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L. Tran-Hung<sup>1</sup>, S. Mathieu<sup>2</sup>, and I. About<sup>1\*</sup>

<sup>1</sup>Laboratoire IMEB-ERT 30, Faculté d'Odontologie, Université de la Méditerranée, 27 Boulevard Jean Moulin, 13355 Marseille Cedex 05, France; and <sup>2</sup>INSERM U559, Faculté de Médecine, Marseille, France; \*corresponding author, Imad.About@odontologie.univ-mrs.fr

# Role of Human Pulp Fibroblasts in Angiogenesis

## ABSTRACT

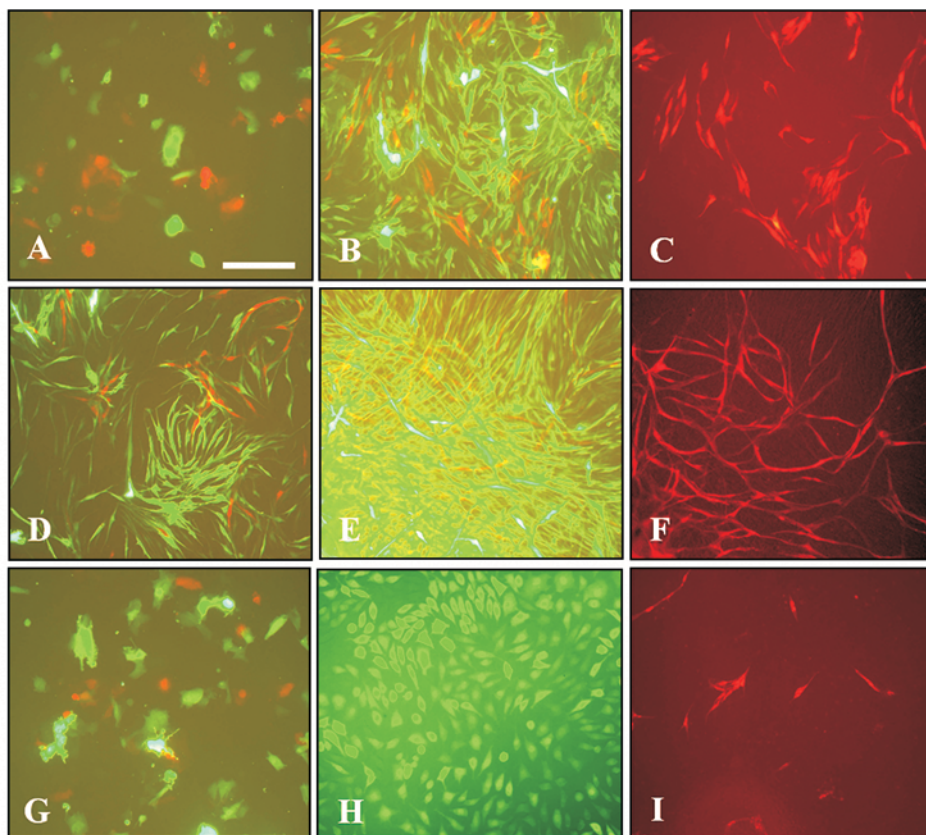
After pulp amputation, complete pulp healing requires not only reparative dentin production but also fibroblast proliferation, nerve fiber growth, and neoangiogenesis. This study was designed to investigate the role of pulp fibroblasts in angiogenesis. Human pulp fibroblasts from third molars co-cultured with human umbilical vein endothelial cells induced the organization of endothelial cells and the formation of tubular structures corresponding to capillaries *in vivo*. The direct contact between both cells was not necessary to induce angiogenesis, and the observed effect was due to soluble factors. This was confirmed with neutralizing antibodies against FGF-2 and VEGF, which decreased the angiogenic effects of these soluble factors. Immunohistochemistry showed that both FGF-2 and VEGF were expressed in human dental pulp fibroblasts, and this expression increased after injury. These results suggest that the pulp fibroblasts secrete angiogenic factors, which are necessary for complete pulp healing, particularly at the pulp injury site.

