Localization of pulp stem cells in chronic pulpitis

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Abstract

Background: Our study aimed to observe the distribution of stem cells in chronic pulpitis and to investigate the expression of specific molecules. Methods: Extracted third molar teeth were collected and divided into two groups. Experiment group was from patients with previous history of spontaneous pain and diagnosed as having chronic pulpitis. Noncarious third molars were used as control group. Dental pulp tissues were collected from these two groups. Real-time RT-PCR test was used to detect the expression of several embryonic and dentinogenic genes in dental pulp. The expression of the mesenchymal cell markers (STRO-1, CD146) and stromal cell-derived factor 1α (SDF-1α)/CXC chemokine receptor 4 (CXCR4) proteins were examined by immunohistochemical and triple immunofluorescence analysis. Results: ThemRNA levels of most embryonic and dentinogenic genes were not statistically different ($p > 0.05$) between the two groups. Immunohistochemical analysis revealed that in inflamed pulp, STRO-1- and CD146-positive cells mainly reside in two specific niches, both adjacent to inflammatory sites: one in the pulp core, and another in odontoblast layer. In contrast, fewer STRO-1- and CD146- positive cells appeared in the vicinity of the root apex. SDF-1α- and CXCR4-positive cells increased in zone I and II, and decreased in zone III, showing a significant correlation with STRO-1. Triple immunofluorescence analysis indicated that most STRO-1-positive cells overlapped with SDF-1α- and CXCR4-positive cells around the perivascular areas near the inflammatory site. Conclusions: This study gave a direct observation of stem cells distribution in chronic pulpitis for the first time and implies the important role of SDF-1α/CXCR4signaling in stem cells-based therapies for reparative dentinogenesis.

Introduction

In healthy tissues, stem cells are found in their specific niche environment. Residing in the dental tissues, pulp stem cell niches have been identified during the last 15 years [1, 2]. By using a combination of marker it was suggested that a population of stem cells reside in the pulp proper, especially at the periphery of main arterioles [1, 3, 4]. The niche in the dental pulp is flexible in order to coordinate stem cell behavior with homeostasis and repair; however, the plasticity of the niche may be co-opted in significant infection and chronic disease.

Irreversible pulpitis is a common and typical inflammatory disease of dental pulp. Root canal treatment remains the only choice when the pulp is too deeply inflamed to expect recovery. Unfortunately, histologic observations of pulp tissue diagnosed as irreversible pulpitis do not show that serious destruction [5]. During last years, new evidence reported that pulpitis-derived stem cells exhibited the similar capability of proliferation and multipotent differentiation capabilities as compared with those from healthy pulps, suggesting that inflamed pulp may not be completely depleted of stem cells but can be repaired [6–8]. Since the pulp stem cell niches are the main resource of tissue regeneration, identifying the niches in the inflamed pulp highlights the potential interplay between inflammatory and regenerative events. In this study, we focus on the localization of stem cells induced to leave their quiescent niche during chronic pulpitis, and investigate the expression of specific molecules.

Materials And Methods

Experimental Specimens

Experimental specimens were obtained from patients after institutional review board–approved informed consent. The patient age rangefrom20 to 35 years old and patients were hospitalized in the Department of Stomatology, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. All specimens were from extracted third molar teeth. In the normal pulp group (control group, n = 7), the teeth were fully erupted, with clinically intact crowns, and were surrounded by clinically healthy periodontal tissue. Three teeth were used for PCR analysis and another four teeth were used for immunohistochemistry analysis. In the inflamed pulp group (experiment group, n = 10), the teeth were diagnosed as chronic pulpitis that presented with previous history of spontaneous pain and lingering pain caused by cold and/or thermal tests. These teeth had clinical and radiographic evidence of deep carious lesions extending toward the pulp chamber. Three teeth were used for PCR analysis, four teeth for immunohistochemistry analysis and three teeth for immunofluorescence analysis.

