**Dlx5** Is a Cell Autonomous Regulator of Chondrocyte Hypertrophy in Mice and Functionally Substitutes for **Dlx6** during Endochondral Ossification

Hui Zhu, Andrew J. Bendall*

Department of Molecular and Cellular Biology, University of Guelph, Guelph, Ontario, Canada

**Abstract**

The axial and appendicular skeleton of vertebrates develops by endochondral ossification, in which skeletogenic tissue is initially cartilaginous and the differentiation of chondrocytes via the hypertrophic pathway precedes the differentiation of osteoblasts and the deposition of a definitive bone matrix. Results from both loss-of-function and misexpression studies have implicated the related homeobox genes **Dlx5** and **Dlx6** as partially redundant positive regulators of chondrocyte hypertrophy. However, experimental perturbations of **Dlx** expression have either not been cell type specific or have been done in the context of endogenous **Dlx5** expression. Thus, it has not been possible to conclude whether the effects on chondrocyte differentiation are cell autonomous or whether they are mediated by **Dlx** expression in adjacent tissues, notably the perichondrium. To address this question we first engineered transgenic mice in which **Dlx5** expression was specifically restricted to immature and differentiating chondrocytes and not the perichondrium. **Col2a1-Dlx5** transgenic embryos and neonates displayed accelerated chondrocyte hypertrophy and mineralization throughout the endochondral skeleton. Furthermore, this transgene specifically rescued defects of chondrocyte differentiation characteristic of the **Dlx5/6** null phenotype. Based on these results, we conclude that the role of **Dlx5** in the hypertrophic pathway is cell autonomous. We further conclude that **Dlx5** and **Dlx6** are functionally equivalent in the endochondral skeleton, in that the requirement for **Dlx5** and **Dlx6** function during chondrocyte hypertrophy can be satisfied with **Dlx5** alone.

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**Introduction**

The adult vertebrate skeleton appears to be a rather uniform bony tissue. This apparent uniformity belies its embryonic origins, as a mosaic structure originating from diverse progenitors that arise in different germ layers, and its development via two distinct ontological processes: intramembranous and endochondral ossification [1,2,3]. In contrast to the direct differentiation of osteoblasts from mesenchymal progenitors that produces the intramembranous bones of the skull and clavicle, the caudal bones of the head, the vertebral column, ribs, and appendicular skeleton develop first as cartilaginous anlagen [4]. Chondroblasts in these skeletal precursors have one of two fates: to undergo a histologically well-defined program of hypertrophy and terminal differentiation in the growing bone or to persist as specialized hypertrophic, and mineralizing chondrocytes. The hypertrophic chondrocyte differentiation pathway is tightly regulated, with numerous signalling pathways providing both positive and negative signals at each step in the differentiation process. Some of these signals, like Ihh or Delta, are made by subpopulations of differentiating chondrocytes [7,8,9]. Others, like members of the TGFB/BMP and Wnt families, are also made by cells in the perichondrium [10,11,12], a tissue that surrounds the growing cartilaginous core and gives rise to the bone collar. These competing extracellular signals induce or repress transcription factors to regulate chondrocyte proliferation and differentiation; the result is coordinated longitudinal bone growth and joint articulation [6,13,14,15,16,17,18,19,20,21].

**Dlx** homeobox genes encode nuclear transcription factors [22,23,24]. In particular, **Dlx5** and **Dlx6** are expressed in all anlagen of the endochondral skeleton. Their expression has been noted in precartilaginous limb bud mesenchyme [25,26] where each overlaps with expression of **Sox9** [27,28]. At later stages of skeletogenesis, when proliferating and differentiating chondrocytes are found in spatially distinct regions of the growth plate, **Dlx5** and **Dlx6** are expressed in the post-mitotic prehypertrophic and hypertrophic zones but not in immature chondroblasts in the resting or proliferating zones [27,28,29]. **Dlx5** and **Dlx6** are also expressed in the perichondrium/periosseum in the long bones as well as ribs and vertebrae [27,30,31,32]. **Dlx5** and doubly deficient **Dlx5**/**Dlx6** mice have revealed requirements for **Dlx5** and **Dlx6** during chondrogenesis [28] and chondrocyte hypertrophy [27,33]. Reciprocally, forced expression of either **Dlx5** or **Dlx6** alone in chicken limb bud micromass cultures stimulated chondrogenesis [28] and misexpression of **Dlx5** in vivo reduced proliferation of epiphysial chondrocytes and resulted in precocious chondrocyte hypertrophy [27,29,34]. Together, these
experiments implicate *Dlx5* and *Dlx6* as partially redundant positive regulators of chondrocyte differentiation. However, to date, perturbations of *Dlx* expression have either not been cell type specific or have been done in the context of endogenous *Dlx5* expression. This general concern is particularly germane when seeking to elucidate a cell-autonomous function for *Dlx5* in chondrocyte hypertrophy given endogenous *Dlx5* expression in both the differentiating chondrocytes and in the perichondrium, the site of synthesis of secreted factors that regulate this process in the chondrogenic core. Here, we first describe a transgenic line of mice in which exogenous *Dlx5* expression is targeted to immature chondrocytes using regulatory elements from the *Col2a1* gene. Tissue-specific misexpression of *Dlx5* accelerated chondrocyte hypertrophy and promoted precocious ossification in the endochondral skeleton of these transgenic mice. Visualization of transgene expression in the absence of endogenous *Dlx5* expression (achieved by crossing the allele onto a *Dlx5*/*−* background) indicated that transgene expression was limited to the chondrogenic core and not the perichondrium. The subsequent rescue of endochondral ossification defects in *Dlx5*/*−/−*; *Col2a1-Dlx5* mice therefore establishes a cell-autonomous function for *Dlx5* during chondrocyte hypertrophy and, furthermore, demonstrates functional equivalence of *Dlx5* and *Dlx6* in the endochondral skeleton.

