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The *HOX* Genes are Expressed, In Vivo, in Human Tooth Germs: In Vitro cAMP Exposure of Dental Pulp Cells Results in Parallel HOX Network Activation and Neuronal Differentiation

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Abstract Homeobox-containing genes play a crucial role in odontogenesis. After the detection of *Dlx* and *Msx* genes in overlapping domains along maxillary and mandibular processes, a homeobox odontogenic code has been proposed to explain the interaction between different homeobox genes during dental lamina patterning. No role has so far been assigned to the *Hox* gene network in the homeobox odontogenic code due to studies on specific *Hox* genes and evolutionary considerations. Despite its involvement in early patterning during embryonal development, the HOX gene network, the most repeat-poor regions of the human genome, controls the phenotype identity of adult eukaryotic cells. Here, according to our results, the *HOX* gene network appears to be active in human tooth germs between 18 and 24 weeks of development. The immunohistochemical localization of specific HOX proteins mostly concerns the epithelial tooth germ compartment. Furthermore, only a few genes of the network are active in embryonal retromolar tissues, as well as in ectomesenchymal dental pulp cells (DPC) grown in vitro from adult human molar. Exposure of DPCs to cAMP induces the expression of from three to nine total *HOX* genes of the network in parallel with phenotype modifications with traits of neuronal differentiation. Our observations suggest that: (i) by combining its component genes, the *HOX* gene network determines the phenotype identity of epithelial and ectomesenchymal cells interacting in the generation of human tooth germ; (ii) cAMP treatment activates the HOX network and induces, in parallel, a neuronal-like phenotype in human primary ectomesenchymal dental pulp cells. J. Cell. Biochem. 97: 836–848, 2006.

Key words: HOX network; tooth germs; neuronal differentiation

In odontogenesis, a crucial role is played by homeobox-containing genes, a superfamily of transcription factors regulating downstream specific effector genes [Jernvall and Thesleff, 2000; Tucker and Sharpe, 2004]. After the detection of *Dlx* and *Msx* genes in overlapping domains along the proximo-distal axis of maxillary and mandibular processes, a homeobox odontogenic code has been proposed to explain the interaction of different types of homeobox genes (*Lhx, Gsc, Barx, Dlx, Msx, Bapx*) during dental lamina patterning [Thomas and Sharpe, 1998]. Mutations in functional domains of homeobox genes (*MSX1* and *PAX 9*) are associated with tooth agenesis in humans [Vastardis et al., 1996; Stockton et al., 2000].

Of all the homeobox-containing genes, a particular role is played by *Hox* genes [Gehring and Hiromi, 1986]. In mice (*Hox* genes) and humans (*HOX* genes) there are at least 39 genes organized in 4 genomic clusters (Fig. 2 #). On the

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basis of sequence similarity and position on the locus, corresponding genes in the four clusters can be aligned with each other and with genes of the *HOM-C* complex of *Drosophila* in 13 paralogous groups [Scott, 1992]. During mammalian development, *Hox* gene expression controls the identity of various regions along the body axis, from the branchial area through to the tail [Graham et al., 1989]. This is achieved according to the rules of temporal and spatial colinearity, with 3′ *Hox* genes expressed early in development and controlling anterior regions, followed by progressively more 5′ genes expressed later and controlling more posterior regions [Dekker et al., 1992]. In particular, 3′ *Hox* genes in groups 1–4 (cervical) primarily control the development of the branchial area and the rhomboencephalon, the embryonic region corresponding to the hindbrain [Lumsden and Krumlauf, 1996]. Central *Hox* genes in groups 5–8 control the thoracic portion of the body, whereas 5′ *Hox* genes in groups 9–13 control the lumbo-sacral region. The *HOX* gene network, comprising the most repeat-poor regions of the human genome [Lander, 2001], is also expressed in normal adult human organs [Cillo, 1994-95]. *Hox* and *homeobox* genes appear to regulate normal development, phenotype cell identity [Cillo et al., 1999], and cell differentiation [Magli et al., 1991; Cantile et al., 2003] in addition to controlling other cellular processes, as proven by the description of congenital [Mortlock and Innis, 1997], somatic [Nakamura et al., 1996], metabolic [Ferber et al., 2002], and neoplastic alterations [Cillo et al., 2001; Abate-Shen, 2002] involving these genes.

Hox genes appear to be inactive in ectomesenchymal cells derived from the neural crest and migrating towards the first pharyngeal arch [Kontges and Lumsden, 1996]. Moreover, from an evolutionary viewpoint, despite being expressed in the first branchial arch in lampreys (jawless fish of the cyclostome family), *Hox* genes are not active in the corresponding pharyngeal arch of jawed vertebrates (gnathostomes) [Trainor and Krumlauf, 2000]. It has therefore been suggested that loss of *Hox* gene expression is important in the transition from jawless to jawed organisms, and that *Hox* genes are not necessary in tooth development [James et al., 2002]. These observations stem from studies on specific genes of the Hox network, such as Hox A2, which establishes the cephalic limit of *Hox* gene expression between rhombomers r1 and r2 and, when absent, enables jaw formation [Prince and Lumsden, 1994]. In contrast to this view, a controversial interpretation has recently implicated endoderm in dentition patterning and proposed teeth evolution as independent from jaw [Smith and Johanson, 2003].

Tooth morphogenesis is the process whereby dental lamina generates tooth through the interaction between epithelial and ectomesenchymal cells in the tooth germ [Jernvall and Thesleff, 2000]. This process is subsequent to pharyngeal arch generation and is strictly connected to cell differentiation. According to the observation that these crucial cellular functions are usually regulated by the *HOX* gene network in the development of several body structures [Magli et al., 1991; Cantile et al., 2003], we have decided to analyze *HOX* gene network expression in human deciduous tooth germs and in retromolar tissue between the 18th and 24th week of in vivo development.

