Published in final edited form as:

Cell Tissue Res. 2015 December ; 362(3): 633–642. doi:10.1007/s00441-015-2248-y.

CXCR4/CXCL12 signaling impacts enamel progenitor cell proliferation and motility in the dental stem cell niche

Tamaki Yokohama-Tamaki^{1,3}, Keishi Otsu², Hidemitsu Harada², Shunichi Shibata³, Nobuko Obara¹, Kazuharu Irie¹, Akiyoshi Taniguchi⁴, Takashi Nagasawa⁵, Kazunari Aoki⁵, Steven R. Caliari⁶, Daniel W. Weisgerber⁶, and Brendan A. C. Harley⁶

Tamaki Yokohama-Tamaki: ttamaki.mfa@tmd.ac.jp; Brendan A. C. Harley: bharley@illinois.edu

¹Division of Histology, Department of Oral Growth and Development, School of Dentistry, Health sciences University of Hokkaido, Hokkaido, Japan

²Division of Oral Histology and Developmental Biology, School of Dentistry, Iwate Medical University, Iwate, Japan

³Maxillofacial Anatomy, Department of Maxillofacial Biology, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan

⁴Biomaterials Center, National Institute for Materials Science, Ibaraki, Japan

⁵Department of Immunobiology and Hematology, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan

⁶Department of Chemical and Biomolecular Engineering, University of Illinois at Urbana-Champaign, Urbana, IL, USA

Abstract

Dental stem cells are located at the proximal ends of rodent incisors. These stem cells reside in the dental epithelial stem cell niche, termed the apical bud. We focused on identifying critical features of a chemotactic signal in the niche. Here, we report that CXCR4/CXCL12 signaling impacts enamel progenitor cell proliferation and motility in dental stem cell niche cells. We report cells in the apical bud express *CXCR4* mRNA at high levels while expression is restricted in the basal epithelium (BE) and transit-amplifying (TA) cell regions. Furthermore, the CXCL12 ligand is present in mesenchymal cells adjacent to the apical bud. We then performed gain- and loss-of-function analyses to better elucidate the role of CXCR4 and CXCL12. CXCR4-deficient mice

Correspondence to: Tamaki Yokohama-Tamaki, ttamaki.mfa@tmd.ac.jp; Brendan A. C. Harley, bharley@illinois.edu.

Electronic supplementary material The online version of this article (doi:10.1007/s00441-015-2248-y) contains supplementary material, which is available to authorized users.

Conflicts of interest The authors indicate no potential conflicts of interest.

<http://www.harleylab.org>

Author contribution Tamaki Yokohama-Tamaki: conception and design, manuscript writing, collection and assembly of all data. Otsu Keishi: provision of materials (mHAT9a cells) and data analysis. Hidemitsu Harada: provision of materials (mHAT9a cells). Nobuko Obara: construction of *CXCL12* mRNA probe. Kazuharu Irie and Shunichi Shibata: data analysis and interpretation of histology. Akiyoshi Taniguchi: data analysis and interpretation of real-time PCR. Takashi Nagasawa, Kazunari Aoki: provision of materials (CXCR4 and CXCL12 knockout mice). Steven R. Caliari: data analysis and interpretation of real-time PCR and transwell cell migration assays. Daniel W. Weisgerber: data analysis and interpretation of alamarBlue assays. Brendan A.C. Harley: provision of study material, data analysis and interpretation, financial support, final approval of manuscript.

contain epithelial cell aggregates, while cell proliferation in mutant incisors was also significantly reduced. We demonstrate in vitro that dental epithelial cells migrate toward sources of CXCL12, whereas knocking down CXCR4 impaired motility and resulted in formation of dense cell colonies. These results suggest that CXCR4 expression may be critical for activation of enamel progenitor cell division and that CXCR4/CXCL12 signaling may control movement of epithelial progenitors from the dental stem cell niche.

Keywords

CXCR4/CXCL12 signal; Tooth; Dental stem cell niche; Migration; Proliferation

Introduction

The incisor of the adult rodent contains tissue-committed stem cells. Dental epithelial stem cells are located in a bulbous epithelial tissue, the apical bud, which is subdivided into four compartments. The innermost compartment is the stellate reticulum (SR), which is surrounded concentrically by the basal epithelium (BE), the inner enamel epithelium (IEE), and the outer enamel epithelium (OEE) (Harada et al. 1999; Harada and Ohshima 2004; see also Figs. 1m, n, 2h, below). These compartments are considered to constitute the dental epithelial stem cell niche. Previous reports have assumed that asymmetric divisions rarely occur in the SR, in which one daughter cell remains as a stem cell while the other moves into the BE (Harada et al. 1999; Harada and Ohshima 2004). As cells migrate between the BE and IEE of the apical bud, they turn into transit-amplifying (TA) cells. Subsequently, TA cells differentiate into ameloblast progenitor cells, which secrete enamel-related proteins on the lateral side of the apical bud. Early studies implicated certain signaling molecules, including FGFs, BMPs, and Notch, in regulation of stem cell proliferation, maintenance, and cell fate decisions in the apical bud (Harada et al. 2002; Thesleff et al. 2007; Wang et al. 2007; Yokohama-Tamaki et al. 2008). However, little information is available regarding the impact of chemokine factors during tooth development in general and, in particular, on the activity of dental epithelial stem cells.

The chemokine receptor CXCR4 is a seven-transmembrane G-protein-coupled receptor for the CXCL12 ligand. CXCL12/CXCR4 interactions are known to play a critical role in directing migration of tissue-specific progenitor cells during embryogenesis. They are also known to play crucial roles in directing neurons, hematopoietic stem cells, and primordial germ line cells (PGCs) to their final destinations (Nagasawa et al. 1996; Tachibana et al. 1998; Libura et al. 2002; O'Hayre et al. 2010). However, the specific functional consequences of CXCR4/CXCL12 signaling in the dental stem cell niche remain poorly understood.

