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; Lanctot *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 mutant 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 (Benouaiche *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 *Dlx* heterozygous parents were determined using the *Dlx5-lacZ* or the *Dlx5;Dlx6*-mutant allele-specific forward primers L-proF and G-proF, respectively, and the *lacZ* reverse primer, with the following sequence:

- *L-proF (Dlx5* allele) 5′CGCAGTAGAAGAACAGC CAC
- *G-proF (Dlx5;Dlx6*-mutant allele) 5′GAGCTATGAC AGGAGTGTTTG
- *KO6 RT-R2 (lacZ* reverse) 5′GGCGATTAAGTTGG GTAACG

$Edn1^{+/-}$ animals were crossbred 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 GADPH, 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 *t*-test could to determine statistical significance could not be done.

- GAPDH Sens 5′TGTCAGCAATGCATCCTGCA
- GAPDH Antisens 5′TGTATGCAGGGATGATGTTC
- Hand2 Sens 5′CCAGCTACATCGCCTACGTC
- Hand2 Antisens 5′TTGCTGCTCACTGTGCTTTT
- Wnt5a Sens 5′AGGAGTTCGTGGACGCTAGA
- Wnt5a Antisens 5′ACTTCTCCTTGAGGGCATCG
- Bmp7 Sens 5′GCGATTTGACAACGAGACCT
- Bmp7 Antisens 5′AGGGTCTCCACAGAGAGCTG
- Dlx3 Sens 5′CGTTTCCAGAAAGCCCAGTA
- Dlx3 Antisens 5′CGTGGAATGGGAAGATGTGT
- Dlx5 Sens 5′CTGGCCGCTTTACAGAGAAG
- Dlx5 Antisens 5′CTGGTGACTGTGGCGAGTTA
- Dlx6-5F Sens 5′CTCAATACCTGGCCCTTCC
- Dlx6-5R Antisens 5′AGAGCGCTTATTCTGAAACCAT
- Meis2 Sens 5′ATCTCAAGGCAAGGGGAAGT
- Meis2 Antisens 5′GAGTAGGGTGTGGGGTCATC
- Pitx1 Sens 5′ATCGTCCGACGCTGATCT
- Pitx1 Antisens 5′CTTAGCTGGGTCCTCTGCAC
- Gsc Sens 5′ACCGATGAGCAGCTCGAA
- Gsc Antisens 5′GCGGTTCTTAAACCAGACCTC
- Edn1 Sens 5′TCCTTGATGGACAAGGAGTGT
- Edn1 Antisens 5′TCGTACCGTATGGACTGGG

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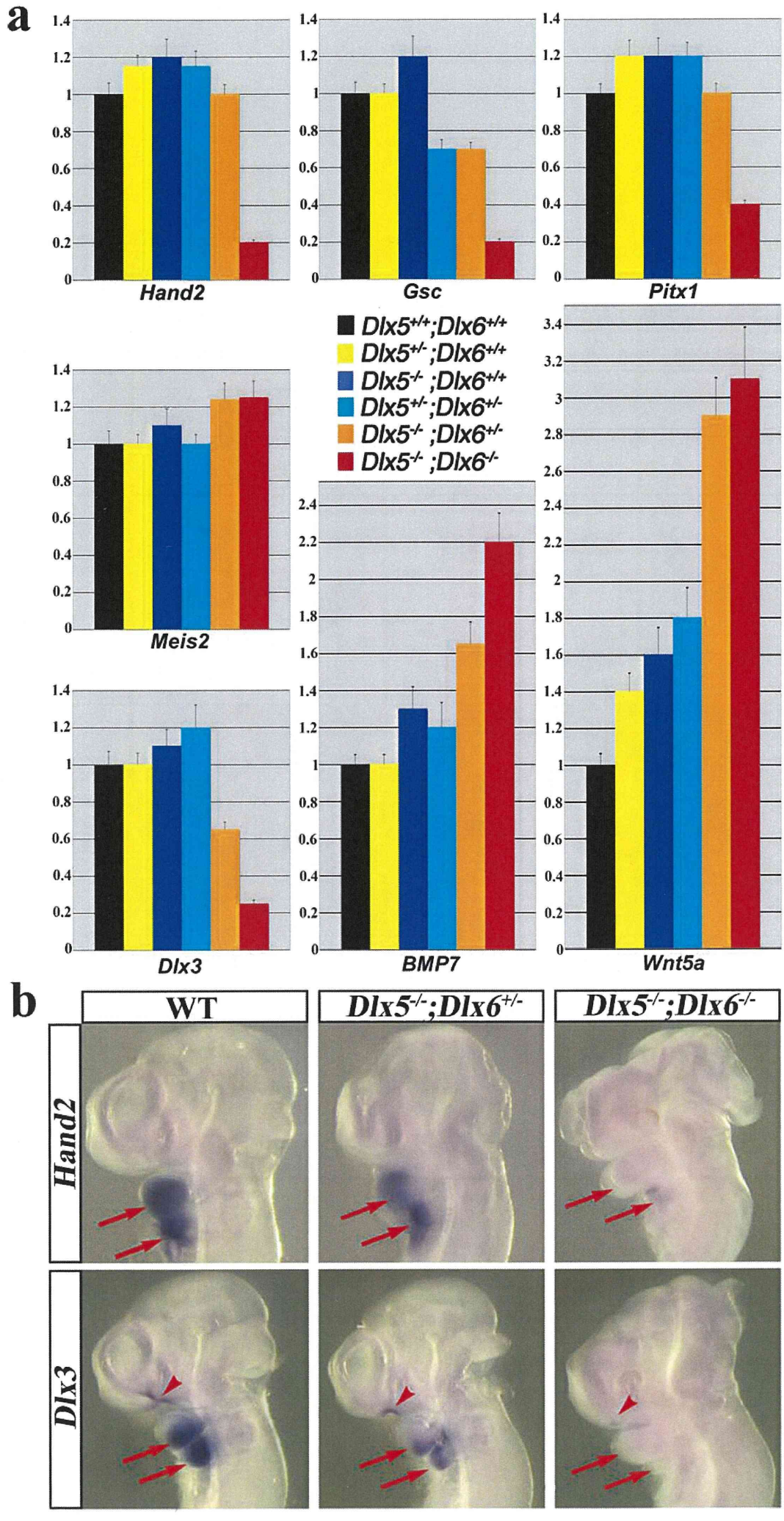
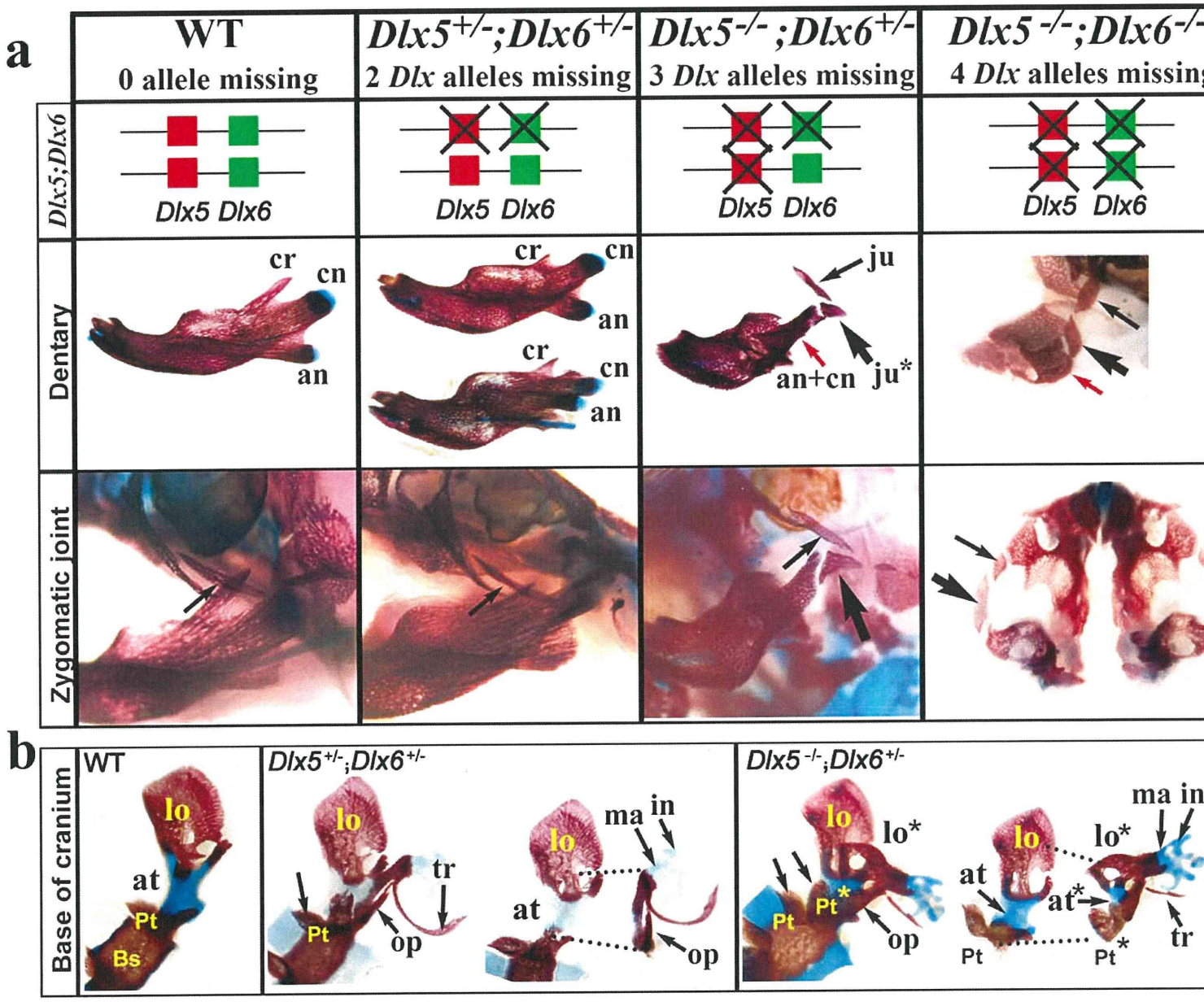
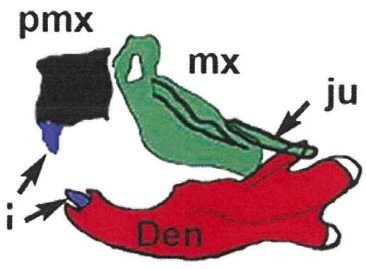
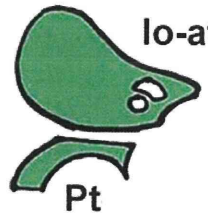
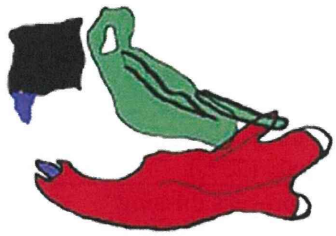
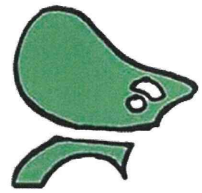
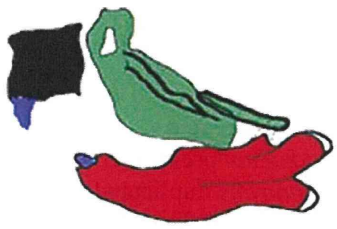
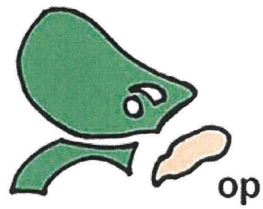
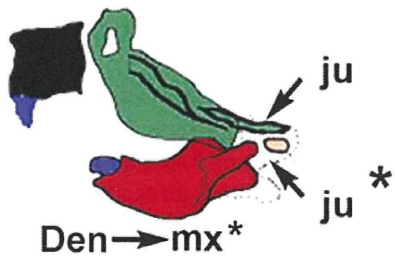
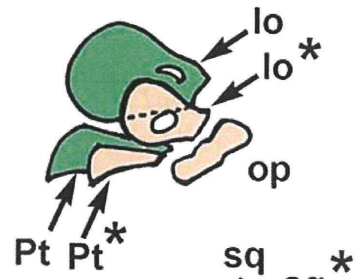
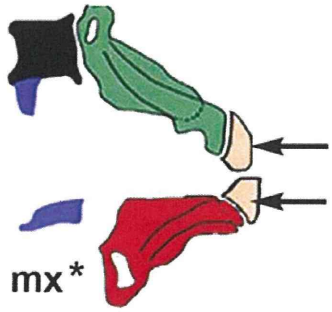
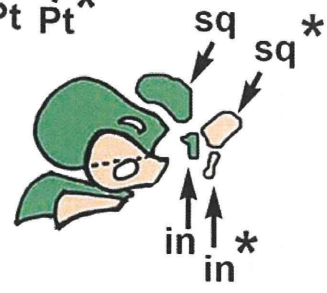


