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Chapter

Historic Background and Current Perspectives in Dental Crown Formation

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Abstract

Understanding the cellular principles of odontogenesis requires an incremental and up-to-date understanding of the sequential molecular embryological processes leading to a complete normal dental formation. This topic review provides a state-of-the-art explanation of these dental morphogenetic processes and the subsequent crown development in normal deciduous and permanent teeth, based on an upgraded version of the "odontogenic homeobox code". The description of these processes is shown from the differential epithelium-ectomesenchyme and epithelium-mesenchyme interaction stand-points, necessary to produce cell-cell and extracellular matrix-cell transformations. These cellular processes lead to the sequential stages of classic histological dental formation, which progressively correspond to the development of dental regions, identities, and forms, to obtain complete deciduous and permanent human dentitions.

Keywords: Odontogenesis, Amelogenesis, Dentinogenesis, tooth root/embryology

1. Introduction

Odontogenesis (dental -or tooth- formation, development, organogenesis or morphogenesis) is the intraoral molecular embryological process in which teeth are formed from specialized embryonic cells until completion of their root formation and eruption [1, 2]. Such formation is a mix of genetically complex processes determined by a series of non-*HOX* homeobox genes, which regulates initial dental histological organization, histodifferentiation, morphodifferentiation, spatial tooth arrangement, amelogenesis, dentinogenesis, periodontal ligament formation and tooth eruption [3]. In this spatial-temporal sequence, the ectodermal cells of the stomodeum are surrounded by the underlying ectomesenchyme -derived from ectoderm-originated cranial neural crest cells- to collaboratively form dental structures, give shape to future dental crowns, and create supporting tooth structures [4]. This sequential process facilitates the formation of specific dental patterns (dental location, structure,

size and shape [type]) and extends until the completion of tooth eruption [1–8]. More than 300 genes are involved in these interactions [8]. The present literature review considers all topics related with the current molecular embryology state-of-the-art on crown formation and gives a baseline to accurately point out critical aspects of dental development that are important in clinical practice.

2. Normal molecular embryology in odontogenesis

As eutherian mammals, humans are heterodonts and diphyodonts [1, 9]. To explain the heterodont origin of teeth, Sharpe (1995) proposed the "odontogenic homeobox code" [10], as a different approach from both Butler's morphogenetic fields (gradient) model (1939–1956) [11–13], and Osborne's clonal model (1978) [14]. Histologically, tooth formation requires simultaneous formation of multiple tissues closely interrelated. Dental patterning is a 3D gradual expression of several and restricted non-*HOX* homeobox genes in specific craniofacial (mandibular and maxillary) ectomesenchymal cell clusters controlled by the same genes expressed inside dental mesenchyme, to develop either uni-, bi- or multicuspid dental patterns [10, 15–17]. This divided tooth formation origin is genetically orchestrated according to the "clock and wavefront" model of vertebrate segmentation [18, 19], currently understood as the "spatio-temporal dynamics of gene expression patterns" [20–22]. Tooth identity interacting genes follow Sharpe's "code", similar to homeobox codes in other developmental systems [10, 15, 16, 23, 24].

The presence of epithelial-ectomesenchymal (EEI) and epithelial-mesenchymal interactions (EMI) through cell-cell and extracellular matrix-cell transformations is essential. Cross-talk between these tissue layers is mediated by diffusible signaling molecules (growth factors), required for normal cell survival and differentiation [25]. These interactions use similar genetic expression cascades in a repetitive manner that depends on several non-*HOX* homeobox genes [10]: *LHX6* and *8, MSX1* and *2, PAX9, RUNX2, DLX1* and *2, BARX1* and *2,* and *ALX3* [3, 24, 26–28]. These genes control fundamental processes of organogenesis by coding transcription factors needed for the formation in dental crown and root of regulatory proteins, cell signaling molecules, and structural proteins essential for multiple cellular processes -proliferation, condensation, adhesion, migration, differentiation and secretion- in the establishment of a totally differentiated tooth [29]. These series of interactions requires the completion of sequential embryological phases, which almost match the histological phases and stages of tooth development: proliferation and cell migration (dental initiation phase: dental lamina-bud stages); cellular differentiation (dental histo -or cyto- differentiation phase: cap-initial bell stages); and formation of specialized dental tissues -enamel and dentin structural protein secretion- (dental morphodifferentiation phase: late bell stage) [26, 30].

A synthesis of the molecular embryological process of tooth crown formation and development appears below, with emphasis on the gene-protein interactions present in each step.

3. Crown formation

3.1 Primary epithelial band (PEB)

(**Figure 1**) This stage, called Dental Placode (DPL) later on, starts when a portion of the ectoderm (future PEB) begins to proliferate in the surroundings of the

Primary Epithelial Band (Epithelial Thickening)
Initiation - Determination of Tooth Region

a.

Medial Nasal Process
Nasal Cavity
Lateral Nasal Process
Nasomaxillary groove
Vestibular Lamina
Dental Lamina
Nasal Septum
Maxillary Swelling
Mandibular Swelling
1)
2)

b. Intermaxillary Process
Canine Vestibular Ridge
Epithelial Bulges
Molar Vestibular Ridge (MVR)
Cheek Furrow Ridge
Epithelial Accessory Cap
MC
Lateral MVR
Medial MVR
1)
2)
dCFR
ITVE
MC
Mandibular Swelling
Vestibular Lamina
Labial vestibular ridge

c. Mesenchyme
Inductive potential direction
Vestibular
6W
Oral Ectoderm

d. Oral direction
Dental Lamina
Lingual
Vestibular
7W
! " 90°
Dental Placode
Initiation Knot

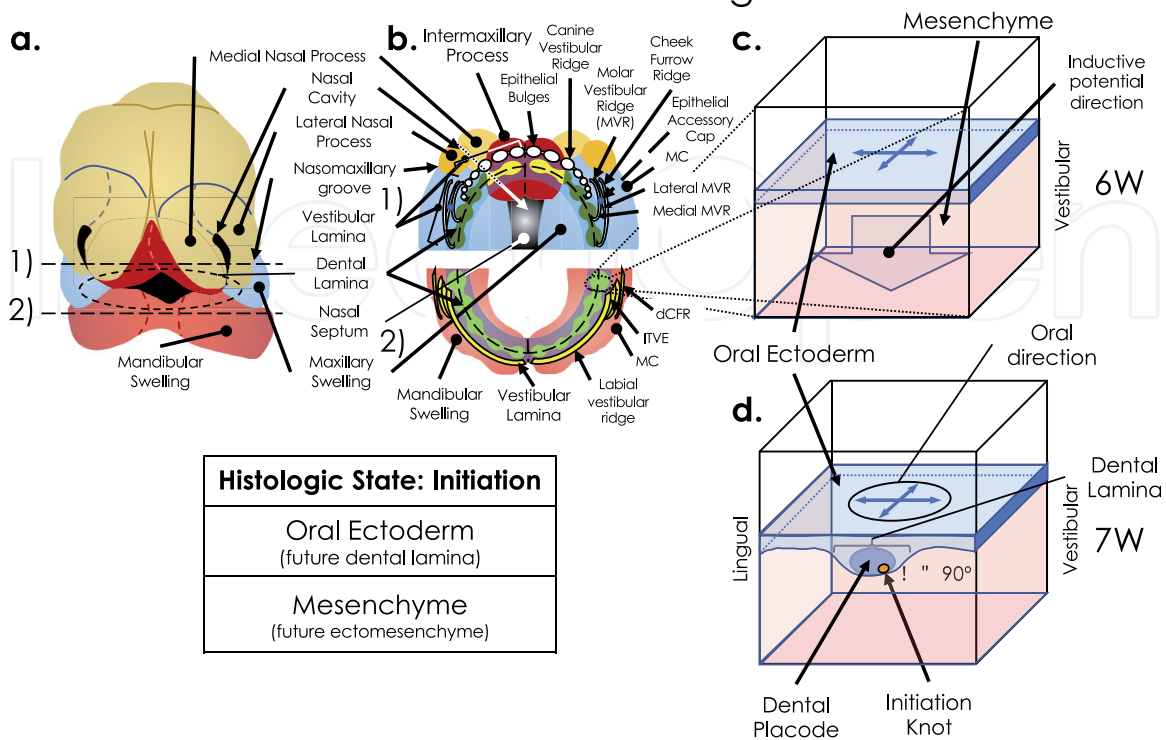| Histologic State: Initiation |
| :---: |
| Oral Ectoderm (future dental lamina) |
| Mesenchyme (future ectomesenchyme) |

