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Spatio-Temporal Dynamics of Gene Expression of the Edn1-Dlx5/6 Pathway During Development of the Lower Jaw

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Summary: The morphogenesis of the vertebrate skull results from highly dynamic integrated processes involving the exchange of signals between the ectoderm, the endoderm, and cephalic neural crest cells (CNCCs). Before migration CNCCs are not committed to form any specific skull element, molecular signals exchanged in restricted regions of tissue interaction are crucial in providing positional identity to the CNCCs mesenchyme and activate the specific morphogenetic process of different skeletal components of the head. In particular, the endothelin-1 (Edn1)-dependent activation of Dlx5 and Dlx6 in CNCCs that colonize the first pharyngeal arch (PA1) is necessary and sufficient to specify maxillo-mandibular identity. Here, to better analyze the spatio-temporal dynamics of this process, we associate quantitative gene expression analysis with detailed examination of skeletal phenotypes resulting from combined allelic reduction of Edn1, Dlx5, and Dlx6. We show that Edn1-dependent and -independent regulatory pathways act at different developmental times in distinct regions of PA1. The Edn1 → Dlx5/6 → Hand2 pathway is already active at E9.5 during early stages of CNCCs colonization. At later stages (E10.5) the scenario is more complex: we propose a model in which PA1 is subdivided into four adjacent territories in which distinct regulations are taking place. This new developmental model may provide a conceptual framework to interpret the craniofacial malformations present in several mouse mutants and in human first arch syndromes. More in general, our findings emphasize the importance of quantitative gene expression in the fine control of morphogenetic events.

Key words: endothelin-1; Dlx; craniofacial development; pharyngeal arches; allelic dosage; cranial neural crest cells; first arch syndromes

INTRODUCTION

Vertebrate jaws are formed through complex morphogenetic processes beginning with the colonization of the first pharyngeal arch (PA1) by Hox-negative cephalic neural crest cells (CNCCs) emigrating from the posterior mesencephalic and rhombencephalic neural folds.
Whereas CNCCs give rise to most chondrocranial and dermocranial elements of the jaws (Clouthier et al., 1998; Couly et al., 2002; Depew and Simpson, 2006; Kontges and Lumsden, 1996; Ruhin et al., 2003), they do not possess, before migration, the topographic information needed to carry out the jaw morphogenesis (Couly et al., 1993). Surgical removal and grafting of small territories of the foregut endoderm at different developmental stages has shown that this epithelium provides to CNCCs part of the topographic information needed to form jaw structures (Couly et al., 1993; Kontges and Lumsden, 1996; Kurihara et al., 1994; Le Douarin and Dupin, 2003; Noden and Trainor, 2005; Trainor and Tam, 1995). The molecular nature of the endodermal signals is only partly known, as experimental evidence suggests that FGFs, BMPs, Edn1, and Shh are surely involved (Benouaiche et al., 2008; Ozeki et al., 2004; Vieux-Rochas et al., 2007).

In this study, we have analyzed mice with combined and/or partial loss of Edn1 and Dlx5, Dlx6 alleles. The Edn1→Dlx5/6→Hand2 signaling is a relevant model to study the spatio-temporal dynamics of gene expression in the PA1 and the consequences for CNCCs specification. Indeed Edn1 is expressed in the endoderm and in the mesodermal core of the mandibular prominence of PA1, whereas Ednra (Edn1 receptor-type A) is broadly expressed by the CNCC-derived PA1 ectomesenchyme and Dlx5, Dlx6 are only expressed in the mesenchyme of the mandibular prominence (Abe et al., 2007; Clouthier et al., 1998, 2000; Ozeki et al., 2004; Ruest et al., 2004, 2005). Loss of Edn1→Ednra signaling results in the down regulation of the two members of the distalless homeobox gene family Dlx5 and Dlx6 (Merlo et al., 2002a; Panganiban and Rubenstein, 2002), and in a homeotic-like transformation of lower into upper jaw structures, similar to that observed upon double inactivation of Dlx5 and Dlx6 (Beverdam et al., 2002; Depew et al., 2002; Fukuhara et al., 2004; Ruest et al., 2004). The constitutive activation of the Edn1→Ednra signaling in the entire PA1 induces a partial transformation of the upper jaw suggesting that PA1 CNCCs are competent to respond to Edn1 signaling.

