MSX2 in ameloblast cell fate and activity
Sylvie Babajko, Muriel de La Dure-Molla, Katia Jedeon, Ariane Berdal

To cite this version:
Sylvie Babajko, Muriel de La Dure-Molla, Katia Jedeon, Ariane Berdal. MSX2 in ameloblast cell fate and activity. Frontiers in Physiology, Frontiers, 2015, 5, pp.510. 10.3389/fphys.2014.00510 . hal-01223840

HAL Id: hal-01223840
https://hal.sorbonne-universite.fr/hal-01223840
Submitted on 3 Nov 2015

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While many effectors have been identified in enamel matrix and cells via genetic studies, physiological networks underlying their expression levels and thus the natural spectrum of enamel thickness and degree of mineralization are now just emerging. Several transcription factors are candidates for enamel gene expression regulation and thus the control of enamel quality. Some of these factors, such as MSX2, are mainly confined to the dental epithelium. MSX2 homeoprotein controls several stages of the ameloblast life cycle. This chapter introduces MSX2 and its target genes in the ameloblast and provides an overview of knowledge regarding its effects in vivo in transgenic mouse models. Currently available in vitro data on the role of MSX2 as a transcription factor and its links to other players in ameloblast gene regulation are considered. MSX2 modulations are relevant to the interplay between developmental, hormonal and environmental pathways and in vivo investigations, notably in the rodent incisor, have provided insight into dental physiology. Indeed, in vivo models are particularly promising for investigating enamel formation and MSX2 function in ameloblast cell fate. MSX2 may be central to the temporal-spatial restriction of enamel protein production by the dental epithelium and thus regulation of enamel quality (thickness and mineralization level) under physiological and pathological conditions. Studies on MSX2 show that amelogenesis is not an isolated process but is part of the more general physiology of coordinated dental-bone complex growth.

**Keywords:** MSX2, transcription factors, ameloblast, differentiation, enamel, teeth

**Abbreviations:** Bp, Base pairs; IEE, internal enamel epithelium.

**Structure and Molecular Mechanisms of Muscle Segment Homeobox (Msx) Genes**

**Homeobox Genes**

Msx2 is a member of the family of divergent homeobox-containing genes homologous to the Drosophila Muscle Segment Homeobox (msh) gene. Evolution including the duplication of the ancestral msh gene, has led to three different genes in mice and two in humans. Homeobox-containing genes share a well-conserved sequence of 183 bp coding for a helix-loop-helix motif of 64 amino acids (Shirasawa et al., 1994). This homeodomain interacts with an A/T-rich DNA sequence thereby conferring transcriptional activity on the proteins carrying it (Gehring et al., 1994). Most homeobox genes are organized in clusters, and this is the case for HoxA, B, C, and D genes that control the development of the trunk spatially and temporally. Other homeobox genes, dispersed around the genome and classified as divergent homeogenes also include the Msx family which is crucial for the development of the head.

**MSX1 and MSX2 Are Transcriptional Regulators**

The homeodomain of homeogenes Msx1 and Msx2 share 98% sequence identity, such that they have similar transcriptional properties (Catron et al., 1996). MSX1 and MSX2 were first reported as transcriptional repressors (Catron et al., 1993, 1995), but their respective activities have not been precisely characterized. They are able to interact with a C/GTAATTG core consensus sequence (Catron et al., 1993). MSX homeoproteins may form either homodimers or heterodimers with other homeoproteins such as those encoded by Dlx (Zhang et al., 1997) and Pax genes (Bendall et al., 1999; Ogawa et al., 2006). The resulting competition for the same promoter sequence may explain, at least in part, their antagonist regulatory activities (Bendall and Abate-Shen, 2000). In addition to the presumed direct interactions via the homeodomain, their N- and C-terminal domains are able to interact with other proteins and thereby also modulate transcription (Catron et al., 1995; Zhang et al., 1996). MSX2 is able to interact with SP3 (Bei, 2009) and with C/EBPα, notably in ameloblasts (Zhou and Snead, 2000). Such physical interactions between MSX2 and C/EBPα enable switching of cell differentiation, as described for osteogenic/adipogenic differentiation in aortic myofibroblasts (Cheng et al., 2003). Transcriptional repression by MSX (reported for MSX1) is also modulated by interactions that drive the nuclear localization of the proteins, as shown for PIAS-1 (Lee et al., 2006) and H3K27me3 (Wang and Abate-Shen, 2012). These various papers show that the target selectivity of MSX1 and MSX2 and their transcriptional activities
are dependent on the nature of the partners they interact with, via binding motifs located outside the homeodomain (Catron et al., 1995; Zhang et al., 1996). The sequence similarity between MSX1 and MSX2 in the N- and C-termini is lower (than in their homeodomains) and this presumably explains the different activities of the two factors. Transcriptional regulations of MSX2 depend on the nature of its partners: the specific combinations involved determine when, where and how the expression of the various MSX2-target genes is modulated.

