Indian hedgehog signaling is required for proper formation, maintenance and migration of Xenopus neural crest

Tristán H. Agüero a, Juan P. Fernández a,b, Guillermo A. Vega López a, Celeste Tríbulo a,b, Manuel J. Aybar a,b,*

a Depto. Biología del Desarrollo, INSIBIO (CONICET-Universidad Nacional de Tucumán), Chacabuco 461, San Miguel de Tucumán, Argentina
b Instituto de Biología “Dr. Francisco D. Barbieri”, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Chacabuco 461, 74000IL, San Miguel de Tucumán, Tucumán, Argentina

A R T I C L E   I N F O

Article history:
Received for publication 29 May 2011
Revised 30 December 2011
Accepted 23 January 2012
Available online 28 January 2012

Keywords:
Neural crest
Specification
Maintenance
Hedgehog
Cell migration
Cyclopamine

A B S T R A C T

Neural crest induction is the result of the combined action at the neural plate border of FGF, BMP, and Wnt signals from the neural plate, mesoderm and nonneural ectoderm. In this work we show that the expression of Indian hedgehog (Ihh, formerly named Banded hedgehog) and members of the Hedgehog pathway occurs at the prospective neural fold, in the premigratory and migratory neural crest. We performed a functional analysis that revealed the requirement of Ihh signaling in neural crest development. During the early steps of neural crest induction loss of function experiments with anti-sense morpholino or locally grafted cyclopamine-loaded beads suppressed the expression of early neural crest markers concomitant with the increase in neural and epidermal markers. We showed that changes in Ihh activity produced no alterations in either cell proliferation or apoptosis, suggesting that this signal involves cell fate decisions. A temporal analysis showed that Hedgehog is continuously required not only in the early and late specification but also during the migration of the neural crest. We also established that the mesodermal source of Ihh is important to maintain specification and also to support the migratory process. By a combination of embryological and molecular approaches our results demonstrated that Ihh signaling drives in the migration of neural crest cells by autocrine or paracrine mechanisms. Finally, the abrogation of Ihh signaling strongly affected only the formation of cartilage derived from the neural crest, while no effects were observed on melanocytes. Taken together, our results provide insights into the role of the Ihh cell signaling pathway during the early steps of neural crest development.

© 2012 Elsevier Inc. All rights reserved.

Introduction

The neural crest (NC), a vertebrate transient and multipotent embryonic cell population, is generated at the lateral borders of the neural plate. The NC cells delaminate from the dorsal neural tube and migrate extensively to several parts of the embryo, giving rise to a wide variety of cell types, including melanocytes, cartilage, connective tissue, neurons and glia of the peripheral nervous system, and neuroendocrine cells (Le Douarin and Kalcheim, 1999). The combined action of FGF, BMP and Wnt signals that originated from the neural plate, mesoderm and nonneural ectoderm are integrated at the neural plate border to induce and specify the neural crest cells (reviewed in Aybar and Mayor, 2002; Basch et al., 2004; Sauka-Spengler and Bronner-Fraser, 2006; Steventon et al., 2005). It has been demonstrated that these secreted molecules mediate tissue interactions during the early steps of neural crest development. These signals first activate neural border specifiers, then the neural crest specifiers are triggered (e.g. Snail1, Snail2, FoxD3, Sox9-10, etc.) during the NC premigratory phase (Meulemans and Bronner-Fraser, 2004), and later these genes are essential for further NC development involving NC specification maintenance or cell migration. Our understanding of the intricate regulatory network remains preliminary. Additional signals are also required to support the initial development of the neural crest, such as the juxtacrine signal Notch/Delta, retinoic acid, or noelin-1 (Barembaum et al., 2000; Endo et al., 2002; Glavic et al., 2004).

The roles and the relative importance of each cell signaling involved in the process of neural crest induction are still controversial. The participation of BMP as a neural crest inducer appears to be insufficient (LaBonne and Bronner-Fraser, 1998; Liem et al., 1995; Marchant et al., 1998; Nguyen et al., 1998) while the role of Wnt as an inducer has been demonstrated for different animal models (Deardorff et al., 2001; Garcia-Castro et al., 2002; Heeg-Truesdell and Labonne, 2006; Saint-Jeannet et al., 1997; Wu et al., 2003). The plurality of signaling molecules required for neural crest induction is not completely understood, but has introduced the idea that some signals could control particular processes or induce specific cell precursors among the multiple cell populations derived from this tissue.

⁎ Corresponding author at: Depto. Biología del Desarrollo, INSIBIO (CONICET-Universidad Nacional de Tucumán), Chacabuco 461, T4000IL, San Miguel de Tucumán, Argentina.
E-mail address: mjaybar@fbqf.unt.edu.ar (M.J. Aybar).

0012-1606/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2012.01.020
Genetic and pharmacological research has demonstrated that Hedgehog (Hh) signaling is essential for the correct development of facial and neural crest-derived structures in the avian embryo (Benouaiche et al., 2008). The experimental reduction of Sonic hedgehog signaling affected the proliferation and survival of cells in the chicken neural tube and cranial neural crest (Ahlgren and Bronner-Fraser, 1999; Ahlgren et al., 2002). Studies conducted in mammalian embryos have shown that the severity of craniofacial defects related to holoprosencephaly correlates with the stage in which interruption in Shh signaling occurs (Chiang et al., 1996; Cordero et al., 2004). In the zebrafish embryo Hh signaling is also required for cranial neural crest morphogenesis and chondrogenesis (Wada et al., 2005). In the absence of Hh signaling through inactivation of the Hh receptor Smoothened (Smoo) a complete loss of cranial skeletal elements in zebrafish embryos was observed (Chen et al., 2001). Cartilage elements were lost or mislocalized after experimental treatments with cyclopamine, an alkaloid antagonist of Hh signaling, administered to whole embryos during the gastrulation period (Eberhart et al., 2006). Recently, through the characterization of con/disf1 mutants, it was demonstrated that multiple and temporally separated Hh-signaling events are required for the development of neurocranial skeleton in zebrafish. In these mutant embryos neural crest cell condensations failed to maintain the expression of key transcription factors for chondrogenesis (Schwand and Ahlgren, 2009).

