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Msx1 and *Dlx5* act independently in development of craniofacial skeleton, but converge on the regulation of Bmp signaling in palate formation

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Abstract

Msx and Dlx homeoproteins control the morphogenesis and organization of craniofacial skeletal structures, specifically those derived from the pharyngeal arches. In vitro Msx and Dlx proteins have opposing transcriptional properties and form heterodimeric complexes via their homeodomain with reciprocal functional repression. In this report we examine the skeletal phenotype of Msx1; *Dlx5* double knock-out (DKO) mice in relationship with their expression territories during craniofacial development. Co-expression of *Dlx5* and *Msx1* is only observed in embryonic tissues in which these genes have independent functions, and thus direct protein interactions are unlikely to control morphogenesis of the cranium. The DKO craniofacial phenotypes indicate a complex interplay between these genes, acting independently (mandible and middle ear), synergistically (deposition of bone tissue) or converging on the same morphogenetic process (palate growth and closure). In the latter case, the absence of *Dlx5* rescues in part the *Msx1*-dependent defects in palate growth and elevation. At the basis of this effect, our data implicate the Bmp (*Bmp7*, *Bmp4*)/Bmp antagonist (*Follistatin*) signal: in the $Dlx5^{-/-}$ palate changes in the expression level of *Bmp7* and *Follistatin* counteract the reduced *Bmp4* expression. These results highlight the importance of precise spatial and temporal regulation of the Bmp/Bmp antagonist system during palate closure.

Keywords: Dlx; Msx; Cleft palate; Bmp; Follistatin; Craniofacial; Pharyngeal arch

1. Introduction

The mammalian face develops from the coordinated growth, morphogenesis and fusion of several embryonic primordia: the frontonasal processes, two maxillary and two mandibular prominences, located around the primitive mouth. The maxillary and mandibular prominences derive from the first pharyngeal arch whose mesenchyme is colonized predominantly by neural crest-derived skeletogenic cells. Ventrally to the mouth, the mandibular arches fuse in the midline and give rise to the skeletal elements of the mandible. Dorsally to the mouth, the maxillary prominences grow and fuse anteriorly with the frontonasal processes, giving rise to the upper lip and the primary palate. The secondary palate develops bilaterally as two shelves that grow from the internal surface of the maxillae and first project vertically along the sides of the tongue (E12–E13). The shelves then elevate and become oriented horizontally, eventually they fuse with each other, with the base of the nasal septum and with the primary palate (E14–E15) (Ferguson, 1998).

The comprehension of the genetic regulation at the basis of craniofacial patterning and morphogenesis is a formidable task

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and is being achieved mainly by the analysis of developmental phenotypes in mutant mouse strains (Wilkie and Morris-Kay, 2001; Francis-West et al., 2003; Couburne and Sharpe, 2003; Richman and Lee, 2003; Thyagarajan et al., 2003; Murray and Schutte, 2004). Among the many gene families that have been implicated, the closely related *Dlx* and *Msx* homeobox transcription factors gene play a central role (Davidson, 1995; Depew et al., 1999; 2002; Merlo et al., 2000; 2003; Bendall and Abate-Shen, 2000; Beverdam et al., 2002; Panganiban and Rubenstein, 2002). *Msx1* and *Msx2* are widely expressed at many embryonic sites, in particular those where epithelial-mesenchyme interactions take place (Davidson, 1995; Bendall and Abate-Shen, 2000). During early head development, *Msx1* and *Msx2* are expressed in migrating neural crest cells, which populate the distal regions of the arches and the frontonasal processes (Bendall and Abate-Shen, 2000; Alappat et al., 2003 and references therein). Similarly, *Dlx* genes are expressed in a proximal-distal combinatorial pattern in the pharyngeal arches (Depew et al., 2002; Merlo et al., 2003). Differential expression of *Dlx* genes in the arches suggest that they confer positional identity on the skeletogenic cells, this notion being supported by the analysis of craniofacial phenotypes in single and double knock-out (DKO) mice (Beverdam et al., 2002; Depew et al., 2002). At later embryonic stages, the partially overlapping expression of the various members of the *Dlx* and *Msx* gene families is thought to mediate epithelial-mesenchymal interactions leading to correct induction, growth and morphogenesis of embryonic structures such as the teeth, the palate, the mandible (Bendall and Abate-Shen, 2000 and references therein).

In the $Msx1^{-/-}$ mice severe craniofacial defects are observed, including palatal cleft, due to failure of the shelves to elevate and fuse, mandible and middle ear ossicle deformity, absence of molars and delayed ossification (Satokata and Maas, 1994). All these defects have been associated to the expression of *Msx1* in the corresponding embryonic territories. In the *Dlx5* null mice, similar skeletal structures are affected, including the palate, the mandible, the middle ear ossicles; these mice exhibit also a generalized delay in the ossification process in the calvaria (Acampora et al., 1999; Depew et al., 1999; Merlo et al., 2000; 2003). The similarity of the skeletal elements affected by *Msx1* or *Dlx5* mutation prompted us to investigate a possible interplay between these genes.

In vitro, the Msx and Dlx proteins appear to have opposing transcription properties with Msx functioning as repressors and Dlx as activators (Alappat et al., 2003; Panganiban and Rubenstein, 2002). In vitro, Msx1, Dlx2 and Dlx5 recombinant proteins form heterodimers via their homeodomain and, by doing so, they reciprocally inhibit their activities (Zhang et al., 1997). Should this observation hold true in vivo, some of the phenotypes observed in $Msx1^{-/-}$ mice may be due to altered Dlx5 activity, and viceversa. In this case, one might expect that in *Msx1*; *Dlx5* DKO mice some of the defects be aggravated or rescued. The essential condition for this regulation to occur is that the two genes be expressed in the same cells at the same time, as proposed by Zhang and coworkers (1997). In this study, we first examined the expression pattern of *Msx1* and *Dlx5* in the craniofacial primordium. Next, we generated *Msx1*; *Dlx5* DKO mice and examined their phenotype compared to those of single mutants. In most structures, the two genes are expressed in separate territories and seem to act independently. On the contrary, the absence of *Dlx5* partially rescues the palatal defects of *Msx1* null mice, although there is no co-expression. We analyzed the signals possibly involved in this regulation and provide data that implicate the Bmp/Bmp-antagonist signal in the process genetically controlled by *Dlx5* and *Msx1* in palate formation.

2. Results

2.1. Frequency of Msx1–Dlx5 DKO mice

$Msx1^{nlacZ}$ mice were crossbred with $Dlx5^{lacZ}$ mice, double heterozygous males and females were obtained at the expected frequency. Crossbreeding of double heterozygotes yielded $Msx1^{-/-}$; $Dlx5^{-/-}$ embryos and pups at a frequency lower than expected (5/98, 5.1 vs. 6.25%, not statistically significant). All delivered DKO, as well as single $Msx1^{-/-}$ and $Dlx5^{-/-}$ animals, died soon after delivery.

2.2. Expression of Dlx5 and Msx1 in craniofacial structures

At the E10.5 stage of development, *Msx1* and *Dlx5* are co-expressed in the distal mandibular arch (Bendall and Abate-Shen, 2000, Fig. 3). We have compared expression of *Msx1* and *Dlx5* at later stages by X-gal staining of serial sections of $Msx1^{nlacZ}$ and $Dlx5^{lacZ}$ heterozygous (normal) embryos at E13.5 and E14.5 (Figs. 1, 6). In situ hybridization on relevant sections was used to confirm the endogenous expression.

