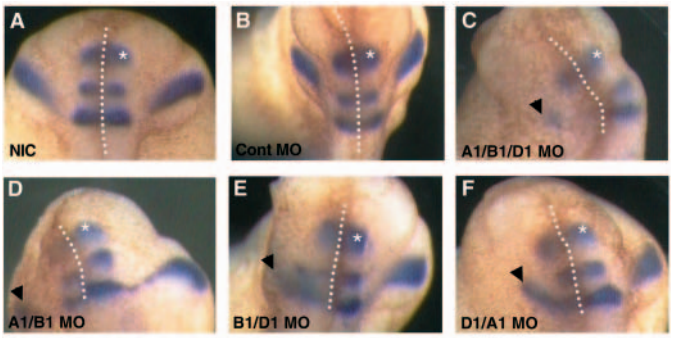

Fig. 3. The triple PG1 knockdown phenotype is more severe than the double knockdowns. Morpholinos for each Hox PG1 gene were injected either in double (D: A1/B1 52%, *n*=25; E: B1/D1 48%, *n*=29; F: D1/A1 64%, *n*=33) or triple (C: A1/B1/D1 94%, *n*=35) combinations into the left-hand side of the embryo and *Krox20* and *Engrailed-2* (*) expression analysed. The hindbrain region of early tailbud stage embryos is shown, with anterior to the top. Non-injected controls (NIC; A), and embryos injected on the left-hand side of the embryo with control morpholino (B) are also shown. Arrowheads indicate neural crest expression.

was co-injected with the triple MO combination the majority of embryos changed from having no *Krox20* expression, or only one faint narrow stripe, to having two stripes or one broad stripe spanning the r3-r5 region (Fig. 4E-G). This indicates that *xHoxd1* can partially rescue the triple knockdown phenotype and bring back some degree of patterning to the r3-r5 region of the hindbrain. We also attempted to rescue the phenotype with *Hoxa1* and *Hoxb1* constructs, but as the overexpression of these genes leads to a loss of the r3 *Krox20* stripe, interpretation of the rescue was difficult.

From these results, it appears that the phenotype of the triple PG1 MO embryos is not just an additive combination of the individual morpholino phenotypes, but is instead a synergistic effect. This indicates that there is a degree of redundancy between these genes in their hindbrain patterning function, and that any of the three genes can fulfil a basic hindbrain patterning role, even in the absence of the others. Therefore we concentrated on the triple PG1 MO combination for a more in depth analysis of Hox PG1 function.

Hox PG1 gene function is necessary for correct hindbrain patterning and segmentation

To further analyse the effect on hindbrain patterning of losing all PG1 function we carried out a detailed marker analysis of embryos injected with all three PG1 morpholinos (Fig. 5). It is clear that although hindbrain patterning is severely affected, as shown by the loss of *Krox20* stripes (Fig. 5G,H), the cells still adopt a neural fate, as the pan neural marker, *Nrp1* (Richter et al., 1990), is unaffected (Fig. 5A,B). In addition the most anterior domain of the embryo, expressing *Otx2* (Pannese et al., 1995) is not altered (Fig. 5C,D), and neither is the midbrain/hindbrain boundary, as shown by *Engrailed-2* expression (Fig. 3C). However, as we move posteriorly along the axis, a transformation of posterior hindbrain into anterior hindbrain becomes apparent. This is shown by the expansion of *Gbx2*. This gene has a stripe of expression at the boundary between the midbrain and hindbrain and in r1 (von Bubnoff et al., 1996), and on the side of the embryo injected with the PG1 morpholinos this stripe is expanded posteriorly (Fig. 5E,F). This posterior expansion was only partially rescued by co-injection of the *Hoxd1* mRNA (75% phenotype, reduced to 58%), indicating that at least one of the other Hox PG1 genes may be necessary for the restriction of *Gbx2* expression. More posterior hindbrain markers, such as *Krox20* and certain Hox genes (see following section), are either severely reduced or lost completely with the triple MO combination. Later analysis of the hindbrain using a neural specific antibody (Jones and Woodland, 1989) and confocal imaging indicated that not only was the patterning of the hindbrain affected, but also its morphology. In the triple MO injected side there were no clear rhombomere boundaries and the hindbrain appeared to be shorter and thinner compared with the control side (Fig. 5I,J). The midbrain also looked slightly affected but this could be a secondary effect due to the shortened hindbrain. Thus PG1 function is necessary for the correct segmentation of the hindbrain, as well as being involved in its patterning.

Another interesting aspect of the triple PG1 knockdown is the loss of segmented *MyoD* expression (Fig. 5H), indicating that the somites are also affected. This effect is specific and the mechanisms underlying it are currently under investigation.

