Multiple functions of Dlx genes

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ABSTRACT Dlx genes comprise a highly conserved family of homeobox genes homologous to the distal-less (Dll) gene of Drosophila. They are thought to act as transcription factors. All Dlx genes are expressed in spatially and temporally restricted patterns in craniofacial primordia, basal telencephalon and diencephalon, and in distal regions of extending appendages, including the limb and the genital bud. Most of them are expressed during morphogenesis of sensory organs and during migration of neural crest cells and interneurons. In addition, Dlx5 and Dlx6 are expressed in differentiating osteoblasts. Gene targeting of Dlx1, Dlx2, Dlx3 and Dlx5 in the mouse germ-line has revealed functions in craniofacial patterning, sensory organ morphogenesis, osteogenesis and placental formation. However, no effect on limb development has yet been revealed from gene inactivation studies. A role for these genes in limb development is however suggested by the linkage of the Split Foot/Hand Malformation human syndrome to a region containing DLX5 and DLX6. As for most transcription factors, these genes seem to have multiple functions at different stages of development or in different tissues and cell types.

KEY WORDS: Dlx, craniofacial, limb, osteogenesis, brain development.

Introduction

The Distal-less (Dll) gene of Drosophila encodes a homeodomain protein expressed in leg primordia of the thoracic segments and in anterior regions of the Drosophila embryo (Cohen et al., 1989; O’Hara et al., 1993). During insect limb development Dll is expressed in the center of the outgrowing leg primordium and in the distal segments of the leg, throughout the entire larval stage (Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997). Distal-less mutant Drosophila show various extents of size reduction and dysmorphogenesis of distal segments of the legs in the adult fly, indicating the Dll activity is required during early larval stages for the development of the entire limb and for correct proximo-distal (PD) organization (Cohen and Jurgens, 1989; Cohen et al., 1989). Homeotic genes of the Bithorax complex repress Dll transcription in the abdominal segments (Vachon et al., 1992). Presumably this mechanism is at the basis of the absence of legs in posterior Drosophila abdominal segments (Carrol, 1994; Panganiban et al., 1997).

In anterior regions of the Drosophila embryo, Dll is expressed in the antenarial, maxillary and labial primordia. Dll-mutant flies show abnormalities of these appendages consistent with a PD growth and morphogenesis defect (Sunkel and Whittle, 1987; Cohen, 1990). It has been shown that Dll is activated by the HOM gene deformed, in the maxillary primordium. In the insect, therefore, there is a different regulation of Dll in gnathal and thoracic segments.

Dll-related genes have been identified and cloned in several species, from Hydra to man. In the mouse, there are six known Dlx genes arranged as pairs facing each other through the 3’ end and located near Hox clusters (Simeone et al., 1994; Nakamura et al., 1996; McGuinness et al., 1996; Liu et al., 1997). The spatial expression of these genes in vertebrates is somehow reminiscent of that observed in insects. Namely they are expressed during limb bud development and in head structures (pharyngeal arches, olfactory epithelium, etc.). Although recent data obtained by gene targeting in the mouse begin to cast some light on the role of distal-less-related genes in mammals, their function and mode of action, as well as the regulatory cascades of which they are part, remain still to be defined.

Sequence, structure and organization of Dlx genes in vertebrates

Vertebrate Dlx genes share a highly conserved homeodomain (Fig. 1A) with the Drosophila distal-less gene. Dlx genes in mouse...
and man are linked in pairs, in a tandem convergent configuration, in the following order: Dlx1 and Dlx2; Dlx5 and Dlx6; Dlx3 and Dlx7. The only exception appears to be the DLX4 gene, that is located on the same chromosomal region as DLX3 and DLX7, but for which no tandem partner has been reported. Within each pair, one member shows a higher degree of homology to one gene of another pair, rather than the other gene on the same pair. This has led to a subdivision of the Dlx gene family in two subfamily, one including Dlx1, -6, -7, and -4, the other including Dlx2, -5, -3 (Fig. 1B). These data can be interpreted as an indication of an initial distal-less duplication event that occurred in the early chordates, that yielded an ancestor tandem, and a series of subsequent duplications of the entire tandem to yield the mammalian configuration. The analysis of distal-less related genes in different vertebrate species substantially supports this hypothesis (Stock et al., 1996).