The three members of the Hh family of secreted factors, Sonic hedgehog (Shh), Indian hedgehog (Ihh, orthologous to the mammalian Indian hedgehog and formerly called Banded hedgehog), and Desert hedgehog (Dhh, orthologous to the mammalian Desert hedgehog and formerly called Cephalic hedgehog) were found in Xenopus embryos (Ekker et al., 1995). Shh is the most studied member in all species, plays crucial roles in animal developmental processes and in the homeostasis of adult tissues (Chari and McDonnell, 2007; Ruiz i Altaba et al., 2002; Wang et al., 2007). In Xenopus, Shh is required for neural tube patterning (for a review see Ruiz i Altaba et al., 2003), during limb development and regeneration (Endo et al., 1997; Endo et al., 2000; Yakushiji et al., 2009), in hypaxial muscle development (Martin et al., 2007), axonal guidance (Gordon et al., 2010), and during amphibian intestinal remodeling (Ishizuya-Oka et al., 2006). On the other hand, Ihh and Dhh displayed novel and distinct expression patterns. Interestingly, Ihh expression was observed in peripheral regions of the neural plate and, at that time, it was implicated in the direct induction of the cement gland (Ekker et al., 1995) and in endochondrial ossification but the precise role of Ihh signaling in Xenopus neural crest development has yet to be elucidated.

Here, we investigated the role of the Ihh signaling pathway in the early development of the neural crest in Xenopus embryos. Single and double in situ hybridization revealed that Ihh and different components of the Hh pathway are expressed in the prospective neural crest. Gain- and loss-of-function experiments showed that Ihh signaling is specifically involved in the early induction of the neural crest precursors while the specification of Shh-dependent structures remains unaffected. A detailed analysis was carried out to establish the temporal requirement of Ihh signaling. A combined embryological and molecular approach enabled us to show the participation of Ihh during neural crest migration. Collectively, we describe findings that offer an insight into the Ihh signaling role during neural crest induction, specification and migration in the amphibian embryo.

**Material and methods**

**Embryonic manipulation, RNA microinjection and lineage tracing**

Embryos were obtained by standard procedures (Aybar et al., 2003) and staged according to Nieuwkoop and Faber (1967). Tissue dissections were performed as previously described (Aybar et al., 2003; Bonano et al., 2008); mRNA synthesis, microinjection, and lineage tracing were performed as described (Aybar et al., 2003; Bonano et al., 2008). In vitro transcribed mRNA was synthesized from pT7TSXbhh (Ekker et al., 1995), pT7TSXbhhN (Lai et al., 1995), and pT7TSXbhhΔN-C (Lai et al., 1995) by standard procedures.

**Cyclopamine treatments**

In order to assess Hh activity, resin (Bio-Rex RG 501-8X resin, Bio-Rad, USA) or Heparin-Sepharose (Amersham, USA) beads were soaked with 20–50 μM of cyclopamine (N-aminoethyl aminoacryl dihydrocinamoyl cyclopamine, LC Laboratories, USA) and grafted into embryos as previously described (Bonano et al., 2008; Honore et al., 2003). Cyclopamine-soaked beads were implanted in the right neural fold region (stages 11.5, 12, 14, and 18), or in the right branchial arch region (stage 23) and the final position of beads was carefully examined under a stereoscopic microscope after the in situ hybridization procedure. BSA- or vehicle-soaked beads were grafted as control in sibling embryos at the same position.

**Morpholino antisense oligonucleotide and DNA constructs**

A morpholino antisense oligonucleotide (IhhMO) was designed and synthesized against Xenopus laevis Ihh including the initiation start site (from +1 to +24 bp) with the sequence 5'-CAGCGAACAAGTGGAATTCTCAG-3' (Gene Tools, LLC). Doses of 10–25 ng/embryo were microinjected with a lineage tracer into one blastomere at the 8- or 16-cell stage embryos. A control antisense morpholino oligonucleotide (CoMO) composed of a random sequence (5'-CCCTTACCTCAGTACTTTATA-3', Gene Tools, LLC) was injected as a control. The Ihh ORF from pT7TSXhh was used as a template for DNA constructs and in vitro mRNA synthesis. For the rescue of morpholino knock-down experiments a DNA construct was prepared by changing only the codon sequence targeted by the IhhMO antisense oligonucleotide. Special care was taken to mutate the DNA sequence without changing the wild type Ihh amino acid sequence. This construct, named CRIhh (CR, Codon Replacement), introduced 6 mismatches in the nucleotide sequence recognized by the morpholino and was made by high fidelity PCR using the following primers: 5'-GGATCCATGCAACTACCCAAAGTCGTTCTGCTTCTC-3' (underlined, BamHI restriction site) and 5'-GTCTAGACTTCGCTGCGATGCT-3' (underlined, NcoI restriction site). The PCR products were purified, A-tailed and cloned into pT7G0TA (pCR II-TOPO) to produce pT7G0TA-Ihh. The PCR products were directionally inserted into BamHI and NcoI pCS2+EGFP vector to produce pCS2+EGFP-Ihh. Both fusion constructs were sequenced on both strands at junction sites by automated DNA sequencing.

In order to test the efficiency of IhhMO in vivo, a IhhGFP fusion was generated by high fidelity PCR using pT7TSXhh as the template and the following primers: 5'-GGATCCATGCAACTACCCAAAGTCGTTCTGCTTCTC-3' (underlined, BamHI restriction site) and 5'-CCATGGTGCCTCCGACCTTTGACAGCGTTTCTACCCAAAGTCGTTCTGCTTCTC-3' (underlined, NcoI restriction site). A fragment containing the N-region of Ihh encoding 196 amino acid residues of Ihh was cloned into the pTOPO-TA vector, amplified and then cloned directionally into BamH1 and Ncol pcS2 + EGFP vector to produce the IhhGFP construct. In vitro transcribed mRNA of this construct was co-injected with different amounts of IhhMO and the GFP fluorescence was observed under a Leica fluorescence stereomicroscope. An additional construct, named CRIhhGFP, was synthesized to complement the testing of IhhMO efficacy. This construct carries 6 mismatches and conserves the amino acid coding of with. The IhhGFP construct was used as a template to amplify by high fidelity PCR a 5'-region that was fused to GFP. The primers used were: 5'-GGATCCATGCAACTACCCAAAGTCGTTCTGCTTCTC-3' (underlined, BamHI restriction site; lowercase and bold letters indicate mismatched bases) and 5'-CCATGGTGCCTCCGACCTTTGACAGCGTTTCTACCCAAAGTCGTTCTGCTTCTC-3' (underlined NcoI restriction site).
In situ hybridization, TUNEL and immunohistochemistry

