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1 Exploiting teeth as a model to study basic features of signaling 2 pathways

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12 **Keywords:** Tooth, signaling pathways, developmental biology, epithelial-
13 mesenchymal interactions, stem cells, SHH, FGF, WNT, TGF β ,
14 Notch, organ cultures, microfluidics, tissue recombination,
15 experimental embryology

18 **Abstract**

19 Teeth constitute a classical model for the study of signaling pathways and their roles in
20 mediating interactions between cells and tissues in organ development, homeostasis and
21 regeneration. Rodent teeth are mostly used as experimental models. Rodent molars have
22 proved fundamental in the study of epithelial-mesenchymal interactions and embryonic
23 organ morphogenesis, as well as to faithfully model human diseases affecting dental tissues.
24 The continuously growing rodent incisor is an excellent tool for the investigation of the
25 mechanisms regulating stem cells dynamics in homeostasis and regeneration. In this review,
26 we discuss the use of teeth as a model to investigate signaling pathways, providing an
27 overview of the many unique experimental approaches offered by this organ. We discuss how
28 complex networks of signaling pathways modulate the various aspects of tooth biology, and
29 the models used to obtain this knowledge. Finally, we introduce new experimental
30 approaches that allow the study of more complex interactions, such as the crosstalk between
31 dental tissues, innervation and vascularization.

32 **Introduction**

33 Teeth are excellent models for studying complexity in biology. From the first stages of tooth
34 development, crosstalk between epithelial and mesenchymal cells initiate the morphological
35 changes that drive tooth formation (1). In adult teeth, homeostasis and regeneration are
36 guaranteed by the cooperation between hard and soft tissues, as well as resident stem cells
37 and their crosstalk with innervation and vascularization (2, 3). The basic features of teeth are
38 highly conserved throughout evolution (4), as tooth-like structures have been discovered in
39 creatures as old as 400million year old, where single tooth plates were used to crush and

40 shred food (5). In particular, the mechanisms underlying tooth development, homeostasis
41 and regeneration are highly conserved between humans and rodents (6). Rodents possess
42 two classes of teeth: molars and incisors. Rodent molars are homologous to human teeth,
43 and this similarity is reflected in the high conservation of the molecular networks that
44 regulate their development. Rodent and human molars develop through the same
45 morphological stages, and their final anatomy is highly similar, with few morphological and
46 cellular differences (6). The crosstalk between epithelium and mesenchyme that is
47 characteristic of molar development is also the basis for the development of many organs,
48 such as hair follicles, limb, glands, lung and kidney (7, 8). These properties have therefore
49 made the rodent molar an optimal model for the study of morphogenesis and epithelial-
50 mesenchymal interactions. In contrast to molars, rodent incisors grow continuously thanks to
51 the presence of populations of adult dental epithelial (DESCs) and mesenchymal (DMSCs)
52 stem cells (2, 9). As such, the rodent incisor became a preeminent model for the study of stem
53 cells, along with other continuously growing organs such as hairs and nails (10).

54 The tooth organ is composed of a unique combination of hard and soft tissues. The enamel is
55 the hardest tissue in the human body, and it covers the crown of the tooth (Figure 1). It
56 consists of highly organized hydroxyapatite crystals, as well as by minor fractions of protein
57 matrix and water (11, 12), which are all fundamental for the exceptional mechanical and
58 buffering properties of enamel (13-15). Enamel is supported by dentin, another highly
59 mineralized tissue formed by carbonated hydroxyapatite, collagen, non-collagenous proteins
60 and proteoglycans (Figure 1) (16). The core of the tooth is occupied by the dental pulp, a
61 connective tissue that conveys innervation and vascularization (Figure 1). Nerve fibers from
62 the trigeminal ganglion enter the pulp cavity from the apical foramen and extend until the

63 dentinal tubules, transmitting pain and sensitivity (17). The dental pulp is highly vascularized,
64 providing oxygen, nutrients and blood-borne factors to sustain tooth survival (18). The tooth
65 is anchored to the surrounding alveolar bone via the periodontium, a unique tissue that offers
66 stability to the tooth by absorbing the masticatory loads (19, 20). Both the dental pulp and
67 the periodontium host resident populations of mesenchymal stem cells that are activated in
68 response to injury (21). All these major anatomical features are shared by human and mouse
69 molars. The tooth integrates the functionality of common tissues present in various organs
70 (e.g. innervation, vascularization), and highly specialized tissues unique to the tooth organ
71 (e.g. enamel, dentin, periodontium). This aspect makes the tooth an exceptional model to
72 study communications between different cell and tissue types, both in physiology and in
73 pathological conditions in which these interactions are disrupted. Moreover, dental
74 pathologies affect the entire population, making the study of tooth biology an important
75 clinical need. Caries leads to the degradation of enamel and, if not properly treated, progress
76 into the dentin and the dental pulp (22). Similarly, the periodontium is often subject to
77 periodontitis, i.e. the infection and inflammation of the periodontium, which is a leading
78 cause of tooth loss (23). Finally, many mutations and syndromes cause dental defects, ranging
79 from tooth agenesis (i.e. lack of teeth) to defective formation of hard tissues (6), all conditions
80 for which satisfactory treatments are not yet available (24).

