Regulation of Msx genes by a Bmp gradient is essential for neural crest specification

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Summary

There is evidence in *Xenopus* and zebrafish embryos that the neural crest/neural folds are specified at the border of the neural plate by a precise threshold concentration of a Bmp gradient. In order to understand the molecular mechanism by which a gradient of Bmp is able to specify the neural crest, we analyzed how the expression of Bmp targets, the Msx genes, is regulated and the role that Msx genes has in neural crest specification.

As Msx genes are directly downstream of Bmp, we analyzed Msx gene expression after experimental modification in the level of Bmp activity by grafting a bead soaked with noggin into *Xenopus* embryos, by expressing in the ectoderm a dominant-negative Bmp4 or Bmp receptor in *Xenopus* and zebrafish embryos, and also through Bmp pathway component mutants in the zebrafish. All the results show that a reduction in the level of Bmp activity leads to an increase in the expression of Msx genes in the neural plate border. Interestingly, by reaching different levels of Bmp activity in animal cap ectoderm, we show that a specific concentration of Bmp induces msx1 expression to a level similar to that required to induce neural crest. Our results indicate that an intermediate level of Bmp activity specifies the expression of Msx genes in the neural fold region.

In addition, we have analyzed the role that msx1 plays on neural crest specification. As msx1 has a role in dorsoventral patterning, we have carried out conditional gain- and loss-of-function experiments using different msx1 constructs fused to a glucocorticoid receptor element to avoid an early effect of this factor. We show that msx1 expression is able to induce all other early neural crest markers tested (snail, slug, foxd3) at the time of neural crest specification. Furthermore, the expression of a dominant negative of Msx genes leads to the inhibition of all the neural crest markers analyzed. It has been previously shown that snail is one of the earliest genes acting in the neural crest genetic cascade. In order to study the hierarchical relationship between msx1 and snail slug we performed several rescue experiments using dominant negatives for these genes. The rescuing activity by snail and slug on neural crest development of the msx1 dominant negative, together with the inability of msx1 to rescue the dominant negatives of slug and snail strongly argue that msx1 is upstream of snail and slug in the genetic cascade that specifies the neural crest in the ectoderm. We propose a model where a gradient of Bmp activity specifies the expression of Msx genes in the neural folds, and that this expression is essential for the early specification of the neural crest.

Key words: Neural crest, Msx genes, Bmp gradient, slug, snail, foxd3, *Xenopus*, Zebrafish

Introduction

The neural crest originates at the border between the neural plate and the future epidermis. It gives rise to numerous and diverse cell types, including much of the peripheral nervous system, the craniofacial skeleton, and pigments cells (for a review, see Labonne and Bronner-Fraser, 1999; Mayor and Aybar, 2001). Although considerable progress has been made recently in the molecular characterization of neural crest-inducing factors, relatively little is known about the genetic cascade of transcription factors that determine the specification of the neural crest at the neural plate border.

Diffusible proteins such as Bmp, Wnt, FGF and retinoic acid play an important role in neural crest induction (for a review, see Aybar and Mayor, 2002; Knecht and Bronner-Fraser, 2002). It has been shown in chick and amphibians that an interaction between neural plate and epidermis is able to induce neural crest cells (Moury and Jacobson, 1990; Selleck and Bronner-Fraser, 1995; Mancilla and Mayor, 1996). There is strong evidence that supports the role of Wnt factors in neural crest induction (reviewed by Yanfeng et al., 2003). In chick embryos, Wnt could be one of the inductive signals produced by the epidermis (García-Castro et al., 2002). It has also been shown that the addition of Bmp to chick neural plate is able to induce neural crest cells (Liem et al., 1995). Experiments in *Xenopus* and zebrafish support a role for Bmp signals during neural crest induction. It has been shown in these animal
models that a gradient of Bmp is able to specify the neural plate border, including neural crest cells (Wilson et al., 1997; Marchant et al., 1998; Nguyen et al., 1998; LaBonne and Bronner-Fraser, 1998). However, in order to fully induce neural crest additional signals are required. These additional signals are Wnts, Fgf and retinoic acid (Saint-Jeannet et al., 1997; LaBonne and Bronner-Fraser, 1998; Lekven et al., 2001; Deardorff et al., 2001; Mayor et al., 1997; Villanueva et al., 2002). A recent model proposes that the threshold value of a Bmp gradient specifies the anterior neural fold, which in a second step is transformed into neural crest cells by the posteriorizing signals of Wnt, FGF and retinoic acid (Villanueva et al., 2002; Aybar and Mayor, 2002).

Once the neural crest is specified by these secreted molecules, a genetic cascade of transcription factors is activated in the prospective neural crest cells. Several genes that code for transcription factors have been found to be expressed in the neural crest (for a review, see Mayor and Aybar, 2001; Aybar et al., 2002), including snail (Mayor et al., 1993; Essex et al., 1993; Linker et al., 2000), slug (Mayor et al., 1995), zic5 (Nakata et al., 2000), foxd3 (Sasai et al., 2001; Dirksen and Jamrich, 1995), twist (Hopwood et al., 1989), sox9 (Spokony et al., 2002) and sox10 (Aoki et al., 2003; Honoré et al., 2003). Although the hierarchical relationship between these transcription factors has not been established, snail seems to lie furthest upstream in the genetic cascade of neural crest development (Aybar et al., 2003).

The connection between the inductive molecules and the transcription factors activated in the neural crest has not been elucidated. As described above, one of the molecules that has a clear role in neural crest induction is Bmp. To link the inductive molecules to the transcription factors, we have started to analyse the role that downstream targets of Bmp could have on neural crest specification. Most of the Bmp target genes identified to date encode homeobox proteins, including msx1 (Suzuki et al., 1997), msx2, vent1 (Gawantka et al., 1999), vent2 (Onichtchouk et al., 1996) and dlx5 (Miyama et al., 1999). In this work, we have studied how Msx gene expression is controlled in the neural folds and the role of msx1 in neural crest development.