Antisense probes containing Digoxigenin-11-UTP or Fluorescein-12-UTP were prepared for Ihh (Ekker et al., 1995), Gli3 (Marine et al., 1997), Ptc1 (Koenbernick et al., 2001; Perron et al., 2003), Gli2 (Koenbernick et al., 2001; Perron et al., 2003), Snai1 (Mayor et al., 1995), Snai2 (Ayar et al., 2003), FoxD3 (Sasai et al., 2001), Mso1 (Triulzo et al., 2003), Sox2 (Dr. R.M. Grainger, personal communication), Tpr2 (Aoki et al., 2003), Pintailavis (also known as FoxA4a), (Ruiz i Altarriba et al., 1993), XK81a (Jonas et al., 1998), Paraxis (Carpio et al., 2004) and Nkx6.2 (EST clone X461615, kindly donated by Dr. Naoto Ueno) by in vitro transfection. Specimens were prepared, hybridized, and stained as previously described (Ayar et al., 2003). As control, sense probes were synthesized and hybridized and did not produce any staining. Apoptosis was detected by TUNEL staining according to the procedure previously described (Hensey and Gautier, 1998; Tribulio et al., 2004). For cartilage staining, embryos were fixed at stages 45–47 and processed as previously described (Bonano et al., 2008). Rabbit Polyclonal anti-phosphohippohostine-3 (Upstate Biotechnology) was used to analyze mitotic cells according the method previously described (Bonano et al., 2008; Turner and Weintraub, 1994).

Results

Expression of Ihh and Hh signaling pathway components in the neural crest

Although the expression pattern of Ihh has already been reported for Xenopus embryos (Ekker et al., 1995), we decided to analyze and compare it with the expression of different components of the Hh signaling pathway and with specific neuronal crest gene markers. Single and double in situ hybridizations were performed in embryos at different stages in order to supplement previously published expression patterns (Fig. 1). At the end of gastrulation (stage 12.5) the expression of Ihh was observed in low levels in the dorsal anterior region of the embryo. This expression occurred mostly in the anterior neural plate and at the lateral border (Fig. 1A, arrowheads). At the midneural stage (stages 14–19) the expression increased in the neural plate and neural plate border (Fig. 1B) but was not observed along the midline (arrow). Sibling embryos stained with sense Ihh probes showed no labeling (Fig. 1B, inset). Transversal sections of stage 16 embryos revealed that Ihh expression occurred mainly in the internal ectodermal layers corresponding to the neural plate and prospective neural crest and in the somitic mesoderm (Fig. 1). At stage 14, a double in situ hybridization of Ihh and the neural crest marker FoxD3 showed a clear overlapping expression of both genes in the neural crest region (Fig. 1M) in whole mount and in sectioned embryos (Fig. 1M'). At stages 21–23, neural crest cells migrate and the expression of Ihh was detected in the three migrating cranial neural crest streams (Fig. 1C, arrowheads) and in the otic and optic vesicles. From stage 22 onwards, Ihh expression appeared localized in a conspicuous chevron-shaped band within each somite (Fig. 1C). We also analyzed the expression of other members of the Hedgehog signaling pathway. The whole-mount in situ hybridization spatial analysis of Patched-1 (Ptc1) showed that this receptor is expressed in early neurula stage embryos in the anterior region of the neural plate and neural plate border (Fig. 1D). Transverse sections revealed Ptc1 expression in the internal ectodermal layer reminiscent of Ihh expression pattern (Fig. 1J). Smoothed (Smo) transcripts were detectable at low levels from early neurula stage (stage 12.5) onwards in a pattern similar to Ihh (Figs. 1E and F). At mid-neurula stages (stages 15–19) the transverse sections showed Smo expression in the internal ectodermal layer and in the underlying mesoderm (Fig. 1K). By stage 22, expression was localized in the head region in the optic and otic vesicles and in the cephalic migratory neural crest streams (Fig. 1G). The expression of Hedgehog effector genes Gli2 and Gli3 was also analyzed by single and double in situ
hybridization. Gli3 expression appeared in the neural fold region (Fig. 1H) and in the internal ectodermal layer at midneurula stages (Figs. 1L and O). Gli2 transcripts were observed in the neural plate and neural plate border overlapping with the expression of the FoxD3 marker in the most lateral domain of Gli2 expression (Figs. 1N, N'). Gli3 expression was found more lateral than Gli2 and overlapping with FoxD3 expression in its central domain of expression (Figs. 1O and O').

We also analyzed the expression of Ihh and members of the Hedgehog pathway by RT-PCR in neural crest, intermediate mesoderm or non-neural ectoderm isolated from embryos at mid-neurula stage (stage 16). As the intermediate mesoderm interacts with the neural crest region during neurulation (Bonano et al., 2008; Steventon et al., 2009), we considered it interesting to establish the expression of Hedgehog components in this tissue. The results confirmed that Ihh and members of the Hedgehog signaling pathway Ptc1, Smo, and Gli3 genes are expressed in the neural crest tissue (Fig. 2A). Ihh, Ptc1, Smo and MyoD expression was detected in the intermediate mesoderm. Moreover, we independently analyzed Ptc1 expression levels by qPCR as readout of Hedgehog signaling in different regions of stage 13 embryos. We were able to detect Ptc1 expression that is indicative of an active Hedgehog signaling pathway in the neural crest tissue. In comparison with the neural crest Ptc1 levels, the notochord, intermediate mesodermal tissue, and the non-neural ectoderm showed almost similar expression levels. The neural plate showed the highest expression level, reaching almost 3-fold the neural crest level. We conclude that Ihh and the Hedgehog Smo and Gli genes are expressed in the neural crest, that the Ihh signaling is active in the neural crest and that the Ihh signal is also available from the subjacent mesoderm.
Ihh signaling is required for neural crest initial development

The expression patterns of Ihh and Hedgehog pathway components strongly suggest that Ihh signaling could have a role during the initial steps of neural crest development. We investigated the activity of Ihh by examining the effects of knocking down its expression using an antisense morpholino oligonucleotide directed against the initial codons of Ihh (IhhMO). We first analyzed the expression of GFP fusion constructs. IhhGFP-injected embryos (Figs. 3A and A') and control morpholino- and IhhGFP-co-injected embryos (Figs. 3B and B') showed GFP fluorescence. Then we analyzed IhhMO oligonucleotide efficiency and confirmed that IhhMO was able to inhibit the translation of the GFP-tagged form of Ihh in a dose-dependent manner (IhhGFP, Figs. 3C and D). We also prepared an IhhGFP fusion construct carrying a 6-bp mutation in the morpholino recognition site (CRihhGFP). The expression of this construct was not affected when it was coinjected with IhhMO at the same doses (Figs. 3E and F).