81 **Development of the tooth**

82 Tooth development proceeds through a series of well-defined morphological stages both in
83 humans and rodents. Odontogenesis starts during early embryonic development (embryonic
84 day of development 10.5 - E10.5 in mice, 6 weeks in humans) with the primary dental lamina
85 forming as a thickening of oral epithelium at the site of the future tooth row. Dental placodes

86 form then along the dental lamina (Figure 2) (1). These epithelial structures establish
87 reciprocal interactions with ectodermal-derived neural crest mesenchymal cells, which
88 migrate from the dorsal part of the neural tube (1, 25, 26). Both epithelial and mesenchymal
89 cells possess the capacity to initiate tooth development (27, 28). Recent evidence suggests
90 that a transient epithelial signaling center, called initiation knot, might direct the first phases
91 of tooth development (29). At later stages, the dental mesenchyme maintains the inductive
92 potential, guiding the progression through the sequential stages of odontogenesis (1, 25). The
93 dental placode invaginates into the underlying mesenchyme and forms an epithelial bud (bud
94 stage, E12.5-E13.5 in mice, 6-8 weeks in humans. Figure 2). At this stage, a cluster of non-
95 dividing epithelial cells at the tip of the bud forms the primary enamel knot, a putative
96 signaling center that guides further tooth morphogenesis (8). The continuous proliferation
97 and growth of the dental epithelium around the condensed mesenchyme leads to the
98 epithelial cap configuration (cap stage, E14.5-E15.5 in mice, 11 weeks in humans. Figure 2).
99 The tooth then acquires the bell configuration (E16.5-E18.5 in mice, 12 weeks in humans) as
100 additional signaling centers, named *secondary enamel knots*, start to form in the epithelium
101 in correspondence to the future cusps (8). Between the cap and the bell stages,
102 cytodifferentiation occurs. Four different epithelial layers can be distinguished: outer enamel
103 epithelium (OEE), stellate reticulum (SR), stratum intermedium (SI) and inner enamel
104 epithelium (IEE) (1, 30). At the tip of the cusps, mesenchymal cells from the dental papilla
105 differentiate into odontoblasts that produce and secrete dentin. Shortly after, IEE cells facing
106 odontoblasts differentiate into enamel-secreting ameloblasts (Figure 2). Ameloblasts and all
107 other epithelial cell populations participate in enamel maturation. Upon completion of
108 enamel formation, these cells form the reduced enamel epithelium, which is lost when the
109 tooth erupts into the oral cavity. The dental epithelium is also responsible of the formation

110 of the roots. The region where IEE and OEE join forms the cervical loop, the site at which root
111 formation is initiated. The IEE and OEE form the *Hertwig's epithelial root sheath* (HERS), which
112 in turn guide root formation (30). The HERS continues to grow apically to shape the root and
113 induces the differentiation of the adjacent dental pulp mesenchymal cells into odontoblasts.
114 These odontoblasts produce the root dentin, while HERS disaggregates into epithelial islands
115 allowing the contact between cells of the dental follicle and the root dentin. HERS cells and
116 mesenchymal cells from the dental follicle cooperate to generate cementum, the hard tissue
117 that allows the anchoring of the tooth roots to the periodontal ligament (31, 32).

118 In contrast to molars, soon after the bud stage the developing rodent incisors rotate antero-
119 posteriorly and become parallel to the long axis of the jaws. This rotation is accompanied by
120 a morphological reorganization along the labial-lingual axis, as only the labial epithelium will
121 give rise to the enamel-forming epithelium, while the lingual side will form the root analogue
122 (33). Populations of epithelial and mesenchymal stem cells are maintained at the posterior
123 end of the organ and drive its continuous growth (Figure 1). The continuous growth of the
124 rodent incisor is also supported by the intense and continuous remodeling of the
125 periodontium (34). Mature rodent incisors are thus characterized by anatomically distinct and
126 molecularly defined territories, where cell proliferation, differentiation, and maturation
127 events can be easily analyzed (35).

128 **Signaling pathways and tooth development**

129 All major signaling pathways, i.e. Sonic Hedgehog (SHH), Fibroblast Growth Factor (FGF),
130 Transforming Growth Factor β / Bone Morphogenetic Protein (TGF β /BMP), WNT, Notch,
131 parathyroid hormone-related protein (PTHrP)/ PTHrP receptor (PPR) (PTHrP/PPR) signaling,

132 and Hippo-YAP/TAZ pathway are recurrently involved in the regulation of tooth development,
133 from the definition of the dental placodes to the latest stages of mineralization and tooth
134 eruption. Ligands and receptors from the various pathways mediate the interactions between
135 the dental epithelium and the neural crest derived mesenchyme and create molecular
136 gradients as well as barriers within dental tissues that result in the activation of specific
137 genetic programs and transcription factors. The latter are the final mediators of tooth
138 morphogenesis, cytodifferentiation and mineralization.

139 SHH, FGF, BMP and Wnt signaling cascades are all initiated by soluble ligands, which can signal
140 both locally via autocrine or paracrine mechanisms, or at long distance, creating morphogen
141 gradients (36). SHH signaling is initiated by three possible soluble ligands, Sonic Hedgehog
142 (Shh), Desert Hedgehog (Dhh) and Indian Hedgehog (Ihh). These ligands bind to their receptor
143 Patched1 (Ptch1), relieving Smoothed (Smo) inhibition and ultimately modulating gene
144 expression via Gli transcription factors (37).

145 The FGF signaling pathway is activated by eighteen secreted proteins that can interact with
146 four tyrosine kinase FGF receptors (FGFRs), which in turn modulate several intracellular
147 cascades, including RAS-MAPK, PI3K-AKT, PLC γ , and STAT pathways. Interaction of FGF
148 ligands with their receptors is strongly regulated by interactions with other proteins and
149 components of the extracellular matrix (38).

