Hoxc-Dependent Mesenchymal Niche Heterogeneity Drives Regional Hair Follicle Regeneration

Graphical Abstract

Highlights
- Distinct skin mesenchymal niches are uniquely marked by regional Hoxc expression
- Epigenetic disruption of Hoxc expression leads to ectopic hair regeneration
- Single Hoxc genes can reprogram a dormant mesenchymal niche into an active niche
- Niche-expressed Hoxc genes activate epithelial SCs through Wnt signaling

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In Brief
Yu et al. show that Hoxc gene expression in mesenchymal niches controls epithelial stem cell activation via Wnt signaling. Hoxc expression patterns vary at the tissue level, suggesting that the regenerative landscape of the skin is governed by mesenchymal niche plasticity.
Hoxc-Dependent Mesenchymal Niche Heterogeneity Drives Regional Hair Follicle Regeneration

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SUMMARY

Mesenchymal niche cells instruct activity of tissue-resident stem and progenitor cell populations. Epithelial stem cells in hair follicles (HFs) have region-specific activity, which may arise from intrinsic cellular heterogeneity within mesenchymal dermal papilla (DP) cells. Here we show that expression of Hoxc genes is sufficient to reprogram mesenchymal DP cells and alter the regenerative potential of epithelial stem cells. Hoxc gene expression in adult skin dermis closely correlates with regional HF regeneration patterns. Disrupting the region-specific expression patterns of Hoxc genes, by either decreasing their epigenetic repression via Bmi1 loss or inducing ectopic interactions of the Hoxc locus with an active epigenetic region, leads to precocious HF regeneration. We further show that a single Hoxc gene is sufficient to activate dormant DP niches and promote regional HF regeneration through canonical Wnt signaling. Altogether, these results reveal that Hoxc genes bestow mesenchymal niches with tissue-level heterogeneity and plasticity.

INTRODUCTION

Mesenchymal cells ubiquitously exist in metazoan organs and play a central role in tissue function during normal homeostasis and disease. In tissues undergoing continuous regeneration, distinct mesenchymal niches play a dominant role in regulating parenchymal stem cell (SC) activity (Chen et al., 2016; Morrison and Scadden, 2014; Sailaja et al., 2016). Mesenchymal niche cells often comprise heterogeneous cell lineages (Donati and Watt, 2015). Underneath the skin epidermis, the dermal fibroblasts arise from different embryonic lineages and have distinct fates during homeostasis or wound healing (Driskell et al., 2013; Rinkevich et al., 2015). In small intestine, a rare mesenchymal cell population under the intestinal epithelium is essential for in vivo SC activity (Aoki et al., 2016). The mouse lung contains multiple mesenchymal populations with distinct developmental origins that are locally organized into multiple domains (Kumar et al., 2014). In most tissues, the molecular mechanisms and principles governing organ-level mesenchymal niche heterogeneity and functional diversity are still largely unknown.

A discrete population of specialized mesenchymal cells located at the base of the hair follicles (HFs), called the dermal papilla (DP), is essential for hair follicle stem cell (HFSC) activity (Driskell et al., 2011; Morgan, 2014). Mouse dorsal skin HFs undergo a cyclic growth phase (anagen), a regression phase (catagen), and a resting phase (telogen). The activation of HFSCs and the transition from telogen to anagen rely on signals emanating from DP, such as FGF, Wnt, BMP, and Shh (Greco et al., 2009; Kishimoto et al., 2000; Rendl et al., 2008; Woo et al., 2012). Freshly isolated DP or in vitro cultured low-passage DP cells can induce de novo HF formation when implanted elsewhere, with the hair type determined by the source of the DP (Jahoda et al., 1984; Oliver, 1970). This indicates that DP cells are intrinsically heterogeneous and contain hard-wired positional memory that in turn instructs the overlaying epithelial cell activity. However, the molecular basis for this DP heterogeneity and inductive ability are unknown. Here we show that expression of Hoxc genes encodes the organ-level positional identity of DP cells and functionally controls the regional HF regeneration pattern through Wnt signaling.
RESULTS

Regional Expression of Hoxc Genes in Dermis Correlates with the Regeneration Pattern of HFSCs

The skin is a complex landscape, with regions of HFs exhibiting different regenerative abilities (Wang et al., 2017) (Figure 1A). Whole-mount analysis using K14-H2BGFP transgenic mice to mark epithelial cells showed that after the morphogenesis, ear skin HFs remain in prolonged telogen; at the same time points, dorsal skin HFs exhibit multiple rounds of spontaneous anagen entries (Figures S1A and S1D). To power HF regeneration, HFSCs on dorsal skin undergo activation in early anagen, while ear skin HFSCs remain quiescent without detectable proliferation (Figure 1B). To stringently test whether ear skin HFSCs are dormant, we used K5-iTA, teto-H2BGFP mice for label-retaining experiments (Tumbar et al., 2004). 3-week-old mice were fed doxycycline for 4 weeks to turn off H2BGFP protein synthesis. To enrich epithelial cells showed that after the morphogenesis, ear skin HFSCs remain quiescent without detectable proliferation (Figure 1B). To stringently test whether ear skin HFSCs are dormant, we used K5-iTA, teto-H2BGFP mice for label-retaining experiments (Tumbar et al., 2004). 3-week-old mice were fed doxycycline for 4 weeks to turn off H2BGFP protein synthesis. To enrich epithelial cells, we separated epithelial cells (GFP+) and dermal cells (GFP−) from P50 telogen Wt/Koa ear skin, Wt/Wt ear skin, and Wt/Wt dorsal skin (Figure S2A). qPCR analysis of epithelial (Krt1, Krt10, and Krt14) and dermal (Pdgfra, Vimentin, S100a4, and Akip2) cell signature genes confirmed the specificity of the FACS-isolated cells (Figure S2B). P50 telogen samples were used to ensure that the expression profile comparisons reflect the differences between chosen skin regions and genetic backgrounds, instead of hair cycle differences. Because Koa is a dominant mutation, here we focused on $\geq 2 \times$ upregulated genes shared by regenerative skin regions (Wt/Koa ear skin and Wt/Wt dorsal skin) compared to the dormant skin region (Wt/Wt ear skin). There are 332 such genes in adult epithelial cells and 309 such genes in adult dermal cells. Then, based on what is known about the importance of breakpoints influencing the expression of neighboring genes after chromosomal inversion (Montavon et al., 2011), we narrowed our focus to the commonly upregulated genes that are within $\geq 1$ Mb of the Koa distal and proximal breakpoints on chromosome 15 (Figures 1C and 2C; Tables S2 and S3).

Using this screening strategy, we pinpointed Hoxc4–Hoxc6, Hoxc8, and Hoxc9 in dermal cells (Figure 1D) and Hoxc8 in epithelial cells (Figure 2C) that are commonly enriched in Wt/Koa ear skin and Wt/Wt dorsal skin compared to Wt/Wt ear skin. The Hoxc cluster contains 9 genes: Hoxc4,5,6,8,9,10, 11,12,13. Here our unbiased transcriptome screen revealed that Wt/Wt ear skin dermal cells lack expression of all Hoxc genes, while dorsal skin dermal cells express Hoxc4–Hoxc10. To determine whether the Hoxc cluster follows spatial collinearity in skin at the whole-organ level and whether Hoxa, Hoxb, and Hoxd clusters show similar expression patterns, we carried out RNA-seq analysis of P50 Wt/Wt ear, dorsal, and tail skin dermal cells, representing the anterior-posterior skin axis. Within skin dermal cells, only Hoxc cluster genes show clear spatial collinearity with robust expression levels (Figure 1E). Dorsal skin dermal cells express Hoxc4–Hoxc10, while tail skin dermal cells express Hoxc10–Hoxc13. The differential expression pattern of the Hoxc cluster genes was validated by qPCR using isolated skin dermal cells from different anterior-posterior regions (Figure 1F).

