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Experimental Design for the Innervation of Tooth Forming from Implanted Cell Re-associations

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1. Introduction

Tooth engineering may refer to either dental tissue regeneration or to the reformation of a complete organ attached to surrounding bone [1–4]. In case of whole tooth organ engineering the use of embryonic dental cells allowed to search for the simplest experimental protocol to be developed in order to maintain and express odontogenic cell potentialities. This was achieved after implantation under the skin or in the kidney capsule or in the jaw of adult mice of cultured re-associations between pellets of dissociated single epithelial and mesenchymal cells [5–7]. The main points that actually need further research concern the identification of non dental cells able to engage in whole tooth formation [8–12], anchoring of the newly formed tooth root to surrounding alveolar bone [3,9,13], and innervation of the engineered organ [14].

The innervation of dental and peridental tissues has been extensively investigated during tooth development in the mouse [15–19]. The timing and pattern of tooth innervation are controlled by diffusible signaling molecules and ultimately by epithelial-mesenchymal interactions [15,20]. Tooth innervation is particularly important in signaling damage in the crown, but also may interfere with several functions including the regulation of blood flow, tissue homeostasis, immune cell function, inflammation, and healing [21,22]. The innervation of engineered tooth germs does not occur spontaneously after implantation, but requires immunodepressed conditions: cyclosporin A (CsA) treatment of the host mice or implantation under the skin of immunocompromised Nude mice [23]. New questions arising from this previous work are discussed in this chapter. They concern a) the nature of relationships between axons and other cells in the dental pulp (odontoblasts, glial cells and blood vessels: endothelial cells and pericytes) in the case of cell re-associations implanted in Nude mice, b) the differential innervation of the dental and peridental mesenchymes, and c) experimental attempts to avoid

the use of CsA or related molecules to achieve dental pulp innervation [23]. For this purpose, specific cell re-associations were designed and analyzed, after *in vitro* culture and further implantation in adult mice, by means of histology, transmission electron microscopy and immunofluorescence. After two weeks of implantation, the cell re-associations reached a stage corresponding to a first lower molar at post-natal day 4 (PN4), when taking into account the crown development and stage of matrix deposition and mineralization [24,25]. However, axons reached the odontoblast layer, which in physiological conditions, occurs at PN7 only [23]. For this reason, the status of innervation in cell re-associations implanted for two weeks was compared to the situation in mouse first lower molar at PN7.

2. Cellular network mediating sensory function in the dental mesenchyme

Interactions between axons and other cells have been investigated in the human dental pulp [26]. The situation in tooth forming from implanted cell re-associations is not known. To analyze it, re-associations between dissociated dental embryonic epithelial and mesenchymal cells were cultured for seven days, then trigeminal ganglia were put in contact with the re-associations and cultured overnight before implantation in Nude mice (for detailed method and scheme of the protocol, see [23]). This protocol was selected because it allows the development of a whole tooth organ (Figure 1A-E; [24]), in conditions where the cellular heterogeneity in the dental mesenchyme can be maintained as it is in physiological conditions [25]. The localization of axons and glial cells, their relationships, and also with neighbor cells will be discussed in three complementary contexts: 1) with odontoblasts (Figures 2 and 4), 2) with microvessels present in the odontoblast layer (Figure 5) and 3) with blood vessels in the dental and peridental mesenchymes (Figures 6 and 8). These observations will be compared to what exists in the mesenchyme of a molar tooth at a similar developmental stage (PN7).

2.1. Innervation in the odontoblast layer

After implantation for two weeks in Nude mice, cell differentiation was achieved in the crown part of the tooth (Figure 1A-C) as it occurs after implantation in ICR mice (Charles River CD-1, [24]) or during molar development (Figure 1F-H, J). In implanted cell re-associations as in molar at PN7, odontoblasts were functional and secreted predentin and dentin constituents in the crown (compare Figure 1C, E with Figure 1G, H) and root portions (compare Figure 1A, E with Figure 1J). The cells were elongated with a nucleus in basal position (Figure 1B, G, H). Odontoblast cell processes were present in the dentinal tubules (Figure 1B, C, G, H). These cell processes are involved in the secretion and permanent mecanosensing in the matrix (for review, see [27–29]). The innervation was analyzed using antibodies to peripherin and neurofilament-200 (NF200) [18,30]. After two weeks of implantation of cell re-associations in Nude mice, axons positive for peripherin (Figure 2B) reached the layer of odontoblasts, which are positive for nestin (Figure 2A-E) or vimentin (Figure 2F). The same was observed in the first molar at PN7 (Figure 2G-L). Similarly, antibodies to NF200 allowed the detection of axons in the dental mesenchyme and reaching the odontoblasts of teeth forming from implanted cell re-associations (Figure 2A, C) as in molars at PN7 (Figure 2I). NF200 is a marker for myelinated

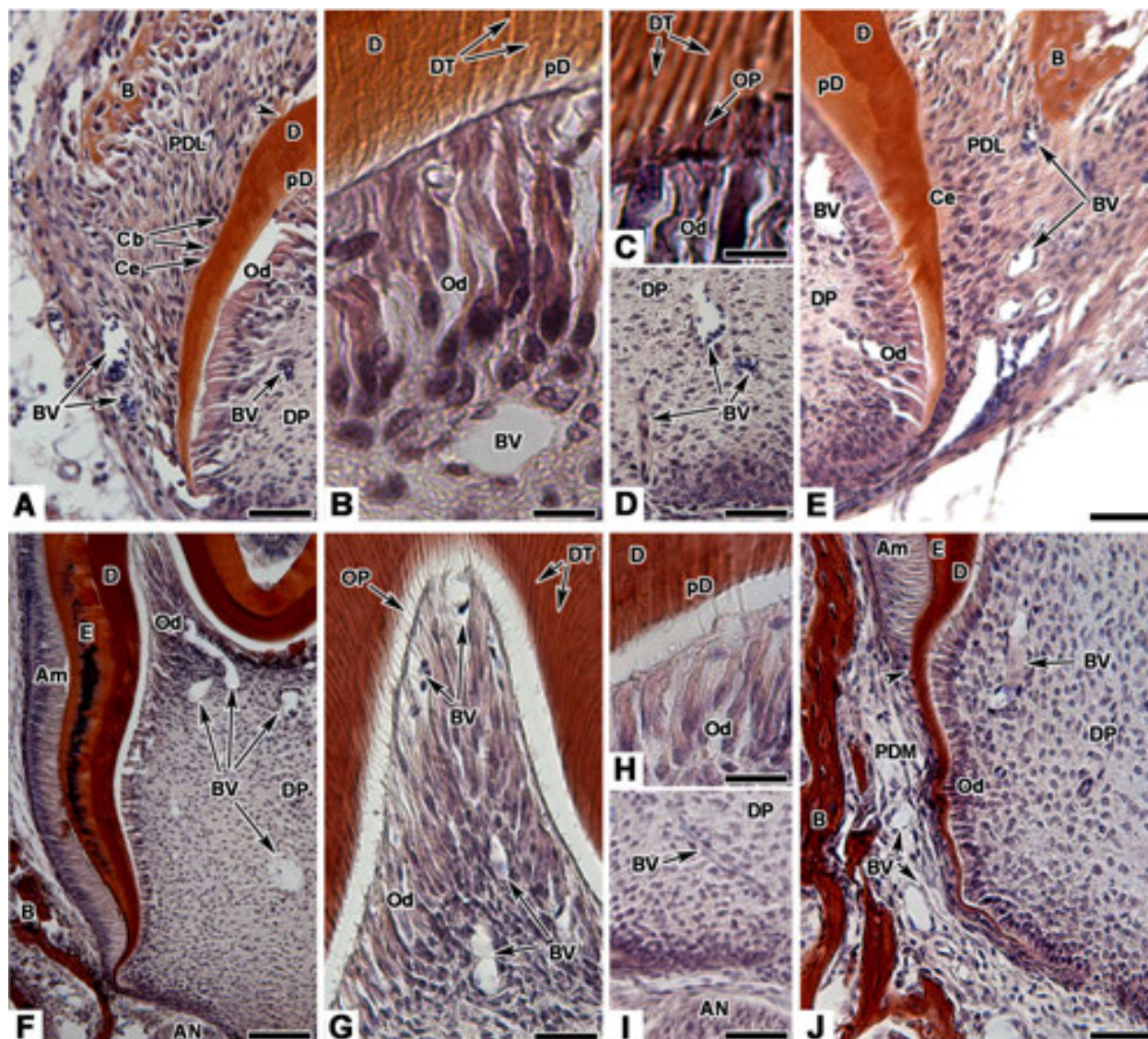


Figure 1. Histology of cultured cell re-associations implanted for two weeks under the skin of Nude mice (A-E) and of the first lower molar of ICR mice at post-natal day 7 (PN7) (F-J). The protocols for cell dissociation, re-association, culture, implantation and innervation have been detailed elsewhere [23]. All procedures were designed in compliance with the recommendations of the European Union (2010/63/EU) for the care and use of laboratory animals [23]. For histology, samples were fixed in Bouin-Hollande and demineralized in 15% EDTA before their inclusion in paraffin. Serial sections (7 μ m) were stained with Mallory's stain. After implantation of cell re-associations for two weeks under skin, crown was developed and root initiated (A, E), as in the developing molar at PN7 (F, J). Arrowheads showed the limit between crown and root in implanted cell re-associations (A), as in molar at PN7 (J). In implanted cell re-associations as in molars at PN7, blood vessels (BV) were present in the dental pulp (DP) (compare D, E with F, I, J) and reached the odontoblast (Od) layer (compare B with G). In both cases, odontoblasts were elongated and polarized, as seen from the position of their nucleus, opposite to the secretory pole (compare B with G, H). These cells were also functional, secreted predentin (pD) and dentin (A, B, E and H). Dentinal tubules (DT) and odontoblast cell processes (OP) were observed in the dentin of implanted cell re-associations as in molars at PN7 (compare B, C with G, H). In both types of samples, ameloblasts (Am) were elongated, polarized and secreted enamel (J, see [23]). Newly formed bone was present at the periphery of implanted cell re-associations (A, E) corresponding to alveolar bone in molar at PN7 (F, J). In contact with the external surface of root dentin, cementoblasts (Cb) were observed in implanted cell re-associations (A). These cells were functional and deposited cementum (Ce) (A, E). Periodontal ligament (PDL) fibers were attached to the root by cementum and extended until reaching newly formed bone (A, E). AN: Alveolar nerve; B: Bone; D: Dentin; E: Enamel. Scale bars=80 μ m (F); 40 μ m (A, D, E, F, G, I, J) and 20 μ m (B, C, H).