**Results**

**Generation of Transgenic Mice and Characterization of Transgene Expression**

*Dlx5* and its *ci*-linked parologue *Dlx6* function as positive regulators of both chondrogenesis and chondrocyte hypertrophy in the endochondral skeleton [27,28,29,33]. To further investigate the function of *Dlx5* during chondrocyte hypertrophy *in vivo*, we generated transgenic mice in which a *Dlx5* cDNA was expressed under control of the promoter and intron 1 enhancer of the *Col2a1* gene (Fig. 1A) so as to target *Dlx5* expression to immature chondrocytes following their differentiation from pluripotent mesenchymal precursors. Following pro-nuclear injection, we obtained four *Col2a1-Dlx5* transgenic founders; these four founders (or their hemizygous offspring) had a variable number of copies of the transgene, as measured by semi-quantitative PCR from genomic DNA (Fig. 1B). The three founders with the highest number of copies (more than ten) were recovered dead as neonates and their transgenic alleles are referred to as *Col2a1-Dlx5*/*+, Col2a1-Dlx5*/*2*, and Col2a1-Dlx5*/*3*. The viable founder with allele *Col2a1-Dlx5*/*2* had fewer than four copies as a hemizygote and was used to establish a stable transgenic line expressing epitope-tagged *Dlx5* (Fig. 1C).

To examine the tissue distribution of transgene expression, we examined embryos following whole mount *in situ* hybridization with a *Dlx5* riboprobe. *Dlx5* expression was visualized in a number of locations that normally express the definitive chondroblast marker *Col2a1* (Fig. 2). In particular, and in contrast to non-transgenic littermates, *Dlx5* expression was apparent in somites along the entire rostro-caudal axis and in rib cartilage and throughout the skeletal anlagen of the limb, where it closely matched expression of the endogenous *Col2a1* gene in the stylopod and zeugopod (Fig. 2A–E). In contrast, at E12.5 ectopic expression of *Dlx5* was most obvious in the phalanges, where it coincided with endogenous *Col2a1* expression. Endogenous expression of *Dlx5* in the otic vesicle, mandibular arch, branchial arches 2 and 3, or in a proximal anterior mesodermal domain in the limb was not altered.

Figure 1. Generation of *Col2a1-Dlx5* transgenic mice. (A) Transgene design. Numbering of promoter and intron elements is with respect to the *Col2a1* transcrption start site. The start codon of *Col2a1* in exon 1 has been mutated to prevent translation in this exon [56]. The asterisk indicates the *Flag* sequence 5′ to the murine *Dlx5* open reading frame, bpA, poly-adenylation sequence from the bovine *Growth Hormone* gene; SA, splice acceptor. Half arrows indicate the approximate location of primers for genotyping: *Col2a1* forward (Cf) plus *Dlx5* reverse (Dr), and for RT-PCR: *Flag* forward (Ff) plus Dr. (B) Transgene copy number. Semi-quantitative PCR was used to compare the approximate transgene copy number in transgenic founder mice: *2+/−* and *3+/−* represent dead hemizygous founders and *19+/−* is a hemizygote neonate from the stable *Col2a1-Dlx5*/*2* line. Control lanes from the left are: wild type DNA (0), wild type DNA mixed with *p30003020Col2a1-Dlx5* at 1 copy per genome equivalent (1) or 10 copies per genome equivalent (10). Amplification of a genomic fragment of the single copy gene *Ihh* was used to judge relative amplification of the transgene. (C) Specific amplification of expressed *Flag-Dlx5* sequence from transgenic embryos in a reverse transcription-dependent manner. RT-PCR analysis of five F4 generation embryonic day (E) 17.5 embryos from a *wt x Col2a1-Dlx5*/*8* mating demonstrates stable heritable expression of the *Col2a1-Dlx5* transription unit. The first lane in the bottom panel (+) shows a positive PCR control for the minus RT experiment. Non-adjacent lanes from the same gel have been spliced together to generate the figure. Lane numbers refer to individual embryos.

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in transgenic embryos. To confirm that Dlx5 was being expressed ectopically in immature chondroblasts, we examined tissue sections following in situ hybridization. Indeed, Dlx5 was expressed throughout the Col2a1-expressing zones of the long bones and vertebrae (Fig. 2F–I and data not shown), including the resting and proliferating zones of the long bone epiphyses, where its expression is not usually detectable (compare Fig 2H,I). Moreover, in the hypertrophic zone, where Col2a1 transcription is normally down regulated, we saw a parallel decrease in Flag-Dlx5 transcript abundance (compare Fig. 2G,I). To view transcription of the transgene in the absence of endogenous Dlx5 expression, we introduced the Col2a1-Dlx5<sup>6/−</sup> allele into a Dlx5<sup>6/−</sup>/Dlx5<sup>6/−</sup> background. Section in situ hybridization to the long bones of the limbs of Dlx5<sup>6/−/6/−</sup>; Col2a1-Dlx5<sup>6/−</sup> embryos revealed that transgene expression was restricted to the cartilaginous core of the skeletal anlage and was not expressed in the surrounding perichondrium (Fig. 2J,K). In summary, expression of the Col2a1-Dlx5<sup>6/−</sup> allele faithfully replicated endogenous Col2a1 gene expression in chondrocytes and resulted in ectopic Dlx5 expression in immature and proliferating chondroblasts.

