Cxcr4 is transiently expressed in both epithelial and mesenchymal compartments of nascent hair follicles but is not required for follicle formation

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Abstract: Hair follicle (HF) morphogenesis relies on the coordinated exchange of signals between mesenchymal and epithelial compartments of embryonic skin. Chemokine receptor *Cxcr4* expression was recently identified in dermal condensates (DCs) of nascent HFs, but its role in promoting HF morphogenesis remains unknown. Our analyses confirmed *Cxcr4* expression in condensate cells, and additionally revealed transient *Cxcr4* expression in incipient epithelial hair placodes. Placodal *Cxcr4* appeared prior to detection in DCs, representing a switch of expression between epithelial and mesenchymal compartments. To explore the functional role of this receptor in both compartments for early HF formation, we conditionally ablated *Cxcr4* with condensate-targeting *Tbx18*^*cre* knock-in and epidermis-targeting *Krt14-cre* transgenic mice. Conditional knockouts for both crosses were viable throughout embryogenesis and into adulthood. Morphological and biochemical marker analyses revealed comparable numbers of HFs forming in knockout embryos compared to wild-type littermate controls in both cases, suggesting that neither dermal nor epithelial *Cxcr4* expression is required for early HF morphogenesis. We conclude that *Cxcr4* expression and chemokine signaling through this receptor in embryonic mouse skin is dispensable for HF formation.

Key words: dermal papilla cells – hair follicle morphogenesis – hair follicle stem cells – mesenchymal-epithelial interactions – stem cell niche

Background

Mesenchymal-epithelial interactions drive diverse morphogenetic events during embryogenesis, including development of the skin and its associated appendages (1–3). Hair follicle (HF) formation in particular is known to rely on signaling crosstalk between these two compartments, and is thought to involve specialized mesenchymal dermal condensate (DC) cells and epithelial placode cells (1). However the precise identity, timing and mechanisms of all exchanged signals remain unknown. In an effort to characterize potentially relevant signaling pathways involved in HF morphogenesis, we examined the unique expression patterns of chemokine receptor CXCR4 in both condensate and placode during early stages of initiation and downgrowth. CXCR4 is a well-studied receptor previously implicated in multiple developmental and disease paradigms including organogenesis, stem cell maintenance, cancer and HIV, wherein CXCR4 interactions with ligand CXCL12 promote biological processes such as directed cell migration, cell survival, and adhesion (4–6). In order to assess the functional role of CXCR4 in HF morphogenesis we used the *cre/loxP* recombination system in genetically modified mice to conditionally ablate this gene in both epithelial and mesenchymal compartments during embryogenesis and assessed HF development.

Questions addressed

Is *Cxcr4* expression in placodes and dermal condensates required for hair follicle morphogenesis?

Experimental design

To investigate patterns of *Cxcr4* expression in embryonic mouse skin we used immunofluorescence on sections and qRT-PCR on sorted cells. More detailed information is available in Data S1.

Results

Chemokine signaling has been previously implicated in hair follicle (HF) morphogenesis, including recent descriptions of *Cxcr4* expression in dermal condensates (DCs) of embryonic HFs (7, 8). To study the expression patterns of *Cxcr4* in the HF mesenchyme in more detail, we used immunofluorescence staining on back skin sections from embryos aged 14.5 days (E14.5), E16.5 and E18.5 during the three main HF formation waves. In E14.5 sections we observed CXCR4 in condensates of stage 1 follicles (Fig. 1a, arrow). At E16.5, CXCR4 was present in DCs of downgrowing HFs and newly formed stage 1 follicles (Fig. 1b). At E18.5, we observed CXCR4 in condensates/dermal papillae of stage 1–5 follicles (Fig. 1c). High levels of *Cxcr4* expression were confirmed by qRT-PCR analysis of FACS-isolated condensate cells at E14.5, compared to whole epidermis and dermis (Fig. 1d). The related chemokine ligand *Cxcl12* is expressed in the embryonic dermis and DCs (Fig. 1d).

