Published in final edited form as:

Differentiation. 2009 April ; 77(4): 395–411. doi:10.1016/j.diff.2008.10.015.

The chemokine SDF-1/CXCL12 regulates the migration of melanocyte progenitors in mouse hair follicles

Abdelhak Belmadani*, Hosung Jung, Dongjun Ren, and Richard J. Miller

Department of Molecular Pharmacology and Biological Chemistry, Northwestern University Medical School, 303 East Chicago Avenue, Ward 8-296, Chicago, IL 60611, USA

Abstract

Mouse skin melanocytes originate from the neural crest and subsequently invade the epidermis and migrate into the hair follicles (HF) where they proliferate and differentiate. Here we demonstrate a role for the chemokine SDF-1/CXCL12 and its receptor CXCR4 in regulating the migration and positioning of melanoblasts during HF formation and cycling. CXCR4 expression by melanoblasts was upregulated during the anagen phase of the HF cycle. CXCR4-expressing cells in the HF also expressed the stem cell markers nestin and LEX, the neural crest marker SOX10 and the cell proliferation marker PCNA. SDF-1 was widely expressed along the path taken by migrating CXCR4-expressing cells in the outer root sheath (ORS), suggesting that SDF-1-mediated signaling might be required for the migration of CXCR4 cells. Skin sections from CXCR4-deficient mice, and skin explants treated with the CXCR4 antagonist AMD3100, contained melanoblasts abnormally concentrated in the epidermis, consistent with a defect in their migration. SDF-1 acted as a chemoattractant for FACS-sorted cells isolated from the anagen skin of CXCR4–EGFP transgenic mice *in vitro*, and AMD3100 inhibited the SDF-1-induced migratory response. Together, these data demonstrate an important role for SDF-1/CXCR4 signaling in directing the migration and positioning of melanoblasts in the HF.

Keywords

CXCR4 receptors; SDF-1; Migration; Melanocyte precursors; Melanocytes

1. Introduction

The embryonic neural crest (NC) gives rise to progenitor cells originating in the dorsal part of the neural tube that are capable of differentiating into several lineages, including melanocytes of the skin and hair (Le Douarin and Dupin, 1993). Exactly how NC cells become committed to the melanocyte lineage and what factors control their survival, proliferation, migration and differentiation are key questions, the answers to which may be

of great importance for understanding the mechanisms underlying several melanocyte-related pathologies such as melanoma.

Mouse NC-derived melanoblasts, the precursors for melanocytes, emerge from the dorsal neural tube at around embryonic day 8.5 (E8.5), migrate along a dorsolateral pathway between the dermatome and the overlying ectoderm, and from E10.5 migrate ventrally through the developing dermis. At E14.5, they begin to invade the overlying epidermis and then migrate into the developing hair follicles (HFs), and become closely coupled to HF development and cycling (Slominski and Paus, 1993).

The different stages of development of HFs in the skin have been extensively documented (Hardy, 1992; Panaretto, 1993; Oro and Scott, 1998; Mikkola and Millar, 2006). HFs contain different cell populations, including dermal papilla (DP) cells derived from the mesenchyme, and epithelial cells, originally derived from the surface epithelium (Hardy, 1992). The initial signal that triggers HF formation results from interactions between the epithelium and the mesenchyme leading to the formation of the placodes and the follicle DP. The epithelial cells in close contact with the DP make up the highly proliferative matrix of the hair bulb. In response to cues derived from the DP, matrix cells rapidly divide and differentiate into keratinized hair shafts. Once formed, the postnatal HF undergoes periods of HF cycling. In mice, a complete dorsal hair cycle lasts approximately 25 days. The HF undergoes periods of active growth (anagen), in which a small number of HF stem cells proliferate, followed by a phase of regression involving apoptosis of the matrix cells (catagen) and a phase of inactivity (telogen) (Stenn and Paus, 2001; Fuchs et al., 2001; Botchkareva et al., 2003; Wilson et al., 1994).

Hair follicle stem cells persist throughout the lifetime of the organism and are located in a niche-like structure called the bulge (Cotsarelis et al., 1990; Morris and Potten, 1999). Bulge cells in young and adult mice are multipotent. They give rise to all epithelial cell lineages within the intact follicle during normal hair cycling, and can be recruited to transiently contribute to the epidermis in response to stimuli such as wounding (Blanpain et al., 2004; Morris and Potten, 1999; Oshima et al., 2001; Taylor et al., 2000). Like HF stem cells, putative melanocyte stem cells are also located in the bulge (Nishimura et al., 2002) and give rise to proliferating melanoblasts that migrate within the outer root sheath (ORS) towards the hair matrix (Botchkareva et al., 2001).

In the mouse HF melanocytes are located in the inner and uppermost layers of the hair matrix and in contact with the DP (Hirobe, 1995). The development of melanocytes in the hair matrix is also under cyclical control in which melanogenesis and anagen are tightly coupled. During HF morphogenesis melanoblasts enter the placodes of the developing HF, proliferate and become melanogenically active in synchrony with the onset of hair fiber formation around postnatal day 4 (Jordan and Jackson, 2000a; Mayer, 1973; Botchkareva et al., 2003). Melanocytes in the hair matrix proliferate in anagen, differentiate to produce melanin pigment that is transferred to keratinocytes of the growing hairs, and then die by apoptosis during catagen (Tobin et al., 1998).

It is unclear what signaling pathways and factors regulate melanocyte migration and development. Several mouse models have proved useful in approaching this question. For example, mutations in the microphthalmia-associated transcription factor (MITF) result in the complete loss of NC melanocytes early in development (Nakayama et al., 1998; Opdecamp et al., 1997). The PAX3 (*Spotch*) mutation results in melanocyte deficiencies, while mutations in c-kit (*Dominant spotting*) do not apparently affect commitment to the melanocyte lineage, but result in a subsequent failure of melanoblasts to survive past early stages of development (Hou et al., 2000; Mackenzie et al., 1997; Wehrle-Haller and Weston, 1995). Furthermore, signaling mediated by the c-kit receptor in mice has been shown to be crucial when melanocyte precursors invade the epidermis from the dermis (Nishikawa et al., 1991; Yoshida et al., 1996, 2006). Migration of early melanoblasts within the dermis requires both c-kit as well as endothelin-3 and endothelin receptor B function (Yoshida et al., 1996). However, the mechanisms involved in the directed migration of melanoblasts allowing them to find their way from the epidermis to the hair matrix bulb during HF development and postnatal hair cycling are unknown. It appears that melanoblasts migrate along the ORS, a finding that may suggest that this cell layer provides important cues which guide these cells towards the hair bulb.

Chemokines are small-secreted proteins that exert their effects by activating a family of G-protein-coupled receptors (GPCRs). Chemokines have been shown to play several fundamental roles in the control of leukocyte development and migration (Tran and Miller, 2003). Moreover, signaling by the chemokine stromal cell-derived factor-1 (SDF-1/CXCL12) via its receptor CXCR4 appears to regulate the migration and the development of stem/progenitor cells that form many tissues (Zou et al., 1998). In addition to stem cells in the bone marrow, these include stem cells that give rise to germ, muscle and endothelial cells. Furthermore, chemokines play an important role in the development of the nervous system. For example, mice that lack either CXCR4 receptors or SDF-1 show abnormal development of the internal granule layer of the cerebellum (Zou et al., 1998), and the dentate gyrus of the hippocampus (Lu et al., 2002). Recently, we demonstrated that the chemokine SDF-1 and its receptor CXCR4 also play an important role in the development of certain NC derivatives (Belmadani et al., 2005). We found that CXCR4 and SDF-1 are expressed by ventrally migrating trunk NC cells and that SDF-1 acts as a chemoattractant for these cells. Furthermore, we showed that disruption of SDF-1/CXCR4 signaling in CXCR4 mutant mice produces deficits in the migration of DRG neural progenitors. We have now examined the expression patterns of SDF-1 and CXCR4 during HF development and demonstrate a role for CXCR4 signaling in the migration of melanoblasts into the HF. These results further demonstrate the widespread role for SDF-1/CXCR4 signaling in the regulation of stem cell migration and development.

2. Materials and methods

2.1. Animals

CXCR4 ko (Zou et al., 1998) and wild-type litter mates and the following transgenic animals were used in this study: Nestin-EGFP transgenic mice (provided by Dr. Anjen Chenn, Northwestern University (Tran et al., 2007), CXCR4-EGFP and SDF-1-EGFP

bacterial artificial chromosome (BAC) transgenic mice (kindly provided by Dr. Mary Beth Hatten and the Gene Expression Nervous System Atlas (GENSAT) project; NINDS contract N01Nso2331 to Rockefeller University, NY, <http://www.gensat.org/index.html>). The generation of BAC transgenic mice expressing EGFP via the CXCR4 or SDF-1 promoters is described in the GENSAT website. Expression of EGFP in these mice has been shown to be identical to endogenous gene expression as examined by *in situ* hybridization (<http://www.gensat.org/index.html>).

CXCR4-EGFP/SDF-mRFP double transgenic mice were obtained by breeding CXCR4-EGFP with SDF-mRFP mice. SDF-mRFP mice were generated as follows: The SDF-1 containing BAC clone (RP23-203H21) was obtained from invitrogen. To generate a SDF-1 BAC reporter vector, monomeric red fluorescence protein 1 (mRFP1) was inserted immediate downstream of the SDF-1 coding sequence by λ -Red mediated recombineering with slight modifications (Lee et al., 2001). An mRFP1-FRT-KAN-FRT targeting cassette was generated by self-ligation of blunt-ended BglII-SmaI fragment of pIGCN21 and then replacing EGFP coding sequence with that of mRFP1 (Lee et al., 2001). The targeting cassette was amplified by PCR using the following chimeric primers, 3' of which were homologous to targeting cassette and 5' of which were homologous to the last exon of SDF1 α : 3. for upstream, 5'-CATTGACCCGAAATTAAAGTGGATCCAAGAGTACCTGGAGAAAGCTTTAAACAAGCCGGTCGCCACCATGGCCTCC-3'; for downstream, 5'-CACTGCCCTTGCATCTCCACGGATGTCAGCCTTCCTCGGGGGTCTACTGGAAAGCTATTCCAGAAGTAGTGAGGA-3. The primers were designed to target mRFP1 immediately downstream of the SDF-1 α coding sequence and upstream of the poly (A) site. The stop codon of SDF-1 α was deleted to generate an SDF1-mRFP1 fusion construct. In this way, the splicing sites for SDF-1 β and γ were disrupted, so SDF-1 α -mRFP1 would be expressed in any cell where any of the isoforms of SDF-1 are expressed. Transgenic mice were generated by the Center for Genetic Medicine, Northwestern University. All mice were bred in the local animal facilities and maintained on a 12-h dark/light cycle (7 AM/7 PM) with food and water ad libitum. Animal-related procedures were approved by the Northwestern University animal care and use committee (ACUC).

