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Original Article

Dlx5 regulates chondrocyte differentiation at multiple stages

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ABSTRACT Endochondral ossification, in which cartilaginous templates are progressively replaced by marrow and bone, represents the dominant mode of development of the axial and appendicular skeleton of vertebrates. Chondrocyte differentiation within the cartilaginous core of these skeletal elements is tightly regulated, both spatially and temporally. Here, we describe the expression of *Dlx5* in the cartilaginous core of limb skeletal elements in chicken and mouse embryos. We find that *Dlx5* is one of the earliest genes expressed in condensing limb mesenchyme that will give rise to the limb skeleton. Later, when proliferating and differentiating chondrocytes are found in spatially distinct regions of the cartilaginous model, *Dlx5* is expressed in the zone of hypertrophy and in proliferating chondrocytes that are poised to differentiate. Consistent with this pattern of expression, we show that forced expression of *Dlx5* potentiates early and late chondrocyte differentiation and inhibits proliferation in cultured cells. Examination of the limbs of mutant *Dlx5* mouse embryos revealed that they displayed a delay in chondrocyte maturation compared with wild type littermates. Taken together, our data reveal a positive role for *Dlx5* during multiple stages of chondrocyte differentiation and, along with previous studies of *Dlx5* and osteogenesis, identify *Dlx5* as a general regulator of differentiation in the mouse skeleton.

KEY WORDS: *Dlx5, chondrogenesis, homeobox gene, chondrocyte differentiation, expression pattern*

Introduction

The vertebrate axial and appendicular skeleton develops by endochondral ossification wherein cartilaginous models prefigure the future bones (Erlebacher*, et al.*, 1995; Cohen, 2000; Olsen*, et al.*, 2000; de Crombrugghe*, et al.*, 2001; Wagner and Karsenty, 2001)**.** Conversely, the majority of bones in the skull, as well as the clavicle, develop by intramembranous ossification in the absence of a cartilaginous intermediate (Cohen, 2000; Hall, 2001). Development of the endochondral skeleton begins with the local condensation of undifferentiated mesenchyme into prechondrogenic aggregates. Cells in the core of these condensations differentiate into chondroblasts while those at the periphery form a specialized structure, the perichondrium, which surrounds the growing cartilaginous core (Hall and Miyake, 2000). The growth of each skeletal element is initially driven by proliferation of the perichondrial cells and the small round chondroblasts they enclose. Subsequently, chondrocytes at the center of the model (diaphysis) exit the cell cycle and undergo differentiation to a hypertrophic state. Continued growth of each skeletal element then proceeds by the proliferation of chondroblasts at the ends (epiphyses) of each individual skeletal element and their controlled maturation through morphologically distinguishable and spatially-ordered zones of increasingly more differentiated stages from the articular ends towards the center of the shaft. Thus, small round chondrocytes at the epiphyses undergo defined transitions to mitotically-active radially flattened chondrocytes, then to post-mitotic and enlarged pre-hypertrophic cells that are committed to terminal differentiation, and finally to hypertrophic and terminally differentiated mineralizing chondrocytes in the diaphysis. Coincident with chondrocyte hypertrophy, cells in the perichondrium differentiate and form a bony collar, initiating a cascade of events that includes invasion of the hypertrophied cartilage by vascular and marrow-forming cells and the deposition of trabecular bone matrix by osteogenic cells (Caplan and Boyan, 1994; Cancedda*, et al.*, 1995; Olsen*, et al.*, 2000). Chondrocyte differentiation and osteogenesis are therefore intimately linked during endochondral ossification with chondrocyte hypertrophy being a prerequisite for the mineralization of skeletal elements.

Abbreviations used in this paper: CEF, chicken embryo fibroblast; DMEM, Delbecco's modified Eagle's medium; dpc, days post-coitum; FBS, fetal bovine serum; RCAS, replication-competent avian retrovirus.

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The maturation of chondrocytes is tightly regulated, both temporally and spatially, with numerous signaling pathways providing both positive and negative signals at each step in the differentiation process. Members of the TGF-β superfamily act at multiple stages of chondrogenesis to positively regulate the recruitment of multipotent mesenchymal cells to the chondrogenic lineage as well as their subsequent proliferation (Francis-West*, et al.*, 1999; Pizette and Niswander, 2000), while acting as negative regulators of terminal differentiation (Duprez*, et al.*, 1996; Serra*, et al.*, 1997; Zou*, et al.*, 1997). *Wnt4* is expressed in cells flanking the periarticular region and induces maturation of proliferating chondroblasts to post-mitotic, prehypertrophic cells, that express *Ihh* (Hartmann and Tabin, 2000). The Ihh signal is relayed from the prehypertrophic cells back to the periarticular perichondrium, where it induces the secretion of PTHrP (Amizuka*, et al.*, 1994; Karaplis*, et al.*, 1994; Lanske*, et al.*, 1996; Lee*, et al.*, 1996; Vortkamp*, et al.*, 1996; Weir*, et al.*, 1996). The PTHrP signal is in turn received by cells in the flattened zone that express *PTH/PTHrP receptor* and prevents them from becoming prehypertrophic (Karaplis*, et al.*, 1994; Lanske*, et al.*, 1996; Vortkamp*, et al.*, 1996). Since *Ihh*-positive cells are committed to hypertrophy, this negative feedback loop involving *Ihh* and *PTHrP* thus regulates the size of the mitotically active chondrocyte pool. *Wnt5b* expression in the prehypertrophic zone reveals a more mature subpopulation of prehypertrophic chondrocytes whose terminal differentiation to hypertrophy is negatively regulated by Wnt5a signals from the perichondrium (Hartmann and Tabin, 2000).

In contrast to our extensive knowledge of the signaling pathways that modulate chondrogenesis and chondrocyte differentiation, there have been fewer functional studies of the transcription factors that act downstream of these signals to regulate the maturation of chondrocytes. *Sox9*, endcoding a HMG-domain transcription factor, is one of the first genes expressed in precartilaginous condensing mesenchyme (Wright*, et al.*, 1995). Sox9 directly activates *Col2α1* which encodes the major structural protein of definitive chondroblasts (Bell*, et al.*, 1997; Ng*, et al.*, 1997) and is therefore a key regulatory protein in early chondrocyte differentiation (Healy*, et al.*, 1999). *Cbfa-1*, encoding a member of the runt domain family, is required for differentiation to the prehypertrophic state (Inada*, et al.*, 1999; Kim*, et al.*, 1999), and can also induce terminal differentiation (Takeda*, et al.*, 2001). The pocket proteins p107 and p130 are required for cell cycle withdrawal and chondrocyte hypertrophy (Rossi*, et al.*, 2002) and unspecified members of the CREB bZIP family also appear to play roles in chondroblast proliferation and progression to prehypertrophy (Long*, et al.*, 2001). The function of other transcription factors in chondrocytes is not well understood.