Because the dermal cells we used for RNA-seq and qPCR validation contain multiple dermal lineages, such as DP and dermal fibroblasts, the cell type-specific expression patterns of the enriched Hoxc genes were further determined using RNA in situ hybridization assays. Hoxc4, Hoxc6, and Hoxc8 are highly expressed in DP cells of Wt/Koa ear skin and Wt/Wt dorsal skin but are absent from Wt/Wt ear skin (Figures 2A and 2B). The expression of Hoxc genes in DP is region dependent, but not hair cycle dependent. In anagen and telogen dorsal skin, the expression of Hoxc4, Hoxc6, and Hoxc8 can be detected in DP using RNA in situ (Figure 2B). This result is confirmed by qPCR using FACS-isolated DP cells from dorsal skin at either anagen or telogen (Figure S2D). Hoxc4–Hoxc6, Hoxc8, and Hoxc9 are expressed by both telogen and anagen dorsal skin DP cells, albeit at slightly different levels (Figure 2C). However, even hair plucking-induced anagen entry in ear skin failed to induce Hoxc gene expression in ear skin DP (Figure S2E). This confirms the region-dependent expression, but not the hair cycle-dependent expression, of Hoxc genes in DP cells. The expression of Hoxc genes is not exclusively in DP cells. Dermal fibroblasts also express detectable Hoxc4, Hoxc6, and Hoxc8, as revealed by RNA in situ (Figures 2A and 2B). This was confirmed by qPCR.
Figure 1. Differential Expression of Hoxc Genes Is Uniquely Correlated with the Regional Regeneration Pattern of HFSCs

(A) Schematic diagram of the empirical scope used to compare HF growth patterns in different skin regions.

(B) Fluorescent whole-mount images of ear and dorsal skin from K14-H2BGFP mice at different postnatal time points. Ki67 staining indicates proliferating cells. Scale bars, 50 μm.

(C) Venn diagram displaying genes commonly upregulated in dermal cells of P50 telogen Wt/Wt dorsal skin and Wt/Koa ear skin compared to Wt/Wt ear skin based on RNA-seq analysis. Chromosome 15 inversion is highlighted with a red dashed line. Upregulated genes are represented as red dots on chromosome 15 and blue dots on the other chromosomes. Hoxc genes are the only commonly upregulated genes ± 1 Mb from both Koa breakpoints.

(D) qPCR of Hoxc genes in dermal cells of P50 telogen Wt/Wt dorsal skin, Wt/Koa ear skin, and Wt/Wt ear skin. Data reflect mean ± SD from 3 mice in 3 independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001.

(E) RNA-seq read data of Hox cluster genes in dermal cells of P50 Wt/Wt ear, dorsal, and tail skin.

(F) qPCR of Hoxc genes in dermal cells from P21 Wt/Wt ear, dorsal, and tail skin. Data reflect mean ± SD from 3 mice in 3 independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001.

See also Figures S1 and S2 and Tables S1 and S3.
using FACS-isolated DP and non-DP dermal cells from P50 telogen dorsal skin (Figure 2D). Akp2 was used as a DP-specific signature gene (Sennett et al., 2015), and none of the Hoxc genes show exclusive expression pattern in DP similar to Akp2.

By using the Koa mutant, we narrowed our focus to Hoxc genes that uniquely demonstrate a spatial collinear expression pattern in skin dermal cells at the whole-organ level. In particular, their expression levels in DP correlate with the regenerative activity of HFSCs in different skin regions (Figure 2E).

**Upregulated Hoxc Genes Lead to Ectopic Regional HF Regeneration**

To investigate whether the observed correlative Hoxc expression pattern plays a functional role in HF regenerative activity, we took advantage of mice with only one copy of the Hoxc cluster still exhibiting the wild-type phenotype (Suemori and Noguchi, 2000). We reasoned that if deleting the Hoxc cluster from the Koa allele could convert the Wt/Koa mice back to the wild-type phenotype, then the Hoxc cluster is responsible for the Koa phenotype. We used CRISPR/Cas9 to knock out the Hoxc cluster from the Koa allele to generate Wt/Koa_Hoxc KO mice. Because Hoxc13 and Hoxc4 reside at either end of the Hoxc cluster, guide RNAs (gRNAs) were designed to target Hoxc13 exon1 and Hoxc4 exon1, respectively (Figures 3A and S3A). The resulting successful deletion of the genomic sequence between Hoxc13 and Hoxc4 was confirmed by genotyping and sequencing (Figures 3D and S3B).

The Wt/Koa_Hoxc KO mice lost the Koa phenotype and appeared to be like wild-type (Figure 3B). Besides losing the obvious elongated ear skin hair shaft (Figure 3K), the Wt/Koa_Hoxc KO ear skin HFs stay in telogen at P39 after morphogenesis, just like the Wt/Wt ear skin HFs (Figures 3F and 3G). qPCR using FACS-isolated dermal cells from P50 telogen ear skin confirmed the downregulated expression of Hoxc4–Hoxc6, Hoxc8, and Hoxc9 in Wt/Koa_Hoxc KO mice compared to Wt/Koa mice (Figure 3C). This conclusively shows the Hoxc cluster bestows the Wt/Koa phenotype.

However, the Hoxc cluster we deleted contains 9 Hoxc genes, 2 microRNAs, lncRNA (Hotair), and other potential regulatory elements such as enhancers (Figure S3C). It is thus possible that loss of elements other than Hoxc genes confers the Wt/Koa phenotype. In our examination of numerous founder lines, we were intrigued to find that one founder line with Hoxc cluster deletion on the Koa allele still exhibited a partial Koa phenotype.
phenotype, instead of the expected wild-type phenotype. To repair Cas9-induced double-strand DNA breaks, the non-homologous end joining generates random sequences at the junction between the Hoxc13 and the Hoxc4 exons in each of the knockout lines. Sequencing revealed that compared to Wt/ Koa_Hoxc KO, this line with the unexpected phenotype had 8 fewer nucleotides at the junction between Hoxc13 exon1 and Hoxc4 exon1, thus creating a de novo Hoxc fusion gene consisting of the C-terminal DNA binding domain of the Hoxc4 gene and a chimeric N-terminal co-factor binding domain from both Hoxc4 and Hoxc13 (Figure 3D). We therefore named this line Wt/Koa_ Hoxc KO, Fusion (Figures 3D and S3D). The 966 bp mRNA transcript of the new Hoxc fusion gene was detected in Wt/Koa_ Hoxc KO, Fusion mice (Figures 3E and S3E). Hair cycle analysis revealed that HFs of Wt/Wt and Wt/Koa_Hoxc KO ear skin were dormant, while HFs of Wt/Koa and Wt/Koa_Hoxc KO, Fusion ear skin were undergoing regeneration, even though the Wt/Koa_ Hoxc KO, Fusion ear skin HFs cycled at a lower percentage compared to Wt/Koa ear skin (Figures 3F and 3G).

The de novo Hoxc fusion gene of the Wt/Koa_Hoxc KO, Fusion mouse line consists of sequences mostly coming from the Hoxc4 gene (Figure 3D). Given that Hoxc4, but not Hoxc13, was among the candidate genes identified in our transcriptome screen, it is thus possible that the increased expression of Hoxc4 alone is responsible for the Koa phenotype. Therefore, we used CRISPR/Cas9 to generate Wt/Koa_Hoxc4 KO mice. Two gRNAs targeting exon1 and exon2 of Hoxc4 were used (Figure S3F). The resulting successful deletion of the genomic sequence encoding Hoxc4 was confirmed by genotyping and sequencing (Figures S3G and S3H). Unexpectedly, Wt/Koa_ Hoxc4 KO mice showed no significant phenotypic difference from Wt/Koa mice (Figures 3J and 3K). The elongated ear skin hair shaft and spontaneous HF regeneration phenomenon persisted in the absence of Hoxc4 in the Koa mice. Even though there was no Hoxc4 expressed in Wt/Koa_Hoxc4 KO mice ear skin dermal cells, there was compensatory upregulation of other Hoxc genes (Hoxc5, Hoxc6, Hoxc8, and Hoxc9) (Figure 3H). This result suggests that multiple Hoxc genes function redundantly to regulate regional HF regeneration.