Within the PA1 of E10.5 mouse embryos Dlx genes are expressed in nested proximo/distal domains: Dlx1 and Dlx2 in the proximal and distal maxillary and mandibular prominences, Dlx5 and Dlx6 in the entire mandibular prominence, while Dlx3 only in a medio/distal territory of the mandibular prominence (Depew et al., 2002; Merlo et al., 2000). The most informative data on the role of Dlx genes in PA1 patterning come from the analysis of mice carrying single or multiple inactivating mutations for Dlx1, Dlx2, Dlx5, and Dlx6. In Dlx5, 6 double mutant mice, lower jaw cartilages and bones are transformed and acquire the shape typical of upper jaw elements. Furthermore, in Dlx5, 6 double null mice, vibrissae and palatine rugae are symmetrically present in the upper and lower jaw, suggesting that an homeotic transformation has taken place (Beverdam et al., 2002; Depew et al., 2002). In Dlx1, 2 double null mice the proximal maxillary region develops abnormal skeletal elements reminiscent of the reptilian upper jaw (Depew et al., 2005; Qiu et al., 1997). These observations have led to the proposition that the combinatorial expression of Dlx genes by PA1 CNCCs determine their relative position and their capacity to give rise to different skeletal elements (Depew and Simpson, 2006; Depew et al., 2005; Merlo et al., 2000).

Several genes have been shown to act downstream of Dlx5 and Dlx6, including Gsc, Pitx1, Wnt5a, Dlx3, Meis2, and the bHLH transcription factor Hand2 (Beverdam et al., 2002; Depew et al., 1999, 2002; Merlo et al., 2000, 2002a). A further set of candidate targets of Dlx5/6 have been recently identified (Jeong et al., 2008). Several of the proposed targets might be directly regulated by Dlx5/6 (e.g., Gbx2, Hand2) as their promoters harbor Dlx-binding regulatory elements (Charite et al., 2001; Jeong et al., 2008).

Integrating quantitative gene expression data with observed phenotypes we propose that Edn1 signaling occurs in two phases: (1) early in development, Edn1 activates the Dlx5/6→Hand2 pathway in postmigratory CNCCs. (2) Late in development, distinct regulations can be recognized in distinct regions of the mandibular prominence: in a more proximal region Dlx5/6 are activated independently from Edn1 and their expression is not associated with Gsc. More distally Dlx5/6 expression depends on Edn1 signaling and results in the activation of downstream genes including Gsc and Pitx1. Hand2 is expressed only in the medio/distal region of the mandibular prominence and its expression depends upon at least three different, regionally restricted, regulations. We conclude that the organization of latero/proximal PA1 structures depends on the quantitative, gene-dosage dependent, regulation of the Edn1→Dlx5/6→Gsc, Pitx1, etc... pathway, while medio/distal lower jaw morphology depends on Hand2 expression. Our findings may also provide the developmental framework in which to elucidate and functionally characterize the molecular lesions, yet to be identified, causing or associated with those human first arch syndromes (FAS) affecting the proximal arch.

RESULTS

Edn1 Allelic Dosage and Dynamics of Dlx and Hand2 Expression in PA1

To better define the role of Edn1/Ednra signaling in the control of mandibular morphogenesis, we examined the effects of allelic reduction of Edn1 on the expression of key regulators of PA1 patterning. First, we measured by RT-qPCR the abundance of Dlx2, 3, 5, 6, and Hand2 transcripts in the dissected mandibular prominence (the ventral segment of the PA1) of WT and Edn11/1 E9 embryos. At this stage of development CNCCs are still migrating, but most of them have already colonized the mandibular region (Couly et al., 2002; Couly et al., 1993; Le Douarin et al., 2004). In Edn11/1 mandibular
prominences, Edn1 expression was reduced by 38% compared to WT, while Dlx5, Dlx6, and Dlx3 levels were reduced respectively of 35, 36, and 24%. Dlx2 and Hand2 were virtually unchanged (Fig. 1a).

