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Original Article

Deferoxamine enhances the migration of dental pulp cells via hypoxia-inducible factor 1α

Rong Du, Junjun Zhao, Yang Wen, Yaqin Zhu, Long Jiang

Department of General Dentistry, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine; College of Stomatology, Shanghai Jiao Tong University; National Center for Stomatology; National Clinical Research Center for Oral Diseases; Shanghai Key Laboratory of Stomatology, Shanghai, China

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Abstract: In previous studies, we found that deferoxamine (DFO) improved the migration of dental pulp cells (DPCs). The present study aimed to determine whether the effects of DFO on the migration of DPCs were regulated via hypoxia-inducible factor 1α (HIF-1α). Recombinant adenovirus vectors carrying short hairpin RNA (shRNA) targeting the human HIF-1α gene (pAd-GFP-shRNA-HIF-1α) and green fluorescent protein (GFP) were constructed. The expression of HIF-1α was inhibited by pAd-GFP-shRNA-HIF-1α at messenger RNA and protein levels. The secretion of stromal cell-derived factor 1α (SDF-1α) or vascular endothelial growth factor (VEGF) in DPCs treated with 10 μM DFO was higher than that in the control condition. The migration of DPCs was enhanced by 10 μM DFO. However, the effects of DFO on DPCs were partially reversed by silencing the HIF-1α gene in enzyme-linked immunosorbent assay or migration assay. Cumulatively, we conclude that DFO upregulated the secretion of SDF-1α or VEGF in DPCs and improved the migration of DPCs through HIF-1α.

Keywords: Deferoxamine, migration, dental pulp cells, hypoxia-inducible factor 1α

Introduction

Maintaining pulp vitality and function depends on dental pulp healing after its exposure owing to dental trauma or deep caries. The formation of reparative dentin has been considered a successful marker of dental pulp healing. The processes involved in reparative dentinogenesis are extremely complex and comprise a series of cellular activities that respond in cascade signal molecules [1, 2]. Therefore, the migration of dental pulp stem cells (DPSCs) toward the injured site is a critical step in the formation of reparative dentin. In our previous studies, we found that deferoxamine (DFO), a chelating agent used in the treatment of iron toxicity and hemochromatosis, could increase the expression of hypoxia-inducible factor 1α (HIF-1α) and improve the repair ability of dental pulp cells (DPCs), particularly the migratory ability of DPCs [3, 4]. Therefore, DFO might be a novel agent of pulp capping with the ability to improve reparative dentinogenesis [3].

Although we suspect that HIF-1α is a pivotal regulator factor, the precise molecular mechanism underlying the effect of DFO on the migration of DPCs remains unknown.

HIF-1α is an essential factor for adaptation to lower oxygen tension [5]. Under hypoxic conditions, HIF-1α is expressed in tissue and improves cells' ability to survive and adapt to new microenvironments via the modulation of expression of various downstream target genes involved in metabolism [6], angiogenesis [7], and stemness [8]. Further, HIF-1α plays an important role in postinjury tissue repair processes [9]. To verify the roles of HIF-1α in DPC migration, further research is required, particularly on some specific target genes in DPCs regulated by HIF-1α that need additional recognition.

Various cytokines are involved in the migration of cells, such as stromal cell-derived factor 1α (SDF-1α) [10] and vascular endothelial growth factor (VEGF) [11]. In previous studies, we found that SDF-1α could attract $CXCR4^+$ DPCs toward the damaged sites in dental pulp [12]. Akazawa et al. [13] reported that SDF-1α produced by DPCs played an important role in homeostasis,

whereas another study found that the expression of VEGF in DPCs enhanced the migratory ability of cells in three-dimensional spheroids [14]. Both SDF-1α and VEGF are regulated by HIF-1α in several damaged organs or hypoxic microenvironments [15, 16].