Among all Hoxc genes, our RNA-seq data indicated that Hoxc8 shows a much higher expression level compared to the others (Figure 1E; Tables S2 and S3). To investigate whether Hoxc8 has a dominant function among all expressed Hoxc genes, we used CRISPR/Cas9 to generate Wt/Koa_Hoxc8 KO mice. Two gRNAs targeting 5’ and 3’ flanking regions of Hoxc8 exons were used (Figure S3I). The resulting successful deletion of the genomic sequence encoding Hoxc8 was confirmed by genotyping and sequencing (Figures S3J and S3K). The loss of Hoxc8 expression in Wt/Koa_Hoxc8 KO mice still leads to compensatory upregulation of other Hoxc genes (Hoxc4–Hoxc6 and Hoxc9) in ear skin dermal cells (Figure 3I). However, Wt/Koa_Hoxc8 KO mice showed a slight but significant reduction in ear skin hair shaft length and a decreased percentage of HFs capable of anagen re-entry compared to Wt/Koa (Figures 3J and 3K). At P39, about 50% of the HFs in the Wt/Koa ear skin re-entered anagen, while less than 20% of the HFs in the Wt/Koa_Hoxc8 KO ear skin did (Figure 3J).

When all mutant strains were combined, there was a clear dosage effect of the Hoxc genes: $Hoxc(4 + 5 + 6 + 8 + 9) > Hoxc(5 + 6 + 8 + 9) > Hoxc(4 + 5 + 6 + 9) > Hoxc_Fusion > No Hoxc$. The extent of the hair shaft length and the percentage of HFs undergoing regeneration are the direct results of the combined number and strength of Hoxc genes expressed regionally.

**Hoxc Genes Function Specifically in DP to Control HF Regeneration**

Our knockout strategy offered conclusive results regarding the function of Hoxc genes in skin, but it didn’t address the question of their cell type specificity. To determine in which cell type Hoxc genes exert their functions, and to unequivocally test whether increased expression of Hoxc genes leads to ectopic activation of ear skin HFs, we used lentivirus-mediated overexpression of individual Hoxc genes in ear skin.

Genetic manipulation of dermal cells has traditionally been challenging; in particular, overexpression in DP cells to test gene function in vivo has not been previously reported. We discovered that because ear skin HF morphogenesis only initiates after birth, it is possible to inject lentivirus-containing solution using an insulin syringe into P1–P2 ear skin dermis, and the resulting infected dermal fibroblast will continue development, with some forming DP (Figure S4A). First, we injected lentivirus expressing only H2BRFP into the dermis of P1 Wt/WT ear skin (Figure 4A). At P21, a mixture of dermal cells within a large area was infected as marked by H2BRFP expression. These included DP cells and dermal fibroblasts (Figure 4B). Both the DP-infected and the DP-uninfected HFs remain dormant after morphogenesis, indicating the experimental procedure does not perturb the normal regional HF regeneration pattern in ear skin (Figure 4C). Then we injected lentivirus expressing Hoxc4, Hoxc6, and Hoxc8 cDNA individually into ear skin dermis. The expression of corresponding Hoxc genes was validated by qPCR using lentivirus-infected fibroblasts in vitro (Figure S4B). Hoxc gene and H2BRFP gene are linked by the 2A self-cleaving peptide sequence, so the expression of H2BRFP can be used to mark cells expressing the indicated Hoxc protein in vivo. Overexpression of one of the three Hoxc genes leads to an obvious prolonged first anagen at P21 and spontaneous regeneration at P28 (Figures 4A–4D and S4C). The result is even visible from the elongated hair shaft produced by HFs within the infected ear skin region (Figure 4E). Spontaneous anagen re-entry was confirmed by Ki67 staining in the secondary hair germ of ear skin HFs and old bulge attached to the newly generated anagen follicle (Figure 4D).

Only HFs with lentivirus-infected DPs exhibited phenotype, while neighboring HFs surrounded by infected dermal fibroblasts were dormant. Quantitatively, ~40% to 90% of HFs with DP cells expressing Hoxc4/6/8 stayed in anagen at P21, while 0% of the neighboring HFs with no DP cells expressing Hoxc4/6/8 stayed in anagen (Figure 4C). This serves as direct evidence that Hoxc genes function specifically in DP cells to drive HF regeneration, and it implies that some yet unknown DP-specific co-factors or DP unique epigenetic modifications are necessary to function together with Hoxc genes to activate HF regeneration.

We next set out to determine whether Hoxc expressed in the epithelial cells could lead to ectopic HF regeneration. To do so, we used an *in utero* lentivirus injection system to overexpress Hoxc4/6/8 in epithelial cells specifically (Figures 4F and S4D) (Beronja et al., 2010). Robust and specific expression of
Figure 3. Increased Expression of Hoxc Genes Leads to Ectopic HFSC Regeneration in Koa Ear Skin

(A) Schematic diagram of CRISPR/Cas9-mediated deletion of the Hoxc cluster on the Koa allele. Hoxc13-gRNA and Hoxc4-gRNA target Hoxc13 exon1 and Hoxc4 exon1, respectively. Koa1, Koa2, and Koa3 are primers used for Koa allele genotyping. Del_F and Del_R are primers used for Hoxc cluster deletion identification.

(B) Representative photo of P40 Wt/Wt, Wt/Koa, and Wt/Koa_Hoxc KO mice.

(C) qPCR of P50 (Telogen) Ear Skin Dermal Cells

(D) Hoxc13 and Hoxc4

(E) L1 and L2

(F) % Total Hair Follicle

(G) P39 Ear Skin

(H) qPCR of Hoxc genes in P50 (Telogen) dermal cells

(I) qPCR of Hoxc genes in P50 (Telogen) dermal cells

(J) % Total Hair Follicle

(K) P39 Ear Skin

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**Regional Expression of the Hoxc Genes Is Regulated by an Antagonistic Switch between Repression and Activation of Epigenetic Modification**

To understand how the regional expression patterns of Hoxc genes are achieved, we first used chromatin immunoprecipitation sequencing (ChiP-seq) to look at acetylation of 27th amino acid in histone H3 (H3K27ac), a marker for active enhancer and transcriptional activity (Creighton et al., 2010). In freshly isolated dermal cells from P50 telogen Wt/Wt ear skin (Figure S2A), no enrichment of H3K27ac was detected at the Hoxc cluster, consistent with lack of expression; in contrast, robust peaks of this marker are observed in the 3’ region of the Hoxc cluster in dorsal skin dermal cells (Figures 5A and S5A). Second, we looked at H3K27 trimethylation (H3K27me3), a marker for polycomb-dependent gene repression, which antagonizes H3K27ac (Tie et al., 2009). Concomitantly, we found that H3K27me3 spreads across the entire Hoxc cluster in adult ear skin dermal cells but was restricted to the 5’ region of the Hoxc cluster in adult dorsal skin dermal cells (Figures 5A and S5A). These heavy decoration of H3K27ac accompanied by the absence of H3K27me3 spanning Hoxc4–Hoxc10 observed in adult dorsal skin dermal cells explains the selective expression of Hoxc4–Hoxc6 and Hoxc8–Hoxc10 in dorsal skin from our RNA-seq analysis. It also indicates the possibility of a chromatin barrier inside the Hoxc cluster demarcating the activation and the repression domains in adult dorsal skin dermal cells. We tested this hypothesis by analyzing the ChiP-seq profile of CCCTC binding factor (CTCF), which is enriched at barriers between topologically associated domains (TADs) (Narendran et al., 2015). In adult dorsal skin dermal cells, we found three occupied CTCF binding sites inside the Hoxc cluster (Figure 5A). The rostral CTCF binding site marks the discontinuity point of H3K27me3 modification at the intergenic region between Hoxc11 and Hoxc10 in dorsal skin dermal cells. This indicates the CTCF binding event within the Hoxc cluster might forge a topological barrier that can insulate the activation region from the repression region and therefore lead to the region-specific expression of the Hoxc cluster genes.