According to our results the *HOX* gene network is active in human tooth germs. Locus D and paralogous group 6 *HOX* genes display the highest number of active genes. Furthermore, only a few genes of the network are active in retromolar tissues. The immunohistochemical localization of specific HOX proteins mostly concerns the epithelial tooth germ compartment. The expression of the HOX network in primary ectomesenchymal human dental pulp cells (DPC) grown in vitro from adult human molar, concerns three *HOX* genes whereas most of the network genes remain silent. To understand whether the expression of the *HOX* gene network can be altered in the dental ectomesenchymal compartment, we exposed DPCs to cyclic AMP which induced: (i) an increase, from three to nine, of the total number of active *HOX* genes in the network; (ii) phenotype modifications with traits of neuronal differentiation.

Our observations suggest that, by combining its component genes, the HOX network determines the phenotype identity of epithelial and ectomesenchymal cells interacting in the generation of human tooth germs. Furthermore, in ectomesenchymal human dental adult pulp cells, cAMP treatment activates the HOX network and induces a neuronal-like phenotype.

MATERIALS AND METHODS

Tissue Samples

Human fetal samples were obtained from legally approved therapeutic abortions at the Department of Pathology, University of Naples Federico II. The sampling was carried out under the control of the University's Guidelines for Human Experimentation (autopsy protocol). Informed consensus was obtained from all the subjects involved in the experiments and the study protocols were reviewed and approved by the University Ethical Committee. The age of the fetuses was calculated from anamnestic and ultrasonographic data, it ranges from 18 to 24 gestational weeks. Tissues were dissected, typically within 2 h after death. Lower and/or upper jaw were removed. Blocks of the upper and lower jaw were sectioned, including the deciduous tooth germs of incisors and first molars. We also removed blocks of mandibular branch as a control tissue free of tooth germ. Each tissue sample was split into two upright parts, one to be fixed and the other part to be snap-frozen in liquid nitrogen and stored at −80° until used for RNA extraction. After fixation (24 h at 4°C) the half blocks were exposed to neutral buffered 10% (w/v) EDTA solution for 3 weeks at 4°C. After dehydration, tissue samples were embedded in wax at 54°C, and 3–5 μm thick sections were cut using a special D profile knife for hard tissue cutting. Paraffin sections were placed on gelatine-coated glass slides and dried overnight at 37°C. Sections were counterstained with hematoxylin-eosin for morphological examination. Figure 1 was obtained from one such section.

RNA Extraction and Analysis

Total cellular RNA was extracted by the guanidinium thiocyanate technique [Chirgwin et al., 1979]. Four micrograms of total RNA were subjected to cDNA synthesis for 1 h at 37°C using the "Ready to go You-Primer First-Strand Beads" kit (Amersham Biosciences cod. 27-9264-01) in a reaction mixture containing 0.5 μg oligo-dT (Amersham Biosciences cod. 27-7610-01).

PCR Amplification

PCR amplification of cDNA was performed in a reaction mixture (Pure Taq Ready to go PCR-beads Amersham Biosciences cod. 27-9558-01) containing 4 μl of cDNA sample and different primer sets (20 p/mol each). The sense/anti-sense *HOX* primers for PCR were designed as previously reported [Cantile et al., 2003]. The sense/anti-sense *NeuroD1* primers for PCR were designed according to the following sequences: *NeuroD1* sense primer 3′-5′-GTTATGAGACTATCACTGCTCAGGACC-anti-sense 5′-3′-AGAAGTTGCCATTGATGCTGAGC (Gene Bank accession n°AF045152). To prevent genomic DNA contamination, primer sense and anti-sense were designed to frame a sequence that crossed at least one intron on the genes. The co-amplification of each specific gene and human β-actin gene, as an internal control, was achieved using two primer sets in a single reaction mixture. We selected two pairs of β-actin primers to obtain amplified fragments with different molecular weight (149 and 433 bp), to be used alternatively in the co-amplification reaction. Duplex-PCR products were separated by ethidium 1.2% agarose gel electrophoresis.

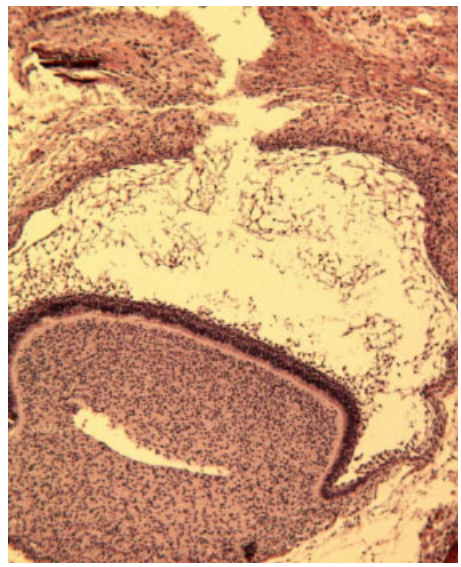

Fig. 1. Ematoxilin-eosin (40×) on paraffin section of early bell stage human tooth germ (UI week 18). Half tooth germs identified through histomorphology have been processed for RNA extraction. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Primary Human Dental-pulp Cells

Primary human dental-pulp cells (DPC) were obtained from patients undergoing routine extractions of third molars (18–30 years of age, both males and females) in the Department of Oral and Maxillofacial Sciences, University "Federico II" of Naples. The use of human tissue

was approved by the Human Institutional Board of the University of Naples. A relatively large amount of tissue is obtained from a single extracted tooth. Dental pulps were dissected from extracted teeth. Tissue pieces were washed with phosphate-buffered saline (PBS) and mechanically dissociated. Tissue fragments where then trypsinized for 20–30 min with 0.25 μg/mL trypsin, and were further mechanically dissociated with fire-polished Pasteur pipettes, and collected into culture medium (DMEM) supplemented with 10% FBS (Gibco), 2 mM glutammine, penicillin (100 U/mL), streptomycin (100 μg/mL), and then explanted into tissue culture dishes. Cells from these explants were cultivated in culture medium in a humidified atmosphere containing 5% CO_2 at 37°C. Confluent cultures were subcultured by trypsinization. Cells of the sixth passage were used in all experiments.