Msx genes, vertebrate homologues of Drosophila msh (muscle segment homeobox) genes, are good candidates for Bmp targets. Three different Msx genes have been identified in the mouse (Shimeld et al., 1996), and at least two of them, msx1 and msx2, have been isolated from human (Jabs et al., 1993), Xenopus (Su et al., 1991) and zebrafish (Ekker et al., 1997). Bmp4 protein can induce or upregulate expression of Msx genes in the epidermis, dental mesenchyme, hindbrain, neural tube, limb bud, paraxial ectomesoderm, oral primordia and ventral mesoderm (Vainio et al., 1993; Graham et al., 1994; Liem et al., 1995; Wang and Sassoon, 1995; Shimeld et al., 1996; Watanabe and Le Douarin, 1996; Barlow and Francis-West, 1997; Suzuki et al., 1997; Yamamoto et al., 2000).

The expression of Msx genes is complex. In Xenopus embryos, msx1 is initially expressed in ventral mesoderm and ectoderm, but later becomes restricted to the neural folds and dorsal neural tube (Suzuki et al., 1997). It has been shown that msx1 can act as a ventralizing factor of the mesoderm and as an inhibitor of nodal signaling (Yamamoto et al., 2000; Yamamoto et al., 2001). Despite its expression in the neural fold, its function in neural crest development has not been analyzed.

In order to understand how msx1 expression is regulated in the prospective neural crest, we proceeded to inhibit Bmp activity and then analyzed the expression of msx1. We inhibited Bmp activity by grafting into Xenopus embryos beads soaked with noggin, by expressing a dominant-negative form of Bmp or a dominant negative of its receptor in Xenopus embryos, or by using several Bmp/Smad zebrafish mutants. Our results show that inhibition of Bmp activity leads to an increase in msx1 expression in Xenopus and zebrafish embryos. In addition, by generating different levels of Bmp activity in animal cap ectoderm, we show that there is a specific concentration of Bmp that can induce msx1 expression. To study msx1 function in neural crest specification and development, we carried out conditional gain- and loss-of-function experiments using different msx1 constructs fused to a glucocorticoid receptor element. Our results show that activation of msx1 induces an expansion of the neural crest territory, as analyzed by the expression of snail, slug and foxd3, whereas expression of an msx1 dominant negative suppresses all neural crest markers analyzed. By performing rescue experiments of the Msx genes dominant-negative with inducible forms of snail and slug, we show that msx1 lies upstream of the Snail family of genes in the genetic cascade of neural crest specification. We propose a model whereby Msx genes are induced by a gradient of Bmp activity and that this induction is essential for neural crest specification.

Materials and methods

Xenopus and zebrafish embryonic manipulation

Embryos were obtained from adult Xenopus laevis by standard hormone-induced egg laying and artificial fertilization (Villanueva et al., 2002). Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967) and animal caps were carried out using eyebrow knives as indicated in Aybar et al. (Aybar et al., 2003). Zebrafish mutant embryos were obtained by intercrossing heterozygous parents; alleles used were svrf<sup>c300</sup>, shpr<sup>c24</sup> and snpf<sup>568r</sup>.

Morpholino treatment in zebrafish

In order to inhibit mesoderm formation, the following combination of two different spadetail (spt) and notail (ntl) morpholinos (MO) was injected at the one-cell stage zebrafish embryo. The spt MO was a kind gift from Sharon Amacher and Bruce Draper. The mix of MO was a kind gift from Kate Lewis. The mix used had the final concentrations of: ntl MO 1 mg/ml; spt MO#2 0.075 mg/ml; spt MO#1 0.75 mg/ml. The ntl MO sequence has been previously published (Nasevicius and Ekker, 2000) and the spt MO sequences are: spt MO 1, 5'-AGCCTGACATTATTAGCCTTCCTCTA-3'; and spt MO 2 5'-GATGTCCTCTAAAGAAATATGTCAG-3'.

Plasmid constructs

Inducible DNA constructs of Msx genes were prepared by fusing the entire coding regions of msx1 (amino acid residues 1-294, Fig. 5A) to the ligand-binding domain of the human glucocorticoid receptor (GR, amino acid residues 512-777). A dominant-negative DNA construct (dnmsx) was prepared by fusing the homeodomain region of msx1 (amino acid residues 156-294) to the GR element (Fig. 5A). Coding sequences were amplified by PCR with a high fidelity polymerase (Roche Molecular Biochemicals, Mannheim, Germany) and the following primers: msx1, 5'-ATGGGAGATTGGTTGTATGGATGCCGC-3' and 5'-GAATCTGAGATCGGCGGACACAG-3'; and Dnmmsx1, 5'-GAATCTGAGATCGGCGGACACAG-3' and 5'-GAATCTGAGATCGGCGGACACAG-3'. The PCR products were purified and cloned into pGEM-T Easy
vector (Promega), EcoRI/SalI-digested and ligated with a SacI/Vhol-digested GR fragment into a pCS2+ vector digested with EcoRI/Vhol. Both fusion constructs were sequenced on both strands at junction sites by automated DNA sequencing (BRC, Cornell University, Ithaca, NY, USA). ΔBmpr and CM-Bmp4 constructs were kindly donated by Dr K. W. Cho.