In the current study, adenoviruses containing short hairpin RNA (shRNA) and the green fluorescent protein (GFP) were constructed and transfected into DPCs to knock down HIF-1α gene expression ($DPCs^{HIF\text{-}1\alpha\text{-}}$). The empty vectors were transfected into DPCs ($DPCs^{pAd}$) as negative controls. SDF-1α and VEGF secretions in the supernatant were detected using enzyme-linked immunosorbent assay (ELISA) after DPCs, $DPCs^{pAd}$, or $DPCs^{HIF\text{-}1\alpha\text{-}}$ were treated with DFO. Additionally, the migratory ability of DPCs, $DPCs^{pAd}$, or $DPCs^{HIF\text{-}1\alpha\text{-}}$ treated with DFO was investigated using the scratch wound healing assay and transmigration assay. The result of this study furthers the understanding of the mechanism underlying HIF-1α regulation of DPCs regarding migration processes and may provide theoretical support for DFO application as a potential novel pulp capping agent.

Materials and methods

Cell culture and transfection

This study was approved by the Independent Ethics Committee of Shanghai Ninth People's Hospital affiliated to Shanghai Jiao Tong University, School of Medicine (protocol no. 201546).

DPCs were obtained from 23 premolars taken from 12 orthodontic patients, including eight males and four females, aged 16-22 years according to the previously describe method [17]. After the first passage, DPCs were cultivated in DMEM and 10% v/v FBS. The culture medium was changed every 4 days. Passages 3-5 of cells were utilized for subsequent experiments.

The shRNA oligos sequence was designed and synthesized according to the human HIF-1α gene sequence (GenBank accession no. NM_001530.3). The effective target sequences were 5'-CACCGCTGGAGACACAATCATATCTCGAAAGATATGATTG TGTCTCCAGC-3' (forward) and 5'-AAAAGCTGGAGACACAATCATATCTTTC GAGATATGATTGTGTCTCCAGC-3' (reverse). A recombinant adenovirus vector carrying the GFP, the shRNA-HIF-1α targeting the human HIF-1α gene (pAd-GFP-shRNA-HIF-1α), or the control adenovirus (pAd-GFP) were constructed by Novobio Biotechnology (Shanghai, China). Based on the virus titer, DPCs were transfected with pAd-GFP-shRNA-HIF-1α ($DPCs^{HIF\text{-}1\alpha\text{-}}$) or pAd-GFP ($DPCs^{pAd}$) at a MOI (PFU/cell) of 100 when they achieved 80% confluency. After infection for 48 h, GFP expression was observed using a fluorescence microscopy.

Identify of the knockdown effect

DPCs were transfected with either pAd-GFP-shRNA-HIF-1α or pAd-GFP. After 48 h, the messenger RNA (mRNA) level of HIF-1α was determined by quantitative real-time polymerase chain reaction (qRT-PCR). The total RNA content of DPCs, $DPCs^{HIF\text{-}1\alpha\text{-}}$, or $DPCs^{pAd}$ was determined by Trizol (Invitrogen, Carlsbad, LA) and complementary DNA was synthesized with the RT Reagent kit (TaKaRa, Tokyo, Japan). Interested mRNA content in each sample was calculated using a comparative ΔΔCt (ΔCt gene-ΔCt control) value method. Relative changes in the differential expression of the genes mentioned above were determined by a $2^{-\Delta\Delta Ct}$ method [18]. HIF-1α primer sequences included 5'-CCCACCGCTGAAACGC-3' (forward) and 5'-ACTATTAGGC TCAGGTGAACTTTGT-3' (reverse). β-actin primer sequences included 5'-TCCTTCCT GGGCATGGAGT-3' (forward) and 5'-CAGGAGGAGCAATGATCTTGAT-3 (reverse).

After 48 h transfection, DPCs, $DPCs^{HIF\text{-}1\alpha\text{-}}$ or $DPCs^{pAd}$ were then treated with 10 μM of DFO (Sigma-Aldrich, Saint Louis, MO) for another 48 h. The protein level of HIF-1α was detected using western blotting. The process of western blotting was performed as described in the previous study [19]. Target proteins were blocked and incubated with mouse anti-human HIF-1α antibody (1:1,000 dilution; Abcam, Cambridge, England) and mouse anti-human β-actin antibody.