Vieux-Rochas *et al.* - Suppl. Figure 1



Vieux-Rochas et al. - Suppl. Figure 2

Edn1/Dlx allelic configuration	Lower jaw transformation	Proximal PA duplication
Wild-Type		
1 *Dlx* allele missing		
2 *Dlx* alleles or 1 *Dlx* and 1 *Edn1* alleles missing		
3 *Dlx* alleles or 2 *Dlx* and 1 *Edn1* alleles missing		
4 *Dlx or* 2 *Edn1* alleles missing		

Vieux-Rochas *et al.* - Supplementary Figure 4

Suppl. Table 1: Summary of reported craniofacial malformations present in *Dlx5/Dlx6* and *Edn1/Ednra* mutants.

Mice genotype	Craniofacial malformations	References
$Dlx5^{+/+}$; $Dlx5^{+/-}$; $Dlx6^{+/+}$; $Dlx6^{+/-}$; $Edn1^{+/+}$; $Edn1^{+/-}$; $EdnrA^{+/+}$; $EdnrA^{+/-}$	None	(Acampora *et al.*, 1999; Depew *et al.*, 1999; Jeong *et al.*, 2008; Merlo *et al.*, 2002b; Ozeki *et al.*, 2004; Ruest *et al.*, 2005)
$Dlx5^{-/-}$	Coronoid process missing Hypoplastic condylar process *Os paradoxicum* present Nasal capsule Otic capsule Exencephaly (25%)	(Acampora *et al.*, 1999; Depew *et al.*, 1999; Merlo *et al.*, 2002b)
$Dlx6^{-/-}$	Coronoid process missing Hypoplastic condylar process Os *paradoxicum* present	(Jeong *et al.*, 2008)
$Dlx5^{-/-}$;$Dlx6^{+/-}$	Coronoid process missing Angular process fused to the condylar process Jugal bone duplicated *Os paradoxicum* present *Lamina obturans* duplicated Pterygoid bone duplicated	(Depew *et al.*, 2005)
$Dlx5^{-/-}$;$Dlx6^{-/-}$	Lower jaw transformed into an upper jaw *Lamina obturans* duplicated Jugal bone duplicated Pterygoid bone duplicated Presence of *vibrissae* in the lower jaw	(Beverdam *et al.*, 2002; Depew *et al.*, 2002)
$Edn1^{-/-}$	Lower jaw transformed into an upper jaw but transformed structures are smaller *Lamina obturans* duplicated Jugal bone duplicated Pterygoid bone duplicated Presence of *vibrissae* in the lower jaw	(Ozeki *et al.*, 2004)
$EdnrA^{-/-}$	Lower jaw transformed into an upper jaw but transformed structures are smaller *Lamina obturans* duplicated Jugal bone duplicated Pterygoid bone duplicated Presence of *vibrissae* in the lower jaw	(Ruest *et al.*, 2005)
$EdnrA^{Edn1/+}$	Upper jaw transformed into a lower jaw Meckel's cartilage duplicated *Malleus* duplicated	(Sato *et al.*, 2008b)

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Review

Evolving maps in craniofacial development

Yorick Gitton[a,1], Églantine Heude[a,1], Maxence Vieux-Rochas[a,2], Laurence Benouaiche[a,b], Anastasia Fontaine[a], Takahiro Sato[c], Yukiko Kurihara[c], Hiroki Kurihara[c], Gérard Couly[a,b], Giovanni Levi[a,*]

[a] *Évolution des Régulations Endocriniennes, CNRS UMR 7221, Muséum National, d'Histoire Naturelle, 75231 Paris Cedex 05, France*
[b] *Service de Chirurgie Plastique, Maxillofaciale et Stomatologie, Hôpital Necker-Enfants Malades, 149, rue de Sèvres, 75015 Paris, France*
[c] *Department of Physiological Chemistry and Metabolism, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*

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ABSTRACT

The shaping of the vertebrate head results from highly dynamic integrated processes involving the growth and exchange of signals between the ectoderm, the endoderm, the mesoderm and Cephalic Neural Crest Cells (CNCCs). During embryonic development, these tissues change their shape and relative position rapidly and come transiently in contact with each other. Molecular signals exchanged in restricted regions of tissue interaction are crucial in providing positional identity to the mesenchymes which will form the different skeletal and muscular components of the head. Slight spatio-temporal modifications of these signalling maps can result in profound changes in craniofacial development and might have contributed to the evolution of facial diversity. Abnormal signalling patterns could also be at the origin of congenital craniofacial malformations. This review brings into perspective recent work on spatial and temporal aspects of facial morphogenesis with particular focus on the molecular mechanisms of jaw specification.