Figure 1.
Primary epithelial band (6-7th IU week) – Deciduous dentition. Embryo head: a. frontal view and b. coronal view: 1) A horizontal maxillary section (formed by maxillary swellings and the intermaxillary process of the medial nasal processes) and 2) a horizontal mandibular section can be identified. Note the different components of vestibular lamina as well as the combined formation of maxillary dental lamina at lateral incisor level (from intermaxillary process and maxillary swellings, respectively); tooth forming site: c. At 6th IU week, the oral ectoderm and mesenchyme starts to differentiate, guided from an inductive potential coming from the ectoderm; d. At 7th IU week, the epithelial band thickens to form the dental placode, containing a transient initiation knot. ITVE: Irregularly thickened vestibular epithelium; dCFR: Differentiated mandibular cheek furrow ridge; MC: Mouth corner.

stomodeum during the sixth intrauterine (IU) week, under FGF8 influence -component of the "clock and wavefront" model that brings periodic pulses of Notch, FGF and WNT signaling [19, 31]. FGF8 acts as a strong inducer of *LHX6* and *8* (formerly *LHX7* or *L3*) gene expression in the embryonic molar region [32]. *LHX* genes encode transcription factors that determine cell fate specification and differentiation in maxillary and mandibular processes' oral ectomesenchyme, palatal shelves and forebrain [32–35].

The PEB invades the underlying mesenchyme, dividing it in Vestibular Lamina (VL) and Dental Lamina (DL). VL generation and cell proliferation is induced by the EGF family (with EGFR internalization) and FGF2 [36]. FGFR1 expressed strongly in both VL and DL/enamel organ [36]. Expression of PCNA (a DNA scaffold for DNA replication proteins) and pRb (an initiator of cell cycle) -intensely localized in VL stratum basale- were found [36]. Both proteins are related with temporal and spatial expression of cytokines and receptors in the PEB, VL, DL/enamel organ, and ectomesenchyme [36]. pRb expression corresponded to CK10 expression in the keratinizing VL [36].

The primary dentition begins to form from the DL inside the embryonic mandibular -first- and maxillary processes in the seventh IU week [30]. At 45–48 IU days,

FGFR1 up-regulates pRb to induce cell proliferation, keratocyte differentiation, and exfoliation of keratinizing VL cells to form the oral vestibule space [36]. Simultaneously, DL -under FGFR1 regulation- forms several partially discontinuous thickenings of oral ectoderm inside the different dental fields (in a dorsal-ventral [molar-first] pattern) [36–38]. At these locations, LHX6 and 8 transcription factors [39, 40] restrict the expression of *GSC* gene to the caudal mesenchyme, establishing the oral–aboral polarity in dental arches [41–43]. These thickenings grow inward in the future dental arch mesenchyme [37], to give origin to the cellular component of deciduous dental germs formation [44]. At this point, a shift of inductive potential from the oral ectoderm to the underlying ectomesenchyme occurs [37].

In the ectomesenchyme, *BARX1* gene is expressed in restricted areas of head and neck mesenchyme [45, 46]. Molar tooth papillae -derived from ectomesenchyme- form under its expression [45]. *GLI1* gene (from the SHH signaling pathway) is also expressed [47–49]. The mesenchymal induction of two members of the TGFβ family -BMP4 and Activin βA- induce overlying epithelial cells to form the DPL [49].

Several subsets of cells inside the DPL become quiescent and form a series of early signaling centers -Initiation Knots (IK's)-. These centers govern epithelial budding and folding, and adjacent ectomesenchyme condensation [9, 50]. These cells start to migrate centripetally and condense to form a non-proliferative mature signaling center. Soon after, bud formation begins with a burst of epithelial cell proliferation in the surroundings of, and oriented away, from the IK's [50], followed by centripetal cell movement and cell intercalation that possibilitate cell rearrangement inside the bud [51]. Tooth bud size and the number of IK cells are regulated by the EDA/EDAR/ EDARADD pathway [50, 52]. Among the genes expressing inductive signals from these DPL non-proliferative cells clusters are *p21, SHH, EDAR, DKK4, FGF4*, and *FGF20* [53–60]. Cell cycle regulatory protein p21 mediates growth arrest at specific stages in the cell cycle by binding to and inhibiting cell division control proteins CDK1 [61, 62], and CDK2 [63]. The levels of EDA (mainly) and FGF signaling -present both in DPL (FGF4 and 9) and newly formed ectomesenchyme (FGF3)- help defining tooth number [32, 64–68]. Epithelial expression of FGFR2c, FGF4 and 9 regulates mesenchymal proliferation, while epithelial proliferation is promoted by mesenchymal expression of FGF3, 10 (both important in dental morphogenesis [tooth number] -mostly FGF3- and tooth size) and FGFR2b. In addition to FGF signaling, TWIST1 modulates FGF signaling within DPL and ectomesenchyme, defining tooth germ size and cusp formation [69].

3.2 Dental germ (bud) (DG)

(**Figure 2**) By the eighth IU week, the odontogenic epithelium located in the innermost region of the DL enters a mitosis phase in aboral direction (away from the oral cavity and inside future mandibular and maxillary bones) and begins to invade the subjacent mesenchyme, forming outbreaks of identical cells (epithelial or dental germs -DGs-) that will shape all enamel organs [39]. Each DG is connected to the DL through a pedicle -gubernaculum dentis or gubernacular canal- [70]. The DG's and ectomesenchyme are separated at this stage by a basement membrane [71]. Cell cycle regulatory proteins CCNA2 -from *CCNA2* gene, which form a complex with CDK2-, Cyclin D1 -encoded by *CCND1* gene-, and Ki-67 -encoded by *MKI67* gene- in molar dental epithelial and mesenchymal cells, and Ki-67 and Cyclin A2 (in lesser amount) in both incisors' EO and dental mesenchyme (with some Ki-67 negative cells in the EO bulbous protrusion) start to be expressed at this time [72]. A Cyclin D1-CDK