To assess the participation of Ihh signaling during neural crest induction, embryos were injected at the 8-cell stage in one dorsal blastomere with wtIhhMO (25 ng/embryo) (Figs. 3G–U) or an equivalent amount of a control morpholino (CoMO, Fig. 3V) to the neural plate border rescued neural crest induction without affecting midline marker expression at the injected site (Fig. 3R, 84% n=32), while embryos injected with wtIhhMO with wtnihh (not shown) and a Snail2-promoter driven CRihh construct restored to normal the expression levels of the neural crest markers FoxD3 and Snail2 (Fig. 3P, 86%; n=77and 3Q, 81%, n=76). In order to rule out the possibility that Ihh knock-out could affect the specification of the embryonic midline, we simultaneously evaluated by double in situ hybridization the expression of the FoxD3 marker gene along midline markers Nkx6.2 and Pintallavis (FoxA4a). We observed that while neural crest expression of FoxD3 was strongly reduced due to the expression of midline marker remained unaffected (Fig. 3T, 84% n=32 and 3S, 85% n=35, respectively). The effect on neural crest induction was rescued by the co-injection of CRihh and by the microinjection of Shh mRNA targeted to the neural plate border. In both cases, the increase in Ihh activity or the ectopically targeted activity of Shh at the neural plate border rescued neural crest induction without affecting embryonic midline specification (Fig. 3T, 93% n=30 and Fig. 3U, 89% n=28).

We also analyzed the effects on neural crest specification of the Ihh dominant negative construct IhhDN-C. This construct was found to block in vivo the activity of Ihh and Nihh (Lai et al., 1995). We observed that the overexpression of IhhDN-C mRNA (1–3 ng/embryo) diminished the expression of FoxD3 (Fig. 4A, 59% n=49) and Snail2 neural crest markers (Fig. 4B, 55%, n=56) and increased the neural plate and epidermis territories expressing Sox2 (Fig. 4C, 48% n=34) and XK81a (Fig. 4D, 51% n=39), respectively. The effect of IhhDN-C was rescued when wtnihh and Nihh were coinjected (Fig. 4E, 81% n=53 and Fig. 4F, 62% n=55). Taken together, the effect of IhhMO and IhhDN-C overexpression indicates that Ihh signaling is required during the initial steps of neural crest formation.

In order to establish whether Ihh signaling might participate in the control of neural crest specification, we microinjected Ihh and Nihh mRNAs into the animal pole of a dorsal blastomere of 4-cell stage embryos. Nihh is an active N-terminal fragment of Ihh that mimics its function (Lai et al., 1995). Ihh injection led to an expansion of the neural crest territory analyzed by the expression of FoxD3 (Fig. 5A, 71% n=62), Snail2 (Fig. 5B, 69% n=65) and Snail1 (not shown, 77% n=43). This effect also produced a concomitant reduction in the Sox2 (Fig. 5C, 57% n=67) neural plate marker and the Xk81a epidermal
marker (Fig. 5D, 55% n = 64). Similar effects on neural crest expression were observed when we overexpressed CRIhh (not shown, 84% n = 67) and Nihh mRNAs (Figs. 5E–H, FoxD3 61% n = 80; Snail2 58% n = 72; Sox2 60% n = 53, and Xk81a 55% n = 54). With the overexpression of wtIhh, double in situ hybridization for Sox2 and Xk81a showed that the expansion of neural crest territory was accompanied by a concomitant reduction in the neural plate and epidermal domains (Fig. 5I, 68% n = 28). The Snail2 promoter-driven overexpression of Ihh (Fig. 5J) also led to a marked increase in neural crest territory evidenced by the expression of the FoxD3 marker (Fig. 5K, 100% n = 64 and Fig. 5L 97%, n = 36). No changes were observed in the expression of the midline marker Nkx6.2 in these α3000CRIhh-injected embryos (Fig. 5L).

These observations suggest that the expansion of the neural crest as a result of the ectopic expression of Ihh is produced by the transformation of the epidermis and of part of the neural plate region into prospective neural crest cells.

Ihh signaling is not involved in the control of cell proliferation or apoptosis in the neural crest

It has been previously shown that the apoptotic process is involved in the development of the neural crest (Honore et al., 2003; Tribulo et al., 2004). Moreover, the Hedgehog cell signaling pathway has been implicated in the control of cell proliferation in many cell systems also including neural crest cells (Ahlgren et al., 2002; Dyer and Kirby, 2009; Fu et al., 2004; Marcelle et al., 1999, etc.). We observed significant changes in the area expressing neural crest markers when we activated or blocked the expression or function of Ihh. There is a possibility that these changes were produced by changes in the cell proliferative status or by changes in the apoptotic process. To rule out such possibility we therefore decided to investigate cell proliferation and apoptosis during Ihh gain or loss of function.

The microinjection of Ihh or IhhMO produced no detectable changes in the cell proliferation status assessed by the detection of a phosphorylated form of H3 histone (Fig. 6An=2 4 ;Fig. 6C n=26). We were also unable to find significant changes in apoptotic cell death evaluated by TUNEL staining between the control side and the treated side of embryos injected with CoMO (0% increase in TUNEL staining, n=20, not shown). Taken together, these results led us to conclude that Ihh signaling does not play a role in the control of cell proliferation or apoptosis, and that the alterations observed in the neural crest territories after changes in the activity of the Ihh pathway could represent ectodermal cell fate decisions.

