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Mouse tooth engineering: a step-by-step approach.

Research Professor Thesis

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Content

Abstract

Odontogenesis is controlled by reciprocal epithelial-mesenchymal interactions mediated by an interposed basement membrane and by diffusible signaling molecules. The mouse tooth germ at Embryonic Day (ED) 14 was used as a model.

After studying complementary aspects of odontogenesis, the aim of the work summarized here was to engineer a whole tooth. Searching for the simplest way to achieve this goal, a biomimetic approach was developed. A set of specific complementary protocols was successively designed. These protocols, their rationale and the main results are briefly discussed.

Dental epithelial and mesenchymal tissues were enzymatically separated and their cells were further dissociated. Tissues/cells or cells/cells were then re-associated, cultured and implanted under the skin of adult mice. The plasticity of embryonic dental epithelial and mesenchymal cells at ED14 allowed their adjustment to each other and adaptation to new environmental conditions as an example of self-organization. Tooth histogenesis as well as cytological functional differentiations of odontoblasts and ameloblasts took place. Several cell sources, when tested to replace dental mesenchymal cells, were not competent.

The main results showed that from ED14 to ED18, mesenchymal cells progressively lost their competence to engage in odontogenesis. It was also lost within few hours, when ED14 dental mesenchymal cells have been pre-cultured *in vitro*. Other organs/models might help to understand this limitation. After implantation, the vascularization was restored and the host mediated it. Vascularization allowed the mineralization of dentin, enamel and cementum. In the crown, mineralized matrices showed the same chemical and physical characteristics as *ex vivo*. Innervation could also be achieved in the dental mesenchyme, but it required immuno-suppression. In the peridental mesenchyme, immuno-suppression was not necessary, illustrating a differential regulation of the innervation in both tissues.

Introduction

In the embryo, tooth germ consists in an epithelium (the dental epithelium and later the enamel organ) and an ecto-mesenchyme (the dental papilla and later the dental pulp). Odontogenesis is controlled by reciprocal epithelial-mesenchymal interactions (Ruch et al., 1995). More simple than the incisor, the first lower molar in mouse is a very common model to study complementary aspects of odontogenesis. Our results and others from the literature allowed us, in 2004, to engage in a new field: “Whole tooth engineering”. We first focused on epithelial-mesenchymal interactions during early stages of engineering. At about the same time, other groups had engaged in similar studies (Ohazama et al., 2004; Komine et al., 2007; Honda et al., 2008; Arany et al., 2009).

The main results about whole tooth engineering, and development of specific protocols as summarized below, are based on a background of 12 publications.

Our aim was to search for the simplest way to engineer a whole organ. For that purpose, a biomimetic approach was developed and several specific complementary processes were successively investigated, including:

1) The maintenance of odontogenic properties in cultured and implanted dental cell-tissue and cell-cell re-associations and their limits in this system.

2) The search for other mesenchymal cell sources, where the ability to respond the epithelially-derived signals (competence) would be maintained

3) The comparison of the mesenchymal cell heterogeneity in re-associations and the tooth at similar developmental stages.

4) The restoration of the vascularization.

5) The comparison of the crown and root matrices in re-associations vs tooth.

6) The restoration of the innervation.

Methodological aspects are listed below and detailed in publications. The design of specific protocols, their justification and the main results are discussed along the text.

Material and methods

Materials

1) Mice
   - ICR mice (C57/BL6) (Charles River Laboratories)
   - GFP mice (C57/BL6) (IGBMC, Strasbourg),
   - Nude CD1 mice (Charles River Laboratories)
   - Sema3A+/− mutant mice (Chronobiotron, Strasbourg)
2) Embryonic first lower molar, mostly at embryonic day (ED) 14
3) Trigeminal ganglion isolation from newborn mice and postnatal (PN) day 0-3.
4) Clonal mesenchymal cell lines (17IA4 and 705IC5), derived from ED18 mouse molars by limiting dilution (Provided by Prof A. Poliard, Univ. Paris Descartes).

Methods (detailed in the original publications)

1) Trypsin dissociation of dental tissues.
2) Enzymatic dissociation and filtration of dental epithelial and mesenchymal cells.
3) Cultures of tooth germs, dental tissues and trigeminal ganglions (on semi-solid medium) and dental cells or cell lines (in supplemented DMEM/F12 liquid medium).
4) Under-skin implantation of cultured re-associations
5) Histology
6) Cryosectioning
7) Immunofluorescent staining
8) Cell proliferation assay (BrdU incorporation)
9) *In situ* hybridization
10) Computer-aided 3D reconstructions
11) RT-PCR
12) Transmission electron microscopy
13) X-ray microanalysis
14) Electron diffraction microanalysis

Ethics: When using animals, experimental protocols were performed according to the current authorization delivered by the “Ministère de l’Enseignement Supérieur et de la Recherche” and the law valid for the research projects within the Czech Republic.

Results

The first step was to check the feasibility of reforming a tooth *in vitro* from tissue-cell and cell-cell re-association.

Epithelial-mesenchymal interactions

Tooth morphogenesis, epithelial histogenesis, as well as odontoblast and ameloblast differentiation are controlled by reciprocal epithelial-mesenchymal interactions. These interactions are mediated by an interposed basement membrane and by diffusible signaling molecules (Yoshiba et al., 1998; Sahlberg et al., 1999; Lesot et al., 2002). Their tuning involves time/space variations in the composition of the basement membrane and associated molecules (Walma and Yamada, 2020).

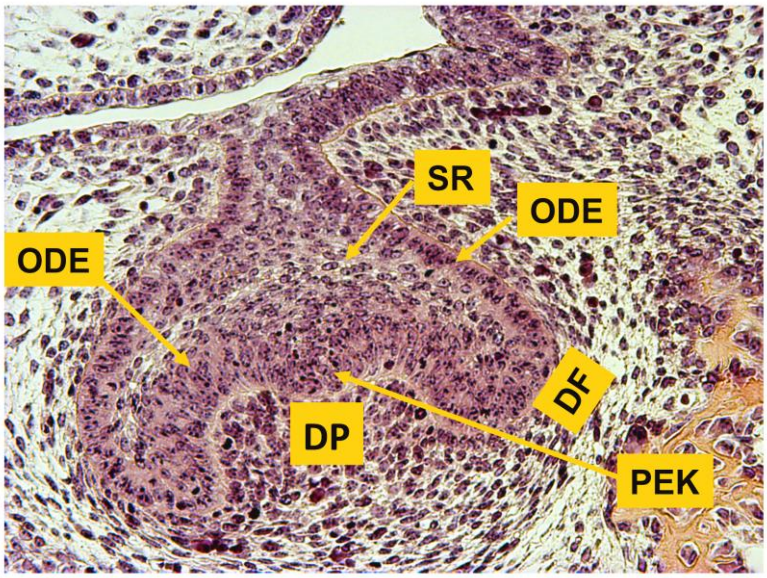

Figure 1: Mouse first lower molar at the cap stage (ED14). Histological section showing the inner (IDE) and outer dental epithelium (ODE), the stellate reticulum (SR) located in between the IDE and ODE and the primary enamel knot (PEK). The mesenchyme includes the dental papilla (DP) and the dental follicle (DF). (Svandova et al., 2020)

Embryonic dental cells were used to test the possibility of tooth engineering. For that purpose, dental mesenchyme and enamel organ from mouse first lower molars at embryonic day (ED)14 were enzymatically dissociated (Hu et al., 2005b). At this early cap stage, the enamel organ consists of four cell types forming the inner dental epithelium (IDE), primary enamel knot (PEK), outer dental epithelium (ODE) and the stellate reticulum (SR) (**Fig. 1**) (Lesot and Brook, 2009). The specific fate of epithelial cells in the different

compartments may be specified by differential cell-cell (Obara et al., 2004) and cell-matrix interactions (Lesot et al., 2002; Fukumoto et al., 2006), as well as by signaling molecules (Xu et al., 2003; O'Connell et al., 2012**)**. All along the different protocols described below, these four compartments will be called (dental) epithelium/epithelial cells. Similarly, mesenchyme/mesenchymal cells will correspond to cells from the dental papilla plus dental follicle (**Fig. 1**) (Svandova et al., 2020).