Dlx5, one of six known *Dlx* homeobox genes in mammals and birds (Simeone*, et al.*, 1994; Ferrari*, et al.*, 1995), is expressed in a variety of differentiating tissues during vertebrate embryogenesis, a feature of all *Dlx* gene expression (reviewed in Bendall and Abate-Shen, 2000; Merlo*, et al.*, 2000; Zerucha and Ekker, 2000). Studies of *Dlx5* function during osteogenesis have been prompted by the observation of *Dlx5* expression in tissues that undergo both intramembranous and endochondral ossification (Simeone*, et al.*, 1994; Zhao*, et al.*, 1994; Ferrari*, et al.*, 1995; Chen*, et al.*, 1996; Davideau*, et al.*, 1999a; Davideau*, et al.*, 1999b). Correlative studies of *Dlx5* expression in calvarial cells from the skull show

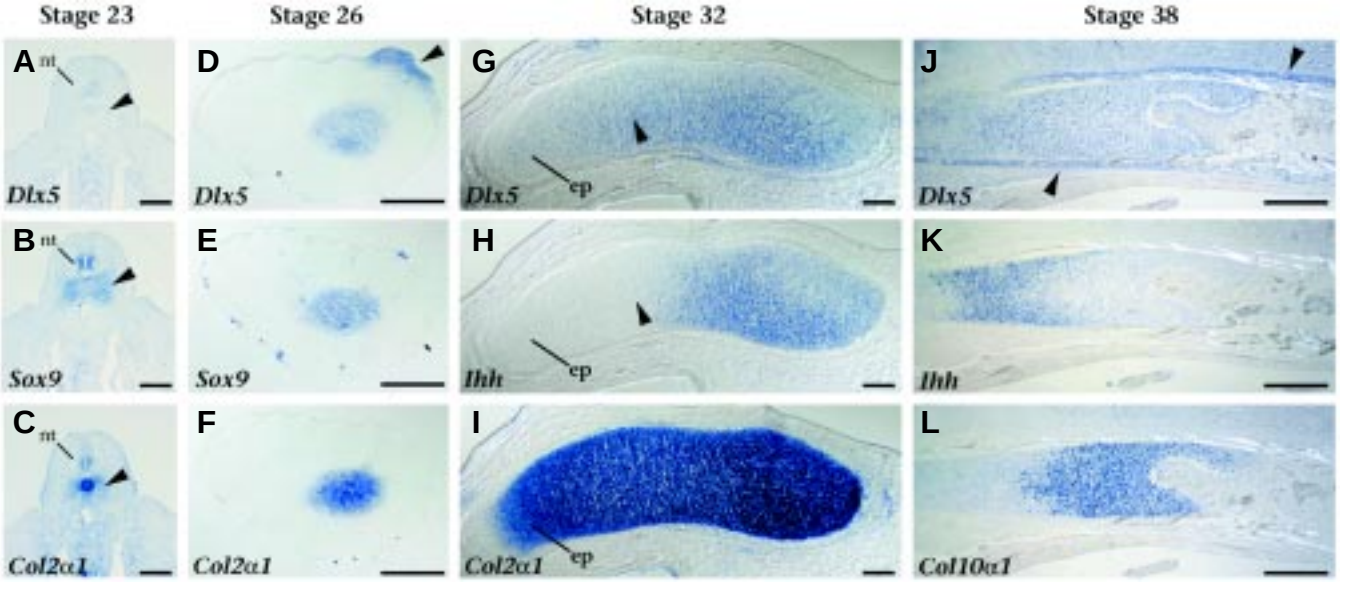

Fig. 1. *Dlx5* expression during chondrogenesis in chicken embryos. In situ *hybridization of* Dlx5 *(A,D,G,J)* Sox9 *(B,E),* Ihh *(H,K),* Col2α1 *(C,F,I), and* Col10α1 *(L) on adjacent transverse sections of chicken embryos at stage 23 (somites and neural tube) and longitudinal sections at stages 26 (whole limb bud), 32 (stylopod), and 38 (proximal humerus).* **(A-C)** *Overlapping expression of* Sox9 *and* Col2α1, *but not* Dlx5, *in the perinotochordal chondrogenic condensation (arrowheads) at stage 23.* **(D-F)** *Overlapping expression of* Dlx5, Sox9, *and* Col2α1 *in the cartilaginous mesenchymal condensation in a stage 26 forelimb bud. Note the additional domain of* Dlx5 *expression at the anterior margin of the wing (arrowhead in D).* **(G-I)** *At stage 32 the* Dlx5 *expression domain overlaps with* Ihh *expression in prehypertrophic chondrocytes but also includes chondrocytes that do not express* Ihh *(arrowhead in G,H).* Col2α1-*positive chondrocytes at the epiphysis do not express* Dlx5. **(J-L)** *At stage 38 the* Dlx5 *expression domain overlaps both the* Ihh *and* Col10α1 *expression domains which are themselves mutually exclusive.* Dlx5 *is also expressed in the perichondrium (J, arrowheads). Abbreviations: ep, epiphysis; nt, neural tube. Scale bars: 0.2 mm (A-C), 0.1 mm (D-I), 0.5 mm (J-L).*