Dental Germ (Bud)
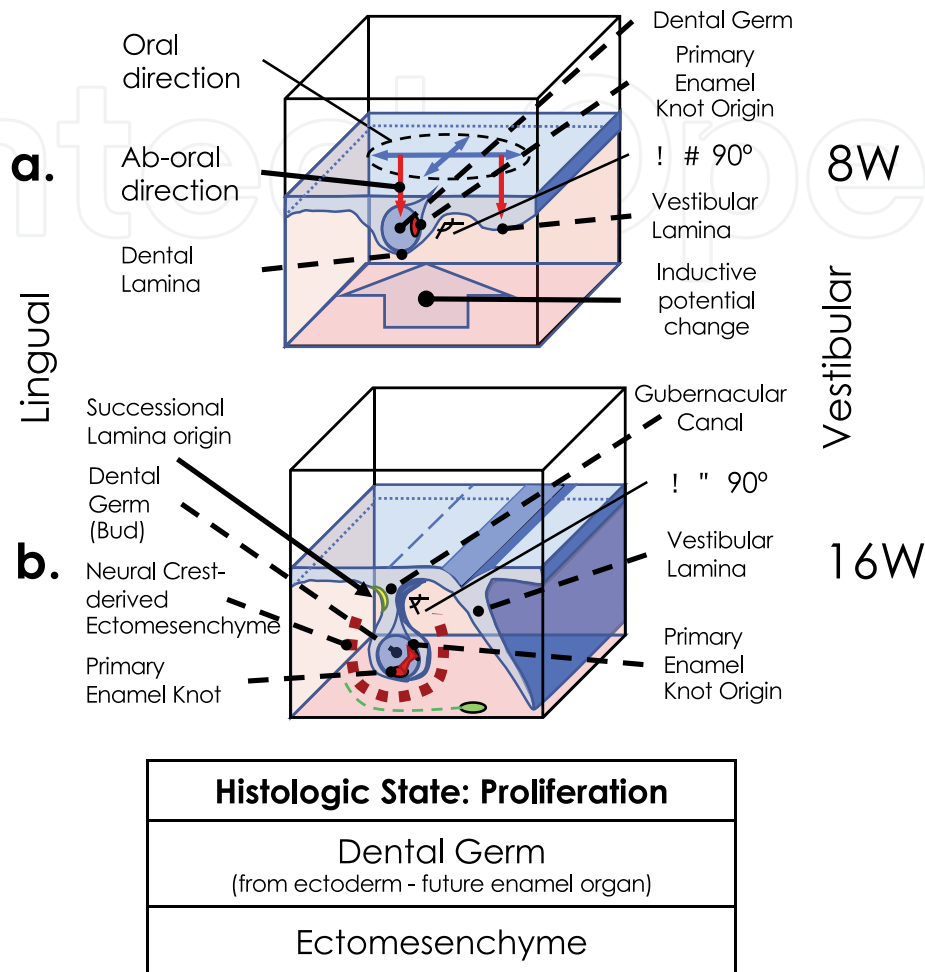## Determination of Tooth Identity

Figure 2.
Dental germ (8-16th IU week) – Deciduous dentition. Tooth forming site: a. At 8th IU week, the dental placode starts to grow in specific points in an aboral direction to form dental germs, due to local changes in the expression of PAX9, MSX1–2, DLX2–5, SHH, and cell cycle regulatory proteins. The forming ectomesenchyme also expresses MSX1–2 (regulated by PAX9), DLX1–2 (molar region), BARX1 (molars and bicuspids), and GLI2 and ALX3 (incisor region). At this point, a change in the direction of the inductive potential takes place; b. At 16th IU week, the dental germ is fully formed, containing a definitive enamel knot. Ectomesenchymal cells surrounding the dental germ start to condense to form the future dental papilla. A successional lamina appears lingual to the dental germ to give rise to the permanent tooth replacement.

complex formation (with CDK2, which compete with canonical CDK4 or 6 [73]) is needed to regulate transition from G1 to S phase [72]. However, upregulation of p21 slightly precedes the cessation of cell proliferation of the forming Enamel Knot (EK -cell cluster located in the center of each internal enamel epithelium-) by CDK2 inhibition [55].

Several key genes also initiate expression. *PAX9* gene (encoding PAX9 transcription factor) is a key control element during odontogenesis initiation. It is specifically expressed in all prospective tooth sites before any morphological odontogenetical signs [2, 5–8, 26, 29, 30, 74–79]. *MSX1* and *2* genes encode regulatory proteins that act as transcriptional repressors inside the cell nucleus [80]. *MSX1* gene is related with the development of ectodermal derivatives, with a strong expression in dental

mesenchyme [1, 2, 4–8, 10, 26, 29, 30, 74–79]. *MSX2* gene also participates from the onset of odontogenesis in both oral epithelium and ectomesenchyme [81]. In order to regulate their effects, MSX homeoproteins form inactive homodimers or heterodimers with DLX homeoproteins (2 and 5) [82–84]. PAX9 homeoprotein has a genetic epistasis with MSX1 protein, being able to directly regulate *MSX1* gene expression and interact with MSX1 protein to enhance its ability to transactivate *MSX1* and mesenchymal *BMP4* gene expression during tooth development [83, 84]. Both genes are regulated by OSR2 transcription factor, responsible of downregulating tooth number [85]. *SHH* gene also participates in epithelial cell proliferation involved in tooth bud formation [86]. In addition, *AXIN2* gene expression was observed in epithelium and mesenchyme of mandible, maxilla and lateral nasal processes. *AXIN2* expression in the incisors and molar buds is located in spherical cell balls at the anterior part of the tooth bud where the EK's form, as well as in the surrounding mesenchyme [87].

Simultaneously, ectomesenchyme condensation surrounding these ectodermal cell bursts express several transcription factors, which differentiate the forming individual condensations from the rest of mesenchyme [88]. *DLX1* and *2* gene expression is found in proximal ectomesenchymal regions where molar teeth are located [15]. *GLI2* gene is responsible of maxillary incisors normal development, while *GLI3* gene is responsible of molars and mandibular incisors size, and maxillary incisors development at the epithelial thickening stage [86]. *PITX2* gene acts as an exclusive marker for developing tooth epithelium and is involved in tooth orientation regulation [25, 89–92], BMP4 restrict both *PAX9* expression to the dental mesenchyme and *PITX2* expression to the dental epithelium [93].

The DG cells also produce glycosaminglycans [94]. Versican (coded by *VCAN* gene) V0/V1 is involved in cell proliferation, while V2 isoform is associated with histodifferentiation phase [94]. These two molecules act as scaffolds for extracellular matrix formation [95], and in EEI in early dentinogenesis [96]. Several glycoproteins such as TN-C -that modulate with anti-adherent effects the cell-matrix interactions [97, 98]- and TN-X -a TN-C complementary protein [99, 100]- are produced also. TN-C is found widely in loose connective tissue, with a regulating role in collagen deposition and indirect binding and bridging of these fibrils [97, 99, 101]. FN, collagen type III, and other chondroitin sulfates are also found [102]. CPNE7 expression -important in EMI- is also seen in dental epithelium [103]. This variation in intracellular molecular contents causes cell turgor and marks the initiation of DG histodifferentiation phase [104]. Trigeminal nerve fibers first appear in the DF at late DG stage. Some nerve fibers from PRPH-reactive neurons are located within tooth germ ectomesenchymal cells [105].

When fully formed around the 16th IU week, deciduous DG's are composed by ectodermal cells groups that will differentiate into future Enamel Organs (EO's), surrounding condensed ectomesenchyme cells that give shape to Dental Papillae (DPa's). These structures will form enamel (from EO), dentin and pulp (from DPa). The mesenchyme surrounding both cell groups is called Dental Follicle (DF) or Sac. This cell group further differentiates in alveolar bone, cementum and periodontal ligament [106].