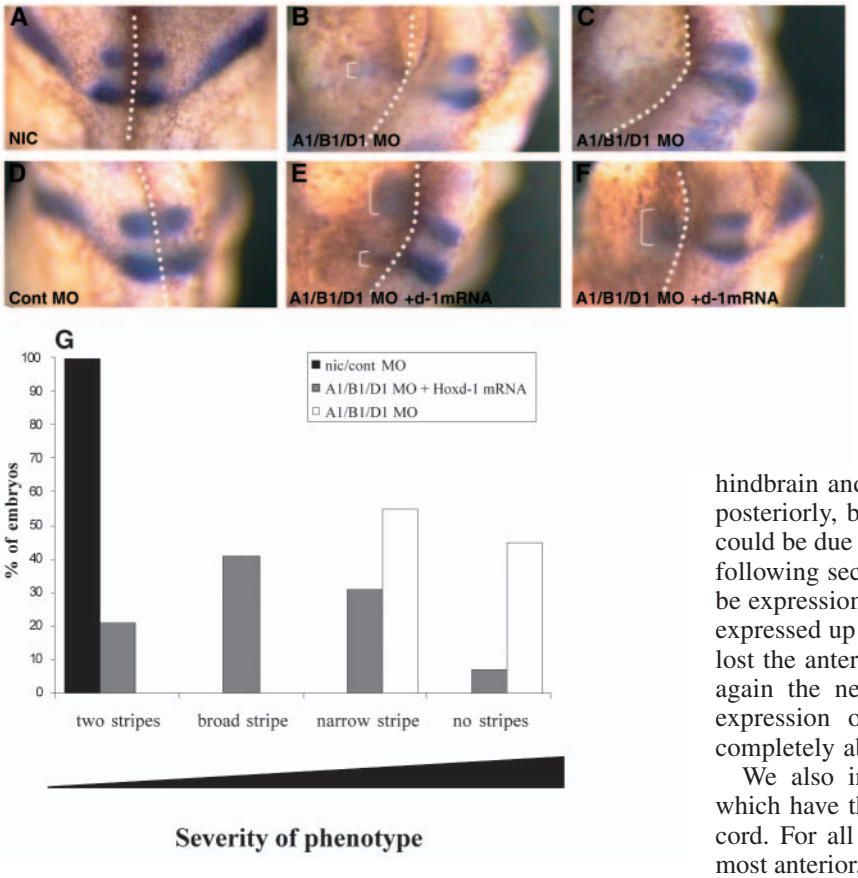

Fig. 4. *Hoxd1* mRNA can partially rescue the triple PG1 morpholino phenotype. Early tailbud stage embryos were injected on the left-hand side (lhs) with all three PG1 morpholinos (A1/B1/D1 MO) either alone (B,C), or in combination with *Hoxd1* mRNA (E,F). Non-injected controls (NIC) and control morpholino (Cont MO) are also shown (A,D). Anterior is to the top. G: *Krox-20* expression is partially rescued by *Hoxd1* RNA. Numbers of embryos with two stripes (E), one broad stripe (F), one narrow stripe (B) or no stripes (C) of *Krox20* expression were scored. Co-injection of *Hoxd1* RNA partially rescued the *Krox20* expression (n=29), which was either absent or very much reduced in embryos injected only with A1/B1/D1MO (n=18).

The function of Hox PG1 genes is necessary for the correct establishment of the 'Hox code'

To investigate whether the hindbrain defects that we observe could be due to a disruption in the 'Hox code', we analysed the expression of several Hox genes from different paralogous groups in embryos injected with the triple PG1 MO combination (Fig. 6). It is known that Hox-Hox cross-regulation occurs (Hooiveld et al., 1999), and as the PG1 genes are the first to be expressed in the embryo, and are expressed in a wide domain, they could play a role in the regulation of the other Hox genes. When we examined the expression of the PG1 genes themselves, *Hoxb1* and *Hoxa1* expression was downregulated (shown for *Hoxb1*, Fig. 6A,B), but the expression of *Hoxd1* was more variable, being reduced in only one third of embryos (n=24) and at other times being unaffected. This indicates that auto- and cross-regulation of these genes play a role in the maintenance of expression, but that external factors are also involved, particularly for *Hoxd1*.

When the other Hox genes were analysed it became apparent that the function of the PG1 genes is necessary for the establishment of the 'Hox code'. Both PG2 genes, *Hoxa2* and *Hoxb2*, normally expressed up to the anterior boundary of r2 and r3, respectively (Pasqualetti et al., 2000) were almost completely absent (shown for *Hoxa2*, Fig. 6C,D). For PG3 the effect was less uniform; *Hoxa3* and *b3* [expressed up to the r4/r5 boundary and in the neural crest derived cells of the 3rd pharyngeal arch (Godsave et al., 1994)] were severely reduced, with only the most posterior expression remaining (shown for *Hoxa3*, Fig. 6E,F). The effect on *Hoxd3* was less severe; its lateral stripe of expression was lost and its hindbrain and neural tube expression was fainter and shifted posteriorly, but slightly expanded laterally (Fig. 6G,H). This could be due to the inability of the neural crest to migrate (see following section) and thus the faint, expanded domain could be expression in the premigratory neural crest cells. *Hoxd4* is expressed up to the r6/r7 boundary and when PG1 function is lost the anterior expression domain is shifted posteriorly and again the neural crest stripe is lost (Fig. 6I,J). Thus, the expression of Hox genes in r2-r7 is either reduced or completely absent.

We also investigated several more posterior Hox genes, which have their anterior expression boundaries in the spinal cord. For all of these genes (*Hoxa5*, *Hoxc6* and *Hoxb9*) the most anterior, spinal cord expression was lost in the triple PG1 knockdown, but the diffuse mesodermal expression (in particular of *Hoxa5* and *Hoxc6*), and more posterior spinal cord expression, was unaffected (Fig. 6K-P).