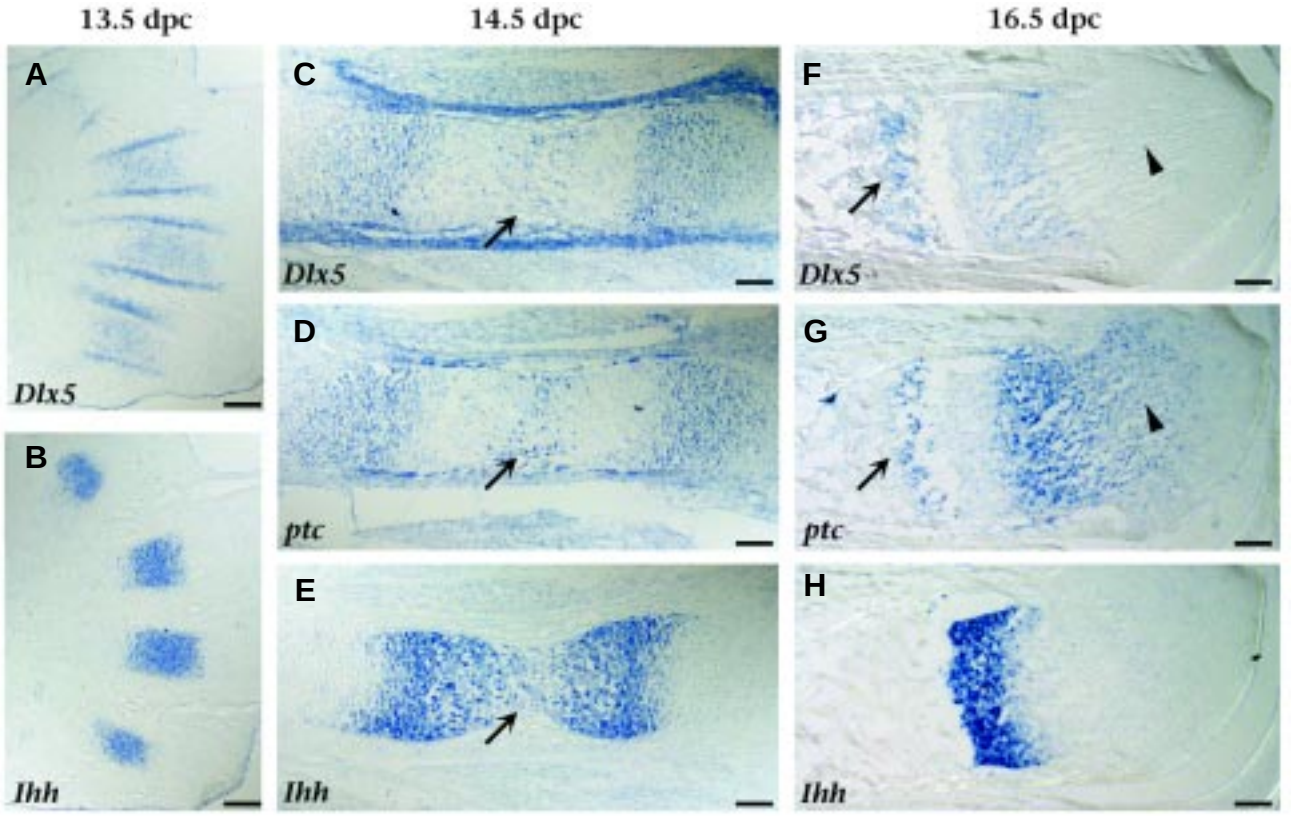

Fig. 2. *Dlx5* expression in the embryonic mouse appendicular skeleton. In situ *hybridization of* Dlx5 *(A,C,F)* ptc *(D,G), and* Ihh *(B,E,H), on adjacent longitudinal sections of mouse embryo forelimbs at 13.5 dpc (A,B autopod), 14.5 dpc (C-E, humerus), and 16.5 dpc (F-H, distal femur).* **(A,B)** *At 13.5 dpc, the* Dlx5 *expression domain overlaps with* Ihh *expression in prehypertrophic chondrocytes but also includes chondrocytes that flank the* Ihh *domain, as well as the perichondrium.* **(C-E)** *At 14.5 dpc the* Dlx5 *expression domain overlaps with that of* ptc *and, like* ptc, *is mostly excluded from the* Ihh*-positive domain but is expressed in the hypertrophic zone. (Arrows indicate the hypertrophic zone in C,D,E).* Dlx5 *expression also continues in the perichondrium.* **(F-H)** *Low level expression of* Dlx5 *continues in the flattened zone and the cartilage-bone interface at the growth plates of long bones at 16.5 dpc where it overlaps with* ptc. *(Arrows indicate the hypertrophic zone in F,G). Expression of* Dlx5 *is not detectable in the* Ihh*-positive prehypertrophic zone nor in proliferating cells that express low levels of* ptc *(Arrowheads indicate the proliferative zone in F,G). Scale bars: 0.1 mm.*

increased expression of *Dlx5* as osteogenic cells mature (Ryoo, *et al.*, 1997). The transcriptional effect of *Dlx5* on specific osteogenic genes is somewhat conflicting however as *Dlx5* has variously been shown to activate (Miyama, *et al.*, 1999), have little effect on (Newberry, *et al.*, 1998), or repress (Ryoo, *et al.*, 1997) osteocalcin expression. Genetic evidence from *Dlx5*$^{-/-}$ mice support the notion that *Dlx5* may be a repressor of osteocalcin *in vivo* (Acampora, *et al.*, 1999) although it is not clear whether this effect is direct or indirect. A second osteoblast-specific gene, bone sialoprotein, has recently been shown to be responsive to *Dlx5* (Benson, *et al.*, 2000). *Dlx5* expression during appendicular skeletogenesis has been noted in precartilaginous limb bud mesenchyme of mammals and birds (Zhao, *et al.*, 1994; Ferrari, *et al.*, 1995) and, later, in the perichondrium/periosteum of more mature cartilages in the limb as well as ribs and vertebrae (Simeone, *et al.*, 1994; Zhao, *et al.*, 1994; Chen, *et al.*, 1996; Acampora, *et al.*, 1999). A recent gain-of-function study suggests that *Dlx5* is a positive regulator of chondrocyte hypertrophy in the chicken embryo (Ferrari and Kosher, 2002).

Here, we describe the temporal expression pattern of *Dlx5* in the chondrogenic core of the developing limb skeleton in chicken and mouse embryos. While *Dlx5* is one of the earliest genes expressed in condensing limb mesenchyme, at later stages of skeletogenesis *Dlx5* is expressed in differentiating chondrocytes and the proliferating cells that abut the prehypertrophic zone. Consistent with this pattern of expression, we show that *Dlx5* is an inhibitor of proliferation and a positive regulator of chondrocyte differentiation in cultured cells. Moreover, mice that are homozygous for targeted disruption of *Dlx5* displayed a delay in chondrocyte differentiation with a concomitant reduction in ossification of the long bones. Together, our data reveal a conserved role for *Dlx5* as a positive regulator of chondrocyte differentiation in vertebrates and, together with other studies linking *Dlx5* function to osteoblast maturation, identify *Dlx5* as a multifunctional regulator of differentiation in the skeleton.

Results

Expression of Dlx5 *during Chondrogenesis in Chicken and Mouse Embryos*

Dlx5 expression has been observed in the mesenchyme of early limb buds and, later, in the perichondrium/periosteum of developing skeletal elements in both chicken and mouse embryos (Simeone, *et al.*, 1994; Zhao, *et al.*, 1994; Ferrari, *et al.*, 1995; Chen, *et al.*, 1996; Acampora, *et al.*, 1999). To understand the timing of *Dlx5* expression with respect to the commitment of limb mesenchymal cells to the chondrogenic lineage we examined adjacent sections from early chicken embryo limbs for the expression of *Dlx5*, *Sox9*,

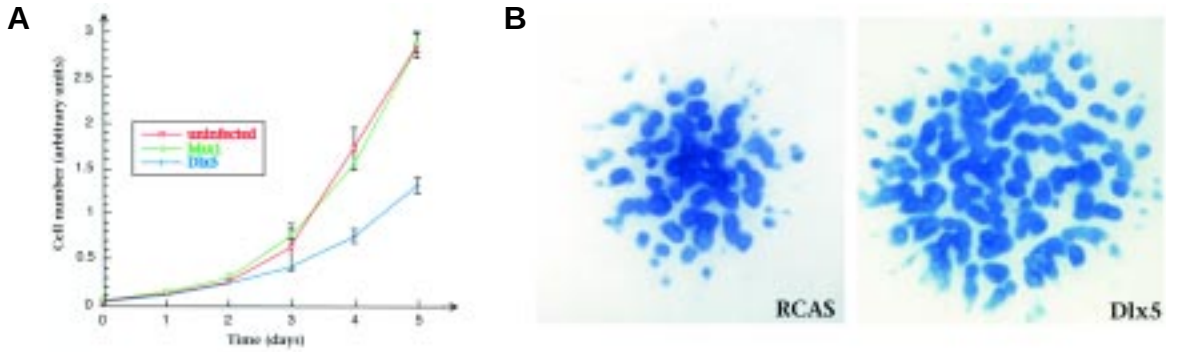

Fig. 3. *Dlx5* inhibits proliferation and stimulates recruitment to the chondrogenic lineage. (A) Dlx5 *reduces proliferation in primary fibroblasts. Chicken embryo fibroblasts were uninfected (uninfected), or infected with* RCAS-Dlx5 *(Dlx5) or* RCAS-Msx1 *(Msx1) and cultured in 4% fetal calf serum. Cell number was estimated by measuring the optical density of Napthal blue-black stained cells and is expressed in arbitrary units. Error bars represent one standard deviation of assays done in triplicate. Representative data are shown from assays repeated a minimum of three times.* **(B)** Dlx5 *stimulates recruitment of pluripotent mesenchyme to the chondrogenic pathway. Micromass assay using stage 21-22 chicken embryo limb bud mesenchyme infected with* RCAS-Dlx5 *(right panel, Dlx5) shows enhanced chondrogenic differentiation compared with* RCAS-*infected mesenchyme (left panel, RCAS). Chondrogenic nodules were visualized with alcian blue staining. Assays were done in duplicate and repeated at least three times; a representative assay is shown.*