As real-time PCR analysis suggested CXCR4 was highly expressed in the apical bud, we monitored the expression of *CXCR4* mRNA in cells derived from the apical bud. In order to investigate the role of CXCR4 and CXCL12 in the epithelial stem cell niche, we observed the incisors of mouse in which each gene was knocked out. In order to further characterize the roles of CXCR4 in dental epithelial cells, we performed knockdown (KD) analysis in

vitro. Here, we investigated changes in cell proliferation and motility in response to CXCL12 in CXCR4 KDs, and we report a significant role of CXCR4/CXCL12 signaling in the dynamics of dental epithelial stem cells.

Materials and methods

Mice

CXCR4 and CXCL12 knockout mice have been described previously (Nagasawa et al. 1996; Tachibana et al. 1998). Embryonic day 17.5 and 18.5 (E17.5 or E18.5) knockout mice were fixed in 4 % paraformaldehyde (PFA) in phosphate-buffered saline (PBS), pH 7.4, and then embedded in paraffin. Histological analysis was performed on 4- μ m sections. Length of the lower incisor and mandibular were measured (Atchley et al. 1985; Yoshida et al. 2013) using Image J (NIH, MD, USA). Wild-type littermates were used as controls. E14, 16, 18 and post-natal day 4 (PN4) Institute of Cancer Research (ICR) mice were purchased from CLEA Japan (Tokyo, Japan). Mice were unfixed or fixed in 4 % PFA in PBS, pH 7.4, and embedded in O.C.T. compound (Sakura Finetek Japan, Tokyo, Japan). All experimental protocols conformed to international guidelines and were approved by the Animal Study Committee of the Health Sciences University of Hokkaido (No. 077) and the Tokyo Medical and Dental University (No. 0150181A).

Quantitative real-time RT-PCR analysis

Lower incisors (PN4) and tooth germs (E14–E18) were dissected from the mandibular with scalpels (Fig. 1a). Dental epithelium was removed mechanically from an incisor with forceps after incubation in 2 % collagenase at 4 °C for 3 h. The apical bud was cut with 18-G needles on the dotted line (Fig. 1b). Total RNA was extracted from the apical bud (Fig. 1b, arrow) and whole tooth germs (E14, E16, E18 and PN4) using TRIzol reagent (Ambion, Austin, TX, USA). Single-strand complementary DNA (cDNA) synthesis was performed using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) from 1 μ g total RNA in a final volume of 20 μ l. Real-time PCR was carried out on an ABI PRISM 7900HT system (Applied Biosystems, Carlsbad, CA, USA). Reactions contained 15 ng template cDNA, 0.3 μ M each forward and reverse primers, and 5 μ l QuantiTect SYBR Green PCR master mix (Qiagen) in 10 μ l total volume. RT-PCR detection of specific transcripts was performed using the following primer sets (Table 1). PCR parameters were as follows: 50 °C for 2 min; 95 °C for 15 min; 40 cycles of 94 °C for 15 s and 60 °C for 30 s; and 72 °C for 30 s. The mRNA level of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control. Three independent experiments were performed in triplicate to obtain reported values. (C_t) values were calculated for control and experimental groups as previously described (Yokohama-Tamaki et al. 2011). One-way analysis of variance (ANOVA) was performed, and significance was calculated using Tukey's comparison test. A *p* value of less than 0.05 was considered statistically significant.

In situ hybridization analysis

In situ hybridization was performed on 10- μ m frozen sections. A 519-bp fragment of CXCR4 cDNA (NCBI Probe: Pr196582.1) was inserted into the pCRII vector (Invitrogen/Life Technologies, Carlsbad, CA, USA). A 304-bp fragment of murine CXCL12 cDNA

(NM_001012477, nucleotides 43–346) was inserted into the pT7T3alpha-18 vector (Invitrogen). Antisense and sense riboprobes for *CXCR4* and *CXCL12* mRNAs were synthesized and digoxigenin (DIG)-labeled according to the manufacturer's instructions (Roche Diagnostics, Basel, Switzerland). Samples were cut into 10- μ m frozen sections and hybridized at 63 °C. In situ hybridization was performed as previously described (Yokohama-Tamaki et al. 2008, 2011).

Immunostaining

Paraffin sections were cut at 5- μ m thickness. Antibodies and dilution were used as follows: Ki-67 (rabbit, 1:1000; Vector Laboratories, Burlingame, CA, USA), HRP-anti rabbit IgG (1:500; Zymed, San Francisco, CA, USA). The signals were visualized using diaminobenzidine (DAB) buffer tablets (Merck, Darmstadt, Germany). Sections were counterstained with hematoxylin. We counted Ki-67-positive cells and total cell number from each of 3 sections, cutting 5- to 8- μ m serial transverse sections from the apical end to 150 μ m ahead to the incisal edge. Three independent experiments were performed in triplicate.

Frozen sections were cut at 8- to 10- μ m thickness. The fresh frozen sections were fixed in periodate-lysine-2 % paraformaldehyde (PLP) solution overnight at 4 °C. The frozen sections of 6-week adult mice were cut by the film sectioning method (Kawamoto 2003). Antibodies and dilution were used as follows: *CXCR4* (goat, 1:300; Gene Tex, Irvine, CA, USA), *CXCL12* (goat, diluted 1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Alexa Fluor 488, Alexa Fluor 546 (1:1000; Molecular Probes, Eugene, OR, USA). Nuclear counterstaining was performed with DAPI (1:1000; Invitrogen). The images were acquired using a fluorescence microscope and an FLUOVIEW FV10i multiphoton confocal laser microscope (Olympus, Tokyo, Japan). Images to be compared directly with each other were processed in an identical manner using Adobe Photoshop, version CS4 (Adobe Systems, San Jose, CA, USA). Each staining was repeated five times on separate samples.