A-fibers and previous observations by transmission electron microscopy showed that such fibers could not be detected in implanted re-associations [23], probably due to the too short period of implantation. For this reason and despite the positive reaction to anti-NF200 antibodies, all further detections of axons were performed using antibodies to peripherin.

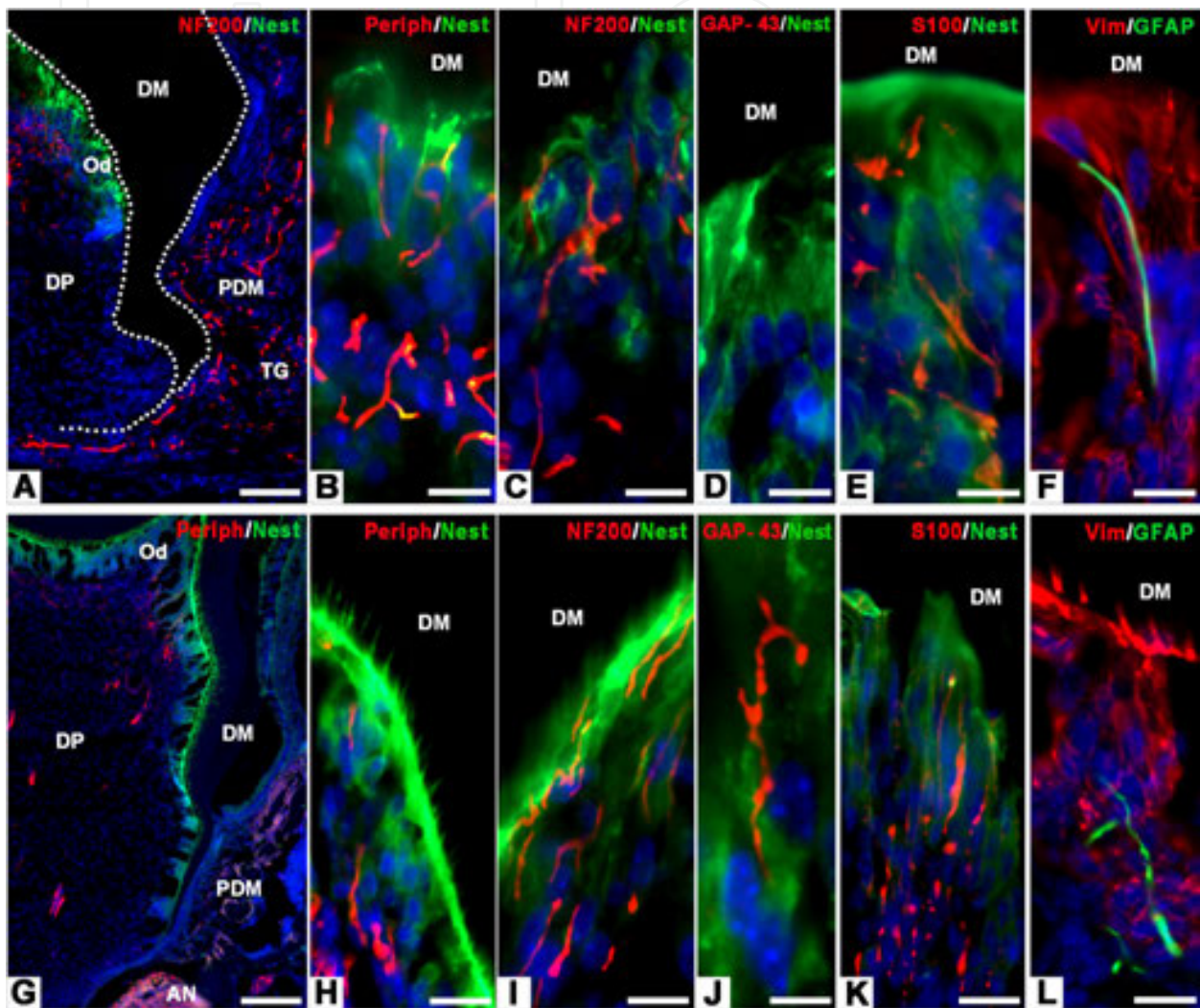


Figure 2. Innervation in the odontoblast layer of cell re-associations implanted for two weeks in Nude mice (A-F), compared to first lower molar at PN7 (G-L). Odontoblasts (Od) were labeled with anti-nestin (A-E and G-K) or anti-vimentin antibodies (F, L). Axons were labeled for peripherin (B, G, H) or NF200 (A, C, I) and glial cells using antibodies to either S100-beta (E, K) or GFAP (F, L). Growth cones were labeled for GAP-43 (D, J). In case of implanted re-associations, axons coming from the trigeminal ganglion (TG) entered the dental mesenchyme and reached the odontoblast layer, as seen after staining for NF200 (A). Similarly, the dental mesenchyme of first lower molar at PN7 was innervated by alveolar nerve (AN) as shown after staining for peripherin (G). Higher magnifications showed that axons positive for either peripherin or NF200 were present in the odontoblast layer, but did not enter the dentinal tubules, neither in implanted cell re-associations (B, C), nor in molars at PN7 (H, I). Growth cones were present in the odontoblast layer of molars at PN7 (J), but not in the case of implanted cell re-associations (D). S100-beta positive glial cells were detected in the odontoblast layer of implanted cell re-associations (E), as in intact molars at PN7 (K). The same was observed for GFAP positive glial cells (compare F with L). DM: dental matrix; DP: dental pulp; PDM: peridental mesenchyme. Scale bars=80 μm (A, G) and 20 μm (B-F and H-L).

Axons had not yet reached the odontoblast processes present in predentin/dentin, neither in cell re-associations implanted for two weeks (Figure 2A-C), nor in the molar at PN7 (Figure 2H, I). In the mouse first lower molar, the presence of axons in dentinal tubules was only detected at PN10, and still very few were observed there [23]. Nagahama et al., [31] reported that a subodontoblastic nerve plexus develops at about PN11 in the first molar, and dentinal innervation still continue to increase in the crown after tooth eruption (PN20). In the crown of mature human primary teeth, nerve endings terminate in or near the odontoblast layer, with a small number penetrating into dentin [32]. Odontoblasts and neighbor axons have been suggested to form a mechanosensory complex (for review see [29]). This raises the question of who is doing what when axons are also present in dentinal tubules [33]. Although Carda and Peydró [33] reported the existence of membrane densifications, similar to synapses in between odontoblast processes and nerve endings, there is a general agreement in the literature that true synapses do not exist in between the two cell types. Instead, appositions with a narrow extracellular gap have been reported [34,35]. Such apposition was also observed by transmission electron microscopy in implanted re-associations (Figure 3A, arrows in A') as well as in molars at PN7 (Figure 3E, arrows in E'). The question on how signals sensed by odontoblasts are transmitted to axons remains unsolved [29].

GAP-43 is an intracellular growth-associated protein. It was suggested to participate in neuronal pathfinding and branching during development and regeneration, by integrating plasma membrane and cytoskeletal responses to extracellular signals [36]. Staining for GAP-43 allows the visualization of growth cone, at the tip of axons [37,38]. More surprisingly, GAP-43 is also expressed by Schwann cells in rat molars [39]. *In vitro* and *in vivo* observations showed that, when related to Schwann cells, GAP-43 was expressed by their precursors and by non-myelinating mature cells [40]. Double stainings for GAP-43 and nestin did not show the presence of GAP-43 in the odontoblast layer in implanted re-associations (Figure 2D), while it was present there in the first molar at PN7 (Figure 2J). GAP-43, in the re-associations, was only detected in the peridental mesenchyme as discussed below. The reasons for this absence of GAP-43 in most parts of the dental mesenchyme from implanted cell re-associations will have to be further investigated. It might have important consequences on later stages of tooth innervation [41]. It will thus be necessary to go further by looking at the situation when cultured cell re-associations are implanted in CsA-treated mice. Indeed, CsA can interfere with GAP-43 expression [42].