and *Col2α1*. The condensation of *Sox9*-expressing mesenchyme at the early limb bud stage of vertebrate embryos marks the commitment of mesenchymal cells to the chondrogenic lineage and represents the earliest visible stage of skeletal development. In the chicken embryo this process begins around Hamburger and Hamilton stage 23 (stage 23) and is accompanied by the expression of *Col2α1*, a direct target gene of *Sox9*, which marks definitive chondroblasts. There was strong overlap in the expression of *Dlx5*, *Sox9*, and *Col2α1* in the limb between stages 23 and 26 (Fig. 1 D-F and data not shown), indicating that expression of *Dlx5* is an early event in the chondrogenic differentiation of limb mesenchyme. Notably, we did not observe *Dlx5* expression accompanying *Sox9* and *Col2α1* expression in perinotochordal chondrocytes during the condensation of vertebral bodies (Fig. 1 A-C). These chondrocytes are of a different origin than those of the limb, having migrated from the sclerotome of adjacent somites (Christ and Wilting, 1992). An absence of *Dlx5* expression thus distinguishes axial chondrogenic condensations from those in the limbs.

By stage 32, three morphologically distinct zones of chondrogenic cells are apparent: small, round chondroblasts at the epiphyses, prehypertrophic cells with large and clear cytoplasms in the diaphysis, and radially flattened cells between. By this time, *Dlx5* expression had become restricted to cells in the flattened zone as well as the prehypertrophic zone. This was evidenced by a *Dlx5* expression domain that encompassed, and was somewhat larger than, the domain of *Ihh* expression in the diaphysis of growing cartilages. In contrast, the proliferating chondroblasts at the epiphysis did not express detectable levels of *Dlx5* (Fig. 1 G-I). By stage 38, prehypertrophic chondrocytes have differentiated and now express *Col10α1* which is a marker of hypertrophy (Linsenmayer, *et al.*, 1991; Yokouchi, *et al.*, 1995). Expression of *Dlx5* continued in the *Ihh*-positive prehypertrophic zone and was also found to persist in the hypertrophic zone where its expression overlapped with *Col10α1* (Fig. 1 J-L). *Dlx5* expression was also seen in the perichondrium at this time (Fig. 1J). Thus, *Dlx5* expression coincides with the initial differentiation of limb mesenchyme to the chondrocyte lineage but subsequently becomes restricted to the mitotically active flattened zone and regions of hypertrophy.

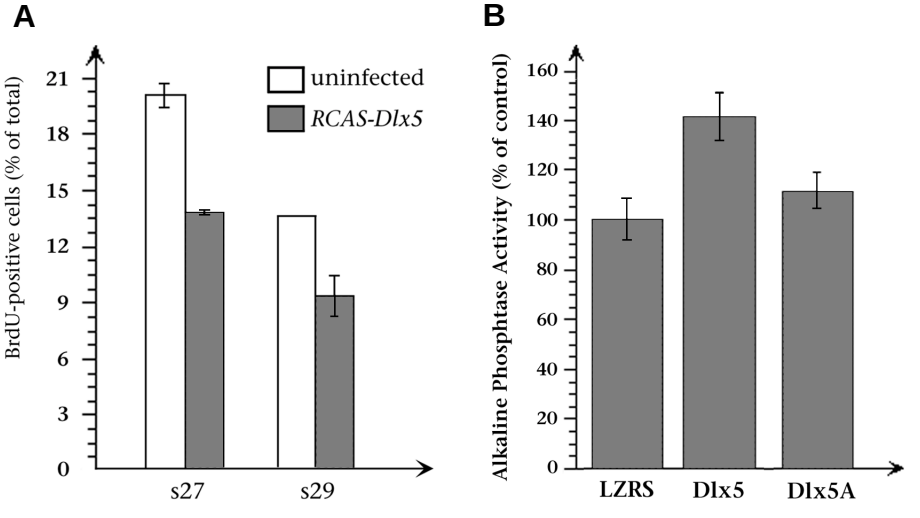

Fig. 4. *Dlx5* stimulates terminal differentiation in chondrocytes. (A) *Reduced proliferative index in the epiphysis following misexpression of* Dlx5. *BrdU-positive cells in the uninfected (clear bars) or* RCAS-Dlx5-*infected (grey bars) radii of stage 27 and stage 29 embryos. Error bars indicate one standard deviation.* **(B)** Dlx5 *stimulates terminal differentiation of chondrocytes. TMC23 chondrocytes were infected with* LZRSΔ, LZRSΔ-Dlx5 *(Dlx5), or* LZRSΔ-Dlx5A *(Dlx5A) then cultured under permissive conditions for differentiation. Alkaline phosphatase activity was measured from cell lysates; error bars represent one standard deviation of assays done in triplicate.*

We next examined *Dlx5* expression in mouse embryos at developmental stages comparable to those studied in chicken. As early as 9.5 days *post-coitum* (dpc), and continuing through 12.5 dpc, *Sox9* and *Col2α1* expression could be detected in migrating sclerotomal cells and perinotochordal chondroblasts but this was not accompanied

by *Dlx5* expression. However, expression of *Sox9*, *Col2α1*, and *Dlx5* was seen in overlapping domains in the limb mesenchyme of these early limb bud stages, being most obvious at 12.5 dpc (data not shown). Thus, early expression of *Dlx5* in condensed limb mesenchyme appears to be conserved in the mouse. At 13.5 dpc, the *Dlx5* expression domain resembled that seen in chick forelimbs at stage 32 where it encompassed, but was broader than, the prehypertrophic domain of *Ihh* expression. *Dlx5* was also expressed in the perichondrium at this stage in the mouse limb (Fig. 2 A,B). By 14.5 dpc *Dlx5* expression partially overlapped, but was mostly excluded from, the region of highest *Ihh* expression. *Dlx5* was expressed in the flattened zone and in the hypertrophic zone where *Ihh* expression is down-regulated, but not throughout the prehypertrophic zone (Fig. 2 C,E). Since this pattern of expression is reminiscent of that described for *ptc*, a target gene of Ihh signaling, we directly compared expression of *Dlx5* and *ptc* at this stage. The *Dlx5* expression domain was essentially indistinguishable from that of *ptc* in the chondrogenic core of skeletal elements with *Dlx5* being additionally expressed at high levels in the perichondrium (Fig. 2 C,D). Low level expression of *Dlx5* occupied the flattened zone at the growth plates of long bones at 16.5 dpc. Expression of *Dlx5* was not detectable in the prehypertrophic zone and was more restricted than *ptc* expression at this stage since *Dlx5* expression was also not detectable in proliferating cells that express low levels of *ptc* (Fig. 2 F,G). Note that *Dlx5* was also expressed with *ptc* in the narrow cartilage-bone interface where terminally differentiated chondrocytes are being replaced by endosteal osteoblasts (Fig. 2 F-H). Examination of other sections confirmed that cells in this region were positive for *Col10α1* (data not shown) suggesting that at least some of these *Dlx5*- and *ptc*-expressing cells were hypertrophic chondrocytes.