**MSX1 AND 2 PRESENT REDUNDANT AND NON-REDUNDANT FUNCTIONS**

MSX functions are significant in morphogenesis in which specific developmental patterns control distinct stages and events. The involvement of MSX in morphogenesis was discovered in work with limb buds and ectodermal appendages such as teeth (Satokata and Maas, 1994; Houzelstein et al., 1997; Satokata et al., 2000). There are now a vast number of human and transgenic mouse gene mutations available (Table 1) providing evidence that both MSX1 and MSX2 are essential for skeletal morphogenesis and differentiation. The expression profiles of Msx homeobox genes may overlap; there may be redundancy between MSX1 and MSX2 as they display structural conservation, according to anatomical site (Sharpe, 1995). This is the case in limbs (and the resulting appendicular skeleton) but not in craniofacial morphogenesis and differentiation as illustrated by the selective phenotype of Msx mutants.

Non-redundant roles of MSX are exemplified in teeth. Both MSX1 and MSX2 are expressed as soon as dental lamina is initiated and their expression continues until the end of tooth formation, but in different areas (Thesleff, 2003). Transgenic mouse models have been used to characterize their respective function in dental development: MSX1 drives early tooth morphogenesis, whereas MSX2 is involved later in development (Beï et al., 2004; Thesleff, 2006). MSX1 gene mutations are associated with tooth agenesis in both human and mouse species (Vastardis et al., 1996; Houzelstein et al., 1997). Msx2 null mutants survive and display variable bone and dental phenotypes, especially in areas in which development is driven by epithelial-mesenchymal cell communications (Satokata et al., 2000). The different phenotypes associated with either MSX1 or MSX2 transgenic models strongly indicate that they do not have the same functions in tooth development; their respective molecular actions and partners need to be characterized.

**MSX2: A KEY ELEMENT OF THE TRANSCRIPTIONAL NETWORK DETERMINING AMELOBLAST GENE EXPRESSION AND AMELOBLAST LIFE-CYCLE**

**MSX2, A KEY FACTOR FOR ENAMEL GENE EXPRESSION**

A number of studies have investigated the regulation of enamel gene transcription. They have identified a number of factors, including MSX2, controlling the transcription of enamel genes (Table 1). The amelogenin gene was the first to be studied because it encodes the most abundant enamel matrix protein. Amelogenin gene repression by MSX2 appears to be indirect through interaction with C/EBPα (Figure 1) (Zhou et al., 2000; Xu et al., 2007a). Interaction between MSX2 and C/EBPα abolishes the activating activity of C/EBPα on amelogenin transcription. MSX2/DLX heterodimers may also be involved in modulating amelogenin expression (Lézot et al., 2008). Indeed, DLX2 and DLX3 have been shown to control amelogenin expression (Lézot et al., 2008; Athanassiou-Papaefthymiou et al., 2011). The selective role of each DLX homeoprotein may be influenced by the other DLX family members expressed in ameloblasts (Lézot et al., 2008). MSX2 transcriptional modulations have been documented for other key enamel genes, notably those encoding enamel matrix proteins (enamelin and ameloblastin) and the two main proteases (MMP20 and KLK4) (Berdal et al., 1993; Molla et al., 2010). MSX2 is also able to repress the expression of calbindin-D28k, a vitamin D-dependent calcium-handling protein, by direct interaction with its proximal promoter (Boñaños et al., 2012). Several partners of MSX2 identified in osteoblasts also influence ameloblast gene expression: for example, RUNX2 differentially regulates enamelin and Klk4 gene expression. Interestingly, its

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**Table 1 | MSX mutations in human and corresponding experimental models.**