Cell culture

The cell line mHAT9a was originally established from the apical bud of mouse incisor (Otsu et al. 2011). mHAT9a cells were grown in Dulbecco's modified Eagle medium/F12 (DMEM/F12) containing L-glutamine, sodium pyruvate (Mediatech, Manassas, VA, USA), 100 U/ml penicillin/streptomycin and 10, 50, or 100 ng/ml CXCL12 protein or 1 ng/ml bFGF (PeproTech, Rocky Hill, NJ, USA).

CXCR4 knockdown

Knockdown of mouse CXCR4 was performed using SureSilencing shRNA Plasmids against CXCR4 (KM03149N) (SABiosciences; Qiagen). We performed real-time PCR analysis of each of the four plasmids provided to determine its efficiency in suppressing CXCR4 expression in mHAT9a, and ultimately chose clone ID 2 (5'-CGTCCATTTCAATAGGATCTT-3') for all subsequent experiments. For the negative control shRNA, we chose one of the four plasmids that did not affect CXCR4 expression levels, clone ID 1 (5'-CCTCTCCTGTTACTGCATCAT-3'). We did not use the control vector NC (5'-GGAATCTCATTTCGATGCATAC-3') provided as a control in the kit.

mHAT9a cells were electroporated with 1 µg plasmid DNA using the Neon Transfection System (Invitrogen) under the following settings: 1400 V, 10 ms/pulse, three pulses. Stable CXCR4 KD mHAT9a cells (CXCR4 KD) and negative-control shRNA mHAT9a cells (NC) were selected for 3 weeks in medium containing 500 µg/ml G418 (Mediatech).

Transwell migration assay

We added 10,000 cells in 100 µl DMEM/F-12 medium supplemented with 10 % FBS to the upper transwell chambers (8 µm pores; Corning Costar, Cambridge, MA, USA). Then, 600 µl of medium with or without 50 ng/ml recombinant murine CXCL12 (SDF-1α) protein (PeproTech) was added to the lower chamber of Transwell system. After 3, 6, 12, and 24 h of incubation at 37 °C in a humidified atmosphere containing 5 % CO₂, cells were fixed in 4 % PFA in PBS and stained with hematoxylin. Migrating cells were counted under an inverted microscope. Three independent experiments were performed in triplicate to obtain reported values.

Cell proliferation assay

Cell proliferation activity was determined using the alamarBlue assay (Invitrogen) (Caliari and Harley 2011). Untransfected, NC and CXCR4 KD mHAT9a cells were plated in a 96-well plate at a density of 10,000 cells/well in 100 µl DMEM/F12 medium containing 10 % FBS along with 0, 10, 50, or 100 ng/ml CXCL12 protein. Cells were cultured for 7 days, with alamarBlue assays performed daily. Briefly, cells were incubated for 3 h at 37 °C in a mixture of 90 µl medium and 10 µl alamarBlue solution. The reduction of resazurin to the fluorescent by-product resorufin by metabolically active cells was measured on an F200 spectrophotometer (Tecan) at 540(52)/580(20) nm (excitation/emission). Three independent experiments were performed in quadruplicate.

Statistics

One-way analysis of variance (ANOVA) was performed, and significance was calculated using Tukey's comparison test. Results are given as mean values ± the standard error of the mean (SE). A *p* value less than 0.05 was considered statistically significant.

Results

Expression of CXCR4 and CXCL12 mRNAs and proteins in the apical bud during incisor development

We performed real-time PCR analysis to compare expression levels of *CXCR4* and *CXCL12* mRNAs in the apical bud from neonatal mice and each whole incisor germ of E14, E16, E18, and PN4 wild-type mice (Fig. 1c, d). *CXCR4* mRNA expression in apical buds (ab) was 9.6-fold higher compared to whole developing incisor germs, whereas there was no significant difference in the expression level of *CXCR4* mRNA between E14 and PN4 incisors (Fig. 1c). *CXCL12* mRNA expression gradually increased as incisor development progressed, but levels of *CXCL12* mRNA were significantly lower in the apical bud relative to the whole developing incisors (Fig. 1d). Next, we visualized the distribution of *CXCR4* and *CXCL12* mRNAs in developing incisors by in situ hybridization. At E14, *CXCR4* mRNA was expressed on the labial and lingual sides of the IEE (Fig. 1e). *CXCL12* mRNA

was expressed at modest levels at the mesenchymal cells under the oral epithelium and dental follicle surrounding the apical end (Fig. 1f). At E16, *CXCR4* transcript was present at slightly decreased levels, and expression was localized in the labial cervical loop epithelium of the apical bud (Fig. 1g), while *CXCL12* mRNA was localized in the dental follicle (Fig. 1h). In developing incisors from E18 and PN4 samples, intense *CXCR4* signals were present in the BE and TA region (Fig. 1i, k). A few cells in the SR and OEE expressed *CXCR4* mRNA (Fig. 1m). At E18 and PN4, *CXCL12* mRNA was present in the dental follicle, also in the dental papilla cells on the lateral side of the apical bud (Fig. 1j, l). Here, *CXCL12* mRNA in the mesenchyme was adjacent to *CXCR4* mRNA expression sites (Fig. 1n).

Immunohistochemical analysis indicated that the distribution of CXCR4 and CXCR12 protein matched that suggested by relative expression of CXCR4 and CXCL12 mRNA in newborn mice (Supplemental Fig. 1s, t). Correspondingly, the signals also were detected in the 6-week fully grown mice (Fig. 1o–r). Taken together, these expression patterns suggest that CXCR4/CXCL12 signaling may play a critical role during incisor development.