Temporal analysis of Ihh requirement for neural crest induction

Antisense morpholino technology provides a very specific way of inhibiting the expression of a single gene. However, this approach is limited since it is difficult to control the timing of morpholino activity. In order to precisely control the moment of Ihh signaling blocking we used the Hedgehog-specific antagonist cyclopamine. Cyclopamine is a steroidal alkaloid found in the plant Veratrum californicum which interrupts the hedgehog pathway activation by binding directly to the Smoothened (Smo) transmembrane receptor (Chen et al., 2002).

We assessed the inhibition of Ihh signaling by using cyclopamine specifically delivered at the neural plate border at three different moments of development. To achieve this goal acryl beads soaked with different amounts of cyclopamine were grafted into the right prospective neural plate border of stage 11.5 (Figs. 7A and B), stage 12 (late gastrula, Figs. 7C–G), and midneurula stage embryos (stage 14, Figs. 7H–J). Embryos were cultured until different stages and the expression of gene markers was analyzed by in situ hybridization. Treatment of stage 11.5 embryos with 20 μM cyclopamine-soaked beads markedly downregulated Snail1 (Fig. 7A, 64% n = 22), Snail2 (Fig. 7B, 56% n = 18) and Mesp1 (Fig. 7C, 66% n = 18) expression in the neural crest territory. A similar effect on the expression of the same marker was observed when cyclopamine-soaked beads were grafted at stage 12 embryos (Fig. 7D, FoxD3, 67% n = 41, and Fig. 7E, Snail2, 56% n = 43, respectively). The pharmacological inhibition by

---

**Fig. 3.** Ihh is required for neural crest specification. (A–F) In vivo efficiency of Ihh antisense morpholino oligonucleotide (IhhMO). Dorsal views of Xenopus laevis embryos under a fluorescence stereoscopic microscope, anterior side is on the left. White arrows indicate the injected side. (A–F) Fluorescence and clear field images of each embryo are superposed and shown in merged images. (A and A') Embryo injected with mRNA encoding FoxD3GFP (1 ng/embryo) showing GFP fluorescence. (B and B') Embryo injected with IhhGFP mRNA (1 ng/embryo) and CoMO (30 ng/embryo). (C, C', D and D') Embryos injected with IhhGFP mRNA (1 ng/embryo) and IhhMO (C and C', low dose (1), 10 ng/embryo; D and D', high dose (2), 20 ng/embryo). No embryo shows GFP fluorescence at a high dose of IhhMO. (E and E') Embryo injected with CRhGFP mRNA showing GFP fluorescence. (F and F') Embryo injected with CRhGFP mRNA and a high dose (2) of IhhMO (20 ng/embryo). The expression of CRhGFP was not affected by the presence of the morpholino oligonucleotide. (G–V) Analysis of IhhMO effects on neural crest specification. Dorsal views of Xenopus laevis embryos, anterior side is on the left. Arrows indicate the injected side. (G and H) IhhMO-injected embryos show inhibition of FoxD3 and Snail2 neural crest markers. (I and J) Expression of the neural plate marker Sox2 and the epidermal marker XK81a is expanded on the IhhMO-injected side. The brackets indicate the width of neural plate (I), and the width of neural plate plus neural crest domain (J). (K) Double in situ hybridization for Sox2 and XK81a genes evidenced that IhhMO led to the reduction in the prospective neural crest domain in the injected side. The brackets indicate the width of the neural crest domain. (L and M) Expression of the mesodermal marker Paraxis. No effect was observed in a dorsal blastomere of 8–16 cell embryos. (L) The targeting of IhhMO to the mesoderm by microinjection of the vegetative region of blastomere D1.1 produced a reduced Paraxis expression in the injected side (M). (N and O) Co-injection of IhhMO and CRhGFP mRNA rescues FoxD3 and Snail2 expression, respectively. (P and Q) The co-injection of CRhGFP specifically driven by the Snail2 promoter (α3000CRhGFP) rescues the expression of FoxD3 and Snail2 neural crest markers. (R and S) The microinjection of IhhMO into a dorsal blastomere of stage 16 embryos decreases the expression of FoxD3 in the neural crest but produces no effects on the midline markers (double in situ hybridization, white arrowheads, Nkx6.2 and Pintallavsis-FoxA4a). (T and U) The effects of IhhMO on the expression of the neural crest marker FoxD3 is rescued by the directed co-injection of CRhGFP (Q) or 5hh to the neural fold region (U). (V) CoMO-injected embryos show normal expression of FoxD3.
cyclopamine produced an increase in the expression of the neural plate marker Sox2 (Fig. 7F, 63% n=44) and XK81a (Fig. 7G, 61% n=39) expressed in tissues adjacent to the neural crest. Although the cyclopamine-soaked beads were implanted into the right neural plate border and this location is far from the embryo midline, we decided to test for possible effects on the midline gene marker Nkx6.2 (Fig. 7H). We observed no effects on Nkx6.2 expression while cyclopamine local treatment only reduced the neural crest expression of FoxD3 (Fig. 7H, 96% n=39). No effect on the expression of the neural crest marker FoxD3 was observed when BSA-soaked beads were grafted at stage 12 (Fig. 7I, 86% n=21).

When cyclopamine-soaked beads were grafted on the neural crest region at the midneurula stage (stage 14), a weaker effect was observed on the treated side of the embryos and the expression of neural crest markers FoxD3 and Snail2 showed only a mild but consistent downregulation (Fig. 7J, 59% n=39, and Fig. 7K, 70% n=30). No effects on the expression of FoxD3 was found when a BSA-soaked bead was grafted on stage 14 embryos (Fig. 7L, 86% n=21).

The analysis of the expression of the components of the Hedgehog pathway suggested that this cell signaling could be active at the neural plate border during the earliest stages of neural crest induction.
We decided to assess the effects of cyclopamine in neural plate border explants by analyzing the expression of early specification neural crest gene markers (Fig. 7M). We assessed Pax3 and Sox10 gene markers as they are considered a neural plate border specifier and neural crest specifier, respectively (Meulemans and Bronner-Fraser, 2004). The neural plate border explants were dissected out from stage 11 embryos and incubated in 20 μM cyclopamine until stage 13 when they were processed for RT-PCR. Cyclopamine-treated explants showed a reduced expression of both Pax3 and Sox10 markers.