**KEY WORDS:** Angiogenesis, pulp, fibroblasts, endothelial cells, reparative dentinogenesis.

## INTRODUCTION

Angiogenesis occurs during embryonic development, chronic inflammation, tumor growth, and wound healing. It is a complex process with extracellular matrix remodeling, secretion of proteolytic enzymes, endothelial cell migration and proliferation, capillary differentiation, and anastomosis (Folkman and Shing, 1992). This process is regulated by the interplay of numerous cytokines and growth factors. Among all the pro-angiogenic factors, vascular endothelial growth factor (VEGF) is considered the most essential for the differentiation of the vascular system (Ferrara, 1996); basic fibroblast growth factor (FGF-2) is known to stimulate angiogenesis *in vivo* and is thought to play a significant role in neovascularization of damaged or traumatized tissue (Gerwins *et al.*, 2000). In pathological conditions, such as moderate caries lesions, growth factors can be released from the dentin to stimulate the pre-existing odontoblasts to secrete reactionary dentin (Tziafas *et al.*, 2000; Smith and Lesot, 2001; Goldberg and Smith, 2004). However, when the caries lesion is severe or in deep cavity preparation, in addition to the damage to the odontoblasts, a necrotic zone associated with partial destruction of the pulp can be observed. This destruction involves several cell types, including odontoblasts, pulp fibroblasts, vascular endothelial cells, and nerve fibers. Several studies have shown that growth factors released from the dentin under these conditions can be involved in pulp healing. Among these growth factors, VEGF and FGF-2 were identified in the dentin (Roberts-Clark and Smith, 2000) and may play a role in angiogenesis.

The dental pulp is a highly vascularized tissue, and exposed cavity preparations result in subsequent injury to pulp tissue, including blood vessels and pulp fibroblasts. It has been well-established that injured endothelial cells release signaling molecules to initiate inflammatory reactions and the healing process. Previous studies have shown that the dental pulp has a high regenerative potential. After surgical pulp amputation, healing can occur, with hard-tissue formation in germ-free animals, independently of a local acidic environment (Tsuji *et al.*, 1987; Inoue and Shimono, 1992). In agreement with these studies, we have shown that progenitor pulp cells can be activated in their tissue of origin after pulp amputation (Tecles *et al.*, 2005), and these cells migrate to the injury site in response to injury to endothelial cells (Mathieu *et al.*, 2005). Many studies have been devoted to the study of reparative dentin secretion and have demonstrated the role played by growth factors and signaling molecules from the dentin and enamel in this process (Goldberg and Smith, 2004). However, after pulp amputation, complete pulp healing requires not only reparative dentin production but also neoangiogenesis and nerve fiber growth. Pulp cells express VEGF in healthy and pathological situations such as irreversible pulpitis, suggesting a role for pulp cells in angiogenesis (Artese *et al.*, 2002). The aim of this work was to investigate the interaction of pulp fibroblasts with the vascular system by a study of the possible role of pulp cells in angiogenesis, particularly after injury.



**Figure 1.** Co-culture of human umbilical vein endothelial cells and human pulp fibroblasts. Human pulp fibroblasts and L929 fibroblastic cell line were fluorescence-labeled by transduction with the Enhanced Green Fluorescent Protein; endothelial cells were labeled with the Discosoma Red Fluorescent Protein-2. In co-cultures, pulp fibroblasts and endothelial cells remained separated after 3 hrs (A). Endothelial cells started to elongate and form closed structures at 24 hours (B,C). At 48 hrs, most endothelial cells were organized (D), and at 6 days, they formed a complete tubular network (E,F). Endothelial cells co-cultured with L929 cells remained separated from each other after 3 hrs (G), and no organization was found even after day 6 (H,I). Green and red fluorescence was simultaneously examined (A,B,D,E,G,H). The other photographs (C,F,I) were visualized by the red fluorescence only. Scale bar = 200  $\mu\text{m}$ .

## MATERIALS & METHODS

### Materials

All materials for cell culture—including culture media, human umbilical vein endothelial cells, and Matrigel—were purchased from Cambrex (Cambrex Bio Science, Walkersville, MD, USA). Chemicals were obtained from Sigma-Aldrich (Sigma Chemicals Corp., St. Louis, MO, USA), unless otherwise stated.