RNA microinjection, lineage tracing and dexamethasone induction

Dejellied *Xenopus* embryos were placed in 75% NAM containing 5% Ficoll and one blastomere of two-cell stage embryos was injected with differing amounts of capped mRNA containing 1-3 μg/μl lysine fixable fluorescein dextran (40,000 MWT; FLDX, Molecular Probes) as a lineage tracer. For the inhibition of Bmp activity, the dominant negatives were injected in one animal blastomere of eight- to 16-cell stage embryo. For animal cap assays, mRNA was injected into the animal side of the two blastomeres of two-cell stage embryos. Approximately 8-12 nl of diluted RNA was injected into each embryo. Ethanol-dissolved dexamethasone (10 μM) was added to the culture medium at stages 12 or 17, and maintained until the embryos were fixed. To control the possible leakage of inducible chimeras, a sibling batch of embryos were cultured without dexamethasone and processed for in situ hybridization. One-cell stage swr/bmp2b mutant zebrafish embryos derived from crosses of swr mutant homozygous adults (Nguyen et al., 1998) were microinjected with either chordin mRNA (Miller-Bertoglio et al., 1997) or a dominant negative Bmp type I receptor (ΔBmpr) mRNA (Graff et al., 1994), as previously described (Nguyen et al., 2000).

Noggin treatment

Acrylic beads (Sigma) were incubated overnight with 100 μg/ml of noggin protein (a kind gift from R. Harland). The beads where grafted into embryos at the appropriate stage and the expression of several markers was later analyzed by in situ hybridization. PBS-soaked beads were used as controls.

In situ hybridisation

For *Xenopus* embryos, antisense probes containing digoxigenin-11-UTP (Roche Biochemicals) were prepared for *msx1* (Suzuki et al., 1997), *XsnaIl* (Essex et al., 1993; Aybar et al., 2003), *fox3* (Sasai et al., 2001), *Xslug* (Mayor et al., 1995), *sox2* (Kishi et al., 2000) and cytokeratin *Xk8IA* (Jonas et al., 1985) by in vitro transcription. Specimens were prepared, hybridized, and stained according to Harland (Harland, 1991) with the modifications described in Mancilla and Mayor (Mancilla and Mayor, 1996). Zebrafish embryos were fixed overnight in 4% paraformaldehyde. Antisense RNA probes for zebrafish gene *msxb* (Ekker et al., 1997) was synthesized from cDNAs using digoxigenin (Boehringer Mannheim) as a label. Hybridization was done as previously described (Jowett and Lettice, 1994).

RNA isolation and RT-PCR analysis

Total RNA was isolated from embryonic tissue by the guanidine thiocyanate/phenol/chloroform method (Chomczynski and Sacchi, 1987), and cDNAs were synthesized using AMV reverse transcriptase (Roche Biochemicals) and oligo(dT) primer. Primers for *Xslug* and *H4* have previously been described (Aybar et al., 2003). The primers used to analyse *Xenopus msx1* expression that amplify a 156 bp product were 5'-GCTAAAA ATGGCTGCTAAG-3' and 5'-AGGTGGCCTGTGAAGATG-3'. PCR amplification with these primers was performed over 28 cycles, and the PCR products were analysed on 1.5% agarose gels. As a control, PCR was performed with RNA that had not been reverse-transcribed to check for DNA contamination. Quantitation of PCR bands was performed using ImageJ software (NIH, USA) on 8-bit greyscale JPG files and the values were normalized to the H4 levels from the same sample and expressed for comparison as relative intensities (sample/H4×10).

Results

Dynamic expression of *msx1* within the neural crest

Although the expression pattern of *msx1* has previously been published in *Xenopus* and zebrafish embryos (Suzuki et al., 1997; Ekker et al., 1997), we decided to analyse its expression pattern and compare it with Bmp4 expression. Single and double in situ hybridization was performed in embryos at different stages (Fig. 1). The expression of Bmp4 has been already described in *Xenopus* embryos (Schmidt et al., 1995), and a dorsal (Fig. 1A) and lateral view (Fig. 2B) are shown for comparison with *msx1* (*msxe* – Zebrafish Information Network) expression at the mid gastrula stage (13). In *Xenopus* embryos, from the early until the mid-gastrula stage (12), the expression of *msx1* can be detected in the lateral and ventral ectoderm, as previously described (Suzuki et al., 1997). However, from the mid-gastrula stage onwards a progressive reduction in the level of *msx1* was observed (Fig. 2A). In *Xenopus*, the expression of *msx1* is first observed at the mid-gastrula stage (13), from which point upwards a progressive reduction in the level of *msx1* is observed, with the strongest levels being detected in the region of the developing neural crest (Fig. 2A).

![Fig. 1. Msx genes are expressed in the neural crest region of *Xenopus* and zebrafish embryos. (A-G) *Xenopus* embryos. Stage 13. (H-K) Zebrafish expression. Bud and five-somites stage. Anterior is upwards. (A,B) Bmp4 expression. (C-G) *msx1* expression. Arrowhead indicates anterior neural fold; asterisk indicates prospective neural crest. (A) Dorsal view, showing strong expression in the anterior neural fold. (B) Lateral view, showing strong expression in the anterior neural fold, intermediate in the prospective neural crest and weaker in the ventral ectoderm. Arrow in B indicates expression in the ventral side. (C) Dorsal view of a double in situ hybridization for *msx1* (purple) and *Xslug* (blue) of a mid/late gastrula stage embryo. Note the overlapping in the expression of both genes in the prospective neural crest region (square) in the whole embryo (C), in the sectioned embryo (F) and in a higher magnification (G). (D) Dorsal view showing the strong expression of *msx1* in the neural folds. (E) Lateral view showing expression in the neural folds. (H) Dorsal view showing expression of *msxb* in the neural plate border. (I) Lateral view showing expression in the neural plate border. (J) Flat mount showing expression in the prospective neural crest region. (K) Higher magnification of J.](Image 314x253 to 570x521)
observed in the ventral ectoderm, resulting in a specific concentration of the label in the prospective neural crest (Fig. 2D,E). At this stage (13) a double in situ hybridization of msx1 and the neural crest marker Xslug shows a clear overlap in their expression domains both in whole mount (Fig. 2C,G), as well as in sectioned embryos (Fig. 2F). The msx1 expression domain includes, but is wider than, the neural crest territory, probably being expressed in prospective placodes and the neural plate border. A similar restriction from the ventral ectoderm to the neural plate border in the expression of msxb was found (Fig. 2H-K), confirming previous publications (Ekker et al., 1997; Cornell and Eisen, 2000). It should be mentioned that it is not clear whether msxb is the orthologue of msx1 (Ekker et al., 1997).