**Figure 2. Transgene expression in the endochondral skeleton.** (A–E) Whole mount in situ hybridization of Dlx5 (A–C) or Col2a1 (D,E) probes to wild type (wt) and Col2a1-Dlx5<sup>6/−/−</sup> transgenic (t/+) littermates at E11.5 and E12.5. Arrows point to somites in panel A. Arrowhead points to digit 2 in panel C. (F–I) Section in situ hybridization of Col2a1 (F,H) or Dlx5 (G,I) riboprobes to adjacent sections of the femur of wild type (F,G) or Col2a1-Dlx5<sup>6/−/−</sup> transgenic embryos (H,I) at E14.5. Arrowheads indicate the Col2a1-positive proliferating zones, arrows point to the resting zones, and the bracket demarcates the hypertrophic zone in panels G and I. Proximal is to the right. (J,K) Section in situ hybridization of Col2a1 (J) or Dlx5 (K) riboprobes to adjacent sections of the tibia of Dlx5<sup>6/−/−</sup>; Col2a1-Dlx5<sup>6/−/−</sup> transgenic embryos at E14.5. Proximal is to the right. md, mandibular arch; ot, otic vesicle; pam, proximal anterior mesoderm. Scale bar = 1 mm in A, 0.5 mm in B–E,J,K, 0.2 mm in F–I.

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Acceleration of Chondrocyte Hypertrophy Following Forced Expression of Dlx5 in Chondroblasts

To examine the functional consequences of misexpressing Dlx5 in chondrocytes, we first examined skeletal preparations of neonates at postnatal day zero (P0) or P1 after staining cartilage and mineralizing bone with alcian blue and alizarin red respectively. All three dead founders, or viable neonates bearing the Col2a1-Dlx5<sup>t19</sup> allele, examined this way had a common phenotype of hypermineralization of the endochondral skeleton (Fig. 3). In its most severe form, seen in three non-viable founders, hypermineralization resulted in smaller stature (compare Fig. 3A, B). The extent of hypermineralization, as estimated from the extent of alizarin red staining and generalized skeletal dysmorphology, followed the allelic series Col2a1-Dlx5<sup>t19</sup> < Col2a1-Dlx5<sup>t119/19</sup> < Col2a1-Dlx5<sup>t119/119</sup>. The hemizygous neonates showed variable degrees of expanded mineralization of the supra- and basioccipital bones at birth, such that these bones were sometimes fused in two to two separate skull components in turn and uncovered a differential sensitivity to transgene expression in the axial and appendicular skeleton.

The basioccipital, occipital and supraoccipital bones of the rostral skull arise from the most cranial somites [35]. Eventually, the occipital bones fuse to surround the foramen magnum but the rostral skull arise from the most cranial somites [35]. Eventually, the occipital bones fuse to surround the foramen magnum but the rostral skull arise from the most cranial somites [35]. Eventually, the occipital bones fuse to surround the foramen magnum but the rostral skull arise from the most cranial somites [35]. Eventually, the occipital bones fuse to surround the foramen magnum but the rostral skull arise from the most cranial somites [35]. Eventually, the occipital bones fuse to surround the foramen magnum but the rostral skull arise from the most cranial somites [35]. Eventually, the occipital bones fuse to surround the foramen magnum but the rostral skull arise from the most cranial somites [35]. Eventually, the occipital bones fuse to surround the foramen magnum but the rostral skull arise from the most cranial somites [35]. Eventually, the occipital bones fuse to surround the foramen magnum but the rostral skull arise from the most cranial somites [35]. Eventually, the occipital bones fuse to surround the foramen magnum but the rostral skull arise from the most cranial somites [35]. Eventually, the occipital bones fuse to surround the foramen magnum but the rostral skull arise from the most cranial somites [35]. Eventually, the occipital bones fuse to surround the foramen magnum but the rostral skull arise from the most cranial somites [35]. Eventually, the occipital bones fuse to surround the foramen magnum but the rostral skull arise from the most cranial somites [35]. Eventual...
Figure 3. Dose-dependent hypermineralization in the endochondral skeleton of Col2a1-Dlx5 transgenic neonates. (A,B) Lateral views of a wild type CD-1 P0 pup (A) and two hemizygous Col2a1-Dlx5 transgenic pups (B), both found dead shortly after birth. (C,D) Dorsal view of the caudal skull of wild type (C) and t19/+ (D) neonates at postnatal day zero (P0) following staining with alcin blue and alizarin red. The arrow points to an area of fusion between the basioccipital and exoccipital bones in panel D. (E,F) Lateral views of a wild type (E) and t1/+ transgenic founder (F) at P0 after staining with alcin blue and alizarin red. (G) Bubble graph of the ossification index of multiple litters of t19/+ hemizygotes and their wild type littermates. Following staining with alcin blue and alizarin red, a score was assigned to each embryo or neonate that reflected the extent of occipital ossification relative to a typical wild type at that stage: 1 = no brain case (exencephalic), 2 = brain case intact but no ossification of the supraoccipital (SO) apparent, 3 = smaller SO than is seen in a majority of wild type specimens (which were assigned a score of 4); 5 = obviously smaller distance between the SO and exoccipital (EO), or between the EO and the basioccipital (BO), compared to a majority of wild type; 6 = unilateral touching of SO and EO or of EO and BO; 7 = bilateral contact between SO and EO, or between the EO and the BO, or fusion of any of these bones. Bubble size is proportional to the number of neonates with a given score; the smallest circles represent a single individual, the largest circle represents 17 individuals. (H–J) Ventral views of wild type (H), hemizygous (I) and homozygous (J) embryos with the Col2a1-Dlx5t19 allele at P1 following alizarin red staining. Arrowheads point to precociously mineralized vertebrae in panel I. (K,L) Ventral views of wild type (K), and Col2a1-Dlx5t1/19 transgenic founder (L) at P0 following alcin blue and alizarin red staining. The most caudal thoracic (T13) and sacral (S4) vertebrae are marked with white asterisks. (M–P) Limb skeleton preparations from neonates with the genotypes as shown following alcin blue and alizarin red staining. at, atlas; at/ax*, fused atlas and axis; ax, axis; BO, basioccipital; D, dentary, EO, exoccipital; fe, femur; fi, fibula; h, humerus; IP, interparietal; IP*, interparietal bone with expanded mineralization; r, radius; sc, scapula; SO, supraoccipital; S/EO*, fused supraoccipital and exoccipital bones; t, tibia; u, ulna; wt, wild type. Scale bar = 5 mm in A,B, 1 mm in C,D, 2 mm in E,F,K–P, 0.5 mm in H–J.

