BMP-Mediated Functional Cooperation between Dlx5;Dlx6 and Msx1;Msx2 during Mammalian Limb Development

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Abstract

The Dlx and Msx homeodomain transcription factors play important roles in the control of limb development. The combined disruption of Msx1 and Msx2, as well as that of Dlx5 and Dlx6, lead to limb patterning defects with anomalies in digit number and shape. Msx1;Msx2 double mutants are characterized by the loss of derivatives of the anterior limb mesoderm which is not observed in either of the simple mutants. Dlx5;Dlx6 double mutants exhibit hindlimb ectodactyly. While the morphogenetic action of Msx genes seems to involve the BMP molecules, the mode of action of Dlx genes still remains elusive. Here, examining the limb phenotypes of combined Dlx and Msx mutants we reveal a new Dlx/Msx regulatory loop directly involving BMPs. In Msx1;Dlx5;Dlx6 triple mutant mice (TKO), beside the expected ectodactyly, we also observe the hallmark morphological anomalies of Msx1;Msx2 double mutants suggesting an epistatic role of Dlx5 and Dlx6 over Msx2. In Msx2;Dlx5;Dlx6 TKO mice we only observe an aggravation of the ectodactyly defect without changes in the number of the individual components of the limb. Using a combination of qPCR, ChiP and bioinformatic analyses, we identify two Dlx/Msx regulatory pathways: 1) in the anterior limb mesoderm a non-cell autonomous Msx-Dlx regulatory loop involves BMP molecules through the AER and 2) in AER cells and, at later stages, in the limb mesoderm the regulation of Msx2 by Dlx5 and Dlx6 occurs also cell autonomously. These data bring new elements to decipher the complex AER-mesoderm dialogue that takes place during limb development and provide clues to understanding the etiology of congenital limb malformations.

Introduction

The developing vertebrate limb is widely adopted as a model to study cell-cell signaling, pattern formation and morphogenesis, and has provided a wealth of knowledge of the function and regulation of specific transcription factors and signaling molecules [1,2,3]. The phenotype of a large set of mutant mice with limb defects led to the identification of genes and signaling pathways essential for normal limb development. The spatio-temporal organization of the complex network of signaling and transcriptional regulations has been elucidated only in part. In brief, genes and regulatory modules can be related to the activation/maintenance/regression of three signaling systems: a) Sonic hedgehog (SHH) and the Zone of Polarizing Activity (ZPA), for the control of digit patterning along the antero-posterior axis [4,5], b) Fibroblast Growth Factors (FGFs) and the Apical Ectodermal Ridge (AER), for the control of proximo-distal growth and for ZPA maintenance [7,8,9,10,11], and c) Lmx1B, in the mesoderm, and Wnt7a and En-1, in the ectoderm, for dorso-ventral specification [12,13,14,15,16]. The three signaling systems are organized in precise time- and space-restricted manners, and are integrated in self-regulatory modules that assure the acquisition or the correct digit complements, limb morphogenesis and overall growth [4,17,18,19]. The signaling molecules of the Bone Morphogenetic Protein (BMP) class have been proposed to link
these three systems together [20,21,22,23,24,25], thus they are regarded as key players in the coordination of limb patterning, morphogenesis and growth along the three axes.

Proximo-distal limb development and digit extension is directly controlled by the signaling activity of the AER via expression of morphogens of the FGF family [7,8,10,26] and their regulation by other signaling molecules such as SHH and BMP antagonists [20,21,27,28]. In this scenario, the Dlx and Msx homeobox transcription factors, expressed in the AER and the mesoderm of the limb buds, play an important morphogenetic role, although their functions and regulations are yet to be defined at the molecular level. Dlx5;Dlx6 double knock-out (DKO) mice are characterized by loss of the central digit(s) and/or fusion with the lateral ones [29,30]; these mice constitute a model of the human congenital defect ectrodactyly or Split Hand Foot Malformation type I, a condition linked to genomic alterations encompassing the Dlx5/Dlx6 region, and for which point mutations in the DNA-binding domain of Dlx5 have recently been found [31]. The double inactivation of Msx1 and Msx2 genes results in moderate-penetrance polydactyly of the forelimbs (FL) and oligodactyly of the hindlimbs (HL) (loss of the anterior digits and part of the tibia) in the zygopod [32,33,34].

Studies on the cellular and molecular functions of Dlx and Msx proteins during limb development have met with difficulties owing to several reasons. First, none of the single knockout for Dlx5, Dlx6, Msx1 or Msx2 shows evident limb defects [29,32,35,36,37,38,39,40,41], suggesting some degree of functional redundancy. Second, members of the Dlx and Msx families are known to control cell proliferation and differentiation of osteoblasts [35,56], and the AER and ectoderm [29,30,57,58]. Dlx5;Dlx6 or Msx1;Msx2 double knock-out (DKO) mice are co-expressed in the AER and, later on, in the limb mesoderm, and 2) a non-cell autonomous regulatory loop between the AER and the anterior limb mesoderm.

Materials and Methods

Mouse Strains and Breeding

All animal procedures were reviewed and approved by the Ethical Committee of the University of Torino and University of Genova, the Italian Ministry of Health and the French Ministère de l’Enseignement supérieur et de la Recherche. No surgery or other manipulation on adult animals was used in this study. All efforts were made to minimize suffering. Generation and genotyping of the Dlx5<sup>−/−</sup>; Msx2<sup>−/−</sup> (hereafter named Dlx5<sup>−/−</sup>/Msx2<sup>−/−</sup>; Dlx5<sup>−/−</sup>;Msx2<sup>+/−</sup>), Msx1<sup>−/−</sup>; Dlx5<sup>−/−</sup>;Msx2<sup>−/−</sup> (hereafter named Dlx5<sup>−/−</sup>/Msx2<sup>−/−</sup>; Msx1<sup>−/−</sup>/Msx2<sup>−/−</sup>), have been previously reported [29,32,35,39]. These mutations were maintained on a C57Bl6/Dba F1 mixed genetic background, throughout. TKO embryos and newborn were obtained by crossbreeding either the Msx1<sup>−/−</sup>/Msx2<sup>−/−</sup> or the Msx2<sup>−/−</sup>/Msx2<sup>−/−</sup> single heterozygous parents with the Dlx5<sup>−/−</sup>/Msx2<sup>−/−</sup> double heterozygous ones, and then crossing the triple heterozygotes. Following mating, the day of the vaginal plug was considered as embryonic age 0.5 (E0.5).

