Original Article

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

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A R T I C L E   I N F O

Article history:
Received 24 June 2022
Received in revised form 24 September 2022
Accepted 29 September 2022

Keywords:
Animal model
Blood supply
Dentinogenesis
Innervation
Osteopontin
Tooth replantation

A B S T R A C T

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.

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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 calcificated 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

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.

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Peer review under responsibility of the Japanese Society for Regenerative Medicine.

https://doi.org/10.1016/j.reth.2022.09.011
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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-Sppflm1Blh/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 to clarify the role of OPN in the process of reparative dentin formation following tooth replantation using the unerupted teeth with short roots in the 2W group as well as the erupted teeth with long roots in the 3W group. 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), and 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: M7249). The avidin-biotin peroxidase complex (Vector Laboratories, Burlingame, CA) 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.

2.6. In situ hybridization

Section in situ hybridization was performed as previously described [18]. Digoxigenin-labeled probe for Col1α1 [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/3D Bon 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 pulpdentin 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.

3. Results

3.1. Pulpal healing was achieved in the Opn KO 2W group, exhibiting newly-differentiated OBLC differentiation and reparative dentin formation

The root formation of replants progressed for 3–14 days after tooth replantation in the Opn KO 2W and WT 2W groups (Fig. 1a), although the length of the roots of the replants was shorter than that of untreated control developing teeth (Fig. 1b). 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. 1b). On day 3 in the Opn KO 2W and WT 2W groups, the odontoblasts exhibited degenerated features or an exudative lesion occurred beneath the coronal dentin. On day 7 in the Opn KO 2W and WT 2W groups, the revascularization was completed and the inflammatory reaction ceased throughout the dental pulp to achieve pulpal healing (Fig. 1c, d, g, h). In the WT 2W and Opn KO 2W groups, Nestin-positive newly-differentiated OBLCs were arranged beneath the reparative dentin (Fig. 1k–l). The filamentous structures also showed Nestin-positive reaction in addition to the OBLCs during days 3–5 (Fig. 1k and l). 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. 1e, i), and the pulp-dentin border of the whole pulp expressed Col1a1 in both the WT 2W and Opn KO 2W groups on day 7 (Fig. 1f, j). On day 56 in the Opn KO 2W group, Nestin-positive OBLCs were arranged along the pulpdentin border, although the pulp cavity was reduced in size (Fig. 2a–d).

3.2. Pulpal healing was not achieved in the Opn KO 3W group

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. 2e). The tertiary dentin area of the Opn KO 3W group significantly decreased compared with that of the Opn KO 2W and WT 2W groups. In contrast, a severe inflammatory reaction occurred to disturb pulpal healing in the Opn KO 3W group (Fig. 2f and g). On day 3 in the WT 3W group, the odontoblasts exhibited degenerated features beneath the coronal dentin (Fig. 2h). On day 7 in the WT 3W group, the revascularization was completed and the inflammatory reaction ceased throughout the dental pulp to achieve pulpal healing (Fig. 2i). The Opn KO 3W group exhibited impaired reparative dentin formation with a significantly low percentage of Nestin-positive newly-differentiated OBLCs during

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Table 1

| 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 (H&E) | (4) | (4) | (4) | (3) | (3) | 18 |
| | Col1a1 (ISH) | 1 | 1 | 0 | 0 | 3 |
| | Ki67 (H&E) | (4) | (4) | (3) | (3) | 0 | (14) |
| | CD31 (H&E) | (3) | (3) | (3) | (3) | 0 | (12) |
| | PCP 9.5 (H&E) | (3) | (3) | (3) | (3) | 0 | (12) |
| Opn KO 3W Replantation | µCT | (3) | (3) | (3) | (3) | 0 | (12) |
| | H&E | 6 | 10 | 10 | 0 | 33 |
| | Nestin (H&E) | (3) | (4) | (6) | (3) | 0 | (16) |
| | Ki67 (H&E) | (4) | (4) | (4) | (3) | 0 | (14) |
| | CD31 (H&E) | (3) | (3) | (3) | (3) | 0 | (12) |
| | PCP 9.5 (H&E) | (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 (H&E) | (4) | (4) | (4) | (3) | 0 | (15) |
| | Col1a1 (ISH) | 1 | 1 | 1 | 0 | 3 |
| | Ki67 (H&E) | (4) | (4) | (3) | (3) | 0 | (14) |
| | CD31 (H&E) | (3) | (3) | (3) | (3) | 0 | (12) |
| | PCP 9.5 (H&E) | (3) | (3) | (3) | (3) | 0 | (12) |
| WT 3W Replantation | µCT | (3) | (3) | (3) | (3) | 0 | (12) |
| | H&E | 3 | 3 | 3 | 3 | 0 | 12 |
| | Nestin (H&E) | (3) | (3) | (3) | (3) | 0 | (12) |
| | Ki67 (H&E) | (3) | (3) | (3) | (3) | 0 | (12) |
| | CD31 (H&E) | (3) | (3) | (3) | (3) | 0 | (12) |
| | PCP 9.5 (H&E) | (3) | (3) | (3) | (3) | 0 | (12) |