<table>
<thead>
<tr>
<th>Pathology</th>
<th>Human OMIM</th>
<th>Mutant mice Phenotype</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MSX1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ectodermal dysplasia 3, Witkop type</td>
<td>189600</td>
<td>Loss of function (Msx1−/−)</td>
<td>Satokata and Maas, 1994</td>
</tr>
<tr>
<td>Orofacial cleft 5</td>
<td>60874</td>
<td>Tooth agenesis, cleft palate</td>
<td>Houzelstein et al., 1997</td>
</tr>
<tr>
<td>Tooth agenesis, with or without orofacial cleft</td>
<td>106600</td>
<td>Gain of function (transgenic mice) craniofacial bone morphogenesis</td>
<td>Nassif et al., 2014</td>
</tr>
<tr>
<td><strong>MSX2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Loss of function</td>
<td>168550</td>
<td>Loss of function (Msx2−/−)</td>
<td>Satokata et al., 2000</td>
</tr>
<tr>
<td>Parietal foramina with cleidocranial dysplasia</td>
<td></td>
<td>Bone defects</td>
<td>Alou et al., 2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tooth</td>
<td>Molla et al., 2010</td>
</tr>
<tr>
<td>Gain of function</td>
<td>604757</td>
<td>Gain of function (transgenic mice)</td>
<td>Liu et al., 1995</td>
</tr>
<tr>
<td>Craniosynostosis, type 2</td>
<td></td>
<td>Premature suture closure, Ectopic cranial bone</td>
<td>Shao et al., 2005</td>
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<tr>
<td></td>
<td></td>
<td>Cardiovascular calcification</td>
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Table 2 | Transcriptional regulations of the major enamel genes.

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Transcription factors</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amelogenin</td>
<td>Msx2</td>
<td>Zhou et al., 2000; Xu et al., 2007a</td>
</tr>
<tr>
<td></td>
<td>C/EBPa</td>
<td>Zhou and Snead, 2000</td>
</tr>
<tr>
<td></td>
<td>NFY</td>
<td>Xu et al., 2006</td>
</tr>
<tr>
<td></td>
<td>C/EBPa</td>
<td>Xu et al., 2007b</td>
</tr>
<tr>
<td></td>
<td>Dlx2</td>
<td>Lézot et al., 2008</td>
</tr>
<tr>
<td></td>
<td>Dlx2 and FoxJ1</td>
<td>Venugopalan et al., 2011</td>
</tr>
<tr>
<td></td>
<td>Oct-1</td>
<td>Xu et al., 2010</td>
</tr>
<tr>
<td></td>
<td>Pitx2</td>
<td>Li et al., 2013, 2014</td>
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<tr>
<td></td>
<td>Clock genes</td>
<td>Lacruz et al., 2012a; Zheng et al., 2013</td>
</tr>
<tr>
<td></td>
<td>Runx2</td>
<td>Athanassiou-Papaefthymiou et al., 2011</td>
</tr>
<tr>
<td></td>
<td>Dlx3</td>
<td>Mitsiadis et al., 2008</td>
</tr>
<tr>
<td></td>
<td>Tbx1</td>
<td>Dhamija and Krebsbach, 2001</td>
</tr>
<tr>
<td>Ameloblastin</td>
<td>Cbfa1 (Runx2)</td>
<td>Dhamija and Krebsbach, 2001</td>
</tr>
<tr>
<td>Enamelin</td>
<td>B-catenin/LEF1</td>
<td>Tian et al., 2010</td>
</tr>
<tr>
<td></td>
<td>Runx2</td>
<td>Athanassiou-Papaefthymiou et al., 2011</td>
</tr>
<tr>
<td></td>
<td>Dlx3</td>
<td>Lee et al., 2010</td>
</tr>
<tr>
<td>MMP20</td>
<td>Runx2 and OdAM</td>
<td>Zhang et al., 2007</td>
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<tr>
<td></td>
<td>c-Jun (AP1)</td>
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<tr>
<td>KLK4</td>
<td>Clock genes</td>
<td>Zheng et al., 2013</td>
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<tr>
<td></td>
<td>Runx2 and OdAM</td>
<td>Athanassiou-Papaefthymiou et al., 2011</td>
</tr>
<tr>
<td></td>
<td>Dlx3</td>
<td></td>
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<tr>
<td>Calbindin D 28k</td>
<td>Msx2</td>
<td>Bolaños et al., 2012</td>
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</tbody>
</table>

For Msx2 expression is modulated either directly by various intracellular factors or indirectly by secreted factors, resulting in finely tuned levels of MSX2 that control enamel gene expression and ameloblast cell fate.

