Dlx Genes in Skeletal Development and Maintenance

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Skeletal shape and mechanical properties define, to a large extent, vertebrate morphology and physical capacities. During development, skeletal morphogenesis results from dynamic communications between chondrocytes, osteoblasts, osteoclasts and other cellular components of the skeleton. Later in life, skeletal integrity depends from the regulatory cascades that assure the equilibrium between bone formation and resorption. Finally, during aging, skeletal catabolism prevails over anabolism resulting in progressive skeletal degradation. These cellular processes depend on the transcriptional cascades that control cell division and differentiation in each cell type. Most *Distal-less* (*Dlx*) homeobox transcription factors are directly involved in determining the proliferation and differentiation of chondrocytes and osteoblasts and, indirectly, of osteoclasts. The role of these genes in the maintenance of bone integrity has been only partially studied. Dlx genes appear to be involved in several bone pathologies including, for example, osteoporosis. Indeed, at least five large-scale GWAS studies aimed to detect loci associated to human bone mineral density (BMD) have identified a known *DLX5/6* regulatory region within chromosome 7q21.3 in proximity of SEM1/FLJ42280/DSS1 coding sequences, suggesting that *DLX5/6* expression is critical in determining healthy BMD.

Keywords: Dlx genes ; skeleton ; bone ; osteoblasts ; osteoclasts ; homeobox genes

1. The *Distal-less* gene family in vertebrates

The *Distal-less* (*Dll)* gene of *Drosophila* encodes a homeodomain protein that is one of the first to be expressed during leg primordia and cephalic development [1][2][3]. Adult *Dll Drosophila* mutants present reduction and dysmorphogenesis of distal segments of most appendages including legs, antennae and mouth parts, indicating that the *Dll* activity is required for appendage proximo-distal (PD) organization during early larval stages [1][4][5]. In vertebrates, *Dlx* genes share a homeodomain, homologous to that of *Drosophila Dll*. During evolution, a linked pair of *Dlx* genes was generated through ancestral tandem duplication after the divergence of chordates and arthropods but prior to that of vertebrates. This bigenic *Dlx* cluster was then duplicated twice at the same time as the two genomic duplications that led to the four *Hox* clusters of existing vertebrates. Finally, the pair of *Dlx* genes linked to the *HoxC* cluster was lost in mammals [6][7]. Therefore, the present mammalian genome include six *Dlx* genes linked in three bigenic pairs, in a tandem convergent configuration facing each other through the 3' end: *Dlx*1/*Dlx*2; *Dlx*3/*Dlx*4 (the latter previously named *Dlx7*) and *Dlx*5/*Dlx*6 [3].

2. Dlx genes in skeletal morphogenesis, differentiation and remodelling

During early development *Dlx* genes are expressed by Cranial Neural Crest Cells (CNCCs), but not by mesodermal precursors or by postmigratory Neural Crest Cells of the trunk, Accordingly, *Dlx* genes play a major morphogenetic role on craniofacial bones while *Hox* genes determine the identity and shape of axial and appendicular bones. These early morphogenetic functions of *Dlx* genes are the result of highly dynamic exchanges of regulatory signals between the different cellular components of the developing embryo [8]. Later in development, *Dlx2, Dlx3, Dlx5* and *Dlx6* assume new functions in different organs; in particular in the post-natal skeleton these genes are involved in the control chondrogenesis and osteogenesis.

Starting at mid gestation, *Dlx2, Dlx3, Dlx5* and *Dlx6* are expressed in most sites of cartilage condensation and in differentiating osteoblasts in areas of bone formation [9][10][11][12]. The expression of *Dlx* genes persists then throughout life both in precursor Bone Marrow Mesenchymal Stem Cells (BM-MSCs) and in differentiated chondrocytes and osteoblasts suggesting their involvement in bone remodelling.