ELISA

DPCs, $DPCs^{HIF\text{-}1\alpha\text{-}}$, or $DPCs^{pAd}$ were seeded in 24-well plates at 1×10^5/well. The supernatant was collected after the cells were treated with 10 μM of DFO for 48 h. The cells cultured in the medium without adding DFO for 48 h was used as a negative control. The secretion of SDF-1α

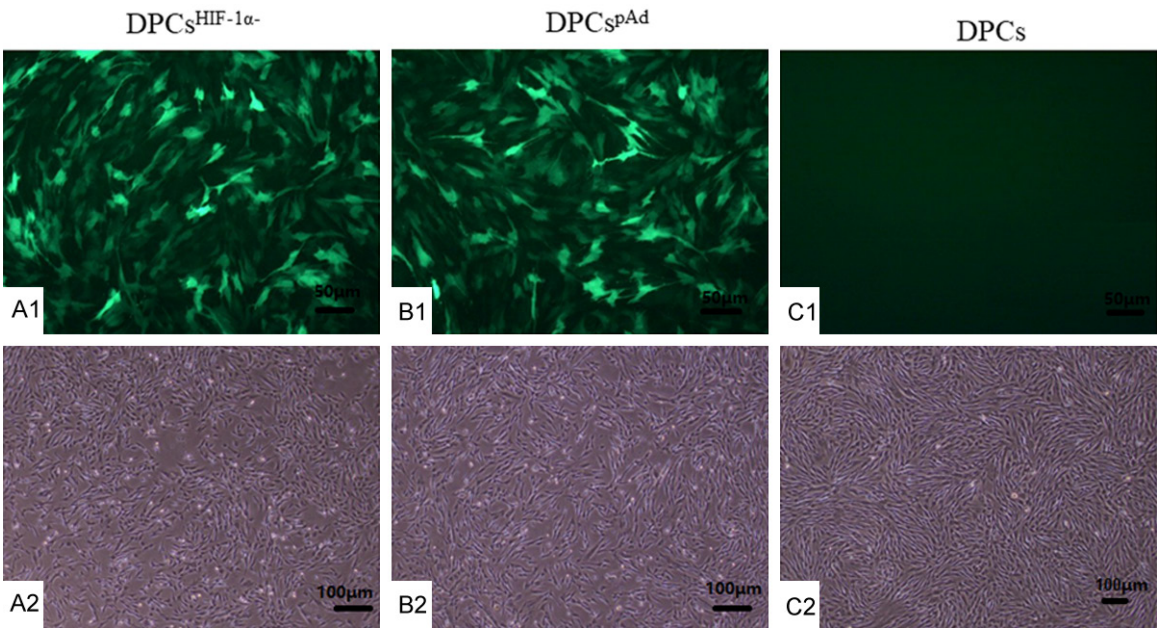

Figure 1. DPCs were transfected with pAd-GFP-shRNA-HIF-1α for 48 h. (A1: Fluorescence microscopy. A2: Inverted phase-contrast microscopy). DPCs were transfected with pAd-GFP for 48 h (B1: Fluorescence microscopy. B2: Inverted phase-contrast microscopy). The control group can be observed in (C1 and C2) (100 ×).

and VEGF levels was measured by ELISA kits (Shanghai Yuanxiang Medical, Shanghai, China), following the manufacturer's instructions.

Scratch wound healing assay

DPCs, DPCs$^{HIF\text{-}1\alpha\text{-}}$, or DPCs^{pAd} were seeded in six-well plates at 1 × 10^5/well and cultured in DMEM containing 10% FBS. When the cells achieved 100% confluency, a wound scratch was created on the surface of the plate using a yellow pipette tip. Some shedding cells were washed gently with PBS and discarded. Next, the culture media containing 10 μM DFO were added to different wells. After treatment for 24 h, the migration of cells into the wound scratch was observed. The number of cells in the wound scratch area was recorded using an inverted phase-contrast microscope.

