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Developmental Expression Pattern

Expression of *Dlx5* and *Dlx6* during specification of the elbow joint

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ABSTRACT The onset of elbow joint formation in the developing limb is characterized morphologically by the conversion of differentiated chondrocytes at the site of incipient joint formation into the densely packed flattened cells of the joint interzone. However, experimental studies have indicated that the elbow joint is specified well before joint interzone formation by a distinctive population of precursor cells located at the site in the developing limb bud at which the elbow joint will subsequently form. Here we show that during specification of the elbow joint in the chick limb bud, the homeodomain transcription factors *Dlx5* and *Dlx6* are highly expressed by a discrete group of cells that encompass the prospective elbow joint. The *Dlx5*- and *Dlx6*-expressing cells at the prospective elbow joint are located where the differentiating humerus branches into the radius and ulna. Thus, *Dlx5* and *Dlx6* are the earliest molecular markers of the presumptive elbow joint yet described. The onset of *Dlx5* expression in the region of the presumptive elbow joint is shortly followed by the initiation of expression amongst the *Dlx5*-expressing cells of *Gdf5*, which encodes a secreted signaling molecule that is involved in regulating the onset of joint formation. These results suggest that *Dlx* genes may be involved in specification of the elbow joint and/or in providing positional information that specifies the site at which the elbow joint will form.

KEY WORDS: *joint development, Dlx5, Dlx6, Gdf5, skeletal development, limb development*

The onset of formation of synovial joints in the developing vertebrate limb is initiated by the segmentation of continuous cartilaginous skeletal rudiments into two or more separate elements. For example, the humerus, radius and ulna are initially formed as an uninterrupted Y-shaped unit in which the radius and ulna branch from the humerus and the separation of this single Y-shaped rudiment into three distinct elements occurs as a result of the formation of the elbow joint between the distal end of the humerus and the proximal ends of the radius and ulna.

Joint formation is initiated by the conversion of differentiated chondrocytes at sites of presumptive joints into narrow bands of densely packed flattened cells called the joint interzone (Mitrovic, 1977, 1978; Craig *et al.,* 1997; Koyama *et al.,* 1995; Hartmann and Tabin, 2001; Lizarraga *et al.,* 2002). In recent years, several secreted signaling molecules including Gdf5 (Storm and Kingsley, 1996), Gdf6 (Settle *et al.,* 2003), Wnt-14 (Hartmann and Tabin, 2001; Guo *et al.,* 2004), stanniocalcin (Stasko and Wagner, 2001) and noggin (Brunet *et al.,* 1998), as well as transcription factors including Cux1 (Lizarraga *et al.,* 2002), ERG-3 (Iwamoto *et al.,* 2000) and δEF1 (Takagi *et al.,* 1998) have been implicated in regulating joint interzone formation.

Although the formation of the joint interzone is the first morphological sign of overt joint formation, experimental studies have indicated that joints, in particular the elbow joint, may be specified very early in limb development well before interzone formation occurs (Holder, 1977). Holder (1977) has found that when the prospective elbow joint is removed at an early stage of development of the chick limb bud, the elbow joint fails to form and the humerus is subsequently fused with the radius and ulna. This suggests that joint formation is dependent on cells present in the prospective elbow joint region that had been specified to form a joint very early in development (Holder, 1977). Virtually nothing is known about the mechanisms involved in specification of the elbow joint or about the genes and signals involved in joint specification or in determining the position at which the joint will develop. It has been suggested that homeobox genes, which provide patterning cues in multiple developing systems, are likely candidates for specifying the locations at which joints will form (Yokouchi *et al.,* 1991; Brunet *et al.,* 1998; Pacifici *et al.,* 2002, 2005).

Abbreviations used in this paper: AER, apical ectodermal ridge; pej, prospective elbow joint; UTR, untranslated region.

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In the present study we show that during early stages of chick limb development well before the onset of overt joint formation the homeobox-containing genes *Dlx5* and *Dlx6* are highly expressed by a discrete group of cells that encompass the prospective elbow joint where the differentiating humerus rudiment branches into the radius and ulna anlage. The onset of *Dlx5* expression in the region of the presumptive elbow joint is shortly followed by the initiation of expression amongst the *Dlx5*-expressing cells of *Gdf5*, which encodes a secreted signaling molecule that is involved in regulating the onset of joint formation (Storm and Kingsley, 1996; Settle *et al.,* 2003). These observations suggest that *Dlx5* and *Dlx6* may provide positional information that specifies the site at which the elbow joint interzone will subsequently form.