MxS2 IS INVOLVED IN CELL PROLIFERATION

MxS2 is present throughout the process of ameloblast differentiation/maturation although its expression decreases during the secretory stage and may modulate enamel gene expression differently depending on the combination of transcription factors present (Figure 2). Very few studies report gene expression modulations during the maturation stage (Lacruz et al., 2012b), such that the function of MSX2 during the terminal differentiation of ameloblasts is less clear.

Msx2 is not only expressed in the inner dental epithelium but throughout the entire enamel organ as evidenced in the rodent apical loop (Jiang and Wang, 2010). In Msx2−/− mouse dental epithelium, proliferation of stellate reticulum cells and stratum...
intermediate cells increases. At these early stages, Msx2 expression is induced by the key transcription factors, DPYS14 and BCL11B (Ctip2), also determinant of cell proliferation (Figures 1, 2) (Golonzhka et al., 2009; Yasukawa et al., 2013). Msx2 expression and Dlx2 expression are also indirectly up-regulated by PITX2 via BMP4, which are also expressed in proliferative cells (Venugopalan et al., 2011; Cao et al., 2013) (Figure 1). As a result of the various Msx2 expression modulations by early factors, Msx2 is expressed in undifferentiated inner enamel epithelial (IEE) cells and down-regulated when ameloblast cells overtly differentiate (Mackenzie et al., 1991, 1992; Maas and Bei, 1997).

LESSONS FROM NON-CONDITIONAL Msx2 MUTANTS—STRENGTHS AND LIMITATIONS

Most of the data described above result from in vitro studies. However, in vivo models are also informative about MSX2 function because they include cell-cell communications.

Msx2−/− MOUSE DENTAL PHENOTYPE ILLUSTRATES THE PLEIOTROPIC ROLE PLAYED BY Msx2

At this time, several targets have been identified in enamel within their physiological context. Dental morphogenesis in Msx2−/− mice is abnormal: cusp generation is affected by enamel knot disorganization (Figure 3D) (Bei et al., 2004); defects are observed in the enamel (Molla et al., 2010) and other dental tissues; and roots are malformed with the root epithelium overexpressing enamel proteins, especially amelogenins and ameloblastin. Also, jaw osteoclast activity is decreased locally around the dental follicle; eruption failure and pseudo-odontogenic tumor devolution of the tooth germs culminate in the third mandibular molars (Aioub et al., 2007). This phenotype is coherent with the pattern of Msx2 expression in many cells forming the tooth and periodontal bone. Indeed, its expression starts from the very beginning of odontogenesis, first in the ectoderm and mesoderm from the gastrulation stage, and subsequently in neural crest cells and oral epithelium (Bendall and Abate-Shen, 2000). During root formation, Msx2 is again expressed in epithelial cells (Hertwig root sheath and later epithelial Malassez rests) as well as in dental and periodontal mesenchyme (Yamashiro et al., 2003; Molla et al., 2010). As alveolar bone and tooth development are linked, it is important to note that differentiating and differentiated osteoblasts express Msx2 (Dodge et al., 1996; Mirzayans et al., 2012). Finally, periodontal osteoclasts express Msx2 and do so with regional gradients related to both dental crown and root growth (Aioub et al., 2007) (Figure 3).
**Msx2 CONTROLS MORPHOGENESIS VIA EPITHELIAL Bmp4 LEVELS AND THE ASSOCIATED DEATH PROGRAM IN ENAMEL KNOT**

Early tooth development in Msx2 null mice is normal, and only a small number of effectors are modified: epithelial Bmp4 expression decreases whereas expressions of Fgf4, the cyclin-dependent kinase (cdk) inhibitor gene p21, and Shh are not modified (Beil et al., 2004). Furthermore, Bmp4 expression is not altered in the mesenchymal compartment. MSX2 intervenes in epithelial-mesenchymal cross-talk, leading to odontogenesis. Mesenchymal Bmp4 stimulates Msx2 and Cdk p21 expression in the enamel knot; MSX2 in turn stimulates Bmp4 expression in epithelial cells. MSX2 in vitro cooperates in the Bmp4-mediated programmed cell death pathway (Irsasena and Kessler, 2002), and Msx2 overexpression stimulates Bmp4 increasing apoptosis (Marazzi et al., 1997; Wu et al., 2003). Thus, the regulatory loop initiated by MSX2 is determinant for dental cell signaling and communication and consequently tooth morphogenesis.