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Fig. 1. μCT images (a), quantitative analyses of mesial and distal root length in μCT images (b), H&E-stained sections (c, d, g, h), Col1α1 in situ hybridization (e, f, i, j), and Nestin immunohistochemistry (k–r) of replanted teeth 3 (a-1, a-5, c, g, e, i, k, o), 5 (a-2, a-6, l, p), 7 (a-3, a-7, d, h, f, j, m, q), and 14 days (a-4, a-8, n, r) after operation in OpnKO 2W (a-1–4, c–f, k–n) and WT 2W (a-5–8, g–j, o–r) groups. (a) The root formation of replants progresses during days 3–14. (b) A significant difference was observed in the length of the mesial root between the control and replanted teeth in the OpnKO 2W group on day 5. (c, d, g, h) On day 3 in the OpnKO 2W and WT 2W groups, the odontoblasts show degenerated features (*) or an exudative lesion (E) occurs beneath the coronal dentin, with an artificial detached distribution of their layer from the predentin. On day 7 in the OpnKO 2W and WT 2W groups, the revascularization (arrows) is completed and the inflammatory reaction ceases throughout the dental pulp to achieve pulpal healing. (e, f, i, j) The timing of Col1α1 mRNA expression in the root pulp of the OpnKO 2W group is faster compared with that of the WT 2W group on day 3. The pulp-dentin border of the whole pulp expresses Col1α1 in both the WT 2W and OpnKO 2W groups on day 7. (k–r) In the OpnKO 2W and WT 2W groups, Nestin-positive newly-differentiated OBLCs are arranged beneath the reparative dentin. Scale bars – (a) 1000 μm, (e, f, i, j, k–r) 250 μm, (c, d, g, h) 100 μm. B, bone; D, dentin; DP, dental pulp; OBLC, odontoblast-like cells.
postoperative days 7–14. In contrast, in the WT 3W group, Nestin-positive newly-differentiated OBLCs were arranged beneath the reparative dentin (Fig. 2k–r). 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. 2j).

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 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. 3a–c). 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. 3c).

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

CD31 is a cell adhesion molecule highly expressed on endothelial cells and concentrated at the junctions between them to be used as a general marker for blood vessels Fig. 4a, c, e, g, i, k, m, o), whereas PGP9.5 is highly expressed in neurons to be used as a general marker for neuronal tissue including peripheral nerves (Fig. 4b, d, f, h, j, l, n, p). In the Opn KO 3W group, pulp healing was disturbed with a failure of pulpal revascularization and reinnervation (Fig. 4i–l). 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. 4q). In contrast, the Opn KO 2W group achieved revascularization and reinnervation within the dental pulp (Fig. 4a–d). The existence of reestablished blood vessels was confirmed by the presence of CD31-positive endothelial cells during days 3–7 (Fig. 4a, c, e, g, m, o).

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 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. Furthermore, the Nestin-positive filamentous structures appear in the pulp in which increasing numbers of proliferative cells and rich revascularization occur. These findings may indicate a correlation between the up-regulation of Nestin expression and activation of the proliferative potential of dental pulp stem/progenitor cells. 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 form 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 subodontoblastic layer is different from the other pulp cells with respect to their origin. The former is of neural crest 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.

### Author contributions

KS-B contributed to data curation; formal analysis; investigation; methodology; validation; writing—original draft preparation. SM, KS, MN, and HI-Y contributed to investigation; validation; writing—review & editing. HO contributed to conceptualization; data curation; investigation; methodology; supervision; validation; writing—original draft preparation.

### Declaration of competing interest

The authors declare no conflicts of interest related to this study.

### Acknowledgments

The authors cordially thank Dr. T. Komori for providing Col1a1 plasmids, Ms. Y. Abe and Ms. M. Kawachi for their technical assistance, and Enago (www.enago.jp) for the English language review. Dr. K. Suzuki-Barrera was supported by Scholarship for Japanese Emigrants and their Descendants in Latin America and the Caribbean: Program for Developing Leaders in Nikkei Communities, JICA-Japan. This work was supported by Grants-in-Aid for Scientific Research (B) (no. 17H04366 to HO) and Challenging Research (Pioneering) (no. 20K21672 to HO) from the Japan Society for the Promotion of Science.

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