### Cell Culture and Transduction

Human pulp fibroblasts were prepared from immature third molars as described previously (About *et al.*, 2000). Briefly, the teeth were obtained from 16-year-old adolescents in compliance with French legislation (informed patients' and parents' consent, and Institutional Review Board approval of the protocol used). After extraction, the teeth were washed and the apical portions removed. The extirpated dental pulp was minced, and explants were cultured in 100-mm-diameter culture dishes (Becton Dickinson Labware, Lincoln Park, NJ, USA). Confluent cultures were collected by trypsinization and subcultured. The cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 UI/mL penicillin, 100  $\mu\text{m}$

streptomycin, and 0.25  $\mu\text{g}/\text{mL}$  amphotericin B (Fungizone). Human umbilical vein endothelial cells from a single donor were routinely cultured in EBM-2 medium supplemented with 2% fetal bovine serum and growth factors at 37°C in a 95% air plus 5%  $\text{CO}_2$  atmosphere until 8 passages.

Human pulp fibroblasts and the L929 fibroblastic cell line (NCTC, Paisley, UK) were fluorescence-labeled by transduction with Enhanced Green Fluorescent Protein as reported previously (Naldini *et al.*, 1996; Mathieu *et al.*, 2004). Similarly, endothelial cells were labeled with Discosoma Red Fluorescent Protein-2.

### Effect of Direct Contact between Pulp Fibroblasts and Endothelial Cells

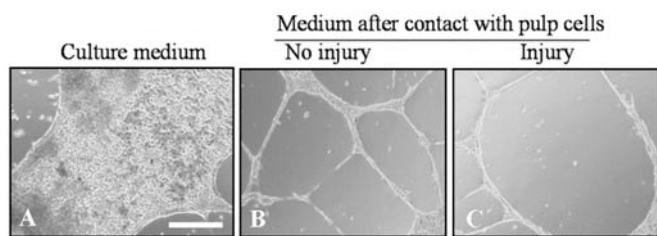
Matrigel (250  $\mu\text{L}$ ) was poured into a 24-well culture plate and allowed to solidify (37°C, 1 hr). Human pulp fibroblasts or L929 fibroblasts were co-cultured with endothelial cells at a ratio of 80%/20% on a Matrigel extracellular matrix in EGM-2 Bullet Kit medium supplemented with 0.5% fetal bovine serum. Angiogenesis was examined under a fluorescence-equipped phase-contrast microscope (Carl Zeiss Axiovert200, Göttingen, Germany).

### Effect of Indirect Contact between Pulp Fibroblasts and Endothelial Cells

For studies of indirect contact, pulp fibroblasts were cultured in EGM-medium without fetal bovine serum. Injuries to fibroblasts were performed mechanically, with sterile scalpels used to disrupt the fibroblast monolayer. The conditioned media obtained after a contact period of 5 hrs with intact or injured cells were then used for the culture of endothelial cells on Matrigel extracellular matrix. The culture medium without contact with pulp fibroblasts was used as control. Endothelial cells ( $4 \times 10^5$  cells/well) were seeded on the Matrigel and cultured in the conditioned media described above. After incubation with these media, the endothelial cell organization was observed with a phase-contrast microscope and quantitatively evaluated by measurement of the tubular perimeters in 30 fields of view *per* group (Salani *et al.*, 2000). To eliminate the pitfalls associated with the use of this assay, we applied random selection of the fields for analysis. Tubular perimeters were analyzed with Scion Image (Scion Corporation, Frederick, MD, USA) software.

### Neutralizing Assays

Two neutralizing antibodies (R&D Systems, Lille, France) at a final concentration of 20  $\mu\text{g}/\text{mL}$  (determined by R&D Systems to give a minimum of 50% neutralization) were used: anti-human vascular endothelial growth factor (anti-VEGF) and anti-human



**Figure 2.** Effect of indirect contact between pulp fibroblasts and endothelial cells. Morphological features of endothelial cells incubated after 24 hrs in control medium without contact with pulp fibroblasts (A), and after contact with conditioned media from intact pulp cells (B) and injured pulp fibroblasts (C). The capillary network of capillary-like structure formation on the Matrigel was photographed in fresh medium under a phase-contrast microscope. Scale bar = 200  $\mu\text{m}$ .

basic fibroblast growth factor (anti-FGF-2). Neutralizing antibodies were added to the conditioned media, either separately or together, from injured pulp fibroblasts. This conditioned medium was then used for the culture of endothelial cells as described above. The medium obtained from injured pulp fibroblasts without neutralizing antibodies was used to indicate the baseline value. The effect of pre-incubating this medium with neutralizing antibodies on tubular perimeters was expressed as a percentage of this baseline value.