Through our initial immunofluorescence studies we uncovered CXCR4 staining also in epithelial placodes (Fig. 1b,c; arrowheads). With additional stainings we confirmed epithelial CXCR4 co-localization with placode marker EDAR at E16.5 and E18.5 (Fig. S1), indicating expression in stage 0 follicles confined to the epithelial compartment. Analyses of skin sections of earliest first-wave follicles at E14.0 corroborated CXCR4 co-localization with EDAR in nascent placodes (Fig. 1e). Finally, with whole-mount immunofluorescence stainings for CXCR4/EDAR of E15.5 *Sox2*^*GFP* skin
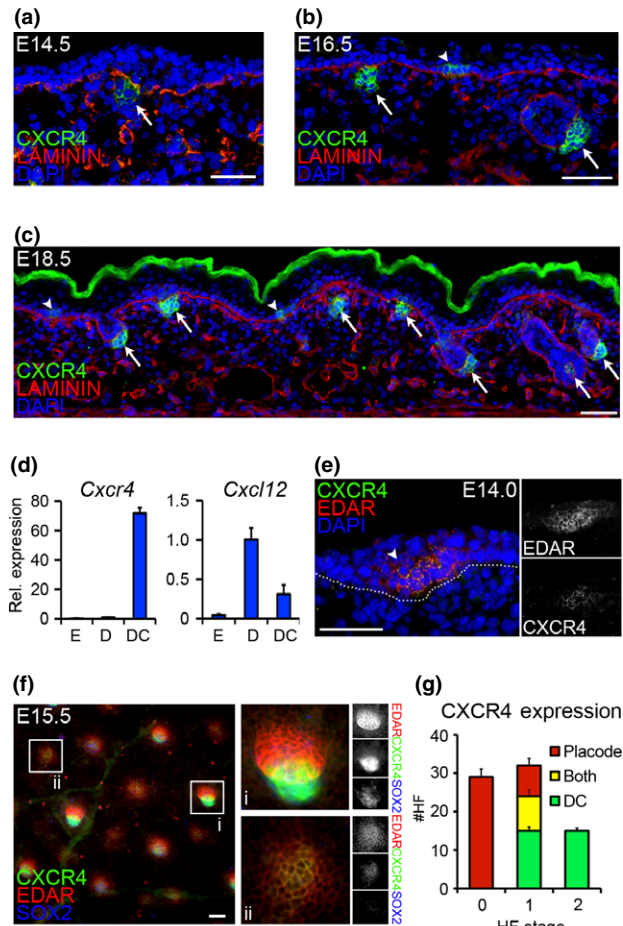
Experimental Dermatology

Figure 1. *Cxcr4* expression in embryonic skin. (a-c) Immunofluorescence staining for CXCR4 shows differential expression in epithelial placodes (arrowheads) and mesenchymal dermal condensates (arrows) of forming HFs at E14.5, E16.5, and E18.5. LAMININ labels basement membranes; Dapi highlights nuclei. (d) qRT-PCR of FACS sorted cells at E14.5 shows high *Cxcr4* expression in dermal condensates ('DC'), compared to embryonic dermis ('D') and epidermis ('E'). *Cxcr4* ligand chemokine *Cxcl12* is expressed in mesenchymal cells. (e) Immunofluorescence of E14.0 skin confirms CXCR4 expression in EDAR+ placodes of earliest forming HFs. (f) EDAR (placode marker) and SOX2 (condensate marker) expression highlight the stage- and compartment-specific localization of CXCR4 expression. In more advanced stage 2 follicles (i) CXCR4 co-localizes with SOX2. In early stage 0 follicles (ii) CXCR4 co-localizes with EDAR. (g) Quantification of CXCR4 localization in early forming HFs by stage at E15.0 (mean and SD of 3 embryos). CXCR4 expression switches from epithelial to mesenchymal cells. Dotted line denotes basement membrane. Scale bar = 50μm.

