Original Article

Role of osteopontin in the process of pulpal healing following tooth replantation in mice

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ABSTRACT

Introduction: The role of osteopontin (OPN) following severe injury remains to be elucidated, especially its relationship with type I collagen (encoded by the *Col1a1* gene) secretion by newly-differentiated odontoblast-like cells (OBLCs). In this study, we examined the role of OPN in the process of reparative dentin formation with a focus on reinnervation and revascularization after tooth replantation in *Opn* knockout (KO) and wild-type (WT) mice.

Methods: Maxillary first molars of 2- and 3-week-old-*Opn* KO and WT mice (*Opn* KO 2W, *Opn* KO 3W, WT 2W, and WT 3W groups) were replanted, followed by fixation 3–56 days after operation. Following micro-computed tomography analysis, the decalcified samples were processed for immunohistochemistry for Ki67, Nestin, PGP 9.5, and CD31 and *in situ* hybridization for *Col1a1*.

Results: An intense inflammatory reaction occurred to disrupt pulpal healing in the replanted teeth of the *Opn* KO 3W group, whereas dental pulp achieved healing in the *Opn* KO 2W and WT groups. The tertiary dentin in the *Opn* KO 3W group was significantly decreased in area compared with the *Opn* KO 2W and WT groups, with a significantly low percentage of Nestin-positive, newly-differentiated OBLCs during postoperative days 7–14. In the *Opn* KO 3W group, the blood vessels were significantly decreased in area and pulp healing was disturbed with a failure of pulpal revascularization and reinnervation.

Conclusions: OPN is necessary for proper reinnervation and revascularization to deposit reparative dentin following severe injury within the dental pulp of erupted teeth with advanced root development.

Keywords: Animal model, Blood supply, Dentinogenesis, Innervation, Osteopontin, Tooth replantation

Abbreviations

M1 first molars
GFP green fluorescent protein
H&E hematoxylin and eosin
H2B histone 2B
KO knockout
MSCs mesenchymal stem cells
μCT micro-computed tomography
OPN osteopontin
OBLCs odontoblast-like cells
SCs Schwann cells
SCAP stem cells derived from the apical papilla
VEGF vascular endothelial growth factor
WT wild-type

1. Introduction

Dental pulp is a highly specialized mesenchymal tissue with a remarkable capacity for repair and regeneration [1]. Pulpal healing with tertiary dentin indicates that the original pulp tissue is replaced by a different tissue with the deposition of a calcified scar [2]. After tooth injury, the formation of tertiary dentin adjacent to preexisting dentin is a secretory response regulated by odontoblasts or odontoblast-like cells (OBLCs). There is an intimate relationship between reactionary dentinogenesis with nerve fiber sprouting following injuries, such as dental caries [3]. This indicates that crosstalk exists between matrix secretory activity by odontoblasts and dynamic neuronal responses. Pulpal nerve fibers exhibit neuroplasticity with sprouting, degenerative, or regenerative processes depending on various scenarios, such as injury or physiological events [4-8]. The response

of blood vessels is to initiate angiogenic events as a consequence of hypoxia, because the secretory activity of the odontoblasts is associated with the positional and ultrastructural changes of pulpal capillaries [9]. The mechanisms that regulate multi-event responses with tertiary dentinogenesis, reinnervation, and revascularization within the dental pulp following exogenous injury remain to be fully elucidated.

Tooth replantation is a severe injury that induces the death of most odontoblasts because of the interruption of the neurovascular supply to the dental pulp. Although pulpal healing with reinnervation and revascularization has been shown to occur in humans [10] and experimental animals [11, 12], there are many factors that affect this successful outcome. The lack of proper oxygenated medium is decisive for the survival of odontoblast-lineage cells and the occlusal force during and/or after the operation is detrimental to the cells [12]. Furthermore, the width and length of the roots are important predictors of pulpal healing with a significant association between the stage of root development or a favorable ratio of the broad apical foramen/short root canal and proper pulpal healing [10, 13]. Therefore, the evaluation and comparison of these factors are important to provide insight into the manner in which dental pulp can (or not) succeed in its own proper healing following tooth replantation.

Understanding how tertiary dentinogenesis and OBLC differentiation occur after tooth replantation is fundamental for evaluating strategies and predicting outcomes for dentin-pulp complex regeneration. The regulation of matrix mineralization and cell adhesion is associated with the presence of non-collagenous proteins, especially small integrin-binding ligand N-linked glycoproteins, which are composed of bone sialoprotein, dentin sialophosphoprotein, dentin matrix protein-1, matrix extracellular phosphoprotein, and osteopontin (OPN) [14]. OPN is localized in the boundary between tertiary dentin and preexisting dentin in mice [15]. Immunocompetent cells, such as macrophages and dendritic cells, secrete OPN, which is deposited at the dentin-predentin interface before OBLC differentiation and after tooth transplantation [16]. In addition, OPN is essential for type I collagen secretion by new OBLCs to form reparative dentin after an injury, such as cavity preparation [17]. However, the role of OPN during reparative dentinogenesis and its interplay with the neurovascular response after severe injury, such as tooth replantation, is not yet fully understood. In this study, we determined the role of OPN in the process of reparative dentin formation with special focus on reinnervation, revascularization, and different root development stages following tooth replantation in *Opn* knockout (KO) and wild-type (WT) mice.