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We next sought to determine whether precocious mineralization of the endochondral skeleton was due to an underlying acceleration of chondrocyte differentiation. We examined hematoxylin and eosin stained sections through the head, trunk and limbs at various stages (Fig. 5). At E16.5, before a mineralization phenotype was apparent in the caudal skull, advanced hypertrophy of the basioccipital bone was apparent. The lateral edges of the basioccipital bone contain small, rounded chondroblasts in wild type embryos, whereas hypertrophic chondrocytes occupied the entire element in transgenic littermates (Fig. 5A,B). Transverse sections through the vertebrae of Col2a1-Dlx5t19/+ transgenic neonates confirmed that mineralization had occurred throughout the vertebrae, whereas the ossification centres of the centrum and neural arches of wild type pups were separated by blocks of cartilaginous tissue that contained both radially flattened and hypertrophic chondrocytes (Fig. 5C,D).

To further confirm the basis of the phenotype, we examined the growth plates of three-week-old mice after H&E staining. There were no gross distortions in the overall architecture of the growth plates of transgenic weanlings (n = 3) compared to wild type littermates (n = 4), with both proliferating and hypertrophic zones being of similar size, although dividing chondrocytes in the proliferative zone were stacked somewhat less regularly in transgenic mice (Fig. 5E,F). Thus, forced expression of Dlx5 in immature chondrocytes resulted in accelerated chondrocyte hypertrophy in the axial skeleton and a more subtle effect in the growth plate of limbs.

To further confirm the basis of the phenotype, we examined the expression of markers of prehypertrophic and hypertrophic chondrocytes in transverse sections through the limbs and vertebrae at times before an overt effect on mineralization was apparent. Consistent with the idea that Dlx5 promoted precocious ossification via accelerated chondrocyte hypertrophy, expression of both prehypertrophic (Ihh) and hypertrophic (Col10a1) markers was expanded in the limbs and vertebrae of Col2a1-Dlx5t19/+ embryos compared with wild type littermates (Fig. 6). In summary, expression of Dlx5 in immature chondrocytes promoted chondrocyte hypertrophy and endochondral ossification.

The Chondrocyte Hypertrophy Function of Dlx5 Is Cell Autonomous and Is Sufficient to Rescue Endochondral Ossification in Dlx5/6 Null Embryos