Because the Hoxc cluster is not directly disrupted by the distal breakpoint in Koa inversion, it is puzzling how the Koa mutant acquires ectopic expression of Hoxc genes. With the assistance of previous published HiC data (data from the Dr. Peter Fraser Laboratory and Dixon et al., 2012), we noticed that the proximal and distal Koa breakpoints were located in two TADs, respectively, which are stable across cell types (Figure S5C). We also found that our CTCF ChiP-seq analysis using adult Wt/Wt ear skin dermal cells shows similar CTCF occupancy at the boundaries of these two TAD domains (data from transcription factor binding sites by ChiP-seq from the Encyclopedia of DNA Elements/Ludwig Cancer Research [LICR TFBS]) (Figure S5C). In Wt/Wt ear skin dermal cells, the TAD domain containing the proximal breakpoint (proximal TAD) is enriched with H3K27ac but lacks H3K27me3, illustrating that proximal TAD is an activation regulatory landscape; in contrast, the TAD domain containing the distal breakpoint (distal TAD) is enriched with robust H3K27me3 coverage but lacks H3K27ac, reflecting the repressed expression status of the Hoxc cluster within this TAD (Figure 5B). After chromatin inversion, distal TAD in Wt/Koa ear skin dermal cells shows distinct H3K27ac enrichment in the Hoxc cluster (Figure 5B). Because the Wt/Koa ear skin dermal cells contain both WT and Koa alleles, H3K27me3 is not significantly decreased compared to cells from Wt/Wt skin (Figure S5B). These data suggest that the Koa inversion disrupted proximal and distal TADs near breakpoints and brought the Hoxc cluster close to the activation regulatory landscape in proximal TAD.

To directly test whether there is ectopic interaction between the Hoxc cluster and the active domain in proximal TAD, we used circularized chromosome conformation capture (4C) with Hoxc4 as bait. In Wt/Koa ear skin dermal cells, there are clear ectopic interactions of Hoxc4 with the remaining active regulatory landscape in proximal TAD; however, all interactions of Hoxc4 remained inside distal TAD with the repressive domain in Wt/Wt ear skin (Figure 5B). Using Hoxc4 as bait, the normalized interaction score at 0.5 M downstream of the distal breakpoint is not significantly different between Wt/Wt and Wt/Koa ear skin dermal cells, but at 0.5 M upstream of the proximal breakpoint, there is a significant difference, indicating ectopic interactions in the Wt/Koa mutant that are absent from the Wt/Wt cells (Figure 5C). Based on these results, we can conclude that the chromatin inversion disrupted two TADs and enabled ectopic interaction of the Hoxc cluster with an active regulatory
Figure 4. Hoxc Genes Function Specifically in DP to Control HF Regeneration

(A) Diagram displaying workflow of lentivirus-mediated Hoxc overexpression in ear skin dermal cells.

(B) Fluorescent whole-mount images of dermal overexpression of either Hoxc8-2A-H2BRFP (left) or H2BRFP alone (right) in ear skin of K14-H2BGFP mice. DPs are marked by alkaline phosphatase (AP) staining, shown as black dots in bright-field images and indicated with arrows in fluorescent images.

(C) Percentage of anagen HFs at P21 upon dermal overexpression of Hoxc4, Hoxc6, and Hoxc8. HFs without DP infected by lentivirus are quantified and shown separately with corresponding DP-infected groups. All data reflect mean ± SD from 3 mice in 3 independent experiments. *p < 0.05; **p < 0.01.

(D and E) Fluorescent whole-mount images (D) and surface view image (E) of Hoxc8-2A-H2BRFP overexpressed in ear skin dermis of K14-H2BGFP mice. Anagen re-entry at P28 is indicated by Ki67 staining (in white). Old bulge (marked as Bu) attached to newly regenerated anagen follicle is marked by the arrowhead. HFs with DP infected by lentivirus marked by an H2BRFP signal are highlighted with yellow dashed lines; HFs without DP infected by lentivirus are marked in white dashed lines. Elongated hair shafts in the dermis-infected region are marked by the arrow in (E).

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landscape near the proximal breakpoint and that this alters the expression of Hoxc genes in the Koa mutant.

To functionally test whether the polycomb-dependent gene repression controls the regional expression of the Hoxc cluster in Wt/Wt skin, we analyzed Bmi1 knockout (KO) mice (Molofsky et al., 2003). Bmi1 is a component of the PRC1 complex (van der Lugt et al., 1996). H3K27me3 is believed to play a key role in regulating PRC1-mediated repression (Simon and Kingston, 2009). Ear skin hair shafts on Bmi1 KO mice were evidently longer compared to those on wild-type mice, a phenotype similar to Hoxc gene overexpression in ear skin (Figure 5D). The mRNA levels of Hoxc4–Hoxc9 are significantly elevated in ear skin dermal cells upon Bmi1 deletion (Figure 5E). We also detected elevated levels of Hoxc11 and Hoxc12 in dorsal skin dermal cells upon Bmi1 deletion (Figures S5D and S5E). However, because there are already multiple Hoxc genes expressed in Wt/Wt dorsal and tail skin dermis, there is no obvious HF growth phenotype in Bmi1 KO dorsal and tail skin (Figure S5F). Altogether, this confirms that polycomb-dependent gene repression controls the regional expression pattern of the Hoxc cluster, which in turn regulates the HF regeneration landscape.

Niche-Expressed Hoxc Genes Control HFSC Activation through Wnt Signaling
To gain insights into how Hoxc genes expressed in DP cells can activate epithelial HFSCs, we performed RNA-seq analysis of dermal cells from P50 telogen skin and Wt/Wt ear skin (Table S4). Bioinformatics analysis using the functional gene annotation tool revealed Wnt signaling pathway genes prominently enriched among the 216 genes changed by R23 in dermal cells between Wt/Wt dorsal skin and Wt/Wt ear skin (Figure 6A). Next, we used Axin2-CreER; Rosa-stop-mTmG mice to examine whether Wnt signaling activity is different in telogen dorsal versus ear skin HFSCs. Axin2 is a canonical Wnt signaling target gene, and its expression is often used to indicate the Wnt signaling level (Lim et al., 2016). In P21 dorsal skin

Figure 5. Regional Expression of the Hoxc Genes Is Regulated by an Antagonistic Switch between Repression and Activation of Epigenetic Modification
(A) Normalized ChIP-seq read densities for the indicated proteins and histone modifications. FACS-isolated dermal cells from P50 telogen skin are used for experiments. Red boxes highlight Hoxc genes. The gray dashed line highlights the barrier of antagonistic epigenetic modification. Red arrowheads point to the CTCF binding sites near the barrier.
(B) Comparisons of 3D conformation, normalized ChIP-seq read densities, and 4C-interaction profiles of regions near Koa breakpoints. FACS-isolated dermal cells from P50 telogen skin are used for experiments. HiC map of the locus from mouse embryonic stem cell (mESC) cells (red contact maps, image generated with the 3D genome browser, http://promoter.bx.psu.edu/hi-c/view.php) is shown. Koa proximal and distal breakpoints (indicated by the gray dashed line) are located in two highlighted TAD domains (transparent blue). Red boxes represent Hoxc genes. The green arrowhead indicates the Hoxc4 viewpoint used in 4C experiments. The red arrowhead indicates ectopic interaction with the activation regulatory landscape.
(C) Statistical analysis of 4C interaction from the Hoxc4 viewpoint to the regions outside of both proximal and distal Koa breakpoints. ***p < 0.001.
(D) Representative surface view images of P35 ear skin in WT and Bmi1 KO. Scale bars, 1 mm.
(E) qPCR of Hoxc genes in dermal cells of P50 telogen Wt ear skin and Bmi1 KO ear skin. Data reflect mean ± SD from 3 mice in 3 independent experiments. **p < 0.01; ***p < 0.001. See also Figure S5.