2. Methods

2.1. Mice

All animal experiments were conducted in compliance with ARRIVE guidelines and a protocol that was reviewed by the Institutional Animal Care and Use Committee and approved by the President of Niigata University (SA00780). Information regarding the animals is available from a previous study [17]. Male and female *Opn*$^{-/-}$ (B6.Cg-*Spp1*^{tm1Blh}/J) and wild-type (WT: C57BL/6J) mice were obtained from the Jackson Laboratories (Bar Harbor, ME) and Charles River Laboratories of Japan (Yokohama, Japan), respectively.

2.2. Tooth Replantation

Two- and three-week-old animals were used for tooth replantation, which were referred to as 2W and 3W groups, respectively. The upper right first molars (M1) of *Opn* KO and WT mice were extracted under deep anesthesia following an intraperitoneal injection of a mixed solution (0.05–0.1 mL/10 g) of Domitor® (1.875 mL: Nippon Zenyaku Kogyo Co, Ltd, Koriyama, Japan), midazolam (2 mL: Sandoz KK, Tokyo, Japan), Vetorphale® (2.5 mL: Meiji Seika Pharma Co, Ltd, Tokyo, Japan), and physiological saline (18.625 mL), using a pair of modified dental tweezers. The tooth was immediately repositioned in the

original socket. The animals were divided into four groups: *Opn* KO 2W, *Opn* KO 3W, WT 2W, and WT 3W groups.

2.3. Micro-computed tomography (μCT) Analysis

μCT analysis (Elescan; Nippon Steel Texeng. Co., Ltd, Tokyo, Japan) was used to examine the stages of root development and the morphological changes of the replants in the *Opn* KO 2W and WT 2W groups at 3, 5, 7, and 14 days following tooth replantation. The CT settings were as follows: pixel matrix, 512 × 512 × 256; slice thickness, 20.67 μm; projection number, 900 × 32; magnification, × 5.3; voltage, 63 kV; and electrical current, 101 μA. The maxillae were reconstructed using a software program (TRI/3D Bon, Ratoc System Engineering, Tokyo, Japan) to evaluate the three-dimensionally reconstructed views of the maxillae including the upper first molars (M1). The untreated teeth from the *Opn* KO 2W and WT 2W groups were used as the control groups.

2.4. Tissue preparation

Tissues were collected from three to ten animals at intervals of 3, 5, 7, 14, and 56 days after tooth replantation (n = 84) as shown in Table 1. At each stage, the animals were perfused with 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) under deep anesthesia following an intraperitoneal injection of a mixed solution (0.05–0.1 mL/10 g) of Domitor®, midazolam, Vetorphale®, and physiological saline and each maxilla was immersed in the same fixative. The procedure for tissue preparation was previously described [17].

2.5. Immunohistochemical analysis

Immunohistochemistry was performed essentially as described in our previous report [17] with a mouse anti-Nestin monoclonal antibody diluted 1:200 (Millipore, Temecula, CA; catalog number: MAB353). The Envision + Horseradish Peroxidase System (Dako Japan, catalog number: K5027) was used along with a rabbit anti-human PGP9.5 antibody diluted to 1:400 (Ultra Clone Ltd., Isle of Wight, UK; catalog number: RA95101), a rat anti-mouse CD31 antibody diluted to 1:50 (BD Pharmingen, San Diego, CA; catalog number: 553370), and a rat anti-Ki67 monoclonal antibody diluted to 1:100 for the cell proliferation assay (Dako Japan, Tokyo, Japan; catalog number: M7249). The avidin-biotin peroxidase complex (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA) formation was determined using biotinylated anti-rabbit or anti-rat immunoglobulin G (Vector Laboratories; catalog number: BA-4000).

2.6. In situ *hybridization*

Section *in situ* hybridization was performed as previously described [18]. Digoxigenin-labeled probe for *Col1a1* [19], which encodes type I collagen protein, was prepared according to the manufacturer's instructions (Roche Diagnostics Corp, Indianapolis, IN).

2.7. Statistical analysis

The root length of the upper first molar was measured in μCT images using Tri-bone Software and Image J software (Image J 1.45s; National Institutes of Health, Bethesda, MD). The percentage of Nestin-positive perimeters in the total perimeter of the pulp-dentin border was calculated using Image J software. Data were obtained from samples of *Opn* KO and WT teeth (Table 1) and one optimal section was selected from each tooth. For the cell proliferation assay based on Ki67 immunohistochemistry, one optimal section was selected from each tooth (Table 1). For the analysis of hard tissue and blood vessel areas, hematoxylin and eosin (H&E) stained sections were prepared from the samples of *Opn* KO and WT teeth (Table 1) and analyzed using Image J and WinRoof software (WinRoof Version 7.4; Mitani Corporation, Tokyo, Japan). The root length, the percentage of hard tissue and blood vessel areas, Nestin-positive perimeter, and Ki67-positive cell density among different groups were compared using a one-way analysis of variance followed by

Bonferroni's multiple comparisons test after the confirmation of data normality and homogeneity of variance (SPSS 21.0.0.0 for Windows; SPSS Japan, Tokyo, Japan). The samples showing no normal distribution were compared using the Kruskal-Wallis test followed by Bonferroni's test for multiple comparisons. Data were reported as the mean + SD and p denotes the p-value.