150 TGF β /BMP signaling is activated by ligands belonging to the TGF β superfamily, which include
151 TGF β I-III, ten BMPs, and activins, among others (39). TGF β ligands bind to a large set of
152 receptors (ALK1-7, BMPRI-II, ACTRII/B, TGF β RII), activating the SMAD signaling cascade (39).

153 Wnt signaling is a highly conserved pathway, activated in mammals by 19 different ligands.
154 These ligands interact with Frizzled receptors (FZD), leading to the activation of canonical or
155 non-canonical downstream signaling cascades. Activation of the canonical Wnt pathway
156 results in the nuclear localization of β -catenin, which in turn regulates the transcription of
157 Wnt target genes (40). Non-canonical Wnt signaling results in the activation of JNK, RHOA or
158 PLC downstream effectors (41).

159 The Notch pathway has a different mode of action as its ligands are membrane-bound. This
160 signaling pathway is driven by five trans-membrane-bound ligands (i.e., Jagged1, Jagged2,
161 Delta1, Delta-like3 and Delta-like4) that interact with four Notch receptors (i.e., Notch1,
162 Notch2, Notch3 and Notch4) (42-45). Ligand-receptor interactions trigger the cleavage of the
163 receptor and the subsequent nuclear translocation of its intracellular domain (46). This leads
164 to the activation of Notch downstream genes (44) (47, 48). The Notch pathway thus
165 constitutes a fundamental cell communication mechanism that enables neighboring cells to
166 adopt different fates by creating sharp molecular boundaries (49, 50).

167 The Hippo-YAP pathway perceives and responds to the physical organization of cells in tissues
168 and coordinates these physical cues with classic growth-factor-mediated signaling pathways.
169 The Hippo pathway consists of a core kinase cascade in which the transcriptional co-activators
170 YAP/TAZ are phosphorylated and inactivated by either their exclusion from the nucleus or
171 their enhanced degradation. The nuclear activity of YAP/TAZ regulates cell and organ growth
172 (51).

173 The parathyroid hormone-related protein (PTHrP) is locally acting autocrine/paracrine ligand,
174 which interacts with its receptor, the PTH/PTHrP receptor (PPR), to activate multiple

175 heterotrimeric G proteins and trigger intracellular signaling pathways . These include
176 primarily Gs/cAMP/protein kinase A (PKA), Gq/PLC/Ca²⁺/PKC signaling, as well as
177 G12/13/RhoA/phospholipase D and the mitogen-activated protein kinase (MAPK)
178 (extracellular signal-regulated kinase, ERK1/2) signaling cascades (52).

179 *Initiation*

180 A precise equilibrium of signals from different signaling pathways establishes the territories
181 where teeth develop, as well as the number of teeth and their class, from very early
182 developmental stages (6, 53). The dental epithelium is already defined before initiation by
183 the expression of the transcription factor *Pitx2* (Figure 2) (54, 55), and its deletion leads to
184 the arrest of tooth development before placode formation (56, 57). The pattern of dentition
185 is also established before the onset of odontogenesis, as competing gradients of FGF and BMP
186 ligands determine the exact localization and class of the different teeth (53, 58-60). BMP4
187 directs the shape of incisors and FGF8 the shape of molars (53). These ligands induce the
188 expression of specific set of genes in the mesenchyme, namely *Msx1* and *Msx2* in the incisors
189 mesenchyme, and *Dlx1* , *Dlx2* and *Barx1* in the molars mesenchyme (Figure 2) (1). The specific
190 combination of these transcription factors determines the development of the tooth germs
191 toward an incisorform or molariform shape (61). SHH and FGF signaling are fundamental
192 drivers of the early phases of odontogenesis. *Shh* is expressed in the dental epithelium at
193 E11.5, and *Shh*-expressing cells attract *Fgf8*-expressing epithelial cells to the dental placode
194 (62). Both these pathways are necessary for the progression of odontogenesis, as their
195 inhibition leads to the arrest of tooth development (62, 63). Wnt/ β -catenin also plays a major
196 role in the initial phases of tooth development. Inhibition of epithelial Wnt signaling blocks
197 tooth development at the placode stage (64), while inhibition of mesenchymal Wnt signaling

198 arrests odontogenesis at the bud stage (65). Conversely, constitutive activation of Wnt/ β -
199 catenin in the oral epithelium induces the formation of ectopic teeth (66, 67).

200 *Morphogenesis*

201 At the bud stage, FGF and BMP signaling are needed to induce the expression of the
202 transcription factors *Pax9* and *Msx1* in the dental mesenchyme. *Pax9* and *Msx1* themselves
203 regulate the expression of *Fgf3*, *Fgf10* and *Bmp4* (68, 69), mediating the progression of
204 odontogenesis (Figure 2). At the bud-cap transition, the dental epithelium is characterized by
205 the appearance of the enamel knots, putative signaling centers localized in correspondence
206 of the future cusps (8). Cells within the enamel knot do not proliferate and produce WNTs,
207 SHH, BMPs and FGFs, which in turn modulate the proliferation of adjacent epithelial cells,
208 leading to the formation of dental cusps (Figure 2) (70). Notch signaling also plays an
209 important role in the determination of the number and shape of cusps. The Notch ligand
210 *Jagged2* (*Jag2*) is expressed in the dental epithelium under the control of mesenchyme-
211 derived FGFs and BMPs, and loss of *Jag2* leads to the formation of smaller cusps via
212 deregulation of *Bmp4*, *Pax9* and *Barx1* expression (71). Enamel knots and cusp formation are
213 also strongly modulated by the activity of the TNF family ligand ectodysplasin A (EDA). The
214 expression of *Eda* is regulated by Wnt signaling, and EDA in turn regulates the expression of
215 *Fgf20* (72). Mice carrying *Eda* deletions have smaller enamel knots, defective dental epithelial
216 invagination and flat cusps (73). During the cap and bell stages, proliferation of the
217 progenitors residing in the cervical loops sustain the elongation of the dental epithelium.
218 FGF10 from the dental mesenchyme plays an important role in this process, as loss of FGF10
219 impairs the generation of new epithelial progenitors and induces apoptosis of cells located in
220 the cervical loops (Figure 2) (74).