Contents

1. Plasticity and regenerative potential of CNCCs

The contribution of Cephalic Neural Crest Cells (CNCCs) to head development was initially recognized by Wilhelm His, Sr. while studying the development of olfactory structures of the chick neurula [1]. He coined the term 'Zwischenstrang' (German for 'Middle chord') to describe a transient, rapidly moving, cell contingent ingressing between the primary germ layers.

The formation of head cartilages and bones was long considered as a mesodermal fate. Yet, as soon as 1893, shortly after Neural Crest Cells (NCCs) were described, Platt demonstrated their contribution to facial chondro-skeletogenesis [2,3]. However, it took more than five decades for this concept to be accepted [4].

Abbreviations: CNCCs, Cephalic Neural Crest Cells; CPM, cranial paraxial mesoderm; Edn1, Endothelin-1; Ednra, Endothelin-1 receptor type A; Mc, Meckel's cartilage; NCCs, Neural Crest Cells; OAVS, oculo-auriculo-vertebral spectrum; PA1, PA2, pharyngeal arch 1,2; pq, palatoquadrate; RA, retinoic acid; RAEs, retinoic acid embryopathies; SpM, lateral splanchnic mesoderm.

* Corresponding author at: UMR 7221 CNRS/MNHN, 7, rue Cuvier, 75005 Paris, France. Tel.: +33 1 40793621; fax: +33 1 40793621.
E-mail address: glevi@mnhn.fr (G. Levi).

[1] These authors have equally contributed to this paper.

[2] Present address: National Research Center Frontiers in Genetics, School of Life Sciences, Ecole Polytechnique Fédérale (EPFL), Lausanne, Switzerland.

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Cell lineage experiments led initially to an over-representation of fate restriction of CNCCs in head morphogenesis supporting the view that pre-migratory CNCCs are irreversibly restricted in their topographic and tissular fate [5]. It took extensive transplantation experiments to challenge this 'neural-crest pre-patterning model', leading to the consensus view that while early target territories are assigned to pre-migratory CNCCs, these cells retain until late stages of migration the capacity to alter their specification according to local signals [6,7].

The fact that CNCCs exhibit a large repertory of differentiated phenotypes which can be individually induced by external factors [8], supported the notion that late events determine head morphogenesis.

Now considered the 'fourth' germ layer [9], NCCs can be seen as an embryonic stem cell reserve which, in the head, contributes to the formation of most chondro- and dermatocranial elements and other connective tissues (for reviews see for example [10,11]).

Possibly, the most interesting characteristic of CNCCs is their high level of plasticity [12]. While truncal Neural Crest Cells maintain an irreversible memory of their rostro-caudal origin [13], CNCCs have the remarkable capacity to substitute for one-another and replace missing cellular contingents until late in neurulation.

The plasticity of CNCCs is so extensive that drastic bilateral extirpation of the neural folds from the prosencephalic to the anterior rhombomeric level can be completely rescued by grafting small fragments of CNCCs presumptive territory [14–20]. Independently from their anterior–posterior origin within the prosencephalic/rhombomeric neural plate, these small contingents of CNCCs proliferate and finally give rise to apparently normal craniofacial structures. The capacity to replace missing contingents of CNCCs is, however, limited to cells deriving from anterior *Hox*-negative territories. Hox-positive truncal NCCs cannot compensate for missing CNCCs. Actually, *Hox* gene expression precludes any engagement into the chondroskeletogenic lineage which remains a peculiarity of CNCCs [21]. Remarkably, *Hox* gene expression appears to be repressed by molecular signals produced by pharyngeal arch cells [22]. The central questions to understand the origin of cephalization could well be how the anterior limit of *Hox* gene expression has appeared during craniate evolution and how, then, the *Hox*-negative domain has expanded [23,24].

As, before delamination, *Hox*-negative CNCCs deriving from different anterior territories can substitute for each other, they are considered as a 'group of equivalence' [15].

How is CNCC plasticity maintained late in neurulation? To answer this question, the fate of CNCCs was monitored in function of their emigration timepoint by substitutive transplantations of presumptive mesencephalic CNCCs [25]. Collectively, these data led to propose that, upon completion of migration, a signal from leader CNCCs prevented further colonization by trailer cells. While the molecular nature of this interaction remains unknown, live cell tracings provides valuable insight showing direct physical interactions among chains of migratory CNCCs [26].

2. Short-distance interactions involved in shaping the head

Transplantation of small territories of the endoderm deriving from different rostro-caudal levels induce outgrowth of ectopic cartilages and associated bony elements [16,27]. Remarkably, the morphology of these structures displays characteristic features of either nasal, jaw or throat cartilages, depending upon the rostro-caudal origin of the endodermal graft. It appears therefore, that regionalized endodermal cues can direct CNCCs towards a specific morphogenetic pathway and that a map of the facial skeleton is inscribed in the endoderm. For example, surgical deletion and/or grafting experiments of the ventral part of the rostral-most endoderm of the avian neurula have shown that this territory is necessary and sufficient to instruct CNCCs to form the mesethmoid which is the ventral cartilage of the nasal capsule. This specific patterning interaction is mediated through the Sonic Hedgehog (Shh)-*Gli1* signalling pathway [27]. Interestingly, this interaction appears to be triggered by the arrival of CNCCs, which induce a strong increase in endodermal *Shh* expression. Similarly, *Fgf8* induction by incoming CNCCs had been considered a triggering event in craniofacial patterning [16,27]. One important question is how these interactions are maintained during the simultaneous migration of CNCCs and both the growth and displacement of endodermal territories. Indeed, the foregut endoderm builds as a continuous rolling-mat as more extra-embryonic materials gets engulfed from lateral and posterior fields [28–30]. The endoderm then elongates rostrally as new territories form ('endodermal zones' [27]).

Patterning of CNCCs depends as well upon cues from ectodermal cells. For example, in the frontonasal process, postmigratory CNCCs organize around developing olfactory pits and receive instructions to build nasal cartilages and bones. This homing site behaves as an organizer involving Fgf, Bmp and Wnt signalling [31].

Which molecular cues are instructing CNCCs towards their morphogenetic program and how a signalling map is generated in cephalic epithelia are, therefore, central questions to understand head morphogenesis. Mutational analysis in zebrafish has provided several candidate genes for endoderm-derived instructive cues in craniofacial patterning (see for instance [32]). Among those, signalling cascades involving Shh, Fgf, Wnt and Bmp have been shown to be involved in the control of CNCCs migration, survival, apoptosis, tissue-specific terminal differentiation and patterned structural arrangement. As these results have been recently reviewed [33,34], we will focus here on the involvement of Endothelin-1 (Edn1) signalling in defining patterning maps as this pathway has not, so far, received as much attention as others.

3. Endothelin-1-dependent activation of *Dlx* genes and the specification of jaw identity

Although the nature of signals involved in instructing CNCCs remains still to be completely elucidated, evidence suggests that Fgfs, Bmps, Edn1 and Shh are surely involved [27,35,36]. In particular, Edn1 signalling plays a pivotal role for the specification of jaw identity: in its presence a lower jaw is formed; in its absence only maxillary structures are generated. Loss of Edn1 → Ednra (Endothelin-1 receptor type A) signalling results in the downregulation of *Dlx5* and *Dlx6*, leading to the transformation of lower into upper jaw structures similar to that observed after the double inactivation of these genes [37–40]. Conversely, the constitutive activation of *Ednra* induces the transformation of maxillary into mandibular structures with duplicated Meckel's cartilages and dermatocranial jaws constituted by four opposing dentary bones [41] (Fig. 1). A similar transformation is obtained by forcing the expression of *Hand2*, a downstream target of the Edn1 pathway, in the *Ednra* domain. These skeletal transformations are accompanied by neuromuscular remodelling with either the absence or duplication of the masseter muscle. Thus, within the first pharyngeal arch (PA1), CNCCs are competent to form both mandibular and maxillary structures and an Edn1 switch is responsible for the choice of either morphogenetic program.