Transmigration assay

Transwell inserts (8-μm pore; Corning, Boston, MA) in 24-well plates were prepared for transmigration assay. The wells of the plate were supplemented with 600 μL of culture medium containing 10 μM DFO, and the upper chambers were seeded with DPCs, DPCs$^{HIF\text{-}1\alpha\text{-}}$, or DPCs^{pAd} (1 × 10^5 cells/well). After incubation in 5% CO_2 atmosphere at 37°C for 48 h, the Transwell upper chambers were immersed in 4% paraformaldehyde for 20 min. The cells on the upper polycarbonate membrane were removed using a cotton swab. The transmigrated cells were quantified through 0.1% crystal violet (Sigma-Aldrich) staining (12). The ability of cell migration was assessed with the optical density (OD) value of eluent. The OD value was obtained at 630 nm.

Statistical analysis

The analysis of data was performed with the Statistical Analysis System version 8.2 software program (SAS Raleigh, NC). All results were described as means ± standard deviations. Statistical significance among the groups was determined by one-way analysis of variance (ANOVA), and the Bonferroni post-hoc test was used for multiple comparisons. A *p*-value of less than 0.05 was considered to be statistically significant.

Results

No significant differences in morphology among the DPCs$^{HIF\text{-}1\alpha\text{-}}$, DPCs^{pAd}, and DPCs

DPCs in all groups were observed after transfection using an inverted phase-contrast microscope. It was noted that they had grown normally and there were no significant differences

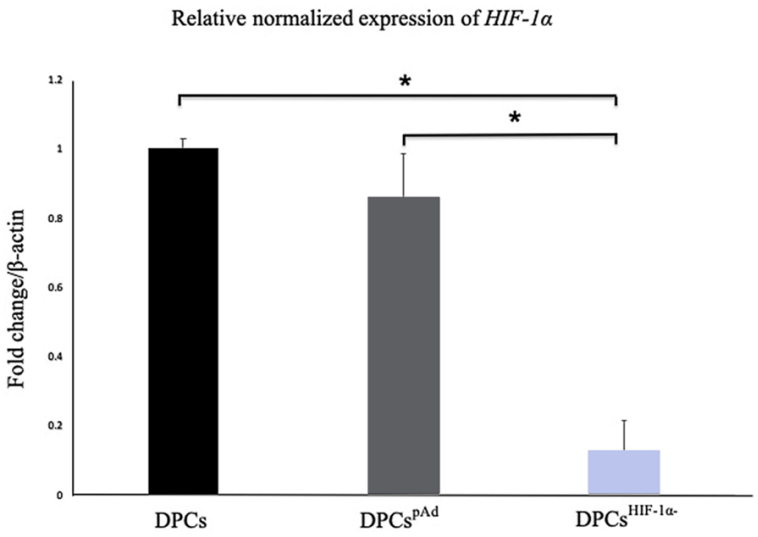

Figure 2. At 48 h after transfection, the mRNA level of HIF-1α was downregulated. Values are presented as means ± standard deviations. Groups were compared using one-way ANOVA. *P < 0.05, n = 3.