Table 1. Number of Animals in Each Experiment and for Statistical Analysis after Tooth Replantation

		Experiment	3D	5D	7D	14D	56D	Total
***Opn* KO**	2W Replantation	μCT	(3)	(3)	(3)	(3)	0	(12)
		H&E	4	4	3	3	6	20
		Nestin (IHC)	(4)	(4)	(4)	(3)	(3)	(18)
		Col1a1 (ISH)	1	1	1	0	0	3
		Ki67 (IHC)	(4)	(4)	(3)	(3)	0	(14)
		CD31 (IHC)	(3)	(3)	(3)	(3)	0	(12)
		PGP 9.5 (IHC)	(3)	(3)	(3)	(3)	0	(12)
	3W Replantation	μCT	(3)	(3)	(3)	(3)	0	(12)
		H&E	6	10	10	7	0	33
		Nestin (IHC)	(3)	(4)	(6)	(3)	0	(16)
		Ki67 (IHC)	(3)	(4)	(4)	(3)	0	(14)
		CD31 (IHC)	(3)	(3)	(3)	(3)	0	(12)
		PGP 9.5 (IHC)	(3)	(3)	(3)	(3)	0	(12)
WT	2W Replantation	μCT	(3)	(3)	(3)	(3)	0	(12)
		H&E	4	3	3	3	0	13
		Nestin (IHC)	(4)	(4)	(4)	(3)	0	(15)
		Col1a1 (ISH)	1	1	1	0	0	3
		Ki67 (IHC)	(4)	(4)	(3)	(3)	0	(14)
		CD31 (IHC)	(3)	(3)	(3)	(3)	0	(12)
		PGP 9.5 (IHC)	(3)	(3)	(3)	(3)	0	(12)
	3W Replantation	μCT	(3)	(3)	(3)	(3)	0	(12)
		H&E	3	3	3	3	0	12
		Nestin (IHC)	(3)	(3)	(3)	(3)	0	(12)
		Ki67 (IHC)	(3)	(3)	(3)	(3)	0	(12)
		CD31 (IHC)	(3)	(3)	(3)	(3)	0	(12)
		PGP 9.5 (IHC)	(3)	(3)	(3)	(3)	0	(12)
								84

3. Results

3.1. Pulpal healing was achieved in the Opn *KO 2W group, but not the* Opn *KO 3W group*

The root formation of replants progressed for 3–14 days after tooth replantation in the *Opn* KO 2W and WT 2W groups (Fig. 1a–h), although the length of the roots of the replants was shorter than that of untreated control developing teeth (Fig. 1i, j). A significant difference was observed in the length of the mesial root between the control and replanted teeth in the *Opn* KO 2W group on day 5 (Fig. 1i). The occurrence of hard tissue was calculated based on Nestin-positive (tertiary dentin) or Nestin negative hard tissue areas in the total pulp area (Fig. 1k). The tertiary dentin area of the *Opn* KO 3W group significantly decreased compared with that of the *Opn* KO 2W and WT groups. On day 3 in the *Opn* KO 2W and WT groups, the odontoblasts exhibited degenerated features beneath the coronal dentin, with an artificial detached distribution of their layer from the predentin. On day 7 in the *Opn* KO 2W and WT groups, the revascularization was completed and the inflammatory reaction ceased throughout the dental pulp to achieve pulpal healing (Fig. 1l–q). In contrast, a severe inflammatory reaction occurred to disturb pulpal healing in the *Opn* KO 3W group (Fig. 1n, o).