Skeletal Preparation and β-galactosidase Detection

Cartilage staining (with Alcian Blue) of E14.5 embryos as well as bone and cartilage staining (with Alcian Blue and Alizarine Red) of E18.5 embryos were carried out as previously described [35]. For β-galactosidase expression analysis E10.5 embryos were fixed for 15–30 min in 2% paraformaldehyde in PBS, while E14.5 embryos were fixed for 15–30 min in 4% PFA. X-gal staining was performed as described [35]. For detection of β-gal on limb sections, E10.5 and E11.5 FLs and HLs were fixed with 4% paraformaldehyde for 8–12 hrs at 4°C, washed in PBS, cryoprotected with 30% sucrose, frozen at −70°C, sectioned (thickness 11 µm) and stained as described [35].

Whole-mount RNA in situ Hybridization

For RNA:RNA whole-mount in situ hybridization (WMISH), embryos at the desired age were dissected in cold RNase-free PBS, fixed in cold 4% PFA for 12–16 hrs, rinsed with PBS, permeabilized by treatment with proteinase-K, prehybridized and hybridized as previously published [60]. Digoxigenin (DIG)-UTP (Roche)-labeled antisense RNA probes were used, synthesized by in vitro transcription with conventional methods. WMISH was carried out following described procedures [61], the signal was detected using an alkaline phosphatase-conjugated anti-DIG antibody and developed with the chromogenic mix NBT-BCIP (Roche). With each probe, at least two normal and two mutant specimens were examined. The Dlx5 probe comprised 780 bp and was linearized with EcoRI and transcribed with T7 RNA polymerase [57]. The Dlx6 probe is a 350 bp fragment spanning exons 3–4 [53]. The Msx1 probe was a 550 bp 3’ spanning the homeodomain, containing exon 1; the Msx2 probe corresponded to 378 bp in the first exon of Msx2 cDNA, the Foxp probe corresponded to most of the mouse coding sequence, the Bmp4 probe (a kind gift from B. Hogan) contains the 3’ UTR and most of the coding sequence from a mouse cDNA [65], the probe for Gremlin corresponded to the entire murine coding sequence (a kind gift from R. Zeller). After hybridization, the signal was revealed with the NBT-BCIP chromogenic reaction.
RNA Quantification by Real-time PCR

Embryonic FLs and HLs at the age E11 were dissected in cold RNase-free PBS, the anterior and posterior halves were separated and pooled in RNA-later (Ambion). Pools of two half limbs from the same embryos were used to extract total RNA, using the Tissue Lyser II reagent followed by elution through RNA microkit plus (Qiagen). cDNA synthesis was done using standard conditions, 3 ng of each cDNA sample were used to carry out qPCRs on a CFX96 equipment (BioRad) using the SybrSafe system (Invitrogen). Samples were analyzed in technical triplicates, and for each genotype (except for the Msx1+/−/Dlx5+/−/Dlx6−/−) biological triplicates could be analyzed. Primer sequences were designed with the Primer Express online tool. RNA quality, primer efficiency and correct size were tested by RT-PCR and agarose gel electrophoresis. Standard curve were performed using WT cDNA of the p63 promoter [57]. Eight but have been shown to respond to Dlx expression with activation of the p63 promoter [62,63].

Chromatin Immunoprecipitation

Genome-wide Identification of Dlx Binding Sites and Target Genes

We used the Position-Weight matrix (PWM) provided by JASPAR under accession PH0024.1. The score of a site was computed with standard log-likelihood ratios, using as null model the nucleotide frequencies computed over the whole intergenic fraction of the mouse genome. We considered for further analysis putative sites scoring 50% of the maximum possible score or better. We selected among the sites identified above the ones that are conserved in at least two of 8 vertebrate species (genome sequence version in parenthesis): mouse (mm9), human (hg19), cow (bosTaurus), opossum (monDom5), platypus (ornAna1), chicken (galGal3), frog (xenTro2), zebrafish (danRer6) and lamprey (petMar1). A site is defined as conserved with species S if it lies in a region of the mouse genome which is aligned with a region of the S genome and the aligned sequence in S is a site according to the same definition used for mouse sites. All genomic sequences and pre-computed “Net” alignments were obtained from UCSC.

A ranked list of putative Dlx target genes was obtained from the sites determined above by associating each site conserved in at least one species to its closest Refseq mRNA, and then selecting the sites located either within 10 kb upstream of the TSS, or within the non-coding portion of the first exon, or in the first intron. We then associated to each putative target a score equal to the sum of the conservation scores (number of species) of its associated sites.

Chromatin Immunoprecipitation

A DLX5-myc expression vectors (OriGene, USA) containing the full-length human DLX5 cDNA with an in-frame insertion of the myc-TAG at the C-terminus, was used as described [66]. The Q178P point mutation [31] was inserted in the DLX5-myc expression vector indicated above, by site directed mutagenesis and sequence verified (Bio-Fab Research, Rome).

The U2Os human osteosarcoma cells were used; these cells express low or undetectable levels of DLX5 mRNA endogenously, but have been shown to respond to Dlx expression with activation of the p63 promoter [37]. Eight μg of the DLX5-myc expression vectors were used for transfections, which yielded an efficiency of 35% (number of myc-positive cells over total counted nuclei).

Results

Msx and Dlx Coexpression Analyses, in silico

Previous evidences indicate that Dlx5 binds to homeodomain-responsive elements in a proximal region of the Msx2 promoter, and thereby regulates its transcription [62,63]. To further support this possibility, we have used a human-mouse co-expression network, generated using published profiling datasets [67,68] and found that DLX5 and MSX genes are connected, i.e. each ranks in the first 1% of the co-expression lists of the other (p<0.01, data not shown).