It is interesting to observe that the effects of Ednra constitutive activation are mostly restricted to PA1 despite the fact that *Ednra* is broadly expressed by the CNCC-derived ectomesenchyme including, for example, the frontonasal prominence. This suggests that the competence to respond to Edn1/Ednra signalling is limited to a sub-

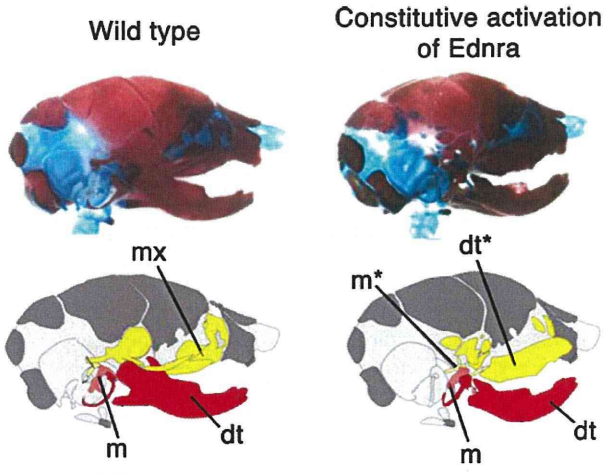

Fig. 1. Constitutive activation of Ednra results in the transformation of maxillary into mandibular structures. Representative images and schematic drawings of the dermatochranium of E18.5 normal and mutant mice in which the endothelin receptor A is constitutively activated. Note the transformation of the maxillary arch into a duplicated dentary bone with duplication of the malleus. *Abbreviations*: dt, dentary; dt*, duplicated dentary; m, malleus; m*, duplicated malleus; mx, maxillary arch. Adapted from [41].

population of *Hox*-negative CNCCs which begins to individualize already during early migration [35,40–45].

Vertebrate *Dlx* genes, targets of Edn1 signalling, are characterized by a homeobox homologous to that of *Drosophila distalless* (*dll*) [46–48]. In the mouse and human genome, there are six *Dlx* genes organized in three bigenic clusters *Dlx1–Dlx2*, *Dlx3–Dlx4* and *Dlx5–Dlx6*. Within PA1 of mouse embryos at 10.5 days of development (E10.5), *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 [38,49]. 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;Dlx6* double mutant mice, lower jaw cartilages and bones are transformed and acquire the shape typical of upper jaw elements. Furthermore, in *Dlx5;Dlx6* double mutant mice, vibrissae and palatine rugae are symmetrically present in the upper and lower jaw suggesting that an homeotic transformation has taken place [37,38]. In *Dlx1;Dlx2* double mutant mice the proximal maxillary region develops abnormal skeletal elements reminiscent of the reptilian upper jaw [50,51].

These observations have led to the suggestion that the combinatorial expression of *Dlx* genes by PA1 CNCCs determine their relative position and their capacity to form different skeletal elements [49,50,52].

Several genes have been shown to act downstream of *Dlx5* and *Dlx6*, including *Gsc*, *Pitx1*, *Wnt5a*, *Dlx3*, *Meis2*, and the bHLH transcription factor *Hand2* [37,38,49,53]. A further set of candidate targets of Dlx5/6 have been recently identified by gene expression profiling of *Dlx5;Dlx6* double null mandibular arches [54]. Several of these genes might be direct targets of Dlx5/6 (e.g. *Gbx2*, *Hand2*) as their promotors harbour Dlx-binding regulatory elements [54,55].

In order to dissect the regulatory events which govern PA1 patterning, many studies have analyzed the effect of either loss-of-function [35,37,38,41,43,53,56–59] or gain-of-function mutants [41]. These genetic experimental settings, however, cannot discriminate between early and late regulatory events.

We recently gained insight into the dynamics of PA1 patterning, by providing quantitative data on the effects of allelic reduction of *Edn1* and *Dlx5;Dlx6* at different developmental stages (Vieux-Rochas, unpublished observations). Our findings are complementary to those recently reported by Ruest and Clouthier [60] using Neural Crest Cell-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 PA1. At early stages of development, up to E9.5 during CNCCs colonization, Edn1 signalling activates *Dlx5/6* expression in CNCCs [35,42]. At later stages, diverse regulations take place in different regions of PA1: in the most proximal part *Dlx5/6* are activated independently from Edn1 and their expression is not associated to *Gsc* activation. More distally *Dlx5/6* activation depends from Edn1 signalling and results in the upregulation of downstream genes including *Gsc* and *Pitx1*.

The nested *Dlx* gene expression pattern should be viewed as the final result of a highly dynamic process, as by E10.5 most CNCCs have migrated to their final position in PA1, have initiated expression of PA-specific genes and are already fate-committed [12,19,61]. The "hinge and caps" architectural and signalling organization of the E10.5 PA1 (proposed in [62]) is likely to be the consequence of patterning events occurring at much earlier stages.

An interesting point is the sensitivity of the proximal part of PA1 to variations in the genetic environment. Indeed, inactivation or allelic reductions of *Edn1*, *Ednra*, *Dlx5* [53,56], *Dlx6* [54], *Gsc* [63], *Pitx1* [64,65], *Gbx2* [66] all show 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.

4. Retinoic acid treatment to study the dynamics of signalling to CNCCs

Retinoic acid (RA), the active metabolite of vitamin A, is essential for normal development of most vertebrate species. RA can also act as a potent teratogen if it is administered during embryonic development in all vertebrates and even in certain invertebrates [67–69]. The malformations induced by RA treatment are very different depending on the dose administered and on the developmental stage at the time of exposure [67–69]. Oral intake of RA during the first weeks of human pregnancy results in highly variable morphological fetal lesions collectively named "Retinoic Acid Embryopathies" (RAEs); these often include severe defects of the jaws and of the middle and external ear [70–73]. Treatment of pregnant mice or of cultured mouse embryos with RA at E8.0 induces fusion and hypoplasia of PA1 and PA2. As most of the structures affected by RA treatment derive from PA1 and PA2, which are colonized by incoming CNCCs [16,24,74–78], it has been repeatedly suggested that CNCCs could be the primary target of RA-induced teratogenesis of facial structures [79–83]. This notion is derived from the fact that most dysmorphic structures in RAE are CNCCs derivatives and was reinforced by initial analyses of animal models suggesting that excess RA could alter CNCC survival and migration [84,85].

However, it has been shown that retinoid-induced defects of PA1 and PA2 occur without any obvious alteration of CNCC migration or apoptosis [80]. More importantly, the RA-mediated transduction machinery is not active in CNCCs shortly after their arrival in the PAs [86]. This notion is further supported by reports showing that CNCCs, although expressing certain RA nuclear receptors [87], may not respond directly to RA under physiological conditions [88–91]. Therefore, CNCCs do not seem to be direct targets of RA.

As mentioned above, grafting and fate mapping experiments have unequivocally shown that molecular signals deriving from the endodermal and ectodermal epithelial linings of PA1, are essential

for patterning the underlying neural crest-derived ectomesenchyme [15,92]. PA epithelia could therefore constitute a possible target of RA activity resulting in distorted signalling to CNCCs.

In support of this hypothesis, it has been shown that treatment of mouse embryos with a general agonist of RA activates ectopic RA signalling in the endoderm and ectoderm lining the first two PAs. This change in epithelial patterning corresponds to a rostral shift of the expression domains of the RA-responsive genes [86].