In the anterior palate region, marked by the vomeronasal organs (VNO), *Msx1,* but not *Dlx5,* is expressed in the mesenchyme, in the chondrogenic tissue surrounding the VNO and in a wide region of the maxilla mesenchyme (Fig. 1B,D). *Dlx5*, but not *Msx1,* is expressed in the VNO and the olfactory epithelium (Fig. 1A,C) (Levi et al., 2003). Co-expression of *Msx1* and *Dlx5* is limited to the most lateral region of the maxillary mesenchyme (Fig. 1A,B). In the posterior palate region, marked by the molar tooth buds, neither *Msx1* not *Dlx5* are expressed. Instead, co-expression of *Msx1* and *Dlx5* is found in the maxilla mesenchyme corresponding to osteogenic areas (Fig. 1C,D). *Msx1* is also strongly expressed in the molar tooth papillae, while *Dlx5* is expressed in the osteogenic mesenchyme surrounding the tooth (Figs. 1, 6) and in the enamel knot cells. Neither *Msx1* nor *Dlx5* are expressed in the dental epithelium (Bendall and Abate-Shen, 2000; Alappat et al., 2003; Merlo et al., 2003). In summary *Msx1* is expressed in the anterior, but not in the posterior, palate (see also Zhang et al., 2002; Nugent and Green, 1998; Givens et al., 2005) while *Dlx5* is not expressed in either the anterior or the posterior palate. We then examined the distribution of *lacZ* expressing cells in the palate, comparing *Msx1* and *Dlx5* heterozygous embryos with the corresponding homozygous ones (Fig. 1E–H). *lacZ* expression is maintained in the correct

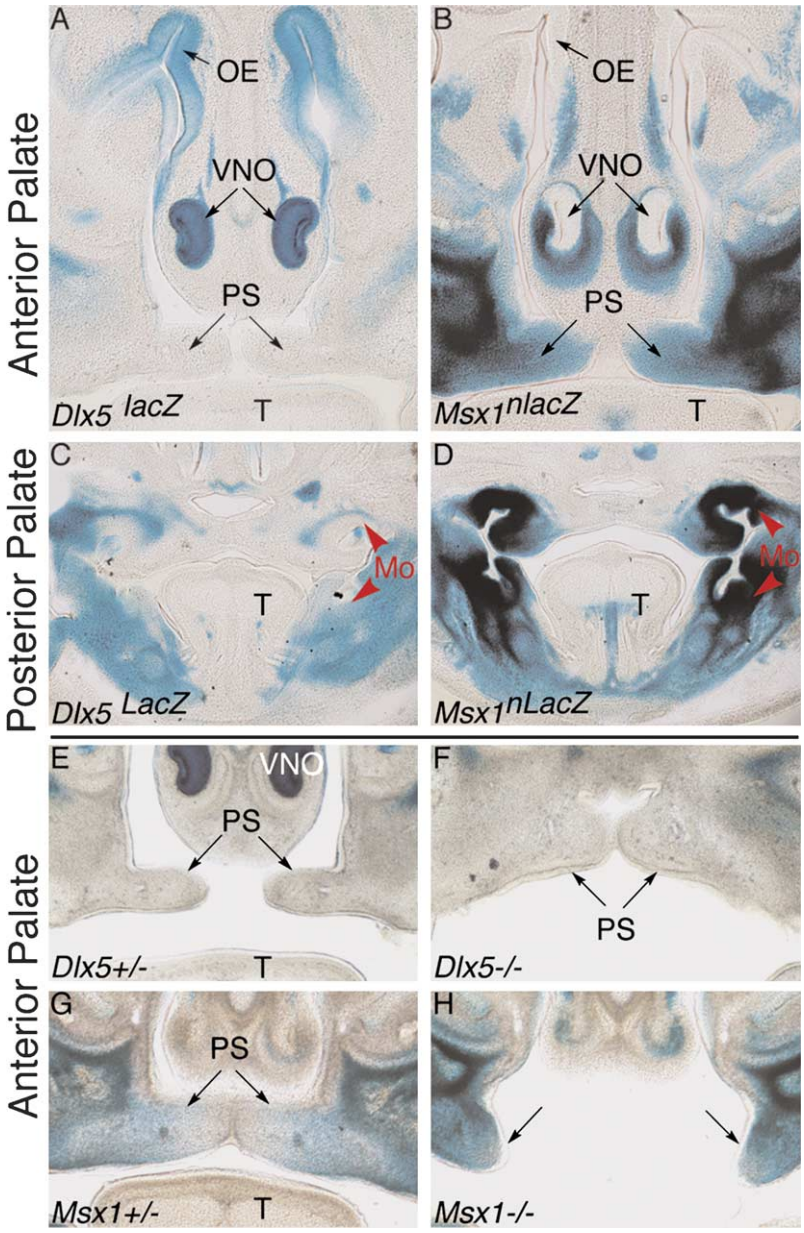


Fig. 1. Expression of *Dlx5* and *Msx1* in craniofacial structures. (Top) expression of $Dlx5^{lacZ}$ and $Msx1^{nlacZ}$ by X-gal staining of frontal sections of normal (heterozygous) E14.5 embryos. The anterior (A,B) and posterior palatal regions (C,D), marked respectively by the VNO and the molar tooth buds, are shown. (Bottom) same as above, applied to compare a normal (on the left) with a mutant (on the right) E14.5 embryo. Palatal shelves are indicated with black arrows, molar buds are indicated with red arrowheads. Genotypes for *Dlx5* and *Mxs1* are reported. Abbreviations: Mo, molar buds; OE, olfactory epithelium, PS, palatal shelves; T, tongue, VNO, vomeronasal organ.

position in both the $Msx1^{-/-}$ and $Dlx5^{-/-}$. Thus, it is unlikely that the palatal phenotypes observed in these mutant mice and the rescue observed in the DKO head be caused by a missing cell population.

2.3. The palatal phenotype

In the *Msx1* null mice the palatal shelves fail to elevate from a vertical to an horizontal position; consequently they do not fuse and remain opened, with the base of the nasal septum is exposed in the oral cavity (Figs. 1H, 2A,G,H) (Satokata and Maas, 1994; Houlzestein et al., 1997). In the *Dlx5* null mice, the palatal shelves are partially formed and elevated but fail to complete growth and to fuse in the midline (Fig. 2E,F) (Acampora et al., 1999; Depew et al., 1999). While the *Msx1* phenotype is fully penetrant, the *Dlx5* one shown in Fig. 2 is the most common of a range of phenotypes; in the more severe (rare) form a near complete opening of the palatal shelves is observed, while in the mildest form (also rare) the shelves grow and elevate to come into contact, however, they fail to fuse. In DKO E13.5 embryos (2/2) the shelves are partly formed and elevated (Fig. 2B), but do not come to contact and fuse. At P0 the shelves are present, elevated, and partially ossified (3/3), although fail to fuse in the midline. This leads to the condition

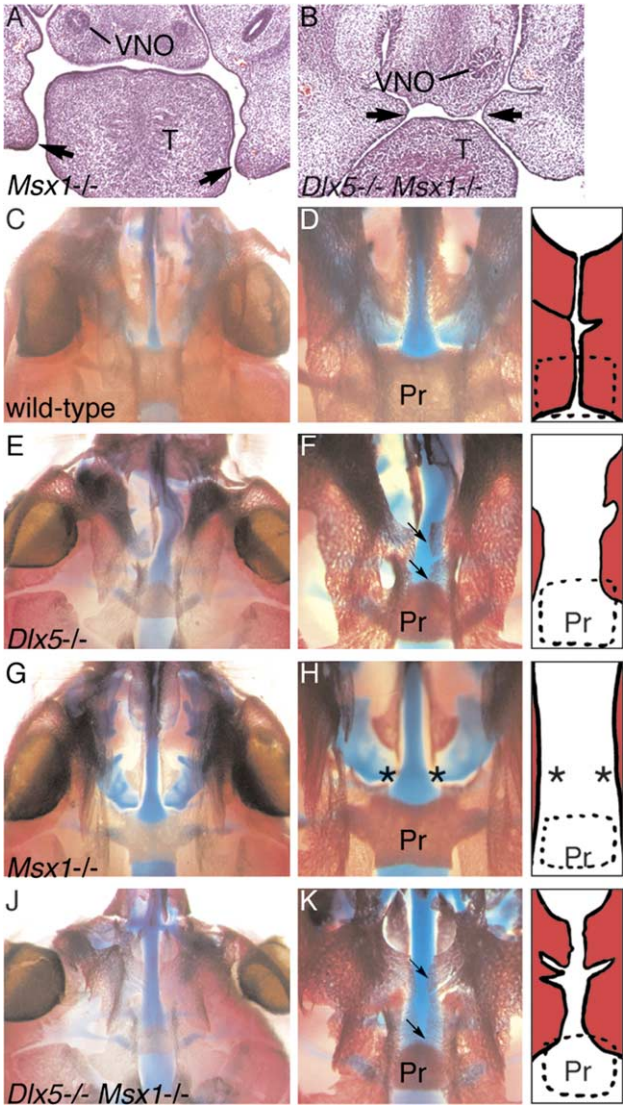


Fig. 2. Rescue of the *Msx1* palatal defects in *Msx1-Dlx5* DKO mice. (A,B) Histology of the palate of $Msx1^{-/-}$ and DKO E13.5 embryos. (C–K) Skeletal preparation of WT (C,D), $Dlx5^{-/-}$ (E,F), $Msx1^{-/-}$ (G,H) and DKO (J,K) mice, at P0. The picture on the right is a higher magnification of the one on the left, detailing the palatal region. The presphenoid bone (Pr) and the palatal shelves (black arrows) are indicated, the absence of the palatal shelves in the $Msx1^{-/-}$ head (H) is indicated with asterisks. A drawing of the palatal shelves is shown on the right, for clarity.

of a mild clefting (Fig. 2J,K) similar to that observed in *Dlx5* null mice. This observation indicates that the severe *Msx1*-related palatal phenotype can be partially rescued in the DKO head.