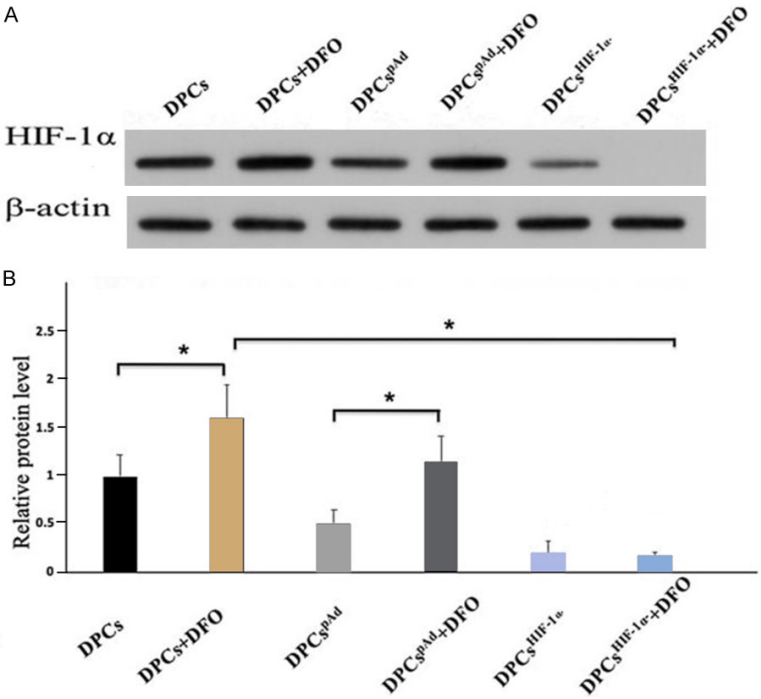

Figure 3. Western blotting results of HIF-1α. Values are presented as means ± standard deviations. Groups were compared using one-way ANOVA. *P < 0.05, n = 3. NS represents no significant difference.

in morphology among the DPCs$^{HIF-1α-}$, DPCs^{pAd}, and DPCs (**Figure 1A-C**). Strong green fluorescence was expressed by both DPCs$^{HIF-1α-}$ and DPCs^{pAd} under the fluorescence microscope (**Figure 1A1** and **1B1**).

The expression of HIF-1α mRNA in the DPCs$^{HIF-1α-}$ was significantly decreased when compared with that in the control group or DPCs^{pAd} group

HIF-1α mRNA relative expression levels in DPCs, DPCs^{pAd}, and DPCs$^{HIF-1α-}$ were assessed (**Figure 2**). Results showed that the expression of HIF-1α mRNA in the DPCs$^{HIF-1α-}$ was significantly decreased when compared with that in the control group or DPCs^{pAd} group ($P < 0.05$).

The expression of HIF-1α in DPCs$^{HIF-1α-}$ was decreased significantly

Western blotting outcomes indicated that DFO enhanced HIF-1α expression in the DPCs group or DPCs^{pAd} group, while there was no significant difference noted when the DPCs$^{HIF-1α-}$ were treated or not treated with DFO. The expression of HIF-1α in DPCs$^{HIF-1α-}$ was decreased significantly, even when treated with 10 μM of DFO for 48 h ($P < 0.05$) (**Figure 3**).

There was no significant difference between the DPCs$^{HIF-1α-}$ and DFO-treated DPCs$^{HIF-1α-}$ groups

The ELISA assay revealed that DFO promoted DPCs to secrete SDF-1α and VEGF. After being treated with 10 μM of DFO for 48 h, the concentration of SDF-1α or VEGF in DPCs or DPCs^{pAd} supernatant was significantly increased when compared with that in the respective control group ($P < 0.05$). However, there was no significant difference between the DPCs$^{HIF-1α-}$ and DFO-treated DPCs$^{HIF-1α-}$ groups (**Figure 4**).