Dlx5 and Dlx6 have shared essential functions in mandibular patterning and AER perdurance and Dlx5/6 null neonates are exencephalic as a result of a failure to ossify the skull vault; in particular, Dlx5/6 neonates lack supraoccipital and interparietal bones at birth [33,36,37,38]. Targeted deletion of both Dlx5/6 also revealed shared functions in the endochondral skeleton. Defects that arise as a result of delayed or absent chondrocyte differentiation vary from the complete absence of mineralised endochondral elements, notably the paired supraoccipital bones, to delayed mineralization throughout the vertebral and appendicular skeleton [33,36]. While the limb and axial skeleton of Dlx6−/− mice has not yet been described, Dlx6 is largely redundant with Dlx5 in patterning the first branchial arch [39]. Further understanding of the functional equivalence of Dlx5 and Dlx6 in endochondral
ossification requires the substitution of one for the other in an in vivo context. We asked whether cartilage-specific expression of Dlx5 could rescue cartilage maturation defects in Dlx5/6 null embryos by crossing Col2a1-Dlx5t19 hemizygous mice (CD-1 background) with heterozygous Dlx5/6+/- mice (C57Bl/6 x DBA background) to specifically reconstitute Dlx5 function in the cartilage of otherwise Dlx5/6 null embryos. Dlx5/6-/-; Col2a1-Dlx5t19/+ neonates showed a rudiment of a mineralized element of varying size, located dorsal to the exoccipital bones, which was interpreted to be supraoccipital in identity (Fig. 7B, n = 4), and partial rescue of the supraoccipital was sometimes unilateral. In contrast, Dlx5/6-/-; Col2a1-Dlx5t19/t19 homozygotes had mineralised supraoccipital bones that were of a similar size to those in Dlx5/6+/- or wild type littermates (Fig. 7C,D, n = 3). Notably, the interparietal, which forms via intramembranous ossification, was not rescued in these animals, nor was transformation of mandibular structures to a maxillary identity (Fig. 7). Finally, we asked whether rescue of endochondral ossification was a general feature of these embryos by examining a more quantitative trait, namely ossification of the vertebral centra. Ossification of the centra of Dlx5/6 null embryos lags that in wild type littermates, being, on average, 76% of that in heterozygous or wild type littermates at E17.5 (Fig. 7E-H). Vertebral mineralization in Dlx5/6-/-; Col2a1-Dlx5t19/+ embryos, however, was indistinguishable from Dlx5/6-/- littermates. Taken together, our data show that Dlx5 can fully compensate for Dlx6 in the endochondral skeleton. While we cannot formally exclude the possibility that Dlx5 was expressed at very low levels in the perichondrium, that were below our detection limits, our results are consistent with a cell autonomous function for Dlx5 during chondrocyte hypertrophy.

**Discussion**

*Dlx5 and Dlx6 as Cell Autonomous Regulators of Chondrocyte Hypertrophy*

We have generated a line of transgenic mice in which the transcription factor Dlx5 is expressed in proliferating chondroblasts. Interestingly, the level of expression of exogenous Dlx5 was similar to that of endogenous Dlx5, as measured by *in situ* hybridization, with both being expressed at much lower levels than Col2a1. Our finding is consistent with the observations of others, using essentially the same Col2a1 sequences [40] and likely reflects the absence of cis-acting sequences that contribute to the higher level expression of Col2a1. We consider this to be an advantage of our mouse model since ectopic expression of Dlx5 is at near physiological levels in Col2a1-Dlx5t19 hemizygotes. Through cell type-specific manipulation of its expression, we provide evidence that Dlx5 is required cell autonomously for chondrocyte hypertrophy and that Dlx6 has a redundant function in this tissue. This is best demonstrated by the fact that restoration of Dlx5 expression in
chondrogenic condensations in the chondrocranium was sufficient to rescue the supraoccipital bones in Dlx5/6-deficient mice. Rescue of the supraoccipital bones in Dlx5/6<sup>2/2</sup>;Col2a1-Dlx5<sup>t19/t19</sup> neonates further indicates that chondrogenic differentiation events prior to activation of Col2a1 are not defective in Dlx5/6 null embryos but rather that the absence of the supraoccipital bones in Dlx5/6<sup>2/2</sup> neonates is due to a subsequent block in chondrocyte differentiation. This chondrogenic function is apparently independent of the role of Dlx5 or Dlx6 as osteoblast differentiation genes or as regulators of craniofacial patterning since Dlx5/6<sup>2/2</sup>;Col2a1-Dlx5<sup>t19/t19</sup> neonates retained intramembranous bone defects and transformation of mandibular to maxillary structures. Similarly, Dlx5 likely has an independent function in the perichondrium where it may promote differentiation to the periosteum. Another study, in which Dlx5 was specifically misexpressed in chondrocytes, documented enhanced chondrocyte hypertrophy in the limb [34]. These tissue-specific manipulations of Dlx5 expression validate the results of more generalized over-expression studies with this gene [29].

Dlx6<sup>2/2</sup> mice display a range of first branchial arch defects that closely resemble those seen in Dlx5<sup>2/2</sup> neonates, but that are milder [39]. The non-additive nature of the patterning defects that occur following the combined deletion of Dlx5 and Dlx6 suggest functional redundancy of these genes in the first branchial arch. Similarly, gain-of-function studies point to a quantitatively equivalent function in stimulating multipotent precursors to differentiate into chondroblasts [28]. The functional equivalence of these genes in a specific tissue had not previously been addressed <em>in vivo</em>; a definitive test of functional equivalency requiring substitution of Dlx5 coding sequences for those of Dlx6. This requirement is satisfied in embryos with combined Col2a1-Dlx5<sup>t19</sup> and Dlx5/6<sup>2/2</sup> alleles and constitutes a test of functional equivalency in cartilage. That both quantitative and qualitative defects in endochondral ossification were rescued by chondrocyte-specific expression of Dlx5 argues that Dlx5 and Dlx6 are functionally interchangeable in chondrocytes and that specific endochondral elements depend on different levels of Dlx5 or Dlx6 activity; vertebral ossification was not completely dependent on either Dlx5 or Dlx6 (since it was not completely blocked in Dlx5/6<sup>2/2</sup> null embryos) and was rescued to wild type levels in hemizygous Col2a1-Dlx5 embryos while complete rescue of the supraoccipital bones required homozygosity of the transgene in a Dlx5/6 null background. Nonetheless, it is quite likely that some functions of the Dlx5-Dlx6 locus will depend on diverged protein functions; some genes behave differently following loss of Dlx6 versus Dlx5 in the mandibular arch, for example [39]. This is not altogether surprising given the striking differences in the amino acid sequences of the Dlx5 and Dlx6 proteins and the differential distribution of their transcriptional activation activities [28].