PAX9 and *MSX1-2* are among several dental patterning genes found in dental mesenchyme [6, 107–109]. Other genes are *DLX1-2* -particularly expressed in maxillary primary molar mesenchyme where they play an important role in maxillary primary molar tooth patterning [15, 23], *BARX1* -involved in multicuspal mesenchymal morphogenesis (molars and bicuspids) [46, 110], and *ALX3* –coding a transcriptional regulator involved in cell-type differentiation and development [24]. Using a different

animal model from McCollum and Sharpe [27, 28], Wakamatsu et al. [24] used 4-tooth-classes animal species (opossum and ferret) to affirm that dental formula differences in humans (that follow the "4-tooth-classes" pattern) were due to a large overlap of the proximal part of the *MSX1* expression and most of the *BARX1* domain in the middle of the mandible primordium [24]. According with their view, *MSX1/BARX1* positive domains correspond to an FGF-dependent activation of *MSX1* overlapping with *BARX1*, which partially produces bicuspid differentiation. Maxillary and mandibular canine correspond to *MSX1*-positive region, and incisors correspond with *MSX1/ALX3* positive region, respectively (**Figure 3**) [24].

Formation of permanent dentition up to second bicuspids started with the derivation of lingual groups of cells from the deciduous enamel organs at the time of deciduous morphodifferentiation (between the 10th and 13th IU week), forming a successional DL under the influence of *SOX2* gene [9, 75]. These lingual cell clusters at each deciduous tooth constitute the successional permanent teeth [76]. The formation of the permanent molars, or additional permanent teeth, originates from the expansion of a subepithelial continual (or additional) DL that comes off the last deciduous DG [14, 76]. Permanent molars represents members of the primary dentition without successional replacement that show later development and eruption [111]. From these two origins, the permanent DG's start to form around the 3–4 months of age and extend their initial formation until the early teen years, when the 3rd molars begin to form (**Figure 3**) [30].

3.3 Dental cap (DC)

(**Figure 4**) The DG invaginates towards the aboral direction, forming a cap-like structure -the EO- that begins to encapsulate the subjacent mesenchyme [71]. This bud-to-cap transition -regulated by FGF3 and RUNX2 [112]- constitutes an epithelial folding deformation phenomenon, fundamental for the differentiation and specialization of dental cell types and the definition of final tooth size and shape, as the dental epithelium elongates and the underlying mesenchyme grows [71].

The epithelial cells of the deepest part in the internal portion of the EO -pre-ameloblasts- begin their histodifferentiation with the formation of the EK's [113–115] -significant non-proliferating tooth signaling centers [50, 116], under the influence of mesenchymal BMP4 [112]. EK's are not derived from the early DPL signaling center, but rather they form de novo at the inner tip of the cap [50]. They are divided into primary EK's -appearing at this stage- and secondary EK's -developing during bell stage- [112, 116]. The number of EK's determine the number of cusps [1, 24, 117, 118] (**Figure 4a** and **b**). EK signaling controls reciprocal EEI -responsible for EK maintenance and morphogenesis-, with proliferation and movement of surrounding EO cells and ectomesenchyme during folding and patterning of dental epithelium and cusps [2, 24, 49, 56, 112]. p21 (protein associated with cell exit from the cell cycle) and Cyclin D1 (a cell cycle enzyme coded by the *CCND1* gene, regulated positively by Rb) -both from cell proliferation-regulating genes-, elongate the cell cycle at G0 phase in the EK [72, 116]. Molar EK cells only express Cyclin D1, while stronger localization of this enzyme and other DNA regulatory genes are found in incisors' dental epithelium compared with dental mesenchyme at this stage [72]. EK's also express in an encapsulated pattern *MSX2* –coding a transcription factor that organize normal cusp generation- plus several extracellular signal molecules belonging to four protein families (BMP, HH, FGF, and WNT), including BMP2, −4 and − 7, SHH, FGF3, −4, −9 and − 20, and WNT3, −10a and -10b [5, 49, 53, 56, 112]. These molecules (mainly
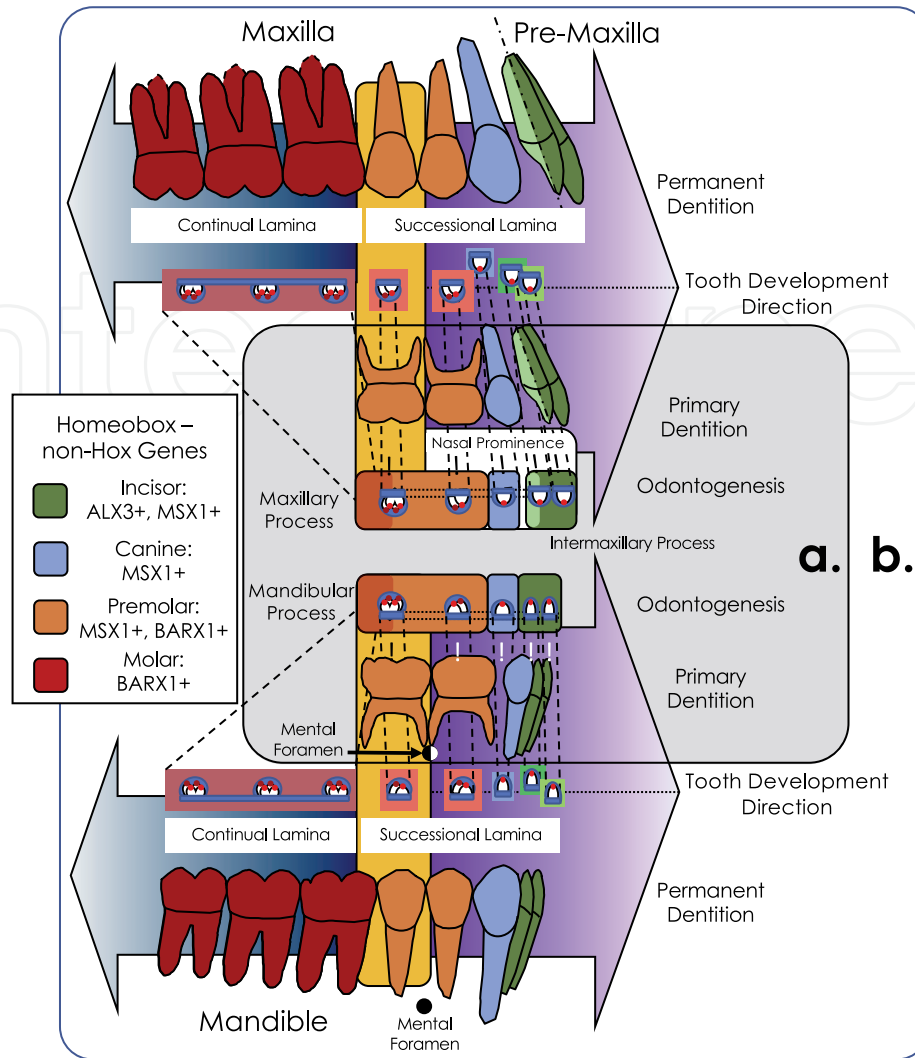
Figure 3.
Homeobox non-Hox genes involved in the development of primary, continual and successional dental laminae.
a. Starting at second deciduous molar dental germ level, primary dentition develops from the dental lamina in
a distal-to-mesial pattern; b. permanent dentition develops from two different origins: The successional lamina
-lingual extension of each deciduous dental germ for incisors, canine and bicuspids-, and the continual lamina -a
distal extension of the deciduous second molar germ that gives origin to permanent molars-. The genes involved
vary according with the level of dentition they belong to (incisor, canine, bicuspid [primary molar], molar),
respectively. (modified from Juuri and Balic [77], Wakamatsu et al., [24]).