Cell Stimulation

To determine the effect of dibutyryl cAMP on DPC proliferation and differentiation, 4×10^5 cells were seeded in 100 mm culture dishes under standard medium conditions. After 3 days of growth, the medium was supplemented with 10 μM db-cAMP (db-cAMP SIGMA), 1 mM isobutylmethyl-xantine (IBMX SIGMA) to inhibit phospodiesterases activity, and with 0.5% of FBS. At appropriate time intervals (72 h), dishes were trypsinized and processed for RNA extraction.

Immunohistochemistry

The immunostainings were performed on paraffin-embedded sections, using a previous step of the step-induced antigen retrieval technique. Before incubation with the primary antibody, the slides were heated in a pressure cooker for 3 min in a solution of 0.01 mol/L sodium citrate. After incubation with the primary antibodies (HOX B6 sc-17171 and HOX D9 sc-8320, Santa Cruz Biotechnology, Inc.) at 1/100 dilution for 1 h, immunodetection was performed with biotinylated anti-mouse immunoglobulins, followed by peroxidase-labeled streptavidine (LSAB-DAKO, Glostrup, Denmark) with diaminobenzidine chromogen as substrate. Sections were counterstained with hematoxylin.

Immunocitochemistry

Human primary DPCs were cultured, as described above, on glass coverslips and fixed in 10% formalin steam in a Petri capsule for 12 h at room temperature, permeabilized in 0.5% Triton X-100 in PBS for 5 min, and incubated with 3% BSA. The coverslips were then incubated with primary antibodies against *neuron specific enolase* (1:100; DAKO, Denmark) and *Neuro D1* (1:100; Santa Cruz Biotechnology, Inc.) for 1 h in a humidified chamber. Immunodetection was performed with biotinylated anti-mouse immunoglobulins, followed by peroxidase-labeled streptavidin (LSAB-DAKO, Glostrup) with diaminobenzidine chromogen as subtrate.

RESULTS

Figure 1 reports the section of a tooth germ from upper incisor at 18 weeks' development. The controlateral halves of specimens morphologically identified in this way were used for RNA extraction.

We detected the expression (RT-PCR) of the whole *HOX* gene network in 15 deciduous tooth germs and in five biopsies of retromolar tissue obtained from fetuses of gestational age between 18 and 24 weeks of embryonal development. The *HOX* gene network appears abundantly expressed in developing dental tissues. Figure 2a reports the expression of the HOX network in tooth germs of lower incisor (LI), upper incisor (UI), lower molar (LM), upper molar (UM), and tissue of the lower jaw (LJ) from the same subject at 20 weeks' embryonal development. The *HOX* gene network appears expressed in the tooth germs in the figure, displaying 18/39 active genes in LI, 23/39 in UI, 24/39 in LM, and 25/39 in UM. Figure 2b reports the expression for RT-PCR of the genes in the HOX D locus referring to the germs in the adjacent figure. All the genes of the HOX D locus are active except *HOX D11*, which is silent in LI, UI, and UM. Whereas *HOX D11* is expressed in LM where the contiguous *HOX D12* gene is not active.

The genes of the HOX network are weakly active in the biopsies of retromolar tissue. Only 5/39 *HOX* genes are active in the sample of retromolar tissue (LJ) reported in Figure 2a, specifically the genes *HOX A9*, *HOX B8*, and *B13* and the genes *HOX D3* and *D4*, whose RT-PCR expression is reported in Figure 2b. Matching results have been obtained from four other retromolar samples (data not shown).

The histograms reported in Figure 3 show the *HOX* genes actively expressed in the tooth

germs analyzed and divided into the four HOX loci (a) and the 13 paralogous groups (b) into which the network can be subdivided. The HOX D locus displays the highest number of active genes (81.08%), while the HOX B locus contains the lowest (44.7%). The HOX A and HOX C loci, respectively, display 52.04% and 48.5% of the active *HOX* genes. As far as the paralogous groups are concerned, the *HOX* genes in paralogous group 6 are more frequently active (as happens in the pharyngeal arch of the lamprey—Cohn, 2002), while the *HOX* genes in paralogous group 2 (including *HOX A2*) are less frequently expressed. In the HOX network,