Then, we carried out a similar analysis on dissected mandibular prominences obtained from E10.5 WT and Edn11/2 embryos. In this case we further subdivided the mandibular prominence into a latero/proximal (LP) and a medio/distal (MD) segment (as shown in Fig. 1b). In the LP segment, Dlx2, Dlx3, Dlx5, and Dlx6 levels were reduced by 35, 50, 35, and 39%, respectively; Hand2 expression was very low and was therefore not considered. In the MD segment the levels of expression of Dlx2, Dlx3, Dlx5, and Dlx6 were not detectably different, while Hand2 transcripts were reduced by 40% (Fig. 2b). In the LP and MD segments of Edn11/2 mandibular prominences, Edn1 transcripts were reduced, respectively by 60 and 40% (Fig. 1c).

Thus, loss of one Edn1 allele reduces the expression levels of Dlx genes in E9 mandibular prominences while at E10.5 Dlx expression is only reduced in the LP part of the mandibular prominence but not in the MD. However, in the MD portion of the E10.5 mandibular prominence, Hand2 expression is detectably reduced, suggesting that Edn1 can regulate Hand2 expression independently from Dlx genes.

Expression of Dlx Target Genes in the Mandibular Prominence of Dlx5;Dlx6 Mutant Embryos

In different regions of the mandibular prominence of PA1 Edn1 and Dlx5/6 signaling could act independently. This led us to analyze the quantitative effects of Dlx5/6 allelic reduction. We first examined how the loss of Dlx5;Dlx6 alleles affected their own level of mRNA expression. In the mandibular prominence of Dlx51/2;Dlx61/2 embryos Dlx5 and Dlx6 mRNAs were reduced, respectively, by 40 and 45%, while in that of Dlx52/2;Dlx62/2 embryos Dlx5 and Dlx6 mRNAs were nearly undetectable (Fig. 2a). To further confirm this finding, we performed in situ hybridization. In Dlx51/2;Dlx61/2 embryos we observed a reduced Dlx5 and Dlx6 signal in the first and second PA, and in the otic vesicle (Fig. 2b). These results confirm that each allele contributes to the pool of transcripts and that mRNA abundance directly reflects allele dosage.

It has been shown that in the mandibular prominence of Dlx51/2;Dlx61/2 embryos, the expression of many target genes is either up- or down-regulated (Beverdam et al., 2002; Depew et al., 2002; Jeong et al., 2008); in particular it appears that Dlx6 directly activates the transcription of Hand2 by binding at its promoter (Charite et al., 2001). We determined the expression level of putative Dlx5;Dlx6 target genes on whole PA1s and on dissected LP and MD segments from embryos with different Dlx5;Dlx6 allelic
FIG. 2. Effect of allelic reduction of \( \text{Dlx5} \) and \( \text{Dlx6} \) on gene expression levels of target genes in PA1 at E10.5. (a) RT-qPCR measurement of \( \text{Dlx5} \) and \( \text{Dlx6} \) transcripts abundance in PA1 of E10.5 WT (black bars), \( \text{Dlx5}^{1/2};\text{Dlx6}^{1/2} \) (blue bars) or \( \text{Dlx5}^{2/2};\text{Dlx6}^{2/2} \) (red bars) embryos. The WT is set = 1, standard deviation is reported. (b) In situ hybridization with \( \text{Dlx5} \) (top) and \( \text{Dlx6} \) (bottom) probes on E10.5 WT (left) and \( \text{Dlx5}^{1/2};\text{Dlx6}^{1/2} \) (right) embryos, showing reduction in mRNA levels in the PAs and otic vesicle of heterozygous embryos. (c) PA1s were dissected from E10.5 embryos with progressive loss of \( \text{Dlx5} \) and \( \text{Dlx6} \) alleles and further divided into LP and MD regions (see Fig. 1), and stored individually. Samples of similar genotype were pooled. The levels of expression of the \( \text{Dlx} \) targets \( \text{Hand2} \), \( \text{Pitx1} \), \( \text{Dlx3} \), \( \text{Gsc} \), and \( \text{Wnt5a} \) were measured by RT-qPCR. The results are color-coded by the number of absent \( \text{Dlx} \) alleles. WT is set = 1.
Allelic reduction of only one or two alleles did not have detectable effects with the exception of Dlx5/6 and Edn1 genes. Loss of one Dlx and one Edn1 allele results in reduction of the coronoid process of the dentary (dashed arrows) and in the appearance of an extra bone between the pterygoid bone and the middle ear (os paradoxicum; green arrows). In Edn1+/-/Dlx5+/-/Dlx6+/- mice (right), we observe fusion of the condylar and angular processes (red arrows) of the dentary bone; appearance of duplicated jugal bones in the proximal mandibular arch (black arrows) and the appearance of the os paradoxicum (green arrows). Note also the appearance of duplicated structures (asterisks) in the lamina obturans/pterygoid region of the base of the cranium, (further dissociated and shown on the right). Abbreviations: an, angular process; cn, condylar process; cr, coronoid process; ds, dentary-squamosal articulation; ju, jugal bone; LO, Lamina Obturans; op, os paradoxicum; pt, pterygoid; tb, tympanic bone; tr, tympanic ring; zy, zygomatic arch.