### Immunohistochemistry

Pulp fibroblasts ( $5 \times 10^5$  cells/mL) were cultured in four-well culture chambers (Falcon, Meylan Cedex, France). Injuries were made with scalpels on confluent cultures as described above. After a five-hour culture period, the slides were fixed in ethanol 70% solution for 1 hr at 4°C. Permeabilization was achieved with 0.5% Triton for 15 min.

Immunohistochemistry was performed on cultured cells in the chambers with anti-FGF-2 and anti-VEGF monoclonal antibodies (R&D Systems, Lille, France). Primary antibodies were diluted in phosphate-buffered saline containing 0.1% bovine serum albumin. Incubation with primary antibodies (10  $\mu\text{g/mL}$ ) was performed overnight at 4°C. Immunostaining was revealed with the use of a labeled streptavidin-biotin kit (LSAB, Dako Corp., Carpinteria, CA, USA), according to the manufacturer's instructions. We created controls by omitting primary antibodies or incubating with unrelated primary antibodies (cytokeratin 19). Blocking experiments were also performed by pre-incubation of anti-FGF-2 and anti-VEGF antibodies with the polypeptides used for immunization. All controls were negative.

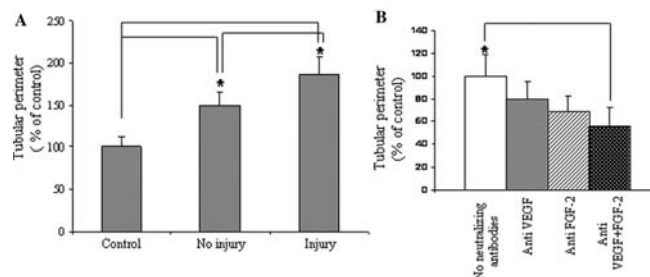
### Statistical Analysis

For quantitative tubular perimeter analysis, 30 view-fields *per* group were analyzed. All experiments were performed in triplicate, and each experiment was reproduced at least 3 times. Error bars reflect the standard deviation, and probability values were assessed by the Mann-Whitney non-parametric test.  $p < 0.05$  was considered significant.

## RESULTS

### Endothelial Cells Co-cultured with Pulp Fibroblasts Organize into Tubular Structures

The co-culture of endothelial cells with pulp fibroblasts induced time-independent morphogenetic changes in the



**Figure 3.** Effects of soluble factors secreted by pulp fibroblasts on the tubular perimeters of capillary-like structures formed by endothelial cells. The quantitative evaluation was done by measurement of the tubular perimeters in 30 fields of view *per* group in 3 independent experiments. Results are expressed as mean  $\pm$  SD. Endothelial cells were seeded on the 24-well culture plate on Matrigel and cultured in conditioned media obtained from intact or injured pulp fibroblasts. Control medium (not in contact with pulp fibroblasts) was also used. After endothelial cells were incubated with these media, the formation of the capillary network was observed by phase-contrast microscopy. (A) The perimeters of tubular structures formed by endothelial cells were measured and expressed as a percentage of the perimeters obtained after the incubation of endothelial cells with the control medium. A significant increase in tube perimeter was observed with the conditioned media from intact and injured pulp fibroblasts. There was a significant increase in the tubular perimeter with the conditioned medium obtained from injured as compared with that obtained from intact fibroblasts ( $*p < 0.05$ ). (B) The addition of FGF-2 or VEGF neutralizing antibodies to the conditioned media obtained from injured pulp fibroblasts resulted in a reduction in the tubular perimeters as compared with those observed without neutralizing antibodies. This difference was statistically significant when both neutralizing antibodies were used. The tubular perimeters were measured and expressed as a percentage of those obtained from injured pulp cells without neutralizing antibodies ( $*p < 0.05$ ).