Positional information versus cell history

During tooth development, the formation of the four epithelial compartments involves differential mitotic activities, differential cell adhesion, apoptosis and cell segregation. Since these activities are regulated in time and space, positional information must be particularly important (Hata et al, 1996; Wolpert, 2002). Positional information and the space-specific distribution of small diffusible signaling molecules, cell-cell and cell-matrix interactions are interdependent. Within a tissue, mechanical forces can signal coupling between cell position and cell fate specification (Chanet and Martin, 2020; Kindberg et al., 2020)

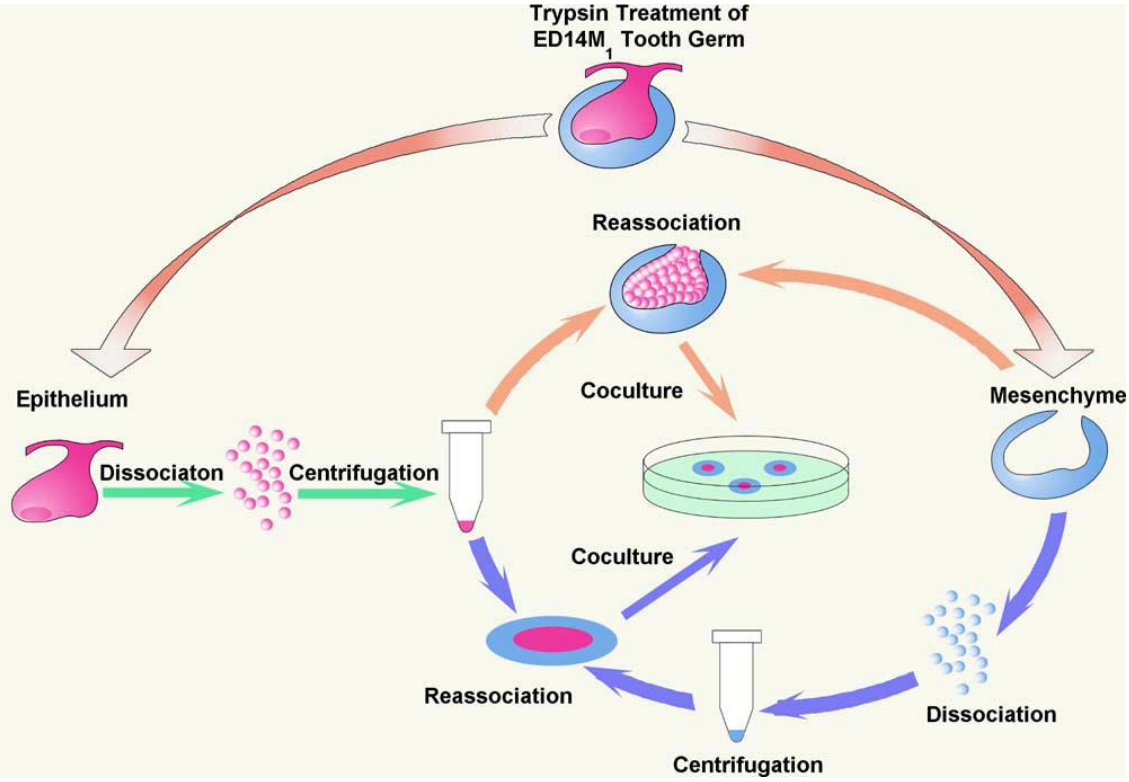

Figure 2: Schematic representation of the experimental procedures for cell-tissue (light brown) and cell-cell (blue) re-associations. Mesenchymal tissues or cells are represented in blue, epitheliums in pink. (Hu et al., 2005b)**.**

As a possible crippling factor, it was important to test whether positional information, as it exists at the cap stage, needs to be memorized to allow tooth engineering. To investigate it, single dental epithelial cells were prepared by trypsin-dissociation and filtration. After trypsin treatment, the hydrolysis of the basement membrane and cell-surface molecules resulted in the loss of positional information of the different epithelial cell groups (Hu et al., 2005b). These cells were pelleted, re-associated to either a dental mesenchyme or dissociated mesenchymal cells and cultured *in vitro* (**Fig. 2**). Although with a different timing when compared to the physiological situation, teeth developed in both types of re-associations showing a characteristic dental epithelial histogenesis, cusp formation, as well as the cytological and functional differentiation of odontoblasts and ameloblasts (Hu et al., 2005a,b).

When re-associations between epithelial cells and an intact mesenchyme had been cultured for 12 to 24 hours *in vitro*, epithelial cells were in contact with a newly formed basement membrane. From 12 hours, mesenchymal cells started to condense as in early stages of odontogenesis (Hu et al., 2005a). This might represent an example of self-organization, independent from any pre-patterning. After 3 days, the shape of the epithelium changed: a first sign of morphogenesis. This was similar to what can be observed during early stages of odontogenesis (Svandova et al., 2020). Epithelial cells thus showed a remarkable plasticity. They rapidly adjusted to their new environment and restored the histological characteristics of IDE, ODE, PEK and SR cells. The rapid progression of these initial steps of histogenesis suggested that initial positional information, possibly linked to cell history, does not need to be memorized: it is progressively set up. The dental mesenchyme, as well as dissociated mesenchymal cells, induced the formation of a PEK indicating that no specific organization in the mesenchyme is initially required for this step (Hu et al., 2005b).

After dissociation, the plasticity of embryonic dental epithelial and mesenchymal cells at ED14 allows their adjustment to each other and to new environmental circumstances. When the specific environment is progressively restored, cells loose their plasticity as they become committed (Kalcheim and Kumar, 2017). This permitted further epithelial-mesenchymal interactions to take place and supported the initiation of tooth formation (Hu et al., 2005a,b and references therein). This first set of data was critical as determining the possibility to continue with this experimental approach and search for further complementary steps.

Crown morphology is altered when dissociated epithelial cells are re-associated with dissociated mesenchymal cells instead of an intact mesenchyme (Hu et al., 2006). It is not known whether the alteration in crown morphogenesis is a consequence of a loss of architecture/organization in the mesenchyme or results from alterations in the signaling mechanisms from the mesenchyme to the epithelium, or both if interrelated. We hypothesized that when dissociated mesenchymal cells were used instead of an intact mesenchyme, the number of mesenchymal cells re-associated to a constant number of epithelial cells might be a limiting factor. To test it, increasing numbers of dental mesenchymal cells were re-associated to an intact epithelium. All the re-associations were cultured for 8 days and then implanted under the skin of adult ICR mice. Results supported our initial hypothesis (Hu et al., 2006).

The period when mesenchymal cells remained competent to engage in tooth formation was investigated as well (Groves et al., 2000). The odontogenic potential of mesenchymal cells as observed at ED14 decreased at ED16 and was lost at ED18 (**Fig. 3**) (Keller et al., 2011). This might result from an increasing number of committed cells at the epithelial-mesenchymal junction. Alternatively, it is possible that mesenchymal cells at ED18 cannot adjust to an ED14 epithelium. This might result from changes in the mesenchymal cells phenotypes when ageing, as observed *in vivo* and also after a pre-culture *in vitro* (Keller et al., 2011; 2012). Similar observations have been reported when studying odontoblast differentiation at later stage (Ruch et al., 1995) and also when investigating the induction of otic placode formation (Groves et al., 2000).

The basement membrane plays a major role in mediating epithelial-mesenchymal interactions in the development of different organs (Lesot et al., 2002; Combes et al., 2015). Its time- and space-specific composition (structural and associated diffusible molecules) determines its ability to mediate interactions (Miner and Yurchenco, 2004). After trypsin treatment, the basement is hydrolyzed. A new one is deposited during the first 24 hours in vitro (Hu et al., 2005b). Supporting this first set of results, it was observed that the odontogenic potential of ED14 mesenchymal cells was lost after a pre-culture *in vitro*. This occurred after 24 hours *in vitro* only and was associated with a rapid change in cell phenotype (Keller et al., 2012). This loss of odontogenic potential might result from a cell selection due to differential adhesion, and a consecutive change in cell heterogeneity (Keller et al., 2011).

The sequence of reciprocal epithelial-mesenchymal interactions

had been investigated in details at a later stage, related to odontoblast differentiation. Results from this study supported this hypothesis. Heterotopic re-associations between dental and non-dental tissues or a dental mesenchyme in contact with either a inner or outer dental epithelium have been cultured. Both types of experiments showed that the mesenchyme controls the composition of the basement membrane, the shape of adjacent epithelial cells and their cell kinetics (Olive and Ruch, 1982; Yoshiba et al., 1998). Interaction between extracellular matrix (ECM) molecules and their cell surface receptors is crucial in tissue morphogenesis (Gumbiner, 1996). We have observed that cultured incisors displayed major modifications in basal lamina deposition. Ultrastructural observations showed that after 6 hours *in vitro* multilayered basal laminae had been deposited on the labial face of incisors. However, this had no further consequence on odontoblast differentiation and even their gradients of differentiation were maintained (Meyer et al.1995). Thus, more important than structural components of the basal lamina, epithelially-derived diffusible molecules (i.e. growth factors) could be involved when cell-surface receptors were progressively expressed by competent preodontoblasts (Begue-Kirn et al.1992; Ruch et al.1995).

Cell sources and the problem of odontogenic competence

Until now, most approaches to tooth tissue engineering have been performed using embryonic dental cells (Hu et al., 2005, 2006; Honda et al., 2008). However, the cell amount necessary for each experiment and their time consuming preparation are limiting parameters. Although this would be required for both the epithelial and mesenchymal compartments of the tooth, mesenchymal cells were tested first. Since it is required for mesenchymal cells induction, embryonic dental epithelium or cells at ED14 have always been used for these experiments (Ohazama et al., 2004; Honda et al., 2008).

Attempts have been made to use two immortalized cell lines derived from the ecto-mesenchyme of mouse molars at ED18 (Priam et al., 2005). When these cells were re-associated with a competent dental epithelium (ED14) and cultured, an epithelial-mesenchymal junction was reconstituted. However, the initial histology of the dental epithelium, as initially visible at ED14, was rapidly lost in the cultured re-associations. These two cell lines failed

to initiate tooth development. Neither odontoblasts nor ameloblasts differentiated. The implantation of cultured re-associations did not improve the results (Keller et al., 2011).