Real-time RT-PCR analysis

Total RNA was extracted from pulp tissue samples by using Trizol reagents (Invitrogen Life Technologies) according to manufacturer's instructions. Aliquots (1 mg) of RNA were reverse transcribed to cDNA (20 ml) with Oligo dT and ReverTra Ace (Toyobo, Osaka, Japan). Real-time PCR analysis was performed with a StepOne™ Real-Time PCR System (Life technologies, US) in 20 ml of an SYBR Premix Ex Taq II solution (TaKaRa Biotechnology Co., Dalian, China) containing 10 mmol/L each of the specific primers. Primers were designed as follows: β-actin (forward: 5'-GTCCACCGCAAATGCTTCTA-3', reverse: 5'-TGCTGTCACCTTCACCGTTC-3', 190 base pairs [bp]); OCT4 (forward: 5'- AGGTATTCAGCCAAACGACCAT-3', reverse: 5'-CACACTCGGACCACATCCTTC-3', 301 base pairs [bp]); NANOG (forward: 5'-CTCCAGCAGATGCAAGAACTCTC-3', reverse: 5'- CATTGGAAGGTTCCCAGTCG-3', 174 base pairs [bp]); SOX-2 (forward: 5'- ATGGGTTCGGTGGTCAAGTC-3', reverse: 5'- GCTCTGGTAGTGCTGGGACAT-3', 183 base pairs [bp]); DSPP (forward: 5'-CATCTCCTAGCAAGATCAAATGTG-3', reverse: 5'-TGGAATATGTAGAAAAACTCTTCCC-3', 190 base pairs [bp]); BSP (forward: 5'-TTAATTTTGCTCAGCATTTTGG – 3', reverse: 5'-CCATTTTCTTCGGATGAGTCAC – 3', 200 base pairs [bp]); KLF4 (forward: 5'-GCTCCATTACCAAGAGCTCATG-3', reverse: 5'- TGGTAAGGTTTCTCACCTGTGT-3', 195 base pairs [bp]); DMP1 (forward: 5'- CTCATGTTCCTTTGGGGATTATC-3', reverse: 5'-TTCCTCTGAGCTAACTTTACTGCC-3', 162 base pairs [bp]); VEGF (forward: 5'-CCGAAACCATGAACTTTCTGC-3', reverse: 5'- GTCCACCAGGGTCTCGATTG – 3', 188 base pairs [bp]). To control variability during amplification because of differences in starting mRNA concentrations, β-actin was used as an internal control. Results data were obtained as threshold cycle (Ct) values. No amplification occurred in the non-template controls. The delta delta Ct method was performed to analyze mRNA expression levels.

Immunohistochemical Staining

Immediately upon extraction, each tooth was fixed in 4% paraformaldehyde at 4 °C for 48 hours. Then the specimens were decalcified with 10% Ethylene Diamine Tetraacetic Acid (EDTA) solution for 8 weeks at 4 °C. Then they were dehydrated in an ethanol series solution and embedded in paraffin. Serial frontal sections (5 μm thick) were cut. For immunostaining, primary antibodies used were Mouse monoclonal anti-human STRO-1 (1 : 10 dilution, R&D Systems Inc., Minneapolis, MN, USA),Rabbit monoclonal anti-human CD146 antibody (1: 200 dilution; Abcam, ab75769), rabbit polyclonal anti-human SDF-1α (1 : 200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit monoclonal anti-human CXCR4 (1 : 200 dilution, Epitomics, California, USA). Sections were subjected to citrate buffer by microwave irradiation for 15 min. Then endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min at room temperature. After washing in phosphate-buffered saline (PBS, pH 7.4) they were incubated with normal goat serum for 30 min at room temperature and then with the primary antibody for 18 hours at 4 °C. Next they were incubated with a biotinylated secondary antibody for 15 min at room temperature. After further rewashing they were incubated with streptavidin-peroxidase (Zhongshan Biotechnology Co., Beijing, China) for 15 min. Finally the sections were developed with 3,3'-diaminobenzidine (Maixin, Fuzhou, China) and confirmed under a microscope. Counterstaining was performed with haematoxylin. Negative controls using PBS instead of the above primary antibody were also prepared. In each specimen, positive cells at the specific region were counted by a blinded observer in 5 randomly selected areas at × 400 magnification. Three sections were counted for every tooth, and the average numbers were subjected to statistical analysis.