Different glial cell types have been observed in the dental and peridental mesenchymes in rodents as in human [43,44]. Glial cells have multiple functions during the development of the peripheral nervous system (PNS) and in repair process [45]. During early PNS development, axonal signals are critical for Schwann cell migration, survival and proliferation [46,47]. In the present study, glial cells were searched for, using antibodies to either S100-beta or glial fibrillary acidic protein (GFAP) (for review, see [46]). In the implanted re-associations, S100-beta and GFAP were detected in the odontoblast layer (Figure 4A, B), as in the intact molar at PN7 (Figure 4F, G). The two antigens showed associations with axons, as seen after double-stainings using anti-peripherin antibodies, in the re-associations (Figure 4A, B) as in the molar at PN7 (Figure 4F, G). Furthermore, S100-beta and GFAP positive cells showed only partial

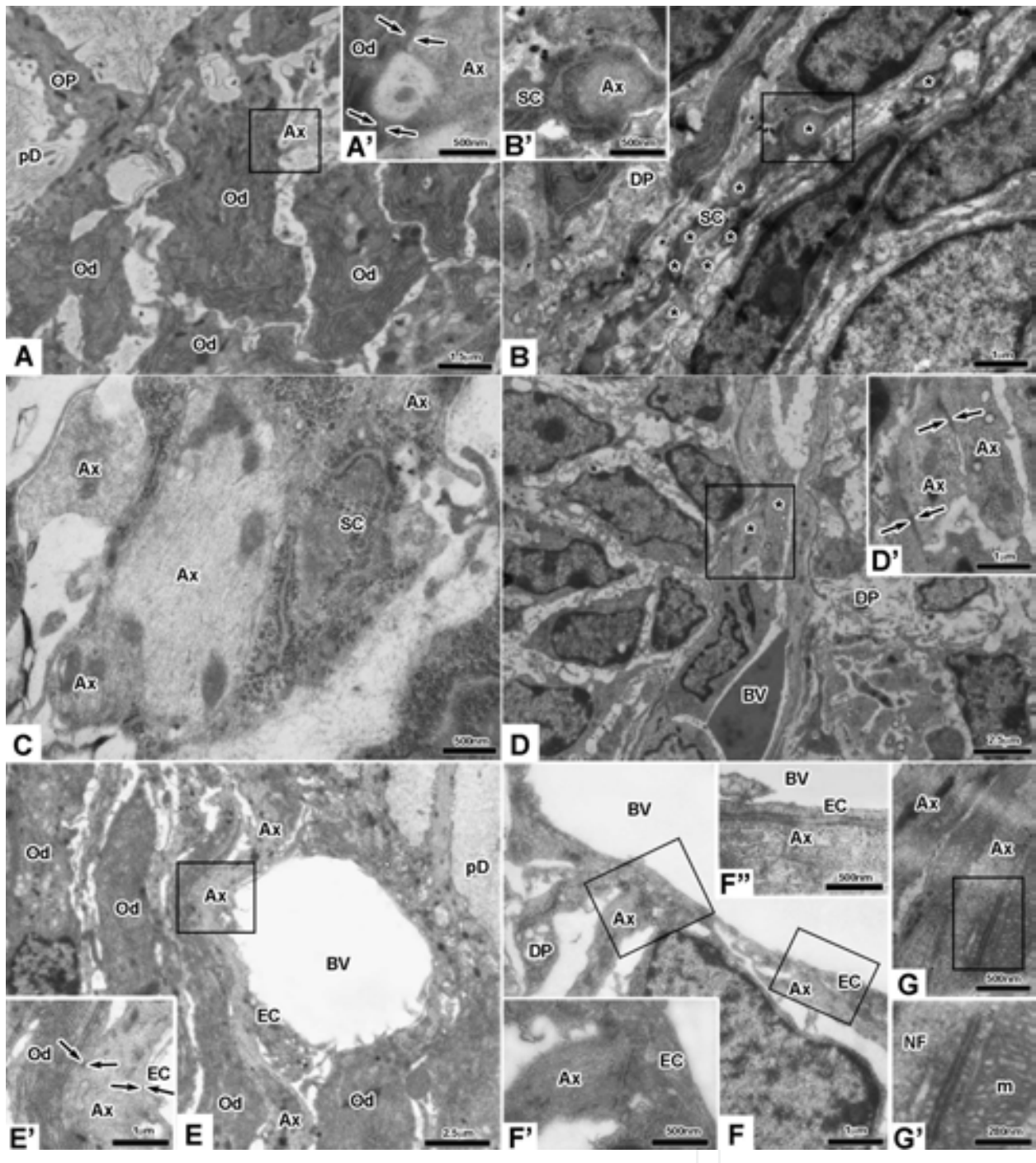


Figure 3. Transmission electron microscopy of cell re-associations implanted for two weeks in Nude mice (A-D') and first lower molar at PN7 (E-G'). Unmyelinated axons (Ax) were present in the odontoblast layer of implanted cell re-associations (A, A'). Arrows in A' showed contacts between an axon and an odontoblast (Od). In the dental pulp (DP) of teeth forming from re-associations, Schwann cells (SC) in contact with or wrapping around axons were seen (B, B', C). In the apical part of the pulp (D, D') unmyelinated axons were present in proximity of blood vessel (BV) and arrows in D' showed contacts between axons. In the odontoblast layer of molar at PN7, an axon showed contact with an endothelial cell (EC) and with an odontoblast (E, arrows in E'). In the dental pulp, axons were in contact with endothelial cells (F, F', F''). Also, a contact between two axons was visualized in molar at PN7 (G, G'). Ax and *: unmyelinated axon; m: mitochondria; NF: neurofilament; OP: odontoblast process; pD: predentin.

co-distribution in the re-associations (arrowheads in Figure 4C-E), as in the molar at PN7 (arrowheads in Figure 4H-J). More GFAP-positive than S100-beta-positive cells were associated with axons. More cells were positive for S100-beta than for GFAP in implanted cell re-associations (Figure 4E) as in intact molars (Figure 4J). These complementary observations indicate that several populations of glial cells are present in implanted cell re-associations, as in a developing molar, in physiological conditions.

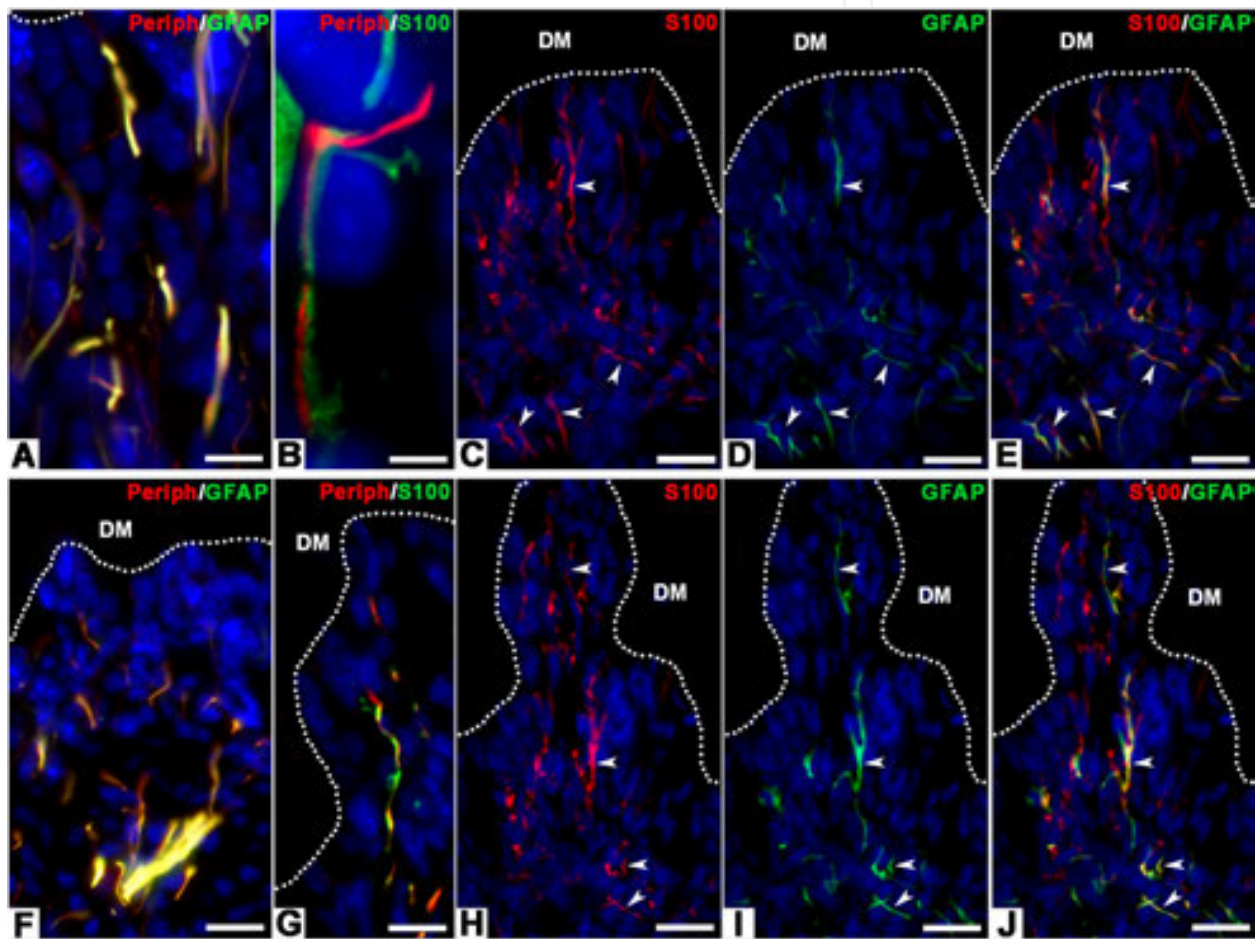


Figure 4. Glial cells in the odontoblast layer of cell re-associations implanted for two weeks in Nude mice (A-E), compared to first lower molars at PN7 (F-J). Axons were visualized with anti-peripherin antibody (A-B and F-G) and glial cells using antibodies to either S100-beta (B, C, E and G, H, J) or GFAP (A, D, E and F, I, J). In implanted cell re-associations as well as in molar at PN7, axons and glial cells were detected in the odontoblast layer (compare A, B with F, G). In the odontoblast layer of implanted cell re-associations, GFAP positive glial cells were associated with axons (A), as in the first lower molar at PN7 (F). The same results were observed for S100-beta positive glial cells (compare B with G). Double stainings for S100-beta and GFAP showed the presence of distinct glial cell types (C-E) as in intact molars at PN7 (H-J). More cells were positive for S100-beta than for GFAP and the two antigens showed only partial co-distribution in implanted cell re-associations (arrowheads in C-E), as in molar at PN7 (arrowheads in H-J). DM: dental matrix. Scale bars=20 μ m (A, C-J) and 8 μ m (B).