Further support for this notion comes from the finding that the Drosophila distal-less gene is located near the HOM-C complex. In human, the DLX3 and DLX7 genes are located on chromosome 17q21, as is Dlx4 (Scherer et al., 1995; Nakamura et al., 1996; Quinn et al., 1997; Morasso et al., 1997), near the HOX-B homeobox gene cluster. The DLX1 and DLX2 genes are linked to the HOX-D gene cluster on chromosome 2 (McGuinness et al., 1996), while the DLX5 and DLX6 genes are linked to the HOX-A cluster on chromosome 7 (Simeone et al., 1994). The same linkage of Dlx genes to Hox clusters is respected in the mouse genome.

It has been observed that the expression of linked Dlx genes is in general very similar or indistinguishable (Simeone et al., 1994; Chen et al., 1996; Ellies et al., 1997a). This suggests that linked Dlx genes may share cis-acting sequences that control their pattern of expression in the embryo and in the adult (Zerucha et al., 2000).

Distal-less proteins are transcription factors

Dlx proteins share similar DNA-binding properties in vitro (Liu et al., 1997; Zhang et al., 1997; Feledy et al., 1999) and are expected to act as homeodomain transcription factors. Transcriptional activation by Dlx3 protein on reporter construct in vitro depends on two subdomains located on either sides of the homeobox (Feledy et al., 1999).

The transcriptional activity of Dlx proteins might be modulated by other proteins. In particular, Msx homeoproteins have been shown to bind to Dlx in vitro. The binding is mediated by their homeodomain, and results in a mutual functional antagonism. It is important to note that this effect can only take place in vivo in cells in which the two proteins are co-expressed. Indeed partial co-expression of Msx and Dlx genes occurs in the apical ectodermal ridge (AER) and underlying mesenchyme of the limb bud and in the pharyngeal arches (Zhang et al., 1997). However the resolution of in situ hybridization experiments is not sufficient to warrant for their cellular coexpression and hence their reciprocal inhibition in vivo. If the hypothesis of mutual inhibition is correct then some of the defects observed in Msx and Dlx genes occurs in the apical ectodermal ridge (AER) and underlying mesenchyme of the limb bud and in the pharyngeal arches (Zhang et al., 1997). However the resolution of in situ hybridization experiments is not sufficient to warrant for their cellular coexpression and hence their reciprocal inhibition in vivo. If the hypothesis of mutual inhibition is correct then some of the defects observed in Msx and Dlx genes occurs in the apical ectodermal ridge (AER) and underlying mesenchyme of the limb bud and in the pharyngeal arches (Zhang et al., 1997). However the resolution of in situ hybridization experiments is not sufficient to warrant for their cellular coexpression and hence their reciprocal inhibition in vivo. If the hypothesis of mutual inhibition is correct then some of the defects observed in Msx (Satokata and Maas, 1994; Houzelstein et al., 1997) or Dlx mutants might be due to improper activation of partner genes. Analysis of Msx / Dlx double mutants will be particularly interesting in this respect.

Dlx genes during limb and appendages development

The expression of the Dll or Dlx homeoproteins seems to be a common features of appendage outgrowth from arthropods to man. Panganiban et al. (1997) have examined the expression of these regulatory genes in protostomes and deuterostomes finding that Dll is expressed along the PD axis of such diverse structures...
as the developing polychaete annelid parapodia, the onychophoran lopopodia, the ascidian ampullae, and even the echinoderm tube feet. In the mouse and chick embryo, the Dlx genes are coexpressed in the AER and in the underlying cells of the progress zone of the developing limb bud (Dollé et al., 1992; Bullfone et al., 1993a; Zhao et al., 1994; Simone et al., 1994; Ferrari et al., 1995; Zhang et al., 1997; Acampora et al., 1999; Ferrari et al., 1999) (Fig. 2A-C). Although there is no evolutionary relation between insect appendages and mammalian limbs, the similarity in terms of territory of expression is striking. Dll/Dlx expression in such diverse appendages could be convergent, but this would have required the independent co-option of Dll/Dlx several times in evolution. Alternatively, appendicular Dll/Dlx expression might have been originated in a common ancestor and been used subsequently to pattern body wall outgrowths in a variety of organisms, including vertebrates. In this regard, it is interesting to note that other non-limb appendages express Dlx genes. For example, the Dlx5 gene is strongly expressed in the external ear lobes of newborn mice (Merlo and Levi, unpublished observations) and in the distal part of the genital bud of the mouse embryo, with a complex pattern reminiscent of that of Hox genes (Fig. 2D,E). This later observation is interesting if we consider that Dll is expressed in the Drosophila genital disk, and that the overall pattern of expression of Dll and of the morphogens wingless and decapentaplegic is similar to that of the leg imaginal disk (Gorfinkiel et al., 1999).