To establish the specificity of this effect of the morpholinos, and to determine the degree to which reintroducing just one of the Hox PG1 genes would restore the Hox code, we utilised the *Hoxd1* morpholino-insensitive mRNA (Fig. 7). When this was injected alone, it induced anterior expansion of the expression of *Hoxb2* (Fig. 7A), but the other Hox genes analysed (*Hoxa3*, *Hoxd4* and *Hoxc6*) were unaffected. When *Hoxd1* mRNA was co-injected with the triple PG1 morpholino combination, however, it rescued the expression patterns of all of these Hox genes (Fig. 7C,F,I,L). This indicates that there may be a degree of redundancy between the PG1 genes and this is borne out by preliminary analyses of injections of the single morpholinos (Fig. 8). These showed that *Hoxa2* and *Hoxb2* expression was still present in all of the single knockdowns (shown for *Hoxb2*, Fig. 8A-D), although *Hoxb2* was slightly reduced with the *Hoxa1* and *Hoxd1* morpholinos. *Hoxa3* hindbrain expression however was reduced with the *Hoxa1* morpholino, and not affected by the other individual morpholinos despite being rescued in the triple PG1 knockdown by the *Hoxd1* mRNA (Fig. 8E-H, Fig. 7D-F). *Hoxd4* was affected by the *Hoxd1* morpholino, which appeared to give a similar phenotype to the triple combination (Fig. 8I-L). Thus different Hox genes appear to be dependant on different Hox PG1 genes, or a combination thereof, but these interactions will require further investigation before they are fully elucidated. However, these data do indicate that PG1 function in general is necessary for the establishment or maintenance of the expression patterns of both anterior and posterior Hox genes. For the more posterior genes this

requirement is restricted to the anterior, ectodermal expression domains, and the expression in the mesoderm and posterior ectoderm is unaffected.

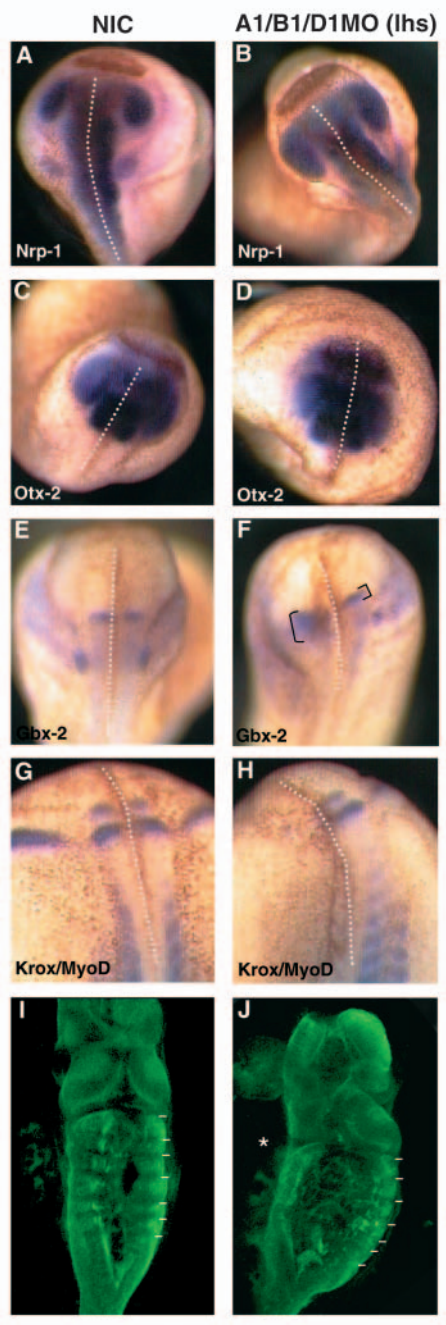

Fig. 5. Triple PG1 knockdown severely affects hindbrain patterning. Morpholinos for each of the Hox PG1 genes (A1/B1/D1MO) were co-injected into the lhs of the embryo (B,D,F,H,J). Non-injected controls are also shown (A,C,E,G,I). The hindbrain region is shown, with anterior to the top. Expression of the neural marker *Nrp1* (A,B: 100%, n=11), the anterior marker *Otx2* (C,D: 100%, n=10), *Gbx2*, which is expressed in r1 (E,F: 77%, n=13) and *Krox20* (G,H: no stripes 41%; one faint stripe 35%, n=71), was analysed. Dotted line indicates the midline. In later embryos (st. 46), the neural antibody 2-G9 was used to show the morphology of the hindbrain [NIC, I; A1/B1/D1MO, injected on the left-hand side (lhs) *, J: 100%, n=5]. Rhombomere boundaries are marked.