2.2. Generation of skin spheres

Postnatal mouse back skin was carefully dissected free of other tissue, cut into 0.3 mm³ pieces using a tissue shopper, washed 3 times in Hanks balanced salt solution (HBSS), and then digested with 0.1% trypsin for 40 min at 37 °C, followed by 0.1% DNAase for 1 min at room temperature. Tissue pieces were then washed twice with HBSS, once with medium (DMEM-F12, 3:1, 1 g/ml⁻¹ fungizone (Gibco-BRL, Carlsbad, CA, USA), 1% penicillin/streptomycin (BioWhittaker, Walkersville, MD, USA) containing 10% rat serum (Harlan Bioproducts, Indianapolis, IN, USA), and twice with serum-free medium. Skin pieces were then mechanically dissociated in medium with the aid of needles of the respective sizes 18, 19, and 21, and the suspension poured through a 40 M cell strainer (Falcon, Franklin Lakes, NJ, USA). Embryonic skin was directly subjected to mechanical dissociation. Dissociated cells were centrifuged at 168 g and resuspended in 5 ml DMEM/F12 (3:1) medium containing B-27 (Gibco-BRL), supplemented with basic fibroblast growth factor (bFGF; 40

ng/ml, R&D Systems Inc., Minneapolis, MN, USA), the recombinant human insulin growth factor (EGF; 20 ng/ml, R&D Systems Inc.). Cells were prepared for FACS sorting to purify EGFP cells and then directly processed for Ca imaging or subjected to immunohistochemistry, *in situ* hybridization, or chemotaxis. In some experiments, FACS-sorted CXCR4-EGFP cells were incubated in fresh medium as above for 7–15 days to examine for self-renewal using sphere formation and serial passaging/subcloning. To induce differentiation, the cultures were switched to medium supplemented with bFGF (2.5 ng/ml), Endothelin-3 (100 μ M) and dibutyl adenosine cAMP (0.5 mM, Sigma), or supplemented with hydrocortisone (1 μ M) and dexametazone (1 μ M) to favor keratinocytes as described in Hirobe (1992), Hirobe and Abe, (2006) and Tamura et al. (1987). After 7–15 d, cultures were again processed for Ca imaging or were subjected to immunohistochemistry.

2.3. Generation of skin explants cultures

About 1 mm³ pieces of back skin were dissected free of other tissues and incubated as described in Jordan and Jackson (2000b).

2.4. Ca imaging

The intracellular free calcium concentration, $(Ca^{2+})_i$ was measured using digital video microfluorimetry as described previously by (Belmadani et al., 2005). Briefly, FACS—sorted cells or cell-derived skin-sphere were plated on PDL-coated glass coverslips for 2 h, rinsed briefly with HEPES buffer (containing the following (in mM): 120 NaCl, 5.4 KCl, 1.6 MgCl₂, 1.8 CaCl₂, 11 glucose, and 25 HEPES, pH 7.4 at 37 °C), and loaded with 2 M fura-2 AM (Molecular Probes, Eugene, OR, USA) in HEPES buffer for 30 min at room temperature. Cultures were then rinsed and kept in the dark in HEPES at room temperature for an additional 30 min to allow for complete dye deesterification. Glass coverslips were then mounted on the stage of a Nikon (Tokyo, Japan) Diaphot inverted epifluorescence microscope equipped for digital fluorescence microscopy. Fluorescence was digitally monitored at 520 nm after excitation at 340 nm (bound Ca^{2+}) and 380 nm (free Ca^{2+}) (20 $\times$ water immersion lens). Ratios of F_{340}/F_{380} were collected before and during treatment with chemokines (e.g., SDF-1, 10–20 nM) and other agents (endothelin-3 (5–10 μ M), stem cell factor (SCF) (100–500 ng/ml), ATP 5–10 μ M) using MetaFluor software from Universal Imaging Corporation (West Chester, PA, USA).

2.5. In situ hybridization

Cultures or 7 μ m skin section were fixed in 4% paraformaldehyde for 1 h and washed in PBS. Probes for SOX10 was a kind gift from Drs. M.E. De Bellard and M. Bronner-Fraser (California Institute of Technology, Pasadena, CA) (De Bellard et al., 2002; Kuhlbrodt et al., 1998). *In situ* hybridization was conducted for 20 h at 70 °C using digoxigenin (DIG)-labeled riboprobes complementary to the coding region of SOX10 (50% formamide, 5 $\times$ SSC, 0.1% Tween 20, 500 g/ml tRNA, 200 g/ml acetylated bovine serum albumin (BSA), and 50 g/ml heparin). Cells were washed four times with 50% formamide, 2 $\times$ SSC, and 0.1% Tween at 70 °C for 20 min and then washed three times using Tris-buffered saline and Tween 20 (TBST; 25 mM Tris-HCl, pH 7.5, 136 mM NaCl, 2.68 mM KCl, and 1% Tween 20). Finally, cells were incubated with 10% lamb serum in TBST buffer for 1 h and then

treated with anti-DIG antibody followed by antibody detection according to the manufacturer's protocol (Roche Products, Welwyn Garden City, UK).

2.6. Fluorescent in situ hybridization (FISH)

This method is similar to the *in situ* hybridization protocol described above with the exception that the probes used were not DIG labeled. After hybridization the slides were washed twice with preheated Solution T (50% formamide, $2 \times$ SSC, 0.1% Tween 20) and then washed with TBST and blocked with 1% H₂O₂ in PBS for 30 min. The slides were then washed 2 times with PBS and then blocked with blocking solution (100 mM Tris-HCl, 150 mM NaCl, 4% goat serum, 0.1% Triton $\times$ 100) for 1 h at room temperature. Horseradish peroxidase (POD)-conjugated sheep anti-digoxigenin (DIG) antibody (1:1000 dilution) in blocking solution was then added and incubated for 1 h. Slides were washed 3 times with TBST and fluorescence was developed using a tyramide signal amplification (TSA) fluorescence procedure (Perkin Elmer-NEN, Boston, MA USA). Sections were incubated in a 1:100 dilution of Cy5 conjugated Tyramide (Perkin Elmer-NEN) for 20 min at room temperature. After FISH, slides were incubated with blocking solution for 1 h and then primary antibody (1:1000 dilution) was added (anti-GFP) and incubated at 4 °C overnight. The slides were then washed 3 times with TBST and incubated with the secondary antibody (rabbit-conjugated AlexaFluor 488, 1:500 dilution) for 1 h. Slides were then washed 3 times with TBST and mounted with Vectashield antifade solution. Slides were analyzed by confocal microscopy (Olympus IX70). As control for the specificity of *in situ* hybridization and TSA amplification, additional sections were subjected to hybridization using sense probes for each receptor.

2.7. Immunohistochemistry

Skin samples from CXCR4, SDF-1, or Nestin transgenic mice of the same age, as well as knockout and control littermates were collected, fixed with 4% paraformaldehyde overnight at 4 °C, then embedded in OCT and frozen on an isopropanol-dry ice slurry. Cryostat sections of 7–10 μ m thickness were obtained and frozen sections were fixed in 4% paraformaldehyde and subjected to immunofluorescence. For immunohistochemistry, antigens were retrieved (steamed) in 10 mM Na citrate (pH 6) for 15 min and then incubated for 45 min in blocking solution (3% BSA, 4% donkey or goat serum, 0.1% Triton) at room temperature. Blocking solution was replaced by the primary antibodies prepared in diluent (3% BSA, 2% donkey or goat serum, 0.1% Triton) at the following concentrations: C11 (rabbit, 1:100, PRB-165P, Covance, San Diego, CA, USA), Cytokeratin 14 (mouse, 1:100, clone LL002, BioTrend, Destin, FL, USA), Cytokeratin 15 (chicken, 1:100, Covance), Cytokeratin 17 (mouse, 1:50, Covance), goat anti-CXCR4 (1:300; Abcam, Cambridge, MA, USA), rabbit anti-GFP (1/300, Santa Cruz, CA, USA), mouse anti-nestin (1:300; BD Biosciences, San Jose, CA, USA), anti-LEX (1:50), goat anti-tyrosinase-related protein (TRP)1 or TRP2 (1:150, Santa Cruz), rabbit anti PCNA (1:1000, Oncogene, San Diego, CA, USA). When staining with mouse antibodies, we used the reagents from the MOM Basic Kit according to the manufacturer's protocol (Vector Labs, Burlingame, CA, USA) to prevent non-specific binding of mouse monoclonal antibodies. Relevant secondary donkey anti-goat or goat anti-rabbit or anti-mouse antibodies conjugated with FITC (Alexa Fluor 488; A11001; Molecular Probes, Carlsbad, CA, USA) or Alexa Fluor 633 (1:500) (T 6391;

Molecular Probes) were used for detection of primary antibodies. Finally, the sections were treated with mounting solution, Prolong Antifade kit (Molecular Probes), on a microscope slide and examined by fluorescence microscopy. For FACS-sorted EGFP cells, Fluorescence immunohistochemistry was performed as described by Belmadani et al. (2005, 2006) with the same antibodies as described above.

2.8. Dunn chamber chemotaxis assay

To assess the chemotaxis of SDF-1 and its inhibition by AMD3100, a CXCR4 antagonist (AIDS Reagent Program, National Institutes of Health, Bethesda, MD), we used a microchemotaxis assay with Dunn chambers (Hawksley Technology, West Sussex, UK). This assay allowed us to assess the migration of cells in response to a gradient of a chemoattractant (Belmadani et al., 2005; Zicha et al., 1997).

2.9. Statistical analysis

Results from at least 3 experiments for the chemotaxis effects of SDF-1 were analyzed by ANOVA with Bonferroni *post hoc* tests. *P* values <0.05 were considered significant.

3. Results

3.1. Expression of CXCR4 and its ligand SDF-1 during embryonic hair follicle development

To determine the expression patterns of CXCR4 and its ligand, the chemokine SDF-1/CXCL12 during HF development in embryonic mouse skin we made use of bacterial artificial chromosome (BAC) transgenic mice expressing EGFP under the control of the CXCR4 or SDF-1 promoters. The generation of these transgenic mice as well as the extent of transgene expression in neural tissues, a major site of SDF-1 and CXCR4 expression (McGrath et al., 1999), are described at the GENSAT project website (<http://www.gensat.org/index.html>) and also in the literature (Bhattacharyya et al., 2008; Tran et al., 2007). Both transgenic mice robustly express EGFP in regions where SDF-1/CXCR4 signaling has been previously demonstrated (Zou et al., 1998; Bagri et al., 2002; Lu et al., 2002; Stumm et al., 2003; Belmadani et al., 2005; Tran et al., 2004, 2007; Tran and Miller, 2005; Chalasani et al., 2003; Pujol et al., 2005; Nagasawa et al., 1996; Tachibana et al., 1998; Ma et al., 1998; Kawabata et al., 1999). For example, EGFP in both mice was found in appropriate structures of the central (CNS), and the peripheral nervous systems (PNS), as well as in the bone marrow and blood vessel endothelium (Bhattacharyya et al., 2008). During mouse embryogenesis, from at least E18, EGFP in SDF-1-EGFP embryos was widely expressed in the dermal mesenchyme directly surrounding developing hair follicles (HFs) and was also localized to the hair follicle dermal papilla (DP), but was excluded from the epidermis (Fig. 1a). EGFP in CXCR4-EGFP embryos was specifically expressed in the hair follicle DP. CXCR4 also appeared to be localized to a few epidermal cells at the mouth of some developing hair follicles (Fig. 1b), and along an epithelial layer of the hair follicle at E19–20 (Fig. 1c). The close juxtaposition and complementary nature of these CXCR4 and SDF-1 expression patterns suggested that CXCR4/SDF-1 signaling might play a role in HF development.