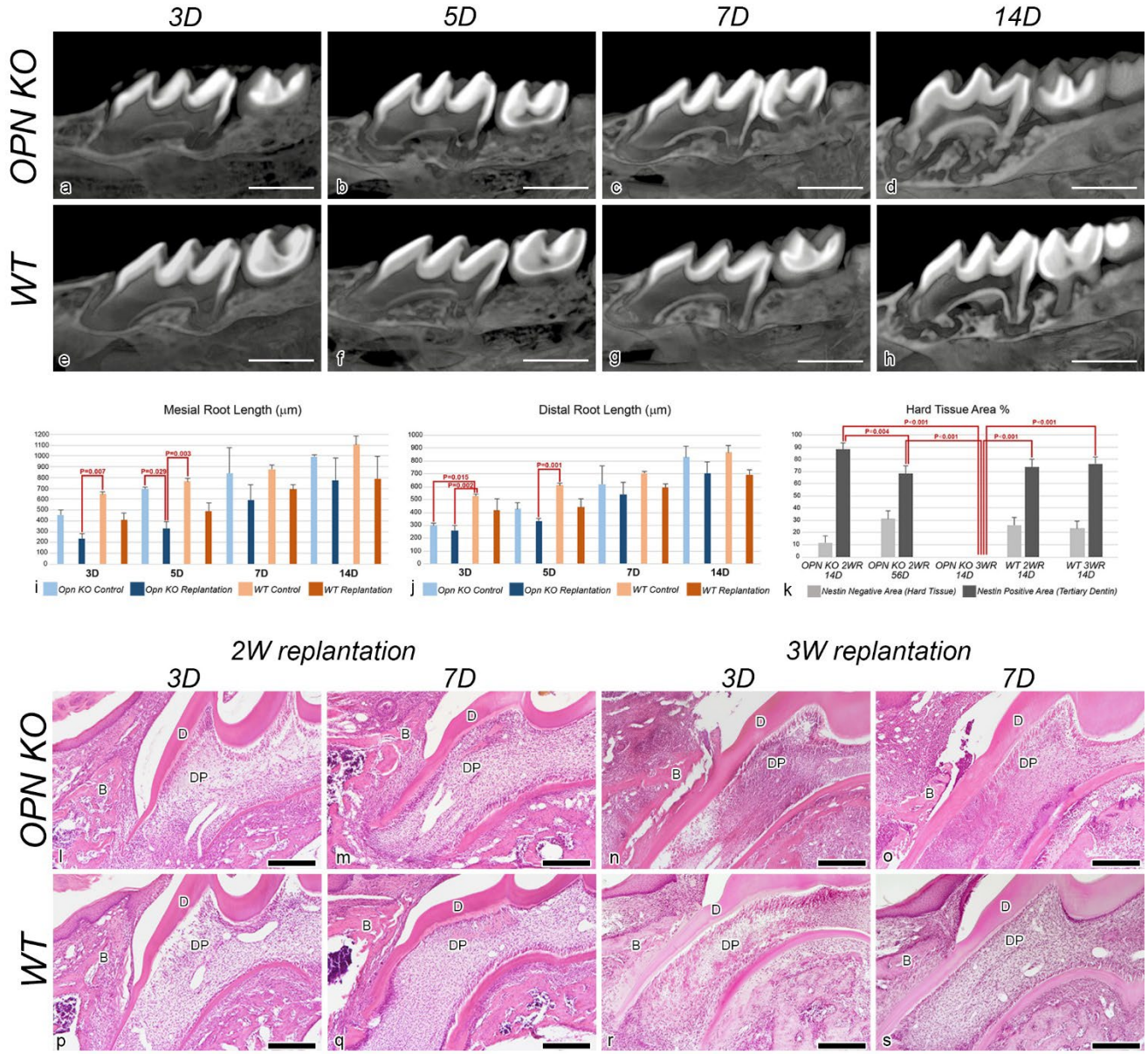

Fig. 1. μCT images (a–h) and H&E-stained sections (l–s) of replanted teeth 3 (a, e, l, n, p, r), 5 (b, f), 7 (c, g, m, o, q, s), and 14 days (d, h) after operation in Opn KO 2W (e–h, l, m), Opn KO 3W (n, o), WT 2W (a–d, p, q), and WT 3W (r, s) groups. Quantitative analyses of mesial (i) and distal root (j) length in μCT images and hard tissue area percentage at 14 and 56 days in Nestin-stained sections (k). (a–h) The root formation of replants progresses during days 3–14. (i, j) A significant difference was observed in the length of the mesial root between the control and replanted teeth in the Opn KO 2W group on day 5. (k) The tertiary dentin of the Opn KO 3W group significantly decreased in area compared with the Opn KO 2W and WT groups. (l–s) On day 3 in the Opn KO 2W and WT groups, the odontoblasts show degenerated features beneath the coronal dentin, with an artificial detached distribution of their layer from the predentin. On day 7 in the Opn KO 2W and WT groups, the revascularization is completed and the inflammatory reaction ceases throughout the dental pulp to achieve pulpal healing. A severe inflammatory reaction occurs which disturbs pulpal healing in the Opn KO 3W group. Scale bars = (a–h) 1000 μm, (l–s) 250 μm. B, bone; D, dentin; DP, dental pulp.

3.2. The Opn KO 2W group exhibits newly-differentiated OBLCs and reparative dentin formation in addition to the WT group

The *Opn* KO 3W group exhibited impaired reparative dentin formation with a significantly low percentage of Nestin-positive newly-differentiated OBLCs during postoperative days 7–14. In contrast, in the WT and *Opn* KO 2W groups, Nestin-positive newly-

differentiated OBLCs were arranged beneath the reparative dentin (Fig. 2a–p, u). The *Opn* KO 2W and WT 2W groups showed a significantly higher occurrence of Nestin-positive newly-differentiated OBLCs along the pulp-dentin border on day 5 compared with the *Opn* KO 3W group. Subsequently, there were no significant differences among the *Opn* KO 2W and WT groups during days 7–14 (Fig. 2u). In addition, the timing of *Col1a1* mRNA expression in the root pulp in the *Opn* KO 2W group was faster compared with that of the WT 2W group on day 3 (Fig. 2q, s), and the pulp-dentin border of the whole pulp expressed *Cola1a1* in both the WT 2W and *Opn* KO 2W groups on day 7 (Fig. 2r, t).