Control of msx1 expression in the prospective neural crest by Bmp signalling

It has been shown that the Msx genes are direct downstream targets of Bmp signalling (Suzuki et al., 1997; Maeda et al., 1997; Yamamoto et al., 2000; Ishimura et al., 2000). We analysed msx1 expression after interfering with Bmp activity. We inhibited Bmp activity in Xenopus embryos (1) by injecting a dominant-negative form of Bmp4 (CM-Bmp4) (Hawley et al., 1995) into one blastomere of an eight- or 16-cell stage embryo, (2) by injecting a dominant-negative form of the Bmp receptor (ΔBmpr) into one blastomere of an eight- or 16-cell stage embryo, or (3) by grafting near the neural folds of a stage 11 embryo a bead soaked with noggin. All these treatments produced an expansion in the msx1 domain (Fig. 2). It should be noted that blocking Bmp signalling does not only expand the territory of msx1 expression, but also increases its level of expression (Fig. 2A,D). As the noggin-soaked bead was grafted at stage 11 and the injections of the dominant-negative mRNA were performed in the ectoderm, no effect on gastrulation or mesodermal patterning was observed. An alternative way to study the effect of decreasing Bmp activity on Msx gene expression is to use different Bmp/Smad zebrafish mutants. The expression of msxb was analysed in wild-type and mutant embryos. In zebrafish wild-type embryos at the five-somites stage, msxb is expressed in two stripes lateral to the dorsal axis (Fig. 3A), similar to what has been described for Xenopus. In swr/bmp2 mutant embryos, the expression of msxb is expanded and moved to an anterior domain (Fig. 3B) (see also Schmid et al., 2000). In smb/smad5 and sbh/bmp7 mutant embryos, msxb expression is greatly and moderately expanded, respectively (Fig. 3C,D). As in these mutant embryos, not only the ectoderm but also the mesodermal patterning are affected. A possible explanation of these results is that the expansion of the neural plate/neural fold markers could be a secondary consequence of a primary expansion of mesoderm, which in turn induces the neural markers in the ectoderm. In order to test whether the expansion of the mesoderm played any role in the expansion of the neural markers described here and in previous publications (Nguyen et al., 1998), we proceeded to inhibit the formation of dorsal mesoderm in some of the mutant embryos and the neural markers were analysed. It has previously described that krox20 (egr2b – Zebrafish Information Network) is characteristically expanded as a ring in swr mutant (Nguyen et al., 1998). We analysed the expression of krox20, as a neural marker, and myod as a mesodermal marker. The expression of both genes can be clearly distinguished in wild type (Fig. 3E) and swr mutants (Fig. 3F), showing the mutant a characteristic expansion of both markers. In order to inhibit mesoderm formation in the swr mutant, we proceeded to inject a mix of ntl (Nasevicius and Ekker, 2000) and spt morpholinos. This injection lead to a total inhibition in the expression of myod (100% of inhibition, n=63), but no effect in the expansion of krox20 was observed (Fig. 3G). These results indicate that the expansion of the neural markers is not dependent on mesodermal patterning.

The swr/bmp2 mutant exhibited an increase in the expression of msxb (Fig. 3B) and a reduction in the expression of neural crest markers (Nguyen et al., 1998). In order to test whether this msxb expression was dependent on remaining Bmp activity in the swr/bmp2 mutant, we injected the embryos with chordin or ΔBmpr mRNA and found similar results. After injection of 50 pg of chordin mRNA, the expression of msxb was significantly reduced, when compared with the un.injected control siblings (Fig. 3H,I), and msxb expression was not
effect on gene expression. Taken together, our experiments using Xenopus and zebrafish embryos strongly argue that Msx gene expression appears to be dependent on a specific level of Bmp signalling.

So far, we have shown that there is no a direct correlation between the level of Bmp activity and the expression of the Msx genes, as a reduction in the first leads to an increase in the expression of the second. As we show in Fig. 1, there are several differences in the expression of Msx genes and bmp4. First, strong expression of bmp4 is detected in the anterior neural plate border, whereas almost no expression of msxl is observed in that region (Fig. 1, arrowhead). The ventral epidermis shows a clear expression of bmp4 (Fig. 1B, arrow), and almost no Msx gene expression (Fig. 1E, arrowhead). The highest level of Msx gene expression can be observed in the posterior neural folds (Fig. 1D,E, asterisk), while the level of Bmp4 expression in that region is intermediate to the expression in the anterior neural plate border and the epidermis (Fig. 1A,B, asterisk). In conclusion, the levels of Bmp4 expression do not match exactly those of msxl. However, the levels of bmp4 expression do not necessarily correlate with Bmp activity levels. Furthermore, it is known that Bmp binding molecules are secreted from the dorsal mesoderm, and in consequence the levels of Bmp activity around the neural plate and neural folds could be lower than those suggested by the levels of Bmp4 mRNA. Taken together, these results prompted us to analyse directly the possibility that msxl transcription is induced at a certain threshold concentration of a Bmp gradient.