Results

During early stages of chick limb bud development (stages 23 through 26), *Dlx5*, which is one of six members of the Dlx family of homeodomain transcription factors, exhibits several discrete domains of expression. The domains *Dlx5* expression include the apical ectodermal ridge (AER) which is directing the outgrowth and patterning of the underlying mesoderm and mesoderm along the anterior and posterior margins of the limb bud (Fig. 1A; see also Ferrari *et al.,* 1995). *Dlx6* a member of the Dlx family which is located immediately contiguous to *Dlx5* on the same chromosome is also expressed in the AER and in the mesoderm at the anterior and posterior margins of the limb bud (Fig. 1B). In addition, at these early stages *Dlx5* and *Dlx6* exhibit a discrete domain of expression in the mid-proximal central core of the wing bud (Fig. 1) which based on fate maps appears to be in the vicinity of the prospective elbow joint. *Dlx5* is also expressed in the vicinity of the prospective knee joint in the leg bud (data not shown).

To pursue further the possible association between *Dlx* gene expression and the presumptive elbow joint, we examined the temporal and spatial pattern of *Dlx5* expression during the initial formation of the Y-shaped humerus, radius and ulna rudiment. In this analysis we compared by *in situ* hybridization the expression of *Dlx5* with that of the cartilage marker type II collagen (*Col2a1*) in adjacent sections of the same limb buds throughout early stages of limb development when elbow joint specification is occurring (Fig. 2). We have found that at these early stages of limb development well before the onset of overt

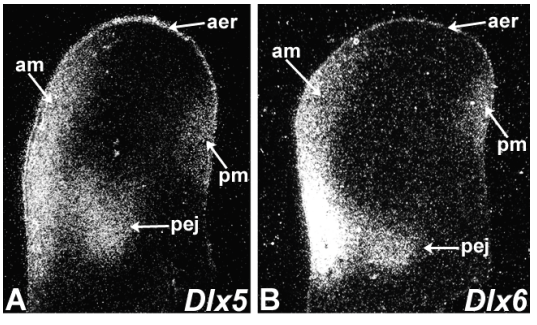

Fig. 1 (Left). Expression domains of *Dlx5* (A) and *Dlx6* (B) in the developing chick wing bud at stage 25 (A) and stage 26 (B). *Both genes are expressed in the apical ectodermal ridge (aer), mesoderm at the anterior (am) and posterior margins (pm) and by a discrete group of cells in the mid-proximal central core that are in the vicinity of the prospective elbow joint (pej).*