Craniofacial Phenotypes of Mice With Combined Loss of Edn1 and Dlx5;Dlx6 Alleles

Dlx and Hand2 genes play important roles in the control of craniofacial morphogenesis. As the loss of one Edn1 allele reduces the expression levels of Dlx and Hand2 genes, we analyzed the skulls of Edn1+/- newborn mice, but no obvious malformation could be detected (not shown); this finding is not surprising as Dlx2+/–, Dlx5+/-, Dlx6+/- and Hand2+/- mice also show only minor craniofacial defects (Acampora et al., 1999; Beverdam et al., 2002, Depew et al., 1999; Robledo et al., 2002, Yanagisawa et al., 2003). As the loss of one Edn1 allele could further reduce the levels of Dlx5/6 and/or Hand2 expression, we examined the craniofacial skeleton of combined Edn1/Dlx mutant mice. We therefore crossedbred Edn1+/- mice with either Dlx5+/- or Dlx5+/-Dlx6+/- mice.

When both one Edn1 and one Dlx allele were lost, we observed a slightly shorter coronoid process of the dentary and the appearance of an os paradoxicum at the base of the cranium, highly reminiscent of that observed in Dlx5+/-;Dlx6+/- or in Dlx5+/- or Dlx6+/- mutants; in each of these allelic configurations two Dlx5/6 alleles are missing (Fig. 3, Supporting Informa-
Inactivation of Ednra fusion, does not, however, express Dlx5/6 ary. The distal-most region of PA1, including the medial embryos, downstream targets expression pattern (Figs. 2 and 3, and Supporting Information Fig. 3). Effect of ednra and Dlx5/6 mutant mice (Fig. 4i–n). Careful analysis of our embryos revealed an additional territory of Gsc expression in the proximal endoderm of PA1 (red arrows, Fig. 5i–k,n), this small territory of expression is independent from both Ednra and Dlx5/6.

**DISCUSSION**

An emerging theme in developmental biology is the importance of quantitative functions shared by related and coexpressed genes. Examples of these are the signaling functions of FGFs expressed in the apical ectodermal ridge (Mariani et al., 2008), the gene-dosage dependent functions of Msx1 and Msx2 for osteogenic differentiation of CNCCs (Han et al., 2007), and the progressive limb phenotypes associated with the combined loss of posterior HoxD alleles and with a gradual increase of expression of the HoxD target Epha3 (Cobb and Duboule, 2005). Our study offers a new example in this direction. One implication of this is that the function of individual genes is best examined upon partial and cumulative gene losses, and within the context of expression of related genes. Indeed, the examination of developmental phenotypes in mice homozygous for recessive mutant mice, although widely used, has serious limitations. One of these is the inability to recognize late-occurring regulations (or phenotypes), in case an early event severely affects morphogenesis, patterning or embryonic viability. Second, we cannot appreciate the phenotypic consequences of reduced gene expression; third we may fail to recognize the dynamics of cell-cell signaling and interactions, as these often require a nearly normal context. Such is the case of Edn1→Dlx signaling at the basis of the homeotic lower jaw transformation, to investigate which many studies have been carried out based on either loss-of-function (Acampora et al., 1999; Beverdam et al., 2002; Clouthier et al., 1998; Depew et al., 1999, 2002; Kurilhara et al., 1994; Ozeki et al., 2004; Sato et al., 2008a; Thomas et al., 1998; Yanagisawa et al., 2003) or gain-of-function mutants (Sato et al., 2008b). Here we provide quantitative data on the effects of allelic reduction of Edn1 and Dlx5/Dlx6 at different developmental stages. Our findings are complementary to those recently reported by Ruest and Clouthier (2009) using CNCC-specific gene deletion and receptor antagonism, and corroborate and extend their major conclusions. We show that, during PA1 development, different Edn1-dependent regulatory pathways act at diverse developmental times in distinct regions of the mandibular prominence. We also show that upon partial
allele loss, the proximal territory of mandibular prominence is the region mainly affected.