FGF4 and WNT10a) and other signal molecules (like CPNE7) are the first inductive signals for pre-odontoblasts differentiation and primordia of other dental pulp cells, normal cusp growth, and patterns of cusp formation that give rise to different dental morphologies in the initial bell stage [1, 30, 56, 103]. Particularly, CPNE7 induces the differentiation of mesenchymal cells (of dental or non-dental origin) into pre-odontoblasts and odontoblasts through EMI [103].

Expression of *LEF1* was also shown in EK's and dental ectomesenchyme at DC and DB stages [116]. This pleiotropic gene codes a member of the LEF/TCF family of transcription factors. It forms a transcriptional regulatory complex with β-catenin to control WNT signaling-mediated cell survival, intercellular adhesion, cell proliferation, pattern formation, axis specification, and cell fate determination -by regulating the cell cycle progression in progenitor cells- [119–127]. In tooth EEI, LEF1 -under direct mediation by BMP4 and WNT10a and b- induce epithelial FGF4 expression, which regulates the induction of mesenchymal FGF3. Reciprocally, FGF3 -

Dental Cap
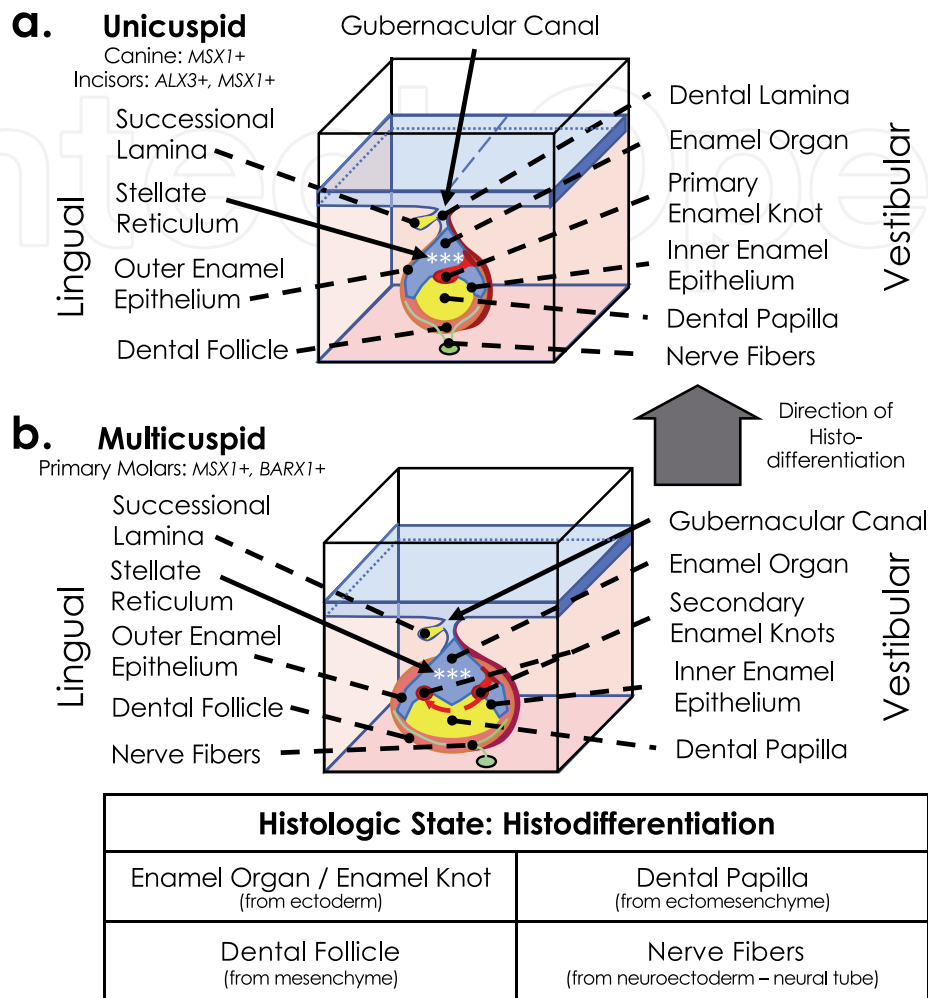## Determination of Tooth Shape

Figure 4.
Dental cap – Deciduous dentition. Tooth forming site: a. Unicuspid tooth formation: After being submitted to an epithelial folding deformation phenomenon -under epithelial FGF3/RUNX2 control-, the dental germ invaginates in an aboral direction and forms the enamel organ and the enamel knot -under epithelial SHH and mesenchymal BMP4-. The ectomesenchyme is partially surrounded by it, assuming the role of dental papilla. Pre-odontoblasts start expression of type I collagen and NCP's genes; b. Multicuspid tooth formation: The primary enamel knot further divides in secondary enamel knots at early bell stage to define the number of cusps.

and possibly BMP4- induce expression of SHH in the epithelium [119, 128, 129]. In addition, it induces the ectomesenchyme competency to form the DPa -a hallmark of the DC stage- [128–130]. It is expressed in crest cells and ectomesenchyme-derived structures during embryogenesis and is critical for EK cell survival [119]. SHH signaling from EK is needed for cervical loop growth and tooth crown patterning regulation during early DB stage, by initiating secondary EK's -expressing almost the same signal molecules as the primary EK's- [112].

The pre-odontoblasts subsequently express *COL1A1* and *COL1A2* genes (which encode the amino-acid chains of type I collagen), whose future constituent protein forms almost 90% of the organic matrix of dentin. In addition, they also express non-collagenous proteins (NCP's) genes such as *DSPP* (which encodes dentin sialophosphoprotein -DSPP-, belonging to the Small Integrin-Binding LIgand Nitrogenous

Glycoproteins [SIBLING] family [131]). DSPP pre-protein is secreted and proteolytically processed in several functional domains to generate DSP, DPP, DGP, and BSP. These proteins perform different functions in dentinal mineralization [106, 131]. Other NCP's include DMP1, SPARC, OPN (from *SPP1* -Secreted Phospho-Protein 1- gene), MEPE (from *MEPE* gene), decorin (encoded by *DCN* gene), biglycan (from *BGN* gene) and two vitamin K-dependent calcium binding proteins of the extracellular bone matrix [132]: MGP (from *MGP* gene) [133, 134], and OSTCN (from *BGLAP* gene). EK apoptosis is the final stage of an EK cell, where induction of p21 and EDAR by BMP4 inhibits EK cell proliferation between G1 and S phase and makes cells responsive to EDA, expressed in the flanking epithelium of the tooth bud [55, 112, 135]. EDA/EDAR/EDARADD signaling regulates the formation and the signaling activity of the EK [64, 112].