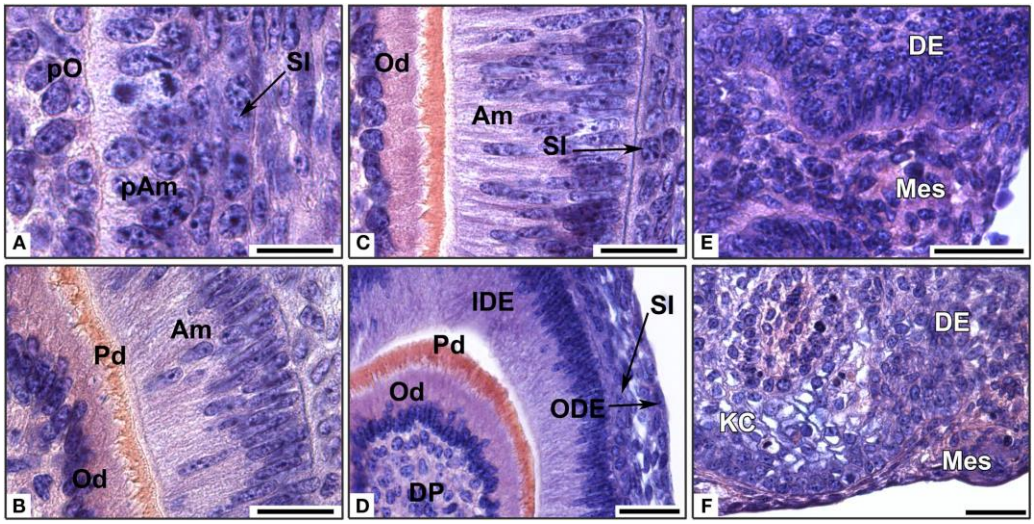

Figure 3: *In vitro* development of re-associations between an ED14 intact dental epithelium and dissociated dental mesenchymal cells from different embryonic stages. Dental epitheliums were re-associated with mesenchymal dental cells from ED14 **(A,B)**, ED16 **(C,D)**, or ED18 **(E,F)**. The re-associations were cultured for 2 days **(E)**, 6 days **(A,C,D,F)**, or 10 days **(B)**. Am, ameloblast; DE, dental epithelium; DP, dental papilla; IDE, inner dental epithelium; KC, keratinized cyst; Mes, mesenchyme; Od, odontoblast; ODE, outer dental epithelium; pAm, preameloblast; Pd, predentin; pO, preodontoblast; SI, stratum intermedium. Bars = 20 µm **(A–C)** and 40 µm **(D–F)**. (Keller et al., 2011).

Since the phenotype of these immortalized cell lines can be very distinct from that of competent embryonic dental mesenchymal cells, Other experiments were performed by increasing their number by *in vitro* expansion. For this purpose, ED14 dental mesenchymal cells cultured up to 4 days without passage. However, already after 24 hours they had lost their odontogenic potential (Keller et al., 2011). To try to understand this observation, possible changes in gene expression, possibly induced by the culture were searched for. RT-PCR was performed to analyze the expression of several transcription factors (Pax9, Dlx5, Lhx6, Lhx7, Msx1, Msx2) and signaling molecules (Bmp2, Bmp4, Fgf3, and Fgf10), all involved in tooth development. Among these, only the expression of Fgf3 and was down-regulated upon culture. This could be critical for tooth engineering since mesenchymal FGF3 has been suggested to act on the epithelium (Nakatomi et al., 2010). FGF1, -2 and -8 can induce Fgf3 expression in the mesenchyme (Bei and Maas, 1998).

Several published data had suggested that FGF2 might improve the results (Russo et al., 1998; Unda et al., 2000; Bianchi et al., 2003; Tsuboi et al., 2003; Arany et al. 2009). Preliminary experiments were performed to test the effects of FGF2. When added to the culture medium, it led to an up-regulation of Fgf3 expression in dental mesenchymal cells from ED14 cultured for 4 days. However, it did not restore their potentiality to engage in tooth formation when re-associated with a competent dental epithelium (Keller et al., 2011).

There is no molecular marker to anticipate the fate of mesenchymal cells in re-associations and to foresee whether they can engage in tooth formation. Several different cell types thus had been tested.

Mouse embryonic stem (ES) cells can be efficiently be committed toward a NC cell-like phenotype and then behave as multipotent NC cells. These cells could then be specified towards classical NC-derived (ES-NC derived) cell types as well as ecto-mesenchymal cells and respond to an embryonic epithelial tissue signaling (Baroffio et al., 1991). Lineage-specific ES cell reporter lines are important for cell tracing: real-time monitoring of *in vitro* differentiation, purification of specific progenitor subpopulations, and longitudinal analysis of their survival and behavior in implanted tissues. Under specific induction ES-NC-derived cells could engage into an osteogenic, neuronal, glial, or vascular program (Acuna-Mendoza et al., 2017). Once specified, these ES-NC-derived cells were re-associated with a dental epithelium at ED14 and tested for their ability to engage in odontogenesis. Again, these cells failed to form a tooth or tooth-like structures (Acuna-Mendoza et al., 2017).

Several teams have found and characterized stem cells present in the dental pulp and in the periodontal ligament (see references in Otsu et al., 2014). The existence of several types of dental and peridental derived “stem” cells indicate they are not “stem” but “progenitor” cells (Svandova et al., 2020). Some encouraging results regarding cell differentiation have been obtained when implanting cultured re-associations between NCLCs derived from iPS cells and an ED14.5 dental epithelium (Otsu et al., 2014).

Up to now, there is only one example where uncommitted non-dental mesenchymal cells engaged in tooth formation. Ohazama et al. (2004) reassociated adult Bone Marrow Stem Cells (BMCs) with an ED10 oral epithelium. At this stage, the epithelium could instruct the mesenchyme to engage in odontogenesis in 10% cases (Mina and Kollar, 1987).

Restoring mesenchymal cells heterogeneity

Epithelial histogenesis is easily visualized. In the mesenchyme the situation is very different. Except for blood vessels (BVs) from ED14,5 as well as odontoblasts and sub-odontoblastic sister cells, which form layers from ED18, the mesenchymal cell organization can hardly be detected. For this reason, very little is known about cell diversity and patterning in this tissue (Svandova et al., 2020 and citations therein). A better knowledge about it might be essential to better understand why, from a certain stage, dental mesenchymal cells failed to engage in tooth engineering. Among other general biological aspects discussed above, the restoration/maintenance of the cell heterogeneity in the dental mesenchyme represents an important criterion to evaluate the protocols developed in this study.

The setting up and dynamic spatial changes in cell heterogeneity determine the fate of cultured and implanted re-associations. A specific study has thus been designed to compare the situation in engineered and developing teeth at similar developmental stages. To this end, the expression of cell surface markers was investigated by immunostaining in: 1) embryonic mouse molars at ED14, as the initial cell source for re-associations, 2) cultured cell re-associations just before their implantation compared to tooth germs at ED18 and 3) cultured cell re-associations implanted for two weeks compared to molars at postnatal day (PN4) (Keller et al., 2012).

Except for markers associated with the vascularization, which cannot be maintained *in vitro*, the staining pattern in the mesenchyme of cultured re-associations was similar to that observed in molars at ED18. After implantation of the cultured re-associations, the re-vascularization and cellular heterogeneity in the mesenchyme were similar to what was observed in developing molars at PN4.

The immunodetection of cell-surface markers demonstrated the existence of several different cell populations in the dental and peridental mesenchymes. The timing of their appearance and their patterning can be different as well (Keller et al., 2012).

After implantation, vascularization still increased the cellular heterogeneity in the mesenchyme, which again was similar to what was observed in developing molars at PN4. Indirectly, the vascularization is involved in the progressive increase in mesenchymal cell heterogeneity, by allowing external cells to enter the mesenchyme (Keller et al., 2012). Implantations in transgenic mice expressing green fluorescent protein (GFP) showed that the host participated in the reconstitution of cell heterogeneity. Not only BVs, but also pericytes and dendritic cells originate from the host. Together with endogenous cells, they form a functional network, which changes at later developmental stages, when the innervation takes place (Kokten et al., 2014). During tooth development, the dynamic of this functional network requires changes in heterotypic cell-cell interactions as well as in signaling pathways (Keller et al., 2012).

The phenotype of mesenchymal cells rapidly changed when they were grown as a monolayer, even without passage. Changes were already visible after 24 hours *in vitro*, which reflected modifications in the balance of cell subpopulations. This might explain the rapid loss of their potential to sustain tooth formation after re-association (Keller et al., 2011).

Vascularization and mineralization in engineered tooth

Since it is involved in tissue oxygenation, metabolites exchanges, mineralization of dental matrices vascularization plays a major role in tooth development. Despite the very important role of blood in the survival of implanted organs, the timing of their development in engineered teeth had not been investigated. BVs were identified by double immunostaining for CD31/Collagen IV and for VEGFR2/Collagen IV (Nait Lechguer et al. 2008). Then three points were investigated: 1) the timing for their appearance in the mesenchyme and enamel organ respectively in the developing molar, 2) the progressive re-vascularization of cultured re-associations after under skin implantation, and 3) the origin of BVs by implanting cultured molars and re-associations in GFP mice.

Ex vivo, BVs entered the dental mesenchyme at the cap stage (ED14,5), and reached the sub-odontoblastic layer at ED18,: when

the first odontoblasts still secreted predentin only and 24 hours before mineralized dentin became apparent. The first BVs entered the enamel organ at the bell stage (ED18). In the epithelial compartment, BVs crossed the basement membrane located in between the dental follicle and the outer dental epithelium, rapidly extended and reached the stratum intermedium 24 hours before ameloblasts became functional and deposited enamel. In cultured teeth and re-associations, some staining for the BV markers persisted in the peridental mesenchyme although BVs, as structures, had disappeared. Nothing could be detected in the dental mesenchyme.