(F) Diagram displaying workflow of lentivirus-mediated Hoxc overexpression in ear skin epidermal cells.
(G and H) Fluorescent whole-mount image (G) and quantifications (H) of anagen HF’s upon overexpression of Hoxc4, Hoxc6, and Hoxc8 in ear skin epidermis of K14-H2BGFp mice. Scale bars, 100 μm. See also Figure S4.
telogen HFs, HFSCs on one side of the bulge are characteristically labeled Axin2+, but there are few Axin2+ HFSCs in ear skin telogen HFs of the same mouse (Figure 6B). Both the first telogen at P21 and the second telogen at P50 were used for this analysis. The same statistically significant differences in Wnt signaling activity between dorsal and ear skin HFSCs were observed (Figures 6B and S6A). The telogen HFs used for analysis were confirmed by lack of Ki67 staining (Figure 6B). Wnt signaling is required for HFSCs activation and HF regeneration (Huelsken et al., 2001; Lien et al., 2014). The lack of Wnt signaling...
in Wt/Wt ear skin HFSCs confirms our RNA-seq result and is consistent with the dormant state of the ear skin HFcs.

To determine whether the Hoxc genes function upstream of Wnt signaling, we used Axin2-CreER; Rosa-stop-mTmG mice to compare the Wnt signaling level in telogen ear skin of Wt/Wt, Wt/Koa, and Wt/Koa_Hoxc KO mice. Compared to less than 10% in Wt/Wt ear skin labeled Axin2+, on average ~60% of HFSCs in Wt/Koa ear skin were labeled Axin2+ (Figure 6C). The Axin2+ HFSCs in the Wt/Koa ear skin do not locate to one side of the bulge, which is different from the pattern observed in Wt/Wt dorsal skin. The endogenous Axin2 mRNA level is also higher in FACS-isolated HFSCs from telogen Wt/Koa ear skin compared to telogen Wt/Wt ear skin (Figure S6B). More strikingly, up to 90% of telogen HFcs in Wt/Koa ear skin have Axin2+ DP, compared to less than 20% in Wt/Wt ear skin (Figure 6C). Ki67 staining was used to demonstrate that telogen HFcs were used for quantification at the indicated stages. Although the Axin2 mRNA level is not different in FACS-isolated dermal cells from Wt/Koa ear skin compared to those from Wt/Wt ear skin, we think this is because DP cells only constitute a small fraction of the collected dermal cells (Figure S6B). Next, we used Wt/Koa_Hoxc KO to determine whether the ectopic activation of Wnt signaling in the Wnt signaling in the Koa allele resulted in complete loss of enhanced Wnt signaling in ear skin HFs and DPcs (Figure 6C). The percentage of both Axin2+ HFSCs and DPcs in Wt/Koa_Hoxc KO ear skin returned to the Wt/Wt level. These results show that Hoxc genes function upstream and can enhance the Wnt signaling level in both DP niche cells and HFSCs.

To gain insights into how Hoxc genes could regulate Wnt signaling, we then performed RNA-seq analysis of dermal cells from P50 telogen Wt/Koa ear skin, Wt/Wt ear skin, and Wt/Wt dorsal skin. Our in vivo overexpression results clearly indicate Hoxc genes function specifically in DPcs to drive HF regeneration. First, we selected Wnt signaling genes that are preferentially expressed in DPcs based on data available on Hair-Gel (Figure S6C) (Sennett et al., 2015). Among these genes, the Wnt amplifier Rspo2 is significantly upregulated in dermal cells of Wt/Koa ear skin and Wt/Wt dorsal skin compared to those of Wt/Wt ear skin, while the Wnt inhibitor Sfrp2 is significantly downregulated (Figure S6D). The RNA-seq results were validated by qPCR using FACS-isolated dermal cells from P50 telogen Wt/Koa ear skin, Wt/Wt ear skin, and Wt/Wt dorsal skin (Figure 6D). The R-spondin (Rspo) family of four secreted proteins, Rspo1–Rspo4, amplifies target cell sensitivity to Wnt ligands by increasing Wnt receptor levels (Kazanskaya et al., 2004). Intradermal injection of recombinant Rspo2 can extend anagen, resulting in elongated hair shafts (Smith et al., 2016) similar to what we observed by overexpressing Hoxc genes in DP. It suggests the differentially expressed Rspo2 could mediate the influence of Hoxc genes on Wnt signaling level. If Hoxc genes regulate region-specific activation of HFSCs through Wnt signaling, then we should be able to bypass Hoxc and directly activate ear skin HFSCs by increasing Wnt signaling. We used Lgr5-CreER; β-catenin-Exon3 mice to activate Wnt signaling in both HFSCs and hair germ cells in ear skin HFcs (Figures 6E and S6E). This activation resulted in proliferation of almost all HFSCs in mutant ear skin and led to the regeneration of HF with new hair shafts (Figure 6E). Our genetic evidence shows a direct causal relationship between the level of Hoxc genes and the Wnt signaling in ear skin, and it placed Hoxc genes upstream of Wnt signal activity in DP cells and HFSCs. Elucidating the molecular mechanism of how the Hoxc gene could be involved in regulating the levels of Rspo2 and Sfrp2 relies on identifying the yet-unknown DP-specific co-factor or DP unique epigenetic landscape that is required for Hoxc gene function in vivo.

To investigate whether Hoxc genes are necessary for Wnt signaling activity in skin regions where they normally express (dorsal and tail skin), we used our intradermal injection of lentivirus expressing short hairpin RNAs (shRNAs) to circumvent embryonic lethality caused by straight KO of the entire Hoxc cluster (Suemori and Noguchi, 2000). Intradermal injection of lentivirus failed to infect DP cells in dorsal skin (Figure S7A). Because intradermal injection of lentivirus can target some DP cells in tail skin (Figures S7C and S7D), we injected a mixture of shRNAs target all 4 Hoxc genes expressed in tail skin (Figures S7B and S7C). The in vivo knockdown efficiencies within infected dermal cells were about 50% downregulation for all 4 Hoxc genes (Figures S7D and S7E). The in vitro knockdown efficiency of each shRNA was more than 90% (Figure S7B). The low knockdown efficiency in vivo could result from a mixture of cells with a different number of shRNAs infected instead of all 4 different shRNAs. Whole-mount analysis based on Ki67 staining to mark anagen yielded no hair cycle differences between DP-infected HFs and nearby DP-uninfected HFs (Figures S7F and S7G). This is consistent with the functional redundancy of Hoxc genes, considering their insufficient knockdown in vivo.

To summarize, by using a chromosome inversion mutant that disrupted the regional HF growth pattern, we revealed the molecular coding system that demarcates different skin regions (Figure 7). The cell intrinsic and epigenetically maintained skin Hoxc code gives rise to mesenchymal niche cell heterogeneity and plasticity. Perturbations of the code result in mesenchymal niche conversion and a reshaped SC regeneration landscape, demonstrating the previously unknown remarkable plasticity of mesenchymal niche cells.