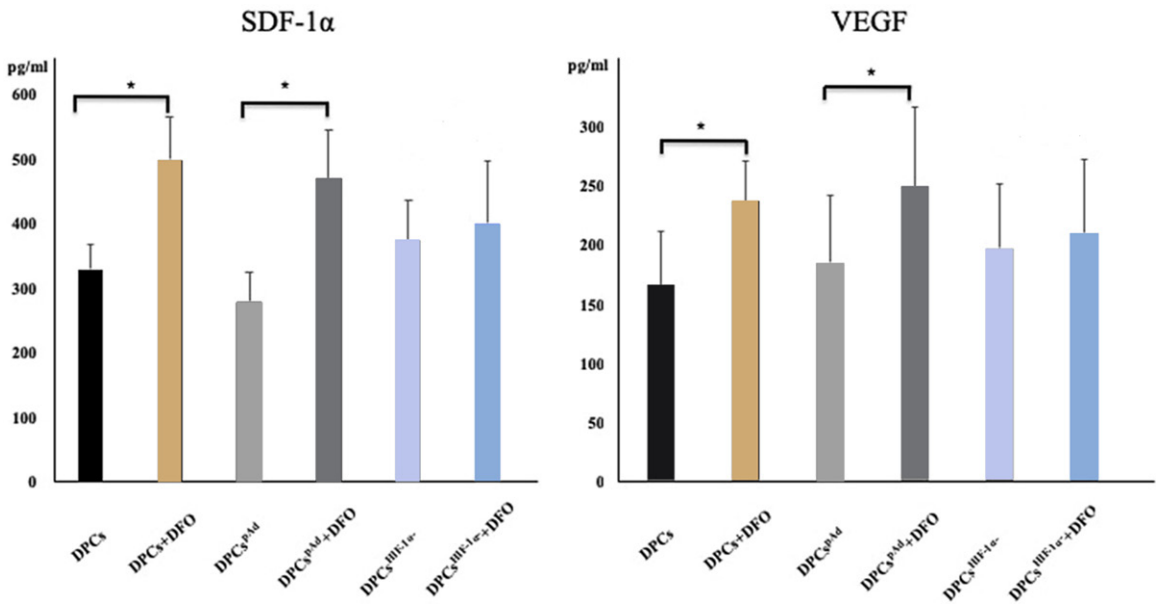

Figure 4. ELISA assay outcomes for SDF-1α and VEGF in DPC supernatant. Values are presented as means ± standard deviations. Groups were compared using one-way ANOVA. *P < 0.05, n = 4. NS represents no significant difference.

The results of scratch wound healing assay indicated that the migration of DPCs was improved by DFO when compared with the control group

The results of scratch wound healing assay indicated that the migration of DPCs was improved by DFO when compared with the control group. More cells migrated toward the scratch in the group of DPCs treated with 10 μM of DFO. However, the results in the DPCs$^{HIF\text{-}1\alpha\text{-}}$ groups were similar, regardless of whether the DPCs$^{HIF\text{-}1\alpha\text{-}}$ were treated with DFO (**Figure 5A** and **5B**).

The OD values of elutes from the DFO group or DPCs^{pAd} treated with DFO group was higher than those of the DPCs group or DPCs^{pAd} group

In the transmigration assay, the number of cells that crossed the pores was measured. After staining with 0.1% crystal violet, cells adhered to the lower side of the polycarbonate membrane were observed under the inverted phase-contrast microscope as shown in **Figure 5C**. The OD values of the elutes from DPCs, DPCs$^{HIF\text{-}1\alpha\text{-}}$, or DPCs^{pAd} treated with DFO were measured and were correlated with the number of transmigrated cells. **Figure 5D** showed that the OD values of elutes from the DFO group or DPCs^{pAd} treated with DFO group was higher than those of the DPCs group or DPCs^{pAd} group ($P < 0.05$), while there was no obvious difference between the OD measurements from the DPCs$^{HIF\text{-}1\alpha\text{-}}$ group and DPCs$^{HIF\text{-}1\alpha\text{-}}$ with DFO group.

Discussion

DFO, a chelating agent used to treat iron toxicity and hemochromatosis [20], has been demonstrated to have some novel applications recently [21]. DFO was found to stimulate osteogenesis and angiogenesis by stabilizing HIF-1α. It has been reported before as well that a bone graft substitute combined with DFO was beneficial in the reconstruction of bone defects [22], and DFO increased the incidence of bony unions by triggering the HIF-1α pathway [23].