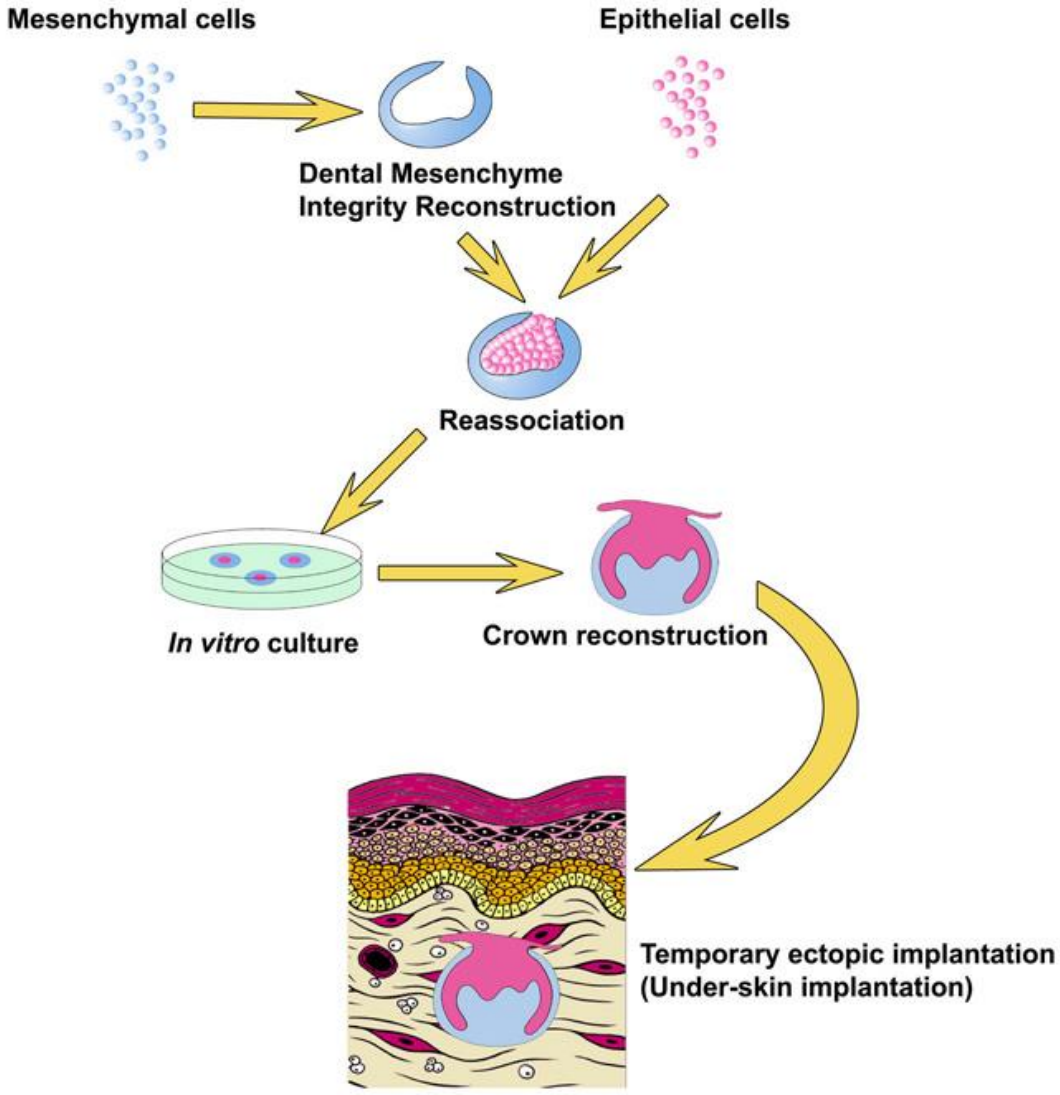

Figure 4: Different steps allowing the vascularization and the mineralization of dental matrices. Dissociated cells from embryonic tooth germs at ED14 are re-associated and co-cultured *in vitro* until tooth crown formation. Then the re-associations are implanted under the skin of adult ICR mice (Hu et al., 2006).

After implantation (see protocol in **Fig. 4**), the dental mesenchyme and enamel organ were re-vascularized in both teeth and re-associations (**Fig. 11**). Implantation of re-associations in GFP mice showed that the re-vascularization involves vessels originating from the host. They allowed organ growth and dental matrices mineralization (Nait Lechguer et al., 2008; 2011).

After two weeks of implantation, in both intact molars and cultured re-associations, α-SMA, a marker of smooth muscle cells, was detected in pericytes associated with large vessels in the apical part of the dental mesenchyme. At that time, CD31-, CD34- and CD146-positive blood vessels were localized all over the dental pulp (Kokten et al., 2014). Pericytes were detected from PN4 in molars *ex vivo*. Pericytes, coming from the host (Keller et al., 2012), and the perivascular environment is a potential source of stem/progenitor cells (Feng et al., 2010; Pang et al. 2016). This study showed that the vascularization can be restored in implanted re-associations, as it exists in physiologically.

Crown formation

Crown formation is characterized by the cytological and functional differentiation of odontoblasts and ameloblasts (Hu et al., 2006). Both could be observed in implanted re-associations (**Fig. 5B**). Compared to the situation *ex vivo*, gradients of cell differentiation were maintained for odontoblasts and for ameloblasts (**Fig. 5B**). Odontoblasts showed characteristic polarized secretion of predentin/dentin (**Figs. 6A, B**). Dentinal tubules containing the odontoblastic processes almost reached the dentin/enamel junction (**Fig. 6C**) (Nait Lechguer et al., 2011).

The next point was to investigate tooth crown, root and peridental ligament formation in implanted re-associations and to compare it with the situation in developing tooth germs (Hu et al., 2006; Nait Lechguer et al., 2009). A special attention was given to the dental matrices: dentin and enamel.

Transmission electron microscopy (TEM) showed the characteristic changes in the cytoplasm of polarized odontoblasts and the organization of the matrix. X-ray microanalysis and electron diffraction further documented the apatite type of mineralization (Nait Lechguer et al., 2011). Predentin/dentin allowed for the induction of ameloblast cytological and functional differentiation.

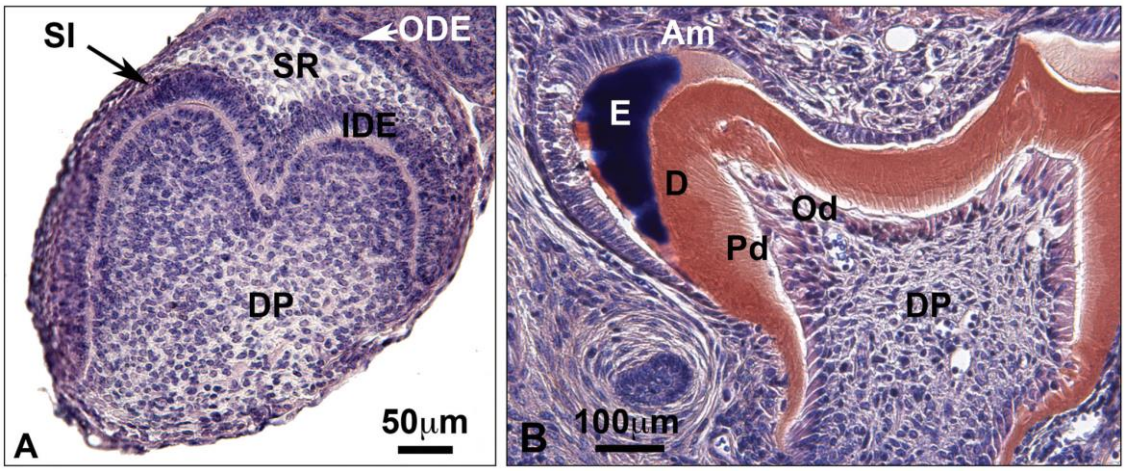

Figure 5: Before implantation in adult mice, cell-cell re-associations were cultured for 8 days (**A**). At this stage, epithelial histogenesis was achieved, showing distinct inner (IDE) and outer (ODE) dental epitheliums, stellate reticulum (SR), and stratum intermedium (SI). Cusps were formed, and the first odontoblasts started to polarize, but most of the mesenchymal cells at the epithelial-mesenchymal junctions were still round pre-odontoblasts (**A**). Two weeks after implantation (**B**), polarized odontoblasts (Od) were functional and secreted predentin (Pd) and dentin (D), in both the crown and the root. Ameloblasts (Am) showed gradients of differentiation, and enamel (E) secretion. DP: Dental pulp. (Nait Lechguer et al., 2011).

In cell-cell re-associations, ameloblast functional differentiation had started before 7 days of implantation. At this stage, the enamel organ was vascularized (Nait Lechguer et al., 2008). Interactions between BVs and cells of the stratum intermedium were observed in implanted re-associations (**Figs. 6F**; Nait Lechguer et al., 2008). After 2 weeks of implantation, ameloblasts were elongated, polarized and secreted enamel proteins (**Figs. 7A, B**). The transport the calcium ions, as mediated by the vascularization, allowed apatite crystals formation in the enamel (Bronckers, 2017). Enamel crystals showed a typical organization with decussation (**Fig. 6E**) and rods-interrods (**Fig. 7C**), as *in vivo* (Shin et al., 2020). TEM showed an intense activity: secretion of stippled material and the presence of numerous coated vesicles involved in a simultaneous resorbing activity as it happens in physiological conditions. It also showed the characteristic organization of enamel rods and inter-rods (**Figs. 7D, E**). For enamel, as for dentin, X-ray microanalysis revealed the presence of a high amount of calcium and phosphorus and electron diffraction showed the crystalline nature of hydroxyapatite (Nait Lechguer et al., 2011).