Notably, the effect of cyclopamine on Sox10 was more intense as the expression of this marker showed approximately a 3.5-fold reduction compared to control explants (Fig. 7M).

Thus, the pharmacological blockade by cyclopamine was able to reproduce the phenotype obtained by the directed microinjection of morpholino oligonucleotides, showing that Ihh activity is required for the early steps of neural crest induction. Furthermore, these results suggest that at midneurula stages the specification of neural crest is still in progress.
Ihh signaling is necessary for the maintenance of neural crest specification

It has been shown that the mesoderm plays a role in neural crest specification and maintenance (Bonano et al., 2008; Bonstein et al., 1998; Mancilla and Mayor, 1996; Marchant et al., 1998; Monsoro-Burq et al., 2003; Steventon et al., 2009). Our results suggest that there is a later requirement for Ihh signaling during specification in the midneurula stages (Fig. 7). We decided to analyze the participation of mesoderm since the early steps of neural crest induction and the participation of Ihh signaling from the late gastrula to the late neurula stages.

NC explants dissected at late gastrula stage (stage 12) and immediately fixed the expressed FoxD3 marker (Fig. 8B). FoxD3 expression was observed both in explants that were dissected out at stage 12 and in explants incubated until stage 16 containing (NC + M) or not (NC) the underlying mesodermal tissue (Figs. 8C and D). When NC + M explants were incubated in the presence of 50 μM cyclopamine the FoxD3 expression was downregulated (Fig. 8E).

A strong expression of FoxD3 was observed in neural crest explants dissected at stage 14 or 17 and fixed immediately (Figs. 8F and J). When equivalent stage 14 neural crest explants with no mesoderm were cultured until the equivalent of stage 18, the expression of FoxD3 was notoriously reduced (Fig. 8G). Conversely, in the neural crest explants containing the subjacent mesoderm (NC + M) the expression of FoxD3 was strong and similar to control explants (Fig. 8H). When these NC + M explants were incubated with 50 μM cyclopamine the expression of FoxD3 almost completely disappeared (Fig. 8I). At later stages, in NC explants that were dissected containing no mesoderm at stage 17 and cultured to equivalent stage 23, the expression of FoxD3 was lost (Fig. 8K), while the explants containing mesoderm conserved the expression of this gene (Fig. 8L). In contrast, in the NC + M explants cultured until stage 23 in 50 μM cyclopamine the expression of FoxD3 was abolished (Fig. 8M).

Taken together, these findings strongly suggest that mesodermal requirement increases from gastrulation to late neurula stages and that Ihh signaling is required during the same period.

Ihh signaling is required for neural crest migration

Ihh transcripts were found to be expressed in the premigratory and migratory neural crest (Figs. 1 and 2). Moreover, our NC explants culture approach revealed that the mesoderm–neural crest relationship is progressively required from early to late neurulation stages to sustain the specification status of neural crest cells (Fig. 8). In these NC explants a Hedgehog signal is also required. All these findings suggest that Ihh signaling might also participate in the migratory process. We blocked the Hedgehog pathway using cyclopamine-loaded beads implanted in the neural crest region of stage 18 embryos, when neural crest cell...
Fig. 9. Ihh signaling is required for neural crest migration. The anterior side of the embryos is on the left. (A) Stage 18 embryos were grafted on the right neural crest region with a 20 μM cyclopamine-soaked bead. Embryos were cultured until stages 21–23 and the expression pattern of marker genes was analyzed. FoxD3 expressing neural crest cells show arrested migration and accumulated laterally to the hindbrain. The leading edge of migration is indicated by dashed lines. (C) Embryos grafted with control BSA-soaked beads show normal neural crest migration compared with the control untreated side (D). (E) Schematic drawing indicating the experimental procedure for the analysis of Ihh participation during neural crest migration. One-cell stage embryos were microinjected with CoMO, IhhMO, α3000CRIhh construct and lineage tracer. When embryos reached stage 17, neural crest explants containing the underlying mesodermal tissue (NC + M) or neural crest tissue alone (NC) were grafted into wild type or IhhMO injected host embryos. (F and F') CoMO-injected NC + M explants show normal migration when transplanted into wild type host embryos. (G and G') IhhMO-injected NC explants grafted into normal wild type host embryos show normal neural crest migration. (H and H') IhhMO- and α3000CRIhh-coinjected NC + M explants grafted into wild type embryos show normal neural crest migration. The CRIhh construct rescued neural crest and produced a normal migratory cell population. The mesodermal source of Ihh is able to support the migration. (I and I') IhhMO- and α3000CRIhh-coinjected NC + M explants grafted onto wild type embryos show normal neural crest migration. (J and J') Wild type NC explants grafted into IhhMO-injected host embryos show normal migration. The neural crest production of Ihh is able to sustain normal cell migration.
migration is about to begin. We observed that cyclopamine produced a clear effect on neural crest cell migration as in the treated side the front of migration, revealed by FoxD3 expression, migrated less than in the control side (Fig. 9G, 72% n = 42). Control BSA- or saline solution-soaked beads produced no effect on neural crest migration (Fig. 9C, 81% n = 33).

As Ihh was also expressed in the mesoderm underlying the neural crest (Figs. 1 and 2), we then wanted to determine the role of this mesodermal source in the migratory process. In order to assess its participation, we performed a transplantation approach transferring neural crest tissue (NC) or neural crest tissue containing the underlying mesodermal tissue (NC + M) to stage 17 wild type- or IhhMO-injected embryos. Donor embryos were microinjected with IhhMO, α3000CRlhh or CoMO and FDA as a lineage tracer and neural crest explants were grafted orthotopically onto host embryos (Fig. 9E). When CoMO-injected neural crest grafts were transplanted to normal wild type embryos a normal cranial neural crest migration pattern was observed (Fig. 9F, 86%, n = 18). Strikingly, when IhhMO-injected NC neural crest explants containing no underlying mesoderm were grafted onto normal host embryos the migration pattern was completely normal, suggesting that the Ihh signal emanating from the subjacent mesoderm rescued neural crest migration (Fig. 9G, 75%, n = 20). The migration of neural crest cells from an IhhMO-injected NC + M graft was rescued by the co-injection of the α3000CRlhh/GFP constructs (Fig. 9H, 78%, n = 18). When lineage tracer-injected NC normal explants were transplanted to IhhMO-treated embryos a normal neural crest migration could be seen (Fig. 9K, 77%, n = 22). In contrast, IhhMO-injected NC grafts transplanted into IhhMO-injected embryos showed no cranial neural crest migration (Fig. 9I, 100%, n = 19). Additionally, the migratory behavior of NC + M explants taken from IhhMO-injected embryos was rescued by the coinjection of α3000CRlhh and a normal migratory pattern in the cephalic neural crest was observed (Fig. 9J, 90%, n = 18).