In previous studies, we found that DFO could enhance the HIF-1α expression in DPCs and improve their proliferation, migration, and odontoblast differentiation [3]. However, there was insufficient evidence to confirm that HIF-1α was involved in the effects of DFO on DPCs. To address this question, the HIF-1α gene expression in DPCs was knocked down by RNA interference. shRNA is a double-stranded RNA sequence able to be cloned into vectors such as adenovirus. When DPCs were transfected by adenovirus containing human HIF-1α shRNA, double-stranded RNA sequences were expressed and then automatically incised into small interference RNA (siRNA). Of note, siRNA

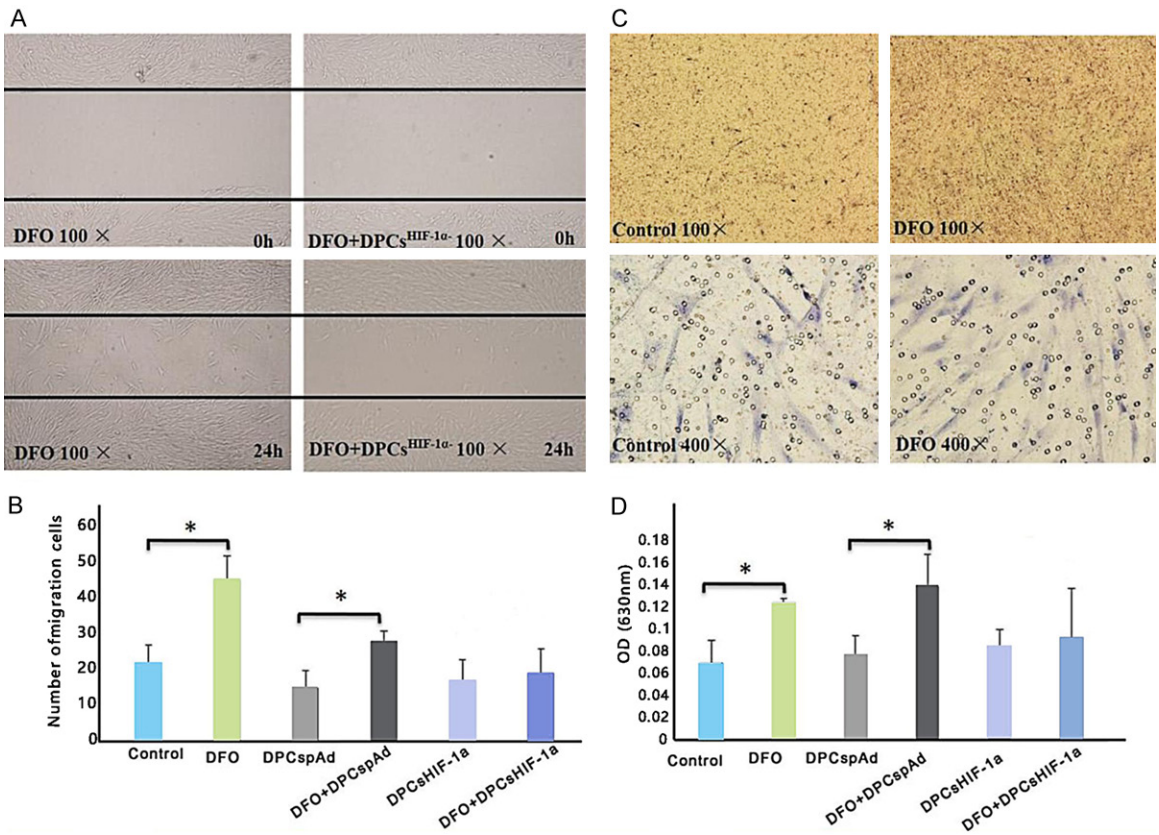

Figure 5. Cell migration assay. A: The effects of DFO on the migration of DPCs determined by generating a scratch in a confluent monolayer of DPCs. In the DFO-treated group, the number of cells in the scratch space was more than that in the group of DPCs$^{HIF-1α-}$ treated with DFO after 24 h (100 ×). B: The numbers of DPCs in the scratch space were counted in different groups. C: The transmigration DPCs were observed under an inverted phase-contrast microscope. D: The OD value in DPCs treated with DFO group was higher than that in the control group. There was no significant difference between the DPCs$^{HIF-1α-}$ and DPCs$^{HIF-1α-}$ +DFO groups. *P < 0.05, n = 4. NS represents no significant difference.

can silence the target gene by mRNA degradation of the HIF-1α gene. In this study, we successfully constructed the vector of shRNA-HIF-1α, which was labeled as pAd-GFP-shRNA-HIF-1α. qRT-PCR and western blot results demonstrated that this vector efficiently inhibited HIF-1α expression in DPCs, even though the DPCs were treated with DFO. This study supports further exploration of the HIF-1α role in situations where DPCs are exposed to hypoxia or DFO.