We set forth to investigate the molecular regulation at the basis *Dlx5*-dependent rescue of the *Msx1* palate phenotype. In $Msx1^{-/-}$ embryos a reduced expression of *Shh*, *Bmp4* and *Bmp2* in the medial edge epithelium (MEE) and mesenchyme of the anterior region has been reported (Zhang et al., 2002). The restoration of *Bmp4* expression in the palate of $Msx1^{-/-}$ mice could partially rescue the growth and elevation of the shelves (Zhang et al., 2002). It seemed therefore likely that the loss of *Dlx5* might infringe on the *Bmp* expression and/or regulation. Since *Dlx5* is not expressed in the *Bmp2-4* territory, such putative function of *Dlx5* should be non-cell-autonomous, and might reasonably include the secreted Bmp antagonist *Noggin, Chordin, Follistatin*. Alternatively, *Msx1* might repress *Dlx5* transcription, and *Dlx5*, in turn, might normally inhibit cell proliferation of the palatal mesenchyme. We tested both of these hypotheses.

2.4. Expression of *Dlx5* and *Dlx6* is not altered in $Msx1^{-/-}$ mice

Expression of *Dlx5* and *Msx1* in the developing head appears to be complementary (Fig. 1). For this reason, the possibility that the Msx1 protein may normally repress *Dlx5* expression in the palate appeared likely. We have examined expression of *Dlx5* in E11 $Msx1^{-/-}$ embryos by whole-mount in situ hybridization. As shown in Fig. 3A–D, expression of *Dlx5* was detected both in the WT and in the $Msx1^{-/-}$ embryos in the expected territories (1st and 2nd pharyngeal arches, ventral cephalic epithelium, olfactory and otic epithelium) with no appreciable difference. These results indicate that *Dlx5* is not under transcription control of *Msx1*. We also examined expression of *Msx1* (Fig. 3E, F) and *Msx2* (not shown) in the pharyngeal arches of E10.5$Dlx5^{-/-}$ embryos. In both WT and mutant embryos *Msx1* is expressed in the expected territories (distal mandibular arch, maxillary arch, mesenchyme of the frontonasal processes, limbs) with no significant difference.

Msx1 could inhibit *Dlx5* expression at a later stage of palate formation. We have therefore examined *Dlx5* expression in sections of E14.5 normal and $Msx1^{-/-}$ heads by in situ hybridization. As shown in Fig. 3G, H, expression of *Dlx5* in the anterior palate is undetectable in both the WT and the $Msx1^{-/-}$ palate. Instead, in the posterior palate, *Dlx5* expression is slightly expanded toward the midline in the $Msx1^{-/-}$ palate (Fig. 3J, K). This region, however, does not overlap with either the *Msx1* or *Bmp4* expression territories (Zhang et al., 2002). Finally, *Dlx5* expression is observed in the lateral maxillary mesenchyme equally in the normal and in the $Msx1^{-/-}$ head, and is unchanged in all other embryonic territories. These results suggest that *Dlx5* expression in vivo is largely independent of *Msx1*.

2.5. Expression of Bmps and Bmp antagonists in Msx1 and Dlx5 mutant embryos

Dlx5 could directly act as an inhibitor of *Bmp4* expression, or could modulate Bmp function by regulating the expression of Bmp antagonists, such as *Noggin*, *Chordin,* or *Follistatin*. *Bmp4* is expressed at sites of fusion between prominences of the head primordium, including the palate (Gong and Guo, 2003). In E13–E14 embryos *Bmp4* is expressed in a restricted domain of the anterior palate mesenchyme, adjacent to the MEE (Fig. 4A,D). Importantly, several genes coding for morphogenetic molecules, including *Bmp2, Bmp4, Bmp7, Follistatin, Shh* and *FGFs*, are expressed in this location. This domain can be considered a signaling center for palate growth. In the $Msx1^{-/-}$ palate at E13.5 and E14.5 *Bmp4*

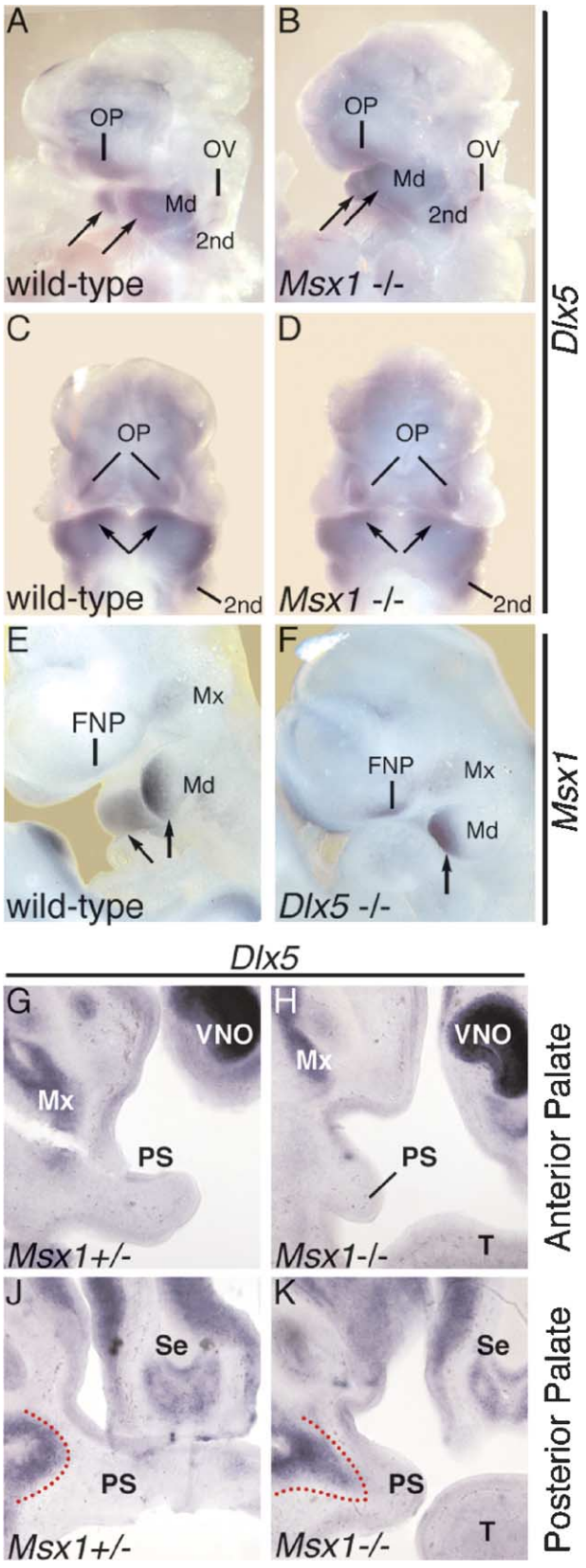


Fig. 3. Expression of *Dlx5* in $Msx1^{-/-}$ embryos. (Top) whole-mount hybridization with *Dlx5* probe on $Msx1^{-/-}$ E10.5 embryos (A–D), and with *Msx1* probe on $Dlx5^{-/-}$ E11embryos (E,F). A,B,E,F, lateral view; C,D, frontal view. *Msx1* and *Dlx5* signal on the 1st arch is indicated (black arrows). *Msx1* hybridization is also observed on the maxillary arch and the frontonasal process. *Dlx5* signal is also observed on the 2nd arch, the olfactory placodes, the otic vesicle. (Bottom) Hybridization with *Dlx5* probe on frontal sections of the anterior (G,H) and posterior (J,K) palate of E14.5 $Msx1^{-/-}$ embryos. Expression is observed in a lateral domain, corresponding to the maxilla. In the $Msx1^{-/-}$ head the *Dlx5* territory is slightly expanded towards the midline (sketched with a dotted red line). Abbreviations: FNP, frontonasal process; Mx, maxillary arch; OP, olfactory placodes; OV, otic vescicle; Se, septum (nasal); 2nd, second (hyoid) arch; other abbreviations as in previous figures.

expression in the anterior region is markedly reduced, however not absent (Zhang et al., 2002; Fig. 4C). Likewise, *Bmp4* expression is reduced in the $Dlx5^{-/-}$ palate (Fig. 4B,E) and is nearly absent in the DKO (Fig. 4E'). To control for tissue preservation and hybridization specificity, the *Bmp4* signal on the incisor tooth dental papillae in the same sections were compared and found to be equal (insets in Fig. 4D,E).