**Figure 6. Expansion of prehypertrophic and hypertrophic marker gene expression in Col2a1-Dlx5 transgenic mice.** (A–D) <em>In situ</em> hybridization of Ihh (A,C) or Col10a1 (B,D) riboprobes to adjacent cryosections of wild type and Col2a1-Dlx5 transgenic femurs at E14.5. Proximal is to the right. (E–H) <em>In situ</em> hybridization of Ihh (E,G) or Col10a1 (F,H) riboprobes to adjacent cryosections of wild type and Col2a1-Dlx5 transgenic vertebral centra at E16.5. Dorsal is up. Scale bar = 0.2 mm for all photomicrographs.

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Figure 7. Chondrocyte-specific expression of Dlx5 rescued endochondral defects in Dlx5/6 null embryos. (A–D) Lateral views of the heads of P0 neonates with the genotypes shown after staining with alcian blue and alizarin red. Asterisk indicates a missing supraocular bone in panel A. Arrows point to supraocular bones in panels B–D. (E–G) Dorsal view of the second to fifth lumbar vertebrae from a Dlx5/6+/- (E), Dlx5/6+/-; Col2a1-Dlx5[19+/+](F), and Dlx5/6+/- (G) embryo at E17.5 following alcian blue and alizarin red staining. Since Dlx5/6+/- embryos are smaller, digital images of L2 to L5 at E17.5 were scaled to the same vertebral size to allow measurements of the relative mineralization of the vertebral bodies. Rostral is at the top. (H) Quantitation of mineralization in the L3 centrum of Dlx5/6+/- (n=9), Dlx5/6+/-; Col2a1-Dlx5[19+/+](n=3), and Dlx5/6+/- (n=9) vertebrae, plotted as average area ± sem. *P<0.05; ns, P>0.05. Scale bar = 2 mm in A–D, 0.5 mm in E,F,G.

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A Hierarchy of Transcription Factors in Chondrocyte Differentiation

Like Dlx5, Runx2 (encoding two isoforms that differ at their amino-termini) is a multifunctional regulator of both chondrocyte and osteoblast differentiation [41] and the two factors have some remarkable parallels in both expression and function. Like Dlx5, Runx2 is expressed in cartilaginous condensations and later, during long bone growth, both genes are expressed in prehypertrophic and hypertrophic chondrocytes. In addition, Dlx5 and Runx2 are expressed in the perichondrium flanking the prehypertrophic and hypertrophic zones and, in both cases, perichondrial expression extends over the proliferating zone ([27,40,42,43,44,45,46,47] and see Fig. 2H). As this study has demonstrated for Dlx5, either Runx2 isom accelerated chondrocyte hypertrophy in the axial and appendicular skeleton when misexpressed in immature cartilage [40,48]. Isoform selective deletion further revealed that Runx2-H has a non-redundant function in chondrocyte maturation [49] and Runx2-I, rather than Runx2-II, is specifically expressed in prehypertrophic and hypertrophic chondrocytes [47]. Runx2 isoforms also induced ectopic chondrocyte hypertrophy in transgenic mice, in which persistent cartilage in the trachea and chondrocranial cartilage was diverged to a hypertrophic fate [40,48], and retroviral-mediated misexpression of Runx2-II converted persistent cartilage to a hypertrophic fate in the chicken hyoid skeleton [50]. In contrast, Dlx5 appears unable to induce ectopic mineralization. None of our four Col2a1-Dlx5 allelic causes ectopic mineralization in persistent cartilage. Even in tissues that normally mineralise, Dlx5 misexpression did not appear to affect the timing of the initial appearance of a calcified matrix, but rather contributed to a subsequent acceleration once begun. Indeed, in the vertebrae, the effects of an accelerated matrix, but rather contributed to a subsequent acceleration once begun. Thus, it is likely that the synthesis of other rate-limiting cofactors for its hypertrophic function. This is an interesting situation given that Dlx5 appears to be a direct upstream regulator of Runx2 during osteoblast differentiation [51,52]. Nevertheless, our data strongly suggest that Dlx5 is not sufficient for transactivation of Runx2 in chondroblasts. Indeed, the signals and upstream transcriptional regulators of Runx2 in chondrocytes are not yet known.

The Mosaic Nature of the Skeleton

The differential sensitivity of the appendicular and axial skeleton to a given level of ectopic Dlx5 expression is not without precedent; non-uniform effects of Col2a1-driven transgenes in the endochondral skeleton have been a hallmark of other studies too. For example, the supracoacipital bone and vertebral skeleton was more sensitive to the effects of a dominant negative PTHrP receptor than the limbs, which only exhibited an effect at the highest number of transgene copies and then only in discrete elements [53]. Furthermore, expression of a constitutively active form of Akt in immature chondrocytes led to accelerated chondrocyte hypertrophy and mineralization in the cranial and vertebral skeleton but delayed hypertrophy in limbs [54]. The differential responses of the axial and appendicular skeleton to Dlx5 and other regulators of cartilage development no doubt reflects differences in the molecular milieu of anatomically distinct chondrocytes and further underscores the mosaic nature of the skeleton.