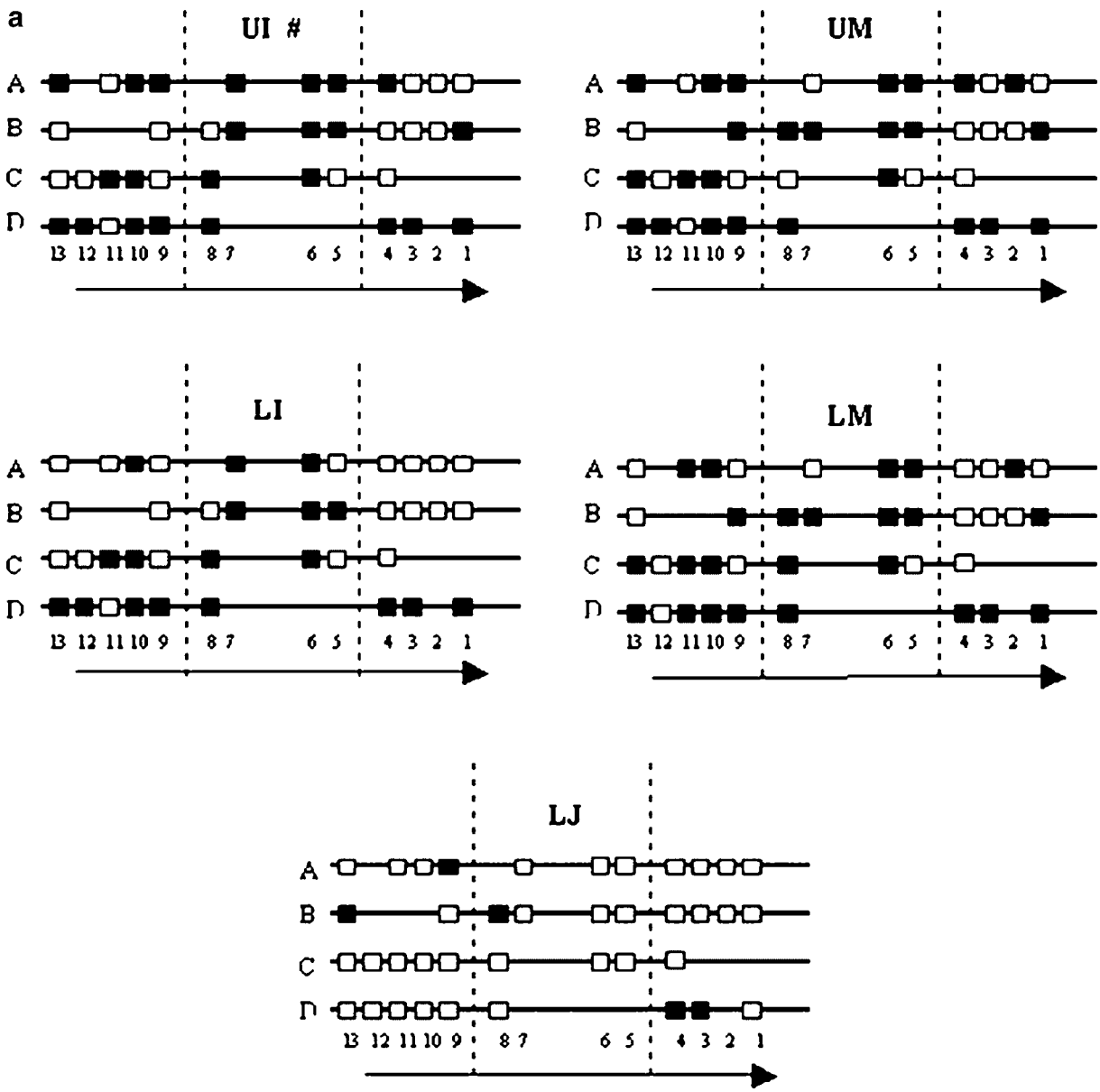

Fig. 2. **a**: Schematic representation of the *HOX* gene network (see the text for details). Expression patterns of the *HOX* gene network (RT-PCR duplex) in human tooth germ RNA from lower (LI) and upper (UI) incisor, lower (LM) and upper (UM) molar, and lower jaw (LJ) from the same subject at 20 weeks of development. Black and white squares indicate active or silent *HOX* genes, respectively. **b**: RT-PCR expression of: *HOX D1*, *HOX D3*, *HOX D4*, *HOX D8*, HOXD9, HOX D10, HOX D11, HOX D12, and *HOX D13*. Control co-amplification of *HOX D1*, *HOX D3*, *HOX D4*, *HOX D8*, HOXD9, *HOX D10* with a 433 bp β-actin primer and of *HOX D11*, *HOX D12*, *HOX D13* with a 149 bp β-actin primer is reported. **Lanes**: lower (LI) and upper (UI) incisor, lower (LM) and upper (UM) molar, and retromolar tissue (LJ). Duplex PCR products were separated by ethidium 1.2% agarose gel electrophoresis.

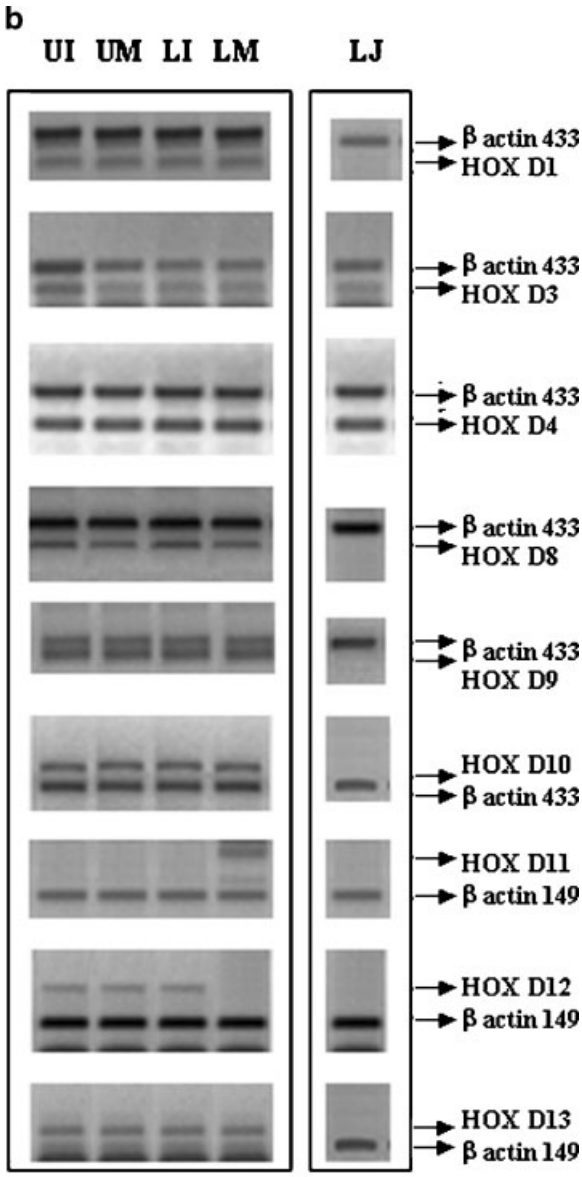

Fig. 2. (*Continued*)

there is a higher number of active thoracic *HOX* genes than either cephalic or lumbo-sacral genes. In any case the active genes of the HOX network in tooth germs exceeds 50% of the analyzed genes.