We then examined the expression of *Bmp7* in the palate of $Dlx5^{-/-}$, $Msx1^{-/-}$ and DKO embryos at E13.5 and E14.5. At E13.5 *Bmp7* is normally expressed in the signaling center of the anterior palate in both the epithelium and the underlying mesenchyme (Fig. 4J,L). *Bmp7* expression extends along the anterior-posterior length of the shelves (Fig. 4F–H), where it is found in the palatal epithelium, but not in the mesenchyme (Fig. 4N,P). Whole-mount in situ hybridization on WT, $Dlx5^{-/-}$ and $Msx1^{-/-}$heads showed a similar level of *Bmp7* expression (Fig. 4F–H); however hybridization on $Dlx5^{-/-}$ palates shows an increase in *Bmp7* signal both anteriorly (Fig. 4K) and posteriorly (data not shown). In the $Msx1^{-/-}$ palate *Bmp7* expression is decreased anteriorly, but increased posteriorly. Finally, *Bmp7* expression was found to be increased in the DKO palate (Fig. 4K'). To control for tissue preservation and specificity of the hybridization, the *Bmp7* signal on the eye lids in the same sections were compared (insets in Fig. 4J,K,M) and the results are summarized in Fig. 7. To confirm the previous finding, *Bmp7* mRNA level in WT, $Msx1^{-/-}$ and $Dlx5^{-/-}$ palatal shelves was quantified by RealTime PCR (Fig. 4N). A 1.5 and a 1.6 fold increase in the *Bmp7* template was observed, respectively, in the $Msx1^{-/-}$ and in the $Dlx5^{-/-}$ RNA sample, in two experiments (with biological replicates). The increased *Bmp7* expression cannot be attributed to a direct *Dlx5* regulation, and therefore a non-cell-autonomous effect is the most likely mechanism.

Expression of the Bmp-antagonist molecules, *Noggin, Chordin*, and *Follistatin* was examined in E13.5 and E14.5 *Dlx5* mutant heads. At E13.5 *Follistatin* expression is normally restricted to a dorsal (nasal) domain of the anterior palate epithelium; in $Dlx5^{-/-}$ palates this expression domain is unchanged (Fig. 5A,B). In the $Msx1^{-/-}$ palate, expression in this region was reduced and irregularly distributed (Fig. 5C). Posteriorly, *Follistatin* is expressed in the palatal epithelium, with higher expression on the tip and on the ventral (oral) domain (Fig. 5E). In the $Dlx5^{-/-}$ palate *Follistatin* expression in the mid-posterior region was reduced and irregularly distributed (Fig. 5F). In the posterior palate of $Msx1^{-/-}$ embryos *Follistatin* expression is detected at levels similar to the WT (Fig. 5G). In DKO embryos, *Follistatin* expression is reduced both anteriorly and posteriorly (Fig. 5D,H). To control for tissue preservation and hybridization specificity, *Follistatin*

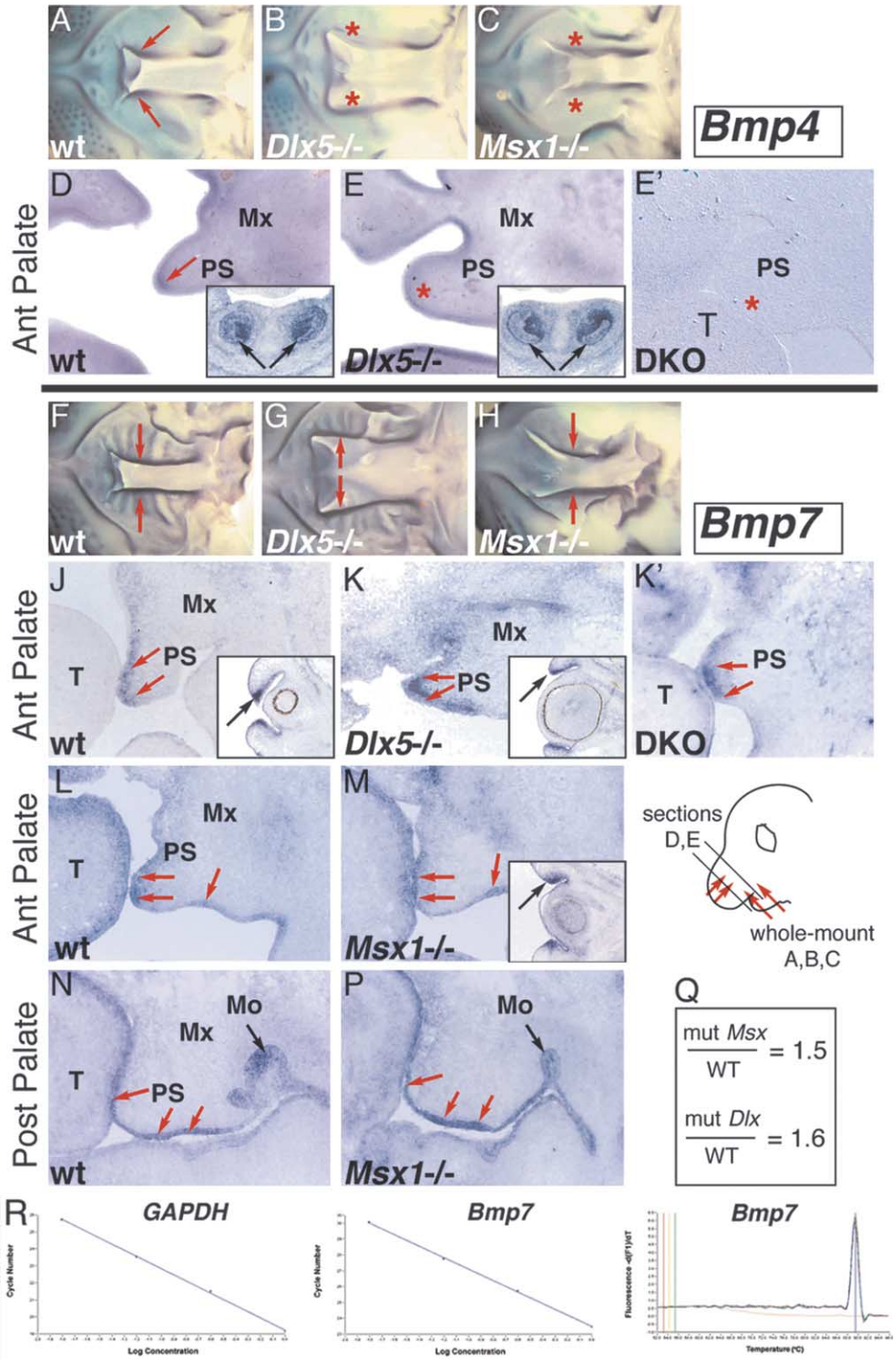


Fig. 4. Expression of *Bmp4* and *Bmp7* in $Dlx5^{-/-}$, $Msx1^{-/-}$ and DKO embryos. (A–C) Whole-mount in situ hybridization for *Bmp4* on the palate of WT (A), $Dlx5^{-/-}$ (B), $Msx1^{-/-}$ (C) E13.5 embryos, in ventral view. *Bmp4* signal in the palatal shelves is indicated with red arrowheads, or red asterisks in case of reduced expression. (D–E') Hybridization for *Bmp4* on frontal sections of WT (D), $Dlx5^{-/-}$ (E) and DKO (E') embryonic heads at E13.5. (F–H) Whole-mount in situ hybridization for *Bmp7* on the palate of WT (F), $Dlx5^{-/-}$ (G) and $Msx1^{-/-}$ (H) embryos at E13.5, in ventral view. The signal in the palatal shelves is indicated with red arrowheads. (J–P) Hybridization with *Bmp7* probe on frontal sections of the palate of WT (J,L,N), $Dlx5^{-/-}$ (K), $Msx1^{-/-}$ (M,P) and DKO (K') embryos. Sections of the anterior (J,K',L,M) and posterior (N,P) palate are shown. Relevant *Bmp7* signal in the palate epithelium and mesenchyme is indicated with red arrows, signal on the molar tooth is indicated with black arrows (N,P). The drawing on the right clarifies the section and the viewing planes adopted. Controls for specific and equal hybridization signal from the same experiments (eye lids for *Bmp7*, incisor teeth for *Bmp4*) are shown in the inserts (lower right). (Q,R) RealTime PCR quantification of *Bmp7* mRNA in WT, $Dlx5^{-/-}$ and $Msx1^{-/-}$ palatal tissue. Regression curves for *GAPDH* and *Bmp7,* and melting curves for *Bmp7* are shown (R), the calculated normalized ratio (mut/wt) is reported. Abbreviations as in previous figures.

signal on the dental epithelium of the molar tooth buds in the same sections were compared (insets in Fig. 5E,H). These results are summarized in Fig. 7.