The phenotype of CXCR4- and CXCL12- deficient incisors

The apical bud forms initially at the end of the incisor at E16 (early bell stage), with the structure of apical bud (SR, BA, IEE, and OEE) clearly observable by E18 (Harada et al. 2002). Consequently, we observed CXCR4- and CXCL12-deficient mice at E17.5–E18.5 to examine alterations in tooth morphology and cell differentiation. *CXCR4*^{-/-} embryos had smaller incisor germs than *CXCR4*^{+/+} (Fig. 2a–e) or *CXCR4*^{+/-} embryos (data not shown), consistent with their reduced body sizes (Nagasawa et al. 1996; Tachibana et al. 1998).

Statistical analysis revealed that the relative length of the *CXCR4*^{-/-} incisors was significantly shorter than the length of the *CXCR4*^{+/+} or *CXCR4*^{+/-} incisors (Fig. 2f). Meanwhile, *CXCL12*^{-/-} embryos had smaller incisor germs than wild-type, although not significantly so (Supplemental Fig. 2o–t). A small epithelial cell aggregation was seen in the SR of CXCR4-deficient apical buds (Fig. 2g, h and i), whereas a similar structure is not seen in the wild-type (Fig. 2j, k and l). We found the aggregates with 62.5 % of *CXCR4*^{-/-} incisor germs (n=8), 16.6 % of *CXCR4*^{+/+} (n=6) and 10 % of *CXCR4*^{+/-} (n=10). Immature odontoblasts were observed in *CXCR4*^{-/-} incisors, but pre-dentin was clearly observed between the IEE and odontoblast cell layers in both CXCR4 mutants and controls (Fig. 2m and n). No distinct morphological defects of pre-ameloblasts were observed (Fig. 2m and n). We also detected markers of ameloblast differentiation (amelogenin protein) and pre-ameloblasts (*Fgf9* and *Shh* mRNAs), as well as a putative stem-cell marker (*Notch1* and *Sox2* mRNA). However, there were no significant differences in marker expression between CXCR4-deficient and wild-type incisors (data not shown), nor between *CXCL12*-deficient and wild-type incisors.

The CXCL12 proteins induce cell spreading around the colonies

To examine the role of CXCL12 proteins in dental epithelial cells, we used mHAT9a cells derived from the apical bud in vitro. We cultured mHAT9a cells in the presence of 1, 50, and 100 ng/ml of CXCL12 proteins (data not shown; 1 ng, 100 ng/ml of CXCL12 proteins) or no or 1 ng/ml of Fgf2 proteins as controls. CXCL12 proteins induced more spread

colonies (Fig. 3a) compared to the controls (Fig. 3b and c). Numerous individual cells were observed at the margins of colonies remarkably at 50 ng/ml of CXCL12 (Fig. 3a). The cell colonies in 1 ng/ml Fgf2 proteins developed faster than the cells with or without CXCL12 proteins, but there were no significant differences in shapes of colonies compared with the control (Fig. 3b and c).

Knockdown of *CXCR4* mRNA alters cell morphology

We then examined whether the effects of CXCL12 proteins were through the CXCR4 in mHAT9a cells. We used shRNA targeting CXCR4 to knock down CXCR4 expression in mHAT9a. Here, *CXCR4* mRNA levels in KDs were reduced by 59 % relative to controls (Fig. 3d). CXCR4 KDs proliferate slowly, creating dense cell colonies (Fig. 3e), and appear to spread less than colonies formed by control cells (Fig. 3f). The cell membrane protrusions were prevalent in control cells, but were rarely observed in CXCR4 KD colonies (Fig. 3f).

Furthermore, we cultured CXCR4 KDs within CXCL12 proteins (1, 50, 100 ng/ml). The protrusions around the KD colonies were observed, but the cells were aggregated at any concentration of CXCL12 proteins (Fig. 3g).

CXCR4/CXCL12 signaling enhances the motility of dental epithelial cells

CXCR4/CXCL12 signaling promotes cell migration and proliferation not only during the inflammatory response but also during embryogenesis (Zou et al. 1998; Ma et al. 1998; Ara et al. 2003). Therefore, we next investigated the effect of CXCR4 and CXCL12 on cell motility using the mHAT9a cells. In transwell migration assays, mHAT9a cells exhibited direction motility towards the lower chamber after incubation for 6 h in the presence of recombinant CXCL12 protein (50 ng/ml). After 24 h, the number of migrating cells increased significantly relative to controls (Fig. 3h) ($p < 0.01$). Next, we compared the motility of stable CXCR4 KD mHAT9a cells to NC control. After 24 h incubation, KD cell migration was significantly reduced compared to NC cells in the presence of CXCL12, and was equivalent to KD cells without any chemotactic signal (Fig. 3i). These results indicate that dental epithelial cells are capable of migrating under control of the CXCR4/CXCL12 signal.