The immunohistochemical localization of the homeoproteins of one of the genes on the HOX D locus (*HOX D9*) and of one gene in paralogous group 6 (*HOX B6*) indicates that the expression, referring to an initial bell stage (UI week 18), mostly concerns the epithelial compartment of the tooth germ whose cell fate is independent of that of the neural crest cells (Fig. 4) [Couly et al., 1998]. In order to understand to what extent the ecto-mesenchymal compartment of the tooth displays a poor HOX network expression pattern, and whether or not this pattern can be altered and with what consequences, we analyzed the expression of the *HOX* gene network in pulp cells obtained in vitro from adult human molar (DPC) and grown as primary culture. The *HOX* gene network appears to be mostly silent in DPC cells, with the exception of the three genes *HOX D3*, *HOX C6*, and *HOX C8*, which are actively expressed (Fig. 5a top). Treatment of the DPC cells with cAMP activates six genes in the HOX network, two cephalic (*HOX B1* and *HOX D4*), three thoracic (*HOX A7*, *HOX B7*, and *HOX B8*), and one lumbo-sacral (*HOX C10*) (Fig. 5a bottom) taking the total number of active genes in the network to nine. After cAMP treatment the network displays three active cervical genes, five active thoracic genes and one active lumbo-sacral gene. Figure 5b reports the RT-PCR expression of the three invariantly active genes in the DPC cells before and after cAMP treatment (top), and the HOX genes which became active following treatment of the DPC cells with cAMP (bottom). Furthermore, cAMP exposure results in the activation of the neurogenic gene *Neuro D1* (Fig. 5b-bottom). Finally, following exposure to cAMP and parallel to the expression of the six HOX genes and *Neuro D1*, the DPC cells display phenotype modification, as shown in Figure 6, passing from a fibroblast-like phenotype (left) to traits of neuronal differentiation (right). In the DPC cells treated with cAMP, neuro-specific proteins are actively expressed, such as neuron-specific enolase (Fig. 6 top right), and the protein product of the *Neuro D1* gene (Fig. 6 bottom right). Matching results have been obtained from three independent primary cultures of DPCs generated from adult human molars.

DISCUSSION

Unlike most of the homeobox containing genes, the genes in the HOX network do not participate only in the initial patterning of embryonal development. They continue to act in later development determining phenotype cell identity [Cillo, 1994–1995; Scott, 1997]. This function, which is invariate in adult eukaryotic cells, probably represents the original function of the HOX network [Hombria and

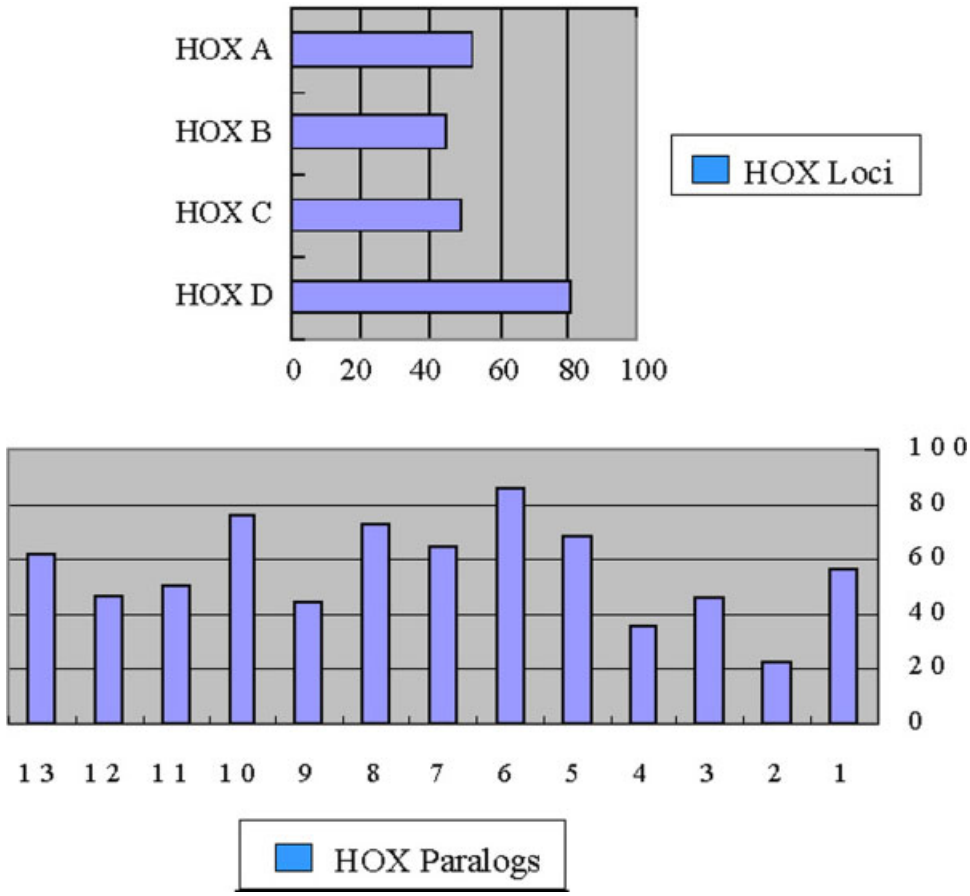

Fig. 3. The histograms represents the total of *HOX* genes expressed in 15 tooth germs, divided into locus HOX A, HOX B, HOX C, and HOX D (top-right) and into the paralogous groups *HOX* genes from 1 to 13 (bottom) into which the HOX network is subdivided. The X-axis on the top histogram and the Y-axis in the bottom histogram represent the percentage of actively expressed *HOX* genes over the total number of *HOX* genes tested.

Lovegrove, 2003], which during early development as well as during organogenesis and adult life, it is displayed through its involvement in differentiation, growth control from cell cycle to apoptosis, and in the main metabolic processes [Cillo et al., 2001]. In the early stages of kidney development, for instance, the metanephric blastema expresses the paralogous group 11 *Hox* genes, *Hox A11*, and *Hox D11* in order to induce the ureter to bud from the Wolffian duct [Wellik et al., 2002]. Whereas, in tubulogenesis, *Hox D11* appears silent before becoming once again active in the final stages of kidney development (from the 23rd week) when the organ acquires its functions, and remains active from birth in the adult kidney (Cantile, Personal Communication).