2.2. Blood vessels-axons relationships in the odontoblast layer

Microvessels are present in the odontoblast layer in re-associations implanted for two weeks in either Nude mice (Figure 5; [23]), or in ICR mice [48]. These microvessels in the odontoblast layer were detected after staining for CD34, collagen type IV or CD146 in implanted cell re-associations (Figure 5A-H) as in molars at PN7 (Figure 5I-P). All three antigens were found to co-distribute, as shown after double stainings for CD146 and collagen type IV (Figure 5Q-S) or for CD34 and collagen type IV (Figure 5T-V). The same was observed in molars at PN7 (data not shown). CD146, a marker of endothelial cells and pericytes, was detected in microvessels from the odontoblast layer in implanted re-associations (Figure 5A, D, F, H), as in molars at PN7 (Figure 5I, L, N, P). However, staining for α -SMA, a marker of smooth muscle cells, was detected in pericytes associated with large vessels in the apical part of the dental mesenchyme [25], but not in the odontoblast layer, neither in implanted cell re-associations nor in molars at PN7 (Figure 6G, K).

The timing of capillaries development and their entering the odontoblast layer (Figure 3E, E') has been correlated with the mineral requirement for dentinogenesis [49]. The relative roles of odontoblasts and capillaries present in this cell layer in the transport of calcium during dentin mineralization has been discussed previously [50]. In the odontoblast layer, double stainings for peripherin and either CD34, or collagen IV, or CD146 showed that very frequently axons were detected in close proximity with capillaries (Figure 5B-D). Such neurovascular relationships were also documented in molars at PN7 (Figure 5J-L). Transmission electron microscopy confirmed these observations and further showed that there exist direct interactions (Figure 3E, E'). Tabata et al., [51] suggested that the innervation of capillaries might be involved in the regulation of blood flow as in larger vessels. Although most pulp axons are sensory ones, both sensory and sympathetic axons can make contacts with dental pulp vessels [34]. Sympathetic axons have been detected in the odontoblast layer, although not extending in dentinal tubules [52].

Double stainings were performed to compare the distribution of glial cells as visualized after staining for S100-beta or GFAP, with that of microvessels visualized after staining for CD34 or CD146 (Figure 5). In implanted re-associations, S100-beta positive cells showed more proximity with microvessels (Figure 5E, F) than GFAP positive glial cells (Figure 5G, H). The same was observed in molars at PN7 (compare Figure 5M, N with Figure 5O, P). This confirms the differential patterning of S100-beta versus GFAP-positive glial cells as discussed above. Furthermore, the stainings for S100-beta in the odontoblast layer of implanted re-associations or molars at PN7 (Figure 5F, M) are in good agreement with observations made in the dental pulp of adult human tooth where the S100-beta positive cells were ensheating blood vessels [26]. Due to the angle of sectioning, several layers of nuclei corresponded to functional odontoblasts at the tip of the cusps (Figure 1G). Typical Schwann cells were not observed by transmission electron microscopy in the odontoblast layer of implanted re-associations or molars at PN7. This fits with previous report showing that in the mouse molar, Schwann cells were present near the base of the odontoblast layer from PN9 and were not detected in the odontoblast layer before PN50-60 [53].

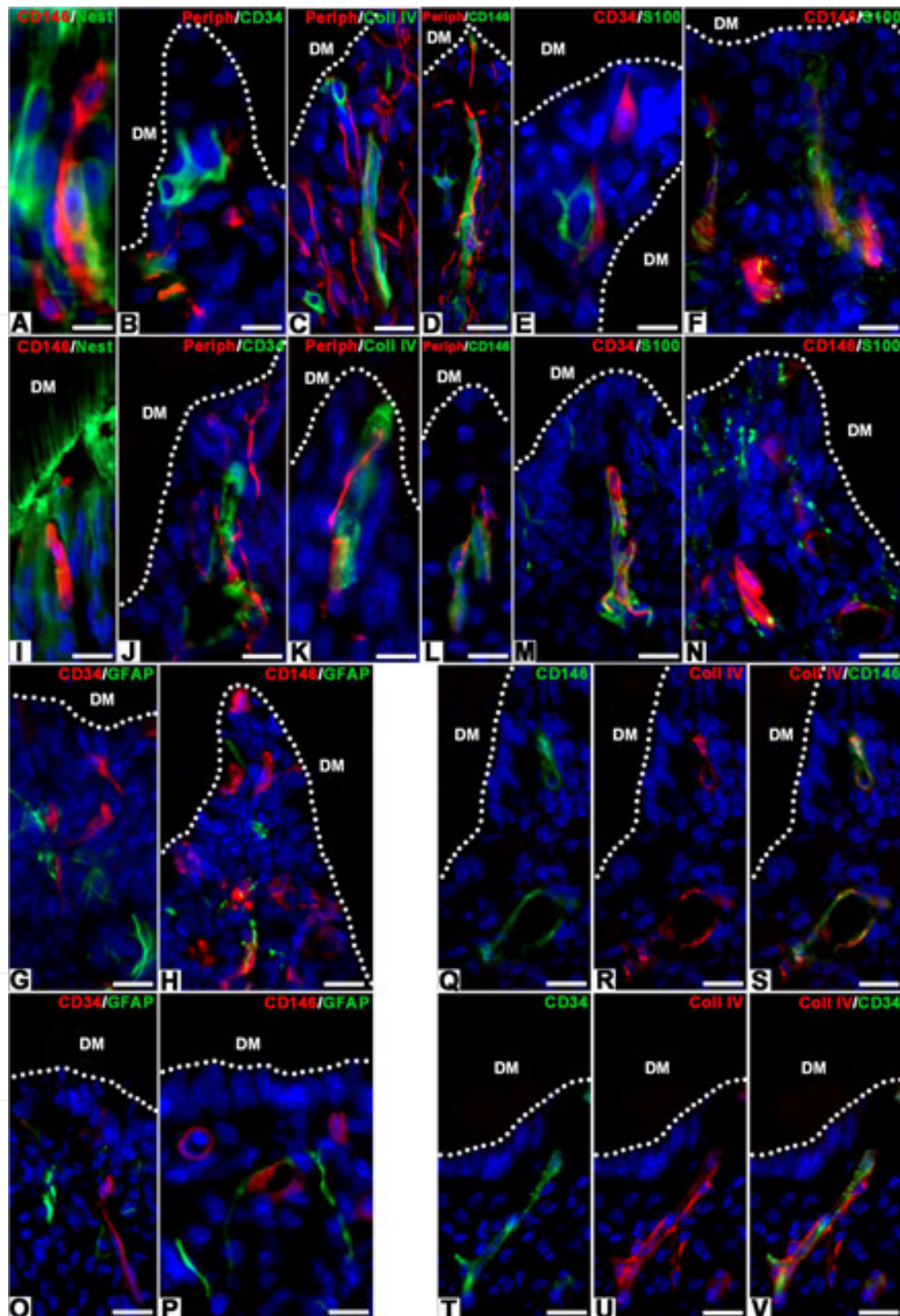


Figure 5. Neurovascular relationships in the odontoblast layer of cell re-associations implanted for two weeks in Nude mice (A-H and Q-V), compared to first lower molar at PN7 (I-P). Odontoblasts were labeled with an anti-nestin antibody (A, I). Blood vessels were either labeled for CD146 (A, D, F, H, I, L, N, P, Q, S), CD34 (B, E, G, J, M, O, T, V),

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-S100-beta (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 S100-beta (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 .

2.3. Blood vessels-axons relationships in the central and apical parts of the dental mesenchyme

The progressive vascularization of the dental mesenchyme has been studied during mouse molar development [48,54] as well as in implanted cell re-associations [25,48]. The vascularization in implanted intact molars or cell re-associations showed similar density but was slightly retarded in re-associations. The implantation of cell re-associations in GFP mice showed that all blood vessels in the implant originated from the host [48].

In the developing molar, pericytes positive for α -SMA were detected only from PN4 in the dental mesenchyme. They were observed in association with large blood vessels at the apical part of the pulp both in the intact molar (Figure 1I; Figure 6K-N) as in cell re-associations implanted for two weeks (Figure 1D; Figure 6G-J; [25]). Pericytes are involved in the development, maturation, stabilization, and remodeling of capillaries and small vessels [55–57]. These cells are also involved in the regulation of capillary blood flow [58]. Still pericytes were suggested to possibly act as mesenchymal stem cells (MSCs) [59–61]. A study performed with mouse incisor as a model showed that pericytes, together with other MSCs-like cells, could participate in tooth growth and repair, giving rise to odontoblasts and odontoblast-like cells [62].

Transmission electron microscopy and double immunostainings for peripherin and CD146 showed close proximity between axons and blood vessels in the central part of the dental pulp of implanted cell re-associations (Figure 3D; Figure 6A), as in intact molars (Figure 3F, F', F''; Figure 6D). Such relationships have also been reported in the case of rat and human teeth by scanning electron microscopy, which showed a dense network of interactions between axons and vessels [63,64]. Transmission electron microscopy also showed contacts between axons in implanted cell re-associations (Figure 3D') as in molar at PN7 (Figure 3G, G'). Blood vessels in the dental pulp are innervated by sensory and sympathetic nerve fibers [17,21]. Both sensory and sympathetic axons would be positive for peripherin. However during development, sensory nerves enter the dental mesenchyme at PN3-4, while sympathetic nerves penetrate this tissue much later, at PN9 [17]. Sympathetic axons are mainly detected in the deeper pulp along the blood vessels [65]. They are involved in vasoregulation [22,66]. Due to their late entering in the dental mesenchyme in physiological conditions, sympathetic nerves should be absent from the re-associations implanted for two weeks.