Root formation

When cultured cell-tissue or cell-cell re-association have been implanted for two weeks, root formation had started (**Figs. 6A, G**) (Hu et al., 2006). The presence of root predentin/dentin and that of cementum illustrated the functional differentiation of odontoblasts and that of cementoblasts (**Figs. 6H, I**) (Nait Lechguer et al., 2011; Keller et al., 2012). Initially cementoblasts secreted collagen at the surface of root dentin (**Figs. 7F, G**).

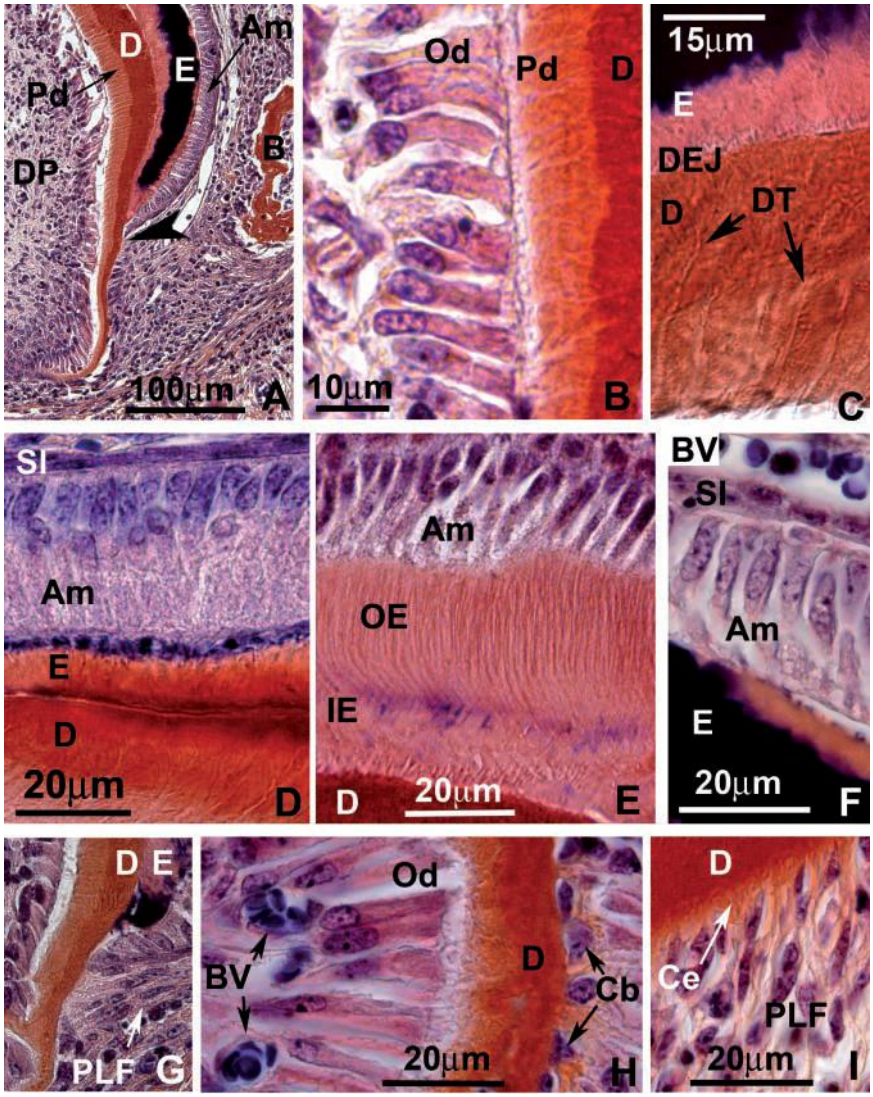

Figure 6: Histology of dental epithelial and mesenchymal cell-cell re-associations cultured for 8 days and implanted for 2 weeks. (**A**) The crown was well developed, and both odontoblasts and ameloblasts (Am) became functional to secrete predentin/dentin (Pd/D) and enamel (E). An arrowhead indicates the sharp crown-root junction. (**B**) Elongated and polarized odontoblasts (Od) secreted predentin and dentin. (**C**) Dentinal tubules (DT) extended toward the dentin/enamel junction (DEJ). (**D**) At an early stage of enamel secretion, ameloblasts formed a monolayer of elongated and polarized cells in contact with the stratum intermedium (SI). (**E**) At a later stage, crystals

showed decussation in the inner part of the enamel (IE), while running parallel in the outer enamel (OE). (**F**) Blood vessels (BV) could be found in direct contact with the stratum intermedium (SI). (**G**) Below the crown-root junction, periodontal ligament fibroblasts (PLF) were already attached to the root and extended until reaching newly formed bone (B), as shown in (A). (**H**) In the root, BVs were observed next to functional odontoblasts. In contact with the external surface of dentin, cementoblasts (Cb) were embedded in extracellular matrix. (**I**) Periodontal ligament fibroblasts attached to the cementum (Ce). (Nait Lechguer et al., 2011)

Peridental ligament

The dental and peridental mesenchymes have distinct origins. At early developmental stage, and in opposition to the dental mesenchyme and follicle cells, interstitial mesenchymal cells, future PDL, do not express markers for neural crests-derived cells (Chai et al., 2000). It has been proposed that at least part of the PDL fibroblasts originate from the stomodeal mesoderm (Cho and Garant, 2000). This might explain their specific properties and differences with the neighboring ecto-mesenchymal odontogenic condensation. The PDL is vascularized and innervated long before the dental mesenchyme.

The PDL forms and differentiates quite late postnatally (Zvackova et al., 2017; Svandova et al., 2020). Even at PN7, the developing PDL does not yet show any characteristic organization. Cells from the PDL produce an ECM mostly consisting in collagen (type I, III, V, VI and XII) fiber bundles (Beertsen et al. 1997; Zvackova et al., 2017). These cells also synthesize molecules regulating collagen fibrillogenesis and the spatial organization of fibers. These include FACITs (fibril associated collagens with interrupted triple helices) and a group of small leucine-rich proteoglycans (SLRPs) (Zvackova et al., 2017). This ECM does not mineralize, which impairs tooth ankylosis during later development (Svandova et al., 2020). PDL collagen fibers interact with the cementum and newly formed bone. When embedded in the cementum on one side (**Figs. 6G, I)** or bone matrix on the other (**Fig. 6A**), these collagen fibers mineralize at their extremities, forming Sharpey's fibers (Hirashima et al., 2020; Svandova et al., 2020).