221 *Cytodifferentiation and mineralization*

222 A key event that characterizes the cap and bell stages is cytodifferentiation. Dental epithelial
223 cells differentiate into ameloblasts, which form enamel, the hardest mineralized tissue of the
224 body. The specification of these cells involves a complex crosstalk between FGFs, BMPs, SHH,
225 the Notch signaling pathway (71, 75, 76) and the transcription factor Tbx1 (Figure 2) (77). FGF
226 is required for the generation of ameloblasts progenitors (74), while SHH activity is necessary
227 for the ameloblasts differentiation (78). Notch signaling plays a key role at this stage. During
228 epithelial cytodifferentiation, Notch receptors and their ligands are expressed in adjacent
229 cells of the dental epithelium, creating sharp boundaries that define dental epithelial cell
230 identity (71, 75, 76, 79, 80). The expression of Notch ligands and receptors in the dental
231 epithelium is modulated by FGF and BMP ligands (71, 80, 81), and Notch signaling modulates
232 the differentiation of the various dental epithelial lineages (71, 82). The Notch ligand Jag2 is
233 expressed in the dental epithelium, and its deletion impairs ameloblastic differentiation and
234 enamel production (71). This effect is partially mediated by the loss of the expression of Tbx1
235 (77). Tbx1 mutations in human patients are associated with DiGeorge Syndrome, a complex
236 human pathology characterized by craniofacial malformations and defective enamel
237 formation. Mice lacking Tbx1 do not have enamel, as ameloblasts fail to differentiate (77).

238 Signaling pathways are also directly involved in amelogenesis, i.e. the formation of enamel.
239 Shh activity is required for the proper terminal differentiation of ameloblasts (78). Wnt/ β -
240 catenin signaling is essential for ameloblast movement during differentiation (83), while
241 constitutive activation of β -catenin in ameloblasts delays their differentiation and leads to
242 the generation of hypomineralized enamel (84). Bcl9 and Pygo2, which are generally
243 considered to be Wnt/ β -catenin transcriptional cofactors, are necessary for the fine

244 structure of enamel, as their deletion leads to the formation of defective enamel. In
245 ameloblasts, these two molecules directly interact with amelogenin and other important
246 enamel proteins, modulating their localization and thus enamel structure (Figure 2) (35).

247 *Root formation*

248 Root development strictly relies on the interaction between epithelial cells from HERS and
249 mesenchymal cells from the dental papilla and the dental follicle (32). Several signaling
250 pathways are involved in this process. Bmp ligands are expressed in the early phases of root
251 formation, and their crosstalk with Shh signaling is necessary for proper HERS formation and
252 root development. Loss of Bmp signaling in the dental epithelium leads to the persistence of
253 Shh-dependent signaling activity in the root, leading to the inhibition of HERS formation and
254 shorter roots (32, 85). The disappearance of Fgf10 expression in the dental mesenchyme is
255 necessary for the initiation of root formation, while exogenous Fgf10 application at the
256 crown-to-root transition inhibits HERS formation (32, 86). Wnt signaling is also fundamental
257 in this process. *Wnt10a*-null mice are affected by taurodontism, a condition characterized by
258 larger pulp chambers and shorter and poorly furcated roots, similar to patients with *WNT10A*
259 mutations (87, 88). Similar phenotypes are induced by the constitutive activation of Wnt
260 signaling in dental follicle cells (89). Recent works highlighted the importance of the
261 PTHrP/PPR signaling axis (52) in the formation of the roots, as deletion of the PPR receptor
262 leads to failure of eruption and the development of significantly truncated roots, which lack
263 periodontal ligaments (90, 91), similar to what observed in human patients with *PTHR1*
264 mutations (92).

265 *Homeostasis and regeneration*

266 Dental stem cell behavior in homeostasis and regeneration is mostly studied in rodent
267 incisors. A complex network of signaling pathways, including FGF, BMP, Notch, and SHH,
268 regulates the maintenance and the differentiation of DESCs and DMSCs. FGF10 and FGF3 are
269 produced in the mesenchyme and promote both the survival and differentiation of DESCs (74,
270 93). FGF9 is produced within the dental epithelium and directly regulates Shh expression,
271 which together with other Hh ligands modulates both DESCs proliferation and differentiation
272 (78, 94). In contrast, BMP and Wnt signaling have antiproliferative and proapoptotic effects
273 on DESCs (95). Incisor DMSCs activation and subsequent differentiation is modulated by
274 nerve-derived Shh (2) as well as Notch signaling (96). Both epithelial and mesenchymal stem
275 cells are essential not only in homeostasis but also during injury repair. Injury to the incisor
276 epithelium leads to the activation of stem and progenitor cells characterized by the
277 expression of Notch1-expressing cells, which contribute to the regeneration of the damaged
278 epithelial layers (82, 97). Recently, Hippo signaling has also been shown to play an important
279 role in regulating DESCs proliferation and differentiation. Hippo-effectors Yap and Taz prevent
280 premature differentiation of DESCs and transit amplifying progenitors via activation of the
281 mTOR signaling pathway, and their deletion leads to loss of cell proliferation and increased
282 cell death in the labial cervical loop area (98).