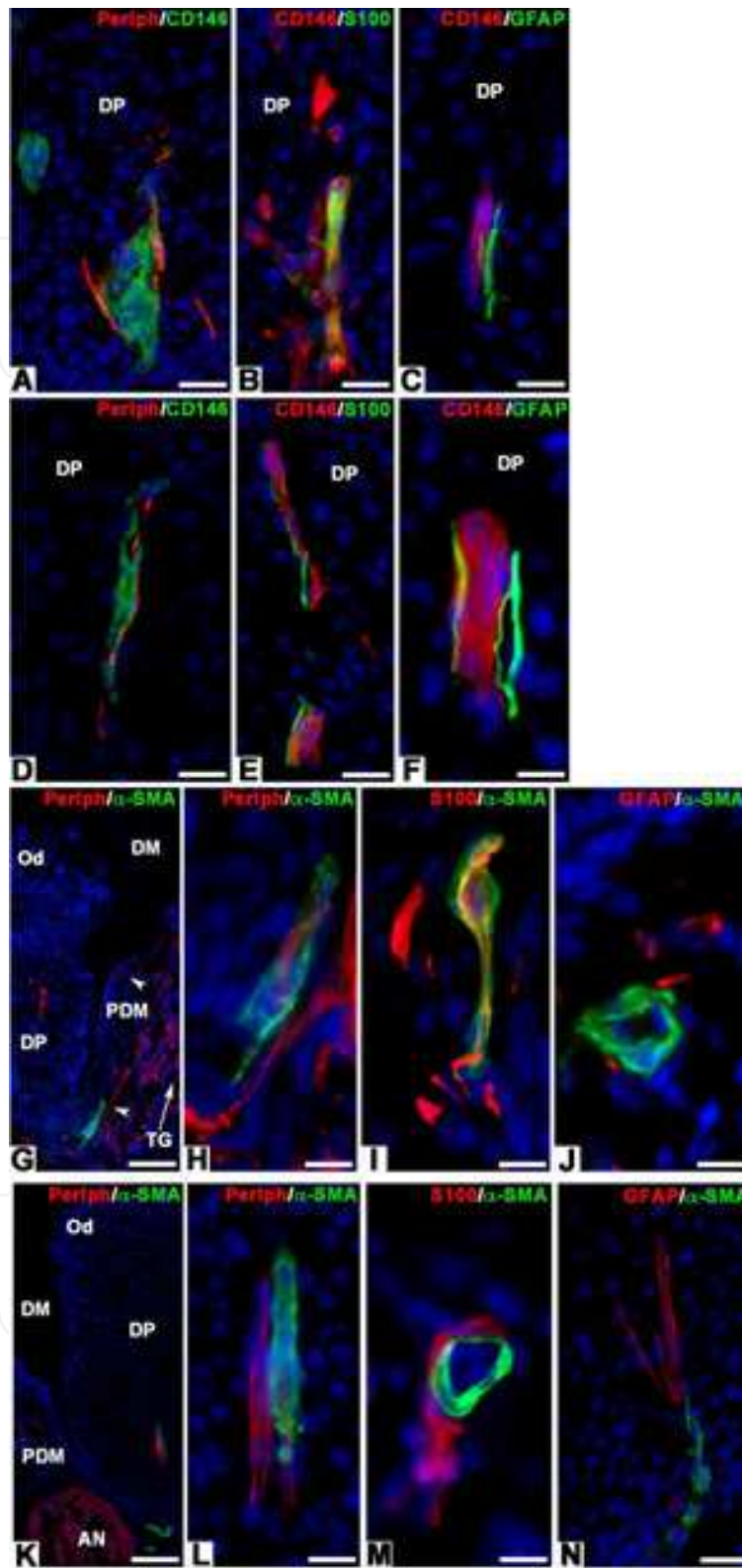


Figure 6. Neurovascular relationships in the central (A-F) and apical parts (G-N) of the dental mesenchyme of cell re-associations implanted for two weeks in Nude mice (A-C, G-J), compared to first lower molar at PN7 (D-F, K-N). Blood vessels were labeled with an anti-CD146 antibody (A-F) in the central part of the dental mesenchyme and with an anti- α -SMA antibody in its apical part (G-N). Axons were detected with an anti-peripherin antibody (A, D, G, H, K, L) and

glial cells using antibodies to either S100-beta (B, E, I, M) or GFAP (C, F, J, N). In implanted re-associations, axons originating from the trigeminal ganglion (TG) extended in the peridental mesenchyme (arrowheads) and dental pulp (G). In the central part of the dental mesenchyme, close relationships were observed between axons and blood vessels in implanted cell re-associations (A) as in the first lower molar at PN7 (D). In both types of samples, more vicinity was observed between blood vessels and S100-beta positive cells than with GFAP positive glial cells (compare B, E with C, F). In implanted cell re-associations pericytes positive for α -SMA surrounded large blood vessels and were detected only in the apical part of the dental mesenchyme (K). In both cases, axons were also detected in close proximity with α -SMA positive blood vessels (H, L). In implanted cell re-associations as in molars at PN7, some S100-beta positive (compare I with M), and GFAP positive glial cells (compare J with N) were detected next to pericytes. However, S100-beta positive cells showed more proximity with pericytes than GFAP positive ones (compare I, M with J, N). AN: alveolar nerve; DM: dental matrix; DP: dental pulp; Od: odontoblast; PDM: peridental mesenchyme. Scale bars=80 μ m (K); 40 μ m (G) and 20 μ m (A-F, H-J, L-N).

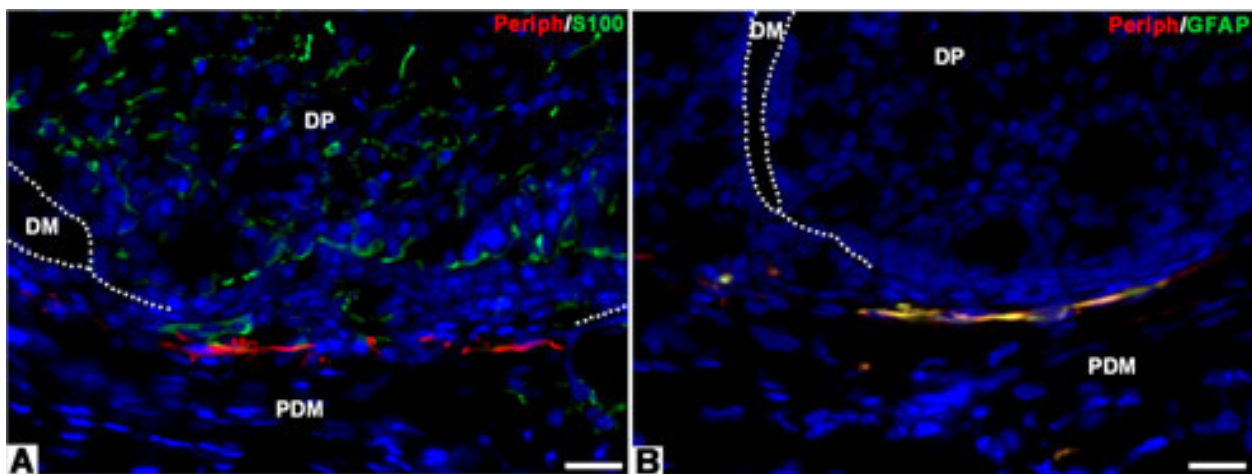


Figure 7. Innervation of cell re-associations implanted for two weeks in ICR mice without cyclosporine A treatment (A, B). Axons were labeled with anti-peripherin antibody (A, B) and glial cells using antibodies to either S100-beta (A) or GFAP (B). In such experimental conditions, axons remained at the border between the peridental mesenchyme (PDM) and dental pulp (DP) but did not enter the dental pulp (A, B). Similarly, GFAP positive glial cells co-localized with axons, at the limit between the peridental mesenchyme and dental pulp of the forming tooth (B). S100-beta positive glial cells showed a broader pattern, being present in close proximity with axons in the peridental mesenchyme, as well in the dental pulp (A). DM: dental matrix. Scale bars=20 μ m.

Neurovascular bundles present in the dental mesenchyme have been suggested to represent a niche for stem cells [59,67]. Immunostainings performed in the human dental pulp showed that STRO-1 positive cells were present in the walls of blood vessels and perineurium surrounding the nerve bundles, while absent in the layer of mature odontoblasts [59]. These authors suggested that the potentialities of this minor stem cell population should be tested to search for a possible ability to differentiate into functional neuronal-like cells. Recently, this was supported by cultures of murine dental pulp stem cells (DPSC) under neuroinductive conditions [68]. Although STRO-1 cannot be searched for in mouse tissues, the immunostaining for peripherin remained negative when cell re-associations (Figure 7A, B) or even intact molars at PN4 were implanted for two weeks in ICR mice in the absence of a trigeminal ganglion [14]. Thus, if dental pulp stem cells can differentiate into neuronal-like cells *in vitro*, this potential was neither expressed in implanted intact molars at PN4 [14] nor in teeth forming

after implantation of cultured cell re-associations [23]. Higuchi et al. [69] have reported the re-innervation of Embryonic Day (ED)18 rat molar germs after they were implanted under the skin of adult Wistar rats for eight weeks. Unfortunately, the early stages when the dental pulp first started to be re-innervated were not analyzed [69]. Nevertheless, these results suggest that, from a certain stage, immunodepressed conditions may no longer be necessary to allow dental pulp innervation.

When cell re-associations were implanted in Nude mice, both S100-beta and GFAP positive cells were detected in the dental pulp, which sometimes could be in close proximity with blood vessels (Figure 6B, C). The same was observed in molars at PN7 (Figure 6E, F). Both in implanted re-associations (Figure 6I, J) and in intact molars (Figure 6M, N), S100-beta positive cells showed more proximity with pericytes than GFAP positive cells. Transmission electron microscopy showed the presence of Schwann cells in the dental pulp of re-associations implanted in CsA-treated mice [23]. When cell re-associations were implanted in ICR mice without CsA treatment, the innervation of the dental mesenchyme was not possible: the dental mesenchyme remained negative for peripherin (Figure 7A, B; [23]). Nevertheless, glial cells expressing S100-beta (Figure 7A), but not GFAP (Figure 7B), were present in the dental mesenchyme. These results agree with previous observations showing that, in human pulp tissues, S100-beta and GFAP are expressed by different cell types, showing distinct patterns [26]. The origin of glial cells present in the dental pulp is not known. Implantation experiments will have to be performed in GFP mice to determine whether S100-beta positive cells have an endogenous origin, come from the host or have a mixed origin, as already observed for other dental pulp cells [25]. Glial cells in dental tissues from rodents appeared quite heterogeneous [43] and in the human dental pulp as well [26]. Schwann cells ensheating axons were observed in the dental pulp of cell reassociations (Figure 3B, B', C). Schwann cells are derived from neural crest cells, and were considered to consist in two types whether myelinating or not, both being involved in the maintenance of axons [70,71]. Differences were also found when comparing Schwann cells in the dental and peridental mesenchymes [43]. These cells show a remarkable plasticity during reparative processes, as being able to dedifferentiate and participate in re-innervation by directing axonal regrowth and re-myelination [45]. Recently, culture conditions were determined, which allowed human dental pulp stem cells to undergo Schwann-like cell differentiation and to support neural outgrowth *in vitro* [72].