It has been previously shown that SDF-1/CXCR4 signaling plays an important role in the development of neural crest cells and their derivatives in the dorsal root ganglia (Belmadani et al., 2005; Zicha et al., 1997). Given our observations on the patterns of expression of SDF-1 and CXCR4 in the developing HF, we tested whether inactivation of this signaling would result in alterations in the development of skin melanoblasts in embryos lacking functional CXCR4 receptors. Animals in which CXCR4 receptors have been inactivated die in late embryogenesis, just prior to birth (Nagasawa et al., 1996; Zou et al., 1998). However, as HF melanoblast development begins prior to this time, we were able to examine the consequences of CXCR4 deletion on initial melanoblast development. We examined the localization of melanoblasts in wild type and CXCR4 KO animals using the melanoblast-specific marker tyrosinase-related protein-2 (TRP2). In wild-type animals at E20 TRP2+melanoblasts were normally and singly distributed throughout the developing HF in the dermis (Fig. 2b,c). In the developing skin of CXCR4 KO animals, this pattern was substantially altered. In this case, TRP2+melanoblasts were generally positioned in the epidermis in irregular clusters and did not enter the HF (Fig. 2e,f), indicating that SDF-1/CXCR4 signaling may be required for the normal migration and the positioning of melanoblasts within the HF.

To determine the role of SDF-1/CXCR4 signaling in melanoblast development in more detail, we examined the behavior of CXCR4–EGFP-expressing cells *in vitro*. We isolated cells from the skin of CXCR4–EGFP transgenic mice at E18–20 and used FACS to sort the cells based on EGFP fluorescence (Fig. 3a). We immunostained FACS-sorted cells with a CXCR4 antibody and showed that EGFP-positive cells all stained for CXCR4 protein ($98 \pm 2\%$) (Fig. 3b,c), however, EGFP-negative cells did not stain at all (Fig. 3b,d). We then examined whether CXCR4–EGFP-expressing cells expressed functional CXCR4 receptors. We carried out calcium (Ca^{2+})*i* imaging studies on FACS-sorted cells from E18–20 CXCR4–EGFP embryos and observed that EGFP-positive cells responded robustly to the addition of SDF-1 (50 nM) (100%). Cells also responded to ATP (100%) an agonist for purinergic receptors, which are expressed by almost all cells and so was used as a positive control at the end of each experiment (Fig. 3e). Importantly, EGFP-negative cells did not respond to SDF-1 (< 1%) but still respond to ATP (100%) (Fig. 3f), confirming the results obtained with the CXCR4 antibody. These data indicate that EGFP expression patterns are a reliable marker for the distribution of functional CXCR4 receptors. Furthermore, immunostaining of FACS-sorted cells for the melanoblast-specific marker tyrosinase-related protein-2 (TRP2) showed that EGFP-positive cells all stained for TRP2 (Fig. 4a). However, EGFP-negative cells did not stain for TRP2 (as in Fig. 12, see below). Interestingly, CXCR4–EGFP-expressing cells also appeared to express several other receptors that have been shown to be important in different aspects of melanoblast development including the tyrosine kinase receptor c-kit and endothelin B receptors. We conducted (Ca^{2+})*i* imaging experiments using stem cell factor (SCF) and endothelin-3 (ET-3). SCF and ET-3, ligands for c-kit and the endothelin B receptors, respectively, are both essential for normal melanocyte development during embryogenesis, and synergistically support survival and growth of melanocytes (Imokawa et al., 2000; Hirobe et al., 2003; Cook et al., 2003). As can be observed in Fig. 4b many cells responded to SCF (84/95:88%) in addition to endothelin-3 (ET-3) (95/95:100%), SDF-1 (100%) and ATP (100%), further indicating that these cells are

melanoblasts. EGFP-negative cells did not respond to SCF or to SDF-1, but still responded to ET-3 (57%) and ATP (100%) (Fig. 4c).

We then addressed the possibility that SDF-1 might act as a chemoattractant migratory cue for melanoblasts as defined by CXCR4 and TRP2 expression among other markers using a microchemotaxis assay (Belmadani et al., 2005; Zicha et al., 1997). We found that melanoblasts migrated towards a source of SDF-1. The effects of SDF-1 were inhibited by the selective CXCR4 antagonist AMD3100, indicating that they were mediated by CXCR4 receptors (Fig. 4d). Overall, these data indicate that CXCR4–EGFP-expressing cells harvested from embryonic skin express functional CXCR4 receptors and that SDF-1 acts as a chemoattractant for these cells.

3.2. Expression of CXCR4 receptors during postnatal hair follicle cycling

To further investigate the localization of CXCR4–EGFP-expressing cells at different stages of the postnatal HF cycle we examined the expression pattern of CXCR4–EGFP during the two first postnatal HF cycles (anagen, catagen, telogen), exploiting the fact that these initial anagen phases are synchronized during the first two postnatal HF cycles, which cover a period of approximately 8 weeks (Wilson et al., 1994). We made observations on skin sections between postnatal days 1 and 45, to allow analysis of both the first and the second postnatal HF cycles. We choose the following ages: P1, P7, P9, P12, P15, and P24, P27, P32 to cover the first and the second anagen, P19, and P38 to cover the first and the second catagen, and P21 and P45 for the first and the second telogen. As shown in Fig. 5, we observed that during the two first anagen phases, EGFP appeared to be expressed in the DP and along an epithelial layer of the HF, presumably representing the outer root sheath (ORS) (see below). Strong expression was also located at the base of the follicle epithelium, presumably representing bulge cells (see below). During catagen, when hair bulb matrix cells undergo regression and degeneration, the number of EGFP-expressing cells decreased, correlating with the reduced size of the HF. During telogen, only a small number of EGFP-positive cells appeared to be located at the base of the follicle epithelium, presumably representing bulge cells or the DP (arrows in f, g, j, k, l). Thus, the degree of CXCR4–EGFP expression appeared to be strongly correlated with the first and the second anagen phases of the HF cycle, a period of intense progenitor cell activity. In the HF, stem/progenitor cells of many types including melanocyte stem cells, are located in the bulge area (Cotsarelis et al., 1990; Morris and Potten, 1999). These cells are induced to proliferate during HF anagen and give rise to proliferating melanoblasts that migrate within the ORS towards the hair matrix (Nishimura et al., 2002).

Immunofluorescence of anagen skin sections for EGFP co-stained with known markers of the bulge area (K15), the ORS (K17) and a nuclear counterstain (DAPI) revealed that CXCR4 is expressed in the ORS (Fig. 6a–f) and the bulge (Fig. 6d–f), areas known to be rich in HF stem/progenitor cells (Cotsarelis et al., 1990; Taylor et al., 2000; Nishimura et al., 2002). These observations suggested that CXCR4 may be expressed by progenitor cells in the HF.

In order to verify that EGFP expression in postnatal HFs really reflects CXCR4 receptor protein expression, we isolated cells from the anagen skin of CXCR4–EGFP transgenic mice

at P7–9 and used FACS to sort the cells based on EGFP fluorescence (Fig. 1a–d, Supplementary data). We immunostained FACS-sorted cells with a CXCR4 antibody and showed that, as with embryonic cells, EGFP-positive cells all stained for CXCR4 protein ($98 \pm 2\%$) (Fig. 1e, f, Supplementary data) and that EGFP-negative cells did not stain at all (Fig. 1g, Supplementary data). We then carried out calcium (Ca^{2+})i imaging studies on FACS-sorted cells from P7–9 CXCR4–EGFP mice and observed that EGFP-positive cells responded robustly to the addition of SDF-1 (50 nM) (100%) and also to ATP (100%) (Fig. 1h, Supplementary data). Importantly, however, EGFP-negative cells did not respond to SDF-1 ($< 1\%$), but still responded to ATP (100%) (Fig. 1i, Supplementary data), confirming the data with CXCR4 antibody staining. Furthermore, immunostaining for CXCR4 on skin sections obtained from P7–9 CXCR4–EGFP transgenic mice showed a strikingly similar staining pattern in the HF to that of the CXCR4–EGFP expression (Figs. 6g,h). It should be noted, however, that as EGFP expressed by the reporter mouse is free to fill the entire cell, whereas the CXCR4 antibody actually detects CXCR4 protein, which is predominantly at the cell surface, an exact correspondence between the two staining patterns would not be expected. Again, as with embryonic skin, these data on postnatal skin confirm that the EGFP expression pattern is a reliable marker for the cellular distribution of functional CXCR4 receptors.

3.3. Expression of *cxcr4* by progenitor cells in the hair follicle

In order to test the hypothesis that CXCR4–EGFP-expressing cells were progenitor cells we analyzed the expression of CXCR4–EGFP together with markers of stem/progenitor cells and the ability of these cells to proliferate and self-renew. We stained CXCR4–EGFP-expressing cells for nestin, an intermediate filament protein, which is highly expressed by neural progenitor cells and some types of neural crest cells (Belmadani et al., 2005) and for Lex (stage-specific embryonic antigen ssea-1), an extracellular matrix-associated carbohydrate frequently used as a stem cell marker (Capela and Temple, 2002). We also stained cells for proliferating cell nuclear antigen (PCNA), which is expressed in the nucleus of proliferating cells. We isolated cells during anagen from the skin of CXCR4–EGFP transgenic mice at P4–7 and sorted them using FACS. As can be seen in Fig. 7, immunostaining for nestin (Fig. 7a) and Lex (Fig. 7b) on FACS-sorted CXCR4–EGFP cells demonstrated that $85 \pm 5\%$ of the cells stained for nestin and 20% stained for Lex. In addition, $63 \pm 3\%$ of the FACS-sorted cells stained for PCNA (Fig. 7c). We examined the self-renewing ability of the FACS-sorted cells using a sphere-forming assay and serial passaging and subcloning. Sorted cells (2×10^3 cells per well on a 24-well culture plate) were cultured in sphere culture medium containing bFGF (40 ng/ml) and EGF (20 ng/ml). After 7 days in culture, cells began to form spheres. By 15 days some spheres still contained many EGFP-positive cells, whereas others contained only a few positive cells. Following dissociation, EGFP cells co-stained for nestin and Lex with similar percentages to those observed for freshly FACS-sorted cells (Fig. 7a,b). Importantly, when cultured at low densities under the same conditions, dissociated EGFP-positive cells formed small spheres indicating the ability of these cells to self-renew (data not shown). Together, these results suggest that some of CXCR4–EGFP-expressing cells located in the postnatal HF possess characteristics of stem/progenitor cells.

Because most of the CXCR4–EGFP-expressing cells of the HF stained for the stem/progenitor marker nestin, we used nestin–EGFP transgenic mice (Tran et al., 2007) to further examine the expression patterns of nestin during the postnatal HF cycle. As can be seen in Fig. 8, EGFP expression in these mice was also localized to areas of the bulge and the ORS during the entire anagen phase (P1, P2, P4, P7, and P26, P30, P33). However, in contrast to CXCR4–EGFP expression, nestin–EGFP expression was not found in the DP. During catagen, the number of ORS nestin–EGFP-expressing cells decreased together with the concomitant reduction in size of the HF. In telogen some nestin–EGFP cells were localized to the bulge area (Insert in e). As with CXCR4–EGFP, the pattern of nestin–EGFP expression correlated strongly with the first and the second anagen phases of the HF cycle. Furthermore, staining for Lex or PCNA on FACS-sorted cells isolated from the anagen skin of P4 nestin–EGFP transgenic mice showed that $30 \pm 5\%$ of the cells stained for Lex and $79 \pm 3\%$ stained for PCNA (data not shown). Li et al. (2003) also observed nestin–EGFP-expressing cells in the HF bulge and suggested that they were progenitor cells. These findings are consistent with our observations on CXCR4–EGFP-expressing cells in the bulge and the ORS, most of which stained for nestin, suggesting that CXCR4–EGFP-expressing cells may serve as stem cells/progenitors in the HF.