The adult skeleton, and bone in particular, is continuously self-regenerating through a process called bone remodelling [13][14]. It has been estimated that in humans most skeletal elements are renewed each decade thanks to the proliferation and differentiation of undifferentiated progenitor cells [14]. Although remodelling is similar for all bones, one should remember that the axial and appendicular skeleton has a different embryonic origin than that of the head: The cephalic skeleton derives exclusively from post-migratory cranial neural crest cells (CNCCs), whereas the rest of the skeleton derives from

mesodermal precursors [15][16]. CNCCs-derived tissues of the head do not express *Hox* genes while axial skeletal elements are *Hox*-positive. Remarkably, progenitor cells assuring bone remodelling maintain the *Hox* positive/negative identity of their original tissue even in the adult [17]. Apparently, therefore, there are distinct sub-populations of cephalic and axial skeletal stem cells.

3. *Dlx* genes in cartilage development and maintenance.

Most *Dlx* genes are involved in cartilage formation and maintenance and play multiple roles in the recruitment of multipotent mesenchymal cells to the chondrogenic lineage and in their subsequent maturation to chondrocytes.

In particular, *Dlx*5 and *Dlx6,* are expressed during chondrogenesis in mouse and avian embryos. *Dlx*5 is strongly expressed by condensing limb mesenchyme early during limb bud development [9][18][19][20][21]. In early chick and mouse embryos (up to E12.5 for the mouse), the expression of *Dlx*5 overlaps that of *Sox9* and type II collagen [21][22], suggesting that *Dlx*5 is an early marker of limb mesenchyme differentiation towards the chondrogenic lineage. Later, during axial chondrogenic condensation, the expression of *Dlx5/6* does not parallel that of *Sox9*. Starting at E13.5, *Dlx*5 is expressed in the mouse growth-plate in proliferating chondrocytes and in Indian hedgehog (*Ihh*)-positive pre-hypertrophic chondrocytes. At E14.5, *Dlx*5 is still expressed in the prehypertrophic zone and continues to be expressed in flattened chondrocytes and in the hypertrophic cartilage zone, even where *Ihh* is downregulated. *Dlx5* is also strongly expressed in the perichondrium and in the growth plate cartilage–bone interface. Later, *Dlx5* expression persists in proliferating chondrocytes and many *Dlx*5-positive cells are present in the prehypertrophic zone .

Comparison of *Dlx*$5^{+/+}$ and *Dlx*$5^{-/-}$ E18.5 growth plates shows that, in the absence of the gene, the proliferating zone is significantly larger while the hypertrophic zone is narrower. These data suggest that Dlx5 acts as a regulator of chondrocyte differentiation and has a smaller effect on the control of proliferation [22].

Several in vitro experiments suggest that Dlx5 is an important regulator of multipotent mesenchymal cell recruitment to the chondroblastic lineage and plays a role in chondrocyte maturation. *Dlx*5 has an anti-proliferative effect on chondrocytes and fibroblasts. Micro-mass assays using chicken embryo limb bud mesenchyme infected with RCAS-*Dlx*5 show an increased chondrogenic differentiation compared to a non-recombinant RCAS-infected mesenchyme. It appears, therefore, that Dlx5 regulates the progressive transition of immature proliferating chondrocytes to pre-hypertrophic and hypertrophic chondrocytes, confirming the notion that Dlx5 acts as a positive regulator of chondrogenic differentiation. n *Dlx*$5/6^{-/-}$ embryos, Col2a1 and Col10a1, markers of pre-hypertrophic and hypertrophic chondrocytes, respectively, were still expressed in articular sections, whereas in *Dlx*$5/6^{+/-}$ embryos, both genes had already been significantly downregulated indicating a severe delay in cartilage differentiation. Both the Fgf/Fgfr3 and the Ihh/Pthrp/Pthrp-R signaling pathways are important for the regulation of chondrocyte proliferation and differentiation, and for the coupling of chondrogenesis to osteogenesis during endochondral ossification . In line with what has been observed for Col2a1 and Col10a1, there is a delay in *Fgfr3* and *Ihh* expression in *Dlx*$5/6^{-/-}$ developing cartilages, resulting in contrasting patterns of *Fgfr3* and *Ihh* expression in control and *Dlx*$5/6^{-/-}$ embryos, while *Pthrp-R* expression is unaffected . Therefore, the expression of most key regulators for chondrogenesis is severely delayed in *Dlx*$5/6^{-/-}$ developing skeletons, resulting in a severe disruption in the differentiation of chondrocytes to *Runx2*-expressing cells [23].