3. Innervation of the periodontium

As a component of the periodontium, the periodontal ligament mediates the attachment of the tooth to surrounding alveolar bone (Figure 1J). The periodontal ligament fibers are the main constituent of the ligament. This tissue is also vascularized (Figure 1J), innervated (Figure 8G) and contains a dense network of mechanoreceptors [73,74]. The periodontal ligament develops in parallel with root formation [75]. During development, the vascularization of the dental sac (prospective peridental mesenchyme) largely precedes the vascularization of the dental mesenchyme [48]. Similarly, nerves come close to the condensing dental mesenchyme long before the periodontium differentiates and becomes innervated [15,76,77].

In the mesenchyme surrounding the forming tooth in implanted re-associations, blood vessels and axons were detected, but showed only a limited degree of associations (Figure 8A, B). The same was observed in molars at PN7 (Figure 8F, G). In implanted re-associations, S100-beta positive and also GFAP-positive cells were detected in association with axons (Figure 8D,E), as in molars at PN7 (Figure 8I, J). In implanted re-associations, there were co-localizations of peripherin and GAP-43 in the peridental mesenchyme, but not in the dental pulp (Figure 8C). However the situation was different in the molar at PN7, where such co-localizations were found in the peridental and dental mesenchymes (Figure 8H).

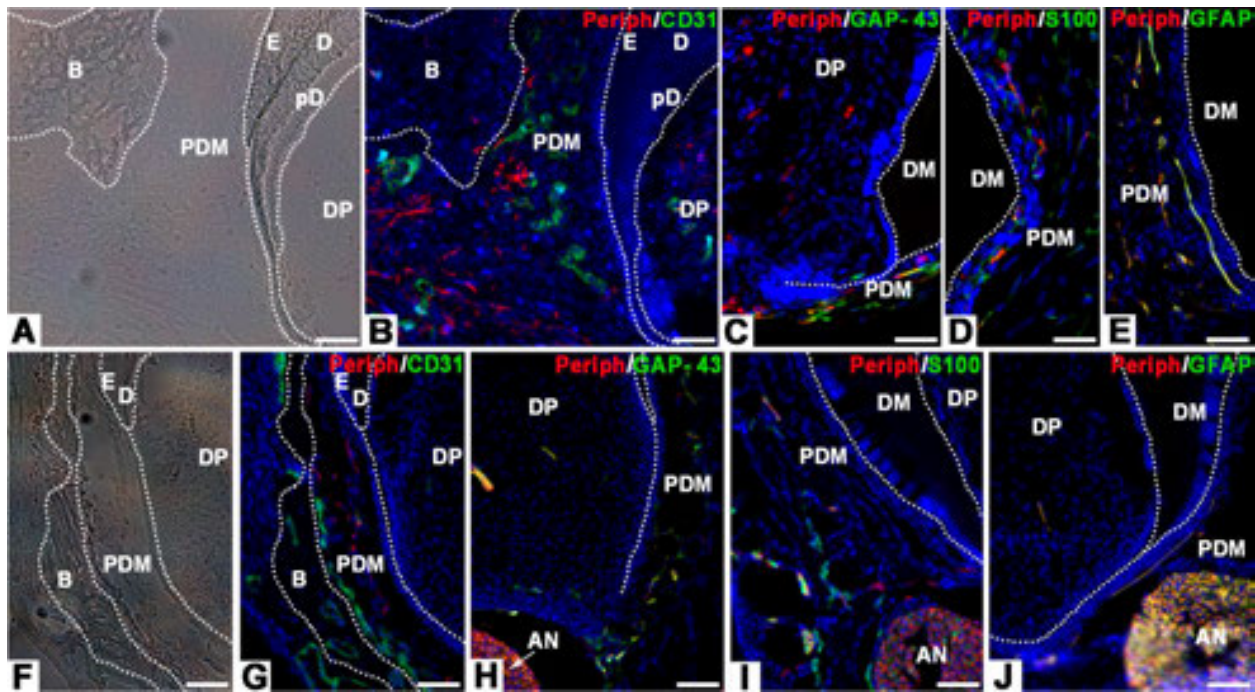


Figure 8. Innervation of the peridental mesenchyme of cell re-associations implanted for two weeks in Nude mice (A-E), compared to first lower molars at PN7 (F-J). Axons were visualized using an antibody to peripherin (B-E, G-J) and their growth cones with an anti-GAP-43 antibody (C, H). Glial cells were labeled using antibodies to either S100-beta (D, I) or GFAP (E, J). Blood vessels were labeled with an anti-CD31 antibody (B, G). Newly formed bone was detected at the periphery of implanted cell re-associations (A, B), analogous to the alveolar bone surrounding the molar at PN7 (F, G). In both cases, axons and blood vessels were observed in the peridental mesenchyme (PDM) between bone and dental tissues (compare B with G). Also, growth cones were detected, which co-distributed with axons in the peridental mesenchyme of implanted cell re-associations (C), as in intact molar at PN7 (H). GAP-43 and peripherin also co-distributed in the dental pulp of molar at PN7 (H). However, GAP-43 was not detected in the dental pulp (DP) of tooth forming from implanted cell re-associations (C). In the peridental mesenchyme of implanted cell re-associations as well as in molars at PN7, different glial cell types were detected after staining for S100-beta (compare D with I) or GFAP (compare E with J). In both cases, these different glial cell types were observed next to axons in the peridental mesenchyme (compare D, E with I, J). AN: alveolar nerve; B: bone; D: dentin; DM: dental matrix; E: enamel; pD: predentin. Scale bars=40 μm (A-C and E-J) and 20 μm (D).

Besides sensory axons, the periodontal ligament also contains sympathetic neurons, which are involved in bone remodeling [78,79]. However, this could not be investigated in the present

experimental design. Implantation of cell re-associations would have to be performed in the jaw, which still raises major difficulties to correctly position the implant, with prospective roots having a correct orientation in the jaw [48]. When cell re-associations had been cultured for eight days, the crown morphology of the forming teeth is well visible (Figure 10B). However, the shape of the material to be implanted is more or less spherical (Figure 10B). It is thus very difficult to avoid a rotation of the samples during their implantation [2], although in some instances, it could be very well achieved [9].

The engineering of tooth root and periodontium (Figure 1E) is now considered as the main point in tooth engineering [80]. Indeed, complementary approaches to address this question are in progress [81–83]. 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 [23]. This indicates that not only the kinetics but also the conditions allowing innervation (immunodepressed conditions) are different in the dental and peridental mesenchymes.

4. Attempts to replace cyclosporin A treatment by using stem cells

The entering of axons in the dental mesenchyme of cell re-associations implanted under the skin of adult mice requires an artificially created immunodepression: treatment of host mice with CsA or implantation in Nude mice [23]. This requirement is not a consequence of a possible change in the mesenchymal cell heterogeneity/behavior during tissue and cell dissociation steps prior to re-association, culture, and implantation. Indeed, the same was observed when implanting intact PN4 molars [14]. The importance of immunomodulation, as observed for the re-innervation in the clinical context of face transplantation [84], has also been reported in a reparative process [85]. The next question was: how to avoid the use of CsA, which actually represents a heavy constraint in view of clinical application [23]?

Stem cells have been suggested to facilitate pulp innervation, possibly by chemoattraction [86]. Before stem cells were taken into account, dental pulp cells were known to interfere with neuron survival and differentiation *in vitro*, which was correlated with their expressing a wide range of neurotrophic factors [87,88]. These included Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), and chemokine ligand (CXCL)12 [88]. Other studies led to propose a potential role of mesenchymal stem cells (MSCs) in immunomodulation, by exerting an immunosuppressive effect on cells from both innate and acquired immunity systems [89–91]. Dental and peridental MSCs also showed such properties [92–95]. Some of these MSCs might originate from pericytes [96], although other origins have also been suspected [62]. The number of pericytes as visualized using antibodies to α -SMA was very limited in re-associations implanted for two weeks (Figure 6G). MSCs from different origins have been shown to stimulate axonal sprouting and tissue innervation [97]. Bone marrow also contains mesenchymal stem cells (BM-MSCs) exhibiting immunomodulatory and regulatory properties [98,99]. BM-MSCs have been shown to stimulate neurite outgrowth, probably due to their expressing NGF and BDNF under specific

experimental conditions [100]. Since bone marrow cells can be easily prepared in large quantities, they were tested for their possible effect in stimulating dental pulp innervation, to possibly avoid the use of CsA.

For this purpose, bone marrow derived cells (BMDCs) [8] were used at passage two [101]. Re-associations between mixed BMDCs/dental mesenchymal cells and an intact dental epithelium from the cap stage at ED14 (Figure 9G) were cultured for eight days *in vitro* (Figure 9H, I) and analyzed for tissue organization and cell differentiation (Figure 10A, C). BMDCs were prepared from GFP mice (Figure 9D) in order to follow their fate in cultured re-associations (Figure 11). Histology of cultured re-associations demonstrated that teeth could develop *in vitro* when mixed BMDCs/mesenchymal cells at a ratio of 50% were re-associated with an ED14 competent dental epithelium and cultured for 8 days (Figure 10A-C), as in the absence of BMDCs (Figure 11F; [24]). Although initially mixed with dental mesenchymal cells, GFP-labeled BMDCs were only detected in the dental mesenchyme and at the periphery of the forming tooth after 6 days of culture (Figure 11A). After 8 days, all BMDCs were located at the periphery of the forming tooth (Figure 11B). This suggests that, in these experimental conditions, BMDCs do not directly participate in the late stages of tooth formation (i.e. odontoblast differentiation). This is further supported by histology showing rather small teeth forming, which can be related to the lower number of dental mesenchymal cells used for the re-associations, when mixed 50:50 with BMDCs (Figure 10D).