283 **Teeth as experimental models**

284 The tooth constitutes a model of excellence to study all the basic processes that underlie
285 organ development, from initiation to morphogenesis, terminal differentiation and
286 mineralization, as well as homeostasis and regeneration (Figure 3). The non-lethality of dental
287 defects and diseases in animal models make teeth an exceptional model for the study of the
288 roles of the different signaling pathways *in vivo*. Defects caused by gene deletions are easily

289 detected in teeth, in particular those that result in arrest of tooth development or in
290 alterations of enamel formation (35).

291 *Developing teeth as prototypical model for epithelial-mesenchymal interactions*

292 The first phases of molar development are strictly controlled by epithelial-mesenchymal
293 interactions. Classical recombination experiments were fundamental to determine that the
294 odontogenic potential, i.e. the ability to induce formation of teeth, shifts between the
295 epithelium and the mesenchyme through development (25, 28, 99). Heterochronic
296 recombination experiments showed that the inductive potential resides in the dental
297 epithelium between E9 and E11, and then it shifts to the dental mesenchyme (25, 28).
298 Heterotypic recombination of dental mesenchyme, isolated from molars beyond E11.5, with
299 non-dental epithelium (e.g. isolated from hair follicles) invariably resulted in the generation
300 of teeth, showing that the dental mesenchyme contained all the signals required to induce
301 odontogenesis (25). Moreover, *In vivo* recombination experiments showed that prior to
302 initiation, the neural-crest derived mesenchyme has the potential to induce the odontogenic
303 program into the oral epithelium (27). Indeed, tooth-like structures developed in mouse-
304 chicken chimeras, where neural crest cells from chicken embryos were replaced by mouse
305 crest cells. This indicates that cranial neural crest cells contain inductive potential and,
306 together with the oral epithelium, contribute to the initiation of tooth formation (Figure 3)
307 (27, 100).

308 *Tooth germ cultures*

309 Embryonic mouse tooth germs can be isolated and cultured as organs in high serum-
310 containing media, as signals exchanged between the epithelium and the mesenchyme allow

311 the progression of tooth development *in vitro* (101). The development of cultured tooth
312 germs is comparable to the *in vivo* tooth development both in terms of structure and function
313 (Figure 3) (101). Tooth organ cultures have thus proved fundamental to study the roles of
314 signaling pathways in the modulation of tissue morphogenesis and cytodifferentiation, as
315 these organotypic cultures allow live imaging (97) as well as easy treatment of the developing
316 tooth germ by electroporation (102), as well as with antibodies, beads releasing growth
317 factors (Figure 3), and small molecules (71, 77, 103-105). Tooth organ cultures lack however
318 important elements such as innervation and vascularization, which are key modulators of
319 many processes, including mineralization and stem cells activation (2, 3). After *in vitro*
320 treatments, cultured tooth germs can be transplanted subcutaneously or in the kidney
321 capsule. In these conditions, tooth germs can receive blood-derived nutrients and signals that
322 are necessary for the full formation of the mineralized tissues (101).

323 *Teeth as model for the study of genetics, genetic therapy and regeneration*

324 Rodent molars constitute optimal models to study the molecular and genetic bases of human
325 tooth development and disease. Mutations affecting tooth development in human patients
326 lead to homologue phenotypes in mice, and most of our knowledge concerning tooth
327 development was obtained via studies on mouse models (6, 88, 106). The existence of
328 discrete morphological stages in odontogenesis provides an easy read out for developmental
329 phenotypes, such as tooth agenesis or arrest of tooth development, induced by alterations of
330 specific signaling pathways (35, 102, 107, 108). In addition, diseases characterized by
331 defective enamel formation, e.g. Amelogenesis Imperfecta, are properly modeled in animal
332 models (6, 109-111). The rodent molar periodontium has composition and behavior similar
333 to the periodontium of human teeth, and has thus been used as a model to study periodontal

334 pathologies and the responses of periodontal structures to mechanical stress (34). Based on
335 this knowledge, tooth organ cultures can then constitute an optimal platform to investigate
336 the genetic treatment of these pathologies via e.g. electroporation (112).

337 Both human and rodent teeth host populations of epithelial and mesenchymal stem cells,
338 which have attracted great attention for their clinical potential. Molars host DMSCs both in
339 the dental pulp and in the periodontium, located in close association with blood vessels (21,
340 113-115). These cells are multipotent, have strong angiogenic and neuroattractive properties
341 (116-118), and are activated in response to injury and other external stimuli (21, 114, 119).
342 Many studies focused on their use in combination with scaffolds to promote regeneration of
343 the dental pulp and the periodontium (117, 120-122). Rare DESCs have been isolated from
344 the periodontium of human teeth and are regarded with great interest for the regeneration
345 of enamel (123). Properties of dental stem cells are however mostly studied in rodent incisors.
346 Incisors grow continuously thanks to the crosstalk between epithelial and mesenchymal stem
347 cell niches, as well as due to their interaction with nerve fibers and blood vessels (2, 9).
348 Lineage tracing and conditional knockout approaches allow the study of the effects of
349 signaling pathways on stem cell function *in vivo* (90, 91, 97, 124). Moreover, the rodent incisor
350 is easily accessible and the cutting of its anterior end does not evoke pain in the animals, thus
351 allowing lowly invasive studies of stem cells reaction to injury (96, 97). Exogenous stem cells
352 can also be injected close to the posterior end of the incisor (125). The periodontium of the
353 rodent incisors remodels continuously to support the continuous growth of the organ,
354 showing a more dynamic behavior than that observed in human teeth and mouse molars (34).
355 Similar to molars, incisors can be cultured in high serum-containing media, as their stem cell
356 populations continue to proliferate and differentiate *in vitro* (126-128). This in turn allows