To explore the possibility that the craniofacial lesions present in RAE could derive from altered signalling from the PA epithelium to CNCCs we performed a series of experiments on both mouse [36] and Xenopus embryos. Timed pregnant mice have been exposed to one single RA pulse at slightly different times of gestation. Craniofacial malformations of the pup only appear when RA is administered to mothers carrying embryos between 9 and 14 somites. In this temporal window CNCCs from rhombomeres 1 and 2 and the mesencephalon reach the PA1 [75]. The defects deriving from a RA treatment performed on embryos of 9 somites affected mostly proximal derivatives of the PA1 such as the proximal part of Meckel's cartilage (Mc) or the malleus, while later treatments affected both proximal and distal structures. These defects were mirrored by the progressive reduction of territories of expression of endodermal and ectodermal signals and by the downregulation of *Dlx* gene expression. Similarly, in Xenopus, RA treatment of embryos produces craniofacial defects only in a narrow temporal interval corresponding to the migration of CNCCs towards the PAs. Remarkably, in this species, RA treatments as short as one minute are sufficient to induce severe craniofacial malformations which can be observed in pre-metamorphic tadpoles several days after RA exposure of the embryos (Vieux-Rochas, unpublished observations). As RA effects are short lived due to the rapid degradation of this molecule, this experimental setting constitutes an interesting tool to study the dynamics of signalling during craniofacial morphogenesis. Our findings imply that for normal craniofacial development it is essential to protect specific signalling regions of the PA1 from the action of RA. Remarkably, the RA-degrading enzymes CYP26A1 and CYP26C1 are expressed in these regions when CNCCs reach the PA1 [93,94].

These findings reinforce the notion that the exact topography and timing of epithelial signalling to CNCC is essential to determine different craniofacial morphotypes [95]. Depending on the exact age of the embryo when RA treatment is performed, we observe the co-variance of several independent craniofacial characters resulting in morphologies that recall the primitive condition of jawed vertebrate skulls where the palatoquadrate (pq) is separated from the braincase. A particularly clear example can be seen at the level of the inner ear. The progressive transformation of the incus into a pq is paralleled by that of the malleus into the proximal part of Mc. These two simultaneous transformations generate an articulation between the proximal part of the transformed Mc and the pq, a typical character of primitive jaws, which, in modern gnathostomes, is transformed into the articulation between the malleus and the incus. Furthermore the co-variance of other characters, such as the appearance of a stylo-hyoid connection can be interpreted as an example of co-evolution of many, not functionally related, characters of the same organism. Similar mechanisms might have been

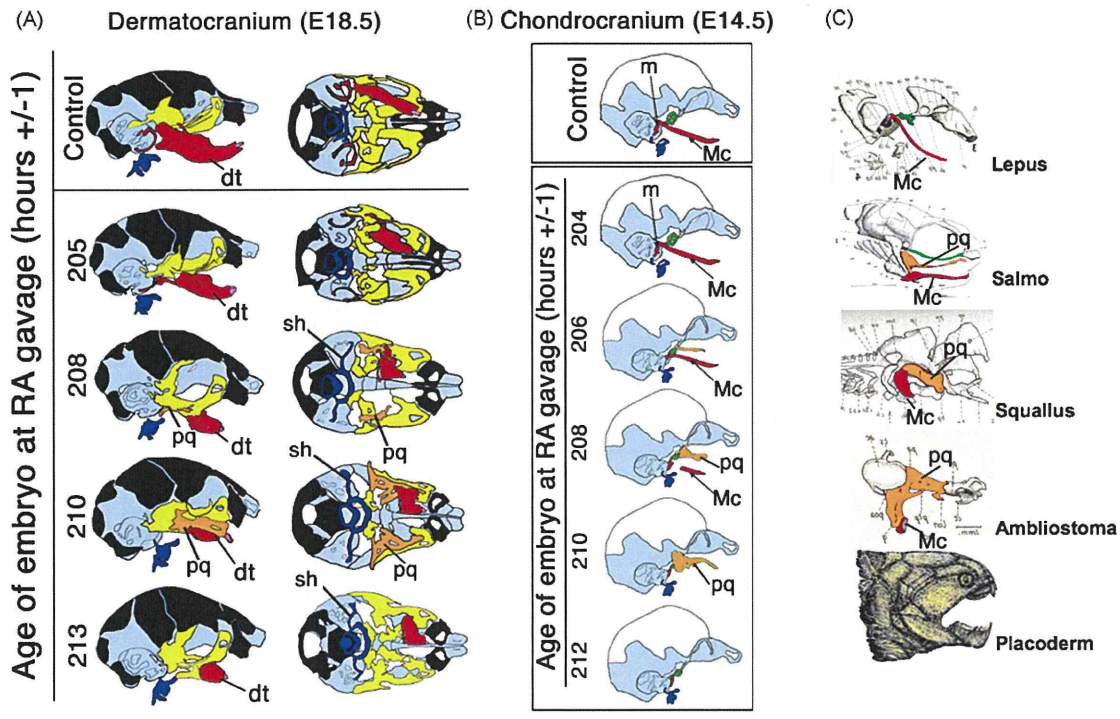

Fig. 2. Craniofacial defects induced by RA treatment.
(A) Series of drawings representing lateral and caudal views of dermatocrania of E18.5 embryos obtained after RA treatment of the mother at the indicated developmental times (expressed in hours post coitum, hpc). The main structures highlighted in colour are: the dentary (dt, red), the palatoquadrate (pq, orange), the derivatives of the maxillary component of the PA1 (yellow) and PA2 derivatives including the hyoid bone and the stylo-hyoid connection (sh, blue). Note the progressive transformation of the dentary starting at 205 hpc accompanied by the appearance of an increasingly large palatoquadrate which, in embryos treated 210 hpc, appears as two closely anastomosed parallel bars. Note also the disappearance of the tympanic ring, the opening of the palate, the deformation of the maxillary complex and of the pterygoid bone. (B) Schematic drawings representing the lateral view of chondrocrania (E14.5) of embryos which received a single RA pulse at the indicated developmental time (expressed in hpc). The main structures highlighted in colour are Meckel's cartilage (Mc, red), the neoformed palatoquadrate (pq, orange), the ala temporalis (at, green), and the hyoidean cartilage (blue). The first defect to develop is a proximal deformation of Mc with the appearance of a medially oriented cartilage bar. The malleus (m) is rapidly lost. The pq becomes progressively more prominent, to occupy most of the maxillary region of the head. (C) Hypothetical parallel between the morphologies obtained by RA treatment and the skeletal organization of different species. Abbreviations: dt, dentary; Mc, Meckel's cartilage; pq, palatoquadrate; sh, stylo-hyoid connection. Adapted from [36].

more generally involved in determining the development and evolution of animal form [36] (Fig. 2).

5. CNCC/mesoderm interactions in defining craniofacial muscle pattern

In contrast to our good understanding of how trunk skeletal muscles are formed, the signalling pathways that control head skeletal muscle morphogenesis are not yet fully elucidated.

While few craniofacial muscles, including the hypobranchial muscles and the tongue muscles develop from the first five somites, most others develop from cranial paraxial mesoderm (CPM) and lateral splanchnic mesoderm (SpM) [96,97] located in the preotic levels of the head. PA1 muscle precursors stream from the CPM and SpM into the neighbouring PA1; they then fill the PA core along with migratory CNCCs. During later PA development, crest cells surround the muscle anlagen, thereby separating the presumptive myoblasts from the overlying surface ectoderm [98–101]. Subsequently, CNCCs give rise to most skeletal elements of the head, to connective tissues and tendons [102,103]. In parallel, mesoderm-derived myoblast cells fuse together to form myofibers, which are attached to a specific CNCCs-derived skeletal element, through CNCCs-derived connective tissue. Each step of myogenesis is reflected by the expression of specific molecular markers including *Tbx1*, *Myf5*, *MyoD*, *Desmin* and *Myosin*.

The molecular mechanisms underlying head muscle patterning – myoblast guidance, positioning and connection to specific attachment sites – begin to be understood [96,104]. Furthermore, the degree to which skeletal muscle specification, differentiation and patterning is intrinsic to muscle (mesoderm) progenitors or controlled by extrinsic environmental signals (e.g. CNCCs) remains an open and fundamental question. Craniofacial shapes are amazingly diverse not only in vertebrates but also within species [e.g. dogs, birds [105,106]]. This diversity might reflect a tight link between the skeletal elements derived from the CNCCs and skeletal muscles of mesodermal origin. Indeed, it has long been suggested that in addition to contributing to the formation of skeletal elements and connective tissue in the head, CNCCs may also be involved in the patterning of the head musculature [5,74,78,98,107–113].

In a recent study [104], genetic perturbations of CNCCs development in mouse embryos and cellular perturbations of CNCCs development in avian embryos have been used to formally demonstrate that although CNCCs are not necessary for the early specification of skeletal muscle progenitors, they later play a crucial role in the migration, positioning and differentiation of cranial muscle precursors in vertebrate embryos. In the absence of CNCCs, other tissues and signals could provide some clues to promote, at least partially, skeletal muscle differentiation in the head.