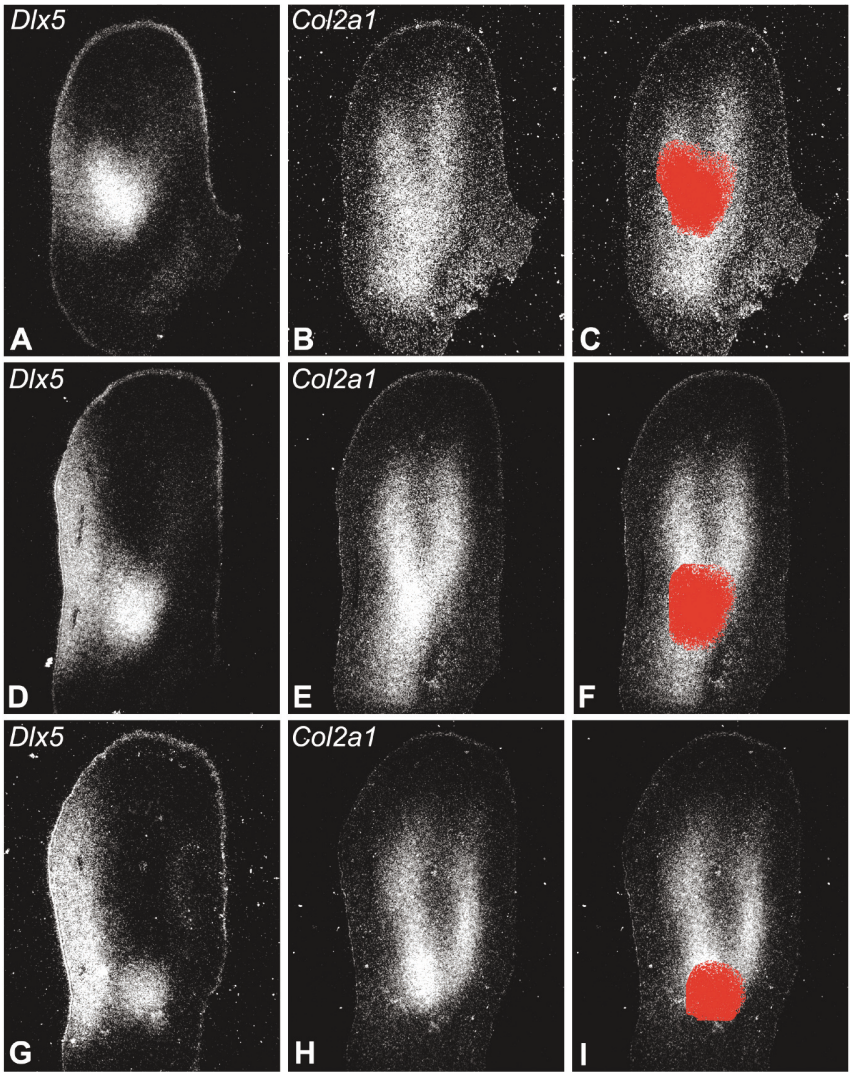

Fig. 2 (Right). *Dlx5* is expressed at the prospective elbow joint during early stages of limb bud development. *Expression of* Dlx5 *and the cartilage marker* Col2a1 *in adjacent sections of the same limb buds at stage 24* **(A,B)**, *stage 25* **(D,E)** *and stage 26* **(G,H)**. *The images in* **(C,F,I)** *were generated by superimposing and aligning the images of adjacent* Dlx5- *and* Col2a1*-expressing sections (A,B) (D,E) (G,H) using Adobe Photoshop, after which the* Dlx5 *expression domain in the vicinity of the prospective elbow joint was selected, pseudocolored red and superimposed on the image of the adjacent* Col2a1 *section. Note that* Dlx5 *is expressed by a discrete group of cells that encompass the prospective elbow joint where the radius and ulna branch from the humerus.*