Inhibition of *CXCR4* mRNA suppresses cell proliferation in the apical bud

In order to elucidate the effect of CXCR4 on cell proliferation, we examined co-localization of CXCR4 proteins and Ki-67, a cell proliferation marker. CXCR4 and Ki-67-double-positive cells were mostly detected in the TA region (Fig. 4a) as well as in the SR regions (Fig. 4a). We subsequently performed immunostaining of Ki-67 in *CXCR4*^{-/-} and *CXCR4*^{+/+} mice as a control. In *CXCR4*^{-/-} mice, Ki-67 positive cells were mostly observed in the TA of the apical bud (Fig. 4b) with a small number of Ki-67-positive cells also seen in the SR (Fig. 4b). By contrast, Ki-67-positive cells were detected in BE, TA and OEE regions in CXCR4 wild-type mice (Fig. 4c). We counted the number of Ki-67-positive cells in the serial transverse sections from the tip of the apical bud to 150 μ m ahead to the incisal edge. In *CXCR4*^{-/-} mice, the percentage of Ki-67-positive cells was reduced (63 %) relative to the level in controls (Fig. 4d). We then investigated whether dental epithelial cell proliferation was dependent on the CXCR4/CXCL12 signal. Changes in alamarBlue fluorescence

intensity were monitored for mHAT9a cells cultured in the presence of 0, 10, 50, and 100 ng/ml of CXCL12 proteins, with fluorescence intensity a reflection of overall cell number (Caliari and Harley 2011). We observed no significant difference in cell number as a function of CXCL12 concentration (data not shown). We next used stable CXCR4 KDs and NCs to examine the role of the CXCR4 receptor. Here, CXCR4 KD cells exhibited significantly decreased growth rate relative to NC cells (Fig. 4e). However, as with the wild-type cells, addition of the CXCL12 protein did not impact proliferation of either CXCR4 KD or NC cells (Fig. 4e).

Discussion

Stem cells reside in tissue-specific microenvironments, termed niches, in order to maintain multipotency and homeostasis of progenitor cells. In this paper, we provide the first published description of the expression and functions of CXCR4/CXCL12 signaling in the dental stem cell niche. We chose to examine the impact of this chemokine based on the results of RT-PCR and histological analysis. Notably, levels of *CXCR4* mRNAs were very high in the apical bud. Furthermore, *CXCR4* signals were gradually localized to the TA region of the incisor as tooth development progressed, whereas *CXCL12* signals were extended to the mesenchymal cells surrounding the apical bud. These results suggest that CXCR4/CXCL12 signaling plays some role in undifferentiated dental epithelial cells in the apical bud, possibly as an epithelial–mesenchymal interaction.

Epithelial stem cell niches are structurally similar to the mouse hair follicle niche and gut epithelial stem cell niche, as well as to the *Drosophila* ovarian follicle stem cell niche. Further, epithelial stem cells are known to interact with their own progeny in each tissue-specific niche (Li et al. 2005; Scadden 2006; Nystul and Spradling 2007; Morrison and Spradling 2008). Therefore, we examined a marker of cell proliferation and noted that these cells frequently divide and migrate in CXCR4-expressing regions of the apical bud. We additionally observed dense cell aggregates in the SR of *CXCR4*^{-/-} mice of the apical bud.

These results lead us to suggest that cell motility in the apical bud might be influenced by CXCR4/CXCL12 signaling. We therefore examined the impact of CXCR4/CXCL12 signaling on cell motility in vitro via analysis of the mHAT9a cell line. Our transwell migration assays revealed that CXCL12 promoted mHAT9a cell chemotaxis, and that migration was significantly reduced by shRNA of CXCR4. These results suggest that CXCR4-expressing dental epithelial progenitor cells can undergo chemotaxis toward sources of CXCL12 secreted by mesenchymal cells around the apical bud in vivo.

Recently, two groups reported that dental pulp stem cells isolated from human tooth could be induced to migrate by CXCL12 (Jiang et al. 2008; Suzuki et al. 2011), lending further credence to our observation. We further report that, in addition to its expression in incisor germs, CXCL12 may also play a role in the motility of undifferentiated mesenchymal cells in mouse incisors. In the developing nervous system, dislocated granule cell aggregates have been found underneath the Purkinje cell layer in *CXCR4*^{-/-} mice, resulting in aberrant laminar structure of the cerebellum (Zou et al. 1998). It is therefore possible that CXCR4/CXCL12 signaling controls the axis of motility from the SR to the exterior of the apical bud.

During chemotaxis, cells must couple the detection of extracellular chemoattractant with creating protrusions in order to undergo directed migration (Theveneau et al. 2010). In our in vitro cultures, CXCL12 enhanced spreading of mHAT9a cells, which have large numbers of protrusions, in a dose-dependent way. By contrast, CXCR4 KD cells tended to form tightly packed colonies and to adopt a less amoeboid morphology. These results suggest that the CXCR4/CXCL12 signaling may induce cellular deformability of the dental epithelial cells. We speculated that the expression of CXCR4 in the dental epithelial stem cell niche might have an important role in the recruitment of progenitor cells from SR or BE region of the apical bud.

We observed that the number of Ki-67 positive cells was reduced in the CXCR4-deficient apical bud, and, furthermore, that CXCR4 KD cells proliferate more slowly than controls. Taken together, these data imply that expression of CXCR4 might be important for cell proliferation in the epithelial stem cell niche. This finding is consistent with previous reports in other tissue-committed stem cells. Notably, CXCR4 and CXCL12 knockout mice exhibit severe reduction of numbers of hematopoietic progenitors in the bone marrow (Sugiyama et al. 2006), neural precursor cells that give rise to neurons, astrocytes and oligodendrocytes in the central nervous system (Ni et al. 2004; Maysami et al. 2006; Brzoska et al. 2012), and PGCs in the gonads (Zou et al. 1998; Ma et al. 1998; Ara et al. 2003). All of these phenotypes have been suggested to arise as a result of impaired migration or homing of cells to a supportive niche. In contrast, according to our results, CXCL12 does not much influence the growth rate of mHAT9a cells. Similar results have been reported for STRO-1- and CD146-positive dental pulp stem cells, neither of which proliferates in response to stimulation by CXCL12 (Miura et al. 2003; Jiang et al. 2008; Suzuki et al. 2011). While there are splice variants of *CXCL12* mRNA, SDF-1 α , β and γ , recent reports have characterized the functional redundancies and interactions between isoforms (David et al. 2002; Jin et al. 2013; Ho et al. 2010). However, the direct effect of CXCL12 on the proliferation of dental epithelial cells remains controversial. One conceivable reason is that the inhibition of CXCR4 expression caused altered plasticity of dental epithelial cells during migration, leading to an alteration of the outside growth of cells.