While there are differences in the expression pattern of *Dlx5* during appendicular skeletogenesis in chicken and mouse limbs, in both species *Dlx5* is expressed in condensing limb bud mesenchyme and, later, in proliferating chondrocytes which flank the prehypertrophic zone. In the chick, *Dlx5* expression also persists in prehypertrophic chondrocytes while in mouse it is expressed in the prehypertrophic zone at early stages but is excluded from this zone at later stages. Thus, *Dlx5* is expressed at a critical point during chondrocyte maturation in both species, namely in chondrocytes just before they commit to terminal differentiation.

Dlx5 *inhibits Cellular Proliferation and stimulates Chondrocyte Differentiation at Multiple Stages*

The expression pattern of *Dlx5* in the cartilage models of chicken and mouse embryo limbs suggests that *Dlx5* may function during chondrocyte differentiation. To test these ideas directly we engineered avian and mammalian retroviral vectors to express *Dlx5* in cultured cells or *in vivo*. As a control, we used a mutated version of *Dlx5* encoding amino acid substitutions in the N-terminal arm of the homeodomain thus rendering a protein that lacks DNA binding activity (*Dlx5A*) (A.B. & C.A.-S., unpublished). We first examined the effect of *Dlx5* expression on proliferation of an uncommitted cell population. We compared the proliferation of chicken embryo fibroblasts infected with a replication-competent avian retrovirus expressing *Dlx5* (*RCAS-Dlx5*) with that of uninfected

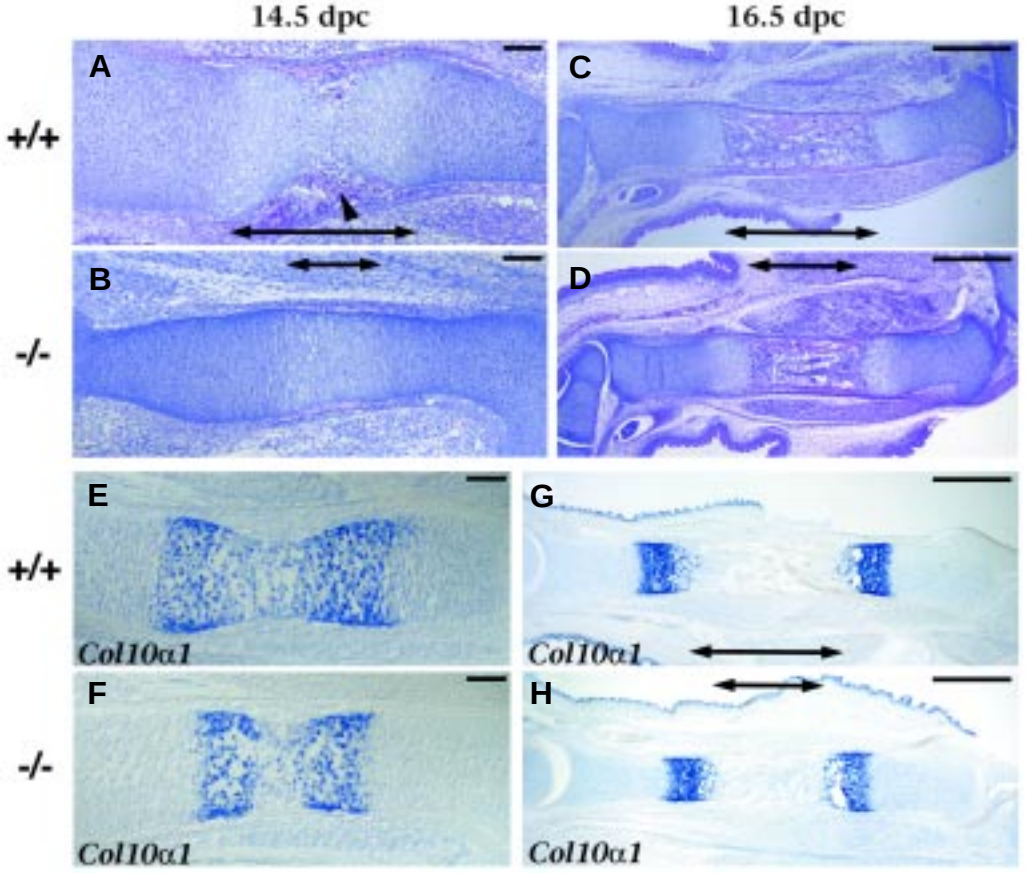

Fig. 5. Chondrocyte maturation is delayed in *Dlx5* mutant mice. *(A-D) Histological analysis of wild-type (A,C) and* Dlx5-/- *mice (B,D). Comparison of the humerus of wild-type* **(A)** *and* Dlx5-/- *littermate* **(B)** *at 14.5 dpc reveals a larger hypertrophic zone (double-headed arrows in A,B) and more extensive bone collar formation (arrowhead in A) in the wild-type embryo. Comparison of the tibia of wild-type* **(C)** *and* Dlx5-/- *littermate* **(D)** *at 16.5 dpc shows a larger ossified zone (double-headed arrows in C,D) in the wild-type embryo. (E-H) Section* in situ *hybridization analysis of wild-type (E,G) and* Dlx5-/- *mice (F,H). Comparison of* Col10α1 *expression in the humerus of wild-type* **(E)** *and* Dlx5-/- *littermate* **(F)** *at 14.5 dpc shows a wider hypertrophic zone in the wild-type embryo. The* Col10α1 *expression domains in the tibia of a wild-type embryo at 16.5 dpc* **(G)** *demarcates a wider ossified zone compared to the* Dlx5-/- *littermate* **(H)**. *All panels show longitudinal sections; proximal is to the right. Representative examples are shown; all limb skeletal elements showed equivalent phenotypes. Scale bars: 0.1 mm (A,B,E,F), 0.5 mm (C,D,G,H).*

CEFs. We found that expression of *Dlx5* resulted in reduced cellular proliferation (approximately 2-fold). This effect was specific to *Dlx5* since infection with retrovirus expressing another homeobox gene (*RCAS-Msx1*) did not significantly inhibit proliferation of these primary cells (Fig. 3A and Hu*, et al.*, 2001).

To gain insights into the role of *Dlx5* as it pertains to chondrogenesis, we examined the effect of *Dlx5* on recruitment of pluripotent mesenchymal cells to the cartilaginous lineage using a micromass assay. Under the conditions of this assay, dispersed pluripotent mesenchymal cells from early stage chicken limb buds will form chondrogenic nodules when cultured at high density (Ahrens*, et al.*, 1977). To examine *Dlx5* function in the initial differentiation of mesenchyme to chondrocytes we infected pluripotent mesenchymal cells from stage 21-22 chicken embryo limb buds with *RCAS* or *RCAS-Dlx5* prior to culturing them at high density. Forced expression of *Dlx5* resulted in approximately twice as many chondrogenic nodules compared with cells infected with the viral vector alone, although individual nodules were not obviously larger in size compared with controls (Fig. 3B). Consistent with its expression in precartilaginous condensations in the limb bud, *Dlx5* thus had a positive effect on early chondrocyte differentiation *in vitro*.