Although head and trunk muscles exhibit the same tissue architecture, their development is remarkably different. The preotic head mesoderm lacks any overt sign of segmentation and never forms somites [114]. In addition, components of the molecular clock that drive trunk mesoderm segmentation and hence somite formation are only transiently expressed in cranial mesoderm [115], which soon after gastrulation merges to form a continuous strip of mesenchyme on either sides of the cranial neural tube [114].

When CPM was grafted next to axial tissues in the trunk, activation of the endogeneous myogenic program in this ectopic position was inhibited, indicating that signals from the axial tissues and surface ectoderm that stimulate myogenesis in the somite are unable to elicit this effect in CPM [116]. Consistent with the notion that myogenic activation differs between head and trunk, distinct regulatory sequences have been found to drive *Myf5* expression in these two regions of the embryo [117,118]. The concept that head and trunk muscle are generated through different regulatory pathways is also reinforced by genetic studies. While skeletal muscle formation in both regions of the embryo requires either *MyoD* or *Myf5* [119], mice lacking both *Myf5* and *Pax3* are completely devoid of trunk muscles yet retain normal head muscles [120]. In addition, mice lacking both *MyoR* and *Capsulin* display a deficit in jaw muscle formation, whereas the trunk musculature remains normal [121]. The specificity of branchiomeric muscle differentiation is also seen by the fact that certain transcription factors are involved in the control of branchiometric myogenesis but do not have an equivalent role on the rest of the axial musculature. For example, in the absence of *Tbx1*, the myogenic determination genes *Myf5* and *MyoD* fail to be normally activated in pharyngeal mesoderm [122] and branchiomeric muscle specification and differentiation are defective in *Pitx2* mutants [123].

In the head, both BMP and the canonical Wnt signalling molecules secreted by the dorsal neural tube act to repress skeletal muscle formation. This may occur via inhibition of the myogenic differentiation of the CPM in the vicinity of the neural tube. By contrast, these same Wnt ligands are required to stimulate myogenesis in the trunk. Moreover, CNCCs secrete both BMP inhibitors (Noggin, Gremlin) and Wnt inhibitors (Frzb), which together induce myogenic differentiation of the CPM in vitro [124]. Therefore, head muscle differentiation is subjected to a complex balance between neural tube-derived inhibitors and CNCCs-derived activators.

6. Human craniofacial malformations deriving from CNCCs lesions

First arch syndromes known also as oto-mandibular dysplasias or mandibulofacial dysostosis correspond to a wide spectrum of human congenital anomalies of latero-facial regions. The malformations associated with these conditions affect predominantly derivatives of the maxillary and mandibular processes of PA1. The abnormal traits display variable quantitative expression and are associated to different conditions including oculo-auriculo-vertebral spectrum (OAVS, OMIM 164210), hemifacial microsomia, Goldenhar, Franceschetti or Treacher Collins syndromes (OMIM 154500) (Fig. 3).

A possible conceptual framework to understand and classify this wide spectrum of first arch malformations is to consider that the skeletal defects result from lesions affecting the specification and differentiation of different contingents of PA1 CNCCs [125]. For example, among human first arch syndromes, we can distinguish maxillary bud pathologies (Franceschetti or Treacher Collins syndromes OMIM 154500) from mandibular bud pathologies (hemifacial microsomia or OAVS, OMIM 164210; Goldenhar syndrome). Association of maxillary and mandibular pathologies is rare and might correspond to earlier defects in CNCCs differentiation. OAVS or hemifacial microsomia is often unilateral, occurs in about 1/5600 birth [125] and displays different severity of first arch defects ranging from moderate hypoplasia to complete agenesis of the jaw with frequently associated muscle anomalies and nervous disorders.

As mentioned before, although the initial specification of CPM cells towards a myogenic program is CNCC-independent, the migration, patterning and differentiation of muscle precursors require extrinsic signals from CNCCs [104]. In line with these findings, OAVS patients present muscular defects associated to the skeletal lesion (see for example [126,127]), however, these initial descriptions where never interpreted in view of recent embryological data on the coordination of CNCCs and CPM early development. Understanding the origin of these muscular defects is very important for the prognosis of surgical intervention on OAVS patients as muscular anomalies cannot be surgically corrected as easily as skeletal malformations.

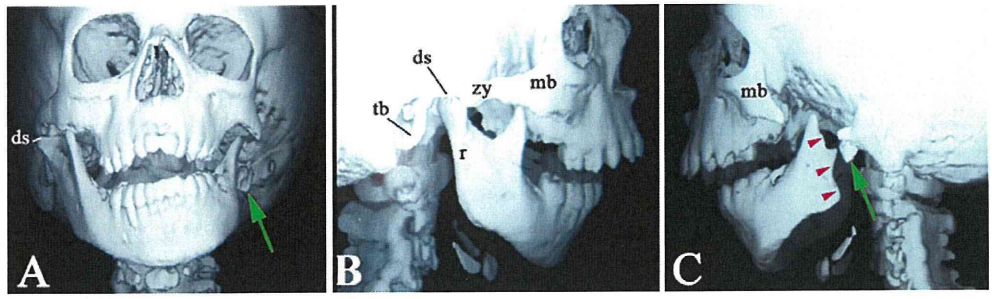

Fig. 3. Example of unilateral mandibulofacial dysostosis.
Frontal (A) and lateral (B, right; C, left) views of a three dimensional reconstruction of facial skeleton of a 6 years old child affected by left mandibulofacial dysostosis with absence of the left ramus (red arrowheads), dentary-squamosal articulation, tympanic bone and proximal zygomatic arch. An ectopic bone (green arrow) is present. The left malar bone is also hypoplastic. The medio-distal part of the mandible including lacteal and definitive teeth is unaffected. *Abbreviations*: ds, dentary-squamosal articulation; mb, malar bone; r, ramus; tb; tympanic bone; zy, zygomatic arch.

Most cases of first arch syndromes are sporadic, but familial inheritance has been reported, in particular a high load score has been found for the 14q32 genomic region which harbours the *Goosecoid* gene (*Gsc*), however, no gross mutation in the coding region was found [128] suggesting a defect in gene regulation.

7. Conclusions

Craniofacial morphogenesis is the result of complex spatio-temporal interactions between the various cellular components of the head. Molecular signals exchanged between CNCCs, mesodermal cells and endo/ectodermal epithelia of the pharyngeal arches are crucial in providing the positional information necessary to shape the different skeletal and muscular components of the head. In particular Edn1 signalling is essential to define maxilo-mandibular identity through the activation of *Dlx* genes. RA treatment of developing embryos perturbs epithelial signalling to CNCCs and has permitted to analyze their contribution to craniofacial morphogenesis. Slight changes in these signalling dynamics can result in profound changes in craniofacial morphology and might have contributed to the evolution of facial diversity. Abnormal signalling patterns could also be at the origin of congenital craniofacial malformations.

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Role of Mesodermal FGF8 and FGF10 Overlaps in the Development of the Arterial Pole of the Heart and Pharyngeal Arch Arteries

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Role of Mesodermal FGF8 and FGF10 Overlaps in the Development of the Arterial Pole of the Heart and Pharyngeal Arch Arteries

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Rationale: The genes encoding fibroblast growth factor (FGF) 8 and 10 are expressed in the anterior part of the second heart field that constitutes a population of cardiac progenitor cells contributing to the arterial pole of the heart. Previous studies of hypomorphic and conditional *Fgf8* mutants show disrupted outflow tract (OFT) and right ventricle (RV) development, whereas *Fgf10* mutants do not have detectable OFT defects.

Objectives: Our aim was to investigate functional overlap between *Fgf8* and *Fgf10* during formation of the arterial pole.

Methods and Results: We generated mesodermal *Fgf8; Fgf10* compound mutants with *MesP1Cre*. The OFT/RV morphology in these mutants was affected with variable penetrance; however, the incidence of embryos with severely affected OFT/RV morphology was significantly increased in response to decreasing *Fgf8* and *Fgf10* gene dosage. *Fgf8* expression in the pharyngeal arch ectoderm is important for development of the pharyngeal arch arteries and their derivatives. We now show that *Fgf8* deletion in the mesoderm alone leads to pharyngeal arch artery phenotypes and that these vascular phenotypes are exacerbated by loss of *Fgf10* function in the mesodermal core of the arches.

Conclusions: These results show functional overlap of FGF8 and FGF10 signaling from second heart field mesoderm during development of the OFT/RV, and from pharyngeal arch mesoderm during pharyngeal arch artery formation, highlighting the sensitivity of these key aspects of cardiovascular development to FGF dosage. (*Circ Res*. 2010;106:495-503.)