357 efficient live imaging, treatments and injury studies (97, 102). The rodent incisor is thus an
358 exceptional model to study the roles of the various signaling pathways on DESCs and DMSCs,
359 both in homeostasis and regeneration.

360 Three-dimensional culture systems started to be used to generate dental tissues starting from
361 single cells. Dental spheroids (dentospheres) were successfully obtained from mouse and
362 human dental epithelial and mesenchymal stem cells (Figure 3) (129-132). Depending on the
363 culture conditions, epithelial dentospheres generated from mouse incisors and molars either
364 showed strong stem cell properties or generated structures characterized by differentiation
365 gradients (129). These dentospheres were shown to model, to a certain extent, some of the
366 basic molecular networks underlying ameloblastic differentiation (132). Dental epithelial and
367 mesenchymal cells can also be combined in three-dimensional systems to generate
368 bioengineered tooth germs (133). Bioengineered teeth have been generated from mouse
369 embryonic dental mesenchymal and epithelial cells. These teeth contained the various dental
370 tissues, while their size was significantly inferior to that of normal teeth (133). To date, no
371 biologically functional bioengineered tooth has been generated from adult cells.

372 *“Organ-on-a-chip”: modeling the interactions between the different dental tissues*

373 Monocultures, organoids and spheroids lack many features necessary for the function of any
374 organ, such as vasculature, innervation, mechanical cues, and immune responses (134).
375 “Organ-on-chip” systems aim to fulfil this need by co-culturing epithelial, mesenchymal,
376 endothelial, and neuronal cells or tissues in communicating chambers (135). Microfluidic
377 devices (Figure 3) have already been used to co-culture developing tooth germs (136), dental
378 stem cells (118), and dental cancer stem cells (137) with trigeminal ganglia, the main mediator

379 of tooth innervation. These studies showed that microfluidics can faithfully recapitulate the
380 *in vivo* situation, thus providing a solid ground for the study of dental tissues in “organ-on-
381 chip” systems (119).

382 **Perspectives**

383 - **Highlight the importance of the field.** In this review we discuss the use of teeth as
384 models to study signaling pathways in organ development, homeostasis and
385 regeneration. Teeth are indeed a prototypical model for the study of morphogenesis,
386 epithelial-mesenchymal interactions, mineralization, and stem cell biology.

387 - **A summary of the current thinking.** Combination of powerful *in vivo* (e.g. transgenic
388 animals) and *in vitro* (e.g. tissue recombinants, long-term whole organ culture)
389 approaches make teeth a unique model for the study of signaling pathways. Complex
390 networks of signaling cascades are involved in tooth initiation, morphogenesis,
391 cytodifferentiation, mineralization, as well as injury and regeneration. Study of these
392 processes has provided important information concerning basic features of signaling
393 pathways, as well as fundamental input for their exploitation for clinical purposes.

394 - **A comment on future directions.** The recent technological advances in three-
395 dimensional culture systems, microfluidic “organ-on-a-chip” platforms, gene editing
396 techniques and nanotechnology are providing new possibilities for the study of
397 signaling cascades and of interactions between the different dental tissues. Moreover,
398 they offer exciting perspectives for regenerative and personalized medicine.