A role for PG1 genes in neural crest development

As Hox PG1 genes are expressed in the cranial neural crest, and PG1 function is clearly also necessary for the expression of certain other Hox genes in this region, we decided to investigate the effects of PG1 knockdown on neural crest and its derivatives. When we examined the expression of *Xslug*, which is expressed in all premigratory and migrating neural crest cells (Linker et al., 2000), we observed that on the triple MO injected side, the *Xslug* domain in the hindbrain region appeared to be upregulated compared with the control side (Fig. 9C). The expression, however, did not extend laterally but was restricted to an area close to the hindbrain. When the *Hoxd1* morpholino insensitive RNA was co-injected with the triple MO combination, the normal expression pattern was restored, with an additional expansion anteriorly (Fig. 9D). This is in keeping with the *Hoxd1* overexpression scenario, where the *Xslug* domain is expanded anteriorly (Fig. 9B). *Xsnail*, another neural crest marker, was also restricted to the area close to the neural tube but in this case the expression level was not significantly altered (Fig. 9E,F). These changes in expression could indicate a role for PG1 genes in neural crest cell migration, as in the knockdown situation the cells seem to remain close to the hindbrain, whereas when *Hoxd1* is overexpressed the *Xslug* positive cells are more widespread. It has been shown that *Xslug*, but not *Xsnail*, is downregulated when the neural crest cells reach their target (Linker et al., 2000). Therefore the apparent upregulation of *Xslug* compared with the control could be because the cells stay close to the hindbrain and thus *Xslug* expression is not downregulated. In later embryos expression of *dll4* in the pharyngeal arches (Papalopulu and Kintner, 1993) is severely reduced, whilst the expression of this gene in the forebrain and eye is still present (Fig. 9G,H). This indicates that PG1 gene function is vital for the development of the pharyngeal arches and this is confirmed by later analysis of the craniofacial structures using alcian blue staining. It can clearly be seen that, on the side injected with the triple PG1 MO combination, the gill cartilages, derived from the 3rd and 4th pharyngeal arches (the branchial arches) are completely missing (Fig. 9K). The ceratohyal, derived from the 2nd pharyngeal arch is still present, but reduced in size. Meckel's cartilage, derived from the 1st pharyngeal arch, is less affected but also appears to be slightly reduced. When the morpholino-insensitive *Hoxd1* RNA is co-injected, the gill cartilages are restored, but remain smaller than in the control side (Fig. 9L).

Thus, in the absence of Hox PG1 function, cranial neural crest cells are specified but they cannot migrate away from the hindbrain. Subsequent development of the pharyngeal arches and their derivatives is severely affected, with the complete loss of the gill cartilages.

Discussion

The complexity of the Hox gene family, and its members' redundancy and cross-reactivity, make it difficult to establish precisely what the function of each individual Hox gene is. However, there is increasing evidence that paralogous groups of Hox genes have shared functions. Therefore, rather than investigate individual Hox genes, we have taken a more global approach and studied the role of a complete paralogous group. The morpholino knockdown approach allowed us to block