Tooth germs originate from the interaction between dental lamina (epithelium) and the ecto-mesenchyme of the first branchial arch [Jernvall and Thesleff, 2000]. The cranial elements of the neural crest give rise to the ecto-mesenchyime involved in odontogenesis, and in the development of the majority of cartilaginous and bone structures of the head and neck [Kontges and Lumsden, 1996]. These cells, although endowed with a certain degree of plasticity, preserve the information on their cell fate, and control the patterning of mesodermal cells, which together migrate into the branchial arches [Le Douarin et al., 2004]. It has been proposed that *Hox* genes control the patterning of neural crest elements [Hunt et al., 1991a]. Several observations suggest that *Hox a2*, the most cephalically expressed *Hox* gene is crucial to develop the structures originating from the first and second branchial arch [Hunt et al., 1991b; Gendron-Maguire et al., 1993; Rijli et al.,

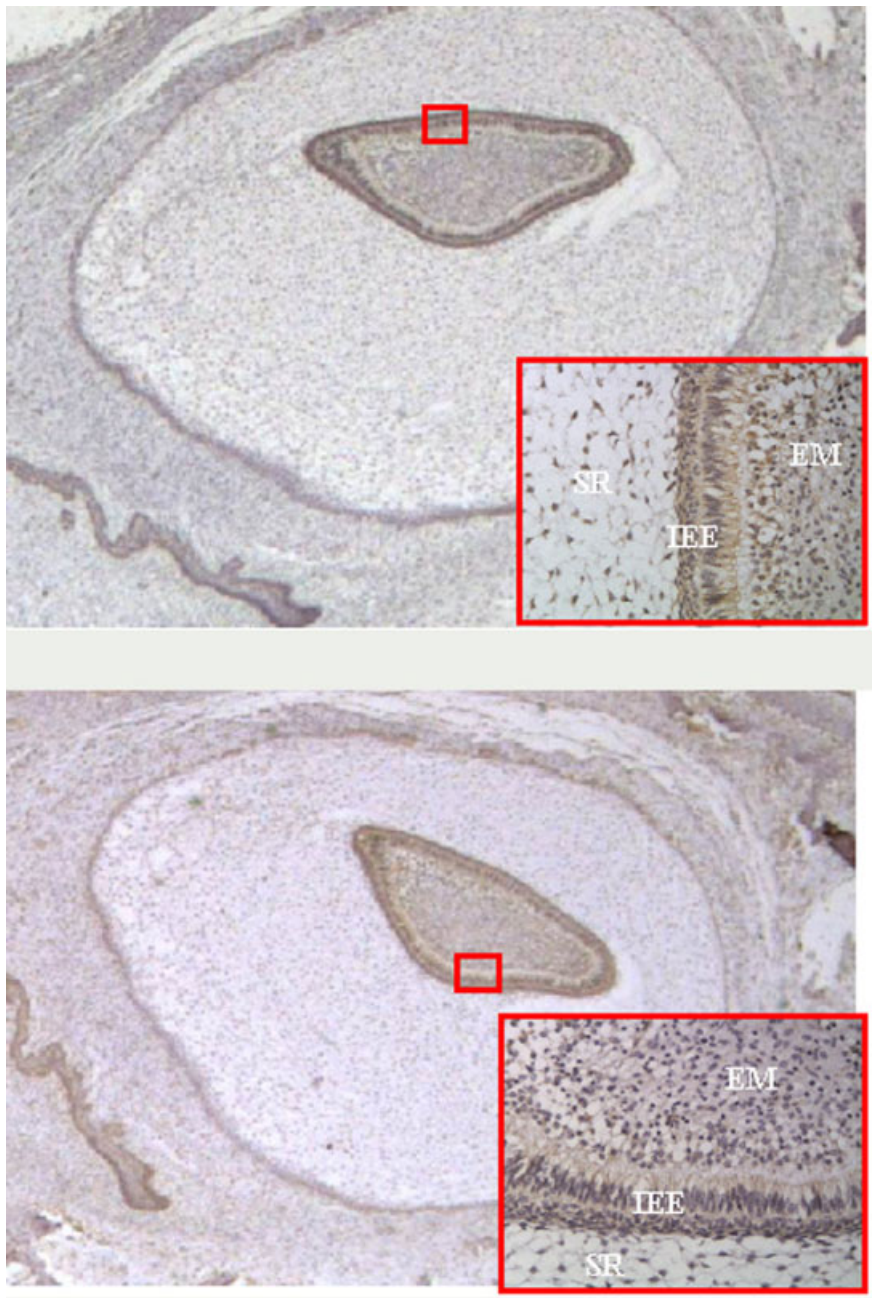

Fig. 4. Immunohistochemical localization of HOX B6 (**top**) and HOX D9 (**bottom**): the immunolabeling concerns, at the early bell stage (UI week 18), the inner enamel epitheilum (IEE), the stratum intermedium (SI), and sparse ectomesenchymal (EM) cells. The satellite reticulum (SR) appears negative. Transversal plane. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

1993]. Although the cells from rhombomers 1 and 2 originally express *Hox a2*, they lose the expression migrating towards the first branchial arch [Prince and Lumsden, 1994]. Whereas the second branchial arch displays a *Hox a2* hyper-expression supporting, according to the principle of posterior prevalence, the hypothesis that *Hox a2* is indispensable to the formation of structures originating from the first branchial arch and that its hyper-expression generates the homeotic transformation of the first arch [Grammatopoulos et al., 2000].