399 **Author contributions**

400 PP, CP, and TM contributed to the writing, reading, and editing of the present review article.

401 All authors have read and agreed to the published version of the manuscript.

402 **Declaration of interests**

403 The authors declare no competing interests.

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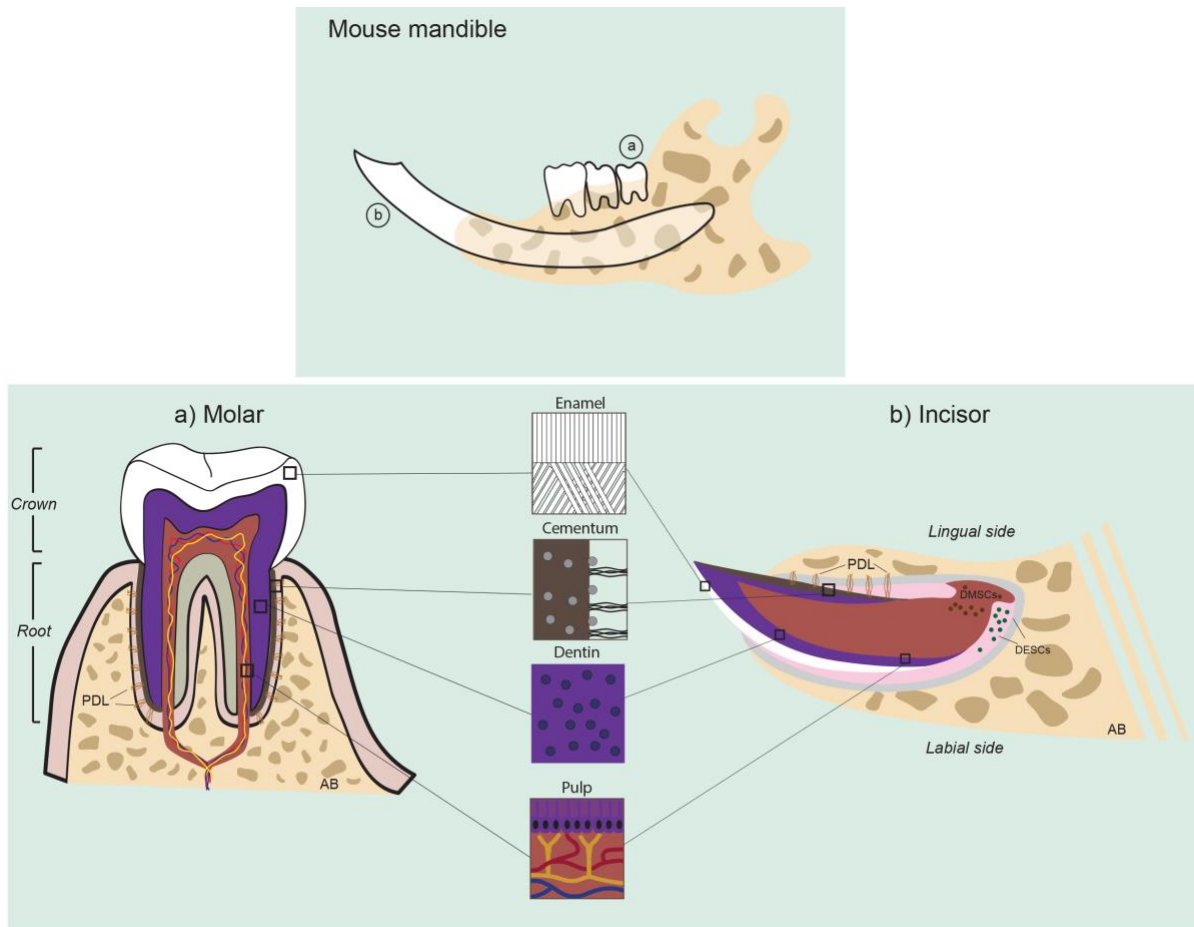
406 the editing of the manuscript.

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409

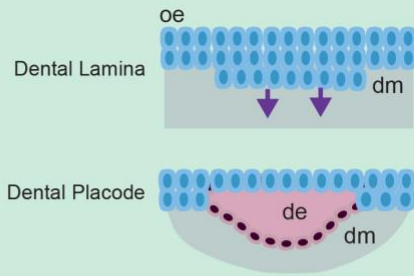
410 **Figures and Figure legends**



411

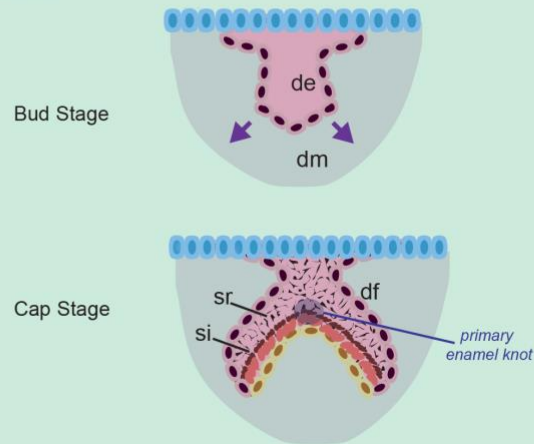
412 **Figure 1.** Schematic representation of the anatomy of rodent molars and incisors.
413 Abbreviations: AB, alveolar bone; DESCs, Dental Epithelial Stem Cells; DMSCs, Dental
414 Mesenchymal Stem Cells; PDL, periodontal ligament. Above: general structure of a mouse
415 lower hemi jaw, and relative localization of the molars and the continuously growing incisor.
416 Below, a): Structure of human molar. The overall features are conserved between human and
417 rodent molars. b) Structure of the continuously growing rodent incisor.

Initiation



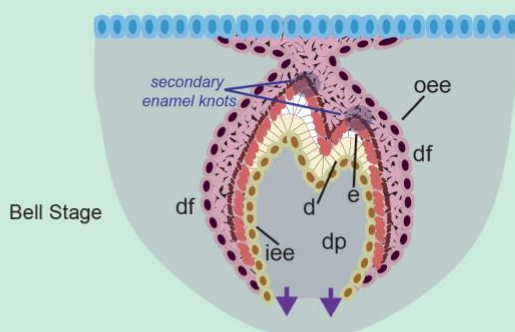
Epithelium	Mesenchyme
BMP	BMP
FGF	ACTIVIN
SHH	Msx1
WNT	Msx2
TNF	Dlx1
Pitx2	Dlx2
	Barx1

Morphogenesis



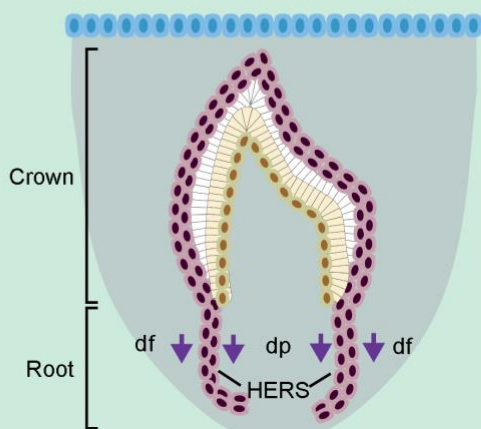
Epithelium	Mesenchyme
BMP	BMP
FGF	FGF
SHH	WNT
WNT	Barx1
NOTCH	Msx1
Pitx2	Msx2
Edar	Pax9
Lef1	Gli1
	Gli2
	Lef1