**ALtered laminin 5α3 patterns affect ameloblast integrity**

In the dental epithelium, laminin 5α3 is expressed in the basal membrane prior to ameloblast differentiation and disappears when cells differentiate (Yoshiba et al., 1998). It has been described as being distributed in a “membrane like” structure localized between the stratum intermedium and ameloblast cell layer. In Msx2−/− mice, ameloblasts are able to achieve terminal differentiation but the integrity of their junctional complexes is affected. In the absence of MSX2, the inner dental epithelium presents rounded and detached ameloblasts with loose intercellular junctions (Beil et al., 2004; Molla et al., 2010) (Figure 4). Laminin 5α3 expression is lower than in wild-type animals, whereas the expression of E-cadherin, β-catenin, and the integrin subunits α5β1 and α6β4 remains unchanged (Beil et al., 2004; Molla et al., 2010). Thus, the MSX2 target gene laminin 5α3 may control the formation of cell-cell-junctions and thus organization of the ameloblastic layer (Zhou et al., 1999). This possibility is supported by a LAMA5 gene mutation in the epidermolysis bullosa characterized by skin fragility and enamel dysplasia (Brooks et al., 2008), as a result of the destruction of dermal and dental epithelia, respectively.

**Prospects for experimental models—from discrete to continuous parameters of enamel quality control**

The physiological conditions are only partly reproduced in vitro.

There have been reported successful in vitro promoter studies in ameloblasts (see above) thanks to establishment of cell models that help to decipher molecular mechanisms (Zhou and Snead, 2000; Zhou et al., 2000; Xu et al., 2007a). However,
Indeed, allometric tooth growth is dependent on (and reversely determines) eruption rate (Castaneda et al., 2011, 2013).

THE RODENT INCISOR, AN “IN VIVO TEST TUBE” FOR ANALYZING GENE AND ENVIRONMENT INTERACTIONS IN ENAMEL

Various experimental approaches have been developed including organotypic cultures (Bronckers, 1983; Bronckers et al., 2009), primary cultures (DenBesten et al., 2005), and hybrid cell cultures (Matsumoto et al., 2011; Jiang et al., 2014). In vivo studies may involve dissections under a microscope which is either manual or, more recently, by laser-capture (Jacques et al., 2014). For growth-limited teeth (three non-renewed molars in rodents), amelogenesis follow-up requires animals of different ages, increasing the sample size required (Onishi et al., 2008). Rodent incisor enamel displays an exceptionally simple structure and is reasonably large. Its continuous growth facilitates the exploration of enamel formation under defined conditions and during defined temporal windows (transgenic mice, environmental, and systemic disturbances) at any animal age (Damkier et al., 2014). The course of amelogenesis is spatially distributed along the longitudinal axis of the tooth. Consequently, extracellular cascades of peptide–peptide and mineral interactions can be sampled along the longitudinal axis of single rodent incisors (Jedeon et al., 2013), and ameloblasts and changing enamel matrix are easily followed (Houari et al., 2014). Protein and mRNA studies are feasible and have included comparisons between incisor samples from test and control conditions in rats (Berdal et al., 1993; Jedeon et al., 2013) and mice (Descroix et al., 2010; Molla et al., 2010).