3.4. Analysis of sdf-1 expression during postnatal hair cycling

If CXCR4-expressing cells in the HF have the characteristics of progenitor cells, we hypothesized that, as in other tissues, they would migrate towards an endogenous source of SDF-1. The question therefore arises as to the localization of SDF-1 during postnatal HF cycling? We therefore examined the distribution of SDF-1 expression using SDF-1–EGFP BAC transgenic mice from P1 to 54 to allow analysis of both the first and the second postnatal hair cycles. As shown in Fig. 9, EGFP expression was detected throughout the HF cycle in the mesenchyme underlying the interfollicular skin and along the potential migration pathway of CXCR4–EGFP cells within the ORS. The expression pattern of SDF-1 seemed to follow a gradient. For example, we observed strong expression along the basal layer of the epidermis, which became less pronounced along the ORS, but was very concentrated at the DP of the HF, where CXCR4–EGFP-expressing cells were also found (Fig. 9). SDF-1 expression was not observed in the epidermis. Moreover, *in situ* hybridization of mouse back skin harvested from wild-type mice during anagen phase at P14 using a probe for the SDF-1 coding region showed that mRNA for SDF-1 was expressed along the HFs. We also performed fluorescence *in situ* hybridization (FISH) together with immunostaining with an EGFP antibody on skin sections harvested from SDF-1–EGFP mice during the anagen phase at P14, and observed colocalization of SDF-1 mRNA expression and SDF-1–EGFP along the HFs (Fig. 2, Supplementary data). In order to precisely identify the sites of SDF-1 protein storage and expression we constructed BAC reporter mice expressing an SDF-1–mRFP fusion protein (see Section 2 and Bhattacharyya et al., 2008). These mice were crossed with CXCR4–EGFP mice to generate a new line of mice expressing both CXCR4–EGFP and SDF-1–mRFP. We observed that SDF-1–mRFP was expressed along the potential migratory path of CXCR4–EGFP-expressing cells in the ORS of the HF (Fig. 9q–s). Furthermore, immunostaining for SDF-1 and CXCR4 proteins using CXCR4–EGFP and SDF-1–EGFP mice showed similar patterns of expression of CXCR4 and SDF-1 (Fig. 3, Supplementary data). All of these data are consistent with the possibility

that SDF-1 acts as a guidance cue for directing the migration of CXCR4-expressing progenitors within the ORS towards the DP. Indeed, this arrangement of sites of SDF-1 expression relative to migrating CXCR4-expressing progenitor cells is similar to that found in other circumstances in which SDF-1 guides the migration of developing stem/progenitor cells (e.g. Belmadani et al., 2005; Lu et al., 2002).

3.5. CXCR4–EGFP-expressing cells exhibit migratory response towards SDF-1

Because we showed that CXCR4–EGFP-expressing cells express functional CXCR4 receptors (Fig. 1, Supplementary data) and that SDF-1 was expressed along the path of their potential migration within the ORS (Fig. 9), we therefore addressed the possibility that SDF-1 might act as a chemoattractant migratory cue for these CXCR4-expressing cells using a microchemotaxis assay. We found that FACS-sorted CXCR4–EGFP-expressing cells migrated towards a source of SDF-1. The effects of SDF-1 were inhibited by the selective CXCR4 antagonist AMD3100, indicating that they were mediated by the CXCR4 receptor (Fig. 10a). We also found that FACS-sorted cells from P4 nestin–EGFP mice produced (Ca^{2+})_i responses to SDF-1 and ATP (Fig. 10b), in a similar fashion to CXCR4-expressing cells. Furthermore, nestin–EGFP cells also migrated towards a source of SDF-1 and this effect was blocked by AMD3100 (Fig. 10c). Thus, nestin-expressing cells also expressed functional CXCR4 receptors, thereby confirming the observed strong correlation between CXCR4 and nestin–EGFP expression during the anagen phase of the HF cycle. Importantly, EGFP-negative cells derived from nestin–EGFP did not respond to SDF-1 in (Ca^{2+})_i imaging assay (data not shown).

3.6. CXCR4-expressing cells of the hair follicles express neural crest markers

We next addressed the question as to the identity and origin of the CXCR4–EGFP progenitor cells in the HF. Because CXCR4 signaling regulates the development of nestin-expressing neural crest (NC)-derived sensory neurons (Belmadani et al., 2005), we hypothesized that nestin and CXCR4-expressing cells in the HF were also derived from the NC. We analyzed the expression of SOX10, which is expressed by NC cells among other cell types (Potter et al., 2001; Hakami et al., 2006) by performing *in situ* hybridization on skin sections from P7 CXCR4–EGFP transgenic mice. We observed expression of SOX10 mRNA within the epithelial cells of the basal layer of the epidermis and the ORS (Fig. 11a), generally coinciding with the pattern of CXCR4-expressing cells. Using FISH with sections from P7 CXCR4–EGFP transgenic mice, we confirmed that SOX10 staining colocalized with CXCR4-expressing cells in the ORS, indicating that CXCR4-expressing cells may be derived from the NC (Fig. 11c,d). We analyzed the expression of SOX10 by FACS-sorted CXCR4–EGFP-expressing cells from P3-4 CXCR4–EGFP transgenic mice using FISH. As can be seen in Fig. 11e,f, we observed that most ($94 \pm 4\%$) of the FACS-sorted CXCR4–EGFP-expressing cells also expressed SOX10, confirming the observations using skin sections (Fig. 11a,c,d), and indicating that CXCR4-expressing cells in the ORS may be derived from the NC.

The NC gives rise to melanocytes in the skin and HF. It has been shown that SOX10 plays a crucial role in the development of NC cells, including NC of the melanocyte lineage (Mollaaghababa and Pavan, 2003; Hakami et al., 2006). SOX10 activates the transcription

of MITF, which in turn acts as a master regulator gene controlling the expression of various melanogenic genes in melanoblasts, including the tyrosinase-related protein-2 (TRP2) (Jiao et al., 2004; Sieber-Blum et al., 2004; Mollaaghababa and Pavan, 2003). We therefore examined the hypothesis that CXCR4–EGFP-expressing cells were melanoblasts. We stained sections from P7 CXCR4–EGFP transgenic mice with either the melanoblast-specific marker tyrosinase-related protein-2 (TRP2), or for ORS-keratin K14, a marker for HF keratinocyte basal cells (Fuchs, 1993; Nelson and Sun, 1983). As shown in Fig. 12a,b most of the EGFP cells of the ORS colocalized with the melanoblast marker TRP2, whereas only a few appeared to stain for the ORS basal keratinocyte marker K14 (Fig. 12c), indicating that most of the EGFP-expressing cells in the ORS are of melanocyte lineage. Next, we stained FACS-sorted CXCR4–EGFP-expressing cells for TRP2 and a pan-keratin antibody (C11) and observed that most ($80 \pm 5\%$) FACS-sorted CXCR4-expressing cells stained for the melanoblast-specific marker TRP2 (Fig. 12e), whereas few ($<5\%$) stained for the keratinocyte marker C11 (data not shown), confirming the findings from skin sections in Fig. 12a,b. In contrast, EGFP-negative cells did not stain for the melanoblast marker TRP2 (Fig. 12d,f). We then conducted $(\text{Ca}^{2+})_i$ imaging experiments in response to endothelin-3 (ET-3) and stem cell factor (SCF). We observed that EGFP+ cells all respond to ET-3 (100%) and to SCF (66%). In contrast, while some EGFP-negative cells also responded to ET-3 (57%), no cells responded to SCF (data not shown). In summary, these results demonstrate that CXCR4-expressing cells in the HF bulge and ORS are probably NC-derived melanoblasts. These cells express functional CXCR4 receptors and SDF-1 acts as chemoattractant for them, thereby suggesting an role for CXCR4/SDF-1 signaling in the migration of melanoblasts from the epidermis towards the bulb of the HF.

It is also interesting to note, that when we FACS-sorted cells from P7 SDF1–EGFP mouse skin some of them also responded to the addition of SDF-1 ($15 \pm 4\%$) and ATP in the $(\text{Ca}^{2+})_i$ imaging assay. However, in contrast to the FACS-sorted CXCR4–EGFP cells they did not stain for nestin (Fig. 4, Supplementary data). These cells probably represent differentiated cells of the DP where CXCR4–EGFP is also expressed (Figs. 1, 5 and 14d). These observations are also consistent with the fact that nestin is not expressed in the lower third of the HF that contains the DP (Fig. 8c,d). Together with the absence of SOX10 staining in the DP (Fig. 11c,d), this data further indicates that CXCR4–EGFP-expressing cells of the DP are not of NC origin.

3.7. CXCR4 expression is markedly reduced in differentiating melanoblasts

To confirm that CXCR4–EGFP-expressing cells in the HF bulge and ORS are NC-derived melanoblasts that can differentiate into melanocytes, and to examine the temporal expression of CXCR4 during melanoblast differentiation, we sought to differentiate these cells into melanocytes *in vitro*. In order to induce these cells to differentiate along the melanocyte lineage we cultured FACS-sorted CXCR4–EGFP-expressing cells in fresh medium to promote their proliferation for 7 days, and then switched to medium supplemented with bFGF (2.5 ng/ml), ET-3 (100 μM) and dibutyryl adenosine cAMP (0.5 mM) or SCF (2 ng/ml) to favor melanocyte differentiation (Hirobe, 1992). In proliferative medium, melanoblasts continued to express CXCR4–EGFP and stained for TRP2 as shown in Fig. 1, (Supplementary data) and Fig. 12e,f, respectively. However, when switched to

medium that promoted the differentiation of melanoblasts we found that CXCR4–EGFP expression and staining with CXCR4 antibody were both markedly reduced after 5 days, and were undetectable after 15 days in culture (Fig. 13a). Under these culture conditions, most of the cells exhibited melanocyte morphology and stained for the mature melanocyte-specific marker TRP1 (Fig. 13b). The cells did not respond to SDF-1 but still responded to SCF (data not shown), and to ET-3 and ATP in the $(Ca^{2+})_i$ imaging assay (Fig. 13c), indicating that CXCR4-expressing cells differentiated into melanocytes that down regulated the CXCR4 receptors. FACS-sorted nestin–EGFP-expressing cells were also cultured under the same conditions as described above for CXCR4–EGFP-expressing cells to induce their differentiation into melanocytes. Under these culture conditions, the cells did not exhibit nestin–EGFP fluorescence or staining for CXCR4 antibody. Cells became positive for the melanocyte marker TRP1 and responded to SCF (data not shown), and to ET-3 and ATP, but not to SDF-1 in the $(Ca^{2+})_i$ imaging assay, (Fig. 5, Supplementary data).

To examine the relevance of these observations *in vivo*, we first immunostained sections of P7–9 CXCR4–EGFP transgenic mice for TRP1 and observed that TRP1 was expressed by two populations of cells in the bulb region of anagen follicles. One population of cells (P1) was observed to be in direct contact with the DP and expressed both CXCR4 and TRP1 (Fig. 13, arrows in d). The second population (P2), was located distally from the DP in the follicle matrix where mature melanocytes are normally found. This population of cells did not appear to express CXCR4 receptors, but still showed strong staining for TRP1 (arrowheads in Fig. 13d), resembling differentiated melanocytes that we obtained in culture (Fig. 13b). We next immunostained P30 nestin–EGFP sections for TRP1 and observed that nestin–EGFP expression did not colocalise with TRP1-positive cells, suggesting that TRP1-positive cells are differentiating or differentiated melanocytes (arrowheads in Fig. 13e). Using P7 SDF–EGFP skin sections immunostained with TRP1 we confirmed the presence of the two population of cells as shown in Fig. 13d (arrows and arrowheads in f, respectively). Of note, DP cells expressed CXCR4 and its ligand SDF-1 (Figs. 1, 5, 9). These results demonstrate that in the bulb melanocytes in direct contact with the DP may undergo proliferation while still expressing CXCR4 receptors. As they move upward away from the DP, they downregulate CXCR4 expression while differentiating into mature melanocytes. Overall, our data indicate that CXCR4–EGFP-expressing cells in postnatal anagen skin, as with embryonic skin, express functional CXCR4 receptors and exhibit melanoblast characteristics, and that SDF-1 acts as a chemoattractant for these cells.