It is known that neural crest can be induced in Xenopus by an intermediate level of Bmp and Wnt signalling (LaBonhe and Bronner-Fraser, 1998; Marchant et al., 1998; Saint-Jeannet et al., 1997; Villanueva et al., 2002). One-cell stage Xenopus embryos were injected with a mixture of between 0 and 500 pg of dominant-negative Bmp4 (CM-Bmp4) mRNA and 50 pg of Wnt5a mRNA. Animal caps were dissected at stage 9, cultured until the equivalent of stage 16 and the expression of Msx genes and the neural crest marker XSlug were analysed by RT-PCR. As expected, XSlug expression was induced at a specific concentration of CM-Bmp4 (100 pg, Fig. 4A,B), lower or higher amounts of CM-Bmp4 failed to induce strong XSlug expression, confirming previous reports of induction of the neural crest by a gradient of Bmp (Morgan and Sargent, 1997; Marchant et al., 1998; Nguyen et al., 1998; LaBonhe and Bronner-Fraser, 1998; Luo et al., 2003). Interestingly, the highest level of msxl expression was also induced at 100 pg of CM-Bmp4 (Fig. 4A,B). Low levels of msxl can be detected at other concentrations, which probably represent the normal epidermal expression observed in untreated animal caps (0 ng of CM-Bmp4 mRNA, Fig. 4A,B). These results strongly support the idea that, like the neural crest markers, msxl transcription is activated by an intermediate concentration in a Bmp gradient.

**msxl is required for neural crest specification**

Given that msxl is expressed in the prospective neural crest territory and that it is induced by the same molecules that induce neural crest, we investigated whether this gene might also function in neural crest development. To overcome the early effects of msxl in mesoderm development, we used the inducible fusion constructs to the ligand-binding domain of glucocorticoid receptor (GR) described previously (Fig. 5A;

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**Fig. 3.** Msxb expression is increased in Bmp/Smad mutant zebrafish embryos. Wild-type and mutant zebrafish embryos were analyzed by whole-mount in situ hybridization for the expression of Msxb at the five-somite stage. Lateral views, anterior is upwards. (A) Wild-type embryos show the characteristic dorsal expression in the embryo. (B) A swr mutant shows an expansion of the Msxb territory in anterior regions. (C) A sbw mutant embryo shows a dramatic ventral expansion in Msxb expression. (D) A snh mutant embryo shows a moderate, lateral expansion in the expression of Msxb, where the two domains of expression can be seen. (E) Flat-mount of a wild-type embryo analyzed for the expression of Krox20 (arrows) and Myod (bracket). Note that the Myod expression is disorganized but can be found in dorsal and ventral sides. (G) swr mutant injected with ntl/splt morpholinos. A complete absence of Myod expression but an expansion in Krox20 (arrows) was observed. (H-J) swr mutant embryos were injected with chordin mRNA and the expression of Msxb was analyzed. Anterior is towards the left. (H) Control uninjected embryos show the characteristic expansion of Msxb expression. (I) Embryos injected with 50 pg of chordin mRNA; note a reduction in Msxb expression. (J) Embryos injected with 200 pg of chordin mRNA exhibit a total inhibition in the expression of Msxb. Each experiment was repeated at least twice, with similar results. Reducing Bmp signaling with ΔBmpr treatment yielded similar results to those shown here for chordin.
To test if the function of msx1 was affected by the GR domain in the fusion protein, we injected the msx-GR and the HDmsx-GR mRNA into one-cell stage embryos, treated with dexamethasone immediately after the injection (stage 5), and analyzed the phenotypes. No difference was detected between the non-inducible and inducible msx1 constructs under these conditions. msx-GR induced ventralization and anterior truncation (Fig. 5B), and HDmsx-GR induced dorsalization (Fig. 5B) as previously published (Yamamoto et al., 2000; Yamamoto et al., 2001). However, when the induction was carried out at the gastrula stage, none of these phenotypes was observed. This indicates that the early function of msx1 is not altered in our fusion proteins, and that late induction of them does not affect dorsoventral mesodermal patterning. Furthermore, because the neural crest is specified around the late gastrula stage, care was taken to induce the fusion constructs just before this stage (stage 12) and analyse its effects at the early neurula stage (stage 18). This is an important experimental approach as it has been shown that the neural plate, but not the neural crest, is already specified at stage 12 (Servetnick and Grainger, 1991; Mancilla and Mayor, 1996; Woda et al., 2003; Glavic et al., 2004).

When mRNA encoding msx-GR was injected into one blastomere of a two-cell stage embryo and then activated at stage 12, the expression of the neural crest markers, slug, snail, and foxd3, was augmented in more than 70% of the injected embryos (Fig. 6A-C). Conversely, the activation at stage 12 of the inducible dominant negative fusion, which contains the Msx gene homeodomain (HDmsx-GR), impaired the expression of slug, snail, and foxd3 in more than 75% of the injected embryos (Fig. 6F-H). These results indicate that msx1 is required for the expression of the neural crest markers. We then analyzed whether the expansion of the neural crest territory was made at the expense of the neural plate or the epidermis. The expression of the neural plate marker Sox2 and the epidermal marker XK81a was analyzed after the activation of msx-GR at stage 12. The results show that there was an inhibition in the expression of Sox2 in about 60% (Fig. 6D) and of XK81a in 63% (Fig. 6E) of the injected embryos. This result indicates that msx1 overexpression can transform the neural plate and epidermal cells that surround the prospective neural crest territory into neural crest cells. We should mention that although many injections were localized at the center of the neural plate or epidermis, we never observed ectopic expression of neural crest markers in those territories. These results suggest that msx1 cannot transform ectodermal cells by itself into neural crest cells, but probably requires additional co-factors that are present near the neural crest region. In support of this observation we never induced the expression of neural crest markers in animal caps injected with msx1 mRNA, as analyzed by RT-PCR (three independent experiments).
Msx genes in neural crest development