Two theories (differentiation and concrescence) explain how these centers can form multicuspid coronal patterns [30]. The theory of differentiation (Cope-Osborn or trituberculate theory) is based on 19th century paleontological and anatomical comparative findings [136, 137]. Phylogenetically, complex multicuspid teeth (initially tricuspid) evolved by differentiation of a unicuspid tooth [30]. The phylogenetic decrease in the number of teeth involved the deletion of the simplest distal teeth, while the remaining teeth underwent further differentiation to produce multicuspid crowns [132, 136, 137]. Osborn et al. (1972-1975), [138] (a different first author, who also proposed the Clone theory of dental formation [14]) stated that this decrease in number involved the deletion of the simplest distal teeth, while the remaining teeth underwent further differentiation to produce multicuspid crowns [138–143]. Also originated in the 19th century, the theory of concrescence (theory of the dimer [Bolk] or of the integrated development) is supported by the dental embryological development that occurs during ontogenesis in different species [30, 144, 145]. According to this theory (partially described by Kükenthal, 1892 and summarized previously to Bolk's work by Adloff, 1916), multicuspid teeth in mammals evolved through the integration of several individual dental primordia (similar to that of reptiles) [30]. This process would integrate two lines of dental primordia in the longitudinal (buccal) and medial (lingual) directions, to form more complex multiple molars. This concrescence of the dental primordia may be accompanied by a shortening of the jaws [137]. It is different from the better-known definition of dental concrescence ("fusion" of adult tooth roots by cementum) [146]. While the concrescence theory explains the multicuspid dental development process, the differentiation theory describes its outcome in adult dental morphology [30].

During the bud-to-cap formation process, the EK's create a spatial deformational anisotropy along the buccal-lingual axis with minimal volume growth, while the remaining epithelial EO cells grow at a higher rate. This epithelial growth increases the internal pressure around the EK's, in addition to the mechanical restrictions occurring at the epithelium-mesenchyme interface. This change might induce the buccal-lingual flattening of tooth germs during cap stage [71].

3.4 Dental bell (DB)

(**Figure 5**) At this stage the future shape of the crown is determined. The DC begins to develop a bone crypt within the ossification zone. The EO acquires the shape of a bell due to the folding and complete cellular differentiation of the internal epithelium of this structure. The DF delimits the external portion of the bell, while the DPa is located towards its internal portion [147].

Dental Bell
Determination of Tooth Shape

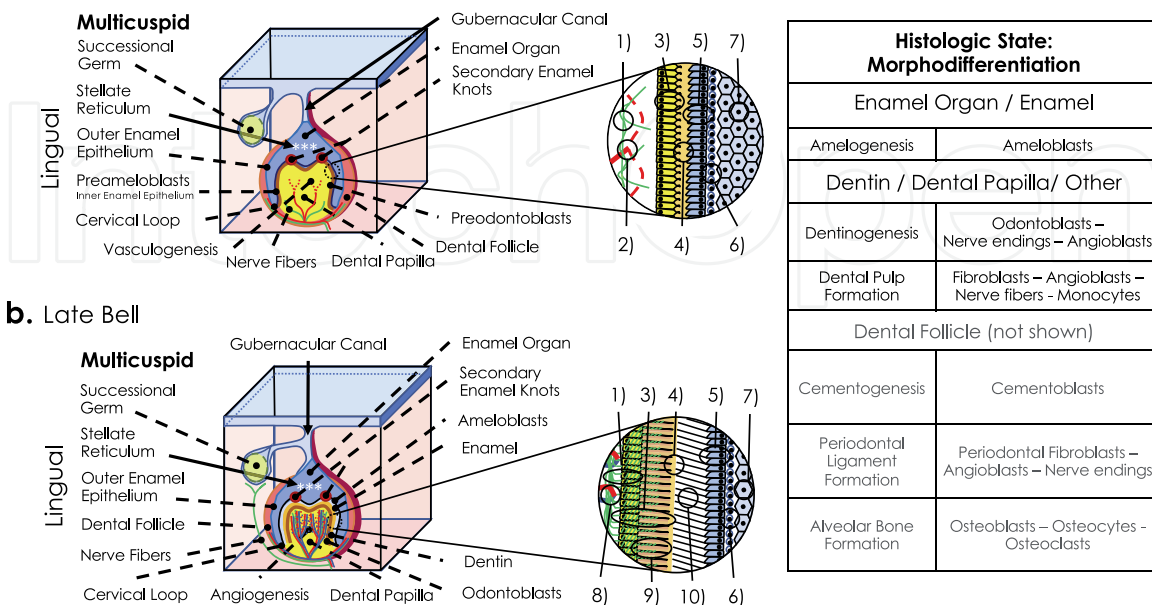**a.** Early Bell

b. Late Bell

Figure 5.
Dental bell – Deciduous dentition. Tooth forming site - Multicuspid tooth formation: a. early bell stage: Starting at the enamel knots level, the newly formed odontoblasts begin to delay mantle dentin. Vasculogenesis is active as well as the growth of nerve fibers. A highly stratified enamel organ can be seen, but no morphodifferentiation changes are visible yet; b. late bell stage: As the delay of dentin and enamel is in process, nerve fibers start to get in contact with odontoblasts to establish the dual role of odontoblasts in tooth formation as both mineralized tissue forming cell and tissue specific neuronal ending. Vascular supply is exquisite for the development of mineralized dentin at this point. 1) nerve fibers; 2) Vasculogenesis; 3) odontoblasts; 4) mantle dentin; 5) Ameloblasts; 6) SI cell; 7) SR cell; 8) angiogenesis; 9) dentin; 10) enamel.

The DB presents two phases from the histological point of view:

3.4.1 Initial or early DB

(**Figure 5a**) This phase continues from the histodifferentiation in the DC stage towards enamel and dentin formation in the DB stage. Histologically, the EO is stratified in four groups [148]:

1. Outer Enamel Epithelium (OEE): cube-shaped epithelial cells located on the periphery of the enamel organ. It is separated from the ectomesenchyme by a basement membrane. These cells express *SLCO4a1* (coding an anion membrane transporter in cell junctions, involved in the transport of sugars and organic acids, metal ions, amine compounds, and estrogen)*, TH* (coding the enzyme tyrosine hydroxylase, which takes part of the pathway that produces catecholamines -group of hormones including dopamine, norepinephrine and epinephrine-) and *AMER1* (coding a regulator protein of the WNT signaling pathway -Adenomatous Polyposis Coli [APC] protein- during cell division and movement) genes, with OEE progenitor gene expression of *FOS* (Fos proto-oncogene or AP-1 Transcription Factor Subunit - coding a leucine zipper protein that dimerize with JUN proteins to form the transcription factor AP-1, a regulator of cell proliferation, differentiation, transformation and cell death), *EGR1* (Early

Growth Response 1 – also a regulator of cell survival, proliferation and death), and *VRTN* (coding vertebrae development associated protein -or vertnin-, which act as a transcriptional repressor functioning independently but in coordination with canonic WNT signaling to regulate dorsal-ventral patterning) [149, 150].

2. Stellate Reticulum (SR): central portion of the enamel organ with turgor cells joined by desmosomes. These cells are marked by *VAT1L, FAM19A4*, and *HEY2* [149].

3. Stratum Intermedium (SI): Two or three layers of flat alkaline-phosphatase-producing cells that are needed for enamel mineralization, marked either by *RAB3IL1, PMCH*, and *CYP2S1* or *PSMB10, C1QB*, and *IBSP* [149]. There is a third layer of cuboidal SI cell that contains *THBD, GNRH1*, and *JPH4* gene expression [149]. The SI progenitors are marked by *CDH6, LRP11* and *CPNE5* genes [149].