To investigate the possible re-innervation of such re-associations, a trigeminal ganglion (TG) was deposited on top of cells/tissue re-associations cultured for 7 days (Figure 9I), and maintained *in vitro* overnight prior to implantation under the skin of ICR mice (Figure 9J). Implants were maintained for two weeks in order to allow direct comparison with previous results obtained after implantation in CsA-treated mice or Nude mice [23]. Histology showed the presence of functional odontoblasts secreting predentin/dentin (Figure 10G) and thus inducing the differentiation of functional ameloblasts secreting enamel (Figure 10E, F). Double stainings for CD31 and peripherin showed the presence of blood vessels in the dental and periodontal mesenchymes (Figure 11C, D, 7:7 samples (100%)). Few axons only were detected in the dental mesenchyme (Figure 11E, 3:7 samples (42%)), while the control re-associations without BMDCs remained negative for peripherin (Figure 11F). However, in these preliminary experiments, axons did not reach the odontoblast layer (Figure 11E).

From the literature, several mechanisms have been proposed to try to explain how exogenous stem cells might stimulate innervation. They include cell replacement, neurotrophic support or immunomodulation and may vary with different target tissues. In the experimental conditions reported here, the possibility of differentiation towards neural cells can be rejected since, already before implantation, bone marrow cells no longer remained present in the dental mesenchyme, which developed during the *in vitro* culture of the re-associations (Figure 11B). Furthermore, the axons present in the dental mesenchyme remained GFP negative (Figure 11E). Nevertheless, these preliminary experiments will have to be completed a) by increasing their number, b) by further testing BMDCs at different passages, and c) by testing sub-populations of these BMDCs.

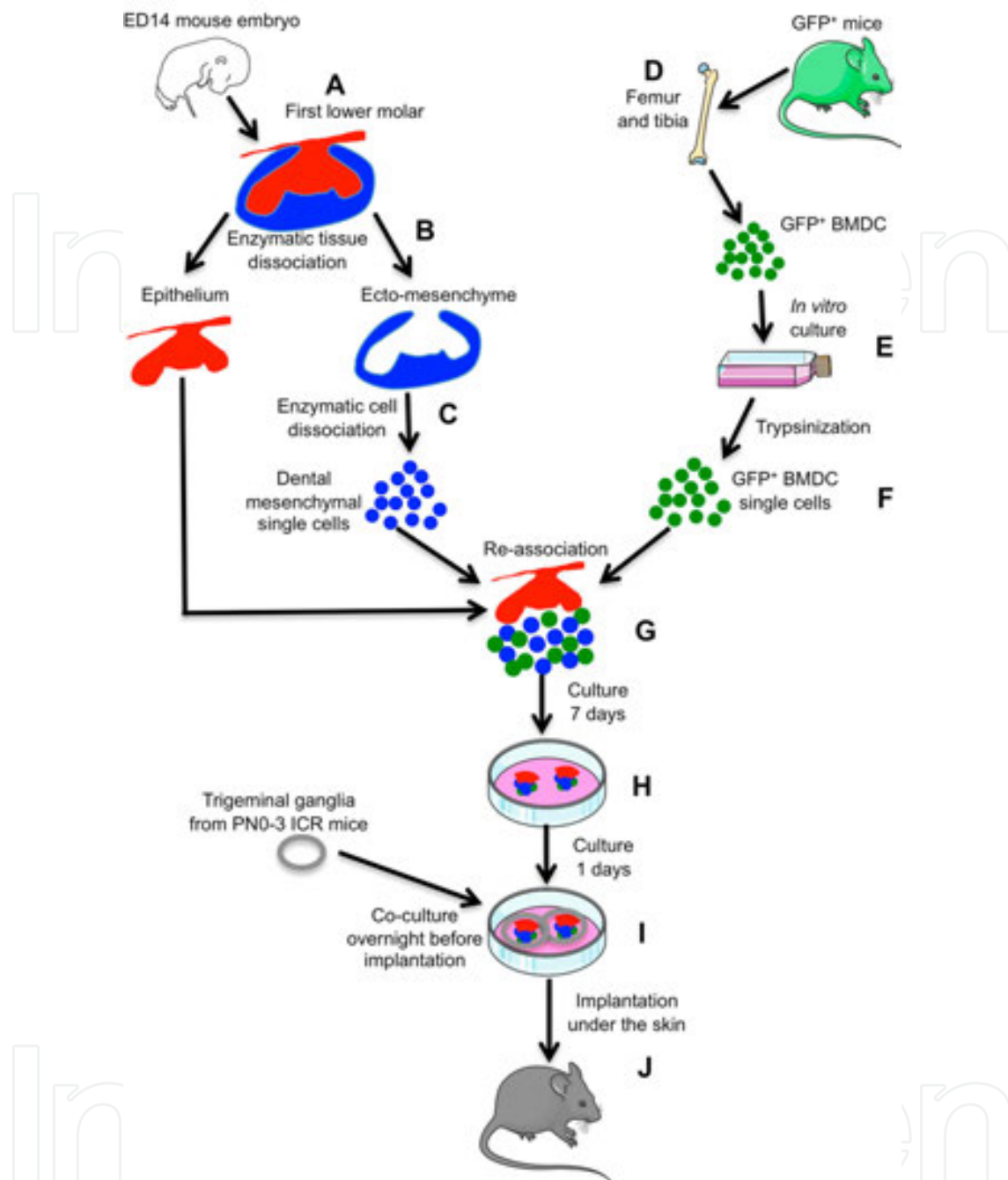


Figure 9. Schematic representation of the experimental procedures to test the effects of bone marrow derived cells on the innervation of implanted cells/tissue re-associations. The mandibular first molars were dissected from ICR embryos at ED14 (A). The dental epithelium and ecto-mesenchyme were dissociated by enzymatic treatment (B). Then dental ecto-mesenchyme was dissociated into single cells (C) and mixed with bone marrow derived cells (50:50) (G). For this purpose, bone marrow derived cells were prepared from femur and tibia bones (D), dissected from adult GFP positive mice (C57BL/6 from the IGBMC, Illkirch, France). The bone marrow derived cells (BDMCs) were cultured *in vitro* and used at passage 2 (E). After trypsinization, BDMCs single cells (F) were mixed with the dental mesenchymal single cells and put in contact with an ED14 intact dental epithelium (G). These re-associations were cultured for 7 days *in vitro* (H) and then a trigeminal ganglion was put on the top of each re-association for a further co-culture overnight (I). These re-associations were implanted between the skin and muscles behind the ears in adult ICR mice and maintained for two weeks (J).