Next, we screened conserved regions of the vertebrate genome for the presence of consensus Dlx DNA-binding sites, as defined by the Dlx5 PWM [69] present in the JASPAR database (accession N° PH0024.1) [70] and reported in Table S3. As the PWM for Dlx5 reported in Jaspar is not highly informative, it is not surprising that a total 565,995 putative binding sites were initially identified in the mouse genome. However, by introducing evolutionary conservation with at least two (out of 8) species examined as a further criterion, this number is reduced to 11,262 sites, and with conservation in three species is further reduced to 4085 (Table S3, complete lists available upon request). The full annotation on the UCSC genome browser is available upon request. As positive controls, the well defined Dlx sites present in the Dlx5/Dlx6 intergenic region [71] and in the Msx2 promoter [62,63] were correctly predicted (Fig. S1). The evolutionary conservation of the sites suggests the presence of positive selection pressure to maintain the sites, confirming their functional relevance.

We then generated a ranked list of putative Dlx targets, based on the position of predicted conserved Dlx sites in the genome, as indicated in the Methods sections. The ranked list contains 3,051 Refseq mRNAs associated to 2,412 unique Entrez gene IDs (available upon request). The Msx1 and Msx2 genes were found in the top 10% of the list of putative Dlx targets, strengthening the possibility that Dlx proteins might directly regulate Msx expression.

Expression of Msx and Dlx Genes during Limb Development

We examined expression of Dlx5, Dlx6, Msx1 and Msx2 in the FL and HL of normal embryos, at E10.5 and E11.5, by X-gal staining of heterozygous embryos carrying an allele with inserted lacZ reporter (Dlx5, Msx1, Msx2), and by WISH (Dlx6) (Fig. 1). WISH for Dlx5, Msx1 and Msx2 have been previously reported with comparable results. In the AER, all four genes are co-expressed starting at E9.5/E10.0. On the contrary, in the limb mesoderm they are expressed with a different time-of-onset. At E10.5 Msx1 and Msx2 are mainly expressed in two mesoderm territories (anterior and posterior) of both the FL and the HL (Fig. 1E–H, S–V), whereas at the same stage the Dlx5 transcript is detected only in the anterior mesoderm of the FL, and not that of the HL (compare Fig. 1A–B with O–P). The Dlx6 transcript is not detected in the mesoderm at this stage (Fig. 1C–D, Q–R). In the HLs, mesodermal expression of Dlx5 starts around E11.5 and is confined to the anterior margin (Fig. 1W–X). At this stage, Msx1 and Msx2 are expressed in the AER and in a larger region...
underneath the AER (Fig. 1K–N, Y-AB). Histological sections of X-gal stained limb buds from Dlx5+/–, Msx1+/– and Msx2+/– embryos (Fig. 1W, W’, Y and AA) reveal that Msx1 and Dlx5 are truly co-expressed in the AER (strong signal in W’ and W”) and in the anterior HL mesoderm (a weak signal is present in W”, indicated with black arrows).

In summary, Msx expression precedes that of Dlx in the HL anterior mesoderm, consequently in this location Dlx genes are unlikely to regulate Msx gene transcription cell-autonomously and Dlx and Msx proteins are unlikely to interact in the anterior limb mesoderm, at early stages.

Msx2 Expression is Downregulated in Dlx5;Dlx6 DKO Limbs

To investigate interactions between the Dlx and the Msx genes during limb development, we used animals DKO for Dlx5;Dlx6 and analyzed the expression of the Msx-lacZ reporter in this genetic background. To do this, we compared the expression patterns of the lacZ reporter between Msx1−/− and Msx1+/+;Dlx5−/−;Dlx6−/− mice (Fig. 2A–H) and between Msx2+/− and Msx2−/−;Dlx5−/−;Dlx6−/− mice (Fig. 2L–P). We observed a clear reduction of Msx2 expression in the anterior mesoderm and AER exclusively in the HLs, whereas neither expression of Msx1 in the HLs nor expression of Msx1 and Msx2 in the FLs were significantly changed (Fig. 2A–P). To further document the reduction of Msx2 expression in the AER of Dlx5;Dlx6 DKO limbs, we carried out WMISH for Msx2 on cryostatic sections of Dlx5;Dlx6 DKO HLs, at E11. Msx2 expression was strongly reduced or absent in the AER and the underlying mesoderm of a central wedge of the mutant limbs, as compared to the same region of normal limbs (Fig. 2Q,R).

Since WMISH is not a quantitative method, we used quantitative Real Time PCR (qRT-PCR) to quantify the reduction of Msx2 mRNA on samples extracted from the HLs of normal and Dlx5;Dlx6 DKO embryos, at E11.5. Limb buds were divided in two halves on the proximo-distal axis, to determine gene expression in the anterior and posterior mesoderm separately. In such samples, the AER cells contributed minimally, while most of the RNA derives from the mesenchyme. Msx2 mRNA abundance was reduced by 60% in the anterior half, and by 50% in the posterior half of Dlx5;Dlx6 DKO HLs from the same embryo, as compared to WT. In the same samples, expression of Msx1 was minimally or not changed (Fig. 2S). We then determined the abundance of Msx and Dlx mRNAs in the anterior half of the HLs of Msx1−/− and Msx2−/− (single homozygous) mutant embryos, as compared to WT. In the Msx2−/− mutant the expression level of Dlx5, Dlx6 and Msx1 was unchanged, whereas in the Msx1−/− mutant the expression levels of Dlx5, Dlx6 and Msx2 were reduced by 65%, 40% and 15%, respectively. Moreover, in the posterior half of the HLs from the Msx1−/− mutant we observed a reduction of 40% in the abundance of Dlx5 and Msx2 mRNA, whereas Dlx6 did not change (Fig. 2T,U).

Limb Phenotype of Msx2;Dlx5;Dlx6 Triple Mutants

To reveal possible functional interactions between the Dlx and the Msx gene products during limb development, we generated TKO mice with genotype Msx1−/−;Dlx5−/−;Dlx6−/− or Msx2−/−;Dlx5−/−;Dlx6−/−. TKO newborns were obtained at a frequency lower than expected, and all died shortly after birth, due to severe craniofacial malformations and consequent breathing impairment. Quadruple knock-out embryos were never obtained in spite of several attempts.

