Editorial

Dlx genes and the maintenance of bone homeostasis and skeletal integrity

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Bone development and bone homeostasis (BH) are highly integrated processes that support skeletal formation, maintenance and repair during the whole lifespan. BH depends on the concerted action of specialized cell populations responsible for either bone apposition (osteoblasts) or resorption (osteoclasts). Osteoblasts and osteoclasts coordinate their action through endocrine and paracrine soluble molecular signals. Similar molecular cues also affect the differentiation of mesenchymal and hematopoietic progenitors influencing the expression and activity of key osteogenic transcription factors.¹

BH requires coordination of three major processes: (1) osteoblast differentiation and bone matrix production, (2) osteoblast-dependent regulation of osteoclastogenesis through the secretion of Receptor Activator of NF- κ B Ligand (RANKL) and osteoprotegerin (OPG²), and (3) cell-autonomous regulation of osteoclast activity.³

Defects in BH are at the origin of common and severe pathologies such as, for example, osteoporosis, a complex disease characterized by a reduction in bone mass with associated bone microarchitectural deterioration and a high risk of fractures. Although environmental factors such as nutrition and mechanical load, as well as lifestyle, may influence bone development and BH, family and twin pair studies have suggested a strong genetic component for these processes.

Bone-related disorders are a major social and economic burden for any health-care system as they affect particularly the aging population, which is increasing in most societies. New insight into the transcriptional programs supporting BH and bone remodelling is, therefore, essential to tackle the challenge posed by the increasing incidence of bone disease.

Mammalian *Dlx* homeobox transcription factors constitute a gene family formed by three bigenic clusters: *Dlx1* and *Dlx2*; *Dlx5* and *Dlx6*; *Dlx3* and *Dlx4*.^{4–6} These six *Dlx* genes are differentially expressed by the cellular components of bone: chondrocytes, osteoblasts and osteoclasts.⁷ *Dlx* transcription factors may well play different and synergic roles in the control of chondrogenesis and/or osteogenesis.^{8,9}

In particular, mutations in *DLX3* result in Tricho-Dento-Osseous syndrome, an autosomal dominant ectodermal dysplasia characterized by curly kinky hair, enamel hypoplasia, taurodontism and increased bone mineral density (BMD)

in intramembranous and endochondral bones.¹⁰ The importance of *DLX3* in bone is also supported by its capacity to regulate directly *in vitro* critical determinants of bone differentiation such as *osteocalcin* (*Ocn*), *Runx2* and *osteopontin*,^{11–13} and by the observation that overexpression of *DLX3* in osteoprogenitors stimulates transcription of osteogenic markers.

Most *in vivo* functional studies on the skeletal regulatory function of *Dlx* genes have been performed either during embryonic development (e.g.: Samee *et al.*⁹) or in organ culture (e.g.: Hsu *et al.*¹⁴) as early lethality of null mutant mice has often hampered the elucidation of their postnatal role.

In this issue of *Cell Death Differentiation*, Isaac *et al.*¹⁵ perform the first *in vivo* study of the postnatal role of *DLX3* in the appendicular skeleton. They generate and analyse mice carrying conditional loss-of-function mutations of *Dlx3* in mesenchymal cells (*Dlx3^{Prx1-cKO}*) or in osteogenic lineage cells (*Dlx3^{OCN-cKO}*). Both models present a significant increase in bone mass throughout their lifespan. In the absence of *Dlx3* endochondral bone formation still takes place at the growth plate, but bone deposition is significantly higher, with trabecular bone extending into the medullary cavity. Cortical bone is thicker, but more porous and with decreased BMD.

By combining *in vivo* gene profiling and *ex vivo* cellular analyses, the authors conclude that the increase in trabecular bone mass observed in *Dlx3^{OCN-cKO}* mice does not arise from impaired osteoclastic activity but from direct enhancement of the bone-forming osteoblast activity with an imbalance in BH in favour of bone apposition. RNA-Seq analysis of *Dlx3^{OCN-cKO}* metaphysis shows upregulation of transcription factors essential for osteoblastogenesis, including *Runx2*,¹⁶ its downstream osteoblast-specific target *Sp7*¹⁷ and *Dlx5/Dlx6*, two positive regulators of chondrocyte and osteoblast differentiation (see Figure 1).^{8,9,18,19}