Triple immunofluorescence analysis

Formalin-fixed paraffin-embedded sections were treated with the same protocol as described above, except that instead of being incubated with 3% H_2O_2, these sections were incubated with 2.5% BSA in PBS for totally1 hour at 37 °C. The sections were incubated with primary antibodies STRO-1 and CXCR4 diluted in 1% BSA in PBS overnight at 4 °C. Sections were washed with PBS with Tween-20 three times and then incubated for 1 hour with a mixture of the secondary antibodies (FITC goat anti-mouse, 1:200; Cy3 goat anti-rabbit, 1:200). Then after blocking with 2.5% BSA for 1 hour at 37 °C again, the sections were incubated with SDF-1α for 1 hour at 37 °C. After washing in PBS with Tween-20, the sections were incubated with AMCA goat anti-rabbit (1:200). Sections were further rewashed with PBS and nuclei were counterstained by incubation with 4',6-diamidino-2-phenylindole. The fluorescent images were observed and photographed using a fluorescence microscope.

Statistical Analysis

All values were expressed as mean values ± standard deviation (SD). All statistical analyses were performed using one-way ANOVA followed by the Student-Newman-Keul's test. Values were compared using multiple comparisons, where p values of 0.05 or less were considered significant.

Results

Differences in mRNA expression profiles between inflamed and normal pulp tissue

To evaluate the renascent characteristics of inflamed pulp tissue, real-time RT-PCR was performed to determine the expression of embryonic and dentinogenic genes between the two groups. As shown in Fig. 1, compared with the control group, the mRNA level of OCT4A was lower in the inflamed pulp group. Although a slight decrease in mRNA levels of NANOG, KLF4, DMP1, DSPP as well as an increase in levels of BSP and VEGF were also found in inflamed pulp samples, the differences between the two groups were not statistically significant ($p > 0.05$).

Expression pattern of MSC markers in normal and inflamed dental pulp

In normal pulps, STRO-1-positive cells were restricted to the pulp core and rarely present in the odontoblast layer or cell-free area. The expression quantity in the root pulp was lower than that in the crown (Table 1 and Supplementary Fig. 1). CD146 expression was restricted to blood vessel walls in the pulp core. The number of CD146-positive cells was also decreased in the root pulp (Supplementary Fig. 1).

In inflamed pulps, we identified expression of given markers within 3 zones. In the crown, zone I was in the cell-rich zone and pulp core beneath the inflammatory site (Fig. 2A). Zone II covered the odontoblast layer and cell-free zone of the pulp near the inflammation site (Fig. 2B). Zone III was localized within the root pulp, close to the apex (Fig. 2C). Cells labeled with anti-STRO-1 and CD146 antibodies were mainly visualized in zone I and II, in close proximity to the inflammation area, indicating an intimate relationship between inflammatory stimuli and stem cells propagation (Figs. 2D, 2E, 2G, 2H). A large quantity of positive stained cells located in the pulp core around the blood vessels and microvessels. According to most authors, the survival of inflamed vital pulp was closely linked to the process of angiogenesis. Moreover, STRO-1 labeling was present in zone II throughout the odontoblast layer providing the evidence that these cells may have odontoginc potential (Fig. 2E).Of note, fewer STRO-1- and CD146- positive cells appeared in the vicinity of the apex in zone III reflected no recruitment of stem cells at the root level (Figs. 2F, 2I). The mean cell counts of STRO-1-positive cells and their corresponding standard deviation values were listed in Table 1.

Partial colocalization of SDF-1α/CXCR4 and STRO-1 in dental pulp

To investigate the molecules involved in the stem cells recruitment, the expression of SDF-1α and its receptor CXCR4 was analyzed by Immunohistochemical Staining. As shown in Fig. 3A, a large number of SDF-1α-positive and CXCR4-positive cells can be observed in inflamed pulp, near the inflammatory infiltrates, whereas, the staining intensity weakened in normal pulp. Then the numbers of SDF-1α-positive

and CXCR4-positive cells in 3 zones in inflamed pulp were counted. As shown in Fig. 3B, the majority of SDF-1α- and CXCR4-positive cells located in zone I and II. STRO-1-positive cells also increased in zone I and II, and decreased in zone III, showing a significant correlation with SDF-1 ($R^2 = 0.841$, $P < 0.05$) and CXCR4 ($R^2 = 0.715$, $P < 0.05$).