The mechanism underlying the aggregation of *CXCR4*^{-/-} dental epithelial cells remains unresolved, but represents an interesting avenue for ongoing experiments to better resolve the developmental basis of the dental epithelial stem cell niche. In conclusion, we propose that CXCR4/CXCL12 signaling impacts the dynamics of enamel progenitor cells from the SR to the exterior of the apical bud. After the release of daughter cells, CXCR4 expression is necessary for cell proliferation in the TA region of mouse incisors. These results have significance not only in resolving mechanisms of action of the native dental epithelial stem cell niche but also in applications looking to mimic critical niche signals for regenerative medicine applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Dr. Stephanie Tsang (University of Illinois at Urbana-Champaign), Dr. Myung-Eun Shin (Duke University Medical Center), and Ms. Yanzhen Li, M.A. (University of North Carolina) for critical discussions; to Mr. Naoyuki Tamaki (IBM Global Services Japan Solution and Services Company ISOL) for technical support in generating beautiful figures; Dr. Yuko Suzuki (Health Sciences University of Hokkaido) for excellent suggestions and for providing materials. This work was supported by JSPS KAKENHI Grant Numbers 19791342, 15K20358 to Y.-T. T. We are also grateful for the funding for this study provided by the Chemistry-Biology Interface Training Program NIH NIGMS T32GM070421 (SRC) as well as the Chemical and Biomolecular Engineering Dept. (BAH), and the Institute for Genomic Biology (BAH) at the University of Illinois at Urbana-Champaign.

References

- Ara T, Nakamura Y, Egawa T, Sugiyama T, Abe K, Kishimoto T, Matsui Y, Nagasawa T. Impaired colonization of the gonads by primordial germ cells in mice lacking a chemokine, stromal cell-derived factor-1 (SDF-1). *Proc Natl Acad Sci USA*. 2003; 100:5319–5323. [PubMed: 12684531]
- Atchley WR, Plummer AA, Riska B. Genetic analysis of size-scaling patterns in the mouse mandible. *Genetics*. 1985; 111:579–595. [PubMed: 4054610]
- Brzoska E, Kowalewska M, Markowska-Zagrajek A, Kowalski K, Archacka K, Zimowska M, Grabowska I, Czerwińska AM, Czarnecka-Góra M, Stremińska W, Jarczyk-Ilach K, Ciemerych MA. Sdf-1 (CXCL12) improves skeletal muscle regeneration via the mobilisation of Cxcr4 and CD34 expressing cells. *Biol Cell*. 2012; 104:722–737. [PubMed: 22978573]
- Caliari SR, Harley BA. The effect of anisotropic collagen-GAG scaffolds and growth factor supplementation on tendon cell recruitment, alignment, and metabolic activity. *Biomaterials*. 2011; 32:5330–5340. [PubMed: 21550653]
- David NB, Sapède D, Saint-Etienne L, Thisse C, Thisse B, Dambly-Chaudière C, Rosa FM, Ghysen A. Molecular basis of cell migration in the fish lateral line: role of the chemokine receptor CXCR4 and of its ligand, SDF1. *Proc Natl Acad Sci USA*. 2002; 99:16297–16302. [PubMed: 12444253]
- Harada H, Ohshima H. New perspectives on tooth development and the dental stem cell niche. *Arch Histol Cytol*. 2004; 67:1–11. [PubMed: 15125018]
- Harada H, Kettunen P, Jung HS, Mustonen T, Wang YA, Thesleff I. Localization of putative stem cells in dental epithelium and their association with Notch and FGF signaling. *J Cell Biol*. 1999; 147:105–120. [PubMed: 10508859]
- Harada H, Toyono T, Toyoshima K, Ohuchi H. FGF10 maintains stem cell compartment in developing mouse incisors. *Development*. 2002; 129:1533–1541. [PubMed: 11880361]
- Ho TK, Tsui J, Xu S, Leoni P, Abraham DJ, Baker DM. Angiogenic effects of stromal cell-derived factor-1 (SDF-1/CXCL12) variants *in vitro* and the *in vivo* expressions of CXCL12 variants and CXCR4 in human critical leg ischemia. *J Vasc Surg*. 2010; 51:689–699. [PubMed: 20206813]
- Jiang L, Zhu YQ, Du R, Gu YX, Xia L, Qin F, Ritchie HH. The expression and role of stromal cell-derived factor-1alpha-CXCR4 axis in human dental pulp. *J Endod*. 2008; 34:939–944. [PubMed: 18634924]
- Jin J, Zhao WC, Yuan F. CXCR7/CXCR4/CXCL12 axis regulates the proliferation, migration, survival and tube formation of choroid-retinal endothelial cells. *Ophthalmic Res*. 2013; 50:6–12. [PubMed: 23549077]
- Kawamoto T. Use of a new adhesive film for the preparation of multi-purpose fresh-frozen sections from hard tissues, whole-animals, insects and plants. *Arch Histol Cytol*. 2003; 66:123–143. [PubMed: 12846553]
- Li CY, Cha W, Luder HU, Charles RP, McMahon M, Mitsiadis TA, Klein OD. E-cadherin regulates the behavior and fate of epithelial stem cells and their progeny in the mouse incisor. *Dev Biol*. 2005; 366:357–366. [PubMed: 22537490]
- Libura J, Drukala J, Majka M, Tomescu O, Navenot JM, Kucia M, Marquez L, Peiper SC, Barr FG, Janowska-Wieczorek A, Ratajczak MZ. CXCR4-SDF1 signaling is activate in rhabdomyosarcoma cells and regulate locomotion, chemotaxis, and adhesion. *Blood*. 2002; 100:2597–2606. [PubMed: 12239174]