We examined the limb skeleton in the TKO animals at two ages: E14.5 (with Alcian-Blue) and E18.5/birth (with Alcian-Blue and Alizarine-Red, which stain, respectively, cartilages and mineralized bones). In Msx2;Dlx5;Dlx6 TKO animals, the HLs were severely affected (Fig. 3G,H) whereas no evident alteration was observed in the FLs (data not shown). The central digit was always missing, rarely (<10%) the two central digits were missing, while the remaining digits extended pairwise towards the opposite (anterior-posterior) sides. Importantly, no loss of anterior digits or of zeugopod elements was ever observed (Fig. 3G,H). The limb phenotype observed in the Msx2;Dlx5;Dlx6 TKO mutant animals is consistent with a significant aggravation of the ectrodactyly phenotype seen in Dlx5;Dlx6 DKO [29,30] (compare Fig. 3G with 3C, and 3H with 3D), without appearance of other recognizable phenotypes. Remarkably a very similar phenotype, although less severe, was observed in animals with genotype Msx2−/−;Dlx5−/−;Dlx6−/−, i.e. in the presence of a single wild-type Msx2 allele (Fig. 3E,F).

Limb Phenotype of Msx1;Dlx5;Dlx6 Triple Mutants

In Msx1;Dlx5;Dlx6 TKO mice the FL were usually normal, although in one case (1/6) anterior polydactyly was observed (data not shown). Of note, polydactyly has been reported for the FLs in Msx1;Msx2 DKO mice with a moderate penetrance [32]. On the contrary, the TKO HLs displayed a severe phenotype consisting in the loss of the tibia and of 3–4 preaxial digits (Fig. 3I,J). Since the HLs of Msx1;Msx2 DKO usually exhibit loss of 1–2 anterior digits and of the tibia [32], we interpreted the TKO phenotype as a combination of ectrodactyly, caused by the loss of Dlx5;Dlx6 [29,30] (Fig 3C,D), and the preaxial adactyly as observed upon the loss of Msx1;Msx2 [32] (Fig. 3K).

The appearance of features of the Msx1;Msx2 DKO phenotype in the Msx1;Dlx5;Dlx6 TKO animals, which retain two functional Msx2 alleles, argues in favor of a severe reduction of Msx2 expression in the TKO limbs, as compared to the Dlx5;Dlx6 DKO.

Indeed, removing a single Msx1 allele in the context of Dlx5;Dlx6 DKO background (i.e. Msx1+/−;Dlx5−/−;Dlx6−/− embryos) results in a 50% reduction of Msx2 mRNA in both the anterior and the posterior half of the embryonic HLs, as compared to WT. Moreover, expression of Msx2 in the anterior and posterior halves of Msx1 homozygous mutant HLs was reduced by 30% compared to Msx1 heterozygous HLs (data not shown). This indicates that reduced Msx2 expression may result from the combination of (1) loss of Dlx5 and Dlx6 (Fig. 2U, Fig. S2), upstream regulators of Msx2, and (2) the loss of Msx1, with the consequent decrease of Msx2 expression (Fig. 2V).

Expression of Bmp4 and Gremlin in Dlx5;Dlx6 Mutant Limbs

The phenotype of the Msx1;Dlx5;Dlx6 TKO HLs shares some similarities with the Msx1;Msx2 DKO phenotype [32]. However, defects in the anterior autopod and zeugopod cannot be simply explained by a direct Dlx5;Dlx6 control on Msx2 expression, since in the presumptive territory of the anterior zeugopod and autopod the expression of Msx1 and Msx2 precedes that of Dlx5 and Dlx6 (Fig. 1). Therefore we hypothesized that a non-cell autonomous regulation should take place, linking a defective AER with Msx2 misexpression in the anterior limb mesoderm. We focused on Bmps as candidate signaling molecules since they are expressed in the AER as well as the anterior and posterior mesoderm [21], and since BMP signaling is known to induce Msx expression in several embryonic territories [20,72].

We carried out WMISH for Bmp4 on Dlx5;Dlx6 DKO embryos at E11 and found that expression is decreased specifically in the central sector of the AER of the mutant HLs (3 of 4 embryos) but not significantly in the anterior mesoderm (Fig. 4C,D). Bmp4
Figure 1. Spatio-temporal expression of Dlx-Msx in developing limbs. A-N, forelimbs. O-AB, hindlimbs, at E10.5 (A-H, O-V) and E11.5 (I-N, W-AB). Whole-mount X-gal staining on FLs and HLs from Dlx5+/−, Msx1+/− and Msx2+/− (lacZ+) heterozygous embryos are shown. Expression of Dlx6 was detected by WMISH on embryonic limbs at the same ages and shown. At E10.5 Msx2 and Msx1 are expressed in the AER and the anterior and posterior mesoderm of HLs and FLs. Dlx5 and Dlx6, at E10.5, are expressed in the AER of HLs and FLs and in the anterior limb mesoderm only of the FLs, but not of the HLs. At later stages (E11.5), Dlx5 and Dlx6 are then expressed in the anterior mesoderm of HLs. Black arrows indicate mesodermal expression. The AER is also indicated. Black asterisks indicate absence of expression. W′,W″ histologic transversal sections of E11.5 HLs from Dlx5−/−
embryos, stained with Xgal. Y’, AA’ histologic transversal sections of HLs from Msx1’+/− (Y’) and Msx2’−/− (AA’) embryos, to compare AER and mesodermal expression between these genes. Section planes and position are reported with red lines (in W, Y and AA). The extent of the Msx1-positive anterior and posterior mesoderm regions, based on the micrographs in AA’ and AC’, are indicated with dashed lines. A strong Dlx5-lacZ signal, overlapping with the Msx1-lacZ and the Msx2-lacZ signal, is detected in the anterior mesoderm (W’, indicated by black arrows).

expression was not significantly changed in the FLs of the same embryos (Fig. 4A,B), as expected considering the lack of any morphological defects in the FLs. As a further control, Bmp4 expression was retained in the pharyngeal arches region (Fig. 4E,F). We also examined the expression of Gremlin in the limbs of E10.5 embryos. Gremlin is a BMP antagonist that participates in a regulatory loop between Shh in the posterior limb mesoderm and FGF4 in the posterior AER, which maintains the AER and ZPA and promotes digit patterning and limb outgrowth [17,73,74]. The anterior-posterior patterning and expression program of Gremlin did not significantly change in either the FLs or the HLs of Dlx5;Dlx6 DKO embryos (Fig. 4G–I). Thus, we concluded that the inactivation of Dlx5 and Dlx6 does not lead to significant alterations of limb antero-posterior patterning. Finally, the expression of Bmp7 was not significantly changed (data not shown).