Taken together, these observations indicate that Ihh signaling is required for the normal migration of cranial neural crest cells. Furthermore, our findings suggest that Ihh could act in both an autocrine and a paracrine manner to support neural crest migration.

**Ihh is required for craniofacial derivatives development**

We decided to investigate the effect of Ihh knockout on neural crest derivatives after the injection of Ihh morpholino antisense oligonucleotides (Fig. 10A). The analysis of cartilage morphology by Alcian blue staining of IhhMO-injected embryos showed a severe reduction in craniofacial cartilages in the BhMO-injected side (Fig. 10A, 66% n = 80). The gross morphology analysis of craniofacial cartilages revealed that neural crest-derived cartilages (Meckel’s, ceratobranchial and ceratohyal cartilages) were markedly reduced. The injection of CoMO showed no effect on craniofacial cartilage size or morphology (Fig. 10F). The effect of IhhMO was rescued by the co-injection of Snail2 promoter-directed Crlhh construct (α3000CRlhh, Fig. 10B, 79% n = 56). We also assessed the late participation of Hh signaling in craniofacial cartilage development. We grafted cyclopamine beads in the right branchial arch region of stage 23 embryos. We observed that in the cyclopamine-treated side the development of craniofacial cartilages was severely affected (Fig. 10D, 95%, n = 44). The control grafting of BSA-loaded beads in the branchial region of sibling embryos produced no effects (Fig 10E, 100%, n = 40). We also observed that the injection of IhhMO produced no effects either on Trp2 expression (Fig. 10I, 100% n = 40) or on melanocyte pattern (Fig. 10G, H, 100% n = 31). In the IhhMO treated embryos no significant differences in melanocyte number or pattern were observed between injected and control sides. These results show that Ihh signaling is required for the normal formation of specific neural crest derivatives such as cartilage but not for the formation of other derivatives like melanocytes.

**Discussion**

Neural crest development is a highly orchestrated process that requires the contribution of various cell signaling pathways and involves the participation of different embryonic tissues. In this study, we showed that early induction, maintenance of specification and migration require Ihh cell signaling. Significant progress has been achieved in understanding the role of Shh in different developmental processes and animal models but the roles of other Hedgehog molecules (i.e. Ihh and Dhh, formerly named Banded hedgehog and Cephalic hedgehog, respectively) during embryogenesis are less known.

We report here the analysis of the Ihh expression pattern and the expression of Hh pathway members Ptc-1, Smo and the effectors Gli2 and Gli3. The comparative analysis of the expression with neural crest and neural plate markers confirmed the expression of these genes in the neural crest during the late gastrula and neurula stages. These results extend and complement the previously reported expression pattern (Ekker et al., 1995) and suggest that new roles could be assumed by Ihh at the neural plate border that are distinct from those attributed to Shh (Brewster et al., 1998; Franco et al., 1999; Lopez et al., 2003; Ruiz i Altaba, 1998).

The expression patterns of Ihh and Hh components in the neural fold suggest that this signaling pathway plays a role in neural crest specification in Xenopus embryos. Ihh knock-down using a specific morpholino oligonucleotide or the pharmacological inactivation by cyclopamine-soaked beads grafted on the neural fold caused depletion of neural crest precursors. In contrast, the overexpression of Ihh produced an increase in the size of the neural crest territory. These results are in agreement with the findings of Franco et al. (1999), who showed an increased expression of neural fold prepattern gene Zic2 after the injection of Ihh or Shh in 2-cell stage embryos. They also reported that Ihh or Shh injection increased the size of the neural plate and reduced the expression of the Snail2 neural crest marker (Franco et al., 1999). This represents an unexpected result as Zic2 gene is anti-neurogenic and a promoter of neural crest induction (Brewster et al., 1998; Nakata et al., 1998). In this work, extreme care was taken to direct Ihh overexpression to the lateral aspect of the neural plate and the neural plate border region by the injection of one dorsal blastomere of 8- to 16-cell stage embryos, or using the Snail2 promoter (α3000, Vallin et al., 2001) to drive the expression of an Ihh construct. Moreover, the cyclopamine-soaked beads were implanted specifically into the neural fold region. These experimental approaches allowed us to assess the participation of Ihh signaling exclusively in the neural crest while the specification of dorsal midline remained unaffected. However, we also showed that the ectopic action of Shh at the neural plate border could rescue the loss of Ihh at the neural fold to support neural crest initial specification. Our results suggest that Ihh possibly has a role in the neural plate that could be different from the one in the neural crest. This suggests the possibility that Ihh could activate molecular mechanisms at the neural fold that support the counterbalance that apparently exists during the specification of ectodermal cells that control the neural versus neural crest fate decision, manifested when one territory increases its size at the expense of another. The lack of change in the cell proliferation or apoptosis status in Ihh- or IhhMO-injected embryos reinforces the participation of the Ihh signaling cascade as a switch in cell fate decisions that take place in the ectoderm during the induction of the neural crest cell population. Conversely, an increased apoptosis rate has been described in chick embryo when Shh was inhibited (Ahlgren and Bronner-Fraser, 1999).