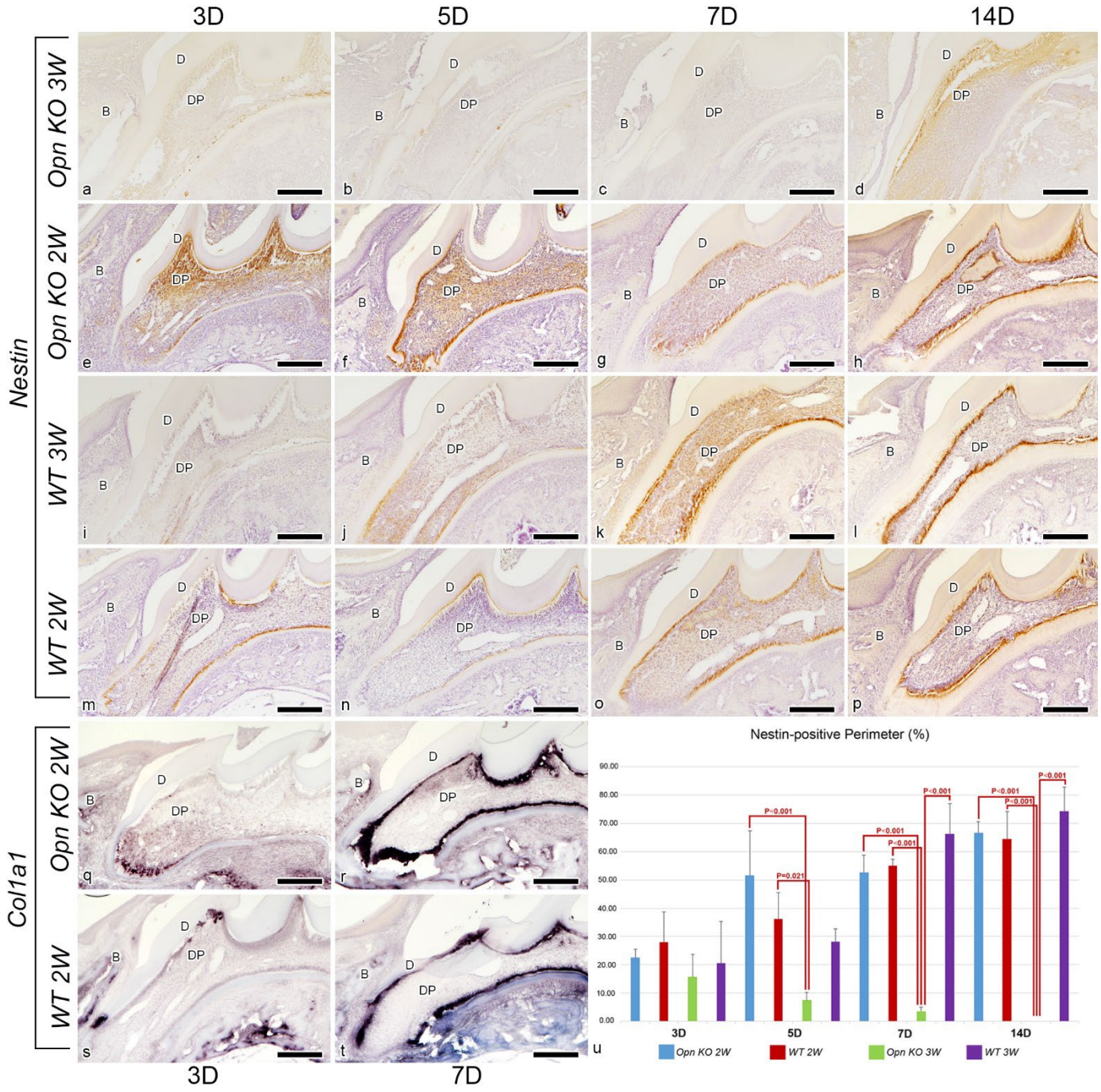

Fig. 2. Nestin immunohistochemistry (a–p) and *Col1a1 in situ* hybridization (q–t) in replanted teeth 3 (a, e, i, m, q, s), 5 (b, f, j, n), 7 (c, g, k, o, r, t), and 14 days (d, h, l, p) after operation in the *Opn* KO 3W (a–d), *Opn* KO 2W (e–h, q, r), WT 3W (i–l), and WT 2W (m–p, s, t) groups. Quantitative analysis of the Nestin-positive perimeter (u). (a–p) The *Opn* KO 3W

group displays impaired reparative dentin formation with a low percentage of Nestin-positive newly-differentiated OBLCs during postoperative days 7–14. In the WT and *Opn* KO 2W groups, Nestin-positive newly-differentiated OBLCs are arranged beneath the reparative dentin. (q–t) The timing of *Col1a1* mRNA expression in the root pulp of the *Opn* KO 2W group is faster compared with that of the WT 2W group on day 3. The pulp-dentin border of the whole pulp expresses *Col1a1* in both the WT 2W and *Opn* KO 2W groups on day 7. (u) Quantitative analysis of the Nestin-positive perimeter shows a significant lower percentage in the *Opn* KO 3W group compared with all other groups during days 7–14. Scale bars = 250 μm. B, bone; D, dentin; DP, dental pulp.

3.3. The Opn *KO 2W group displayed cell proliferation within the dental pulp after replantation*

The analysis of cell proliferation by Ki67 immunostaining confirmed a lack of proper pulp healing in the *Opn* KO 3W group, with significantly decreased cell proliferation activity (Fig. 3a–f) compared with the *Opn* KO 2W and WT 2W groups on day 3 and the *Opn* KO 2W and WT 3W groups on day 5 (Fig. 3g). The cell proliferation activity of the *Opn* KO 2W group tended to be higher compared with that of the WT 2W group. The proliferative activity reached its peak on day 5 in the *Opn* KO 2W group, whereas it occurred on day 3 in the WT 2W group (Fig. 3g).