**Bmp2 and Bmp4 Expression Levels are Synergistically Reduced by Msx and Dlx Mutations**

The WMISH results reported above suggest that Bmp4 expression is unchanged in the anterior mesoderm of the limbs; however this technique is poorly quantitative and cannot reveal minor changes in gene expression level. To further investigate the mechanism that might induce and/or sustain Msx2 expression in the anterior half of the HLs, we determined the relative abundance of Bmp2 and Bmp4 mRNAs by qRT-PCR in the anterior and posterior halves of the embryonic HLs (E11) with different genotypes. Expression of Bmp2 is mainly decreased in the anterior (about 50%), and minimally in the posterior half (20%) of the HLs from Dlx5;Dlx6 DKO embryos (Fig. 4O). Bmp2 expression is similarly reduced in Msx1’−/− HLs. Bmp4 expression is minimally or not affected in the Msx1−/− mutants, while in the Dlx5;Dlx6 DKO Bmp4 expression is reduced of about 30% and 20%, respectively, in the anterior and in the posterior halves (Fig. 4O). Of note, Bmp4 expression is also reduced in Msx2−/− embryonic HLs, less severely in the anterior (30%) than the posterior half (50%) (data not shown). Strikingly we observed a strong synergy between Msx1 and Dlx5;Dlx6 in controlling BMP expression. When combined with the Dlx5;Dlx6 DKO mutation, the loss of a single Msx1 allele was sufficient to reduce Bmp2 and Bmp4 expression to levels lower than 10% of that of control samples, both in the anterior and in the posterior halves of the HLs. Considering that Msx2 is a known target of Bmp2 and Bmp4 in several tissues [72,75,76], this may explain how Msx2 expression is reduced in the Msx1;Dlx5;Dlx6 TKO, and how this mutant shows a limb phenotype with similarities to the Msx1;Msx2 DKO.

Finally, we determined the abundance of Fgf8 and Shh mRNAs in HL samples from the relevant genotypes (Fig. S3). The Abundance of Fgf8 mRNA was slightly reduced in the absence of Dlx5 and Dlx6 (DKO samples), and was further drastically reduced when one Msx1 allele was eliminated in the absence of Dlx5;Dlx6. Similarly, the abundance Shh mRNA (in the posterior half of the limb buds) was slightly reduced in the absence of Dlx5 and Dlx6, and was further reduced when one Msx1 allele was simultaneously eliminated. While reduced Fgf8 expression is not surprising [30], reduced Shh expression is novel and may contribute to the altered digit number and morphogenesis seen in the Msx1;Dlx5;Dlx6 TKO embryos.

**Dlx5 Binds to Conserved Sequences in the Proximity of the Bmp2 and Bmp4 Loci**

To search for possible direct regulations of the Bmp2 and Bmp4 loci by Dlx5, we first explored the genome-wide predictions of conserved Dlxs sites, described above. In the proximity of the Bmp2 locus we identified three Dlx consensus binding sequences, named B2-RE1, located 30 kb upstream of the transcription start site (TSS), B2-RE2 and B2-RE3, located respectively 3.5 kb and 18 kb downstream of the end of the Bmp2 transcript. All these Dlx binding elements are conserved, although not identical, in at least two vertebrate species, and fall within stretches of conserved genomic sequence (Fig. 5A,B). The B2–R3 element was recognized as conserved only between human and mouse, and contains 6 (of 16 bases) mismatches, in non-critical positions. In the proximity of the Bmp4 locus we identified two Dlx consensus sequences, named B4-RE1 and B4-RE2, located respectively 430 bp upstream of the TSS and within the second intron, conserved in at least two mammalian species (Fig. 5A,B). Also these predicted Dlx binding sites fall within stretches of conserved genomic sequence (sequences and genomic locations provided in Tables S4 and S5).

To demonstrate that the Dlx5 protein physically binds to these putative responsive elements, we transfected a human DLX5-myc-tag expression vector [66] and the same one modified to harbor the disease-causing point mutation Q178P [31] in the U2Os osteoblast cells (Fig. 5C). These cells were chosen since they are of human origin, they express low endogenous level of DLX5, have been shown to respond to Dlx5 expression by activation of the p63 and other promoters [57], are of osteoblastic origin (Dlx5 plays a role in late osteoblast differentiation [35]), and are easily transfected. The crosslinked chromatin was immunoprecipitated with an anti-myc antibody, or with anti-acetylated Histone 4 (H4Ac) and subjected to PCR amplification with primers flanking the predicted DLX sites in the human genome. With the exception of the B2-RE3 element (not shown), all the other elements showed an enrichment of PCR amplification products from chromatin of DLX5-myc transfected cells, as compared to the chromatin from cells transfected with the empty vector (Fig. 5D). The same elements immunoprecipitated with anti-H4Ac, suggesting that in these locations the chromatin is available for transcription regulation. Interestingly, the Q178P mutant DLX5 protein did not show any binding to these sequences, thus this disease-causing mutation is associated with loss of DNA-binding activity on the Bmp elements. As further controls, PCR amplification of two sequences containing no identifiable homeodomain-binding sequences (BMP2 exon 3 and BMP4 exon 5), as well as an irrelevant sequence (IL10) did not show any enrichment (not shown). These results reinforce the notion that DLX proteins physically interact with four (out of five) conserved elements close to the Bmp2 and the Bmp4 loci, and might exert a direct transcription regulatory activity, relevant for normal limb development.