Noggin is expressed in chondrogenic condensations, in the olfactory and VN epithelium, and in a restricted region of the maxilla mesenchyme, but not in the palate proper (Fig. 5J). Examination of serial sections of a WT embryo hybridized with *Dlx5* or *Noggin* indicates that expression of these two genes in the maxillary region only partially overlaps (Fig. 5, compare J and L, red arrows) and furthermore *Noggin* expression did not significantly change in $Dlx5^{-/-}$ heads (Fig. 5K). To control tissue preservation and specificity of hybridization, *Noggin* signal on the ventricular zone of the forebrain were compared (insets in Fig. 5J,K). For *Dlx5*, control hybridization on the ganglionic eminence of the forebrain is shown (inset in Fig. 5L). Finally, *Chordin* expression in WT and $Dlx5^{-/-}$ palate was examined and found not to change significantly (data not shown). As further control for the relative position of the head territories and the specificity of the observed differences, we examined expression of the morphogens *Wnt5a* and *Wnt5b*. In E14.5 embryos, *Wnt5a* is expressed in the medial mesenchyme of both the anterior and the posterior palate (Fig. 5M). In $Dlx5^{-/-}$ palatal shelves *Wnt5a* expression is maintained in the corresponding territory (Fig. 5N). *Wnt5b* is expressed in a territory partly overlapping with *Noggin* and *Dlx5* (Fig. 5P). In $Dlx5^{-/-}$ heads *Wnt5b* expression in this domain is reduced (Fig. 5Q) suggesting a generalized dysfunction of the *Dlx5* expressing cells in this region.

2.6. The mandible and middle ear phenotypes

In the developing jaw $Msx1^{nlacZ}$ is expressed at E14.5 in a large mesenchymal domain including the dental papilla, but not in the dental epithelium (Fig. 6B,B'). In equivalent sections $Dlx5^{lacZ}$ is expressed in osteogenic areas around the incisor teeth buds (Fig. 6A,A') and the Meckel's cartilage (data not shown), but not in the dental epithelium and papilla. Thus expression of *Dlx5* and *Msx1* overlaps only in discrete regions of osteogenic mesenchyme. The jaws of WT, $Dlx5^{-/-}$, $Msx1^{-/-}$ and DKO mice, as well as the combined genotypes at P0, were compared (Fig. 6C–H). While both the $Dlx5^{-/-}$ (Fig. 6D) and the $Msx1^{-/-}$ (Fig. 6F) jaws showed the expected defects (Acampora et al., 1999; Depew et al., 1999; Satokata and Maas, 1994), in the DKO jaw both the coronoid process and the molar teeth are missing (Fig. 6H); these defects are related, respectively, to the *Dlx5* and the *Msx1* null phenotypes. Furthermore the DKO jaw is shorter and coarser compared to the WT or to the $Msx1^{-/-}$, although not as short as the $Dlx5^{-/-}$ ones. This could be a reflection of the variability of the $Dlx5^{-/-}$ phenotype (reported in Acampora et al., 1999; Depew et al., 1999).

In the middle and inner ear region of E14.5 embryos $Dlx5^{lacZ}$ is expressed in the malleus and incus (Fig. 6J), while $Msx1^{nlacZ}$ is expressed in the malleus and the cochlea (Fig. 6K). In DKO mice at P0 the malleus lacks the head and is deformed, an *Msx1*-related phenotype and, conversely, an ectopic bone is present, a *Dlx5* related phenotype (Fig. 6M–P). Thus, in the middle ear region both the *Msx1*- and the *Dlx5*-related defects can be recognized in the DKO.

Since, no novel defects or aggravations of known ones is observed in the jaw and middle ear of DKO mice, *Msx1* and *Dlx5* appear to have independent, non-overlapping functions in these structures.

2.7. Other craniofacial phenotypes

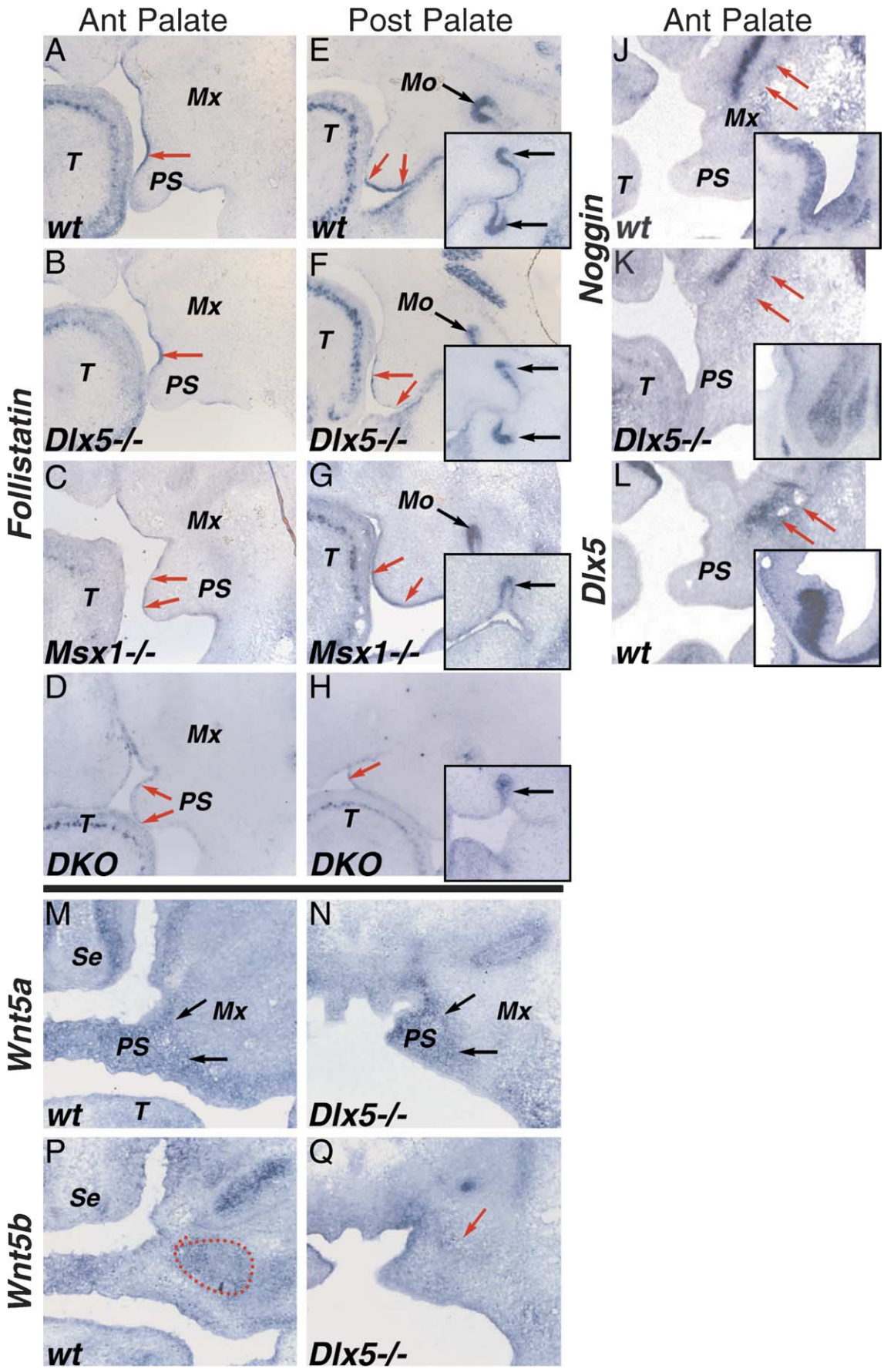
In the DKO mice we observe a number of defects related to those observed in the $Dlx5^{-/-}$ cranium (Table 1 summarized in Table 1). No evident aggravation or rescue of these phenotypes has been observed in the DKO, with the exception of the vault bones which appear smaller, heavily perforated and with irregular borders, the fontanels appear rather open on the midline (see Supplementary material, Fig. S1). *Msx1*, but not *Dlx5,* is expressed in the osteogenic calvaria mesenchyme of the head in E14 embryos. Expression of *Dlx5* in this tissue appears at a later stage (E16) (Acampora et al., 1999; Holleville et al., 2003, and data not shown). Interestingly, both the *Msx1* and *Dlx5* null mice show a delayed deposition of mineralized tissue in their calvaria (Ryoo et al., 1997; Newberry et al., 1998; Oreste-Cardoso et al., 2002; Acampora et al., 1999; Satokata and Maas, 1994). This result might indicate a cooperating function of these genes in osteoblast differentiation. A delayed ossification of the fore- and hind-limbs is also observed in the DKO mice (data not shown).