- Ma Q, Jones D, Borghesani PR, Segal RA, Nagasawa T, Kishimoto T, Bronson RT, Springer TA. Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proc Natl Acad Sci USA*. 1998; 95:9448–9453. [PubMed: 9689100]
- Maysami S, Nguyen D, Zobel F, Pitz C, Heine S, Höpfner M, Stangel M. Modulation of rat oligodendrocyte precursor cells by the chemokine CXCL12. *Neuroreport*. 2006; 31:1187–1190. [PubMed: 16837851]
- Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, Shi S. SHED: stem cells from human exfoliated deciduous teeth. *Proc Natl Acad Sci USA*. 2003; 100:5807–5812. [PubMed: 12716973]
- Morrison SJ, Spradling AC. Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. *Cell*. 2008; 132:598–611. [PubMed: 18295578]
- Nagasawa T, Hirota S, Tachibana K, Takakura N, Nishikawa S, Kitamura Y, Yoshida N, Kikutani H, Kishimoto T. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature*. 1996; 382:635–638. [PubMed: 8757135]
- Ni HT, Hu S, Sheng WS, Olson JM, Cheeran MC, Chan AS, Lokensgard JR, Peterson PK. High-level expression of functional chemokine receptor CXCR4 on human neural precursor cells. *Brain Res Dev Brain Res*. 2004; 152:159–169.
- Nystul T, Spradling AC. An epithelial niche in the *Drosophila* ovary undergoes long range stem cell replacement. *Cell Stem Cell*. 2007; 1:277–285. [PubMed: 18371362]
- O'Hayre M, Salanga CL, Kipps TJ, Messmer D, Dorrestein PC, Handel TM. Elucidating the CXCL12/CXCR4 signaling network in chronic lymphocytic leukemia through phosphoproteomics analysis. *PLoS ONE*. 2010; 22:e11716. [PubMed: 20661426]
- Otsu K, Kishigami R, Fujiwara N, Ishizeki K, Harada H. Functional role of Rho-kinase in ameloblast differentiation. *J Cell Physiol*. 2011; 226:2527–2534. [PubMed: 21792909]
- Scadden DT. The stem-cell niches as an entity of action. *Nature*. 2006; 441:1075–1079. [PubMed: 16810242]
- Sugiyama T, Kohara H, Noda M, Nagasawa T. Maintenance of the Hematopoietic Stem Cell Pool by CXCL12-CXCR4 Chemokine Signaling in Bone Marrow Stromal Cell Niches. *Immunity*. 2006; 25:977–988. [PubMed: 17174120]
- Suzuki T, Lee CH, Chen M, Zhao W, Fu SY, Qi JJ, Chotkowski G, Eisig SB, Wong A, Mao JJ. Induced migration of dental pulp stem cells for *in vivo* pulp regeneration. *J Dent Res*. 2011; 90:1013–1018. [PubMed: 21586666]
- Tachibana K, Hirota S, Iizasa H, Yoshida H, Kawabata K, Kataoka Y, Kitamura Y, Matsushima K, Yoshida N, Nishikawa S, Kishimoto T, Nagasawa T. The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. *Nature*. 1998; 93:591–594. [PubMed: 9634237]
- Thesleff I, Wang XP, Suomalainen M. Regulation of epithelial stem cells in tooth regeneration. *C R Biol*. 2007; 330:561–564. [PubMed: 17631454]
- Theveneau E, Marchant L, Kuriyama S, Gull M, Moepps B, Parsons M, Mayor R. Collective chemotaxis requires contact-dependent cell polarity. *Dev Cell*. 2010; 19:39–53. [PubMed: 20643349]
- Wang XP, Suomalainen M, Felszeghy S, Zelarayan LC, Alonso MT, Plikus MV, Maas RL, Chuong CM, Schimmang T, Thesleff I. An integrated gene regulatory network controls stem cell proliferation in teeth. *PLoS Biol*. 2007; 5:e159. [PubMed: 17564495]
- Yokohama-Tamaki T, Fujiwara N, Shibata S, Wakisaka S, Harada H. The Epithelial - Mesenchymal interaction plays a role in the maintenance of the stem cell niche of mouse incisors via Fgf10 and Fgf9. *Open Biotechnol J*. 2008; 2:111–115.
- Yokohama-Tamaki T, Maeda T, Tanaka TS, Shibata S. Functional analysis of CTRP3/cartducin in Meckel's cartilage and developing condylar cartilage in the fetal mouse mandible. *J Anat*. 2011; 218:517–533. [PubMed: 21371032]
- Yoshida T, Iwata T, Umemoto T, Shiratsuchi Y, Kawashima N, Sugiyama T, Yamato M, Okano T. Promotion of Mouse Ameloblast Proliferation by Lgr5 Mediated Integrin Signaling. *J Cell Biochem*. 2013; 114:2138–2147. [PubMed: 23553959]
- Zou YR, Kottmann AH, Kuroda M, Taniuchi I, Littman DR. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature*. 1998; 393:595–599. [PubMed: 9634238]

Fig. 1.