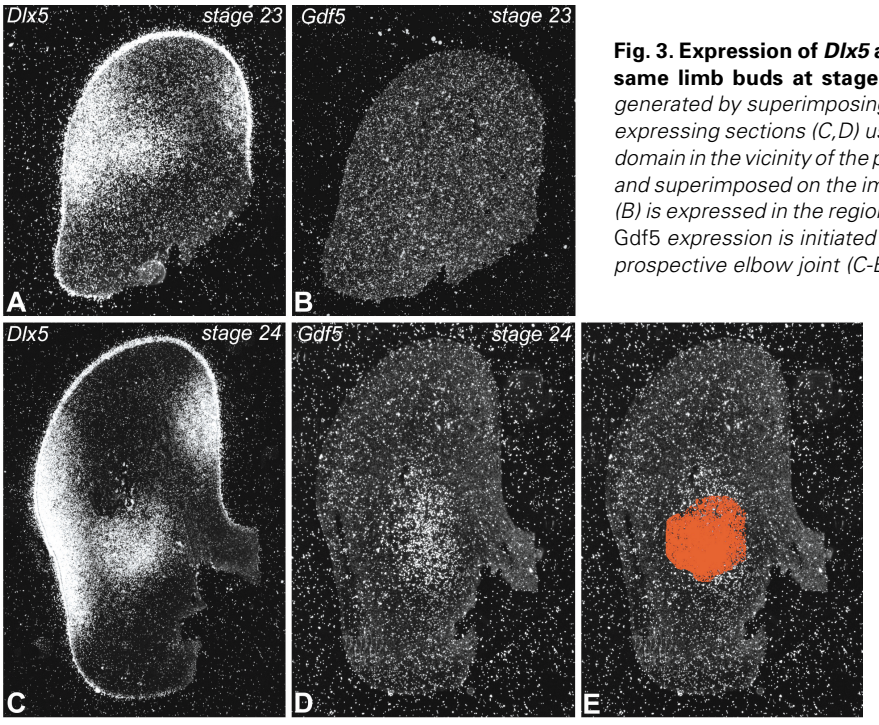

Fig. 3. Expression of *Dlx5* and the joint marker *Gdf5* in adjacent sections of the same limb buds at stage 23 (A,B) and stage 24 (C,D). *The image in* **(E)** *was generated by superimposing and aligning the images of adjacent* Dlx5- *and* Col2a1-*expressing sections (C,D) using Adobe Photoshop, after which the* Dlx5 *expression domain in the vicinity of the prospective elbow joint was selected, pseudocolored red and superimposed on the image of the adjacent* Gdf5 *section.* Dlx5 *(A), but not* Gdf5 *(B) is expressed in the region of the prospective elbow joint at stage 23. At stage 24,* Gdf5 *expression is initiated amongst the* Dlx5*-expressing cells in the vicinity of the prospective elbow joint (C-E).*

joint formation *Dlx5* does appear to be expressed by a discrete group of cells that encompass the region of the limb bud where the elbow joint will subsequently form (Fig. 2A, D, G). The *Dlx5*-expressing cells in the area of the presumptive elbow joint are located where the differentiating humerus branches into the radius and ulna (Fig. 2B, E, H). To more accurately confirm the localization of this *Dlx5* expression domain at the presumptive elbow joint, the images of adjacent *Dlx5*- and *Col2a1*-expressing sections were superimposed in Abode Photoshop and precisely aligned, after which the *Dlx5* expression domain in the vicinity of the prospective elbow joint was selected, psuedocolored red and superimposed on the adjacent *Col2a1*-expressing image. As shown in Figs. 2C, F, I, this analysis confirms that this domain of *Dlx5* expression indeed encompasses the prospective elbow joint where the humerus bifurcates into the radius and ulna.

The *Col2a1* probe (Nah *et al.,* 1988) used to monitor the formation of the Y-shaped humerus, radius and ulna rudiment does not distinguish between two alternatively spliced forms of *Col2a1* transcripts, *Col IIA* which is typically expressed by prechondrogenic as well as chondrogenic cells and *Col IIB* which is expressed by more differentiated chondrocytes (Ryan and Sandell, 1990; Nah and Upholt, 1991; Ng *et al.*, 1993). However, the Y-shaped humerus, radius and ulna rudiment at these early stages of cartilage differentiation is also characterized by the expression of other cartilage markers such as *aggrecan* (Mallein-Gerin *et al.*, 1988).

Our results indicate that *Dlx5* is expressed in the presumptive elbow joint during the period of development when the elbow joint is being specified. To further pursue the association of *Dlx5* with elbow joint formation, we next compared the temporal expression in adjacent sections of the same limb buds of *Dlx5* with that of the secreted signaling molecule *Gdf5*, which is expressed at the onset of joint interzone formation and is one of the earliest known markers of joint formation (Storm and Kingsley, 1996; Merino *et al.,* 1999; Lizarraga *et al.,* 2002). The expression of *Dlx5* is first detectable in the area of the prospective elbow joint at stage 23 (Fig. 3A) at which time *Gdf5* is not expressed in that region (Fig. 3B). However, at about stage 25 shortly after the onset of *Dlx5* expression in the prospective elbow joint region, the expression of *Gdf5* is initiated amongst the *Dlx5* expressing cells (Fig. 3C-E). Subsequently, at the onset of overt elbow joint formation at stage 27, *Gdf5* is expressed in the elbow joint interzone and *Dlx5* expression is downregulated in the elbow joint interzone (data not shown and Lizarraga *et al.,* 2002).