To clarify the possible colocalization of STRO-1 with SDF-1α and CXCR4 in inflamed pulp, triple immunofluorescence was then done. As shown in Fig. 3C, the expression pattern of SDF-1α was similar to that of CXCR4, widely and significantly expressed near the inflammatory area. The intensity of STRO-1 staining was a little lower, mainly detected in cells around perivascular areas. Most STRO-1-positive cells overlapped with SDF-1α- and CXCR4-positive cells noticed around the perivascular areas and the extravascular areas. Together, these results suggested a positive correlation between SDF-1α-CXCR4 and STRO-1, which may indicate that SDF-1α-CXCR4 interaction might act as the molecular basis and increase the directional migration of pulp stem cells.

Discussion

Our study revealed the distribution of pulp stem cells in chronic pulpitis for the first time. During the pathological processes, pulp reactivity occurs in spontaneous attempts at protection and/or repair [2]. Tissue breakdown elicited migration of an important number of stem cells to the lesion site. These cells were concentrated in several specific niches, being activated and utilized in the repair mechanism against dental damage [9]. The balance between the inflammation and reparative processes would determine the extent of pulp inflammation and the viability of the affected tooth [10].

To date, the precise identity of pulp stem cells remains a challenge because of the lack of a single specific stem cell marker. Different immunoselection protocols to characterize these cells were based primarily on their high expression of the STRO-1 and CD146 antigen [1, 3, 8]. Based on these markers, we found that in normal pulp, STRO-1- and CD146-positive cells were restricted to the cell-rich zone and pulp core around blood vessels. This is consistent with previous studies, which provided strong evidence that the perivascular tissue represents the main stem cell niche in the adult pulp. They inferred that the vasculature could provide support for cell survival and differentiation, and thus long-term dentinogenesis in dentin-pulp complex [3, 11]. In addition, real-time PCR analysis revealed that the expression of OCT4 gene was lower in the inflamed pulp; however, the expressions of other embryonic and dentinogenic genes in the inflamed pulp were similar to those in the normal pulp. The results supported the fact that MSCs derived from inflamed human pulps preserve the full capability of proliferation and multipotent differentiation as compared with those from healthy pulps [6, 7].

Then we revealed that in chronic pulpitis, STRO-1- and CD146-positive cells mostly gathered in two specific niches, both adjacent to inflammatory lesions: one in the pulp core, and another in odontoblastic area. This was in agreement with the well-known participation of inflammation-induced chemoattraction in progenitor cell migration in animal models after dental injury [12, 13]. By the tritiated thymidine labeling method, Fitzgerald et al found that odontoblast replacement in the exposed pulp of the monkey's tooth

involved multiple DNA replications and migration of pulpal cells from the deeper pulp [14]. Harichane et al further demonstrated that 48 hours after pulp exposure the PCNA-positive cells mobilized near the exposure site of the rat's molar [15]. These observations lend support to the idea that the inflammatory process could induce rapid recruitment of progenitor cells resident in the pulp stroma to the vicinity of the lesion.

In reparative dentinogenesis, a cascade of events involving cell division, cell migration, and cytodifferentiation must occur before secretion of the matrix can take place [16]. During chronic pulpitis, STRO-1 labeling was present throughout the odontoblast layer. It means that the chronic inflammation leads not only to the migration of stem cells to the lesion but also accelerates their differentiation and maturation. On the one hand, it causes an increase of the number of active odontoblasts and initiation of reparative dentinogenesis. On the other hand, the stem cells exhaustion may lead to tissue senescence at later stage [17].