We next examined the effect of forced expression of *Dlx5* on proliferation and terminal differentiation of chondrocytes. To this end, we infected the nascent limb buds of chicken embryos at stage 17 with the *Dlx5*-expressing avian retrovirus (*RCAS-Dlx5*) and examined the wing skeleton between stages 27 and 29. We detected high levels of *Dlx5* mRNA throughout all infected wing tissues by section *in situ* hybridization at these stages and exogenous Dlx5 protein was expressed at high levels in the infected right hand wings, but not the uninfected left hand wings of treated embryos as detected in protein extracts made from wings at stage 36 (data not shown). To evaluate the proliferative status of chondrocytes in *RCAS-Dlx5*-infected wings, we examined infected embryos at stages 27-29 following BrdU labeling of proliferating cells. Misexpression of *Dlx5* resulted in a lower proportion (by about one third) of proliferating chondrocytes in the epiphysis compared to uninfected controls (Fig. 4A) ($P < 0.025$ at stage 27, $P < 0.1$ at stage 29). Thus, in addition to its general anti-proliferative activity on primary cells *in vitro*, *Dlx5* also inhibits proliferation of chondrocytes in the endochondral skeleton *in vivo*. This latter finding is in agreement with the recent observations of Ferrari and Kosher (2002).

To examine the effect of *Dlx5* on later stages of chondrocyte differentiation, we utilized a characterized cell model, TMC23, which can be induced to undergo terminal differentiation under defined culture conditions (Xu*, et al.*, 1998). We infected TMC23 cells with a replication-defective mammalian retroviral vector encoding *Dlx5* or *Dlx5A* and assessed terminal differentiation by measuring levels of alkaline phosphatase which is secreted by hypertrophic and mineralizing chondrocytes. Infection with *LZRSΔ-Dlx5* enhanced the terminal differentiation of TMC23 cells by approximately 40% compared with *LZRSΔ-Dlx5A* or with the *LZRSΔ* vector (Fig. 4B). This enhanced terminal differentiation was

TABLE 1

ENDOCHONDRAL OSSIFICATION IN THE LONG BONES OF *DLX5* $^{-/-}$ EMBRYOS[a]

		Humerus	Radius	Ulna	Femur	Tibia
Litter 1[b]	-/- (n=2)	0.24 ±0.03	0.28 ±0.05	0.23 ±0.04	0.20 ±0.02	0.23 ±0.03
15.5 dpc	+/+ (n=1)	0.31	0.35	0.33	0.24	0.30
Litter 2	-/- (n=2)	0.21 ±0.02	0.25 ±0.04	0.23 ±0.03	0.19 ±0.01	0.26 ±0.03
15.5 dpc	+/+ (n=2)	0.32 ±0.02	0.37 ±0.02	0.33 ±0.01	0.27 ±0.01	0.34 ±0.01
Litter 3	-/- (n=5)	0.19 ±0.04	0.24 ±0.04	0.21 ±0.03	0.18 ±0.03	0.21 ±0.04
15.5 dpc	+/- (n=3)	0.25 ±0.02	0.28 ±0.03	0.24 ±0.03	0.20 ±0.03	0.24 ±0.01
Summary[c]	-/- (n=9)					
15.5 dpc	+/+, +/- (n=6)	73±5%	78±8%	77±8%	81±8%	80±5%
Litter 4	-/- (n=2)	0.57 ±0.01	0.64 ±0.00	0.62 ±0.00	0.50 ±0.01	0.56 ±0.00
18.5 dpc	+/+ (n=2)	0.61 ±0.01	0.71 ±0.02	0.69 ±0.02	0.54 ±0.00	0.62 ±0.00
Litter 5	-/- (n=2)	0.61 ±0.00	0.67 ±0.02	0.64 ±0.05	0.51 ±0.01	0.59 ±0.01
18.5 dpc	+/+ (n=2)	0.64 ±0.01	0.66 ±0.00	0.64 ±0.02	0.53 ±0.02	0.58 ±0.02
Litter 6	-/- (n=2)	0.57 ±0.01	0.62 ±0.01	0.61 ±0.01	0.43 ±0.03	0.54 ±0.02
18.5 dpc	+/+ (n=1)	0.67	0.71	0.68	0.56	0.62
Litter 7	-/- (n=4)	0.58 ±0.01	0.62 ±0.03	0.60 ±0.02	0.50 ±0.02	0.55 ±0.02
18.5 dpc	+/+ (n=2)	0.64 ±0.03	0.71 ±0.01	0.68 ±0.00	0.57 ±0.02	0.66 ±0.01
Summary	-/- (n=10)					
18.5 dpc	+/+ (n=7)	91±4%	92±6%	92±5%	88±7%	88±9%

[a]Ossification was quantitated as the proportion of total skeletal element length that stains positive with alizarin red S. Independent measurements were made of left and right limbs and averaged. Ossification for each group is expressed as the mean ± s.e.m.

[b]All litters for this analysis contained at least two *Dlx5* homozygotes. Litter 3 contained no wild type embryos; comparison of homozygotes with heterozygotes was still significant.

[c]The summary line represents the combined data for all embryos at each developmental stage and is represented as the percentage of wildtype or heterozygote ossification seen in *Dlx5* $^{-/-}$ littermates (mean ± s.e.m).

specific to chondrocytes since we did not observe a stimulation of differentiation following forced expression of *Dlx5* in an osteoblast cell line (G.H. A.B. & C.A.-S., unpublished data). In summary, our gain-of-function studies reveal that *Dlx5* encodes a general anti-proliferative activity and that it positively regulates chondrocyte differentiation at multiple stages of the pathway, acting at both the recruitment step as well as terminal differentiation.

Chondrogenic Defects in Dlx5$^{-/-}$ *Mice*

Chondrogenic defects in the appendicular skeleton of *Dlx5*$^{-/-}$ mice have not previously been reported (Acampora, *et al.*, 1999; Depew, *et al.*, 1999). In light of our data that *Dlx5* is expressed in those chondrocytes about to commit to terminal differentiation in mouse embryo limbs and that misexpression of *Dlx5* stimulates chondrocyte differentiation *in vitro*, we examined the appendicular skeletons of embryos from *Dlx5* heterozygote crosses. We first examined skeletons that had been stained with alcian blue and alizarin red to reveal the extent of ossification. In all litters examined at 15.5 dpc (n=3 litters), there was a statistically significant reduction in the ossification of all the long bones of the fore- and hindlimbs of *Dlx5* homozygous embryos compared to their wild-type littermates, with the ossified proportion of some limb elements being up to one third less in *Dlx5*$^{-/-}$ embryos ($P < 0.15$, Table 1). In three of four litters examined at 18.5 dpc the reduction in ossification was less pronounced ($P < 0.1$, Table 1) with the fourth litter showing no significant difference between littermates.

To examine the cellular basis for this reduction in ossification, we examined the histology of limbs from wildtype and *Dlx5* homozygote littermates. At 14.5 dpc, the limbs of wildtype embryos contained an obvious zone of hypertrophic cells with early signs of invasion from a thickened bone collar in the middle of the hypertrophic zone (Fig. 5A). In contrast, cartilages from *Dlx5*$^{-/-}$ littermates contained a significantly narrower hypertrophic zone with no sign of invasion (Fig. 5B). Two days later, at 16.5 dpc, the limbs of wildtype embryos had undergone significant ossification in which hypertrophic chondrocytes flanked a central region containing trabecular bone (Fig. 5C). The limbs of *Dlx5*$^{-/-}$ littermates had also begun to mineralize, but the ossified region occupied a smaller proportion of the skeletal element compared with wildtype or heterozygote littermates (Fig. 5D). Cell counts of BrdU-labeled chondrocytes in the growth plates of wild-type and *Dlx5*$^{-/-}$ embryos demonstrated that there was no significant difference in the proportion of proliferating chondrocytes in the absence of *Dlx5* (data not shown).