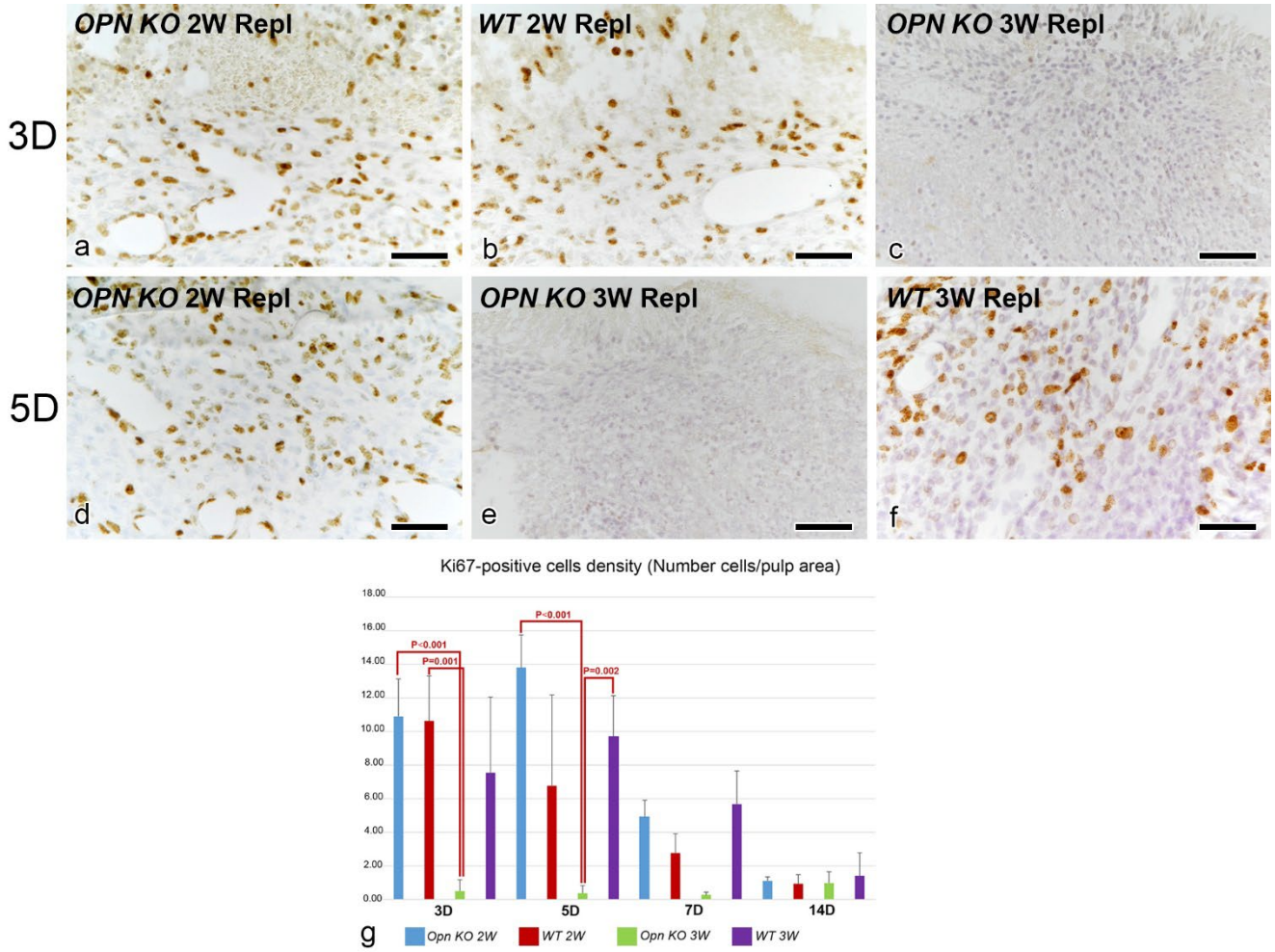

Fig. 3. Ki67 immunohistochemistry (a–f) in the dental pulp 3 (a–c) and 5 days (d–f) after operation in the Opn KO 2W (a, d), Opn KO 3W (c, e), WT 2W (b), and WT 3W (f) groups. Quantitative analysis of Ki67-positive cell density in the dental pulp (g). (a–f) The Opn KO 3W group lacks proliferative activity in the dental pulp, whereas all other groups show active cell proliferation. (g) The Opn KO 3W group exhibits a significant decreased in cell proliferative activity compared with the Opn KO 2W and WT 2W groups on day 3 and the Opn KO 2W and WT 3W groups on day 5. Scale bars = 50 μm.

3.4. Vascularization and innervation are reestablished within the dental pulp in the Opn KO 2W group

In the *Opn* KO 3W group, pulp healing was disturbed with a failure of pulpal revascularization and reinnervation (Fig. 4c, j). The area of blood vessels in the *Opn* KO 3W group was significantly decreased compared with that in the WT groups on day 5, WT 3W group on day 7, and the *Opn* KO 2W and WT 3W groups on day 14 (Fig. 4g). In contrast, the *Opn* KO 2W group achieved revascularization and reinnervation within the dental pulp (Fig. 4a, b, h, i). The existence of reestablished blood vessels was confirmed by the presence of CD31-positive endothelial cells during days 3–7 (Fig. 4a–f).