In spite of their strong expression during early and late phases of limb outgrowth, no limb or genital phenotype has been reported for any of the Dlx deficient mice obtained so far (Qiu et al., 1995; Qi et al., 1997; Acampora et al., 1999, Depew et al., 1999). It is conceivable that the various Dlx genes coexpressed in the AER serve some redundant function. If this is the case, the disruption of more than one Dlx genes might be necessary to uncover their function in limb development.

A strong point in favor of a role of Dlx genes in limb development comes from the genetic analysis of families affected by split hand/foot malformations (SHFM). In man DLX5 and 6 genes are considered as candidate genes for certain types of SHFM since they map to the critical interval of SHFM1 on chromosome 7q21.1 (Sherer et al., 1994). Furthermore, in some families, SHFM with complete penetrance is correlated to deletions, inversions or translocations of the chromosomal region 7q21.3-q21.1 (Sherer et al., 1994). We have recently found (Pfeffer et al., submitted) that the first exon of human and mouse DLX6 genes contain a CAG/CCG (polyglutamine/poly-proline) repeat region strikingly similar to the trinucleotide repeat present in the Huntington’s disease gene. This CAG repeat is polymorphic in the normal human population suggesting that DLX6 could have a role in the control of limb patterning. Mutation analysis of Dlx6-null mutant mice will contribute to answer this question.

**Dlx genes in craniofacial development**

The earliest skeletal elements to appear during mammalian skull development are cartilage structures, evolved from modification of ancient elements of more primitive vertebrates (reviewed in: Hanken and Thorogood, 1993), collectively known as chondrocranium. Part of the chondrocranium gives rise to the skeleton around the nose, eye, inner ear, and the base of the brain, and is known as neurocranium. The chondrocranium derived from the branchial arches gives rise to most of the facial skeleton and is known as splanchnocranium. Most of the chondrocranial elements undergo ossification, but some regress (i.e. Meckel’s cartilage of the first arch). A third component of the skull appears later and originates by intramembranous ossification, as is known as dermotocranium. This type of bone formation is characteristic of the calvaria but is seen also around previously formed chondrocranial elements. In Fig. 3A we summarize the developmental origin and the relative position of skeletal elements of the splanchnocranium.

![Fig. 2. Dlx5 expression in extending appendages in the mouse embryo.](image-url)
Fate-mapping studies in the chicken embryo indicate that the dermato- and splanchnocranial originate from cephalic neural crest (CNC)-derived mesenchyme (Le Douarin, 1982; Noden, 1983; Couly et al., 1993; Koentges and Lumsden, 1996). Craniofacial-forming mesenchyme appears to possess early-established positional information. The complex genetic control of CNC migration and differentiation is gradually being elucidated through the analysis of mutant mice.

Members of the Dlx gene family are expressed early in CNC cells and later in craniofacial mesenchyme (Dollé et al., 1992; Bulfone et al., 1993a; Akimenko et al., 1994; Robinson and Mahon, 1994; Simeone et al., 1994; Ellies et al., 1997b; Qiu et al., 1997; Yang et al., 1998; Depew et al., 1999). At 9.5 days of mouse development, Dlx1 and Dlx2 genes are expressed in both the maxillary (proximal) and the mandibular (distal) component of the first pharyngeal arch, whereas the Dlx3, Dlx5 and Dlx6 are expressed only in the mandibular portion of the first branchial arch.