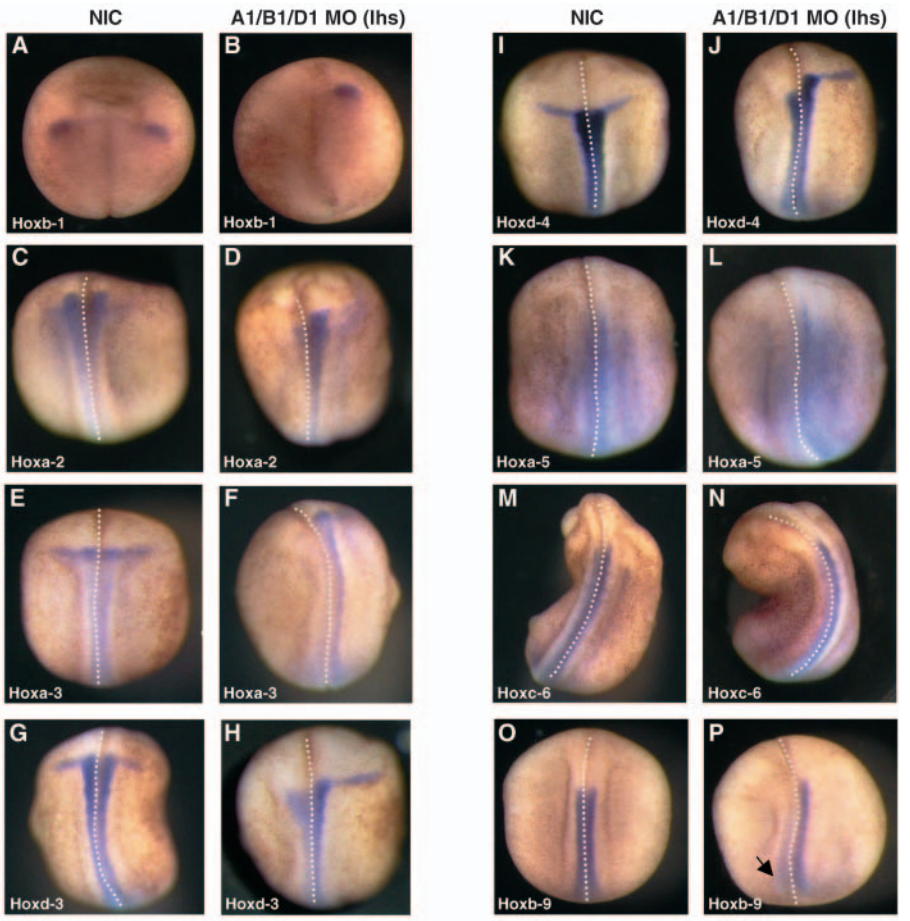

Fig. 6. Knockdown of all three PG1 genes affects more posterior Hox genes. Non injected controls (NIC; A,C,E,G,I,K,M,O) and embryos injected on the left-hand side (lhs) with all three Hox PG1 morpholinos (A1/B1/D1MO; B,D,F,H,J,L,N,P) were analysed for Hox gene expression at early tailbud stage. Embryos are shown from the dorsal side, anterior to the top. *Hoxb1* expression was lost (A,B: 70%, *n*=10), and *Hoxa2* (C,D: 100%, *n*=18) and *Hoxa3* (E,F: 100%, *n*=14) expression was almost completely absent. *Hoxd3* (G,H: 68%, *n*=19) and *Hoxd4* (I,J: 79%, *n*=14) neural tube expression was still present, but the pharyngeal arch expression was lost. The more posterior Hox genes (*Hoxa5*, K,L: 100%, *n*=10; *Hoxc6*, M,N: 71%, *n*=14; *Hoxb9*, O,P: 93%, *n*=14) had reduced neural tube expression in the anterior part of the embryo but their posterior expression domains (arrow) and mesodermal expression were unaffected.

simultaneously the translation of the three *Xenopus laevis* PG1 Hox genes, *Hoxa1*, *Hoxb1* and *Hoxd1*, and assess the roles of this paralogous group in the development of the embryo.

Hindbrain patterning and redundancy of Hox PG1 genes

The literature abounds with information about the effects on hindbrain patterning of single and double knockouts of Hox PG1 members. The data from mouse indicate that *Hoxa1* and *b1*, but not *Hoxd1*, are vital for the correct patterning and formation of the hindbrain and its derivatives. In this study we have shown that, in *Xenopus laevis*, all three Hox PG1 genes are expressed in the presumptive hindbrain region at a time when they could influence hindbrain patterning. This is in contradiction to the situation in mouse, where *Hoxd1* is only expressed in the mesoderm and neural crest (Frohman and Martin, 1992). However, the observed *Hoxd1* expression in the presumptive hindbrain region in *Xenopus* is very low, and is downregulated earlier than the other two paralogous genes, and it is therefore possible that a comparable low level expression in mouse was below detection limits.

When we knock down the function of all three PG1 genes we observe a more severe effect than in either the single or double knockdowns. Fig. 10 shows a schematic for the effect on the hindbrain of the stepwise elimination of Hox PG1 genes, with increasingly more severe phenotypes occurring when there is less PG1 function present. In *Xenopus*, the hindbrain expression of the r3/r5 marker, *Krox20*, is completely lost in the triple knockdown, whereas its expression domain is merely altered (indicating a reduction in the r3-r5 region) when at least one PG1 gene remains functional. Preliminary results indicated that the triple knockdown phenotype is a synergistic, rather than an additive effect, which implies a degree of redundancy between the PG1 genes and, thus, we concentrated on the triple knockdown to elucidate the basic PG1 group function. We cannot therefore rule out effects being due to the double morpholino combinations. However, defects in the hindbrain of *Xenopus* embryos completely deficient in PG1 function were more extensive than any previously reported for single or double PG1 knockouts in other organisms. Expression of markers for r2-r7 was downregulated in the triple knockdown, whereas the expression of an r1 marker, *Gbx-2* was expanded posteriorly, indicating a possible transformation of more posterior hindbrain to an r1 type identity. Later morphological analysis showed that the hindbrain is reduced in size and segmentation is perturbed. This phenotype is actually similar to the situation in zebrafish when the functions of Hox cofactors from the *Meis* and *Pbx* families are compromised (Fig. 10). In *Pbx4* (*lazarus*) mutants that have been injected with a *Pbx2* morpholino (to achieve a total block of early Pbx function) the hindbrain is not segmented and r2-r7 acquire an r1-like identity, referred to as the hindbrain ground state (Waskiewicz et al., 2002). Likewise, when the function of *Meis* is blocked, using two dominant-negative constructs, a similar, non-segmented hindbrain is produced (Choe and Sagerstrom, 2004). The similarity of these phenotypes to the triple PG1 MO phenotype suggests that they could, at least partially, be due to the blocking of PG1 gene function. A recent study in *Xenopus* showed that hindbrain *Krox20* stripes were eliminated when either a *Meis* morpholino or a dominant-negative *Hoxd1* RNA construct (which may also block *Hoxa1*