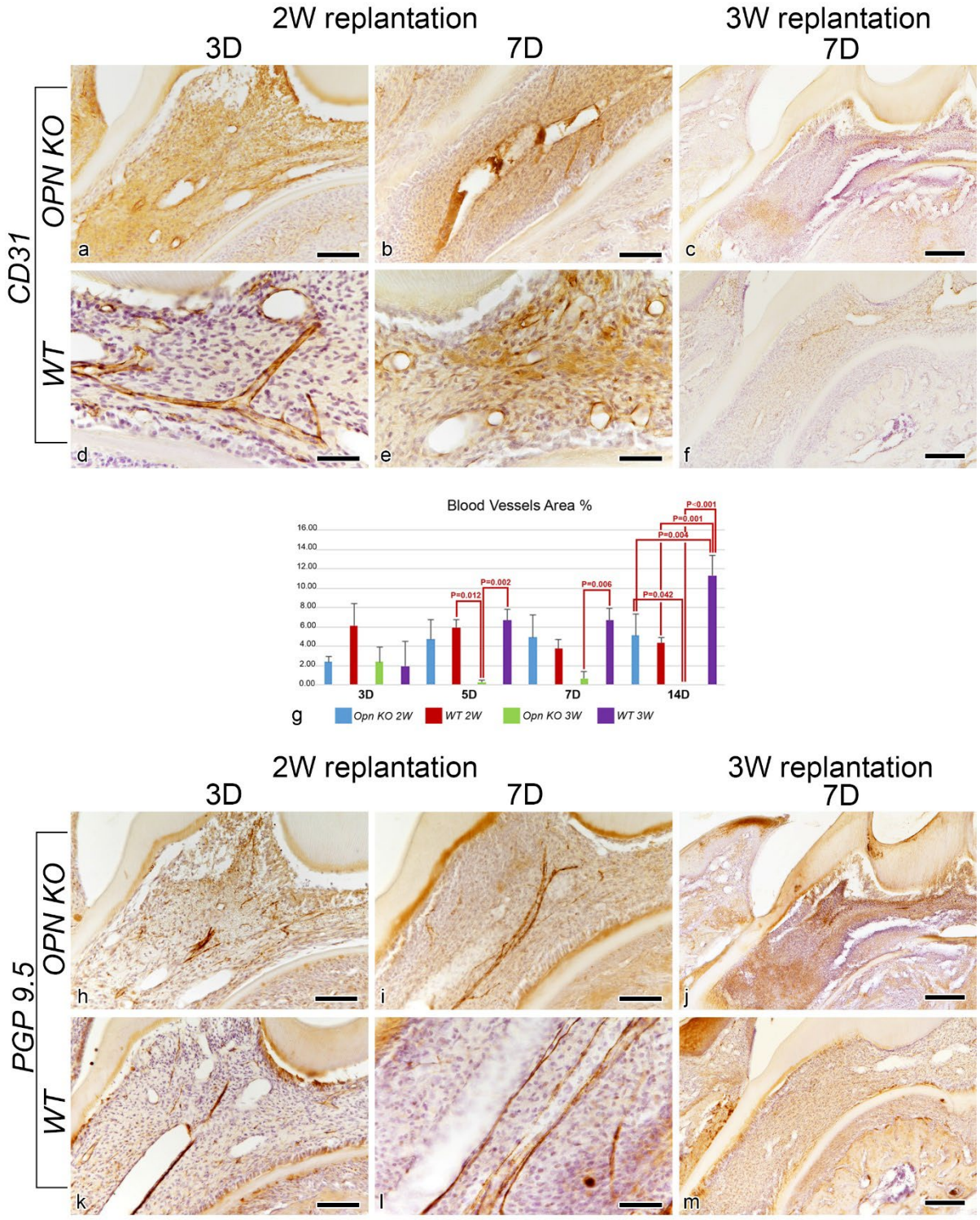

Fig. 4. CD31 (a–f) and PGP 9.5 (h–m) immunohistochemistry in replanted teeth 3 (a, d, h, k) and 7 days (b, c, e, f, i, j, l, m) after operation in the Opn KO 2W (a, b, h, i), Opn KO 3W (c, j), WT 2W (d, e, k, l), and WT 3W (f, m) groups. Quantitative analysis of blood vessel area percentage in H&E-stained sections. (a–f, h–m) In the Opn KO 3W group, pulp healing is disturbed with a failure of pulpal revascularization and reinnervation. The Opn KO 2W group succeeded in revascularization and reinnervation within the dental pulp. (g) The area of blood vessels in the Opn KO 3W group significantly decreased compared with that in the WT groups on day 5, the WT 3W group on day 7, and the Opn KO 2W and WT 3W groups after 14 days. Scale bars = (c, f, j, m) 250 μm, (a, b, h, i, k) 100 μm, (d, e, l) 50 μm.

4. Discussion

4.1. The significance of the experimental design

The main difference between the experimental groups is that the maxillary first molar of the *Opn* KO 2W group is characterized by early root development and non-erupted tooth, whereas the *Opn* KO 3W group contained the erupted tooth with advanced root development. Compared with the *Opn* KO 2W with WT 2W control groups, root development in the former group was delayed compared with the latter group, indicating a different baseline between these groups. Moreover, a significant difference was temporarily observed in the length of the mesial root between the control and replanted teeth in the *Opn* KO 2W group on day 5. The experiment for tooth replantation using 2-week-old mice provides a better environment for replant without serious deleterious effects on Hertwig's epithelial root sheath because of the short length of the replant roots and gingival covering.