**Discussion**

Dlx5, Dlx6, Msx1 and Msx2 genes encode homeodomain transcription factors involved in limb development. The targeted disruption of each of these genes, individually, does not lead to
Figure 2. Reduction of Msx2 expression in Dlx5/Dlx6 DKO HLs. A-P. Whole-mount X-gal staining to detect Msx1 and Msx2 expression in Dlx5/Dlx6 DKO. In the FL (A-D,L) no changes of expression is observed whereas in the HL (E-H, M-P), Msx2 expression is reduced in the AER and in the anterior limb mesoderm of the Dlx5;Dlx6 DKO HLs. Q,R. Sections of WT (Q) and Dlx5;Dlx6 mutant HLs (R) hybridized in situ to detect Msx2.
limb defects, however double $\text{Msx1/Msx2}$ [33,34] and $\text{Dlx5/Dlx6}$ [29,30] mutant mice present severe limb malformations indicating that these genes participate in the control of digit number and morphogenesis. The Dlx and Msx homeodomains show a high degree of sequence similarity and similar DNA binding sequences in vitro [43]. In spite of these similarities, there is no evidence suggesting that these homeoproteins cooperate or interact, except in two specific developmental processes: elevation and fusion of the palatal shelves [60,61] and development of the frontal bone [59]. In this work, we shed light on direct and indirect interactions between these two classes of homeodomain genes during limb development.

### Direct and Indirect Dlx-Msx Regulations during Limb Bud Development

Although $\text{Msx2;Dlx5;Dlx6 TKO}$ mice show an aggravation of the ectrodactyly phenotype, as compared to $\text{Dlx5;Dlx6 DKO}$, they do not present any obvious additional defect. Furthermore, inactivation of either $\text{Dlx5}$; $\text{Dlx6}$ or $\text{Msx2}$ does not modify the level of $\text{Msx1}$ expression, suggesting that the $\text{Msx2;Dlx5;Dlx6 TKO}$ showing a drastically reduced $\text{Msx2}$ signal in the AER and in the underlying mesoderm, but not in a proximal mesoderm territory. S-U. Quantification of the expression of Dlx5, Dlx6, Msx1 and Msx2 mRNAs by qRT-PCR in HLs from Dlx5;Dlx6 DKO (S), Msx2$^{-/-}$ (T) and Msx1$^{-/-}$ (U), relative to WT. The results show a reduction of 45% of $\text{Msx2}$ expression in the Dlx5;Dlx6 DKO HLs compared to WT, but not of $\text{Msx1}$. $\text{Dlx5}$ and $\text{Dlx6}$ expression is downregulated in Msx1 KO HLs but not in Msx2 KO HLs. Expression of the knocked-out genes was also tested, as control, and always found to be reduced to undetectable levels (not shown).

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**Figure 3.** Skeletal preparations of the HLs of single and combined $\text{Msx/Dlx}$ mutant animals. Chondroskeletal preparation of the HLs of E14.5 embryos (micrographs on the left) and full skeletal preparation on newborn animals (micrographs on the right), representing single and combined Dlx;Msx mutant genotypes (indicated on the left). The HLs of $\text{Msx2}^{+/+}$;Dlx5/Dlx6 DKO animals (E,F) display an aggravated ectrodactyly phenotype compared to Dlx5;Dlx6 DKO ones (C,D), with fusion of the external digit (1 with 2, 4 with 5) and hypoplasia of the central digit. $\text{Msx2;Dlx5;Dlx6 TKO}$ HLs (G,H) display a further aggravated ectrodactyly phenotype, with the external digits fused and extended towards the opposite (anterior-posterior) sides, and a complete absence of the central digit. The limbs of $\text{Msx1}^{+/+}$;Dlx5;Dlx6 DKO mice (not shown) show ectrodactyly similar to that observed in Dlx5;Dlx6 DKO mice, whereas $\text{Msx1;Dlx5;Dlx6 TKO}$ mice (I,J) show ectrodactyly and loss of skeletal elements deriving from the anterior mesoderm of the autopod and zeugopod, a phenotype seen in $\text{Msx1;Msx2}$ DKO mutant embryos (K). The stylopod shows no evident defects. The anterior-posterior orientation is shown. The numbers 1–5 indicate the digits (1 is the toe). Asterisks indicate hypoplasia or absence of skeletal structures. The drawings on the left schematically illustrate the Dlx-related (red elements) and the Msx-related (purple elements) skeletal defects, corresponding to the genotypes examined.

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phenotype represents the exclusive contribution of the absence of Msx2 in the Dlx5;Dlx6 mutant context. This leads us to conclude that either Msx2 has a rather minor function in limb development, or that its function is largely compensated by Msx1, in agreement with the limited defects observed in Msx1+/−;Msx2−/− compound mutant animals [32] (Y. Lallemant, unpublished). On the contrary, Msx1;Dlx5;Dlx6 TKO mice display a limb defect which can be interpreted as the sum of the limb anomalies found in the Msx1;Msx2 DKO and in the Dlx5;Dlx6 DKO. These results strongly suggest that the expression of Msx2 is suppressed in this genetic context, and that therefore Dlx5;Dlx6 are genetically upstream of Msx2.

We show that in Dlx5;Dlx6 DKO limbs Msx2 expression is diminished in the central sector of the AER. As starting at E10.5

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**Figure 4. Expression of Bmp4 in Dlx5;Dlx6 DKO limbs.** A-D. Detection of Bmp4 mRNA by WMISH on WT (left) and Dlx5;Dlx6 DKO mutant (right) limbs, at E11. FLs are on the top, HLs are on the bottom. E,F. In situ detection of Bmp4 mRNA in the pharyngeal arches region of WT (left) and Dlx5;Dlx6 DKO mutant embryos (right), at E10.5. While Bmp4 expression in the anterior mesoderm of the FLs (A,B) or the HLs (C,D) is unchanged (green arrowheads), expression in the central wedge of the AER of mutant embryos is diminished in the HLs, but not in the FLs (red arrows in D). G-L. Detection of Gremlin mRNA in FLs (G,H) and HLs (I-L) of WT (left) and Dlx5;Dlx6 DKO mutant embryos (right), at E10.5. While Bmp4 expression in the anterior mesoderm of the FLs (A,B) or the HLs (C,D) is unchanged (green arrowheads), expression in the central wedge of the AER of mutant embryos is diminished in the HLs, but not in the FLs (red arrows in D). Gremlin expression is unchanged both in the FLs (G,H) and in the HLs (I-L) of Dlx mutant embryos (red arrowheads). Genotypes and probes are reported on the top. The Anterior-Posterior (A-P) orientation is indicated. M,N. whole-mount photographs documenting the reduced size of mutant embryos and justifying the slightly reduced size of the mutant limbs, often observed. O. Quantification of Bmp2 and Bmp4 mRNAs by qRT-PCR in the anterior and posterior halves of HLs from embryos with the genotype indicated on the top of each graph, compared to WT.