Discussion

The conversion of differentiated chondrocytes at sites of incipient joint formation into the densely packed cells of the joint interzone is the first morphological indication of joint formation in the skeletal elements of the developing limb and several of the genes and signals that regulate interzone formation have been identified (see Pacifici *et al.,* 2002, 2005 for reviews). However, little or nothing is known about the genes, signals, or mechanisms that specify the positions within cartilage rudiments at which joint interzones will form.

The experimental studies of Holder (1977) indicate that the elbow joint is specified very early in limb development by a distinctive population of precursor cells located at the site at which the elbow joint interzone will subsequently form. When the presumptive elbow joint is removed at an early stage (stage 24) of chick limb bud development well before elbow joint interzone formation (stage 27), the elbow joint fails to form. This indicates that joint formation is dependent on cells in the presumptive elbow joint region that had been specified to form a joint very early in limb development. Furthermore, the studies of Holder (1977) suggest that the presumptive elbow joint may be capable of autonomously differentiating into joint cells, suggesting that the interzone may be derived from a distinct precursor cell type that differentiates according to information acquired at earlier stages. Interestingly, the limbs of *Gli3/Plzf* double knockout mouse embryos lack proximal (stylopod and zeugopod) skeletal elements, but nevertheless possess a single proximal cartilaginous ball of cells resembling a joint that expresses joint markers including *Gdf5*, suggesting the joint

may be derived from a distinct population of precursor cells (Barna *et al.,* 2005).

In the present study we demonstrate that at early stages of chick limb development well before the onset of overt joint formation the homeodomain transcription factors *Dlx5* and *Dlx6* are highly expressed by a discrete group of cells that encompass the region of the limb bud at which the elbow joint will subsequently form. The *Dlx5*- and *Dlx6*-expressing cells at the prospective elbow joint are located where the differentiating humerus branches into the radius and ulna. Thus, Dlx5 and Dlx6 are the earliest molecular markers of the prospective elbow joint yet described. The *Dlx-5*- and *Dlx6*-expressing cells we have identified correspond quite well to the presumptive elbow joint cells whose removal at an early stage results in subsequent fusion of the humerus with the radius and ulna and failure of elbow joint formation (Holder, 1977). This striking correspondence suggests the possibility that the *Dlx*-expressing cells located at the presumptive elbow joint constitute a distinctive population of cells that play an important role in the specification of the elbow joint and/or in providing positional information that specifies the site at which the elbow joint will form.

One possible mechanism by which *Dlx* genes may specify elbow joint formation is by regulating the expression of signaling molecules that control the onset of joint interzone formation. Indeed, we have found that shortly after the onset of expression of *Dlx5* at the presumptive elbow joint, the expression of *Gdf5*, a secreted signaling molecule that regulates the onset of joint interzone formation, is initiated amongst the *Dlx5*-expressing cells. This suggests the possibility that *Dlx* genes may be involved in regulating the initiation of *Gdf5* expression and/or in specifying the site at which *Gdf5* expression initiates.

It has previously been suggested that homeobox genes may determine the position at which joints form (Yokouchi–*et al.,* 1991; Brunet *et al.,* 1998; Pacific *et al.,* 2002, 2005). Interestingly, the expression domain of *Dlx* genes at the prospective elbow joint corresponds to the site where the expression domains of members of the *HoxA* and *HoxD* clusters intersect (Yokouchi *et al.,* 1991). Thus, *Dlx* genes may act in conjunction with *HoxA* and *HoxD* genes in providing positional cues which determine the site of elbow joint formation and/or the site of bifurcation of the humerus.

Experimental Procedures

In situ hybridization was done as previously described (Ferrari *et al.,* 1999, 2002) on serially sectioned limb buds using the following ^{33}P-labeled probes: a 323 bp *Dlx5*-specific probe from the 3' untranslated region (UTR) of chicken *Dlx5* (Ferrari *et al.,* 1995); a 1 kb *Dlx6* probe consisting of 240 bp of exon 3 and 760 bp of 3' UTR from a chicken *Dlx6* genomic clone; a 328 bp *Gdf5*-specific probe prepared as previously described (Lizarraga *et al.,* 2002) and a chicken *Col2a1* probe (Nah *et al.,* 1988). In order to correlate the domains of expression of different genes, adjacent sections (within 20 μm of one another) of the same limb buds were mounted on separate slides and hybridized with different probes.

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