Cytodifferentiation



Epithelium	Mesenchyme
BMP	BMP
FGF	FGF
SHH	WNT
WNT	Barx1
NOTCH	Msx1
Tbx1	Msx2
β -catenin	Pax9
Bcl9/Pygo2	Gli1
	Gli2
	Lef1

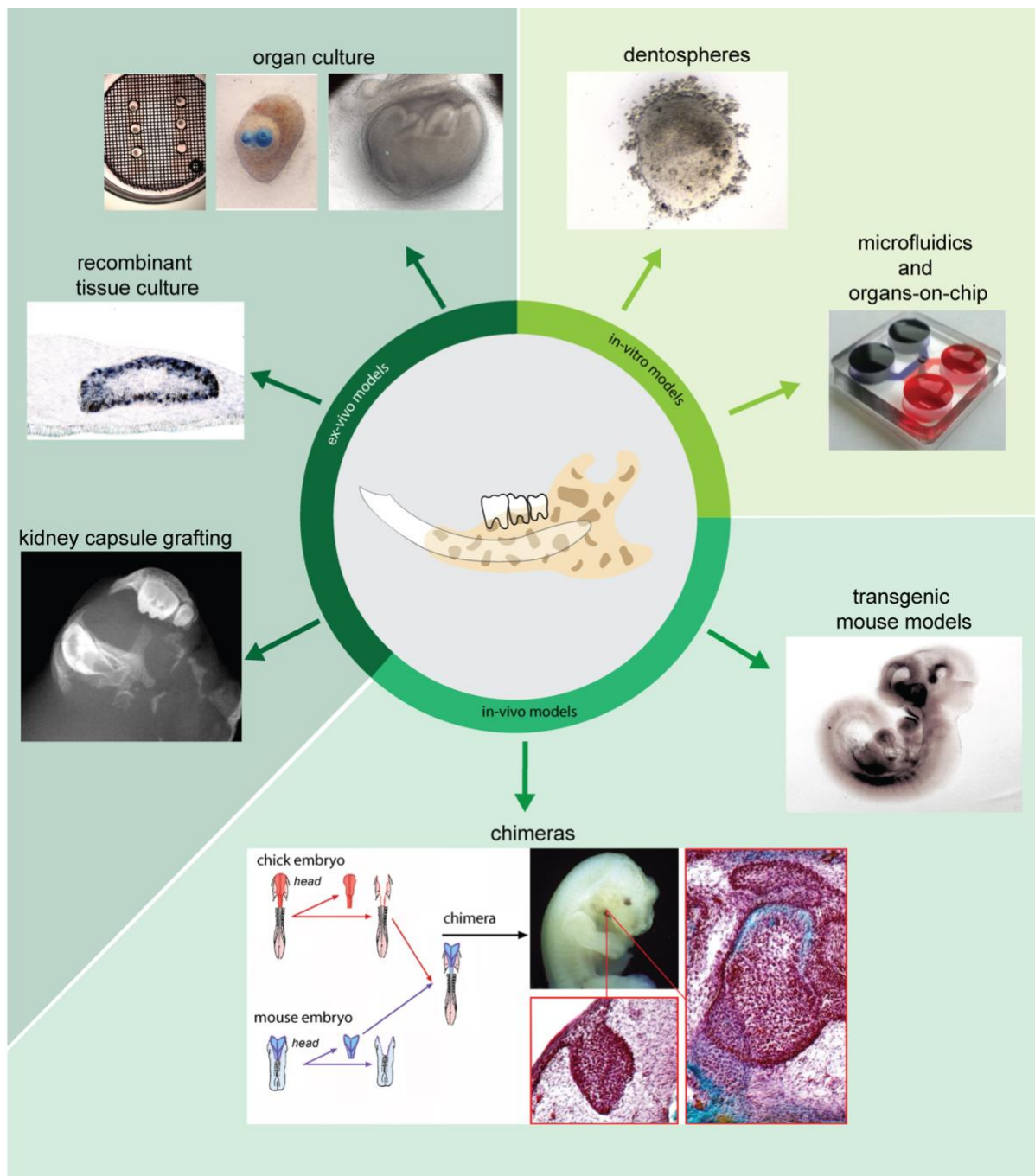
Root formation



Epithelium	Mesenchyme
BMP	BMP
Smad4	TGFb
Msx2	Smad4
SHH	WNT
	FGF
	PTHrP / PPR
	Gli1

419 **Figure 2.** Overview of tooth development. The main signaling pathways (capital letters) and
420 transcription factors (lowercase) involved at the different morphological stages are listed.
421 Abbreviations: d, dentin; de, dental epithelium; df, dental follicle; dm, dental mesenchyme;
422 dp, dental papilla; e, enamel; HERS, Hertwig's Epithelial Root Sheath; iee, inner enamel
423 epithelium; oe, oral epithelium; oee, outer enamel epithelium.

424



425

426 **Figure 3.** Overview of the main *in vivo*, *ex vivo* and *in vitro* experimental approaches for the
 427 study of signaling pathways in dental tissues.

428

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