3.8. Normal positioning of melanoblasts in skin explant preparations is disrupted by the CXCR4 antagonist AMD3100

Our data (Fig. 2) showed that melanoblast distribution is altered in CXCR4 mutant embryos arguing for a role for SDF-1/CXCR4 signaling in their migration. To further confirm that SDF-1/CXCR4 signaling is directly involved in melanoblast migration and normal positioning, preparations of mouse skin explants harvested from E13.5 embryos were grown in the absence or presence of the selective CXCR4 antagonist AMD3100, and the distribution of TRP2+ melanoblasts was determined. During normal development, at E14.5, melanoblasts begin to invade the epidermis (Nishikawa et al., 1991; Kunisada et al., 1998; Jordan and Jackson, 2000b; Kunisada et al., 1996) and then migrate into the developing HFs

during the succeeding 3–4 days. Consistent with this distribution, EGFP–CXCR4-expressing cells were exclusively distributed in the dermal HFs at E18 in CXCR4–EGFP mouse skin sections (Fig. 1). In control skin explant preparations grown for 12 days (corresponding to P5 postnatal mice), most of the TRP2+ cells were distributed in the dermis (Fig. 14a). In contrast, after explants were grown with AMD3100, all TRP2+ cells were clustered in the epidermis (Fig. 14b), further indicating that the migration of TRP2+ cells into the dermis depends on CXCR4 activation. Overall, these data indicate that inactivation of CXCR4 signaling during embryonic development results in the accumulation of MPs in the epidermis.

4. Discussion

Melanoblasts (MPs), a subpopulation of cells derived from the NC, emerge from the dorsal neural tube around embryonic day 8.5 (E8.5) in mice and migrate along a dorsolateral pathway between the dermatome and the overlying ectoderm. From E10.5 MPs migrate ventrally through the developing dermis in response to the effects of endothelin, c-kit and GDNF receptor stimulation (Kunisada et al., 1998; Yoshida et al., 1996; Nishimura et al., 2002). At E14.5, they begin to invade the overlying epidermis partly as a result of local c-kit signaling (Nishikawa et al., 1991; Kunisada et al., 1998; Jordan and Jackson, 2000b; Kunisada et al., 1996) and then migrate into the developing HFs in hairy skin. In regions of non-hairy skin, melanocytes remain in the epidermis. Thus, it appears that the presence of HFs may trigger specific events leading to melanocyte localization within the HFs. We have now demonstrated that MPs express functional CXCR4 chemokine receptors and that the CXCR4 ligand, the chemokine SDF-1 (also known as CXCL12), is abundantly expressed in the skin along the path taken by migrating CXCR4-expressing cells in the ORS. SDF-1 acted as a chemoattractant for these cells, suggesting that it normally guides their migration into the HF, a possibility that is supported by the phenotypes of CXCR4 KO mice and skin explant preparations treated with the selective CXCR4 antagonist AMD3100. It had previously been demonstrated that the tyrosine kinase receptor c-kit and its ligand SCF as well as GDNF and endothelin all played important roles in maintaining the proliferation of MPs and guiding their migration from the NC to the developing skin and HF (Nishimura et al., 2002, 1999; Jordan and Jackson, 2000b; Kunisada et al., 1996, 1998; Nishikawa et al., 1991; Peters et al., 2002; Yoshida et al., 1996; Zou et al., 1998; Tachibana et al., 1998; Ma et al., 1998). Furthermore, c-kit signaling has been shown to produce a generalized non-directed “motogenic” effect on melanoblasts (Hirobe et al., 2003; Peters et al., 2002; Kunisada et al., 1998; Ito et al., 1999). Nevertheless, it has also been clear that the ultimate positioning of MPs within the HF required an unidentified chemotactic factor (Jordan and Jackson, 2000b). Our data suggests that SDF-1 plays this particular role. These results should be considered in the context of several recent reports demonstrating the role of CXCR4 signaling in regulating the migration of stem/progenitor cells in many different tissues (Zou et al., 1998; Bagri et al., 2002; Lu et al., 2002; Stumm et al., 2003; Belmadani et al., 2005; Tran et al., 2004, 2007; Tran and Miller, 2005; Chalasani et al., 2003; Pujol et al., 2005; Nagasawa et al., 1996; Tachibana et al., 1998; Ma et al., 1998; Kawabata et al., 1999). Indeed, analysis of the phenotype of CXCR4 KO mice has revealed deficiencies in the formation of numerous tissues that can be explained by this mechanism. In the case of

NC derivatives, for example, we have previously demonstrated that CXCR4 signaling is important for guiding the migration of neural progenitors for DRG neurons from the neural tube to the nascent DRG (Belmadani et al., 2005).

The cycling of anagen and catagen phases in HF development requires continuous and extensive HF remodeling. To achieve this end, the HF seems to contain several different types of stem cells including epithelial, melanocyte, nestin expressing and other types of stem cells that are important for this process (Oshima et al., 2001; Blanpain et al., 2004; Claudinot et al., 2005; Nishimura et al., 2002; Cotsarelis et al., 1990; Taylor et al., 2000). For example, cells expressing EGFP under the control of the nestin promoter have previously been isolated from the HF and shown to be mainly located in the bulge area during early anagen (Li et al., 2003). At later times these cells collectively migrate within the ORS towards the base of the HF, where they participate in the formation of the new hair (Li et al., 2003), as demonstrated in transplantation experiments (Oshima et al., 2001; Taylor et al., 2000). In our studies, however, nestin-EGFP-expressing cells were localized to both the inner and the outer root sheaths (Figs. 8 and 13e). Moreover, it has been demonstrated that HFs also contain a pool of NC-derived progenitors, which can produce MPs and can also give rise to Merkel cells (Sieber-Blum et al., 2004; Sieber-Blum and Grim, 2004; Wong et al., 2006). The relationship between this group of cells and those HF progenitors that express nestin has not been entirely clear. As we have now demonstrated, many CXCR4-expressing cells also express nestin. These two molecules appear to be colocalized in cells of the ORS but not in the DP, which expressed only CXCR4. It was also demonstrated that nestin-expressing progenitors in the HF could give rise to several other types of progeny including neurons, glia and smooth muscle cells (Li et al., 2003; Amoh et al., 2005b), as described in transplantation experiments (Sieber-Blum et al., 2006; Amoh et al., 2005a). However, their ability to form melanocytes has not been reported. As we have now demonstrated, nestin-expressing cells will also differentiate into melanocytes. Thus, it seems likely that the nestin-expressing progenitors in the HF can act as MPs and express CXCR4, which is involved in their migration within the HF. It will be of interest to see whether CXCR4-progenitors can give rise to other cell types *in vivo*.

Nishimura et al (2002) used transgenic mice carrying a lacZ reporter allele driven by the *Dct* promoter and identified the bulge of the HF as the niche for undifferentiated resting or slowly cycling melanoblasts. These cells can not only generate differentiated melanocytes but are also able to self-renew, as we have also demonstrated, and to populate vacant niches where they return to a quiescent state (Nishimura et al., 2002). This observation further supports the possibility that the HF contains molecules that encourage the directed migration of melanoblasts. Moreover, *Dct-LacZ* positive cells also express SOX10 (Osawa et al., 2005), in agreement with our results (Fig. 11). Our results also show that MPs are at least bipotent, giving rise to cells consisting mostly of melanocytes (TRP1 positive) and a few cells staining with a pankeratin antibody, providing further support for the hypothesis that *Dct-LacZ* positive cells indeed represent multipotent stem cells. It will be of interest to determine whether *Dct-LacZ* positive cells express nestin and CXCR4.

After leaving the bulge region and losing contact with their niche, MPs migrating within the ORS seem to follow a spatial gradient of SDF-1 expression. Thus, we observed strong

expression of the chemokine along the basal layer of the epidermis, which became less intense along the ORS, and was very concentrated in the DP. Once the cells reach the region of the DP they appear to down regulate nestin expression and also to start down regulating CXCR4 receptors as they move upwards away from the DP prior to maturing into differentiated melanocytes. The source of SDF-1 that guides MP migration appears to be mesenchymal cells that line the route of MP migration as well as the DP. Thus, it is possible that in addition to acting as a migratory cue, the constitutive secretion of SDF-1 by the DP may also encourage down regulation of CXCR4 receptors by MPs as they mature into melanocytes and migrate away from the DP.

The proposed role of SDF-1 and CXCR4 expression in MP function exhibits similarities to the effects of SDF-1 in several other situations where it regulates stem cell migration. For example, in the migration of NC progenitors to the DRG, SDF-1 is similarly secreted by mesenchymal cells along the route of progenitor cell migration (Belmadani et al., 2005). In the developing cerebellum, SDF-1 maintains granule cell progenitors in their proliferative niche and their migration away is associated with the down-regulated expression of CXCR4 receptors by granule cell progenitors (Zou et al., 1998; Ma et al., 1998). Similar observations have been made concerning the effects of SDF-1 signaling in the hematopoietic system (Sawada et al., 1998).

As we have also demonstrated, in addition to secreting SDF-1, some of the cells of the DP also appear to express CXCR4. Thus, isolation of SDF-1-expressing cells by FACS included a population of cells that responded to exogenous SDF-1 but did not stain for nestin, consistent with our observations on skin sections from CXCR4–EGFP and nestin–EGFP mice. The expression of SDF-1 and CXCR4 receptors by DP cells also raises the possibility of autocrine responsiveness of these cells to SDF-1 although the precise function of such signaling is not clear from our studies.

In conclusion, we have demonstrated that signaling via the CXCR4 chemokine receptor plays a central role in the positioning of melanoblasts within the hair follicle. It is interesting to note that CXCR4 receptors are also highly expressed in melanoma cells (Muller et al., 2001), which are known to be highly aggressive in terms of their propensity to form metastatic tumors. Hence, it is possible that deregulated CXCR4 signaling is also responsible for this behavior.

Acknowledgements

We thank Dr. Daniel R. Littman (Skirball Institute of Molecular Medicine, New York University Medical Center) for CXCR4 KO mice. The authors thank Dr. R Lavker and Dr. M Sieber-Blum for their critical review of the paper. This work was supported by research grants from the NIH: 5 R01 DA013141-08, R R37 MH040165-23.