**DISCUSSION**

In Waddington’s diagram, if a rolling ball represents a stem or progenitor cell, then the hilly background represents the niche environment. Yamanaka’s paradigm shifting work showed us that the ball could change course by itself but that the background has always remained static. Here we demonstrate that in contrast to traditional view, the landscape is composed of mobile modules controlled by underlying gears and pulleys. Consequently, the shape of the landscape can be changed precisely. In our case, just one transcription factor is enough to change the HF regeneration pattern by modulating the niche cells. In most epithelium tissues undergoing regeneration, heterogeneous mesenchymal cells are often locally organized into multiple domains, so it’s likely that the same principle uncovered here can be generally applied to other tissues. Many other features of skin demonstrate regional regeneration patterns, such as different epidermis thicknesses, pigmentation patterns, and sweat gland distributions (Chang, 2009;...
Chuong et al., 2013). It will be of interest to determine whether the skin Hoxc code unearthed here can also be applied to these diverse tissue growth patterns and the evolutionary change of integuments among different species. A prerequisite to investigate these questions is a deeper understanding of the molecular mechanisms controlling the Hoxc gene spatial collinear expression patterns. To do this, additional analysis of the epigenetic modifications modulated by PRC1/PRC2 complexes, as well as additional functional studies regarding the formation of chromatin barriers, will be needed in future studies.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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Figure 7. Schematic Diagram of Epigenetically Maintained Hoxc Genes Giving Rise to Mesenchymal Niche Cell Heterogeneity and Plasticity

Hoxc genes with a region-specific expression pattern encode the positional identity of DP niche cells and control HFSC regeneration by modulating Wnt signaling. In Wt/Wt dorsal skin, Hoxc4–Hoxc6 and Hoxc8–Hoxc10 are highly expressed in dermal fibroblasts and DP cells. In Wt/Wt ear skin, all Hoxc genes are repressed. In Wt/Koa ear skin, the Hoxc cluster interacts with an epigenetic active region outside the Koa proximal breakpoint upon chromatin inversion, which results in ectopic expression of Hoxc4–Hoxc6, Hoxc8, and Hoxc9 in ear skin dermis. This results in an enhanced Wnt signaling level in Wt/Koa ear skin and consequent HF regeneration.
SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and four tables and can be found with this article online at https://doi.org/10.1016/j.stem.2018.07.016.

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AUTHOR CONTRIBUTIONS

T.C. and Z.Y. conceived the project, designed the experiments, and wrote the manuscript. Z.Y. performed most experiments. F.W. generated CRISPR/Cas9-mediated gene-edited mice. T.C. conducted the in vivo injection. K.J. performed the RNA in situ experiment. N.O. performed tail skin lentivirus infection. Z.X. performed the Wnt pathway-related experiments. H.H. and Z.Y. conducted bioinformatics analysis. D.C. and D.L. contributed to hair cycle analysis, plasmid construct, and in vitro validation. Z.L. contributed to in vitro experiments. X.L., H.L., and R.C. provided the Bmi1 KO mice. R.D. contributed to Bmi1 KO mice analysis. T.K. provided the Koa mutant mice. Y.Y., B.Z., W.X., and Z.D. provided protocols, reagents, and scientific advice.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES


### STAR METHODS

#### KEY RESOURCES TABLE

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Ting Chen (chenting@nibs.ac.cn).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Biological Sciences. All of the animals were handled according to the guidelines of the Chinese law regulating the usage of experimental animals and the protocols (M0020) approved by the Committee on the Ethics of Animal Experiments of the National Institute of Biological Sciences, Beijing. Mice were group housed (up to 5 animals per cage) on a 12:12 hour light-dark cycle, with free access to food and water in individually ventilated specific pathogen free (SPF) cages. All mice used were healthy and were not involved in any previous procedures nor drug treatment unless indicated otherwise. For FACS-related experiment (including RNA-seq, ChIP-seq, 4C-seq, and qPCR), female mice were used to reduce enzyme digestion time and increase cell viability and yield, since female mice tend to have thinner dermis tissue compared to male mice. For other experiment, mice from both sexes were randomly allocated to experimental groups. Our results do not suggest any gender influence on the study outcomes. The age of mice used in this study were indicated in figure and figure legends. All surgery was performed under combination anesthesia involving isoflurane and remifentanly and every effort was made to minimize discomfort and suffering.
K14-H2BGFP mice were kindly provided by Dr. Elaine Fuchs. Koa (RRID: MGI: 3815543) mice were kindly provided by Dr. Tetsuo Kunieda. The K5-tTA (Tumbar et al., 2004), Lef1-RFP (Rendl et al., 2005), β-catenin-Exon3 (Harada et al., 1999), and Bmi1 KO (Lu et al., 2017) mice have all been described previously. TRE-mCMV-H2BGFP (teto-H2BGFP) mice (Stock: 005104), Axin2-CreER (Stock: 018867), Lgr5-GFP-CreER (Stock: 008875), Ai14 mice (Stock: 007914) and Rosa-stop-mTmG mice (Stock: 007576) are from Jackson Laboratories. K14-H2BGFP, K5-tTA, Lef1-RFP, and TRE-mCMV-H2BGFP mice were CD-1 outbred strains. Koa mice in this study were on a hybrid strain of C3H/HeH and CD-1. β-Catenin-Exon3 mice in this study were on a hybrid strain with CD-1. Bmi1 KO mice were on a hybrid strain of C57/BL6 and CD-1.

Cell Culture
All the cell lines were cultured at 37°C in a cell incubator with 5% CO₂. 293FT cells (Thermo Fisher Scientific, Cat #R70007) used for virus package were maintained in DMEM ( Gibco) medium supplemented with 10% (v/v) FBS ( Gibco), 1% (v/v) Pen-strep/L-glut (Lonza), 1% (v/v) 100mM Sodium Pyruvate (Lonza), 1% (v/v) 7.5% sodium bicarbonate (Lonza), and 500 μg/mL G418 (Lonza). Primary mouse tail skin fibroblasts were maintained in DMEM (GIBCO) medium supplemented with 10% (v/v) FBS (GIBCO), 1% (v/v) Pen-strep/L-glut (Lonza), 1% (v/v) 100mM Sodium Pyruvate (Lonza), and 1% (v/v) 7.5% sodium bicarbonate (Lonza).

METHOD DETAILS
Animal Treatments
For label-retaining experiments, 1mg/ml doxycycline water was administered to K5-tTA; tet-o-H2BGFP mice from P21 to P50. For Cre-ER activation, Tamoxifen was dissolved in sunflower oil/10% ethanol. Axin2-CreER; mTmG mice received a single intraperitoneal injection of 50 μl 10mg/ml Tamoxifen solution. For plucking experiment, hair shaft was plucked at P22 on ear skin. 8 days later on P30, ear skin sample was used for RNA in situ hybridization.

Immunofluorescence Staining
For section staining, tissues were embedded in OCT compound, frozen, cryosectioned (20-30 μm), and fixed for 10 min in 4% Paraformaldehyde in PBS. Sections were permeabilized for 10 min in 0.5% Triton (PBST) and blocked for 1 h in blocking buffer (2% normal donkey serum, 1% BSA, and 0.3% Triton in PBS). For whole mount staining, tissues were fixed for 30 min in 4% Paraformaldehyde in PBS and permeabilized for 10 min in 0.5% Triton (PBST) and blocked for 1 h in blocking buffer. The following antibodies were used: Ki67 (ebioscience, 14-5698-82, RRID: AB_10854564), anti-β-catenin (Sigma, C2206, RRID: AB_476831), and anti-K14 (Chen Lab). Tissue and section samples were imaged on a Nikon A1-R confocal microscope. Microscopy data was analyzed using Imaris software with the 3D visualization module (Bitplane). RBG images were assembled in Adobe Photoshop CS3 and panels were labeled with Adobe Illustrator CS6.

Fluorescence-activated Cell Sorting (FACS)
For the label-retaining experiment using K5-tTA; tet-o-H2BGFP mice, ear skin was split into two halves and placed dermis side down to float on a 0.25% trypsin solution (GIBCO, 25300-054) at 37°C for 20 min. Separately, dorsal skin was cut into 6 to 9 pieces and was placed dermis side down to float on a 0.25% trypsin solution at 37°C for 30 min. Epithelial cell suspensions were obtained by scraping the epidermis side gently with a scalpel. The cells were then filtered with strainers (70 μm followed by 40 μm). Cells were then stained for 30 mins with Alex647-CD34 antibody (ebioscience, 50-0341, RRID: AB_10609352) and CD49f-PE (ebioscience, 12-0495-553079, RRID: AB_394609), and biotin-CD117 antibody (ebioscience, 13-1171-82, RRID: AB_466569), and then washed. Cells were then stained for 30 mins with streptavidin-APC (ebioscience, 17-4317-82, RRID: AB_2732902) and then washed. DAPI staining was used to identify and exclude dead cells. Cell analysis and isolations was performed on a BD Aria III cell sorter controlled by FACSDiva software (BD bioscience). FACS analyses were performed using LSII FACS Analyzer (BD bioscience) and then analyzed with FlowJo software (FlowJo LLC).
RNA Isolation and Real-time PCR

Total RNA was isolated from FACS-purified cells lysed with Trizol (Life Technologies) followed by extraction using a direct-Zol RNA mini prep kit (Zymo research). For cDNA synthesis, equal amounts of RNA were added to reverse-transcriptase reaction mix (Vazyme, R222-01). Expression levels were normalized to the expression of PPIB. Real-time PCR was conducted using a CFX96TM Real-Time system (Bio-RAD) with Power SYBRR Green PCR Master Mix (Life Technologies). All primer pairs were designed for the same cycling conditions: 10 min at 95°C for initial denaturing, 40 cycles of 10 s at 95°C for denaturing, 30 s at 61°C for annealing, and 10 s at 65°C for extension. The primers were designed to produce a product spanning exon-intron boundaries in each of the target genes; the sequences are shown in Table S1.