4. Roles of *Dlx* genes in osteoblast differentiation

Dlx3

During endochondral ossification *Dlx*3 is expressed in cells of the osteoblastic lineage and in the inner layer of the periosteum. In E15.5 mouse embryos, *Dlx*3 is present in osteoblast progenitors, in osteoblasts lining bone trabeculae and in osteocytes at early stages of differentiation, whereas mature osteocytes do not express this gene. At E16.5, *Dlx*3 is still expressed by cells surrounding the trabeculae and by endosteal osteoblasts of the diaphyseal cortical bone [24].

Overexpression of *Dlx*3 in MC3T3-E1 cells induced an increased expression of the osteoblast-specific genes *Collagen type1*, *Bone sialo protein*, *Osteopontin* and *Osteocalcin*. ChIP analysis provided evidence of direct binding of Dlx3 onto the *osteocalcin* regulatory region [24].

The tricho-dento osseous syndrome (TDO) is an autosomal dominant disorder characterized by enamel hypoplasia, severe taurodontism, moderately increased trabecular bone mineral density and typical kinky/curly hairs. TDO is linked to chromosome 17q21 markers with no indication of genetic heterogeneity [25][26]. The TDO locus has been mapped to the chromosomal region that includes the *DLX3-4* tandem and, more specifically to a 4bp deletion in the human *DLX*3 gene (*DLX*3 4bp DEL, NT3198 mutation; OMIM 600525) [27][28][29]**.** Beside taurodontism and enamel hypoplasia, TDO patients

carrying this 4bp deletion often present significantly higher bone mineral density in radius, ulna, spines and hips compared to unaffected family members [30]. The 4 bp *DLX3* deletion results in a frameshift that changes the last 97 C-terminal amino acids and gives rise to a novel 119 amino acid C-terminal peptide in the mouse *Dlx3* cDNA, just 3′ to the homeobox. The homeodomain region in both human and mouse *DLX3* genes includes a nuclear localization signal (NLS) [31]. The 4 bp *DLX3* deletion does not alter the structure of the homeobox nor of the NLS regions therefore the nuclear translocation ability of mutant DLX3 protein is unchanged.

The trabecular bone volume and the mineral density of mice carrying the *Dlx3* 4bp deletion is increased both in young and adult individuals. As the rate of bone formation does not increase *in vivo* in these mice, their phenotype was suggested to derive from decreased osteoclast formation and bone resorption due to the increased serum levels of IFN-*γ* [32].

The notion that Dlx3 regulates osteoblast activity is reinforced by the fact that *Dlx3*-deleted bone marrow stroma cells (BMSCs), also show increased osteoblastic differentiation with increased expression of *Runx2* and *Dlx5*. Collectively, these observations suggest that DLX3 is a negative regulator of osteoblastogenesis. This notion is further supported by ChIP analysis on BMSCs, which shows that DLX3 binds to the promoters of *Sp7*, *Dlx5* and *Dlx6,* and *Runx2*, directly modulating their activity (see also [33]).

Dlx5 and Dlx6

*Dlx*5 is expressed by osteoblasts from very early stages of bone development [9][19][34] and persists in these cells throughout life [35]. In perinatal and post-natal bone, *Dlx5* expression is predominantly found at the periphery of diaphyseal bone and in cells surrounded by osteoid within the trabeculae [12][36].

*Dlx*5$^{-/-}$ mice die at birth. Histopathological analysis of their long bones performed in late embryos showed that both trabecular and cortical bone components were affected. By E15.5, *Dlx*5$^{-/-}$ mice present a significant reduction in the ossified portion of long bones. At birth, histological analysis revealed a lesion characterised by the presence of a more complex structure of the endosteal component of the diaphysis, which forms an elaborate mesh of woven bone, and by the reduction of the periosteal bone lamina [12]. Delayed ossification of the parietal, interparietal and superoccipital bones of *Dlx*5$^{-/-}$ mice results in open fontanellae. These morphological findings, both in endochondral and intramembranous bone types, suggest that the absence of *Dlx*5 results in a generalized defect in osteogenesis.