At early stages of CNCCs colonization, Edn1 signaling activates Dlx5/6 expression in CNCCs; accordingly Dlx5/6 mRNAs are reduced at E9 in the mandibular prominence of Edn1 heterozygotes (see Fig. 1). If early Edn1 signaling is abrogated (i.e., in Edn1 or Ednra-null mice), Dlx5/6 fail to be activated in the entire PA1 at least up to E9.5 (Abe et al., 2007; Ozeki et al., 2004; see Fig. 5). This implies that signals that pattern Dlx expres-
At later stages (E10.5) the situation is more complex. Combining our data with results reported in the literature, we propose a model in which the E10.5 mandibular arch is subdivided into four adjacent territories, in which distinct timing and patterns of gene expression are linked to the onset of specific phenotypes (Fig. 5c): (1) in the distal-most region (purple in Fig. 5c), Hand2 expression is independent from both Edn1 and Dlx5/6. Indeed, Hand2 expression is retained in a small distal territory in Edn1-null, Ednra-null and Dlx5/6-null animals (Beverdam et al., 2002; Clouthier et al., 2000; Fukuhara et al., 2004; Ozeki et al., 2004; Ruest et al., 2004), possibly associated with the specific fate of this region to undergo midline fusion (Barbosa et al., 2007). (2) in the MD region of PA1, Hand2 expression can be activated by Edn1 independently of Dlx5/6 as seen from the fact that Edn1 allelic reduction affects Hand2, but not Dlx5/6 expression (see Fig. 1). This Dlx5/6-dependent Hand2 regulation could well take place in the distal Dlx5/6-free region of the E10.5 PA1 (orange in Fig. 5c). (3) In the medial region of PA1 at E10.5 (yellow in Fig. 5c), Hand2 is activated through the established Edn1→Dlx6 pathway most probably involving the reported Dlx6-dependent Hand2 enhancer (Charite et al., 2001). Notably, inactivation of this enhancer results in defects in the medio-distal part of PA1 as suggested by our model (Yanagisawa et al., 2003) and by timed inhibition of Edn1 signaling using Ednra antagonists (Ruest and Clouthier, 2009). (4) Finally, in the proximal part of the E10.5 PA1 (grey in Fig. 5c), Hand2 is never expressed. Within subterritories of this region Dlx5 and Dlx6 can be activated even in the absence of an Edn1 inducing signal. These different sub-territories could confer a regional selectivity, in turn required for the correct unfolding of the lower jaw morphogenetic program.

Allelic reduction of Edn1 results in lower Dlx5/Dlx6 (and Dlx2 and Dlx3) expression in the proximal, but not
in the medio-distal part of the mandibular prominence at E10.5. Therefore, the expression of Dlx genes in the distal PA1 (at early stages) is independent from Edn1. A possible interpretation of these findings is that an initial Edn1 signal is necessary to activate Dlx5/6 expression in incoming CNCCs and that, at later stages, the distal expression of these genes is maintained independently of Edn1 (Fig. 1c). In support of this, Dlx5/6 expression in the proximal PA1 is reactivated at E10.5 even in the absence of Edn1 (Fig. 5a–h; Ozeki et al., 2004), indicating the existence of an Edn1-independent mechanism of Dlx5/6 activation or maintenance in the LP region. The reduced expression of Dlx2 and Dlx3 in the presence of only one Edn1 allele may indicate the possibility of a global Edn1→Dlx control, or of Dlx5/6 regulating the expression of Dlx2 and Dlx3. This hypothesis, however, would need to be specifically tested.