In Fig. 3B–D, we summarize the details the skull defects observed in Dlx1, Dlx2, and Dlx5 deficient mice. Craniofacial defects of Dlx2 mutant mice can be divided in first arch-derived (incus, alisphenoid, maxillary, jugal, squamosal, palatine and pterygoid) and second arch-derived (stapes and styloid) and second arch-derived (stapes and styloid) components of the first pharyngeal arch, whereas the Dlx3, Dlx5 and Dlx6 are expressed only in the mandibular portion of the first branchial arch.

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In the Dlx5 null mutants mice morphological alterations are observed in skeletal elements derived from both the proximal and the distal domains of the first branchial arch (Acampora et al., 1999, Depew et al., 1999). The presence of defects in derivatives of the mandibular arch of Dlx5-/-animals, where also Dlx1, 2, 3 and 6 are expressed at 9.5 dpc suggests that redundancy between Dlx genes is not generalized, but occurs only in specific cases. The results of Dlx1 and Dlx2 knock-outs have led to the proposition that a PD pattern of nested Dlx gene expression might be the basis of PD specification of splanchnocranial skeletal elements (Qiu et al., 1997). Although attractive, this hypothesis appears overly simplified in the light of the Dlx5 knock-out mouse as some craniofacial defects observed in Dlx5-/- mice cannot be simply explained by a PD patterning of arch organization. For example the maxilla and the palate bones are defective both in Dlx2-/- and in Dlx5-/- mice; these bones derive from the proximal part of the maxillary arch where Dlx5 expression is not present at 9.5 dpc (but where is expressed at later stages). In our view, the origin of the molecular patterning within the branchial arches should be seen in a more dynamic perspective. Our data show (Fig. 4) that the territory of expression of Dlx5 in the first branchial arch changes during development. At 9.5 dpc is expressed in the distal part of the mandibular portion of the first arch with low incipient expression in the maxillary arch. In subsequent stages the territory of expression rapidly extends to most of the mandibular and maxillary arch. The expression of the “proximal” Dlx genes (Dlx1, Dlx2) precedes that of the “distal” ones (Dlx3, Dlx5, Dlx6) in the proximal domain of the branchial arches. This dynamic view of Dlx gene expression appears to better explain the morphological defects resulting from Dlx gene disruption in the mouse. A precise knowledge of the timing of expression and of the exact distribution within the arch of sets of genes interacting in a complex spatio-temporal manner will be needed to understand the molecular codes governing craniofacial development. These complex patterns of expression will most probably cause local changes in cell proliferation and/or apoptosis ultimately resulting in the specific morphology of each bone. For example we have found that inactivation of Dlx5 alters the territory with the highest density of proliferating cells within the first branchial
arch. In the absence of Dlx5 this territory results expanded and a new boundary is defined (Acampora et al., 1999). Alteration in cell proliferation might also help in understanding the frequent appearance of additional skeletal elements (referred as “os paradoxicum”, or “strutt”) in Dlx and other mutants (Qiu et al., 1995; Depew et al., 1999; Acampora et al., 1999).

Dlx genes in sensory organs morphogenesis

Several Dlx genes are expressed in the otic vesicle (Robinson and Mahon, 1994; Qiu et al., 1997; Acampora et al., 1999; Depew et al., 1999), the transitory embryonic structure that gives rise to the epithelial and neurosensory component of the vestibular and acoustic organs of the inner ear (Webb and Noden, 1993; Torres and Giraldez, 1998). Dlx5 and -6 expression can be detected as early as the otic pit stage, but is later confined to the dorso-lateral region of the vesicle, which originates the vestibular portion of the inner ear (Fig. 4). Accordingly, mice deficient for Dlx5 show severe dysmorphogenesis of the semicircular canals while little or no defects are observed in the cochlea (Acampora et al., 1999; Depew et al., 1999).

A number of transcription factors are expressed in specific territories of the otic vesicle (Torres and Giraldez, 1998) and may be part of a transcriptional cascades involving Dlx genes. In an effort to identify genes upstream and downstream in the Dlx regulatory cascades that control otic development, mice with targeted inactivation of different genes showing similar inner ear defects may indicate a possible functional relation. For instance, mice deficient for the Nkx-5.1/Hmx3 or for the Dlx5 transcription factors show strikingly similar vestibular defects (Hadrys et al., 1999; Wang et al., 1998; Acampora et al., 1999; Depew et al., 1999). However, in situ hybridization with Dlx5 or with Nkx-5.1 probes on, respectively, Nkx-5.1 or Dlx5 null embryos failed to show changes in their expression profile, making it unlikely that these two genes be part of the same regulatory cascade (Acampora et al., 1999; Zerega and Levi, unpublished observation).