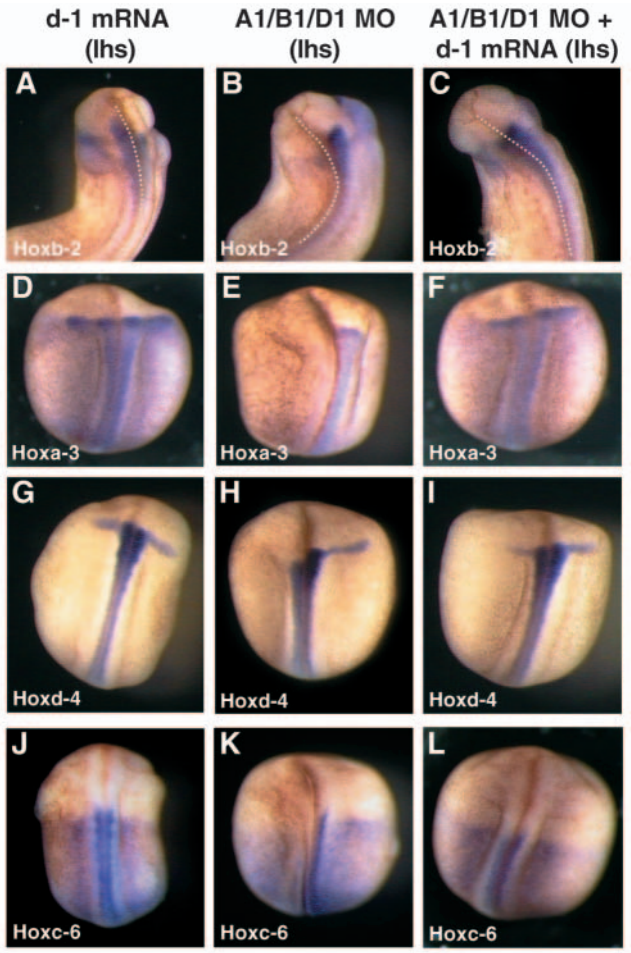

Fig. 7. *Hoxd1* mRNA rescues Hox expression in PG1 knockdown embryos. Embryos injected on the left-hand side (lhs) with *Hoxd1* MO insensitive mRNA (A,D,G,J), all three PG1 morpholinos (B,E,H,K) or a combination of both (C,F,I,L) were analysed for expression of *Hoxb2, Hoxa3, Hoxd4* and *Hoxc6*. Embryos are shown from the dorsal side, anterior to the top. *Hoxb2* expression was expanded posteriorly with *Hoxd1* mRNA (A: 75%, *n*=12), lost with the triple PG1 morpholinos (B: 93%, *n*=28) and rescued when a combination was injected (C: 48% of embryos had downregulated expression, *n*=33). The other Hox genes were not affected by the *Hoxd1* mRNA overexpression. PG1 MO downregulated expression of *Hoxa3* (E: 78%, *n*=9), *Hoxd4* (H: 100%, *n*=9) and *Hoxc6* (K: 78%, *n*=9) and in the rescues this downregulation was reduced to 34%, 20% and 33% for *Hoxa3*, *Hoxd4* and *Hoxc6*, respectively (F,I,L).

and *Hoxb1* function and, thus, be similar to the triple MO situation) were injected into the embryo (Dibner et al., 2004). It has also been noted in one *Hoxa1* knockout study in mouse that segmentation is completely blocked, but again all three of the PG1 genes could be affected, as the truncated *Hoxa1* splice variant still present could perhaps act as a dominant-negative (Chisaka et al., 1992).

The PG1 function in hindbrain could be mediated via other, downstream, Hox genes. For example, when both Hox PG2 genes are knocked out in mouse the rhombomere boundaries in the r2/r3 region are absent (Davenne et al., 1999). Thus the effect that we see with the triple PG1 knockdown on segmentation in this anterior region of the hindbrain (where PG1 genes are not expressed) could be due to the downregulation of *Hoxa2* and *Hoxb2*.

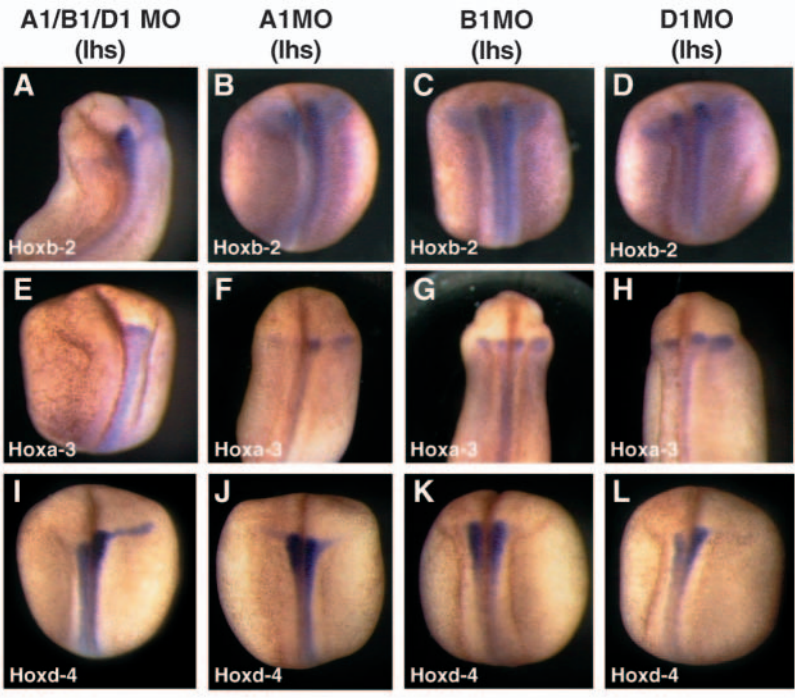

Fig. 8. Effects of single PG1 knockdowns on Hox genes. Embryos injected on the left-hand side (lhs) with the triple PG1 MO combination had reduced *Hoxb2* (A: 93%, *n*=28), *Hoxa3* (E: 100%, *n*=14) and *Hoxd4* (I: 79%, *n*=14). The *Hoxa1* MO weakly downregulated *Hoxb2* (B: 80%, *n*=10) and reduced *Hoxa3* (F: 54%, *n*=11), but not *Hoxd4* (J: 100%, *n*=20). The *Hoxb1* MO had no effect on any of these genes (C: *Hoxb2*, 100%, *n*=10; G: *Hoxa3*, 100% *n*=9; K: *Hoxd4*, 100%, *n*=18). *Hoxd1* MO reduced *Hoxd4* expression (L: 100%, *n*=16), weakly reduced *Hoxb2* (D: 43%, *n*=14), but did not affect *Hoxa3* (H: 100%, *n*=10).