Key Words: second heart field ■ arterial pole defects ■ pharyngeal arch artery defects

Malformations of the arterial pole of the heart account for more than 30% of human congenital heart defects.[1] Remodeling of the outflow tract (OFT) plays a critical role in the maturation of the arterial pole. As the heart matures, cushion tissue is formed in the OFT, as a result of an epithelial–mesenchymal transformation.[2] Rotation and shortening of the OFT are accompanied by fusion of the cushions to form a septum that divides the OFT into the aorta and pulmonary trunk. Subsequent morphogenetic events result in alignment of the aorta with the left ventricle and the pulmonary trunk with the right ventricle (RV).[3] The OFT is derived from the anterior part of the second heart field (SHF), which in the mouse embryo, also contributes to the RV and ventricular septum.[4–6] This field of splanchnic mesoderm initially lies medial to the cardiac crescent and then dorsally and anteriorly to the heart tube. In addition to myocardium, the SHF contributes smooth muscle to the base of the great arteries.[6,7] A second source of cells, namely cardiac neural crest (CNC), migrates from the neuroectoderm of the dorsal neural tube into the OFT and contributes to cushion formation and correct septation and alignment of the aortic and pulmonary outflows.[8–10] CNC also interacts with the mesodermal cells of the SHF.[11] When CNC is ablated in the chick embryo, OFT elongation and looping of the cardiac tube is perturbed, leading to persistent truncus arteriosus and misalignment of the great arteries relative to the ventricles.[10] Many signaling pathways in the SHF, CNC, and pharyngeal region control development of the arterial pole of the heart by affecting specification, proliferation, survival and differentiation of progenitor cells.[12]

SHF mesoderm also plays a role in the formation of the blood vessels that channel blood from the aorta and pulmonary trunk to the body and lungs. These arteries form within the third, fourth, and sixth pharyngeal arches, which are bilateral embryonic structures, consisting of surface ectoderm, inner endoderm, and a mesodermal core expressing SHF markers, that becomes surrounded by CNC. The endothelium of the pharyngeal arch

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From the Department of Developmental Biology (Y.W., S.D.V., M.E.B.), Centre National de la Recherche Scientifique, Unité de Recherche Associée 2578, Institut Pasteur, Paris, France; Department of Pediatric Cardiology (S.M.-T.), Tokyo Women's Medical University, Tokyo, Japan; Developmental Biology Institute of Marseille-Luminy (R.G.K.), Unité Mixte de Recherche 6216 Université de la Méditerranée, Campus de Luminy, Marseille, France; and Departments of Pediatrics, Neurobiology, and Anatomy and Human Genetics (A.M.M.), University of Utah, Salt Lake City. Present address for Y.W.: Department of Developmental Neurobiology, Institute of Development, Aging and Cancer, Tohoku University, Sendai, Miyagi, Japan.

Correspondence to Margaret Buckingham, DPhil, Department of Developmental Biology, URA CNRS 2578, Institut Pasteur, 25 rue du Dr. Roux 75015 Paris, France. E-mail margaret.buckingham@pasteur.fr

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Non-standard Abbreviations and Acronyms	
CNC	cardiac neural crest
DORV	double outlet right ventricle
E	embryonic day
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
OFT	outflow tract
PAA	pharyngeal arch artery
RV	right ventricle
SHF	second heart field
VSD	ventricular septal defect

arteries (PAAs) is formed from the mesodermal core of the arches, whereas the vascular smooth muscle is predominantly CNC-derived.[8,13,14] The PAAs form progressively following the anterior/posterior gradient of pharyngeal arch development, and from embryonic day (E)10.5 in the mouse embryo, remodeling leads to the mature PAA system. Many different cellular and genetic perturbations disrupt formation and /or remodeling of the PAAs.[10]

Fgf10 was identified as a gene expressed in cells that contribute to the OFT and RV in the mouse embryo, leading to the concept of the SHF.[15] However *Fgf10*-null mutants have no evident SHF or PAA defects.[16] *Fgf8* expression overlaps with that of *Fgf10* in the SHF from the cardiac crescent stage.[15] Their expression is restricted to the anterior part of the SHF and is regulated by retinoic acid signaling on the anterior-posterior axis.[17] *Fgf10* transcription extends from the anterior SHF into the mesodermal core of the pharyngeal arches, where *Fgf8* transcripts are difficult to detect. However, $Fgf8^{LacZ/+}$, $Fgf8^{GFP/+}$, and *Fgf8* enhancer-*LacZ* transgenic mice show LacZ or green fluorescent protein (GFP) expression in the anterior SHF and the mesodermal core of the arches, implying that *Fgf8* is transcribed there.[18–20] Unlike *Fgf10*, *Fgf8* is also expressed in pharyngeal endoderm and ectoderm, where its expression is maintained, at least until E9.5.[21] *Fgf8* mutants die at gastrulation[22]; however, *Fgf8* hypomorphs survive to birth with variable OFT defects including persistent truncus arteriosus, transposition of the great arteries, and pharyngeal arch-related phenotypes. The fourth PAA is most severely affected resulting in interrupted or right aortic arch.[23,24] Apoptosis of CNC was noted in these hypomorphs, suggesting fibroblast growth factor (FGF)8 signaling defects in surrounding tissues that directly or indirectly affect CNC, because *Fgf8* is not expressed in these cells. Subsequently, conditional mutagenesis of *Fgf8* with a battery of Cre recombinases, directed to pharyngeal ectoderm, pharyngeal endoderm, and SHF/pharyngeal arch mesoderm, has allowed tissue-specific phenotypes to be determined.[13,19,20,25] Deletion of *Fgf8* in the pharyngeal ectoderm leads to CNC apoptosis, affecting PAA development.[25] The role of mesodermal FGF8 in PAA development has not been reported. Mesodermal and endodermal FGF8 are critical for OFT development, affecting the proliferation, survival, and transcriptional activity of SHF cells and their derivatives, in addition to the function of the CNC. Deletion of *Fgf8* in early cardiogenic mesoderm with *MesP1Cre* causes initial OFT/RV hypoplasia and OFT alignment defects in survivors at incomplete penetrance.[19] This implies that FGF10 may compensate for loss of FGF8 in the precardiac mesoderm.

In this study, we generated compound *Fgf8* and *Fgf10* mutants in the cardiac and pharyngeal mesoderm, using *MesP1Cre*. We show that PAA development is perturbed by mesodermal *Fgf8* deletion. The incidence and severity of PAA and of OFT defects increased with decreasing *Fgf8* and *Fgf10* gene dosage, resulting in severe defects in double *Fgf8*;*Fgf10* homozygous conditional mutants. These results reveal functional overlap of mesodermal FGF8 and FGF10 during SHF/OFT and PAA development, uncovering for the first time a role for FGF10 in the formation of the arterial pole of the heart. They also illustrate the sensitivity of these processes to incremental reductions in the level of FGF.

Methods

Mouse Lines

Mouse care and procedures were in accordance with institutional and national guidelines. The *Fgf8*-conditional mutant, *Fgf10* mutant, and *MesP1Cre* alleles are as previously described.[19,26,27] The $MesP1^{Cre/+}$ line was bred onto an $Fgf10^{+/-}$ background and then onto $Fgf8^{flox/+}$, which was crossed with an $Fgf8^{flox/flox}$;$Fgf10^{+/-}$ line to obtain compound mutants. *Fgf8* and *Fgf10* genotyping by PCR was performed using the following primers: P1, P2, and 5′-GAGCTTGCTGGGGGAAACTTCCTGACTAGG-3′ for $Fgf10^{26}$; and 5′-TGCCTAAGGGGAGAAGGCTGG-3′, 5′-AAATTTAAGCTGTGTAGATTCCATAG-3′, and 5′-GATTTCAGGAGAACAGACCAGAG-3′ primers for *Fgf8*. Numbers of embryos obtained at different stages are summarized in Online Table I (available in the Online Data Supplement at http://circres.ahajournals.org). Total numbers of embryos examined for each genotype at E10.5 and E15.5 to 18.5 are given in Tables 1 and 2, respectively.