HIF-1α is widely accepted as a key transcription factor for the regulaton and stabilization of cellular adaptations to hypoxic stress [24]. In previous studies, hypoxia not only promoted the mineralization of DPCs but also enhanced HIF-1α expression [3, 25]. This allows for the conclusion that HIF-1α might play a crucial role in triggering the restoration of pulp injury.

SDF-1α is a small (8-13 kDa) secreted chemokine protein. It has been confirmed that SDF-1α is upregulated in damaged tissues to attract CXCR4-positive cells, which are mobilized from their niches in response to the stimulation related to tissue/organ damage [26]. Our previous research efforts revealed that the SDF-1α-CXCR4 axis may contribute to the migration of DPCs toward the damage site [12]. It has been reported before as well that HIF-1α induces SDF-1α upregulation in endothelial cells [27]. The present study sought to demonstrate that SDF-1α is regulated by HIF-1α in DPCs. VEGF is a critical regulator of both physiologic and pathologic angiogenesis. Aranha et al. [28] reported that VEGF participated in hypoxic dental pulp revascularization. Amemiya et al. [29] also indicated that the expression of VEGF was increased in beagle dog DPCs, which was regulated by HIF-1α under hypoxic conditions. In

this study, we found that DFO not only promoted DPCs to express HIF-1α but also enhanced the secretion of SDF-1α and VEGF in DPCs. Furthermore, if the HIF-1α gene of DPCs was silenced by shRNA, the effects of DFO on the expressions of SDF-1α and VEGF were also inhibited. The results of this study demonstrate that DFO upregulates the secretion of SDF-1α and VEGF in DPCs via HIF-1α.

In the scratch wound healing assay and transmigration assay, we verified that DFO enhanced the migration of DPCs. However, the effects of DFO on DPCs$^{HIF\text{-}1\alpha-}$ were not notable. Our results indicate that the migration of DPCs was regulated by HIF-1α when the cells were treated with DFO.

The chemotactic migration of SDF-1α has been widely reported [13, 30, 31] and the function of VEGF in migration has also been demonstrated [32, 33]. Therefore, we infer that SDF-1α and VEGF may have roles in the migration of DPCs when DPCs are treated with DFO.

This study still has several limitations owing to the experimental conditions used. For example, we did not do the knockout mice experiment. Moreover, the exact mechanism by which DFO affects DPCs requires further analysis. Likewise, the effects of DFO on the possibility of DFO involvement should also be explored in future studies. We will conduct a more in-depth and comprehensive analysis of these aspects as soon as possible, in order to obtain more robust test results.

In conclusion, we successfully constructed the pAd-GFP-shRNA-HIF-1α vector, which efficiently knocks down HIF-1α expression when DPCs are treated with DFO. Moreover, in our present study, we demonstrated that DFO upregulates the expression of SDF-1α and VEGF in DPCs and enhances the migration of DPCs via HIF-1α. In summary, DFO induces the expression of HIF-1α in DPCs and then HIF-1α upregulates the secretion of SDF-1α and VEGF, enhancing the migration of DPCs. These findings may facilitate further efforts to explore the possibility of DFO involvement in new therapeutic treatments for dental pulp injury.

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Disclosure of conflict of interest

None.

Address correspondence to: Yaqin Zhu and Long Jiang, Department of General Dentistry, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine; College of Stomatology, Shanghai Jiao Tong University; National Center for Stomatology; National Clinical Research Center for Oral Diseases; Shanghai Key Laboratory of Stomatology, Shanghai, China. Tel: +86-021-53315202; +86-13816568151; E-mail: zyq1590@163.com (YQZ); h7gdo0j@163.com (LJ)

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