A peculiarity of the Dlx5 and -6 genes is their early expression in defined regions of the frontonasal ectoderm (Yang et al., 1998; Acampora et al., 1999; Depew et al., 1999), in the olfactory placodes, and subsequently in the olfactory and respiratory epithelium that lines the nasal cavities and the vomeronasal organ of the mouse (Fig. 4). At present, defects affecting the olfactory epithelium in Dlx5 deficient mice have not been unequivocally demonstrated. The olfactory placode, similarly to the otic placode, invaginates and delaminates in a complex series of morphogenetic events, induced in part by signals from the surrounding mesenchyme (Webb and Noden, 1993). Although the Dlx5 gene is expressed very early in both the olfactory and otic placodes, its activity does not seem to be required for their initial specification, since invagination and early morphogenesis takes place normally.

Dlx genes in brain development

Several transcription factors are expressed in subpopulations of neurons in the developing forebrain and olfactory bulb. Their restricted domains of expression define distinct regions of the early forebrain. Among these are members of the Dlx gene family (Price, 1993; Bulfone et al., 1993b; Porteus et al., 1994; Tole and Patterson, 1995). Dlx1 and -2 are expressed in cells of the subcortical telencephalon that migrate across the pallial-subpallial limit and enter the mantle and subventricular zone (SVZ) of the cerebral cortex in 12.5 dpc mouse embryos. Mice with a disrupting mutation of the Dlx1 and -2 genes exhibit a reduction in number and a defective differentiation of both striatal projection neurons and neocortical interneurons (Anderson et al., 1997a,b). This supports the current hypothesis that cortical projection neurons and interneurons are derived from distinct regions of the telencephalon (Anderson et al., 1999).

Later in development Dlx1 and Dlx2 are also expressed in the interneurons of the olfactory bulb (Porteus et al., 1994), cells derived from proliferation and migration from the SVZ. The Dlx1/ Dlx2 knockout mice lack mature periglomerular and granule cells of the olfactory bulbs, represented by GABAergic interneurons. This defect results from a block in the migration and differentiation of SVZ-derived cells from the basal telencephalon (Qiu et al., 1997). The interesting observation is that the development of the two major neuronal cell types in the olfactory bulb, the projection neurons and the inhibitory interneurons, are under distinct genetic control, and express different subset of transcription factors (Bulfone et al., 1998). In addition there is evidence in favor of an anatomically distinct origin of these cells, as is the case for the cortical projection neurons and interneurons (Anderson et al., 1999). Thus,
a model has been suggested in which olfactory bulb projection neurons are generated from progenitors in the ventricular zone of the developing bulb and express transcription factors characteristic of the cerebral cortex, whereas most interneurons in the bulb are generated in the SVZ that express subcortical transcription factors.

The Dlx5 and Dlx6 genes are expressed in the developing forebrain, with a very similar profile (Simeone et al., 1994). Transcripts are detected early in the primordium of the ganglionic eminence, and in the ventral diencephalon. At 12.5 dpc these genes are expressed in the ventral thalamus, in both the medial and lateral ganglionic eminence, and in the basal telencephalic vesicle anterior to the preoptic area. At later stages Dlx5 and Dlx6 are expressed in the SVZ of the olfactory area and in the developing olfactory bulbs. Finally, at birth expression is found also in the olfactory tuberculum and in the neocortex (Simeone et al., 1994; Acampora et al., 1999; Merlo and Levi, unpublished observation).

We have mentioned above that several Dlx genes are expressed in the primordia of the basal ganglia, in overlapping pattern according to the stage of cell differentiation (Liu et al., 1997). Dlx1 and Dlx2 are expressed in the least mature cells both in the ventricular and in the SVZ. In contrast Dlx5 is expressed in cells of the SVZ and in post-mitotic cells of the mantle, but not in the ventricular zone, while Dlx6 expression is higher in the mantle cells (Liu et al., 1997). These data suggest that each Dlx gene may play a specific role in the differentiation of the cell types that compose the basal ganglia.