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Dlx and Msx genes are co-expressed in the AER, it is possible to hypothesize that in this territory Dlx proteins bind directly on the Msx2 promoter, as previously reported [62,63]. On the contrary, in the anterior limb mesenchyme, the expression of Msx1 and Msx2 precedes that of Dlx5 and Dlx6. This precludes the possibility of a direct regulation of Msx genes by Dlx proteins. Nevertheless, our qRT-PCR analyses show that Msx2 expression is reduced in the anterior limb mesoderm of Dlx5;Dlx6 DKO limbs, implying the existence of a non cell-autonomous mode of regulation between the AER and the anterior limb mesoderm. It is possible, therefore, that a diffusible protein, expressed by AER cells in a Dlx-dependent fashion, is required to initiate and/or sustain Msx2 expression in the anterior limb mesoderm.

Bmps as Signaling Relays between Dlx and Msx

Msx genes are well documented downstream effectors of BMP signaling in several developing structures [72,77,78,79,80,81,82]. Enhanced BMP signaling in the limb in mice deficient for the BMP antagonist Gremlin, results in upregulation of both Msx1 and Msx2 expression [73]. Conversely, blocking BMP signaling in the limb ectoderm by ectopic expression of Noggin, another BMP antagonist, results in decreased Msx2 expression [23]. In the chick limb buds, expression of a constitutively-active BMP receptor, or misexpression of Msx1, in the dorsal ectoderm, induces the formation of ectopic AERs [20]. In addition, the combined inactivation of Msx1 and Msx2 leads to a phenotype that mimics, in some aspects, the loss of BMP signaling [32].

Noticeably, Msx genes are also upstream of Bmp4 in several developmental systems. During tooth germ development, mesodermal Msx1 is needed for efficient Bmp4 expression [83,84,85,86]. Palatal development, which is impaired in Msx12/2 mice, can be rescued by a Bmp4-expressing transgene [87]. In the limb itself, Msx genes are required in the mesoderm for maintenance of Bmp4 expression [34]. Thus, BMP signaling is both upstream and downstream of Msx genes, depending on the context and the developing structure. The possibility that Bmp2 and Bmp4 participate in a Dlx-Msx signaling loop between the limb bud AER and mesoderm is clearly in line with previously identified roles of these molecules.

Therefore, Bmps represented likely candidates to mediate a non cell-autonomous regulation between AER-expressed Dlx5;Dlx6 and mesodermal Msx2. Indeed, we find that Bmp2 and Bmp4 expression is significantly reduced in Dlx5;Dlx6 DKO hindlimbs, at E11 when no evident defect is yet visible. Reduction for Bmp2 and Bmp4 in the anterior part of the HL is too high (50 and 30%, respectively) to be accounted for by the ectoderm alone, and rather indicates a downregulation of these Bmps in the mesoderm, too. This could mean that Bmps produced in the ectoderm activate...
Bmps also in the mesoderm, either directly or via Msx1. Indeed, further inactivation of one Msx1 allele in the context of the loss of Dlx5;Dlx6 nearly abolishes Bmp2 and Bmp4 expression, necessarily implying both the ectoderm and the mesoderm (Fig. 4K). This downregulation would explain a reduction in Msx2 expression in the anterior mesenchyme in the Dlx5;Dlx6 mutant limbs, at the same embryonic ages. The direct action of Dlx over Bmp is further supported by our ChIP data which indicate that DLX5, but not its mutated variant Q178P [31], binds to conserved regions near the BMP2 and BMP4 loci. However formal evidence that DLX5 activates BMP2 and BMP4 transcription is still lacking, as no suitable AER-related cell line is available for such experiments. It would be of interest to further investigate whether MSX1 also binds to the same conserved sequences in the BMP2 and BMP4 promoters.

Such a non cell-autonomous mode of regulation is strikingly similar to that occurring during development of the palatal shelves and the tooth primordia, both of which involve diffusion of Bmp between adjacent epithelial/mesodermal cell layers [60,87], and in the case of the tooth germ, an induction of Bmp4 expression in the mesoderm by ectodermal Bmp4 via expression of the Bmp target Msx1 [85,86].

The loss of mesenchymal Bmp2, 4 and 7 expression, on the other side, has been shown to be required for osteogenic differentiation, in a dose-dependent fashion and with Bmp molecules acting in a partially redundant way [88]. In their work, the selective loss of Bmp2 and Bmp4 in the limb mesenchyme affects zeugopod development and skeletogenesis, and less severely the autopod. On a similar note, another work [22] shows that the gradual elimination of Bmp4 from the limb mesenchyme is required to rescue the Grem1−/− phenotype and a normal digit organization, implying a right amount of mesenchyme-derived Bmps is essential for AER function and for autopod morphogenesis. In the Msx1;Dlx5;Dlx6 TKO mutants we observe a quite severe autopod defect (loss of digits) accompanied, however, by less severe zeugopod defect. Thus, it appears that both AER- and mesenchyme-derived Bmps are involved in the Msx;Dlx defects. The possibility that misexpression of AER-Bmps alone directly cause the TKO defects is unlikely. Rather, in light of the well known self-regulatory system of signalling loops comprising FGFs, SHH and BMPs [22], and considering that we observe changes in the expression levels of Fgf8 and Shh upon loss of Msx1, Dlx5 and Dlx6 genes, most likely the overall nature of the limb defects in TKO mutant embryos is a quantitative misregulation of the “slow module” of this loop. Dlx and Msx genes can be regarded as new players in this complex regulation. Since Shh participates in the “slow module”, a more critical role for it could be envisioned, as suggested by resemblance of the phenotype of Msx1;Dlx5;Dlx6 TKO limbs with that exhibited by Shh KO embryos [89].