Allelic reduction of Edn1 affects Hand2 expression in the MD territory suggesting an Edn1-dependent, Dlx-independent regulation of Hand2 which might take place in the Dlx-free region of the distal PA1. Hand2 is expressed in the distal mandibular prominence and its inactivation causes loss of distal skeletal elements of the lower jaw (Yanagisawa et al., 2003). Analysis of the regulatory regions of Hand2 has revealed the presence of an Edn1-responsive enhancer whose activation depends upon binding of Dlx6, although other, yet unspecified, Edn1-dependent proteins could bind to this enhancer (Charite et al., 2001). On the basis of these results, it has been proposed that Hand2 is the final effector of the Edn1→Dlx5/6 regulatory cascade and its level of expression could determine the shape of the distal lower jaw (Sato et al., 2008b; Yanagisawa et al., 2003). However, targeted inactivation of the Edn1/Dlx6-dependent enhancer does not completely abrogate Hand2 expression in the distal part of PA1 suggesting that other, not yet identified, regulatory elements might activate Hand2 expression in PA1 (Yanagisawa et al., 2003). As Hand2 is expressed only in the medio-distal portion of PA1 while Edn1, Ednra and Dlx genes are expressed both in proximal and distal parts of PA1 an active suppression mechanism for Hand2 expression might be acting in the proximal territory.

Considering Hand2 expression and regulation, and the loss of the distal lower jaw in Hand2 null mice (Thomas et al., 1998; Yanagisawa et al., 2003), we conclude that the mandibular arch is subdivided into two Hand2-independent and dependent parts corresponding to the proximal and distal part of the dentary, respectively. This notion is supported by the fact that, forced expression of Hand2 in the whole PA1, including the maxillary arch, induces only transformation of maxillary derivatives into distal mandibular structures (Sato et al., 2008b).

The phenotypes of mice carrying combined Dlx gene mutations, and the nested expression of Dlx genes within the PAs at E10.5 have led to the proposal that Dlx genes might establish maxillo-mandibular identity by providing a Hox-like proximo/distal and upper/lower combinatorial code (Depew et al., 2002, 2005). A more sophisticated model, known as the “hinge-caps” organization of the PA1, has been proposed (Depew and Compagnucci, 2008). Both of these models, however, do not take in account the dynamics of gene expression and cell migration during PA1 development. In our view, the nested Dlx gene expression pattern is likely to be the consequence of patterning events occurring at much earlier stages, as by E10.5 most CNCCs have already migrated to their final position, have initiated expression of PA-specific genes and are fate-committed (Couly et al., 1998; Le Douarin et al., 2004; Le Douarin and Dupin, 2003).

CNCCs of the proximal mandibular prominence appear more sensitive to variations in the genetic environment, than are distal ones: inactivation or allelic reductions of Edn1, Ednra, Dlx5 (Acampora et al., 1999; Depew et al., 1999), Dlx6 (Jeong et al., 2008), Gsc (Yamada et al., 1995), Pitx1 (Bobola et al., 2003; Lancot et al., 1999), Gbx2 (Byrd and Meyers, 2005) all lead to proximal defect of the dentary or of the middle and external ear whereas derivatives of the distal part of the first arch are not affected. Interestingly, Dlx5;Dlx6 are expressed at higher level distally (Figs. 2 and 4) and even allelic reduction of Edn1 results in maintaining their distal expression levels. These findings suggest the existence of a threshold level of expression of Dlx for the activation of targets genes.

Human first arch syndromes (FAS) include a wide spectrum of congenital anomalies characterized by defects of CNCC derivatives, and in most cases proximal and not distal jaw structures are affected (Gorlin, 2001). The abnormal traits are associated with different conditions including for example oculo-auriculo-vertebral spectrum (OAVS, OMIM 164210), hemifacial microsomia, mandibulofacial dysostosis, Goldenhar or Franceschetti syndromes. The consequence is a lateral deviation of the mandible accompanied by an anomaly of the dentary occlusion and hearing deficiency. The phenotypes of FAS are strongly suggestive of a defect of CNCCs, and interestingly, targeted inactivation of genes involved in patterning CNCCs often results in proximal defects of the dentary and/or of the middle and external ear (for a recent review see: Gitton et al., 2010). Based on morphological similarities with mouse mutants models, the involvement of Edn1 and putative targets in FAS has been suggested (Kelberman et al., 2001; Masotti et al., 2008; Singer et al., 1994), but not experimentally proven. Our observation on partial allele losses of the Edn1-Dlx pathway might help explain why human FAS affect proximal, rather than distal, derivatives of PA1.