Predictably, we found little movement of STRO-1- and CD146-positive cells in the root apex, suggesting that it's the supportive microenvironment; especially specific molecules that regulate how pulp stem cell populations participate in tissue maintenance and regeneration. The specific molecules that promote or inhibit pulp stem cell migration are yet not clear. Nevertheless, the presence of certain molecules is well established, and several functional studies suggest important regulatory pathways. Recently, much attention has been paid to SDF-1α and its unique receptor CXCR4 for the migration of adult stem cells to the site of injury, which contributes to the impaired tissue remodeling [18, 19]. In the present study, intense staining of SDF-1α and CXCR4 was observed in zone I and II, adjacent to inflammatory sites. The explanation for this result is that the inflammation responding to tissue damage could produce many factors, such as interleukin-1, tumor- necrosis factor-α, hypoxia-inducible factor-1, etc., which stimulate the expression of SDF-1α. And the increased numbers of CXCR4-positive cells in the inflamed pulp could be due to chemotactic attraction towards the sources of the chemokine [12, 20]. SDF-1 interacting with its G-protein coupled receptor CXCR4 to induce SDF-1/CXCR4 signaling has already been well documented [21]. Furthermore, in our data immunofluorescent co-localization of STRO-1 and SDF-1α/CXCR4-positive cells confirmed the presence of stem cells in the inflammatory progression frontier, providing direct evidence of close relation between stem cells and SDF-1/CXCR4 signaling. Li et al further revealed that SDF-1/CXCR4 axis induced human dental pulp stem cell migration in vitro through FAK/PI3K/Akt and GSK3β/β-catenin pathways [19].

In conclusion, our findings illustrated the distribution of pulp stem cells in chronic pulpitis and indicated a positive correlation between the SDF-1α-CXCR4 axis and stem cells. Further understanding of functional activity of the stem cell niches and specific signaling in pathologic conditions may lead to the development of a new and less invasive strategy for treatment of pulpitis.

Abbreviations

SDF-1α: stromal cell-derived factor 1α; CXCR4: CXC chemokine receptor 4; Ct: threshold cycle; EDTA: ethylene diamine tetraacetic acid; PBS: phosphate-buffered saline; KLF4: kruppel-like factor 4, DMP1: dentin matrix protein-1, DSPP: dentin sialophosphoprotein; BSP: bone sialoprotein; VEGF: vascular endothelial growth factor

Declarations

Acknowledgements

Not applicable.

Authors' contributions

Yan Wu and Jiarong Liu conceived and designed the study. Yan Wu, Caixia Zhou, Xueying Tong and Shue Li performed the research. Yan Wu analyzed the data and drafted the article. Yan Wu and Jiarong Liu revised the article and made the final approval of the version to be submitted.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was reviewed and approved by the Committee of Ethics on Human Experiments of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (No.S169; 3/2/2016). Written informed consent was obtained for all subjects after the nature and possible consequences of the studies were explained.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Table

Due to technical limitations the Tables are available as a download in the Supplementary Files.