Indeed, the *in vitro* analysis of *Dlx5*$^{-/-}$ cells suggests that this transcription factor promotes osteoblast proliferation and differentiation as indicated by the decreased expression of bone differentiation markers and their reduced capacity to generate mineralized nodules *in vitro* [36]. These *Dlx5*$^{-/-}$ osteoblastic defects may depend from *Runx2* dependent or independent pathways. As will be better discussed later, *Dlx5* acts as a transcriptional activator of *Runx2* in bone by binding to its P1 promoter [37] and to a *Runx2* bone specific enhancer; consequently, *Dlx5* inactivation results in decreased *Runx2* expression. *Dlx5* can also activate osteoblast-specific genes such as *ALP* and *osteocalcin* in the absence of *Runx2*, suggesting a *Runx2*-independent pathway. *Osx* expression is also reduced in cultured *Dlx5*$^{-/-}$ osteoblast, possibly through a Dlx5-dependent/Runx2-independent mechanism [38]. Both *osteocalcin* and *BSP*, markers of osteoblast differentiation, are drastically down-regulated in *Dlx5*$^{-/-}$ osteoblasts, as predicted by the analysis of their regulatory regions and by *in vitro* studies [34][39]. Remarkably, no significant difference in trabecular thickness, an indicative parameter of osteoblastic activity, was observed in *Dlx5*$^{-/-}$ mice at birth. This apparent contradiction may depend on the different origin of cortical and trabecular osteoblasts precursors. Indeed, cultured calvaria-derived cells are more similar to cortical than to trabecular osteoblasts from long bones [36].

Simultaneous inactivation of *Dlx*5 and *Dlx*6 results in more pronounced abnormalities of endochondral bones than the single inactivation of *Dlx*5, suggesting a redundant function for the two genes. Serial skeletal sections showed the presence of vascularization and mineralized bone matrix in heterozygous *Dlx*5/6$^{+/-}$ embryos, while, comparable regions in *Dlx*5/6$^{-/-}$ embryos consisted of hypertrophic and calcified chondrocytes, with minimal vascular invasion, and a predominantly cartilaginous matrix. Molecular analysis of comparable E16.5 *Dlx*5/6$^{-/-}$ embryos also suggested a delay in the onset of the osteogenic pathway. Similar *Runx2* expression patterns were observed within the chondrium and perichondrium of *Dlx*5/6$^{+/-}$ and *Dlx*5/6$^{-/-}$ mice, while *osteocalcin* gene expression was dramatically reduced in *Dlx*5/6$^{-/-}$ skeletal sections. Altogether, these results suggest that *Dlx*5/6 have a partially redundant positive role in the osteoblast maturation pathway, and that, in their absence, the accumulation of mature osteoblasts is severely retarded or absent [23].

5. Association between *DLX5/6* and human bone mineral density

DLX5/6 are associated to a type of human ectrodactyly known as Split Hand Foot Malformation Type I (SHFM1) [40]. The inactivation of *Dlx5/6* in the mouse results in a similar limb phenotype reinforcing the notion that these genes are responsible for this disease [23][41]. The characterization of *DLX5/6* regulatory region performed by generating reporter

transgenic animals has permitted to identify 26 tissue-specific enhancers (eDlx#XX) [42] capable to direct the expression of the genes either in the limb, in craniofacial regions and/or in the brain during development. However, the role of these enhancers in the adult is unknown.

Remarkably, at least 5 GWAS studies have found a strong association between adult bone mineral density and SNPs located in the 7q21.3 region that includes eDlx#18 [43][44][45][46][47]. Furthermore, an haplotype controlling bone mineral density and osteoporosis susceptibility has been reported in this locus [48]. This haplotype is constituted by a succession of co-segregating SNPs located in intronic regions close to the gene *SEM1/FLJ4220/DSS1* that is associated to SHFM1 but has no known function; this locus includes upstream enhancers of *DLX5/6*. Collectively, these observations suggest that polymorphisms in SNPs regulating *DLX5/6* expression are involved in bone mineral density (BMD) determination and might be considered susceptibility factors for osteoporosis.

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