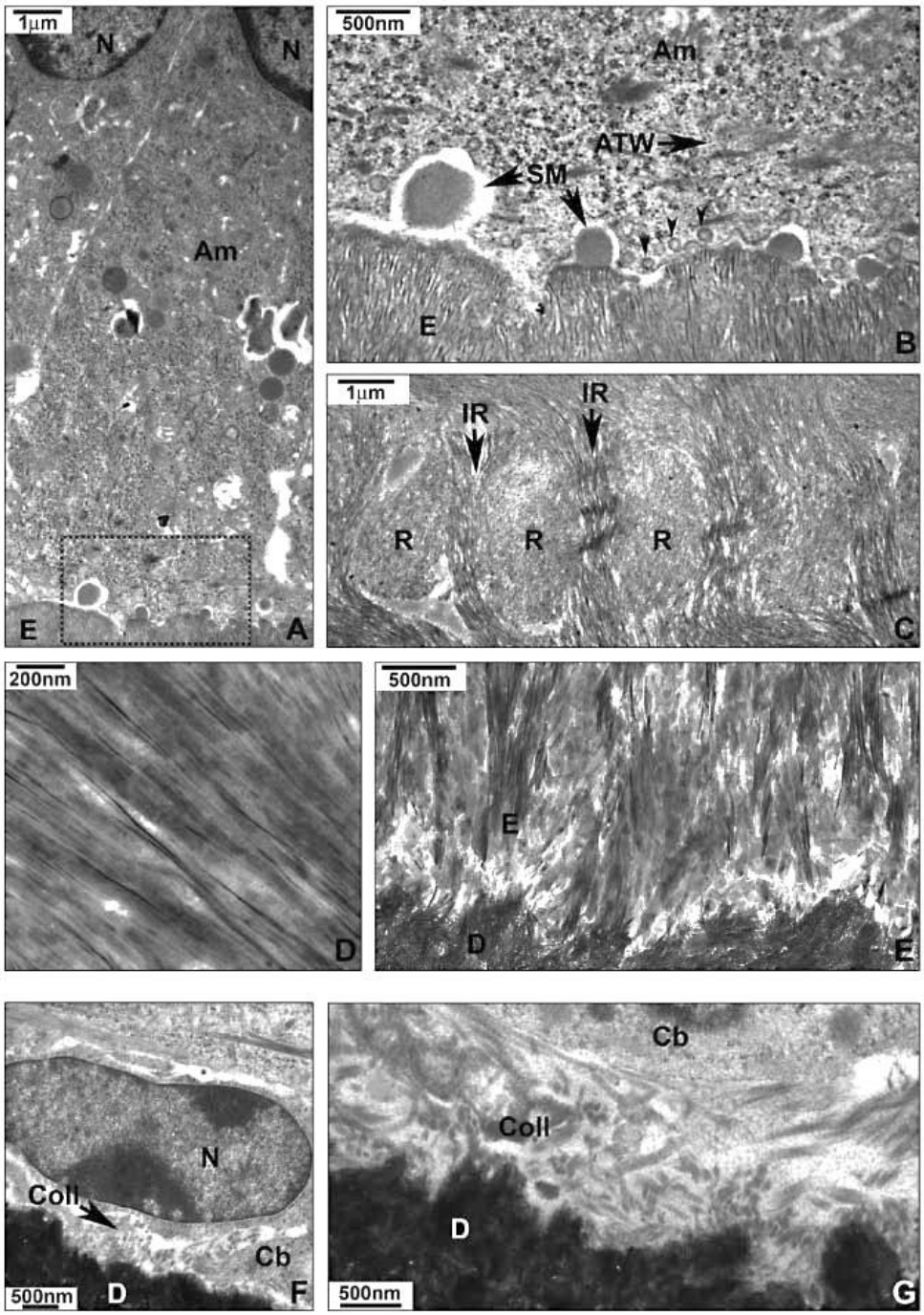

Figure 7: Transmission electron microscopy of ameloblasts-enamel **(A-E)** and of cementoblasts-cementum **(F,G)** in dental epithelial and mesenchymal cell-cell re-associations cultured for 8 days and implanted for 2 weeks. **(A)** Ameloblasts (Am) were elongated, and their nuclei (N) were distant from the secretory pole, in contact with the enamel (E). **(B)** corresponds to the box in **(A)**, showing the secretory pole of ameloblasts. Below the apical terminal web (ATW), intense activity was observed, with the secretion of stippled material (SM) and the presence of numerous coated vesicles (arrowheads) suggesting a simultaneous resorbing activity. (**C)** The enamel showed a characteristic organization of rods (R) and inter-rods (IR). **(D-E)** High magnifications of enamel crystals in the decussation zone **(D)** and near the dentin-enamel junction (E). **(F-G)** Cementoblasts (Cb) secrete collagen (Coll) in contact with root dentin (D). (Nait Lechguer et al., 2011)

Other cells also interact with the surface of cementum: Hertwig's epithelial root sheath (HERS) cells (**Fig. 8**). HERS cells are key cells in root formation. They form an epithelial structure, extending from the apical region of the enamel organ, growing apically, and guiding tooth root formation. Immunostainings for collagen IV and cytokeratin 14 showed that HERS cells were present after the implantation of cultured cell re-associations for two weeks (**Fig. 8**) (Nait Lechguer et al., 2011). Although this is still controversial, they have been suggested to be involved in stimulating both cementogenesis and root dentinogenesis (**Figs. 6H, I; Figs. 7F, G**).

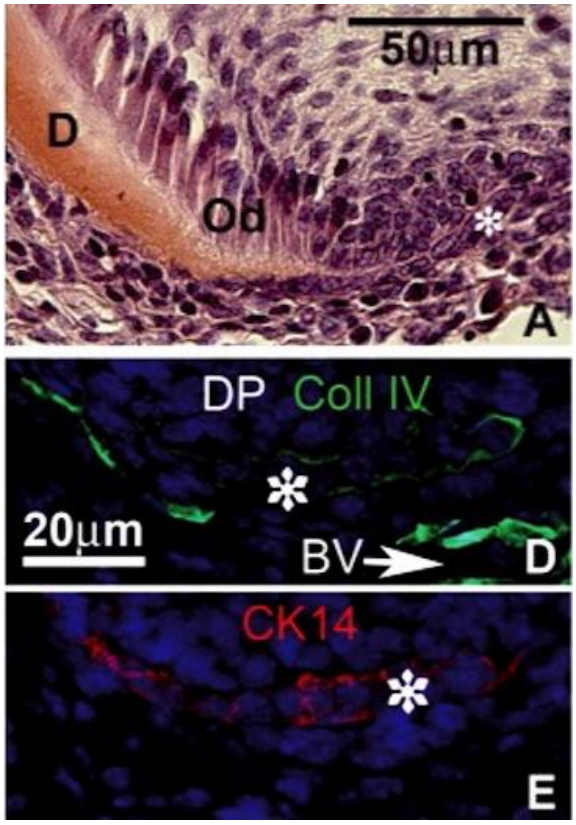

Figure 8: After 2 weeks of implantation, root had begun to develop in implanted re-associations. HERS cells (white star) are hardly seen after histological staining (**A**), but easily detectable after immunostaining for either collagen type IV (**D**) or cytokeratin 14 (**E**).

Innervation

Tooth innervation is mediated by axons originating from the trigeminal ganglia and regulated in time and space (Moe et al., 2008; Luukko and Kettunen, 2014). It is particularly important in signaling damages in the crown, but may also interfere with several functions including the regulation of blood flow, tissue homeostasis, immune

cells function, inflammation and healing. The peripheral nervous system is characterized by an intimate relationship between axons and resident peripheral glial cells, the Schwann cells.

Attempts to innervate cell re-associations, were the most recent part of the work. It was also a very complex one, which required the setting up of complementary experimental approaches.

When implanting cultured cell re-associations under the skin of adult ICR mice, the innervation of the dental mesenchyme did not occur spontaneously (Kokten et al., 2014). To try to solve this problem, cell-cell re-associations were cultured for six days and then co-cultured for 24 hours with trigeminal ganglia before implantation (**Fig. 9**). Double immunostainings using antibodies to peripherin (innervation) and CD31 (vascularization) showed that re-associations were fully vascularized while nerve fibers extended only in peridental tissues. Even after 2 weeks, the dental papilla of implanted re-associations was not innervated (Kokten et al., 2014).

Implantation in cyclosporine A-treated mice

Previous clinical works concerning face transplantation required an immuno-suppressive therapy, performed by using cyclosporine A (CsA) treatment to avoid graft rejection (Siemionow et al., 2011). CsA reversibly inhibits T-lymphocyte function, and unexpectedly, was found to stimulate the re-innervation of transplanted tissues. Attempts were thus made to use this molecule in our context. CsA was added to drinking water of ICR mice. Under these conditions, the dental mesenchyme was re-innervated (Kokten et al., 2024).

When co-implantations were performed in CsA-treated mice, both the cytological and functional differentiation of odontoblasts and ameloblasts did occur. All three constituents of the cytoskeleton (microtubules, microfilaments and intermediate filaments) are involved in the elongation and polarization of both cell types. CsA thus had no negative effect, although it is a specific inhibitor of the calcineurin-NFAT pathway (Li et al., 2011) and calcineurin is known to regulate the organization of the cytoskeleton (Descazeaud et al., 2012). On the other hand, calcineurin is strongly expressed in secretory odontoblasts and ameloblasts, where it has been suggested to correlate with active mineralization phases (Oshima and Watanabe, 2012). Histology and TEM showed that neither dentin nor enamel secretion/mineralization was altered when cell re-associations were implanted in CsA-treated ICR mice, when compared to untreated ones (Kokten et al., 2014).

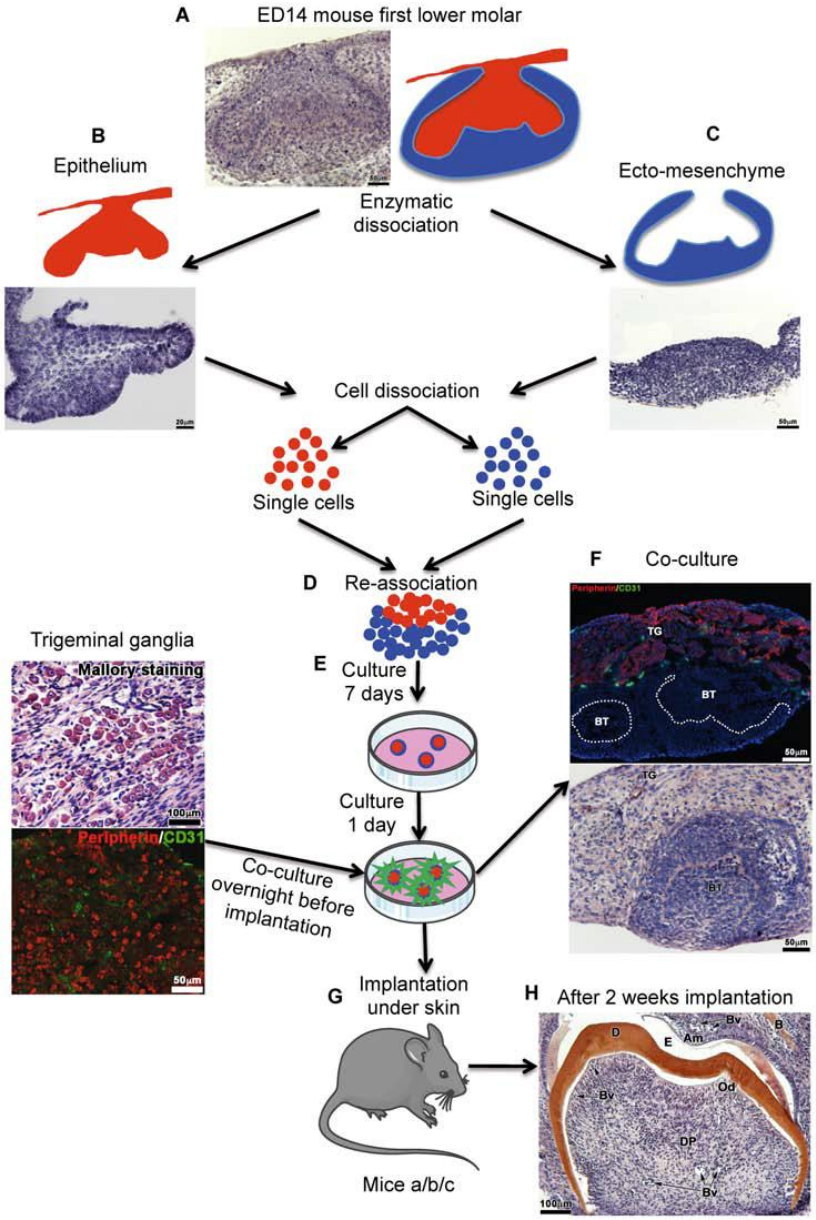

Figure 9: Protocol for the innervation of engineered teeth. The mandibular first molars were dissected from ICR mouse embryos at ED14 (cap stage) (**A**). Then, the dental epithelium (**B**) and ecto-mesenchyme (**C**) were enzymatically separated. Each tissue was dissociated into single cells, which were then re-associated (**D**) and grown on semi-solid cultured medium (**E**). After 7 days *in vitro*, each re-association was co-cultured overnight with trigeminal ganglia from ICR newborn mice (**F**). The eighth day, bioengineered tooth unit and trigeminal ganglia were co-implanted (**G**), between skin and muscles behind the ears in adult ICR. (Kokten et al., 2014)