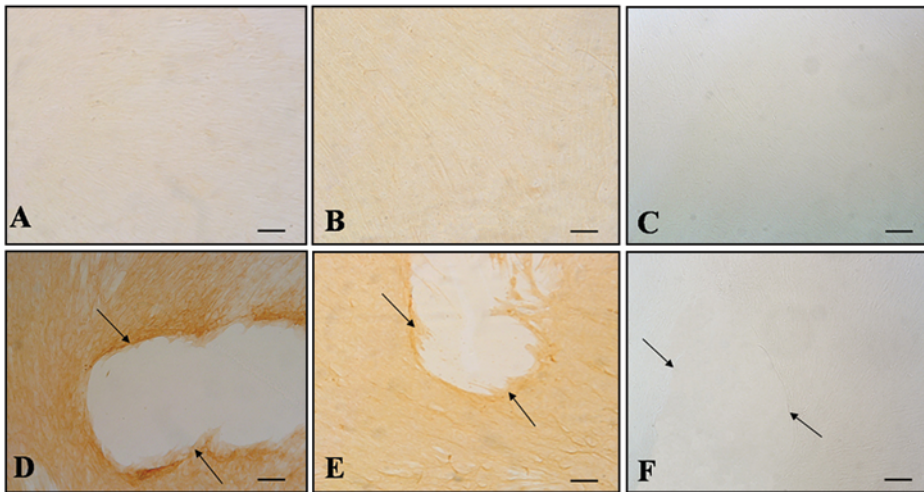
endothelial cells, which organized into capillary-like structures. At 3 hrs, both cell types were observed and appeared to be round (Fig. 1A). The cells spread and started to organize after 24 hrs (Figs. 1B, 1C). At 48 hrs, most cells had formed tubular structures (Fig. 1D). After 6 days, endothelial cells became elongated, forming thin cords of interconnecting cells, and exhibited elongations and branching to form a network of capillary-like structures (Figs. 1E, 1F). Endothelial cells co-cultured with L929 cells remained separated from each other (Fig. 1G), and no tubular organization was found, even after 6 days (Figs. 1H, 1I).

### Pulp Fibroblasts Promote Angiogenesis via Soluble Factors

In most cases, no organization of endothelial cells was found with control medium, which had not been in contact with pulp fibroblasts (Fig. 2A). However, some structural rearrangements of endothelial cells were observed, but the tubular perimeters were small. The conditioned culture medium obtained after 5 hrs of contact with intact or injured pulp fibroblasts induced marked changes in endothelial cell morphology, with structural re-arrangements leading to the formation of capillary-like networks. This was observed with the conditioned media from intact (Fig. 2B) and injured pulp fibroblasts (Fig. 2C). However, there was an increase in the tubular perimeter with the medium obtained from injured pulp fibroblasts, compared with that obtained from intact cells.

The tubular perimeter measurements revealed a significant increase ( $p < 0.05$ ) of 49.95% with the medium obtained from intact pulp fibroblasts over the control medium (without any





**Figure 4.** Expression of FGF-2 and VEGF in human pulp cells. Pulp fibroblasts were cultured in four-well culture chambers. Injuries were made with scalpels on confluent cultures. After a five-hour culture period, immunohistochemistry showed the expression of FGF-2 (A) and VEGF (B) at a low level in pulp fibroblasts. Stronger expression of FGF-2 (D) and VEGF (E) could be observed after injury. Controls with no primary antibodies were negative in intact (C) and injured cells (F). Arrows indicate injury sites. Scale bars = 100  $\mu\text{m}$ .

contact with pulp fibroblasts). Moreover, a significant promotion in tubular perimeter (36.97% increase over medium from intact cells) was observed with the medium from injured pulp fibroblasts ( $p < 0.05$ ) (Fig. 3A).

### Capillary Network Tubular Perimeters Decrease with Neutralizing Antibodies against FGF-2 and VEGF

The addition of each neutralizing antibody to the conditioned media obtained from injured pulp fibroblasts resulted in a reduction in the tubular perimeters, as compared with those observed without neutralizing antibodies. This reduction was 20.86% with anti-VEGF, 31.14% with anti-FGF-2, and 44.13% when both neutralizing antibodies were present together (Fig. 3B). A statistically significant difference was observed when both neutralizing antibodies were used ( $p < 0.05$ ) (Fig. 3B).

### FGF-2 and VEGF Expression in Human Dental Pulp Fibroblasts Increases after Injury

Immunohistochemistry showed that both FGF-2 (Fig. 4A) and VEGF (Fig. 4B) were expressed at a low level in human pulp fibroblasts. This expression increased after injury (Figs. 4D, 4E). Control experiments performed on intact (Fig. 4C) or injured cells (Fig. 4F) showed negative immunoreactivity.