Supplementary Figure Legend

Supplementary Figure 1. Localization of STRO-1- and CD146- positive cells in normal pulp by immunohistochemical analysis. Light microscope images of HE sections show the coronal and apical parts of the pulp (A, B). Cells stained by STRO-1 and CD146 antibody are detected (C-F). Scale bars: (A, B) 250 μm; (C, E) 100 μm; (D, F) 50 μm.
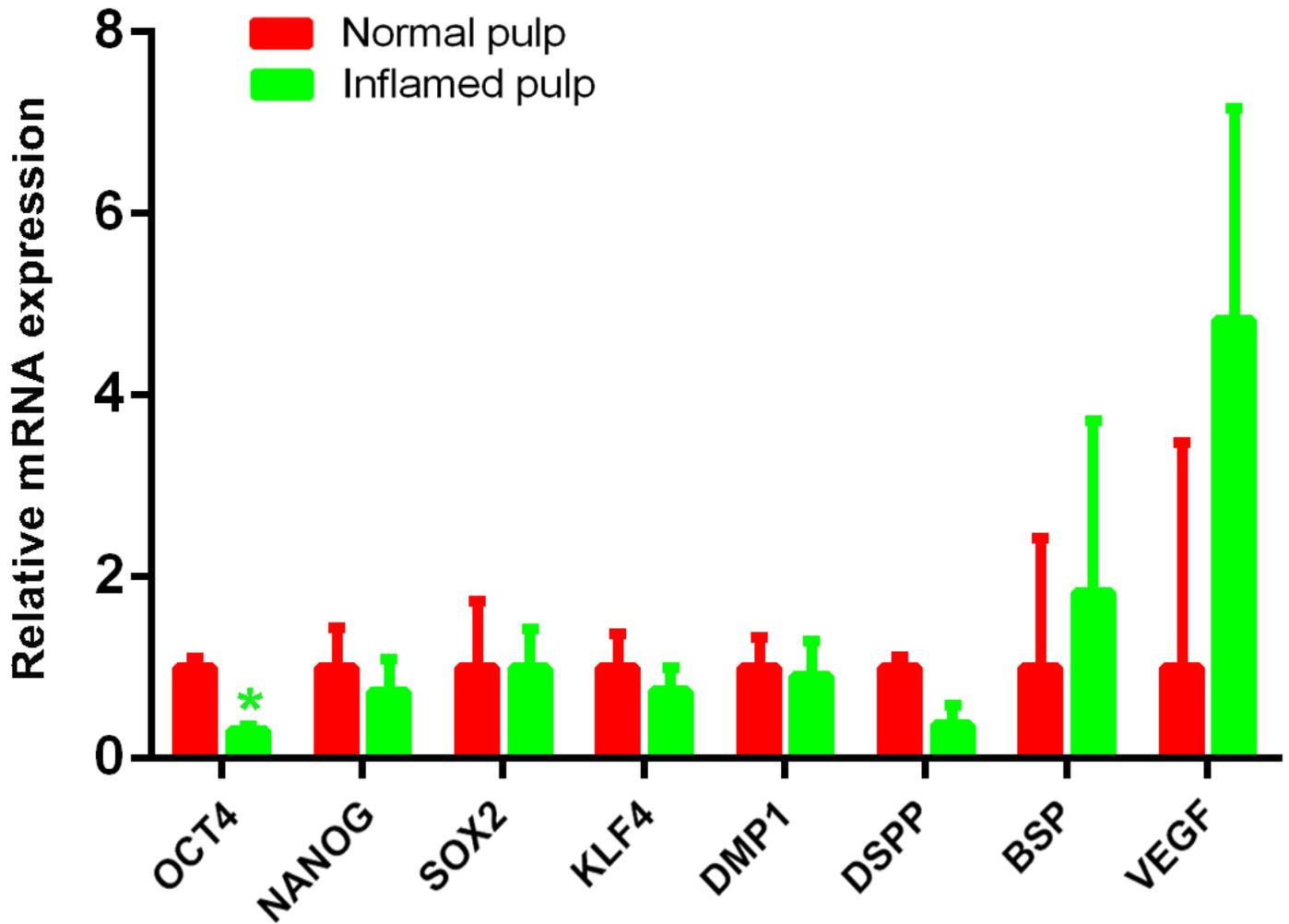
Figures

Figure 1

Real-time PCR analysis on expression of OCT4, NANOG, SOX2, KLF4, DMP1, DSPP, BSP and VEGF. Relative mRNA levels in inflamed pulp group (n=3) were presented as ratios relative to control group (n=3), after normalization for their respective β-actin mRNA expression. Results are reported as the mean±SD of 3 independent experiments. *P < 0.05 was considered statistically significant.

Figure 2

Localization of STRO-1- and CD146- positive cells in inflamed pulp by immunohistochemical analysis. Light microscope images of HE sections show the coronal and apical parts of the pulp (A, B, C). Cells stained by STRO-1 and CD146 antibody are detected in three zones: Zone I is in the central core of the pulp beneath the inflammatory site. Zone II covers the odontoblastic, cell-free and cell-rich areas of the pulp near the inflammatory site. Zone III is localized within the root pulp, close to the apex (D-I). Scale bars: (A–C) 250 µm; (D–I) 50 µm.