We then inhibited msx1 function and examined the effect on the neural plate and epidermis. Embryos were injected with HDmsx-GR mRNA into one blastomere of a two-cell stage embryo, induced at stage 12 and the expression of Sox2 and Xk81a was analyzed. We observed an expansion of Sox2 (Fig. 6I) and Xk81a (Fig. 6J) expression on the injected side of 58 and 71% of the embryos, respectively. Taken together, these results show that the function of msx1 at the late gastrula stage is to specify the fate of the ectodermal cells as neural crest cells, and when its function is inhibited the prospective neural crest cells are transformed into neural plate and epidermis. This function of msx1 is coherent with its expression in the prospective neural crest cells.

To show that the msx1 dominant-negative (XHDmsx1-GR) specifically inhibits msx1 function, we performed rescue experiments. Embryos were injected in one blastomere at the two-cell stage. The inducible constructs were activated at stage 12, and the expression of the neural crest markers, XSlug, foxd3 and XSnail was analyzed at stage 18. The injection of XHDmsx1-GR inhibited in greater than 65% of the embryos the neural crest marker expression (Fig. 6Q, for simplicity only the percentage of XSlug expression are indicated in the graphic, but similar percentages were obtained for foxd3 and XSnail expression). However, the co-injection of XHDmsx1-GR and msx-GR was able to rescue the expression of the neural crest markers in a dose-dependent manner. When XHDmsx1-GR and msx-GR were injected in a proportion of 2:1, the inhibition in the expression of the neural crest markers was reduced to 50% (Fig. 6K-M,Q), but when equal amounts of both constructs were co-injected, the inhibition of the neural crest markers was rescued in more than 80% (Fig. 6N-P,Q). As expected, the injection of msx-GR alone produced an expansion in the expression of the neural crest markers in more than 70% of the embryos (Fig. 6Q). Thus, we conclude that the phenotypic effects of the inducible msx1 dominant-negative reflect a modulation of the natural msx1 target genes.

Hierarchical relationship between msx1 and the Snail family genes

Our results show that msx1 plays a role in the early
specification of the neural crest and, as it is a Bmp target that is involved in neural crest induction, it is likely that msx1 is one of the earliest transcription factors in the genetic cascade of neural crest specification. Many transcription factors are expressed in the prospective neural crest, e.g. snail, slug, zic5, foxd3, ap2, Dlx genes, sox9 and sox10 and all of them may play a role in neural crest specification (Mayor et al., 1993; Essex et al., 1993; Linker et al., 2000; Mayor et al., 1995; Nakata et al., 2000; Dirksen and Jamrich, 1995; Hopwood et al., 1989; Luo et al., 2003; Woda et al., 2003; Aoki et al., 2003; Honoré et al., 2003). However, the hierarchical relationship between these factors has not been elucidated. It was recently proposed that snail is an early gene working upstream of the genetic cascade in neural crest specification (Aybar et al., 2003). Based on these observations, we decided to analyse the relationship between msx1 and snail and slug in neural crest development.

A combination of HDmsx-GR and XSlug-GR was injected into one blastomere at the two-cell stage with different combinations of HDmsx-GR and XSlug-GR (A-G) or XSnail-GR (H-N), induced at stage 12 and the expression of the neural crest markers XSlug (A,D,H,K), foxd3 (B,E,L) and XSnail (C,F,J,M) was analyzed at stage 18. Anterior is towards the right. The injected side detected by fluorescein staining is indicated by an arrowhead. (A-C) Embryos were injected with 500 pg of HDmsx-GR mRNA and 500 pg of XSlug-GR (ratio 1:1). Note the rescue in the expression of the neural crest markers. (D-F) Embryos were injected with 250 pg of HDmsx-GR mRNA and 750 pg of XSlug-GR (ratio 1:3). Note the rescue in the expression of the neural crest markers. (G) Summary of the expression of XSlug. The injected and uninjected side was analyzed for each embryo. Number of embryos analyzed for XSlug expression is 225. Note that the strong rescue (65%) was reached with a ratio of 1:1 for the injected mRNAs. Similar values of rescue were obtained for the other neural crest markers (72% for foxd3, total number is 225; 68% for XSnail, total number is 235). (H) Embryos were injected with 500 pg of HDmsx-GR mRNA and 500 pg of XSnail-GR (proportion of 1:1). Note the rescue in the expression of the neural crest markers. (K-M) Embryos were injected with 250 pg of HDmsx-GR mRNA and 750 pg of XSnail-GR (proportion of 1:3). Note the rescue in the expression of the neural crest markers. (N) Summary of the expression of XSlug. The injected and uninjected side was analyzed for each embryo. Number of embryos analyzed for XSlug expression is 215. Note that the strong rescue (67%) was reached with a ratio of 1:1 for the injected mRNAs. Similar values of rescue were obtained for the other neural crest markers (51% for foxd3, total number is 202; 62% for XSnail, total number is 215).

Essex et al., 1993; Linker et al., 2000; Mayor et al., 1995; Nakata et al., 2000; Dirksen and Jamrich, 1995; Hopwood et al., 1989; Luo et al., 2003; Woda et al., 2003; Aoki et al., 2003; Honoré et al., 2003). However, the hierarchical relationship between these factors has not been elucidated. It was recently proposed that snail is an early gene working upstream of the genetic cascade in neural crest specification (Aybar et al., 2003). Based on these observations, we decided to analyse the relationship between msx1 and snail and slug in neural crest development.