**Figure 6. A dynamic model for Dlx-Msx-Bmp functional interactions during HL development.** Schematic drawing to summarize our results and illustrate our model of functional interaction between Dlx5;Dlx6, Msx1, Msx2, Bmp2 and Bmp4. On the top, a scheme of the limb bud, the AER (in light blue color) and the mesoderm (in pink color) is reported. Below, the proposed dynamic model of gene regulations, shown for an Early (E9.5–E10, on the left) and a Late (E10–E10.5, on the right) phases of HL development, using the same color code as above. The anterior mesenchyme is framed with a dotted black box; the Ant-Post and Prox-Dist directions are shown. Bmp2 and Bmp4 are placed at the interface between the AER and the Ant Mes, to indicate that these are diffusible signaling molecules.

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In conclusion, we propose a model that involves a complex epithelial-mesodermal dialogue between Dlx and Msx (Fig. 6), entailing two distinct modes of regulation in the limb buds: a direct, cell-autonomous, regulation intrinsic to AER cells, and an indirect regulation between the AER cells and the anterior limb mesoderm. We further provide data suggesting that Bmp2 and Bmp4 mediate a non cell-autonomous control of Dlx over Msx, establishing a dialogue between the AER and the anterior mesoderm of the developing limb.

Genotypes, Morphotypes, Gene Dosage and Expression Levels

When comparing the limb phenotypes of the genetic series Dlx5;Dlx6 DKO vs. Msx2+/−;Dlx5−/−;Dlx6−/− vs. Msx2;Dlx5;Dlx6 TKO (Fig. 3C–H), we observe a clear increase in severity of the ectrodactyly defect. This interesting observation can be explained by introducing the notion that Msx2 expression depends on allelic dosage, and that a threshold level of Msx2 expression is critical to drive normal morphogenesis.

The importance of allelic dosage, hence quantitative gene expression, is increasingly being recognized, even when phenotypes are not evident (see [88,90]). As a further example, we detect a reduction of Msx2 mRNA level in the Msx2+/− mice, in which no evident phenotype can be seen. Our explanation, in this case, is that reduced Msx2 expression alone is not sufficient to cause limb malformations due to the presence of two functional Msx1 alleles. Loss of one Msx2 allele in the context of Dlx5;Dlx6 DKO, instead, aggravates the phenotype. We explain this by proposing that Msx2 expression is severely reduced, approaching that of the null condition, due to the combination of a) the genetic inactivation of one allele, and b) the lack of Dlx5;Dlx6 genes.

Likewise, in Msx1;Dlx5;Dlx6 TKO animals, Msx2 expression is further reduced. A residual level is nonetheless observed; however, this is not sufficient to compensate for the loss of Msx1. In conclusion, allelic dosage and quantitative gene expression are crucial factors to be considered in the interpretation of a series of phenotypes, especially when related genes are involved.

Conclusions

In human, the Dlx5 and Dlx6 genes cause the SHFM-type-1 congenital malformation when lost or mutated, while the Msx1 and Msx2 genes cause cleft palate and tooth agenesis. By crossbreeding mutant mouse strains, we show that the Dlx5;Dlx6 and Msx1;Msx2 genes cooperate for normal limb development and morphogenesis. At least two modes of regulation have emerged, one in which Dlx5;Dlx6 control expression of Msx2 cell-autonomously, the other in which the AER and the anterior mesenchyme interact non cell-autonomously, entailing Bmps as signaling molecules. We further show that the BMP2 and BMP4 loci comprise Dlx5-binding elements, occupied by Dlx5. Thus, the highly related homeodomain genes Dlx and Msx are two key players of a novel set of molecular and histological interactions during limb development.

Supporting Information

Figure S1 Top. Location of predicted conserved Dlx binding sites in the Dlx5-Dlx6 intergenic genomic region. Sites are indicated with colour vertical bars (asterisk) and annotated with the species conservation. The chromosomal position and coordinates are also reported. The mammalian genomic conservation is reported on the bottom. The known i56i element is correctly predicted by the PWM bioinformatic approach we have adopted. Bottom. Same as above, relative to the Msx2 proximal promoter. Two known conserved Dlx binding sites are correctly predicted.

Figure S2 Quantification of the Msx2 mRNAs by qRT-PCR in the anterior and posterior halves of HLs from Msx1+/−;Dlx5−/−;Dlx6−/− embryos, relative to the corresponding WT samples (set = 1).

Figure S3 Quantification of the Fgf8 and Shh mRNAs by qRT-PCR in the HLs from Msx1−/− (top left), Dlx5−/−;Dlx6−/− (top right), Msx1+/−;Dlx5+/−;Dlx6+/− (bottom left) and Msx1+/−;Dlx5−/−;Dlx6−/− (bottom right) embryos, relative to the corresponding WT samples (set = 1).

Table S1 Sequences of the oligonucleotides used for real-time qPCR on mouse embryonic tissues.

Table S2 Sequences of the oligonucleotides used for ChIP analysis on the predicted Dlx elements near the human BMP2 and BMP4 loci.

Table S3 The Dlx5 Position-Weight matrix and results of the prediction of Dlx5 binding sites based on genomic conservation.

Table S4 Sequences of the mouse and human conserved genomic regions containing predicted Dlx5 binding sites near the BMP2 locus.

Table S5 Sequences of the mouse and human conserved genomic regions containing predicted Dlx5 binding sites near the BMP4 locus.

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Author Contributions

Conceived and designed the experiments: MV-R PP SA OB MFC AT YL BR GL. Performed the experiments: MV-R KB SM GG SA OB LG. Analyzed the data: MV-R GG PP SA OB LG. Contributed reagents/materials/analysis tools: GG PP OB LG. Wrote the paper: MV-R PP OB YL BR GL GRM.

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