References

- Amoh Y, Li L, Campillo R, Kawahara K, Katsuoka K, Penman S, Hoffman RM. Implanted hair follicle stem cells form Schwann cells that support repair of severed peripheral nerves. *Proc. Natl. Acad. Sci. USA.* 2005a; 102:17734–17738. [PubMed: 16314569]
- Amoh Y, Li L, Katsuoka K, Penman S, Hoffman RM. Multipotent nestin-positive, keratin-negative hair-follicle bulge stem cells can form neurons. *Proc. Natl. Acad. Sci. USA.* 2005b; 102:5530–5534. [PubMed: 15802470]

- Bagri A, Gurney T, He X, Zou YR, Littman DR, Tessier-Lavigne M, Pleasure SJ. The chemokine SDF1 regulates migration of dentate granule cells. *Development*. 2002; 129:4249–4260. [PubMed: 12183377]
- Belmadani A, Tran PB, Ren D, Assimacopoulos S, Grove EA, Miller RJ. The chemokine stromal cell-derived factor-1 regulates the migration of sensory neuron progenitors. *J. Neurosci*. 2005; 25:3995–4003. [PubMed: 15843601]
- Belmadani A, Tran PB, Ren D, Miller RJ. Chemokines regulate the migration of neural progenitors to sites of neuroinflammation. *J. Neurosci*. 2006; 26:3182–3191. [PubMed: 16554469]
- Bhattacharyya BJ, Banisadr G, Jung H, Ren D, Cronshaw DG, Zou Y, Miller RJ. The chemokine stromal cell-derived factor-1 regulates GABAergic inputs to neural progenitors in the postnatal dentate gyrus. *J. Neurosci*. 2008; 28:6720–6730. [PubMed: 18579746]
- Blanpain C, Lowry WE, Geoghegan A, Polak L, Fuchs E. Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. *Cell*. 2004; 118:635–648. [PubMed: 15339667]
- Botchkareva NV, Khlgatian M, Longley BJ, Botchkarev VA, Gilchrist BA. SCF/c-kit signaling is required for cyclic regeneration of the hair pigmentation unit. *Faseb J*. 2001; 15:645–658. [PubMed: 11259383]
- Botchkareva NV, Botchkarev VA, Gilchrist BA. Fate of melanocytes during development of the hair follicle pigmentary unit. *J. Invest. Dermatol. Symp. Proc*. 2003; 8:76–79.
- Capela A, Temple S. LeX/ssea-1 is expressed by adult mouse CNS stem cells, identifying them as nonependymal. *Neuron*. 2002; 35:865–875. [PubMed: 12372282]
- Chalasani SH, Baribaud F, Coughlan CM, Sunshine MJ, Lee VM, Doms RW, Littman DR, Raper JA. The chemokine stromal cell-derived factor-1 promotes the survival of embryonic retinal ganglion cells. *J. Neurosci*. 2003; 23:4601–4612. [PubMed: 12805300]
- Claudinet S, Nicolas M, Oshima H, Rochat A, Barrandon Y. Long-term renewal of hair follicles from clonogenic multipotent stem cells. *Proc. Natl. Acad. Sci. USA*. 2005; 102:14677–14682. [PubMed: 16203973]
- Cook AL, Donatien PD, Smith AG, Murphy M, Jones MK, Herlyn M, Bennett DC, Leonard JH, Sturm RA. Human melanoblasts in culture: expression of BRN2 and synergistic regulation by fibroblast growth factor-2, stem cell factor, and endothelin-3. *J. Invest. Dermatol*. 2003; 121:1150–1159. [PubMed: 14708619]
- Cotsarelis G, Sun TT, Lavker RM. Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. *Cell*. 1990; 61:1329–1337. [PubMed: 2364430]
- De Bellard ME, Ching W, Gossler A, Bronner-Fraser M. Disruption of segmental neural crest migration and ephrin expression in delta-1 null mice. *Dev. Biol*. 2002; 249:121–130. [PubMed: 12217323]
- Fuchs E. Epidermal differentiation and keratin gene expression. *J. Cell Sci. Suppl*. 1993; 17:197–208. [PubMed: 7511614]
- Fuchs E, Merrill BJ, Jamora C, DasGupta R. At the roots of a never-ending cycle. *Dev. Cell*. 2001; 1:13–25. [PubMed: 11703920]
- Hakami RM, Hou L, Baxter LL, Loftus SK, Southard-Smith EM, Incao A, Cheng J, Pavan WJ. Genetic evidence does not support direct regulation of EDNRB by SOX10 in migratory neural crest and the melanocyte lineage. *Mech. Dev*. 2006; 123:124–134. [PubMed: 16412618]
- Hardy MH. The secret life of the hair follicle. *Trends Genet*. 1992; 8:55–61. [PubMed: 1566372]
- Hirobe T. Basic fibroblast growth factor stimulates the sustained proliferation of mouse epidermal melanoblasts in a serum-free medium in the presence of dibutyryl cyclic AMP and keratinocytes. *Development*. 1992; 114:435–445. [PubMed: 1317290]
- Hirobe T. Structure and function of melanocytes: microscopic morphology and cell biology of mouse melanocytes in the epidermis and hair follicle. *Histol. Histopathol*. 1995; 10:223–237. [PubMed: 7756740]
- Hirobe T, Abe H. The slaty mutation affects the morphology and maturation of melanosomes in the mouse melanocytes. *Pigment Cell Res*. 2006; 19:454–459. [PubMed: 16965275]

- Hirobe T, Osawa M, Nishikawa S. Steel factor controls the proliferation and differentiation of neonatal mouse epidermal melanocytes in culture. *Pigment Cell Res.* 2003; 16:644–655. [PubMed: 14629722]
- Hou L, Panthier JJ, Arnheiter H. Signaling and transcriptional regulation in the neural crest-derived melanocyte lineage: interactions between KIT and MITF. *Development.* 2000; 127:5379–5389. [PubMed: 11076759]
- Imokawa G, Kobayashi T, Miyagishi M. Intracellular signaling mechanisms leading to synergistic effects of endothelin-1 and stem cell factor on proliferation of cultured human melanocytes. Cross-talk via trans-activation of the tyrosine kinase c-kit receptor. *J. Biol. Chem.* 2000; 275:33321–33328. [PubMed: 10921922]
- Ito M, Kawa Y, Ono H, Okura M, Baba T, Kubota Y, Nishikawa SI, Mizoguchi M. Removal of stem cell factor or addition of monoclonal anti-c-kit antibody induces apoptosis in murine melanocyte precursors. *J. Invest. Dermatol.* 1999; 112:796–801. [PubMed: 10233774]
- Jiao Z, Mollaaghababa R, Pavan WJ, Antonellis A, Green ED, Hornyak TJ. Direct interaction of Sox10 with the promoter of murine dopachrome tautomerase (Dct) and synergistic activation of Dct expression with Mitf. *Pigment Cell Res.* 2004; 17:352–362. [PubMed: 15250937]
- Jordan SA, Jackson IJ. A late wave of melanoblast differentiation and rostrocaudal migration revealed in patch and rump-white embryos. *Mech. Dev.* 2000a; 92:135–143. [PubMed: 10727853]
- Jordan SA, Jackson IJ. MGF (KIT ligand) is a chemokinetic factor for melanoblast migration into hair follicles. *Dev. Biol.* 2000b; 225:424–436. [PubMed: 10985860]
- Kawabata K, Ujikawa M, Egawa T, Kawamoto H, Tachibana K, Iizasa H, Katsura Y, Kishimoto T, Nagasawa T. A cell-autonomous requirement for CXCR4 in long-term lymphoid and myeloid reconstitution. *Proc. Natl. Acad. Sci. USA.* 1999; 96:5663–5667. [PubMed: 10318941]
- Kuhlbrodt K, Schmidt C, Sock E, Pingault V, Bondurand N, Goossens M, Wegner M. Functional analysis of Sox10 mutations found in human Waardenburg–Hirschsprung patients. *J. Biol. Chem.* 1998; 273:23033–23038. [PubMed: 9722528]
- Kunisada K, Hirota H, Fujio Y, Matsui H, Tani Y, Yamauchi-Takahara K, Kishimoto T. Activation of JAK-STAT and MAP kinases by leukemia inhibitory factor through gp130 in cardiac myocytes. *Circulation.* 1996; 94:2626–2632. [PubMed: 8921810]
- Kunisada T, Yoshida H, Yamazaki H, Miyamoto A, Hemmi H, Nishimura E, Shultz LD, Nishikawa S, Hayashi S. Transgene expression of steel factor in the basal layer of epidermis promotes survival, proliferation, differentiation and migration of melanocyte precursors. *Development.* 1998; 125:2915–2923. [PubMed: 9655813]
- Le Douarin NM, Dupin E. Cell lineage analysis in neural crest ontogeny. *J. Neurobiol.* 1993; 24:146–161. [PubMed: 8445384]
- Lee EC, Yu D, Martinez de Velasco J, Tessarollo L, Swing DA, Court DL, Jenkins NA, Copeland NG. A highly efficient *Escherichia coli*-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. *Genomics.* 2001; 73:56–65. [PubMed: 11352566]
- Li L, Mignone J, Yang M, Matic M, Penman S, Enikolopov G, Hoffman RM. Nestin expression in hair follicle sheath progenitor cells. *Proc. Natl. Acad. Sci. USA.* 2003; 100:9958–9961. [PubMed: 12904579]
- Lu M, Grove EA, Miller RJ. Abnormal development of the hippocampal dentate gyrus in mice lacking the CXCR4 chemokine receptor. *Proc. Natl. Acad. Sci. USA.* 2002; 99:7090–7095. [PubMed: 11983855]
- 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]
- Mackenzie MA, Jordan SA, Budd PS, Jackson IJ. Activation of the receptor tyrosine kinase kit is required for the proliferation of melanoblasts in the mouse embryo. *Dev. Biol.* 1997; 192:99–107. [PubMed: 9405100]
- Mayer TC. The migratory pathway of neural crest cells into the skin of mouse embryos. *Dev. Biol.* 1973; 34:39–46. [PubMed: 4595498]