The fact that *Dlx3* plays an important role in regulating osteoblast activity is further supported by the analysis of *Dlx3*-deleted bone marrow stroma cells (BMSCs), which also shows increased osteoblast differentiation and bone-forming activity associated with increased gene expression of *Runx2* and *Dlx5*. The notion that *DLX3* acts as a negative regulator of osteoblastogenesis by reducing *Runx2*, *Sp7*, *Dlx5* and *Dlx6* gene expression is further supported by ChIP analysis on

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Figure 1 Summary diagram of the effects of *Dlx3* inactivation in osteogenic cells. In osteoblast (lower part of the figure) *Dlx3* inhibits the expression of positive regulators of osteogenesis. *Dlx3* inactivation results in higher levels of expression of *Runx2*, *Sp7*, *Dlx5/6*, and so on, and increases osteogenesis. Cortical bone osteoblasts of *Dlx3*^{-/-} mice present also a higher level of expression of *Opg*, suggesting that, in parallel with increased bone deposition, this bone compartment is also characterized by reduced activity of osteoclasts (upper part of Figure 1). *Dlx3* is, therefore, a negative modulator of osteogenesis at multiple levels; its coordinated action with other activators of osteogenesis, such as *Dlx5*, is required for the maintenance of bone homeostasis

BMSCs, which shows that *DLX3* binds to the promoters of *Sp7*, *Dlx5* and *Dlx6* and *Runx2*, directly modulating their activity (see also Hassan *et al.*¹²).

An interesting concept that emerges from this study is that the combined action of *Dlx3* and *Dlx5/6* generates a regulatory network essential for the maintenance of BH. First of all, the data (both *in vivo* and *in vitro*) indicate that *Dlx3* negatively regulates *Dlx5/6* in bone, whereas previous studies showed that the targeted simultaneous inactivation of *Dlx5* and *Dlx6*²⁰ results in the loss of *Dlx3* expression. Taken together, these results suggest that bone formation and homeostasis implicate reciprocal regulatory loops between *Dlx5/Dlx6* and *Dlx3*. *In vitro* studies have shown that *Dlx3* and *Dlx5* associate with the *Ocn* promoter at the onset of transcriptional activation, concomitant with *Runx2* occupancy, and, while *Dlx3* occupancy on the *Ocn* promoter decreases from osteoblast maturation to mineralization, *Dlx5* occupancy is maximal during the mineralization stage.¹²

Isaac *et al.*¹⁵ demonstrate that *Dlx3* deletion in calvaria osteoblasts results in increased occupancy of *Dlx5* and increased and premature occupancy of *Runx2* on regulatory elements on the *Ocn* promoter. Thus, *Dlx3* deletion could

directly affect the network of molecular switches and promote osteoblastic differentiation and bone-forming activity via an enhancement of the occupancy of bone-activator TFs such as *Dlx5* and *Runx2*. Therefore, *Dlx3* and *Dlx5* seem to have coordinated and opposite roles in the transcription of bone-related genes via molecular switches at their promoter regions.

Inactivation of *Dlx3* results in an increased thickness of cortical bone associated with increased mineral apposition rate in the periosteum, decreased BMD, increased porosity and vascularization. RNA-Seq analysis of cortical bone of mutant mice revealed upregulation of *Opg* and *Mepe*, two major inhibitors of osteoclastogenesis strongly expressed by late-differentiated osteoblasts and osteocytes. ChIP analysis demonstrates that *Dlx3* binds to several sites on the *Opg* promoter. These findings bolster the concept that *Dlx3* inactivation in osteoblasts induces increased levels of *Opg* and *Mepe* in cortical bone, thereby deregulating homeostasis in favour of enhanced bone deposition (see Figure 1). Indeed, *Dlx5*^{-/-} mice have an increased number of osteoclasts while *Dlx5*^{-/-} osteoblasts exhibit decreased *Opg* expression, resulting in a higher Rankl/*Opg* ratio.⁹ It seems plausible that, besides *Dlx5*,⁹ *Dlx3* is also a central regulator of osteoblast/osteoclast coupling. However, conversely to *Dlx5*, *Dlx3* would inhibit osteoblast bone-forming activity via a negative transcriptional control of genes responsible for bone formation, while simultaneously activating bone resorption through osteoblast-activated regulation of osteoclastogenesis. It seems therefore that, throughout life, *Dlx3* is a negative modulator of osteogenesis and that its coordinated action with other TFs, such as *Dlx5*, is required for the maintenance of BH. *DLX3* is therefore an interesting target for future translational research in human skeletal disorders such as osteoporosis.

Conflict of Interest

The authors declare no conflict of interest.

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