3. Discussion

We report on the craniofacial phenotype of *Msx1;Dlx5* DKO mice, compared with the defects in single *Msx1* or *Dlx5* mutants. Based on the specific defect and on their expression pattern, we recognize these categories (see also Table 1):

(A) Non-overlapping defects affecting the same skeletal element, indicating that *Dlx5* and *Msx1* have independent functions (mandible and middle ear).
(B) *Dlx5*-related defects, with no aggravation (ectopic bone, deformed pterigoids, basisphenoid and basioccipital, disorganized foramina of the *ala temporalis*, hypoplasia and asymmetry of the nasal and otic capsules).
(C) *Msx1*-related defects, with no aggravation (absence of molar teeth and alveolar bone, deformation of the squamosal).
(D) Phenotypic rescue (growth, elevation and ossification of the palate). Since *Msx1* and *Dlx5* are not co-expressed, this effect is non-cell-autonomous.
(E) Aggravation of defects affecting elements where genes are co-expressed, indicating a synergistic function (deposition of the calcified bone tissue in the calvaria and phalanges).

Since, the Dlx and Msx homeoproteins are known to form heterodimers in vitro and the interaction leads to abrogation of their DNA-binding and transcriptional activities (Zhang et al., 1997), some of the phenotypes observed in *Msx1* or in *Dlx5*


Ant Palate
Post Palate
Ant Palate
Follistatin
Noggin
Dlx5
Wnt5a
Wnt5b
A
B
C
D
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F
G
H
J
K
L
M
N
P
Q
Mx
T
PS
Mo
Se
wt
Dlx5-/-
Msx1-/-
DKO

mutant animals could be due to altered activity of the cognate protein partner, provided that these genes are co-expressed. With the possible exception of the bone tissue, our data indicate that in vivo functional interactions between Msx1 and Dlx5 are unlikely to occur. The reason for this is two folds: first, their expression territories in the embryonic head at E13–E14 are to a large extent not overlapping; second, in the region of co-expression these two genes carry out independent morphogenetic functions, as demonstrated by the additive feature of the jaw and middle ear phenotypes in DKO mice. The expression of *Msx2* is highly overlapping with that of *Msx1*, while *Msx3* is only expressed in the dorsal neural tube (Alappat et al., 2003); therefore, functional interaction of Dlx5 with any of the Msx homeoproteins in vivo is improbable.

Alternatively, a repressor activity of *Msx1* on *Dlx5* expression was taken into consideration. Msx proteins are mainly transcriptional repressors (Alappat et al., 2003), while Dlx proteins are usually activators (Panganiban and Rubenstein, 2002). If this hypothesis was true, *Dlx5* expression should be augmented or expanded in $Msx1^{-/-}$ mice. However, we did not observe changes in *Dlx5* expression in the absence of *Msx1* and thus this possibility has been ruled out.

3.1. Dlx and Msx genes in craniofacial and palate development

A variety of molecules have been implicated in signaling during morphogenesis of facial primordia, including secreted molecules (Shh, Bmp, Wnt, Fgf) and transcription factors (*Dlx*, *Otx*, *Msx*, *Gli* and *Tbx*) (Wilkie and Morriss-Kay, 2001; Francis-West et al., 2003; Richman and Lee, 2003). Towards the understanding of how these molecules interact, one of the most informative approaches is the observations of skeletal phenotypes in mice with targeted mutations (Thyagarajan et al., 2003). Failure of the palatal shelves to grow, elevate or fuse on the midline is the basis of oro-facial cleft, or palatal cleft, the most frequent craniofacial malformation in babies (Ferguson, 1998). Cleft palate results from the disturbance of tightly controlled events that are regulated by a number of molecules (Houdayer and Bahuau, 1998; Marazita and Mooney, 2004; Johnston and Bronsky, 1995; Prescott et al., 2001; Murray and Schutte, 2004; Kaartinen et al., 1995; Matzuk et al., 1995; Proetzel et al., 1995; Peters et al., 1998; Miettinen et al., 1999; Szeto et al., 1999; Zhao et al., 1999). Mutation or disruption of the *MSX1/Msx1* genes causes in both human and mice oro-facial cleft and tooth agenesis (Satokata and Maas, 1994; Vastardis et al., 1996; Houzelstein et al., 1997; van den Boogaard et al., 2000). In the mouse palatal cleft is associated with a downregulation of *Bmp4* in the anterior palate (discussed below). Disruption of *Dlx* genes also causes palatal cleft (Acampora et al., 1999; Depew et al., 1999; Qiu et al., 1997), although they are not expressed in the palatal mesenchyme. Disruption of *Dlx5* leads to a less severe cleft, as compared to *Msx1*: the shelves are usually present and elevated, however, fail to grow properly and to fuse on the midline. Three mechanisms can be proposed: either *Dlx* genes instruct cells precursor of the palatal *anlage* early in development (i.e. neural crest or arch ectomesenchyme, where they are expressed), or they control expression of secreted diffusable molecules, or the cleft is the consequence of a generalized deformation of the cranium. With respect to this last possibility, recent data indicate that apoptosis of MEE cells, essential for fusion of the shelves, is sensitive to the distance between the shelves (Gurley et al., 2004). It is conceivable that palate closure requires a threshold of physical inter-shelf distance not to be exceeded, a distance that can vary as a consequence of dysmorphologies of the cranium. This could partly explain the $Dlx5^{-/-}$ palatal cleft, in fact its severity correlates with the general severity of other craniofacial defects (unpublished). However, the palatal shelves of the DKO appear more normal than those of either of the single mutants, in spite of the generalized distortion, and therefore, the palatal rescue is not directly related to other craniofacial abnormalities.

The craniofacial phenotypes of *Msx* and *Dlx* mutant mice could be interpreted according to two (non-mutually exclusive) models: one in which *Msx* and *Dlx* confer spatial identity to the ectomesenchymal cells in a cell-autonomous way, the second in which they mediate non-cell-autonomous instructive signals converging on the regulation of specific signaling centers. In this study, we provide evidence for a non-cell-autonomous cooperation between *Dlx5* and *Msx1* during palate growth, elevation and ossification: these genes are expressed in adjacent domains, with *Msx1* but not *Dlx5* present in the palate proper. During organ development, interactions between neighboring tissue layers are crucial for growth, morphogenesis and differentiation (Richman and Tickle, 1992; Thesleff et al., 1995). Palatogenesis as well critically requires interactions between the crest-derived mesenchyme and the overlying epithelium (Slavkin, 1984; Ferguson and Honig, 1984). Recently, a restricted region of the anterior palate is coming to attention as a signaling center. This region expresses a several morphogenetic molecules including *Shh*, *Bmp*, *Wnt*, *Msx1*, but not *Dlx5* or *Dlx6* (unpublished data). Zhang and coworkers (2002) have shown that cell proliferation is reduced in the anterior palatal mesenchyme of $Msx1^{-/-}$ mice, (see also mice Hu et al., 2001),

Fig. 5. Expression of *Follistatin*, *Noggin*, *Wnt5a* and *Wnt5b* in $Dlx5^{-/-}$, $Msx1^{-/-}$ and DKO embryos. (A–H) *Follistatin* expression in the anterior (A,B,C,D) and posterior (E,F,G,H) palate of WT (A,E), $Dlx5^{-/-}$ (B,F), $Msx1^{-/-}$ (C,G) and DKO (D,H) embryos at E13.5. Red arrows indicate the signal on the palatal epithelium. Control hybridizations from the same experiment (molar tooth buds) are shown for each genotype in the lower-right insets. (J–L) Expression of *Noggin* in WT (J) and $Dlx5^{-/-}$ (K) embryonic heads at E13.5, compared to expression of *Dlx5* in adjacent sections of the WT specimen (L). Red arrows indicate *Noggin* signal in the maxillary mesenchyme that overlaps with *Dlx5*. Control hybridizations from the same experiment (ganglionic eminence of the forebrain) are shown for both probes in the lower-right insets. Expression of *Wnt5a* (M,N) and *Wnt5b* (P,Q) in WT (M,P) and $Dlx5^{-/-}$ (N,Q) embryonic heads at E14.5. Expression of *Wnt5a* is observed in the palatal mesenchyme (black arrows in M) and is unchanged in the $Dlx5^{-/-}$ specimen (N). Expression of *Wnt5b* in observed in a mesenchymal domain, lateral to the palatal shelves (sketched in P); In the $Dlx5^{-/-}$ expression is reduced (red arrow in Q). The section and viewing planes are the same as in Fig. 4. Abbreviations as in previous figures.