tissues, where GFP strongly labels condensates (9) and follicles are present in stages 0–2, we confirmed HF-stage dependent CXCR4 expression (Fig. 1f): larger advanced follicles with distinct elongated EDAR domains robustly stained for CXCR4 in condensates (Fig. 1f, insert i), while nascent follicles without obvious condensates displayed small circular EDAR domains that co-localized with CXCR4 (Fig. 1f, insert ii). Stage-specific quantification of compartmental CXCR4 localization revealed that epithelial expression is limited to stage 0 and few stage 1 follicles, whereas mesenchymal CXCR4 is found in stage 1 and stage 2 follicles. Collectively these results indicate that as follicles mature during morphogenesis, *Cxcr4* expression switches from the epithelial to the mesenchymal HF domain.
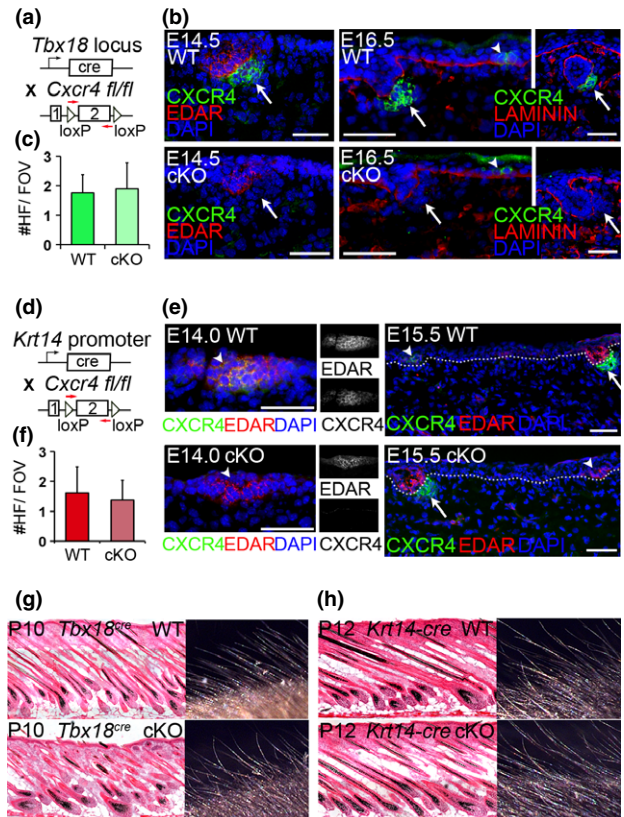
Figure 2. Targeted ablation of *Cxcr4* in placodes or condensates does not affect HF formation. (a) Schematic of *Tbx18cre* and *Cxcr4fl/fl* lines to target dermal condensates at E14.5 for *Cxcr4* ablation. (b) Immunofluorescence staining confirms CXCR4 ablation at the protein level in condensates (arrows) at E14.5 and E16.5 stage 1–2 follicles, while epithelial CXCR4 expression remains intact (arrowheads). (c) Quantification of first-wave HF numbers per field of view (FOV). Comparable HF numbers form in P10 cKO skin compared to controls. (d-f) *Krt14-cre* targets epidermal cells including the placode for *Cxcr4* ablation. (e) Immunofluorescence staining confirms absence of CXCR4 in placodes (arrowheads) as early as E14.0 in cKO skin. Note expression in condensates is still present (arrows). (f) Comparable numbers of first-wave follicles form in P12 cKO skin compared to controls. (g-h) Hematoxylin/eosin staining of skin sections and macroscopic view of external hair shafts. Hair follicles and shafts form normally in both *Tbx18cre* (g) and *Krt14-cre* (h) *Cxcr4fl/fl* cKO pups. c,f: Data are mean and SD of 2 WT and 2 cKO sex-matched littermates (6-10 FOV). Dotted lines denote basement membranes. Scale bar = 50 μm.

To explore the function of *Cxcr4* in placodes and/or condensates of forming HFs, we used separate *cre/loxP* strategies to conditionally ablate expression in the epithelial and mesenchymal compartments. To target DCs, we crossed *Tbx18cre* (10) with *Cxcr4* floxed mice (11) (Fig. 2a). *Cxcr4* ablation in E14.5 conditional knockout (cKO) DCs was confirmed by immunofluorescence (Fig. 2b). Despite early and efficient *Cxcr4* ablation, first-wave HFs formed normally in cKO skin and numbers were comparable to those found in wild-type (WT) littermates in both embryonic skin (Fig. S2a) and in postnatal P10 skin (Fig. 2c,g). To independently confirm that CXCR4 in DCs is dispensable for HF formation we ablated this gene also by crossing *Cxcr4* floxed mice with the *Prx1-cre* transgenic line (Fig. S3a) (12). The *Prx1* promoter is widely active in the ventral skin mesenchyme starting at E11.5, well before HF formation (13, 14). By E14.5 the entire ventral dermis is labeled with a *cre* reporter (Fig. S3b). Despite

broad and early *Cxcr4* ablation in the dermis, HFs were still induced in cKO ventral skin (Fig. S3c, SOX2^{+} DCs in green), and HFs formed normally (data not shown). These data suggest that DC expression of *Cxcr4* is not required for HF formation.