PG1 Hox genes are necessary for the anterior portion of the 'Hox code'

Numerous interactions between various Hox genes have been shown to exist in the embryo (Deschamps et al., 1999; Hooiveld et al., 1999; Maconochie et al., 1997; Melton et al., 2004). Indeed one aspect of colinearity could be the sequential activation of anterior Hox genes that are, in turn, responsible for the activation of the more posterior ones (Hooiveld et al., 1999). Although the majority of the literature in mouse does not support this model, this could be due to a high degree of redundancy between different Hox genes masking the effect in single or double knockout mice. As the PG1 genes are the first to be expressed in the embryo we investigated the possibility that they could initiate a cascade of Hox expression, which would lead to the established 'Hox code'. Although PG1 function is clearly not a prerequisite *per se* for Hox gene

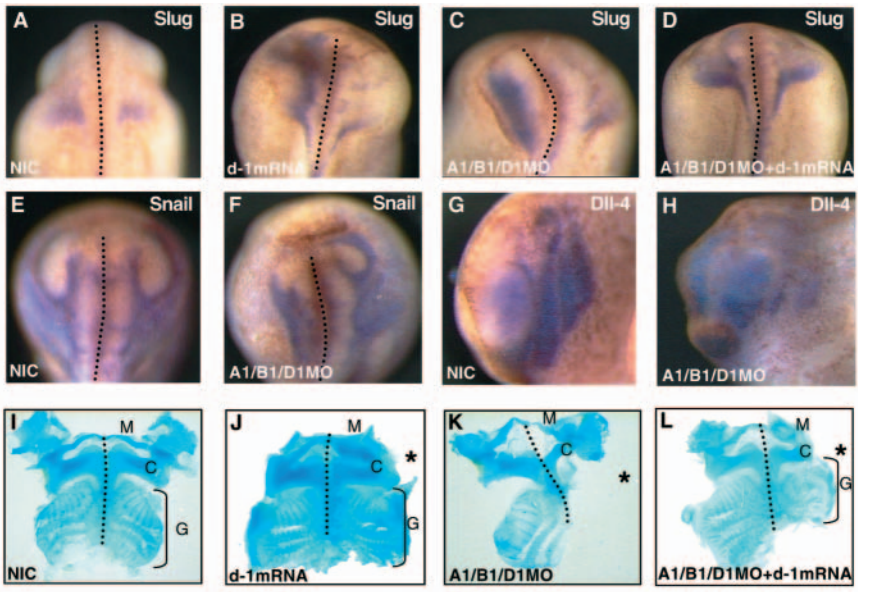

Fig. 9. Knockdown of all three PG1 genes affects the development of neural crest and its derivatives. Non-injected controls (NIC; A,E,G,I) and embryos injected on the left-hand side with *Hoxd1* MO insensitive mRNA (B,J), all three Hox PG1 morpholinos (A1/B1/D1MO; C,F,H,K) or a combination of both (D,L) were analyzed for neural crest gene expression, or stained with alcian blue to show the craniofacial structures. The embryos shown in A-F are early tailbud stage embryos shown from the dorsal side with anterior to the top. With the PG1 MOs, neural crest cells fail to migrate away from the hindbrain, as shown by *Xslug* (C: 86%, *n*=29) and *Xsnail* (F: 100%, *n*=14) expression. This is rescued (22% of embryos with restricted *Xslug* expression, *n*=9) by *Hoxd1* overexpression (D) and simple overexpression leads to an expanded *Xslug* domain (B: 100%, *n*=10). Slightly later tailbud embryos analyzed for *dll4* are shown from the left-hand side, with anterior to the left, and in the triple PG1 knockdown *dll4* expression in the branchial arches is restricted to close to the hindbrain (50%) or completely lost (G,H: 44%, *n*=16). Alcian blue was used to stain the cartilage of stage 49, embryos (I-L) shown from the ventral side to show the craniofacial structures (injected side on the right:*). The complete gill area is missing in the triple morpholino injected embryos (K: 82%, *n*=11) and this is rescued (26% of embryos with missing gill, *n*=19) when *Hoxd1* mRNA is co-injected (L). C, ceratohyal; G, gill cartilage; M, Meckel's cartilage. Dotted line indicates midline.

expression we did uncover a reliance on PG1 function for the correct expression of Hox genes from paralogous groups 2-9. This widespread effect on posterior Hox genes is more severe than that observed in the single or double PG1 knockouts in mouse (Dolle et al., 1993; Maconochie et al., 1997; Rossel and Capecchi, 1999). However, as the triple PG1 knockdown has never previously been investigated, it is possible that the situation in mouse is similar to that in *Xenopus*, with a high degree of functional redundancy between the genes.