RNA in Situ Hybridization

Tissues were harvested and fixed in 10% neutral buffered formalin for 16h, and then dehydrated and embedded in paraffin. Tissue sections were cut at 5-μm thickness, air-dried at room temperature, and processed for RNA in situ detection by using the RNAscope 2.0 FFPE Reagent Kit (RED and BROWN) according to the manufacturer’s instructions (Advanced Cell Diagnostics). RNAscope probes used were as follows: Hoxc4 (NM_013553.2, region 2-1722), Hoxc6 (NM_010465.2, region 2-1565), Hoxc8 (NM_010466.2, region 223-1729), ppib (NM_011149.2, region 98-856).

Generation of Hoxc Deletions Using CRISPER/Cas9

The gRNAs used for Hoxc deletion in the Koa allele were: Hoxc13-gRNA, gaagcttcggctcctaccgtGGG; Hoxc4-gRNA, cttgcaactcggcagccacGGG. The genotyping primers for Hoxc13 deletion in the Koa allele were: Del_F, CGCCACCTCTGGGC TATGTTTACC; Del_R: GGGCTGGACGCGGAGGAGCA. The gRNAs used for Hoxc4 deletion in the Koa allele were: Hoxc4-gRNA, cttgcaactcggcagccacGGG; Hoxc4-4-gRNA-2: cagccgccccggacacgcCGG. The genotyping primers for Hoxc4 deletion in the Koa allele were: Del_F2, GGATCTAGATCATCCACACAGC; Del_R2: TGGGGCAGGGGTGTAAGTTATGTC. The gRNAs used for Hoxc8 deletion in the Koa allele were: Hoxc8-gRNA, agttcgggatccgccggcGGG; Hoxc8-4-gRNA-2: gcggccaagcgcggggtaggAGG. The genotyping primers for Hoxc8 deletion in the Koa allele were: Del_F3, CACAGCCAGCTCCAGCCC; Del_R3: TCTTTCGAGCACATTCATAACAGAG. The detection primers for Hoxc fusion transcript were: Hoxc_Fusion_F, ATGACGACTCTCGCTCTCCTG; Hoxc_Fusion_R, TTACCTCGTGATCTCCTCTGCC. The detection primers for PPIB are listed in Table S1. The full electrophoresis gel images of PCR product can be found in Figure S3. For the analysis of Hoxc fusion transcript, gel area including the target band (Hoxc fusion transcript and PPIB) in Figure S3E were cropped and stitched in Figure 3E.

Lentivirus Vector Construction, Production and Injection

For overexpression of Hoxc4, 6, and 8, full-length Hoxc cDNA was amplified from CD1 mouse cDNA and then inserted into LV-RFP (Beronja et al., 2010). A self-cleaving P2A peptide was introduced between Hoxc and H2B-RFP for simultaneous expression. For knock down of Hoxc10, 11, 12, and 13, shRNA lentiviral constructs were obtained from the RNAi Consortium (TRC) mouse lentiviral library. ShRNAs were then sub-cloned into LV-RFP (Beronja et al., 2010). Sequences of individual shRNA used in experiments are listed here. Hoxc10-shRNA, CCGGCGCAGAATGAAAACCTCAGAAGACTCCAGTTTTCGATTTTCTGCTTGTTTGG; Hoxc11-shRNA, CGGGCGGCCCTCTGATTCTCGAAATCTCGAGATCTCTCATGAGAGGCAGCGCTTTT; Hoxc12-shRNA, CCGGGCTGTCGCTGGTTTCTCGAGAAGGGGGGTTTGAGAGACAGCTTTT; and Hoxc13-shRNA, CCGGGAGCGGCTCATCCCTGTTGAACGCTGTGACAGGATGCGCGCTTTT. For overexpression or knock down of Hoxc in dermis, high titer lentivirus was produced as previously described (Beronja et al., 2010). ~5μl high titer lentivirus (~10^5 cfu/ml) was intradermally injected using insulin syringes with a 29-gauge needle (BD) into the middle of the ear on P1~2. For overexpression of Hoxc cDNA in epidermis, lentivirus was produced and injected using in utero system as previously described (Beronja et al., 2010).

RNA-Seq

RNA from FACS-purified cells was submitted to the sequencing center at the National Institute of Biological Sciences for quantification, RNA-Seq library preparation, and sequencing. The libraries were sequenced on the Illumina HiSeq 2500 platform using the Single-End 1X50-bp sequencing strategy.

ChiP-Seq

The ChiP of dermal cells was conducted based on a previously described protocol with minor variations (Xie et al., 2013). ~5~6 million dermal cells were fixed in 1% formaldehyde for 10 min at 37°C. Crosslinked cells were lysed in lysis buffer (0.3% SDS, 50 mM Tris-HCl, pH = 8.0, 20 mM EDTA, and freshly added protease inhibitors) on ice for 10 min, with vortexing every 2 min. Lysed samples were immediately subjected to 36 cycles of sonication at power 10 with a sonicator (Qsonica, Q700) to generate small chromatin fragments. 5 μg of the H3K27ac (Active Motif, 39133, RRID: AB_2561016), and H3K27me3 (Millipore, 07-449, RRID: AB_310624), and CTCF (Thermal Fisher, PAS-17143, RRID: AB_10982283), antibodies were used for immunoprecipitation. ChiP DNA was submitted to the Biodynamic Optical Imaging Center at Peking University for quantification, ChiP-Seq library preparation, and sequencing. The libraries were sequenced on the Illumina HiSeq 2500 platform using either the Pair-End 2x100-bp or Single-End 1x50-bp sequencing strategy.
4C-Seq

4C experiment was conducted based on a previously-described protocol (Noordermeer et al., 2011) with a few modifications. ~4 million dermal cells were fixed in 1% formaldehyde at room temperature for 10 min while tumbling. Formaldehyde was quenched by adding glycine to a final concentration 0.125 M. Crosslinked cells were lysed in 15 mL lysis buffer at 4 °C for 2 hours (50 mM Tris-HCl (pH 8), 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1% Triton, and freshly added protease inhibitors). NlaIII was used as primary restriction enzyme for overnight digestion at 37 °C. DpnII was used as secondary restriction enzyme for overnight digestion at 37 °C. A total of ~1 mg of each 4C-seq library was amplified using 16 individual PCR reactions with inverse primers including P5/P7 Illumina adapters (the amount of input DNA is not exceeding 200 ng per reaction). Primer sequences are listed below: Hoxc4_view_point_F: AATGATACGCGACCGCCGAACACCTCTTTCCCTACACGACGCTCTTCCGATCTATCACGACAACAACAAAAACCCAGCA GGT; Hoxc4_view_point_R: CAAGCAGAAGACGGCATACGATGATGTGAAATGCCCCGTGA. 4C libraries were submitted to the Biodynamic Optical Imaging Center at Peking University for quantification and sequencing. The libraries were sequenced on the Illumina HiSeq 2500 platform using the Single-End 1x50-bp sequencing strategy.