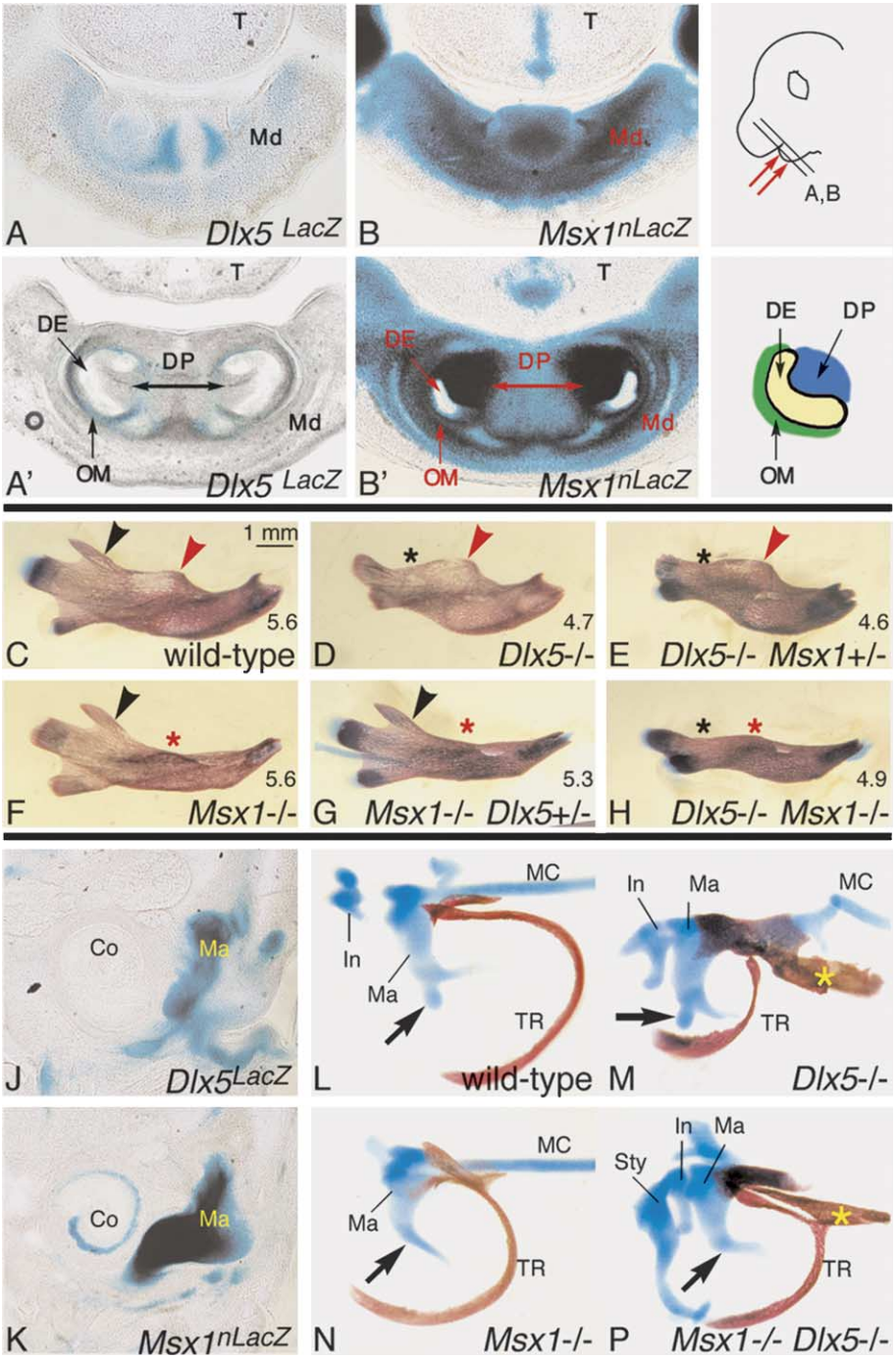

Fig. 6. Craniofacial defects in *Msx1-Dlx5* DKO mice. (Top) the mandible. (A,B') X-gal staining of frontal sections of $Dlx5^{lacZ}$ (A,A') and $Msx1^{nlacZ}$ (B,B') heterozygous embryos at E14.5. The section planes are clarified in the drawing on the right. Expression of *Dlx5* is found in the osteogenic mesenchyme (OM) around the incisor teeth (A'), but not in the dental papilla (DP) (double arrow in A'). Expression of *Msx1* is found in the anterior mesenchyme (B), in the OM of the jaw and in the dental papilla (DP) of the incisor teeth (double arrow in B'). Neither *Dlx5* nor *Msx1* are expressed in the dental epithelium (DE). The clarify the expression territories in A' and B', the position of OM, DE and DP in the developing incisor tooth is sketched on the right. (C–H) Dissected mandible at P0. The normal jaw is shown in C, the DKO is shown in H. Black arrowheads, coronoid process; red arrowheads, molar tooth; asterisks indicate missing structures. (Bottom) the middle ear. (J,K) Expression of $Dlx5^{lacZ}$ (J) and $Msx1^{nlacZ}$ (K) in E14.5 heterozygous embryos. (L–P) Skeletal structures of the middle ear at P0. Black arrows indicate the head of the malleus; yellow asterisks (M,P) indicate the ectopic bone. Note that in L–N the styloid process has been dissected out, and that due to distortions of the cranium, the samples M and P are slightly rotated to allow visualization of the Malleus. Abbreviations: Co, cochlea; DE, dental epithelium; DP, dental papilla; In, incus; Ma, malleus; MC, Meckel's cartilage; Md, mandible; OM, osteogenic mesenchyme; Sty, styloid process; T, tongue; TR, tympanic ring. Scale bar in $C = 1$ mm. Values reported in C–H (on the right) indicate the length of the jaw, in mm.

associated with a downregulation of *Bmp4*, *Bmp2* and *Shh*. Importantly, exogenous expression of *Bmp4* in the same region could restore normal cell proliferation, *Shh* and *Bmp2* expression, and palatal elevation. These results clearly indicate that *Msx1* controls a genetic hierarchy that entails transcription activation of *Bmp4* in a limited co-expression territory, and this in turn controls expression of *Shh* and *Bmp2*.

Table 1
Summary of the skeletal defect in *Dlx5-Msx1* DKO mice

Structure	*Dlx5–Msx1* DKO (1)	*Dlx5* mutant (1)	Expression of *Dlx5* (2)	*Msx1* mutant (1)	Expression of *Msx1* (2)
Mandible	Missing molars and coronoid process	Missing coronoid process	Yes	Missing molars	Yes
Middle ear	Malleus deformed presence of strut	Presence of strut	Yes	Malleus deformed	Yes
Calvaria	Severe delay (3)	Mild delay (3)	Late	Mild delay (3)	Early
Otic capsule	Deformation of the vestibular region	Deformation of the vestibular region	Yes	–	–
Nasal cavity	Deformed, asymmetric	Deformed, asymmetric	Yes	–	–
Lamina obturans	Foramina disorganized or missing	Foramina disorganized or missing	Yes	–	–
Palatal shelves	Formed, elevated, partl. ossified but reduced and unfused	Formed, elevated, partl. ossified but reduced and unfused	No	Severely reduced, not elevated, unfused	Yes
Limb morphology	Normal	Normal	Yes	Normal	Yes
Limb ossification	Delayed (3)	Slightly delayed (3)		Slightly delayed (3)	

(1) Skeletal defects (cartilages and bones) observed at P0. (2) The expression columns combine data from this paper (E13.5 and E14.5) and from published literature. (3) Retardation of mineralized bone deposition

In this work we establish that *Dlx5* inactivation partly rescues the *Msx1*-dependent defect via increased *Bmp7* and reduced *Follistatin* expression. This observation links *Msx1* and *Dlx5* in the regulation of a common signaling system critical for palate morphogenesis, as a result of cell non-autonomous interactions between adjacent cells and tissues; in fact *Bmp4* is expressed in the anterior signaling center, while *Bmp7* and *Follistatin* are expressed in a wider domain, along the antero-posterior length of the epithelium. In $Dlx5^{-/-}$ and DKO embryos *Bmp7* is increased throughout the palate, while *Follistatin* is decreased posteriorly. Furthermore, *Dlx5* and *Dlx6* are not expressed in the anterior palate, where the *Msx1-Bmp4* regulation takes place. Together, these observations suggest that the restoration of palate growth and elevation might result from a recruitment of the central-posterior palate tissue via enhancement of Bmp functions posteriorly.

3.2. Bmp signaling and palate morphogenesis

The expression pattern of *Bmp2* and *−4* is highly reminiscent of that found in other embryonic territories where fusion occurs, such as the frontonasal processes (Gong and Guo, 2003). Midline fusion of the palatal shelves, thus, is one more example of this important function of Bmp molecules. *Bmp4* is also likely to carry out a morphogenetic function in the palatal primordia prior to their fusion, since the palatal shelves of $Msx1^{-/-}$ mice never come in proximity (Zhang et al., 2002). Interestingly, in the corresponding region of the palate the Bmp-antagonist *Follistatin* (Balemans and VanHul, 2002) is only expressed in a restricted dorsal (nasal) domain but not in the signaling center (our data) and might serve the function to restrict the *Bmp2* and *-4* activity to the tip of the shelves.