4. Internal Enamel Epithelium (IEE): cylindrical cells that differentiate into ameloblasts -enamel-producing cells-. This epithelium has an internal basal portion of undifferentiated mesenchymal cells, followed by another cell layer closer to the epithelial surface composed of pre-ameloblasts -elongated cells in ameloblastic differentiation, marked by *COL22A1, VWDE, KIF5C* gene expression-.

An additional cellular region is identifiable in the apical side of the EO at this stage: the Cervical Loop (CL) (after Diamond and Weinmann, [unpublished data]) [151] (precursor of the Hertwig's Epithelial Root Sheath - HERS). This transient structure is formed at the union of both IEE and OEE, and is composed on its central portion of loosely aggregated SR surrounded by SI [152]. Currently, there has been an agreement on the stages of development inside this epithelial zone. While CL refers to an initial enamel-forming phase inside this ectodermal structure, HERS is established when an EMI occurs in the OEE cellular component during dental root formation to give rise to cementoblasts [152]. HERS and its remnants (Malassez' epithelial rests) are involved in the root formation process. HERS apical growth and subsequent cementoblast transformation splits root dentine from the incipient periodontal ligament [152].

Regulation of the programmed EK and IEE cell-cycle phase-timing controls tooth morphology [116]. Secondary EK's follow a precise sequence to determine sites where the epithelial sheet folds and cusp' development starts, with mechanisms of lateral inhibition and central on-off signaling [112]. Their development is regulated by reiterative signals from primary EK's, previously formed secondary EK's, and mesenchymal signals that code different tooth morphologies through an intricated gene expression network [112]. These histodifferentiation changes in crown formation are mediated by various transcription factors (regulatory proteins such as PITX2, LEF1, MSX1, PAX9 and with less effect DLX1-2 and GLI2-3) and extracellular signal molecules/receptors (AXIN1–2; WNT3, −7b, and 10a-b; EDA-EDAR-EDARADD; BMP2,4, and 7; FGF1,2,4, and 8; SHH; HGF; PTC1; SLIT2; and SMO) [2, 4, 8].

Ectomesenchymal BMP4 signaling -expressed after DPa WNT/ß-catenin signaling [92, 153]- is fundamental for ameloblast differentiation [154]. EPFR -a Krüppel-like family (KLF) transcription factor expressed in ameloblast- and odontoblast-lineage cells [155]-, also produces enhanced ameloblast WNT/ß-catenin and BMP4 expression and induces intranuclear ß-catenin accumulation and formation of cell junctions during predifferentiation bell stages [156]. CPNE7 protein expression also raises in DPa ectomesenchymal cells at this stage [103].

3.4.2 Late or advanced DB

(**Figure 5b**) In this phase, changes occur in morphodifferentiation for the formation of enamel, dentin, cementum, periodontal ligament, and alveolar bone. At crown formation stage, CPNE7 is localized in differentiating odontoblasts and odontoblast processes during dentinogenesis [157]. It initiates dentinal hydroxyapatite nucleation, promotes the formation of dentinal tubule-like structures, and stimulates obliteration of dentinal tubules due to its calcium-binding properties [157, 158]. CPNE7 expression is lost when odontoblasts reach full differentiation [103].

Afterwards, odontoblasts produce a new cascade of transcription factors associated with the production of structural dentin proteins (DSPP, DMP1, BSP, OPN, collagen I, III, and V) [2, 4, 8], initially creating a relatively atubular and hypomineralized outer mantle dentin (15– 30 μm thick, without or with a very low content of dentinal tubules) at the dentin-enamel junction (DEJ) [159]. This layer serves to dissipate pressures and forces concentrated in the DEJ [159]. Crown mantle dentin contains phosphorylated proteins in underphosphorylated or non-phosphorylated forms, with particular elastic properties and resilience [159, 160]. This characteristic is due to different NCP's found in this layer compared with circumpulpal dentin [159]. The deposition of mantle dentin acts as a stimulus for IEE cells of the EK's to differentiate into ameloblasts and start producing structural EMP's (Enamel Matrix Proteins) organic matrix. EMP's are divided in amelogenins (90%) and non-amelogenin proteins (remaining 10%). AMEL is formed from two gene isoforms: *AMELX* (90% of the amelogenin) and *AMELY* (remaining 10%). The remaining EMP's are: AMBN (secreted by odontoblasts and HERS cells) involved in regulation of ameloblast adhesion, proliferation, differentiation, enamel mineralization and structural organization [161]; ENAM, necessary for ameloblast adhesion to enamel, crystal elongation and regulation of mineral formation; AMTN, involved in the maturation and hardness of enamel; and TUFT1, implicated in enamel mineralization [2, 4, 8, 162–164]. EMP's will shape the nanocrystalline hydroxyapatite that make up the enamel crystals. EPFN and MSX2 participate conjointly in a network of transcription factors against FST -inhibitor of TGFβ that regulate ameloblast differentiation-, controlling ameloblast life cycle and amelogenesis at later stages [115].

Then, the odontoblasts' dentinal extensions initiate dentin secretion. Only at these locations inside forming teeth, polarized odontoblasts start to produce membrane protrusions that detach and form matrix vesicles, which contribute to initial dentin mineralization [165, 166]. Dentin is deposited concentrically around odontoblastic extensions -dentinal tubules- to form peritubular dentin. Intertubular dentin is deposited between the tubules. After this step, the rest of dentin and enamel layers begin to be deposited by apposition [2, 147].

Enamel formation is divided in secretory and maturational phases. In the first phase, ameloblasts secrete EMP's and favors the organized formation of very long apatite crystals, forming the full thickness of a partially mineralized enamel [163, 167]. To reach its final hardness during the maturation stage, most of the EMP's are disassembled by three enzymes secreted by ameloblasts as the crystal growth continues: MMP20, KLK4 and AMTN. While MMP20 inactivates EMP's during the secretory stage, KLK4 totally breaks down the remaining EMP's during maturation phase [163, 167]. The possible AMTN role is the engagement in proteolytic processing of the enamel matrix, similar to KLK4 [164]. After being removed during dental differentiation, a thin basal-like layer at the interface between the apical cell membranes of ameloblasts and the surface of maturing enamel is reformed (and named enamel

matrix). It is composed mainly from LN5 [168, 169], together with glycoconjugates and highly glycosylated components that constitute a differential hallmark from typical basal laminae [170, 171]. LN5 is encoded by *LAMA5* (or *3*) (found in dental epithelial basal membrane [172]), *LAMB3* (found at basal and apical sides of enamel-secreting ameloblasts of the first molar [173]), and *LAMC2* genes [172, 174, 175]. LN5 forms a protein complex localized in the anchoring filaments underneath the hemidesmosomes [176, 177], which participate in cell-basement membrane adhesion [178, 179]. LN5 is normally required for cell migration and differentiation, and is essential for epithelial morphogenesis, hemidesmosome assembly and stability [174, 180, 181]. In developing teeth, LN5 is expressed by functional ameloblasts, before the initiation of final enamel mineralization and hardening (maturation stage) by cyclic modulations [173].