The early requirement of Hedgehog signaling for a correct development of the neural crest appears to be a feature of amphibian embryos. In the chick embryo the increased activity of Shh abrogates NC induction (Liem et al., 1993; Selleck et al., 1998). In addition, the disruption of dorsal-ventral neural tube patterning by cyclopamine-blocking of Hedgehog signaling after NC ablation induced migratory cardiac neural crest-like cells (Hutson et al., 2009). It has been shown for zebrafish
embryos that most neural crest-derived craniofacial cartilages are lost or mislocalized by treatment with cyclopamine during the gastrulation period, but this requirement of Hh signaling is indirect and acts by specifying the stomodeal ectoderm before the neural crest cells arrive at its vicinity (Eberhart et al., 2006; Wada et al., 2005). Cyclopamine treatments of zebrafish embryos during early neurulation and neural crest specification also resulted in reduced mandibular cartilage (Schwend and Ahlgren, 2009). It has been suggested that although genetic studies in zebrafish have shown that Shh is involved in the formation of craniofacial cartilages, this role could be shared with Shhb (formerly named tiggy-winkle hedgehog, twhh), which is expressed in a similar pattern (Wada et al., 2005). In agreement with the published experimental data in zebrafish studies, we also found that late treatment with cyclopamine disrupts craniofacial cartilage formation. However, more studies are needed to discriminate between different Hedgehog signaling molecules since Shh and Ihh are both expressed in the branchial arch region (Ekker et al., 1995, this work) and also to determine their relative contribution to chondrogenesis.

To additionally understand the participation of Ihh signaling during neural crest development, we evaluated the temporal requirement of this signaling at different times of neural crest formation. Previous studies have demonstrated that the competence window for Xenopus neural crest induction is established from stages 10+ to 13 (Mancilla and Mayor, 1996). Our analysis using cyclopamine-treated stage 11 explants and cyclopamine-soaked beads grafted at stages 11.5, 12 and 14 and neural crest explants taken from stages 12, 14 and 17 embryos showed that Ihh signaling requirement goes beyond the ectodermal competence period and lasts until the midneurula stage. This agrees with previous observations that demonstrated the requirement of BMP, Wnt and Edn1 as maintenance signals from the subjacent mesoderm that sustain the neural crest specification status (Bonano et al., 2008; Steventon et al., 2009). When we analyzed the effects of cyclopamine on neural fold explants at the onset of neural crest induction we were able to establish that both Pax3, a gene considered a neural fold specifier, and Sox10, a neural crest specifier (Meulemans and Bronner-Fraser, 2004), showed a decreased expression. We found that Sox10 expression was more strongly inhibited than Pax3 expression, which suggests that the Ihh signaling pathway is probably able to selectively regulate certain components of the genetic cascade that controls neural crest induction, although this requires further investigation.

Ihh is expressed in the premigratory and migratory neural crest cells in Xenopus embryos. Here we showed that Ihh signaling is continuously required for neural crest migration and that this signal functions in both autocrine and paracrine modes. In contrast, in zebrafish embryos it has been shown that cyclopamine treatment during cranial neural crest migration caused little effects on the development of pharyngeal arch elements, revealing a differential requirement for Hh signaling (Schwend et al., 2011).
In our transplantation assays the Ihh source located in the somitic mesoderm was able to promote and maintain neural crest migration. It has been demonstrated that the mesoderm is involved in the neural crest initial induction (Bonstein et al., 1998; Marchant et al., 1998; Steventon et al., 2009), and in recent years the relationship between neural crest and surrounding tissues has increased in importance since those tissues provide essential signaling molecules required for migration and later development (Ahlgren and Bronner-Fraser, 1999; Bonano et al., 2008; Brito et al., 2006; Kimmel et al., 2001; Steventon et al., 2009).

In this work we showed that early Ihh abolition by the morpholino oligonucleotide and a late cyclopamine treatment in the branchial arch region at the end of neural crest migration (stage 23) impaired the formation of cranial neural crest-derived cartilages, although no changes were found in the generation of other trunk neural crest derivatives such as pigment cells. This observation agrees with a previous report that shows that Xenopus cranial neural crest cells are sensitive to cyclopamine treatment while trunk neural crest cells are resistant to treatment (Dunn et al., 1995). Species-specific requirements for Hh signals appear to be operating since in zebrafish embryos the genetic and pharmacological inhibition of Hh signaling leads directly to loss of dorsal root ganglia neurons derived from trunk neural crest, although there were no obvious effects on the level of expression of the crestin marker, on the pattern of neural crest migration or in the development of pigment cells and fin ectomesenchyme (Ungos et al., 2003).

Our results support an early role for Ihh in the neural crest development signaling based on the use of a specific morpholino to abolish its expression and temporal analysis of cyclopamine local effects. This leads us to propose that, among the multiplicity of signals operating in the neural crest early specification, Ihh signaling might participate in the establishment of different cell subtypes in the premigratory and migratory neural crest. Thus, the initial loss of the neural crest affected the size of cranial neural crest-derived structures, and the late effect of cyclopamine suggest that Ihh is required for the molecular events of craniofacial development, although this possibility requires further investigation. In zebrafish it has been shown that Hh-signaling is also required for the expression of transcription factors involved in chondrogenesis and osteogenesis differentiation (Schwend and Ahlgren, 2009). In addition, in mouse embryos, Ihh (the mammalian homologous of Xenopus Ihh) is expressed in the proximal region of Meckel’s cartilage, which is entirely of neural crest origin, undergoes endochondral ossification and contributes to sclerotic ossicles (Iwasaki et al., 1997). Moreover, Ihh signaling also participates in the endochondral ossification during Xenopus limb formation (Morishii et al., 2005). In summary, our findings demonstrate the embryonic requirement of Ihh signaling for neural crest development. However, further research is necessary to understand the molecular mechanisms underlying its participation in each neural crest event. The transcription factors Gli2 or Gli3, in their activator or repressor forms, appear as interesting candidates acting in the transduction of Ihh signaling in neural crest cells, but such possibility still remains to be explored.

Acknowledgments

We wish to thank Stephen Ekker for the Hedgehog clones, Florence Broders, Yoshiki Sasai, Robert Grainger, Jean-Pierre Saint-Jeannet, Thomas Sargent, Dave Turner, Muriel Perron, Ariel Ruiz i Altaba, and Naoto Ueno for the donation of other cDNAs used in this research. We specially thank Roberto Mayor for discussion and helpful comments, and Mrs. Virginia Méndez for the proofreading. This investigation was supported by grants from ANPCyT-Foncyt to MJA (PICT2007-0692, PICT2010-1224) and CT (PICT2007-0737), and by grants from CIUNT to MJA and CT.

References