- McGrath KE, Koniski AD, Maltby KM, McGann JK, Palis J. Embryonic expression and function of the chemokine SDF-1 and its receptor, CXCR4. *Dev. Biol.* 1999; 213:442–456. [PubMed: 10479460]
- Mikkola ML, Millar SE. The mammary bud as a skin appendage: unique and shared aspects of development. *J. Mammary Gland Biol. Neoplasia.* 2006; 11:187–203. [PubMed: 17111222]
- Mollaaghababa R, Pavan WJ. The importance of having your SOX on: role of SOX10 in the development of neural crest-derived melanocytes and glia. *Oncogene.* 2003; 22:3024–3034. [PubMed: 12789277]
- Morris RJ, Potten CS. Highly persistent label-retaining cells in the hair follicles of mice and their fate following induction of anagen. *J. Invest. Dermatol.* 1999; 112:470–475. [PubMed: 10201531]
- Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, Verastegui E, Zlotnik A. Involvement of chemokine receptors in breast cancer metastasis. *Nature.* 2001; 410:50–56. [PubMed: 11242036]
- Nagasawa T, Nakajima T, Tachibana K, Iizasa H, Bleul CC, Yoshie O, Matsushima K, Yoshida N, Springer TA, Kishimoto T. Molecular cloning and characterization of a murine pre-B-cell growth-stimulating factor/stromal cell-derived factor 1 receptor, a murine homolog of the human immunodeficiency virus 1 entry coreceptor fusin. *Proc. Natl. Acad. Sci. USA.* 1996; 93:14726–14729. [PubMed: 8962122]
- Nakayama A, Nguyen MT, Chen CC, Opdecamp K, Hodgkinson CA, Arnheiter H. Mutations in microphthalmia, the mouse homolog of the human deafness gene MITF, affect neuroepithelial and neural crest-derived melanocytes differently. *Mech. Dev.* 1998; 70:155–166. [PubMed: 9510032]
- Nelson WG, Sun TT. The 50- and 58-kdalton keratin classes as molecular markers for stratified squamous epithelia: cell culture studies. *J. Cell Biol.* 1983; 97:244–251. [PubMed: 6190820]
- Nishikawa S, Kusakabe M, Yoshinaga K, Ogawa M, Hayashi S, Kunisada T, Era T, Sakakura T, Nishikawa S. In utero manipulation of coat color formation by a monoclonal anti-c-kit antibody: two distinct waves of c-kit-dependency during melanocyte development. *Embo J.* 1991; 10:2111–2118. [PubMed: 1712289]
- Nishimura EK, Yoshida H, Kunisada T, Nishikawa SI. Regulation of E- and P-cadherin expression correlated with melanocyte migration and diversification. *Dev. Biol.* 1999; 215:155–166. [PubMed: 10545227]
- Nishimura EK, Jordan SA, Oshima H, Yoshida H, Osawa M, Moriyama M, Jackson IJ, Barrandon Y, Miyachi Y, Nishikawa S. Dominant role of the niche in melanocyte stem-cell fate determination. *Nature.* 2002; 416:854–860. [PubMed: 11976685]
- Opdecamp K, Nakayama A, Nguyen MT, Hodgkinson CA, Pavan WJ, Arnheiter H. Melanocyte development in vivo and in neural crest cell cultures: crucial dependence on the Mitf basic-helix-loop-helix-zipper transcription factor. *Development.* 1997; 124:2377–2386. [PubMed: 9199364]
- Oro AE, Scott MP. Splitting hairs: dissecting roles of signaling systems in epidermal development. *Cell.* 1998; 95:575–578. [PubMed: 9845357]
- Osawa M, Egawa G, Mak SS, Moriyama M, Freter R, Yonetani S, Beermann F, Nishikawa S. Molecular characterization of melanocyte stem cells in their niche. *Development.* 2005; 132:5589–5599. [PubMed: 16314490]
- Oshima H, Rochat A, Kedzia C, Kobayashi K, Barrandon Y. Morphogenesis and renewal of hair follicles from adult multipotent stem cells. *Cell.* 2001; 104:233–245. [PubMed: 11207364]
- Panaretto BA. Gene expression of potential morphogens during hair follicle and tooth formation: a review. *Reprod. Fertil. Dev.* 1993; 5:345–360. [PubMed: 7512272]
- Peters EM, Tobin DJ, Botchkareva N, Maurer M, Paus R. Migration of melanoblasts into the developing murine hair follicle is accompanied by transient c-kit expression. *J. Histochem. Cytochem.* 2002; 50:751–766. [PubMed: 12019292]
- Potter SB, Mollaaghababa R, Hou L, Southard-Smith EM, Hornyak TJ, Arnheiter H, Pavan WJ. Analysis of SOX10 function in neural crest-derived melanocyte development: SOX10-dependent transcriptional control of dopachrome tautomerase. *Dev. Biol.* 2001; 237:245–257. [PubMed: 11543611]
- Pujol F, Kitabgi P, Boudin H. The chemokine SDF-1 differentially regulates axonal elongation and branching in hippocampal neurons. *J. Cell Sci.* 2005; 118:1071–1080. [PubMed: 15731012]

- Sawada S, Gowrishankar K, Kitamura R, Suzuki M, Suzuki G, Tahara S, Koito A. Disturbed CD4+ T cell homeostasis and in vitro HIV-1 susceptibility in transgenic mice expressing T cell line-tropic HIV-1 receptors. *J. Exp. Med.* 1998; 187:1439–1449. [PubMed: 9565636]
- Sieber-Blum M, Grim M. The adult hair follicle: cradle for pluripotent neural crest stem cells. *Birth Defects. Res. C Embryo Today.* 2004; 72:162–172. [PubMed: 15269890]
- Sieber-Blum M, Grim M, Hu YF, Szeder V. Pluripotent neural crest stem cells in the adult hair follicle. *Dev. Dyn.* 2004; 231:258–269. [PubMed: 15366003]
- Sieber-Blum M, Schnell L, Grim M, Hu YF, Schneider R, Schwab ME. Characterization of epidermal neural crest stem cell (EPI-NCSC) grafts in the lesioned spinal cord. *Mol. Cell Neurosci.* 2006; 32:67–81. [PubMed: 16626970]
- Slominski A, Paus R. Melanogenesis is coupled to murine anagen: toward new concepts for the role of melanocytes and the regulation of melanogenesis in hair growth. *J. Invest. Dermatol.* 1993; 101:90S–97S. [PubMed: 8326158]
- Stenn KS, Paus R. Controls of hair follicle cycling. *Physiol. Rev.* 2001; 81:449–494. [PubMed: 11152763]
- Stumm RK, Zhou C, Ara T, Lazarini F, Dubois-Dalcq M, Nagasawa T, Holtt V, Schulz S. CXCR4 regulates interneuron migration in the developing neocortex. *J. Neurosci.* 2003; 23:5123–5130. [PubMed: 12832536]
- 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; 393:591–594. [PubMed: 9634237]
- Tamura A, Halaban R, Moellmann G, Cowan JM, Lerner MR, Lerner AB. Normal murine melanocytes in culture. *In Vitro Cell Dev. Biol.* 1987; 23:519–522. [PubMed: 3610949]
- Taylor G, Lehrer MS, Jensen PJ, Sun TT, Lavker RM. Involvement of follicular stem cells in forming not only the follicle but also the epidermis. *Cell.* 2000; 102:451–461. [PubMed: 10966107]
- Tobin DJ, Hagen E, Botchkarev VA, Paus R. Do hair bulb melanocytes undergo apoptosis during hair follicle regression (catagen)? *J. Invest. Dermatol.* 1998; 111:941–947. [PubMed: 9856800]
- Tran PB, Miller RJ. Chemokine receptors in the brain: a developing story. *J. Comp. Neurol.* 2003; 457:1–6. [PubMed: 12541320]
- Tran PB, Miller RJ. HIV-1, chemokines and neurogenesis. *Neurotox Res.* 2005; 8:149–158. [PubMed: 16260392]
- Tran PB, Ren D, Veldhouse TJ, Miller RJ. Chemokine receptors are expressed widely by embryonic and adult neural progenitor cells. *J. Neurosci. Res.* 2004; 76:20–34. [PubMed: 15048927]
- Tran PB, Banisadr G, Ren D, Chenn A, Miller RJ. Chemokine receptor expression by neural progenitor cells in neurogenic regions of mouse brain. *J. Comp. Neurol.* 2007; 500:1007–1033. [PubMed: 17183554]
- Wehrle-Haller B, Weston JA. Soluble and cell-bound forms of steel factor activity play distinct roles in melanocyte precursor dispersal and survival on the lateral neural crest migration pathway. *Development.* 1995; 121:731–742. [PubMed: 7536655]
- Wilson C, Cotsarelis G, Wei ZG, Fryer E, Margolis-Fryer J, Ostead M, Tokarek R, Sun TT, Lavker RM. Cells within the bulge region of mouse hair follicle transiently proliferate during early anagen: heterogeneity and functional differences of various hair cycles. *Differentiation.* 1994; 55:127–136. [PubMed: 8143930]
- Wong CE, Paratore C, Dours-Zimmermann MT, Rochat A, Pietri T, Suter U, Zimmermann DR, Dufour S, Thiery JP, Meijer D, Beermann F, Barrandon Y, Sommer L. Neural crest-derived cells with stem cell features can be traced back to multiple lineages in the adult skin. *J. Cell Biol.* 2006; 175:1005–1015. [PubMed: 17158956]
- Yoshida H, Kunisada T, Kusakabe M, Nishikawa S, Nishikawa SI. Distinct stages of melanocyte differentiation revealed by analysis of nonuniform pigmentation patterns. *Development.* 1996; 122:1207–1214. [PubMed: 8620847]
- Yoshida S, Shimmura S, Nagoshi N, Fukuda K, Matsuzaki Y, Okano H, Tsubota K. Isolation of multipotent neural crest-derived stem cells from the adult mouse cornea. *Stem Cells.* 2006; 24:2714–2722. [PubMed: 16888282]

- Zicha D, Dunn G, Jones G. Analyzing chemotaxis using the Dunn direct-viewing chamber. *Methods Mol. Biol.* 1997; 75:449–457. [PubMed: 9276291]
- 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]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 1.

Expression patterns of SDF-1 and CXCR4 in the skin during mouse embryogenesis: Paraformaldehyde-fixed sections of skin harvested from E18 and E20 embryos showing SDF-1-EGFP (a) and CXCR4-EGFP (b,c) expression during hair follicle development at day E18 (a,b) and E20 (c). From E18 SDF-1-EGFP is expressed in the dermal cells enveloping the hair follicle (HF) and in the dermal papilla (DP) (a), whereas CXCR4-EGFP is expressed in a region that seems to correspond to the “Bulge” area (B) and in the DP (b). (c) shows GFP expression in the external epithelial layer of the hair follicle at E20. Arrows in (a,b) point to the dermal papilla. Insert in (b) shows a higher magnification ($\times 2$) of the area in box. Scale bar = 100 μm .

Fig. 2.

Distribution and localization of melanoblasts in the embryonic skin of CXCR4 mutant mice: Paraformaldehyde-fixed sections of skin harvested from E18–20 embryos were immunostained for the melanoblast-specific marker tyrosinase-related protein-2 (TRP2) to show the distribution of melanoblasts and their localization in the skin of CXCR4^{+/+} and CXCR4^{-/-} mice. Melanoblasts marked by TRP2 were regularly shaped and localized at about the same distance from the epidermis and the dermis in the HF in CXCR4^{+/+} mice (b,c), but irregularly positioned in the epidermis in CXCR4^{-/-} mice (e,f). (a,d) are 4',6-diamidino-2-phenylindole (DAPI) stained skin sections. (epidermis (ep); dermis (d); hair follicle (HF)). Scale bar = 50 μ m.

Fig. 3.

FACS purification of CXCR4-expressing cells: cells from embryonic skin of CXCR4–EGFP transgenic mice at E18–20 were isolated and sorted by fluorescence-activated cell sorting (FACS) based on EGFP fluorescence. (a) Sample FACS plot of the population of CXCR4–EGFP-expressing cells selected; CXCR4-expressing cells were selected as a highly fluorescent population (R3; right peak). (b–d) Epifluorescence (green) of dissociated embryonic skin cells before and after FACS purification co-stained for CXCR4 (red). (b) Mixed skin cells before FACS purification; only a very small percentage ($1.83 \pm 0.18\%$) of dissociated cells are CXCR4–EGFP-expressing cells that stain for CXCR4 receptors. (c) FACS purification results in an essentially pure population of EGFP-positive cells that all stained for CXCR4 receptors (c). (d) FACS-sorted EGFP-negative cells did not stain for CXCR4 receptors as shown by DAPI counter stain (blue, d). (e,f) Sample $(Ca^{2+})_i$ imaging plots of a population of FACS-sorted cells harvested from the skin of CXCR4–EGFP embryos (E18–20). In (e) CXCR4–EGFP-expressing cells responded to SDF-1, and ATP. SDF-1 and ATP-induced Ca responses were present in 100% of cells tested ($n = 3$ experiments). (f) EGFP-negative cells did not respond to SDF-1 but still responded to ATP. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

SDF-1 induces (Ca^{2+})_i changes and acts as a chemoattractant for melanoblasts of embryonic skin: cells from embryonic skin of CXCR4-EGFP transgenic mice at E18–20 were isolated, sorted by fluorescence-activated cell sorting (FACS) based on EGFP fluorescence and subjected to immunocytochemistry for the melanoblast-specific marker TRP2 (a), (Ca^{2+})_i imaging (b,c) and microchemotaxis (d) assays. (a) Merged image of CXCR4-EGFP expression (green) and immunostaining for TRP2 showing FACS-sorted EGFP cells all stained for TRP2. In (b) CXCR4-EGFP-expressing cells responded to stem cell factor (SCF) (84/95: 88%), and endothelin-3 (ET-3) (95/95: 100%), in addition to SDF-1 and ATP, indicating that they are melanoblasts. (c) EGFP-negative cells did not respond to SCF but still responded to ET-3 (< 10%) and ATP. (d) FACS-sorted EGFP-positive cells as characterized as in (a,b) migrated towards an SDF-1 (50 nM) gradient (120 min). The migration responses by FACS-sorted EGFP-positive cells towards SDF-1 was inhibited by the CXCR4 antagonist AMD3100 (10 μ M) ($n = 3$), indicating that they were mediated by the CXCR4 receptor. * $P < 0.05$, significantly different from controls. ** $P < 0.05$, significantly different from SDF-1-treated groups. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5.