The DPa at this point is starting to convert in a loose connective tissue -containing cells, blood vessels, lymphatics, and nerve fibers-. There is a common and abundant vascular supply that irrigates in a loop-form not only the DPa (future dental pulp), but also the DF (future periodontium and alveolar bone marrow) [182]. A normal blood supply system in the developing tooth guarantees adequate gas exchange, nutrient supply, and waste removal [183]. The development of a mature vascular system -capillaries, arterioles, arteries, and veins- requires fine regulation among hemangioblasts (a mesenchymal stem cell type) and their successors -angioblasts and vascular endothelial cells (EC's), and mural (supporting) cells such as pericytes and vascular smooth muscle cells (VSMC's)- [184]. For this purpose, an EMI is needed to initiate dental germ vasculogenesis. Developmental vasculogenesis is the formation of new blood vessels during embryogenesis from angioblasts (endothelial precursors cells or hemangioblast-derived progenitor cells, a division of splanchnic -visceral- or splanchnopleuric mesoderm, part of lateral mesoderm) at the third IU week [183–185].

Early in embryogenesis, hemangioblasts (without a lumen) are induced by FGF1 and 2 -on a VEGF-dependent manner through stimulation of VEGF expression and upregulation of VEGFR2 signaling [186]- to migrate from the splanchnopleuric mesoderm into the head region, differentiate in angioblasts and organize to form a primitive capillary vascular network to supply the developing brain [183, 184]. Angioblast differentiation depends on paracrine signaling in part due to VEGF and VEGFR1 [183]. Simultaneously, FGF2 and PDGF-BB act synergistically to form stable vessels even after the decrease of angiogenic factors. Mural cells (particularly VSMC) around newformed microvessels have abundant PDGF receptor (PDGFR) protein expression and demonstrate potent PDGF signaling response (to PDGF-BB) to promote mural cell recruitment and maturation [186].

Angioblasts are the first mesenchymal (mesodermally-derived) cells to reach the developing DPa at late DC stage [184, 187]. Vasculogenesis of the dental germ starts at early DB [183]. Migrating angioblasts inside DPa mature to create a network of EC's, aggregate and invade it to form new dental blood vessels with a basement membrane, which provide DPa blood supply [183, 187]. Thin terminal arterioles -and venules, compared with their lumen- enter the pulp, with few collateral circulation [182]. Terminal pulpal capillaries (diameter up to 30 μm), forming a rough and thin vascular network, were observed in the inner region of the DPa of the upper first molar germ at the early DB stage before ectomesenchymal histodifferentiation [188].

Oxygen diffusion to support good oxygen interchange with DPa cells depends on the presence of a vascular network at 200 μm maximum [189]. Oxygen delivery to tissues depends on three conditions: O_2 availability, O_2 arterial blood transportation (O_2% -ca [capacity]-O_2- or O_2 content), and tissue perfusion [190]. In addition, ca-O_2 blood characteristics are determined by the pressure of inspired O_2, ventilation

and gas exchange, the Hb concentration and its affinity for O_2. The O_2 blood carrying capacity is 20 ml of O_2/100 ml of blood [190]. The O_2 saturation (sa-O_2) -% of Hb binding sites filled by O_2 and related with O_2 partial arterial pressure (p-O_2), or O_2 tension- is between 96 and 98% in blood but can vary among tissues [190]. Hypoxia -deprivation of oxygen (and nutrients) by inadequate diffusion at greater distances- is the driving force of vasculogenesis [183]. This event improves VEGF (A -mainly- and B) and HIF1α (proangiogenic factors) expression in hypoxic DPSC's and dental pulp fibroblasts (DPF's), which act on adjacent EC's to promote endothelial precursor invasion on DPa [191]. VEGFA stimulates endothelial cell proliferation by binding to VEGFR2. VEGFA increases pulpal blood flow, capillary hyperpermeability [192], and releases ANG's and other angiogenic factors [193, 194]. Either ANG1 or 2 bind competitively to TIE2 receptor to exert their effects [185]. TIE2 activation of EC's requires ANG1 binding [195], while ANG2 (mostly expressed on EC's) acts as their inhibitor [196].

After odontoblast histodifferentiation but before dentin deposition, the terminal pulpal capillaries gradually approach odontoblasts. The terminal capillary network increased in extent, cover all pulpal horns, their density increase and capillary diameter decrease up to 20 µm [188]. At later developmental stages, new blood vessels are mainly formed by angiogenesis -sprouting of new blood vessels from existing vasculature [185]. At late DB, 10-µm terminal pulpal capillaries invade the odontoblastic layer when DB morphodifferentiation starts in the cuspal region. The capillaries create an irregular vascular network surface with many low-capillary loops facing the predentin, until around 5 µm from it [188]. At this point, the dense network decreases the capillaries diameter to 5 µm [188].

Neuronal cells play an organogenetic regulatory role in addition to their tooth innervation function during embryonic development [105]. Dental pulpal afferent fibers from trigeminal (semilunar or Gasser) ganglion provide mainly dentition nociception in injury or infection [197], and mechanoception [198–200]. Axon guidance and pulpal innervation patterning parallel tooth formation process, and depends on a tight spatiotemporal regulation guided by chemoattraction and chemorepulsion [197].

The first axons reach the deciduous mandibular first molar germ at DC stage [201]. Chemorepulsive signals such as SEMA3A (and its NRP1-PLXNA class receptor complex) inhibit the entrance of axons by its axon repulsion and growth cone collapse properties [202]. Ramification of nerve fibers in the DF base and presence of some peripherin-reactive neuronal cells next to DL and oral epithelium at the buccal side of the tooth germs are recognized [105]. Axons do not enter DPa until final crown shape is completed at the end of early DB stage, just before the beginning of mineralization [197].

Regarding chemoattractant signaling, there are three groups of neurotrophic factors involved [203]: neurotrophins (or neurotrophic growth factors) such as NGF, BDNF, NT3, NT4/5 and their receptors LANR (for all neurotrophins), TRKA (for NGF in nociceptive neurons [204–206], TRKB (for BDNF, NT3 and 4) and TRKC (for NT3 -which mediate neurotrophin signals inside cells [207]); GFL's like GDNF, NRTN, ARTN, PSPN and their receptors GFRα1 (for GNF), GFRα2 (for NRTN), GFRα3 (for ARTN) and GFRα4 (for PSPN); and neuropoietic cytokines such as CNTF and LIF [105, 203, 208].

It has been suggested a role of GFL(GDNF)/RET signaling in promoting DPa/dental pulp innervation. GDNF/GFRα1 complex mediates RET receptor activation [197, 209] and triggers PI3K/AKT, MEK/ERK, SRC and WNT signaling pathways to

regulate neuron cell function [203]. GFL-RET signaling might mediate dental pulpal afferent chemoattraction [197] by upregulation of GDNF and NRTN during the period of tooth innervation [210]. The differential expression of receptors GFRα2 and GFRα4, co-receptor RET, and ETV5 in tooth organogenesis (mainly in SHED) could indicate a change in regulation compared with adult DPSC [203]. After these changes, peripherin positive nerve fibers can be distinguished in the deciduous first molar tooth germ. At late DB stage, neuronal cells are detected within the mesenchymal cells beneath pre-odontoblasts and in the surrounding mesenchyme [105]. This dental primordium, with all its differentiated elements, gives rise to the coronal portion of the tooth.

4. Conclusion

Understanding the dental development process from the molecular embryological point of view -as described in the present synopsis of dental crown formation- allows the clinical dentist to evaluate the presence or absence of these events from the clinical evaluation. This is important to detect and accurately describe events that does not follow the normal pathway of odontogenesis.

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