To investigate the role of *Cxcr4* in the epithelial placode, we crossed *Krt14-cre* (15) with *Cxcr4* floxed mice (Fig. 2d) and confirmed robust CXCR4 ablation in first wave HF placodes with immunofluorescence (Fig. 2e). Continued expression of CXCR4 in DCs highlighted the compartment-specific gene knockout (Fig. 2e). Despite the absence of CXCR4 expression in cKO placodes, embryonic HFs formed normally at E14.5 (Fig. S2b). Quantification of first-wave HFs at postnatal day P12 revealed formation in normal numbers in cKO skin compared to WT littermate controls (Fig. 2f,h). Collectively, these data suggest that *Cxcr4* expression by either the epithelial or mesenchymal compartment is dispensable for normal HF morphogenesis to proceed.

Conclusions

Chemokine signaling has been previously implicated in HF development, and in this study we confirmed the expression of the chemokine receptor *Cxcr4* in newly forming follicles and assessed its function using mouse genetics. We found *Cxcr4* expression in nascent HFs within placodes, which switches to condensates in maturing follicles. Despite its uniquely localized expression, conditional gene ablation revealed that this receptor is dispensable for HF development. In general CXCR4 is thought to interact exclusively with ligand CXCL12 (16), suggesting that other chemokine receptors are unlikely to play a role in functionally compensating the absence of *Cxcr4* through genetic redundancy. However in prior investigations of chemokine function in development, separate signaling cascades were found to cooperate with the *Cxcr4-Cxcl12* axis to drive tissue morphogenesis (17). Therefore it might be interesting in future investigations to examine the gene expression profiles of conditional knockout skin for any compensatory gene changes.

Author Contribution

R.S. and M.R. conceived and designed the experiments, R.S., A.R., and K.D. performed experiments, C.C. contributed essential reagents and tools, R.S. and M.R. analyzed and interpreted the data, R.S. prepared figures, and R.S. and M.R. wrote the manuscript.

Conflict of interests

The authors declare no conflict of interest.

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Supporting Information

Additional supporting data may be found in the supplementary information of this article:

Figure S1. CXCR4 co-localization with EDAR at E16.5 and E18.5. Immunofluorescence staining for CXCR4. CXCR4 in the epithelial compartment overlaps with EDAR in (a) E16.5 and (b) E18.5 embryo back skin. CXCR4 is expressed in EDAR^{+} placode cells of stage 0–1 follicles (arrowheads) and the condensate cells of stage 1–5 follicles (arrows). Asterisk denotes nonspecific staining. Dotted lines denote basement membranes. Scale bar = 50 μm.

Figure S2. Hair follicles form in comparable numbers in *Cxcr4* ablated condensates and placodes at E14.5. Quantification of first-wave HF numbers in embryo skin at E14.5 per field of view (FOV) for *Tbx18*[cre];*Cxcr4*[fl] (a) and *Krt14-cre*;*Cxcr4*[fl] (b) crosses. Data represent mean and SD of 3 WT and 3 cKO embryo littermates.

Figure S3. *Cxcr4* ablation with *Prx1-cre* does not impair hair induction. (a) Schematic of *Prx-cre* and *Cxcr4fl/fl* lines to target the ventral mesenchyme for *Cxcr4* ablation after E11.5. (b) *Prx1-cre* crossed with the red/green switch *cre* reporter. Membrane GFP (mG) is expressed after *cre*-mediated removal of membrane tdTomato (mT) under the *actin-B* promoter. Dotted line denotes basement membrane. (c) Whole-mount immunofluorescence staining for CXCR4 and condensate marker SOX2 of E15.5 WT and cKO skin from embryo flanks. In WT skin and dorsal skin of *Prx1-cre*;*Cxcl4*^{fl/fl} embryos, CXCR4 co-localizes with SOX2 (yellow). In ventral cKO skin CXCR4 is absent in dermal condensates (green). Scale bar = 200 μm.

Data S1. Materials and methods.