The rescue of palatal formation in the DKO can be explained by the increased *Bmp7* expression compensating for the reduction of *Bmp4* (Fig. 7). Indeed, disruption of *Dlx5* leads to an up-regulation of *Bmp7* expression in the palate, also observed in the DKO palate. Bmp7 may serve the dual function of stimulating growth of the palatal mesenchymal cells, a function known for Bmp4 (Zhang et al., 2002), and that of inducing ossification at later stages (Franceschi et al., 2000). The possibility that Bmp7 may functionally substitute for other Bmps is supported by the analysis of skeletal phenotypes in compound *Bmp* mutant mice, showing redundancy in vivo (Zhao, 2002; Goumans and Mummery, 2000; Dudley et al., 1995; Luo et al., 1995; Jena et al., 1997; Solloway and Robertson, 1999); in particular *Bmp4;Bmp7*, but not *Bmp2;Bmp7* or *Bmp5;Bmp7*, double heterozygotes show skeletal abnormalities (Katagiri et al., 1998), an indication that Bmp4 and Bmp7 might synergistically regulate a common mechanism. At the receptor level, several type I and type II subunits bind

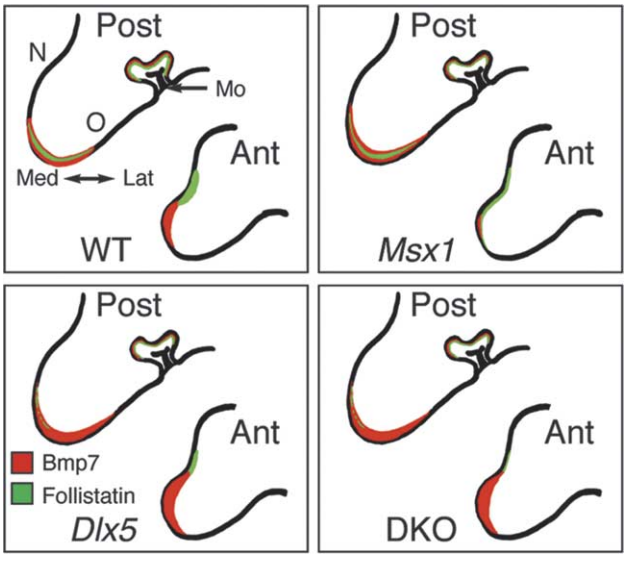

Fig. 7. Schematic drawing of the relative expression territories of *Bmp7* (red) and *Follistatin* (green) in the anterior and posterior palate of WT (upper left), $Msx1^{-/-}$ (upper right), $Dlx5^{-/-}$ (lower left) and DKO (lower right) embryos at E13.5. The medial-lateral (Med, Lat) and the Nasal-Oral (N, O) axes and directions are shown. In the posterior palate expression in the molar tooth (Mo) is also indicated. For a detailed description, see the Section 2.

Bmps and induce Smad phosphorylation (Hogan, 1996; Goumans and Mummery, 2000; Zhao, 2002). Biochemically it is unclear which ligand binds to which receptor complex, and receptor-ligand binding appears to be rather promiscuous. At the transduction level, Bmp4 binds to the Alk3/Alk6 receptors (BMP-R type IA and IB, respectively), while Bmp7 preferentially binds to Alk2 (Aoki et al., 2001); however all these receptors induce activation of Smad8 (Kawai et al., 2000). Thus, in selected biological systems Bmp molecules might be able to compensate each other's activity.

The rescue of palate morphogenesis in the DKO could also be favored by the reduced *Follistatin* expression observed in the *Dlx5*$^{-/-}$ palate (Fig. 7). Follistatin is a diffusible molecule, whose altered concentration may likely affect adjacent territories, including more anterior palatal domains. The effect should be that of a reduced Bmp antagonist activity in the medial and posterior palatal mesenchyme, where *Bmp7* is overexpressed. Functionally, these two effects go in the same direction, since they are expected to potentiate Bmp signaling. The changes in *Bmp7* and *Follistatin* expression in *Dlx5*$^{-/-}$ embryos occur at a location in which *Dlx5* is not expressed, indicating the cell non-autonomous nature of the palatal rescuing. In the *Msx1*$^{-/-}$ palatal shelves *Follistatin* expression is reduced anteriorly but is maintained posteriorly.

In summary, the effect of the *Dlx5* mutation on the Bmp system during palate formation consists in a non-cell-autonomous regulation on the expression of *Bmp7* and *Follistatin* with the result of potentiating the Bmp signal. This strengthens the notion that the tight regulation of expression and activity of this class of molecules is essential in the coordination of cellular events leading to growth, morphogenesis and fusion of embryonic structures. More generally, our findings are an illustration of the importance of non-cell-autonomous signaling between adjacent territories, a less well explored level of regulation during embryonic morphogenesis.

4. Experimental procedures

4.1. Targeted disruption of Dlx5 and Msx1

Mice with targeted disruption of *Dlx5* have been reported (Acampora et al., 1999). The targeted allele has the 1st and 2nd exons replaced by the *lacZ* reporter. The modified null allele, denominated *Dlx5*^{lacZ}, allows for detection of the *Dlx5*-expressing cells by staining for β-galactosidase (β-gal) expression. In all the territories examined β-gal expression recapitulates known *Dlx5* expression. Genotypes of the *Dlx5* mice were determined as described (Acampora et al., 1999). Mice with a targeted inactivation of *Msx1* have been reported (Houzelstein et al., 1997). In the targeted allele the *nlacZ* reporter gene was introduced in phase into the *Msx1*coding sequence. The modified allele denominated *Msx1*^{nlacZ}, allows for the detection of *Msx1*-expressing cells by staining for β-gal. Genotypes of the *Msx1* mice were determined by PCR using the following primers:

WT: primer A: 5′ CGCGCTGGAAAGGGCC
primer B: 5′ CTATTGCCGAGCGCGCG
mutant: primer C: 5′ TTCAGGCTGCGCAACTGTT
primer B (as above)

4.2. Phenotype analysis

Skeletal staining of newborn animals was carried out as previously described (Alizarin and Alcian Blue, Acampora et al., 1999). Whole-mount detection of β-gal was done on E12.5–E14.5 embryos as described (Merlo et al., 2002). For histological detection of β-gal, E13.5 and E14.5 embryos was fixed with 4% PAF 20' RT, followed by washes in PBS, sectioning by vibratome (80 μm) and staining as described (Perera et al., 2004).

In situ hybridization was carried out with DIG-labeled antisense probes for the following genes: *Dlx5*, a 750 bp fragment spanning the coding sequence; *Dlx6*, a 350 bp fragment spanning exons 3 and 4 (Perera et al., 2004); *Msx1*, a 550 bp 3′ spanning the homeodomain; *Msx2*, containing exon 1; *Noggin*, obtained from R. Harland (Berkeley, Univ., CA); *Nhordin*, from Dr J. Belo (Inst Gulbenkian de Cienca, Oeiras, Portugal) and *Bmp4*, from Dr E. Bober (Martin-Luther Univ., Halle Germany). Probes for murine *Bmp7* and *Follistatin* were obtained by RT-PCR from mouse embryo cDNA and corresponded to, respectively, a 511 bp fragment of the 3′UTR and a 534 bp fragment of coding plus 3′UTR sequences. With each probe, at least two normal and three mutant specimens were examined. For the palate, frontal 100 μm vibratome sections or 12 μm cryostatic sections from E13.5–E14.5 embryos were obtained serially. Hybridization on vibratome floating slices or on cryostatic sections was performed as described (Perera et al., 2004; Zhadanov et al., 1995), signal was revealed with BM-Purple (Roche).

4.3. RNA quantification by real-time PCR

E14.5 palatal shelves (6 WT and six mutants, two independent experiments) were accurately dissected, pooled in TriPure Reagent (Roche) and extracted as indicated by the manufacturer. As *Bmp7* is not expressed in nearby tissues, the only source is the medial palatal mesenchyme. RNA quality, primer efficiency and correct size were tested by RT-PCR and agarose gel electrophoresis. RealTime was performed with LightCycler (Roche) using FastStart DNA MasterPLUS SYBR-Green I (Roche). Five microlitres of cDNA were used in each reactions, standard curve were performed using WT cDNA with four calibration points: 1:10; 1:40; 1:160; 1:640. All samples were in duplicates. Specificity and absence of primer dimers was controlled by denaturation curves. GAPDH was used for normalization, calculated using LightCycler Software 3.5.3. Primer sequences are:

GAPDH S 5′ TGTCAGCAATGCATCCTGCA 3′
GAPDH AS 5′ TGTATGCAGGGATGATGTTC 3′
Bmp7 F 5′ GCGATTTGACAACGAGACCT 3′
Bmp7 R 5′ AGGGTCTCCACAGAGAGCTG 3′

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mod.2005.10.007

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