We next examined molecular markers of hypertrophic chondrocytes to establish the basis for this phenotypic difference. At 14.5 dpc, the expression domains of both *Ihh* and *Col10α1* were narrower by approximately 30% in the cartilages of *Dlx5*$^{-/-}$ limbs compared with wildtype and heterozygous littermates (Fig. 5E,F and data not shown), confirming that there is a smaller pool of chondrocytes actively differentiating in the absence of *Dlx5*. At 16.5 dpc, the area demarcated by *Ihh* and *Col10α1* remained about 30% narrower in *Dlx5*$^{-/-}$ embryos compared with wildtype and heterozygous littermates, indicating an ongoing delay in chondrocyte differentiation (Fig. 5G,H and data not shown). In summary, our analysis of mouse embryos demonstrates that *Dlx5* is required for normal levels of chondrocyte differentiation and that reduced ossification in the appendicular skeleton of *Dlx5*$^{-/-}$ mice is consistent with the observed delay in chondrocyte hypertrophy.

Discussion

The observed expression of *Dlx5* in a variety of mineralizing tissues - teeth, intramembranous bones of the skull, and the perichondrium/periosteum of the endochondral skeleton (Simeone, *et al.*, 1994; Zhao, *et al.*, 1994; Ferrari, *et al.*, 1995; Chen, *et al.*, 1996; Ryoo, *et al.*, 1997) – has prompted a close examination of its function during osteogenesis (Ryoo, *et al.*, 1997; Newberry, *et al.*, 1998; Acampora, *et al.*, 1999; Depew, *et al.*, 1999; Miyama, *et al.*, 1999; Benson, *et al.*, 2000). Here, we have shown that *Dlx5* is also expressed in regions corresponding to proliferating and differentiating chondrocytes in the cartilaginous core of appendicular skeletal elements and we provide evidence that it is required for chondrocyte differentiation during endochondral ossification in mice.

We have characterized the expression pattern of *Dlx5* in the chondrogenic core of developing elements of the appendicular skeleton with respect to markers of chondrocyte maturation. *Dlx5* expression is activated early in condensing limb mesenchyme. Indeed, we could not distinguish the temporal order of activation of *Dlx5* and *Sox9*, the earliest known gene to be activated in chondrogenic mesenchyme. We note that *Sox9* expression in early limb buds was not obviously affected following misexpression of *Dlx5* (data not shown); however, the latency period between infection and high level expression of *Dlx5* may have masked a potential effect. Similarly, we were unable to observe differences in *Sox9* expression in *Dlx5*$^{-/-}$ embryos although, in this case, *Dlx6* may have functionally compensated for loss of *Dlx5*. Therefore, while we cannot rule out the possibility that *Dlx5* also regulates recruitment of limb mesenchyme to the chondrocyte lineage by acting upstream of *Sox9* or in a parallel pathway, a direct demonstration of *Dlx* function in recruitment of limb mesenchyme to the chondrogenic lineage *in vivo* remains to be demonstrated and is probably best pursued in mice lacking both *Dlx5* and *Dlx6* function. In contrast, *Dlx5* expression did not accompany *Sox9* and *Col2α1* expression in early vertebral chondrocytes surrounding the notochord. These chondrocytes arise from somitic rather than lateral plate mesoderm, having migrated from the adjacent sclerotome to surround the notochord before differentiating into chondroblasts. Interestingly, the homeobox gene *Nkx3.2/Bapx1* has a reciprocal pattern of expression, being expressed in the perinotochordal mesenchyme prior to its differentiation into chondroblasts but not in *Sox9*-positive condensing limb mesenchyme until overt chondroblast differentiation has occurred (Murtaugh, *et al.*, 2001). Thus, pre-chondrogenic mesenchyme of different origin appears to express unique combinations of regulatory factors.

In skeletal elements of mid-stage chicken embryos, where chondrocytes differentiate in a spatially ordered manner, *Dlx5* expression is restricted to mitotically active chondroblasts in the flattened zone and in post-mitotic prehypertrophic and differentiated hypertrophic chondrocytes, as well as in the perichondrium. The *Dlx5* expression domain initially overlaps with that of *Ihh* in mouse embryo limbs but is subsequently excluded from the *Ihh*-positive pre-hypertrophic zone, being confined to the flattened and hypertrophic zones, and finally the flattened and endosteal front in more mature growth plates. Despite these differences in the expression patterns, in both species *Dlx5* is expressed at a critical time in chondrocyte maturation, namely in the proliferating chondrocytes of the morphologically distinct flattened zone. Cells

that leave this zone activate *Ihh* and become committed to terminal differentiation. Thus, *Dlx5* may regulate a set of target genes that triggers the switch to hypertrophy. We think it unlikely that *Ihh* itself is one of the direct targets of Dlx5 regulation since *Ihh* continues to be expressed in *Dlx5*$^{-/-}$ limbs (Acampora, *et al.*, 1999). The overlapping expression of *Dlx5* with the *Ihh*-target gene *ptc* suggests that, alternatively, *Dlx5* may be responsive to Ihh signaling. We think that this is also unlikely since we observed *Dlx5* expression in the immature chondrocytes of *Ihh*$^{-/-}$ limbs (data not shown). The absence of *Dlx5* expression in the *Ihh*-positive domain of later stage mouse embryonic limbs suggests that once Dlx5 target genes are activated (or repressed) differentiating chondrocytes may not require continuous expression of *Dlx5* for hypertrophy to proceed.

We show that *Dlx5* encodes a general anti-proliferative activity; misexpression of *Dlx5* reduced the proliferative index of both fibroblasts and chondrocytes. This accounts for the absence of detectable *Dlx5* expression in the epiphyseal pool of proliferating chondroblasts and explains our observation that loss of *Dlx5* function in mouse embryos had no effect on the proliferation of chondroblasts in the epiphysis of skeletal elements. Thus, the initial phase of *Dlx5* expression in chondrogenic mesenchyme gives way to a more restricted pattern of expression in spatially separated populations of chondrocytes at different stages of maturation. A pattern of early expression in progenitors that later becomes restricted to specific populations of cells that are undergoing differentiation (or are competent to do so) is also a feature of *Dlx5* gene expression in the forebrain (Simeone, *et al.*, 1994; Liu, *et al.*, 1997; Acampora, *et al.*, 1999; Depew, *et al.*, 1999; Eisenstat, *et al.*, 1999) and underlines a common theme of *Dlx5* gene activity in the regulation of the differentiated cell phenotype in multiple tissues. Consistent with this biphasic expression pattern, we also show that *Dlx5* can function at two discrete points of chondrogenic differentiation, namely the recruitment of pluripotent mesenchyme to the chondrocyte lineage and the terminal differentiation of chondrocytes to a mineralizing and alkaline phosphatase-secreting phenotype.