Materials and Methods

Ethics Statement

Prior institutional approval was received for the animal work described in this study from the University of Guelph Animal Care Committee, in accordance with Canadian Council on Animal Care guidelines.

Generation of Transgenic Mice, Genotyping, and RT-PCR

The murine Dlx5 open reading frame was amplified as a 5’ Flag-tagged HindIII-Not fragment and shuttled into a modified pBlas13 [55]. The bovine polyadenylation (polyA) sequence from p3000b3020Col2a1 [56] was amplified and cloned downstream of Dlx5 as an Not-Xba fragment, and the FlagDlx5-bpA cassette was cloned back into p3000b3020Col2a1 as a HindIII-Sal fragment, replacing the polyA-polyA sequences. The construct was digested with Not and Sal and the linear transgene cassette was microinjected into the pronuclei of fertilized CD-1 oocytes. Genotyping of transgenic embryos was done with forward primer 5’-AACGTTCGCGGACAAGGCA-3’ and reverse primer 5’-GAGCGTTTGCCCATAGAAC-3’, which amplified a 1040 bp fragment. The Col2a1-Dlx5GII’ allele was maintained in a hemizygous state on a CD-1 background; hemizygous animals were occasionally bred to generate homozygous embryos or neonates. Offspring from Col2a1-Dlx5GII’ x Dlx5/N-/- crosses were backcrossed to Dlx5/6 heterozygotes to generate transgene-positive, Dlx5/6 null mice. A 438 bp genomic Ihh fragment was amplified with primers: 5’-ACCGTGTGTTGCCAGGATGTG-3’ and 5’-TACCACAGGCTTGGTCAGTC-3’, Dlx5/6 homozygotes were genotyped with the lacZ primer pair: 5’-GGCGTACACATATTCG-3’ and 5’-TGTGAGCGAGTAAGAC3’ [32]. The presence of FlagDlx5 and β-actin mRNA was determined on total RNA prepared from E16.5 embryos using the Trizol Reagent (Invitrogen). 1 µg of RNA was reverse transcribed (Superscript II, Invitrogen) and PCR amplified (Taq, UBI Life Sciences) with standard protocols. Primer sequences used for RT-PCR were: FlagDlx5 (For 5’-GACTACAAAGGAGGAG-GATGAC-3’ and Rev 5’-GAGCGTTTGCCCATAGAAAG-3’) and β-actin (For 5’-GAAAAATCTGGCACCACACC-3’; Rev 5’-CAGAAAGGAGGAGTGAAGA-3’).

Skeletal Staining

Alcian Blue and Alizarin Red staining was used to visualize cartilaginous and mineralised skeletal tissues respectively of embryos and neonates. Briefly, eviscerated and skinned embryos and neonates. E16.5) bodies were fixed in 95% ethanol over several days. Embryos were stained in 80% ethanol, 20% acetic acid, 0.01% Alcian blue 8GX for 2–4 days, cleared overnight in 1% KOH, stained 1–2 days in 0.001% Alizarin Red S in 1% KOH, then rinsed in 1% KOH, and a graded series of H2O/glycerol to 100% glycerol.
In Situ Hybridisation

Whole mount and cryosection in situ hybridisation was done as described previously [55] with the following antisense riboprobes: Dlx5, a 0.87 kb BamHI – HindIII fragment corresponding to the full length open reading frame [28]; Col2a1, a 0.4 kb cDNA corresponding to nt 1–402 of Genbank X57982; Bsh, a 1.8 kb partial cDNA EcoRII fragment [57], and Col10a1, a 0.86 kb AphiI – Sall fragment containing nt 1351–2215 of Genbank X65121.

Imaging

Images were taken using a MicroPublisher colour digital camera on a Leica MZ12.5 Stereomicroscope with Qcapture software (QImaging) or on a Leica DMRA2 upright microscope with Openlab software (Improvision) and processed using Adobe Photoshop.

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Author Contributions

Conceived and designed the experiments: AJB. Performed the experiments: HZ, AJB. Analyzed the data: HZ, AJB. Contributed reagents/materials/analysis tools: HZ. Wrote the paper: HZ, AJB.