## DISCUSSION

This work is indicative that pulp fibroblasts play an important role in angiogenesis, which is a key step in the healing sequence of the pulp after amputation. This was demonstrated here by the fact that co-cultures of pulp fibroblasts and endothelial cells induced the formation of a network of tubular structures of endothelial cells, indicative of angiogenesis stimulation, and this effect showed a dramatic increase after injury. Angiogenesis has often been studied in cultures of endothelial cells, which have the ability to organize into tubular structures with appropriate angiogenic signals (Kubota *et al.*,

1988). The evaluation of the angiogenic potential of a particular factor can be made after incubation with endothelial cells and the measurement of the dimensions of the tubular structures produced, perimeter, area, or number of branches. In this study, we used tubular perimeters, which have been reported to reflect the angiogenic potential of molecules (Salani *et al.*, 2000). However, the use of tubular perimeters alone was a limitation of the study.

Co-cultures of pulp fibroblasts with endothelial cells induced tubular organization of endothelial cells, while this was not observed in co-cultures of L929 fibroblasts and endothelial cells. This indicates that pulp fibroblasts express angiogenic signals that can act on endothelial cells.

In an attempt to understand if this direct contact was necessary for promoting angiogenesis, we

cultured endothelial cells alone with conditioned media obtained from intact or injured pulp cells. The results showed that angiogenesis promotion was due to soluble factors secreted by pulp fibroblasts. This angiogenic effect was obtained with conditioned culture media after relatively brief contact with intact pulp fibroblasts, and was more dramatic if the medium was obtained after contact with injured cells. The observation of angiogenesis with the conditioned medium from cell cultures without injury is in agreement with that of the co-cultures of both cell types and the immunohistochemical results. The increase of angiogenesis after injury is a very important event for pulp healing, and it is in agreement with the release of angiogenic growth factors from the pulp following orthodontic force (Derringer *et al.*, 1996; Derringer and Linden, 2004).

Immunohistochemistry revealed the expression of VEGF and FGF-2 in pulp fibroblasts. The expression increased shortly after injury (5 hrs), indicating a very rapid response, suggesting a role directly linked to the injury. A confirmation of this angiogenic effect came from neutralizing antibody experiments: Neutralizing antibodies decreased the angiogenic effects of the soluble factors in the conditioned medium from injured pulp fibroblasts. This neutralizing effect was appreciable and was observed with the neutralizing antibodies used separately, indicating that both were released and played a role in angiogenesis. This agrees with the well-known synergistic effects of FGF-2 and VEGF on endothelial cells, in which FGF-2 potentiates the action of VEGF by inducing the expression of VEGF, which in turn increases VEGF receptor expression (Hata *et al.*, 1999). The persistence of angiogenesis after neutralization of FGF-2 and VEGF may be due to the fact that pulp cells secrete other angiogenic signals that remain to be identified.

A previous study has showed that human pulp fibroblasts from healthy and inflamed tissues express VEGF (Artese *et al.*, 2002). VEGF expression and secretion have recently been

reported in MDPC-23 cells in response to adhesive resins (Mantellini *et al.*, 2006) or lipoteichoic acid application, suggesting increased expression of this growth factor under pathological conditions (Telles *et al.*, 2003). This growth factor, together with FGF-2, is sequestered in dentin (Roberts-Clark and Smith, 2000), suggesting a role for these molecules in angiogenesis under caries lesions. The release of such factors due to dentin dissolution in caries lesions or after trauma may potentiate the effects seen in this study.

Our work provides clear evidence that human pulp fibroblasts express both FGF-2 and VEGF, and shows that these molecules exert their angiogenic effects as soluble factors. The release of these factors is very rapid and corresponds well to the pathological changes in the pulp following injury. After pulp injury, the odontoblast progenitor cell migration to the injury site may require newly formed blood vessels. These can be initiated by the secretion of angiogenic growth factors by dental pulp fibroblasts, as demonstrated. Additionally, it has been shown that FGF-2 stimulates adult rat dental pulp cells proliferation (Nakao *et al.*, 2004).

Thus, in addition to reparative dentin secretion, pulp cells are involved in angiogenesis. This study contributes to our understanding of the pathophysiology of the dental pulp and the mechanisms responsible for tooth pulp repair. It opens another research orientation concerning the engineering of vascularized dental pulp tissue.

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