From our data we cannot distinguish when the PG1 genes are required for the expression of the Hox genes examined, although it is likely to be an early input, as the later domains of *Hoxa2* and *b2*, at least, extend beyond the Hox PG1 expression domains. It is clear, however, that without Hox PG1 function the 'Hox code' is severely compromised. Thus, via the control of the 'Hox code', the PG1 genes can eventually exert their influence outside of their own expression domains. This has already been shown for *Hoxa1*, the knockout of which has defects in the r3 domain, which is anterior to the normal *Hoxa1* expression domain (Helmbacher et al., 1998). This mechanism would account for the extent of the defects that we observe in the triple PG1 knockdown. It could also be due to a more anterior expression domain early in development of one or all of the Hox genes, which could be the case for *Hoxb1,* as its early expression is very close to the *Otx-2* domain. Alternatively, these effects could be due to a non cell-autonomous effect. Several signalling pathways influence patterning of the hindbrain, such as the FGF and retinoic acid pathway (Roy and Sagerstrom, 2004; Walshe et al., 2002), and it is feasible that the extent of the phenotypes that we observe may be partly due to a disruption in these signalling pathways. It has also been shown in vitro that certain Hox proteins are able to cross the cell membrane (Amsellem et al., 2003; Chatelin et al., 1996) and thus it is possible that the Hox proteins themselves act non cell-autonomously to pattern the hindbrain.

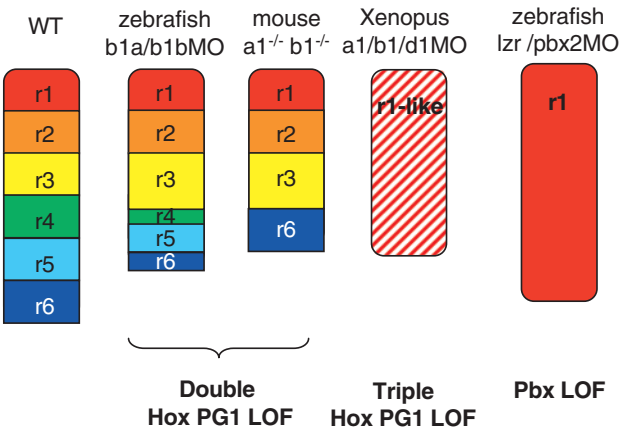

Fig. 10. Schematic for the patterning of rhombomeres 1-6 in the hindbrain in various loss of function (LOF) combinations of Hox PG1 genes, and also the Hox cofactor *Pbx*. There is a successive loss of rhombomeric identity as more PG1 function is removed in the different organisms: zebrafish (McClintock et al., 2002); mouse (Rossel and Capecchi, 1999); and *Xenopus* (this study). The triple PG1 LOF phenotype in *Xenopus* resembles the *Pbx* complete LOF in zebrafish (Waskiewicz et al., 2002).

A role for PG1 genes in neural crest migration and development of the pharyngeal arches

A link between the Hox PG1 genes and craniofacial development has previously been demonstrated, with *Hoxa1* and *-b1* double mutants lacking the 2nd pharyngeal arch and its derivatives as well as some derivatives of the 1st pharyngeal arch (Gavalas et al., 1998; Rossel and Capecchi, 1999). Here we show that in the absence of any PG1 gene function, cranial neural crest cells are still specified but they appear to be unable to migrate away from the neural tube. Conversely, when *Hoxd1*

is overexpressed, migration seems to be enhanced, as seen by the widespread *Xslug* domain.

It is interesting to note that in the *Hoxa1/b1* double knockout in mouse, neural crest cells fail to delaminate from the presumptive r4 territory, although they delaminate normally from the other rhombomeres (Gavalas et al., 2001). This failure could be more widespread in the triple PG1 knockdown, leading to a wholesale block of cranial neural crest cell migration. This could be due to the effect on the Hox code, as many of the Hox genes downregulated are also expressed in the neural crest, or are known to be involved in their patterning (Trainor and Krumlauf, 2001).

Major defects in the craniofacial structures are later seen in embryos lacking PG1 function, which are probably due to the inability of the cranial neural crest cells to migrate. Derivatives of all the pharyngeal arches are affected and most strikingly, the gill cartilages, derived from the 3rd and 4th pharyngeal arches, are completely missing. Effects such as this have not previously been seen in PG1 mutants, where generally only the 1st and 2nd pharyngeal arches are affected (Gavalas et al., 1998). Thus the complete abrogation of PG1 function has identified additional regions of the embryo which are ultimately dependant upon these genes for their formation.

In conclusion, we have knocked down the function of the complete Hox Paralogous Group 1 and illustrated that these genes have a degree of functional redundancy in their role of patterning the *Xenopus* hindbrain. We have demonstrated that PG1 function is essential for the correct establishment of the 'Hox code'. In the absence of PG1 function, and perhaps as a consequence of 'Hox code' disruption, hindbrain segmentation is perturbed and r2-r7 are mis-specified. In addition we have identified a novel requirement for Hox PG1 function in the migration of the cranial neural crest and the development of the pharyngeal arches.

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