Expression patterns of CXCR4 within the postnatal skin of CXCR4-EGFP transgenic mice: Paraformaldehyde-fixed sections of skin illustrating CXCR4-EGFP expression during hair follicle cycling at postnatal days (P): P1, P7, P9, P12, P15, and P24, P27, P32 to cover the first and the second anagen (ana); P19, and P38 to cover the first and the second catagen (cata); and P21 and P45 for the first and the second telogen (telo), respectively. EGFP appeared to be strongly expressed in the dermal papilla (DP) and along an epithelial layer of the hair follicles (HFs) during the anagen phases of the HF cycles. At catagen (P19 and P38) and telogen (P21 and P45) HFs, only a small number of EGFP-positive cells appeared to be located at the base of the follicle epithelium. All panels (a-i) are of the same magnification (scale bar in panel (b) = 100 μ m). Insert shows a higher magnification ($\times 2$) of the boxed area in (c) highlighting CXCR-EGFP-expressing cells probably representing cells of the bulge. Arrows in panels (f,g,j,k,i) point to CXCR-EGFP-expressing cells probably representing cells of the DP or the bulge. Outer root sheath (ORS); bulge region (B); dermal papilla (DP); anagen, (ana); catagen (cata); telogen (telo).

Fig. 6.

CXCR4-EGFP-expressing cells are located in the HF bulge area and the outer root sheath: paraformaldehyde-fixed sections of skin harvested from CXCR4-EGFP mice at P7-9 illustrating immunostaining for CXCR4-GFP, K17 (marker of the outer root sheath (ORS)), K15 (marker of the HF bulge), and CXCR4. (a-c) are merged image of immunostaining for GFP (a) and K17 (b), showing CXCR4-EGFP expression in the ORS. (d-f) are merged image of immunostaining for GFP (a) and K15 (b), showing CXCR4-EGFP expression in the HF bulge area. Thus, CXCR4-EGFP appears to be expressed in the bulge area (B) and the ORS, regions known to be rich of HF stem/progenitor cells. (g,h) are merged images of EGFP expression (green) and immunostaining for CXCR4 (red) showing expression of CXCR4 by cells of an epithelial layer of the HF. (g, longitudinal view of a hair follicle and h, transverse view of hair follicles). Scale bar in panels (g,h) = 50 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 7.

Characterization of CXCR4-expressing cells: Cells from postnatal anagen skin of CXCR4–EGFP transgenic mice at P4–7 were isolated, sorted by fluorescence-activated cell sorting (FACS) based on EGFP fluorescence, and subjected to immunocytochemistry for nestin, Lex and the proliferation cell nuclear factor (PCNA), markers generally used for the identification of progenitor cells. (a–c), illustrates immunostaining of CXCR4–EGFP FACS-sorted cells for nestin (a), Lex (b) and PCNA (c), thus indicating that these cells express markers of progenitor cells.

Fig. 8.

Expression patterns of nestin-EGFP in the postnatal skin of transgenic mice: Confocal images from paraformaldehyde-fixed sections of skin showing nestin-EGFP expression during hair follicle cycling at postnatal day 1, 2, 4, 7 and 26, 30, 33 to cover the first and the second anagen (ana); P19, and P37 to cover the first and the second catagen (cata); and P21 for the first telogen (telo). During anagen (P1, P2, P4, P7, P26, P30, P33) nestin-EGFP is expressed in the bulge area and the ORS, but not in the DP (in contrast to CXCR4-EGFP expression, Fig. 5), but only a small number of EGFP-positive cells appeared to be located at the base of the follicle epithelium at catagen HF at P19 and P37, and telogen HF at P21. Scale bar in panels (a-i) = 100 μ m. Insert shows a higher magnification ($\times 2$) of the boxed area in (e) highlighting nestin-EGFP-expressing cells probably representing cells of the bulge area. anagen, (ana); catagen (cata); telogen (telo).

Fig. 9.

Expression patterns of SDF-1 within the postnatal skin of SDF-1 transgenic mice: Confocal images from paraformaldehyde-fixed sections of skin showing SDF-1-EGFP expression during hair follicle cycling at postnatal days (P): P1, P7, P11, P14 and P24, P28, P33, P35 to cover the first and the second anagen (ana); P19, and P38 to cover the first and the second catagen (cata); and P21 and P41, P44, P46, P50 for the first and the second telogen (telo). (a–p) SDF-1-EGFP is abundantly expressed in dermal cells and in the DP forming a gradient throughout the postnatal HF cycle at anagen, catagen and telogen phases. In (q–s)

we used BAC transgenic mice (SDF-1-mRFP1 mice), which expressed an SDF-1-mRFP fusion protein crossed with CXCR4-EGFP mice to generate a new line of mice expressing both CXCR4-EGFP and SDF-1-mRFP. Confocal images of sections of skin from these mice demonstrate the relative localization of CXCR4-EGFP-expressing cells in anagen HF's (green in q and s) and that of SDF-1-mRFP (red in r and s) showing the production and localization of SDF-1-mRFP protein along the path of CXCR4-expressing cells. Scale bar in panels (a-k, o-p) = 100 μ m, (l-n, q-s) = 50 μ m. anagen, (ana); catagen (cata); telogen (telo). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 10.

SDF-1 induces (Ca^{2+})_i changes and acts as a chemoattractant for CXCR4-EGFP-expressing cells of postnatal skin: CXCR4-EGFP-expressing cells harvested from anagen skin at P4–7 and isolated by FACS migrated towards an SDF-1 (50 nM) gradient (120 min) and this was inhibited by the CXCR4 antagonist AMD3100 (20 μM) ($n = 4$) (a). Consistent with the fact that most of the CXCR4-EGFP-expressing cells stained for nestin, FACS-EGFP-expressing cells harvested from anagen skin of nestin-EGFP mice at P4–7 also responded to SDF-1, and ATP ($n = 3$) (b) in the Ca response assay. Similarly, nestin-EGFP-expressing cells migrated towards an SDF-1 (50 nM) gradient and this was inhibited by AMD3100 (20 μM) ($n = 2$) (c). * $P < 0.05$, Significantly different from respective controls in a and c. ** $P < 0.05$, significantly different from respective SDF-1-treated groups in a and c.

Fig. 11.

Expression of SOX10, a neural crest marker, by CXCR4–EGFP-expressing cells: Skin sections and FACS-sorted skin cells harvested from an anagen postnatal day P7 of CXCR4–EGFP mice were subjected to *in situ* hybridization with a probe specific for SOX10, using digoxigenin (DIG) labeling (a,b,e) and fluorescence *in situ* hybridization (FISH) performed together with immunostaining with an EGFP antibody (c,d,f). (a,b,e) show SOX10 mRNA expression by hair follicles along the outer root sheath (ORS) and the basal layer of the epidermis (a) and by CXCR4–EGFP FACS-sorted cells (e); (b) negative control for SOX10 sense riboprobe. (c,d,f) are merged images of CXCR4–EGFP expression immunostained with EGFP antibody and images processed with FISH to show the colocalization of SOX10 and CXCR4–EGFP. Arrows in c and d, point to the ORS in a longitudinal view of a hair follicle (c), and transverse view of hair follicles (d). Arrowheads in c and d point to the absence of SOX10 staining in the HF matrix. (f) shows FISH for SOX10 and CXCR4–EGFP in P7-CXCR4–EGFP FACS-sorted cells. Scale bar in panels (a–d) = 50 μm, (e,f) = 20 μm.

Fig. 12.

Expression of the melanocyte-specific marker tyrosinase-related protein 2 (TRP2) by CXCR4-EGFP-expressing cells: The expression of TRP2 was carried out using immunohistochemistry on skin sections from P7-CXCR4-EGFP transgenic mice. The overlap of CXCR4-EGFP cells (green) and Alexa 633-labeled TRP2 cells (red) shows that most of the CXCR4-EGFP cells expressed the melanoblast-specific marker TRP2 (a,b) (a, longitudinal view of a hair follicle, (b) transverse view of hair follicles. In (c) immunostaining for the ORS keratinocyte marker K14 was carried out on skin sections in P7-CXCR4-EGFP transgenic mice to show the expression of K14 relative to CXCR4-EGFP cells and observed that few cells expressed the ORS keratinocyte marker K14 (arrow in c). (d-f) immunostaining for TRP2 on P7-CXCR4-EGFP FACS-sorted cells. (d) Merged image of immunostaining for the melanoblast marker TRP2 on mixed skin cells before FACS purification showing only a very small percentage (<2%) of cells that are EGFP-positive (green, arrows in d) and which stain for TRP2 (red, arrows in d). In (e) Most EGFP-positive cells stained for TRP2. (f) EGFP-negative cells as shown by DAPI counterstain (blue) did not stain for TRP2. Scale bar in panels (a-c) = 50 μ m and (c) = 20 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 13.

CXCR4 expression is undetectable in differentiated melanocytes *in vitro* and *in vivo*: (a–b) Confocal images of immunostaining for CXCR4 (a) and for the differentiated melanocyte marker tyrosinase-related protein 1 (TRP1) (b) in differentiated melanocytes. In this experiment, CXCR4–EGFP FACS-sorted TRP2 positive cells were cultured with bFGF (2.5 ng/ml), endothelin-3 (100 μ M) and dibutyryl adenosine cAMP (0.5 mM) or SCF (2 ng/ml) for 15 days to induce differentiation to melanocytes. After 15 days, most of the cells no longer exhibited CXCR4–EGFP fluorescence or staining with CXCR4 antibody (a), became positive for the melanocyte marker TRP1 (b), but did not respond to SDF-1 (c) in the SDF-1-induced (Ca^{2+})_i imaging assay. (d–f) are merged images of EGFP fluorescence (green) with TRP1 fluorescent immunostaining (red) on anagen skin sections from P7-CXCR4–EGFP (d), P30 nestin–EGFP (e) and P7-SDF–EGFP (f). Arrows in (d) point to cells in close contact with and around the dermal papilla (DP) that expressed both CXCR4 and the melanocyte marker TRP1. This was confirmed in merged images of SDF-1–EGFP with TRP1 (arrows in f). In contrast, TRP1+ positive cells not in direct contact with the DP and located in the upper part of the matrix bulb did not appear to exhibit CXCR4–EGFP fluorescence (arrowheads in d–f). Scale bar = 50 μ m.

Fig. 14.

Migration and localization of melanoblasts in skin explant preparations is disrupted by AMD3100, a selective antagonist for CXCR4 receptors: Skin explant cultures prepared from E13.5 mouse embryos were treated with the CXCR4 antagonist AMD3100 (100 μ M) for 48 h and assessed for the melanoblast marker tyrosinase-related protein 2 (TRP2) immunoreactivity after 10 days in culture. Immunostaining for TRP2 showed that most melanoblasts positioned in the dermis (d) in control explants (arrows, a) but accumulated in the epidermis (ep) in AMD3100-treated explants (arrows, b). Scale bar = 100 μ m.