The lack of *Hox* gene expression and the simultaneous expression of other homeobox-containing genes in the patterning of the first

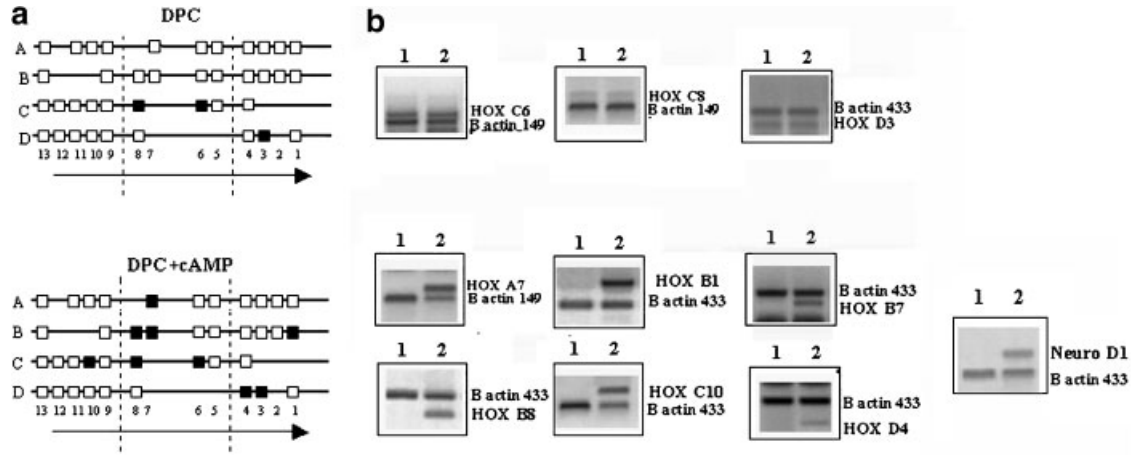

Fig. 5. (a) Expression patterns of the *HOX* gene network in primary human dental pulp cells (DPC) grown in vitro from adult molar. Black and white squares indicate active or silent *HOX* genes, respectively. Top left = the *HOX* gene expressed in untreated DPC cells are *HOX D3*, *HOX C6*, and *HOX C8*. Bottom left = the *HOX* genes expressed in cAMP (10–5 M × 72 h) treated DPC cells are *HOX B1*, *HOX D3*, *HOX D4*, *HOX C6*, *HOX A7*, *HOX B7*, *HOX B8*, *HOX C8* and *HOX C10*. (b) Top = RT-PCR expression of the three invariantly expressed *HOX D3*, *HOX C6*, and *HOX C8* genes in DPC and in cAMP treated DPC cells. Control co-amplification of *HOX D3* and *HOX C6* with a 149 bp β-*bactin* primer and of *HOX C8* with a 433 bp β-*actin* primer is reported. Bottom = RT-PCR expression of the six *HOX* genes activated in DPC cells by cAMP treatment *HOX B1*, *HOX D4*, *HOX A7*, *HOX B7*, *HOX B8*, *HOX C10* plus the neurogenic gene *Neuro D1*. Control co-amplification of *HOX A7* with a 149 bp β-bactin primer and of *HOX B1*, *HOX B7*, *HOX B8*, *HOX C10*, *HOX D4*, and *Neuro D1* with a 433 bp β-*actin* primer is reported. **Lane 1** = untreated DPC cells. **Lane 2** = cAMP treated DPC cells. Duplex PCR products were separated by ethidium 1.2% agarose gel electrophoresis.

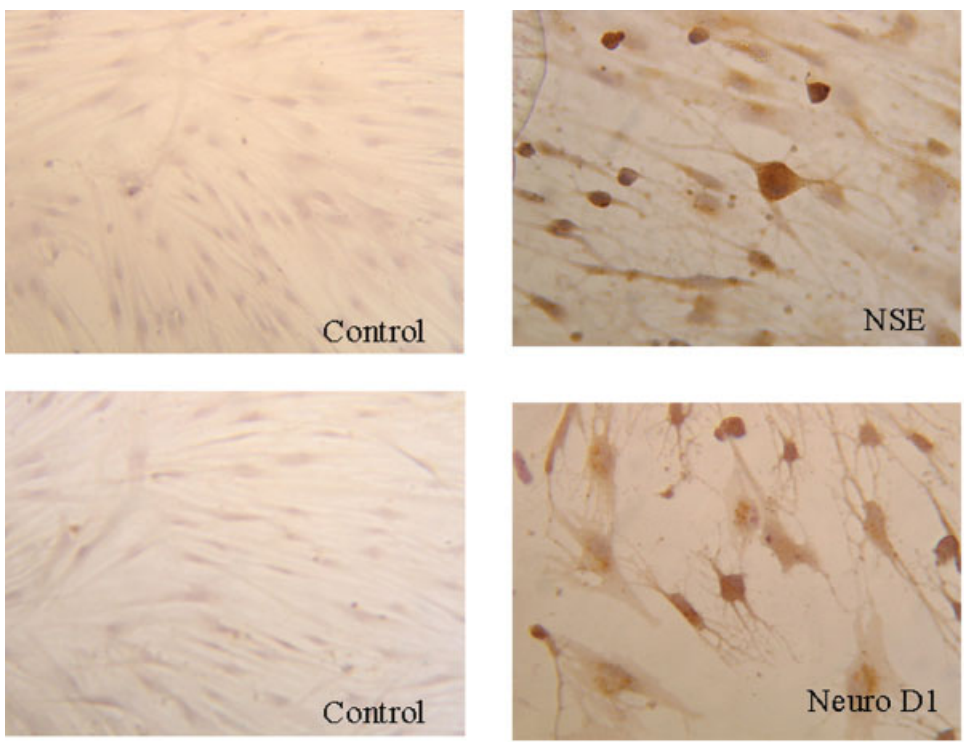

Fig. 6. Top and bottom left = Phenotype identity of untreated DPC cells (20×); Right = positive immunostaining for neuron specific-enolase (top) and neuro D1 (bottom) in cAMP treated DPC cells (40×). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

branchial arch suggested that the *Hox* gene network plays no role in defining the homeobox odonotogenic code [Thomas and Sharpe, 1998]. Whereas, it has also been excluded that *Hox* genes must necessarily be silent in order to induce tooth formation, unlike what is observed in the generation of skeleton structures originating from the first branchial arch [James et al., 2002]. It was therefore concluded that the *Hox* gene network was not involved in odontogenic patterning [James et al., 2002].

Our data indicate that, regardless of what happens during odontogenic patterning of the early phases of tooth development, the *HOX* gene network is active in human tooth germs between the 18th and the 24th week of development. In particular, the expression we describe in this gestational period involving more than 50% of the total number of *HOX* genes tested, mostly seem to concern the epithelial compartment, as is shown by the immunohistochemical localization of the homeoproteins *HOX D9* and HOX *B6*. It is well known that the cell fate of the epithelial compartment is independent of the ecto-mesenchymal elements generated from the neural crest [Couly et al., 1998].