Expression patterns of *CXCR4* and *CXCL12* mRNAs and proteins in developing incisors. **a**, **b** An incisor germ and the inner enamel epithelium. *Arrow* indicates the apical bud. The *dotted line* indicates the border between IEE and the apical bud. **c**, **d** Quantitation of relative *CXCR4* and *CXCL12* mRNA levels in the apical bud and developing incisors (**, $p < 0.01$). **e–n** Expression patterns of *CXCR4* (**e**, **g**, **i**, **k**, **m**) and *CXCL12* (**f**, **h**, **j**, **l**, **n**) mRNAs were analyzed by in situ hybridization. The *dotted line* indicates the outline of the epithelium. In E14 incisors, *CXCR4* mRNA was detected on the labial and lingual sides of the IEE (**e**, *arrows* and an *arrowhead*), whereas *CXCL12* mRNA was detected in the mesenchyme surrounding the enamel organ (**f**, *arrows*). At E16, *CXCR4* and *CXCL12* mRNA expression

patterns became restricted to the apical end of the incisor (**g, h, arrows**). In E18 and PN4 incisors, strong *CXCR4* expression was detected in BE to TA (*arrows*) of the apical bud (**i, k, m**). A few cells in the SR and OEE expressed *CXCR4* mRNA (**m, arrowheads**). *CXCL12* expression was detected in the dental follicle (*arrows*) and dental papilla (*arrowheads*) around the apical bud (**j, l, n**). **o–r** Immuno histochemistry of CXCR4 and CXCL12 proteins in 6 week incisors. **o** CXCR4 signals existed in the TA (**o, arrow**). The signal was detected from the OEE region (**p, arrowhead**) to the end of TA (*arrowhead*). **q** CXCL12 signals in the mesenchyme were adjacent to the apical bud (**q, arrowheads**). The intense signals were detected in the lateral side of the apical bud (**r, arrow**) and dental follicle (**r, arrow heads**). *Scale bar* 100 μ m. Significant differences between samples were determined by Tukey's test. $**p<0.01$. *ab* apical bud, *LA* labial side, *LI* lingual side, *OEE* outer enamel epithelium, *BE* basal epithelium, *TA* transit-amplifying cells, *SR* stellate reticulum

Fig. 2. $CXCR4^{-/-}$ mutant incisors contain epithelial cell aggregations in the apical bud. **a-e** $CXCR4^{-/-}$ and $CXCR4^{+/+}$ heads, mandibulars and incisor germs. **f** Relative length of the lower incisors of $CXCR4^{-/-}$ and $CXCR4^{+/+}$ mice (** $p < 0.01$). **g-n** Hematoxylin and eosin staining of mandibular incisor sections from E18.5 $CXCR4^{-/-}$ and $CXCR4^{+/+}$ mouse incisors. Boxes in (g, j) are shown in higher magnification in subsequent panels (h, k). **i** A transverse section of a $CXCR4^{-/-}$ apical bud and **l** controls. Epithelial cell aggregation was observed in $CXCR4^{-/-}$ apical buds (h arrow, i). Scale bars (a, b) 500 μ m, (d, g) 100 μ m, (h, i) 50 μ m (m) 20 μ m. IEE inner enamel epithelium, BE basal epithelium, SR stellate reticulum, OEE outer enamel epithelium, DP dental pulp, Od odontoblasts, De dentin, Am ameloblasts

Fig. 3.

CXCR4/CXCL12 signaling promotes dental epithelial cell morphology and motility. **a–c** Phase contrast images show mHAT9a cells in CXCL12-treated, untreated (control) and Fgf2-treated culture after 24 h. Spreading cells were present around the colonies within the CXCL12-containing medium (**a**), while not significant in control medium (**b, c**). **d** Expression of *CXCR4* mRNA in mHAT9a cells transfected with vectors expressing either CXCR4 shRNA or negative-control shRNA. **e–g** Phase-contrast images. CXCR4 KD cells formed dense colonies (**e**). Spreading cells were present around the margins of control colonies (**f**, arrows). CXCL12-treated CXCR4 KD cells formed dense colonies (**g**). **h** CXCL12 significantly induced migration by mHAT9a cells after 24 h incubation. **i** The motility of CXCR4 KD cells was suppressed. Data are expressed as mean $\pm$ SEM; $n=3$. Significant differences between samples were determined by Tukey's test. ** $p<0.01$. * $p<0.05$. Scale bars 50 μ m. NC mHAT9a cells expressing negative-control shRNA, KD mHAT9a cells expressing CXCR4 shRNA

Fig. 4.

Epithelial cell proliferation is suppressed in the CXCR4 mutant apical bud. **a** CXCR4/Ki-67 (i.e., proliferating) double staining of the apical bud. *Arrows* indicate CXCR4/Ki-67-double-positive cells in the TA. *Arrowheads* indicate the double-positive cells in the SR. **b, c** Ki-67 staining of CXCR4^{-/-} apical bud (**b**) and control (**c**). *Arrows* indicate Ki-67-positive cells in the TA. *Arrowheads* indicate Ki-67-positive cells in the SR. **d** In E18.5 CXCR4^{-/-} mutant mice, Ki-67-positive cell number was significantly reduced in the apical bud. **e** In CXCR4 KD mHAT9a cells, proliferation was inhibited, but the impact of CXCL12 was small. *Scale bar* 50 μ m. Data are expressed as mean $\pm$ SEM, $n=3$ (**b**) and $n=4$ (**c**). Significant differences between samples were determined by Tukey's test. ** $p<0.01$

Table 1

Primer list for gene expression analysis

DNA	Primer Sequence (5' → 3')	Product size
<i>CXCR4</i>	Forward: CCAGCCCTCCTCCTGACTACTATA Reverse: GGGTAAAGGCGGTCACAGAT	101 bp
<i>CXCL12</i>	Forward: TGTGCTCAGAGACCCTTTCC Reverse: TGGTCCGTCAGGCTACAGA	101 bp
<i>GAPDH</i>	Forward: TGAACGGGAAGCTCACTGG Reverse: TCCACCACCCTGTGCTGTA	306 bp

GAPDH glyceraldehyde-3-phosphate dehydrogenase