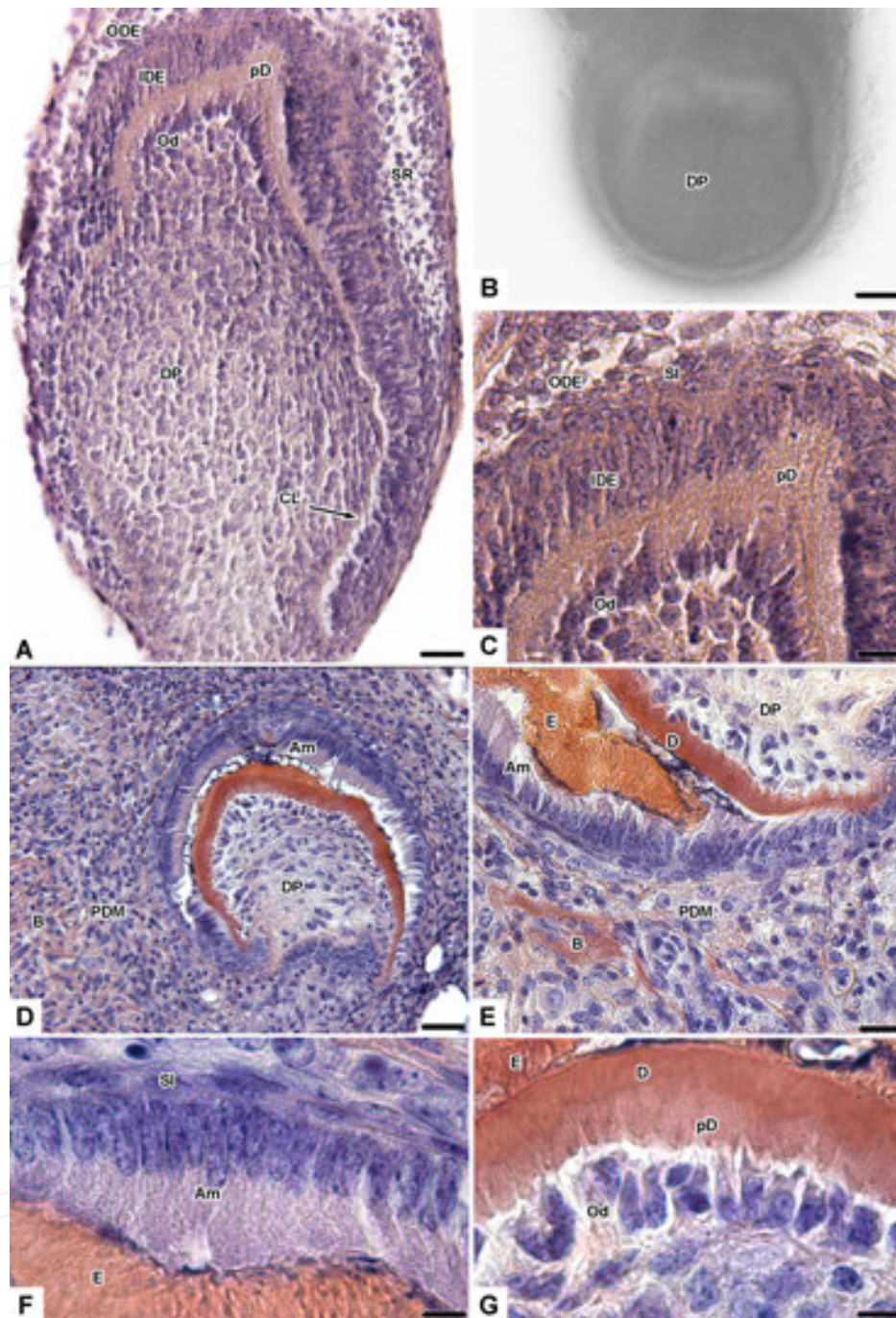


Figure 10. Histology of re-associations between an enamel organ and mixed dental mesenchymal cells and bone marrow derived cells prior to implantation (A-C) and implanted for two weeks under the skin of ICR mice (D-G). Re-associations between mixed BMDCs/dental mesenchymal cells (50:50) and an intact dental epithelium from the cap stage at embryonic days 14 (ED14) were cultured before implantation for two weeks under the skin of ICR mice. After eight days in culture, cells/tissue re-associations showed a characteristic dental epithelial histogenesis, with the presence of the inner (IDE) and outer dental epithelium (ODE) (A,C), the stellate reticulum (SR) (A) and the stratum intermedium (SI) (C). Odontoblasts (Od) were differentiated and induced the differentiation of ameloblasts, which elongated in the IDE (A). Odontoblasts secreted predentin (pD) (A, C). After implantation (D-G), crown (D) and root (E) were developed and newly formed bone (B) was present next to the tooth (E). Ameloblasts (Am) were elongated, polarized and secreted enamel (F). Ameloblasts were in contact with the stratum intermedium (F). Odontoblasts were elongated and polarized with their nucleus opposite to the secretory pole (G). They were functional, secreting predentin/dentin and

dental tubules were visible (G). CL: cervical loop; D: dentin; DP: dental pulp; E: enamel; PDM: peridental mesenchyme. Scale bars = 80 μm (B); 40 μm (A, D); 20 μm (C, E) and 8 μm (F, G).

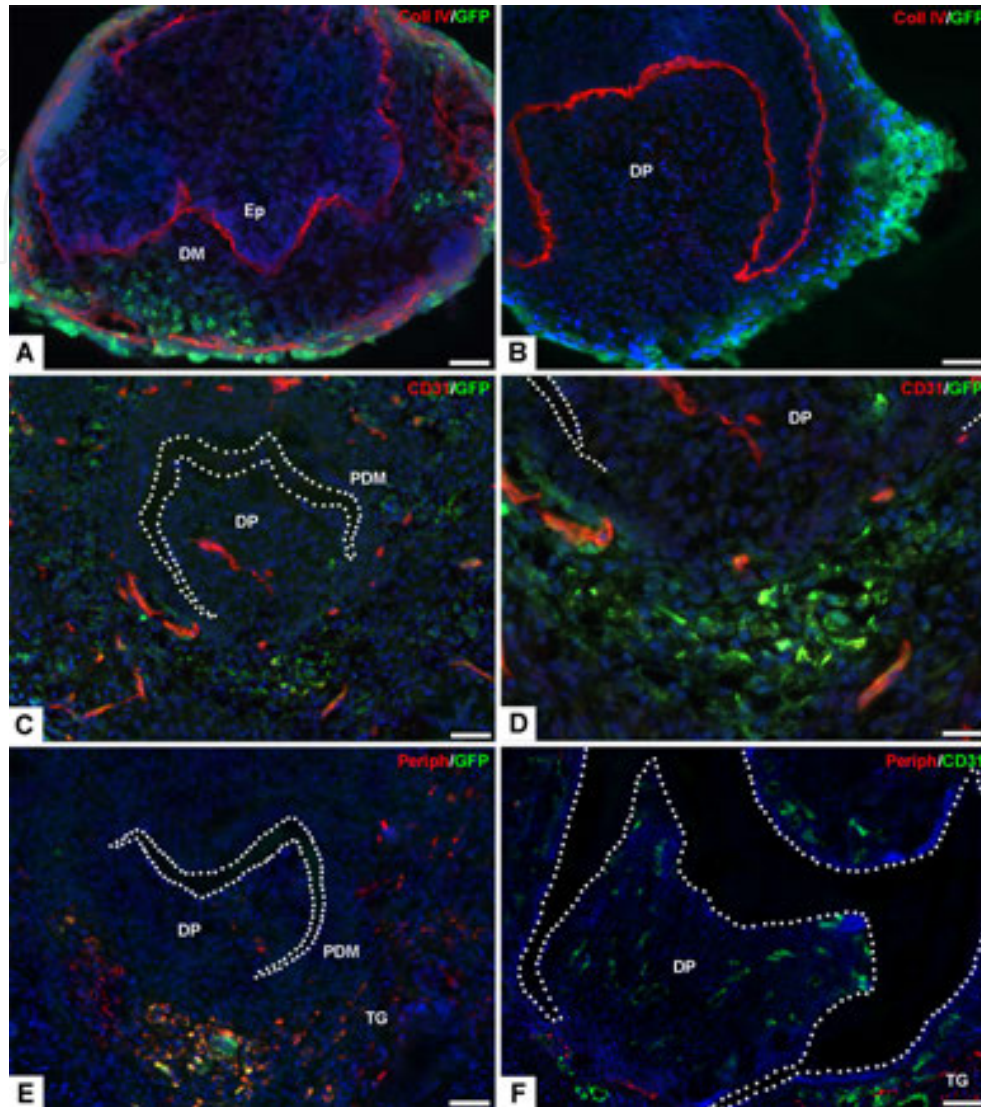


Figure 11. Vascularization and innervation of re-associations between mixed dental cells and bone marrow derived cells and an intact dental epithelium cultured for six days (A), or eight days (B), or cultured and implanted for two weeks with a trigeminal ganglion (C-E), and control re-associations without bone marrow-derived cells (F). Implantations were performed under the skin of ICR mice. The epithelial-mesenchymal junction was labeled with an antibody against collagen type IV (A, B). Blood vessels were labeled with an antibody to CD31 (C, D, F) and axons with an antibody to peripherin (E, F). GFP positive bone marrow derived cells (BMDCs) were labeled with a specific antibody to GFP (A-E). After six days in culture, BMDCs were present in the dental mesenchyme (DM) and at the periphery of the forming tooth (A). After eight days in culture, GFP-positive BMDCs were only detected at the periphery of the forming tooth (B). After two weeks of implantation, blood vessels were present in the peridental tissues and could enter the dental pulp (DP) in cell re-associations with BMDCs (C, D), as in cell re-associations without BMDCs (F). In implanted cell re-associations with BMDCs, axons from the trigeminal ganglion (TG) were detected in the dental pulp (E), while in control re-associations (without BMDCs) axons were observed only at the limit between the peridental mesenchyme (PDM) and dental pulp (F). After implantation for two weeks, GFP positive BMDCs were detected only in the periphery of the forming tooth and not in the dental pulp (C-E). Ep: epithelium; PDM. Scale bars=40 μm (A-C, E, F) and 20 μm (D).

5. Conclusions and prospects

All together, the results presented here show how far it is possible to reproduce the innervation of the dental and peridental mesenchymes in teeth forming from cultured and implanted cell re-associations, as it may occur during odontogenesis. As long as immunodepressed conditions can be maintained (implantation in Nude mice), it is possible within two weeks of implantation to reproduce the innervation of the dental mesenchyme, with axons reaching odontoblasts. Glial cells were present as well, although further work will be necessary to determine their origin. Still some differences exist when comparing the situation in implanted re-associations with developing molars. It will be necessary to determine how far these might result from differences in the kinetic of innervation in the two conditions and whether this would have further consequences after longer implantation period. Longer implantations would also allow determining whether myelinisation can be achieved. Using a method described by Honda et al., [102], implantation in the jaw, next to the alveolar nerve, will have to be tested to try to better approach the physiological situation and see whether tooth innervation can occur in these conditions. At the same time, this might allow the sympathetic innervation of the tooth, thus avoiding parallel experiments where cell re-associations would have to be grown in contact with a superior cervical ganglion.

The method used here will have to be adjusted when new non-dental mesenchymal cell sources will become available to engage in whole tooth engineering. Indeed, dental embryonic cells represent a very convenient tool to design and try to simplify experimental conditions for tooth organ engineering. However, such cells would not be available in a clinical context, so that several other cell sources are being tested (for review see [2,80,103]). Dental embryonic cells were also a convenient model to investigate the rapid loss of odontogenic potentialities with aging and after *in vitro* culture [104]. Despite the presence of a morphogenetic center, the primary enamel knot, the enamel organ at the cap stage has no more odontogenic potential [105]. Previous studies offered the possibility to use younger epithelial sources, when still retaining odontogenic inductive potential as tested for tooth engineering [8]. Obviously the odontogenic potentialities of dental pulp cells rapidly decrease during development and are quite poor in adult tissues. This can be seen from reparative processes, which are quite limited physiologically and lead to osteodentin instead of tubular dentin in most experimental conditions [62,106]. For this reason, the design of experimental conditions where non dental cells can be induced to give rise to odontoblasts secreting tubular dentin [8,59] or to ameloblasts secreting enamel may require important technical adjustments [107–109]. Investigating the innervation of the mesenchyme and more specifically of the odontoblast layer forming in these conditions will be a future challenge.

Abbreviations

α -SMA: alpha smooth muscle actin; BDNF: brain-derived neurotrophic factor; BM-MSc: bone marrow mesenchymal stem cell; BMDC: bone marrow derived cell; CD34: cluster of differen-

tiation 34; CsA: cyclosporin A; CXCL: chemokine ligand; DPSC: dental pulp stem cell; ED: embryonic day; GAP-43: growth-associated protein-43; GDNF: glial-derived neurotrophic factor; GFAP: glial fibrillary acidic protein; GFP: green fluorescent protein; MSC: mesenchymal stem cell; NF200: neurofilament 200; NGF: nerve growth factor; PN: post-natal; PNS: peripheral nervous system; TEM: transmission electron microscopy; TG: trigeminal ganglion.

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