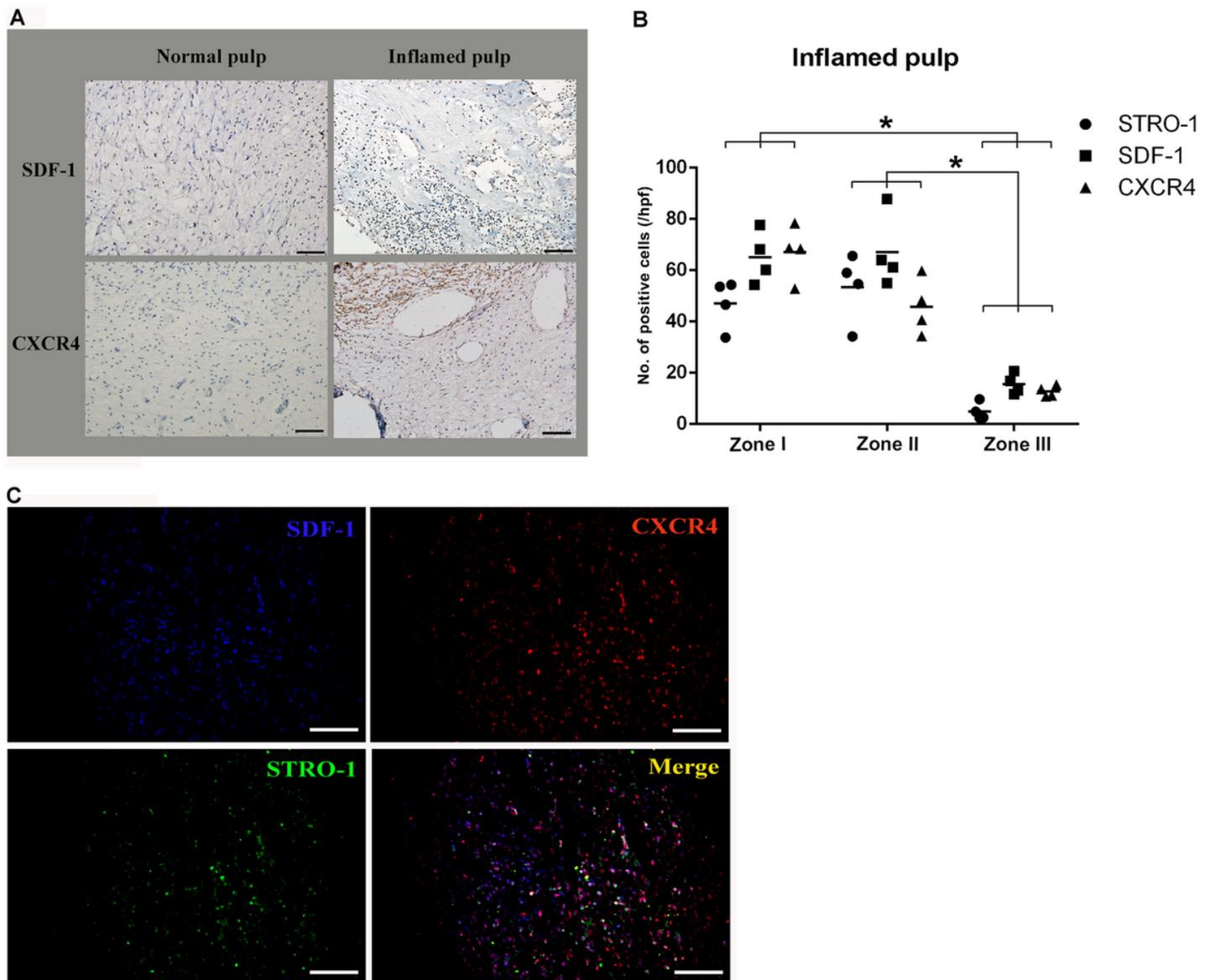## Figure 3

The expression pattern of SDF-1α, CXCR4 and STRO-1 in normal and inflamed pulp. (A) Immunohistochemical staining for SDF-1α and CXCR4 in normal and inflamed pulp. (B) Quantitative analysis of SDF-1α− positive, CXCR4-positive and STRO-1-positive cells in inflamed pulp (/hpf, per high-power field). There were significant differences between zone I and zone III, as well as between zone II and zone III (P <0.05). (C) Triple immunofluorescence staining for SDF-1α, CXCR4 and STRO-1 in inflamed pulp. A mixture of primary antibodies of STRO-1+/CXCR4+ was applied, followed by a mixture of Cy3-

conjugated secondary antibodies (red) to CXCR4 and FITC- conjugated secondary antibodies (green) to STRO-1. Then the addition of primary antibodies of SDF-1α followed by AMCA- conjugated secondary antibodies (blue) to SDF-1α was subsequently performed. Scale bars: 100 μm.

Supplementary Files

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