A final general conclusion of our study is that early morphogenetic signals seem to define “large” territories of the craniofacial anlage while subsequent regulations coordinate much more spatio-temporally defined and diversified structures, to specify more “local” shapes of individual elements of the jaw. Distinct time-specific levels of regulation might help to explain the apparent contradiction between data suggesting that CNCCs specification
requires external signals (Benoaiche et al., 2008; Couly et al., 2002; Le Douarin et al., 2004; Le Douarin and Dupin, 2003; Vieux-Rochas et al., 2007) and data suggesting that CNCCs are instead endowed with cell-autonomous information to generate craniofacial structures (Schneider and Helms, 2003). As expected, early signals (Edn1, FGF8, others) appear more conserved in different animal classes, while subsequent complex regulations might considerably vary from genome to genome and could contribute to jaw diversification in vertebrates.

**METHODS**

**Mouse Mutants**

Animal procedures were approved by National and Institutional ethical committees. Mouse strains were maintained on B6/D2 F1 hybrid genetic background. Edn1 mutant mice were genotyped as indicated (Kurihara et al., 1994). Mice with targeted disruption of Dlx5 or Dlx5;Dlx6 were genotyped as previously reported (Acampora et al., 1999; Beverdam et al., 2002; Merlo et al., 2002b). The genotypes of embryos obtained from mixed heterozygous parents were determined using the Dlx5-lacZ reverse primer, with the following sequence:

- **L-proF** (Dlx5 allele) 5'CGCAGTAGGAAGACGC CAC
- **G-proF** (Dlx5;Dlx6-mutant allele) 5'GAGCTATGAC AGGAGTTTGTG
- **KO6 RT-R2** (lacZ reverse) 5'GGGCGATTAGTGG GTACG

Edn1+/− animals were crossed with Dlx5+/− and Dlx5+/−;Dlx6−/− to generate double and triple heterozygotes, and from these Edn1−/−;Dlx5+/− and Edn1+/−; Dlx5−/−;Dlx6−/− animals were obtained.

**Skeletal Preparations and In Situ Hybridization**

Skeletal staining of E14.5 embryos and newborn animals (Alcian Blue for E14.5 embryos, Alizarin Red/Alcian Blue for newborns) was carried out as previously described (Vieux-Rochas et al., 2007). A minimum of 4, with a maximum of 10, embryos/newborns per genotype were analyzed for skeletal phenotypes, per each genotype.

In situ hybridization was done with DIG-labeled RNA probes corresponding to the antisense sequence of murine Dlx3, Dlx5, Dlx6, Gsc and Hand2 (all previously reported: Charite et al., 2001; Perera et al., 2004; Radoja et al., 2007), using the procedure described by Wilkinson and Nieto (1993). For each probe, at least three normal and three mutant specimens were examined. For semi-quantitative comparisons, all the procedures were carried out in the same vials on littermate embryos; the time of chromogenic reaction was reduced to avoid signal saturation.

**Tissue Collection, RNA Extraction, and RT-qPCR**

E9 or E10.5 embryos were genotyped by PCR on DNA extracted from extra-embryonic tissues. The PA1s were dissected under stereomicroscope using fine scissors, further separated into a proximal and a distal part (see Fig. 5c). The anatomic hallmark was the bulge formed at the PA1 end. Sections were carried out vertically in a rostro-caudal way. Tissues were collected in RNA later (Ambion), pooled according to the genotype, transferred in Tripure Reagent (Roche) and processed for RNA extraction as indicated by the manufacturer. A minimum of three PA1s per genotype were pooled in one sample; two biological replicates were prepared. Each sample was analyzed in duplicates (technical replicates). RNA quality, primer efficiency and correct product size were verified by RT-PCR and agarose gel electrophoresis. qPCR was performed with LightCycler (Roche) using FastStart DNA MasterPLUS SYBR-Green I (Roche). Five microliter of cDNA were used in each reaction, standard curve were done using WT cDNA with four calibration points: TQ; 1:3; 1:9; 1:27. Specificity and absence of primer dimers was controlled by denaturation curves. GAPDH mRNA was used for normalization. Results of mutant tissues are expressed as fold-change relative to the corresponding WT. For each target, the mRNA abundance was calculated relative to GAPDH, using the LightCycler Software 3.5.3, based on the general formula Δ (Δ CT). Because of the limited sample size (two replicates) and the two steps of normalization, the Student's test could to determine statistical significance could not be done.

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LITERATURE CITED