**Dlx genes and osteogenesis**

Unlike all other members of the mammalian Dlx family, the Dlx5 and Dlx6 genes are expressed in all skeletal elements from the time of initial cartilage formation onward (Simeone et al., 1994; Zhao et al., 1994). A further suggestion that Dlx genes might be important for the control of osteogenesis comes from the observation that Dlx5 protein represses osteocalcin gene transcription in cultured calvaria cells via a cis-acting homeodomain-binding site (Ryoo et al., 1999; Merlo and Levi, unpublished observation).

As the Dlx5 mutation that we have generated was characterized by an in-phase insertion of lac-Z, we could analyze in detail the expression of this gene during osteogenesis. We have shown that Dlx5 expression is found in all bones during osteoblast differentiation (see Fig. 2C) and disappears in fully differentiated osteocytes. Its expression is prevalent in periosteal bone, but is also seen in a few cells of the endosteal compartment which might represent osteoblasts at a specific stage of differentiation. Dlx5(-/-) mice show a delayed ossification of dermatocranial bones which resemble that observed in mice in which one copy of Cbfa1, a key regulator of osteoblast differentiation, is inactivated (Otto et al., 1997). However, in Dlx5(-/-) mice, we observe a mild defect of osteogenesis, which suggests that this gene plays a role in osteoblast differentiation and in bone formation (Acampora et al., 1999). As Dlx5 is coexpressed during osteogenesis with Dlx6, it is possible that a more severe effect will be observed when both genes will be inactivated.

A further possible involvement of Dlx genes in development of mineralized tissues comes from the identification of a 4 bp deletion in human DLX3 which correlated with the tricho-dento-osseous (TDO; OMIM 190320) syndrome phenotype in 6 families. This mutation causes e a frameshift and a premature termination codon, resulting in a functionally altered DLX3 protein (Price et al., 1998a,b). TDO is characterized by an autosomal dominant inheritance of enamel hypoplasia and hypocalcification with associated strikingly curly hair. Unfortunately it has not been possible so far to study the in vivo function of Dlx3 in bone development, as the targeted deletion of the mouse Dlx3 gene resulted in embryonic death between 9.5 and 10 dpc, due to placental defects that altered the development of the labyrinthine layer (Morasso et al., 1999). Dlx3 is initially expressed in ectoplacental cone cells and choriclonic plate, and later in the labyrinthine trophoblast of the chorioallantoic placenta, where major defects are observed in the Dlx3(-/-) embryos.

**Dlx genes and hematopoiesis**

Shimamoto et al. (1997) have shown that DLX7 is expressed in normal bone marrow cells and at a particularly high levels in cell lines with the erythroid phenotype. Inhibition of DLX7 gene expression by an antisense oligonucleotide directed against DLX7 in erythroleukemia cell lines reduced the plating efficiency and induced apoptosis. The antisense treatment was accompanied by a reduction in GATA-1 and c-myc mRNA levels. These results suggested that the function of the DLX7 gene may be linked to some aspect of erythropoiesis, possibly in the regulation of apoptosis that occurs during normal erythropoiesis. In our laboratory we have found a severe deregulation of several Dlx genes in acute lymphoblastic leukemia patients which might also suggest a function of these genes in the control of apoptosis (Brigati et al., in preparation).

**Acknowledgements**

We thank Ms. B. Pesce and Mr. S. Mantero for excellent technical help. G.L. was supported by grants from ARSEP, AISM, Consiglio Nazionale delle Ricerche (Progetto Finalizzato “Biotecnologie”) and Ministero della Sanità. The support from Telethon (Italy) for the project: "Use of transgenic mutant mice as a model to study the molecular control of bone development and peripheral myelination and to develop new gene therapy strategies in the embryo" (Project D76) is gratefully acknowledged. This work was partially supported by funds of the European Community to the GENOSPORA project (QLRT-1999-02108). L.P. is the recipient of a fellowship from F.I.R.C. (Fondazione Italiana Ricerca sul Cancro). G.M. is an Assistant Telethon Scientist, “Dulbecco Career” Nº 03/cp from Telethon-Italy.

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