The smaller domain of *Col10α1* expression in *Dlx5*$^{-/-}$ embryo limbs at 14.5 dpc directly implicates *Dlx5* in the normal progression of proliferating to hypertrophic chondrocytes. By 16.5 dpc however, the levels of *Col10α1* appear more like those in wild type littermates. This observation is consistent with the ossification data shown in Table 1 where a larger effect was seen on mineralization in 14.5 dpc *Dlx5*$^{-/-}$ limbs compared with those from 18.5 dpc embryos. Together, these observations suggest the existence of a mechanism that partially compensates for loss of *Dlx5* function at later stages of embryonic skeletogenesis. Such a mechanism may involve the perichondrium/periosteum which is a known source of negative signals for chondrocyte hypertrophy (Di Nino *et al.*, 2001). One consequence of loss of *Dlx5* expression in the perichondrium may therefore be to impair the ability of this tissue to negatively regulate chondrocyte hypertrophy.

In combination with a recent study in chicken embryos (Ferrari and Kosher, 2002), this study reveals the conserved function of *Dlx5* as a positive regulator of chondrocyte differentiation in vertebrates. Furthermore, since *Dlx5* has also been implicated in the positive regulation of osteoblast differentiation (Ryoo, *et al.*, 1997; Miyama, *et al.*, 1999; Benson, *et al.*, 2000), *Dlx5* should now be viewed as a general regulator of differentiation during endochondral ossification. Indeed, we cannot formally exclude the possibility that the observed reduction in ossification of skeletal elements in *Dlx5*$^{-/-}$ embryos in part reflected a loss of *Dlx5* function in osteogenic precursors. The runt domain gene *Cbfa1* also regulates the differentiation of both chondrocytes and osteoblasts. Indeed, it is noteworthy that expression of *Cbfa1* in maturing chondrocytes is quite similar to the pattern described here for *Dlx5* (Kim, *et al.*, 1999). While the appendicular skeleton of *Cbfa1*$^{-/-}$ mice shows a more pronounced delay in chondrocyte hypertrophy and endochondral ossification compared with *Dlx5*$^{-/-}$ embryos (Inada, *et al.*, 1999; Kim, *et al.*, 1999; Takeda, *et al.*, 2001), the *Cbfa1*$^{-/-}$ phenotype is similar to that seen in mice with targeted deletion of both *Dlx5* and *Dlx6* genes (Merlo, *et al.*, 2002; Robledo, *et al.*, 2002). Teasing apart the different contributions to skeletogenesis of multifunctional genes like *Dlx5* will be the focus of further studies.

Materials and Methods

Retroviral Constructs

Avian retroviruses, *RCASBP(A)-Dlx5* and *RCASBP(A)-Dlx5A*, were made by cloning Myc-tagged *Dlx5* or *Dlx5A* into the *Bam*HI and *Hin*dIII sites of the *SLAX13* shuttle vector (Hughes, *et al.*, 1987) then subcloning into *RCASBP(A)* (Hughes, *et al.*, 1987; Fekete and Cepko, 1993) as *Cla*I fragments. Mammalian retroviruses were constructed in a derivative of *LZRSpBMN-Z* (Kinsella and Nolan, 1996) in which the *lacZ* gene was excised to make *pLZRSΔ* (Hu, *et al.*, 2001). Sequences corresponding to the coding region of *Dlx5* or a mutated *Dlx5* derivative, *Dlx5A*, (having mutations K138A, R140A, Y143A in the N-terminal arm of the homeodomain) were engineered to add a Myc epitope at the amino-terminus and subcloned as *Bam*HI-*Hin*dIII fragments into *pLZRSΔ*. The complete sequences of all PCR-amplified constructs were confirmed. Expression of retroviral-expressed proteins was verified by western blot analysis using lysates from infected cells.

Retroviral Gene Transfer and Cell Assays

Replication-competent *Dlx5*-expressing avian retroviruses that were high-titre and active were difficult to grow since *Dlx5* reduces CEF proliferation rates and spontaneous mutants therefore confer a significant growth advantage. Retroviruses used in this study were generated using a modified procedure from that described previously (Fekete and Cepko, 1993). The transfection of CEFs with *RCAS* plasmids was scaled up to a 176 cm^2 dish. Transfected cells were passaged in a single 1:4 split and allowed to reach confluence before collecting viral supernatants in low serum medium. Concentrated viral stocks from this procedure had titres of $1x10^8$ – $2x10^8$ cfu/ml.

Proliferation assays were done with CEFs infected with RCAS retrovirus or left uninfected. Cells were plated at low density in media containing 2% or 4% fetal bovine serum. Cell number was quantitated daily from the optical density of Napthal blue-black stained cells.

Micromass assays were performed essentially as described (Ahrens, *et al.*, 1977). White Leghorn chicken eggs (SPAFAS) were incubated to stage 21-22 (Hamburger and Hamilton, 1951). Forelimb buds were surgically removed and digested with 0.1% trypsin/0.1% collagenase. 10 μl of digested cells ($2x10^7$ cells/ml) were infected with 1 μl of concentrated avian retroviruses (~10^8 cfu/ml) in growth media (F12/DMEM supplemented with 10% FBS) containing 8 μg/ml polybrene. Infected cells were incubated for 4 days and chondrocyte differentiation was visualized by staining with 0.5% Alcian Blue 8GX (pH 1).

Replication-defective mammalian retroviruses were made in Phoenix ecotropic retroviral packaging cells (ATCC) by transfection of the relevant *pLZRSΔ* plasmids using Lipofectamine Reagent (Life Tech.). Viral supernatants were collected 24 hours after transfected cells reached confluence. Target cells were seeded one day prior to infection at low density ($1 x 10^4/cm^2$)

and infected with viral supernatants on two consecutive days at a multiplicity of infection of 20-50; 4 μg/ml polybrene was added to enhance infectivity. TMC23 cells (Xu, *et al*., 1998) were grown in DMEM supplemented with 10% FBS. Differentiation was induced by treating confluent cells with 50 μg/ml ascorbic acid and 4 mM β-glycerol phosphate for 16 days.

Retroviral Infection of Chicken Embryos and Proliferation Assays

Retrovirus injection of chicken embryos was done as previously described (Bendall, *et al*., 1999). Expression of exogenous proteins was confirmed by homogenizing wings in high salt buffer (50 mM Tris, pH 8, 500 mM NaCl, 1% NP-40) followed by western blotting of extracts and detection with anti-Myc antiserum. *Proliferation assays:* For chicken, BrdU was injected into the membranous space around chicken embryos (1.5 mg per egg) and the embryo fixed 80 min later. For mouse, BrdU in 7 mM NaOH, 0.9% NaCl was injected intraperitoneally into pregnant mice (50μg/g body weight) and mothers were sacrificed 30 min later. Embryonic mouse and chicken limbs were dissected, fixed overnight in Bouin's fixative, and embedded in paraffin. BrdU immunohistochemistry was as follows. Sections were deparafinized in xylene and rehydrated, trypsinized in 0.125% trypsin for 50 min at 37°C, and denatured with 2N HCl for 30 min at room temperature. Primary antibody incubation with 1:75 anti-BrdU (BD Immunocytometry Systems) was for 30 min at room temperature followed by secondary antibody incubation with 1:200 biotinylated anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc.) for 45 min at room temperature. Signal was detected with ABC and Nova Red kits (Vector Labs).

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