Locus *HOX D* genes appear to be mostly active during odontogenesis together with locus *HOX A* genes. These loci have already been involved with epithelial–mesenchymal interaction during development of several human organs. Concerning paralogous groups *HOX* genes, thoracic groups such as 6, 5, 8, and 10 appear to be active in tooth germs. *HOX* genes located in these paralogous groups interact with several genes playing important role in human odontogenesis.

The mandibular mesenchymal compartment displays a minimal expression of the *HOX* gene network (5/39 active genes in the lowe jaw in Fig. 2). Similarly, pulp cells (of ecto-mesenchymal origin) DPC express only 3/39 active *HOX* genes. Moreover, the in vitro treatment of DPC cells with cAMP results in both an increase (from three to nine) in the number of active *HOX* genes in the network and a phenotype modification with traits of neuronal morphology and activation of neurogenic markers. This confirms the possibility to induce the expression of the *HOX* gene network in the ecto-mesenchyme concomitantly with the occurrence of phenotype modification and differentiation of ecto-mesenchymal elements in neuronal-like cells.

Based on the expression of *HOX* D locus genes in human tooth germs, we postulate a crucial role of this locus in human dentition. Locus D *HOX* genes are located on chromosome 2q31–32, in contiguity with the genes *Dlx-1* and *Dlx-2*. Mammals display six *Dlx* genes arranged as three convergently-transcribed bigene clusters, with each cluster located on a different Hox-containing chromosome. Analysis of the eight described zebrafish *Dlx*-genes have suggested a duplication evolution in common with *Hox* genes [Stock, 2005]. Furthermore, the subdivision of active *HOX* genes in tooth germs according to paralogous groups (Fig. 3) indicates that paralogous group 6 *HOX* genes represent the most active paralogous group genes in the whole HOX network. From the evolutionary point of view, in lampreys the *HoxL6* gene (homologue of the paralogous group 6 *HOX* genes) not only is the *Hox* gene most frequently active in the mandibular arch of developing embryos, but its transcripts also co-localize with Dlx, a marker of lamprey cranial neural crest [Neidert et al., 2001]. Therefore, locus D and paralogous group 6 *Hox* genes might physically and functionally interact with Dlx homeobox genes, which are crucial in neurogenesis and odontogenesis.

During embryonal development, *Hox D* genes play an important role in limb and digit generation [Zakany et al., 2004]. The chromosomal area 2q 31–33 including the HOX D locus, and the *Dlx 1* and *Dlx 2* genes also houses: (a) a global cis-regulatory DNA control region able to coordinate the expression of HOX D and contiguous phylogenetically unrelated genes [Spitz et al., 2003]; (b) a series of neurogenic-related genes involved with neuronal cell conversion [including *neuro D1*, Niwa et al., 2004]; (c) at least two noncoding RNA genes, involved in a series of important cell and developmental pathways, a prostate specific miRNA (*PGCEM1*) [Petrovics et al., 2004], and the gene miR-10b located inside the HOX D locus between *HOX D4* and *HOX D8* [Mansfield et al., 2004]; (d) the cAMP effector genes *CREB1*, *CREB2*, and *cAMP GEFII* [Montminy, 1997].

cAMP treatment of DPC pulp cells activates the neurogenic gene *Neuro D1* plus six HOX genes in the network, one of which *HOX D4*, is active in every samples of Figure 2a. Thus *HOX D4* appears to be an epithelial marker activated by cAMP during neuronal differentiation of DPC cells. *Neuro D1* belongs to the "atonal"

family of transcription factors responsible for regulating the generation of a protosensory organ in *Drosophila* [Niwa et al., 2004] and is known to act in the conversion of epithelial cells into neurons [Lee et al., 1995]. Our data suggest its involvement in the neuronal differentiation of ecto-mesenchymal cells. There is increasing evidence in biology in favor of the concept of physically joined gene sets acting together to regulate crucial cell functions [Misteli, 2004].

The chromosomal region 2q31–33 thus appears to be: (i) involved in the epithelial–mesenchymal interaction typical of human organogenesis (ref); (ii) implicated in modifications of the epithelial–mesenchymal interaction with neuroendocrine differentiation of the characteristics of advanced human prostate adenocarcinoma [Cantile et al., 2004]; (iii) evolutionarily one of the genome areas most highly conserved over the last 500 million years [Sabarinadh et al., 2004]. We therefore assume that the expression of genes localized in this chromosomal area plays a crucial role in the phenotype determination of the epithelial compartment of tooth germ during the interaction with ecto-mesenchyme. The results of this interaction include the activation of a neurogenic programme involved in the conversion of these cell types into neuronal cell elements occurring in a coordinated way during organogenesis [Niwa et al., 2004], and in an altered way during human carcinogenesis [Cantile et al., 2004]. In the chromosomal area 2q31–33, moreover, alterations occur which generate human pathologies: structural [synpolydactyly—Bosse et al., 2000, mesomelic displasia Kantaputra type—MDK—Kantaputra et al., 1992; Fujimoto et al., 1998]; proliferative [prostate adenocarcinoma—Cantile et al., 2005]; neurological [autism—Bacchelli et al., 2003].

In conclusion, our data point to the involvement of the *HOX* gene network in human odontogenesis and suggest that its expression mostly concerns the epithelial compartment of the tooth germ. The expression of the *HOX* gene network is minimal in the ecto-mesenchymal mandibular compartment as well as in primary DPCs. cAMP exposure of human adult pulp cells induces a substantial increase in the expression of the *HOX* gene network, in parallel with the induction of neuronal phenotype. The involvement of the *HOX D* locus genes both in characterizing the epithelial component of tooth germ and in the neuronal conversion of pulp cells allows us to postulate that the HOX D locus and the neurogenic gene programme, within which the locus is localized on chromosome 2, play an important role in epithelial–mesenchymal interactions.

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