MSX2 IS A MORPHOGEN FOR ENAMEL, CONTROLLING ITS THICKNESS

The murine incisor “in vivo test tube” has been used in studies of MSX2 in differentiated ameloblasts. Msx2 heterozygous (Msx2+/−) mice are a unique model for investigating MSX2 dose effects. Ameloblasts survive and differentiate appropriately in Msx2+/− mice (unlike those in Msx2−/− mice) and the amounts of Msx2 mRNA are half those in wild-type mice. Enamel gene studies have revealed a specific overabundance of amelogenin mRNA in Msx2+/− mice. The enamel phenotype in Msx2+/− mice included increased thickness and, more specifically, a thicker outer prismatic enamel layer and larger prism diameter (Molla et al., 2010; Figure 5). This suggests that MSX2 determines enamel thickness in vivo. Indeed, a rigorous analysis of the pattern of Msx2 expression during the secretion stage revealed an inverse relationship between Msx2 mRNA levels and enamel thickness (Molla et al., 2010) as similarly described for enamel thickness and amelogenin levels (Snead et al., 1988). This is also the case for DLX2 (Lézot et al., 2008) in which an inverse correlation between DLX2 levels and thickness was shown through quantitative measurements of incisors. Also, decreased production of enamel proteins during the enamel maturation stage is associated with a significant up-regulation of Msx2 (Figure 5), in accordance with in vitro data showing the MSX2 transcriptional repression of amelogenin expression. On the contrary, ameloblast alterations observed in Msx2−/− mice result in deficient enamel protein production (especially amelogenin) and result in hypoplastic enamel without visible prismatic structures (Molla et al., 2010—see Figure 3F).
FIGURE 5 | Physiological levels of MSX2 and its target-genes, amelogenin and calbindin-D28k. Msx2 expression during the ameloblast life-cycle is inversely related to amelogenin abundance. The figure is a compilation of published findings (Molla et al., 2010) and illustrates two significant examples of gene expression level fluctuations (amelogenin—green; and calbindin-D28k—gray) in ameloblasts during amelogenesis and their relationships with Msx2 expression (red). (1) The presecretion/secreton/maturation sequence. Msx2 down-regulation is related to down-regulation of amelogenin and calbindin-D28k from the presecretion stage to the secretion stage. (2) During the secretion stage controlling enamel thickness; amelogenin mRNA production in ameloblasts depends on their sites or localization (Snead et al., 1988), leading to the differing thickness of enamel across the tooth. The patterns of Msx2 and Dlx2 expression are the converse of that of amelogenin (Lézot et al., 2008). We established that MSX2 indeed contributes to enamel thickness inhibition in vivo (Molla et al., 2010). Enamel thickness as determined by scanning electron microscopy in mandible incisor of 3-month old mice is greater under Msx2 haploinsufficiency than in controls (here Msx2+/− compared to wild-type Msx2+/+ mice). This Msx2 haploinsufficiency is also associated with overexpression of both amelogenin (Molla et al., 2010) and calbindin-D28k (Bolaños et al., 2012). The relationship between calbindin-D28k and MSX2 is more complex because calbindin-D28k abundance abruptly increases for a second time during the maturation stage. However, the partners of MSX2 at this stage are still unknown and its activity on gene expression has not been extensively investigated.

CONCLUSION
Integrative physiological networks underlying amelogenesis are just emerging. Experimental progress in the field of enamel has mainly been based on gene disrupting strategies to describe developmental circuits which drive enamel formation. However, only precise quantitative and continuous studies allow appropriate analysis of the interplays that determine enamel thickness and quality. This is the case for studies addressing the constitutive regulation of ameloblast activity, illustrated here by the MSX and DLX homeoprotein pathways. They provide clues on how enamel acquires differential thickness depending on its anatomical location. They modulate expression of key genes involved in amelogenesis (Lézot et al., 2002, 2008; Molla et al., 2010; Bolaños et al., 2012). It is thus very likely that these factors are able to transmit effects of many environmental factors, whether regional or systemic (including the availability of calcium, vitamin D, fluoride, or even pollutants) that also affect the final enamel composition and quality (Berdal et al., 1993; Jedeon et al., 2013; Houari et al., 2014).

In summary, the dental cell and enamel system illustrates how MSX homeoproteins may be reiteratively enrolled in a single organ. Throughout the cell life-cycle, cooperation between particular transcription factors in a stage-specific manner controls the expression of a number of genes. Such MSX2-target genes encode growth factors, junctional complexes, matrix proteins, and other proteins involved in enamel mineralization. Consistent with reiterative up- and down-regulations, MSX2 drives particular events; some in early development, some during growth and, finally, some in conditions of homeostasis in adults with the effect decreasing with age. MSX2 is exemplary of the integrative schemes of a single transcription factor making multiple contributions to the physiological or pathological development of complex organs, composed of many cells of different types, as described here in the dental-bone complex.
FUNDING
This work was funded by the University Paris-Diderot, the French National Institute of Health and Medical Research (INSERM) and the National Research Agency (ANR): OSTEODIVERSITY (SVSE 1-2012).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 11 June 2014; accepted: 08 December 2014; published online: 05 January 2015.


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