4.2. The role of OPN in the process of reparative dentinogenesis

The tertiary dentin in the *Opn* KO 3W group was significantly decreased in area compared with the *Opn* KO 2W and WT groups, with a significantly low percentage of Nestin-positive newly-differentiated OBLCs present during postoperative days 7–14. OPN deficiency inhibits OBLC differentiation to induce no hard tissue formation in the replants with advanced root formation. OPN is deposited at the boundary between the preexisting and reparative dentin after cavity preparation in mice, resulting in a role for OPN in type I collagen secretion [17]. However, the *Opn* KO 2W group showed OBLC differentiation. A reduction in root length can accelerate pulp regeneration by improving the revascularization of the replanted teeth [20]. The favorable ratio of broad apical foramen/short root canal is important, since the foramen is the primary access route for blood vessels and nerves to the coronal dental pulp. If the apical foramen is too small, it may prevent cell migration, revascularization, and reinnervation. Moreover, ischemic pulp healing depends on the prevalence of reinnervation and revascularization and the absence of bacterial invasion [21]. This is based on the difference between the experimental groups (non-erupted versus erupted replants) and the gingival covering that protects from oral microflora in the case of the *Opn* KO 2W group. The *Opn* KO 2W and WT 2W groups exhibited significantly higher numbers of Nestin-positive newly-differentiated OBLCs along the pulp-dentin border on day 5 compared with the *Opn* KO 3W group. Subsequently, there were no significant differences between the *Opn* KO 2W and WT groups during days 7–14. With respect to cavity preparation, reparative dentin formation is disturbed because of the loss of *Col1a1* mRNA expression in the *Opn* KO mice. In contrast, reactionary dentin formation is quite normal even in these mice, indicating that OBLCs require OPN for the secretion of type I collagen, whereas OPN is not necessary for the surviving odontoblasts responsible for reactionary dentin [17]. As mentioned above, reparative dentin formation occurred in the *Opn* KO 2W group in the present study. The stem/progenitor cells for OBLCs may be different between the mild injury models, such as cavity preparation, and severe injury models, such as tooth replantation. The former model provides localized injury beneath the disrupted dentin, whereas the latter model results in the total death of odontoblasts throughout the dental pulp. Furthermore, tooth replantation results in the degeneration of the subodontoblastic layer and the central pulp tissue in addition to the odontoblasts. Dental pulp stem/progenitor cells are localized within the perivascular niche in the subodontoblastic layer and the central pulp tissue. In fact, our previous studies using prenatal labeling methods with BrdU and Tet-OP-histone 2B (H2B)-green fluorescent protein (GFP) mice demonstrated the occurrence of label-retaining cells in these niches [2]. These findings suggest that other types of stem/progenitor cells commit themselves to OBLCs in the *Opn* KO 2W group.

During early root development, the apical papilla harbors mesenchymal stem cells (MSCs), which are stem cells derived from the apical papilla (SCAP), which are capable of becoming OBLCs to produce dentin *in vivo* [22], enlightening the potential role of SCAP in pulpal healing and regeneration. The first molar of the *Opn* KO 2W group is the developing tooth with immature roots containing many SCAP at the apical portion. SCAP-derived OBLCs may not need OPN for type I collagen secretion. This notion is supported by the previous evidence that the coronal subodontoblstic layer is different from the other pulp cells with respect to their origin. The former is of neuronal ectodermal origin, whereas the latter is of neural crest origin [23]. SCAP can easily reach the coronal pulp chamber because of the short distance. Further studies are needed to identify the multiple types of stem/progenitor cells for OBLCs. The timing of *Col1a1* mRNA expression in the root pulp of the *Opn* KO 2W group is faster compared with that of the WT 2W group on day 3, and the pulp-dentin border of the whole pulp expressed *Col1a1* mRNA in both the WT 2W and *Opn* KO 2W groups on day 7. These findings support the above notion. In the early stage of healing, the activity of *Col1a1* mRNA in the *Opn* 2W group suggests high SCAP activity. The cell proliferative activity of the *Opn* KO 2W group tended to be higher compared with that of the WT 2W group, however, peak proliferative activity in the WT 2W group is faster (on day 3) compared with that in the *Opn* KO 2W group (on day 5). The delayed healing process in the *Opn* KO 2W group compared with that in the WT 2W group may be attributed to an abnormal immune response resulting from *Opn* deficiency [24].

4.3. Relationship between OPN and angiogenesis/axonal regeneration

In the *Opn* KO 3W group, the blood vessels were significantly decreased in area and the pulp healing was disturbed with a failure of pulpal revascularization and reinnervation. Angiogenesis is important for organ growth and repair, as hypoxia following injury induces complex and specific pro-angiogenic responses within the dental pulp where vascular endothelial growth factor (VEGF) participates in the revascularization process [25]. The consequence of vascular disruption during hypoxia triggers an increase of MSC migration and expression of OPN in osteocytes [26]. In the *Opn* KO mice with advanced root development, pulp healing is disturbed with a failure of pulpal revascularization. OPN enhances angiogenesis directly through the activation of the PI3K/Akt pathway and improves the expression of VEGF [27], confirming an important role of OPN during angiogenesis in tissue regenerative events. Revascularization is an important event during reinnervation, in which the distal stump becomes vascularized in response to the macrophage-induced VEGF signal with Schwann cells (SCs) migrating along the vasculature [28]. A peripheral nerve injury triggers a Wallerian degeneration process, which includes a multicellular response primarily from SCs, blood vessels, and immunocompetent cells [29]. The dental pulp also exhibits neuroplasticity, and nerve fibers display a prominent and progressive axonal degeneration in a Wallerian-like scheme under physiological root resorption [7]. In the present study, the axonal regeneration was impaired within dental pulp of the *Opn* KO 3W group with advanced root development. Previous studies demonstrated that SCs express OPN in the degenerating distal nerve stump during the first days following injury [30]. OPN was significantly upregulated after peripheral nerve injury to promote proliferation and inhibition of SC apoptosis [31]. These findings indicate the importance of OPN during axonal regeneration in peripheral nerves with a special role in the survival of SCs, which are fundamental in providing the necessary signals and spatial cues to the injured nerves.

5. Conclusions

OPN has an important role during pulp regeneration, since the re-establishment of innervation and vascularization are major processes during pulp healing. The present

study also demonstrated that OPN is necessary for proper reinnervation and revascularization to deposit reparative dentin after severe injury within the dental pulp in erupted teeth with advanced root development.

Conflicts of interest: The authors declare no conflicts of interest related to this study

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