A combination of HDmsx-GR and XSlug-GR was injected into one blastomere of two-cell stage embryos, induced at stage 12 and the expression of XSlug, foxd3 and XSnail was analysed. Different proportions of mRNA of the two constructs were injected. As expected the injection of HDmsx-GR alone produced a strong inhibition in the expression of the neural crest markers (Fig. 7G); however, co-injection of XSlug-GR rescued this effect in a dose-dependent manner (Fig. 7A-G). The injection of XSlug-GR leads to an expansion of the neural crest territory (Fig. 7G), as previously reported (Aybar et al., 2003). Note that when equal amounts of HDmsx-GR and XSlug-GR are used, XSlug expression is rescued in about 70% of the embryos (Fig. 7A,G). Similar results were obtained for other neural crest markers (foxd3, Fig. 7B, 72% rescued; XSnail, Fig. 7C, 68% rescued). However, in the opposite experiment, co-injection of a dominant negative slug
msx1 function lies upstream of XSlug function in the genetic cascade of neural crest specification.

We performed similar rescue experiments with XSnail. Embryos were co-injected with HDmsx-GR and XSnail-GR and a dose-dependent rescue of the effect on neural crest markers was observed (Fig. 7H-N). Once more, the optimal rescue was obtained when equal amounts of both mRNA were used, reaching a nearly 70% rescue in the expression of XSlug, foxd3, and XSnail. Again, compared with this level of rescue, we failed to produce a significant rescue by the co-injection of a dominant-negative XSnail (XSnailZnF-GR) (Aybar et al., 2003) and msx-GR (less than 10% rescued for the expression of XSlug, n=104). These results support the idea that msx1 is upstream of XSnail in the cascade of specification of the neural crest. Thus, as XSnail is one of the earliest genes to be expressed and to work in the neural crest genetic cascade, we propose that msx1 could be the earliest gene to be activated in this cascade.

Discussion
Control of Msx gene transcription by Bmp signalling
It has been established that Msx genes are a direct target of Bmp signalling in several animal models, and that Msx gene promoter elements respond to Smad proteins that are directly activated by the Bmp ligand (Suzuki et al., 1997; Takahashi et al., 1997; Gonzalez et al., 1998; Alvarez-Martinez et al., 2002). In addition, at early stages of ectodermal patterning in Xenopus and zebrafish, the Msx genes and the Bmp factors are expressed in the same region, supporting the idea that the first are regulated by Bmp activity. However, at later stages there is not a perfect superposition between Msx genes and Bmp factor expression domains. At the completion of gastrulation, the highest level of Bmp expression is found in the anterior neural fold, where no Msx gene is detected (Suzuki et al., 1997; Schmidt et al., 1995) (this study, Fig. 1A,D). In addition, Msx and Bmp genes in different animal models are expressed in a variety of tissues or regions of developing embryos, which, interestingly, are not always coincident in those tissues (Vainio et al., 1993; Graham et al., 1994; Liem et al., 1995; Wang and Sassoon, 1995; Shimeld et al., 1996; Watanabe and Le Douarin, 1996; Barlow and Francis-West, 1997; Suzuki et al., 1997; Yamamoto et al., 2000; Yamashiro et al., 2003; Lord et al., 1995; D’Alessio and Frasch, 1996; Ishikini et al., 1997). The discrepancies between the Bmp expression domain and its downstream target the Msx genes can be explained by taking into account that the site of Bmp expression does not necessarily correspond to the site of its activity. First, Bmp ligands are diffusible proteins. Second, Bmp activity is under the repressive control of several factors that block its activity, such as the Bmp antagonists chordin, noggin, follistatin, short gastrulation, etc. (Piccolo et al., 1996; Zimmerman et al., 1996; Hemmati-Brivanlou et al., 1994). Several lines of evidence in Xenopus and zebrafish support the idea that interaction between Bmps, secreted by the mesoderm and the ectoderm, and Bmp antagonists, produced by the dorsal mesoderm, generate a ventrodorsal gradient of Bmp activity in the ectoderm, which is required to specify its dorsoventral pattern (Barth et al., 1999; Neave et al., 1997; Nguyen et al., 1998; Nguyen et al., 2000; Marchant et al., 1998; Luo et al., 2001a; Luo et al., 2001b; Wilson et al., 1997; LaBonne and Bronner-Fraser, 1998; Morgan and Sargent, 1997). It should be mentioned that this Bmp gradient model has no experimental support for patterning the chick ectoderm (Streit et al., 1998; Faure et al., 2002).

We present evidence that supports the idea that a specific level of Bmp activity leads to Msx gene transcription. In addition, we show that this level of Bmp activity corresponds exactly with the level that is able to induce neural crest cells. Thus, Msx gene transcription is dramatically increased in the ectoderm by a level of Bmp activity intermediate to the level required to induce neural plate or epidermis. This high level of Msx genes, induced at a precise and intermediate threshold of Bmp signaling, is required to induce neural crest cells.

There are many reports that support the idea that a gradient of Bmp activity divides the ectoderm into neural plate, neural crest and epidermis. The molecular mechanism by which different levels of Bmp are able to activate the transcription of different genes and in turn specify different tissues is unknown. One possibility is that Bmp activates all its downstream target genes in a linear way; thus, the gradient of Bmp should be transformed into a gradient of all of its targets genes, including Msx genes, with high and low levels in the ventral and dorsal sides, respectively. Then this gradient of Msx genes should specify the neural crest at an intermediate level by an unknown mechanism. In this work, we show that this is not the case for Msx genes, as this gene is induced by a precise and intermediate level of Bmp activity. Thus, we rule out the alternative that the neural crest is specified by an intermediate concentration of Msx protein within a gradient. Instead, we support the idea that the gradient of Bmp is immediately transformed into the activation of Msx genes at a precise threshold.