QUANTIFICATION AND STATISTICAL ANALYSIS

RNA-Seq Alignment, Analysis and Visualization

For analysis, raw transcriptome sequence data were mapped to the mouse genome (GRCm38/mm9) using TopHat (v2.0.13) with default settings to produce a reference-guided transcript assembly. Cufflinks (v2.2.1) was used to normalize expression levels for each sample to fragments per kilobase of transcript per million mapped reads (FPKM). Cufflinks (v2.2.1) was used to quantify changes in gene expression between the P50 telogen dermal cells (K14-H2BGFP-) of Wt/Wt ear and dorsal skin. Genes with significantly different expression levels (p < 0.01 and ≥ 2X) were chosen for further analysis. Gene ontology (GO) analysis of genes was done using DAVID (Database for Annotation, Visualization and Integrated Discovery), and -log10 (p value) were plotted to show term significance. RNA-Seq signal tracks were presented by Integrative Genomics Viewer (IGV) software.

ChIP-Seq Alignment, Analysis and Visualization

All sequenced reads were filtered, and then de-barcoded. Low-quality ends were trimmed. Reads were then aligned to mouse genome (mm9) using Bowtie2. Normalization among different samples was achieved using HOMOER with default settings and normalize reads for each sample to fragments per kilobase of transcript per million mapped reads (RPKM). Three biological replicates were sequenced for each population. Average RPKM of H3K27me3 modification on Chr15:102.8M-102.9M in P50 ear dermal cells (K14-H2BGFP-) between Wt/Wt and Wt/Koa were calculated and compared using unpaired two-tailed Student’s t tests implemented in Prism5 (GraphPad). ChIP-Seq signal tracks were presented by Integrative Genomics Viewer (IGV) software.

4C-Seq Alignment, Analysis and Visualization

Raw sequencing reads were trimmed using cutadapt, with 4C reading primer sequence and primary restriction site ligated to the 5’ end. The processed reads were aligned to the mouse reference genome (mm9) by bowtie with no mismatches and unique match allowed. Reads in a region directly surrounding the 2 kb up- and downstream viewpoint were excluded. A library file of fragments is generated based on the first and second restriction sites used for the 4C template preparation using a custom perl script. A list of raw reads number per fragment was counted using aligned reads. Then the 4C signal was transformed to a binary value (0 or 1) for each fragment, depending on whether the normalized read count was below or above a certain threshold of 10 counts. Fragments that fulfill the threshold criteria are termed hits. Then the hit percentage of a window of 10 fragment was calculated as an estimate of the contact frequency. Wiggle track format (WIG) or GFF file was generated for visualizing and future analysis purposes. For statistical analysis of 0.5M upstream of proximal breakpoint or 0.5M downstream of distal breakpoint, the fragment counts of the two regions were normalized to fragment counts on chromosome15 and defined as normalized 4C interaction score. Comparisons were performed using unpaired two-tailed Student’s t tests implemented in Prism5 (GraphPad). Statistically significant differences between groups are noted in figures with asterisks (**p < 0.001). 4C-Seq signal tracks were presented by Integrative Genomics Viewer (IGV) software.

Statistics

For “in vivo” experiment, the number of independent experimental replicates are indicated in figure legends, with n representing n experimental replicates using at least n animals. For qPCR experiment, hair cycle analysis, virus injection experiment, Axin2CreER labeling experiment, Lgr5CreER activation experiment, and K5Tα labeling experiment, one mouse was used in each experiment for each group. For RNA-seq, ChIP-seq, and 4C-seq, 1-5 mice were used in each experiment to obtain enough cells. For “in vitro” experiment, the number of independent experimental replicates are indicated in figure legends, with n representing n experimental replicates.

For Figure 1, expression level of Hoxc genes was calculated from 9 mice in 3 experimental replicates (Figures 1D and 1F). For Figure 2, expression level of Hoxc genes and 4kp2 was calculated from 6 mice in 3 experimental replicates (Figures 2C and 2D). For Figure 3, expression level of Hoxc genes was calculated from 6 mice in 3 experimental replicates (Figures 3C, 3H, and 3I); hair cycle was quantified from 18 mice in 3 experimental replicates (3F, 3J). For Figure 4, percentage of anagen hair follicle was calculated from 24 mice in 3 experimental replicates (Figures 4C and 4H). For Figure 5, normalized 4C interaction score was calculated from 6 mice
expression level of Hoxc genes was calculated from 6 mice in 3 experimental replicates (Figure 5F). For Figure 6, percentage of labeled HFSCs and percentage of HFs with labeled DP were calculated from 15 mice in 3 experimental replicates (Figures 6B and 6C); expression level of sfrp2 and rspo2 was calculated from 9 mice in 3 experimental replicates (Figure 6D); percentage of HFs with Ki67+ cells and percentage of new hair shafts were calculated from 6 mice in 3 experimental replicates (Figure 6E). For Figure S1, Ratio of GFP high percentage (P50/P21) was calculated from 6 mice in 3 experimental replicates (Figure S1B); hair cycle was quantified from 54 mice in 3 experimental replicates (Figure S1D). For Figure S2, expression level of indicated genes was calculated from 3 mice in 3 experimental replicates (Figure S2B). For Figure S4, expression level of Hoxc genes was calculated from 3 independent experimental replicates (Figure S3B), with ~0.5 million 3T3 cells for each qPCR. For Figure S5, RPKM and average reads density were calculated from 3 replicates ChIP-seq data using ~15 mice (Figure S5B); expression level of Hoxc genes was calculated from 6 mice in 3 experimental replicates (Figure S5E). For Figure S6, percentage of labeled HFSCs was calculated from 6 mice in 3 experimental replicates (Figure S6A); expression level of axin2 was calculated from 6 mice in 3 experimental replicates (Figure S6B). For Figure S7, expression level of Hoxc genes was calculated from 3 independent experimental replicates (Figure S7B), with ~0.5 million skin dermal fibroblast cells for each qPCR; expression level of Hoxc genes was calculated from 6 mice in 3 independent experimental replicates (Figure S7E); hair cycle was quantified from 14 mice in 7 experimental replicates (Figure S7G).

Double-blind study was used in the quantification of hair cycle in Figure S7G. For other experiments, randomization and experimenter blinding were not necessary, given the lack of ambiguity in the phenotypes observed. Sample size was not predetermined. No data or subjects were excluded in all experiments.

Data are presented as mean value ± the standard deviation (SD). Statistical analyses were performed using unpaired two-tailed Student’s t tests implemented in Prism5 (GraphPad). P value was calculated with a confidence interval of 95% to indicate the statistical significance between groups. A P value < 0.05 was considered statistically significant. Statistically significant differences between groups are noted in figures with asterisks (*p < 0.05, **p < 0.01, ***p < 0.001).

DATA AND SOFTWARE AVAILABILITY

All software used in this study is freely or commercially available (see the Key Resources Table). The accession number for the RNA-seq, ChIP-seq, and 4C-seq data reported in this study is NCBI GEO: GSE116723. Resources of all other cited data are listed below:

NPC HiC-seq data (from Peter Fraser lab, available in 3D Genome Browser: http://promoter.bx.psu.edu/hi-c/view.php?method=Hi-C&species=mouse&assembly=mm9&source=inside&tissue=NPC&type=HindIII-raw-Fraser2016&resolution=40&c_url=&transfer=&chr=chr15&start=0&end=103520000&sessionID=&browser=none);

Neuron HiC-seq data (from Peter Fraser lab, available in 3D Genome Browser: http://promoter.bx.psu.edu/hi-c/view.php?method=Hi-C&species=mouse&assembly=mm9&source=inside&tissue=Neuron&type=HindIII-raw-Fraser2016&resolution=40&c_url=&transfer=&gene=&chr=chr15&start=-43360000&end=213680000&sessionID=&browser=none);

mESC HiC-seq data (GEO: GSE35156); mESC CTCF ChIP-seq data (GEO: GSM918748).