Scoring OFT and RV Morphology

Right-sided views of the OFT and RV in embryos at E9.5 were scored according to four parameters, OFT length, the angle between the proximal and distal regions of the OFT, the size of the RV, and the extent of looping estimated by the angle of the OFT across the ventral body of the embryo. Scoring was performed by two independent observers blinded to genotype. There was remarkably little interobserver variability in the scores of individual embryos of all genotypes (not shown). Embryos were classified as normal, mild, moderate, and severe depending on the score (2 points for each parameter, with a maximum of 8 points: <2 points, severe; <4 points, moderate; <6 points, mild; ≥ 6, normal).

Histological Analysis and Ink Injection

Longitudinal sections (10 μm) were made in the OFT and pharyngeal arch region after paraffin embedding of E10.5 embryos. To visualize PAA formation, India ink was injected into the left ventricle or umbilical vein of E10.5 embryos using drawn glass pipettes.

Quantitative Real-Time PCR

The third to sixth pharyngeal arch region was dissected from embryos at 24 to 26 somite stages and stored in RNAlater (Qiagen) at -20°C until use. Total RNA was isolated from a pool of six samples for each genotype using RNeasy Mini Kit (Qiagen). The first strand cDNA was synthesized using SuperScript II Reverse Transcriptase (Invitrogen). Quantitative PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems) with the StepOnePlus Real-Time PCR system (Applied Biosystems). Reverse transcription and quantitative PCR were repeated 3 times, and *Gapdh* was used to normalize gene expression. Primer sequences were 5′-TGAAAGGCGGATACTTG-

Table 1. Incidence of Abnormal PAAs in *Fgf8;Fgf10* Compound Mutants With *MesP1Cre* at E10.5

Fgf8;Fgf10 Genotype	No. of Embryos Examined	Second PAA			Third PAA			Fourth PAA			Sixth PAA			PAA Total	
			Presence			Absence			Absence			Absence			
		Normal	Uni	Bi	Normal	Uni	Bi	Normal	Uni	Bi	Normal	Uni	Bi	Normal	Abnormal
flox/+;+/+	10	10	0	0	10	0	0	10	0	0	8	0	2	8	2
flox/+;+/−	14	14	0	0	14	0	0	13	1	0	14	0	0	13	1
flox/+;−/−	3	3	0	0	3	0	0	3	0	0	3	0	0	3	0
flox/flox;+/+	16	15	0	1	11	4	1	12	4	0	15	0	1	8	8
flox/flox;+/−	13	11	2	0	8	5	0	8	5	0	13	0	0	6	7
flox/flox;−/−	7	4	1	2	4	2	1	1	4	2	7	0	0	0	7

PAAs examined after ink injection as shown in Figure 3. Unilateral (Uni) or bilateral (Bi) defects were scored as abnormal in all cases. χ^2 test was applied in comparisons of the following examples in the table. Second PAA: $^*P<0.05$, *Fgf8*$^{flox/flox}$ vs *Fgf8*$^{flox/flox}$;*Fgf10*$^{-/-}$; fourth PAA: $^{**}P<0.01$, *Fgf8*$^{flox/flox}$ vs *Fgf8*$^{flox/flox}$;*Fgf10*$^{-/-}$; $^*P<0.05$, *Fgf8*$^{flox/flox}$;*Fgf10*$^{+/-}$ vs *Fgf8*$^{flox/flox}$;*Fgf10*$^{-/-}$; PAA total: $^*P<0.05$, *Fgf8*$^{flox/flox}$ vs *Fgf8*$^{flox/flox}$;*Fgf10*$^{-/-}$ and *Fgf8*$^{flox/flox}$;*Fgf10*$^{+/-}$ vs *Fgf8*$^{flox/flox}$;*Fgf10*$^{-/-}$.

GAC-3′ and 5′-TGTCCGGTACCTGAGCTTCT-3′ for *Pea3*; 5′-GGACACAGATCTGGCTCACGA-3′ and 5′-CGTGGCTACAGGACGACAAC-3′ for *Erm*; *Isl1* primers were as designed by Liao et al.[28]

Results

Fgf10 expression is observed in the anterior SHF and pharyngeal mesoderm, whereas *Fgf8* is expressed in pharyngeal endoderm and ectoderm in addition to these mesodermal tissues. To generate compound mutants for *Fgf8* and *Fgf10* in this mesoderm, we deleted the *Fgf8* conditional allele (*Fgf8*^{flox}) with *MesP1*$^{Cre/+}$. *Fgf10*-null homozygotes are not viable after birth, and we therefore used the *Fgf10* heterozygotes (*Fgf10*$^{+/-}$) for crosses (see Online Table I, *Fgf8*$^{flox/+}$;*Fgf10*$^{+/-}$;*MesP1*$^{Cre/+}$× *Fgf8*$^{flox/flox}$;*Fgf10*$^{+/-}$). In the following text, *MesP1*$^{Cre/+}$ is included in all genotypes.

OFT and RV Morphology Is Affected by Reducing *Fgf8* and *Fgf10* Dosage

In *Fgf8;Fgf10* double heterozygous (*Fgf8*$^{flox/+}$;*Fgf10*$^{+/-}$) (Figure 1B) and *Fgf8*$^{flox/+}$;*Fgf10*$^{-/-}$ embryos (Figure 1C), OFT and RV morphology is normal at E9.5, similar to that of *Fgf8*$^{flox/+}$ embryos (Figure 1A). *Fgf8*$^{flox/flox}$ embryos have hypomorphic OFTs and RVs (Figure 1D through 1F), as previously reported with a *MesP1Cre* at E9.5.[19] When *Fgf10* dosage was decreased in addition to *Fgf8* deletion (Figure 1G through 1L), more severe phenotypes were observed, including hypoplasia of the second and third pharyngeal arches (Figure 1I and 1L). This was also illustrated by sections of the OFT at E10.5 showing normal presence of CNC and initiation of epithelial–mesenchymal transition before cushion formation in control *Fgf8;Fgf10* double heterozygotes (Figure 1M). In contrast, CNC invasion and epithelial–mesenchymal transition were notably compromised in *Fgf8;Fgf10* double homozygous mutants (Figure 1O) and already affected in *Fgf8*$^{flox/flox}$ mutants (Figure 1N).

To confirm and quantify this, we scored OFT and RV morphology on the basis of OFT length, the angle between the proximal and distal regions of the OFT, RV size and extent of looping, by a blind test. Phenotypes were classified as normal, mild, moderate, and severe, depending on the score (Figure 2A). The number of abnormal *Fgf8*$^{flox/flox}$ embryos was less than in our previous report, probably because in that case the *Fgf8*$^{flox/-}$ cross was analyzed.[19] The important point, shown in Figure 2A, is that increasingly affected embryos were observed as *Fgf* dosage is reduced. No severe phenotypes were observed with *Fgf8* deletion alone, whereas no normal hearts were observed in *Fgf8;Fgf10* double homozygous mutants. This result shows that FGF10 functions with FGF8 in OFT and RV development. To verify that FGF signaling was attenuated in *Fgf8;Fgf10* double homozygous mutants, we performed quantitative PCR analysis for FGF target genes, *Pea3* and *Erm*, using RNA isolated from the pharyngeal region (including SHF mesoderm, endoderm,

Table 2. Incidence of Abnormal Heart and PAAs in *Fgf8;Fgf10* Compound Mutants With *MesP1Cre* at E15.5 to E18.5

Fgf8;Fgf10 Genotype	No. of Embryos Examined	Heart			Pharyngeal Arch Arteries				
		Normal	TGA/DORV	VSD	Normal	Short or Absent LCC	ARSA	RtAA	Narrow AoA
flox/+;+/+	7	7	0	0	7	0	0	0	0
flox/+;+/−	13	13	0	0	13	0	0	0	0
flox/+;−/−	4	4	0	0	4	0	0	0	0
flox/flox;+/+	11	9	1	2	8	2	1	1	0
flox/flox;+/−	13	8	1	5	9	2	2	1	1
flox/flox;−/−	5	1	1	3	1	2	2	0	1

One or more arterial pole or PAA malformations were scored as abnormal in all cases. χ^2 test was applied in comparisons of the following examples in the table. Heart: $^*P=0.018$, *Fgf8*$^{flox/flox}$ vs *Fgf8*$^{flox/flox}$;*Fgf10*$^{-/-}$; PAAs: $^*P=0.049$, *Fgf8*$^{flox/flox}$ vs *Fgf8*$^{flox/flox}$;*Fgf10*$^{-/-}$. AoA indicates aortic arch; ARSA, aberrant origin of right subclavian artery; RtAA, right aortic arch; TGA, transposition of the great arteries.