References

1. Cohen MM Jr (2000) Merging the old skeletal biology with the new. I. Intramembranous ossification, endochondral ossification, ectopic bone, secondary cartilage, and pathologic considerations. J Craniofac Genet Dev Biol 20: 84–93.
2. de la Fuente L, Helms JA (2005) Head, shoulders, knees, and toes. Dev Biol 282: 294–306.
3. Mackie EJ, Ahmed YA, Tatarczuch L, Chen KS, Mirams M (2000) Endochondral ossification: how cartilage is converted into bone in the developing skeleton. Int J Biochem Cell Biol 32: 46–62.
4. Hall BK, Miyake T (2000) All for one and one for all: condensations and the initiation of skeletal development. Bioessays 22: 130–147.
5. Archer CW, Francis-West P (2003) The chondrocyte. Int J Biochem Cell Biol 35: 401–404.
6. Pacifici M, Koyama E, Iwamoto M (2005) Mechanisms of synovial joint and articular cartilage formation: recent advances, but many lingering mysteries. Birth Defects Res (Part C) 75: 257–248.
7. Karsenty G (2003) The complexities of skeletal biology. Nature 423: 316–318.
8. Lanske B, Karaplis AC, Lee K, Luz A, Vortkamp A, et al. (1996) Regulation of rate of cartilage differentiation by Indian hedgehog and PTH-related protein. Science 273: 615–622.
9. Lanske B, Karaplis AC, Lee K, Luz A, Vortkamp A, et al. (1996) PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone capture growth. Science 273: 603–666.
10. Crowe R, Zikherman J, Niswander L (1999) Delta-1 negatively regulates the transition from prehyapatytic to hypertrophic chondrocytes during cartilage formation. Development 126: 987–998.
11. Zou H, Wieser R, Massague J, Niswander L (1997) Distinct roles of type I bone morphogenetic protein receptors in the formation and differentiation of cartilage. Genes Dev 11: 2191–2203.
12. Kronenberg HM (2003) Developmental regulation of the growth plate. Nature 423: 332–336.
13. de Crombrugghe B, Lefebvre V, Nakashima K (2001) Regulatory mechanisms in the pathways of cartilage and bone formation. Curr Opin Genet Dev 11: 527–532.
14. Eames BF, de la Fuente L, Helms JA (2003) Molecular ontogeny of the skeleton. Development 129: 4371–4386.
15. Eames BF, de la Fuente L, Helms JA (2003) Pattern mammalian jaw primordium by regulating both lower jaw-specific and craniofacial, vestibular and bone defects in mice lacking the Distal-less-related gene Dlx5. Development 126: 3795–3809.
16. Cesare MC, Noamani B, Abberly DE, Zhi H, Levi G, et al. (2006) Dlx5- and Dlx6-mediated chondrogenesis: Differential domain requirements for a conserved function. Mech Dev 123: 819–830.
17. Ferrari D, Sumoy L, Gannon J, Sun H, Brown AM, et al. (1995) The expression pattern of the Distal-less homeobox-containing gene Dlx-5 in the developing chick limb bud suggests its involvement in apical ectodermal ridge activity, pattern formation, and cartilage differentiation. Mech Dev 52: 257–264.
18. Inada M, Yasui T, Nomura S, Miyake S, Deguchi K, et al. (1999) Maturational disturbance of chondrocytes in Dlx5–/– mice. Dev Dyn 214: 279–290.
19. Kim IS, Otto F, Zabel B, Mundlos S (1999) Regulation of chondrocyte differentiation by Chx11. Mech Dev 80: 159–170.
45. Enomoto H, Enomoto-Iwamoto M, Iwamoto M, Nomura S, Himeno M, et al. (2000) Cbfa1 is a positive regulatory factor in chondrocyte maturation. J Biol Chem 275: 8695–8702.

46. Choi KY, Lee SW, Park MH, Bae YC, Shin HI, et al. (2002) Spatio-temporal expression patterns of Runx2 isoforms in early skeletogenesis. Exp Mol Med 34: 426–433.

47. Zhang S, Xiao Z, Luo J, He N, Mahlosi J, et al. (2009) Dose-Dependent Effects of Runx2 on Bone Development. J Bone Miner Res 10.1359/jbmr.090502.

48. Ueta C, Iwamoto M, Kanatani N, Yoshida C, Liu Y, et al. (2001) Skeletal malformations caused by overexpression of Cbfa1 or its dominant negative form in chondrocytes. J Cell Biol 153: 87–99.

49. Xiao ZS, Hjelmeland AB, Quarles LD (2004) Selective deficiency of the "bone-related" Runx2-II unexpectedly preserves osteoblast-mediated skeletogenesis. J Biol Chem 279: 20307–20313.

50. Eames BF, Sharpe PT, Helms JA (2004) Hierarchy revealed in the specification of three skeletal fates by Sox9 and Runx2. Dev Biol 274: 188–200.

51. Lee MH, Kim YJ, Kim JH, Park HD, Kang AR, et al. (2003) BMP-2-induced Runx2 expression is mediated by Dlx5, and TGF-β1 opposes the BMP-2-induced osteoblast differentiation by suppression of Dlx5 expression. J Biol Chem 278: 34387–34394.

52. Lee MH, Kim YJ, Yoon WJ, Kim JI, Kim BG, et al. (2005) Dlx5 specifically regulates Runx2 type II expression by binding to homeodomain-response elements in the Runx2 distal promoter. J Biol Chem 280: 35379–35387.

53. Schipani E, Lamske B, Hanzehinm J, Luz A, Kovacs CS, et al. (1997) Targeted expression of constitutively active receptors for parathyroid hormone and parathyroid hormone-related peptide delays endochondral bone formation and rescues mice that lack parathyroid hormone-related peptide. Proc Natl Acad Sci USA 94: 13689–13694.

54. Rokutaanda S, Fajita T, Kanatani N, Yoshida CA, Komori H, et al. (2009) Akt regulates skeletal development through GSK3, mTOR, and FoxOs. Dev Biol 326: 78–93.

55. Zhu H, Bendall AJ (2006) Dlx3 is expressed in the ventral forebrain of chicken embryos: implications for the evolution of the Dlx gene family. Int J Dev Biol 50: 71–75.

56. Zhou G, Garofalo S, Mukhopadhyay K, Lefebvre V, Smith CN, et al. (1995) A 182 bp fragment of the mouse proα1(II) collagen gene is sufficient to direct chondrocyte expression in transgenic mice. J Cell Sci 108: 3677–3684.

57. Echelard Y, Epstein DJ, St-Jacques B, Shen L, Mohler J, et al. (1993) Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. Cell 75: 1417–1430.