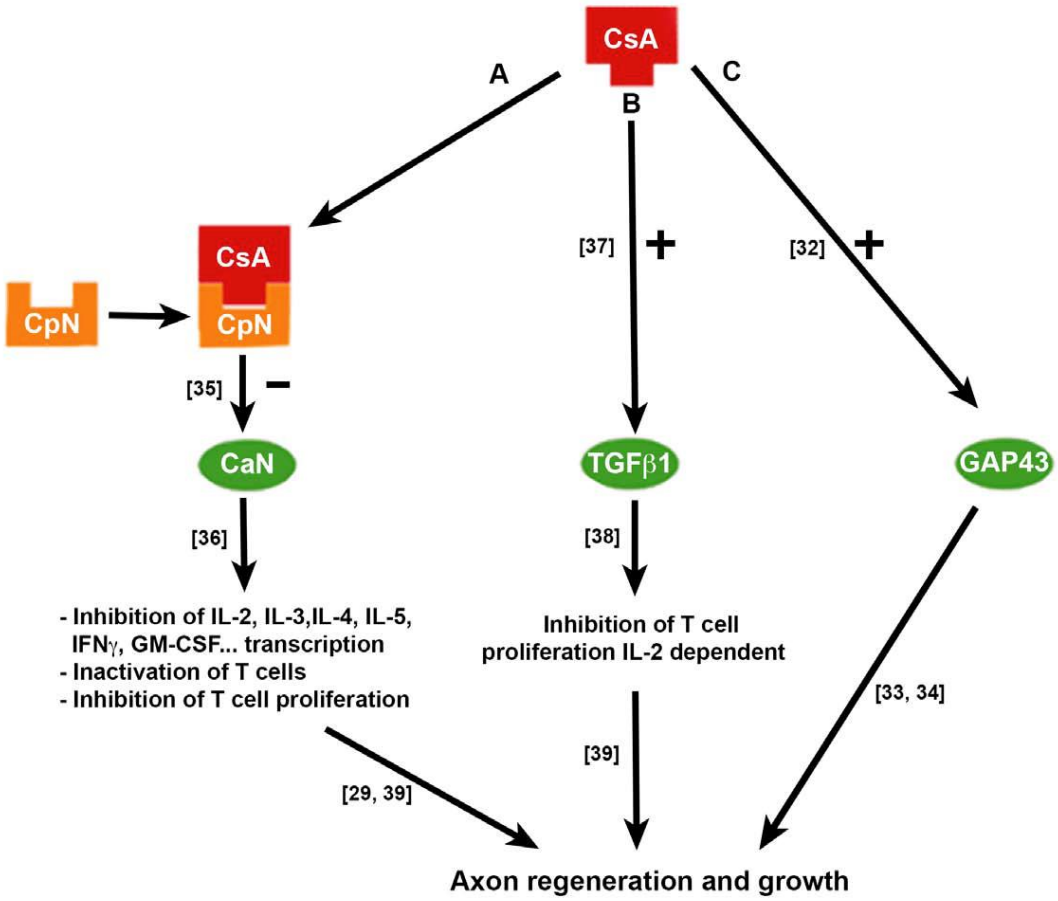

Figure 10: Mechanisms of action of cyclosporine A (CsA). Three different mechanisms have been proposed in the literature (A, B, C). (A) In the cytoplasm of T cells, CsA binds to cyclophylin (CpN) to form a complex. This complex binds and blocks the function of the enzyme calcineurin (CaN). Consequently, T cells do not produce some cytokines, which were necessary for full T cell activation. Furthermore, this pathway inhibits the proliferation of T cells. (B) Alternatively, CsA may increase transforming growth factor-beta1 (TGF-b1) transcription in interleukin-2 dependent T cells. This pathway also induces the inhibition of proliferation of T cells. In both cases (A, B), the inhibition of T cells enhances axonal regeneration. (C) CsA increases the expression of growth-associated protein-43 (GAP-43) expression in axonal growth cones and thus may have a direct effect on axonal extension. IL, interleukin; IFN-c, interferon gamma; GM-CSF, granulocyte macrophage-colony stimulating factor. (Kokten et al., 2014)

After two weeks of implantation in CsA-treated ICR mice, axons reached the odontoblast layer, extended in betwen odontoblasts, almost in contact with predentin. At that time, the cell-cell re-associations reached a stage corresponding to that of a first lower

molar at PN4, when taking into account the crown development: matrix deposition and mineralization (Keller et al., 2012). In physiological condition, axons reach the odontoblast layer at PN7 only (Kokten et al., 2014). CsA not only allowed the innervation, but also accelerated it, when compared to physiological situation.

Immunostaining for S100β protein showed that Schwann cells were present in both the peridental and dental mesenchymes of implanted cell re-associations (Kokten et al., 2014). For unknown reason, only unmyelinated axons were detected when using TEM. In physiological conditions, both myelinated and unmyelinated axons are present in the dental mesenchyme of rodent molar.

Implantation in Nude mice

CsA has multiple effects, including its interfering with three pathways possibly stimulating nerve growth (**Fig. 10**). Attempts were thus made to check whether immuno-suppression by itself could interfere with the innervation. To evaluate it, cell-cell re-associations cultured with trigeminal ganglia were co-implanted in immuno-deficient Nude mice (**Fig. 11**). In these conditions as well, the dental mesenchyme was innervated already after one week of implantation and axons reached the odontoblast layer after two weeks (**Fig. 11**). This demonstrated that immuno-suppression is sufficient to allow the innervation of the dental mesenchyme. (Kokten et al., 2014).

Other questions sill remained, which concerned the interactions of axons and glial cells with BVs in the dental and peridental mesenchymes, and with microvessels present in the odontoblast layer to allow comparison between the three locations. Capillaries development and their entering the odontoblast layer correlate with mineral requirement for dentinogenesis. Odontoblast cell processes extend in the matrix through dentinal tubules and are involved in mechanosensing, mediated by the dentinal fluid (**Fig. 6c**) (Svandova et al., 2020).

Neurovascular interactions in the dental mesenchyme

When cell re-associations were implanted for two weeks in Nude mice, S100β– and GFAP-positive glial cells were detected in the dental pulp. Some of these were in close proximity with BVs. Both in implanted re-associations and in intact molars at PN7, S100β-positive cells showed more proximity with pericytes than GFAP-positive cells (Kokten et al., 2014).

In conditions where the innervation of the dental mesenchyme was not possible, still glial cells expressing S100β, but not GFAP, were detected there (Kuchler-Bopp et al., 2018). The heterogeneity of glial cells as seen here agrees with observations made in the human dental pulp. Glial markers, S100β and GFAP, appear to be developmentally regulated independently from each other (Grundmann et al., 2019).

Neurovascular interactions in the peridental mesenchyme

The periodontal ligament develops in parallel with root formation (Svandova et al., 2020). In the peridental mesenchyme of implanted re-associations as in molars at PN7, both S100β-positive and GFAP-positive cells were detected in association with axons. GAP-43, a major growth cone protein, is involved in neuronal pathfinding. Its localization was compared with that of axons (peripherin). In re-associations implanted for 2 weeks, both antigens co-localized in the peridental mesenchyme, but not in the dental mesenchyme. In molar at PN7 however, such co-localizations were observed in both mesenchymes. The presence of axons in the peridental mesenchyme of engineered teeth has been observed after implantation in ICR mice i.e. in conditions where the dental mesenchyme cannot be innervated. This indicates that not only the kinetics but also the conditions allowing innervation (immune-suppression) are different in the dental and peridental mesenchymes.

Interactions of axons with microvessels in the odontoblast layer

Double staining for peripherin and either CD34, collagen IV, or CD146 illustrated very frequent neurovascular relationship in the odontoblast layer (**Fig. 11**). This observation in re-associations implanted for two weeks in Nude mice was similar to what exists in molars at PN7 (Kokten et al., 2014). From the literature, the question on how signals sensed by odontoblasts are transmitted to axons remains unsolved.

Antibodies to either S100β (**Figs. 11E, F, M, N**) or (GFAP) were used to search for glial cells (**Figs. 11 G, H, O, P**). In the dental mesenchyme, S100β-positive cells showed more proximity with microvessels than GFAP-positive ones. In the odontoblast layer, both antigens were detected in close association with axons.