The molecular mechanism by which Msx genes are expressed only at intermediate levels of Bmp activity is unknown. One possibility is the presence of Bmp receptors with different affinities for its ligands and with different downstream targets, being Msx genes a specific target for a specific receptor. Another alternative is the activation at high levels of Bmp activity of a repressor of Msx gene transcription. This alternative is supported by the observation of an early and transient expression of msx1 and msxb in the ventral ectoderm, which could be inhibited at later stages by this hypothetical repressor. Additional experiments are required to distinguish between these and other alternatives.

Role of Msx genes in neural crest specification
Our gain- and loss-of-function experiments show that Msx genes are required for the early specification of the neural crest. Inhibition of Msx gene function at the time of neural crest specification by use of an inducible dominant negative, leads to inhibition in the expression of the earliest neural crest markers known like snail, slug and foxd3. Activation of Msx genes just prior to neural crest specification leads to an expansion of the endogenous neural crest territory; however, we never observed isolated regions of neural crest marker induction within the neural plate or epidermis. This result suggests that Msx genes work together with other factors,
present in the neural plate border, to specify neural crest cells. This explanation is also supported by our inability to induce neural crest markers in animal caps injected with Msx genes. Taken together, we propose that the Bmp gradient induces at the neural plate border the expression of Msx genes and another factor, and that both are required to activate the genetic cascade of neural crest specification. This additional factor could also be activated by the Wnts, Fgf or retinoic acid signaling, as it is known that they are required for neural crest induction (LaBonne and Bronner-Fraser, 1998; Villanueva et al., 2002; Saint-Jeannet et al., 1997; Luo et al., 2001a; Luo et al., 2001b). One possible candidate for this additional factor could be pax7, as it is known that it is expressed at the neural plate border in a domain slightly broader than the neural crest territory, and it is able to activate the expression of neural crest markers (Bang et al., 1999; Mayor et al., 2000). In our animal cap gradient experiment, Wnt signaling was required to induce neural crest and msxl expression (Fig. 4E), as widely reported (LaBonne and Bronner-Fraser, 1998; Villanueva et al., 2002; Saint-Jeannet et al., 1997; Luo et al., 2001a; Luo et al., 2001b; Honoré et al., 2003). In addition, it has been observed that there is a synergistic effect between Bmp and Wnt signaling in the induction of Msx genes in culture cells (Willert et al., 2002). The ability of Msx genes to induce neural crest is time dependent. When the activation of Msx genes is performed before gastrulation, it does not promote neural crest development, but instead promotes epidermal development (Suzuki et al., 1997); when Msx genes are activated after gastrulation (stage 17), once the neural crest is specified (Mancilla and Mayor, 1996; Aybar and Mayor, 2002), no increase in neural crest markers is observed (not shown).

Once the neural crest genetic cascade is activated by Msx genes, the expression of specific genes that are able to confer neural crest identity is induced. One of the earliest genes in this cascade seems to be snail (Aybar et al., 2003), as it is the only gene identified so far whose expression in animal caps is able to specifically induce the expression of early and late neural crest markers. The expression of genes such as Meis, Pbx, foxd3 and Zic family members, not only trigger the expression of neural crest markers, but also induce the expression of neural plate markers (Sasai et al., 2001; Nakata et al., 2000; Mizuseki et al., 1998; Nagai et al., 1997; Nakata et al., 1997; Nakata et al., 1998; Maeda et al., 2002). In addition, snail seems to lie upstream of slug in the neural crest genetic cascade (Aybar et al., 2003). In this work, we rescued the effect of an Msx gene dominant negative by snail or slug co-injection, but we were not able to rescue the effect of a slug or snail dominant negative by msxl co-expression. Taken together, these results strongly support the conclusion that Msx genes are upstream of snail/slug in the specification of the neural crest cells. This conclusion is consistent with the fact that Msx genes are a direct target of Bmp, which is one inducer of the neural crest.

It should be noted that the expression of Msx genes includes the prospective neural crest territory, but also encompasses cells adjacent to the neural crest. Those adjacent cells could be placodal and dorsal neural tube cells. Thus, although we have demonstrated a clear role for Msx genes in early neural crest specification, other experiments remain to be performed to investigate the role of Msx genes in other neural fold cells types. Interestingly, there is evidence that indicates that the preplacodal field, which is adjacent to the neural crest, is also specified by a precise threshold concentration of the Bmp gradient (A. Glavic and R.M., unpublished).

Loss of function of Msx genes in the mouse, by knocking out the gene or use of antisense oligonucleotides, produced a wide range of phenotypes, many of them related to the development of neural crest derivatives (Foerst-Potts and Sadler, 1997; Jumlongras et al., 2001; Satokata and Maas, 1994). However, analysis of these results is complicated by the presence of three Msx genes, which could operate in a redundant manner. In addition to the early role of Msx genes in neural crest specification, these genes play a role in the control of apoptosis (Gomes and Kessler, 2001; Marazzi et al., 1997). The more complex pattern of expression within the neural folds observed at neurula stages is probably related to its apoptotic function in the neural crest (C.T., M.J.A. and R.M., unpublished).

In conclusion, the dynamic expression of Msx genes during embryonic development is probably a consequence of a complex system of transcriptional regulation and reflects the multiple functions that this gene plays in several developmental processes. We have unravelled one of its roles in early neural crest development and have shown how its expression is controlled in these cells.

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