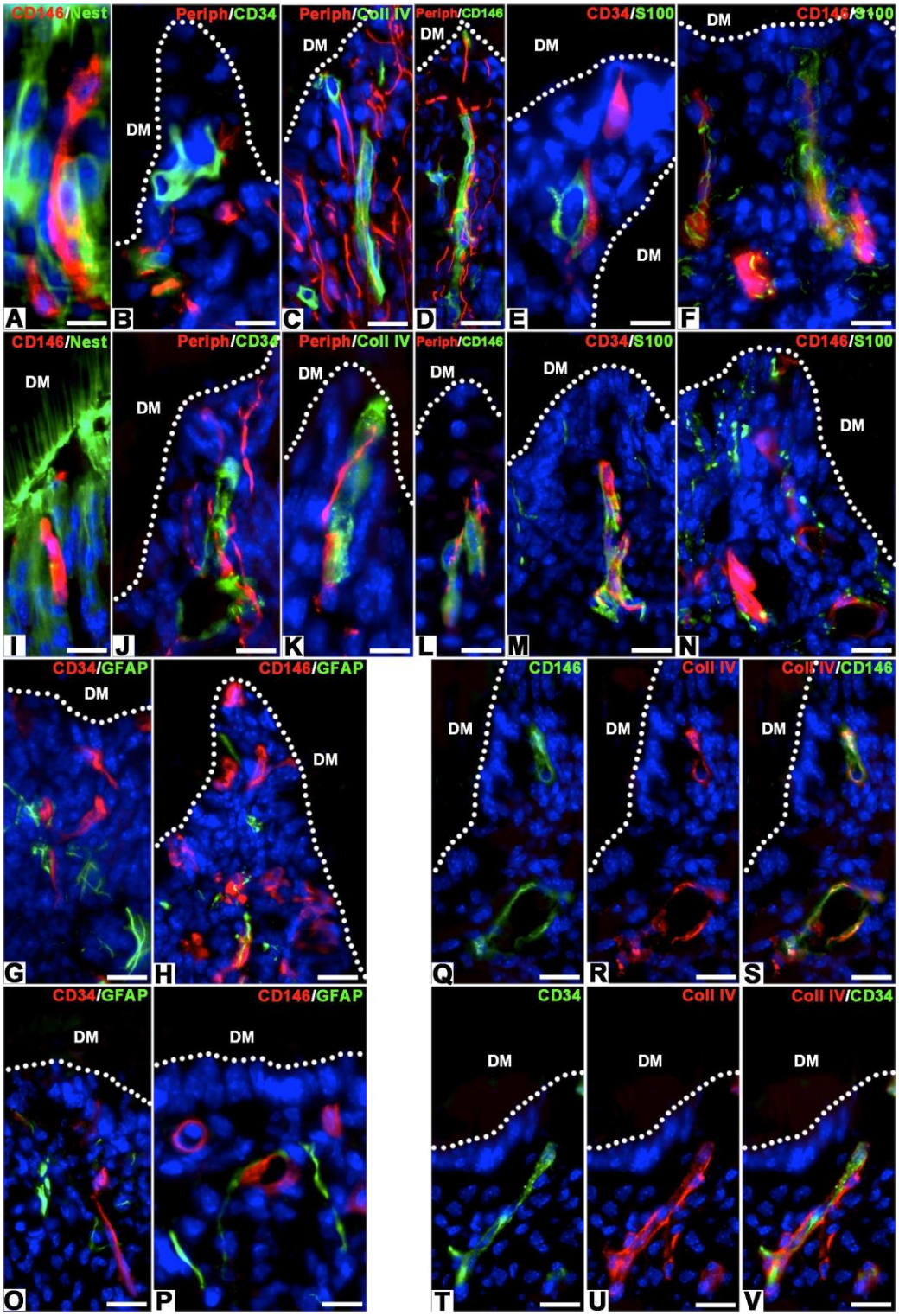

Figure 11: Neurovascular relationships in the odontoblast layer of cell re-associations implanted for two weeks in Nude mice (**A-H** and **Q-V**), compared to first lower molar at PN7 (**I-P**). Odontoblasts were labeled with an anti-nestin antibody (**A, I**). were either labeled for CD146 (**A, D, F, H, I, L, N, P, Q, S**), CD34 (**B, E, G, J, M, O, T, V**) or collagen type IV (**C, K, R, S, U, V**). Axons were labeled with anti-peripherin antibody (**B-D, J-L**) and glial cells using either anti-S100β (**E, F, M, N**) or anti-GFAP antibodies (**G, H, O, P**). Blood vessels were present in the odontoblast layer in implanted cell re-associations (**A**), as in the first lower molar at PN7 (**I**). In both cases also, axons were detected in

the odontoblast layer and showed close proximity with blood vessels (compare **B-D** with **J-L**). CD146 (**Q**) and collagen type IV (**R**) were detected in the same blood vessels (**S**) and the same was observed when comparing the localization of CD34 and collagen type IV (**T-V**). In the odontoblast layer of implanted cell re-associations, as in molar at PN7, glial cells were detected after staining for S100β (compare **E, F** with **M, N**) or for GFAP (compare **G, H** with **O, P**). In both cases, a certain proportion of glial cells were detected next to blood vessels positive with CD34 (compare **E, M** with **G, O**) or CD146 (compare **F, N** with **H, P**). DM: dental matrix. Scale bars=20 µm. (augmented from Kokten et al., 2014)

However, there was only a partial co-distribution of cells positive for S100β and those positive for GFAP. The number of cells positive for S100β was higher than GFAP-positive ones. More GFAP-positive than S100β-positive cells were associated with axons. These observations from implanted re-associations as well as from molars at PN7, confirm that there exist several populations of glial cells.

NRP1/ Semaphorin 3A

According to the literature, Semaphorin 3A (Sema3A) and its receptors (NRP1, NRP2) are involved in the inhibition of axon growth in the dental mesenchyme. A study was designed to determine the effect of Sema3A on the innervation of bioengineered teeth (Kuchler-Bopp et al., 2018). For this purpose, three complementary functional approaches were tested.

- A first step was the analysis of the innervation of the dental pulp of molars from newborn (PN0) control mice (Sema3A+/+) and mice deficient for Sema3A (Sema3A−/−). Axons were detected by immunostaining for peripherin and neurofilament-200 (NF200). Although restricted to the peridental mesenchyme in Sema3A+/+ mice at PN0, axons entered the dental pulp in Sema3A−/− mice.

- Then, ED14 tooth germs from either Sema3A−/− or Sema3A+/+ mice were cultured together with trigeminal ganglia and implanted for two weeks in adult ICR mice. The dental pulps of tooth germs from Sema3A−/− mice were innervated, but not those originating from Sema3A+/+ mice.

- A “Membrane Targeting Peptide NRP1” (MTP-NRP1) suppressing the inhibitory effect of Sema3A, had previously been identified (Roth et al., 2008). The injection of this peptide at the site of implantation of cultured cell re-associations allowed their innervation. Depending from the cellular context (peridental or dental), NRPs can be important for regulating immune response and inhibiting it (Kuchler-Bopp et al., 2018). Regarding the heterogeneity

of glial cells populations, the results were the same when implanting re-associations from Sema3A-/- mice in ICR mice or from Sema3A+/+ in Nude mice (Kokten et al., 2014).

Altogether, these data showed that the inhibition of a single axon repellent molecule, Sema3A, allows for the innervation of the dental mesenchyme in bioengineered teeth (Kuchler-Bopp et al., 2018).

Conclusions and open questions

Given the very slow development of a tooth in human, the study and results summarized here did not aim to address clinical aspects. The goal was to find out protocols and experimental conditions to reproduce the physiological steps of odontogenesis, hoping that clinicians could take advantage of it for regenerative medicine.

Along this work, the main results showed that:

From ED14 to ED18, mesenchymal cells progressively lose their competence to engage in odontogenesis. The competence of ED14 mesenchymal cells is also lost after a pre-culture *in vitro*.

After implantation of cultured cell-cell re-associations, the vascularization can be restored. Endothelial cells and pericytes originate from the host.

After implantation, odontoblasts and ameloblasts engage in functional differentiation. They secrete dentin and enamel constituents. The two mineralized matrices show the same characteristics as *ex vivo*. In the root portion, cementoblasts become functional as well.

The restoration of the innervation can be achieved in the dental mesenchyme, but requires immuno-suppression. In the peridental mesenchyme, this is not necessary, illustrating a differential regulation of the innervation.

Open questions

The development of these experimental approaches allowed checking previous descriptive data about cellular events, which take place during odontogenesis. It also raised new questions.

These include the search for easily accessible competent cells. Despite their small amount, embryonic dental cells still remain the only available cell source. Other organs/models might help to understand this limitation.

The innervation of re-associations was the most complex point to be achieved. The design of a simplified protocol would first require a better identification and understanding of the limiting parameters. It might help to come closer to the physiological situation and better understand the differential regulation of the innervation in the dental/peridental mesenchymes.

All along this study, a reproducible observation was very intriguing. In implanted cell-cell re-associations, tooth formation was accompanied by that of bone and a interposed PDL. In a

physiological context, mesenchymal cells involved in all three tissues/organ have two distinct origins (recalled in Svandova et al., 2020). Even if a contamination of the dental/follicular mesenchymes dissected at ED14 by cells from the prospective PDL cannot be excluded, all cell types have been mixed after trypsin dissociation. It is difficult then to imagine how mesenchymal cells might segregate according to their origins when all positional information has been lost. Implantation in GFP mice would have shown a possible involvement of host cells in these mesenchymal compartments. When combined with multiple double immunostainings, it should then be possible to characterize the cell types possibly originating from the host.

The cellular heterogeneity varies with time and also within regions of the mesenchyme. Cell-matrix and cell-cell interactions are permanently readjusted during tooth development (Zvackova et al., 2017; Svandova et al., 2020). In opposition to obvious epithelial histogenesis, the situation in the mesenchyme is much more discrete. Although remaining unknown, distinct functional networks must exist within the dental and peridental mesenchymes (Keller et al., 2012). Most of it could be reproduced in the experimental approaches described here.

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